Turun yliopisto
University of Turku

A SYSTEMS BIOLOGY ANALYSIS OF PP2A FUNCTIONS IN CANCER CELLS

Otto Kauko

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1264 | Medica - Odontologica | Turku 2017

University of Turku

Faculty of Medicine

Department of Pathology

Turku Doctoral Programme of Molecular Medicine

Turku Doctoral Programme of Biomedical Sciences

Turku Centre for Biotechnology

Supervised by

Prof. Jukka Westermarck, MD-PhD
Turku Centre for Biotechnology
Department of Pathology
University of Turku
Turku, Finland

Reviewed by

Prof. Anne-Claude Gingras
Lunenfeld-Tanenbaum Research Institute
Mount Sinai Hospital
Toronto, Canada

Prof. Lars-Gunnar Larsson
Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet
Stockholm, Sweden

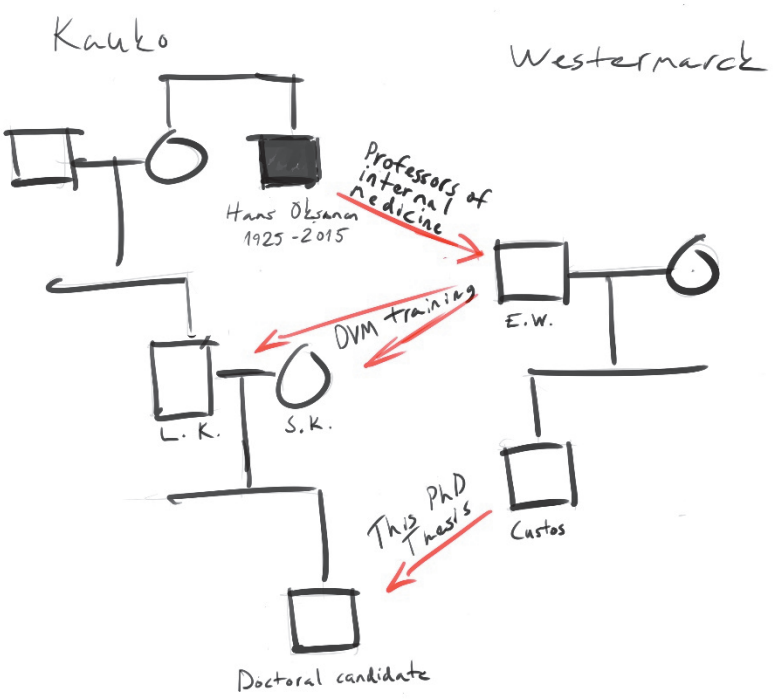
Opponent

Prof. Jussi Taipale
Department of Biosciences and Nutrition
Karolinska Institutet
Stockholm, Sweden

Cover photo by Otto Kauko

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

ISBN 978-951-29-6686-8 (PRINT)
ISBN 978-951-29-6687-5 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2017



To the memory of my great uncle Hans Oksanen and to continuity

Otto Kauko

A Systems Biology Analysis of PP2A Functions in Cancer Cells

University of Turku, Faculty of Medicine, Department of Pathology, Turku Doctoral Programme of Molecular Medicine, Turku Doctoral Programme of Biomedical Sciences, Turku Centre for Biotechnology

ABSTRACT

Cancer is characterized by aberrant activation of phosphorylation signalling cascades. However, despite the critical role of phosphatases in protein phosphorylation, their contribution to cancer cell signalling is only emerging. Notably, Protein phosphatase 2A (PP2A) has a well-established tumor suppressor function but it is poorly understood which of its many targets are relevant for this function. This is partly due to the wide range of activities that PP2A participates in and partly due to the fact that PP2A activity regulation, as well as the deregulation in cancer, occurs via many auxiliary subunits and endogenous inhibitor proteins.

In this MD-PhD thesis, we have used various systems biology approaches, including phosphoproteomics, high throughput drug sensitivity screening, and transcriptomics to study the functions of the most frequently mutated PP2A subunit, PPP2R1A, as well as three of its endogenous inhibitor proteins, CIP2A, PME1, and SET in cancer cells.

This study demonstrates that PP2A reactivation is poorly tolerated by several types of cancer cells and results in downregulation of multiple oncogenic pathways, as well as induction of senescence. Specifically, CIP2A is a regulator of MYC transactivation in basal type breast cancers and our results indicate multiple cooperative mechanisms by which PP2A regulates MYC. Analysis of PP2A dephosphorylome also provided novel insights into general organization of phosphorylation signalling and emphasized the role of PP2A inhibition in the nucleus.

By combining the phosphoprotemics data with cancer cell responses to over 300 drugs, we have identified mechanistically distinct types of interactions between drug sensitivity and PP2A activity. We further validated that inhibition of PP2A in KRAS mutant lung cancers confers resistance to MAPK pathway inhibitors including the combination of Raf and MEK inhibitors.

Together, these findings provide new evidence to support PP2A reactivation as cancer therapeutic strategy and to support evaluating PP2A activity as a predicitive marker for cancer therapy responses.

Keywords: PP2A, CIP2A, MYC, protein phosphorylation, drug resistance, labe-free phosphoproteomics

Otto Kauko

Systeemibiolginen analyysi PP2A:n toiminnoista syöpäsoluissa

Turun Yliopisto, Lääketieteellinen tiedekunta, Patologian laitos, Turun Molekyylilääketieteen tohtoriohjelma, Turun Biolääketieteen tohtoriohjelma, Turun Biotekniikan keskus

TIIVISTELMÄ

Syövälle ominainen piirre on fosforylaation perustuvien signaalipolkujen poikkeava aktivoituminen. Huolimatta fosfataasien keskeisestä tehtävästä proteiinien fosforylaatiossa, niiden merkityksestä syöpäsolujen signlaalinvälityksessä on vasta vähän tietoa. Erityisesti Proteiinifosfataasi 2A:lla (PP2A) on selkeästi osoitettu olevan kasvunestäjproteiini-ominaisuuksia, mutta se mitkä PP2A:n monista kohdeproteiineista ovat tärkeitä syövän kannalta tunnetaan huonosti. Osittain tämä johtuu PP2A:n toimintojen moninaisuudesta ja osittain siitä, että PP2A:n säätely, sekä syövissä esiintyvät säätelyn häiriöt, tapahtuvat ylimääräisten alayksiköiden ja inhibiittoriproteiinien kautta

Tässä väitöskirjatutkimuksessa olemme tutkineet PP2A:n yleisimmin mutatoituneen alayksikön, PPP2R1A:n, sekä kolmen inhibiittoriproteiinin, CIP2A:n, PME-1:n ja SETin, toimintoja syöpäsoluissa käyttäen erilaisia systeemibilogisia lähestymistapoja, mukaan lukien fosfoproteomiikkaa, lääkeherkkyysseulontaa ja transkriptomi-analyysiä.

Tämä tutkimus osoittaa, että monet syöpäsolut sietävät huonosti PP2A:n uudelleen aktivoimista, joka johtaa useiden onkogeenisten signaalipolkujen estymiseen ja senesenssin käynnistymiseen. Tuloksemme viittaavat siihen, että PP2A säätelee MYC-onkogeeniä useilla toisiaan tukevilla mekanismeilla, ja CIP2A:lla on merkitystä MYCin transaktivaation säätelyssä basaalityypin rintasyövässä. PP2A:n defosforylomista saatu tieto auttaa myös ymmärtämään yleisellä tasolla fosforylaatiosignaloinnin järjestäytymistä soluissa ja osoittaa, että PP2A:n inhibitiolla on keskeinen merkitys tumassa.

Yhdistämällä fosfoproteomiikan ja syöpäsolujen vasteet yli 300 lääkkeelle olemme tunnistaneet useita mekanismeiltaan erilaisia yhteisvaikutuksia PP2A:n aktiivisuuden ja lääkeherkkyyksien välillä. PP2A:n estämisestä aiheutuva resistenssin MAPK-signaalipolun inhbiittoreille KRAS-mutaatioita kantasvissa syöpäsoluissa vahvistettiin lisätutukimuksilla. PP2A:n estäminen teki solut resistenteiksi myös MEK ja RAF inhibiittorien yhdistelmälle.

Yhdessä nämä tulokset puoltavat PP2A:n reaktivaatiota syövän hoitostrategiana ja PP2A:n aktiivisuuden määrittämistä syöpähoitojen ennustekijänä.

Avainsanat: PP2A, CIP2A, MYC, proteiinien fosforylaatio, lääkeresistenssi, leimaton fosfoproteomiikka

TABLE OF CONTENTS

ABBREVIATIONS

AKT	Protein kinase B
AML	Acute myeloid leukemia
APK	Atypical protein kinase
ATP/ADP/AMP	Adenosine tri/di/monophosphate
AurB	Aurora kinase B
BCR-ABL	Breakpoint cluster region - Abelson fusion protein
CAN	Copy number alteration
CDK1, CDK2	Cyclin-dependent kinase 1, 2
CIP2A	Cancerous inhibitor of Protein phosphatase 2A
CK2α	Casein kinase 2, alpha subunit
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
E2F1	Transcription factor E2F1
EGFR	Epidermal growth factor receptor
ELK	Eukaryotic like protein kinase
EPK	Eukaryotic protein kinase
Fbxw7	F-box/WD repeat-containing protein 7
HEK	Human ebryonic kidney cell
HMEC	Human mammary epithelial cell
HNRNPA2B1	Heterogeneous Nuclear Ribonucleoprotein A2/B1
IER3	Immediate early response 3
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KSHV	Kaposi's sarcoma-associated herpesvirus
LOH	Loss of heterozygosity
MAPK	Mitogen activate protein kinase
MCV	Merkel cell polyomavirus
mTOR	mammalian target of rapamycin
MYC	C-MYC proto-oncogene
PI3K, PIK3CA	Phosphatidylinositol 3-kinase, catalytic subunit alpha
Plk1	Polo-like kinase 1
PME-1	Protein phosphatase methyl esterase 1
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B, Calcineurin

PP2C	Protein phosphatase 2C
PP6	Protein phosphatase 6
PSTP	Protein serine/threonine phosphatase
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
PTPA	Phosphotyrosyl Phosphatase Activator
RAF (BRAF)	RAF serine/threonine-protein kinases
RAS (H,K,N)	RAS-family small GTPases
RB	Retinoblastoma protein
SET	SET nuclear proto-oncogene
SH2	Src homology 2 domain
SNP	Single nucleotide polymorphism
SRC	SRC proto oncogene non-receptor tyrosine kinase
SV40	Simian vacuolating virus 40
T-ALL	T-cell acute lymphoblastic leukemia
TCGA	The Cancer Genome Atlas
TKI	Tyrosine kinase inhibitor
TP53	Tumor protein p53

LIST OF ORIGINAL PUBLICATIONS

I. Niemelä M, **Kauko O**, Sihto H, Mpindi J-P, Nicorici D, Pernilä P, Kallioniemi O-P, Joensuu H, Hautaniemi S, and Westermarck J; CIP2A signature reveals the MYC dependency of CIP2A-regulated phenotypes and its clinical association with breast cancer subtypes. *Oncogene 31(39): 4266-4278, 2012.*

II. **Kauko O**, Laajala TD, Jumppanen M, Hintsanen P, Suni V, Haapaniemi P, Corthals G, Aittokallio T, Westermarck J, and Imanishi S; Label-free quantitative phosphoproteomics with novel pairwise abundance normalization reveals synergistic RAS and CIP2A signaling. *Scientific Reports 17(5): 13099, 2015.*

III. **Kauko O**, Imanishi SY, Kulesskiy E, Laajala TD, Yetukuri L, Padzik A, Jumppanen M, Haapaniemi P, Yadav B, Suni V, Varila T, Corthals G, Wennerberg K, Aittokallio T and Westermarck J; Systemic map of Protein Phosphatase 2A (PP2A)-regulated dephosphorylome and drug responses in cancer cells. *Manuscript.*

1. INTRODUCTION

Cancer is a universal feature of multicellular life. Multicellularity presents an organism with a unique challenge: the need to suppress the fitness of individual cells, which has been perfected during 3 billion years, for the benefit of the whole organism. If this regulation fails even in one cell, the result may be cancer.

To achieve this goal, multicellular organisms have developed a variety of regulatory mechanisms that limit cell growth in response to cell-to-cell communication and other extracellular stimuli[1, 2]. Indeed, many of the genes that malfunction in human cancers have emerged in the first multicellular animals and are involved in signal transduction[3, 4]. Another group of cancer genes with even longer evolutionary history are the genes involved in maintenance of DNA fidelity.[3] In order for a tumor to become lethal however, it needs also the ability to invade distant organs and form metastases[5]

Interestingly, there seems to be little correlation with body size and cancer incidence. This phenomenon has been named Peto's paradox after the epidemiologist Sir Richard Peto who first noted it[6]. It is a paradox not only because the cell number varies by several orders of magnitude between animals or because larger animals tend to live longer, but also because cells in larger animals have had to undergo many more cell divisions leading to accumulation of errors in DNA; Despite the similar lifetime risk of getting cancer, humans have been estimated to undergo 10^5 times more mitoses than mice during their lifetime.[7] Despite the considerable variety in the number and identity of required genetic alterations between tumor types[8], the importance of the cell divisions in accumulation of genetic errors is evident even in the comparison of life-time cancer risk in different human tissues; the analysis by Tomasetti an Vogelstein[9] suggests that perhaps more than half of all cancers can be explained by the number of cell divisions that have happened in these tissues.

An extreme example of Peto's paradox is the size difference between honey bee *(Apis Mellifera)* and blue whale (*Balaenoptera musculus*). Naturally occurring ovary tumors have been reported in honey bee queens[10] (Figure 1.) whereas blue whale is not known to be exceptionally susceptible to cancer, despite weighing about 10 million times more than a honey bee queen and having a comparable life span – at the time when it is born[11, 12]. After the birth, age and weight can increase by nearly two orders of magnitude each. [8]

Although other factors, such as the higher metabolic rate, may contribute to cancer in smaller organisms[13], the inevitable conclusion from these staggering odds is that larger and longer-lived animals need more efficient mechanisms for controlling cell growth. Elephants, for example, have 20 copies of the tumor suppressor gene TP53 resulting in more efficient DNA damage response. This property makes elephant cells more likely to undergo apoptosis following DNA damage than human cells.[14] Another example is provided by the experimental transformation of normal cells into cancer cells. Transformation of human cells is considerably more difficult than transformation of mouse cells, and the key difference is the specific requirement for Protein phosphatase 2A (PP2A) inactivation in transformation of many types of human cells[15, 16].

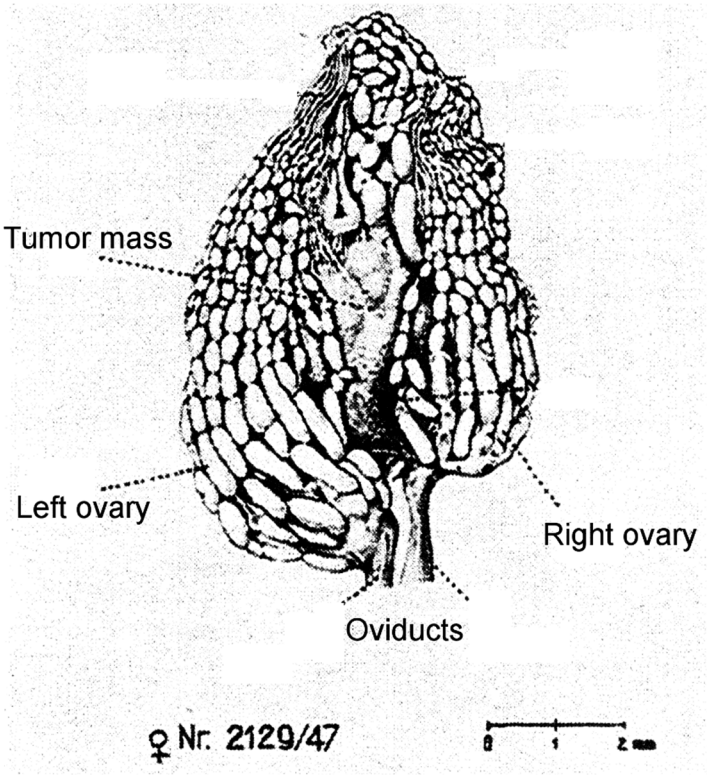


Figure 1. Honey bee queen ovary tumor. Depiction of an ovary tumor found on one year old honey bee queen, modified from Fyg, 1963[10]. The tumor was initiated in the right ovary and grows to the space between the ovaries.

In addition to experimental transformation, the tumor suppressor role of PP2A is supported by the cancer genomics literature accumulated over the recent years. However, whereas transformation experiments have implicated PP2A in

regulation of several oncogenic pathways, cancer genomics data suggests that the key tumor suppressor function of PP2A may be linked to maintenance of genome stability. In this review of literature, I will discuss the role of PP2A in phosphorylation signalling, its tumor suppressor functions in the light of current cancer genomics evidence, and the therapeutic implications of PP2A deregulation in cancer.

Identification of the key PP2A targets mediating its tumor suppressor functions is complicated by the breadth of its substrate base. Therefore, in the experimental part of this thesis we have used various systems biology approaches to gain an unbiased view on the effects of PP2A activity manipulation. The findings presented in this thesis provide novel insight into PP2A functions, expand on previously reported regulation of MYC, and highlight the role of PP2A in drug resistance.

2. REVIEW OF LITERATURE

2.1. Protein phosphorylation

Protein phosphorylation is the most common post-translational modification of proteins[17]. Phosphorylation refers to the transfer of a phosphate group to a suitable amino acid on a protein. The resulting phospho-amino acid carries a 2-charge and possesses unique structural properties compared to the non-modified amino acids, which can be exploited in cellular signal transduction, protein stability regulation, protein-protein-interactions, and allosteric control of enzymatic activity[18]

Majority of phosphorylation events occur on hydroxyl group containing amino acids. Both transfer of a phosphatae group from ATP to hydroxyl-group containing amino acids to generate a phosphoester bond formation and the hydrolytic cleavage of this bond are essentially irreversible reactions in physiological conditions. The phosphoester bond of a phosphorylated amino acid retains significant portion of the bond energy that is released by cleavage of the terminal phosphate from ATP. What drives protein phosphorylation, and makes it irreversible, is the high ATP/ADP ratio in living cells.[18] Dephosphorylation reaction is made irreversible by the release of the phosphoester bond energy. However, despite having a high bond energy, phosphoester bonds are very stable. Negative charge of the phosphate group contributes to the stability of the bond by repelling the attacking nucleophiles.[19] This generates an energy barrier that necessitates specialized enzyme catalysis for the hydrolysis reaction. This stability, together with irreversibility of the reactions, is basis for controlling the phosphorylation and dephosphorylation reactions and makes phosphate modification so amenable to signal transduction and allosteric regulation of protein functions. An important implication from these concepts is that not just protein phosphorylation but also dephosphorylation can function as an activation mechanism in allosteric control of protein function, checkpoint signalling, and initiation of signalling cascades.[20-22]

2.1.1. Phosphorylated residues

Vast majority of protein phosphorylation occurs in the form of phosphate esters of the hydroxyl group containing amino acids: serine, threonine, and

tyrosine. When tyrosine phosphorylation was discovered by Hunter and Sefton, quantification of phosphorylated amino acids in chicken cells suggested a very low abundance of phosphotyrosines: 0.04% of all phosphorylated residues. The phosphotyrosine fraction was increased to 0.3% by Rous sarcoma virus mediated infection that introduced active Src-tyrosine kinase into the cells. Yet, the majority of the phosphorylation events occurred on serine (92%) and threonine (8%) residues[23]. Mass spectrometry-based analysis of the phosphoproteome by Olsen et al. increased the sensitivity of phosphotyrosine detection, and permitted re-evalution of percentages of each phosphor-amino acid, resulting in 1.8% Y, 86.4% S, and 11.8% T[24]. Discrepancies between the different methods have been attributed to the signalling state of the cells, low basal state of phosphorylation, sensitivity of the methods, and higher stability of pS/T[24]. Later higher coverage studies have reported similar ratios (0.4% Y, 84.1% S, and 15.5% T out of 38229 phosphorylation events), however, phospho-tyrosine antibody enrichment can increase the fraction of identified phospho-tyrosine residues by an order of magnitude[25]. The phosphorylation sites recorded in PhosphoSitePlus® (www.phosphosite.org) 2014 update were 16.7% Y, 58.5% S, and 24.9% T out of 247826 non-redundant phosphorylation sites in total[26], although comparison of different data sets suggests that the data on tyrosine phosphorylation has higher coverage than serine/threonine data[25] .

The fraction of phosphothreonines has also been growing with the increased coverage of phosphoproteome, suggesting that they tend to have lower basal level of phosphorylation than serines on average. The early analyses have been limited to identifying a limited number of highly abundant phosphosites, and these studies seem to have overestimated serine/threonine ratio compared to higher coverage data[25].Phosphorylation sites are more likely to be found on disordered regions of proteins than corresponding non-phosphorylated amino acids[27]. This tendency is far more pronounced for serines and threonines than tyrosines[26], which may indicate that tyrosines are more often involved in allosteric control of proteins. However, this property of phosphotyrosines may also reflect the enrichment of all tyrosines in globular domains[28].

Serines and threonines are more likely to form clustered phosphorylation sites [29], which has been associated with regulation of protein-protein interactions[30]. Although nonphosphorylated serines may also cluster, surrounding sequences of known phosphorylation sites are significantly more enriched in serines

compared to the surrounding sequences of nonphosphorylated S, T, and Y residues[28]. No clear division exists between the functions mediated by serine and threonine phosphorylation and comparative genetics analysis suggests that they are to some extent interchangeable[31]. However, some differences exist between phosphoserines and threonines; distribution of phosphothreonines between disordered and structured domains is intermediate between phosphoserines and phosphotyrosines[26]

Basic amino acids, histidine and, to lesser extent, lysine and arginine are also known to undergo phosphorylation on a small number of proteins. However, the phosphoamidate bond in these phosphorylated residues is more labile than phosphoester and is susceptible to hydrolysis in acidic conditions.[32] Partly owing to this lability and the fact that liquid chromatography of most modern mass spectrometry set ups is operated at acidic conditions, histidine phosphorylation is difficult to study and it may be more widespread than what the current literature suggests; in a specific cellular fraction, basic nuclear proteins, it may account for up to 6% of the total phosphorylation[33]. Recently, development of phosphohistidien specific antibodies has enabled the discovery of hundreds of hisitidine-phosphorylated proteins, suggesting a potential involvement of histidine phosphorylation in cell cycle regulation, RNA processing, and ribosome related functions[34]. A special case of histidine phosphorylation is the two-component system, that is found in bacteria, fungi, and plants but is absent in metazoan. Two-component histidine kinases are a class of membrane proteins that function e.g. as chemotactic receptors. The kinase autophosphorylates a histidine residue and the phosphate is then relayed to an aspartate residue and further to a different histidine residue in a different domain/subunit from where it finally reaches an aspartate on the substrate protein.[35] Although not structurally related, human nucleoside diphosphate kinases also form histidine phosphate intermediates from where the phosphate is further transferred to a histidine or aspartate in the substrate[36]. Phosphohistidine can be dephosphorylated by serine/threonine phosphatases PP1, PP2A, and PP2C *in vitro.* The rate of phosphohistidine dephosphorylation on H4 by PP1 and PP2A was comparable to phosphoserine dephosphorylation Phosphorylase A[37]. Although these PSTPs constitute majority of the phosphohistidine phosphatase activity *in vitro*, phosphohistidine specific phosphatases, such as PHPT1, have also been described[38]. Lastly, Cysteine phosphorylation has been observed in bacteria[39].

2.1.2. Kinases and phosphatases

Protein phosphorylation occurs when an enzyme called kinase transfers the terminal phosphate group from ATP to a phosphorylatable amino acid on protein. This phosphate is removed by another class of enzymes, protein phosphatases. A significant portion of the human genome is dedicated to the phosphorylation machinery. A characterization of the kinase complement in human genome by Manning et al. identified 518 protein kinases, including 478 eukaryotic protein kinases (EPK), and 40 atypical protein kinases (APK)[40]. Earlier report by Kostich et al. identified 510 EPKs[41]. The analysis by Manning et al., which benefited from non-publicly available data from different sources, was also able to identify kinases not present in the first analysis. Comparison of the results suggests that most of the additional kinases reported by Kostich et al. are either duplicates or likely pseudokinases and comparison analysis resolved the conflicts in favour of Manning et al. Generally the agreement was found good (99%) on the 428 EPKs retaining the conserved residues in their catalytic domain, however, some atypical kinases may have been missed by both analyses. One of them is a secreted atypical protein kinase FAM20C[42]. The number of kinases in human kinome is increased to 539 with the inclusion of 20 lipid kinases[43]

Structure-based analysis suggests that evolutionary origins of phosphatases are more diverse, and the division between protein phosphatases and metabolic enzymes that catalyse reactions on small molecule substrates is less obvious, than with protein kinases. In some cases structurally diverse phosphatases have converged on similar substrate specificity while some families of phosphatases exhibit considerable divergence in their substrates[44]. The 2015 update of Human Dephosphorylation Database (DEPOD) lists 239 (228 active) phosphatases[45], including 108 phosphotyrosine phosphatases (PTP) and 38 serine/threonine phosphatases (PSTP). The initial analysis of PTPs in human genome of by Alonso et al. identified 107 Tyrosine phosphatases, of which 81 are catalytically active towards pY. PTPs are similar to tyrosine kinases in terms of number, subcellular localization, and protein interaction motifs [46]. However, the number of serine/threonine phosphatases is about an order of magnitude lower than the number of corresponding kinases. This lack of diversity in the S/T dephosphorylation is made up by the modular structure of phosphatase complexes, where multiple mutually exclusive regulatory subunits determine the subcellular localization and substrate specificity[47] In contrast, tyrosine

kinases and phosphatases are multidomain proteins, and similar functions are provided by different domains of the same protein[46]

2.1.3. Evolution of protein phosphorylation

Catalysed phosphate ester bond formation and cleavage reactions have been present already during the early nucleic acid evolution and thus predate the emergence of proteins. That, together with the selection of ATP as the major energy storing molecule, may have contributed to the dominant role of the phosphate modification on proteins in cellular signalling[18]. Although protein phosphorylation occurs in all living organisms, the eukaryotic, and especially metazoan phosphorylation machinery has undergone significant diversification.

2.1.3.1. Evolution of kinases and phosphatases

Eukaryotic protein kinases evolved from eukaryotic like protein kinases (ELK) in prokaryotes. Catalytic mechanism conserved between ELKs and EPKs but whereas the ELKs are more akin to metabolic enzymes with high turnover, EPKs have evolved sophisticated regulatory mechanisms. One of these is the conserved activation segment that typically assumes ordered conformation following a phosphorylation event. Thus, majority of kinases are activated by phosphorylation. In addition, EPKs have additional substrate recognition elements[48]

Tyrosine kinases and some mitogen activated protein kinases (MAPK) are specific to metazoans.[49] Closest unicellular relatives of the metazoans are choanoflagellates, that, even though unicellular, exhibit colonial behaviour with rudimentary differentiation in tasks perfomed by the members of the colony. Analysis of the choanoflagellate M.brevicollis genome reveals that they possess some MAPKs but e.g. and JNK the MAPKKK Raf are exclusive to metazoans[50]. M.brevicollis also possesses an elaborate tyrosine signalling machinery with 128 kinases and 38 phosphatases, however, it bears very little similarity to bilaterian metazoan phosphotyrosine signalling, and appears to have developed largely independently [51]. PI3K-family lipid kinases have also expanded during metazoan evolution from one kinase regulating vesicular traffic in yeast to eight kinases and multiple regulatory subunits in vertebrates that regulate diverse signal transduction events[52].

Phosphatases have undergone parallel diversification. Interestingly, PTPs and SH2-domains, that specifically interact with phosphorylated tyrosines, seem to have existed before the first tyrosine kinases.[53] It has been suggested that they play a role in phosphotyrosine signalling also in fungi where some serine/threonine kinases possess limited tyrosine kinase activity[53, 54]. PTPs and SH2 domains have then experienced rapid diverisification and coevolution with the tyrosine kinases after their emergence.[53]

The evolution of serine and threonine dephosphorylation is more evident in the regulatory subunits. One copy of PP2A B56-family regulatory subunit gene is found in primitive eukaryotes, and it has been duplicated in early metazoan evolution and 5 isoforms are found in most vertebrates. Further diversification between the isoforms has also happened in vertebrates[55]. Similarly, the yeast *Saccharomyces cerevisiae* has only one B55 homolog, Cdc55[56], whereas the human B55-family contains 4 members, out of which the β- and γ-isoforms are mainly expressed in brain[57].

Protein phosphatase 1 (PP1) has larger number of regulatory subunits than PP2A and while some of them are widespread among all eukaryotes, this evolutionarily related class of proteins has expanded significantly in metazoans with 7 new subunit families (R9, R10, R12-16). Duplication has occurred at least once in each of these families in vertebrates and the inhibitor-1 (R1-family) is only present in vertebrates. For some of these proteins, the putative PP1-regulatory function is based on the evolutionary origin and conservation of the catalytic subunit binding motifs. In addition to the primary regulöatory subunits, unrelated proteins converging to PP1 regulatory function in bilaterian metazoan lineage have also been identified.[58]

2.1.3.2. Evolution of phosphorylation sites

Allosteric regulation of proteins requires both specific phosphorylation and dephosphorylation activity as well as highly specific structural reorganization on the substrates part. Evolution of such complex traits can only occur sequentially.

Human genome bears evidence of selective forces acting on potentially phosphorylatable residues after the emergence of a kinase activity. The number of tyrosine residues in the proteome has been decreasing with the expansion of tyrosine kinome during metazoan evolution, suggesting a selective pressure against tyrosines whose phosphorylation is harmful[59]. While other factors, such as the increase in genomic GC-content especially in warm blooded animals, may

have contributed to this trend[60], the loss of tyrosines was more pronounced in proteins not known to be tyrosine phosphorylated[59]. Additionally, similar relationship was observed between threonines and serine/threonine kinome.

However, once the kinase activity is present, new phosphorylation sites can also evolve in place of previously non-phosphorylatable residues. There is evidence that phosphorylation sites involved in conserved allosteric activation have evolved, at least in part, from aspartates and glutamates that are structurally similar to phosphorylated serines and threonines. With a detailed comparative genomics analysis, Pearlman and others showed that there is a bias in distant homologs to have aspartate and glutamate in place of phosphorylatable serine. In addition, substitutions between acidic and phosphorylatable amino acids exhibited clade specific patterns, or emerged in some paralogs after duplication[31].

Phosphorylated amino acid residues tend to be located in disorderd and surface regions of proteins. When this tendency is taken into account, they are significantly more conserved than corresponding nonphosphorylated residues[61]. The conservation is also more evident on the phosphosites with known function, or on the sites that are found in the context of known kinase specificity determinants, suggesting that significant portion of non-conserved phosphorylation may be non-functional[62]. However, localization of phosphorylation sites that interact with phosphopeptide binding motifs is more flexible than localization of phosphosites involved in allosteric regulation. An example is provided by a comparison of CDK1 target sites across 32 fungal species. Majority of CDK1 target sites are clustered in disordered regions and are enriched for binding partners of 14-3-3 motifs. The exact position of these phosphosite clusters is poorly conserved between different fungals species.[30] Furthermore, a related kinase Ime2 phosphorylates distinct but closely located sites on same substrates in meiosis, and these phosphorylations mediate similar functions that CDK1 target sites mediate in mitosis.[63]

The evolutionary conservation of phosphorylation sites is more pronounced for serine: Serines have the highest mutation frequency of all amino acids. However phosphorylated serines exhibit a higher degree of conservation than phosphothreonines. Higher rate of change in phosphothreonines has been suggested to indicate a more ancient evolutionary origin of phosphoserine signalling.[64] Accordingly, a comparison within the mammalian lineage shows that human proteome has experienced a net gain of phosphorylated threonines

since the divergence from mice.[61] Interestingly, several major phosphatases have a preference for threonine, whereas a majority of kinases prefer serine[65, 66]. This suggests that serines have, on average, a higher chance to be phosphorylated and contributes to the above described phenomenon of serines being overrepresented in mass spectrometry based phosphoproteomics analyses. It is worth noting, although the causality is not established, that the increase in phosphothreonine complement co-occurs with a more important role of phosphatase regulation of oncogenic pathways in the transformation of human cells compared to mouse cells.[15]

In kinases, the specificity between serine and threonine is largely determined by one residue in the vicinity of the catalytic site and the switch in this specificity seems to have occurred independently in multiple kinases throughout the evolution[67]. Similarly, switching between serines and threonines has also frequently occurred in substrates[31]. In addition to kinases and phosphatases, selectivity between serines and threonines is a property of phosphopeptide interaction motifs[68].

2.1.4. Deregulation of protein phosphorylation signalling in cancer

Given the essential role of phosphorylation signalling in translating extracellular signals in to cellular functions, it is not surprising that these phosphorylation events are deregulated in cancer.

Tyrosine kinase Src was the first oncogene to be discovered[69] and most frequent amino acid substitution mutation, present nearly 10% of all human cancers according to COSMIC data base, is the activating valine to glutamate mutation in BRAF kinase codon 600 (Figure 2, cancer.sanger.ac.uk[70]). First attempts to catalogue frequently mutated genes in cancer noted the overrepresentation of kinases[71]. Kinases remain significantly enriched in the more recent large scale cancer genomics analysis by Fleuren et al. that has utilized copy number data and more sophisticated methods to distinguish between driver and passenger mutations[42]. There is a particularly strong overrepresentation of tyrosine kinases, with nearly half of the receptor tyrosine kinases harbouring likely driver mutations in cancers, and also of kinases belonging to MAPK cascades. The PI3K/Akt also stands out when the lipid kinases are included in the analysis. As discussed earlier, these are the kinases that have emerged during the evolution of multicellular life in metazoan lineage and are involved in transducing signals from extracellular space into the cell.

Copy number alterations frequently target regulators of phosphorylation signalling. A 5000 sample pan-cancer analysis of copy number alterations suggested that phosphatases are implicated in regions of copy number loss in cancer[72]. Correspondingly, an earlier analysis of over 3000 samples suggested association between kinases and amplification of genomic DNA[73]

In addition, mutations have been shown to abolish and generate new phosphorylation sites as well as alter specificity determinants in kinases and in target proteins with the potential to generate novel kinase-substrate combinations.[74] The mutations on the substrate protein genes, either destroying phosphorylation sites or altering specificity determinants, represent large categories of mutations that potentially affect phosphorylation signalling, however, their functional impact remains to be assessed.

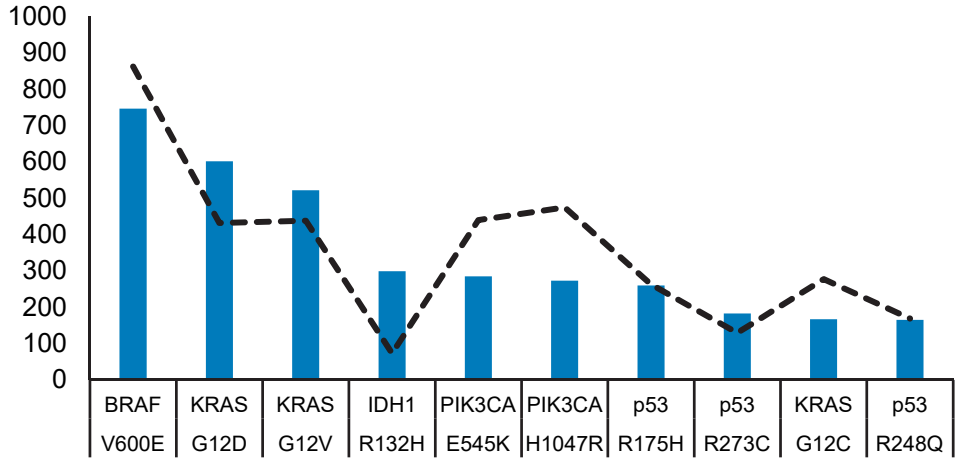


Figure 2. Ten most frequent missense mutations in human cancers. This figure is based on COSMIC v76 genome-wide data comprising 18186 samples (cancer.sanger.ac.uk)[70]. The most frequent mutation in this data set is the activating valine to glutamate mutation at BRAF codon 600, whereas the combined frequency of all codon 12 mutations in its direct upstream regulator, KRAS, is even higher. The top ten list also has two activating mutations of the lipid kinase PIK3CA. The dotted line represents the incidence of mutations weighted by SEER 2015 Cancer incidence estimates[75] to account for over and underrepresentation of certain cancer types in COSMIC database.

2.2. PP2A structure and function

PP2A was initially defined as one of the four serine/threonine phosphatases, PP1, PP2A, PP2B, and PP2C. This classification was based on 1) inhibition by endogenous inhibitor proteins and small molecule inhibitors, 2) requirement of divalent cations for catalytic activity, and 3) the ability to dephosphorylate certain substrates. These approaches identifyíed PP2A as a major constituent to total serine/threonine phosphatase activity, along with PP1 and PP2C.[76] However, what was classified as PP2A before the gene-centric analyses is likely to have included also other related phosphatases. Nonetheless, PP2A has very large number of substrates and it plays a role in wide range of biological functions[77]. The diverse functions of PP2A are under elaborate control involving a large number of mutually exclusive regulatory subunits and endogenous inhibitor proteins, as well as post-translational modifications (Figure 3). In addition, it has been suggested that the activation of the newly synthesized C-subunit by PTPA is coupled to the biogenesis of the holoenzyme in order to restrict the promiscuous activity of the free C-subunit[78, 79].

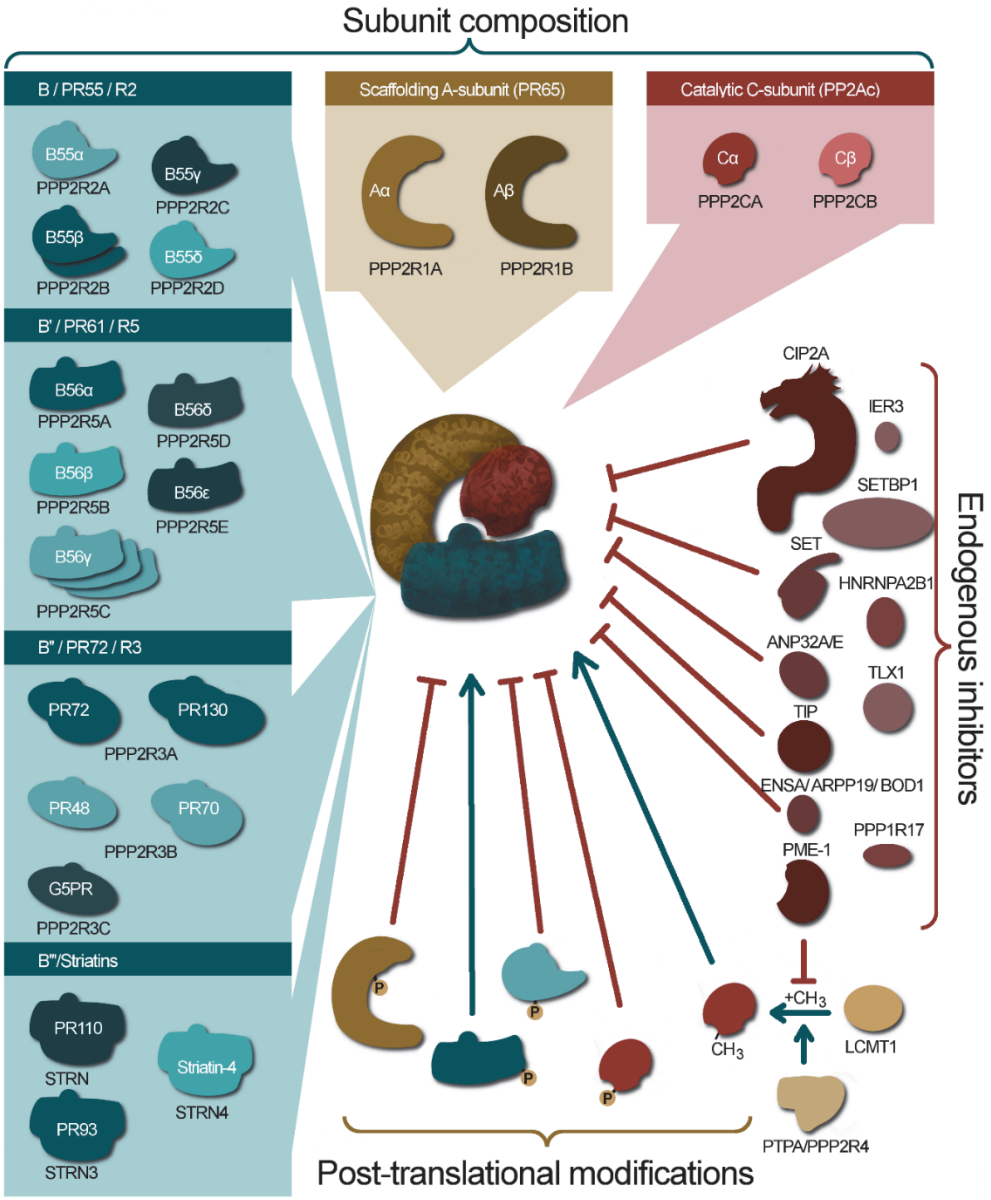


Figure 3. Canonical PP2A subunits and mechanisms of activity regulation. PP2A activity is controlled by subunit composition of the holoenzyme, endogenous inhibitor proteins, and post-translational modifications of the subunits.

2.2.1. Catalytic C-subnunits and Scaffolding A-subunits

In humans, the catalytic C-subunits of PP2A are encoded by two different genes, PPP2CA and PPP2CB, and their 309 amino acid long sequences are 98% identical.[80] There are also two isoforms of the 65 kilodalton scaffolding A-

subunit, that have 87% identical amino acid sequence and are encoded by the genes PPP2R1A and PPP2R1B.[81] For both A- and C-subunit, the alpha isoform is dominant, comprising roughly 90% of the total amount of these subunits under the conditions analysed thus far.[76, 81-83] In line with their essential role, knockout of either PPP2CA or PPP2R1A in mice is embryonic lethal[84, 85], whereas knockout of PPP2CB produced no obvious phenotype[86].

The catalytic subunits are very highly conserved across the metazoan lineage[87]. Both catalytic subunits have similar affinity towards A- and B-subunits and similar catalytic activities when bound to the same A- and B-subunits.[88] It is not known if the catalytic subunit isoforms have distinct substrates, however, some tissue specific differences in their relative expressions have been reported[89], and differences in their subcellular localization patterns have also been reported[90].

Distinct functions have been reported for the A-subunit isoforms[91]. The Aβ has lower affinity towards B55-family subunits and C-subunits than Aα. Aα is expressed at high level in all tissues, whereas the expression of Aβ is low in most tissues and moderately high in testis. Aα exhibits a high level of conservation throughout the vertebrate evolution whereas Aβ is more varied.[83]

PP2A exists in cells mainly as a dimer composed of A- and C-subunit or a trimer containing a variable B-subunit in addition to A- and C-subunits[76], though the C-subunit can also be found in complexes that do not include the A- or B-subunits, notably with alpha4 (IGBP1), an interaction that conserved from yeast to mammals[92]. The B-subunit is thought to contribute to the substrate specificity and to localize PP2A activity to relevant sites. However, the division between B-subunits, endogenous inhibitors, and other interacting proteins is not always clear, and a widespread substrate recognition sequence has only been described for B55 and B56-family subunits thus far[93, 94]. Moreover, even in the absence of systematic analysis, there is evidence that some of the subunits, inhibitors, and interaction partners are shared with other phosphatases.[95-97] For example, the regulation of carboxyterminal methylation of the C-subunit is at least partly shared between PP2A, PP4, and PP6; LCMT-1 has been shown to methylate all of them[98] and an interaction has also been reported between PPP4C and PME-1[99]. Binding to Alpha4 is another shared interaction between PP2A, PP4, and PP6[100] and crosslinking mass spectrometry analysis suggests that the Aα subunit of PP2A can form a hybrid complex with PP4 subunits PPPP4R3A and PPP4C via interactions that are similar to PP2A B56γ and C-subunit

binding[101]. PP2A inhibitors ANP32A, SET, PPP1R17, and TLX1 also interact with PP1, and they are referred to as subunits in the context of PP1[58].

2.2.2. Regulatory B-subunits

A total of 15 genes are generally considered as B-subunit genes. The proteins encoded by these genes belong into 4 structurally distinct families, B/B55/R2, B'/B56/PR61/R5, B''/PR72/R3, and B'''/PR93/PR110/Striatins. (Figure 3.) Although there is no obvious sequence similarity between the B-subunit families, binding assays of B56-subunit fragments identified a bipartite A-subunit binding domain that exhibited limited conservation between B55, B56, and PR72 families[102]. This domain was not found in Striatins, however, there is a similar sequence in Striatins and the B56 family subunits that corresponds to the first amino acids in the N-terminal part of the shared A-subunit binding motif.[103] Striatin also has other more N-terminal regions that contribute to PP2A binding[104].

The first B-subunits to be characterized were the α- and β-isoforms of the B55-family[105]. The β-subunit and the later identified γ-subunit[106] are mostly expressed in brain[57]. The last member of this B-subunit family is the δ-isoform, which is most similar to the α-isoform[107]. Both of them are ubiquitously expressed and despite differences in their subcellular localizations[107], the δ-isoform shares some of the critical mitotic functions of the α-isoform[108].

In addition to the 55 kilodalton B55-subunits, PP2A was purified as a trimer containing A, C, and a 72-kDa B-subunit in early experiments[76]. Characterization of this subunit suggested that it is expressed as 130 kDa and 72 kDa variants sharing the C-terminus[109]. These proteins, encoded by PPP2R3A gene, were named as as PR130 and PR72. PR48, encoded by PPP2R3B, was identified as a binding partner of Cdc6 in a yeast-2-hybrid screen and subsequent analysis revealed the role as B-subunit based on the homology with PR72/PR130[110]. A 70 kDa homologue of PR48 was identified in *Xenopus laevis* and a subsequent analysis of the human PPP2R3B gene suggested that the 70 kDa variant, PR70, is also expressed in humans. These variants share the C-terminal region and the methionine in the beginning of the PR48 is not conserved in *X.laevis*.[111] The PR59 protein sometimes listed as a member of this family is a mouse protein that is highly similar to human PR48/PR70[110, 112]. G5PR, encoded by PPP2R3C, was identified as a binding partner of GANP in a yeast-2-hybrid screen its sequence

is most closely related to the PR72-family subunits. Functional analyses suggest that it is a PP2A subunit, and also an interaction partner of PP5[96].

Splicing variants of the PR72-family have received more attention, perhaps because of the nomenclature that more clearly distinguishes the variants. However, alternative splicing variants with distinct functions, such as mitochondria specific localization of PP2A, have been reported also for B55- and B56-subunits[113, 114].

A two hybrid experiment by McCright and Virshup using A-subunit as a bait identified a third type of B-subunit with an approximate molecular weight of 56 kilodaltons, and no obvious sequence similarity to B55 and PR72 subunits[115]. In addition to the initially identified α-, β-, and γ-isoforms, the family contains two more functional genes coding for δ- and ε-isoforms, the former of which is larger than the other members of this family with an approximate molecular weight of 70 kilodaltons.[114, 116] Evolutionarily, γ and δ isoforms are more closely related and their common ancestor diverged from the common ancestor of α,β, and ε in early metazoan evolution. However, significant diversification between the isoforms has occurred during vertebrate evolution.[55] A shared property of the γ- and δ-subunits is the mainly nuclear localization whereas the others are cytoplasmic[114].

Striatin and SG2NA, encoded by STRN and STRN3, were identified as the fourth B-subunit family based on immunoprecipitation experiments and sequence similarity to B56 subunits that resulted in antibody cross reactivity.[103] Zinedin, encoded by STRN4 is a third member of the striatin-family and has a very high degree of homology with the N-terminal half of Striatin and SG2NA that contains the PP2A binding regions. SG2NA is ubiquitously expressed but the expression of Striatin and Zinedin is mainly restricted to brain.[117] Out of all B-subunits, the role of striatins is least well established, and while Striatin and SG2NA bind to PP2A in the absence of other B-subunits, they also seem to be members of the same protein complex and targets of PP2A.[118] Goudreault et al. performed a complex set of iterative affinity purification and mass spectrometry experiments to characterize this complex, referred to as STRIPAK. In addition to striatins and the A- and C-subunit of PP2A, the STRIPAK complex contains Mob3 and STRIP proteins. Interestingly, the PP2A A- and C-subunits also bound to other members of STRIPAK, and the striatins also interacted with each other.[119]

2.2.3. Proteins with subunit-like functions

A number of proteins have been shown, with varying levels of evidence, to bind to the C-subunit or A/C-dimer forming catalytically active complexes. These interaction have been reviewed extensively by Guergnon et al.[120] and include a number of kinases. In addition, many viral proteins bind to the A-subunit in a competitive fashion with the endogenous B-subunits.

Interestingly, sequence similarities have been observed between SV40 small t-antigen and CK2α-kinases. Although B-subunits and small t-antigen bind to overlapping regions of A-subunit, the binding motif in small t-antigen and, by extension, CK2α-kinases lacks significant similarity with the shared A-subunit binding motif of B-subunits.[102] Casein kinase 2 alpha (CK2α) has been shown to interact with PP2A via a motif that is shared with the small t-antigen. Binding of CK2α to PP2A core enzyme enhanced MEK dephosphorylation and limited the effect of RAS in transformation.[121] A similar motif is also found in Casein kinase 2 alpha 2 (CK2α') and binding of CK2α' to PP2A core enzyme has been shown to inhibit its activity towards Tau.[122] However, CK2α and CK2α' were not analysed in parallel. Therefore, it is not known whether CK2α-kinases have opposite effect on PP2A activity or whether these results can be explained by restricted substrate specificity.

Another group of kinases with limited sequence similarity to SV40 small t-antigen PP2A binding region are the Janus kinases.[123] Mutating one of the two putative PP2A binding sequences on JAK2 has been shown to impair PP2A-JAK2 interaction.[124] JAK2 has been shown to transiently interact with PP2A following IL-11 stimulation[123]. In addition, IL3 stimulation has been reported to promote PP2A-JAK2 interaction, resulting in transient inhibition of PP2A activity and phosphorylation of the catalytic subunit Y307, detected by a generic phosphotyrosine antibody from a PP2A C-subunit immunoprecipitate[125]. However, it has also been suggested that the complex formation with JAK2 directs PP2A to specific targets as it negatively regulates JAK2 and STAT5 signaling induced by IL-3 treatment.[125] Another kinase with multiple interactions with PP2A core dimer is the Src non-receptor tyrosine kinase. Like JAK2, activation of Src first stimulates the PP2A interaction. Using the same methodology as with JAK2, Src was also shown to phosphorylate the Y307 of the C-subunit resulting in transient inhibition of PP2A activity and this phosphorylation has been shown to reduce the binding between PP2A and these kinases.[124, 126] However, although the net effect of the Y307

phosphorylation was a reduction in binding between Src and PP2A A/C-dimer, the phosphorylation promoted the interaction with the Src SH2 domain[126]. Also similarly to JAK2, PP2A is a negative regulator of the Src activity and downstream targets[127].

The PP2A catalytic subunit has been shown to interact with SH3-domains on GPR54[128] and the SH3 domain of Src also contributes to the binding to PP2A[126]. In addition, a sequence similar to SH3 core motif was found on the two regions of AMBRA1 that were shown to mediate binding to PP2A catalytic subunit. AMBRA1 was also shown to contribute to the regulation of MYC S62 dephosphorylation by PP2A[129]

B-cell receptor associated protein α4, encoded by IGBP1 gene, was identified as a binding partner of PP2A based on the evolutionary conservation of previously reported interaction in yeast. The yeast homolog of α4, Tap42, plays a role in TOR signalling[130], and this function seems to be conserved also in mammals.[131] α4 binds directly to the PP2A C-subunit, however, there are conflicting reports on the biochemical properties of this dimer.The dimer has been reported to retain phoshataseactivity towards some targets, including Mid1 in an *in vivo* setting[92, 132]. In contrast, structural analysis suggests that the conformation of the C-subunit active site is perturbed upon α4 binding and the dimer is catalytically inactive. Ectopically expressed α4 has been reported to sequester the C-subunit resulting in PP2A inhibition, and it has been suggested that α4 functions as PP2A inhibitor[133] and this also the proposed mode of action in mTOR signalling pathway. Ectopic overexpression of α4 has also been shown to contribute to transformation[134]. However, this artificial overexpression may not be representative of the physiological functions of α4 and it seems more likely that α4 acts as a chaperone that stabilizes the incompletely folded C-subunit[135]. This physiological stabilization is likely to involve only a small fraction of the C-subunit, yet it seems to be essential for the assembly of active holoenzymes[97, 136]. It is possible that the catalytically inactive C-α4 dimer still possesses some functions. A catalytically inactive splice variant of C-subunit has been reported and this variant exhibits increased affinity towards α4[137]. A catalytic-activity-independent role also been proposed for the C-subunit in the recruitment of Condensin II to chromosomes[138].

2.2.4. Endogenous PP2A inhibitor proteins

An important group of PP2A interacting proteins are the endogenous inhibitor proteins that, like the subunits, are encoded by separate genes. The endogenous phosphatase inhibitors often function as downstream mediators of kinase pathways or in specific phases of the cell cycle to promote cell growth and may only regulate a subset of phosphatases targets[139]. Discovery of novel inhibitor proteins has strengthened this concept also for PP2A. To date, at least 14 structurally and functionally heterogeneous proteins have been labelled as inhibitors of PP2A based on their ability to interact with PP2A, and to inhibit dephosphorylation carried out by PP2A (Table 1). Many of these proteins have been reported to also carry out PP2A independent functions.

Table 1. Endogenous inhibitor proteins of PP2A

Inhibitor	Aliases	Mode of inhibition	References
SET	I2PP2A	Interacts with C-subunit	140, 141
ANP32A	I1PP2A		140
ANP32E	Cpd1		142
PME-1	PPME1	Demethylates C-subunit, removes MN^{2+} ions required for catalytic activity, and stabilizes the inactive conformation of C-subunit	143-145
CIP2A	KIAA1524	Binds to B56-family subunits sequestering them from the holoenzyme	146, Wang, Okkeri, et al. *In press*
TIP	TIPRL	Binds to C-subunit, mutations in Aα-subunit enhance interaction	147, 148
ARPP19		B55α/δ, in mitosis, activated by Greatwall kinase	149
ENSA		B55α/δ, in mitosis, activated by Greatwall kinase	150
BOD1		B56-family, in mitosis	151
TLX1	HOX11	Interacts with C-subunit	152, 153
PPP1R17	G-substrate		95, 154
IER3		B56-family, following ERK activation	155
SETBP1		PP2A inhibition requires binding to SET	156
HNRNPA2B1		PP2A inhibition enhanced by binding to SET	157

2.2.5. Regulation of PP2A activity by post-translational modifications

Although the B-subunits interact mainly with the A-subunit, the C-terminus of the catalytic subunit has an important role in the selection of B-subunits. Selectivity is achieved in part by post translational modifications, namely methylation of the C-terminal L309 and, possibly, phosphorylation of T304 and Y307. It has been suggested that methylation of the L309 is coupled to the PP2A holoenzyme assembly and catalytic subunit activation which may be a mechanism to limit the promiscuous activity of PP2A lacking regulatory subunits.[158]

2.2.5.1. Methylation of the catalytic subunit C-terminus

Methylation of the L309 has been reported to be required for B55-family subunit binding and to enhance the binding of some B56-family subunits[159, 160]. Methylation is catalysed by LCMT1 and it seems to require that the C-subunit is in active conformation[161]. The activation of the C-subunit is catalysed by PTPA that, at least in yeast, targets the AC-dimer and is dependent on the crosstalk with PME-1[78]. PME-1 is responsible for removing the L309 methylation but it also stabilizes the catalytic subunit in inactive conformation.[143-145] This process has been reported to evict the manganese ions required for catalytic activity from the C-subunit[143]. The model, in which interaction with PME-1 is required for PTPA function, implicates PME-1 also in the biogenesis of PP2A holoenzymes. However, in the context of cancer cell signalling, elevated expression of PME-1 results in PP2A inhibition and it correlates with cancer stage in glioblastoma and endometrial cancer[99, 162]. Furthermore, reduced methylation of the C-subunit also contributes to transformation[163, 164]

2.2.5.2. Phosphorylation of the C-subunit

PP2A is inhibited by phosphorylation of the catalytic subunit at Y307 by a wide range of tyrosine kinases. The phosphorylation was measured by radiolabeling and the Y307 was confirmed as the primary phosphorylation site *in vitro* by deletion mutants[165], although other tyrosine phosphorylations have also been reported in PhosphositePlus database[26]. The phosphorylation at Y307 inhibits the catalytic activity of the purified C subunit as well as AC-dimer[165]. In addition, targeted mutagenesis studies suggest that it is likely to prevent the binding of B56α, β, and ε subunits[159] and inhibit methylation, further restricting the holoenzyme formation[159, 160]. It may also enhance the interaction with SET[166], however, this finding is depdendent on quantification of Y307 phosphorylation

by an antibody that may no be specific for the phosphorylated form (Egon Ogris, personal communication)

Catalytic subunit is also phosphorylated at a threonine residue resulting in diminished activity towards the target protein MBP. Functional studies suggest T304 as the phosphorylated residue, and this is also the only phosphorylated threonine reported in PhosphositePlus database[26] Phosphomimetic T304D mutation specifically prevents B55-family subunit binding in human[159] and mouse cells[160] and abolishes essential PP2A functions in yeast cells[167]. PP2A C-subunit has been shown to be transiently phosphorylated at unspecified residue following a treatment of cells with TPA, an activator of Protein kinase C, a serine/threonine kinase. This phosphorylation coincided with a drop in phosphatase activity towards Histone H3[115]. Neither aspartate nor alanine mutant of T304 significantly affected the methylation efficiency of L309[159, 160].

2.2.5.3. Phoshoregulation of other subunits.

In addition to C-subunit, phosphorylation has been reported to occur on T268, S303, and S314 of the A-subunit. Phosphorylation of these sites reduces the interaction between A- and C-subunit resulting in lower PP2A activity towards multiple substrate proteins.[168]

Inhibitory phosphorylations have been identified on B55α S167[169] and on *Schizosaccharomyces pombe* B56 subunits[170]. Activating phosphorylations also occur on B56-family subunits[171, 172].

Lastly, phosphorylation activates or enhances the activity of several endogenous inhibitor proteins, including ARPP19[149], ENSA[150], Bod1[151], IER3[155], ANP32A[173], and SET[174]. A well characterized example is the phosphorylation of ARPP19 and ENSA by Greatwall kinase that specifically suppress PP2A complexes with B55α and B55δ. Inhibition of these phosphatase activities is essential for initiation and maintenance of mitosis[149, 150].

2.3. PP2A as a tumor suppressor

2.3.1. Experimental transformation of human cells

Interest in PP2A's tumor suppressor functions stems from two independent series of findings: 1) Carcinogenic properties of small molecule phosphatase inhibitors[175-177] and 2) transforming activity of SV40 virus. The experimental

transformation using SV40 early genes has become an important research tool for studying PP2A subunits, regulators, and targets, and these findings will be discussed in the chapter below.

When the transformation is defined as increased proliferation, gained ability to grow in anchorage-independent manner, and gained ability to form tumors in immunocompromised mice, a distinction arises between human and rodent cells; rodent cells can be transformed by expressing SV40 large t-antigen, whereas human cells require both large and small t-antigens[16]. SV40 small t-antigen interacts with two cellular proteins that were identified as the A- and C-subunits of PP2A[178]. The interaction with C-subunit was mediated by the A-subunit, and the interaction between A-subunit and small t-antigen was blocked by the B-subunit. Binding of small t-antigen to AC-dimer inhibited its activity towards a subset of target proteins[179], and mutations in the small t-antigen that interfere with AC-dimer binding and inhibition of its catalytic activity have been shown to be less efficient in transformation[180].

2.3.1.1. Minimum requirements for transformation

The transformation of human cells has proven to be quite difficult and has in many experiments relied, at least partly, on carcinogen-induced or spontaneously arising alterations. Expressing both large and small t-antigens of the SV40 virus readily confers the ability to grow in an anchorage independent manner to primary human fibroblasts isolated from foreskin, and this was shown to be dependent on small t-antigen's ability to inhibit PP2A[181]. However, without active telomerase, cells are significantly less efficient in forming colonies in soft agar and rapidly enter crisis. Therefore, Hahn and others have suggested that freshly isolated human epithelial cells and fibroblasts can be readily transformed by expression of SV40 large t-antigen together with telomerase and constitutively active HRAS, which enhances soft agar colony formation and is required for tumor formation in xenograft experiments[182]. Although not known at the time, the constructs used in these experiments also contained SV40 small t-antigen gene, and the PP2A inhibition by this small t-antigen was later shown to be essential component in this transformation model[183, 184]. The effect of large t-antigen has been attributed to inhibition of tumor suppresors TP53 and RB, however, other functions are likely to contribute[185]. Also the small t-antigen has other cellular targets, some of which interact with shared sequences of the small and large t-antigens[181]. Nevertheless this model has proven to be useful in many follow-up experiments

for identification of PP2A subunits and regulators that are responsible for the tumor suppressor functions of PP2A as well as downstream pathways whose activation they prevent.

2.3.1.2. PP2A subunits implicated in transformation

Structural analysis shows that small t-antigen binding to A-subunit is mutually exclusive with binding of B56-family subunits and also restricts the access to C-subunit catalytic site. However, compared to B56-subunits, small t-antigen has lower affinity towards A-subunit and it seems inefficient at replacing them in an *in vitro* setting[186]. This is in contrast to an earlier report showing that overexpression of small t-antigen in cells efficiently competed with B55α and to lesser extent with B56γ3 subunits that were also ectopically expressed at a similar level[187]. In addition, overexpression of B56γ3 but not B55α prevented the transforming activity of small t-antigen. Furthermore, downregulation of B56γ3 by shRNA was able to partially replace small t-antigen in transformation[187]. These findings support the view that small t-antigen competes with B-subunits rather than targets the functions of the AC-dimer. A potential resolution for the conflicting results from *in vitro* and *in vivo* experiments is the differential sensitivity of some B-subunits and small t-antigen to the catalytic subunit methylation. Small t-antigen binding is not affected by methylation[163], which is required for B55α binding and enchances B56-family and PR72/PR130 subunit binding[159, 160]. In line with these findings, downregulation of C-subunit methylation by PME-1 overexpression or LCMT-1 inhibition enhances the B56γ-shRNA mediated transformation[163].

In addition to B56γ, silencing of B56α, PR72/PR130[164], or B55β[188] B-subunits as well as A-subunit beta[91] have been shown to contribute to transformation. Moderate downregulation of the alpha isoforms of A- and C-subunits also contribute to transformation, however, efficient depletion of these proteins is detrimental to the cells[164]. In addition to these PP2A subunits, downregulation of PP2A activator PTPA[164], overexpression of PP2A inhibitor CIP2A[146], overexpression of α4 (which was associated with reduced catalytic activity of PP2A)[189] or treatment with small molecule phosphatase inhibitor okadaic acid[187] can partially replace small t-antigen in transformation.

2.3.1.3. Pathways activated by PP2A inhibition in transformation

Although expression of activated HRAS and inhibition of certain PP2A functions represents the minimum number of changes required for transformation of

immortalized cells, other components in the same signalling systems, acting either upstream or downstream of RAS and PP2A, can replace them in the HEK-TER transformation model. Furthermore, different cell types in humans have different requirements on which oncogenic pathways need to be activated by, or in combination with, PP2A inhibition to achieve transformation[155].

In human mammary epithelial cells (HMECs), PP2A inhibition by SV40 small t-antigen can be replaced by expressing a constitutively active form of PIK3CA or of its two downstream effectors, AKT and Rac1. Furthermore, functional PI3K signaling was shown to be required for small t-antigen mediated transformation in these cells.[190] Although these results demonstrated that activation of PI3K signaling is needed in combination with RAS for transformation, RAS itself also contributes to the activation PI3K signaling. Using RAS mutants deficient in RAF, RAL, or PI3K activation, Rangarajan et al. have shown that the ability of RAS to activate PI3K, in combination with small t-antigen, is needed for transformation of HMECs and HEKs, and it enhances the transformation of fibroblasts.[15]

MYC has been shown to cooperate with small t-antigen in the transformation of immortalized HMECs and fibroblasts. However, although the combination of MYC overexpression and small t-antigen promoted the ability to grow in anchorage independent manner, it did not support xenograft tumor formation[185, 190]. In the transformation of fallopian tube secretory epithelial cells, MYC also supported *in vivo* tumor growth, albeit less efficiently than activated RAS.[191] In the study by Wei at al., Myc did not cooperate with RAS in the anchorage independent growth of fibroblasts, however, it increased the colony size of the cells that also expressed small t-antigen[185]. In contrast, Yeh et al. have reported that while overexpression of wild type Myc did not contribute the transformation of HEK293 cells or fibroblasts, overexpression of MYC with the stabilizing T58A mutation fully replaced SV40 small t-antigen.[192] Disregarding the possibility that T58A mutations would alter MYC functions, Wei et al. found more synergy between MYC and PP2A inhibition whereas Yeh et al. found synergy between MYC and RAS activation. However, Sablina et al. also used T58A mutant in the transformation of HEK293 cells and observed no significant response in anchorage independent growth and no *in vivo* tumor formation, even though both of these properties were conferred by small t-antigen in the same study[164]. Instead, the T58A mutant required the cooperation of activated AKT and β-catenin to partially replicate small t-antigens effect. Accordingly, depletion of endogenous MYC, AKT or β-catenin attenuated small t-antigen mediated transformation[164]. One factor that may

contribute to the differences in the MYC mediated transformation is the passage number of the cells; late passage HMECs have been reported to have higher expression of endogenous MYC, assumed to be caused by random alterations acquired during the cell culture[190].

The role of different PP2A subunits, as well as PTPA and CIP2A, in transformation has been attributed to their ability to regulate MYC, Akt, and β-catenin pathways.[146, 164, 188, 193] The beta isoform of the scaffolding subunit is an exception. Although a loss of function can partially replace SV40 small t-antigen in transformation, unlike the alpha isoform, it does not bind to small t-antigen. Instead, RalA protein is a key target for Aβ-containing PP2A and required for transformation caused by Aβ loss functions[91]

In addition, regulation of CDK2 by PP2A may have a minor transformation suppressing effect. The evidence supporting this is the cooperation between Cyclin E, a cofactor of CDK2, and small t-antigen induction of proliferation in density arrested fibroblasts,[194] and ability of Cyclin dependent kinase inhibitor 1B/p27 to replace small t-antigen in anchorage independent growth[195].

In conclusion, these experiments collectively suggest that inhibition of PP2A contributes to transformation via multiple pathways, including MYC and PI3K/Akt. Furthermore, RAS activation and PP2A inhibition cooperate in the regulation of these pathways, however, there are cell type specific differences in their relative contributions to pathway activation.

2.3.1.4. Limitations of the transformation model

Most of the transformation experiments studying PP2A have utilized large t-antigen, which inhibits p53 and Rb-proteins and enables the cells to bypass oncogene-induced senescense.[15] PP2A is implicated in regulation of both of these proteins, as well as senescence, but this side of PP2A's tumor suppressor functions has likely been masked by the effect of large t-antigen in the transformation experiments. Accordingly, it has been shown that although PP2A inhibition is not required for transformation of mouse cells, small t-antigen promotes transformation if the concentration of large t-antigen is limiting.[196]

Another limitation is the translatability of the results to real cancers. Perhaps owing to its ability to regulate multiple oncogenic pathways simultaneously, PP2A has been identified as one of the minimum requirements for transformation. However, as will be discussed in more detail below, the cancer

genomics data suggests that other activating mutations are far more common in these pathways and they are also distributed independently of PP2A inhibiting alterations..

2.3.2. Regulation of p53 and senescence by PP2A

One of the mechanisms by which p53 promotes senescence is a p21 mediated inhibition of CDK2. Phosphorylation of Rb by CDK2 inhibits its interaction with cell cycle associated transcription factor E2F1.[197] Activation of p53-p21 axis also downregulates E2F1 and CIP2A protein levels in breast cancer cells, while inhibition results in their overexpression[198]. Thus one of the p53 functions seems to be to promote another key tumor suppressor, PP2A. However, this regulation also goes to the other direction: PP2A-B56δ removes an inhibitory T55 phosphorylation on p53, resulting in induction of p21 expression[199]. The overexpression of E2F1 and CIP2A following p53-p21 inhibition is reinforced by positive feedback loop, and this feedback was associated with resistance to senescence induction. Mechanistically, E2F1 directly enhances CIP2A transcription and CIP2A prevents E2F1 dephosphorylation by the PP2A-B55α complex[198].

Interestingly, although MYC activation can induce senescence, suppression of MYC has been associated with senescence induction in multiple different cancer cell lines[200]. In melanoma cells, MYC is highly expressed compared to benign nevi and its expression has been associated with the evasion of RAS-MAPK pathway activation associated senescence induction that is prominent in benign nevi but not metastatic melanoma.[201] The regulation of MYC and senescence sensitivity in this setting has been associated with B56α-mediated dephosphorylation of MYC S62 and positive feedback loop between CIP2A and MYC[202]. The MYC-mediated senescence tolerance has also been associated with phosphorylation of S62 by CDK2[203], and CDK2 has been shown to protect mouse embryonic fibroblasts from MYC induced senescence[204]. These two mechanisms of senescence tolerance are probably connected; PP2A inhibition may also contribute to the CDK2 activation[194], and the feedback loop between CIP2A and E2F1 has been reported to involve MYC[205].

Another indication of the overlapping tumor suppressor functions of PP2A and p53 is provided by a knock-in mouse model of cancer derived E64D mutation of PPP2R1A. This mutation increases lung cancer incidence but completely loses its effect on dominant negative p53 background.[85] In addition, some cancer

derived mutations in PPP2R5C gene have been shown to promote cancer cell proliferation only in p53 expressing cells[199].

PPP2R1A and p53 mutations exhibit a tendency towards co-occurrence in endometrial cancer and both are likely to be early events in endometrial cancer[206]. Furthermore, this relationship co-occurrence was mainly caused by the hotspot mutations of PPP2R1A. Both PPP2R1A and p53 mutations are associated with serous type endometrial cancer[207], and the tendency towards co-occurrence can also be seen in the previously published data on this subtype (Figure 4). These findings suggest that, similarly to the SV40-mediated transformation, PP2A and p53 inhibition cooperate also in endometrial cancer. These findings may also indicate increased overlap of their tumor suppressor functions compared to mice, which may be an adaption to safeguard against cancer in humans[8]. However, this co-occurrence may also reflect the ability of cells to tolerate PPP2R1A mutations that have been associated with whole genome duplications[72] and will be discussed in more detail together with the PPP2R2A deletions later in this review.

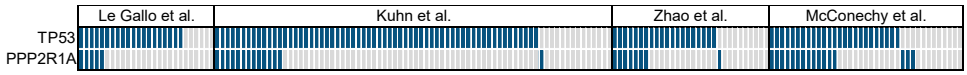


Figure 4. Co-occurrence of p53 and PPP2R1A mutations in serous endometrial cancer. The data were extracted from 4 independent data sets[208-211]. Fisher's exact test p-value for the co-occurrence is 0.0238

2.3.3. Cell cycle regulation

Related to senescence regulation, transformation studies have also hinted at a role for PP2A in cell cycle regulation via the Rb-family pocket proteins. Again, separate inhibition of Rb in these experiments may have masked some of PP2A's effects. Rb-family pocket proteins, Rb, p130, and p107, are negative regulators of the cell cycle, and PP2A has been shown to remove inhibitory phosphorylations on these proteins to enable cell cycle stop at G1.[212] However, another distinct PP2A-mediated mechanism has also been described by Naetar et al.[213] They have shown that transient inhibition of PP2A during G2 shortens the following G1 phase and denies the entry to G0 by indirect deregulation of pocket proteins. This was attributed to MYC mediated overexpression of Cyclin E, a cofactor of CDK2, in mitosis. Accordingly, ectopic MYC expression was shown to be sufficient for overriding the G0 stop, and the process was also dependent on RAS.

2.3.4. PP2A in mitosis

In addition to pocket protein and G0 regulation, PP2A functions as an antagonist to the key mitotic kinases and it is required for multiple critical processes, the deregulation of which may lead into chromosomal segregation errors or DNA damage[214]. Many of these functions are coregulated by PP1, characterized by feedback loops, and dependent on controlled sequential activations of these phosphatases and their kinase antagonists. A simplified overview of PP2A's involvement is presented below.

A global upregulation of protein phosphorylation occurs in mitosis[215]. A recent study by Grallert et al.[170] on fission yeast *Schizosaccharomyces pombe* suggests that the bulk activities of PP2A-B55, PP2A-B56 and PP1 are regulated by inhibitory phosphorylations in mitosis. Reactivation of the phosphatases in late mitosis was reported to happen sequentially, starting with autodephosphorylation of PP1, which activates PP2A-B55 that then mediate the dephosphorylation of B56 subunits, the activation of which occurs with a delay due to opposing Plk1 activity.

However, the phosphorylation site on B55-subunits was not identified and it has been suggested that this regulation may also involve the mitotic inhibitor proteins of PP2A-B55[216]. Normal mitotic entry and progression requires the phosphorylation of mitotic targets of Cdk1, and this is enabled by Inhibition of PP2A[217]. PP2A is inhibited by Greatwall kinase that phosphorylates the endosulfines Arpp19 and ENSA that function as specific inhibitors of PP2A-B55δ[149, 150]. In mammalian cells, the major B-subunit in PP2A complexes opposing Cdk1 is the related B55α[169], and both B55α and B55δ are reactivated upon, and coregulate, the mitotic exit[108].

In early mitosis, Plk1 phosphorylates cohesin complexes holding the sister chromatids together, causing them to dissociate. Shugoshin-associated PP2A-B56 opposes this phosphorylation at centromeres until proper kinetochore-microtubule attachment is achieved[218, 219]. Depletion of PP2A A-subunits was shown to interfere with the centromeric cohesion of sister chromatids[218]. Centromeric PP2A-B56 relocates towards kinetochores and participates in the stabilization of kinetochore-microtubule attachments by dephosphorylating Plk1 and Aurora B targets. Codepletion of B56-family subunits was shown to induce frequent chromosome misalignments in metaphase, suggesting that impaired PP2A-B56 function may result in whole chromosome gains and losses in cancer[220]. In addition to dephosphorylating their targets, PP2A-B56 is also

implicated in regulation of localization of Plk1 and Aurora B[220, 221]. PP2A-B56 localization to kinetochores is mediated by binding to BubR1 and this interaction, in turn, is mediated by phosphorylation of Plk1 target site on BubR1. Accordingly, BubR1 has also been shown to stabilize the kinetochore-microtubule attachments by controlling Aurora B activity via PP2A-B56.[222, 223] PP2A-B56 functions at centromere and kinetochore are controlled by another inhibitor protein, Bod1, which shares sequence similarity with the endosulfines. Bod1 was shown to be required for sister chromatid cohesion and proper localization of Plk1[151]. Once all kinetochore-microtubule attachments have been established, spindle assembly checkpoint signalling is silenced. PP2A-B56 complexes initiate these signalling events by antagonizing Aurora B.[224] Completion of spindle assembly checkpoint initiates anaphase and chromosome segregation. Following segregation, chromosomes are decondensed and the nuclear envelope is reassembled. These events are triggered by dephosphorylation gradient generated by PP1 and PP2A phosphatases following spatial separation of chromosomes from Aurora B at the midzone.[21] Grallert et al. have propose that the above mentioned mitotic functions, even though critically important for proper chromosomal segregation, represent strictly localized transient PP2A activations during the global inhibition of these phosphatases.[170]

As will be discussed in more detail later in this review, the most frequent genomic alterations of PP2A subunits in cancer have been associated with a potential mutator phenotype. Furthermore, these alterations involve loss of the B55α-subunit or impaired interaction between Aα-subunit and B55/B56 subunits, suggesting that the ability to progress through mitosis in an orderly fashion is at the core of PP2A tumor suppressor function.

2.3.5. Deregulation of MYC in cancer

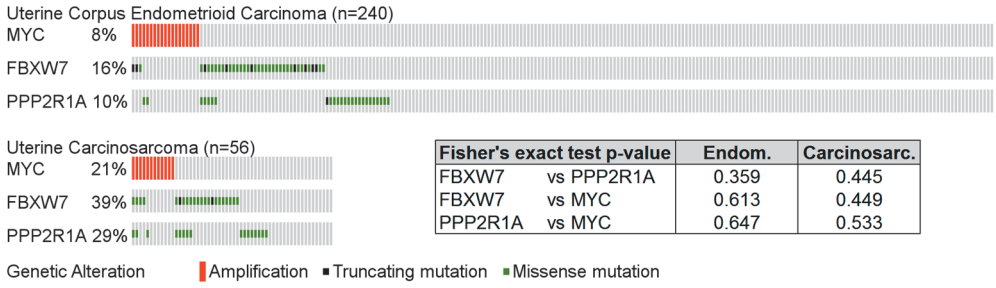
MYC is one of the most commonly amplified genes in human cancers[72]. In Burkitt's lymphoma, MYC is frequently activated by translocations to immunoglobulin heavy chain locus that place it under the control of an active enhancer[225, 226]. In cervical cancer, the most common integration site of HPV genome elements is in the vicinity of MYC. This integration has been reported to occur in 9,7% of HPV positive cervical carcinomas and often involves HPV promoter sequences that increase MYC expression.[227] Furthermore, the 8q24 chromosomal region upstream of MYC contains multiple independent cancer risk loci, including the SNP rs6983267, which has been associated with higher

cancer burden than any other variant in humans.[228] This SNP determines the binding of TCF7L2 transcription factor downstream of Wnt-signaling, and the region containing the SNP functions as an enhancer for MYC expression in colorectal cancer.[229, 230] Although this 1Mb region upstream of MYC is void of any genes, it codes multiple long non-coding RNAs (lncRNA) that regulate MYC expression[231]. One of these lncRNAs, CCAT2, overlaps with the rs6983267 and may engage in positive feedback loop with Wnt-signaling. The risk allele at rs6983267 was shown to increase the expression of CCAT2.[232] While the effect of this SNP on MYC expression is modest[229, 230], deletion of this enhancer region in mouse ablates the oncogenic potential of MYC[233].

In contrast, MYC mutations are rare in cancer. The stabilizing T58A mutations have become a research tool commonly used in transformation experiments based on the mutations found in viral MYC oncogenes and in endogenous MYC in Burkitt's lymphoma.[234] Recurrent T58I substitutions in Burkitt's lymphoma have also been observed[235]. However, MYC codon 58 mutations are exceedingly rare in other cancers; the COSMIC database v77 (cancer.sanger.ac.uk)[70] only lists 3 instances of T58A and one T58I mutation. Some clustering of mutations has been observed in the region surrounding T58 and S62, and the combined rate of these mutations seems to be fairly high in Burkitt's lymphoma[235]. These mutations are likely to affect the ubiquitinylation by Fbxw7, however, some of them have additional effects that are likely contribute to their selection[236]. Clustering of mutations at lower frequency has also been observed in another phosphodegron motif, located between residues 244-248, in lymphomas. Some of these phenocopy also the degradation- independent effects of the mutations at residues 58-62[237].

While the MYC stabilizing mutations are rare, MYC stabilization is not.[238] Following proliferative stimulus, MYC stabilization lasts longer than transcriptional upregulation[239], and constitutive stabilization occurs in cancers. For example, MYC overexpression in melanoma compared to benign nevi has been attributed to the increased stability[202]. Proteolytic regulation of MYC is complex, and several components of this regulatory machinery have also been shown to be differentially expressed in cancers[240]. However, while the differential expression of the multiple regulators may contribute to MYC stability, the contribution of any one component is usually a fraction of total stabilization[240], and it is difficult to identify all the MYC stabilizing alterations that have been selected in these cancer cells.

One of the ubiquitin ligases associated with MYC stability regulation, Fbxw7, is frequently mutated in human cancers.[241] The Fbxw7 mutations have also been associated with MYC stabilization in some cancers[242]. In addition, PP2A deregulation is moderately frequent in some cancers and will be discussed in more detail later in this review. However, while both Fbxw7 and PPP2R1A mutations are common in certain endometrial cancer subtypes, they seem to be distributed independently of MYC amplifications and each other (Figure 5), suggesting that factors other than MYC regulation contribute to the selection of these mutations.



Fisher's exact test p-value		Endom.	Carcinosarc.
FBXW7	vs PPP2R1A	0.359	0.445
FBXW7	vs MYC	0.613	0.449
PPP2R1A	vs MYC	0.647	0.533

Figure 5. Occurrence of MYC amplifications, FBXW7 mutations, and PPP2R1A mutations in Uterine Corpus Endometrioid Carcinoma and Uterine Carcinosarcoma. The data sets were generated by TCGA research network http://cancergenome.nih.gov/. The data and the statistical calculations were retrieved from cBioportal[243, 244].

In summary, these findings invariably suggest that the primary mechanism by which the thus far characterized genomic events increase MYC expression is transcriptional. The mutations affecting ubiquitinylation mediated degradation are a contributing factor in certain lymphomas, but rare in solid tumors. However, the selection of genomic events targeting Fbxw7, PP2A, and other components of ubiquitinylation machinery is likely also influenced by MYC-independent functions of these proteins.

2.3.5.1. Regulation of MYC stability by PP2A

Regulation of MYC by PP2A is often attributed to dephosphorylation of S62 and the resulting destabilization of MYC[192]. In this model, phosphorylation of T58 is a priming event for S62 dephosphorylation that leads to ubiquitinylation by Fbxw7. This has also been proposed as the mechanism by which T58A mutation stabilizes MYC. However, the stabilizing effect of this mutations exceeds that of the whole phosphodegron deletion, as well as the that resulting from Fbw7 depletion, suggesting that other mechanisms besides degradation are mediated

by this region of MYC.[240] Despite the transforming potential, the T58A mutation also has a qualitatively different effects to MYC overexpression, as demonstrated by impaired activation of reporter gene expression[245]. However, there seems to be some selectivity in how this mutation affects MYC targets. Interestingly, the T58A mutation enables the evasion of p53 mediated apoptosis, and the significance of this finding is further supported by reciprocal distribution of certain MYC mutations with p53 mutations.[236]

The role of S62 dephosphorylation in MYC degradation has been also questioned[240], and like T58, its function is probably not limited to stability regulation[203]. In addition, it may promote certain MYC functions by regulating the subnuclear partitioning[239], which may affect stability indirectly[240]. Furthermore, multiple other phosphatases may contribute to the dephosphorylation of S62, and one of them, Scp1, seems to dephosphorylate the S62 of both T58A and wild type MYC.[246]

Although PP2A has been shown to regulate MYC stability and S62 phosphorylation[192], the regulation of MYC stability by PP2A should not be equated to S62 dephosphorylation alone. In addition to S62 dephoshorylation, PP2A negatively regulates the upstream kinases, including ERK[173]. In addition, the tumor suppressor function of PP2A B55β-subunit has been attributed to regulation of S62 via PDK1-Pim1 axis[188, 247]. PP2A also removes the inhibitory S9 phosphorylation on GSK3β, which, in addition to T58 phosphorylation[248], inhibits β-catenin, which has been linked to transcriptional regulation of MYC by multiple mechanisms.[230, 249] Although the T58/S62 containing degron regulated by Fbw7/USP28 is arguably an important one, there are multiple other ubiquitinylation sites, as well as ubiquitinylation independent stability regulating elements on MYC. Furthermore, a number of phosphorylation sites have been associated with MYC stability[250, 251], and it has been suggested that the regulation of MYC stability by PP2A may not be limited to the T58/S62 phosphodegron motif.[240]

Lastly, even though there is a wealth of correlative data on MYC protein expression and PP2A inhibition, the causality may also be the other way round. A positive feedback loop between MYC and PP2A inhibitor CIP2A has been reported by different research groups in gastric cancer[252], melanoma[202], and CML cells[205]. The significance of the feedback loop between MYC and CIP2A is further supported by coregulated gene expression[146, 253] and shared physiological functions.[239] In addition, MYC also promotes the transcription of

other PP2A inhibitor proteins, including SET and HNRNPA2B1[254, 255]. Importantly, inhibition of SET by small molecules or depletion of either SET or CIP2A results in MYC downregulation and suppression, and these treatments are generally poorly tolerated by cancer cells[146, 252, 253, 256-258]. Furthermore, the response to these treatments has been shown to depend on their ability to downregulate MYC[259]. Interestingly, CIP2A seems to contribute only to a subset of MYC functions, and unlike MYC depletion, CIP2A depletion is well tolerated by normal cells[239, 260]. These findings suggest that although PP2A deregulation may not be the genetic driver alteration behind MYC overexpression, some MYC-driven cancers exhibit a non-oncogene-addiction type relationship with low PP2A activity due to its role in MYC stabilization and transactivation.

2.3.6. Regulation of PI3K/AKT/mTOR signalling

PP2A negatively regulates AKT by participating in the dephosphorylation of S473 and T308[171, 261]. PP2A also dephosphorylates the inhibitory S9 phosphorylation on GSK3β, which is a target of AKT[248, 262]. PP2A and mTOR have been shown to regulate each other by diverse mechanisms. mTOR inhibits PP2A by phosphorylating the C-subunit at an unspecified residue and PP2A has been implicated in the dephosphorylation of the canonical targets of mTOR, p70S6K and 4E-BP1[263]. Peterson and others have suggested that this inhibition of PP2A may actually be the primary mode of mTOR's action towards these substrates.[263] A similar PP2A-mediated mechanism has been described for regulation of IRS-1 by mTOR[264], and regulation of the S170 autophosphorylation site on MAP4K3[265]. Regulation of PP2A by mTOR may also involve Alpha4 and the endogenous inhibitors of PP2A, namely CIP2A and TIPRL[131, 266, 267]. PP2A in turn can regulate mTOR via AKT as well as via feedback loop involving MAP4K3[265].

Both PIK3CA and PPP2R1A mutations can promote transformation in place of small t-antigen[190, 193]. Rare B56-family subunit mutations have also been reported in human overgrowth syndromes that are associated with PI3K/AKT pathway mutations.[268] The recurrent PPP2R1A mutations in endometrial cancer have been shown to interfere with dephosphorylation of established PP2A targets in AKT/mTOR signalling[148]. Furthermore, B55α, the B-subunit associated with AKT T308 phosphorylation[269], is recurrently deleted in human cancers[72]. However, PPP2R1A mutations seem to be distributed independently of PI3K/AKT pathway mutations[206]. Although there is apparent mutual exclusivity between PTEN and PPP2R1A mutations in endometrial cancer, this is a result

from their association with endometrioid and serous type endometrial cancers, respectively. The mutual exclusivity is not observed in subtype-specific analysis.[209] PIK3CA activating mutations are among the most frequent alterations in human cancers (Fig. 2), and PTEN mutations are also far more frequent than PP2A subunit mutations[241]. Although PPP2R1A mutations may contribute to PI3K/AKT pathway activation in cancer, the selective advantage provided by these mutations seems to differ from the more frequent activating mutations on this pathway.

2.3.7. Mutual antagonism of PP2A and BCR-ABL fusion protein in CML

90% of chronic myeloid leukemia cases harbour the BCR-ABL fusion gene, which has been shown to exert its effects in part by SET-mediated inhibition of PP2A[270]. This inhibition requires interaction between BCR-ABL and JAK2, and is independent of ABL kinase activity[271]. In BCR-ABL expressing cells, PP2A reactivation suppresses the downstream signalling of BCR-ABL as well as limits the proliferation and *in vivo* maliganant potential of these cells. PP2A was also shown to promote BCR-ABL degradation in SHP-1 dependent manner, suggesting that downregulation of PP2A activity is a key mechanism for sustained BCR-ABL signaling[270]. Although BCR-ABL was shown to upregulate SET via JAK2, the constitutively active JAK2 mutation in myeloproliferative diseases has been associated with SET activation by PKC-mediated phosphorylation at S9, although direct inhibition of PP2A by JAK2 mediated phosphorylation of the C-subunit Y307 may also have contributed to the reduction of PP2A activity.[174] Results from several preclinical studies suggest that PP2A activating drugs, including FTY720 and OP449 that inhibit the SET-PP2A interaction, possess therapeutic potential in CML. Although these drugs are likely to have other cellular targets, such as the sphingosine receptors for FTY720, rescue of the cytotoxic effect by okadaic acid treatment suggested a mainly PP2A mediated mechanism. Particularly, PP2A reactivation may enhance the effect of BCR-ABL inhibitors CML, and overcome resistance.[271-273]

BCR-ABL also promotes CIP2A expression in kinase activity dependent manner. High CIP2A expression at diagnosis, and failure to downregulate CIP2A by Imatinib, were associated with disease progression[256]. However, 2nd generation TKIs targeting BCR-ABL were also efficient in treatment of patients with high CIP2A expression at diagnosis, which was attributed to the ability to prevent the formation of a positive feedback loop between E2F1,CIP2A, and MYC by an unknown mechanism[205]. RNAi mediated silencing of CIP2A has also been

associated with reduced proliferation, increased apoptosis and suppression of BCR-ABL[205, 274].

Interestingly, recurrent SETBP1 stabilizing mutations have been reported in the rare atypical CML, which lacks the characteristic BCR-ABL fusion gene. A recurrent atypical CML-derived SETBP1 stabilizing mutation, G870S, was shown to contribute to PP2A inhibition[275]. While other oncogenic properties of SETBP1 may contribute to the selection of mutant cells[276], reciprocal occurrence of these mutations and BCR-ABL fusions suggests that, for CML cells, PP2A activity may generate a selective pressure that needs to be overcome by a specific adaptation.

2.4. PP2A inhibition in human cancers

2.4.1. Contribution of PP2A inhibition by tumor inducing viruses to human cancers

PP2A subunits are targets for many viral proteins, and some of these viruses have been causally linked to human cancers[277] However, despite the requirement for PP2A inhibition in SV40-mediated *in vitro* –transformation, there is no conclusive evidence to establish the causal link between PP2A inhibition and naturally occurring human cancers.

SV40 was discovered as a contaminant of polio vaccines which led millions of people to be exposed to the virus in U.S. alone in 1950's [278]. Soon thereafter, SV40 virus transforming activity in human tissue cultures [279] and the ability to induce tumors in rodents[280, 281] were discovered. The potential connection between SV40 and human cancers has subsequently been studied extensively, and results have been controversial. However, it seems likely that faulty PCR methods and cross reactivity of antibodies targeting other polyoma viruses have produced false positive findings. This, together with the lack of epidemiological evidence, suggest that, despite the widespread exposure, SV40 has an insiginifcant, if any, contribution to human cancers[282, 283].

In addition to SV40, other polyoma virus genomes contain genes for small and middle t-antigen that interact with PP2A. One of them, Merkel cell polyoma virus (MCV), has been established as causative agent of a rare human cancer, Merkel cell carcinoma[284]. However, although MCV small t-antigen interacts with PP2A, this interaction is dispensable for transforming activity of the MCV small

t-antigen, which has been linked to activation of S6 and 4E-BP1 signaling[285]. The PP2A interaction motif has been mapped to the opposite surface of the MCV small t-antigen as the transforming activity, and compared to SV40 small t-antigen it replaced a limited number of B-subunits[286]. JCV and BKV, also members of polyomaviridae, possess genes for PP2A interacting t-antigens.[287, 288] However, these proteins have not been characterized in the context of transformation and it is not known whether they behave more like the MCV or SV40 small t-antigen. While these viruses are able to induce tumors in rodents, the connection to human cancers is uncertain, and cancer induction would likely require immunocompromised condition.[289]

Kaposi's sarcoma associated herpes virus (KSHV) is the causative agent of Kaposi's sarcoma[290] and associated with certain lymphomas[291]. KSHV protein LANA has been shown to bind B56- and possibly B55-family subunits of PP2A [292]. However, the significance of this binding is not clear. LANA interacts with other cellular targets including Rb and p53, and a number of pathways are also dysregulated by other KSHV proteins[293].

Human papillomaviruses are associated with by far the highest cancer burden of all cancer promoting viruses[294, 295]. Human papillomavirus virus 16 E7 oncoprotein has been shown to activate AKT signalling via PP2A mediated mechanism[296]. It can also partially replace small t-antigen in the HEK-TER transformation model, however, this second study by White et al. was unable to find any evidence for the interaction with PP2A[297]. UBR4 was identified as a potential binding partner mediating a subset of E7 functions. In some instances, the effect of E7 resembled SV40 small t-antigen and in others the phenotype was similar to murine polyoma virus small t-antigen, which lacks the transforming activity in this model, but still exerts part of its functions via PP2A. Considering that SV40 small t-antigen also induces AKT phosphorylation[298] and activated variant AKT's upstream regulator, PIK3CA, can even replace small t-antigen in the transformation of human mammary epithelial cells[190], it seems that these viral proteins have some convergent functions but there probably are differences in their cellular interaction partners.

In summary, out of the viral proteins interacting with PP2A, only the transformation activity of SV40 small t-antigen has been linked to PP2A. However, SV40 is unlikely to contribute to human cancers. In contrast, MCV, KSHV, and HPV16 have been linked to cancer but it is uncertain at best, that their cancer promoting functions would depend on PP2A inhibition.

2.4.2. Review of the cancer genomics literature on PP2A subunit inactivation in human cancers

Cancer associated genetic and epigenetic alterations rarely affect the catalytic subunits of PP2A. Majority of the alterations occur on the genes encoding the A-subunits and certain B-subunits of PP2A, and only impair a subset of PP2A's functions. Alterations in any one subunit are relatively rare (Figure 6); most of the PP2A subunit genes do not have obvious driver mutations, and most copy number alterations of PP2A subunit genes are likely to be "passenger" alterations that have been co-amplified or deleted with other genes located in same chromosomal location. The two alterations with strong evidence from large scale cancer genomics studies are recurrent point mutations in PPP2R1A gene[241, 299, 300] and homozygous deletions of PPP2R2A gene[72, 301].

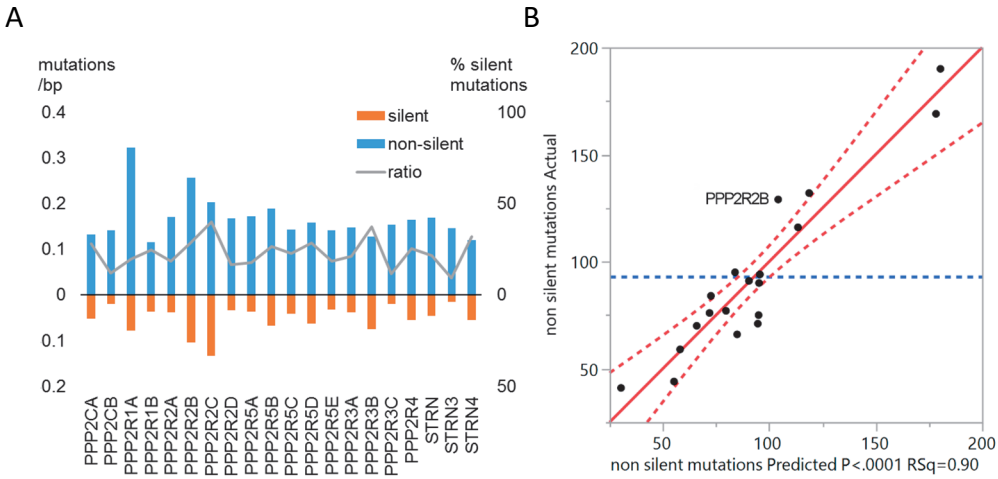


Figure 6. Frequency of PP2A subunit mutations in human cancers. A. frequency of mutations per base pair in COSMIC v77 data (cancer.sanger.ac.uk)[70]. Ratio of silent to non-silent mutations is represented with line (right axis) B. Multivariate analysis of PP2A subunit mutation frequency including gene length, replication time, chromosomal conformation (HiC index), and gene expression as covariates based on the analysis by Lawrence et al[302]. This analysis attempts to model the background mutation frequency more accurately and it is able to explain 90% of the variation in mutation frequency between PP2A subunit genes. PPP2R2B has a moderately high silent mutation rate (A), however, in this multivariate analysis it also exhibited the largest deviation from expected mutation rate, suggesting that some PPP2R2B mutations may provide a selective advantage to the cancer cells.

However, functional studies have linked also non-recurrent mutations in multiple subunits to impaired trimer assembly, failure to dephosphorylate specific targets, increased cancer cell proliferation, and transformation[91, 199, 303,

[304]. Furthermore, as discussed in more detail below, different subunits are inactivated by different mechanisms: PPP2R1A has recurrent point mutations, PPP2R2A is commonly deleted, PPP2R2B is inactivated by promoter methylation, and PPP2R1B mRNA frequently undergoes aberrant splicing in cancers. Studies focusing on one type of alterations are, therefore, likely to underestimate the combined frequency of genetic and epigenetic PP2A dysregulation in cancers.

2.4.2.1. PPP2R1A (Scaffolding subunit Aα)

PPP2R1A is the most commonly mutated PP2A subunit coding gene and has been identified as a likely cancer gene in multiple large scale cancer genomics studies[241, 299, 300]. Recurrent mutations have been reported to occur at low frequency in ovarian cancer[305-308] and endometrial cancer, where they associate strongly with serous type cancer (15-40% of the patients)[208, 210, 211, 306, 308], carcinosarcoma (9/42, 21.4%)[209], and possibly with clear cell carcinoma[309] subtypes. Rare, but recurrent, PPP2R1A mutations are also found in other cancers, such as vulvar squamous cell carcinoma[310] and colorectal cancer (Figure 7B).

Recurrent cancer-associated mutations of PPP2R1A are concentrated on two hotspots of mutations in exons 5 and 6 (Figure 7). This type of distribution is more common for gain-of-function mutations, which has led has led some researchers to suggest an oncogenic role for PPP2R1A[299, 305]. However, it is more likely that these mutations inhibit a subset of PP2A functions by interfering with the trimer assembly.[303, 304, 311]

Interestingly, a similar pattern of hotspot mutations is found in a related phosphatase, PP6. Recurrent mutations have been reported in the catalytic subunit gene PPP6C in melanoma[312, 313] and basal cell carcinoma[314]. Some of these mutations disrupt the catalytic activity of the C-subunit, but a subset of these mutations interferes with the interaction between catalytic subunit and SAPS-family regulatory subunits, that share homology with the PP2A B56-family B-subunits[315, 316]

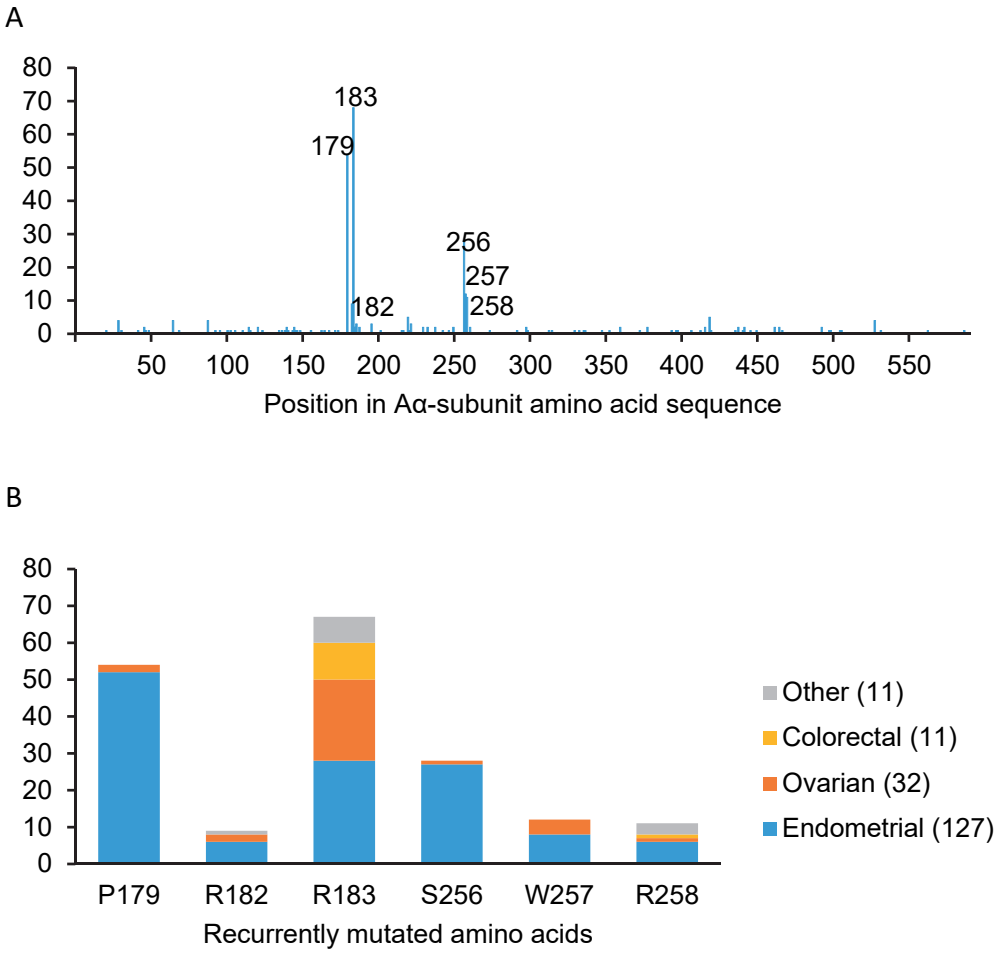


Figure 7. Clustering of missense mutations in PPP2R1A gene A) Missense mutations of PPP2R1A reported in COSMIC v77 (cancer.sanger.ac.uk)[70]. The mutations in codon 419, curated from a report by Martin et al.[317], were excluded as likely artefacts. These mutations had suspiciously high rate (73%) in a panel of 22 head and neck squamous cell carcinoma cell lines, were not reported in the original article by Martin et al., and are not corroborated by larger studies on the same cancer type[318]. B) Distribution of recurrent missense mutations in PPP2R1A by cancer type

Three recurrent mutations, P179L, R182W, and R258H, have also been observed as *de novo*-mutations in intellectual disability patients[311]. Another study found P179L and R182W *de novo*-mutations in undiagnosed severe developmental disability patients[319]. These mutations are located in the regions that are thought to mediate B-subunit binding.[304] All three mutations retain binding to B56δ, and P179L is also able to bind PR72. Binding to other B56-family members was reduced and binding to B55-family members was lost[311]. In contrast, an

experimental P179A mutant retains some B55α-binding capability whilst impairing B56γ binding[320]. Although the P179L, R182W, and R258H mutations don't map to the C-subunit interaction surface, all of them interfere with C-subunit binding. Some trimer assembly still occurs, however, P179L and R182W mutants also reduce the catalytic activity of the trimer.[321] All six recurrent mutations are found in endometrial cancer, P179 being the most frequently mutated amino acid. In contrast, ovarian and colorectal cancer mutations are predominantly found in codon for R183, suggesting that these mutations may result in different phenotype (Figure 7B). A recent study by Haesen et al. has characterized 10 different substitutions to these 6 amino acids that recurrently occur in endometrial cancer[148]. These mutants resulted in lost binding to B55α/β subunits, reduced binding to C-subunits, and exhibited varied effects towards B56- and PR72-family subunits. All mutations retained B56δ binding, and most resulted in increased binding to STRN-family subunits, possibly because these subunits are less dependent on C-subunit interaction. However, Haesen et al. have suggested that these mutations act as gain of function mutations via increased binding to PP2A inhibitor TIPRL, resulting substrate trapping complexes.[148] Interestingly, the codon 183 mutations seemed to produce most potent deregulation of AKT and mTOR pathways, as well as highest increase in anchorage independent and xenograft tumor growth, which may explain why they are found in other cancers as well.

In addition to hotspot mutations, several other mutations cluster to the B-subunit interface and may impair binding of specific subunits[303, 304]. A well-studied example of this is the E64D mutation, which has been shown to impair B56-family subunit binding[322] and contribute to transformation[193]. In a knock-in mouse model this mutation increased benzopyrene induced lung cancer incidence[85] and KRAS G12D driven lung cancer progression[304]

2.4.2.2. PPP2R1B (Scaffolding subunit Aβ)

PPP2R1B mutations have been reported to occur at moderate frequency in some cancers, however, current large scale sequencing data suggests that the incidence of PPP2R1B somatic mutations in cancers is very low (Figure 6). The early estimates were based on small data sets, did not consider background mutation frequency, and were inflated in part by inclusion of a single nucleotide variant resulting in G90D substitution, which is a low frequency germ line polymorphism[323, 324]. Intrestingly, however, a report by Esplin et al.[325] suggests, that this variant has reduced binding to B56γ and is associated with elevated

breast cancer risk. Similarly, some cancer-derived mutations of PPP2R1B have been shown to interfere with C- or B-subunit binding and to promote transformation[91, 326, 327].

PPP2R1B is located on 11q23 chromosomal region that frequently undergoes LOH in many solid tumors and hematological malignancies. Although inhibition of PPP2R1B contributes to transformation, loss of function of both PPP2R1B alleles is likely to be required for the malignant phenotype[91]. Homozygous alterations are rare and other tumor suppressor in this chromosomal location are likely to contribute to the selection of these copy number losses[328-330].

However, in addition to mutations and copy number alterations, epigenetic and post-transcriptional mechanisms may contribute to PPP2R1B inactivation together with LOH. Methylation of the remaining allele has been reported to inactivate PPP2R1B in B-cell lymphomas, albeit rarely[331]. Several studies have also reported cancer associated aberrant splicing of PPP2R1B mRNA. Aberrant splicing variants, that were not present in the corresponding normal tissue, have been detected in hepatocellular carcinoma, and these splicing variants had a tendency to associate with LOH in PPP2R1B locus[332]. In B-cell chronic lymphocytic leukemia study, copy number loss did not affect the expression level of PPP2R1B mRNA. However, the expression was generally lower than in B-cell controls, and aberrantly spliced variants skipping exons 9 or 2-3 were frequently expressed.[333] Exon3 skipping has also been reported in CLL[329]. Importantly, a recurrent deletion affecting PPP2R1B exon 9 on genomic DNA has been reported in breast cancer. This deletion preserves the reading frame and is predicted to impair B-subunit binding[328].

2.4.2.3. PPP2R2A (B55α)

PPP2R2A is located in 8p21 chromosomal region, which frequently undergoes LOH in cancers. Furthermore, in a 5000 sample pan-cancer data set and a 2000 sample breast cancer data set PPP2R2A gene is the center of a deletion peak, with a small fraction of samples harbouring a homozygous deletion (1% of all breast cancers).[72, 301]. In breast cancer, homozygous PPP2R2A deletions associate with Luminal B subtype, and are a marker of poor prognosis[301, 334]. Low frequency homozygous deletions have also been observed in prostate cancer[335, 336], and primary plasma cell leukemia[337], and nonsense mutations have been reported to contribute to loss of protein expression in AML[338]

The PPP2R2A gene is located close to PPP2CB gene, which is often co-deleted with PPP2R2A. However, it is uncertain whether loss of PPP2CB contributes to the selective advantage of these cancer cells. The Cβ subunit encoded by this gene constitutes only a small fraction of total catalytic subunit[82], and the knockdown of Cβ does not significantly contribute to cell transformation, whereas even partial knockdown of the alpha isoform promotes transformation[164].

PPP2R2A encodes for the abundantly expressed B55α subunit with multiple functions. B55α is the major B-subunit in the PP2A complexes opposing CDK1 in mitosis[108, 217], and it may act as a tumor suppressor by inhibiting AKT via dephosphorylation of T308[269]. However, PPP2R2A depletion does not contribute to transformation in HEK-TER model[164]. Furthermore, PPP2R2A seems to be essential for some cancer cells, e.g. pancreatic[339]. In addition, binding of adenovirus E4orf4 to B55α, resulting in inhibition of its functions, promotes cell death[340]. Although this may be an outcome of artificially high overexpression that would not occur in normal Adenovirus infection[341], it is nevertheless a result of potent inhibition of B55α function.

This dualistic property of PPP2R2A, being required in some cancer cells and deleted in others, may suggest that PPP2R2A is involved in maintenance of chromosomal stability and genome duplications. Dewhurst and others[342] have shown that cells rarely survive whole genome duplications, however, once the whole genome duplication has occurred, these cells have higher tolerance of chromosomal instability and are likely to experience copy number alterations in specific chromosomal locations. They also showed that in colorectal cancer, whole genome duplication is generally an early event and may provide a selective advantage to the cells that survived it.[342] Deletions of PPP2R2A associate with whole genome duplications. Similar association was also found for PPP2R1A mutations, leading Zack and others to suggest a causal link between PP2A aberrations and whole genome duplications[72]. Additional support for this view is provided by computational approach developed by Youn and Simon[343]. This method seeks to time the alterations in cancer genomes, and this analysis identified PPP2R2A deletions as a potential mutator alteration; timing of PPP2R2A deletions coincides with an overall increase in the copy number alteration rate. Lastly, loss of PPP2R2A has been shown to impair homologous recombination repair[344]. These findings suggest, that PPP2R2A deletions are selected, not because they promote oncogenic signalling, but because they promote and enable chromosomal instability.

2.4.2.4. PPP2R2B (B55β)

PPP2R2B was identified as one of the genes, whose expression is induced in breast cancer cell lines by HDAC inhibitor and DNA methyltransferase inhibitor combination[345]. Later studies have shown that inactivation of PPP2R2B by increased methylation occurs in ductal carcinoma in situ and in invasive breast cancer[346], as well as in colorectal cancer[188]. In nasopharyngeal carcinoma cell lines, PPP2R2B methylation was associated with acquired PI3K/mTOR inhibitor resistance[247]. Another study found frequent PPP2R2B methylation in laryngeal squamous cell carcinoma, however, the methylation occured at comparable level in healthy tissue of the epiglottis[347]. In addition, methylation has been observed in approximately 10% of gliomas, but normal tissue was not analysed in this study[348] In glioblastoma multiforme, PPP2R2B is located in the vicinity of a recurrent chromosomal breakpoint and its downregulation is associated with poor survival[349].

Inactivation and frequent methylation may be partly explained by tissue specific expression of PPP2R2B; In normal tissues, expression of PPP2R2B is mainly restricted to brain[57]. However, putative tumor suppressor functions have also been described, suggesting that the methylation may be selected for[188, 247, 345]. In addition, PPP2R2B is one of the subunit genes implicated in transformation[188] and has the 2nd highest mutation frequency per base pair (Figure 6). Two single nucleotide polymorphisms in this gene have been associated with breast cancer risk[350] and prognosis[351] and a third one with lung cancer risk[352].

2.4.2.5. PPP2R5C (B56γ)

Despite being the prototypical tumor suppressor B-subunit[187], there are few cancer associated mutations mutations in the PPP2R5C gene coding for B56γ. However, functional analysis of PPP2R5C mutations suggests that many of them promote proliferation and for a subset of these mutations, the increase in proliferation was associated with impaired ability to dephosphorylate p53 T55[199].

Interestingly, there is a strong allelic bias in CNA events involving the intronic SNP, rs6575883, of PPP2R5C in breast cancer[353]. However, Ito et al.[354] have reported a frequent 1bp insertion in the intron 1 of PPP2R5C gene. A total of 4 samples with this heterozygous insertion also had deletion in PPP2R5C gene, and in all cases the deleted allele was the one with frameshift insertion. This suggests, that rather than inactivating a tumor suppressor, these copy number

alterations preserve the functional allele. Because only exons were sequenced in the study by Ito et al., the association between the exon 1 frameshift insertion and the intronic SNP rs6575883 could not be studied.

2.4.3. Endogenous inhibitor proteins

2.4.3.1. Cancer associated genomic alterations in PP2A inhibitors

Many of the PP2A inhibitors are considered oncogenic. In the cancer genomics literature there is strong evidence for the oncogenicity of SETBP1 and TLX1. SETBP1 alterations are found in several, but relatively rare, myeloid lineage malignancies[275, 355]. Both SETBP1 stabilizing mutations and translocations that increase its expression have been associated with reduced PP2A activity[156, 275], however, PP2A independent mechanisms are likely to contribute to the oncogenic function of SETBP1[276, 356]. TLX1 is frequently overexpressed by translocation events in ALL and directly regulates the transcription several genes involved in mitotic control resulting chromosomal segregation errors[357]. Inhibition of PP2A has been described as a contributing mechanism to cell cycle deregulation by TLX1[152, 153]. A recurrent splice site mutation has been reported for HNRNPA2B1 in adult T-cell leukemia/lymphoma.[358] Function of the resulting truncated protein has not been characterized. Recurrent gene fusions have also been described for SET in haematological malignancies[359, 360] and for HNRNPA2B1 in prostate cancer[361-363]. However, the HNRNPA2B1 fusion is likely to only contribute regulatory regions to its fusion partner ETV1.[361] Although the recurrent SET-NUP214 fusion retains PP2A interacting regions[141, 360], the fusion has not been shown to enhance PP2A inhibition. Instead, it results in nuclear localization of NUP214, which is a shared property with other NUP214 fusion with DEK.[364] A proposed mechanism for the oncogenicity of these NUP214 fusions is the resulting upregulation of HOXA9 and HOXA10 genes that interferes with cell differentiation[365]. Although CIP2A has been reported as fusion partner with Mixed lineage leukaemia gene (MLL) in one AML case[366], this fusion only involved the C-terminal fragment of CIP2A and it is uncertain, whether this fragment retains PP2A inhibitory activity. Interestingly however, MLL is recurrently undergoes fusions with other genes[367] and MLL-ENL fusion protein has been shown to interact with SET-PP2A complex[368]. In addition, SNP rs2278911 in CIP2A has been associated with altered HBV or HCV associated hepatocellular carcinoma risk[369].

2.4.3.2. PP2A inhibitor protein overexpression

Although most PP2A inhibitors do not harbour driver alterations, many of them are overexpressed in cancers and their expression tends to correlate in tumor samples (Figure8). Hierarchical clustering of the correlation matrix suggests that the expressions of five potentially oncogenic inhibitors, SET, CIP2A, TIP, ANP32E, and HNRNPA2B1, form a cluster with similar expression patterns across 21 cancer types. Expression of CIP2A and SET has been associated with proliferative status of the cells[260, 370]. CIP2A, SET and HNRNPA2B1 are also regulated by MYC [252, 254, 255]. Both CIP2A and SET have been shown to promote MYC stabilization whereas PP2A promotes its degradation[146, 192, 257]. Together, these reports suggest that the positive feedback between PP2A inhibition and MYC may extend beyond the positive feedback loop that has been described between CIP2A and MYC, where CIP2A promotes MYC stability and MYC enhances the expression of CIP2A[202, 205, 252]. . Overexpression of one or more of these inhibitor proteins, relative to normal tissue, has been reported in most tumor types. In addition, the expression often carries prognostic information.

SET expression correlates with ovarian cancer stage[371] and CLL progression[372]. High SET expression has been associated with poor prognosis in AML[373], colorectal cancer[374], and non-small cell lung cancer[375]. Although SET is a potent inhibitor of PP2A, multiple functions have been reported for SET and it has been implicated in the negative regulation of tumor suppressors p53[376] and NM23-H1[377] via PP2A-independent mechanisms.

CIP2A is expressed at very low level in non-proliferating normal cells and overexpression occurs almost ubiquitously in cancers[260, 378]. CIP2A mRNA expression is a strong survival indicator in the TCGA pan-cancer data[379]. CIP2A protein expression has been associated with poor prognosis in gastric cancer[252], CML[256], AML[380], tongue cancer[381], ovarian cancer[382], kidney cancer[383], hepatocellular cancer[384], bladder cancer[385], HER2-negative breast cancer[198], melanoma[386], pancreatic cancer[387], head and neck cancer[388], and cancer of the bile duct[389]. Association has also been found in some[390, 391]. but not all [392] colorectal cancer studies. A meta-analysis of 342 non-small cell lung cancer prognostic markers identified CIP2A as one of the 26 markers consistently associated with prognosis in more than one data set[393]. CIP2A has been almost exclusively studied in the context of PP2A inhibition, however, it has been suggested that CIP2A may also promote cell cycle progression via direct interaction with Plk1 and NEK2[394, 395].

HNRNPA2B1 has a high expression in most normal tissues[361] and it is further elevated in many cancers.[396, 397] Elevated HNRNPA2B1 expression has been associated with poor prognosis in non-small cell lung cancer[398] and liver cancer[399]. HNRNPA2B1 participates in pre-mRNA processing and splicing regulation, the deregulation of which may contribute to its oncogenicity. However, the RNA-binding domain has also been reported to bind PP2A and inhibit it cooperatively with SET. Expression of the PP2A binding domain alone was shown to increase cell proliferation[157]

ANP32E is belongs to a family at least 8 genes (ANP32A-H), and the PP2A interacting region is shared by the A and the E isoforms.[321] While ANP32A has been suggested to suppress transformation[400], this property seems to be independent of the interaction with PP2A.[321] In contrast, ANP32E has been associated with poor prognosis in myeloma[401]. This increased expression was associated with copy number gains, and a comparison of mRNA expression and gene copy numbers in gastric cancer also identified ANP32E as a gene whose expression is strongly increased by copy number gains[402]. ANP32E is located in the 1q21 chromosomal location that frequently undergoes copy number gains in cancers, however other likely drivers of this amplification have been identified[72]

Increased expression of TIP (TIPRL) has been reported in hepatocellular cancer[403]. TIP also has an emerging oncogenic role due to its preferential binding to the mutant Aα frequently found in endometrial cancers.[148] In addition to PP2A, TIP also associates with PP4 and PP6[147]

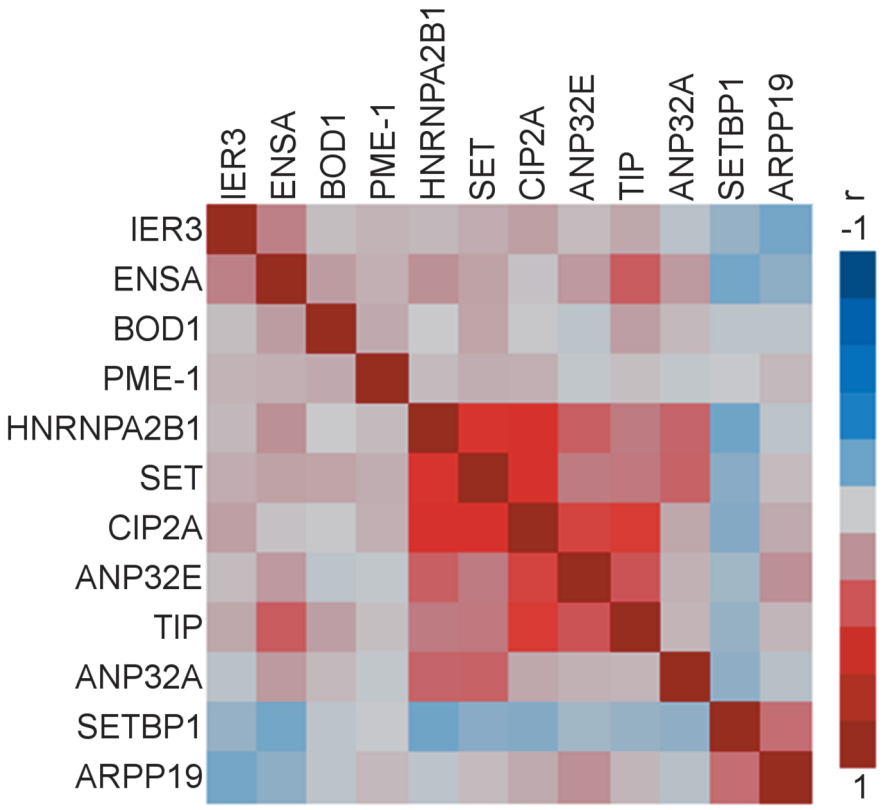


Figure 8. Co-expression of PP2A inhibitors. Hierarchical clustering (average linkage) of Pearson's correlations of endogenous PP2A inhibitor mRNA expression in TCGA pan-cancer data set containing 9755 samples from 21 different cancer types. The data were downloaded from UCSC cancer genomics browser[404] in May 2016. Five PP2A inihibitors, SET, CIP2A, TIP, ANP32E, and HNRNPA2B1 form a cluster with correlated expression patterns. HOX11/TLX1 and PPP1R17/G-substrate were excluded due to not having detectable expression in large number of samples.

2.4.4. C-subunit Y307 phosphorylation

Catalytic subunit is negatively regulated by phosphorylation at Y307. However, recently, concerns have been voiced over the limited specificity of commercially available antibodies towards the Y307 phosphorylated C-subunit (Egon Ogris, personal communication). Therefore, the studies using this methodology are reviewed separately below.

Following studies have used radiolabeling or immunoprecipitation coupled with a generic phosphotyrosine antibody. The Y307 is phosphorylated *in vitro* by multiple tyrosine kinases including $p60^{v\text{-}src}$, $p56^{lck}$, IGR, EGFR[165], and Jak2[124]. The phosphorylation by receptor tyrosine kinases was shown to be ligand

dependent[165] and Y307 has been shown to undergo transient phosphorylation after serum or EGF stimulation of serum starved fibroblasts and in 10T1/2 fibroblasts transformed by p60$^{v\text{-}src}$ [405]. Y307 is dephosphorylated by PTP1B[405] but it also undergoes autodephosphorylation[165]. Consistently, inhibition of PP2A by small molecule inhibitor Okadaic acid increased Y307 phosphorylation[165]. Phosphorylation at T304 was also increased with another phoaphatase inhibitor, Microcystin-LR , suggesting that it may also be subject to autodephosphorylation[409]. Y307 inhibition may also interfere with methylation; phosphomimetic Y307D and Y307E mutants are not methylated[159, 160]. However, it is not known whether the PP2A complexes that are formed despite of Y307 phosphorylation still possess catalytic activity.

Following paragraph lists the studies using the potentially unspecific pY307 antibody. Increased Y307 phosphorylation has been reported in HER2-positive breast cancers[406], Jak2 V617F mutant myeloproloferative diseases[174], murine 32Dcl3 –cells expressing Bcr-Abl fusion gene[270], in colorectal cancer cell lines compared to normal mucosa[408], and in CLL compared to normal B-cells[166]. Y307 phosphorylation has been associated with poor prognosis in metastatic colorectal cancer[411] and breast cancer[412]. Microystin-LR was shown to increase Y307 with a simultaneous decrease in C-subunit methylation[407], whereas treatment with PP2A activating compound Forskolin has been reported to decrease phosphorylation[408].Correlation between Y307 phosphorylation and the expression levels of PP2A inhibitors SET[156, 270, 413], CIP2A[256, 412], and SETBP1[156] has also been reported.

Although it may be difficult to quantify how the inhibitor proteins affect autodephosphorylation, the existence of autodephosphorylation mechanism has been validated by other methods[165], suggesting that mechanisms for PP2A inhibition are not isolated from each other. Particularly, the correlation and prognostic value of multiple markers of PP2A inhibition suggest that low PP2A activity is a characteristic outcome of oncogenic signaling in aggressive cancers.

2.5. PP2A as a therapeutic target in cancer

2.5.1. PP2A inhibition

As described above, PP2A is involved in the regulation of cell cycle checkpoints and multiple mitotic processes. Consequently, inhibition of PP2A by small

molecule phosphatase inhibitors is toxic to the cells and the rapidly proliferating cells may be more susceptible to PP2A inhibition. This is the rationale behind PP2A inhibition as a cancer therapy and a number of preclinical studies have addressed the cancer cell killing potential of PP2A inhibitors, either alone or in combination with other genotoxic compunds.[414] PP2A small molecule inhibitors have also been reported to promote the activation and expansion of natural killer cells in metastatic melanoma mouse model[415].

Fostriecin is one of the most PP2A-specific of the natural compound phosphatase inhibitors, although it will also inhibit the related PP4 and possibly PP6 at comparable efficiency[416]. Following the promising preclinical results, it entered to a phase I clinical trial, however, the trial failed due to the low *in vivo* stability of fostriecin[417]. Another study was also cancelled due to limited supply and difficulties with the synthesis[418]. Development of more stable derivatives has been explored[419], but none have completed clinical trials. Interestingly, another naturally occurring phosphatase inhibitor, cantharidin, is a major constituent in Chinese traditional medicine treatment Mylabris, which is prepared form Chinese blister beetle. Cantharidin and its derivatives have been used for cancer treatment in China since in the past three decades and modest survival benefits have been reported.[420] However, more data from controlled trials are required.

2.5.2. Reactivation

As discussed above many PP2A inhibiting mechanisms are connected to the proliferative status of the cell under physiological conditions and cooperatively inhibit PP2A in cancer cells. Thus, even in the absence of genomic alteration of PP2A subunits, PP2A activity is suppressed in cancer cells. A large body of literature shows that PP2A reactivation is generally poorly tolerated by cancer cells and results in deactivation of many oncogenic pathways. Thus PP2A reactivation is increasingly being considered as a potential cancer therapeutic[421]. PP2A reactivation can be achieved by compounds that directly bind to PP2A and promote its phosphatase activity or by inhibitors of the endogenous inhibitor proteins.

2.5.2.1. Direct PP2A activators

Forskolin is derived from a plant *Coleus forskohlii* and its anticancer properties have been investigated over three decades ago[422], but no significant advances

seem to have been made since. Forskolin has been reported to activate PP2A via cyclic AMP signaling[423] and it continues to be used as a research tool for exploring pharmacologic activation of PP2A in cancer cells. However, other mechanisms may be involved as 1,9,-dideoxyforskolin has been shown to produce comparable effect to Forskolin, FTY720, or SET knockdown without activating cyclic AMP signaling in NK cells[424, 425] and CML cells[270, 271].

Tricyclic antipsychotic drugs have recently been discovered as novel PP2A activators. Perphenazine is an approved antipsychotic drug that was indentified as a potential therapeutic agent in T-ALL in high throughput screening. Perphenazine was shown to bind Aα-subunit of PP2A and reduce the phosphorylation of many etablished PP2A targets. However this effect is likely to require higher concentrations than the antipsychotic effect, and the safety of these has not been tested.[426] Also other phenothiazine class drugs have been shown to activate PP2A[427]. Derivatives of these drugs decoupling the antipsychotic effects from the anticancer effects have been developed, and one of these compounds, RTC-5, has been shown to be effective in lung cancer xenograft model[428].

2.5.2.2. SET inhibitors

FTY720, also known as Fingolimod, is an anti-inflammatory drug approved for the treatment of multiple sclerosis. FTY720 has also been shown to promote PP2A activity[429] and this has been mainly attributed to the disruption of SET-PP2A interaction[430]. FTY720 is a sphingosine analogue and also a target of sphingosine kinases 1-4. However, unlike sphingosine, the interaction of FTY720 with sphingosine receptors leads to transient activation followed by their internalization and downregulation, which is the basis of the immunosuppressive functions[431]. A nonphosphorylatable derivative of FTY720 retains the SET interaction and anticancer properties in Lewis lung cancer and $JAK2^{V617F}$ leukemia mouse models[174, 430]. It also suppresses BCR-ABL function in CML cells regardless of concomitant sphingosine kinase inhibition[432]. However, the anticancer use of FTY720 is likely to require higher doses and longer treatments, and the adverse effects associated with such treatments have not been properly assessed. Derivatives of FTY720 are also being developed to address these concerns.[433]

OP449 is a peptide analogue derived from ApoE protein that has been shown to interact with SET. OP449 has been reported to activate PP2A more potently than

FTY720 and exhibit selective cell killing activity to CLL cells compared to normal B-cells, as well as to limit the xenograft growth of Ramos NHS cells.[372, 434] It was also effective in several AML, CML, breast cancer, prostate cancer, and pancreatic cancer cell lines, and reduced the tumor burden in AML, breast cancer, prostate cancer, and pancreatic cancer xenografts[257, 259, 272, 435]

2.5.2.3. CIP2A inhibitors

Cancer specific expression, prognostic significance, and dispensability in most normal tissues makes CIP2A an appealing drug target[260, 378]. Consequently, multiple compounds have been reported to achieve their cancer cell killing effect in part by downregulation of CIP2A. However, for the most part, mechanistic details have been lacking, and no rescue experiments have been presented. A study by Liu et al. reported that Celastrol, a Chinese traditional medicine –derived compound, binds to CIP2A covalently leading to its degradation[436]. However, the downregulation of CIP2A seems to be associated with the overt toxicity of this compound at high concentrations and a sub lethal proliferation deficit similar to RNAi was not achieved (unpublished observation).

2.5.2.4. Countering kinase inhibitor resistance by PP2A reactivation

Although the PP2A genomic alterations do not seem to be selected due the activation of specific oncogenic pathways, low PP2A activity is a widespread, if not inevitable result of oncogenic signaling in rapidly proliferating cancer cells. Specifically, many kinase cascades have a built-in mechanism for transient PP2A inhibition: For example, ERK activates PP2A inhibitor IER3[155] and JAK2 directly binds to PP2A and introduces an inhibitory phosphorylation, likely at Y307[124]. Additional inhibition of PP2A has been shown to prolong ERK activation in response to transient signals[115] and to contribute to constitutive signaling by $JAK2^{V617F}$ in AML cells[174].

Kinase inhibitor resistance often involves reactivation of the targeted pathway. In lung cancer, resistance to EGFR small molecule inhibitors most commonly occurs via resistance mutation in the EGFR or by activating mutations in the downstream RAS-MAPK pathway[437]. Similarly in colorectal cancer, majority of the resistance to anti-EGFR antibody therapy is caused by activating mutations in RAS-MAPK pathway[438]. However, in a significant proportion of resistant tumors, the mechanism of resistance has not been identified. Functional genomics screens have shown that under the selective pressure of the drug, otherwise rare[439] or even deleterious[440] alterations may be selected because

they enable the reactivation of the targeted pathway. Importantly, these alterations are selected in a situation where PP2A activity is suppressed. Because PP2A participates in multilevel regulation of a wide range of kinase pathways with oncogenic potential[77, 441], it could simultaneously regulate feedback mechanisms and collateral resistance pathways, thus shaping the selective pressures on the resistance mutations.

Therefore, potentially most promising application for PP2A activators would be a combination therapy with kinase inhibitors. Some preliminary results on combining PP2A activation with kinase inhibition have already been reported. Synergistic effect was reported between the SET inhibitor OP449 and Imatinib in CML cell lines, between OP449 and FLT3 or JAK2 inhibitors in AML cell lines[272], as well as between FTY720 and multi kinase inhibitor sorafenib in hepatocellular cancer cell lines[442]. Furthermore, FTY720 has been reported to specifically eradicate the quiescent CML stem cells with low BCR-ABL activity and intrinsic resistance to Imatinib[271].

3. AIMS OF THE STUDY

PP2A is known to exert multilevel regulation on many pathways. Specifically, participation of PP2A in the feedback signalling may have autoantagonistic effects, suggesting that specific phosphorylation events regulated by PP2A may have context dependent functions.[77, 115, 443] The common theme in this thesis has been the use of various systems biology approaches to study how PP2A activity manipulation affects cancer cell functions and drug sensitivity. The specific aims of the subprojects are listed below.

I. Characterization of CIP2A-regulated transcriptional changes. Identification of CIP2A regulated transcriptional changes in clinically relevant setting.

II. Development of normalization method for label-free phosphoproteomics that enables the analysis of data with large unidirectional shifts.

III. Systematic characterization of PP2A regulated phosphorylation changes. Identification of drugs whose sensitivity is determined by PP2A activity

4. MATERIALS AND METHODS

The aim of this thesis has been to study PP2A functions by manipulating its activity in various cancer cell lines followed by systems biology analyses to characterize the effects of PP2A inhibition and reactivation. Various bioinformatics and statistics approaches were utilized to inform the targeted functional validations by various molecular biology techniques. The methods used in this thesis are tabulated below.

Method	Used in publication
PP2A activity manipulation	
CRISPR/Cas9 mediated knockin	III
CRISPR/Cas9 mediated knockout	III
siRNA knockdown	I, II, III
Okadaic acid treatment	I, II
Systems biology analyses	
Genome-wide gene expression microarray	I
Label free LC-MS/MS (phospho)proteomics analysis	II, III
High throughput drug sensitivity and resistance testing	III
Bioinformatics and statistics	
Enrichment analysis, pathway analysis, GSEA	I, II, III
Hierarchical and soft clustering	I, II, III
Kaplan-Meier survival analysis	II
Kinase target prediction, motif analysis	II, III
Targeted analyses	
Cell viability assay using WST-1	III
Colony formation assay	I, II, III
FISH	I
Immunohistochemical staining	I
RT-qPCR	I, III
Senescence associated β-galactosidase staining	III
Transwell migration assay	I
Western blotting	I, II, III
Wound healing assay	I
Supporting methods	

Agarose gel electrophoresis	III
Bacterial transformartion and plasmid purification	III
cloning by PCR	III
lentivirus infection	III
Restricition enzyme digestion and cloning	III
TiO_2 enrichment of phosphopeptides	II, III
Trypsin digestion	II, III

Cell culture

All the cancer cell lines used in this thesis have been cultured in DMEM or RPMI medium containing 10% FBS, 2 mM glutamine, 50 I.U./ml penicillin, and 50 µg/ml streptomycin as listed below. All cell lines have been tested for Mycoplasma contamination.

Cell line	**Cancer type**	**Medium**
MCF-7	Breast cancer	RPMI + 10µg/ml insulin, 100µM NEAA
MDA-MB-231	Breast cancer	DMEM
HeLa	Cervical cancer	DMEM
CW-2	Colorectal cancer	RPMI
HCA7	Colorectal cancer	DMEM
NIC-H747	Colorectal cancer	RPMI
AGS	Gastric cancer	RPMI
A549	Lung adenocarcinoma	DMEM
NCI-H2122	Lung adenocarcinoma	RPMI
NCI-H460	Lung adenocarcinoma	RPMI

RNAi and Genome editing

To inhibit or reactivate PP2A, we have used siRNA-mediated silencing of subunits or endogenous PP2A inhibitors, respectively, and CRISPR/Cas9 mediated knock-in and knockout modifications on the PPP2R1A gene coding for the major scaffolding subunit of PP2A.

Lung cancer cells were transfected with Lipofectamine RNAiMAX and 100 pmol of siRNA in 2300µL final volume, and the other cells were transfected with Oligofectamine and 250 pmol of siRNA in 1000µl final volume according to manufacturer's instructions (Life Technologies). Downstream analyses and experiments were performed 72h after transfection.

The CRISPR/Cas9 mediated knockout was performed using sequential lentivirus mediated delivery of lentiCas9-Blast plasmid (Addgene plasmid # 52962) lentiGuide-Puro plasmid (Addgene plasmid # 52963) followed by antibiotic selections. These plasmids were a gift from Feng Zhang[444]. The linear gRNA expression vector for the knock-in was generated according to a previously published protocol[445]. The gRNA vector, together with 150 base single stranded knock-in template (300ng each), were transfected to Cas9 expressing cells using Lipofectamine RNAiMAX and the siRNA transfection protocol. For both knockout and knock-in, clones were established and modifications were screened by electrophoresis based methods and confirmed by sanger sequencing.

Microarray and RT-qPCR

In order to study the thus far uncharacterized CIP2A functions, we performed a genome-wide gene expression analysis on CIP2A depleted HeLa cells using Illumina HT 12 v3 microarray (Illumina) and validated selected results using RT-qPCR assays designed by Universal Probe library design tool (Roche Applied Science). For these analyses, the RNA were extracted with NucleoSpin RNA II kit (Macherey-Nagel) reverse transcribed with M-MuLV RNase H- reverse transcriptase (Finnzymes). RT–PCR reactions were run using Applied Biosystems 7900HT Fast Sequence Detection System and TaqMan Universal Master Mix II, no UNG (Applied Biosystems). Same approach has been used for RT-qPCR validation of siRNA knockdowns.

The microarray analysis was performed at the Finnish functional genomics centre, University of Turku and Åbo Akademi University. Microarray data were extracted with Illumina BeadStudio software. The data were analysed with the lumi Bioconductor package (Bioconductor) followed by quantile normalization[446]. Probes with expression values at the background level (detection $p<0.05$) were excluded from further analyses and genes with an absolute fold change of ≥1.3 were considered as differentially regulated.

Label-free phosphoproteomics analysis

In order to gain a better understanding of PP2A regulated phosphorylation changes, we have utilized label-free phosphoproteomics approach with the following workflow:

Cells were collected by scraping in ice cold PBS and snap frozen. Cell pellets were lysed in a buffer containing 8 M urea, 50 mM Tris pH 7.5, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 60 mM B-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, Roche complete protease inhibitor cocktail tablet, and 5 µM pepstatin A. Lysates were sonicated and centrifuged at 100000g for 30 min. The supernatant was collected and diluted to 5mg/ml with 8 M urea and 50 mM Tris-HCl pH 8.5, after spiking in 10 µl of 1 µg/µl bovine α-casein (Sigma-Aldrich). The proteins were reduced with 200mM DTT, alkylated with 1M iodoacetamide, followed by quenching with 1M DTT, and subjected to digestion for 18h at 37°C with 690 µl (to 300µl of lysate) of 50 mM Tris-HCl pH 8.5 containing 20 µg of sequencing grade modified trypsin (Promega). After digestion, peptides were acidified with 10µl of 10% TFA and frozen. Peptides were desalted and TiO_2 enrichment was carried out similarly as before[447].

LC-MS/MS analysis was performed at the Turku Proteomics Facility, University of Turku and Åbo Akademi University using an EASY-nLC 1000 nanoflow LC instrument coupled to a Q Exactive quadrupole-orbitrap mass spectrometer (Thermo Fisher Scientific). Samples were analysed both before and after TiO_2 enrichment and peptide identifications were searched with Mascot (v2.4.1) via Proteome Discoverer (v1.4.0.288, Thermo Fisher Scientific). PhosphoRS v3.0 was used for phosphosite localization. In order to increase the number of identified phosphopeptides, we also used recently developed SimSpectraST search[448] and these two search results were merged. The FDR rate for SimSpectraST was controlled against a target-decoy library similarly to PhosphoRS, and the localizations were scored according to the scheme outlined in II Supplementary table 13. Label-free quantification was performed separately for non-enriched and enriched data using Progenesis LC-MS (v4.1). The non-enriched data were normalized in Progenesis with median centering normalization and the TiO_2 enriched data were normalized with Pairwise normalization described in article II[379] and results section.

Bioinformatics analyses

For an unbiased interpretation of the systems biology data generated by above described methods, we have used Ingenuity pathway analysis and Gene set enrichment analysis[449]. The phosphorylation site data were further analysed with NetworKIN, NetPhorest[450], GPS 2.0[451], and Motif-X[452] kinase target prediction tools. To study the clinical relevance of CIP2A expression in cancer, we have analysed the expression of CIP2A regulated genes in two publicly available breast cancer gene expression data sets[453, 454] and studied the interaction between CIP2A expression and KRAS or NRAS expression or KRAS mutational status in patient survival in TCGA pan-cancer data set generated by TCGA research network (http://cancergenome.nih.gov/) and downloaded from UCSC cancer genomics browser[404].

Drug sensitivity and resistance testing

As a complementary approach to characterize PP2A regulated pathways critical to cancer cell proliferation, we have used high throughput drug sensitivity and resistance testing. Cell number (500-1000 cells / 384-plate well) was optimized based on saturation of the CTG readout and the screening was performed as described before[455]. The differential drug sensitivity score (DSS)[456] was calculated for each siRNA compared to controls. Cosine similarity was calculated between sensitivity changes for each drug and the LC-MS/MS based measurement of PP2A activity to identify PP2A dependent drug groups.

Selected drugs and drug combinations were studied further in colony formation assays and cell viability assays based on WST-1 cell proliferation reagent. Cell numbers and colony formation time were optimized for each cell line based on saturation of WST-1 signal of area coverage. Colony formation assays in the II and III articles were performed using ColonyArea quantification[457].

Statistical analyses

For the statistical test based on the assumption that the data is normally distributed, the approximate normality has been checked by visual inspection. T-tests were performed as two-tailed. Statistical calculations in the literature review part and for the unpublished results have been performed with JMP®

Pro 12.2.0 (SAS Institute inc., USA) unless stated otherwise. Limma analysis of differentially regulated phosphopeptides was peformed in R using the limma package[458]. The unsupervised hierarchical clustering and soft clustering were performed in R. Mfuzz package was used for soft clustering[459].

5. RESULTS

5.1. CIP2A regulates MYC activity

CIP2A depletion has been shown to reduce S62 phosphorylated and total MYC protein expression[146]. We have confirmed the effect of CIP2A on protein expression, and the parallel analysis of MYC mRNA suggests that CIP2A mainly contributes to the post-translational regulation of MYC (I Supplementary fig. 2). As a further support for the PP2A-mediated mechanism, we show that MYC downregulation by CIP2A depletion can be partially rescued by simultaneous depletion of B55α or B56β subunits (I Fig. 4C). However, functional consequences of this regulation have not been explored in detail prior to this study.

In order to gain insight on the CIP2A functions, we have analysed genome wide gene expression changes associated with CIP2A depletion in HeLa cells. The data were collected from five independent sample pairs at 3d and 5d time points and subjected to rigorous statistical analysis, resulting in 134 differentially regulated genes, that are referred to as CIP2A signature (I fig. 1B,C, I Supplementary table 1) Out of the 134 mRNAs identified in the primary screen 12 were selected for validation in RT-qPCR with two independent siRNAs, attesting that the majority of the effects we are reporting are likely due to on-target silencing of CIP2A. These mRNAs were also validated in MCF7 breast cancer cell line and AGS gastric cancer cell line (I Fig 1D, Supplementary fig 1A-C).

Ingenuity pathway analysis and gene set enrichment analysis (GSEA) suggested that CIP2A signature contains MYC regulated genes (I Fig. 2). We measured the expression of the selected CIP2A target genes also after MYC RNAi and 9/12 of these genes were regulated similarly by both CIP2A and MYC (I Fig. 5A). In addition we analysed the expression of 4 previously validated MYC target genes and these were also regulated similarly, although not quite as potently, by CIP2A (I Fig. 5C). Furthermore, the depletion of B55α or B56β subunits that rescued MYC expression, also partially rescued the expression of 6 randomly selected shared target genes of MYC and CIP2A (I Fig. 5E) These findings further support the view that CIP2A is a PP2A inhibitor, although the possibility that CIP2A would act independently from PP2A cannot be excluded from the these data alone. As an additional indication that CIP2A regulates MYC activity, CIP2A depletion also reduced the signal form MYC/E-box reporter assay (I Fig. 5D).

5.2. CIP2A associates with basal type and ERBB2-positive breast cancer

Expression of selected CIP2A signature genes with consistent expression change at 3 and 5 day timepoints was studied in a previously published breast cancer transcriptome data set[454]. Hierarchical clustering of the signature gene expression identified a subgroup containing the majority of the basal and ERBB2+ breast cancers in this data set (I Fig. 6A). Similar clustering was seen in another previously published breast cancer gene expression data set[453] (I Supplementary fig. 7). Immunohistochemistry analysis of CIP2A expression was performed on a tissue microarray of 1028 breast cancer samples[460] and elevated CIP2A expression was significantly more frequent in the basal and ERBB2+ cancers than in other breast cancer subtypes (I Fig. 6B, I Supplementary fig 8A). In addition, gene expression data on 39 breast cancer cell lines were downloaded from National Cancer Institute's cancer Bioinformatics Grid (caBIG). Highest CIP2A mRNA was found in cell lines that were derived from basal type tumors (I Fig. 6D). Lastly, expression of selected shared target genes of MYC and CIP2A were also regulated by CIP2A depletion in basal type breast cancer -derived MDA-MB-231 cell line (I Fig. 6E)

MYC overexpression, commonly resulting from amplification, has also been associated with basal type and EGFR or ERBB2 overexpressing breast cancers[461]. Therefore, we analysed the association between MYC amplifications and CIP2A expression on a subset of the tissue microarray comprising 144 samples. Interestingly, we observed a strong tendency towards co-occurrence between high CIP2A expression and MYC amplifications (I Fig. 6C, I Supplementary fig 8B)

5.3. Pairwise normalization of label free phosphoproteomics data

Although MYC regulation seems to be central mechanism by which CIP2A affects transcription of genes, we have also identified MYC independent functions, such as the regulation of JNK2 expression or transwell migration (I Fig. 3). Furthermore, because the suggested mechanism of CIP2A is to inhibit dephosphorylation by PP2A, some CIP2A effects could be easily missed by transcriptomics analysis. Therefore, in the subsequent projects we have turned to mass spectrometry based phosphoproteomics as the primary research tool to elucidate CIP2A and PP2A functions.

The most frequently used normalization method in label free phosphoprotemics is the median centering normalization which is not suitable for handling large uni-directional phosphorylation changes (II Fig. 1B). PP2A is a major constitutent to total serine/threonine phosphatase activity in cells[76] and we suspected that manipulation of PP2A activity would affect a significant portion of all phosphorylation sites, thus compromising the assumptions that centering normalization methods are based on. We therefore developed a novel normalization methodology, named pairwise normalization, that attempts to normalize the phosphopeptide abundance to the abundance of the nonphosphorylated peptides prior to phosphopeptide enrichment, which is assumed to stay more constant (Supplementary fig. 1) This requires an additional step of analysing the sample prior to phosphopeptide enrichment and calculating the ratios for each phosphopeptide that was identified and quantified both before and after enrichment (II Fig 2). The median of these ratios is then used as a global correction factor for the phosphopeptides quantified in the enriched sample.

5.3.1. Pairwise normalization increased the accuracy of PP2A phosphoproteome analysis

In order to assess the performance of the pairwise normalization, we prepared samples treated with phosphatase inhibitor Okadaic acid (OA) as well as siRNA targeting either CIP2A or the three forms of RAS (H,K, and N). Following the phosphoproteomics analysis outlined in II Fig 2A, the data were subjected to median centering, quantile, or the newly developed pairwise normalizations. The treatments were expected to result in increase (OA) or decrease (CIP2A and RAS) of global phosphorylation, however this was only evident after pairwise normalization (II Fig. 3). When ability of different normalizations to distinguish between treatments were compared, quantile normalization performed poorly compared to others, and struggled especially with the okadaic acid treated samples that exhibited the highest magnitude phosphorylation changes (II Fig. 4A,C,D, II Supplementary table 7). Lastly, the performance of different normalization methods was assessed by comparison to phosphospecific antibody based quantification. Whereas some conclusions, e.g. clustering of CIP2A and RAS phosphoproteomes together, was robust to the selection of normalization method (II Fig. 4), pairwise normalization consistently exhibited the highest correlation to western blots (II Fig. 5C,D), and when the direction of regulation was considered, pairwise normalization had significantly higher

agreement with western blots than other normalizations (II Fig. 5B) Furthermore, we have also analysed the activity of two central downstream effectors of RAS, namely AKT and ERK, by quantifying the phosphorylation of their target proteins. Subtle donwregulation was observed following RAS and CIP2A knockdown in the pairwise normalized data, but not in the median centering normalized data (II Fig. 6C).

The reproducibility between replicates sample was similar with median centering and pairwise normalization (II Supplementary fig. 1A), suggesting that largest benefit of the pairwise normalization is obtained when applied to data where some samples exhibit large phosphorylation changes. Therefore, we have also considered potential indicators of these large scale changes. Following the test treatments, the expression of majority of proteins stayed relatively constant, whereas significant fraction of phosphosites were differentially regulated (II table 1, II Supplementary Fig 6.) One indicator for unidirectional large scale phosphorylation changes is the skewing of the fold change distribution that was clearly seen in the OA treated sample and occurs regardless of the normalization method used (Supplementary fig. 4C). Pairwise normalization was applied to the data of the third project. Global phosphorylation changes in these data are evident from the skewing of the fold change distributions (III Fig. 1B) but this is only reflected in the average fold changes if pairwise normalization is used, suggesting that also in these data pairwise normalization reveals biological insights that would otherwise be masked by the normalization bias.

5.4. RAS and CIP2A have overlapping functions

CIP2A and RAS depletion produced similar phosphorylation changes and this similarity was not limited to AKT and ERK targets (II Fig. 4A,D, II Fig. 6A, II Supplementary fig. 6). In contrast, the quantified protein level changes varied more; RAS depletion regulated many more proteins, several of which were involved in carbohydrate metabolism and other metabolic pathways (II Fig. 6A, II Supplementary fig 6, II Supplementary fig. 7.).

In order to explore the functional implications of the overlapping phosphoregulation, we performed a series of colony formation assays on HeLa, as well as 3 colorectal cancer cell lines. We observed a general trend of synergy between CIP2A and KRAS knockdown in the impairment of colony formation

ability in these cells (II Fig. 7B,C). The effect of CIP2A and KRAS was similar to the triple RAS knockdown and addition of CIP2A knockdown to the triple RAS knockdown did not increase the effect any further (II Supplementary fig. 10). We also performed a survival analysis on the TCGA pan-cancer data set. CIP2A expression was found to be a strong survival indicator in pan-cancer data and synergistic survival effect was observed between CIP2A expression and KRAS expression, NRAS expression, or KRAS mutations (II Figure 7A). Distribution CIP2A high and CIP2A low patients into good and poor prognosis cancer types was controlled and is unlikely to contribute to the observed survival effect of CIP2A in KRAS mutant cancers (II Supplementary table 11).

5.5. Depletion of PPP2R1A, CIP2A, PME-1 or SET causes large unidirectional phosphorylation changes

In order to study the PP2A dephosphorylome we have targeted the main structural subunit Aα, encoded by PPP2R1A gene, and three endogenous inhibitor proteins, CIP2A, PME-1 and SET, by RNAi in HeLa cells. CIP2A was targeted with 4 siRNAs and the other genes with 3 siRNAs in triplicates. Each siRNA targeted a different region of the mRNA and the knodowns were validated using western blotting and RT-qPCR. The resulting phosphorylation changes were analysed by label- free phosphoprotemics analysis coupled with pairwise normalization.

In accordance with their suggested biological functions, depletion Aα-subunit resulted in increased phosphorylation in most differentially regulated phosphosites and the depletion of inhibitor proteins resulted dephosphorylation (III Fig. 1A). This trend was also evident from the average fold change of all quantified peptides, as well as from the skewing of the fold change distribution (III Fig. 1B)

Out of the tested inhibitor proteins, SET caused the largest changes, downregulating nearly a third of all phosphopeptides at 5% FDR (III Fig. 1C). SET also regulated the majority of the phosphopeptides regulated by CIP2A or PME-1 (III Fig. 1D). Interestingly, PPP2R1A and the inhibitor proteins regulated a largely non-overlapping set of phosphopeptides (III Fig. 1D).

5.5.1. PP2A activation and inhibition regulate different targets

We further studied the different types of phosphosite regulation in the test samples by soft clustering approach developed by Futschik and Carlisle[462]. Also this analysis suggested that Aα and inhibitor protein depletion, resulting in PP2A inhibition and activation, respectively, regulated largely different phosphosites (III Fig. 2A). Clusters 1 and 2 are characterized by upregulation following Aα depletion and clusters 3-6 are characterized by downregulation following inhibitor protein depletion. This suggests that majority of phosphosites exist predominantly in dephosphorylated or phosphorylated state and as a result the average phosphorylation on these sites can only be upregulated or downregulated. This concept is also supported by the observation that cluster 1 and 2 had significantly higher fraction of threonines (III Supplementary fig. 1), which is consistent with preference of many phosphatases, and especially PP2A, to dephosphorylate threonine, as well as with the preference of majority of kinases for serine[65, 66].

Using Igenuity pathway analysis tool, we have studied the enrichment of certain pathways into different clusters. For example, the ingenuity pathway PTEN signalling is enriched in the clusters 1 and 2, suggesting that it has a low basal level of phosphorylation and potential for upregulation (III Fig. 2B, III Fig. 3A). In contrast, ERK/MAPK signalling is enriched in the cluster 3, suggesting that the ERK pathway phosphorylations have higher basal level of phosphorylation and more potential for downregulation. We used NetworKIN[450] to study the regulation of the downstream effectors of these pathways. Similar enrichment was observed in predicted targets of AKT and ERK (III Fig. 2D) and this is also consistent with our previous report (II Fig 6C). Multiple other cancer relevant pathways were also regulated by PPP2R1A or inhibitor protein depletion. Phospholipase C signalling was enriched in cluster 1-3 and PKC targets in clusters 1-2 (III Fig. 2B,D), suggesting that this pathway is activated by PP2A inhibition. Interestingly, PKC has been shown promote PP2A inhibition via activation of SET, suggesting a positive feedback loop[174].

5.5.2. PP2A activity in cytoplasm and nucleus

None of the clusters were regulated exclusively by any one inhibitor protein, however, in clusters 4-6, regulation was strongest by SET and weakest by CIP2A (III Fig. 2A). These clusters were also enriched for nuclear proteins (III Fig. 2E), which is consistent with the subcellular localization of the targeted proteins.

CIP2A has a predominantly cytoplasmic localization, whereas PME-1 and SET are mainly nuclear (III Supplementary fig. 3B). Localization of PPP2R1A, and by extension PP2A, is predominantly cytoplasmic, again consistently with the lowest fraction of nuclear proteins in clusters 1 and 2. In addition to the differences between clusters, this trend was also observed in the gene specific differentially regulated peptides: PME-1 and SET had the highest fraction of nuclear targets and PPP2R1A had the lowest (III Supplementary fig. 3A). Furthermore, this finding is supported by the enrichment of PKC targets into clusters 1 and 2, and CK2α targets into clusters 5 and 6 (III Fig. 2D). The targets of these kinases have been shown to be predominantly cytoplasmic and nuclear, respectively[463].

5.6. PP2A regulates MYC activity by multiple mechanisms

Results from the first project strongly suggest that CIP2A is involved in regulation of MYC activity in cell culture models and naturally occurring breast cancers. Therefore, we have explored the mechanisms by which PP2A could influence MYC activity also in the phosphoproteomics data.

These results are summarized in III Fig. 3B, and include the expected regulation of S62 phosphorylation[146, 192]. In addition to the dephosphorylation of this residue, the phosphoprotemics data suggests that PP2A negatively regulates its phosphorylation via the upstream kinase ERK2[464]. Furthermore, PPP2R1A depletion potently induced the inhibitory S9 phosphorylation on GSK3β that has been implicated in MYC T58 phosphorylation and reduced MYC stability[248]. All these phosphorylation changes are concordant with the previous report (II Fig. 5A,B) as well as previously published literature[173, 192, 248]. Phosphorylation at other sites has been associated with MYC stability regulation, and it has been suggested that PP2A may regulate also these sites[240]. Examples in our data include S279 and S293[250, 251].

Although the regulation of MYC by PP2A is often interpreted in terms of MYC stability, the above described phosphorylation events at T58/S62 also likely to affect transactivation by MYC[240]. As an additional mechanism by which PP2A could regulate MYC activity, we observed altered phosphorylation on S2/S11 of MAX, an obligate dimerization partner of MYC. These sites were dephosphorylated potently by SET knockdown and phosphorylation of them has been shown to promote MYC-MAX dimerization[465].

Importantly, according to previous literature, these mechanism would be cooperative rather than antagonistic.

5.7. PP2A activity determines cancer drug responses

The phosphoproteomics analysis demonstrated that PP2A activity manipulation simultaneously alters the activity of many cancer relevant pathways, and in many pathways this regulation occurs on multiple levels of the signal transduction. As cancer drug resistance often involves reactivation of key signalling events via collateral resistance pathways or by relieving negative feedback, we explored the effect of PP2A activity manipulation on cancer drug sensitivity by high throughput drug sensitivity and resistance testing (DSRT)[455, 456].

One average, PPP2R1A depletion made the cells more resistant and SET depletion more sensitive to a total of 238 drugs currently used or investigated as potential cancer therapy (III Fig. 4E). PME-1 and CIP2A did not significantly alter the average sensitivity, however, for many drugs the inhibitor proteins exhibited responses that correlated with the strength of PP2A activity regulation (III Fig. 4 B-D). Especially PME-1 and SET exhibited highly correlated responses, exemplified by topoisomerase and HDAC inhibitors (III Fig. 4F,G, III Supplementary fig. 5)

General PP2A dependency of the drug responses was studied by analysing the correlation between PP2A activity estimate derived from the differentially regulated phosphosites (III Fig. 1C) and drug sensitivity changes for each drug. We identified Aurk inhibitors, JAK2 inhibitors and mTOR inhibitors as the drug groups that consistently correlated with PP2A activity (III Fig. 4A-D). Sensitivity to JAK2 and Aurk inhibitors correlated with PP2A activity (III Fig. 4B,C). mTOR inhibitors exhibited negative correlation with PP2A activity, mainly due to sensitization in PPP2R1A depleted cells (III Fig. 4A,D). In addition, there was trend towards increased resistance to rapalogs in CIP2A depleted cells (one sample t-test p=0.06). The response to Temsirolimus is shown as an example (Figure 9).

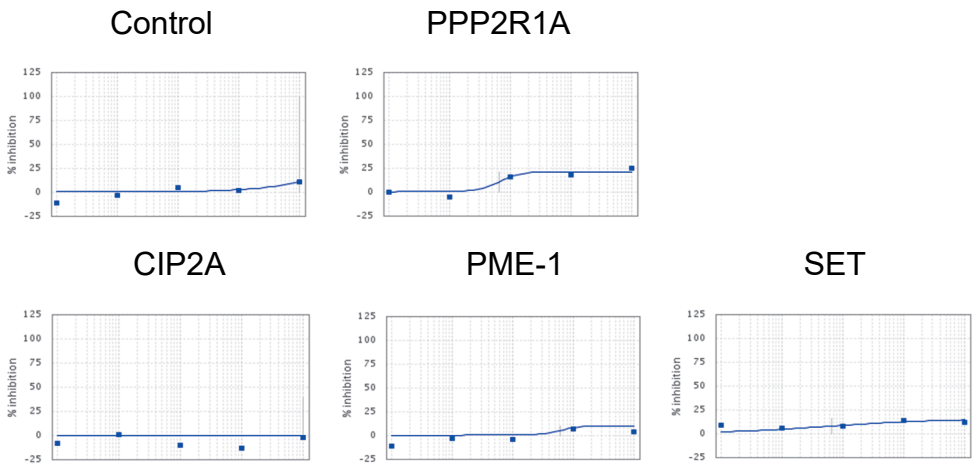


Figure 9. Response of HeLa cells to Temsirolimus. Temsirolimus was used at 1nM-10μM concentrations with 10-fold intervals. Representative siRNAs for each gene are shown. The response is sublethal up to 10μM conncetration reaching approximately 25% suppresion of cell viability in PPP2R1A depleted cells

The findings from HeLa cells suggest, that drugs exhibiting most synergy with PP2A activation are kinase inhibitors. However, HeLa cells are not a good model for aberrant kinase pathway activation; cancer initiating event in HeLa cells has probably been the integration of HPV18 genome in the vicinity of MYC[466]. In contrast, RAS is positioned upstream of multiple kinase cascades[467], and synergy between RAS activation and PP2A inhibition in the regulation of these pathways has been reported in various contexts by a large body of literature[15, 91, 164, 213], including the second project of this thesis[379]. KRAS mutant lung cancers are associated with significant mortality and have proven to be difficult to treat with targeted cancer therapeutics. Therefore, we extended the DSRT analysis to a KRAS mutant lung cancer cell line, A549, that was screened with a library of 230 kinase inhibitors. SET was excluded from the analysis because SET depletion alone was sufficient to inhibit the proliferation (III Supplementary fig. 6)

Similarly to HeLa cells, PPP2R1A depletion made the A549 cells more resistant on average, whereas PME-1 depletion resulted increased the average sensitivity to kinase inhibitors. CIP2A depletion did not alter the average sensitivity significantly, however, CIP2A and PME-1 depletions resulted in highly correlated sensitivity changes (III Fig. 5A,C). In this screen, MEK/ERK inhibitors, Aurk inhibitors, and EGFR inhibitors were identified as drug groups that consistently exhibited differential response to PP2A activation and inhibition (III Fig. 5 A,D-F). However, in the case of MEK/ERK inhibitors this was mainly a caused by

increased resistance following PPP2R1A depletion, whereas the EGFR inhibitors were only affected by the inhibitor protein depletion, resulting in sensitization (III Fig. 5 D,E). Only Aurk inhibitors displayed strong bidirectional sensitivity change similarly to HeLa cells (III Fig. 5 F).

Subset of the inhibitors in this screen were validated in two other KRAS mutant cell lines, NCI-H2122 and NCI-H460. Results from NCI-H2122 cells were concordant with the A549 cells, however, NCI-H460 cells, which harbour a homozygous E64D mutation in PPP2R1A, differed from the other two and were characterized by lower magnitude sensitivity changes on average (III Fig. 5G,H)

5.7.1. PP2A inhibition is a resistance mechanism to MAPK pathway inhibitors in KRAS mutant lung adenocarcinoma.

Depletion of PPP2R1A in A549 cell consistently increased resistance to the 11 tested MEK inhibitors, as well as the ERK inhibitor SCH772984 (III Fig. 5 A,E). This resistance was confirmed for MEK inhibitor Trametinib and SCH772984 by secondary cell viability readout (III Fig. 6A) and by colony formation assay (III Fig. 6B). Also NCI-H2122 were desensitized to MEK inhibitors and SCH772984 by PPP2R1A depletion (III Fig. 5G). In contrast, NCI-H460 cells did not exhibit this desensitization (III Fig.5G, III Fig. 6C). Furthermore, in NCI-H460 and PPP2R1A depleted A549 cells, Trametinib only caused a partial cytostatic response (III Supplementary fig. 7).

Using CRISPR/Cas9, we attempted to reintroduce a wild type allele into NCI-H460, however, this resulted in an unexpectedly strong tendency of the cells to undergo senescence and we were unable generate a cell line with homozygous WT allele (III Fig. 6D,E, III Supplementary fig. 9). Nevertheless, introducing one WT allele resulted in minor sensitization to Trametinib (III Supplementary fig. 10). A549 cells have 4 copies of PPP2R1A. Using CRISPR/Cas9, we also generated A549 cells with one or two PPP2R1A alleles knocked out. These cells exhibited stable gene dose-dependent sensitivity to Trametinib (III Supplementary fig. 8).

MEK inhibition has been show to relieve a number of negative feedback loops, which may lead into activation of resistance mechanisms[468-470]. Particularly, involvement of RAF in the resistance signalling creates a situation, where combined MEK and RAF inhibition is synergistic[469]. Therefore we also tested the pan-RAF inhibitor LY3009120 alone and in combination with Trametinib. This MEK and RAF inhibitor combination proved to be more potent than either of them alone (III Fig. 6F,G,I), however in PPP2R1A depleted A549 cells, this

combination only achieved partial cytostatic response over a wide concentration range (III Fig. 6G,H).

MEK inhibitor resistance in lung cancer cells often involves activation of AKT/mTOR signalling. Therefore, we tested the effect of non-ATP-competitive AKT and mTOR inhibitors, Perifosine and Temsirolimus, on the Trametinib sensitivity. Similarly to HeLa, either of these two inhibitors alone was well tolerated at high 10μM concentration. However, this concentration sensitized to Trametinib partially cancelling the resistance granted by PPP2R1A depletion (III Fig. 6J). Importantly, under Temsirolimus treatment, Trametinib became cytotoxic also in PPP2R1A depleted cells at submicromolar concentrations (III Fig. 6K).

Western blot analysis (III Fig. 6L) suggests that the above described resistance to Trametinib in PPP2R1A depleted cells does not involve ERK reactivation. Instead, we AKT induction and partial rescue of S6 signaling and MYC expression. Addition of mTOR inhibitor temsirolimus shut down S6 signaling and blocked AKT induction but did not have a noticeable effect on MYC. PPP2R1A depletion also increased S9 phosphorylation on GSK3β regardless of AKT activation status.

6. DISCUSSION

PP2A is a major constituent to serine/threonine phosphatase activity and has a wide substrate base, including a number of oncogenic kinases[77, 441]. Previous literature has mainly focused on specific dephosphorylation events carried out by PP2A. However, PP2A is known to participate in multilevel and even autoantagonistic regulation of many interconnected pathways[77]. Because systems level understanding of PP2A functions is rudimentary, my thesis work has utilized various systems biology approaches, including transcriptomics, phosphoproteomics, and high-throughput drug sensitivity testing, in addition to taking advantage of the publicly available cancer genomics data, to study the functions of PP2A, and specifically CIP2A, in cancer cells. The work presented here constitutes a first systematic attempt to characterize PP2A functions in cancer cells.

6.1. MYC and PP2A

6.1.1. CIP2A regulates MYC activity

Genomewide transcriptomics analysis of CIP2A depleted cells identified MYC-dependency of majority of CIP2A regulated gene expression changes, thus providing a functional validation for the previously reported MYC stability regulation and shared phenotypes between MYC and CIP2A[146]. Furthermore, both CIP2A expression and CIP2A target gene signature associated with basal type breast cancer, which has been associated with MYC amplification and overexpression[461]. Together, these findings strongly suggest that CIP2A overexpression is not merely a passenger but shapes the transcriptional landscape of these tumors. The association of CIP2A with basal type breast cancer is consistent with a more recent report by Janghorban et al.[257], and together these finding constitute solid clinical evidence of CIP2A's oncogenic role. The notion that CIP2A exerts its functions by regulating MYC activity, gains further support from the recent study by Myant et al.[239] where MYC mediated phenotype in DNA-damage induced intestinal regeneration model was shown to be dependent on CIP2A. While this study demonstrated the physiological relevance, it also highlighted the context dependency, of MYC regulation by CIP2A; CIP2A is dispensable for normal tissue function and regulates specific fraction of MYC at nuclear lamina in response to DNA damage. Together these

findings establish CIP2A as a potential therapeutic target in MYC-driven malignancies.

6.1.2. MYC-PP2A feedback loops

We initially hypothesized that CIP2A overexpression, resulting in MYC stabilization, would exhibit a mutually exclusive relationship with MYC amplification. However, we found the opposite to be true. This suggests a positive feedback loop between CIP2A and MYC and a couple of studies had already alluded to the existence of such feedback via transcriptional regulation of CIP2A by MYC[202, 252]. Recently, a third study by Lucas et al[205] suggested that this feedback is also dependent on E2F1, which is consistent with CIP2A-E2F1 feedback loop associated with senescence tolerance in breast cancer cells[198] and with CIP2A-MYC feedback loop that has similarly been associated with suppression of oncogene-induced senescence in melanoma[202]. It is also telling that the initial finding on the regulation of MYC activity by CIP2A was made in HeLa cells, where MYC, together with the HPV18 enhancer sequences, has undergone several fold amplification[466]. While MYC has been shown to promote the expression of the PP2A B-subunit B56δ in a negative feedback loop[248], it also promotes the expression of the inhibitor proteins SET and HNRNPA2B1[254, 255]. Together, these findings suggest that MYC promotes PP2A inhibition as a positive feedback mechanism to sustain its functions in cancer cells. Furthermore, the phosphoproteomics data presented in the third project provides further mechanistic insight into the other half of MYC by PP2A feedback: although MYC may promote B56δ expression in specific context, its downstream mediator GSK3β can also be inhibited by PP2A inhibition, suggesting that PP2A activity manipulation affects MYC activity by multiple cooperative mechanisms.

6.2. Suppression of senescence by PP2A inhibition

PP2A reactivation is generally poorly tolerated by cancer cells. An indication of this was the unexpectedly strong induction of senescence in NCI-H460 after reverting one allele of the homozygous PPP2R1A E64D mutation back to WT in these cells. This finding is consistent with the previously reported suppression of senescence by CIP2A[198]. Severity of this phenotype in NCI-H460 cells after reverting a somatic mutation provides new insights into PP2A suppression in cancer cells. As discussed in the literature review part, PP2A is inhibited by

multiple cooperative mechanisms, whose genomic determinants may be difficult to track. The poor tolerance of PPP2R1A WT allele in NCI-H460 suggests that cancer cells are fine-tuned by evolutionary forces to certain level of PP2A suppression to sustain the cancer cell viability and proliferation. Supporting this view are the findings that although B55α is recurrently deleted in some cancers[72, 301], it is needed in other cancers to sustain cell viability[339].

Suppression of senescence seems to be a shared phenotype between MYC and different modes of PP2A inhibition. The senescence tolerance mediated by MYC has been associated specifically with the senescence induced RAS-MAPK activation and it has been suggested as the mechanistic explanation behind the cooperation of MYC and RAS in malignant transformation[471]. RAS has also been shown to cooperate with PP2A inhibition in transformation. However, these studies have involved suppression of TP53 and Rb to block senescence, and the cooperation has been attributed to activation of several shared downstream pathways[15, 91, 164]. This regulation of oncogenic signalling cascades represents another side of the PP2A tumor suppressor functions and it is very consistent with the extensive overlap of CIP2A and RAS regulated phosphoproteomes reported in the second project of this thesis[379].

6.3. PP2A dephosphorylome

6.3.1. Pairwise normalization for label free phosphoproteomics

In order to study the PP2A dephosphorylome, we first needed to develop a novel normalization method for label-free phosphoproteomics to address the problem arising from a large scale unidirectional phosphorylation changes[215, 472], such as those associated with PP2A activity manipulation. Alternative methods for controlling large unidirectional changes in phosphorylation include spike-in internal controls[473], however inaccuracies in protein quantification from concentrated urea buffer and in spiking small volumes represent significant problems for this method, especially in larger sample sets that are a key application area for label-free analysis[474]. Using a combination of bioinformatics, statistical, and western blotting analyses, we have demonstrated that the novel pairwise normalization outperformed the conventional normalization methods

Skewing of the fold change distribution was identified as a potential indication of large scale unidirectional phosphorylation changes and this becomes especially evident in the larger data set of the third project, thus providing further support for the rationale behind pairwise normalization.

6.3.2. Unidirectionality of the phosphorylation changes

One of the novel findings from PP2A dephosphorylome analysis was the widespread uni-directionality of the phosphorylation changes; although significant fraction of the detected phosphosites could be altered by PP2A activity manipulation, individual phosphosites tended to be either upregulated by PP2A inhibition or dephosphorylated following PP2A activation. One interpretation for these findings is that for most sites, kinase/phosphatase balance favors one extreme, i.e. phosphosites have a tendency to predominantly exist in phosphorylated or dephosphorylated state. Thus, the effect of PP2A depends on what pathways are on, as dephosphorylated residues cannot be dephosphorylated any further. Tyrosine phosphorylation occurs often in the early steps of signalling cascades where in the basal state phosphatase activity greatly exceeds kinase activity and this situation is reversed in a switch like fashion following a transient activation[475]. Tyrosine kinases then activate widespread serine/threonine signaling that can also integrate signals from other sources and tend to exhibit higher basal level of phosphorylation on average[24, 476]. However, the results presented here suggest, that at level of individual phosphorylation sites, also serine and threonine phosphorylation occurs in a binary switch-like fashion.

It is possible that the apparent similarity between CIP2A and RAS regulated phosphoproteomes in the second project, as well as between the three inhibitor proteins in the third project, is also a function of basal state of phosphorylation, i.e. their depletion can only deactivate pathways that were previously activated. Furthermore, we are more likely to detect sites that were phosphorylated in control samples and then dephosphorylated following the siRNA treatments, than we are to detect sites that had low basal state of phosphorylation to begin with. It is thus conceivable, that some of the specific targets of RAS or PP2A inhibitor proteins are not detected by this methodology. Nevertheless, we have observed the similar regulation on the well-established key downstream pathways of RAS, namely MAPK and AKT pathways. Furthermore, we found synergy between CIP2A and KRAS in cancer cell growth and patient survival, and the saturation of the colony formation response with combination of either

CIP2A and KRAS or all three forms of RAS further suggests regulation of overlapping pathways. Previous work has shown that these proteins cooperate in transformation[146]. The effect of CIP2A overexpression in transformation can be generalized to PP2A inhibition by a large body of literature. Importantly, these studies collectively suggest that RAS activation and PP2A inhibition regulate overlapping, rather than complementary, pathways in transformation. E.g. activation of PIK3CA in HMECs can replace PP2A inhibition by SV40 small t-antigen[190], whereas the combination of PIK3CA and RAL-GEFs can replace RAS in human embryonic kidney cells[15]. Intact PI3K signalling is still required for the transforming activity of RAS in HMECs[15] and suppression of PP2A activity towards RALA promotes the transformation of human embryonic kidney cells[91]. Similarly, MYC overexpression has been reported to synergize with PP2A inhibition in fibroblasts[185] and RAS activation in human embryonic kidney cells[192]. The regulation of cell cycle progression and bypassing G0 stop represents another setting where PP2A inhibition cooperates with RAS to induce MYC-mediated signaling[213].

6.3.3. PP2A activity gradient between nucleus and cytoplasm

Despite the similarities in the inhibitor protein regulated phosphoproteomes, we also discovered interesting differences. One of them is the differential preference for, and potency of dephosphorylation of, proteins located in the nucleus. The phosphorylation levels of PP2A-regulatable phosphosites tended to increase from cytoplasm to nucleus. In many signalling pathways, the sequential activation of kinases happens in the vicinity of the cell membrane, form where the terminal kinase or an activated transcription factor is translocated to nucleus[477]. However, recent mathematical models suggest, that if a protein phosphorylated at the membrane travels to nucleus under constant phosphatase activity, it has an increasing chance to get dephosphosrylated, the further it moves from the membrane towards nucleus[478]. This is the opposite of what we observed, however, the findings were very consistent with the known subcellular localization of PP2A inhibitor proteins: the nuclear inhibitors, SET and PME-1, had a higher fraction of nuclear protein in their targets compared to PPP2R1A and CIP2A. Furthermore, the nuclear inhibitors exerted stronger control over many phosphosites in the nuclear proteins. Therefore, we propose that this spatial organization of PP2A activity regulation contributes to signal propagation in cells, and these findings provide novel insight into how

PP2A inhibitor overexpression may contribute to sustained oncogenic signalling in cancer cells.

6.4. PP2A dependent drug responses

We have used high throughput drug sensitivity screening as a complementary method to study PP2A functions. A number of parallels can be drawn between the phosphoproteomics and drug sensitivity data. For example, the overall effect of PPP2R1A was opposite to the inhibitor proteins responses. SET depletion results in larger changes than depletion of PME-1 or CIP2A. Subcellular localization may also be a factor; PME-1 and SET are predominantly nuclear, and compared to CIP2A, higher fraction of their phosphoproteome is nuclear. Consistently, only PME-1 and SET, but not CIP2A, sensitized to topoisomerase inhibitors, which are likely to exert their effect in the nucleus.

Majority of phosphosites and many drug sensitivities exhibited unidirectional change. Examples of the drug groups with uni-directional change include EGFR and MEK inhibitors in A549 cells, as well as HDAC and ATP-competitive mTOR inhibitors in HeLa cells. AKT and mTOR pathways seem to have low basal state of phosphorylation and potential for upregulation, suggesting that these pathways only have a minor contribution to the proliferation of HeLa cells. This also explains why mTOR and PI3K/mTOR inhibitors are well tolerated at high concentrations and why there is not much room for sensitization by cooperative PP2A reactivation. The paradoxical result of sensitization by PP2A inhibition, therefore, is likely a result of promoting proliferation by first turning on these pathways, and subsequently turning them off by the drug treatment. The relative sensitization is caused by the increased ability of the cells to take advantage of these pathways at low PP2A activity state. While these results highlight the role of PP2A in AKT/mTOR signalling, it is conceivable that in AKT/mTOR dependent cancer, such as EGFR mutant lung cancer[479]. As will be discussed below, AKT/mTOR signalling, and its regulation by PP2A, becomes relevant also in KRAS mutant lung cancer treated with MAPK pathway inhibitors[468].

The drugs whose sensitivity mirrors PP2A activity change to both directions, then, represent a special case. One group of these drugs were the Aurora kinase inhibitors. A number of mitotic processes are tuned by opposing activities of Aurora B and PP2A, where the spatial control contributes to

kinase/phosphatase balance[21, 218, 221]. JAK2 on the other hand, engages in complex reciprocal regulation with PP2A. In addition to promoting SET activity towards PP2A, JAK2 directly interacts with PP2A[174]. It has been suggested that JAK2 both inhibits PP2A by transient phosphorylation of C-subunit and recruits it to its own downstream targets as a negative feedforward mechanism[125]. It is thus conceivable that in these special cases the response to the combined targeting of PP2A and AurB or JAK2 exhibits more gradation than just turning on or off certain phosphorylations.

6.4.1. PP2A inhibition confers MAPK pathway inhibitor resistance in KRAS mutant lung cancer

We have identified PP2A inhibition as novel resistance mechanism to MAPK inhibitors in KRAS mutant lung cancer cells. Furthermore, PP2A inhibition also conferred resistance to the synthetic lethal combination of MEK and RAF inhibitors[469]. Our findings suggest, that the resistance is caused via multi-pathway mechanism.

Although AKT/mTOR signalling only had a minor contribution to cell viability in both HeLa and A549 cells, AKT and mTOR inhibitors sensitized the A549 cells to MEK inhibition, suggesting that suppressed MAPK pathway activity increases the dependency on AKT/mTOR signalling. This is consistent with previously reported MEK inhibitor resistance mechanisms lung cancer involving RTK mediated activation of AKT/mTOR signalling[468, 470]. In addition, the mechanisms that sustained cell viability in PPP2R1A depleted cells even at micromolar concentrations of MEK inhibitor seemed to be dependent on mTOR and, to lesser extent, AKT. This observation is consistent with the view that PPP2R1A depleted cells can take advantage of AKT-mTOR pathway activation, resulting in relative sensitization to the drugs targeting this pathway (Fig. 9). However, while the MEK inhibitor produced cytotoxic effect in the presence of mTOR inhibitor, PPP2R1A depleted cells were still less sensitive to the MEK inhibition. The ability of PP2A inhibition to partially rescue MYC expression was not affected by mTOR inhibitor, suggesting that MYC upregulation is a contributing mechanism to MEK inhibitor resistance.

Importantly, the PP2A-suppression associated resistance to MEK inhibitor was confirmed by the analysis of a relevant cell line with a well characterized PPP2R1A mutation. These findings demonstrate that PP2A inhibition is a novel multipathway resistance mechanism to MAPK inhibitors in lung

adenocarcinomas harbouring KRAS activating mutations, and strongly suggest that the predictive potential of PP2A activity status should be evaluated in further studies.

7. SUMMARY

The work presented in this thesis constitutes a first attempt to systematically understand targets and target mechanisms of PP2A tumor suppressor activity. More specifically, results reveal for the first time regulation of MYC target genes by CIP2A in a clinically relevant setting. Further, the phosphoproteomics analysis indicated multiple cooperative mechanisms of MYC regulation by PP2A.

Significant fraction of the whole phosphoproteome can be affected by inhibiting or activating PP2A, and PP2A participates in multilevel regulation of many known oncogenic pathways. Further analysis of PP2A dephosphorylome suggested that the effects of PP2A inhibition and activation occur mostly on non-overlapping sites. This suggests that for most phosphosites, kinase/phosphatase balance strongly favors either dephosphorylated or phosphorylated state, and e.g. increased activation of PP2A phosphatase function can only affect sites that were phosphorylated. We also noticed a gradient of increased phosphorylation from cytoplasm to nucleus in the PP2A responsive phosphorylation sites. This finding is consistent with known localization subcellular patterns of PP2A inhibitors and suggests that spatial regulation of PP2A activity by inhibitor proteins contributes signal transduction in cells.

We have described different types of interactions between PP2A activity and drug sensitivity. Majority of the drug responses exhibited a correlation with PP2A activity, and we have validated PP2A inhibition as resistance mechanism to MAPK pathway inhibitors in KRAS mutant lung cancer. Importantly, this resistance was not cancelled by the combination of RAF and MEK inhibitors.

Together, the findings presented in this thesis strongly suggest that PP2A reactivation, either alone or in combination with kinase inhibitors, holds therapeutic potential and that PP2A acitivity status carries predictive information on cancer therapy responses.

As a methodological advance this thesis presents a novel normalization method for label free phosphoproteomics, called pairwise normalization, and demonstrates that it increases the accuracy of quantification in the data sets described here.

8. ACKNOWLEDGEMENTS

This work has been carried out in Turku Centre for Biotechnology and I want to warmly thank the staff and personnel, and Professors Riitta Lahesmaa and John Ericksson, for making the Centre a great place to work at.

I want to thank my supervisor Professor Jukka Westermarck for trusting me with an important project and for maintaining an enthusiastic and encouraging atmosphere when something did not go as in Stromsö. I also want to express my deepest gratitude for your commitment to my training and career advancement whether or not it benefited you.

I am very grateful to Professor Susumu Imanishi, who became the second supervisor of my thesis when we had to address analytical problems before getting to the biological ones. Your devotion to this project has also kept me motivated.

I want to thank Professors Anne-Claude Gingras and Lars-Gunnar Larsson for reviewing my thesis and especially for the valuable scientific insight on the last manuscript.

I would like to thank my opponent, Professor Jussi Taipale, for accepting the invitation and for the invitation.

I am grateful to all the collaborators that have made this research possible. I would like to thank Professor Tero Aittokallio for all the scientific insight and other help, and the group members, Bhagwan Yadav, Petteri Hintsanen, and especially Daniel Laajala, for the critically important support in statistics and bioinformatics. I want to thank Krister Wennerberg for an exciting collaboration and access to the high throughput drug screening, and Evgeny Kulesskiy for running the screens with me. I have enjoyed working with you in Helsinki. I am grateful to the local collaborators Pekka Haapaniemi, Professor Garry Corthals, and Veronika Suni, who have enabled the phosphoproteomics project together with Susumu and to Artur Padzik for the help with lentiviruses and CRISPR. I thank Professor Sampsa Hautaniemi, Professor Olli-Pekka Kallioniemi, John Patrick Mpindi, and Daniel Nicorici for the bioinformatics effort on the first project and Professor Heikki Joensuu, Harri Sihto, and Paula Pernilä for the analysis and access to a valuable clinical resource.

Thanks to all Westermarck lab members past and present! I want to specifically thank Minna Niemelä for my initiation to the field, Amanpreet Kaur for peer

support and for sharing the lab with me for the longest time, Xi Qiao for enjoyable early morning discussions on parenting, Mikael Jumppanen for helping me a lot in a short time, Laxman Yetukuri for being the bioinformatician that I always wanted, Taina Kalevo-Mattila for keeping things running and doing a lot of stuff for me, and Tiina Arsiola for keeping different things running and also doing a lot of stuff for me. Thank you to Juha Okkeri – If I was more accurate or you were more agile our badminton games would not have been as tight, and probably not as enjoyable either. Thank you to Anni Laine, Christian Rupp, and Karolina Pavic for sharing your expertise and thank you to Nora Mäkelä, Oxana Denisova, Anna Lipsanen, Christophe Come, Johanna Jukkala, Taru Varila, Tuuli Halonen, Inga Pukonen, Yuba Pokharel, and Anchit Khanna for the many memorable moments in and outside of the Westermarck lab. Thanks to the next generation, Joni Merisaari, Srikar Nagelli, and Mukund Sharma for making the lab lively again and best of luck in your future work.

I am very grateful for the financial support that this work has received from the Turku Graduate School for Biomedical Sciences (TuBS), Finnish medical foundation, Finnish Cultural Foundation, Instrumentarium Science Foundation, University of Turku, The Cancer Society of Finland, Emil Aaltosen säätiö, The Maud Kuistila Memorial Foundation, K.Albin Johanssons stiftelse, The Paulo Foundation, Waldemar von Frenckells stiftelse, Oskar Öflunds Stiftelse, Suomalais-Norjalainen lääketieteen säätiö, and Ida Montinin säätiö.

I feel privileged and proud to have been part of the TuBS before the homogenization of the graduate programs, and I want thank to Professor Olli Lassila and Nina Widberg for arranging all the great scientific and extracurricular activities.

I thank my thesis committee, Professor Lea Sistonen, Attila Gyenesei, and Susumu for all the advice that you have given. I would like thank Outi Irjala for guiding me through the bureaucratic procedures required for graduation.

I want to thank Professor Johanna Ivaska and the research organization that she commands, and Panu Jaakkola and his group, Krista Rantanen, Heidi Högel, and, Petra Miikkulainen, for the many scientific and non-scientific interactions and for contributing to a pleasant work atmosphere.

I take this opportunity to also thank my friends and relatives who have not contributed to this thesis at all (except Kari who killed one hypothesis) but who have contributed immensely to my quality of life during these years.

I want to thank my parents, Saana and Lassi, for their unconditional support in all my choices and for raising a kid with an inquisitive nature and interest for natural phenomena. I want to thank my parents, my siblings Julle, Hanna, and Pinja, and my extended family, Raija and Ville, for all the support and for being great grandparents, aunts and uncles to our kids. I also thank my father for many interesting discussions on comparative biology (e.g. Fig. 1)

Lastly, I want to thank my wife, Leni, for the past 14 years and for taking the next leap with me. Thank you also for taking the main responsibility in the two most important and successful collaborations of my life. Most importantly, I want to thank our sons, Konsta and Tuukka, for teaching me more about life than I thought was possible. Nothing has affected my life as much as you three have, and nothing will come even close to being as important to me as you are.

Turku, December 2016

Otto Kauko

9. REFERENCES

1. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
2. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
3. Domazet-Loso, T. & Tautz, D. Phylostratigraphic tracking of cancer genes suggests a link to the emergence of multicellularity in metazoa. *BMC Biol* **8**, 66 (2010).
4. Chen, H., Lin, F., Xing, K. & He, X. The reverse evolution from multicellularity to unicellularity during carcinogenesis. *Nat Commun* **6**, 6367 (2015).
5. Lazebnik, Y. What are the hallmarks of cancer? *Nat Rev Cancer* **10**, 232-233 (2010).
6. Peto, R., Roe, F.J., Lee, P.N., Levy, L. & Clack, J. Cancer and ageing in mice and men. *Br J Cancer* **32**, 411-426 (1975).
7. Rangarajan, A. & Weinberg, R.A. Opinion: Comparative biology of mouse versus human cells: modelling human cancer in mice. *Nat Rev Cancer* **3**, 952-959 (2003).
8. Leroi, A.M., Koufopanou, V. & Burt, A. Cancer selection. *Nat Rev Cancer* **3**, 226-231 (2003).
9. Tomasetti, C. & Vogelstein, B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* **347**, 78-81 (2015).
10. Fyg, W. 152 (Laboratoire de recherches apicoles, 1963).
11. Roston, R.A., Lickorish, D. & Buchholtz, E.A. Anatomy and age estimation of an early blue whale (Balaenoptera musculus) fetus. *Anat Rec (Hoboken)* **296**, 709-722 (2013).
12. Kahya, Y., Gençer, H.V. & Woyke, J. Weight at emergence of honey bee (Apis mellifera caucasica) queens and its effect on live weights at the pre and post mating periods. *Journal of Apicultural Research* **47** (2008).
13. Ames, B.N., Shigenaga, M.K. & Hagen, T.M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci U S A* **90**, 7915-7922 (1993).
14. Abegglen, L.M. et al. Potential Mechanisms for Cancer Resistance in Elephants and Comparative Cellular Response to DNA Damage in Humans. *Jama* **314**, 1850-1860 (2015).
15. Rangarajan, A., Hong, S.J., Gifford, A. & Weinberg, R.A. Species- and cell type-specific requirements for cellular transformation. *Cancer Cell* **6**, 171-183 (2004).
16. Chang, L.S., Pan, S., Pater, M.M. & Di Mayorca, G. Differential requirement for SV40 early genes in immortalization and transformation of primary rat and human embryonic cells. *Virology* **146**, 246-261 (1985).
17. Khoury, G.A., Baliban, R.C. & Floudas, C.A. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Scientific Reports, Published online: 13 September 2011; | doi:10.1038/srep00090* (2011).
18. Hunter, T. Why nature chose phosphate to modify proteins. *Philos Trans R Soc Lond B Biol Sci* **367**, 2513-2516 (2012).
19. Kamerlin, S.C., Sharma, P.K., Prasad, R.B. & Warshel, A. Why nature really chose phosphate. *Q Rev Biophys* **46**, 1-132 (2013).
20. Zheng, X.F., Kalev, P. & Chowdhury, D. Emerging role of protein phosphatases changes the landscape of phospho-signaling in DNA damage response. *DNA Repair (Amst)* **32**, 58-65 (2015).
21. Afonso, O. et al. Feedback control of chromosome separation by a midzone Aurora B gradient. *Science* **345**, 332-336 (2014).
22. Chan, G., Kalaitzidis, D. & Neel, B.G. The tyrosine phosphatase Shp2 (PTPN11) in cancer. *Cancer Metastasis Rev* **27**, 179-192 (2008).
23. Hunter, T. & Sefton, B.M. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. *Proc Natl Acad Sci U S A* **77**, 1311-1315 (1980).

24. Olsen, J.V. et al. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. *Cell* **127**, 635-648 (2006).

25. Sharma, K. et al. Ultradeep human phosphoproteome reveals a distinct regulatory nature of tyr and ser/thr-based signaling. *Cell Rep* **8**, 1583-1594 (2014).

26. Hornbeck, P.V. et al. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res* **43**, D512-520 (2015).

27. Gao, J. & Xu, D. Correlation between posttranslational modification and intrinsic disorder in protein. *Pac Symp Biocomput*, 94-103 (2012).

28. Iakoucheva, L.M. et al. The importance of intrinsic disorder for protein phosphorylation. *Nucleic Acids Res* **32**, 1037-1049 (2004).

29. Schweiger, R. & Linial, M. Cooperativity within proximal phosphorylation sites is revealed from large-scale proteomics data. *Biol Direct* **5**, 6 (2010).

30. Holt, L.J. et al. Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science* **325**, 1682-1686 (2009).

31. Pearlman, S.M., Serber, Z. & Ferrell, J.E., Jr. A mechanism for the evolution of phosphorylation sites. *Cell* **147**, 934-946 (2011).

32. Ciesla, J., Fraczyk, T. & Rode, W. Phosphorylation of basic amino acid residues in proteins: important but easily missed. *Acta Biochim Pol* **58**, 137-148 (2011).

33. Matthews, H.R. Protein kinases and phosphatases that act on histidine, lysine, or arginine residues in eukaryotic proteins: a possible regulator of the mitogen-activated protein kinase cascade. *Pharmacol Ther* **67**, 323-350 (1995).

34. Fuhs, S.R. et al. Monoclonal 1- and 3-Phosphohistidine Antibodies: New Tools to Study Histidine Phosphorylation. *Cell* **162**, 198-210 (2015).

35. Attwood, P.V. Histidine kinases from bacteria to humans. *Biochem Soc Trans* **41**, 1023-1028 (2013).

36. Wagner, P.D. & Vu, N.D. Histidine to aspartate phosphotransferase activity of nm23 proteins: phosphorylation of aldolase C on Asp-319. *Biochem J* **346 Pt 3**, 623-630 (2000).

37. Kim, Y., Huang, J., Cohen, P. & Matthews, H.R. Protein phosphatases 1, 2A, and 2C are protein histidine phosphatases. *J Biol Chem* **268**, 18513-18518 (1993).

38. Ek, P. et al. Identification and characterization of a mammalian 14-kDa phosphohistidine phosphatase. *Eur J Biochem* **269**, 5016-5023 (2002).

39. Sun, F. et al. Protein cysteine phosphorylation of SarA/MgrA family transcriptional regulators mediates bacterial virulence and antibiotic resistance. *Proc Natl Acad Sci U S A* **109**, 15461-15466 (2012).

40. Manning, G., Whyte, D.B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298**, 1912-1934 (2002).

41. Kostich, M. et al. Human members of the eukaryotic protein kinase family. *Genome Biol* **3**, Research0043 (2002).

42. Fleuren, E.D., Zhang, L., Wu, J. & Daly, R.J. The kinome 'at large' in cancer. *Nat Rev Cancer* **16**, 83-98 (2016).

43. Duong-Ly, K.C. & Peterson, J.R. The human kinome and kinase inhibition. *Curr Protoc Pharmacol* **Chapter 2**, Unit2.9 (2013).

44. Li, X., Wilmanns, M., Thornton, J. & Kohn, M. Elucidating human phosphatase-substrate networks. *Sci Signal* **6**, rs10 (2013).

45. Duan, G., Li, X. & Kohn, M. The human DEPhOsphorylation database DEPOD: a 2015 update. *Nucleic Acids Res* **43**, D531-535 (2015).

46. Alonso, A. et al. Protein tyrosine phosphatases in the human genome. *Cell* **117**, 699-711 (2004).

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

48. Taylor, S.S. & Kornev, A.P. Protein kinases: evolution of dynamic regulatory proteins. *Trends Biochem Sci* **36**, 65-77 (2011).

49. Manning, G., Plowman, G.D., Hunter, T. & Sudarsanam, S. Evolution of protein kinase signaling from yeast to man. *Trends Biochem Sci* **27**, 514-520 (2002).

50. King, N. et al. The genome of the choanoflagellate Monosiga brevicollis and the origin of metazoans. *Nature* **451**, 783-788 (2008).

51. Manning, G., Young, S.L., Miller, W.T. & Zhai, Y. The protist, Monosiga brevicollis, has a tyrosine kinase signaling network more elaborate and diverse than found in any known metazoan. *Proc Natl Acad Sci U S A* **105**, 9674-9679 (2008).

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

53. Pincus, D., Letunic, I., Bork, P. & Lim, W.A. Evolution of the phospho-tyrosine signaling machinery in premetazoan lineages. *Proc Natl Acad Sci U S A* **105**, 9680-9684 (2008).

54. Schieven, G., Thorner, J. & Martin, G.S. Protein-tyrosine kinase activity in Saccharomyces cerevisiae. *Science* **231**, 390-393 (1986).

55. Sommer, L.M., Cho, H., Choudhary, M. & Seeling, J.M. Evolutionary Analysis of the B56 Gene Family of PP2A Regulatory Subunits. *Int J Mol Sci* **16**, 10134-10157 (2015).

56. Healy, A.M. et al. CDC55, a Saccharomyces cerevisiae gene involved in cellular morphogenesis: identification, characterization, and homology to the B subunit of mammalian type 2A protein phosphatase. *Mol Cell Biol* **11**, 5767-5780 (1991).

57. Strack, S., Zaucha, J.A., Ebner, F.F., Colbran, R.J. & Wadzinski, B.E. Brain protein phosphatase 2A: developmental regulation and distinct cellular and subcellular localization by B subunits. *J Comp Neurol* **392**, 515-527 (1998).

58. Ceulemans, H., Stalmans, W. & Bollen, M. Regulator-driven functional diversification of protein phosphatase-1 in eukaryotic evolution. *Bioessays* **24**, 371-381 (2002).

59. Tan, C.S. et al. Positive selection of tyrosine loss in metazoan evolution. *Science* **325**, 1686-1688 (2009).

60. Su, Z., Huang, W. & Gu, X. Comment on "Positive selection of tyrosine loss in metazoan evolution". *Science* **332**, 917; author reply 917 (2011).

61. Chen, S.C., Chen, F.C. & Li, W.H. Phosphorylated and nonphosphorylated serine and threonine residues evolve at different rates in mammals. *Mol Biol Evol* **27**, 2548-2554 (2010).

62. Landry, C.R., Levy, E.D. & Michnick, S.W. Weak functional constraints on phosphoproteomes. *Trends Genet* **25**, 193-197 (2009).

63. Holt, L.J., Hutti, J.E., Cantley, L.C. & Morgan, D.O. Evolution of Ime2 phosphorylation sites on Cdk1 substrates provides a mechanism to limit the effects of the phosphatase Cdc14 in meiosis. *Mol Cell* **25**, 689-702 (2007).

64. Creixell, P., Schoof, E.M., Tan, C.S. & Linding, R. Mutational properties of amino acid residues: implications for evolvability of phosphorylatable residues. *Philos Trans R Soc Lond B Biol Sci* **367**, 2584-2593 (2012).

65. Pinna, L.A. & Donella-Deana, A. Phosphorylated synthetic peptides as tools for studying protein phosphatases. *Biochim Biophys Acta* **1222**, 415-431 (1994).

66. Pinna, L.A. & Ruzzene, M. How do protein kinases recognize their substrates? *Biochim Biophys Acta* **1314**, 191-225 (1996).

67. Chen, C. et al. Identification of a major determinant for serine-threonine kinase phosphoacceptor specificity. *Mol Cell* **53**, 140-147 (2014).

68. Durocher, D. et al. The molecular basis of FHA domain:phosphopeptide binding specificity and implications for phospho-dependent signaling mechanisms. *Mol Cell* **6**, 1169-1182 (2000).

69. Martin, G.S. The hunting of the Src. *Nat Rev Mol Cell Biol* **2**, 467-475 (2001).

70. Forbes, S.A. et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* **43**, D805-811 (2015).

71. Futreal, P.A. et al. A census of human cancer genes. *Nat Rev Cancer* **4**, 177-183 (2004).

72. Zack, T.I. et al. Pan-cancer patterns of somatic copy number alteration. *Nat Genet* **45**, 1134-1140 (2013).

73. Beroukhim, R. et al. The landscape of somatic copy-number alteration across human cancers. *Nature* **463**, 899-905 (2010).

74. Creixell, P. et al. Kinome-wide decoding of network-attacking mutations rewiring cancer signaling. *Cell* **163**, 202-217 (2015).

75. Howlader, N. et al. in http://seer.cancer.gov/csr/1975_2011/ (National Cancer Institute. Bethesda, MD, 2014).

76. Cohen, P. The structure and regulation of protein phosphatases. *Annu Rev Biochem* **58**, 453-508 (1989).

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

78. Hombauer, H. et al. Generation of active protein phosphatase 2A is coupled to holoenzyme assembly. *PLoS Biol* **5**, e155 (2007).

79. Fellner, T. et al. A novel and essential mechanism determining specificity and activity of protein phosphatase 2A (PP2A) in vivo. *Genes Dev* **17**, 2138-2150 (2003).

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

81. Hemmings, B.A. et al. alpha- and beta-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure. *Biochemistry* **29**, 3166-3173 (1990).

82. Hendrix, P. et al. Analysis of subunit isoforms in protein phosphatase 2A holoenzymes from rabbit and Xenopus. *J Biol Chem* **268**, 7330-7337 (1993).

83. Zhou, J., Pham, H.T., Ruediger, R. & Walter, G. Characterization of the Aalpha and Abeta subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution. *Biochem J* **369**, 387-398 (2003).

84. Götz, J., Probst, A., Ehler, E., Hemmings, B. & Kues, W. Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Calpha. *Proc Natl Acad Sci U S A* **95**, 12370-12375 (1998).

85. Ruediger, R., Ruiz, J. & Walter, G. Human cancer-associated mutations in the Aalpha subunit of protein phosphatase 2A increase lung cancer incidence in Aalpha knock-in and knockout mice. *Mol Cell Biol* **31**, 3832-3844 (2011).

86. Gu, P., Qi, X., Zhou, Y., Wang, Y. & Gao, X. Generation of Ppp2Ca and Ppp2Cb conditional null alleles in mouse. *Genesis* **50**, 429-436 (2012).

87. Orgad, S. et al. The structure of protein phosphatase 2A is as highly conserved as that of protein phosphatase 1. *FEBS Lett* **275**, 44-48 (1990).

88. Zhou, J., Pham, H.T. & Walter, G. The formation and activity of PP2A holoenzymes do not depend on the isoform of the catalytic subunit. *J Biol Chem* **278**, 8617-8622 (2003).

89. Lüss, H. et al. Regional expression of protein phosphatase type 1 and 2A catalytic subunit isoforms in the human heart. *J Mol Cell Cardiol* **32**, 2349-2359 (2000).

90. Gotz, J., Probst, A., Mistl, C., Nitsch, R.M. & Ehler, E. Distinct role of protein phosphatase 2A subunit Calpha in the regulation of E-cadherin and beta-catenin during development. *Mech Dev* **93**, 83-93 (2000).

91. Sablina, A.A. et al. The tumor suppressor PP2A Abeta regulates the RalA GTPase. *Cell* **129**, 969-982 (2007).

92. Murata, K., Wu, J. & Brautigan, D.L. B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. *Proc Natl Acad Sci U S A* **94**, 10624-10629 (1997).

93. Hertz, E.P. et al. A Conserved Motif Provides Binding Specificity to the PP2A-B56 Phosphatase. *Mol Cell* **63**, 686-695 (2016).

94. Cundell, M.J. et al. A PP2A-B55 recognition signal controls substrate dephosphorylation kinetics during mitotic exit. *J Cell Biol* **214**, 539-554 (2016).

95. Endo, S. et al. Molecular identification of human G-substrate, a possible downstream component of the cGMP-dependent protein kinase cascade in cerebellar Purkinje cells. *Proc Natl Acad Sci U S A* **96**, 2467-2472 (1999).

96. Kono, Y. et al. MCM3-binding GANP DNA-primase is associated with a novel phosphatase component G5PR. *Genes Cells* **7**, 821-834 (2002).

97. Kong, M., Ditsworth, D., Lindsten, T. & Thompson, C.B. Alpha4 is an essential regulator of PP2A phosphatase activity. *Mol Cell* **36**, 51-60 (2009).

98. Hwang, J., Lee, J.A. & Pallas, D.C. Leucine Carboxyl Methyltransferase 1 (LCMT-1) Methylates Protein Phosphatase 4 (PP4) and Protein Phosphatase 6 (PP6) and Differentially Regulates the Stable Formation of Different PP4 Holoenzymes. *J Biol Chem* **291**, 21008-21019 (2016).

99. Wandzioch, E. et al. PME-1 modulates protein phosphatase 2A activity to promote the malignant phenotype of endometrial cancer cells. *Cancer Res* **74**, 4295-4305 (2014).

100. Chen, J., Peterson, R.T. & Schreiber, S.L. Alpha 4 associates with protein phosphatases 2A, 4, and 6. *Biochem Biophys Res Commun* **247**, 827-832 (1998).

101. Herzog, F. et al. Structural probing of a protein phosphatase 2A network by chemical cross-linking and mass spectrometry. *Science* **337**, 1348-1352 (2012).

102. Li, X. & Virshup, D.M. Two conserved domains in regulatory B subunits mediate binding to the A subunit of protein phosphatase 2A. *Eur J Biochem* **269**, 546-552 (2002).

103. Moreno, C.S. et al. WD40 repeat proteins striatin and S/G(2) nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A. *J Biol Chem* **275**, 5257-5263 (2000).

104. Gordon, J. et al. Protein phosphatase 2a (PP2A) binds within the oligomerization domain of striatin and regulates the phosphorylation and activation of the mammalian Ste20-Like kinase Mst3. *BMC Biochem* **12**, 54 (2011).

105. Mayer, R.E. et al. Structure of the 55-kDa regulatory subunit of protein phosphatase 2A: evidence for a neuronal-specific isoform. *Biochemistry* **30**, 3589-3597 (1991).

106. Zolnierowicz, S. et al. Diversity in the regulatory B-subunits of protein phosphatase 2A: identification of a novel isoform highly expressed in brain. *Biochemistry* **33**, 11858-11867 (1994).

107. Strack, S., Chang, D., Zaucha, J.A., Colbran, R.J. & Wadzinski, B.E. Cloning and characterization of B delta, a novel regulatory subunit of protein phosphatase 2A. *FEBS Lett* **460**, 462-466 (1999).

108. Manchado, E. et al. Targeting mitotic exit leads to tumor regression in vivo: Modulation by Cdk1, Mastl, and the PP2A/B55alpha,delta phosphatase. *Cancer Cell* **18**, 641-654 (2010).

109. Hendrix, P. et al. Structure and expression of a 72-kDa regulatory subunit of protein phosphatase 2A. Evidence for different size forms produced by alternative splicing. *J Biol Chem* **268**, 15267-15276 (1993).

110. Yan, Z., Fedorov, S.A., Mumby, M.C. & Williams, R.S. PR48, a novel regulatory subunit of protein phosphatase 2A, interacts with Cdc6 and modulates DNA replication in human cells. *Mol Cell Biol* **20**, 1021-1029 (2000).

111. Stevens, I. et al. Identification and characterization of B"-subunits of protein phosphatase 2 A in Xenopus laevis oocytes and adult tissues. *Eur J Biochem* **270**, 376-387 (2003).

112. Voorhoeve, P.M., Hijmans, E.M. & Bernards, R. Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. *Oncogene* **18**, 515-524 (1999).

113. Dagda, R.K., Zaucha, J.A., Wadzinski, B.E. & Strack, S. A developmentally regulated, neuron-specific splice variant of the variable subunit Bbeta targets protein phosphatase 2A to mitochondria and modulates apoptosis. *J Biol Chem* **278**, 24976-24985 (2003).

114. McCright, B., Rivers, A.M., Audlin, S. & Virshup, D.M. The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J Biol Chem* **271**, 22081-22089 (1996).

115. Braconi Quintaje, S. et al. Role of protein phosphatase 2A in the regulation of mitogen-activated protein kinase activity in ventricular cardiomyocytes. *Biochem Biophys Res Commun* **221**, 539-547 (1996).

116. Tanabe, O. et al. Molecular cloning of a 74-kDa regulatory subunit (B" or delta) of human protein phosphatase 2A. *FEBS Lett* **379**, 107-111 (1996).

117. Benoist, M., Gaillard, S. & Castets, F. The striatin family: a new signaling platform in dendritic spines. *J Physiol Paris* **99**, 146-153 (2006).

118. Moreno, C.S., Lane, W.S. & Pallas, D.C. A mammalian homolog of yeast MOB1 is both a member and a putative substrate of striatin family-protein phosphatase 2A complexes. *J Biol Chem* **276**, 24253-24260 (2001).

119. Goudreault, M. et al. A PP2A phosphatase high density interaction network identifies a novel striatin-interacting phosphatase and kinase complex linked to the cerebral cavernous malformation 3 (CCM3) protein. *Mol Cell Proteomics* **8**, 157-171 (2009).

120. Guergnon, J. et al. PP2A targeting by viral proteins: a widespread biological strategy from DNA/RNA tumor viruses to HIV-1. *Biochim Biophys Acta* **1812**, 1498-1507 (2011).

121. Hériché, J.K. et al. Regulation of protein phosphatase 2A by direct interaction with casein kinase 2alpha. *Science* **276**, 952-955 (1997).

122. Pérez, M. & Avila, J. The expression of casein kinase 2alpha' and phosphatase 2A activity. *Biochim Biophys Acta* **1449**, 150-156 (1999).

123. Fuhrer, D.K. & Yang, Y.C. Complex formation of JAK2 with PP2A, P13K, and Yes in response to the hematopoietic cytokine interleukin-11. *Biochem Biophys Res Commun* **224**, 289-296 (1996).

124. Yokoyama, N., Reich, N.C. & Miller, W.T. Determinants for the interaction between Janus kinase 2 and protein phosphatase 2A. *Arch Biochem Biophys* **417**, 87-95 (2003).

125. Yokoyama, N., Reich, N.C. & Miller, W.T. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J Interferon Cytokine Res* **21**, 369-378 (2001).

126. Yokoyama, N. & Miller, W.T. Inhibition of Src by direct interaction with protein phosphatase 2A. *FEBS Lett* **505**, 460-464 (2001).

127. Yokoyama, N. & Miller, W.T. Protein phosphatase 2A interacts with the Src kinase substrate p130(CAS). *Oncogene* **20**, 6057-6065 (2001).

128. Evans, B.J. et al. Physical association of GPR54 C-terminal with protein phosphatase 2A. *Biochem Biophys Res Commun* **377**, 1067-1071 (2008).

129. Cianfanelli, V. et al. AMBRA1 links autophagy to cell proliferation and tumorigenesis by promoting c-Myc dephosphorylation and degradation. *Nat Cell Biol* **17**, 20-30 (2015).

130. Yan, G., Shen, X. & Jiang, Y. Rapamycin activates Tap42-associated phosphatases by abrogating their association with Tor complex 1. *Embo j* **25**, 3546-3555 (2006).

131. Grech, G. et al. Igbp1 is part of a positive feedback loop in stem cell factor-dependent, selective mRNA translation initiation inhibiting erythroid differentiation. *Blood* **112**, 2750-2760 (2008).

132. Liu, J., Prickett, T.D., Elliott, E., Meroni, G. & Brautigan, D.L. Phosphorylation and microtubule association of the Opitz syndrome protein mid-1 is regulated by protein phosphatase 2A via binding to the regulatory subunit alpha 4. *Proc Natl Acad Sci U S A* **98**, 6650-6655 (2001).

133. Wong, P.M., Feng, Y., Wang, J., Shi, R. & Jiang, X. Regulation of autophagy by coordinated action of mTORC1 and protein phosphatase 2A. *Nat Commun* **6**, 8048 (2015).

134. Chen, L.P. et al. α4 is highly expressed in carcinogen-transformed human cells and primary human cancers. *Oncogene* **30**, 2943-2953 (2011).

135. Jiang, L. et al. Structural basis of protein phosphatase 2A stable latency. *Nat Commun* **4**, 1699 (2013).

136. Reid, M.A. et al. The B55α subunit of PP2A drives a p53-dependent metabolic adaptation to glutamine deprivation. *Mol Cell* **50**, 200-211 (2013).

137. Migueleti, D.L., Smetana, J.H., Nunes, H.F., Kobarg, J. & Zanchin, N.I. Identification and characterization of an alternatively spliced isoform of the human protein phosphatase 2Aα catalytic subunit. *J Biol Chem* **287**, 4853-4862 (2012).

138. Takemoto, A. et al. The chromosomal association of condensin II is regulated by a noncatalytic function of PP2A. *Nat Struct Mol Biol* **16**, 1302-1308 (2009).

139. Shenolikar, S. Protein phosphatase regulation by endogenous inhibitors. *Semin Cancer Biol* **6**, 219-227 (1995).

140. Li, M., Guo, H. & Damuni, Z. Purification and characterization of two potent heat-stable protein inhibitors of protein phosphatase 2A from bovine kidney. *Biochemistry* **34**, 1988-1996 (1995).

141. Li, M., Makkinje, A. & Damuni, Z. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* **271**, 11059-11062 (1996).

142. Costanzo, R.V. et al. Anp32e/Cpd1 regulates protein phosphatase 2A activity at synapses during synaptogenesis. *Eur J Neurosci* **23**, 309-324 (2006).

143. Xing, Y. et al. Structural mechanism of demethylation and inactivation of protein phosphatase 2A. *Cell* **133**, 154-163 (2008).

144. Ogris, E. et al. A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A. *J Biol Chem* **274**, 14382-14391 (1999).

145. Longin, S. et al. An inactive protein phosphatase 2A population is associated with methylesterase and can be re-activated by the phosphotyrosyl phosphatase activator. *Biochem J* **380**, 111-119 (2004).

146. Junttila, M.R. et al. CIP2A inhibits PP2A in human malignancies. *Cell* **130**, 51-62 (2007).

147. McConnell, J.L., Gomez, R.J., McCorvey, L.R., Law, B.K. & Wadzinski, B.E. Identification of a PP2A-interacting protein that functions as a negative regulator of phosphatase activity in the ATM/ATR signaling pathway. *Oncogene* **26**, 6021-6030 (2007).

148. Haesen, D. et al. Recurrent PPP2R1A mutations in uterine cancer act through a dominant-negative mechanism to promote malignant cell growth. *Cancer Res* (2016).

149. Gharbi-Ayachi, A. et al. The substrate of Greatwall kinase, Arpp19, controls mitosis by inhibiting protein phosphatase 2A. *Science* **330**, 1673-1677 (2010).

150. Mochida, S., Maslen, S.L., Skehel, M. & Hunt, T. Greatwall phosphorylates an inhibitor of protein phosphatase 2A that is essential for mitosis. *Science* **330**, 1670-1673 (2010).

151. Porter, I.M., Schleicher, K., Porter, M. & Swedlow, J.R. Bod1 regulates protein phosphatase 2A at mitotic kinetochores. *Nat Commun* **4**, 2677 (2013).

152. Kawabe, T., Muslin, A.J. & Korsmeyer, S.J. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. *Nature* **385**, 454-458 (1997).

153. Riz, I. & Hawley, R.G. G1/S transcriptional networks modulated by the HOX11/TLX1 oncogene of T-cell acute lymphoblastic leukemia. *Oncogene* **24**, 5561-5575 (2005).

154. Chung, C.Y., Koprich, J.B., Endo, S. & Isacson, O. An endogenous serine/threonine protein phosphatase inhibitor, G-substrate, reduces vulnerability in models of Parkinson's disease. *J Neurosci* **27**, 8314-8323 (2007).

155. Letourneux, C., Rocher, G. & Porteu, F. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *Embo j* **25**, 727-738 (2006).

156. Cristobal, I. et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. *Blood* **115**, 615-625 (2010).

157. Vera, J. et al. Heterogeneous nuclear ribonucleoprotein A2 is a SET-binding protein and a PP2A inhibitor. *Oncogene* **25**, 260-270 (2006).

158. Sents, W., Ivanova, E., Lambrecht, C., Haesen, D. & Janssens, V. The biogenesis of active protein phosphatase 2A holoenzymes: a tightly regulated process creating phosphatase specificity. *FEBS J* (2012).

159. Longin, S. et al. Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit. *J Biol Chem* **282**, 26971-26980 (2007).

160. Nunbhakdi-Craig, V. et al. Expression of protein phosphatase 2A mutants and silencing of the regulatory B alpha subunit induce a selective loss of acetylated and detyrosinated microtubules. *J Neurochem* **101**, 959-971 (2007).

161. Stanevich, V. et al. The structural basis for tight control of PP2A methylation and function by LCMT-1. *Mol Cell* **41**, 331-342 (2011).

162. Puustinen, P. et al. PME-1 protects extracellular signal-regulated kinase pathway activity from protein phosphatase 2A-mediated inactivation in human malignant glioma. *Cancer Res* **69**, 2870-2877 (2009).

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

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

165. Chen, J., Martin, B.L. & Brautigan, D.L. Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science* **257**, 1261-1264 (1992).

166. Zonta, F. et al. Lyn sustains oncogenic signaling in chronic lymphocytic leukemia by strengthening SET-mediated inhibition of PP2A. *Blood* **125**, 3747-3755 (2015).

167. Evans, D.R. & Hemmings, B.A. Important role for phylogenetically invariant PP2Acalpha active site and C-terminal residues revealed by mutational analysis in Saccharomyces cerevisiae. *Genetics* **156**, 21-29 (2000).

168. Kotlo, K. et al. PR65A phosphorylation regulates PP2A complex signaling. *PLoS One* **9**, e85000 (2014).

169. Schmitz, M.H. et al. Live-cell imaging RNAi screen identifies PP2A-B55alpha and importin-beta1 as key mitotic exit regulators in human cells. *Nat Cell Biol* **12**, 886-893 (2010).

170. Grallert, A. et al. A PP1-PP2A phosphatase relay controls mitotic progression. *Nature* **517**, 94-98 (2015).

171. Kim, K.Y. et al. Adiponectin-activated AMPK stimulates dephosphorylation of AKT through protein phosphatase 2A activation. *Cancer Res* **69**, 4018-4026 (2009).

172. Shouse, G.P., Nobumori, Y., Panowicz, M.J. & Liu, X. ATM-mediated phosphorylation activates the tumor-suppressive function of B56γ-PP2A. *Oncogene* **30**, 3755-3765 (2011).

173. Yu, L.G., Packman, L.C., Weldon, M., Hamlett, J. & Rhodes, J.M. Protein phosphatase 2A, a negative regulator of the ERK signaling pathway, is activated by tyrosine phosphorylation of putative HLA class II-associated protein I (PHAPI)/pp32 in response to the antiproliferative lectin, jacalin. *J Biol Chem* **279**, 41377-41383 (2004).

174. Oaks, J.J. et al. Antagonistic activities of the immunomodulator and PP2A-activating drug FTY720 (Fingolimod, Gilenya) in Jak2-driven hematologic malignancies. *Blood* **122**, 1923-1934 (2013).

175. Suganuma, M. et al. Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter. *Proc Natl Acad Sci U S A* **85**, 1768-1771 (1988).

176. Suganuma, M. et al. Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. *Cancer Res* **50**, 3521-3525 (1990).

177. Fujiki, H. & Suganuma, M. Tumor promoters--microcystin-LR, nodularin and TNF-alpha and human cancer development. *Anticancer Agents Med Chem* **11**, 4-18 (2011).

178. Pallas, D.C. et al. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. *Cell* **60**, 167-176 (1990).

179. Yang, S.I. et al. Control of protein phosphatase 2A by simian virus 40 small-t antigen. *Mol Cell Biol* **11**, 1988-1995 (1991).

180. Mungre, S. et al. Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation. *J Virol* **68**, 1675-1681 (1994).

181. Porras, A. et al. A novel simian virus 40 early-region domain mediates transactivation of the cyclin A promoter by small-t antigen and is required for transformation in small-t antigen-dependent assays. *J Virol* **70**, 6902-6908 (1996).

182. Hahn, W.C. et al. Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464-468 (1999).

183. Yu, J., Boyapati, A. & Rundell, K. Critical role for SV40 small-t antigen in human cell transformation. *Virology* **290**, 192-198 (2001).

184. Hahn, W.C. et al. Enumeration of the simian virus 40 early region elements necessary for human cell transformation. *Mol Cell Biol* **22**, 2111-2123 (2002).

185. Wei, W., Jobling, W.A., Chen, W., Hahn, W.C. & Sedivy, J.M. in Mol Cell Biol, Vol. 23 2859-2870 (2003).

186. Chen, Y. et al. Structural and biochemical insights into the regulation of protein phosphatase 2A by small t antigen of SV40. *Nat Struct Mol Biol* **14**, 527-534 (2007).

187. Chen, W. et al. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* **5**, 127-136 (2004).

188. Tan, J. et al. B55beta-associated PP2A complex controls PDK1-directed myc signaling and modulates rapamycin sensitivity in colorectal cancer. *Cancer Cell* **18**, 459-471 (2010).

189. Chen, L.P. et al. alpha4 is highly expressed in carcinogen-transformed human cells and primary human cancers. *Oncogene* **30**, 2943-2953 (2011).

190. Zhao, J.J. et al. Human mammary epithelial cell transformation through the activation of phosphatidylinositol 3-kinase. *Cancer Cell* **3**, 483-495 (2003).

191. Karst, A.M., Levanon, K. & Drapkin, R. Modeling high-grade serous ovarian carcinogenesis from the fallopian tube. *Proc Natl Acad Sci U S A* **108**, 7547-7552 (2011).

192. Yeh, E. et al. A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol* **6**, 308-318 (2004).

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

194. Sotillo, E., Garriga, J., Kurimchak, A. & Graña, X. Cyclin E and SV40 small T antigen cooperate to bypass quiescence and contribute to transformation by activating CDK2 in human fibroblasts. *J Biol Chem* **283**, 11280-11292 (2008).

195. Miller, J.P. et al. p27kip1 protein levels reflect a nexus of oncogenic signaling during cell transformation. *J Biol Chem* **287**, 19775-19785 (2012).

196. Bikel, I. et al. SV40 small t antigen enhances the transformation activity of limiting concentrations of SV40 large T antigen. *Cell* **48**, 321-330 (1987).

197. Laine, A. & Westermarck, J. Molecular pathways: harnessing E2F1 regulation for prosenescence therapy in p53-defective cancer cells. *Clin Cancer Res* **20**, 3644-3650 (2014).

198. Laine, A. et al. Senescence sensitivity of breast cancer cells is defined by positive feedback loop between CIP2A and E2F1. *Cancer Discov* **3**, 182-197 (2013).

199. Nobumori, Y. et al. Characterization of B56gamma tumor-associated mutations reveals mechanisms for B56gamma-PP2A tumor suppressor activity. *Mol Cancer Res* (2013).

200. Wu, C.H. et al. Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation. *Proc Natl Acad Sci U S A* **104**, 13028-13033 (2007).

201. Zhuang, D. et al. C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. *Oncogene* **27**, 6623-6634 (2008).

202. Mannava, S. et al. PP2A-B56alpha controls oncogene-induced senescence in normal and tumor human melanocytic cells. *Oncogene* **31**, 1484-1492 (2012).

203. Hydbring, P. et al. Phosphorylation by Cdk2 is required for Myc to repress Ras-induced senescence in cotransformation. *Proc Natl Acad Sci U S A* **107**, 58-63 (2010).

204. Campaner, S. et al. Cdk2 suppresses cellular senescence induced by the c-myc oncogene. *Nat Cell Biol* **12**, 54-59; sup pp 51-14 (2010).

205. Lucas, C.M. et al. Second generation tyrosine kinase inhibitors prevent disease progression in high-risk (high CIP2A) chronic myeloid leukaemia patients. *Leukemia* **29**, 1514-1523 (2015).

206. Gibson, W.J. et al. The genomic landscape and evolution of endometrial carcinoma progression and abdominopelvic metastasis. *Nat Genet* **48**, 848-855 (2016).

207. O'Hara, A.J. & Bell, D.W. The genomics and genetics of endometrial cancer. *Adv Genomics Genet* **2012**, 33-47 (2012).

208. Kuhn, E. et al. Identification of molecular pathway aberrations in uterine serous carcinoma by genome-wide analyses. *J Natl Cancer Inst* **104**, 1503-1513 (2012).

209. McConechy, M.K. et al. Use of mutation profiles to refine the classification of endometrial carcinomas. *J Pathol* **228**, 20-30 (2012).

210. Zhao, S. et al. Landscape of somatic single-nucleotide and copy-number mutations in uterine serous carcinoma. *Proc Natl Acad Sci U S A* **110**, 2916-2921 (2013).

211. Le Gallo, M. et al. Exome sequencing of serous endometrial tumors identifies recurrent somatic mutations in chromatin-remodeling and ubiquitin ligase complex genes. *Nat Genet* **44**, 1310-1315 (2012).

212. Kurimchak, A. & Grana, X. PP2A holoenzymes negatively and positively regulate cell cycle progression by dephosphorylating pocket proteins and multiple CDK substrates. *Gene* **499**, 1-7 (2012).

213. Naetar, N. et al. PP2A-Mediated Regulation of Ras Signaling in G2 Is Essential for Stable Quiescence and Normal G1 Length. *Mol Cell* **54**, 932-945 (2014).

214. Foley, E.A. & Kapoor, T.M. Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. *Nat Rev Mol Cell Biol* **14**, 25-37 (2013).

215. Dephoure, N. et al. A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci U S A* **105**, 10762-10767 (2008).

216. Bollen, M. Cell cycle: It takes three to find the exit. *Nature* **517**, 29-30 (2015).

217. Vigneron, S. et al. Greatwall maintains mitosis through regulation of PP2A. *Embo j* **28**, 2786-2793 (2009).

218. Kitajima, T.S. et al. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature* **441**, 46-52 (2006).

219. Riedel, C.G. et al. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature* **441**, 53-61 (2006).

220. Foley, E.A., Maldonado, M. & Kapoor, T.M. Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. *Nat Cell Biol* **13**, 1265-1271 (2011).

221. Qian, J., Beullens, M., Lesage, B. & Bollen, M. Aurora B defines its own chromosomal targeting by opposing the recruitment of the phosphatase scaffold Repo-Man. *Curr Biol* **23**, 1136-1143 (2013).

222. Suijkerbuijk, S.J., Vleugel, M., Teixeira, A. & Kops, G.J. Integration of kinase and phosphatase activities by BUBR1 ensures formation of stable kinetochore-microtubule attachments. *Dev Cell* **23**, 745-755 (2012).

223. Kruse, T. et al. Direct binding between BubR1 and B56-PP2A phosphatase complexes regulate mitotic progression. *J Cell Sci* **126**, 1086-1092 (2013).

224. Nijenhuis, W., Vallardi, G., Teixeira, A., Kops, G.J. & Saurin, A.T. Negative feedback at kinetochores underlies a responsive spindle checkpoint signal. *Nat Cell Biol* **16**, 1257-1264 (2014).

225. Erikson, J., ar-Rushdi, A., Drwinga, H.L., Nowell, P.C. & Croce, C.M. Transcriptional activation of the translocated c-myc oncogene in burkitt lymphoma. *Proc Natl Acad Sci U S A* **80**, 820-824 (1983).

226. Taub, R. et al. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc Natl Acad Sci U S A* **79**, 7837-7841 (1982).

227. Hu, Z. et al. Genome-wide profiling of HPV integration in cervical cancer identifies clustered genomic hot spots and a potential microhomology-mediated integration mechanism. *Nat Genet* **47**, 158-163 (2015).

228. Sur, I., Tuupanen, S., Whitington, T., Aaltonen, L.A. & Taipale, J. Lessons from functional analysis of genome-wide association studies. *Cancer Res* **73**, 4180-4184 (2013).

229. Pomerantz, M.M. et al. The 8q24 cancer risk variant rs6983267 shows long-range interaction with MYC in colorectal cancer. *Nat Genet* **41**, 882-884 (2009).

230. Tuupanen, S. et al. The common colorectal cancer predisposition SNP rs6983267 at chromosome 8q24 confers potential to enhanced Wnt signaling. *Nat Genet* **41**, 885-890 (2009).

231. Xiang, J.F., Yang, L. & Chen, L.L. The long noncoding RNA regulation at the MYC locus. *Curr Opin Genet Dev* **33**, 41-48 (2015).

232. Ling, H. et al. CCAT2, a novel noncoding RNA mapping to 8q24, underlies metastatic progression and chromosomal instability in colon cancer. *Genome Res* **23**, 1446-1461 (2013).

233. Sur, I.K. et al. Mice lacking a Myc enhancer that includes human SNP rs6983267 are resistant to intestinal tumors. *Science* **338**, 1360-1363 (2012).

234. Pulverer, B.J. et al. Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. *Oncogene* **9**, 59-70 (1994).

235. Bhatia, K. et al. Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. *Nat Genet* **5**, 56-61 (1993).

236. Hemann, M.T. et al. Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. *Nature* **436**, 807-811 (2005).

237. Chakraborty, A.A. et al. A common functional consequence of tumor-derived mutations within c-MYC. *Oncogene* **34**, 2406-2409 (2015).

238. Junttila, M.R. & Westermarck, J. Mechanisms of MYC stabilization in human malignancies. *Cell Cycle* **7**, 592-596 (2008).

239. Myant, K. et al. Serine 62-Phosphorylated MYC Associates with Nuclear Lamins and Its Regulation by CIP2A Is Essential for Regenerative Proliferation. *Cell Rep* **12**, 1019-1031 (2015).

240. Thomas, L.R. & Tansey, W.P. Proteolytic control of the oncoprotein transcription factor Myc. *Adv Cancer Res* **110**, 77-106 (2011).

241. Kandoth, C. et al. Mutational landscape and significance across 12 major cancer types. *Nature* **502**, 333-339 (2013).

242. King, B. et al. The ubiquitin ligase FBXW7 modulates leukemia-initiating cell activity by regulating MYC stability. *Cell* **153**, 1552-1566 (2013).

243. Cerami, E. et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* **2**, 401-404 (2012).

244. Gao, J. et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* **6**, pl1 (2013).

245. Gupta, S., Seth, A. & Davis, R.J. Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. *Proc Natl Acad Sci U S A* **90**, 3216-3220 (1993).

246. Wang, W. et al. SCP1 regulates c-Myc stability and functions through dephosphorylating c-Myc Ser62. *Oncogene* **35**, 491-500 (2016).

247. Qian, X.J. et al. Inhibition of DNA methyltransferase as a novel therapeutic strategy to overcome acquired resistance to dual PI3K/mTOR inhibitors. *Oncotarget* **6**, 5134-5146 (2015).

248. Liu, L. & Eisenman, R.N. Regulation of c-Myc Protein Abundance by a Protein Phosphatase 2A-Glycogen Synthase Kinase 3β-Negative Feedback Pathway. *Genes Cancer* **3**, 23-36 (2012).

249. Morin, P.J. beta-catenin signaling and cancer. *Bioessays* **21**, 1021-1030 (1999).

250. Popov, N., Schulein, C., Jaenicke, L.A. & Eilers, M. Ubiquitylation of the amino terminus of Myc by SCF(beta-TrCP) antagonizes SCF(Fbw7)-mediated turnover. *Nat Cell Biol* **12**, 973-981 (2010).

251. Guo, Z. et al. PIM inhibitors target CD25-positive AML cells through concomitant suppression of STAT5 activation and degradation of MYC oncogene. *Blood* **124**, 1777-1789 (2014).

252. Khanna, A. et al. MYC-dependent regulation and prognostic role of CIP2A in gastric cancer. *J Natl Cancer Inst* **101**, 793-805 (2009).

253. Niemela, M. et al. CIP2A signature reveals the MYC dependency of CIP2A-regulated phenotypes and its. *Oncogene* **31**, 4266-4278 (2012).

254. Pippa, R. et al. MYC-dependent recruitment of RUNX1 and GATA2 on the SET oncogene promoter enhances PP2A inactivation in acute myeloid leukemia. *Oncotarget* (2016).

255. David, C.J., Chen, M., Assanah, M., Canoll, P. & Manley, J.L. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* **463**, 364-368 (2010).

256. Lucas, C.M. et al. Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukemia is a critical determinant of disease progression. *Blood* **117**, 6660-6668 (2011).

257. Janghorban, M. et al. Targeting c-MYC by antagonizing PP2A inhibitors in breast cancer. *Proc Natl Acad Sci U S A* **111**, 9157-9162 (2014).

258. Mukhopadhyay, A. et al. Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *Faseb j* **23**, 751-763 (2009).

259. Farrell, A.S. et al. Targeting inhibitors of the tumor suppressor PP2A for the treatment of pancreatic cancer. *Mol Cancer Res* **12**, 924-939 (2014).

260. Ventela, S. et al. CIP2A promotes proliferation of spermatogonial progenitor cells and spermatogenesis in mice. *PLoS One* **7**, e33209 (2012).

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

262. Zhou, X.W., Winblad, B., Guan, Z. & Pei, J.J. Interactions between glycogen synthase kinase 3beta, protein kinase B, and protein phosphatase 2A in tau phosphorylation in mouse N2a neuroblastoma cells. *J Alzheimers Dis* **17**, 929-937 (2009).

263. Peterson, R.T., Desai, B.N., Hardwick, J.S. & Schreiber, S.L. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. *Proc Natl Acad Sci U S A* **96**, 4438-4442 (1999).

264. Hartley, D. & Cooper, G.M. Role of mTOR in the degradation of IRS-1: regulation of PP2A activity. *J Cell Biochem* **85**, 304-314 (2002).

265. Yan, L. et al. PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signaling to mTOR. *Mol Cell* **37**, 633-642 (2010).

266. Puustinen, P. et al. CIP2A oncoprotein controls cell growth and autophagy through mTORC1 activation. *J Cell Biol* **204**, 713-727 (2014).

267. Nakashima, A. et al. A positive role of mammalian Tip41-like protein, TIPRL, in the amino-acid dependent mTORC1-signaling pathway through interaction with PP2A. *FEBS Lett* **587**, 2924-2929 (2013).

268. Loveday, C. et al. Mutations in the PP2A regulatory subunit B family genes PPP2R5B, PPP2R5C and PPP2R5D cause human overgrowth. *Hum Mol Genet* **24**, 4775-4779 (2015).

269. Kuo, Y.C. et al. Regulation of phosphorylation of Thr-308 of Akt, cell proliferation, and survival by the B55alpha regulatory subunit targeting of the protein phosphatase 2A holoenzyme to Akt. *J Biol Chem* **283**, 1882-1892 (2008).

270. Neviani, P. et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell* **8**, 355-368 (2005).

271. Neviani, P. et al. PP2A-activating drugs selectively eradicate TKI-resistant chronic myeloid leukemic stem cells. *J Clin Invest* **123**, 4144-4157 (2013).

272. Agarwal, A. et al. Antagonism of SET using OP449 enhances the efficacy of tyrosine kinase inhibitors and overcomes drug resistance in myeloid leukemia. *Clin Cancer Res* (2014).

273. Kiyota, M. et al. FTY720 induces apoptosis of chronic myelogenous leukemia cells via dual activation of BIM and BID and overcomes various types of resistance to tyrosine kinase inhibitors. *Apoptosis* **18**, 1437-1446 (2013).

274. Wang, J. et al. CIP2A is overexpressed and involved in the pathogenesis of chronic myelocytic leukemia by interacting with breakpoint cluster region-Abelson leukemia virus. *Med Oncol* **31**, 112 (2014).

275. Piazza, R. et al. Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. *Nat Genet* **45**, 18-24 (2013).

276. Oakley, K. et al. Setbp1 promotes the self-renewal of murine myeloid progenitors via activation of Hoxa9 and Hoxa10. *Blood* **119**, 6099-6108 (2012).

277. Chen, K.F. et al. Development of erlotinib derivatives as CIP2A-ablating agents independent of EGFR activity. *Bioorg Med Chem* **20**, 6144-6153 (2012).

278. SWEET, B.H. & HILLEMAN, M.R. The vacuolating virus, S.V. 40. *Proc Soc Exp Biol Med* **105**, 420-427 (1960).

279. KOPROWSKI, H. et al. Transformation of cultures of human tissue infected with simian virus SV40. *Acta Unio Int Contra Cancrum* **19**, 362-367 (1963).

280. Eddy, B.E., Borman, G.S., Grubbs, G.E. & Young, R.D. Identification of the oncogenic substance in rhesus monkey kidney cell culture as simian virus 40. *Virology* **17**, 65-75 (1962).

281. Rabson, A.S., O'Conor, G.T., Kirschstein, R.L. & Branigan, W.J. Papillary ependymomas produced in Rattus (Mastomys) natalensis inoculated with vacuolating virus (SV40). *J Natl Cancer Inst* **29**, 765-787 (1962).

282. Poulin, D.L. & DeCaprio, J.A. Is there a role for SV40 in human cancer? *J Clin Oncol* **24**, 4356-4365 (2006).

283. Shah, K.V. SV40 and human cancer: a review of recent data. *Int J Cancer* **120**, 215-223 (2007).

284. Feng, H., Shuda, M., Chang, Y. & Moore, P.S. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* **319**, 1096-1100 (2008).

285. Shuda, M., Kwun, H.J., Feng, H., Chang, Y. & Moore, P.S. Human Merkel cell polyomavirus small T antigen is an oncoprotein targeting the 4E-BP1 translation regulator. *J Clin Invest* **121**, 3623-3634 (2011).

286. Kwun, H.J. et al. Restricted protein phosphatase 2A targeting by Merkel cell polyomavirus small T antigen. *J Virol* **89**, 4191-4200 (2015).

287. Bollag, B., Hofstetter, C.A., Reviriego-Mendoza, M.M. & Frisque, R.J. JC virus small T antigen binds phosphatase PP2A and Rb family proteins and is required for efficient viral DNA replication activity. *PLoS One* **5**, e10606 (2010).

288. Rundell, K., Major, E.O. & Lampert, M. Association of cellular 56,000- and 32,000-molecular-weight protein with BK virus and polyoma virus t-antigens. *J Virol* **37**, 1090-1093 (1981).

289. Dalianis, T. & Hirsch, H.H. Human polyomaviruses in disease and cancer. *Virology* **437**, 63-72 (2013).

290. Chang, Y. et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**, 1865-1869 (1994).

291. Carbone, A. & Gloghini, A. KSHV/HHV8-associated lymphomas. *Br J Haematol* **140**, 13-24 (2008).

292. Shamay, M. et al. A protein array screen for Kaposi's sarcoma-associated herpesvirus LANA interactors links LANA to TIP60, PP2A activity, and telomere shortening. *J Virol* **86**, 5179-5191 (2012).

293. Järviluoma, A. & Ojala, P.M. Cell signaling pathways engaged by KSHV. *Biochim Biophys Acta* **1766**, 140-158 (2006).

294. Viens, L.J. et al. in MMWR Morb Mortal Wkly Rep, Vol. 65 661-666 (2016).

295. Arbyn, M. et al. EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. *Int J Cancer* **131**, 1969-1982 (2012).

296. Pim, D., Massimi, P., Dilworth, S.M. & Banks, L. Activation of the protein kinase B pathway by the HPV-16 E7 oncoprotein occurs through a mechanism involving interaction with PP2A. *Oncogene* **24**, 7830-7838 (2005).

297. White, E.A. et al. Papillomavirus E7 oncoproteins share functions with polyomavirus small T antigens. *J Virol* **89**, 2857-2865 (2015).

298. Yuan, H., Veldman, T., Rundell, K. & Schlegel, R. Simian virus 40 small tumor antigen activates AKT and telomerase and induces anchorage-independent growth of human epithelial cells. *J Virol* **76**, 10685-10691 (2002).

299. Vogelstein, B. et al. Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).

300. Lawrence, M.S. et al. Discovery and saturation analysis of cancer genes across 21 tumour types. *Nature* **505**, 495-501 (2014).

301. Curtis, C. et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346-352 (2012).

302. Lawrence, M.S. et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature* **499**, 214-218 (2013).

303. Kamburov, A. et al. Comprehensive assessment of cancer missense mutation clustering in protein structures. *Proc Natl Acad Sci U S A* **112**, E5486-5495 (2015).

304. Walter, G. & Ruediger, R. Mouse model for probing tumor suppressor activity of protein phosphatase 2A in diverse signaling pathways. *Cell Cycle* **11**, 451-459 (2012).

305. Jones, S. et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science* **330**, 228-231 (2010).

306. McConechy, M.K. et al. Subtype-specific mutation of PPP2R1A in endometrial and ovarian carcinomas. *J Pathol* **223**, 567-573 (2011).

307. Rahman, M. et al. PPP2R1A mutation is a rare event in ovarian carcinoma across histological subtypes. *Anticancer Res* **33**, 113-118 (2013).

308. Shih Ie, M. et al. Somatic mutations of PPP2R1A in ovarian and uterine carcinomas. *Am J Pathol* **178**, 1442-1447 (2011).

309. Hoang, L.N. et al. Targeted mutation analysis of endometrial clear cell carcinoma. *Histopathology* **66**, 664-674 (2015).

310. Trietsch, M.D. et al. CDKN2A(p16) and HRAS are frequently mutated in vulvar squamous cell carcinoma. *Gynecol Oncol* **135**, 149-155 (2014).

311. Houge, G. et al. B56delta-related protein phosphatase 2A dysfunction identified in patients with intellectual disability. *J Clin Invest* **125**, 3051-3062 (2015).

312. Hodis, E. et al. A landscape of driver mutations in melanoma. *Cell* **150**, 251-263 (2012).

313. Krauthammer, M. et al. Exome sequencing identifies recurrent somatic RAC1 mutations in melanoma. *Nat Genet* **44**, 1006-1014 (2012).

314. Bonilla, X. et al. Genomic analysis identifies new drivers and progression pathways in skin basal cell carcinoma. *Nat Genet* **48**, 398-406 (2016).

315. Gold, H.L. et al. PP6C Hotspot Mutations in Melanoma Display Sensitivity to Aurora Kinase Inhibition. *Mol Cancer Res* **12**, 433-439 (2014).

316. Hammond, D. et al. Melanoma-associated mutations in protein phosphatase 6 cause chromosome instability and DNA damage owing to dysregulated Aurora-A. *J Cell Sci* **126**, 3429-3440 (2013).

317. Martin, D. et al. The head and neck cancer cell oncogenome: a platform for the development of precision molecular therapies. *Oncotarget* **5**, 8906-8923 (2014).

318. Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature* **517**, 576-582 (2015).

319. Study, D.D.D. Large-scale discovery of novel genetic causes of developmental disorders. *Nature* **519**, 223-228 (2015).

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

321. Haesen, D., Sents, W., Ivanova, E., Lambrecht, C. & Janssens, V. Cellular inhibitors of Protein Phosphatase PP2A in cancer. *Biomedical Research* **01/2012**, SI 197-211 (2013).

322. Ruediger, R., Pham, H.T. & Walter, G. Disruption of protein phosphatase 2A subunit interaction in human cancers with mutations in the A alpha subunit gene. *Oncogene* **20**, 10-15 (2001).

323. Campbell, I.G. & Manolitsas, T. Absence of PPP2R1B gene alterations in primary ovarian cancers. *Oncogene* **18**, 6367-6369 (1999).

324. Suraweera, N. et al. Mutations within Wnt pathway genes in sporadic colorectal cancers and cell lines. *Int J Cancer* **119**, 1837-1842 (2006).

325. Esplin, E.D. et al. The glycine 90 to aspartate alteration in the Abeta subunit of PP2A (PPP2R1B) associates with breast cancer and causes a deficit in protein function. *Genes Chromosomes Cancer* **45**, 182-190 (2006).

326. Ruediger, R., Pham, H.T. & Walter, G. Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene. *Oncogene* **20**, 1892-1899 (2001).

327. Tamaki, M., Goi, T., Hirono, Y., Katayama, K. & Yamaguchi, A. PPP2R1B gene alterations inhibit interaction of PP2A-Abeta and PP2A-C proteins in colorectal cancers. *Oncol Rep* **11**, 655-659 (2004).

328. Calin, G.A. et al. Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms. *Oncogene* **19**, 1191-1195 (2000).

329. Zhu, Y. et al. PPP2R1B gene in chronic lymphocytic leukemias and mantle cell lymphomas. *Leuk Lymphoma* **41**, 177-183 (2001).

330. Hemmer, S. et al. Alterations in the suppressor gene PPP2R1B in parathyroid hyperplasias and adenomas. *Cancer Genet Cytogenet* **134**, 13-17 (2002).

331. Poretti, G. et al. Chromosome 11q23.1 is an unstable region in B-cell tumor cell lines. *Leuk Res* **35**, 808-813 (2011).

332. Chou, H.C. et al. Alterations of tumour suppressor gene PPP2R1B in hepatocellular carcinoma. *Cancer Lett* **253**, 138-143 (2007).

333. Kalla, C. et al. Analysis of 11q22-q23 deletion target genes in B-cell chronic lymphocytic leukaemia: evidence for a pathogenic role of NPAT, CUL5, and PPP2R1B. *Eur J Cancer* **43**, 1328-1335 (2007).

334. Beca, F., Pereira, M., Cameselle-Teijeiro, J.F., Martins, D. & Schmitt, F. Altered PPP2R2A and Cyclin D1 expression defines a subgroup of aggressive luminal-like breast cancer. *BMC Cancer* **15**, 285 (2015).

335. Liu, W. et al. Homozygous deletions and recurrent amplifications implicate new genes involved in prostate cancer. *Neoplasia* **10**, 897-907 (2008).

336. Cheng, Y. et al. Evaluation of PPP2R2A as a prostate cancer susceptibility gene: a comprehensive germline and somatic study. *Cancer Genet* **204**, 375-381 (2011).

337. Mosca, L. et al. Genome-wide analysis of primary plasma cell leukemia identifies recurrent imbalances associated with changes in transcriptional profiles. *Am J Hematol* **88**, 16-23 (2013).

338. Shouse, G., de Necochea-Campion, R., Mirshahidi, S., Liu, X. & Chen, C.S. Novel B55alpha-PP2A mutations in AML promote AKT T308 phosphorylation and sensitivity to AKT inhibitor-induced growth arrest. *Oncotarget* (2016).

339. Hein, A.L. et al. PR55alpha Subunit of Protein Phosphatase 2A Supports the Tumorigenic and Metastatic Potential of Pancreatic Cancer Cells by Sustaining Hyperactive Oncogenic Signaling. *Cancer Res* **76**, 2243-2253 (2016).

340. Li, S. et al. The adenovirus E4orf4 protein induces G2/M arrest and cell death by blocking protein phosphatase 2A activity regulated by the B55 subunit. *J Virol* **83**, 8340-8352 (2009).

341. Mui, M.Z. et al. Identification of the adenovirus E4orf4 protein binding site on the B55alpha and Cdc55 regulatory subunits of PP2A: Implications for PP2A function, tumor cell killing and viral replication. *PLoS Pathog* **9**, e1003742 (2013).

342. Dewhurst, S.M. et al. Tolerance of whole-genome doubling propagates chromosomal instability and accelerates cancer genome evolution. *Cancer Discov* **4**, 175-185 (2014).

343. Youn, A. & Simon, R. Using passenger mutations to estimate the timing of driver mutations and identify mutator alterations. *BMC Bioinformatics* **14**, 363 (2013).

344. Kalev, P. et al. Loss of PPP2R2A inhibits homologous recombination DNA repair and predicts tumor sensitivity to PARP inhibition. *Cancer Res* **72**, 6414-6424 (2012).

345. Keen, J.C. et al. Epigenetic regulation of protein phosphatase 2A (PP2A), lymphotactin (XCL1) and estrogen receptor alpha (ER) expression in human breast cancer cells. *Cancer Biol Ther* **3**, 1304-1312 (2004).

346. Muggerud, A.A. et al. Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. *Breast Cancer Res* **12**, R3 (2010).

347. Paluszczak, J. et al. Frequent hypermethylation of WNT pathway genes in laryngeal squamous cell carcinomas. *J Oral Pathol Med* **43**, 652-657 (2014).

348. Majchrzak-Celińska, A., Słocińska, M., Barciszewska, A.M., Nowak, S. & Baer-Dubowska, W. Wnt pathway antagonists, SFRP1, SFRP2, SOX17, and PPP2R2B, are methylated in gliomas and SFRP1 methylation predicts shorter survival. *J Appl Genet* **57**, 189-197 (2016).

349. Leone, P.E. et al. Integration of global spectral karyotyping, CGH arrays, and expression arrays reveals important genes in the pathogenesis of glioblastoma multiforme. *Ann Surg Oncol* **19**, 2367-2379 (2012).

350. Vazquez, A. et al. A genetic variant in a PP2A regulatory subunit encoded by the PPP2R2B gene associates with altered breast cancer risk and recurrence. *Int J Cancer* **128**, 2335-2343 (2011).

351. Jamshidi, M. et al. Germline variation in TP53 regulatory network genes associates with breast cancer survival and treatment outcome. *Int J Cancer* **132**, 2044-2055 (2013).

352. Dong, J. et al. Association analyses identify multiple new lung cancer susceptibility loci and their interactions with smoking in the Chinese population. *Nat Genet* **44**, 895-899 (2012).

353. Van Loo, P. et al. Allele-specific copy number analysis of tumors. *Proc Natl Acad Sci U S A* **107**, 16910-16915 (2010).

354. Ito, T. et al. Activation of ERK/IER3/PP2A-B56γ-positive feedback loop in lung adenocarcinoma by allelic deletion of B56γ gene. *Oncol Rep* **35**, 2635-2642 (2016).

355. Makishima, H. et al. Somatic SETBP1 mutations in myeloid malignancies. *Nat Genet* **45**, 942-946 (2013).

356. Vishwakarma, B.A. et al. Runx1 repression by histone deacetylation is critical for Setbp1-induced mouse myeloid leukemia development. *Leukemia* **30**, 200-208 (2016).

357. De Keersmaecker, K. et al. The TLX1 oncogene drives aneuploidy in T cell transformation. *Nat Med* **16**, 1321-1327 (2010).

358. Kataoka, K. et al. Integrated molecular analysis of adult T cell leukemia/lymphoma. *Nat Genet* **47**, 1304-1315 (2015).

359. von Lindern, M. et al. Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol Cell Biol* **12**, 3346-3355 (1992).

360. Kim, J. et al. Molecular characterization of alternative SET-NUP214 fusion transcripts in a case of acute undifferentiated leukemia. *Cancer Genet Cytogenet* **201**, 73-80 (2010).

361. Tomlins, S.A. et al. Distinct classes of chromosomal rearrangements create oncogenic ETS gene fusions in prostate cancer. *Nature* **448**, 595-599 (2007).

362. Miyagi, Y. et al. ETS family-associated gene fusions in Japanese prostate cancer: analysis of 194 radical prostatectomy samples. *Mod Pathol* **23**, 1492-1498 (2010).

363. Attard, G. et al. Heterogeneity and clinical significance of ETV1 translocations in human prostate cancer. *Br J Cancer* **99**, 314-320 (2008).

364. Fornerod, M. et al. Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene* **10**, 1739-1748 (1995).

365. Van Vlierberghe, P. et al. The recurrent SET-NUP214 fusion as a new HOXA activation mechanism in pediatric T-cell acute lymphoblastic leukemia. *Blood* **111**, 4668-4680 (2008).

366. Coenen, E.A. et al. KIAA1524: A novel MLL translocation partner in acute myeloid leukemia. *Leuk Res* **35**, 133-135 (2011).

367. Meyer, C. et al. in Leukemia, Vol. 27 2165-2176 (2013).

368. Adler, H.T., Nallaseth, F.S., Walter, G. & Tkachuk, D.C. HRX leukemic fusion proteins form a heterocomplex with the leukemia-associated protein SET and protein phosphatase 2A. *J Biol Chem* **272**, 28407-28414 (1997).

369. Li, Y. et al. HapMap-based study of CIP2A gene polymorphisms and HCC susceptibility. *Oncol Lett* **4**, 358-364 (2012).

370. Carlson, S.G. et al. Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms' tumor. *J Am Soc Nephrol* **9**, 1873-1880 (1998).

371. Ouellet, V. et al. SET complex in serous epithelial ovarian cancer. *Int J Cancer* **119**, 2119-2126 (2006).

372. Christensen, D.J. et al. SET oncoprotein overexpression in B-cell chronic lymphocytic leukemia and non-Hodgkin lymphoma: a predictor of aggressive disease and a new treatment target. *Blood* **118**, 4150-4158 (2011).

373. Cristobal, I. et al. Overexpression of SET is a recurrent event associated with poor outcome and contributes to protein phosphatase 2A inhibition in acute myeloid leukemia. *Haematologica* **97**, 543-550 (2012).

374. Cristobal, I. et al. Deregulation of the PP2A inhibitor SET shows promising therapeutic implications and determines poor clinical outcome in patients with metastatic colorectal cancer. *Clin Cancer Res* **21**, 347-356 (2015).

375. Liu, H. et al. Overexpression of PP2A inhibitor SET oncoprotein is associated with tumor progression and poor prognosis in human non-small cell lung cancer. *Oncotarget* **6**, 14913-14925 (2015).

376. Kim, J.Y. et al. Inhibition of p53 acetylation by INHAT subunit SET/TAF-Ibeta represses p53 activity. *Nucleic Acids Res* **40**, 75-87 (2012).

377. Fan, Z., Beresford, P.J., Oh, D.Y., Zhang, D. & Lieberman, J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* **112**, 659-672 (2003).

378. Khanna, A. & Pimanda, J.E. Clinical significance of cancerous inhibitor of protein phosphatase 2A in human cancers. *Int J Cancer* **138**, 525-532 (2016).

379. Kauko, O. et al. Label-free quantitative phosphoproteomics with novel pairwise abundance normalization reveals synergistic RAS and CIP2A signaling. *Sci Rep* **5**, 13099 (2015).

380. Barragán, E. et al. CIP2A high expression is a poor prognostic factor in normal karyotype acute myeloid leukemia. *Haematologica* **100**, e183-185 (2015).

381. Bockelman, C. et al. High CIP2A immunoreactivity is an independent prognostic indicator in early-stage tongue cancer. *Br J Cancer* **104**, 1890-1895 (2011).

382. Bockelman, C. et al. Prognostic role of CIP2A expression in serous ovarian cancer. *Br J Cancer* **105**, 989-995 (2011).

383. Ren, J. et al. Expression of CIP2A in renal cell carcinomas correlates with tumour invasion, metastasis and patients' survival. *Br J Cancer* **105**, 1905-1911 (2011).

384. He, H., Wu, G., Li, W., Cao, Y. & Liu, Y. CIP2A is highly expressed in hepatocellular carcinoma and predicts poor prognosis. *Diagn Mol Pathol* **21**, 143-149 (2012).

385. Xue, Y. et al. CIP2A is a predictor of survival and a novel therapeutic target in bladder urothelial cell carcinoma. *Med Oncol* **30**, 406 (2013).

386. Shi, F., Ding, Y., Ju, S., Wu, X. & Cao, S. Expression and prognostic significance of CIP2A in cutaneous malignant melanoma. *Biomarkers* (2013).

387. Wang, L. et al. CIP2A expression is associated with altered expression of epithelial-mesenchymal transition markers and predictive of poor prognosis in pancreatic ductal adenocarcinoma. *Tumour Biol* **34**, 2309-2313 (2013).

388. Ventelä, S. et al. CIP2A is an Oct4 target gene involved in head and neck squamous cell cancer oncogenicity and radioresistance. *Oncotarget* **6**, 144-158 (2015).

389. Xu, P., Huang, Q., Xie, F., Xu, X.L. & Shao, F. Increased expression of CIP2A in cholangiocarcinoma and correlation with poor prognosis. *Hepatogastroenterology* **60**, 669-672 (2013).

390. Wiegering, A. et al. CIP2A influences survival in colon cancer and is critical for maintaining Myc expression. *PLoS One* **8**, e75292 (2013).

391. Teng, H.W. et al. CIP2A is a predictor of poor prognosis in colon cancer. *J Gastrointest Surg* **16**, 1037-1047 (2012).

392. Bockelman, C. et al. CIP2A overexpression is associated with c-Myc expression in colorectal cancer. *Cancer Biol Ther* **13**, 289-295 (2012).

393. Lindskog, C., Edlund, K., Mattsson, J.S. & Micke, P. Immunohistochemistry-based prognostic biomarkers in NSCLC: novel findings on the road to clinical use? *Expert Rev Mol Diagn* **15**, 471-490 (2015).

394. Jeong, A.L. et al. Cancerous Inhibitor of Protein Phosphatase 2A (CIP2A) Protein Is Involved in Centrosome Separation through the Regulation of NIMA (Never In Mitosis Gene A)-related Kinase 2 (NEK2) Protein Activity. *J Biol Chem* **289**, 28-40 (2014).

395. Kim, J.S., Kim, E.J., Oh, J.S., Park, I.C. & Hwang, S.G. CIP2A modulates cell-cycle progression in human cancer cells by regulating the stability and activity of Plk1. *Cancer Res* **73**, 6667-6678 (2013).

396. Zhou, J. et al. Differential expression of the early lung cancer detection marker, heterogeneous nuclear ribonucleoprotein-A2/B1 (hnRNP-A2/B1) in normal breast and neoplastic breast cancer. *Breast Cancer Res Treat* **66**, 217-224 (2001).

397. Yan-Sanders, Y., Hammons, G.J. & Lyn-Cook, B.D. Increased expression of heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP) in pancreatic tissue from smokers and pancreatic tumor cells. *Cancer Lett* **183**, 215-220 (2002).

398. Zech, V.F., Dlaska, M., Tzankov, A. & Hilbe, W. Prognostic and diagnostic relevance of hnRNP A2/B1, hnRNP B1 and S100 A2 in non-small cell lung cancer. *Cancer Detect Prev* **30**, 395-402 (2006).

399. Mizuno, H. et al. Heterogeneous nuclear ribonucleoprotein A2/B1 in association with hTERT is a potential biomarker for hepatocellular carcinoma. *Liver Int* **32**, 1146-1155 (2012).

400. Kadkol, S.S., Brody, J.R., Pevsner, J., Bai, J. & Pasternack, G.R. Modulation of oncogenic potential by alternative gene use in human prostate cancer. *Nat Med* **5**, 275-279 (1999).

401. Walker, B.A. et al. A compendium of myeloma-associated chromosomal copy number abnormalities and their prognostic value. *Blood* **116**, e56-65 (2010).

402. Tsukamoto, Y. et al. Genome-wide analysis of DNA copy number alterations and gene expression in gastric cancer. *J Pathol* **216**, 471-482 (2008).

403. Song, I.S. et al. Inhibition of MKK7-JNK by the TOR signaling pathway regulator-like protein contributes to resistance of HCC cells to TRAIL-induced apoptosis. *Gastroenterology* **143**, 1341-1351 (2012).

404. Cline, M.S. et al. Exploring TCGA Pan-Cancer data at the UCSC Cancer Genomics Browser. *Sci Rep* **3**, 2652 (2013).

405. Chen, J., Parsons, S. & Brautigan, D.L. Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts. *J Biol Chem* **269**, 7957-7962 (1994).

406. Wong, L.L., Zhang, D., Chang, C.F. & Koay, E.S. Silencing of the PP2A catalytic subunit causes HER-2/neu positive breast cancer cells to undergo apoptosis. *Exp Cell Res* **316**, 3387-3396 (2010).

407. Sun, Y. et al. Microcystin-LR induces protein phosphatase 2A alteration in a human liver cell line. *Environ Toxicol* **29**, 1236-1244 (2014).

408. Cristobal, I. et al. Hyperphosphorylation of PP2A in colorectal cancer and the potential therapeutic value showed by its forskolin-induced dephosphorylation and activation. *Biochim Biophys Acta* **1842**, 1823-1829 (2014).

409. Guo, H. & Damuni, Z. Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A. *Proc Natl Acad Sci U S A* **90**, 2500-2504 (1993).

410. Li, M. & Damuni, Z. Okadaic acid and microcystin-LR directly inhibit the methylation of protein phosphatase 2A by its specific methyltransferase. *Biochem Biophys Res Commun* **202**, 1023-1030 (1994).

411. Cristobal, I. et al. Phosphorylated protein phosphatase 2A determines poor outcome in patients with metastatic colorectal cancer. *Br J Cancer* **111**, 756-762 (2014).

412. Rincon, R. et al. PP2A inhibition determines poor outcome and doxorubicin resistance in early breast cancer and its activation shows promising therapeutic effects. *Oncotarget* **6**, 4299-4314 (2015).

413. Irie, A., Harada, K., Araki, N. & Nishimura, Y. Phosphorylation of SET protein at Ser171 by protein kinase D2 diminishes its inhibitory effect on protein phosphatase 2A. *PLoS One* **7**, e51242 (2012).

414. Kalev, P. & Sablina, A.A. Protein phosphatase 2A as a potential target for anticancer therapy. *Anticancer Agents Med Chem* **11**, 38-46 (2011).

415. Kawada, M. et al. Specific inhibitors of protein phosphatase 2A inhibit tumor metastasis through augmentation of natural killer cells. *Int Immunopharmacol* **3**, 179-188 (2003).

416. Swingle, M., Ni, L. & Honkanen, R.E. Small-molecule inhibitors of ser/thr protein phosphatases: specificity, use and common forms of abuse. *Methods Mol Biol* **365**, 23-38 (2007).

417. de Jong, R.S. et al. Phase I and pharmacokinetic study of the topoisomerase II catalytic inhibitor fostriecin. *Br J Cancer* **79**, 882-887 (1999).

418. Le, L.H. et al. Phase I and pharmacokinetic study of fostriecin given as an intravenous bolus daily for five consecutive days. *Invest New Drugs* **22**, 159-167 (2004).

419. Buck, S.B. et al. Fundamental role of the fostriecin unsaturated lactone and implications for selective protein phosphatase inhibition. *J Am Chem Soc* **125**, 15694-15695 (2003).

420. Wang, G.S. Medical uses of mylabris in ancient China and recent studies. *J Ethnopharmacol* **26**, 147-162 (1989).

421. Perrotti, D. & Neviani, P. Protein phosphatase 2A: a target for anticancer therapy. *Lancet Oncol* **14**, e229-238 (2013).

422. Agarwal, K.C. & Parks, R.E., Jr. Forskolin: a potential antimetastatic agent. *Int J Cancer* **32**, 801-804 (1983).

423. Feschenko, M.S., Stevenson, E., Nairn, A.C. & Sweadner, K.J. A novel cAMP-stimulated pathway in protein phosphatase 2A activation. *J Pharmacol Exp Ther* **302**, 111-118 (2002).

424. Trotta, R. et al. The PP2A inhibitor SET regulates natural killer cell IFN-gamma production. *J Exp Med* **204**, 2397-2405 (2007).

425. Trotta, R. et al. The PP2A inhibitor SET regulates granzyme B expression in human natural killer cells. *Blood* **117**, 2378-2384 (2011).

426. Gutierrez, A. et al. Phenothiazines induce PP2A-mediated apoptosis in T cell acute lymphoblastic leukemia. *J Clin Invest* **124**, 644-655 (2014).

427. Chien, W. et al. Activation of protein phosphatase 2A tumor suppressor as potential treatment of pancreatic cancer. *Mol Oncol* **9**, 889-905 (2015).

428. Kastrinsky, D.B. et al. Reengineered tricyclic anti-cancer agents. *Bioorg Med Chem* **23**, 6528-6534 (2015).

429. Matsuoka, Y., Nagahara, Y., Ikekita, M. & Shinomiya, T. A novel immunosuppressive agent FTY720 induced Akt dephosphorylation in leukemia cells. *Br J Pharmacol* **138**, 1303-1312 (2003).

430. Saddoughi, S.A. et al. Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumour suppression via activation of PP2A-RIPK1-dependent necroptosis. *EMBO Mol Med* **5**, 105-121 (2013).

431. Ingwersen, J. et al. Fingolimod in multiple sclerosis: mechanisms of action and clinical efficacy. *Clin Immunol* **142**, 15-24 (2012).

432. Neviani, P. et al. FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest* **117**, 2408-2421 (2007).

433. Patmanathan, S.N., Yap, L.F., Murray, P.G. & Paterson, I.C. The antineoplastic properties of FTY720: evidence for the repurposing of fingolimod. *J Cell Mol Med* **19**, 2329-2340 (2015).

434. Christensen, D.J. et al. Apolipoprotein E and peptide mimetics modulate inflammation by binding the SET protein and activating protein phosphatase 2A. *J Immunol* **186**, 2535-2542 (2011).

435. Hu, X. et al. Inhibition of Pten deficient Castration Resistant Prostate Cancer by Targeting of the SET - PP2A Signaling axis. *Sci Rep* **5**, 15182 (2015).

436. Liu, Z. et al. Cancerous inhibitor of PP2A is targeted by natural compound celastrol for degradation in non-small-cell lung cancer. *Carcinogenesis* (2013).

437. Cortot, A.B. & Jänne, P.A. Molecular mechanisms of resistance in epidermal growth factor receptor-mutant lung adenocarcinomas. *Eur Respir Rev* **23**, 356-366 (2014).

438. Misale, S., Di Nicolantonio, F., Sartore-Bianchi, A., Siena, S. & Bardelli, A. Resistance to anti-EGFR therapy in colorectal cancer: from heterogeneity to convergent evolution. *Cancer Discov* **4**, 1269-1280 (2014).

439. de Bruin, E.C. et al. Reduced NF1 expression confers resistance to EGFR inhibition in lung cancer. *Cancer Discov* **4**, 606-619 (2014).

440. Kemper, K. et al. Intra- and inter-tumor heterogeneity in a vemurafenib-resistant melanoma patient and derived xenografts. *EMBO Mol Med* **7**, 1104-1118 (2015).

441. Millward, T.A., Zolnierowicz, S. & Hemmings, B.A. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem Sci* **24**, 186-191 (1999).

442. Ahmed, D. et al. FTY720 (Fingolimod) sensitizes hepatocellular carcinoma cells to sorafenib-mediated cytotoxicity. *Pharmacol Res Perspect* **3**, e00171 (2015).

443. Cullis, J. et al. The RhoGEF GEF-H1 is required for oncogenic RAS signaling via KSR-1. *Cancer Cell* **25**, 181-195 (2014).

444. Sanjana, N.E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-784 (2014).

445. Balboa, D. et al. Conditionally Stabilized dCas9 Activator for Controlling Gene Expression in Human Cell Reprogramming and Differentiation. *Stem Cell Reports* **5**, 448-459 (2015).

446. Bolstad, B.M. in R package version 1.26.1 (

447. Imanishi, S.Y. et al. Reference-facilitated phosphoproteomics: fast and reliable phosphopeptide validation by microLC-ESI-Q-TOF MS/MS. *Mol Cell Proteomics* **6**, 1380-1391 (2007).

448. Suni, V., Imanishi, S.Y., Maiolica, A., Aebersold, R. & Corthals, G.L. Confident Site Localization Using a Simulated Phosphopeptide Spectral Library. *J Proteome Res* (2015).

449. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550 (2005).

450. Horn, H. et al. KinomeXplorer: an integrated platform for kinome biology studies. *Nat Methods* **11**, 603-604 (2014).

451. Xue, Y. et al. GPS 2.0, a tool to predict kinase-specific phosphorylation sites in hierarchy. *Mol Cell Proteomics* **7**, 1598-1608 (2008).

452. Chou, M.F. & Schwartz, D. Biological sequence motif discovery using motif-x. *Curr Protoc Bioinformatics* **Chapter 13**, Unit 13.15-24 (2011).

453. Miller, L.D. et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci U S A* **102**, 13550-13555 (2005).

454. Enerly, E. et al. miRNA-mRNA integrated analysis reveals roles for miRNAs in primary breast tumors. *PLoS One* **6**, e16915 (2011).

455. Pemovska, T. et al. Individualized systems medicine strategy to tailor treatments for patients with chemorefractory acute myeloid leukemia. *Cancer Discov* **3**, 1416-1429 (2013).

456. Yadav, B. et al. Quantitative scoring of differential drug sensitivity for individually optimized anticancer therapies. *Sci Rep* **4**, 5193 (2014).

457. Guzman, C., Bagga, M., Kaur, A., Westermarck, J. & Abankwa, D. ColonyArea: an ImageJ plugin to automatically quantify colony formation in clonogenic assays. *PLoS One* **9**, e92444 (2014).

458. Ritchie, M.E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* **43**, e47 (2015).

459. Kumar, L. & M, E.F. Mfuzz: A software package for soft clustering of microarray data. *Bioinformation* **2**, 5-7 (2007).

460. Joensuu, H. et al. Amplification of erbB2 and erbB2 expression are superior to estrogen receptor status as risk factors for distant recurrence in pT1N0M0 breast cancer: a nationwide population-based study. *Clin Cancer Res* **9**, 923-930 (2003).

461. Xu, J., Chen, Y. & Olopade, O.I. MYC and Breast Cancer. *Genes Cancer* **1**, 629-640 (2010).

462. Futschik, M.E. & Carlisle, B. Noise-robust soft clustering of gene expression time-course data. *J Bioinform Comput Biol* **3**, 965-988 (2005).

463. Linding, R. et al. Systematic discovery of in vivo phosphorylation networks. *Cell* **129**, 1415-1426 (2007).

464. Harwood, F.C., Shu, L. & Houghton, P.J. mTORC1 signaling can regulate growth factor activation of p44/42 mitogen-activated protein kinases through protein phosphatase 2A. *J Biol Chem* **283**, 2575-2585 (2008).

465. Koskinen, P.J., Vastrik, I., Makela, T.P., Eisenman, R.N. & Alitalo, K. Max activity is affected by phosphorylation at two NH2-terminal sites. *Cell Growth Differ* **5**, 313-320 (1994).

466. Adey, A. et al. The haplotype-resolved genome and epigenome of the aneuploid HeLa cancer cell line. *Nature* **500**, 207-211 (2013).

467. Rajalingam, K., Schreck, R., Rapp, U.R. & Albert, S. Ras oncogenes and their downstream targets. *Biochim Biophys Acta* **1773**, 1177-1195 (2007).

468. Ebi, H. et al. Receptor tyrosine kinases exert dominant control over PI3K signaling in human KRAS mutant colorectal cancers. *J Clin Invest* **121**, 4311-4321 (2011).

469. Lamba, S. et al. RAF suppression synergizes with MEK inhibition in KRAS mutant cancer cells. *Cell Rep* **8**, 1475-1483 (2014).

470. Turke, A.B. et al. MEK inhibition leads to PI3K/AKT activation by relieving a negative feedback on ERBB receptors. *Cancer Res* **72**, 3228-3237 (2012).

471. Hydbring, P. & Larsson, L.G. Tipping the balance: Cdk2 enables Myc to suppress senescence. *Cancer Res* **70**, 6687-6691 (2010).

472. Riley, N.M. & Coon, J.J. Phosphoproteomics in the Age of Rapid and Deep Proteome Profiling. *Anal Chem* **88**, 74-94 (2016).

473. Wang, Y.T. et al. An informatics-assisted label-free quantitation strategy that depicts phosphoproteomic profiles in lung cancer cell invasion. *J Proteome Res* **9**, 5582-5597 (2010).

474. de Graaf, E.L., Giansanti, P., Altelaar, A.F. & Heck, A.J. Single step enrichment by Ti4+-IMAC and label free quantitation enables in-depth monitoring of phosphorylation dynamics with high reproducibility and temporal resolution. *Mol Cell Proteomics* (2014).

475. Hunter, T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **80**, 225-236 (1995).

476. Cohen, P. Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem Sci* **17**, 408-413 (1992).

477. Hunter, T. Signaling--2000 and beyond. *Cell* **100**, 113-127 (2000).

478. Meyers, J., Craig, J. & Odde, D.J. Potential for control of signaling pathways via cell size and shape. *Curr Biol* **16**, 1685-1693 (2006).

479. Fumarola, C., Bonelli, M.A., Petronini, P.G. & Alfieri, R.R. Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. *Biochem Pharmacol* **90**, 197-207 (2014).