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Review article

Cancer Stem Cell Phosphatases

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Abstract:

Cancer stem cells (CSCs) are involved in the initiation and progression of human malignancies by enabling cancer tissue self-renewal capacity and constituting the therapy-resistant population of tumor cells. However, despite the exhausting characterization of CSC genetics, epigenetics, and kinase signaling, eradication of CSCs remains an unattainable goal in most human malignancies. While phosphatases contribute equally with kinases to cellular phosphoregulation, our understanding of phosphatases in CSCs lags severely behind our knowledge about other CSC signaling mechanisms. Many cancer-relevant phosphatases have recently become druggable, indicating that further understanding of the CSC phosphatases might provide novel therapeutic opportunities. This review summarizes the current knowledge about fundamental, but yet poorly understood involvement of phosphatases in the regulation of major CSC signaling pathways. We also review the functional roles of phosphatases in CSC self-renewal, cancer progression, and therapy resistance; focusing particularly on hematological cancers and glioblastoma. We further discuss the small molecule targeting of CSC phosphatases and their therapeutic potential in cancer combination therapies.

Keywords: SHP2, PTEN, PP2A, B56, B55, PTP1B, DUSP1, DUSP4, DUSP6, WIP1, WNT, β-catenin, APC, PI3K, AKT, Hedgehog, NOTCH, JAK-STAT, MYC, tumor suppressor, SHP099, TNO155, RMC-1630, LB100, SMAP, DT-061, iHAP1, FTY720, BCI-215, SET, PME-1, CIP2A, ARPP19, NEDD4-1, WWP1

Introduction

In normal adult tissues, stem cells (SCs) confer the capacity for tissue regeneration and homeostasis [1]. The self-renewal capacity of SCs, and generation of differentiated cellular progenies, is a prerequisite for tissue maintenance and repair throughout an organism´s lifespan. Based on cancer stem cell hypothesis, cancers evolve from an analogous small population of cells designated as cancer stem cells (CSCs), or as tumor initiating cells (TICs, or tumor-cell-of-origin) [2, 3]. Neither CSCs or TICs can be definitely recognized by markers, but are rather defined by their functional properties such as the capacity for long-term self-renewal and tumor initiation [3, 4]. While in normal SCs, differentiation into one or multiple cell lineages is another prime character, in CSCs the tumor initiating capacity and recapitulation of different cancer cell lineages are fundamental functional properties and contribute to intratumor heterogeneity [5]. Although CSCs display differential gene expression profiles and exhibit expression of certain cell surface markers as compared with SCs [6-8], most CSC surface markers are present in human embryonic stem cells or adult stem cells [9]. Similarly, CSCs and SCs from the same tissue are regulated by similar molecular mechanisms [10, 11].

As phosphorylation-dependent signaling pathways are often deregulated in human cancers, it is not surprising that deregulated phosphosignaling plays an important role also in self-renewal, proliferation, survival and differentiation of CSCs [12]. The CSC signaling pathways crosstalk, and the CSC niche microenvironment determines what signaling pathways are activated. In addition to their importance for CSC self-renewal, proliferation, and survival, phosphorylation-dependent signaling pathways promote resistance to many therapeutics. Importantly, the therapy tolerance in cancer develops initially by non-genetic mechanisms including phosphorylation-dependent signaling rewiring [13, 14]. Simultaneous inhibition of several kinase pathways has shown a marginal success in targeting CSCs with acquired

chemoresistance [15-17]. However, findings from clinical resistance of most cancer types to kinase inhibitors suggest that these compounds and their combinations have failed to sufficiently eradicate CSCs [18].

The current understanding of the roles of phosphatases in regulation of key CSC functions is still relatively poor. However, since phosphatases regulate most CSC-related phosphorylation-dependent pathways [18-20], further understanding of phosphatase-mediated regulation of CSCs will provide novel approaches to inhibit the critical CSC functions, including CSC non-genetic therapy tolerance. This is particularly appealing as many phosphatases have recently become druggable by small molecules [18, 21-23]. In this work, we review the current knowledge of phosphatases in CSCs. In particular, we focus on phosphatase-mediated regulation of signaling pathways which are strongly implicated in CSCs: WNT, PI3K-AKT, Hedgehog (HH), NOTCH, and JAK-STAT. We also review the current knowledge of the functional importance of phosphatases in selected cancer types, and discuss potential phosphatase targeted therapies to overcome therapy resistance in CSCs.

Phosphatase families

Based on their amino acid sequence, the majority of human protein phosphatases are classified as protein serine/threonine phosphatases (PSPs) and tyrosine phosphatases (PTPs) (Fig. 1) [24]. Since nearly 70% of all human phosphoregulation targets either serine or threonine residues, PSPs constitute a very powerful phosphoproteome regulatory mechanism. Based on the catalytic mechanism, PSPs are subdivided into phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), or aspartate-based phosphatases. PSPs, the majority of which function as dimeric or trimeric protein complexes, consist of a catalytic subunit complexed with either one or two scaffolding subunits and a substrate determining regulatory subunit (Fig. 1). Instead, PTPs function as monomeric

enzymes, and based on the number of genes which encode the catalytic subunits, PTPs are the largest family of phosphatases (Fig. 1). On the basis of the sequence of their catalytic domains, PTPs are classified into three distinct subfamilies: cysteine, aspartate and histidine-based PTPs [24]. Cysteine-based PTPs include receptor tyrosine phosphatases (PTPRs), non-receptor tyrosine phosphatases (PTPNs), and CDC25 phosphatases. A subclass of cysteine-based PTPs are dual-specificity protein phosphatases, targeting either serine/threonine and tyrosine residues (DUSPs), or tyrosine and phosphatidylinositol (3,4,5)-trisphosphate (PTEN). For more detailed biochemistry of phosphatases, characteristics of the phosphatase families, and their broader roles in cancer, the reader is encouraged to study the recent reviews [25-28].

Oncogenic and tumor suppressor phosphatases

The functional assignment of a phosphatase as either an oncoprotein or a tumor suppressor is defined by the functional role of the phosphosite they target [23]. Certain tumor suppressor and oncogenic phosphatases have recently become druggable, with reactivating and inhibiting small molecules, respectively [21-23, 28]. Because there is limited information about the functional relevance of phosphatases in specific CSC phenotypes, first we briefly summarize the overall cancer relevance of the most important tumor suppressor and oncogenic phosphatases, focusing on those which will be later discussed in more details in regard to the CSC pathways they regulate.

Protein phosphatase 2A (PP2A) is a heterotrimeric protein complex consisting of a catalytic subunit (PP2Ac or C), a scaffold subunit (PR65 or A), and one of the alternative regulatory B subunits. There are α and β isoforms for both the catalytic and scaffolding subunits. Moreover, there are four B subunit families, each with various isoforms or splice variants. Such variability in PP2A holoenzyme composition results in a family of functionally distinct

phosphatases which cover broad substrate specificity and control diverse cellular functions [29-31].

In addition to strong evidence that inhibition of PP2A drives human cell transformation [32, 33], recent studies have provided compelling evidence for PP2A-mediated tumor suppression in mouse models [34-36]. These studies demonstrate that even partial inhibition of PP2A activity is sufficient to drive mouse tumorigenesis [36]. Inhibition of PP2A activity in human neoplasms occurs through various mechanisms. A prevalent genetic mechanism for PP2A inhibition is haploinsufficiency of *PPP2R4*, which encodes the PP2A activator PTPA [36], whereas point mutations in the scaffolding A subunit *PPP2R1A* are observed in relatively high frequency in some specific cancer types [37]. The non-genetic PP2A inhibition mechanisms are very common across most cancer types and include overexpression of PP2A inhibitory oncoproteins such as CIP2A, NOCIVA, PME-1, SET, and ARPP19 [38, 39]. Very importantly and highlighting the relevance of PP2A complex composition, the PP2A complexes containing STRN B subunits are oncogenic due to their negative role in regulation of the tumor suppressor Hippo pathway upstream of YAP [40-42].

Based on the original discovery that some antipsychotic phenothiazines are direct PP2A reactivators [43], two different series of PP2A reactivating drugs (iHAPs and SMAPs) have recently been developed [22, 23, 44-46]. Importantly, these compounds selectively activate only certain tumor suppressive PP2A complexes, namely PP2A-B55 and PP2A-B56, and thereby have strong antitumor effects, without affecting other PP2A functions that might cause systemic side-effects [22, 23, 44, 46]. Whereas iHAPs efficiently kill T-ALL cells [44], series of SMAPs have been shown to be effective towards numerous types of cancer [20, 23, 45, 47]. PP2A reactivation globally promotes kinase inhibitor sensitivity [20, 48], and

SMAP-elicited PP2A reactivation combined with kinase inhibitors results in a significant tumor regression in *KRAS*-driven lung cancer *in vivo* [20]. Additionally, the FTY720 (Fingolimod), an FDA approved immunosuppressant drug, also activates PP2A by targeting interaction between SET and PP2Ac, and is effective in several types of leukemic cells *in vitro* and *in vivo* [23, 49-52]. FTY720 is phosphorylated in cells by sphingosine kinases, and this is required for the immunosuppressive effects, while SET inhibition is mediated by non-phosphorylated pool of FTY720. Importantly, development of non-phosphorylatable analogues of FTY720, devoid of direct immune-modulatory activities, has been reported. Similar to FTY720, they restore PP2A activity by disruption of the PP2A-SET complex, and inhibit growth of leukemic cells [23, 49, 53, 54].

PTEN is a tumor suppressor phosphatase with pleiotropic roles in tumor hallmarks such as cell proliferation, cellular senescence, metabolism, and regulation of tumor microenvironment [26, 55]. *PTEN* is one of the most commonly mutated tumor suppressor genes, and if not mutated, largely suppressed or down-modulated [26, 55]. Similar to PP2A, PTEN is a haploinsufficient tumor suppressor and its partial loss results in sustained phosphoinositide 3-kinase (PI3K) activation and tumorigenesis [56]. PTEN is a dual-specificity phosphatase with both protein and lipid phosphatase activity. Its lipid phosphatase activity targets the phosphatidyl-inositol-3,4,5-phosphate (PIP_3) which is critical for PI3K/AKT activation, and thereby malignant growth and survival [26, 57]. The cancer relevance of the protein phosphatase activity of PTEN is still elusive. PTEN dephosphorylates Ser, Thr, and Tyr residues in substrates such as focal adhesion kinase (FAK) and cAMP responsive-element binding protein (CREB) [55]. Inhibition of PTEN phosphatase activity activates the nonreceptor tyrosine kinase SRC and drives HER2 inhibitor resistance in breast cancer cells [58]. On the other hand, a recent study has

demonstrated that while inhibition of PTEN lipid phosphatase activity promotes PI3K-driven mammary tumorigenesis *in vivo*, co-inhibition of its protein phosphatase activity blocks tumorigenesis via regulation of the glucocorticoid receptor [59].

Recent evidence indicates that similar to PP2A, PTEN tumor suppressor functions could be pharmacologically reactivated through inhibition of its endogenous inhibitors [23, 60, 61]. Two NEDD4 family HECT domain E3 ubiquitin ligases, NEDD4-1 and WWP1, inhibit PTEN. Two recent studies demonstrated that their negative impact on PTEN stability and membrane localization is impaired by treatment with indole-3-carbinol (I3C), a natural indolecarbinol compound derived from cruciferous vegetables [23, 60, 61]. I3C induces apoptosis in cells expressing wild-type PTEN, but not in cells with mutant or null PTEN, and results in potent antitumor effects *in vivo*. These studies provide evidence for small molecule druggability of PTEN [23, 60, 61].

SHP2 (Src homology phosphatase 2), encoded by *PTPN11*, is a protein tyrosine phosphatase involved in different signaling pathways and induced by various stimuli such as growth factors and cytokines [62, 63]. Patients with Noonan syndrome, and juvenile myelomonocytic leukemia (JMML), a childhood myeloproliferative neoplasm (MPN), have germline mutations in *PTPN11,* whereas somatic mutations in *PTPN11* account for 34% of non-syndromic JMMLs. The gain of function mutations in *PTPN11* have also been found in a small percentage of patients with myelodysplastic syndrome (MDS), *de novo* acute myeloid leukemia (AML) [64]. Moreover, a recent study unraveled the important contribution of *Ptpn11* mutations in bone marrow microenvironment and leukemogenesis [65]. *Ptpn11* activating mutations in mesenchymal stem/progenitor cells induce production of the CC chemokine CCL3 (also known as MIP-1α), which recruits monocytes. Consequently, the

HSCs are hyperactivated by interleukin-1β produced by monocytes, leading to initiation and development of MPN. Interestingly, CCL3 receptor antagonists reverse the MPN development induced by the *Ptpn11*-mutated bone marrow microenvironment. These findings unravel the key role of *Ptpn11* mutations in the bone marrow microenvironment in leukemogenesis [65].

SHP2 is essential for KIT-induced myeloproliferative disease (MPD) through activation of the PI3K/AKT signaling pathway. Consistently, a SHP2 inhibitor has been shown to augment anti-tumor efficacy of PI3K inhibition in KIT-induced MPD *in vivo* [66]. In addition to hematological cancers, somatic *PTPN11* mutations are found in 5-7% of glioblastoma (GB) cases [67]. Moreover, SHP2 promotes RAS activity and is required for growth of *KRAS*-mutant non-small-cell lung cancer *in vivo,* and its inhibition restores sensitivity to ALK inhibitors [68]. SHP2 blockade enhances sensitivity of *KRAS*-amplified gastroesophageal tumor models to MEK inhibition [69]. These findings, together with the recent development of selective allosteric and small-molecule SHP2 degraders using the proteolysis-targeting chimera (PROTAC) concept [23, 70-74] suggest that SHP2 inhibition is an attractive therapeutic strategy in several cancer types.

DUSPs are a large subgroup of cysteine-based PTP superfamily, which dephosphorylate both tyrosine and serine/threonine residues in mitogen-activated protein kinases (MAPKs) [27]. DUSP1 has some tumor suppressor properties as its overexpression attenuates oncogenic behavior in gallbladder cancer *in vitro* and *in vivo* [75]. DUSP1 is also upregulated in low-grade, but downregulated in high-grade prostate carcinomas [76, 77]. On the other hand, DUSP1 negatively regulates the pro-apoptotic JNK and P38 MAP kinases, triggers evasion from JNK-mediated apoptosis and provides survival benefit and supports oncogenicity [78, 79]. DUSP6 targets specifically ERK1/2 kinases and its expression is

increased in breast cancer, GB and acute lymphatic leukemia [27, 80-82]. *DUSP6* depletion or its pharmacologic inhibition reduces cell proliferation and induces apoptotic cell death [83-85], indicating an oncogenic role for DUSP6.

Regulation of cancer stem cell signaling pathways by phosphatases

WNT pathway and phosphatases in cancer stem cells

The soluble ligand WNT induces β-catenin pathway, which is important for normal embryonic development via Frizzled and LRP receptors. WNT controls body axis patterning, cell fate decision, cell proliferation and migration. WNT signaling is also responsible in controlling tissue renewal and regeneration in adult vertebrate stem cells [86, 87]. Activation of WNT signaling in CSCs has been shown in a variety of cancers [88], as well as in CSC-mediated breast cancer metastases [89].

The PP2A-B56 complex inhibits WNT/β-catenin by multiple mechanisms [90, 91]. PP2A dephosphorylates the inhibitory serine 9 phosphorylation in GSK3β, which results in proteasomal degradation of β-catenin, and inhibition of several β-catenin stemness target genes such as MYC. A recent study confirmed that in the CSC context, PP2A-B56 is the phosphatase responsible for GSK3β dephosphorylation as well [92]. In addition, the PP2A catalytic subunit PP2Acα colocalizes with β-catenin at the plasma membrane, which prevents β-catenin translocation and β-catenin-mediated transactivation [93, 94]. There is also evidence that the PP2A/B56 complex inhibits WNT signaling via Axin-mediated regulation of β-catenin activity without affecting β-catenin stability [95]. Interestingly, two recent studies indicated a potential WNT-PP2A feedback loop, resulting in maximal WNT pathway activity. In these studies, GSK3β inhibition was found to suppress protein

276 expression of PP2A-A, B56 and C subunits in GB stem cells (GSCs) [92], whereas WNT
277 treatment inhibited PP2A activity in cancer cells [96].
278 MYC, a WNT/β-catenin target gene, is a master regulator of stem cell self-renewal [97-99].
279 MYC is directly inhibited by PP2A-B56-mediated GSK3β activation, as GSK3β is a MYC
280 threonine 58 kinase, and this phosphorylation triggers proteosomal MYC degradation [100,
281 101]. PP2A-B56 inhibition particularly stabilizes the expression of serine 62 phosphorylated
282 MYC (pS62MYC) [102-104]. In APC-deficient intestinal crypts, MYC is essential for stem
283 cell expansion downstream of β-catenin activation [99]. On the other hand, in regenerating
284 intestinal crypts, the PP2A-B56 inhibitor protein CIP2A [105] increased expression of serine
285 62 phosphorylated MYC (pS62MYC), and supported MYC-mediated transcriptional activity
286 [102]. Functionally, CIP2A deficient mice were unable to regenerate their intestine in
287 response to irradiation. In line with these results, hyperproliferative skin lesions in mouse
288 model of hypomorphic deletion of B56α subunit displayed increased levels of pS62MYC
289 [106]. It was further shown that this leads to acceleration of papilloma initiation through
290 enhancement of the number of skin stem cells. This is most likely due to cell intrinsic effects
291 of PP2A inhibition in keratinocytes, as siRNA mediated depletion of B56α was found to
292 increase proliferation of keratinocytes *in vitro*. In addition to skin, the hypomorphic B56α
293 mouse model exhibited elevated clonogenicity of bone marrow stem cells [106]. Importantly,
294 in both mouse studies, increased PP2A activity selectively inhibited pS62MYC protein pool,
295 without any notable effects on total MYC protein expression [106, 107]. These results
296 somewhat question the *in vitro* observed role for serine 62 phosphorylation in regulating
297 overall MYC protein stability [102-104]. On the other hand, the results indicate for an
298 interesting possibility to suppress the oncogenic form of MYC (pS62MYC) by PP2A
299 reactivation *in vivo,* without deleterious effects on tissue homeostasis observed with total
300 MYC inhibition [108]. Further support for the importance of PP2A inhibition in supporting

WNT/MYC-mediated self-renewal was obtained from human embryonic stem cells (hESC). PP2A activity was gradually increased during hESC differentiation, and inhibition of PP2A by semi-selective okadaic acid [109] was able to sustain hESC self-renewal in the absence of basic fibroblast growth factor (bFGF) [110]. Mechanistically, the effects of PP2A inhibition on hESC self-renewal were through increased activity of GSK3β-MYC and AKT pathways [110]. Together, these findings delineate that PP2A-B56 plays a central role in stem cell self-renewal and proliferation via regulation of WNT pathway and MYC activity [90]. Furthermore, regarding the tumor suppressor activity of PP2A-B56 [34, 111-113], targeting the PP2A-B56 inhibitors CIP2A and SET, which are overexpressed in multiple cancers [38, 114-120], could provide a novel approach for eradication of WNT-driven CSCs. Importantly, MYC and β-catenin are most likely required for the self-renewal and survival of the proliferating CSC/LSC pool but not for their quiescence. In line with this, it has been demonstrated that PP2A-mediated regulation of MYC and β-catenin is necessary for the transcriptional activation of G1/S and M mediators, which is necessary for the re-entry of CSC/LSC into the cell cycle and self-renewal but not cell cycle exit [121].

There is also evidence for the regulation of the non-canonical WNT signaling by PP2A. Conditional reprogramming of human ectocervical, breast and prostate cancer cells induces their stem cell-like properties through β-catenin activation via a non-canonical pathway that is independent of WNT and AKT/GSK-3β. In turn, this β-catenin-dependent transcription and induction of stem cell-like behavior is largely regulated by PP2A [122].

In addition to PP2A, recent studies imply for an important role for SHP2 in regulating WNT pathway in CSCs. SHP2 is overexpressed in CSCs of chemoresistant hepatocellular carcinomas (HCCs), and in recurrent HCCs from patients [123]. Further investigation revealed that SHP2 facilitates liver CSC self-renewal via nuclear translocation of β-catenin.

β-catenin translocation in *SHP2*-depleted spheroids was associated with decreased GSK3β serine 9 phosphorylation whereas no effects were observed on GSK3β tyrosine 216 phosphorylation. This indicates that SHP2 indirectly regulates GSK3β phosphorylation via a serine/threonine phosphatase such as PP2A [123]. SHP2 impacts WNT signaling via dephosphorylation of parafibromin/CDC73 as well [123-126] (Fig. 2). Originally, it was found that SHP2-mediated dephosphorylation of parafibromin results in inhibition of its tumor suppressor activity at RNA polymerase II-associated factor (PAF) complex and its binding to β-catenin, and thereby induction of expression of WNT target genes [125]. Importantly, related to loss-of-function *SHP2* mutations found from patients with Leopard syndrome, it was shown that these mutations inhibit SHP-mediated parafibromin dephosphorylation *in vitro* [124]. Even though no direct evidence has been shown for the impact of SHP2-mediated parafibromin regulation and its effects on WNT signaling in stem cell context, a recent study has found that parafibromin competitively interacts with β-catenin and GLI, thereby potentiating transactivation of WNT- and HH-target genes in a mutually exclusive manner [126]. Parafibromin binds to the NOTCH intracellular domain (NICD), enabling concerted activation of WNT and NOTCH-target genes [126]. These transitions between different stem cell pathway effectors were regulated by SHP2 [126], indicating that SHP2 efficiently coordinates several critical stem cell and CSC pathway activities through parafibromin (Fig. 2).

PI3K/AKT and phosphatases in cancer stem cells

PI3K/AKT signaling pathway is a master regulator of tumorigenesis, with increasing evidence for its contribution to CSCs [127]. Activation of PI3K/AKT in CSCs is associated with radioresistance in prostate cancer and tumorigenicity of breast, colorectal, and HCC as reviewed earlier [127]. In this vein, inhibition of PI3K/AKT/mTOR activity by specific inhibitors

decreases the CSC population in a variety of human malignancies including GB, breast cancer, pancreatic carcinoma, prostate cancer and lung carcinoma [128-132]. Of phosphatases with CSC relevance, PI3K/AKT signaling is directly regulated by PTEN [55] and PP2A [133] (Fig. 2). PI3K/AKT signaling is indirectly regulated by many other phosphatases, as this pathway exhibits cross-talks with many other phosphorylation-dependent signaling pathways.

PTEN is a master regulator of PI3K/AKT by its unique capacity to dephosphorylate PIP_3, critical for PI3K activation [26, 55]. Mechanistically, PI3K/AKT activation due to PTEN loss promotes self-renewal through modulation of several downstream pathways. For instance, suppression of PTEN by microRNA-216a promotes AKT-mediated phosphorylation of FOXO3a and GSK-3β, which in turn supports the maintenance of stem-like populations and clonogenic potential in HCC [134]. PTEN inhibition promotes crosstalk between the WNT/β-catenin and PI3K/AKT pathways and drives self-renewal induced by Aryl hydrocarbon receptor and the cytochrome P450 1A1 activation [135]. Moreover, PTEN suppresses PI3K/AKT/mTOR-mediated maintenance of stemness through induction of chemokine receptor type 4 (CXCR4) and STAT3 activation [130]. Additionally, *PTEN* loss in prostate epithelial cells, and the subsequent activation of the PI3K/AKT/IL-6 axis activates STAT3. In this setting, AKT induces Iκβ degradation, which permits nuclear translocation of NF-κβ and induction of IL-6 transcription, which promotes self-renewal and tumorigenesis via STAT3 activation [136]. Banasavadi-Siddegowda *et al*. have reported that downregulation of *PTEN* via protein arginine methyltransferase-5 (PRMT5)-mediated methylation is required for the maintenance of primary GB neurospheres via AKT/ERK [137]. Importantly, PTEN regulates cell cycle progression in CSCs via the PI3K/AKT pathway and this is not limited to the control of quiescence [138]. Peng *et al*. have shown that a high number of cyclin D1+

cells were residing in the bone marrow of an AML mouse model with PTEN deficiency. Interestingly, after administration of rapamycin, leukemia cancer stem cells (L-CSCs) were depleted and normal hematological stem cells (HSCs) were restored, providing evidence for the involvement of the PI3K/mTOR pathway [138].

PP2A is a well-established direct AKT phosphatase [133, 139, 140]. However, PP2A´s role in regulating AKT dephosphorylation and activity specifically in the CSC context is poorly understood. In colorectal CSC, the natural compound silibinin was shown to inhibit stemness properties via inhibition of AKT phosphorylation. Interestingly, during prolonged culture of CSC spheres, the sphere growth correlated with increased AKT phosphorylation and decreased PP2A activity, partly rescued by silibinin treatment [141]. Interestingly, a recent study has identified a potential functional link between the major PI3K/AKT phosphatases, PP2A and PTEN, in liver TICs. The CBP inhibitor ICG001 and RNAi-mediated depletion of *CBP* reduced anchorage independent growth in human HCC cell lines and murine TICs associated with reduction of PTEN, AKT and β-catenin phosphorylation. ICG001 increased PP2A activity and ICG001-elicited serine dephosphorylation of PTEN was preempted by co-treatment with okadaic acid [142], which is an unselective PP2A inhibitor.

Of the other CSC relevant phosphatases, there is evidence for interaction between leukemia-associated mutant *Shp2* with Gab2, an important scaffolding protein in PI3K/AKT/mTOR signaling in juvenile myelomonocytic leukemia, and elevated PI3K/AKT/mTOR pathway activity in *Ptpn11*-mutant leukemic cells [143].

<u>HH pathway and phosphatases in cancer stem cells</u>

HH pathway is activated by binding of a soluble ligand Sonic Hedgehog (SHH) to Patched cell surface receptor, resulting in dimerization with Smoothened receptor, and consequent

release and nuclear translocation of the GLI transcription factor (Fig. 2) [144]. HH pathway is active in stem cells during prenatal development and controls cellular proliferation, differentiation and migration [145]. Reactivation of HH signaling has been observed in CSCs of various primary malignancies like breast cancer, GB, pancreatic adenocarcinoma, multiple myeloma and chronic myeloid leukemia, as well as in metastases [12, 144].

Using a stochastic model of GSC, it was shown that nutritional deprivation increased the expression of GSC-specific biomarkers, the cells acquired higher invasive and angiogenic properties and exhibited resistance to multiple anticancer compounds. This nutritional stress was associated with activation of HH and WNT signaling pathways via stabilizing of GLI and β-catenin through modulation of the GSK3β/AKT axis. As an AKT pathway phosphatase, depletion of *PTEN* potentiated the expression of both GLI and β-catenin which resulted in increased neurosphere formation, and GSC-specific biomarker expression [146] (Fig. 2). Therefore, PTEN potentially coordinates activities of two critical CSC pathways, WNT and HH.

WIP1 is a member of the PP2C family of Ser/Thr protein phosphatases and is encoded by *PPM1D*, a P53 target gene induced by various types of DNA damage [147] (Fig. 1). WIP1 is amplified/overexpressed in certain human malignancies [148]. WIP1 was shown to increase transcriptional activity, nuclear translocation, and protein stability of GLI (Fig. 2) [149]. Specifically, it was shown that regulation of the transcriptional activity of GLI1 by WIP1 depends on its phosphatase activity and is independent of P53 [149]. Moreover, WIP1 is essential for tumor growth and self-renewal *in vitro* and xenograft growth mediated by HH activation, and blockade of HH potentiates the effects of WIP1 inhibition on reducing tumor growth [149]. The P53-independent function of WIP1 as positive regulator of the SHH signaling was later confirmed in neuronal precursors, and in two independent HH-driven medulloblastoma (MB) models *in vivo* [150]. In both the *in vivo* models, genetic *Wip1*

deletion had radical effects on *de novo* MB tumorigenesis. These finding suggest that WIP1 plays a key role in sustaining tumor growth and CSC self-renewal mediated by the HH pathway. As a potentially druggable phosphatase [148, 151], WIP1 inhibition could constitute a novel treatment strategy for MB.

NOTCH and phosphatases in cancer stem cells

In normal stem cells, NOTCH pathway has an important role in regulation of self-renewal properties and differentiation states. NOTCH signaling is dysregulated in many cancers [152] and contributes to maintenance of their stemness properties [153]. PTEN functionally interacts with NOTCH in breast cancer. In breast cancer cells which are resistant to the anti-HER2 mAb trastuzumab, NOTCH-1 expression is increased and drives tumor recurrence. NOTCH-1 expression directly suppresses *PTEN* expression, and its inhibition resulted in PTEN induction and PTEN-mediated reversion of trastuzumab resistance [154] (Fig. 2). On the other hand, loss of PTEN in mammary fibroblasts promotes ErbB2-dependent mammary tumorigenesis. PTEN deletion was shown to trigger abnormal alveolar side-branching, which results in an expansion of the mammary epithelial stem cell (MaSC) enriched basal/myoepithelial population and an increase in *in vitro* stem cell activity. Mechanistically, PTEN depletion in tumor-associated fibroblasts down-regulated JAGGED-1, a transmembrane inhibitory ligand for the NOTCH3 receptor [155]. Moreover, reintroduction of JAGGED-1 within the PTEN-null fibroblasts preempted the observed increase in colony forming activity, which indicates that stromal JAGGED-1 has a key role in regulation of MaSC properties. Consistent with this, breast cancer patients with both low stromal JAGGED-1 and PTEN represent a shorter recurrence time compared to those whose tumors express low levels of either alone. These findings reveal a stromal PTEN-to-JAGGED-1 axis in maintaining the MaSC population and preventing breast cancer tumorigenesis [155].

As an indication for the potential role for PP2A in regulation of NOTCH signaling, *Drosophila* PP2A catalytic subunit was found to inhibit casein kinase 2 (CK2)-mediated effects in NOTCH signaling and to induce similar phenotype with *NOTCH* mutation [156]. More recently, the same group demonstrated that the regulatory PP2A B-subunit which mediates this regulation was human B56 homologue widerborst [157]. Moreover, in human NOTCH-dependent T-ALL cell line KOPTK1, PP2A reactivation by phenothiazines potently synergized with NOTCH inhibition by γ-secretase inhibitor GSI in cell killing [43]. In addition to PP2A-mediated regulation of NOTCH activity indicated by these studies, a recent study identified transcription factor IER5 as a direct NOTCH target, and PP2A B55α subunit as one of the IER5 suppressed genes downstream of NOTCH [158]. NOTCH and IER5 were epistatic in regulation of at least some NOTCH target genes further indicating PP2A-mediated control of NOTCH activity. Regulation of NOTCH signaling by other CSC phosphatases is yet obscure.

JAK/STAT and phosphatases in cancer stem cells

Janus-activated kinase/signal transducer and activator of transcription (JAK/STAT) pathway promotes transcription of genes involved in stem cell maintenance, hematopoiesis and neurogenesis [159, 160]. In addition to hematological malignancies, dysregulation of JAK/STAT pathway has been detected in stem-like cells in breast cancer, prostate cancer and GB models [161-163], and in CSC-mediated colon cancer metastasis [164].

Function of JAK effectors, the STAT transcription factors, are heavily regulated by both tyrosine and S/T phosphorylation. Several protein tyrosine phosphatases such as SHP1, SHP2, TCPTP45, DUSP3 and PTP1B have been shown to regulate different components of the JAK/STAT pathway, and contribute to its dysregulation in different cancer types [165].

475 However, there is a paucity of studies using CSCs and demonstrating the role of
476 phosphatases in JAK/STAT pathway regulation. Nevertheless, considering that most
477 hematological cancers could be classified as diseases of CSC dysfunction, and the critical
478 roles of JAK/STAT pathway in hematological CSCs, the previously identified roles of tyrosine
479 phosphatases in JAK/STAT regulation most probably translate to CSC biology [166, 167].
480 Most recently, a study demonstrated that homozygous myeloid cell lineage specific deletion
481 of *Ptp1b* induced AML and this was dependent on increased JAK/STAT activity [168]. A
482 recent study demonstrated that TICs have enhanced phosphorylation levels of STAT3
483 serine 727 because of PP2A inactivation [169]. Moreover, the PP2A-STAT3^{S727}-Col XVII
484 pathway increased the ability of TIC to establish malignant tumors in murine models of lung
485 cancer with pleural effusion, and spontaneous colon cancer metastasis [169]. Moreover,
486 PP2A-regulated JAK2 might have a role in CSC survival and self-renewal through induction
487 of SET and ß-catenin activation [49, 170].

488 ## Cancer relevance of cancer stem cell phosphatases

489 Due to their key roles in cancer therapy resistance and tumor recurrence, CSCs have strong
490 clinical implications. CSCs have been shown to be intrinsically more resistant to various
491 therapies in a wide range of malignancies, and constitute the self-renewing population of
492 cancer cells with a tumor-initiating capacity. Therefore, therapeutic targeting of CSCs has
493 attracted great attention. Whereas previous chapters focused on the role of phosphatases
494 in specific CSC pathways, below, we summarize the roles of phosphatases in controlling
495 tumor-initiating capacity, and therapy resistance of CSCs in selected cancer types.

496 **CSC phosphatases in hematological malignancies**

497 Hematological neoplasms are the human cancer types in which CSCs have the most
498 established role in tumor initiation, malignant progression and therapy resistance. All
499 hematological cell types are derived from either myeloid or lymphoid stem cells, and based

on the current understanding, leukemias and lymphomas are caused by defects in these stem cell populations, or in immature progenitor populations of hematological cells derived from HSCs [171, 172].

PTEN plays a key role in self-renewal of normal and leukemic HSCs. Loss of PTEN depletes normal HSCs and promotes leukemogenesis by generation of leukemia-initiating cells (LICs) [173, 174]. In line with the critical role of PTEN in suppressing the AKT/mTOR activity, several AKT and mTOR inhibitors have been shown to alleviate leukemogenesis in *Pten*-depleted cancer models [173, 174]. Additionally, β-catenin activation, and a t(14;15) chromosome translocation, favor formation of PTEN-deficient LICs and subsequent leukemogenesis [175]. In line with its haploinsufficient tumor suppressor activity, *Pten* heterozygous mice develop spontaneous T-cell leukemias and lymphomas [173, 174, 176, 177]. The effects of PTEN depletion on initiation of hematological (stem cell) malignancies in mouse models is dependent on the moment in mouse development when PTEN is depleted, and the cell type in which the depletion occurs. PTEN depletion during embryonal development leads to T-cell acute lymphoblastic leukemia and lymphoma [175, 178], whereas in adulthood it induces myeloid malignancies [179, 180]. Together with emerging therapeutic strategies to reactive haploinsufficient PTEN [23, 61, 181], these findings highlight PTEN pathway as an attractive therapeutic target to devise effective leukemia therapies without damaging the normal stem cell pool.

Inhibition of PP2A is strongly implicated in leukemogenesis. By series of elegant studies, Perrotti and co-workers have shown that non-genetic PP2A inhibition promotes propagation of a therapy resistant chronic myeloid leukemic stem cell population [49, 117, 182]. Mechanistically, the oncogenic BCR/ABL kinase inhibits PP2A activity by promoting SET expression, which in turn enhances the BCR/ABL signaling activity by preventing dephosphorylation of ERK, STAT5, AKT, MYC and BAD [117]. Furthermore, PP2A inhibition

is needed for CML-quiescent leukemic stem cell survival through the regulation of the G1/S proteins cyclin D2/cyclin-dependent kinase 6 [183], and a more recent study identified micro-RNA MIR300 as an additional mechanism for PP2A activation through inhibition of SET in quiescent CML-LSCs [184]. Indicative of therapeutic relevance, it was demonstrated that the PP2A activating compounds such as FTY720 [185], eliminate both proliferating and quiescent LSCs in CML, with the latter responsible for disease relapse [49, 182, 184]. Importance of SET, and its potential direct druggability in leukemic cells was supported by studies in which FTY720 derivatives, devoid of immunosuppressive activity, were found to efficiently kill leukemia cells by inhibiting the SET-PP2A interaction [49, 52, 53]. In addition to SET, another PP2A-B56 inhibitor CIP2A [29, 186] is overexpressed in CML, and its levels in diagnostic samples were shown to strongly predict progression of the disease to blast crisis [116]. Mechanistically, CIP2A expression promoted MYC levels, and the BCR/ABL kinase activity in CML cells [116]. More recently, a novel CIP2A splicing variant NOCIVA was discovered and high expression of NOCIVA was found to predict for poor patient outcomes both in AML and CML [39].

The cAMP-regulated phosphoprotein 19 (ARPP19) is a PP2A-B55 inhibitor protein important in mitosis. ARPP19 is highly expressed in embryonic tissues and its expression is decreased during development, suggesting that ARPP19 regulates stemness [187]. In keeping with this, ARPP19 is part of leukemic stem cells (LSC) signature in AML [188, 189]. Moreover, it was one of the three genes involved in phenotypic LSC signature, which predicted poor-prognosis in an AML cohort [188]. Although a recent follow-up study did not directly address the role of ARPP19 in AML LSCs, it found that high diagnostic ARPP19 levels predicted patient relapse from standard AML chemotherapy independently of known genetic AML risk factors [190].

549 Many druggable PTPs are also implicated in stem cell-derived leukemias and lymphomas
550 [25]. The clinical importance of SHP2 is strongly emphasized by high frequency of activating
551 mutations in hematological cancers (reviewed in [25, 167]), and preclinical efficacy of small
552 molecule SHP2 inhibitor in mouse AML models with different genetic and epigenetic
553 backgrounds [191]. Related to stem cell pathways discussed above, ablation of Gab2 in
554 *Ptpn11*-mutant mice decreased splenomegaly and myeloid cell infiltration in
555 nonhematopoietic organs, and normalized excessive myeloid differentiation of stem cells
556 [192]. Moreover, the acute leukemia progression of myeloproliferative neoplasm (MPN) was
557 reduced in the double mutant mice and their survival was prolonged [192]. Similar findings
558 were observed after treatment of the *Ptpn11*-mutant mice with rapamycin, a potent mTOR
559 inhibitor. Collectively, these results identify SHP2 as a candidate therapeutic target in
560 hematological cancers with small molecule inhibitors which are currently in clinical trials in
561 several solid cancer types [23, 73].
562 In mice with p53-mutant background, deletion of *Ptpn1* (coding for PTP1B) results in
563 emergence of predominantly B cell lymphomas [193], whereas inactivating mutations are
564 found from some human B cell lymphomas such as Hodgkin lymphomas [194]. Consistent
565 with stem cell characteristics of B-cell lymphomas, *Ptpn1*-/- mice predisposed to B-cell
566 lymphomas displayed increased numbers of immature Pre-B cells in their bone marrow
567 [193]. More recently, myeloid lineage specific deletion *of Ptpn1* was shown to result in
568 development of mouse AML accompanied with an increase in immature myeloid blast cells
569 in the peripheral blood [168]. These results highlight the dual function of PTP1B as an
570 oncogenic phosphatase in many solid cancers, but as a tumor suppressor in hematological
571 cancers [25]. Therefore, a caution about potential hematological effects of any future PTP1B
572 targeted therapies [23, 73] is fully warranted.

573 Polycythemia vera (PV) is a clonal hematopoietic stem cell disorder characterized by gain-
574 of-function mutation in *JAK2* kinase (JAK2V617F). Using induced pluripotent stem cells-
575 derived CD34+ progenitor-enriched cultures from JAK2V617F+ PV patient, Stetka *et al*.
576 recently showed that JAK2V617F abrogates activation of the proapoptotic P38/JNK MAP
577 kinases [195]. Mechanistically, the expression of DUSP1, which inactivates P38/JNK, was
578 high in these cells, and its RNAi-mediated knockdown augmented DNA damage response
579 and apoptosis. These results suggest that high expression of DUSP1 in the JAK2V617F+
580 PV progenitors is a protection mechanism against DNA damage and promotes their survival,
581 and further identify DUSP1 as a potential druggable target in PV [196]. On the other hand,
582 DUSP4 was found to be downregulated either by promoter hypermethylation or by gene
583 deletion in more than 80% of human DLBCL cases, which recognizes it as a human
584 lymphoma tumor suppressor phosphatase [197]. Low expression of DUSP4 also served as
585 a significant poor prognosis factor in DLBCL patients treated with standard chemotherapy.
586 Interestingly, as opposed to the role of JNK as a pro-apoptotic DUSP target in some other
587 malignancies, JNKs may also have oncogenic activities [198], and the authors provided
588 compelling evidence that JNKs are the targets of tumor suppressor activity of DUSP4 in
589 DLBCL cells [197]. Together with evidence for epigenetic silencing of another DLBCL tumor
590 suppressor phosphatase SHP1 [199], epigenetic restoration of expression of DUSP4 and
591 SHP1 could thus provide a therapeutic opportunity to target CSCs in DLBCL patients.

592 **CSC phosphatases in GB**

593 GB is a grade IV astrocytoma with a very poor median survival due to its rapid growth,
594 angiogenesis, invasiveness and therapeutic resistance [200, 201]. GB was among the first
595 cancer types in which the importance of CSCs for disease recurrence and therapy
596 resistance was established [4]. However, due to several conflicting reports, and because
597 molecular markers were not sufficient to selectively and sensitively define GSCs, the

definition of GSCs has been difficult to explicitly define. Rich and co-workers recently proposed that GSCs should rather be defined functionally based on their capacity for self-renewal and establishing heterogenic tumors *in vivo* [4]. Similar to definitive characteristics of GSCs, there has also been conflicting views about the origin of GSCs. While some reports identify them as reprogrammed astrocytes, most evidence suggests that the origin of GSCs are glial stem cells that reside in the subventricular zone of the brain [4, 202]. These cells generate neural progenitors and neuroblasts, and among phosphatases, PTEN [203, 204] and PP2A [205, 206] have been implicated in inhibiting the self-renewal and proliferation of these neural progenitor cell types. CIP2A was also recently shown to support neural stem cell fate of the neural crest cells which give rise to neuroblastomas [207].

PTEN is mutated in about 35% of GB tumors and represent one of the most frequent genetic alterations in GB [201]. Genetic deletion of *PTEN* was originally shown to co-operate with loss of P53 in driving mouse GB development [208]. Loss of PTEN also synergizes with several other oncogenic events in development of murine GB, which shares high functional similarities with human GB [209, 210]. Furthermore, by using a mouse model in which three tumor suppressors, *Nf1*, *Trp53*, and *Pten* were genetically deleted in adult committed neural and oligodendrocytes progenitors, Parada and co-workers showed that these adult subventricular zone cells were reprogrammed to two distinct type of GSCs with human GSC resemblance [211]. An important conclusion from these studies was that the cell of origin in which the tumor suppressors were inactivated defines the type of GB. More recently, in an attempt to address the fundamental differences in molecular mechanisms by which mouse and human cells undergo malignant transformation [32, 212], it was demonstrated that also neural stem cells (NSCs) derived from PTEN-depleted human embryonic stem cells underwent neoplastic transformation towards a GSC phenotype [213]. Mechanistically, the tumor suppressor role of PTEN in the human NSCs was proposed to be mediated by nuclear

PTEN-mediated direct inhibition of transcription factor CREB, and subsequent inhibition of PAX7 transcription [213]. Very interestingly, protein but not lipid phosphatase activity of PTEN was required for suppression of both NSC cell migration and CREB phosphorylation. Whether protein phosphatase activity of PTEN is critical for other GSC functions remains to be revealed, however, these results highlight that in addition to its lipid phosphatase activity toward PIP3, protein phosphatase activity of PTEN might have tumor suppressor role in NSCs as well. Together, these results indicate a fundamental role for PTEN as a tumor suppressor phosphatase which suppresses GSCs functions in GB.

Whereas PP2A subunit genes were found genetically intact in almost all sequenced human GBs [48], high expression of three PP2A inhibitor proteins PME-1, ARPP-19 and CIP2A correlate with disease aggressiveness in GB [118, 214, 215]. Additionally, these PP2A inhibitor proteins were overexpressed in GSC cell lines [47, 118], and inhibition of either CIP2A or PME-1 has been shown to inhibit clonogenic growth of primary GSC lines [48, 118]. Similar to other cancer types [216], the overexpression of CIP2A in GSC cells was stimulated by constitutive CHK1 kinase activity via GSC transcription factor STAT3 [118]. Therefore, constitutive DNA damage, which is very apparent in GB [217], could promote viability of GSC by this recently discovered feed-forward loop between CHK1, STAT3 and CIP2A. Further supporting the role of CIP2A-regulated PP2A-B56 complexes as GSC tumor suppressors, the mRNA expression of B56α and B56γ was downregulated in GBs versus normal samples [118]. Of a potential therapeutic relevance, a recent study demonstrated that pharmacological activators of PP2A-B56, the SMAPs [46], killed CSCs very efficiently *in vitro*, regardless of their transcriptional subtype or mutational background [47]. *In vivo*, orally dosed SMAP therapy significantly inhibited growth of intracranial GB cells with CSC properties, and extended the lifespan of the tumor bearing mice by 70% [47]. Moreover, in line with the results indicating that PP2A reactivation overcomes kinase inhibitor resistance

in other cancer types [20, 49, 218, 219], PME-1-mediated PP2A inhibition was found to drive kinase inhibitor resistance in GSCs [48]. On the contrary to these studies which clearly implicate the pharmacological PP2A reactivation as a potential GSC-targeted therapeutic approach, a recent study demonstrated that high PP2Ac catalytic activity in GB significantly correlates with a poor patient survival [220]. These seemingly paradoxical findings can be explained by the fact that whereas reactivation of certain tumor suppressive PP2A complexes by SMAPs [22] or by inhibition of PP2A inhibitor proteins [48, 118] targets specific CSC survival pathways, inhibition of total PP2A activity by chemical PP2A inhibitors such as okadaic acid [220] and LB100 derivatives [221] induces a widespread S/T phosphorylation imbalance and GSCs cannot compensate it. Nevertheless, the results obtained by okadaic acid or LB100 should be treated with caution in relation to PP2A CSC functions, because these compounds also efficiently inhibit related oncogenic phosphatase PP5 [23, 109, 222].

In summary, including the evidence about SHP2, DUSPs and CDC25 [72, 79, 223], it is clear that phosphatases have important pathogenic roles in GSCs. Due to the lack of any major advance in GB therapies in the last 15 years, and the central role of GSCs in GB therapy resistance [4], the recent development of orally bioavailable small molecules targeting GSC phosphatases [23, 47, 72, 73] might provide new therapeutic opportunities for these patients. Especially, regarding the plasticity of GSCs, and the role of SHP2 and PP2A in therapy-induced signaling rewiring and GSC drug responses [19, 20, 48, 72, 224], their blockade in combination with existing therapies could inhibit drug tolerance in GB.

CSC phosphatases in other cancer types

PTEN loss in prostate stem/progenitor cells triggers prostate cancer tumor initiation and invasion [225]. Moreover, constitutive activation of the RAS/MAPK signaling pathway co-

674 operates with *PTEN* loss to favor metastasis in prostate cancer stem/progenitor cells [226].
675 Interestingly, CIP2A was recently implicated in prostate CSCs [115]. Furthermore, our
676 unpublished findings suggest that those PrCa tumors that harbor both complete loss of
677 PTEN, and overexpression of PP2A inhibitor protein PME-1 (i.e. double tumor suppressor
678 phosphatase inhibition), are particularly aggressive [227].
679 Testicular cancers are considered to be of stem cell origin [228], and express the pluripotent
680 stem cell marker OCT4 [228, 229]. CIP2A was recently shown to be co-expressed with
681 OCT4 in 95% of a small number of testicular cancer samples, and OCT4 was shown to drive
682 CIP2A gene promoter activity in testicular cancer cells *in vitro* [229]. Interestingly, CIP2A
683 and MYC double positivity was detected in testicular cancers, but MYC was not expressed
684 in normal CIP2A positive testicular stem cells, spermatogonia [230]. Moreover, double
685 positivity for OCT4 and CIP2A associated with poor differentiation in head and neck
686 squamous cell carcinoma (HNSCC) cells, and a worse survival in radiotherapy treated
687 HNSCC patients [229]. The PP2A inhibitor SET was recently shown to drive stemness
688 characteristics in gastric cancer cells via E2F1. Although the study did not utilize gastric
689 CSCs, the authors demonstrated that SET promoted stem cell-like sphere growth of
690 established cell lines and its overexpression increased expression of stem cell associated
691 CD44 [231]. Interestingly, inhibition of SET by OP499 selectively inhibited spheroid growth
692 of gastric cancer cells and had significant antitumor effect in a xenograft model.
693 In small cell lung cancer (SCLC), protein kinase A (PKA) activity is required for propagation
694 of SCLC stem cells and its genetic ablation, or inhibition by pharmacological reactivation of
695 PP2A was found to suppress SCLC expansion in cell culture and *in vivo* [232]. In non-small-
696 cell lung cancer (NSCLC) cells, P38 kinase activity has been shown to suppress CSC
697 properties [233]. A recent study by Deng *et al*. has shown that elevated expression of WIP1
698 correlates with reduced levels of activated P38, and increased expression of CSC markers

699 in NSCLC tissues. Functionally, WIP1 was found to promote NSCLC CSC properties by
700 inhibiting P38 activity, and pharmacologic inhibition of WIP1 inhibited CSC properties in
701 NSCLC cells *in vitro* and *in vivo* [234].
702 Examples of phosphatases implicated in breast cancer stem cells (BCSC) include different
703 DUSPs and PTEN. Boulding *et al*. documented that DUSP1, 4 and 6 differentially contribute
704 to the formation of $CD44^{hi}/CD24^{lo}/EpCAM^{+}$ BCSCs [80]. Similar to its putative tumor
705 suppressor role in DBCL [197], DUSP4 loss has been functionally linked to BCSC
706 phenotypes, breast cancer progression, and therapy resistance [235-238]. Another
707 interesting indication for the roles of DUSPs in BCSC was published by Semenza's
708 laboratory recently [239]. They demonstrated that chemotherapy-induced HIF1 tuned DUSP
709 expression balance through inhibition of DUSP16 and induction of DUSP9. As a result,
710 breast cancer cells cultured in stem cell like conditions displayed decreased p-ERK and
711 increased P38 MAPK activity, resulting *in vivo* in Nanog-mediated BCSC enrichment [239].
712 The epithelial-mesenchymal transition (EMT) increases self-renewal capability of tumor cells
713 [240]. As shown by a recent study, restoration of PTEN expression reverses EMT, and
714 inhibits CSC capacity of breast cancer cells via dephosphorylation and downmodulation of
715 the adaptor protein Abi1 [241]. Prognosis of patients with advanced-stage endometrial
716 cancer remains poor [242]. A recent study demonstrated that DUSP6 promotes expression
717 of CSC markers ALDH1, NANOG, SOX2 and OCT4A, and enhances sphere formation
718 ability of endometrial cancer cell lines [243]. In patients with endometrial cancers, elevated
719 expression of DUSP6 associates with a shorter progression-free and overall survival. These
720 results suggest that DUSP6 could be a therapeutic target to eliminate CSCs in endometrial
721 cancer [243].
722
723

Conclusions

Phosphatases play major roles in CSC-mediated self-renewal during cancer initiation and contribute to therapy resistance in several cancer types. Despite this, our understanding of the role of phosphatases in CSC is lagging severely behind of kinases and other cellular signaling mechanisms. Therefore, characterization of the roles of many entirely unstudied phosphatases in CSC and/or further clarification of the precise roles of the already established important oncogenic and tumor suppressor phosphatases might have a transforming impact. Considering recently emerging pharmacological opportunities for phosphatase modulation [22, 23, 71, 73], and role for many of these druggable phosphatases in CSCs, it will be important to evaluate whether the phosphatase targeting compounds would open novel therapeutic opportunities for CSC-targeted therapies. Regarding non-genetic therapy-induced drug tolerance [13, 14], recent studies have demonstrated a profound impact for phosphatase modulators in cancer therapy responses [19, 20, 23, 48, 49, 51, 70, 73, 120, 151, 218, 219, 221]. This further indicates that combinations of CSC phosphatase targeted therapies, with existing cancer therapies could provide clinically meaningful benefits. Especially in the case of CSC pathways for which there are no currently approved targeted therapies, such as HH and NOTCH, further understanding how phosphatase targeting interacts with these pathways might open entirely novel therapeutic opportunities. In theory, it could also be possible to combine the phosphatase targeting therapies based on the knowledge how different CSC pathways co-operate in the CSC apoptosis resistance and emerging information how phosphatases regulate these pathways (Fig. 2).

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Figure legends

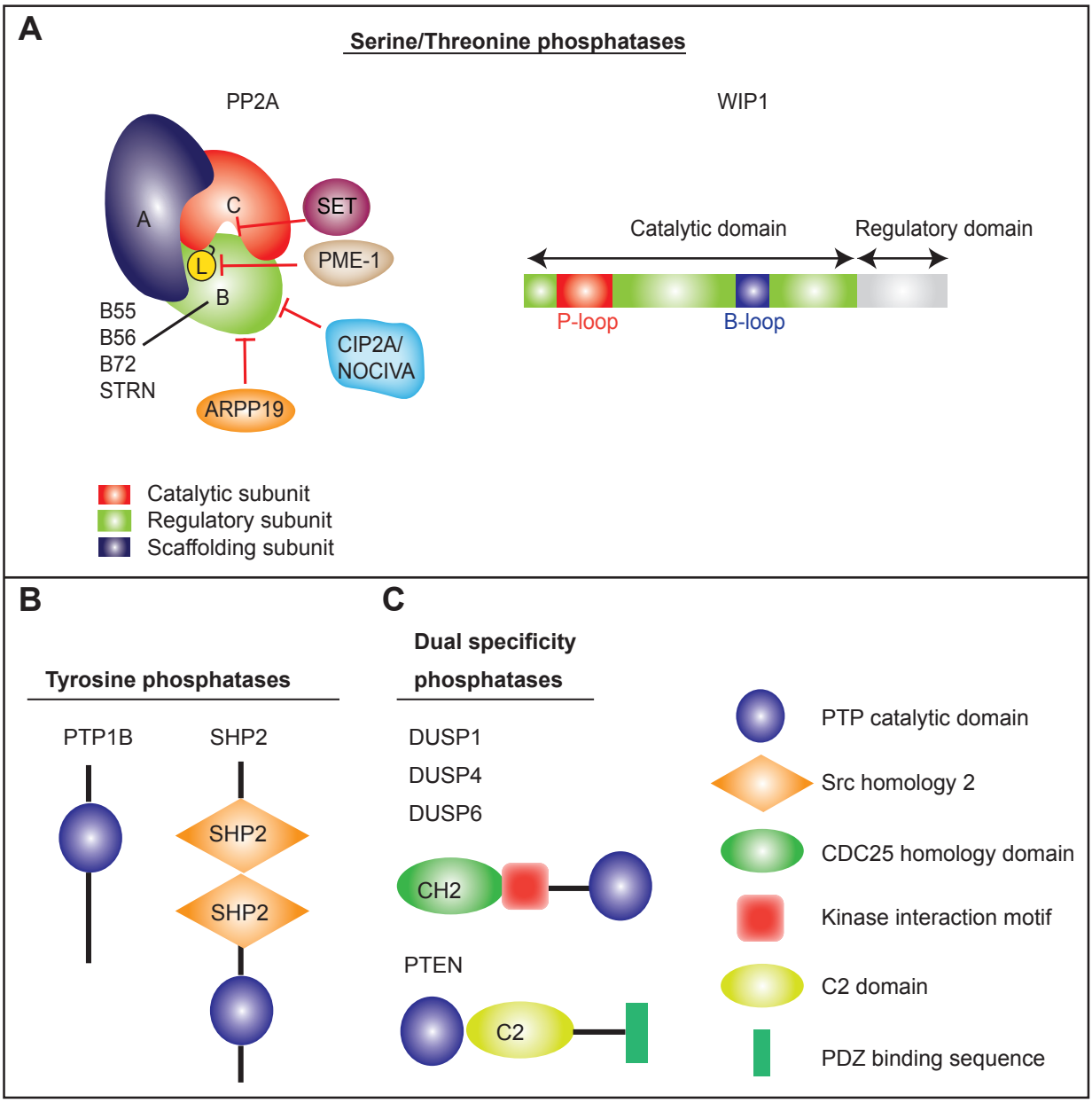
Figure 1. Families of cancer stem cell phosphatases. Based on their amino acid substrates, phosphatases are divided into **(A)** serine/threonine phosphatases, **(B)** tyrosine phosphatases (PTPs) or **(C)** dual-specificity phosphatases that are capable of dephosphorylating both serine/threonine and tyrosine residues or tyrosine residues and lipids. **(A)** PP2A is a serine/threonine phosphatase that functions as a trimeric complex in which a member from any four B-subunit families (B55, B56, B72 or STRN) defines the substrate specificity. PP2A complexes are inhibited by oncogenic inhibitor proteins PME-1, SET, CIP2A/NOCIVA and ARPP19. WIP1 (PPM1D) instead is a monomeric serine/threonine phosphatase consisting of a catalytic and a regulatory domain. **(B)** Representative examples of cancer stem cell relevant non-receptor type PTPs which are monomeric proteins encoded by a single gene but composed of indicated functional domains that are relevant for their activity regulation and substrate recognition. **(C)** Representative examples of cancer stem cell relevant dual specificity phosphatases. Whereas DUSPs dephosphorylate both tyrosines and serine/threonines on mitogen-activated protein kinases, PTEN dephosphorylates a lipid, phosphatidylinositol PIP_3 and tyrosines, serines, or threonines.

Figure 2. Phosphatase-mediated regulation of cancer stem cell signaling pathways. Phosphatases involved in each of the depicted pathways are colored yellow. Direct regulation of the signaling pathway is shown with a connective line between a phosphatase and a specific target protein, whereas those phosphatases that either have been shown to

indirectly regulate phosphorylation, or the direct dephosphorylating activity is unclear, are presented adjacent to their target proteins.

Author contributions: Conceptualization (MM, JW), Data mining (MM, TA, JW), Manuscript writing (MM, TA, JW), Figure drawing (MM, TA).

Figure 1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Serine/Threonine phosphatases
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SET
L
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B55
B56
B72
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NOCIVA
ARPP19
WIP1
Catalytic domain
Regulatory domain
P-loop
B-loop
Catalytic subunit
Regulatory subunit
Scaffolding subunit
B
Tyrosine phosphatases
PTP1B
SHP2
SHP2
SHP2
C
Dual specificity
phosphatases
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CH2
PTEN
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PTP catalytic domain
Src homology 2
CDC25 homology domain
Kinase interaction motif
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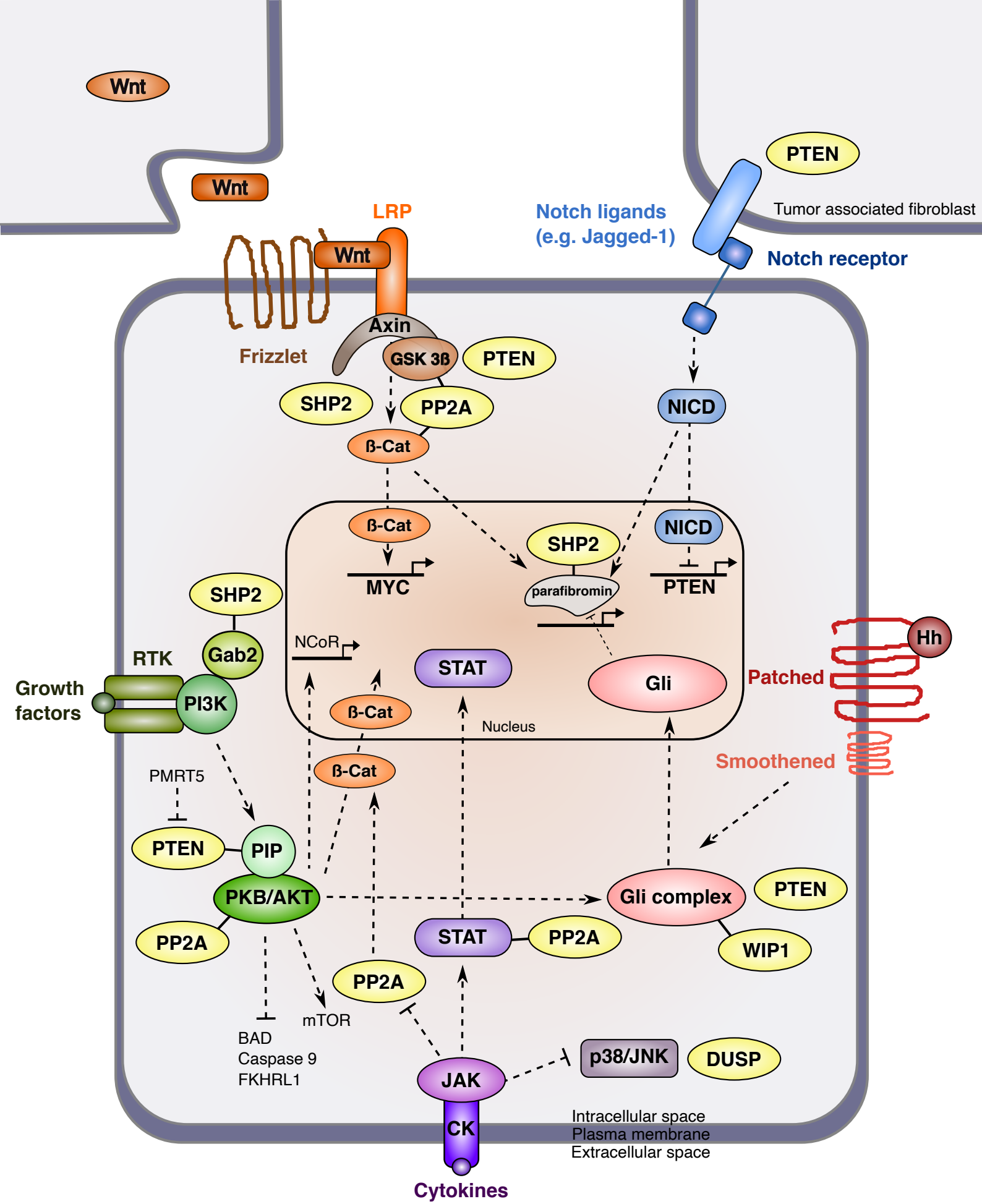
PDZ binding sequence


Wnt
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Axin
Frizzlet
GSK 3ß
PTEN
SHP2
PP2A
ß-Cat
PTEN
Tumor associated fibroblast
Notch ligands
(e.g. Jagged-1)
Notch receptor
NICD
ß-Cat
MYC
SHP2
parafibromin
NICD
PTEN
NCoR
STAT
Gli
Nucleus
ß-Cat
ß-Cat
SHP2
Gab2
RTK
Growth
factors
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PMRT5
PTEN
PIP
PKB/AKT
PP2A
PP2A
mTOR
BAD
Caspase 9
FKHRL1
Hh
Patched
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CK
p38/JNK
DUSP
Intracellular space
Plasma membrane
Extracellular space
Cytokines

Figure 2