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# GCIP/CCNDBP1, a Helix–Loop–Helix Protein, Suppresses Tumorigenesis

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**Abstract** Deletions and/or loss of heterozygosity (LOH) on chromosome 15 (15q15 and 15q21) have been found in several human tumors, including carcinomas of the colorectum, breast, lung, prostate, and bladder, suggesting the presence of potential tumor suppressor gene(s) in this particular region of chromosome 15. GCIP also called CCNDBP1, DIP1, or HHM, localized at chromosome 15q15, is a recently identified helix–loop–helix leucine zipper (HLH-ZIP) protein without a basic region like the Id family of proteins. In this study, we reported that the expression of GCIP was significantly downregulated in several different human tumors, including breast tumor, prostate tumor, and colon tumors. In human colon tumors, both mRNA and protein expression levels of GCIP were decreased significantly compared to the normal tissues. Treatment of colon cancer cells SW480 with sodium butyrate (NaB), which induces colon cancer cell differentiation, can induce the upregulation of GCIP expression, suggesting that the protein functions as a negative regulator in cell proliferation. Overexpression of GCIP in SW480 colon cancer cell line resulted in a significant inhibition on tumor cell colony formation, while silencing of GCIP expression by siRNA can promote cell colony formation. Furthermore, overexpression of GCIP inhibited the transcriptional activity of cyclin D1 promoter and the expression of cyclin D1 protein in the cell. Finally, we demonstrate that GCIP specifically interacts with one of the class III HDAC proteins, SirT6, which is important for maintaining genome stability. Together, our data suggest a possible function of GCIP in tumor suppression. J. Cell. Biochem. 100: 1376–1386, 2007. 

**Key words:** CCNDBP1; GCIP/DIP1/HHM; SirT6; tumor suppressor; cyclin D1; colon cancer

Cell growth and differentiation are complex and well-organized processes in which cells respond to stimuli from the environment by carrying out genetic programs. Basic helix–loop–helix (bHLH) transcription factors play a key role in cell growth, proliferation, and differentiation by controlling the expression of genes involved in cell lineage commitment, cell fate determination, proliferation, and survival through homo- or hetero-dimerization and binding to "E box" sequences [Massari and Murre, 2000]. The HLH proteins can be classified into several groups based on their tissue distribution, partner choice, DNA-binding properties, and structural features. The first group is the E proteins, the products of E2 genes (E12, E47, E2-5, E2-2, and HEB), also called class A. The E-proteins serve as a heterodimer partner for the tissue-specific class B of bHLH proteins [Murre et al., 1989; Lassar et al., 1991; Hu et al., 1992; Kee et al., 2000]. The second group is the tissue-specific class B bHLH proteins that bind E-box DNA sequences and regulate tissue-specific cell growth and differentiation [Chakraborty et al., 1991; Weintraub, 1993; Hamamori et al., 1997; Bounpheng et al., 2000]. Examples include the

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myogenic bHLH family of proteins (MyoD, Myogenin, Myf-5, MRF4), the cardiogenic family of proteins (eHand and dHand), the neurogenic family of proteins (Neurogenin, NeuroD, Mash-1, Mash-2, Hes, NSCL), and the hematopoietic family of bHLH proteins (SCL/TAL-1, Lyl-1, and ABF-1). The third group is the members that have a leucine zipper motif localized in the carboxyl-terminal of the bHLH region, such as the *Myc* family of proteins [Blackwell et al., 1990]. Members of the *Myc* proto-ongene family encode transcription factors that regulate multiple aspects of cell functions, including proliferation, differentiation, transformation, and apoptosis [Dang et al., 1999]. The fourth group of HLH protein is the Id family of proteins that lack a basic region prior to the HLH domain. This family of proteins forms a complex with class A and some class B factors and is unable to bind to DNA. The Id family of proteins acts as dominant negative inhibitors for class B factors, functioning as positive regulators of cell growth and as negative regulators of cell differentiation [Benezra et al., 1990; Norton and Atherton, 1998; Lasorella et al., 2000]. Mice deficient in the Id family of proteins have profound defects in neurogenesis, angiogenesis, vascularization of tumor xenografts, impaired immune responses, and tumorigenesis [Lyden et al., 1999; Rivera et al., 2000; Rivera and Murre, 2001; Ruzinova and Benezra, 2003; Sikder et al., 2003a].

GCIP (CCNDBP1), an HLH leucine zipper protein without a basic region, was initially identified and characterized in our lab and others to interact with Grap2, cyclinD1 (DIP1), and as a human homolog of MAID protein (HHM) [Terai et al., 2000; Xia et al., 2000; Yao et al., 2000]. GCIP encodes a 360-amino acid protein with a calculated molecular mass of 40 kDa. It contains an HLH domain without a basic region like the Id family. On the other hand, GCIP also contains putative leucine-zip domains in its structure similar to the *Myc* family of proteins. Northern blot analysis detected a 1.3-kb transcript in all tissues examined, with highest expression in heart, muscle, peripheral leukocytes, and brain. Mouse GCIP which is named as Maid (Maternal Id-like molecule), was isolated from a subtraction cDNA library enriched for maternal transcripts that are still present in the mouse 2-cell stage embryo. It may model the translational and transcriptional regulation of gene expression during the transition from gamete to embryo [Hwang et al., 1997]. Our data from Western blot and RT-PCR experiments indicated that GCIP is expressed mostly in differentiating or terminally differentiated cellular stage, with a reduced expression in growing and proliferating cells such as other reported negative regulators of cell growth [Xiang et al., 2002] overexpression of GCIP in transgenic mice decreases susceptibility to chemical hepatocarcinogenesis [Ma et al., 2006].

Constitutive activation of the Wnt-signaling pathway is known to play a crucial role in colon cancer. β-Catenin and Tcf4 are the downstream effectors of the Wnt signaling cascade. In colorectal cancer, mutations in Wnt cascade genes such as APC and β-catenin lead to β-catenin inappropriately accumulating in nuclei where it forms active complexes with lymphoid enhancer factor-1 (LEF-1)/T-cell transcription factors, and ultimately activation of transcription of downstream target genes, such as c-*Myc* and cyclin D1 [Bienz and Clevers, 2000; Hovanes et al., 2001; Clevers, 2004].

GCIP is localized on chromosome 15q15, a region frequently deleted or loss of heterozygosity (LOH), in different tumors, including colorectum, breast, lung, and bladder tumor [Natrajan et al., 2003]. To understand the potential functions of GCIP as a potential tumor suppressor on chromosome 15, we further examined the expression patterns of GCIP and its potential role in cell cycle and cell signal transduction. In this study, we demonstrated that the expression levels of GCIP at both mRNA and protein level were significantly decreased in colon tumor using Northern blot analysis with matched Normal/Tumor expression array and immunocytochemistry with human tissue arrays. Overexpression of GCIP in SW480 colon cancer cells inhibits tumor cell colony formation on soft agar. Decreasing GCIP expression by specific small interfering RNAs (siRNAs) promoted colony formation, suggesting GCIP could function as a potential tumor suppressor in colon carcinogenesis. Furthermore, sodium butyrate (NaB), an agent inducing cell differentiation (inhibiting cell proliferation) in SW480, increased GCIP expression, further supporting the notion that GCIP could function as a regulator of cell proliferation and differentiation. In addition, GCIP specifically interacts with one of the class III nicotinamide adenine dinucleotide (NAD)-dependent

deacetylases, SirT6. Transient overexpression of GCIP in the cell inhibited the transcriptional activity of cyclin D1 promoter, as well as significantly inhibited the expression of endogenous cyclin D1 protein. Together, these data suggest that GCIP may function as an important regulator in colon cancer cell tumorigenesis.

## MATERIALS AND METHODS

### Cell Culture

The colon cancer cell line SW480 and monkey kidney cell line Cos-7, were obtained from ATCC (Rockville, MD). Both were maintained in a 37°C incubator with 5% $CO_2$ humidified air in Dulbecco's minimal modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum.

### Northern and Western Blot Analyses

Total RNA was isolated using TRIZOL reagent (Invitrogen) and separated on 1% agarose-formaldehyde gel. RNA was transferred by capillary blotting overnight onto Hybond+ membrane (Amersham-Pharmacia Biotech) in 10× SSC. GCIP probe was labeled with [$\alpha$-$^{32}$P] dCTP (Amersham) using the Random Primes DNA Labeling system (Invitrogen), and hybridized according to the manufacturer's protocol. Prehybridization and hybridization were done in 10 ml of Rapid Hyb buffer (Amersham) at 68°C for 1 h and overnight, respectively. The blot was washed two times in 2× SSC with 0.1% SDS at room temperature for 30 min, followed by two washes in 0.1× SSC with 0.1% SDS at 68°C for 30 min. We stripped the GCIP probe and re-probed the blot with β-actin probe using the same protocol. Blots were exposed 3 h to overnight on BioRad Phosphorimager screen.

Cells were lysed by 4 g/L trypsin containing 0.2 g/L EDTA, then collected after washing twice with phosphate buffered saline (PBS, pH 7.4). Total protein was extracted by using TRIZOL reagent (Invitrogen) and dissolved in 1% SDS by incubating at 50°C. Each sample was diluted by 1:20, thus the resulting 0.05% SDS protein will not interfere protein quantification. Protein concentration was measured by Pierce's BCA Assay Kit. Protein samples (20 μg each) were separated by 10% SDS–PAGE and electroblotted to Hybond-P membranes (Amersham). The blots were blocked with 50 g/L nonfat milk in TBST washing buffer for 1 h at room temperature and then incubated at 4°C overnight with primary antibodies. After washing, the blots were labeled with HRP-conjugated secondary antibodies and detected by ECL (PIERCE). For repeated blots using the same membranes, membranes were striped in strip buffer (2% SDS, 100 mM beta-mercaptoethanol, 50 mM Tris, pH 6.8) and incubated at 50°C for 30 min with shaking. The blot was rinsed multiple times in TBST and then could be used as described above. The P21, CyclinD1, Actin antibody were bought from Santa CruZ Biotechnology, Inc.

### Match Normal/Tumor Expression Array and Tissue Immunohistochemical Staining

Matched cDNA array from human normal (N) and tumor (T) tissues were obtained from Clontech. The membrane was blotted by using [$\alpha$-$^{32}$P]dCTP-labeled GCIP probe to show the expression of GCIP in colon normal and tumor tissue. We then stripped the GCIP probe and re-probed the membrane with [$\alpha$-$^{32}$P] dCTP-labeled Ubiquitin to demonstrate that equal amount of cDNAs between the normal/tumor (N/T) samples were loaded to the membrane. The manufacturer's protocol was followed in each step.

Human colon and rectal cancer tissue and normal tissue array was obtained from IMGENEX. The specific primary rabbit anti-GCIP antibody was produced using the GST-C-terminal domain of GCIP and was affinity purified. The primary antibody was used at 1:1,000 in block serum. Rabbit ABC staining kit was purchased from Santa Cruz Biotechnology. Immunohistochemical staining follows the manufacturer's protocol from Santa Cruz Biotechnology. The slides were evaluated by pictures taken under a Nikon Digital camera Dxm1200 microscope. The immunohistochemical staining in tissues was scored according to the following guidelines: 0 for no staining; 1 for weak staining; 2 for mediate staining; and 3 for strong staining in the colon samples. The data were used for *t*-test statistics analysis. The results were considered statistically significant at $P < 0.05$.

### Luciferase Reporter Assays

Forty-eight hours after transfection of the plasmids, the cells in a 24-well plate were lysed and harvested in 200 μl reporter lysis buffer (Promega), and cell lysates were assayed for

luciferase activities. The luciferase assay was carried out using luciferase assay kit (Promega) and Packard Topcount Scintillation Counter. Extracts were also assayed for β-galactosidase activity with the Galacto-Light Plus™ beta-Galactosidase Reporter Gene Assay Systems (Tropix). Each extract was assayed three times, and the mean relative light unit (RLU) was corrected by values obtained from an extract prepared from nontransfected cells. The relative luciferase activity was calculated as RLU/β-galactosidase.

### Soft Agar Assay

SW480 colon carcinoma cells were cultured in DMEM containing 10% fetal calf serum and transfected by pCMV-Tag2B vector, the pCMV-Tag2B-GCIP plasmid, and the siRNA plasmid specific for GCIP. Transfected cells will be selected with G418 for 7 days. After selection, cells will be analyzed for colony growth by suspended in 0.3% agarose medium containing DMEM+5% FBS and layered onto a 2.5-ml bed of 0.6% agarose in a 35-mm dish with grids. Plates were incubated for 2–3 weeks, and the number of colonies >100 μm will be counted.

### In Vivo Protein Binding Assays and In Vitro Co-Immunoprecipitation Assays

For construction of glutathione S-transferase (GST)-GCIP protein, full-length GCIP was subcloned into pGEX4T-1 vector by PCR. GST-GCIP fusion proteins were purified as recommended by the manufacturer (Amersham Pharmacia Biotech) and used in an in vitro protein-binding assay.

In an effort to examine GCIP and SirTs protein interactions in vivo, Cos-7 cells were transfected with pCMVTag control expression plasmids or with expression plasmids containing Flag-SirT1-7 separately. Following 48 h after transfection, collected cells were washed with PBS and resuspended in 400 μl ice-cold cytoplasmic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA, 1 mM DTT, 1.0 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mg/ml benzamidine) and incubated for 15 min on ice. Ten percent NP-40 (12.5 μl in amount) was added, the reaction was centrifuged at 12,000 rpm for 1 min, and the supernatant was collected as the cytoplasmic extract. The pellet was resuspended in 25 μl nuclear extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1.0 mM EDTA pH 8.0, 1.0 mM EGTA pH 7.0) and incubated on ice for 30 min with intermittent vortexing. The nuclear extract was collected following centrifugation. The cytoplasmic and nuclear extract were mixed and diluted in binding buffer (1× PBS pH 7.2, 10% Glycerol, 1 mM $MgCl_2$, 0.2 mM ATP, 0.1% BSA, 0.01% Triton X-100, 1 mM DTT, 1 mM EGTA). GST-GCIP was added to each sample, and the sample was incubated for an additional 1 h at 4°C. After being washed with binding buffer three times, the final bead pellet was resuspended in SDS loading buffer and subjected to protein gel electrophoresis, followed by transfer to a nylon membrane. Western analysis was carried out as described above by using an anti-Flag monoclonal antibody to detect the presence of the SirTs protein.

For GCIP and SirTs protein coimmunoprecipitation assays, a GCIP rabbit polyclonal antibody was added to each sample in IP buffer (10 mM HEPES, pH 7.6, 250 mM NaCl, 0.25% NP-40, 5 mM EDTA), and the mixture was incubated at 4°C for 1 h. Protein A-agarose beads (20 μl) were added to each sample subsequently, and the sample was incubated for an additional 1 h at 4°C. After being washed with nuclear lysis buffer, the final bead pellet was resuspended in SDS loading buffer and subjected to protein gel electrophoresis and Western blot as described above.

## RESULTS

### Decreased or Loss of Expression of GCIP (CCNDBP1) in Human Tumor Tissues

Altered expression of genes in tumor tissue compared to normal tissue suggests the involvement of such genes in carcinogenesis. GCIP is localized on chromosome 15q15, a region found with deletions in several human tumors, including carcinomas of the colorectum, breast, lung, and the bladder. To examine the expression pattern of GCIP in different human tumor and corresponding normal tissues, we first performed an initial database search to examine the differential expression patterns of the gene in Oncomine database (http://www.oncomine. org) [Rhodes et al., 2004]. We found that the expression levels of GCIP were significantly decreased in several cancer tissues with tumor progression and metastasis, including breast tumors, prostate tumors, and colon tumors (Data not shown, see Oncomine website for

details). To examine the potential role of this gene in tumorigenesis, we further performed Northern blot analysis with Matched human Normal/Tumor expression array (CLONTECH Laboratories, Inc.) using the $^{32}P$ labeled specific GCIP cDNA probe. As shown in Figure 1, the expression level of GCIP mRNA was significantly decreased in 9 of 11 matched colon tumor samples (Fig. 1A, top, N, normal tissues; T, tumor tissues from the same patient). As a control, human ubiquitin cDNA probe was used in the matched Normal/Tumor array to show that equal amounts of mRNA were loaded between normal and tumor samples (Fig. 1A, bottom). The significant decrease of GCIP mRNA expression level prompted us to examine whether GCIP can function as a negative regulator (suppressor gene) in human colon cancer cells and in cell proliferation pathway.

To further determine whether GCIP expression at protein level is also decreased in colon tumor tissues compared to normal colon tissues, we analyzed the expression of GCIP proteins in 58 human colon and rectal cancer tissue samples and matched normal tissues by immunohistochemistry using specific anti-GCIP antibody (Tissue Array slide from IMGENEX Corporation). Immuno-histochemical staining results showed that GCIP protein expression was decreased significantly in colon and rectal tumor tissues (Fig. 1B(c,d), Tumor) as compared to normal colon samples (Fig. 1B(a,b), Normal). Among the 58 tissue samples, the expression level of GCIP protein was decreased significantly in 50% of the tumor samples (29 out of 58 samples) (Fig. 1C). The anti-GCIP antibody shows excellent specificity for endogenous and overexpressed GCIP protein in the cell nucleus (Fig. 1D). Together, our data demonstrate that the expression of GCIP is significantly decreased at both mRNA and protein levels in colon cancer samples as compared to normal colon tissues, suggesting that GCIP functions as a potential tumor suppressor in colon tumorigenesis.

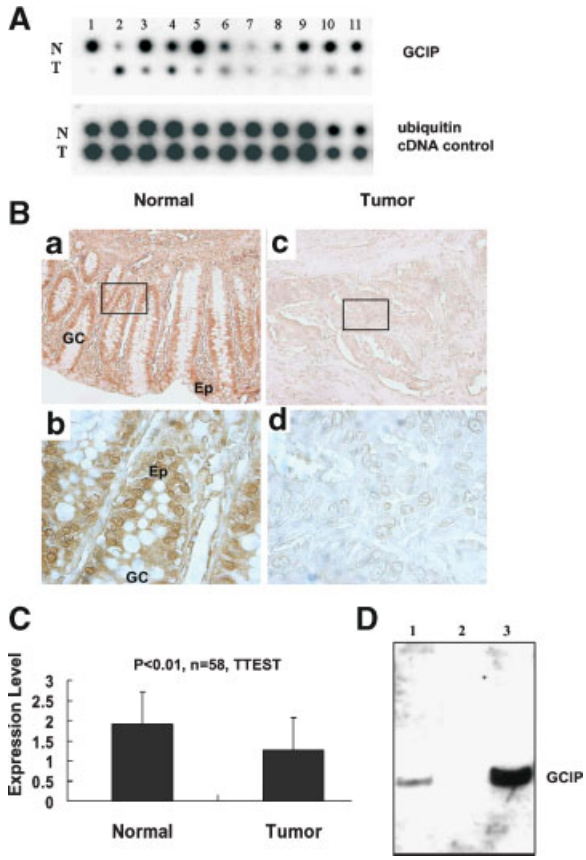


**Fig. 1.** Decreased or loss of expression of GCIP in colon tumors. **A**: The expression of GCIP mRNA was significantly decreased or lost in colon tumors. The $^{32}P$ labeled GCIP cDNA probe and human ubiquitin cDNA control probe were hybridized to the matched Normal/Tumor expression array (product from CLONTECH Laboratories, Inc.), which includes amplified cDNA from 11 colon tumors and corresponding normal tissues from individual patients. GCIP mRNA level significantly decreased in 7 out of 11 samples. **B**: GCIP protein expression decreases in colon tumor. Immunohistochemical staining for GCIP in colon normal tissue (a,b) and corresponding tumor tissue (c,d) from the identical patients. In normal colon tissue, the expression of GCIP is higher in epithelium (Ep), but lower in goblet cell [Kasbohm et al., 2005]. Note that GCIP expression is significantly reduced in colon tumor tissues (c,d) as compared to normal tissues (a,b). **C**: Relative expression levels of GCIP protein in colon tumor tissues and corresponding normal tissues determined by immunohistochemical staining method were evaluated by *t*-test. The expression level of GCIP protein detected in the colon tumor tissues is significantly lower than that from the corresponding normal colon tissue ($P < 0.01$, $n = 58$, *t*-test). **D**: Western blot analysis of GCIP protein demonstrated the specificity of anti-GCIP antibody. Nuclear extracts from SW480 cells transfected with control vector (pCMV-Tag 2B) (**lane 1**) and GCIP (**lane 3**). As a control, cytoplasmic extract from SW480 cell without transfecion was shown in lane 2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

## Sodium Butyrate (NaB) Inhibits Colon Cancer Cell Proliferation and Increases the Expression of GCIP

Butyrate is a short-chain fatty acid naturally generated in the colon by the anerobic bacterial fermentation of dietary fiber [Topping and Clifton, 2001]. Butyrate was shown to be a trophic factor for normal colonic epithelial cells

both in vivo and in vitro [Scheppach et al., 1992; Gibson et al., 1999]. In contrast, sodium butyrate was shown to inhibit cell proliferation and induce cell differentiation in cancer colonic epithelial cells [Ho et al., 1994; Benard and Balasubramanian, 1997; Navarro et al., 1997; Archer et al., 1998; Vincan et al., 2000]. To investigate the possible involvement of GCIP in colon cancer cell proliferation and differentiation, we treated the SW480 cells with 5 mM sodium butyrate (NaB) and then examined the expression of GCIP during NaB-induced cell differentiation using specific antibody of GCIP and Western blot analysis.

As shown in Figure 2, the expression of GCIP was significantly increased during the differentiation of SW480 cells induced by NaB. The amount of GCIP protein increased approximately threefold after 12 h induction of the cells by NaB (Fig. 2B). Previous studies indicated that NaB inhibited colon cancer cell growth and induce cell differentiation by stimulating the expression of p21 protein [Archer et al., 1998; Vaziri et al., 1998; Orchel et al., 2003]. As a control, we found that the expression of GCIP correlates very well with the expression of p21 during the differentiation of SW480 cells induced by NaB (Fig. 2A, middle). Together, these data suggest that GCIP is highly expressed in the differentiated cell stage and that GCIP expression is downregulated in tumorigenesis and in proliferating cells.

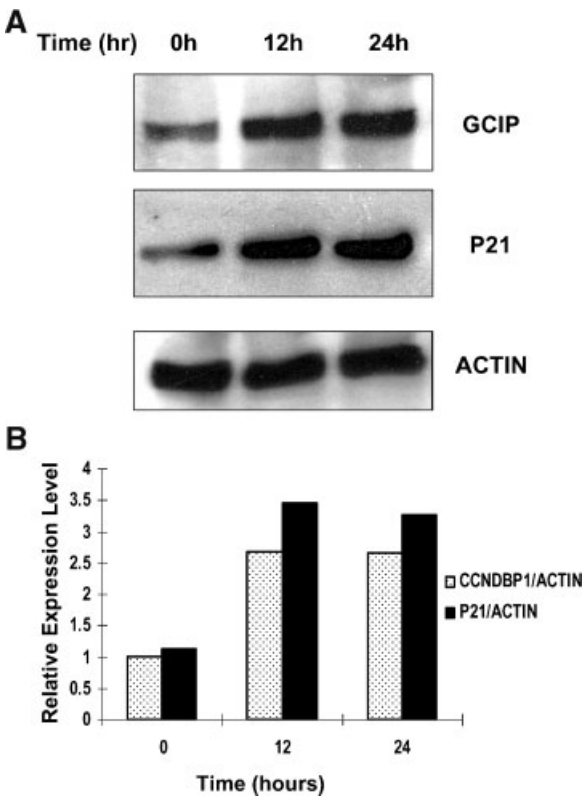


**Fig. 2.** Induced expression of GCIP/CCNDBP1 and p21 in SW480 cells by 5 mM NaB. **A**: Western blot analysis of GCIP and p21 expression in SW480 cells after treatment with 5 mM NaB. Equal aliquots of protein extracted from SW480 cells 0, 12, and 24 h after NaB treatment were electrophoresed and the proteins were transferred to a Hybond-P membranes. The membranes were incubated with an anti-p21 antibody, anti-GCIP antibody, anti-actin antibody, and the specific binding of the antibodies was detected using ECL system; result showed that GCIP protein level increased as well as P21 during the differentiation induced by NaB. **B**: Relative values summarized from Western blots showed the expression level of GCIP and P21 increase 2.5 to 3.5-fold after 12 and 24 h NaB treatment.

### GCIP Regulates Cell Proliferation and Suppresses the Tumorigenicity of SW480 Colon Cancer Cells

To explore the role of GCIP in cell proliferation and tumorigenesis, we use small interference RNAs (siRNAs) to inhibit GCIP expression in cells and then examine how GCIP affects cell proliferation. For stable expression of siRNAs within cells, the U6 promoter was cloned in front of GCIP gene specific targeting sequence (19 nt sequences GACTCAATGAGGCAGCTGT from GCIP cDNA separated by a 9 nt spacer from the reverse complement of the same sequence) and five thymidines (T5) as termination signal (Fig. 3A). SW480 cells were transfected with pCMV-Tag2B vector, pU6-GCIP siRNA, and pCMV-Tag2B-GCIP. Total RNA and proteins were extracted from the transfected and control cells, Northern blot and Western blot analyses were performed to examine the expression levels of GCIP mRNA and GCIP protein (Fig. 3B,C). As shown in Figure 3B,C, GCIP mRNA and protein were significantly decreased in the cells transfected with pU6-GCIPsiRNA, suggesting that pU6-GCIPsiRNA could efficiently decrease the expression of GCIP mRNA and protein. Similar data were obtained for monkey kidney cells (COS-7) transfected with the same plasmids (data not shown). Two other siRNA constructs for GCIP had no effect on both mRNA and protein expression and are used as control for specific siRNA knockdown of GCIP (data not shown).

To examine the effect of GCIP in tumorigenesis, we examined how GCIP regulated ancho-

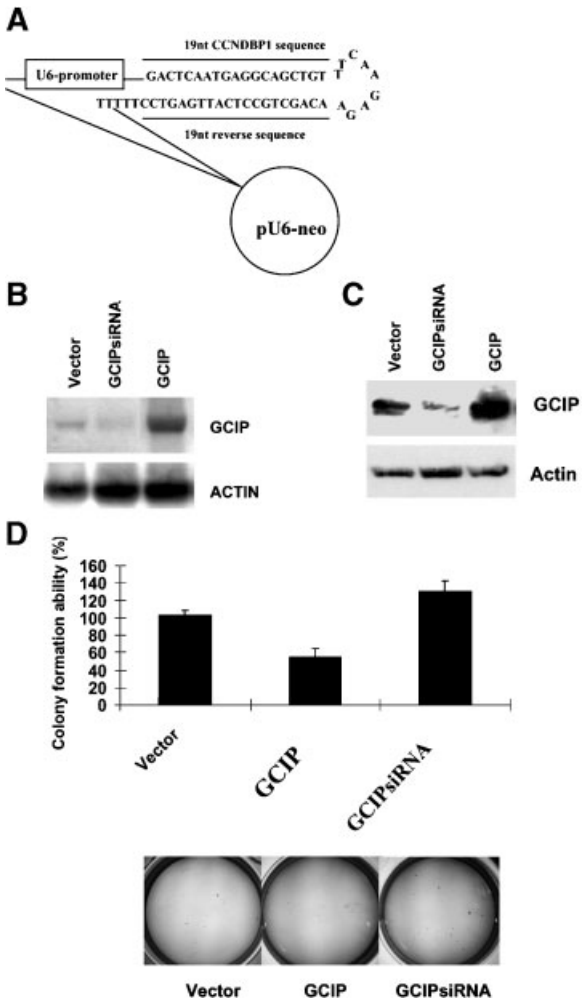


**Fig. 3.** Downregulation of GCIP expression promotes tumor cell colony formation. **A**: Constructs of GCIP small interference RNA. **B**: Northern blot analysis of SW480 cells transfected with pCMV-Tag2B vector, pU6-GCIPsiRNA, and pCMV-Tag2B-GCIP. GCIP RNA was decreased in the cells transfected with pU6-GCIPsiRNA but increased in the cells transfected with pCMV-Tag2B-GCIP. **C**: Western blot analysis of SW480 cells transfected with pCMVTaq2B vector, pU6-GCIPsiRNA, and pCMV-Tag2B-GCIP. GCIP protein was decreased in the cells transfected with pU6-GCIPsiRNA but increased in the cells transfected with pCMV-Tag2B-GCIP. **D**: GCIP suppresses anchorage-independent growth of SW480 colon cancer cells. SW480 cells were stably transfected with pCMV-Tag2B-GCIP (column 2), pU6-GCIPsiRNA (column 3), or empty pCMV-Tag2B (column 1), plated in soft agar, and assayed for colony formation after 3 weeks. Results showed that overexpression of GCIP inhibited SW480 cell colony formation on soft agar while downregulation of GCIP expression by siRNA promoted tumor cell colony formation.

rage independent cell growth through soft agar assays. SW480 cells stably transfected with pCMV-Tag2B control vector, pU6-GCIPsiRNA, and pCMV-Tag2B-GCIP plasmid, respectively, were analyzed and compared in soft agar assay. As shown in Figure 3D, overexpression of GCIP inhibited SW480 cell colony formation on soft agar assay by 60%, while downregulation of GCIP by specific siRNA significantly increased the colony formation of colon cancer cells by 130%, suggesting that GCIP can function as a suppressor gene for tumor cell colony formation in the soft agar assays.

### GCIP Inhibits the Transcriptional Activity of Cyclin D1 Promoter and the Expression of Cyclin D1

In our previous studies, cyclin D1 has been shown to associate with GCIP [Xia et al., 2000]. Cyclin D1 is well known to be the downstream target gene of cell proliferation pathway. To understand the potential mechanism of GCIP inhibition of cell proliferation and tumorigenesis, we examined how GCIP affects the expression of cyclin D1, a key molecule involved in cell cycle progression and tumorigenesis. First, we investigated how GCIP regulates the transcriptional activation of cyclin D1 by transfecting SW480 cells with the pCMV-Tag2B vector, pCMV-Tag2B-GCIP plasmid, or pU6-GCIP-siRNA plasmids, together with the cyclin D1 promoter reporter. Cell extract were assayed for luciferase or beta-galactosidase activities 48 h later. As shown in Figure 4A, overexpression of GCIP can markedly inhibit cyclin D1 transcriptional activity, while decreasing GCIP expression by specific siRNA in the cells can alleviate the inhibitory effect of cyclin D1 transcription (Fig. 4A). Based on the inhibitory effect of GCIP on the transcriptional activity of cyclin D1 promoter in the cell, we further examined whether GCIP could inhibit the expression of cyclin D1 protein in the cell. Cells were transfected with pCMV-Tag2B vector, pCMV-Tag2B-GCIP, or pU6-GCIPsiRNA, respectively. Total proteins were extracted and separated by SDS–PAGE. Western blot analysis was performed to examine the expression levels of the GCIP, cyclin D1, and actin. As shown in Figure 4B, GCIP protein was decreased in the cells transfected with pU6-GCIPsiRNA, confirming that pU6-GCIPsiRNA could efficiently decrease GCIP expression level (Fig. 4B). Downregulation of GCIP greatly increased the expression level of cyclinD1 protein in the cells transfected with pU6-GCIPsiRNA, while overexpression of GCIP dramatically decreased the

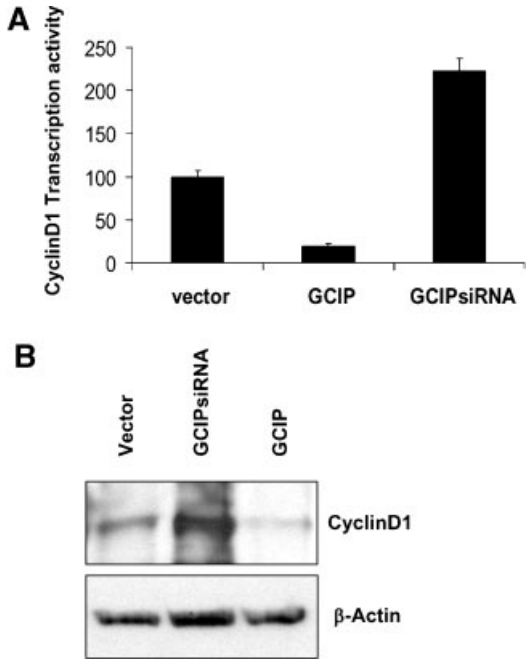


**Fig. 4.** GCIP inhibits the transcriptional activity of cyclin D1 promoter and the expression of cyclin D1 protein. **A**: GCIP inhibits transcriptional activation of cyclin D1 promoter. SW480 cells were plated and transiently transfected with pCMV-Tag2B vector, pCMV-Tag2B-GCIP plasmid, or pU6-GCIPsiRNA, together with the cyclin D1 reporter. Overexpression of GCIP markedly inhibited cyclinD1 promoter transcriptional activity while decreasing GCIP expression can upregulate cyclin D1 transcription. **B**: Inhibition of cyclin D1 protein by GCIP. SW480 cells were plated and transiently transfected with pCMV-Tag2B vector, pCMV-Tag2B-GCIP, or pU6-GCIPsiRNA plasmid, respectively. Cell extracts were assayed for expression of cyclin D1, and β-actin with specific antibodies using Western blot analysis 72 h later. Result showed that overexpression of GCIP markedly inhibited cyclinD1 expression while decreasing GCIP expression by siRNA significantly increased the expression of cyclin D1 protein in the cells.

expression of cyclin D1 in cells transfected with pCMV-Tag2B-GCIP (Fig. 4B), suggesting an important role of GCIP in regulating cyclin D1 expression and tumorigenesis.

### GCIP Interacts With the Class III HDACs, Sirtuin 6 (SirT6), In Vitro, and in the Cells

In our previous study, GCIP was found to function as a transcriptional repressor. And this transcription inhibition activity was not affected when cells were incubated with either of the two HDAC inhibitors, trichostatin A (TSA) and sodium butyrate (NaB) [Ma et al., 2006]. Both TSA and NaB are well-known HDAC inhibitors and were able to strongly promote transcriptional activation at indicated concentration. GCIP functions as a transcriptional repressor through TSA/NaB insensitive pathways, suggesting that GCIP-induced transcriptional inhibition is independent on the class I and class II HDACs, which are sensitive to the inhibition by TSA and NaB.

To further understand whether the class III TSA/NaB-insensitive HDACs, also known as Sirtuins (SirT1–7), are involved in GCIP-induced transcriptional regulation, we examined whether GCIP might act as a transcription repressor through recruitment of Sirtuins. Sirtuins are identified as NAD-dependent deacetylases and their catalytic activities are not inhibited by TSA and NaB. The physical interaction between GCIP and Sirtuins was tested by GST protein pull down assays and by co-immunoprecipitation. As showed in Figure 5, FLAG-tagged SirT1–7 was expressed in mammalian cells and the expression of SirT1–7 proteins was detected by using anti-Flag monoclonal antibody (Fig. 5, top). Only SirT6 protein but not other Sirtuins (SirT1-5 and SirT7) was

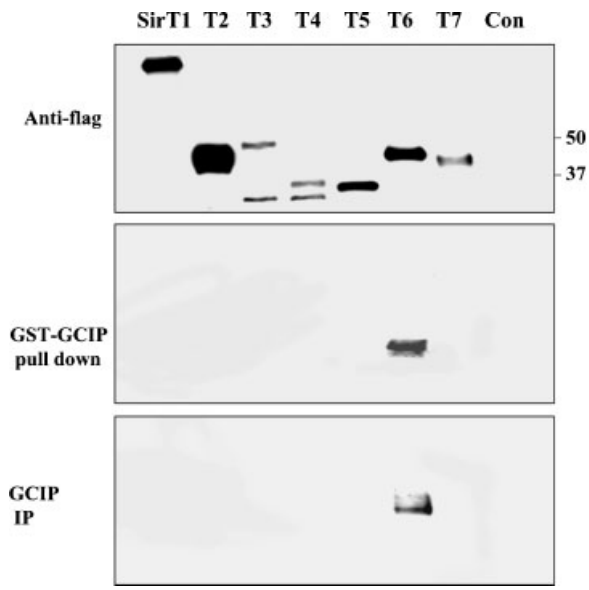


**Fig. 5.** GCIP/CCNDBP1 interacts with SirT6 in vitro and in the cells. Cos-7 cells were transfected with Flag-tagged SirT1-7. The expression of SirT proteins (SirT1–7) was detected by immunoblots of cell lysates using anti-flag antibody (**upper panel**). Interaction of recombinant GST-GCIP protein with SirTs proteins was examined using GST-GCIP pull down assays. Only Flag-SirT6 was retained and pulled down by GST-GCIP (**medium panel**). The SirT6 protein was visualized by Western blot against Flag-tagged SirT6. The GCIP-SirT6 interaction was confirmed by co-immunoprecipitation of the endogenous GCIP and SirTs (**lower panel**). Only Flag-tagged SirT6 was associated with GCIP and immunoprecipitated by specific GCIP antibody.

pulled down by the GST-GCIP fusion protein and by co-immunoprecipitation with antibody against endogenous GCIP protein in mammalian cells (Fig. 5, middle and bottom), suggesting that GCIP specifically interacts with SirT6 protein to form protein complex in mammalian cells (Fig. 5, middle and bottom).

## DISCUSSION

Helix–loop–helix [Hermeking et al., 2000] proteins have been shown to control commitment and differentiation in multiple cell lineages in organisms ranging from Drosophila to human [Kageyama and Nakanishi, 1997; Norton, 2000]. These proteins share a common sequence motif of helix–loop–helix dimerization domain. The bHLH proteins are intimately associated with positive regulation in cell differentiation and lineage commitment. The Id family of proteins does not possess a basic DNA-binding domain and functions as a dominant-negative regulator of basic HLH proteins; they are important parts of signaling pathways involved in development, cell cycle, and tumorigenesis [Sikder et al., 2003b; Zebedee and Hara, 2001]. GCIP is a new helix–loop–helix leucine zipper (HLH-ZIP) protein without a basic DNA-binding domain. Overexpression of GCIP reduced the cyclin D-dependent protein kinase (CDK4) activity and the phosphorylation of retinoblastoma (RB) protein, and consequently, the E2F1-mediated transcription activity in cell cycle progression [Xia et al., 2000]. High level expression of GCIP in terminally differentiated tissues and the inhibition of E2F1 transcription activation suggest that GCIP could play an important role in controlling cell differentiation and proliferation. Unlike Id genes which have been identified as potential protooncogenes [Ruzinova and Benezra, 2003; Sikder et al., 2003b; Wang et al., 2003], GCIP has an inhibitory effect on colon tumorigenesis. Our data demonstrate that GCIP was downregulated in colon tumor samples both at mRNA and protein levels. Overexpression of GCIP can inhibit anchorage-independent growth of SW480 colon cancer cells in a soft agar colony formation assay. The role of GCIP to be a tumor suppressor is also supported by its genomic location in chromosome 15q14-15, a common region deleted or LOH in several types of human tumors, including the colorectal [Tomlinson et al., 1999; Park et al., 2000], breast [Wick et al., 1996; Gonzalez et al., 1999], mesothelioma [Balsara et al., 1999; De Rienzo et al., 2001], pancreatic [Rigaud et al., 2000], bladder [Natrajan et al., 2003], and gastric tumor [Chun et al., 2000].

Our previous data indicated that GCIP might play an important role in controlling cell differentiation and proliferation [Xia et al., 2000]. In this study, we found GCIP could inhibit cyclin D1 transcriptional activity and the expression of cyclin D1 in the cell, suggesting that GCIP may regulate tumorigenesis by controlling the expression of cyclin D1 and affect the cell proliferation signaling pathway.

Butyric acid that is the short-chain fatty acids produced by microbial fermentation in the colon has been shown to inhibit cancer cell proliferation and induce tumor cell differentiation through a mechanism of action still not completely understood. In our studies, we demonstrated that NaB can induce the expression of GCIP in SW480 cells similar to the p21 protein after treatment of the cancer cells by NaB, suggesting that GCIP may play an important role in NaB-induced cancer cell differentiation. GCIP, like the p21 protein, may provide a mechanism of action for butyric acid-inhibited cancer cell proliferation.

GCIP was found to function as a transcriptional repressor in our previous study. Expression of GCIP in transgenic mouse liver decreases susceptibility of the mice to chemically-induced hepatocarcinogenesis [Ma et al., 2006]. This repressor function is insensitive to TSA/NaB [Ma et al., 2006]. In this study, we demonstrated that GCIP specifically interacts with one of the class III HDAC proteins SirT6. However, we were unable to prove that GCIP acts as a transcription repressor through interaction with SirT6 since the deacetylase activity of SirT6 has not been confirmed yet [Liszt et al., 2005], although other Sirtuins such as SirT1 have been confirmed to act as a transcription repressor through its NAD-dependent HDAC activity. A new published article in Cell [Mostoslavsky et al., 2006] reports that deficiency in SIRT6 results in genome instability through the DNA base excision repair pathway. Other than acting as a transcription repressor to cyclinD1, GCIP may inhibit tumorigenesis through interaction with the chromatin-associated protein SIRT6, then promotes resistance to DNA damage and suppresses genomic instability.

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