Biochemical Pharmacology 83 (2012) 47–56

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

DIF-1 inhibits the Wnt/β-catenin signaling pathway by inhibiting TCF7L2 expression in colon cancer cell lines

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ARTICLE INFO

Article history:
Received 11 August 2011
Accepted 3 October 2011
Available online 8 October 2011

Keywords:
Wnt/β-catenin signaling pathway
Differentiation-inducing factor-1 (DIF-1)
TCF7L2
Colon cancer cell
Cyclin D1

ABSTRACT

We previously reported that differentiation-inducing factor-1 (DIF-1), a morphogen in *Dictyostelium discoideum*, inhibits the proliferation of human cancer cell lines by inducing β-catenin degradation and suppressing the Wnt/β-catenin signaling pathway. To determine whether β-catenin degradation is essential for the effect of DIF-1, we examined the effect of DIF-1 on human colon cancer cell lines (HCT-116, SW-620 and DLD-1), in which the Wnt/β-catenin signaling pathway is constitutively active. DIF-1 strongly inhibited cell proliferation and arrested the cell cycle in the G_0/G_1 phase via the suppression of cyclin D1 expression at mRNA and protein levels without reducing β-catenin protein. TCF-dependent transcriptional activity and cyclin D1 promoter activity were revealed to be inhibited via suppression of transcription factor 7-like 2 (TCF7L2) expression. Luciferase reporter assays and EMSAs using the TCF7L2 promoter fragments indicated that the binding site for the transcription factor early growth response-1 (Egr-1), which is located in the −609 to −601 bp region relative to the start codon in the TCF7L2 promoter, was involved in DIF-1 activity. Moreover, RNAi-mediated depletion of endogenous TCF7L2 resulted in reduced cyclin D1 promoter activity and protein expression, and the overexpression of TCF7L2 overrode the inhibition of the TCF-dependent transcriptional activity and cyclin D1 promoter activity induced by DIF-1. Therefore, DIF-1 seemed to inhibit the Wnt/β-catenin signaling pathway by suppressing TCF7L2 expression via reduced Egr-1-dependent transcriptional activity in these colon cancer cell lines. Our results provide a novel insight into the mechanisms by which DIF-1 inhibits the Wnt/β-catenin signaling pathway.

1. Introduction

The Wnt/β-catenin signaling pathway plays a number of key roles in embryonic development and maintenance of homeostasis in matured tissues. It is well known that the constant activation of Wnt/β-catenin signaling can lead to cancer development [1–4]. Notably, there are numerous reports on the involvement of the Wnt/β-catenin signaling pathway in colorectal cancers. Most colorectal cancers contain somatic mutations in adenomatous polyposis coli (APC) or β-catenin, which are members of the Wnt/β-catenin signaling pathway, resulting in constitutive activation of target gene transcription by an accumulation of nuclear β-catenin [5–8]. Numerous target genes of Wnt/β-catenin signaling are proto-oncogenes that have been directly implicated in cancer development [9–11]. Among them, TCF7L2 has been shown to be involved in tumor formation, and the β-catenin/TCF7L2 complex is described as a master switch that controls the proliferation and differentiation of intestinal epithelial cells [12–14]. Therefore, anti-cancer drugs that suppress transcriptional activity dependent on the β-catenin/TCF7L2 complex may be of significant therapeutic value for colon cancer therapy.

Differentiation-inducing factors (DIFs) were identified in *Dictyostelium discoideum* as morphogens required for stalk cell differentiation [15,16]. In the DIF family, DIF-1 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone) was the first to be identified. DIF activity is not limited to *Dictyostelium* and has been shown to strongly inhibit the proliferation of human cells [17–19]. Previously, we reported that DIFs inhibit the Wnt/β-catenin signaling pathway via glycogen synthase kinase-3β (GSK-3β)

Abbreviations: DIF, differentiation-inducing factor; GSK-3β, glycogen synthase kinase-3β; TCF7L2, transcription factor 7-like 2; Egr-1, early growth response-1; APC, adenomatous polyposis coli; RT-PCR, reverse transcription-polymerase chain reaction; RNAi, RNA interference; EMSA, electrophoretic mobility shift assay.

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doi:10.1016/j.bcp.2011.10.001

activation followed by β-catenin degradation, leading to cell cycle arrest at the G_0/G_1 phase via the suppression of cyclin D1 expression in various human cells [20–27]. However, the effect of DIFs on human colon cancer cells, in which the Wnt/β-catenin signaling pathway is constitutively activated by inhibition of β-catenin destruction mechanisms, has not been elucidated.

In this study, we examine the effect of DIF-1 on human colon cancer cell lines that contain mutations in β-catenin (HCT-116) and APC (DLD-1 and SW-620).

2. Materials and methods

2.1. Chemicals and antibodies

DIF-1 was synthesized as described elsewhere [15,16]. MG132 was purchased from the Peptide Institute, Osaka, Japan. SB216763 was purchased from BIOMOL international (Farmingdale, NY). TOPflash (TCF reporter plasmid) and FOPflash (negative control for TOPflash) were purchased from Upstate Biotechnology (Lake Placid, NY). Wild-type and mutant cyclin D1 pGL3 basic luciferase reporter constructs were a generous gift from Drs. O. Tetsu and F. McCormick, University of California, San Francisco. The polyclonal anti-cyclin D1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti-Egr-1 antibody was purchased from Cell Signaling Technology (Danvers, MA). The monoclonal anti-β-catenin antibody was purchased from BD Biosciences (San Jose, CA). The monoclonal anti-α-tubulin antibody was purchased from Calbiochem (Darmstadt, Germany). The monoclonal anti-GAPDH antibody was purchased from Abcam (Cambridge, UK). The monoclonal anti-TCF7L2 antibody was purchased from MILLIPORE (Temecula, CA).

2.2. Cell culture

Human colon cancer cell lines HCT-116 (expressing wild-type APC and mutant β-catenin), DLD-1 and SW-620 (expressing mutant APC and wild-type β-catenin) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 0.1 μg/ml streptomycin.

2.3. Cell proliferation assay

HCT-116 cells (3×10^4 cells/well), DLD-1 cells (5×10^4 cells/well) and SW-620 cells (7.5×10^4 cells/well) were seeded in 24-well plates and treated with or without various concentrations of DIF-1 for specific periods. Cells were harvested by trypsin/EDTA treatment and counted using a Coulter Counter (Beckman Coulter, Indianapolis, IN).

2.4. Flow cytometric analysis

Cells were suspended in a hypotonic solution containing 50 μg/ml propidium iodide (PI), 0.1% sodium citrate and 0.1% Triton X-100. PI-stained samples (1×10^5 cells) were analyzed for fluorescence with a Becton-Dickinson FACSCalibur (Franklin Lakes, NJ).

2.5. Western blotting

Western blotting was performed as described elsewhere [21]. Samples were separated by 12% SDS–PAGE and then transferred to a polyvinylidene difluoride membrane using a semidry transfer system (1 h, 12 V). Immunoreactive proteins were visualized by treatment with a detection reagent (LumiGLO; Cell Signaling Technology). Densitometric analysis was performed using NIH Image J Software.

2.6. Purification of nuclear proteins

Nuclear proteins were isolated from cells cultured in 60-mm dishes using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) followed by Western blot and EMSA analyses.

2.7. RT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA). Using 1 μg RNA, the expression of cyclin D1, TCF7L2, Egr-1, GAPDH mRNA was analyzed by RT-PCR. The following primers were used: TCF7L2 (forward: 5′-ACG AGG GCG AAC AGG AGG AG-3′, reverse: 5′-TGG GCG AGA GCG ATC CGT TG-3′) and Egr-1 (forward: 5′-GGT CAG TGG CCT AGT GAG C-3′, reverse: 5′-TGC TGT CGT TGG ATG GCA C-3′). Other primer sets are described elsewhere [21].

2.8. RNAi

TCF7L2 Select Stealth™ RNAi was purchased from Invitrogen. Transfection of dsRNA (100 nM) was performed using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. A Stealth™ RNAi negative control, which is the GC-matched scrambled sequence, was also purchased from Invitrogen.

2.9. Construction of the TCF7L2 reporter plasmid

The 5′-flanking region of the human *TCF7L2* gene (−1306/−1 bp relative to the start codon) was PCR-amplified from human genomic DNA followed by cloning into pGL3-Basic firefly luciferase reporter vector. A series of deletion plasmids (−869/−1, −629/−1, −604/−1, −578/−1, −434/−1, −223/−1 bp) were generated by PCR using TCF7L2-Luc (−1306/−1 bp) as a template.

2.10. TCF7L2-overexpressing plasmid

Total cellular RNA was extracted from HCT-116 cells and TCF7L2 cDNA was obtained by RT-PCR. The cDNA (GenBank accession number FJ010167) was verified by DNA sequencing and subcloned into pcDNA3 (Invitrogen).

2.11. Luciferase reporter assay

Cells were transfected with luciferase reporter plasmids and pRL-SV40, a Renilla luciferase expression plasmid as a control for transfection efficiency, using Lipofectamine Plus reagent (Invitrogen). Then, cells were cultured for 24 h followed by stimulation with DIF-1 at the indicated periods. Luciferase activity was determined with a luminometer (Lumat LB 9507, Berthold Technologies, Barsinghausen, Germany) and normalized to Renilla luciferase activity.

2.12. EMSA

Complementary oligonucleotides corresponding to the −634/−605 and −618/−589 bp regions in the TCF7L2 promoter were synthesized (5′-GGC GCC CGA AAG GAT CAT TGT TAG CCG CCC-3′ and 5′-CAT TGT TAG CCG CCC CCG CCC CGC CCA CCC-3′) and labeled at the 3′-end with biotin using a Biotin 3′ End DNA Labeling Kit (Pierce). Nuclear protein extracts (5 μg) were incubated with biotin-3′-labeled oligonucleotides with or without a 100-fold molar excess of unlabelled oligonucleotide as a competitor. To identify DNA binding to protein, nuclear protein extracts were incubated with 500 ng anti-Egr-1 or anti-α-tubulin antibodies prior to labeled probe addition. The anti-α-tubulin antibody was

used as an irrelevant control. Samples were separated by electrophoresis on a 7% native polyacrylamide gel, and biotin-labeled bands were detected with a Phototope®-Star Kit (New England Bio Labs, Ipswich, MA).

2.13. Statistics

Results were expressed as the mean ± S.E. Differences between values were statistically analyzed using a Student's *t*-test or one-way ANOVA with Bonferroni post hoc tests (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Antiproliferative effect of DIF-1 on human colon cancer cells

DIF-1 exhibits a powerful anti-proliferative effect and inhibits the Wnt/β-catenin signaling pathway in various human cell species [20–27]. Therefore, we first examined whether DIF-1 also inhibit the proliferation of human colon cancer cell lines (HCT-116, DLD-1, and SW-620), in which the Wnt/β-catenin signaling pathway is constitutively active. As shown in Fig. 1A, DIF-1 strongly inhibited cell proliferation in a dose-dependent manner, while the response to DIF-1 varied among cell lines. Next, we examined the cell cycle distribution using flow cytometric analysis. DIF-1 treatment significantly increased cell numbers in the G_0/G_1 phase, decreasing cell numbers in the S and G_2/M phases (Fig. 1B), indicating that DIF-1 induced G_0/G_1 arrest in human colon cancer cell lines. These results were consistent with our previous studies and suggested that DIF-1 is also active in human colon cancer cell lines.

3.2. DIF-1 induces proteolysis of cyclin D1 by activation of GSK-3β in colon cancer cells

Because DIF-1 induces G_0/G_1 cell cycle arrest by the suppression of cyclin D1 expression via the activation of GSK-3β [22,23,27], we analyzed the effect of DIF-1 on cyclin D1 in HCT-116 cells. As shown in Fig. 2A and B, DIF-1 reduced the amount of cyclin D1 protein in time- and dose-dependent manners. Ubiquitin-proteasome inhibitor MG132 treatment prevented cyclin D1 reduction (Fig. 2C). Because GSK-3β has been reported to trigger cyclin D1 proteolysis [28,29], we investigated whether GSK-3β was involved in the DIF-1-induced degradation of cyclin D1 using GSK-3β inhibitors, lithium chloride and SB216763. As shown in Fig. 2D, pre-treatment with lithium chloride (30 mM, 3 h) or SB216763 (20 μM, 3 h) attenuated the effect of DIF-1, indicating that GSK-3β was also involved in DIF-1-induced cyclin D1 degradation in HCT-116 cells. Although the time requirement varied among cell lines, DIF-1 also induced cyclin D1 degradation through the GSK-3β activation in DLD-1 and SW620 cells (data not shown).

Among the three cell lines analyzed, HCT-116 cells were the most sensitive to DIF-1. Therefore, we used HCT-116 cells in subsequent experiments.

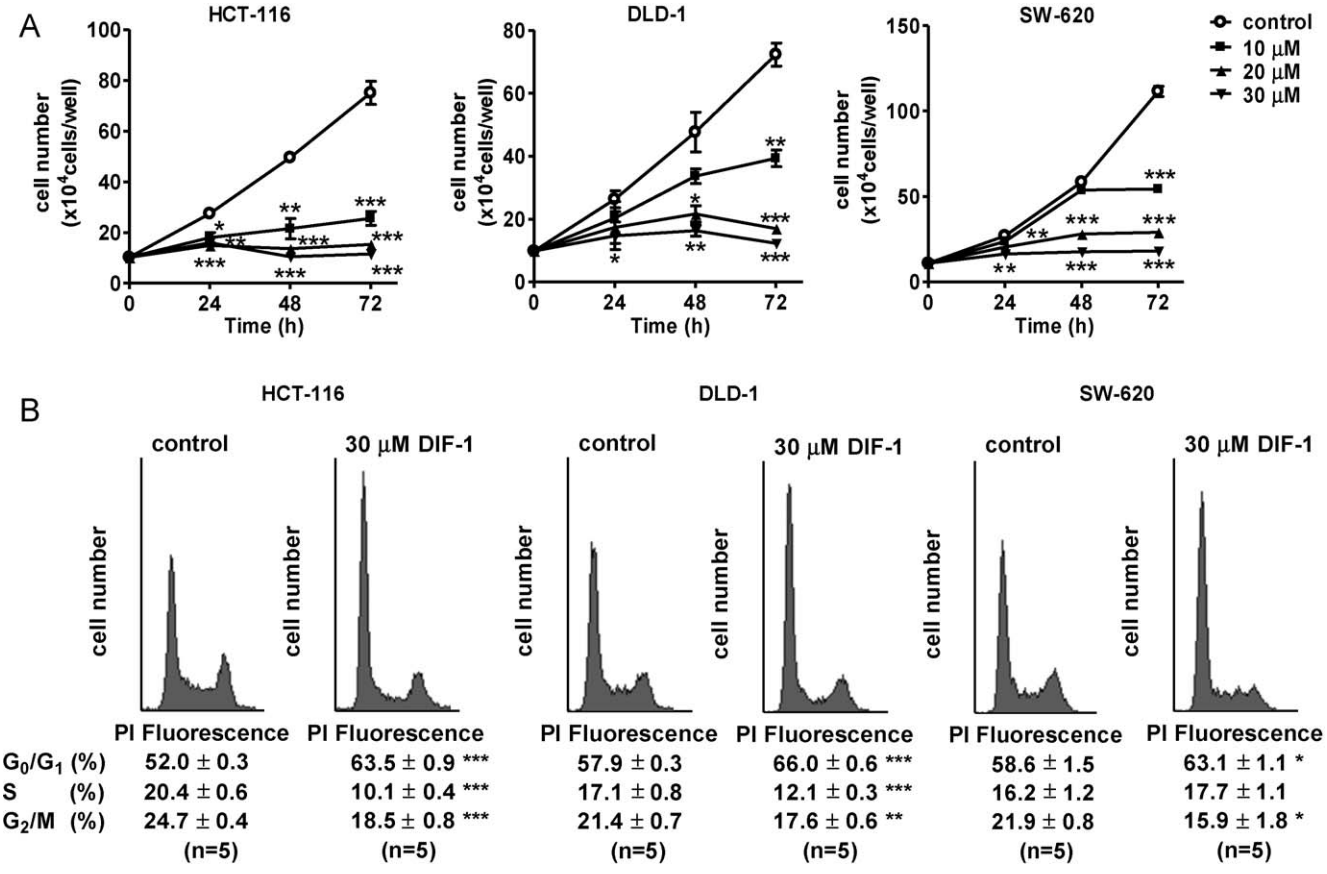

Fig. 1. Antiproliferative effect of DIF-1 on human colon cancer cells.
(A) Cell proliferation assay. HCT-116, DLD-1 and SW-620 cells were seeded on a 24-well plate and incubated with various concentration of DIF-1. Cells were harvested by trypsin/EDTA treatment at the indicated times and then counted. Values are the means ± S.E. from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control. (B) Flow cytometry. HCT-116, DLD-1 and SW-620 cells were incubated with DIF-1 (30 μM) for 24 h and then harvested by trypsin/EDTA treatment. Cells were stained with propidium iodide (PI) and nuclear fluorescence was measured. The percentages of cells in the various cell cycle phases are shown. The results are the means ± S.E. from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control.

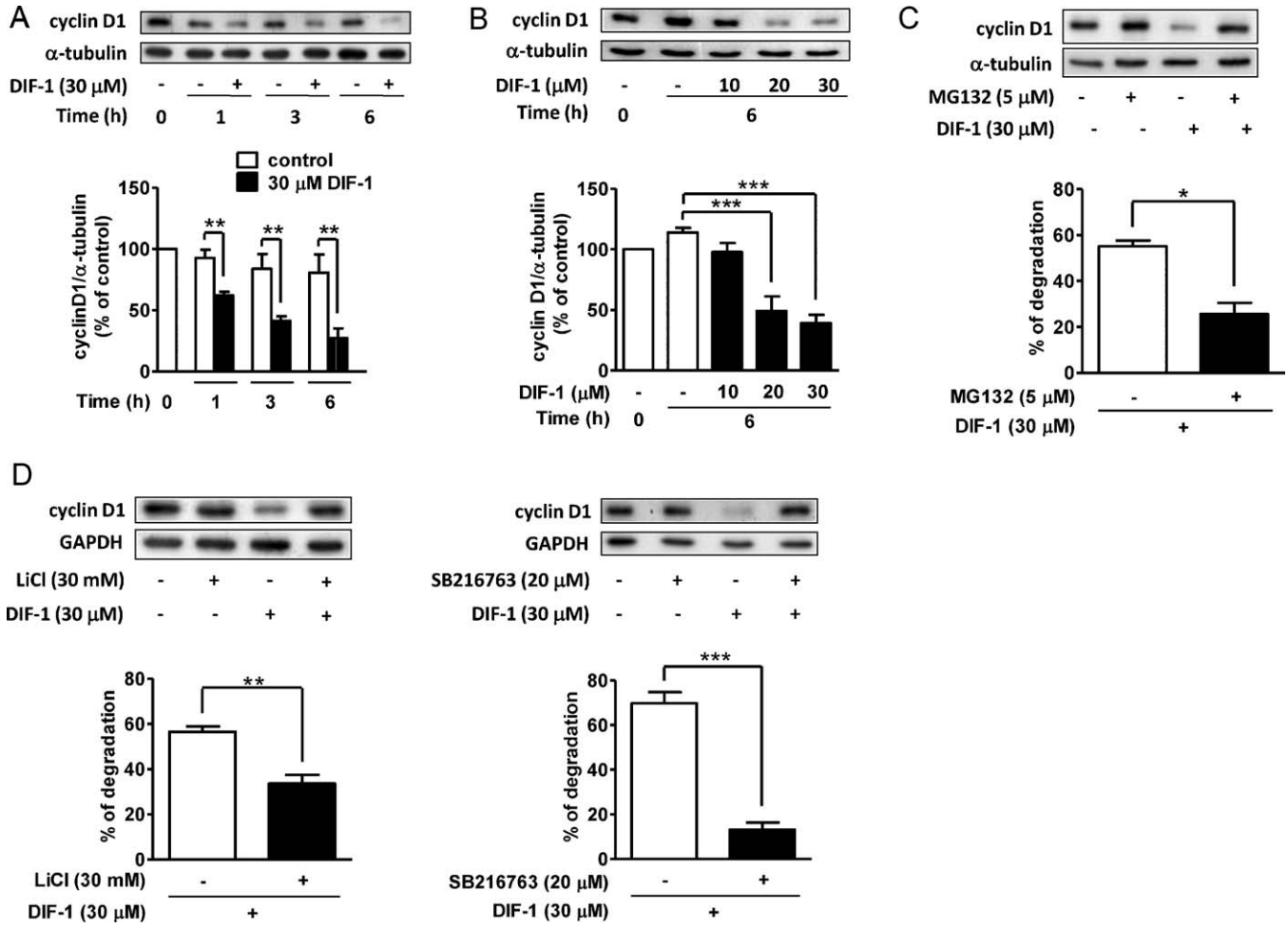

Fig. 2. DIF-1-induced proteolysis of cyclin D1 in human colon cancer cells.
(A) Time-course. HCT-116 cells were incubated with or without DIF-1(30 μM) for the indicated periods. (B) Dose dependency. HCT-116 cells were incubated with various concentrations of DIF-1 for 6 h. (C) The effect of the proteasome inhibitor MG132. HCT-116 cells pretreated with MG132 for 1 h were incubated with or without DIF-1 (30 μM) for 6 h. (D) HCT-116 cells were pretreated with or without 30 mM LiCl or 20 μM SB216763 for 3 h and then incubated with or without DIF-1 (30 μM) for 6 h. Samples were subjected to Western blot analysis using anti-cyclin D1 and anti-α-tubulin or GAPDH antibodies. Protein bands were quantified and shown as percentages of the control level at time 0 (A and B) or as percentages of the degraded amounts (C and D). Values are the means ± S.E. from three independent experiments and statistically analyzed using Student's *t*-test (A, C, D) or one-way ANOVA with a Bonferroni post hoc test (B). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.3. DIF-1 reduces cyclin D1 promoter activity without affecting the amount of β-catenin protein in HCT-116 cells

Next, we examined the effect of DIF-1 on cyclin D1 mRNA levels and found that DIF-1 reduced the mRNA expression in a dose-dependent manner (Fig. 3A). Because the cyclin D1 gene (*CCND1*) is a Wnt/β-catenin signaling target gene [30], the effect of DIF-1 on TCF-dependent transcriptional activity was examined using TOPflash and FOPflash, a TCF reporter plasmid and its negative control. As shown in Fig. 3B, DIF-1 significantly reduced TOPflash activity after 6 h of stimulation without affecting FOPflash activity. We also examined the effect of DIF-1 on cyclin D1 promoter activity using a wild-type promoter and a mutant promoter that lacks the TCF consensus binding site located at −81/−75 bp [23,30]. DIF-1 significantly reduced wild-type promoter activity, while no significant effect on mutant promoter activity was observed (Fig. 3C). Because we previously reported that DIF-1 reduces TCF transcriptional activity via β-catenin degradation [23], the effect of DIF-1 on the amount of β-catenin protein was also examined. As shown in Fig. 3D and E, DIF-1 showed no significant effect on total or nuclear β-catenin protein levels, indicating that DIF-1 suppressed TCF-dependent transcriptional activity and cyclin D1 promoter activity without affecting the amount of β-catenin protein.

3.4. DIF-1 represses the transcription of TCF7L2 in HCT-116 cells

To elucidate the mechanism by which DIF-1 suppressed TCF-dependent transcriptional activity without affecting the β-catenin protein level, we next examined the effect of DIF-1 on TCF7L2 expression, which is known as the main transcription factor in the Wnt/β-catenin signaling pathway. Although the amount of TCF7L2 increased according to the culture period of the control cells, the amount of TCF7L2 was significantly reduced by DIF-1-treatment in time- and dose-dependent manners (Fig. 4A and B). The effect of DIF-1 on the TCF7L2 mRNA levels was also examined and a reduction in TCF7L2 mRNA by DIF-1 in a dose-dependent manner was observed (Fig. 4C).

Next, we examined the effect of DIF-1 on TCF7L2 promoter activity. The 5′-flanking region of the human TCF7L2 gene was obtained by PCR and cloned into a luciferase reporter plasmid (pGL3-Basic). As shown in Fig. 4D, although TCF7L2 promoter activity increased by approximately 2-fold by 24 h incubation, DIF-1 reduced it to under the basal activity. These results indicate that

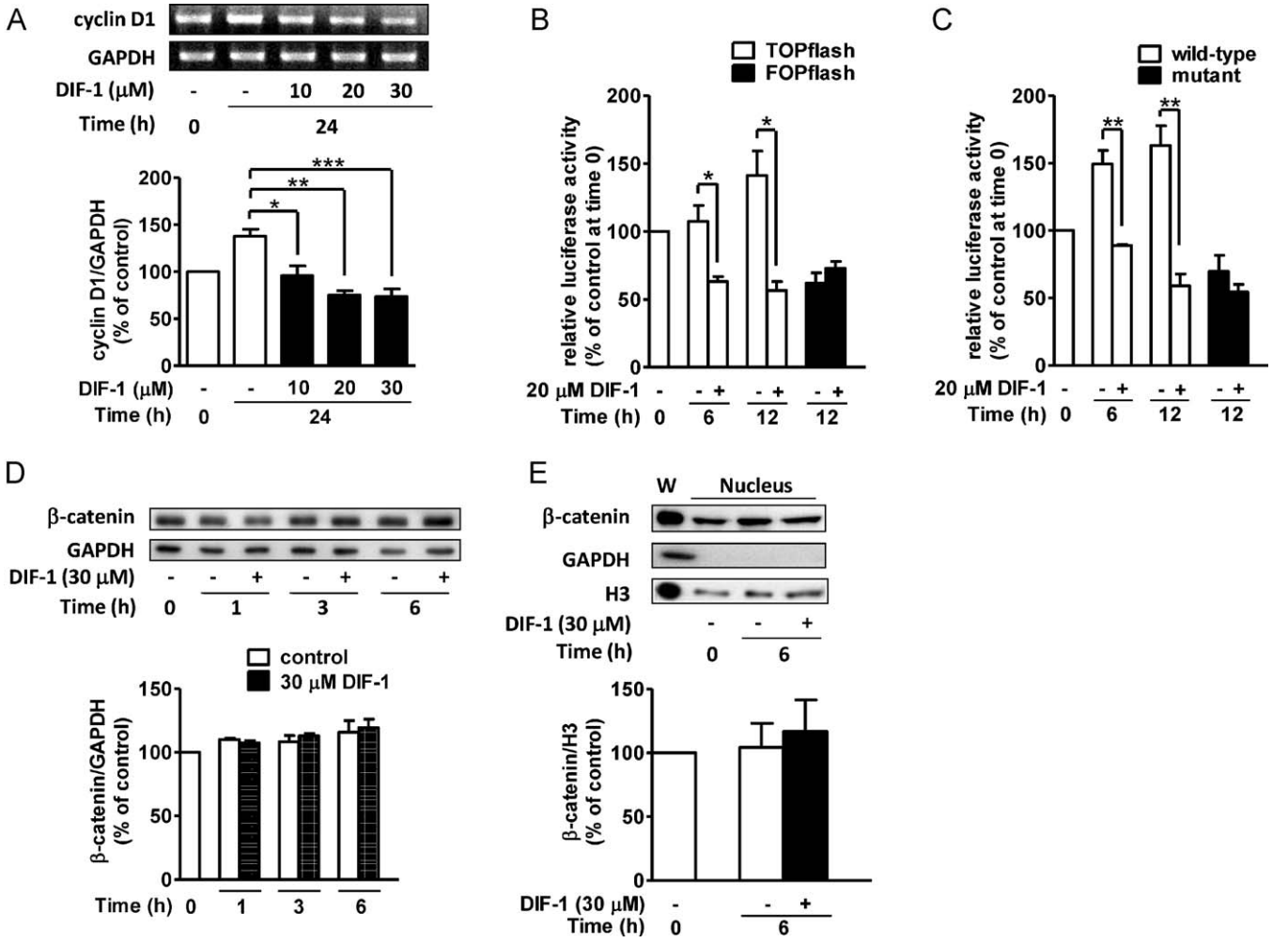

Fig. 3. Effect of DIF-1 on cyclin D1 mRNA expression in HCT-116 cells.
(A) HCT-116 cells were incubated with or without various concentration of DIF-1 for 24 h. Total RNA (1 μg) was subjected to RT-PCR to analyze cyclin D1 and GAPDH expression. PCR cycle numbers were 26 for cyclin D1 and 20 for GAPDH. mRNA expression levels were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) TOPFlash or FOPFlash was co-transfected with pRL-SV40 into HCT-116 cells. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for the indicated periods. Luciferase activity is shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments performed in duplicate. $^{*}P < 0.05$. (C) HCT-116 cells were co-transfected with luciferase reporter vectors (pGL3 containing wild-type or mutant cyclin D1 promoters) and pRL-SV40. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for the indicated periods. Luciferase activity is shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments performed in duplicate. $^{**}P < 0.01$. (D) HCT-116 cells were incubated with or without DIF-1 (30 μM) for the indicated periods. Protein samples were subjected to Western blot analysis using anti-β-catenin and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments. (E) HCT-116 cells were incubated with DIF-1 (30 μM) for 6 h and then nuclear proteins were isolated. Protein samples were subjected to Western blot analysis using anti-β-catenin, anti-GAPDH and anti-histone H3 antibodies. Protein bands were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments.

DIF-1 decreased the TCF7L2 protein level by suppressing TCF7L2 mRNA transcription in HCT-116 cells.

3.5. DIF-1 represses TCF7L2 promoter activity by suppressing Egr-1-dependent transcriptional activity in HCT-116 cells

To locate the DIF-1 responsive region(s) in the TCF7L2 promoter, a promoter deletion assay was performed. As shown in Fig. 5A, when constructs longer than 629 bp (−1306/−1 bp, −869/−1 bp, and −629/−1 bp) were transfected, the activity of TCF7L2 promoters increased by 1.5–2-fold after 24 h incubation. However, the effect was almost completely inhibited by DIF-1. Therefore, we examined the effect of DIF-1 on TCF7L2 promoter activity using further deletions in the −629/−1 bp promoter region (−604/−1 bp and −578/−1 bp) to reveal the minimal responsive region in the TCF7L2 promoter. As shown in Fig. 5B, the effect of DIF-1 was attenuated by transfection of a −604/−1 bp construct, suggesting that the responsive element to DIF-1 may exist between −629 and −604 bp.

Next, an EMSA was performed using two probes (probe 1, −634/−605 bp; and probe 2, −618/−589 bp) to examine the effect of DIF-1 on the binding of nuclear proteins to this region. We paid particular attention to the putative Egr-1 binding site, located at −609/−601 bp, because the Egr-1 transcription factor has been reported to modulate TCF7L2 promoter activity [31]. As shown in Fig. 5C, both probes bound to nuclear proteins (lane 2) and the addition of a 100-fold molar excess of each unlabeled probe resulted in a significant reduction of the bands (lane 4). The binding of probe 2, which contained the putative Egr-1 binding site, was markedly decreased in nuclear protein extracts from DIF-1-treated cells, suggesting that DIF-1 inhibited the DNA-binding activity of Egr-1. To study the involvement of Egr-1, a supershift assay was performed using an anti-Egr-1 antibody. As shown in Fig. 5D, although a supershifted band was not observed, an evident

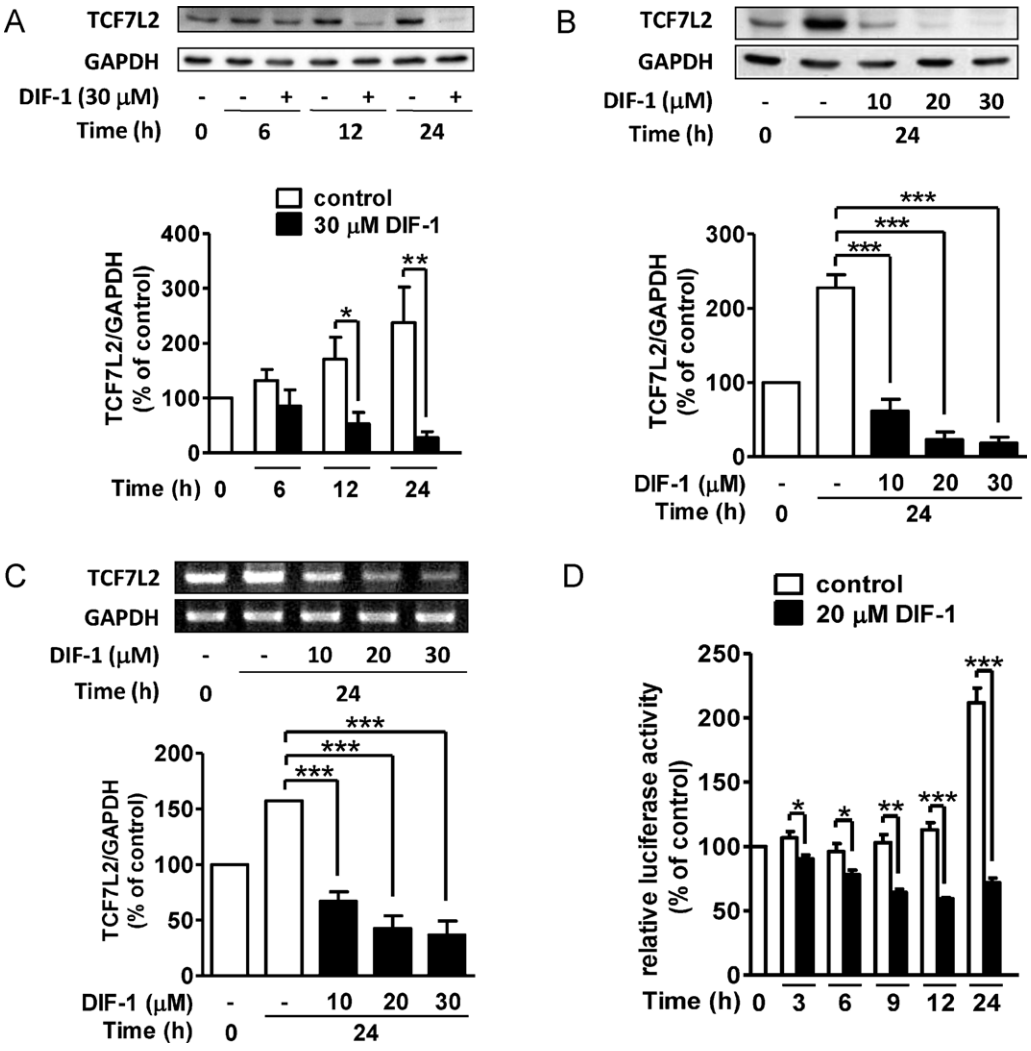

Fig. 4. DIF-1 represses the expression of TCF7L2 in HCT-116 cells.
(A) HCT-116 cells were incubated with or without DIF-1 (30 μM) for the indicated periods. Protein samples were subjected to Western blot analysis using anti-TCF7L2 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) HCT-116 cells were incubated with or without various concentrations of DIF-1 for 24 h. Protein bands were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{***}P < 0.001$. (C) HCT-116 cells were incubated with or without various concentrations of DIF-1 for 24 h. Total RNA (1 μg) was subjected to RT-PCR to analyze TCF7L2 and GAPDH expression. PCR cycle numbers were 23 for TCF7L2 and 20 for GAPDH. mRNA expression levels were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{***}P < 0.001$. (D) HCT-116 cells were co-transfected with a TCF7L2 promoter construct (−1306/−1 bp) and pRL-SV40. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for the indicated periods. Luciferase activity is shown as percentages of the control level. Values are the means ± S.E. from three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

reduction of the shifted band was observed with the addition of the anti-Egr-1 antibody.

Subsequently, the effects of DIF-1 on Egr-1 protein and mRNA levels were examined by Western blot and RT-PCR analyses, respectively. We found that DIF-1 significantly reduced total (Fig. 5E) and nuclear (Fig. 5F) Egr-1 protein levels, as well as mRNA levels (Fig. 5G). These observations indicated that DIF-1 suppressed mRNA expression and reduced the amount of Egr-1 protein, resulting in the reduction of TCF7L2 promoter activity.

3.6. DIF-1 inhibits the Wnt/β-catenin signaling pathway by repressing TCF7L2 expression in HCT-116 cells

Furthermore, we depleted endogenous TCF7L2 by RNAi to confirm the involvement of TCF7L2 in cyclin D1 expression. As shown in Fig. 6A, cyclin D1 protein amount was significantly decreased by TCF7L2 depletion. The effect of TCF7L2 depletion on the cyclin D1 promoter activity was also analyzed using a wild-type promoter and a mutant promoter that lacks the TCF consensus binding site. The activity of wild-type promoter was significantly suppressed by TCF7L2 depletion, whereas it did not have significant effect on mutant promoter activity (Fig. 6B). These results indicated that cyclin D1 expression was promoted by TCF7L2 in HCT-116 cells. Next, we examined the effect of DIF-1 using TCF7L2-overexpressing HCT-116 cells. As shown in Fig. 6C, although the expression level of TCF7L2 in the cells transfected with empty pcDNA3 was reduced after 12 h stimulation with DIF-1, it was weakened in the cells transfected with pcDNA3/TCF7L2. Subsequently, we examined the effect of DIF-1 on TCF-dependent transcriptional activity and cyclin D1 promoter activity. Compared with the cells transfected with empty pcDNA3, the effect of DIF-1 on TOPflash activity and cyclin D1 promoter activity was attenuated in TCF7L2-overexpressing cells (Fig. 6D). These observations indicated that DIF-1-induced reduction of TCF7L2 was associated with the inhibition of the Wnt/β-catenin signaling pathway and cyclin D1 promoter activity in HCT-116 cells.

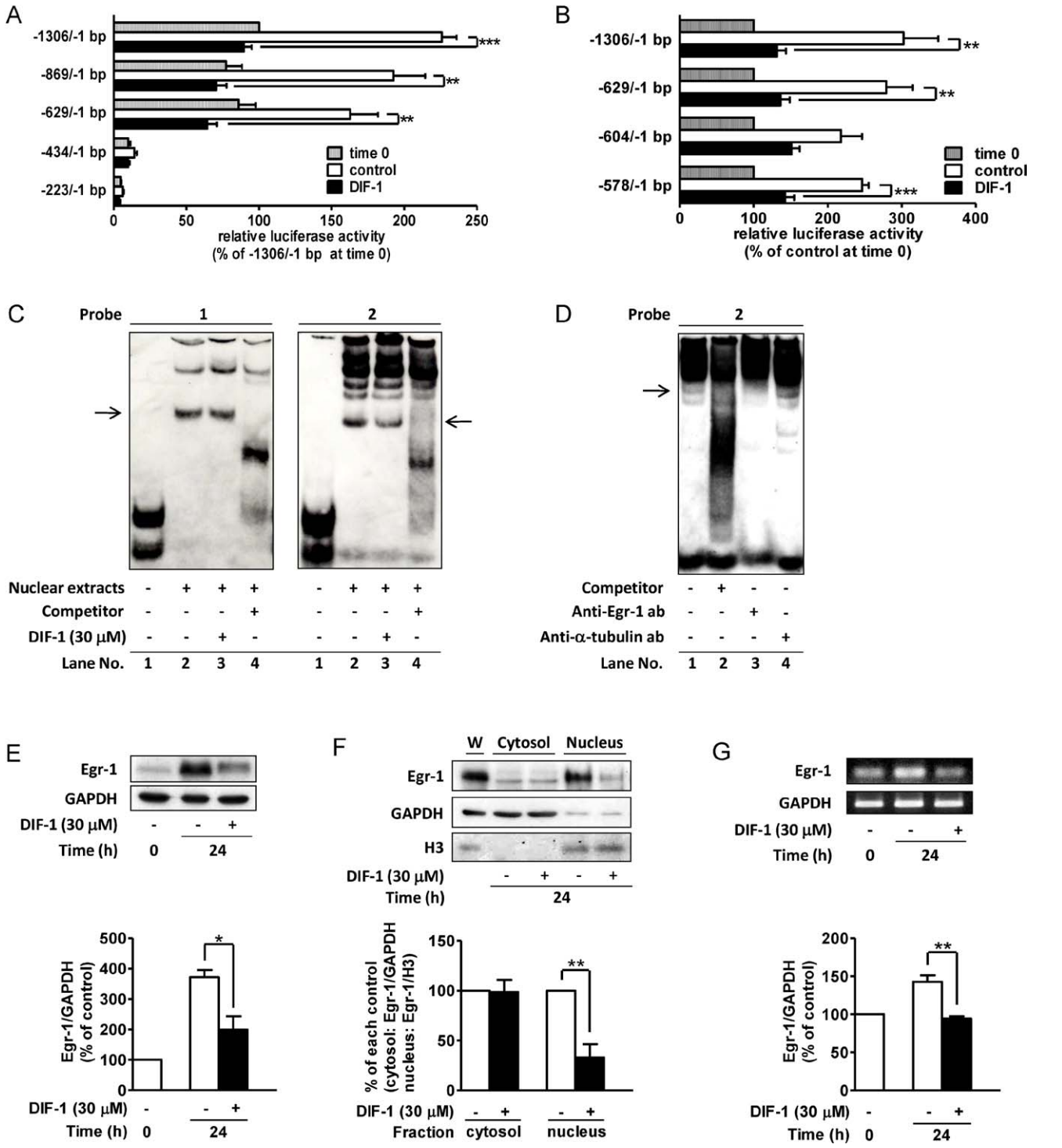

Fig. 5. Identification of the responsive region to DIF-1 in the TCF7L2 promoter in HCT-116 cells.
(A) The indicated 5′-flanking region of the TCF7L2 promoter construct (−1306/−1, −869/−1, −629/−1, −434/−1, −223/−1 bp) and pRL-SV40 were co-transfected into HCT-116 cells. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for 24 h. Luciferase activity is shown as percentages of the level of the −1306/−1 bp construct at time 0. Values are the means ± S.E. from three independent experiments performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$. (B) The indicated 5′-flanking region of the TCF7L2 promoter construct (−1306/−1, −629/−1, −604/−1, −578/−1 bp) and pRL-SV40 were co-transfected into HCT-116 cells. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for 24 h. Luciferase activity is shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments performed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Nuclear protein was extracted from HCT-116 cells with or without DIF-1-treatment (30 μM) for 24 h and an EMSA was performed. A representative result from four independent experiments is shown. Arrows indicate specific bands. (D) Supershift assay. Nuclear protein was incubated with or without an anti-Egr-1 antibody or an anti-α-tubulin antibody. A representative result from three independent experiments is shown. Arrows indicate specific bands. (E) HCT-116 cells were incubated with or without DIF-1 (30 μM) for 24 h. Protein samples were subjected to Western blot analysis using anti-Egr-1 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control level at time 0. Values are the means ± S.E. from three independent experiments. $^{*}P < 0.05$. (F) HCT-116 cells were incubated with DIF-1 (30 μM) for 24 h followed by cytosolic and nuclear protein isolation. Protein samples were subjected to Western blot analysis using anti-Egr-1, anti-GAPDH and anti-histone H3 antibodies. Protein bands were quantified and shown as percentages of the control. Values are the means ± S.E. from three independent experiments and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{**}P < 0.01$. W, Whole cell lysate. (G) HCT-116 cells were incubated with or without DIF-1 (30 μM) for 24 h. Total RNA (1 μg) was subjected to RT-PCR to analyze Egr-1 and GAPDH expression. PCR cycle numbers were 23 for Egr-1 and 20 for GAPDH. mRNA expression levels were quantified and shown as percentages of the control level at time 0. The result is representative of three experiments. Values are the means ± S.E. from three independent experiments. $^{**}P < 0.01$.

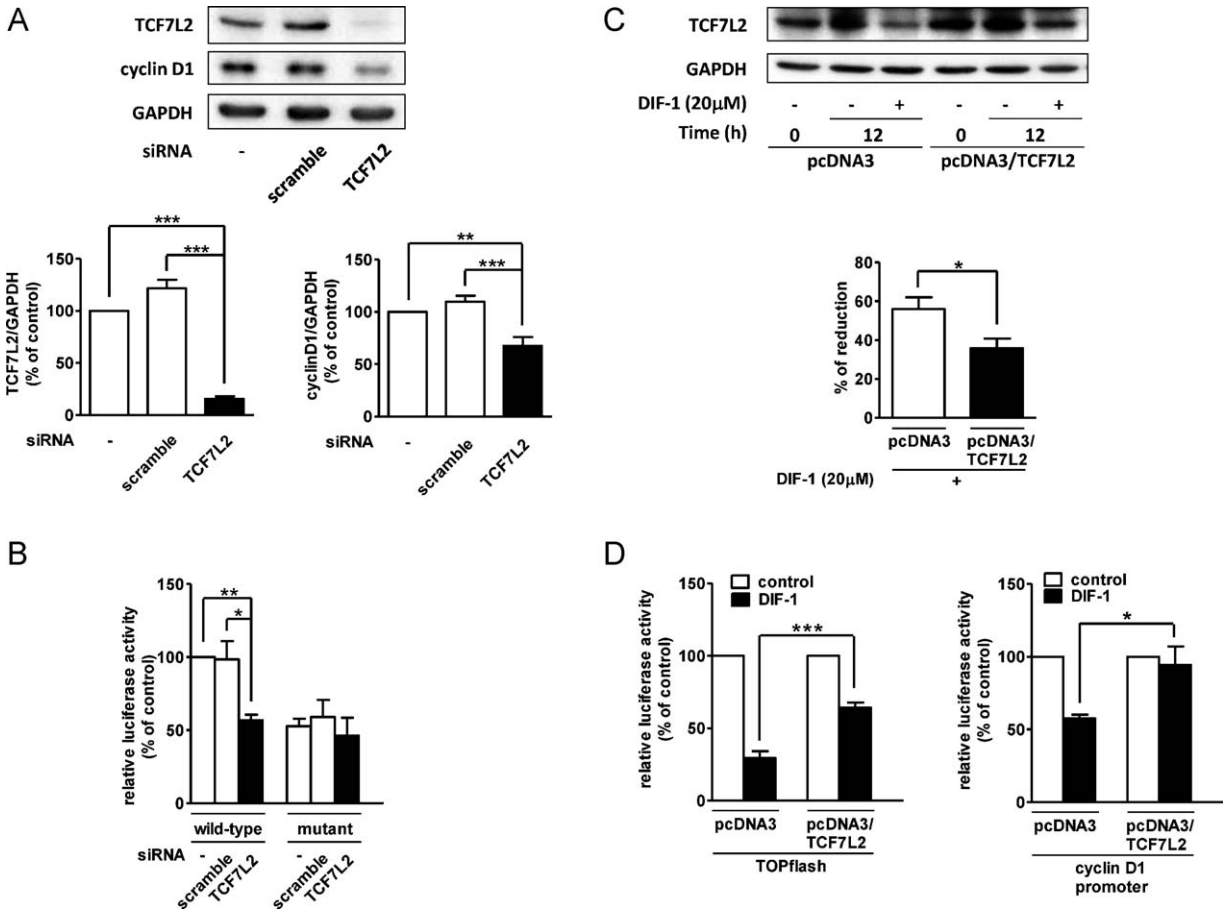

Fig. 6. DIF-1 inhibits the Wnt/β-catenin signaling pathway by repressing TCF7L2 expression in HCT-116 cells.
(A) HCT-116 cells were transfected with scrambled control or TCF7L2 siRNAs. After 48 h incubation, protein samples were subjected to Western blot analysis using anti-TCF7L2, anti-cyclin D1 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control. Values are the means ± S.E. from three independent experiments and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{**}P < 0.01$; $^{***}P < 0.001$. (B) HCT-116 cells were transfected with scrambled control or TCF7L2 siRNAs (100 nM). After 24 h incubation, HCT-116 cells were co-transfected with wild-type or mutant cyclin D1 promoter constructs and pRL-SV40 and then incubated for 24 h. Luciferase activity is shown as percentages of the control level. Values are the means ± S.E. from three independent experiments performed in duplicate and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) pcDNA3 or pcDNA3/TCF7L2 was transfected into HCT-116 cells. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for 12 h. Protein samples were subjected to Western blot analysis using anti-TCF7L2 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the reduced amount. Values are the means ± S.E. from three independent experiments. $^{*}P < 0.05$. (D) TOPFlash (left) or cyclin D1 promoter (right) and pcDNA3 or pcDNA3/TCF7L2 were co-transfected with pRL-SV40 into HCT-116 cells. After 24 h incubation, cells were stimulated with or without DIF-1 (20 μM) for 12 h. Luciferase activity is shown as percentages of the each control level. Values are the means ± S.E. from three independent experiments performed in duplicate. $^{*}P < 0.05$; $^{***}P < 0.001$.

3.7. Effect of DIF-1 on TCF7L2 expression in DLD-1 cells

To elucidate whether the same mechanism was involved in the suppression of cyclin D1 transcription in other human colon cancer cell lines, the effect of DIF-1 on TCF7L2 expression was examined in DLD-1 cells. We used the DLD-1 cell line because the cells express mutant APC and wild-type β-catenin, whereas HCT-116 cells express wild-type APC and mutant β-catenin. DIF-1 reduced the amount of TCF7L2 protein in time- and dose-dependent manners (Fig. 7A and B), and also suppressed TCF7L2 mRNA expression (Fig. 7C). These results indicated that DIF-1 also suppressed TCF7L2 expression at mRNA and protein levels in DLD-1 cells.

4. Discussion

We previously reported that DIF-1 inhibits the proliferation of human cancer cell lines via the suppression of cyclin D1 expression and the Wnt/β-catenin signaling pathway by β-catenin degradation via GSK-3β activation. In this study, we examined the effect of DIF-1 on human colon cancer cell lines (SW-620, DLD-1, and HCT-116), in which the Wnt/β-catenin signaling pathway is constitutively activated by mutations in proteins involved in the β-catenin destruction complex. DIF-1 strongly inhibited cell proliferation and induced cyclin D1 degradation via GSK-3β activation, and moreover suppressed cyclin D1 mRNA expression and promoter activity without affecting the amount of β-catenin protein. Further examination revealed that DIF-1 strongly suppressed TCF7L2 expression by inhibiting promoter activity via a decrease in Egr-1 protein in the nucleus, which resulted in inhibition of the Wnt/β-catenin signaling pathway. Therefore, our results suggested that DIF-1 induced cyclin D1 protein degradation via GSK-3β activation and inhibited the Wnt/β-catenin signaling pathway, thereby inhibiting cyclin D1 mRNA expression, by the suppression of TCF7L2 expression via Egr-1 in colon cancer cells.

The *Egr-1* gene belongs to a group of early response genes, and stimulation by numerous environmental signals, including growth factors and hormones, rapidly induces *Egr-1* gene expression [32]. Egr-1 has various biological functions by regulating the expression of diverse genes including growth factors, cytokines receptors, adhesion molecules and proteases. The role of Egr-1 in cancer development and growth is controversial. For example, Egr-1 has been shown to regulate several tumor suppressor genes, and Egr-1 expression inhibits the proliferation of several types of human cancer cells [33–35]. However, other reports show that Egr-1

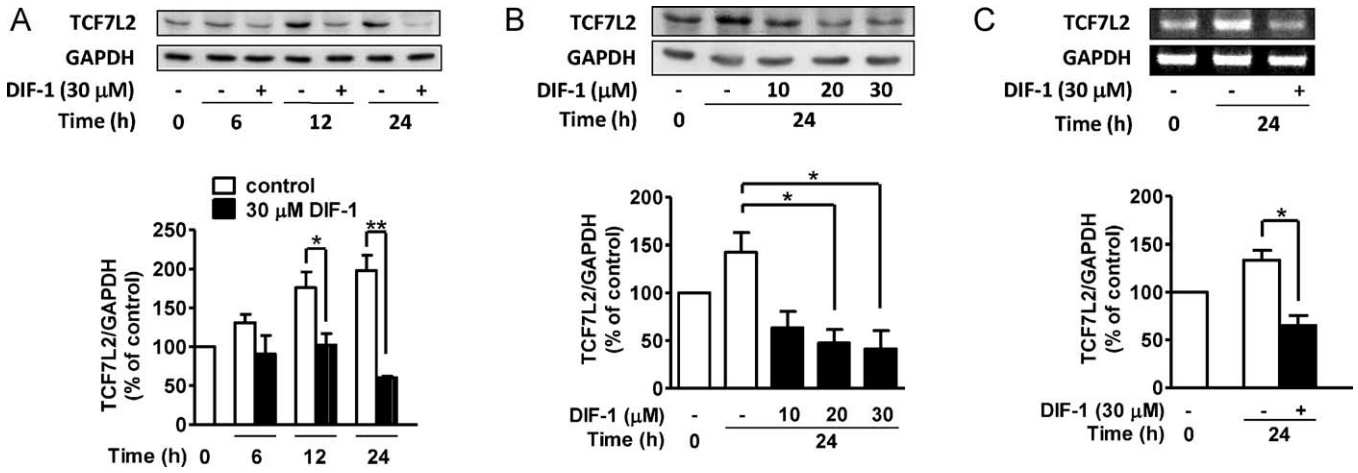

Fig. 7. Effect of DIF-1 on TCF7L2 expression in DLD-1 cells.
(A) DLD-1 cells were incubated with or without DIF-1 (30 μM) for the indicated periods. Protein samples were subjected to Western blot analysis using anti-TCF7L2 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control at time 0. Values are the means ± S.E. from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) DLD-1 cells were incubated with or without various concentrations of DIF-1 for 24 h. Protein samples were subjected to Western blot analysis using anti-TCF7L2 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentages of the control at time 0. Values are the means ± S.E. from three independent experiments and statistically analyzed using a one-way ANOVA with a Bonferroni post hoc test. $^{*}P < 0.05$. (C) DLD-1 cells were incubated with or without DIF-1 (30 μM) for 24 h. Total RNA (1 μg) was subjected to RT-PCR to analyze TCF7L2 and GAPDH expression. PCR cycle numbers were 23 for TCF7L2 and 20 for GAPDH. mRNA expression levels were quantified and shown as percentages of the control level at time 0. The result is representative of three experiments. Values are the means ± S.E. from three independent experiments. $^{*}P < 0.05$.

promotes the proliferation and survival of cancer cells [36–39]. In human colon cancer cell lines, it has been reported that curcumin inhibits proliferation via suppression of epidermal growth factor receptor gene expression by reducing Egr-1 activity [40]. Moreover, Fahmy et al. showed that Egr-1 inhibition by DNAzymes represses neovascularization in the rat cornea, and found that Egr-1 has a crucial role in endothelial cell proliferation and tumor angiogenesis [41]. These observations are consistent with our previous report that showed DIF-1 inhibits human endothelial cell proliferation and angiogenesis *in vitro* and *in vivo* [27]. Although we could not determine the mechanism by which DIF-1 suppressed Egr-1 mRNA levels, the inhibition of transcriptional Egr-1 activity may be a mechanism of DIF-1, which induces anti-tumor effects.

To identify the responsive element for DIF-1, we generated reporter plasmids that contained varying lengths of the 5′-flanking region of the *TCF7L2* gene and found that the binding site for Egr-1, located from −609 to −601 bp, was a possible responsive element for DIF-1. Saegusa et al. also reported that Egr-1 regulates TCF7L2 promoter activity and indentified the responsive element for Egr-1 between −786 and −778 bp [31]. We could not determine the reason why a different region in the TCF7L2 promoter was responsive to Egr-1. Since the previous study used a different type of carcinoma cell line (endometrial carcinoma cells), the regulation mechanism of TCF7L2 transcription may be different among cancer cell lines. Further studies are required to solve this issue.

Constitutive activation of the Wnt/β-catenin signaling pathway may trigger the formation of colon cancers. Therefore, anti-cancer drugs that suppress the transcriptional activity of the Wnt/β-catenin signaling pathway may be of therapeutic value for colon cancer therapy. For this purpose, although several compounds have been developed and examined, the majority of Wnt/β-catenin signaling inhibitors are in preclinical stages of development [42–46]. In the present study, we show that DIF-1 significantly reduces the transcriptional activity of the Wnt/β-catenin signaling pathway by suppressing TCF7L2 expression, indicating that DIF-1 may possess an anti-tumor effect against colon cancer, which involves the dysregulation of β-catenin. Moreover, we previously reported that DIF-1 exhibits anti-angiogenic effects independently of the Wnt/β-catenin signaling pathway. Therefore, DIF-1 may be a promising candidate compound to treat colon cancer by suppression of the Wnt/β-catenin signaling pathway and angiogenesis.

Acknowledgements

We thank Drs. O. Tetsu, and F. McCormick (University of California, San Francisco) for kindly providing wild-type and mutant cyclin D1 pGL3 basic luciferase reporter plasmids.

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and Grants-in-Aid for graduate students at Kyushu University. We would like to thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University.

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