Wnt Signaling Inhibits Forkhead Box O3a-induced Transcription and Apoptosis through Up-regulation of Serum- and Glucocorticoid-inducible Kinase 1*§

Manuel Dehner†, Michel Hadjihannas‡, Jörg Weiske‡, Otmar Huber‡, and Jürgen Behrens‡†

From the †Department of Experimental Medicine II, Nikolaus-Fiebiger-Center for Molecular Medicine, University of Erlangen, Glueckstrasse 6, 91054 Erlangen, Germany and the ‡Department of Laboratory Medicine and Pathobiology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany

In human cancers, mutations in components of the Wnt signaling pathway lead to β-catenin stabilization and result in augmented gene transcription. HCT116 colon cancer cells carry stabilizing mutations in β-catenin and exhibit an elevated activation of Wnt signaling. To clarify the role of an overactive Wnt signaling, we used DNA microarray analysis to search for genes whose expression is up-regulated after knockdown of the wild type adenomatous polyposis coli (APC) tumor suppressor in HCT116 cells, which further enhances Wnt signaling activation. Serum and glucocorticoid-inducible kinase 1 (SGK1) was among the most up-regulated genes following APC knockdown through small interfering RNA. Up-regulation of SGK1 in response to small interfering RNA against APC was inhibited by concomitant knockdown of β-catenin. Quantitative real time reverse transcription-PCR, Western blot, and chromatin immunoprecipitation analyses confirmed that SGK1 is a direct β-catenin target gene. SGK1 negatively regulates the pro-apoptotic transcription factor Forkhead box O3a (FoxO3a) via phosphorylation and exclusion from the nucleus. We show that Wnt signaling activation results in FoxO3a exclusion from the nucleus and inhibits expression of FoxO3a target genes. Importantly, FoxO3a mutants that fail to be phosphorylated and therefore are regulated by SGK1 are not influenced by activation of Wnt signaling. In line, knockdown of SGK1 relieves the effects of Wnt signaling on FoxO3a localization and FoxO3a-dependent transcription. Finally, we show that induction of Wnt signaling inhibits FoxO3a-induced apoptosis. Collectively our results indicate that evasion of apoptosis is another feature employed by an overactive Wnt signaling.

The serine/threonine kinase SGK1 is transcriptionally up-regulated in response to a variety of external stimuli including growth factors such as insulin and hepatocyte growth factor as well as steroid hormones, in particular aldosterone (1–5). SGK1 activity is stimulated by 3-phosphoinositide-dependent kinase 1/2-mediated phosphorylation and is tightly linked to the phosphatidylinositol 3-kinase pathway (6). In this respect, SGK1 resembles PKB/Akt, with which it also shares specific substrates, namely members of the Forkhead box transcription factor (FoxO) family. SGK1 phosphorylates and inhibits the nuclear localization and transcriptional activity of the pro-apoptotic FoxO3a/FKHRL1 (7, 8). This results in increased cell growth and survival (9–11). Indeed, it is currently believed that induction of SGK1 by environmental stress stimuli allows the cell to survive under such adverse conditions, most likely through its capacity to inhibit FoxOs (7, 12). FoxO target genes include Bcl2-interacting mediator (BIM) and p27kip1, which promote apoptosis and inhibit cell cycle progression respectively (13, 14). In line, studies in Caenorhabditis elegans show a role of the SGK1/FoxO pathway in stress tolerance (15).

The canonical Wnt signaling pathway can also suppress apoptosis (16–19), but the underlying molecular mechanisms are not entirely clear. Wnts are secreted factors that bind to Fz/LRP receptors, leading to the stabilization of the nucleocytoplasmic protein β-catenin. Stabilization of β-catenin enhances its nuclear translocation and cooperation with members of the TCF/Lef family of transcriptional regulators to induce transcription of target genes such as c-Myc, survivin, cyclinD1, and axin2/conductin (17, 20–23). In the absence of Wnt signals, β-catenin is found in the so-called β-catenin destruction complex that includes the tumor suppressor APC, scaffold proteins axin and axin2/conductin, and the kinases casein kinase 1ε and glycogen synthase kinase-3β, which promote the phosphorylation of β-catenin at the N-terminal serine and threonine residues (24–30). Phosphorylated β-catenin is earmarked for rapid degradation in proteasomes. Disregulated Wnt signaling is a hallmark of many cancers, especially colorectal cancer. Activation of Wnt signaling in the majority of colorectal tumors is achieved through mutations in APC that compromise the integrity of the β-catenin destruction complex (31, 32). In a small percentage of colorectal cancers, mutations in β-catenin itself lead to its stabilization and increased transcriptional activity (33, 34).
tional activity (33). In cancer cells that carry mutated β-catenin but wild type APC, the magnitude of Wnt-signaling may be dampened because of binding and sequestration of mutated β-catenin into the cytoplasm by functional APC, axin, and axin2/ conductin (34–37). Indeed, we have previously noticed that expression of the Wnt target gene axin2/conductin can be further activated through the siRNA-mediated knockdown of APC in HCT116 cells, which carry mutated β-catenin but wild type APC (38). This indicates that wild type APC can suppress Wnt signaling even in the presence of mutated β-catenin. The different Wnt signaling activation levels in these two genetic settings, represented by APC or β-catenin mutations, might serve as a dosage switch to diversify the response of target genes.

Using DNA microarrays, we identified SGK1 to be up-regulated upon knockdown of APC in HCT116 cells in a β-catenin-dependent manner. Activation of Wnt signaling by APC depletion or strong overexpression of β-catenin led to nuclear exclusion of the SGK1 substrate FoxO3a and diminished FoxO-transcription or strong overexpression of 10% CO2. For reporter assays, the cells were transfected with polyethylenimin in 24-well tissue culture plates with the indicated activity (33). In cancer cells that carry mutated β-catenin but wild type APC, the magnitude of Wnt-signaling may be dampened because of binding and sequestration of mutated β-catenin via XmaI and BamHI. YFP-FoxO3a was constructed with the amplifying primers: 5’-TCCCCCGGAGGGTGAAACTGAGGCTGC-3’ (5’ primer) and 5’-CGGATCCGGAGGAAGTCCCGTGGAGG-3’ (3’ primer) and the product was ligated into myF-C2

Analysis of Gene Expression—Total RNA was prepared from DLD1-dnTCF and HCT116 cells using peqGOLD Trifast® reagent (Peqlab, Erlangen, Germany) following the manufacturer’s protocol. cDNAs were generated from 1 µg of RNA using Superscript™ II reverse transcriptase (Invitrogen) and random hexamer primer, according to the recommendations of the manufacturer. In addition 40 units of RNase inhibitor were added (RNaseOUT™; Invitrogen).

Quantitative real-time RT-PCR analysis—Quantitative real-time RT-PCR was performed using specific sense and antisense primers in a 25-µl reaction volume containing 12.5 µl of Absolute™ QPCR SYBR® Green Mix (ABgene, Hamburg, Germany), 0.25 pmol of each primer, and 1 µl of cDNA. The amplification number of cycles was 50, and the reaction took place for 20 s at 95 °C, 20 s at 57 °C, and 40 s at 72 °C, with an initial step of 95 °C for 12 min (hot start) and a final step of 72 °C for 2 min. Oligonucleotide primers were the following: for SGK1, 5’-GACCTCACCACCTAGGAGCC-3’ (5’ primer) and 5’-CTGGAAGAAGAGTGAGGCCC-3’ (3’ primer); for Foxin2, 5’-GAACATTTGCCAAACCGTG-3’ (5’ primer) and 5’-CTCTGGAGCTGTTCCTACTGCC-3’ (3’ primer). The resulting amplification products were cloned into the NotI site of pcDNA-FLAG. For YFP-tagged SGK1, PCR was performed with the primers: 5’-ATAGAATTGCGCCCGTACGTTGAAAACGTGGCTGC-3’ (5’ primer) and 5’-TATTGGTCGACCCAGCAGAACAGGAACTCAG-3’ (3’ primer) and for FoxO4 5’-ATAGAATTGCGCCCGTACGTTGAAAACGTGGCTGC-3’ (5’ primer) and 5’-TATTGGTCGACCCAGCAGAACAGGAACTCAG-3’ (3’ primer) and for FoxO4 5’-ATAGAATTGCGCCCGTACGTTGAAAACGTGGCTGC-3’ (5’ primer) and 5’-TATTGGTCGACCCAGCAGAACAGGAACTCAG-3’ (3’ primer). The resulting amplification products were cloned into the NotI site of pcDNA-FLAG.

MATERIALS AND METHODS

Cell Culture and Transfection—All cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria) at 37 °C in a humidified atmosphere of 10% CO2. For reporter assays, the cells were transfected with polyethylenimin in 24-well tissue culture plates with the indicated constructs and the following DNA amounts: reporters, 25 ng; regulators, 50 ng; and RSV-βGal (encodes the β-galactosidase (39)), 50 ng, to normalize for transfection efficiency. FoxO-dependent reporters were activated by transfection of 20 ng of FoxO3a/4. The amount of DNA for each transfection was adjusted by the addition of empty pcDNA3.1 vector. Luciferase activity was assayed 24 h after transfection and normalized to β-galactosidase activity as previously described (40). Transfections of siRNAs (100 nmol) were performed in 6-well tissue plates using TransIT TKO (Mirus, Madison, WI), as recommended by the manufacturers. After 24 h the medium was changed, and 48 h post-transfection the cells were harvested.

Plasmids and siRNAs—The following plasmids and siRNAs were described previously: CMV-APC, RFP-APC, RFP-Wnt3a, RFP-LRP6da, YFP-β-catenin, and YFP-S45A β-catenin (34); S33A β-catenin (41); siRNA against GFP, APC, and β-catenin (38); TOPflash and FOPflash (42); and pcDNA3.1-FLAG (26). Commerially available vectors of the c-Jun system pFR-Luc, pFAC-Jun, and pFCE-MEK from STRATAGENE (product 219000), the triple mutant of FoxO3a (FLAG-FoxO3a-TM) from addgene (plasmid 8361), and the siRNA against SGK1 (SGK Validated Stealth™ RNA interference Duponk) from Invitrogen were used. The reporter constructs BIM-luc and p27Kip-luc were kind gifts from Rene H. Medema, and FLAG-Asef2 was a kind gift from A. Grohmann. Plasmids with the cDNAs of SGK1 (identification code IRAp970E014D), FoxO3a (identification code IRAp969FO0851D), and FoxO4 (identification code IRAmp995HO213Q) were purchased from RZPD (Berlin, Germany). To create FLAG-tagged constructs of SGK1 and FoxO4, we used cDNA clones as templates together with the following primers: for SGK1 5’-ATAAGAATTGCGCCCGTACGTTGAAAACGTGGCTGC-3’ (5’ primer) and 5’-TATTGGTCGACCCAGCAGAACAGGAACTCAG-3’ (3’ primer) and for FoxO4 5’-ATAAGAATTGCGCCCGTACGTTGAAAACGTGGCTGC-3’ (5’ primer) and 5’-TATTGGTCGACCCAGCAGAACAGGAACTCAG-3’ (3’ primer). The resulting amplification products were cloned into the NotI site of pcDNA-FLAG.

DNA Microarrays—Microarrays analysis on Affymetrix HG-U133 (DLD1-dnTCF4) and HG-U133 Plus 2.0 (HCT116 siRNAs) GeneChips (Affymetrix, Santa Clara, CA) were carried out as described previously (41) using Microarray Suite 5.0 (Affymetrix; statistical algorithm), Data Mining Tool 3.0 (Affymetrix), and MS Excel and Access software.

Western Blotting—The cells were cultured for 24 h (reporter assays) or 48 h (experiments with RNA interference), and Western blotting was performed as described previously (38). The primary antibodies rabbit anti-SK1 (Biomol), mouse anti-β-actin (Sigma), and mouse anti-GFP (Roche Applied Science), as well as peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Cambridgeshire, UK) were used.
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RESULTS

Wnt Signaling Overactivation Leads to the Up-regulation of SGK1—TOP/FOP reporter assays showed that moderate β-catenin-dependent transcription in HCT116 is increased following the knockdown of APC and is restored to moderate levels when β-catenin is concomitantly knocked down (supplemental Fig. S1). Knockdown efficiency was validated by Western blotting (38). To identify genes activated following APC knockdown, we performed a DNA microarray analysis. For this, HCT116 cells were transfected with either control siRNA (siGFP) or a siRNA against APC (siAPC) or siRNAs against both APC and β-catenin (siAPC/β-cat). RNA was isolated 48 h after transfection and used to probe Affymetrix HG-U133 Plus 2.0 microarrays. In line with our previous results (38), moderate levels of axin2 in HCT116 cells were increased after knockdown of APC by 5.5-fold. This increase was significantly prevented by concomitant knockdown of β-catenin (down to 2.2). SGK1 was found to be regulated in a very similar pattern as axin2, i.e. up-regulated by siAPC 6.0-fold and down-regulated again when β-catenin was concomitantly transfected by 3.0-fold. Quantitative real time PCR confirmed the microarray analysis (Fig. 1A). To substantiate whether SGK1 is a Wnt/β-

according to the manufacturer’s instructions. For detection of Conductin/Axin2, the mouse C/G7 antibody (22) (concentrated hybridoma supernatant at 1:100 dilutions) was used. The bands were visualized with enhanced chemiluminescence on a LAS-3000 (FUJIFILM) and quantified using Aida Image Analyzer v. 3.52 software.

Chromatin Immunoprecipitation (ChIP)—ChIP was performed essentially as described earlier (47, 48). Fifty micrograms of DNA were incubated in 300 ml of radioimmune precipitation assay buffer overnight at 4 °C, with 2 μg of anti-TCF-4 (H-125X) (Santa Cruz Biotechnology), 2 μg of anti-β-catenin (clone14) (BD Biosciences), or anti-IgG (Santa Cruz Biotechnology) antibodies. Following precipitation of immune complexes, a 212-bp region of the human SGK1 promoter was amplified (94 °C for 5 min; 25 cycles of 94 °C for 15 s, 64 °C for 20 s, and 72 °C for 30 s; followed by a final 5-min extension at 72 °C) using the oligonucleotides: forward 5’-CTTTCCAGCCAGACTCCAGG-3’ and reverse 5’-CTGGCACAAGCTCGTTAATACC-3’. PCR products were separated by electrophoresis on an 8% polyacrylamide gel and visualized by ethidium bromide staining.

Immunofluorescence Microscopy—293T and DLD1 cells were grown on glass coverslips, fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 for 5 min, and incubated with rabbit anti-β-catenin (H102, Santa Cruz, CA) or rabbit anti-FLAG (Sigma) antibodies according to the manufacturer’s instructions. DLD1 cells were fixed in 3% paraformaldehyde for 5 min followed by 10 min of incubation at −20 °C in ice-cold methanol, permeabilized with 0.5% Triton X-100 for 15 min, and incubated with rabbit anti-FoxO3a (75D8) (product 2497, Cell Signaling Technology) or rabbit/mouse anti-FLAG (Sigma) antibodies according to the manufacturer’s instructions. Appropriate Cy3/Cy2-labeled secondary antibodies (Jackson Immunoresearch) were used to visualize primary antibodies. The nuclei were stained with bis-benziminde (Hoechst 33258; Sigma) for 5 min. Cell micrographs were obtained with a CCD camera (Visirion, Munich, Germany) on a Zeiss Axioptan 2 imaging microscope (Zeiss, Oberkochen, Germany).

TUNEL Assay—24 h after transfection, supernatants and trypsinized cells were collected and pelleted. Staining of free DNA 3′OH ends was performed with APO-Brd™ TUNEL assay kit (A23210; Invitrogen) following the manufacturer’s instructions. The cells were analyzed by fluorescence-activated cell sorting. Cell fluorescence was measured with a FACSScan flow cytometer (BD Bioscience, Heidelberg, Germany).

RESULTS

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FIGURE 1. Up-regulation of SGK1 by overactivation of Wnt signaling. A, left panel, quantitative real time RT-PCR showing relative expression of SGK1 and Axin2 in HCT116 cells after knockdown of APC (siAPC) or concomitant knockdown of APC and β-catenin (siAPC + siβ-catenin) as well as in siGFP transfected controls. Right panel, fold repression of SGK1, Axin2 and glyceraldehyde-3-phosphate dehydrogenase (control) expression in DLD1 cells after induction of dominant-negative TCF4 (dnTCF4) by the addition of doxycycline, as determined by quantitative real time RT-PCR. The error bars reflect the standard error from at least three independent experiments. B, Western blots showing SGK1 and Axin2 protein levels in HCT116 cells after knockdown of APC and β-catenin (left panels), in DLD1 cells after induction of dnTCF4 with doxycycline (middle panels), and in Rat2-MV7 control cells and Wnt-1 expressing Rat2 cells (right panels). The numbers below individual bands reflect quantification of the relative expression of SGK1 and Axin2 following normalization to β-actin. For easy comparison controls (siGFP, + dox and MV7) were set to a value of 1.
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catenin target gene, we induced the expression of a dominant-negative version of TCF4 (dnTCF4) in DLD1 colon cancer cells, which has been previously shown to inhibit β-catenin-mediated transcription (22, 49). In these cells, SGK1, like axin2 expression levels, were repressed after the induction of dnTCF4 (Fig. 1A). Western blot analysis showed that protein levels of axin2 and SGK1 were modulated in a similar manner as the mRNA levels after siAPC and siβ-cat transfection in HCT116 and after the induction of dnTCF4 in DLD1 cells (Fig. 1B). In addition, SGK1, similar to axin2, was up-regulated in Rat2 cells with constitutive activation of Wnt signaling because of stable expression of the Wnt1 ligand (Rat2-Wnt1) (22, 50), as compared with control Rat2-MV7 cells (Fig. 1B).

**SGK1 Is a Direct β-Catenin Target Gene**—Promoter-reporter assays showed that a -2886-bp fragment of the putative SGK1 promoter was activated after knockdown of APC and inhibited by knockdown of β-catenin by siRNAs in HCT116 cells (Fig. 2, A and B). Furthermore, the putative SGK1 promoter was activated following transfection of a stabilized version of β-catenin in HCT116 cells (Fig. 2B). Conversely, the SGK1 promoter reporter was inhibited in DLD1-dnTCF4 cells after induction of dnTCF4 by doxycycline and transfection of APC in SW480 cells that carry mutated APC (Fig. 2B). Reporter constructs for Wnt signaling activation (TOP) and controls (FOP) as well as Axin2 promoter reporter were used to verify induction and inhibition of Wnt signaling-dependent transcription in the various cellular systems used above (Fig. 2B). Importantly, ChIP analyses in DLD1-dnTCF4 indicate that β-catenin and TCF4 binds to a fragment of the putative SGK1 promoter and that the binding of β-catenin is abolished following induction of the dnTCF4 by the addition of doxycycline (Fig. 2C). Additionally, β-catenin, TCF4 as well as LEF1 are bound to the SGK1 promoter in SW480 cells (Fig. 2C). β-Catenin is found in a complex with LEF-1 or TCF4 at the promoter as revealed by consecutive immunoprecipitations where immunocomplexes obtained by anti-β-catenin antibodies were reimmunoprecipitated with antibodies to TCF4 or LEF-1. These data strongly demonstrate that SGK1 is a direct transcriptional target gene of the Wnt/β-catenin signaling pathway.

**Wnt Signaling Inhibits the Nuclear Localization of FoxO3a through SGK1**—SGK1 phosphorylates FoxO transcription factors and inhibits their activity by nuclear exclusion. In light of our results, we sought to determine whether the activation of Wnt signaling would affect FoxO activity. After transfection in 293T cells, FoxO3a was uniformly present in the nucleus and cytoplasm. When SGK1 was cotransfected, FoxO3a was restricted to the cytoplasm (Fig. 3A) (7, 51). Treatment with H₂O₂ enhanced the nuclear localization of FoxO3a, as previously reported (Fig. 3A) (52). Importantly, activation of the Wnt signaling through cotransfection of either Wnt3A, a dominant-active LRP6 coreceptor (LRP6da, (34)), or β-catenin led to the exclusion of FoxO3a from the nucleus. The cotransfection of an unrelated protein (Asef2) had no effect on FoxO3a localization (Fig. 3A). To determine whether nuclear exclusion of FoxO3a by Wnt activators required FoxO3a phosphorylation, we employed a triple mutant of FoxO3a in which SGK1 phosphorylation sites are mutated (FoxO3a-TM) (52). As expected, this mutant appeared to be prominently localized in the nucleus as compared with wild type FoxO3a. SGK1 coexpression or H₂O₂ treatment did not affect the nuclear localization of FoxO3a-TM (Fig. 3A). Importantly, cotransfection of Wnt activators did not inhibit the nuclear localization of FoxO3a-TM, indicating that Wnt signaling induces the nuclear exclusion of FoxO3a through phosphorylation. In addition, knockdown of APC in 293T cells abolished the nuclear localization of FoxO3a but not that of FoxO3a-TM (Fig. 3B). These results indicate that activation of Wnt signaling either through expression of
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Wnt activators or after depletion of the endogenous APC leads to the nuclear exclusion of FoxO3a in a phosphorylation-dependent manner.

To substantiate our findings in a colon cancer cellular setting, we made use of the DLD1-dnTCF4 cells. In the absence of doxycycline, both exogenous and endogenous FoxO3a was clearly found to be localized mostly in the cytoplasm and absent from the nucleus, as seen in Fig. 4 (A and B, respectively). Following 24 h of doxycycline treatment, there was a drastic shift of both exogenous and endogenous FoxO3a into the nucleus of DLD1 cells (Fig. 4). Doxycycline treatment had no effect on either exogenous or endogenous FoxO3a in DLD-TR7 control cells (data not shown). Biochemical fractionation showed that activation of dnTCF4 led to an increase of endogenous FoxO3a in the nucleus when normalized to Lamin A/C expression levels, which was further enhanced by treatment of the cells with H2O2 (supplemental Fig. S1).

To investigate whether SGK1 can reverse the nuclear localization of FoxO3a following inhibition of Wnt signaling, we transfected FLAG-tagged SGK1 in DLD1-dnTCF4 cells and subsequently inhibited the Wnt signaling by doxycycline treatment. The majority of cells expressing SGK1-FLAG showed a cytoplasmic localization for exogenous FoxO3a (Fig. 4A). Importantly transfection of SGK1 also reversed the nuclear localization of endogenous FoxO3a (Fig. 4B). We sought to determine whether SGK1 mediates the nuclear exclusion of FoxO3a. For this we transfected DLD1-dnTCF4 kept in the absence of doxycycline with a siRNA against SGK1 or control siRNA. Efficient knockdown of SGK1 induced the nuclear accumulation of exogenous FoxO3a (Fig. 4A and supplemental Fig. S1). Specifically, whereas in only 10% of the cells expressing exogenous FoxO3a and control siRNA, FoxO3a localized in the nucleus, ~50% of the cells cotransfected with a siRNA against SGK1 showed a nuclear localization for exogenous FoxO3a. To determine the effects of SGK1 knockdown on endogenous FoxO3a, we cotransfected a siRNA against SGK1 together with GFP to monitor transfected cells. In line, only 20% of nontransfected cells showed nuclear staining for endogenous FoxO3a, whereas in 65% of the cells transfected with siRNA against SGK1, endogenous FoxO3a localized in the nucleus (Fig. 4B). These findings clearly demonstrate that an aberrantly activated Wnt/β-catenin signaling enforces the cytoplasmic localization of FoxO3a through SGK1.

Wnt Signaling Inhibits FoxO-dependent Transcription through SGK1—Because Wnt signaling could inhibit the nuclear localization of FoxO3a, we asked the question whether it would also alter FoxO-dependent transcription. To that end, we employed a FoxO-dependent promoter reporter construct (8×DBE). As expected, the basal activity of this reporter in HCT116 cells increased after transfection of FoxO3a and was strongly reduced following the cotransfection of SGK1. Interestingly, similarly to SGK1, the cotransfection of wild type or dominant-active S45A β-catenin reduced activation, whereas the transfection of APC increased the activity of the reporter by FoxO3a (Fig. 5A). Note that in these experiments FoxO3a protein levels remained unchanged (Fig. 5A). In addition, β-catenin could inhibit the activity of FoxO4 in this reporter assay, whereas APC promoted activation (supplemental Fig. S1). In striking contrast, the cotransfection of β-catenin, APC, or SGK1 had no influence on the activation of the reporter when the phosphorylation dead mutant FoxO3a-TM was used in this assay (Fig. 5B). These data indicate that the modulation of FoxO-dependent transcription by Wnt activators and repressors requires the phosphorylation of FoxO3a. We also observed that β-catenin decreased the FoxO3a-dependent transcription in a dose-dependent manner (supplemental Fig. S1). As a negative control, we used a c-Jun-dependent reporter, which remained unchanged in response to the various treatments used in our
reporter assay. (Fig. 5C; see also supplemental Fig. S1 for further control reporters).

We were interested to see whether modulation of Wnt signaling after knockdown of endogenous components of the pathway would also alter FoxO-dependent transcription. Indeed, in HCT116 cells, the activity of FoxO3a on the 8×DBE was diminished after Wnt signaling activation through APC knockdown. Reciprocally, Wnt signaling inhibition through β-catenin knockdown in these cells increased reporter activation by FoxO3a, demonstrating that endogenous components of the Wnt pathway are involved in the regulation of FoxO activity (Fig. 5D).

To determine whether Wnt signaling influences FoxO3a-dependent transcription through the up-regulation of SGK1, we reduced FoxO3a-dependent transcription by either β-catenin transfection or knockdown of APC and additionally knocked down SGK1 via siRNA. The efficient knockdown of SGK1 using two different siRNAs increased FoxO3a-dependent reporter activity, which could be reduced again by cotransfection of SGK1 (Fig. 5, E and F, and supplemental Fig. S1). Importantly, knockdown of SGK1 abolished the negative effects of activated Wnt signaling on FoxO3a-mediated transcription (Fig. 5, E and F). To specifically investigate SGK1 function in relation to Wnt signaling and FoxO3a, we cotransfected APC and SGK1 and measured FoxO3a-dependent transcription. Although APC was able to counteract the repression of FoxO-dependent transcription by β-catenin, it had no effect on the repression by exogenous SGK1 (Fig. 5G). Our data strongly support the notion that Wnt signaling can counteract FoxO-dependent transcription via the up-regulation of SGK1.

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FIGURE 4. Wnt signaling regulates localization of exogenous and endogenous FoxO3a through SGK1 in DLD1 colon cancer cells. A, DLD1-dnTCF4 cells were transfected with FoxO3a (YFP fusion) alone or together with SGK1 (FLAG fusion) or siRNA against SGK1 (siSGK), as indicated on the left. Wnt signaling was either left in an active state (−dox) or inhibited by induction of dnTCF4 with doxycycline (+dox), as indicated within the panels. The cells were processed for YFP fluorescence or FLAG immunofluorescence as indicated within panels. The bar charts adjacent to panels show the percentage of cells with a nuclear (N) or cytoplasmic (C) distribution of transfected FoxO3 in response to individual treatments and transfections as indicated. B, DLD1-dnTCF4 cells were left untransfected or transfected with SGK1 (FLAG fusion) or with a siRNA against SGK1 together with GFP to mark transfected cells (siSGK1 + GFP), as indicated on the left. Wnt signaling was either left in an active state (−dox) or inhibited by induction of dnTCF4 (+dox), as indicated within panels. The cells were processed for endogenous FoxO3a, FLAG immunofluorescence or GFP fluorescence as indicated within panels. In the lower panels the dotted circles indicate the positions of the nucleus as revealed by 4′,6′-diamino-2-phenylindole staining (not shown). The bar charts adjacent to the panels show the percentage of cells with nuclear (N) or cytoplasmic (C) distribution of endogenous FoxO3a. At least 100 cells were counted per experiment from two independent experiments.
thought to be mediated through transcriptional activation of FoxO3a target genes, the pro-apoptotic BIM and cell cycle regulator p27^KIP1. To assess the role of Wnt signaling in the regulation of these FoxO target genes, we measured the relative expression of both BIM and p27^KIP1 by real time RT-PCR in HCT116 cells. As seen in Fig. 6A, transfection of FoxO3a induced the expression of both BIM and p27^KIP1, which was abrogated upon cotransfection of wild type/S45A β-catenin or SGK1. In agreement, expression of both target genes increased after inhibition of Wnt/β-catenin signaling in DLD1 cells (Fig. 6B). To confirm that the changes observed were due to changes in transcriptional activity at the promoters of these target genes, we analyzed promoter reporter constructs for both BIM and p27^KIP1. As has been reported before, FoxO3a expression induced the activity of both reporters (53). This activation was repressed when wild type/S45A β-catenin or SGK1 were coexpressed (Fig. 6C). In line, the expression of APC further increased the activity of these promoter constructs (Fig. 6C). Because FoxO3a can induce apoptosis, we reasoned that if Wnt signaling can influence FoxO3a-dependent transcription, it could possibly alter apoptosis induced by FoxO3a. We found that inhibition of Wnt signaling in DLD1-dnTCF4, which induces nuclear localization of FoxO3a (Fig. 4B), led to apoptosis as determined by propidium iodide staining and fluorescence-activated cell sorter analysis as well as by M30 CytoDEATH™ (Roche Applied Science) staining (supplemental Fig. S1 and data not shown, respectively). Furthermore, we transiently overexpressed FoxO3a in HCT116 cells and using TUNEL assay determined the extent of apoptosis. Twenty-four hours after FoxO3a transfection, nearly half of the cell population was undergoing apoptosis. Coexpression of β-catenin or SGK1 reduced apoptosis by ∼30–40 and 70%, respectively, whereas the coexpression of APC further promoted FoxO3a-induced apoptosis (Fig. 6D). These results further substantiate a repressive role of active Wnt signaling in FoxO-mediated transcription and apoptosis.

**DISCUSSION**

In the present study we show that activation of the Wnt signaling pathway induces expression of SGK1 and leads to nuclear exclusion of FoxOs, consequently inhibiting FoxO-mediated transcription and apoptosis. These results place SGK1 at the center of a cross-talk between Wnt signaling and the action of FoxOs, which might have important consequences in development and cancer.
This indicates that β-catenin-dependent transcription is important for the up-regulation of SGK1. The finding prompted us to analyze the expression of SGK1 in other cells following modulation of the Wnt signaling. In DLD1 colon cancer cells induced to express a dominant-negative TCF that inhibits signaling, SGK1 expression was drastically down-regulated. Additionally, we observed high SGK1 expression in Wnt1 expressing Rat2 cells as compared with control cells. These findings further support our conclusion that SGK1 is a target of the Wnt pathway. In line with our results, immunohistochemistry revealed that SGK expression levels in colonic adenomas, which show strong Wnt signaling, are increased as compared with healthy tissue (54). Promoter reporter experiments showed up-regulation of the SGK1 promoter by β-catenin, and ChIP analyses further confirm that the transcriptional activation of SGK1 by Wnt signaling is a direct one, demonstrating the presence of β-catenin and TCF4 as well as LEF1 at the endogenous SGK1 promoter. These data additionally point to a β-catenin-dependent regulation of SGK1 in our HCT116 cellular system. Taken together, our results strongly indicate that SGK1 is a direct target gene of β-catenin.

Wnt Signaling and FoxO—Several functions have been proposed for SGK1, and the question arises of which of these might be related to Wnt signaling. Renal sodium retention is defective in SGK1 knock-out mice, reflecting the role of SGK1 in regulating sodium transporter activity. There has been no evidence so far that Wnt signaling plays a role in this process. Under stress conditions and after growth factor withdrawal, SGK1 appears to play a protective role by promoting cell survival and inhibiting apoptosis (10, 12, 52). Among key substrates of SGK1, which are relevant for its anti-apoptotic effects, are members of the FoxO transcription factor family. SGK1 inhibits FoxOs through phosphorylation and nuclear exclusion, which in turn leads to a reduced transcription of pro-apoptotic target genes. Our data show that Wnt signaling interferes with FoxO-induced apoptosis, most likely by blocking its transcriptional activity. The activation of the Wnt pathway interferes with the nuclear localization of FoxO3α and reduces transcription from artificial FoxO-dependent reporter constructs as well as the expression of known FoxO target genes BIM and p27KIP1 and inhibits reporters driven by promoters of both genes. Moreover, SGK1 seems to be required for the effects of Wnt signaling on FoxO-dependent transcription. In recent years Forkhead box proteins including FoxOs have been described to have characteristics of tumor suppressors (55, 56). It has been shown that the loss of FoxO genes result in modest neoplasia phenotypes (56). Interestingly, a study using APC<sup>MIM</sup> mice, which carry truncating mutations in the APC gene and are genetically susceptible to colorectal cancers demonstrated that APC<sup>+</sup>/Min Fox1<sup>−/−</sup> mice displayed increased tumor load in the colon compared with APC<sup>+</sup>/Min Fox1<sup>+</sup> (57). This suggests that the loss or reduction of FoxO activity synergizes with an active Wnt signaling to promote tumor formation and maintenance. Our results propose that an activated Wnt signaling can actually promote FoxO inhibition, thus taking advantage of the anti-apoptotic consequence of low FoxO activity.

It has recently been shown that the β-catenin homolog BAR-1 in <i>C. elegans</i> is required for FoxO (Daf-16)-dependent
Wnt Signaling Regulates SGK1

It was suggested that β-catenin activates FoxO-dependent transcription by directly interacting with FoxO transcription factors. As shown in Figs. 5 and 6, in our experimental conditions β-catenin had rather the opposite effect, i.e. β-catenin repressed FoxO-dependent reporters. The reason for this discrepancy is presently unclear. Essers et al. (58) also showed that β-catenin overexpression enhanced the FoxO-induced arrest in G1, something that seems counterintuitive when considering the relationships between β-catenin overexpression, Wnt signaling activation, and cancer progression. It is possible that different levels of effectors (FoxO, β-catenin) were achieved in these studies that would result in either activation or inhibition. Our experiments with siRNA against APC and β-catenin confirm the results we obtained in overexpression experiments. Importantly, we could show that various activators of the Wnt signaling inhibit the nuclear localization of exogenous and endogenous FoxO3α, which is in line with an overall repressive action of Wnt signaling on FoxOs.

FoxOs appear to play a role in stem cell maintenance and might counter c-Myc, which is a central target gene of the Wnt pathway (55). Given the repressive effects of Wnt signaling on FoxOs elucidated in our study, we propose that Wnt and FoxO pathways act antagonistically with respect to cell transformation.

Acknowledgments—We thank M. Sachs, F. Kuphal, and L. Klein-Hitpass (Essen) for assistance in microarray analysis.

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