

p27^{Kip1} nuclear localization and cyclin-dependent kinase inhibitory activity are regulated by glycogen synthase kinase-3 in human colon cancer cells

Q Wang¹, Y Zhou¹, X Wang¹ and BM Evers^{*1,2}

The cellular mechanisms regulating intestinal differentiation are poorly understood. Sodium butyrate (NaBT), a short-chain fatty acid, increases p27^{Kip1} expression and induces cell cycle arrest associated with intestinal cell differentiation. Here, we show that treatment of intestinal-derived cells with NaBT induced G0/G1 arrest and intestinal alkaline phosphatase, a marker of differentiation, activity and mRNA expression; this induction was attenuated by inhibition of glycogen synthase kinase-3 (GSK-3). Moreover, treatment with NaBT increased the nuclear, but not the cytosolic, expression and activity of GSK-3 β . NaBT decreased cyclin-dependent kinase CDK2 activity and induced p27^{Kip1} expression; inhibition of GSK-3 rescued NaBT-inhibited CDK2 activity and blocked NaBT-induced p27^{Kip1} expression in the nucleus but not in the cytoplasm. In addition, we demonstrate that NaBT decreased the expression of S-phase kinase-associated protein 2 (Skp2), and this decrease was attenuated by GSK-3 inhibition. Furthermore, NaBT increased p27^{Kip1} binding to CDK2, which was completely abolished by GSK-3 inhibition. Overexpression of an active form of GSK-3 β reduced Skp2 expression, increased p27^{Kip1} in the nucleus and increased p27^{Kip1} binding to CDK2. Our results suggest that GSK-3 not only regulates nuclear p27^{Kip1} expression through the downregulation of nuclear Skp2 expression but also functions to regulate p27^{Kip1} assembly with CDK2, thereby playing a critical role in the G0/G1 arrest associated with intestinal cell differentiation.

Cell Death and Differentiation (2008) 15, 908–919; doi:10.1038/cdd.2008.2; published online 8 February 2008

The mucosal lining of the intestinal tract serves a number of important physiologic functions and is in a constant state of self-renewal. Intestinal stem cells, localized to the crypts, differentiate into one of four primary cell types with absorptive enterocytes making up the predominant terminally differentiated cell type in the villus.¹ In the process of differentiation, enterocytes acquire structural features of mature cells, such as microvilli, and express specific gene products such as intestinal alkaline phosphatase, a brush border enzyme.² The differentiated enterocytes then undergo a process of programmed cell death and are extruded into the gut lumen. The cellular mechanisms triggering differentiation and subsequent apoptosis of these epithelial cells are poorly understood.

For immature intestinal enterocytes to differentiate, the cells need to be in the G1 phase of the cell cycle.³ The mammalian cell cycle is regulated by the sequential activation and inactivation of a highly conserved family of cyclin-dependent kinases (CDKs).⁴ Progression through early to mid-G1 is dependent on CDK4 and possibly CDK6, and progression through late G1 and the S phase requires activation of CDK2. The activities of the CDKs can be inhibited by the binding of CDK inhibitory proteins including Cip/Kip family (p21^{Waf1}, p27^{Kip1}, and p57^{Kip2}) and INK4 family

(p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c}, and p19^{Ink4d}). p27^{Kip1} is regulated post-transcriptionally by proteolytic degradation. CDK2 binds to p27^{Kip1} and phosphorylates p27^{Kip1} on threonine 187. The phosphorylated p27^{Kip1} is recognized by S-phase kinase-associated protein 2 (Skp2), a component of the SCF complex, and subsequently degraded by ubiquitin–proteasome pathway.⁵ Butyrate, a short-chain fatty acid produced in the colonic lumen, is a histone deacetylase (HDAC) inhibitor that induces intestinal cell G0/G1 arrest and differentiation. Butyrate-induced intestinal cell differentiation is associated with the upregulation of p27^{Kip1}.⁶

Glycogen synthase kinase-3 (GSK-3) is an Akt substrate that is inhibited upon phosphorylation by Akt. GSK-3 has been implicated in multiple biological processes by phosphorylation of a broad range of substrates, including several transcription factors such as c-Myc, c-Jun and c-Myb and the translation factor eIF2B. Two isoforms of GSK-3, GSK-3 α and GSK-3 β , have been identified. As a downstream target of the phosphatidylinositol-3 kinase/AKT (PI3-kinase/Akt) pathway, GSK-3 activity regulates cell proliferation and differentiation.^{7,8} Previously, we have shown that inhibition of the PI3-kinase pathway enhances sodium butyrate (NaBT)-mediated intestinal cell differentiation.⁹

¹Department of Surgery, The University of Texas Medical Branch, Galveston, TX, USA and ²Sealy Center for Cancer Cell Biology, The University of Texas Medical Branch, Galveston, TX, USA

*Corresponding author: BM Evers, Department of Surgery, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0536, USA. Tel: 409 772 5612; Fax: 409 747 4819; E-mail: mevers@utmb.edu

Keywords: p27^{Kip1}; CDK; GSK-3; Skp2; human colon cancer cells; differentiation; NaBT

Abbreviations: CDK, cyclin-dependent kinase; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK-3, glycogen-synthase kinase-3; NaBT, sodium butyrate; PI3-kinase, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RNase, ribonuclease; Skp2, S-phase kinase-associated protein 2; β -gal, β -galactosidase

Received 03.8.07; revised 18.12.07; accepted 04.1.08; Edited by M Blagosklonny; published online 08.2.08

In this study, we investigated the role of GSK-3 in the process of intestinal cell differentiation using the human colon cancer cell line, HT29, which displays a multipotent phenotype and represents a well-characterized model of intestinal differentiation.^{9–11} We show that GSK-3 activity is required for NaBT-induced G0/G1 arrest, CDK2 inhibition, decreased Skp2 expression and increased p27^{Kip1} expression in the nucleus and binding to CDK2; expression of the constitutively active form of Akt or inhibition of GSK-3 significantly inhibited intestinal cell differentiation induced by NaBT. Our results indicate that GSK-3 regulates cell cycle progression, which may be through the regulation of nuclear p27^{Kip1} expression and binding to CDK2 in a Skp2 ubiquitination-dependent manner. Collectively, these results further indicate a contributory role for the PI3-kinase/Akt/GSK-3 pathway in the process of intestinal cell differentiation.

Results

Inhibition of GSK-3 attenuates NaBT-induced cell cycle arrest and HT29 cell differentiation. HT29 cells accumulate at the G0/G1 cell cycle checkpoint and differentiate to an enterocyte-like phenotype after treatment with NaBT.¹² Since GSK-3 contributes to the inhibition of cell cycle progression in differentiating osteoblasts,¹³ we tested whether GSK-3 plays a role in NaBT-induced HT29 cell cycle inhibition. As shown in Figure 1a, treatment with NaBT induced cells to accumulate at the G0/G1 cell cycle checkpoint as expected. Treatment with lithium chloride (LiCl), which inhibits GSK-3 in a Mg²⁺ competitive manner,¹⁴ increased the proportion of cells in the S phase. This increase may come from the increased Myc as GSK-3 β negatively regulates Myc protein expression¹⁵ and Myc is able to induce S-phase entry.¹⁶ Treatment with the combination of LiCl and NaBT reversed NaBT-mediated G1 cell arrest. Similar results were obtained after treatment with SB-216763, a potent inhibitor of GSK-3¹⁷ (data not shown). These results suggested that GSK-3 may play a role in NaBT-induced G1 arrest. Also, we noted that treatment with a combination of NaBT and LiCl resulted in an obvious increase in the percentage of cells at the G2/M phase, which is consistent with findings by Jin *et al.*¹⁸ using human leukemia cells. To determine whether NaBT resulted in cell death during the 24 h treatment period, protein was extracted to assess for either increased PARP cleavage or active caspase-3. As shown in Figure 1b, there was no increase in PARP cleavage and active caspase-3 until 48 h after NaBT treatment.

An important early event in the terminal differentiation of cells is their withdrawal from the cell cycle.^{11,19} Since GSK-3 plays a role in cell cycle arrest, we postulated that inhibition of GSK-3 may inhibit differentiation. Therefore, we examined the effects of GSK-3 inhibitors on the induction of NaBT-mediated intestinal alkaline phosphatase expression, a marker of intestinal differentiation. HT29 cells were pretreated with LiCl (Figure 1c and d) or SB-216763 (Figure 1e and f) at various concentrations for 30 min followed by treatment with NaBT. LiCl dose-dependently inhibited NaBT-induced intestinal alkaline phosphatase activity and expression. Consistent with

these results, SB-216763 blocked induction of intestinal alkaline phosphatase activity and mRNA expression by NaBT. Taken together, our results indicate that GSK-3 plays an important role in NaBT-mediated intestinal cell differentiation.

Akt regulates intestinal alkaline phosphatase activity and expression induced by NaBT.

Glycogen synthase kinase-3 is inactivated when it is phosphorylated downstream of Akt. Hence, it would be predicted that activation of Akt by PI3-kinase would be associated with inhibition of GSK-3 and, subsequently, inhibition of intestinal cell differentiation. To test this hypothesis, HT29 cells were infected with an adenovirus encoding the activated myristoylated form of Akt (Ad-Akt) or the adenoviral control vector encoding β -galactosidase (β -gal) at a multiplicity of infection (MOI) of 10 pfu/cell. Infection was carried out for 1 h followed by the replacement of fresh medium and an additional 24 h of incubation. Cells were treated in the presence or absence of NaBT and protein and RNA extracted for western and northern blot analysis, respectively (Figure 2). Infection with Ad-Akt increased expression of phosphorylated Akt, Akt and phosphorylated GSK-3 β protein (Figure 2a). As shown in Figure 2b and c, infection of HT29 cells with the Ad-Akt adenoviral vector alone had no effect on intestinal alkaline phosphatase activity and mRNA expression; however, infection with the Ad-Akt vector resulted in inhibition of intestinal alkaline phosphatase activity and mRNA expression induced by NaBT compared with NaBT and infection of the control (β -gal) adenovirus, which suggests that signaling through the PI3-kinase/Akt pathway regulates intestinal cell differentiation induced by NaBT treatment.

Treatment with NaBT increases the expression and activity of GSK-3 β in the nucleus.

To test whether GSK-3 was influenced by NaBT treatment, GSK-3 β activity was assayed by measuring the phosphorylation of recombinant tau, a well-characterized substrate of GSK-3.²⁰ Because GSK-3 β is located in both the cytosolic and nuclear compartments of cells but predominantly in the cytoplasmic compartment during the G1 phase,²⁰ nuclear and cytoplasmic proteins were fractionated from control and NaBT-treated cells and examined for GSK-3 β activity. NaBT treatment resulted in an obvious increase in the activity of nuclear GSK-3 β (Figure 3a). Since GSK-3 inhibition attenuated NaBT-mediated G1 arrest, these findings indicate a role for GSK-3 in NaBT-induced cell cycle arrest.

To analyze the mechanisms for increased GSK-3 β activity by NaBT treatment, Ser9-phosphorylated and Tyr216-phosphorylated GSK-3 β protein expression was determined by western analysis. Ser9 phosphorylation of GSK-3 β decreased GSK-3 β activity whereas Tyr216 phosphorylation increased GSK-3 β activity.²¹ NaBT treatment increased nuclear expression levels of total GSK-3 β expression and Tyr216-phosphorylated GSK-3 β expression without affecting their expression in the cytosol (Figure 3b). Interestingly, NaBT treatment increased Ser9-phosphorylated GSK-3 β protein expression in both the cytosolic and nuclear fractions. Similar results were obtained following treatment with other HDAC inhibitors, trichostatin A (TSA) and apicidin (Supplementary

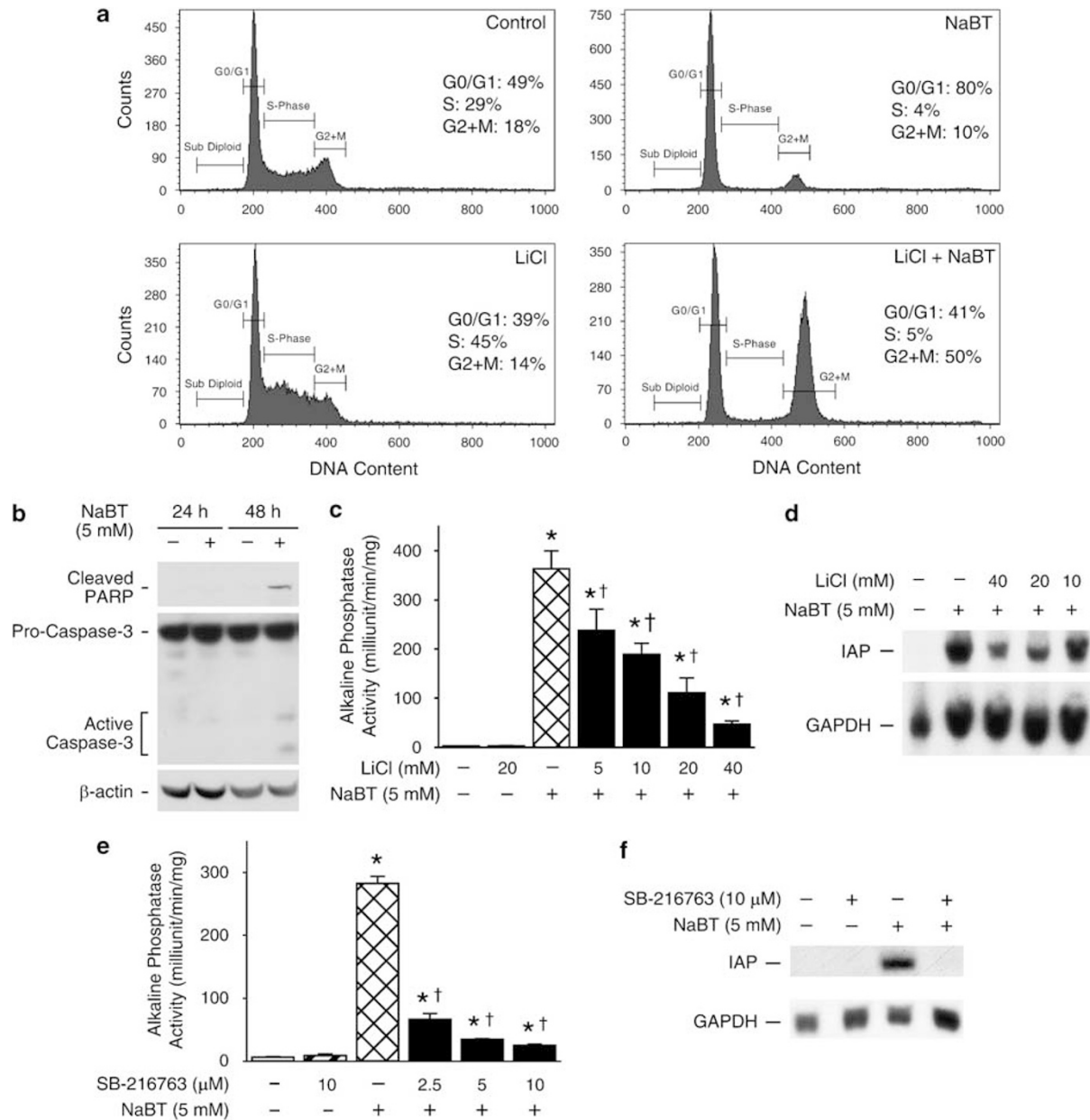


Figure 1 Effect of GSK-3 inhibition on NaBT-induced G0/G1 arrest and intestinal alkaline phosphatase activity and expression. (a) HT29 cells were pretreated with or without 10 mM LiCl for 30 min followed by combination treatment with 5 mM NaBT for 24 h. Cells were trypsinized, ethanol-fixed and subjected to propidium iodide staining for quantification of DNA content by flow cytometry. The percentage of cells in each phase of the cell cycle was determined. (b) HT29 cells were treated with NaBT (5 mM) for 24 h or 48 h. Both floating and attached cells were harvested, and whole-cell protein lysates were prepared for western blot analysis using anticlaved PARP or active caspase-3. The same membranes were stripped and reprobed with antiactin antibody. (c and e) HT29 cells were pretreated with or without 10 mM LiCl or 10 μ M SB-216763 for 30 min followed by combination treatment with 5 mM NaBT for 24 h. Cells were lysed and intestinal alkaline phosphatase activity was determined. (d and f) Total RNA was extracted from cells and northern blot analysis for intestinal alkaline phosphatase mRNA expression was performed. Hybridization was performed using radiolabeled cDNA probes specific for intestinal alkaline phosphatase and GAPDH. (Data represent mean \pm S.D.; * P < 0.05 versus control; † P < 0.05 versus NaBT alone.)

Figure 1a–c). In addition, HDAC inhibitors increased the activity of GSK-3 β in the nucleus as demonstrated by *in vitro* kinase assays (Supplementary Figure 1d). Our results suggest that HDAC inhibitors increase nuclear GSK-3 β activity regardless of the phosphorylation at Ser9.

Inhibition of GSK-3 overcomes NaBT-induced CDK2 inhibition. Progression through G1 is dependent on CDK2

and CDK4.⁴ To determine whether GSK-3 regulation of NaBT-mediated G1 cell cycle arrest occurs through CDK2 or CDK4 inhibition, HT29 cells were pretreated with LiCl (Figure 4a) or SB-216763 (Figure 4b) followed by combination treatment with NaBT for 24 h. Cell lysates were immunoprecipitated using anti-CDK2 or anti-CDK4 antibodies, respectively. CDK2 or CDK4 activity was determined by an *in vitro* kinase assay. Treatment with

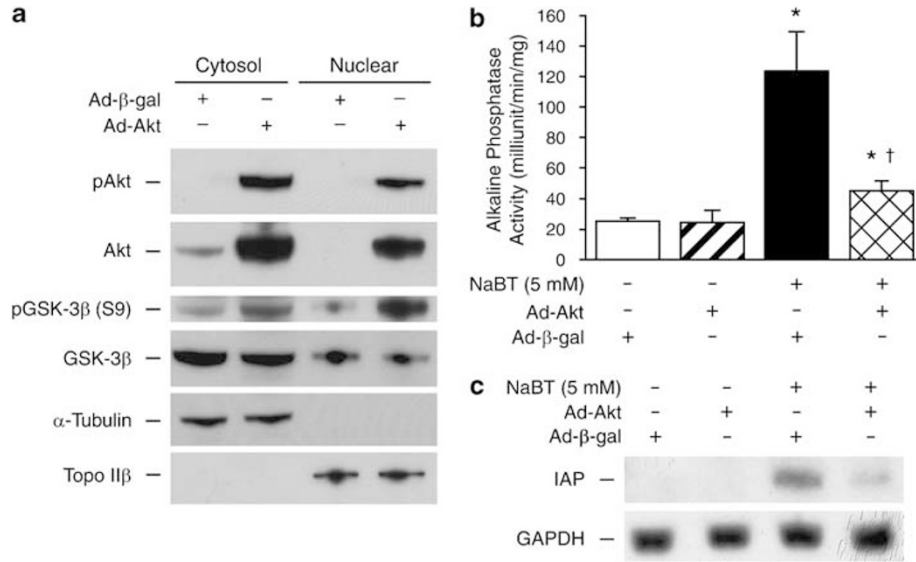


Figure 2 Overexpression of Akt inhibits NaBT-induced intestinal alkaline phosphatase activity and expression. HT29 cells were infected with a recombinant adenovirus encoding the myristoylated activated form of Akt or vector control encoding β-gal at an MOI of 10 pfu/cell. After 24 h, cells were treated with NaBT (5 mM) for 24 h and then extracted for RNA and protein. **(a)** Cytosol and nuclear proteins were extracted and resolved on SDS-PAGE and blotted with anti-phospho-Akt, -Akt, phospho-GSK-3β and GSK-3β. Membranes were stripped and reprobed with anti-α-tubulin and topoisomerase IIβ (Topo IIβ) for purity of the cytosolic and nuclear fractions, respectively, and equal loading. **(b)** Intestinal alkaline phosphatase activity was measured using an alkaline phosphatase determination kit from Sigma. (Data represent mean ± S.D.; **P* < 0.05 versus control; †*P* < 0.05 versus NaBT alone.) **(c)** Total RNA (40 μg) was fractionated, transferred to nitrocellulose membranes and probed with a labeled intestinal alkaline phosphatase cDNA; blots were stripped and reprobed with GAPDH

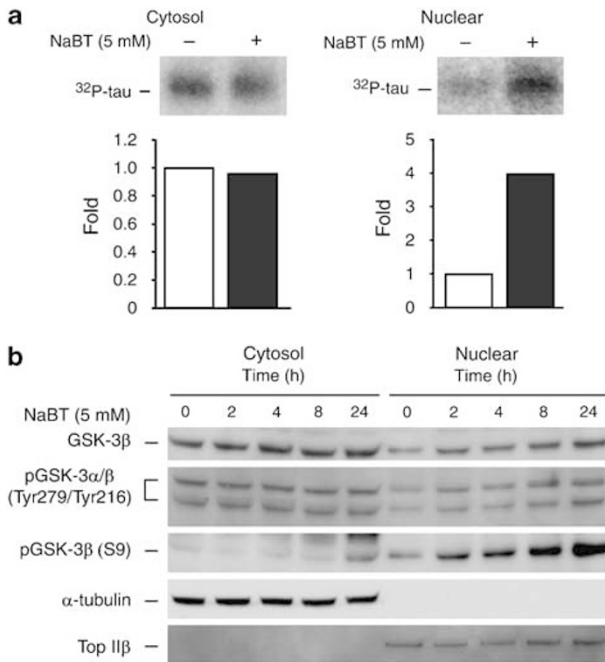


Figure 3 NaBT treatment activates GSK-3β in the nucleus. **(a)** HT29 cells were treated with (+) or without (-) 5 mM NaBT for 24 h, and harvested at the end of the treatment. Cytosol and nuclear fractions were prepared and GSK-3β activity was assayed by *in vitro* kinase assay using tau protein (³²P-tau) as substrate. Phosphorylated tau protein signals were quantitated densitometrically and expressed as fold change with respect to untreated control groups. **(b)** HT29 cells were treated with 5 mM NaBT for various times. Cytosolic and nuclear protein fractions were extracted and western blot was performed and resolved on SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies to GSK-3β, phospho-GSK-3β (Ser9), phospho-GSK-3α/β (Tyr278/Tyr216), α-tubulin or topoisomerase IIβ (Topo IIβ)

NaBT alone inhibited CDK2 activity (upper panels) but increased CDK4 activity (lower panels); inhibition of GSK-3 using LiCl or SB-216763 significantly attenuated NaBT inhibition of CDK2 activity. Previously, we showed that treatment with NaBT increased expression and activity of GSK-3β in the nucleus. Together, our results suggest that GSK-3β contributes to NaBT-mediated G1 cell cycle arrest through CDK2 inhibition.

GSK-3β regulates nuclear p27^{Kip1} protein expression. To further examine the mechanisms responsible for NaBT-mediated G1 arrest and CDK2 inhibition, cell cycle-regulatory mRNA expression was analyzed by ribonuclease (RNase) protection assay (RPA). Treatment with NaBT increased p57, p21^{Waf1} and p19 mRNA expression but decreased p53 and p16 (Figure 5a) and cyclin A and cyclin D1 mRNA expression (Figure 5b); however, treatment with LiCl slightly increased p21^{Waf1} mRNA expression but did not affect the expression levels of the other genes. Similar results were obtained when cells were treated with SB-216763 (data not shown). These results suggest that GSK-3 regulation of NaBT-mediated cell cycle arrest may not be through the transcriptional regulation of cell cycle-related genes.

Treatment with NaBT results in intestinal cell cycle arrest in G0/G1, which is correlated with p21^{Waf1} and p27^{Kip1} induction.²² To further analyze the mechanisms underlying GSK-3-associated cell cycle arrest, the expression of p21^{Waf1} and p27^{Kip1} protein was examined in HT29 cells treated with NaBT in the presence or absence of LiCl or SB-216763. Addition of LiCl (Figure 5c) or SB-216763 (Figure 5d) attenuated the induction of p27^{Kip1} but not p21^{Waf1} protein expression, suggesting that p27^{Kip1} participates in the cell cycle transitions regulated by GSK-3.

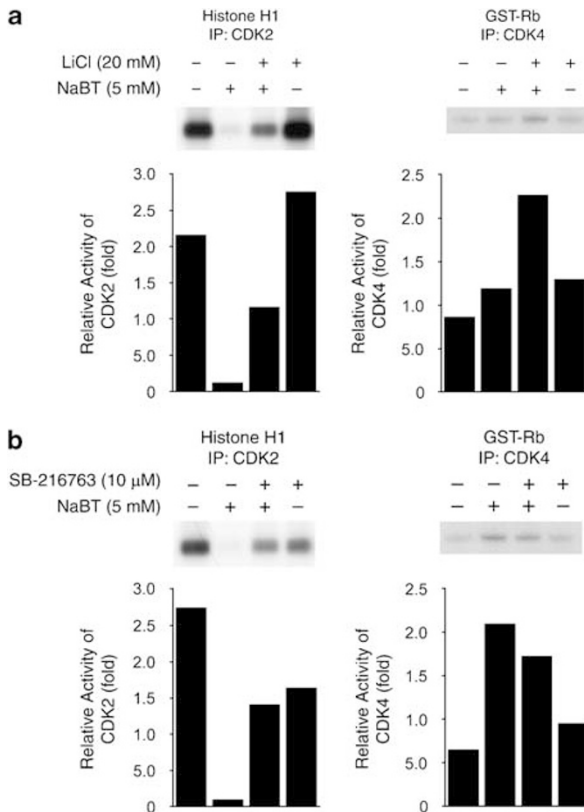


Figure 4 Inhibition of GSK-3 rescues NaBT-decreased CDK2 kinase activity. HT29 cells were pretreated with (+) or without (-) 10 mM LiCl (**a**) or 10 μM SB-216763 (**b**) for 30 min followed by combination treatment with 5 mM NaBT for 24 h. Protein extracts were immunoprecipitated (IP) with anti-CDK2 or anti-CDK4 antibodies. Resultant immune complexes were analyzed for CDK2 activity using histone H1 as substrate (upper panel) or for CDK4 activity using glutathione S-transferase retinoblastoma protein (GST-Rb) as substrate (lower panel). Phosphorylated histone H1 or GST-Rb protein signals were quantitated densitometrically and expressed as fold change with respect to untreated control groups

Once $p27^{Kip1}$ accumulates in the nucleus, it binds to CDK2 and inhibits CDK2 activity and eventually induces cell cycle arrest. We next determined $p27^{Kip1}$ protein expression in the cytosolic and nuclear fractions. NaBT (5 mM) increased $p27^{Kip1}$ protein expression in the cytosol from 4 to 24 h and in the nucleus at 24 h after treatment (Figure 6a). NaBT at dosages of 1.25–5 mM and 2.5–5 mM increased $p27^{Kip1}$ expression at 24 h in the cytosolic and nuclear fractions, respectively (Figure 6b). Purity of the fractions was confirmed by α -tubulin expression in the cytosol and topoisomerase II β expression in the nuclear fraction. Addition of LiCl (Figure 6c) or SB-216763 (Figure 6d) completely blocked NaBT-increased $p27^{Kip1}$ nuclear expression without affecting $p27^{Kip1}$ expression in the cytosol, suggesting a specific GSK-3 regulation of nuclear $p27^{Kip1}$ expression.

To further demonstrate the role of GSK-3 in the regulation of nuclear $p27^{Kip1}$ expression, we transfected cells with siRNA directed to GSK-3 β (Figure 6e). RNAi-mediated suppression of GSK-3 β attenuated nuclear $p27^{Kip1}$ induction by NaBT without affecting cytosolic $p27^{Kip1}$ induction. Immunoblotting confirmed that expression of GSK-3 β was significantly inhibited by GSK-3 β siRNA after transfection.

To confirm the role of GSK-3 in the regulation of nuclear $p27^{Kip1}$ expression, HT29 cells were infected with an adenovirus encoding the activated form of GSK-3 β (Ad-HA-GSK-3 β S9A) or the adenoviral control vector encoding β -galactosidase (Ad- β -gal) at an MOI of 10 pfu/cell. Infection was carried out for 1 h followed by the replacement of fresh medium and an additional 24 h of incubation. Cytosol and nuclear proteins were extracted and western blotting performed for analysis of $p27^{Kip1}$ expression. Infection of HT29 cells with the Ad-HA-GSK-3 β S9A adenoviral vector resulted in increased $p27^{Kip1}$ in the nuclear fraction (Figure 6f) without affecting the $p27^{Kip1}$ level in the cytosol, compared with infection of the control (β -gal) adenovirus. Overexpression of the active form of GSK-3 β was confirmed by western blotting using anti-HA antibody and *in vitro* kinase assay using tau protein as the substrate. These data suggest a critical role of GSK-3 in the regulation of nuclear $p27^{Kip1}$ expression. Together, our results indicate that GSK-3 participates in the regulation of the cell cycle through specific regulation of nuclear $p27^{Kip1}$ protein expression.

Involvement of Skp2 in GSK-3 β -mediated nuclear $p27^{Kip1}$ accumulation. Our results (Figure 5) indicate that NaBT-mediated induction of $p27^{Kip1}$ expression is likely dependent on a post-transcriptional mechanism. Recent evidence has shown that the Skp2 nuclear ubiquitination-dependent pathway plays an important role in regulation of nuclear $p27^{Kip1}$ expression.⁵ To determine if Skp2 may regulate $p27^{Kip1}$ expression in differentiating intestinal cells, we first examined the nuclear levels of Skp2. Treatment of HT29 cells with NaBT significantly inhibited Skp2 expression (Figure 7a and b); this inhibition was partially attenuated by LiCl (Figure 7c) or SB-216763 (Figure 7d), suggesting that GSK-3-mediated nuclear $p27^{Kip1}$ accumulation is through the decrease of Skp2 expression in the nucleus. To confirm the role of Skp2 in the regulation of nuclear $p27^{Kip1}$ expression, we transfected HT29 cells with siRNA directed to Skp2. Suppression of Skp2 increased nuclear $p27^{Kip1}$ expression in HT29 cells as compared to cells transfected with non-targeting control siRNA (Figure 7e). Immunoblotting analysis confirmed that expression of Skp2 was significantly inhibited by Skp2 siRNA.

To further confirm the role of GSK-3 in the regulation of Skp2 expression, we first transfected cells with siRNA directed to GSK-3 β . The suppression of GSK-3 β partially reversed nuclear Skp2 reduction by NaBT as compared to cells transfected with non-targeting control siRNA (Figure 7f). Next, we infected HT29 cells with Ad-HA-GSK-3 β S9A or Ad- β -gal at an MOI of 10 pfu/cell. Nuclear protein was extracted and immunoprecipitation performed using anti-Skp2 antibody. As shown in Figure 7g, infection of HT29 cells with the Ad-HA-GSK-3 β S9A adenoviral vector reduced Skp2 protein levels in the nuclear fraction compared with infection of the control (β -gal) adenovirus, suggesting that GSK-3 activity negatively regulates nuclear Skp2 expression resulting in the accumulation of nuclear $p27^{Kip1}$.

GSK-3 β regulates $p27^{Kip1}$ binding to CDK2. We have shown that NaBT increased $p27^{Kip1}$ protein expression, inhibited CDK2 activity and increased CDK4 activity

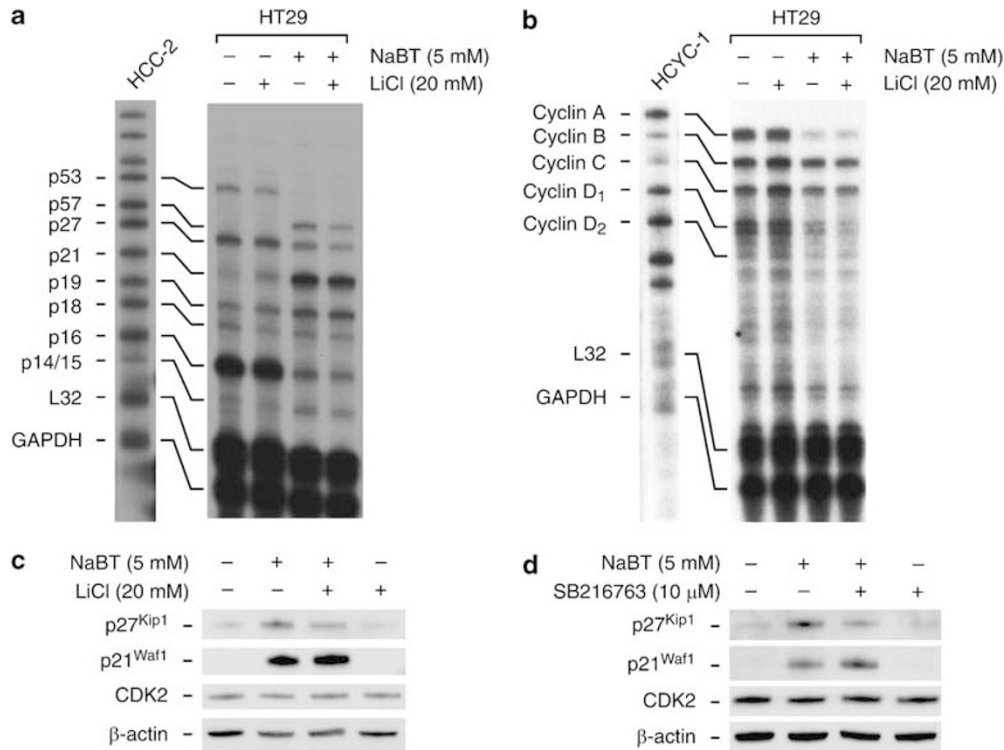


Figure 5 Determination of cell cycle mRNA expression. (a and b) Ribonuclease (RNase) protection assays were performed using RNA from HT29 cells treated with either 5 mM NaBT, 20 mM LiCl or combination of NaBT and LiCl for 24 h, hybridized with multi-probes for cell cycle-dependent kinase inhibitors (a: hCC-2) or cyclins (b: hCYC-1). (c and d) HT29 cells were pretreated with (+) or without (-) 10 mM LiCl (c) or 10 μM SB-216763 (d) for 30 min followed by combination treatment with 5 mM NaBT for 24 h. Whole-cell protein extracts were resolved on SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies to p27^{Kip1}, p21^{Waf1}, CDK2 or β-actin

(Figure 4). We next investigated p27^{Kip1} binding to CDK2 and CDK4. Extracts from control or NaBT-treated cells were immunoprecipitated with anti-CDK2 or anti-CDK4 antibodies or normal IgG as control, and the amount of p27^{Kip1} in the immune complexes assayed by western blotting using anti-p27^{Kip1} antibody. As shown in Figure 8a, NaBT treatment increased the amount of p27^{Kip1} in the complexes immunoprecipitated with anti-CDK2 but not in the complexes immunoprecipitated with anti-CDK4. Reprobing the filters with anti-CDK2 and anti-CDK4 antibodies confirmed that the immunoprecipitates from control and NaBT-treated cells contained the same amounts of CDK2 and CDK4 as expected from the analysis of the total content of these proteins in the cells. Thus, NaBT appears to produce a selective increase of p27^{Kip1} binding to CDK2.

To analyze whether inhibition of GSK-3 affects the association of p27^{Kip1} with CDK2, HT29 cells were pretreated with LiCl or SB-216763 followed by combination treatment with NaBT for 24 h; whole-cell extracts were immunoprecipitated with an anti-CDK2 antibody and the amount of p27^{Kip1} in the immune complexes was assayed by western blotting using an anti-p27^{Kip1} antibody. Treatment with GSK-3 inhibitors LiCl (Figure 8b) or SB-216763 (Figure 8c) blocked p27^{Kip1} binding to CDK2. These results suggest that GSK-3 is essential for NaBT-increased p27^{Kip1} binding to CDK2.

To further confirm the role of GSK-3 in the regulation of p27^{Kip1} association with CDK2, HT29 cells were infected with an adenovirus encoding the activated form of GSK-3β

(Ad-HA-GSK-3βS9A) or the Ad-β-gal at an MOI of 10 pfu/cell. Infection was carried out for 1 h followed by the replacement of fresh medium and an additional 24 h of incubation. Whole-cell protein was extracted and immunoprecipitation performed using an anti-CDK2 antibody. As shown in Figure 8d, infection of HT29 cells with the Ad-HA-GSK-3βS9A adenoviral vector resulted in an increased amount of p27^{Kip1} in the complexes immunoprecipitated with anti-CDK2 compared with infection of the control (β-gal) adenovirus, which suggests that GSK-3β is not only necessary in NaBT-mediated p27^{Kip1} binding to CDK2 but is also sufficient alone to increase the association of p27^{Kip1} and CDK2 in HT29 cells. Collectively, our results reveal an important role of GSK-3β in the regulation of p27^{Kip1} nuclear localization and CDK2 inhibitory activity.

Discussion

The PI3-kinase/Akt/GSK-3 signaling pathway has been implicated in the regulation of cell growth, apoptosis and differentiation of various cell types.^{9,23,24} Previously, we have shown that the inhibition of PI3-kinase enhances enterocyte-like differentiation of the HT29 and Caco-2 human colon cancer cells suggesting a role for PI3-kinase inhibition in intestinal cell differentiation.⁹ In our present study, we demonstrate that inhibition of GSK-3 by complementary approaches (i.e., chemical inhibition and constitutively active Akt overexpression) attenuates brush-border enzyme activity and expression, a measure of enterocyte-like differentiation,

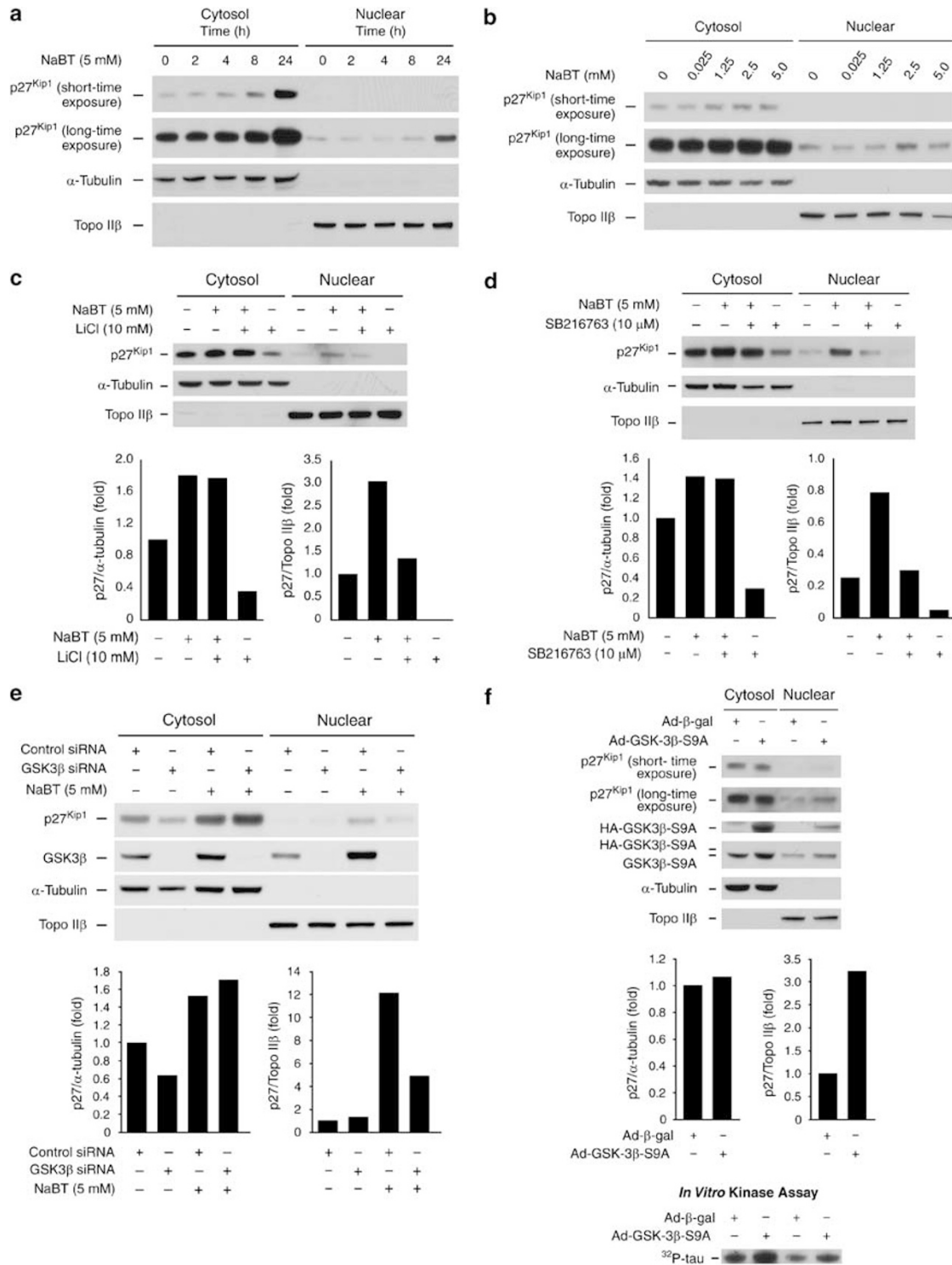


Figure 6 GSK-3 β regulation of nuclear p27^{Kip1} expression. (a and b) HT29 cells were treated with NaBT (5 mM) over a time course (a) or with various concentrations for 24 h. Cytosolic and nuclear protein fractions were extracted and western blot was performed and resolved on SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies to p27^{Kip1}, α -tubulin or topoisomerase II β (Topo II β). (c and d) HT29 cells were pretreated with (+) or without (-) 10 mM LiCl (c) or 10 μ M SB-216763 (d) for 30 min followed by combination treatment with 5 mM NaBT for 24 h. Cytosol and nuclear proteins were extracted for analysis of p27^{Kip1} protein expression. (e) HT29 cells were transfected with siRNA directed to GSK-3 β or control siRNA. Twenty-four hours after transfection, cells were treated with NaBT for an additional 24 h. Cytosol and nuclear proteins were extracted for analysis of p27^{Kip1} protein expression. Knockdown of GSK-3 β expression was confirmed by western blotting using anti-GSK-3 β antibody. (f) HT29 cells were infected with a recombinant adenovirus encoding the active form of GSK-3 β (Ad-HA-GSK-3 β S9A) or vector control encoding β -gal (Ad- β -gal) at an MOI of 10 pfu/cell. After 48 h incubation, cytosol and nuclear protein were extracted and western blotting performed using anti-p27^{Kip1}, anti-HA and anti-GSK-3 antibodies. Membranes were stripped and re-probed using anti- α -tubulin or topoisomerase II β (Topo II β) antibodies, respectively. GSK-3 β activity was assayed by *in vitro* kinase assay using tau protein (³²P-tau) as substrate (bottom panel). p27^{Kip1} signals were quantitated densitometrically and expressed as fold change with respect to α -tubulin or Topo II β

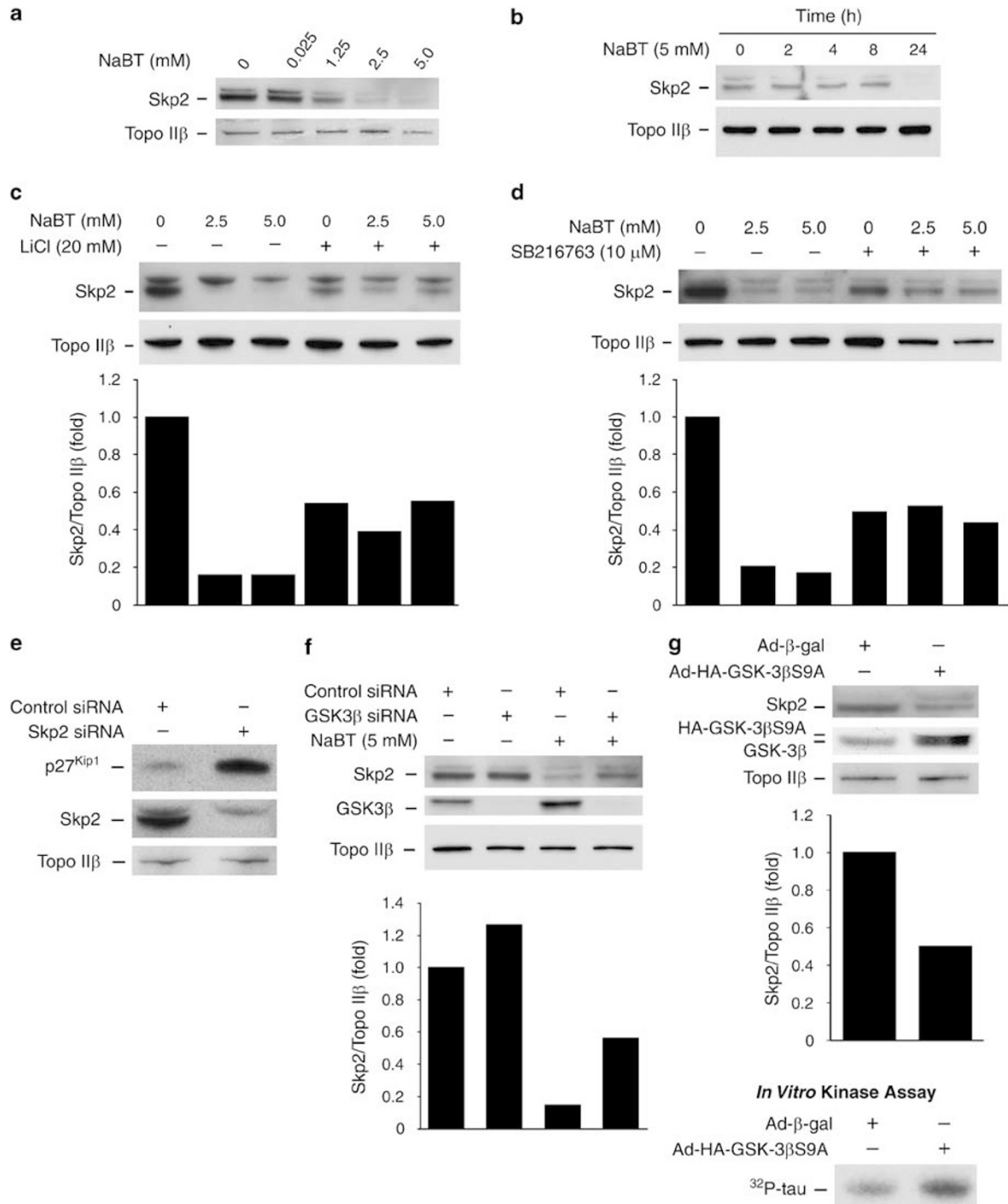


Figure 7 GSK-3 increases nuclear p27^{Kip1} through downregulation of the Skp2 ubiquitin ligase pathway. **(a and b)** HT29 cells were treated with various concentrations of NaBT for 24 h **(a)** or with NaBT (5 mM) over a time course **(b)**. Nuclear protein fractions were extracted and western blot was performed and resolved on SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies to Skp2 or Top IIβ. **(c and d)** HT29 cells were pretreated with (+) or without (-) 10 mM LiCl **(c)** or 10 μM SB-216763 **(d)** for 30 min followed by combination treatment with NaBT for 24 h. Nuclear protein was extracted for analysis of Skp2 protein expression. **(e)** HT29 cells were transfected with siRNA directed to Skp2 or control siRNA and incubated for 48 h. Nuclear protein was extracted and resolved on SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies to p27^{Kip1} and Skp2. Membranes were stripped and re-probed using anti-Top IIβ antibody as a loading control. **(f)** HT29 cells were transfected with siRNA directed to GSK-3β or control siRNA. Twenty-four hours after transfection, cells were treated with NaBT for an additional 24 h. Nuclear protein was extracted for analysis of p27^{Kip1} protein expression. Knockdown of GSK-3β expression was confirmed by western blotting using anti-GSK-3β antibody. **(g)** HT29 cells were infected with a recombinant adenovirus encoding the active form of GSK-3β (Ad-HA-GSK-3βS9A) or vector control encoding β-gal (Ad-β-gal) at an MOI of 10 pfu/cell and incubated for 48 h. Nuclear protein was extracted and resolved on SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies to Skp2 and GSK-3β. Membranes were stripped and re-probed using anti-Top IIβ antibody as a loading control. GSK-3β activity was assayed by an *in vitro* kinase assay using tau protein (³²P-tau) as substrate (bottom panel). Skp2 signals were quantitated densitometrically and expressed as fold change with respect to Top IIβ

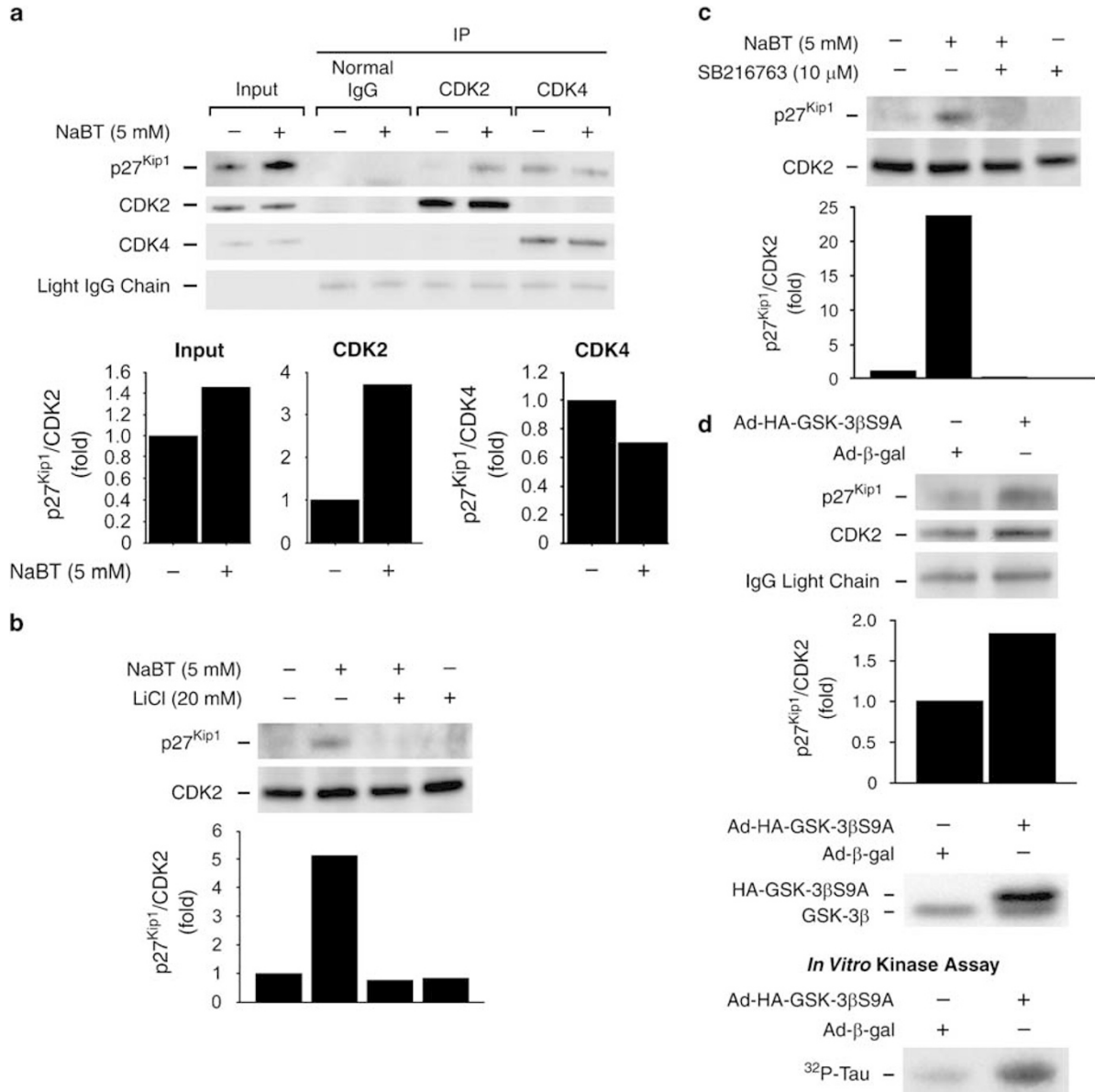


Figure 8 GSK-3 β regulation of p27^{Kip1} association with CDK2. (a) HT29 cells were treated with (+) or without (-) 5 mM NaBT for 24 h. Protein extracts were immunoprecipitated (IP) with anti-CDK2 or anti-CDK4 antibodies. Normal rabbit IgG was used as the control. The CDK2- or CDK4-associated p27^{Kip1} in the resultant immune complexes was analyzed by western blotting using anti-p27^{Kip1} antibody. Membranes were stripped and re-probed using anti-CDK2 or anti-CDK4 antibody as loading controls. (b and c) HT29 cells were pretreated with (+) or without (-) 10 mM LiCl (b) or 10 μ M SB-216763 (c) for 30 min followed by combination treatment with 5 mM NaBT for 24 h. Protein extracts were immunoprecipitated (IP) with an anti-CDK2 antibody. The CDK2-associated p27^{Kip1} in the resultant immune complexes was analyzed by western blotting using anti-p27^{Kip1} or antibody. Membranes were stripped and re-probed using an anti-CDK2 antibody as a loading control. (d) HT29 cells were infected with a recombinant adenovirus encoding the active form of GSK-3 β (Ad-HA-GSK-3 β S9A) or vector control encoding β -gal (Ad- β -gal) at an MOI of 10 pfu/cell. After 48 h incubation, whole-cell protein was extracted and immunoprecipitated (IP) with anti-CDK2 antibody (upper panel). The CDK2-associated p27^{Kip1} in the resultant immune complexes was analyzed by western blotting using anti-p27^{Kip1} antibody. Membranes were stripped and re-probed using anti-CDK2 antibody as a loading control. Overexpression of HA-tagged GSK-3S9A was confirmed by western blotting using anti-GSK-3 β antibody (lower panel). GSK-3 β activity was assayed by an *in vitro* kinase assay using tau protein (³²P-tau) as substrate (bottom panel). p27^{Kip1} signals were quantitated densitometrically and expressed as fold change with respect to CDK2 or CDK4

in the intestinal-derived HT29 cell line. Consistent with these results, infection of HT29 cells with recombinant adenovirus encoding constitutively active PI3-kinase significantly abrogated the NaBT-induced activity of intestinal alkaline phosphatase.²⁵ Taken together, our results suggest that intestinal cell differentiation is regulated by PI3-kinase/Akt/GSK-3 signaling pathway.

Cell proliferation and differentiation are traditionally perceived as reciprocal processes with cell-cycle withdrawal required for terminal differentiation.⁶ p27^{Kip1} plays an important role in the regulation of cell cycle progression and induction of intestinal cell differentiation.^{6,26} Genetic deletion of p27^{Kip1} but not p21^{Waf1} has been shown to impact intestinal cell differentiation whereas forced p27^{Kip1} expression leads to

differentiation.²⁷ These reports suggest that p27^{Kip1} is more important than p21^{Waf1} in the regulation of intestinal cell differentiation. In agreement with these findings, we showed that inhibition of GSK-3 β attenuated NaBT-mediated intestinal cell differentiation and, more importantly, that inhibition of GSK-3 β blocked NaBT-mediated nuclear p27^{Kip1} expression while overexpression of the active form of GSK-3 β increased nuclear p27^{Kip1} expression suggesting an important role in the regulation of intestinal cell differentiation through the regulation of nuclear p27^{Kip1} expression. Previously, we have shown that inhibition of the PI3-kinase pathway or overexpression of PTEN (phosphatase and tensin homolog deleted on chromosome 10) increases p27^{Kip1} and enhances intestinal cell differentiation.^{9,28} Consistent with our results in intestinal cells, activation of the PI3-kinase/Akt signaling pathway by CD28 and inactivation of GSK-3 by LiCl resulted in down-regulation of p27^{Kip1} and cell cycle progression in primary human T cells.²⁹

Recently, HDAC inhibitors have been shown to decrease the phosphorylation of Akt, and decrease the phosphorylation of GSK-3 β at Ser9.^{30,31} Ser9 phosphorylation of GSK-3 β decreases GSK-3 β activity whereas Tyr216 phosphorylation increases GSK-3 β activity.²¹ HDAC inhibitors decreased Akt phosphorylation significantly in the cytosol with only a minor effect noted in the nucleus. The induction of GSK-3 β activity in the nucleus may not be due to the dephosphorylated Akt, which should result in the dephosphorylation of GSK-3 β at Ser9, as treatment of HT29 cells with HDAC inhibitors actually increased GSK-3 β phosphorylation at Ser9. Although HDAC inhibitors increased total GSK-3 β protein expression as well as GSK-3 β phosphorylation at Tyr216, the mechanisms of GSK-3 β activation in the nucleus by HDAC inhibitors, which may involve novel pathways or additional regulatory elements for GSK-3 β , remain to be delineated further.

Nuclear GSK-3 β expression is related to cell cycle progression. GSK-3 β is predominantly cytoplasmic during the G1 phase, but a significant fraction enters the nucleus during the S phase.³² We showed that NaBT increased nuclear GSK-3 β expression and activity and induced G1 cell cycle arrest, which was attenuated by GSK-3 inhibition. Our results demonstrate a role of GSK-3 β in the regulation of G1 cell cycle progression. In addition to attenuation of G1 arrest, treatment with GSK-3 inhibitors increased the percentage of cells in the G2/M phase. It is possible that GSK-3 may regulate G2/M through the regulation of Chk1 phosphorylation, an important regulator of G2/M checkpoint, as SB-216763 and LiCl have been shown to enhance the Chk1 phosphorylation and G2/M arrest by etoposide.¹⁸ Another possibility is the involvement of nuclear factor- κ B (NF- κ B) regulation. Fluxes in GSK-3 β in the nucleus at critical periods may be related to the well-documented capacity of nuclear GSK-3 β to activate NF- κ B.⁷ Inhibition of NF- κ B has shown to increase the percentage of cells in the G2/M phase in HT29 cells.³³ Nuclear GSK-3 β has been shown to phosphorylate cyclin D1, thus resulting in the export of cyclin D1 from the nucleus.³² Cyclin D1 plays distinct roles in cell cycle progression through the G1 phase. In addition, cyclin D1 negatively regulates p27^{Kip1} expression through the regulation of p27^{Kip1} proteolysis.³⁴ Our results showing an increase of p27^{Kip1} nuclear accumulation and CDK2 inhibition by nuclear GSK-3 β suggest that multiple

mechanisms are involved in the GSK-3 β regulation of cell cycle progression through the G1 phase.

Histone deacetylase inhibitors, such as NaBT, induce cell cycle arrest in the G1 phase associated with the induction of cell cycle-dependent kinase inhibitors (e.g., p27^{Kip1} and p21^{Waf1}). NaBT can regulate p27^{Kip1} expression at either the transcriptional⁶ or the post-transcriptional level.³⁵ In our current study, we showed that NaBT increased p27^{Kip1} protein expression without affecting p27^{Kip1} mRNA expression, which suggested a post-transcriptional regulation of p27^{Kip1} by NaBT in HT29 cells. Focusing on p27^{Kip1} protein turnover and localization, several pathways have been suggested to regulate these cellular events.⁵ The pathway that has been best described involves the SCFSkp2 nuclear ubiquitination-dependent turnover. CDK2 phosphorylates p27^{Kip1} at T187 in late G1 phase and initiates Skp2-dependent p27^{Kip1} ubiquitination and proteasome degradation. Skp2 has been found to be important in tumor progression, and increased degradation of p27 by the Skp2-dependent mechanism is correlated with increasing colon cancer cell aggressiveness.³⁶ In our study, we showed that GSK-3 β regulation of nuclear p27^{Kip1} passes through negative regulation of Skp2 expression. GSK-3 β may regulate Skp2 expression through the inhibition of Notch signaling as Notch signaling has been shown to induce Skp2 expression in lymphoblastic leukemia cell lines³⁷ and phosphorylation by GSK-3 β downregulates Notch activity.³⁸ As activation of the PI3-kinase pathway increases Skp2 expression,³⁹ it is possible that PI3-kinase increases Skp2 expression through GSK-3 inhibition.

In addition to the Skp2-dependent ubiquitination pathway, there are other possible post-transcriptional mechanisms that regulate the stability of p27^{Kip1} and its binding to CDK2 in nucleus. For example, GSK-3 has been shown to phosphorylate p27^{Kip1} at the S160 and S161 positions resulting in increased p27^{Kip1} stability.⁴⁰ It is possible that NaBT treatment may result in the phosphorylation of p27^{Kip1} at S160 and S161 in the nucleus through GSK-3 activation leading to nuclear p27^{Kip1} accumulation and increased p27^{Kip1} binding to CDK2. Currently, however, there are no antibodies against S160 or S161 that can be used to test this possibility. Whether phosphorylation of p27^{Kip1} at S160 or S161 affects p27^{Kip1} binding to CDK2 remains to be elucidated.

In conclusion, our results support a contributory role for the PI3-kinase/Akt/GSK-3 pathway in the differentiation process of intestinal cells. Importantly, these data show that nuclear GSK-3 β decreased Skp2 expression and thus increased nuclear p27^{Kip1} accumulation and p27^{Kip1} binding to CDK2. Inhibition of CDK2 contributes to NaBT-mediated G1 cell cycle arrest and subsequently, to NaBT-mediated intestinal cell differentiation (summarized in Figure 9). Our present study identifies a novel mechanism whereby GSK-3 β affects nuclear p27^{Kip1} proteolysis and participates in the regulation of cell cycle progression in intestinal cells.

Materials and Methods

Materials. Sodium butyrate, TSA and LiCl were purchased from Sigma Chemical Company (St Louis, MO, USA). The GSK-3 inhibitor, SB-216763, was purchased from Tocris (Ellisville, MO, USA). Recombinant tau protein was from Panvera (Madison, WI, USA). Apicidin was from Calbiochem (San Diego, CA, USA). Adenovirus vectors encoding β -gal (Ad- β -gal; control) and the myristoylated active

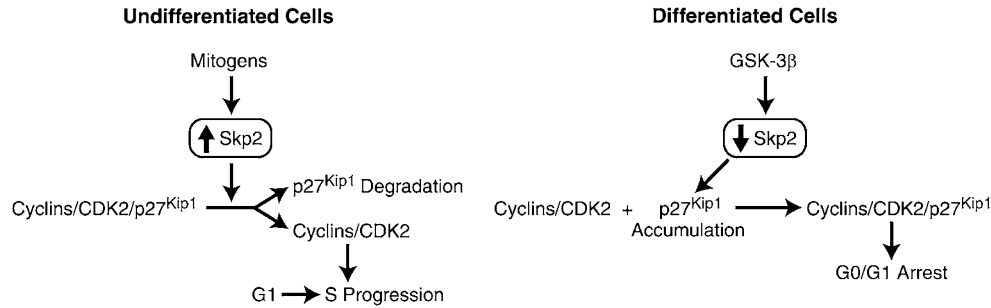


Figure 9 GSK-3 β /Skp2/p27^{Kip1}/CDK2 pathway model. Treatment with NaBT increases nuclear GSK-3 β expression and activity resulting in decreased Skp2 expression, leading to increased p27^{Kip1} expression, inhibition of CDK2 and G1 cell cycle arrest

form of Akt (Ad-Akt) were kindly provided by Dr. Wataru Ogawa (Kobe University School of Medicine, Chuo-ku, Japan). The adenovirus vector encoding the HA-tagged catalytically active mutant of GSK3 β (HA-GSK-3 β S9A) was provided by Dr. Morris J Birnbaum (University of Pennsylvania School of Medicine, Philadelphia, PA). Non-targeting control siRNA SMARTpool and the SMARTpool for targeting Skp2 and GSK-3 β were purchased from Dharmacon Inc. (Lafayette, CO, USA). [γ -³²P] ATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NY, USA). Nitrocellulose filters for northern blots were from Sartorius (Göttingen, Germany). The human intestinal alkaline phosphatase cDNA probe (pcD98#7) was from the ATCC (Rockville, MD, USA). The constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was obtained from Ambion (Austin, TX, USA) and used to ensure the integrity of the RNA samples analyzed by northern blot. Total RNA was isolated using Ultraspec RNA (Biotex Laboratories, Houston, TX, USA). RNase protection experiments were performed using the RPA-III kit from Ambion. Polyvinylidene difluoride (PVDF) membranes for western blots were purchased from Bio-Rad Laboratories (Hercules, CA, USA), and X-ray film was purchased from Eastman Kodak (Rochester, NY, USA). The enhanced chemiluminescence (ECL) system for western immunoblot analysis was from Amersham (Arlington Heights, IL, USA). Tissue culture media was obtained from GIBCO-BRL (Grand Island, NY, USA). All other reagents were of molecular biology grade and purchased from either Sigma or Amresco (Solon, OH, USA).

Antibodies. Rabbit anti-Akt, anti-phospho-Akt (Ser473) and rabbit anti-phospho-GSK-3 β (Ser9) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Rabbit anti-HA and rabbit anti- β -actin antibodies were from Sigma. Mouse monoclonal anti-GSK-3 β (clone 7), mouse monoclonal anti-p27^{Kip1} (clone 57), mouse anti-human Top II β (clone40/Topoll β) and mouse monoclonal anti-p21^{Waf1} (clone SX118) antibodies were purchased from BD Biosciences (San Diego, CA, USA). Mouse anti-phospho-GSK-3 (Tyr278/Tyr216) (clone 5G-2F) was from Upstate (Temecula, CA, USA). Rabbit anticlaved PARP antibody (ab4830) was from Abcam Inc. (Cambridge, MA, USA). Polyclonal anti-CDK2 (M2), anti-CDK4 (H-22), anti- α -tubulin (B-7), anti-caspase-3 (H-277) and anti-Skp2 (H-435) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and treatment. The human colon cancer cell line, HT29, was maintained in McCoy's 5A supplemented with 10% of fetal calf serum. The cells were pretreated with inhibitors for 30 min and then treated with NaBT in the presence or absence of inhibitors.

Subcellular fractionation and western blot analysis. Nuclear and cytosolic fractions were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL, USA). Cytosolic protein (80 μ g) or nuclear protein (20 μ g) was resolved on a 10% polyacrylamide gel and transferred to PVDF membranes as previously described.²⁸ Filters were incubated for 1 h at room temperature in blotting solution. Membranes were incubated overnight with primary antibodies followed by blotting with a horseradish peroxidase-conjugated secondary antibody for 1 h and visualized using an ECL detection system.

RNA isolation, northern blot analysis and RNase protection assays. Total RNA was isolated using the UltraspecTM RNA reagent. RNA (30 μ g) was run in 1.2% agarose/formaldehyde gels and transferred to supported nitrocellulose. Membranes were hybridized with a random-primed ³²P-labeled

human intestinal alkaline phosphatase cDNA probe. After hybridization with the GAPDH probe, a control for equality of RNA loading, membranes were washed again and signals detected by autoradiography. RiboQuant MultiProbe RNase Protection Assay System was used for the detection of multiple, specific mRNA species. ³²P-labeled antisense RNA probes were prepared using the Human Apoptosis hCC-2 and hCYC-1 Template Sets and hybridization performed according to the manufacturer's protocol.

Alkaline phosphatase enzyme activity assay. Protein was extracted from cells with lysis buffer and concentrations were determined. Cell lysates (20 μ l) were used to determine alkaline phosphatase activity by a commercially available kit (Sigma) as we have previously described.¹¹

Cell cycle analysis. The cell cycle distribution was determined by flow cytometric analysis of propidium iodide-labeled cells. Briefly, HT29 cells were harvested with trypsin. Cells were collected, washed twice with ice-cold PBS and fixed in ice-cold 70% ethanol. Cells were then washed twice with ice-cold PBS, resuspended in PBS containing 100 U/ml RNase A, incubated at 37°C for 30 min, stained with propidium iodide (20 μ g/ml) and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA), as we have previously described.¹¹

In vitro kinase assays. The activity of CDK2, CDK4 and GSK-3 was measured as we have described previously.^{11,20} Briefly, CDK2, CDK4 or GSK-3 β was immunoprecipitated from cytosolic (100 μ g of protein) or nuclear extracts (25 μ g of protein). Kinase activity was measured by incubating immunoprecipitated CDK2, CDK4 or GSK-3 β in 40 μ l of kinase buffer with either 4 μ g recombinant tau protein (to measure GSK-3 β -associated kinase activity) or 5 μ g of histone H1 (to measure CDK2-associated kinase activity) or GST-Rb (to measure CDK4-associated kinase activity) at 30°C for 30 min. The samples were then heated to 95°C for 5 min and resolved by 10% SDS-PAGE. The gels were dried, and the phosphorylated proteins were visualized by autoradiography.

Statistical analysis. Alkaline phosphatase activities were analyzed using the Kruskal–Wallis test due to heterogeneous variability among treatment groups in each experiment. All tests were assessed at the 0.05 level of significance. All statistical computations were carried out using SAS[®].

Acknowledgements. We thank Wataru Ogawa (Kobe University School of Medicine, Chuo-ku, Japan) for the adenovirus encoding β -gal (Ad- β -gal) and the myristoylated activated form of Akt (Ad-Akt), Morris J Birnbaum (University of Pennsylvania School of Medicine, Philadelphia, PA) for adenovirus vector encoding HA-tagged catalytically active mutant of GSK3 β (HA-GSK-3 β S9A). We also thank Karen Martin for manuscript preparation and Tatsuo Uchida for statistical analysis. This work was supported by grants RO1 DK48498, RO1 CA104748, R37 AG10885 and PO1 DK35608 from the National Institutes of Health.

- Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. *Am J Anat* 1974; **141**: 537–561.
- Traber PG. Differentiation of intestinal epithelial cells: lessons from the study of intestine-specific gene expression. *J Lab Clin Med* 1994; **123**: 467–477.

3. Carethers JM. Cell checkpoints and enterocyte differentiation: a recipe for sequential stages focus on "Caco-2 intestinal cell differentiation is associated with G1 arrest and suppression of CDK2 and CDK4". *Am J Physiol* 1998; **275**: C1191–C1192.
4. Sherr CJ. Mammalian G1 cyclins. *Cell* 1993; **73**: 1059–1065.
5. Koff A. How to decrease p27Kip1 levels during tumor development. *Cancer cell* 2006; **9**: 75–76.
6. Litvak DA, Evers BM, Hwang KO, Hellmich MR, Ko TC, Townsend Jr CM. Butyrate-induced differentiation of Caco-2 cells is associated with apoptosis and early induction of p21Waf1/Cip1 and p27Kip1. *Surgery* 1998; **124**: 161–169; discussion 169–170.
7. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 2000; **406**: 86–90.
8. Marcus EA, Kintner C, Harris W. The role of GSK3beta in regulating neuronal differentiation in *Xenopus laevis*. *Mol Cell Neurosci* 1998; **12**: 269–280.
9. Wang Q, Wang X, Hernandez A, Kim S, Evers BM. Inhibition of the phosphatidylinositol 3-kinase pathway contributes to HT29 and Caco-2 intestinal cell differentiation. *Gastroenterology* 2001; **120**: 1381–1392.
10. Heerdt BG, Houston MA, Augenlicht LH. Potentiation by specific short-chain fatty acids of differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res* 1994; **54**: 3288–3293.
11. Ding QM, Ko TC, Evers BM. Caco-2 intestinal cell differentiation is associated with G1 arrest and suppression of CDK2 and CDK4. *Am J Physiol* 1998; **275**: C1193–C1200.
12. Haton C, Lebrun F, Benderitter M, Griffiths NM. Maintenance of differentiation capacity of HT-29 cells after radiation exposure. *Int J Radiat Biol* 2005; **81**: 211–220.
13. Smith E, Coetzee GA, Frenkel B. Glucocorticoids inhibit cell cycle progression in differentiating osteoblasts via glycogen synthase kinase-3beta. *J Biol Chem* 2002; **277**: 18191–18197.
14. Phiel CJ, Klein PS. Molecular targets of lithium action. *Annu Rev Pharmacol Toxicol* 2001; **41**: 789–813.
15. Gregory MA, Qi Y, Hann SR. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J Biol Chem* 2003; **278**: 51606–51612.
16. Santoni-Rugiu E, Falck J, Mailand N, Bartek J, Lukas J. Involvement of Myc activity in a G(1)/S-promoting mechanism parallel to the pRb/E2F pathway. *Mol Cell Biol* 2000; **20**: 3497–3509.
17. Coghlan MP, Culbert AA, Cross DA, Corcoran SL, Yates JW, Pearce NJ *et al*. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* 2000; **7**: 793–803.
18. Jin ZH, Kurosu T, Yamaguchi M, Arai A, Miura O. Hematopoietic cytokines enhance Chk1-dependent G2/M checkpoint activation by etoposide through the Akt/GSK3 pathway to inhibit apoptosis. *Oncogene* 2005; **24**: 1973–1981.
19. Zavitz KH, Zipursky SL. Controlling cell proliferation in differentiating tissues: genetic analysis of negative regulators of G1- > S-phase progression. *Curr Opin Cell Biol* 1997; **9**: 773–781.
20. Watcharasit P, Bijur GN, Zmijewski JW, Song L, Zmijewska A, Chen X *et al*. Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. *Proc Natl Acad Sci USA* 2002; **99**: 7951–7955.
21. Forde JE, Dale TC. Glycogen synthase kinase 3: a key regulator of cellular fate. *Cell Mol Life Sci* 2007; **64**: 1930–1944.
22. Evers BM, Ko TC, Li J, Thompson EA. Cell cycle protein suppression and p21 induction in differentiating Caco-2 cells. *Am J Physiol* 1996; **271**: G722–G727.
23. Krasilnikov MA. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. *Biochemistry (Mosc)* 2000; **65**: 59–67.
24. Ptasznik A, Beattie GM, Mally MI, Cirulli V, Lopez A, Hayek A. Phosphatidylinositol 3-kinase is a negative regulator of cellular differentiation. *J Cell Biol* 1997; **137**: 1127–1136.
25. Tureckova J, Vojtechova M, Kucerova D, Velek J, Tuhackova Z. Sodium butyrate-mediated differentiation of colorectal cancer cells: regulation of PKCbeta1 by PI 3-kinase. *Int J Mol Med* 2005; **15**: 329–335.
26. Deschenes C, Vezina A, Beaulieu JF, Rivard N. Role of p27(Kip1) in human intestinal cell differentiation. *Gastroenterology* 2001; **120**: 423–438.
27. Quaroni A, Tian JQ, Seth P, Ap Rhys C. p27(Kip1) is an inducer of intestinal epithelial cell differentiation. *Am J Physiol Cell Physiol* 2000; **279**: C1045–C1057.
28. Wang Q, Zhou Y, Wang X, Chung DH, Evers BM. Regulation of PTEN expression in intestinal epithelial cells by c-Jun NH2-terminal kinase activation and nuclear factor-kappaB inhibition. *Cancer Res* 2007; **67**: 7773–7781.
29. Appleman LJ, van Puijenbroek AA, Shu KM, Nadler LM, Boussiotis VA. CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. *J Immunol* 2002; **168**: 2729–2736.
30. Alao JP, Stavropoulou AV, Lam EW, Coombes RC. Role of glycogen synthase kinase 3 beta (GSK3beta) in mediating the cytotoxic effects of the histone deacetylase inhibitor trichostatin A (TSA) in MCF-7 breast cancer cells. *Mol Cancer* 2006; **5**: 40.
31. Chen CS, Weng SC, Tseng PH, Lin HP. Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes. *J Biol Chem* 2005; **280**: 38879–38887.
32. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998; **12**: 3499–3511.
33. Rouet-Benzineb P, Aparicio T, Guilmeau S, Pouzet C, Descatoire V, Buysse M *et al*. Leptin counteracts sodium butyrate-induced apoptosis in human colon cancer HT-29 cells via NF-kappaB signaling. *J Biol Chem* 2004; **279**: 16495–16502.
34. Jonason JH, Gavrilova N, Wu M, Zhang H, Sun H. Regulation of SCF(SKP2) ubiquitin E3 ligase assembly and p27(KIP1) proteolysis by the PTEN pathway and cyclin D1. *Cell cycle* 2007; **6**: 951–961.
35. Chen JS, Faller DV. Histone deacetylase inhibition-mediated post-translational elevation of p27KIP1 protein levels is required for G1 arrest in fibroblasts. *J Cell Physiol* 2005; **202**: 87–99.
36. Timmerbeul I, Garrett-Engle CM, Kossatz U, Chen X, Firpo E, Grunwald V *et al*. Testing the importance of p27 degradation by the SCFskp2 pathway in murine models of lung and colon cancer. *Proc Natl Acad Sci U S A* 2006; **103**: 14009–14014.
37. Dohda T, Maljukova A, Liu L, Heyman M, Grandner D, Brodin D *et al*. Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines. *Exp Cell Res* 2007; **313**: 3141–3152.
38. Espinosa L, Ingles-Esteve J, Aguilera C, Bigas A. Phosphorylation by glycogen synthase kinase-3 beta down-regulates Notch activity, a link for Notch and Wnt pathways. *J Biol Chem* 2003; **278**: 32227–32235.
39. Auld CA, Caccia CD, Morrison RF. Hormonal induction of adipogenesis induces Skp2 expression through PI3K and MAPK pathways. *J Cell Biochem* 2007; **100**: 204–216.
40. Surjit M, Lal SK. Glycogen synthase kinase-3 phosphorylates and regulates the stability of p27kip1 protein. *Cell cycle* 2007; **6**: 580–588.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)