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Glucose-Induced β-Catenin Acetylation Enhances Wnt Signaling in Cancer

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SUMMARY

Nuclear accumulation of β-catenin, a widely recognized marker of poor cancer prognosis, drives cancer cell proliferation and senescence bypass and regulates incretins, critical regulators of fat and glucose metabolism. Diabetes, characterized by elevated blood glucose levels, is associated with increased cancer risk, partly because of increased insulin growth factor 1 signaling, but whether elevated glucose directly impacts cancer-associated signal-transduction pathways is unknown. Here, we show that high glucose is essential for nuclear localization of β-catenin in response to Wnt signaling. Glucose-dependent β-catenin nuclear retention requires lysine 354 and is mediated by alteration of the balance between p300 and sirtuins that trigger β-catenin acetylation. Consequently β-catenin accumulates in the nucleus and activates target promoters under combined glucose and Wnt stimulation, but not with either stimulus alone. Our results reveal a mechanism by which high glucose enhances signaling through the cancer-associated Wnt/β-catenin pathway and may explain the increased frequency of cancer associated with obesity and diabetes.

INTRODUCTION

"The dose makes the poison," wrote Paracelsus in the 16th century, and this is especially relevant for diabetics in reference to glucose. Less known, although widely accepted, is the fact that certain cancers are found with an increased frequency in the obese and/or diabetic population. Although it is increasingly important and strongly supported by epidemiological evidence (Gao and Yao, 2009; Gerber, 2009; Hillon et al., 2010; Khandekar et al., 2011; Renehan et al., 2008), little is known of the mechanistic origins of the diabetes- and obesity-cancer link.

High insulin levels, as an adaptation to insulin resistance at the onset of diabetes or as a result of exogenous administration, may promote cell growth and cancer by acting through the insulin growth factor receptor (IGFR) family (Pisani, 2008). However, hyperinsulinemia appears to be secondary to hyperglycemia and might not explain the increased cancer risk in the nondiabetic obese or hyperglycemic populations. An alternative possibility is that high serum glucose levels may directly modulate cancer-related signaling pathways, especially given the increased-glucose-consumption characteristic of cancer cells (Warburg et al., 1967).

The Wnt/β-catenin pathway is a global regulator of embryonic development, is required for tissue renewal in postembryonic animals (for example, in the maintenance of stem cells in intestinal crypts [Korinek et al., 1998]), and, when deregulated, can promote senescence bypass (Delmas et al., 2007), aberrant cell growth, and cancer (Barker et al., 2009; Kinzler and Vogelstein, 1996). As such, nuclear accumulation of β-catenin is one of the most widely recognized markers of malignancy. Significantly, a strong genetic association between specific polymorphisms in the *TCF7L2* (TCF4) gene, an effector of the Wnt pathway, and diabetes has been described (Lyssenko, 2008; Saxena et al., 2006), suggesting that Wnt/β-catenin may represent a link between diabetes and cancer.

The connections between Wnt signaling and diabetes, although increasingly recognized, are not straightforward. Upon glucose ingestion, the body responds by secreting insulin to lower blood glucose levels; failure to do so leads to diabetes. The earliest response to glucose ingestion is secretion by enteroendocrine cells of incretin hormones, which are required for normal glucose-dependent insulin secretion and also act on extrapancreatic tissues to control the global body energy balance (McIntosh et al., 2009). Glucose-dependent insulinotropic peptide (GIP) accounts for 75% of incretin plasma levels (Nauck et al., 1993) and is responsible for pancreatic glucose-dependent insulin secretion; its signaling is lost in diabetes (Flatt and Green, 2006). Wnt/β-catenin increases the expression of incretin genes *GIP* and *GCG* (proglucagon) in enteroendocrine cells, reviewed in García-Jiménez (2010), and modulates incretin signaling in pancreatic beta cells (Liu and Habener, 2008). Thus, control of global metabolism by Wnt signaling might be mediated at least partially through increased expression and signaling of incretins.

Wnt-induced inactivation of Glycogen synthase kinase 3β (GSK3β) leads to β-catenin stabilization (Wu and Pan, 2010) and is required for β-catenin entry into the nucleus, where it binds members of the T cell factor (TCF)/lymphoid enhancer factor (LEF) such as LEF-1 to activate the transcription of target

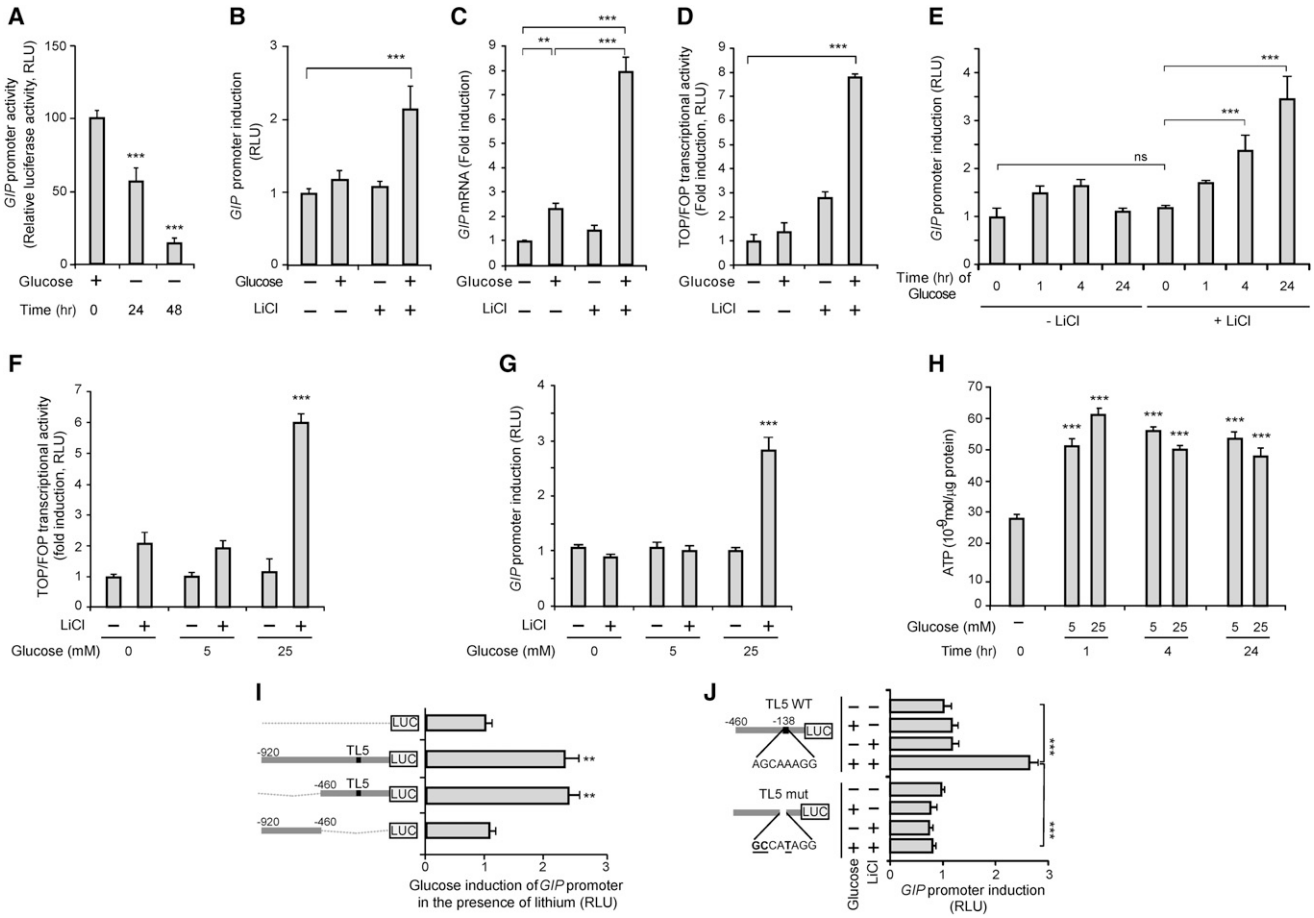

Figure 1. Glucose Enhances *GIP* Expression, Acting through WNT or LiCl Effectors

(A) STC-1 cells transfected with *GIP*-luciferase reporter were deprived of glucose as indicated.

(B) Cells deprived of glucose for 36 hr were stimulated with glucose and/or LiCl. Glucose concentration was 25 mM in all experiments unless otherwise indicated, and LiCl was 20 mM.

(C) qRT-PCR of *GIP* mRNA in cells treated as in (B). Values normalized with endogenous control (18S) are referred to as fold induction over untreated cells.

(D) TOPFlash/FOPFlash ratio after induction by glucose ± LiCl.

(E) Time course: cells cultured ± LiCl were stimulated with glucose for the times indicated.

(F) TOPFlash/FOPFlash ratio in response to glucose 5 mM or 25 mM.

(G) Transcriptional activity of *GIP* promoter as in (F).

(H) Time course of ATP levels after glucose 5 mM or 25 mM induction.

(I and J) Glucose induction of *GIP*-luciferase reporter on mutants: deletions (I), point mutation at Wnt-responsive TL5 element (J). Relative luciferase units (RLU) was calculated as fold induction relative to the corresponding control in all experiments. Values represent mean ± SEM; n ≥ 3. Statistical analysis in all the work was via ANOVA, n ≥ 3 unless otherwise stated. See also Figure S1.

genes (Clevers, 2006). The mechanisms that allow nuclear accumulation of β-catenin are poorly understood.

Here, we show that nuclear accumulation of stabilized β-catenin requires glucose in a wide range of human tumor-derived cell lines. Upon Wnt stimulation, glucose promotes the formation of a LEF-1/β-catenin complex that associates with the acetylase p300 and displaces the SIRT1 deacetylase, leading to increased β-catenin acetylation, its nuclear accumulation, and transcription activation. Consistently, the lysine 354 mutation in β-catenin abolishes glucose amplification of Wnt-dependent transcription. These results highlight a key mechanism that ties glucose levels to Wnt/β-catenin signaling, with important implications for both cancer and glucose homeostasis.

RESULTS

Wnt or LiCl Induction of *GIP* Gene Expression Requires Glucose

Glucose is the physiological stimulus for GIP secretion, but whether glucose induces *GIP* transcription is not known. The effect of glucose depletion on basal *GIP* expression was examined in enteroendocrine STC-1 cells transfected with a *GIP* promoter-luciferase reporter (García-Martínez et al., 2009) (Figure 1A). Glucose deprivation for 24 hr or 48 hr led to 50% and 80% reduction in basal *GIP* promoter activity, respectively. The effect of glucose addition on *GIP* expression was then evaluated and compared to the effect of lithium chloride (LiCl), which

mimics Wnt-3a signaling in STC-1 cells to induce *GIP* expression through a LEF/Tcf element (García-Martínez et al., 2009). Surprisingly, neither LiCl nor glucose alone induced the *GIP* promoter, but in the presence of LiCl, glucose induced a 2.2-fold increase in *GIP*-promoter activity (Figure 1B). Likewise, combined glucose and LiCl increased endogenous *GIP* messenger RNA (mRNA) by 8-fold (Figure 1C), whereas either stimulus alone promoted a very modest induction of endogenous *GIP*, as determined using quantitative RT-PCR (qRT-PCR). These results suggest that glucose targets molecules regulated by Wnt or LiCl and explain why induction of *GIP* by glucose has previously been so difficult to observe in enteroendocrine STC-1 cells.

A LEF/Tcf Response Element Mediates Glucose Induction of the *GIP* Promoter

If glucose regulates the Wnt signaling pathway, it should alter the responsiveness of a consensus Wnt-dependent element. TOPFlash, bearing eight copies of the consensus LEF/Tcf binding site or the mutated FOPFlash reporters (Korinek et al., 1998), was transfected into STC-1 cells. LiCl modestly increased the TOP/FOP ratio 2.5-fold (Figure 1D), whereas glucose alone was unable to activate this promoter. However, glucose substantially amplified the effect of LiCl, increasing the TOP/FOP ratio up to 8-fold. Transcriptional synergy between glucose and LiCl was significant between 1 and 4 hr and increased up to 24 hr (Figure 1E). Importantly, glucose was also required for activation of the *GIP* promoter by LiCl or Wnt (Figures S1A and S1B available online), and the synergy was independent of the order of addition. Importantly, only concentrations that mimic hyperglycemia (a glucose of 25 mM and not 5 mM) synergized with LiCl for activation of the TOPFlash (Figure 1F) or *GIP* (Figure 1G) reporters. Because both concentrations of glucose increased ATP levels to a similar extent (Figure 1H) and independently of the presence of LiCl (Figures S1C and S1D), the results suggest that the transcriptional synergy observed with glucose and LiCl or Wnt is specific to high glucose and is not accounted for by increased ATP production.

Because LiCl induces *GIP* expression in enteroendocrine cells through stabilization of the Wnt effector β-catenin that is targeted to the proximal *GIP* promoter (García-Martínez et al., 2009), we asked whether glucose also acted through this natural Wnt-responsive element. To this end, we transfected a series of *GIP* promoter deletion mutants and challenged them with glucose. Deletion of the promoter region containing the previously reported Wnt-dependent TL5 element abrogated the response to glucose (Figure 1I). Mutagenesis of the TL5 element confirmed that its integrity is required for glucose induction (Figure 1J). Thus, high glucose enhances LiCl- or Wnt-dependent *GIP* expression through molecules that bind the Wnt-dependent TL5 element. Significantly, the effects of the WNT glucose combination were not restricted to the *GIP* promoter, but were also observed on TOPFlash and other WNT-responsive genes (see also below).

Glucose Is Required for Nuclear β-Catenin Accumulation upon Its Stabilization by LiCl or Wnt-3a

Given that Wnt-3a (or LiCl) induces *GIP* promoter activity and cytosolic accumulation of the strong transcriptional coactivator β-catenin, confocal microscopy was used to study whether glucose regulates β-catenin subcellular distribution. The results (Figure 2A) indicate that β-catenin staining was restricted to focal adhesions in cells cultured in the absence of LiCl or Wnt and glucose (control) and that glucose alone did not alter the location of β-catenin. Lamin B (red) staining delimits the nuclear membrane. Wnt-3a or LiCl alone displaced β-catenin from the cell periphery and increased β-catenin accumulation in the cytoplasm, but not in the nucleus. Strikingly, addition of glucose (25 mM) to cells cultured with Wnt-3a resulted in substantial nuclear accumulation of β-catenin. Nuclear accumulation of β-catenin occurred early, between 1 hr and 4 hr after glucose addition, and was reproduced upon the addition of glucose to cells treated with LiCl, although the proportion of β-catenin retained in the cytoplasm was higher than with Wnt-3a.

Western blotting of fractionated cell extracts (Figure 2B) confirmed that LiCl alone induced elevated cytoplasmic β-catenin, but nuclear accumulation of β-catenin required both glucose and LiCl (Figure 2B). Lamin B1 and GAPDH are shown as nuclear and cytoplasmic fractionation controls, respectively, and total ERK is shown as the loading control. Only 25 mM of glucose (not 5 mM) triggered nuclear accumulation of β-catenin in the presence of LiCl (Figure S2A), consistent with the transcriptional induction of the TOPFlash and *GIP* reporters (Figures 1F and 1G). Thus, LiCl and Wnt-3a induce cytosolic β-catenin accumulation, but nuclear accumulation requires high glucose in addition.

Both LiCl and Wnt-3a induce Ser9 phosphorylation of GSK3β to inhibit GSK3β-driven β-catenin degradation and lead to β-catenin cytosolic accumulation (time courses, Figures S2B and S2C). However, nuclear accumulation of β-catenin required the addition of high glucose for at least 1 hr and remained up to 24 hr (Figure 2C). Importantly, alternative GSK3β inhibitors, BIO and SB 216763 instead of Wnt or LiCl, also cooperated with glucose to induce β-catenin nuclear accumulation (Figure S2D) and transcriptional activity (Figure S2E). Consistently, expression of a kinase-inactive (KI) mutant of GSK3β also led to β-catenin-activated transcription in cells cultured with high glucose (Figure S2F). Neither LiCl nor high-glucose effects were mediated through altered Akt phosphorylation (Figure S2G), compared with insulin used as positive control. Moreover, glucose alone did not inactivate GSK3β, because neither pGSK3β (Ser 9) nor β-catenin levels were increased unless LiCl was also present at any time or dosage (Figures S3A–S3B).

Thus, LiCl or Wnt-3a inhibits GSK3β, inducing cytosolic accumulation of β-catenin, which allows high glucose to induce nuclear β-catenin accumulation, and neither cytosolic nor nuclear β-catenin accumulation relies on Akt activation.

Importantly, glucose-induced nuclear accumulation of β-catenin (Figure S3A) occurred between 1 hr and 4 hr, matched the time required for transcriptional induction as shown in Figure 1E, and was specific to high glucose, in that using glutamine as an alternative carbon source did not substitute for glucose in promoting nuclear accumulation of β-catenin (Figure S3C). Glucose targeted β-catenin specifically and did not alter the control of other transcription factors by growth factors, such

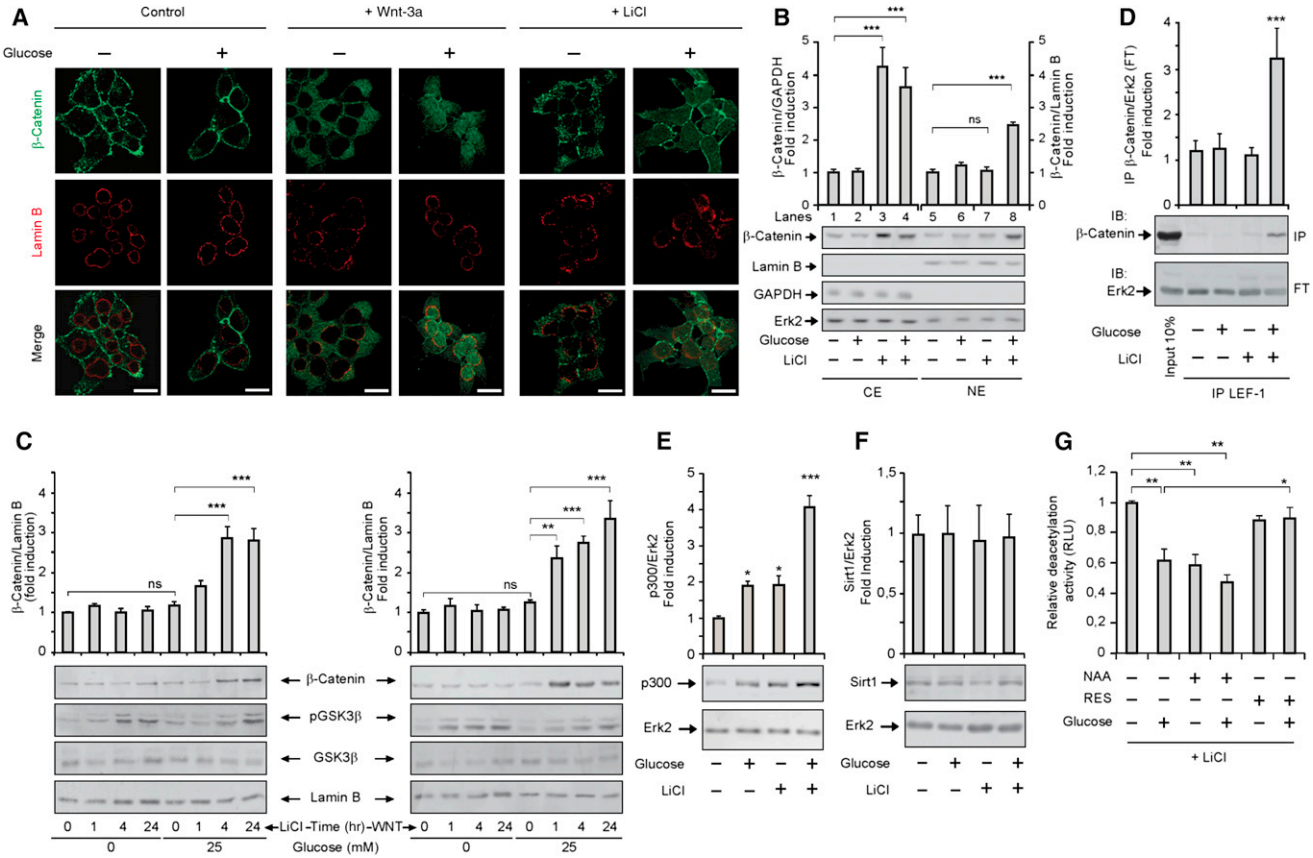

Figure 2. Glucose Promotes LEF-1/β-Catenin Interaction and Nuclear Accumulation

STC-1 cells cultured as in Figure 1B.

(A) β-catenin and Lamin B, confocal imaging. Bars represent 25 μm.

(B) β-catenin accumulation in cytoplasmic (CE) or nuclear (NE) extracts, representative western blot and statistical analysis. Values represent mean ± SEM in (B)–(G), n ≥ 3. Fractionation and loading controls: GAPDH (cytoplasmic), Lamin B (nuclear), and Erk2 (both).

(C–F) Representative western blots and statistical analysis of nuclear extracts from STC-1 cultured as indicated.

(C) Time course upon LiCl or WNT-3A addition.

(D) Anti-LEF-1 immunoprecipitation, Input (10%); Erk2 in the flow through serves as loading control.

(E) p300 levels.

(F) SIRT1 levels.

(G) NAD^+-dependent deacetylase activity in STC-1 cells cultured with sirtuin inhibitor NAA (300 μM) or enhancer RES (50 μM). Relative luciferase units (RLU) was calculated as fold induction relative to the corresponding control. See also Figures S2– S5.

as FoxO1 regulation by Insulin or Smads regulation by transforming growth factor β (TGFβ) (data not shown).

Taken together, the results thus far indicate that neither Wnt or LiCl alone nor high glucose alone can trigger nuclear accumulation of β-catenin. However, high glucose will promote nuclear accumulation of β-catenin and activation of its target promoters if β-catenin is stabilized by prior inactivation of GSK3β with Wnt-3a or LiCl.

Upon Cytosolic β-Catenin Accumulation, Glucose Favors β-Catenin and LEF-1 Interaction and Nuclear Retention

β-catenin is unable to bind directly to DNA but activates incretin expression through an element that binds Wnt effectors such as LEF-1. Combined glucose and LiCl do not increase LEF-1 expression (J.M.G.-M. and C.G.-J., unpublished data) but lead to nuclear accumulation of β-catenin, despite lacking a classical nuclear localization signal (NLS). Because LEF-1 possesses its own NLS (Huber et al., 1996) and is able to interact with β-catenin, we asked whether glucose favored LEF-1 and β-catenin interaction as a possible mechanism for the nuclear accumulation of β-catenin. Immunoprecipitation experiments indicated that LiCl and glucose cooperate to selectively favor nuclear LEF-1 and β-catenin interaction, and neither glucose nor LiCl alone allowed complex formation (Figure 2D). Overexpression of LEF-1 in LiCl-stimulated cells induced nuclear accumulation of β-catenin, even in the absence of glucose (Figure S4A), and increased *GIP*-promoter activity (Figure S4B), whereas a dominant negative LEF-1 (DN-LEF-1) unable to bind β-catenin blocked the induction of *GIP* transcription by LiCl (Figure S4C) or β-catenin (Figure S4D). Thus, the availability of LEF-1 may be critical for LEF-1 and β-catenin interaction and nuclear accumulation.

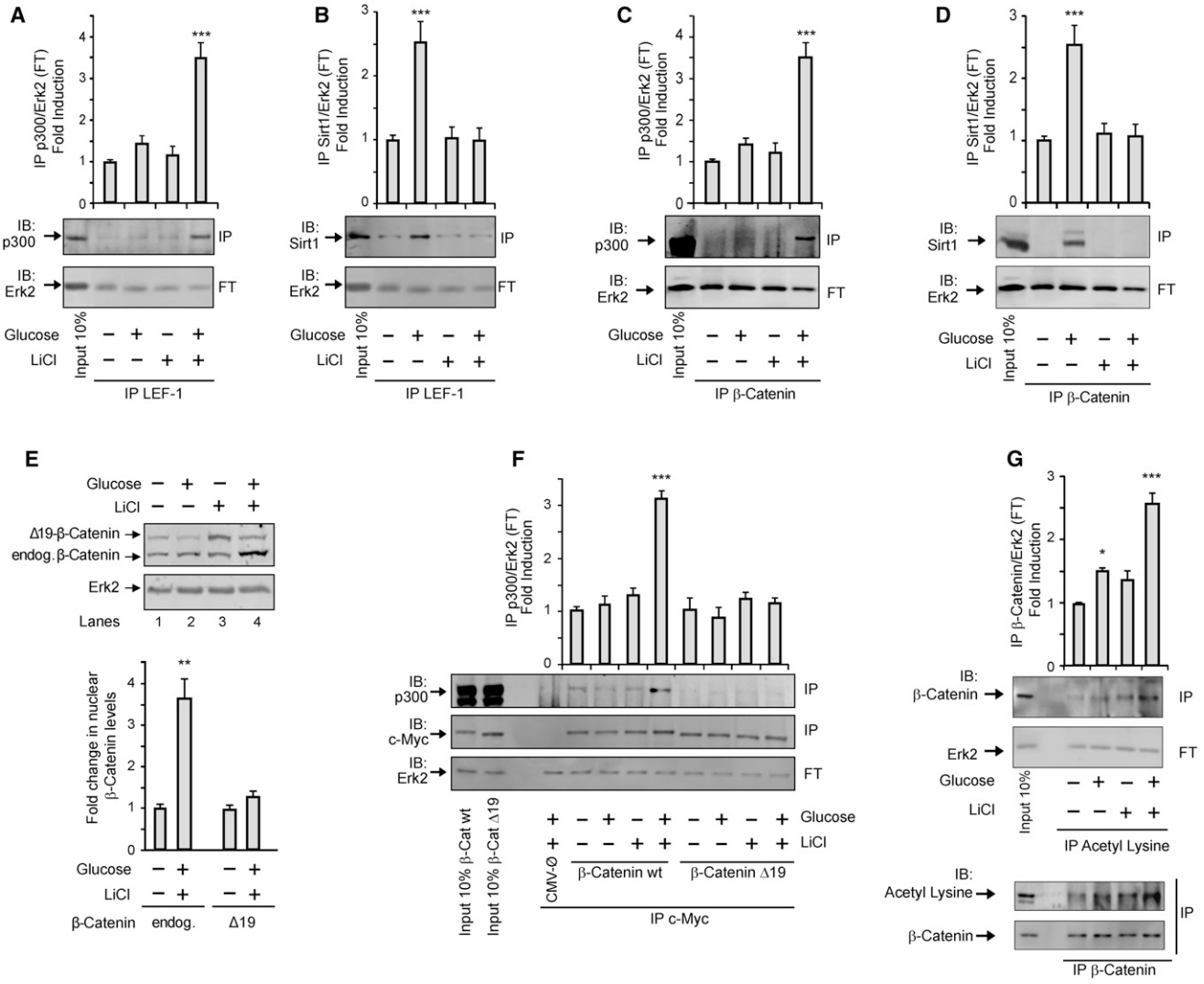

Figure 3. Glucose and LiCl Induce Nuclear Accumulation of Acetylated β-Catenin through Interactions with p300 and Sirtuins

Representative immunoblots (IB) with the antibodies indicated on the left of each panel and quantification; values represent mean ± SEM, n = 3 in all panels.

(A–D, F, and G) Immunoprecipitation of STC-1 nuclear extracts with the antibodies indicated at the bottom (e.g., IP LEF-1 in A). Input (10%) and Erk2 in flow through (as loading control) are shown.

(E) Nuclear extracts from STC-1 cells transfected with myc-tagged Δ19 β-catenin and treated as indicated.

(F) Nuclear extracts from STC-1 cells transfected with myc-tagged WT or Δ19 β-catenin.

Glucose and LiCl Cooperate to Increase Nuclear p300 Levels and Inhibit Sirtuin Activity

Given that acetylation of β-catenin through p300 or CBP (Wolf et al., 2002; Xu and Kimelman, 2007) and its deacetylation by SIRT1 (Firestein et al., 2008) impacts its oncogenicity, we examined whether combined LiCl and glucose treatment altered the levels of the acetylase p300 and the deacetylase SIRT1. Importantly, combined LiCl and glucose additively increased p300 levels (Figure 2E), and although SIRT1 levels were unaffected (Figure 2F), combined LiCl and glucose cooperated to reduce sirtuin activity by 40% (Figure 2G). Glucose or LiCl alone did not reduce sirtuin activity (data not shown). Upon LiCl treatment, the effect of glucose on sirtuin activity was largely abolished by the sirtuin enhancer resveratrol (RES) and was reproduced by the sirtuin inhibitor nicotinamide (NAA). Together, these results suggest that combined glucose and LiCl treatments cooperate to profoundly alter the acetylation balance by increasing levels of the acetylase p300 and reducing the deacetylase activity of sirtuins.

Glucose and LiCl Cooperate to Favor LEF-1, β-Catenin, and p300 Interactions, β-Catenin Acetylation, and Nuclear Accumulation

Given that both p300 and SIRT1 activities control the localization of other transcription factors, their interactions with LEF-1 were examined in anti-LEF-1 immunoprecipitates (Figure 3A).

Glucose and LiCl cooperated to increase the level of p300/LEF-1 complexes by 3.5-fold, consistent with results shown in Figure 2E, whereas single treatment did not have a significant influence. By contrast, SIRT1/LEF-1 complexes were increased 2.5-fold with glucose alone compared to cells untreated or treated with LiCl alone (Figure 3B). Strikingly, LiCl disrupted the SIRT1/LEF-1 complexes induced by glucose, most likely owing to competition for LEF-1 binding between β-catenin (accumulated by LiCl) and SIRT1. Glutathione S-transferase (GST)-LEF-1 pull-down confirmed the immunoprecipitation results (Figures S5A and S5B) obtained using LEF-1 antibodies. Thus, upon LiCl treatment, glucose promotes the formation and nuclear retention of LEF-1 complexes with β-catenin and p300, but not with SIRT1.

Immunoprecipitates of nuclear β-catenin were then examined. Strikingly, β-catenin and p300 interaction was detected only in cells treated with both glucose and LiCl and not in control cells or cells with a single glucose or LiCl treatment (Figure 3C). SIRT1 and β-catenin interactions increased with glucose alone more than 2-fold, but LiCl disrupted SIRT1 interactions (Figure 3D). Collectively, the results indicate that combined glucose and LiCl treatment increases p300-LEF-1-β-catenin interaction while preventing SIRT1-LEF-1 and SIRT1-β-catenin interaction and suggest that increased acetylation by p300 may be required for nuclear retention of the LEF-1/β-catenin complex.

Endogenous β-catenin underwent nuclear accumulation following glucose and LiCl stimulation; by contrast, a β-catenin mutant unable to interact with LEF-1 (Δ19 β-catenin) (Orsulic and Peifer, 1996; Prieve and Waterman, 1999) was unable to accumulate in the nucleus (Figure 3E), suggesting that β-catenin/LEF-1 interaction was required for nuclear retention.

The requirement of LEF-1 for mediating the p300/β-catenin interaction was examined in a western blot for p300 on anti-myc immunoprecipitates from cells transfected with Myc-tagged wild-type (WT) or Δ19β-catenin mutant and treated with LiCl, glucose, both, or none (Figure 3F). Whereas ectoptic WT β-catenin bound p300 upon exposure of the cells to both glucose and LiCl (like endogenous β-catenin), conditions that promote LEF-1 and β-catenin interaction and nuclear accumulation of β-catenin, the Δ19 β-catenin mutant was unable to bind p300 under any conditions. Given that p300 binds the C terminus of β-catenin (Hecht et al., 2000) more than 300 amino acids away from the region missing in Δ19 β-catenin (arms 5–6), our results suggest that LEF-1/β-catenin complexes recruit p300 more efficiently than does β-catenin alone. Thus, the LEF-1 and β-catenin interaction promoted by the combination of glucose and LiCl appears critical for stabilizing p300 interaction and nuclear retention.

To test whether β-catenin acetylation is increased under the conditions required for nuclear accumulation, we immunoprecipitated nuclear extracts with acetyllysine antibody and detected β-catenin (Figure 3G, upper panels) or immunoprecipitated with anti-β-catenin and detected anti-acetyllysine (Figure 3G, lower panels). In both cases, acetylated β-catenin was increased in the nucleus of cells cultured under combined glucose and LiCl treatment, conditions wherein p300 interaction is favored and SIRT1 interaction is decreased.

p300 and SIRT1 Activities Mediate Nuclear Accumulation of and Transcriptional Activation by β-Catenin

Overexpression of Flag-p300 upon LiCl-induced β-catenin accumulation increased β-catenin nuclear accumulation as much as glucose did (Figure 4A). Conversely, C646, a specific inhibitor of p300 acetyltransferase activity, abolished glucose-induced nuclear accumulation and also acetylation upon LiCl treatment (Figure 4B), indicating that p300 interaction and activity is a limiting step required for β-catenin nuclear accumulation.

Figure 4C shows that manipulation of the acetylation balance by inhibition of the deacetylase activity of sirtuins with NAA mimics glucose induction of β-catenin nuclear accumulation and acetylation, whereas RES-enhanced sirtuin activity blocks glucose-induced nuclear accumulation and acetylation of β-catenin. Immunocytochemistry (Figure 4D) revealed that β-catenin located at focal adhesions in control cells cultured in the absence of LiCl, whereas LiCl induced cytoplasmic accumulation of β-catenin. In the presence of LiCl, both NAA and glucose result in similar nuclear accumulation of β-catenin, but RES prevents nuclear accumulation of β-catenin induced by glucose. Importantly, small interfering RNA (siRNA)-mediated SIRT1 depletion largely reproduced the effects of glucose, allowing nuclear β-catenin accumulation in the presence of LiCl (Figure 4E). Thus, upon LiCl- or Wnt-3a-induced accumulation of β-catenin, high glucose appears to promote nuclear retention by increasing β-catenin acetylation, resulting from its actions on both acetylases and deacetylases.

The impact of acetylation on the transcriptional functionality of Wnt effectors was then analyzed. TOPFlash or FOPFlash reporters were transfected into STC-1 cells cultured in the presence of LiCl, and the acetylation balance was manipulated with glucose, NAA, and/or RES (Figure 4F). In LiCl-stimulated cells, sirtuin inhibition mimics transcriptional induction by glucose through the LEF/Tcf elements present in the TOPFlash reporter, whereas RES effectively blocks the glucose-driven induction. The results indicate that inhibition of sirtuins by the combination of glucose and LiCl enhances Wnt-dependent transcriptional activation and suggest that similar mechanisms may alter transcription from the natural *GIP* promoter that bears a Wnt-responsive element. Figure 4G shows that in the presence of LiCl, NAA mimics glucose induction of the *GIP* promoter and RES blocks the glucose-dependent induction. These results are consistent with transcriptional activity of Wnt effectors being induced via glucose-driven acetylation of β-catenin by p300 and the inhibition of sirtuins. The Δ19 β-catenin mutant that is unresponsive to the glucose and LiCl combination lacks one lysine, K354. Significantly, the K354R β-catenin mutant expressed in cells treated with glucose and LiCl was unable to interact with GST-LEF-1 (Figure S5C) and when immunoprecipitated did not interact with p300 (Figure S5D; compare with Δ19 and WT β-catenin in both cases). Consequently, the K354R β-catenin mutant exhibited reduced acetylation in response to glucose and LiCl (Figure 5A) or after p300 overexpression (Figure 5B), was not retained in the nucleus (Figures 5A and 5C) despite its cytosolic accumulation (data not shown), and failed to induce *GIP* transcription upon treatment with glucose and LiCl (Figure 5D). Mutant K345R β-catenin previously identified as being

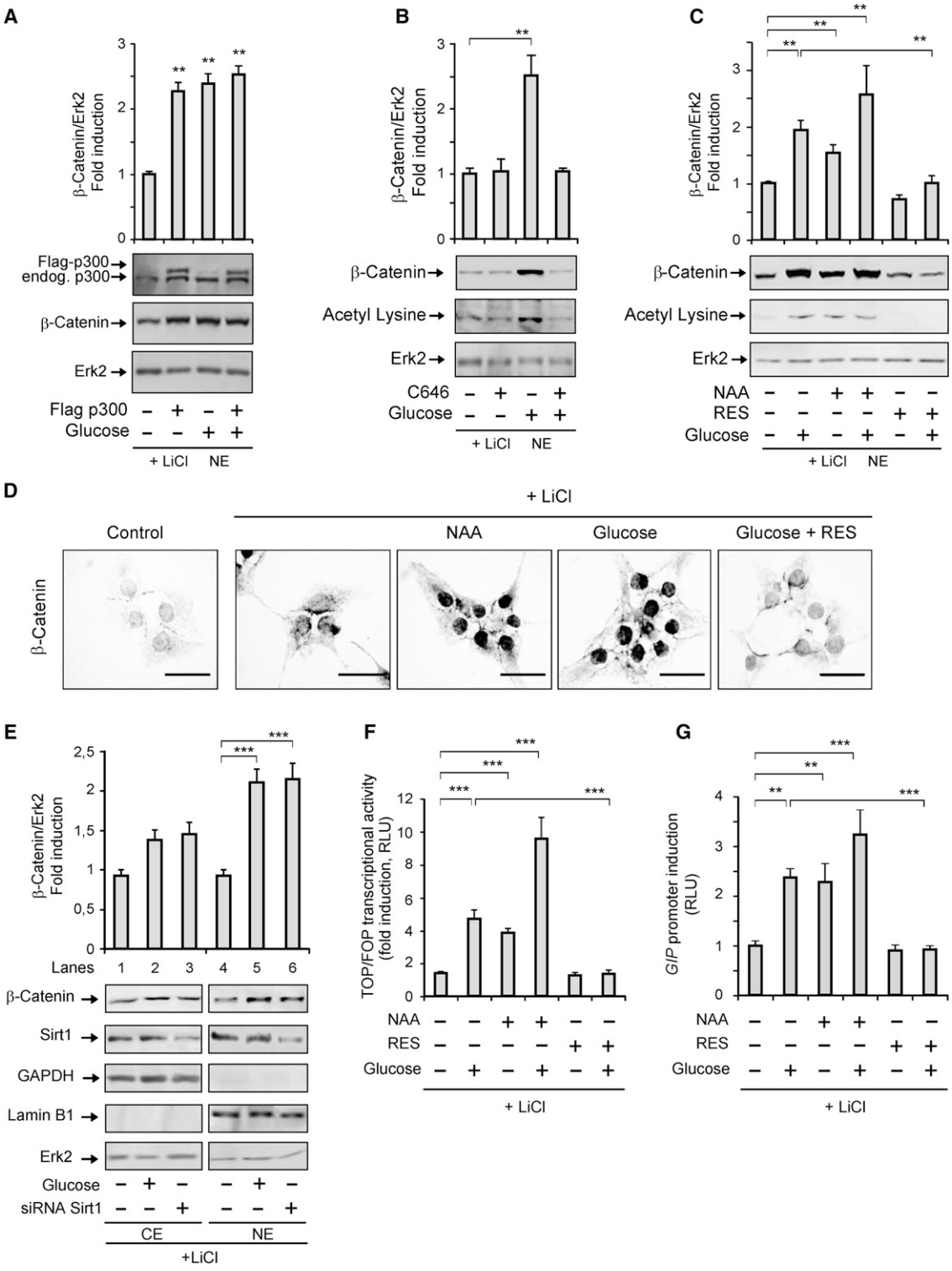

Figure 4. Glucose and LiCl Cooperation to Increase p300 and Inhibit SIRT1 Mediates Nuclear Accumulation and Transcriptional Activation by β-Catenin

(A–C, E) Fractionated extracts: Nuclear (NE) and cytoplasmic extracts (CE) from STC-1 cells cultured with LiCl and treated as indicated were analyzed by western blotting for β-catenin nuclear accumulation. Erk2 is the loading control. Values represent mean ± SEM (n ≥ 3).

(A) Cells transfected with Flag-tagged p300.

(B) Inhibition of p300 by C646 (5 μM) abolishes β-catenin nuclear accumulation (upper panel) and acetylation (middle) by glucose and LiCl.

(legend continued on next page)

acetylated by p300 but with little effect on transcription (Lévy et al., 2004) served as the control. Targeting of β-catenin K354 by p300 and SIRT1 was confirmed by immunoprecipitations with anti-acetyllysine (Figure S5E) and detection of c-*myc*-tagged β-catenin, or vice versa (Figure S5F). WT β-catenin, but not K354R mutant β-catenin, was acetylated by p300 in cells cultured with LiCl as shown previously; acetylation was enhanced by NAA inhibition of SIRT activity and was blocked by inhibition of p300 with C646 or overexpression of SIRT1. Thus, K354 plays a critical and highly specific role in promoting β-catenin nuclear accumulation and transcriptional activation in response to LiCl and glucose.

Glucose Amplifies Wnt-Dependent Transcription, Favoring In Vivo Binding of LEF-1 and β-Catenin to Their Chromatin Sites in Target Promoters

The in vivo effects of glucose on the endogenous *GIP* promoter, as well as on other genes (Figures 5E and 5F), were analyzed by chromatin immunoprecipitation (ChIP) followed by PCR. Representative semiquantitative PCRs on immunoprecipitates using LEF-1 and β-catenin antibodies are shown for the *GIP* promoter (Figure 5E, top); the lower panel corresponds to a control intronic sequence of *GIP* without TCF/LEF elements, and the bottom panel shows a statistical analysis of qPCRs for *GIP* performed on immunoprecipitates from five biological replicates. Figure 5F shows similar results for the promoters of other candidate genes regulated by glucose, namely the proglucagon gene *GCG* that encodes another incretin, GLP-1, and a critical proliferation regulator, *cyclin* D, where *GAPDH* was used as a control non-Wnt-regulated gene. The qPCR data (bottom) represents a statistical analysis of qPCRs for *GCG* or *cyclin* performed on immunoprecipitates from three biological replicates. At the *GIP*, *GCG*, and *cyclin* D promoters, both LEF-1 and β-catenin were poorly bound in the absence of LiCl. Single treatment with glucose or LiCl alone induced only minor increases in the binding of both LEF-1 and β-catenin. By contrast, in the presence of LiCl, glucose strongly enhanced their binding to these promoters. Results are presented on a log2 scale. Taken together, upon LiCl treatment, glucose increased the binding of LEF-1 and especially that of β-catenin on different Wnt target genes.

Thus, glucose enhances Wnt-dependent gene expression, allowing nuclear retention of acetylated β-catenin and enhancing productive binding to its target promoter elements on multiple Wnt-responsive genes.

Glucose Amplifies Wnt/LiCl Signaling in Tumor Cells of Human Origin

Nuclear β-catenin is a well-known marker of malignancy in a wide range of cancers. High glucose consumption directed toward glycolysis is the metabolic hallmark of cancer cells. We reasoned that high glucose uptake by cells could contribute to the tumor phenotype by promoting nuclear accumulation of β-catenin to activate proliferation-related genes. We assayed several tumor-derived cell lines to determine whether their intranuclear β-catenin accumulation was dependent on glucose (Figure 6A). Our data revealed that in the presence of LiCl, all tumor-derived cell lines tested, including enteroendocrine (STC-1), colon (HT-29), pancreas (AsPC-1), ovary (OVCAR3), and breast (MDA-MB-231), respond to high glucose by accumulating β-catenin in the nucleus. We then analyzed whether increased LEF-1 and β-catenin binding to the *cyclin* D promoter was dependent on glucose. Figure 6B shows a representative semiquantitative PCR on chromatin immunoprecipitates from HT-29 cells; Figure 6C shows statistical analysis of qPCR on chromatin immunoprecipitated from three biological replicates. Increased binding of both LEF-1 and β-catenin at the *cyclin* D promoter follows combined treatment with glucose and LiCl in HT-29 colon cancer cell lines. Ovarian cancer cell lines have also been examined with the same result (A.C.-C., J.M.G.-M., and C.G.-J., unpublished results). Thus, high glucose amplifies Wnt signaling by ensuring high intranuclear LEF-1/β-catenin accumulation and binding to its target promoters.

DISCUSSION

Understanding how glucose triggers increased incretin expression to regulate the insulin response is of fundamental importance, because deregulation of insulin secretion leads to diabetes, and deregulation of insulin signaling predisposes individuals to cancer (Efeyan and Sabatini, 2010; Gualberto and Pollak, 2009). Although increased insulin or IGFR signaling is likely to play a key role in the diabetes-cancer link, nutrients and energy may signal in cancer through metabolic targets such as mammalian target of rapamycin (Morton et al., 2011). Because targeting the metabolic sensor AMPK with metformin diminishes cancer mortality among diabetics (Dowling et al., 2007), many anticancer drugs directed toward metabolic pathways are being developed (Tennant et al., 2010). Importantly, elevated serum-glucose levels in diabetes are associated with increased cancer risk (Giovannucci et al., 2010; Vigneri, 2009), which suggests that components of the glucose homeostasis mechanism impact key cancer-associated signaling pathways. Epidemiological studies strongly link glycemic index to cancer risk (Gnagnarella et al., 2008), yet the precise mechanisms underpinning these important observations remain to be fully elucidated. Our results indicate that elevated glucose itself enhances Wnt signaling, a well-characterized cancer-associated pathway, by targeting nuclear accumulation of its effector β-catenin. Thus, although glucose is known to regulate a range of transcription factors (e.g., FoxO1 and Pdx-1), the results presented in this

(C) Sirtuin inhibition with NAA, 300 μM, or enhancement with RES, 50 μM, tunes nuclear accumulation and acetylation of β-catenin.
(D) Immunocytochemistry: nuclear accumulation of β-catenin by NAA (300 μM) or glucose, and glucose-effect block by RES (50 μM). Bars represent 25 μm.
(E) SIRT1 siRNA transfection of STC-1 cells (+) leads to nuclear accumulation of β-catenin; scrambled RNA (−) is shown as a control. GAPDH and Lamin B: fractionation controls.
(F and G) Luciferase assays on STC-1 cells transfected with indicated reporters in response to NAA or RES.
(F) TOPFlash/FOPFlash ratio.
(G) *GIP*-promoter activity. Relative luciferase units (RLU) expressed as fold induction relative to the corresponding control. Values represent mean ± SEM ($n \geq 3$).

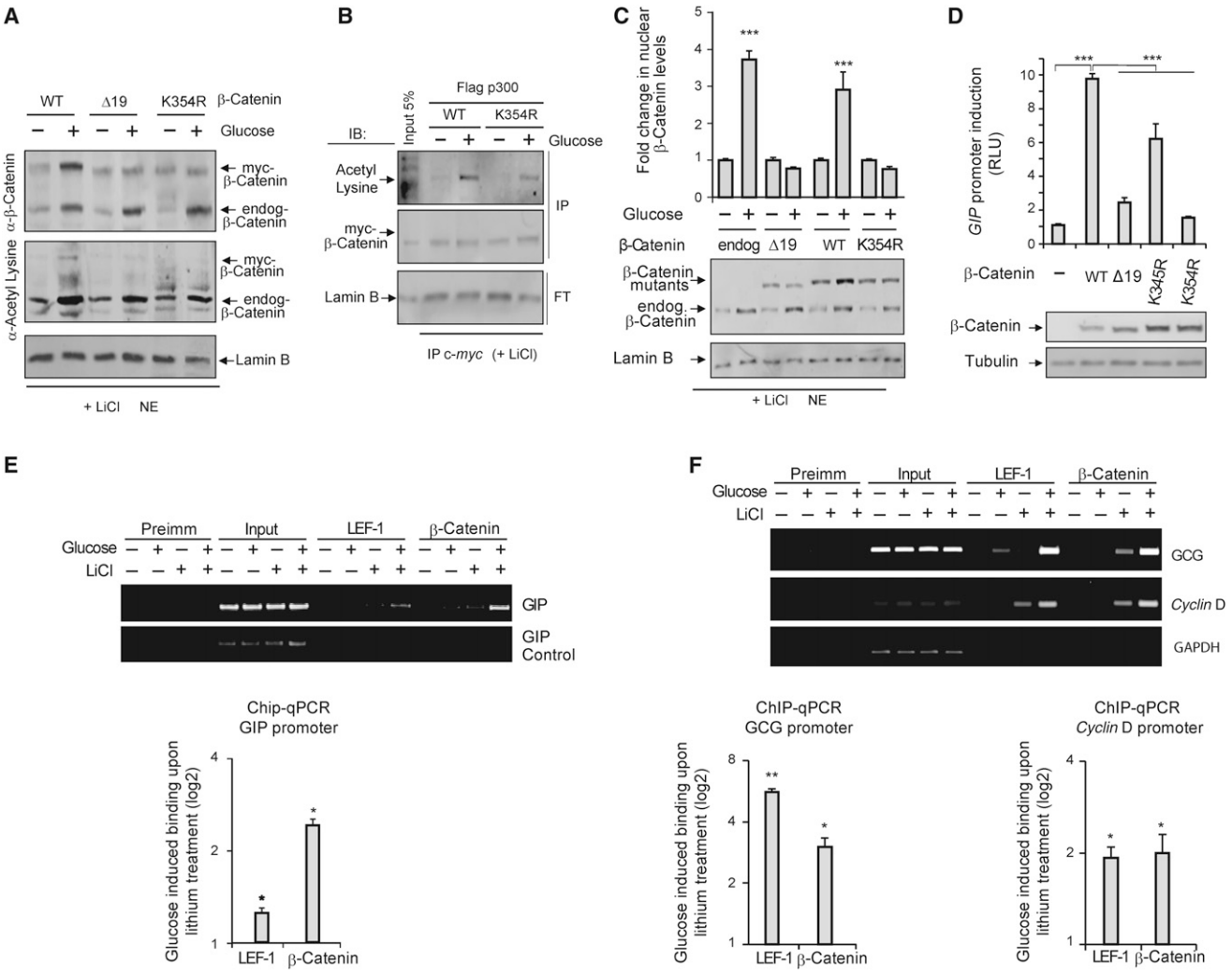

Figure 5. Combined Glucose and LiCl Effects Require Lysine 354 of β-Catenin and Increase In Vivo Binding to Chromatin of LEF-1 and β-Catenin

(A–C) Nuclear extracts (NE) of STC-1 cell transfected with myc-tagged versions of β-catenin: WT, Δ19, or K354R mutants cultured with LiCl and treated as indicated analyzed for β-catenin nuclear accumulation; (A) and (C) show the analysis in crude nuclear extracts, and (B) in immunoprecipitates of the NE using c-*myc* antibody after cotransfection with Flag-tagged p300 expression vector.

(D) Cells as in (A) were cotransfected with *GIP*-luciferase reporter. Relative luciferase units (RLU) were calculated as in Figure 4G. The western blot shown is representative and shows the expression levels of the mutants.

(E and F) ChIP from STC-1 cells treated as indicated followed by semiquantitative PCRs (top panels) and qPCR (graphs).

(E) ChIP at the TL5-containing region of *GIP* the promoter using a TCF/LEF-free region at intron 2 of *GIP* as a negative control.

(F) ChIP at the *proglucagon* (*GCG*), *cyclin* D, and *GAPDH* promoters as indicated. *GAPDH* is used as a control gene not regulated by Wnt. Preimmune serum and ChIP from input chromatin are shown as controls. For qPCRs, normalized values were calculated as fold induction of samples treated with LiCl in the presence versus absence of glucose and presented as mean ± SEM (n = 5) on a log2 scale. See also Figure S5.

work highlight a specific impact of glucose on Wnt/β-catenin signaling.

Despite the importance of Wnt/β-catenin signaling for development, tissue renewal, and cancer, the mechanisms by which β-catenin enters and is retained in the nucleus under Wnt stimulation are poorly understood. Given that β-catenin lacks a classical NLS, its nuclear entry is likely to depend on its interactions with other molecules, such as LEF-1, with their own NLS. Although we and others (Kim and Hay, 2001) have observed that overexpression of LEF-1 results in nuclear entry of β-catenin, the physiological stimulus for nuclear accumulation of β-catenin has not previously been identified. That acetylation might be implicated was indicated by studies in which overexpressed p300 or CBP could interact with the C terminus of β-catenin (Hecht et al., 2000), leading to lysine acetylation, binding to TCF/LEF factors (Lévy et al., 2004), nuclear entry of β-catenin (Wolf et al., 2002), and increased oncogenic potential (Ma et al., 2005). However, mutation of the acetylated residue

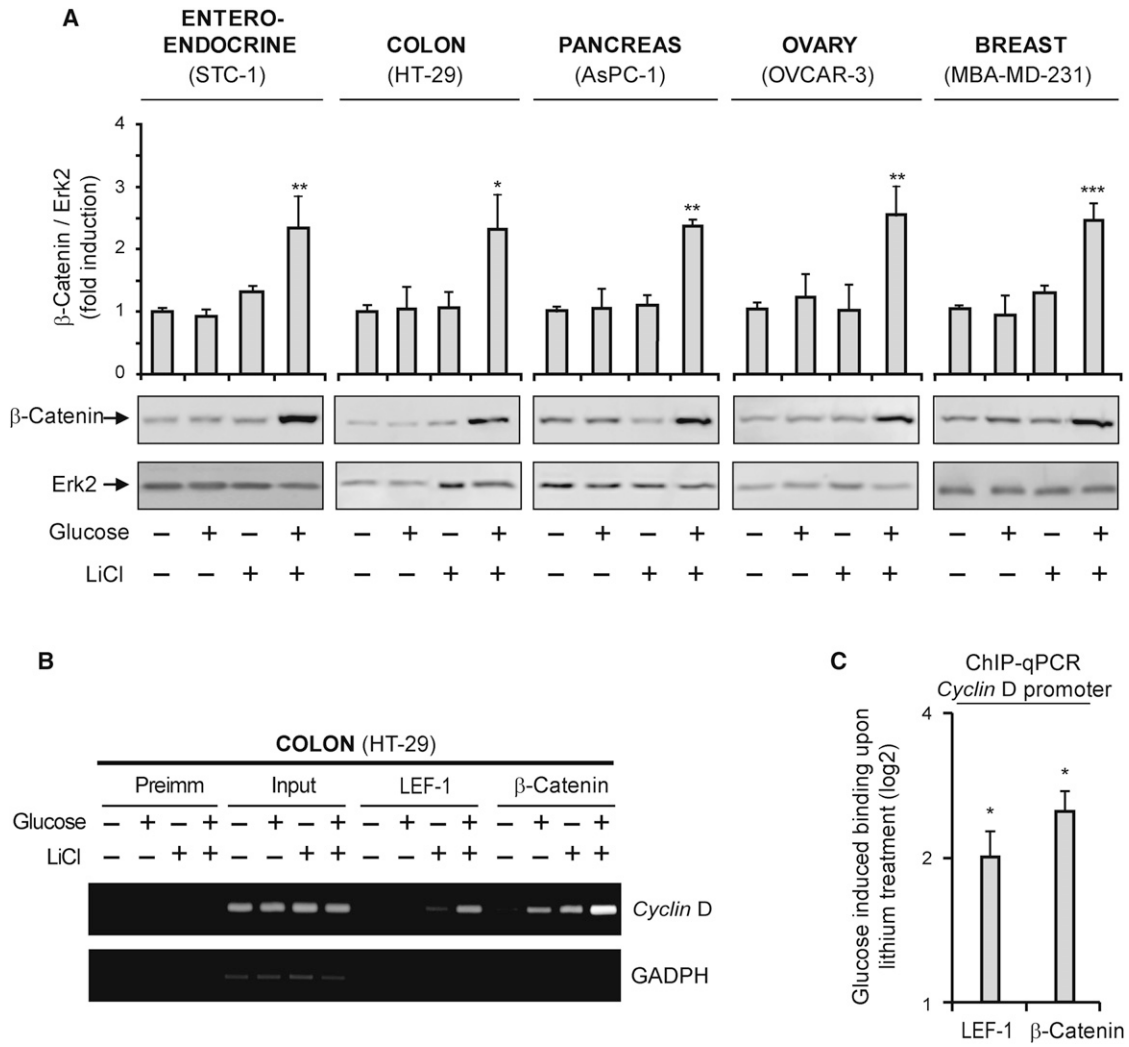

Figure 6. Glucose Is Required for Intranuclear β-Catenin Accumulation in Human Tumor-Derived Cell Lines and Enhances Binding of LEF-1 and β-Catenin to Target Promoters

(A) Western blot analysis of β-catenin, p300, and ERK2 from indicated cell lines cultured ± LiCl and/or glucose, showing representative gels and statistical analysis, values represent mean ± SEM, (n = 3).

(B and C) Chromatin was immunoprecipitated from HT-29 colon cancer cells. ChIP was followed by semiquantitative PCR (B) or qPCR (C) as in Figure 5. Normalized values are presented as mean ± SEM (n = 3) on a log2 scale.

identified, K345, did not affect β-catenin-driven transcription activation. Our results indicate that the physiological trigger underlying nuclear accumulation of β-catenin in cooperation with Wnt or LiCl is high glucose. In the presence of LiCl, glucose performs two complementary functions directed toward enhancing nuclear accumulation of acetylated β-catenin. First, it increases p300 expression and enhances its interactions with LEF-1 and β-catenin; second, it inhibits sirtuin deacetylase activity. Together, these events lead to increased β-catenin acetylation and nuclear accumulation. Moreover, we identify K354 as an essential residue for glucose induction of β-catenin nuclear accumulation. K354 lies within the region absent in the Δ19 mutant, a region previously identified as being critical for β-catenin interaction with LEF-1 (von Kries et al., 2000). The effects are highly specific, because glucose cannot be substituted by another carbon source such as glutamine and the effects of other growth factors such as Insulin or TGFβ are independent of glucose (data not shown).

Collectively, the data presented are consistent with a model in which p300/CBP acetylation of lysine residues in the armadillo repeats increase binding of TCF/LEF, leading to β-catenin nuclear accumulation. In this respect it is possible that β-catenin may treadmill between the nucleus and the cytoplasm and that its recruitment to DNA via its acetylation-enhanced interaction with LEF-1 would lead to its effective nuclear retention. Our results are also consistent with LEF-1 promoting interaction of β-catenin with p300, thereby facilitating the probable formation of a tripartite nuclear complex that would mediate gene regulation.

Interestingly, SIRT1 interaction with both β-catenin and LEF-1 is increased in response to high glucose, but these complexes

are disrupted in the presence of LiCl. Although the precise molecular mechanisms have not been explored here, we view it as probable that SIRT1 acts as a competitor for interaction between LEF-1 and β-catenin. Such a scenario would be advantageous, because it would ensure that β-catenin nuclear accumulation would only occur on receipt of a simultaneous Wnt and high-glucose signal; the low level of β-catenin observed in the absence of a Wnt signal would be insufficient to activate gene expression even in the presence of glucose, because it would not be able to displace SIRT1 from LEF-1.

Although SIRT1 may play a role via stable and direct binding to LEF-1 or β-catenin, it is also clear that its catalytic activity is particularly important. SIRT1 deacetylates β-catenin to decrease its oncogenic capacity (Firestein et al., 2008), and SIRT1 depletion, or inhibition using NAA, in cells treated with LiCl mimics glucose-driven nuclear retention of β-catenin for promoting transcriptional activation. Thus, the effect of glucose is likely to be mediated, at least partially, via the inhibition of sirtuins. The reduction in sirtuin activity obtained in cells treated with NAA or with glucose and LiCl is greater than that obtained in other cells with NAA at doses 10- to 30-fold higher (Audrito et al., 2011), although it is understandably less than the inhibition obtained using NAA in vitro under chemically defined conditions with purified protein (Sauve and Schramm, 2003). Because tumor cells exhibit enhanced glycolysis, obtaining much lower energy per molecule of glucose than normal cells through respiration, under LiCl stimulation high glucose uptake may speed up glycolysis, leading to rapid turnover of the NAD^+ obtained by lactic acid production. Thus, high glucose may promote the depletion of NAD^+, thereby limiting the amount of this essential sirtuin cofactor and consequently the deacetylase activity of sirtuins.

Finally, our results also imply that the glucose responsiveness of enteroendocrine cells will require an autocrine or paracrine source of either Wnt or an alternative β-catenin-stabilizing signal to enable glucose-dependent incretin expression and subsequent secretion for insulin release by the pancreas. Identification of the source and nature of signals that stimulate the Wnt/β-catenin pathway to cooperate with glucose in mediating incretin production is a key issue. Given that cancer cells are a well-characterized source of autocrine Wnt signaling (Bafico et al., 2004), the impact of β-catenin accumulation on tumor progression may be critically dependent on glucose availability. This notion is reinforced by our finding that glucose and LiCl synergize to promote nuclear accumulation of β-catenin in a wide range of cell lines derived from diverse cancer types and by the glucose requirement observed in macrophages for autocrine activation of the Wnt/β-catenin pathway (Anagnostou and Shepherd, 2008). LEF/β-catenin-driven gene expression in a high-glucose microenvironment could be used by tumor cells to promote proliferation and/or invasion. Wnt signaling and its modulation by nutrients such as glucose may reveal relatively unexplored targets in metabolic disease.

EXPERIMENTAL PROCEDURES

Antibodies

The antibodies used were as follows: anti-LEF-1, β-catenin, Myc, p300, total ERK2, Lamin B (Santa Cruz Biotechnology), acetylated lysine, pGSK3β (Cell Signaling), GAPDH (Abcam), Sirt1 (Millipore), biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA), and anti-β-catenin (BD Biosciences). Fluorescein isothiocyanate or Texas red were conjugated for immunofluorescence.

Plasmids

GIP luciferase, Super8XTOPFlash, Super8XFOPflash, and KI GSK3β (K85R) have been described previously (García-Martínez et al., 2009). Expression vectors for HA-LEF-1 and WT and mutant Δ19 Myc-β-catenin were gifts from Marian L. Waterman, Flag-p300 from Colin Goding, and DN-LEF-1 from Lionel Larue. The QuikChange Kit (Stratagene) was used for site-directed mutagenesis. Primers used in this work are described in Table S1.

Cell Culture and Transfections

Tumor enteroendocrine STC-1, ovary carcinoma OVCAR-3, mammary adenocarcinoma MDA-MB-231, and colorectal adenocarcinoma HT-29 cells were cultured in Dulbecco's modified Eagle's medium and pancreatic carcinoma AsPC-1 in RPMI. Both media were supplemented with 10% fetal bovine serum. Cells starved of glucose and/or glutamine for 24–36 hr were stimulated as indicated. Cells were transfected with JetPEI Polyplus reagent (Genycell Biotech) and treated as indicated 24 hr later. Recombinant Wnt-3a proteins were from PeproTech. The GloMax 96 Luminometer (Promega) and Dual-Luciferase kit were used. Fractionation was as in Andrews and Faller (1991).

Immunoprecipitations

Protein A/G-coated magnetic beads (Invitrogen) loaded with the indicated antibody were incubated with nuclear extracts O/N at 4°C. After three washes, bound proteins were analyzed via western blotting.

ChIP was performed as described (García-Martínez et al., 2009). The primers are listed in Table S1.

Immunocytochemistry and Immunofluorescence

Cells were fixed with 4% paraformaldehyde in PBS (pH 7.4), permeabilized with 0.5% Triton X-100, and blocked with 5% BSA. Immunocytochemistry was performed following the immunoperoxidase procedure (ABC kit, Vector Laboratories). Immunofluorescence was performed by mounting cells on ProLong Gold (Molecular Probes).

SIRT1 Activity

SIRT1 Activity was measured using a SIRT-Glo Assay kit (Promega), following the manufacturer's instructions. After glucose starvation, cells were stimulated as indicated. For each reaction, 1 μg protein was incubated with SIRT-Glo Reagent Mix at room temperature for 30 min, and the product was measured via luminescence.

siRNA Interference

Cells transfected with SIRT-1 siRNA or scrambled oligoribonucleotides (Invitrogen) using jetPRIME Polyplus reagent (Genycell Biotech) were starved of glucose 6 hr posttransfection for 18–24 hr more and stimulated as indicated for 24 hr more.

ATP Levels

Total ATP was measured using an ELITEN ATP Assay System. Cells were lysed with TE (10 mM Tris, 4 mM EDTA [pH 7.5]) and heated for 7 min at 95°C, and cell debris was pelleted at 14,000 rpm for 3 min. Lysate totaling 30 μl was incubated with ATP reagent and luminescence normalized with protein. A standard curve was generated by using known ATP concentrations.

qRT-PCRs

Primers used are specified in Table S1; 18S ribosomal RNA primers served as a nonregulated control. Relative expression was calculated using the Ct method, expressed as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The PCR efficiency was ~100%. Reagents and detection systems were from Applied Biosystems.

Statistical Analysis

Results (main and supplemental) are presented as fold induction, mean ± SEM, from at least three biological replicas for luciferase assays, western blots

and RT-PCR, or five biological replicas for ChIP. Tests for significance were ANOVA and Bonferroni's post hoc test. Differences were considered statistically significant if *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.11.022.

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