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ORIGINAL ARTICLE

Differentiation-inducing factor-1 enhances 5-fluorouracil action on oral cancer cells inhibiting E2F1 and thymidylate synthase mRNAs accumulation

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Abstract
Purpose Differentiation-inducing factor-1 (DIF-1) is a morphogen originally identified in the amoebozoan *Dictyostelium discoideum*. In mammalian cells, it has been shown to activate GSK3β, which in turn is expected to reduce levels of β-catenin and cyclin D1, thus mediating DIF-1 antiproliferative properties. Since this could alter the expression and activity of E2F1 transcription factor and consequently those of the prognostic marker/chemotherapy target thymidylate synthase (TS), we evaluated (1) whether DIF-1 could effectively regulate these genes, (2) whether it could interfere with cell viability, and (3) whether DIF-1 activity could enhance the efficacy of the TS inhibitor 5-fluorouracil (5-FU).
Methods We investigated the effects of DIF-1 in continuous human cell lines derived from two oral tumor histotypes (corresponding to an adenosquamous and a squamous carcinoma) and a gingival epithelium. We evaluated mRNA accumulation by means of quantitative real-time PCR and efficacy of drugs on cell viability by means of MTT assay.
Results DIF-1 inhibited the accumulation of E2F1 mRNA and reduces TS mRNA levels in tumor cell lines, but did not alter mRNA levels in the gingival counterpart. As a result, it inhibited proliferation preferentially of tumor cell in time- and concentration-dependent manner. Moreover, it enhanced cytotoxic effects of 5-FU only in tumor cell, whereas reduced them in the gingival counterpart.
Conclusions These findings suggest a tumor-specific action of DIF-1 on oral carcinoma cells. Thus, interfering with E2F1 and TS transcription, DIF-1 potentiates TS enzymatic inhibitors.

Keywords Differentiation-inducing factor · Thymidylate synthase · E2F1 · Oral cancer

Introduction

Differentiation-inducing factor-1 (DIF-1) is a dichlorinated phenyl-alkyl-ketone that controls cell differentiation and morphogenesis in *Dictyostelium discoideum* [1]. DIF-1 and, to a greater extent, DIF-3 (a monochlorinated analog and main metabolite of DIF-1) are necessary and sufficient to induce stalk cell differentiation both in vivo and in monolayer assays at nanomolar concentrations. DIFs are known to regulate STATc [2], a member of the STAT family, by indirectly controlling its translocation to the nucleus, and dimB [3], a member of the bZIP family of transcription factors, by inducing its phosphorylation. However, both the DIFs receptor and the involved kinase have yet to be identified. RblA, the *D. discoideum* ortholog of the retinoblastoma protein (pRb), is involved in the regulation of the response to DIF, and $rblA^{null}$ cells show a three to tenfold increase in their sensitivity to DIF [4].

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Interestingly, DIFs have shown antiproliferative activity in vitro on different tumor cell lines, including those of leukemic [5], gastric [6] and oral squamous carcinoma (OSCC) origin [7]. In mammalian cells, DIFs block the cell cycle in the G_0–G_1 transition through glycogen synthase kinase 3β (GSK3β) activation. This kinase negatively affects cyclin D1 transcription through β-catenin phosphorylation [8] and also promotes cyclin D1 proteasome-dependent degradation [9]. In turn, cyclin D1 inhibition maintains pRb in a hypophosphorilated/active form, repressing the activity of E2F family members [10, 11]. Recent evidence suggests that E2F1 is also transcriptionally controlled by the Wnt/β-catenin pathway [12]. Thymidylate synthase (TS) is one of the S-phase-specific targets of the E2F1/pRb pathway [13]. It is a key-enzyme in the synthesis of DNA, being the unique intracellular de novo source of deoxythymidylate (dTMP), which is an essential precursor for DNA biosynthesis [14]. TS represents a relevant pharmacological target for the chemotherapy of many tumors, and its inhibitors, such as 5-fluorouracil (5-FU), pemetrexed or raltitrexed, are clinically used for gastrointestinal, lung and oral carcinomas, among other types of solid tumors [15].

Aims of our study were to assess i) the potential modulatory effect of DIF-1 on E2F1 and TS mRNA expression, and ii) whether it can enhance the efficacy of 5-FU on two oral carcinoma cell lines (CAL 27 [16, 17] and HSC-2 [18]) and a naturally immortalized line from gingival epithelium (S–G [19]).

Materials and methods

Human cell lines culture

CAL 27 (tongue adenosquamous carcinoma), HSC-2 (mouth squamous cell carcinoma) and S–G (gingival nontumorigenic keratinocytes) cell lines (kindly provided by Prof. H. Babich, Yeshiva University, New York, NY, U.S.A.) were cultured in 75 cm^2 flasks (TPP AG, Switzerland) in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G, 40 μg/ml gentamycin sulfate and 2.5 μg/ml amphotericin B (all from Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37°C in a humidified 5% CO_2 atmosphere. Cells were harvested twice a week with 0.25% porcine trypsin solution (from Sigma-Aldrich).

Cell growth kinetics study

To determine the growth rate, 1×10^5 cells of each cell line were seeded into 25 cm^2 flasks (TPP AG) with 5 ml of growth medium. Viable cell counts were performed through trypan blue exclusion staining. Given two measurements of a growing quantity, q_1 at time t_1 and q_2 at time t_2, doubling time (T_d) of cell lines was calculated as:

$$T_d = (t_2 - t_1) \times \left[\frac{\log(2)}{\log\left(\frac{q_2}{q_1}\right)} \right].$$

Quantitative real-time polymerase chain reaction (qPCR)

Cell lines were seeded at a density of 1×10^5 per well in 24-well plates (TPP AG) and cultured in RPMI-1640 alone or supplemented with 25, 50 or 75 μM DIF-1. Each experiment was done in triplicate. After 24 h, total RNA was extracted from each sample with TRIreagent solution (Sigma-Aldrich) according to manufacturer's instructions. One microgram of RNA was purified from contaminating DNA through a Deoxyribonuclease I kit and retrotranscribed with Random Hexamer Primer according to M-MuLV Reverse Transcriptase manufacturer's instructions (all reagents were from Fermentas International Inc., Canada). The relative cDNA quantification of E2F1, TS and β-Actin, as reference gene, was done using a fluorescence-based real-time detection method, with the previously described primers sequences and cycling conditions [20]. Each measurement was done in duplicate.

Proliferation assay

Cell lines were seeded at a density of 5×10^2 per well in 96-well plates (TPP AG). After 1 day, culture medium was replaced and supplemented with serial dilutions (from 12.5 to 50 μM) of DIF-1 (kindly provided by Dr R. R. Kay, MRC, Cambridge, UK), 5-FU (Sigma-Aldrich) or a combination 1:1 of them (ranging from 6.25 to 25 μM). As control were used ethanol and water, respectively. After 24, 48 and 72 h of incubation, cell viability was evaluated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay with three independent experiments.

Cell cycle

Cell cycle analysis was evaluated by flow cytometry, through propidium iodide labeling. Cells (2.5×10^5) were seeded on 100 mm dishes (TPP AG). After 1 day, culture medium was replaced and supplemented with 6.25, 12.5 and 25 μM DIF-1. 48 h later, cells were harvested and washed twice in PBS. Cells in suspension were spun at 1,000 rpm for 5 min. Cellular pellet was fixed in 1 ml 70% ethanol for 30 min at 4°C. Cells were washed, treated with 0.1 mg/ml RNAse A for 15 min and resuspended in an appropriate volume of propidium iodide at a final

concentration of 50 μg/ml for 3 h at 4°C. For each sample, 5×10^4 events were acquired on a CyAn ADP flow cytometer (Becton–Dickinson). Each experiment was done in triplicate.

Statistical analysis

The fold change in gene expression levels was evaluated using the $2^{-\Delta\Delta Ct}$ method, while cell viability was expressed as arithmetic mean percentage of the control ± standard error. One-way ANOVA and Newman–Keuls multiple comparison test (for post-ANOVA comparisons) were used to evaluate the statistical significance of the differences for all data. Correlation analysis was performed with Pearson's correlation test. All analyses were carried out with GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA, http://www.graphpad.com), with $P < 0.05$ as the significance cut off.

The nature of interaction between DIF-1 and 5-FU was evaluated by combination index (CI) method. DIF-1 was combined with 5-FU at a fixed ratio that spanned IC10, IC25 and IC50. Data analysis was performed using the Calcusyn software (Biosoft, NY, USA).

Results

DIF-1 regulates E2F1 and TS mRNA levels

At the exponential growth phase, CAL 27, HSC-2 and S–G cells had similar proliferation rates with comparable doubling times (~30 ± 2 h).

We examined the effects of DIF-1 at a molecular level, by studying the accumulation of E2F1 and TS mRNAs. After exposure to DIF-1 for 24 h, CAL 27 and HSC-2 cell lines exhibited lower levels of E2F1 mRNA that significantly correlated with the concentration of DIF-1 ($P < 0.0001$) (Fig. 1a). Adenosquamous CAL 27 cells showed significant decrease in E2F1 mRNA at all the DIF-1 concentrations. In the HSC-2 line, the only significant response was observed at 75 μM DIF-1 (−90%), while it only decreased to 50% of the untreated value at 50 μM DIF-1. No significant changes in the E2F1 mRNA levels were observed in the S–G control line.

TS mRNA accumulation was also negatively affected by increasing DIF-1 concentration ($P < 0.0001$), though with different dose responses (Fig. 1b). CAL 27 cells showed a reduction in TS mRNA after exposure to 25 μM (−35%) and 50 μM DIF-1 (−70%). No further inhibition was obtained at 75 μM. On the contrary, HSC-2 cells TS mRNA level decreased at 75 μM DIF-1 (−85%) only, while no decrements were observed in the control S–G cells.

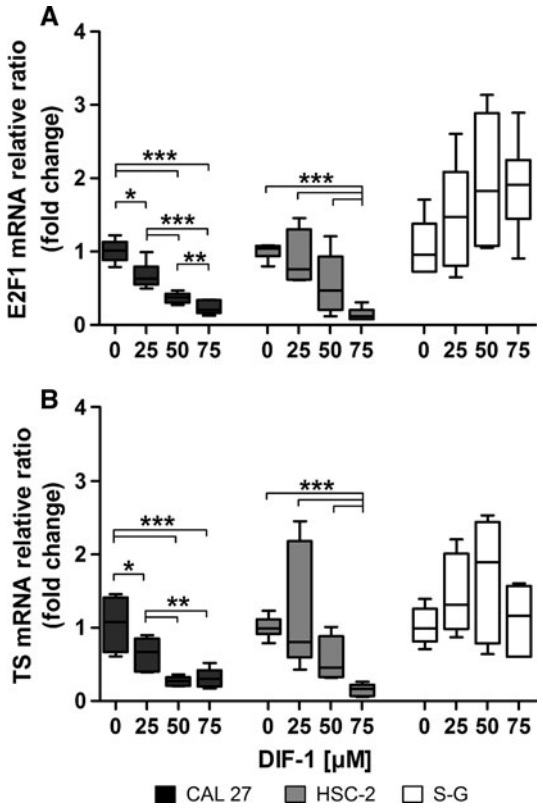

Fig. 1 Changes in E2F1 and TS mRNAs expression after 24 h of DIF-1 treatment evaluated by qPCR. Tumor-derived CAL 27 and HSC-2 cell lines had both E2F1 (**a**) and TS (**b**) mRNA expression significantly depleted in a concentration-dependent manner. Gingival epithelium-derived S–G cell line was not affected even at the highest concentration. *Horizontal lines* in the *middle* represent median values, and *upper* and *lower bars* point the distance from the 10th to 90th percentile from the median, respectively. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

Assessment of DIF-1 effective concentrations on cell viability

In order to evaluate the antiproliferative action of DIF-1 on oral cell lines, we treated growing CAL 27, HSC-2 and S–G cells with increasing amounts of DIF-1 and measured the viability by a colorimetric assay at different times. The antiproliferative activity of DIF-1 was concentration and time dependent. The tested concentrations of DIF-1 did not affect cell proliferation after 24 h of incubation (not shown), while a significant antiproliferative effect in tumor cells was seen after 48 and 72 h (Fig. 2a, b). The sensitivity to DIF-1 antiproliferative action varied among cell lines: at 48 h, DIF-1 significantly inhibited cell growth at 25 μM only in HSC-2 cells. At the highest concentration (50 μM), the average cell viability was reduced to about half for CAL 27 and HSC-2, while only a mild inhibitory effect was observed in the S–G cell line (Fig. 2a). After 72 h of incubation, DIF-1 greatly reduced HSC-2 cell viability at

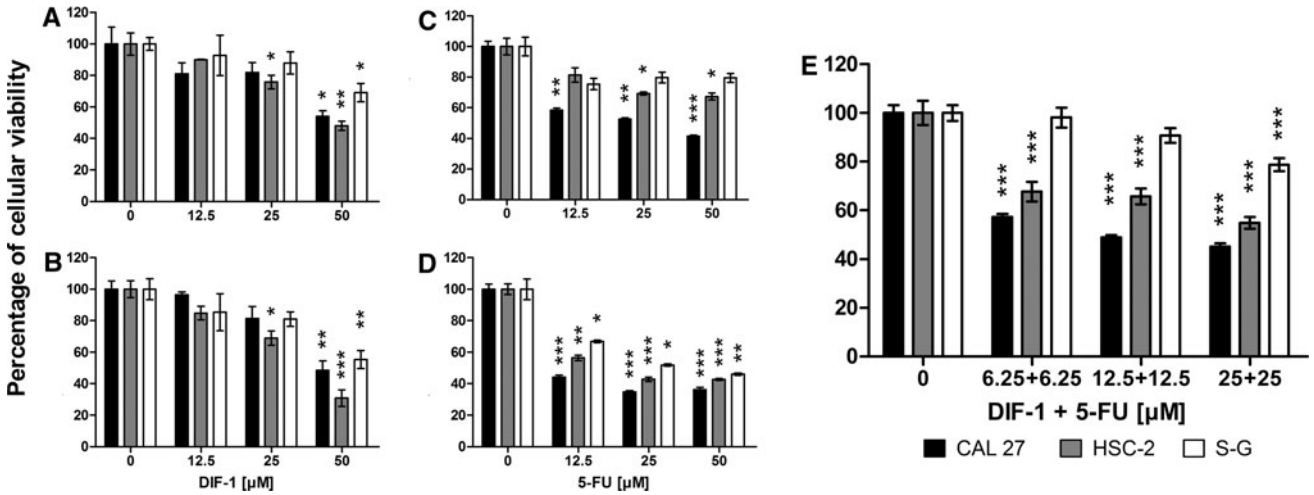

Fig. 2 DIF-1-mediated cell lines viability impairment. It was concentration and time dependent; DIF-1 action was higher in tumor cell lines (CAL 27 and HSC-2) than in a gingival epithelial cell line (S–G). After 48 h of treatment, **a** DIF-1 determined about 50% of growth inhibition in CAL 27 and HSC-2 cells, although only at the highest concentration; 25–30% of impairment was also achieved by both S–G and HSC-2 at 50 and 25 μM, respectively. After 72 h, **b** DIF-1 effect on all cell lines was sharper than 48 h. Only tumor lines exceeded the 50% of viability inhibition. Similarly to DIF-1, 5-FU induced a concentration- and time-dependent (**c** 48 h; **d** 72 h) inhibition of viability in all cell lines tested. 5-FU effect was strongest than that of DIF-1, but in the same concentration range. DIF-1 enhances 5-FU cytotoxicity (**e**). If compared to 5-FU alone (**c**), its association with DIF-1 results in slightly better results with halved 5-FU concentrations. All data derived from the arithmetical mean of three different experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus untreated cells (control)

concentrations of 25 and 50 μM. The highest DIF-1 concentration significantly inhibited both CAL 27 and S–G proliferation to approximately half of the control value (Fig. 2b).

DIF-1 affects cell viability at the same concentration range of 5-FU

In a similar experiment, we compared DIF-1 antiproliferative action to that of 5-FU, one of the most used TS enzymatic inhibitor. Although 24 h exposure time to 5-FU did not significantly affect the viability of any cell line (not shown), prolonged exposure times reduced it (Fig. 2c, d). After 48 h of incubation, CAL 27 line was significantly affected at all tested concentrations, while in HSC-2 cells viability was decreased only at 25 and 50 μM 5-FU. Conversely, no significant difference was observed in the S–G control (Fig. 2c). After 72 h of incubation with 5-FU, cell proliferation was strongly inhibited in all cell lines. CAL 27 cells were the most strongly affected, even at the lowest concentration, while HSC-2 proliferation decreased to about a half of the control value only at higher concentrations and S–G showed the lowest response (Fig. 2d).

DIF-1 enhances 5-FU tumor cytotoxic activity

We evaluated whether DIF-1 could act as enhancer for 5-FU. After 48 h of incubation, the viability of both tumor cell lines was significantly diminished: association of DIF-1 and 5-FU resulted in the same or slightly better inhibition than 5-FU alone, but with halved 5-FU concentrations. On the contrary, S–G cell line viability is slightly increased if compared with that obtained treating cells with the same 5-FU concentration alone (Fig. 2e).

As known, high DIF-1 concentrations could block cell cycle in G_0/G_1 phase, while 5-FU acts in S phase. Hence, we assessed whether the DIF-1 doses employed for the combination test were able to alter the cell cycle of our lines. No significant differences were found in the percentage of cells in G_0/G_1 phase (60–70%) or in the amount of those in apoptosis (1–5%). Moreover, the CI analysis revealed that DIF-1 and 5-FU were synergic to strongly synergic in their cytotoxic action at the considered doses but only on tumor lines (ID10: 0.09 and 0.26; ID25: 0.24 and 0.27; ID50: 0.61 and 0.80, for CAL 27 and HSC-2, respectively).

Discussion

In the present study, we employed one nontumorigenic gingival epithelium and two tumor-derived cell lines (with similar proliferation rates) to investigate the amoebozoan-derived DIF-1 effects. Cell viability was most heavily influenced in neoplastic cells, with limited effects in the gingival-derived line, where cells were only mildly affected at the highest DIF-1 concentrations and longest incubation times. At a molecular level, the action of DIF-1 was

even more tumor specific: reduction in cell viability is paralleled by a comparable reduction in E2F1 and TS mRNAs only in HSC-2 and CAL 27 cells, while mRNA levels do not seem to correlate with cell viability in the control cells used (Fig. 3). This observation suggests a possible involvement of multiple pathways in the antiproliferative action of DIF-1.

DIF-1 has been shown to mediate the degradation of β-catenin and cyclin D1 through GSK3β activation. Interestingly, aberrations of Wnt/β-catenin pathway are commonly

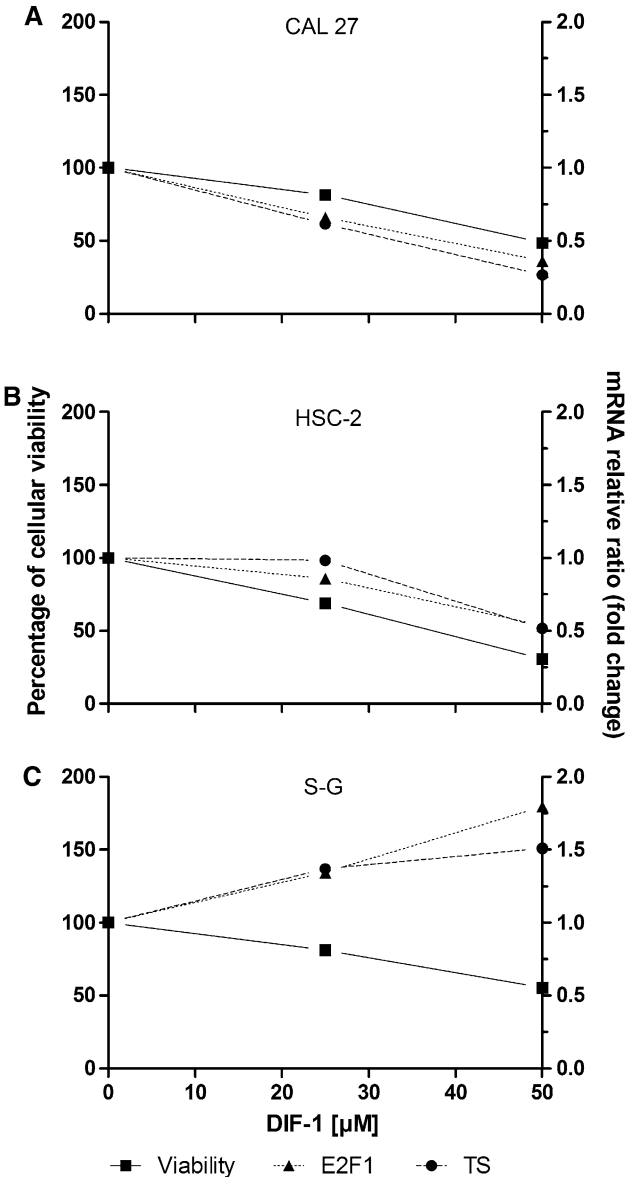

Fig. 3 Comparison between cell viability (*straight line*) and mRNA expression (*dotted lines*) confirms DIF-1 tumor specificity. Neoplastic cell lines show a direct correlation between viability and mRNA relative ratio of both E2F1 and TS (**a**, **b**), as showed by the paralleled decreasing trend of both variables. Otherwise in gingival epithelium-derived *line variables* are inversely correlated, plotting as *divergent lines* (**c**)

associated with tumor development [21], and this pathway is known to be widely deregulated in oral cancers but not in the normal epithelium [22]. The decrease in β-catenin potentiates the effect on cyclin D1 by reducing its de novo synthesis [7–9]. Cyclin D1-dependent kinases action liberates E2F1 from complexes with pRb, thus activating its function. E2F1 promotes proliferation through its modulatory effects on S-phase-specific gene expression.

In addition, effects on E2F1 transcription are expected, as T-cell factor (TCF) is an effector of β-catenin and the presence of TCF consensus sites within the E2F1 gene promoter has been reported [12]. We have now shown that E2F1 and hence TS mRNAs are indeed tumor-specifically down-regulated by DIF-1. Moreover, it could also explain the relative lack of antiproliferative action in the control cells, where, upon DIF-1 treatment, E2F1 would be down-regulated at the protein level only (Fig. 4).

E2F1 and TS are both involved in the control of metazoan cell proliferation. While E2F1 is the effector of the G_1–S transition controlled by pRb, TS is involved in the purine biosynthesis, fueling a metabolic pathway for the synthesis of DNA [10, 11, 13]. In the last decades, TS has been targeted by different antineoplastic compounds. Among these, 5-FU and its analogs (alone or in combination) have been widely used in a variety of malignancies including gastrointestinal, breast, nonsmall cell lung and head and neck cancers [15]. However, several studies suggested that higher levels of TS in tumor cells might decrease the responsiveness of antitumor drugs targeting TS, leading to drug resistance, particularly for the gastrointestinal neoplasms [23]. Although in oral cancers the inverse correlation between TS enzyme levels and treatment efficacy of 5-FU or analogs is still debated, a lower TS expression is generally considered a good predictive marker [14, 23]. Reduction in TS level could thus improve antitumor effect of TS-inhibitors, as previously shown [24, 25]. Here, we demonstrated that the association DIF-1/5-FU can inhibit the cell viability of both squamous and adenosquamous cell carcinoma lines at halved concentrations than 5-FU alone, without affecting and even protecting that of nontumor cells. At the evaluated doses, DIF-1 did not alter the cell cycles of considered cell lines, despite the G_0/G_1 block of several other lines has been already demonstrated [7, 26–28]. CAL 27 and HSC-2 cell lines had a massive DIF-1-mediated down-regulation of both E2F1 and TS genes only at higher concentrations than those employed in the combination experiment. Moreover, by the analysis of fixed ratio Combination Index, DIF-1 and 5-FU had a synergic action at the considered doses, but we can deduce from growth curves an antagonism at higher doses, probably due to the occurrence of the expected cell cycle disruption. It could be a further clue for the

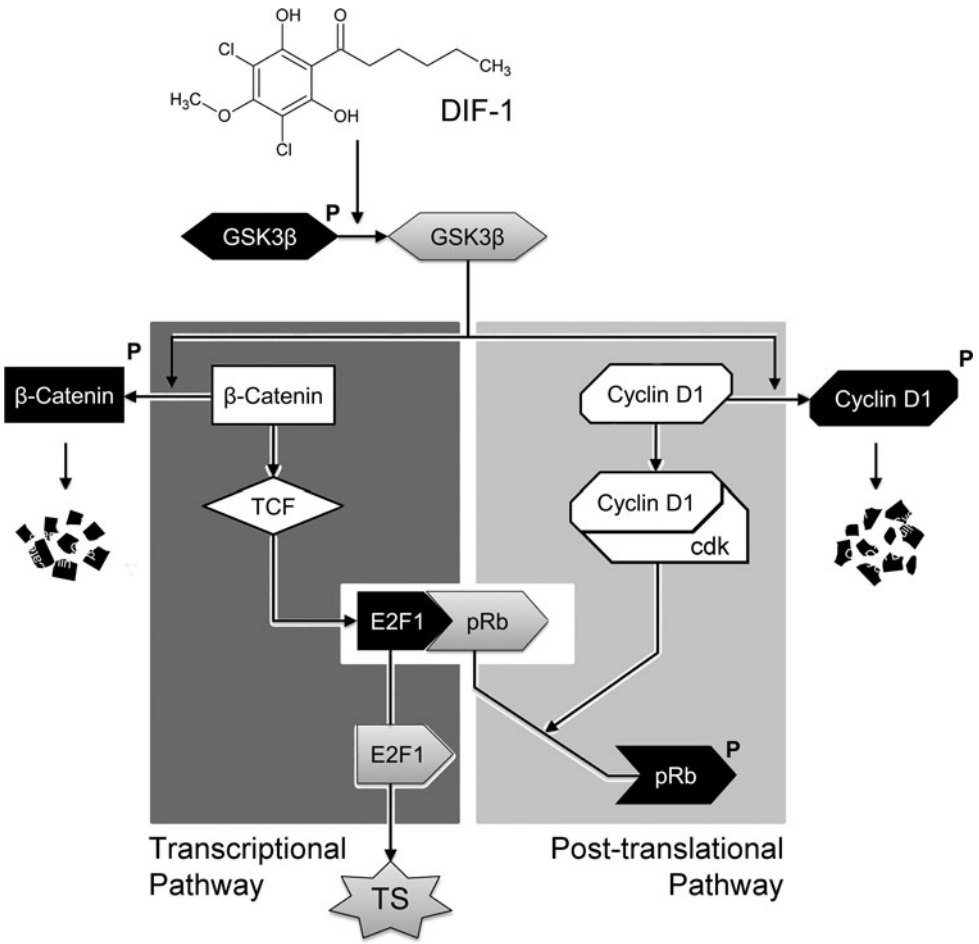

Fig. 4 Proposed DIF-1 signaling pathway. DIF-1 induces dephosphorylation of GSK3β by unknown mechanisms. Active GSK3β inhibits cyclin D1 and mediates the concomitant β-catenin/TCF impairment, further worsening cyclin D1 depletion. Cyclin D1 reminders are less able to inactivate pRb and induce E2F1 release. The DIF-1-mediated β-catenin/TCF inhibition impairs E2F1 transcription, prejudicing its overall availability regardless to the pRb repression activity. Both transcriptional and posttranslational pathways of E2F1 lead to the impairment of S-phase genes (TS) transcription. *P* indicates phosphorylation. *Darkened boxes* denote the deactivated protein form

involvement of TS as molecular target at both transcriptional and posttranslational levels.

Although in vivo data about toxicological and pharmacokinetics profiles are not yet evaluated, DIF-1 interference with E2F1 and TS transcription could offer the opportunity to develop more compliable and tumor-selective strategies, reducing the dose of TS enzymatic inhibitors characterized by important dose-correlated adverse effects.

It should be noted that DIF1 activity was observed only at micromolar concentrations, generally difficult to reach in vivo. This aspect provides an incentive to develop DIF-1 analogs with higher activities at lower concentrations.

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