Colorectal Carcinogenesis: Connecting K-RAS–Induced Transformation and CREB Activity In Vitro and In Vivo

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Abstract

Oncogenic transformation is often associated with an increased expression of the cAMP response element binding (CREB) transcription factor controlling the expression of genes involved in cell proliferation, cell cycle, apoptosis, and tumor development, but a link between K-RAS¹²-induced transformation and CREB has not yet been determined. Therefore, the constitutive and/or inhibitor-regulated mRNA and protein expression of CREB and signal transduction components and growth properties of parental fibroblasts, K-RAS¹²-transformed counterparts, shCREB K-RAS¹² transfectants and human colon carcinoma cells were determined. Increased CREB transcript and protein levels accompanied by an enhanced CREB activity was detected in K-RAS¹²-transformed murine fibroblasts and K-RAS¹²-mutated human tumor cells, which is dependent on the MAPK/MEK, PI3K, and/or PKC signal transduction. Immunohistochemical (IHC) staining of colorectal carcinoma lesions and murine tumors, with known KRAS gene mutation status, using antibodies specific for CREB and phospho-CREB, revealed a mechanistic link between CREB expression and K-RAS¹²-mutated colorectal carcinoma lesions when compared with control tissues. Silencing of CREB by shRNA and/or treatment with a CREB inhibitor (KG-501) reverted the neoplastic phenotype of K-RAS¹² transformants as demonstrated by a more fibroblast-like morphology, enhanced apoptosis sensitivity, increased doubling time, decreased migration, invasion and anchorage-independent growth, reduced tumorigenesis, and enhanced immunogenicity in vivo. The impaired shCREB-mediated invasion of K-RAS¹² transformants was accompanied by a transcriptional downregulation of different matrix metalloproteinas (MMP) coupled with their reduced enzymatic activity.

Implications: CREB plays a key role in the K-RAS¹²-mediated neoplastic phenotype and represents a suitable therapeutic target for murine and human K-RAS¹²-induced tumors.

Introduction

The cAMP response element binding (CREB) molecule represents an important member of bZIP-containing transcription factors activated by multiple signal transduction pathways in response to external stimuli, such as hormones, growth factors, and stress. These trigger the phosphorylation of CREB at position Ser-133 located in the kinase-inducible domain (KID) and its subsequent association with the coactivator, the CREB-binding protein CBP (1) through the KID-interacting (KIX) domain in CBP (2). CREB binds to the consensus 8-bp palindrom CRE sequence localized within the promoters and enhancers of many genes (3–5), resulting in the initiation of CREB-controlled gene expression.

Depending on the phosphorylation pattern mediated by cAMP-(Ser-133) or Ca²⁺–driven (Ser-142) activation cascades (6) CREB can promote pleiotropic, but distinct activities in different cell types, including altered metabolism, cell cycle, apoptosis, migration, invasion as well as proliferation (7–10). Thus, CREB controls critical cellular processes and is involved in the regulation of immortalization and malignant transformation (11). Recently, CREB was found to be overexpressed in many solid tumors of distinct histology when compared with normal adjacent tissues (12–14) and hematologic malignancies (3, 15, 16), which could be linked in some cases to tumor progression (14, 17), decreased time to relapse, and decreased event-free survival of patients (18). On the basis of the important role of tumor progression, CREB represents a promising target for cancer therapy. Therefore, several strategies have been developed to inhibit CREB phosphorylation, CREB–DNA, or CREB–CBP interaction, which are currently tested in CREB-overexpressing tumors (19, 20).

Although the CREB activity is regulated by distinct pathways involved in tumorigenesis, such as the PKA, MAPK, PI3K/Akt, and CaMK II signal transduction (21), a direct link between CREB and oncogene-mediated transformation has not yet been described. Therefore, oncogenic RAS has been shown to promote neoplastic transformation by engaging a variety of effector mechanisms (22). The RAS-dependent signaling events include the RAS–MAPK, RAS–PI3K, RAS–RHO, GTPase, and RAS–RHO GTPase pathways (23, 24), which control cellular proliferation, differentiation, and survival. In order to gain novel insights into
the role of CREB activation in the malignant phenotype. In vitro models of ras-transformed cells represent suitable tools for such analyses. Because K-ras genes are often mutated in tumors of distinct histology, which is frequently associated with a poor prognosis and worse clinical outcome of patients (25, 26) as well as resistance to targeted therapies (15, 27), it was analyzed whether CREB contributes to the K-RASV12-transformed phenotype of murine fibroblasts and human K-RASV12-mutated colorectal carcinoma cell lines and lesions.

### Materials and Methods

#### Cell culture and inhibitor treatment

The murine fibroblast cell line NIH3T3 was purchased from the ATCC, while K-RAS-transformed NIH3T3 cells were generated and maintained as previously described (28, 29). Eight human colorectal carcinoma cell lines (Colo320, HT29, RKO, SW480, Caco2, Colo206F, HCT116, and SW403) with known ras gene status and the normal colon epithelial cell line (CoN) obtained from the ATCC were used.

Cells were treated with inhibitors as indicated in Supplementary Table S1.

#### Transfections

A total of $4 \times 10^5$ cells per well were seeded into 6-well plates 24 hours prior to transfection with 1.5 μg/well of the Sac-digested murine CREB1-specific shRNA-encoding plasmid (SABioscience) using PolyFect (Qiagen) according to the manufacturer's instructions. A nonsense (NC) construct served as a control. The cell monolayer was incubated overnight at 37°C in complete medium and puromycin-resistant colonies were selected in medium supplemented with 3 μg puromycin/mL. Individual clones were picked after 3 weeks and subsequently expanded in individual wells.

#### cDNA synthesis, qPCR, and RT-PCR

RNA was isolated using the NucleoSpin II kit (Macherey-Nagel) according to the manufacturer's instructions and then subjected to cDNA synthesis followed by qPCR or RT-PCR with target-specific primers (Supplementary Table S2A and S2B) as recently described (30). The PCR were performed in triplicates. For qPCR the relative mRNA expression levels for specific genes were normalized to the signal generated from GAPDH, while for RT-PCR amplification products were separated in 1.5% agarose gels.

#### DNA extraction and sequencing

DNA extraction from cells was performed as recently described and subjected to pyrosequence analysis using the Pyromark kit (Qiagen) according to the manufacturer's instructions (31).

#### Western blot analysis

Thirty micrograms of protein per lane was subjected to Western blot analysis as recently described (30) using the target-specific primary antibodies anti-CREB, anti-phospho(Ser-133)-CREB, anti-Akt, anti-phospho(Ser473)-Akt, anti-ERK, anti-phospho(Thr202/Tyr204)-ERK (Cell Signaling Technology), and anti-β-actin (Sigma) in combination with suitable horseradish peroxidase (HRP)–conjugated secondary antibodies (Cell Signaling Technology) followed by visualization with the LumiLight Western Blotting Substrate (Roche) and recording with a LAS3000 system (Fujifilm). The immunostaining signals were subsequently quantified with the ImageJ software based on the measurement of the relative intensity. Values were further normalized to β-actin levels of the respective blots.

The endogenous RAS expression and activity was determined using a RAS pull-down assay according to the manufacturer's instructions as recently described (32). Cytosolic and membrane proteins were isolated with the cell fractionation kit (Cell Signaling Technology). GAPDH was used as a cytosolic marker and pan-Cadherin as a membrane marker.

#### 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay

The proliferation rate was determined using a XTT-based assay. Briefly, $5 \times 10^4$ cells per well in 96-well plates were seeded in complete medium without phenol red and incubated at 37°C for the time points indicated before XTT reagent (Cell Proliferation Kit II) was added for 4 hours according to the manufacturer's instructions. Cell proliferation was quantified by determination of the absorbance of the oxidized XTT solution at 570 nm using an ELISA reader system (Dynex).

#### Carboxyfluorescein diacetate succinimidyl ester staining

Cells were stained using the CellTrace CFSE Cell Proliferation Kit (Molecular Probe). CFSE-stained cells were harvested 48 and 96 hours after seeding and directly subjected to flow cytometry (FACS Calibur; BD) according to the manufacturer's instructions. Carboxyfluorescein diacetate succinimidyl ester (CFSE) profiles were analyzed using the CellQuest software (BD).

#### BrdUrd incorporation assay

A total of $5 \times 10^4$ cells per well were seeded into a 96-well plate and the bromodeoxuryridine (BrdUrd) incorporation was measured with a BrdUrd proliferation assay kit (Roche) following the manufacturer's instructions.

#### Cell-cycle analysis

For synchronization, cells were starved in complete EMEM containing 0.5% FBS for 48 hours before EMEM with 10% FBS was added for the indicated times. After trypsinization, cells were washed twice with PBS and fixed overnight in 70% ethanol at 4°C. Fixed cells were washing twice with PBS, RNA was digested with RNase A (30 μg/mL) for 30 minutes in the dark at room temperature followed by the addition of 20 μg/mL 7-aminomatinycin D for 1 hour in the dark. Cell-cycle distribution was analyzed by flow cytometry on a FACS Calibur. The amount of cells in the different cell-cycle phases was determined by the ModFit software (Verity Software House).

#### Apoptosis assays

For quantification of apoptosis, $1 \times 10^5$ cells were stained with the annexin V kit (MBL) according to the manufacturer's instructions. In addition, the cleaved caspase-3 was determined by flow cytometry with an antibody recognizing the active form of caspase-3 (FITC Active Caspase-3 Apoptosis Kit; BD). Cells treated with the caspase-3 inhibitor Z-DEVD-FMK (BD) for 24 hours served as a control. The activity of caspase-3 and caspase-7 was further measured with the fluorescence-based Apo-ONE homogeneous Caspase-3/7 Assay (Promega).

#### Mitochondrial membrane potential

Cells were incubated for 24 hours and the cell pellet was stained with the JC-1 dye (Invitrogen). CCCP was used as a positive control.
Figure 1.
Constitutive and inhibitor-regulated CREB mRNA and protein expression pattern in NIH3T3 and K-RAS^{V12}-transformed murine fibroblasts and/or human colorectal carcinoma cell lines. A, CREB mRNA expression levels of NIH3T3 and K-RAS^{V12} transformants. CREB transcription was determined in NIH3T3 cells and K-RAS^{V12} counterparts by real-time quantitative PCR with specific primers as described in Materials and Methods. The bar chart in the panel displays the relative expression levels normalized to the corresponding β-actin level in the indicated cell lines. Data shown represent mean values obtained from three independent biologic replicates. B, representative immunostainings for CREB and p-CREB Ser133. Total cellular extracts were separated in 10% SDS–PAGE gels and blotted onto nitrocellulose membranes before staining with specific antibodies was performed. White lines indicate that intervening lanes have been spliced out. The protein expression was quantified using the ImageJ software. The results are represented in bar charts and normalized to β-actin. C, human colorectal carcinoma cell lines were subjected to qPCR and D, Western blot analysis as described above. The mutation status of the ras and B-Raf genes of the human colorectal carcinoma cell lines is summarized in the table below the Western blot analysis. (Continued on the following page.)
Table 1. Expression pattern of CREB in colorectal carcinoma lesions in comparison with the K-RAS12 status

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Wound-healing assay

Confluent cells were incubated for 24 hours in 0.5% FBS containing medium, before a wound was generated by scratching the cell monolayer with a 100-μL pipette tip. After washing with PBS, fresh culture medium supplemented with 0.5% FBS was added and the wound closure was monitored over time as described previously (33). The wound area was measured at every time point using the MetaVue software (Molecular Devices).

Migration and invasion assay

For determination of the cell migration, 1 × 10^5 cells cultured in medium supplemented with 1% FBS were seeded into the upper well, whereas medium containing 10% FCS was added to the lower chamber of the Transwell chamber system (Corning). After incubation for 18 hours at 37°C, nonmigrated cells of the top insert were completely removed, whereas cells on the bottom insert surface were lysed with the CellTiter-Glo (Promega), before the ATP content was measured in a luminometer (Berthold). For invasion assays, the polycarbonate membranes were coated with Matrigel (30 μg/well; BD).

Cell adhesion assay

Varying concentrations of fibronectin prepared in PBS with Ca^{2+} and Mg^{2+} were used to coat 96-well plates (50 μL/well). After incubation overnight at 4°C, plates were rinsed four times with 200-μL PBS with Ca^{2+}/Mg^{2+}/well, and blocked with 200 μL/well of 1% BSA for 1 hour at room temperature. A total of 5 × 10^4 cells per well were allowed to adhere to the plate for 1.5 hours at 37°C. The nonadherent cells were removed by gently washing the plate four times with PBS. The number of adherent cells/well was quantified by measuring the cellular ATP content as described above.

In vivo tumorigenicity

A total of 1 × 10^6 cells per mouse were subcutaneously injected into the flank of DBA-1 and Fox1;nu/nu mice (male and female, age: 6 weeks to 3 month; Harlan). Tumor growth was monitored three times a week by caliper measurement and tumor volume was determined. Animals were housed under standard conditions. The animal care committee of Martin Luther University [Halle (Saale), Germany] approved all experiments. At the end of the

(Continued.) Both K-RAS^{V12} cell lines are homozygous. E. K-RAS^{V12} and F. NIH3T3 cells were left untreated or treated for 24 hours with the signal transduction inhibitors H89, LY294002, PD98059, R031-8220 before Western blot analysis was performed using respective antibodies. G. cells left untreated or treated with the farnesyl transferase inhibitor tipifarnib for 48 hours with the indicated concentrations before Western blot analysis was performed as described above. H. the localization of RAS was analyzed under treatment with tipifarnib. Cells were incubated for 48 hours with the indicated concentrations of tipifarnib and cells were fractionated into cytosolic and membrane proteins. GAPDH and pan-Cadherin were used as fraction markers. I. cells were incubated for 48 and 72 hours with increasing concentrations of tipifarnib and the viability was measured with annexin V-FITC/PI staining following FACs analysis.
experiments (after 35 to 42 days), tumors were isolated, paraffin-embedded, and stored until use.

**Determination of the immune cell populations in peripheral blood**

Heparinized blood samples were prepared and analyzed as previously described (33).

**Gelatin zymography**

To determine the activity of matrix metalloproteinases (MMP), aliquots of cell-conditioned medium were analyzed by gelatin zymography as described previously (34).

**Statistical analysis**

A two-sided Student t test or ANOVA was used for the statistical analysis of data, with a \( P < 0.05 \) considered as significant. SD, *, \( P < 0.05 \); **, \( P < 0.01 \); ns, not significant.

**Results**

Upregulation of CREB expression by mutated RAS\(^{V12}\) transformatnts

In order to determine whether K-RAS\(^{V12}\)-mediated transformation modulates CREB expression, qPCR and Western blot analysis of K-RAS\(^{V12}\)-transformed cells and respective controls were performed.
Figure 3.
Reduced migration and cell motility of NIH3T3 cells and RASV12 transformants due to CREB gene silencing. A, scratch assays of NIH3T3 and K-RASV12 cells and shCREB transfectants were performed and representative photos of the wound distance were taken at each time point as indicated. The numbers indicate the scratch area setting (0 hours) to 100%. The magnification is demonstrated by the bar (80 μm). The bar charts represent the mean and SD from three independent experiments. Microscope: Leica DM IRB. B, the cell motility of the cell lines was determined by Transwell assays as described in Materials and Methods and is represented in a histogram as percentage of migrated cells. The data represent the mean of three independent experiments using triplicates. The black triangle indicates increasing CREB knockdown grade in the case of the shCREB clones as shown in Fig. 2A. C, cells were incubated with the indicated concentration of KG-501 for 24 hours and migration potential was than analyzed. D, invasiveness was determined by Matrigel invasion assay. The frequency of invasiveness is given as percentage and represents the mean of three independent experiments. E, cells were incubated with the indicated concentration of KG-501 for 24 hours and invasion potential was analyzed by using Matrigel coated Transwell inserts. F, the expression of different MMPs and HAS was analyzed by qPCR. Data show the mean of three independent experiments. nd, not determined. G, MMP activity was determined by gelatin zymography. White lines indicate that intervening lanes have been spliced out.
Figure 4.
Altered growth properties after CREB silencing. A, for the determination of the generation time by XTT assay, 5 x 10^3 cells per well were seeded into a 96-well plates and proliferation was determined 24, 48, 72, and 96 hours after seeding according to the manufacturer's protocol. Three independent experiments were performed in triplicates and the data were presented in bars. The black triangle indicates the increased CREB silencing in the case of the shCREB clones as shown in Fig. 2A. (Continued on the following page.)
An increased CREB transcription was found in K-RAS\textsuperscript{V12}-transformed cells when compared with controls (Fig. 1A). This effect was even more pronounced at the protein level with a 2- to 6-fold upregulation of both unphosphorylated as well as phosphorylated CREB protein in the respective K-RAS\textsuperscript{V12} transformants (Fig. 1B). The K-RAS\textsuperscript{V12}-mediated increase in CREB activity of murine fibroblasts was accompanied by an activation of major RAS-induced signal transduction pathways (Fig. 1E and F, DMSO-treated lines).

In addition, the CREB expression and function was analyzed in eight human colorectal carcinoma cell lines with known K-ras status. Normal colon epithelial (CoN) cells served as a control. Although CoN cells expressed only marginal CREB mRNA and protein levels, a heterogeneous, but higher transcript (Fig. 1C) and protein levels (Fig. 1D) were detected in all eight colorectal carcinoma cell lines analyzed with the highest p-CREB levels found in the K-RAS\textsuperscript{V12}-mutated SW403 and SW480 cell lines (Fig. 1D). However, it is noteworthy that Caco2 cells lacking a K-RAS mutation demonstrated an increased CREB expression and activation when compared with CoN cells. This might be due to other common mutations of tumor-suppressor genes found in colorectal carcinoma, such as p53, APC, and SMAD4, which have been identified in Caco2 cells (35). Indeed, SMAD4 mutations have been shown to activate CREB (36). Murine fibroblasts overexpressing a wild-type K-RAS showed a lesser increase of phosphorylated CREB than cells with mutated K-RAS\textsuperscript{V12} or K-RAS\textsuperscript{D19} (Supplementary Fig. S1A).

### Inhibition of CREB expression by signal transduction inhibitors

In order to get deeper insights into the link between RAS and CREB, K-RAS\textsuperscript{V12} mutants and control cells were treated with different inhibitors affecting the major RAS-dependent signaling pathways (Fig. 1E–G). Treatment of K-RAS\textsuperscript{V12} cells with different farnesyl inhibitors block the CREB phosphorylation, but to a different extent. Tipifarnib demonstrated the strongest effects on K-RAS\textsuperscript{V12} cells with a dose-dependent downregulation of unphosphorylated and phosphorylated CREB (Fig. 1G). Under tipifarnib treatment for 48 hours the inhibition of RAS farnesylation caused a loss of membrane bound RAS and an increased cytosolic localization (Fig. 1H), which could explain the loss of CREB phosphorylation. This was accompanied by an increased sensitivity of K-RAS\textsuperscript{V12} cells to tipifarnib when compared with untransformed cells as determined by cell viability (Fig. 1I). Inhibitors targeting the MAPK and Akt pathway caused a downregulation of the CREB activity in K-RAS\textsuperscript{V12} transformants (Supplementary Fig. S1B and S1C). Similar to murine K-RAS\textsuperscript{V12} cells treatment of RAS-mutated SW480 cells with the MEK1/2 inhibitor trametinib significantly decreased, while other inhibitors increased the p-CREB expression levels (Supplementary Fig. S1D). Trametinib had negative consequences for the cell proliferation and cell-cycle progression as well as the cell viability (Supplementary Fig. S1E–S1G).

### Association of CREB expression and activity in human colorectal carcinoma lesions with known ras mutation status

To confirm the ras-mediated modulation of CREB in situ, 23 colorectal carcinoma lesions and adjacent normal colon epithelium were analyzed for CREB and p-CREB expression using immunohistochemistry followed by correlation of these results to the K-ras phenotype of the lesions. A nuclear and partial cytoplasmic CREB staining was found in both wt and K-RAS\textsuperscript{V12} colorectal carcinoma lesions analyzed, while normal colon epithelium and tumor-infiltrating immune cells expressed marginal CREB protein. However, in contrast to wt K-RAS colorectal carcinoma lesions expressing low to marginal levels of p-CREB, a heterogeneous CREB expression pattern in K-RAS\textsuperscript{V12} colorectal carcinoma lesions was demonstrated with a high positive p-CREB staining for most tumor areas (Supplementary Fig. S2A and S2B and Table 1). On the basis of the CREB expression pattern obtained in \textit{in vitro} models of RAS-mediated transformation and \textit{in situ} on colorectal carcinoma lesions, a molecular link between mutated K-RAS\textsuperscript{V12} and CREB activity was postulated.

### Phenotypic alterations of the K-RAS\textsuperscript{V12}-transformed cells in response to CREB silencing

In order to understand the function of K-RAS\textsuperscript{V12}-induced CREB expression and activation, CREB was silenced by shRNA in parental NIH3T3 cells and K-RAS\textsuperscript{V12} transformants. NC constructs served as a control. Stable expression of shCREB caused a downregulation of CREB transcription up to 60% and of CREB protein to approximately 90% in both parental NIH3T3 cells and K-RAS\textsuperscript{V12}-transformed fibroblast, while the NC constructs only slightly modulate the CREB expression levels (Fig. 2A). CREB silencing did not or only marginally affect the transcription of the sequence related ATF-1 gene, whereas an approximately 2-fold increase of CREM transcription was detected in shCREB transfectants underlining the specificity of CREB targeting (Fig. 2B). Neither the expression nor the activity of RAS were changed in K-RAS\textsuperscript{V12}, transformed fibroblasts by CREB silencing (Fig. 2C). Despite the presence of the mutation (Fig. 2D), CREB silencing caused an altered morphology of K-RAS\textsuperscript{V12} cells and reverted its neoplastic phenotype characterized by focus formation to a more fibroblast-like morphology (Supplementary Fig. S3A). These results were in line with a reduced focus formation capacity of K-RAS\textsuperscript{V12} transformants at low FBS concentrations (Supplementary Fig. S3B).

### Effect of CREB-dependent silencing on migration, adherence, and invasion properties of K-RAS\textsuperscript{V12} transformants

Due to the shCREB-mediated phenotypic changes, altered growth properties of K-RAS\textsuperscript{V12}-transformed cells were suggested.

(Continued)
Therefore, the role of CREB on cellular functions contributing to tumor progression, migration, adhesion, and invasion capacity was investigated in NIH3T3 cells and K-RASV12 transformants and shCREB transfectants. The wound-healing assay revealed a total loss or a reduced wound-healing rate upon CREB silencing (Fig. 3A). Using the Transwell system, an increased migration of K-RAS-transformants than of NIH3T3 cells was found, while CREB silencing caused an approximately 50% decrease in the migration capacity of both cell lines (Fig. 3B). Interestingly, the disruption of the CREB–CBP interaction using the inhibitor KG-501 caused a strong, dose-dependent inhibition of migrating K-RASV12 cells, which was already significant in the presence of 5 μmol/L KG-501 reacting 80% downregulation in the presence of 25 μmol/L of this drug (Fig. 3C). In contrast, low concentrations of KG-501 did not affect the migration capacity of NIH3T3 cells, while it was only approximately 20% reduced in these cells in the presence of 25 μmol/L KG-501. Furthermore, the altered migration capacity could be linked to an altered expression of AHNK and cortactin (Supplementary Fig. S4D).

Next to the altered migration rate of untransformed versus transformed cells, NIH3T3 cells exhibit a lower adherence rate even at high fibronectin concentrations when compared with K-RASV12 mutants exhibiting a strong concentration-dependent adherence to fibronectin (Supplementary Fig. S4A). This process is at least partially linked to CREB, because CREB-silenced K-RASV12-transformed cells showed a reduced adherence independent of the given fibronectin concentrations, which is comparable with untreated NIH3T3 cells. Under cultivation on Ultra Low Cluster Plates K-RASV12 but not NIH cells forms spheroids, which were smaller with reduced serum concentrations and CREB deficiency (Supplementary Fig. S4B). This is in line with the lack of NIH3T3 cells to form colonies in soft agar. In contrast, K-RASV12-transformed cells could grow under these conditions and form a high number of soft agar colonies (Supplementary Fig. S4C), which were 5- to 9-fold reduced upon CREB silencing.

In addition, a diminished invasion capacity was found in K-RASV12 cells by CREB silencing (Fig. 3D) as well as in a dose-dependent manner by KG-501 treatment (Fig. 3E). The invasion potential of NIH3T3 cells was increased by cultivation with K-RASV12-conditioned medium (Supplementary Fig. S5C), while the inhibition of MMP activity by GM-6001 decreased the invasion potential (Supplementary Fig. S5D).

To further elucidate the molecular mechanisms underlying the shCREB-mediated inhibition of invasion, the expression and function of selected MMPs were determined. K-RASV12-transformed cells expressed higher MMP-2 and MMP-9 mRNA levels than NIH3T3 cells, which were reduced by CREB silencing (Fig. 3F).

Transcriptional downregulation of MMP expression by CREB silencing

To investigate the processes leading to an altered MMP transcription, the MMP-2 and MMP-9 promoter activity was analyzed in both NIH3T3 cells and RAS transformants and their CREB-silenced counterparts. As shown in Supplementary Fig. S5A, an enhanced MMP promoter activity was found in K-RASV12 transformants, which could be downregulated by shCREB. To characterize the CREB-dependent transcriptional downregulation of the MMP promoter activity in detail promoter constructs with different deletions/mutations of the CRE-binding sites were analyzed. Although no CRE or half-CRE sites have been identified in the MMP-2 promoter by in silico analysis, a putative CREB-binding site was postulated at position—660 bp. Deletion of a 50-bp sequence (−695 to −644 bp) containing this postulated CREB binding site and in addition a STATX-binding site decreased the MMP-2 promoter activity between 13% and 20% when compared with the wt promoter. MMP-9 promoter mutants with point mutations in the three half CRE sites exert distinct activity. Although the mutation in half CRE site 1 showed no significant effect on the basal promoter activity, mutations in the half CRE sites 2 and 3 negatively interfere with the promoter activity, but not in all cell lines analyzed. A mutation in the half CRE site 2 only decreased the promoter activity in K-RASV12-mutated cell lines by approximately 40% but not in NIH3T3 cells. In contrast, the mutation in the half CRE site 3 impaired the promoter activity in all cell lines tested, but to a distinct extent (Supplementary Fig. S5B and S5C). This CREB-dependent transcriptional downregulation of MMPs was accompanied by a loss or reduced proteolytic activity for MMP-2 and MMP-9 upon CREB silencing as determined by gelatin zymography of the culture supernatants (Fig. 3G).

Reduced proliferation and cell-cycle progression upon CREB silencing

On the basis of the CREB silencing-mediated effects on the migration, adhesion, and invasion capacity in particular in K-RASV12 cells when compared with control cells, a link between CREB expression and the K-RAS-transformed phenotype was suggested. Despite K-RASV12 cells had an increased doubling time and proliferation rate when compared with NIH3T3, the effect of CREB suppression was similar resulting in increased doubling times (Fig. 4A) and reduced cell proliferation rates of both NIH3T3 and K-RASV12 transformants (Fig. 4B and D). In contrast, the G1–S cell-cycle arrest was more pronounced in shCREB NIH3T3 cells than in shCREB K-RASV12 cells (Fig. 4C), which was accompanied by an inhibition of cyclin B1 and D1 mRNA expression in CREB-silenced NIH3T3 and K-RASV12 cells when compared with respective controls (Fig. 4E). In line with these results, the shCREB-mediated decreased proliferation and G1–S cell-cycle arrest (Supplementary Fig. S1E and S1F) an increased cell death (Supplementary Fig. S1G) was found in human colorectal carcinoma cells treated with trametinib. Cultivation under serum reduction caused growth reduction in the CREB-deficient cell lines, while K-RASV12 cells were less influenced (Supplementary Fig. S3E).

Altered apoptosis sensitivity of CREB-silenced cells

The altered growth properties of NIH3T3 cells and K-RASV12 transformants upon CREB silencing might be accompanied by changes in the apoptosis sensitivity of these cells. Using annexin V/PI-staining, a significant dose-dependent increase in the frequency of early and late apoptotic cells was detected upon CREB silencing (Fig. 5A). However, the shCREB-mediated apoptosis was more pronounced in NIH3T3 cells when compared with K-RASV12 transformants. This was associated with a downregulation of bcl-2 and bcl-xl and a strong increase of bax mRNA levels upon CREB silencing (Fig. 5B). These data were further supported by higher levels of cleaved caspase-3/7 in CREB-silenced transfectants (Fig. 5C and D), while the caspase-3 inhibitor Z-DEVD-FMK reduced apoptosis (Fig. 5E). The reduced CREB expression further
decreased mitochondrial membrane potential in NIH3T3, but not in K-RASV12 cells (Fig. 5F).

K-RASV12-induced tumorigenicity is CREB-dependent

On the basis of the in vitro results, an effect of CREB silencing on the K-RASV12-induced in vivo tumorigenicity was suggested. To test this hypothesis, K-RASV12-transformed cells, their CREB-silenced counterparts as well as parental fibroblasts were subcutaneously injected into DBA-1 mice. As expected, parental NIH3T3 cells exert no tumorigenic potential (data not shown), whereas all mice injected with K-RASV12-transformed cells developed tumors within 2 weeks. In contrast, the frequency of tumor formation was 60%
Figure 6. Altered tumorigenicity upon CREB silencing. A, for the analysis of in vivo tumorigenicity $1 \times 10^5$ cells were injected s.c. into DBA-1 mice (n for K-RAS$^{V12}$ and shCREB = 10, n for NC cells = 5), and mice were monitored three times a week for tumor development. B, tumor growth was measured by a caliper at least two times a week. Mice were killed 6 weeks after s.c. injection and tumor end volume was measured at the removed tumor. (Continued on the following page.)
Link of K-Ras Mutations to CREB in Tumor Cells

Table 2. Differences of K-RASV12 and NIH3T3 cells

<table>
<thead>
<tr>
<th></th>
<th>NIH3T3</th>
<th>NIH3T3 shCREB</th>
<th>K-RASG12V</th>
<th>K-RASG12V shCREB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Fibroblast-like</td>
<td>Fibroblast-like</td>
<td>Spindle-like, nonplanar cell structure</td>
<td>Less spindle-like than K-RASG12V</td>
</tr>
<tr>
<td>Focus formation</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Contact inhibition</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>RAS activity</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ERK/AKT signaling</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>n.d.</td>
</tr>
<tr>
<td>Generation time</td>
<td>22 h</td>
<td>20 h</td>
<td>19 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Cyclin B/D expression</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cell cycle after serum depletion</td>
<td>+</td>
<td>Arrest</td>
<td>+</td>
<td>Arrest</td>
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<tr>
<td>Apoptosis</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Caspase-3 activation</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Mitochondrial potential</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Woundhealing</td>
<td>+++</td>
<td>+/−</td>
<td>++</td>
<td>+</td>
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<tr>
<td>Migration</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Invasion</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+</td>
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<td>+++</td>
<td>+</td>
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<td>MMP-9 secretion</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Tumor development DBA-1 mice</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>++</td>
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<tr>
<td>Tumor development nude mice</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Adhesion</td>
<td>++</td>
<td>−</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Anchorage independent growth</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Spheroid formation</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Proliferation under serum reduction</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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</tbody>
</table>

NOTE: −, no ability; +, weak ability; ++, moderate ability; ++++, strong ability; n.d., not determined.

Reduced by injecting shCREB K-RASV12 cells (Fig. 6A). This was accompanied by a reduced tumor volume and size when compared with K-RASV12 transfectants (Fig. 6B). Interestingly, the mice injected with K-RASV12 cells had a lower frequency of effector cells in the peripheral blood when compared with mice injected with the shCREB K-RASV12 cells (Fig. 6E). These results suggest that CREB silencing interferes with the tumorigenicity by at least partially enhancing their immunogenicity.

To further test this hypothesis, K-RASV12 cells and their shCREB transfectants were injected into nu/nu mice and the tumor formation and growth was again monitored over time. As shown in Fig. 6C and D, the effect of shCREB silencing on tumor growth was not as pronounced as in DBA-1 mice, suggesting that the shCREB-mediated tumor regression is associated with immune responses. Both cell lines developed higher tumor end volumes in the nude mice than in the DBA-1 mice (Fig. 6F). Tumors of comparable size had more established blood vessels in the wt group (Supplementary Fig. S6A). The phosphorylation of CREB-Ser133 was markedly decreased in the tumors of shCREB origin (Supplementary Fig. S6B). These date suggest that the shCREB-mediated reduced tumorigenicity might be due to alterations in their immunogenicity when compared with K-RASV12 transfectants.

Discussion

Activation of CREB has been demonstrated in human tumors, suggesting a role of this transcription factor in the malignant transformation process (11). CREB is regulated by distinct pathways known to be activated in tumors. However, whether the activation of CREB is directly driving the malignant phenotype of cells or whether this is a secondary effect due to the activation of upstream pathways has not yet been clarified and was addressed in this study using murine in vitro models of K-RASV12 transformation. An increased expression and constitutive activation of CREB was found in K-RASV12-transformed cells. This might be due to an activation of the different effector pathways, including the MAPK pathway, and confirmed the recent results describing an increased phosphorylation of ERK1/ERK2 in K-RASV12 transfectants (32). Furthermore, an activation of CREB was also found in K-RASV12-mutated human colorectal carcinoma cell lines and tumor lesions when compared with normal colon epithelium. However, a direct correlation was not that obvious because these cell lines harbor other mutations, such as B-raf, p53 APC, and SMAD2. The latter could also affect CREB activity. Furthermore, there also exist evidences that CREB could be activated by different upstream kinases depending on the cell types analyzed (21). The CREB activation was associated with PI3K, MAPK, and PKC, but not PKA signal transduction processes as demonstrated in the inhibitor experiments. A dose-dependent CREB downregulation by a farnesyl inhibitor was shown for the first time, which extends the activity of CREB on RAS transformants. It is noteworthy that changes in the CREB activity may not only be a result of RAS mutations, but also of an altered tumor microenvironment (37).

(Continued) C, for the analysis of in vivo tumorigenicity 1 × 10⁶ cells were injected s.c. into athymic Fox-1nu/nu mice (n = 5). Animals were monitored three times a week for tumor development. D, tumor growth was measured by a caliper at least two times a week. Mice were killed 4 weeks after s.c. injection and tumor end volumes in the nude mice than in the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d. for the nude mice and after 42 days for the DBA-1 mice. The black line is the mean value of every column. A complete tumor regression occurred in the DBA-1 mice n.d.
In addition to the K-RASV12 transformants described in this study an upregulated CREB expression and activation was detected in human tumors of distinct origin and appear to contribute to the initiation, progression, and metastatic potential of tumors as well as to a reduced patients’ survival (38). In some of these tumor entities, mutations in the ras gene frequently occur, such as colon and lung carcinoma (25, 26, 39), but a direct link between RAS overexpression and/or mutation and CREB activity has not yet been determined. Higher levels of p-CREB expression were found in K-RASV12-mutated colorectal carcinoma lesions, which should be confirmed by a higher number of lesions analyzed.

However, the importance of CREB, its targets, and associated pathways has recently been underscored by the identification of mutations in the CBP gene in acute lymphoblastic leukemia both at diagnosis or acquired at relapse, which caused an impaired histone acetylation and transcriptional regulation of CREB targets and therapy resistance. These data underline the functional and clinical significance of the CREB/CBP-controlled gene expression in malignancies (40).

Because overexpressed CREB might act as a proto-oncogene resulting in altered growth properties a direct inhibition of CREB by shRNA might cause a reversion of the malignant phenotype of tumor cells. Indeed, silencing of CREB in RASV12-transformed cells decreased CREB and p-CREB protein expression without changes in the expression and function of RAS, which was accompanied by a reversion of the transformed morphology. Inhibition of CREB by shRNA in K-RASV12 transfectants also effectively suppressed the anchorage-independent growth, transformation, migration, and invasion. The latter was accompanied by an inhibition or downregulation of the expression and activity of selected MMPs, known to induce cell migration and invasion (41) as well as by a reduced adherence to fibronectin. These data in line with the MMP-2- and MMP-9-mediated increased invasion potential in vitro and in vivo and enhanced angiogenesis in vivo (12, 42). Increased MMP-2 and MMP-9 promoter activity was found upon oncogenic Ras transformation when compared with untransformed controls. This was dependent on CREB because mutations of the three putative CREB-binding sites in the MMP-9 promoter resulted in a significantly decreased activity, while a deletion of the single CRE site decreased the MMP-2 promoter activity by 20%. Although the binding of CREB to the MMP-2 and MMP-9 promoter had already been shown in different studies (43, 44), this effect has not yet been linked to RAS transformation. These data are in line with reports demonstrating that CREB protein elimination decreased the activities of MMP in NSCLC (45) and melanoma (42).

On the basis of these results, CREB might be involved in an enhanced tumorigenicity of K-RASV12 transformants. Indeed, the CREB-silenced K-RASV12 cells exerted a reduced in vivo growth directly linking CREB to the neoplastic phenotype. This effect appears to be due to an increased immunogenicity of shCREB transfectants, because the incidence of tumor formation and tumor size was more increased by injecting these cells into immunodeficient nu/nu mice. These results were strengthened by a decreased frequency of effector T cells in mice injected with K-RASV12 when compared with their shCREB transfectants. This might be mediated by an increased expression of MHC class I surface antigens in shCREB K-RASV12 transformants (personal communication). Thus, modulation of CREB expression and/or activity might be a potential therapeutic strategy of tumors for growth inhibition (38). This hypothesis is supported by (i) the use of the KIX/KID inhibitor KG-501 leading to a reduced proliferation and migration capacity as well as (ii) by using a dominant negative A-CREB protein inhibiting the DNA binding of the CREB TF family. In this model, A-CREB expression caused an 80% reduction of papilloma formation, reduced growth, and induced apoptosis of v-H-RAS-transformed cells, whereas H-ras mutations or v-H-RAS-transformed cells required CREB function for viability (46). In addition, metastasis formation was suppressed by a dominant-negative CREB mutant (47), while overexpression of CREB increased metastasis formation (48). CREB overexpression is linked with a poor prognosis and a decreased survival rate (49). Many tumor entities showed an increased CREB expression and a higher activity and phosphorylation compared with nonmalignant tissues. We therefore analyzed the effect of CREB knockdown on the in vivo tumorigenicity. The number of tumor-bearing mice was significantly decreased and tumor growth was delayed. Similar results were observed in a nude mice model injected with melanoma cells expressing a dominant negative CREB (44). This could also be shown for an artificial CREB model, when the Ser133 is mutated (12).

CREB is a transcription factor that ubiquitous controls cell proliferation, migration, and cell survival independent from the KRAS mutation status of the cell, while it mediates typical oncogene transformed phenotype abilities such as invasion, migration, tumorigenicity, and anchorage-indepentant growth (Fig. 6G). Thus, CREB represents a promising candidate for pharmacologic evaluation and for therapeutic intervention in particular for the treatment of RAS-induced tumors to revert their transformed phenotype (Table 2).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Steven, C.V. Recktenwald, B. Hiebl, B. Seliger
Development of methodology: A. Steven, C.V. Recktenwald
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Steven, M. Heiduk, C.V. Recktenwald, B. Hiebl, C. Wickenhauser, C. Massa
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Steven, M. Heiduk, C. Wickenhauser, C. Massa, B. Seliger
Writing, review, and/or revision of the manuscript: A. Steven, C. Wickenhau- se, B. Seliger
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Hiebl, B. Seliger
Study supervision: C.V. Recktenwald, B. Seliger

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Colorectal Carcinogenesis: Connecting K-RAS–Induced Transformation and CREB Activity *In Vitro* and *In Vivo*

André Steven, Max Heiduk, Christian V. Recktenwald, et al.


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