Supplemental text to:

Targeting Glycogen Synthase Kinase 3 for Therapeutic Benefit in Lymphoma

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Additional Materials and Methods

Antibodies, and other reagents

The GSK3 inhibitor 9-ING-41 was a gift from Daniel Schmitt, Actuate Therapeutics Inc.; its characteristics including the toxicity and some safety data in mice have been previously described.[12,14-16] Other common agents were from Sigma-Aldrich. Antibodies for immunoblotting, including anti-human GSK3α (Cat#4337), GSK3β (Cat# 12456), phospho-GSK3α-S21/GSK3β-S9 (Cat#9327) were purchased from Cell Signaling. Mouse monoclonal anti-GSK3β (clone 7/ GSK3β, Cat# 610201, BD Biosciences), anti-α-tubulin (clone MD1A, Cat# T9026) and rabbit anti-pericentrin (Cat# ab4448) antibodies used for immunofluorescence were from Sigma and Abcam, respectively. Alexa 488 or Alexa 565 conjugated secondary antibodies were products of Life Technologies.

Apoptosis Assay

Cells were seeded at $5x10^5$ cells/well in 24 well plates, and incubated with 9-ING-41 at indicated concentrations for 48 hours. The cells were then stained with FITC conjugated Annexin V (Life Technologies) and Propidium Iodide followed by analysis on a BD FACS Calibur flow cytometer.

DNA Cell Cycle

Cells were fixed and permeabilized with cold ethanol, treated with RNase, and stained with propidium iodide (Sigma). Stained cells were run on a BD FACS Calibur using CELLQuest PRO Software (Becton Dickinson). Data were analyzed with FlowJo v.X software (Tree Star Inc).

Proliferation Assay

Cells were seeded at $1x10^4$ cells/well in 96 well plates, and incubated with 9-ING-41 at indicated concentrations for 48 hours followed by pulsing with tritium labeled thymidine for overnight before being analyzed for thymidine uptake.

Western Immunoblotting

Western analysis was performed as previously described and developed on a LI-COR Odyssey CLX imager using LI-COR reagent system.

Quantitative PCR

Total mRNAs from lymphoma cell lines were isolated using an RNAeasy kit (Qiagen) and cDNAs synthesized with a Superscript III cDNA synthesis kit (Life technology). The qPCR were then performed on an ABI 7500 Real-Time PCR Systems (Applied Biosystems) using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen).

Drug IC50 calculation

We calculated the IC_{50}s of 9-ING-41 on cell survival and proliferation using an on-line IC50 calculation tool (https://www.aatbio.com/tools/ic50-calculator/).

Immunohistochemistry staining

Immunohistochemistry (IHC) on 5 μm thick paraffin sections was performed according to standard protocol. Briefly, tissue sections on slides were deparaffinized with xylene, rehydrated with series of alcohol, followed by antigen retrieval in citrate buffer (pH 6.0). The resulting slides were endogenous peroxidase quenched with 30% hydrogen peroxide. Slides were then incubated with anti-GSK3b antibody (BD, 1:150) at room temperature for 2 hours, washed three times with Tris-buffered saline (5 min each) and incubated with biotinylated anti-mouse secondary antibody (1:200) for 1h at room temperature. After treating the slides with HRP-conjugated ABC complex (Vectastain, Vector Laboratories) for 1h at room temperature, color

was developed with 3,39-diamino Benzidine (DAB, Vector Laboratories) counterstained with methylene blue, mounted with DPX and examined and imaged on a Nikon Eclipse Ti microscope. negative controls were performed on patient samples without addition of primary antibody.

Immunofluorescence staining for centrosome and spindle localization of GSK3β

For the coimmunostaining of GSK3β and pericentrin at centrosomes (**Figure 5C-5J**), we used an incomplete fixation method by fixing the cells on cytospin slides with 3% paraformaldehyde in PBS for 5 min at room temperature. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 10 min followed by blocking with 5% BSA in PBS for 1 hour and immunostained with mouse anti-GSK3β mAb and rabbit anti-pericentrin overnight at 4^0C. The cells were then washed and further stained with fluorochrome conjugated secondary antibodies.

All other immunostainings of GSK3β and α-Tubulin on cytospin preparations of lymphoma cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature followed by permeabilization and the same staining steps as above. The cells were analyzed and imaged on a conventional Zeiss microscope or a Zeiss LSR 780 confocal microscope.

Determining the prognostic value of GSK3α or GSK3β expressing in lymphoma patients

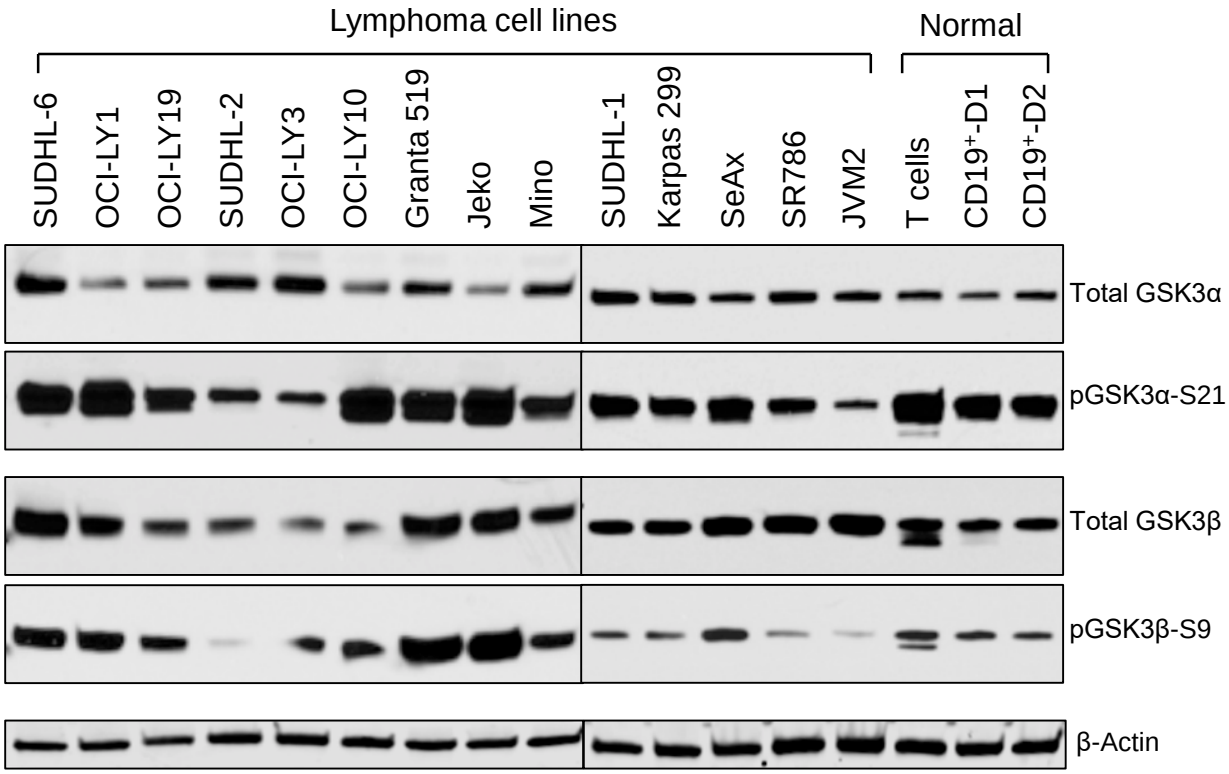
The data files of RNA-Seq on 546 DLBCL patients were downloaded from of Dr. Staudt's lab website (https://lymphochip.nih.gov/signaturedb/) were analyzed in JMP software. First, we select those 234 RNA-Seq samples with matching clinical data, we then performed a receiver operating characteristics (ROC) curve analysis to determine high and low expression group cutoffs of Log2RPKM values for both GSK3α and GSK3β. Kaplan-Meier curve analysis on survivorship for each group pair was then performed.

Supplemental Figures

Supplemental Figure 1. Western blotting analysis of the total and phosphorylated GSK3α and GSK3β proteins in various lymphoma cells lines. ECL instead of Li-COR exposure system was used.

Supplemental Figure 2. Analyzing the prognostic values of GSK3α and GSK3β mRNA expression in DLBCL patients. (**A**) RNA-Seq data from Schmitz et al[18] on a cohort of 234 DLBCL patients were re-grouped into GSK3α-high, GSK3α-low, GSK3β-high, and GSK3β-low groups using ROC analysis determined cutoffs. (**B**) Kaplan-Meier survival curve showing GSK3α high expression group (n=172) had a shorter median OS of 7.8 years (95% OS: 7.2-8.4 years) while GSK3α low expression group (n=62) had a longer median OS of 8.9 years (95% OS: 8.2-10.1years), p=0.03. (**C**) Kaplan-Meier survival curve showing GSK3β high expression group (n=170) had a shorter median OS of 7.8 years (95% OS: 7.2-8.2 years) while GSK3β low expression group (n=64) had a longer median OS of 9.7 years (95% OS: 8.6-11.5 years), p=0.0005.

Supplemental Figure 1



Supplemental Figure 2