The TGF-β co-receptor endoglin modulates the expression and transforming potential of H-Ras

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Abstract

Endoglin is a co-receptor for transforming growth factor-β (TGF-β) that acts as a suppressor of malignancy during mouse skin carcinogenesis. Because in this model system H-Ras activation drives tumor initiation and progression, we have assessed the effects of endoglin on the expression of H-Ras in transformed keratinocytes. We found that TGF-β1 increases the expression of H-Ras at both mRNA and protein levels. The TGF-β1-induced *H-Ras* promoter transactivation was Smad4-independent, but mediated by the activation of the TGF-β type I receptor ALK5 and the Ras-mitogen-activated protein kinase (MAPK) pathway. Endoglin attenuated stimulation by TGF-β1 of both MAPK signalling activity and H-Ras gene expression. Moreover, endoglin inhibited the Ras/MAPK pathway in transformed epidermal cells containing a H-Ras oncogene, as evidenced by the levels of Ras-GTP, phospho-MEK and phospho-ERK as well as the expression of c-fos, a MAPK downstream target gene. Interestingly, in spindle carcinoma cells, that have a hyperactivated Ras/MAPK pathway, endoglin inhibited ERK phosphorylation without affecting MEK or Ras activity. The mechanism for this effect is unknown but strongly depends on the endoglin extracellular domain. Because the MAPK pathway is a downstream mediator of the transforming potential of Ras, the effect of endoglin on the oncogenic function of H-Ras was assessed. Endoglin inhibited the transforming capacity of H-Ras(Q61K) and H-Ras(G12V) oncogenes in a NIH3T3 focus formation assay. The ability to interfere with the expression and oncogenic potential of H-Ras provides a new face of the suppressor role exhibited by endoglin in H-Ras-driven carcinogenesis.

Introduction

Endoglin (CD105) is an auxiliary receptor for transforming growth factor-β (TGF-β) that is highly expressed in endothelial cells of the tumor vasculature and at much lower levels in tumor cells [1]. So far, most of the studies on endoglin have focused on endothelial cells, as it plays an important role in vascular development, remodelling and homeostasis [2]. In addition to the pro-angiogenic role of endoglin, there is evidence supporting its involvement in cancer progression by its direct function on the tumor cells themselves [3]. Thus, in cultured human prostate cancer cells, loss of endoglin expression enhances cell migration and invasiveness [4,5]. Also, we have shown that endoglin plays an invasion suppressor role during mouse skin chemical carcinogenesis [6]. In this model, successive applications of an initiator and a promoter of carcinogenesis results in the outgrowth of benign papillomas that progress to malignant squamous cell carcinomas (SCC), and subsequently to highly undifferentiated spindle cell carcinomas (SpCC). Tumors are initiated by an activating mutation in the *H-Ras* gene [7], and increases in the copy number and/or expression of mutated *H-Ras* are associated with progression to carcinomas [7-9]. Although TGF-β1 acts as a tumor suppressor at early stages of carcinogenesis, it also promotes malignancy by inducing an epithelial-mesenchymal transition (EMT) associated with progression to SpCCs [10,11]. While the current evidence suggests that endoglin acts in endothelial cells as a molecular switch by balancing the signalling of TGF-β through the type I receptors ALK1 and ALK5 [12], endoglin acts in epidermal cells attenuating TGF-β/ALK5 signaling [6]. Interestingly, membrane-bound endoglin is inactivated by shedding during progression from SCC to SpCC allowing an increased TGF-β signalling and malignant progression [6]. Thus, both transgenic mice with targeted expression of TGF-β1 to the epidermis [11] and endoglin heterozygous ($Eng^{+/-}$) mice [13,14] show a vast acceleration of malignant progression and enhanced development of SpCCs after chemical carcinogenesis.

Since in epidermal carcinogenesis TGF-β1 promotes malignant progression and this process is associated with increased H-Ras levels, we asked whether TGF-β1 regulates *H-Ras* gene expression and whether endoglin modulates this TGF-β1 effect in transformed keratinocytes.

Materials and Methods

Cell culture conditions and treatments.

The epidermal mouse cell lines PDV and MCA-3D [15] were cultured in Ham´s F-12 medium supplemented with amino acids and vitamins (Gibco, Rockville MD). The cell lines B9 and CarC were derived from mouse skin chemically-induced carcinomas [8,16]. The mouse NIH 3T3 cell line was obtained from ATCC (Manassas, VA, USA) and cultured in DMEM medium. Clones of PDV cells stably transfected with an expression vector encoding shRNA that silences mouse endoglin (shEng2 and shEng4) as well as pooled PDV cells stably transfected with an endoglin-unrelated sequence (shCont) were used [6]. Stable transfectant clones of the CarC cell line expressing human L-endoglin (L-Eng1 and L-Eng2) or transfected with an empty vector (pcDNA3) have been described [6]. CarC cells retrovirally infected with the viral plasmids pWZL (empty vector), pWZL-L-Eng FL (full length endoglin), pWZL-L-Eng ΔCt (lacking the cytoplasmic domain) and pWZL-L-Eng ΔPDZ (lacking the PDZ domain) were generated as described [17]. HA-tagged pWZL-L-Eng ΔEC (lacking the extracellular domain) was generated using HA-TMCT-Endo construct previously described [18]. Retrovirally transduced CarC cells were cultured in DMEM containing 400 micrograms/ml hygromycin B (Invitrogen). All culture media were supplemented with 10% foetal bovine serum (FBS; Invitrogen) and 80 μg/ml Gentamycin. Cells were maintained at 37ºC in a 5% CO_2 humidified atmosphere. For TGF-β1 treatment, cells were incubated with human recombinant TGF-β1 (R&D Systems) at a final concentration of 10 ng/ml for the

indicated times. Treatments with the chemical inhibitor of MEK UO125 (Calbiochem) were carried out at a concentration of 5 μM in 0.1% dimethyl sulfoxide (DMSO).

Plasmids, expression vectors, transfections and reporter assays.

The expression vector encoding HA-tagged full length endoglin in pDisplay vector (Invitrogen) has been reported [18]. Expression vectors encoding HA-tagged constitutively active ALK5 (T204D) and kinase deficient ALK5 (K232R), were provided by Dr. Liliana Attisano (University of Toronto, Canada). Plasmid pMEXneo encoding a dominant-negative H-Ras mutant (RasN17) was described by Feig and Cooper [19]. Plasmids encoding dominant negative versions of Raf1 and HA-tagged MEK1 were provided by Dr. S. Lavandero (University of Chile, Chile). Expression vectors encoding human Smad2 and Smad3 have been described [20]. A dominant negative Smad4 in pCMV was provided by Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, USA). For short hairpin RNA (shRNA)-mediated knockdown of endoglin, double-stranded oligonucleotides encoding shRNA, that silence the mouse endoglin gene, and inserted into the pSUPER-GFP vector [6] was used. The pSUPER-GFP vector containing an endoglin-unrelated sequence was used as a negative control. The retroviral vector pWZL and the endoglin derived constructs L-Eng FL, L-Eng ΔCt, L-Eng ΔPDZ, and HA-L-Eng ΔEC described above are based on the pBABE retrovirus, which uses an internal ribosomal entry site to drive hygromycin resistance [21]. Amplification of the viruses was carried out in the packaging cell line 293-T.

The reporter vector pGL2-Ha-Ras containing the rat Ha-Ras proximal promoter region -1500/-80 (Supplementary Fig. 1) was kindly provided by Dr. Mikheev (Fred Hutchinson Cancer Research Center, University of Washington, USA) [22]. The reporters (SRE)-luc, containing a serum-responsive element, and cyclin A-luc driven by the promoter of cyclin A, were provided by Dr. A. Corbí (Centro de Investigaciones Biológicas, Madrid, Spain). The reporter pCMV-Gal4-Elk1 (1-147) containing an Ets-like transcription factor (ELK) 1

activation domain that confers MAPK specificity was purchased from Stratagene (La Jolla, CA). The pcFos-luc reporter, whose luciferase expression is driven by c-fos promoter [23], was constructed in pGL3 vector (Promega). The Smad3-responsive promoter construct $(CAGA)_{12}$-Luc was provided by Dr. Peter ten Dijke [24].

Cells were transfected with Superfect (Qiagen) according to manufacturer´s instructions. Reporter assays were performed as described [25] in the presence or absence of TGF-β1, as indicated. Basically, cells were pretreated with TGF-β1, transfected with the reporter vector and then incubated with TGF-β1. One day after transfection, luciferase activity was determined. All transfections were performed in combination with pSV40-LacZ encoding beta-galactosidase, as a control for transfection efficiency and normalization. Luciferase activity was determined by using a commercial kit (Promega), whereas beta-galactosidase activity was determined using the Galacto-Ligth Kit (Applied Biosystem,USA). Light emission was measured in a TD20/20 luminometer (Promega, Madison, WI). The experiments were performed in triplicates at least three times and representative experiments are shown in the figures.

Antibodies, pull-down and Immunoblotting assays

The broad specific anti-Ras monoclonal antibody was purchased from R&D Systems (clone #342404). For immunodetection of endoglin proteins, the mAbs MJ7/18 and P4A4 which recognize epitopes within the extracellular domains of murine and human endoglin, respectively were used [14]. Recombinant proteins with the HA epitope (endoglin, ALK5, and MEK1,2-DN) were detected with 12CA5 mAb (Boehringer Mannheim). Recombinant Smad proteins with the Flag epitope were detected with anti-Flag M2 mAb (Sigma). The antibodies against phospho ERK1,2, ERK1,2, phospho-MEK1,2 and MEK1,2 were purchased from Cell Signaling. Anti-c-Fos (sc-52) and anti-Raf1 (C12; sc-133) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-α-tubulin

antibody was purchased from Sigma. The secondary antibody coupled to horseradish peroxidase (HPO) was purchased from DAKO (Barcelona, Spain). To analyze the level of active Ras in cell lysates, the ability of Ras-GTP to bind the Ras-binding domain of Raf-1 (RBD) was used in pull-down assays as previously described [26] and using a commercial kit (Cytoskeleton, Denver, CO, US). For immunoblotting experiments, proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes as described [25]. Membranes were blocked with 4% milk (diluted in Tris-buffered saline and 0.5% Tween 20), and incubated with the appropriate antibody at 4°C overnight. The targeted proteins were detected by enhanced chemiluminescence as indicated by the manufacturer (Pierce).

Semiquantitative RT-PCR analysis

Total RNA was extracted from keratinocytes with Trizol (Gibco BRL, Grand Island, NY). To evaluate the transcript levels of Ha-Ras and endoglin, total RNA was subjected to RT-PCR using Ha-Ras [9] and endoglin [27] specific primers. The cycling parameters for the PCR reactions were as follows: 10 min at 94ºC, 30 cycles of 94°C for 15 sec, 55°C for 15 sec and 72°C for 90 sec, followed by 10 min extension at 72°C. The PCR of the housekeeping gene GADPH was also performed to ensure equivalency in all samples.

Foci formation assay

Foci formation assays were performed in mouse NIH3T3 fibroblasts. For transformation with H-RasQ61K, cells were transfected using Superfect reagent (Qiagen) with plasmids bearing human L-Endoglin in pDisplay [18], si-SuperGFP-Endoglin [6] and H-RasQ61K pAL8-Ras [28]. Next day, each plate was splitted in three, and incubated with DMEM containing 5% DCS (Donor calf serum, Invitrogen). For transformation with H-Ras-G12V, cells were infected with retrovirus bearing H-Ras-G12V and endoglin. Retroviruses were obtained using the Linx packaging cell line. NIH 3T3 infected plates were centrifuged at 1,500 rpm for one hour at room temperature and then incubated at 32ºC for 6-8 hours. Then, plates were

transferred to 37ºC. Both, H-RasQ61K and H-Ras-G12V transduced cells were incubated for around two weeks changing media every two days. Then, cells were fixed and stained with crystal violet, and foci were counted.

Statistics

Data are given as means ±SD from at least three independent experiments. When necessary, statistical significance was evaluated using the Students' t-test. Differences were considered to be significant at a value of $p<0.05$.

Results

TGF-β1 stimulates H-Ras promoter activity in premalignant and malignant keratinocytes

PDV transformed keratinocytes express both normal and oncogenic versions of the *H-Ras* gene and produce well differentiated SCC upon injection in mice [15]. Chronic treatment of PDV cells with TGF-β1 promotes an EMT and the conversion to a spindle cell tumor phenotype [10]. Since progression from SCC to SpCC is associated with increased levels of activated H-Ras [8,29], we analyzed in PDV cells the effect on *H-Ras* gene expression of a chronic treatment with TGF-β1. As shown in Figure 1A, a series of sequential increases in H-Ras protein levels were observed in PDV cells incubated with TGF-β1, reaching a plateau after ~3 days of treatment. In agreement with these data, a similar kinetics of H-Ras stimulation was obtained by analyzing the mRNA levels by semiquantitative RT-PCR and the activity of an exogenous H-Ras promoter [22] (Fig. 1B). This stimulatory effect on H-Ras was also observed in other epidermal cell lines, such as MCA3D premalignant keratinocytes and B9 squamous carcinoma cells (Fig. 1C).

TGF-β1 stimulation of the H-Ras promoter is mediated by the ALK5/Ras/MAPK signalling pathway

TGF-β signals through Smad-dependent and Smad-independent pathways [30]. To find out the signalling pathway that mediates TGF-β1-induced transactivation of the H-Ras promoter, PDV cells were co-transfected with the H-Ras promoter and plasmids encoding different normal or mutant components of the Smad and Ras/MAPK pathways. Constitutively active TGF-β type I receptor ALK5 (ALK5-T204D) enhanced basal H-Ras promoter activity to the same level as treatment with TGF-β, while kinase dead ALK5 (ALK5-K232R) blocked the TGF-β1-induced H-Ras promoter transactivation (Fig. 1D), indicating that activation of ALK5 is necessary for TGF-β1 stimulation of the H-Ras promoter. The behavior of the H-Ras promoter when overexpressing Smad2/Smad3 or a dominant negative Smad4 was similar to that of the control empty vector (Fig. 1D), suggesting the involvement of an Smad4-independent pathway. In contrast, stimulation of the H-Ras promoter by TGF-β1 depends on a fully active Ras/MAPK pathway since expression of a dominant-negative form of Ras, Raf or MEK1,2, or treatment with UO125, a pharmacological inhibitor of MEK1,2, completely blocked the TGF-β1 effect (Fig. 1E).

Endoglin interferes with TGF-β1-induced Ras/MAPK activation and TGF-β1-stimulated H-Ras gene expression

Because stimulation of *H-Ras* expression by TGF-β1 occurs via ALK5, and endoglin negatively modulates TGF-β1/ALK5 signalling in epidermal cells [6], we reasoned that endoglin should modulate this TGF-β1 effect. To test this hypothesis, increasing amounts of exogenous endoglin were transfected in PDV cells and the activity of *H-Ras* promoter determined before and after treatment with TGF-β1 (Fig. 2A). As expected, PDV cells expressing low (endogenous) levels of endoglin [13] showed a 2.8-fold increase of the *H-Ras* promoter activity upon treatment with TGF-β1. This stimulatory effect decreased in a dose-dependent fashion as increasing levels of human endoglin were expressed. These results suggest that endoglin interferes with TGF-β1-dependent stimulation of the *H-Ras* promoter.

To assess whether the stimulation of H-Ras mRNA levels by TGF-β1 are regulated by endoglin, a semiquantitative RT-PCR analysis was carried out in control and human endoglin-transfected PDV cells. As shown in Fig. 2B, overexpression of exogenous endoglin blocked the 3-fold stimulation of H-Ras transcript levels observed after treatment with TGF-β1.

Because TGF-β1-dependent *H-Ras* transactivation is mediated by Ras-MAPK signalling activity (Fig. 1E), we investigated whether endoglin was able to modulate this pathway. To this aim, the effect of TGF-β1 on the activity of reporter constructs specific for the MAPK signalling pathway was analyzed in the presence or absence of endoglin. These reporter genes included (SRE)-luc containing a serum-responsive element, pCMV-Gal4-Elk1, comprising an Ets-like transcription factor (ELK1) activation domain, and p-cFos containing the c-Fos promoter. All three reporter genes were activated by TGF-β1 in the absence but not in the presence of exogenous endoglin (Fig. 2C). Interestingly, endoglin also decreased basal activity of these reporter genes, suggesting that the TGF-β co-receptor not only inhibits stimulation of this pathway by TGF-β1 but also inhibits the basal MAPK signalling activity. Alternatively, endoglin inhibition of the basal reporter activity could be explained by an endoglin effect on autocrine TGF-β responses. As a positive control, endoglin abolished the TGF-β1 stimulation of the Smad3-responsive construct (CAGA)12-Luc (Fig. 2C). As a negative control, endoglin did not affect the basal activity nor the TGF-β1-induced inhibition of the p38-dependent and ERK-independent promoter activity of cyclin A, further supporting the specificity of endoglin within the MAPK pathway.

Endoglin inhibits the MAPK pathway in transformed epidermal cells.

We have previously shown in PDV cells that TGF-β1 transiently stimulates extracellular signal-regulated kinase (ERK1,2) phosphorylation and translocation from the cytoplasm to the nucleus, with a maximum at 30 min, whereas the upstream Ras activation was a much earlier event [26]. In order to analyze whether endoglin interferes basal ERK1,2

phosphorylation and/or the kinetic profile of ERK1,2 activation by TGF-β1, we utilized PDV cells in which endoglin expression can be markedly reduced (50-70%) by shRNA interference [6]. Figure 3A shows that specific suppression of endoglin expression associates with increased basal levels of phospho-ERK1,2 (~2-fold) and enhanced c-Fos expression; also, no changes in the total protein levels of ERK1,2 were observed. In addition to an enhanced basal Erk1,2 activation, the endoglin knockdown led to increased TGF-β1-induced Erk1,2 phosphorylation (Fig. 3B). By contrast, overexpression of human endoglin reduced basal and TGF-β1-induced levels of phospho-ERK1,2 in PDV cells (data not shown). Of note, the relative intensity of the two ERK isoforms varied among different experiments, probably due to the particular electrophoretic and exposure conditions used in each case. Next, the upstream components of the MAPK pathway were analyzed. Suppression of endoglin expression in PDV cells increased both basal and TGF-β-estimulated Ras and MEK activities, as evidenced by the levels of active Ras-GTP, phospho-MEK1,2 and phospho-ERK1,2 (Fig. 3C), suggesting that endoglin interferes the upstream components of the Ras/Raf/MEK/ERK pathway. As expected, the TGF-β-dependent stimulation was evident in all the samples (shCont and shEng), as demonstrated by comparative densitometric analysis of the bands (Fig. 3C, histogram on the right). The TGF-β-dependent upregulation of the MAPK components occurred even with Ras-GTP (Fig. 3C, overexposed gel), in spite of the fact that the time point selected for this analysis (15 min) was optimized for ERK activation and it is known that maximal Ras activation occurs at an earlier time. Of note, in PDV cells, due to the H-Ras mutant allele, an enhanced basal activation of Ras, as compared to normal keratinocytes, was observed (data not shown), in agreement with a previous report [26].

The effect of endoglin was also assessed on the highly aggressive spindle carcinoma cell line CarC that expresses undetectable levels of membrane-associated endoglin [6]. CarC contains the same mutated H-Ras oncogene as PDV cells, but, while PDV expresses normal H-Ras

protein, CarC has lost the normal H-Ras allele [15,31] and represents a step further in malignant progression with respect to PDV [32]. As shown in Figure 4A, two different clones of CarC cell transfectants stably expressing endoglin displayed reduced levels of phospho-ERK1,2 (~50%) and c-Fos as compared to mock transfectants, while no significant change was observed in the expression of total ERK1,2 proteins. At variance with PDV cells, endoglin expression did not affect the levels of active Ras (Ras-GTP) or MEK1,2 (phospho-MEK1,2) (Fig. 4A), suggesting that the TGF-β co-receptor directly inhibits ERK1,2 phosphorylation. In order to map the endoglin domain involved in this inhibitory effect, different endoglin truncated constructs were expressed in CarC cells (Fig. 4B). Endoglin constructs L-Eng-ΔCt and L-Eng-ΔPDZ lacking the whole cytoplasmic domain or the PDZ binding motif located at the carboxy-terminus, respectively, diminished the levels of phospho-ERK1,2 and c-fos in a similar fashion as the full length endoglin construct. Interestingly, expression of the construct (HA)L-Eng-ΔEC, lacking the extracellular domain of endoglin, was unable to inhibit the phosphorylation of ERK1,2 and the expression of c-Fos (Fig. 4B), and this effect was not mediated by the HA tag of the construct (data not shown). These results suggest that the extracellular, but not the cytoplasmic domain of endoglin is involved in the inhibition of ERK1,2 phosphorylation.

Endoglin inhibits transformation of NIH3T3 cells by oncogenic H-Ras.

Because endoglin appears to modulate ERK signalling activity and ERK is a downstream mediator of the transforming potential of Ras, we wondered whether endoglin could interfere with H-Ras oncogenic function. To address this, we assessed whether endoglin affects the transforming potential of the H-RasQ61K oncogene, which has normal glutamine 61 mutated to lysine. NIH3T3 fibroblasts express similar amounts of endogenous endoglin as PDV cells [13]. Therefore, besides the parental cells, we used NIH3T3 fibroblasts in which endoglin levels were downregulated by shRNA interference as well as NIH3T3 cells transfected with

the mouse endoglin cDNA to enhance endogenous endoglin levels. As shown in Figure 5, shRNA-mediated downregulation of endoglin increased (~30%) the already robust capacity of H-RasQ61K for cellular transformation [33]. By contrast, endoglin overexpression decreased the H-RasQ61K transforming capacity at a similar extent. In order to asses that this effect of endoglin was not restricted to a particular H-Ras oncogene, we also used H-RasG12V in which normal glutamine 12 was mutated to valine. As shown in the inset of Figure 5, endoglin expression also significantly reduced the transforming capacity of H-RasG12V. These results suggest that in addition to regulate TGF-β-induced H-Ras gene expression and MAPK signalling activity, endoglin modulates the oncogenic potential of H-Ras.

Discussion

TGF-β1 induces H-Ras gene expression in keratinocytes through activation of the Ras/MAPK pathway. In this report we show that chronic exposure of mouse transformed keratinocytes to TGF-β1 upregulates *H-Ras* gene expression concomitantly to stimulation of cell migration/invasiveness and EMT [10,34,35]. This result is in line with a previous study by Xie and colleagues showing that treatment of mouse mammary gland epithelial cells with TGF-β1 for 24 h increased about 2.5-fold *H-Ras* expression, as determined by microarray analyses [36]. The effect of the growth factor on *H-Ras* expression appears to be independent of Smad4 signaling but dependent on Ras/MAPK signaling activity. This conclusion is supported by experiments showing that in PDV cells expression of a Smad4 dominant-negative form did not affect the TGF-β1 dependent activation of the H-Ras promoter, whereas dominant-negative forms of either Ras, Raf, or MEK1,2 as well as a MEK1,2 pharmacological inhibitor abolished the TGF-β effect (Fig. 1D, E). It should be taken into account, however, that Smad4 does not need to be involved in all effects mediated by R-Smads [37,38]. Thus, the effect of TGF-β1 on *H-Ras* expression may be independent of

Smad4, but may require the cooperation of Smad2/3 and the Ras/MAPK pathway. The involvement of MAPK signaling activity is similar to other TGF-β cell responses associated with malignancy, such as the induction of EMT [36] or upregulation of the matrix proteinases urokinase (uPA) and MMP-9 collagenase [39,40]. Altogether, these results point to activation of MAPK as a key signalling event for TGF-β1 to push malignant progression in epidermal carcinogenesis. This dependency on MAPK activity for TGF-β1 to upregulate *H-Ras* expression also suggests that it might be mediated by Ets related and/or Sp1 transcription factors, whose putative binding motifs are widely distributed along the *H-Ras* gene promoter (Supplementary Fig. 1). Indeed, a sequence within the *H-Ras* promoter known as the H-Ras response element (HRE) is known to bind Ets transcription factors, and most of Ets family proteins are major nuclear effectors of the Ras/MAPK signalling pathway [41,42]. In addition, the transcription factor Sp1, which is also activated through MAPK, can bind consensus GC-rich motifs in the *H-Ras* promoter and is required for H-Ras gene expression [43,44]. Accordingly, TGF-β1 activates Sp1 and, in turn, Sp1 transactivates the H-Ras promoter in PDV cells (data not shown), suggesting that at least Sp1 appears to mediate stimulation of *H-Ras* gene expression by TGF-β1 in transformed keratinocytes.

The early changes in ERK activity were analyzed as one of the primary downstream signals in response to TGF-β, which, in turn, may account for the long term effects on H-Ras expression and promoter activity. However, it should be noted that although there is an early response of MAPK activity to TGF-β, in agreement with previous reports [45,46], this peak response occurs in a regular cyclic mode during long term exposure of PDV cells to TGF-β [26]. These results suggest that there may be a coupling between the changes in Erk activity and *H-Ras* expression throughout the whole period of incubation.

Differential regulation of the MAPK pathway by endoglin in PDV versus CarC transformed keratinocytes. Endoglin attenuated the TGF-β1 cell response on *H-Ras* expression (Fig. 2).

This is not surprising since we have shown that endoglin inhibits both basal and TGF-β1 stimulated ALK5-dependent cell responses in PDV cells [6], and, conversely, downregulation of endoglin expression in these transformed keratinocytes leads to increased basal and TGF-β1-stimulated Smad3 [6] and Ras/MAPK (Fig. 3) activities. Furthermore, endoglin was shown to inhibit ERK activity in both PDV and CarC transformed epidermal cell lines (Figs. 3 and 4), but the mechanism involved appears to be different in each cell type. In PDV cells, endoglin inhibited the basal activity of the entire Ras/MEK/ERK pathway, a fact likely dependent on endoglin inhibition of intrinsic ALK5 activity [6]. This is in agreement with reports showing that membrane-associated endoglin physically interacts with type I TGF-β receptors impairing ALK5 activation [18,47] and inhibiting stimulation of Smad3 [48] and ERK1,2 by TGF-β1 [49,50] in different cell types. In CarC cells, on the other hand, endoglin appeared to directly reduce constitutive ERK phosphorylation without affecting the activity of upstream components of the pathway (Fig. 4A).

A schematic model depicting the differences between PDV and CarC cells is shown in Figure 6. The major difference between CarC and PDV cells might be due to the strong constitutive activity of the Ras/MAPK pathway in the former cell line that somehow confers resistance to the endoglin effect at this level. Indeed, CarC cells have a hyperactivated MAPK pathway due to the presence of a mutated H-Ras oncogene and absence of a normal H-Ras allele which increases the dosage of mutated to normal Ras protein expression within the cells [15,31]. Also, CarC spindle cells have downregulated ALK5 and TβRII levels with respect to PDV cells [6]. Nevertheless, CarC cells do respond to TGF-β stimulation of Smad3, although at a lower rate than PDV cells, and endoglin is able to inhibit TGF-β-mediated Smad3 activation in CarC cells [6]. Taken together, these results suggest that blocking endoglin expression would restore normal TGF-β signaling through Ras/ERK/fos/H-Ras in PDV cells, whereas

over-expression of endoglin would block any remaining TGF-β receptor signaling in CarC cells, which would reduce Smad3 signaling.

Involvement of additional cross-talks between the MAPK and the Smad pathways. In order to understand the whole picture, we should take into account the existence of additional cross-talks at different levels between the Ras/MAPK and Smad pathways. Thus, MAPK-mediated phosphorylation appears to have a dual role in Smad2/3 regulation. Mitogens and hyperactive Ras result in ERK-mediated phosphorylation of Smad3 that inhibits its activity [51]. By contrast, ERK-dependent phosphorylation of Smad2 enhances its transcriptional activity [52]. Therefore, through this mechanism, the hyperactivated Ras/ERK pathway may contribute to a reduced TGF-β/Smad3 signalling in CarC cells. Furthermore, Arany and coworkers have found that ERK activation by both TGF-β and oncogenic Ras is reduced in primary mouse embryo fibroblasts null for Smad3 [53]. Interestingly, endoglin expression in CarC cells significantly decreased basal Smad3 activity, as monitored by the $(CAGA)_{12}$-Luc reporter construct, but has no major effect on basal Smad2 activity [6]. These results suggest that the endoglin-mediated reduction of ERK phosphorylation may involve Smad3 inactivation, similarly as in Smad3 knockout cells [53]. Alternatively, this effect might be mediated by the activation of a MAPK phosphatase [54]. Because endoglin is known to be phosphorylated at Ser/Thr residues [18,55,56], it remains to be determined whether endoglin may be a substrate of MEK or may directly interact with MEK or ERK1/2, thus interfering with ERK1/2 phosphorylation by MEK.

Endoglin protein domains involved in ERK inhibition. Endoglin also inhibits the basal phosphorylation of ERK1,2 in myoblasts and endothelial cells that apparently have normal Ras genes [49,50]. The latter authors have suggested that in endothelial cells this effect is mediated by endoglin internalization through interaction of its cytoplasmic domain with β-arrestin. The mechanism by which endoglin inhibits ERK signalling activity in CarC cells is

presently unknown, but it seems to be different from endothelial cells since it depends on the extracellular domain and not on the endodomain (Fig. 4B). Nonetheless, the possible involvement of the transmembrane domain in this inhibitory effect of endoglin, remains to be investigated. In this regard, the localization of TGF-β receptors (and also tyrosine kinase receptors) in lipid rafts is essential for MAPK activation [57 and references therein]. A similar requirement may exist for the inhibitory effect exerted by the TGF-β coreceptor, involving both the extracellular and transmembrane domains of endoglin. Thus, the observed discrepancy between endothelial and epidermal cells could be attributed to the different distribution of endoglin on the membrane microdomains and their distinct routes of internalization.

Endoglin modulates the oncogenic potential of H-Ras. Consistent with the inhibition of ERK1,2 phosphorylation, a relevant finding of this work is the ability of endoglin to protect NIH3T3 fibroblasts from H-Ras oncogenic transformation (Figure 5). Arany and coworkers have shown that Smad3-deficient embryo fibroblasts are safeguarded against viral H-Ras transformation [53]. This observation is in line with our previous results since endoglin inhibits both basal and TGF-β-stimulated Smad3 signalling in epidermal cells [6]. Whether the protector effect exhibited by endoglin is mediated by reduced Ras farnesylation and suppression of the Ras/JNK MAPK pathway, as it is the case of Smad3-deficient fibroblasts [53], remains to be investigated. The inhibitory effect of endoglin on H-Ras oncogenic transformation seems to be a novel and important clue of its suppressor role in epidermal carcinogenesis [6], a system in which tumorigenesis is driven by H-Ras activation [58]. Two major consequences of endoglin inactivation during tumor progression [6] would be to facilitate TGF-β-stimulated expression of H-Ras and to augment the oncogenic capacity of mutated H-Ras. Thus, TGF-β/endoglin and Ras cooperate to modulate malignant progression.

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LEGENDS TO FIGURES

Figure 1. TGF-β enhances *H-Ras* expression in transformed keratinocytes by activating the Ras-MAPK pathway. Keratinocytes were incubated in the presence or absence of TGF-β1 for the indicated times.

A. Analysis of Ha-Ras protein levels. PDV cells were incubated in the presence or absence of TGF-β1 and the Ha-Ras protein levels were visualized by western blot. Normalized Ha-Ras protein levels were measured relative to α-tubulin.

B. Kinetic study of the TGF-β1-dependent transcriptional regulation of H-Ras. PDV cells were incubated in the presence or absence of TGF-β1. For analysis of H-Ras mRNA levels, total RNA was extracted and subjected to semiquantitative RT-PCR and H-Ras transcript levels were normalized relative to GAPDH (top). For H-Ras promoter activity assays, one day before completion of TGF-β treatment, treated and untreated cells were transfected with a reporter construct driven by the H-Ras promoter and the luciferase activity was measured 24 h later (bottom).

C. Analysis of H-Ras promoter activity in the carcinoma cell lines MCA3D, PDV and B9. Cells were transfected with a reporter construct driven by the H-Ras promoter and incubated for 24hrs. Then, cells were treated in the presence or absence of TGF-β1 for 2 days and the luciferase activity was measured. *Statistically significant differences respect to untreated cells ($p<0.005$).

D and **E**. Analysis of the signalling pathway involved. The luciferase experiments were performed in triplicates at least three times and representative experiments are shown in the figures. RLU, relative luciferase units.

D. Analysis of ALK5 and Smad involvement. PDV cells were cotransfected with a reporter construct driven by the H-Ras promoter and expression vectors (EV) coding for the

constitutively active ALK5 (ALK5-T204D), the kinase dead form of ALK5 (ALK5-K232R), Smad2/Smad3, or the dominant negative (DN) form of Smad4. Twenty four hours after transfection, cells were incubated or not with TGF-β1 for 1 day and the luciferase activity was measured (histogram on the left). Right, the ectopic expression of Flag-tagged Smad4-DN, Smad2 and Smad3 in transfected PDV cells was revealed by Western blot analysis using anti-Flag antibodies. The ectopic expression of HA-tagged ALK5 (T204D) and ALK5 (K232R) in transfected PDV cells was revealed by Western blot analysis using anti-HA antibodies. As a negative control, transfection with an empty vector (V) was carried out. As a loading control, the presence of α-tubulin is included.

E. Analysis of Ras-MAPK involvement. PDV cells were cotransfected with a reporter construct driven by the H-Ras promoter and expression vectors coding for dominant negative (DN) forms of Ras, Raf, or MEK1,2. A chemical inhibitor of ERK (UO125) was also used. Twenty four hours after transfection, cells were treated or not with TGF-β1 for 1 day and the luciferase activity was measured (histogram on the left). A control transfection with an empty vector, as well as a control incubation with the vehicle (0.1% DMSO) used in the treatment with UO125, were included. Right, the ectopic expression of Ras-DN, Raf1-DN or HA-tagged MEK1,2-DN in transfected PDV cells was revealed by Western blot analysis using specific antibodies against Ras, Raf and HA. Note that polyclonal antibodies against Ras and Raf1 also recognize the endogenous proteins, but the signals are substantially higher upon expression of the DN forms of Ras and Raf1. As a loading control, the presence of α-tubulin is included. V, empty vector; MAPK-EV, expression vector encoding components of the MAPK pathway.

Figure 2. Endoglin inhibits the induction of H-Ras by TGF-β1

A. Effect of endoglin on the stimulation of H-Ras promoter activity by TGF-β1. PDV cells were cotransfected with a reporter construct driven by the H-Ras promoter and increasing

amounts of an expression vector coding for human endoglin. After 24hrs, cells were treated in the presence or absence of TGF-β1 for 2 days. Then, cells were lysed and the luciferase activity was measured. The inset shows the recombinant expression of endoglin in transfected PDV cells, as evidenced by Western blot analysis using anti-endoglin antibodies. As a loading control, the presence of α-tubulin is included.

B. Effect of endoglin on the TGF-β1 stimulation of H-Ras transcript levels. PDV cells were transfected or not with endoglin and incubated or not with TGF-β1 for 7 days as indicated. Total RNA was extracted and subjected to semiquantitative RT-PCR to determine endoglin, H-Ras and GAPDH levels. Normalized H-Ras transcript levels relative to GAPDH are shown in the histogram.

C. Effect of endoglin on the activation of MAPK target genes by TGF-β1. PDV cells were cotransfected with the reporters SRE-luc, containing a serum-responsive element, pCMV-Gal4-Elk1, that confers MAPK specificity, pcFos-luc containing the c-fos promoter, and a reporter construct driven by the cyclin A promoter, as well as an endoglin expression vector, as indicated. As controls, mock transfections in the presence of an empty vector (V) or transfections with the Smad3-responsive promoter construct $(CAGA)_{12}$-Luc were carried out. Twenty four hours after transfection, cells were treated or not with TGF-β1 for 1 day, and, then, the luciferase activity was measured.

Figure 3. Endoglin modulates basal and TGF-β-dependent activation of MAPK in transformed keratinocytes.

A. Effect of endoglin knockdown on basal ERK phosphorylation. Western blot analysis of endoglin (Eng), phospho-ERK1,2 (pERK1,2), total ERK1,2 and c-Fos in PDV cells stably transfected with shRNA endoglin (shEng2 and shEng4) or shRNA control (shCont). No differences between shCont and mock transfected cells were observed in several independent experiments (data not shown). Anti-α-tubulin antibodies were used as a control for protein

loading. Normalized pERKs levels relative to total ERK proteins are shown in the histogram. This is a representative experiment out of three.

B. Effect of endoglin knockdown on TGF-β-dependent ERK phosphorylation. shEng2 and shCont cells were incubated or not with TGF-β1 for the times indicated. Total cellular lysates were subjected to Western Blot analysis using specific monoclonal antibodies to phospho-ERK1,2 and total ERK1,2. Normalized pERKs levels relative to total ERK proteins are shown in the histogram. This is a representative experiment out of three.

C. Effect of endoglin knockdown on basal and TGF-β-induced Ras and MEK activities. Cells were incubated or not with 10 ng/ml TGF-β1 for 15 minutes. Western blot analyses of phospho-MEK1,2, total MEK1,2, phospho-ERK1,2 and total ERK1,2, in PDV cells stably transfected with shRNA endoglin (shEng2 and shEng4) or shRNA control (shCont) are shown. The levels of total Ras and active Ras-GTP proteins were determined in total cell lysates by precipitating with RBD-Sepharose, followed by Western blotting with a pan-Ras monoclonal antibody. Low (LE) and high (HE) exposures of the Ras-GTP signal are shown. Anti-β-actin antibodies were used as a control for protein loading. Normalized Ras-GTP levels versus total Ras, pMEKs levels relative to total MEK and pERKs levels relative to total ERK proteins are shown in the histogram. An arbitrary value of 1 was assigned to untreated shCont samples. This is a representative experiment.

Figure 4. Endoglin inhibits ERK phosphorylation in spindle carcinoma cells with hyperactivated MAPK activity.

A. Total cellular lysates from CarC cell transfectants stably expressing human endoglin (L-Eng1 and L-Eng2) and control mock transfectants were subjected to Western blot analysis using specific monoclonal antibodies to endoglin (Eng), phospho-MEK1,2, total MEK1,2, phospho-ERK1,2, total ERK1,2 and c-Fos. The levels of total Ras and active Ras-GTP proteins were determined in the cell lysates by precipitating with RBD-Sepharose followed by

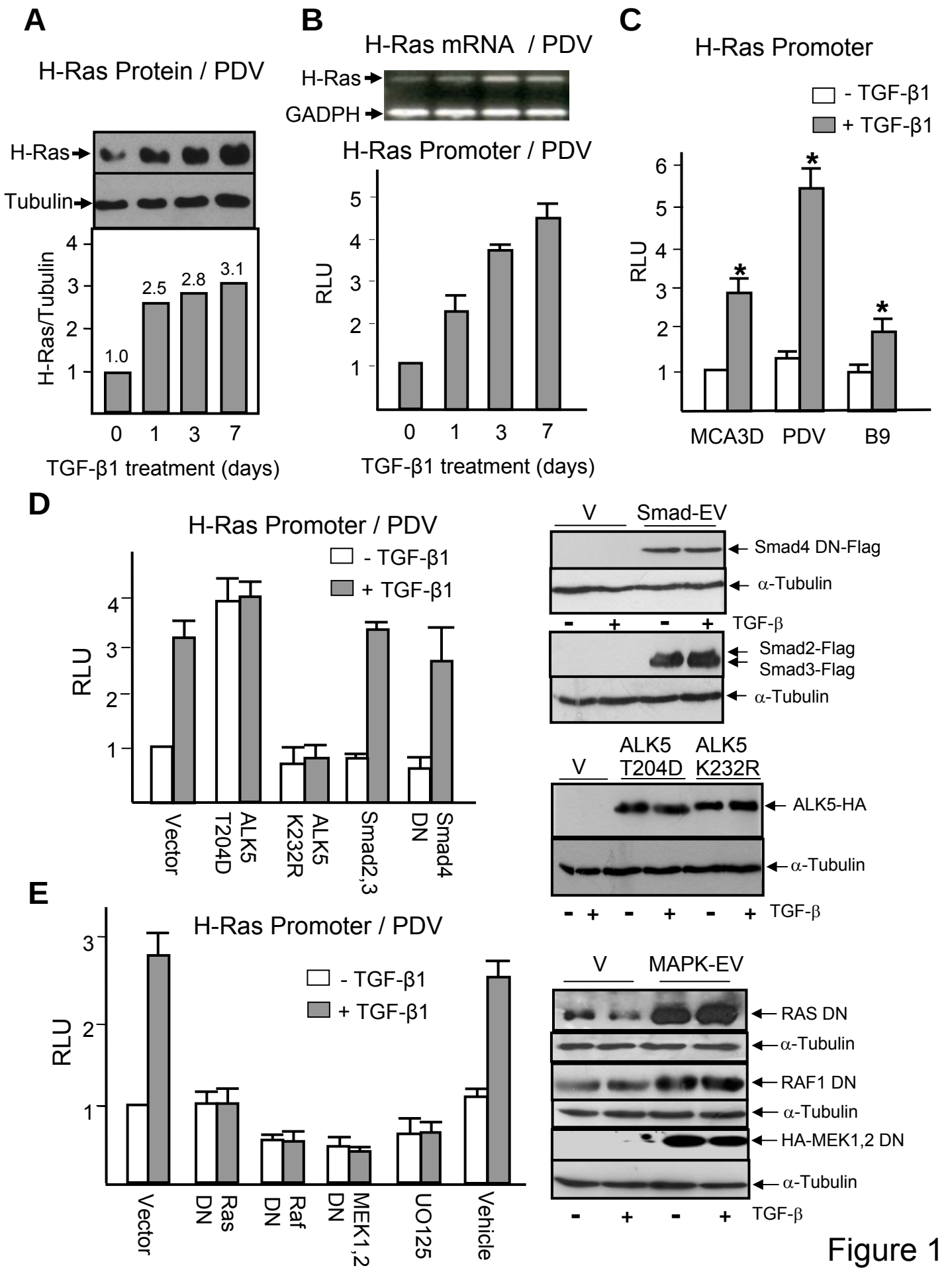
Western blotting with a pan-Ras monoclonal antibody. α-tubulin was used as a control for protein loading. A representative experiment out of three is shown. Inhibition of pERK 1/2 and c-fos levels in the presence of endoglin was observed (*). Normalized pERKs levels relative to total ERK proteins are shown in the histogram.

B. Inhibition of MAPK activity by endoglin in spindle carcinoma cells depends on the extracellular domain. Total cellular lysates from CarC cells retrovirally infected with the constructs pWZL (empty vector), L-Eng FL (full length endoglin), L-Eng ΔCt (lacking the cytoplasmic domain), L-Eng ΔPDZ (lacking the PDZ domain), and the HA-tagged L-Eng ΔEC (lacking the extracellular domain) were subjected to Western blot analysis using specific monoclonal antibodies to endoglin (Eng), phospho-ERK1,2, total ERK1,2, and c-Fos, and α-tubulin. Normalized pERKs levels relative to total ERK proteins are shown in the histogram. A representative experiment out of two is shown.

Figure 5. Effect of endoglin on the transforming activity of the H-Ras oncogene.

Mouse NIH 3T3 fibroblasts were transfected with expression vectors encoding human endoglin (pDisplay-HA-Endoglin), shRNA specific for mouse endoglin (shEng2) and H-RasQ61K (pAL8-Ras). Next day, each plate was splitted in three, and incubated with DMEM containing 5% donor calf serum for 2-3 weeks. Then, cells were fixed and stained with crystal violet, and foci were counted. This is a representative experiment out of three. Figures on top of the bars stand for the mean number of foci in each condition. Statistically significant differences respect to cells transformed with H-RasQ61K alone were observed (*$p<0.005$; **$p<0.0005$). In the inset a representative experiment is shown in which NIH 3T3 fibroblasts were infected with retrovirus bearing H-Ras-G12V and human endoglin. Transduced cells were incubated at 37ºC for around two weeks changing media every two days. Then, cells were fixed and stained with crystal violet, and foci were counted.

Figure 6. Hypothetical model for the role of endoglin on the Ras/MAPK pathway in transformed keratinocytes. TGF-β binds to the signalling receptor complex formed by the serine threonine kinase receptors type I (RI) and type II (RII) and endoglin. In turn, this receptor complex can activate two different signalling pathways: i) the Smad pathway by phosphorylating the receptor activated Smad3 protein; and ii) the Ras/MAPK pathway leading to the Raf/MEK/ERK phosphorylation cascade. In PDV cells, the TGF-β-induced activation of the Ras/MAPK pathway leads to the upregulated expression of *H-Ras*. In PDV and CarC cells, endoglin interferes with the TGF-β-dependent Smad3 activity; conversely, ERK phosphorylates and inactivates Smad3. In PDV transformed keratinocytes, containing a normal to mutated H-Ras gene dosage of ~2:1 [15], endoglin interferes with basal and TGF-beta-dependent Ras activation and downstream components. However, in CarC spindle cells, homozygous for the mutant H-Ras allele [15], endoglin inhibits ERK phosphorylation, but not the upstream hyperactivated (black arrows) Ras/Raf/MEK components. H-Ras*, oncogenic H-Ras mutant. Dashed grey arrows indicate a weaker signal than straight arrows. The involvement of mitogenic growth factors and their corresponding tyrosine kinase receptors that activate the MAPK pathway, the association of phospho-Smad3 with Smad4 as well as other Ras family members different from H-Ras have been omitted for simplification. For further details, see the discussion section.


A
H-Ras Protein / PDV
H-Ras
Tubulin
H-Ras/Tubulin
1.0
2.5
2.8
3.1
0 1 3 7
TGF-β1 treatment (days)
B
H-Ras mRNA / PDV
H-Ras
GADPH
H-Ras Promoter / PDV
RLU
0 1 3 7
TGF-β1 treatment (days)
C
H-Ras Promoter
- TGF-β1
+ TGF-β1
RLU
MCA3D PDV B9
D
H-Ras Promoter / PDV
- TGF-β1
+ TGF-β1
RLU
Vector
ALK5 T204D
ALK5 K232R
Smad2,3
Smad4 DN
V
Smad-EV
Smad4 DN-Flag
α-Tubulin
TGF-β
Smad2-Flag
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ALK5 T204D
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ALK5-HA
α-Tubulin
TGF-β
E
H-Ras Promoter / PDV
- TGF-β1
+ TGF-β1
RLU
Vector
Ras DN
Raf DN
MEK1,2 DN
UO125
Vehicle
V
MAPK-EV
RAS DN
α-Tubulin
RAF1 DN
α-Tubulin
HA-MEK1,2 DN
α-Tubulin
TGF-β

Figure 1

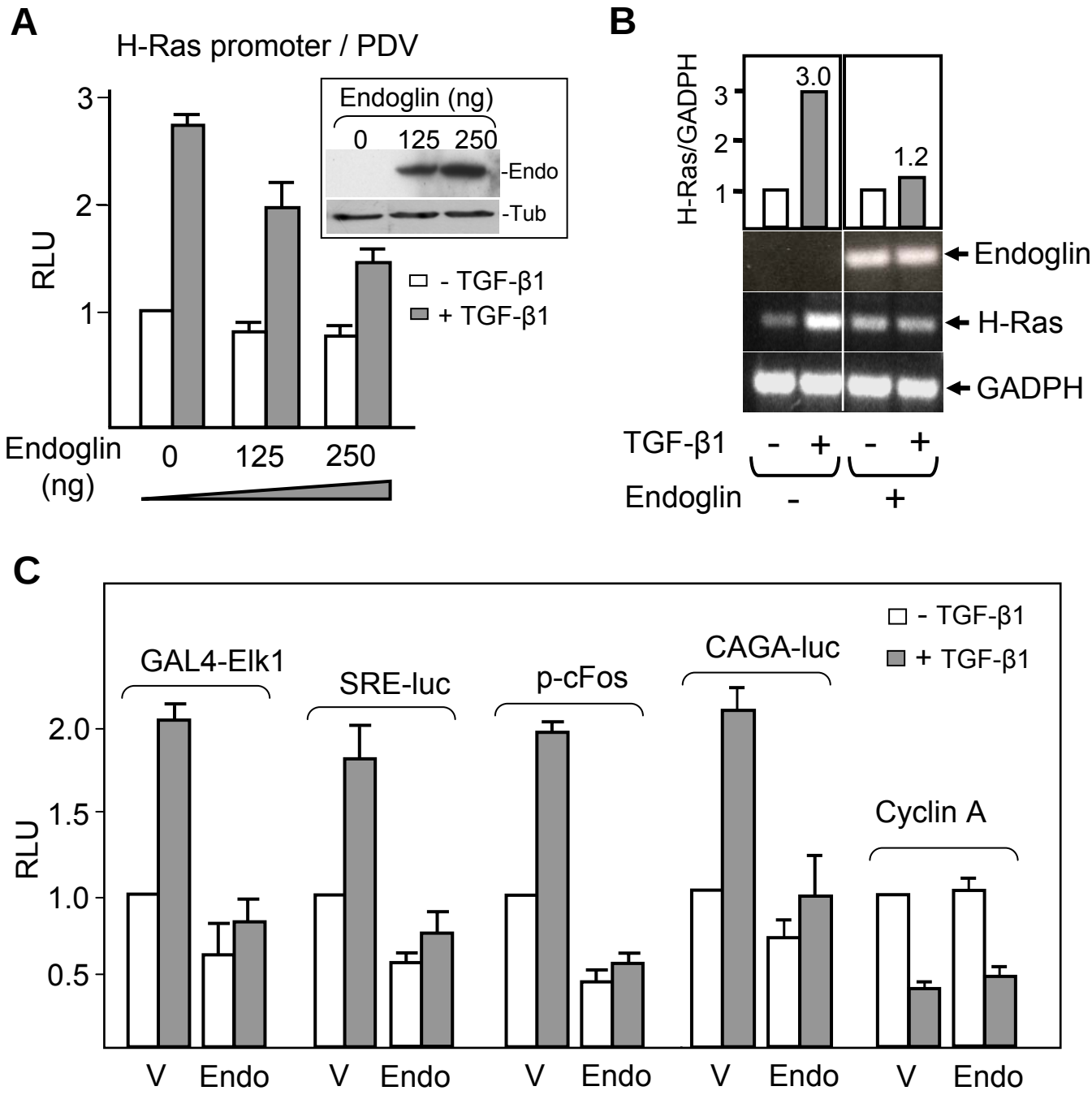

A
H-Ras promoter / PDV
RLU
3
2
1
Endoglin (ng)
0
125
250
-Endo
-Tub
- TGF-β1
+ TGF-β1
Endoglin (ng)
0
125
250
B
H-Ras/GADPH
3.0
1.2
Endoglin
H-Ras
GADPH
TGF-β1
- + - +
Endoglin
- +
C
GAL4-Elk1
SRE-luc
p-cFos
CAGA-luc
Cyclin A
- TGF-β1
+ TGF-β1
RLU
2.0
1.5
1.0
0.5
V
Endo
V
Endo
V
Endo
V
Endo
V
Endo

Figure 2

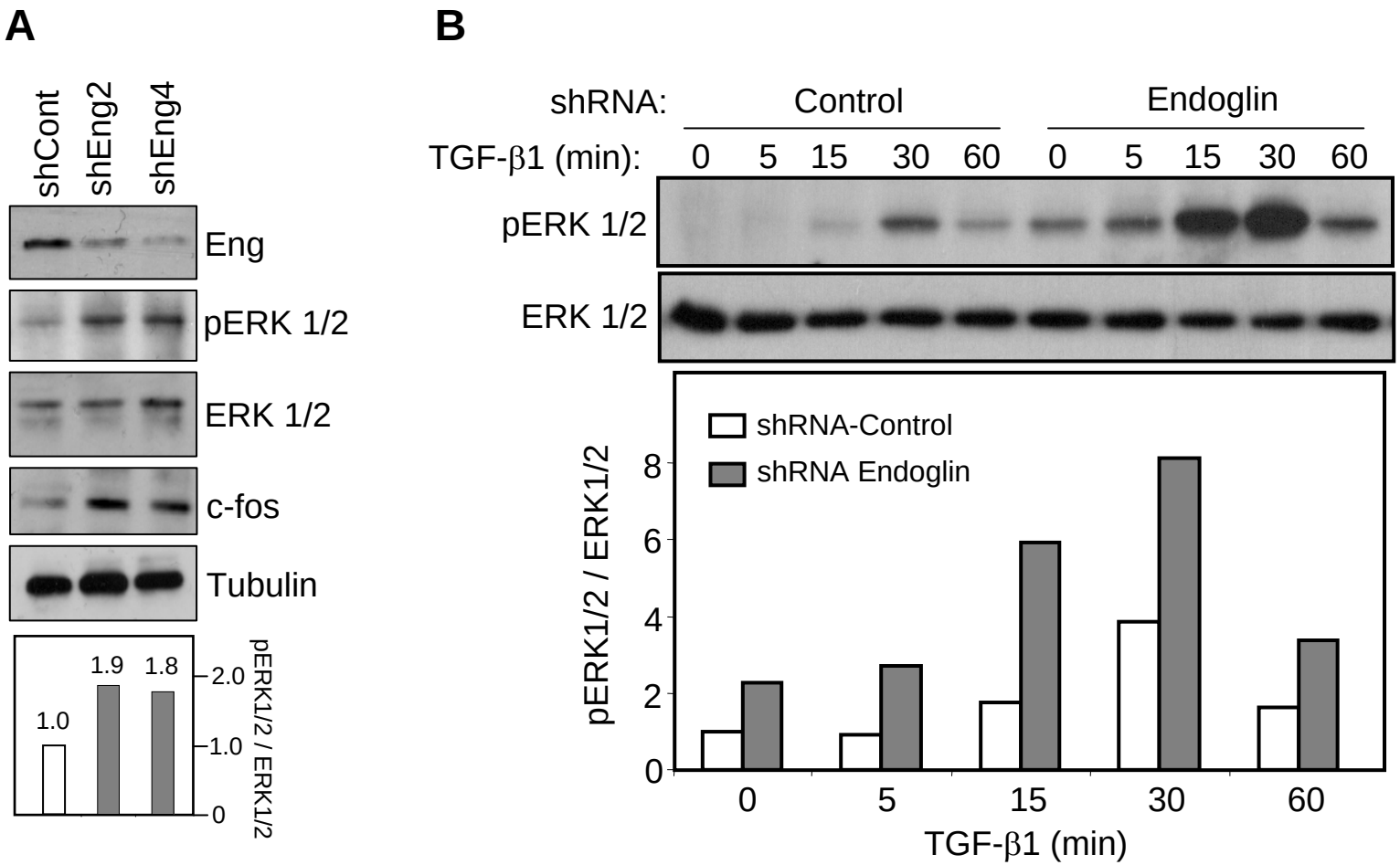

A
shCont
shEng2
shEng4
Eng
pERK 1/2
ERK 1/2
c-fos
Tubulin
1.0
1.9
1.8
2.0
1.0
0
pERK1/2 / ERK1/2
B
shRNA:
Control
Endoglin
TGF-β1 (min):
0 5 15 30 60 0 5 15 30 60
pERK 1/2
ERK 1/2
shRNA-Control
shRNA Endoglin
pERK1/2 / ERK1/2
0
2
4
6
8
0 5 15 30 60
TGF-β1 (min)

Figure 3

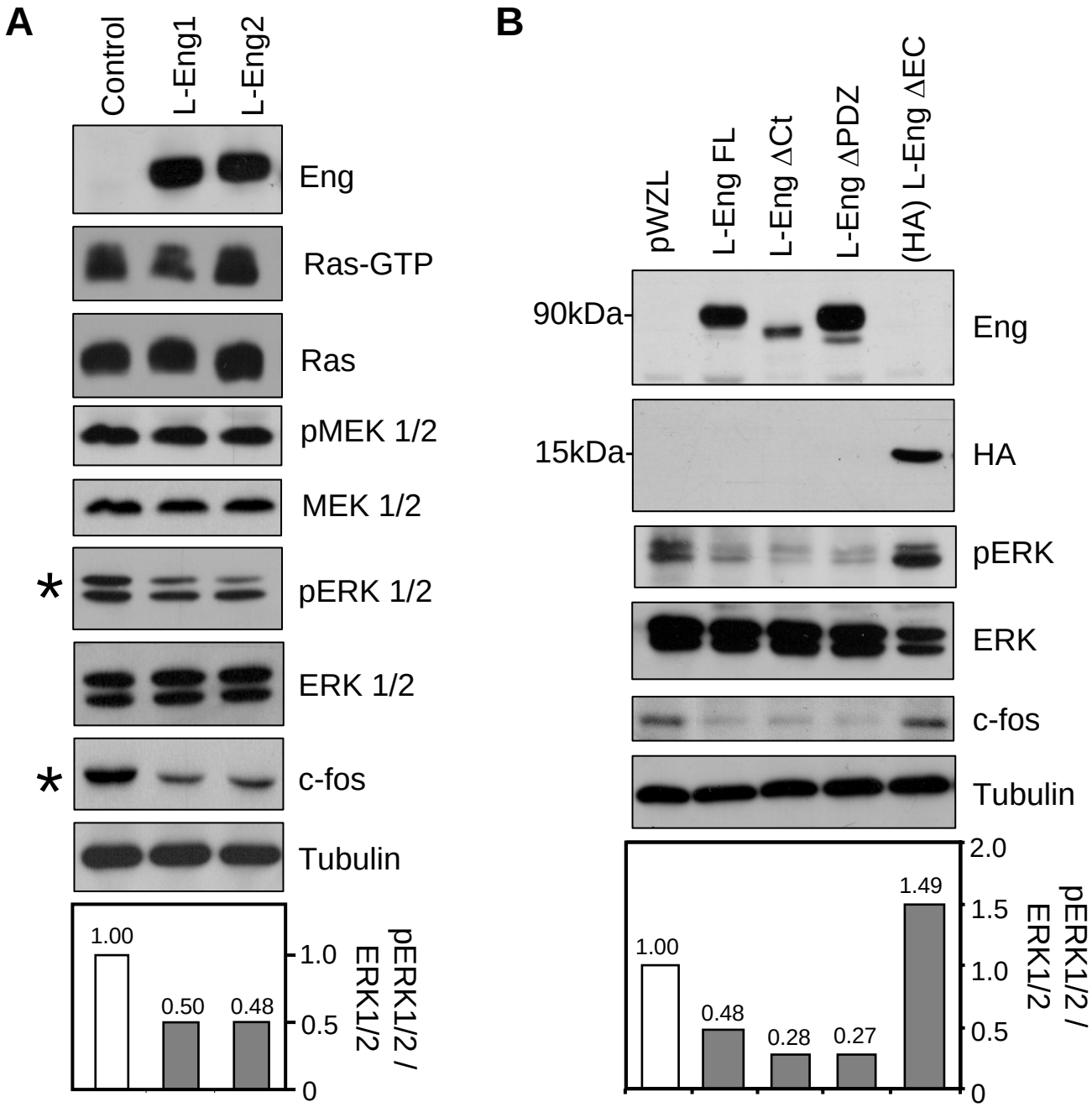

A
Control
L-Eng1
L-Eng2
Eng
Ras-GTP
Ras
pMEK 1/2
MEK 1/2
pERK 1/2
ERK 1/2
c-fos
Tubulin
*
*
1.00
0.50
0.48
1.0
0.5
0
pERK1/2 / ERK1/2
B
pWZL
L-Eng FL
L-Eng ΔCt
L-Eng ΔPDZ
(HA) L-Eng ΔEC
90kDa-
15kDa-
Eng
HA
pERK
ERK
c-fos
Tubulin
1.00
0.48
0.28
0.27
1.49
2.0
1.5
1.0
0.5
0
pERK1/2 / ERK1/2

Figure 4

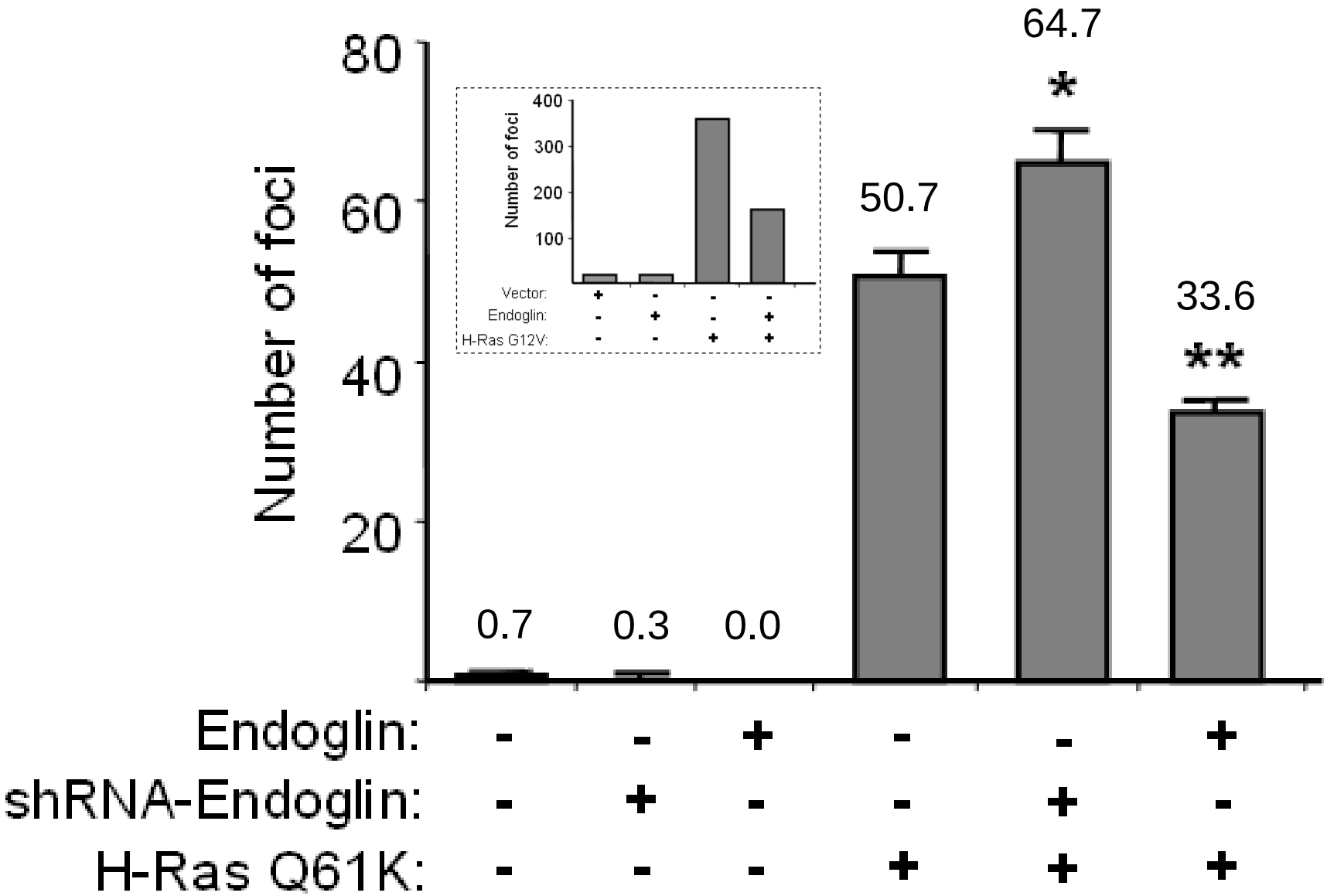
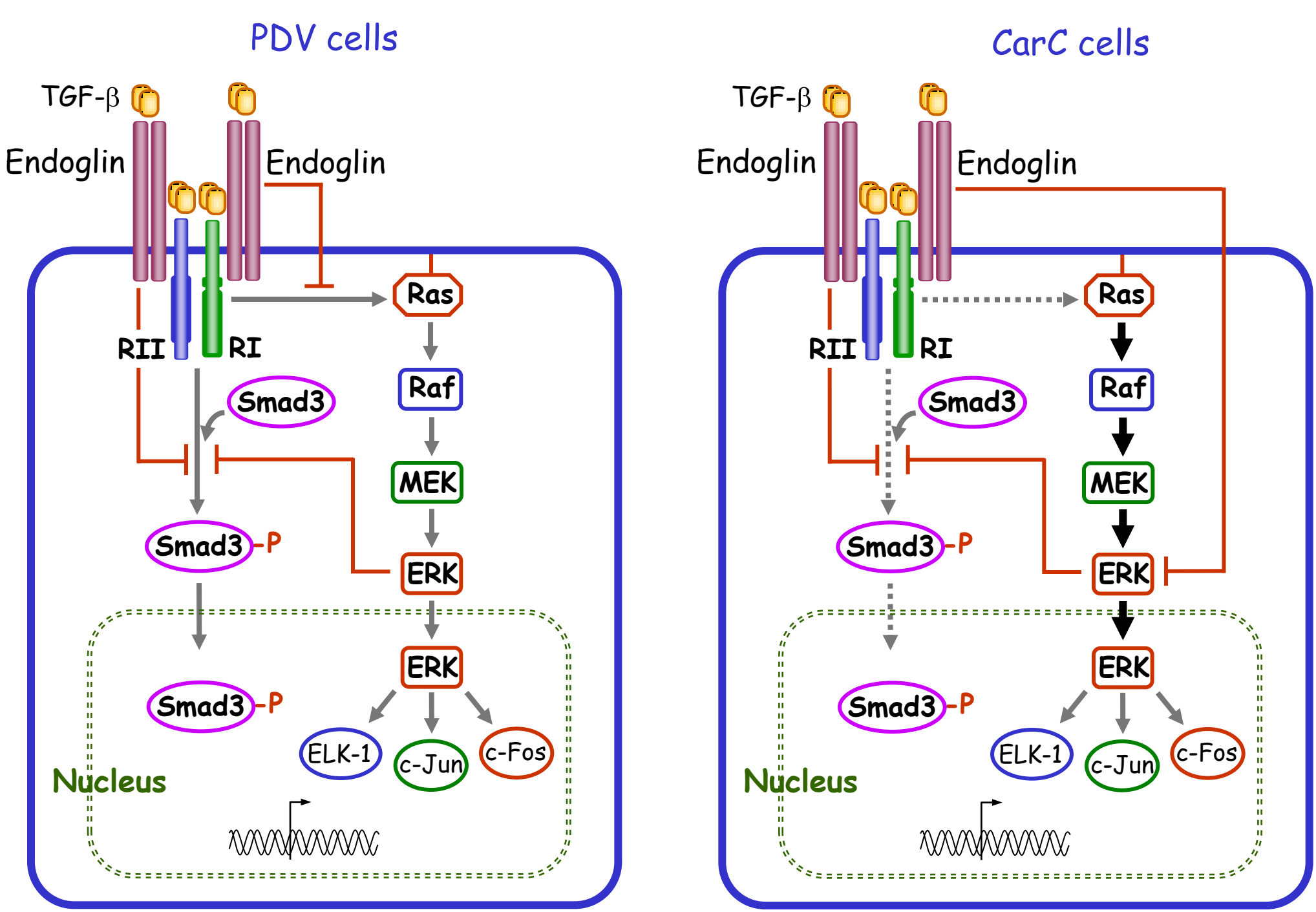

Number of foci
80
60
40
20
0.7
0.3
0.0
50.7
64.7
*
33.6
**
Number of foci
400
300
200
100
Vector:
Endoglin:
H-Ras G12V:
Endoglin:
shRNA-Endoglin:
H-Ras Q61K:

Figure 5


PDV cells
TGF-β
Endoglin
Endoglin
RII
RI
Ras
Raf
MEK
ERK
Smad3
Smad3-P
Smad3-P
ERK
ELK-1
c-Jun
c-Fos
Nucleus
CarC cells
TGF-β
Endoglin
Endoglin
RII
RI
Ras
Raf
MEK
ERK
Smad3
Smad3-P
Smad3-P
ERK
ELK-1
c-Jun
c-Fos
Nucleus