c.−67G>C) and examined their impact on the $p16^{INK4a}$ 5′UTR activity using two luciferase-based reporter vectors, pGL3-basic and pGL3-promoter, that differ in basal transcription level and that were transfected into the melanoma-derived WM266−4 and in the breast cancer-derived MCF7 cells. The $p16^{INK4a}$ 5′UTR variants cloned into the pGL3-promoter plasmid type were also tested in two additional p16-null, melanoma-derived cell lines G361 and SK-Mel-5. Luciferase activity and mRNA levels were quantified to assess the impact of the mutations both at transcriptional and post-transcriptional levels. The wild type 5′UTR sequence, containing a reported SNP (c.−33G>C) and a known melanoma-predisposing mutation (c.−34G>T), were included as controls. Results revealed that the variants at −21 and −34 severely reduced the reporter activity. The variants at −56 and at −25&−180 exhibited a milder impact, while results with c.−67G>C were dependent on the plasmid type. Quantification of the luciferase mRNA indicated that the effects of the variants were mainly post-transcriptional. Using a bicistronic dual-luciferase reporter plasmid, we confirmed that c.−21C>T and c.−34G>T had a severe negative impact in both cell lines. We also applied a polysomal profiling technique to samples heterozygous for the 5′UTR variants, including patient-derived lymphoblasts and the analysis of allelic imbalance indicated that in addition to the c.−21C>T variant, the c.−56T>G and c.−67G>C variants also reduced mRNA translation efficiency. Overall, our results suggest that the c.−21C>T sequence variant negatively impact on $p16^{INK4a}$ 5′UTR activity, acting mainly at a post-transcriptional level, and can thus be of clinical significance in the melanoma proneness. We propose that these variants should be considered as potential mutations.

656 MiniSOX9, a dominant-negative isoform of the transcription factor SOX9 in colon tumour cells

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Background: Inherited and acquired changes in pre-mRNA splicing have been demonstrated to play a significant role in human diseases, especially in cancer. Splice variants, found predominantly in tumours, have a clear diagnostic value and may provide potential drug targets. Deciphering the mechanisms underlying aberrant splicing in cancer may provide crucial insight to our understanding of malignant transformation. The transcription factor SOX9 is over-expressed in tumours of many origins and particularly in colon (Blache et *al*, 2004 and Lu et *al*, 2008). However, the anti-proliferative activity of SOX9 (Bastide et *al*, 2007) as well as its direct stimulation of the tumour suppressor CEACAM1 (Zalzali et *al*, 2008) and repression of the carcinoembryonic antigen (Jay et *al*, 2005) clearly indicate that SOX9 acts as a tumour suppressor. Therefore, its anti-oncogenic activity is inhibited in colon tumour expressing SOX9. This assumption led to the discovery of MiniSOX9, a truncated version of the transcription factor SOX9.
Material and Methods/Results: MiniSOX9 results from the retention of the second intron of the SOX9 gene, leading to a protein devoid of the transactivation domain. MiniSOX9 behaves as a SOX9 dominant-negative inhibitor and stimulates the canonical Wnt pathway. We showed that MiniSOX9 is strongly expressed in intestinal tumours of APCdelta14 mice. In addition, MiniSOX9 is present at high levels in human colon cancer samples whereas it is almost undetectable in the surrounding healthy tissues.
Conclusions: Our data point to an unexpected oncogenic activity produced from the *SOX9* locus and gives a new perspective on its role in colon cancer.

Reference(s)

Bastide, P., Darido, C., Pannequin, J., Kist, R., Robine, S., Marty-Double, C., Bibeau, F., Scherer, G., Joubert, D., Hollande, F., et al. (2007). J Cell Biol 178, 635–648.
Blache, P., van de Wetering, M., Duluc, I., Domon, C., Berta, P., Freund, J.N., Clevers, H., and Jay, P. (2004). J Cell Biol 166, 37−47.
Jay P., Berta P., Blache P. (2005). Cancer Res. 65, 2193–2198.
Lu, B., Fang, Y., Xu, J., Wang, L., Xu, F., Xu, E., Huang, Q., and Lai, M. (2008). Am J Clin Pathol 130, 897–904.
Zalzali, H., Naudin, C., Bastide, P., Quittau-Prevostel, C., Yaghi, C., Poulat, F., Jay, P., and Blache, P. (2008). Oncogene 27, 7131–7138.

657 Protein kinase C and epidermal growth factor receptor signalling mediate growth stimulation by neurotensin in the colon carcinoma cell line HCT116

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Background: In addition to receptor tyrosine kinases, G protein-coupled receptors may also stimulate cell proliferation. The aim of the present study was to examine the signalling pathways mediating growth stimulation by neurotensin, which activates G_q-coupled receptors, in the colon carcinoma cell line HCT116.
Material and Methods: Cells were plated at a density of 50,000/cm^2 in medium containing 10% serum and cultured overnight, followed by serum starvation for 24 hours before stimulation with neurotensin. DNA synthesis was determined by pulse labelling for 3 hours with radiolabelled thymidine 12 hours after neurotensin addition. Activation of phospholipase C was determined as inositol phosphate accumulation in cells labelled with radioactive inositol for 24 hours. Phosphorylation of extracellular signal-regulated kinase (ERK), Akt, and the epidermal growth factor receptor (EGFR) was determined by Western blotting using phospho-specific antibodies.
Results: Neurotensin dose-dependently stimulated inositol phosphate accumulation and DNA synthesis. Preincubation with a protein kinase C (PKC) inhibitor, GF109203X, inhibited neurotensin-stimulated DNA synthesis. Both basal and neurotensin-stimulated DNA synthesis was reduced following preincubation with inhibitors of the EGFR tyrosine kinase (AG1478 and gefitinib), as well as by inhibitors of phosphoinositide 3-kinase (wortmannin) and the ERK kinase, MEK (PD98059). Neurotensin-stimulated ERK phosphorylation was inhibited by preincubation with the PKC inhibitor, but was not affected by inhibition of the EGFR tyrosine kinase. In contrast, neurotensin-induced phosphorylation of Akt was not affected by inhibition of PKC. Chelation of intracellular calcium, inhibition of matrix metalloproteases with GM6001, and inhibition of the EGFR with cetuximab or gefitinib suppressed neurotensin-stimulated phosphorylation of Akt.
Conclusions: The present results suggest that neurotensin stimulates ERK through PKC, while Akt phosphorylation is mediated by Ca^{2+}-dependent activation of matrix metalloproteases, which promote release of ligands that activate the EGF receptor. Furthermore, the results suggest that activation of the EGF receptor is required for neurotensin to exert an optimal growth stimulatory effect in HCT116 cells.

658 USP1-regulated FANCD2/FANCI monoubiquitination controls the DDB1-dependent degradation of phosphorylated CHK1

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Background: The maintenance of genetic stability in response to genotoxic stress and stalled replication forks depends on the timely regulated and interconnected action of cell cycle checkpoints, which delay cell cycle progression, and DNA repair mechanisms, which eliminate DNA lesions. The delayed mitotic entry of DNA damaged cells mainly depends on the ATR-mediated phosphorylation and activation of the checkpoint kinase CHK1. Previous works have identified that termination of CHK1 signalling during recovery requires SCF^{bTrCP}-dependent destruction of CHK1 mediator CLASPIN, a process impeded by the deubiquitinating enzyme ubiquitin-specific protease 7 (USP7). Here we describe a new pathway involved in turning off activated CHK1 in human cells involving the USP1 deubiquitinase.
Materials and Methods: *Cells*: For our experiment we used HeLa cells, U2OS, 293T and wild-type SV40-immortalized MRC5 fibroblasts as well as Fanconi anemia cells and their ectopically corrected counterpart. DNA damage was induced by exposure to UVC, DNA crosslinkers and replication inhibitors.
Transfections: Proteins depletion was obtained by siRNA transfection performed with Oligofectamine and experiments were carried out 48 h to 72 h later.
Proteins analysis: Chromatin fractionation, proteins extraction and analysis by Western blot were performed following standard methods.
Immunofluorescence: Two days after transfection, cells grown on glass coverslips were pre-extracted with 0.5% Triton X-100 in PBS for 5 min at RT before fixation in 4% paraformaldehyde during 15min and processed following standard procedures. Cells were examined at a magnification of x630 using a fluorescence microscope.
Results: We show that depletion of USP1 deubiquitinase decreases the amount of phosphorylated and total CHK1. We demonstrate that CHK1 inhibition in USP1-depleted cells is the mainly the consequence of high levels of monoubiquitinated FANCD2/FANCI and we establish that monoubiquitinated FANCD2/FANCI stimulates a DDB1-dependent degradation of phosphorylated CHK1.
Conclusions: Combining our observations with previously published data, we propose a model in which CHK1 is necessary to activate the DNA damage-induced FANCD2/FANCI monoubiquitination that, in turn, shuts down CHK1 activation. This pathway is likely compromised in Fanconi anemia contributing to the clinical and cellular phenotype of this rare syndrome that associates cancer predisposition and genetic instability.