What turns CREB on? And off? And why does it matter?

Cellular and Molecular Life Sciences

André Steven¹, Michael Friedrich¹, Paul Jank², Nadine Heimer¹, Jan Budczies³, Carsten Denkert², Barbara Seliger¹

¹ Institute for Medical Immunology, Martin Luther University Halle-Wittenberg, 06112 Halle (Saale), Germany
² Institute of Pathology, Philipps University Marburg, 35043 Marburg, Germany
³ Institute of Pathology, University Clinic Heidelberg, 69120 Heidelberg, Germany

Corresponding author: Prof. Dr. Barbara Seliger
Martin Luther University Halle-Wittenberg
Institute for Medical Immunology
Magdeburger Str. 2
06112 Halle (Saale), Germany

Telephone: (+49) (345) 557 - 1357
Fax: (+49) (345) 557 - 4055
E-mail: barbara.seliger@uk-halle.de

Note: The listed references in this document are included under their indicated number in the main manuscript.
### Supplementary Material

#### Supplementary Tables

Supplementary Table 1: Modulation of CREB protein in tumors and its clinical relevance. Phrases in italics reflect outcomes that differ from the majority of the literature.

| Carcinoma                      | CREB expression in cancer vs normal | Clinical relevance and prognosis                                                                 | References |
|--------------------------------|-------------------------------------|---------------------------------------------------------------------------------------------------|------------|
| ALL                            | CREB overexpression                 | n/a                                                 | [159]      |
|                                | p-CREB overexpression               | poorer survival                                    | [25]       |
| AML                            | CREB overexpression                 | less favorable prognosis                           | [19]       |
| breast cancer                  | increased (p-)CREB                  | poor prognosis, metastasis, nodal involvement      | [160]      |
|                                | increased CREB                      | n/a                                                 | [20]       |
|                                | increased (p-)CREB                  | n/a                                                 | [32]       |
| colon cancer                   | increased p-CREB                    | n/a                                                 | [161]      |
|                                | increased (p-)CREB                  | n/a                                                 | [131]      |
| ESCC                           | CREB overexpression                 | correlation with lymph node metastasis and tumor-node-metastasis (TNM) stage                     | [9]        |
| gastric cancer                 | increased p-CREB                    | n/a                                                 | [162]      |
|                                |                                    | poor survival                                      | [163]      |
| glioblastoma/glioma            | increased p-CREB                    | poor survival                                      | [164]      |
|                                | increased CREB                      | promotes proliferation                              | [139]      |
|                                | CREB overexpression                 | shorter OS and PFS                                 | [61]       |
|                                | CREB overexpression                 | correlation with tumor grading                      | [133]      |
|                                | CREB overexpression                 | correlation with tumor grading, poorer survival    | [18]       |
| hepatocellular carcinoma       | increased CREB                      | n/a                                                 | [165]      |
|                                | increased CREB                      | high CREB leads to shorter DFS and OS              | [166]      |
|                                | increased (p-)CREB                  | high (p)CREB leads to shorter DFS and OS           | [167]      |
| Hodgkin’s lymphoma             | decreased CREB                      | high expression of CREB correlates with favorable prognosis | [168]      |
| kidney, renal cell carcinoma, clear cell | increased (p-)CREB | CREB increased migration and invasion                | [15]       |
|                                | increased CREB                      | correlation with higher TNM stages                  | [169]      |
| laryngeal cancer               | CREB overexpression                 | association with cancer differentiation, tumor stage, and lymphatic metastasis                    | [17]       |
| leukemia, lymphatic            | CREB overexpression                 | poor outcome                                       | [170]      |
| Cancer Type                  | CREB Status                  | n/a Values                                      | References |
|-----------------------------|------------------------------|-------------------------------------------------|------------|
| leukemia, myeloid           | CREB overexpression          | n/a induction of aberrant myelopoiesis          | [171]      |
| lung cancer                 | increased (p-)CREB           | CREB is significantly upregulated               | [172]      |
|                             | increased p-CREB/CREB        | overexpression of CREB or p-CREB was related to a lower probability of survival. | [173]      |
| medulloblastoma             | increased p-CREB             | p-CREB favorable prognosis                      | [229]      |
| meningioma                  | increased p-CREB             | correlation with angiogenesis + recurrence      | [174]      |
| NSCLC                       | increased p-CREB             | favorable prognosis for smokers and squamous cell carcinoma | [175]      |
| prostate cancer             | increased p-CREB and p-CREB/CREB ratio | n/a                                             | [176]      |
| ovarian cancer              | increased CREB               | promotes cell proliferation                      | [177]      |
| SCLC small cell lines carcinoma | increased p-CREB          | promotes cell proliferation                      | [11]       |
| skin cancer, malignant melanoma | increased p-CREB        | melanoma progression                            | [178]      |
|                             | increased p-CREB             | tumor growth and metastasis                      | [179]      |
|                             | increased p-CREB             | increased p-CREB correlates with metastasis     | [180]      |
| thyroid cancer              | CREB overexpression          | n/a                                             | [181]      |

n/a = not analyzed
Supplementary Table 2: Known stimuli modulating CREB activity and downstream signal pathways in human und murine tumor cells.

| Stimulus                        | CREB residue | Protein kinases | Cell model, tissue                     | References |
|---------------------------------|--------------|-----------------|----------------------------------------|------------|
| **Growth factor signaling/kinases** |              |                 |                                        |            |
| c-KIT/MC1R                       | n/a          | PKA, ERK        | melanoma cells, h                       | [182]      |
| EGF                             | Ser133       | ERK1/2, AKT     | IGROV1 cells, h (ovarian cancer)        | [183] [219]|
| HER-2/neu receptor               | n/a          | n/a             | metastatic breast cancer tissue, h      | [184]      |
| Serum                           | Ser133       | ERK 1/2, p38    | Caco-2 cells, h                         | [185]      |
| **Steroid hormone signaling**    |              |                 |                                        |            |
| 17β estradiol                    | Ser133       | n/a             | TNBC cells, h                          | [186]      |
| Calcitriol                      | n/a          | PI3K/AKT        | HeLa cells, THP-1 cells, h              | [187]      |
| Corticosteroids                 | n/a          | n/a             | 4B cells, h (hypothalamic cell line)    | [188]      |
| **Peptide (hormone) signaling**  |              |                 |                                        |            |
| Catecholamine                    |              | cAMP/PKC        | SKOV3 cells, h (ovarian cancer)         | [189]      |
| **Cytokines**                    |              |                 |                                        |            |
| IL-1β                            | Ser133       | ERK1/2          | gastric cancer, h                       | [224]      |
| **NO and oxidative stress**      |              |                 |                                        |            |
| ER stress                        | Ser131       | n/a             | MDA-MB231, h (breast cancer)            | [190]      |
| H$_2$O$_2$                       | Ser133       | PKA, ERK        | C10 cells, h (colorectal adenocarcinoma)| [191]      |
| H$_2$O$_2$                       | Ser121       | n/a             | K562 cells, h (myelogenous leukemia), L-40(lymphoblasts)| [192] |
| Hypoxia                          | Ser133       | PKA             | T cell lymphoma, m                      | [193]      |
| Hypoxia                          | Ser131       | n/a             | MDA-MB231, h (breast cancer)            | [190]      |
| **Viral, bacterial and plant components** |       |                 |                                        |            |
| Acacetin                        | n/a          | ERK1/2          | B16F10 cells, m (melanoma)              | [194]      |
| Angelica sinensis polysaccharides| n/a          | ROCK1           | T47D, Hs578T, h (breast cancer)         | [195]      |
| Compound                          | Phosphorylation Site | Kinase or Pathway | Cell Line, Species | Reference |
|----------------------------------|----------------------|-------------------|-------------------|-----------|
| Diosmetrin                       | n/a                  | ERK1/2            | B16F10 cells, m (melanoma) | [194]     |
| Sulforaphene                     | Ser133               | MSK1              | Eca109, h (esophageal cancer) | [196]     |
| Yessotoxin                       | Ser133               | mTOR              | leukemia cells, h   | [197]     |
| **Phospholipids and lipid signaling** |                      |                   |                   |           |
| Placental total lipid            | Ser133               | p38 MAPK          | B10F10 cells, m (melanoma) | [198]     |
| **Environmental stress factors** |                      |                   |                   |           |
| DNA damage                       | Ser121               | ATM               | HEK293T, MeWo, HELA, h | [199]     |
| FCS depletion                    | Ser133               | JNK               | HCT116, h (colon cancer) | [200]     |
| Glucose deprivation              | Ser121               | n/a               | U2OS cells, h (osteosarcoma) | [201]     |
| Glucose deprivation              | Ser129               | GSK3α             | PC12 cells, r (pheochromocytoma), F9 cells (teratocarcinoma) | [202]     |
| IR                               | Ser108, 111, 114     | n/a               | HEK293T, MeWo, HELA, h | [199] [203] |
| UVB                              | Ser133               | p38               | SKM1 (acute myeloid leukemia), *in vivo*, m | [204]     |
| **Ion channels and intracellular Ca²⁺ signaling** |                      |                   |                   |           |
| KCl                              | Ser142               | nuclear CaMK II   | PC12 cells, r (pheochromocytoma) | [205]     |
| **Chemotherapeutics**            |                      |                   |                   |           |
| 4-Hydroxytamoxifen               | n/a                  | AKT               | MCF-7, SKBR-3, h (breast cancer) | [206]     |
| Doxorubicin                      | Ser133               | ERK               | malignant mesothelial, h | [207]     |
| “genotoxic stress”/DNA damage    | Ser270               | HIPK2             | K562 cells, h (myelogenous leukemia), SH-SY5Y, h (neuroblastoma cells) | [158]     |
| Quinaldic acid                   | Ser133               | AKT               | HT-29, LS180, Caco-2, h (colorectal carcinoma) | [208]     |
| Retinoic acid                    | Ser133               | n/a               | neuroblastoma cells, h | [209]     |

n/a = not specified; Species: m = mouse, h = human, r = rat
Supplementary Table 3: Structural alterations of the CREB1 gene in different tumor entities.

| Tumor entity                                          | n     | Genetic alterations (order)                   | Frequency of mutation rate [%] | Study                      |
|-------------------------------------------------------|-------|-----------------------------------------------|-------------------------------|----------------------------|
| bladder cancer                                       | 408   | deep deletion > amplification                 | 1.5                           | TCGA, [210]                |
| breast invasive carcinoma                            | 996   | amplification > deep deletion > missense mutation = fusion | 1.3                           | TCGA, panCancer Atlas      |
| cervical squamous cell carcinoma and endocervical adenocarcinoma | 191   | deep deletion > missense mutation            | 4.0                           | TCGA, provisional          |
| esophageal adenocarcinoma                            | 265   | amplification                                 | 3.0                           | TCGA, [211]                |
| esophageal carcinoma                                 | 184   | amplification > deep deletion                 | 2.7                           | TCGA, provisional          |
| head and neck squamous cell carcinoma                 | 504   | deep deletion > amplification > truncating mutation | 1.6                           | TCGA, provisional          |
| kidney renal clear cell carcinoma                    | 448   | amplification > deep deletion                 | 1.3                           | TCGA, provisional          |
| kidney renal papillary cell carcinoma                | 280   | deep deletion > amplification = missense mutation | 1.4                           | TCGA, provisional          |
| lung adenocarcinoma                                  | 230   | amplification > missense mutation            | 1.7                           | TCGA, [212]                |
| lung squamous cell carcinoma                         | 469   | amplification > deep deletion = missense mutation | 1.3                           | TCGA, panCancer Atlas      |
| metastatic prostate adenocarcinoma                   | 444   | amplification                                 | 4.0                           | [213]                      |
| neuroendocrine prostate cancer                       | 114   | amplification                                 | 12.0                          | [214]                      |
| ovarian serous cystadenocarcinoma                    | 311   | amplification > deep deletion                 | 5.0                           | TCGA, provisional          |
| pan-lung cancer                                      | 1144  | amplification > missense mutation > deep deletion > in-frame mutation | 1.3                           | [215]                      |
| prostate adenocarcinoma                              | 1013  | amplification > missense mutation > deep deletion | 1.8                           | [216]                      |
| stomach adenocarcinoma                              | 393   | amplification > missense mutation > deep deletion > truncating mutation | 3.0                           | TCGA, provisional          |
| uterine corpus endometrial carcinoma                 | 242   | missense mutation > amplification > deep deletion = truncating mutation | 2.9                           | TCGA, provisional          |
| uveal melanoma                                       | 80    | deep deletion                                 | 1.3                           | TCGA, panCancer Atlas      |

n = number of samples

The cBioPortal database (https://cbioportal.org/) was used for the analysis of the mutation load in different tumors. The study with the highest number of samples was chosen. Only tumor entities with a mutation rate > 1.0% and > 50 samples were included. The most common genetic alterations are listed first.
### Supplementary Table 4: Mutation rate of CREB1 in different tumor cell lines.

| Cell line | Tumor entity | CREB alterations | Other notable mutations |
|-----------|--------------|------------------|-------------------------|
| Capan-1   | mixed, ductal adenocarcinoma from liver metastasis | amplification | ERBB2 amplification, MYC amplification, KRAS (G12V), TP53 (A159V) |
| J82       | bladder cancer, transitional cell carcinoma | amplification | PTEN deep deletion, BCL2L1 amplification, TP53 (E271K) |
| MJ        | cutaneous T cell lymphoma | amplification | MYC amplification, ERBB4 amplification, MYB (Y629H) |
| MOTN-1    | T cell leukemia, T cell large granular lymphocytic leukemia | amplification | MYC amplification, BRCA1 amplification |
| NCI-H661  | lung cancer, large cell carcinoma | amplification | KRAS amplification, CCNE1 amplification, TP53 (R158L, S215I) |
| OVKATE    | ovarian cancer, adenocarcinoma | amplification | KRAS amplification, CCND1 amplification, TP53 (R282W) |
| RD        | rhabdomyosarcoma | amplification | BRCA2 deep deletion, MYC amplification, NRAS (Q61H), TP53 (R248W) |
| ALLSIL    | hematopoietic and lymphoid tissue mixed cancer types | deep deletion (homodeleted) | TP53 deep deletion, NOTCH1 (L1593P) |
| EC-GI-10  | esophagus carcinoma | deep deletion (homodeleted) | ERBB2 amplification, MYC amplification, TP53 (Y234C, R273L) |
| ME1       | acute myeloid leukemia, hematopoietic and lymphoid tissue | deep deletion (homodeleted) | TP53 deep deletion, CCND1 amplification, NRAS (Q61H) |
| MPP 89    | mesothelioma | deep deletion (homodeleted) | TP53 deep deletion, ATM deep deletion, RB1 (V654M) |
| OCI-LY19  | B cell lymphoma | deep deletion (homo deleted) | MYC amplification, FGFR1 amplification, NRAS (Q61K), CREBBP (D1435E) |
| OVCAR-8   | ovarian cancer, adenocarcinoma | deep deletion (homozygous deleted) | MYC amplification, B2m deep deletion, ERBB2 (G776V), TP53 (X126_splice) |
| BT474     | breast cancer, ductal carcinoma | truncating mutation (Y252*) | ERBB2 amplification, CCND1 amplification, BRCA2 (S3094*), TP53 (E285K) |
| KCL-22    | chronic myeloid leukemia in blast crisis, Philadelphia chromosome-positive CML | truncating mutation (R95Tfs*14) | USP6 deep deletion, PIK3CA(E545G), CREBBP (Q2045*) |
| HCC70     | breast cancer, ductal carcinoma | missense mutation (R298Q) | TERT amplification, RICTOR amplification, PTEN (F90Lfs*9), TP53 (R248Q) |
| LoVo      | colorectal cancer, large intestine | missense mutation (P75Q) | DUSP22 deep deletion, B2m deep deletion, KRAS (G13D), APC (R1114*) |
| MDA-MB-453| breast cancer, ductal carcinoma | missense mutation (E319K) | ERBB2 amplification, MYC amplification, PIK3CA(H1047R), PTEN (E307K) |
| SNU175    | colorectal cancer, large intestine, adenocarcinoma | missense mutation (T324A) | ERCC2 deep deletion, SUFU deep deletion, KRAS (A59T), EGFR (A864V) |
| SW1116    | colorectal cancer, large intestine, adenocarcinoma | missense mutation (L234V) | KRAS (G12A), TP53 (A159D), SMAD2 deep deletion |

For the analysis, CREB-mutated tumor cell lines were screened in cBioPortal with the dataset “Cancer Cell Line Encyclopedia (Novartis/Broad, Nature 2012)”
**Supplementary Table 5: Small molecule inhibitors targeting the interaction between CREB and CBP (KID – KIX) and their *in vitro* and/or *in vivo* use.**

| Inhibitor                                                                 | Cas-No.          | Cancer entity/cell line                  | Used concentration & incubation time | Reference |
|---------------------------------------------------------------------------|------------------|------------------------------------------|--------------------------------------|-----------|
| Naphthol-AS-E-phosphate (KG-501)                                          | 18228-17-6       | ALL pheochromocytoma                     | 10 – 50 µM, 1 -2 d                   | [25]      |
|                                                                           |                  | A549 (lung adenocarcinoma)               | 1 – 10 µM, n/a                        | [101]     |
|                                                                           |                  | PC12 (pheochromocytoma)                  | 10 µM, 72 h                           | [217]     |
|                                                                           |                  | U-87MG (glioblastoma)                    | 10 µM, 72 h                           | [218]     |
|                                                                           |                  | HBMEC                                    | 25 µM, 4 h                            | [100]     |
|                                                                           |                  | HER-2/neu+ BC, KRASV12                   | 25 µM, 24 h                           | [32, 131] |
|                                                                           |                  | Human lung cancer                        | 1 – 10 µM, 24 h                        | [104]     |
|                                                                           |                  | NSCLC/HUVEC                              | 5 – 20 µM, 96 h                        | [95]      |
|                                                                           |                  | NSCLC/HUVEC                              | 10 µM, 24 h                           | [95]      |
| Naphthol-AS-MX-phosphate                                                  | 1596-56-1        | Human lung cancer cells                  | 5 – 20 µM, 96 h                        | [104]     |
| Naphthol-AS-TR-phosphate                                                  | 2616-72-0        | Human lung cancer cells                  | 5 – 20 µM, 24 h/96 h                  | [104]     |
| 3-(3-Aminopropoxy)-N-[2-[[3-[(4-chloro-2-hydroxyphenyl)amino]carbonyl]-2-naphthalenyl]oxyethyl]-2-naphthalenecarboxamide hydrochloride (666-15) | 1433286-70-4     | BC cells                                 | 1 nM – 1 µM, 72 h                        | [219] [111] |
|                                                                           |                  | MDSC                                     | 100 nM, n/a                           | [220]     |
|                                                                           |                  | Neuroblastoma                            | 5 µM, 12 h                            | [132]     |
|                                                                           |                  | PDAC, *in vivo* (mouse)                  | 10 mg/kg BW/d for 3 weeks             | [221]     |
|                                                                           |                  | *in vivo* (mouse)                        | 10 mg/kg BW/d (5 times a week over three weeks) | [105]     |
|                                                                           |                  | N2A cells (neuroblastoma)                | 5 µM, 12 h                            | [227]     |
| N-(4-cyanophenyl)-3-hydroxy-2-naphthamide (XX-650-23)                     | 117739-40-9      | AML cell lines                           | 0.01 – 10 µM, 48 h                    | [108]     |
|                                                                           |                  | AML cell lines                           | 0.1 – 10 µM, 48 h                     | [109]     |
|                                                                           |                  | *in vivo* (mouse)                        | 2 mg/kg (single dosis)                | [230]     |
| N-(4-Chlorophenyl)-3-hydroxy-2-naphthamide (Luciferase inhibitor III)     | 92-78-4          | HEK293T                                  | 10 – 100 µM, n/a                      | [94]      |
|                                                                           |                  | HEK293T                                  | 1 – 100 µM, 2 – 4.5 h                 | [222]     |

The different inhibitors were tested on different murine or human cell lines at varying concentrations and for different time points. n/a = not specified.
Supplementary Figures

Supplementary Figure 1:

phosphorylation see Table 1 & Supplementary Table 2

Acetylation
O glycosylation
Phosphorylation
Ubiquitination
SUMOylation
**Supplementary figure legends**

Supplementary Figure 1: Domain structure of CREB and important aa residues.

The scheme shows the longest CREB1 protein isoform with 341 aa. Important amino acid residues that can be posttranslational modified are numbered. Most serine residues that can be phosphorylated (blue) are localized in the KID but also in the glutamine rich Q2 domain. Ubiquitination (orange) or SUMOylation (brown) is possible through lysine site-chains in the bZIP, while lysine connected with acetylation (green) are mainly found in the KID and the α region. O glycosylation (purple) is possible in the Q2 domain.

Supplementary Figure 2: CREB is a central player in gene regulation.

The transcription factor CREB is of central importance in oncogenesis. Several signal transduction pathways (e.g., PI3K/AKT, RAS/MEK, cAMP/PKA) may lead to activation of CREB phosphorylation, causing dimerization of CREB and binding to the CRE-DNA element. On the one hand, CREB regulates protein-coding genes, such as bcl-2, as well as noncoding genes of miRNAs or long noncoding RNAs. In the latter case, a negative feedback loop is also possible because some of the CREB-regulated miRNAs themselves can target CREB mRNA. Furthermore, posttranslational modifications of the CREB protein can significantly influence its activity. In addition to the abovementioned phosphorylation, this also includes modifications by ubiquitination or SUMOylation. The activity and function of CREB, such as the promotion of angiogenesis, are thus influenced not only by the expression level but also by the PTMs of CREB.