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Targeting glycogen synthase kinase 3 for therapeutic benefit in lymphoma

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Abstract:
Targeting the B-cell receptor and PI3K/mTOR signaling pathways has shown meaningful but incomplete anti-tumor activity in lymphoma. Glycogen Synthase 3 (GSK3) kinase α and β are two homologous and functionally overlapping serine/threonine kinases that phosphorylate multiple protein substrates in several of key signaling pathways. To date, no agents targeting GSK3 have been approved for lymphoma therapy. We show that lymphoma cells abundantly express GSK3α and GSK3β compared to normal B- and T-lymphocytes at both mRNA and protein levels. By use of a new GSK3 inhibitor 9-ING-41 and by genetic deletion of GSK3α and GSK3β genes using CRISPR/CAS9 knockout, GSK3 was demonstrated to be functionally important to lymphoma cell growth and proliferation. GSK3β binds to centrosomes and microtubules, and lymphoma cells treated with 9-ING-41 become arrested in mitotic prophase, supporting the notion that GSK3β is necessary for the progression of mitosis. By analyzing recently published RNA-Seq data on 234 DLBCL patients, we found higher expression of either GSK3α or GSK3β correlate well with shorter overall survival. These data provide rationale for testing GSK3 inhibitors in lymphoma patient trials.

Conflict of interest: COI declared - see note

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Targeting Glycogen Synthase Kinase 3 for Therapeutic Benefit in Lymphoma

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Key Points

1. Glycogen Synthase 3 (GSK3) is overexpressed in and functionally exploited by lymphoma cells.
2. New GSK3 inhibitor 9-ING-41 induces apoptosis and cell cycle arrest at mitotic prophase by targeting centrosomes and microtubule bound GSK3β.

Abstract

Targeting the B-cell receptor and PI3K/mTOR signaling pathways has shown meaningful but incomplete anti-tumor activity in lymphoma. Glycogen Synthase 3 (GSK3) kinase α and β are two homologous and functionally overlapping serine/threonine kinases that phosphorylate multiple protein substrates in several of key signaling pathways. To date, no agents targeting GSK3 have been approved for lymphoma therapy. We show that lymphoma cells abundantly express GSK3α and GSK3β compared to normal B- and T-lymphocytes at both mRNA and protein levels. By use of a new GSK3 inhibitor 9-ING-41 and by genetic deletion of GSK3α and GSK3β genes using CRISPR/CAS9 knockout, GSK3 was demonstrated to be functionally important to lymphoma cell growth and proliferation. GSK3β binds to centrosomes and microtubules, and lymphoma cells treated with 9-ING-41 become arrested in mitotic prophase, supporting the notion that GSK3β is necessary for the progression of mitosis. By analyzing recently published RNA-Seq data on 234 DLBCL patients, we found higher expression of either GSK3α or GSK3β correlate well with shorter overall survival. These data provide rationale for testing GSK3 inhibitors in lymphoma patient trials.

Introduction

Lymphoma is the sixth most common cancer[1] and diffuse large B-cell lymphoma (DLBCL) remains the most common non-Hodgkin lymphoma (NHL). Although therapies have improved, there remain unmet needs for the DLBCL and T-cell non-Hodgkin lymphoma (TCL) patients not currently cured as well as most mantle cell (MCL) patients that still die of their disease. Recent research has focused on targeting hyperactivated signal pathways that are essential to lymphoma cell growth.[2] Initial success with this strategy has been demonstrated with the Bruton's tyrosine kinase (BTK) inhibitors ibrutinib[3] and acalabrutinib[4] the PI3K inhibitors idelalisib[5] and copanilisib[6] the mTORC1 inhibitor everolimus,[7] and the immune modulatory agent lenalidomide.[8] However, the single-agent/single-target strategy often leads to the development of drug resistance. Thus, multi-agent regimens have been developed with increased response rates but at higher cost and more side effects. There remains a need for new agents those that target multiple signaling pathways simultaneously.

Glycogen Synthase 3 (GSK3) kinase α and β are highly homologous and functionally overlapping serine/threonine kinases encoded by independent genes *GSK3A* and *GSK3B*, respectively. Given their functional redundancy, we refer to both as GSK3 unless otherwise specified. GSK3 phosphorylates more than 100 different protein substrates that are components of many key cellular pathways including Wnt/β-catenin, Hedgehog, Notch, NF-κB, and PI3K/Akt;[9-11] therefore, GSK3 has widespread potential impact on health and disease. Furthermore, PI3K/AKT/GSK3β signaling also governs glucose hypermetabolism in cancer cells.[12] A characteristic feature of all aggressive lymphoma types and relapsed low-grade NHL is increased glucose metabolism as vividly demonstrated by 2-deoxy-2-[fluorine-18] fluoro-D-

glucose-PET imaging (FDG-PET). Targeting pathways of glucose hypermetabolism offers potential for therapeutic benefit in NHL.[13]

We hypothesized that targeting GSK3 may be a new strategy in lymphoma treatment by interfering with tumor growth and survival through pathways not affected by standard therapies. The purpose of this study is to understand whether GSK3α and GSK3β are upregulated in lymphoma cells, determine if these kinases are essential to lymphoma cell survival, and whether inhibiting GSK3 results in any anti-lymphoma activity. We also investigate the potential mechanisms by which GSK3 inhibitors kill lymphoma cells and explore the therapeutic potential for a novel GSK3 inhibitor 9-ING-41.

Materials and Methods

Primary lymphoma cells and lymphoma cell lines

Lymphoma tissue samples were obtained from patients after written informed consent. This Lymphoma SPORE biospecimens protocol was approved by the Mayo Clinic Institutional Review Board in accordance with the Declaration of Helsinki. All primary patient samples were biopsy tissues from spleen or lymph nodes. Fresh tissue samples were gently dissociated into cell suspension and subjected to Ficoll-Paque density gradient centrifugation. Primary lymphoma cells were then used directly for proliferation assay, or stored in -80°C for Western analysis later after lymphoma diagnosis confirmation.

All lymphoma cell lines used in this study were purchased from ATCC (Manassas, VA) or DSMZ (Braunschweig, Germany). DLBCL lines were cultured in IMDM medium supplemented with 10% human serum (Sigma-Aldrich); TCL and MCL lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum. The Jeko cell line used for xenograft modeling in mice were stably expressed with Firefly luciferase (Fluc) through lenti-viral transduction. Cell lines are periodically checked for the absence of mycoplasma infection, and authenticated by either home brew SNP-based PCR method or short tandem repeat profiling through ATCC.

CRISPR/CAS9 approach to deleting GSK3α and/or GSK3β

Guide RNAs (gRNAs) for targeting the 1st coding exons of both *GSK3α* and *GSK3β* genes were designed using a web tool (http://crispr.mit.edu/). The gRNA sequences (*GSK3α*: GACAGATGCCTTTCCGCCGC; *GSK3β*: CGGCTTGCAGCTCTCCGCAA), were cloned into the px458 vector (Addgene) carrying a co-expressing GFP. The constructs were nucleofected into lymphoma cells using a nucleofection kit (Lonza, Basel, Switzerland). Thirty-six hours post

nucleofection, GFP expressing single cells were sorted into 96 well plates at 1 cell/well on an Aria II FACS sorter. After the expansion of single cell subclones in culture for two weeks, each subclone was genotyped by PCR and Sanger's DNA sequencing.

Lymphoma Xenograft modeling in mice

All mouse work was conducted in compliance with the Mayo Clinic Institutional Animal Care and Use Committee (IACUC) protocol. NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice used in our experiments were purchased from the Jackson Laboratory (Bar Harbor, Maine). Eight to ten mice of same sex at 8 weeks of age were subcutaneously injected with $5x10^6$ Fluc expressing Jeko cells in the right flanks. Tumor engraftment was verified by imaging 4 days after Jeko cell inoculation. The tumor engrafted mice were randomly grouped into control and treatment groups, then untreated or treated with 9-ING-41 by IP injection as indicated. Tumor volumes were measured by an IVIS Imager (Xenogen, Alameda, CA) 20 minutes after IP injection of 200ul of 15mg/ml D-leuciferin (GoldBio, St. Louis, MO) and anesthetized with 2.5% isoflurane. All imaging variables were kept consistent for comparativeness. The experiment terminated when the largest tumor met the size limit of the IACUC protocol.

Detailed Materials for antibodies and chemicals, and Methods for cell apoptosis, proliferation, Western blotting, immunofluorescence staining, immunohistochemistry staining, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), cell cycle analysis, and RNA-Seq data analysis are described in the supplemental Methods (available on the *Blood* Web site).

Results

GSK3α and GSK3β are overexpressed in lymphoma cells.

To determine if GSK3 would constitute an effective therapeutic target in lymphoma, we examined the expression status of GSK3α and GSK3β in purified human normal B and T cells and DLBCL, MCL, and TCL lymphoma cell lines. By RT-qPCR, we found that most lymphoma lines showed higher but variable levels of GSK3α and GSK3β mRNAs compared to the lower expression in normal lymphocytes (**Figure 1A**). These results suggest that the transcription of GSK3 is enhanced in most of lymphoma cell lines. We next analyzed GSK3 protein expression by Western blotting; likewise, most lymphoma lines (except Ly-19) showed strong expression of GSK3α protein compared to weak expression in B and T lymphocytes (**Figure 1B**, in green). Similarly, GSK3β protein was also strongly expressed in all lymphoma cell lines but very weakly expressed (visible after long exposure; not shown) in normal lymphocytes (**Figure 1B**, in red). Our data suggest that GSK3 proteins are indeed overexpressed in most B- and T-lymphoma cell lines. However, the levels of mRNA and proteins of a given cell line are not always in perfect agreement. This is not surprising since the steady state levels of protein and mRNA are determined by many factors that govern not only their production, but also their stability.

Since phosphorylation of GSK3α at Serine 21 and GSK3β at Serine 9 position each render the protein functionally inactive, we wondered if GSK3α and GSK3β are phosphorylated at these sites in lymphoma cells. By Western blotting, both proteins were variably phosphorylated across our lymphoma line panel similar to normal lymphocytes. We did not see any unique pattern that is specific to lymphoma lines (**Supplemental Figure 1**) suggesting the baseline phosphorylation of GSK3 is unlikely significant in lymphoma. Taken together, our

results suggest that both GSK3α and GSK3β are indeed abundantly expressed in most lymphoma cell lines compared to normal lymphocytes, supporting the potential for targeting GSK3 for therapeutic benefit.

GSK3α and GSK3β are functionally important in lymphoma cells.

Given that both GSK3α and GSK3β are overexpressed in lymphoma cells, and that both enzymes are implicated in multiple signaling pathways critical for cell functions, we examined whether GSK3α and GSK3β are functionally supporting the survival and proliferation of lymphoma cells. To that end, we took two complementary approaches – the first utilized a new GSK3 inhibitor 9-ING-41 and the second used knockout of *GSK3A* and *GSK3B* genes using CRISPR/CAS9. Treatment of TCL and MCL lines with low doses of 9-ING-41 for 48 hours induced apoptosis (**Figure 2A**); the DLBCL lines required higher concentrations (**Figure 2B**). In contrast, we did not detect significant apoptosis in purified normal unstimulated T lymphocytes or peripheral blood mononuclear cells even at a concentration of 10.0 μM of 9-ING-41. We further calculated the inhibitory concentrations of 9-ING-41 at half of the maximal effect (IC_{50}) on cell survival of various lymphoma cell lines (**Table 1)**. Our data indicate that 9-ING-41 can specifically induce lymphoma cell apoptosis without affecting normal lymphocytes.

To test the role of GSK3 in lymphoma cell proliferation, we performed the thymidine incorporation assay in the presence or absence of 9-ING-41. The proliferation rate of all TCL and MCL lines was profoundly inhibited in the presence of 9-ING-41 concentrations as low as 1.0 μM; the DLBCL lines required slightly higher concentrations. We also calculated the IC_{50} of 9-ING-41 on cell proliferation for various lymphoma cell lines (**Table 1)**. Our data suggest that GSK3 activity is indeed important for the proliferation and survival of lymphoma cells.

In a second approach, we used CRISPR/CAS9 knockout technique to genetically delete *GSK3A* and *GSK3B* genes. After transient expression of the construct carrying CAS9-T2A-GFP and gRNA specific *GSK3A* or *GSK3B* genes exon 1 sequences, GFP expressing single cells were sorted into 96-well plate by flow sorting. After 2-3 weeks in culture, single cell subclones carrying unique modification in *GSK3A, GSK3B,* or both genes were genotyped for the gene deletion and western blot verified for the protein depletion. As summarized in **Table 2**, several *GSK3A* null knockout subclones were readily obtained from all 5 cell lines tested, and *GSK3B* null subclones were also obtained from Ly-1 cell lines. However, after analyzing 24-36 single cell subclones from Ly-19, Jeko, Mino, and Karpas 299 cell lines, no knockout subclones were detected; i.e., all survived clones were either wildtype or carried heterozygous mutations suggesting *GSK3B* null cells from those cell lines likely died during culture. Given that CRISPR/CAS9 is a highly efficient approach for biallelic deletion of *GSK3B* gene in Ly-1 cells (19/24, 79%) but no *GSK3B* null clones in any other lymphoma lines tested (0/24, 0/24, 0/36, 0/26, 0%), our data support the notion *GSK3B* is necessary for the survival of lymphoma cells in these cell lines. Using shRNA knockdown we also observed similar results showing that *GSK3B* knockdown is lethal in several lymphoma lines except Ly-1 (data not shown). Interestingly, unlike other lines that all died following *GSK3B* knockout, we derived multiple Ly-1 subclones with homozygous deletion of *GSK3A, GSK3B*, or both genes. Thus, Ly-1, a GCB-type DLBCL cell line, likely possesses a unique, unexplained compensatory mechanism(s) that prevents the cells from dying upon *GSK3B* deletion. Taken together, we have shown pharmacologically and genetically that *GSK3B* function is indeed necessary for the proliferation and survival of lymphoma cells. This suggests that targeting *GSK3B* has potential for lymphoma treatment.

GSK3 inhibition blocks G2/M progression in lymphoma cells.

Our finding that 9-ING-41 strongly inhibits proliferation *in vitro* prompted us to examine the effect of 9-ING-41 on lymphoma cell cycle kinetics. After 9-ING-41 treatment, we consistently observed cell cycle blockage at G2/M after as little as 24 hours of treatment in all lines tested (**Figure 3A**), suggesting that GSK3 activity is required for successful progression of mitosis. To further determine whether this G2/M arrest specifically resulted from GSK3β inhibition, we examined the cell cycle profile of parental (wildtype), *GSK3A*, *GSK3B*, and *GSK3A/B* knockout Ly-1 subclones. In the absence of treatment, both parental wildtype and *GSK3A*-null Ly-1 cells showed normal cell cycle profiles, while *GSK3B* and *GSK3A/B* knockout subclones exhibited increased cells in G2/M (**Figure 3B**). In addition, *GSK3A/B* double knockout subclones also showed increased levels of polyploid (>4N) cells, possibly due to defective mitosis. These cell cycle abnormalities of *GSK3B* -null and *GSK3A/B* -null Ly-1 cells are subtle and have little impact on the survival of the Ly-1 progeny, likely through Ly-1 cell line specific compensatory mechanisms. In summary, 9-ING-41 treatment phenocopies the effect of *GSK3B* single or *GSK3A/B* double deletion on cell cycle progression suggest that *GSK3B* is critical for lymphoma cell cycle G2/M progression, and 9-ING-41 is a potent cell cycle blocking agent for lymphoma cells.

GSK3 inhibition arrests lymphoma cells at the prophase stage of mitosis.

Although cells arrested in G2/M appear as a single DNA content (4N) peak on a flow cytometry histogram (**Figure 3A**), there are actually at least 5 sequential steps (M1-M5) in G2/M critical to successful cell division. These include prophase (M1, chromosome condensation, mitotic spindle formation starts), prometaphase (M2, nuclear membrane breakdown, centrosome polarization), metaphase (M3, chromosome pairs align middle plane), anaphase (M4, daughter

chromatids separate), telophase (M5, reformation of daughter nuclei), and the final cytokinesis (separation of two daughter cells).[17] Each of these discrete steps has a unique identifiable nuclear morphology on Wright's stained cells (depicted in **Figure 4A**). We examined the morphology of untreated and 9-ING-41 treated Jeko cells to determine at what stage they become arrested. As shown in **Figure 4B** (left panel), all M1-M5 mitotic steps were readily identified in untreated mitotic Jeko cells; however, in 9-ING-41 treated cells (right panel) a large fraction of cells showed the morphology of condensed chromosomes and reduced cytoplasmic staining resembling prophase (M1) cells without identifiable cells with M2-M5 morphology. By differential counting of 100 mitotic cells, we found all stages (M1-M5) of mitotic cells readily identifiable in untreated cells; only prophase (M1) cells were accounted for in 9-ING-41 treatment mitotic cells (**Figure 4C**). Similar results (data not shown) were observed in other lymphoma lines including DHL-6, Ly-3, Mino and Karpas 299. These observations support the conclusion that GSK3 activity is necessary for the progression of mitotic prophase.

GSK3β is localized to centrosomes and mitotic spindles of lymphoma cells.

Knowing that GSK3 inhibition leads to mitotic arrest at prophase, we next questioned the involvement of GSK3β in two key prophase events, centrosome polarization and mitotic spindle formation. We examined the subcellular localization of GSK3β protein in interphase Jeko or Ly-1 cells by immunofluorescence staining. During interphase, GSK3β is prominently localized in the nucleus and centrosome-like pair dots in the cytoplasm of Jeko cells (**Figure 5A-B**). To further demonstrate that those cytoplasmic pair dots were indeed centrosomes, we first co-stained wt Ly-1 cells for GSK3β protein and the centrosome marker pericentrin using an incomplete fixation protocol and found the cytoplasmic GSK3β dots were perfectly colocalized with pericentrin (**Figure 5D-5F**), suggesting these GSK3β bright dots (in green, **Figure 5A-C &**

5F) are indeed centrosomes. We further demonstrated that the anti-GSK3β antibody staining was specific to GSK3β protein by showing its absence in *GSK3B*-null Ly1 cells (**Figure 5G-J**). Therefore, we conclude that GSK3β is localized to centrosomes and the nucleus in interphase cells.

To determine the intracellular localization of GSK3β in mitotic cells, we analyzed GSK3β localization in normal mitotic Jeko cells. GSK3β staining (in green, **Figure 5K-L**) exhibited a "firework-like" pattern with polarized centrosomes in the middle and mitotic spindles or microtubules extending outwards. The staining is specific to GSK3β since such staining is absent in *GSK3B* deficient mitotic cells (**Figure 5O-P**). Using a separate stain for microtubule marker α-tubulin, we show that α-tubulin gives a staining pattern similar to that of GSK3β (in red, **Figure 5M-N**) suggesting that GSK3β is localized to microtubules during mitosis. These observations collectively suggest that GSK3β protein is specifically localized to centrosomes and mitotic spindles during mitosis. Next, we determined the localization of GSK3β in 9-ING-41 treated Jeko cells (**Figure 5Q-R**). We found similar firework-like GSK3β staining patterns in all prophase cells without any altered GSK3β localization. Taken together, these data indicate that GSK3β localized to centrosomes and nucleus during interphase (**Figure 5A-B**), and to centrosomes and mitotic microtubules during mitosis (**Figure 5K-L**). Treatment of 9-ING-41 did not alter the localization of GSK3β, nor affect centrosome polarization or microtubule formation; therefore, 9-ING-41 is likely to inhibit microtubule function at a later step preventing the progression of prophase.

GSK3 expression and targeting in primary cells from lymphoma patients.

Having demonstrated that 9-ING-41 potently inhibits lymphoma proliferation and survival in lymphoma cell lines, we wanted to learn if this remains true in patient samples. To

that end, we examined the expression of GSK3α and GSK3β proteins in primary lymphoma cells freshly isolated from patients with MCL, high grade B-cell lymphoma, follicular lymphoma grade 3B, DLBCL, or angioimmunoblastic TCL. All 5 samples had stronger expression of GSK3α and GSK3β proteins compared to normal blood B-cell controls (**Figure 6A**). These patient cells were similarly responsive to the antiproliferative effects of 9-ING-41 (**Figure 6B**). To complement our data on limited fresh patient samples, we further probed paraffin samples from patients of a different cohort with various lymphoma types for GSK3β protein expression by IHC. As shown in **Figure 6C**, we found GSK3β overexpression in all samples with variable intensity. Similar to our results, RNA-Seq analysis on primary DLBCL patient samples derived from a public database at Gene Expression Profile Interactive Analysis (http://gepia.cancer-pku.cn), also showed increased expression of GSK3α and GSK3β in lymphoma than normal lymphocytes.

Given our findings that GSK3α and GSK3β are overexpressed in lymphoma, we hypothesized the overexpression of either gene product may correlate with patients' clinical outcome. To that end, we analyzed recently published RNA-Seq data[18] on a cohort of 234 DLBCL patients with clinical survival data [median follow-up was 10.5 years (95% CI: 7.9-not reached)]. A receiver operating characteristics (ROC) curve analysis was performed to dichotomize the GSK3α and GSK3β expression to establish the optimal cutoffs (10.5 for GSK3α, and 8.6 for GSK3β) for high and low expression grouping. As shown in the supplemental Figure 2 that GSK3α high expression group (≥10.5, n=172) had an OS of 7.8 years (95% OS: 7.2-8.4) while to GSK3α low expression group (<10.5, n=62) had a significantly (p=0.03) higher OS of 8.9 years (95% OS: 8.2-10.1). Similarly, GSK3β high expression group (≥8.6, n=170) had an OS of 7.8 years (95% OS: 7.2-8.2) while to GSK3β low expression group (<8.6, n=62) had a

significantly (p=0.0005) higher OS of 9.7 years (95% OS: 8.6-11.5). The results suggest that overexpression GSK3α or GSK3β each correlates with poorer clinical outcome. In addition, the grouping data also show that the majority of DLBCL patients are segregated in high expression group for either GSK3α or GSK3β further validating our conclusion that GSK3α and GSK3β are generally overexpressed in lymphoma.

Targeting GSK3 in mouse xenografts of human lymphoma.

Given that GSK3 inhibitor 9-ING-41 effectively inhibits the proliferation and survival of lymphoma cells *in vitro*, we wanted to know if the inhibitor would also have anti-lymphoma activity *in vivo*. To that end, we established an MCL xenograft mouse model by subcutaneously injecting NGS mice with Jeko cells expressing the firefly luciferase reporter gene Fluc. In two independent experiments, eight and ten mice with engrafted tumors verified by imaging (typically 4 days after tumor inoculation) randomly served as controls or received 9-ING-41 treatment 40mg/kg every other day IP (**Figure 7A**). As shown in **Figure 7B**, the control (untreated) group mice had large tumors with marked luciferase activities by the day17; however, the 9-ING-41 treated mice had smaller tumors with much lower luciferase activity. Our data demonstrated that 9-ING-41 has single-agent anti-tumor activity in a mouse model of MCL.

Discussion

GSK3α and GSK3β are functionally related multifunctional serine/threonine protein kinases implicated in many signaling pathways that support the proliferation of lymphomas.[11] Glucose hypermetabolism is a hallmark of active lymphoma and GSK3 is a key regulator of glucose metabolism through the PI3K/mTOR and several TRAF3-regulated pathways. Cellular GSK3 status also influences the effects of other kinase inhibitors,[19] making GSK3 an attractive target for lymphoma therapeutics.

Herein we show that both GSK3α and GSK3β are overexpressed in lymphoma subtypes considered to be glucose-avid. GSK3 activity can be inhibited *in vitro* with a novel GSK3 inhibitor 9-ING-41, or by genetic deletion of *GSK3α* and *GSK3β* using CRISPR/CAS9. Each approach resulted in effective killing of lymphoma cells. One mechanism of this toxicity that we identified is the requirement for GSK3β in mitotic spindle function. 9-ING-41 treatment induced cell cycle arrest at prophase likely due to defective mitotic spindle function, preventing cell cycle progression to prometaphase. Our data demonstrate that GSK3 is critical for the proliferation and survival of lymphoma in general and MCL and TCL in particular. Karmali et al have also recently reported that 9-ING-41 treatment *in vitro* can induce apoptosis in a set of lymphoma cell lines different than the ones we studied.[20] Their studies, in addition to our mechanistic insights into the roles of GSK3 in lymphoma biology, provide rationale for studies in patients with lymphoma.

GSK3α and GSK3β have been reported to be overexpressed in various solid tumors[21-34] and GSK3β overexpression correlates with an inferior prognosis in endometrial cancer.[35] Increased intracellular pools of GSK3β have been found in pancreatic cancer cells[36] and nuclear accumulation of GSK3β is associated with poor cell differentiation.[31] Consistent with our

findings, Pérez-Benavente et al reported that the transcription factor and proto-oncogene JunB is strongly upregulated in anaplastic lymphoma kinase (ALK)-positive anaplastic large cell TCL (ALCL), and its phosphorylation is mediated by GSK3β.[37] Therefore, it is conceivable that these enzymes may play different roles in different cancers.

There have been other reports regarding the association of GSK3 with centrosomes. Mbom et al reported that both GSK3β and NEK2 phosphorylate β-catenin, also localized to mitotic centrosomes.[38] GSK3 reduces the stability of Securin, an important regulator of mitosis, by phosphorylating and consequent ubiquitination of the protein.[39] Lee et al showed that GSK3β physically binds to and phosphorylates hBora, another important mitotic factor, critical for Aurora A kinase to activate entry into mitosis.[40] We describe here that GSK3β binds to centrosomes and mitotic spindles, two key features of mitotic prophase, and 9-ING-41 treatment leads to cell cycle arrest at mitotic prophase. The exact molecular mechanism of this cell cycle arrest requires further exploration. Furthermore, the 9-ING-41 effects likely extend well beyond the cell cycle arrest. Indeed, it is well known that other signaling pathways involving GSK3 including Notch[41], Wnt[42], and Hedgehog[43] pathways are critical in many lymphoma subtypes, and key components in these pathways are frequently mutated or aberrantly expressed. Our RNA-Seq data on GSK3-deficient Ly-1 cell clones revealed significantly reduced expression of components of many pathways including glycolysis (data not shown) confirming our initial hypothesis that the multifaceted GSK3 is a promising new multifunctional lymphoma target.

In addition to its role in the proliferation and survival of tumor cells, inhibition of GSK3 has recently been implicated in modulating the function of CD8 cytotoxic T cells through the downregulation of PD-1 expression[43] and the function of NK cells[44] in mouse models. Taylor et

al has recently further shown that the GSK3 inhibitor SB415286 could effectively suppress the growth of mouse EV4 lymphoma xenografts in vivo by reactivation of CD8 T-cells.[45]

To our knowledge, we are the first to comprehensively demonstrate that not only lymphoma cell lines but also primary patient samples are sensitive to GSK3 inhibition and to describe potential mechanisms of that inhibition. Our data not only demonstrate the relevance of targeting GSK3 but also the potential for the new GSK3 inhibitor 9-ING-41 to be used in lymphoma therapy. The comparison of 9-ING-41 treatment, and GSK3 knockout support the notion that 9-ING-41 specifically targets predominantly GSK3. GSK3 was one of the first protein serine/threonine kinases discovered[46] and although there are many different GSK3 inhibitors available,[47,48] none are approved for cancer. Only lithium carbonate is FDA approved for the indication of bipolar disorder.[49] Since lithium is neither specific nor particularly potent in inhibiting GSK3 activity, it is unlikely to be an effective anti-lymphoma drug. Given our data showing the direct role of GSK3 on tumor proliferation and survival and its possible role in rejuvenating tumor-killing T cells and NK cells, it appears that GSK3 inhibitors such as 9-ING-41 may constitute a unique class of drug that could target lymphoma and other cancers through several parallel mechanisms. Our data along with others[9] lay the groundwork for the phase 1 trial of 9-ING-41 (NCT03678883) which has activated.

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Authorship

Contribution: XW, TW designed the experiments, analyzed and interpreted results, and wrote the manuscript; XW, MS, JL, KN, LZ, conducted experiments, analyzed data; JA, YL, JK, KW analyzed data and generated figures; AN, SA, KWP, GB, DB, FG, DS discussed and interpreted results, read and endorsed the manuscript.

Conflict-of-interest Disclosure

XW, MS, KN, JL, JA, YL, LZ, KWP, AN, SA, JK, KW, GB, TW: none; FG, DS, are employees and stockholders of Actuate Therapeutics Inc.; DB is a member of Actuate Therapeutics Scientific Advisory Board and is a stockholder.

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Table 1. 9-ING-41 inhibitory concentrations at 50% of the maximum (IC_{50}) on cell survival and cell proliferation in various lymphoma cell lines.

	Lymphoma line	IC_{50} (μM) on Cell survival	IC_{50} (μM) on Cell proliferation
DLBCL	OCI-LY1	3.05	0.69
	OCI-LY19	2.21	1.96
	OCI-Ly3	3.76	1.03
	SU-DHL6	1.58	0.84
MCL	Granta-519	0.77	0.38
	Jeko	1.28	0.94
	Mino	0.55	0.72
TCL	Karpas-299	3.34	0.26
	MyLa	0.69	0.19
	SeAx	1.60	0.38

Table 2. Effect of GSK3α and/or GSK3β knockout using CRISPR/Cas9 approach on the survival of various lymphoma cell lines

Lymphoma line	Null knockout clones/total clones screened		
	GSK3α	GSK3β	GSK3α/β
OCI-LY1	9/17	19/24	6/13
OCI-LY19	20/28	0/24	nd
Jeko	7/12	0/24	nd
Mino	5/12	0/36	nd
Karpas299	12/20	0/26	nd

nd: not done

Figure Legend

Figure 1. GSK3α and GSK3β mRNA and proteins are overexpressed in lymphomas (A) Real-time PCR quantitation showing GSK3α and GSK3β mRNAs are overexpressed in lymphoma lines in comparison to low expression in normal B or T lymphocytes. (B) Western blot images demonstrating GSK3α and GSK3β proteins are also abundantly expressed in various lymphoma lines in comparison to purified normal B or T lymphocytes.

Figure 2. GSK3 is essential for lymphoma cell proliferation and survival. Unstimulated peripheral blood B- and T- lymphocytes isolated from a healthy donor were used as normal control. Pro-apoptotic effect of the GSK3 inhibitor 9-ING-41 in various MCL and TCL lines **(A)** and DLBCL lines **(B). (C)** Cell proliferation profile of various lymphoma cell lines upon treatment with 9-ING-41. Results **(A-C)** are from 3 independent experiments.

Figure 3. GSK3 inhibition or deletion in lymphoma cells leads to cell cycle arrest in G2/M. **(A)** Cell cycle profile of 3 representative cell lines Jeko, Mino, and OCI-Ly3 after 24 hour treatment with 0, 1.0 and 2.0 μM 9-ING-41. **(B)** Cell cycle profiles of parental Ly-1 cells and GSK3α, GSK3β, GSK3αβ knockout subclones. Inset: Western blot image showing the depletion of GSK3α and GSK3β protein in the knockout Ly-1 subclones.

Figure 4. Inhibition of GSK3 by 9-ING-41 leads to mitotic prophase arrest. (A) Cartoon depiction of the sequential steps (M1-M5) during mitosis (purchased from Shutterstock and modified). **(B).** Representative Wright stain images of Jeko cells untreated and treated with 1.0

uM 9-ING-41 for 24 hours. Various mitotic stage cells (M1-M5) are readily identified in untreated cells (left panel) while only prophase (M1) cells in large number are seen in 9-ING-41 cells (right panel). **(C).** Bar chart showing the number of mitotic M1-M5 cells identified when 100 untreated or 9-ING-41 treated Jeko cells were counted. Similar results (data not shown) were observed in at least 4 different lymphoma cell lines.

Figure 5. GSK3β localized to centrosomes. (A) An immunofluorescence image showing GSK3β is localized to the nucleus and centrosome pairs in interphase Jeko cells. **(B)** A close-up (magnification) image of that shown in **(A)**. **(C-F)** single or multi-channel images of co-immunostaining of GSK3β and pericentrin in wild-type Ly-1 cells showing their colocalization to the centrosomes. **(G-H)** single or multi-channel images of co-immunostaining of GSK3β and pericentrin in GSK3β null in Ly-1 cells showing the staining is specific to GSK3β. **(K)** An immunofluorescence image showing GSK3β (green) localized to firework like structures resembling to mitotic spindles in mitotic Jeko cells. **(L)** A close-up image of a mitotic cell shown in **(K)**. **(M)** An overlay image showing the microtubule structure by α-tubulin (red) staining and DNA (in blue). **(N)** A close-up image of that shown in **(M)**. **(O-P)** Images showing that spindle structure staining of GSK3β is absent in GSK3β null Ly-1 cells. **(Q)** An immunofluorescence image showing GSK3β localized to mitotic spindle structure and polarized centrosomes in 9-ING-41 treated Jeko cells. **(R)** A close-up image of a representative mitotic cell shown in **(Q)**.

Figure 6. Aberrant expression of GSK3 proteins in primary lymphoma patient (P) cells and their proliferative response to 9-ING-41. (A) Immunoblot of GSK3α and GSK3β proteins showing overexpression in patient samples vs normal B-cell control. P1: MCL; P2: High grade

B-cell lymphoma; P3: follicular large B-cell lymphoma 3B; P4: DLBCL; P5: angioimmunoblastic T-cell lymphoma. **(B)** 9-ING-41 inhibited proliferation in all 5 patient samples. **(C)** Immunohistochemistry staining of GSK3β on paraffin tissue sections of patients with various lymphoma. Representative images show the spectrum of GSK3β (in brown) overexpression in different lymphoma samples. Methylene blue counterstaining (in blue) shows cells negative for GSK3β in the background and in antibody negative control panel. Images were collected under 40x magnification.

Figure 7. Anti-lymphoma effect of 9-ING-41 *in vivo* in Jeko derived xenograft mouse model. **(A)** Experimental design showing 9-ING-41 treatment schedule and dosage. **(B)** Bioluminescence images of xenograft bearing mice untreated or treated with 9-ING-41. The images shown were collected at the end of the experiment (day 17). The experiment was done twice both showed similar results.

Figure 1.

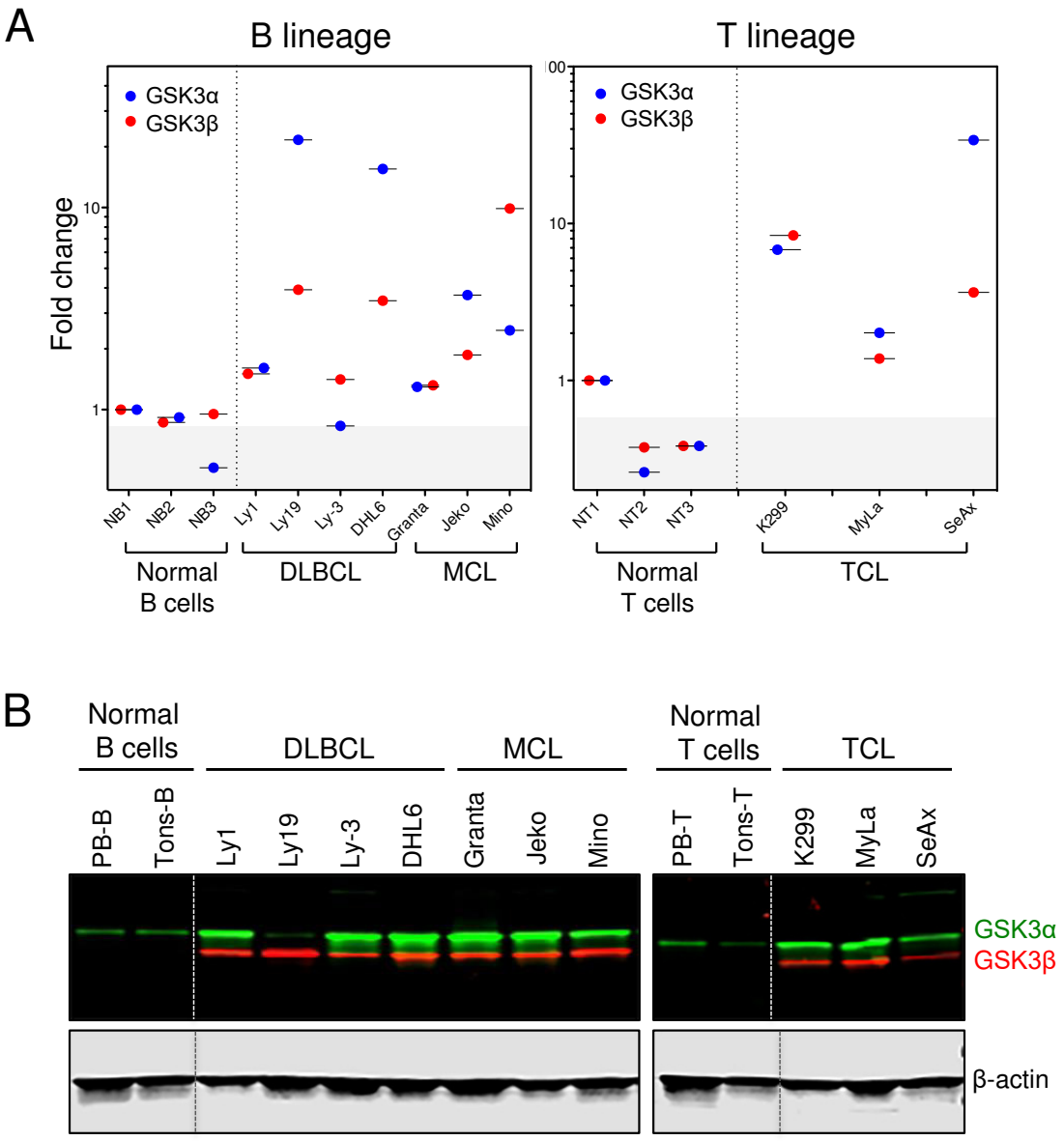
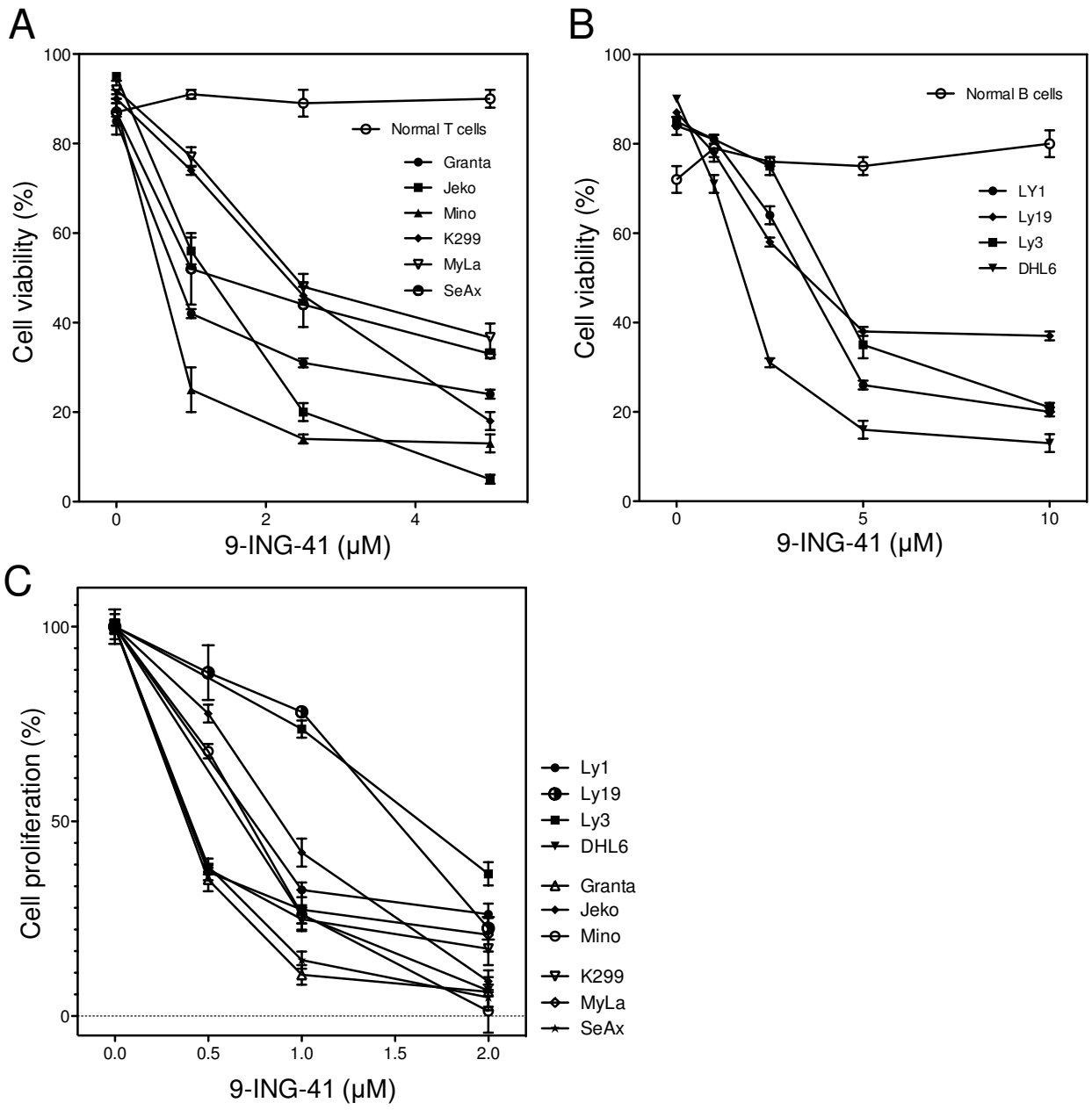


Figure 2



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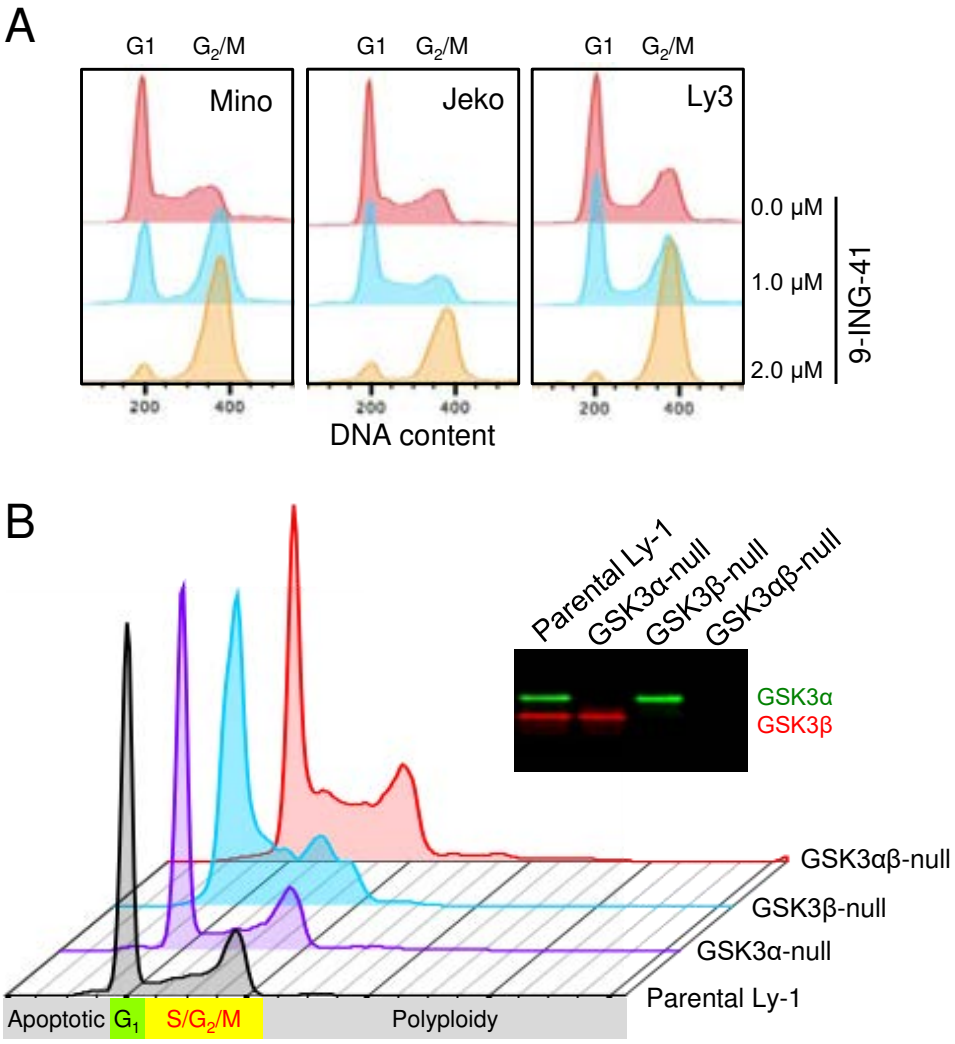


Figure 4

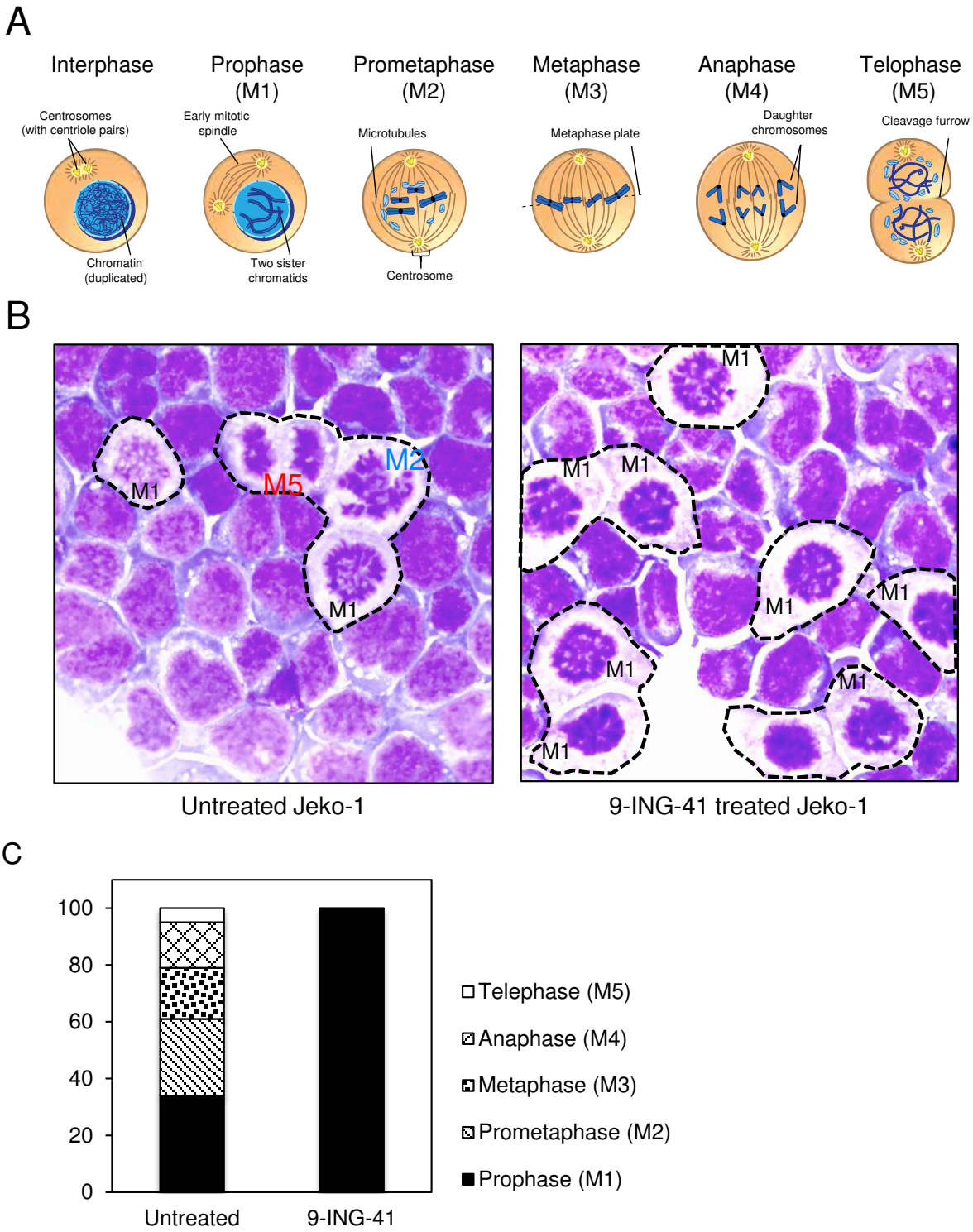


Figure 5

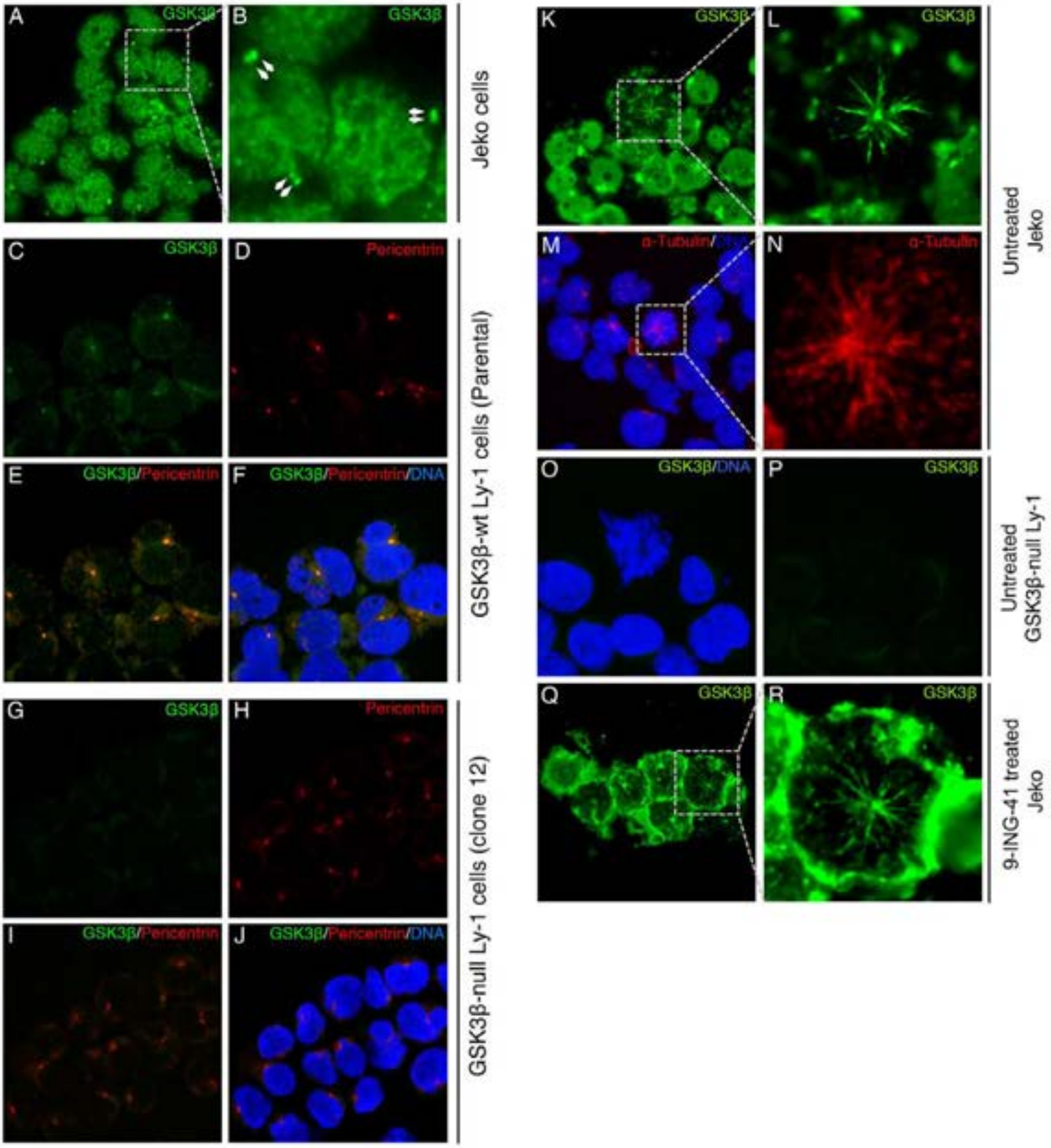


Figure 6

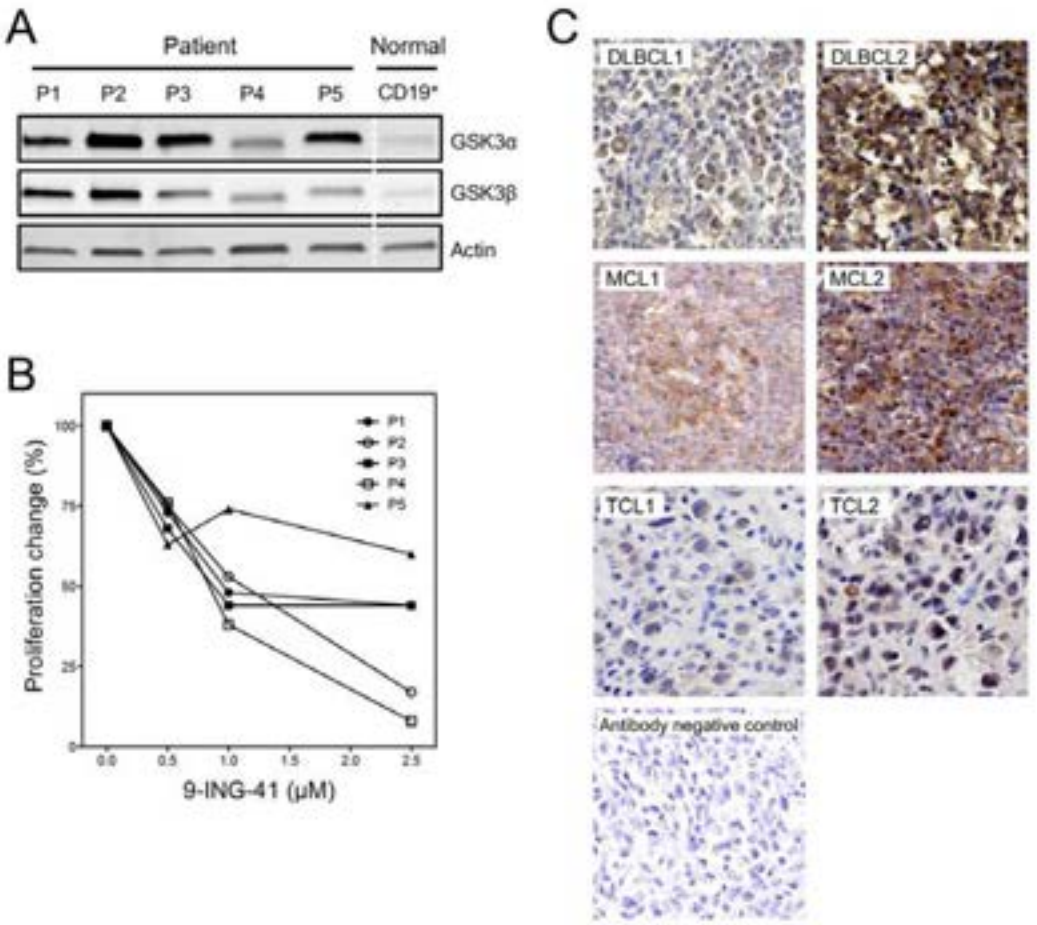
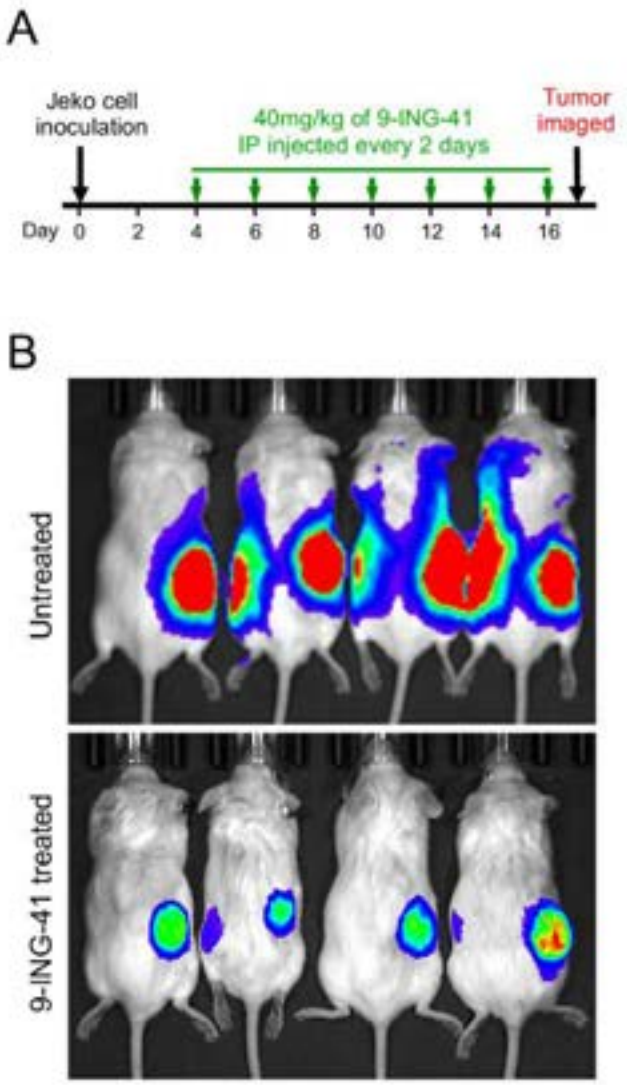


Figure 7



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Targeting glycogen synthase kinase 3 for therapeutic benefit in lymphoma

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