



ALMA MATER STUDIORUM  
UNIVERSITÀ DI BOLOGNA

ARCHIVIO ISTITUZIONALE  
DELLA RICERCA

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

GSK-3: a multifaceted player in acute leukemias.

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

A.M. Martelli, C.E. (2021). GSK-3: a multifaceted player in acute leukemias. LEUKEMIA, 35(7), 1829-1842 [10.1038/s41375-021-01243-z].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/827585> since: 2021-07-06

*Published:*

DOI: <http://doi.org/10.1038/s41375-021-01243-z>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

## **GSK-3: a multifaceted player in acute leukemias**

**Alberto M. Martelli <sup>1</sup>, Camilla Evangelisti<sup>1</sup>, Francesca Paganelli <sup>1,2,3</sup>, Francesca Chiarini <sup>2,3</sup>, James A. McCubrey <sup>4\*</sup> (orcid id# 0000-0001-6027-3156)**

<sup>1</sup>Department of Biomedical and Neuromotor Sciences, University of Bologna, 40126 Bologna, Italy.

<sup>2</sup> CNR Institute of Molecular Genetics "Luigi Luca Cavalli-Sforza" Unit of Bologna, 40136 Bologna, Italy.

<sup>3</sup> IRCCS Istituto Ortopedico Rizzoli, 40136 Bologna, Italy.

<sup>4</sup> Department of Microbiology & Immunology, Brody School of Medicine, East Carolina University, Greenville, NC 27834, USA.

\*Correspondence: [mccubreyj@ecu.edu](mailto:mccubreyj@ecu.edu)

## **Abstract**

Glycogen synthase kinase 3 (GSK-3) consists of two isoforms ( $\alpha$  and  $\beta$ ) 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.  $\beta$ -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 <sup>1</sup>. However, GSK-3 regulates other physiological functions, including cell proliferation, differentiation, apoptosis, and embryonic development <sup>2,3</sup>.

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 <sup>4</sup>. 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 <sup>5</sup>.

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 <sup>5</sup>. 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  $\alpha$  (51-kDa) and  $\beta$  (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) <sup>6</sup>. 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<sub>2</sub>-terminal lobe. In addition, a glycine-rich domain (73% glycine) is present only in the NH<sub>2</sub>-terminal  $\alpha$  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 <sup>7</sup>. Although GSK-3 $\alpha$  and GSK-3 $\beta$  are ubiquitously expressed in tissues and organs and share some common substrates <sup>8</sup>, 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 <sup>9</sup>. Regarding its subcellular localization, GSK-3 is mainly a cytosolic enzyme, however there are pools of mitochondrial <sup>10</sup> and nuclear GSK-3 <sup>11</sup>. Nuclear GSK-3 continuously shuttles from the nucleus to the cytoplasm and vice-versa <sup>12</sup>. Importantly, an aberrant increase in the GSK-3 $\beta$  nuclear pool has been linked to upregulation of nuclear factor- $\kappa$ B (NF- $\kappa$ B)-mediated gene transcription in some cancer settings, including acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) <sup>13,14</sup>.

At variance with most protein kinases, GSK-3 $\alpha$  and GSK-3 $\beta$  are constitutively active, as autophosphorylation of GSK-3 $\alpha$  at Tyr279 and of GSK-3 $\beta$  at Tyr216, which increases the enzymatic activity, is observed under resting conditions <sup>15</sup>. However, kinases capable of phosphorylating the GSK-3 paralogs at tyrosine residues have been identified, including p60 Sarcoma (p60 Src) <sup>15</sup> and mitogen-activated protein kinase kinase (MEK) <sup>16</sup>. In contrast, phosphorylation at Ser21 (GSK- $\alpha$ ) and Ser9 (GSK-3 $\beta$ ) inactivates GSK-3 <sup>17</sup>. (**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) <sup>18</sup>. 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 <sup>19</sup> (**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-3 $\alpha/\beta$  at Ser21/9, thereby upregulating the enzymatic activity <sup>20</sup>. 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 <sup>21</sup> (**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)<sup>22</sup>,  $\beta$ -catenin<sup>23</sup>, cyclin D1<sup>24</sup>, c-Myc<sup>25</sup>, and myeloid leukemia cell differentiation protein 1 (Mcl-1)<sup>26</sup>. Moreover, two GSK-3 substrates, p70S6K and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), are involved in protein translation<sup>27, 28</sup> (**Figure 1b**). GSK-3 substrates are recognized by specific E3 ubiquitin ligases through phosphorylated motifs (phosphodegron motifs) and targeted for proteasomal degradation<sup>29</sup>. Therefore, in several cancer settings GSK-3 acts as a negative regulator of Wnt/ $\beta$ -catenin, growth factor/tyrosine kinase receptor, Sonic Hedgehog, and G-protein-coupled receptor signaling networks, thereby behaving as an oncosuppressor kinase<sup>5</sup>. 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/ $\beta$ -catenin network, as it controls the turnover of the free pool of cytoplasmic  $\beta$ -catenin<sup>5</sup>. Indeed, GSK-3 $\beta$  is part of multiprotein destruction complex that includes adenomatous polyposis coli (APC), casein kinase 1 $\alpha$  (CK1 $\alpha$ ), Axis inhibition protein 1 (Axin1), and  $\beta$ -catenin itself. CK1 $\alpha$  phosphorylates  $\beta$ -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  $\beta$ -catenin, as GSK-3 does not bind  $\beta$ -catenin directly<sup>30</sup>. Phosphorylated  $\beta$ -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 <sup>31</sup> (**Figure 2**). In contrast, when GSK-3 activity is inhibited by upstream kinases,  $\beta$ -catenin migrates to the nucleus, where it activates specific pro-oncogenic transcriptional programs <sup>32</sup>. Therefore, GSK-3 acts as a key suppressor of the Wnt/ $\beta$ -catenin signaling pathway <sup>2</sup> that is frequently overactive in acute leukemias <sup>33</sup>.

Given that genetic ablation of either GSK-3 $\alpha$  or GSK-3 $\beta$  did not lead to  $\beta$ -catenin accumulation in an embryonic stem cells context, the two paralogs have been considered to be redundant in this respect <sup>34</sup>. However, more recent findings seem to indicate the GSK-3 $\alpha$  and GSK-3 $\beta$  could play a cell-dependent differential role in the degradation of  $\beta$ -catenin <sup>35</sup>. 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 <sup>36</sup>. 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 <sup>37</sup>. 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-3 $\alpha$  and GSK-3 $\beta$  with almost equal potency <sup>37</sup>. 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 <sup>4</sup>. However, paralog-selective inhibitors such as compound 27, BRD0705, and compound 28\_14 (that target GSK-3 $\alpha$  <sup>35, 38, 39</sup>) or TWS199 and BRD3731 (that target GSK-3 $\beta$  <sup>35, 40</sup>) have been identified. 6-bromoindirubin 3'-oxime (BIO) is a hemi-synthetic derivative of indirubins found in edible mollusks and plants which inhibits GSK-3 $\beta$  <sup>41</sup>.

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 <sup>42, 43</sup>. GSK-3 inhibitors have entered clinical trials for both neurodegenerative disorders and cancer, however a general lack of therapeutic activity was reported <sup>44, 45</sup>.

### **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<sup>+</sup> cells <sup>46</sup>) as well as peripheral blood neutrophils and platelets in more than 90% of patients taking LiCl <sup>47</sup>. Furthermore, LiCl increases transplantable HSCs in mice <sup>48</sup>. These studies implicated GSK-3 as an important regulator of HSC homeostasis. Moreover, canonical Wnt/ $\beta$ -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 <sup>49</sup>.

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 <sup>50</sup>. In a subsequent investigation, it was reported that BIO activated  $\beta$ -catenin in cord blood CD34<sup>+</sup> cells, thereby upregulating two  $\beta$ -catenin transcriptional targets critical for HSC self-renewal, i.e. c-Myc and Homeobox protein B4 <sup>51</sup>. Moreover, GSK-3 $\beta$  inhibition by BIO resulted in delayed ex-vivo expansion of CD34<sup>+</sup> 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 <sup>51</sup>. Overall, these findings suggest the involvement of GSK-3 $\beta$  in the preservation of HSCs and their interactions with the BM microenvironment. Therefore, methods for the inhibition of GSK-3 $\beta$  activity may be useful for clinical ex-vivo expansion of CD34<sup>+</sup> 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  $\beta$ -catenin activity <sup>52</sup>. 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  $\alpha$  (TNF $\alpha$ ) and IL-1 $\beta$ ] that attenuated HSC expansion through induction of p38MAPK activation, a well-established negative regulator of HSC self-renewal <sup>53</sup> and of GSK-3 <sup>19</sup>. Accordingly, HSC expansion could be restored by the concomitant use of a p38MAPK inhibitor (SB203580) <sup>52</sup>.

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  $\beta$ -catenin-dependent manner, consistently with a role for Wnt signaling in HSC homeostasis <sup>54</sup>. 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  $\beta$ -catenin knockout. These findings implied that GSK-3 regulated both Wnt/  $\beta$ -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 <sup>55</sup>. In general, it has long been thought that there exists a functional redundancy between *Gsk3 $\alpha$*  and *Gsk3 $\beta$*  alleles in the regulation of Wnt/ $\beta$ -catenin signaling in mouse embryonic cells <sup>34</sup>. However, a recent study demonstrated that GSK-3 $\beta$  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-3 $\alpha$  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 <sup>56</sup>. This observation suggested that

GSK-3 $\beta$  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-3 $\beta$  and GSK-3 $\alpha$  uniquely contribute to AML development in mice by affecting Wnt/Akt/mTOR signaling or metabolism (mainly mitochondrial activity), respectively <sup>56</sup>. The Wnt/ $\beta$ -catenin pathway was a major pathway up-regulated in cells who had lost GSK-3 $\beta$ . Interestingly, both the transcriptome and the epigenomic profile of the *Gsk3 $\beta$*  knock-out mice displayed similarities to the BM of patients with MDS. Furthermore, the authors were able to define a specific *Gsk3 $\beta$*  molecular signature that then allowed for a molecular discrimination among MDS and AML patient transcriptomes. Therefore, this *Gsk3 $\beta$*  signature may have the potential to serve as both a diagnostic and a prognostic biomarker <sup>56</sup> of MDS. Overall, the findings by Guezguez et al. <sup>56</sup> suggest that some the signaling pathways regulated by GSK-3 $\beta$  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 *Gsk3 $\alpha$* , 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 <sup>57</sup>.

Several molecular alterations of key components of signaling pathways contribute to AML pathogenesis and progression <sup>58</sup>. 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 <sup>33</sup>. 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 <sup>59</sup>. 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 it was possible to correlate low levels of *GSK3A* with resistance to the FLT3-ITD inhibitor, AC220 <sup>60</sup>. Drug-resistance was due to activation of the Wnt/ $\beta$ -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. <sup>61</sup> 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<sup>Kip1</sup>. The decreased levels of p27<sup>Kip1</sup> 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- $\beta$  predominated over GSK3- $\alpha$ . 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/ $\beta$ -catenin and Notch signaling activation, as we have highlighted previously <sup>50</sup>.

However, in a subsequent study carried out in human AML cell lines and primary samples it was demonstrated that the specific loss of GSK-3 $\alpha$  induced the expression of genes and morphological changes consistent with myeloid maturation, thereby leading to differentiation of AML cells <sup>62</sup>. Moreover, GSK-3 $\alpha$ -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-3 $\alpha$ , and not GSK-3 $\beta$ ,

was critical for driving leukemic cell proliferation and survival. Interestingly, loss of GSK-3 $\alpha$  did not impact on  $\beta$ -catenin stabilization <sup>62</sup>.

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-3 $\beta$  and an oncogenic nuclear GSK-3 $\beta$ . Indeed, the existence of an aberrant pool of nuclear GSK-3 $\beta$  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-3 $\beta$  enhances AML colony formation and AML growth in murine models. Mechanistically, the nuclear GSK-3 $\beta$  pool promotes nuclear localization of the NF- $\kappa$ B 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<sup>+</sup> HSCs lack this pool of nuclear which has clinical significance as it strongly correlates to a worse AML patient outcome <sup>13</sup>. It could be therefore hypothesized that when GSK-3 $\beta$  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-3 $\beta$  constantly shuttles between the cytoplasm and the nucleus, although it predominantly localizes to the cytoplasm <sup>11</sup>. It is also worth remembering that GSK-3 $\beta$  lacks a classical nuclear export signal. For its nuclear export GSK-3 $\beta$  relies on its interactions with Frequently rearranged in advanced T-cell lymphomas protein 1 and 2 whose expression levels are variable in cancer cells <sup>63</sup>. Therefore, further investigations on the regulation of GSK-3 $\beta$  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.<sup>13</sup> with the data reported by Banerji and coworkers on the pro-leukemic role of GSK-3 $\alpha$ .<sup>62</sup> 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<sup>13</sup> need to be further clarified. For example, it is unclear how high levels of nuclear GSK-3 $\beta$  resulted in NF- $\kappa$ B translocation to the nucleus, as the authors could not demonstrate a concomitant decrease in the levels of the NF- $\kappa$ B inhibitor, I $\kappa$ B. Moreover, they found that high levels of nuclear GSK-3 $\beta$  led to increased phosphorylation of the p65 subunit of NF- $\kappa$ B at the Ser563 residue. Although this phosphorylation has long been considered to promote nuclear translocation and activation of NF- $\kappa$ B, recent evidence seems to indicate that it could have an inhibitory effect<sup>64</sup>, therefore its relevance in AML cells needs to be addressed further.

In AML cell lines GSK-3 $\beta$  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<sup>65</sup>. If GSK-3 $\beta$  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<sup>66-68</sup>. Indeed, PU.1 is a key factor at two bifurcations of hematopoiesis, i.e. myeloid vs. erythroid cells, and monocytes vs. granulocytes<sup>69</sup>. Interestingly, more than 40% of AML patients display low PU.1 expression<sup>70</sup>, 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 <sup>71</sup>. It is not known where phosphorylation of PU.1 by GSK-3 $\beta$  takes place. PU.1 is mainly nuclear in AML primary cells, as demonstrated by immunofluorescence which could document only a faint cytoplasmic staining <sup>72</sup>. Therefore, it might be that nuclear GSK-3 $\beta$  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 <sup>73</sup>. The different roles of GSK-3 paralogs in AML are listed in Table 1.

### **Targeting GSK-3 signaling in AML**

Given that both GSK-3 $\alpha$  and GSK-3 $\beta$  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 <sup>51, 66, 67, 74</sup>. In contrast, the inhibitors did not display cytotoxicity towards healthy HSCs <sup>67</sup>.

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-3 $\alpha/\beta$  inhibition are very limited as monotherapy <sup>75</sup>.

The findings on GSK-3 $\alpha$  as the key paralog sustaining growth of AML cells <sup>62</sup> have led to the development of two  $\alpha$  isoform-selective inhibitors. The first of these inhibitors to be released was BRD0705 <sup>35</sup>. BRD0705 takes advantage of the GSK-3 $\beta$ -Asp133  $\rightarrow$  GSK-3 $\alpha$ -Glu196 switch in the kinase hinge for achieving specificity towards the - $\alpha$  isoform. This difference translates into topological changes within the ATP-binding pocket and the adjacent hydrophobic selectivity pocket. Importantly, BRD0705 did not stabilize  $\beta$ -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 <sup>35</sup>. The second inhibitor, G28\_14, displayed IC<sub>50</sub> values of 33 nM and 218 nM against GSK-3 $\alpha$  and -3 $\beta$ , respectively, which corresponds to a 6.6-fold isoform-selectivity <sup>39</sup>. 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/ $\beta$ -catenin signaling <sup>39</sup>. Nevertheless, given the recent findings indicating GSK-3 $\beta$  as a key isoform in AML cells <sup>13</sup>, the usefulness of the  $\alpha$  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, <sup>76</sup>, but lack efficacy in other AML subtypes. Si et al. <sup>77</sup> 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 <sup>78</sup>. 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-3 $\beta$  activation, as NDRG2 increased the interactions between GSK-3 $\beta$  and PP2A, thereby facilitating the dephosphorylation of GSK-3 $\beta$  at S9 by PP2A <sup>79</sup>.

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 <sup>80</sup>). Four patients attained disease stability with no increase in circulating blasts for  $\geq 4$  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<sup>+</sup> CD38<sup>-</sup> 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 <sup>80</sup>.

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 GSK-3 $\beta$  negatively impacted the expression and transcriptional activity of RAR via phosphorylation at Ser445, hence GSK-3 inhibition promoted myeloid differentiation <sup>81</sup>. 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 <sup>82</sup>. 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/ $\beta$ -catenin pathway that is involved in drug-resistance and proliferation of leukemic cells <sup>83-85</sup>. Therefore, in this case, GSK-3 behaves as a tumor suppressor intrinsic to malignant cells. Nevertheless, GSK-3 $\beta$  has been reported to act as a key effector involved in sustaining an immunosuppressive leukemic BMM. It has been indeed demonstrated that GSK-3 $\beta$  is overexpressed in natural killer (NK) cells of the BMM <sup>86</sup>. Either genetic or pharmacological

inhibition of GSK-3 $\beta$  resulted in increased production of TNF $\alpha$  in NK cells, via NF- $\kappa$ B 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 <sup>86</sup>. Therefore, GSK-3 $\beta$  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-3 $\beta$  <sup>87</sup> and this might result in pro-cancer effects <sup>88</sup>.

### **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 <sup>89</sup>. 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 <sup>90, 91</sup>, while in T-ALL survival rate are 80% for children and 40-50% for adults patients <sup>92</sup>. 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 <sup>93</sup>. 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-3 $\beta$  for regulating survival in Jurkat T-ALL cells <sup>94</sup>. 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-3 $\beta$  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 <sup>94</sup>. 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 <sup>95</sup>. Nevertheless, the authors were unable to demonstrate conclusively that GSK-3 $\beta$  phosphorylated c-Myb, although they could detect changes in the phosphorylation status of c-Myb upon pharmacological inhibition of GSK-3 $\beta$  <sup>94</sup>.

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-3 $\beta$  in primary cells from pediatric ALL patients was demonstrated long before it was observed in AML <sup>14</sup>. Treatment with GSK-3 pharmacological inhibitors resulted in a decrease in nuclear GSK-3 $\beta$  levels without interfering with cytoplasmic GSK-3 $\beta$ . This was accompanied by inhibition of NF- $\kappa$ B p65 transcriptional activity which negatively impacted on the

expression of the NF- $\kappa$ B target gene, survivin <sup>14</sup>. The role of GSK-3 related to the NF- $\kappa$ B pathway was subsequently confirmed in T- and B-ALL cell lines <sup>96</sup>.

Another GSK-3 target shared by both AML and T-ALL cells is XIAP <sup>97</sup>. XIAP was identified in a study on calcineurin (CN) (also known as PP3CA), a Ca<sup>2+</sup>-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 <sup>98</sup>. CN was found to directly interact with GSK-3 $\beta$  in T-ALL cells, thereby leading to increased catalytic activity of GSK-3 $\beta$ , most likely via increased autophosphorylation at the Tyr216 residue <sup>97</sup>. 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-3 $\beta$  (that is indicative of a higher enzymatic activity) compared with samples that were less sensitive to inhibition <sup>97</sup>. 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-3 $\beta$ , 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-3 $\beta$  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-3 $\beta$  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-3 $\beta$  inhibitors such as TWS199<sup>40</sup> or BRD3731<sup>35</sup> could display the same anti-leukemic effects as BIO and result in lower toxicity.

Very recently, a key role for GSK-3 $\alpha$  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<sup>99</sup>. However, the development of resistance to asparaginase is not uncommon<sup>100</sup>. Hinze and coworkers<sup>101</sup> 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  $\beta$ -catenin activation, but rather by Wnt-dependent inhibition of GSK-3 $\alpha$  that led to stabilization of proteins (Wnt/STOP), as documented by the use of either shRNA to GSK-3 $\alpha$  or a GSK-3 $\alpha$  selective inhibitor, BRD075. In contrast, shRNA to GSK-3 $\beta$  was ineffective, while the GSK3 $\beta$ -selective inhibitor BRD3731 displayed only modest effects. Mechanistically, it was demonstrated that the Wnt pathway activation inhibited



GSK-3 $\alpha$ -controlled protein ubiquitination and proteasomal degradation, a well-known catabolic source of asparagine <sup>101</sup>, 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  $\beta$ -catenin <sup>102, 103</sup>, but could also unleash anti-cancer signaling via inhibition of GSK-3 $\alpha$ . 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 <sup>104</sup>. However, the levels of c-Myc were not assessed in the work by Hinze et al. <sup>101</sup>. 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-3 $\beta$  seems to act as a tumor promoter in T-ALL, however GSK-3 $\alpha$  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 <sup>60, 97, 101</sup>. 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 microenvironment, including immune cells. Our knowledge of GSK-3 $\beta$  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 <sup>105</sup>.

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<sup>106</sup> and proteolysis-targeted chimeras (PROTAC)<sup>107</sup>, 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.

**Author contributions** AMM contributed to the literature search data and wrote the paper. FP, CE, and FC contributed to the literature search and drew the figures. JAM was responsible for the concept and critical revision of this paper.

**Conflicts of Interest** The authors declare no conflict of interest.

## References

1. Yeaman SJ, Armstrong JL, Bonavaud SM, Poinasamy D, Pickersgill L, Halse R. Regulation of glycogen synthesis in human muscle cells. *Biochem Soc Trans.* 2001; 29: 537-41.
2. McCubrey JA, Rakus D, Gizak A, Steelman LS, Abrams SL, Lertpiriyapong K, *et al.* Effects of mutations in Wnt/ $\beta$ -catenin, hedgehog, Notch and PI3K pathways on GSK-3 activity- Diverse effects on cell growth, metabolism and cancer. *Biochim Biophys Acta.* 2016; 1863: 2942-76.
3. Dey S, Brothag C, Vijayaraghavan S. Signaling Enzymes Required for Sperm Maturation and Fertilization in Mammals. *Front Cell Dev Biol.* 2019; 7: 341.

4. Ahmad F, Woodgett JR. Emerging roles of GSK-3 $\alpha$  in pathophysiology: Emphasis on cardio-metabolic disorders. *Biochim Biophys Acta-Mol Cell Res.* 2020; 1867: 118616.
5. Nagini S, Sophia J, Mishra R. Glycogen synthase kinases: Moonlighting proteins with theranostic potential in cancer. *Semin Cancer Biol.* 2019; 56: 25-36.
6. Cole AR. GSK3 as a Sensor Determining Cell Fate in the Brain. *Front Mol Neurosci.* 2012; 5: 4.
7. Cervello M, Augello G, Cusimano A, Emma MR, Balasus D, Azzolina A, et al. Pivotal roles of glycogen synthase-3 in hepatocellular carcinoma. *Adv Biol Regul.* 2017; 65: 59-76.
8. Itoh S, Saito T, Hirata M, Ushita M, Ikeda T, Woodgett JR, et al. GSK-3 $\alpha$  and GSK-3 $\beta$  proteins are involved in early stages of chondrocyte differentiation with functional redundancy through RelA protein phosphorylation. *J Biol Chem.* 2012; 287: 29227-36.
9. Kerkela R, Kockeritz L, Macaulay K, Zhou J, Doble BW, Beahm C, et al. Deletion of GSK-3 $\beta$  in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. *J Clin Invest.* 2008; 118: 3609-18.
10. Yang K, Chen Z, Gao J, Shi W, Li L, Jiang S, et al. The Key Roles of GSK-3 $\beta$  in Regulating Mitochondrial Activity. *Cell Physiol Biochem.* 2017; 44: 1445-59.
11. Bechard M, Dalton S. Subcellular localization of glycogen synthase kinase 3beta controls embryonic stem cell self-renewal. *Mol Cell Biol.* 2009; 29: 2092-104.
12. Evangelisti C, Chiarini F, Paganelli F, Marmioli S, Martelli AM. Crosstalks of GSK3 signaling with the mTOR network and effects on targeted therapy of cancer. *Biochim Biophys Acta-Mol Cell Res.* 2020; 1867: 118635.
13. Ignatz-Hoover JJ, Wang V, Mackowski NM, Roe AJ, Ghansah IK, Ueda M, et al. Aberrant GSK3 $\beta$  nuclear localization promotes AML growth and drug resistance. *Blood Adv.* 2018; 2: 2890-2903.
14. Hu Y, Gu X, Li R, Luo Q, Xu Y. Glycogen synthase kinase-3 $\beta$  inhibition induces nuclear factor-kappa $\beta$ -mediated apoptosis in pediatric acute lymphocyte leukemia cells. *J Exp Clin Cancer Res.* 2010; 29: 154.

15. Goc A, Al-Husein B, Katsanevas K, Steinbach A, Lou U, Sabbineni H, et al. Targeting Src-mediated Tyr216 phosphorylation and activation of GSK-3 in prostate cancer cells inhibit prostate cancer progression in vitro and in vivo. *Oncotarget*. 2014; 5: 775-87.
16. Takahashi-Yanaga F, Shiraishi F, Hirata M, Miwa Y, Morimoto S, Sasaguri T. Glycogen synthase kinase-3 $\beta$  is tyrosine-phosphorylated by MEK1 in human skin fibroblasts. *Biochem Biophys Res Commun*. 2004; 316: 411-5.
17. Dajani R, Fraser E, Roe SM, Young N, Good V, Dale TC, et al. Crystal structure of glycogen synthase kinase 3  $\beta$ : structural basis for phosphate-primed substrate specificity and autoinhibition. *Cell*. 2001; 105: 721-32.
18. Kaidanovich-Beilin O, Woodgett JR. GSK-3: Functional Insights from Cell Biology and Animal Models. *Front Mol Neurosci*. 2011; 4: 40.
19. Chiara F, Rasola A. GSK-3 and mitochondria in cancer cells. *Front Oncol*. 2013; 3: 16.
20. Lambrecht C, Libbrecht L, Sagaert X, Pauwels P, Hoorne Y, Crowther J, et al. Loss of protein phosphatase 2A regulatory subunit B56 $\delta$  promotes spontaneous tumorigenesis in vivo. *Oncogene*. 2018; 37: 544-52.
21. Tang XL, Wang CN, Zhu XY, Ni X. Protein tyrosine phosphatase SHP-1 modulates osteoblast differentiation through direct association with and dephosphorylation of GSK3 $\beta$ . *Mol Cell Endocrinol*. 2017; 439: 203-12.
22. de Groot RP, Auwerx J, Bourouis M, Sassone-Corsi P. Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the Drosophila kinase shaggy. *Oncogene*. 1993; 8: 841-7.
23. Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P. Binding of GSK3 $\beta$  to the APC- $\beta$ -catenin complex and regulation of complex assembly. *Science*. 1996; 272: 1023-6.
24. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3 $\beta$  regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev*. 1998; 12: 3499-11.
25. Leis H, Segrelles C, Ruiz S, Santos M, Paramio JM. Expression, localization, and activity of glycogen synthase kinase 3 $\beta$  during mouse skin tumorigenesis. *Mol Carcinog*. 2002; 35: 180-5.

26. Tong J, Wang P, Tan S, Chen D, Nikolovska-Coleska Z, Zou F, et al. Mcl-1 Degradation Is Required for Targeted Therapeutics to Eradicate Colon Cancer Cells. *Cancer Res.* 2017; 77: 2512-21.
27. Shin S, Wolgamott L, Yu Y, Blenis J, Yoon SO. Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation. *Proc Natl Acad Sci USA.* 2011; 108: E1204-13.
28. Shin S, Wolgamott L, Tcherkezian J, Vallabhapurapu S, Yu Y, Roux PP, et al. Glycogen synthase kinase-3 $\beta$  positively regulates protein synthesis and cell proliferation through the regulation of translation initiation factor 4E-binding protein 1. *Oncogene.* 2014; 33: 1690-9.
29. Robertson H, Hayes JD, Sutherland C. A partnership with the proteasome; the destructive nature of GSK3. *Biochem Pharmacol.* 2018; 147: 77-92.
30. Dajani R, Fraser E, Roe SM, Yeo M, Good VM, Thompson V, et al. Structural basis for recruitment of glycogen synthase kinase 3 $\beta$  to the axin-APC scaffold complex. *EMBO J.* 2003; 22: 494-501.
31. Stamos JL, Weis WI. The  $\beta$ -catenin destruction complex. *Cold Spring Harb Perspect Biol* 2013; 5: a007898.
32. Jung YS, Park JI. Wnt signaling in cancer: therapeutic targeting of Wnt signaling beyond  $\beta$ -catenin and the destruction complex. *Exp Mol Med.* 2020; 52:183-91.
33. McCubrey JA, Steelman LS, Bertrand FE, Davis NM, Abrams SL, Montalto G, et al. Multifaceted roles of GSK-3 and Wnt/ $\beta$ -catenin in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention. *Leukemia.* 2014; 28: 15-33.
34. Doble BW, Patel S, Wood GA, Kockeritz LK, Woodgett JR. Functional redundancy of GSK-3 $\alpha$  and GSK-3 $\beta$  in Wnt/ $\beta$ -catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev Cell.* 2007; 12: 957-71.
35. Wagner FF, Benajiba L, Campbell AJ, Weiwer M, Sacher JR, Gale JP, et al. Exploiting an Asp-Glu "switch" in glycogen synthase kinase 3 to design paralog-selective inhibitors for use in acute myeloid leukemia. *Sci Transl Med.* 2018; 10:eaam8460.
36. Kitanaka N, Hall FS, Uhl GR, Kitanaka J. Lithium Pharmacology and a Potential Role of Lithium on Methamphetamine Abuse and Dependence. *Curr Drug Res Rev.* 2019; 11: 85-91.

37. Takahashi-Yanaga F. Activator or inhibitor? GSK-3 as a new drug target. *Biochem Pharmacol.* 2013; 86: 191-9.
38. Neumann T, Benajiba L, Goring S, Stegmaier K, Schmidt B. Evaluation of Improved Glycogen Synthase Kinase-3 $\alpha$  Inhibitors in Models of Acute Myeloid Leukemia. *J Med Chem.* 2015; 58: 8907-19.
39. Wang Y, Dou X, Jiang L, Jin H, Zhang L, Zhang L, et al. Discovery of novel glycogen synthase kinase-3 $\alpha$  inhibitors: Structure-based virtual screening, preliminary SAR and biological evaluation for treatment of acute myeloid leukemia. *Eur J Med Chem.* 2019; 171: 221-34.
40. Ding S, Wu TY, Brinker A, Peters EC, Hur W, Gray NS, et al. Synthetic small molecules that control stem cell fate. *Proc Natl Acad Sci USA.* 2003; 100: 7632-7.
41. Jiang J, Zhao M, Zhang A, Yu M, Lin X, Wu M, et al. Characterization of a GSK-3 inhibitor in culture of human cord blood primitive hematopoietic cells. *Biomed Pharmacother.* 2010; 64: 482-6.
42. Tolosa E, Litvan I, Hoglinger GU, Burn D, Lees A, Andres MV, et al. A phase 2 trial of the GSK-3 inhibitor tideglusib in progressive supranuclear palsy. *Mov Disord.* 2014; 29: 470-8.
43. Matsunaga S, Fujishiro H, Takechi H. Efficacy and Safety of Glycogen Synthase Kinase 3 Inhibitors for Alzheimer's Disease: A Systematic Review and Meta-Analysis. *J Alzheimers Dis.* 2019; 69: 1031-9.
44. del Ser T, Steinwachs KC, Gertz HJ, Andres MV, Gomez-Carrillo B, Medina M, et al. Treatment of Alzheimer's disease with the GSK-3 inhibitor tideglusib: a pilot study. *J Alzheimers Dis.* 2013; 33: 205-5.
45. Gray JE, Infante JR, Brail LH, Simon GR, Cooksey JF, Jones SF, et al. A first-in-human phase I dose-escalation, pharmacokinetic, and pharmacodynamic evaluation of intravenous LY2090314, a glycogen synthase kinase 3 inhibitor, administered in combination with pemetrexed and carboplatin. *Invest New Drugs.* 2015; 33: 1187-1196.
46. Ballin A, Lehman D, Sirota P, Litvinjuk U, Meytes D. Increased number of peripheral blood CD34<sup>+</sup> cells in lithium-treated patients. *Br J Haematol.* 1998; 100: 219-21.
47. Boggs DR, Joyce RA. The hematopoietic effects of lithium. *Semin Hematol.* 1983; 20: 129-38.
48. Joyce RA. Sequential effects of lithium on haematopoiesis. *Br J Haematol.* 1984; 56: 307-21.

49. Luis TC, Ichii M, Brugman MH, Kincade P, Staal FJ. Wnt signaling strength regulates normal hematopoiesis and its deregulation is involved in leukemia development. *Leukemia*. 2012; 26: 414-21.
50. Trowbridge JJ, Xenocostas A, Moon RT, Bhatia M. Glycogen synthase kinase-3 is an in vivo regulator of hematopoietic stem cell repopulation. *Nat Med*. 2006; 12: 89-98.
51. Holmes T, O'Brien TA, Knight R, Lindeman R, Shen S, Song E, et al. Glycogen synthase kinase-3 $\beta$  inhibition preserves hematopoietic stem cell activity and inhibits leukemic cell growth. *Stem Cells*. 2008; 26: 1288-97.
52. Li J, Zhang L, Yin L, Ma N, Wang T, Wu Y, et al. In Vitro Expansion of Hematopoietic Stem Cells by Inhibition of Both GSK3 and p38 Signaling. *Stem Cells Dev*. 2019; 28: 1486-97.
53. Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K, et al. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med*. 2006; 12: 446-51.
54. Huang J, Zhang Y, Bersenev A, O'Brien WT, Tong W, Emerson SG, et al. Pivotal role for glycogen synthase kinase-3 in hematopoietic stem cell homeostasis in mice. *J Clin Invest*. 2009; 119: 3519-29.
55. Huang J, Nguyen-McCarty M, Hexner EO, Danet-Desnoyers G, Klein PS. Maintenance of hematopoietic stem cells through regulation of Wnt and mTOR pathways. *Nat Med*. 2012; 18: 1778-85.
56. Guezguez B, Almakadi M, Benoit YD, Shapovalova Z, Rahmig S, Fiebig-Comyn A, et al. GSK3 Deficiencies in Hematopoietic Stem Cells Initiate Pre-neoplastic State that Is Predictive of Clinical Outcomes of Human Acute Leukemia. *Cancer Cell*. 2016; 29: 61-74.
57. Patel SA, Gerber JM. A User's Guide to Novel Therapies for Acute Myeloid Leukemia. *Clin Lymphoma Myeloma Leuk*. 2020; 20: 277-88.
58. Shafer D, Grant S. Update on rational targeted therapy in AML. *Blood Rev*. 2016; 30: 275-83.
59. Ruvolo PP, Qiu Y, Coombes KR, Zhang N, Neeley ES, Ruvolo VR, et al. Phosphorylation of GSK3 $\alpha$ / $\beta$  correlates with activation of AKT and is prognostic for poor overall survival in acute myeloid leukemia patients. *Biochim Biophys Acta-Clin*. 2015; 4: 59-68.



60. Hou P, Wu C, Wang Y, Qi R, Bhavanasi D, Zuo Z, et al. A Genome-Wide CRISPR Screen Identifies Genes Critical for Resistance to FLT3 Inhibitor AC220. *Cancer Res.* 2017; 77: 4402-13.
61. Wang Z, Smith KS, Murphy M, Piloto O, Somervaille TC, Cleary ML. Glycogen synthase kinase 3 in MLL leukaemia maintenance and targeted therapy. *Nature.* 2008; 455: 1205-9.
62. Banerji V, Frumm SM, Ross KN, Li LS, Schinzel AC, Hahn CK, *et al.* The intersection of genetic and chemical genomic screens identifies GSK-3 $\alpha$  as a target in human acute myeloid leukemia. *J Clin Invest.* 2012; 122: 935-47.
63. He L, Fei DL, Nagiec MJ, Mutvei AP, Lamprakis A, Kim BY, et al. Regulation of GSK3 cellular location by FRAT modulates mTORC1-dependent cell growth and sensitivity to rapamycin. *Proc Natl Acad Sci USA.* 2019; 116: 19523-9.
64. Pradere JP, Hernandez C, Koppe C, Friedman RA, Luedde T, Schwabe RF. Negative regulation of NF- $\kappa$ B p65 activity by serine 536 phosphorylation. *Sci Signal.* 2016; 9: ra85.
65. Mishra M, Thacker G, Sharma A, Singh AK, Upadhyay V, Sanyal S, et al. FBW7 inhibits myeloid differentiation in acute myeloid leukemia via GSK3-dependent ubiquitination of PU.1. *Mol Cancer Res.* 2021;19:261-73.
66. Song EY, Palladinetti P, Klamer G, Ko KH, Lindeman R, O'Brien TA, et al. Glycogen synthase kinase-3 $\beta$  inhibitors suppress leukemia cell growth. *Exp Hematol.* 2010; 38: 908-21.
67. Hu S, Ueda M, Stetson L, Ignatz-Hoover J, Moreton S, Chakrabarti A, et al. A Novel Glycogen Synthase Kinase-3 Inhibitor Optimized for Acute Myeloid Leukemia Differentiation Activity. *Mol Cancer Ther.* 2016; 15: 1485-94.
68. Gupta K, Stefan T, Ignatz-Hoover J, Moreton S, Parizher G, Sauntharajah Y, et al. GSK-3 Inhibition Sensitizes Acute Myeloid Leukemia Cells to 1,25D-Mediated Differentiation. *Cancer Res.* 2016; 76: 2743-53.
69. Takei H, Kobayashi SS. Targeting transcription factors in acute myeloid leukemia. *Int J Hematol.* 2019; 109: 28-34.
70. Antony-Debre I, Paul A, Leite J, Mitchell K, Kim HM, Carvajal LA, et al. Pharmacological inhibition of the transcription factor PU.1 in leukemia. *J Clin Invest.* 2017; 127: 4297-313.

71. Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, et al. Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat Genet.* 2004; 36: 624-30.
72. Pianigiani G, Betti C, Bigerna B, Rossi R, Brunetti L. PU.1 subcellular localization in acute myeloid leukaemia with mutated NPM1. *Br J Haematol.* 2020; 188: 184-7.
73. He L, Gomes AP, Wang X, Yoon SO, Lee G, Nagiec MJ, et al. mTORC1 Promotes Metabolic Reprogramming by the Suppression of GSK3-Dependent Foxk1 Phosphorylation. *Mol Cell.* 2018; 70: 949-60.
74. Lee YC, Shi YJ, Wang LJ, Chiou JT, Huang CH, Chang LS. GSK3 $\beta$  suppression inhibits MCL1 protein synthesis in human acute myeloid leukemia cells. *J Cell Physiol.* 2021; 236:570-86.
75. Rizzieri DA, Cooley S, Odenike O, Moonan L, Chow KH, Jackson K, et al. An open-label phase 2 study of glycogen synthase kinase-3 inhibitor LY2090314 in patients with acute leukemia. *Leuk Lymphoma.* 2016; 57: 1800-6.
76. Thomas X. Acute Promyelocytic Leukemia: A History over 60 Years-From the Most Malignant to the most Curable Form of Acute Leukemia. *Oncol Ther.* 2019; 7: 33-65.
77. Si J, Mueller L, Collins SJ. GSK3 inhibitors enhance retinoic acid receptor activity and induce the differentiation of retinoic acid-sensitive myeloid leukemia cells. *Leukemia.* 2011; 25: 1914-8.
78. Gupta K, Gulen F, Sun L, Aguilera R, Chakrabarti A, Kiselar J, et al. GSK3 is a regulator of RAR-mediated differentiation. *Leukemia.* 2012; 26: 1277-85.
79. Park S, Han HT, Oh SS, Kim DH, Jeong JW, Lee KW, et al. NDRG2 Sensitizes Myeloid Leukemia to Arsenic Trioxide via GSK3 $\beta$ -NDRG2-PP2A Complex Formation. *Cells.* 2019; 8:495.
80. Ueda M, Stefan T, Stetson L, Ignatz-Hoover JJ, Tomlinson B, Creger RJ, et al. Phase I Trial of Lithium and Tretinoin for Treatment of Relapsed and Refractory Non-promyelocytic Acute Myeloid Leukemia. *Front Oncol.* 2020; 10: 327.
81. Zassadowski F, Pokorna K, Ferre N, Guidez F, Llopis L, Chourbagi O, et al. Lithium chloride antileukemic activity in acute promyelocytic leukemia is GSK-3 and MEK/ERK dependent. *Leukemia.* 2015; 29: 2277-84.

82. Cancilla D, Rettig MP, DiPersio JF. Targeting CXCR4 in AML and ALL. *Front Oncol.* 2020; 10: 1672.
83. Hu K, Gu Y, Lou L, Liu L, Hu Y, Wang B, et al. Galectin-3 mediates bone marrow microenvironment-induced drug resistance in acute leukemia cells via Wnt/ $\beta$ -catenin signaling pathway. *J Hematol Oncol.* 2015; 8: 1.
84. Takam Kanga P, Dal Collo G, Cassaro A, Bazzoni R, Delfino P, Adamo A, et al. Small Molecule Inhibitors of Microenvironmental Wnt/ $\beta$ -Catenin Signaling Enhance the Chemosensitivity of Acute Myeloid Leukemia. *Cancers* 2020; 12:2696.
85. Ruan Y, Kim HN, Ogana H, Kim YM. Wnt Signaling in Leukemia and Its Bone Marrow Microenvironment. *Int J Mol Sci.* 2020; 21:6247.
86. Parameswaran R, Ramakrishnan P, Moreton SA, Xia Z, Hou Y, Lee DA, et al. Repression of GSK3 restores NK cell cytotoxicity in AML patients. *Nat Commun.* 2016; 7: 11154.
87. Graham JA, Fray M, de Haseth S, Lee KM, Lian MM, Chase CM, et al. Suppressive regulatory T cell activity is potentiated by glycogen synthase kinase 3 $\beta$  inhibition. *J Biol Chem.* 2010; 285: 32852-9.
88. Saleh R, Elkord E. FoxP3<sup>+</sup> T regulatory cells in cancer: Prognostic biomarkers and therapeutic targets. *Cancer Lett.* 2020; 490: 174-85.
89. Vadillo E, Dorantes-Acosta E, Pelayo R, Schnoor M. T cell acute lymphoblastic leukemia (T-ALL): New insights into the cellular origins and infiltration mechanisms common and unique among hematologic malignancies. *Blood Rev.* 2018; 32: 36-51.
90. Hunger SP, Lu X, Devidas M, Camitta BM, Gaynon PS, Winick NJ, et al. Improved survival for children and adolescents with acute lymphoblastic leukemia between 1990 and 2005: a report from the children's oncology group. *J Clin Oncol* 2012.; 30: 1663-9.
91. Winter SS, Dunsmore KP, Devidas M, Wood BL, Esiashvili N, Chen Z, et al. Improved Survival for Children and Young Adults With T-Lineage Acute Lymphoblastic Leukemia: Results From the Children's Oncology Group AALL0434 Methotrexate Randomization. *J Clin Oncol.* 2018; 36: 2926-34.
92. Raetz EA, Teachey DT. T-cell acute lymphoblastic leukemia. *Hematology Am Soc Hematol Educ Program.* 2016; 2016: 580-8.
93. Gokbuget N. How should we treat a patient with relapsed Ph-negative B-ALL and what novel approaches are being investigated? *Best Pract Res Clin Haematol.* 2017; 30: 261-74.

94. Zhou F, Zhang L, van Laar T, van Dam H, Ten Dijke P. GSK3 $\beta$  inactivation induces apoptosis of leukemia cells by repressing the function of c-Myb. *Mol Biol Cell*. 2011; 22: 3533-40.
95. Weathington NM, Snavely CA, Chen BB, Zhao J, Zhao Y, Mallampalli RK. Glycogen synthase kinase-3 $\beta$  stabilizes the interleukin (IL)-22 receptor from proteasomal degradation in murine lung epithelia. *J Biol Chem*. 2014; 289: 17610-9.
96. Wang XJ, Xu YH, Yang GC, Chen HX, Zhang P. Tetramethylpyrazine inhibits the proliferation of acute lymphocytic leukemia cell lines via decrease in GSK-3 $\beta$ . *Oncol Rep*. 2015; 33: 2368-74.
97. Tosello V, Bordin F, Yu J, Agnusdei V, Indraccolo S, Basso G, et al. Calcineurin and GSK-3 inhibition sensitizes T-cell acute lymphoblastic leukemia cells to apoptosis through X-linked inhibitor of apoptosis protein degradation. *Leukemia*. 2016; 30: 812-2.
98. Lee JU, Kim LK, Choi JM. Revisiting the Concept of Targeting NFAT to Control T Cell Immunity and Autoimmune Diseases. *Front Immunol*. 2018; 9: 2747.
99. Radadiya A, Zhu W, Coricello A, Alcaro S, Richards NGJ. Improving the Treatment of Acute Lymphoblastic Leukemia. *Biochemistry*. 2020; 59: 3193-200.
100. Lee JK, Kang S, Wang X, Rosales JL, Gao X, Byun HG, et al. HAP1 loss confers l-asparaginase resistance in ALL by downregulating the calpain-1-Bid-caspase-3/12 pathway. *Blood*. 2019; 133: 2222-32.
101. Hinze L, Pfirrmann M, Karim S, Degar J, McGuckin C, Vinjamur D, et al. Synthetic Lethality of Wnt Pathway Activation and Asparaginase in Drug-Resistant Acute Leukemias. *Cancer Cell*. 2019; 35: 664-76.
102. Chiarini F, Paganelli F, Martelli AM, Evangelisti C. The Role Played by Wnt/ $\beta$ -Catenin Signaling Pathway in Acute Lymphoblastic Leukemia. *Int J Mol Sci*. 2020; 21: 1098.
103. Evangelisti C, Chiarini F, Cappellini A, Paganelli F, Fini M, Santi S, et al. Targeting Wnt/ $\beta$ -catenin and PI3K/Akt/mTOR pathways in T-cell acute lymphoblastic leukemia. *J Cell Physiol*. 2020; 235: 5413-28.
104. Borga C, Foster CA, Iyer S, Garcia SP, Langenau DM, Frazer JK. Molecularly distinct models of zebrafish Myc-induced B cell leukemia. *Leukemia*. 2019; 33: 559-62.

105. Galluzzi L, Spranger S, Fuchs E, Lopez-Soto A. WNT Signaling in Cancer Immunosurveillance. *Trends Cell Biol.* 2019; 29: 44-65.
106. Nomura S, Takahashi H, Suzuki J, Kuwahara M, Yamashita M, Sawasaki T. Pyrrothiogatain acts as an inhibitor of GATA family proteins and inhibits Th2 cell differentiation in vitro. *Sci Rep.* 2019; 9: 17335.
107. Martin-Acosta P, Xiao X. PROTACs to address the challenges facing small molecule inhibitors. *Eur J Med Chem.* 2021; 210: 112993.

## FIGURE LEGENDS

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

**a:** Structural domains of GSK-3 $\alpha$  and GSK-3 $\beta$ . **b:** The two isoforms, when active are autophosphorylated at Tyr residues (279 for GSK-3 $\alpha$  and 216 for GSK-3 $\beta$ ). However, there are proteins kinases capable of phosphorylating GSK-3 $\alpha/\beta$  at tyrosine residues (p60 Src, MEK). The tyrosine residues are dephosphorylated by SHP-1. Phosphorylation at Ser21 (GSK-3 $\alpha$ ) or Ser9 (GSK-3 $\beta$ ) 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 facilitated 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,  $\beta$ -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  $\beta$ -catenin/WNT signaling.**

Active (i.e. tyrosine phosphorylated) GSK3 $\alpha/\beta$  is part of the destruction complex which targets  $\beta$ -catenin to destruction via the proteasome. Other components of the complex include APC, Axin1, CK1 $\alpha$ , and  $\beta$ -catenin itself. CK1 $\alpha$  phosphorylates  $\beta$ -catenin at Ser45. This phosphorylation event primes  $\beta$ -catenin for subsequent phosphorylation by GSK3 $\alpha/\beta$  at multiple residues (Ser33, Ser37, and Thr41). Once phosphorylated by GSK-3,  $\beta$ -catenin is recognized by the FBXW7/SCF complex and targeted for proteasomal degradation. Abbreviations: APC, adenomatous polyposis coli; CK1 $\alpha$ , casein kinase 1 $\alpha$ ; FBXW7, F-box/WD repeat-containing protein 7; GSK-3, glycogen synthase kinase 3; SCF, SKP-Cullin-Fbox.

**Figure 3. Nuclear GSK-3 $\beta$  increases NF- $\kappa$ B-dependent transcription of Bcl-xL and XIAP.**

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

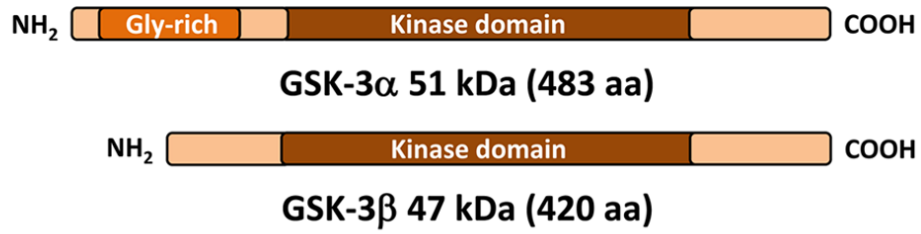
**Figure 4. The transcription factor PU.1 is a substrate of GSK-3 $\beta$  in human AML cell lines.**

**a:** active (i.e. tyrosine phosphorylated) GSK-3 $\beta$  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 phosphos phosphorylation of GSK-3 $\beta$  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-3 $\alpha$ .**

GSK-3 $\alpha$ -phosphorylated proteins are recognized and ubiquitinated 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-3 $\alpha$  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**



**b**

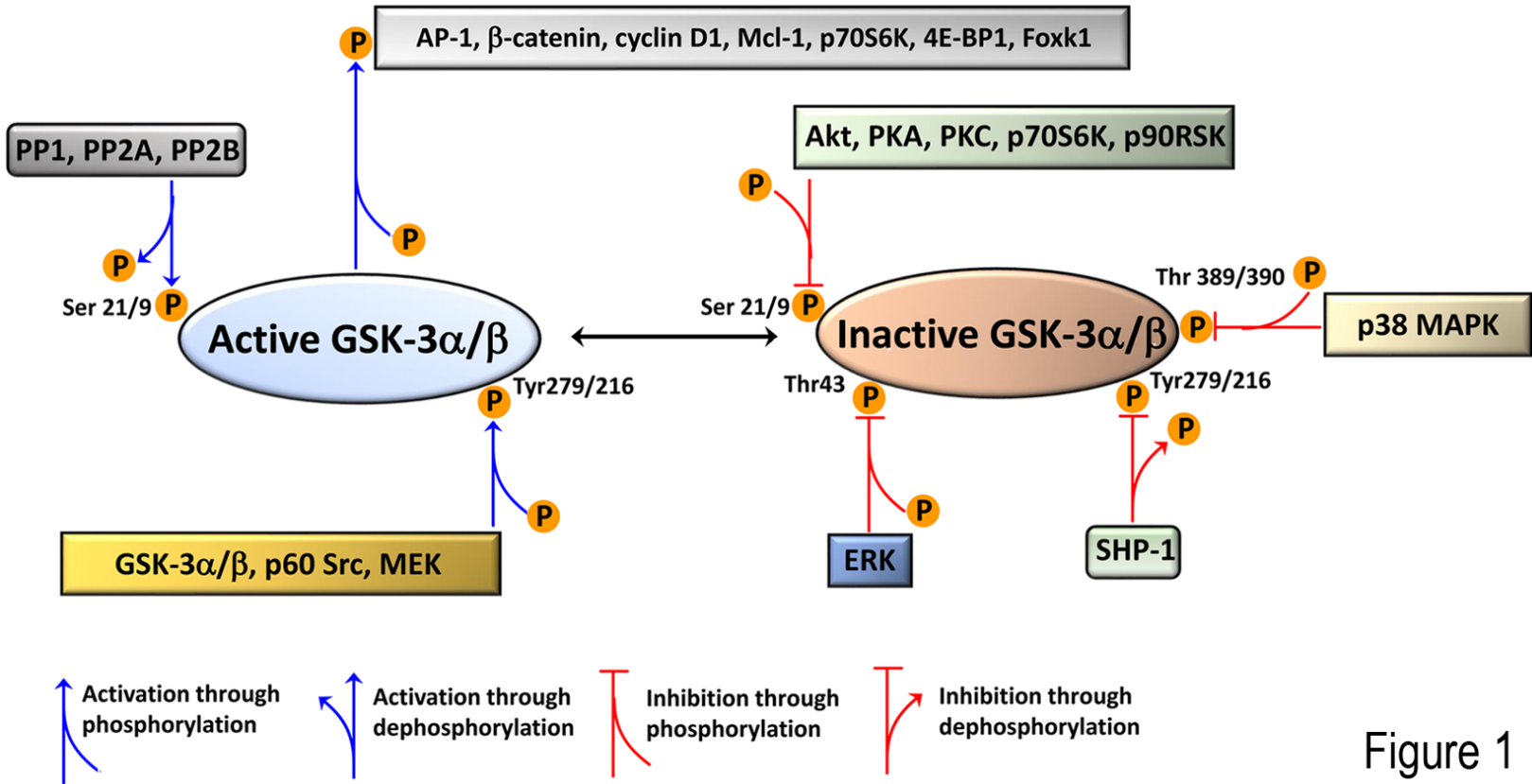


Figure 1



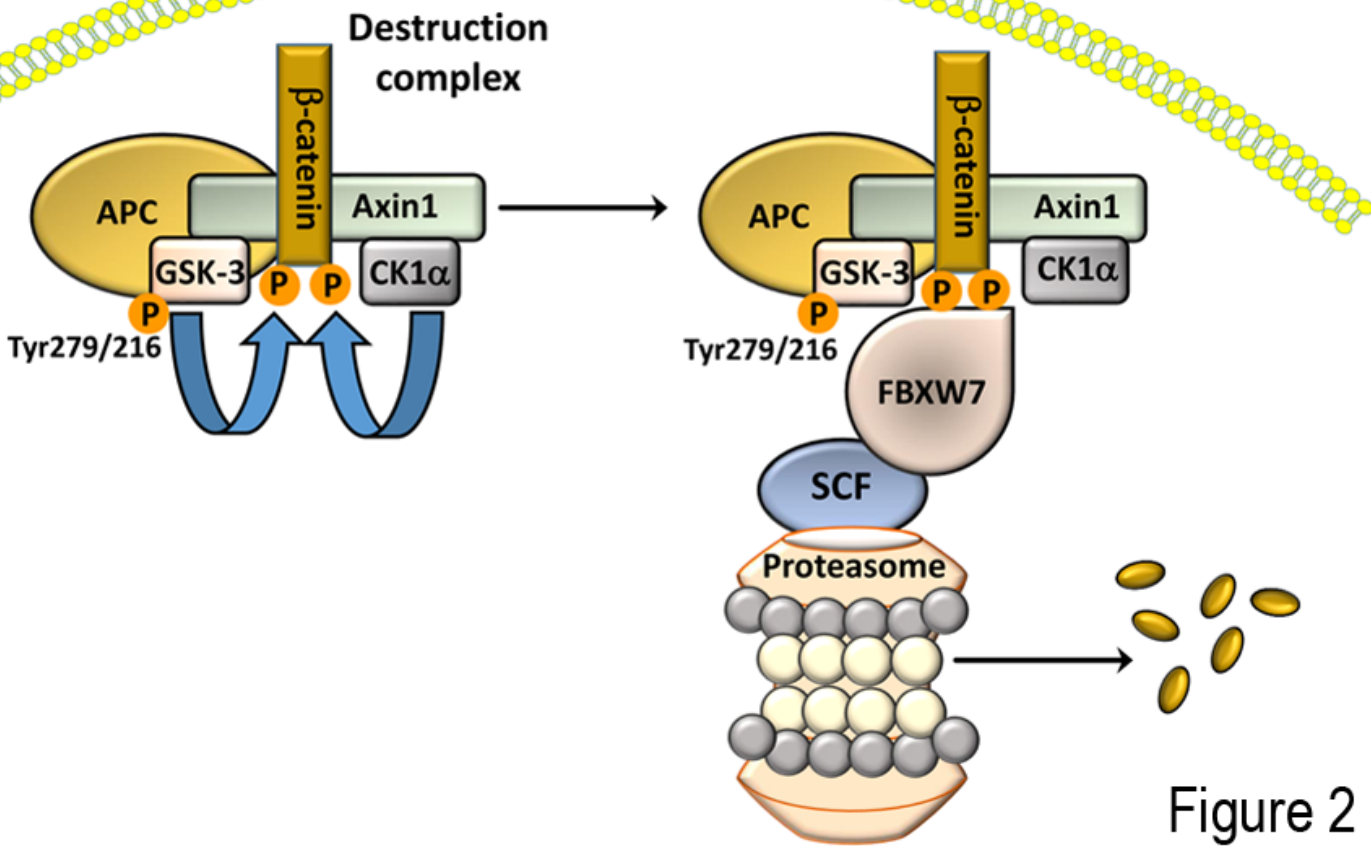


Figure 2

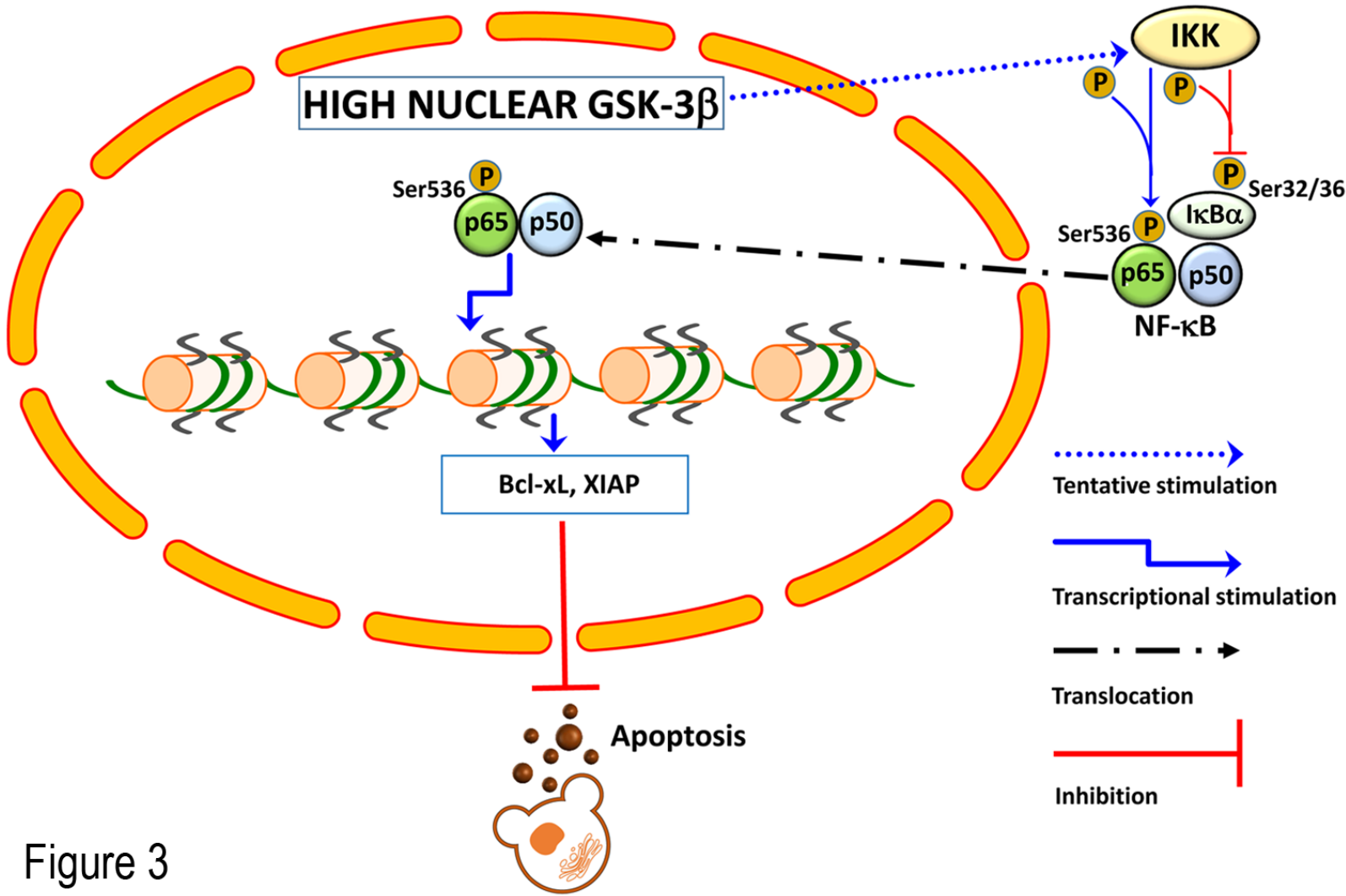


Figure 3

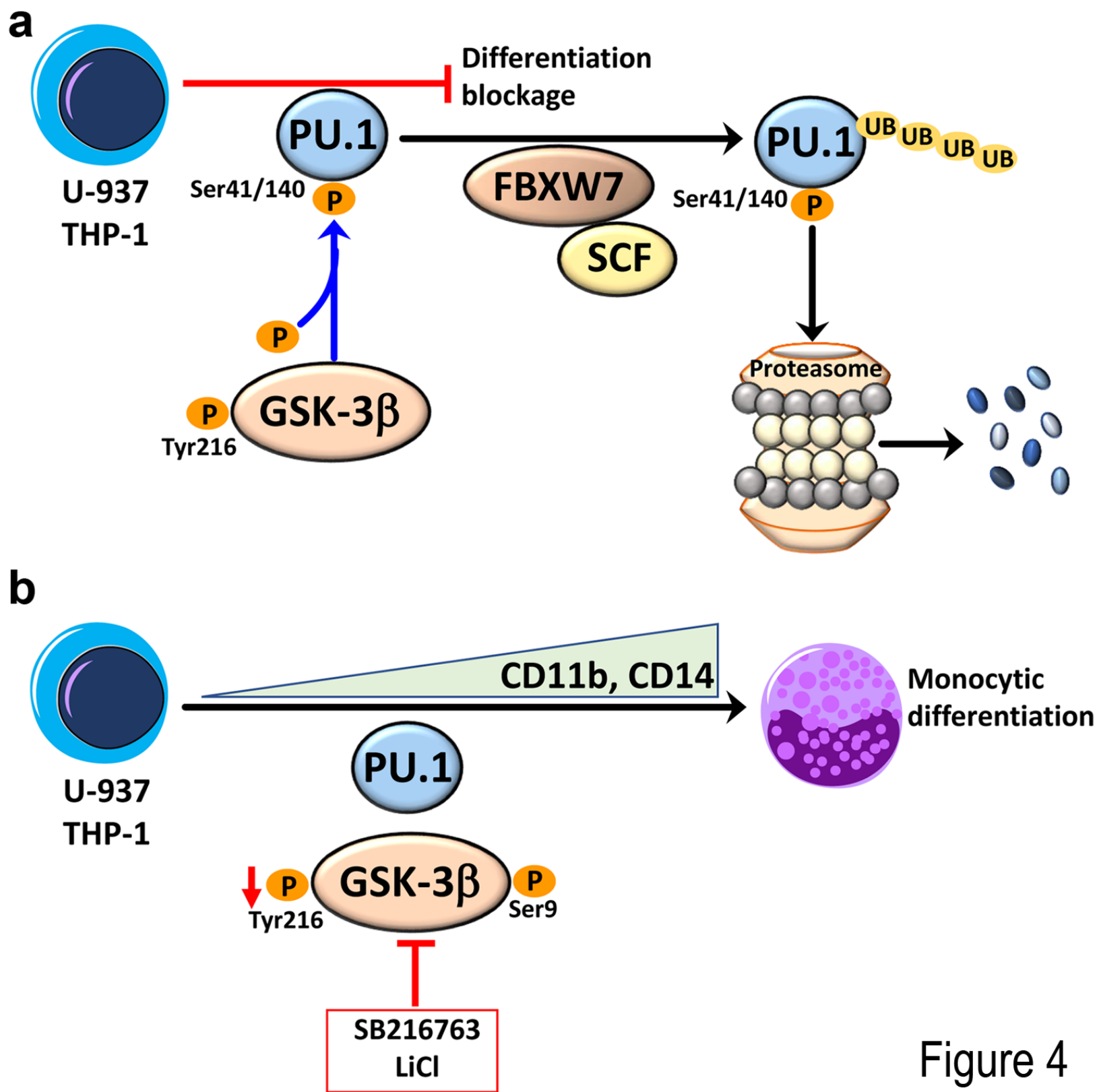


Figure 4

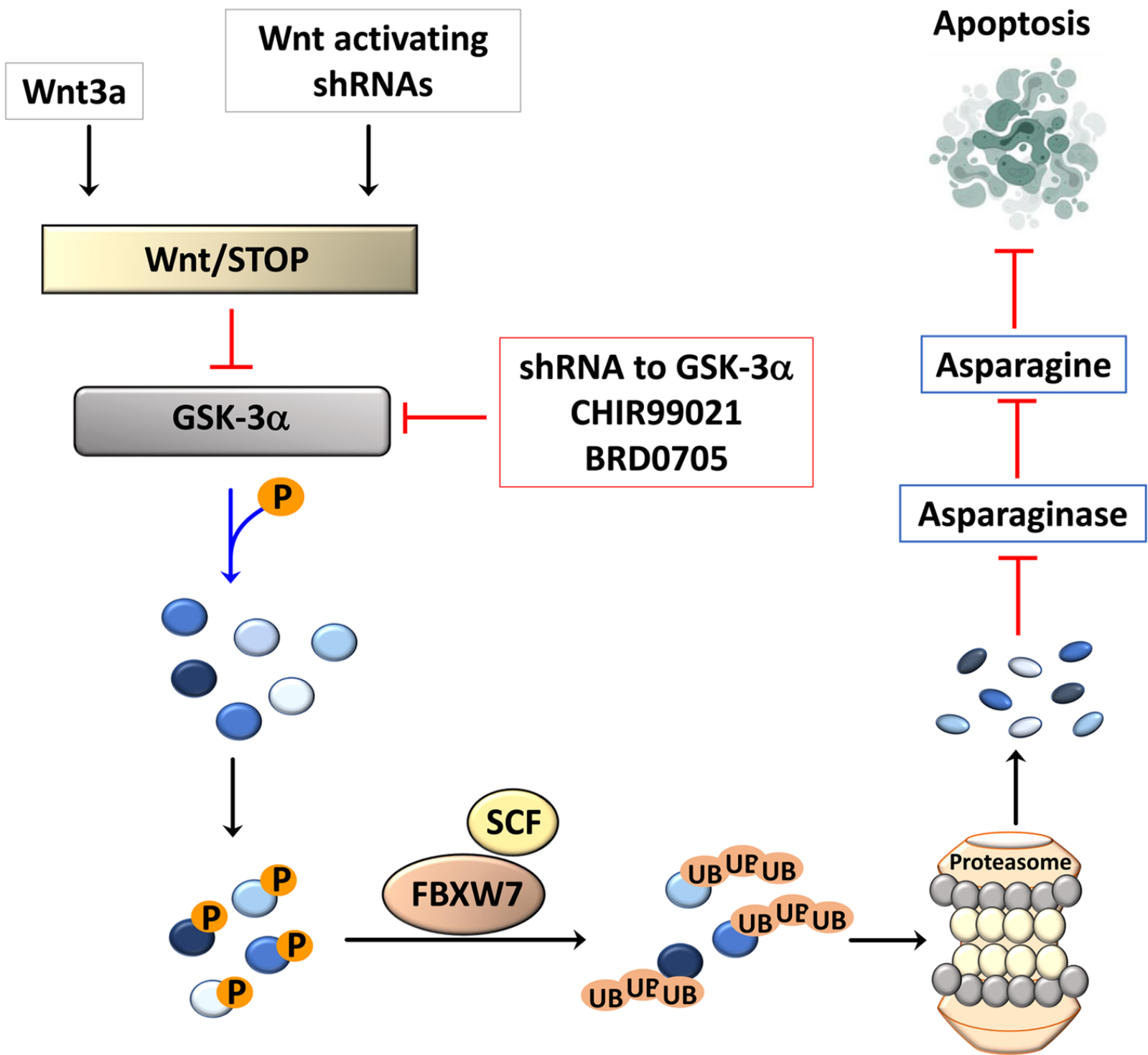


Figure 5

**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-3 $\alpha$                | Tumor suppressor | Genetic                    | Activation of Wnt/ $\beta$ -catenin signaling, decreased sensitivity to AC220 | 60        |
| AML (MLL)        | GSK-3 $\beta$ >GSK-3 $\alpha$ | Tumor promoter   | Genetic or pharmacological | Up-regulation of p27 <sup>Kip1</sup>  | 61        |
| AML              | GSK-3 $\alpha$                | Tumor promoter   | Genetic or pharmacological | Induction of myelomonocytic differentiation                                   | 62        |
| AML              | GSK-3 $\beta$ (nuclear)       | Tumor promoter   | Genetic or pharmacological | Decreased NF- $\kappa$ B p65-dependent transcription of Bcl-xL and XIAP       | 13        |
| AML              | GSK-3 $\beta$                 | Tumor promoter   | Pharmacological            | Enhancement of monocytic differentiation via PU.1                             | 65        |
| T-ALL            | GSK-3 $\beta$                 | Tumor promoter   | Genetic or pharmacological | Decreased expression of c-Myb-dependent Bcl-2 and survivin                    | 94        |
| B-ALL, T-ALL     | GSK-3 $\beta$ (nuclear)       | Tumor promoter   | Genetic or pharmacological | Decreased NF- $\kappa$ B p65-dependent transcription of survivin              | 14,96     |
| T-ALL            | GSK-3 $\beta$                 | Tumor promoter   | Genetic or pharmacological | Decreased expression of caspin, Mcl-1, survivin, XIAP, c-Myb.                 | 97        |
| B-ALL, T-ALL     | GSK-3 $\alpha$                | 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- $\kappa$ B, nuclear factor  $\kappa$ B; XIAP, X-linked inhibitor of apoptosis protein.