**Mareike Herrmann**

# Analysis of sAC expression and function as a co-factor of CREB

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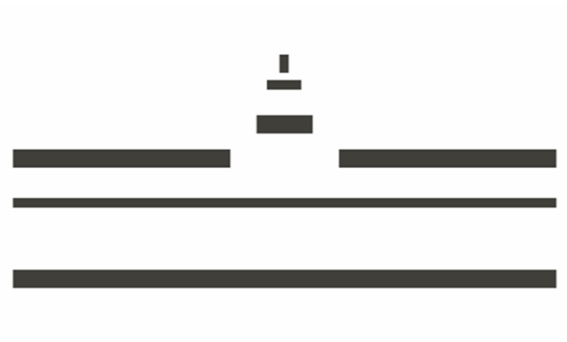

# Analysis of sAC expression and function as a co-factor of CREB

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- 2012 -

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*Für meine Eltern*

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**Abbreviations**

| | |
|---|---|
| A | adenosine |
| Ac | acetate |
| Acc# | accession number |
| ACE | angiotensin-converting enzyme |
| ADCY | adenylyl cyclase |
| AS | antisense strand |
| $AT_1$ | angiotensin II receptor type 1 |
| ATF | activating transcription factor |
| ATP | adenosine triphosphate |
| bp | basepairs |
| BRE | TFIIB recognition element |
| BRP44 | brain protein 44 |
| bZIP | basic leucine zipper |
| C | cytosine |
| $Ca^{2+}$ | calcium |
| CAD | coronary artery disease |
| CaMK IV | calcium-calmodulin dependent protein kinase IV |
| cAMP | cyclic adenosine monophosphate |
| CBP | CREB-binding protein |
| C/EBP | CCAAT/enhancer binding protein |
| ChIP | chromatin immunoprecipitation |
| $Cl^-$ | chloride |
| Co-IP | co-immunoprecipitation |
| COS7 | transformed african green monkey kidney fibroblast cells |
| CPE bind | cytoplasmatic polyadenylation element binding factor |
| CRE | cAMP response element |
| CREB | cAMP response element binding protein |
| CTCF | CCCTC binding factor |
| CV-1 | african green monkey kidney cells |
| CVD | cardiovascular disease |
| Da | dalton |
| DBP | diastolic blood pressure |
| dNTP | deoxynucleotide triphosphate |
| DPE | downstream promoter element |

| | |
|---|---|
| EA.hy926 | human vascular endothelial cells |
| e.g. | “exempli gratia” for example |
| e.i. | “id est” that is |
| ENaC | endothelial sodium channel |
| et al. | “et alia” and others |
| Exo I | exonuclease I |
| FI | fold induction |
| g | gram |
| G | guanine |
| GAPDH | glycerine-aldehyde-3-phosphate-dehydrogenase |
| GATA1 | transcription factor with affinity for the sequence GATA |
| GRA | glucocorticoid remediable aldosteronism |
| GRE | glucocorticoid response element |
| GWAS | genome-wide association studies |
| h | hours |
| HAT | histone acetyltransferase |
| $HCO_3^-$ | hydrogen carbonate |
| HE | 2-hydroxyestradiol |
| HEK293T | human embryonic kidney cells |
| HeLa | human cervix carcinoma cells |
| HepG2 | human hepatocellular carcinoma cells |
| HNF-3 alpha | hepatocyte nuclear factor-3 alpha |
| hRP27 | human ribosomal protein 27 |
| i.e. | “id est“ that is to say |
| IHKE | immortalized human kidney epithelial cells |
| INR | initiator element |
| k | kilo |
| $K^+$ | potassium |
| l | liter |
| LB | lysogeny broth |
| LD | linkage disequilibrium |
| $Li^{2+}$ | lithium |
| luc | luciferase gene |
| L-Zip | leucine zipper |
| M | Mol |
| µ | mycro |
| mcs | multiple cloning site |

| | |
|---|---|
| $Mg^{2+}$ | magnesium |
| min | minutes |
| miRNA | microRNA |
| mmHg | millimetres of mercury |
| $Mn^{2+}$ | mangan |
| MolHap | molecular haplotype |
| MolProMD | Münster Molecular Functional Profiling for Mechanism Detection study |
| $mpkCCD_{c14}$ | mouse cortical collecting duct cells |
| MR | mineralocorticoid receptor |
| MSK1 | mitogen and stress activated protein kinase 1 |
| n | nano |
| $Na^+$ | sodium |
| NCOR2 | nuclear receptor co-repressor 2 |
| NKCC | $Na^+/K^+/2Cl^-$ cotransporter |
| ns | not significant |
| OD | optical density |
| p | probability of error |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PDE | phosphodiesterase |
| Per1 | circadian clock protein Period 1 |
| pH | “potentia hydrogenii” |
| PIC | preinitiation complex |
| PKA | protein kinase A |
| PMA | phorbol 12-myristate 13-acetate |
| Pol II | RNA polymerase II |
| RAAS | renin angiotensin aldosterone system |
| RLU | relative light units |
| RT | room temperature |
| RT-PCR | reverse transcription polymerase chain reaction |
| s | seconds |
| sAC | soluble adenylyl cyclase |
| SAP | shrimp alkaline phosphatase |
| SBP | systolic blood pressure |
| SDS | sodium dodecyl sulfate |

| | |
|---|---|
| sgk | glucocorticoid inducible kinase |
| SMRT | silencing mediator of retinoic acid and thyroid hormone receptor |
| SNP | single nucleotide polymorphism |
| SP1 | specificity protein 1 |
| SS | sense strand |
| SV40 | simian vacuolating virus 40 |
| T | thymine |
| $T_A$ | Annealing temperature |
| TAF | TBP-associated factor |
| TBP | TATA-box binding protein |
| TF | transcription factor |
| TFIIA/B/D/E/F/H | transcription factor IIA/B/D/E/F/H |
| THP1 | human acute monocytic leukemia cells |
| $T_M$ | melting temperature |
| tmAC | transmembrane adenylyl cyclase |
| TSS | transcription start site |
| u | units |
| USF | upstream transcription factor |
| UTR | untranslated region |
| w/o | without |
| wt | wild type |
| Zn | zinc |

**Abstract**

The soluble adenylyl cyclase (sAC) catalyzes the generation of cAMP in specified microdomains of the cytoplasm and the nucleus. In the current study we demonstrated that sAC is involved in aldosterone-mediated gene expression of cAMP-regulated genes such as the *epithelial $Na^+$ channel* (*ENaC)* and the *$Na^+/K^+$-ATPase*, both mediating the aldosterone-induced increase of $Na^+$ currents across the membrane. We identified sAC as a co-factor of the cAMP response element binding protein (CREB), binding directly to DNA with regulative impact on transcriptional activity mediated by cAMP response elements using co-immunoprecipitation, chromatin immunoprecipitation and reporter gene assays.

We identified two distinct promoter regions by reporter gene assays to drive *sAC* transcription: the 5´-flanking region in combination with exon 1, harboring the core promoter (5´-promoter) and an intronic promoter located within intron 4. We demonstrated that these two alternative promoter regions regulate *sAC* transcription in a cell type- and differentiation-specific manner. The intronic promoter displayed the strongest transcriptional activity in vascular endothelial cells (EA.hy926) whereas in kidney cells (IHKE and HEK293T), the 5´-promoter was transcriptionally more active. The intronic promoter was specifically activated by aldosterone, while the 5´-promoter was activated by the cAMP-dependent transcription factors CREB and sAC itself. We defined the transcription factors C/EBP alpha and beta as cell type-specific modulators of sAC transcription in endothelial and kidney cells. Accordingly, we identified three isoforms of sAC (50, 70 and 80 kDa) via western blot analysis with different expression patterns in endothelial and kidney cells.

Screening of 4 kb of the *sAC* 5'-flanking region in 60 patients with cardiovascular disease (MolProMD study) led to the identification of seven genetic variants, three of which are in a strong linkage disequilibrium resulting in two molecular haplotypes: MolHap1 [$Ins^{-2356} – C^{-2181} – T^{-2092}$] and MolHap2 [$Del^{-2356} – T^{-2181} – G^{-2092}$]. Using reporter gene assays, we demonstrated allele-specific transcriptional activity of MolHap1 and MolHap2. SP1 was identified as a factor that modulates this allele-specific transcriptional activity.

Our results indicate that the sAC gene promoter is polymorphic in CVD patients, leading to altered sAC expression. This may result in a dysregulation of aldosterone-mediated gene expression by sAC acting as a co-factor of CREB.

To evaluate the genetic impact of sAC gene variants at the population level a large, well characterized study population should be genotyped.

# 1 Introduction

## 1.1 Blood pressure regulation

### 1.1.1 Blood pressure

Blood pressure is one of the principal vital signs. It is defined as the pressure exerted by circulating blood upon the walls of arteries at the level of the heart. Since systolic blood pressure (SBP) is defined as the peak of cardiac contraction and diastolic blood pressure (DBP) as the peak of cardiac relaxation, SBP displays the maximal value, dependent on the intensity of the heart deflating, whereas DBP displays the minimal value, dependent on arterial elasticity and blood volume. Blood pressure continually adapts to differing requirements during the day, such as exercise, stress, diet, disease, and sleep. The physiological factors which regulate blood pressure are especially the heart rate, blood volume and arterial stiffness. Average daily blood pressure is tightly regulated by several organs, such as the hypothalamic-pituitary-adrenocortical axis, lung, kidney, heart, and vasculature and fluctuates substantially with behavior. The complex regulation is dependent on various factors and interaction between the nervous system and hormonal control feedback loops (Guyenet, 2006), as well as the interaction of genes and environment. As a result, blood pressure regulation is not completely understood (Herrera and Coffman, 2012). Pathophysiological dysregulation of blood pressure leads to hypertension and, therefore, to an increased risk of cardiovascular disease, which was initially shown in the 1951s by the population based “Framingham heart study” (Dawber et al., 1951).

### 1.1.2 Hypertension

Hypertension is one of the predominant health problems worldwide (Whitworth, 2003; Wolf-Meier et al., 2003). It is a frequent, chronic and age-related disorder of the vascular system, in which the blood pressure value is to high. Hypertension is defined as SBP ≥140 mmHg and/or DBP ≥90 mmHg (table 1, Mancia et al., 2007).

**Table 1: Systolic and diastolic blood pressure thresholds**

Classification of blood pressure values (Adapted from Mancia et al., 2007)

| Category | SBP (mmHg) | DBP (mmHg) |
|---|---|---|
| Optimum | <120 | <80 |
| Normal | 120-129 | 80-84 |
| High-normal | 130-139 | 85-89 |
| Hypertension grade 1 (mild) | 140-159 | 90-99 |
| Hypertension grade 2 (moderate) | 160-179 | 100-109 |
| Hypertension grade 3 (severe) | >180 | <110 |
| Isolated systolic hypertension | 140-149 | <90 |

SBP: systolic blood pressure, DBP: diastolic blood pressure, mmHg: millimetres of mercury

Hypertension is classified into three different grades dependent on blood pressure values (table 1). Both, systolic and diastolic blood pressure levels are associated with cardiovascular morbidity and mortality (Lewington et al., 2002). Slight increases in blood pressure constitute a higher risk for cardiovascular diseases, whereas reduction of SBP by 10 mmHg and DBP by 5 mmHg is associated with 40% lower risk of fatal stroke and 30% lower risk of fatal myocardial infarction (Rosendorff et al., 2007).

Hypertension is classified as primary or essential hypertension and secondary hypertension. In the case of essential hypertension, which is true for 90% of hypertensives an organic reason is not observable (Staessen et al., 2003). 10% of hypertensives suffer from secondary hypertension, which is characterized by organic diseases or genetic mutations in single genes, which lead to high blood pressure.

Hypertension leads to arteriosclerosis, stroke and myocardial infarction, as well as renal and myocardial failure (Lewington et al., 2002; Staessen et al., 2003). One billion people worldwide suffer from hypertension, whereas by 2025 this number will increase to 1.56 billion people. Nearly 8 million people die worldwide from hypertension-related diseases (Lee and Cooper, 2009). Overall hypertension concerns 25% to 35% of the adult population. Furthermore the prevalence of hypertension increases with age, affecting between 60% to 70% of those beyond the seventh decade of life (Staessen et al., 2003). SBP continues to rise until the eight decade of life, whereas DBP only rises until the age of 50 years (Staessen et al., 2003).

The substantial influence of lifestyle, inheritance and environmental factors on blood pressure was shown in the 1951 by the “Framingham heart study”. Hypertension is inducible through stress, alcohol consumption, smoking, high caloric diet, drugs, and pregnancy (Dawber et al., 1951). Additionally numerous risk factors for developing

hypertension are known, including insufficient physical exercise, high body mass index, high salt intake and the individual genetic background.
In summary, hypertension arises from heterogeneous environmental and genetic factors, which are not completely understood yet (Staessen et al., 2003).

### 1.1.3 Genetics of hypertension

The importance of the genetic background of hypertension was shown by several family-based and twin studies, in which the inheritance of hypertension has been estimated to be 30% to 50% (Luft, 2001). These studies also illustrate that even individuals with the same environmental and genetic background show different phenotypes, as justified by epigenetic modifications to the related genes (Drewell et al., 2000).
Since the genetic background of blood pressure contributes 30% to 50% of the risk to be affected, it is important to identify the involved genes, which may predict the risk for developing the disease and help to develop antihypertensive drugs (Tanira and Balushi, 2005). Mostly, hypertension is a polygenic disease, i.e. it depends on a large number of genes, each of which takes part in the regulation of blood pressure, with exception of monogenic forms, such as glucocorticoid remediable aldosteronism (GRA, Pizzolo et al., 2005) or liddle disease (Hansson et al., 1995). Apart from spontaneous mutations, genetic variants display the heterogeneity of the gene pool of a species. The genome is polymorphic to facilitate phenotypic differences between the individuals. Different genetic variants, with different frequencies, influence gene expression according to their position within the gene.
A widely used approach to identify genetic variants associated with hypertension is to sequence a specific gene from diseased individuals and search for variants existing with a higher frequency in affected individuals (Tabor et al., 2002).
Linkage analysis is used to explain the inheritance of genotype and phenotype of the disease in pedigrees, representing information on gene frequency, mode of inheritance and penetrance (Tabor et al., 2002).
Association analysis is performed with large study collectives, comparing cases and controls for probable risk alleles. The advantage of such studies is based on the greater statistical power to detect genes even those with smaller effects (Tabor et al., 2002).
Genome-wide association studies (GWAS) are non-hypothesis driven approaches giving the possibility to identify previously unknown genetic loci.
Several recently unidentified genetic variants, which influence systolic and diastolic blood pressure, were identified in a genome-wide association study of 200,000 European

individuals (International Consortium for Blood Pressure Genome-Wide Association Studies, 2011). If these genetic variants are located in coding regions of the gene, the function of the protein can be affected, as shown for the “mineralocorticoid receptor activating mutation”, which leads to hypertension by a higher constitutive mineralocorticoid receptor (MR) activity, caused by a mutation in the coding region of the MR (Geller et al., 2000). Additionally, variants in the regulatory regions of a gene, which lead to hypertension by affecting the amount of the protein, were illustrated for the angiotensinogen gene promoter (Brand-Herrmann et al., 2004). Since hypertension is a polygenic trait, where the factors and signaling pathways involved are not completely understood, it remains important to gain new insights into the molecular basis of blood pressure regulation and potential new therapeutic pathways.

### 1.1.4 Pathophysiology of hypertension

As already mentioned, blood pressure is regulated by various genetic and environmental factors (Franklin, 2001). The kidneys play a pivotal role in blood pressure regulation (Guyton, 1990; Keller et al., 2003), shown e.g. for the hypertensive rat model, in which renal sodium excretion is impaired (Bianchi and Ferrari, 1992). Transplantation of a kidney from a normotensive to a hypertensive rat reduces the blood pressure of the hypertensive rat, pointing to the fundamental role of the kidney in blood pressure regulation (Rettig et al., 1990). Salt sensitivity is a common finding in hypertensives, resulting from dysregulation of sodium excretion by the kidneys modified by endocrine or neurocrine factors (DiBona, 2000) or various mutations in membrane proteins, such as cytoskeleton proteins or ion transporters (Manunta et al., 2001).
The complete set of mechanisms regulating blood pressure is not fully understood, but depends mainly on vasoconstriction and vasodilation, sodium and fluid balance, which are regulated on various levels.

### 1.1.5 Renin angiotensin aldosterone system (RAAS)

In the kidney, the RAAS is an important system for blood pressure regulation (Brewster and Perazella, 2004). Changes in blood pressure are sensed in the glomerular afferent arterioles, so that renin is released from the juxtaglomerular apparatus when blood pressure decreases. Renin enzymatically cleaves angiotensinogen into the decapeptide angiotensin I. The proteolytic angiotensin-converting enzyme (ACE) is localized in the

vascular endothelium and cell membranes of the kidneys, brain, heart, and lung, where it converts angiotensin I into the octapeptide angiotensin II. Angiotensin II acts primarily via the angiotensin II receptor type 1 ($AT_1$), which leads to an increase in $Na^+$ reabsorption and arteriolar vasoconstriction, thereby leading to blood pressure elevation (figure 1).
Additionally angiotensin II induces aldosterone secretion from the zona glomerulosa of the adrenal gland (Laragh et al., 1960) and other tissues (Garty, 1992).
The mechanism of aldosterone signaling is not fully elucidated. Which effects are mediated by genomic pathways is still controversial, involving transcription and translation, and non-genomic pathways, such as second messengers and kinase cascades (Funder, 2006). The MR mediates the genomic pathway of aldosterone signaling (Bonvalet, 1998). However, MR downstream signaling is not completely defined. In porcine coronary vascular smooth muscle cells, aldosterone induces a $Ca^{2+}$-dependent increase of intracellular cAMP and a time-dependent phosphorylation of CREB, which can not be blocked by inhibition of the MR (Christ et al., 1999). In the genomic pathway aldosterone-activated MR leads to the expression of genes, which are involved in $Na^+$-reabsorption in the distal tubule of the kidney. This is mediated by increased expression of $Na^+/K^+$-ATPase and $Na^+/H^+$-exchanger in renal distal tubule cells (Reilly and Ellison, 2000). The MR can also act via non-genomic pathways, by direct stimulation of membrane translocation of ENaC, in addition to the increasing effect on ENaC expression on the genomic pathway. ENaC is expressed in the apical membrane of renal tubule principal cells. Active ENaC permits $Na^+$ transport between the lumen of the renal collecting duct and the blood. Increasing $Na^+$ concentration leads to activation of the $Na^+/K^+$-ATPase, which exchanges two $Na^+$ ions from the cytosol for three $K^+$ ions from the extracellular lumen (Rossier and Stutts, 2009). This depolarization of the apical membrane through opened ENaC leads to reabsorption of NaCl and, therefore, increases blood pressure. In addition, ENaC is proposed to be the key mediator of aldosterone-dependent blood pressure control in the vascular endothelium (Kusche-Vihrog et al., 2008).

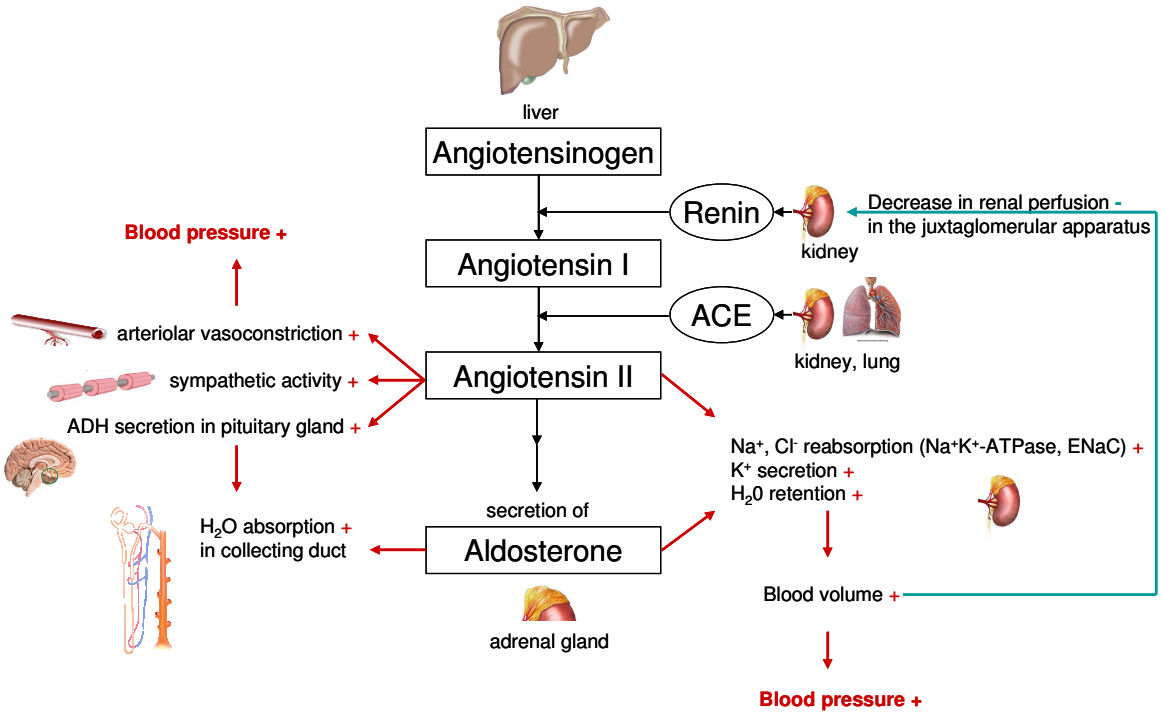



**Figure 1: Schematic representation of the renin angiotensin aldosterone system (RAAS)**
Angiotensinogen is produced in the liver, cleaved by renin into angiotensin I and subsequently to angiotensin II by the angiotensinogen converting enzyme (ACE). Angiotensin II leads to the release of aldosterone and together with aldosterone has several vasoconstrictive effects, which consequently lead to an increase in blood pressure.

## 1.2 sAC and blood pressure regulation

### 1.2.1 Crosstalk between aldosterone- and cAMP-signaling

The mechanism of aldosterone signaling has not been fully elucidated, but there is evidence of a crosstalk with the cyclic adenosine monophosphate (cAMP) signaling pathway (Le Menuet et al., 2001; Ouvrard-Pascaud et al., 2005), depending on the cell system and the context in which it is examined (Grossmann et al., 2010). A $Ca^{2+}$-dependent increase in intracellular cAMP concentration and of CREB phosphorylation was found in vascular smooth muscle cells after incubation with aldosterone (Christ et al., 1999). Aldosterone also stimulated cAMP production in rat inner medullar collecting ducts (Sheader et al., 2002) and in bovine endothelial aortic cells (Leopold et al., 2007). It has been suggested, that stimulation of an adenylate cyclase mediates the aldosterone-stimulated cAMP generation (Sheader et al., 2002), potentially linking aldosterone to sAC signaling.

Another potential influence of aldosterone on CREB-mediated gene expression was shown in juxtaglomerular cells, where aldosterone activated the gene expression of *renin*. Renin and aldosterone form a part of the RAAS system (Klar et al., 2002).
In HeLa cells, a MR-dependent effect of cAMP on $Na^+/K^+$-ATPase expression was shown. $Na^+/K^+$-ATPase expression is regulated via a promoter involving a cAMP response element (CRE) and, therefore, can be upregulated via cAMP. This activation can be repressed by the MR, probably through the interaction of aldosterone with cAMP-inducible transcription factors (Ahmad and Medford, 1995). In contrast, in MR-knockout mice, treatment with aldosterone led to enhanced cAMP concentrations (Haseroth et al., 1999). Comparable results were shown regarding the vasopressin-inducible gene expression of hepatocyte nuclear factor-3 alpha (HNF-3 alpha), in which aldosterone inhibited the cAMP-induced activation (González-Núñez et al., 2004).
In the zona glomerulosa of the adrenal gland, aldosterone secretion is stimulated via $Ca^{2+}$ or cAMP. Additionally, MR-mediated activating effects on gene expression were shown to be inducible by cAMP (Nordeen et al., 1994; Nordeen et al., 1995; Lim-Tio and Fuller 1998). This enhanced MR effect on gene expression seems to be dependent on the basal promoter structure and increases with the number of glucocorticoid response element (GRE) motifs. Consequently, cAMP and aldosterone act synergistically on GRE motifs (Massaad et al., 1999).
Furthermore, expression of the serum and glucocorticoid-inducible kinase (sgk), which is important in activation of certain $K^+$, $Na^+$, and $Cl^-$ channels, was enhanced by stimulation with cAMP or aldosterone, but the combination of both did not yield an additive effect (Snyder et al., 2004).
To summarize, there is a crosstalk between aldosterone and cAMP signaling in many cell system pathways. Nevertheless, numerous aspects of this crosstalk remain unclear and which factors are involved still needs to be examined.

### 1.2.2 sAC protein function

cAMP signaling is the most widely used cellular signaling pathway. In 1971, Earl Sutherland was awarded the Nobel Prize for identifying cAMP as a mediator of cellular regulation of metabolic processes. cAMP mediates cellular responses to external stimuli, modulates cell growth and differentiation, and modulates several physiological pathways, including metabolism, apoptosis, migration, development, ion transport, pH regulation, and gene expression (Robison et al., 1968).

In the mid 1990s, a family of nine membrane-associated adenylyl cyclases (ADCY1-9), which catalyze the accumulation of cAMP out of adenosine triphosphate (ATP), was characterized. These transmembrane adenylyl cyclases (tmACs) differ in their expression patterns. Furthermore they are differentially regulated by G-proteins in response to hormones and neurotransmitters and could be activated by forskolin (Taussig and Gilman, 1995; Sunahara et al., 1996). Forskolin is applied to increase intracellular cAMP levels via stimulation of tmACs. tmACs are expressed in organisms from prokaryotes to higher eukaryotes and their catalytic domain is highly conserved. In 1999 an additional soluble form of adenylyl cyclase (ADCY10, sAC) was characterized in mammals, which is independent of forskolin or G proteins (Buck et al., 1999). sAC is the only enzyme that can sense differences in $CO_2$ concentrations in mammals and acts, therefore, as a catalytic sensor. Its catalytic domains show higher conservation to cyanobacterial adenylyl cyclase than to mammalian tmACs (Chen et al., 2000).

Two isoforms of sAC are currently described in rats, a full length (187 kDa) and a truncated form (50 kDa) with a 10 to 20-fold higher activity compared to the full length form (Buck et al., 1999), arising from alternative splicing (Jaiswal and Conti, 2001). Both isoforms comprise two heterologous catalytic domains (C1 and C2), whereas the C-terminus of the full length form additionally comprises several putative regulatory domains, such as an autoinhibitory region (Gordeladze et al., 1981), a conical P-loop, and a leucine zipper sequence (Buck et al., 1999, figure 2).

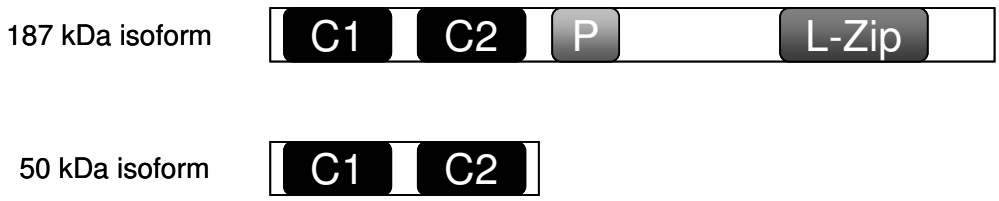



**Figure 2: Isoforms of rat sAC**

The 187 kDa isoform of sAC contains two catalytic domains (C1, C2), a P-loop (P) sequence, and a leucin zipper (L-Zip). The truncated 50 kDa isoform of sAC consists only of the catalytic domains.

Since there are several isoform predictions, which contain only the C2 domain, it remains to be investigated whether they are generated via alternative splicing or alternative promoter usage in different tissues (Farrell et al., 2008; Geng et al., 2005; Schmid et al., 2007). A knockout mouse model of sAC exists, in which the exons encoding C1 were removed, but still retains the C2 domain and the non-catalytic C-terminus (Esposito et al., 2004). This mutation was sufficient to induce male infertility, since sAC function is

important in sperm capacitation (Sinclair et al., 2000; Xie et al., 2006; Hess et al., 2005; Farrell et al., 2008). In addition, female mice exhibited increased cholesterol and triglyceride levels and both sexes exhibited a slight increase in the heart rate. Lack of both catalytic isoforms is lethal (Esposito et al., 2004). From this result it may be inferred that in order to function in tissues, apart from testis, it is sufficient for sAC to contain the C2 domain.

To catalyze cyclization of ATP to cAMP, sAC needs two divalent metal cations in its catalytic center. $Ca^{2+}$ increases the enzyme affinity for ATP (Jaiswal and Conti, 2003) and $HCO_3^-$ stimulates sAC activity by allosteric changes of the enzyme that lead to closure of the active site, recruitment of $Mg^{2+}$, and rearrangement of the phosphates of substrate ATP (Neer, 1978). In the primary step of cAMP generation, the first divalent metal cation $Ca^{2+}$ binds to the gamma-phosphate of ATP. This interacts with specific residues of the catalytic center, resulting in an “open state”. Furthermore, the second divalent metal cation $Mg^{2+}$ binds to the alpha-phosphate of ATP. This interaction of a set of catalytic residues leads to a “closed state”. This conformation change from open to close induces esterification of the alpha-phosphate of the ribose and cyclisation takes part by release of the beta- and gamma-phosphate (Tresguerres et al., 2011).

cAMP mediates a large composite of functions and, therefore, needs to be locally regulated. sAC plays an important role in this regulation, since it generates the local demand of cAMP in special microdomains that are in close proximity to cAMP targets throughout the cell (Chen et al., 2000; Zippin et al., 2004, figure 3). For this regulation, A-kinase-anchoring proteins are necessary, which tether protein kinase A (PKA) to the specific microdomains (Beene and Scott, 2007; Carnegie et al., 2009). Phosphodiesterases (PDEs) avoid cAMP cross communication between the microdomains by degradation of cAMP (Houslay, 2010; Calebiro et al., 2010 a; Calebiro et al., 2010 b).

In the kidney, cAMP regulates several ion-transport processes in the nephron. Several studies suggested the appearance of different sAC isoforms in the kidney, which potentially mediate different processes (Farrell et al., 2008; Geng et al., 2005; Paunescu et al., 2008; Pastor-Soler et al., 2003; Hallows et al., 2009). sAC location is mostly distributed in cells of the medullary and cortical thick ascending loop of henle, cells of the distal tubule, and cells of the collecting duct (Pastor-Soler et al., 2003; Hallows et al., 2009). In the thick ascending loop of henle, $Na^+/K^+$-ATPase, which was shown to be regulated by sAC (Hallows et al., 2009), supplies the energy for the apical $Na^+/K^+/2Cl^-$ cotransporter (NKCC, Fenton and Knepper, 2007). In response to cAMP, phosphorylated NKCC2 has been shown to be located in the apical membrane of the thick ascending loop of henle (Ortiz, 2006; Caceres et al., 2009). High amounts of $HCO_3^-$ may stimulate NaCl

and water absorption (Loon and Wilcox, 1998). In the distal tubule, reabsorption of NaCl, $Mg^{2+}$, $Ca^{2+}$ and $K^+$ and also secretion of $K^+$ takes place. sAC senses elevations in luminal pH and $HCO_3^-$ concentrations and restores the original low pH (Pastor-Soler et al., 2003). A potential role of sAC in transepithelial $Na^+$ transport was shown (Hallows et al., 2009) in mouse cortical collecting duct cells ($mpkCCD_{c14}$). Due to their expression of ENaC and $Na^+/K^+$-ATPase in their apical and basolateral membrane, $mpkCCD_{c14}$ cells are considered to be most similar to distal tubule principal cells (Bens et al., 1999; Vandewalle, 2002). Besides the effects of sAC in the kidney, sAC also plays an important role in inflammation. In immune cells, sAC signaling is important for activation of the small GTPase Rap1, which regulates qualitative T cell responses (Bivona et al., 2004). An abundance of sAC was found in leukocytes (Geng et al., 2005) and neutrophils (Han et al., 2005), in which sAC mediates tumor necrosis factor-induced release of $H_2O_2$, which induces cell death (Ho et al., 2011).

To gain new insights into the regulation of sAC and to find possible explanations for sAC dysfunction, the regulation of the level of transcription needs to be investigated.

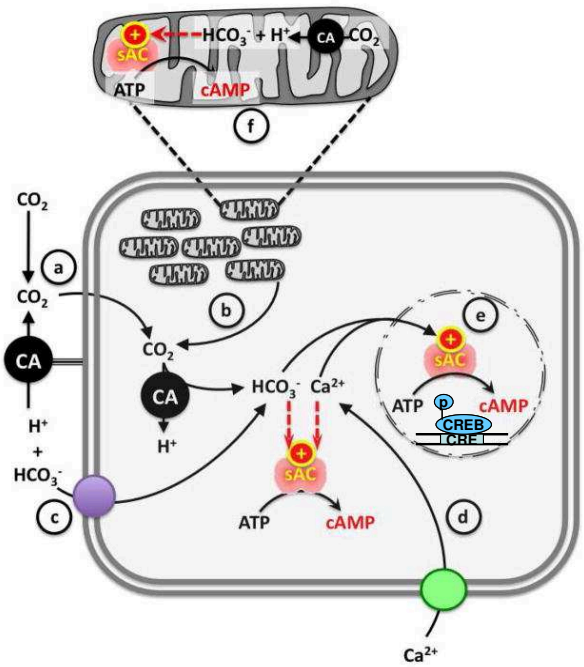



**Figure 3: sAC signaling in special microdomains**

sAC is localized in the cytoplasm, the nucleus (e) and in mitochondria (f). sAC can be activated by $Ca^{2+}$ (d) or $HCO_3^-$ derived from carbonic anhydrase (CA)-dependent hydration of (a) external and (b) metabolic $CO_2$ and $HCO_3^-$ that enter via membrane transporters (c). Active sAC can mediate phospohorylation of the transcription factor CREB in the nucleus (e). In mitochondria, sAC has been shown to be activated by metabolically generated $CO_2$ via CA (f). (Adapted from Tresguerres et al., 2011, with kind permission)

### 1.2.3 sAC gene structure

The *sAC* gene spans a large domain of 105 kb on chromosome 1 (figure 4). The exon structure is conserved in mammals. It consists of 33 exons, of which a region in exon 5 and upstream of exon 24 is alternatively transcribed. The 5´-untranslated region (5´-UTR) includes exon 1 and a part of exon 2. The 3´-untranslated region (3´-UTR) includes the back of exon 33. Three possible alternative translational start sites are located in exon 2, exon 5 and upstream of exon 24 (http://www.ensembl.org/index.html). In rats, the existence of two different sAC transcript variants is described, the full length transcript and a splice variant in which exon 11 is spliced out leading to a reading frame shift and a stop codon in exon 11. The full length transcript is translated into the full length protein form (187 kDa) and the spliced transcript is translated into the truncated isoform (50 kDa).

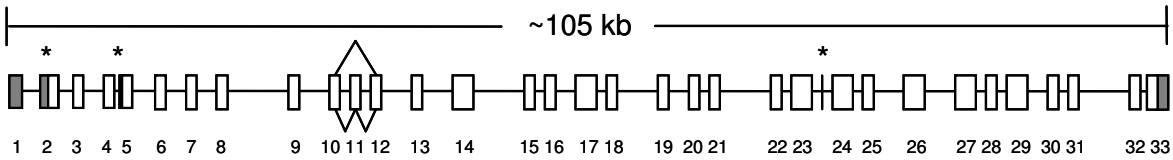



**Figure 4: *sAC* gene structure**

The human *sAC* gene contains 33 exons and spans 105 kb on chromosome 1. Asterisks mark potential translational start sites. Two alternative transcripts exist, which result from alternative splicing. In the upper section exon 11 is spliced out, leading to a reading frame shift and an alternative stop codon in exon 12. Grey boxes: untranslated regions, black boxes: alternatively transcribed regions, kb: kilobasepairs

### 1.2.4 CREB as target of sAC signaling

In mammals, the most comprehensively characterized transcriptional response to cAMP is mediated by the transcription factor CREB. CREB is highly conserved in mammals, which is indicative of the importance of its function (Mayr and Montminy, 2001). Dysfunction of CREB is involved in many diseases, such as cancer (Drozdov et al., 2011) and dementia (Müller et al., 2011). Furthermore CREB plays a role in inflammation (Chava et al., 2012) and was shown to be downregulated in vascular disease (Lösel et al., 2004).

CREB belongs to the basic leucine zipper (bZIP) transcription factor superfamily, which binds to CRE motifs with the recognition sequence 5´-TGACGTCA-3´. It mediates both, basal and PKA-inducible transcription through two separate and independently active domains. These are the kinase-inducible domain (Q1) and the constitutive activation

domain (Q2/CAD), both of which interact with the basal transcription factor (TF) TAF130, a component of the TFIID and TFIIB complex. For the activation of CREB, interaction with the CREB-binding protein (CBP) is required (Kim et al., 2000).
The kinase-inducible domain Q1 can be activated via phosphorylation of serin at position 133. Phosphorylation can be operated through signaling of transmembrane adenylyl cyclase, which produces cAMP leading to activation of cytosolic PKA and translocation of the catalytic units of PKA into the nucleus. However, the sAC also plays an essential role in CREB phosphorylation, which is not completely elucidated until now (Zippin et al., 2010; Xu et al., 2011). Members of the CREB/ATF (activating transcription factor) family and Fos/Jun family are leucine zipper containing proteins with sequence similarity in their DNA-binding domain and can produce different transcriptional regulators by heterodimerization (Brindle and Montminy, 1992). CREB can dimerize with itself, with other proteins without leucine zipper domains or with the transcription factor CCAAT-enhancer-binding protein (C/EBP) beta, which contains a leucine zipper domain (Park et al., 1993).

## 1.3 Gene expression control

### 1.3.1 Levels of expression regulation

Phenotypic differences of cells, as well as response and adaptation to environmental impact are regulated on the level of gene transcription and post-transcriptional and translational modifications.
Under- or overproduction of one ore more proteins can cause several diseases, such as cancer, immune disorders or hypertension. This shows the importance of a specific gene regulation.
The human genome exhibits 22,500 protein coding genes, whereas almost every gene is regulated by distinct promoter regions (Dübel et al., 2010). Gene promoters are defined as a specific DNA sequence, to which the RNA polymerase binds and signals, where transcription should begin (Roberts, 1969). For most human genes the exact and complete localization of the promoter regions remains unknown (Eckhardt et al., 2006). It can be located in the 5´-flanking region, the 5´-UTR or intronic regions of the gene over long distances. In eukaryotes, 90% of the gene regions are intronic, which often harbor regulatory elements, whereas only 10% of the gene regions consist of coding exonic regions.

In addition to gene regulation, there are several levels to control the amount and functionality of proteins in the cell. 1) Transcription can only take place in active euchromatin and not in condensed heterochromatin, densely packed around histones. Recruitment by transcription factors and chromatin modifying complexes can lead to accessibility of transcriptional start sites (TSSs). 2) Genes can be epigenetically modified by histone acetylation or methylation, which leads to transcriptional activity or inactivity. 3) mRNA can be processed by splicing events or degraded by RNA interference (Kornblihtt, 2006; Lin et al., 2008). 4) The last step of control is the regulation of the amount of protein itself, due to posttranslational modifications including phosphorylation, glycosylation, ubiquitinylation and several other processes (Shenoy and Rockman, 2011). These modifications can influence both the protein function and its localization. All of these processes are connected via negative or positive feedback loops.

### 1.3.2 *Cis*- and *trans*-regulatory elements

Elements which are located and coded by the DNA and influence transcriptional activity of promoter sequences are called *cis*-regulatory elements. These include enhancers, silencers and insulators (Riethoven, 2010). Enhancer and silencer elements can act independently of position and distance to the promoters they control (Atchison, 1988; Kermekchiev et al., 1991). They are able to mediate their activating or repressing effects when *trans*-regulatory elements, such as transcription factors bind to *cis*-regulatory elements. Insulators are able to block undesirable promoter enhancer interaction and enable the compartmentalization of the genome in differentially active domains (Lutz et al., 2003). There are two different insulators according to their function (Geyer, 1997, figure 5). Enhancer blocking insulators are sequences between promoter and enhancer or promoter and silencer, on which the insulator can bind to block the interaction between the promoter and the corresponding gene (Geyer and Corces, 1992; Kellum and Schedl, 1992). This may be caused by chromatin loops with insulator elements and factors on the basis of these loops (de Laat and Grosveld, 2003). Based on this sterical distance, interactions between promoter and enhancer or promoter and silencer are prevented (Schedl and Broach, 2003). The impact of the enhancer on the corresponding promoter may be mediated by histone modification, which will be passed along the DNA strand and blocked by insulator binding (Felsenfeld et al., 2004). Barrier insulators isolate genes from the repressing effect of bordering heterochromatin (Sun and Elgin, 1999). In mammals, all insulator elements are associated with the CCCTC-binding factor (CTCF, Ohlsson et al., 2001).

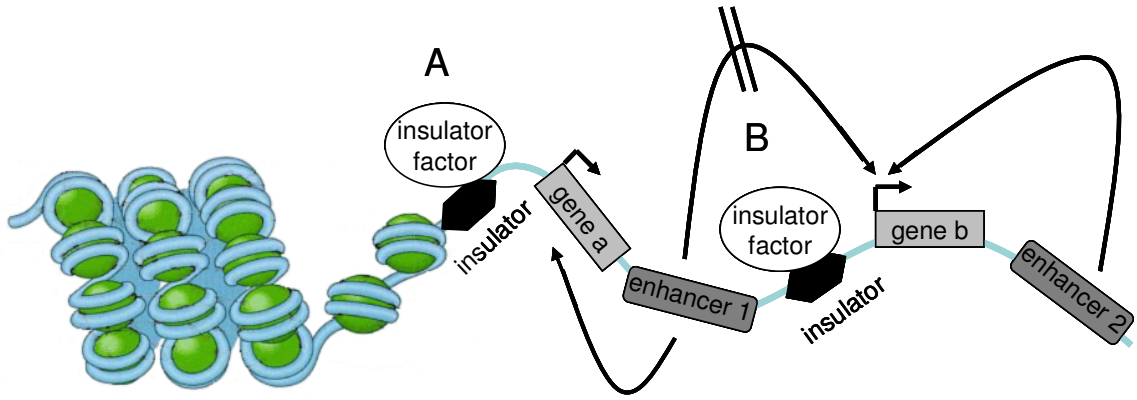



**Figure 5: Schematic representation of barrier insulator function and enhancer blocking insulator function**

**A** Barrier insulators isolate active chromatin and inactive chromatin domains from each other. **B** Enhancer can activate non-directionally transcription (enhancer 2/gene b) of different genes. Insulator factors, which are bound between enhancer (enhancer 1) and promoter of ascertained genes (gene b) block the enhancing function.

The functionality of enhancers and silencers depends on both, their accessibility and the relative amount of active transcription factors. Transcription factors are proteins that bind specifically to short DNA sequences of 5 to 20 bp and influence the transcription rate of definite genes. They can activate or repress the recruitment of RNA polymerase II (Pol II) to promoter regions. One promoter region typically contains 10 to 50 binding sites for 5 to 15 different transcription factors (Arnone and Davidson, 1997), which are the elementary units of promoter regulation. These modules are highly variable between different genes to ensure specificity of transcription (Wray et al., 2003) and are related to gene function (Lee et al., 2005). Genes that code for proteins involved in complex processes present a large amount of conserved transcription factor binding sites. On the other hand housekeeping genes, genes that are expressed in all tissues under various conditions, present restricted transcription factor binding sites as they underlie a less specific regulation (Farré et al., 2007).

In most cases, transcription factors interact in synergy. In other cases, they interact antagonistically, when different transcription factors recognize the same recognition site with different affinities or overlapping sites (Masquilier and Sassone-Corsi, 1992). Hence, the repression of gene transcription occurs when repressors compete with activators by blocking the activation site (Wang et al., 1997) or by direct interaction with general transcription factors (Song et al., 1995). The activity of transcription factors can be regulated by controlling the synthesis and degradation of the factor or by regulating its activation. Rapid regulation of transcription factors is mediated by phosphorylation, ligand binding, and interaction with other proteins (Dadarlat and Skeel, 2011).

It is important that regulation of gene transcription can be adapted to environmental processes (Titze and Machnik, 2010). To ensure appropriate and correct gene expression, interaction of transcription factors with their recognition site should be tightly regulated. Genetic variations, such as single nucleotide polymorphisms (SNPs) in these recognition sites can lead to default gene regulation and probably influence the amount of protein with potential disease causing effects.

### 1.3.3 Promoter assembling

Transcriptional regulation is dependent on *cis*-acting factors (DNA and Chromatin), *trans*-acting factors (transcription factors and associated proteins), the basal transcription machinery including TATA binding proteins and Pol II, three dimensional structures and time (van Driel et al., 2003).

Transcriptional initiation, characterized most completely for TATA promoters, starts with the binding of the basal transcription factor II D (TFIID), which binds to sequences that contain TATA boxes and initiator elements (INR) at a given distance range (figure 6). TFIID recruits the TATA-box binding protein (TBP) and eight to twelve different TBP-associated factors (TAFs), to build the Pol II initiation complex. The N-terminal domain of TFIIB helps to position the DNA on the surface of the polymerase. Next, TFIIE enters and recruits TFIIH, which acts as helicase and ATPase to help unwind the DNA double strand. TFIIF captures the wrapping of the DNA around the preinition complex. Finally, the DNA strand can enter the active site of the polymerase, which synthesizes the RNA strand and at the end of synthesis rewinds the DNA strand (Kim et al., 2005, Baumann et al., 2010).

There are two functional components of promoter assembling, the basal core promoter and the proximal promoter. The basal core promoter, where RNA polymerase complex is recruited, typically comprises 70 to 80 bp, harboring core promoter elements, such as the TATA-box, INR, downstream promoter element (DPE) or B recognition element (BRE), which are located within a given distance to the TSS (table 2).

**Table 2: Sequence and frequency of core promoter element in vertebrates**

| Core element | Bp position relative to TSS | consensus sequence | frequency in promoters |
|---|---|---|---|
| TATA | < -31 to -26 | TATAWAAR | 10 to 16% |
| INR | -2 to +4 | YYANWYY | 55% |
| DPE | +28 to +32 | RGWYV | 48% |
| BRE | -37 to -32 | SSRCGCC | 12 to 62% |

INR: initiator element, DPE: downstream promoter element, BRE: B recognition element, TSS: transcriptional start site, nucleotide abbreviation: T: thymine, A: adenosine, G: guanine, C: cytosine, W: weak (A or T), R: purine (A or G), Y: pyrimidine, V: not T (A, C or G), S: strong (C or G). (Adapted from Heintzman and Ren, 2007)

In contradiction to earlier reports on promoter elements, only 10% to 16% of all promoters harbor a TATA box, comprising the sequence TATAAA 26 to 30 bp upstream of the TSS (Bajic et al., 2004; Butler and Kadonaga, 2002). BRE promoters harbour an upstream extension of a subset of TATA boxes. TATA less promoters typically contain an INR and a DPE 30 nucleotides downstream of the TSS (Burke and Kadonga, 1997; Gershenzon and Ioshikhes, 2005) and several CpG repeats, called CpG islands. Transcription from CpG islands initiates from multiple weak start sites, often distributed over a region of 100 nucleotides, in contrast to transcription from a single site, bound by the majority of transcription factors. Since TATA-less promoters have no intrinsic specificity for TFIID recruitment, activators like SP1 bind to the GC rich regions and recruit the TFIID complex (Pugh and Tijan, 1991; Butler and Kadonaga, 2002; Hilton and Wang, 2003; Wierstra, 2008).

Most core promoters do not contain a single TSS but rather an array of closely located initiation sites. Alternative TSS can be localized in other exons or alternative first exons are included. Differentially regulated alternative promoter systems are the basis of cell type-specific transcription of genes (Carninci et al., 2006). This leads to differences in the N-terminus of the proteins, and may lead to alterations in protein levels, functions, and localization (Ayoubi and Van De Ven, 1996). The proximal promoter located upstream of the core promoter is related to transcription factors that confer specificity of transcription.

In eukaryotes, there is no transcriptional activity from a promoter in absence of specific transcription factors. The interaction of transcription factors with the basal transcription machinery is mediated by co-activator proteins (Heintzman and Ren, 2007).

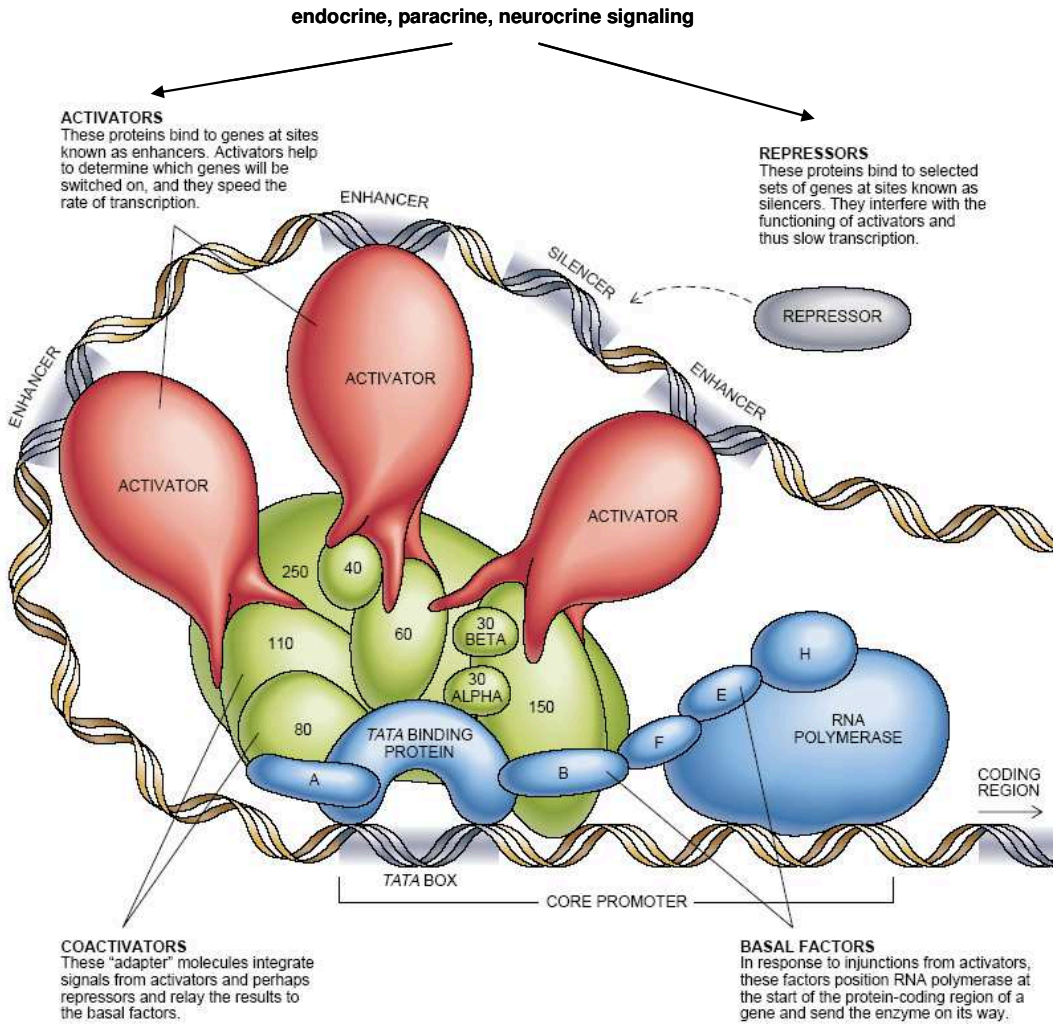



**Figure 6: Control levels of eukaryotic transcription at TATA promoters**

Endocrine, paracrine, and neurocrine factors lead to activation of *trans*-regulatory factors, which bind to *cis*-regulatory elements, such as enhancers or silencers. Binding of activating factors (red) leads to DNA looping and interaction with RNA polymerase II co-activators (green, named according to their molecular masses in kilodalton). Initially, the basal transcription factor TFIID enters TATA box sequences in the core promoter and recruits the TATA binding protein. Next, basal transcription factors TFIIB, TFIIE, TFIIH, and TFIIF assemble to form the TFII complex, which interacts with RNA polymerase II to initiate basal transcription (blue). TF: transcription factor. (Adapted from Tjian, 1995, with kind permission)

## 1.4 Aim and design of the study

The steroid hormone aldosterone contributes to blood pressure regulation by activating the MR, thereby enhancing sodium reabsorption in the distal tubule of the kidney. In the vascular endothelium, aldosterone is a pivotal regulator of blood pressure by the RAAS. Since sAC has been reported to hamper the aldosterone effect in kidney collecting duct cells (Hallows et al., 2009), a transferable regulative effect of sAC on blood pressure in the kidney and the vascular endothelium was postulated. Thereby sAC could potentially act via the genomic or the non-genomic aldosterone pathway.

In this study, special emphasis was put on the role of sAC as a co-activator of the transcription factor CREB and the regulation of genes that are under transcriptional control of aldosterone. Therefore, the interaction of sAC with CREB was studied via co-immunoprezipitation (Co-IP). Determination of the sAC contribution on CRE motifs in different cell types were analyzed by chromatin immunoprecipitation (ChIP). By use of a CRE control luciferase reporter vector, a specific inhibitor of sAC (KH7), and a sAC expression vector, the modifying effect of sAC action on CRE elements were examined. The effect on downstream target genes of sAC signaling was analyzed by quantitative reverse transcription real time PCR.

Since several studies suggested multiple forms of sAC protein, appearance of these isoforms were analyzed in kidney and endothelial cells and the cellular localization of sAC isoforms were defined.

To elucidate the molecular basis of sAC promoter function and regulation, which was unknown prior to this study, the promoter of sAC was characterized in kidney cells (immortalized human kidney epithelial cell line [IHKE], and human embryonic kidney cell line [HEK293T]), and in human vascular endothelial cells (EA.hy926). Reporter gene assays of the 5´-flanking region, a part of the 5´-UTR (exon 1), and intron 4 were performed to detect *cis*-regulatory regions. Potential *cis*-regulatory elements were identified by *in silico* analysis of transcriptionally active regions. Cotransfection with *cis*-active factors was performed and the obtained results confirmed by ChIP assay. Furthermore, patients with cardiovascular disease were screened for variants in the 5´-flanking region, which were tested for their impact on transcriptional activity.

# 2 Material

## 2.1 Chemicals

| | |
|---|---|
| Acidic acid | Roth, Karlsruhe |
| Acrylamide-Bisacrylamide 30% (37, 5:1) (AA/BA) | Merck, Darmstadt |
| Acetylsalicylic acid | Sigma-Aldrich, Steinheim |
| Agar (Bacto$^{TM}$) | BD Bioscience, Heidelberg |
| Agarose | Biozym Scientific, Oldendorf |
| Aldosterone | Sigma-Aldrich, Steinheim |
| Ammonium persulfate (APS) | Sigma-Aldrich, Steinheim |
| Betaine | Sigma-Aldrich, Steinheim |
| Boridic acid | Roth, Karlsruhe |
| Bromphenol blue | Sigma-Aldrich, Steinheim |
| 8-Bromoadenosine-3´,5´-cyclic monophosphate (8-Br-cAMP) | Biolog, Bremen |
| Calcium chloride ($CaCl_2$) | Sigma-Aldrich, Steinheim |
| Caseine | Sigma-Aldrich, Steinheim |
| Chloroform | Fluka Reidel.de Haën, Seelze |
| Coomassie Brilliant Blue R-250 | Roth, Karlsruhe |
| Cobalt(II) chloride ($CoCl_2$) | Merck, Darmstadt |
| Deoxycholic acid | Sigma-Aldrich, Steinheim |
| 4',6-diamidino-2-phenylindole (DAPI) | Sigma-Aldrich, Steinheim |
| Dimethyl sulfoxide (DMSO) | Merck, Darmstadt |
| dNTPs (dATP, dCTP, dGTP, dTTP) | Fermentas, St. Leon-Rot |
| 1,4 Dithiothreitol (DTT) | Roth, Karlsruhe |
| Ethanol | Merck, Darmstadt |
| Ethidium bromide | Roth, Karlsruhe |
| Ethylenediamine-tetraacetic acid (EDTA) | Merck, Darmstadt |
| Ethyleneglycol-tetraacetic acid (EGTA) | Merck, Darmstadt |
| Ficoll | Fluka Reidel.de Haën, Seelze |
| Formaldehyde 37% | Roth, Karlsruhe |
| Formamide | AppliChem, Darmstadt |
| Gelatin | Sigma-Aldrich, Steinheim |
| Glacial acetic acid | Roth, Karlsruhe |
| L-Glutamine | Sigma-Aldrich, Steinheim |

| | |
|---|---|
| Glycerol | Roth, Karlsruhe |
| Glycine | Roth, Karlsruhe |
| HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) | Roth, Karlsruhe |
| 2-hydroxyestradiol (HE) | Steraloids, Newport, USA |
| Imidazole | Roth, Karlsruhe |
| Interleukin-1β (IL-1 β) | Calbiochem, Darmstadt |
| Isopropylalcohol | Merck, Darmstadt |
| KH7 | Sigma-Aldrich, Steinheim |
| Lithium chloride (LiCl) | Merck, Darmstadt |
| Magnesium chloride hexahydrate ($MgCl_2$) | Roth, Karlsruhe |
| Manganese(II) chloride ($MnCl_2$) | Sigma-Aldrich, Steinheim |
| β-Mercaptoethanol | Serva, Heidelberg |
| Methanol | Roth, Karlsruhe |
| 3-(N-Morpholino)propanesulfonic acid (MOPS) | Sigma-Aldrich, Steinheim |
| N',N',N'-Tetramethylendiamine (TEMED) | Roth, Karlsruhe |
| Nonidet P-40 | Sigma-Aldrich, Steinheim |
| Paraformaldehyde, powder (95%) (PFA) | Sigma-Aldrich, Steinheim |
| Phenylmethylsulphonyl fluoride (PMSF) | Roth, Karlsruhe |
| Phorbol-12-myristate-13-acetate (PMA) | Sigma-Aldrich, Steinheim |
| Potassium chloride (KCl) | Merck, Darmstadt |
| Protease inhibitor cocktail with EDTA (Complete) | Roche Diagnostics, Mannheim |
| Sodium acetate (NaAc) | Merck, Darmstadt |
| Sodium bicarbonate ($NaHCO_3$) | Sigma-Aldrich |
| Sodium chloride (NaCl) | Roth, Karlsruhe |
| Sodium deoxycholate | Simga-Aldrich |
| Sodium dodecyl sulfate (SDS) | Roth, Karlsruhe |
| Tris-(hydroxymethyl)-aminomethane (Tris-base) | Roth, Karlsruhe |
| Triton X-100 | Roth, Karlsruhe |
| Tryptone (Bacto$^{TM}$) | BD Bioscience, Heidelberg |
| Tween-20 | Roth, Karlsruhe |
| Xylene xyanole | Roth, Karlsruhe |
| Yeast extract (Bacto$^{TM}$) | BD Bioscience, Heidelberg |

## 2.2 Sera and media

| | |
|---|---|
| Dulbecco's modified eagle's medium (DMEM) | Sigma-Aldrich, Steinheim |
| Dulbecco's phosphate buffered saline (PBS) | PAA, Pasching |
| Fetal bovine serum (conditioned) (FBS) | PAA, Pasching |
| Fetal calf serum (FCS), iron-supplemented | Cell Concepts, Umkirch |
| Roswell Park Memorial Institute 1640 medium (RPMI) | Sigma-Aldrich, Steinheim |

## 2.3 DNA and protein marker

| | |
|---|---|
| GeneRuler 100 bp DNA ladder | Fermentas, St. Leon-Rot |
| GeneRuler 1 kb DNA ladder | Fermentas, St. Leon-Rot |
| Precision Plus Protein Dual Color Standard Plus | BioRad, Munich |
| Precision Plus Protein Western C | BioRad, Munich |

## 2.4 Enzymes and antibiotics

| | |
|---|---|
| Ampicillin | Roth, Karlsruhe |
| BigDye3.1 | Applied Biosystems, Foster City, USA |
| GoTaq DNA Polymerase | Promega, Mannheim |
| Penicillin/Streptomycin | PAA, Pasching |
| Proteinase K | Fermentas, St. Leon-Rot |
| Restriction endonucleases | Fermentas, St. Leon-Rot |
| RiboLock RNase inhibitor | Fermentas, St. Leon-Rot |
| Shrimp Alkaline Phosphatase | Fermentas, St. Leon-Rot |
| Spectinomycin | Sigma-Aldrich, Steinheim |
| TdT terminal transferase | Roche Diagnostics, Mannheim |
| Trypsine-EDTA (0.05%) | Gibco, Karlsruhe |

## 2.5 Consumables and kits

| | |
|---|---|
| BCA protein Assay Kit | Thermo Fischer, Bonn |
| ChIP-IT® Control qPCR Kit – Human | Active Motif, Tegernheim |

| | |
|---|---|
| CL-X Posure™ Film | Thermo Fischer, Bonn |
| Gateway LR Clonase II Enzyme Mix | Invitrogen, Karlsruhe |
| High Pure PCR Product Purification Kit | Roche Diagnostics, Mannheim |
| KAPA-HiFi PCR Kit | PEQLAB, Erlangen |
| Immobilon-P Transfer Membrane (PVDF) | Millipore, Bedford, USA |
| LightShift Chemiluminescent EMSA Detection Kit | Thermo Fischer, Bonn |
| LR Clonase II Enzyme Mix | Invitrogen, Karlsruhe |
| Lipofectamine™ 2000 | Invitrogen, Karlsruhe |
| Luciferase Assay System | Promega, Mannheim |
| Magnetic Protein-G beads | Invitrogen, Karlsruhe |
| M-MuLV Reverse Transcriptase | Fermentas, St. Leon-Rot |
| Nanofectin | PAA, Pasching |
| NucleoSpin Plasmid | Macherey-Nagel, Düren |
| NucleoSpin RNA II | Macherey-Nagel, Düren |
| Passive Lysis Buffer (5 x) | Promega, Mannheim |
| pCR8/GW/TOPO TA Cloning | Invitrogen, Karlsruhe |
| PureLink™ HiPure Plasmid DNA Purification Kit | Invitrogen, Karlsruhe |
| QIAamp DNA Blood Mini Kit | Qiagen, Hilden |
| QIAquick Gel Extraction Kit | Qiagen, Hilden |
| QuikChange site-directed mutagenesis | Agilent Technologies, Waldbronn |
| siRNA control duplex (low GC) | Invitrogen, Karlsruhe |
| SuperScript III Reverse Transcriptase | Invitrogen, Karlsruhe |
| SuperSignal West Chemiluminescent Substrate Pico/Femto | Thermo Fischer, Bonn |
| tRNA | Roche Diagnostics, Mannheim |
| Whatman Paper 3MM Chr. | Biometra, Göttingen |
| Pipette tips 0.1 µl - 1000 µl | Sarstedt, Nürnbrecht |
| Reaction tubes 0.2 ml - 2 ml | Eppendorf, Hamburg |
| | Biozym, Hess. Oldendorf |
| 15 ml/50 ml tubes | Greiner, Kremsmünster |
| | Nunc, Wiesbaden |
| Petri dishes | Sarstedt, Nürnbrecht |
| Plastics for cell culture | Greiner, Kremsmünster |
| PCR plates, microtiter plates | Abgene, Hamburg |

## 2.6 Antibodies

| Antibody | Host | Manufacturer |
|---|---|---|
| β-actin | rabbit | Cell Signaling, Frankfurt am Main |
| CREB | rabbit | Cell Signaling, Frankfurt am Main |
| CREB | mouse | Cell Signaling, Frankfurt am Main |
| CREB-p | rabbit | Nanotools, Teningen |
| sAC | rabbit | Deciphergen, Aurora, USA |
| anti-mouse | sheep | GE Healthcare UK Ltd, Little Chalfont Buckinghamshire, UK |
| anti-rabbit | donkey | GE Healthcare UK Ltd, Little Chalfont Buckinghamshire, UK |
| anti-tubulin | donkey | GE Healthcare UK Ltd, Little Chalfont Buckinghamshire, UK |

## 2.7 Plasmids and vectors

| Plasmid/vector | Description | Manufacturer/gift of |
|---|---|---|
| pCR8/GW/TOPO | cloning vector | Invitrogen, Karlsruhe |
| pGL3-Basic | reporter gene vector | Promega, Mannheim |
| pGL3-Control | reporter gene vector | Promega, Mannheim |
| pGL3-Promoter | reporter gene vector | Promega, Mannheim |
| pRC/CMV | expression vector | Dr. Dimitris Kardassis, Heraklion, Greece |
| CREB-pRC/CMV | expression vector | Dr. Vincent Coulon, Montpellier, France |
| SP1-pRC/CMV | expression vector | Dr. Birgit Gellersen, Hamburg |
| pSG5 | expression vector | Dr. Birgit Gellersen, Hamburg |
| C/EBP alpha-pSG5 | expression vector | Dr. Birgit Gellersen, Hamburg |
| C/EBP beta-pSG5 | expression vector | Dr. Birgit Gellersen, Hamburg |
| CRE control vector | reporter gene vector | Dr. Elwyn Isaac, Leeds, UK |
| Bacterial artificial chromosome (BAC) IRCMp5012D1214D Acc#.:BC117366.1 | full length cDNA clone | BACPAC Resource Center, Oakland, USA |

## 2.8 Reportergene constructs of sAC promoter (according to Acc#.:NM_018417.3)

| | |
|---|---|
| -3715/+250 | Reporter gene construct bearing 3715 bp of the 5´-flanking region and 250 bp of exon 1 |
| -3528/+250 | Reporter gene construct bearing 3528 bp of the 5´-flanking region and 250 bp of exon 1 |
| -3016/+250 | Reporter gene construct bearing 3016 bp of the 5´-flanking region and 250 bp of exon 1 |
| -2139/+250 | Reporter gene construct bearing 2139 bp of the 5´-flanking region and 250 bp of exon 1 |
| -1516/+250 | Reporter gene construct bearing 1516 bp of the 5´-flanking region and 250 bp of exon 1 |
| -1320/+250 | Reporter gene construct bearing 1320 bp of the 5´-flanking region and 250 bp of exon 1 |
| -1112/+250 | Reporter gene construct bearing 1112 bp of the 5´-flanking region and 250 bp of exon 1 |
| -991/+250 | Reporter gene construct bearing 991 bp of the 5´-flanking region and 250 bp of exon 1 |
| -490/+250 | Reporter gene construct bearing 490 bp of the 5´-flanking region and 250 bp of exon 1 |
| -2139/-77 w/o exon 1 | Reporter gene construct bearing 2062 bp of the 5´-flanking region |
| -1516/-77 w/o exon 1 | Reporter gene construct bearing 1439 bp of the 5´-flanking region |
| -1320/-77 w/o exon 1 | Reporter gene construct bearing 1243 bp of the 5´-flanking region |
| -1112/-77 w/o exon 1 | Reporter gene construct bearing 1035 bp of the 5´-flanking region |
| -991/-77 w/o exon 1 | Reporter gene construct bearing 914 bp of the 5´-flanking region |
| -490/-77 w/o exon 1 | Reporter gene construct bearing 413 bp of the 5´-flanking region |
| -77/-250 w/o (exon 1) | Reporter gene construct bearing 250 bp of the 5´-untranslated region and 77 bp of the 5´-flanking region |
| +16197/+16377 (intron 4) | Reporter gene construct bearing 180 bp of intron 4 |

## 2.9 Bacteria (*E. coli*)

| Strain | Genotype | Manufacturer |
|---|---|---|
| | derivatives of *E.coli* W strains | |
| Mach1 | ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR(rK- mK+) | Invitrogen, Karlsruhe |

## 2.10 Eucaryotic cells

| Line | Origin | Reference |
|---|---|---|
| COS7 | African green monkey | ATCC no.: CRL-11268 |
| EA.hy926 | Human vascular endothelium | Edgell et al., 1983 |
| HEK293T | Human embryonic kidney | ATCC no.: CRL-11268 |
| HepG2 | Human hepatocellular carcinoma | ATCC no.: HB-8065 |
| IHKE | Immortalized human kidney epithelium | Haugen et al., 1989 |
| THP1 | Human monocytes | ATCC no.: TIB-202 |

## 2.11 Laboratory equipment

| Instrument | Specification | Manufacturer |
|---|---|---|
| Autoclave | FVS-2 | Fedegari, Albuzzano, Italy |
| | Systec VX-75 | Systec, Wettenberg |
| Cell counter | Casy Model TT | Innovatis, Bielefeld |
| Centrifuge | Multifuge 3SR | Heraeus, Hanau |
| | 5415C | Eppendorf, Hamburg |
| | 5417R | Eppendorf, Hamburg |
| | 5810R | Eppendorf, Hamburg |
| | J2-21M/E Beckman | Coulter, Krefeld |
| $CO_2$-Incubator (eukaryotic cells) | MCO-18AIC | Sanyo, Munich |
| Developing machine | Optimax | Protec, Oberstenfeld |
| Gel electrophoresis chamber | Mini PROTEAN | BioRad, Munich |
| | StarPhoresis | Starlab, Ahrensburg |
| Gel imaging | AlphaImagerEC | Alpha Innotech Corp, San Leandro, USA |

| | | |
|---|---|---|
| Incubator shaker (bacteria) | Shaker Series 25 | New Brunswick Scientific, Nürtingen |
| Luminometer | Sirius V12 | Berthold Detection Systems, Pforzheim |
| Microbiological incubator | B 6120 | Heraeus, Hanau |
| Microscope | Axiovert 40 CFL | Zeiss, Jena |
| | Axioplan 2 | Zeiss, Jena |
| pH-Meter | Calimatic 766 | Knick, Dülmen |
| Power supply | PowerPackBasic | BioRad, Munich |
| Spectrophotometer | Nanophotometer | Implen, Munich |
| Sequence detection system | 7500 ABIprism | Applied Biosystems, Foster City, USA |
| Shaker | GFL 3006 | GFL, Großburgwedel |
| Sonicator | Bioruptor UCD-200 | Diagenode, Liège, Belgium |
| Sterile hood (bacteria) | Class II type EF | Clean air Techniek B.V., Woerden, The Netherlands |
| Sterile hood (eukaryotic cells) | HS 12 | Heraeus, Hanau |
| Tank blot chamber | Mini Trans-Blot Cell | BioRad, Munich |
| Thermocycler | PTC-225, DNA Engine Tetrad (2) | MJ Research, Miami, USA |
| Vortexer | Bio Vortex V1 | Kisker, Steinfurt |
| | VortexGenie2 | Bender&Hobein, Zurich, Switzerland |
| Waterbath | GFL 1083 | GFL, Großburgwedel |

# 3 Methods

## 3.1 Molecular biological methods

All standardized molecular methods were performed as described in “Molecular Cloning” (Sambrook and Russel, 2001) or in manufacturers’ instructions. Modifications in protocols are indicated where applicable.

### 3.1.1 Isolation of nucleic acids

#### 3.1.1.1 Isolation of genomic DNA

Genomic DNA of white blood cells was isolated using the QIAamp DNA Blood Kit (Qiagen) according to manufacturers’ protocol. 200 µl of human EDTA-treated whole blood were mixed with 20 µl proteinase K and 200 µl binding buffer, incubated at 56°C for 10 min, and loaded onto the spin columns, allowing the DNA to bind to the silica-gel membrane. After two washing steps, the DNA was eluted in $dH_2O$ (pH 7 - 8.5) or TE buffer and stored at -20°C.

#### 3.1.1.2 Isolation of total RNA

Total RNA was extracted from cultured cells using the NucleoSpin RNA II Kit (Macherey-Nagel) according to manufacturers’ protocol. Briefly, ~5 x $10^6$ cultured cells were washed twice with dulbecco´s phosphate buffered saline (PBS) and lysed with 350 µl lysis buffer (1% β-mercaptoethanol). By filtration through a filter column, clearance of the lysate was conducted. 350 µl of ethanol were added to achieve optimal binding conditions. Subsequently, the lysate was loaded onto the RNA binding column and the membrane was desalted. Digestion of DNA was performed by addition of DNase for 15 min. After three washing steps, the RNA was eluted in RNase-free water and held at 4°C or was stored at -80°C.

#### 3.1.1.3 Isolation of plasmid DNA

Isolation of plasmid DNA from *E. coli* cultures was performed using the NucleoSpin Plasmid Kit (Macherey-Nagel). 2 ml of an overnight culture of transformed *E. coli* were centrifugated and the pellet was lysed in resuspension buffer containing RNase A for 5 min at RT. Neutralization buffer was added and centrifugated to clear the lysate. The DNA was loaded and bound to a silica membrane. The plasmid DNA was eluted in TE buffer after two washing steps and held at 4°C or was stored at -20°C.
Preparation of transfection grade endotoxin-free plasmid DNA from *E. coli* cultures was performed with the PureLink HiPure Plasmid DNA Purification Kit (Invitrogen) as described in manufacturers' protocol. 100 ml of *E. coli* cells of an overnight culture were centrifugated and the pellet was resuspended in an RNase A containing buffer. Subsequently, the cells were lysated by addition of a lysis buffer for 5 min, the lysate was cleared using a precipitation buffer and centrifugated (12,000 x g, 10 min, RT). The supernatant was cleared from bacterial endotoxins by an additional incubation step with Endotoxin Removal Buffer A and one washing step with Endotoxin Removal Buffer B. The cleared lysate was loaded onto a pre-equilibrated column, washed and eluted. Afterwards, the DNA was precipitated by addition of isopropanol (70% v/v) and centrifugation (15000 x g, 30 min, 4°C). After washing with ethanol (70% v/v), the DNA was air-dried and resuspended in TE buffer. Plasmid DNA was held at 4°C and stored at -20°C.

**Endotoxin Removal Buffer A**
50 mM MOPS, pH 7.0
750 mM sodium chloride
10% (w/v) Triton X-100
10% (v/v) isopropyl alcohol

**Endotoxin Removal Buffer B**
100 mM sodium acetate, pH 5.0
750 mM sodium chloride
1% (w/v) Triton X-100

### 3.1.2 Photometric measurement of nucleic acid concentration

Measurement of concentration and purity of nucleic acids was performed photometrically with the nanophotometer (Implen). The particular elution buffer served as blank. An optical density (OD) of 1 at 260 nm represents a concentration of 50 µg/ml of dsDNA or 40 µg/ml of RNA. The $E_{260}/E_{280}$ ratio indicates the degree of purity of the nuclei acid, with a value of 1.9 in the case of pure DNA and a value under 2.0 in the case of pure RNA.

### 3.1.3 Polymerase chain reaction (PCR)

The PCR is conducted to amplify DNA fragments *in vitro* with two specific oligonucleotides (primers) using a thermo resistant DNA polymerase as first introduced by Mullis et al. (Mullis et al., 1986). Semiquantitative PCRs were performed with GoTaq DNA polymerase (Promega) or KAPAHiFi polymerase (PeqLab). Taq DNA polymerase was used for standard PCRs, which needs a relatively low elongation time. An enzyme with a proofreading function, such as the High Fidelity polymerase, was used to assure amplicon sequence identity to template DNA for cloning of transfection vectors.
All oligonucleotide sequences used for single amplifications are listed in the appendix (table A1).

**Standard PCR reaction**

5 ng genomic DNA
10 µM sense primer (SS)
10 µM antisense primer (AS)
200 µM of each dNTP
1 M betaine
1x Taq polymerase buffer
0.6 U Taq polymerase
add nuclease-free $H_2O$ to 25 µL

**Standard PCR program**

| | | |
|---|---|---|
| Initial denaturation | 95°C, 5 min | |
| Denaturation | 95°C, 1 min | 25-35 cycles |
| Annealing* | X°C, 45 sec | |
| Elongation | 72°C, 1 min/kb | |
| Terminal elongation | 72°C, 10 min | |

*Annealing temperature ($T_A$) depended on primer melting temperature ($T_M$) and was calculated as $([T_{M(SS)} + T_{M(AS)}]/2) - 2 = T_A$.
$T_M$ was calculated using the following algorithm (Nakano et al., 1999):
$T_M = (wA+xT)*2 + (yG+zC)*4 - 16.6*\log_{10}(0.050) + 16.6*\log_{10}([Na^+])$
(w,x,y,z are the number of the bases A,T,G,C in the sequence, respectively)

Four PCR modifications were applied when necessary:

#### 3.1.3.1 Real time PCR

To account for a small amount of template DNA, real time PCR was used. The samples were amplified in triplicate by real-time PCR (using the Stratagene MxPro detection system) in a final volume of 25 µl using Absolute QPCR SYBR Green ROX Mix (Thermo Scientific). Melting curve analysis was performed using Dissociation Curves software

(Stratagene) to ensure that only a single product was amplified. Specificity of the reactions was confirmed by 2.5% agarose gel electrophoresis. Results were obtained using Stratagene MxPro sequence detection software and evaluated using Excel (Microsoft). Specific primer sequences for amplification are exposed in table 3.

**Table 3: Oligonucleotide sequences for real time PCR**

| Description | Sequence 5'-3' | Reference |
|---|---|---|
| mineralocorticoid receptor SS | GATTTGGCGAGACCAGAGCA | De-An et al., 2010 |
| mineralocorticoid receptor AS | AAGCGAACGATACCAGAAACTACA | De-An et al., 2010 |
| $Na^+/K^+$-ATPase alpha SS | TGTCCAGAATTGCAGGTCTTTG | Murphy et al., 2004 |
| $Na^+/K^+$-ATPase alpha AS | TGCCCGCTTAAGAATAGGTAGGT | Murphy et al., 2004 |
| $Na^+/K^+$-ATPase beta SS | ACCAATCTTACCATGGACACTGAA | Murphy et al., 2004 |
| $Na^+/K^+$-ATPase beta AS | CGGTCTTTCTCACTGTACCCAAT | Murphy et al., 2004 |
| ENaC alpha SS | CCTCTGTCACGATGGTCACCCTCC | Bergann et al., 2009 |
| ENaC alpha AS | CAGCAGGTCAAAGACGAGCTCAG | Bergann et al., 2009 |
| soluble adenylyl cyclase SS | CTGAGCAGTTGGTGGAGATCCTC | Schmid et al., 2007 |
| soluble adenylyl cyclase AS | CAGCCAGTCCTATCTTGACTCGG | Schmid et al., 2007 |
| glyceraldehyde-3-phosphate dehydrogenase SS | CTGCACCACCAACTGCTTAGCAC | BY999181.1 |
| glyceraldehyde-3-phosphate dehydrogenase AS | CACCACCATGGAGAAGGCTGGGG | BY999181.1 |

#### 3.1.3.2 Touch down PCR

To ensure the enrichment of specific PCR products the annealing temperature was gradually decreased, starting at 5-10°C over the calculated primer annealing temperature. The annealing temperature was reduced by 2°C every second cycle until the calculated annealing temperature was reached, followed by 25 cycles at the final annealing temperature (Don et al., 1991).

#### 3.1.3.3 Nested PCR

To generate a higher amount and specificity of a weak PCR signal, nested PCR war used. Amplified PCR products from a first PCR reaction were used as templates for a second

PCR reaction by using a second set of primers located within the first amplicon. PCR products from the first PCR amplification were extracted from agarose gels (chapter 3.1.6) or directly used as templates.

#### 3.1.3.4 Thermal gradient PCR

To determine the optimal annealing temperature for a set of oligonucleotides, the single PCR reaction was performed over a range of defined annealing temperatures (Chang and Lee, 2005).

### 3.1.4 Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase PCR was performed to synthesize cDNA by using Superscript III (Invitrogen) or M-MuLV Reverse Transcriptase (Fermentas) according to manufacturers' instructions. Total RNA (0.5 - 1 µg) was mixed with 1 µl oligo ($dT_{18-20}$), 1-2 µl dNTPs (10 mM each), 20-40 U RNase Inhibitor (RiboLock, Fermentas; RNaseOUT, Invitrogen) and incubated with the particular reverse transcriptase. The RNA was reversely transcribed into cDNA at 50°C (Superscript III) or at 37°C (M-MuLV) for 60 min. The reaction was inactivated at 70°C for 15 min (Superscript III) or for 10 min (M-MuLV). To control if synthesis was successful a diagnostic PCR of human ribosomal protein 27 (hRP27) was routinely performed. To detect endogenous expression of sAC, cDNA was used as template for amplification with specific primers (appendix, table A2) in a semiquantitative PCR.

### 3.1.5 DNA modifications

#### 3.1.5.1 Restriction

Up to 1 µg DNA was restricted using 1 unit of the appropriate restriction endonuclease. Reaction buffer and $dH_2O$ were added to a total volume of 20 µl, incubated at 37°C (or different optimal temperature dependent on the enzyme) for 1 h. The restriction enzyme was heat-inactivated at 70°C for 10 min. Restriction efficiency of DNA was controlled by agarose gel electrophoresis (chapter 3.1.6).

#### 3.1.5.2 Dephosphorylation

To avoid relegation of linearized plasmid DNA with compatible ends, shrimp alkaline phosphatase (SAP) was used for dephosphorylation of the 5'-ends. Digestion reaction was mixed with 1 unit SAP and 10 x reaction buffer and $dH_2O$ was added to a total volume of 25 µl. Reaction mixture was incubated at 37°C for 30 min and heat-inactivated at 65°C for 10 min.

### 3.1.6 Agarose gel electrophoresis

To determine the size of DNA fragments, agarose gel electrophoresis was used. DNA migrates in an electric field because of the negatively charged phosphate backbone. Agarose concentrations of 0.8% to 3%, depending on fragment size, were applied in 1 x TAE buffer. Ethidium bromide was added to the gel solution at a concentration of 0.05 µg/ml to visualize DNA double-strands by use of the AlphaImager (Alpha Innotech Corporation) gel documentation system.

**50 x TAE buffer**
40 mM Tris base
1 mM EDTA
5.71% glacial acetic acid

**6 x loading buffer**
0.02% (w/v) bromphenole blue
0.02% (w/v) xylene xyanole
30% (v/v) glycerol
20 mM Tris-HCl, pH 7.6
2 mM EDTA

### 3.1.7 Purification of PCR products

#### 3.1.7.1 Column purification

To reach a high purification grade of DNA fragments for subsequent applications like sequencing or cloning, the High Pure PCR Product Purification Kit (Roche) was used. The PCR reactions were mixed with binding buffer, loaded onto the silica membrane column and washed twice. DNA was eluted in 10 mM Tris-HCl (pH 8.5).

#### 3.1.7.2 Gel extraction

Gel extraction was performed using the QIAquick Gel Extraction Kit (Qiagen). After agarose gel electrophoresis, the DNA fragments were resectioned from 0.8% agarose gels. Gel slices containing DNA were mixed with solubilization buffer QG (pH 7.5) and heated at 50°C for 10 min until agarose dissolves. The probes were mixed with one gel volume of isopropanol (100%) and loaded onto the silica membrane column. After two washing steps, DNA was eluted in EB buffer (10 mM Tris-HCl, pH 8.5).

#### 3.1.7.3 DNA precipitation

DNA precipitation was performed to concentrate the amount of DNA. The sample was mixed with 10% volume of 3 M NaAc (pH 5.2) and one volume isopropanol (100%), incubated at -80°C for 2 h and centrifuged twice (maximal speed, 20 min, 4°C). After two washing steps with ice-cold ethanol (70%), the pellet was air-dried and the DNA resuspended in an appropriate volume of nuclease-free $dH_2O$.

#### 3.1.7.4 ExoSAP clean-up

A rapid one-step PCR clean-up for subsequent sequencing reactions was performed with ExoSAP clean up. A mixture of exonuclease I (Exo I) (Fermentas) and SAP (Fermentas) was used to digest small single-stranded fragments (e.g. primers) and to remove dNTPs. 1 µl of ExoSAP mixture was added to a PCR product (5 µl) and was incubated at 37°C for 30 min. Heat inactivation of enzymes was performed at 80°C for 15 min.

**ExoSAP mixture**
20 U Exonuclease I (*E. coli*)
10 U SAP
add $dH_2O$ to 100 µl

### 3.1.8 Sequencing

For detection and localization of genetic variants in the MolProMD study and to ascertain sequence accuracy of DNA fragments and plasmid constructs, samples were sequenced (both strains) using an automated ABI 3730 fluorescence sequencer with BigDye terminator chemistry (PE Applied Biosystems).

### 3.1.9 Construction of reporter gene constructs

To generate reporter gene constructs, promoter fragments were amplified using extracted DNA from clone IRCMp5012D1214D, bearing *sAC* wild type (wt) sequence, as template. Deletion constructs of the *sAC* 5'-flanking region were amplified using one antisense primer at position +250 bp and sense primers (table 4) generating serial deletion constructs. Promoter constructs lacking the untranslated exon 1 were generated using one antisense primer at postion -77 and sense primers (table 4). The construct of the intron 4 promoter was designed from position +16197 to +16377 (table 4). Constructs harbouring MolHap1 and MolHap2 were generated from position -2436 to -1993 (table 4).
For transient transfection assays, amplified PCR fragments were introduced in the entry vector pCR8/GW/TOPO (Invitrogen, figure 7). The basis of this cloning technique is the site-specific recombination property of bacteriophage λ (Landy, 1989). Recombination occurs at attachment sequences of phage DNA (*att*P) and bacteria DNA (*att*B). The introduced PCR fragment is flanked by *att*L sequences. The vector was subsequently transformed into competent Mach1 (Invitrogen) bacterial cells (chapter 3.3.1.3) and the plasmid isolated and purified (chapter 3.1.1.3).
Constructs with the right 5' to 3'-orientation were cloned into the promoter-less luciferase reporter gene vector pGL3-Basic (Promega, figure 8) or into the pGL3-Promoter vector (Promega, figure 8), harbouring the simian vacuolating virus 40 (SV40) promoter for preinitiation complex (PIC). The modified pGL3-Basic destination vector, bearing artificial *att*R sites, was mixed with the entry vector and incubated with the LR clonase enzyme allowing the exchange of the gateway cassette in combination with the *sAC* promoter fragment. For verification of accurate insert size and orientation (5'-3'), plasmids were double digested with sequence-specific endonucleases (chapter 3.1.5.1). Sequencing (chapter 3.1.8) of generated plasmids for transfection assays was performed to control sequence correctness and identity.

**Standard pCR8/GW/TOPO cloning reaction**

1 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$)
1 µl pCR8/GW/TOPO cloning vector (10 ng/µl)
4 µl purified insert
incubation for 5 min at RT

**LR clonase reaction**

100 ng entry vector
150 ng destination vector
2 µl LR Clonase
add TE buffer to 8 µl
incubation for 1 h at 25°C
add 1 µl Proteinase K
incubation for 10 min at 37°C

**Table 4: Oligonucleotide sequence for *sAC* promoter deletion constructs**

| Description | Sequence 5' to 3' | Position | Ref. Acc# |
|---|---|---|---|
| sAC -490 SS | TGTTAGAAAACACACCAGCCTCT | -490 | NM_018417.3 |
| sAC -991 SS | TCAGTGCTGCTGTTTCCTCA | -991 | NM_018417.3 |
| sAC -1112 SS | AAGAAGCTGCTTGGGGTAGA | -1112 | NM_018417.3 |
| sAC -1320 SS | GAGGTTGCAGTGAGCTGAG | -1320 | NM_018417.3 |
| sAC -1516 SS | TGCCTGAAATCCCAGCACTT | -1516 | NM_018417.3 |
| sAC -3016 SS | GGCTCCGTTGTGAGGAGAGA | -3016 | NM_018417.3 |
| sAC -3528 SS | TGCCTGGTCCATGATAAGTGTT | -3528 | NM_018417.3 |
| sAC -3715 SS | TGCTGAAGAGACTGAGAAATGGGTAGT | -3715 | NM_018417.3 |
| sAC -490 SS | TGGCTTTTCCTCAGCCCTGGA | +250 | NM_018417.3 |
| sAC -490 SS | CCCTGACCCTTGCCTCAAATGTG | -77 | NM_018417.3 |
| sAC intron 4 SS | GCAGGTATGGGGGCTTACTAAGATA | +16197 | NM_018417.3 |
| sAC intron 4 AS | AGATTGATCCCCAGGGCA | +16377 | NM_018417.3 |
| sAC MolHap SS | TTGTAGCATTTAGATACAATCATAGGC | -2436 | NM_018417.3 |
| sAC MolHap AS | CCTTTGCACTCCAGCCTG | -1993 | NM_018417.3 |

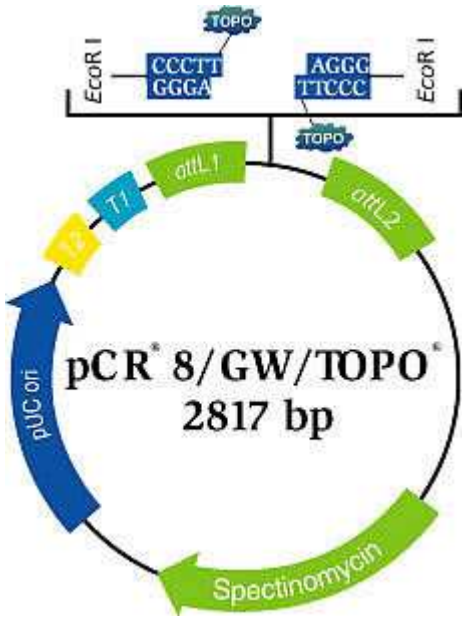
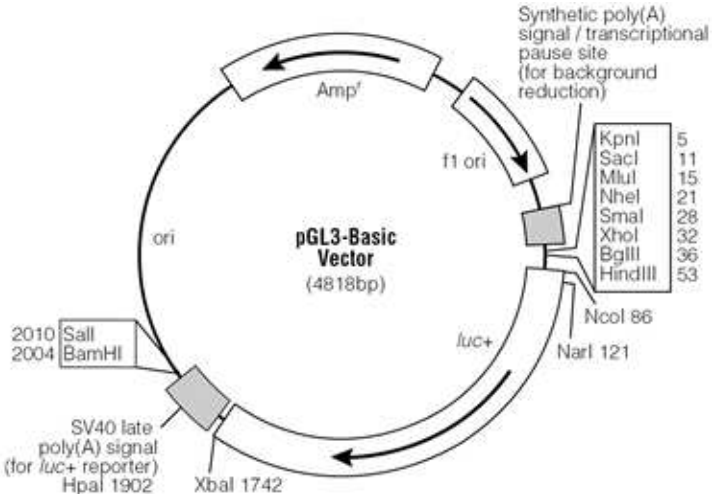



**Figure 7: pCR8/GW/TOPO vector circle map**

The pCR8/GW/TOPO vector functioned as entry vector of the amplified construct sequences and contains attL sequences for recombination.



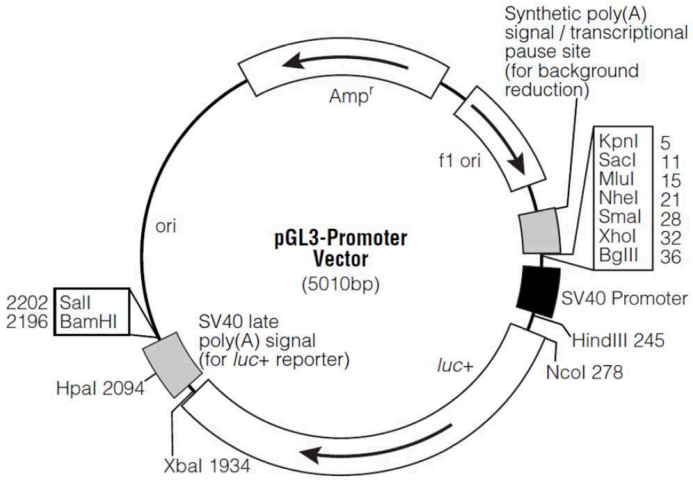



**Figure 8: The pGL3-vector circle maps**

The pGL3-Basic vector lacks eukaryotic promoter and enhancer sequences, the pGL3-Control vector possesses a complete promoter with SV40 promoter and enhancer sequences, and the pGL3-Promoter vector contains a minimal SV40 promoter without an enhancer upstream of the luciferase gene. Putative promoter or enhancer sequences were introduced in 5' to 3' orientation into the pGL3-Basic or pGL3-Promoter vector, respectively. luc+: cDNA encoding the modified firefly luciferase. Ampr: gene conferring ampicillin-resistance in *E. coli*. f1 ori: origin of replication derived from filamentous phage. ori: origin of plasmid replication in *E. coli*. Arrows within luc+ and the Ampr gene indicate the direction of transcription. The arrow in f1 ori indicates the direction of ssDNA strand synthesis. SV40: simian vacuolating virus 40.

### 3.1.10 Site directed mutagenesis

The detected genetic variants in the 5´-flanking region of sAC at positions -2356, -2181 and -2092 were introduced by QuikChange site-directed mutagenesis (Stratagene) following the manufacturers' instructions. The wt promoter construct (-3016/+250) served as PCR template. Oligonucleotide primers were designed to generate a mutant plasmid containing the nucleotide exchange. PCR conditions included a 6-7 min elongation step each cycle instead of the final elongation step. The amplified product was treated with methylation-sensitive DpnI endonuclease, to digest the parental DNA template strand and to select for mutation-containing synthesized DNA. After transformation and plasmid extraction, correctness of the exchanged nucleotides was checked by automated sequencing.

**Standard mixture**

10x reaction buffer

125 ng of primer 1 (sense strand)

125 ng of primer 2 (antisense strand)

10 ng of dsDNA template plasmid

1 µl of dNTP mix

2.5 u of *PfuTurbo* DNA polymerase (2.5 u/µl)

$dH_2O$ to 50 µl

10 units of *Dpn*I (10 u/µl)

### 3.1.11 ChIP assay

To investigate interaction of a specific DNA sequence with *trans*-acting factors, ChIP assay was performed by using a modified protocol (Boyd et al. 1998; Liu et al., 2000). The basic steps of this technique are crosslinking of proteins with the DNA and precipitation of bounded chromatin using selected specific antibodies. To identify DNA fragments associated with the protein of interest amplification with specific primers was performed. About $10^7$ cells were fixed by adding formaldehyde to a final concentration of 1% (v/v) and incubated for 15 min at RT. Cells were washed twice with ice-cold PBS (Sigma) and lysed for 10 min at RT. Nuclei were isolated followed by DNA sonification using a Bioruptor (Diagenode) until the chromatin had an average size of 300 to 500 bp (≤45 min, 0.5 s interval, 200 W, 4°C). Size of chromatin fragments was routinely controlled using agarose gel electrophoresis. After centrifugation, the supernatant was incubated with rabbit pre-immune serum for 30 min at 4°C and subsequently incubated with freshly prepared magnetic protein-G beads (blocked with BSA and tRNA 1 h, 4°C) for 30 min at 4°C. The samples were centrifugated, the supernatant was transferred to low-binding tubes and 4 µg of selected antibody anti-sAC (Deciphergen), anti-CREB (Cell Signaling), anti-CREB-p (Nanotools), and IgG (Active Motif) were added and incubated over night at 4°C. The next day, samples were incubated with freshly prepared magnetic protein-G beads for 3 h at 4°C. After washing with wash buffer I, II and III, the antibody/protein/DNA complex was eluted from the beads. Crosslinks were reversed at 67°C over night and proteins were digested using proteinase K (2 h, 37°C). The DNA was extracted by phenol/chloroform/isoamyl alcohol extraction, resuspended in nuclease-free $dH_2O$ and used for PCR analysis. Amplification of DNA was performed with primers for a CRE control sequence in the C-FOS gene locus (ChIPAb+ CREB, Millipore, Lundblad et al.,

1998; Dalley et al., 1999; Impey et al., 2004) and in *sAC* exon 1 (oligonucleotide sequences are listed in table 5).

**Cellular lysis buffer**
10 mM Tris pH 8.0
10 mM NaCl
0.2% (v/v) NP-40
Roche Complete
proteinase inhibitor

**Nuclear lysis buffer**
50 mM Tris pH 8.0
10 mM EDTA
1% (w/v) SDS
Roche Complete
proteinase inhibitor

**Dilution buffer**
20 mM Tris pH 8.0
2 mM EDTA
150 mM NaCl
1% (w/v) Triton X-100
Roche Complete
proteinase inhibitor

**Wash buffer I**
20 mM Tris pH 8.0
2 mM EDTA
50 mM NaCl
1% (w/v) Triton X-100
0.1% (w/v) SDS
Roche Complete
proteinase inhibitor

**Wash buffer II**
10 mM Tris pH 8.0
1 mM EDTA
0.25 mM LiCl
1% (v/v) NP-40
1% (w/v) Deoxycholic acid

**Wash buffer III**
20 mM Tris pH 7.6
50 mM NaCl

**Elution buffer**
10 mM $NaHCO_3$
1% (w/v) SDS

**Table 5: Oligonucleotide sequences for ChIP**

| Description | Sequence 5'-3' | Ref. Acc# |
|---|---|---|
| CRE control ChIP (C-FOS) SS | GGCCCACGAGACCTCTGAGACA | NP_004370 |
| CRE control ChIP (C-FOS) AS | GCCTTGGCGCGT GTCCTAATCT | NP_004370 |
| sAC exon 1 ChIP SS | GGCCTCCTCTCCTGTCTT | NM_018417.3 |
| sAC exon 1 ChIP AS | AGGTCTGGCTTTTCCTCAGC | NM_018417.3 |

## 3.2 Protein biochemical methods

### 3.2.1 Extraction of proteins

#### 3.2.1.1 Extraction of cellular protein extract

Cells were washed in ice cold PBS, centrifuged and lysed in lysis buffer. To remove cellular debris, samples were centrifuged again (12000 x g, 5 min, 4°C). Pre-heated 4 x SDS-PAGE sample buffer was added to the supernatants and they were heated to 95°C for 5 min. Protein samples were aliquoted and stored at -70°C.

| **Lysis buffer** | **4 x SDS sample buffer** |
|---|---|
| 150 mM sodium chloride | 200 mM Tris-HCl, pH 6.8 |
| 1% Triton X-100 | 8% (w/v) SDS |
| 0.5% sodium deoxycholate | 0.4% (w/v) bromphenol blue |
| 0.1% SDS | 40% (v/v) glycerol |
| 50 mM Tris, pH 8.0 | |

#### 3.2.1.2 Extraction of nuclear protein extract

Nuclear proteins were extracted by a modified protocol of Schreiber et al. (Schreiber at al., 1989). A total of $10^7$ cells were washed twice with ice-cold PBS, scraped and centrifuged (5000 x g, 2 min, 4°C). Pellets were resuspended in a low salt buffer (500-800 µl) and allowed to swell for 15 min on ice. After addition of 25-75 µl NP-40 (10% solution) and incubation for 5 min at RT, lysed cells were centrifuged (5000 x g, 5 min, 4°C). The supernatant containing the cytosolic protein was removed and stored at -80°C, while the pellets were resuspended in 50-150 µl of a high salt buffer. After incubation of 3 h, cellular debris were centrifuged twice (24,000 x g, 1 h, 4°C) and the nuclear protein extracts were aliquoted on ice, snap frozen in liquid nitrogen and stored at -80°C.

**Low salt buffer**
10 mM HEPES, pH 7.9
10 mM KCl
1 mM DTT
1.5 mM $MgCl_2$
Roche Complete proteinase inhibitor

**High salt buffer**
20 mM HEPES, pH 7.9
0.2 mM EDTA pH 8.0
1 mM DTT
420 mM NaCl
1.5 mM $MgCl_2$
0.5 mM PMSF
25% (v/v) glycerol
Roche Complete proteinase inhibitor

### 3.2.2 Protein quantification

Quantification of the protein content was determined using the BCA Protein Assays Kit (Thermo Fischer). The measurement of a series of dilutions with known concentrations of BSA served as standard curve. Protein concentrations were measured photometrically and calculated with reference to the standard curve.

### 3.2.3 SDS polyacrylamide gel electrophoresis (PAGE)

A 10% SDS gel was used for separation of protein samples as described by Rittenhouse and Marcus (Rittenhouse and Marcus, 1984). The anionic detergent SDS denatures secondary and non-disulfide-linked tertiary structures and leads to a negative charge of a protein in relation to its mass, thus the migration distance of the protein in the gel is assumed to be directly proportional to the protein size. Protein samples were incubated in SDS sample buffer at 95°C for 10 min. After incubation on ice for 5 min, samples ran on a stacking gel (4% polyacrylamide) at 80 V and were separated in the following 10% stacking gel at 100 V. Running of the gel was controlled using a prestained marker.

**Stacking gel (4%)**
560 µl AA/BA, 30%
675 µl 0.5 M Tris-HCl, pH 6.8
675 µl 0.5 M imidazole, pH 6.8
75 µl SDS, 10%
40 µl APS, 10%
5 µl TEMED
add $dH_2O$ to 4.2 ml

**Running gel (10%)**
2.5 ml AA/BA, 30%
1.9 ml 1.5 M Tris-HCl, pH 8.8
75 µl SDS, 10%
25 µl APS, 10%
5 µl TEMED
add $dH_2O$ to 7.5 ml

**1 x SDS running buffer**

25 mM Tris base

102 mM glycine

1% (w/v) SDS

### 3.2.4 Coomassie blue staining

Visualization of protein bands was performed by incubation of the gel for 1 h in coomassie blue staining solution for 30 min, followed by two washing steps with destaining solution (each 30 min).

**Coomassie staining solution**

0.25% (w/v) Coomassie Brilliant Blue R-250

45% (v/v) methanol

10% (v/v) acetic acid

add $dH_2O$

**Destaining solution**

45% methanol

10% acetic acid

add $dH_2O$

### 3.2.5 Western blot (wet blot)

For immunologic detection of proteins, the protocol of Towbin et al. was used (Towbin et al., 1979). The protein extracts were transferred from the SDS gel (chapter 3.2.3) to a polyvinylidene difluoride membrane, which was activated for 5 min in methanol and equilibrated in blotting buffer. Briefly, the membrane was placed onto the gel and covered with two sheets of whatman-paper on each site. The blots were run for 1 h at 100 V using cooling units. After blotting, membranes were saturated in blocking buffer over night at 4°C.

Immunodetection of proteins of interest was performed by incubation of the membrane with specific antibody for 1 h at RT with following concentration: anti-sAC (Deciphergen; 1:1000; rabbit), anti-CREB (Cell Signaling, 1:1000 rabbit; Cell Signaling, 1:1000 mouse), anti-CREB-p (Nanotools, 1:1000 rabbit) and β-tubulin (Thermo Scientific, 1:1000). Horseradish-peroxidase-coupled secondary antibodies (GE Healthcare, UK Ltd) were given for 45 min (RT) at following dilutions: anti-mouse 1:5000 and anti-rabbit of 1:5000. After extensive washing, membranes were incubated for 5 min in SuperSignal West Chemiluminescent Substrate (Pico or Femto, Thermo Scientific) and exposed to CL-X Posure Film (Thermo Fischer). β-tubulin served as gel loading control.

| 1 x Blotting buffer | Blocking solution | Washing solution (1 x TBS-T) |
|---|---|---|
| 25 mM Tris base | 4% (w/v) casein | 100 mM Tris base |
| 192 mM glycine | in 1 x TBS-T | 1.5 mM NaCl |
| 10% methanol | | 0.03% (v/v) Tween-20 |

### 3.2.6 Co-immunoprecipitation

To identify potential protein/protein interactions co-immunoprecipitation (Co-IP) was performed. 100 µl of freshly prepared whole cell lysate (chapter 3.2.1.1) was incubated for 2-3 h with an appropriate amount of the first antibody at RT. 5-10 µl protein G sepharose beads were added and incubated for 1 h at RT. The samples were centrifugated (2000 g, 5 min, RT) and the pellet was washed for at least three times with washing buffer. Next, the samples were heated with SDS sample buffer at 95°C for 10 min, followed by SDS-PAGE (chapter 3.2.3) and western blot analysis with the second antibody (chapter 3.2.5).

| Washing buffer | Elution buffer |
|---|---|
| 20 mM Tris | Wash buffer + 4% SDS |
| 0.2%Triton | |
| water up to 50 ml | |

## 3.3 Cell biological and microbiological methods

### 3.3.1 Prokaryotic cells

#### 3.3.1.1 Cell culture and storage

Bacteria were used for the generation and amplification of plasmid DNA. Cultivation was performed at 37°C either in liquid lysogeny broth (LB) medium or on LB agar plates. Antibiotics were applied for specific selection of transformed bacteria. For long-term storage, overnight cultures were centrifuged, the pellets were resuspended in LB Medium with 15% (v/v) glycerol and frozen in liquid nitrogen and stored at -80°C.

**LB Medium**
10 g Bactotryptone
10 g NaCl
5 g Yeast extract
add $dH_2O$ to 1000 ml, pH 7.0
Autoclave at 121°C for 120 min

**LB Agar**
15 g Bacto Agar in 1000 ml LB Medium
add appropriate antibiotics
after cool down to 56°C

#### 3.3.1.2 Generation of chemically competent cells

Transformable competent *E. coli* bacterial cells were generated according to a modified protocol by Hanahan (Hanahan, 1983). 200 ml of LB-Medium was inoculated with *E. coli* cells, which grown at 37°C to an $OD_{600}$ of 0.5. Cells were incubated for 20 min in an ice bath and harvested by centrifugation (4000 x g, 15 min, 4°C). The pellet was resuspended in 10 ml of a $MnCl_2$-transform buffer and incubated on ice for 10 min. After centrifugation (3000 x g, 10 min, 4°C), the cells was resuspended in 7.4 ml of $MnCl_2$-transform buffer and mixed gently, followed by dropwise addition of 560 µl DMSO. Aliquots of 100 µl were snap frozen in liquid nitrogen and stored at -80°C. The transformation efficiency of generated competent cells was routinely controlled by transformation of the pUC19 vector.

**$MnCl_2$-transform buffer**
10 mM HEPES, pH 6.8
15 mM $CaCl_2$
20 mM KCl
55 mM $MnCl_2$

#### 3.3.1.3 Transformation of DNA

An aliquot of competent *E.coli* cells of 100 µl was thawed on ice and incubated with 50 ng of DNA for 25 min on ice, heat-shocked for 45 sec at 42°C and briefly cooled down on ice for 1 min. 250 µl of pre-warmed LB-Medium was added and the cells were incubated at 37°C for 45 min. 100-150 µl of the cells were plated onto antibiotic agar plates and incubated at 37°C over night.

### 3.3.2 Eukaryotic cells

#### 3.3.2.1 Cell culture

The human vascular endothelial cell line EA.hy926 (kind gift of Edgell et al., 1983) and the human embryonic kidney cell line HEK293T were maintained in DMEM (Sigma-Aldrich) with 10% conditioned fetal calf serum (PAA, Cölbe, Germany), penicillin (100 units/ml), streptomycin (100 ng/ml), and L-Glutamine (2 mmol/ml, all Sigma-Aldrich). For cultivation of HEK293T iron-supplemented FCS was used (Cell Concepts). Immortalized human kidney epithelial (IHKE) cells were maintained in Dulbecco's modified Eagle's medium/Ham's-F12 enriched with 1% bovine calf serum (PAA), 100 units/ml penicillin, 100 ng/ml streptomycin, 2 mmol/ml L-glutamine, 10 ml/l Insulin-transferrin-sodium selenite media supplement, 1.25 g/l $NaHCO_3$, 55 mg/l sodium pyruvate, 10 µg/l human epidermal growth factor (all Sigma-Aldrich, Munich, Germany) and 15 mmol/l N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; Merck). The human hepatocellular carcinoma cell line HEPG2 and the monocytic cell line THP1 were maintained in RPMI 1640 medium containing 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM/ml L-Glutamine. For cultivation of THP1 monocytes 1 x modified Eagle's medium amino acid solution (Sigma) was added. THP1 cells were kept at a concentration of 0.5 to 1 x $10^6$/ml. When state of confluence was reached, cells were detached from surface by trypsination and splitted at appropriate ratios for further cultivation. Cells were cultivated at least for two passages before used for experiments. The number of passages did not exceed 40 in any case.
For stimulation experiments, cells were incubated with 500 µM 8-Br-cAMP (Biolog), 10 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich), 1 µM, 10 µM, 30 µM and 60 µM KH7 (Sigma Aldrich), 120 µM 2-hydroxyestradiol (HE, steraloids) and 1 nM aldosterone (Sigma Aldrich) for 24 h.

#### 3.3.2.2 Storage

For long term storage cells were washed twice with PBS, trypsinated, and transferred to fresh medium. After centrifugation, cells were placed on ice and resuspended in 90% (v/v) fetal calf serum mixed with 10% DMSO. Cells were stored at -80°C and transferred to liquid nitrogen the next day. Thawing of cells occurred as fast as possible, using a waterbath at 37°C. Cells were washed with PBS to remove DMSO from the freezing medium. After centrifugation cells were transferred into pre-warmed medium.

#### 3.3.2.3 Transient transfection

EA.hy926 and IHKE cells were transfected using Nanofectin (PAA) according to manufacturers' protocol. Nanofectin consists of a positively charged polymer with DNA-binding capacity, which is embedded into a porous nanoparticle. 500 ml medium with $10^5$ cells/well was transferred into 24-well plates an transfected the next day. Two hours prior transfection medium was changed.

For both, EA.hy926 and IHKE cells, 1 µg DNA and 3.2 µl Nanofectin solution was incubated in 50 µl NaCl solution for 10 min at RT. The diluted Nanofectin particles were added drop wise to the diluted DNA and gently vortexed. After incubation for 30 min at RT, the transfection complexes were added drop wise to the cell medium. Transfection reagent was removed by change of medium after 3 h. Stimulation of the cells was performed during change of medium. Cells were harvested 24 h post transfection with 100 µl passive lysis buffer (Promega) and luciferase activity was determined using a sirius singletube luminometer (Berthold detection systems). 20 µl of cell lysate were routinely diluted with 75 µl luciferase substrate. The pGL3-Control vector, which harbors a competent SV40 viral promoter and an additional enhancer upstream of the *luciferase* gene, served as positive control. The promotor-less pGL3-Basic vector served as empty shuttle vector control. Transfection of the CRE control vector (figure 9) was used as an index of the cAMP-dependent signaling pathway. Transfection experiments were repeated at least three times, in triplicates for each plasmid.

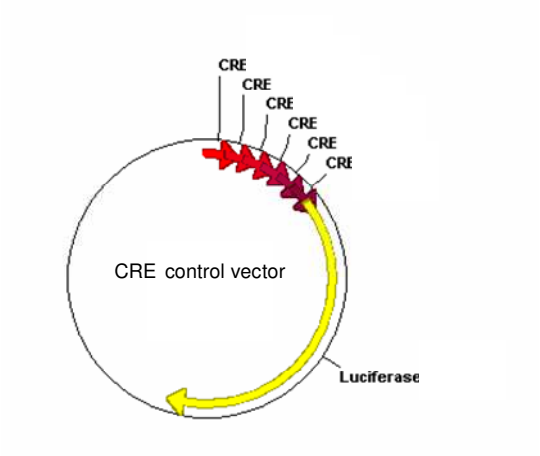



**Figure 9: Schematic representation of the CRE control vector**

pADneo2 reporter plasmid containing the firefly luciferase gene under the transcriptional control of multiple units of CRE (pADneo2-C6-BGL) was used to measure CRE-mediated transcriptional activity (Isaac et al., 2007).

### 3.3.2.4 Cotransfection

For cotransfection experiments overexpression of the proteins CREB and SP1, which were cloned into the pRC/CMV expression vector and C/EBP alpha and C/EBP beta, which were cloned into the pSG5 expression vector (figure 10) were performed to analyze the possible effect on transcription of the cotransfected reporter gene construct. The expression vector and reporter gene plasmids were transfected in a 3:1 ratio.

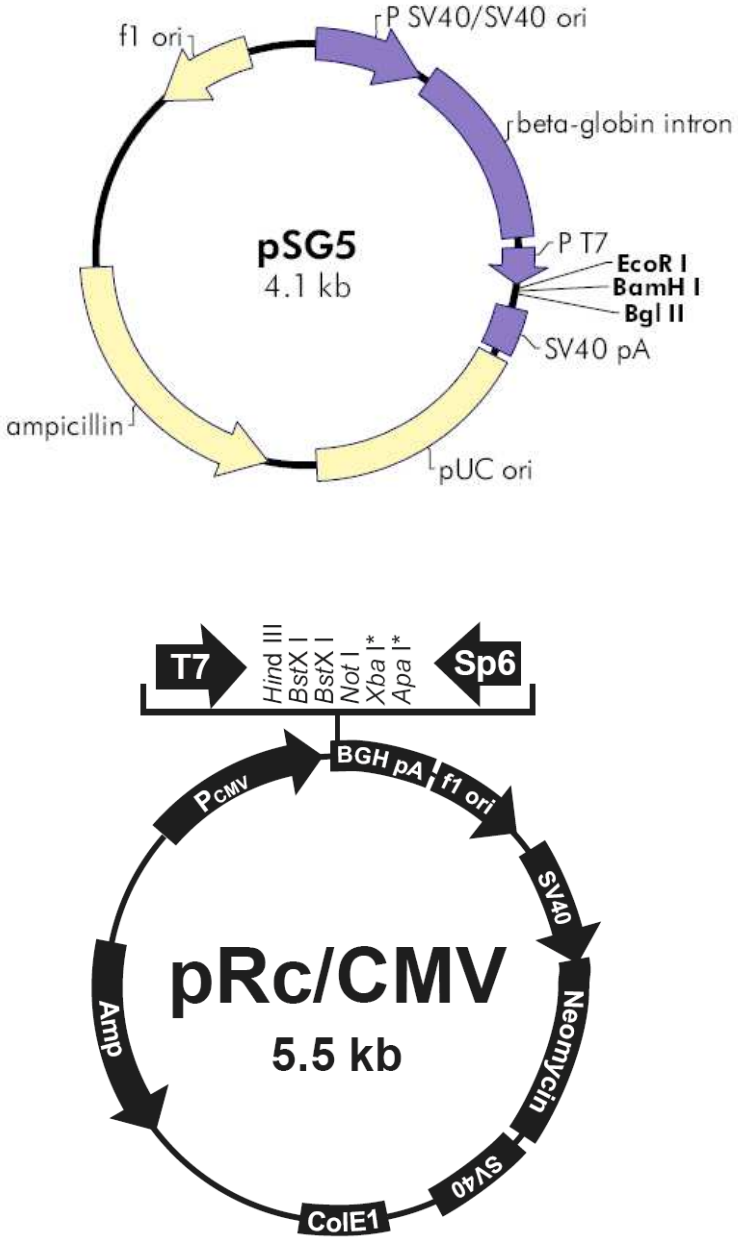



**Figure 10: Expression vector circle maps**

Map of the expression vector pSG5 and pRc/CMV. Amp: gene conferring ampicillin-resistance in *E. coli*. Neomycin: gene conferring neomycin-resistance in *E. coli*. ori: origin of plasmid replication in Arrows within genes indicate the direction of transcription. SV40: simian vacuolating virus 40. pA: polyA signal.

### 3.4 *In silico* analyzes of putative transcription factor binding sites

Prediction of TFBS was performed by *in silico* analysis using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and AliBaba2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html, Grabe, 2002; Messeguer et al., 2002). The standard settings were used for the data algorithms. Both programs use information on binding sites of the eukaryotic TRANSFAC database.

### 3.5 Statistical methods

P-values were calculated using the scientific analysis and presented with the computer program “Graph Pad Prism 4.0/5.0”. Significance was calculated by unpaired, two-tailed t-test (C.I.:95%). The significance levels were set at ***$p<0.001$, **$p<0.01$, and *$p<0.05$.

### 3.6 Study population

The current investigation was based on the Münster Molecular Functional Profiling for Mechanism Detection (MolProMD) Study. The Münster MolProMD Study is a prospective study of patients with CVD (e.g. with myocardial infarction, essential hypertension), aimed at studying the molecular mechanism of CVD. The study was approved by the ethics committee of the Medical Faculty, Westphalia Wilhelms-University of Münster and written informed consent was obtained from all study subjects. Genomic DNA from patients of this study was used for the detection of genetic variants by sequencing as well as for subcloning and generation of gene promoter reporter vectors (Dördelmann et al., 2008).

# 4 Results

## 4.1 Gene regulatory function of sAC

### 4.1.1 sAC acts as a co-factor of CREB

#### 4.1.1.1 sAC interacts with CREB

Initial studies have shown the importance of sAC for CREB phosphorylation and activation. Co-localisation of sAC with CREB has been shown by immunofluorescence. Further, $HCO_3^-$ induces CREB phosphorylation via sAC activation (Zippin et al., 2004).
To determine if sAC and CREB interact physically, Co-IP was performed in EA.hy926 and IHKE cells (figure 11). Co-IP is implemented to identify protein/protein interactions by using target protein-specific antibodies. Interaction partners of the target protein can subsequently be identified by western blot analysis. Precipitation of whole cell lysates of EA.hy926 and IHKE cells was performed with a specific CREB antibody. The captured complex was separated with SDS-gelelectrophoresis and detected with a sAC antibody, to identify potential CREB/sAC interactions. To ensure antibody specificity a sAC-peptide was used as negative control.

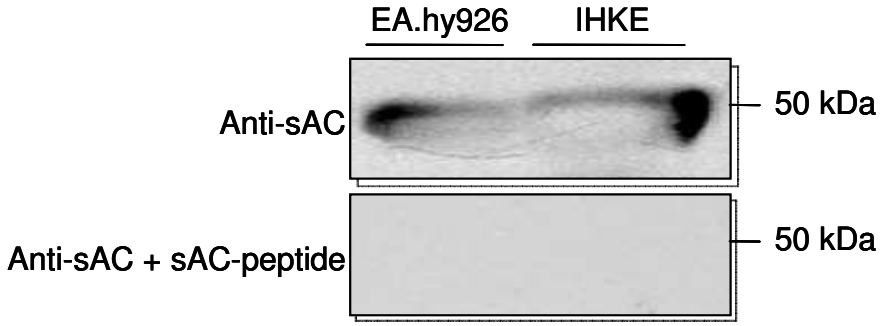



**Figure 11: Co-IP of CREB and sAC**
Co-IP in whole cell lysates of EA.hy926 and IHKE cells was performed with a CREB antibody. Detection with a sAC antibody in a western blot showed a specific band for sAC at ~50 kDa. The lysates were precipitated with a CREB antibody and the captured complex was precipitated with sAC in the absence (upper panel) or presence (lower panel) of 10 mg/ml antigen peptide. Co-IP: co-immunoprecipitation, sAC: soluble adenylyl cyclase, CREB: cAMP response element binding protein, kDa: kilodalton.

Detection of the CREB-immunoprecipitated complex with a sAC antibody showed a specific signal at ~50 kDa in both cell lines (figure 11). The applied sAC peptide prevented the sAC antibody from binding to the precipitated complex pointing to the specificity of the sAC antibody.

#### 4.1.1.2 sAC is bound together with CREB on chromatin

To evaluate the role of sAC in gene expression regulation via the cAMP-regulated transcription factor CREB, the localization of sAC on chromatin was analyzed (figure 12). ChIP assays were performed to investigate protein/DNA interactions *in vivo*. The basic steps of this technique are (1) fixation of protein/DNA complexes, (2) sonification of the DNA, (3) immunoprecipitation with specific antibodies recognizing sAC, CREB, and CREB-p, and (4) detection of the precipitated DNA via PCR.

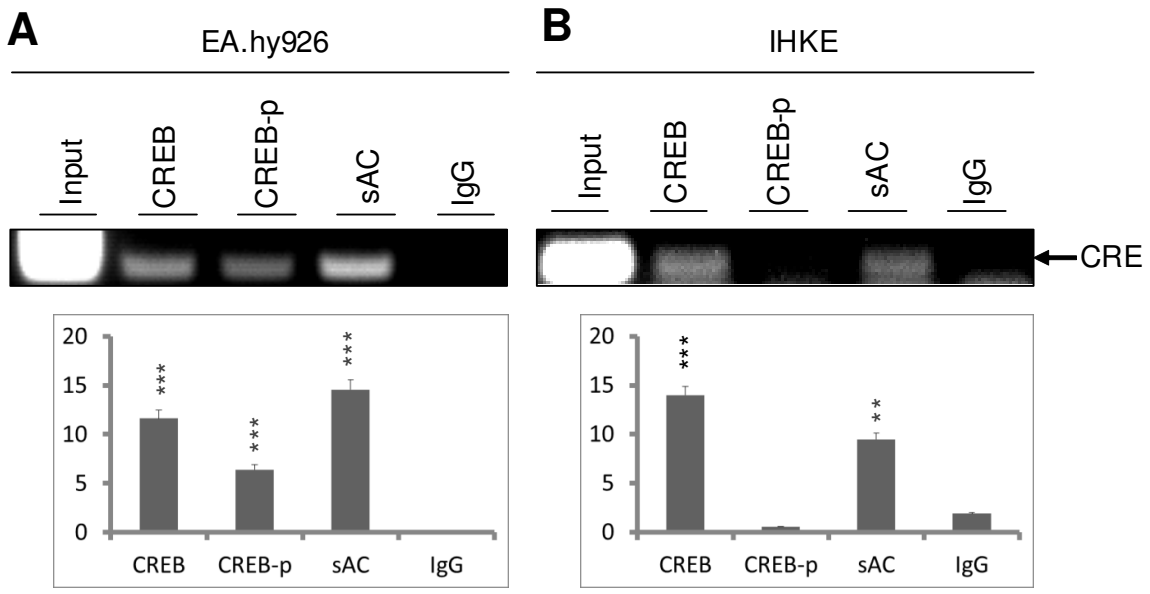



**Figure 12: sAC binding at CRE element**

Chromatin immunoprecipitation analysis in EA.hy926 (**A**) and IHKE (**B**) cells demonstrates *in vivo* binding of the transcription factor CREB and sAC on a CRE consensus site. Binding of CREB and sAC was detected in both cell lines, whereas a binding of the phosphorylated CREB-p occurred exclusively in EA.hy926 cells. DNA was precipitated with specific antibodies against CREB, CREB-p, and sAC. Input-DNA (Input), immunoprecipitated DNA, and DNA, which was precipitated with IgG as control, were amplified with specific primers for a CRE control element in the C-FOS gene. Gelelectrophoresis was performed and band intensities were three times quantified densitometrically. The input signal was defined as 100%, signal intensities are indicated as percentage of input. Level of significance was determined in reference to IgG. *** $p<0.001$, ** $p<0.01$.

In EA.hy926 cells, sAC showed a signal intensity of 15% compared to a 12% signal intensity of CREB, to the CRE consensus sequence (figure 12A). CREB-p was detected at lower intensities. In IHKE cells, a signal intensity of 10% of sAC and 15% of CREB was detected and no binding of CREB-p was observed (figure 12B). Taken together, these findings demonstrate sAC binding in complex with CREB on a CRE consensus sequence in EA.hy926 and IHKE cells.

#### 4.1.1.3 Transcriptional activity driven by CRE sites depends on sAC

Since sAC showed binding capacities at the CRE consensus sequence (figure 12), CRE activation was investigated to determine if it depends partially on sAC abundance. A CRE control vector was used comprising a set of six CREs upstream of the *luciferase* reporter gene to measure the CRE-mediated transcriptional activity. Inhibitors of sAC were tested for their potential to prevent CRE-mediated transcriptional activity. KH7 is a sAC-specific inhibitor (figure 13). 2-hydroxyestradiol (HE, figure 9), which is often used as sAC-inhibitor (Pastor-Soler et al., 2003; Luconi et al., 2005; Pierre et al., 2009), was shown to be a general inhibitor of class III AC activity, since it inhibited the tmAC with an $IC_{50}$ value of ~2 µM (Steegborn et al., 2005).

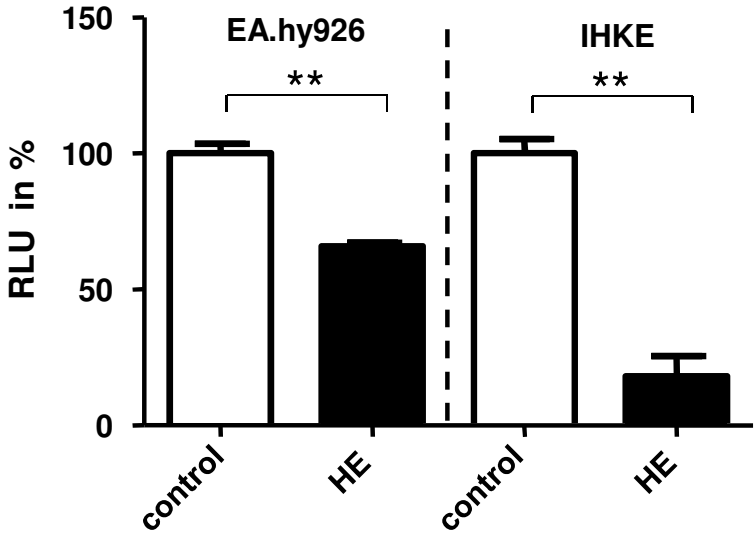



**Figure 13: HE affects CRE-mediated transcriptional activity**

The inhibition of sAC and tmACs resulted in a decreased transcriptional activity of the CRE control vector. In EA.hy926 cells, treatment with HE reduced transcriptional activity to 60% of initial activity. In IHKE cells, HE reduced transcriptional activity to 20% of initial activity. IHKE and EA.hy926 cells were transfected with a CRE control vector, comprising six CREs upstream of the *luciferase* gene. Transfected cells were treated with 120 µM of the inhibitor 2-hydroxyestradiol (HE) for 24 h or with equal volumes of ethanol (control). Transcriptional activity of control was defined as 100%. ** $p<0.01$. HE: 2-hydroxyestradiol. Transcriptional activity was assessed as relative light units (RLUs).

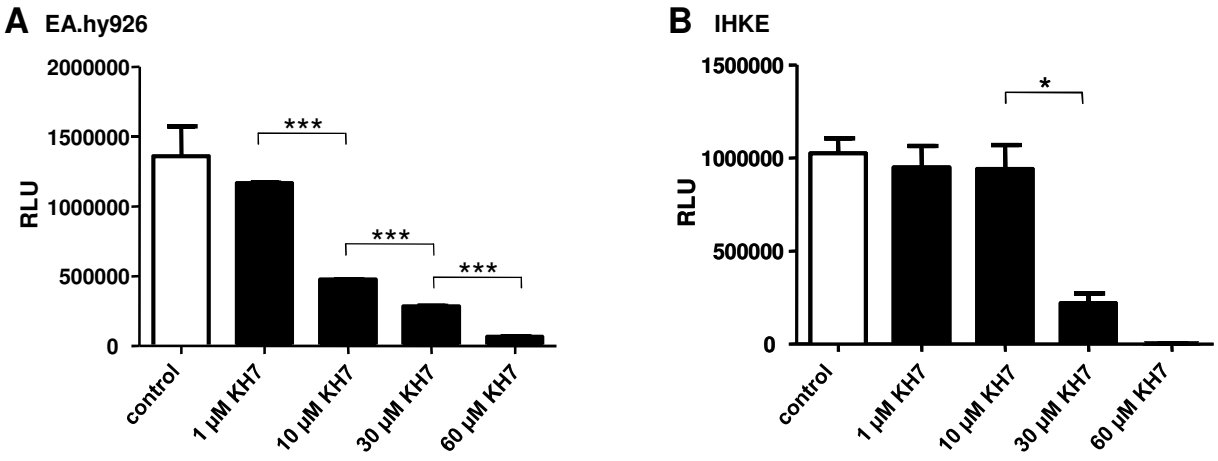



**Figure 14: Inhibition of sAC by KH7 decreases CRE-mediated transcriptional activity in a dose-dependent manner**

Treatment with different doses of sAC-specific inhibitor KH7 in EA.hy926 (**A**) and IHKE (**B**) cells led to dose-dependent decrease of CRE-mediated transcriptional activity. EA.hy926 and IHKE cells were transfected with a CRE control vector, with six CRE elements upstream of the *luciferase* gene and treated for 24 h with increasing doses of the sAC-specific inhibitor KH7 or with equal volumes of DMSO (control). *** $p<0.001$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

Inhibition with HE (figure 13) and KH7 (figure 14) significantly (HE, $p<0.01$; KH7, $p<0.001$ and 0.05) reduced transcriptional activity driven by CREs. Inhibition of sAC with KH7 showed a significant dose-dependent decrease of CRE-mediated transcriptional activity in EA.hy926 cells (figure 14A). In contrast in IHKE cells, inhibition of sAC with 1 µM and 10 µM KH7 had no effect on transcriptional activity driven by CREs (figure 14B). 30 µM and 60 µM KH7 showed significant reduction of CRE-mediated transcriptional activity in both cell lines ($p<0.001$, $p<0.05$).

To test whether the inhibiting effect of KH7 results from a lack of cAMP or from the inhibition of the sAC enzyme directly, the cells were treated with cAMP to compensate the lack of cAMP due to sAC inhibition (figure 15). Compensation of sAC inhibition with cAMP stimulation did not overcome the inhibiting effect of KH7 on CRE-mediated transcriptional activity, since transcriptional activity is decreased ~3-fold compared to control ($p<0.01$, $p<0.05$). These effects were observed in a similar manner for both the IHKE cells and the EA.hy926 cells.

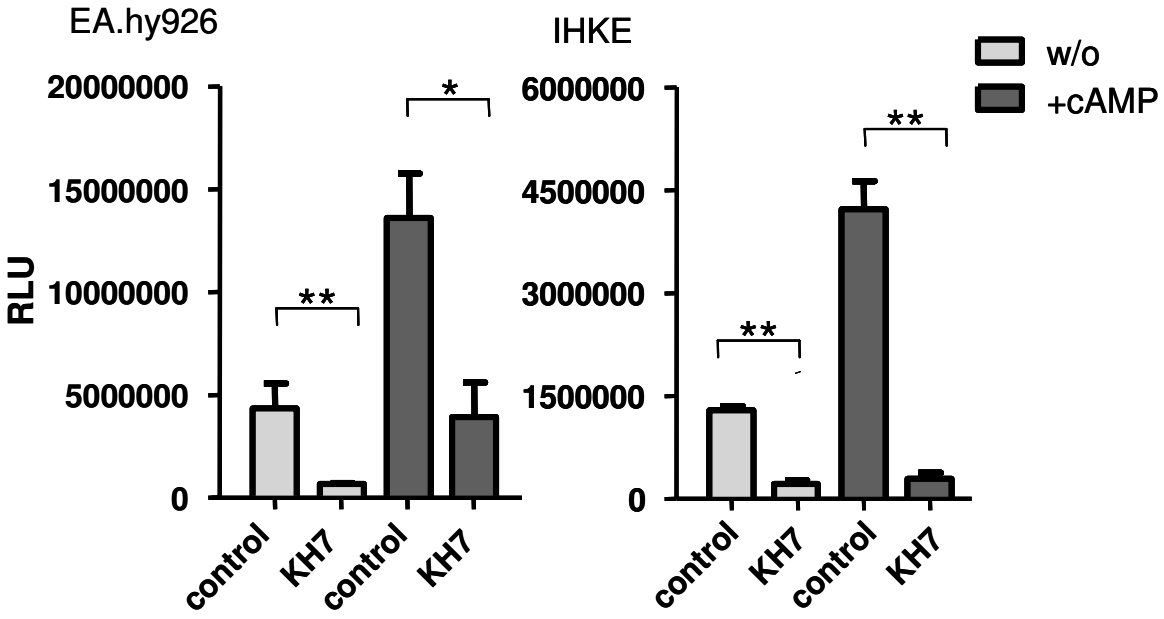



**Figure 15: sAC influences cAMP-independent CRE-mediated transcriptional activity**
Transcriptional activity of CRE elements decreased after treatment with 30 µM of sAC inhibitor KH7. Treatment with cAMP increased the overall CRE-mediated transcriptional activity, whereas it did not overcome the inhibiting effect of KH7 on CRE-mediated transcriptional activity. EA.hy926 and IHKE cells were transfected with a CRE control vector, comprising six CREs upstream of the *luciferase* gene and treated with (+cAMP) or without 0.5 mM 8-Br-cAMP (w/o) for 24 h. ** $p<0.01$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

### 4.1.2 sAC affects expression of genes involved in aldosterone signaling

#### 4.1.2.1 Aldosterone-mediated activation of CRE sites is blocked by inhibition of sAC

There is some evidence that aldosterone takes part in cAMP-regulated gene expression (Christ et al., 1999). Therefore, we analyzed the effect of aldosterone stimulation on the transcriptional activity of the CRE control vector (figure 16).

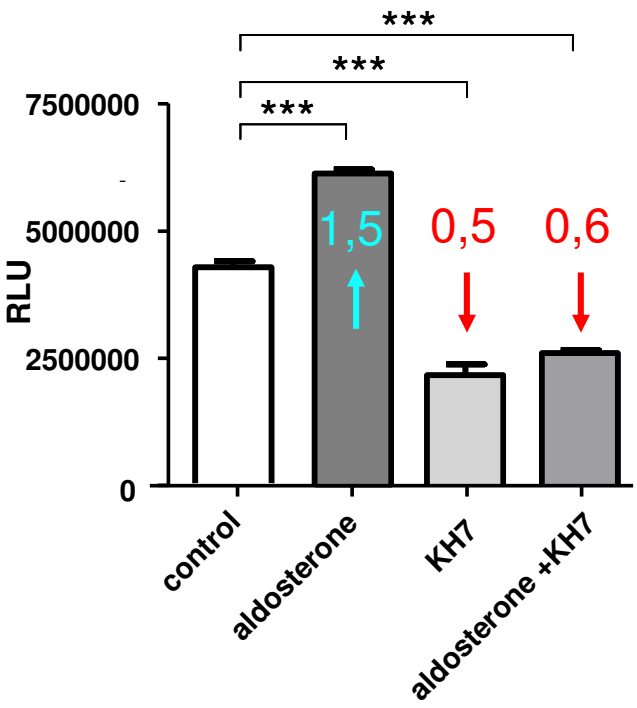
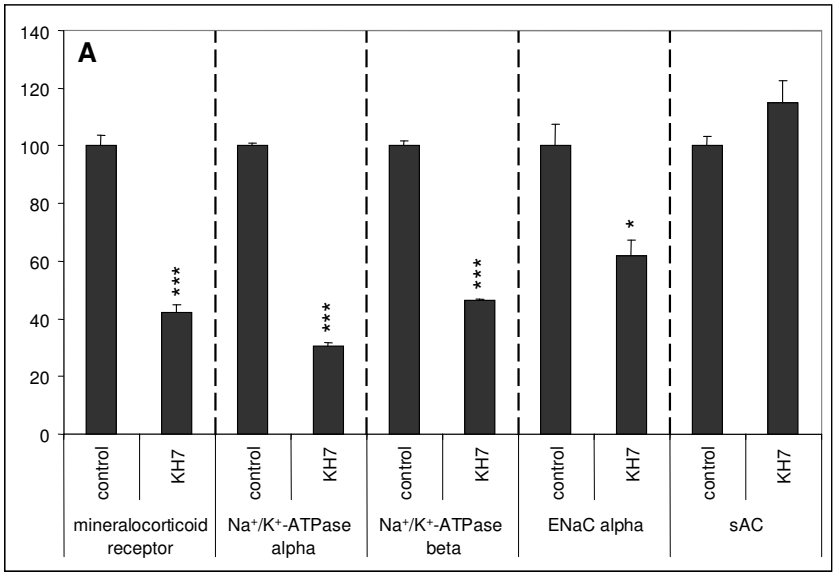



**Figure 16: Aldosterone influences sAC enhanced CRE-mediated transcriptional activity**
Aldosterone had an activating effect on CRE-mediated transcriptional activity. EA.hy926 cells were transfected with a CRE control vector, comprising six CREs upstream of the *luciferase* gene and treated with 1 nM aldosterone and 30 µM KH7. *** $p<0.001$. Transcriptional activity was assessed as relative light units (RLUs).

Treatment of cells with aldosterone increased CRE-mediated transcriptional activity 1.5-fold ($p<0.001$). The elevated transcriptional activity, resulting from aldosterone stimulation was significantly ($p<0.001$) decreased to 0.6-fold after incubation with KH7 (figure 16).

#### 4.1.2.2 Aldosterone-regulated genes are influenced by sAC

To determine the regulatory effect of sAC on genes involved in aldosterone signaling, we analyzed the expression pattern of the *mineralocorticoid receptor*, *$Na^+/K^+$-ATPase alpha*, *$Na^+/K^+$-ATPase beta*, *ENaC alpha* and *sAC* via real time PCR after treatment with aldosterone and KH7 (figure 17).


A
140
120
100
80
60
40
20
0
***
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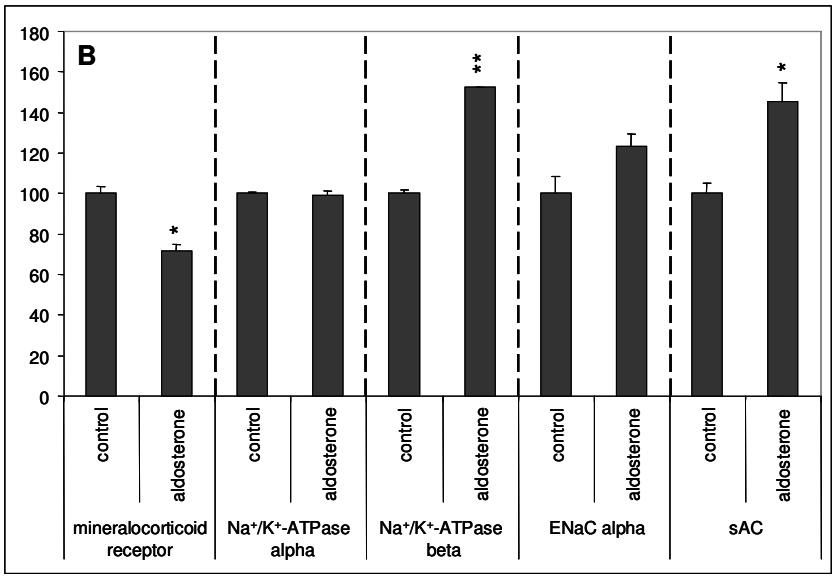
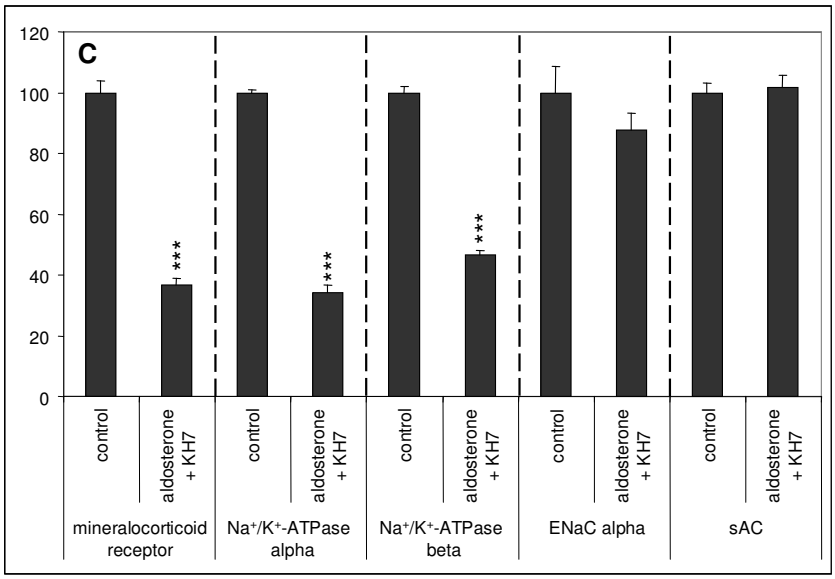
*
control
KH7
mineralocorticoid receptor
Na+/K+-ATPase alpha
Na+/K+-ATPase beta
ENaC alpha
sAC


B
180
160
140
120
100
80
60
40
20
0
*
**
*
control
aldosterone
mineralocorticoid receptor
Na+/K+-ATPase alpha
Na+/K+-ATPase beta
ENaC alpha
sAC


C
120
100
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control
aldosterone + KH7
mineralocorticoid receptor
Na+/K+-ATPase alpha
Na+/K+-ATPase beta
ENaC alpha
sAC

**Figure 17: Inhibition of sAC leads to reduction of aldosterone-regulated gene expression**
Changes in the expression of genes involved in aldosterone signaling after treatment with 60 µM of sAC inhibitor KH7 and 1 nM aldosterone were analyzed by reverse transcription real time PCR in EA.hy926 cells. **A** Treatment with KH7 resulted in a downregulation of the *mineralocorticoid receptor*, *$Na^+/K^+$-ATPase alpha*, *$Na^+/K^+$-ATPase beta*, and *ENaC alpha*. **B** Treatment with aldosterone resulted in an upregulation of *$Na^+/K^+$-ATPase beta*, *ENaC alpha*, and *sAC* as well as in a downregulation of the *mineralocorticoid receptor* transcript and had no effect on the expression of the *$Na^+/K^+$-ATPase alpha*. **C** Treatment of KH7 in combination with aldosterone hampered the aldosterone effect on the expression of all analyzed genes. *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Normalization of all amplifications was performed in reference to glycerine-aldehyde-3-phosphate-dehydrogenase (GAPDH).

Aldosterone enhanced the expression of *$Na^+/K^+$-ATPase beta* ($p<0.01$), *ENaC alpha*, and *sAC* ($p<0.05$), inhibited the expression of *mineralocorticoid receptor* ($p<0.05$), and had no effect on the expression of *$Na^+/K^+$-ATPase alpha* (figure 17B). Inhibition of sAC by KH7 resulted in a significant decrease ($p<0.001$; $p<0.05$) of expression of all analyzed genes, except *sAC* (figure 17A). Furthermore sAC inhibition did prevent the effect of aldosterone on the expression of these genes, since all analyzed genes displayed only ~50% of expression after treatment with aldosterone and KH7 (figure 17C) compared to treatment with aldosterone exclusively (figure 17B).

## 4.2 Transcriptional regulation of sAC

### 4.2.1 Endogenous expression of sAC

Since sAC expression was shown to be modulated by aldosterone (figure 17B), we investigated the transcriptional regulation of *sAC*. To identify cell lines that endogenously express *sAC* under basic and different stimulatory conditions (cAMP, PMA), the expression of *sAC* in the following cell lines was investigated: human hepatocellular carcinoma cell line (HepG2), human vascular endothelial cells (EA.hy926), human embryonic kidney 293T cell line (HEK293T), immortalized human kidney epithelial cell line (IHKE), and human acute monocytic leukemia cells (THP1, figure 18).
EA.hy926, HEK293T and IHKE cell lines showed endogenous expression of *sAC*. To determine if sAC is involved in cAMP signal transduction, the influence of cAMP on sAC mRNA expression in all cell lines was analyzed. In HepG2, EA.hy926, IHKE, and THP1 cells stimulation with cAMP increased *sAC* expression, whereas in HEK293T cells cAMP

treatment led to decreased *sAC* expression. Stimulation with PMA increased *sAC* expression exclusively in HepG2 and HEK293T cells.

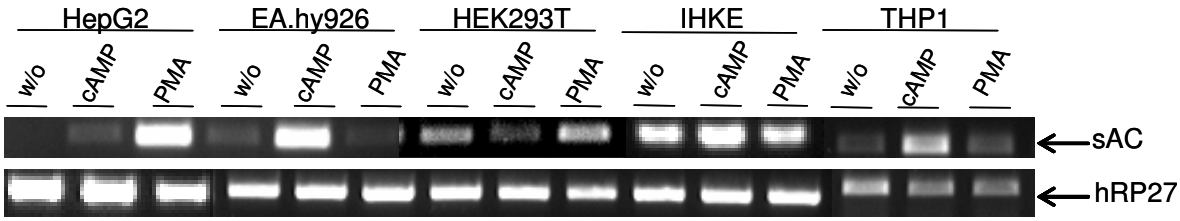


**Figure 18: Endogenous expression of *sAC***

*sAC* expression was detected in EA.hy926, HEK293T and IHKE cells. Stimulation with cAMP led to upregulation in HepG2, EA.hy926, IHKE and THP1 cells, whereas cAMP stimulation led to downregulation in HEK293T cells. In HepG2 cells, endogenous expression of *sAC* was upregulated by PMA. RNA was isolated after treatment with $5 \times 10^{-4}$ M cAMP or $10^{-8}$ M PMA for 24 h. Amplification with specific primers for human ribosomal protein 27 (hRP27) served as loading control. HepG2: human hepatocellular carcinoma cell line, EA.hy926: human vascular endothelial cells, HEK293T: human embryonic kidney 293T cell line, IHKE: immortalized human kidney epithelial cell line, THP1: human acute monocytic leukemia cells, w/o: unstimulated cells.

### 4.2.2 *sAC* transcription in endothelial and kidney cells

#### 4.2.2.1 Distinct sAC isoform expression in kidney and endothelial cells

To study sAC protein expression and localization in endothelial and kidney cells, western blot analysis was performed to detect possible differences of sAC protein expression in cell extract and nuclear extract of endothelial and kidney cells (figure 19).

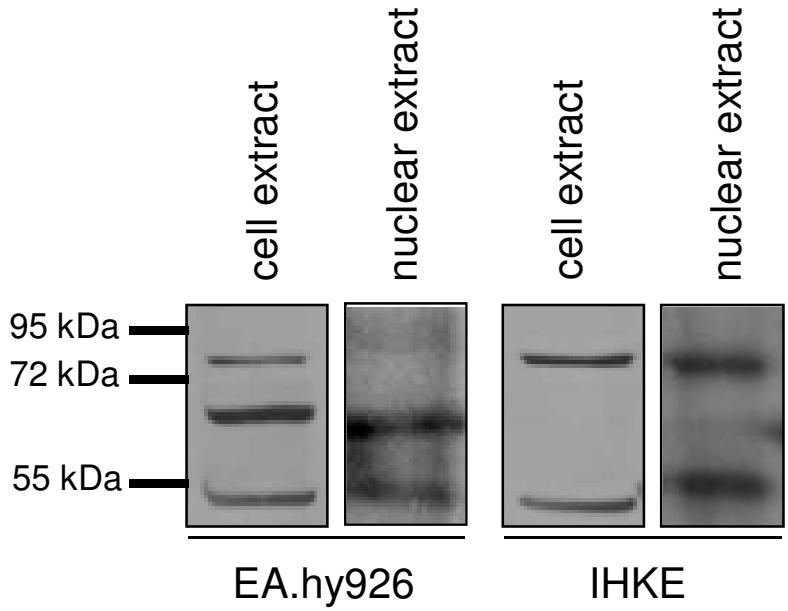


**Figure 19: sAC is endogenously expressed in endothelial and kidney cells and localized in the nucleus**

Detection of sAC using a specific sAC antibody in whole cell extracts of EA.hy926 cells showed three distinct bands at 50, 70 and 80 kDa. EA.hy926 nuclear extract displayed a signal at 50 and 70 kDa. Nuclear and whole cell extract of IHKE displayed a signal at 50 and 80 kDa.

In both cell lines sAC protein could be detected in the cell extract as well as in the nuclear extract. We observed isoforms at 50 kDa, at 80 kDa and additionally at 70 kDa in EA.hy926 cells, which has not been described so far. While all three isoforms were expressed in the whole cell extract, the 80 kDa isoform could not be detected in the nuclear extract of EA.hy926 cells. In contrast to the findings in EA.hy926 cells the 50 kDa and the 80 kDa isoforms were found in the cell and the nuclear extract in the IHKE cells, while in both extracts the 70 kDa isoform, which could be observed in both extracts of EA.hy926, could not be found (figure 19).

#### 4.2.2.2 *sAC* promoter structure in endothelial and kidney cells

Since differences in the expression pattern of *sAC* could be detected in endothelial and kidney cells (figure 19), we investigated the transcriptional regulation in endothelial (EA.hy926) and kidney (IHKE, HEK293T) cell lines. To identify transcriptional active promoter portions, we cloned the region -3715 to +250 into the promoterless pGL3-Basic vector, harboring the *luciferase* reporter gene downstream of the multiple cloning site

(mcs), to detect potential promoter activity of the 5´-flanking region (figure 20). Expression of luciferase protein resulted from transcriptional activity of the specific *sAC* promoter fragment. Transcriptional activity was determined as relative light units of the permitted light resulting from the chemical reaction of oxidation of luciferin catalyzed by luciferase. (Greer and Szalay, 2002). By deleting parts of the 5´-end of the constructs, we created serial deletion constructs. We performed transient transfection assays in EA.hy926, IHKE, and HEK293T cells.

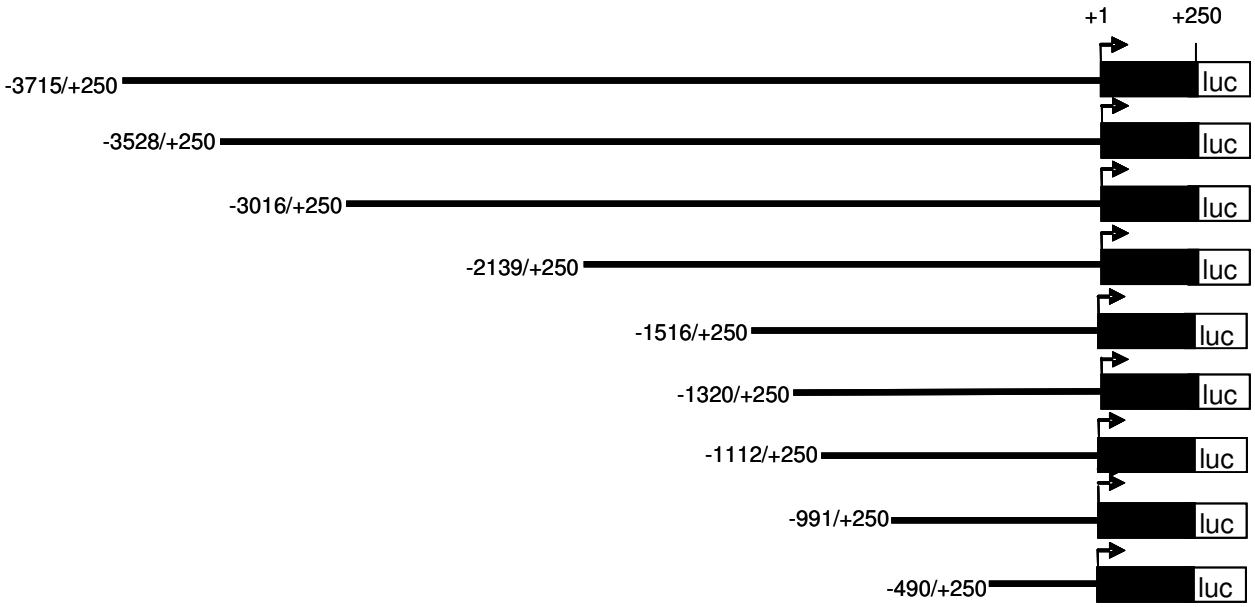


**Figure 20: Schematic representation of *sAC* promoter deletion constructs**

3715 bp of the 5´-flanking region of the *sAC* gene harboring 250 bp of exon 1 were cloned into the pGL3-Basic vector upstream of the reporter gene *luciferase* and serial deletion constructs were generated. Sequence positions are shown according to TSS (Acc#.: NM_018417.4). The arrow indicates the TSS. The black box indicates a part of the untranslated exon 1. The white box indicates the *luciferase* gene. luc: *luciferase*.

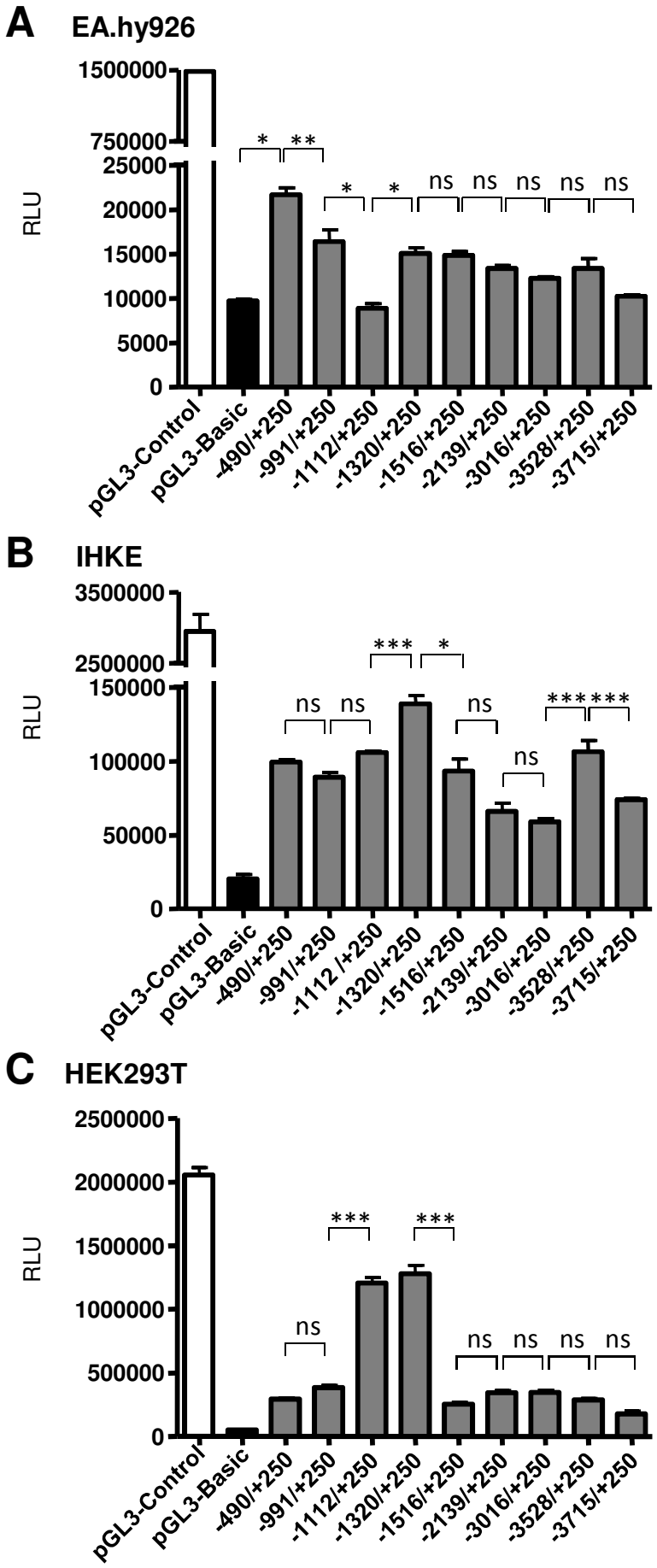
A
EA.hy926
RLU
1500000
750000
25000
20000
15000
10000
5000
0
* **
* * ns ns ns ns ns
pGL3-Control
pGL3-Basic
-490/+250
-991/+250
-1112/+250
-1320/+250
-1516/+250
-2139/+250
-3016/+250
-3528/+250
-3715/+250
B
IHKE
RLU
3500000
2500000
150000
100000
50000
0
*** *
ns ns ns ns ******
pGL3-Control
pGL3-Basic
-490/+250
-991/+250
-1112 /+250
-1320/+250
-1516/+250
-2139/+250
-3016/+250
-3528/+250
-3715/+250
C
HEK293T
RLU
2500000
2000000
1500000
1000000
500000
0
*** ***
ns ns ns ns ns
pGL3-Control
pGL3-Basic
-490/+250
-991/+250
-1112/+250
-1320/+250
-1516/+250
-2139/+250
-3016/+250
-3528/+250
-3715/+250

**Figure 21: *sAC* promoter structure in endothelial and kidney cell lines**
Deletion constructs of the *sAC* 5´-flanking region were transiently transfected into EA.hy926 (**A**), IHKE (**B**), and HEK293T (**C**) cells to identify transcriptionally active promoter regions within the 5'-flanking region. In the endothelial cell line EA.hy926, the construct -490/+250 harbored the highest transcriptional activity, whereas in the kidney cell lines IHKE and HEK293T the strongest transcriptional activity was detected in construct -1320/+250. In IHKE cells, the construct -3528/+250 also showed a significantly higher transcriptional activity compared to the surrounding constructs. White bars display transcriptional activity of the pGL3-Control vector, with a strong viral SV40 promoter; black bars display basic transcriptional activity of the pGL3-Basic vector. *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns: not significant. Transcriptional activity was assessed as relative light units (RLUs).

The 5´-flanking region of *sAC* showed transcriptional activity in endothelial, as well as in kidney cell lines, the overall transcriptional activity being lower in the endothelial cell line EA.hy926. The transcriptional activity significantly ($p<0.01$) peaked in the shortest construct -490/+250, but showed only 2-fold higher transcriptional activity compared to pGL3-Basic, and increases with longer construct length in EA.hy926 cells (figure 21A). In HEK293T the construct -1320/+250 showed a very strong transcriptional activity with 75% of transcriptional activity compared to pGL3-Control (figure 21C). The highest transcriptional activity in both kidney cell lines, IHKE and HEK293T, was observed for the construct -1320/+250 ($p<0.001$) whereas only in the IHKE cell line the transcriptional activity did show another significant peak at the construct -3528/+250 ($p<0.001$), pointing to a more expanded promoter region (figure 21B).

#### 4.2.2.3 Regulatory effect of exon 1 on transcriptional activity of the *sAC* promoter

The 5´-UTR often has significant impact on transcriptional control of gene expression (Pickering and Willis, 2005). To analyze if *sAC* expression is regulated via regions in the 5´-UTR, we generated *sAC* promoter deletion constructs lacking the 5´-untranslated region of exon 1 (figure 22).

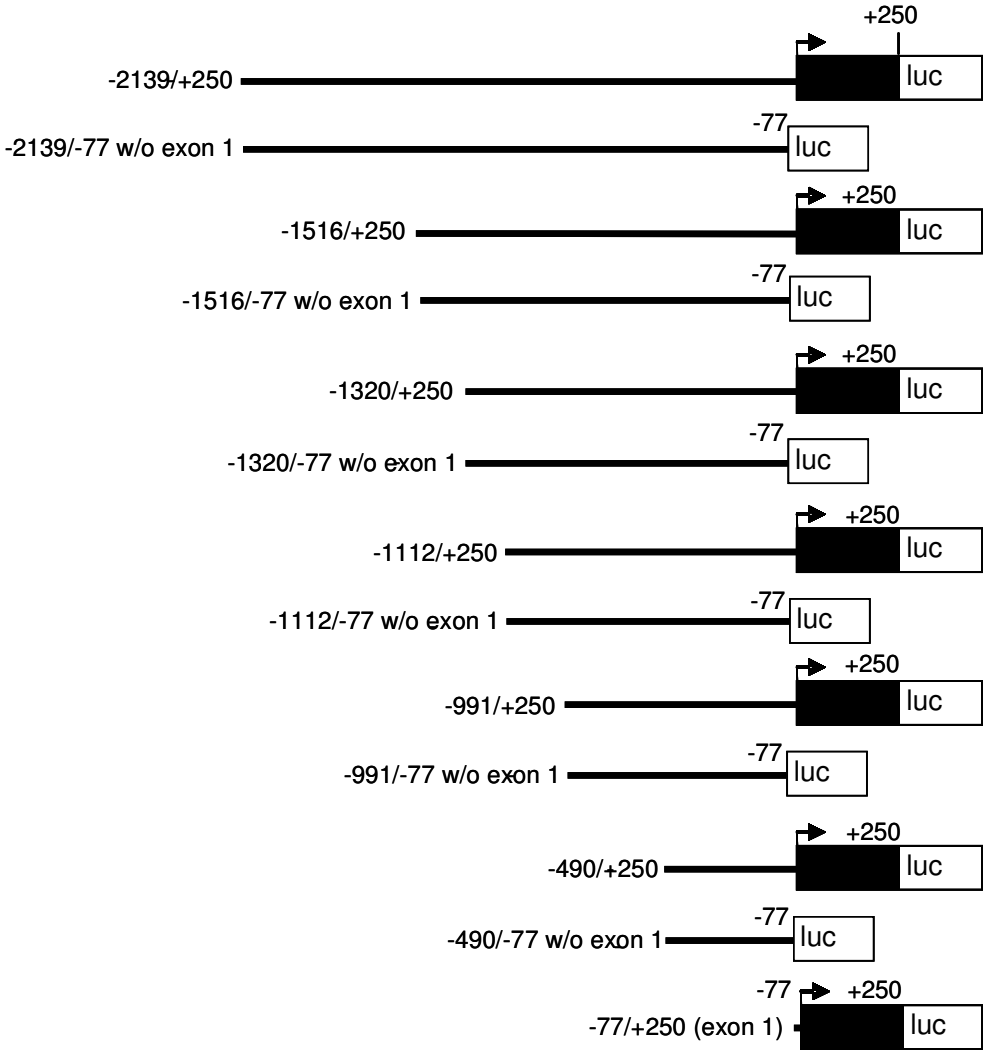


**Figure 22: Schematic representation of *sAC* promoter fragments with or without exon 1**

Deletion constructs were designed as described in figure 20 and exon 1 was deleted or exon 1 alone (-77/+250) cloned upstream of the luciferase gene in the pGL3-Basic vector. Sequence positions are shown according to TSS (Acc#.: NM_018417.4). luc: luciferase.

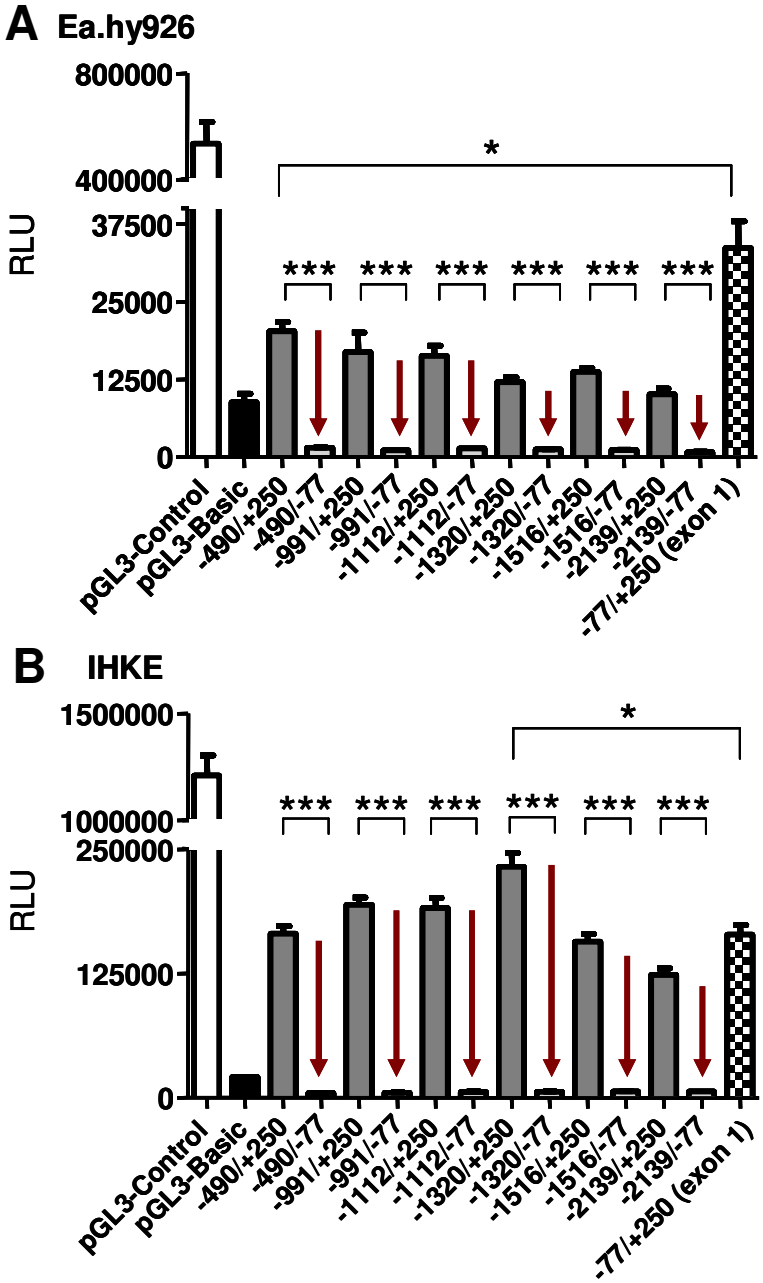


**Figure 23: Exon 1 comprises essential *cis*-regulatory elements**

sAC promoter fragments with exon 1 (position -77 to +250 bp relative to TSS [Acc#.: NM_018417.4]) and promoter fragments without exon 1 were transiently transfected into EA.hy926 (**A**) and IHKE (**B**) cells. Comparison of constructs lacking exon 1 with constructs containing exon 1 revealed a total inhibition of transcriptional activity when exon 1 was excised (marked with red arrows). The construct -77/+250 (exon 1) held a high transcriptional activity in both cell lines. White bars display transcriptional activity of pGL3-Control vector, black bars display basic transcriptional activity of pGL3-Basic vector. *** $p<0.001$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

To investigate the effect of the 5´-UTR on transcriptional activity of the *sAC* promoter in different cell lines, constructs including the 5´-UTR (+250), constructs lacking the 5´-UTR (-77) and a deletion construct representing the 5´-UTR alone (-77/+250 [exon 1]) were designed (figure 22). Deletion of the 5´-UTR resulted in a significant ($p<0.001$) inhibition of the transcriptional activity for all constructs in both cell lines compared to constructs including the 5´-UTR. The construct representing the isolated 5´-UTR (-77/+250 [exon 1]) showed high transcriptional activity in both cell lines. In EA.hy926 cells, the 5´-UTR (-77/+250 [exon 1]) construct displayed significant ($p<0.05$) higher transcriptional activity compared to the other promoter constructs, harboring additional 5´-flanking regions (figure 23A). In IHKE cells, construct -77/+250 (exon 1) showed a significantly lower ($p<0.05$) transcriptional activity compared to the highest transcriptional activity of the construct -1320/+250 (figure 23B).

#### 4.2.2.4 Alternative promoter region positioned in intron 4

Since an alternative translational start site in exon 5 was predicted by the group of Geng (Geng et al., 2005), it was investigated whether an alternative promoter upstream of exon 5 exists. Therefore, the region comprising intron 4 (+16197/+16377) was cloned into the pGL3-Basic vector (figure 24).

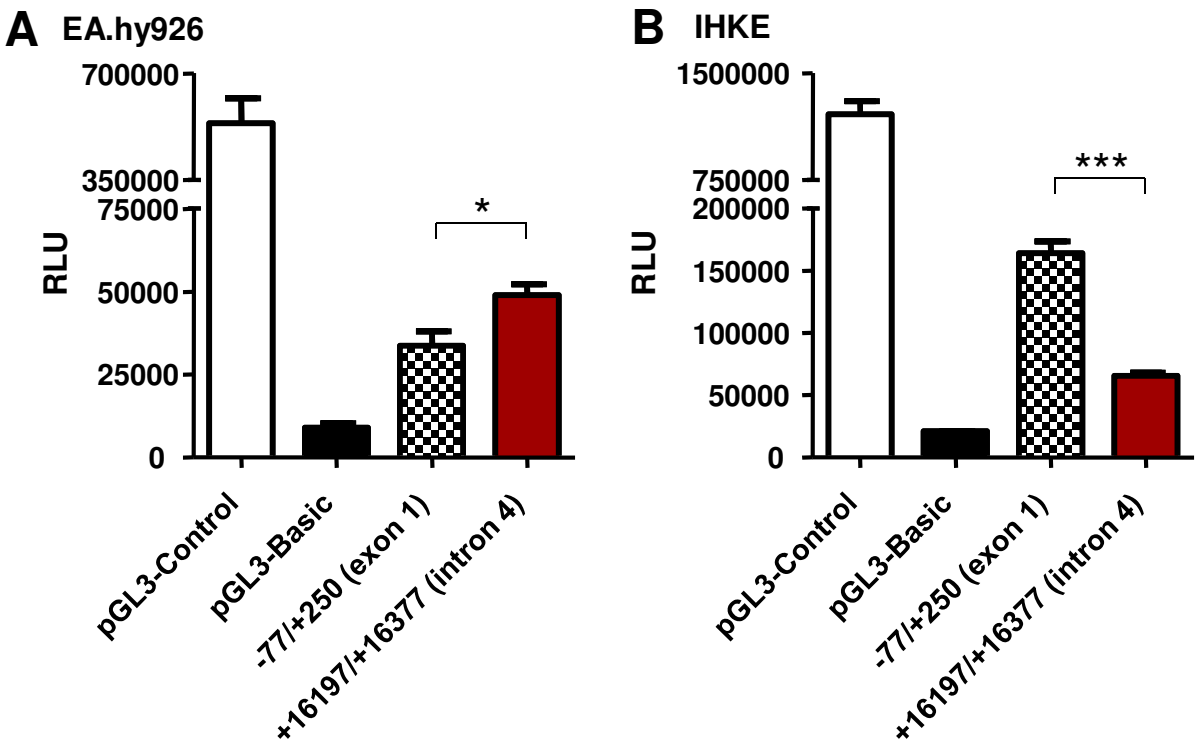


**Figure 24: Intron 4 holds independent transcriptional activity**

*sAC* promoter constructs of exon 1 (-77/+250) or intron 4 (+16197/+16377) were transiently transfected in EA.hy926 and IHKE cells. **A** The intron 4 construct (red bar) exhibited a higher transcriptional activity compared to the exon 1 construct in EA.hy926 cells ($p<0.05$). **B** By contrast, the exon 1 construct showed a significantly higher transcriptional activity in IHKE cells ($p<0.001$). White bars display transcriptional activity of the pGL3-Control vector, black bars display basic transcriptional activity of the pGL3-Basic vector. *** $p<0.001$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

Transfection experiments including the *sAC* intron 4 construct demonstrated existence of a second intronic promoter region, independent of the 5´-promoter located in exon 1. The intron 4 construct showed the highest transcriptional activity ($p<0.05$) compared to the 5´-promoter in EA.hy626 cells (figure 24A). In IHKE cells, the intron 4 construct showed a lower transcriptional activity ($p<0.001$), compared to the 5´-promoter (figure 24B).

To investigate whether differential usage of a potential alternative promoter positioned in intron 4 resulted in independent usage of an alternative TSS in exon 5 in EA.hy926 and IHKE cells, we performed reverse transcriptase real time PCR (figure 25). A region covering exon 2 to 3 was amplified and compared to transcript including exon 32 to 33. Differences in the amounts of transcript representing exon 2 to 3 and exon 32 to 33 would point to an individual and independent TSS besides TSS1 (Acc#.: NM_018417.4). In both

cell lines transcripts representing exon 32 to 33 were detected, while exon 2 to 3 was absent in EA.hy926 (figure 25).

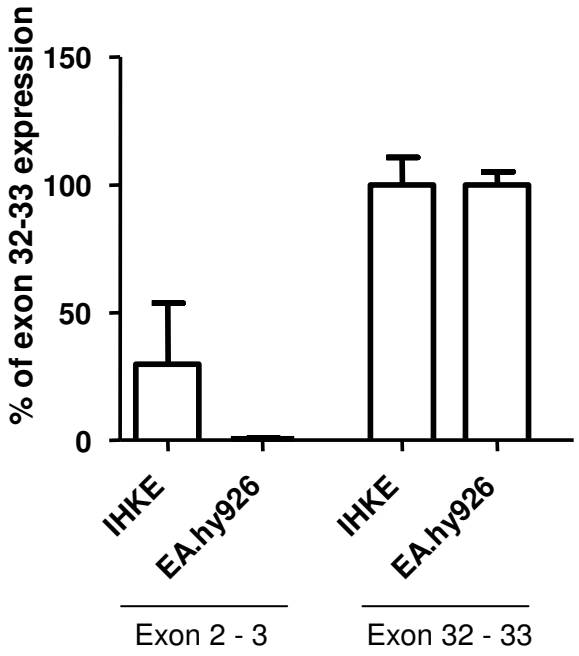


**Figure 25: Different transcripts in EA.hy926 and IHKE cells**

Amplification of a region covering exon 2 to exon 3 was performed by reverse transcriptase real time PCR. Amplification of a region covering exon 32 to 33 was set as 100 %. The region in front of intron 4 (exon 2 to exon 3) was expressed in IHKE cells but not in EA.hy926 cells. Normalization of all amplifications was performed in reference to GAPDH.

### 4.2.3 Transcriptional regulation by aldosterone

We identified two promoter regions in EA.hy926 and IHKE cells, which showed cell type-specific differences in transcriptional activity (5´-promoter and intronic promoter, figure 23). Since it has been shown that inhibition of sAC results in an abrogation of the aldosterone effect, hampering the activation of the $Na^+/K^+$-ATPase in renal epithelial collecting duct cells (Hallows et al., 2009), we tested the potential influence of the mineralocorticoid hormone aldosterone on the activity of the two promoter regions, located in the 5´-promoter and the intronic promoter.

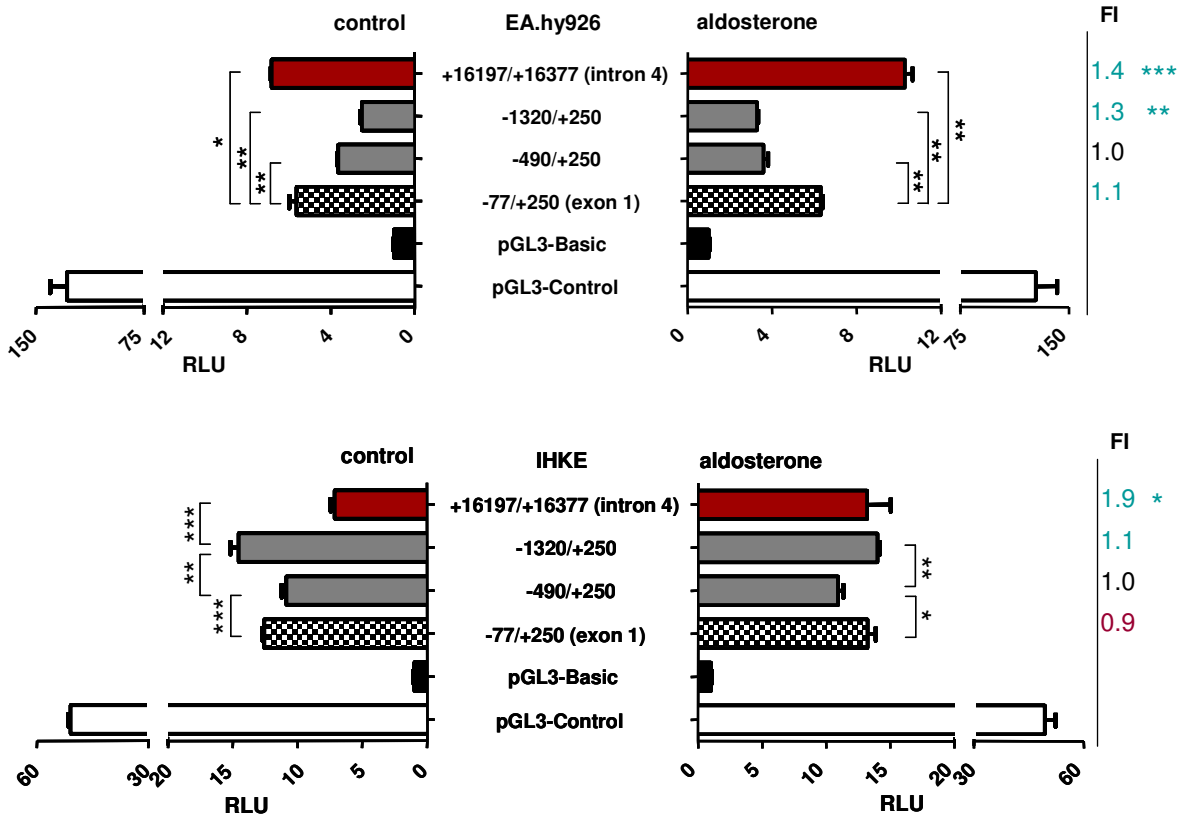


**Figure 26: Aldosterone stimulates the transcriptional activity of the *sAC* promoter**

Selected *sAC* promoter constructs were transiently transfected into EA.hy926 and IHKE cells and stimulated with 1 nM aldosterone for 24 h or treated with ethanol as control. Stimulation with aldosterone led to a significant increase of transcriptional activity of intron 4 construct (red bars) in both cell lines. In EA.hy926 cells, the transcriptional activity of the construct -1320/+250 was also enhanced by aldosterone treatment. Transcriptional activity of exon 1 was not affected by aldosterone treatment in either cell line. Transcriptional activity of the pGL3-Basic vector was defined as 1. Fold induction (FI) of transcriptional activity with aldosterone stimulation relative to unstimulated conditions is shown on the right. White bars display transcriptional activity of the pGL3-Control vector, black bars display basic transcriptional activity of pGL3-Basic vector. *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

Treatment of both cell lines with aldosterone enhanced transcriptional activity of the intron 4 construct in IHKE cells 1.9-fold ($p<0.05$) and in EA.hy926 cell 1.4-fold ($p<0.001$). In EA.hy926 cells, the -1320/+250 construct also showed a slight activation ($p<0.01$) by aldosterone treatment (FI: 1.3, figure 26). To test whether this transcriptional activation of the *sAC* promoter results in increased amounts of expressed sAC protein, western blot analysis was performed using EA.hy926 extract. The slight increase in the amount of sAC protein after treatment with aldosterone was in accordance with the effect on transcriptional activity (figure 27).

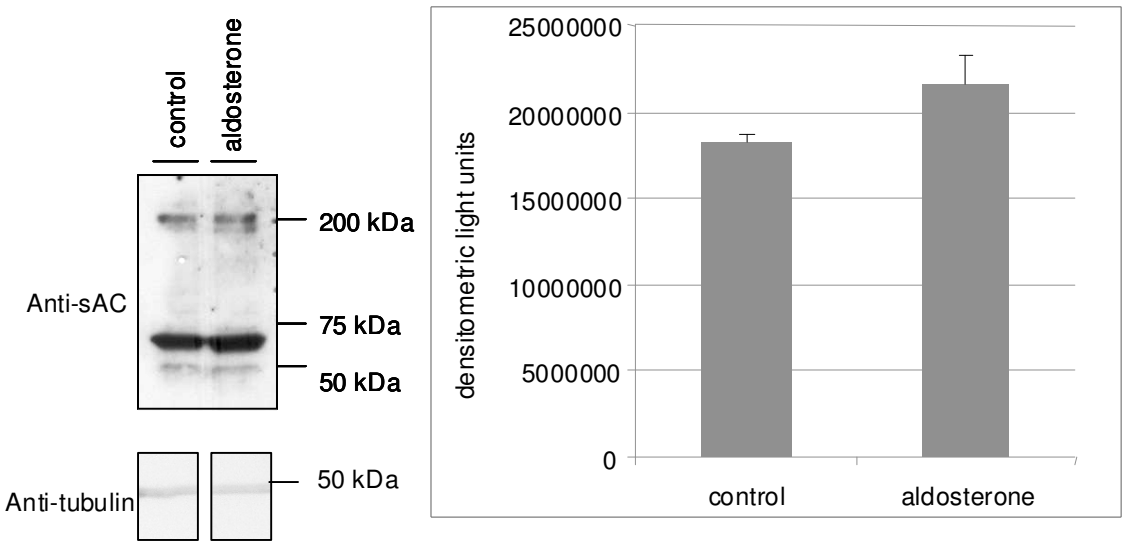


**Figure 27: Aldosterone induces an increase of sAC protein expression in EA.hy926 cells**
EA.hy926 cells were treated with 1 nM aldosterone or equal amounts of ethanol as control for 24 h. Western blot analysis with a sAC specific antibody and an antibody against tubulin as control was performed and intensities of all bands on 50, 70, 80 and 180 kDa were analysed densitometrically (shown right). Treatment with aldosterone led to an increased amount of sAC protein. kDa: kilodalton.

### 4.2.4 Transcriptional regulation by CREB

Since sAC is an important protein of the cAMP signaling pathway, the influence of cAMP-dependent transcription factors on transcriptional regulation of the *sAC* gene was examined. *In silico* analysis revealed several CREB binding sites, whereas the best prediction of a CRE could be found in exon 1 at position +138 (Zhang et al., 2005). Therefore, cotransfection experiments of the exon 1 *sAC* promoter construct were conducted in the presence of overexpressed CREB.

A strong induction of the transcriptional activity of the exon 1 promoter constructs was observed in the presence of overexpressed CREB (figure 28). A 2.5-fold induction ($p<0.01$) by CREB overexpression compared to empty shuttle control was observed in EA.hy926 cells, while a 1.7-fold induction ($p<0.001$) was observed in IHKE cells.

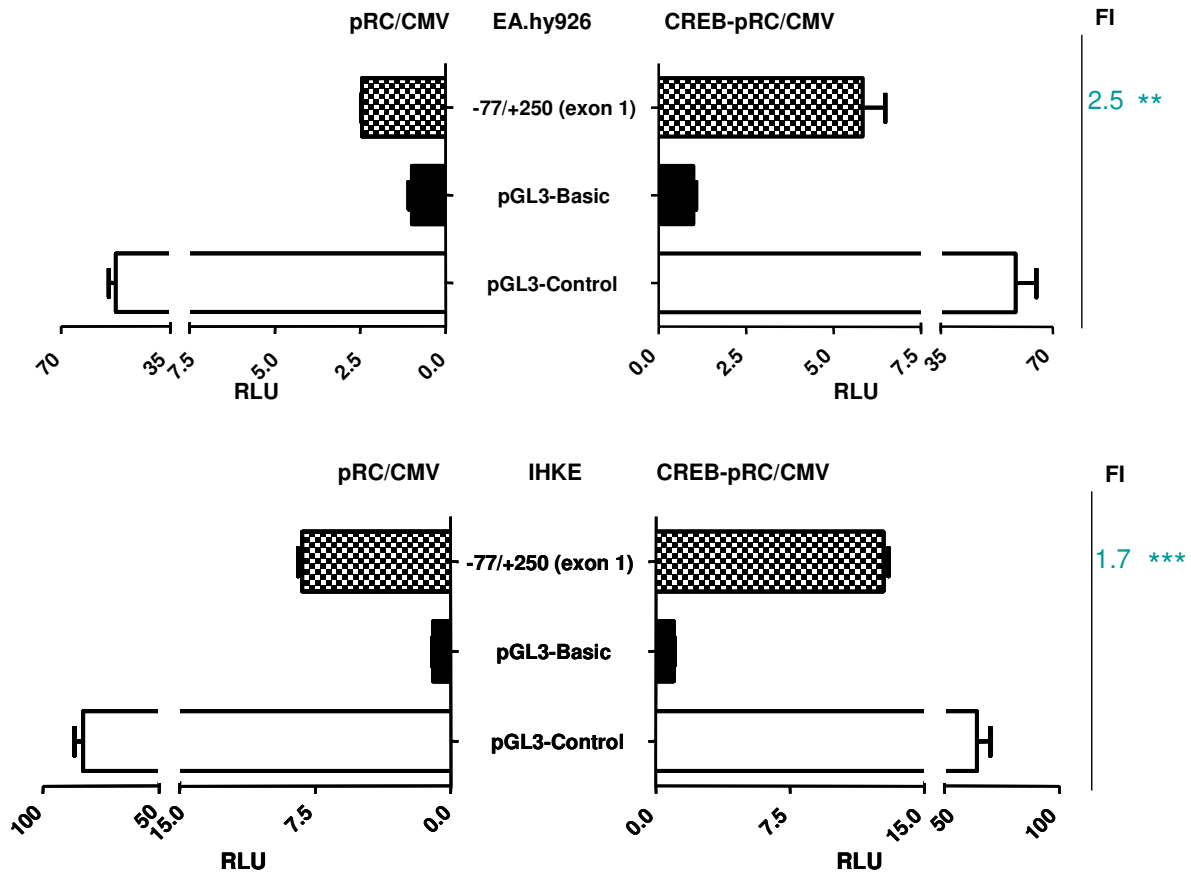


**Figure 28: CREB overexpression enhanced transcriptional activity of the *sAC* exon 1 promoter fragment**

Significant induction of the transcriptional activities of the exon 1 construct was observed upon overexpression of CREB in EA.hy926 (FI: 2.5) and IHKE (FI: 1.7) cells. The empty vector pRC/CMV served as shuttle control (left). Transcriptional activity of the pGL3-Basic vector was defined as 1. Fold induction (FI) was calculated as transcriptional activity of exon 1 in presence of CREB relative to the empty vector control and is shown on the right. White bars display transcriptional activity of the pGL3-Control vector, black bars display basic transcriptional activity of pGL3-Basic vector. *** $p<0.001$, ** $p<0.01$. Transcriptional activity was assessed as relative light units (RLUs).

To confirm the result that the transcriptional activity of exon 1 is regulated by the transcription factor CREB *in vivo*, a ChIP assay was performed (figure 29). Using a CREB antibody and subsequent amplification of a PCR product harboring region +156 to +244 of exon 1, *in vivo* binding of CREB was confirmed within exon 1 of the *sAC* gene. A precipitation with a CREB antibody and IgG was performed, which served as the negative control. The signal intensity was controlled by amplification of 10% and 1% Input-DNA.

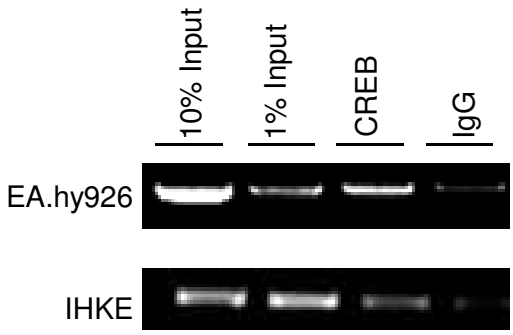


**Figure 29: CREB binds to a region of the *sAC* 5´-UTR *in vivo***

Chromatin immunoprecipitation analysis in EA.hy926 and IHKE cells demonstrated binding of the transcription factor CREB to the *sAC* 5´-UTR under basic conditions. Input-DNA, immunoprecipitated DNA, and DNA that was treated with IgG as control, were amplified with specific primers for exon 1 and analyzed by gelelectrophoresis.

### 4.2.5 Transcriptional regulation by sAC

Since it was shown that activation of sAC by $HCO_3^-$ leads to increased CREB phosphorylation (Zippin et al., 2004), the effect of sAC overexpression on transcriptional activity of exon 1 was examined. A sAC expression vector was designed, expressing the full length cDNA. Cotransfection experiments of the construct harboring *sAC* exon 1 and the sAC expression vector were performed. Transcriptional activity of exon 1 was significantly enhanced 1.6-fold ($p<0.001$) in EA.hy926 cells and 2-fold ($p<0.001$) in IHKE cells (figure 30).

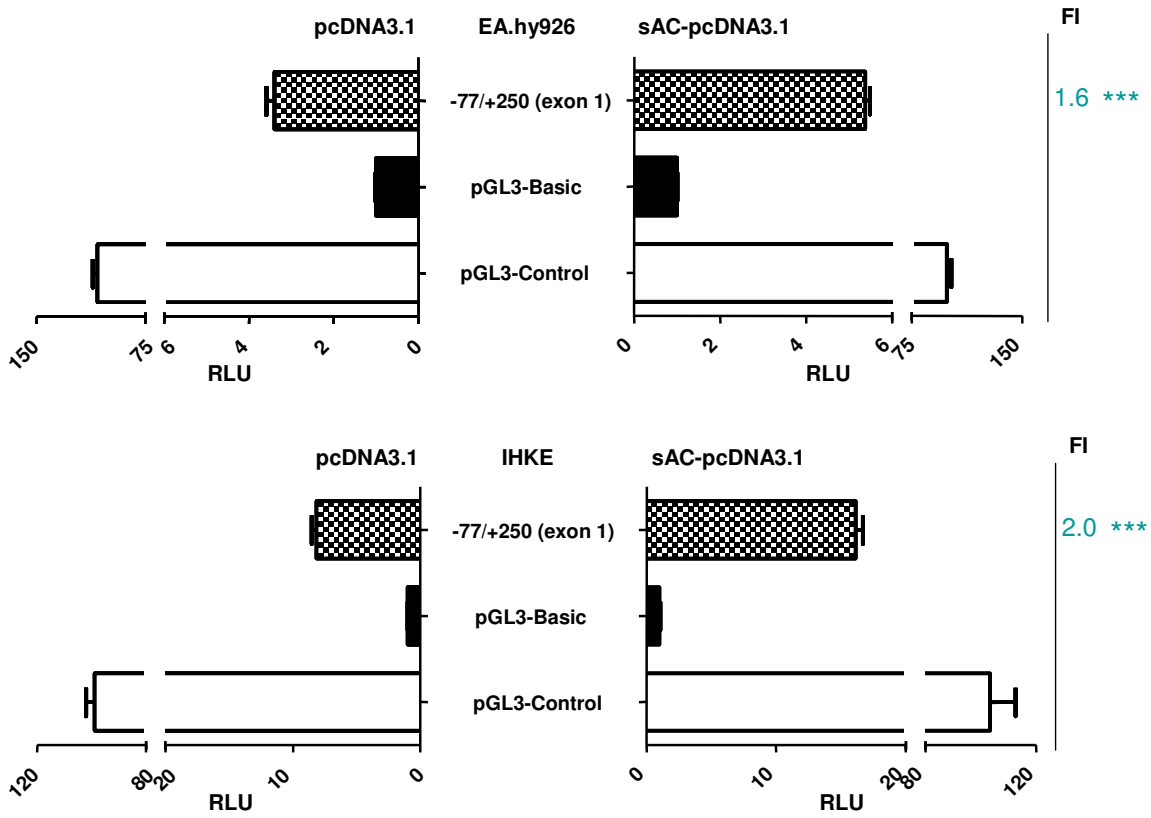


**Figure 30: sAC overexpression enhanced transcriptional activity of the *sAC* exon 1 promoter fragment**

A significant induction of the transcriptional activity of the *sAC* exon 1 construct was observed upon the overexpression of sAC in EA.hy926 (FI: 1.6) and IHKE (FI: 2.0) cells. The empty vector pcDNA3.1 served as shuttle control (left). Transcriptional activity of the pGL3-Basic vector was defined as 1. Fold induction (FI) of transcriptional activity of sAC overexpression relative to empty vector control is shown on the right. White bars display transcriptional activity of the pGL3-Control vector, black bars display basic transcriptional activity of the pGL3-Basic vector. *** $p<0.001$. Transcriptional activity was assessed as relative light units (RLUs).

### 4.2.6 Transcriptional regulation by C/EBPs

In addition to predictions for CREB binding sites, *in silico* analysis revealed several C/EBP binding clusters at positions -1175 to -1128, -470 to -454 and two binding sites between positions -77 and +250. C/EBPs build a family of transcription factors, which can homodimerize or heterodimerize for example with CREB (Park et al., 1993). Similar to CREB, C/EBPs can be regulated by phosphorylation, leading to activation or repression of transcription, dependent on the cellular context (Lynch et al., 2011). In this respect, the role of C/EBPs in the regulation of *sAC* gene expression was examined in EA.hy926 and IHKE cells (figure 31).

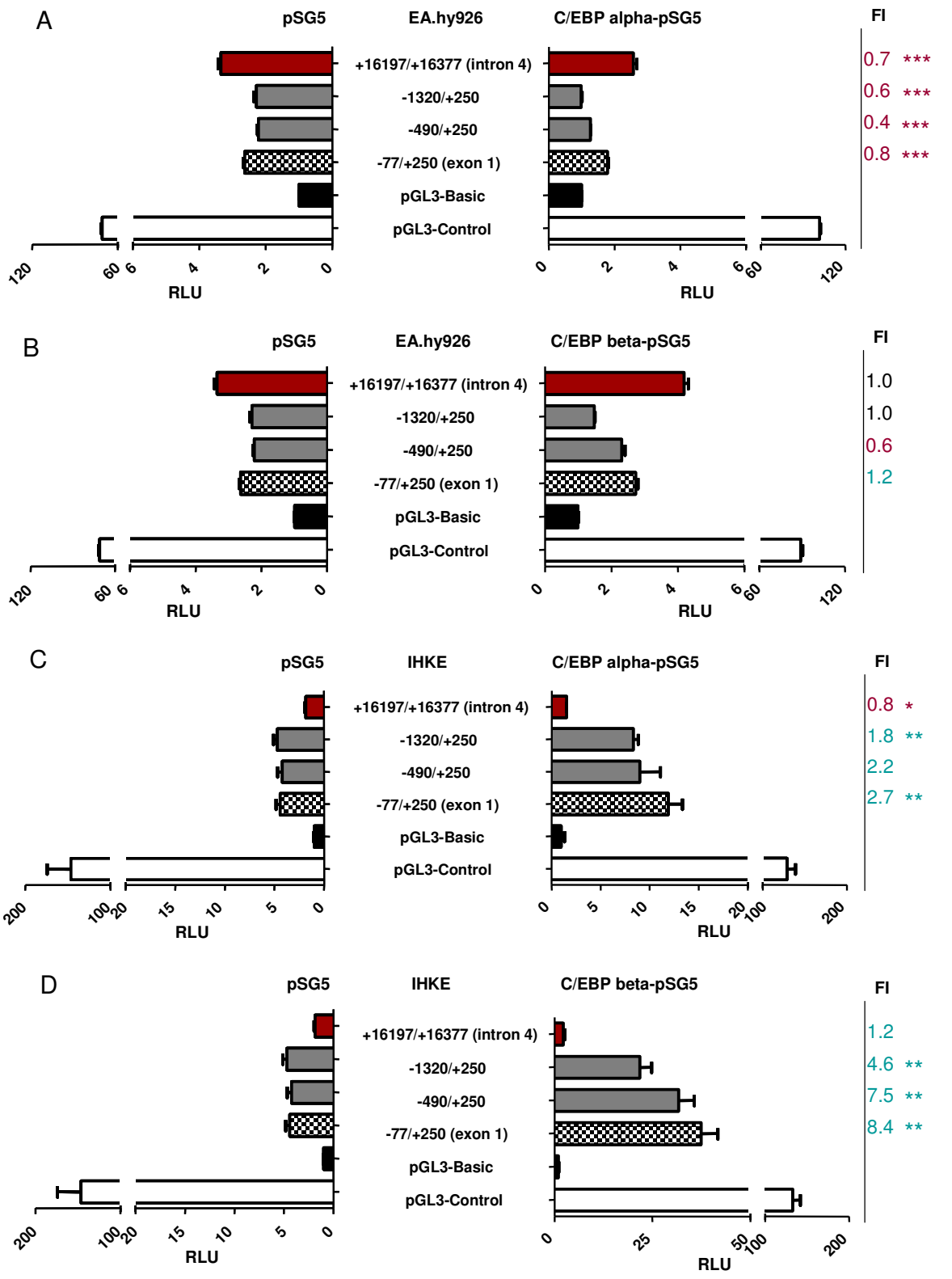


**Figure 31: Effect of C/EBP alpha and C/EBP beta on transcriptional activity of *sAC* promoter constructs**

The overexpression of C/EBP alpha in EA.hy926 cells (**A**) led to 0.4 to 0.8-fold decreased transcriptional activity of all tested *sAC* promoter constructs. The overexpression of C/EBP beta had no significant influence on transcriptional activity in EA.hy926 cells (**B**). In IHKE cells,

overexpression of C/EBP alpha (**C**) led to 1.8 to 2.7-fold increase of transcriptional activity of all constructs containing the 5´-UTR promoter (-77/+250, -490/+250, -1320/+250). A decrease of transcriptional activity was observed for the intron 4 construct. C/EBP beta overexpression increased the transcriptional activity of constructs harboring the 5´-UTR promoter (-77/+250, -490/+250, -1320/+250) 4.6 to 8.4-fold in IHKE cells, whereas no effect could be detected on the construct harboring intron 4 (**D**). The empty vector pSG5 served as shuttle control (left). Transcriptional activity of the pGL3-Basic vector was defined as 1. Fold induction (FI) of transcriptional activity of C/EBP alpha or C/EBP beta overexpression relative to empty vector control is shown on the right. White bars display transcriptional activity of the pGL3-Control vector, black bars display basic transcriptional activity of the pGL3-Basic vector. *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

In IHKE cells, transcriptional activity of constructs harboring the 5´-UTR promoter (-77/+250, -490/+250, -1320/+250), was significantly increased by overexpression of C/EBP alpha, whereas the strongest fold induction of 2.7 ($p<0.001$) was observed for the -77/+250 (exon 1) construct (figure 31C). Expression of C/EBP beta led to a stronger induction of transcriptional activity of constructs harboring the 5´-UTR promoter (-77/+250, -490/+250, -1320/+250) compared to C/EBP alpha, with the highest FI of 8.4 ($p<0.01$) for the -77/+250 (exon 1) construct (figure 31D). Overexpression of C/EBP alpha and beta did not alter transcriptional activity of the intron 4 construct. In contrast, in EA.hy926 cells the transcription factor C/EBP alpha led to a slight decrease (mean FI: 0.6125, $p<0.001$) of transcriptional activity of all tested *sAC* promoter constructs (figure 31A). Cotransfection with C/EBP beta did not significantly affect *sAC* promoter activity in EA.hy926 cells (figure 31B).

## 4.3 Determination of genetic variants within the *sAC* 5´-UTR promoter

### 4.3.1 Polymorphic promoter structure

Genetic variants, which reside in the promoter region, can influence transcriptional activity (Brand-Herrmann et al., 2004). After the determination of *sAC* regulatory regions, they were analyzed to find if genetic variants reside in the 5´-flanking region of the *sAC* gene. DNA of 60 CVD patients from the Münster MolProMD Study were screened. 4,000 bp were sequenced, since the 3´-end of the neighboring gene, coding for *brain protein 44* (*BRP44*) is located 4,000 bp upstream of the 5´-end of the *sAC* gene. We identified seven genetic variants, three of which were newly detected (blue). In addition, three variants

were in a nearly complete linkage disequilibrium (LD) determined in a LD plot, shown in red rectangles (figure 32).

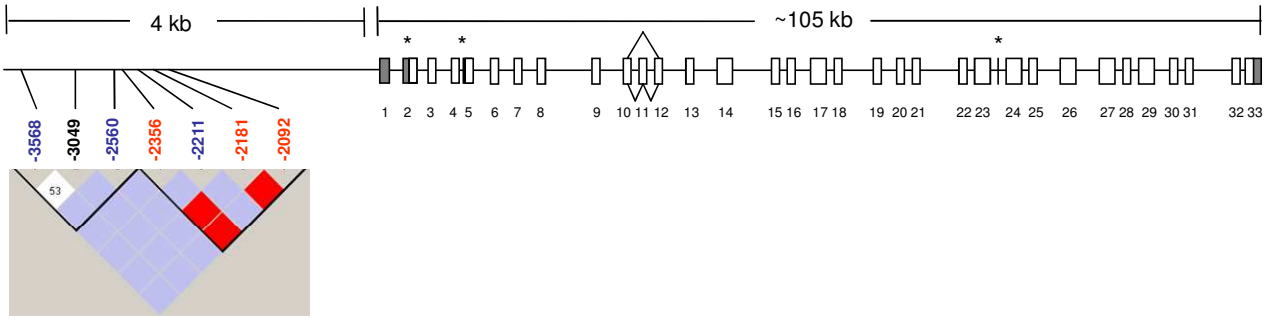


**Figure 32: Polymorphic promoter structure of *sAC***

The *sAC* gene spans 105 kb and is located on chromosome 1. Four kb of the 5´-flanking region were sequenced in 60 patients with cardiovascular disease and seven genetic variants were identified, three of which were newly detected (dark blue). The LD plot was performed using Haploview. The second block of the LD Plot comprises three of the variants at positions -2356, -2181 and -2092 in a nearly complete linkage disequilibrium (red boxes). The untranslated regions are shown as grey boxes, alternatively transcribed regions are shown in black, asterisks indicate putative alternative translational start sites.

The three genetic variants at position -2356, -2181, and -2092 cosegregate and generate two molecular haplotypes: Haplotype 1 (MolHap1) [$Ins^{-2356}$ – $C^{-2181}$ – $T^{-2092}$] with the major allele combination and haplotype 2 (MolHap2) [$Del^{-2356}$ – $T^{-2181}$ – $G^{-2092}$] with the minor allele combination (figure 33).

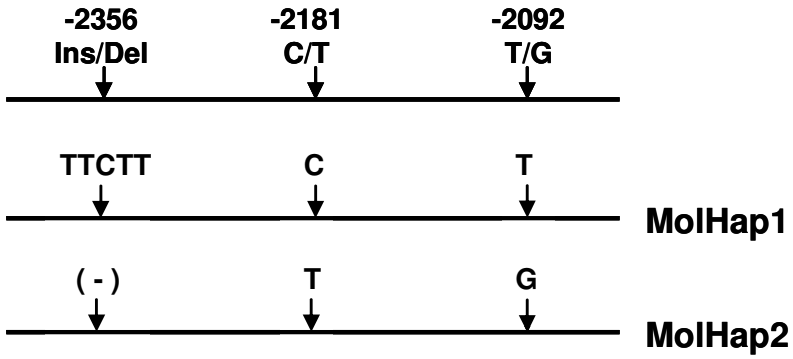


**Figure 33: Molecular haplotypes within the human *sAC* promoter region**

The variants at positions -2356, -2181 and -2092 are in an almost complete linkage disequilibrium and generate two molecular haplotypes MolHap1: [$Ins^{-2356}$ – $C^{-2181}$ – $T^{-2092}$] and MolHap2: [$Del^{-2356}$ – $T^{-2181}$ – $G^{-2092}$].

### 4.3.2 *In silico* analysis of identified genetic variants

Both haplotype-sequences were analyzed for potential transcription factor binding sites *in silico* using the net based program AliBaba 2.1 (http://www.gene-regulation.com/pub/databases.html). This program accesses the transcription factor binding site database Transfac 7.0. (figure 34).

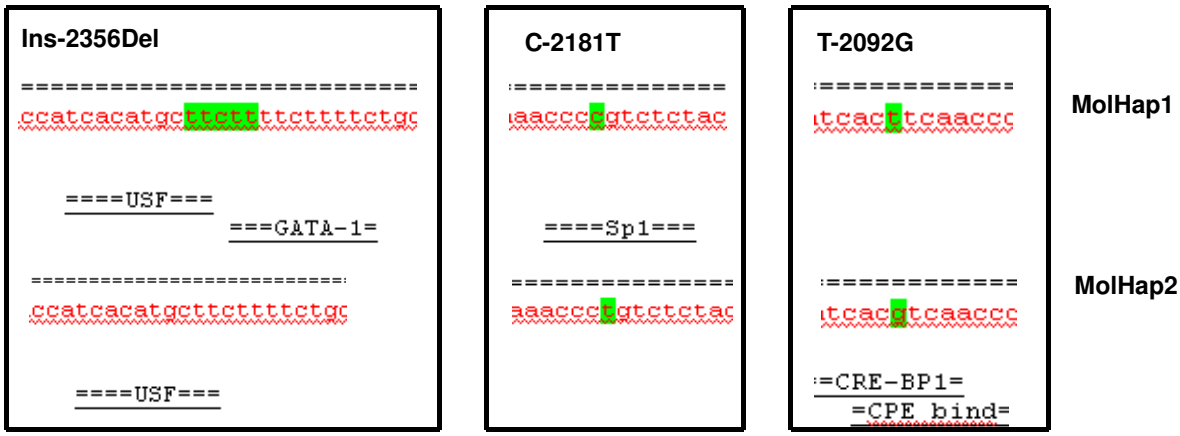


**Figure 34: Putative transcription factor binding sites of MolHap1 and MolHap2**
Sequence of molecular haplotypes MolHap1 [Ins$^{-2356}$ – C$^{-2181}$ – T$^{-2092}$] and MolHap2 [Del$^{-2356}$ – T$^{-2181}$ – G$^{-2092}$] were analyzed using AliBaba 2.1. Positions of variants are marked in green. Predicted transcription factors are displayed beneath their recognition site. USF: upstream transcription factor; GATA 1: transcription factor with affinity for the sequence GATA; Sp1: specificity protein 1; CRE-BP1: CREB, cAMP response element binding protein 1; CPE bind: cytoplasmatic polyadenylation element binding factor.

Predicted binding patterns of potential transcription factors differed between the haplotypes (figure 34). For both, MolHap1 and MolHap2, the -2356 Ins/Del represented a putative binding site for upstream transcription factor (USF), which is linked to the lipid metabolism (Wu et al., 2010). Insertion of the sequence TTCTT at position -2356 resulted in the prediction of binding of the erythrocyte-specific transcription factor GATA-1. Introduction of the -2181C allele created a putative binding site for SP1, whereas the minor allele lacked this binding site. The transcription factor Sp1 belongs to a Cys2His2 zinc finger domain family, which is ubiquitously expressed (Solomon et al., 2008). No binding was predicted for the -2092T allele, but the -2092G allele showed a binding site for CREB and the CPE binding factor, which mediates polyadenylation of 3´-untranslated regions of mRNA (Hake and Richter, 1994).

### 4.3.3 Transcriptional activity of MolHaps

We analyzed transcription activity of MolHap1 and MolHap2 using reporter gene assays. Therefore, we cloned the sequence spanning Ins/Del-2356, C/T-2181, and T/G-2092 into the reporter gene vector pGL3-Promoter, which contains a minimal SV40 in front of the *luziferase* gene, to compensate for the lack of the core promoter located in *sAC* exon 1. Subsequently the constructs were transfected in HEK293T cells. MolHap2 showed a significantly ($p<0.01$) higher transcriptional activity compared to MolHap1 (figure 35).

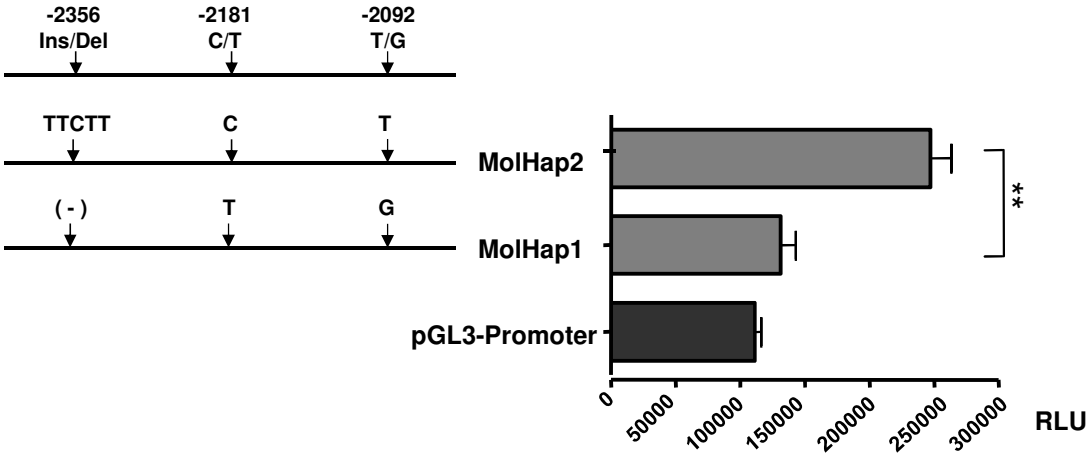


**Figure 35: Transcriptional activity of MolHap1 and MolHap2**

A 406 bp fragment of the *sAC* promoter comprising the MolHap1 [$Ins^{-2356} – C^{-2181} – T^{-2092}$] or the MolHap2 [$Del^{-2356} – T^{-2181} – G^{-2092}$] sequence was cloned into the pGL3-Promoter vector. MolHap2 showed an ~2-fold higher transcriptional activity compared to MolHap1. Dark grey bars display the pGL3-Promoter vector transcriptional activity. ** $p<0.01$. Transcriptional activity was assessed as relative light units (RLUs).

### 4.3.4 Allele-specific transcriptional regulation by the transcription factor SP1

In contrast to MolHap2, MolHap1 showed a binding site for the transcription factor SP1, which plays an important role in transcription initiation of TATA-less promoters (Hilton and Wang, 2003). Overexpression of SP1 was performed to detect its effect on transcriptional activity of MolHap1 and MolHap2 in HEK293T cells (figure 36).

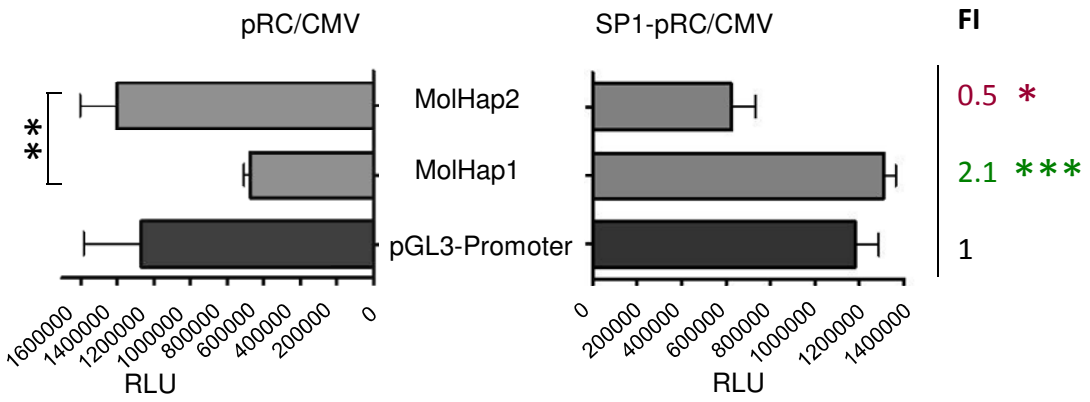


**Figure 36: Overexpression of SP1 alters transcriptional activity of MolHap1 and MolHap2**
Transient cotransfection of constructs harboring MolHap1 and MolHap2 were performed in HEK293T cells. pRC/CMV (left) served as the empty shuttle control. SP1 overexpression upregulated the transcriptional activity of MolHap1 2.1-fold and decreased the transcriptional activity of MolHap2 by 50%. Light grey bars display the *sAC* constructs transcriptional activity; dark grey bars display the pGL3-Promoter vector transcriptional activity. *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Transcriptional activity was assessed as relative light units (RLUs).

MolHap1, harboring a potential binding site for SP1, showed a 2.1-fold higher transcriptional activity upon overexpression of SP1 ($p<0.001$). The transcriptional activity of MolHap2 decreased by 0.5-fold due to SP1 overexpression ($p<0.05$, figure 36).

# 5 Discussion

In the present study, effects of sAC on aldosterone-regulated gene expression were analyzed. We examined the physical interaction of sAC and CREB in Co-IPs and assessed the constitution of a transcriptional module of sAC and CREB in ChIP assays in endothelial and kidney cells. In addition, we analyzed the impact of sAC on CRE motifs using reporter gene assays and on the expression of genes involved in aldosterone signaling using reverse transcription real time PCR. Since *sAC* expression itself is considerably modulated by aldosterone, we determined functional *sAC* promoter sequences and analyzed this significance in aldosterone-mediated *sAC* regulation. Therefore, reporter gene analyses were implemented to identify active promoter portions and the impact of *trans*-acting factors of *sAC* in endothelial and kidney cells, as well as the impact of genetic variants within the 5´-flanking region on transcriptional activity.

## 5.1 sAC acts as a co-factor of CREB

Recent studies suggested that sAC is an important factor involved in CREB phosphorylation. The group of Zippin (Zippin et al., 2004) reported elevated $HCO_3^-$ levels leading to sAC activation, subsequently increasing CREB phosphorylation. Accordingly, it has been shown that phosphorylation on serine 133 is a prerequisite for the function of CREB as transcriptional activator (Al-Tawashi et al., 2012). Using Co-IP, we demonstrated the existence of a CREB/sAC complex, which underlies the importance of sAC in CREB-mediated transcriptional activation.

Further, our ChIP assays in EA.hy926 and IHKE cells revealed that this CREB/sAC complex interacts with CRE sequences *in vivo*. The confirmation of binding of CREB-p together with sAC using ChIP assay remains complicated, because sometimes phosphatase inhibition is not efficient enough to detect phosphorylated proteins. Application of the sAC-specific inhibitor KH7 in transient transfections using a CRE reporter vector suggested that sAC is a major factor involved in CRE-mediated transcriptional activation. To eliminate KH7 effects on cellular cAMP production by sAC, we artificially elevated cellular cAMP levels using 8-Br-cAMP. Notably, external cAMP could not compensate inhibition of CRE-mediated transcriptional activity by KH7. Together with the findings of Zippin et al. (Zippin et al., 2004) these results suggest that sAC facilitates the local demand of cAMP, required for CREB to act as transcriptional activator directly in the nucleus. Most recently, several groups independently reported (Bacskai

et al., 1993; Rich et al., 2000; Rich et al., 2001; Zaccolo and Pozzan, 2002) that cAMP generated by tmACs remains close to the site of synthesis. Cytosolic cAMP generated by tmACs activates PKA, leading to translocation of PKA catalytic subunits into the nucleus and to CREB phosphorylation (Al-Tawashi et al., 2012). Subsequently, CREB-p recruits the co-activators CBP or p300, which both possess histone acetyl transferase activity resulting in chromatin remodeling, transcription factor binding, and transcription initiation (Wurm et al., 2012).

There is evidence that an additional signaling pathway for CREB phosphorylation different from cytoplasmatic PKA signaling exist. In fact, the local demand of cAMP was shown to be generated by the sAC in special microdomains. Interestingly, appearance of the PKA holoenzyme in nuclei was shown, modifying the existing dogma of cAMP-PKA signaling in the nucleus (Sample et al., 2012).

Other protein kinases localized directly in the nucleus, such as the “mitogen and stress activated protein kinase 1” (MSK1) and the “calcium-calmodulin-dependent protein kinase IV” (CaMK IV, Bok et al., 2007) were shown to be involved in CREB phosphorylation (Vermeulen et al., 2003; Ko et al., 2005). Therefore, MSK1 and CaMK IV may act as effectors for CREB phosphorylation based on sAC-generated cAMP. Interestingly, sAC has been reported to be induced by oxidative stress (Acin-Perez et al., 2009), such as MSK1. In addition, sAC can be induced by elevated intracellular calcium levels (Han et al., 2005), such as CaMK IV demonstrating that these factors are involved in the same metabolic pathways.

Our results suggest that besides the classical signaling pathway leading to CREB phosphorylation via tmACs, another important signaling pathway involving sAC as a transcriptional co-activator of CREB, and nuclear localized protein kinases, exists.

## 5.2 sAC is involved in the genomic aldosterone pathway

To elucidate the role of sAC as a potential co-activator of CREB, we investigated the impact of sAC on gene expression regulation of selected target genes. Since an impact of sAC on aldosterone-mediated $Na^+$ currents was shown (Hallows et al., 2009), which could be due to an influence of sAC on the expression of genes involved in aldosterone signaling, we focused on genes which play central roles in aldosterone signaling. Therefore, we analyzed the expression of the *MR*, *$Na^+/K^+$-ATPase alpha*, *$Na^+/K^+$-ATPase beta*, and *ENaC alpha* in the endothelial cell line EA.hy926 by reverse transcription real time PCR. An impact of CREB on transcriptional activation of these genes has been reported (Ahmad and Medford, 1995; Listwak et al., 1996; Dagenais et al., 2001;

Matlhagela et al., 2005). We demonstrated an essential role of sAC on the expression of all these genes, since the expression decreased after sAC inhibition. Furthermore, we were able to show that the aldosterone-induced expression of *$Na^+/K^+$-ATPase beta* and *ENaC alpha* was blocked by inhibition of sAC, suggesting an interaction between the aldosterone signal transduction system and sAC in gene expression regulation.

In contrast to our results, the group of Verhovez (Verhovez et al., 2012) did not detect effects of aldosterone (10 nM aldosterone for 10, 18 or 24 h) on any transcript in human umbilical vein endothelial cells and human coronary artery endothelial cells using a gene expression microarray. Notably, the authors used a firefly luciferase based assay to investigate the functionality of the MR in their cell lines and could not provide evidence for a fully functional MR. In contrast, we used endothelial EA.hy926 cells, in which a functional MR was already shown to exist (Pojoga et al., 2012). In addition, an intracellular signaling cascade that precedes the classical genomic response to aldosterone exists in EA.hy926 cells (Wildling et al., 2009).

The MR-mediated aldosterone effects are referred as genomic effects (Bonvalet, 1998). Genomic signaling of aldosterone involves aldosterone binding to the intracellular MR and subsequent translocation of the MR complex into the nucleus. Accordingly, aldosterone is considered to be the main ligand of the MR, which is highly expressed in the kidney (Krozowski et al., 1989; Lombès et al., 1990), but also in other tissues, where RAAS signaling occurs, such as the vascular endothelium (Edwards et al., 1988; Funder et al., 1988). This MR-mediated transcriptional regulation exerts effects which are observed several hours after stimulation. In addition, rapid and nongenomic effects of aldosterone, which occurs within minutes, have been reported (Chai et al., 2005).

*MR* expression is regulated via two distinct promoter regions, called P1 and P2 (Zennaro et al., 1996) giving rise to a tissue-specific regulation of aldosterone. The developmental-specific promoter P2 was shown to be induced by aldosterone in a dose-dependent manner, whereas promoter P1, which was shown to be active in all MR expressing tissues, was unaffected by aldosterone stimulation (Viengchareun et al., 2007).

In our study, we detected a moderate, but still significant repressing effect of aldosterone on the *MR* expression in endothelial cells. This observation could be based on epigenetic modifications of the *MR* promoter P2 in EA.hy926 cells resulting in a transcriptional inactive heterochromatin structure. Interestingly, an interaction of the MR with the co-repressor “nuclear receptor co-repressor 2” (NCOR2) on the promoter region P2 (Wang et al., 2004) has been reported. NCOR2, also known as “silencing mediator of retinoic acid and thyroid hormone receptor” (SMRT), contains multiple autonomous repression domains (Chen and Evans, 1995), which facilitate the recruitment of histone deacetylase to the promoter leading to epigenetic silencing of promoter P2 (Nagy et al., 1997).

In kidney collecting duct cells, the most important aldosterone effectors are ENaC and $Na^+/K^+$-ATPase, which mediate the aldosterone-induced increase of $Na^+$ currents across the membrane (Summa et al., 2001). ENaC consists of three different subunits: ENaC alpha, ENaC beta and ENaC gamma, each of which comprises two transmembrane helices and one extracellular loop (Loffing and Schild, 2005). Amiloride is an ENaC-specific antagonist, which acts independently of the presence of aldosterone (Kusche-Vihrog et al., 2008). It is known that corticosteroids are important regulators of the amiloride-sensitive $Na^+$ transport in the collecting duct, distal colon, and in airway epithelia (Verity et al., 2001). Interestingly, long-term treatment with aldosterone via the genomic pathway has been shown to increase the expression of ENaC alpha (Verity et al., 2001; Mick et al., 2001), while ENaC beta and ENaC gamma are constitutively expressed in the kidney (Masilamani et al., 1999; Loffing et al., 2000; Loffing et al., 2001). Corresponding to ENaC alpha subunit regulation in the kidney, we observed a slight activation upon aldosterone stimulation on ENaC alpha expression in endothelial cells.

Only recently, ENaC expression has been reported to involve the circadian clock protein Period 1 (Per1). Aldosterone itself has been shown to take part in circadian regulation and can be used to reset circadian clocks in cultured cells. The extent of target gene activation by aldosterone furthermore depends on the circadian status of the cell (Gumz et al., 2010). Since we analyzed aldosterone effects on target genes after 24 hours, our experimental setup maybe not be suitable to detect aldosterone effects on ENaC alpha expression. Accordingly, cells in culture would perhaps need a circadian clocks reset (Kaeffer and Pardini, 2005) and expression levels should be determined at different time points (Storch et al, 2002) to detect potential aldosterone effects on genes under the control of circadian regulation.

The integral membrane $Na^+/K^+$-ATPase holoenzyme is composed of three subunits, a large catalytic subunit (alpha), a smaller glycoprotein subunit (beta), responsible for formation and structural integrity of the $Na^+/K^+$-ATPase holoenzyme, and a modulatory gamma subunit (McDonough et al., 1990). We detected an enhancing effect of aldosterone on the expression of *$Na^+/K^+$-ATPase beta* in EA.hy926 cells, supporting the results obtained by the group of Derfoul (Derfoul et al., 1998) in african green monkey kidney cells (CV-1) and by the group of Kolla (Kolla et al., 1999) in transformed african green monkey kidney fibroblast cells (COS7). Both groups were able to show an activating effect of aldosterone on *$Na^+/K^+$-ATPase beta* gene expression. In addition, Kolla et al. (Kolla et al., 1999) demonstrated an activating effect on the expression of *$Na^+/K^+$-ATPase alpha* in COS7, which is in contrast to our findings in EA.hy926 cells, where no significant influence of aldosterone on *$Na^+/K^+$-ATPase alpha* expression could be detected. It is conceivable that aldosterone has different, cell type-specific effects on the

regulation of the $Na^+/K^+$-ATPase holoenzyme subunits. Aldosterone effects on the expression of *$Na^+/K^+$-ATPase beta* might influence formation and structural integrity of the holoenzyme in endothelial cells, while the aldosterone-mediated decrease of the catalytic alpha subunit expression directly reduce the $Na^+/K^+$-ATPase enzyme activity in the kidney.

### 5.3 Different transcriptional regulation of *sAC* in kidney and endothelial cells

Since we were able to show that aldosterone enhances sAC mRNA and protein expression, we analyzed how these effects are mediated on the transcriptional level. Until now, the structure and regulation of the sAC protein is not completely understood. Several studies suggested the appearance of diverse isoforms in human cells (Geng et al., 2005; Schmid et al. 2010).

We provided evidence for the existence of at least two sAC isoforms (~80 kDa and ~50 kDa), which are expressed cell type-specifically in kidney and endothelial cells. Both isoforms have also been reported by Geng et al. (Geng et al., 2005) in HEK293 and Caco-2 cells. In endothelial cells, the 80 kDa isoform was located exclusively in the cytoplasm, but was detected in nuclear extracts and whole cell lysates of the kidney cell line. This specific isoform was also shown to be located in human airway epithelial cells (Schmid et al., 2010). The 50 kDa isoform, which potentially represents a more active splice variant of sAC (Chaloupka et al., 2006), was ubiquitously expressed in both cell lines. Notably, we also detected another 70 kDa band exclusively in endothelial cells, which has not yet been reported.

These isoforms could arise from alternative splicing or alternative translational start sites. The *sAC* gene spans 105 kb and contains several large intronic regions, which potentially contain transcriptionally active regions. An alternative translational start site is located in exon 5 (Geng et al., 2005). The differential usage of individual translational start sites is potentially enabled by alternative located promoter regions. The concept of alternative promoter systems thereby might serve for tissue- or differentiation-specific regulation (Liu, 2010) and might result in different isoform expression.

To define transcriptionally active regions in the *sAC* promoter, serial deletion constructs of the 5´-flanking region including exon 1, which harbors a part of the 5´-UTR, were generated and transiently transfected into two kidney cell lines (IHKE, HEK293T) and an endothelial cell line (EA.hy926). We define the location of the *sAC* core promoter to the untranslated exon 1, which likely represents the region required for assembling of the PIC and Pol II recruitment, since deletion of this region led to total abrogation of *sAC* transcriptional activity of all promoter deletion constructs. We also detected a cell type-

specific effect in *sAC* gene expression regulation. In kidney cells, the strongest transcriptional activity was detected for the construct -1320/+250, whereas in the endothelial cells the core promoter in exon 1 showed the highest transcriptional activity. We detected an additional region with significant transcriptional activity, represented by the deletion construct -3528/+250, in the immortalized kidney cell line IHKE, in contrast to the embryonic kidney cells HEK293T where transcriptional activity appears more restricted. This observation points to a differentiation-specific regulation of the *sAC,* which is probably required since the cAMP demand needs to be regulated during differentiation.
Additionally to the identified 5´-promoter located upstream of the translational start site in exon 2, intronic *cis*-active elements upstream of the alternative translational start site in exon 5 were detected. This alternative promoter region was designated as intronic promoter. Both promoter regions seem to be regulated in a cell type-specific manner, since the 5´-promoter showed stronger transcriptional activity compared to the intronic promoter in the kidney cells, while the intronic promoter displayed the strongest transcriptional activity in the endothelial cells. This distinctive feature may be the molecular basis for an orchestrated expression of different isoforms in the kidney and the vascular endothelium. An alternative promoter usage which led to tissue-specific isoform expression has been shown e.g. for the estrogen receptor alpha by the group of Ishii et al. (Ishii et al., 2010). Alternative promoter utilization in distinct cell types is reasonable in cell type-specific regulation via *trans*-active factors (Mullen et al., 2011).

## 5.4 sAC expression regulation

### 5.4.1 Modulation of sAC expression by cAMP

Combinations of different *trans*-acting factors, regulatory elements, and co-activating factors lead to cell type-specific transcriptional activity (Carninci et al., 2006).
Since sAC is an important component of cAMP signaling, it seems obvious that transcriptional regulation is modulated by cAMP-dependent factors. The most important cAMP-dependent transcription factor is CREB. As already discussed, CREB was identified as a target of sAC signaling (chapter 5.1). Zhang et al. (Zhang et al., 2005) used ChIP-on-chip analysis to determine the existence of a CRE site within exon 1 at position +138 of the *sAC* gene, a region which we designated as *sAC* core promoter (chapter 5.4.1).
We were able to identify CREB as a transcriptional activator of *sAC* by reporter gene and ChIP assays. Our results suggest that sAC may function as a co-activator of the

transcription factor CREB. sAC may also be involved in a feedback regulation, acting on its own promoter, since we observed similar effects for sAC overexpression, compared to CREB overexpression.

CREB is known to form homo- or heterodimers, the heterodimerization capacity of CREB being restricted to other proteins without a leucine zipper domain, with the exception of C/EBP beta (Park et al., 1993). C/EBP beta belongs to a family of transcription factors composed of six members, called C/EBP alpha to C/EBP zeta. C/EBP proteins interact with the CCAAT motif (Zuo et al., 2006), of which clusters are present for example in the *sAC* core promoter region and the upstream promoter at positions -1175 to -1128, -470 to -454 and -77 to +250.

We tested the regulative capacity of different C/EBP family members in coexpression experiments and identified C/EBP beta as a strong activator of the *sAC* 5´-promoter in kidney cells. In endothelial cells, cotransfection experiments with C/EBP beta only showed a slight increase in transcriptional activity of 5´-promoter constructs.

Overexpression of C/EBP alpha showed controversial effects in both cell lines. In IHKE cells, the transcriptional activity of deletion constructs harboring the 5´-promoter was significantly increased by C/EBP alpha overexpression, whereas the transcriptional activity of these constructs was decreased in EA.hy926 cells.

Neither C/EBP beta nor C/EBP alpha showed an effect on transcriptional activity with respect to the *sAC* intronic promoter. This leads to the suggestion that C/EBP alpha and beta serve as cell type-specific regulators of the *sAC* 5´-promoter.

Once they bound to DNA via their leucine zipper domain, C/EBPs can recruit co-activators such as CBP (Kovács et al., 2003), which can open the chromatin structure and recruit basal transcription factors as it is known for CREB (Kim et al., 2000). Even though all C/EBP isoforms share substantial sequence identity > 90 % in their leucine zipper domain, they exert different cell type-specific functions (Lekstrom-Himes and Xanthopoulos, 1998). C/EBP alpha is responsible for both adipogenesis and normal adipocyte function (Wang et al., 1995). C/EBP beta is involved in the regulation of genes participating in immune and inflammatory responses (Zhang et al., 2010). C/EBP family members can act as transcriptional activators as well as transcriptional repressors depending on their dimerization partners (McFie et al., 2006), suggesting an interaction of C/EBP alpha with a co-activator in IHKE cells and an interaction with a co-repressor on the 5´-promoter in EA.hy926 cells. The function of C/EBP family members is modulated via phosphorylation (Buck and Chojkier, 2007). Therefore, C/EBPs are potential phosphorylation targets in sAC signaling and the regulation of sAC expression via C/EBPs points to a feedback regulation of the *sAC* promoter, which is in good accordance with our CREB overexpression results.

### 5.4.2 Modulation of sAC expression by aldosterone

In addition to transcription factors of cAMP-mediated pathways, transcription factors involved in aldosterone signaling also seem to be potential transcriptional regulators of *sAC*. Recently, sAC has been shown to play a pivotal role in aldosterone signaling, since inhibition of sAC blocked the aldosterone-mediated $Na^+$ currents in the kidney (Hallows et al., 2009). In endothelial cells, aldosterone plays a role in the pathogenesis of endothelial dysfunction, since an aldosterone-mediated activation of the RAAS increases blood pressure and plays an important role in end-organ damage (Xavier et al., 2008).

In our analysis, aldosterone predominantly enhanced the transcriptional activity of the intronic *sAC* promoter in endothelial cells as well as in kidney cells, in contrast to cAMP-dependent factors, which act on the untranslated region of the 5´-promoter. This aldosterone-stimulated transcriptional activity of the intronic *sAC* promoter results consequently in increased sAC mRNA and protein expression levels.

We demonstrated differential regulation of sAC expression in kidney and endothelial cells by two independent promoter regions (5´-promoter, intronic promoter), located in close vicinity to two different translational start sites (exon 2, exon 5, Acc#.: NM_018417.4), which possibly explain the different isoform expression in these cells.

## 5.5 Genetic variants influence *sAC* promoter activity

In the current study, we screened a 4,000 bp region upstream of the TSS and were able to show that the *sAC* 5´-flanking region is polymorphic. Genetic variants, which are located nearby the TSS, have been shown to influence gene expression (Telgmann et al., 2009). In addition, genetic variants within *cis*-active regions may change the binding pattern of *trans*-active elements, by alteration of their binding affinity to DNA (Hagedorn et al., 2009; Johnson et al., 2012).

We identified seven genetic variants within 4,000 bp of the 5´-flanking region of *sAC* in 60 MolProMD patients, including three novel SNPs. All identified genetic variants are located upstream of position -2092. This result suggests that both *cis*- and *trans*-acting elements downstream of position -2092 might be highly conserved, because of their essential function for transcriptional activity of the *sAC* promoter. Three genetic variants were in a nearly complete linkage disequilibrium generating two molecular haplotypes, MolHap1 and MolHap2. Alleles, which reside in a high linkage disequilibrium often execute a common function.

MolHap2 showed a ~2-fold higher transcriptional activity than MolHap1, which could be due to altered interaction with *trans*-acting factors. The T allele at position -2181 disrupts a potential SP1 binding site. In accordance with the *in silico* analysis, we showed that SP1 activates the transcriptional activity of MolHap1 containing the SP1 binding site, but suppresses transcriptional activity of MolHap2. SP1 acts as an important transcriptional activator of TATA-less promoters, while it binds with its zinc fingers to GC-rich promoter regions (Hilton and Wang, 2003). Even though the GC content of the analyzed region is only ~48%, the 5´-flanking region of the *sAC* gene lacks a TATA motif, but several SP1 binding sites and is, therefore, a potential target of SP1-initiated transcription.
These results suggests that in some CVD patients a dysregulation of *sAC* transcriptional activity could occur, which possibly leads to dysregulation of aldosterone-mediated gene expression (chapter 5.2). This may play a role in the differential development of CVD.

## 5.6 Conclusion

It is known, that aldosterone contributes to the development of hypertension (Funder, 2011). In fact, high serum plasma aldosterone levels may predispose normotensive subjects to the risk for the development of incident hypertension (Vasan et al., 2004). In the current study, we were able to demonstrate that sAC is involved in genomic aldosterone signaling. Our results also indicate that sAC, acting as a co-factor of CREB, influences aldosterone-mediated gene expression and potentially affects $Na^+$ currents across the cell membrane in the kidney and the endothelium.
Based on this, sAC may represent a new drug target for disorders in which aldosterone signaling is impaired. To balance a deficiency of aldosterone synthesis, overexpression of sAC could balance the effect on aldosterone-induced gene expression. If a pathophysiological aldosterone overexpression occurs, e.g. in the case of hyperaldosteronism (Funder, 2011), sAC inhibition could potentially downregulate the aldosterone-induced gene expression.
Hypertension is a complex multifactorial trait, in which the individual genetic predisposition, in combination with environmental factors and lifestyle, plays an essential role. Consequently, the risk to develop hypertension can be influenced in a distinct range by a healthy lifestyle, physical exercise, low salt intake and less mental stress, constricted by the genetic background as an uninfluenceable factor (Staessen et al., 2003).
We were able to show that the *sAC* gene promoter is polymorphic in CVD patients, which leads to altered sAC expression. The analysis of genetic variants, their functional relevance and distribution in the population, may lead to a better understanding of the

pathophysiology of hypertension and development of CVD. Genome-wide gene expression analyzes, in combination with whole genome sequencing could help to determine the interactions between genetic and environmental factors and to obtain a more detailed insight into human gene regulation and the impact of the individual variable genome structure. The development of a genetic risk profile for hypertension could contribute to disease prevention and might help to develop individually optimized therapeutic treatment strategies.

# 6 Outlook

sAC has been identified as a co-activator of CREB and an effector of aldosterone-mediated gene expression. We demonstrated interaction of sAC with CREB on CRE sites using ChIP analyzes. The underlying molecular mechanism of sAC binding to DNA has to be evaluated in detail. sAC and CREB RNA interference experiments and subsequently ChIP analyzes could be used to test if sAC binds to DNA independently of CREB. Identification of further interaction partners of sAC by Co-IP would lead to new insights in sAC signaling.

Another part of this work was the functional characterization of the human *sAC* promoter and identification of regulatory regions, which were shown to depend on the cell type and differentiation status. Two autonomous promoter regions were identified to be differentially regulated by cAMP and aldosterone signaling. In addition, different isoform expression of sAC with various distribution in the nucleus and the cytoplasm had been identified in endothelial and kidney cells, which may originate from alternative and independent TSSs. 5´-RACE experiments should be performed to identify potentially unknown TSSs in addition to the TSS in exon 1 (Acc#.: NM_018417.4). The sequence of exon 5 of the *sAC* gene reveals a putative alternative translational start site (Geng et al., 2005), which could lead to the different isoform expression. The exact peptide sequences of these isoforms are unknown and could be examined using mass spectrometry or peptide sequencing. Functional assays would determine the catalytic activity of these isoforms.

To improve understanding of how sAC levels change in physiological and pathological conditions, a more comprehensive study of the transcriptional regulation of *sAC* is needed. A knockout mouse model of sAC exists, which demonstrates the consequences of a lack of the sAC protein. Female knockout mice exhibited increased cholesterol and triglyceride levels and both sexes exhibited a slight increase in the heart rate (Farrell et al., 2008).

We screened the 5´-flanking region of *sAC* in 60 CVD patients and identified genetic variants, which change transcriptional activity of the *sAC* promoter. To elucidate the role of sAC as a genetic factor that influences the predisposition to develop hypertension a larger study cohort should be genotyped for *sAC* promoter variants and related to hypertension-associated phenotypes.

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# 8 Appendix

**Table A1: Oligonucleotides used for sequential analysis of the *sAC* promoter**

| Description | Sequence 5' to 3' | Ref. Acc# |
|---|---|---|
| sAC seq 1 SS | CCATTTGTCCGTAATAAACCA | NM_018417.3 |
| sAC seq 1 AS | TTTTGAGACAGCGTCTCCCT | NM_018417.3 |
| sAC seq 2 SS | CCTGAATTTGAATCCCAGCTC | NM_018417.3 |
| sAC seq 2 AS | TTCAGTGGGAGTGCAGAGC | NM_018417.3 |
| sAC seq 3 SS | GCTCCGTTGTGAGGAGAGAGAC | NM_018417.3 |
| sAC seq 3 AS | GGTCTCCTAAGCCCCTCTTG | NM_018417.3 |
| sAC seq 4 SS | TTTATTAAAAATTTATGTGAGCTTGG | NM_018417.3 |
| sAC seq 4 AS | CTTTGCACTCCAGCCTCG | NM_018417.3 |
| sAC seq 5 SS | GTTGCAGTGAGCTGAGATCG | NM_018417.3 |
| sAC seq 5 AS | TAACACAGTGCCTGGTCCAC | NM_018417.3 |
| sAC seq 6 SS | TTCAAGAGAGCAAAATGAGGATAAT | NM_018417.3 |
| sAC seq 6 AS | CCTATTTGCACGGTTTCTGAA | NM_018417.3 |
| sAC seq 7 SS | AATTGAAGGTAGACCCAGAAAGT | NM_018417.3 |
| sAC seq 7 AS | GGTTTCCACAACTCACACCA | NM_018417.3 |
| sAC seq 8 SS | AGCTCTCCCTAAGGGGATTG | NM_018417.3 |
| sAC seq 8 AS | TTCAAACAAAAATTTACCTCACAAA | NM_018417.3 |
| sAC seq 9 SS | CCAGTCAGAAAGGGCAGGTA | NM_018417.3 |
| sAC seq 9 AS | AGAATAATGTCACCCGGCCT | NM_018417.3 |
| sAC seq 10 SS | TAGACAGACATGGCGCTTCA | NM_018417.3 |
| sAC seq 10 AS | GTTCCAAACCGGCAGCTTAC | NM_018417.3 |
| sAC seq 11 SS | TACCTCTTGAAGGGGGCTCT | NM_018417.3 |
| sAC seq 11 AS | TCTCATTCCAAGGTGCTCCC | NM_018417.3 |
| sAC seq 12 SS | TTATCTTTCGGGCCTCATTC | NM_018417.3 |
| sAC seq 12 AS | AACGACACAGACACACATGTGGA | NM_018417.3 |
| sAC seq 13 SS | ATTGATACGGCTCCGATGAG | NM_018417.3 |
| sAC seq 13 AS | GGACTGGCCCATAGTCAGAA | NM_018417.3 |
| sAC intron 4 SS | TTTTGGAGGAGACATCCTGAA | NM_018417.3 |
| sAC intron 4 AS | CCAGCTGCCGTAGGATTTAT | NM_018417.3 |

**Table A2: Oligonucleotides used for diagnostic PCR of *sAC* transcript**

| Description | Sequence 5' to 3' | Ref. Acc# |
|---|---|---|
| sAC exon 2 SS | GGACTGGCCCATAGTCAGAA | NM_018417.3 |
| sAC exon 3 AS | AGCAGTGCCATGTACATGG | NM_018417.3 |
| sAC exon 4 SS | TTTTGGAGGAGACATCCTGAA | NM_018417.3 |
| sAC exon 5 AS | GTAGCCTGGAGATCCATGGA | NM_018417.3 |
| sAC exon 32 SS | CTGTATATTAATGGGAGATG | NM_018417.3 |
| sAC exon 33 AS | GACCAATGGCTTCAGACGATC | NM_018417.3 |

# 9 Conferences

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Herrmann M, Schmitz B, Guske K, Roosterman D, Brand SM, Brand E. Functional analysis of soluble adenylyl cyclase promoter molecular haplotypes. 77th Annual Meeting Deutsche Gesellschaft für Experimentelle und Klinische Pharmakologie und Toxikologie e.V. Frankfurt a. M. 30.03.–01.04. 2011

Herrmann M, Guske K, Schmitz B, Salomon A, Brand SM, Brand E. Transcriptional regulation of soluble adenylyl cyclase (sAC) and sAC binding to cAMP response element (CRE) binding sites. 21st European Meeting on Hypertension and Cardiovascular Prevention, Mailand 17.-20.06.2011. Oral presentation.

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Herrmann M, Guske K, Schmitz B, Salomon A, Roosterman D, Brand SM, Brand E. Function of sAC as a transcriptional regulator of aldosterone mediated genes and sAC expression. 22st European Meeting on Hypertension and Cardiovascular Prevention, London 26.-29.04.2012. Oral presentation.

# 10 Publications

Salomon A, Schmitz B, Herrmann M, Rötrige A, Fabritius C, Morange PE, Cambien F, Tiret L, Trégouët DA, Pap T, Brand E and Brand SM. Regulation of transcription factor HIVEP1 by inflammatory cytokines and statins. Manuscript ready for submission.

Herrmann M, Salomon A, Schmitz B, Roosterman D, Guske K, Schelleckes M, Brand SM and Brand E. Soluble adenylyl cyclase transcriptionally regulates genes involved in aldosterone signalling. Manuscript in preparation.

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