Vol 455|30 October 2008|doi:10.1038/nature07284

Glycogen synthase kinase 3 in *MLL* leukaemia maintenance and targeted therapy

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Glycogen synthase kinase 3 (GSK3) is a multifunctional serine/threonine kinase that participates in numerous signalling pathways involved in diverse physiological processes. Several of these pathways are implicated in disease pathogenesis, which has prompted efforts to develop GSK3-specific inhibitors for therapeutic applications. However, before now, there has been no strong rationale for targeting GSK3 in malignancies. Here we report pharmacological, physiological and genetic studies that demonstrate an oncogenic requirement for GSK3 in the maintenance of a specific subtype of poor prognosis human leukaemia, genetically defined by mutations of the *MLL* proto-oncogene. In contrast to its previously characterized roles in suppression of neoplasia-associated signalling pathways, GSK3 paradoxically supports *MLL* leukaemia cell proliferation and transformation by a mechanism that ultimately involves destabilization of the cyclin-dependent kinase inhibitor p27$^{\mathrm{Kip1}}$. Inhibition of GSK3 in a preclinical murine model of *MLL* leukaemia provides promising evidence of efficacy and earmarks GSK3 as a candidate cancer drug target.

GSK3 is a serine/threonine kinase that functions in numerous signalling pathways initiated by diverse stimuli[1]. Originally studied for its role in glycogen metabolism and insulin action, GSK3 has subsequently been shown to have central functions in many cellular and physiological processes including transcription, cell cycle division, apoptosis, cell fate determination and stem cell maintenance, among others[1–3]. GSK3 is constitutively active in resting cells, showing a preference for primed substrates[4], and is functionally inactivated after phosphorylation by various kinases in response to different stimuli[3,5]. Given its various contributions and the diversity of putative substrates, many levels of regulation help confer GSK3 signalling specificity, which varies among cell types and their states of differentiation.

GSK3 functions in several pathways implicated in human diseases, which has prompted efforts to develop specific inhibitors for therapeutic applications. GSK3 facilitates non-insulin-dependent diabetes by the inactivation of glycogen synthase[3,6], and may have a role in promoting various inflammatory processes through the activation of the transcription factor nuclear factor-κB by, at present, undefined mechanisms[7,8]. GSK3-mediated hyperphosphorylation of tau (also known as MAPT), a component of neurofibrillary tangles, may facilitate Alzheimer's disease and other neurodegenerative disorders[9]. In cancer cells, however, signalling pathways that are normally suppressed by GSK3—such as Wnt and Hedgehog, which are involved in embryonic cell fate determination and normal stem cell maintenance—are aberrantly activated[10–13]. This underscores the normal role of GSK3 in mediating phosphorylation of substrates such as β-catenin (Wnt signalling), MYCN (Hedgehog signalling) and JUN, which leads to their destruction and/or inactivation, thus inhibiting signals that otherwise promote proliferation and self-renewal[14–16] (Supplementary Fig. 1). Consistent with these molecular functions, GSK3 inhibition significantly enhances maintenance of embryonic stem cell pluripotency and haematopoietic stem cell repopulation after bone marrow transplantation[17,18], although the specific pathways for these effects remain undefined. Despite its inhibitory roles in pathways implicated in cancer pathogenesis, there has so far been no compelling rationale for the targeting of GSK3 as a therapeutic approach in malignancies. Here we demonstrate a paradoxical and unexpected role for GSK3 in cancer maintenance, and we establish GSK3 as a potential selective therapeutic target in a genetically distinctive and poor prognosis subset of acute leukaemia.

GSK3 inhibition induces G1 arrest of *MLL* leukaemia cells

A small-scale screen was conducted to identify compounds that specifically blocked the growth of genetically defined subsets of leukaemia cells. Thirty compounds (Supplementary Table 1) that target principal kinases or other enzymes were screened for differential dose-responses in various cell lines (Supplementary Table 2). These cell lines represent human leukaemias harbouring a variety of chromosomal translocations that create distinctive chimaeric fusion proteins implicated in disease pathogenesis. The leukaemia cell lines were comparably sensitive to most of the tested compounds (data not shown). However, cell lines that expressed *MLL–AF4* or *MLL–AF5*, the highly related fusion oncogenes created by t(4;11) or t(5;11) chromosomal translocations, respectively, showed enhanced sensitivity to GSK3-IX, a GSK3 inhibitor that also targets cyclin-dependent kinases (CDKs; Fig. 1a; for clarity, only two representative control cell lines are shown). Their proliferation was inhibited at a half-maximal inhibitory concentration (IC_{50}) of 0.3–2 μM, a concentration range comparable to that which promotes expansion of haematopoietic stem cells *in vitro*[18], but tenfold lower than the toxicity levels for non-*MLL* leukaemia cell lines (Fig. 1a) and normal bone marrow progenitors (see later). In contrast, the CDK inhibitors roscovitine (Fig. 1a), flavopiridol and olomoucine (data not shown) had similar IC_{50} values for all cell lines, suggesting that the inhibitory effects of GSK3-IX on *MLL* cell lines resulted from GSK3, not CDK, blockade. Further studies with SB216763 (a widely used maleimide-containing GSK3 inhibitor with a relatively higher IC_{50} than GSK3-IX) and with alsterpaullone (which has a similar inhibition profile as GSK3-IX)

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confirmed that *MLL* leukaemia cells were differentially sensitive to GSK3 inhibition (Fig. 1b and data not shown). Increased β-catenin levels correlated with effective GSK3 inhibition, which did not alter MLL oncoprotein abundance or function (Supplementary Fig. 2a, b).

Cell cycle analyses showed a marked reduction in G1–S phase progression of *MLL* leukaemia cells after 24 h of inhibitor treatment, whereas non-*MLL* leukaemia cells were only minimally affected (Fig. 1c). More prolonged incubation with inhibitor (6 days) was associated with cell death, as evidenced by a substantial increase in sub-G0/G1 DNA content (Supplementary Fig. 3a, b). These data suggest that GSK3, which is constitutively active in normal resting cells, paradoxically supports the proliferation and sustained survival of a genetically defined subset of leukaemia.

GSK3 dependence is a general feature of *MLL* transformed cells

Murine transformation models were used to characterize the role of GSK3 in *MLL* leukaemia further. Transduction of *MLL* oncogenes into primary murine myeloid progenitors induces aberrant *Hox* gene expression[19,20], leading to enhanced self-renewal *in vitro* and acute myeloid leukaemias *in vivo* that accurately model the features of human *MLL* leukaemia[21] without altering GSK3 levels or activity (Supplementary Fig. 3c, d). Culture of *MLL*-transduced myeloid progenitors with a GSK3 inhibitor reduced their clonogenic potentials and proliferation (Fig. 2a). This contrasted with progenitors immortalized by other fusion oncogenes (Fig. 2a and data not shown), which showed no adverse growth effects with 10 μM SB216763 treatment, as was also the case for primary myeloid progenitors (Supplementary Fig. 4). Inhibition of GSK3 primarily resulted in proliferative arrest of *MLL* transformed cells, but prolonged exposure induced morphological features of myeloid differentiation (Fig. 2b, c) and reduced expression of c-Kit (also known as KIT) (data not shown), a phenotypic marker of normal progenitors and *MLL* leukaemia stem cells[22]. Mouse B cell progenitors transformed by *MLL–AF4*, but not by other oncogenes, also showed markedly reduced proliferation in 10 μM SB216763 (Fig. 2d). These data suggest that GSK3 dependence may be a primary consequence and general feature of *MLL* transformation in several haematopoietic lineages. Furthermore, expression of a constitutively active mutant of the protein kinase AKT, which phosphorylates GSK3 and negatively regulates its kinase activity, resulted in suppression of cell growth and clonogenic potentials of mouse myeloid and B cell progenitors transformed by *MLL* oncogenes (Supplementary Fig. 5), providing support that *MLL* transformed cells are dependent on GSK3 for continued proliferation and maintenance of their transformed phenotypes *in vitro*.

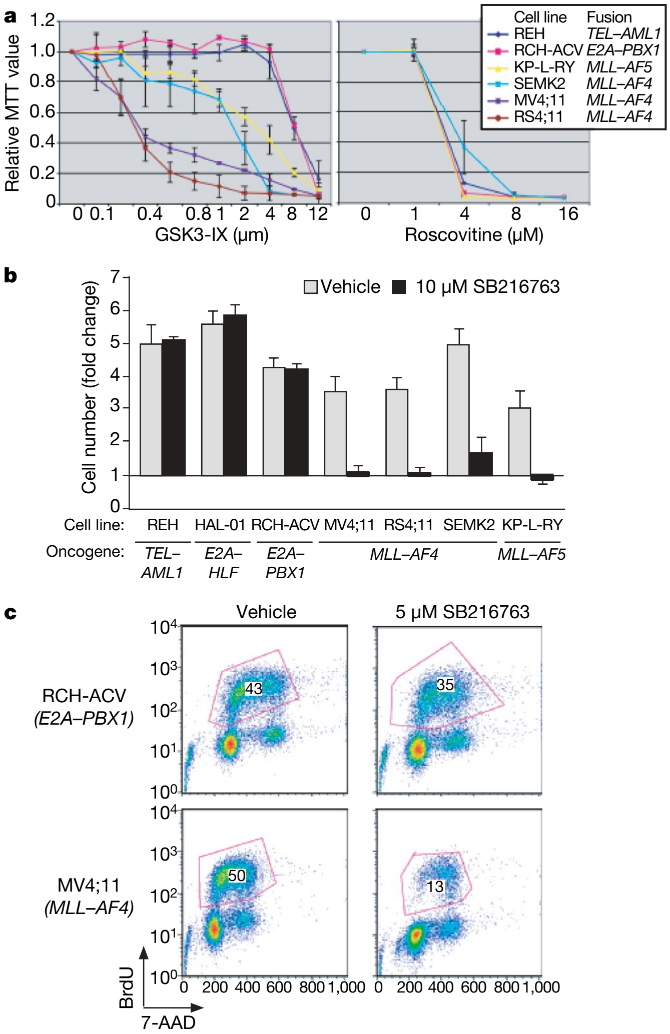

Figure 1 | Sensitivity of *MLL* leukaemia cell lines to GSK3 inhibition. a, The growth of human leukaemia cell lines was assessed after 3 days culture in the presence of the indicated concentrations of GSK3-IX (left panel) or roscovitine (right panel). The results are expressed as the cell numbers relative to those without drug treatment, and represent the mean of three independent experiments (± s.e.m.). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. **b**, Human leukaemia cell lines were cultured in the absence or presence of 10 μM SB216763 for 2 days. The results of a representative experiment are expressed as the fold change in cell number compared to day 0 (± s.e.m., $n = 3$). **c**, Human leukaemia cell lines were cultured in the presence of 5 μM SB216763 for 24 h, and BrdU incorporation was quantified by fluorescence-activated cell sorting (FACS) analysis.

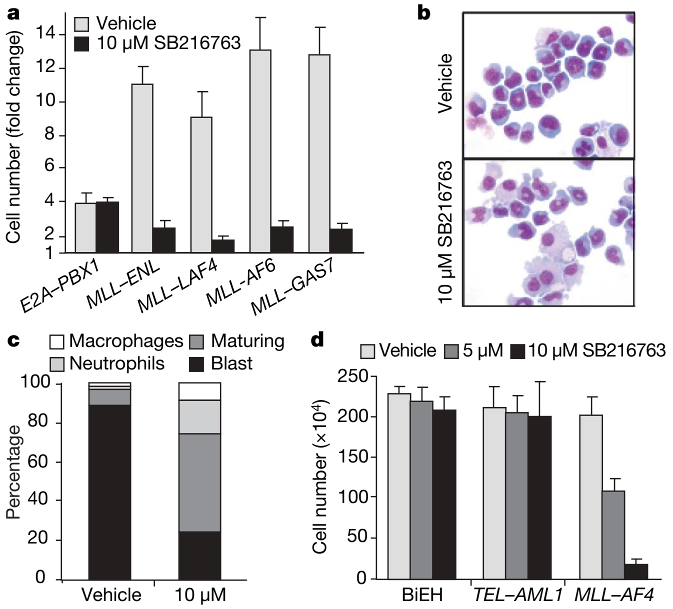

Figure 2 | Sensitivity of *MLL*-transformed mouse B and myeloid progenitors to GSK3 inhibition. a, The growth of myeloid progenitors transformed by various oncogenes was assessed after 3 days of culture in the presence or absence of a GSK3 inhibitor. The results of a representative experiment are expressed as the fold change in cell number compared to day 0 (± s.e.m., $n = 3$). **b**, **c**, The morphological features of *MLL–ENL* transformed myeloid progenitors were assessed after 4 days of culture in the presence or absence of GSK3 inhibitor. Original magnification, ×40. The bar graph (**c**) indicates the mean number of cells with the indicated morphological features ($n = 3$). **d**, The growth of B lymphoid progenitors transformed by *E2A–HLF* and *BCL2* (BiEH)[37], *TEL–AML1* or *MLL–AF4* oncogenes was assessed after 3 days of culture in the absence or presence (5 μM or 10 μM) of SB216763. The results of a representative experiment are expressed as the fold change in cell number compared to day 0 (± s.e.m., $n = 3$).

GSK3α/β isoforms cooperatively maintain *MLL* transformation

To investigate further the GSK3 requirement, myeloid progenitors were isolated from fetal livers of *Gsk3b*$^{-/-}$ mice (embryonic day (E)16 embryos), transduced with retroviral vectors encoding *MLL* or unrelated oncogenes (Fig. 3a), and then serially replated in methylcellulose culture to assess their self-renewal properties. *Gsk3b*$^{-/-}$ cells were capable of sustaining the enhanced self-renewal typically induced by *MLL* oncogenes, and did not show reduced clonogenic potentials compared with wild-type cells transduced with the same *MLL* oncogenes (Supplementary Fig. 6) despite a 50% reduction in overall GSK3 activity levels (Supplementary Fig. 7d). Thus, GSK3-β was not required to initiate *MLL* transformation *in vitro*. However, *MLL*-transformed *Gsk3b*$^{-/-}$ cells showed markedly increased sensitivity to pharmacological GSK3 inhibition (Fig. 3b, c), which was reversed by the forced expression of exogenous GSK3-β (Fig. 3d). In contrast, *Gsk3b*$^{-/-}$ cells transformed by other fusion oncogenes (Fig. 3b and data not shown) were unaffected by a several fold higher concentration of inhibitor. Thus, genetic reduction of GSK3-β levels, by knockout or short-hairpin-RNA-mediated knockdown (Supplementary Fig. 7a), in *MLL*-transformed myeloid progenitors resulted in increased sensitivity to pharmacological GSK3 inhibition.

Persistence of the transformed phenotype but enhanced inhibitor sensitivity in the absence of GSK3-β suggested that the two GSK3 isoforms probably have redundant roles in *MLL* transformation. Thus, GSK3-α knockdown studies were performed in myeloid progenitors, which resulted in efficient reduction of GSK3-α protein levels in wild-type as well as *Gsk3b*$^{-/-}$ cells, (Fig. 4a) accompanied by further decrease in total GSK3 activity to less than 20% of wild-type cells (Supplementary Fig. 7d). Unlike GSK3-β knockout or knockdown cells, *MLL*-transformed cells deficient for GSK3-α (*Gsk3a*^{KD}) did not show differences in growth or heightened sensitivity to GSK3 inhibitors (Supplementary Fig. 7a, b). However, myeloid progenitors deficient for both GSK3 isoforms (*Gsk3b*$^{-/-}$ *Gsk3a*^{KD}) showed a marked impairment in clonogenicity and proliferation compared to wild-type cells transformed by *MLL* oncogenes (Fig. 4b, c), and were unable to sustain long-term growth in culture. In contrast, the growth of cells transformed by other leukaemia oncogenes was unaffected by the compound deficiency of both GSK3 isoforms (Fig. 4b, c) despite substantially decreased GSK3 activity (Supplementary Fig. 7d). *Gsk3b*$^{-/-}$ *Gsk3a*^{KD} cells transduced with *MLL* oncogenes also had a more differentiated myeloid morphology (Fig. 4d) and phenotype (not shown). Notably, *MLL–ENL* transformed cells lacking both GSK3 isoforms were unable to induce leukaemia in transplanted mice (Fig. 4e). Thus, GSK3 isoforms cooperatively maintain critical features of the *MLL* transformed phenotype, although GSK3-β serves a predominant role.

Efficacy of GSK3 inhibition in a mouse model of *MLL* leukaemia

A mouse model of *MLL*-associated leukaemia was used to assess whether GSK3 inhibition *in vivo* would affect the course of disease.

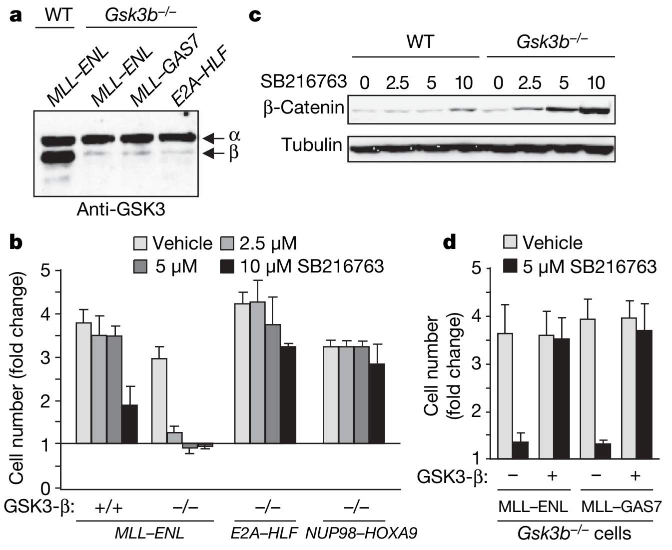

Figure 3 | Genetic ablation of GSK3-β hypersensitizes *MLL*-transformed cells to pharmacological GSK3 inhibition. **a**, Western blot analysis demonstrates the amounts of GSK3 protein isoforms in wild type or *Gsk3b*$^{-/-}$ myeloid progenitors transformed by the indicated oncogenes. **b**, Wild type (+/+) or *Gsk3b*$^{-/-}$ (−/−) myeloid progenitors transformed by various oncogenes were incubated in the presence of the indicated concentrations of SB216763. Cell numbers were enumerated on day 2 and expressed as the fold change compared to day 0 (± s.e.m. of triplicate analyses). **c**, Western blot analysis demonstrates the relative amounts of β-catenin after treatment with the indicated concentrations (μM) of inhibitor in wild type (WT) or *Gsk3b*$^{-/-}$ myeloid progenitors transformed by *MLL–ENL*. **d**, *Gsk3b*$^{-/-}$ myeloid progenitors transformed by *MLL* oncogenes were stably transduced with Flag–GSK3-β (+) or vector (−), and then incubated in the presence or absence of 5 μM SB216763. Cell numbers were enumerated on day 2 and expressed as the fold change compared to day 0 (± s.e.m. of triplicate analyses).

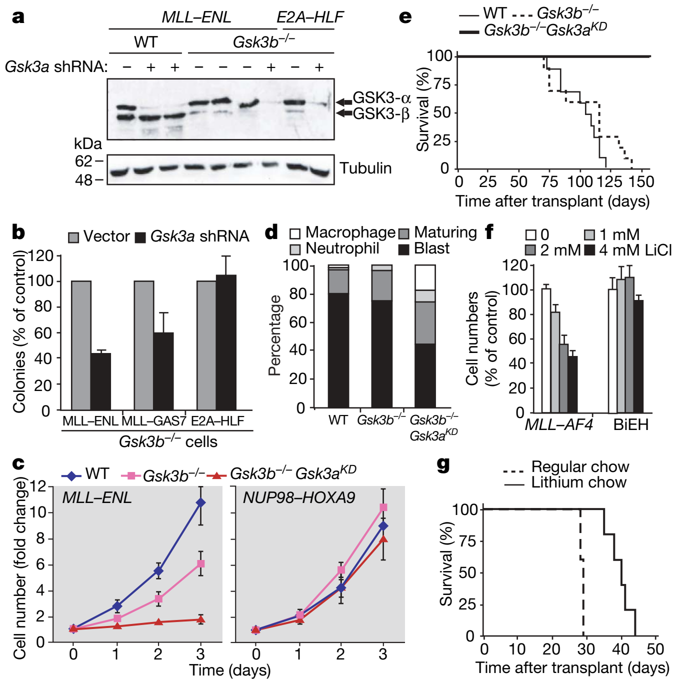

Figure 4 | Compound genetic deficiency of GSK3-α and GSK3-β impairs the growth and leukemogenicity of *MLL*-transformed cells. **a**, Western blot analysis was performed on wild type (WT) and *Gsk3b*$^{-/-}$ myeloid progenitors transformed by the indicated oncogenes (top) and transduced by lentiviral vectors lacking (−) or expressing (+) *Gsk3a* shRNA . **b**, Myeloid progenitors transformed by the indicated oncogenes were acutely transduced with lentiviral vectors lacking or expressing *Gsk3a* shRNA and then plated in methylcellulose medium. Colonies were enumerated after 5 days, and the mean (± s.e.m.) numbers of three independent determinations are expressed relative to vector alone. **c**, Proliferation of myeloid progenitors (WT, *Gsk3b*$^{-/-}$ or *Gsk3b*$^{-/-}$ *Gsk3a*^{KD}) transformed by *MLL–ENL* (left panel) or *NUP98–HOXA9* (right panel) was assessed at the indicated days in liquid culture (± s.e.m. of triplicate analyses). **d**, The morphological features of *MLL–ENL* transformed myeloid progenitors with the indicated genotypes were assessed after 4 days of culture. The bar graph indicates the mean number of cells with the indicated morphological features ($n = 3$). **e**, Survival curves are shown for cohorts of mice transplanted with cells (WT, *Gsk3b*$^{-/-}$ or *Gsk3b*$^{-/-}$ *Gsk3a*^{KD}) stably transduced with *MLL–ENL* (10 mice each). **f**, Cell numbers were determined after 3 days of culture in the indicated concentrations of lithium chloride. **g**, Survival curves show significantly different latencies ($P < 0.001$) for the development of acute leukaemia in cohorts of mice transplanted with *MLL–AF4* leukaemia cells (5×10^4) and maintained on normal or lithium carbonate (0.4%) laced chow as indicated.

Mice transplanted with *MLL–AF4*-transformed B cell precursors developed a fatal aggressive leukaemia within 29 days, characterized by massive infiltration of the bone marrow, spleen and liver, with leukaemic blasts (data not shown). However, treatment with lithium carbonate, which has been extensively used to modulate *in vivo* GSK3 kinase activity[23] and impairs *MLL* leukaemia cell proliferation *in vitro* (Fig. 4f), resulted in a significant prolongation of survival (Fig. 4g and Supplementary Fig. 7e). These results indicate that sensitivity of *MLL*-transformed cells to GSK3 inhibition is not restricted to *in vitro* environments, and provide evidence of therapeutic efficacy.

p27$^{\text{Kip1}}$ mediates the response to GSK3 inhibition

Cell cycle arrest in response to GSK3 inhibition suggested that cell cycle regulators may be downstream targets of GSK3 signalling in *MLL*-transformed cells. Western blot analysis implicated the CDK inhibitor (CDKI) p27$^{\text{Kip1}}$ as the levels of this significantly increased in human *MLL* leukaemia cells (Fig. 5a) and murine transformed progenitors (Fig. 5b) after inhibitor treatment, which is temporally consistent with the onset of cell cycle arrest (Fig. 1c and Supplementary Fig. 3a). Conversely, p27$^{\text{Kip1}}$ levels did not increase in control cells (Fig. 5a, b), which continued to actively cycle in the presence of inhibitor (Supplementary Fig. 3 and data not shown). GSK3, either directly or indirectly, negatively regulates p27$^{\text{Kip1}}$ protein stability because inhibitor treatment increased the p27$^{\text{Kip1}}$ half-life without inducing increased messenger RNA levels (Supplementary Fig. 8). β-Catenin levels increased substantially in *MLL*-transformed but also in control cells in response to GSK3 inhibition, whereas levels of p21, another CDKI, were not altered (Fig. 5a, b). Knockdown of p27$^{\text{Kip1}}$ resulted in substantial reductions of p27$^{\text{Kip1}}$ protein (Fig. 5c), and prevented the growth arrest otherwise induced by GSK3 inhibitor in *MLL*-transformed myeloid progenitors (Fig. 5d). Thus, p27$^{\text{Kip1}}$ is a critical downstream mediator of the cell cycle arrest associated with GSK3 inhibition in *MLL* transformed cells.

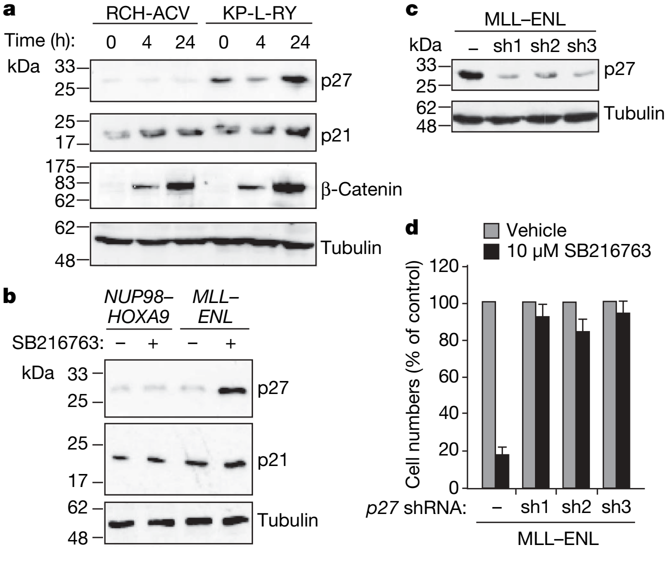

Figure 5 | GSK3 maintains *MLL* transformation through suppression of p27$^{\text{Kip1}}$. **a**, Human leukaemia cell lines (RCH-ACV and KP-L-RY) were treated with 10 μM SB216763 for the indicated times and protein levels were assessed by western blot analysis. **b**, Murine myeloid progenitors transformed by the indicated oncogenes were cultured in the presence (+) or absence (−) of SB216763 (10 μM) for 24 h in liquid culture, and then subjected to western blot analysis. **c**, Western blot analysis demonstrates the p27$^{\text{Kip1}}$ (upper panel) or β-tubulin (lower panel, loading control) protein levels in *MLL–ENL* transformed myeloid progenitors stably transduced with lentiviral vectors expressing shRNAs specific for p27$^{\text{Kip1}}$ (denoted as sh1 to sh3). **d**, Myeloid progenitors transformed by *MLL-ENL* and stably transduced with lentiviral vectors lacking (−) or expressing one of three different p27$^{\text{Kip1}}$ shRNAs were cultured for 3 days in the presence or absence of 10 μM SB216763. Viable cell numbers are expressed relative to the numbers obtained with lentiviral vector transduced cells (± s.e.m. of triplicate analyses).

Discussion

GSK3 maintenance of a genetically distinctive subset of acute leukaemia establishes an enabling role for this multifunctional kinase in oncogenesis. This contrasts with its well-characterized function to suppress signalling pathways that otherwise promote proliferation and self-renewal, a role thwarted in colon cancer and other cancers with oncogenic mutations of β-catenin that abrogate its GSK3-mediated phosphorylation and subsequent destruction on the Wnt pathway[24]. Similarly, hyperactivation of AKT is implicated in cancer pathogenesis through enhanced survival and proliferation[25]. In contrast, *MLL*-transformed cells are sustained by GSK3 and consistently antagonized by GSK3 inhibitors of varying selectivity and specificity (Supplementary Fig. 1), and also adversely affected by constitutively active AKT, a physiological inhibitor of GSK3 activity. Genetic and pharmacological studies confirm the requirement of GSK3 to maintain *MLL*-mediated transformation and leukaemogenesis in preclinical murine models. Thus, GSK3 can promote oncogenesis and does not have an exclusively suppressive role in cancer pathogenesis.

The mechanism by which GSK3 supports *MLL*-oncogene-induced proliferation and transformation is mediated through the destabilization of p27$^{\text{Kip1}}$, a CDKI with established roles in tumour suppression[26]. The *p27^{Kip1}* and *p18^{Ink4c}* genes have been shown to be direct transcriptional targets of MLL[27], a histone methyltransferase that positively maintains gene expression through covalent chromatin modification[20]. In endocrine neoplasias, this tumour suppressor circuit that normally maintains CDKI expression is abrogated by mutations or loss of menin (also known as multiple endocrine neoplasia)[27], a critical component of the MLL histone methyltransferase complex[28,29]. MLL oncoproteins also activate p27$^{\text{Kip1}}$ expression[30], which would seem to be counterproductive for leukaemia pathogenesis. Our results indicate a potential mechanism for the suppression of p27$^{\text{Kip1}}$ either directly or indirectly by GSK3, which provides a permissive cellular context for *MLL*-mediated transformation. However, phosphorylation of p27$^{\text{Kip1}}$ by GSK3 has recently been shown to enhance its stability and prohibit cell cycle progression in the absence of growth factors[31], which contrasts with increased p27$^{\text{Kip1}}$ levels after GSK3 inhibition in *MLL*-transformed cells. Paradoxically increased p27$^{\text{Kip1}}$ expression has also been observed in a myeloma cell line on GSK3 inhibition as part of a paracrine/autocrine feedback loop involving IL-6 signalling and forkhead transcription factors[32]. Therefore, the functional relationships of GSK3 with p27$^{\text{Kip1}}$ seem complex and cell context dependent. Nevertheless, our studies link these factors on a pathogenic pathway that is critical for maintenance of *MLL* leukaemia.

The observed dependence on GSK3 provides a potential therapeutic target in a genetically distinctive subset of leukaemia defined by mutations of the *MLL* proto-oncogene. *MLL* is activated by a substantial array of chromosomal aberrations in diverse haematological disorders that account for approximately 5%–10% of sporadic leukaemias in adults and children[33]. Independent of their association with other high-risk features, *MLL* aberrations are often predictive of poor clinical outcome[34], which warrants a search for new treatment approaches. GSK3 has not previously been considered as a therapeutic target in cancer. In fact, its normally suppressive roles in Wnt, hedgehog and Notch pathway signalling have raised the theoretical possibility that GSK3 inhibition may increase the risk of neoplasia. However, chronic administration of lithium, a relatively nonspecific GSK3 inhibitor used for the treatment of bipolar disorders, has not been associated with increased cancer risk[35]. Notably, GSK3 is a specific *in vivo* modulator of haematopoietic stem cell activity, and GSK3 inhibitors enhance haematopoietic stem cell repopulation in mice after bone marrow transplantation[17]. Thus, like PTEN[36], GSK3 has converse roles in normal versus leukaemia stem cell maintenance, which may confer significant therapeutic selectivity. Our preclinical studies using lithium carbonate to target GSK3 in a murine model of *MLL* leukaemia provide promising evidence of efficacy. The paradoxical sensitivity of *MLL* leukaemias to GSK3 inhibition earmarks this multifunctional kinase as a therapeutic target, and provides a rationale to develop inhibitors with suitable pharmacodynamic properties for clinical trials to determine whether GSK3 inhibition may have therapeutic efficacy in a distinctive subset of poor prognosis leukaemia.

METHODS SUMMARY

Cell cultures and inhibitor assays. Human leukaemia cell lines, and transformed mouse myeloid or B cell precursors, were cultured in medium (with appropriate supplements) containing kinase inhibitors at the indicated concentrations. Cell viabilities were determined by trypan-blue dye exclusion and cell growth was quantified using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays. Cell proliferation was determined by measuring BrdU incorporation. Flow cytometry was used to assess cell cycle status on the basis of propidium iodide staining and to quantify apoptosis on the basis of annexin V staining[27].

Transformation and leukaemogenesis assays. Myeloid progenitors were transduced with retroviral vectors as described previously[21] with minor modifications, and were cultured in liquid or semi-solid medium supplemented with cytokines. B cell progenitors were transduced as described previously[37] and co-cultured on neo-resistant-irradiated OP9 stromal cells. After continuous passage and adaptation to liquid culture, immortalized cells were used for injections of syngeneic mice, and cell lines generated by explantation of splenocytes collected from leukaemic mice were used for GSK3 inhibitor studies. For knockdown studies, transformed progenitors were transduced with shRNA lentiviral constructs, selected for drug resistance *in vitro* and then evaluated for growth in the presence of GSK3 inhibitors. For *in vivo* studies, myeloid progenitors (wild type or *Gsk3b*$^{-/-}$) transformed by *MLL–ENL* were transduced with lentiviral knockdown constructs, selected for drug resistance and then transplanted (10^6 cells) by intravenous injection into sub-lethally irradiated severe combined immunodeficient (SCID) mice. For lithium treatment, irradiated mice were transplanted with *MLL–AF4* leukaemic B cell progenitors (50,000 cells) and maintained on 0.4% lithium-carbonate-containing chow with saline water.

Protein assays. Protein extracts were prepared by cell lysis in buffer containing protease inhibitors, subjected to SDS–PAGE and analysed by western blot using primary antibodies as indicated throughout.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 9 April; accepted 18 July 2008.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank R. Roth for providing AKT constructs, P. J. Roach for providing GSK3 constructs, D. G. Gilliland for providing a TEL-AML1 construct, M. Iwasaki for NUP98-HOXA9 cells, M. Ambrus and C. Nicolas for technical assistance, and members of the Cleary laboratory for discussions. We acknowledge support from the Children's Health Initiative of the Packard Foundation, PHS grants CA55029 and CA116606, the Leukemia and Lymphoma Society, the Williams Lawrence Foundation and a Developmental Research Award from the Stanford Cancer Center.

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METHODS

Mice. C57BL/6 and SCID mice were obtained from the breeding facility of the Stanford University Veterinary Service Center. $Gsk3b^{+/-}$ mice (provided by G. R. Crabtree with permission from J. R. Woodgett) were maintained on a CD1 genetic background. All experiments on mice were performed with the approval and in accordance with Stanford's Administrative Panel on Laboratory Animal Care.

Inhibitors. The GSK3 inhibitors SB216763 (Sigma), GSK3-IX and alsterpaullone (EMDbiosciences) were dissolved in dimethylsulphoxide and used at the indicated concentrations. All other inhibitors (EMDbiosciences) were dissolved in dimethylsulphoxide and used at the concentrations indicated in Supplementary Table 1.

Cell cultures. All human leukaemia cell lines (Supplementary Table 2) were maintained in R10 medium (RPMI1640 supplemented with 10% FBS, 1% L-glutamine and penicillin/streptomycin). Immortalized mouse myeloid cells were maintained in R20/20 medium (RPMI1640 with 20% FCS, 20% WEHI-conditioned medium, l% L-glutamine and penicillin/streptomycin). Immortalized mouse B cells were cultured in OP9 medium (MEMα+GlutaMax 1 with 10% FBS, 1% L-glutamine, penicillin/streptomycin and 2 μM β-mercaptoethanol) containing 1 ng ml^{-1} IL-7 when necessary. All culture medium was obtained from Gibco.

DNA constructs and virus production. Retroviral constructs (MSCV vector) encoding *MLL–ENL*, *MLL–LAF4*, *MLL–AF6*, *MLL–GAS7*, *E2A–PBX1*, *NUP98–HOXA9* and *E2A–HLF* were reported previously[21,28,37–40]. Retroviral constructs encoding *CA–AKT*, *ER–CA–AKT*[41], and wild-type or S9A mutant GSK3B were constructed by cloning the respective complementary DNAs into MSCV using standard cloning techniques. Retrovirus production was performed as described previously[42]. Oligonucleotides for specific shRNA knockdown of *Gsk3a*, *Gsk3b* or $p27^{Kip1}$ (sequences in Supplementary Table 3) were designed using PSICOLIGOMAKER 1.5 software (http://web.mit.edu/jacks-lab/protocols_table.html), and cloned into pSicoR-Puro or pSicoR-Hygromycin lentiviral vectors. Lentiviral stocks were produced as described previously[43].

Murine progenitor transformation assays. Myeloid progenitor transformation was performed as described previously[21] with minor modifications. In brief, c-Kit$^+$ cells were isolated from the bone marrow of 4–8-week-old C57BL/6 mice or E16 fetal livers ($Gsk3b^{-/-}$ mice) using an auto-MACS and anti-c-Kit beads (Miltenyi Biotech). The c-Kit$^+$ cells were spinoculated with retroviral supernatant in the presence of 5 μg ml^{-1} polybrene for 2 h at 1,350*g* and at 32 °C. After spinoculation and after overnight culture, cells were plated in methylcellulose medium (M3231; Stem Cell Technologies) containing 20 ng ml^{-1} stem cell factor, 10 ng ml^{-1} IL-6, granulocyte–macrophage colony-stimulating factor (GM–CSF), and IL-3 (R&D Systems) with appropriate antibiotic selection. After 5–7 days of culture, colonies were counted, pooled, and then 10^4 cells were replated in the same medium but without antibiotic. At the end of the fourth round, cells were transferred to R20/20 medium to establish continuous cell lines.

B cell progenitors were transduced as described previously[37] with minor modifications. Transduced cells were co-cultured on neo-resistant-irradiated OP9 stromal cells. After continuous passage and adaptation to liquid culture, immortalized cell lines were used for injections of syngeneic recipient mice. *MLL–AF4* B cell precursor leukaemia cell lines were generated by explantation of splenocytes collected from leukaemic mice.

Transduction of immortalized mouse cells. Immortalized mouse cells (20,000) were transduced with retroviral or lentiviral constructs by spinoculation at 2,500*g* or 1,200*g* for 2 h at 32 °C. Transduced cells were then resuspended in 200 μl of R20/20 or OP9 medium and transferred to 96-well plates. After overnight incubation at 37 °C, myeloid cells were plated in methycellulose medium containing IL-3, IL-6, GM-CSF and stem cell factor. Transduced B cells were plated in methylcellulose medium containing IL-7.

Leukaemogenesis assays. Myeloid progenitors (wild type or $Gsk3b^{-/-}$) transformed by *MLL–ENL* were transduced with different lentiviruses, selected for drug resistance and then transplanted (10^6 cells) by intravenous injection into sub-lethally irradiated (2 Gy) C.B-17 *scid*/*scid* mice (6–8–weeks-old). For lithium treatment, irradiated (1 Gy) C57BL/6 mice were transplanted with *MLL–AF4*-transformed B cell progenitors (50,000 cells) and maintained on 0.4% lithium-carbonate-containing chow with saline water (Harlan Teklad). Lithium treatment was initiated 3 days before transplantation and continued for 30 days, at which point treatment was withheld for 5 days to allow recovery from drug-induced diuresis, and was then resumed. Development of acute leukaemia was confirmed by blood smear, peripheral blood leukocyte counts, FACS analyses and/or histology.

Flow cytometry. Staining of cells for FACS analysis was performed as previously described[42] using conjugated antibodies obtained from either BD Pharmingen or eBioscience. Cell cycle assays using propidium iodide staining, and apoptosis assays using annexin V staining, were performed as described[28]. BrdU incorporation was determined using the BrdU flow kit (BD Pharmingen) according to the manufacturer's instructions.

Cell proliferation and MTT assays. Cultured cells (10,000–20,000) were plated in 96-well plates in R10, R20/20 or OP9 medium (100 μl) containing different concentrations of the indicated kinase inhibitors (Supplementary Table 1) and incubated at 37 °C. The numbers of viable cells were determined by trypan-blue dye exclusion at the indicated times using a haemocytometer. For MTT assays, cells were cultured for 3–4 days and then quantified using a cell proliferation kit 1 under conditions recommended by the manufacturer (Roche).

Western blot. Cells were lysed in buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA) containing protease inhibitors (complete mini protease inhibitors; Roche). Proteins (40 μg) were subjected to SDS–PAGE and analysed by western blot using primary antibodies specific for GSK3 (Upstate Biotechnology), β-catenin (Upstate Biotechnology), phosph-GSK3 (Cell Signalling), AKT (Cell Signaling), tubulin (Sigma), p27 or p21 (Santa Cruz Biotechnology).

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