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Rap1-suppressed tumorigenesis is concomitant with the interference in Ras effector signaling

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Abstract Expression of Rap1 blocks epithelial growth factor-induced extracellular signal-regulated kinases (ERKs) activation. However, recent studies demonstrated that Rap1 mediates ERKs activation induced by nerve growth factor. The anti-oncogenic effect of Rap1 has been reported but its mechanism remains unclear. To evaluate the correlation between the anti-transforming effect and the activation of ERKs, we transfected rap1 cDNA into Hep3B cells and selected stable transfectants. The Rap1 transfectants completely lost their intrinsic tumorigenicity in Balb/c nude mice. Both insulin and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-stimulated ERK activations were also blocked. Our findings suggest that Rap1-suppressed tumorigenicity is concomitant with ERKs inhibition.

Key words: Rap1; Extracellular signal-regulated kinase; GTPase; Mitogenesis; Insulin; 12-*O*-Tetradecanoyl phorbol-13-acetate

1. Introduction

Rap1 is a small GTPase that was identified in a screen for Ras homologous proteins [1] and independently as a suppressor of v-Ki-ras transformation [2]. It has an effector domain identical to the effector domain of Ras. The active form of Rap1 was found to bind to most Ras effectors, e.g. Raf-1 and B-raf, the Ral guanine nucleotide exchange factors (RalGEFs) RalGDS, Rgl and Rlf [3–5]. This has prompted the speculation that Rap1 may antagonize Ras functions by sequestering Ras effectors in a non-productive complex. For instance, overexpression of active Rap1 inhibits Ras-induced germinal vesicle breakdown in *Xenopus* oocytes, and in mammalian cells antagonizes the Ras-dependent activation of extracellular signal-regulated kinase (ERK) pathway [6,7]. Several studies have demonstrated that there is substantial similarity in the upstream signaling mechanism that regulates both Ras and Rap1 activation. Ras-GTP binding is stimulated by Ras-GEF SOS [8]. The carboxyl-terminal domain of SOS contains a proline-rich region that directs its association with the SH3 domain of the small adapter protein, GRB2 [9,10]. In analogy, the formation of GTP-bound Rap1 results from the specific interaction with Rap1 GEF C3G [11,12]. The proline-rich regions of C3G are responsible for association with the central SH3 domain of the adapter protein, CrKII [13]. Identification of these associated proteins has led to the hypothesis that Rap1 proteins have other physiological roles in addition to Ras-antagonizing effects. In PC12 cells, it was suggested that cAMP activates Rap1, and this activation induces association of Rap1 and B-Raf, leading to the activation of ERK [14]. Furthermore, Rap1 mediates sustained ERK activation induced by nerve growth factor through a Ras-independent pathway in these cells [15]. Zwartkruis et al. have shown that Rap1 is also rapidly activated following stimulation of a large variety of growth factor receptors, including receptor tyrosine kinase for platelet-derived growth factor and epithelial growth factor (EGF), but not insulin. 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA), which mimics diacylglycerol, also induces Rap1 activation. In their studies, activation of endogenous Rap1 failed to affect Ras-dependent ERK activation [16].

Insulin induces a variety of physiological responses including stimulation of cell proliferation and modulation of specific gene expression in its target cells [17,18]. It elicits these effects by binding to specific cell surface receptor, which activates the protein tyrosine kinase intrinsic to the β-subunit of the receptor [19]. Two immediate substrates of insulin receptor, insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) have been identified [20,21]. These IRS proteins can be phosphorylated by activated insulin receptor and then act as docking proteins to bind the p85 regulatory subunit of phosphatidylinositol-3 kinase and growth factor receptor binding protein 2 (GRB2) through their Src homology-2 domains [22,23]. The GRB2 then physically interacts with Ras-GEF, SOS. Binding of GRB2 and SOS to IRS-1 provides a functional complex necessary for Ras activation. Once in the active GTP-bound state, Ras directly associates and activates Raf-1 kinase. In turn, activated Raf-1 phosphorylates mitogen-activated/extracellular signal-regulated kinase kinase (MEK), which is an immediate upstream activator of ERKs [24,25]. The phorbol ester, TPA, has been shown to mimic insulin-like effects on glucose transport, glucose oxidation and lipogenesis in adipocytes and some other cell types [26]. It has been well documented that insulin and TPA activate Ras/Raf-1/MEK/ERKs in many cells. With respect to Rap1, these two agents seem to have different effects. In Rat-1 cells, TPA activates endogenous Rap1 and this activation fails to affect Ras-dependent ERK activation, while insulin has no effect on Rap1 activity [16]. In Chinese hamster ovary (CHO) cells, insulin induces a time-dependent uncoupling of Raf-1 from Rap1 with a concomitant association of Raf-1 with Ras, which then activates

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Abbreviations: ERK, extracellular signal-regulated kinase; MEK, mitogen-activated/extracellular signal-regulated kinase kinase; IRS, insulin receptor substrate; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; GEF, guanylnucleotide exchange factor

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the downstream Raf-1/MEK/ERK cascade [27]. We have observed that TPA mimics insulin effects in the stimulation of proto-oncogene expression, cell proliferation and the suppression of hepatitis B surface antigen expression in human hepatoma Hep3B cells [28]. To verify the effects of insulin and TPA upon Rap1 activity, we transfected wild-type Rap1 cDNA into Hep3B cells and selected stable transfectants. This approach allowed us to study the role of Rap1 upon ERK activation while stimulating with insulin or TPA, through the amplification of Rap1 signal by expressing Rap1 in Hep3B cells.

It has been intensively discussed whether rap genes are anti-oncogenes [29]. Jelinek and Hassel have shown that rap1 gene induced the reversion of polyomavirus middle T antigen-transformed Rat-2 cells [30]. Most evidences supported the conclusion that overexpression of rap1 genes does induce reversion of malignant phenotypes in certain tumor cell lines [31,32]. However, an oncogenic effect of Rap1b has been reported in Swiss 3T3 cells, in which Rap1 increases DNA synthesis and cAMP exerts a stimulatory effect on cell growth [33]. With these rather paradoxical results, it will be interesting to verify the correlation between either the anti-oncogenic effect and ERKs inactivation or oncogenic effect and ERKs activation. In this paper, we attempted to verify the effect of Rap1 upon ERKs activation by insulin or TPA and the correlation between the oncogenic or anti-oncogenic effect of Rap1 and ERKs activation in Hep3B cells. We found that expression of Rap1 reduced the saturation density of Hep3B cells and suppressed their intrinsic tumorigenicity. Furthermore, the activation of ERKs by insulin or TPA was blocked in Rap1 transfectants. Taken together, in Hep3B cells, Rap1 plays a negative role on ERKs activation by either insulin or TPA and this ERKs inactivation is concomitant with the suppression of tumorigenesis.

2. Materials and methods

2.1. Cell culture

Human hepatoma Hep3B cells were kindly given by Dr. B. Knowles (Wista Institute, PA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal calf serum (FCS, Gibco) in an atmosphere of 5% CO_2, 95% air at 37°C.

2.2. Calcium-phosphate DNA transfection and G418 selection of Rap1 stable transfectants

Human rap1/krev-1 cDNA was transfected into Hep3B cells using the calcium-phosphate method as described [34]. The transfected cells were then selected using 1 mg/ml of G418. After 2 weeks of G418 selection, surviving colonies were isolated.

2.3. Western blot analysis of ERKs or tyrosine phosphorylation pattern

Hep3B cells were seeded at a density of 2×10^6 cells/100 mm plate with 10% FCS. After 16 h, the culture medium was changed to serum-free DMEM for 48 h. The serum-starved cells were then treated with 10^{-9} M or 10^{-7} M insulin for 10 min. The cells were then washed in ice-cold PBS (pH 7.4) and lysed using lysis buffer containing 50 mM HEPES (pH 7.8), 4 mM EDTA, 2 mM EGTA, 5 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin and 1% Triton X-100. Cell lysate was centrifuged at $100\,000\times g$ for 30 min at 4°C. Supernatant (100 μg of protein) was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrotransferred to nitrocellulose in transfer solution containing 25 mM Tris, 192 mM glycine, 20% methanol. The blot was incubated overnight in buffer containing 25 mM Tris (pH 8.0), 125 mM NaCl, 0.05% Tween 20, 0.1% sodium azide and 3% skim milk. The nitrocellulose paper was then probed with anti-ERKs polyclonal antibody or anti-phosphotyrosine antibodies (PY20, PY69) for 3 h at room temperature and then washed three times with Tris-buffered saline (25 mM Tris (pH 8.0), 125 mM NaCl). Mobility shift was detected using the enhanced chemiluminescence detection system (Amersham).

3. Results

3.1. Expression of Rap1 reduces the saturation density of Hep3B cells in culture

The 1.8 kb of rap1 cDNA was constructed into a pCD2 vector containing a neomycin resistance gene under the regulation of an SV40 early promoter. After transfection into the human hepatoma cell line Hep3B, Rap1 stable transfectants were selected using 1 mg/ml G418. The surviving colonies were recovered after 2 weeks of selection. Expression of rap1 gene was confirmed by Northern blotting using rap1 cDNA as a probe. Two different clones (Rap1-3, Rap1-12) were chosen for following studies according to their different expression levels of rap1 mRNA. Another control clone was also selected, which was transfected with a mock vector containing only a neomycin resistance gene under the regulation of an SV40 promoter.

We first investigate whether expression of Rap1 changes the growth property of Hep3B cells. Hep3B derivatives were cultured in 24-well plates with DMEM containing 10% FCS. As seen in Fig. 1A, the growth rates of parental cells and Rap1

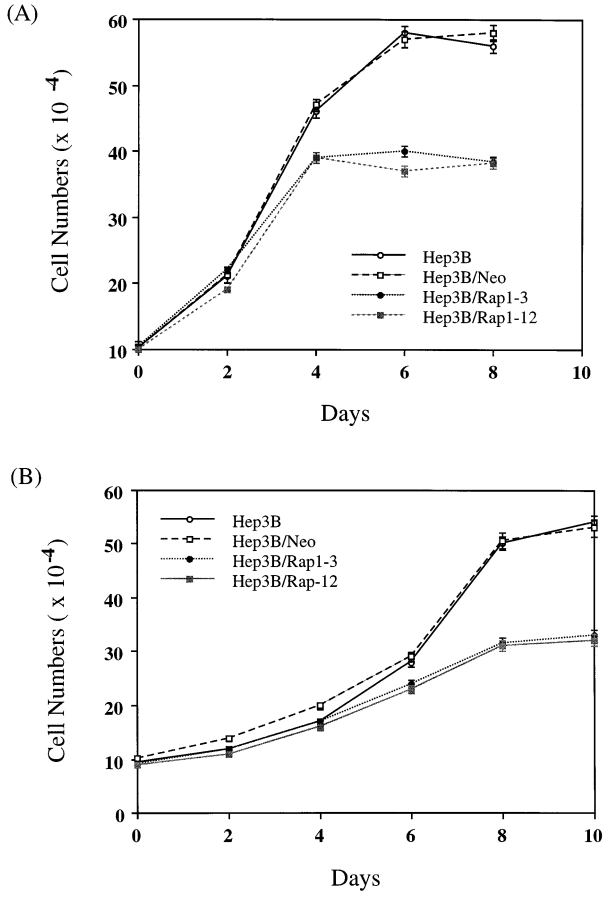

Fig. 1. Growth properties of Hep3B, Hep3B/Neo, Hep3B/Rap1 cells. The derivatives of Hep3B cells were seeded in 24-well plates with DMEM containing (A) 10% FCS or (B) 0% FCS. The culture medium was changed every 2 days. The cell numbers were counted using hemocytometry every 48 h.

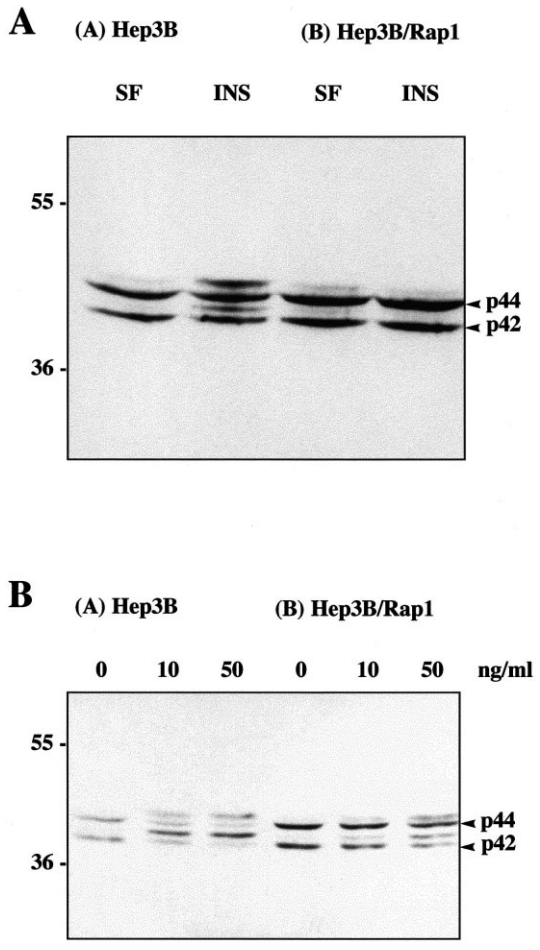

Fig. 2. ERKs activation induced by (A) insulin or (B) TPA in both Hep3B and Hep3B/Rap1 cells. Hep3B and Hep3B/Rap1-3 cells were treated with (A) 10^{-8} M insulin for 10 min or (B) 10 and 50 ng/ml TPA for 5 min after 48 h serum starvation. Cell extracts were harvested and analyzed in 10% SDS–PAGE. The mobility property of ERKs was detected using anti-ERKs antibody as described in Section 2. SF, control cells; INS, insulin-treated cells.

transfectants were similar for the first 4 days. After 4 days in culture, Rap1 transfectants became confluent and their cell numbers did not increase until the end of culture, while cell number of parental Hep3B cells kept increasing until the day 6, then became saturated. The saturation densities of two Rap1-transfected clones were reduced by 40% comparing to that of control cells. As Hep3B cells can steadily divide even in a serum-free culture condition, we also evaluated growth properties of Hep3B derivatives in a serum-free medium. A similar phenomenon was found, as shown in Fig. 1B. This phenomenon is not a non-specific effect because Hep3B/Neo control cells have the same saturation density as Hep3B parental cells.

Table 1
Suppression of tumorigenesis by Rap1 in human hepatoma Hep3B cells

Cells	Dose (cell numbers)	Tumorigenesis in Balb/c nude mice
Hep3B	2×10^6	5/9
	10^7	8/8
Hep3B/Rap1-3	2×10^6	0/5
	10^7	0/5
Hep3B/Rap1-12	2×10^6	0/4
	10^7	0/3

Hep3B, Hep3B/Rap1-3 and Hep3B/Rap1-12 cells were subdermally injected into the upper backs of Balb/c nude mice with different doses. The tumor formation was measured at the day 49 after inoculation.

3.2. Expression of Rap1 suppresses the tumorigenicity of Hep3B cells

As Rap1 reduces the saturation density, we further observed whether Rap1 suppresses the tumorigenicity of Hep3B cells. Two different Rap1 transfectants (Rap1-3 and Rap1-12) and parental Hep3B cells were inoculated into the upper back of Balb/c nude mice at two cell densities. As seen in Table 1, parental Hep3B cells induced 100% tumorigenesis, while two Rap1 clones lost this oncogenic effect.

3.3. Expression of Rap1 inhibits insulin- or TPA-induced ERKs activation

We then investigated the effect of Rap1 upon ERKs activation by insulin and TPA. Cells were treated with 10^{-8} M insulin for 10 min after 48 h serum starvation. As seen in Fig. 2A, 10^{-8} M insulin stimulated the phosphorylation of ERKs, which induces mobility shifts of ERK-1 and ERK-2 in an

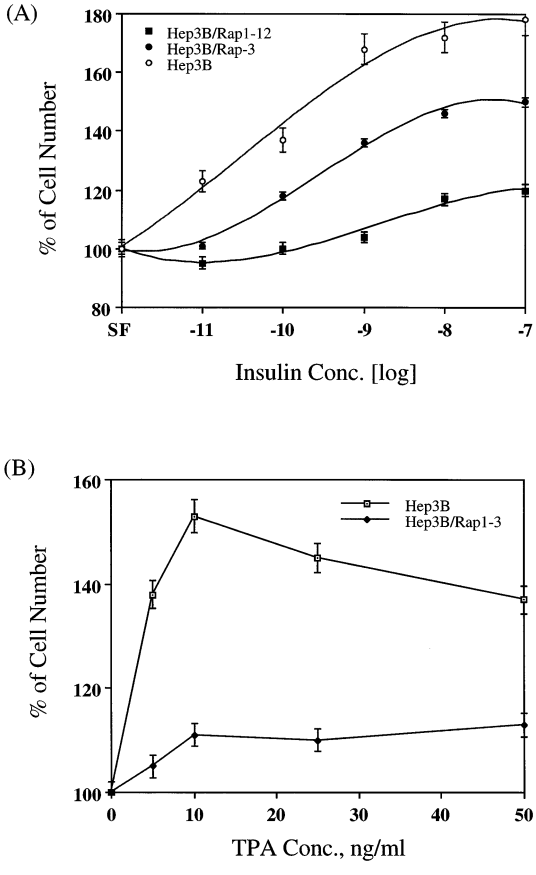

Fig. 3. Mitogenesis induced by (A) insulin or (B) TPA in Hep3B and Hep3B/Rap1 cells. The derivatives of Hep3B cells were seeded in 24-well plates. After 48 h serum starvation, these cells were treated with various concentrations of (A) insulin or (B) TPA for another 48 h. Cell numbers were counted using hemocytometry. Percentage of cell number was determined in the insulin-treated groups, as compared with the serum-free DMEM groups (SF) which were considered as 100% in cell number.

acrylamide gel. However, insulin could not induce a mobility change in Rap1-expressing cells. Hep3B and Rap1 transfectants were treated with different concentrations of TPA. As seen in Fig. 2B, 10 or 50 ng/ml TPA induced the phosphorylation of ERK-1 and ERK-2 proteins after 5 min treatment, however, this effect was reduced in Rap1A cells.

3.4. Expression of Rap1 also inhibits insulin- and TPA-induced cell proliferation

Both insulin and TPA stimulate proliferation of Hep3B cells. To investigate the effect of Rap1 on the mitogenesis of these two agents, Hep3B and Hep3B/Rap1 cells were seeded in 24-well plates. They were treated with various concentrations of either insulin or TPA for 48 h after serum starvation. As shown in Fig. 3A, insulin stimulated proliferation of Hep3B cells in a dose-dependent manner, but the mitogenic effect was diminished in two Rap1 clones. Rap1-12 clone, which expressed a higher level of rap1 mRNA than Rap1-3 clone, has more dramatic reduction in the mitogenic response. The reduction of TPA-induced cell proliferation was also observed in Rap1 transfectants, 20 ng/ml of TPA induced a 50% increase in Hep3B cells after 48 h treatment, while in Rap1-3 clone, the mitogenic effect was diminished to 10% (Fig. 3B).

4. Discussion

Loss of contact inhibition is a parameter of oncogene-induced transformation in cultured cells, and this phenomenon can be measured from the increase of saturation density. We found that expression of Rap1 reduced the saturation density of Hep3B/Rap1-3 and Rap1-12 by 40% compared to parental Hep3B cells. This decrease provided evidence to support a reversion-inductive activity of Rap1 in transformed cells. Furthermore, expression of Rap1 completely suppressed the tumorigenicity of parental Hep3B cells. Caamano et al. have shown that overexpression of rap1 gene partially suppresses the tumorigenicity of a lung cancer cell line Calu-6 [32]. In polyomavirus middle T antigen-transformed Rat-2 cells, Rap1 also induces reversion of transformation [30]. However, the mechanism of this anti-transforming effect remains unclear. Constitutively activated MEK induces transformation of NIH3T3 cells [35]. These observations have indicated that activation of the Raf/MEK/ERKs pathway may be sufficient to trigger transformation in certain cells. If signalings through the ERK pathway were involved in maintaining the transformation state, then blockage of Ras/ERKs activation could be useful in inhibiting the transformed phenotype. Chen et al. have demonstrated that addition of cAMP or expression of a mutant-activated $G_{\alpha s}$ (Q227L) inhibited the tumorigenesis of MCF-7 human breast cancer cells and showed that the ERK signaling pathway may be involved in regulation of transformed phenotypes [36]. An oncogenic effect of Rap1b has been reported in Swiss 3T3 cells, in which Rap1 increases DNA synthesis and cAMP exerts a stimulatory effect on cell growth [33]. It is not surprising that overexpression of Rap1b exhibits mitogenic and oncogenic phenomena in a cell line where cAMP is known to be a positive regulator of cell growth. Interestingly, the mitogenic action of Rap1b is only revealed in the presence of other growth factors, i.e. insulin/insulin-like growth factor 1, which activates the Ras/ERK pathway. This synergistic behavior implies a collaboration between Ras-dependent and cAMP-dependent signaling events. It would be interesting to verify the activation of ERKs in the Rap1b-expressing cells. In this paper, we showed for the first time that Rap1 completely abolishes tumorigenicity of human hepatoma cells in nude mice and the concomitance between tumorigenic inhibition and ERK activation. It has long been known that cAMP inhibits cell proliferation in certain cells, while stimulating in others. Rap1 is activated by signals that raise the intracellular level of cAMP [37,38], suggesting that Rap1 may be intimately linked with some of the cAMP biological effects. It is very likely that introduction of Rap1 may amplify the 'basal' cAMP signal in Hep3B cells, which may inhibit ERK activation and cause the reversion of transformed phenotype.

The physiological role of Rap1 in regulating intracellular signaling appears to be enigmatic and can be either positive or negative dependent upon the cell context. Expression of a constitutively active Rap1 (RapV12) inhibited the ERK activation induced by LPA and EGF in Rat-1 fibroblasts [7]. These results implied that Rap1 activation serves to modulate Ras signaling. However, Zwartkruis et al. have shown that EGF activated Rap1 in Rat-1, and this activation did not affect ERK-2 activation in Rat-1 cells [16]. These data suggested that Rap1 can be activated by growth factors, and this activation is not necessarily related to the Ras/Raf/MEK/ERK pathway. These paradoxical data may be explained by the possibility that activation of endogenous Rap1 does not interfere with growth factors-induced Raf-1/MEK/ERK activation, because it is unlikely that growth factors activate Rap1 to repress Ras effector signaling. However, overexpression of active Rap1 may interfere in Ras signaling, presumably by trapping Raf-1 in an inactive complex. Nevertheless, Okada et al. have shown that insulin induced a time-dependent decrease in the amount of GTP bound to endogenous Rap1 in CHO cells. This inactivation of Rap1 was associated with an insulin-stimulated decrease in the amount of Rap1-Raf-1 complex. Concomitant with the inactivation of Rap1 and decrease in Rap-Raf-1 binding, a rapid insulin-stimulated dissociation of the CrkII-C3G complex was observed. Activation of insulin receptor kinase may induce the tyrosine phosphorylation of CrkII-C3G complex. The uncoupling of CrkII from C3G allowed conversion of Rap1 from the active GTP-bound conformational state to the inactive GDP form. The inactivation of Rap1 released the bound Raf-1, which can then associate with GTP-bound Ras, resulting in the activation of the Raf-1/MEK/ERK cascade [27]. These results suggest that endogenous Rap1 antagonizes Ras function by preventing Raf-1 coupling with Ras, and this antagonizing effect can be regulated by insulin. In our study, we showed that expression of a wild-type Rap1 inhibited insulin-induced ERK activation and cell proliferation. Inhibition of the Ras/Raf/MEK/ERK pathway blocked insulin-induced cell proliferation in Hep3B cells (Yea-Lih Lin, unpublished data). This phenomenon implies that wild-type Rap1 can function as an antagonist to Ras in Hep3B cells.

With respect to TPA, the involvement of Rap1 in ERK activation or inactivation remains unclear. In Rat-1 fibroblasts, TPA activates Rap1 and this activation does not contribute or affect ERK-2 activation, while expression of active Rap1 blocks the activation of c-fos promoter by K-Ras or TPA, but not by Raf-1 in transient transfected NIH3T3 cells [39]. Here, we show for the first time that expression of Rap1 inhibits TPA-induced ERK activation. We have found that

TPA stimulates MEK/ERKs and cell proliferation through a Ras-dependent pathway. These results further suggest that Rap1 interferes with Ras effects in terms of ERKs activation and cell proliferation in Hep3B cells.

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