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Interdependent action of RalGEF and Erk in Ras-induced primitive endoderm differentiation of F9 embryonal carcinoma cells

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Previous work by us and others has implicated a role for Ral guanine exchange factors (RalGEFs) in Ras-induced cell growth and oncogenic transformation. Here we show for the first time that RalGEFs are involved in Ras-induced differentiation as well. Expression of oncogenic Ras in F9 embryonal carcinoma (EC) cells is known to induce differentiation to a primitive endoderm (PrE)-like phenotype, but the downstream signal transduction mechanisms involved are unclear. We found that PrE differentiation is induced by the Ras effector domain mutants, RasV12G37 and RasV12E38, but not by RasV12C40. Accordingly, expression of constitutively active forms of RalGEF (Rlf-CAAX) or Raf1 (Raf-CAAX) is sufficient to induce differentiation. Inhibition of RalGEF activity by expression of dominant negative Ral completely abolishes Rlf-CAAX- and RasV12G37-induced differentiation, while it reduces differentiation by RasV12 and Raf-CAAX. Finally, while Rlf-CAAX does not increase Erk activity, inhibition of MEK blocks both Ras- as well as Rlf-CAAX-induced differentiation, suggesting that RalGEFs induce PrE differentiation in a manner depending on basal MEK or Erk activity. Based on these results we conclude that Ras induces PrE differentiation of F9 EC cells via an interplay of Erk- and RalGEF-mediated pathways.

Keywords: Ras; RalGEF; Erk; F9 embryonal carcinoma; primitive endoderm

Introduction

The recent finding that certain effector domain mutants of oncogenic Ras discriminate between various downstream pathways has given insight into the contribution of each pathway to Ras-mediated effects, such as gene expression and transformation (Bos, 1997). Of the three recognized effectors of Ras, Raf1 binds specifically to RasV12E38, RalGEFs to RasV12G37 and PI3-kinase to RasV12C40 (White *et al*., 1995; Rodriguez-Viciana *et al*., 1997). Expression of these mutants separately induces no or little transformation, while they strongly synergize in transformation upon coexpression (White *et al*., 1995; Rodriguez-Viciana *et al*., 1997). Thus, the different Ras effector pathways may each induce a specific subset of biological responses that build up the complex transformed phenotype.

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We recently found that Ras functions as a regulator of extraembryonic endoderm differentiation *in vitro* (Verheijen *et al*., 1999). The formation of extraembryonic endoderm is the earliest developmental process in the mammalian embryo and takes place in the blastocyst around day 4.5 p.c., when cells of the inner cell mass differentiate into primitive endoderm (PrE). Despite the importance of extraembryonic endoderm in establishing a proper environment for embryonic development, little is known about the signals involved in the differentiation of extraembryonic endoderm. F9 embryonal carcinoma (EC) cells are a suitable *in vitro* model system to study the formation of extraembryonic endoderm (Strickland and Madavi, 1978). We recently found that expression of oncogenic Ras triggers differentiation of F9 EC cells to PrE, while Ras activity inhibits subsequent differentiation towards parietal endoderm (Yamaguchi-Iwai *et al*., 1990; Verheijen *et al*., 1999). Here, we analysed the signalling pathways involved in Ras-mediated PrE differentiation in further detail. We show that Ras induces PrE differentiation via an interdependent action of RalGEFs and Erk.

Results and discussion

To identify the effector(s) of Ras involved in PrE differentiation, we transiently transfected F9 EC cells with β-galactosidase (β-gal) together with effector domain mutants of RasV12. By determining the morphology and the expression of differentiation markers, such as Troma-1 (Kemler *et al*., 1981) and SSEA-1 (Solter and Knowles, 1978) of β-gal expressing cells, the differentiation state of the transfected cells was investigated, as described before (Verheijen *et al*., 1999). When F9 EC cells were transfected with control plasmids, they remained undifferentiated, as reflected in their morphology, the absence of the differentiation marker Troma-1 (Figure 1), and the presence of the stem cell marker SSEA-1 (not shown). However, when cells were transfected with oncogenic RasV12, approximately 80% of the transfected cells had differentiated to a PrE-like phenotype, based on the enlarged and flattened morphology, the expression of Troma-1 (Figure 1) and the disappearance of SSEA-1 (not shown). Transfection of F9 EC cells with RasV12G37 or RasV12E38 strongly induces PrE differentiation, in contrast to transfection with RasV12C40 (Figure 1b). The ability of RasV12G37 and RasV12E38 to separately mimic RasV12-induced differentiation of

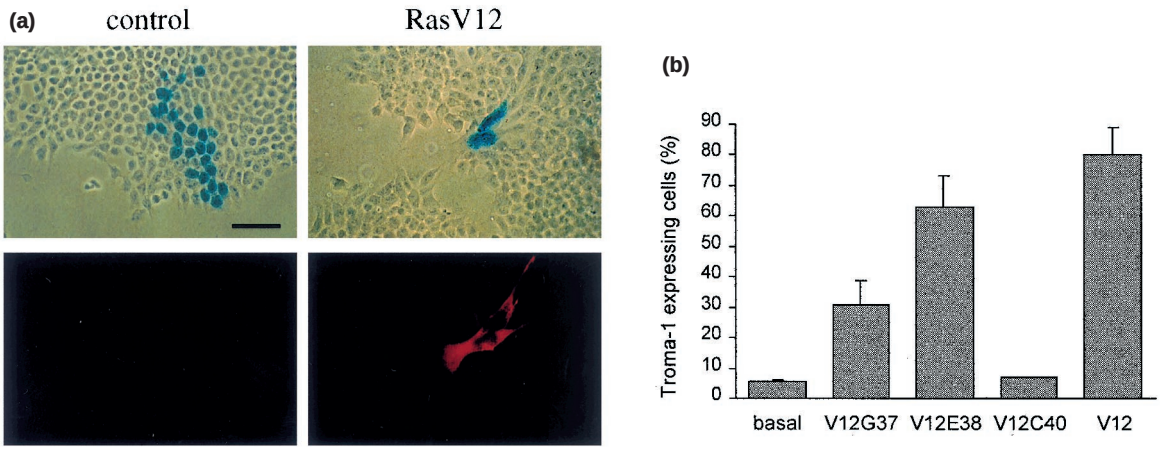

Figure 1 The Ras domain effector mutants RasV12G37 and RasV12E38 induce PrE differentiation of F9 embryonal carcinoma cells. F9 EC cells were transiently transfected either with β-gal or β-gal and either RasV12, RasV12G37, RasV12E38 or RasV12C40, as depicted. Cells were stained for Troma-1 as shown in (**a**) for cells expressing either β-gal (control) or β-gal and RasV12. Bar, 50 μm. (**b**) Percentage of β-gal expressing cells positive for Troma-1. Expression of Troma-1 correlated with the appearance of a PrE-like morphology and reduction in SSEA-1 expression (not shown). Data represents the mean $\pm$ s.d. of at least three independent experiments

F9 EC cells is in marked contrast to their effect on oncogenic transformation in which the single effector domain mutants do not mimic the effect of RasV12 (reviewed in Bos, 1997).

Since RasV12C40 selectively binds and activates the Ras effector PI-3 kinase (Rodriguez-Viciana *et al*., 1997), its inability to induce differentiation indicates that PI3-kinase and downstream targets like PKB and Rac are not directly responsible for differentiation induced by Ras. The differentiating potential of RasV12G37, which binds RalGEFs (Rodriguez-Viciana *et al*., 1997), suggests a role for Ral and RalGEFs in Ras-mediated PrE differentiation. Accordingly, transfection of F9 EC cells with Rlf-CAAX, a constitutively active RalGEF (Wolthuis *et al*., 1997), induced PrE differentiation in contrast to transfection of the catalytically inactive form Rlf-ΔCAT-CAAX (Figure 2a). Importantly, a constitutive GTP-bound, and therefore putative active form of Ral, RalV23 (Wolthuis *et al*., 1997), did not induce differentiation. This is in agreement with our recent observations that RalV23 could not mimic Rlf-CAAX in activation of the c-*fos* promoter (Wolthuis *et al*., 1997), and suggests either that Ral is not involved in Rlf-induced PrE differentiation or that RalV23 does not mimic endogenously activated Ral as previously discussed (Bos, 1997). RasV12E38, a mutant which couples to Raf1 (Rodriguez-Viciana *et al*., 1997), also induces differentiation of F9 cells, suggesting that Raf1 is involved in differentiation by Ras as well. This is in agreement with our previous findings that constitutively active Raf1 or MEK is sufficient to trigger PrE differentiation (Verheijen *et al*., 1999). Rlf-CAAX and a suboptimal amount of Raf-CAAX cooperate in the induction of PrE differentiation (Figure 2b). Thus, Ras induces differentiation via both Raf1 as well as RalGEF mediated pathways.

To determine the importance of the RalGEF/Ral pathway for differentiation by Ras and Raf1, we first transfected F9 cells with a dominant negative form of Ral, RalN28, thereby blocking the action of RalGEFs (Miller *et al*., 1997; Wolthuis *et al*., 1997). Accordingly, RalN28 completely abolished Rlf-CAAX-induced PrE differentiation (Figure 2c), while it did not inhibit differentiation induced by retinoic acid, an *in vitro* inducer of PrE differentiation (Strickland and Madavi, 1978). Importantly, RalN28 completely abolished differentiation by RasV12G37, indicating that differentiation by RasV12G37 is indeed mediated by RalGEFs. PrE differentiation by RasV12, RasV12E38 or Raf-CAAX was partly inhibited by RalN28, suggesting that RalGEF contributes to Ras- and Raf1-induced PrE differentiation, but is not strictly required.

Previous work has shown that the Raf1/Erk and the RalGEF/Ral cascades are separate pathways, for instance, RasV12G37, RalGDS or Rlf-CAAX do not activate Erk (Wolthuis *et al*., 1997; White *et al*., 1996; Okazaki *et al*., 1997) and induction of gene expression by Rlf-CAAX occurs in an Erk-independent manner (Wolthuis *et al*., 1997). The role of Erk in Rlf-CAAX-induced PrE differentiation was investigated by either transfection of an interfering mutant of MEK, MEK-A222 (Pagès *et al*., 1994), or by incubation with the MEK inhibitor PD98059 (Dudley *et al*., 1995). Both MEK-A222 as well as PD98059 did not induce differentiation by itself, nor did they inhibit RA-induced differentiation. However, PD98059 completely inhibited RasV12-induced differentiation (Figure 3a). Transfection with MEK-A222 did not completely abolish RasV12-induced differentiation, probably due to the inability of MEK-A222 to completely inhibit RasV12-induced Erk activation (not shown). Surprisingly, inhibition of Erk activation by either PD98059 or MEK-A222 completely abolished differentiation induced by both Rlf-CAAX (Figure 3a) as well as RasV12G37 (not shown). This indicates that Erk is involved in RalGEF-mediated PrE differentiation. However, Rlf-CAAX did not activate Erk (Figure 3b). Furthermore, Raf-CAAX at a concentration that induces comparable differentiation as Rlf-CAAX (Figure 3c), strongly activates Erk. Together with our observation that prolonged incubation with PD98059 reduced the basal level of Erk activity to approximately 40% of the control (Figure 3b), this suggests that Rlf

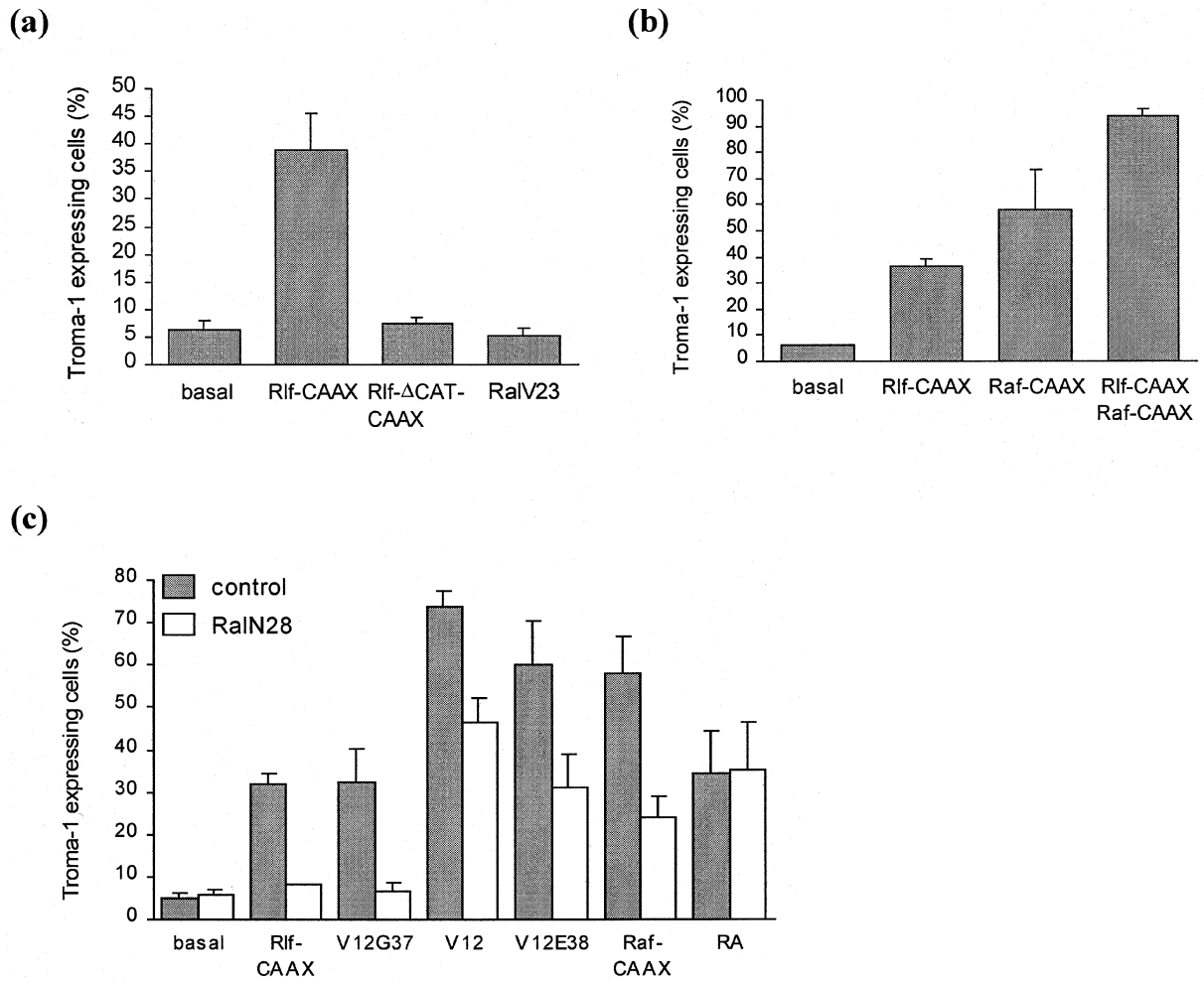

Figure 2 Role of RalGEFs in Ras-induced PrE differentiation (**a**) Rlf-CAAX induces PrE differentiation. F9 EC cells were transfected either with β-gal (basal) or with β-gal and either Rlf-CAAX, inactive Rlf-ΔCAT-CAAX orRalV23. (**b**) Additive effect of Rlf-CAAX and Raf-CAAX on PrE differentiation. F9 EC cells were transfected either with β-gal (basal) or with β-gal and either Rlf-CAAX, Raf-CAAX (0.1 μg) or both, as depicted. (**c**) F9 EC cells were transfected either with β-gal (basal) or with β-gal, RalN28 and depicted constructs, and left untreated or treated for 2 days with 1 μM RA as indicated. For further information, see legend to Figure 1

requires basal MEK or Erk activity to trigger differentiation.

The identity of the factor(s) inducing PrE differentiation in the early mouse embryo is still unknown. RA is a strong inducer of F9 EC cells *in vitro* and induces activation of Ras (Verheijen *et al*., 1999) and Ral (not shown) in these cells. However, inhibition of Erk or RalGEF did not interfere with RA-induced PrE differentiation nor did transfection with dominant negative Ras, RasAsn17 (data not shown). This suggests that RA can induce PrE differentiation via Ras-independent pathways. Our observation that both the Ras/Erk pathway as well as the Ras/RalGEF pathway have the capacity to induce PrE differentiation *in vitro*, suggests that these signalling cascades play a major role in PrE differentiation *in vivo*. Interestingly, Cheng *et al*. (1998) recently showed by introducing a null mutation into the mouse Grb2 gene, that Grb2-Ras signalling is required for endoderm differentiation *in vivo*. Based on our results, we conclude that Ras induces endoderm differentiation (at least *in vitro*) via an interplay of two pathways: a RalGEF mediated pathway which requires basal MEK or Erk activity to induce differentiation, and the Raf1/Erk cascade which requires RalGEF activity for optimal induction of differentiation (see Figure 4). The downstream components involved in RalGEF-induced PrE differentiation are unclear. A possible target might be c-*jun*, since ectopic expression of c-*jun* in F9 EC cells is sufficient for differentiation to PrE and RalGEFs might in principle be able to regulate JNK activity via the putative Ral effector RalBP (Feig *et al*., 1996). Leppä *et al*. (1998), recently reported that induction of c-*jun* by the Erk pathway is required for JNK-induced neuronal differentiation of PC12 cells. We observed that MEK or Erk activity is required for Rlf-induced PrE differentiation of F9 EC cells. Interestingly, differentiation induced by ectopic expression of c-*jun* could not be inhibited by PD98059 (data not shown). Whether there is a role for Erk-induced c-*jun* expression in RalGEF-mediated PrE differentiation or whether, presently unknown, MEK substrates other than Erk are involved in this differentiation process remains to be determined.

We observed that Raf1-induced differentiation could be partly inhibited by RalN28. Assuming that RalN28 completely abolishes RalGEF activity this indicates that RalGEFs are at least contributing to differentia-

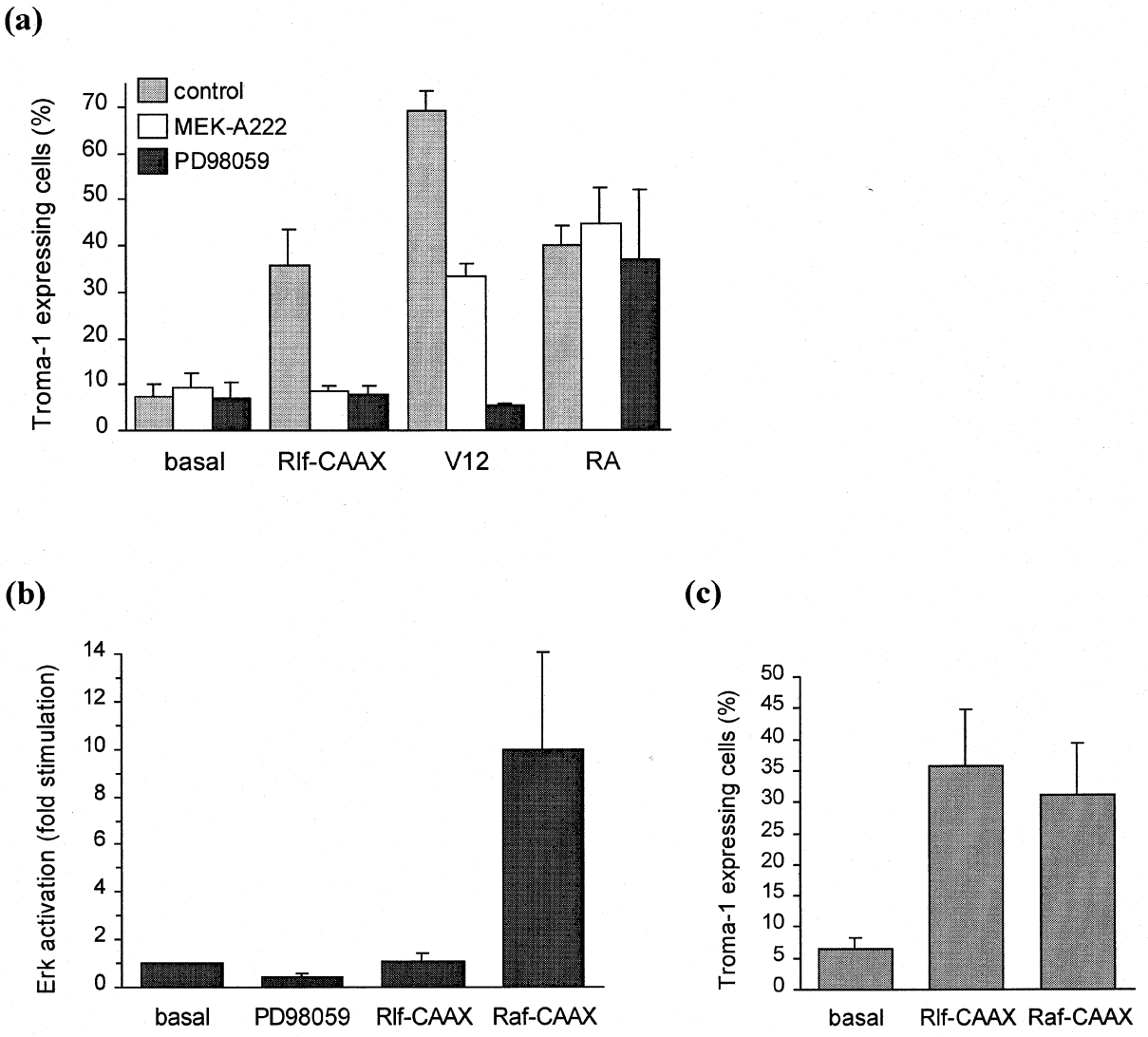

Figure 3 Role of Erk in Ras-induced PrE differentiation. (**a**) F9 EC cells were transfected either with β-gal (basal) or with β-gal and either Rlf-CAAX, RasV12, or MEK-A222, as indicated. Subsequently, the cells were left untreated, treated with 50 μM PD98059 and/or treated with 1 μM RA for 2 days. Shown is the percentage of β-gal expressing cells positive for Troma-1. (**b**) Rlf-CAAX does not activate Erk in F9 cells. F9 cells were transfected with hemagglutinin-tagged Erk (0.6 μg), treated with 50 μM PD98059 for 2 days, or cotransfected with Rlf-CAAX (0.6 μg) or Raf-CAAX (0.06 μg). The activity of HA-Erk was assayed using MBP as a substrate, as previously described (Verheijen and Defize, 1995). Data are shown as fold increase of Erk activity relative to unstimulated controls. (**c**) PrE differentiation of F9 cells which were treated as in (**b**). Show is the percentage of β-gal expressing cells positive for Troma-1. For further information, see legend to Figure 1

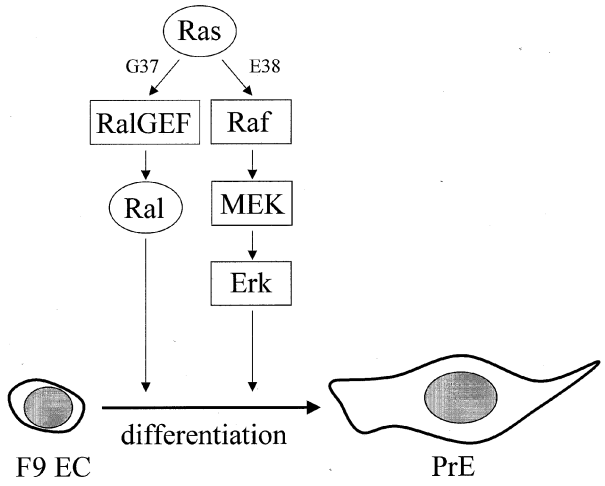

Figure 4 Model for Ras-induced PrE differentiation of F9 embryonal carcinoma cells. (For description see Results and discussion)

tion by Raf1. If RalN28 does not completely inhibit RalGEF activity, differentiation by Raf1 may be fully dependent on RalGEF activity, so that in the presence of RalN28 Raf1 utilizes residual RalGEF activity to trigger differentiation. The requirement of RalGEFs in the action of Raf1 is also observed in Raf1-induced transformation of NIH3T3 fibroblasts, which was reported to be inhibited by RalN28 (Urano *et al*., 1996). The inhibition of Raf1-induced differentiation and transformation by RalN28 might be explained by the requirement of basal RalGEF activity levels for optimal Raf1/Erk-induced differentiation and transformation. It is also possible that RalN28 inhibits RalGEF activity induced by a Raf1-mediated autocrine loop. This is not unprecedented, since Raf1 mediates autocrine activation of JNKs in NIH3T3 cells (McCarthy *et al*., 1997). However, transfection of F9 EC cells with constitutively active mutants of Ras, Raf1 or Rlf does not differentiate the surrounding cells

(e.g. Figure 1a), suggesting that putative secreted factors are not sufficient to trigger differentiation on their own.

Materials and methods

Materials

All-*trans* RA was purchased from Sigma; PD98059 from Calbiochem, and [γ-^{32}P]ATP from Amersham ('s Hertogenbosch, The Netherlands). Monoclonal antibodies against SSEA-1 were a gift from D Solter (Wistar Institute, Philadelphia, PA, USA), monoclonal antibodies against TROMA-1 were a gift from R Kemler (Max-Planck-Institute for Immunobiology, Freiburg, Germany). The RasV12 and respective effector domain mutants were kindly provided by J Downward (Rodriguez-Viciana *et al*., 1997). Raf-CAAX was a gift from C Marshall (Leevers *et al*., 1994), MEK-A222 was a gift from J Pouysségur (Pagès *et al*., 1994). The expression plasmids PMT2-HA-Rlf-CAAX, PMT2-HA-Rlf-ΔCAT-CAAX, PMT2-HA-RalN28 and PMT2-HA-RalV23 (Wolthuis *et al*., 1997), as well as pSV2LacZ and pSG5-HA-p42MAP (Verheijen *et al*., 1999), have been described elsewhere.

Cell culture, induction of differentiation and transfection

F9 EC cells were obtained from the ATCC and cultured as described (Verheijen *et al*., 1999). Transfections were performed usinig lipofectamin (Verheijen *et al*., 1999) with 0.3 μg pSV2-lacZ and 0.3 μg of indicated constructs, unless noted otherwise.

Activation of Erk

For determination of Erk activity epitope-tagged p42 HA-MAP kinase was immunoprecipitated with protein A-Sepharose beads coupled to monoclonal antibody 12CA5 (Verheijen and Defize, 1995). After the kinase reaction with MBP as a substrate, the reaction mix was subjected to SDS-polyacrylamide gel electrophoresis. Phosphorylation of MBP was measured using a PhosphorImager and ImageQuant software (Molecular Dynamics).

β-galactosidase staining and immunofluorescence

Cells were washed twice with PBS, fixed with 2% paraformaldehyde, incubated with an X-Gal staining solution (PBS with 0.8 mg/ml X-Gal (Gibco – BRL), 1 mg/ml $K_3FE(CN)_6$, 1.3 mg/ml $K_4FE(CN)_6$), and subsequently incubated with 20 mM NH_4Cl for 30 min. Subsequent antibodies incubations for detection of SSEA-1 and Troma-1 were performed as described previously (Verheijen *et al*., 1999).

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