Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

Full paper

Differentiation-inducing factor-3 inhibits intestinal tumor growth *in vitro* and *in vivo*

Naoya Kubokura [a, b], Fumi Takahashi-Yanaga [a, c, *], Masaki Arioka [a], Tatsuya Yoshihara [a], Kazunobu Igawa [d], Katsuhiko Tomooka [d], Sachio Morimoto [a], Yoshimichi Nakatsu [e], Teruhisa Tsuzuki [e], Yusaku Nakabeppu [f], Takayuki Matsumoto [g], Takanari Kitazono [b], Toshiyuki Sasaguri [a]

[a] *Department of Clinical Pharmacology, Faculty of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan*
[b] *Department of Medicine and Clinical Science, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan*
[c] *Global Medical Science Education Unit, Faculty of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan*
[d] *Department of Molecular and Material Science, Institute for Materials Chemistry and Engineering, Kyushu University, Kasuga, 816-8580, Japan*
[e] *Department of Medical Biophysics and Radiation Biology, Faculty of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan*
[f] *Division of Neurofunctional Genomics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka, 812-8582, Japan*
[g] *Division of Gastroenterology, Department of Internal Medicine, School of Medicine, Iwate Medical University, Morioka, 020-0023, Japan*

A B S T R A C T

Differentiation-inducing factor-1 (DIF-1) produced by *Dictyostelium discoideum* strongly inhibits the proliferation of various types of cancer cells by suppression of the Wnt/β-catenin signal transduction pathway. In the present study, we examined the effect of differentiation-inducing factor-3 (DIF-3), a monochlorinated metabolite of DIF-1 that is also produced by *D. discoideum*, on human colon cancer cell lines HCT-116 and DLD-1. DIF-3 strongly inhibited cell proliferation by arresting the cell cycle at the G_0/G_1 phase. DIF-3 reduced the expression levels of cyclin D1 and c-Myc by facilitating their degradation via activation of GSK-3β in a time and dose-dependent manner. In addition, DIF-3 suppressed the expression of T-cell factor 7-like 2, a key transcription factor in the Wnt/β-catenin signaling pathway, thereby reducing the mRNA levels of *cyclin D1* and *c-Myc*. Subsequently, we examined the *in vivo* effects of DIF-3 in *Mutyh*$^{-/-}$ mice with oxidative stress-induced intestinal cancers. Repeated oral administration of DIF-3 markedly reduced the number and size of cancers at a level comparable to that of DIF-1. These data suggest that DIF-3 inhibits intestinal cancer cell proliferation *in vitro* and *in vivo*, probably by mechanisms similar to those identified in DIF-1 actions, and that DIF-3 may be a potential novel anti-cancer agent.

1. Introduction

The Wnt/β-catenin signaling pathway plays essential roles in cell proliferation and differentiation, embryonic development, maintenance of tissue homeostasis, and several other biological processes. In cell proliferation, activation of the Wnt/β-catenin pathway results in upregulation of its target genes, such as *cyclin D1* and *c-myc*, which play key roles in the initiation and progression of G_1 phase in the cell cycle (1, 2).

Under physiological conditions, the activity of the Wnt/β-catenin pathway is tightly controlled by the β-catenin destruction complex in which glycogen synthase kinase-3 (GSK-3) phosphorylates β-catenin to induce its proteasomal degradation in cooperation with axin and adenomatous polyposis coli (APC). However, mutations in components of the destruction complex or β-catenin itself can lead to hyperactivation of the Wnt/β-catenin pathway and promotion of cancer development (3–7). Indeed, the Wnt/β-

catenin pathway is constitutively active in most colorectal cancers, as well as in other types of malignant tumors (8—12).

Differentiation-inducing factors (DIFs) were identified in *D. discoideum* as morphogens required for differentiation to stalk cells (13, 14), and have been shown to strongly inhibit the proliferation of mammalian cells, regardless of whether they are normal or cancerous (7,15—25). DIF-1 [1-(3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone] was the first identified member of the DIF family. We have reported that DIF-1 activates GSK-3β to phosphorylate and induce degradation of cyclin D1, c-Myc, and β-catenin, which leads to inhibition of *cyclin D1* and *c-Myc* transcription (16—23). Recently, we found that DIF-1 inhibits the expression of T-cell factor 7-like 2 (TCF7L2), a key transcription factor in the induction of Wnt target genes, which might explain why DIF-1 suppresses the proliferation of colon cancer cells with constitutive activation of β-catenin signaling (22—24). Indeed, repeated oral administration of DIF-1 inhibits oxidative stress-induced intestinal tumor growth *in vivo* in a dose-dependent manner (24).

D. discoideum metabolizes DIF-1 to the monochlorinated analogue DIF-3 [1-(3-chloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone] (14). We have previously reported that DIF-3 also exerts a strong antiproliferative effect on the human cervical cancer cell line HeLa by inducing cyclin D1 degradation and inhibiting *cyclin D1* mRNA expression (16). In some types of cancer cells, DIF-3 has been reported to exert more powerful antiproliferative effects than DIF-1 (16, 25).

Therefore, in the present study, we examined whether DIF-3 exerts antiproliferative effects on human colon cancer cell lines HCT-116 and DLD-1, which exhibit constitutive inactivation of the β-catenin destruction complex, and analyzed downstream components of the Wnt/β-catenin signal transduction pathway. Furthermore, we examined the effect of repeated oral administration of DIF-3 on intestinal cancer in *Mutyh*-deficient (*Mutyh*$^{-/-}$) mice (26), which lack the MutY homolog (MUTYH), a mammalian DNA glycosylase that initiates base excision repair, and are thus susceptible to oxidative stress-induced carcinogenesis (27—29).

2. Materials and methods

2.1. Chemicals and antibodies

DIF-1 and DIF-3 were synthesized as described previously (13, 14). MG132 was purchased from the Peptide Institute (Osaka, Japan). SB216763 was purchased from BIOMOL International (Farmingdale, NY, USA). A monoclonal and a polyclonal anti-cyclin D1 antibodys were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A monoclonal anti-c-Myc antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). A monoclonal anti-α-tubulin antibody was purchased from Calbiochem (Darmstadt, Germany). A monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Abcam (Cambridge, UK). A monoclonal anti-TCF7L2 antibody was purchased from Merck Millipore (Billerica, MA, USA). A monoclonal anti-β-catenin antibody was purchased from BD Biosciences (San Jose, CA, USA).

2.2. Cell culture

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

2.3. Cell proliferation assay

HCT-116 or DLD-1 cells were seeded in 24-well plates (5×10^4 cells/well) and treated with various concentrations of DIF-1 or DIF-3 for the indicated periods. Cells were harvested by trypsin/EDTA treatment and counted using an automated cell counter (TC10; Bio-Rad, Tokyo, Japan).

2.4. Flow cytometry

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 FACSCalibur (Becton—Dickinson, Franklin Lakes, NJ, USA).

2.5. Western blotting

Samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a poly-vinylidene fluoride membrane using a semidry transfer system (1 h at 12 V). Immunoreactive proteins were visualized by treatment with a detection reagent (LumiGLO; Cell Signaling Technology). Densitometric analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Real-time quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen). Reverse transcription was carried out using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, DriveRockville, MD, USA). Quantitative PCR was performed using TaqMan® Universal Master Mix II, TaqMan® Gene Expression Assays (*cyclin D1*, Hs0075553_m1; *c-Myc*, Hs00153408_m1; *GAPDH*, Hs99999905_m1), and an ABI 7500 Real-Time PCR System (Applied Biosystems). PCR conditions were initial denaturation for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The expression levels of target genes were calculated from the ΔΔCt values. *GAPDH* was used as an internal control.

2.7. Plasmids

TOPflash (TCF reporter plasmid) and FOPflash (negative control for TOPflash) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). A cyclin D1 pGL3 basic luciferase reporter plasmid was a generous gift from Drs. O. Tetsu and F. McCormick (University of California, San Francisco, CA, USA). A TCF7L2 overexpression plasmid was generated as described previously (22).

2.8. 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, Life Technologies). After 24 h, the cells were stimulated with DIF-3 for the indicated periods. Luciferase activity was determined with a luminometer (Lumat LB 9507; Berthold Technologies, Barsinghausen, Germany) and normalized against *Renilla* luciferase activity.

2.9. Intestinal tumor model

Induction of intestinal tumor formation (adenomas and carcinomas) in *Mutyh*$^{-/-}$ mice was carried out by a previously reported method (26). Briefly, KBrO$_3$ dissolved in water at a concentration of

2 g/L was administered to 4-week-old mice for 12 weeks. At 16 weeks of age, the mice were randomly divided into two groups (eight mice including four male mice and four female mice in each group). Mice in the test group were orally administered DIF-3 suspended in a 0.25% methylcellulose solution once a day for 5 days/week over 4 weeks. Control mice received the vehicle only. The body weight of the mice was monitored weekly. At 20 weeks of age, all mice were sacrificed to obtain blood and intestinal samples. Blood samples were analyzed for blood cell counts by a Celltac-alpha MEK-6358 (Nihon Kohden, Tokyo, Japan). Intestines were fixed in 4% formaldehyde, and the tumors were observed under a microscope. Images of the tumors were obtained and analyzed using ImageJ software.

2.10. Histological analyses

Tumors of 1.0–2.0 mm in diameter, which had developed 3.0–5.0 cm distal from the pylorus, were resected from formaldehyde-fixed intestines. Samples were embedded in paraffin and subjected to hematoxylin-eosin (HE) and immunohistochemical staining. Briefly, the sections were incubated with the anti-TCF7L2 antibody (1:2000 dilution) or polyclonal anti-cyclin D1 antibody (1:100 dilution) overnight at 4 °C, followed by incubation with the secondary antibody (Histofine, Nichirei, Tokyo, Japan) for 1 h. The sections were then analyzed with a Biozero microscope (Keyence, Osaka, Japan).

2.11. Statistical analyses

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis of differences between two mean values was performed using the Student's t-test. Multiple mean values were compared by one-way analysis of variance with the Dunnett's multiple comparison test (GraphPad Prism 5.0; GraphPad Software, La Jolla, CA, USA). P-values of less than 0.05 were considered statistically significant.

2.12. Ethics statement

This study protocol was approved by the Committee of Ethics on Animal Experiments at Kyushu University (Permit Number: A22-046-0). Animal handling and procedures were carried out in compliance with the Guidelines for Animal Experiments, Kyushu University, and the Law (No. 105) and Notification (No. 6) of the Japanese Government. All surgeries were performed under sodium pentobarbital anesthesia while making all efforts to minimize suffering.

3. Results

3.1. Antiproliferative effect of DIF-3 on human colon cancer cells

First, we examined the effect of DIF-3 on the proliferation of the human colon cancer cell lines, HCT-116 and DLD-1. As shown in
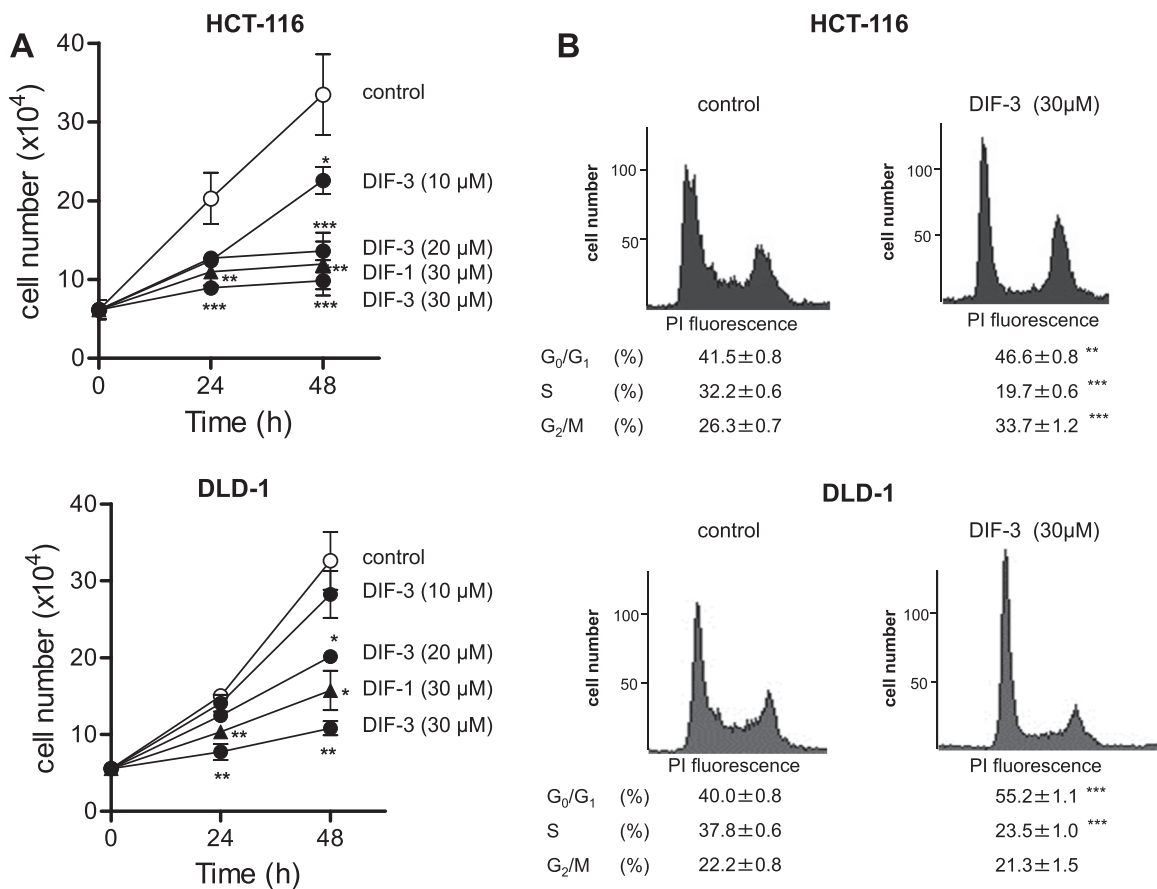

Fig. 1. Antiproliferative effect of DIF-3 on human colon cancer cells. (A) Cell proliferation assay. HCT-116 and DLD-1 cells were seeded on a 24-well plate and treated with the indicated concentrations of DIF-1 or DIF-3 for the indicated periods. (B) Flow cytometric analysis. Cells were treated with DIF-3 (30 μM) for 24 h and then harvested by trypsin/EDTA treatment. The cells were stained with PI and nuclear fluorescence was measured by flow cytometry. The percentages of cells in the various cell cycle phases are shown. The results are the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.
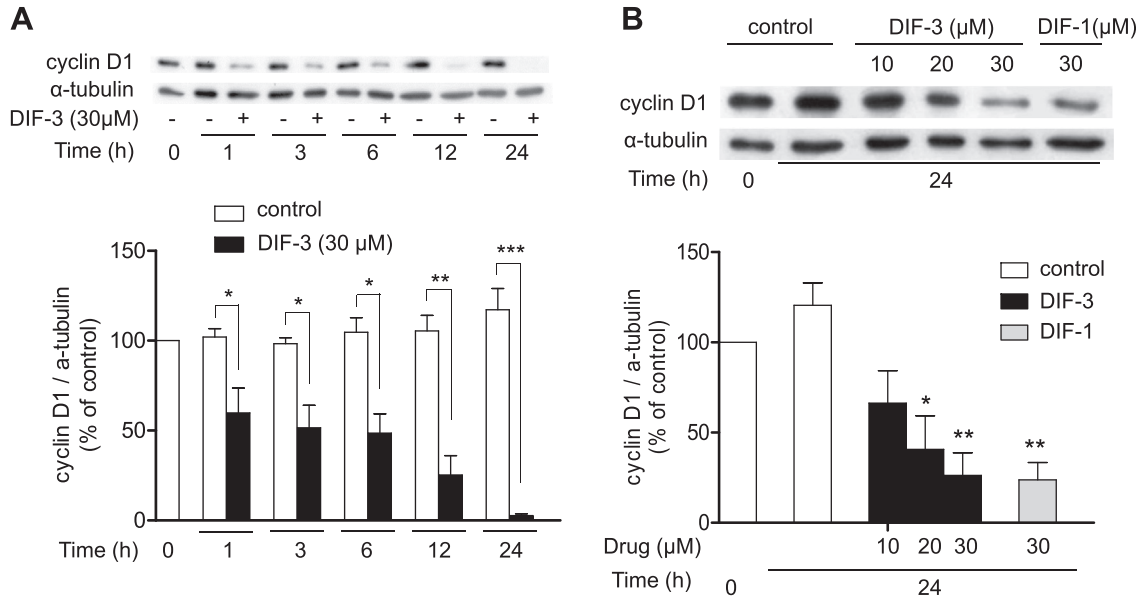
Fig. 2. DIF-3 suppresses the expression of cyclin D1 in HCT-116 cells. (A) Time course. HCT-116 cells were treated with or without DIF-3 (30 μM) for the indicated periods. (B) Dose dependency. HCT-116 cells were treated with the indicated concentrations of DIF-1 or DIF-3 for 24 h. Protein samples were subjected to Western blot analysis using anti-cyclin D1 and anti-α-tubulin antibodies. Protein bands were quantified and are shown as the percentage of the control level at time 0. The results are the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

Fig. 1A, DIF-3 strongly inhibited proliferation in a dose-dependent manner in both cell lines. The effect of DIF-3 was slightly stronger than that of DIF-1, and HCT-116 cells were more sensitive to DIF-3 than DLD-1 cells. Flow cytometry showed that treatment with DIF-3 significantly increased the number of cells in G_0/G_1 phase and decreased those in S phase in both cell lines (Fig. 1B). Although DIF-3 also significantly increased the number of cells in G_2 phase when HCT-116 cells were employed, this effect was not observed by using DLD-1 cells. These results indicated that DIF-3 arrested the cell cycle at G_0/G_1 phase.

3.2. DIF-3 induces proteolysis of cyclin D1 and c-Myc in HCT-116 cells via activation of GSK-3β

Because DIF-3 induces cell cycle arrest at G_0/G_1 phase by suppressing cyclin D1 expression in HeLa cells (16), we examined the effect of DIF-3 on the expression of cyclin D1 in HCT-116 cells, in which β-catenin is constitutively active. As shown in Fig. 2A and B, a rapid and marked reduction in the amount of cyclin D1 protein in a time and dose-dependent manner was induced by DIF-3. Furthermore, treatment with DIF-3 resulted in a strong reduction in the
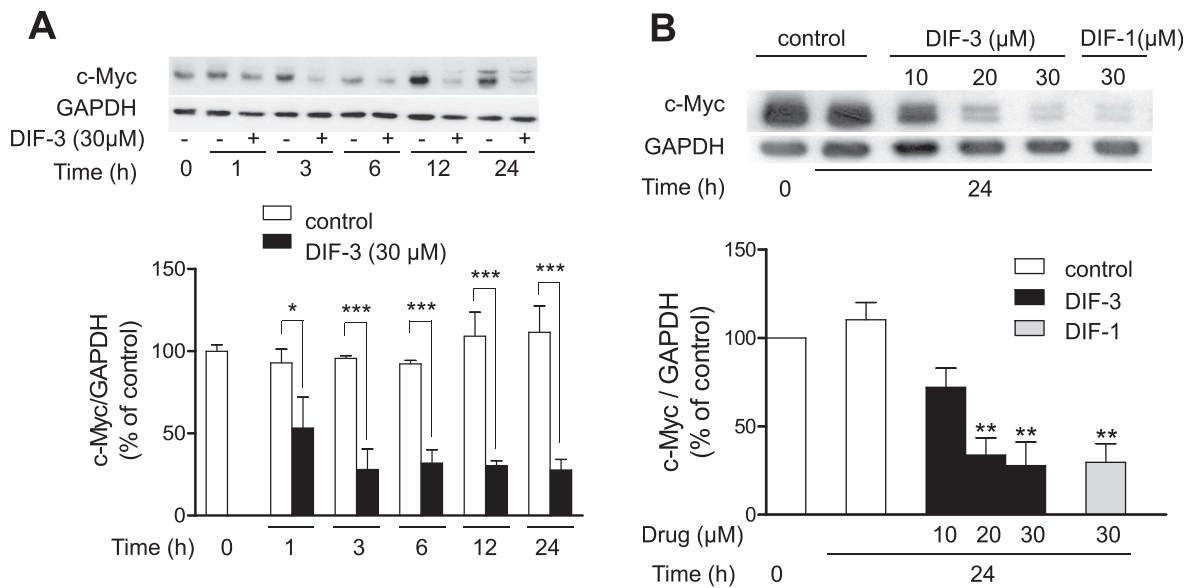

Fig. 3. DIF-3 suppresses the expression of c-Myc in HCT-116 cells. (A) Time course. Cells were treated with or without DIF-3 (30 μM) for the indicated periods. (B) Dose dependency. Cells were treated with the indicated concentrations of DIF-1 or DIF-3 for 24 h. Protein samples were subjected to Western blot analysis using anti-c-Myc and anti-GAPDH antibodies. Protein bands were quantified and are shown as the percentage of the control level at time 0. The results are the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.
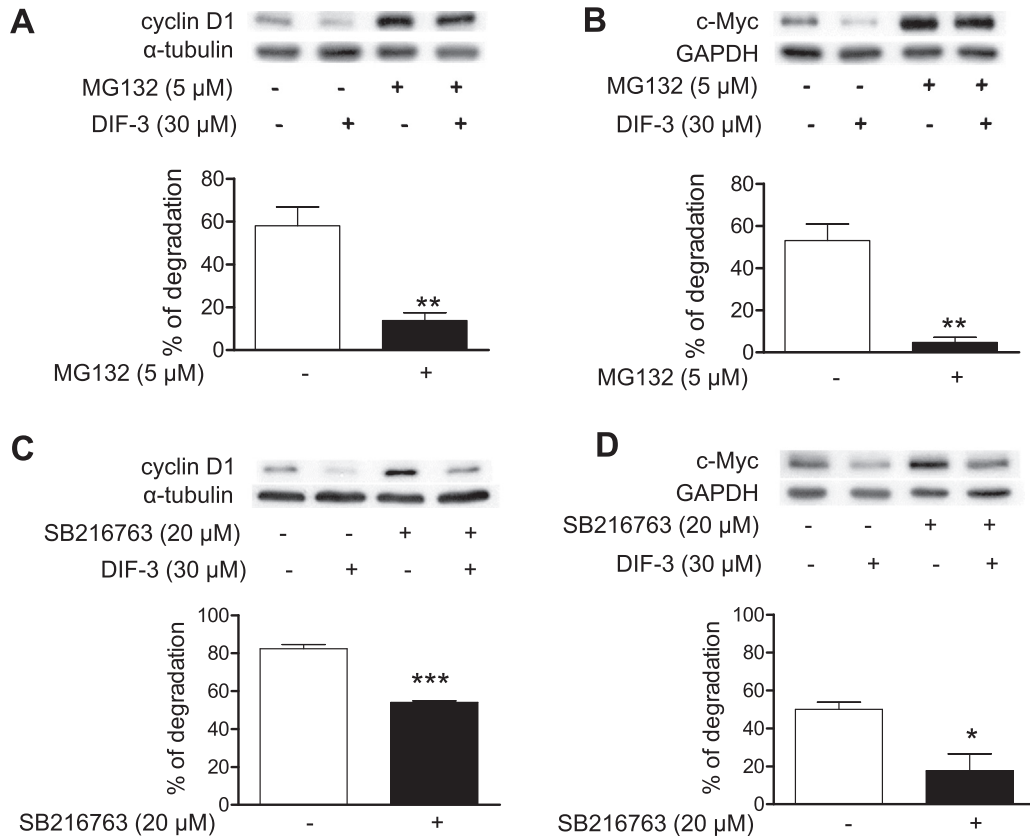
Fig. 4. DIF-3 induces degradation of cyclin D1 and c-Myc in HCT-116 cells. Cells pretreated with MG132 for 1 h (A and B) or SB216763 for 3 h (C and D) were treated with or without DIF-3 (30 μM) for 1 h. Samples were subjected to Western blot analysis using anti-cyclin D1 and anti-α-tubulin antibodies (A and C) or anti-c-Myc and GAPDH antibodies (B and D). Protein bands were quantified and are shown as the percentages of the degraded amounts. Values are the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.
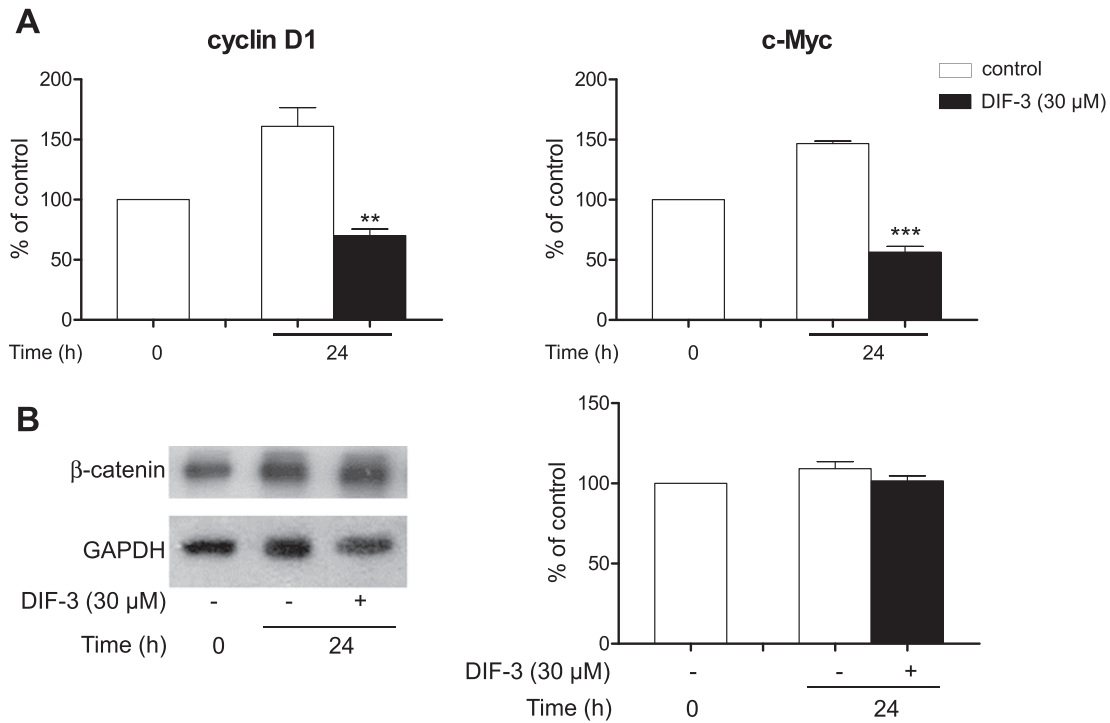

Fig. 5. DIF-3 represses the expression of *cyclin D1* and *c-Myc* mRNA without decreasing the amount of β-catenin protein in HCT-116 cells. (A) mRNA expression. Cells were treated with or without 30 μM DIF-3 for 24 h. Total RNA was isolated and subjected to quantitative PCR to analyze *cyclin D1*, *c-Myc*, and *GAPDH* mRNA expression. The levels of mRNA expression were quantified and are shown as the percentage of the control level at time 0. (B) β-catenin protein. Cells were treated with or without DIF-3 (30 μM) for 24 h. Protein samples were subjected to Western blot analysis using anti-β-catenin and anti-GAPDH antibodies. Protein bands were quantified and are shown as the percentage of the control level at time 0. Values are the mean ± SEM of three independent experiments. **$P < 0.01$, ***$P < 0.001$ vs. control.

expression levels of c-Myc in a time and dose-dependent manner (Fig. 3A and B). DIF-3 decreased the expression of cyclin D1 and c-Myc to a level comparable to the reduction induced by DIF-1 (Figs. 2B and 3B).

To determine the mechanisms of the DIF-3-induced reduction in the expression levels of cyclin D1 and c-Myc, we examined the effect of MG132, a ubiquitin-proteasome inhibitor, on the actions of DIF-3. As shown in Fig. 4A and B, pretreatment with MG132
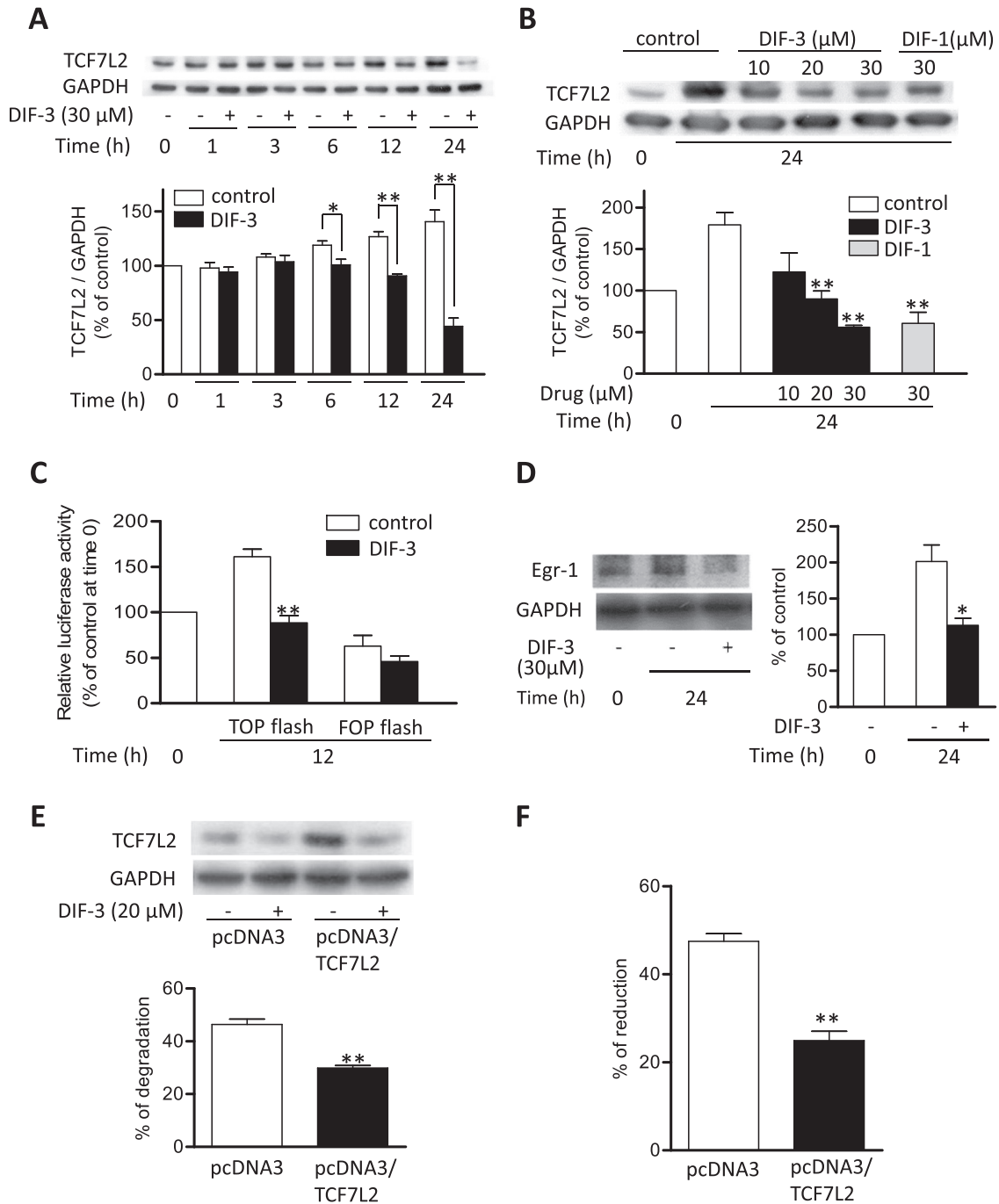

Fig. 6. DIF-3 inhibits the expression of TCF7L2 and suppresses TCF-dependent transcription in HCT-116 cells. (A) Time course. Cells were treated with or without DIF-3 (30 μM) for the indicated periods. (B) Dose dependency. Cells were treated with or without the indicated concentrations of DIF-1 or DIF-3 for 24 h. Protein samples were subjected to Western blot analysis using anti-TCF7L2 and anti-GAPDH antibodies. Protein bands were quantified and are shown as the percentage of the control level at time 0. (C) Effects of DIF-3 on TCF-dependent transcriptional activity. TOPFlash or FOPFlash were co-transfected with pRL-SV40 into HCT-116 cells. After 24 h of incubation, the cells were stimulated with 20 μM DIF-3 for 12 h. Luciferase activity is shown as the percentage of the control level at time 0. (D) Egr-1 protein. Cells were treated with or without DIF-3 (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 are shown as the percentage of the control level at time 0. (E) TCF7L2 overexpression. The pcDNA3 or pcDNA3/TCF7L2 plasmid was transfected into HCT-116 cells. After 24 h, the cells were treated with or without DIF-3 (20 μM) for 12 h. Protein samples were subjected to Western blotting using anti-TCF7L2 and anti-GAPDH antibodies. Protein bands were quantified and shown as percentage of the decrease in the amount of TCF7L2. (F) Cyclin D1 promoter activity using TCF7L2-overexpressed cells. Cyclin D1 promoter and pcDNA3 or pcDNA3/TCF7L2 plasmids were co-transfected with pRL-SV40 into HCT-116 cells. After 24 h of incubation, the cells were treated with or without DIF-3 (20 μM) for 12 h. Data are shown as percentages of the reduction in luciferase activity. Values are the mean ± SEM of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

markedly attenuated the effects of DIF-3, indicating that DIF-3 accelerated ubiquitin-proteasome-dependent proteolysis of cyclin D1 and c-Myc. Because GSK-3β has been reported to trigger proteolysis of cyclin D1 and c-Myc (30, 31), we determined whether GSK-3β was involved in the DIF-3-induced degradation of cyclin D1 and c-Myc using the GSK-3β inhibitor SB216763. As shown in Fig. 4C and D, pretreatment with SB216763 (20 μM for 3 h) attenuated the effect of DIF-3, indicating the involvement of GSK-3β in the DIF-3-induced degradation of cyclin D1 and c-Myc.

3.3. DIF-3 suppresses TCF-mediated transcription in HCT-116 cells

Interestingly, DIF-3 not only accelerated the proteolysis of cyclin D1 and c-Myc, but also reduced their mRNA levels (Fig. 5A). These effects could not be explained by inhibition of β-catenin, because the mutant β-catenin expressed in HCT-116 cells is resistant to the β-catenin destruction complex. In fact, DIF-3 had no effect on the expression level of β-catenin (Fig. 5B).

To solve this enigma, we examined the effect of DIF-3 on the expression of TCF7L2, a key transcription factor in the Wnt/β-catenin signaling pathway. The amount of TCF7L2 increased gradually over time in control cells, but it was markedly reduced by DIF-3 in a time and dose-dependent manner (Fig. 6A and B). Consistently, the TCF-mediated transcription measured by the TOPflash assay was strongly suppressed in cells treated with DIF-3 (Fig. 6C). Furthermore, we investigated the effect of DIF-3 on early growth response-1 (Egr-1), because we had previously found that DIF-1 suppresses TCF7L2 expression by decreasing the expression

of Egr-1, a transcription factor of TCF7L2 (22). As shown in Fig. 6D, treatment with DIF-3 for 24 h reduced the protein level of Egr-1.

Next, we overexpressed TCF7L2 to confirm its involvement in the action of DIF-3. As shown in Fig. 6E, stimulation with DIF-3 for 12 h reduced the expression level of TCF7L2 in cells transfected with the empty pcDNA3 vector, whereas this effect was attenuated in cells transfected with pcDNA3/TCF7L2. Subsequently, we examined the effect of DIF-3 on cyclin D1 promoter activity. Compared with cells transfected with the empty pcDNA3 vector, the effect of DIF-3 was attenuated in cells overexpressing TCF7L2 (Fig. 6F). These results suggested that the DIF-3-induced reduction of TCF7L2 was associated with the transcriptional inhibition of cyclin D1 mRNA in HCT-116 cells.

3.4. DIF-3 inhibits Wnt/β-catenin signaling pathway-related proteins in DLD-1 cells

We next examined the mechanism by which DIF-3 inhibited the proliferation of DLD-1 cells in which the function of the β-catenin destruction complex is impaired by mutant APC. As demonstrated in Fig. 7, similar to its effect on HCT-116 cells, DIF-3 markedly reduced the expression levels of cyclin D1, c-Myc, and TCF7L2 in DLD-1 cells.

3.5. Inhibitory effect of DIF-3 on oxidative stress-induced tumors in Mutyh$^{-/-}$ mice

To investigate the anti-tumor effect of DIF-3 in vivo, we used mice deficient for MUTYH (Mutyh$^{-/-}$), an enzyme that prevents the
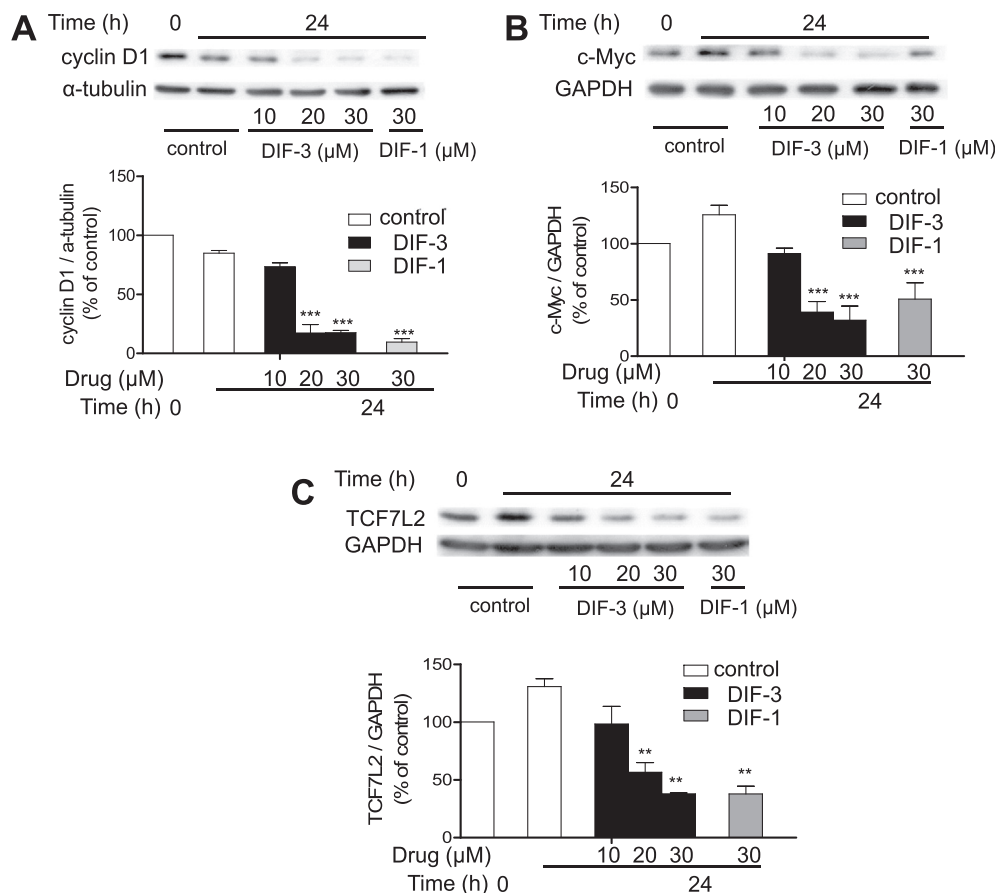

Fig. 7. Effect of DIF-3 in DLD-1 cells. DLD-1 cells were treated with the indicated concentrations of DIF-1 or DIF-3 for 24 h. Protein samples were subjected to Western blot analysis using anti-cyclin D1 (A), anti-c-Myc (B), or anti-TCF7L2 (C) antibodies. Protein bands were quantified and are shown as the percentage of the control at time 0. Values are the mean ± SEM of three independent experiments. **$P < 0.01$, ***$P < 0.001$ vs. control.

formation of oxidative stress-induced DNA lesions. Previous studies have shown that MUTYH deficiency may be involved in the development of colorectal adenomas and carcinomas in humans (27—29). Furthermore, we have previously reported dramatic increases in the occurrence of oxidative stress-induced carcinomas in the small intestines of $Mutyh^{-/-}$ mice in comparison with normal mice (24, 26).

Twelve weeks of treatment with 0.2% KBrO$_3$, a strong oxidant, induced numerous intestinal tumors in $Mutyh^{-/-}$ mice (Fig. 8A). To evaluate the effect of DIF-3 on these tumors, DIF-3 or the vehicle only were orally administered to $Mutyh^{-/-}$ mice for 4 weeks.

Treatment with DIF-3 (150 mg/kg/day for 4 weeks) markedly reduced the number of intestinal tumors, especially the number of large tumors with a diameter of >2.0 mm (Fig. 8A). There were no differences in the appearance, activity, body weight, or blood cell counts between DIF-3-treated mice and the controls (Supplementary Figure 1).

Subsequently, we performed immunohistochemical analyses of the tumors. As shown in Fig. 8B, the numbers of TCF7L2-and cyclin D1-positive nuclei in tumors were significantly decreased in the DIF-3-treated group compared with the control, which was consistent with the in vitro experimental results.
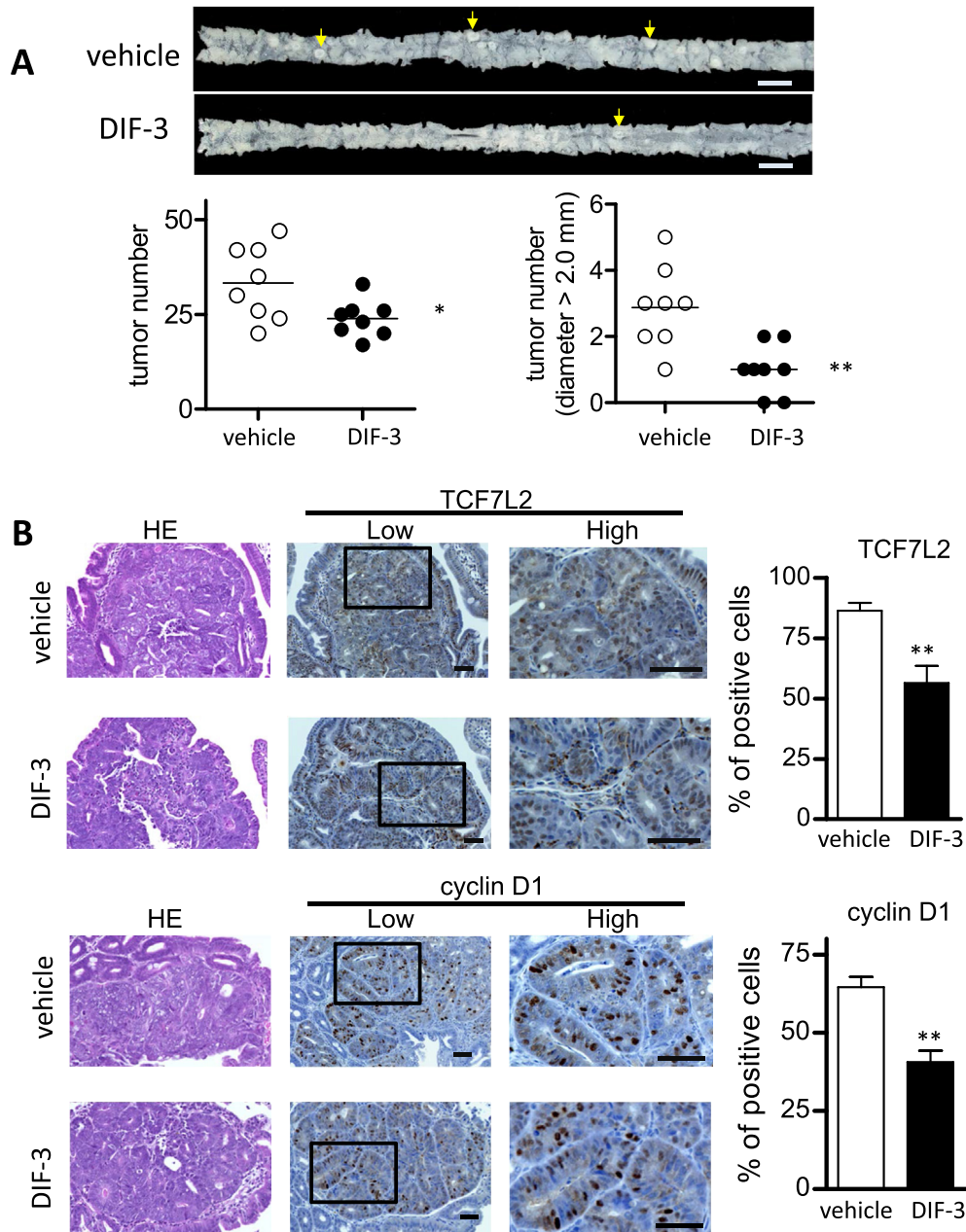

Fig. 8. Effect of DIF-3 on intestinal tumors induced by KBrO$_3$ in $Mutyh^{-/-}$ mice. (A) Upper panels: proximal region of the small intestines. Arrows indicate tumors of >2.0 mm in diameter. Scale bar: 1 cm. Lower left panel: tumor numbers were plotted for each mouse. Lower right panel: the number of tumors of >2.0 mm in diameter was plotted for each mouse. Horizontal bars indicate the mean of each group (n = 8). *P < 0.05, **P < 0.01 vs. vehicle. (B) Histological analyses of intestinal tumors. Tumor samples (n = 5 in each group) were stained with anti-TCF7L2 (upper) or anti-cyclin D1 (lower) antibodies. Black square indicates the magnified area. Scale bar: 50 μm. More than 100 nuclei were counted in each tumor sample and percentages of stained nuclei was shown. Values are shown as the mean ± SEM of five individual samples. **P < 0.01 vs. vehicle.

4. Discussion

Based on our earlier investigations on the DIF family, we hypothesized that DIFs arrest the cell cycle by inducing degradation of proteins essential for cell proliferation such as cyclin D1 and β-catenin. Degradation of β-catenin further prevents TCF-mediated transcription of several genes required for cell proliferation (15–21). According to this hypothesis, the action of DIFs appears to require an intact β-catenin destruction complex. Therefore, we speculated that DIFs would be unable to inhibit colorectal cancer cell proliferation because of the impaired functions of the β-catenin destruction complex in most colorectal cancers, including MUTYH-associated polyposis, which are caused by mutations in its components such as β-catenin and APC (7–12,27–29). However, in contrast to this hypothesis, we found that DIF-1 strongly inhibited the proliferation of colorectal cancer cells through suppression of TCF7L2 transcription by downregulation of Egr-1 levels in the nucleus, resulting in the inhibition of the Wnt/β-catenin signaling pathway (22). Consistent with our study, Saegusa et al (32) reported that TCF7L2 promoter activity is regulated by Egr-1. According to these observations, downregulation of Egr-1 expression might be one of the important actions of DIF-1 in suppression of the Wnt/β-catenin signaling pathway.

Thus, we determined whether only DIF-1 inhibits the proliferation of colon cancer cells irrespective of the β-catenin destruction complex or whether this action is shared by other DIF family members, particularly DIF-3, because DIF-3 exerts stronger effects than DIF-1 on suppression of human cancer cell proliferation (16, 25). Specifically, we investigated whether DIF-3 can inhibit the proliferation of colorectal cancers with impaired β-catenin destruction machinery with respect to its effect on TCF7L2. DIF-1 and DIF-3 demonstrated essentially the same effects. DIF-3 also had an antiproliferative effect on HCT-116 and DLD-1 cells, and reduced the expression levels of cyclin D1, c-Myc, and TCF7L2 despite the mutant β-catenin or APC. In addition, DIF-3, but not DIF-1, led to a significant increase in the number of HCT-116 cells in G_2 phase, whereas this effect was not seen in DLD-1, HeLa or bovine aortic endothelial cells in which proliferation is strongly inhibited by DIF-3 (16). Therefore, cell cycle arrest at G_2/M phase may not be essential for the action of DIF-3. Moreover, DIF-3 exhibited an anti-tumor effect in *Mutyh*$^{-/-}$ mice, a useful animal model for colorectal adenocarcinoma, without apparent adverse drug reactions. Oral administration of DIF-3 markedly reduced the number of intestinal tumors, especially that of large tumors (>2.0 mm in diameter), suggesting that DIF-3 has potential as a novel anti-cancer agent.

We expected that the anti-tumor effect of DIF-3 on colorectal cancer cells, if any, would be stronger than that of DIF-1, because it has been reported that DIF-3 has more powerful effects than DIF-1 to inhibit the proliferation of HeLa cells (16) and the human leukemia cell line K562 (25). However, in the present study, the efficacy of DIF-3 was comparable to that of DIF-1 in HCT-116 and DLD-1 cells. Moreover, the *in vivo* effects of DIF-3 in the oxidative stress-induced intestinal tumor model appeared to be weaker than those of DIF-1 (24). Although we cannot exactly explain the reason for these results, we speculate that the presence or absence of mutations in the β-catenin destruction complex could have caused the difference in sensitivity to DIF-1 and DIF-3 between the cell lines. The β-catenin destruction complex is impaired in HCT-116 and DLD-1 cells, but it is intact in HeLa and K562 cells. Therefore, the anti-tumor effect of DIF-3 may be more dependent on suppression of β-catenin signaling than that of DIF-1. Further studies are required to examine this issue.

Similar to DIF-1, we assume that DIF-3 is a potential lead compound as a novel oral anti-cancer drug with unique mechanisms of action. However, to identify and develop an optimal compound, it is critical to identify the target molecule(s) of the DIF family. Despite our and others' efforts, the target molecule(s) of the DIF family, which mediate their antiproliferative effect on cancer cells, have not been identified. Shimizu *et al.* have suggested that PDE1 (calmodulin-dependent cyclic nucleotide phosphodiesterase) may be a target of DIFs (33). In addition, we have reported that mitochondrial malate dehydrogenase may be one of the target molecules of DIF-1 (34). However, the effects of DIFs on these molecules does not appear to correlate with their antiproliferative effects (33, 34). Identification of these target molecule(s) may not only reveal the precise mechanisms of action of the DIF family, but also facilitate the design of novel anticancer drugs with unique mechanisms of action.

Conflicts of interest

The authors indicated no potential conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2015.03.005.

References

(1) Sherr CJ. Cancer cell cycles. Science. 1996;274:1672–1677.
(2) Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer. 2008;8: 976–990.
(3) Barnes DM, Gillett CE. Cyclin D1 in breast cancer. Brest Cancer Res Treat. 1998;52:1–15.
(4) Nesbit CE, Tersak JM, Prochownik EV. MYC oncogenes and human neoplastic disease. Oncogene. 1999;18:3004–3016.
(5) Utsunomiya T, Doki Y, Takemoto H, Shiozaki H, Yano M, Sekimoto M, et al. Correlation of β-catenin and cyclin D1 expression in colon cancers. Oncology. 2001;61:226–233.
(6) Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. Minireview: cyclin D1: normal and abnormal functions. Endocrinology. 2004;145:5439–5447.
(7) Takahashi-Yanaga F, Sasaguri T. Drug development targeting the glycogen synthase kinase-3β (GSK-3β)-mediated signal transduction pathway: inhibitors of the Wnt/β-catenin signaling pathway as novel anticancer drugs. J Pharmacol Sci. 2009;109:179–183.
(8) Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781–810.
(9) Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, et al. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science. 1997;275:1787–1790.
(10) Satoh S, Daigo Y, Furukawa Y, Kato T, Miwa N, Nishiwaki T, et al. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat Genet. 2000;24:245–250.
(11) McDonald SA, Preston SL, Lovell MJ, Wright NA, Jankowski JA. Mechanisms of disease: from stem cells to colorectal cancer. Nat Clin Pract Gastroenterol Hepatol. 2006;3:267–274.
(12) Segditsas S, Tomlinson I. Colorectal cancer and genetic alterations in the Wnt pathway. Oncogene. 2006;25:7531–7537.
(13) Morris HR, Taylor GW, Masento MS, Jermyn KA, Kay RR. Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*. Nature. 1987;328:811–814.
(14) Morris HR, Masento MS, Taylor GW, Jermyn KA, Kay RR. Structure elucidation of two differentiation inducing factors (DIF-2 and DIF-3) from the cellular slime mould *Dictyostelium discoideum*. Biochem J. 1988;249:903–906.
(15) Miwa Y, Sasaguri T, Kosaka C, Taba Y, Ishida A, Abumiya T, et al. Differentiation-inducing factor-1, a morphogen of dictyostelium, induces G_1 arrest and differentiation of vascular smooth muscle cells. Circ Res. 2000;86:68–75.
(16) Takahashi-Yanaga F, Taba Y, Miwa Y, Kubohara Y, Watanabe H, Hirata M, et al. Dictyostelium differentiation-inducing factor-3 activates glycogen synthase kinase-3β and degrades cyclin D1 in mammalian cells. J Biol Chem. 2003;278: 9663–9670.

(17) Yasmin T, Takahashi-Yanaga F, Mori J, Miwa Y, Hirata M, Watanabe Y, et al. Differentiation-inducing factor-1 suppresses gene expression of cyclin D1 in tumor cells. Biochem Biophys Res Commun. 2005;338:903–909.

(18) Matsuzaki E, Takahashi-Yanaga F, Miwa Y, Hirata M, Watanabe Y, Sato N, et al. Differentiation-inducing factor-1 alters canonical Wnt signaling and suppresses alkaline phosphatase expression in osteoblast-like cell lines. J Bone Min Res. 2006;21:1307–1316.

(19) Takahashi-Yanaga F, Mori J, Matsuzaki E, Watanabe Y, Hirata M, Miwa Y, et al. Involvement of GSK-3β and DYRK1B in differentiation-inducing factor-3-induced phosphorylation of cyclin D1 in HeLa cells. J Biol Chem. 2006;281: 38489–38497.

(20) Mori J, Takahashi-Yanaga F, Miwa Y, Watanabe Y, Hirata M, Morimoto S, et al. Differentiation-inducing factor-1 induces cyclin D1 degradation through the phosphorylation of Thr^{286} in squamous cell carcinoma. Exp Cell Res. 2005;310:426–433.

(21) Yoshihara T, Takahashi-Yanaga F, Shiraishi F, Morimoto S, Watanabe Y, Hirata M, et al. Anti-angiogenic effects of differentiation-inducing factor-1 involving VEGFR-2 expression inhibition independent of the Wnt/β-catenin signaling pathway. Mol Cancer. 2010;9:245.

(22) Jingushi K, Takahashi-Yanaga F, Yoshihara T, Shiraishi F, Watanabe Y, Hirata M, et al. DIF-1 inhibits the Wnt/β-catenin signaling pathway by inhibiting TCF7L2 expression in colon cancer cell lines. Biochem Pharmacol. 2012;83:47–56.

(23) Jingushi K, Nakamura T, Takahashi-Yanaga F, Matsuzaki E, Watanabe Y, Yoshihara T, et al. Differentiation-inducing factor-1 suppresses the expression of c-Myc in the human cancer cell lines. J Pharmacol Sci. 2013;121:103–109.

(24) Takahashi-Yanaga F, Yoshihara T, Jingushi K, Igawa K, Tomooka K, Watanabe Y, et al. DIF-1 inhibits tumor growth in vivo reducing phosphorylation of GSK-3β and expressions of cyclin D1 and TCF7L2 in cancer model mice. Biochem Pharmacol. 2014;89:340–348.

(25) Kubohara Y. Effects of differentiation-inducing factors of *Dictyostelium discoideum* on human leukemia K562 cells: DIF-3 is the most potent anti-leukemic agent. Eur J Pharmacol. 1999;381:57–62.

(26) Sakamoto K, Tominaga Y, Yamauchi K, Nakatsu Y, Sakumi K, Yoshiyama K, et al. MUTYH-deficient mice are susceptible to spontaneous and oxidative stress induced intestinal tumorigenesis. Cancer Res. 2007;67:6599–6604.

(27) Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, et al. Inherited variants of MYH associated with somatic G: C–>T: a mutations in colorectal tumors. Nat Genet. 2002;30:227–232.

(28) Sampson JR, Dolwani S, Jones S, Eccles D, Ellis A, Evans DG, et al. Autosomal recessive colorectal adenomatous polyposis due to inherited mutations of MYH. Lancet. 2003;362:39–41.

(29) Sieber OM, Lipton L, Crabtree M, Heinimann K, Fidalgo P, Phillips RK, et al. Multiple colorectal adenomas, classic adenomatous polyposis, and germ-line mutations in MYH. N Engl J Med. 2003;348:791–799.

(30) Diehl JA, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev. 1997;11:957–972.

(31) Gregory MA, Qi Y, Hann SR. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. J Biol Chem. 2003;278:51606–51612.

(32) Saegusa M, Hashimura M, Kuwata T, Hamano M, Watanabe J, Kawaguchi M, et al. Transcription factor Egr1 acts as an upstream regulator of β-catenin signaling through up-regulation of TCF4 and p300 expression during trans-differentiation of endometrial carcinoma cells. J Pathol. 2008;216:521–532.

(33) Shimizu K, Murata T, Tagawa T, Takahashi K, Ishikawa R, Abe Y, et al. Calmodulin-dependent cyclic nucleotide phosphatase (PDE1) is a pharmacological target of differentiation-inducing factor-1, an antitumor agent isolated from Dictyostelium. Cancer Res. 2004;64:2568–2571.

(34) Matsuda T, Takahashi-Yanaga F, Yoshihara T, Maenaka K, Watanabe Y, Miwa Y, et al. Dictyostelium differentiation-inducing factor-1 binds to mitochondrial malate dehydrogenase and inhibits its activity. J Pharmacol Sci. 2010;112:320–326.