

Ser/Thr Phosphatases: The New Frontier for Myeloid Leukemia Therapy?

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1. Introduction

Myeloid leukemias are characterised by mutation and altered expression of a range of tyrosine kinases. Over 90% of chronic myeloid leukemias (CML) harbour the Philadelphia chromosome, resulting in expression of the BCR/ABL fusion protein, a constitutively active tyrosine kinase that is essential for survival of the CML cells. Acute myeloid leukemia (AML) is a heterogeneous disease characterised by mutations and dysregulation of a range of tyrosine kinases including the receptors Fms-like tyrosine kinase (Flt-3), c-KIT and platelet derived growth factor receptor (PDGFR). Tyrosine kinases represent powerful therapeutic targets, as the archetypical example of imatinib has shown for CML. However, many patients develop resistance to imatinib and other second generation inhibitors. Furthermore, trials of tyrosine kinase inhibitors for AML have thus far proven disappointing. Thus novel therapeutic targets are needed in order to improve the survival of myeloid leukemia patients.

Oncogenic tyrosine kinases induce activation of a variety of signaling pathways required for the growth and survival of leukemia cells, such as the Ras/MAPK, PI3K/Akt, and JAK/STAT pathways. In addition to protein kinases, the rate and duration of protein phosphorylation is tightly regulated by the activity of protein phosphatases, and in normal cells the reversal of protein phosphorylation by phosphatases is essential for providing the fine-tuning of signaling pathways and maintaining a balance in cellular physiology. While much of the focus for targeted therapies in leukemia therapy has concentrated on the kinases responsible for phosphorylation events, relatively little attention has been given to the role that protein phosphatases play. However, research over the past decade has now begun to highlight the importance of protein phosphatases in leukemia and their potential as targets for novel therapies. In particular, the ser/thr phosphatase PP2A has emerged as an important tumor suppressor in myeloid leukemias and strategies aimed at reactivating this complex enzyme show great promise for a new generation of leukemia therapies.

2. Protein phosphorylation in cellular signaling

Signal transduction via a network of cellular communication pathways enables modulation of essential cellular functions such as proliferation, differentiation, survival, adhesion, motility and death. Phosphorylation is the most common mechanism for the propagation of

intracellular signals. The net phosphorylation state relies on a delicate balance between protein kinases, which catalyse phosphate addition, and protein phosphatases, catalysing phosphate removal. The role of protein kinases in the cellular signaling pathways controlling biological functions has been extensively studied and protein kinases are currently the pharmaceutical industry's second largest drug target (Cohen 2002). In contrast, the role of phosphatases in disease has only recently come to the forefront of research.

Proteins are primarily phosphorylated on Serine (Ser), Threonine (Thr) and Tyrosine (Tyr) residues, each accounting for approximately 86, 12 and 2% of the human phosphoproteome respectively (Olsen *et al.* 2006). Around 2% of the human genome encodes a protein kinase gene, totalling 518 genes, of which 428 are known or predicted to phosphorylate Ser or Thr residues, and 90 encode protein tyrosine kinases (Alonso *et al.* 2004; Manning *et al.* 2002). In contrast, the human genome only encodes 147 phosphatase catalytic subunit genes. Of these, 107 encode a protein tyrosine phosphatase (PTP), a number that is comparable with the opposing tyrosine kinase genes (Alonso *et al.* 2004). Interestingly, while 98% of phosphoprotein sites are Ser and Thr residues, only a handful of the total protein phosphatases are specific for these amino acids (Moorhead *et al.* 2007). The unique way in which Ser/Thr phosphatases are regulated explains the difference in the number of Ser/Thr phosphatase catalytic subunits compared to PTPs. The evolution of PTPs has developed through the addition of discrete modular domains onto a core catalytic domain that define the function of the enzyme. In contrast, Ser/Thr phosphatases consist of a relatively simple catalytic subunit that reversibly binds to additional regulatory or interacting partner proteins which target the complex to specific subcellular locations and substrates, and ultimately control their activity.

3. Classification of protein phosphatases

Protein phosphatases can be classified into three main classes based on characteristics such as sequence, structure and phosphoamino-acid specificity. According to substrate specificity the largest phosphatase class is the ser/thr specific phosphoprotein phosphatase (PPP) family including PP1, PP2A, PP2B and PP4-PP7. The metallo-protein phosphatase dependant (PPM) family, made up mainly of PP2C, also functions against serine and threonine residues. Protein tyrosine phosphatases (PTP) form the second group and the aspartate-based or dual specificity protein phosphatases (DUSPs) the third. Genetic sequencing and analysis maintained these rules of partition, however it has recently been shown that particular Ser/Thr phosphatases can also dephosphorylate Tyr residues and various enzymes that fall into the dual specificity category based on their genetic sequence can selectively function on Ser, Thr, Tyr, phosphoinositides or RNA (Alonso *et al.* 2004; Begley and Dixon 2005).

4. PTPs in myeloid leukemias

The role of PTPs in cancer has been recently reviewed (Jiang and Zhang 2008; Julien *et al.* 2011; Lopez-Ruiz *et al.* 2011; Ruela-de-Sousa *et al.* 2011) and will not be discussed in detail here. However it should be noted that PTPs can act as either tumor suppressors or oncogenes in both solid tumors and leukaemias. For example, SHP1, a non-receptor PTP, displays tumor suppressive properties. SHP1 associates with a number of signaling molecules including CD5, the IL-3 receptor, CD22, the B-cell receptor, c-KIT and BCR/ABL

(Bruecher-Encke *et al.* 2001; Lorenz *et al.* 1996; Zhang *et al.* 2000), and these interactions exert primarily inhibitory effects on the signaling pathways. Silencing of the SHP1 gene via promotor methylation has been identified as a common event in a range of leukemias and lymphomas, and has been suggested as a potential marker for disease progression (Chim *et al.* 2004; Johan *et al.* 2005; Oka *et al.* 2002; Zhang *et al.* 2000). Other PTPs implicated as tumor suppressors in myeloid leukemias include PTEN, PTPN2, DEP-1, and DUSP-16. In contrast, SHP2 displays oncogenic properties as it promotes growth and survival pathways due to its dephosphorylating activity toward negative regulators of the Ras/Erk and PI3K/Akt signaling pathways (Neel *et al.* 2003). Somatic mutations in the SHP2 gene occur in around 30% of sporadic juvenile myelomonocytic leukemia (JMML) cases (Tartaglia *et al.* 2003), 6% of childhood acute lymphoblastic leukemia (ALL) (Tartaglia *et al.* 2004) and 5% of AML (Bentires-Alj *et al.* 2004; Tartaglia and Gelb 2005; Tartaglia *et al.* 2004; Tartaglia *et al.* 2005). Other PTPs with potential oncogenic roles in leukemia include CD45, Cdc25, and DUSP-7.

5. Ser/Thr phosphatases

In the early 1980s Ingebritsen and Cohen utilised a number of characteristics of phosphatases to pioneer nomenclature classes for the various enzymes (Ingebritsen and Cohen 1983a). Biochemical assays, sensitivity to endogenous inhibitors and the limited knowledge of substrate specificity at the time were originally used to classify the Ser/Thr phosphatases as either type 1 (PP1) or type 2 (PP2). PP1 specifically dephosphorylates the β subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of the two small heat- and acid-stable proteins, termed inhibitor-1 (I-1) (Nimmo and Cohen 1978) and inhibitor-2 (I-2) (Foulkes and Cohen 1980). PP2 phosphatases preferentially dephosphorylate the α -subunit of phosphorylase kinase and are insensitive to I-1 and I-2 (Cohen 1989; Ingebritsen and Cohen 1983a; Ingebritsen and Cohen 1983b). PP2 phosphatases could in turn be subclassified into three distinct enzymes, PP2A, PP2B, and PP2C in a number of ways, but most simply by their metal-ion requirement. PP2A does not require a metal ion, however, PP2B and PP2C are Ca^{2+} and Mg^{2+} dependent respectively (Ingebritsen and Cohen 1983a; Ingebritsen and Cohen 1983b; Moorhead *et al.* 2007). More recently the Ser/Thr phosphatases were re-defined according to their structurally distinct gene family as part of the phosphoprotein phosphatase (PPP) family comprising PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7 catalytic subunits, or protein phosphatase Mg^{2+} - or Mn^{2+} -dependent (PPM) family primarily composed of the PP2C catalytic subunit. The majority of Ser/Thr phosphatase activity in vivo is accounted for by the PPP family members PP1, PP2A and PP2B, together with PP2C of the PPM family (Barford *et al.* 1998). In the majority of cases they operate in hetero-oligomeric complexes interacting with an increasing diversity of targeting and regulatory subunits (Gallego and Virshup 2005).

6. Protein phosphatase 2A (PP2A)

The past decade has seen a surge in research into the PPP family member, PP2A, most notably due to the discoveries indicating its important role as a tumor suppressor. In particular PP2A has been implicated as a major player in myeloid leukemias and as a potential target for novel therapeutic strategies. In order to study the role of PP2A in leukemogenesis, understanding the structure and function of this complex enzyme is imperative, and as such is summarised below.

6.1 Structure and regulation of PP2A

PP2A is not a single phosphatase, but essentially encompasses a group of oligomeric enzymes consisting of a well conserved catalytic and structural subunit, together with the addition of one of a variety of regulatory subunits (Fig. 1). It makes up 1% of total cellular proteins and along with PP1, accounts for over 90% of serine/threonine phosphatase activity in the cell (Eichhorn *et al.* 2009). The PP2A core enzyme consists of a structural subunit (PP2A-A/PR65) and a catalytic subunit (PP2Ac). In mammals, two distinct genes (α and β) encode closely related versions of both the A (Hemmings *et al.* 1990) and C subunits (Arino *et al.* 1988). A third regulatory subunit (PP2A B) binds to the AC heterodimer, and determines both the substrate specificity and cellular localisation of PP2A holoenzyme complexes. Three B subunit families have been identified to date: B/B55/PR55 (Mayer *et al.* 1991; Strack *et al.* 1999; Zolnierowicz *et al.* 1994), B'/B56/PR61 (McCright *et al.* 1996; McCright and Virshup 1995), B''/PR72/130/PR70/48 (Hendrix *et al.* 1993; Stevens *et al.* 2003; Yan *et al.* 2000). Regulation of PP2A activity is accomplished primarily by members of its regulatory subunits. An additional level of regulation is introduced by post-translational modification of the catalytic subunit, which can undergo methylation and phosphorylation (Janssens *et al.* 2008), as well as the interaction with a vast array of other cellular and viral proteins such as SET, CIP2A and the SV40 small T antigen (Janssens and Goris 2001; Sablina and Hahn 2008). The use of specific PP2A inhibitors, PP2A activators and molecular genetics tools both *in vitro* and *in vivo* have exposed a role for PP2A in cell morphology, cell cycle regulation, development and apoptosis.

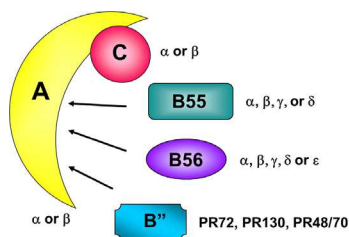


Fig. 1. Schematic of PP2A holoenzymes

6.2 PP2A in cellular signaling

PP2A has been implicated in a wide range of cellular signaling pathways, many of which are involved in cellular proliferation, apoptosis and differentiation, and as such are important in tumorigenesis. Since PP2A is one of the most abundant cellular phosphatases, it is not surprising that PP2A can exert opposing roles on similar pathways by acting at different levels. Recent work indicates that this substrate specificity is mediated by distinct PP2A complexes.

6.2.1 PP2A and MAPK signaling

A major function of PP2A is regulation of the MAPK signaling pathway. Interestingly, PP2A can exert both inhibitory and activating effects in a context-dependent manner (Fig. 2). PP2A acts negatively via the dephosphorylation of MEK (Heriche *et al.* 1997; Sontag *et al.* 1993) and ERK both *in vitro* and in mammalian cells (Alessi *et al.* 1995; Wang *et al.* 2003; Zhou *et al.* 2002). Specific knockdown of B56 β and B56 γ , but not B55 family subunits in NIH3T3 mouse fibroblasts increases basal ERK activation and prolongs ERK signal during stimulation in the

absence of pMEK (Fig. 3A) (Letourneux *et al.* 2006). More recent evidence indicates that PP2A activates Ras-dependent MAPK signaling at the level of Raf-1 and its scaffolding protein Kinase Suppressor of Ras-1 (KSR1) (Abraham *et al.* 2000; Jaumot and Hancock 2001; Kubicek *et al.* 2002; Ory *et al.* 2003). Dephosphorylation of KSR1 (Ser329) and Raf-1 (Ser259) by B55 α -containing PP2A complexes induces membrane translocation and increases the kinase activity of both proteins in several mammalian cell models, including NIH3T3, COS and HEK293 cells (Fig. 3B) (Abraham *et al.* 2000; Adams *et al.* 2005; Dougherty *et al.* 2005; Ory *et al.* 2003). Taken together, these studies provide biochemical mechanisms for how PP2A functions as a negative and positive regulator of MAPK signaling, depending on the specific regulatory subunit and substrate involved.

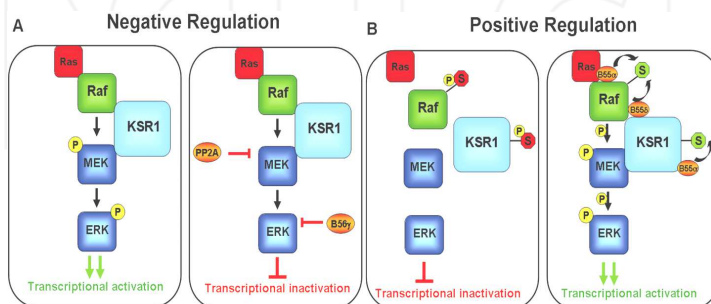


Fig. 2. Schematic overview of MAPK signaling regulation by PP2A

A) PP2A holoenzymes containing B56 family members negatively regulate MAPK signaling by inactivating MEK and ERK. **B)** In the inactive state, Raf and KSR1 are phosphorylated on Ser259 and Ser329, respectively, and are located within the cytoplasm. Upon stimulation, B55 α -containing PP2A complexes dephosphorylate these residues, which results in protein activation and translocation to the plasma membrane. This facilitates the Ras/Raf interaction and induces transcriptional activation.

6.2.2 PP2A and PI3K/Akt signaling

The normal function of Akt is tightly modulated by phosphorylation events on Thr308 or Ser473 (Sarbasov *et al.* 2005; Vanhaesebroeck and Alessi 2000), and PP2A is the major phosphatase targeting these residues *in vitro* (Borgatti *et al.* 2003; Ivaska *et al.* 2002; Resjo *et al.* 2002). Over-expression of B55 α -containing PP2A holoenzymes in the pro-lymphoid FL5.12 cell line substantially dephosphorylates Akt at Thr308 and results in subsequent growth suppression (Kuo *et al.* 2008). Conversely, A α downregulation impairs Akt phosphorylation in neuronal cells, implicating PP2A as a positive regulator of the PI3K/Akt survival signaling cascade (Strack *et al.* 2004). An intriguing study by Andrabi *et al.*, demonstrated that Akt can act as a pro- or anti-apoptotic protein depending on environmental stimuli, and this is governed by PP2A (Andrabi *et al.* 2007). The specific PP2A regulatory subunits controlling this apparent switch remain undefined; however, this study reinforces the importance of understanding PP2A regulated cell signaling in a context dependent manner.

6.2.3 PP2A and Wnt/ β -catenin signaling

The function of PP2A in Wnt/ β -catenin signaling is similar to its role in the MAPK pathway, with individual PP2A subunits exerting either positive or negative effects (Fig. 3). B56

family members associate with APC (Seeling *et al.* 1999; Yamamoto *et al.* 2001) and axin (Hsu *et al.* 1999; Li *et al.* 2001) to impair Wnt signaling; a function that is critical for normal dorsal/ventral axis formation in *Xenopus* development (Li *et al.* 2001). Further studies demonstrate that association of the PP2A PR72 subunit with Naked cuticle is critical for the inhibitory function of this protein on the Wnt pathway (Fig. 3A) (Creyghton *et al.* 2005).

PP2A is also an important positive regulator of Wnt signaling (Bajpai *et al.* 2004; Gotz *et al.* 2000; Ratcliffe *et al.* 2000; Willert *et al.* 1999). Loss of function analysis suggests that B56 ϵ is required for Wnt-mediated development in *Xenopus* embryogenesis (Yang *et al.* 2003). Purified B55 α -containing PP2A holoenzymes directly dephosphorylate β -catenin *in vitro*. Accordingly, specific knockdown of B55 α in SW480 colon cancer cells significantly elevates β -catenin phosphorylation, which induces protein degradation and inhibits the Wnt pathway (Zhang *et al.* 2009). Surprisingly, PR130 opposes the action of PR72 and modulates Wnt signal transduction by restricting the ability of Naked to function as a Wnt inhibitor (Fig. 4B) (Creyghton *et al.* 2006). These studies illustrate an excellent example whereby specific PP2A regulatory subunits determine holoenzyme function and provide the fine control on important cellular processes.

6.2.4 PP2A and p53 regulation

The tumor suppressor, p53, plays a critical role in mediating cellular responses to various types of stress, such as DNA damage, by inducing growth arrest or programmed cell death. The stability and activity of p53 is regulated by phosphorylation which, under normal cellular growth conditions, targets the protein for proteasome-mediated degradation (Vogelstein *et al.* 2000). PP2A B56 γ holoenzymes dephosphorylate p53 on Ser37 (Dohoney *et al.* 2004) and Thr55 (Li *et al.* 2007) following γ radiation; an event which stabilises p53 in response to DNA damage and contributes to apoptosis in mammalian cells. Furthermore, ataxia-telangiectasia mutated (ATM) directly phosphorylates and specifically regulates B56 γ and B56 δ . Phosphorylation of B56 γ 3 at Ser510 after DNA damage increases B56 γ -PP2A complexes, and directs PP2A phosphatase activity toward p53, activating its tumor-suppressive functions (Shouse *et al.* 2010). Conversely, RNAi knockdown of B56 γ reduces p53 stability and inhibits cell death (Li *et al.* 2007). B56 ϵ also regulates the p53-dependent apoptotic pathway by controlling the stability of p53 protein (Jin *et al.* 2010).

Other findings implicate an important role for B56 α -containing PP2A complexes in p53 degradation. One target of p53, cyclin G, recruits B56 α into a quaternary complex with the E3 ubiquitin ligase, mouse double minute 2 (Mdm2) (Okamoto *et al.* 1996; Okamoto *et al.* 2002). The subsequent dephosphorylation and activation of Mdm2 leads to ubiquitination and degradation of p53 (Haupt *et al.* 1997), thus allowing the cell to proliferate. In this context, PP2A serves as a negative regulator.

6.2.5 PP2A and c-Myc regulation

PP2A also plays a prominent role in controlling the accumulation of the proto-oncoprotein, c-Myc (Yeh *et al.* 2004). Aberrant regulation of c-Myc has been linked to transformation in up to 70% of human tumors; therefore tight control of this protein is crucial for maintaining cellular homeostasis (Nesbit *et al.* 1999). c-Myc stability is regulated, in part, through phosphorylation at two residues, Ser62 and Thr58 (Sears *et al.* 2000). Whilst ERK-mediated phosphorylation at Ser62 stabilises c-Myc, specific B56 α -containing PP2A complexes reverse these effects, leading to destabilisation and ubiquitin-mediated degradation (Arnold and

Sears 2006). In addition, a novel PP2A-interacting protein, designated cancerous inhibitor of PP2A (CIP2A), was found to selectively target the catalytic activity of PP2A-B56 α associated with c-Myc, and protect c-Myc from Ser62 dephosphorylation. Accordingly, depletion of CIP2A results in significantly increased PP2A activity measured from c-Myc immunoprecipitates and correlates with c-Myc destabilisation (Junttila *et al.* 2007).

Collectively, these results illustrate the dynamic interaction of the PP2A holoenzyme with signaling cascades involved in fundamental cellular processes such as proliferation, survival and development. The fact that PP2A is involved in both the negative and positive regulation of these pathways highlights the exquisite nature of PP2A modulation and underscores the importance of investigating specific complexes when determining PP2A function. Identification of critical subunits that are aberrantly regulated during transformation may ultimately lead to the development of novel treatments for cancer patients.

6.3 PP2A as a tumor suppressor

The fundamental evidence that implicated PP2A as a tumor suppressor was the discovery that okadaic acid, a tumor promoter (Fujiki and Suganuma 2009; Suganuma *et al.* 1988), potently inhibits the phosphatase activity of PP2A (Bialojan and Takai 1988; Haystead *et al.* 1989). In addition, the oncogenic polyomavirus middle and small tumor (ST) antigens, along with the simian virus 40 (SV40) ST antigen, transforms mammalian cells by inhibiting PP2A (Pallas *et al.* 1990). Transformation of the normal human fibroblast kidney epithelial cell line, HEK293, required several key genetic elements; human telomerase catalytic subunit, an oncogenic allele of H-Ras, and the SV40 large T (LT) and ST antigens (HEK-TER) (Hahn *et al.* 1999). Whilst expression of LT enables the cells to bypass senescence, complete tumor formation requires the addition of ST and thus inhibition of PP2A (Yu *et al.* 2001). Accordingly, ST mutants lacking the PP2A binding domain fail to induce tumorigenic transformation of HEK-TER cells (Hahn *et al.* 2002). Structural insights reveal that ST interacts with the PP2A-A subunit which overlaps B56 subunit binding site and results in its displacement from the core enzyme. Functionally, expression of ST activates Akt signaling in human cells *in vitro* (Rodriguez-Viciano *et al.* 2006; Yuan *et al.* 2002; Zhao *et al.* 2003). Taken together, these observations indicate that complete transformation of human cells requires the perturbation of PP2A, for example by ST.

A somewhat confusing aspect of PP2A function in cancer arises because PP2A plays important roles in promoting cell cycle progression and cell survival (Li *et al.* 2002; Lin *et al.* 1998; Mayer-Jaekel *et al.* 1993), which are functions usually associated with tumor initiation and progression rather than suppression. Therefore, cellular transformation will most likely occur through the disruption of PP2A holoenzymes that normally exert negative regulation on oncogenic pathways.

Whilst some contradiction exists as to the importance of PP2A scaffolding subunits in cancer development, several mutations have been identified in spontaneously arising human cancers. Notably, somatic alterations of the gene encoding A β (PPP2R1B) have been detected in up to 8-15% of colon, 15% of lung and 13% of breast cancers (Calin *et al.* 2000; Takagi *et al.* 2000; Tamaki *et al.* 2004; Wang *et al.* 1998). Mutations of the more abundant A α subunit have been observed, albeit at a lower frequency (Calin *et al.* 2000) and cancer-associated A subunit mutants exhibit differential defects in binding to the B and C subunits, which correlates with impaired PP2A activity (Chen *et al.* 2005; Ruediger *et al.* 2001a;

Ruediger *et al.* 2001b; Sablina *et al.* 2007). Even in the absence of mutations, reduced protein expression of A α has been found in 25 out of 58 brain tumors (Colella *et al.* 2001). Decreased levels of A β have also been observed in 16 of 32 cancer cell lines derived from human lung, colon and breast cancer, as well as primary glioblastoma and B-CLL patient samples compared to normal tissue (Kalla *et al.* 2007; Suzuki and Takahashi 2003; Takagi *et al.* 2000; Zhou *et al.* 2003). Importantly, Ruediger *et al.*, recently generated knock-in mice with cancer associated A α mutations, and A α knockouts, both of which exhibited increased incidence of lung cancer when treated with benzopyrene, thus supporting the role of PP2A as a tumor suppressor (Ruediger *et al.* 2011).

Pivotal studies using the HEK-TER transformation model demonstrated that suppression of B56 γ , but not B55 α , functionally mimicked the introduction of ST and resulted in partial tumorigenic transformation (Chen *et al.* 2004; Moreno *et al.* 2004). Moreover, depletion of B56 γ containing complexes leads to activation of the anti-apoptotic Akt pathway (Chen *et al.* 2005). These observations were the first to demonstrate that PP2A complexes containing B56 γ modulate the phosphorylation of substrates associated with transformation. Loss of B56 γ has been demonstrated in some human cancers. Decreased expression was identified in primary human melanoma samples compared to melanocytic nevi (Deichmann *et al.* 2001), and in human lung cancer cell lines where subsequent overexpression of B56 γ reversed the tumorigenic phenotype (Chen *et al.* 2004). Reduced transcript levels of B56 γ have also been documented in patients with aggressive B-CLL compared to those with stable disease (Falt *et al.* 2005). A mutation in B56 γ has also been observed in lung cancer, and this was shown to disrupt the interaction of B56 γ with p53 (Shouse *et al.* 2010). In contrast, higher expression levels of B56 γ 1 mRNA were reported in human melanoma cell lines compared to normal melanocytes (Francia *et al.* 1999).

Recently the HEK293T system was further utilised to systematically examine all PP2A regulatory subunits (Sablina *et al.* 2010). In addition to B56 γ , suppression of B56 α , PR72/PR130, and PTPA (protein phosphatase 2A activator), replaced the expression of ST in transformation. Interestingly, the effects on signaling pathways differed depending on the regulatory subunit suppressed. Knockdown of B56 γ and PTPA, but not B56 α or PR72/PR130, led to enhanced Akt phosphorylation, while knockdown of B56 α , PR72/PR130, and PTPA, but not B56 γ , resulted in increased c-Myc expression. Moreover, suppression of B56 γ and PTPA led to increased β -catenin activity, whereas PR72/PR130 suppression decreased β -catenin activity (Sablina *et al.* 2010). Overexpression of CIP2A in the HEK-TER model can also replace ST in inducing transformation (Junttila *et al.* 2007), and increased CIP2A has been detected in myeloid leukemia patient samples (Wang and Li 2011), gastric and colon cancer samples (Khanna *et al.* 2009; Li *et al.* 2008; Soo Hoo *et al.* 2002).

6.4 PP2A in myeloid leukemias

An emerging body of evidence implicates PP2A as an important tumor suppressor in myeloid leukemias (Table 1). In particular, recent work from our laboratory and others has revealed a common theme in myeloid leukemias: inactivation of PP2A by leukemia associated tyrosine kinases (Fig. 3). Importantly, PP2A inhibition is essential for leukemias driven by these oncogenic tyrosine kinases, as re-activation of PP2A results in dephosphorylation, and thus deactivation, of the kinase and subsequent inhibition of leukemogenesis. Hence, re-activation of PP2A is an attractive strategy for leukemia therapy.

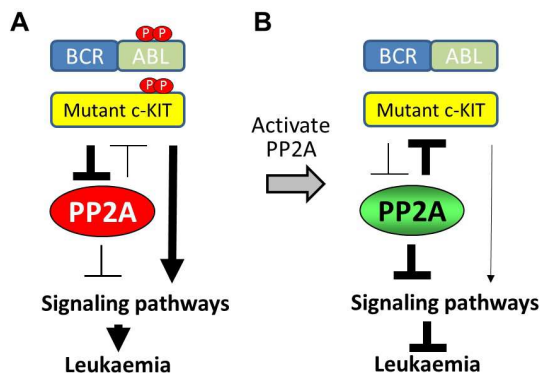


Fig. 3. Model of PP2A inhibition in myeloid leukemias. (A) PP2A activity is inhibited by BCR/ABL and oncogenic mutant c-KIT, enabling the activation of signaling pathways leading to leukemogenesis. (B) Pharmacological activation of PP2A induces de-activation (dephosphorylation) of BCR/ABL and c-KIT, and inhibition of downstream signaling pathways, leading to inhibition of leukemogenesis.

6.4.1 PP2A inhibition in CML

Functional inactivation of PP2A by BCR/ABL is essential for the development of CML-BC and Ph⁺ ALL, and is thought to result from upregulation of the endogenous PP2A inhibitor SET (I₂PP2A or TAF-1 β) protein (Neviani *et al.* 2007; Neviani *et al.* 2005). Although its physiological function remains incompletely understood, abnormal expression of SET mRNA has been documented in solid tumors and haematological disorders (Carlson *et al.* 1998; Fornerod *et al.* 1995; Li *et al.* 1996). Expression of BCR/ABL in mouse myeloid precursor cells stimulates SET expression and correlates with a loss of PP2A activity (Neviani *et al.* 2005). Conversely, inhibition of BCR/ABL with imatinib treatment dramatically reduces SET expression, which results in the restoration of PP2A activity back to untransfected levels and results in the dephosphorylation of several substrates which are shared by BCR/ABL and PP2A (Neviani *et al.* 2005). These include STAT5, ERK1/2, Akt, BAD and JAK2 (Calabretta and Perrotti 2004; Janssens and Goris 2001). Importantly, molecular or pharmacological reactivation of PP2A resulted in growth suppression, enhanced apoptosis, restored differentiation, and decreased *in vivo* leukemogenesis of imatinib-sensitive and -resistant BCR/ABL⁺ cell lines and primary CML-BC cells. Furthermore, reactivation of PP2A, by overexpression of PP2AC or 1,9-dideoxyforskolin treatment (a cAMP-independent PP2A activator) (see section 6.5.1), reduced the activity and expression of BCR/ABL in both imatinib-sensitive and -resistant BCR/ABL cells, suggesting that BCR/ABL itself is a target for PP2A activity. Indeed, reactivation of PP2A results in PP2AC association with BCR/ABL via the SHP1 tyrosine phosphatase, BCR/ABL dephosphorylation, and proteasomal degradation (Neviani *et al.* 2005). These findings establish an important link between an oncogenic kinase and a tumor suppressing phosphatase, and indicate that pharmacologic enhancement of PP2A is a powerful therapeutic strategy for imatinib-resistant CML. Taken together, these results suggest that SET-dependent inhibition of PP2A is required for the transduction of aberrant mitogenic, survival and anti-differentiation signals that contribute to the development of CML from the chronic phase into blast crisis (Neviani *et al.* 2005).

Phosphatase	Leukaemia	Expression profile, prognosis, other	Reference
Ser/Thr PPs			
PP2A	CML	BCR/ABL inhibits PP2A activity via ↑ SET exp'n in CML-BC & BCR/ABL+ cell lines; Forskolin & FTY720 inhibit IM-sensitive and resistant BCR/ABL+ leukaemogenesis	(Neviani, Santhanam et al. 2005; Neviani, Santhanam et al. 2007; Samanta, Chakraborty et al. 2009; Salas, Ponnusamy et al. 2011)
	AML, APL	↑ exp'n regulatory subunits during differentiation of myeloid cell lines	(Uzunoglu, Uslu et al. 1999; Yuksel, Saydam et al. 2002)
	AML	SET translocations in AML	(Li, Makkineje et al. 1996)
		↓ activity in AML-M1	(Yamamoto, Suzuki et al. 1999)
		↓ activity in AML blasts	(Yang, Li et al.)
		↓ activity correlates with ↑ pAkt & poor prognosis/high risk karyotype patients	(Gallay, Dos Santos et al. 2009)
		↑ pY ²⁹⁴ -PP2A-C, ↓ subunit exp'n in AML blasts	(Cristobal, Garcia-Orti et al. 2011)
		Forskolin induced PP2A activation induces apoptosis in AML cell lines	(Cristobal, Garcia-Orti et al. 2011)
		↑ CIP2A in newly diagnosed & relapsed AML patients	(Wang, Li et al.)
		Mutant c-KIT inhibits activity & ↓ subunit exp'n; FTY720 inhibits leukaemogenesis	(Roberts KG 2010)
		↓ PP2A-B55α exp'n poor prognostic marker	(Ruvolo, Qui et al. 2011)
		↑ SETBP1 inhibits PP2A & associated with poor prognosis in elderly patients	(Cristobal, Blanco et al.)
		↓ activity associated with chemoresistance	(Jiffar, Kuninna et al. 2004)
		Antitumour agent, erucylphosphohomocholine, induces apoptosis via PP2A mediated ERK dephosphorylation in AML cell lines and primary cells	(Martelli, Papa et al.)
		↑ PP2A-B56γ exp'n in AML patients	(Zheng, Chen et al.)
		↓ PP2A-Cα in del(5q) AML contributes to lanelomide sensitivity	(Wei, Chen et al. 2009)
I-ALL	I-ALL	HRX fusion protein interacts with SET	(Adler, Nallaseth et al. 1997)
		PPP2R2B (Aβ) target for mir-19 in Notch-1 induced I-ALL	(Mavrikis, Wolte et al. 2010)
		HOX11 interacts with PP2A & inhibits activity	(Kawabe, Muslin et al. 1997; Riz and Hawley 2005)
		Cu-culture with stromal cells protects B-ALL cells from chemotherapeutics due to ↓ PP2A activity & ↑ pAkt	(Wang, Fairney et al. 2004)
		FTY720-induced PP2A activation inhibits leukaemogenesis	(Liu, Zhao et al. 2008)
CLL	CLL	↓ exp'n & alternative splicing of PPP2R2B (Aβ) & ↓ activity	(Kalla, Scheuermann et al. 2007)
		PDE4 inhibitors apoptosis by activating PP2A	(Moon and Lerner 2003)
		Altered PP2A activity & exp'n during differentiation of HL-60 cells	(Nishikawa, Omay et al. 1994; Bhoola and Hammond 2000; Luo, Huang et al. 2006; Ouyang, Xu et al. 2008; Xu, Ou-Yang et al. 2008)
PP1	APL, AML	Altered activity, subcellular distribution and gene exp'n during differentiation	(Omay, Ogasawara et al. 1995; Uzunoglu, Uslu et al. 1999; Bhoola and Hammond 2000; Luo, Huang et al. 2006)
	AML, ALL and B-CLL	↓ activity in poorly differentiated AML-M1, ALL and B-CLL compared to blasts from more differentiated AML patients	(Yamamoto, Suzuki et al. 1999)
	T-ALL	HOX11 interacts & inhibits PP1	(Kawabe, Muslin et al. 1997; Riz and Hawley 2005)
PP2B	AML	↑ activity in well differentiated AML blasts	(Yamamoto, Suzuki et al. 1999)

Table 1. Ser/Thr Phosphatases in Leukaemia

6.4.2 PP2A inhibition in AML

Oncogenic c-KIT signaling also requires inhibition of PP2A for leukemogenesis (Roberts *et al.* 2010). c-KIT is a receptor tyrosine kinase that activates similar proliferation, differentiation and survival signaling pathways as BCR/ABL, such as the PI3K, ERK and JAK/STAT pathways (Linnekin 1999). Gain-of-function c-KIT mutations occur in a range of malignancies, including AML, systemic mastocytosis, testicular seminoma, gastrointestinal stromal tumors (GIST) and melanomas, making c-KIT an excellent target for anti-cancer therapies (Kitamura and Hirota 2004; Masson and Ronnstrand 2009). These mutants induce hyperphosphorylation of c-KIT and constitutive activation of downstream signaling pathways (Masson and Ronnstrand 2009). Activating c-KIT mutations occur in up to 48% of core binding factor-AML (CBF-AML) patients, and are associated with increased relapse

and reduced survival (Muller *et al.* 2008). While imatinib has shown remarkable success in treating c-KIT+ GIST patients (Demetri *et al.* 2002), the majority of c-KIT mutations expressed in AML patients are intrinsically resistant to imatinib, and as such, many CBF-AML and systemic mastocytosis patients are unresponsive to imatinib therapy (Cairolì *et al.* 2005; Pardanani *et al.* 2003). Our laboratory has recently shown that myeloid precursor cells expressing oncogenic mutant c-KIT receptors display significantly reduced PP2A activity compared to c-KIT-negative or WT-c-KIT expressing cells (Roberts *et al.* 2010). Inhibition of PP2A by mutant c-KIT is associated with reduced protein expression of PP2A subunits, together with altered expression of the endogenous PP2A inhibitory protein SET. Overexpression of PP2A-A caused growth inhibition and apoptosis, suggesting that PP2A inhibition is functionally important in c-KIT mediated leukemogenesis. Importantly, reactivation of PP2A resulted in dephosphorylation (and hence deactivation) of the mutant c-KIT receptor itself, as well as inactivation of downstream signaling proteins required for c-KIT induced leukemogenesis (e.g. Akt, ERK and STAT5) (Roberts *et al.* 2010). Furthermore, activation of PP2A by the pharmacological agent FTY720, inhibited leukemogenesis driven by mutant c-KIT (See section 6.5.2).

A number of recent studies further support a tumor suppressive role for PP2A in AML. Reduced expression of the PP2A-B55 α subunit and increased Akt phosphorylation in AML patient blasts was associated with shorter complete remission (Ruvolo *et al.* 2011). Hyperphosphorylation of PP2Ac, known to inhibit PP2A activity, has been observed in 78% of AML patients. This was associated with enhanced expression of PP2A inhibitors SET, CIP2A, and/or SET binding protein 1 (SETBP1) (Cristobal *et al.* 2011). Reduced expression of PP2A-A and a number of PP2A regulatory subunits was also observed in a number of patients. Increased expression of SETBP1 was also shown to be induced by a novel translocation t(12;18)(p13;q12) involving ETV6 in a patient with AML (Cristobal *et al.* 2010). SETBP1 overexpression protected SET from protease cleavage, leading to the formation of a SETBP1-SET-PP2A complex that results in PP2A inhibition, promoting proliferation of the leukemic cells. SETBP1 was further shown to be overexpressed in over 27% of AML patients and correlated with significantly shorter overall survival, in particular in patients over 60 years (Cristobal *et al.* 2010). Thus SETBP1 confers a negative prognostic impact and may be a predictive factor in any future clinical trials with PP2A activators. Increased expression of another PP2A inhibitory protein, CIP2A, has also been observed in diagnosis and relapse AML patients compared to patients in remission or healthy controls (Wang and Li 2011).

6.5 Targeting PP2A for anti-leukemia therapy

6.5.1 Forskolin

Forskolin, a diterpene isolated from the roots of *Coleus forskohlii*, is primarily known to stimulate the adenylate cyclase system, which results in elevated levels of cyclic AMP (cAMP) and subsequent activation of protein kinase A (PKA) (Seamon and Daly 1981; Seamon *et al.* 1981). The anticancer properties of this compound were initially demonstrated through potent inhibition of growth and tumor colonisation of the highly metastatic BL6 melanoma cell line (Agarwal and Parks 1983). Further studies indicated its potential use against ALL (Gutzkow *et al.* 2002) and CML cell lines (Taetle and Li-en 1984). However, more recent findings demonstrate that forskolin also activates PP2A (Feschenko *et al.* 2002); a mechanism which contributes to induction of apoptosis in B-CLL, CML-BC and AML patient samples (Cristobal *et al.* 2011; Moon and Lerner 2003; Neviani *et al.* 2005). Neviani *et*

al., was the first to highlight the therapeutic relevance of using PP2A-activators to specifically target leukemia cells (Neviani *et al.* 2005). Forskolin inhibited the *in vivo* leukemogenesis of imatinib sensitive and resistant BCR/ABL+ 32Dcl3 cells in mice, resulting in significantly prolonged survival. Furthermore, treatment with 1,9-dideoxy-forskolin, which lacks adenylate cyclase activity, impaired the clonogenic potential of BCR/ABL+ 32Dcl3 cells to a similar degree as forskolin, suggesting that the anti-leukaemic effects of forskolin and its derivative depends on the induction of PP2A activity rather than cAMP. Restoration of PP2A activity with forskolin was also found to inhibit Akt and ERK activity, block proliferation and induce caspase-dependant apoptosis in AML cell lines (Cristobal *et al.* 2011; Neviani *et al.* 2005). Furthermore, forskolin had an additive effect with common AML induction therapy drugs Idarubicin and Ara-c (Cristobal *et al.* 2011).

6.5.2 FTY720

FTY720 was first synthesised by structural modifications of myriocin (ISP-1), a fungal metabolite isolated from *Isaria sinclairii* culture broth (Fujita *et al.* 1994), and is structurally similar to sphingosine (Albert *et al.* 2005; Kiuchi *et al.* 2005). It is effectively phosphorylated *in vivo* by SphK2 to yield the biologically active FTY720-phosphate (FTY720-P) (Brinkmann *et al.* 2002; Zemann *et al.* 2006). Interaction of FTY720-P with one of the five known S1P receptors induces receptor internalisation and degradation (Graler and Goetzl 2004; Matloubian *et al.* 2004). The S1P signal is required for the migration of lymphocytes from secondary lymphoid tissues back into the efferent lymphatics and systemic circulation (Cyster 2005; Matloubian *et al.* 2004) and prolonged S1PR downregulation by FTY720-P inhibits the immune response by sequestering functional lymphocytes within secondary lymphoid organs (Brinkmann *et al.* 2002; Mandala *et al.* 2002). The use of FTY720 as an immunomodulator is currently being evaluated in Phase III trials for multiple sclerosis (Cohen *et al.* 2009; Takabe *et al.* 2008).

A more recent mechanism of action identified for FTY720 is its activation of purified PP2A trimers *in vitro* (Matsuoka *et al.* 2003) and as loss of PP2A phosphatase activity contributes to the pathophysiology of BCR/ABL-driven leukemias (Neviani *et al.* 2007) a logical prediction would be that restoration of PP2A levels reverses the leukaemic phenotype. Indeed, an extensive study has shown that pharmacological reactivation of PP2A with FTY720 inhibits the proliferation, enhances apoptosis, restores differentiation and impairs colony formation of imatinib-sensitive and -resistant BCR-ABL+ cell lines and CML-BC patient blasts (Neviani *et al.* 2007). Notably, FTY720 promotes BCR/ABL tyrosine dephosphorylation and proteolytic degradation, together with reduced phosphorylation of the PP2A targets Akt, ERK1/2 and STAT5 (Neviani *et al.* 2007). Co-treatment with okadaic acid or transduction of SV40 ST reverses the enhancement of PP2A activity and restores substrate phosphorylation, strongly indicating that FTY720 functions through a PP2A-dependent mechanism.

Importantly, the *in vitro* efficacy of FTY720 translated into an *in vivo* model markedly suppressing both imatinib-sensitive (WT) and -resistant (T315I) BCR/ABL+ leukemogenesis (Neviani *et al.* 2007). After 4 weeks of treatment, saline-treated mice contained a large number of undifferentiated myeloid cells within the peripheral circulation, representing an overt AML phenotype with extensive blast infiltration of distal organs. In contrast, FTY720-treated mice displayed undetectable levels of BCR/ABL+ cells in the systemic circulation and secondary organs. Accordingly, these effects were sustained long term with 80% and 50% of WT and T315I BCR/ABL+ FTY720-treated mice, respectively, still alive at 27 weeks

and showing no signs of leukemia. In contrast, all saline-treated mice were sacrificed 5 weeks post-tumor cell injection (Neviani *et al.* 2007). No toxic side effects were observed with administration of FTY720, highlighting the safety and therapeutic relevance of utilising PP2A-activating drugs in leukemia patients.

FTY720 also activates PP2A in mutant c-KIT myeloid cells, leading to growth inhibition and induction of apoptosis *in vitro* (Roberts *et al.* 2010). Activation of PP2A is required for the anti-leukaemic effects, as PP2A inhibition with okadaic acid inhibits these effects. FTY720-induced PP2A activity results in inhibition of c-KIT phosphorylation, and inactivation of downstream signaling proteins regulated by both c-KIT and PP2A. Importantly, FTY720 also inhibited the *in vivo* tumor growth of mutant c-KIT myeloid cells in a syngeneic mouse model (Roberts *et al.* 2010). Thus FTY720 may also be a useful therapeutic agent for CBF-AML patients harbouring activating c-KIT mutations.

7. Other serine/threonine phosphatases in myeloid leukemia

7.1 PP1

PP1 activity has been found to alter according to AML blast differentiation. Yamamoto *et al.*, found that AML patients with well differentiated leukemia had higher PP1 activity than patients with poorly differentiated AML-M1 (Yamamoto *et al.* 1999). AML blast PP1 activity also correlated with patient prognosis where individuals with significantly low PP1 activity had lower overall survival than those with high PP1 activity, with a median survival for each group of 8 and 27 months respectively (Nishikawa *et al.* 1994). Thus low PP1 activity may be a prognostic indicator of poor prognosis. The functional consequence of low PP1 activity in AML is not known, however PP1 was recently found to dephosphorylate Akt at T405, and PP1 overexpression induced Akt dephosphorylation, promoted cell survival and inhibited differentiation (Xiao *et al.* 2010). PP1 also promotes survival by negative regulation of p53 (Li *et al.* 2006), and is implicated in regulating Wnt/ β -catenin (Jiang *et al.* 2009), TGF- β (Shi *et al.* 2004), and NF- κ B (Li *et al.* 2008) signaling pathways. Luo *et al.*, showed that arsenic sulfide, a therapy historically used for treating CML in China, inhibited proliferation and induced differentiation of a human APL HL-60 cell line down a monocytic pathway. This correlated with increasing PP1 and PP2A activity, and co-treatment with a concentration of OA that inhibits both phosphatases suppressed the arsenic sulfide induced differentiation (Luo *et al.* 2006). Furthermore, while the mechanism was unknown at the time, other studies had previously shown similar effect in an APL NB4 cell model (Bai and Huang 1998; Lu and Wang 2002) and in chronic myeloid leukemia K562 cells (Yin *et al.* 2004). These studies suggest that PP1 plays a role in the growth inhibition and differentiation of AML, and may also provide a useful prognostic tool and/or therapy target.

7.2 PP2B

PP2B (calcineurin) is probably best known for its role in immunity where it is activated upon T cell receptor stimulation. Indeed, calcineurin is a well established target of prophylactic agents used in transplantation, such as Cyclosporin A and FK506, where calcineurin inhibition suppresses IL-2 production. Calcineurin was recently implicated as an important oncogene in lymphoid leukemias (Medyouf *et al.* 2007; Medyouf and Ghysdael 2008; Muller and Rao 2007). Sustained calcineurin activity was observed in human B- and T-cell lymphomas and in a range of mouse models of lymphoid malignancies. Moreover,

expression of constitutively active mutant calcineurin favored leukemia progression, while treatment with Cyclosporin A or FK506 induced apoptosis of leukemic cells and rapid tumor clearance, and significantly improved mouse survival (Medyouf *et al.* 2007). Thus inhibiting calcineurin is a potential therapeutic strategy for lymphoid leukemias. In contrast, a specific role for calcineurin in myeloid leukemias has not been reported, however one study investigating its activity in AML patients of different FAB subtype found that calcineurin activity is relatively low in leukemic blasts arresting at the stage of early pluripotent stem cells, and increases during the course of myelomonocytic commitment and maturation (Yamamoto *et al.* 1999).

7.3 PP2C

Like PP2B, little information exists regarding the role of PP2C in myeloid leukemia. One study revealed it had low expression in AML patient blood cells and its activity and expression were relatively constant in various leukemic cell types from AML, ALL, and CLL patients (Yamamoto *et al.* 1999), suggesting it may not play a remarkable role in leukemogenesis.

8. Conclusions

There is no doubt that PP2A acts as a tumor suppressor in myeloid leukemias, and targeting its re-activation, either directly or via inhibition of an endogenous inhibitory protein such as SET, is a promising therapeutic strategy. Whether the pre-clinical promise will translate to improved survival of myeloid leukemia patients is currently unknown, but clinical trials of PP2A activators are eagerly awaited. Emerging studies indicate that other phosphatases may also play important roles in myeloid leukemias, and future studies aimed at deciphering the molecular mechanisms regulating these phosphatases and their downstream targets is sure to identify further targets for novel therapies.

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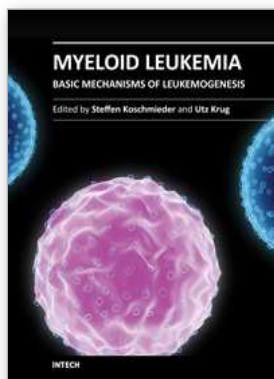
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The current book comprises a series of chapters from experts in the field of myeloid cell biology and myeloid leukemia pathogenesis. It is meant to provide reviews about current knowledge in the area of basic science of acute (AML) and chronic myeloid leukemia (CML) as well as original publications covering specific aspects of these important diseases. Covering the specifics of leukemia biology and pathogenesis by authors from different parts of the World, including America, Europe, Africa, and Asia, this book provides a colorful view on research activities in this field around the globe.

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