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GSK-3: a multifaceted player in acute leukemias

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Abstract

Glycogen synthase kinase 3 (GSK-3) consists of two isoforms (α and β) that were originally linked to glucose metabolism regulation. However, GSK-3 is also involved in several signaling pathways controlling many different key functions in healthy cells. GSK-3 is a unique kinase in that its isoforms are constitutively active, while they are inactivated mainly through phosphorylation at Ser residues by a variety of upstream kinases. In the early 1990s GSK-3 emerged as a key player in cancer cell pathophysiology. Since active GSK-3 promotes destruction of multiple oncogenic proteins (e.g. β-catenin, c-Myc, Mcl-1) it was considered to be a tumor suppressor. Accordingly, GSK-3 is frequently inactivated in human cancer via aberrant regulation of upstream signaling pathways. More recently, however, it has emerged that GSK-3 isoforms display also oncogenic properties, as they up-regulate pathways critical for neoplastic cell proliferation, survival, and drug-resistance. The regulatory roles of GSK-3 isoforms in cell cycle, apoptosis, DNA repair, tumor metabolism, invasion, and metastasis reflect the therapeutic relevance of these kinases and provide the rationale for combining GSK-3 inhibitors with other targeted drugs. Here, we discuss the multiple and often conflicting roles of GSK-3 isoforms in acute leukemias. We also review the current status of GSK-3 inhibitor development for innovative leukemia therapy.

Introduction

Glycogen synthase kinase-3 (GSK-3) is an evolutionary conserved, constitutively active, broad specificity serine/threonine kinase involved in the control of multiple signaling pathways. GSK-3 was originally discovered as the enzyme which, by phosphorylating and inactivating glycogen synthase, opposes glucose conversion into glycogen [1]. However, GSK-3 regulates other physiological functions, including cell proliferation, differentiation, apoptosis, and embryonic development [2, 3].

Over the last two decades GSK-3 has emerged as a kinase critically involved in several human disorders, including type 2 diabetes, cardiovascular diseases, chronic inflammation, bipolar disorder, Alzheimer's disease, and cancer [4]. Regarding cancer, GSK-3 was initially considered a tumor suppressor. However, more recent investigations have disclosed an oncogenic role for GSK-3 in some cancer settings, including hematological malignant disorders [5].

It should be considered that several GSK-3 inhibitors have been synthesized over the years and represent emerging tools for possible clinical intervention in human disorders, especially in combination with other treatments [5]. Here, after providing a brief overview of GSK-3 signaling and inhibitors, we summarize the current knowledge on GSK-3 relevance in the pathophysiology of acute leukemias. Moreover, we illustrate how GSK-3 inhibitors might be employed in the future for improving the outcome of this class of malignant disorders.

GSK-3 signaling

GSK-3 comprises the α (51-kDa) and β (47–kDa) isoforms, or more correctly, paralogs, as they are homologous proteins encoded for by different genes (*GSK3A* and *GSK3B* in mammals, located on chromosome 19 and chromosome 3, respectively) [6]. GSK-3 paralogs display a bi-lobal architecture, consisting of a large COOH-terminal globular domain, responsible for the kinase activity, and a small ATP-binding NH_2-terminal lobe. In addition, a glycine-rich domain (73% glycine) is present only in the NH_2-terminal α isoform (**Figure 1a**). The two paralogs display 98% amino acid sequence identity within their kinase domains, while sharing ~ 85% amino acid overall sequence homology. Nevertheless, the isoforms share only 36% similarity in the last 76 amino acids of their COOH-terminal regions [7]. Although GSK-3α and GSK-3β are ubiquitously expressed in tissues and organs and share some common substrates [8], they also exhibit distinct biological roles. Indeed, the loss of one paralog could not be fully compensated for by the other, as demonstrated by gene ablation studies in mice [9]. Regarding its subcellular localization, GSK-3 is mainly a cytosolic enzyme, however there are pools of mitochondrial [10] and nuclear GSK-3 [11]. Nuclear GSK-3 continuously shuttles from the nucleus to the cytoplasm and vice-versa [12]. Importantly, an aberrant increase in the GSK-3β nuclear pool has been linked to upregulation of nuclear factor-κB (NF-κB)-mediated gene transcription in some cancer settings, including acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) [13, 14].

At variance with most protein kinases, GSK-3α and GSK-3β are constitutively active, as autophosphorylation of GSK-3α at Tyr279 and of GSK-3β at Tyr216, which increases the enzymatic activity, is observed under resting conditions [15]. However, kinases capable of phosphorylating the GSK-3 paralogs at tyrosine residues have been identified, including p60 Sarcoma (p60 Src) [15] and mitogen-activated protein kinase kinase (MEK) [16]. In contrast, phosphorylation at Ser21 (GSK-α) and Ser9 (GSK-3β) inactivates GSK-3 [17]. (**Figure 1b**). Several upstream kinases phosphorylate the Ser21/9 residues, including Akt, protein kinase A, protein kinase C, p70 ribosomal S6 kinase (p70S6K), and p90 ribosomal S6 kinase (p90RSK) [18]. Furthermore, phosphorylation at Thr43 by extracellular signal-regulated protein kinase (ERK) or at Thr389/390 by p38 mitogen-activated protein kinase (p38MAPK) results in GSK-3 inhibition, as these events facilitate subsequent phosphorylation at Ser 21/9 [19] (**Figure 1b**). The effects of the kinases on GSK-3 are counterbalanced by protein phosphatases, including protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and protein phosphatase 2B (PP2B) that dephosphorylate GSK-3α/β at Ser21/9, thereby upregulating the enzymatic activity [20]. Moreover, the phosphorylated tyrosine residues are targeted by tyrosine protein phosphatase Src homology-2 (SH2) domain-containing phosphatase 1 (SHP-1) which inhibits GSK-3 activity [21] (**Figure 1b**).

Although nearly 100 substrates of GSK-3 have been identified, only a handful of them have been validated in physiological settings. Nevertheless, this fact underscores the importance of GSK-3 in regulating a myriad of different cellular processes. GSK-3 prefers substrates that have been already phosphorylated (primed) by other kinases, including p38MAPK, ERK, 5′-adenosine monophosphate-activated protein kinase, and c-Jun N-terminal kinase. Among GSK-3 substrates, several play important roles in cancer cell biology, such as activator protein-1 (AP-1) [22], β-catenin [23], cyclin D1 [24], c-Myc [25], and myeloid leukemia cell differentiation protein 1 (Mcl-1) [26]. Moreover, two GSK-3 substrates, p70S6K and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), are involved in protein translation [27, 28] (**Figure 1b**). GSK-3 substrates are recognized by specific E3 ubiquitin ligases through phosphorylated motifs (phosphodegron motifs) and targeted for proteasomal degradation [29]. Therefore, in several cancer settings GSK-3 acts as a negative regulator of Wnt/β-catenin, growth factor/tyrosine kinase receptor, Sonic Hedgehog, and G-protein-coupled receptor signaling networks, thereby behaving as an oncosuppressor kinase [5]. However, as we will highlight in this review, there are contexts where active GSK-3 leads to degradation of anti-cancer molecules, hence GSK-3 behaves as an oncogenic kinase in some settings.

For the scopes of this review, it is important to underscore that GSK-3 plays a pivotal role in the canonical Wnt/β-catenin network, as it controls the turnover of the free pool of cytoplasmic β-catenin [5]. Indeed, GSK-3β is part of multiprotein destruction complex that includes adenomatous polyposis coli (APC), casein kinase 1α (CK1α), Axis inhibition protein 1 (Axin1), and β-catenin itself. CK1α phosphorylates β-catenin at Ser45 residue, thereby priming it for subsequent phosphorylation by GSK-3 at Ser33/37 and Thr41. APC and Axin1 facilitate the interactions of GSK-3 with β-catenin, as GSK-3 does not bind β-catenin directly [30]. Phosphorylated β-catenin is then recognized by the F-box/WD repeat-containing protein 7 (FBXW7), i.e. the substrate recognition motif of multimeric E3 ligase

SCF (SKP-Cullin-Fbox) and targeted for proteasomal degradation [31] (**Figure 2**). In contrast, when GSK-3 activity is inhibited by upstream kinases, β-catenin migrates to the nucleus, where it activates specific pro-oncogenic transcriptional programs [32]. Therefore, GSK-3 acts as a key suppressor of the Wnt/β-catenin signaling pathway [2] that is frequently overactive in acute leukemias [33].

Given that genetic ablation of either GSK-3α or GSK-3β did not lead to β-catenin accumulation in an embryonic stem cells context, the two paralogs have been considered to be redundant in this respect [34]. However, more recent findings seem to indicate the GSK-3α and GSK-3β could play a cell-dependent differential role in the degradation of β-catenin [35]. Therefore, these observations suggest that alterations in the function of the two paralogs may have varying impacts on human cancer pathophysiology.

GSK-3 inhibitors

Since GSK-3 is a kinase at the crossroad of many signal transduction networks playing key roles in cancer cells, it has attracted the attention of both the academic community and pharmacological companies for the development of selective inhibitors. However, the development of GSK-3 inhibitors as cancer therapeutics has been hampered by the very large number of GSK-3 substrates, whose targeting might disrupt cell functions of vital importance for healthy cells. The first identified GSK-3 inhibitor was LiCl, which is not specific [36]. Subsequently, synthetic ATP-competitive GSK-3 inhibitors were isolated. Early drugs (e.g. SB216763) were not GSK-3 specific, as they also inhibited cyclin-dependent kinases [37]. More recently, AR-A014418, CHIR99021, and LY2090314 have been identified as

GSK-3 inhibitors. These inhibitors are more potent and specific, however, they target both GSK-3α and GSK-3β with almost equal potency [37]. Indeed, the amino acid sequence around the ATP-binding pocket of the two GSK-3 paralogs is nearly identical, hence drugs targeting this domain fail to discriminate [4]. However, paralog-selective inhibitors such as compound 27, BRD0705, and compound 28_14 (that target GSK-3α [35, 38, 39]) or TWS199 and BRD3731 (that target GSK-3β [35, 40]) have been identified. 6-bromoindirubin 3′-oxime (BIO) is a hemi-synthetic derivative of indirubins found in edible mollusks and plants which inhibits GSK-3β [41].

Tideglusib (NP031112) is the first, non-ATP-competitive GSK-3 inhibitor which has been tested in patients with neurological disorders, such as supranuclear palsy and Alzheimer's disease [42, 43]. GSK-3 inhibitors have entered clinical trials for both neurodegenerative disorders and cancer, however a general lack of therapeutic activity was reported [44, 45].

The role of GSK-3 in hematopoietic stem cells (HSCs)

It has long been known that LiCl, which inhibits GSK-3, increases circulating HSCs (identified as $CD34^+$ cells [46]) as well as peripheral blood neutrophils and platelets in more than 90% of patients taking LiCl [47]. Furthermore, LiCl increases transplantable HSCs in mice [48]. These studies implicated GSK-3 as an important regulator of HSC homeostasis. Moreover, canonical Wnt/β-catenin signaling plays key roles in the maintenance of HSC homeostasis. Evidence suggests that Wnt regulates HSC physiology in a dose-dependent manner, as mild levels of Wnt activation enhance hematopoiesis, whereas a high Wnt activity impairs HSC function [49].

Early findings demonstrated that administration of a GSK-3 inhibitor (CHIR99021) to recipient mice transplanted with either mouse or human HSCs, improved megakaryocyte and neutrophil recovery, as well as the recipient survival. This resulted in enhanced long-term repopulation [50]. In a subsequent investigation, it was reported that BIO activated β-catenin in cord blood $CD34^+$ cells, thereby upregulating two β-catenin transcriptional targets critical for HSC self-renewal, i.e. c-Myc and Homeobox protein B4 [51]. Moreover, GSK-3β inhibition by BIO resulted in delayed ex-vivo expansion of $CD34^+$ cells, although it enhanced the preservation of stem cell activity in long-term culture with bone marrow (BM) stroma. These effects were due to impaired cell cycling, decreased apoptosis, and increased adherence of HSCs to BM stroma. The improved adherence to stroma was mediated via upregulation of CXCR4 [51]. Overall, these findings suggest the involvement of GSK-3β in the preservation of HSCs and their interactions with the BM microenvironment. Therefore, methods for the inhibition of GSK-3β activity may be useful for clinical ex-vivo expansion of $CD34^+$ cells for transplantation.

More recently, it was reported that the use of a mild dosage of CHIR99021 resulted in the expansion of purified murine HSCs in vitro through the induction of a moderate level of β-catenin activity [52]. However, if the experiments were repeated in a setting where HSC purification was not performed, CHIR99021 also stimulated myeloid cells to produce inflammatory cytokines [Tumor Necrosis Factor α (TNFα) and IL-1β] that attenuated HSC expansion through induction of p38MAPK activation, a well-established negative regulator of HSC self-renewal [53] and of GSK-3 [19]. Accordingly, HSC expansion could be restored by the concomitant use of a p38MAPK inhibitor (SB203580) [52].

However, a correct interpretation of the findings emerging from the aforementioned studies is limited by the use of GSK-3 inhibitors that are far from being specific. More accurate information on the roles of GSK-3 in general and of its paralogs in particular in HSCs was obtained in investigations based on genetic ablation.

The disruption of *Gsk3* in the BM transiently expanded the pool of HSCs in a β-catenin–dependent manner, consistently with a role for Wnt signaling in HSC homeostasis [54]. In contrast, in assays of long-term HSC (LT-HSC) function, disruption of *Gsk3* progressively depleted HSCs through activation of mechanistic target of rapamycin (mTOR). The LT-HSC depletion was prevented by mTOR inhibition and exacerbated by β-catenin knockout. These findings implied that GSK-3 regulated both Wnt/ β-catenin and mTOR signaling in HSCs. These pathways promote HSC self-renewal and lineage commitment, respectively, such that *Gsk3* knock-out in the presence of the mTOR inhibitor, rapamycin, expanded the LT-HSC pool. Accordingly, the same group subsequently demonstrated that LiCl could increase the number of LT-HSCs in vivo when combined with rapamycin [55]. In general, it has long been thought that there exists a functional redundancy between *Gsk3α* and *Gsk3β* alleles in the regulation of Wnt/β-catenin signaling in mouse embryonic cells [34]. However, a recent study demonstrated that GSK-3β deletion in HSCs resulted in a pre-neoplastic state consistent with human myelodysplastic syndrome (MDS, i.e. an increase in dysplastic granulocytes along with reduced erythrocyte, monocyte, and lymphocyte numbers and no change in platelet levels) while GSK-3α deletion had no effects. Of note, when BM cells from primary recipients were serially transplanted into secondary mice, the animals developed an identical hematopoietic disorder [56]. This observation suggested that

GSK-3β deletion generates self-renewing cells that could be functionally defined as MDS-initiating cells (MDS-ICs) as they are capable of sustaining MDS in vivo. However, transcriptome and functional studies revealed that both GSK-3β and GSK-3*α* uniquely contribute to AML development in mice by affecting Wnt/Akt/mTOR signaling or metabolism (mainly mitochondrial activity), respectively [56]. The Wnt/β-catenin pathway was a major pathway up-regulated in cells who had lost GSK-3β. Interestingly, both the transcriptome and the epigenomic profile of the *Gsk3β* knock-out mice displayed similarities to the BM of patients with MDS. Furthermore, the authors were able to define a specific *Gsk3β* molecular signature that then allowed for a molecular discrimination among MDS and AML patient transcriptomes. Therefore, this *Gsk3β* signature may have the potential to serve as both a diagnostic and a prognostic biomarker [56] of MDS. Overall, the findings by Guezguez et al. [56] suggest that some the signaling pathways regulated by GSK-3β are critical for the evolution of MDS from healthy HSCs in both pediatric and adult patients, which then allows a permissive state for additional genetic lesions, such as loss of *Gsk3α*, thereby leading to AML development.

GSK3 signaling in AML

AML is an aggressive and widely heterogeneous disorder characterized by the clonal proliferation of HSC or progenitor cells of the myeloid lineage. The AML field has seen major advances from the standpoint of disease pathobiology, however therapeutic advances remain quite limited. AML is still a devastating disease with poor patient outcome, as current chemotherapy protocols mostly lead to only initial remission.

Therefore, there is an urgent requirement for the development of alternative targeted treatment strategies to widen the therapeutic windows, especially for older patients [57].

Several molecular alterations of key components of signaling pathways contribute to AML pathogenesis and progression [58]. Among these, GSK-3 can be considered as a central hub in a variety of signaling networks involved in leukemic cell proliferation, survival, and drug-resistance [33]. As underscored above, the role of GSK-3 paralogs in cancer development is controversial and AML makes no exception to the rule. Regarding the tumor suppressor role of GSK-3 in AML, it has been shown that patients with elevated levels of Ser 21/9 phosphorylated, hence inactive, GSK-3 displayed a lower overall survival and complete remission incidence, compared with patients with low levels. This finding implied that the levels of Ser 21/9 p-GSK-3 could be used as a negative prognostic factor in an intermediate cytogenetic AML subgroup [59]. However, a limit of this study is that there was no attempt to identify the GSK-3 paralog which could act as a tumor suppressor. In another study is was possible to correlate low levels of *GSK3A* with resistance to the FLT3-ITD inhibitor, AC220 [60]. Drug-resistance was due to activation of the Wnt/β-catenin signaling.

On the other hand, an important role for GSK-3 as a tumor promoter in AML was firstly highlighted by Wang et al. [61] who demonstrated that GSK-3 activity was critical in sustaining mixed-lineage leukemia gene (MLL) leukemia cell transformation and proliferation, via decreased expression of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$. The decreased levels of $p27^{Kip1}$ were not the consequence of down-regulated mRNA synthesis, therefore they might be dependent on decreased protein stability, although this was not formally demonstrated. Nevertheless, using a series of elegant genetic manipulation techniques, the authors were able to demonstrate that murine *MLL–ENL* transformed myeloid progenitors lacking both GSK-3 isoforms were unable to induce leukemia in transplanted mice. Therefore, it was concluded that GSK-3 isoforms cooperated to maintain critical features of the MLL-transformed phenotype, although GSK3-β predominated over GSK3-α. Likewise, GSK-3 pharmacological inhibition acted on leukemic stem cells (LSCs), without interfering with the growth of healthy HSCs, thereby supporting the hypothesis that GSK-3 could represent an effective candidate for drug targeting in this leukemia setting. Accordingly, mice with MLL-like leukemia showed an increase of about 40-50% in lifespan when treated with LiCl. This observation is intriguing, as it had previously been demonstrated that LiCl enhanced the repopulating capacity of HSCs transplanted in NOD/SCID mice, through Wnt/β-catenin and Notch signaling activation, as we have highlighted previously [50].

However, in a subsequent study carried out in human AML cell lines and primary samples it was demonstrated that the specific loss of GSK-3α induced the expression of genes and morphological changes consistent with myeloid maturation, thereby leading to differentiation of AML cells [62]. Moreover, GSK-3α-specific suppression led to impaired leukemic cell growth and proliferation, induction of apoptosis, loss of colony formation in semi-solid medium, as well anti-leukemic activity in vivo. In contrast, ectopic expression of a *GSK3A* cDNA insensitive to the effects of the shRNA rescued the alterations on colony formation, thereby reinforcing the hypothesis that in this setting GSK-3α, and not GSK-3β, was critical for driving leukemic cell proliferation and survival. Interestingly, loss of GSK-3α did not impact on β-catenin stabilization [62].

A possible explanation for the contradictory roles of GSK-3 in cancer including AML, might be related to an imbalance between a pro-apoptotic cytoplasmic GSK-3β and an oncogenic nuclear GSK-3β. Indeed, the existence of an aberrant pool of nuclear GSK-3β was recently reported in AML cells, where it drives leukemic cell survival and drug-resistance. Moreover, the nuclear, but not the cytoplasmic, fraction of GSK-3β enhances AML colony formation and AML growth in murine models. Mechanistically, the nuclear GSK-3β pool promotes nuclear localization of the NF-κB subunit, p65, thereby enhancing transcription of pro-survival genes, including B-cell lymphoma-extra-large (Bcl-xL) and X-linked inhibitor of apoptosis protein (XIAP) (**Figure 3**). Importantly, healthy $CD34^+$ HSCs lack this pool of nuclear which has clinical significance as it strongly correlates to a worse AML patient outcome [13]. It could be therefore hypothesized that when GSK-3β is upregulated and localizes to the nucleus, the kinase acts as tumor promoter, whereas when it localizes to the cytoplasm, it could display pro-apoptotic effects. In this context, it is important to emphasize that GSK-3β constantly shuttles between the cytoplasm and the nucleus, although it predominantly localizes to the cytoplasm [11]. It is also worth remembering that GSK-3β lacks a classical nuclear export signal. For its nuclear export GSK-3β relies on its interactions with Frequently rearranged in advanced T-cell lymphomas protein 1 and 2 whose expression levels are variable in cancer cells [63]. Therefore, further investigations on the regulation of GSK-3β transport in and out of the nucleus in AML cells should be performed to better clarify this issue, as it is not easy to reconcile the findings from Ignatz-Hoover et al. [13] with the data reported by Banerji and coworkers on the pro-leukemic role of GSK-3α [62]. The difference could not be related to the cell models used, as both the groups analyzed a similar panel of human AML cell lines in addition to primary AML samples. Moreover, some other aspects of the work of Ignatz-Hoover and coworkers [13] need to be further clarified. For example, it is unclear how high levels of nuclear GSK-3β resulted in NF-κB translocation to the nucleus, as the authors could not demonstrate a concomitant decrease in the levels of the NF-κB inhibitor, IκB. Moreover, they found that high levels of nuclear GSK-3β led to increased phosphorylation of the p65 subunit of NF-κB at the Ser563 residue. Although this phosphorylation has long been considered to promote nuclear translocation and activation of NF-κB, recent evidence seems to indicate that it could have an inhibitory effect [64], therefore its relevance in AML cells needs to be addressed further.

In AML cell lines GSK-3β is also capable of phosphorylating the PU.1 transcription factor at Ser41 and Ser140, thereby leading to its recognition by FBXW7 and subsequent degradation via the proteasome [65]. If GSK-3β was pharmacologically inhibited, PU.1 was not degraded and the leukemic cells were able to differentiate along the monocytic lineage (**Figure 4**).

These findings are in agreement with previous observations showing that GSK-3 inhibitors led to differentiation of AML cells towards a more mature phenotype [66-68]. Indeed, PU.1 is a key factor at two bifurcations of hematopoiesis, i.e. myeloid vs. erythroid cells, and monocytes vs. granulocytes [69]. Interestingly, more than 40% of AML patients display low PU-1 expression [70], while an 80% reduction of PU.1 expression via homozygous knockout of an enhancer located 14 kb upstream of PU.1 led to AML development in mice [71]. It is not known where phosphorylation of PU.1 by GSK-3β takes place. PU.1 is mainly nuclear in AML primary cells, as demonstrated by immunofluorescence which could document only a faint cytoplasmic staining [72]. Therefore, it might be that nuclear GSK-3β phosphorylates PU.1, thereby facilitating its nuclear export and subsequent proteasomal degradation. This would not be unprecedented, as it has been reported for another target of GSK-3, i.e. forkhead/winged helix family k1 [73]. The different roles of GSK-3 paralogs in AML are listed in Table 1.

Targeting GSK-3 signaling in AML

Given that both GSK-3α and GSK-3β have been reported to play key roles in AML cell pathophysiology, it is not surprising that early GSK-3 inhibitors have been tested as potential innovative treatments in preclinical AML models. However, these inhibitors do not differentiate the two isoforms. Overall, it has been reported that treatment of AML cells with GSK-3 inhibitors resulted in a slower proliferation, enhanced apoptosis, and lowering of drug-resistance [51, 66, 67, 74]. In contrast, the inhibitors did not display cytotoxicity towards healthy HSCs [67].

These pre-clinical studies have led to a phase II clinical trial (ClinicalTrials.gov NCT01214603) where LY2090314 was tested in 20 AML patients. Despite the encouraging preliminary results regarding the good tolerance of the drug, LY2090314 was ineffective from a clinical point of view as no complete or partial remissions were observed. These findings indicate that the clinical benefits of GSK-3α/β inhibition are very limited as monotherapy [75].

The findings on GSK-3α as the key paralog sustaining growth of AML cells [62] have led to the development of two α isoform-selective inhibitors. The first of these inhibitors to be released was BRD0705 [35]. BRD0705 takes advantage of the GSK-3β-Asp133 → GSK-3α-Glu196 switch in the kinase hinge for achieving specificity towards the -α isoform. This difference translates into topological changes within the ATP-binding pocket and the adjacent hydrophobic selectivity pocket. Importantly, BRD0705 did not stabilize β-catenin, thereby mitigating a potential concern related to GSK-3 inhibition. The drug induced myeloid differentiation and decreased colony formation in AML cells, with no apparent detrimental effects in healthy HSCs. Furthermore, the inhibitor impaired leukemia initiation and prolonged survival in murine AML models [35]. The second inhibitor, G28_14, displayed IC_{50} values of 33 nM and 218 nM against GSK-3α and -3β, respectively, which corresponds to a 6.6-fold isoform-selectivity [39]. G28_14 suppressed cell survival by impairing cell proliferation by up to 90% in two human AML cell lines. Moreover, surface marker expression (CD11b, CD11c, and CD14) analysis demonstrated that G28_14 induced terminal differentiation of AML cells. Importantly, also G28_14 did not activate Wnt/β-catenin signaling [39]. Nevertheless, given the recent findings indicating GSK-3β as a key isoform in AML cells [13], the usefulness of the α isoform-selective inhibitors in AML setting remains to be determined.

Targeting GSK-3 has been also proposed as a strategy for sensitizing non-acute promyelocytic leukemia (APL) AML cells to drugs [all-trans retinoic acid (ATRA) and arsenic trioxide (ATO)] that are highly effective in APL patients, [76], but lack efficacy in other AML subtypes. Si et al. [77] were the first to demonstrate that a combination of ATRA with LiCl or other GSK-3 inhibitors significantly increased ATRA-mediated leukemic cell differentiation and displayed strong anti-leukemic activity both in APL and non-APL AML cells. A subsequent study showed that the retinoic acid receptor (RAR) is a target of GSK-3 and that GSK-3 could impact on the expression and transcriptional activity of the RAR in non-APL cells [78]. The sensitivity of non-APL AML cells to ATO was shown to be dependent on the levels of N-Myc downstream-regulated gene 2 (NDRG2). NDRG2-overexpressing

U937 cells (U937-NDRG2) showed a higher sensitivity to ATO than mock-transfected U937 cells (U937-Mock). Mechanistically, NDRG2 overexpression was associated with Mcl-1 degradation through GSK-3β activation, as NDRG2 increased the interactions between GSK-3β and PP2A, thereby facilitating the dephosphorylation of GSK-3β at S9 by PP2A [79].

These preclinical studies have led to a trial where LiCl was administered in combination with ATRA to 12 relapsed/refractory non-APL AML patients (ClinicalTrials.gov NCT01820624 [80]). Four patients attained disease stability with no increase in circulating blasts for ≥4 weeks. Target serum LiCl concentration was achieved in all patients and correlated with GSK-3 inhibition in leukemic cells, while immunophenotypic changes associated with myeloid differentiation (increased expression of CD11b, CD14, and CD15) were observed in five patients. The treatment led to a reduction in the $CD34^+$ $CD38^-$ LSC compartment both in vivo and in vitro. It was concluded that, although well tolerated, the combination of LiCl and ATRA had limited clinical activity which might be due to the weak GSK-3 inhibition by LiCl [80].

For the sake of completeness, it should be reminded LiCl and other GSK-3 inhibitors restored, in ATRA-resistant APL cells, the expression of ATRA target genes and the ATRA-induced differentiation. Indeed, it was demonstrated that that GSK-3β negatively impacted the expression and transcriptional activity of RAR via phosphorylation at Ser445, hence GSK-3 inhibition promoted myeloid differentiation [81]. These findings demonstrate that LiCl has the potential to reactivate ATRA-dependent transcriptional activation and differentiation in ATRA-resistant APL cells and might be a useful drug for treating APL patients who have lost sensitivity to ATRA.

GSK-3 and the leukemic BM microenvironment (BMM) in AML

The BMM provides a home for malignant cells and is responsible for disease relapse as well as drug-resistance. Therefore, the targeting of the interactions between leukemic cells and those of the BMM might provide new therapeutic avenues [82]. At present little is known about the roles of GSK-3 in the leukemic BMM or niche. GSK-3 is inactivated via molecules secreted by BMM cells, thereby leading to up-regulation of the Wnt/β-catenin pathway that is involved in drug-resistance and proliferation of leukemic cells [83-85]. Therefore, in this case, GSK-3 behaves as a tumor suppressor intrinsic to malignant cells. Nevertheless, GSK-3β has been reported to act as a key effector involved in sustaining an immunosuppressive leukemic BMM. It has been indeed demonstrated that GSK-3β is overexpressed in natural killer (NK) cells of the BMM [86]. Either genetic or pharmacological inhibition of GSK-3β resulted in increased production of TNFα in NK cells, via NF-κB activation. This resulted in up-regulation of intercellular adhesion molecule-1 on AML cells, thereby increasing NK-AML conjugate formation and enhanced AML cell killing [86]. Therefore, GSK-3β inhibition might lead to benefits to AML patients by improving the NK cell activity in the leukemic BMM microenvironment. However, the consequences of GSK-3 targeting should be evaluated in other immune cells of the BMM, as T regulatory cell suppressive activity is potentiated by inhibition of GSK-3β [87] and this might result in pro-cancer effects [88].

GSK-3 signaling and targeting in ALL

ALL originates from clonal expansion of transformed T-cell (T-ALL) or B-cell (B-ALL) and is characterized by a marked biological and clinical heterogeneity [89]. Chemotherapy remains the most effective therapeutic approach against ALL, attaining a survival rate of 85% for children and 20-40% for adults B-ALL patients [90, 91], while in T-ALL survival rate are 80% for children and 40-50% for adults patients [92] Recently, treatment of relapsed/refractory B-ALL has been revolutionized by the approval of new therapeutic strategies, including the CD19 bispecific antibody Blinatumomab, the conjugated CD22 antibody Inotuzumab, and chimeric antigen receptor (CAR) T-cell immunotherapy [93]. Nevertheless, these strategies may lead to severe adverse effects, pose a heavy economic burden, and are not effective in T-ALL. Therefore, identification of novel therapies to support ALL conventional chemotherapy is needed. This is especially true of T-ALL where targeted treatment options remain limited.

GSK-3 has been implicated in the pathophysiology of both B-ALL and T-ALL via various signaling pathways. A GSK-3-related signaling intermediate identified in ALL, but not in AML, is c-Myeloblastosis (c-Myb), which is a main target of GSK-3β for regulating survival in Jurkat T-ALL cells [94]. c-Myb was found to be interact and cooperate with the transcription factor Lymphoid Enhancer–Binding factor-1 in the activation of B-cell lymphoma 2 (Bcl-2) and survivin genes. GSK-3β inactivation by pharmacological inhibition or knock-down via shRNA reduced the expression of c-Myb by promoting its ubiquitination-mediated degradation, thereby inhibiting the expression of c-Myb-dependent Bcl-2 and survivin [94]. Accordingly, proteasome inhibition could partially rescue c-Myb degradation. At first glance this finding might appear surprising as active GSK-3 is generally considered to be a positive regulator of proteasome activity, however there are reports showing that phosphorylation by GSK-3 could result in stabilization of the substrate, rather than destabilization [95]. Nevertheless, the authors were unable to demonstrate conclusively that GSK-3β phosphorylated c-Myb, although they could detect changes in the phosphorylation status of c-Myb upon pharmacological inhibition of GSK-3β [94].

There are some aspects of GSK-3 signaling that are shared by both AML and ALL cells. For example, it is interesting that an aberrant nuclear accumulation of GSK-3β in primary cells from pediatric ALL patients was demonstrated long before it was observed in AML [14]. Treatment with GSK-3 pharmacological inhibitors resulted in a decrease in nuclear GSK-3β levels without interfering with cytoplasmic GSK-3β. This was accompanied by inhibition of NF-κB p65 transcriptional activity which negatively impacted on the expression of the NF-κB target gene, survivin [14]. The role of GSK-3 related to the NF-κB pathway was subsequently confirmed in T- and B-ALL cell lines [96].

Another GSK-3 target shared by both AML and T-ALL cells is XIAP [97]. XIAP was identified in a study on calcineurin (CN) (also known as PP3CA), a Ca^{2+}-activated protein phosphatase which dephosphorylates nuclear factor of activated T-cell (NFAT) proteins, a family of transcription factors involved in many aspects of activated T-cell physiology [98]. CN was found to directly interact with GSK-3β in T-ALL cells, thereby leading to increased catalytic activity of GSK-3β, most likely via increased autophosphorylation at the Tyr216 residue [97]. Treatment with GSK-3 pharmacological inhibitors resulted in cytotoxicity in T-ALL cell lines and T-ALL xenografts. Interestingly, T-ALL cell lines and xenografts displaying a more immature phenotype (pro/pre T-ALL subgroup) were more sensitive to GSK-3 inhibition and exhibited a lower ratio of p-Ser9/p-Tyr216 GSK-3β (that is indicative of a higher enzymatic activity) compared with samples that were less sensitive to inhibition [97]. GSK-3 inhibition with BIO negatively impacted on the expression levels of some proteins known to be targets of GSK-3, including claspin, Mcl-1, survivin, XIAP, and c-Myb. However, maximal downregulation of these proteins was observed when BIO was combined with a CN inhibitor (CN585), presumably because CN585 decreased the phosphorylation levels of p-Tyr216 GSK-3β, although this was not demonstrated. While the downregulation of claspin and survivin was due to impaired gene transcription (as indicated by a decrease in the levels of their mRNAs), a proteasome inhibitor substantially rescued XIAP downregulation. Therefore, this report is another example of a setting where active GSK-3β results in protein stabilization through proteasome inhibition.

Importantly, the BIO plus CN585 combined treatment was effective also in vivo in pre-clinical models of T-ALL. Overall, also this study supports the concept that GSK-3β acts as a tumor promoter in T-ALL by upregulating several pro-oncogenic proteins via a variety of mechanisms and indicates a possible novel treatment for relapsed/refractory T-ALL patients. However, it should be pointed out that the authors reported signs of toxicity in mice upon prolonged use of the two drugs. In the future, it would be important to test whether selective GSK-3β inhibitors such as TWS199 [40] or BRD3731 [35] could display the same anti-leukemic effects as BIO and result in lower toxicity.

Very recently, a key role for GSK-3α in causing resistance to asparaginase has been reported in ALL cells. Asparaginase, an enzyme which deaminates the nonessential amino acid asparagine, has long been used for treating ALL patients, in combination with chemotherapeutic drugs. The use of asparaginase has improved the outcome of both T-ALL and B-ALL patients [99]. However, the development of resistance to asparaginase is not uncommon [100]. Hinze and coworkers [101] demonstrated that in human B-ALL and T-ALL cells lines, Wnt signaling activation sensitized leukemic cells to asparaginase. This effect was not mediated by β-catenin activation, but rather by Wnt-dependent inhibition of GSK-3α that led to stabilization of proteins (Wnt/STOP) , as documented by the use of either shRNA to GSK-3α or a GSK-3α selective inhibitor, BRD075. In contrast, shRNA to GSK-3β was ineffective, while the GSK3β-selective inhibitor BRD3731 displayed only modest effects. Mechanistically, it was demonstrated that the Wnt pathway activation inhibited

GSK-3α-controlled protein ubiquitination and proteasomal degradation, a well-known catabolic source of asparagine [101], thereby leading to apoptosis of leukemic cells treated with asparaginase. A drug combination consisting of asparaginase and BRD075 displayed synthetic lethality in a murine model xenografted with human T-ALL or B-ALL PDXs characterized by resistance to asparaginase, but not in healthy HSCs (**Figure 5**). These findings are particularly interesting as they show that Wnt activation in ALL cells does not only result in pro-oncogenic effects through β-catenin [102, 103], but could also unleash anti-cancer signaling via inhibition of GSK-3α. However, it should be also considered that in pancreatic and colorectal cancers activation of Wnt/STOP signaling led to an increase in the levels of c-Myc, thereby enhancing ribosome biogenesis. c-Myc is a critical driver of cancer proliferation as well as a key effector in ALL development [104]. However, the levels of c-Myc were not assessed in the work by Hinze et al. [101]. We believe this is a critical experiment that needs to be performed, as stimulation of Wnt/STOP in ALL cells might lead to pro-leukemic effects via enhanced c-Myc activity.

In general, GSK-3β seems to act as a tumor promoter in T-ALL, however GSK-3α is involved in drug-resistance to asparaginase in both T-ALL and B-ALL (see Table 1).

Conclusions and future developments

Aberrant GSK-3 signaling has been implicated in the development and progression of several types of neoplasia, nevertheless therapeutic intervention has been hampered by the fact that GSK-3 paralogs can function as either cancer suppressors or promoters based on the cancer cell-type and context. Moreover, given the multiple effects of this ubiquitously expressed kinase, alterations in its homeostasis affect innumerable cellular pathways in healthy cells.

As we have summarized here, GSK-3 seems to predominantly act as a tumor promoter in acute leukemias. Therefore, from a theoretical point of view, paralog-selective GSK-3 inhibitors might be considered useful molecules to add to our growing arsenal of targeted drugs effective against acute leukemias. However, much work needs to be done to fully understand the complex interactions involving this kinase and the best ways to utilize GSK-3 inhibitors to the benefit of patients diagnosed with acute leukemias, while maintaining normal function of the kinase in normal cells. It is unlikely that GSK-3 inhibitors will work in monotherapy, they should be more effective when combined with other drugs, as we have highlighted here [60, 97, 101]. Moreover, biomarkers that could indicate which patients could benefit the most from therapeutics targeting GSK-3 still await identification.

Yet another critical step will be to gain a better understanding of the interactions involving GSK-3 that take place between leukemic cells and cells of the tumor microenviroment, including immune cells. Our knowledge of GSK-3β roles in regulating anticancer immune responses is evolving rapidly. Such an issue might be of fundamental importance, given the conflicting results obtained with GSK-3 inhibitors in different types of tumors, that might be related to different responses of the immunomodulatory cells of the cancer microenvironment [105].

Further investigations of the mechanisms underlying the complex roles of GSK-3 in acute leukemias should also provide insights toward other molecules, interacting with GSK-3, that would serve as targets more amenable to a therapeutic intervention than GSK-3 itself. Indeed, the landscape of targeted therapy in hematological cancers is rapidly changing. Although most of the early targeted drugs have met with a limited success, the introduction of small-molecule protein-protein/DNA interaction (PPI/PDI) disruptors [106] and proteolysis-targeted chimeras (PROTAC) [107], have changed the definition of 'druggable' over the last years. Therefore, transcription factors and other proteins with no enzymatic activity, previously considered as 'undruggable', can now be successfully targeted. Investigations on the signaling pathways regulated by GSK-3 might lead to the discovery of additional targets that could then be exploited for improving the outcome of acute leukemia patients thanks to the use of these novel technologies.

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Conflicts of Interest The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Structural domains and regulation of GSK-3 isoform activity.

a: Structural domains of GSK-3α and GSK-3β. **b**: The two isoforms, when active are autophosphorylated at Tyr residues (279 for GSK-3α and 216 for GSK-3β). However, there are proteins kinases capable of phoshorylating GSK-3α/β at tyrosine residues (p60 Src, MEK). The tyrosine residues are dephosphorylated by SHP-1. Phosphorylation at Ser21 (GSK-3α) or Ser9 (GSK-3β) inhibits their enzymatic activity. The Ser residues are targeted by a variety of upstream kinases, including Akt, PKA, PKC, p70S6K, p90RSK. Phosphorylation at Ser residues is facilated by phosphorylation at Thr residues by ERK and p38 MAPK. Protein phosphatases (PP1, PP2A, PP2B) dephosphorylate the Ser residues. When active, GSK-3 isoforms phosphorylate several substrates that are usually targeted for destruction at the proteasome (AP-1, β-catenin, cyclin D1, Mcl-1, p70S6K, 4E-BP1, Foxk1). Abbreviations: AP-1, activator protein-1; 4E-BP1, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1; ERK, extracellular signal-regulated protein kinase; GSK-3, glycogen synthase kinase 3; Foxk1, forkhead/winged helix family k1; Mcl-1, myeloid leukemia cell differentiation protein 1; MEK, mitogen-activated protein kinase kinase; PKA, protein kinase A; PKC, protein kinase C; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; p38 MAPK, p38 mitogen-activated protein kinase; p60 Src, p60 Sarcoma; p70S6K, p70 ribosomal S6 kinase; p90RSK, p90 ribosomal S6 kinase; SHP-1, Src homology-2 (SH2) domain-containing phosphatase 1.

Figure 2. GSK-3 is a critical negative regulator of β-catenin/WNT signaling.

Active (i.e. tyrosine phosphorylated) GSK3α/β is part of the destruction complex which targets β-catenin to destruction via the protesome. Other components of the complex include APC, Axin1, CK1α, and β-catenin itself. CK1α phosphorylates β-catenin at Ser45. This phosphorylation event primes β-catenin for subsequent phosphorylation by GSK3α/β at multiple residues (Ser33, Ser37, and Thr41). Once phosphorylated by GSK-3, β-catenin is recognized by the FBXW7/SCF complex and targeted for proteasomal degradation. Abbreviations: APC, adenomatous polyposis coli; CK1α, casein kinase 1α; FBXW7, F-box/WD repeat-containing protein 7; GSK-3, glycogen synthase kinase 3; SCF, SKP-Cullin-Fbox.

Figure 3. Nuclear GSK-3β increases NF-kB-dependent transcription of Bcl-xL and XIAP.

Increased levels of nuclear GSK-3β results in translocation to the nucleus of phosphorylated NF-kB which up-regulates the transcription of genes encoding for the anti-apoptotic proteins, Bcl-xL and XIAP. It is still unclear how GSK-3β promotes NF-κB nuclear translocation, as the expression levels of the NF-κB inhibitor, IκB, were similar in AML samples displaying a high concentration of GSK-3β within the nucleus when compared with samples with low levels. Abbraviations: Bcl-xL, B-cell lymphoma-extra-large (Bcl-xL); GSK-3, glycogen synthase kinase 3; IκB, inhibitor of κB; IKK, IκB kinase; NF-κB, nuclear factor-κB; XIAP, X-linked inhibitor of apoptosis protein.

Figure 4. The transcription factor PU.1 is a substrate of GSK-3β in human AML cell lines.

a: active (i.e. tyrosine phosphorylated) GSK-3β phosphorylates PU.1 at Ser 41/140. Phosphorylated PU.1 is recognized and ubiquitinated by the FBXW7/SCF complex, leading to its degradation by the proteasome. Therefore, monocytic differentiation of U937 and THP-1 cells is blocked. **b**: treatment with GSK-3 inhibitors (LiCl, SB216763) decreases tyrosine phosphosphorylation of GSK-3β and increases phosphorylation at Ser9, hence the kinase is inactive. As a consequence, PU.1 is not degraded and acts as a key effector of monocytic differentiation. Abbreviations: FBXW7, F-box/WD repeat-containing protein 7; GSK-3, glycogen synthase kinase 3; SCF, SKP-Cullin-Fbox.

Figure 5. Wnt/STOP signaling activation sensitizes ALL cells to asparaginase via inactivation of GSK-3α.

GSK-3α-phosphorylated proteins are recognized and unbiquitinated mainly via the FBXW7/SCF complex, then targeted to the proteasome. This provides an alternative catabolic source of asparagine that circumvents the pro-apoptotic effects of asparaginase in B-ALL and T-ALL cells. When Wnt/STOP signaling is stimulated (for example by Wnt3a or Wnt activating shRNAs) or GSK-3α is genetically or pharmacologically inhibited, the production of asparagine is substantially lowered and the pharmacological effects of asparaginase are restored. Abbreviations: FBXW7, F-box/WD repeat-containing protein 7; GSK-3, glycogen synthase kinase 3; SCF, SKP-Cullin-Fbox; Wnt/STOP, Wnt-dependent stabilization of proteins.

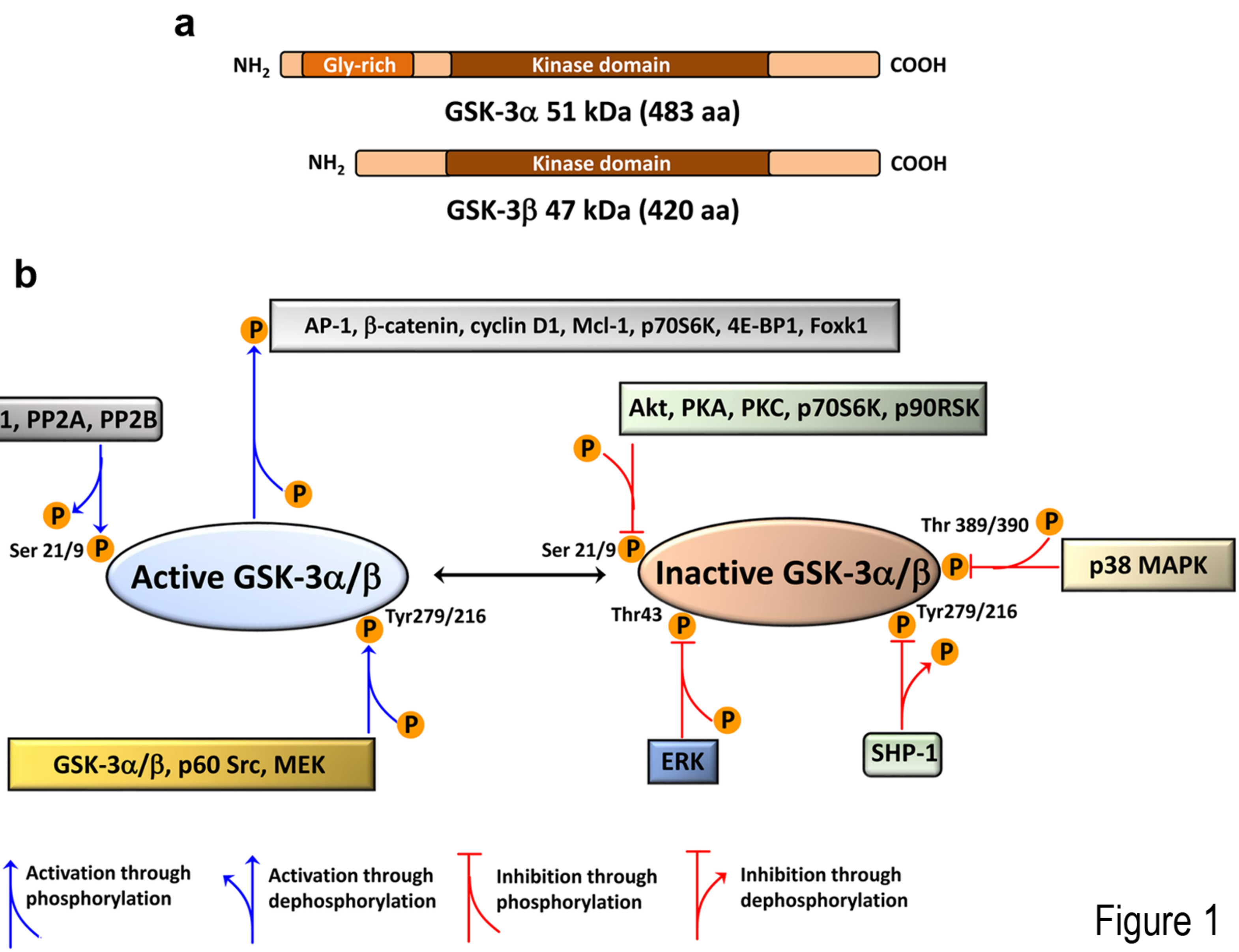

a
NH2
Gly-rich
Kinase domain
COOH
GSK-3α 51 kDa (483 aa)
NH2
Kinase domain
COOH
GSK-3β 47 kDa (420 aa)
b
AP-1, β-catenin, cyclin D1, Mcl-1, p70S6K, 4E-BP1, Foxk1
PP1, PP2A, PP2B
Akt, PKA, PKC, p70S6K, p90RSK
Ser 21/9
Active GSK-3α/β
Tyr279/216
Ser 21/9
Inactive GSK-3α/β
Thr 389/390
p38 MAPK
Thr43
Tyr279/216
GSK-3α/β, p60 Src, MEK
ERK
SHP-1
P
Activation through phosphorylation
Activation through dephosphorylation
Inhibition through phosphorylation
Inhibition through dephosphorylation

Figure 1

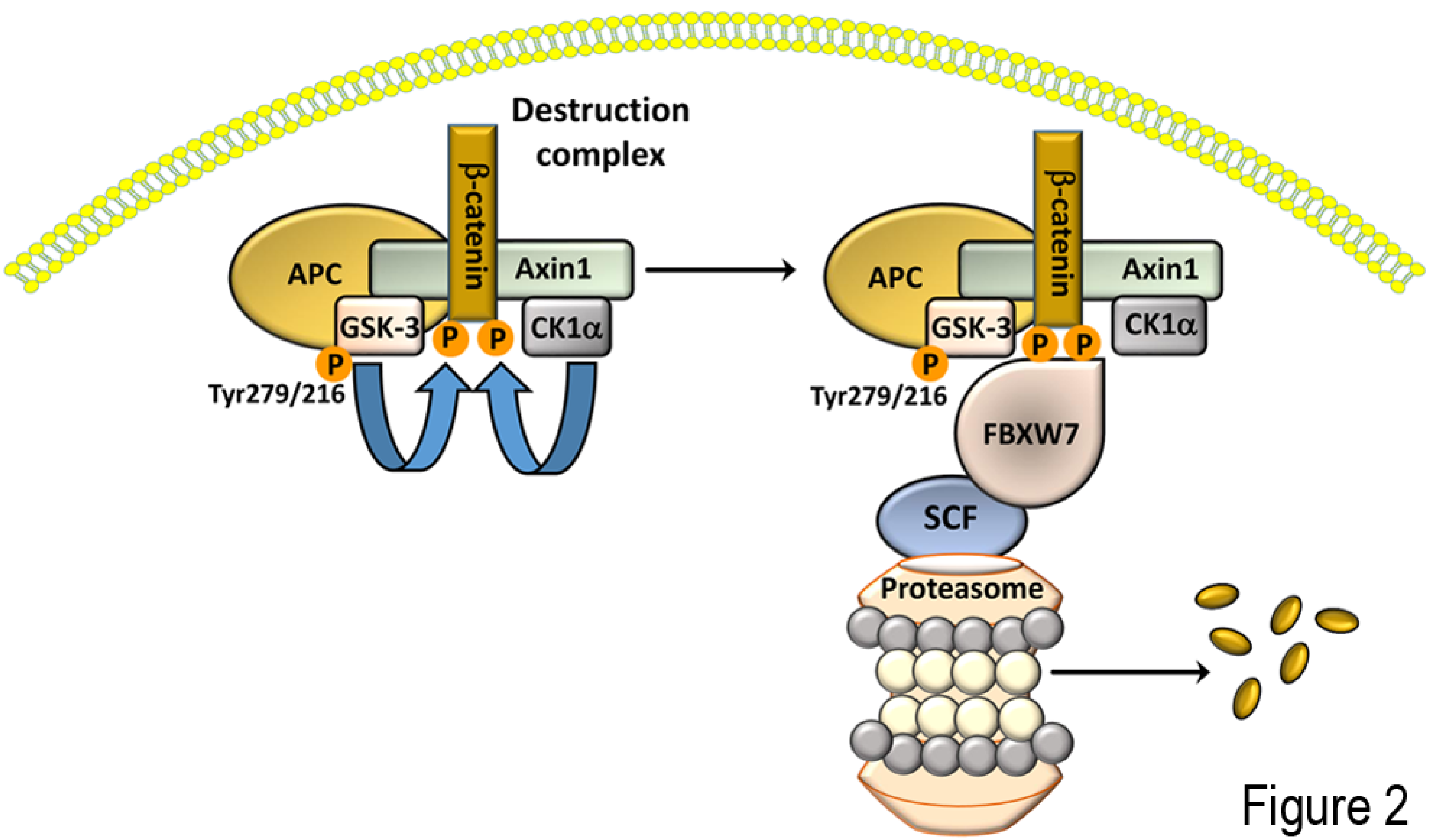
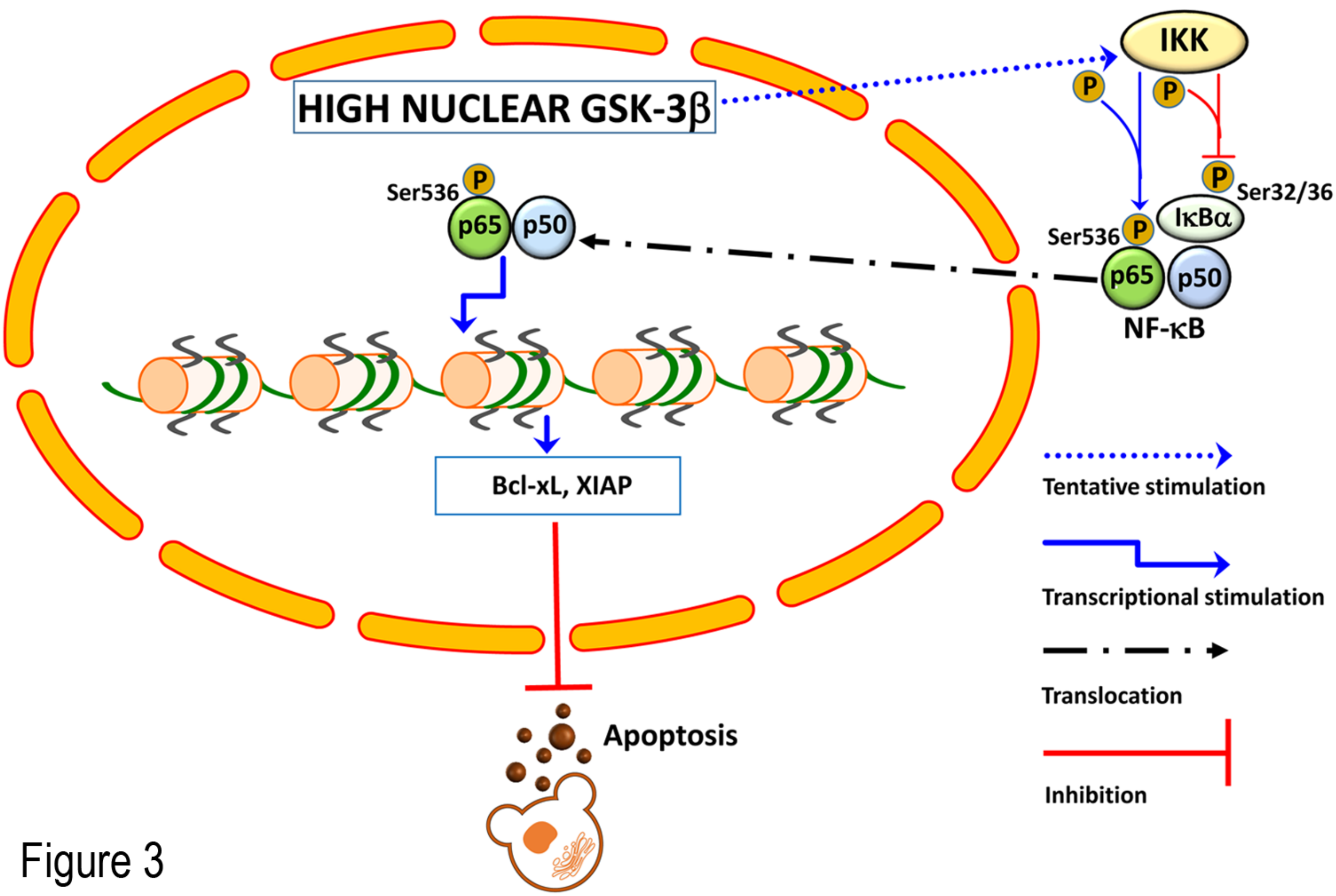

Destruction
complex
APC
β-catenin
Axin1
GSK-3
CK1α
P
P
P
Tyr279/216
APC
β-catenin
Axin1
GSK-3
CK1α
P
P
P
Tyr279/216
FBXW7
SCF
Proteasome

Figure 2



HIGH NUCLEAR GSK-3β
IKK
P
P
P
Ser32/36
IκBα
Ser536
P
p65
p50
NF-κB
Ser536
P
p65
p50
Bcl-xL, XIAP
Apoptosis
Tentative stimulation
Transcriptional stimulation
Translocation
Inhibition

Figure 3

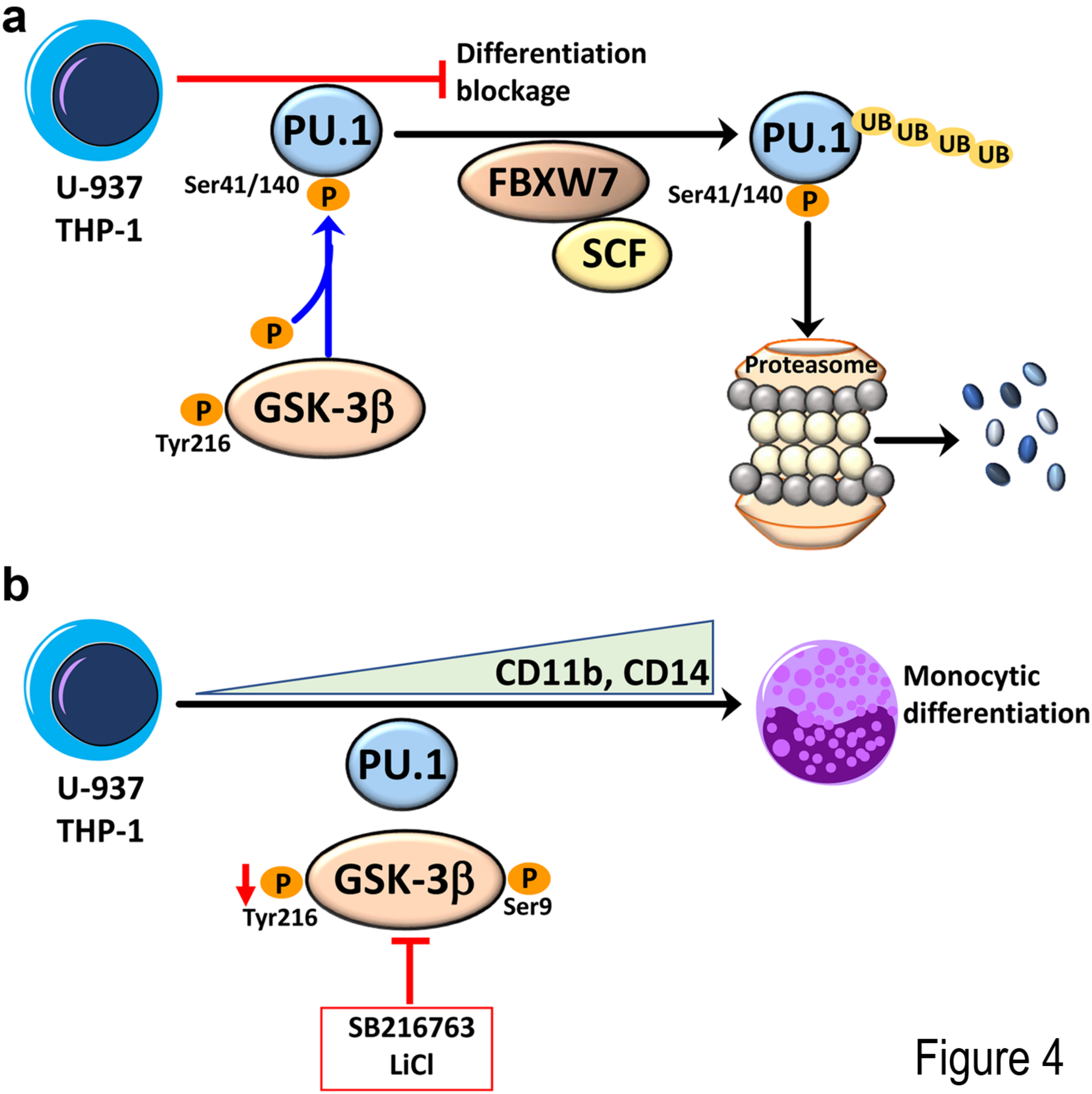
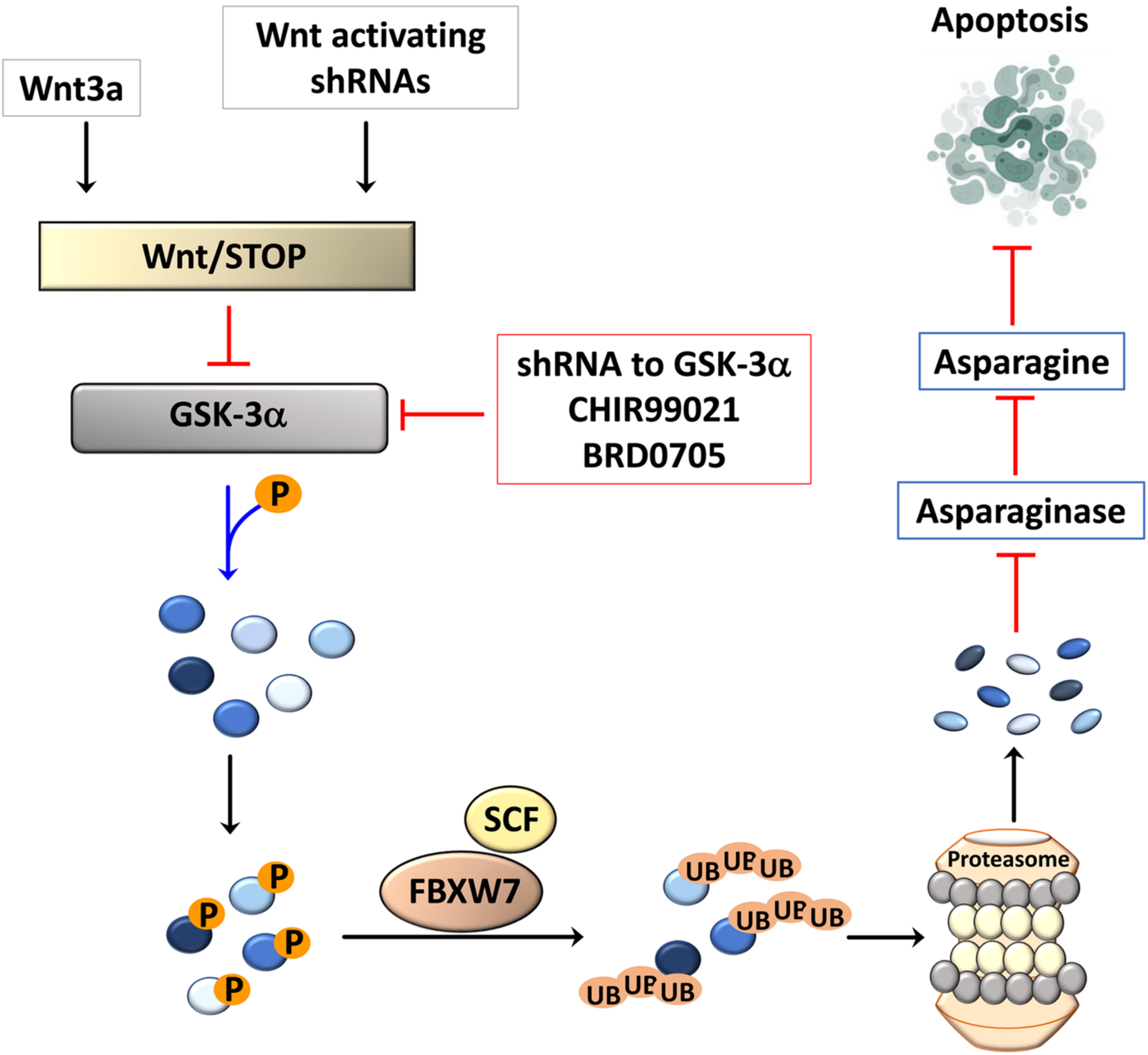

a
Differentiation
blockage
PU.1
U-937
THP-1
Ser41/140
P
FBXW7
SCF
PU.1
UB UB UB UB
Ser41/140
P
P
GSK-3β
P
Tyr216
Proteasome
b
CD11b, CD14
Monocytic
differentiation
U-937
THP-1
PU.1
P
GSK-3β
P
Tyr216
Ser9
SB216763
LiCl

Figure 4



Wnt3a
Wnt activating shRNAs
Apoptosis
Wnt/STOP
GSK-3α
shRNA to GSK-3α
CHIR99021
BRD0705
Asparagine
Asparaginase
P
SCF
FBXW7
UB
Proteasome

Figure 5

Table 1. Involvement of GSK-3 isoforms in acute leukemias and effects of their inhibition

Type of leukemia	Isoform involved	Function	Type of inhibition	Effects of inhibition	Reference
AML	GSK-3α	Tumor suppressor	Genetic	Activation of Wnt/β-catenin signaling, decreased sensitivity to AC220	[60]
AML (MLL)	GSK-3β >GSK-3α	Tumor promoter	Genetic or pharmacological	Up-regulation of $p27^{Kip1}$	[61]
AML	GSK-3α	Tumor promoter	Genetic or pharmacological	Induction of myelo-monoytic differentiation	[62]
AML	GSK-3β (nuclear)	Tumor promoter	Genetic or pharmacological	Decreased NF-κB p65-dependent transcription of Bcl-xL and XIAP	[13]
AML	GSK-3β	Tumor promoter	Pharmacological	Enhancement of monocytic differentiation via PU.1	[65]
T-ALL	GSK-3β	Tumor promoter	Genetic or pharmacological	Decreased expression of c-Myb-dependent Bcl-2 and survivin	[94]
B-ALL, T-ALL	GSK-3β (nuclear)	Tumor promoter	Genetic or pharmacological	Decreased NF-κB p65-dependent transcription of survivin	[14,96]
T-ALL	GSK-3β	Tumor promoter	Genetic or pharmacological	Decreased expression of claspin, Mcl-1, survivin, XIAP, c-Myb.	[97]
B-ALL, T-ALL	GSK-3α	Tumor promoter	Genetic or pharmacological	Activation of Wnt/STOP signaling, restoring of glutaminase sensitivity	[101]

Abbreviations: Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra-large; c-Myb, c-Myeloblastosis; GSK-3, glycogen synthase kinase 3; Mcl-1, myeloid leukemia cell differentiation protein 1; MLL, mixed-lineage leukemia; NF-κB, nuclear factor κB; XIAP, X-linked inhibitor of apoptosis protein.