

GSK3 β inactivation induces apoptosis of leukemia cells by repressing the function of c-Myb

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ABSTRACT Glycogen synthase kinase 3 β (GSK3 β) regulates diverse physiological processes, including metabolism, development, oncogenesis, and neuroprotection. GSK3 β kinase activity has been reported to be critical for various types of cancer cells, but the mechanism has remained elusive. In this study we examine the mechanism by which GSK3 β regulates the survival of leukemia cells. We demonstrate that upon GSK3 β kinase inhibition different types of leukemia cells show severe proliferation defects as a result of apoptosis. The transcription factor c-Myb is found to be the main target of GSK3 β inhibition in cell survival. GSK3 β inactivation reduces the expression of c-Myb by promoting its ubiquitination-mediated degradation, thereby inhibiting the expression of c-Myb-dependent antiapoptotic genes *Bcl2* and *survivin*. Coimmunoprecipitation, reporter assays, chromatin immunoprecipitation, and knockdown studies show that c-Myb needs to interact and cooperate with transcription factor LEF-1 in the activation of *Bcl2* and *survivin* and that both transcription factors are required for cell survival. These data reveal an as-yet-unknown mechanism by which GSK3 β controls cell survival.

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INTRODUCTION

GSK3 β , a multifunctional serine/threonine (Ser/Thr) kinase, was originally discovered as a key enzyme in the control of glucose metabolism. In addition, it was identified as a component of the Wnt signaling pathway, forming a complex with APC and Axin, which inactivates the pathway in the absence of Wnt ligands by phosphorylating and degrading β -catenin. In the presence of Wnt ligand this complex is disrupted (Harwood, 2001). GSK3 β is also involved in Alzheimer's disease since it associates with presenilin and phosphorylates the microtubule-associated protein tau (Takashima *et al.*, 1998; Baki *et al.*, 2004). Disruption of the murine GSK3 β gene results in embryonic lethality due to severe liver degeneration, and the GSK3 β -

deficient fibroblasts are more sensitive to tumor necrosis factor- α -induced apoptosis as a result of reduced nuclear factor (NF)- κ B activity (Hoeflich *et al.*, 2000). In certain pancreatic cancers, prostate cancers, and gliomas the activity of GSK3 β is required for cancer cell survival (Ougolkov *et al.*, 2005; Kotliarova *et al.*, 2008; Vene *et al.*, 2008; Korur *et al.*, 2009; Mai *et al.*, 2009; Mamaghani *et al.*, 2009). In addition, in leukemia cells GSK3 β can enhance proliferation and/or survival (Holmes *et al.*, 2008; Wang *et al.*, 2008). The mechanism of these GSK3 β functions remains elusive and might depend on cell type, context, and nature of the oncogenic mutations.

The transcription factor c-Myb was discovered as a protooncogene and is expressed predominantly in immature hematopoietic cells (Westin *et al.*, 1982; Luscher and Eisenman, 1990; Thompson and Ramsay, 1995). Forced expression of c-Myb inhibits erythroid differentiation, whereas antisense c-Myb oligonucleotides cause growth arrest and inhibition of hematopoietic colony formation (Clarke *et al.*, 1988; Gewirtz and Calabretta, 1988; Shivdasani and Orkin, 1996). c-Myb-deficient mice show normal hematopoiesis in the yolk sac but defective hematopoiesis in the fetal liver (Mucenski *et al.*, 1991). Oncogenically activated versions of c-Myb have been found in two different avian leukemia viruses—avian myeloblastosis virus and E26—which can transform immature hematopoietic cells in vitro and induce acute myeloid or erythroid leukemia in chickens (Beug *et al.*, 1979; Radke *et al.*, 1982; Oh and Reddy, 1999). c-Myb regulates hematopoietic gene expression in combination with other transcription factors, cofactors, or accessory proteins, such as core-binding factor/PEBP2, C/EBP α , C/EBP ϵ , PU.1, and cAMP

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Abbreviations used: Bax, BCL2-associated X protein; Bcl2, B-cell CLL/lymphoma 2; Bcl-x_L, BCL2-like 1; Bim, BCL2-like 11 (apoptosis facilitator); C/EBP, CCAAT/enhancer binding protein; DKK1, dickkopf homologue 1; GSK3 β , glycogen synthase kinase 3 β ; HA, hemagglutinin; LEF-1, lymphoid enhancer-binding factor-1; shRNA, small hairpin RNA.

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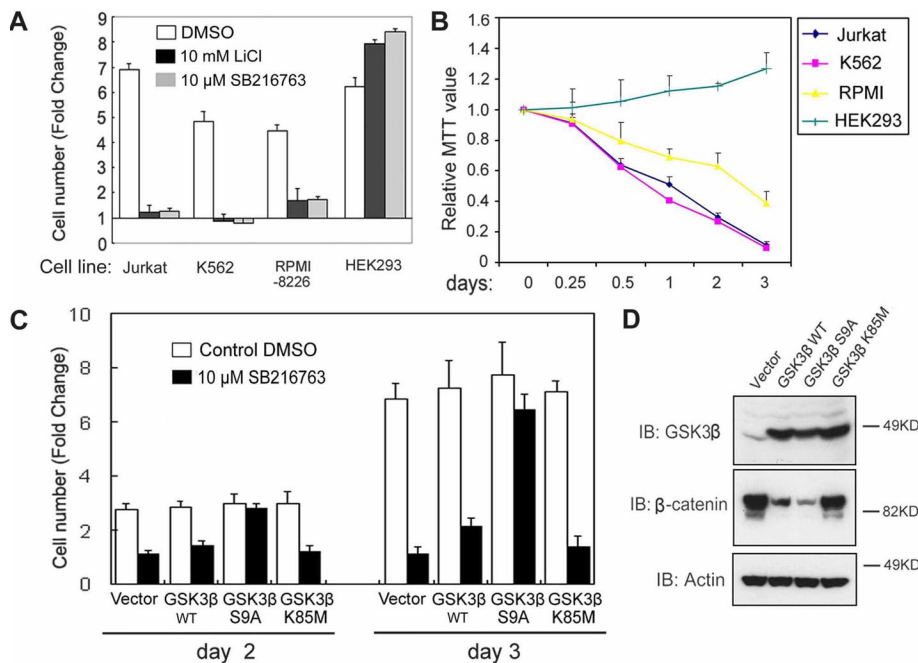


FIGURE 1: GSK3 β inactivation causes growth inhibition of leukemia cells. (A) Jurkat, K562, RPMI 8226, and HEK293 cells were treated with or without LiCl (10 mM) or SB216763 (10 μ M). Cell numbers were determined with a CASY model TT cell counter (Schärfe System, Reutlingen, Germany) on days 0 and 3; the fold change is depicted. (B) Cells were treated with or without SB216763 as described, and their viability was measured with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI) at indicated time points. The relative viability (\pm SB216763) is depicted. (C) Jurkat cells were infected with lentivirus expressing GSK3 β WT, GSK3 β S9A, and GSK3 β K85M and treated with or without SB216763 (10 μ M). Cell numbers were determined as described for A. (D) Immunoblotting shows the expression levels of wild-type and mutant GSK3 β in the experiment shown in C and verification of their effects on β -catenin expression. Data are represented as mean and SD of triplicates and are representative of at least two independent experiments.

response element-binding (CREB)-binding protein (Hernandez-Munain and Krangel, 1995; Dai *et al.*, 1996; Verbeek *et al.*, 1999). Oncogenic changes in *c-Myb* have also been found in colorectal and breast cancers (Ramsay and Gonda, 2008). However, the signaling pathway(s) regulating *c-Myb* activity are largely unclear.

Lymphoid enhancer-binding factor-1 (LEF-1), a member of the high-mobility group box family, was originally identified as a pre-B and T lymphocyte-specific protein that regulates the T cell receptor α enhancer (Giese *et al.*, 1991; Travis *et al.*, 1991). By binding to the minor groove it was found to induce a sharp bend in the DNA, thereby facilitating the assembly of a higher-order multiprotein enhancer complex containing other lymphoid-specific proteins and Ets-1, PEBP2- α , and ATF/CREB (Giese *et al.*, 1992; Love *et al.*, 1995). LEF-1 is also an essential regulator of canonical Wnt/ β -catenin signaling and a leukemogenesis factor (Behrens *et al.*, 1996; Hsu *et al.*, 1998; Petropoulos *et al.*, 2008; Gutierrez *et al.*, 2010).

In the present study, we examined the mechanism by which GSK3 β maintains leukemia cell growth. In different types of leukemia cells, GSK3 β kinase inhibitors caused severe proliferation defects due to apoptosis. We found the survival genes *Bcl2* and *survivin* to be inhibited upon GSK3 β inactivation, as a result of decreased binding of *c-Myb* and LEF-1 to their promoters. GSK3 β inactivation increased *c-Myb* degradation, and overexpression of *c-Myb* could block the inhibitory effect of GSK3 β inactivation on cell proliferation. We also show that *c-Myb* and LEF-1 interact and cooperate in the activation of *Bcl2* and *survivin* in leukemia cells and that both are required for cell survival. These data reveal an

as-yet-unknown function of GSK3 β and suggest that the effect of GSK3 β on leukemia cell proliferation depends on its regulation of *c-Myb*.

RESULTS

GSK3 β activity is required for the survival of leukemia cells

To examine the role of GSK3 β activity in leukemia cells, we treated acute T cell leukemia Jurkat, chronic myelogenous leukemia K562, and myeloma RPMI-8226 cells with the GSK3 β kinase inhibitor LiCl or SB216763. Both inhibitors strongly reduced the proliferation of these cells as measured by cell counting and cell viability analysis. In contrast, proliferation of HEK293 cells was not inhibited (Figure 1, A and B). The kinase activity of GSK3 β was found to be critical for the proliferation of the leukemia cells since overexpression of the constitutively active GSK3 β mutant S9A rescued the growth inhibition by SB216763, whereas wild-type GSK3 β and the kinase-dead mutant K85M did not (Figure 1, C and D).

We next tested whether the decreased viability observed after SB216763 or LiCl treatment in the leukemia cells was due to increased apoptosis. Indeed, as shown in Figure 2A, in both Jurkat and K562 cells, SB216763 treatment strongly increased the amount of annexin V-positive cells. Similar results were obtained in Jurkat, K562, and RPMI-8226 when the amount of sub-G1 cells was measured (Supplemental Figure S1 and unpublished data). The levels of active caspase 3 were also increased after SB216763 or LiCl treatment in these three cell lines (Figure 2B). This demonstrates that GSK3 β activity protects leukemia cells from apoptosis.

GSK3 β inactivation inhibits the activation of the survival genes *Bcl2* and *survivin* by *c-Myb* and LEF-1

To elucidate the mechanism by which GSK3 β regulates proliferation and survival of leukemia cells, we first analyzed the expression of various proliferation- and apoptosis-related genes. Quantitative real-time PCR (qRT-PCR) assays in Jurkat and K562 cells showed that LiCl or SB216763 strongly reduced the mRNA levels of the apoptosis-related genes *Bcl2*, *survivin*, *c-Myc*, and *CEBP α* (Figure 3A and unpublished data). In contrast, the levels of *Bim*, *Bax*, and *Bcl-x_L* did not show any difference, whereas the cell cycle inhibitors *p21* and *p27* showed up-regulation and down-regulation, respectively (Supplemental Figure S2). Activation of the Wnt target genes *DKK1* and *Axin2* by LiCl or SB216763 indicated that Wnt signaling was strongly induced under these conditions (Figure 3A). Western analysis confirmed that the protein levels of *Bcl2* and *survivin* also were inhibited by GSK3 β inactivation in Jurkat, K562, and RPMI-8226 cells (Figure 3B).

Because *Bcl2* and *survivin* are target genes of the transcription factors *c-Myb* and LEF-1, respectively, whereas *c-Myc* is regulated by both *c-Myb* and LEF-1, we next investigated whether GSK3 β inhibition affects the binding of these transcription factors to the *Bcl2* and *survivin* promoters. Chromatin immunoprecipitation (ChIP) analysis showed that after SB216763 treatment, both

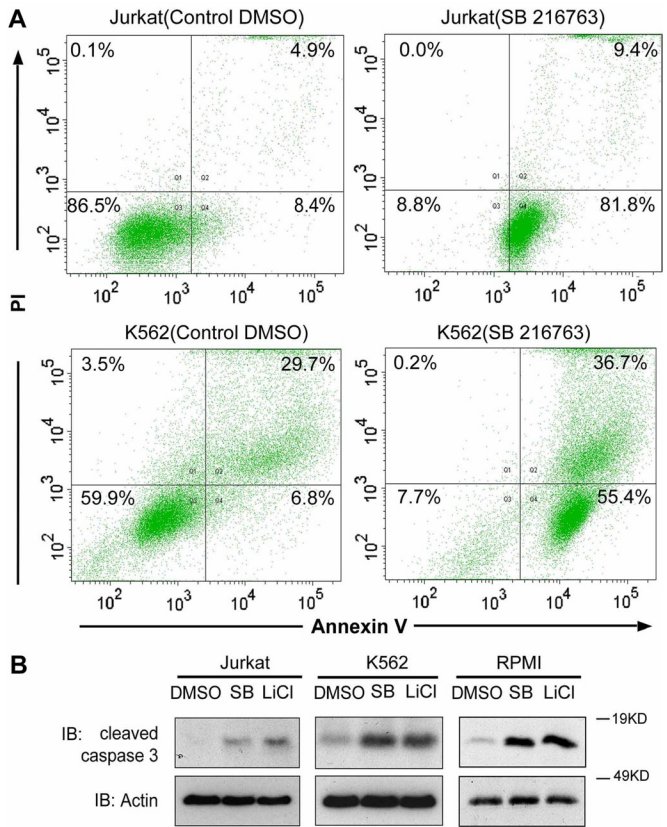


FIGURE 2: GSK3 β inactivation induces apoptosis in leukemia cells. (A) Jurkat and K562 cells were treated with or without SB216763 (10 μ M) for 20 h, stained with annexin V and PI, and analyzed by fluorescence-activated cell sorting. (B) Jurkat, K562, and RPMI 8226 cells were treated with SB216763 (SB; 10 μ M) or LiCl (10 mM) for 20 h, and cell lysates were analyzed by immunoblotting.

the binding of c-Myb to the *Bcl2* promoter and the binding of LEF-1 to the *survivin* promoter were dramatically decreased (Figure 3, C and D). Moreover, LEF-1 also bound to the *Bcl2* promoter, whereas c-Myb also bound to *survivin* promoter, and these interactions were reduced upon SB216763 treatment as well. Taken together, GSK3 β inactivation suppresses the transcriptional activation of apoptosis-related genes *Bcl2* and *survivin* by c-Myb and LEF-1.

GSK3 β inactivation reduces c-Myb protein levels and promotes its ubiquitination

We next tested the effect of GSK3 β inactivation on the c-Myb and LEF-1 proteins. Of importance, in all leukemia cells examined, the levels of c-Myb were reduced upon SB216763 or LiCl treatment, whereas the levels of LEF-1 did not show significant differences (Figure 4A and Supplemental Figure S3). This decrease in c-Myb was already prominent after 4 h of GSK3 β kinase inhibition and coincided with Wnt signaling activation as measured by β -catenin induction, which indicates that c-Myb might be regulated by GSK3 β kinase activity directly (Supplemental Figure S4). Consistent with results published in the literature, we could not detect endogenous c-Myb expression in HEK293 cells (Supplemental Figure S3) (Corradini *et al.*, 2005; Zhao *et al.*, 2009), which might explain why the proliferation of these cells is not inhibited by GSK3 β (Figure 1, A and B). Of importance, and consistent with results obtained with pharmacological GSK3 β inhibitors, short hairpin RNA (shRNA)-me-

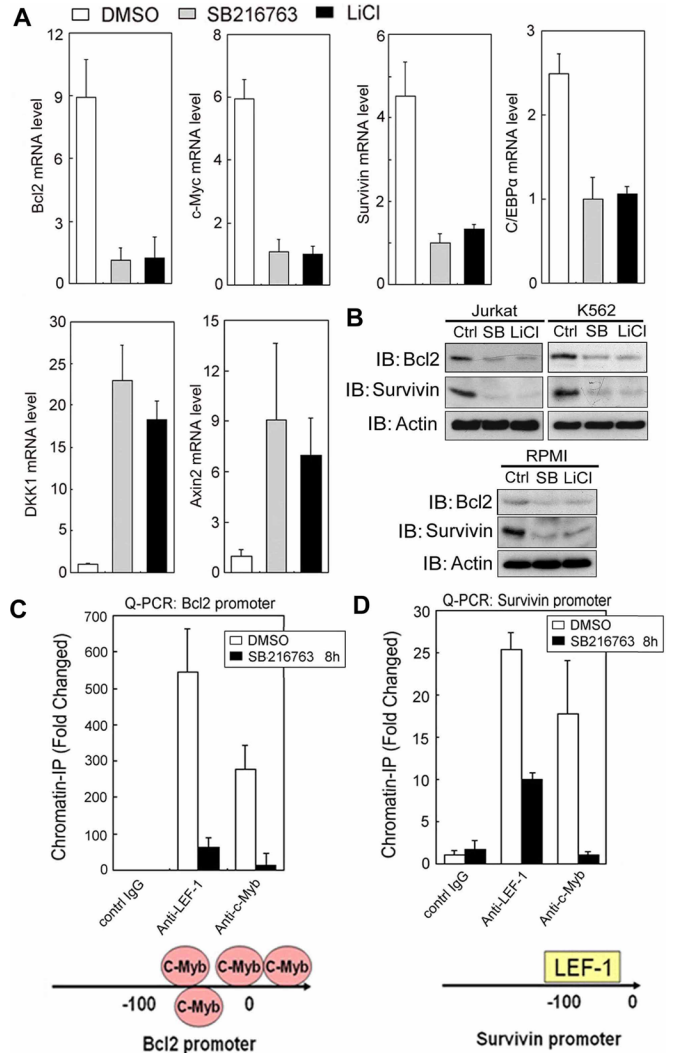


FIGURE 3: GSK3 β inactivation inhibits the expression of *Bcl2* and *survivin*. (A) Jurkat cells were treated with SB216763 (10 μ M) or LiCl (10 mM) for 20 h, and RNA was reverse transcribed and analyzed by qRT-PCR. Values and error bars represent the mean and SD of triplicates and are representative of at least two independent experiments. (B) Jurkat, K562, and RPMI 8226 cells were treated with SB216763 (SB) (10 μ M) or LiCl (10 mM) for 20 h, and cell lysates were analyzed by immunoblotting. (C, D) Jurkat cells were treated with or without SB216763 (10 μ M) for 8 h. Cells were lysed, and ChIP was performed with anti-LEF-1, anti-c-Myb, or a control antibody (immunoglobulin G [IgG]). The precipitated promoter fragments of *Bcl2* (C) and *survivin* (D) were amplified by qRT-PCR (top). The positions of the identified LEF-1- and c-Myb-binding sites in the promoters are shown in a sketch map (bottom). Values and error bars represent the mean and SD of triplicates and are representative of at least two independent experiments.

diated depletion of GSK3 β in all three kinds of leukemia cell lines leads to an obvious decrease in c-Myb and up-regulation of cleaved caspase 3 (Figure 4B). c-Myb overexpression could rescue the inhibition of cell proliferation by SB216763 in Jurkat cells, demonstrating that c-Myb is a critical growth-regulatory target of GSK3 β kinase activity in these cells (Figure 4C). Moreover, in a small-scale screen in which 52 kinase inhibitors were tested for their ability to inhibit Jurkat cell proliferation, almost all of the inhibitors that suppressed GSK3 β kinase activity were found also to decrease the levels of c-Myb and to suppress proliferation (Supplemental Figure S5).

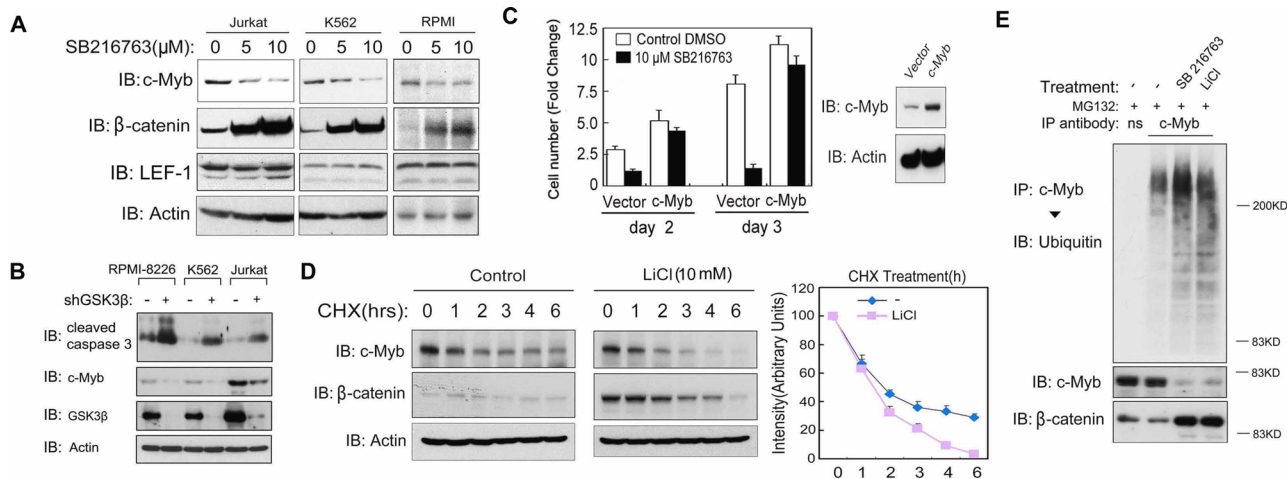


FIGURE 4: The c-Myb protein level is critical for GSK3 β -dependent cell survival. (A) Jurkat, K562, and RPMI 8226 cells were treated with or without 5 or 10 μ M SB216763 for 8 h, and cell lysates were analyzed by immunoblotting. (B) RPMI 8226, K562, and Jurkat cells were infected with lentivirus expressing GSK3 β shRNA or a nonspecific control shRNA. Four days after infection, cells were harvested for immunoblotting analysis. (C) Jurkat cells were infected with lentiviral-overexpressed c-Myb or a control vector and after 48 h treated with or without SB216763 (10 μ M) for another 2 or 3 d. Cell numbers were determined at days 0, 2, and 3 with a CASY Model TT cell counter (left). Data are represented as mean and SD. The overexpression of c-Myb was verified by immunoblotting (right). (D) Jurkat cells were treated with cycloheximide (20 μ g/ml) in the absence or presence of LiCl (10 mM) for the indicated time points, and cell lysates were analyzed by immunoblotting. For the quantification on the right, the c-Myb protein level at time 0 was set at 100%. (E) Jurkat cells were treated with or without SB216763 (10 μ M) or LiCl (10 mM) for 6 h, after which MG132 (10 μ M) was added for another 4 h. c-Myb and nonspecific control immunoprecipitations were analyzed by SDS-PAGE and immunoblotting.

We subsequently investigated how GSK3 β inactivation could lead to a rapid decrease (within 4 h) in the amount of c-Myb. qRT-PCR analysis of c-Myb mRNA in SB216763- or LiCl-treated Jurkat and K562 cells revealed only significant down-regulation after 8 h (unpublished data), which is probably an indirect consequence of decreased c-Myb autoinduction (Nicolaidis *et al.*, 1991). Inhibition of de novo protein synthesis by cycloheximide (CHX) showed that c-Myb degraded faster in cells treated with SB216763 or LiCl (Figure 4D and unpublished data). In addition, we found that the ubiquitination of c-Myb was increased after GSK3 β inactivation (Figure 4E). Proteasome inhibition by MG132 could partially rescue c-Myb degradation (unpublished data). This suggests that GSK3 β kinase may protect c-Myb against proteasome-mediated degradation by inhibiting its ubiquitination.

LEF-1 and c-Myb cooperatively regulate transcription of *Bcl2* and *survivin*

Because both the LEF-1 and c-Myb fractions bound to the *survivin* and *Bcl2* promoters were decreased upon GSK3 β inactivation (Figure 3, C and D), whereas only the (total) c-Myb levels were reduced, we next examined whether c-Myb and LEF-1 could cooperate in LEF-1- or c-Myb-dependent transcription activation. Indeed, both c-Myb and LEF-1 were able to activate the LEF-1-responsive reporter LEF-Luc and c-Myb-responsive pTRHR-Luc, and the activation was strongly increased when the two proteins were coexpressed (Figure 5, A and B). Moreover, knockdown of c-Myb reduced the activation of LEF-Luc by LEF-1, and knockdown of LEF-1 reduced the activation of pTRHR-Luc by c-Myb (Figure 5, C and D). We next examined whether c-Myb was required for LEF-1 binding to and/or activation of the endogenous *survivin* and *Bcl2* promoters and vice versa. CHIP assays in c-Myb-depleted or LEF-1-depleted cells showed that both c-Myb and LEF-1 were required for optimal binding of each of them to the *Bcl2* and *survivin* upstream regulatory

regions in Jurkat cells. c-Myb knockdown in fact completely blocked the binding of LEF-1 to the *Bcl2* promoter, whereas LEF-1 knockdown strongly impaired the binding of c-Myb to the *survivin* promoter (Figure 5E). Coimmunoprecipitation assays showed that endogenous c-Myb and LEF-1 can bind each other, suggesting that they form a complex regulating these survival genes (Figure 5F). Moreover, qRT-PCR analysis in the c-Myb or LEF-1 knockdown cells showed that both c-Myb and LEF-1 are required for the expression of *Bcl2* and *survivin* (Figure 5G), whereas overexpression of c-Myb or LEF-1 increased the mRNA levels of these two survival genes (Supplemental Figure S6). Together, these data show that c-Myb and LEF-1 interact and cooperate in the activation of *Bcl2* and *survivin* in leukemia cells.

c-Myb and LEF-1 are both required for leukemia cell survival

The results described earlier suggested that both c-Myb and LEF-1 are critical in GSK3 β -dependent cell survival. We therefore examined the effect of c-Myb and LEF-1 knockdown on cell proliferation and cell death. Knockdown of c-Myb or LEF-1 was found to strongly inhibit the proliferation of Jurkat and K562 cells (Figure 6A and Supplemental Figure S7). Western analysis showed that this inhibition was accompanied by a decrease in the levels of *Bcl2* and *survivin* and a strong increase in active caspase 3 (Figure 6B). The amount of annexin V-positive cells also increased (Figure 6C). These data demonstrate that by cooperatively activating *Bcl2* and *survivin*, both c-Myb and LEF-1 are essential factors responsible for GSK3 β activity-mediated cell survival in leukemia cells.

DISCUSSION

In this study, we analyzed the effect of GSK3 β inactivation in several kinds of leukemia cell lines. In all of these cells GSK3 β inhibitors

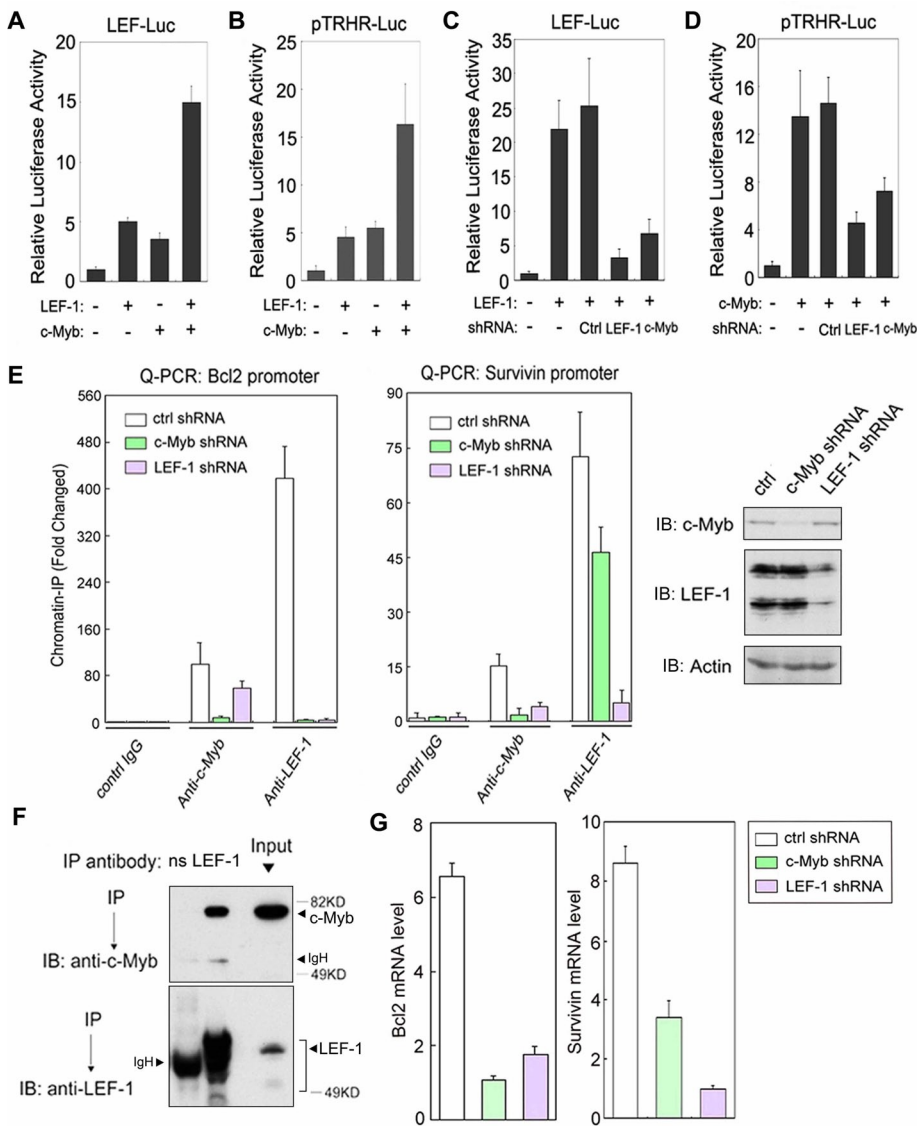


FIGURE 5: LEF-1 and c-Myb cooperatively regulate transcription. (A–D) K562 cells were transiently transfected with the indicated plasmids and analyzed for luciferase assay after 36 h. Data are represented as mean and SD. (E) Jurkat cells were infected with lentivirus expressing c-Myb or LEF-1 shRNA or a nonspecific control (ctrl) shRNA. After 60 h, cells were lysed and ChIP was performed with anti-LEF-1, anti-c-Myb, or a control antibody (IgG). The precipitated promoter fragments were amplified by qRT-PCR (left). Data are represented as mean and SD. The knockdown efficiency of the c-Myb and LEF-1 shRNAs was validated by immunoblotting analysis (right). (F) To detect the interaction between c-Myb and LEF-1 in Jurkat cells, LEF-1 and nonspecific control (ns) immunoprecipitations (IPs) were analyzed by SDS–PAGE and immunoblotting (IB). (G) RNA from the Jurkat cells treated as described for E was analyzed by qRT-PCR analysis. Values and error bars represent the mean and SD of triplicates and are representative of at least two independent experiments.

induced apoptosis and reduced the expression of the survival factors Bcl2 and survivin. Moreover, GSK3 β was found to control Bcl2 and survivin expression by preventing ubiquitin-dependent degradation of c-Myb and thereby to stimulate c-Myb– and LEF-1–dependent transcriptional activation. In contrast to the leukemia cells examined, we found that GSK3 β inactivation did not affect the proliferation of HEK293 cells, which do not express c-Myb (Supplemental Figure S3). Previous studies showed that GSK3 β can control cell proliferation and/or survival by positively regulating NF- κ B function (Hoeflich *et al.*, 2000; Ougolkov *et al.*, 2005) or by destabilizing the cyclin-dependent kinase inhibitor p27Kip1 (Wang *et al.*, 2008).

However, we found no evidence that GSK3 β altered NF- κ B activity and p27Kip1 stability in the cell lines that we investigated (unpublished data). Our results therefore reveal a novel mechanism by which GSK3 β stimulates cell survival and proliferation and suggest that the levels of c-Myb can be a determining factor in GSK3 β inhibition–induced apoptosis of certain leukemia cell lines.

We show here that in leukemia cells GSK3 β inactivation promotes the ubiquitination of c-Myb. Because phosphorylation of c-Myb by NLK can regulate its ubiquitin-dependent degradation (Kanei-Ishii *et al.*, 2004; Ramsay and Gonda, 2008), we tested the effect of GSK3 β inactivation on c-Myb phosphorylation status. Of interest, c-Myb threonine phosphorylation was significantly increased after treatment with SB216763 or LiCl, whereas phosphorylation on serine and tyrosine remained unchanged (unpublished data). However, it remains to be established whether GSK3 β kinase activity protects c-Myb against proteasome-mediated degradation by inhibiting its threonine phosphorylation, and whether NLK is involved in this. We could not detect a stable interaction between endogenous GSK3 β and c-Myb.

Because GSK3 β inactivation is a direct consequence of Wnt signaling, we also examined whether other components of the Wnt signaling pathway affect the levels and ubiquitination of c-Myb in leukemia cells. Consistent with a previous report that Wnt1 induces ubiquitination and proteasome-dependent degradation of c-Myb (Kanei-Ishii *et al.*, 2004), Wnt1 and Wnt3a indeed decreased c-Myb protein level in Jurkat cells, but wild-type β -catenin and a constitutively active mutant did not (Supplemental Figure S8A). Ubiquitination of c-Myb was also increased by Wnt1 and decreased by DKK1 but not by β -catenin (Supplemental Figure 8B and unpublished data). This indicates that GSK3 β is the key component of the Wnt pathway in the control of c-Myb protein and that the negative regulation by GSK3 β occurs independent of canonical Wnt/ β -catenin signaling.

By decreasing the level of c-Myb, inhibition of GSK3 β affects the function of the transcription factor LEF-1. Here we show that c-Myb and LEF-1 cooperatively bind to and activate the growth-regulatory genes *Bcl2*, *survivin*, and *c-Myc* in leukemia cells and thereby protect these cells against apoptosis. Overexpression of LEF-1 and c-Myb also showed cooperative effects in HEK293 cells (unpublished data). However, in HEK293 cells endogenous c-Myb is undetectable (Supplemental Figure S3) (Corradini *et al.*, 2005; Zhao *et al.*, 2009). Of interest, the other two TCF/LEF family members, TCF1 and TCF4, which play a similar role as LEF-1 in Wnt signaling, did not interact with or regulate the transactivation of c-Myb (unpublished data), indicating that the cooperation

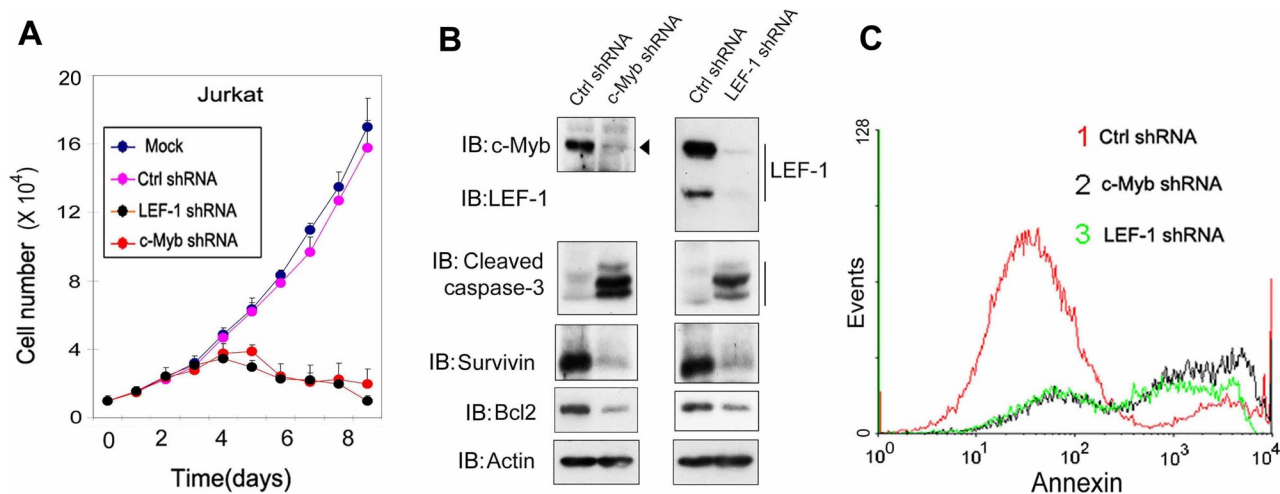


FIGURE 6: C-Myb and LEF-1 are both required for the survival of leukemia cells. (A) Jurkat cells were infected with lentivirus expressing c-Myb or LEF-1 shRNA, or nonspecific control (ctrl) shRNA or were mock infected. The cell numbers were counted at the indicated days. (B) Lysates of Jurkat cells treated as described for A were isolated 60 h after infection and analyzed by immunoblotting. (C) Jurkat cells treated as described for A were harvested 60 h after infection, stained with annexin V and PI, and analyzed by fluorescence-activated cell sorting.

between LEF-1 and c-Myb is rather specific in controlling the growth of leukemia cells.

As a component of multiple signaling pathways, GSK3 β has been implicated in metabolic defects, developmental problems, cancers, and neuroprotective disorders. Therefore, various types of GSK3 β kinase inhibitors are being tested for their specificity and suitability in therapeutic applications. Our results suggest that the level of c-Myb is a determining factor in the response of leukemia cells to GSK3 β kinase inhibitors, which is of particular interest for the therapy of leukemia and cancers that have c-Myb amplifications.

MATERIALS AND METHODS

Reagents and plasmids

SB216763 was obtained from Tocris Bioscience (Ellisville, MO). The expressing vectors for LEF-1, GSK3 β WT, GSK3 β K85M, and LEF-luciferase and TopFlash-luciferase transcriptional reporter constructs were described previously (Zhang *et al.*, 2006; Zhou *et al.*, 2008). GSK3 β S9A (Guo *et al.*, 2008) was kindly provided by Xiao-Fan Wang (Duke University, Durham, NC) and HA-c-Myb and pTHR-Luci (Saether *et al.*, 2007) by Odd Stokke Gabrielsen (University of Oslo, Oslo, Norway). Lentiviral viral vectors expressing shRNA targeting human LEF-1, c-Myb, and GSK3 β were obtained from Sigma-Aldrich (St. Louis, MO) Mission shRNA (LEF-1, TRCN0000020163; c-Myb, TRCN0000040062; GSK3 β , TRCN0000000822). The lentiviral vectors that direct the ectopic expression of LEF-1 and c-Myb were generated by inserting the LEF-1 and c-Myb cDNA fragments into pLV.CMV.bc.puro by PCR and *Mlu*I and *Xho*I digestion. Lentiviral vectors for ectopic expression of GSK3 β WT, GSK3 β S9A, and GSK3 β K85M mutants were generated by cloning the corresponding cDNAs into pLV.CMV.bc.puro FLAG vector by PCR and *Sal*I and *Xba*I digestion. All the primers used for making constructs are listed in Supplemental Table S1.

Cell culture

RPMI 8226, Jurkat, and K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. HEK293T cells were cultured in DMEM supplemented with 10% fetal calf serum

and L-glutamine. All the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Reporter assay

Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and harvested after 36 h to determine luciferase activities using a PerkinElmer (Waltham, MA) luminometer. In all transfections, 20 ng of a *Renilla* expression vector was cotransfected as an internal transfection control. Each experiment was performed in triplicate, and the data represent the mean \pm SD of three independent experiments after normalization to *Renilla* activity.

Flow cytometry (fluorescence-activated cell sorting)

Aliquots of 10⁶ cells were washed twice with phosphate-buffered saline (PBS) and resuspended in binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, pH 7.5, 0.14 M NaCl, 2.5 mM CaCl₂). Propidium iodide (PI) (3.3 μ g/ml)/annexin V-fluorescein isothiocyanate (0.55 μ g/ml) was added, and the samples were incubated for 10 min at room temperature in the dark. Fluorescence was detected using BD LSR II flow cytometry (BD Biosciences, San Diego, CA). The number of cells that were negative for PI and positive for annexin V was scored as apoptotic. The results were analyzed by BD FACSDiva software.

Immunoprecipitation and immunoblotting

As previously described (Zhang *et al.*, 2004, 2006), cells were lysed with 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Sigma-Aldrich) for 10 min at 4°C. After centrifugation at 12,000 \times g for 15 min, the lysates were immunoprecipitated with antibodies and protein A-Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 3 h at 4°C. Thereafter, the precipitates were washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and the immune complexes were eluted with sample buffer containing 1% SDS for 5 min at 95°C and analyzed by SDS-PAGE.

Immunoblotting was performed as described (Zhou *et al.*, 2008) using secondary anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase (GE Healthcare, Buckinghamshire, United Kingdom) and visualized by chemiluminescence.

Antibodies used for immunoprecipitation and immunoblotting were as follows: LEF-1 (C12A5; Cell Signaling, Beverly, MA), c-Myb (clone 1-1; Upstate, Millipore, Billerica, MA), β -catenin (BD Transduction Laboratories, Lexington, KY), β -actin (A5441; Sigma-Aldrich), Bcl2 (C-2; Santa Cruz Biotechnology, Santa Cruz, CA), survivin (sc-17779; Santa Cruz Biotechnology), cleaved caspase 3 (Asp175; 5A1E; Cell Signaling), and ubiquitin (P4D1; Santa Cruz Biotechnology).

Ubiquitination detection

Cells were washed with PBS and lysed in two pellet volumes of RIPA buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, and 1% SDS) supplemented with protease inhibitors and 10 mM *N*-ethylmaleimide. Lysates were sonicated, boiled at 95°C for 5 min, diluted to RIPA buffer containing 0.1% SDS, and then centrifuged at 4°C ($16 \times 10^3 g$ for 15 min). The supernatant was incubated with c-Myb antibody and protein A-Sepharose for 3 h at 4°C. After extensive washing, bound proteins were eluted with 2 \times SDS sample buffer and separated on SDS-PAGE, followed by Western blotting.

qRT-PCR

Total RNA was isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). A 1- μ g amount of RNA was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). qRT-PCR was performed by SYBR Green incorporation (Applied Biosystems, Foster City, CA) using a StepOne Plus real-time PCR system (Applied Biosystems). Results were normalized to those obtained with glyceraldehyde-3-phosphate dehydrogenase and presented as fold induction over control cells. All the primers used for qRT-PCR are listed in Supplemental Table 1.

Chromatin immunoprecipitation assay

Cells were chemically cross-linked by addition of 1/10 volume of freshly prepared 11% formaldehyde solution to the culture medium and incubated for 15 min at room temperature. Cells were subsequently rinsed twice with PBS, harvested using a silicon scraper, lysed (1% SDS, 50 mM Tris, pH 8.0, 5 mM EDTA, and proteinase inhibitors), and sonicated to obtain DNA fragments of ~300–500 base pairs on average. Samples were then centrifuged at 14,000 rpm for 10 min. The supernatant was diluted (20 mM Tris, pH 8.0, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, proteinase inhibitors) and then incubated overnight at 4°C with 2.5 μ g of antibodies (anti-c-Myb and anti-LEF-1). The immunocomplex was collected with 50 μ l of protein A beads by 1-h coinubation and then washed sequentially with TSE I (0.1% SDS, 20 mM Tris, pH 8.0, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, proteinase inhibitors), TSE II (0.1% SDS, 20 mM Tris, pH 8.0, 2 mM EDTA, 1% Triton X-100, 500 mM NaCl, proteinase inhibitors), LiCl buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.25 mM LiCl, 0.1% NP-40, 1% deoxycholate sodium), and TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). The bound immunocomplex was eluted with 400 μ l of fresh elution buffer (25 mM Tris, pH 8.0, 10 mM EDTA, 0.5% SDS) by heating at 65°C with occasional vortex for 15 min, and cross-linking was reversed by overnight incubation at 65°C. Whole-cell-extract DNA (input fraction obtained from the sonication step) was also treated for cross-linking reversal. Immunoprecipitated DNA and whole-cell-extract DNA were then purified by treatment with RNaseA, proteinase K, and multiple

phenol:chloroform:isoamyl alcohol extraction. All of the samples were examined by qRT-PCR. All of the primers used for ChIP assay are listed in Supplemental Table 1.

Statistical analysis

Statistical analyses were performed with a two-tailed unpaired *t* test. *p* < 0.05 was considered statistically significant.

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