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Glycogen Synthase Kinase-3 β Is a Prosurvival Signal for the Maintenance of Human Mast Cell Homeostasis

Madeleine Rådinger,¹ Daniel Smrž, Dean D. Metcalfe, and Alasdair M. Gilfillan

Homeostasis of mature tissue-resident mast cells is dependent on the relative activation of pro- and antiapoptotic regulators. In this study, we investigated the role of glycogen synthase kinase 3 β (GSK3 β) in the survival of neoplastic and nonneoplastic human mast cells. GSK3 β was observed to be phosphorylated at the Y²¹⁶ activating residue under resting conditions in both the neoplastic HMC1.2 cell line and in peripheral blood-derived primary human mast cells (HuMCs), suggesting constitutive activation of GSK3 β in these cells. Lentiviral-transduced short hairpin RNA knockdown of GSK3 β in both the HMC1.2 cells and HuMCs resulted in a significant reduction in cell survival as determined with the MTT assay. The decrease in stem cell factor (SCF)-mediated survival in the GSK3 β knockdown HuMCs was reflected by enhancement of SCF withdrawal-induced apoptosis, as determined by Annexin V staining and caspase cleavage, and this was associated with a pronounced reduction in SCF-mediated phosphorylation of Src homology 2 domain-containing phosphatase 2 and ERK1/2 and reduced expression of the antiapoptotic proteins Bcl-xl and Bcl-2. These data show that GSK3 β is an essential antiapoptotic factor in both neoplastic and nontransformed primary human mast cells through the regulation of SCF-mediated Src homology 2 domain-containing phosphatase 2 and ERK activation. Our data suggest that targeting of GSK3 β with small m.w. inhibitors such as CHIR 99021 may thus provide a mechanism for limiting mast cell survival and subsequently decreasing the intensity of the allergic inflammatory response. *The Journal of Immunology*, 2011, 187: 000–000.

Chronic allergic inflammation is characterized by increased mast cell infiltration and population in the affected tissues. Mast cell burden in these tissues is dependent not only on migration of mast cells/mast cell precursors into these sites but also on pro-survival and antiapoptotic signaling pathways. Processes, including the migration and survival of tissue mast cells, are tightly regulated by stem cell factor (SCF), the ligand for the growth factor receptor KIT (1–3). KIT, which is a member of the growth factor receptors with inherent tyrosine kinase family activity (4, 5), undergoes dimerization and autophosphorylation following SCF-induced ligation. Constitutive activation of KIT activity, through a point mutation (D816V) in the KIT catalytic domain is considered a hallmark of the myeloproliferative disorder mastocytosis (6–8). This disease is characterized by dysregulated growth of mast cells and elevated mast cell numbers in associated skin lesions and tissues such as bone marrow (7). In addition to the documentation of more mast cells harboring the D816V mutation within tissues of mastocytosis patients, the rapidly dividing HMC1.2 human mast cell line also expresses this mutation (9).

Autophosphorylation of KIT at specific tyrosine residues within the cytosolic domain induces recruitment of Src homology 2 domain-containing signaling proteins, which leads to activation of these and other molecules required for transducing KIT-mediated responses, including mast cell growth and survival (10, 11). We have recently provided evidence to support a role for PI3K-regulated mammalian target of rapamycin in cell survival in both human and mouse mast cells (12). Furthermore, the transcription factor FOXO3a has been shown to regulate SCF-mediated survival in mouse mast cells by repressing the expression of proapoptotic proteins via a PI3K-dependent pathway (13). It is clear, however, that other antiapoptotic pathways exist that may contribute to mast cell homeostasis. For example, in the LAD2 human mast cell line, cell survival can be maintained in the absence of SCF despite the lack of an activating mutation in KIT (14). Furthermore, mouse mast cells can proliferate and survive in the presence of IL-3 but in the absence of SCF.

We have previously determined that glycogen synthase kinase 3 β (GSK3 β), a ubiquitously expressed, conserved serine/threonine protein kinase, contributes to various Fc ϵ RI/KIT-mediated mast cell functions including cytokine production and KIT-mediated chemotaxis (15). As GSK3 β has also been implicated in tumor growth (16), we thus investigated whether, as for PI3K and mammalian target of rapamycin, GSK3 β also contributes to the homeostasis of normal and neoplastic mast cells. In this study, we report that gene knockdown of GSK3 β by short hairpin RNA (shRNA) and blocking of its activity by the small molecule GSK3 β inhibitor CHIR 99021 substantially reduces the ability of the HMC1.2 cell line to expand and survive in culture. Furthermore, we show that the ability of SCF to maintain the survival of terminally differentiated primary cultures of human mast cells (HuMCs) is markedly reduced as a consequence of induction of apoptosis following GSK3 β knockdown. Thus, GSK3 β appears to represent an important mast cell pro-survival signal. Targeting GSK3 β accordingly may provide a mechanism for modulating

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Abbreviations used in this article: GS, glycogen synthase; GSK3 β , glycogen synthase kinase 3 β ; HuMC, human mast cell; PI, propidium iodide; PLC γ , phospholipase C γ ; SCF, stem cell factor; shContr, scrambled short hairpin RNA; shGSK3 β , short hairpin RNA for glycogen synthase kinase 3 β ; SHP2, Src homology 2 domain-containing phosphatase 2; shRNA, short hairpin RNA.

mast cell survival and apoptotic pathways in neoplastic mast cells and potentially mast cells participating in the allergic inflammatory response.

Materials and Methods

Mast cell culture

HuMCs were developed from CD34⁺ peripheral blood progenitor cells in StemPro-34 culture medium containing StemPro supplement (Invitrogen Life Technologies), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), recombinant human IL-3 (30 ng/ml, first week only), recombinant human IL-6 (100 ng/ml), and recombinant human SCF (100 ng/ml) (PeproTech, Rocky Hill, NJ). Experiments were conducted 7–9 wk after the initiation of HuMC cultures. The CD34⁺ cells were obtained from normal volunteers under a protocol (98-I-0027; principal investigator, Dr. A. Kirshenbaum) approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board and with appropriate informed consents. The growth factor-independent human mast cell line HMC1.2 was cultured in IMDM medium supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml).

Lentivirus shRNA transfection of cell lines and transduction of HuMCs

The following GSK3 β -targeted shRNA were purchased from Sigma-Aldrich (St. Louis, MO): 5'-CCGGGTGTGGATCAGTTGGTAGAAAC-TCGAGTTTCTACCACTGATCCACACTTTT-3' (TRCN0000010552) (construct A); 5'-CCGGGACACTAAAGTGATTGGAAATCTCGAGATT-TCCAATCACTTTAGTGTCTTTT-3' (TRCN0000040002) (construct B); and 5'-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTG-CTCTTCATCTTGTGTTT-3' (SHC002) (control nontarget control vector). Packaging vector (MissionLentiviral packaging mix; Sigma-Aldrich), pLKO1 transfer vectors with GSK3 β shRNA (Sigma-Aldrich), or scrambled control shRNA were cotransfected into 293T packaging cells with FuGENE6 transfection reagent (Roche, Indianapolis, IN) as described (15). The transfected 293T cells were grown in DMEM containing FBS (10%), L-glutamine (4 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Following 16–19 h of transfection, medium was removed and replaced with fresh DMEM. After 62–65 h of transfection, virus was collected by centrifugation, and the virus pellet was resuspended in 3 ml prewarmed StemPro medium (Invitrogen Life Technologies). Transduction of HuMCs was conducted by transferring the 3 ml resuspended virus to a T75 culture flask containing 3 to 4 $\times 10^6$ HuMCs in 15 ml supplemented StemPro cell culture medium (Invitrogen Life Technologies) or 2 to 3 $\times 10^6$ HMC1.2 cells in 10 ml IMDM medium. Two days postinfection, the medium was changed to virus-free medium, and antibiotic selection was initiated (0.2 μ g/ml puromycin [Sigma-Aldrich] for HuMCs and 2 μ g/ml puromycin for HMC1.2 cells). Experiments were conducted on days 7 to 8 posttransduction. We have previously demonstrated that this construct does not affect the expression of other signaling proteins including phospholipase C γ (PLC γ)₁, Lyn, and NF- κ B in HuMCs (15).

Cell activation

HuMCs were starved in cytokine-free media overnight following rinsing with HEPES buffer containing 0.04% BSA and activated by addition of SCF (30 ng/ml) for the times noted in the figure legends. Where indicated, cells were pretreated with the Src family tyrosine kinase inhibitor PP2 (3 μ M), the PLC γ inhibitor U73122 (1 μ M; EMD Biosciences), or the PI3K inhibitor wortmannin (100 nM; Calbiochem) 20 min prior to the activation with SCF.

Immunoblotting

Cell lysates were prepared as described (17, 18). Aliquots of lysates were loaded onto a 4–12% NuPage BisTris gel (Invitrogen Life Technologies). Proteins were separated by electrophoresis and then transferred onto nitrocellulose membranes. The proteins were probed with the following phospho-specific Abs from Cell Signaling Technology (Beverly, MA): anti-phospho-Akt (p-S⁴⁷³), anti-phospho-GSK3 β (p-S⁹), anti-phospho-glycogen synthase (p-S⁶⁴¹), anti-phospho-Src homology 2 domain-containing phosphatase 2 (SHP2) (p-Y⁵⁸⁰), and anti-phospho-ERK (p-T²⁰²/Y²⁰⁴). The anti-phospho-GSK3 α/β (p-Y²⁷⁹/Y²¹⁶) Ab was from Invitrogen. Total Syk and KIT Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). The Abs specific for Mcl-1, Bcl-xL, Bcl-2, Bim, Bid, or Bad were from Cell Signaling Technology. Immunoreactive proteins were visualized by probing with a secondary HRP-conjugated Ab. To normalize protein loading, identically loaded samples were probed for Syk or total

KIT. To quantify changes in protein phosphorylation, the ECL films were scanned using a Quantity One scanner (Bio-Rad, Hercules, CA).

Cell survival assay: trypan blue method

An aliquot of suspended cells (20 μ l) was mixed with 20 μ l trypan blue solution. Cells were counted, and viability was assayed by exclusion of trypan blue dye using a hemocytometer. In some experiments, HMC1.2 cells (2 $\times 10^5$ cells in 1 ml media) were cultured with or without the GSK3 β inhibitor (CHIR 99021 [3–30 μ M]; AH Diagnostics, Aarhus, Denmark) for 24, 48, or 72 h. Cell viability was then assessed by trypan blue exclusion.

Cell survival assay: MTT colorimetric assay

To assess mast cell survival, HuMCs, transduced with scrambled shRNA (shContr) or shRNAs for GSK3 β (shGSK3 β), were starved in cytokine-depleted medium for 20 h. Cells (1 $\times 10^5$ /well) were then seeded in 96-well plates and stimulated with SCF for 24 h. The percentage of viable cells was assessed with an MTT-based colorimetric assay (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, after 24 h in culture, MTT solution was added, and cells were incubated for an additional 3.5 h at 37°C. The MTT was solubilized, and the absorbance was measured at 570 nm.

Apoptosis assay

Apoptosis was evaluated by flow cytometry using cellular Annexin V binding (Annexin V, FITC apoptosis detection kit I; BD Biosciences, San Jose, CA). In brief, cells were stained with Annexin V and propidium iodide (PI) according to the manufacturer's instructions and then analyzed using FACScan flow cytometry (BD Biosciences). All PI-positive cells were considered dead. PI-negative and Annexin V-positive cells were considered early apoptotic cells, and the remaining double-negative cells were considered viable.

Proliferation assay: BrdU incorporation

Cell proliferation was determined by the BrdU (a thymidine analog) assay that measures its incorporation into DNA. HMC1.2 cells, transduced with shContr or shGSK3 β , were cultured overnight in IMEM without FBS and resuspended in IMEM containing 10% FBS. Cells were then incubated for 24 h at 1.5 $\times 10^5$ cells/100 μ l in 96-well plates. Incorporation of BrdU into the shContr- or shGSK3 β -transduced HMC1.2 cells was assessed using a BrdU cell proliferation assay kit (Calbiochem, San Diego, CA) as described (19).

Statistical analysis

Data are presented as the mean $\pm$ SEM. Significant difference between two groups was analyzed by unpaired Student *t* test. A *p* value <0.05 was considered statistically significant.

Results

Knockdown of GSK3 β decreases survival of the HMC1.2 human leukemia mast cell line

To explore the potential role of GSK3 β as a mast cell survival/antiapoptotic signal, we adopted a lentivirus-mediated knockdown approach (15), as GSK3 β deficiency in mice results in an embryonic lethal phenotype (20). We initially examined the consequences of GSK3 β knockdown in the SCF-independent HMC1.2 mast cell line. As shown in Fig. 1A, GSK3 β -targeted shRNA substantially decreased the expression of GSK3 β in the HMC1.2 cells without reducing the expression of the internal controls KIT and Syk. GSK3 β activity is regulated by the phosphorylation status of Y²¹⁶ and S⁹. The Y²¹⁶ residue is reported to be constitutively phosphorylated in resting cells, thus maintaining GSK3 β in an active state, whereas the phosphorylation of the S⁹ site usually follows growth factor stimulation and activation of the PI3K–Akt signaling pathway (21–23). As shown in Fig. 1A, Y²¹⁶ and S⁹ are constitutively phosphorylated in the HMC1.2 cells, and, as expected, the phosphorylation of these residues is substantially reduced in cells treated with the GSK3 β -targeted shRNA.

Having successfully knocked down GSK3 β in the HMC1.2 cells, we next determined the consequences of GSK3 β knock-

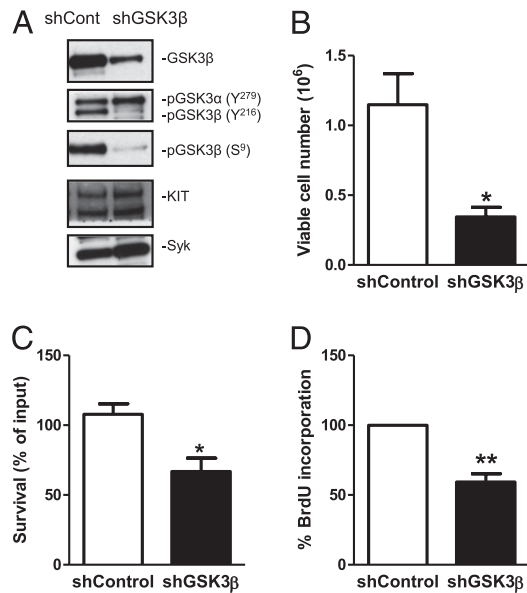


FIGURE 1. Antiproliferative effect of GSK3 β knockdown in HMC1.2 cell line. *A*, The HMC1.2 cell line was transduced with shContr or shGSK3 β . Whole-cell extracts were prepared and immunoblotted with anti-GSK3 β , anti-phospho-GSK3 α/β (p-Y²⁷⁹/Y²¹⁶), anti-p-GSK3 β (S⁹), anti-Syk, or anti-KIT Abs. *B*, Cell viability was assessed by trypan blue dye exclusion. Cell survival was assessed by means of an MTT assay in which the starting cell number was 1×10^5 cells/well (*C*), and proliferation was assessed by BrdU incorporation (*D*). In all cases, $n = 3-5$. * $p < 0.05$, ** $p < 0.01$ for comparison with shContr-transduced HMC1.2.

down on the proliferation and/or survival of HMC1.2 cells. As shown in Fig. 1*B*, GSK3 β knockdown significantly decreased the number of viable HMC1.2 cells as assessed by trypan blue exclusion 9 d posttransduction. In addition, when cell survival was determined by the MTT assay during a 24-h period, GSK3 β knockdown significantly decreased HMC1.2 cells' survival (Fig. 1*C*). To determine whether this reduction may, in part, reflect a decrease in proliferation rate, HMC1.2 cells, transduced with shRNA targeting GSK3 β or with scrambled control shRNA, were cultured overnight in the absence of FBS, resuspended in media containing FBS for 24 h, and cell proliferation monitored by BrdU incorporation. As shown in Fig. 1*D*, BrdU incorporation was significantly reduced in the GSK3 β knockdown in HMC1.2 cells compared with the scrambled control shRNA-treated cells. Taken together, these data suggest that GSK3 β is required for HMC1.2 cell survival, but this, in part, may reflect a requirement for GSK3 β in cell proliferation. To further investigate this potential role for GSK3 β in mast cell homeostasis, we next determined the manifestations of GSK3 β knockdown in mature HuMCs, which represent a terminally differentiated nondividing cell population that, unlike HMC1.2 cells, requires SCF for survival.

Expression and phosphorylation of GSK3 β in primary human mast cells

We first confirmed the ability of GSK3 β -targeted shRNA to downregulate GSK3 β expression and phosphorylation in SCF-challenged and quiescent HuMCs. Cells, starved of cytokines overnight and treated with control or GSK3 β -targeted shRNA, were either unchallenged or challenged with SCF for 2 min and cell lysates assessed for expression and phosphorylation of GSK3 β . Despite overnight starvation of SCF, and as was observed in the HMC1.2 cells, GSK3 β was found to be constitutively phosphorylated at position Y²¹⁶ in resting HuMCs and no consistent increase in the phosphorylation of this residue was observed

in cells rechallenged with SCF (Fig. 2*A*). Regardless, the expression of GSK3 β and, as a consequence, GSK3 β phosphorylated at Y²¹⁶ was markedly reduced in HuMCs treated with GSK3 β -targeted shRNA. In contrast to the HMC1.2 cells, there was minimal constitutive phosphorylation of the S⁹ residue of GSK3 β in resting primary HuMCs. However, this phosphorylation was enhanced in SCF-challenged cells (Fig. 2*A*). As expected, this phosphorylation was markedly reduced in cells treated with GSK3 β -targeted shRNA. Nevertheless, the inability of SCF alone to further enhance the observed constitutive phosphorylation of GSK3 β at Y²¹⁶ again suggests that GSK3 β may be constitutively active in the resting state in HuMCs and that this activity cannot be further enhanced through KIT activation. Furthermore, the lack of reduction of the phosphorylation of GS by SCF (Fig. 2*B*), a response that could be reduced by GSK3 β -targeted shRNA (15), suggests that the phosphorylation of GSK3 β at S⁹ in the HuMCs minimally impacted GSK3 β activity, at least over the time frame examined.

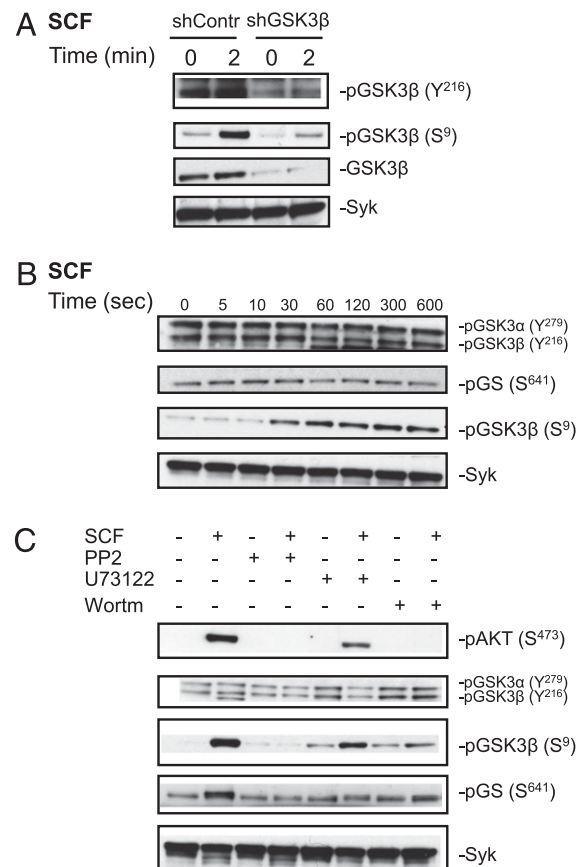


FIGURE 2. Expression and activity of GSK3 β inhibition in primary HuMCs. *A*, HuMCs, transduced with shContr or shGSK3 β , were starved overnight in SCF-depleted media and then stimulated with SCF for 2 min as described in *Materials and Methods*. Whole-cell extracts were prepared and immunoblotted with anti-p-GSK3 β (Y²¹⁶), anti-p-GSK3 β (S⁹), or anti-GSK3 β Abs. The level of Syk demonstrates equivalent protein loading of samples. *B*, Kinetics of SCF-mediated phosphorylation of GSK3 β in HuMCs. Whole-cell extracts were prepared and immunoblotted with anti-p-GSK3 α/β (p-Y²⁷⁹/Y²¹⁶), anti-p-GS (pGS) (S⁶⁴¹), or anti-p-GSK3 β (S⁹) Abs. The level of total Syk demonstrates equivalent protein loading of the samples. *C*, HuMCs were preincubated with a Src inhibitor (PP2), a PLC γ inhibitor (U73122), or a PI3K inhibitor (wortmannin [Wortm]) for 20 min prior to stimulation with SCF for 2 min as described in *Materials and Methods*. Whole-cell extracts were prepared and immunoblotted with anti-p-Akt (S⁴⁷³), anti-p-GSK3 β (Y²¹⁶), anti-p-GSK3 β (S⁹), or anti-p-GS (S⁶⁴¹) Abs. The level of Syk demonstrates equivalent protein loading of samples.

We have previously determined that phosphorylation of the downstream substrate for GSK3 β , GS, is moderately enhanced in HuMCs following a combination of KIT and Fc ϵ RI-mediated activation (15). Hence, we examined the phosphorylation status of p-GSK3 β (Y²¹⁶) and GS (S⁶⁴¹) in HuMC over a 10-min time period during which maximal SCF-induced KIT phosphorylation is observed (24). As can be seen from Fig. 2B, there was no consistent increase in phosphorylation of either of these respective GSK3 β and GS residues. These data overall are thus consistent with the conclusion that the constitutive phosphorylation and activity of GSK3 β observed in the HMC1.2 and HuMC cells is regulated at least in part independently of KIT activity.

To provide further support for this conclusion, we investigated whether inhibitors of known signaling processes downstream of KIT block the constitutive phosphorylation of GS and GSK3 β , including PP2, an Src family tyrosine kinase inhibitor also capable of inhibiting KIT phosphorylation (24); U71322, a PLC γ 1 inhibitor (25); and wortmannin, a selective PI3K inhibitor (25). As expected, and to serve as a positive control, PP2 inhibited SCF-mediated PI3K activation, as indicated by the attenuation of the phosphorylation of Akt. Wortmannin primarily inhibited Akt phosphorylation (Fig. 2C). Although all three agents variably inhibited the increase in phosphorylation of GSK3 β at S⁹ and the observed increase in phosphorylation in GS at S⁶⁴¹ in these experiments, they had minimal effect on the constitutive phosphorylation of GSK3 β at Y²¹⁶ and GS at S⁶⁴¹. These data suggest that although KIT activates PLC γ 1 (25), tyrosine kinases, and PI3K, the constitutive activation pathway is independent of these signaling processes. These data further support the conclusion that the constitutive activation of GSK3 β observed in mast cells is independent of KIT activation.

GSK3 β is required for SCF-mediated HuMCs survival

To next determine the outcome of GSK3 β knockdown on HuMC survival, two different shRNA constructs were selected based on their relative abilities to decrease GSK3 β expression in HuMCs as described (15) and as shown in Fig. 3A. Knockdown of GSK3 β with these constructs resulted in a significant reduction in viable cells as determined by trypan blue viability count ($68.9 \pm 8.6\%$ [construct A] and $31.2 \pm 10.3\%$ [construct B] versus $91.3 \pm 2.7\%$ [control construct] remaining viable cells; $n = 3$, $p < 0.01$) (Fig. 3B) and with the MTT assay ($54.4 \pm 10.4\%$ and $25.7 \pm 7.1\%$ versus $95.8 \pm 3.3\%$ for survival of cell input; $n = 3$, $p < 0.05$ and $p < 0.001$, respectively) (Fig. 3C). Based on these parameters, the decrease in survival of the cells treated with the two different GSK3 β constructs thus correlated to the degree of GSK3 β knockdown observed in Fig. 3A. Taken together, these data indicate that, although the requirements for GSK3 β in the maintenance of neoplastic mast cells may be partly dependent on effects on cell division, at least in mature primary cultured HuMCs, it principally functions as an antiapoptotic signal for the maintenance of mast cell survival.

GSK3 β knockdown increases SCF withdrawal-induced apoptosis in HuMCs

To provide further evidence to support the role of GSK3 β as an antiapoptotic factor, we next determined whether the degree of knockdown of GSK3 β similarly correlated to the respective markers of apoptosis and cell death, Annexin V and PI staining. The GSK3 β -targeted shRNA-treated cells were grown in SCF-depleted medium for 20 h to increase the sensitivity of the as-

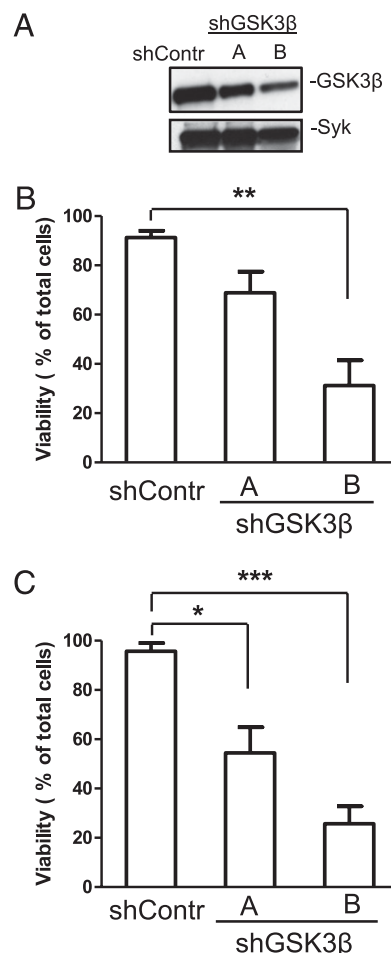


FIGURE 3. GSK3 β is required for HuMC survival. A, HuMCs transduced with shContr or shRNA for two different constructs of GSK3 β (shGSK3 β A and shGSK3 β B) and probed with anti-GSK3 β and Syk protein. Transduced cells were then starved overnight, and cell viability was measured by trypan blue test (B), or cells (1×10^5 /well) starved overnight were treated with SCF (100 ng/ml) for 24 h (C) and the number of cells surviving assessed with the MTT assay and expressed as percent survival of input as described in *Materials and Methods*. In all cases, $n = 3$ to 4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison with shContr-transduced HuMCs.

say. Under these conditions, the shGSK3 β -treated HuMCs displayed a significant increase in apoptosis compared with the scrambled control-treated cells (construct A: $39.9 \pm 2.7\%$ and construct B: $54.5 \pm 3.9\%$ versus $25.6 \pm 3.1\%$ apoptotic cells; $n = 4$ to 5 , $p < 0.001$) (Fig. 4A). Fig. 4B shows representative scatter plots of HuMCs transduced with shContr or shRNA for two different constructs of GSK3 β starved in SCF-depleted media for 20 h and stained for Annexin V and PI. Caspase-3, which is activated upon cleavage, plays a dominant role in the extrinsic apoptotic pathway. To further establish the role of GSK3 β in the extrinsic apoptotic pathway, we therefore next examined cleaved caspase 3 by Western blotting. As can be seen in Fig. 4C and 4D, the increase in apoptotic cells was associated with an increase in cleaved caspase-3 in the GSK3 β knockdown cells compared with control-treated cells. Taken together, these data support a role for GSK3 β as a key regulator of an antiapoptotic signaling pathway required for mast cell homeostasis. These data further support the conclusion that the antiapoptotic signals provided by GSK3 β act in conjunction with those initiated by SCF, rather than being directly regulated by SCF-mediated signaling.

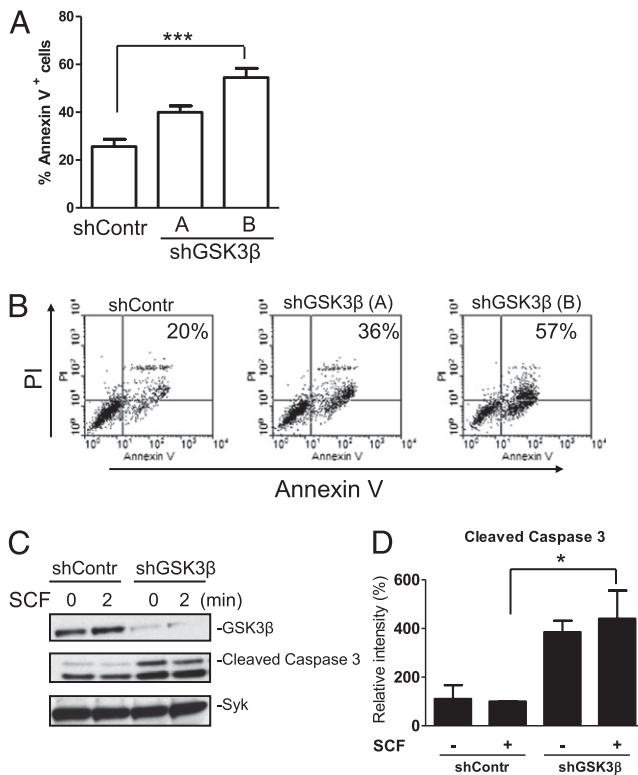


FIGURE 4. GSK3 β knockdown induces apoptosis in HuMC. *A*, HuMCs transduced with shContr or shRNA for GSK3 β (shGSK3 β A and B) were starved for 20 h, and apoptosis was measured by Annexin V staining. *B*, Representative scatter plots of HuMCs transduced with shContr or shRNA for two different constructs of GSK3 β starved in SCF-depleted media for 20 h and stained for Annexin V (x-axis) and PI (y-axis). The percent represents total Annexin V⁺ cells (percent Annexin V⁺ cells in upper and lower right quadrants). shControl and shGSK3 β -transduced cells (construct B) were starved 15–20 h in SCF-depleted medium and then stimulated with SCF for 2 min. *C*, Whole-cell extracts were prepared and immunoblotted with anti-GSK3 β , anti-cleaved caspase-3 Abs. The level of Syk demonstrates equivalent protein loading of samples. Data in *D* were generated by scanning blots from three to four independent experiments for cleaved caspase-3 and normalizing to responses obtained at 2 min with SCF stimulation in shContr cells; $n = 3$ –5. * $p < 0.05$, *** $p < 0.001$ for comparison with shContr-transduced HuMCs.

Downregulation of GSK3 β inhibits SHP2 and ERK1/2 but not PI3K activation

As discussed (26, 27), an intact PI3K signaling axis is required for mast cell homeostasis. We therefore next examined whether the similar requirement for GSK3 β for the maintenance of mast cell homeostasis could be explained by potential positive-feedback regulation of PI3K activity. We thus examined if the SCF-dependent phosphorylation of Akt was inhibited in the GSK3 β knocked-down HuMCs compared with the scrambled control-treated cells. As expected, and by means of a control, both the phosphorylation of Y²¹⁶ of GSK3 β and the phosphorylation of S⁹ of GSK3 β were significantly reduced in the knockdown cells compared with control shRNA-treated cells (Fig. 5A–C). In contrast, there was no significant difference in the phosphorylation of Akt (S⁴⁷³) (Fig. 5A, 5D) following SCF stimulation in the GSK3 β knockdown cells compared with control cells. These observations indicate that the decrease in survival of HuMCs following knockdown of GSK3 β was not due to positive feedback regulation of PI3K by GSK3 β , but requires the regulation of other critical signaling elements required for mast cell homeostasis.

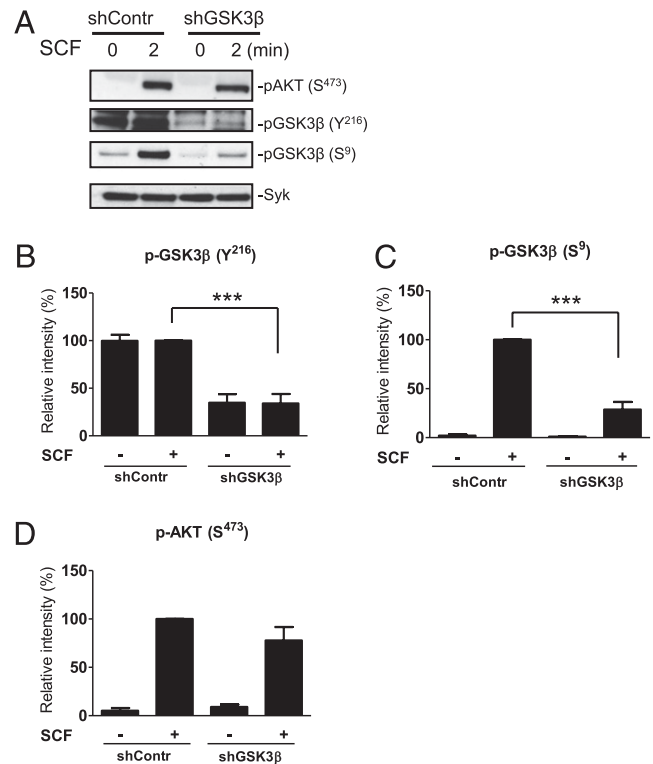


FIGURE 5. Effect of GSK3 β knockdown on SCF-mediated PI3K activation in primary HuMCs. *A*, HuMCs, transduced with scrambled shRNA or shGSK3 β , were stimulated with SCF for 2 min as described in *Materials and Methods*. Whole-cell extracts were prepared and immunoblotted with anti-p-Akt (S⁴⁷³), anti-phospho-GSK3 α/β (p-Y²⁷⁹/Y²¹⁶), or anti-p-GSK3 β (S⁹) Abs. The level of Syk demonstrates equivalent protein loading of samples. A representative blot is shown in figure. *B–D*, Data were generated by scanning multiple blots as shown in *A* and normalizing to the response obtained at 2 min with SCF stimulation. In all cases, $n = 3$ to 4. *** $p < 0.001$ for comparison with SCF response in shContr-transduced HuMCs.

In addition to PI3K, studies conducted in mouse bone marrow-derived mast cells suggest that SHP2 is also required for SCF-mediated mast cell proliferation and survival through the regulation of Rac/JNK (28). Furthermore, SHP2/Ras activation has also been shown to be critical for growth factor-induced ERK activation through an undefined mechanism (29). We thus investigated whether the impact of GSK3 β deficiency on HuMC survival may be explained by a downregulation of SHP2 and/or ERK activity. As can be seen in Fig. 6A–C, SCF-mediated activation of both SHP2 and ERK was significantly reduced in the GSK3 β knockdown cells compared with the control cells. It is also of note that the constitutive phosphorylation of SHP2 but not ERK tended to also be lower in GSK3 β knockdown cells. Taken together, these data indicate that the constitutive activation of GSK3 β maintains mast cell homeostasis by providing a permissive signal allowing phosphorylated KIT to recruit SHP2 and ERK.

Knockdown of GSK3 β downregulates expression of Bcl-2 family member antiapoptotic proteins

Growth factor-induced apoptosis is regulated by the interplay of prosurvival and proapoptotic members of the Bcl-2 family proteins (30, 31). Studies have demonstrated that hyperactivation of phospho-ERK enhances cell survival by inducing expression of the prosurvival molecules Bcl-2 and Bcl-xL and suppression of the proapoptotic BH3-only protein Bim (32, 33). Similarly, gain-of-function SHP2 mutant-expressing cells have elevated levels of

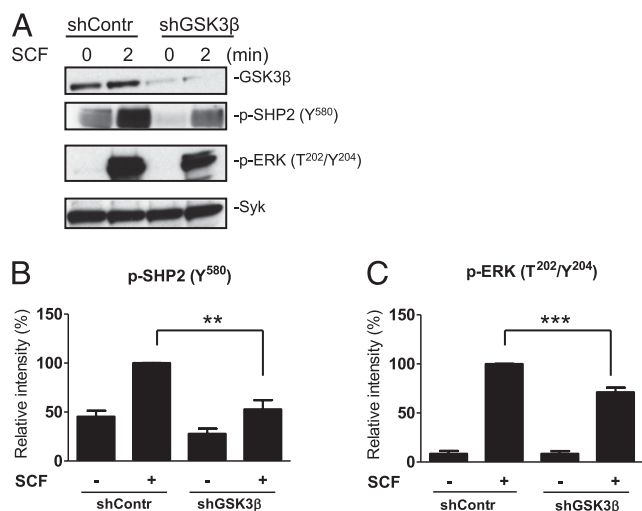


FIGURE 6. GSK3 β knockdown reduces SCF-mediated SHP2 and ERK activation in primary HuMCs. A, HuMCs, transduced with scrambled shRNA or shGSK3 β , were stimulated with SCF for 2 min as described in *Materials and Methods*. Whole-cell extracts were prepared and immunoblotted with anti-GSK3 β , anti-p-SHP2 (Y⁵⁸⁰), or anti-p-ERK (T²⁰²/Y²⁰⁴) Abs. The level of Syk demonstrates equivalent protein loading of samples. A representative blot is shown in figure. B–D, Data were generated by scanning the multiple blots as in A and normalizing to the response obtained at 2 min with SCF stimulation in shContr-transduced HuMCs. In all cases, $n = 3$ to 4. ** $p < 0.01$, *** $p < 0.001$ for comparison with SCF response.

both Bcl-2 and Bcl-xL and reduced levels of Bim (32), which has previously been shown to be critical for growth factor deprivation-induced mast cell apoptosis (34). As we observed that GSK3 β contributed to the regulation of ERK and SHP2 activity, we examined the expression of both antiapoptotic (Mcl-1, Bcl-xL, and Bcl-2) and proapoptotic (Bim, Bid, and Bad) proteins in the SCF-starved and nonstarved HuMCs following treatment with the GSK3 β -targeted and control shRNA. As shown in Fig. 7A, GSK3 β knockdown, if anything, increased the expression of Mcl-1, whereas SCF starvation reduced the expression of this antiapoptotic protein. However, the expression of Bcl-xL and Bcl-2 were reduced by both SCF starvation and GSK3 β knockdown, with further reduction observed with the combination of these approaches. In contrast, whereas SCF-enhanced the expression of Bim (Fig. 7B) but partially reduced the expression of Bid and Bad, GSK3 β knockdown reduced the expression of all three proteins. Nevertheless, the data as a whole suggested that GSK3 β acts as a prosurvival signal for human mast cells by regulating the expression of the antiapoptotic proteins Bcl-xL and Bcl-2 through the activation of ERK and SHP2. The reduced expression of the proapoptotic proteins following GSK3 β knockdown (Bim, Bid, and Bad) and, to a certain extent, by SCF starvation (Bid and Bad) was somewhat counterintuitive but may be a consequence of caspase cleavage (35, 36).

A small-molecule GSK3 β inhibitor decreases survival of the HMC1.2 human leukemia mast cell line

Finally, based on our conclusion that GSK3 β was an important mast cell prosurvival signal, we investigated whether a small molecule inhibitor of GSK3 β could be employed to reduce HMC1.2 survival. To date, CHIR 99021 is the most selective inhibitor of GSK3 β reported (37, 38). As can be seen in Fig. 8, this compound dose-dependently reduced the number of viable HMC1.2 cells after 24, 48, and 72 h in culture, thus providing evidence for

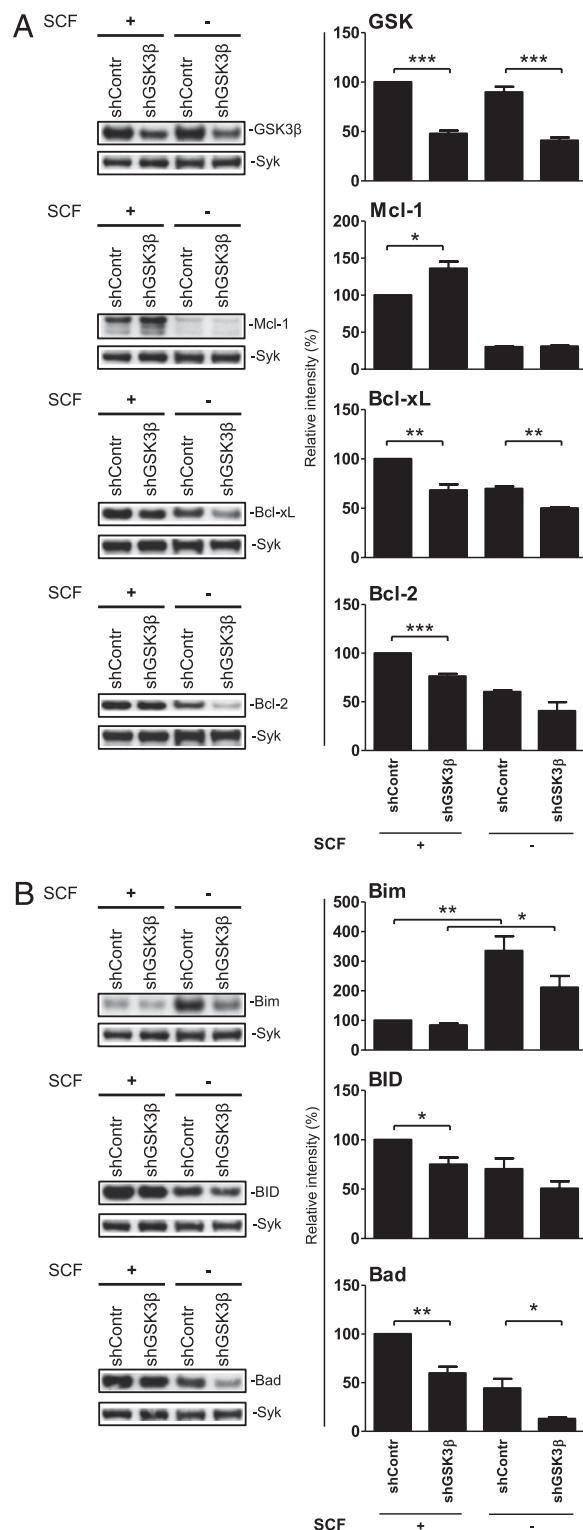


FIGURE 7. Effect of GSK3 β knockdown and SCF starvation on pro- and antiapoptotic proteins in primary HuMCs. HuMCs transduced with shContr or shGSK3 β A were (SCF⁻) or were not (SCF⁺) starved for 20 h. Whole-cell extracts were prepared and immunoblotted with GSK, Mcl-1, Bcl-xL, Bcl-2 (A), or Bim, BID, or Bad-specific (B) Abs. Immunoblotting with Syk-specific Ab was used as sample protein loading control. The relative intensities of the immunoreactive proteins (left panels) were normalized to Syk and evaluated (right panels). In all cases, $n = 3$ to 4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison with SCF response.

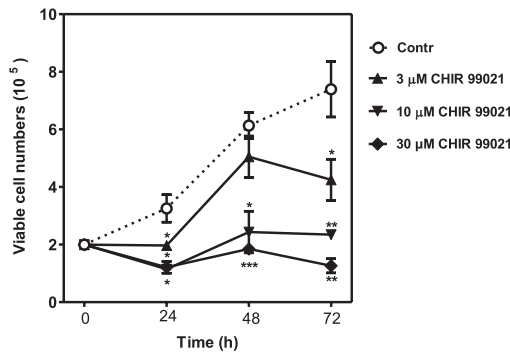


FIGURE 8. Effects of CHIR 99021 on HuMC1.2 cell line viability. HMC1.2 cells were cultured in the absence or presence of GSK3 β inhibitor (CHIR 99021; 3–30 μ M), and viability cell count was assessed by trypan blue dye exclusion after 24, 48, or 72 h. All data are presented as the mean $\pm$ SEM of three independent experiments conducted in triplicate. * p < 0.05, ** p < 0.01, *** p < 0.001 versus vehicle-treated control cells.

the potential of reducing mast cell burden by small-molecule targeting of GSK3 β .

Discussion

In this paper, we describe studies that support the conclusion that GSK3 β represents an essential prosurvival and/or antiapoptotic signaling element in human mast cells and that this may be a consequence of the dependency of SCF-mediated SHP2 and ERK activation, known mast cell survival signals, on GSK3 β activity.

Mast cell homeostasis represents a balance between mast cell proliferation/survival signal, and mast cell proapoptotic signals (13, 39). In mature terminally differentiated human mast cells, SCF-mediated KIT activation skews this balance to prosurvival. Similarly, the ability of recognized KIT inhibitors to downregulate the expansion and survival of HMC1.1 and 1.2 cells (40) indicates that the myeloproliferative/survival potential of these cells is, at least in part, a consequence of the presence of activating mutations in KIT in these cell lines. As would be expected for a putative prosurvival/antiapoptotic signal, we observed that GSK3 β is constitutively active in the HMC1.2 cells (Fig. 1) and in HuMCs (Fig. 2), as reported (15). This was evidenced by the phosphorylation of the activating Y²¹⁶ residue in GSK3 β and GS at S⁶⁴¹ under resting conditions in both the HMC1.2 cells and HuMCs. It is tempting to speculate that the constitutive activation of GSK3 β that we observed in the HMC1.2 cell line and the HuMCs may be a consequence of KIT activity. We have observed, for example, that there is a slight enhancement of GS phosphorylation at position S⁶⁴¹ in HuMCs coactivated via KIT and the Fc ϵ RI. However, the inconsistency of this response in HuMCs challenged with SCF alone (Fig. 2) and the lack of an increase in phosphorylation of GSK3 β at the activating Y²¹⁶ residue in response to SCF would suggest that GSK3 β activity in these cells is largely regulated independently of SCF. This conclusion is further supported by the limited ability of inhibitors of SCF-mediated signaling events to attenuate the constitutive GSK3 β activity.

The kinase(s) responsible for the phosphorylation of GSK3 β at Y²¹⁶ in the human mast cells remain(s) unclear. However, the studies conducted with PP2 would suggest that Src kinase family members are not responsible. It is evident, however, that the regulation of GSK3 β activity at any point in time represents a balance between negative and positive regulatory pathways (21). Although it is apparent that SCF can induce the phosphorylation of GSK3 β at the inhibitory site (S⁹), this was not associated with

a decrease in the phosphorylation of GS at S⁶⁴¹, which we have demonstrated requires the presence of GSK3 β for optimal phosphorylation in mast cells (15). From these data, we conclude that there is net constitutive activation of GSK3 β , and, based on the shRNA knockdown studies, it is evident that the constitutive GSK3 β activity present in both HMC1.2 cells (Fig. 1) and the HuMCs (Fig. 3) is essential for their survival.

The requirement for GSK3 β in mast cell homeostasis may be dependent on both its regulation of cell division and of antiapoptotic pathways. Although the BrdU assay conducted in the HMC1.2 cells indicates that the dependency of the survival of these cells on GSK3 β could be partly explained by prevention of cell division, the studies conducted on the nonproliferating HuMCs would indicate that GSK3 β also plays a major role in the prevention of mast cell apoptosis. This conclusion is supported by the close correlation between GSK3 β expression and HuMC survival as determined by trypan blue exclusion and the MTT assay (Fig. 3) and by the close correlation between the degree of GSK3 β knockdown and the indices of apoptosis, Annexin V staining, and caspase cleavage in the SCF-starved HuMCs (Fig. 4).

GSK3 β has been described to regulate multiple cellular events including cell growth, cell survival, metabolism, gene expression, and apoptosis (21, 41–43). However, in certain cases, including the regulation of growth factor- and mitogen-mediated responses, the described roles appear paradoxical in that GSK3 β may both positively and negatively regulate cellular processes through tightly coupled activation and/or inactivation. For example, in a variety of cell types including eosinophils (41), GSK3 β has been suggested to support cell survival downstream of the PI3K/Akt pathway. However, in studies conducted in both hematopoietic cells and in neuronal cells, it has been proposed that apoptosis induced by growth factor withdrawal or PI3K inhibition is mediated by GSK3 β (44–46). Furthermore, GSK3 β inhibition has been suggested to modulate radiation resistance in certain cancers as well as promoting tumor growth through stabilization of B-catenin (16, 47). Thus, as with the regulation of GSK3 β activity, GSK3 β -regulated responses may also represent a fine balance between negatively regulated and positively regulated signaling responses.

Our previous studies in which we explored the regulation of mast cell chemotaxis and cytokine production by GSK3 β (15) also indicate that the role(s) of GSK3 β in mast cell function are complex. Our results suggested, for example, that constitutively activated GSK3 β must be considered in the context of regulation of signaling events in addition to the potential roles of receptor-mediated upregulation and/or downregulation of GSK3 β activity (15). Certainly, in the current study, the constitutive activity of GSK3 β we observed, the inability of SCF to enhance this response, and the requirement for ongoing GSK3 β activity for cell survival would suggest that GSK3 β is also a prerequisite signal, rather than an inducible signal, for mast cell homeostasis.

Although PI3K and PI3K-regulated prosurvival pathways are recognized to be critical for mast cell homeostasis, our observation that the phosphorylation of AKT is unaffected by GSK3 β knockdown (Fig. 5) suggests that GSK3 β does not regulate cell proliferation/survival through the feedback regulation of PI3K activity. However, based on our previous results (15), it is possible that PI3K may, in part, contribute to the regulation of GSK3 β activity. It thus appears more likely that the ability of GSK3 β to function as a prosurvival factor is related to its requirement for the ability of SCF to induce SHP2 and ERK phosphorylation (Fig. 6) and potentially JNK phosphorylation (15). In mouse bone marrow-derived mast cells, SHP2, through the regulation of Rac/JNK, has been shown to be required for SCF-mediated mast cell prolifera-

tion and survival (28) and to be critical for growth factor-induced ERK activation (48). SHP2 is a ubiquitously expressed non-receptor protein tyrosine phosphatase that participates in signaling events downstream of receptors for growth factors, cytokines, hormones, and control cell growth, differentiation, migration, and death (48). Thus, activation of SHP2 and its association with Gab1 is critical for sustained ERK activation downstream of several growth factor receptors and cytokines (48). The necessity for GSK3 β in regulation of the MAPKs ERK and JNK in the manner described above may be due to priming of regulatory components of the MAPK pathways by GSK3 β (15). Consequently, a deficiency in GSK3 β would result in an inability of SCF, or indeed other stimuli, to regulate cellular responses via the MAPKs. However, it is also possible that SHP2 acts as a GSK3 β substrate, because GSK3 β -knockdown cells showed an impaired SCF-mediated activation of SHP2. As both SHP2 and ERK have been demonstrated to regulate the expression of antiapoptotic Bcl-2 family proteins (32, 33), our observation that GSK3 β knockdown resulted in the downregulation of these proteins (Fig. 7) provided evidence for the mechanism by which GSK3 β may function as a prosurvival signal in human mast cells. Such a mechanism would involve the requirement of SHP2 and ERK for constitutively active GSK3 β to promote cell survival through expression of the Bcl-2 family members Bcl-2 and Bcl-xL.

In summary, the data presented in this study provide evidence that GSK3 β is a key regulator of mast cell homeostasis in both neoplastic and primary cultured human mast cells through prevention of apoptosis. The data also indicate that the myeloproliferative capacity of the neoplastic HMC1.2 cells at least in part requires GSK3 β activity and that a small-molecule inhibitor (CHIR 99021) of GSK3 β activity effectively reduces HMC1.2 cell survival (Fig. 8). Thus, targeting GSK3 β may provide a mechanism for modulating mast cell survival and apoptotic pathways in myeloproliferative disorders as well as in the allergic inflammatory response.

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Disclosures

The authors have no financial conflicts of interest.

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