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The mucin MUC4 is a transcriptional and post-transcriptional target of K-ras oncogene in pancreatic cancer. Implication of MAPK/AP-1, NF- κ B and RalB signalling pathways

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Abstract

The membrane-bound mucin MUC4 is a high molecular weight glycoprotein frequently deregulated in cancer. In pancreatic cancer, one of the most deadly cancers in occidental countries, MUC4 is neo-expressed in the preneoplastic stages and thereafter is involved in cancer cell properties leading to cancer progression and chemoresistance. K-ras oncogene is a small GTPase of the RAS superfamily, highly implicated in cancer. K-ras mutations are considered as an initiating event of pancreatic carcinogenesis and K-ras oncogenic activities are necessary components of cancer progression. However, K-ras remains clinically undruggable. Targeting early downstream K-ras signaling in cancer may thus appear as an interesting strategy and MUC4 regulation by K-ras in pancreatic carcinogenesis remains unknown. Using the Pdx1-Cre; LStopL-K-ras^{G12D} mouse model of pancreatic carcinogenesis, we show that the *in vivo* early neo-expression of the mucin Muc4 in pancreatic intraepithelial neoplastic lesions (PanINs) induced by mutated K-ras is correlated with the activation of ERK, JNK and NF-κB signalling pathways. *In vitro*, transfection of constitutively activated K-ras^{G12V} in pancreatic cancer cells led to the transcriptional upregulation of *MUC4*. This activation was found to be mediated at the transcriptional level by AP-1 and NF-κB transcription factors *via* MAPK, JNK and NF-κB pathways and at the post-transcriptional level by a mechanism involving the RalB GTPase. Altogether, these results identify MUC4 as a transcriptional and post-transcriptional target of K-ras in pancreatic cancer. This opens avenues in developing new approaches to target the early steps of this deadly cancer.

Keywords:

MUC4, K-ras, RalB, pancreatic cancer, transcription, AP-1.

1. Introduction

Pancreatic cancer is the 4th cause of death by cancer in the world [1] with a median survival lower than 6 months and an extremely low five-year survival rate (5%). This dramatic outcome can be explained by a late diagnosis and a lack of efficient therapies. At the time of diagnosis, only 10-15 % of patients are eligible for surgical resection. For the other patients (85%), palliative gemcitabine-based chemotherapy remains the main option [2] but radio/chemo-resistance is frequent in pancreatic carcinoma (less than 30% of patients respond positively). Pancreatic carcinogenesis follows a hyperplasia/metaplasia/*in situ* carcinoma/invasive carcinoma development sequence. Pancreatic ductal adenocarcinoma (PDAC), the most frequent type of exocrine pancreatic cancer (85%), arises from ductal precursor lesions called Pancreatic Intraepithelial Neoplasia (PanIN), graded from hyperplasia (PanIN-1A/B) to *in situ* carcinoma (PanIN-3) [3]. Understanding mechanisms of initiating and progression of PanINs into adenocarcinoma may lead to identification of new therapeutic targets and diagnostic markers. Among histologic, cytologic and genetic alterations characterizing PanINs, K-ras mutations [4] and neoexpression of the mucin MUC4 have been described to occur as early as PanIN-1A stage in humans [5].

K-ras, a member of the Ras protein family is a small (21 kDa) guanosine tri-phosphate hydrolase protein (GTPase), ubiquitarily expressed [6]. Ras protein is a transducer of external stimuli from growth factors to signalling pathways through plasma membrane (notably tyrosine kinase receptors). Ras plays important roles in proliferation, apoptosis, senescence, migration, survival or angiogenesis. K-ras cycles between two states: an inactive state, bound to guanosine di-phosphate (GDP), and an active state, bound to GTP. Mutated K-ras is blocked into GTP-bound state and loses its ability to return to an inactivated state, inducing an upregulation of numerous downstream signalling pathways and cancer progression [7]. K-ras mutations are frequently observed in cancer (30% of cancer), especially in pancreatic cancer where mutations in K-ras (mainly at codons 12 and 13) are nearly systematic (> 90%) [8]. Moreover, mutated K-ras is detected as early as PanIN-1A and participates in both initiation and progression of pancreatic carcinogenesis [9]. However, K-ras is still an undruggable clinical target [10].

Mucins are high molecular weight glycoproteins divided in two groups: (i) secreted mucins which participate in mucus architecture and epithelial protection and (ii) membrane-bound mucins that are implicated in cell-cell and cell-extra-cellular matrix interactions. Mucin aberrant overexpression is often associated with tumor progression [11]. MUC4 is a membrane-bound mucin expressed at the surface of epithelial cells in digestive-, respiratory- and reproductive tracts. Moreover, MUC4 is neo-expressed in pancreatic carcinogenesis as early as PanIN-1A stage with a gradual increase toward pancreatic adenocarcinoma, whereas it is not expressed in healthy pancreas [5].

In pancreatic cancer, MUC4 expression is associated with a poor prognosis [11]. It has been shown that MUC4 is involved in biological properties of cancer cells. Recent studies in our laboratory showed a direct interaction between MUC4 and its membrane partner, the oncogenic receptor ErbB2, also often overexpressed in pancreatic cancer [12]. Additionally, the involvement of MUC4 in gemcitabine resistance

of pancreatic cancer cells [13] and its regulation by miRNAs [14] indicate that the oncogenic mucin MUC4 is a potential cancer biomarker and a promising therapeutic target. Understanding MUC4 regulation in the early steps of carcinogenesis will be useful for future therapies notably in pancreatic cancer.

MUC4 neo-expression and mutation in K-ras oncogene being early concomitant events and K-ras mutation being implicated in initiation of pancreatic carcinogenesis, we hypothesized that K-ras directly regulates MUC4. In this report, we show for the first time that indeed MUC4 is a target of K-ras signalling and that both transcriptional and post-transcriptional mechanisms are involved.

2. Material and methods

2.1 Mouse models

Pdx1-Cre (C57Bl/6 background) transgenic mice were obtained from the Mouse Models of Human Cancer Consortium (MMHCC, USA). LStopL-Kras^{G12D} (C57Bl/6 background) transgenic mice were obtained from Dr D. Tuveson (Cold Spring Harbor Laboratory, NY, USA). Mice were maintained as heterozygous lines and crossed to obtain Pdx1-Cre;LStopL-K-ras^{G12D} mice. Genomic DNA was extracted using the Nucleospin Tissue kit (Macherey Nagel, Hoerd, Germany) and Cre and K-ras^{G12D} alleles were analysed by PCR. [Cre: (forward) CCT GGA AAA TGC TTC TGT CCG, (reverse) CAG GGT GTT ATA AGC AAT CCC; K-ras: (forward K ras^{WT}) GTC GAC AAG CTC ATG CGG GTG, (forward LStopL-K-ras^{G12D}) AGC TAG CCA CCA TGG CTT GAG TAA GTC TGC A, (reverse) CCT TTA CAA GCG CAC GCA GAC TGT AGA]. Pdx1-Cre;LStopL-K-ras^{G12D} and Pdx1-Cre;K-ras^{WT} control mice were sacrificed by cervical dislocation at 3, 5, 9 and 12 months of age before dissection. All procedures were in accordance with the guideline of Animal Care Committee (Comité Ethique Expérimentation Animale Nord Pas-de-Calais, #AF042008 and #00422.01).

2.2 Immunohistochemistry

Sections of five micrometers of paraffin embedded pancreatic tissues from Pdx1-Cre;LstopL-K ras^{G12D} mice and Pdx1-Cre;K-ras^{WT} were cut and deparaffinized using graded xylene-ethanol-water baths. Antigen sites were retrieved by heating in citrate buffer pH 6.0 (Dako Real™ Target Retrieval Solution) in a microwave for 20 min at 700 W or in a pressure cooker for 90 s at 100°C followed by endogenous peroxidase inactivation in 3 % (v/v) hydrogen peroxide for 30 min. A permeabilisation/saturation bath was performed by incubation in Teng-T 1X buffer (10 mM Tris-HCl pH=8.0 containing 5 mM EDTA, 150 mM NaCl, 0.25 % (w/v) gelatine, 0.01 % (v/v) Tween® 20, pH 8.0) (permeabilisation and saturation), or in D-PBS-BSA 3 % (w/v) solution (saturation). Thereafter, sections were incubated overnight using anti-Muc4 1G8 (mouse, sc33654 Santa Cruz, 1/200), JNK and phospho JNK (rabbit, 9258 and 9251S, Cell Signaling, 1/100 and 1/50 respectively), p42/44 MAPK and phospho p42/44 MAPK (rabbit, 9102 and 4377, Cell signalling, 1/500 and 1/100 respectively), p65 NF-κB and phospho p65 NF-κB (rabbit, sc7151X and sc33020, Santa Cruz, 1/500 and 1/100 respectively) antibodies at 4 °C. Then, the slides were incubated for 1 h with corresponding biotinylated secondary antibodies followed by incubation with Vectastain Elite solution (PK6100, Vector). Staining was done by incubation in 0.5 mg/ml of 3,3'-diaminobenzidine (DAB), 0.02 % (v/v) H₂O₂ in 30 mM imidazole, and 1 mM EDTA (pH 7.0). A counter-stain with haematoxylin (Sigma) and lithium carbonate was performed. Finally, after dehydration by graded H₂O-ethanol-xylene baths, sections were mounted using Pertex (Monting medium for light microscopy, Histolab). Controls were performed in the same conditions without primary antibodies. Each staining was performed on tissues from five different mice at every age. IHC stainings were scored by an expert gastrointestinal tract (GIT)

pathologist (FR). Results are evaluated as no/weak/strong staining and expressed as % of normal or PanINs stained tissue.

2.3 Cell culture

Human pancreatic cancer cell lines BxPC3 and Capan-2 were cultured as previously described ([50] and [17]). The human immortalised pancreatic cell line HPNE was a gift from D. M. Ouellette (UNMC, Omaha, Nebraska) and was cultured in 1/3 of M-3 Base medium (INCELL) and 2/3 of Dulbecco's modified Eagle's medium (Gibco, Life Technologies) supplemented with 5 % (v/v) of foetal bovine serum (Gibco, Life Technologies), 100 U/ml of penicillin, 100 µg/ml of streptomycin (Invitrogen, Life Technologies) and 1 ng/ml of recombinant EGF (Gibco, Life Technologies). All cell lines were maintained in an incubator at 37 °C with 5 % CO₂.

2.4 Transfection and luciferase assays

Transient transfection experiments were performed 24 h after seeding using Effectene™ (Qiagen) as previously described [51] using 0.25 µg of expression vectors. PBABE-puro-K-ras^{G12V} was obtained from W. Hahn (Addgene plasmid # 9052). pBABE-puro was obtained from H. Land, J. Morgenstern and B. Weinberg (Addgene plasmid # 1764). PZIP and pZIP-ras15A plasmids were obtained from J. Merchant (University of Michigan, USA). NF-κB and AP-1 luciferase assays were performed as previously described using synthetic promoters containing respectively three κB and AP-1 binding sites [13]. In co-transfection studies, 1 µg of pGL3-MUC4 promoter reporter vectors were co-transfected with 0.25 µg of either the expression vector pBABE-K-ras^{G12V} or corresponding empty vector. Results were expressed as fold induction of luciferase activity in cells transfected with K ras^{G12V} expression vector compared with that obtained with the empty vector. Transient inhibition of MUC4 (L-004577-00), K-ras (L005069-00), Erk1 (MAPK3, L-003514-00), Erk2 (MAPK1, L-003555-00), JNK1 (MAPK8, L-003592-00), JNK2 (MAPK9, L-003505-00) and NF-κB (p105 NF-κB, L-003520-00 and L-003918-00) and control non-targeting siRNA (NT, D-001810-02) was carried out using siRNA from Dharmacon (Thermo Scientific) as previously described [52].

2.5 Western blotting

Total protein extracts were performed as previously described [53] and quantified using bicinchoninic acid method following the manufacturer's instruction (Pierce). Western blot experiments were performed as previously described [52] on nitrocellulose membrane (0.2 µm, Schleicher et Shüll, Life Technologies). Membranes were incubated with antibodies against MUC4 (8G7) (1/200, SC-53945 Santa Cruz), phospho-Erk1/2 (Thr202/Tyr204) (1/500, #4276, Cell Signaling), Erk1/2 (1/500, #4696, Cell Signaling), NF-κB Phospho-p65 (Ser536) (1/200, #3033, Cell Signaling), NF-κB p65 (1/500, #E498, Cell Signaling), phospho-SAPK/JNK (Thr183/Tyr185) (dilution 1/500, #9251, Cell Signaling), SAPK/JNK (1/500, #3258, Cell Signaling), β-actin (#A5441, 1/5000, Sigma-Aldrich, St Quentin Fallavier, France). Antibodies were diluted in 5 % (w/v) nonfat dry milk in 1X Tris-Buffered Saline containing Tween-20 (TBS-T), except for MUC4

and β actin that were diluted in 1X TBS-T, and incubated overnight at 4°C. Peroxidase-conjugated secondary antibodies (Sigma-Aldrich) were used and immunoreactive bands were visualized using the Super Signal® West Pico chemoluminescent substrate (Thermo Scientific) and visualized using the LAS4000 apparatus (Fujifilm, Courbevoie, France).

2.6 Real time reverse transcription PCR (qRT-PCR)

Total RNA were extracted using NucleoSpin RNAII kit (Machery-Nagel) 48 h after transfection. Reverse transcription was performed with 1 μ g of RNA extract using M-MLV-RT enzyme (Moloney-Murine Leukemia Virus, Promega) with oligo(dT)₁₅ (20 mM, Promega) and dNTP (2 mM, Eurogentec). QRT-PCR was performed using the SsoFast Evagreen Supermix kit (biorad) as previously described [13].

2.7 Chromatin immune-precipitation assays (ChIP)

For ChIP analysis, 10⁶ cells (for each IP) were fixed with 1 % (v/v) formaldehyde for 10 min at room temperature. Cross-linking was blocked with 0.125 M glycine for 5 min and cells were washed with cold 1X D-PBS (Dulbecco-PBS) and with 1X D-PBS containing protease inhibitors (10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 0.2 mM EDTA and 0.5 mM PMSF). Cells were collected by centrifugation at 685 x g for 5 min at 4 °C and resuspended in lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂ and 0.1 % (v/v) Nonidet P40) containing protease inhibitors and incubated for 10 min on ice. Chromatin extracts were sonicated twice at 200 W with the Bioruptor system (Diagenode) for 10 pulses of 30 s each with 30 s rest between each pulse. Sonicated extracts were centrifuged at 10000 x g for 10 min at 4 °C and the supernatant was collected. An aliquot of the total supernatant was removed as input control and the rest was fractionated and immunoprecipitated overnight on a rotating platform at 4 °C with 3 μ g of the target antibody: c-fos (SC-52), c-Jun (SC-44), p65 NF- κ B (SC-7151X) (Santa Cruz) or normal rabbit IgGs (Upstate Biotechnology). A mixture of magnetic beads coupled to protein G (1/4) and protein A (3/4) (100-02D and 100 03D, Life Technologies) were incubated overnight with 5 volumes of blocking solution (0.5 % (w/v) BSA in 1X D-PBS). For each immune-precipitated extract, 3 h incubation with 100 μ l of beads was performed. Beads were isolated by magnetic field, washed four times with low salt buffer solution (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1 % (v/v) SDS, 1 % (v/v) Triton X-100), washed with high salt buffer solution (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 0.1 % (v/v) SDS, 1 % (v/v) Triton X-100) and 1X TE solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-Na₂). Beads were isolated by magnetic field and centrifuged 3 min at 960 x g to remove supernatant. Cross-linking complexes were eluted following 15 min incubation at 65 °C in 210 μ l of elution buffer (5 mM Tris-HCl, pH 8.0, 25 mM EDTA, 10 % (v/v) SDS) and was vortexed every 2 min. Beads were removed by 1 min centrifugation at 16000 x g and supernatants were transferred into new tubes and 200 μ l of sterile water were added to supernatants and input fraction. Reversion of cross-linking was performed overnight at 65 °C by addition of 8 μ l of 5 M NaCl. Chromatin-associated proteins were digested with Q protease (19155, Qiagen) at 50 °C for 1 h and the DNA was purified with the Wizard® DNA Clean-up System (Promega). Real time PCR analyses were performed with 1 μ l of chromatin using SsoFast Evagreen Supermix kit (BioRad) and the CFX96 real time PCR system

(Bio-Rad) using the following primers: -427/-304 Forward TCT TCC CCA TTC TTC CCA GC, Reverse AGG TCA CAC TGG AAA TGG GT; - 196/-105 Forward GGC TGA GGA GAG AAA AGG GT, Reverse CCA AGT GCG TTT CTC CGA AG; *MUC4* intron6/7 (irrelevant region) Forward GTA GAG AAA CAG GGC ATA GGA and Reverse CCT GTG TTT TCT TCC ACC CAC. Chromatin enrichment was normalized to that of input and expressed as ratio of IgG intensity.

2.8 Statistical analyses

At least three independent experiments were performed using independent biological samples (produced from different transfections). The different graphs show the average of results of at least 3 independent experiments \pm standard error means (SEM).

Statistical analyses were performed using the Graphpad Prism 4.0 software (Graphpad softwares Inc., La Jolla, CA, USA). Differences in data of two samples were analyzed by Student's t-test or ANOVA test with selected comparison using Tukey's post-hoc test, and were considered significant for the following P-values: * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$. For paired tumour and adjacent non-tumour samples, paired t test were performed.

3. Results

3.1 *in vivo* MUC4 expression and *in vitro* upregulation by K-ras

We evaluated Muc4 expression by immunohistochemistry (IHC) in the transgenic K-ras^{G12D} mouse model of pancreatic cancer. The haematoxylin-eosin staining of Pdx1-Cre;K-ras^{G12D} mice shows PanINs formation that increase of grade, size and number with time (3 to 12 months), whereas no PanIN formation is observed in Pdx1-Cre;K-ras^{WT} control mice at any age (Figure 1A). As expected, most of the PanIN formed are early lesions PanIN1A/1B. We show that Muc4 is neo-expressed (apical membrane and cytoplasmic staining) in PanIN cells (weak staining 100%) at every age whereas it is not expressed in healthy pancreatic exocrine tissues from control mice at any age (Figure 1A, supplemental Figure 1A). This expression pattern reproduces MUC4 overexpression in human pancreatic tumours (Supplemental figure 2; [15]).

We then investigated K-ras involvement in *MUC4* transcriptional regulation in BxPC3 (wild type (WT) K-ras) and CAPAN-2 (constitutively mutated activated K-ras^{G12V}) human pancreatic cancer cell lines. Transient overexpression of K-ras^{G12V} or the corresponding control vector was performed. By qRT-PCR analysis, we confirmed K-ras^{G12V} enrichment in BxPC3 (21.3-fold) and in the CAPAN-2 (4.7-fold) cell lines (Figure 1B). As expected, an increased protein level of MUC4 was observed in both transfected cell lines with the G12V activated K-ras mutant (Figure 1C) whereas decreased MUC4 was observed in cells transfected with inactivated form of K-ras (G15A mutant) (Figure 1D). Altogether, these results indicate that *in vivo* K-ras and MUC4 are co-expressed in the same PanIN stages and that *in vitro* activated K-ras oncogene up-regulate MUC4 expression in pancreatic cancer cells.

3.2 MAPK, JNK and NF-κB pathways regulate MUC4 expression

We then checked whether MAPK, JNK and NF-κB pathways, known to be activated by K-ras [16], were altered in the mouse model of pancreatic carcinogenesis. Immunostaining of pancreatic tissues from Pdx1-Cre;LStopL-K-ras^{G12D} and Pdx1-Cre; K-ras^{WT} control mice were performed at 3, 5, 9 and 12 months. We show a nuclear staining of phospho p42/44 MAPK and a nuclear and light cytoplasmic staining of p42/44 MAPK in PanIN cells as early as 3 months of age (39-49% weak staining and 51-61% strong staining) whereas no staining was observed in surrounding normal exocrine tissue and in healthy pancreas of control mice at any age (Figure 2 A-F, supplemental Figure 1B). We also show a gradual increase of phospho-JNK nuclear staining in PanINs between 3 and 12 months of age whereas no staining was observed in PanIN-surrounding normal tissue and in healthy pancreas of control mice at any age (Figure 2 G-I). Total JNK expression showed a nuclear and a light cytoplasmic staining at all ages in both transgenic mice (Figure 2 J-L). Finally, we show a strong nuclear staining of phospho p65 NF-κB in 100% of PanIN cells in Pdx1-Cre;LStopL-K-ras^{G12D} mice and 72-81% weak staining in cells of Pdx1-Cre; K-ras^{WT} control mice. We also observed a gradual increase of p65 NF-κB cytoplasmic staining in PanINs whereas only a light nuclear staining was observed in surrounding normal exocrine tissue and in healthy pancreas of control mice at any

age (Figure 2 M-R, supplemental Figure 1C). Negative control was performed without primary antibodies (supplemental Figure 3). These results show activation of MAPK, JNK, and NF- κ B pathways in PanINs preneoplastic lesions.

Analysis of p42/44 MAPK, JNK and p65 NF- κ B activation was also investigated *in vitro* in CAPAN-2 (Figure 3A) and BxPC3 (supplemental Figure 4) cell lines transfected with K-ras^{G12V} vector or control vector. The results indicate an activation of p65 NF- κ B (3.02-fold) and p42/44 MAPK (1.76-fold) by mutated K-ras^{G12V} but not JNK kinase (0.92-fold) (Figure 3A). These results suggest that *in vitro* K-ras^{G12V} activates p42/44 MAPK and NF- κ B pathways in pancreatic cancer cells. In order to investigate the involvement of these pathways on MUC4 expression, transient inhibition of p42/44 MAPK, JNK and NF- κ B using specific siRNAs was performed in CAPAN-2 cells (supplemental Figure 5A and B). We show a statistically significant ($p < 0.001$) decrease in *MUC4* mRNA expression after knocking-down expression of p42 MAPK, p44 MAPK, NF- κ B (p50 and p65 subunit), and JNK (1 and 2) suggesting the involvement of these pathways in MUC4 expression (Figure 3B).

3.3 MUC4 expression is regulated by K-ras signaling at the promoter level

We then investigated the implication of K-ras, NF- κ B and p42/44 MAPK signalling pathways at the *MUC4* promoter level. For that, K-ras^{G12V} or control vectors were co-transfected with *MUC4* promoter deletion constructs covering both the proximal and distal promoters of *MUC4* (Figure 3C) [17]. Overexpression of K-ras^{G12V} in CAPAN-2 cells induced a significant increase of luciferase activities of the -461/-1 ($p < 0.001$) and -1187/-1 ($p < 0.05$) *MUC4* promoter regions (Figure 3D). Interestingly, significant increase of luciferase activity of the -3135/-2570 and -2779/-2570 distal constructs and of the -1187/-1 proximal construct was observed in normal immortalized HPNE K-ras^{G12D} stably transfected cells compared to corresponding control cells (Figure 3D). These results indicate that K-ras is able to transcriptionally activate *MUC4* promoters in a cell-specific manner.

C-Fos, c-Jun (components of AP-1 transcription factor) and NF- κ B are transcription factors downstream MAPK, JNK and NF- κ B pathways. C-fos expression was investigated by immunohistochemistry in Pdx1-Cre; K-ras^{G12D} mouse model. C-fos expression was observed in the nuclear compartment in PanINs at every age (100% of cells) but was not detected in healthy pancreas of control mice (Figure 4A, supplemental Figure 1E). Specific c-jun staining could not be obtained with any of the c-jun antibodies assayed. Co-transfection of K-ras^{G12V} or control vectors with κ B-LUC or AP-1-LUC reporter vectors were performed in BxPC3 and CAPAN-2 cancer cell lines. The luciferase activity analysis indicated that κ B-LUC and AP-1-LUC reporter vector activities were significantly increased in both cell lines, suggesting the activation of AP-1 and NF- κ B transcription factors by mutated K-ras in pancreatic cancer cells (Figure 4B). We then evaluated the direct binding of AP-1 and NF- κ B on *MUC4* promoters. Chromatin immuno-precipitation experiments in CAPAN-2 cells were performed at the proximal region (-427/-304 and -196/-105) containing putative AP-1 and NF- κ B binding sites [17]. We showed the occupancy of AP-1 at -427/-304 by both

components (c-fos and c-jun). We also observed the AP-1 and NF- κ B occupancy at -196/-105 *MUC4* promoter region by c-fos, c-jun and p65 NF- κ B transcription factors respectively (Figure 4C). No binding was observed using an irrelevant region spanning introns 6 and 7 of *MUC4* gene.

Altogether, these results indicate the implication of MAPK, JNK and NF- κ B pathways in *MUC4* induction by mutated K-ras. This transcriptional regulation is mediated by AP-1 and NF- κ B transcription factors *via* their binding to AP-1 and κ B response elements in *MUC4* promoters.

3.4 The Ral GTPase pathway is involved in *MUC4* regulation

Importance of Ral GTPase pathway has been shown in K-ras transformation of human cells [18]. *In vivo* overexpression of RalB GTPase was confirmed in human pancreatic tumours compared to adjacent non tumoral tissues using analysis of 2 PDAC datasets of NCBI Gene Expression Omnibus (GEO,GSE 16515, $p=0.0003$ and GSE 28735, $p<0.0001$) (Figure 5A). Moreover, expression of Ral GTPase was investigated in pancreatic tissues from Pdx1-Cre;LStopL-K-ras^{G12D} mice and showed a strong cytoplasmic overexpression in PanINs in 3 to 12 months-old mice (100%) whereas only a light nuclear staining was observed in normal surrounding exocrine tissue and in healthy pancreas of control mice at any age (49-56% weak staining) (Figure 5B, supplemental Figure 1D). Silencing of RalA and RalB GTPases was performed in BxPC3 and CAPAN-2 cells to study their implication on *MUC4* expression. Western-Blot analysis of *MUC4* expression showed an absence of *MUC4* protein expression following RalB silencing in both cell lines (Figure 5C and data not shown) whereas *MUC4* mRNA level was not altered (Figure 5D and data not shown). RalA silencing had no effect on *MUC4* expression. These results suggest the implication of RalB GTPase in *MUC4* regulation by K-ras at a post-transcriptional level.

4. Discussion

In this manuscript, we showed that K-ras^{G12D} leads to an increase of Muc4 expression *in vivo* during the early steps of pancreatic carcinogenesis (PanINs) and that Kras activates MUC4 expression *in vitro* both at the transcriptional level via MAPK and NF-κB pathways and at the post-transcriptional level via RalB pathway. The Pdx1-Cre;LStopL-K-ras^{G12D} transgenic mouse is a well-described model to decipher early steps of pancreatic carcinogenesis known to reproduce human histological lesions in carcinogenetic sequence [19]. Using this mutated K-ras-induced model, we confirmed the early neo-expression of the mucin Muc4 in PanINs. These results recapitulate the pattern of MUC4 expression in both human and mouse PanINs lesions [5].

MUC4 expression is often deregulated in human cancers including oesophagus, gallbladder, salivary gland, lung and pancreas [5,12,20–23]. In pancreatic carcinogenesis, the MUC4 mucin is early neoexpressed and plays important roles in cancer cell properties [13,24]. MUC4 is a potential biomarker for pancreatic cancer and a potential therapeutic target against this pathology. Deciphering MUC4 regulation may be useful for future strategies targeting this mucin in the early steps of pancreatic carcinogenesis but also for other MUC4 overexpressing cancers [5,12,20–23].

K-ras is a major oncogene, frequently mutated in human cancers (COSMIC database <http://www.sanger.ac.uk/genetics/CGP/cosmic/>). Genetic alteration of *K-ras* is associated with a poor prognosis in pancreatic cancer [25], where K-ras mutations are nearly systematic [26]. K-ras is known to activate numerous signalling pathways [16]. In a tumour context, oncogenic activities induced by K-ras signalling are implicated in a large panel of cancer cells properties [7]. In particular, K-ras, like MUC4, is involved in chemoresistance of pancreatic cancer cells [13,27]. As a K-ras target, MUC4 could be a mediator of K-ras-induced chemoresistance.

In this report, we show that recruitment of p42/44 MAPK pathway by K-ras that leads to the upregulation of MUC4 expression. The p42/44 MAPK pathway is complex and mediates cancer-associated biological properties such as proliferation and migration [28]. Notably in pancreatic cancer, MAPK is necessary for initiation and maintenance of the tumour [9,29]. It is also implicated in migration and metastatic processes *via* up-regulation of metalloproteases [30,31]. MUC4 is implicated in migration and invasion properties of pancreatic cancer cells suggesting that migration and metastasis processes induced by MAPK activities under K-ras mutation could depend on MUC4 expression [24].

JNK pathway is a stress-responsive pathway, frequently implicated in tumorigenic development, which supports Ras oncogenic stress in cancer cells [32,33]. K-ras mutations are considered as the initiating event of pancreatic carcinogenesis. However, K-ras signalling is not sufficient for advanced carcinogenesis as other mutations and alterations are required [34]. We show here *in vivo* a gradual increase of JNK pathway activation in PanINs over time and a sustained activation of JNK pathway in pancreatic cancer cells *in vitro* suggesting that JNK activation could be required to support K-ras-induced PanINs progression. Previous work in our laboratory showed that P42/44 MAPK and JNK pathways are upregulated following MUC4

interaction with the ErbB2 oncogenic receptor [24]. Altogether, these data suggest a potential role for the mucin MUC4 in the amplification loop of the K-ras-activated signalling pathway that promotes carcinogenesis.

We showed the involvement of NF- κ B pathway in MUC4 regulation by the K-ras oncogene. The NF- κ B pathway is an essential signal from oncogenic Ras to allow its transforming activity through activation of the PI3K-AKT-IKK signalling pathway [35,36]. NF- κ B pathway is implicated in survival, angiogenesis and chemoresistance of tumour cells and is found constitutively activated in pancreatic cancer [37,38]. In this context, NF- κ B pathway is involved in PDAC development by reinforcing K-ras signalling *via* a positive feedback loop [39,40]. Moreover, Swartz and colleagues showed gradual increase in MUC4 expression during PanINs to PDAC development, suggesting a link between gradual increase in both K-ras signalling (MAPK and NF- κ B) and MUC4 expression in pancreatic carcinogenesis [5].

C-Fos is the main effector activated by p42/44 MAPK pathway [41] and c-jun, the main effector of the JNK pathway. Both are components of AP-1 transcription factor [42]. We show here direct binding of c-Fos, c-jun and p65 NF- κ B subunit on *MUC4* promoters. In addition, connections between AP-1 and NF- κ B has been described supporting the potential cooperation of these pathways in MUC4 activation by K-ras signalling [43]. Previous works have shown cooperation between c-Jun and PEA3 transcription factors on *MUC4* promoter supporting the important role of c-Jun transcription factor [44]. Implication of K-ras signalling in PEA3 transcription activity has not yet been described. Moreover, ERK pathway can also modulate a large panel of effectors such as Smad4, E2F1 and STAT1, all implicated in *MUC4* regulation [45–47]. The involvement of these effectors, downstream of K-ras, on MUC4 regulation remains to be determined.

Ral pathway is implicated in the regulation of endocytosis, exocytosis, cellular trafficking, immune response and in the pathological context of tumour cell proliferation [48]. In pancreatic cancer, RalGTPase plays an important role in Ras-transformation, anchorage independency, survival against oncogenic stress and radiotherapy resistance [18,49,50]. RalA and RalB are GTPases from the Ras-super family activated by K-ras signalling [16]. Ral guanine-nucleotide-exchange factors (Ral-GEFs) were shown to bind to Ras-GTP and lead to activation Ral-GTPases by promoting its GTP-bound state [51]. In this work, using human PDAC datasets and Pdx1-Cre;LStopL-K-ras^{G12D} mouse model of pancreatic carcinogenesis, we describe for the first time an overexpression of RalGTPases in PanINs and inhibition of MUC4 expression under RalB GTPase silencing. These results suggest the implication of the Ral pathway in MUC4 regulation by K-ras at the post-transcriptional level. Implication of MUC4 in Ral-mediated cancer properties has not been studied so far. In the future, it will be interesting to decipher the cellular mechanism of MUC4 post-transcriptional regulation by Ral pathway.

In conclusion, we show in this work a cellular mechanism linking K-ras, the main driver of pancreatic carcinogenesis to the mucin MUC4, an important actor of pancreatic cancer. This link involves MAPK, JNK and NF- κ B pathways at the transcriptional level *via* AP-1 and p65 NF- κ B subunit recruitment on *MUC4* promoters and RalB pathway at the post-transcriptional level. Since K-ras is still a clinically undruggable

target, increasing knowledge on MUC4 as an important K-ras target for cancer progression may lead, in the future, to the development of new efficient therapies in K-ras driven epithelial cancers.

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6. Author contribution

Conceived and designed the experiments: RV, IVS, NJ.

Performed the experiments: RV, BD, NS, AV & DMM.

Analyzed the data: RV, FR, IVS & NJ.

Wrote and edited the paper: RV, NP, IVS & NJ.

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Figure legends

Figure 1: **Effect of mutated K-ras on *in vivo* and *in vitro* Muc4 expression.** (A) Hematoxylin and eosin staining and IHC were performed to study Muc4 expression in pancreatic tissue from Pdx1-Cre;LStopL-K-ras^{G12D} (3 and 12 months) and Pdx1-Cre;K-ras^{WT} control mice (12 months), representative of five mice per age. (B) K-ras^{G12V} enrichment was measured by qRT-PCR in BxPC3 and CAPAN-2 cells following transfection with K-ras^{G12V} or control pBABE vector. K-ras^{G12V} relative expression was normalized to endogenous K-ras wild type allele. (C) MUC4 expression level was analyzed by western blotting in K-ras^{G12V} transfected cells. (D) MUC4 expression level was analysed by western blotting in K-ras^{G15A} transfected cells. Three independent experiments were performed using independent biological samples.

Figure 2: **Activation of signalling pathways in Pdx1-Cre;LStopL-K-ras^{G12D} and control mice.** Expression of phospho p42/44 (A-C), p42/44 (D-F), phospho JNK (G-I), JNK (J-L), phospho p65 (M-O) and p65 (P-R) was analysed by IHC in pancreatic tissues from Pdx1-Cre;LStopL-K-ras^{G12D} (3 and 12 months) or control mice (12 months). Five mice were analysed per age.

Figure 3: **K-ras activated signalling pathways are involved in MUC4 transcriptional regulation.** (A) Western blot analysis of p42/44 MAPK, JNK and NF-κB phosphorylation and expression in K-ras^{G12V} transfected CAPAN-2 cell line. Phosphorylation level was normalized to constitutive state and indicated as a ratio. (B) MUC4 relative expression in CAPAN-2 cells following silencing of MUC4, p42 MAPK, p44 MAPK, p50 and p65 NF-κB, JNK1, and JNK2 by siRNA. (C) MUC4 promoters schematic representation. Promoters region inserted in pGL3-luciferase reporter vector are indicated with black arrows. Amplified regions used for ChIP assays are indicated with white arrows. (D) Relative luciferase activities of MUC4 promoters in CAPAN-2 cells transfected with K-ras^{G12V} (left) (normalized to cell co-transfected with corresponding empty vector) or in HPNE-Kras^{G12D} compared to HPNE cells (right) (normalized to pGL3 control vector). Three independent experiments were performed using independent biological samples.

Figure 4: **MUC4 promoters regulation by K-ras oncogene via AP1 and NF-κB transcription factors.** (A) C-Fos expression in pancreatic tissues from Pdx1-Cre;LStopL-K-ras^{G12D} (3 and 12 months) or control mice (12 months) was studied using IHC. Five mice were analysed per age. (B) AP-1-Luc and κB-Luc activities in BxPC3 and CAPAN-2 cells transfected by K-ras^{G12V} or control vectors. (C) ChIP of MUC4 proximal (-427/-304 and -196/-105) promoter regions by c-Fos, c-Jun or p65 NF-κB in CAPAN-2 cells. Chromatin enrichment was normalized to that of input and expressed as ratio of IgG intensity. Amplification of an irrelevant region located in introns 6 and 7 of MUC4

gene was analysed as a negative control. Three independent experiments were performed using independent biological samples.

Figure 5: Implication of RalB pathway in MUC4 post-transcriptional regulation. (A) Immunohistochemical analysis of RalGTPases expression in pancreatic tissues from Pdx1-Cre;LStopL-K-ras^{G12D} (3 and 12 months) or control mice (12 months). (B) Relative RalB expression was analysed in pancreatic tumours compared to adjacent non tumour tissues from two human PDAC datasets of the NCBI Gene Expression Omnibus (GEO,GSE 16515, p=0.0003 and GSE 28735, p<0.0001). (C) MUC4 relative expression was analyzed by western blotting in CAPAN-2 cells following silencing by siRNA targeting RalA or RalB. (D) MUC4 relative expression was analyzed by qRT-PCR in CAPAN-2 cells after silencing targeting RalA or RalB by siRNA (normalized to GAPDH expression).

Supplemental figure legends

Supplemental figure 1: Scoring of immunohistochemical stainings performed in Pdx1-Cre;LStopL-K-ras^{G12D} and control mice. Scoring of Muc4 (A), phospho p42/44 MAPK (B), phospho p65 NF- κ B (C), RalA/B (D) and c-fos (E) immunohistochemical staining in PanINs formed in Pdx1-Cre;LStopL-K-ras^{G12D} mice and normal exocrine pancreatic tissue from control mice at 3, 5 9 and 12 months. Results are expressed as % of normal or PanINs stained tissue in five different mice at every age. IHC scoring was performed by an expert GIT pathologist.

Supplemental figure 2: MUC4 expression in human adenocarcinoma and adjacent tissues. Analysis of *MUC4* expression in tumour and adjacent non-tumour tissues from GSE 16515 and GSE 28735 datasets of NCBI Gene Expression Omnibus. For paired tumour and adjacent non-tumour samples, paired t test were performed (**p<0.001).

Supplemental figure 3: Negative controls of immunohistochemical staining in Pdx1-Cre;LStopL-K-ras^{G12D} mice without primary antibodies.

Supplemental figure 4: Signalling pathways by K-ras^{G12V} in BxPC3 cells. Western blot analysis of p42/44 MAPK, JNK and NF- κ B phosphorylation and expression in K-ras^{G12V} transfected BxPC3 cell line. Phosphorylation level was normalized to constitutive state and indicated as a ratio.

Supplemental figure 5: Signalling pathways analysis following silencing by siRNA. (A) Analysis of p42/44 MAPK, p50 and p65 NF- κ B and β -actin expression in CAPAN-2 cell following silencing of p42 MAPK, p44 MAPK, p50 NF- κ B, p65 NF- κ B, JNK 1 or JNK 2. Band intensities were analysed and normalised to β -actin intensity. This ratio was expressed and normalized to that of NT ratio. (B) Western blot analysis of MUC4 expression following silencing by siRNA in CAPAN-2 cells. (C) Western blot analysis of RalA/B expression following silencing by siRNA in BxPC3 and CAPAN-2 cells.

A

Pdx1-Cre; K-ras^{WT}

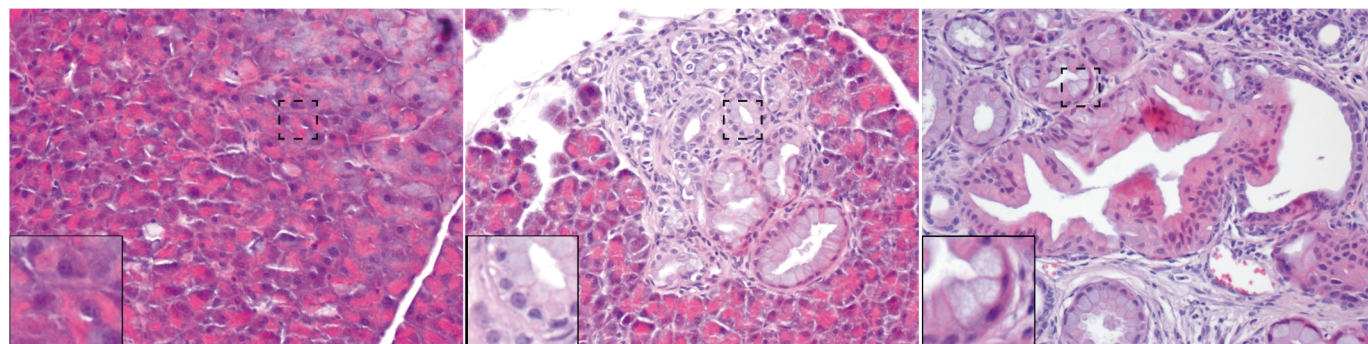
Pdx1-Cre; LStopL-K-ras^{G12D}

12 months

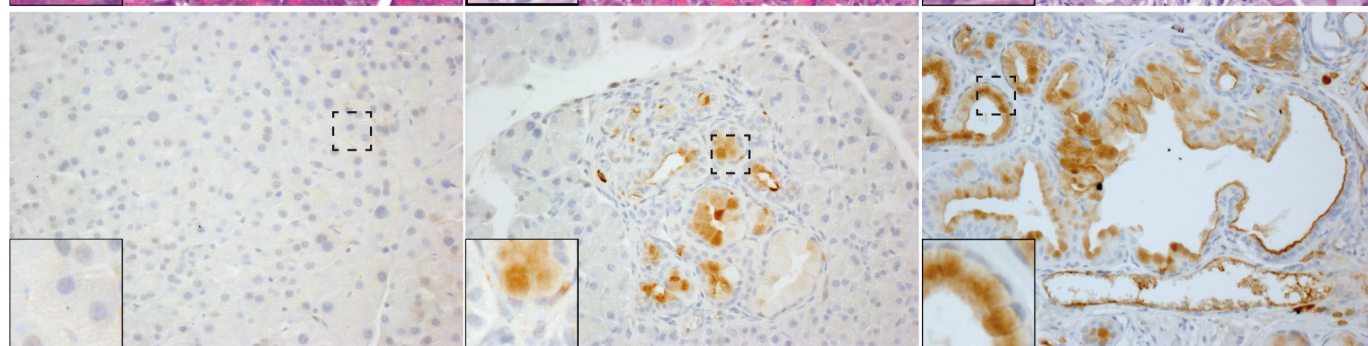
3 months

12 months

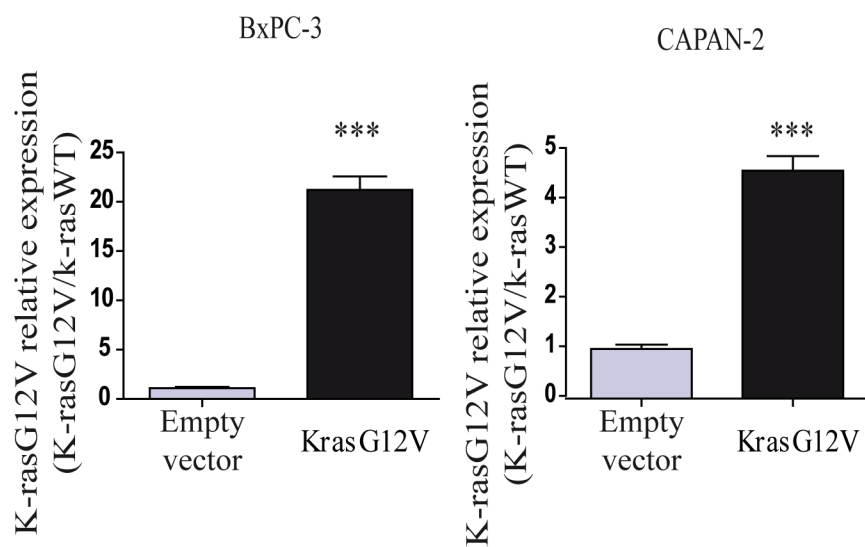
HE



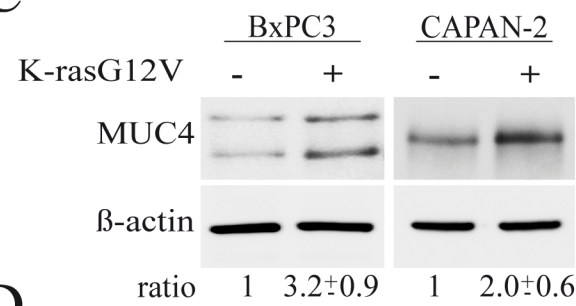
Muc4



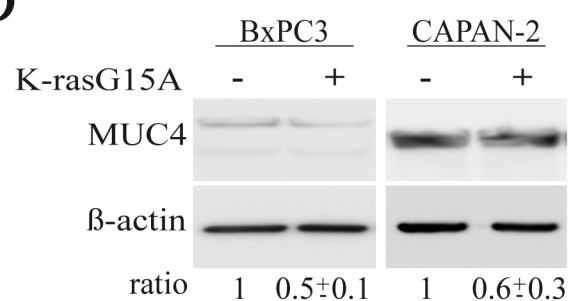
B

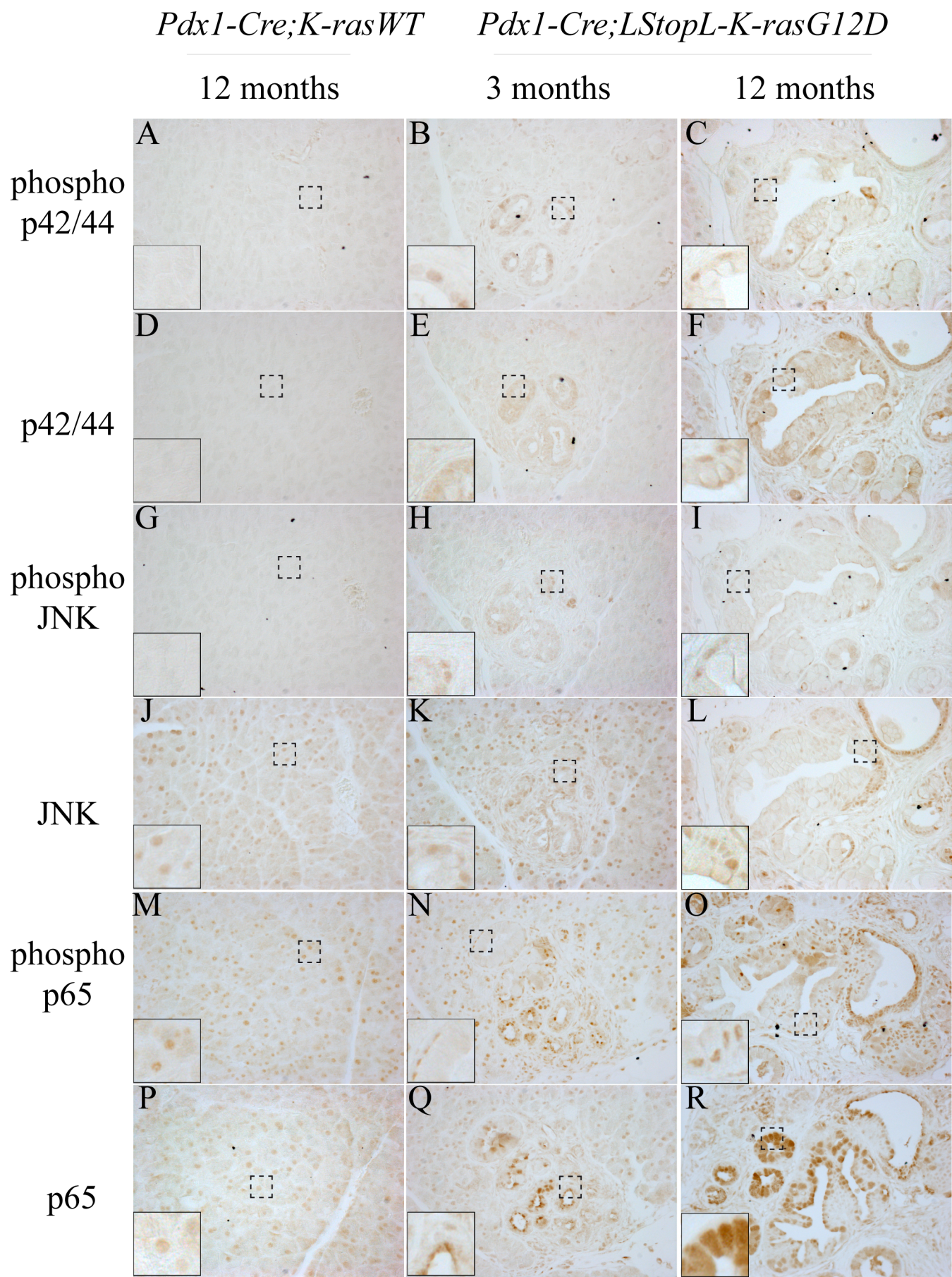


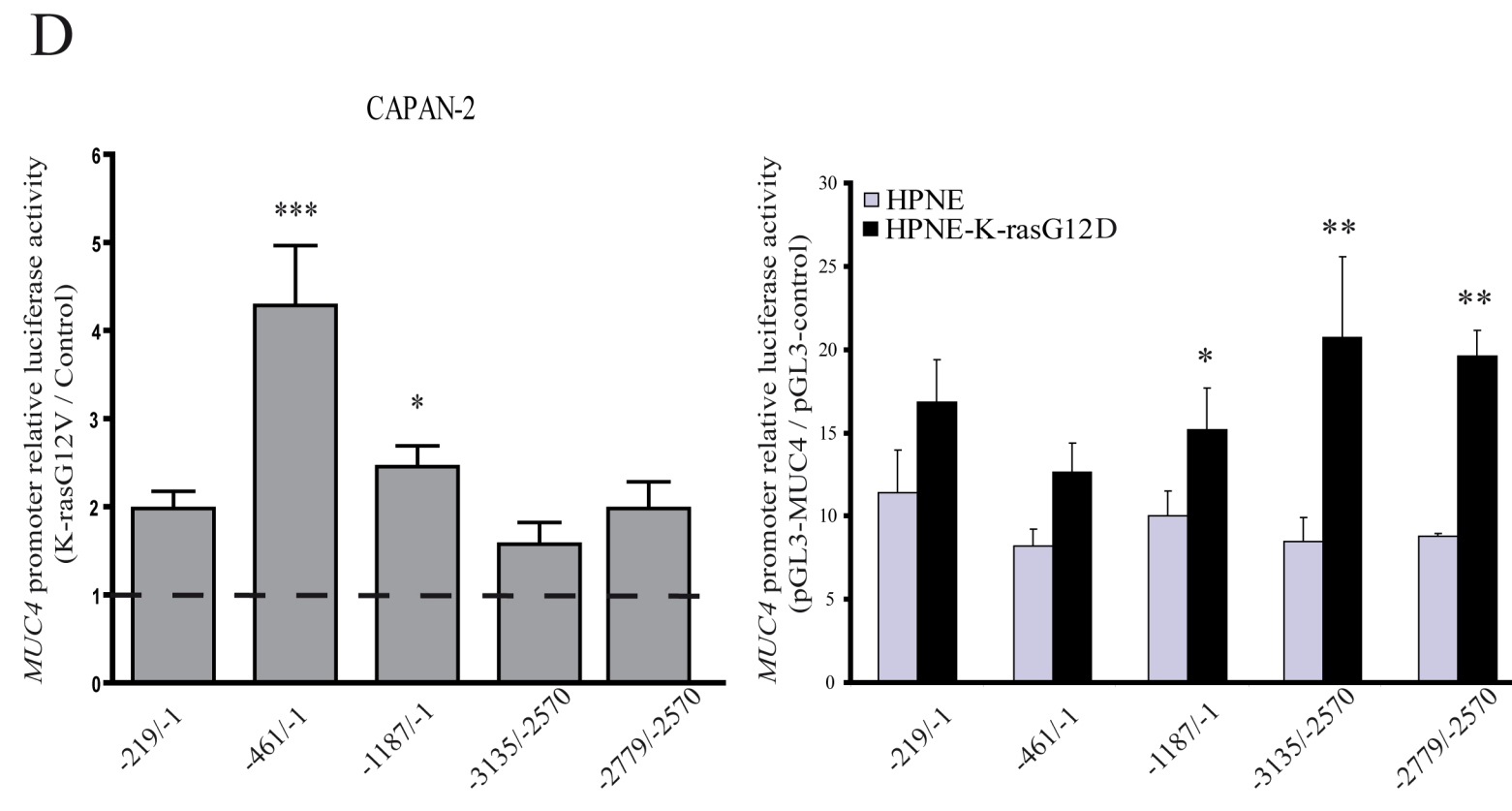
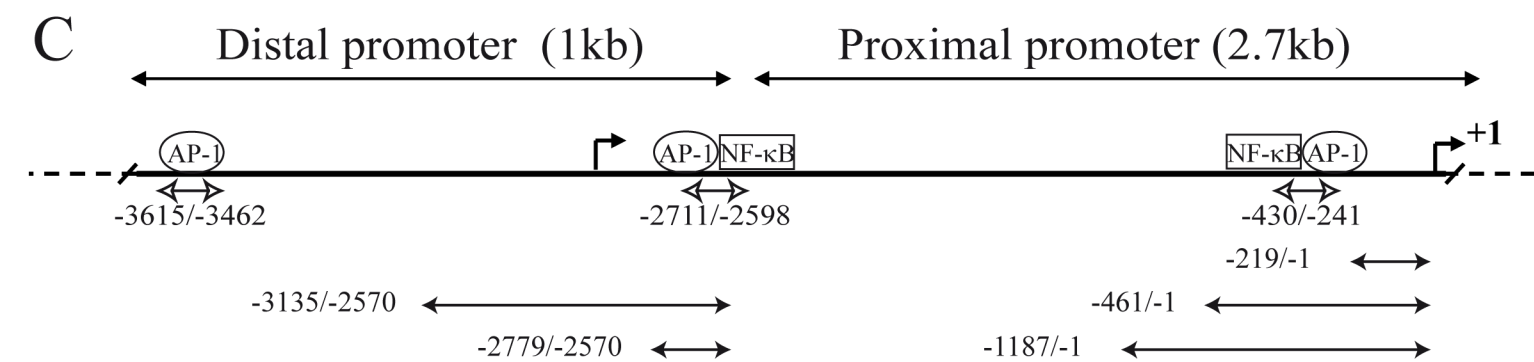
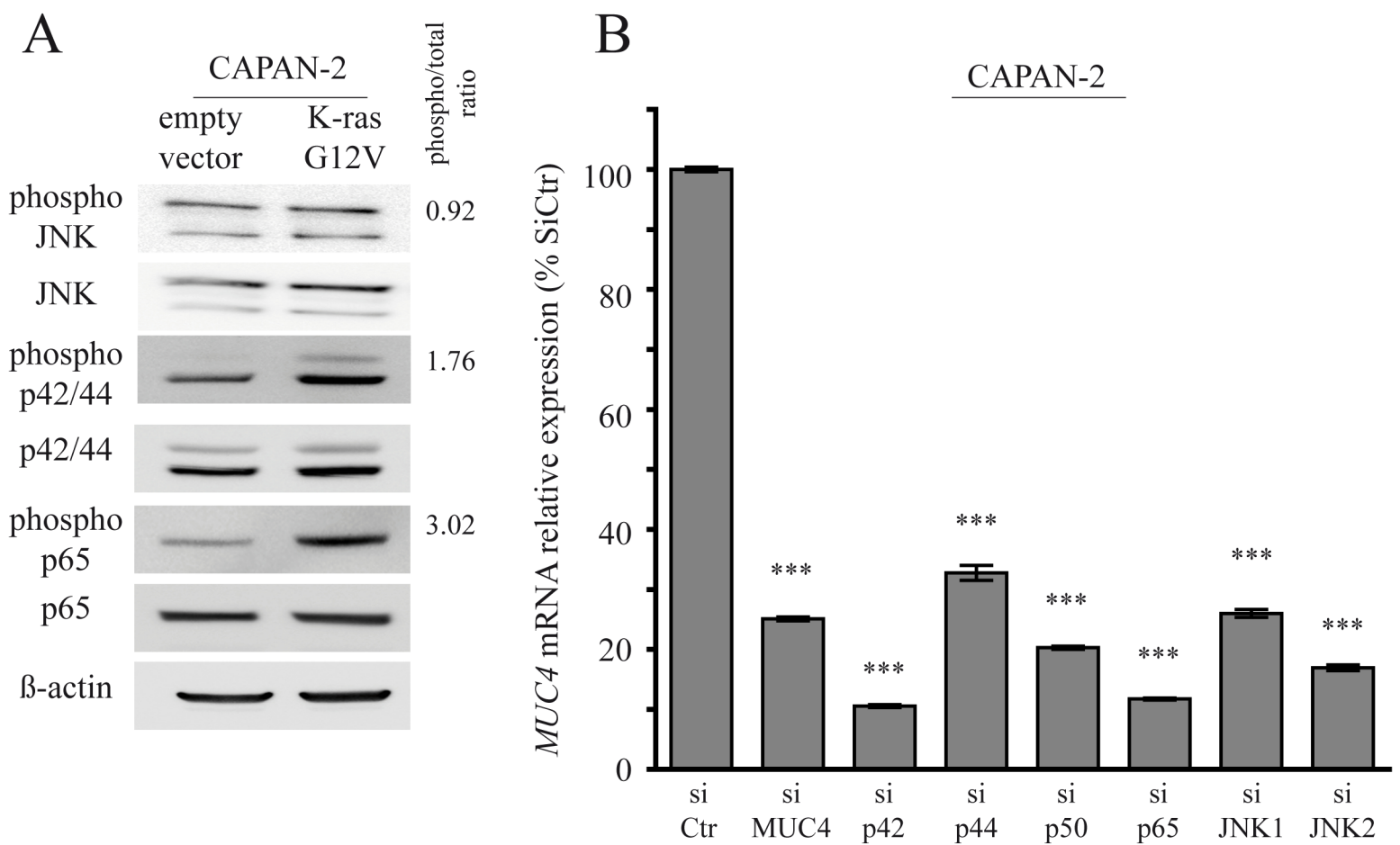
C

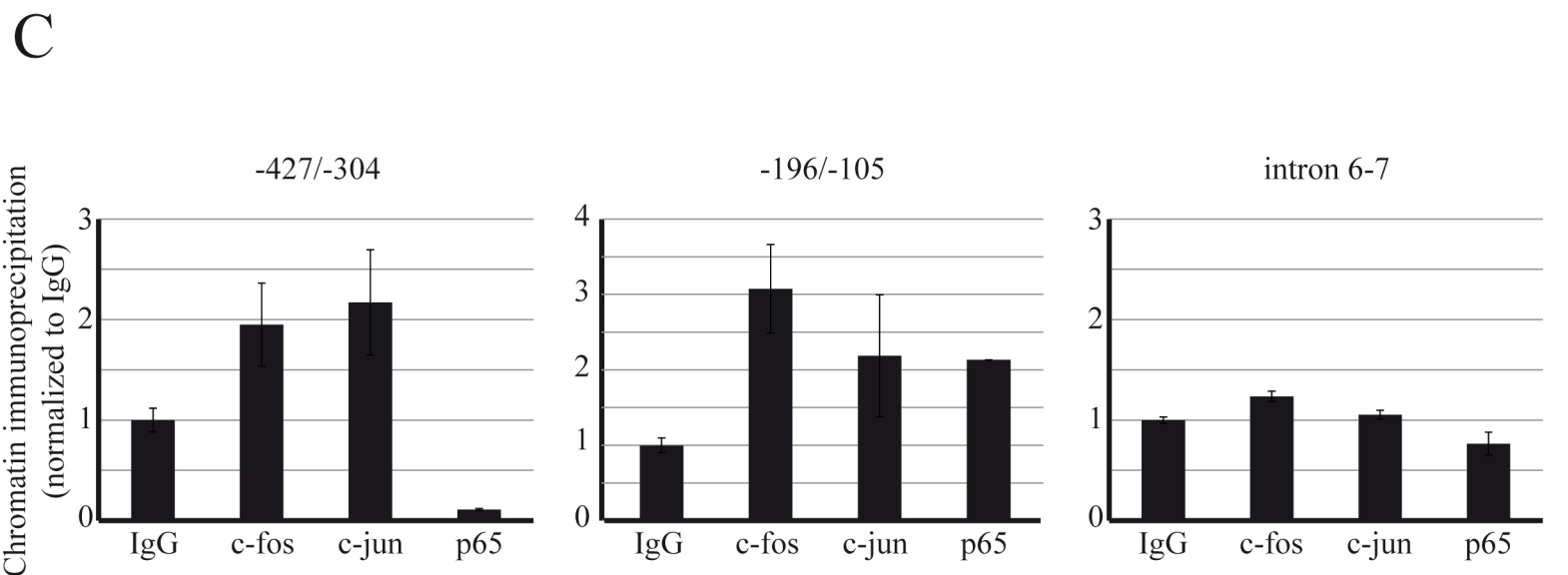
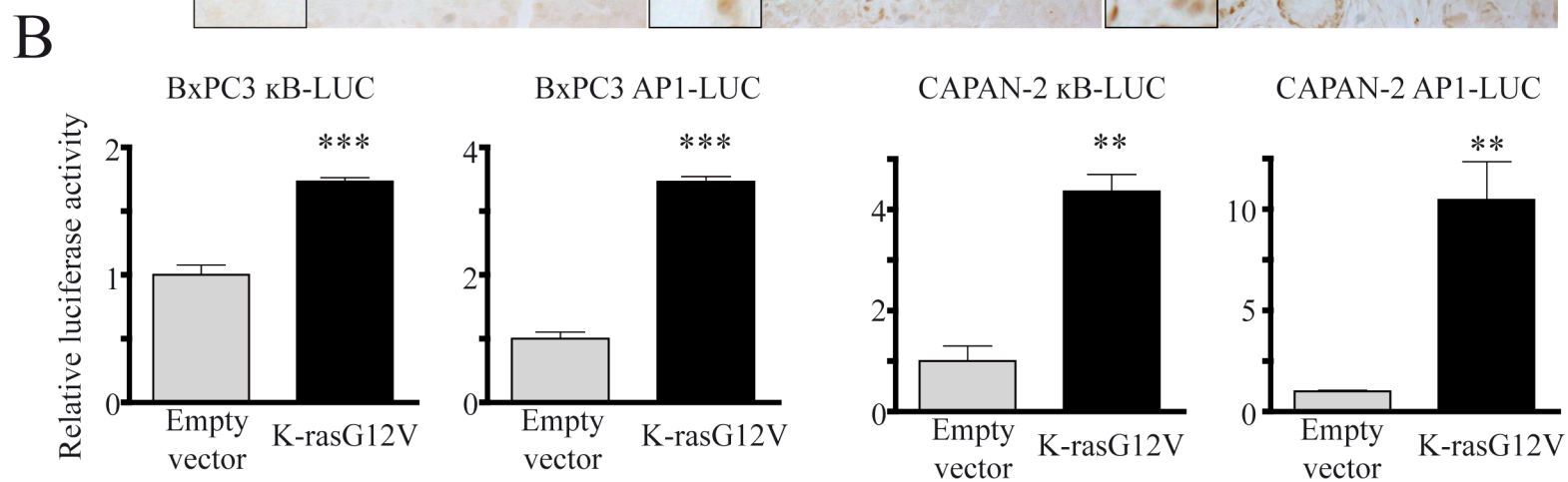
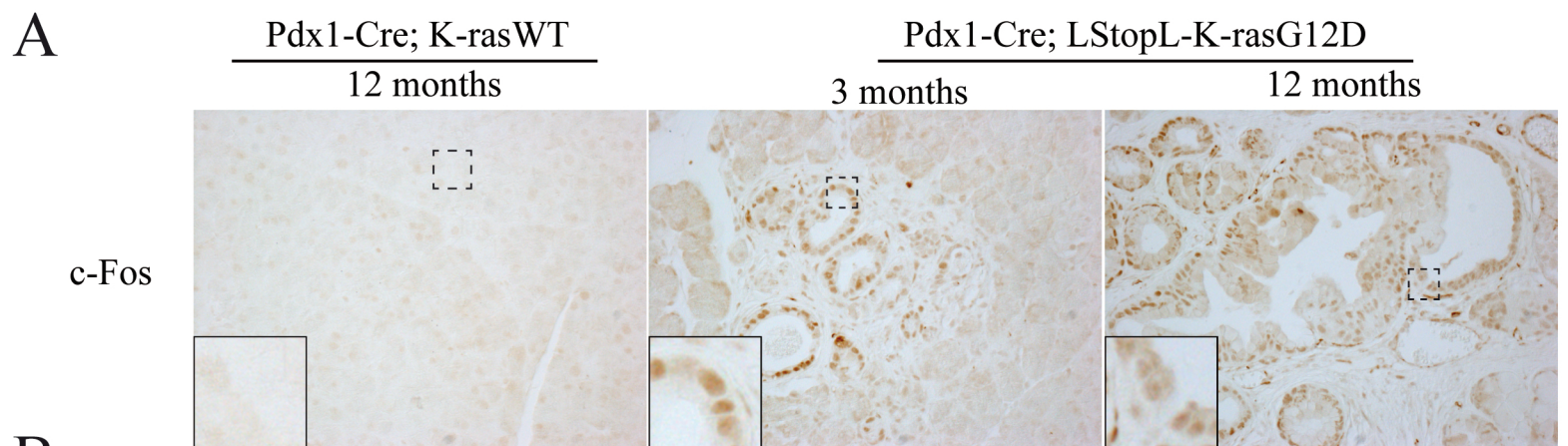


D

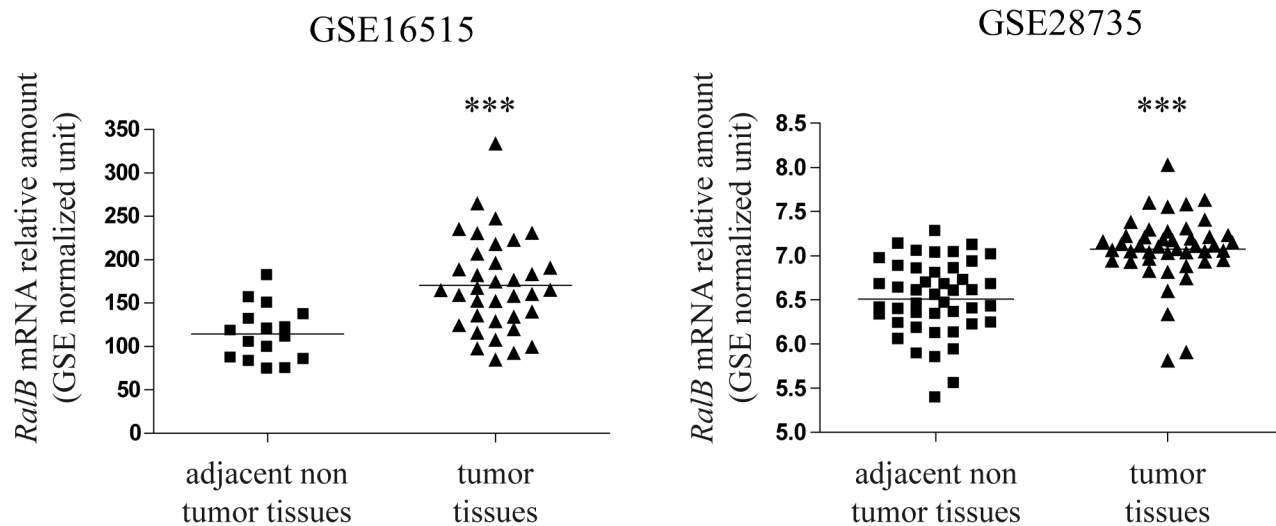








A



B

Pdx1-Cre;K-rasWT

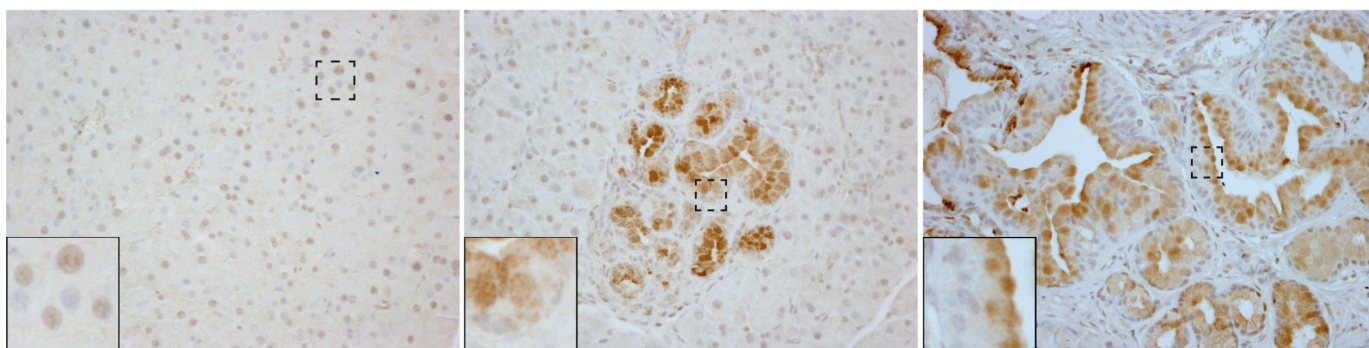
12 months

Pdx1-Cre;LStopL-K-rasG12D

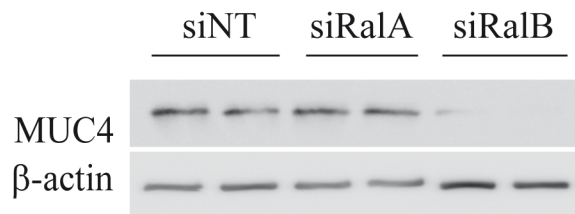
3 months

12 months

RalA/B



C



D

