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► **To cite this version:**

Valérie Fauquette, Michael Perrais, Sylvain Cerulis, Nicolas Jonckheere, Marie-Paule Ducourouble, et al.. The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells: implications for the proliferation/differentiation balance in the cells. *Biochemical Journal*, 2005, 386, pp.35 - 45. hal-02905757

**HAL Id: hal-02905757**

**<https://hal.science/hal-02905757>**

Submitted on 29 Sep 2020

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**The antagonistic regulation of human MUC4 and ErbB-2 genes by the Ets protein PEA3 in pancreatic cancer cells. Implications for the proliferation/differentiation balance in the cells.**

REVISED

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Page heading title: MUC4 and ErbB-2 transcriptional regulation by the Ets factor PEA3

Abbreviations used: DAC, ductal adenocarcinoma; WD, well-differentiated; PD, poorly differentiated; ECM, extra cellular matrix; RT-PCR, Reverse Transcriptase PCR; WT, wild type; MT, mutated type; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; TBE, Tris-Borate-EDTA.

Keywords: mucin, transcription, carcinogenesis, MUC4, PEA3, ErbB-2.

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## **ABSTRACT**

The human transmembrane mucin MUC4 is aberrantly expressed in 75% of pancreatic ductal adenocarcinomas (DAC) whereas no expression is found in normal pancreas. Therefore MUC4 appears as a useful biological marker for the diagnosis of DAC. Since rat Muc4, was shown to interact with ErbB-2 tyrosine kinase receptor and to either promote cell survival and differentiation or cell proliferation, it is postulated that MUC4 may also participate in pancreatic carcinogenesis. Our aim was to investigate in parallel the role of the Ets factor PEA3 in *MUC4* and *ErbB-2* transcriptional regulation in pancreatic cancer cells. Two *MUC4* expressing well-differentiated (WD) (CAPAN-1 & CAPAN-2) and one *MUC4* non expressing poorly differentiated (PANC-1) cell lines were used. The three cell lines express ErbB-2 at different levels. By co-transfection and site-directed mutagenesis, we show that PEA3 is a transactivator of *MUC4* promoter and that the -216 and -2368 PEA3 binding sites of *MUC4* promoter are essential. We also demonstrate that PEA3 acts in synergy with c-Jun and Sp1 to transactivate the proximal region of *MUC4* promoter and increase *MUC4* mRNA levels in WD cells. These results suggest that *MUC4* is a new target gene of the Ets factor PEA3 in pancreatic cancer cells. Conversely, PEA3 represses the transcriptional activity of two fragments of *ErbB-2* promoter in a dose-dependent manner and decreases the endogenous *ErbB-2* mRNA levels in WD cell lines. Thus, PEA3, by its capacity to up-regulate the epithelial marker MUC4 and to down-regulate the ErbB-2 oncogene, appears as a key regulator of the differentiation/proliferation balance in pancreatic cancer cells.

## INTRODUCTION

Pancreatic cancer is an especially devastating form of cancer, with only 3% of patients still alive five years after diagnosis. It constitutes the fifth leading cause of cancer-related death in Western countries. The poor prognosis of this disease is linked to the fact that it affects a rather unaccessible organ and remains asymptomatic until well advanced. Its diagnosis is therefore made at a late stage where peritoneal dissemination and liver metastasis have already occurred. Moreover pancreatic cancer is difficult to treat because of its resistance to chemotherapy and radiotherapy. A better understanding of the molecular mechanisms of pancreatic carcinogenesis may lead to the definition of new therapeutic targets and efficient chemotherapeutic interventions. Pathologically, most pancreatic cancers correspond to ductal adenocarcinoma (DAC) that develop from ductal epithelium. Hruban *et al.* [1] recently proposed a progression model for pancreatic cancer from intraepithelial neoplasia to invasive DAC similar to the adenoma-carcinoma sequence described in the colon. In the pancreatic carcinogenetic sequence, *K-ras* activation and *ErbB-2* overexpression are the earliest genetic events [2].

Mucins are high molecular weight O-glycoproteins encoded by at least ten genes in humans [3], that exhibit an aberrant pattern of expression in various human malignancies. The common structural feature of all mucins is the presence of tandemly repeated amino acid regions that serve as docking sites for O-glycans. Two groups of mucins are now distinguished [3]: (i) secreted mucins including gel-forming (MUC2, MUC5AC, MUC5B and MUC6) and non-gel-forming (MUC7) mucins; (ii) membrane-bound mucins that are thought to participate in cell-cell and cell-extracellular matrix (ECM) interactions or in cell signaling [4,5]. Human *MUC4* belongs to this last group. It encodes a large transmembrane mucin characterized by the presence of a repetitive domain rich in serine and threonine in its N-terminal region, two EGF-like domains, a transmembrane domain and a short cytoplasmic tail in its C-terminal part [6]. *MUC4* is the human homologue of rat SMC/Muc4, a heterodimeric glycoprotein made of a mucin subunit ASGP-1 tightly bound to a transmembrane subunit ASGP-2. ASGP-2 contains two EGF-like domains in its extracellular part, one of which, EGF1, acts as a ligand for the ErbB-2/Neu oncogene [4]. By analogy, *MUC4* is thought to interact with the HER2/ErbB-2 tyrosine kinase receptor.

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Several studies have shown that mucin genes are dysregulated in pancreatic cancer. While *MUC4* is not expressed in normal pancreas [7], up to 75% of pancreatic DAC and of pancreatic cancer cell lines show *de novo* expression of *MUC4* at the mRNA level [8]. Recently, Swartz *et al* [9] demonstrated a gradual expression of *MUC4* at the protein level in pancreatic carcinogenesis with 17% of pancreatic intraepithelial neoplasia type 1 positive for *MUC4* whereas in DAC the percentage rises to 89%. The rare expression of *MUC4* in non neoplastic lesions of the pancreas was also confirmed. According to its high specificity for pancreatic cancer, *MUC4* could be used as a biological marker for the histopathological diagnosis of pancreatic adenocarcinoma and for its early detection in the peripheral blood [10]. Moreover *MUC4* is one of the few genes identified as an attractive target for novel therapeutic strategies in pancreatic cancer [11]. However at this time, the molecular mechanisms leading to *MUC4* dysregulation in pancreatic carcinoma are still poorly understood.

In order to decipher these mechanisms, we have previously characterized *MUC4* promoter and showed that it contains a GC-rich and TATA-less proximal regulatory region and a distal regulatory region flanked by a TATA box [12]. Several consensus binding sites for the Ets transcription factors (5'-GGAA/T-3') were found both in the proximal and distal regions of *MUC4* promoter. Among those, the proximal putative *cis*-element (P<sub>216</sub>) is conserved in the rat *Muc4* promoter [13]. The Ets transcription factors have in common a 85-amino-acid helix-turn-helix DNA binding domain called the ETS domain. Interestingly, the Ets factors have been implicated in a wide range of cellular processes such as differentiation, proliferation and transformation [14]. Recently, Perez *et al.* [15] have demonstrated that PEA3, a founding member of an Ets protein subfamily, was capable of transactivating *rMuc4* promoter in the mouse mammary epithelial cell line HC11. This effect relies in part on a direct binding of PEA3 to its proximal *cis*-element P<sub>339</sub> (5'-AGGAA-3') that is not perfectly conserved in the human *MUC4* promoter (5'-AGGAG-3'). In light of earlier data on the regulation of *ErbB-2* by PEA3 in COS cells [16] and in ovarian cancer cell lines [17], we undertook to investigate in parallel the effects of PEA3 on *MUC4* and *ErbB-2* regulation in human pancreatic cancer cell lines. As an experimental model, we used two well-differentiated (WD) cell lines, CAPAN-1 and CAPAN-2, that express high levels of *MUC4* mRNA

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and a poorly differentiated (PD) cell line that does not express *MUC4*, PANC-1 [12]. The 3 cell lines express ErbB-2 protein at different levels. The aim of our study was to demonstrate whether in pancreatic cancer cell lines, PEA3 co-regulates *MUC4* and *ErbB-2* that encode 2 membrane-bound protein partners.

## MATERIALS AND METHODS

*Cell cultures.* Pancreatic cancer cell lines CAPAN-1 and CAPAN-2 were purchased from ATCC (American Type Culture Collection), and PANC-1 from ECACC (European Collection of Animal Cell Cultures). The cells were cultured as previously described [12].

*Transient transfections.* Cells were passed at  $0.5 \times 10^6$  cells/well the day before being transfected by pGL3 basic vector (Promega, France) containing a deletion mutant of *MUC4* promoter upstream of the luciferase gene [12] using Effectene reagent as previously described [18], or by pXP2 vector containing a deletion fragment of ErbB-2 promoter [19] upstream of the luciferase gene (kind gift of Dr R. Winkler, Université de Liège, Belgium). Total cell extracts were prepared after a 48h incubation at 37°C using 1X RLB buffer according to the manufacturer's instructions (Promega, France). The luciferase activity and the protein content of the cell extract were measured as previously described [12]. Results were first expressed as luciferase activity/ $\mu$ g of protein. In order to take into account transfection efficiency, corrected results were obtained by dividing the luciferase activity/ $\mu$ g protein by those of the positive control plasmid (pGL3-control vector). Final results are expressed as fold of induction of the pGL3-control vector. In co-transfection studies, cells were transfected with pGL3 *MUC4* promoter deletion mutants in the presence of an expression vector encoding either mPEA3 (pSV-HA-mPEA3, kind gift of Dr J.L. Baert, Institut Pasteur de Lille, France), ER81 (pSV-ER81), ERM (pSV-ERM, both kind gifts of Pr Y. de Launoit, Université Libre de Bruxelles, Belgium), Sp1 (pCMV-Sp1, kind gift of Dr J.M. Horowitz, Raleigh, USA), or c-Jun (pRSV-c-Jun). Results were expressed as fold induction relative to the co-transfection performed in the presence of the corresponding empty expression vectors. Each combination of plasmids was assayed in triplicate in three separate experiments. Data were analyzed by Student's t test with differences less than 0.05 considered significant.

*Site-directed mutagenesis.* The P<sub>216</sub> PEA3 binding site was mutated from AGGAA to AAAAA in the -219/-1 deletion mutant using the Quick Site Directed Mutagenesis Kit (Stratagene, France) according to the manufacturer's instructions. The mutated plasmid DNA was purified and sequenced on both strands before being used in cell transfection experiments.

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Nuclear extract preparation and Western-blotting. Nuclear and cytoplasmic extracts from cell lines of interest were prepared as previously described [20] and kept at -80°C before use. Protein content was determined using the BCA method. Nuclear or cytoplasmic proteins (25µg) were separated by 10% SDS-PAGE, followed by an electrotransfer onto a 0.2 µm nitrocellulose membrane (Schleicher & Schuell, Germany). The membranes were first incubated with specific primary antibodies [anti-PEA3 mouse monoclonal (sc-113), anti-c-Jun rabbit polyclonal (sc-44) and anti-Sp1 goat polyclonal antibody (sc-59) all from Santa Cruz Biotechnology (Tebu, France); anti-ErbB-2 mouse monoclonal antibody (NCL-CB11) from Novo Castra (Tebu, France)] before a second incubation with a peroxidase-conjugated secondary antibody (PEA3, c-Jun, erbB-2) or alkaline phosphatase conjugated IgG (Sp1). Immunoreactive bands were visualized using the chemiluminescence Super Signal system (PEA3, c-Jun, ErbB-2) and Hyperfilm ECL (Amershampharmabiotech, UK) or (Sp1) Nitrotetrazolium Blue Chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies, France). The autoradiograms were scanned and a densitometry analysis was performed using the Clara Vision Gel Smart-Gel Analysis software. Values were expressed as arbitrary units.

Electrophoretic Mobility Shift Assays (EMSA). Binding studies were performed with double stranded oligonucleotides containing the P<sub>216</sub> PEA3 binding site of the *MUC4* proximal promoter (sense oligonucleotide: 5' CGGTGTTTTCTTTCCTGGTGG 3'). The oligonucleotides were annealed before being phosphorylated at the 5'-end with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Biosciences, France) and T4 polynucleotide kinase. The binding reaction was performed by preincubating 9 µg of nuclear extract in 10 mM HEPES pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 1 mM DTT, 12.5% glycerol and 2 µg polyd(I,C) in a final volume of 20 µl for 10 min at room temperature. Then the radiolabelled probe (90,000 c.p.m.) was added to the reaction mixture and incubated for an additional 30 min at room temperature as previously described [21]. Competition experiments using a 100-fold molar excess of wild type or mutated (sense oligonucleotide: 5'CGGTGTTTTCTTIGGTGGTGG 3') cold probe were performed as described before [21]. Supershift experiments were performed by adding 2 µl of an anti-PEA3 or anti-ErbB-2 antibody to the reaction mixture together with the radiolabelled probe [21]. The reaction was stopped by adding



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2 µl of 10X loading buffer. Samples were run on a 6% non denaturing polyacrylamide gel (200V for 3 hours at 4°C) with 0.5X TBE (Tris Borate EDTA) buffer. Gels were dried and autoradiographed. The PEA3 fusion protein was prepared using the pSV-HA-mPEA3 expression vector and the TNT T7 Quick Coupled Transcription/Translation System (Promega, France) according to the manufacturer's instructions. Binding reaction was performed by using 5 µl of the PEA3 fusion protein preparation and the experimental protocol reported by Perez et al [15].

Reverse Transcriptase(RT)-PCR analysis. Total RNAs from pancreatic cancer cells were prepared using the RNeasy mini-kit (Qiagen SA, France) as already described [12]. cDNA was synthesized from 1.5 µg RNA using the Advantage-RT for PCR kit (BD Bioscience, France). To evaluate *MUC4* expression, PCR was performed on 2 µl of cDNA using previously described primers [12]. To evaluate *PEA3* expression, a specific pair of primers was designed (E1Af: 5' ACCATGGCGAGCAGTGCCTT 3', E1Ar: 5' TCCGACAGCTGGTGTGGTA 3') that amplifies a 288 bp fragment outside of the ETS domain, that starts in exon 6 and ends in exon 8 of the human gene. PCR was carried out in a final reaction volume of 50 µl containing 5 µl of cDNA, 2µl of AmpliTaq (Roche, France), 10 pmoles of each primer and 1.5 mM MgCl<sub>2</sub> using the following program: denaturation at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min; primer annealing at 57°C for 1 min, and elongation at 72°C for 1 min. Final elongation was conducted at 72°C for 5 min. To check the specificity of the primers, the 300 bp band was gel-purified before being sequenced using the ABI PRISM 377 XL automatic sequencer and the ABI PRISM Ampli Taq FS Big Dye Terminator cycle sequencing ready reactions Kit (Applied Biosystems, France). The nucleotide sequence was analyzed by the EMBL FASTA Server [22] showing a 100% identity with the sequence of PEA3/E1AF mRNA (GenBank Accession Number D12762). To evaluate *ErbB-2* expression we used the following pair of primers (NAU2126: 5' CGCGAGCTCGGAGGACGAGTGTGTGGG 3' and NAU2127: 5' CGCGGTACC-CAGAATGCCAACCACCGC 3') that amplifies a 485 bp fragment that showed a 100% identity with the human c-erb-B2 mRNA (GenBank Accession Number X03363). The PCR program was as followed: denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30s;

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primer annealing at 60°C for 30s, and elongation at 72°C for 30s. Final elongation was conducted at 72°C for 5 min. *28S* or *β actin* were used as internal controls.

*Immunohistochemistry.* Cells were passed at  $1.5 \times 10^6$  cells / 75 cm<sup>2</sup> flask 48h before being transfected with 4 µg of vector as described before. Forty-eight hours later, the cells were trypsinized, centrifuged and washed once with 1X PBS. The pellet was fixed in 4% formaldehyde, embedded in paraffin and 3 µm sections were prepared. Immunohistochemistry was performed on serial sections of the same blocks using an automatic immunostainer (ES, Ventana Medical System, Strasbourg, France) as previously described [23]. The monoclonal anti-MUC4 antibody, that recognized the tandem repeat region of the protein [9], was used at a 1/10000 dilution.

## RESULTS

**Expression of MUC4, ErbB-2 and PEA3 in pancreatic cancer cells.** Expression of the different partners studied in this report was evaluated by RT-PCR or Western-blotting. As already shown [12], *MUC4* mRNA were found only in the WD cell lines CAPAN-1 and CAPAN-2 (Fig. 1A). PEA3 expression was first evaluated by RT-PCR using a specific pair of primers designed to amplify a 5' region of human *PEA3* cDNA outside of the conserved ETS domain. The results showed that the 3 cell lines express PEA3 mRNA (Fig 1A), at an apparently lower level in PANC-1 cells. To look for statistically significant differences, a *PEA3/β actin* ratio was calculated on 2 independent experiments and a t test performed, showing that CAPAN-1 (mean ratio (SD): 0.865 (0.007)), CAPAN-2 (0.945 (0.106)) and PANC-1 cells (0.616 (0.220)) express similar levels of *PEA3*. We also examined PEA3 expression at the protein level by Western-blotting using nuclear (lanes 1-3) and cytosolic (lanes 4-6) extracts prepared from the three cell lines (Fig. 1B). PEA3 is found at higher levels in CAPAN-1 (lane 1) and CAPAN-2 (lane 2) nuclear extracts when compared with PANC-1 (lane 3). On the contrary, PANC-1 cytosolic extracts (lane 6) contain higher amounts of PEA3 than CAPAN-1 (lane 4) or CAPAN-2 (lane 5). Finally, we controlled the levels of ErbB-2 protein expressed by the 3 cell lines by Western blotting (Fig. 1B). The densitometry analysis of the bands showed that CAPAN-1 cells expressed the lowest levels of ErbB-2 ( $p < 0.05$  vs CAPAN-2,  $n=2$ ) whereas CAPAN-2 and PANC-1 expressed similar levels (mean ErbB-2/actin ratio (SD): 0.89 (0.014) for CAPAN-1; 1.50 (0.009) for CAPAN-2 and 1.11 (0.24) for PANC-1).

### PEA3 selectively transactivates the promoter of *MUC4*

The promoter of *MUC4* contains several canonical Ets binding sites (5'-AGGAA-3') located in the proximal transcriptional unit ( $P_{216}$  &  $P_{349}$ ) upstream of the transcription start site (Fig. 2, upper panel) and in the distal transcriptional unit upstream ( $P_{2950}$ ,  $P_{2872}$ ,  $P_{2647}$ ) or downstream ( $P_{2368}$ ) of the transcription start site (Fig. 2, lower panel). To study the effects of the Ets protein PEA3 on *MUC4* promoter, we co-transfected a panel of deletion mutants covering both promoters of *MUC4* [12] in the presence of an expression vector encoding full-length PEA3. In CAPAN-1 cells (Fig. 3A, white

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bars), PEA3 strongly transactivates the proximal promoter especially the -219/-1 region containing the PEA3 site P<sub>216</sub>. A stronger effect is not seen with longer fragments outlining the importance of this site. Mutagenesis of P<sub>216</sub> (5'-AGGAA-3' to 5'-AAAAA-3') led to a 60% reduction of the PEA3 transactivating effect on the -219/-1 region (Figure 3A, MT -219/-1). When the luciferase activities of wild type (WT -219/-1) and mutant (MT -219/-1) constructs were compared in the absence of PEA3, we observed a 70% reduction of activity for the mutated form (Fig. 3B). This indicates that the PEA3 P<sub>216</sub> site acts as an important positive regulatory element required for *MUC4* promoter activity in CAPAN-1 cells.

In CAPAN-2 cells (Fig. 3A, black bars), which express lower levels of *MUC4* mRNA (see Fig. 1A), the transactivating effect of PEA3 on the -144/-1 and -219/-1 regions was 50% less intense than in CAPAN-1 cells. Interestingly, the response to PEA3 in these cells increased as longer fragments of *MUC4* promoter were transfected. These results suggest that positive elements for PEA3 regulation are present in the -461/-219 region of the promoter. Accordingly, the mutation of the conserved PEA3 site P<sub>216</sub> did not significantly affect the transcriptional activity of the -219/-1 region in CAPAN-2 cells (Fig. 3B). PEA3 also induces the luciferase activity driven by the -3135/-2307 region of *MUC4* promoter in CAPAN-1 (4-fold) and CAPAN-2 (2-fold) cells (Fig. 3A) whereas it does not modify the activity of the -3135/-2572 region. The -3135/-2307 region contains one additional Ets binding site (P<sub>2368</sub>) and two closely-linked AP-1 *cis*-elements compared to the -3135/-2572 region. Thus these sites are probably crucial for PEA3 transactivating effect in those two cell lines.

Finally, the weakest effects of PEA3 were observed in the *MUC4* non-expressing cell line PANC-1 (Fig. 3A, hatched bars) in which the transactivating effect of PEA3 remained less than 2 fold except for the -386/-1 region of the proximal promoter where it reached 2.7 (2.7 ± 0.8). Nevertheless, this value was significantly lower than those obtained in CAPAN-2 cells (3.8 ± 0.57, p<0.05, Student's t test).

To check whether PEA3 transactivating effect on *MUC4* promoter is shared by other members of its sub-family, co-transfection studies were carried out using expression vectors encoding ER81 or ERM both in CAPAN-1 and CAPAN-2 cells. Three fragments of *MUC4* promoter corresponding

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to either the -3135/-2307, -219/-1 or -461/-1 region were chosen. In CAPAN-1 cells, ER81 and ERM did not transactivate the proximal nor the distal regions of *MUC4* promoter thus demonstrating an effect restricted to PEA3. On the contrary, in CAPAN-2 cells ER81 and ERM are at least as potent transactivators as PEA3 on the 3 regions of *MUC4* promoter (data not shown).

We then checked whether PEA3 could bind to the P<sub>216</sub> binding site by gel retardation analysis. As shown in figure 4A one retarded complex (lanes 1 and 6, arrow #1) was obtained when incubating CAPAN-1 or CAPAN-2 nuclear extracts with the T<sub>76</sub> radiolabelled probe containing the P<sub>216</sub> site (5'-AGGAA-3'). Competition experiments carried out with a 100-fold excess of cold T<sub>76</sub> oligonucleotide led to a substantial decrease of the shifted band (lanes 2 and 7) whereas an oligonucleotide with a mutated PEA3 binding site (T<sub>76MT</sub> : 5'-ACCAA-3') did not modify the binding (lanes 3 and 8). Therefore the data indicate that an Ets protein is involved in the formation of the retarded complex. Surprisingly, no supershift was observed when an anti-PEA3 antibody was added in the binding reaction (lanes 4 and 9) instead a strong increase of the retarded band was noticed, being more pronounced with CAPAN-2 nuclear extracts (compare lane 9 to lane 6, and lane 4 to lane 1). The addition of an irrelevant antibody (anti-ErbB-2) to the medium did not modify the retarded band formation (lanes 5 and 10). To show that PEA3 binds directly to the P<sub>216</sub> site, we produced a fusion protein encoding a HA-mPEA3 fusion protein and checked its ability to bind to the T<sub>76</sub> probe. As shown in figure 4B, the high mobility band (arrow #2, lane 2) represents a specific DNA-protein complex. The upper band is not specific as it was also found in the control reaction containing no added DNA plasmid (lane 1, n.s.). The specificity of complex #2 was confirmed by performing cold competition with a 100-fold excess of T<sub>76</sub> that led to a total loss of the shifted band (lane 3). On the contrary, when the T<sub>76MT</sub> oligonucleotide with a mutated PEA3 binding site was used, the intensity of the shift band decreased very slightly (lane 4). This shows that PEA3 can bind specifically to the P<sub>216</sub> site in *MUC4* promoter.

#### **c-Jun and Sp1 transactivating effects on the promoter of *MUC4***

PEA3 is often considered as a weak transcription factor that needs to cooperate with partners such as Sp1, c-Jun to efficiently transactivate its target genes [14]. Moreover, both the proximal and

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distal promoters of *MUC4* have several consensus putative *cis*-elements for AP-1 and Sp1 transcription factors (see Fig. 2). To show whether Sp1 and/or AP-1 cooperate with PEA3 to activate *MUC4* transcription, we cotransfected the -461/-1 and -219/-1 regions of the proximal promoter and the -3135/-2307 region of the distal promoter with expression vectors encoding PEA3, Sp1 or c-Jun, respectively (Fig. 5). The -461/-1 region that is the most responsive to PEA3 in CAPAN-2 cells (see Fig. 3A) is also stimulated by Sp1 (3-fold induction; Fig. 5A, black bars) or c-Jun (*idem*). Interestingly, coexpression of Sp1 greatly increases the transactivating effect of PEA3 on the -461/-1 region in CAPAN-2 cells (12-fold;  $p < 0.001$  versus PEA3 alone). This is not observed on the shorter -219/-1 region (Fig. 5B) suggesting that the upstream Sp1 and the P<sub>349</sub> PEA3 *cis*-elements (Fig. 2) are required for this synergistic effect. An additive effect is seen between c-jun and PEA3 on the -461/-1 region ( $p < 0.05$ ) becoming synergistic on the shorter -219/-1 in CAPAN-2 cells (10-fold induction,  $p < 0.01$ ). Since this last region does not contain a canonical AP-1 *cis*-element, these data suggest that c-Jun acts indirectly probably by increasing PEA3 binding to the P<sub>216</sub> binding site as previously proposed [24]. On the contrary, Sp1 does not increase the transactivating effect of PEA3 on the -461/-1 or the -219/-1 regions in CAPAN-1 cells (Figs. 5A & 5B, white bars). A weak synergistic effect is noticed between c-Jun and PEA3 on the -219/-1 region in these cells (9-fold induction). In PANC-1 cells (hatched bars), the weak transactivating effect of PEA3 on the 2 proximal regions of *MUC4* promoter is not modified by the coexpression of either Sp1 or c-Jun. Taken together, these results indicate that strong synergistic effects between PEA3/Sp1 or PEA3/c-jun occur on *MUC4* proximal promoter in CAPAN-2 cells.

Concerning the distal promoter of *MUC4*, a 4-fold activation by c-Jun is obtained on the -3135/-2307 region in CAPAN-1 cells (Fig. 5C) making this transcription factor as potent as PEA3. Interestingly, c-Jun and PEA3 act in synergy to transactivate this region both in CAPAN-1 (17-fold induction,  $p < 0.001$ ) and CAPAN-2 cells (10-fold,  $p < 0.05$ ). This effect is not seen on the -3135/-2572 region (data not shown). Sp1, alone or with PEA3, does not modify the luciferase activity driven by the -3135/-2307 region in either cell line (Fig. 5C). Taken together, these results outline the important role of the P<sub>2368</sub> PEA3 site and of the two adjacent AP-1 *cis*-elements (see Fig. 2) to convey synergistic activation of *MUC4* distal promoter in both WD cell lines. c-Jun could either act

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directly by binding to its *cis*-elements and/or indirectly by increasing the binding of PEA3 to its cognate element.

### **PEA3 and its partners regulate *MUC4* mRNA levels in pancreatic cancer cells**

The response of the -3135/-2307 region to PEA3 and c-Jun in CAPAN-1 and CAPAN-2 cells, and those of the -461/-1 and -219/-1 regions to PEA3 and Sp1 and PEA3 and c-Jun in CAPAN-2 cells respectively, prompted us to evaluate whether these two combinations of transcription factors also affected the level of endogenous *MUC4* mRNA in WD pancreatic cancer cells. To this end, we transiently cotransfected these cells as described before and isolated total RNA 48h later. RT-PCR (Fig. 6, upper panel) and the densitometry analysis of the bands (Fig. 6, lower panel) show that (i) PEA3, Sp1 and c-jun alone did not modify *MUC4* mRNA levels in both WD cell lines; (ii) PEA3 + Sp1 strongly increased *MUC4* expression in CAPAN-1 cells by 221%; (iv) PEA3 + c-Jun increased *MUC4* expression by 160% both in CAPAN-1 and CAPAN-2 cells. None of these combinations was able to induce the expression of *MUC4* in the PD PANC-1 cells (data not shown). The absence of induction of endogenous *MUC4* expression with 0.2 µg of PEA3 expression vector was unexpected, and thus thought to be dose-dependent. Therefore higher amounts of the expression vector (0.5, 1 or 2 µg) were assayed and the level of *MUC4* mRNA was measured as previously described. The results confirmed that hypothesis as in CAPAN-1 cells a high activation (5.7 fold) was obtained with 1 µg of PEA3 expression vector. In CAPAN-2 cells, an induction was also observed but remained weaker with a maximal value of 2.1 fold with 1 µg of expression vector. In conclusion, our results demonstrate that PEA3 alone, in a dose-dependent manner, or in association with c-Jun and/or Sp1, is a potent activator of *MUC4* expression in the WD CAPAN-1 and CAPAN-2 cells.

### **PEA3 regulates *MUC4* apomucin expression in pancreatic cancer cells.**

Having shown that PEA3 transactivates *MUC4* promoter in CAPAN-1 and CAPAN-2 cells and induces *MUC4* mRNA levels in these cell lines, we undertook to evaluate its effects at the protein level. To this end, cells were transfected with either an empty expression vector or with pSV-HA-

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mPEA3, and then processed for immunostaining with a specific anti-MUC4 antibody. In CAPAN-1 mock cells, 38 % of the cells were positively labeled with anti-MUC4 antibody (Fig. 7A). The staining was restricted to the cytoplasm. Transfection with PEA3 expression vector led to an increase of the percentage of positive cells to 60% accompanied by an increase of the staining intensity (Fig. 7C). In CAPAN-2 mock cells, 53 % of the cells were positively labeled with the anti-MUC4 antibody (Fig. 7A), a percentage that was not significantly modified to 56 % in the PEA3-transfected cells (Fig. 7D). The immunostaining intensity was weaker in CAPAN-2 cells than in CAPAN-1 cells. In conclusion, our results showed that PEA3 is also an activator of MUC4 apomucin expression in CAPAN-1 cells.

### **PEA3 and its partners are expressed in pancreatic cancer cells**

Having demonstrated a strong correlation between the transactivating effect of PEA3 on *MUC4* promoter activity (Fig. 3A) and the level of *MUC4* mRNA expressed by the three pancreatic cancer cell lines (Fig. 1), we undertook to determine the level of expression of PEA3 and its partners in these cells. As previously shown, PEA3 is found at higher levels in the nuclear extracts of the WD cell lines than in those of the PD PANC-1 cells. These results suggest a cytosolic sequestration of PEA3 in PANC-1 cells that may prevent it to exert its transcriptional effects. Moreover, since the different levels of *MUC4* expression in the three cell lines may be due to a different pattern of expression of c-Jun, and/or Sp1 that cooperate with PEA3, we determined their levels of expression in the same nuclear extracts by Western-blotting (Fig. 8). c-Jun is mainly found under the dimeric form (band b) in the three cell lines. Interestingly, the intensity of the band corresponding to the dimeric form of c-Jun is much stronger in CAPAN-1 (lane 3) and CAPAN-2 (lane 2) cells than in PANC-1 (lane 1) suggesting that c-Jun is engaged in active transcriptional complexes in *MUC4* positive cell lines. The amount of Sp1 (band c) is similar in the three cell lines.

### **PEA3 down regulates the expression of ErbB-2 in pancreatic cancer cells**

Since MUC4 is the ligand of ErbB-2 we wanted to check whether they were both positively co-regulated by PEA3. To this aim, co-transfection experiments were performed using two proximal



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fragments of the human *ErbB-2* promoter i.e. the p255 and the p537 fragments [19]. The p255 contains a conserved consensus PEA3 binding site (5'-AGGAA-3') near the TATA box and several core elements (5'-GGAA-3') for Ets proteins; the p537 contains one more PEA3 putative binding site nearby a GATA-1 and an AP-1 binding sites [19]. The transfection experiments showed that in CAPAN-1, CAPAN-2 and PANC-1 cells, PEA3 repressed the transcriptional activity of the two fragments of *ErbB-2* promoter in a similar way (Fig. 9A). This suggests that the proximal PEA3 consensus binding site in the p255 fragment is sufficient to mediate PEA3 inhibitory effect on *ErbB-2* promoter. Moreover, the down-regulation of *ErbB-2* promoter by PEA3 is dose-dependent, as increasing amounts of PEA3 repress the promoter activity of the p255 region up to 90% in the three cell lines. In conclusion, these results indicate that in contrast to what was observed on *MUC4* promoter, PEA3 is a potent transrepressor of the human *ErbB-2* promoter in pancreatic cancer cell lines.

The inhibition of *ErbB-2* expression by PEA3 was confirmed at the mRNA level. In CAPAN-1 cells and, to a lower extent in CAPAN-2 cells, endogenous *ErbB-2* mRNA levels substantially decreased when PEA3 was overexpressed in the cells (Fig. 9B). The maximum decrease was 75-80% in CAPAN-1 cells (for 1  $\mu$ g expression vector) and 50% in CAPAN-2 cells (for 2  $\mu$ g expression vector, Fig. 9C). Thus expression level in cells correlated well with regulatory data at the promoter level. When one compared the levels of expression of PEA3, MUC4 and ErbB-2 (Fig. 1) in the 3 cell lines, it came to our attention that the WD CAPAN-1 cells expressed both high levels of PEA3 and MUC4 and the lowest levels of ErbB-2. On the contrary, the PD PANC-1 cells that contained low levels of active PEA3, express ErbB-2 but no MUC4.

## DISCUSSION

The aim of this study was to better understand the mechanisms leading to *MUC4* aberrant expression in pancreatic cancer cell lines, and especially to further characterize the transcription factors and the *cis*-regulatory elements involved in the control of *MUC4* expression. In this study we focused our attention on the Ets transcription factor PEA3 for two reasons. First, both the distal and proximal promoters of *MUC4* contain numerous binding sites for Ets transcription factors, among those the P<sub>216</sub> is conserved in human [12] and rat [13] promoters. Second, PEA3 is a key transcriptional regulator of the proto-oncogene *ErbB-2* [16,17]. Moreover, the fact that ratMuc4 was shown to be a ligand of the rat homologue of ErbB-2 (p185neu) [4] suggests a link between MUC4, PEA3 and ErbB-2.

Our data demonstrate that PEA3 acts as a potent transactivator of both regions of *MUC4* promoter in the *MUC4*-expressing cell lines and that its transactivating effect is correlated to the level of *MUC4* mRNA expressed by these cells. We also showed by EMSA experiments that PEA3 binds to the P<sub>216</sub> PEA3 element of *MUC4* promoter and consequently acts directly. The fact that the interaction between PEA3 and its *cis*-element, is stronger in the presence of an anti-PEA3 antibody could rely on a relief of PEA3 DNA binding autoinhibition by preincubation with the antibody as previously described [25]. These data are in agreement with a recent work showing that PEA3 transactivates the proximal promoter of rat *Muc4* in mammary cancer cells [15]. Thus PEA3 appears as an important factor that could upregulate *MUC4* expression in tumor cells.

Chotteau-Lelièvre *et al.* [26] previously showed using *in situ* hybridization that in adult mice PEA3 expression is restricted to the brain, skeletal muscles and colon while during development PEA3 is widely expressed. An important point was therefore to check PEA3 expression in pancreatic cancer cells in order to document its biological role in the control of *MUC4* expression. Here we show for the first time that PEA3 is expressed in pancreatic cancer cells, as recently shown in human breast [16] and colon cancer cell lines [27]. Moreover, PEA3 is present at high levels in the nuclear compartment of *MUC4* positive WD cell lines allowing it to exert its transactivating effect. In the *MUC4*-negative PD PANC-1 cells, the level of PEA3 mRNA is similar to those observed in the *MUC4* positive cells. However, in PANC-1 cells, PEA3 protein

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is mostly found in the cytosol compartment and did not transactivate *MUC4* promoter. Thus it may be hypothesized that in PANC-1 cells the transduction pathway(s) involved in PEA3 activation, translocation and binding to DNA is/are altered, preventing it from exerting its transactivating effect or that *MUC4* locus is inactivated by epigenetic mechanisms [28]. Bojovic and Hassell [25] have previously suggested that PEA3 is found in mammalian cells in a dynamic equilibrium between 2 states: one able and one unable to bind to DNA. The transition between the active and inactive states depends on post-translational modifications such as phosphorylation [29] and/or on interactions with protein partners. Altogether, the differences seen in the transactivating effect and in the intracellular distribution of PEA3 between *MUC4*-expressing and non expressing cell lines suggest the involvement of PEA3, and possibly of other Ets proteins, in the regulation of *MUC4* transcription in pancreatic tumors. The PEA3-mediated increase of *MUC4* endogenous expression in CAPAN-1 and CAPAN-2 cells was dose-dependent and became efficient at high doses of PEA3. This is in agreement with the fact that PEA3 is considered as a weak transactivator that often cooperates with other transcription factors to regulate the expression of target genes [30]. Among the already known partners of PEA3, we focused our attention on Sp1 and c-Jun transcription factors. C-Jun is a major member of the AP-1 family, which regulates a variety of cellular processes such as proliferation, differentiation and apoptosis [31]. Our results indicate that PEA3 and c-Jun strongly transactivate the distal region -3135/-2307 of *MUC4* promoter in CAPAN-1 and CAPAN-2 cells and the proximal region -219/-1 in CAPAN-2 cells. The activation of the promoter was associated with an increase of endogenous *MUC4* expression in these cells, especially in CAPAN-1 cells. Moreover, the fact that the amounts of c-Jun engaged in active transcriptional complexes are higher in CAPAN-1 and CAPAN-2 than in PANC-1 cells further document the key regulatory role of this factor in *MUC4* expression. The underlying mechanisms resulting in different levels of c-Jun activity are currently unknown. Differences in the activity of c-Jun N-terminal kinase (JNK) pathway, responsible for the activation of c-Jun by phosphorylation [31], seem unlikely since the MEKK1-JNK pathway is active in PANC-1 cells [32]. An involvement of the extracellular signal-regulated kinase (ERK) pathway that is known to play an important role in PANC-1 proliferation [33] could not be currently ruled out. Interestingly, both PEA3 and its co-

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factor c-Jun transcriptional activities are regulated by the MAPK pathway. JNK can induce AP-1 activity by phosphorylation of c-Jun [31]. Similarly, PEA3 activity is positively regulated by the ERK and JNK pathways [29]. Further studies are required to determine the nature of the signaling pathway(s) that regulate PEA3 activity in pancreatic cancer cells and whether PEA3 is a downstream target gene of ErbB-2.

Our results demonstrate that PEA3 transactivates the *MUC4* promoter in cooperation with c-Jun in WD pancreatic tumor cells. These results are consistent with previous data showing that c-Jun synergizes with PEA3 to transactivate the matrilysin promoter in kidney epithelial cells [27]. Moreover the activity of several promoters including those of metalloproteinase-13 [25], and cyclooxygenase-2 (*COX-2*) [34] requires closely spaced *cis*-elements for PEA3 and AP-1 suggesting that these transcription factors function synergistically. PEA3 alone induces the expression of several metalloproteinase genes such as collagenase-IV by MCF-7 cells, thus promoting the degradation of the ECM [35]. Therefore, the Ets transcription factors and especially PEA3 are involved in the invasiveness of tumor cells [36]. However the function of PEA3 may not be limited to this step of tumorigenesis, since it also regulates the expression of other genes such as *COX-2* [34], *ErbB-2* [16-17] and *MUC4* (this report), which are known to modulate cell proliferation and/or apoptosis [37,38]. Our working hypothesis was that PEA3 may induce the expression of both *MUC4* and *ErbB-2* simultaneously thereby promoting the formation of a stable complex between these two proteins at the membrane level and consequently the phosphorylation of ErbB-2, followed by activation of intracellular signaling pathways. These pathways are either linked to epithelial cell proliferation and cell transformation [38] or to epithelial cell differentiation, depending on the presence of neuregulin, an ErbB-3 ligand [39].

In normal epithelial cells, rat Muc4 is apically localized [39,40] whereas the ErbB-2 tyrosine kinase receptor is present at the basolateral membrane together with the other ErbB receptors. This localization depends on a specific interaction between the C-terminal end of ErbB-2 and the PDZ domain of ERBIN [41], and occurs only when ErbB-2 is not phosphorylated. Recently Ramsauer *et al.* [40] demonstrated that rat Muc4 was able to trigger ErbB-2 translocation from the basolateral to the apical membrane of polarized human colon carcinoma Caco-2 cells. This

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relocalization prevents the receptor to interact with partners (other ErbB receptors) to form an array of homo- and heterodimers and promotes the differentiated state of the cell. Interestingly, our results indicate that PEA3 could act as both an activator of *MUC4* expression and a repressor of *ErbB-2* expression in WD pancreatic cancer cells. Such a repression of ErbB-2 expression by PEA3 has already been observed in breast and ovarian cancer cell lines [17]. This inhibitory function seems to be restricted to PEA3 since ESX (Epithelial restricted with Serine boX)/Elf-3, an epithelial-specific Ets protein, transactivates the *ErbB-2* promoter in mammary cells [42], and is capable of conferring aggressive and metastatic properties to the non tumorigenic human mammary epithelial MCF-12A cells [43]. Moreover, Brembeck *et al.* [44] have previously demonstrated that ESX suppresses the promoter activity of keratin 4, that plays a key role in the early differentiation of the esophageal squamous epithelium. These data suggest a link between up-regulation of ErbB-2, promotion of cell transformation and inhibition of differentiation. By contrast, the stable expression of PEA3, that represses *ErbB-2* promoter activity in breast cancer cells [17], did not induce a cell transformation in MCF-12A cells [43]. Therefore, several lines of evidence suggest that PEA3 down-regulates *ErbB-2* oncogene expression in tumor cells. The fact that concomittantly, PEA3 up-regulates the expression of *MUC4*, an epithelial marker merely expressed in differentiated cells [39,40] suggests that PEA3 promotes the differentiation of pancreatic cancer cells. Our results thus favor the hypothesis that PEA3 could be one of the key regulators of the differentiation/proliferation balance in pancreatic cancer cells (Fig. 10).

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#### **ACKNOWLEDGEMENTS**

We are indebted to Danièle Petitprez, Dominique Demeyer, Christine Mouton, Annette Leclercq and Brigitte Hémon for their excellent technical help. We also thank Drs. S. Aubert and M.C. Copin for their help in interpreting the immunohistochemical data. This work was supported by grants from l'Association de Recherche contre le Cancer (n°5785 to IVS) and le Comité du Pas-de-Calais de la Ligue Nationale contre le Cancer (to IVS). N. Jonckheere is a recipient of a Centre Hospitalier Régional Universitaire de Lille – Région Nord Pas-de-Calais Ph.D. fellowship and V. Fauquette of a Ministère de la Recherche et de la Technologie Ph.D. fellowship.

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## LEGENDS TO FIGURES

- **Figure 1**

(A) **Analysis of the expression of *MUC4* and *PEA3* mRNAs in pancreatic tumor cells by RT-PCR.** Cells were cultured in 75 cm<sup>2</sup> flasks to 80% confluence. RT-PCR was performed using specific primer pairs as described in the Materials and Methods section. 28S or  $\beta$  actin were used as internal controls. PCR products were separated on 1.5% agarose gel containing ethidium bromide in 1X TBE buffer. The size of the PCR products were as follows: *MUC4*: 596 bp; 28S: 231 bp;  $\beta$  actin: 661 bp.

(B) **Expression of ErbB-2 and *PEA3* proteins by Western-blotting in pancreatic cancer cells.** Cells were cultured in 75 cm<sup>2</sup> plates at 100% confluence before being scrapped in PBS buffer. Cytosolic and nuclear extracts were prepared as described in Material and Methods and then separated on 10% SDS-PAGE (25  $\mu$ g). Proteins were electrotransferred onto nitrocellulose and the blot was probed with a specific anti-human *PEA3* antibody. For ErbB-2 analysis total cellular extracts were used (see Materials and Methods) and separated by 8% SDS-PAGE (20  $\mu$ g).
  
- **Figure 2**

**Schematic representation of the proximal and distal regulatory regions of *MUC4* promoters.** The numbering refers to the A of the first ATG, and the main *cis*-elements for Sp1, AP-1, AP-2 and *PEA3* (Pxxx) are shown. The broken arrows represent the transcriptional start site locations. The nucleotide sequence and the orientation of the *PEA3 cis*-elements are boxed.
  
- **Figure 3**

***PEA3* transactivates *MUC4* promoter in a cell-specific manner in pancreatic tumor cell lines.** (A) The *MUC4*-pGL3 deletion mutants were transiently transfected in CAPAN-1, CAPAN-2 and PANC-1 cells in the presence of 0.2  $\mu$ g of pSV-m*PEA3* expression vector or empty pSV. The MT -219/-1 fragment represents the -219/-1 region in which the *PEA3 cis*-element P<sub>216</sub> was mutated. Results are expressed as fold induction of luciferase activity relative to the empty expression vector. Values are the mean of 3 independent experiments where cotransfections were run in triplicate. The error bar represents the standard deviation (SD) of the mean.  $\blacktriangle$ , p<0.05; \*, p<0.01. (B) Effect of mutation of the P<sub>216</sub> site on the luciferase activity driven by the -219/-1 region in CAPAN-1 and CAPAN-2 cells. The luciferase activity of the wild type region (WT -219/-1) was arbitrarily set at 100%. Results are expressed as the mean and SD of 3 independent experiments.

- **Figure 4**

**DNA binding of PEA3 to the P<sub>216</sub> cis-element of MUC4 proximal promoter.**

(A) Nuclear extracts from CAPAN-1 and CAPAN-2 cells were used in EMSA experiments as described in the Material and Methods. A specific retarded complex (arrow #1) was seen both in CAPAN-1 (lane 1) and CAPAN-2 cells (lane 6). Specificity was assessed by addition of a 100-fold excess of a unlabelled wild type probe (lanes 2 and 6), or mutated probe (lanes 3 and 7). Supershift experiments were carried out by adding 2 µl of an anti-PEA3 antibody (lanes 4 and 9) or an anti-ErbB-2 antibody (lanes 5 and 10).

(B) The DNA binding activity of a full-length mouse PEA3 protein was evaluated by EMSA. 5 µl of the transcription-translation reaction performed without plasmid DNA (control reaction, lane 1) or 5 µl of mPEA3 protein (lanes 2 and 3) were incubated with a radiolabelled T76 probe. A non specific DNA-protein complex (# n.s.) is observed both in lanes 1 and 2. The specific complex (#2) corresponds to the high mobility band that disappeared upon addition of an excess of cold probe (lane 3) but decreased only weakly upon addition of an excess of the mutated probe (lane 4).

- **Figure 5**

**PEA3 acts in synergy with c-Jun or Sp1 to transactivate different regions of MUC4 promoter.** PEA3, Sp1, and c-Jun were transiently cotransfected alone or in combination in pancreatic tumor cells with fragments encoding the -461/-1 (A), -219/-1 (B) or the -3135/-2307 (C) regions of MUC4 promoters. Results are expressed as fold induction relative to the empty expression vector. Values are the mean of 3 independent experiments where cotransfections were run in triplicate. ▲, p<0.05; \*\*, p<0.001 versus PEA3 alone.

- **Figure 6**

(A) **Effects of c-Jun, Sp1 or PEA3 alone or in combination on the expression of endogenous MUC4.** Cells were cultured in 40 mm dishes at 40 % confluence before being transfected with 0.2 µg of expression vectors encoding PEA3, Sp1 and/or c-Jun or the empty expression vector (C: control). Analysis of MUC4 mRNA was assessed by RT-PCR as described in Materials and Methods. 28S was used as an internal control.

(B) **Representation of the level of MUC4 mRNA expressed by the cell lines.** Results are expressed as the MUC4/28S ratio after densitometry analysis of the bands. The ratio obtained with the empty expression vector was set at 1. The data shown are the mean ± range of 2 independent experiments.

- **Figure 7**  
**Effect of PEA3 on MUC4 apomucin expression in CAPAN-1 and CAPAN-2 cells.** Immunohistochemistry was performed as described in the Materials and methods section. CAPAN-1 cells transfected by 4  $\mu$ g of a control empty vector (A) or PEA3 expression vector (C). CAPAN-2 cells transfected by 4  $\mu$ g of a control empty vector (B) or PEA3 expression vector (D). Magnification x 200.
- **Figure 8**  
**Analysis of the expression of c-Jun and Sp1 in nuclear extracts of PANC-1 (lane 1), CAPAN-2 (lane 2) and CAPAN-1 (lane 3) cells by Western Blot.** Nuclear proteins (20  $\mu$ g) were separated on 10% SDS-PAGE before being transferred onto nitrocellulose. The blot was stained using specific anti c-Jun or anti Sp1 antibody. Migration of the molecular weight (MW) standard is shown on the right hand side. The arrows point at (a) the monomeric c-Jun (around 40 kDa); (b) dimeric c-Jun; (c) Sp1 protein ( $\approx$ 105 kDa).
- **Figure 9**  
(A) **Effects of increasing amounts of PEA3 expression vector on the promoter activity and the expression of human *ErbB-2* promoter.** Expression vector (0.2 to 2  $\mu$ g) was transiently cotransfected with deletion fragments of *ErbB-2* promoter in CAPAN-1, CAPAN-2 and PANC-1 cells. Results are expressed as in Figure 3A.  $\blacktriangle$ ,  $p < 0.05$ ; \*,  $p < 0.01$  and \*\*,  $p < 0.001$  vs controls.  
(B) **Effects of increasing amounts of PEA3 expression vector on the expression of endogenous *ErbB-2*.** Cells were cultured in 40 mm dishes at 40 % confluence before being transfected with 0.2 to 2  $\mu$ g of expression vectors encoding PEA3 or with empty expression vector. Analysis of *ErbB-2* mRNA was assessed by RT-PCR as described in Materials and Methods. *28S* was used as an internal control.  
(C) **Representation of the level of *ErbB-2* mRNA expressed.** Results are expressed as described in figure 7B and are the mean  $\pm$  range of the 2 independent experiments.
- **Figure 10**  
**Model for the roles of PEA3 both on the regulation of *MUC4* and *ErbB-2* expression in relationship with the proliferation-differentiation balance in pancreatic cancer cells.** (+: stimulating effect, -: inhibiting effect, ?: not determined).

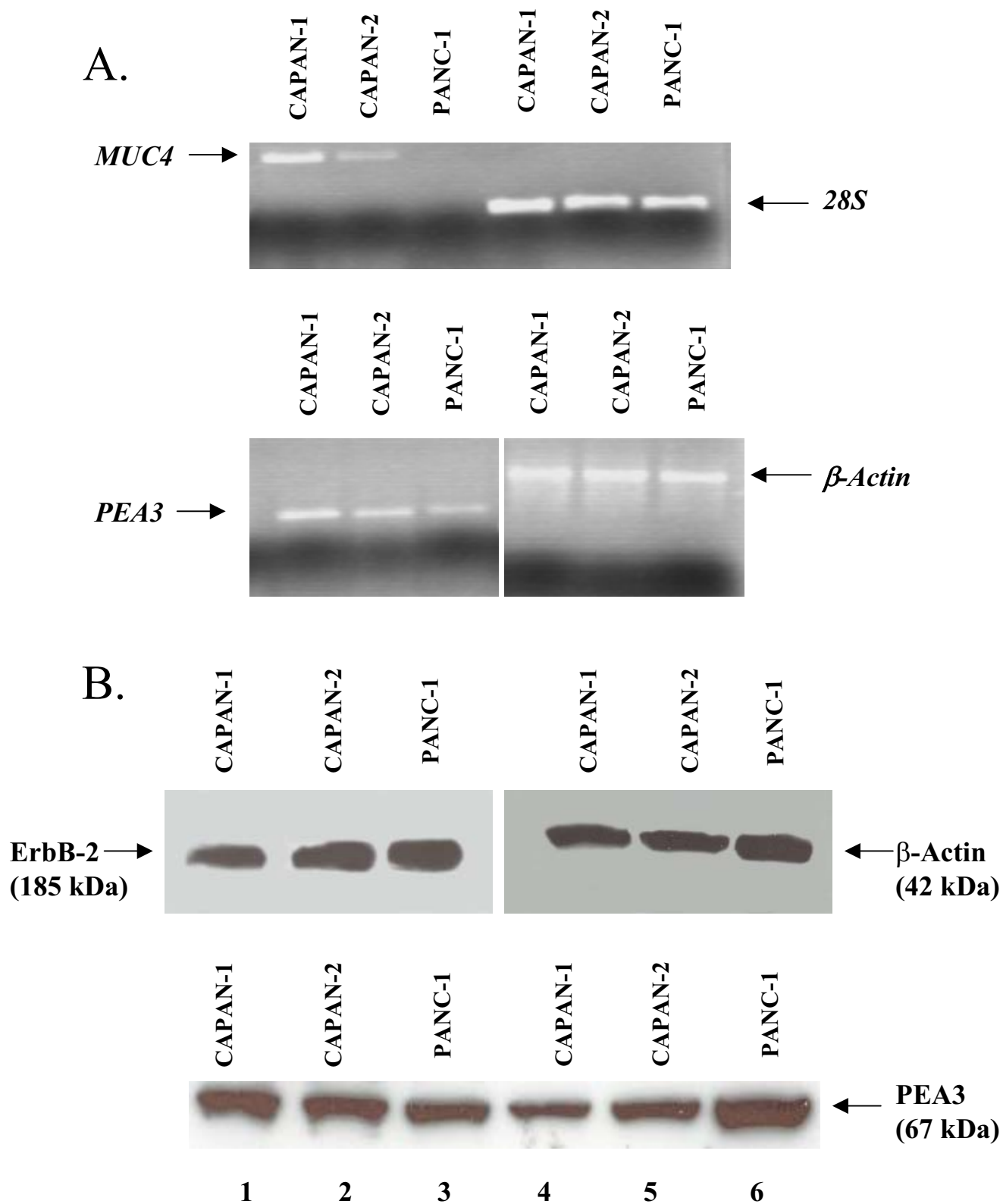


Figure 1

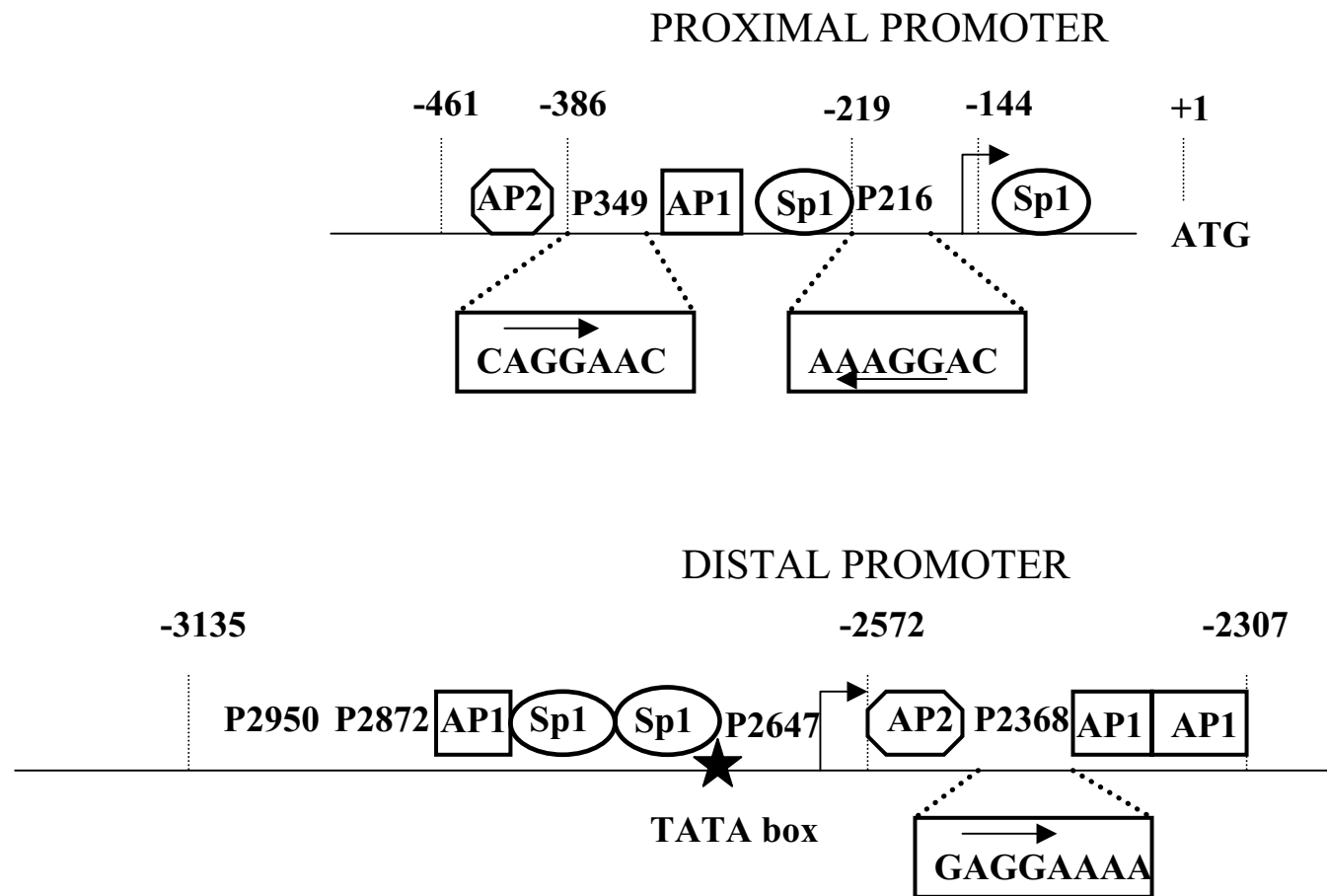


Figure 2

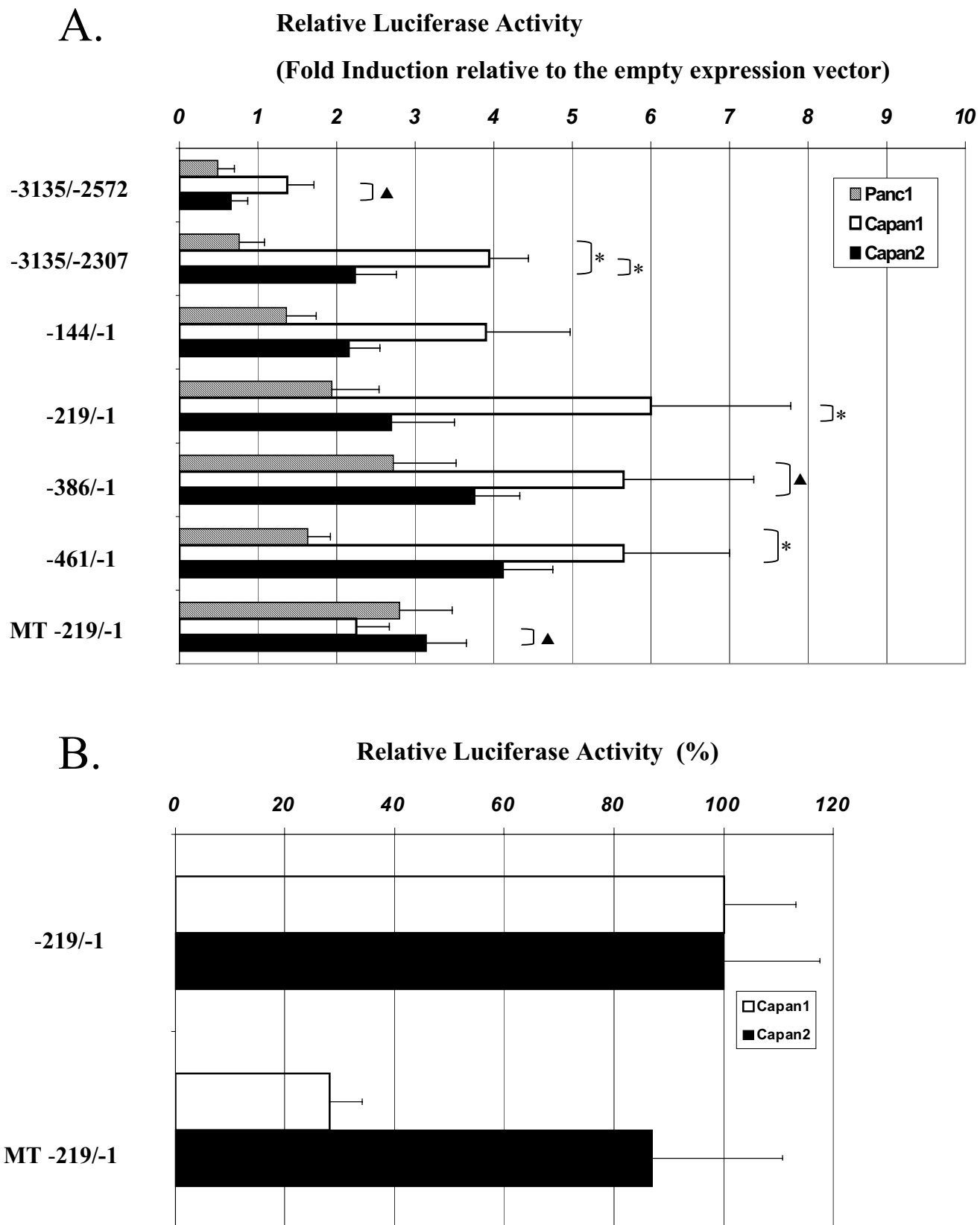


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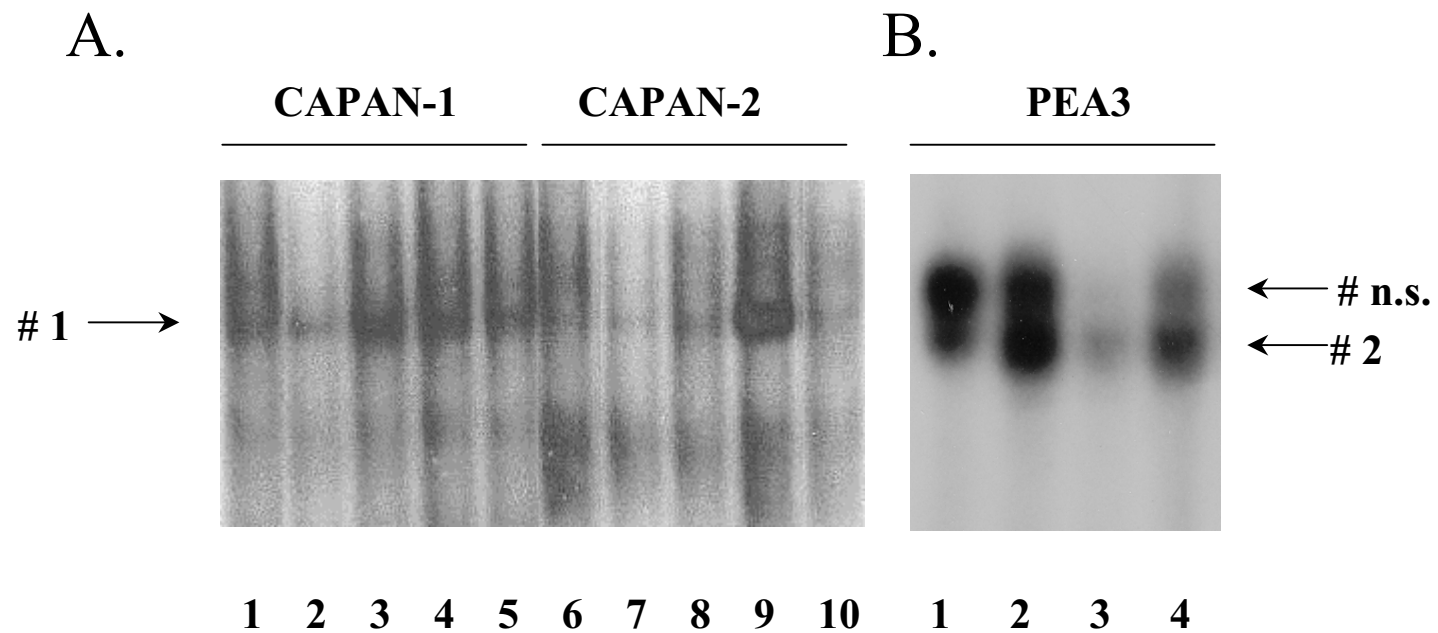


Figure 4



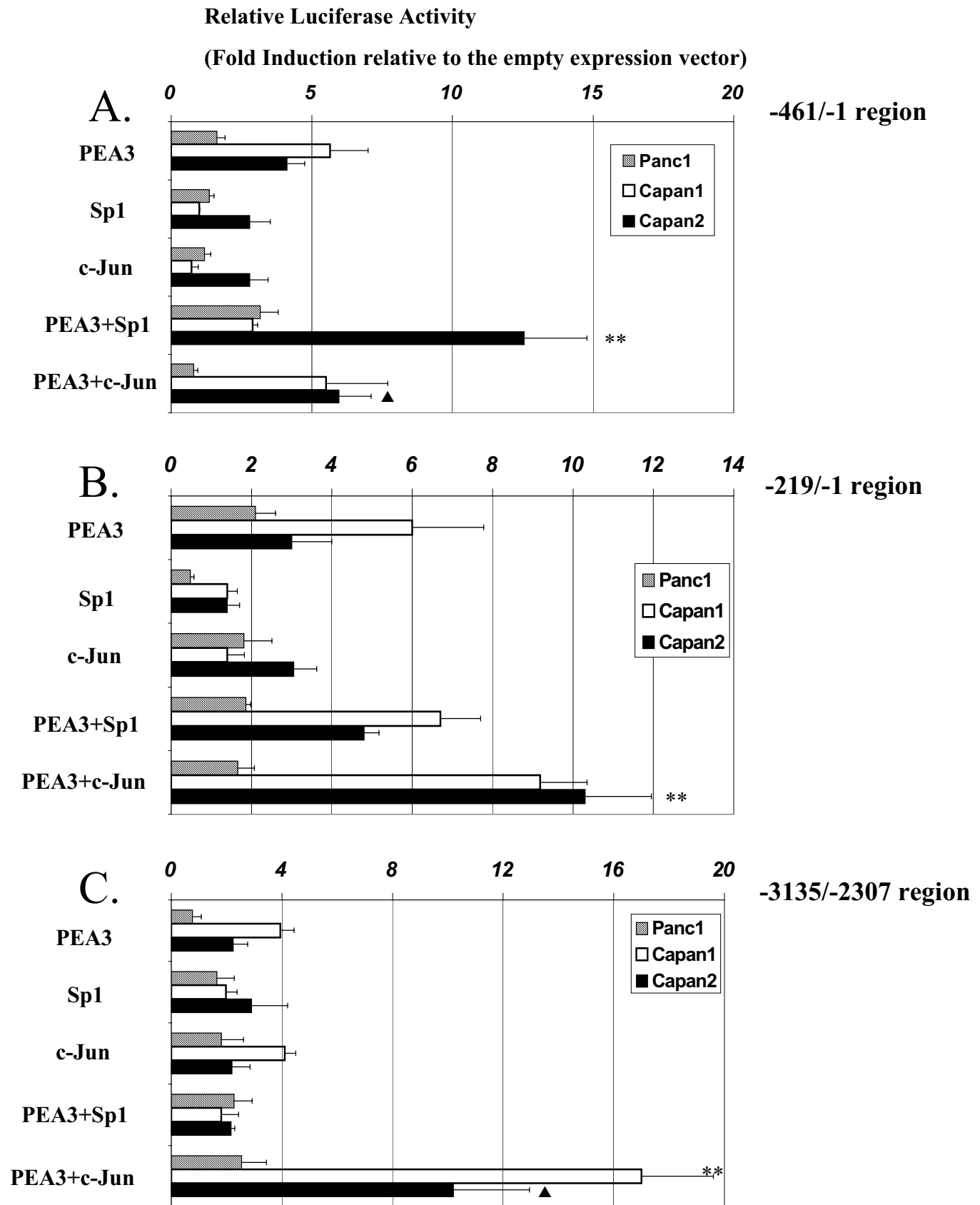


Figure 5

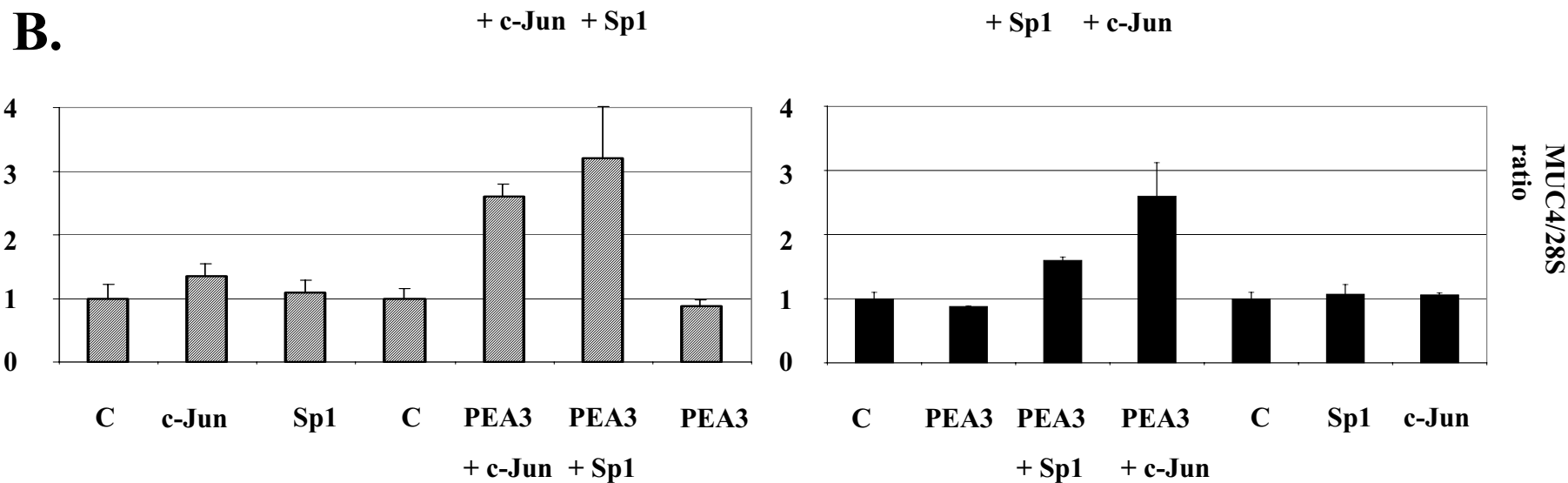
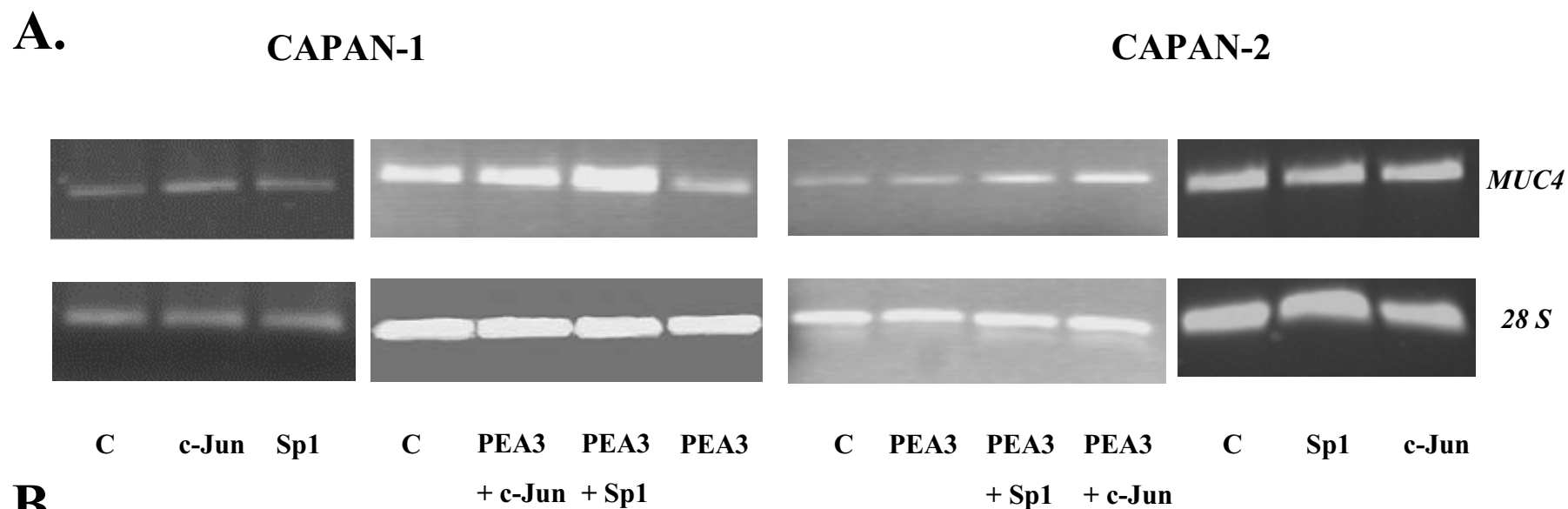


Figure 6

**CAPAN-1**

**CAPAN-2**

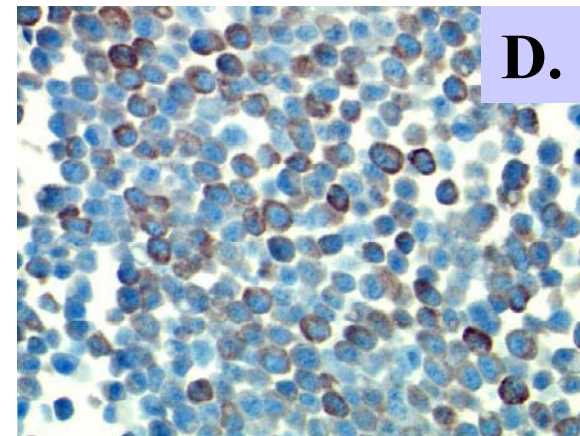
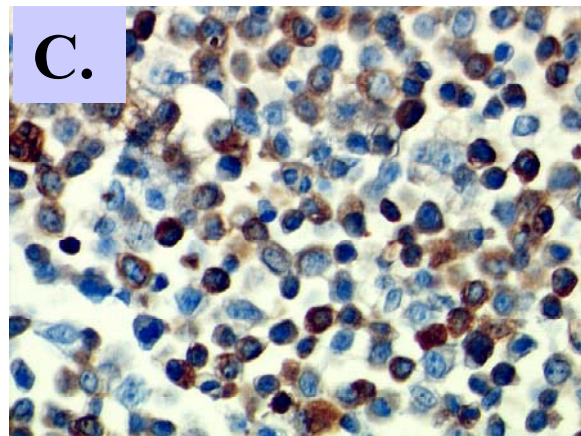
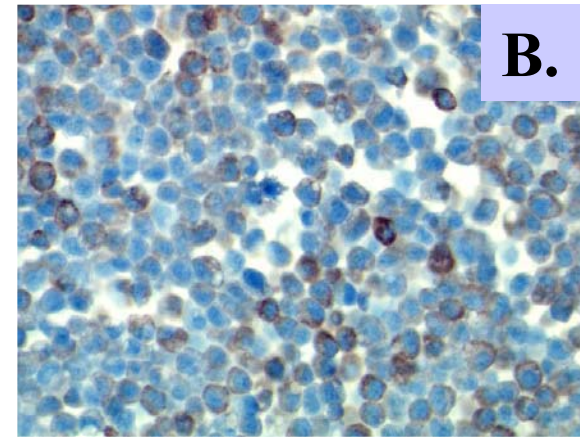
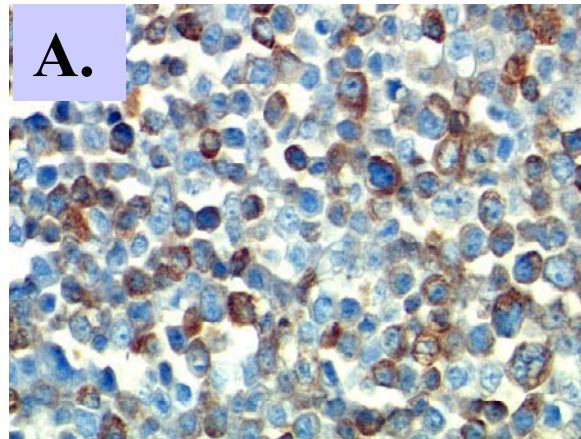


Figure 7

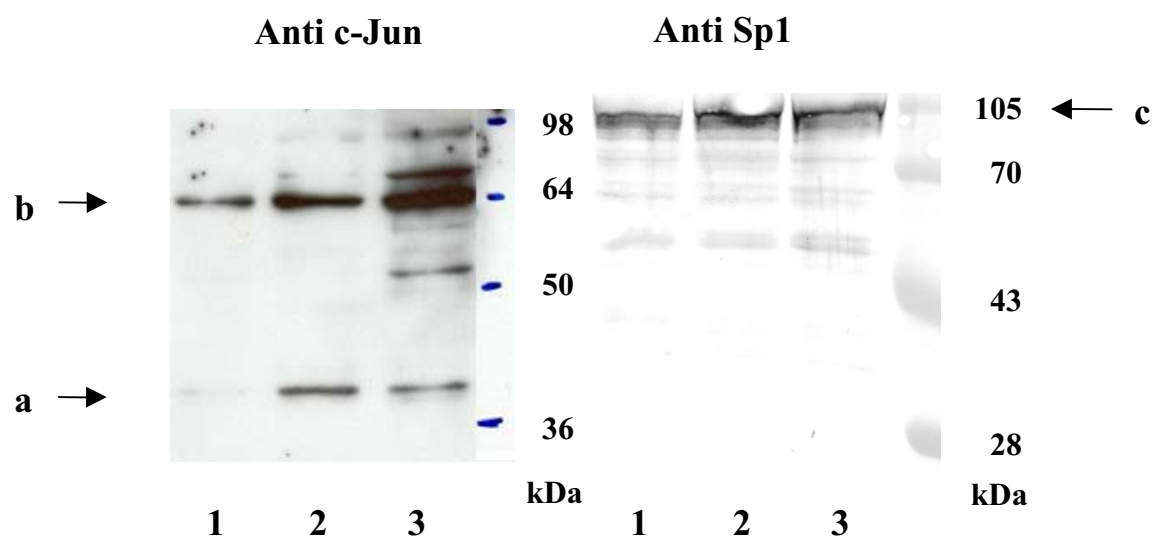


Figure 8

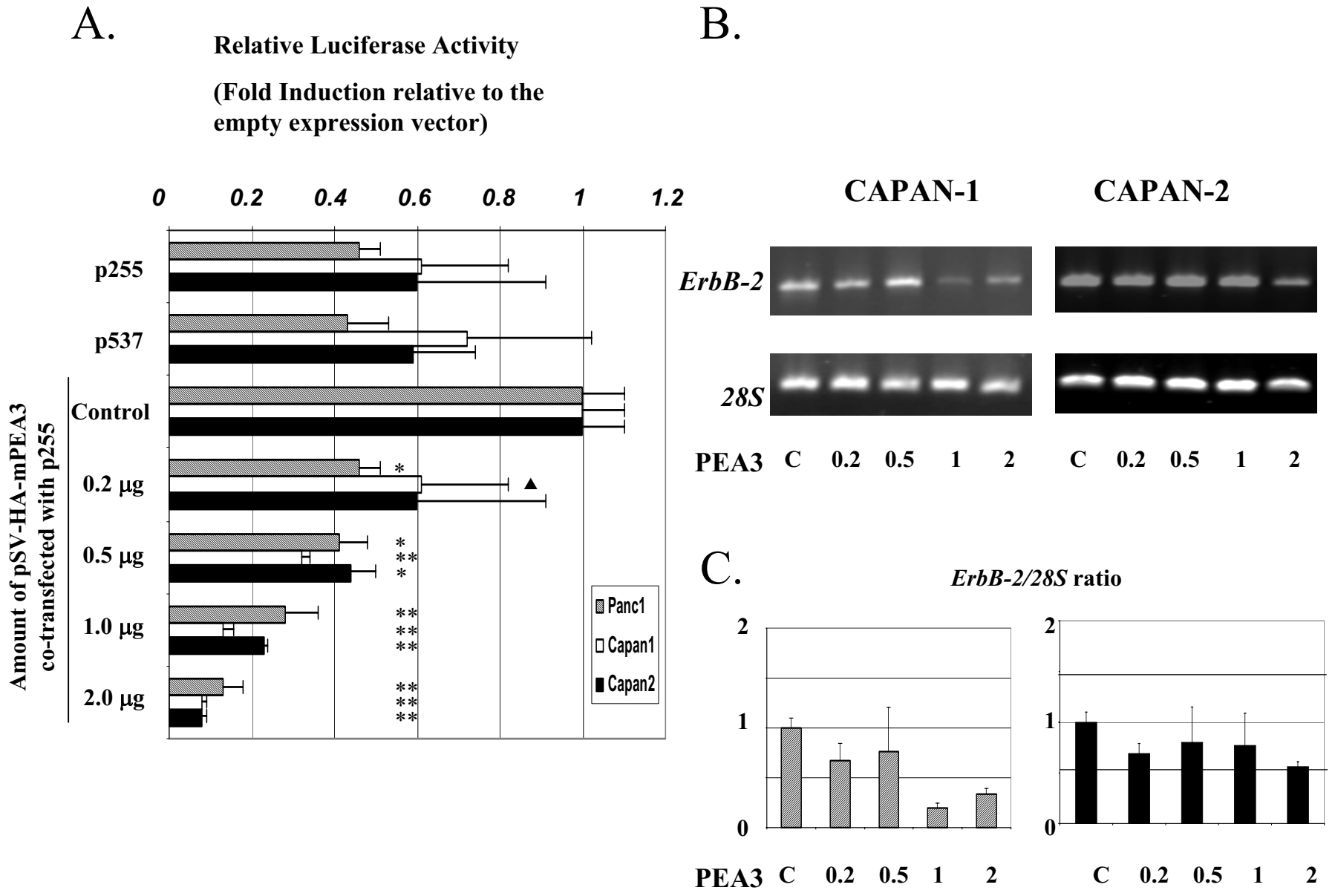


Figure 9

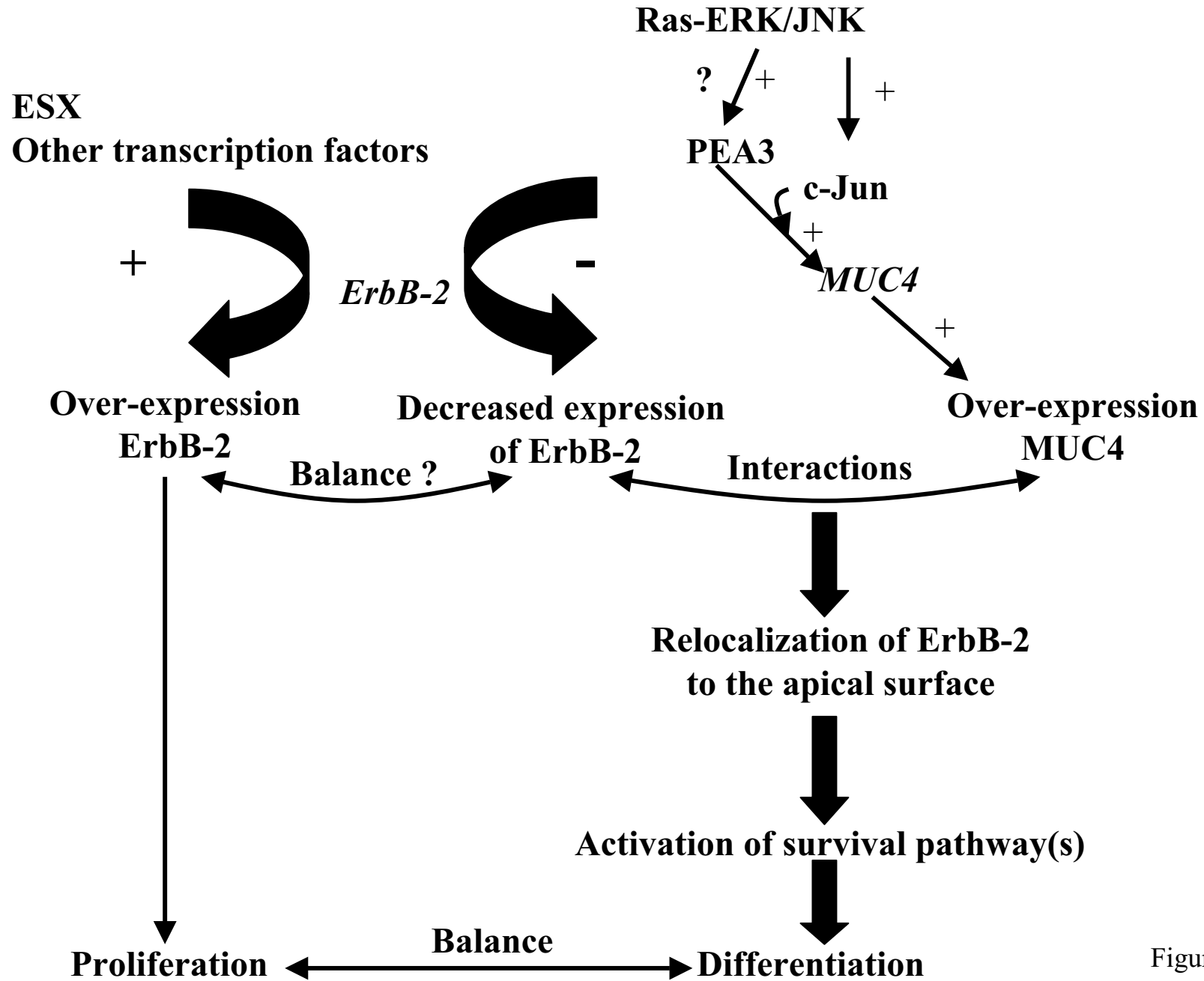


Figure 10