Investigations on the Regulation of 5-Lipoxygenase Gene Expression by DNA Methylation and Histone Deacetylation/Acetylation

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Meiner Familie

„Das Runde muss in das Eckige“

Sepp Herberger, 1954

CONTENTS

1 Introduction

1.1 Lipoxygenases

Lipoxygenases (LOs) are dioxygenases that catalyze the conversion of polyunsaturated fatty acids, containing a series of cis double bonds, to hydroperoxy fatty acids. They are widely expressed in plants, fungi and animals but not in bacteria or yeast. The different enzymes are named after the position of the carbon C, which is oxygenated. When necessary the stereoconfiguration is specified, e.g. 12*R*-LO or 12*S*-LO. Because of the existence of several 12-lipoxygenases in mammalians, with distinct sequences, catalytic activities and function, these are named after the prototypical tissues of their occurrence, e.g. platelet, leukocyte, or epidermal 12-LO (Yamamoto et al. 1997).

Up to date five human LOs are described, the reticulocyte-type 15(*S*)-LO (Sigal et al. 1988), platelet-type 12(*S*)-LO (Izumi et al. 1990), epidermis-type 12(*R*)-LO (Boeglin et al. 1998), epidermis-type 15(*S*)-LO (Brash et al. 1997) and 5(*S*)-LO (Matsumoto et al. 1988). The first mammalian LO was identified in 1974 in human platelets by Hamberg et. al. (Hamberg and Samuelsson 1974), the 5-LO by Borgeat in 1976 (Borgeat et al. 1976).

1.2 5-Lipoxygenase

5-Lipoxygenase (5-LO) catalyzes the conversion of arachidonic acid (AA) to 5(*S*)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and the further reaction to the unstable epoxide leukotriene A4 (LTA4), (5(*S*)-6-oxido-7,9,11-trans-14-cis-eicosatetraenoic acid (Samuelsson 1983; Ford-Hutchinson 1994; Ford-Hutchinson et al. 1994), corresponding to the two distinct enzyme activites – the oxygenase activity and the LTA4 synthase activity respectively.

1.2.1 Structure and enzyme activity

So far no crystal structure of 5-LO exists. Based on the rabbit reticulocyte 15-LO crystal structure, as well as in conjunction with primary and secondary structure information, a model of 5-LO was predicted. As for all lipoxygenases, the protein consists of a C-terminal catalytic domain (aa 115 to 673) and an N-terminal β-barrel domain (aa 1 to 114) (Hammarberg et al. 2000; Hemak et al. 2002). The C-terminal domain is composed of α-helices and contains the catalytic iron, in its inactive ferrous form (Fe^{2+}). The iron acts as electron acceptor and donor during the lipoxygenase reaction mechanism. For catalysis, it needs to be restored into the

active ferric form by redox reaction with fatty acid hydroperoxides, i.e. 5-HPETE, 12-HPETE and 13-hydroperoxyoctadecaenoic acid (Rouzer and Samuelsson 1986; Riendeau et al. 1989; Hammarberg et al. 2001). Selenium-dependent glutathione peroxidases (GPx) reduce the cellular peroxide content and are therefore potent endogenous suppressors of 5-LO activity (Weitzel and Wendel 1993; Werz and Steinhilber 1996).

The residues essential for iron binding (Zhang et al. 1993; Hammarberg et al. 1995), substrate positioning (Gillmor et al. 1997) and nuclear import (Jones et al. 2002; Jones et al. 2003) have been identified in the catalytic domain. The catalytic domain represents ~80% of the lipoxygenase mass, but it is the β-barrel region that regulates the action of this domain.

The β-barrel domain is composed of two 4-stranded antiparallel beta sheets and is one the defining members of the PLAT (Polycystin-1, Lipoxygenase, α-Toxin) domain family (Bateman and Sandford 1999). This domain shares some common features with C2 domains, e.g. calcium-dependent phospholipid binding, but may best be considered a distinct subset of the C2 family (Chahinian et al. 2000). Binding of Ca^{2+} to the 5-LO β-barrel domain leads to phosphatidylcholine (PC) selectivity (Kulkarni et al. 2002) and is essential for nuclear membrane translocation (Chen and Funk 2001) and membrane association (due to the higher PC content).

Moreover 5-LO contains a functional Src homology (SH)3-binding motif, a short, proline-rich region, suggesting a role for 5-LO in tyrosine kinase signalling (Lepley and Fitzpatrick 1994). By means of ATP-affinity column chromatography binding of ATP to the residues of Trp75 and Trp201 has been shown (Wiseman 1989; Denis et al. 1991). Among LOs only 5-LO can bind ATP (Zhang et al. 2000) and is activated by nucleotides (Ochi et al. 1983; Falgueyret et al. 1995; Noguchi et al. 1996).

1.2.2 Subcellular distribution of 5-LO

Depending on the cell type 5-LO in resting cells either localizes in the cytosol or within the soluble compartment of the nucleus. Upon cell activation the cytosolic enzyme translocates to the ER or the outer nuclear membrane, whereas the nuclear enzyme translocates to the inner nuclear envelope (Luo et al. 2003). Import of 5-LO depends on both, the nuclear localization sequences and the phosphorylation site Ser271 (Luo et al. 2003), association with the nuclear membrane depends on calcium binding to the C2-like domain.

1.2.3 Phosphorylation of 5-LO

Protein phosphorylation is a regulatory mechanism for transduction of extracellular signals leading to the activation and redistribution of numerous cellular enzymes and transcription factors. Recently p38 MAPK-regulated MKs and ERK1/2 have been reported to phosphorylate and activate 5-LO (Werz et al. 2000; Werz et al. 2002). Cell stress induced Ca^{2+}-independent phosphorylation of 5-LO at Ser271 by MK-2/3. ERK2 can phosphorylate 5-LO at Ser663 in vitro, also independently of calcium. Both pathways have been shown to be involved in the translocation of 5-LO to the nucleus (Lepley and Fitzpatrick 1996; Boden et al. 2000).

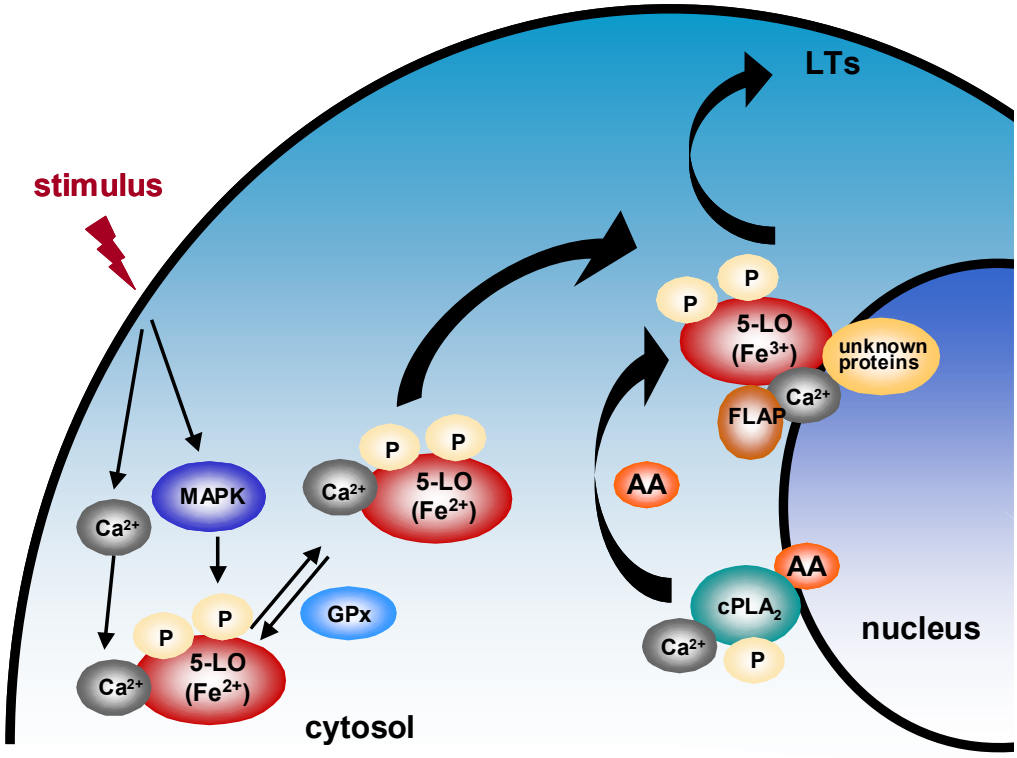


Fig. 1 Activation of 5-lipoxygenase

The figure above is summarizing the activation of 5-LO in a cellular context. Cell stimuli lead to elevated intracellular calcium levels and/or stress induced activation of protein kinases. Upon calcium binding and/or phosphorylation, as well as conversion of the inactive ferrous iron to its active ferric form by fatty acid hydroperoxides, the activated enzyme translocates to the nuclear membrane. There 5-LO colocalizes with $cPLA_2$, FLAP and LTC_4S. AA is released from phospholipids by $cPLA_2$ and presented by FLAP to 5-LO, initiating the leukotriene biosynthesis.

1.2.4 Cytosolic phospholiase A_2 ($cPLA_2$)

A critical parameter for 5-LO activity is the availability of substrate. Phospholipase A_2 comprises a superfamily of enzymes that hydrolyse the ester bond of phospholipids at the sn-2 position (Dennis 1997). Stimulated peritoneal macrophages from $cPLA_2$ knockout mice do not produce prostaglandin E_2 or leukotriene B_4 and C_4 (Bonventre et al. 1997; Uozumi et al. 1997). In leukocytes cytosolic phospholipase A_2, a protein of 85 kDa, provides AA for leukotriene synthesis, depending on the cell type and stimulus (Leslie 1997). Similar to 5-LO, Ca^{2+} and/or phosphorylation lead to an activation of $cPLA_2$ (Clark et al. 1991; Lin et al. 1993; Kramer et al. 1996).

1.2.5 5-Lipoxygenase-activating protein (FLAP)

FLAP is an 18 kDa membrane-bound protein that was discovered as a target of the LT biosynthesis inhibitor MK886 (Miller et al. 1990). MK886 inhibited LT synthesis in intact leukocytes, but not in cell homogenates, thus not inhibiting 5-LO enzyme activity itself. In cotransfection studies with 5-LO and FLAP, LT synthesis was dependent on the presence of FLAP, 5-LO expression alone did not result in any LT production (Dixon et al. 1990). Also FLAP-deficient macrophages from knock-out mice failed to produce any leukotrienes (Byrum et al. 1997). It was shown, that FLAP can bind AA and serves as an AA transfer protein facilitating the substrate presentation for 5-LO (Mancini et al. 1993). A model was proposed where AA-binding FLAP forms a heterodimer with 5-LO (Abramovitz et al. 1993), but direct binding to 5-LO was never shown.

1.3 Mediators of the 5-LO pathway

1.3.1 Leukotrienes

As lipid mediators, leukotrienes are involved in the signaling between cells, in host defence and inflammatory response. 5-LO is basically expressed in inflammatory cells such as polymorphonuclear cells, leukocytes, eosinophils, monocytes/macrophages, mast cells, dendritic cells, and B-lymphocytes. When overproduced, they also contribute to a variety of diseases, including asthma (Drazen et al. 1994), fibrosis (Wilborn et al. 1996), atherosclerosis (Spanbroek et al. 2003), allergic hyper-responsiveness (Rachelefsky 1997), ulcerative colitis (Rachelefsky 1997), psoriasis (Iversen et al. 1997), rheumatoid arthritis (Griffiths et al. 1995) and ischemic reperfusion injury (Noiri et al. 2000).

After the conversion of AA to 5-HPETE and LTA4, LTA4, depending on the cell type, is either converted to LTB4 by LTA4 hydrolase (LTA4H) or conjugated with glutathione by LTC4 synthase, forming the **cysteinyl leukotriene (CysLT)** LTC4. The successive amino acid cleavage of LTC4 results in LTD4 and LTE4. Together these cysLTs have been called "the slow reacting substance of anaphylaxis" (SRS-A), because of their spasmogenic effects .

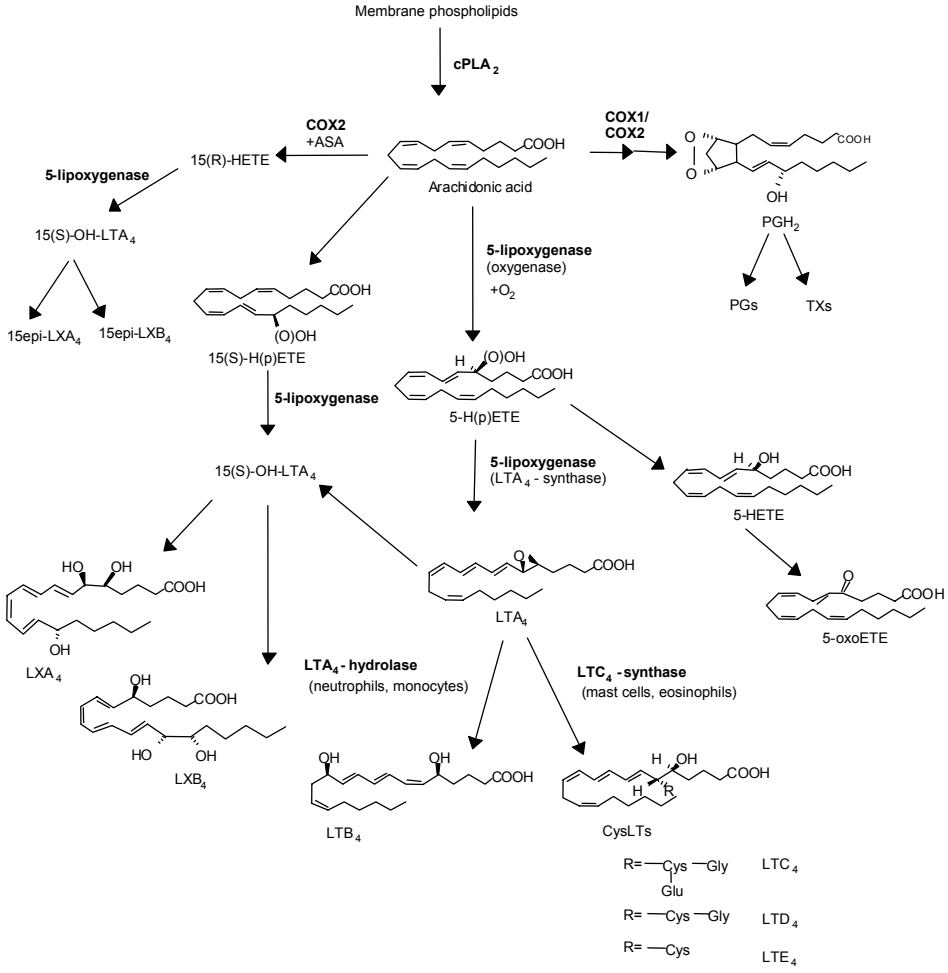


Fig. 2 The 5-LO pathway

They lead to smooth muscle contraction (Allen et al. 1992), thus promoting bronchoconstriction (Samuelsson 1983). Additionally they promote plasma exudation (Dahlen et al. 1981), stimulate mucus secretion (Marom et al. 1982), and recruit eosinophils (Michelassi et al. 1982). They are also reported to reduce myocardial contractibility and coronoary blood flow (Underwood et al. 1996).

LTB4 is a potent neutrophil chemoattractant and stimulator of leukocyte adhesion to endothelial cells (Samuelsson 1983; Peters-Golden and Brock 2001). In contrast to 5-LO, which is only expressed in haematopoetic cells, LTA4H is ubiquitously expressed. During

inflammation, LTB4 is produced in all kinds of tissue, due to transcellular metabolism of LTA4 (Sala et al. 1996).

Leukotrienes act at distinct GPCRs. The **cysteinyl leukotriene receptors CysLT1** and **CysLT2** mediate the actions of LTC4 and LTD4. CysLT1 is found on airway smooth muscle cells (Lynch et al. 1999) and vascular endothelial cells (Gronert et al. 2001), promoting bronchoconstriction. The CysLT2 receptor is detected within pulmonary veins, in spleen, Purkinje fibers of the heart and discrete regions of adrenal gland (Heise et al. 2000).

LTB4 binds with high affinity to the **B-LT1 receptor** mainly expressed on leukocytes, with low affinity to the B-LT2 receptor, which is expressed in wide variety of tissues (Tager and Luster 2003). The B-LT1 receptor is reponsible for the chemotactic response to LTB4, whereas the function of **B-LT2** is still unknown (Yokomizo et al. 2000; Yokomizo et al. 2001).

1.3.2 Lipoxins

Besides in the generation of proinflammatory mediators 5-LO is also involved in the production of endogenous anti-inflammatory lipid mediators, the lipoxin family (Serhan et al. 1999). **Lipoxins** (lipoxygenase interaction products, LXs) are conjugated trihydroxytetraene-containing eicosanoids (Serhan 2002). They are generated by transcellular biosynthesis during cell-cell interaction. In an interplay between activated leukocytes and platelets, leukocyte 5-LO catalyzes the formation of LTA4 and platelet 12-LO transforms LTA4 to LXA4 and LXB4 (Serhan and Sheppard 1990; Serhan and Romano 1995). In epithelial and endothelial cells 15-LO generates 15*S*-HETE and in neutrophils 5-LO proceeds the production to LXs (Serhan et al. 1984). The third route in LX production is triggered after aspirin treatment (Claria and Serhan 1995). Acetylation of COX2 switches the catalytic activity of COX2 to a 15-LO, resulting in 15*R*-HETE, which is further transformed by 5-LO to 15-epi-LXA4 and 15-epi-LXB4, also called aspirin triggered lipoxins (ATL). The beneficial actions of NSAIDs, including prevention of myocardial infarction may be in part due to the biosynthesis of ATL. Anti-inflammatory actions of LXs and ATL include inhibition of leukocyte-mediated injury, stimulation of macrophages, clearance of apoptotic neutrophils, repression of proinflammatory cytokine production, inhibition of cell proliferation and migration and others (for detailed review see (McMahon and Godson 2004)). Bioactions of lipoxins are mediated either via the GPCR ALXR (Badr et al. 1989; Brady et al. 1990; Maddox et al. 1997; Gronert et al. 1998; Bandeira-Melo et al. 2000; McMahon et al. 2000; Gronert et al. 2001; McMahon

et al. 2002), with LXA4 as specific ligand, or by competitve antagonism at the Cyc-LT1 receptor (Badr et al. 1989; McMahon et al. 2000; Gronert et al. 2001).

1.4 Pharmacological inhibitors

Pharmacological intervention on behalf of LTs is possible during either their formation or via receptor antagonism to prevent their biological action.

Although **glucocorticoids** have been demonstrated to inhibit phospholipases and eicosanoid formation in many experimental setups, high dose glucocorticoid therapy did not significantly suppress LT formation (Claesson and Dahlen 1999).

1.4.1 5-LO inhibitors

5-LO inhibitors can be classified into three different groups, namely redox-active compounds, iron-ligand inhibitors with weak redox-active properties and non-redox-type inhibitors.

Redox-active inhibitors reduce the catalytic active ferric iron to the inactive ferrous form (Fe^{2+}). This group of inhibitors consists of phenols like nordihydroguaretic acid, caffeic acid, or flavonoids and coumarins. Many of these inhibitors show severe side effects like methemoglobin formation or are rapidly metabolized (McMillan and Walker 1992; Ford-Hutchinson et al. 1994).

The second group of inhibitors, the **iron chelating substances**, consist of hydroxamic acid and N-hydroxy-urea derivatives. Because of the rapid metabolization of the hydroxamate group to an inactive carboxylate group, the development of inhibitors was focused on N-hydroxy-urea derivates like A-64077 (zileuton). Up to date, zileuton (Zyflo®) is the only 5-LO inhibitor on the market for the treatment of asthma. Only low benefits for the treatment of other diseases were observed with zileuton.

The poor selectivity and low bioavailability of both groups of inhibitors lead to the development of **non-redox-type inhibitors**. These substances contain methoxyalkylthiazol or methoxytetrahydropyran structures, competing with AA for the binding site of 5-LO. ZD 2138 and ZM 230487 are selective and orally active 5-LO inhibitors (Crawley et al. 1992). In human leukocytes and whole blood assays LT-biosynthesis was inhibited with IC50 values of about 20-50 nM (Smith et al. 1995). However, they failed in clinical trials of chronic inflammatory diseases (Kusner et al. 1994). Both compounds were less active in cell homogenates than in intact cells (Smith et al. 1995). Addition of glutathione or DTT reversed

the loss of inhibitory activity, indicating that the peroxide level defines the potency of both inhibitors (Werz et al. 1998). In other studies with ZM 230487 or the related Merck compound L-739,010, the potency of the inhibitors depended on the stimulus and phosphorylation status of 5-LO (Fischer et al. 2003).

These data suggest, that the inhibitory potency of 5-LO inhibitors depends on the physiological conditions 5-LO is activated under. In vitro and ex vivo testing of potential 5-LO inhibitors should therefore include the different activation pathways of 5-LO, by using physiological stimuli and priming agents, leading to either calcium or phosphorylation dependent 5-LO activation, as well as consider different peroxide levels within the cells.

1.4.2 Dual COX and 5-LO inhibitors

Both enzymes are co-expressed and up-regulated during inflammation and their products, i.e. PGE2 and LTB4 are elevated in inflammed tissues. So there is much reason to develop substances inhibiting both enzymes at the same time. Several substances are currently evaluated in experimental setups (RWJ-63556, ER-34122) or clinical trials for different inflammatory diseases, including S-2474 against arthritis. One very promising substance is Licofelone (ML-3000), a pyrrolizidine derivate and an arachidonic acid mimetic, which has already been tested in phase III trials with patients with osteoarthritis. No severe side effects, like hepatotoxicity were found and the compound demonstrated an excellent gastrointestinal profile, better than NSAIDs and equivalent to selective COX2-inhibitors (Wallace et al. 1994; Celotti and Durand 2003; Charlier and Michaux 2003).

Hyperforin is the major lipophilic constiuent of Hypericum perforatum (St. John´s worth). The acylphloroglucinol derivate has recently been demonstrated to inhibit 5-LO in human PMNL (IC50 1-2 µM in intact cells) and COX1 in platelets (IC50 0.3 or 3 µM dependent on the stimulus). Experiments with crude enzymes revealed that hyperforin acts as an direct inhibitor of 5-LO in an uncompetitive manner. The mechanism how 5-LO is inhibited is still unkonwn. COX2, 12-LO and 15-LO product formation was not influenced

1.4.3 FLAP inhibitors

So far MK886, BAY-X-1005, MK-0591 have been designed and their properties and potency has been characterized (reviewed in (Devillier et al. 1999; Drazen et al. 1999; Werz 2002)). Both MK886 and BAY-X-1005 show impaired efficacy in whool blood assays, probably do the presence of exogenous AA (Rouzer et al. 1988; Steinhilber et al. 1993; Werz et al. 1997) and the high affinity of the inhibitors to plasma proteins (Charleson et al. 1994).

1.4.4 Receptor antagonists

The orally active **Cyc-LT1 antagonists** montelukast (Singulair®), pranlukast (Ultair®) and zafirlukast (Accolate®) have been tested in several clinical trials and shown to reduce asthma exacerbations and to improve pulmonary function (Nathan and Kemp 2001).
The **LTB4 receptor antagonists** CP-105.696 and SC-41930 reduce arthritis index and ankle bone destruction in IL-1 accelerated collagen-induced arthrithis and reduce artherosclerosis lesion progression in mice (Kuwabara et al. 2000; Aiello et al. 2002).

1.5 5-LO gene and protein expression

The cDNA for the human 5-lipoxygenase was first cloned by Dixon et al. from DMSO differentiated HL-60 cells. The cDNA contained a 673 amino acid long open reading frame, encoding for a 78 kDa large protein (Dixon et al. 1988). In parallel, Matsumoto could clone the cDNA for 5-LO from a lung and placenta λgt11 cDNA library. He described a 34 bp 5′-noncoding region and a 442 bp 3′ noncoding region (Matsumoto et al. 1988).
The human 5-LO gene was first characterized by Funk and Hoshiko (Funk et al. 1989). The 5-LO gene is unique compared to the other human lipoxygenase genes in several respects, including its distinct separate chromosomal location, on chromosome 10q11.2 instead of 17p13, and its size, 4-7 times larger than the other LO genes (Funk et al. 2002). It consists of 14 exons separated by 13 introns, spanning around 82 kb. The extent of the 5′- and 3′-regulatory sequences is unknown.

1.5.1 The 5-LO promoter

The 5′-flanking region has first been characterized by C. Funk and S. Hoshiko (Funk et al. 1989; Hoshiko et al. 1990). By nuclease S1 protection and primer extension experiments a major TIS in leukocytes at –65 in relation to ATG was identified. Other minor transcription initiation sites at –107, -97, -89, -62, -66, -35, and –34 were described by the one or other method. Alternative transcripts, 3.1, 4.8, 6.4, and 8.6 kb in length instead of 2.7 kb, have been reported in brain tumor cells and DMSO differentiated HL-60 cells (Boado et al. 1992), possibly due to aberrant splicing events.
The sequence comprising about 5900 bp upstream of the ATG displayed promoter activity in reporter gene assays in both 5-LO negative and 5-LO positive cell lines, HeLa and HL-60 cells respectively. The promoter study performed by Hoshiko in HeLa cells also revealed two positive regulatory sequences (-5900 to –3700 and –931 to –854) and two negative regulatory

elements (-3499 to –1557 and –727 to –292). The stretch of –179 to –56 was essential for full promoter activity and contained the sequence involved in induction by PMA treatment, as well as the 5-tandem consensus Sp1/Egr1 binding site (Sp1 binding to –GGGCGG- and Egr1 to -GCGGGGGCG-). In gel shifts, Sp1 binding from HeLa cell extracts was shown to the DNA stretch –212 to –88 (containing 6 Sp1 consensus binding sites / GC boxes). Different bands appeared with a nuclear HL-60 extract. Later it was shown that PMA treatment of HUVEC cells triggers Egr1 expression and binding to the 5-tandem Sp1/Egr1 binding site (In et al. 1997).

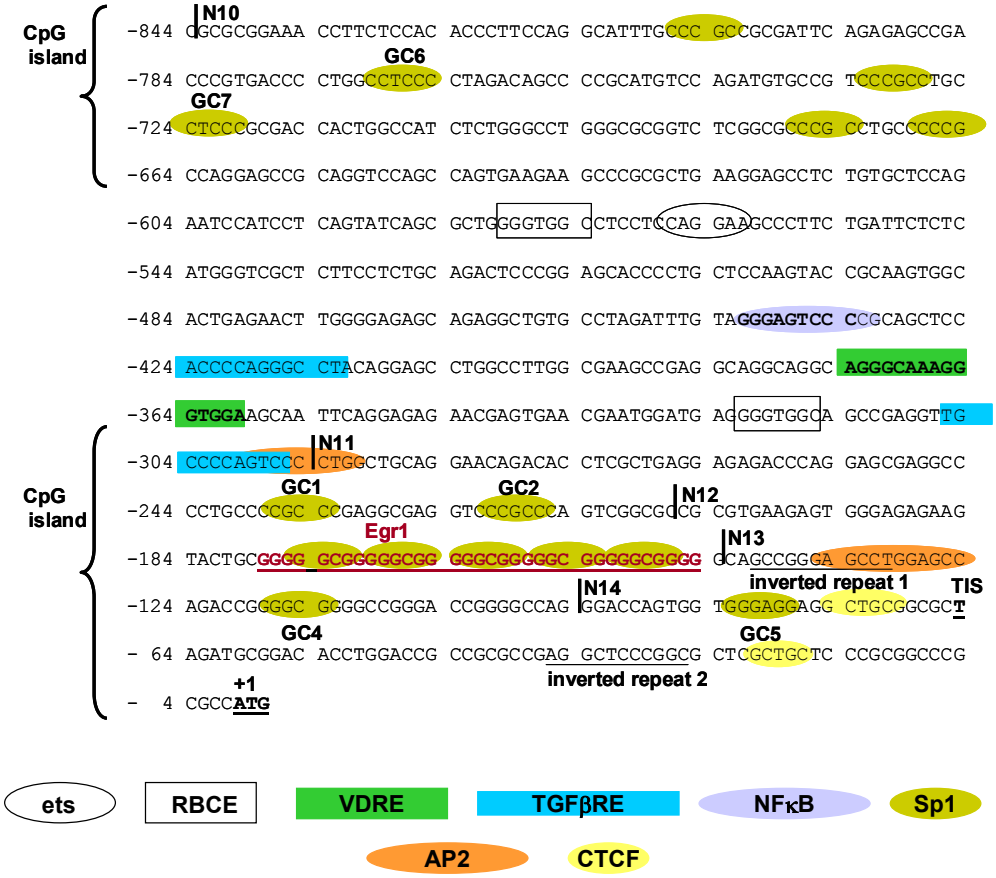


Fig. 3 Overview of the proximal 5-LO promoter. *Indicated are the different transcription factor binding sites, CpG islands and reporter gene constructs (N10, N11, N12, N13). The functional significance of the putative binding sites for AP2 (Imagawa et al. 1987), CTCF (Filippova et al. 2002), NFκB (Lenardo and Baltimore 1989), RBCE (Kim et al. 1992; Chen et al. 1994), ets/PU.1 (Pahl et al. 1993; Suzuki et al. 1998; Voo and Skalnik 1999) and the TGFβ response element (Marigo et al. 1994) is still unknown. Egr1/Sp1 binding has been shown to GC1, GC2, the 5-tandem binding site, GC4 and GC5 (In et al. 1997; Silverman et al. 1998; Dishart et al. 2005). The more distal binding sites are of interest for future studies. VDR binding was investigated in (Sorg et al. 2006).*

The analyzed promoter sequence contains several consensus binding sites for different transcription factors, including Sp1, Sp3, Egr1, Egr2, glucocorticoid receptors, NFκB, GATA, myb and AP family members (Hoshiko et al. 1990). For not all of them functionality could be shown so far. The c-myb protein downregulates 5-LO gene expression and inhibits macrophage differentiation (Habenicht et al. 1989). In vitro binding to the consensus motif in the 5-LO promoter at –1840 to –1852 (TAACGG) was demonstrated (Ponton et al. 1997). Sequence analysis with databases like TRANSFAC® suggest the presence of several putative response elements for nuclear receptors, including the VDR, retinoid Z receptor α (RZRα) as well as the retinoic acid related orphan receptor α(RORα), TGFβ, Smads, ATF-2, hFast-1, Oct-1 and p53. Binding of RORα1 and RZRα to a putative RZR RE at -1521 to -1510 has been demonstrated before. The promoter activity as well as 5-LO protein expression in B-lymphocytes was repressed by melatonin, a ligand of RZRα (Steinhilber et al. 1995).
The promoter lacks TATA and CCAAT boxes as well as an Inr element. Conspicuous is the high GC content and the high number of Sp1 consensus binding sites (GGGCGG). These are characteristics of so-called housekeeping genes, being constitutively expressed with little regulation. However 5-LO gene expression takes place primarily in cells of myeloid lineage and is regulated on multiple levels.

1.5.2 Mouse 5-LO promoter

In 2001 the **mouse 5-lipoxygenase** promoter was cloned and characterized (Silverman et al. 2002). Though different alignment programs failed to align or to find similarities of the human and mouse promoter, a few features of both promoters were almost identical. The first 292 bp were identified as the core promoter region containing a major TIS at -52 bp and three minor start sites, comparable to the human promoter. Both promoters lack the TATA motif, TFIIB recognition elements or initiator sequences and are GC-rich. They both contain Sp1/Sp3, PU.1/SPI-1 and GATA binding sites in similar promoter regions. The only Sp1 binding site located at -184 bp to –189 bp seems orthologous to the 5-tandem GC box of the human promoter (-145 to-179) and mutation of the site also results in a loss of promoter activity in reporter gene assay. Sp1 binding, but not Egr1 binding to this site was shown in gel shifts. The lack of any Egr1 binding site and functional response of the promoter to Egr1 over-expression suggest that Sp1 and Sp3 are more important in the regulation of 5-lipoxygenase than Egr1. GATA binding was not detectable in gel shifts, but PU.1 binding.

1.5.3 Naturally occuring mutations of the 5-LO promoter

Naturally occurring mutations of the 5-tandem Sp1 binding sites within the human 5-LO promoter were identified, such as the deletion of one or two, or addition of one GC box within the 5-fold Sp1/Egr1 binding motif (In et al. 1997). In reporter gene assays with transfected HeLa cells the promoter activity was reduced independently of the genotype of the mutation, whereas in SL2 cells, which do not express Sp1 and Egr1, the activity was proportional to the number of Sp1/Egr1 sites (In et al. 1997; Silverman et al. 1998). In gel shift assays, binding of both transcription factors to the mutated sites was slightly reduced. Binding of recombinant Sp1 and Egr1 to the 5-fold GC box (-179 to -147 in relation to ATG, also GC0) was demonstrated by DNase I footprints (Silverman et al. 1998). In the same experiments Sp1 could also protect DNA in the regions covering -117 to -109 (GC4) and -224 to -218 bp (GC2) covering two other Sp1 consensus binding sites. No other Egr1 binding sites were detected in these experiments. In SL2 cells, the over-expression of Sp1 and Egr1 in combination with a reporter gene construct covering the 5-LO promoter from -229 bp up to the translational start site resulted in an about 30-fold induction of CAT-activity. Expression of Egr1 or Sp1 alone lead to a significantly lower promoter induction. Interestingly, Sp1 over-expression showed a stronger effect on the promoter construct lacking the 5-fold GC box, only covering –129 bp up to the translational start site. The role of the GC-boxes was studied more closely in 5-LO positive MM6 cells (Dishart et al. 2005). A new Sp1 binding site close to the major transcription site was identified. Incubation with the antibiotic mithramycin, which binds to GC-rich sequences, reduced 5-LO expression and activity during differentiation in MM6 cells with $1,25(OH)_2D_3$ and TGFβ. In DNase I footprinting Sp1 binding to the GC-rich parts of the promoter was demonstrated. The levels of Sp1, Sp3 and Egr1 did not change upon $1,25(OH)_2D_3$/TGFβ differentiation of the cells, whereas 5-LO expression is only found after the differentiation. Together the data suggest that at the early time points Sp1/Sp3 and Egr1 are involved in the basal transcriptional regulation of 5-LO, but that differentiation with $1,25(OH)_2D_3$/TGFβ also involves another type of mechanism.

In a study with asthma patients treated with the selective 5-LO inhibitor ABT-761, a zileuton derivative, patients carrying a mutation of the 5-tandem site in the 5-LO promoter in both alleles showed no response to the anti-asthma treatment. The patients with a 5-fold GC box on both alleles showed an average change in the forced expiratory volume in the first second (FEV1) of +18.8±3.6%, patients (n=64) carrying both, one mutation and one wild-type site, improved by 23.3±6.0% (n=40). The 10 patients with the mutations on both alleles, did not benefit from the treatment, indicated by the average change of FEV1 of –1.2±2.9% (Drazen et

al. 1999). In further studies with Japanese asthma patients the 5-LO promoter mutations did not associate either aspirin-induced (AIA) or aspirin-tolerant asthma (ATA) patient groups nor with urinary LTE4 and LTC4S activity (Kawagishi et al. 2002). In a Caucasian population the 5-LO Sp1 polymorphism was not associated with asthma or asthma-related phenotypes either (Sayers et al. 2003). In a recent study performed within Korean population, no significant differences in the frequencies of the 5-LO promoter polymorphisms were observed between ASA and AIA patients or the control group, but there was a strong association with airway hyperresponsiveness in aspirin-intolerant-asthma patients (Kim et al. 2005).

Recently clinical, population genetic, cell biological and mouse studies indicate the participitation of the 5-LO pathway in atherogenesis. In one study 5-LO promoter genotypes were linked to atherosclerosis (Dwyer et al. 2004). In the Los Angeles atherosclerosis study the Sp1 promoter polymorphism (for carriers of two variant alleles) was associated with increased intima-media thickness. Increased intake of n-3 fatty acids could blunt the effect whereas increased intake of AA enhanced the atherogenic effect.

The results from the Los Angeles atherosclerosis study rather suggest an upregulation of 5-LO expression, than a down-regulation found in the reporter gene assays due to the promoter polymorphism. Possible explanations for this paradoxia are versatile. Transfection of HeLa and SL2 cells, both not expressing endogenous 5-LO, may not reflect the in vivo process of inflammation. Also other enzymes involved in inflammtory processes like 15-LO or COX2, may profit from reduced 5-LO expression and have impact in the promotion of atherosclerosis (Cyrus et al. 2001; Burleigh et al. 2002). Also the functionality of 5-LO promoter genotypes with respect to LT formation in relevant leukocyte populations has not been clearly demonstrated in asthma or cardiovascular disease patients.

Though the 5 LO promoter contains several features of so-called housekeeping genes 5-LO expression occurs in a cell specific manner. The expression is mainly limited to cells derived from the bone marrow like granulocytes, monocytes, macrophages, mast cells, and B-lymphocytes (Steinhilber 1999). Besides relevant levels of 5-LO were detected in epidermal Langerhans cells (tissue-like macrophages) (Spanbroek et al. 1998) and cultured human skin keratinocytes (Janssen-Timmen et al. 1995).

1.5.4 Stimuli of protein expression

Different inflammatory stimuli increase 5-LO mRNA levels in leukocytes, e.g. transforming growth factor-β (TGFβ), granulocyte-monocyte colony-stimulating factor (GM-CSF), interleukin-3, oxidized low-densitiy lipoprotein (LDL), phorbol esters (PMA),

Ca^{2+}-ionophores (Silverman and Drazen 1999). In HL-60 cells, a promyeloic leukemia cell line, differentiation with DMSO, retinoic acid (RA) or $1,25(OH)_2D_3$ increased 5-LO levels (Bennett et al. 1993; Piechele et al. 1993). In the myeloid cell lines, MM6 and HL-60, differentiation of the cells with calcitriol ($1,25(OH)_2D_3$) and TGFβ results in a strong upregulation of 5-LO gene expression, on the level of mRNA, protein and enzyme activity (Brungs et al. 1994; 1995). In HL-60 cells treatment with 24 nM of $1,25(OH)_2D_3$ alone increased 5-LO mRNA levels 4-fold, the protein amount about 14-fold, protein activity in intact cells 14-fold and in cell homogenates 38-fold. TGFβ (1 ng/ml) alone showed no effect, but lead to a further increase of 5-LO mRNA, protein and protein activity when combined with $1,25(OH)_2D_3$.

In MM6 cells, differentiated with 50 nM $1,25(OH)_2D_3$ and 1 ng/ml TGFβ, the effects were even stronger. The 5-LO mRNA levels increased up to 64-fold, the protein amount 128-fold and the protein activity more than 500-fold. Furthermore, it was demonstrated that the upregulation of 5-LO mRNA levels depended on protein synthesis, e.g. of transcription factors. More detailed investigations on the mRNA level in MM6 cells demonstrated that mRNA half-life was not affected by the combined treatment of $1,25(OH)_2D_3$ and TGFβ. By exon/intron specific RT-PCR it was further specified that TGFβ and $1,25(OH)_2D_3$ increase primary transcripts about 5-fold and mature 5-LO mRNA 42-fold. Cycloheximid inhibited the effect of both agents on the maturation, but only the action of TGFβ on the primary transcripts, indicating a $1,25(OH)_2D_3$ impact on the transcriptional level in the presence of TGFβ induced proteins. On the post-transcriptional level, including transcript elongation and/or maturation cycloheximid blocked the effects of both agents (Harle et al. 1998).

Most known biological effects of $1,25(OH)_2D_3$ are “genomic” effects, mediated through specific and high affinity binding to the nuclear $1,25(OH)_2D_3$ receptor (VDR). Transcriptional transactivation involves the binding of the receptor to a response element in proximity to the TIS of a responding gene. The 5-LO promoter contains putative VDREs in the proximal promoter area, located at -374 to –327 in relation to ATG (Carlberg 1995). Binding of the receptor heterodimer VDR-RXR was demonstrated in vitro, in gel shifts and DNase I footprints, as well as in vivo, in chromatin immunoprecipitation assays (ChIP). However the elements showed no functionality in response to $1,25(OH)_2D_3$ in transient reporter gene studies (Uhl et al. 2002; Sorg et al. 2006), nor did a stably transfected 5-LO promoter reporter gene construct comprising –6,144 to +150 in relation to ATG. This finding is supporting the results of nuclear run-off assays, in which $1,25(OH)_2D_3$ did not increase transcriptional activity (Harle et al. 1999). In a new approach the complete 5-LO gene was

screened for putative VDRE. Gel shift studies, reporter gene assays and chromatin immunoprecipitation revelead the existance of at least 3 functional VDRE, one located further upstream in the promoter (at -2,250), one in intron 4 (at about +42,000) and one in intron 5 (at about +50,600), which may cooperatively mediate the $1,25(OH)_2D_3$ response (Seuter et al. 2006).

1.5.5 DNA methylation and 5-LO

Expression of several genes with GC-rich promoters has been shown to be regulated by DNA methylation. Depending on the GC content GC-rich sequences can be characterized as so-called CpG islands, which are of a minimum length of 200 bp, have a minimum GC-content of 50% and an observed/expected ratio of GCs greater than 0.6. In housekeeping genes CpG islands stay unmethylated, whereas in tissue-specific genes DNA methylation of the promoter leads to transcriptional silencing of the gene (Singal and Ginder 1999).

The 5-LO gene contains at least two CpG islands, covering the proximal promoter area -1052 bp to –633 bp and –266 bp to +278 bp in relation to the ATG of 5-LO (compare www.ebi.ac.uk/emboss/cpgplot/index.html, examining the sequence of the homo sapiens chromosome 10 contig NT_033985 position 3267921 to 3274901).

Recently it was demonstrated that 5-LO gene expression in the 5-LO negative cell lines U937 and HL-60TB is linked to DNA methylation of the proximal 5-LO promoter (Uhl et al. 2002). Treatment of the cells with the demethylating agent 5-aza-2`-deoxycytidine upregulated the 5-LO mRNA levels in both cell lines. Subsequent treatment with $1,25(OH)_2D_3$ and TGFβ also resulted in prominent 5-LO protein activity. Bisulfite sequencing of genomic DNA of both cell lines revealed that the 5-LO core promoter is heavily methylated in both cell lines, but not in the 5-LO positive cell line HL-60. AdC partly demethylated the core promoter, thus the degree of methylation/demethylation correlates to the level of gene expression. In reporter gene assays in vitro methylation of the most active deletion variant N10, comprising the 5-LO core promoter region, almost abolished the promoter activity. These data suggest that DNA methylation is at least one possible mechanism in the cell specific expression of 5-LO. Apparently the regulation by DNA methylation is unrelated to the regulation by TGFβ and $1,25(OH)_2D_3$ (Uhl et al. 2002).

Transcriptional repression by DNA methylation can occur on several levels: i) the binding of methylation sensitive transcription factors may be inhibited (Watt and Molloy 1988) and/or ii) so-called methyl-CpG DNA binding proteins (Boyes and Bird 1991; Hendrich and Bird 1998) recognize the methylated sequences and recruit other corepressors like histone and chromatin

modifying enzymes, e.g. histone deacetylases (HDAC), transforming the transcriptional active chromatin into condensed chromatin (Jones et al. 1998; Nan et al. 1998; Ng et al. 1999; Wade et al. 1999; Zhang et al. 1999; Sarraf and Stancheva 2004). Several histone deacetylase inhibitors have been identified or synthesized. Trichostatin A (TsA), a hydroxamic acid isolated from *streptomyces hygroscopius,* inhibits HDACs in nanomolar concentrations and evokes histone acetylation. After TsA treatment, an upregulation of 5-LO mRNA levels in MM6 cells occurs and 5-LO promoter activity is induced in reporter gene assays after transient transfection (Klan et al. 2003).

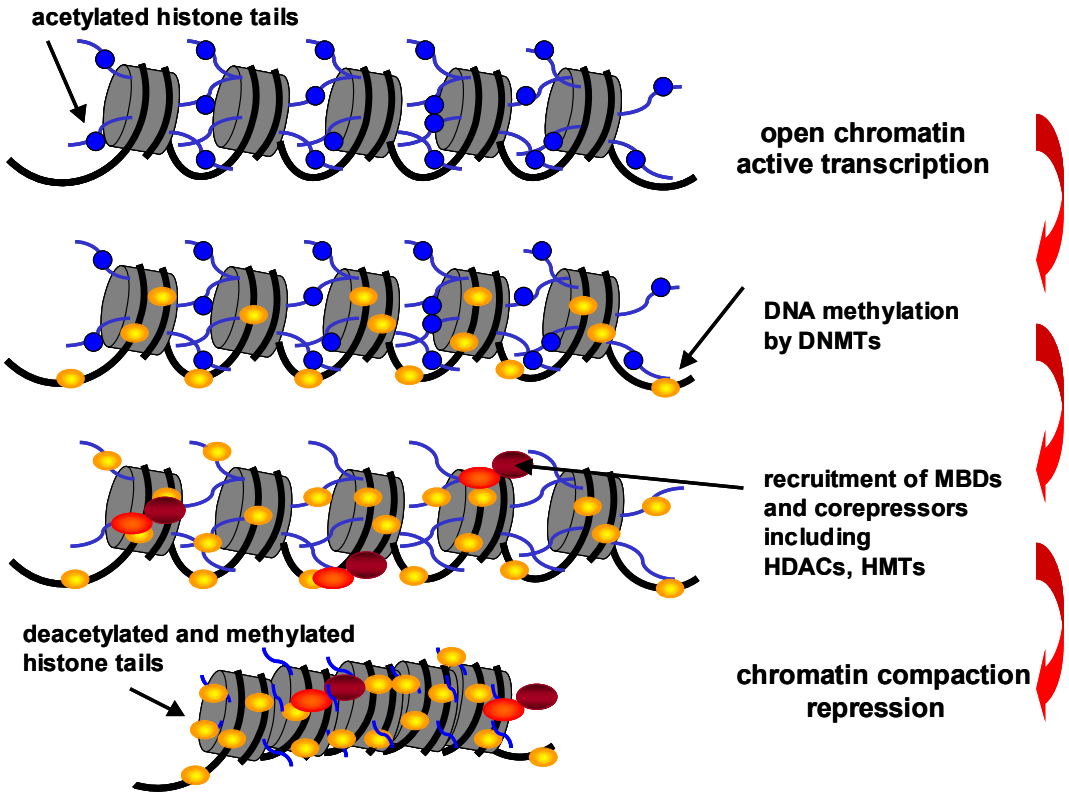


Fig. 4 Transcriptional repression by DNA methylation and histone deacetylation.

TsA also induced promoter activity of in vitro methylated 5-LO reporter gene constructs, but it never reached the activity level of the unmethylated and TsA treated constructs, indicating that the methylation dominates over the TsA-sensitive histone deacetylation. Other reports describe an upregulation of neuronal 5-LO mRNA levels after treatment with valproic acid, an other HDAC inhibitor with potential demethylating properties (Phiel et al. 2001; Manev and Uz 2002; Detich et al. 2003; Yildirim et al. 2003; Zhang et al. 2004). Together these data suggest a link between DNA methylation and chromatin remodeling in the regulation of 5-LO transcription (see Fig. 4).

1.6 Epigenetic Mechanisms

In addition to the information encoded by the DNA sequence, so-called epigenetic information can be stably inherited through many cell generations.

1.6.1 DNA methylation

One type of epigenetic information comprises the addition of a methyl group to the 5´-position of the cytosine ring of CpG dinucleotides, the DNA methylation (Naveh-Many and Cedar 1982). About 70% of the CpG dinucleotides are methylated in vertebrates (Ehrlich et al. 1982). CpG islands, clusters of CpG dinucleotides, are mainly unmethylated and are found in approx. 60% of human promoters (Antequera and Bird 1993). Methylation of promoter CpG islands leads to subsequent inactivation of the associated genes.

1.6.1.1 DNA Methyltransferases

The methylation patterns are introduced by a set of DNA methyltransferases, which are subdivided into the two groups of de novo methyltransferases (DNMT3a, DNMT3b) introducing cytosine methylation in formerly unmethylated CpGs and the maintenance methyltransferase DNMT1, copying an existing methylation pattern during the DNA replication from one strand to the other. So far three potential mechanism of triggering de novo methylation are understood. First, DNMTs might recognize DNA or chromatin itself, by a PWWP domain, a DNA sequence-independent interacting domain (Qiu et al. 2002; Shirohzu et al. 2002; Ge et al. 2004). Secondly site-specific transcriptional repressors can recruit DNMTs. The myc protein has been described to recruit DNMT3a to the p21 promoter, leading to subsequent de novo methylation of the promoter (Fuks et al. 2001; Brenner et al. 2005). Recently RNAi gene silencing has been linked to DNA methylation. After the introduction of double-stranded RNA corresponding to the promoter sequence of a gene, the target gene was silenced accompanied by de novo methylation of the promoter sequence (Kawasaki and Taira 2004; Morris et al. 2004). Also recruitment of DNMT3A and DNMT3B via interaction with the histone methyltransferase SETDB1 (Li et al. 2006) or SUV39H1 (Fuks et al. 2003) have been described.

1.6.1.2 Methyl-CpG-binding proteins

The fact that methylated CpG islands are associated with transcriptional repression lead to the search for factors being able to read and interpret this epigenetic information. The first protein

discovered, to bind to methylated DNA and mediating transcriptional repression was MeCP1, methyl-CpG-binding protein 1 (Meehan et al. 1989). Later it was shown, that MeCP1 is a protein complex, consisting of MBD2 as the methyl-CpG-binding protein and the chromatin remodeling complex Mi2-NuRD, which contains the histone deacetylases HDAC1 and 2, MBD3, the chromatin remodeller Mi-2, other proteins like RbAp46/48, metastasis-associated protein 2, Sin3a and SAP30 and SAP18 (Feng and Zhang 2001).

MBD (methyl-CpG-binding domain) proteins selectively bind to methylated DNA and recruit chromatin remodelling and transcriptional repressor complexes, thereby establishing a repressive chromatin state. So far, there are at least five mammalian MBD proteins: MeCP2, MBD1, MBD2, and MBD3 for transcriptional repression and MBD4 (also known as MED1) primarily for mismatch repair as a thymine glycosylase (Hendrich and Bird 1998). Sequence similarity between MBD proteins is limited to the methyl-binding domain itself. Only MBD2 and MBD3 display about 70 % sequence similarity. This diversity predicts different functions, but biochemical analysis could also show different behavior in the ability to bind to methylated DNA. Only MeCP2, MBD1, MBD2 and MBD4 bind to methylated DNA. They all drive transcriptional repression due to the presence of a transcriptional repressor domain (TRD) interacting with other proteins.

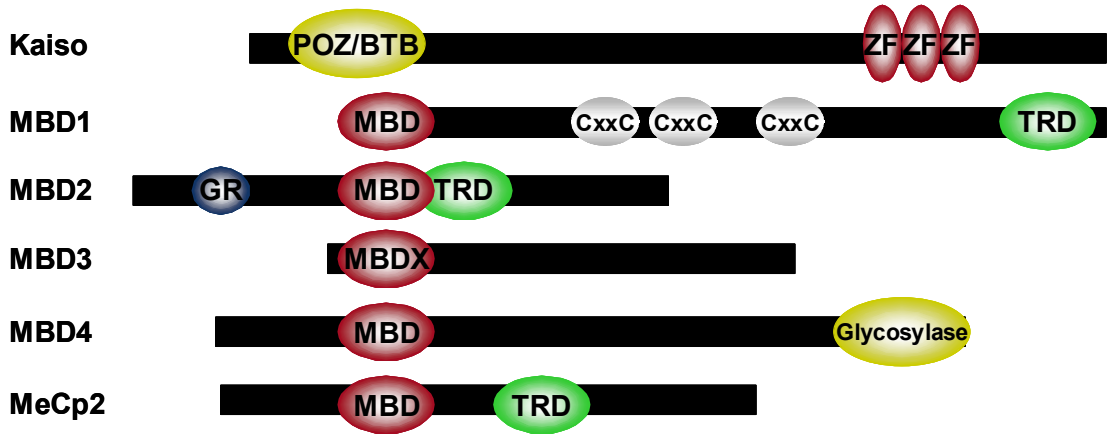


Fig. 5 Methyl-binding domain proteins. *MBD1, 2, 3, 4 and MeCP2 bind to methylated DNA via the well conserved methyl-binding-domain (MBD). MBD1 additionally binds to unmethylated DNA via one of its zinc-binding domains (CxxC). The transcriptional repression domain (TRD) mediates transcriptional silencing due to interaction with corepressors. Because of amino acid changes MBD3 is unable to bind to methylated DNA and requires interaction with MBD2. MBD4 contains an additional glycosylase domain for DNA repair. Kaiso lacks a MBD and binds to unmethylated and methylated DNA via the zinc-finger domain (ZF). The POZ domain mediates transcriptional repression (Klose and Bird 2006).*

MeCP2 is able to bind to a single symmetrically methylated CpG (Lewis et al. 1992) and mutations within the MBD of MeCP2 causes the Rett syndrome (Amir et al. 1999). Recently Klose et. al. found a $[A/T]_{\geq 4}$ run close to methylated CpG essential for high-affinity binding at selected sites and at known MeCP2 target regions in the Bdnf and Dlx6 genes (Klose et al. 2005). MeCP2 interacts with the transcriptional repressor complex Sin3 containing histone deacetylases (HDAC1/2) (Jones et al. 1998; Nan et al. 1998). The recruitment of HDACs by MBD proteins links another epigenetic mechanism of transcriptional regulation with DNA methylation, namely histone modifications (for details see below). MeCP2 has also been shown to associate with histone methyltransferase activity, targeting Lys9 on the histone H3 tail, a modification linked to repressed chromatin (Lachner and Jenuwein 2002; Fuks et al. 2003) and Dnmt1 (Kimura and Shiota 2003). In another study MeCP2 alone was capable of mediating chromatin compaction independent of HDAC activity and DNA methylation (Georgel et al. 2003). Horike et. al. described a silent-chromatin loop formation by MeCP2, which was absent in MeCP2-null mice (Horike et al. 2005) and independent of DNA methylation. Also binding of MeCP2 to unmethylated DNA in vitro has been described (von Kries et al. 1991; Buhrmester et al. 1995), as well as its capability of repressing transcription from both methylated and unmethylated DNA (Meehan et al. 1992; Nan et al. 1997; Kaludov and Wolffe 2000). Another group discovered a regulating function of MeCP2 in RNA splicing (Young et al. 2005), further suggesting functions besides its role in DNA methylation.

MBD1 binds to symmetrically methylated DNA and is able to repress promoter activity even from a 3-kb distance (Ng et al. 2000; Fujita et al. 2003). In addition to the TRD, MBD1 contains up to three zinc-coordinating CXXC domains, depending on the splice variants. The third CXXC domain has been shown to bind specifically to non-methylated CpGs and to induce DNA methylation independent transcriptional silencing (Fujita et al. 2000; Jorgensen et al. 2004).

Unlike MBD2 and MeCP2, MBD1 does not appear to interact with HDAC1 or HDAC2, since coimmunoprecipitation failed to show an interaction (Ng et al. 2000). Still TsA relieved the transcriptional repression. Later it was shown, that MBD1 can interact with the histone methyltransferase Suv39h1 and the methyl-lysine binding protein HP1 via its methyl-binding domain and that Suv39h1 associates with HDAC1/2 (Fujita et al. 2003).

In a yeast two-hybrid screening interaction between the transcriptional domain of MBD1 and MCAF (MBD1-containing chromatin-associated factor), a cofactor of the histone methyltransferase SETDB1, was found (Fujita et al. 2003). MCAF itself contains two

conserved domains, one binding MBD1, another binding the transcription factor Sp1. The factor seems to facilitate Sp1-mediated transcription, but when binding to MBD1, it blocks transcription. MBD1 recruits SETDB1 through interaction with MCAF1 to form HP1-condensed heterochromatin (Ichimura et al. 2005).

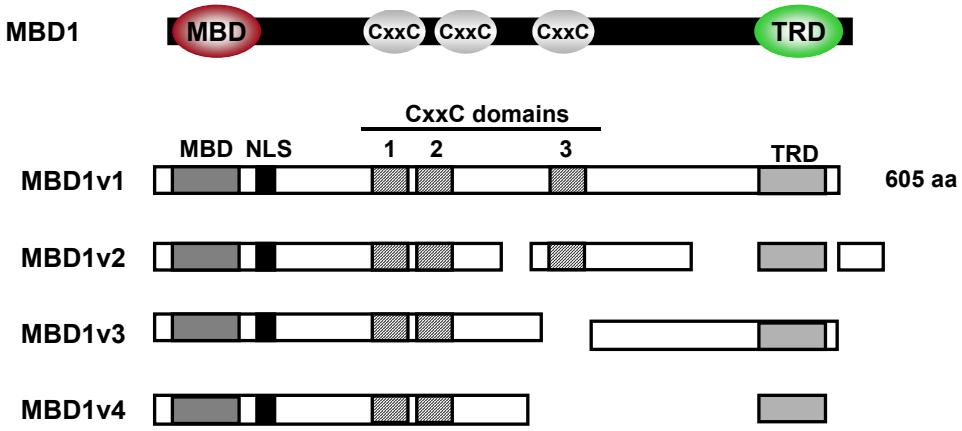


Fig. 6 Different splicing variants of MBD1.

During S phase of the cell cycle **MBD1** has been demonstrated to form a complex with the histone H3 K9 methylase, SETDB1, and the chromatin assembly factor CAFp150 , which in turn is associating with the DNA replication machinery (Sarraf and Stancheva 2004). This may indicate a special role for MBD1 during the cell cycle in linking DNA methylation to histone modification and establishment of gene silencing.

MBD3 is the smallest member of the MBD family. Mammalian MBD3 has lost its ability to bind to methylated DNA, due to a mutation of two highly conserved amino acids (Wade et al. 1999). It is a component of the Mi2-NuRD complex (Zhang et al. 1999), being part of the MeCP1 complex, mentioned above. MBD3 is crucial to normal mammalian development, as MBD3 knockout mice fail to develop to term (Hendrich et al. 2001).

MBD4 can bind to methylated DNA, but first seemed not involved in transcriptional repression. Instead it plays a role in the repair of methyl-CpG/TpG mismatches that can arise from sponaneous deamination (Hendrich et al. 1999). New reports now suggest repressional activity for MBD4 at methylated genes (Kondo et al. 2005). Association of MBD4 with the methylated promoters of $p16^{INK4\alpha}$ and hMLH1 was demonstrated in vivo. The repression is HDAC dependent as HDAC1 and Sin3a directly bind to MBD4.

Splice variants exist for all MBDs. **MBD2** occurs as a full-length protein (MBD2a) and as a N-terminal truncation, that arises through the usage of an alternative translational start codon. The N-terminus of MBD2, lacking in MBD3, contains a repeat of glycine and arginine residues, whose function is still unknown. The truncated MBD2b was reported to demethylate

DNA, but these experiments could not be confirmed by others. MBD2 together with MBD3 is part of the MeCP1 complex (Bhattacharya SK 1999).

MBD2 knock out mice displayed a weak phenotype. Overall methylation and imprinting was not effected in these mice. In reporter gene assays with mouse fibroblast cell lines, established from wildtype and Mbd2-null mice, significantly repression of the was found with the Mbd2 deficient cell lines, indicating a possible redundancy between MBD proteins in the repression of methylated promoters (Hendrich et al. 2001).

So far no sequence specificity was found for MBD2, giving raise to the question weather MBD2 randomly binds to methylated DNA. Sekimata et al could identify a protein binding to MBD2, MIZF (MBD2-interacting zinc finger) with a specific 5 bp recognition sequence for DNA, CGGAC (Sekimata et al. 2001; Sekimata and Homma 2004). The same motif was found in the retinoblastoma gene, in vivo binding of MIZF to the Rb promoter was shown and reduction of the Rb promoter activity in reporter gene assays after cotransfection of MIZF (Sekimata and Homma 2004). From their experiments it seems reasonable, that MIZF recruits MBD2 and potentially the HDAC containing Mi2-NuRD repressor complex to specific target sequences and allows sequence-dependent repression.

Kaiso is a structurally unrelated protein, which has also been shown to bind methylated CGCGs (CGCGCCCAAACG) through its three Kruppel-like C2H2 zinc fingers (Prokhortchouk et al. 2001). The protein belongs to the family of POZ (pox virus and zinc finger) zinc finger transcription factors, which are implicated in development and cancer (reviewed in (Bardwell and Treisman 1994; Albagli et al. 1995)). Most family members act as transcriptional repressors and are characterized by an N-terminal protein-protein interaction POZ domain and a C-terminal DNA-binding domain, that consists of one or more Kruppel-like C2H2 zinc fingers. Kaiso was originally discovered in a yeast two-hybrid system with p120 as a bait (Daniel and Reynolds 1999). Up to date Kaiso is the only POZ-ZF protein that has been shown to have dual specificity DNA binding. It can bind methylated CpG dinucleotides or a specific non-methylated DNA sequence (TCCTGCNA), with the minimal core sequence CTGCNA, via its zinc fingers 2 and 3 (Daniel et al. 2002). P120 intervenes with the sequence specific DNA binding ability of Kaiso (Daniel et al. 2002), . Recently Kaiso was identified as a component of the human NCoR corepressor complex (Yoon et al. 2003) and repression by Kaiso requires the active NCoR complex, which associates HDAC3. Furthermore Kaiso can interact with the human enhancer blocker CTCF, decreasing the insulator activity of CTCF (Defossez et al. 2005).

Besides Kaiso, two so far uncharacterized proteins, **ZBTB4** and **ZBTB38**, containing the Kaiso-like zinc fingers, are reported to bind methylated DNA in vitro and in vivo. When transfected in mouse cells, they colocalize with foci of heavily methylated satellite DNA. In chromatin immunoprecipitation both of these proteins bind to the methylated allele of H19/Igf2. They repress the transcription of methylated templates in transfection assays. Unlike Kaiso, they can bind single methylated CpGs and are tissue-specifically expressed, suggesting non-overlapping functions (Filion et al. 2006).

1.6.2 Histone Code

The fundamental subunit of chromatin is a nucleosome, consisting of 146 bp of DNA wrapped around an octamer of histone proteins, an $(H3/H4)_2$ tetramer assembled with two H2A/H2B dimers (Luger 2003). Consecutive nucleosomes form the "beads-on-a-string" fiber of 11 nm, which can further be compacted into a 30 nm fiber through the incorporation of linker histone H1 protein (Wong et al. 1998; Wolffe 1999).

The flexible higher order structures of chromatin link to the activation state of chromatin. Gene expression occurs at transcriptionally active and structurally accessible states, whereas repression is linked to a structurally condensed state. During the last years more and more different post-translational modifications of the N-termini of histone proteins have been described, among them acetylation of lysines (Grunstein 1997; Sterner and Berger 2000), methylation of lysine and arginine residues (Zhang and Reinberg 2001), phosphorylation of serine and threonine (Nowak and Corces 2004), ubiquitinylation (Davie and Murphy 1990) and sumoylation (Nathan et al. 2003) of lysines, ADP ribosylation (Adamietz and Rudolph 1984), glycosylation (Liebich et al. 1993), biotinylation (Hymes et al. 1995) and carbonylation (Wondrak et al. 2000). These modifications can be highly reversible, such as lysine acetylation and serine and threonine phosphorylation, or more stable, such as lysine and arginine methylation (Zhang and Reinberg 2001; Lachner and Jenuwein 2002). The figure below depicts the variety of modifications of the different residues and the proteins with histone-methyltransferase activity (for review see (Margueron et al. 2005)).

Experimental data suggest, that specific post-translational patterns influence the establishment of subsequent modifications on the same histone tail or on neighbouring tails, resulting in either an accessible or rather condensed chromatin structure. Following this "histone code" hypothesis (Strahl and Allis 2000; Fischle et al. 2003), specific modifications and their implications for gene expression have been described. Elevated H3/H4 acetylation levels of a gene display transcriptional activity. Furthermore the methylation of H3K4 facilitates

subsequent acetylation of H3 and H4 by the acetyltransferase p300. Methylation of H3K9 on the other hand inhibits acetylation (Wang et al. 2001) and leads to heterochromatin formation (Rea et al. 2000; Bannister et al. 2001; Lachner et al. 2001; Nakayama et al. 2001).

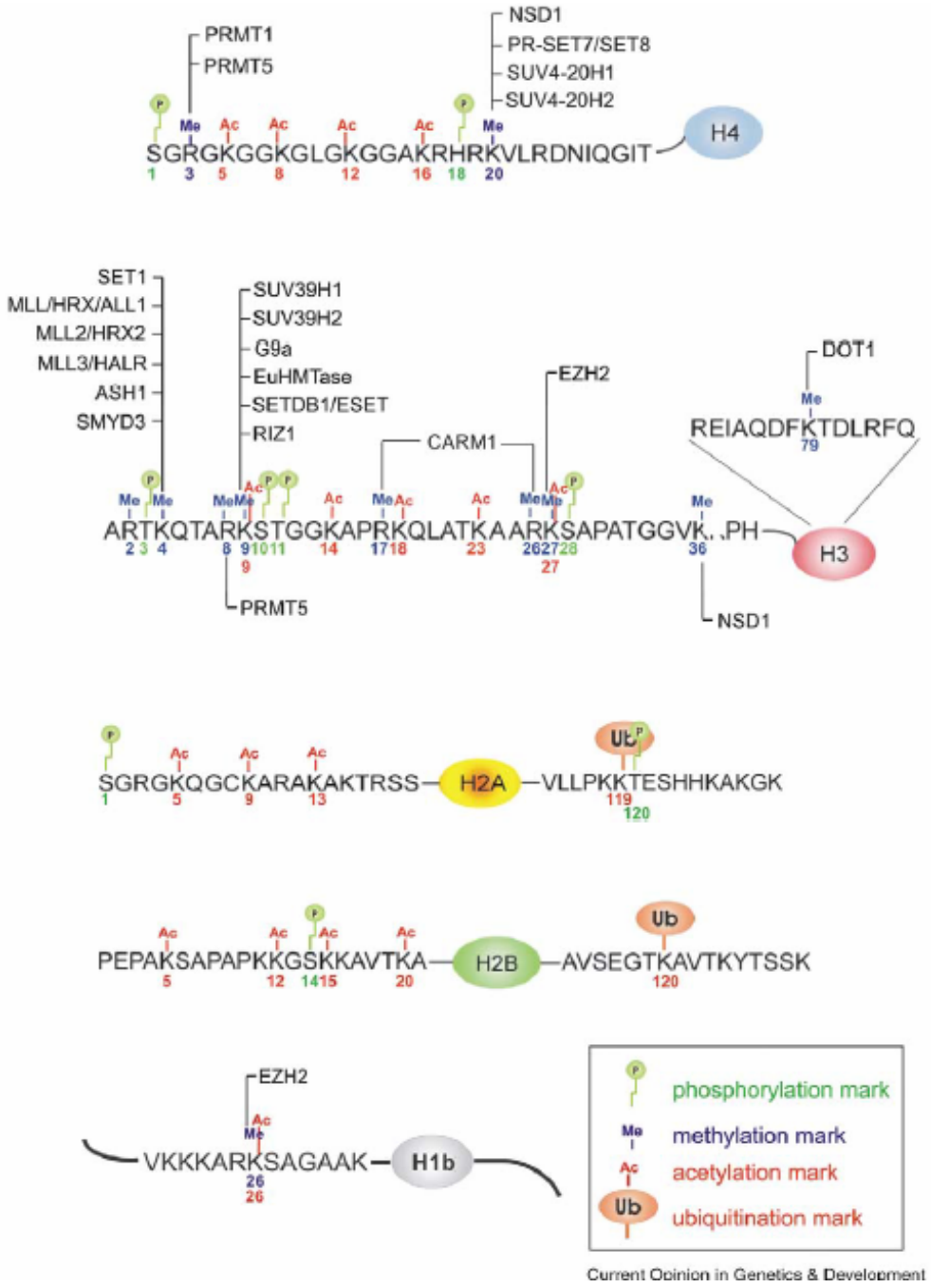


Fig. 7 Post-translational modifications of histones. *Depicted are phosphorylation, methylation, acetylation and ubiquitination sites, as well as the so far known histone methyltransferases and their target sites [figure from (Margueron et al. 2005)].*

One explanation of how histone acetylation implicates active gene expression is the change of histone charges resulting in different interaction with DNA and other nucleosomes, e.g.

elevated acetylation leads to repulsion of DNA entailing a more open chromatin structure. Another model involves the interpretation of the histone modifications by effector molecules. The chromodomain protein HP1 (heterochromatin protein) exclusively binds to di/trimethylated H3K9 (Jacobs and Khorasanizadeh 2002), polycomb protein specifically to trimethylated H3K27 (Cao et al. 2002; Wang et al. 2004). These molecules further spread the histone code. HP1, e.g. can recruit SUV39H1, a histone methyltransferase, presumably methylating the H3 tail of the adjacent nucleosomes, mediating further HP1 binding and spreading of heterochromatin (Aagaard et al. 1999). Acetylated histones recruit positive effectors of transcription, e.g. components of the basal transcription machinery, and other bromodomain proteins (recognizing acetylated lysine) such as p300 and CBP, two histone acetyltransferases, possibly further spreading the acetylation marks and open chromatin structure (Ragvin et al. 2004).

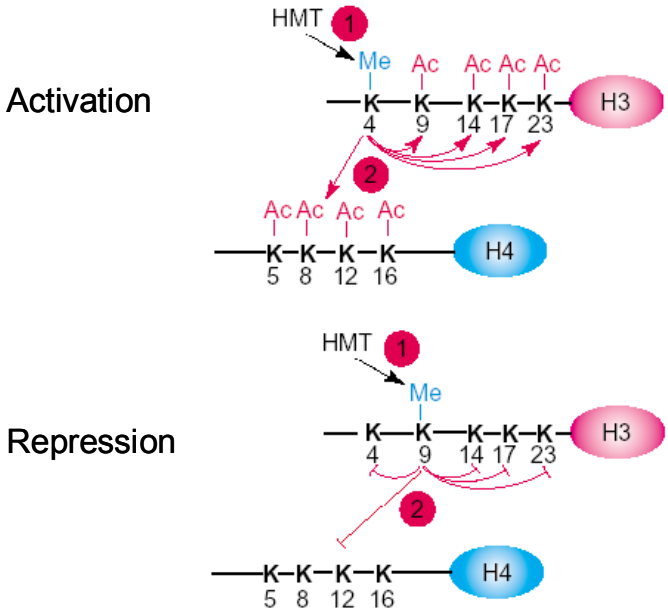


Fig. 8 Example of interaction of different histone modifications. *Methylation of lysine 4 of histone protein H3 facilitates subsequent acetylation of K9, 14, 17 and 23 of H3, as well as acetylation of K5, 8, 12 and 16 of H4. Acetylation leads to transcriptional activation. Methylation of H3K9 on the other hand inhibits subsequent methylation and triggers transcriptional repression [figure from (Margueron et al. 2005)].*

It still remains unclear how cells remember their during the differentiation chosen gene expression profile and carry on the information to the next generation after cell division. The DNA methylation pattern is inherited by the action of DNMT1, recognizing a hemimethylated site and methylating the opposite DNA strand in an appropriate manner. During replication

DNA and histones have to be physically separated during the polymerase transit. How the distribution of old and new histones to both daughter strands works remains unclear.

1.6.3 The "H3 barcode hypothesis"

Besides the genetic and histone code, Hake and Allis have recently proposed the "H3 barcode hypothesis" (Hake and Allis 2006). The hypothesis is based on the existence of three different H3 variants and their possible different biological function. In a study with different mammalian cell lines Hake and colleagues could demonstrate that the expression levels of the H3 variants varied, independent of cell growth, cell cycle state or chromosomal diploidy, suggesting different incorporation patterns during tissue specific differentiation of the cells. Additionally the variants carried different post-translational modifications, which are either known as marks for transcriptionally active chromatin as for H3.3 (acetlyation of K9, K14, K27, K18, K23 and dimethylation of K36, K72) or for silenced chromatin as for H3.2 (di/trimethylation of K27). H3.1 was enriched in both active and repressive marks (K9 methylation and K14 acetylation) (Hake et al. 2006).

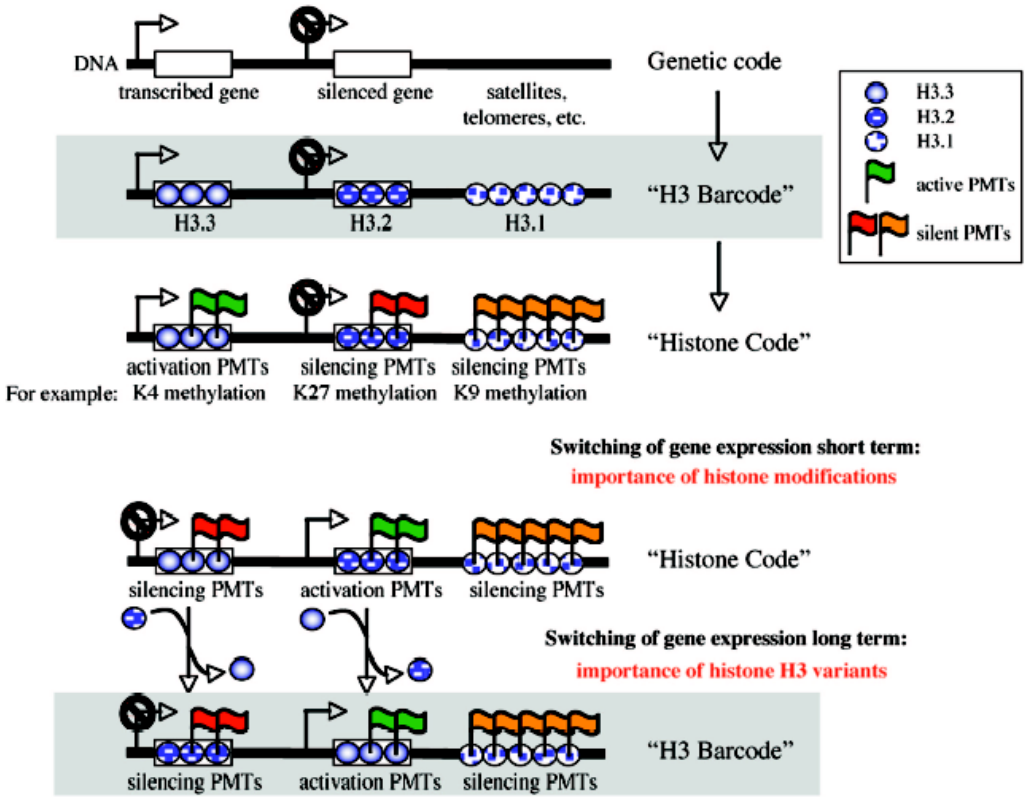


Fig. 9 Graphical combination of the genetic code, the H3 barcode and the histone code. *Incorporation of the three different H3 variants separates the genome into active chromatin (H3.3) or constitutively repressed (H3.2) or facultative repressed chromatin (H3.1). [figure from (Hake and Allis 2006)].*

The hypothesis suggests that the incorporation of different H3 variants separates the chromatin into active chromatin (incorporation of H3.3), constitutive (H3.2) or facultative (H3.1) heterochromatin domains and serves as a long-term epigenetic memory, whereas post-translational modifications of histone proteins rather influence the regulation of short-term gene expression.

1.7 Histone acetylation/deacetylation

As described above histone proteins are subject to dynamic and reversible lysine acetylation. Histone acetyltransferases (**HAT**) catalyze the transfer of acetyl groups from acetyl-CoA to lysine residues of target proteins, while histone deacetylases (**HDACs**) remove them. Acetylation of histone proteins leads to an open and transcriptionally active chromatin structure, whereas deacetylation ends in chromatin compaction. Important positions for acetylation are lysine 9 (K9) and K14, K17, K23 of H3, and K5, K8, K12, and K16 of H4.
The acetylation status of proteins can change protein function, e.g. the DNA binding, transcriptional activation and repressor activity of transcription factors, stability, nuclear localization and coactivator interaction.
Beside histone proteins, other targets of HATs/HDACs have been identified, suggesting substrate and/or function specificity, e.g. transcription factors such as p53 (Juan et al. 2000), GATA-1 EKLF, HNF-4, Sp1 and Sp3 (Doetzlhofer et al. 1999; Ammanamanchi et al. 2003), structural proteins such as tubulin (Hubbert et al. 2002; Matsuyama et al. 2002; Palazzo et al. 2003), Hsp70 (Johnson et al. 2002) and Hsp90 (Kovacs et al. 2005; Murphy et al. 2005). Also the basal transcription machinery itself is targeted, deacetylation of TAFI68 by mSir2a inhibits PolI-dependent transcription (Muth et al. 2001).

1.7.1 HDACs

According to their yeast counterparts, HDACs are divided into three different classes. **Class I** contains HDACs 1, 2, 3 and 8, homologs of the yeast protein RPD3. HDAC1, 2, 3 are ubiquitously expressed, HDAC8 in cells showing smooth muscle differentiation. **Class II** comprises HDACs 4, 5, 6, 7, 9, 10, which are more similar to the yeast histone deacetylase (Hda1), and are tissue-specific expressed. Class I and class II HDACs contain the same conserved catalytic domain, but class II HDACs are two to three times larger in size than class I HDACs. HDAC11 has too low sequence similarity to be classified in either of both classes (Gao et al. 2002).

Class III members are homologs of the silent information regulator (Sir2), also called sirtuins. The sirtuin deacetylases contain a 275 aa catalytic domain, which is unrelated to that of HDACs. They require NAD as substrate and operate via a different mechanism (Frye 2000). Nicotinamide is liberated from NAD^+ while the acetyl group of the substrate is transferred to cleaved NAD^+, generating O-acetyl-ADP-ribose. This class of deacetylases appears not to have histones as their primary substrates and is not inhibited by compounds that inhibit class I and class II HDACs. Nicotinamide inhibits SIR2 action (Bitterman et al. 2002; Anderson et al. 2003).

HDAC1 binds **HDAC2** to form the catalytic core of the Sin3, Mi-2/NuRD/NRD and CoREST complexes, whereas **HDAC3** is the catalytic subunit of the NCoR and SMRT complexes (Cress and Seto 2000; Ng and Bird 2000; Grozinger and Schreiber 2002). These protein complexes are necessary for HDAC activity (Carmen et al. 1999; Hu et al. 2000; Lee et al. 2004) and serve as corepressors for several transcriptional repressors (Zhang et al. 1999; Guenther et al. 2001). The class II HDACs 4, 5, and 7 cannot be activated by the SMRT/NCoR complex. They are not active deacetylases, but recruit the HDAC3/SMRT/NCoR complex (Fischle et al. 2001; Fischle et al. 2002).

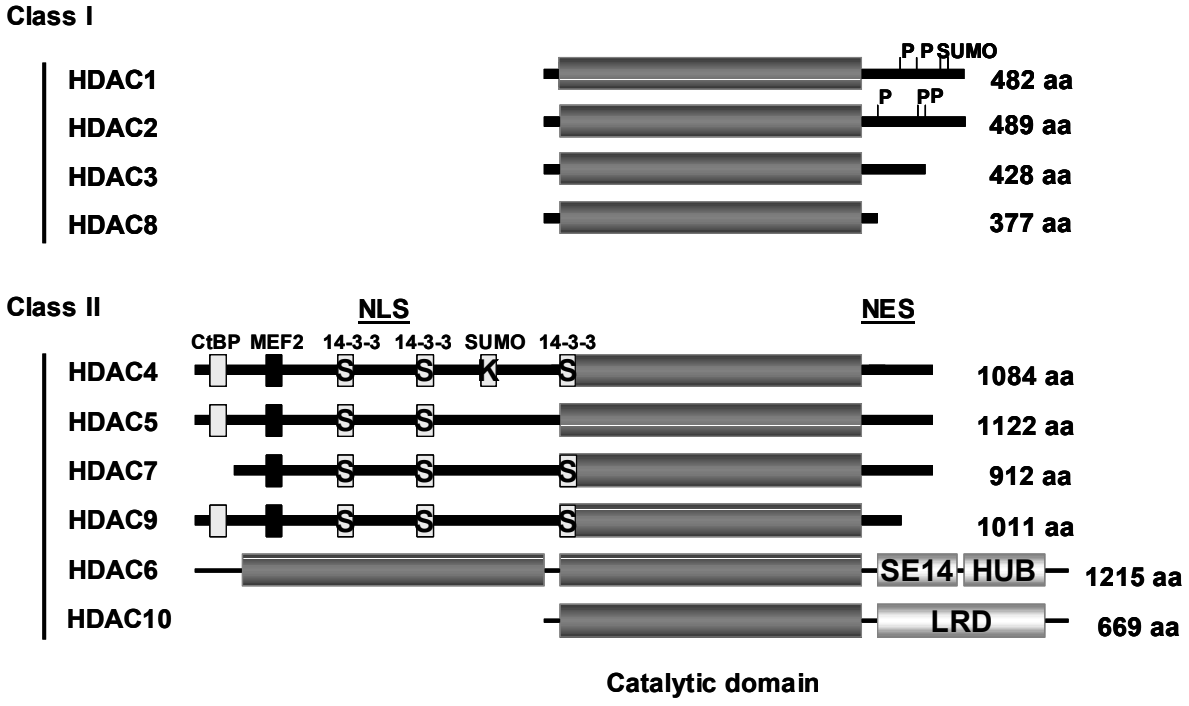


Fig. 10 Structures of class I/II histonedeacetylases. *Class I HDACs are homologs of the yeast protein Rpd3, class II HDACs are homologs of the Hda1 yeast protein. They all share the same catalytic domain, but class II HDACs are 2-3 times larger than class I HDACs.*

HDAC1, 2, and 3 also cooperate with other chromatin and transcriptional regulators, such as ATP-dependent chromatin remodelers (Underhill et al. 2000; Battaglioli et al. 2002;

Kuzmichev et al. 2002), DNA methyltransferases (Fuks et al. 2001; Burgers et al. 2002; Di Croce et al. 2002), histone methyltransferases (van der Vlag and Otte 1999; Czermin et al. 2001; Tie et al. 2001) and topoisomerase II (Tsai et al. 2000; Lupo et al. 2001). Except for HDAC3 (Fischle et al. 2001; Fischle et al. 2002; Yang et al. 2002), which contains both a nuclear import and export signal, all class I HDACs are exclusively located in the nucleus (Johnstone 2002). There is evidence that **HDAC8** has also other targets than histone proteins. The potent HDAC8 inhibitor SB-379278-A did not affect the cellular histone acetylation status or SV40 promoter activity in the human colon carcinoma cell line SW620 (Hu et al. 2003). Waltregny et al. could also show an exclusive expression of HDAC8 in vivo in cells showing smooth muscle differentiation (Waltregny et al. 2004).

HDAC8 associates with the smooth muscle isoform of α-actin in primary human smooth muscle cells. So far it is unclear, if HDAC8 deacetylase activity is involved in the regulation of any smooth muscle cytoskeletal protein. Repression of HDAC8 expression however, alters the morphology of the cells and reduces smooth muscle contractility (Waltregny et al. 2005).

Class II HDACs are structurally and functionally different from class I HDACs. They are divided into two subclasses, IIa (HDAC4, 5, 7, 9) and IIb (HDAC6, 10, 11). IIa members interact with transcription factors including MEF2, BCL6, PLZF and TR2, with transcriptional corepressors such as NCoR, SMRT, CtBP and with HP1, the methyl-lysine-binding protein (Bertos et al. 2001; Fischle et al. 2001; Khochbin et al. 2001; Lemercier et al. 2002; McKinsey et al. 2002). Class II HDACs can shuttle between the nucleus and the cytosol.

Class IIa HDACs are regarded as regulators of myogenesis. They bind to the myocyte enhancer factor-2 (MEF2) and repress specific gene expression in a calcium-dependent manner (Miska et al. 1999; Sparrow et al. 1999; Lu et al. 2000; Youn et al. 2000). Similar to class IIa HDACs the transcription factor MEF2 is highly expressed in muscle cells, neural cells and T-cells. Upon recruitment of class IIa HDACs, gene expression is repressed. Activation by calcium signaling leads to a release of HDACs and activation of transcription (Grozinger and Schreiber 2000; McKinsey et al. 2000; Youn et al. 2000). There is also evidence for an essential role of MEF2 and HDACs in the development and function of the heart. The deletion of **HDAC9** in mice leads to cardiac hypertrophy (Zhang et al. 2002). A mutation of MEF2A has been linked to human coronary artery disease and myocardial infarction (Wang et al. 2003). Class IIa HDAC5 and HDAC9 act as repressors of stress-induced heart hypertrophy (Chang et al. 2004).

HDAC6 is mainly localized in the cytosol. It contains two catalytic domains, both having histone deacetylase activity, but only the C-terminal domain is possessing tubulin deacetylase activity (Grozinger et al. 1999; Haggarty et al. 2003). HDAC6 functions as a α-tubulin deacetylase and regulates microtubule-dependent cell motility (Hubbert et al. 2002; Haggarty et al. 2003; Zhang et al. 2003). Additionally it was discovered, that HDAC6 associates with ubiquitinated proteins (Seigneurin-Berny et al. 2001; Hook et al. 2002) and is a component of the aggresome, where misfolded proteins are processed (Kawaguchi et al. 2003). The tubulin activity of HDAC6 is also involved in the regulation of HIV-1 infection and Env-mediated syncytia formation. Overexpression of HDAC6 prevented HIV-1 envelope-dependent cell fusion and infection without affecting the expression and distribution of HIV-1 receptors (Valenzuela-Fernandez et al. 2005). Another protein regulated by reversible acetylation is chaperone Hsp90. Inactivation of HDAC6 results in Hsp90 hyperacetylation and the loss of chaperone activity. Hsp90 is necessary for the glucocorticoid receptor (GR) to assume its competent ligand binding conformation, and hyperacetylation of the heat shock protein results in GR defective ligand binding, nuclear translocation and transcriptional activation (Bali et al. 2005; Kovacs et al. 2005; Murphy et al. 2005).

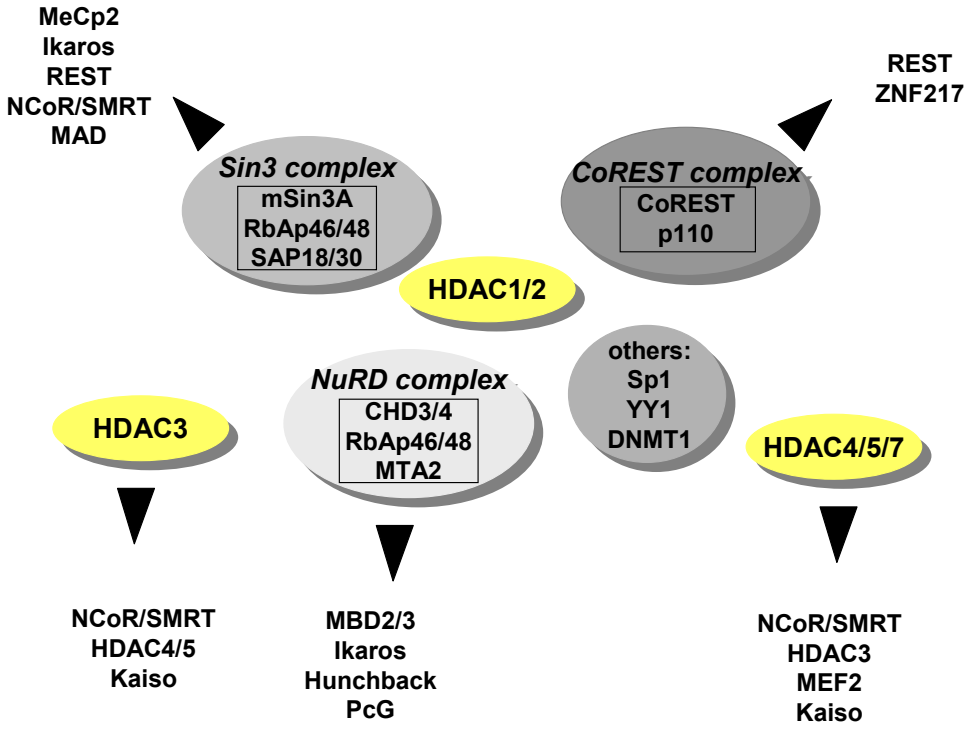


Fig. 11 Various interactions of HDAC proteins. *HDAC1 and HDAC2 are found in the three main corepressor complexes Sin3, NuRD and CoREST, which are recruited to promoters or DNA binding domains of proteins. HDAC1/2 also interact directly with DNA binding proteins such as YY1, Sp1 and others. HDAC3, 4, 5, and 7 associate with the NCo/SMRT corepressor complexes, with the MEF2*

transcription factor family, or the methyl-CpG-binding protein Kaiso. The corepressor complexes Sin3, CoRest and Mi-2/NuRD can further associate with MBD proteins, linking again, histone deacetylation to DNA methylation.

1.7.2 Regulation of HDAC activity

As reviewed in (Burke and Baniahmad 2000; Wade 2001; Grozinger and Schreiber 2002; Sengupta and Seto 2004) the activity of HDACs is regulated via several mechanism. **I)** HDACs may interact directly with DNA binding proteins, which specifically bind to a certain promoter, e.g. HDAC1 and HDAC2 bind to YY1 (Yang et al. 1996), Rb-binding protein (Brehm et al. 1998), and Sp1/Sp3 (Sun et al. 2002). **II)** Instead of direct interaction with a DNA binding protein, HDACs can be recruited via protein-protein interaction to a DNA binding protein, e.g. as a component of the Sin3a complex. This complex is recruited by nuclear hormone receptors in the absence of ligand. Upon ligand binding, the receptor changes its confirmation and HATs instead of HDACs form a complex with the receptor. **III)** HDACs and HATs can compete for the same binding site of a transcription factor, e.g. HDAC4 and p300 compete for the same binding site of MEF2 (Youn et al. 2000). **IV)** Class II HDACs 4, 5, and 7 shuttle between the nucleus and the cytosol (Grozinger and Schreiber 2000). After phosphorylation at their N-termini, binding to MEF is interrupted and the proteins are exported from the nucleus (McKinsey et al. 2000). Subsequent recruitment of HATs by MEF leads to acetylation and activation of MEF target promoters (Lu et al. 2000). **V)** Post-translational modifications such as phosphorylation, also play an important role in the regulation of HDAC activity. Phosphorylation of HDAC1 by CK2 promotes enzymatic activity as well as the ability to form protein complexes (Pflum et al. 2001). Also HDAC2 is phosphorylated by CK2 (Tsai and Seto 2002), which is necessary for enzymatic activity and complex formation with Sin3a and Mi-2/NuRD/NRD. Furthermore Sun et al. could show the association of Sp1/Sp3 with HDAC1 and phosphorylated HDAC2 in human breast cancer cells (Sun et al. 2002). Phosphorylation of HDAC8 by PKA reduces deacetylase activity and leads to H3/H4 hyperacetylation (Lee et al. 2004). Phosphorylation of HDAC4 and 5 inhibits binding to MEF and results in nuclear export (Lu et al. 2000; Lu et al. 2000). Besides phosphorylation, sumoylation of HDAC1 (Colombo 2002; David et al. 2002), HDAC4, and HDAC6 has been described (Kirsh et al. 2002). Though the studies for HDAC1 are contradictory, sumoylation of HDAC4 enhances deacetylase activity. Sumoylation of MEF2 itself inhibits transcription. HDAC4 potentiates sumoylation of MEF2, but is inhibited by the sumoylation of HDAC4 itself (Gregoire and Yang 2005).

1.7.3 Histone deacetylase inhibitors

The active site of HDACs I and II consist of a gently curved tubular pocket with a wider bottom (Finnin et al. 1999). The removal of the acetyl group occurs via a charge-relay system, which requires a Zn^{2+} ion, bound to the bottom of the pocket, coordinated by several histidine and aspartate residues. HDACi function by replacing the Zn^{2+} ion. TsA is the most potent reversible HDACi currently known, with an IC50 in the nanomolar range, perfectly fitting to the active site (Yoshida et al. 1990). The **hydroamic acid functional group** coordinates the zinc ion, the aliphatic chain makes van der Waals contacts within the channel leading to the catalytic center and the cap group has contact with residues on the rim of the pocket. Class I and class II HDACs are all equally sensitive to TsA (Marks et al. 2001; Marmorstein 2001; Yoshida et al. 2001; Johnstone 2002). Similar to the natural compound TsA, other hydroxamic acid group containing substances have been developed, e.g. suberoyl anilide hydroxamic acid (SAHA), NVP-LAQ824, PDX101, oxamflatin and others.

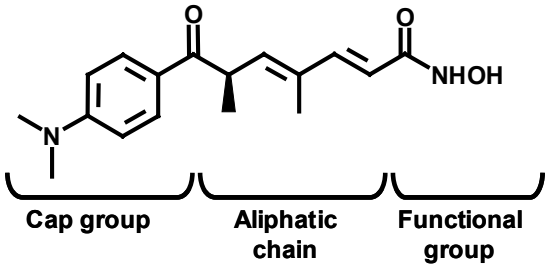


Fig. 12 Trichostatin A - Structural properties of HDAC inhibitors. *Trichostatin A contains a hydroxamic acid functional group, which chelates the zinc ion in the catalytic center of the histone deacetylases. The aliphatic chain mimics the lysine and has an optimal length of 5-6 carbons. The cap group interacts with the residues surrounding the rim of the catalytic cave.*

A second group of inhibitors comprises **short fatty acids**, as butyrate, phenylbutyrate and the anticonvulsant valproic acid (VPA), which are less efficient inhibitors than TsA with IC50 values in the millimolar range. **Cyclic tetrapeptide antibiotics**, including the natural compound trapoxin, and benzamides, e.g. MS-275 and Cl-994 (p-N-acetyl dinaline) comprise two other groups of inhibitors.

All HDACs react equally sensitive to the different HDACi. Exceptions are HDAC6, which is sensitive to TsA, but not to trapoxin B (Furumai et al. 2001). Class I HDACs are five times more efficiently inhibited by VPA, than class II HDACs (Gottlicher et al. 2001). HDAC4 is less sensitive to butyrate. The benzamide MS-27-275 preferentially inhibits HDAC1 with an IC50 around 0.3 μM, compared to the inhibition of HDAC3 with an IC50 of 8 μM (Hu et al. 2003). The novel synthetic compounds SK-7041 and SK-7046, with the hydroxamic acid

group from TsA and a pyridyl ring from the benzamide MS-275, preferentially target HDAC1 and HDAC2 (Park et al. 2004).

Unexpectedly HDACi selectively alter the expression of only 2-10% of genes in cultured transformed cells (Van Lint et al. 1996; Mariadason et al. 2000; Glaser et al. 2003; Mitsiades et al. 2004). In a newer microarray study with SAHA and depsipepetide the expression of a higher number of genes, at least 22%, was regulated by both substances within an 16 h interval (Peart et al. 2005). Diverse HDACi seem to activate a common set of genes.

HDACi induce cancer cell cycle arrest, growth inhibition, differentiation, and programmed cell death (Johnstone 2002; Marks et al. 2004; Dokmanovic and Marks 2005). For this reason different HDACi are in clinical trials for cancer treatment, among them sodium phenyl-butyrate, SAHA, LAQ824, depsipeptide, MS-275, Cl-944, yroxamide, PXD101 and valproic acid (Dokmanovic and Marks 2005; Liu et al. 2006).

While all class I and class II HDACs are equally sensitive to TsA, **valproic acid** preferentially inhibits class I HDACs. HDAC2 is about 5-fold more efficiently inhibited than HDAC5 and HDAC6 (Gottlicher et al. 2001), HDAC6 and HDAC10 are not inhibited by VPA (IC50 > 20 mM) (Gurvich et al. 2004). In contrast to other HDACi, VPA also induces proteasomal degradation of HDAC2 (Kramer et al. 2003). Valproic acid has also been shown to induce 5-LO expression in murine hippocampus and in the human neuron-like cell lines NT2 and NT2-N (Yildirim et al. 2003; Zhang et al. 2004).

Nicotinamide (vitamin B3), as an inhibitor of class III deacetylases is often used to treat anxiety, osteoarthritis and psychosis and is in clinical trials for treatment of cancer and type I diabetes (Luo et al. 2001; Vaziri et al. 2001; Bitterman et al. 2002).

1.8 Gene regulation by Sp1 and Sp3

1.8.1 Structure of Sp transcription factors Sp1 and Sp3

Sp proteins form a subgroup of Sp/Kruppel-like factors (KLFs). The family of Sp transcription factors comprises by now eight members, Sp1 to Sp8, and is characterized by the presence of a particular combination of three conserved Cys2His2 zinc fingers at the C-terminus, which form the DNA-binding domain of these factors. Sp1 and Sp3 are the best examined family members. Besides the zinc fingers Sp1 and the long isoforms of Sp3 contain two well conserved N-terminal transcription activation domains, A and B (Courey and Tjian 1988; Courey et al. 1989; Pascal and Tjian 1991; Suske 1999). The C domain of Sp1 consists of highly charged amino acids. The D domain in Sp1 enables the formation of high order

complexes and synergistic activation together with the A and B domains and is not found in Sp3 (Hagen et al. 1994; Dennig et al. 1996). Whereas Sp1 possesses an N-terminal inhibitory domain, the inhibitory domain of Sp3 is close to the zinc finger domain (De Luca et al. 1996; Dennig et al. 1996). All Sp proteins contain a N-terminal Sp box, an endoproteolytic cleavage site (Su et al. 1999).

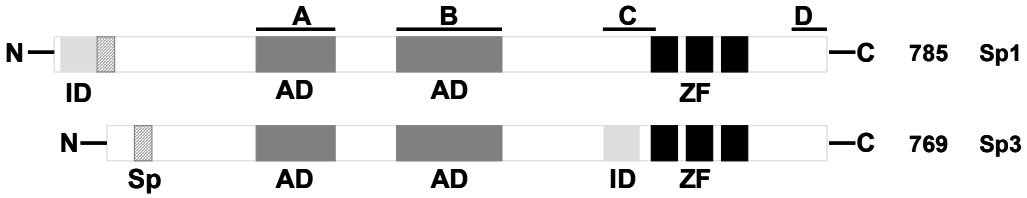


Fig. 13 Domains of Sp1 and Sp3. *(AD) activation domain, (ID) inhibitory domain, (ZF) zinc finger.*

1.8.2 DNA affinity

Sp1 and Sp3 are ubiquitously expressed, Sp4 predominantly in neuronal cells and some epithelial cells. Except for Sp2, all family members bind to GC-rich elements such as the GC-box (GGGGCGGGG) and the related GT/CACCC-box (GGTGTGGGG), found in promoters of housekeeping genes, tissue-specific expressed genes, viral genes and cell cycle regulated genes with identical affinity (Hagen et al. 1994; Suske 1999; Black et al. 2001). A study demonstrated that Sp1 can also bind to a GC box in a nucleosome, though the binding affinity is 10- to 20-fold reduced compared to a naked DNA (Li et al. 1994).

Sp1 forms a tetramer and then associates with the binding site, thus providing an enlarged interface for protein interactions (Mastrangelo et al. 1991). This synergistic transcriptionally activation is also seen in promoters with multiple Sp1 binding sites, Sp1 loops intervening DNA between distal and proximal promoter sites (Pascal and Tjian 1991; Su et al. 1991). The synergistical activation is not exhibited by Sp3 (Yu et al. 2003). Sp3 is unable to form oligomers (Mastrangelo et al. 1991; Pascal and Tjian 1991; Yu et al. 2003). On the other hand, Sp3 forms more stable complexes at adjacent binding sites than multimeric Sp1-DNA complexes and displaces Sp1 from these adjacent binding sites, possibly explaining the repressive effects of Sp3 by disturbing Sp1 dependent synergistic transactivation (Yu et al. 2003). However the p21 promoter contains six Sp1 binding sites and Sp3 activates the promoter stronger than Sp1 (Sowa et al. 1999; Gartel et al. 2000).

Regarding the effects of CpG methylation, the reports are controversial. In some studies DNA methylation does not interfere with Sp1 binding (Holler et al. 1988; Clark et al. 1997; Zhu et al. 2003), in others CpG methylation reduces Sp1 binding in gel shifts (Clark et al. 1997;

Mancini et al. 1999). In the p21 promoter methylation of adjacent residues of the GC box reduced Sp1 binding stronger than methylation of the central CpG (Clark et al. 1997; Zhu et al. 2003). In reporter gene assays with the p16 and SNRPN promoter, Sp1 cotransfection induced promoter activity despite in vitro methylation of the promoter constructs (Fujita et al. 2000).

1.8.3 Transcriptional control by Sp1/Sp3

Sp1 and Sp3 may act as negative or positive regulators of gene expression. The general mechanism for Sp protein dependent transactivation involves DNA-binding and subsequent interaction with components of the basal transcription machinery for constitutive expression (Pugh and Tjian 1990; Smale et al. 1990; Tanese et al. 1991; Emami et al. 1998). Furthermore, the CRSP complex, "cofactors required for Sp1 activation", a multiunit cofactor complex regulates transcription by mediating signals between enhancer-bound factors and the core transcriptional machinery (Ryu and Tjian 1999; Ryu et al. 1999; Taatjes et al. 2002). The protein complex consists of subunits, which are shared by other mediator complexes, for example the DRIP complex (vitamin D interacting proteins) or the analogous TRAP complex (thyroid hormone receptor associated proteins) (Rachez and Freedman 2001). Different activators seem to induce different conformational changes upon binding to selective subunits of the complex thus allowing promoter selectivity (Taatjes et al. 2004). Also the composition of the complex defines its actions, for example, the CRSP/Med2 complex was isolated, lacking both subunits Med220 and Med70. The complex was able to potentiate transcription in response to Sp1, but not to VDR (Taatjes et al. 2004; Taatjes and Tjian 2004).

1.8.3.1 Expression ratio of Sp1/Sp3

The most simple mechanism of regulation depends on the relative levels of Sp1 and Sp3 protein expression. In Caco-2 cells for example, the MAO B promoter is regulated by Sp1 and Sp3. Sp3 acts as an repressor on the promoter. With differentiation of Caco-2 cells, Sp3 expression decreases and MAO-B expression increases (Wong et al. 2003). Similarly the human secretin promoter is regulated by the Sp1/Sp3 protein ratio. Higher Sp1 levels induced transcription whereas higher Sp3 levels repressed the promoter activity (Pang et al. 2004). The basal regulation of 15-lipoxygenase is dependent on Sp1 interaction with the promoter, whereas Sp3 decreases promoter activity (Tang et al. 2004).

1.8.3.2 Interaction with other proteins

Since Sp proteins can also interact with multiple other DNA-binding proteins and coactivators/corepressors the transcriptional control further strongly depends on the promoter and cellular context. This is described by the multiple reports regarding the regulation of the p21 promoter by Sp1/Sp3/KLF4 DNA binding to the six proximal GC-rich elements and different protein interactions in different cell lines [reviewed in (Safe and Kim 2004)]. Amongst others activation of p21 expression was induced by p53 in several cell lines (Zhang et al. 2000; Koutsodontis et al. 2001), by calcineurin-induced NFAT1 and NFAT2 (Santini et al. 2001), by c-jun in HepG2 cells and repressed in human embryonic epithelial 293 cells all via distinct GC-sites within the p21 promoter (Kardassis et al. 1999; Wang et al. 2000).

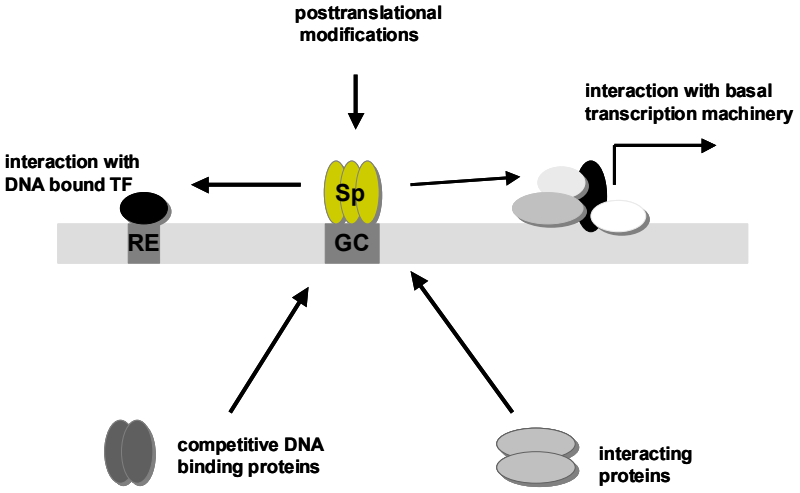


Fig. 14 Multiple regulation of Sp mediated transcriptional control.

Other transcription factors interacting with Sp1 include Ahr, Arnt, GATA-1, GATA-2, GATA-3, NF-YA, VHL, MyoD, PML. HTLF, E2F1, YY1, MDM2, c-jun, AP-2, myc, NFAT-1, HD protein, cyclin A, Oct-1, TBP, HNF3, HNF4, p53, MEF2C, SMAD2, SMAD3, SMDA4, Msx1, several viral proteins, Rb, E1a, p107, ZBP89, DNMT1 (Song et al. 2001; Song et al. 2001), histone modifying proteins such as HDAC1 (Doetzlhofer et al. 1999; Kang et al. 2005), p300 (Mastrangelo et al. 1991; Jang and Steinert 2002; Huang et al. 2005) and nuclear receptors such as ER, AR, PR, RAR, RXR, PPARγ, VDR, the orphan nuclear receptors SF-1 (steroidgenic factor-1) and COUP-TFII (chicken ovalbumin upstream promoter transcription factor-II [reviewed in (Safe and Kim 2004)].

Nuclear receptors instead of binding to their response element, can interact with other transcription factors, e.g. Sp1 to activate gene expression. $1,25(OH)_2D_3$ upregulates p27 expression by inducing complex formation of a VDR/Sp1 complex. Sp1 in this case functions as an anchor protein for VDR (Huang et al. 2004; Cheng et al. 2006).

Recently an interaction between the corepressors SMRT, NCoR and BCoR has been described between the zinc finger domain and the Sp1 inhibitory domain, giving another possible explanation for the repressive effects of Sp1 in some contexts (Lee et al. 2005).
Multiple reports describe the recruitment of HDAC1 and HDAC2 by Sp1 and/or Sp3 to gene promoters. The histone deacetylase inhibitor TsA induces expression of the human telomerase reverse transcriptase (hTERT) gene (Hou et al. 2002), the human luteinizing hormone receptor gene (Zhang and Dufau 2002; 2003), the TGFβ type II receptor (Huang et al. 2005), insulin-like growth factor binding protein-3 gene (Choi et al. 2002) and others. The effect is usually reversed by mutation of a specific Sp1 binding site in the proximal promoter area. TsA treatment leads to a release of HDAC1 and Sp3 from the Sp1/Sp3/HDAC1/p300 transcriptional complex from the IGFBP-3 promoter in Hep3B cells and increases Sp1 binding. TsA also induces acetylation (Huang et al. 2005) or phosphorylation of Sp1 (Choi et al. 2002).

1.8.3.3 Sp3

In vivo four isoforms of **Sp3** are expressed that differ in the extent of the N-terminal part, deriving from alternative translational start sites and not from splicing events. The two slow migrating isoforms form bands around 78/80 kDa, lacking the A domain, the long isoforms around 115 kDa. None of the isoforms become glycosylated as Sp1, but all become sumoylated at lysine 551 within the inhibitory domain of Sp3. Sumoylation renders all isoforms inactive (Ross et al. 2002; Sapetschnig et al. 2002; Spengler et al. 2005). The long isoforms act as transcriptional activators whereas the small isoforms are inactive (Sapetschnig et al. 2004) and can compete with Sp1 binding or promoter-specific transcription factors, thereby repressing transcription (Hagen et al. 1994; Kennett et al. 1997; Kumar and Butler 1997; Majello et al. 1997; Kennett et al. 2002). However it remains unclear why Sp3 acts as an activator/repressor on some promoter settings, but not on others.

1.8.3.4 Posttranslational modifications

Braun et. al. formerly described the acetylation of the same lysine residue of **Sp3** that becomes sumoylated (Braun et al. 2001). In vitro CBP and p300, but not PCAF could acetylate Sp3. Originally the acetylation event was discussed to be involved in the inhibitory function of Sp3, since the lysine residue 553 (KEE motif) is essential for silencing function (Braun et al. 2001). However in other reports the acetylation of Sp3 increases the transactivation activity. E.g. in the late passage of MCF7 cells, Sp3 acted as an transcriptional

repressor of the TGFβ receptor expression because of a higher Sp3/Sp1 ratio, whereas TsA-induced acetylation of Sp3 recovers TGFβ expression by transactivation of Sp3 (Ammanamanchi and Brattain 2001; Braun et al. 2001; Ammanamanchi et al. 2003). It is possible that acetylation and sumoylation of the same lysine residue regulate Sp3 transcriptional activity (Ross et al. 2002; Sapetschnig et al. 2002; Sapetschnig et al. 2004; Spengler et al. 2005). No phosphorylation of Sp3 has been described so far.

Phosphorylation of **Sp1** can occur at multiple sites by many kinases both in the N- and C-terminus. Phosphorylation of Sp1 is induced by various signals including viral infection, growth factors, certain drugs, cytokines, mechanical stress, etc (Jackson et al. 1990; Lin et al. 1997; Chun et al. 1998; Alroy et al. 1999; Black et al. 1999; Ray et al. 1999; Kim and DeLuca 2002; Pan and Hung 2002; Bonello and Khachigian 2004).

Phosphorylation by phosphatidylinositol-3-kinase (PI3-K) e.g. increases phosphorylation of Sp1 in several prostate cancer cells lines and enhances binding to the promoter of the vascular endothelial growth factor (VEGF) (Pore et al. 2004). Phosphorylation by PKA (Lee et al. 2003), PKC (Rafty and Khachigian 2001), cyclin A-cyclin-dependent kinases (Fojas de Borja et al. 2001; Haidweger et al. 2001; Banchio et al. 2004) and mitogen-activated protein kinase (Onishi et al. 2001; Milanini-Mongiat et al. 2002) have also been described and linked to increased DNA binding and transcription activity of Sp1. As reviewed by Chu et. al phosphorylation does not always increase the DNA-binding ability of Sp1 and induced transactivation can occur independently of an enhanced DNA-binding affinity (Lin et al. 1997; Reisinger et al. 2003; Chu and Ferro 2005). Sp1 phosphorylation also increases ubiquitinylation and subsequent proteolysis, decreasing Sp1 nulear protein levels (Leggett et al. 1995; Mortensen et al. 1997). Since Sp1 can be phosphorylated at various sites or combination of sites, phosphorylation leads to a range of changes in protein function, further depending on interacting proteins, the promoter and cellular context.

Similar to Sp3, TsA induced acetylation of Sp1 resulted in increased transcription of the TGFβ II receptor (Huang et al. 2005). Trichostatin A treatment altered a multiprotein complex consisting of Sp1, NF-Y, HDAC1, p300 and PCAF. Whereas p300 and PCAF recruitment was increased, HDAC1 interaction was decreased. The authors could also show, that acetylation of Sp1 was dependent on PCAF histone acetyltransferase activity and that PCAF can acetylate Sp1 in vivo. Acetylation of Sp1 was also induced after treatment of various human cancer cell lines with the DNA topoisomerase II poison TAS-103, but only in p300 expressing cells. The treatment increased p300 expression and interaction with Sp1, resulting in induced promoter activity of the SV40 promoter (Torigoe et al. 2005). In an earlier study it

was demonstrated that the acetyltransferase region of p300 interacts with the DNA binding domain of Sp1 and stimulates DNA binding of Sp1 rather physically than by acetylation (Suzuki et al. 2000).

Concerning serine/threonine O-linked β-N-acetylglucosamine glycosylation, reports describe different consequences for Sp1 function. For example, glycosylation protects from subsequent degradation after phosphorylation, indicating a reciprocal relationship between phosphorylated and glycosylated Sp1 (Han and Kudlow 1997; Wells et al. 2001; Spengler and Brattain 2006). The modification intervenes the Sp1 interaction with TATA-binding protein-associated factor (TAF110) (Roos et al. 1997) and in some contexts inhibits Sp1-dependent transactivation (Yang et al. 2001), in others increases Sp1 transactivation (Roos et al. 1997).

Recently sumoylation of Sp1 has been described and linked to transcriptional repression of Sp1. The sumoylation of lysine 16 inhibits the N-terminal cleavage of Sp1 within a negative regulatory domain. The authors suggest an inhibiting role of the non-cleaved Sp1 in transcription via recruitment of corepressors to the uncleaved binding domain (Spengler and Brattain 2006).

The multiple interactions of Sp1/Sp3 with other DNA binding proteins, coactivators/corepressors, DNA and histone modifying proteins, point out, how complex a promoter in a specific cell context may be regulated.

2 Aims of the present study

As outlined in the introduction, the mechanism how 5-LO gene transcription is regulated, is not yet fully understood. Besides the prominent upregulation triggered by differentiation of cells of myeloid origin by $1,25(OH)_2D_3$ and TGFβ (Brungs et al. 1994; 1995; Harle et al. 1998; 1999; Uhl et al. 2002; Sorg et al. 2006), which only partly depends on the transcriptional level, transcription factors binding to GC-rich sequences including Sp1/Sp3 and Egr1 (In et al. 1997; Silverman et al. 1998; Dishart et al. 2005), as well as proteins involved in the regulation by DNA methylation and histone deacetylation (Uhl et al. 2002; Klan et al. 2003), e.g. methyl-CpG-binding proteins and HDACs, are/could be recruited to the 5-LO promoter under various conditions.

One aim of this study was to investigate how the histone deacetylase inhibitor TsA induces 5-LO promoter activity and gene expression. Early studies indicated the presence of relevant elements within the core promoter region, possibly Sp1 binding sites (Klan et al. 2003). An upregulation of gene expression by histone deacetylase inhibitors suggests the recruitment of HDACs, often in combination with HATs, within the proximal promoter area. Treatment with HDACi in some cases change the composition of multiprotein-complexes, e.g. consisting of Sp1/NF-Y/HDAC1/p300, as shown for the TGFβII receptor (Huang et al. 2005). Also posttranslational modifications of transcription factors by HDACs or HATs in response to HDACi have been described, e.g. acetylation or phosphorylation of Sp1/Sp3 after TsA treatment (Choi et al. 2002; Ammanamanchi et al. 2003; Huang et al. 2005), changing the transactivation or binding properties of the transcription factors.

Since 5-LO expression and DNA methylation of the 5-LO core promoter correlate in 5-LO positive and 5-LO negative cell lines (Uhl et al. 2002), proteins involved in the regulation by DNA methylation, such as methyl-CpG-binding proteins, should be identified and the potential binding sites within the core promoter isolated. In order to investigate the influence of DNA methylation on the binding affinity of Sp1, gel shift studies were performed with methylated DNA stretches.

3 Materials and Methods

3.1 Cell lines

Mono-Mac-6 cells

Human acute monocytic leukemia cell line; differentiation to macrophages possible; cells were kindly provided by H. W. L. Ziegler-Heitbrock, Inst. of Immunology, Univ. of Munich. The cells show 5-LO expression after differentiation with TGFβ and $1,25(OH)_2D_3$.

HeLa cells

Established from human epitheloid cervix carcinoma; later diagnosis changed to adenoma carcinoma; cells were obtained from Dr. W. E Müller, Pharmacological Institute, Biocenter, Frankfurt. The cells show no 5-LO expression, there is no effect of TGFβ and $1,25(OH)_2D_3$ treatment on 5-LO expression.

3.2 Cell culture

Mono Mac 6 cells were cultured in RPMI-1640 medium supplemented with 2 mM Glutamine, 1 mM sodium pyruvate, 1 x nonessential amino acids, 10 µg/ml insulin, 1 mM oxalacetic acid, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) fetal bovine serum. Cells were maintained at 37 °C in an humified atmosphere of 5% CO_2. Cultures were seeded at 2 x 10^5 cells/ml. In some experiments TGFβ (2 ng/ml) and $1,25(OH)_2D_3$ (50 nM) were added for induction of 5-LO expression and cell differentation. TGFβ1 was purified from outdated platelets as described by Werz et. al. (Werz et al. 1996). If indicated, cells were treated 6 h with TsA, 330 nM, purchased from Sigma (T8552, solved in ethanol).

HeLa cells were grown in Dulbecco`s modified Eagle`s medium (DMEM) supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin and 100 units/ml penicillin. If indicated, cells were treated 24 h with TsA, 330 nM, or 24 h with 1 mM valproic acid (Aldrich, Cat.: 22,425-1), or with 5 mM nicotinamide (Sigma, solved in DMSO).

3.3 Preparation of whole cell extracts for DNA affinity purification assay

MM6 cells were seeded at 2 x 10^5 cells/ml. After 4 days the cells were collected by centrifugation at 1200 rpm at RT for 3 min. The pellets were rinsed with PBS (pH 7.4) and collected in one falcon tube. Following centrifugation the cells were resuspended in 10 ml of NETN buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.25% NP-40) and lysed for 15 min on ice. After sonication (3 x 5 sec) and cooling on ice for another 30 min the

cell debris was removed through centrifugation (10,000 rpm at 4 °C for 10 min) and the supernatant was stored at -70 °C. Protein content was determined with Bradford assay (BioRad Protein Assay).

HeLa cells were seeded 1-2 x 10^6 cells/180 cm^2, equivalent to splitting the cells 1:10 every 4 days. After 72 h the cells were harvested. For this the cells were washed twice with PBS (pH 7.4) at RT, scraped off in PBS buffer and collected in a falcon tube. After centrifugation at 1,200 rpm for 3 min, 1 ml of ice-cold NETN buffer per collected dish was added and the cells were lysed for 15 min on ice. The cell debris was removed through centrifugation (10,000 rpm at 4 °C for 10 min) and the supernatant was stored at -70 °C.

3.4 Preparation of nuclear and cytosolic cell fractions

To check for protein expression after transfection, cells were harvested and fractionated into nuclear and non-nuclear compartments. Adherent HeLa cells were first washed twice with PBS (pH 7.4), then scraped off the cell culture dishes and transferred to falcon tubes. After centrifugation at 1200 rpm (200 g) at RT for 5 min the pellet is resuspended in 300 µl of NP-40 buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40) containing protease inhibitors (1 mM PMSF, 1 µg/ml STI, 10 mg/ml Leupeptin), lysed on ice for 10 min and subsequently centrifuged at 2700 rpm (800 g) at 4 °C for 10 min. The supernatant is the cytosolic fraction and stored at –20 °C. The pellet (nuclear fraction) is resuspended in 50 µl of TKM buffer (50 mM Tris-HCl pH 7.4, 250 mM Sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA), sonicated for 3 x 5 sec and stored at –20 °C. For Western blot analysis usually the nuclear fraction was prepared. Different probes were normalized to the cell number of harvested cells, the volumes were adjusted with MQ to a volume of 20 µl, 5 µl of 5 x Laemmli buffer (250 mM Tris-HCl pH 6.8, 5 mM EDTA, 50% Glycerol, 10% SDS, 0.05% BPB, 10% β-Mercaptoethanol) were added and the samples boiled for 5 min at 95 °C.

3.5 Preparation of nuclear extracts for gel shift assays and DAPA

Nuclear extracts were prepared according to Shapiro (Shapiro et al. 1988). Depending on the cell type and the growth conditions about 0.2 – 1 x 10^9 cells were harvested by centrifugation at 170 g, 10 min, 4 °C. Cells were washed twice in cold PBS (pH 7.4). HeLa cells were washed twice with ice cold PBS before scraping the cells off the cell culture dishes. The pellet was resuspended in a small volume of PBS and centrifuged at 300 g for 10 min, after which the packed cell volume

(PCV) was measured. The pellet was resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl and Complete Protease Inhibitor Complex® (CPIC, Roche) to 5 times the PCV and allowed to swell on ice for 10 min. The cells were pelleted by centrifugation at 300 g for 10 min at 4 °C, and resuspended in hypotonic buffer (volume corresponding to 2 x PCV). Cells were broken by Dounce homogenization: first tight pestle; then loose pestle after adding 0.1 volume of sucrose restore buffer (50 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, CPIC, and sucrose 67.5% (w/v)). The broken cells were quickly centrifuged at 10,000 rpm for 1 min. The viscous pellet (containing nuclei) was resuspended in 2.1 ml nuclear resuspension buffer (20 mM HEPES, pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 2 mM DTT, 25% glycerol, 0.1 volume saturated (at 4 °C) ammonium sulfate and CPIC), shaken for 30 min at 4 °C, during this treatment nuclei are disrupted. The resulting solution was cleared by centrifugation for 90 min at 150,000 g, and nuclear proteins were precipitated with ammonium sulfate (0.39 g/ml, stirred on ice for 20 min). The precipitate was collected by centrifugation at 85,000 g for 20 min. The pellet was resuspended in 0.6 ml dialysis buffer (20 mM HEPES, pH 7.9, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT and CPIC) and dialyzed for 2 times 2 h in >200 volumes of dialysis buffer. The final nuclear protein extract was stored at –70 °C.

3.6 SDS-PAGE and Western blot

If not otherwise indicated 10% polyacrylamide gels were run at 170 V for 60 min. Proteins were electroblotted onto Millipore Immobilon-FL PVDF membrane using the BioRad Mini Protean system. Membranes were blocked in a mixture of Li-Cor Blocking buffer and PBS (1:1) for 1 h at RT and then incubated overnight at 4 °C with primary antibody solution. After washing 4 x 5 min in PBS-Tween 0.1%, the membranes were incubated 30 min at RT in secondary antibody and subsequently washed another four times for 5 min in PBS-Tween 0.1% and finally 5 min in PBS. The membranes were scanned in the Li-Cor Odyssey scanner at 169 µm resolution and high quality.

Primary antibodies

Sp1 (sc-059)	anti-rabbit, anti-goat	1:1000	Santa Cruz / Heidelberg
Sp3 (sc-644)	anti-rabbit	1:1000	Santa Cruz / Heidelberg
HDAC2 (sc-7899)	anti-rabbit	1:1000	Santa Cruz / Heidelberg
HDAC3 (sc-11417)	anti-rabbit	1:1000	Santa Cruz / Heidelberg
HDAC8 (sc-17778)	anti-rabbit	1:1000	Santa Cruz / Heidelberg

p300 (sc-32244)	anti-mouse	1:1000	Santa Cruz / Heidelberg
PCAF (sc-6300)	anti-gaot	1:1000	Santa Cruz / Heidelberg
MBD1 (sc-25262)	anti-mouse	1:1000	Santa Cruz / Heidelberg
MBD2 (sc-9397)	anti-goat	1:1000	Santa Cruz / Heidelberg
MBD3 (sc-9402)	anti-goat	1:1000	Santa Cruz / Heidelberg
MeCP2 (sc-20700)	anti-rabbit	1:1000	Santa Cruz / Heidelberg
Anti-acetyl-Lysine (06-933)	anti-rabbit	1:5000	Upstate / Hamburg
Anti-HDAC1 clone 2E10 (05-614)	anti-mouse	1:2000	Upstate / Hamburg
Anti-MBD2/3 (07-199)	anti-rabbit	1:5000	Biomol / Hamburg
MBD1 (ab3753)	anti-rabbit	1:1000	Abcam / UK

Secondary antibodies

Donkey anti-rabbit (#611-732127)	IRDye800CW	1:10000	Rockland
Donkey anti-goat (#A-21084)	Alexa680	1:10000	Molecular Probes
Goat anti-rabbit (926-32230)	IRDye800CW	1:10000	Odyssey InfraredImaging
Goat anti-mouse (927-30021M)	Alexa680	1:10000	Odyssey InfraredImaging
Goat anti-rabbit (926-32221)	IRDye680	1:10000	Odyssey InfraredImaging
Goat anti-mouse (927-30020R/C)	IRDye800CW	1:10000	Odyssey InfraredImaging

3.7 Plasmids

3.7.1 5-LO promoter reporter gene vectors

The cloning of the basic 5-LO promoter luciferase reporter gene constructs N0 to N14 was done by Niko Klan and is described in (Klan et al. 2003; Sorg et al. 2006). N0 contains the promoter region from +53 bp up to -6079 bp in relation to the major transcription initiation site (-12 bp to -6144 bp in relation to the ATG). It was cloned by digesting the plasmid K1 (containing the fragment -6079 to +409 of the 5-LO gene (provided by Shigeru Hoshiko, Japan) first with *BstE*II, subsequently blunting with T4 DNA polymerase, digesting with *Kpn*I and finally ligating the obtained fragment into the promoterless luciferase reporter vector pGL3Basic (Promega), which was opened with *Kpn*I and *Sma*I.

The plasmids N1-N11 were constructed by digestion of N0, using *Kpn*I in combination with either *Afl*II (N1), *Van*911 (N2), *Nde*I (N3), *Pvu*II (N5), *Eco*1471 (N6), *EcoR*I (N7), *EcoR*V (N8), *Pme*I p(N9), *Pau*I (N10) and *Bst*XI (N11). Overhangs were blunted by T4 DNA polymerase and the plasmids religated with T4 DNA ligase.

Constructs N12 to N16 and GC0 were obtained by PCR deletion using N10 as the template. The primers used in the PCR reaction are described in Tab. 1. Following 18 temperature

cycles, the PCR product was treated with *Dpn*I, DNA ends were phosphorylated by T4 polynucleotide kinase and ligated by T4 DNA ligase.

Several mutations of transcription factor binding sites and methylation sites were introduced by **site-directed mutagenesis** according to the instructions of the QuikChange II site-directed mutagenesis kit of Stratagene. The primers used for the PCR reaction had a minimum length of 25 bp, a minimum GC content of 40% and an annealing temperature of at least 78 °C, carrying the mutation in the middle of the sequence. Primers, if possible, ended with a G or C and were purified by HPLC. For the introduction of a point mutation, the PCR reaction was run for 12 cycles, for the mutation of several base pairs, for 16 cycles. The PCR product was treated with *Dpn*I to digest template DNA and subsequently transformed to supercompetent SURE® E.coli (Stratagene). The primers used for the different mutations are listed in Tab. 1.

The following mutations of potential Sp1 binding sites were done by site-directed mutagenesis: Mutation of the two proximal GC boxes, GC4 (-113 bp to –118 bp in relation to the ATG of 5-LO) and GC box 5 (-78 bp to -83 bp in relation to the ATG of 5-LO) in construct N13, the mutation of GC5 in N14, resulting in the reporter gene constructs N13GC4, N13GC5, N13GC45 and N14GC5.

The same single mutations were generated in the larger 5-LO promoter plasmids N10 and GC0 (a deletion variant of N10, missing the 5-tandem Sp1 binding site at –147 bp to –176 bp in relation to 5-LO), generating the constructs N10GC4, N10GC5, and GC0GC5.

To produce the plasmids GC0GC4 and GC0GC45 a different cloning strategy was chosen. Because of a lack of restriction sites, it was not possible to cut fragments out of the already existing mutated plasmids and to religate them into the larger plasmids. For this reason the fragments were generated by PCR. Basically, two each other **flanking PCR** products were obtained, one containing the wished mutations of the GC boxes, already introduced in either N13GC4 or N13GC45, another containing the adjacent fragment of GC0, needed for the ligation into GC0. The plasmid GC0 was opened by the restriction enzymes *Not*I and *Nco*I, the PCR fragments were digested with either *Not*I (GC0 as a template) or *Nco*I (N13GC4/N13GC45 as a template), phosphorylated at the blunt ends and ligated both into GC0. The primers used for the PCR reaction are shown under “Primers for PCR product 1 and 2” in Tab. 1 below.

The triple mutants GC0GC145 and GC0GC245 were obtained using the same strategy, using GC0GC1 or GC0GC2 and N13GC45 as templates. GC0GC1245 was generated using N10GC12 and N13GC45 as templates.

Mutations of the more distal binding sites of Sp1, including the sites from -238 bp to -233 bp (GC box 1), -216 bp to -221 bp (GC box 2), -721 bp to -726 bp (GC box 7), -766 bp to -771 bp (GC box 6), were introduced into N10 and GC0 using site-directed mutagenesis, resulting in the constructs N10GC1, N10GC2, N10GC6, N10GC7 and GC0GC1, GC0GC2. Mutations of the two potential Sp1 binding sites GC8 (-887 bp to -892 bp in relation to ATG) and GC9 (-1002 bp to -1007 bp) within the reporter gene construct N9 were also introduced by site-directed mutagenesis generating the reporter gene constructs N9GC8 with an additional *BamH*I site, N9GC9 with an additional *Nco*I site and N9GC89 respectively. To further introduce the five-fold mutation of GC1, GC2, GC4, GC5 and the deletion of GC0 into these constructs the *BstX*I/*Bgl*II fragment from construct GC0GC1245 was inserted, replacing the natural *BstX*I/*Bgl*II fragments from the plasmids via restriction digest, leading to the constructs N9GC01245, N9GC8GC01245, N9GC8GC01245 and N9GC89GC01245.
The most distal Sp1 binding site (GC box 10, from -1002 bp to -1007 bp) was mutated in N8 via site-directed mutagenesis resulting in construct N8GC10.
To investigate the influence of the methylation sites within the proximal promoter area, mutations of these sites were introduced into construct N13. Construct N13MutMethIII was achieved via site-directed mutagenesis, N13MutMethI and N13MutMethII via two flanking PCR products which were cloned into the *Not*I/*Nco*I opened plasmid N13.
Additionally CpG sites within the inverted repeat structure of the proximal 5-LO promoter were mutated to check for methylation effects in the secondary DNA structure (Hoshiko et al. 1990). These mutations were both introduced via site-directed mutagenesis into N13, resulting in the reporter gene constructs N13MutInvRep1 and N13MutInvRep2. Primers used for the methylation relevant mutations are stated in the table below.

3.7.2 Expression plasmids

The expression plasmids pETM1, pETM3, pETM4 for the methyl-CpG-binding proteins Mbd1 (AF072240, coding for the murine isoform Mbd1a), Mbd2 (AF072243, using the second ATG coding for the murine isoform Mbd2b) and Mbd3 (AF072248) of murine origin and pCMV-HA-MeCP2 of rat origin (NM_022673) were kindly provided by Adrian Bird, University of Edinburgh, UK. H. Leonhardt, from the Max Delbruck center in Berlin, Germany, kindly send us the expression plasmid of mouse Dnmt1, pEMT. From M. Szyf, McGill University, Montreal we have received pHis-dMTase (human MBD2b) and from F. Li, University of Giessen, Germany, the plasmids pcDNA3.1HisDnmt3a and pcDNA3.1HisDnmt3b1, containing the murine coding sequences.

Plasmid	Forward primer for PCR deletion	Reversed primer for PCR deletion
N12	CGCGTGAAGAGTGGGAGAGAAGTACTGCGG	TATCGATAGAGAAATGTTCTGGCA
N13	CAGCCGGGAGCCTGGAGCCAGACC	See N12
N14	AGGGACCAGTGGTGGGAGGAGGCT	See N12.
N15	GCTAGATGCGGACACCTGGACCGC	See N12
N16	GGCTCCCGGCGCTCGCTGCTC	See N12
GC0	CAGCCGGGAGCCTGGAGCCAGACC	CCGCAGTACTTCTCTCCCACTCTTCACGCG
Plasmid	**Forw. primer for site-directed mutagenesis**	**Rev. primer for site-directed mutagenesis**
N13GC4	AGCCAGACCGGCCCGGGGCCGGG	CCCGGCCCCGGGCCGGTCTGGCT
N13GC5	GGACCAGTGTGCCAGGAGGCTGCGGC	GCCGCAGCCTCCTGGCACCACTGGTCC
N13GC45	see N13GC5; N13GC4 as template	see N13GC5; N13GC4 as template
N14GC5	see N13GC5	see N13GC5
N10GC1	GCCCCTGCCCCGGGCGAGGCGAG	CTCGCCTCGCCCGGGGCAGGGGC
N10GC2	GAGGCGAGGTCCCGGGCAGTCGGCG	CGCCGACTGCCCGGGACCTCGCCTC
N10GC12	see N10GC2; N10GC1 as template	see N10GC2; N10GC1 as template
N10GC4	see N13GC4	see N13GC4
N10GC5	see N13GC5	see N13GC5
N10GC6	CGACCCGTGACCCCTGGCCTGAGGTAGACAGCCC	GGGCTGTCTACCTCAGGCCAGGGGTCACGGGTCG
N10GC7	CGTCCCGCCTGCCCGGGGCGACCACTGGC	GCCAGTGGTCGCCCCGGGCAGGCGGGACG
GC0GC1	see N10GC1	see N10GC1
GC0GC2	see N10GC2	see N10GC2
GC0GC5	see N13GC5	see N13GC5
N9GC8	GAGGCGGGCGCCAGGAGTGGATCCGAACCTGGG	CCCAGGTTCGGATCCACTCCTGGCGCCCGCCTC
N9GC9	CCCAGCCGCGGGAAGCCATGGAGGAGCGCGC	GCGCGCTCCTCCATGGCTTCCCGCGGCTGGG
N9GC89	see N9GC8	see N9GC8
N8GC10	GACTTCACATCCCCGGGATCCCACGCACGGTGAGC	GCTCACCGTGCGTGGGATCCCGGGGATGTGAAGTC
N13MutInvRep1	CTCTATCG ATACAGCTGGGAGCCTGGAGCC	GGCTCCAGGCTCCCAGCTGTATCGATAGAG
N13MutInvRep2	CGAGGCTCCCGACCCTCGCTGCTC	GAGCAGCGAGGGTCGGGAGCCTCG
N13MutMethIII	CAGACTGGGGCGGGGCTGGGACCTGGGCCAG	CTGGCCCAGGTCCCAGCCCCGCCCCAGTCTG
Plasmid	**Primer for flanking PCR product 1**	**Primer for flanking PCR product 2**
GC0GC4	Template: GC0	Template: N13GC4
	for TCGGTGCGGGCCTCTTCGCTATTACGCCAG	for CAGCCGGGAGCCTGGAGCCAGACCG
	rev CCGCAGTACTTCTCTCCCACTCTTCACGCG	rev CCAGGAACCAGGGCGTATCT
GC0GC45	Template: GC0	Template: N13GC45
	see GC0GC4	see GC0GC4
	see GC0GC4	see GC0GC4
GC0GC12	Template: N10GC12	Template: N13
	see GC0GC4	see GC0GC4
	see GC0GC4	see GC0GC4
GC0GC145	Template: GC0GC1	Template: N13GC45
	see GC0GC4	see GC0GC4
	see GC0GC4	see GC0GC4
GC0GC245	Template: GC0GC2	Template: N13GC45
	see GC0GC4	see GC0GC4
	see GC0GC4	see GC0GC4
GC0GC1245	Template N10GC12	Template N10GC12
	see GC0GC4	see GC0GC4
	see GC0GC4	see GC0GC4
N13MutMethII	Template N13	Template N13

	for TCGGTGCGGGCCTCTTCGCTATTACGCCAG	for GCTAGATGCGGACACCTGGACCGCC
	rev ACCACAGCCTCCTCCCACCACTGGTC	rev GCCAACCGAACGGACATTTC
N13MutMethI	Template N13	Template N13
	for TCGGTGCGGGCCTCTTCGCTATTACGCCAG	for TGCTGAGGCTCCCGGCGCTTGCTGCTCC
	rev CAGCAGTCCAGGTGTCCACATCTAG	rev CCAGGAACCAGGGCGTATCT
	Forward primer for oligo insertion	**Reversed primer for oligo insertion**
pSG5mcsNEU	AATTCCCATGGCATGCGGATCCGGTACCACT AGTCCCGGGA	GATCTCCCGGGACTAGTGGTACCGGATCCGCA TGCCATGGG
	Forward primer for PCR amplification	**Reversed primer for PCR amplification**
pSG5HDAC1	TCCCCCGGGATGGCGCAGACGGCAGGGCAC	GGAAGATCTTCAGGCCAACTTGACCTCCTCCTTG
pSG5Hdac2m	TCCCCCGGGATGGCGTACAGTCAAGGAGGCGG	GAAGATCTTCAGGGGTTGCTGAGCTGTTCTG
pSG5HDAC2h	TCCCCCGGGATGGCGTACATGCAAGGAGGCGC	GAAGATCTTCAGGGGTTGCTGAGCTGTTCTG
pSG5HDAC3	CGGGATCCCCATGGCCAAGACCGTGGCC	GGACTAGTCCACTCTTAAATCTCCACATCGCTTTCC
pSG5HDAC8	CGGAATTCATGGAGGAGCCGGAGGAACCG	CCCAAGCTTCTAGACCACATGCTTCAGATTCCCTTTG
pSG5Mbd1m	TCCGGAATTCATGGCTGAGTCCTGGCAGGACT	TGGAAGATCTCTCTCCTACAAAACTTCTTCTTTCAAC TGC
pSG5Mbd2m	TCCGGAATTCGCCATGGACTGCCCGGCCCTCCC	TGGAAGATCTTGCACTGCACCGGAAGGGCCCTGC
pSG5Mbd3m	TCCGGAATTCATGGAGCGGAAGAGGTGGGAGTGCC	TGGAAGATCTCTACACTCGCTCTGGCTCCGGCTCTTC
pSG5Dnmt1m	AGGAATGGCAGACTCAAATAGATCCCCAAGATC	CTAGTCCTTGGTAGCAGCCTCCTCTTTTGC
pSG5DNMT3b1	TCCGGAATTCCAGGAAACAATGAAGGGAGACAGCAG	GCGGATCCCTATTCACAGGCAAAGTAGTCCTTCAACG

Tab. 1 Primers used for cloning. *All oligonucleotides were synthesized by Sigma-Genosys (Steinheim).*

Further on, different plasmids expressing histone deacetylases (human HDAC1, HDAC3 [pcDNAFlagHDAC3], HDAC8, as well as mouse Hdac2 [pME18S-HDAC2]) were kindly provided by E. Seto, from the H. Lee Moffit Cancer Center, University of South Florida, USA. pEVR2/CMV-Sp1, pRC/CMV-Sp3 and pRC/CMV-Sp4 were a gift of by G. Suske, University of Marburg, Germany.

For co-expression studies together with 5-LO promoter reporter gene constructs it was reasonable to use expression plasmids with the same vector background. For this reason the expression plasmids were cloned into the high copy expression vector pSG5 from Stratagene, containing a SV40 promoter. First the multiple cloning site of the pSG5 vector was extended. For this the pSG5VDR plasmid (a gift of C. Carlberg, University of Kuopio, Finland, containing the vitamin D receptor coding sequence) was opened with *EcoR*I and *Bgl*II and a double stranded oligo filling up the *EcoR*I and *Bgl*II site, and additionally containing restriction sites for *Nco*I, *BamH*I, *Kpn*I, *Spe*I, and *Sma*I was ligated into the backbone of the plasmid.

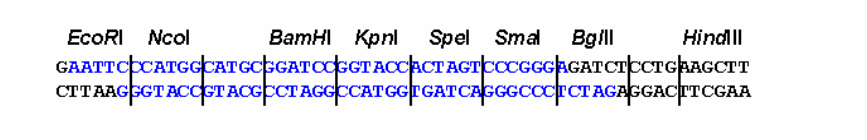


Fig. 15 Extention of the multiple cloning site of pSG5mcsNEU

PCR fragments covering the coding sequences of human HDAC1 (Acc. No. NM_004964), HDAC3 (Acc. No. NM_003883) and HDAC8 (Acc. No. NM_018486) were respectively inserted via the restriction sites *Sma*I/*Bgl*II, *BamH*I/*Spe*I and *EcoR*I/HindIII into pSG5mcsNeu. Human HDAC2 (Acc. No. NM_001527) was cloned from HeLa cDNA using *Sma*I and *Bgl*II for inserting the PCR fragment into pSG5mcsNeu.

The coding sequence of Dnmt1 (Acc. No. AF175432) was amplified by PCR and introduced via the *Sma*I site into the pSG5mcsNEU. Dnmt3b1 (Acc. No. AF068626) were subcloned via PCR amplification into pSG5mcsNeu using *EcoR*I and *BamH*I sites. Murine Dnmt3a (Acc. No. AF068625) was excised from pSX173 with *Xba*I and *BamH*I and ligated to the pSG5mcs opened with *Spe*I and *BamH*I.

The coding sequences of the murine methyl-CpG-binding proteins were also introduced into pSG5mcsNeu using PCR amplification and the restriction sites of *EcoR*I and *Bgl*II in the mcs.

The HA-tagged MeCP2 cds (Acc. No. NM_022673) was excised from pCMV-HA-MeCP2 with *EcoR*I and ligated into the *EcoR*I opened pSG5mcsNeu vector, resulting in pSG5mcsMeCP2.

Plasmid sequences were confirmed by DNA sequencing. Restrictions enzymes were purchased from either MBI Fermentas, New England Biolabs or Promega, Pfu DNA Polymerase from Promega, T4 DNA Ligase from NEB and T4 Polynukleotide Kinase from MBI Fermentas.

3.8 Preparation of plasmid DNA

Larger amounts of plasmid DNA, especially for transfections, were prepared using the Nucleobond AX 2000®-System (Macherey-Nagel). According to the manufacturer 500 ml of bacterial culture is harvested. After lysing the cells by a NaOH/SDS solution, chromosomal DNA and proteins are precipitated with a potassium acetate solution and the plasmid DNA is further purified via a silica based anion exchange chromatography.

3.9 In vitro methylation

Plasmid DNA or PCR products were incubated over night at 37 °C with *Hpa*II methylase., M. *Hha*I, M. *Sss*I or the combination of M. *Hpa*II and M. *Hha*I at 0.8 unit/µg pDNA in either 50 mM Tris-HCl, 5 mM 2-Mercaptoethanol, 10 mM EDTA, pH 7.5, for the methylation with M. *Hpa*II or M. *Hha*I, or 10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9, for the methylation with M. *Sss*I each supplemented with 800 µM of S-adenosylmethionine. Complete methylation was confirmed by either *Hha*I, *Hpa*II of *BstU*I restriction digest of the plasmids. For transfections the methylated pDNA was purified by phenol-chloroform extraction, PCR products by NucleoSpin® Extract columns (Macherey-Nagel). HpaII methylase was purchased from MBI Fermentas, HhaI M. and SssI M. from New England Biolabs.

3.9.1 Phenol-Chloroform extraction

The methylation reaction is supplemented with 50 µl of 3 M sodium acetate and adjusted with MQ to a total volume of 500 µl. An equal volume of phenol (pH 7.6-8.0, equilibrated with TE buffer), chloroform and isoamylcalcohol (25:24:1) is added. The mixture is vortexed for 1 min and centrifuged for 5 min at 12,000 g. The upper, aqueous phase is transferred to a fresh tube and an equal volume of chloroform and isoamyl alcohol (24:1) is added. The mixture is vortexed for 30 sec and centrifuged at 12,000 g for 2 min. The upper phase is transferred to a fresh tube again, the same volume of ice-cold isopropyl alcohol is added to precipitate the DNA. For complete precipitation the samples are incubated at –20 °C for 1 h and centrifuged at 4 °C for 45 min at 12,000 g. The pellet is rinsed with ice-cold ethanol (70%) and dried in the laminar flow. Finally the pellet is solved in 50 µl sterile MQ.

3.10 Transfection methods

3.10.1 Lipofection of HeLa cells

Before transfection, cells were plated into a 24-well tissue culture plate at a density of 4×10^4 cells per well for 24 h, so that 60-80% of the cells were confluent at the time of transfection. Plasmid DNA of the luciferase reporter gene construct (0.4 µg) and internal standard pCMVSEAP (0.02 µg) were diluted into 50 µl of serum free DMEM and incubated with Plus® reagent (Invitrogen) for 15 min. Precomplexed plasmid DNA was mixed with 25 µl of 1:50 in serum free DMEM diluted Lipofectin® reagent (Invitrogen) and incubated for 30 min

at room temperature. Meanwhile the medium of the cells was replaced by 200 µl of fresh serum free medium and the DNA-PLUS-Lipofectin reagent complex was added to the cells and incubated for 3 or 5 h at 37 °C in 5% CO_2. Then 1 ml of medium containing 15% (v/v) FCS was added. 24 h after transfection the medium was removed, replaced by 100 µl of DMEM medium and luciferase activity was determined.

3.10.2 Calcium phosphate transfection method for HeLa cells

For reporter gene assays cells were, 24 h prior to transfection, plated into a 24-well tissue culture plate at a densitiy of 4 x 10^4 cells per well, so that 60-80% of the cells were confluent at the time of transfection. For the transfection 0.8 µg plasmid DNA of the luciferase reporter gene construct, and 0.02 µg of internal standard pCMVSEAP were used per well. For the preparation of the precipitate 20 µg of the reporter gene plasmid and 0.5 µg of pCMVSEAP were diluted in 450 µl of sterile MQ and mixed with 50 µl of 2.5 M $CaCl_2$ solution. The mixture is then added dropwise (within 1 min) to 500 µl of HeBS buffer pH 7.08 (50 mM HEPES, 28 mM NaCl, 1.5 mM Na_2HPO_4) while bubbling air into the suspension in order to mix instantly DNA and phosphate buffer. After another min of incubation time 40 µl of the calcium phosphate-DNA precipitation per well are added to the cells. The medium was changed 16 h after transfection. Usually 24 h later the cells were harvested. The medium was again changed, 100 µl of fresh medium were added and luciferase activity was determined. If at all, cells were incubated directly after changing the medium.

3.10.3 Electroporation of MM6 cells

According to Niko Klan (Klan and Steinhilber 2003), 48 h to 72 h before transfection, cells were split to 2 x 10^5 cells/ml and cultured at 37 °C in an humified atmosphere of 5% CO_2. At the time of transfection, cells were harvested by centrifugation at 1200 rpm for 5 min at RT and washed twice with RPMI-1640, containing neither FCS nor other additives. Then cells were resuspended at a density of 40 x 10^6 cells/ml in RPMI-1640 medium without any additives. 0.3 ml of cell suspension were placed into a 0.4 cm electroporation cuvette. 40 µg of plasmid DNA (reporter gene construct) and 1 µg of internal standard (pCMVSEAP) were dissolved in water to a final volume of 30 µl and added to the cell suspension. Cells and plasmids were preincubated for 5 min at RT before electroporation at 975 µF and 200 V in the Biorad Gene Pulser II. Immediately after the pulse, the cuvettes were placed on ice for 20 min. Finally the transfected cells were transferred to 10 ml of cell culture medium. Cells were harvested 6 h later, since the Luciferase signal peaked at 6 h after transfection.

3.11 Reporter gene assays

To determine the activity of the different 5-LO promoter constructs under various conditions cell lysates were assayed for luciferase activity by measuring light emission in a Microlumat Plus LB96V EG&G Berthold Luminometer. The light emission was integrated for 5 sec when measuring the luciferase signal whereas the signal of the internal standard (SEAP activity) was only integrated for one sec. Considering different transfection efficiencies the luciferase activity was normalized to the SEAP activity.

3.11.1 Luciferase assay

MM6 cells were harvested 6 hours after transfection, emptying the cell culture flasks into 10 ml falcon tubes, centrifuging the cells for 2 min at 1200 rpm at RT. Due to increased cell death after the stressing electroporation procedure highest luciferase signals are achieved after 6 h after transfection and are decreasing when cells are collected at later times. 33 µl of the medium is saved for the SEAP assay, the rest of the medium is decanted and the falcons are put upside down on a cellulose to dry the pellets. Cells are resuspended in 100 µl of PBS and lysed in 100 µl of lysis buffer of the Steady- or Bright-Glow™ luciferase assay system (Promega) containing the luciferase substrate luciferin, which is converted by Firefly-luciferase to oxyluciferin under the emission of light. The lysated cells are transferred to a white 96 well plate, which is measured in the luminometer.
HeLa cells are harvested, if not otherwise indicated, 24 h after removing the Calcium phosphate precipitation and changing the medium. 33 µl of medium are saved for the SEAP assay. Then, the medium is replaced by 100 µl of fresh HeLa medium, 100 µl of lysis buffer are added and the procedure is followed as described above for the MM6 cells.

3.11.2 SEAP (Secreted Placental Alkaline Phosphatase) assay

The activity of the internal standard was determined using the Phospha-Light™ system kit from Applied Biosystems. According to the manufacturers recommendations 33 µl of the medium of the transfected cells are mixed with 100 µl of dilution buffer and incubated at 65 °C for 30 min to heat inactivate endogenous alkaline phosphatases. To measure the activity of the transfected SEAP, 50 µl of the conditioned medium are transferred to a 96 well plate, preincubated for 5 min at RT with 50 µl assay buffer (containing inhibitors for endogenous alkaline phosphatases) and finally incubated for 20 min with 50 µl of reaction buffer

containing the Tropix CSPD® chemoluminescent substrate for the secreted alkaline phosphatase. Light emission is measured and the signal is integrated for one sec.

3.12 Gel shifts

All oligonucleotides were synthesized by Sigma-Genosys (Steinheim). For methylation studies, either the inner CpG dinucleotide was methylated at the 5´-position of the cytosine or CpG dinucleotides surrounding the Sp1 binding sites were methylated . Oligos were dissolved in sterile MQ at 100 µM concentrations. For annealing, 1 µl the forward and 1 µl of the complementary reversed strand oligo were mixed in 98 µl MQ and heated up to 95 °C for 5 min, then the probes were cooled down at 0.02 °C/sec to room temperature.

3.5 µl of the double-stranded oligonucleotides were labeled at the 5´-ends using T4 polynucleotide kinase in 20 µl of reaction volume containing 10 µCi of [γ-^{32}P]-ATP (GE Healthcare). After stopping the reaction with 29 µl of TE buffer pH 8.0, 1 µl of EDTA pH 8.0 (0.5 M) and heat inactivation at 68 °C the radio-labeled double-stranded oligonucleotides were purified through G-25 microspin columns (GE-Healthcare), and stored at –20 °C. 1-10 µg of nuclear extract, and/or Sp1 protein (150-300 ng, Promega) were preincubated in binding buffer (50 mM Tris-HCl, 2.5 mM DTT, 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 20% glycerol) for 10 min at RT before the labeled oligonucleotide probe (40,000 cpm to 150,000 cpm; as indicated in the figures) was added to a total reaction volume of 20 µl. For competition studies, a 100-fold molar excess of unlabeled oligonucleotide was added to the reaction mixture prior to the addition of radio-labeled probe. EMSA reactions were resolved on 4-20% pre-run (200 V for 60 min in an ice bath) nondenaturing polyacrylamide TBE gels (BioRad premade) which were electrophoresed at 100 V for 110 min in an ice bath. Gels were dried under vacuum before exposure, or exposed directly to Fuji Super RX film at –20 °C or to a FLA 3000 imaging plate at RT for the indicated exposure times.

Forward strands of EMSA probes 5´ to 3´	
CTGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCAG	5 x GC box
CTGCGGGGG**O**GGGGG**O**GGGGG**O**GGGGG**O**GGGGG**O**GGGGGCAG	5 x GC box methylated
CAGACCGGGGCGGGGCCGGGACCGGGGC	Consensus Sp1 site / GC box 4
CAGAC**O**GGGGCGGGGC**O**GGGAC**O**GGGGC	Consensus Sp1 site / GC box 4 methylated around the Sp1 binding motif
CAGACCGGGG**O**GGGGCCGGGACCGGGGC	Consensus Sp1 site / GC box 4 methylated within the Sp1 binding motif

GACCGGGGCCAGGGACCAGTGGTGGGAGGAGGCTGCGGCGC	Non-consensus Sp1 site / GC box 5 41 bp
GAC**O**GGGGCCAGGGACCAGTGGTGGGAGGAGGCTG**O**GG**O**GC	Non-consensus Sp1 site / GC box 5 41 bp methylated around the Sp1 binding motif
AGTGGTGGGAGGAGGCTGCGGCGCT	Non-consensus Sp1 site / GC box 5 20 bp
GATCGAACTGACCGCCCGCGGCCCGT	Ap2 binding site as unspecific competitor
ATTCGATCGGGGCGGGGCGAGC	classical Sp1 binding motif as specific competitor

Tab. 2 Forward strands of EMSA probes 5´ to 3´

3.13 DNA affinity purification assays (DAPA)

This assay is based on the immobilization of protein-DNA complexes via binding of a biotinylated oligonuceotide to a streptavidin matrix. Oligonucleotides, only the forward strand was biotinylated at the 5´-end, were synthesized by Sigma-Genosys (Steinheim). Sense and antisense oligonucleotides were dissolved in 10-fold annealing buffer (0.5 M NaCl, 0.2 M Tris-HCl pH 8.0) to an end concentration of 50 µM, heated for 5 min at 95°C and annealed by decreasing the temperature down to RT in steps of 0.02°C/sec.

200 µl of whole cell extract were incubated with 200 µl of binding buffer H (100 mM KCl, 20 mM HEPES [pH 7.8], 20% glycerol, 1 mM DTT, 0.1% NP40) and 2 µl of the biotinylated double-stranded oligo mixture for one hour on ice. After adding 50 µl of equilibrated streptavidin-coupled agarose beads (Sigma, S1638) incubation was continued for 30 min at 4 °C on a spinning wheel. The beads were washed four times with 500 µl of binding buffer by swaying the tubes 6-8 times and finally boiled with 20 µl of MQ and 5 µl of 5 x Laemmli sample buffer (250 mM Tris-HCl pH 6.8, 5 mM EDTA, 50% Glycerol, 10% SDS, 0.05% BPB, 10% β-Mercaptoethanol). The proteins were separated via SDS-PAGE.

Forward strands of DAPA probes 5´ to 3´	
CTGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCAG	5 x GC box
CAGACCGGGGCGGGGCCGGGACCGGGGC	Consensus Sp1 site / GC box 4
CAGACCATCTGCAGCCCGGGACCGGGGC	Consensus Sp1 site / GC box 4 mutated
GACCGGGGCCAGGGACCAGTGGTGGGAGGAGGCTGCGGCGC	Non-consensus Sp1 site / GC box 5
GACCGGGGCCAGGGACCAGTGGTGATCTGCAGCTGCGGCGC	Non-consensus Sp1 site / GC box 5 mutated

Tab. 3 Forward strands of DAPA probes

3.14 Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed as described by Väisänen (Vaisanen et al. 2005). In brief, nuclear proteins were crosslinked to genomic DNA by adding formaldehyde directly to the medium to a final concentration of 1% (v/v), for 10 min at 37°C. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating at room temperature for five minutes on a rocking platform. The medium was removed and the cells were washed twice with ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4 \cdot 2H_2O$).

After lysis in Pipes buffer (5 mM Pipes (pH 8.0), 85 mM KCL, 0.5% NP-40 plus protease inhibitors) and SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1) plus protease inhibitors) cells were sonicated to DNA fragments of 300-1000 bp in length. The cellular debris was removed by centrifugation and the lysates were diluted 1:10 (v/v) in ChIP dilution buffer (16.7 mM Tris-HCl (pH 8.1), 0,01% (w/v) SDS, 1.1% (v/v), Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors).

To remove unspecific background, the chromatin suspensions were incubated with a salmon sperm DNA / protein A agarose slurry (Upstate Biotechnology, Lake Placid, NY, USA) at 4°C for 30 min with agitation. The samples were centrifuged and the recovered chromatin solutions were incubated over night at 4°C with 5 µl of the indicated antibodies. The immuno-complexes were collected with 60 µl of protein A agarose slurry at 4°C for two hours with rotation. The beads were pelleted by centrifugation at 4°C for one minute at 100g and washed sequentially for five minutes by rotation with 1 ml of the following buffers: low-salt wash buffer (20 mM Tris-HCl (pH 8.1), 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high-salt wash buffer (20 mM Tris-HCl (pH8.1), 0.1% SDS. 1% Triton X-100, 2 mM EDTA, 500 mM NaCl) and LiCl wash buffer (10 mM Tris-HCl (pH 8.1), 0.25 mM LiCl, 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, 1 mM EDTA). Before elution the beads were washed twice with TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) The immuno-complexes were then eluted by adding 250 µl of elution buffer (1% SDS, 100 mM $NaHCO_3$) and incubated at room temperature for 15 min with rotation. After centrifugation, the supernatant was collected and the elution was repeated. The supernatants were combined and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and incubating at 65°C overnight. The remaining proteins were digested by adding proteinase K (final concentration 40 µg/ml) and incubation at 45°C for one hour. The DNA was recovered by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, by volume) and precipitated

with 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol using glycogen as a carrier.

Subsequently, the immuno-precipitated genomic DNA was used as a template for PCR. 10 ng of each immunoprecipitated DNA was used as template for the PCR reactions with the following profile: preincubation at 94°C for 5 min, 40 cycles denaturation at 95°C for 30 seconds, annealing at primer-specific temperature for 30 seconds and elongation at 72°C for 30 seconds, with one final incubation at 72°C for ten minutes. The PCR products, loaded with SybrGreen, were separated by electrophoresis through 2.0% agarose and the gel images scanned on a Fuji FLA3000 reader. Antibodies for Sp1, [(H-225) sc-14027], Sp3 [(D-20) sc-644] and RNA polymerase II [polII (n-20) sc-899] were purchased from Santa Cruz Biotechnology, Heidelberg. Antibody for acetylated histone H4 from Upstate, Lake Placid, H4 #06-866. PCR reactions were run in an IQ-cycler, Biorad, Hercules (USA).

PCR primer sequences	**Location within the 5-LO promoter (in relation to 5-LO TIS)**
5′-CACAAACCCAAGACAGTATGAGGAGATG-3′ 5′-CACGGGTCGGCTCTCTGAATCG-3′	-1049 to -714
5′-CGATTCAGAGAGCCGACCCGTG-3′ 5′-CTTCCACCCTTTGCCCTGCCTG-3′	-735 to -292
5′-GCAGGCAGGCAGGGCAAAGGGTGGAAG-3′ 5′-AGCAGCGAGCGCCGGGAG-3′	-318 to +52
5′-AGGAACAGACACCTCGCTGAGGAG-3′ 5′-GAGGCTGAGGTAGATGTAGTCGTCAGTG-3′	-219 to +143

Tab. 4 PCR primer sequences for ChIP and their location within the 5-LO promoter

4 Results

4.1 Regulation of 5-LO gene expression by histone deacetylation

4.1.1 5-LO promoter activity is induced by the histone deacetylase inhibitor TsA

In Mono Mac 6 (MM6) cells 5-lipoxygenase mRNA levels are induced 10.6-fold after treatment with the histone deacetylase inhibitor trichostatin A (TsA). In reporter gene assays we could also show that TsA induces 5-LO promoter activity, both in HeLa and in MM6 cells, independent of transforming growth factor (TGFβ) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Klan et al. 2003). Since the activity of the core promoter fragment N10, comprising the region -843 bp to -12 bp (in relation to the 5-LO ATG), was strongly induced by TsA, successive deletion variants, of the 5-LO promoter, N11 to N16, were transfected into HeLa cells and stimulated with TsA for 24 h, in order to identify the region entailing HDAC activity.

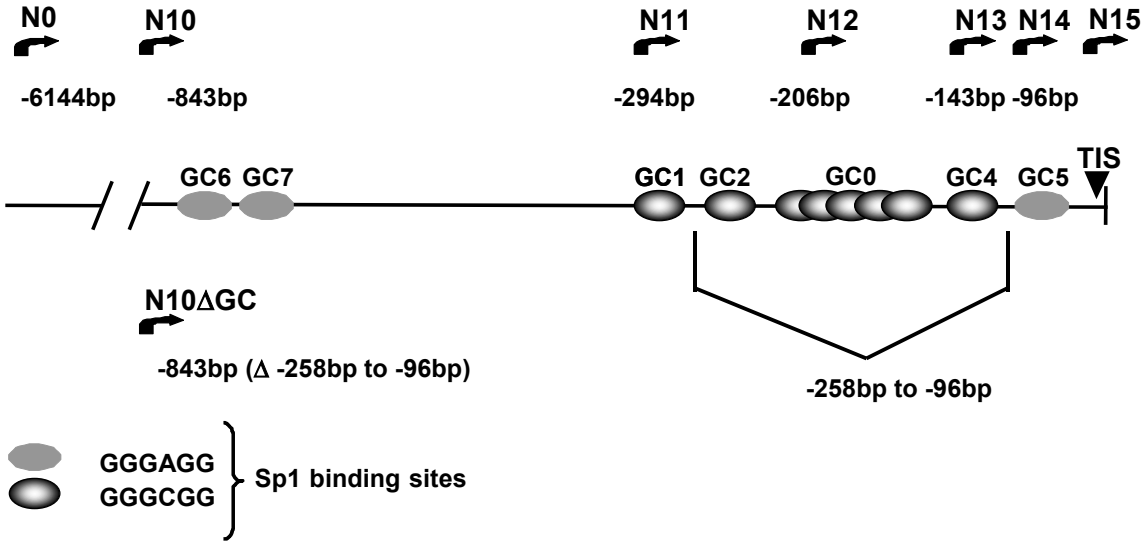


Fig. 16 Schematic overview of the 5-LO promoter and reporter gene deletion variants. *The largest reporter gene construct N0 comprises the sequence -6144 bp to –12 bp of the 5-LO promoter sequence in relation to the ATG of 5-LO. The most active promoter construct is the plasmid N10, containing eight consensus Sp1 binding sites and three non-consensus binding sites. N10ΔGC is a special deletion variant of N10, lacking the central Sp1 binding sites. Further depicted are successive deletion variants of the promoter construct N10, differing in size and GC box content. N15, the smallest construct depicted, just covers the major transcription initiation site (TIS).*

N11 is still containing the sequences –294 bp to –12 bp, N12 –206 bp to –12 bp, N13 –143 bp to –12 bp, N14 –96 bp to –12, N15 –67 bp to –12 bp (just comprising the major transcription initiation site), and N16, lacking the TIS, only comprising the sequence from –35 bp to –12 bp

(all in relation to the 5-LO ATG). N10ΔGC (also N10dGC) is a deletion variant of N10 lacking the sequences –258 bp to –96 bp, including the central Sp1/Egr1 binding sites (compare the chart of Fig. 16). The reporter gene data show an induction of the larger promoter constructs N10, N11, N12 and N13, whereas the smaller constructs, N14 to N16, do not respond to the histone deacetylase inhibitor treatment (see Fig. 17).

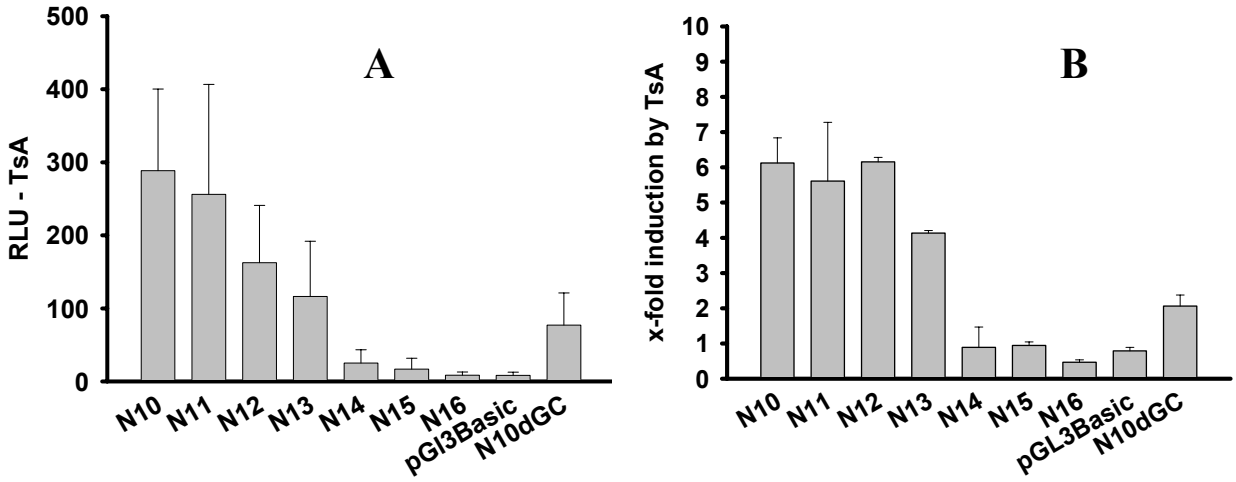


Fig. 17 Induction of 5-LO promoter activity of successive deletion variants by TsA in reporter assays in HeLa cells. *The cells were seeded in 24 well plates and transfected with 0.8 µg of the promoter luciferase reporter gene constructs together with receptor expression vectors pSG5hRXR and pSG5hVDR (0.1 µg each) and 0.02 µg of pCMVSEAP per well by calcium phosphate method. The medium was changed 16 h after transfection and the cells were incubated either with solvent or with TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency by cotransfection of pCMVSEAP. A: Relative activity in untreated cells B: Inductions by TsA, expressed with respect to the untreated and transfected cells.*

It is possible, that the loss of induction by TsA is due to the loss of promoter function, since the shorter reporter gene constructs only have weak promoter activity. Nevertheless, construct N14 is still 5-fold more active than the promoterless construct pGl3Basic.

4.1.2 Mutations of Sp1 binding sites relieve the response to TsA

Besides in length, the deletion variants also differ in their content of Sp1 binding sites. In silico analysis of of the 5-LO promoter reveals eight different consensus binding sites within the proximal promoter area of –294 bp. Sp1 binding to and transactivation of the proximal promoter area has been shown in DNase I footprints and gel shift assays for the stretches -179 bp to -147 bp (containing the 5xGC box), -224 bp to -218 bp (GC2), -118 bp to -109 bp

(GC4) and -85 bp and -66 bp (GC5) as well as in reporter gene assays (In et al. 1997; Silverman et al. 1998; Dishart et al. 2005).

In different genes, e.g. p21, TGFβ type II receptor, induction of promoter activity by TsA has been linked to Sp1 binding sites within the promoter sequence (Sowa et al. 1997; Ammanamanchi and Brattain 2001; Ammanamanchi et al. 2003). Reporter gene assays with the 5-LO constructs suggest, that the response to TsA is dependent on an element still present in construct N13, comprising -143 bp to –12 bp in relation to the 5-LO ATG, but not in the shorter construct N14, comprising only –96 bp to –12 bp. For this reason mutations of the two Sp1 binding sites, GC box 4 (still present in N13, but not in N14) and GC box 5 (still present in N14), have been introduced via site-directed mutagenesis into the minimal promoter constructs N13 and N14. The resulting plasmids N13GC4 (GC box 4 is mutated), N13GC5 (mutated GC box 5), the double mutated construct N13GC45 and N14GC5 have been transfected into HeLa cells and their activity as well as their response to TsA have been measured (see Fig. 18).

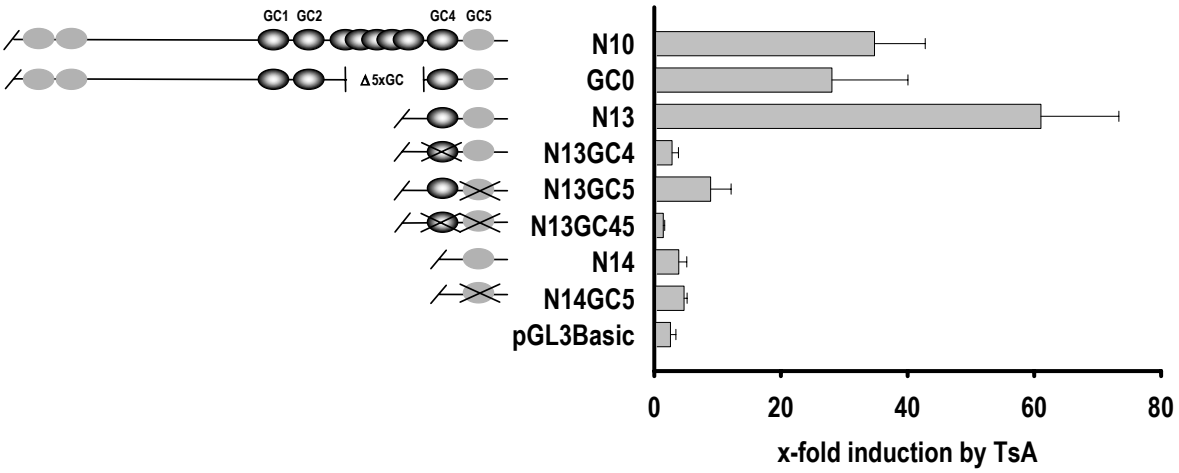


Fig. 18 Point mutations of Sp1 binding sites relieve response of 5-LO promoter constructs to the histone deacetylase inhibitor TsA in HeLa cells. *The cells were transfected by the calcium phosphate precipitation method with the wild–type or mutated reporter gene constructs (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then, luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of six independent experiments after normalization for transfection effeciency. Inductions are expressed with respect to the untreated cells.*

Trichostatin A induces the promoter activity of plasmid N10 up to 34.8-fold. Construct GC0, lacking the 5-fold GC box, is slightly less induced, 28.0-fold, and the shorter deletion variant N13, also lacking the tandemized GC box, in these experiments is strongly induced up to

61.0-fold. The mutation of GC box 4 in N13GC4 attenuates the response to TsA comparable to the level of the shorter construct N14, lacking GC box 4 at all (inductions 2.8-fold and 3.9-fold respectively). Mutation of GC box 5 also strongly decreases the activating effect of TsA, but not to the same extent (8.9-fold induction of N13GC5). Looking at the activity of the constructs itself (without TsA treatment, see Fig. 19) mutation of GC box 4 almost reduces the promoter activity to the level of the promoterless pGl3Basic construct. Mutation of GC5 enhances promoter activity 2.1-fold, the double mutation results in a loss of promoter activity. When the cells are treated with TsA, mutation of GC box 4 in N13 again abolishes promoter activity, mutation of GC5 also reduces promoter activity (3.2-fold).

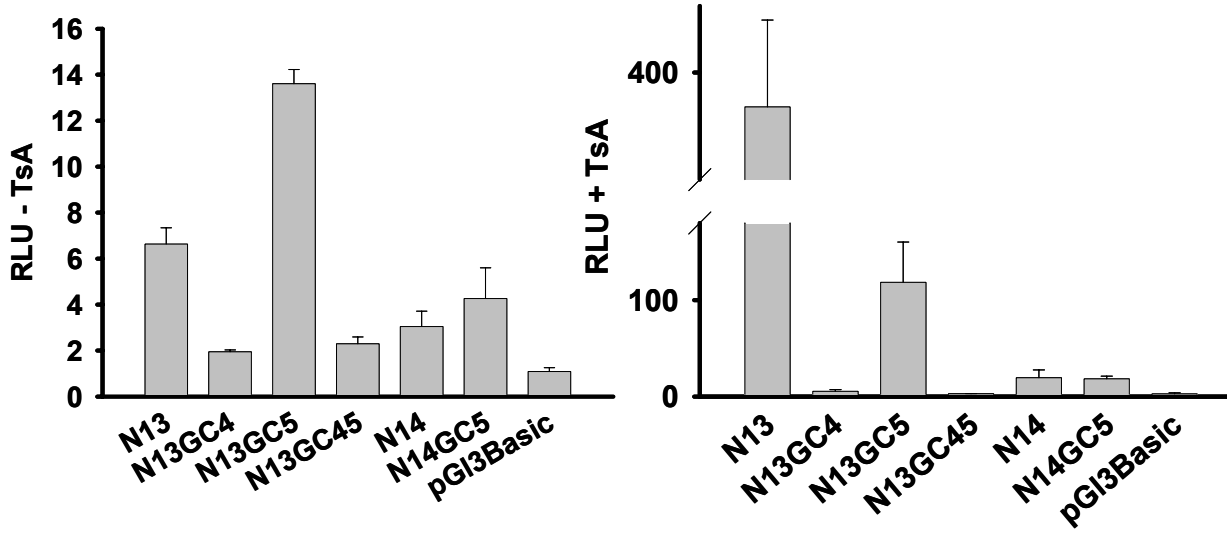


Fig. 19 Relative activity of the GC box-mutated constructs N13 and N14 in HeLa cells. *The cells were transfected by the calcium phosphate precipitation method with the wild–type or point mutated reporter gene constructs (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or with TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of six independent experiments after normalization for transfection effeciency. Inductions are expressed with respect to the untreated cells.*

From these data we conclude, that the proximal Sp1 binding sites GC4 and GC5 recruit HDAC activity. In the minimal promoter construct N13, GC box 4 is an activating element, whereas GC box 5 has repressing features. It seems, that in HeLa cells an transcriptional activator preferably binds to GC box 4, whereas some repressor is recruited to GC box 5. After the treatment with the histone deacetylase inhibitor TsA the formerly repressive function of GC box 5 is reversed into an activating function.

4.1.3 Response to TsA in a larger promoter context

The same mutations of the Sp1 binding sites GC4 and GC5 were cloned into the larger and most active 5-LO promoter construct N10, to investigate the relevance and functionality of these sites in a larger promoter context. In addition to GC4, GC5, and the 5-fold GC box, N10 contains several more putative Sp1 binding sites (compare Fig. 3 page 9). The consensus motifs GC1 (-133 to -139 bp) and GC2 (-116 to -139 in relation to 5-LO ATG), still present in the CpG island comprising the core promoter region, and two non-consensus motifs in the distal CpG island, GC6 (-770 to -765) and GC7 (-720 to -725) were also introduced into N10, resulting in the constructs N10GC6, N10GC7, N10GC1 and N10GC2. GC0 lacks the 5-fold GC box. Sp1 binding to element GC2 had been demonstrated before in DNase I footprints (Silverman et al. 1998).

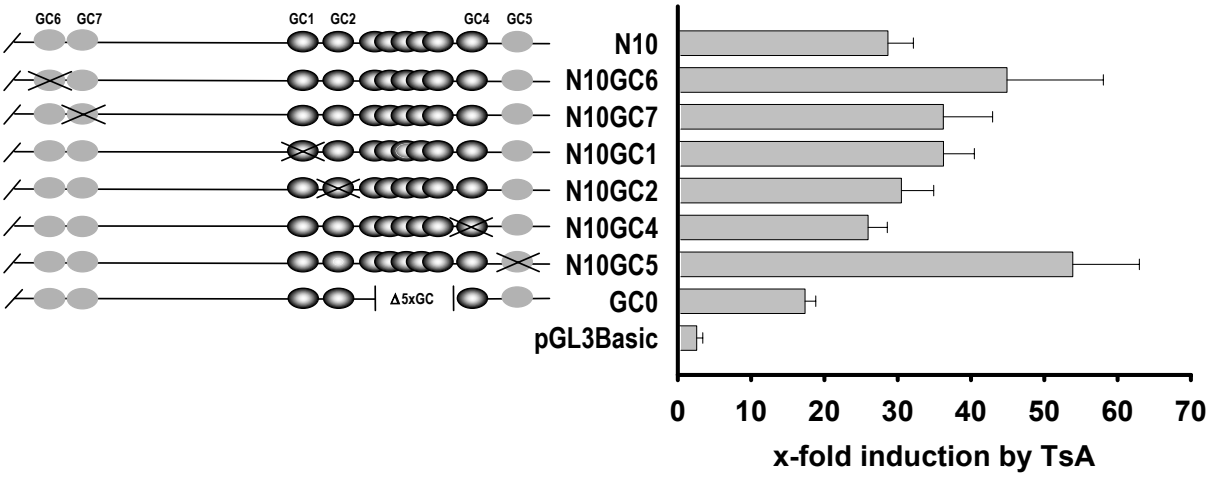


Fig. 20 Mutation of Sp1 binding sites in the reporter gene construct N10 and their response to TsA. *HeLa cells were transfected by the calcium phosphate precipitation method with the wild–type or mutated reporter gene constructs (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of five independent experiments after normalization for transfection effeciency. Inductions are expressed with respect to the untreated cells.*

The mutation of the different GC boxes within the larger promoter context of construct N10 showed weaker influence on the response to TsA than in the minimal promoter plasmid N13 (see Fig. 20). The mutations of GC7 and GC1 do not efficiently influence the promoter response to TsA. Mutation of the distal non-consensus sites GC6 and especially of the proximal site GC5 even increase the effect of TsA. Only the deletion of the 5-tandem GC box and mutation of GC box 4 decrease the response to the histone deacetylase inhibitor (42% and

11% respectively). In the larger promoter context the impact of discrete Sp1 binding sites is less pronounced than in the minimal promoter context of N13.

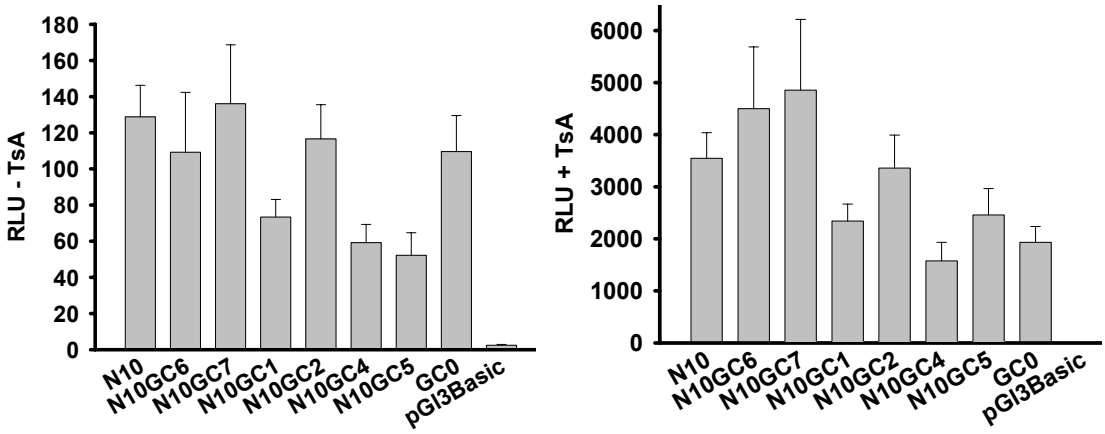


Fig. 21 Relative activity of the GC box-mutated constructs cloned from N10. *HeLa cells were transfected by the calcium phosphate precipitation method with the wild–type or mutated reporter gene constructs (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of five independent experiments after normalization for transfection effeciency.*

The relative activity of the mutated constructs is only reduced, when GC4, GC5 and GC1 are mutated (44%, 54%, 40% respectively; with TsA treatment 35%, 56%, 31%, 46% (GC0) repectively). Also in the larger promoter context GC box 4 is necessary for the full promoter activity. In contrast to the data with the mutated sites in N13, in N10 also GC box 5 and GC box 1 are important for full promoter activity.

4.1.4 Investigations on the role of particular Sp1 binding sites

In order to examine the interdependence of discrete Sp1 binding sites, the particular mutations were combined in the reporter gene construct GC0, already lacking the 5-tandem GC box. The combination of different mutations in the complete construct N10 was impossible to accomplish, due to the presence of the 5-fold GC box, the resulting high GC content and the lack of restriction sites.

The generated constructs were termed according to the mutations carrying, e.g. GC0GC4 is equivalent to construct GC0 with an additional mutation in GC box 4. GC0GC12 is equivalent to GC0, holding extra mutations in the binding sites GC 1 and GC 2. The plasmids

were transfected into HeLa cells and the promoter activity was determined in the luciferase assay (see Fig. 22).

In these experiments the deletion of the 5-fold GC box significantly decreased the promoter activity, which was not the case in an other series of experiments. Mutations of any other Sp1 binding site further reduced the promoter activity, especially the mutation of GC4. The combination of two and more mutated binding site resulted finally in a complete loss of promoter activity (compare GC0GC1245).

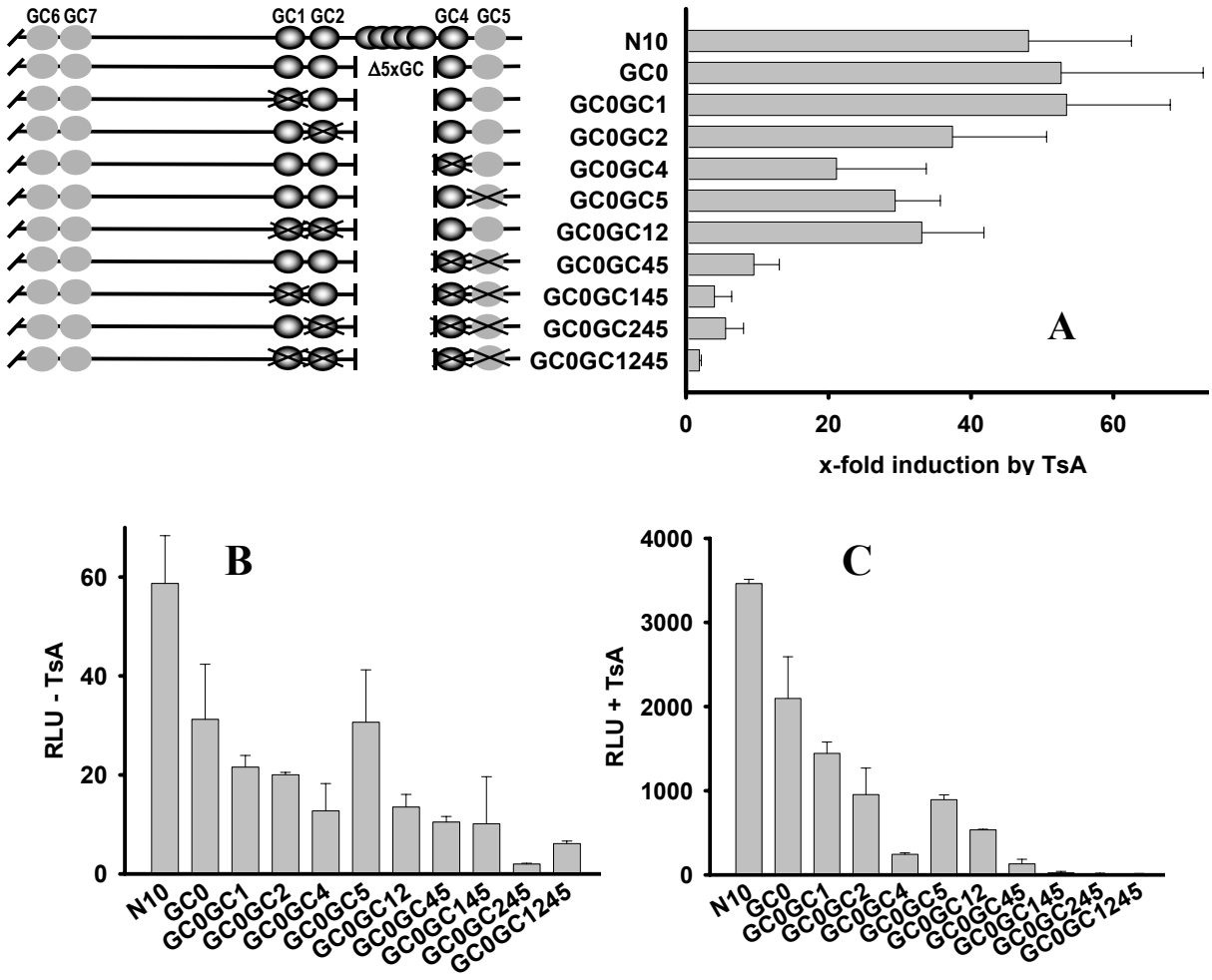


Fig. 22 Mutation of Sp1 binding sites in the reporter gene construct GC0, relative activity and response to TsA. *HeLa cells were transfected by the calcium phosphate precipitation method with the wild–type or mutated reporter gene constructs (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency. Inductions are expressed with respect to the untreated cells. A: x-fold induction by TsA, B and C RLUs without and with TsA treatment respectively.*

The response to trichostatin A was reduced strongest when GC4 was additionally mutated. The combined mutations of GC4 and GC5 further decreased the effect of TsA, any extra mutation eliminates the effect.

Together these results underline the importance of GC box 4 for the full promoter activity of 5-LO in HeLa cells in the minimal construct N13 and in the larger promoter constructs N10 and GC0, as well as the relevance of this site in respect to the recruitment of histone deacetylase activity. The results also suggest an additive effect of the particular sites in the promoter activation, independent of any stimulus.

4.1.5 Reporter gene assays with mutated constructs in MM6 cells

The same experiments were performed in the 5-LO expressing cell line Mono Mac 6, to investigate the influence of the cellular context on the promoter activity and the response to the histone deacetylase inhibitor TsA. Mutation of the GC boxes GC1, GC2 and GC5 in construct N10 do not influence the response of the 5-LO promoter to TsA. Mutation of GC4 strongly reduces the induction by TsA, so does the deletion of the tandem GC box, but to a lesser extent (58% versus 32%). These data are in accordance with the results achieved in HeLa cells (compare Fig. 23 with Fig. 20).

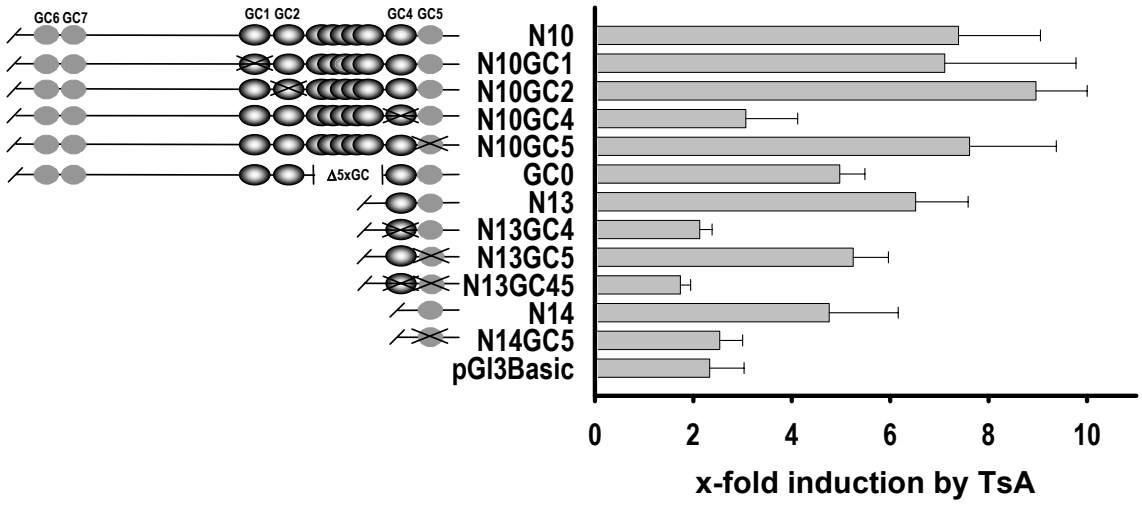


Fig. 23 Induction of 5-LO promoter constructs by histone deacetylase inhibitor trichostatin A in MM6 cells. *72 h before transfection cells were seeded at 2 x 10^5 cells/ml. For the electroporation procedure the cells were suspended at a density of 40 x 10^6 cells/ml in RPMI-1640 medium without any additives. 0.3 ml of the cells suspension were placed into a 0.4 cm electroporation cuvette. 40 µg of reporter gene plasmid and 1 µg of internal standard pCMVSEAP were dissolved in MQ and added to the suspension. Electroporation was performed in a Biorad Gene Pulser at 975 µF and 200 V. The transfected cells were transferred to 10 ml of cell culture medium and incubated with 330 nM TsA.*

After 6 h, the cells were harvested and luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of 2-4 independent experiments after normalization for transfection efficiency. Inductions are expressed with respect to the transfected but untreated cells.

Looking at the minimal promoter constructs, in MM6 cells, TsA induces the shortest construct N14 to the same extent as construct N13 (see Fig. 23). This finding is in contrast to HeLa cells, in which N13 is 15.6 x higher induced than N14 (compare Fig. 20). The higher induction in HeLa cells is probably caused by the different incubation times used for the two cell lines. Mono Mac 6 cells could only be incubated with TsA for 6 h (due to the low survival rate of the cells after the electroporation procedure and the fast decay of luciferase activity), whereas HeLa cells were incubated for 24 h before the promoter activity was determined. As in HeLa cells, the mutation of GC4 in construct N13 attenuates the TsA response, but in contrast to, the mutation of GC5 does not alter the TsA response. Interestingly the same mutation in N14 leads to a loss of induction by TsA. Also in contrast to the results obtained in HeLa cells, both GC boxes are required for full promoter activity in N13, irrespective to the treatment with the histone deacetylase inhibitor (see Fig. 24).

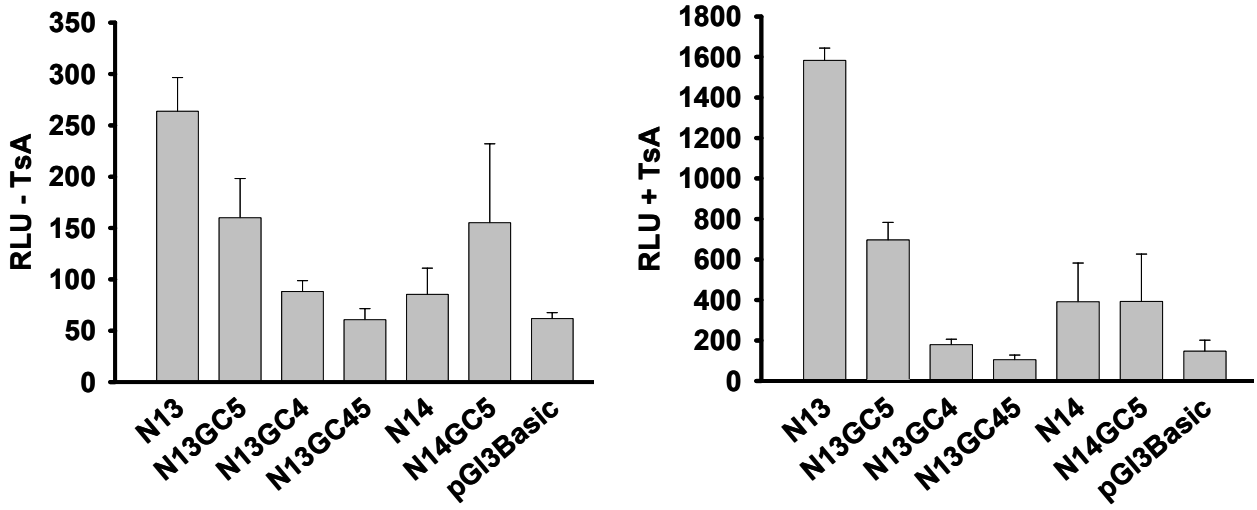


Fig. 24 Relative promoter activity of mutated construct N13 in MM6 cells*. The electroporation procedure was performed as described in Fig. 23. Each experiment was performed in triplicates. Results are presented as mean + SE of 3 independent experiments after normalization for transfection effeciency.*

Within the larger promoter context of construct N10 the mutation of GC box 4 does not result in a decrease of promoter activity. Only after treatment with TsA the activity is significantly reduced, when GC box 4 is mutated.

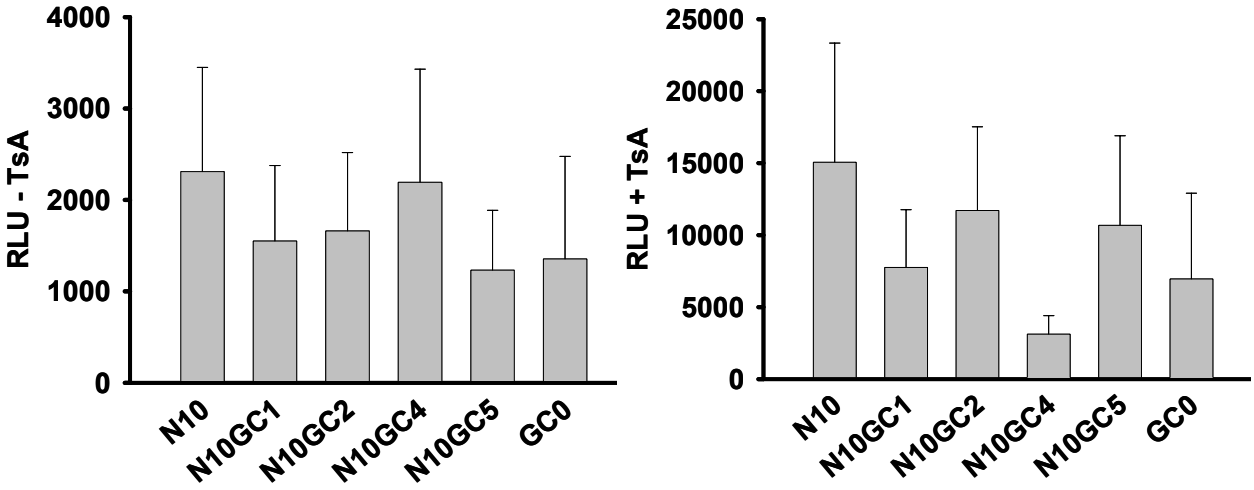


Fig. 25 Relative promoter activity of mutated construct N10 in MM6 cells. *The electroporation procedure was performed as described in Fig. 23. Each experiment was performed in triplicates. Results are presented as mean + SE of 2-4 independent experiments after normalization for transfection effeciency.*

These results suggest a similar role for GC box 4 in both cell lines. Possibly other factors are recruited to the non-consensus site GC5, whose presence in MM6 cells is also necessary for full promoter activity.

4.1.6 Activity and TsA response of more distal promoter parts

Reporter gene assays with successive deletion variants of the 5-LO promoter revealed positively and negatively regulated DNA stretches (Hoshiko et al. 1990; Sorg et al. 2006). In HeLa cells as in MM6 cells, N10 displays the highest promoter activity. The promoter activity of construct N9 (the next larger reporter gene construct, extended by 135 bp, see Fig. 26) is already decreased by 50% compared to the activity of N10. This part of the 5-LO promoter sequence is part of a second CpG island, including two more potential Sp1 binding sites, GC8 and GC9. Activity and response of the plasmids N7, N8 and N9 to the histone deacetylase inhibitor TsA were determined in reporter gene studies. These experiments were only performed in HeLa cells.

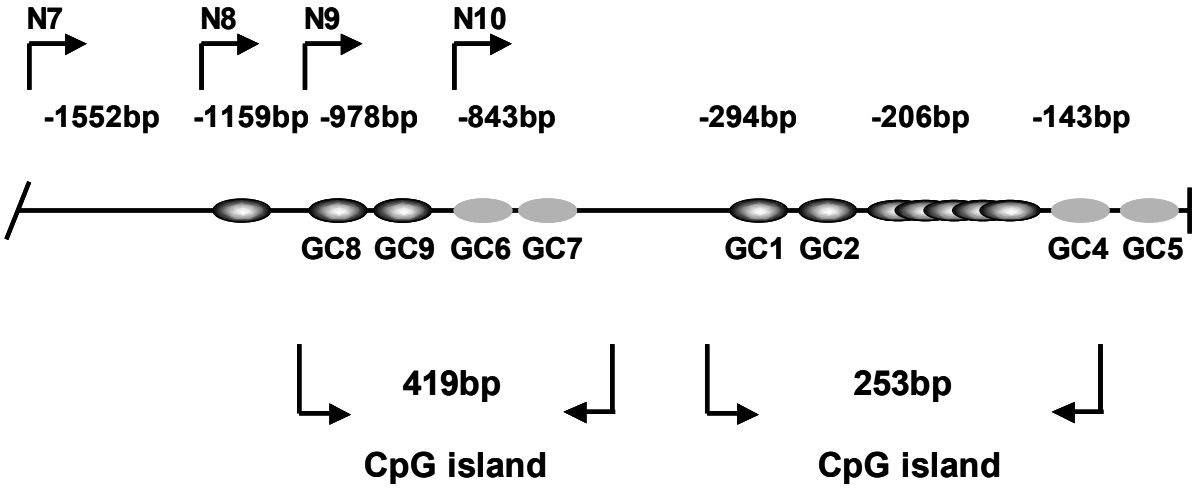


Fig. 26 Schematic overview of the reporter gene constructs N7, N8 and N9. *Covered by these plasmids are more distal promoter parts. In the distal end of N10 starts a second CpG island, containing additional Sp1 binding sites. CpG islands are characterized by an above average GC content, a minimum length of 200 bp and an observed/expected presence of CpG greater than 0.6.*

The distal extension of the promoter plasmids reduces promoter activity (see Fig. 27). N9 already looses 50% of the promoter activity of N10. N8 and N7 display comparable promoter acitivity to N9, indicating the presence of repressive elements within the promoter extension of N9, comprising an additional sequence of 135 bp compared to N10. Additional treatment of the less active promoter plasmids with TsA results in an increase of promoter activtiy, and suggests recruitment of HDAC activity in this promoter region. However their promoter activity does not reach the level of N10 (data not shown).

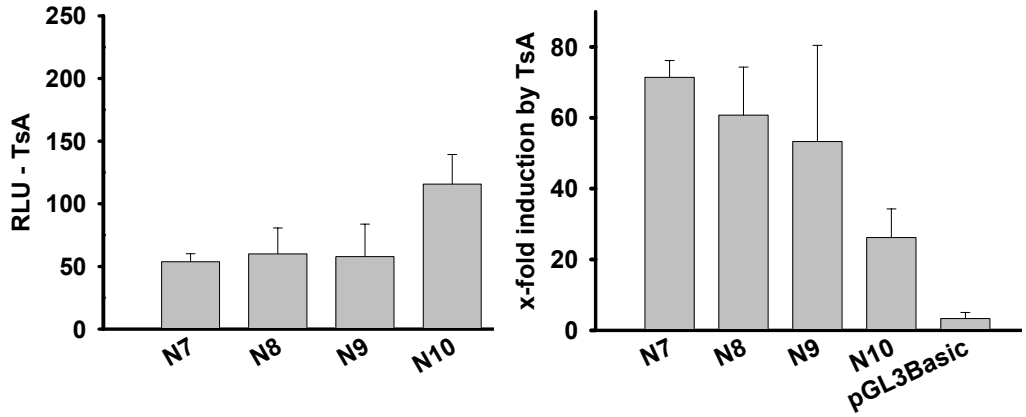


Fig. 27 Relative activtiy and response to TsA of the reporter gene constructs N7, N8 and N9. *HeLa cells were transfected by the calcium phosphate precipitation method (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of*

three independent experiments after normalization for transfection effeciency. Inductions are expressed with respect to the transfected but untreated cells.

To examine the influence of the two potential GC boxes in construct N9, both sites were mutated to inhibit transcription factor binding. Both mutations resulted in a decrease of promoter activity, indicating their activating character (see Fig. 28).

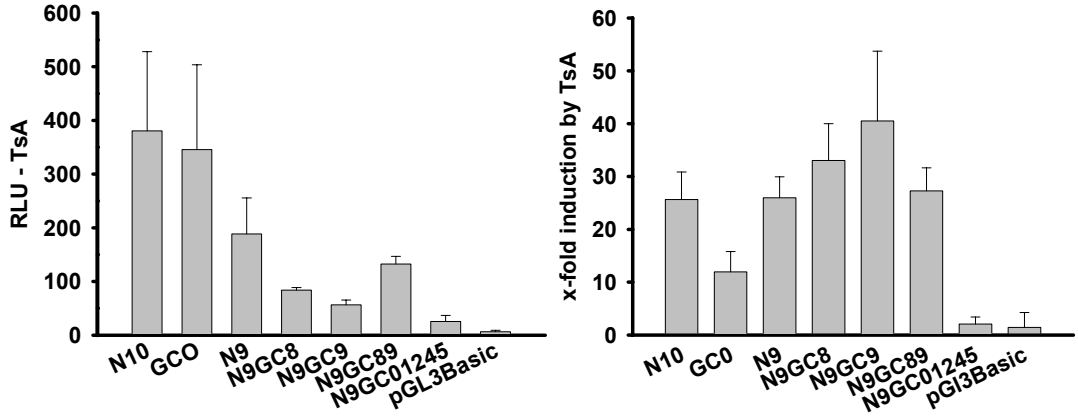


Fig. 28 Mutation of Sp1 binding sites in the reporter gene construct N9 reduces promoter activtiy and enhances the response to TsA. *HeLa cells were transfected by the calcium phosphate precipitation method (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency. Inductions are expressed with respect to the transfected but untreated cells.*

Additional deletion of the 5-fold GC box and the other proximal Sp1 binding sites GC1, GC2, GC4 and GC5 in construct N9GC01245, reduced promoter activity almost to the level of the promoterless plasmid pGl3Basic. (N9GC01245: 25 RLUs, pGl3Basic: 6 RLUs; N9: 188 RLUs). The single mutations of GC8 and GC9 rather increased the promoter response to TsA, whereas the mutation of the proximal sites (GC0GC1245) abolished the induction by TsA. These results underline the importance of the proximal GC boxes for basal and induced promoter activity. The elements GC8 and GC9 do not seem to be responsible for the repressive effects of construct N9, nor do they seem to recruit HDAC activity.

4.1.7 Time dependent induction of promoter activity after TsA treatment

HeLa cells were transfected with the highly inducible reporter gene plasmids N10 and N13, as well as with the promoterless luciferase construct pGl3Basic and the SV40 promoter containing plasmid pGl3Prom as a positive control. After the removal of the calcium phosphate precipitate, the cells were incubated with or without 330 nM trichostatin A for either 4 h, 8 h, 12 h of 24 h. Then the promoter activity was determined.

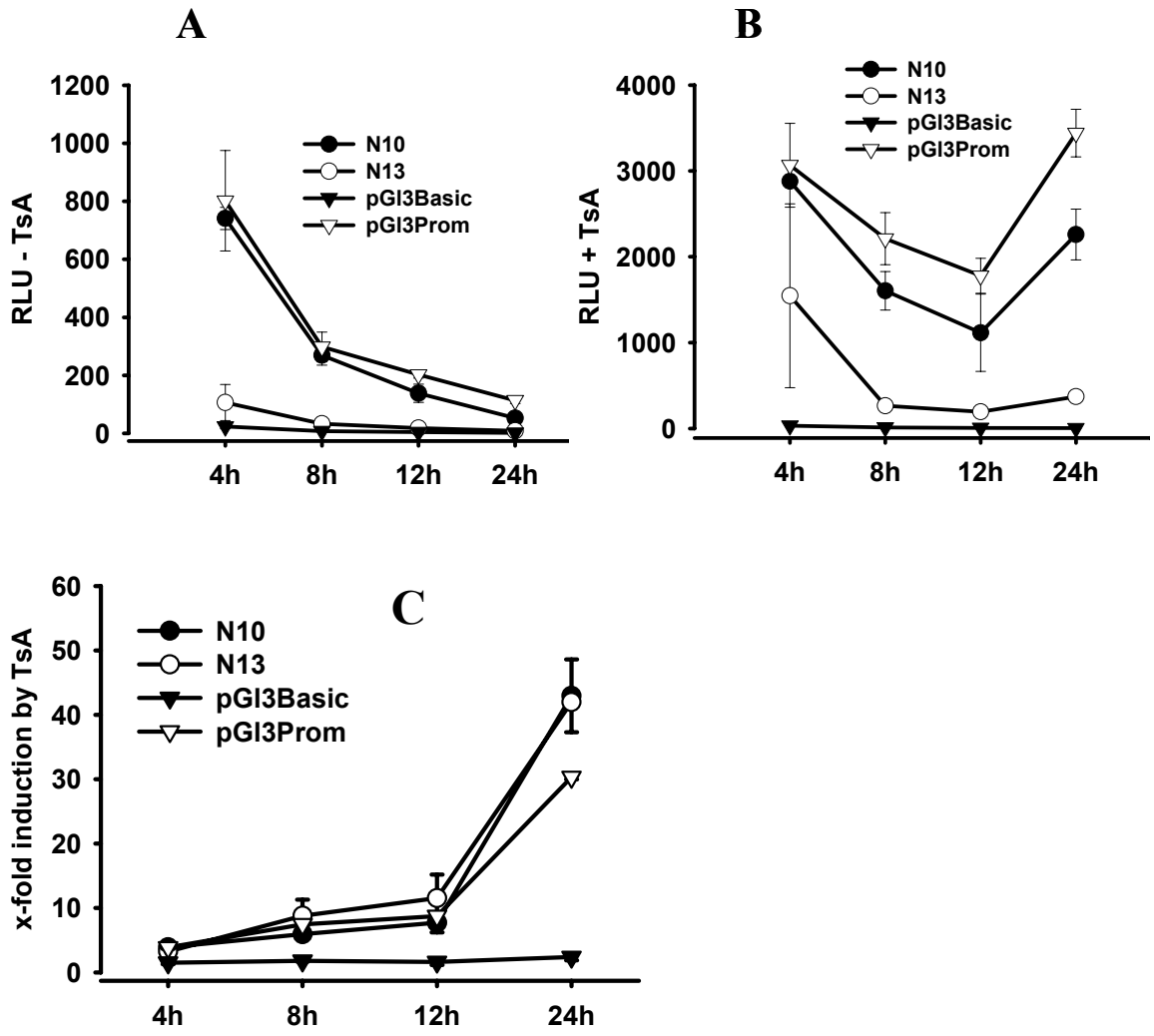


Fig. 29 Time dependent induction of promoter activity in reporter assays after TsA treatment. *HeLa cells were transfected by the calcium phosphate precipitation method (0.8 µg of reporter gene construct, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with solvent (A) or TsA (330 nM) for 4 h, 8 h, 12 h or 24 h (B). Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency. Inductions are expressed in respect to untreated cells (C).*

Without TsA treatment promoter activity is steadily decreasing. When the cells were incubated with TsA the basal level of activity is highly increased already after 4 h, then the activity starts to decay. After 24 h, construct N10 and pGl3Prom, containing multiple Sp1 binding sites (8 versus 6) gain back their full activity, but not the minimal construct N13. These data indicate an early activation of the 5-LO promoter and prolonged transcriptional activity after HDACi treatment.

4.1.8 Effects of valproic acid and nicotinamide on 5-LO promoter activity

As outlined in the introduction, three different families of histone deacetylases are distinguished. Class I HDACs consist of HDAC1, 2, 3 and 8; whereas class II consists of HDAC4, 5, 6, 7, 9, 10 and 11. Members of a third class, also called sirtuins, are structurally unrelated to the human class I and class II HDACs and consist of homologues of the highly conserved yeast Sir2 protein, NAD^+-dependent deacetylases. Nicotinamide, a product of the Sir2 deacetylation reaction, is an inhibitor of Sir2 activity, both in vivo and in vitro.

The activity of class I and class II HDACs is inhibited, amongst others, by hydroxamic and short-chain fatty acids. The hydroxamic acid trichostatin A inhibits class I and class II HDACs, whereas the short-chain fatty acid valproic acid preferentially inhibits class I HDACs (Gottlicher et al. 2001; Gurvich et al. 2004).

In order to identify the histone deacetylases involved in the regulation of the 5-LO promoter, the three inhibitors were tested in reporter gene assay (see Fig. 30 A). The 5-LO promoter construct N10 was transfected into HeLa cells and either incubated together with 330 nM TsA, 1 mM VPA (IC50 in HeLa cells ~1 mM, data not shown) or 5 mM Nicotinamide for 24 h.

TsA activated the 5-LO promoter strongest, 14-fold after 24 h, followed by valproic acid, 8-fold. Nicotinamide showed no effect (1.5-fold). Since VPA also significantly induces 5-LO promoter activity, the recruitment of class I HDACs seems evident, whereas the recruitment of sirtuins is beyond question. In the next experiment, the effect of VPA on the different deletion variants and mutants of construct N13 were tested.

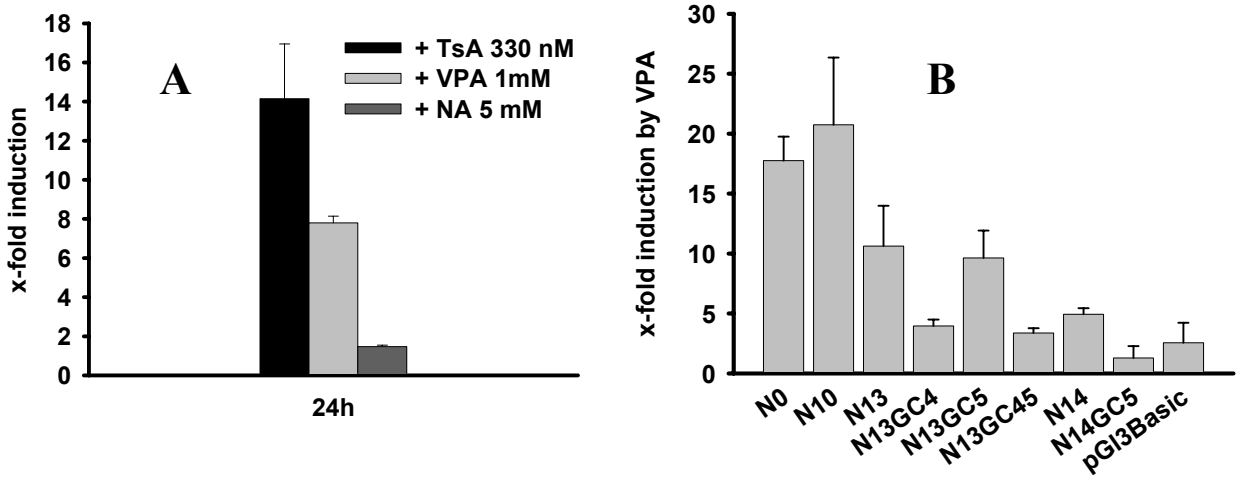


Fig. 30 Effect of different histone deacetylase inhibitors on 5-LO promoter activity. *HeLa cells were transfected by the calcium phosphate precipitation method (0.8 µg of reporter gene construct N10, 0.02 µg of pCMVSEAP per well). The medium was changed 16 h after transfection and the cells were incubated either with DMSO, TsA (330 nM), NA (5 mM) or VPA (1 mM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of 3-4 independent experiments after normalization for transfection effeciency. Inductions are expressed in respect to untreated cells. A: Reporter gene construct N10 was transfected into HeLa cells and incubated with the different HDAC inhibitors. B: The indicated plasmids were transfected and cells were incubated with 1 mM VPA.*

The single mutation of GC box 4 in N13GC4 and the mutation of both GC boxes reduced the activation by valproic acid down to the level of the promoterless plasmid pGl3Basic, comparable to the former experiments in HeLa cells (see Fig. 18). The mutation of GC5 had no infuence on basal of induced promoter activity (data not shown).

4.1.9 Protein expression of Sp1 and Sp3 in HeLa and MM6 cells

Whole cellular protein extracts of TsA treated and untreated HeLa and MM6 cells were prepared and examined by Western blot analysis, as described in "Materials and Methods" 3.3 and 3.6. The protein expression of both transcription factors was unchanged after HDACi treatment in both cell lines.

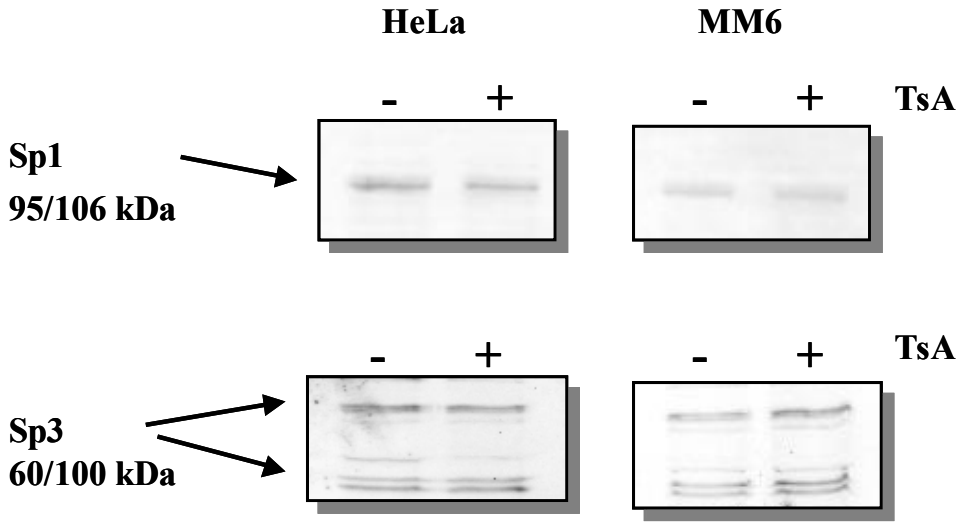


Fig. 31 Endogenous protein expression of Sp1 and Sp3 in HeLa and MM6 cells. *The cells were treated with or without 330 nM TsA for 24h before cell harvest. The preparation of the cell extracts is described in 3.3. The protein content was determined with Bradford assay and same amounts of treated and untreated extracts (10 µg) were loaded for SDS-PAGE and Western blot analysis (see 3.6). Antibodies: anti-rabbit anti-Sp1 and anti-rabbit anti-Sp3.*

4.1.10 Coexpression studies with Sp1 and Sp3 in reporter gene assay

Since the activating effect of the histone deacetylase inhibitors is related to the presence of two Sp1 binding sites, GC4 and GC5, binding of Sp1 and Sp3 to the different mutants of the 5-LO promoter reporter gene construct N13 was tested. For this, expression plasmids for human Sp1 and Sp3 were cotransfected together with the promoter plasmids into HeLa cells and the promoter activity was determined. We expected a further increase of promoter activity at least by Sp1 since activation by this transcription factor has been shown before in SL2 cells, which lack endogenous Sp1 or Sp3 expression.

In HeLa cells, the cotransfection of Sp1 or Sp3 alone did not change promoter activity, which may be due to the high endogenous expression levels of both proteins. On the other hand promoter activity of construct N13 was induced after the cotransfection, when the cells were additionally treated with the histone deacetylase inhibitor trichostatin A, suggesting that TsA somehow enhances the binding or the transactivation by Sp1 and to a lesser degree by Sp3. In these experiments both proteins act as activators on the 5-LO gene expressionl. Sp3 in these experiments does not compete with Sp1 binding.

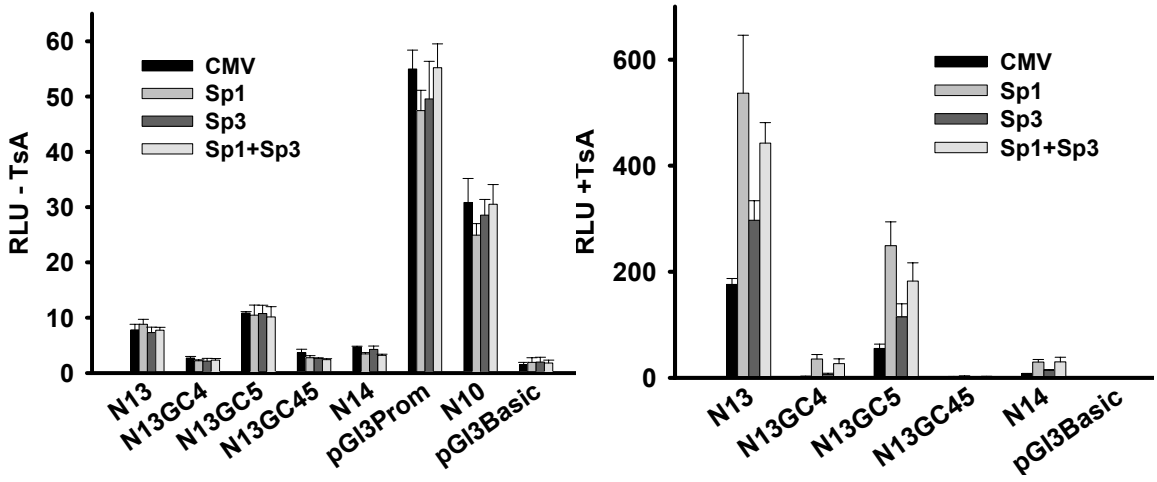


Fig. 32 Cotransfection of Sp1 and Sp3 to 5-LO promoter constructs. *HeLa cells were transfected by the calcium phosphate precipitation method with 0.8 µg of the indicated promoter plasmids and 0.1 µg of either the empty pCMV construct or the expression plasmids pCMVSp1 or/and pCMVSp3. The medium was changed 16 h after transfection and the cells were incubated either with solvent or TsA (330 nM) for 24 h. Then luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of 3 independent experiments after normalization for transfection effeciency by cotransfection of pCMVSEAP.*

The promoter activity of the mutant N13GC4 is strongest induced after Sp1 cotransfection when the cells are treated with TsA, 11.7-fold, followed by N13GC5, 4.5-fold, and N14, 3.9-fold. The effects on N13 and N10 are much weaker, 3.0-fold and 1.4-fold induction by Sp1 cotransfection respectively. Protein overexpression was checked in Western blot analysis (data not shown).

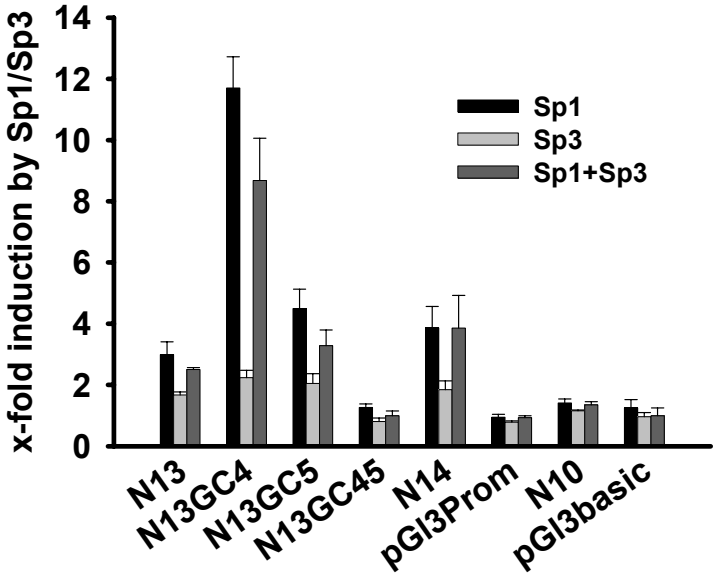


Fig. 33 x-fold induction by Sp1/Sp3 cotransfection. *Experiments were performed as described above in* ***Fig. 32****.*

4.1.11 Effect of TsA on DNA binding affinity of Sp1 and Sp3

Since the endogenous expression levels of the transcription factors Sp1 and Sp3 were not influenced by the treatment with the histone deacetylase inhibitor TsA, it was of interest to examine their binding affinities in response to TsA. A DNA affinity purification assay was established and binding of Sp1 and Sp3 to the proximal GC boxes including the 5-fold tandemized GC box, the consensus site GC4 and the non-consensus site GC5 was analyzed. Specific protein binding to the probes was checked by mutating the GC boxes and by incubating the cell extract only with beads (without a DNA probe).

Forward strands of DAPA probes 5´ to 3´		
CTGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCAG	5 x GC box	5xGC
CAGACCGGGGCGGGGCCGGGACCGGGGC	Consensus Sp1 site / GC box 4	GC4
CAGACCATCTGCAGCCCGGGACCGGGGC	Consensus Sp1 site / GC box 4 mutated	GC4M
GACCGGGGCCAGGGACCAGTGGTGGGAGGAGGCTGCGGCGC	Non-consensus Sp1 site / GC box 5	GC5
GACCGGGGCCAGGGACCAGTGGTGATCTGCAGCTGCGGCGC	Non-consensus Sp1 site / GC box 5 mutated	GC5M

Tab. 5 DNA probes used in DNA affinity purification assay. *Five different probes were investigated covering the proximal Sp1 binding sites of the 5-LO promoter. Depicted are the forward strands of the probes; 5xGC, covering the 5-fold GC box, GC4, covering the consensus binding site, GC4M, covering the mutated GC box 4, GC5 and GC5M, covering the non-consensus binding site either with the wild-type or mutated sequence.*

2 µl of biotinylated DNA probes were incubated with 200 µl of whole cellular extract in 200 µl of binding buffer H (100 mM KCl, 20 mM HEPES [pH 7.8] 20% glycerol, 1 mM DTT, 0.1% NP40) on ice for 1 h. Then 50 µl of streptavidin-coupled agarose beads (Sigma) were added to the mixture and incubated on a spinning wheel for another 30 min at 4°C. After washing the beads four times with 500 µl of binding buffer H the probes were boiled with 20 µl of MQ and 5 µl of 5x laemmli sample buffer (250 mM Tris-HCl pH 6.8, 5 mM EDTA, 50% Glycerol, 10% SDS, 0.05% BPB, 10% β-mercaptoethanol). The supernatant containing the proteins were separated by SDS-PAGE. The use of nuclear extracts instead of the whole-cell extracts resulted in unspecific protein binding.

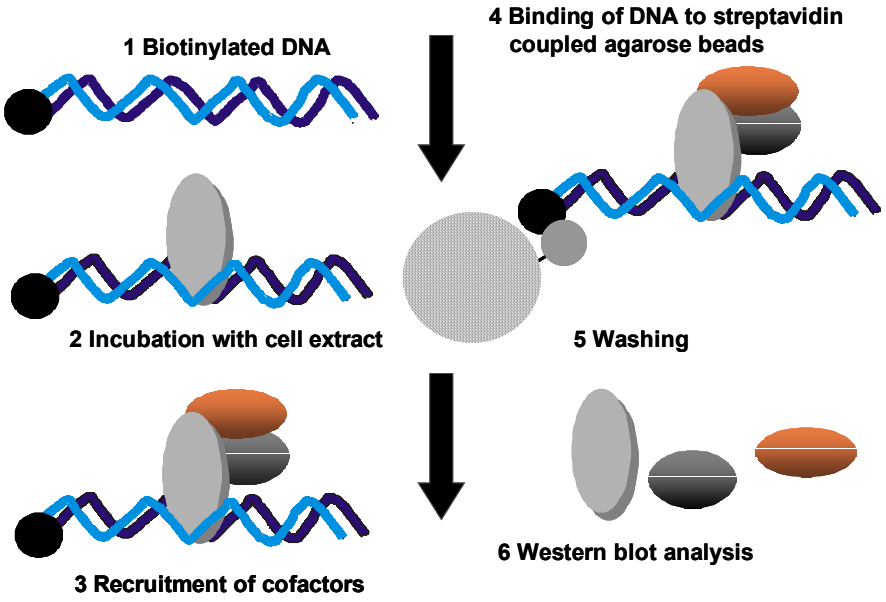


Fig. 34 Principle of DNA affinity purification assay. *Biotinylated DNA probes, either oligonucleotides or PCR fragments, are incubated with cellular extract. Depending on the protocol whole-cell extracts or nuclear cell extracts can be utilized. DNA binding proteins theoretically recruit cofactors. When these DNA-protein complexes have formed, the binding reaction is incubated with the streptavidin-coupled matrix, e.g. with agarose beads. This step is followed by washing steps with binding buffer, in order to reduce unspecific protein binding. To improve the specificity of protein binding, different salt concentrations, tensides or competitor DNA, such as salmon sperm DNA or synthetic polymers like poly(dA-dT)•poly(dA-dT), can be added to the binding reaction itself or to the washing steps. The proteins specifically binding can either be eluted from the beads, also using different salt concentrations or the complete mixture can be boiled with leammli buffer. The captured proteins are separated and identified by SDS-PAGE.*

The binding affinity of Sp1 in this assay neither with HeLa cell extract nor with MM6 cell extract seemed to be changed after the treatment with the histone deacetylase inhibitor (see Fig. 35). In HeLa cells the binding of Sp1 to the non-consensus binding site is less prominent than to the other sites, but independently of trichostatin A treatment. The DNA affinity of Sp3 was also unchanged after HDACi treatment and did not differ between the probes (see Fig. 36).

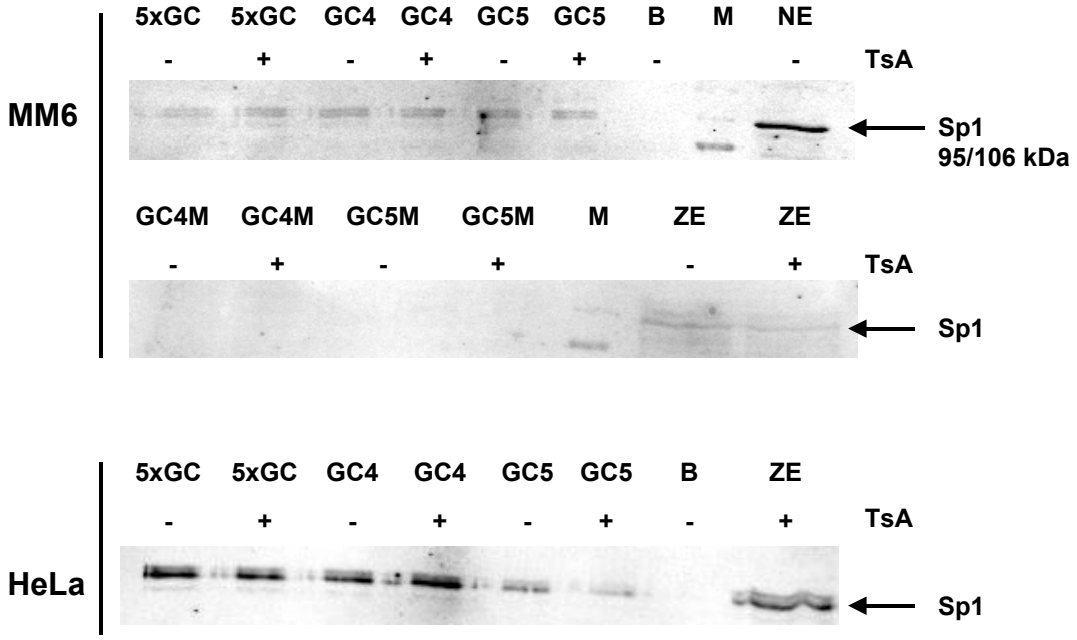
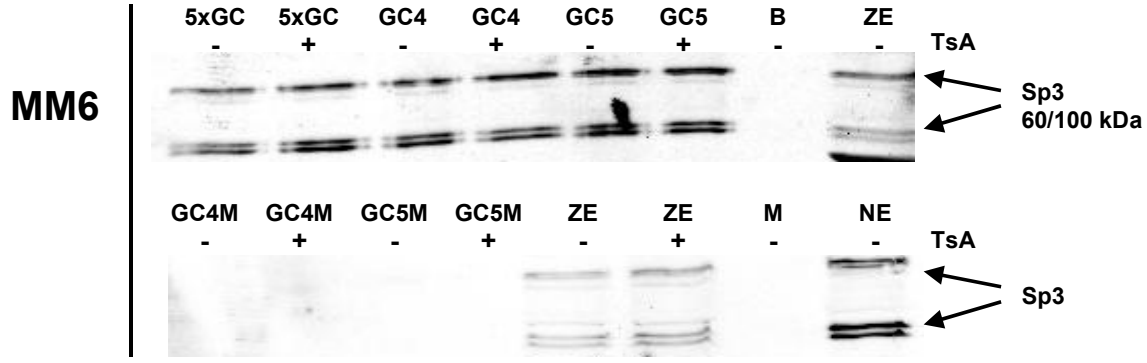


Fig. 35 DNA affinity purification assay with HeLa and MM6 cell extracts for Sp1. *200 µl of whole-cell extract were incubated with 200 µl of binding buffer H and 2 µl of double-stranded biotinylated oligonucleotides for one hour on ice. The cell extracts were gained as described in "Materials and Methods". The cells were either treated with TsA (330 nM) for 24h or with solvent. After adding 50 µl of equilibrated streptavidin-coupled agarose beads, the incubation was continued on a spinning wheel for 30 min at 4°C. The beads were washed four times with 500 µl of binding buffer and after taking off the supernatant the samples were boiled with 25 µl of 1x Laemmli sample buffer for 5 min at 95 °C. The DNA-binding proteins were separated by SDS-PAGE and analyzed by Western blot for the presence of Sp1. One representative example of at least two independent experiments is shown. 5xGC: 5-fold GC box as DNA probe; GC4: GC box 4 as probe; GC4M: DNA probe covering the mutated Sp1 binding site GC4; GC5 and GC5M respectively. M: All Blue Protein Marker (Biorad); ZE: sample of whole-cell extract, used in the DAPA; NE: nuclear extract; B: beads only.*



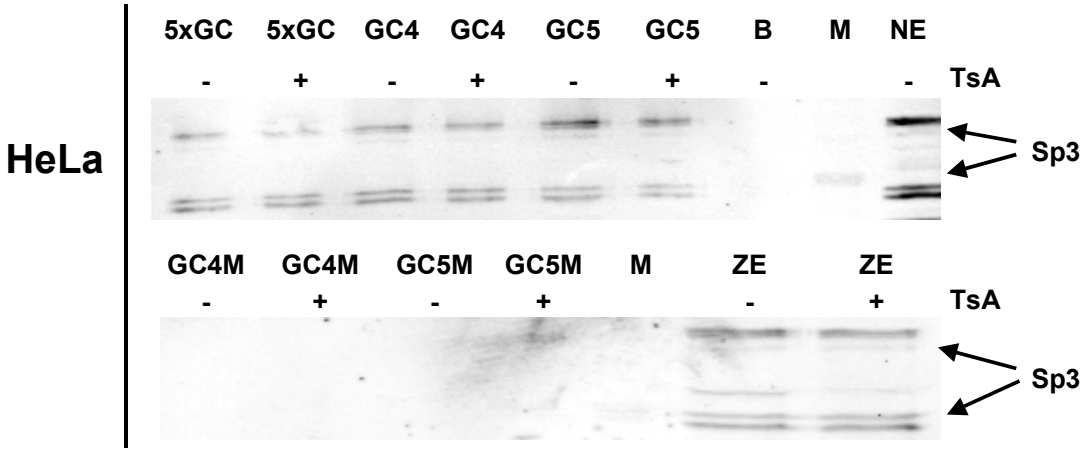


Fig. 36 DAPA with HeLa and MM6 cell extracts for Sp3. *The assay procedure is described in Fig. 35. The DNA-binding proteins from the supernatant were separated by SDS-PAGE and analyzed by Western blot for the presence of Sp3. One representative example of at least two independent experiments is shown. 5xGC: 5fold GC box as DNA probe; GC4: GC box 4 as probe; GC4M: DNA probe covering the mutated Sp1 binding site GC4; GC5 and GC5M respectively. M: All Blue Protein Marker (Biorad); ZE: sample of whole-cell extract, used in the DAPA; NE: nuclear extract; B: beads only.*

4.1.12 In vivo binding of Sp1 and Sp3 to the 5-LO promoter in MM6 cells

In chromatin immunoprecipitation (ChIP) assays the effect of TsA on the histone acetylation status of the 5-LO promoter and the in vivo binding of RNA polymerase II, Sp1 and Sp3 cells was examined in MM6 cells. These data were kindly provided by Dr. Sabine Seuter. Since the promoter activity in the reporter gene assays highly depends upon the proximal GC-rich sequences containing most Sp1 binding sites, ChIP analysis focused on the first 1000 bp just upstream of the TIS.

ChIP assays were performed as described by Väisänen (Vaisanen et al. 2005). In brief, nuclear proteins were crosslinked to genomic DNA by adding formaldehyde directly to the medium (1%, 10 min at 37°C). After lysation in Pipes buffer (5 mM Pipes pH 8.0, 85 mM KCL, 0.5% NP-40 plus protease inhibitors) and SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1 plus protease inhibitors) cells were sonicated to DNA fragments of 300-1000 bp in length. The chromatin was then diluted in ChIP dilution buffer. After incubating the chromatin resuspensions with a salmon sperm DNA / protein A agarose slurry to remove unspecific background the recovered chromatin solutions were incubated over night at 4°C with the indicated antibodies. The immuno-complexes were collected with

protein A agarose slurry. The beads were pelleted, washed with increasing salt concentrations and finally the immuno-complexes were eluted. The crosslink was reversed and the remaining proteins digested by adding proteinase K.

Genomic DNA fragments were recovered using phenol-chloroform extraction. Subsequently the immuno-precipitated genomic DNA was used as a template for PCR. The amount of generated PCR product reflects the abundance of DNA binding proteins. The PCR products, loaded with SybrGreen, were separated by electrophoresis through 2.0% agarose and the gel images scanned on a Fuji FLA3000 reader (for a more detailed description of the procedure and the primer sequences used for the PCR reaction see 3.14 in "Meterials and Methods").

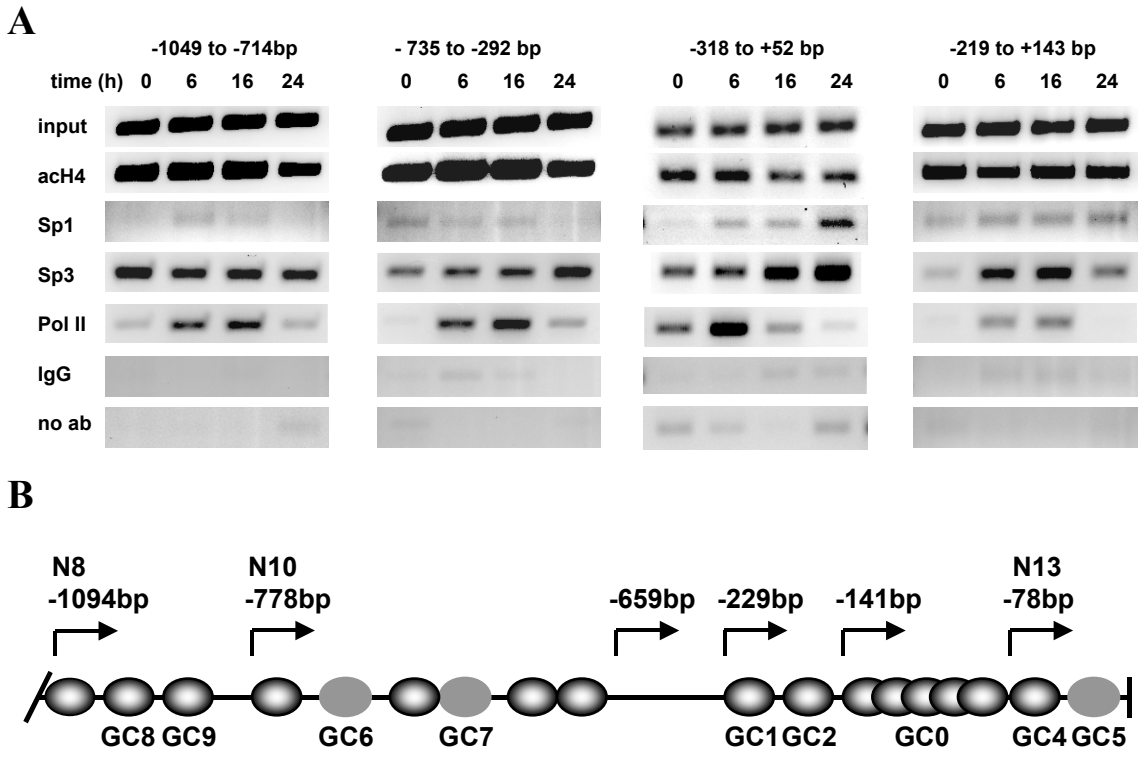


Fig. 37 Chromatin immunoprecipitation assay of the 5-LO promoter in MM6 cells. *A: Depicted are the gel scans of the obtained PCR products covering the proximal promoter region after immunoprecipitation with the antibodies against RNA polymerase II, the transcription factors Sp1 and Sp3 as well as with an antibody against acetylated histone H4 protein, giving information about the activation status of the promoter. As controls, PCRs were run with DNA from recovered chromatin directly after reversing the cross-linking (input bands), after precipitation with IgG (IgG bands), and after immunoprecipitation without any antibody (no ab bands) to exclude unspecific bands. B: The four regions examined in the ChIP are equivalent to the GC rich proximal promoter area, containing*

several Sp1/Sp3 binding sites. Please note, that the numbering is in relation to the major TIS (=+1) of the 5-LO promoter and not in relation to ATG.

The high acetylation status of histone H4 (acetylation of K5, K8, K12, K16), without any TsA treatment indicates an open and transcriptional active chromatin structure of the 5-LO gene promoter area. Nevertheless the 5-LO mRNA expression level in MM6 cells without any treatment is very low (Klan et al. 2003). RNA polymerase II binding within the examined area increases clearly already after 6 h of TsA treatment and exists without any treatment only in the area surrounding the TIS (-318 to +52 bp). Sp1 binding seems to decrease in the two distal promoter parts, whereas the binding in the proximal areas increases with time. Sp3 behaves similarly, within the most distal area the protein slightly looses its affinity, whereas in all other regions the binding is strongly enhanced.

From these in vivo data we can conclude that in MM6 cells, TsA treatment does not increase the acetylation status of the 5-LO promoter and the relaxation state of chromatin, but clearly enhances Sp1 and Sp3 binding, especially within the area surrounding the TIS, including the proximal GC boxes 1, 2, 4, 5 and the tandemized box. Due to the high GC content of the proximal 5-LO promoter, it was not possible to investigate binding to more precise regions or even discrete Sp1 binding sites. Nevertheless, the data also show in vivo binding of Sp1 and Sp3 to the more distal promoter parts including the putative binding sites GC6, GC7, GC8, GC9 and others.

4.1.13 Cotransfection of expression plasmids of class I HDACs

In coimmunoprecipitation studies complex forming between Sp1 and HDAC1 and HDAC2 has been shown in different cell lines, e.g. in the pancreatic cancer cell lines BxPC-3, MIA PaCa-2 cells (Zhao et al. 2003), and in UK Pan-1 cells (Huang et al. 2005). The same publications also suggest a disruption of this complex after treatment with the histone deacetylase inhibitor TsA. Preliminary data by C. Katryniok proofed the recruitment of HDAC1 and HDAC2 by Sp1 both in HeLa and in MM6 cells, but TsA treatment does not alter the complex formation. She also investigated, a possible interaction between Sp3, HDAC1 and HDAC2 in HeLa and in MM6 cells. In both cell lines HDAC1 coimmunoprecipitated with HDAC2, but none of both proteins with Sp3.

An other approach to verify the recruitment of preferentially class I HDACs are coexpression studies with 5-LO reporter gene constructs. Expression plasmids containing the human HDAC1, HDAC2, HDAC3, HDAC8 were cotransfected with the promoter plasmid N13,

containing the TsA responding Sp1 binding sites GC4 and GC5, into HeLa cells. To assure sufficient amounts of Sp1 protein in the cells, the expression plasmid for human Sp1 was additionally transfected. In HeLa cells, HDAC1, 2 and 3 are expressed endogenously, whereas HDAC8 is not (data not shown). Protein expression after transfection was checked by Western blot analysis (data not shown).

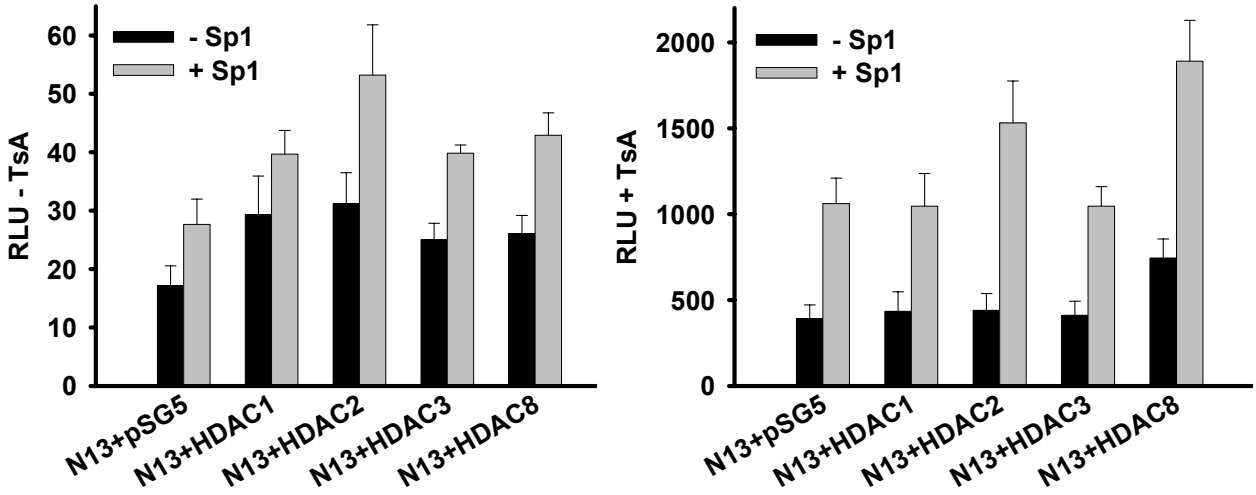


Fig. 38 Cotransfection of HDACs: Relative activity of N13. *Hela cells were transfected by the calcium phosphate precipitation method with 0.8 µg of reporter gene construct N13, with 0.1 µg of pEVR2/CMV-Sp1 or empty pCMV and with 0.1 µg of expression plasmids for the human class I histone deacetylases or the empty control plasmid pSG5. 16 h after transfection the medium was changed and the cells were treated with solvent of trichostatin A (330 nM) for 24 h. Then luciferase activity was determined. Results are presented as mean + SE of 3 independent experiments after normalization for transfection effeciency.*

In untreated cells the additional cotransfection of the histone deacetylases interestingly resulted in an increase of promoter activity and not as assumed in a reduction. This effect was independent of Sp1 cotransfection. When the cells were treated with the histone deacetylase inhibitor the activating effect of the cotransfection was relieved and as shown before the transactivation by Sp1 was induced. These data do not provide any evidence that class I HDACs are directly recruited to the proximal 5-LO promoter.

4.1.14 DNA affinity purification assay: Possible recruitment of HDACs and HATs

An other experimental approach to investigate the recruitment of HDACs to the 5-LO promoter was achieved by DNA affinity purification assays. The same assay conditions used to show specific Sp1 and Sp3 binding were applied and the recruitment to oligonucleotides covering GC box 4 and GC box 5, or containing the mutated Sp1 binding sites was investigated. Additional to the recruitment of HDACs, the potential binding of histone

acetyltransferases (HATs) was investigated. Interaction between Sp1 and p300 has been described in Hep3B cells at the IGFBP-3 promoter (Choi et al. 2002), between Sp1, p300 and PCAF in Mia, PaCa-2 and UK Pan-1 cells (Huang et al. 2005).

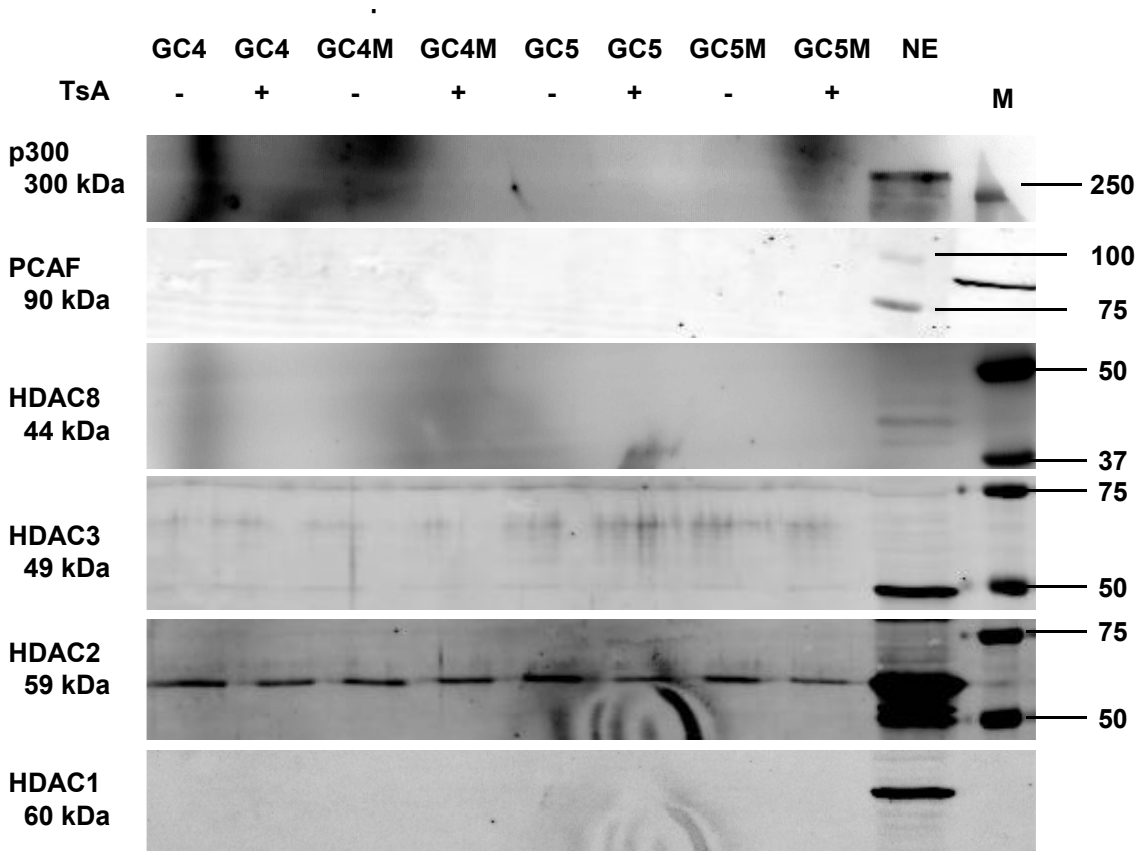


Fig. 39 DNA affinity purification assay with HeLa cell extracts. *200 µl of whole-cell extract were incubated with 200 µl of binding buffer H and 2 µl of double-stranded biotinylated oligonucleotides for one hour on ice. The cell extracts were gained as described in 3.3 in "Materials and Methods". The cells were either treated with TsA (330 nM) for 24 h or with solvent. After adding 50 µl of equilibrated streptavidin-coupled agarose beads, the incubation was continued on a spinning wheel for 30 min at 4 °C. The beads were washed four times with 500 µl of binding buffer and after taking of the supernatant boiled with 25 µl of 1x Laemmli sample buffer for 5 min at 95 °C. The DNA-binding proteins and potential coactivators or corepressors from the supernatant were separated by SDS-PAGE and analyzed by Western blot with the indicated antibodies. One representative example of at least two independent experiments is shown. GC4: GC box 4 as probe; GC4M: DNA probe covering the mutated Sp1 binding site GC4; GC5 and GC5M respectively. M: All Blue Protein Marker (Biorad;) NE: nuclear cell extract. Antibodies: anti-mouse anti-HDAC1 clone 2E10, anti-rabbit anti-HDAC2, anti-rabbit anti-HDAC3, anti-mouse anti-HDAC8; anti-mouse anti-p300, anti-goat anti-PCAF.*

Under the specific binding conditions applied in this assay, originally chosen to proof Sp1 and Sp3 binding, neither p300 nor the histone acetyltransferase PCAF was recruited to the

different oligonucleotides. Also the identification of HDAC1, HDAC3 and HDAC8 failed in this assay. The only evidence for other protein binding eventually by Sp1 or Sp3 could be presented for HDAC2. However, recruitment of HDAC2 appeared not to be specific, since it was found binding to the wild-type probes as well as to the mutated probes and even only to the agarose beads. It seems that the binding conditions for each protein have to be adjusted to proof specific DNA binding or recruitment by DNA-binding proteins.

4.1.15 Investigations on the acetylation status of Sp1 and Sp3

Inhibition of histone deacetylases shifts the balance from histone deacetylase acitvity towards higher histone acetyltransferase activity. Histone acetyltransferases, such as CBP/p300, PCAF, GCN5, do not only acetylate histone proteins, but also transcription factors, including p53, GATA-1, YY1, STAT3 NF-κB and others [reviewed in (Glozak et al. 2005)]. The acetylation of Sp1 eventually results in an increased DNA binding affinity as well as in a higher transactivation activity [reviewed in (Li et al. 2004)]. The acetylation of Sp3 renders the transcription factor a transcriptional activator in the regulation of TGFβ type II receptor (Ammanamanchi and Brattain 2001; Ammanamanchi et al. 2003). In the assumption, Sp1/Sp3 might be acetylated upon TsA treatment, I applied the DNA affinity purification assay to investigate the acetylation status of Sp1 and Sp3 after binding to the 5-LO promoter probes. In the Western blot analysis of the fished proteins, I first detected the transcription factor binding, then incubated the extensively washed membrane with the anti-acetyl-lysine antibody and the equivalent second antibody.

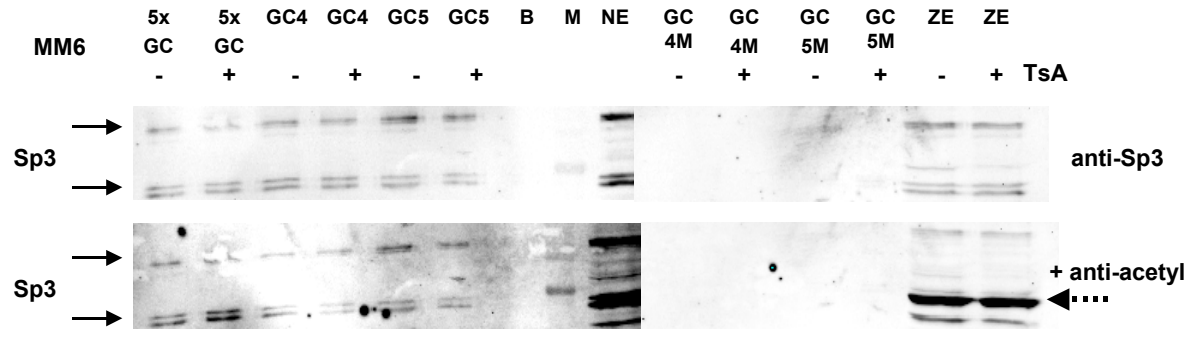


Fig. 40 DNA affinity purification assay for Sp3 with MM6 cell extract. *200 µl of whole-cell extract were incubated with 200 µl of binding buffer H and 2 µl of double-stranded biotinylated oligonucleotides for one hour on ice. The cell extracts were gained as described in "Materials and Methods". The cells were either treated with TsA (330 nM) for 24 h or with solvent. After adding 50 µl of equilibrated streptavidin-coupled agarose beads, the incubation was continued on a spinning wheel*

for 30 min at 4 °C. The beads were washed four times with 500 µl of binding buffer and after taking off the supernatant the samples were boiled with 25 µl of 1x Laemmli sample buffer for 5 min at 95 °C. The DNA-binding proteins were separated by SDS-PAGE and analyzed by Western blot for the presence of Sp3 (anti-rabbit, sc-644, Santa Cruz). Then, the membrane was incubated with anti-acetyl-lysine antibody (anti-rabbit anti-acetyl-lysine #06-933, Upstate) to check for acetylated protein binding. 5xGC: 5-fold GC box as DNA probe; GC4: GC box 4 as probe; GC4M: DNA probe covering the mutated Sp1 binding site GC4; GC5 and GC5M respectively. M: All Blue Protein Marker (Biorad); ZE: sample of whole-cell extract, used in the DAPA; NE: nuclear extract; B: beads only.

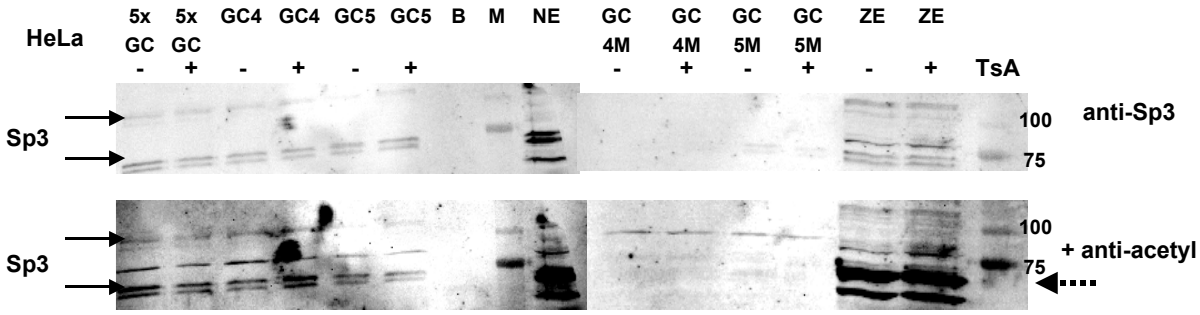


Fig. 41 DNA affinity purification assay for Sp3 and acetylated Sp3 protein with HeLa cell extract. *DAPA and Western blot analysis were performed as described in Fig. 40.*

As in the experiments before, in HeLa and in MM6 cells, trichostatin A did not influence the binding of Sp3 to the different GC boxes of the proximal 5-LO promoter. The incubation with the anti-acetyl-lysine antibody did not change the signals of the Sp3 bands from the pulldown, but intensified a band overlapping the migration band of the smaller isoforms from the cell extracts itself. However, this finding was independent of TsA treatment of the cell extracts.

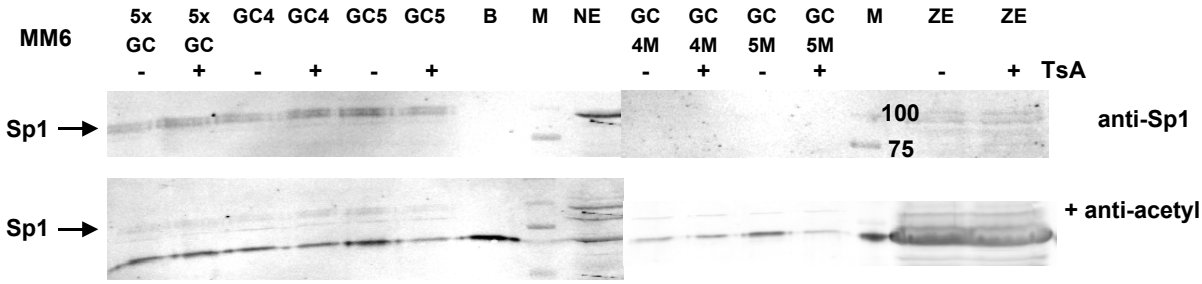


Fig. 42 DNA purification affinity assay for Sp1 with MM6 cell extract. *DAPA and Western blot analysis were performed as described in Fig. 40. Antibodies: anti-rabbit anti-Sp1, sc-059 (Santa Cruz); anti-rabbit anti-acetyl-lysine, #06-933 (Upstate).*

The same experiment was repeated and instead of Sp3, Sp1 was detected. Sp1 binding was not influenced by TsA. After incubating the membrane with the anti-acetyl-lysine antibody, additional bands appeared in lanes of the cell extracts, as well as in the lanes of the pulled samples. Nevertheless, these bands did not overlap with Sp1 and did also appear in the negative control (beads only), therefore resulting from some unspecific protein binding to the agarose beads.

4.2 Regulation of the 5-lipoxygenase promoter by DNA methylation

The expression of 5-lipoxygenase in myeloid cell lines is regulated by DNA methylation, as was shown by Uhl and Klan et al. (2002). In the 5-LO negative cell lines U937 and HL-60TB the 5-LO promoter was heavily methylated within the proximal promoter region, whereas it was completely unmethylated in the 5-LO positive cell line HL-60. Treatement of U937 and HL-60TB cells with the DNA methylase inhibitor AdC (5-aza-2`-deoxycytidine) could induce 5-LO expression and led to a demethylation of the promoter as shown by bisulfite sequencing. Both in HeLa (5-LO negative) and in MM6 cells (5-LO positive), reporter gene constructs of the 5-LO core promoter showed strong activity, which was almost abolished after in vitro methylation with *Sss*I methylase, recognizing and methylating all CpG dinucleotides.

4.2.1 **DNA methylation reduces promoter activity in reporter gene studies**

In order to localize relevant methylation or transcription factor binding sites within the core region of the 5-LO promoter which could be involved in the repression of promoter activity by DNA methylation, reporter gene constructs differing in length and CpG dinucleotide content were in vitro methylated, purified by phenol-chloroform extraction and transfected into HeLa cells. Besides *Sss*I methylase recognizing all potential CpG dinucleotides, M. *Hpa*II and M. *Hha*I were used for methylation (as well as their combination), only partially methylating the promoter because of their more specific recognition sites, -CCGG- and –CGCG-, respectively. 8 h after the calcium phosphate transfection, the medium was changed, cells were incubated with either ethanol or trichostatin A (330 nM) for 24 h and reporter gene activity was determined.

As in former experiments, in vitro methylation led to a strong reduction of 5-LO promoter activity. Methylation with *Sss*I methylase reduces promoter activity to the level of the promoterless reporter gene plasmid pGl3Basic. Also M. *Hpa*II and M. *Hha*I inhibit promoter activity, though to a lesser extent. Besides pGl3Basic as negative control, pGl3Prom was used as positive control. The reporter gene plasmid contains pGl3Basic as backbone (as the 5-LO constructs) and the SV40 (simian virus) promoter instead of the 5-LO promoter. Since this promoter lacks *Hpa*II and *Hha*I methylation sites, one should expect no alterations in promoter activity after methylation with M. *Hpa*II and M. *Hha*I. Nevertheless, *Hpa*II methylation leads to a significant decrease of promoter activity of pGl3Prom (16.7–fold), indicating, that methylation of the plasmid backbone can influence promoter activity too, possibly via action of methyl-CpG-binding proteins from more distal plasmid parts.

Methylation of all CpG dinucleotides by *Sss*I methylase showed the strongest repression of promoter activity, 50.8-fold for the largest construct N10, comprising –778 bp to +53 bp in

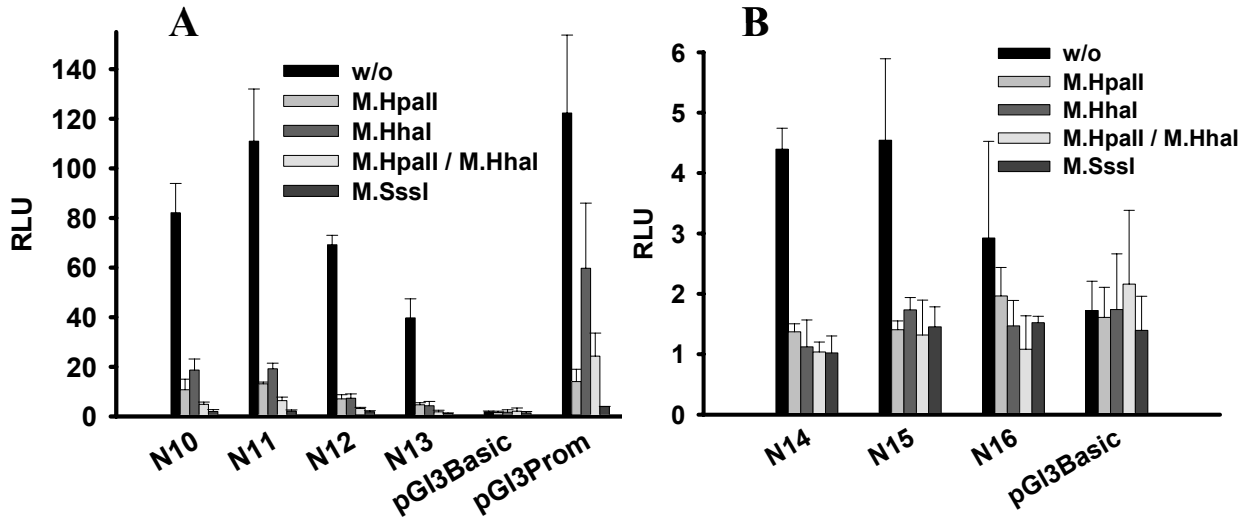


Fig. 43 ***DNA methylation reduces promoter activity in reporter gene assays.*** *After in vitro methylation with HpaII, HhaI or SssI methylase, plasmids were transfected into HeLa cells by the calcium phosphate method. Medium was changed 8 h after transfection, Reporter gene activity was measured 24 h later. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency by cotransfection of pCMVSEAP. Reductions are expressed with respect to unmethylated plasmids.*

relation to the major TIS, 63.3-fold, 41.6-fold, 35.1-fold, 21–fold for N11, N12, N13 and N14 respectively, and only 4-fold and 2.2-fold for the minimal promoter constructs N15 and N16. The smaller the deletion variants are, the smaller is the loss of promoter activity caused by the different methylation patterns (N14 to N16). This may be due to the basically lower promoter activity of the shorter constructs, the adjusting ratio of the number of methylation sites for the different methylases or may indicate the special relevance of the methylation sites within the proximal promoter area.

Plasmid	Promoter	HpaII M. sites / x-fold reduction	HhaI M. sites / x-fold reduction	SssI M. sites / x-fold reduction
N10	-843 bp to –12 bp	6 / 11.6*	10 / 5.2*	64 / 50.8*
N11	-294 bp to –12 bp	5 / 8.5*	4 / 6.1	31 / 63.3*
N12	-206 bp to –12 bp	5 / 11.2**	3 / 10.8**	21 / 41.6**
N13	-143 bp to –12 bp	5 / 9.1*	3 / 23.4	15 / 35.1*

N14	-96 bp to –12 bp	1 / 9.4	3 / 20.7	10 / 21.0
N15	-67 bp to –12 bp	1 / 3.2	2 / 2.7	8 / 4.0
N16	-34 bp to –12 bp	1 / 2.1	1 / 1.8	3 / 2.2
pGl3Prom	SV40	0 / 16.7*	0 / 5.5	10 / 58.5*

Tab. 6 Depicted are the number of methylation sites and the x-fold reduction caused by the specific methylation patterns. *The data were achieved in the same experiments as in Fig 43. Reductions are expressed with respect to unmethylated plasmids. *, P<0.05 **, P<0.01, by paired two-sided Student´s t test. The positions of the promoter sequences included in the different plasmids are in relation to the 5-LO ATG.*

4.2.2 Reduction of promoter activity correlates with methylated CpG sites

In the larger luciferase reporter gene constructs (N10, N11, N12, and N13) the decrease in promoter activity is dependent on the number of methylated CpGs within the promoter region. Fig. 44 is showing the correlation between the number of methylation sites and the reduction by DNA methylation. Clearly the reduction in promoter activity is increasing with the number of methylation sites.

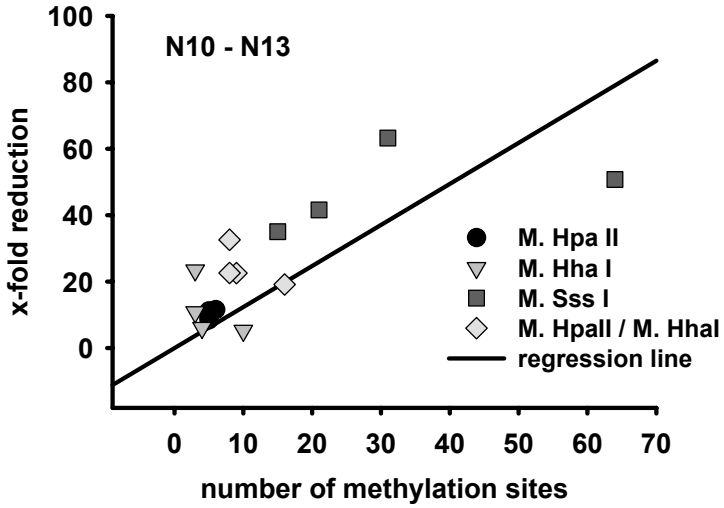


Fig. 44 The reduction in promoter activity of the larger promoter constructs N10, N11, N12 and N13 correlates with the number of methylation sites. *Data were achieved in the same experiments as in Fig.43. Plotted are x-fold reductions in relation to the number of methylation sites. The linear regression line was drawn through the origin.*

4.2.3 TsA induces 5-LO promoter activity dependent on the degree of methylation

In vivo, so-called methyl-CpG-binding proteins can bind to methylated DNA sequences and either directly or indirectly recruit histone deacetylases. As mentioned before, endogenous 5-LO gene expression in MM6 cells is induced after TsA treatment (Klan et al. 2003). So far no studies concerning the methylation status of the 5-LO promoter in MM6 cells exist. Since treatment with $1,25(OH)_2D_3$ and TGFβ strongly induces the protein expression in MM6 cells, but not in U937 or HL-60-TB cells (in both cells lines the promoter is methylated), we assume that the promoter is not methylated in MM6 cells. In order to investigate, if DNA methylation promotes recruitment of HDACs to the 5-LO promoter, HeLa cells were additionally stimulated with TsA when transfected with the methylated promoter constructs.

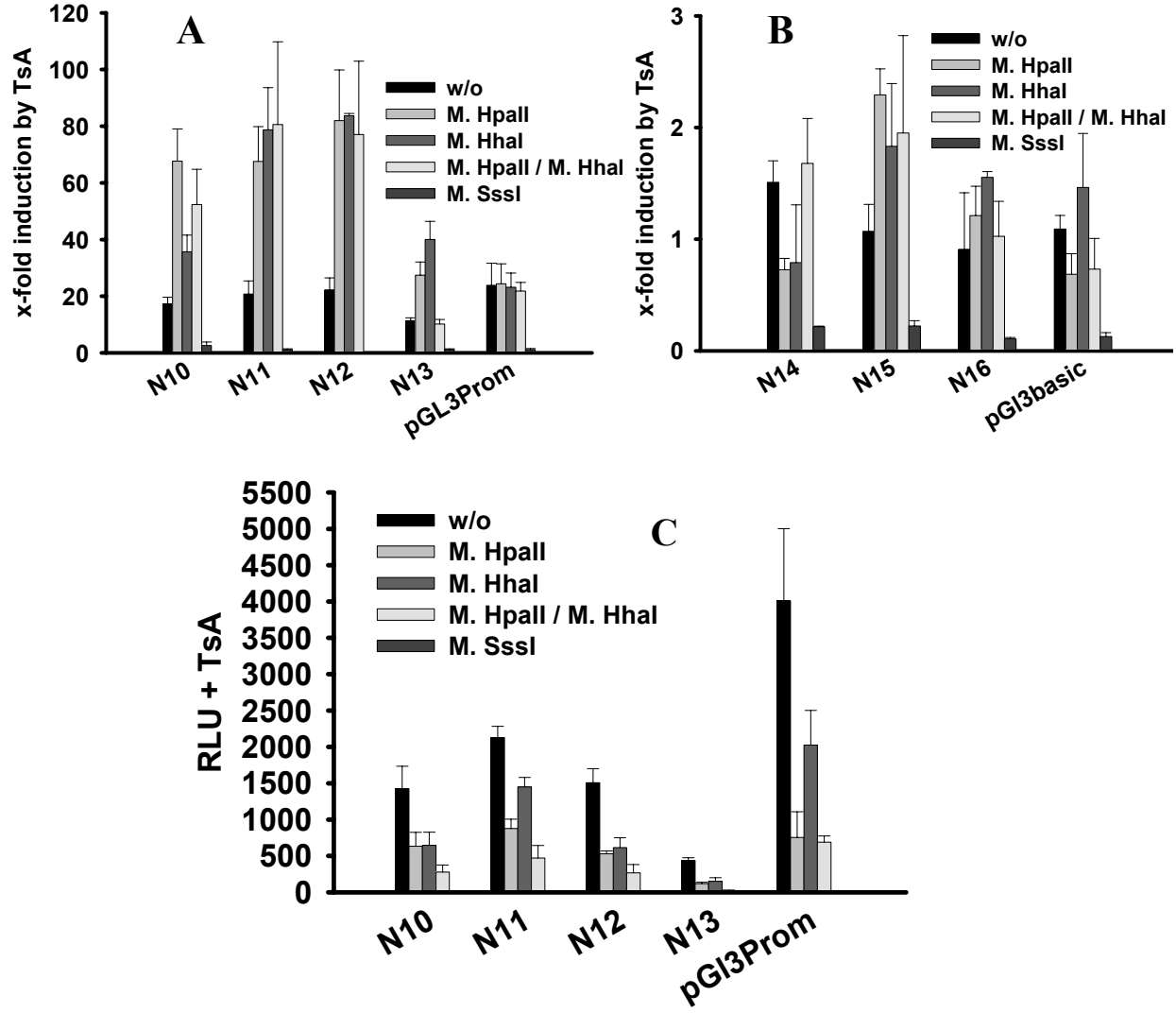


Fig. 45 TsA induces promoter activity of unmethylated and partially methylated reporter gene constructs. *After in vitro methylation with HpaII, HhaI or SssI methylase, plasmids were transfected into HeLa cells by the calcium phosphate method. Medium was changed 8 h after transfection, cells were incubted with TsA 330 nM for 24 h. Each experiment was performed in triplicates. Results are*

presented as mean + SE of at three independent experiments after normalization for transfection effeciency by cotransfection of pCMVSEAP. Inductions by TsA are expressed with respect to untreated cells (A,B). C: relative promoter activites after TsA treatment.

Promoter activity of the larger unmethylated promoter constructs N10 to N13 is increased after treatment with TsA, whereas the small constructs N14, N15 and N16 are not affected. This finding locates the effect of TsA to the more distal promoter area upstream of N14. Complete DNA methylation is abolishing promoter activity as well as any induction by TsA. Either TsA treatment alone is not sufficient to reactivate the promoter or the regulation is independent of any HDAC recruitment at this state of methylation.

Partial methylation of the promoter fragments by M. *Hpa*II and M. *Hha*I on the other hand stimulates activation by the histone deacetylase inhibitor, indicating a recruitment of HDACs to the promoter and the potential of activation of the promoter. Still, the methylated constructs never reach the original promoter activity of the unmethylated constructs.

4.2.4 Cotransfection of different methyl-CpG-binding proteins

Methylated DNA is recognized by methyl-CpG-binding proteins (MBD proteins). In order to identify MBD proteins binding to the 5-LO promoter, we intensified reporter gene assays. In cotransfection studies with methylated promoter plasmids and expression plasmids for murine Mbd1, Mbd2, Mbd3 and rat MeCP2, we expected the methyl-CpG-binding proteins to further reduce promoter activity, either by competing with other transcription factors or by recruitment of corepressors, including HDACs. The coding sequences of the different MBDs were cloned into pSG5, to ensure the same expression levels of the different proteins (for details see "Materials and Methods", 3.7.2). Protein expression was controlled by Western blot analysis. We could not detect endogenous MBD1 or MeCP2 in HeLa cells.

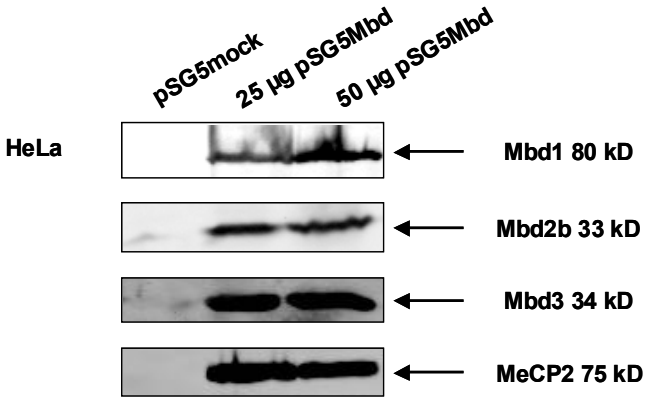


Fig. 46 Protein overexpression of methyl-CpG-binding proteins in HeLa cells. *24 h before transfection, HeLa cells were seeded in 14.5 cm ∅ dishes, so that 60-80% of the cells were confluent*

at the time of transfection. Cells were either transfected with 50 µg of pSG5mock per dish or with 25 µg or 50 µg of expression plasmid pSG5Mbd1, pSG5Mbd2, pSG5Mbd3 or pSG5MeCP2 by calcium phosphate transfection. 16 h after transfection the medium was changed, 24 h later the cells were harvested and fractionated into the cytosolic and nuclear compartments, as described in "Materials and Methods", 3.4. For SDS-PAGE and Western blot analysis the different probes were normalized to the cell number of harvested cells. The nuclear fractions were blotted.

In the reporter gene assays with methylated construct N10, only the cotransfection with Mbd1 leads to a further reduction in promoter activity. The effect is strongest after *Hpa*II methylation, but also exists after methylation with M. *Hha*I and M. *Sss*I, see Fig. 47. The same is true for construct N13, indicating a possible binding site within this proximal promoter region.

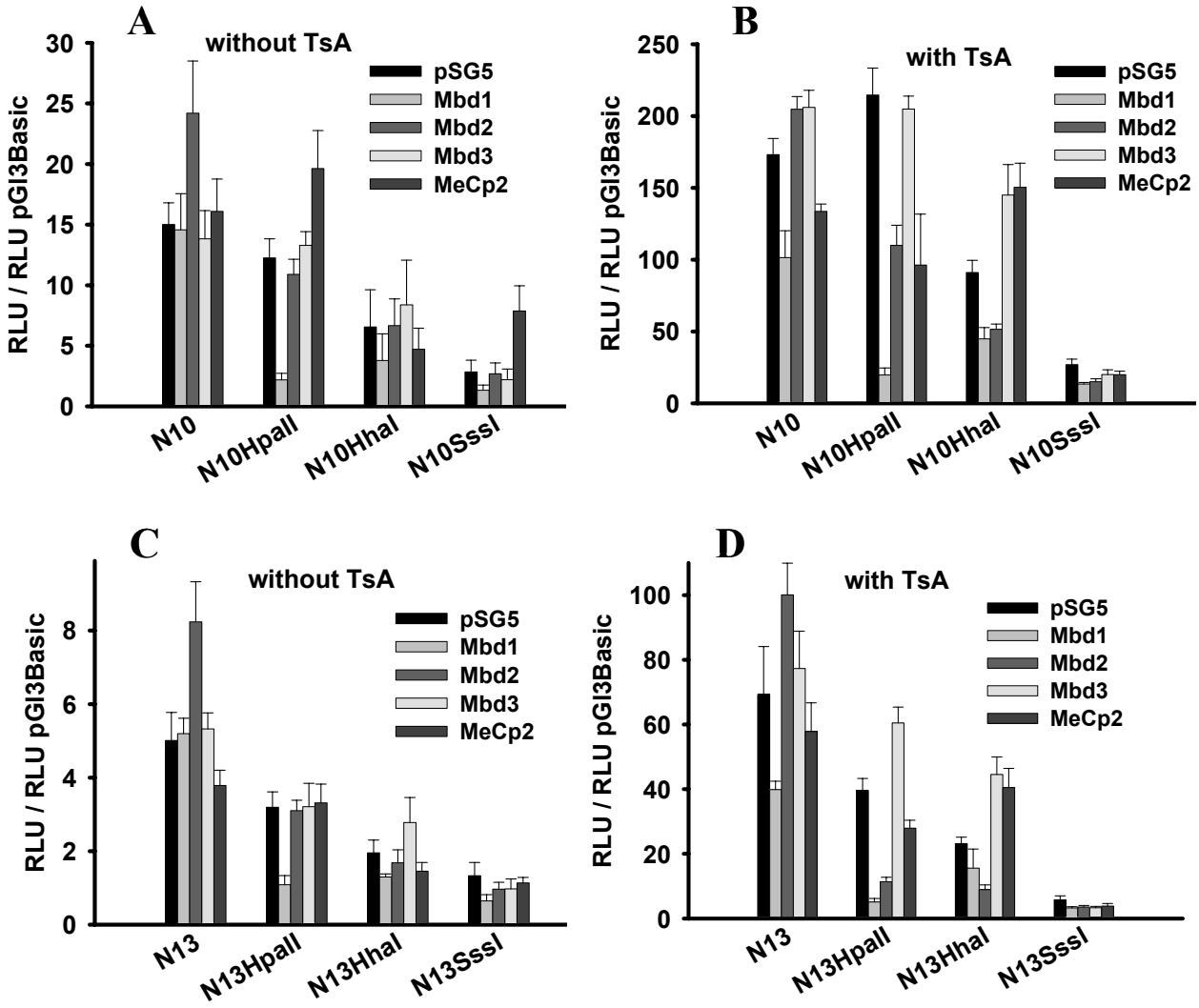


Fig. 47 Relative Activity of methylated reporter gene constructs N10 and N13 cotransfected with methyl-CpG-binding proteins. *After in vitro methylation of the reporter gene plasmids and purification with phenol-chloroform extraction, the constructs were transfected into HeLa cells by calcium phosphate method. The cells were seeded in 24 well plates and transfected with 0.8 µg of the*

methylated reporter gene plasmids with 0.02 µg of pCMVSEAP and 0.1 µg of the expression plasmids. Medium was changed 8 h after transfection, cells were incubted with solvent or TsA, 330 nM, for 24 h. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency by cotransfection of pCMVSEAP. This time, luciferase activity was determined using the Bright Glow® Luciferase Assay System (Promega), ensuring higher activities (about 10-fold) of the methylated constructs, due to the different buffer conditions during the luciferase assay. Also RLUs had to be normalized to pGl3Basic because the cotransfection caused backbone effects on pGl3Basic.

Additional treatment of the cells with TsA does not relieve the reduction in promoter activity caused by Mbd1. This finding is according to (Fujita et al. 2003), suggesting an alternative pathway in the repression by Mbd1 independent of HDACs. Interestingly, when the cells are treated with the histone deacetylase inhibitor TsA Mbd2 decreases the promoter activity.

4.2.5 Mutation of methylation sites within the proximal promoter of 5-LO

Observations so far suggest the presence of relevant methylation sites and a possible recruitment of Mbd1 to the proximal promoter area covered in the reporter gene construct N13.

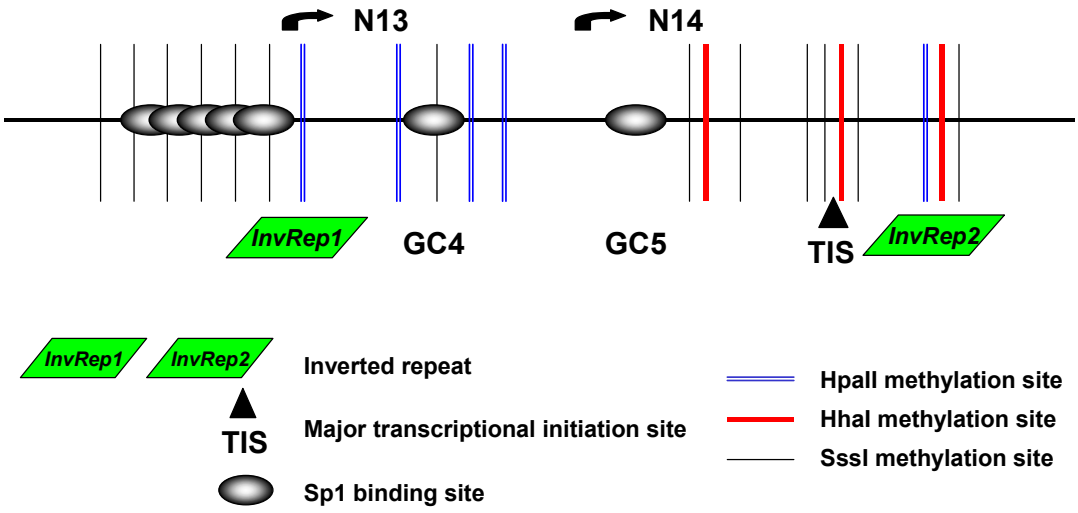


Fig. 48 Schematic overview of the proximal 5-LO promoter covered by the reporter gene constructs N13 and N14. *Depicted are the different methylation sites for M. SssI, M. HpaII and M. HhaI, the inverted repeats and the Sp1 binding sites.*

To identify the potential binding site for Mbd1, different methylation sites within N13 were mutated to inhibit methylation and binding of Mbd1. Construct N13 lacks the 5-fold GC box,

but still contains the two Sp1 binding sites, GC box 4 and GC box 5. Additionally, two inverted repeats are present in this proximal promoter area, whose functions are still unclear. The *Hpa*II methylation sites were of special interest, since Mbd1 cotransfection showed its strongest effects when the reporter gene constructs were methylated with M. *Hpa*II (mutations in N13Rep1, N13Rep2 and N13MMIII). In gel shift assays, methylation of the *Hpa*II sites around GC box 4 diminished Sp1 binding (see Fig. 54), but binding of other nuclear, so far unknown proteins was enhanced (mutated in N13MutMethIII). Methylation of the *Hha*I site between GC box 5 rather increased Sp1 binding, no additional protein binding was seen (mutated in N13MutMethII). The relevance of the sites surrounding the TIS or within the inverted repeats shall be investigated by the mutations in N13MutMethI, N13InvRep1 and N13InvRep2.

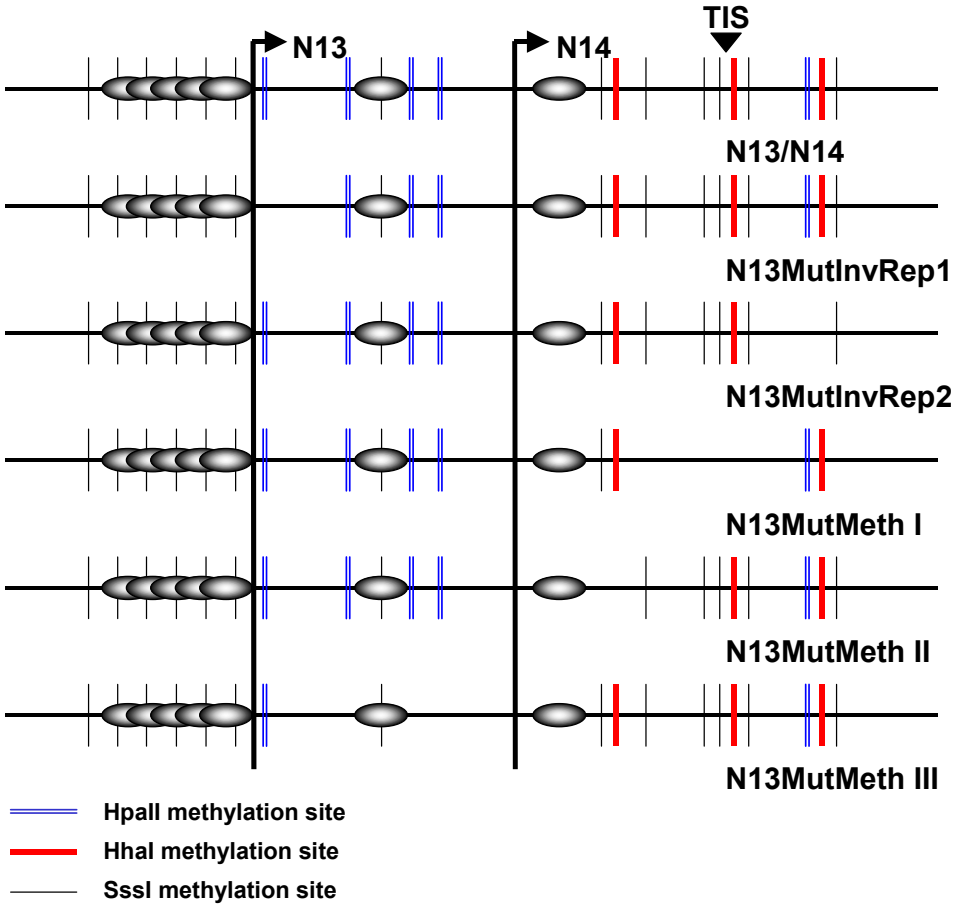


Fig. 49 Mutations of the different methylation sites within N13. *In N13MutInvRep1 and N13MutInvRep2 methylation sites within the sequences of the inverted repeast were point mutated. In the constructs N13MutMethI – III methylation sites of larger stretches of the promoter were mutated, including the HpaII sites surrounding GC box 4, the methylation sites surrounding GC box 5 and the methylation sites surrounding the transcription start site.*

Since the mutations could also affect basal promoter activity, by disabling transcription factor binding, changes in promoter activity of the unmethylated constructs have to be considered. The point mutation of the *Hpa*II methylation site within the inverted repeat 1 sequence significantly reduces the promoter activity, as well as the mutation of the sites beside GC box 5 in N13MutMethII (N13MMII). The other mutations do not significantly change promoter activity. Also, the different mutations show no significant changes in response to TsA treatment, underlining the dependency of the TsA effect on only GC box 4.

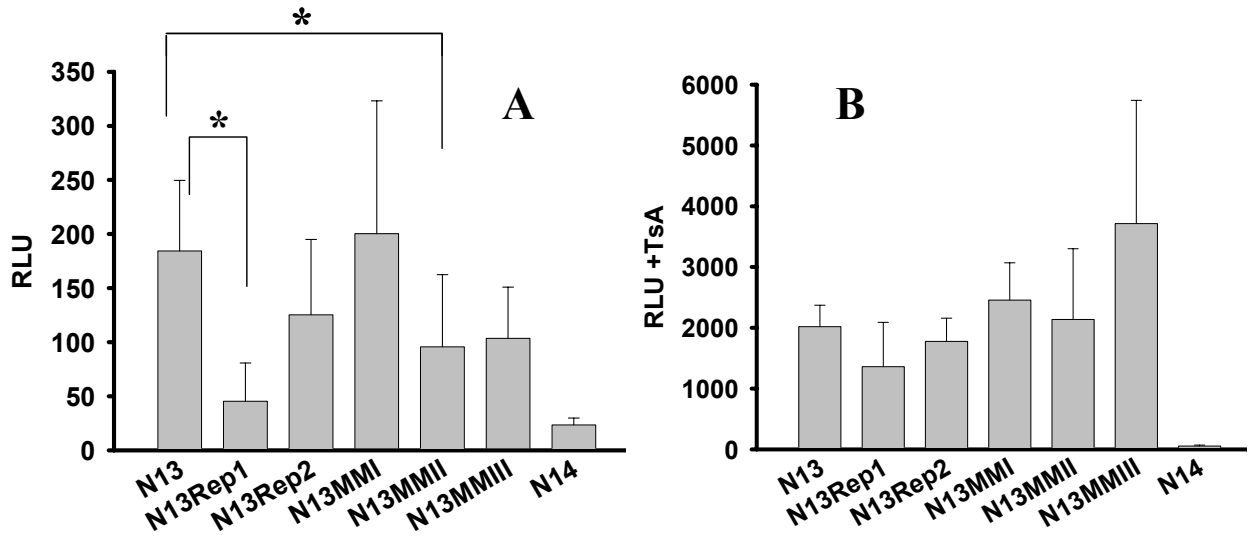


Fig. 50 Relative Activity of the mutated, but unmethylated N13 constructs. *HeLa cells were seeded in 24 well plates and transfected with 0.8 µg of the purified plasmids with 0.02 µg of pCMVSEAP by calcium phosphate method. The medium was changed 8 h after transfection. Cells were incubated with TsA (330 nM) or ethanol. 24 h later, the reporter gene activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency by pCMVSEAP. *, P<0.05 **, P<0.01, by paired two-sided Student´s t test.*

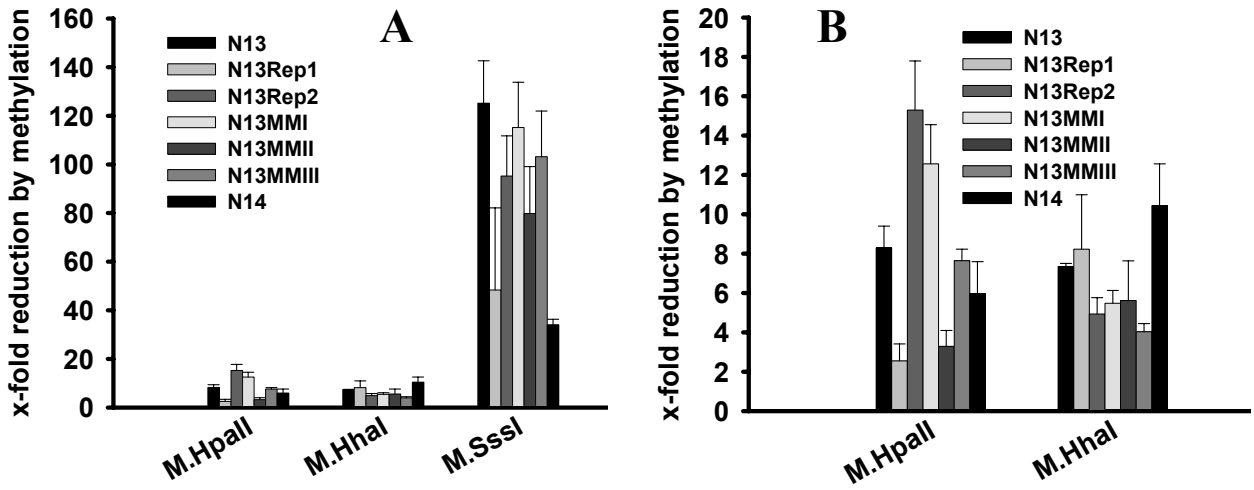


Fig. 51 X-fold reduction by DNA methylation of the different mutated N13 constructs. *After in vitro methylation with M. HpaII, M. HhaI or M. SssI the plasmids were purified using phenol-chloroform extraction. HeLa cells were seeded in 24 well plates and transfected with 0.8 µg of the purified plasmids with 0.02 µg of pCMVSEAP by calcium phosphate method. The medium was changed 8 h after transfection. Cells were incubated with TsA (330 nM) or ethanol. 24 h later, the reporter gene activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency by pCMVSEAP. *, P<0.05 **, P<0.01, by paired two-sided Student´s t test.*

Methylation of the plasmids itself reduces promoter activity. In respect to the different mutations, reduction of promoter activity by *Hha*I methylation does only significantly differ between the constructs N13 and N13MutMethIII (N13MMIII).

Also the mutations in N13Rep1 and N13MutMethII (N13MMII) are releasing the repressive effects of DNA methylation by M. *Hpa*II, and in N13Rep2, carrying a mutation of the *Hpa*II site upstream of the TIS, *Hpa*II methylation even further reduces promoter activity. Only the mutations in N13MMII do significantly decrease the reduction by *Sss*I methylation compared to N13.

The additional cotransfection of the expression plasmid of the murine CpG-binding protein Mbd1 leads to a further decrease in promoter activity of the unmethylated and methylated constructs. Mbd1 strongly effects *Hpa*II methylated constructs, though *Sss*I methylation and coexpression of Mbd1 together have the biggest impact on promoter activity. In comparison to N13, Mbd1 significantly reduces promoter activtiy of N14 (**) when the plasmids were methylated with M. *Hpa*II (*, P<0.05 **, P<0.01, by paired two-sided Student´s t test), but not of the other constructs.

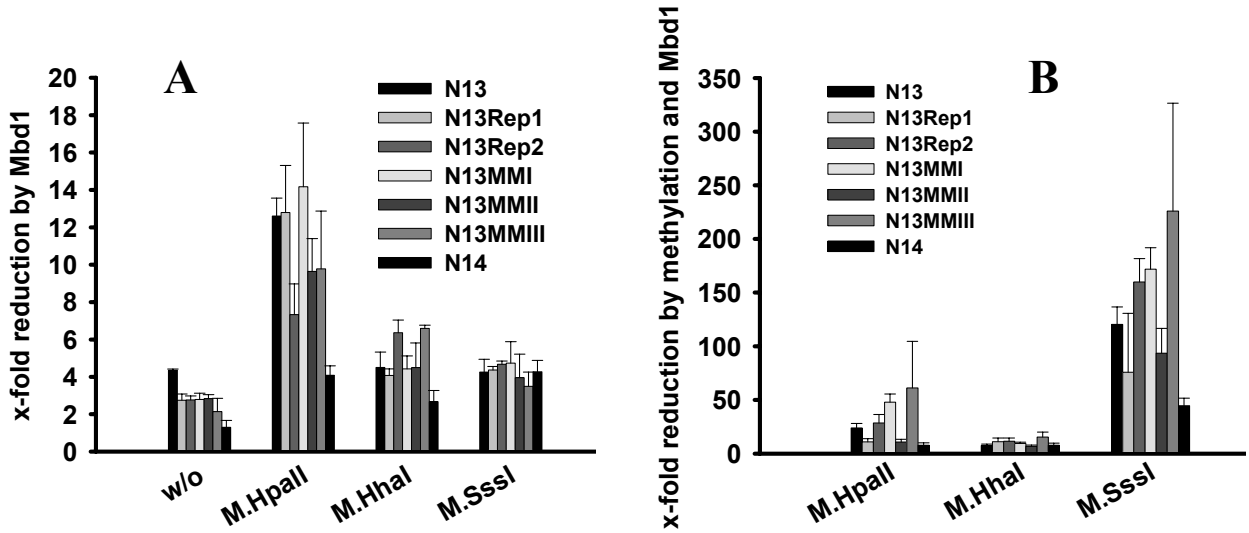


Fig. 52 X-fold reduction by Mbd1 cotransfection. Mbd1 alone reduces promoter activity of unmethylated and methylated mutated N13 constructs. Mbd1 enhances promoter repression by DNA methylation. *After in vitro methylation with M. HpaII, M. HhaI or M. SssI the plasmids were purified using phenol-chloroform extraction. HeLa cells were seeded in 24 well plates and transfected with 0.8 µg of the purified plasmids with 0.02 µg of pCMVSEAP and 0.1 µg of pSG5mock or pSG5Mbd1 by the calcium phosphate method. The medium was changed 8 h after transfection. 24 h later, the reporter gene activity was determined. Each experiment was performed in triplicates. Results are presented as mean + SE of three independent experiments after normalization for transfection effeciency by pCMVSEAP.*

4.2.6 Influence of DNA methylation on in vitro Sp1 binding

Within the proximal 5-LO promoter area three different Sp1 binding sites exist. For all of them, in vitro binding of Sp1 has been shown before (In et al. 1997; Silverman et al. 1998; Dishart et al. 2005). Mutation or deletion of theses sites results in a loss of promoter activity in reporter gene assays (see Fig. 22). As outlined in the introduction (see page 33) different studies have shown interference between DNA methylation and Sp1 binding. In some cases the binding of Sp1 to methylated Sp1 binding sites was reduced, in others methylation of adjacent sites diminished binding of the transcription factor, or methylation showed no influence at all.

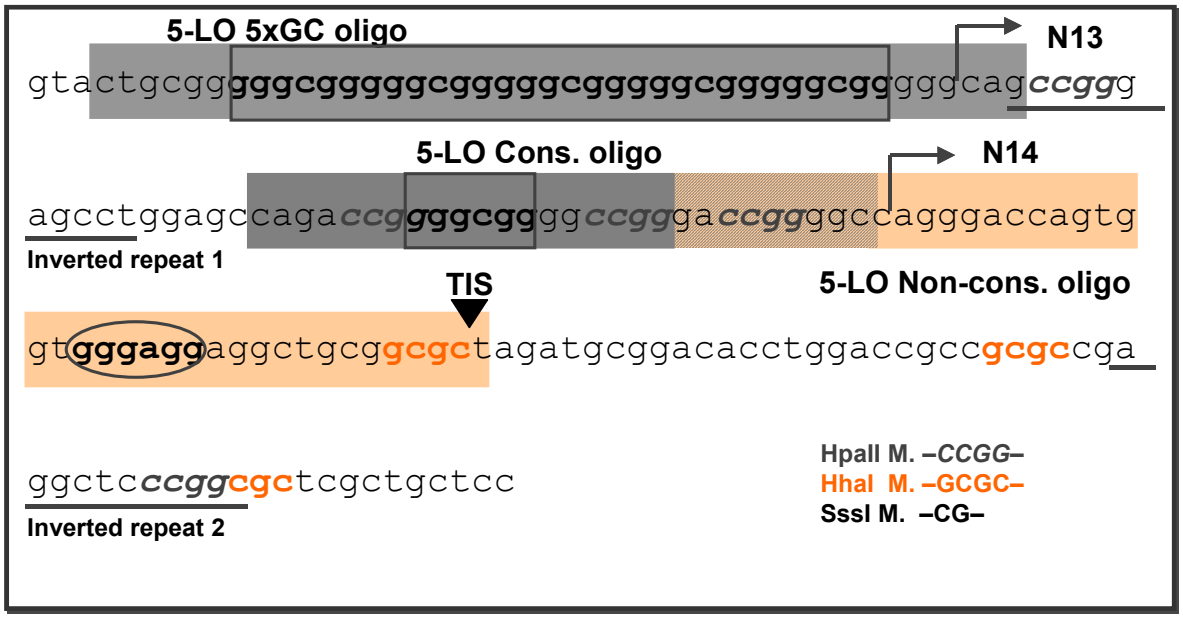


Fig. 53 Overview of the proximal 5-LO promoter sequence and oligonucleotides used as probes in gel shift assays. *To test the influence of DNA methylation on Sp1 binding within the 5-LO promoter context, three different oligonucleotides were designed, covering the three proximal Sp1 binding sites: the 5 x GC oligo, the consensus oligo and the non-consensus oligo, indicated by the grey boxes. Parts of the consensus and non-consensus oligos are overlapping. In the 5 x GC oligo, all five GC boxes were methylated. Methylation of the consensus oligo is possible at three M. HpaII methylation sites surrounding GC box 4 and within the binding site itself. The non-consensus oligo was methylated at both ends, equivalent to a methylation by M. HpaII and M. HhaI.*

In order to investigate the influence of DNA methylation on Sp1 binding sites of the 5-LO promoter, oligonucleotides covering the different sites were ordered and binding to the unmodified or methylated probes was tested in gel shift assays. Either the CpG dinucleotides of the core binding site/s itself were methylated or the CpGs surrounding the GC boxes were methylated. Following this guideline, the 5 x GC oligo was either synthesized completely unmethylated, or all five CpG dinucletides within the five Sp1 core motifs were methylated at the 5´ position of the cytosines. Fig. 53 and Tab. 7 below depict the location of the probes within the 5-LO promoter and the relevant sequences modified by DNA methylation.

Forward strand of double-stranded probes 5´ to 3´	
CTGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCGGGGGCAG	**5 x GC oligo**, covering 5 x GC box, unmethylated
CTGCGGGGG**O**GGGGG**O**GGGGG**O**GGGGG**O**GGGGG**O**GGGGGCAG	**5 x GC *Sss*I M. oligo**, all Sp1 sites methylated
CAGACCGGGGCGGGGCCGGGACCGGGGC	**Consensus oligo**, covering GC box 4
CAGAC**O**GGGGCGGGGC**O**GGGAC**O**GGGGC	**Consensus *Hpa*II M.**, methylated around

	GC box 4 (three *Hpa*II M. sites)
CAGACCGGGG**O**GGGGCCGGGACCGGGGC	**Consensus *Sss*I M.**, methylated within the Sp1 binding motif itself
GACCGGGGCCAGGGACCAGTGGTGGGAGGAGGCTGCGGCGC	**Non-consensus oligo**, Sp1 site / GC box 5, 41 bp
GAC**O**GGGGCCAGGGACCAGTGGTGGGAGGAGGCTG**O**GG**O**GC	**Non-consensus H/H**, Sp1 site / GC box 5, 41 bp, methylated around the Sp1 binding motif (*Hpa*II and *Hha*I M. site)
AGTGGTGGGAGGAGGCTGCGGCGCT	**Non-consensus 20 bp**, Sp1 site / GC box 5, Missing the *Hpa*II M. site
GATCGAACTGACCGCCCGCGGCCCGT	**Unspecific competitor**, Ap2 binding site
ATTCGATCGGGGCGGGGCGAGC	**Specific competitor**, classical Sp1 binding motif

Tab. 7 Sequences of the forward strands of the equivalent gel shift probes. *"O" represents a methylated cytosine of a CpG dinucleotide.*

The consensus oligo was tested either completely unmodified, methylated within the GC box (GC box 4 in reporter gene assays), or with the three *Hpa*II M. sites surrounding the GC box. In case of the last Sp1 binding site (GC box 5 in reporter gene assays) a methylation of the transcription factor binding site itself is not possible, since the sequence covers a non-consensus binding motif -GGGAGG-. Nevertheless the methylation of the residues within the *Hpa*II M. and *Hha*I M. sites surrounding the binding site was possible. As an additional control, a shorter version of the non-consensus oligo was used, only 20 bp long, missing the *Hpa*II M. site.

After annealing the single-stranded primers (for details see "Materials and Methods", page 52), oligonucleotides were labeled at the 5´-ends using T4 polynucleotide kinase. Radiolabelled double-stranded oligos then were purified with Microspin G-25 columns (Amersham Biosciences) and stored at –20 °C. 1.25 µg to 10 µg of nuclear extract and/or Sp1 protein (150-300 ng) was incubated with labelled oligonucleotide probe in binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 20% glycerol) and 0.5 µg poly(dAdT)•poly(dAdT) for 30 min at RT in a total reaction volume of 20 µl. For competition studies a 25-, 50- or 100-fold molar excess of unlabelled Sp1 consensus oligonucleotide (purchased from Promega) was added to the reaction mixture prior to the addition of radiolabelled probe. The reactions were usually resolved on 4-20% pre-run non-denaturating polyacrylamide TBE gels (BioRad premade) which were electrophoresed at 100 V for 110 min. Gels were dried under vacuum before exposure, or exposed directly to Fuji Super RX film at –20 °C or to a FLA 3000 imaging plate at RT for the indicated exposure times.

4.2.7 Sp1 binding to the methylated consensus binding site is diminished

In the first experiments I determined if Sp1 binding to the consensus binding site (GC box 4) was influenced by DNA methylation. 2.5 µg or 5.0 µg of NE of MM6 cells shifted the unmethylated probe and three bands appeared (arrowheads, see Fig. 54). All three complexes could be competed out by the cold competitor (lanes 4 and 5). Recombinant Sp1 protein alone or mixed with 2.5 µg of nuclear extract produced an even more prominent band (lane 6 and lane 7), running on the height of the slowest migrating band, shifted with only nuclear extract.

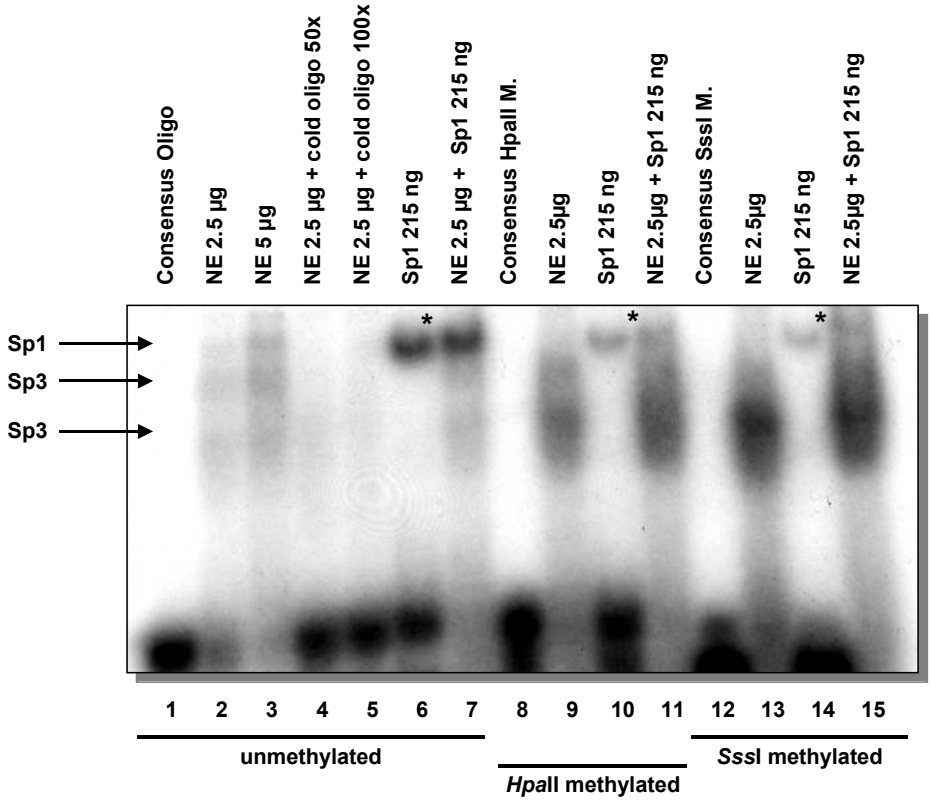


Fig. 54 Gel Shifts with the unmethylated and methylated consensus oligo comprising GC box 4. *After the labeling with ^{32}P-ATP and T4 Polynucleotide Kinase the unmethylated or methylated probes were incubated with 2.5 or 5.0 µg of nuclear extract generated from MM6 cells. For competition studies specific competitor in 50- or 100-fold excess was added to the incubation mixture. Binding of recombinant Sp1 protein to the probes was checked either in combination with nuclear extract or alone. 240,000 cpm of the different probes were used for the binding reactions, the gel was directly exposed to Fuji Super RX film at –20 °C for 2 h. * band generated with Sp1 recombinant protein.*

Nuclear extract shifted the consensus *Hpa*II M. probe, methylated around the Sp1 binding motif, even resulting in a stronger band pattern (lanes 9-11). The slowest migrating band almost disappeared, the faster complexes became more evident, indicating binding of other

proteins than Sp1. These effects were even more prominent with the consensus *Sss*I M. probe, in which the Sp1 binding motif itself is methylated (lanes 13-15). Recombinant Sp1 binding clearly decreased with methylation.

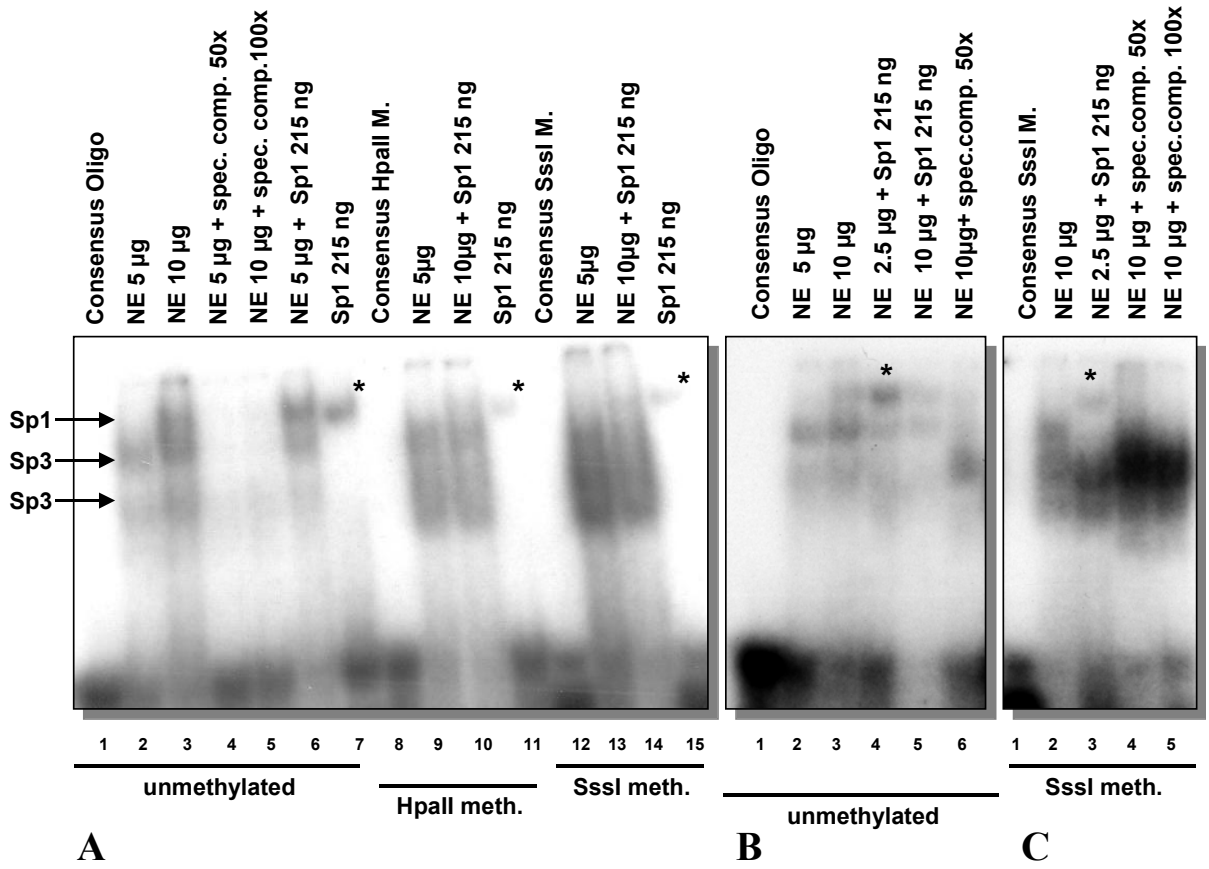


Fig. 55 Gel Shifts with the unmethylated/methylated consensus oligo, comprising GC box 4, using nuclear extract of HeLa cells. *A, B, C: After the labeling, the probes were incubated with 5.0 µg or 10.0 µg of nuclear extract generated from HeLa cells. For competition studies specific competitor in 50- or 100-fold excess was added to the incubation mixture. Binding of recombinant Sp1 protein to the probes was checked either in combination with nuclear extract or alone. 240,000 cpm of the different probes were used for the binding reactions, the gel was directly exposed to Fuji Super RX film at -20°C for 1 h. * band generated with Sp1 recombinant protein. Presented are the results from two different experiments, A versus B and C (B unmethylated probe, C methylated probe).*

The same experiment was repeated with nuclear extract from HeLa cells (see Fig. 55). The same bands appeared (indicated by arrowheads), which could be competed out by the specific Sp1/Sp3 competitor, for the unmethylated probe (A, lane 4 and 5). Again, methylation of the probes clearly decreased Sp1 binding (compare A, lanes 7, 11, 15), and the faster migrating bands were enhanced. In a second experiment (B and C), the fastest migrating band could not

be competed out by the specific Sp1 competitor (B, lane 6), indicating protein binding other than any Sp1 family member, even to the non-methylated probe. Interestingly this band was strongly enhanced, when the probe was methylated and when competitor was added to the binding reaction (see fig. C).

4.2.8 Sp1 binding to the non-consensus binding site increases with methylation

Concerning the non-consensus Sp1 binding site, results from EMSA look quite different. The increasing amounts of nuclear extract only slightly lead to increased protein binding with the non-methylated probe. Only a faint shift appeared on the migration level comparable with recombinant Sp1 protein (indicated by the asterisk), but two strong bands on a faster level (indicated by arrowheads). Competition with specific unlabeled Sp1 competitor enhanced the middle band, instead of diminishing the signal, indicating that Sp1 is not dominating in this protein-DNA complex. Also the binding of recombinant Sp1 protein to the non-consensus probe is weak and is increased when the probe is methylated (compare lane 5 and lane 11).

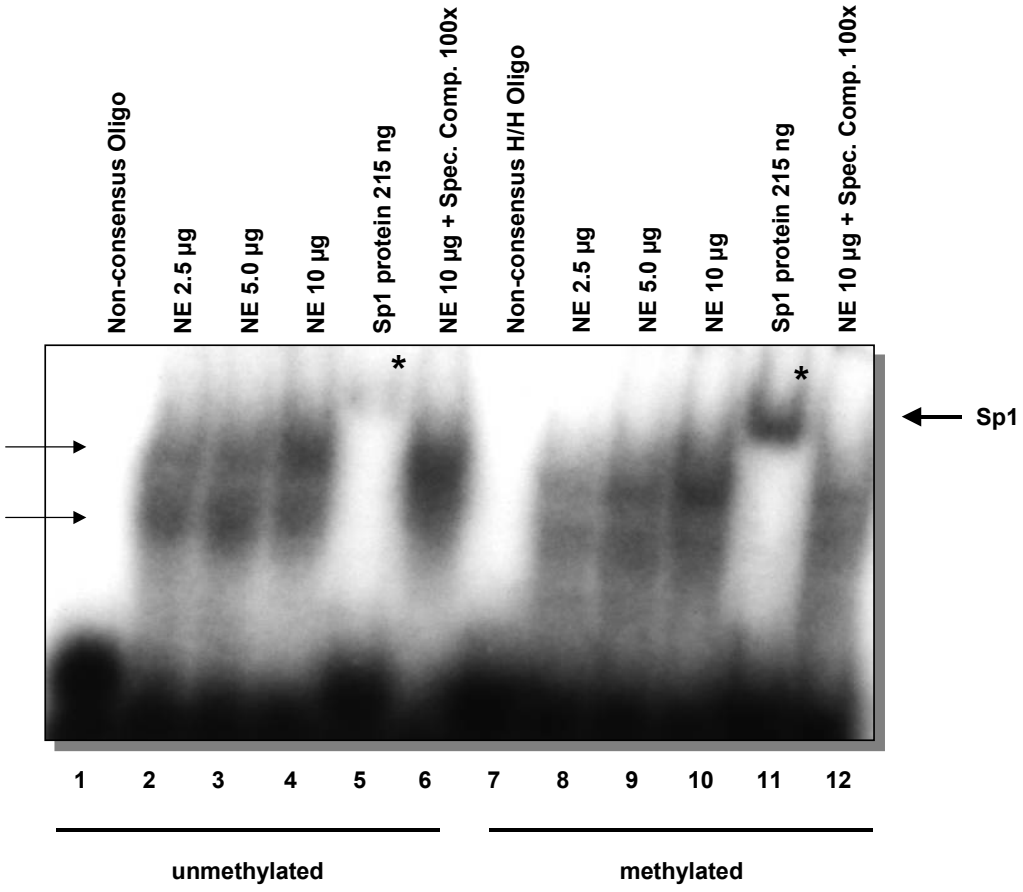


Fig. 56 Gel Shifts with the unmethylated and methylated non-consensus oligo comprising GC box 5.
After labeling, the probes were incubated with 2.5 µg, 5.0 µg or 10.0 µg of nuclear extract generated

*from HeLa cells. For competition studies specific competitor in 100-fold excess was added to the reaction mixture. Binding of recombinant Sp1 protein to the probes was checked without addition of nuclear extract. 250,000 cpm of the different probes were used for the binding reactions, the gel (5% TBE) was directly exposed to Fuji Super RX film at -20°C for 1 h. * band generated with recombinant Sp1.*

In a control experiment the binding of Sp1 to a shorter probe (non-consensus 20 bp), also containing the non-consensus motif was tested (see Fig. 57). Clearly the binding of the recombinant protein is more efficient to the shorter probe (compare lane 4 and lane 8), suggesting some hindrance of protein binding to the longer probe.

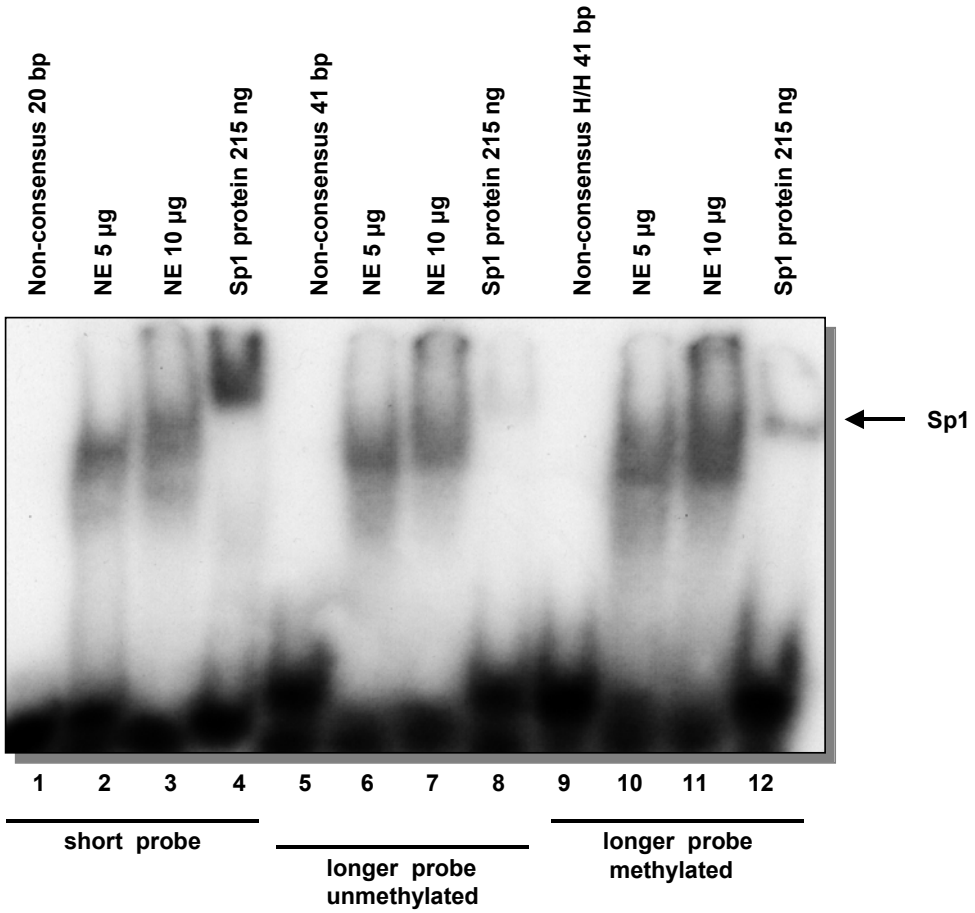


Fig. 57 Gel Shifts with oligonucleotides covering the non-consensus Sp1 binding site (GC-box 5). *After end-labeling the double-stranded oligos with ^{32}P-ATP and T4 Polynucleotide Kinase, the probes were incubated at RT for 30 min either with nuclear extract from HeLa cells or with recombinant Sp1 protein. 250,000 cpm of the different probes were used for the binding reactions, the gel (5% TBE) was directly exposed to Fuji Super RX film at -20°C for 1h.*

4.2.9 Sp1 binding to the 5-fold GC box is attenuated by DNA methylation

Finally protein binding to the 5-fold GC box was examined (see Fig. 58). With 10 µg and 20 µg of HeLa nuclear extract three bands shifted with the non-methylated probe. Adding an excess of specific Sp1 competitor only competed the slowest band out (lanes 4, 5). Cold probe competed all bands out, excluding unspecific protein binding (lane 6). The addition of recombinant Sp1 protein to the binding reaction in combination with nuclear extract slightly enhanced the slowest migrating band (lane 7). Sp1 alone resulted in a distinct band (lane 8), on about the same level as the slow migrating band. Nuclear extract in combination with the methylated probe resulted in about five protein-DNA complexes (indicated by the arrowheads), the highest comparable to recombinant Sp1 binding (lane 10). The competition with specific Sp1 competitor enhanced the three fastest complexes (lane 12) and abrogated the slow migrating complexes, indicating again other protein binding than Sp1 or Sp1 family members. Sp1 binding itself is strongly diminished to the methylated band (lane 15). Similar results were achieved with MM6 extracts (data not shown).

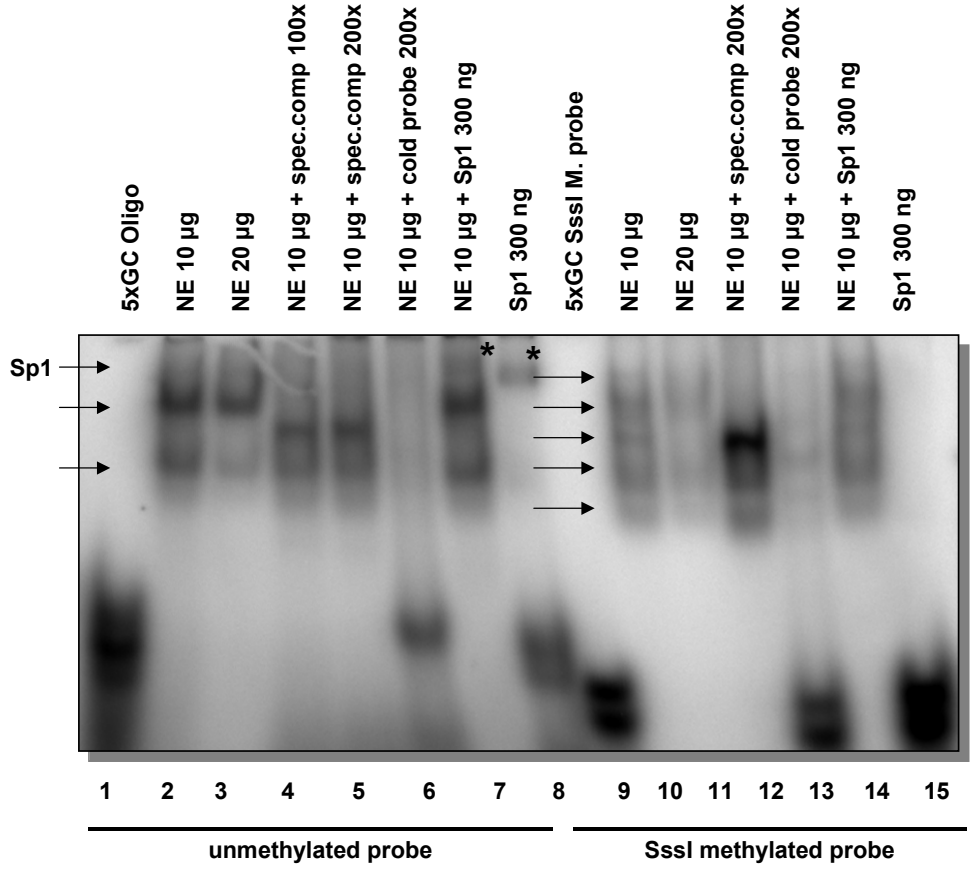


Fig. 58 Sp1 binding to the 5-fold GC box. *After end-labeling the double-stranded oligos with ^{32}P-ATP and T4 Polynucleotide Kinase, the probes were incubated at RT for 30 min either with nuclear extract*

*from HeLa cells or with recombinant Sp1 protein. 120,000 cpm of the different probes were used for the binding reactions, the gel was directly exposed to a FLA 3000 imaging plate for 17 h at RT. * band generated with recombinant Sp1.*

To conclude from theses experiments, Sp1 binding to the methylated consensus binding site is attenuated, but still possible. Additionally, methylation induces the binding of an unidentified protein. Methylation of the 5-fold GC box results in the same effects, Sp1 binding is diminished and binding of an unknown protein enhanced. In case of the non-consensus binding site, Sp1 binding is increased upon methylation of surrounding residues, rather due to secondary effects, like interference with other transcription factor binding or changes in the secondary structure of DNA. Sadly supershift experiments with Sp1 or Sp3 antibody did not work. To reveal what other proteins besides Sp1 bind to the different probes, especially after DNA methylation, gel shift assays including supershift studies with antibodies against MBD proteins should be performed.

5 Discussion

5.1 Regulation of 5-LO gene expression by histone deacetylation

The 5-LO promoter lacks TATA and CCAAT boxes as well as an Inr element (Hoshiko et al. 1990). As underlined by the presence of two CpG islands covering the proximal promoter area and the major transcription start site, the promoter is highly GC-rich (compare Fig. 3 page 10). Several Sp1 binding sites, including a 5-fold tandemized Sp1/Egr1 element, convey basal promoter activity (In et al. 1997; Silverman et al. 1998; Dishart et al. 2005). Though carrying these characteristics of so-called housekeeping genes, 5-LO is tissue-specifically expressed, mainly in cells of myeloid origin (Steinhilber 1999). Recent results suggest that DNA methylation is involved in the tissue-specific expression pattern of 5-LO or at least in the loss of 5-LO expression in myeloid cancer cell lines HL-60TB and U937. TGFβ and $1,25(OH)_2D_3$ induce 5-LO expression in the monocytic cancer cell line MM6 and HL-60, but not in HL-60TB, a subtype of HL-60 cells characterized by the lack of 5-LO expression, or in U937 cells. Since in these cell lines the 5-LO promoter is heavily methylated, it seems that demethylation is a prerequisite for 5-LO expression and TGFβ/$1,25(OH)_2D_3$ treatment alone cannot induce expression (Uhl et al. 2002; Klan et al. 2003).

DNA methylation and histone modifications of the nucleosomal core histone proteins H3 and H4 interact in the transcriptional shut down of genes. Methyl-CpG-binding domain proteins bind to methylated DNA sequences and recruit corepressor complexes including HDACs (Jones et al. 1998; Nan et al. 1998) or associate with histone methyltransferases (Sarraf and Stancheva 2004). Also direct interaction between the de novo DNA methyltransferase DNMT3a and HDAC1 have been reported (Fuks et al. 2001), as well as complex formation between DNMT1, HDAC1, Rb and E2F1 (Robertson et al. 2000) and between DNMT1 and HDAC2 (Rountree et al. 2000). The histone methyltransferase ESET/SETDB1 can repress transcription in a HMT activity-independent manner by associating with HDAC1/2 (Yang et al. 2003). Not only DNA methylation triggers histone modifications, such as deacetylation, but also histone modifications trigger DNA methylation and so far it is not clear what happens first. Studies in Neurospora and Arabidopsis indicate, that histone methylation directs DNA methylation (Tamaru and Selker 2001; Jackson et al. 2002; Tamaru et al. 2003). Methylation of lysine 9 of histone H3 induces DNA methylation in mice at pericentric heterochromatin (Lehnertz et al. 2003). After targeted gene disruption of DNA methyltransferases, the tumor suppressor gene p16 was reactivated after promoter demethylation in the HCT116 cell line. After several cell passages the gene was silenced again, independently of DNA methylation,

but associated with H3K9 methylation (Bachman et al. 2003). Only in even later passages the gene was remethylated. Recently the interaction between the histone methyltransferase SETDB1 and the DNA methyltransferase DNMT3a was described (Li et al. 2006). Mutskov studied the events involved in the silencing of transgenes. He describes early loss of acetylation of H3 and H3K4 methylation, which leads to gene repression. Later H3K9 becomes methylated followed by DNA methylation of the transgene (Mutskov and Felsenfeld 2004). Also the promoter of the tumor supressor gene RASSF1A in human mammary epithelial cells is silenced after initial histone inactivation followed by DNA methylation (Strunnikova et al. 2005).

These links between DNA methylation and histone modifications suggest a similar interaction of both pathways in the regulation of 5-LO gene expression. In the hippocampus of mice and in human Caco-2 cells, a colon carcinoma cell line, valproic acid and butyrate, two short fatty-acid histone deacetylase inhibitors, induced 5-LO mRNA expression (Wachtershauser et al. 2000; Yildirim et al. 2003). In MM6 and HeLa cells, the potent inhibitor trichostatin A induced 5-LO gene expression in reporter gene assays of non-methylated and in vitro methylated plasmids. Also the mRNA level of 5-LO in MM6 cells is increased after TsA treatment, though to a lesser extent as after differentiation with TGFβ/1,25$(OH)_2D_3$ for four days, (11- versus 43-fold). In order to identify the region responsible for the strong induction of 5-LO promoter activity in reporter gene assays, successive deletion variants of the most active reporter gene construct N10 were transfected into HeLa cells and the effect of TsA was investigated. Clearly the larger constructs N10 (-843 bp to –12 bp in relation to ATG), N11, N12 and N13 were induced by the HDACi TsA (4- to 6-fold) (Klan et al. 2003), whereas the shorter constructs N14 (-96 bp tp –12 bp) to N16 were not. Since N13 (-143 bp to –12 bp) already lacks the 5-fold tandemized GC box, TsA independently of this element induces 5-LO promoter activity. Besides in length, N13 and N14 differ in the presence of two functional Sp1 binding sites, termed GC4 and GC5. GC4 represents a consensus Sp1 binding site (5′-GGGCGGG-3′), whereas GC5 comprises the non-consensus motif (5′-GGGAGG-3′). Sp1 binding to both sites was verified in gel shift assays, as well as in DNase I footprints (Hoshiko et al. 1990; In et al. 1997; Silverman et al. 1998; Dishart et al. 2005). Furthermore mutations of the Sp1 binding sites caused a reduction of promoter activity in reporter gene assays in MM6 cells, indicating their functionality in basal promoter activity (Dishart et al. 2005). Several reports have described the recruitment of HDAC activity by transcription factors, including Sp1 and Sp3. The p21 gene expression is upregulated by the HDACi superoylanilide hydroxamic acid in NIH3T3 cells (Xiao et al. 1999). Both Sp1 and Sp3

binding to a proximal GC site are required for this upregulation, whereas in human osteosarcoma cells MG63 Sp3 alone mediates the TsA response (Sowa et al. 1999). One proximal Sp1 binding site in the human luteinizing hormone receptor (hLHR) gene promoter recruits a histone deacetylase-mSin3A complex (Zhang and Dufau 2002). Often the GC site involved in the HDACi response, usually a proximal Sp1 binding site, also participates in the regulation of basal promoter activity.

Mutational analysis in reporter gene assays indeed confirmed the involvement of Sp1/Sp3 binding sites in the regulation of the 5-LO promoter by the histone deacetylase inhibitor TsA. Mutation of GC4 in the still highly inducible construct N13, attenuated the response to TsA comparable to the response of construct N14. Mutation of GC5 was less efficient. The double mutation completely eliminated the reponse to TsA, indicating an interaction between both sites. The fact that the mutation of GC4 without any treatment reduces promoter activity in HeLa cells, whereas mutation of GC5 rather activates promoter activity, suggests recruitment of some kind of repressor by GC5-binding factors. TsA treatment in HeLa cells reverses the repressing function of GC5, indicating the recruitment of HDAC activity via this binding site. Since the TsA response depends on GC4, it is resonable that the recruited HDAC(s) somehow influence GC4-binding factors.

The influence of the particular binding sites was subsequently investigated in the larger promoter context of the most active reporter gene construct N10. In this promoter context the single GC box mutations were relatively insufficient in suppressing the TsA effect. The mutation of GC4, having the strongest impact in the shorter construct N13 decreased the TsA triggered promoter activation only by 11%, compared to 95% in construct N13. The deletion of the tandem GC box (GC0) reduced the TsA response after all by 42%. The data indicate a cooperation for HDAC recruitment between the discrete GC sites in the intact promoter context. However mutations of GC box 4, GC5 and GC1 reduce promoter activity by up to 54%, independent of TsA treatment, thus underlining their relevance in basal 5-LO promoter activity.

Successive combinatorial mutation of the separate Sp1 binding sites in construct GC0, already lacking the five tandemized GC box, revealed the importance and additive influence of each site for full promoter activity. The mutation of each site resulted in a decrease of promoter activity and the mutation of several sites at once enhanced this effect in an additive matter. Only mutation of GC5 alone did not change the promoter activity. The combined mutations of the proximal sites GC1, GC2, GC4, GC5 and the additional deletion of GC0 in the construct N9, containing parts of a second CpG island with more potential Sp1/Sp3 binding sites, were

sufficient to abolish the promoter activity of this construct, giving further evidence of the necessity of the proximal GC sites for basal activity. The double mutation of GC4 and GC5 introduced into GC0 strongly attenuated the inducing effect of TsA by about 82% emphasizing their relevance in HDAC recruitment (compare Fig. 20 page 60).

Reporter gene analysis in MM6 cells, showed similar results. The mutation of GC4 alone, both in the minimal and larger promoter context, neutralized the activating effects of TsA. In MM6 cells both GC box 4 and GC box 5 revealed activating properties independently of HDACi treatment. Possibly HDACs and HATs are recruited at the same time to establish dynamic histone acetylation of promoter-bound histones. Acetylation of histone proteins is a dynamic process, regulated by the opposing effects of HATs and HDACs. For about 15% of the core histones rapid acetylation and deacetylation occurs ($t_{1/2}$ = 7 min for monoacetylated histone H4 and $t_{1/2}$ = 3-7 min for deacetylation). Other core histone proteins are acetylated and deacetylated at a slower rate of $t_{1/2}$ = 200-300 min and $t_{1/2}$ = 30 min respectively (Davie 1997).

In MM6 cells, the histone acetyltransferases as activating factors could overbalance HDACs, whereas in HeLa cells, HDACs tip the scales in the promoter regulation. As examined in ChIP assay the high acetylation degree of histone H4 in MM6 cells even without any TsA treatment supports the idea of histone acetyltransferase activity dominating over HDAC activity (compare Fig. 37 page 77). So far no studies exist on the histone acetylation status of the 5-LO gene in HeLa cells. The DNA methylation status of the 5-LO promoter in MM6 or HeLa cells is still unclear. However since 5-LO gene expression in MM6 cells is inducible with 1,25$(OH)_2D_3$ and TGFβ, but not in HeLa cells, we assume a methylation free promoter sequence in MM6 cells and a methylated sequence in the 5-LO negative HeLa cell line.

In MM6 and HeLa cells the 5-LO promoter contains two negatively regulated stretches, one covering the sequence -5396 to –4895 and the other –914 to –779 bp in relation to the major TIS (Sorg et al. 2006). The second area overlaps with the more distal CpG island in the reporter gene construct N9. TsA treatment induces the promoter activity of N9, but does not completely overcome the repressive character of this promoter region. Mutation of the two GC boxes present in the additional promoter sequence of this construct reduces promoter activity, but does not relieve the response to TsA, whereas the deletion/mutation of the proximal sites GC0, GC1, GC2, GC4 and GC5 (see construct N9GC01245) clearly abolishes the effect. This finding demonstrates, that HDACs are rather recruited to the proximal than to the distal promoter area and further suggests involvement of other repressors in the distal promoter area.

Since the protein levels of Sp1 and Sp3 in HeLa and MM6 cells did not change upon TsA treatment, the effect of their transactivation activity was investigated by cotransfection of Sp1 and Sp3 with the 5-LO promoter mutants (Fig. 32 page 72). The cotransfection alone did not influence promoter activity, maybe due to already high endogenous Sp1 and Sp3 protein levels. Only with TsA treatment, both proteins induced the promoter activity of construct N13, indicating direct influence of TsA treatment on the transactivation capability of Sp1 and Sp3, possibly by posttranslational modifications or changing interacting protein partners. Interestingly neither Sp1 nor Sp3 could induce the promoter activity of N10, independently of TsA treatment. Sp1 and Sp3 only seem to influence the two proximal GC sites in front of the TIS. Regarding the different GC box mutants of N13, promoter activity was reduced after mutation of either GC box, but stronger activated by cotransfection than N13 itself. When GC4, the activating element in HeLa cells, was mutated, Sp1 cotransfection showed the strongest impact in relative promoter activation (11.7-fold induction). Possibly Sp1/Sp3 compete with a repressor binding to GC box 5.

In vitro binding of Sp1 and Sp3 to the 5-fold GC box, GC4 and GC5 was unchanged after HDACi treatment as investigated in DAPA. However, in vivo binding of Sp1 and Sp3 increased upon TsA treatment, especially in the proximal promoter area, containing the elements investigated in DAPA. In chromatin immunoprecipitation assays the acetylation status of the 5-LO promoter, RNA polymerase II binding, Sp1 and Sp3 binding were investigated in MM6 cells after TsA treatment. Especially in the promoter area containing all proximal GC sites binding of Sp1 and Sp3 is increased. Sp1 does not seem to associate with the more distal regions (-1049 to -318), whereas Sp3 does very well, also without HDACi treatment (compare Fig. 37 page 77).

The divergence between in vitro and in vivo binding affinities of Sp1 and Sp3 to the 5-LO promoter after TsA treatment might be explained by changes in chromatin structure, such as acetylation of H3 and H4, resulting in a more relaxed chromatin structure facilitating Sp1 and Sp3 binding. Interestingly already in untreated Mono Mac 6 cells the examined promoter region contains heavily acetylated nucleosomal histone protein H4. Unlikely TsA renders the chromatin structure even more accessible for transcription factor binding. It has been demonstrated that acetylation to 46% of maximal site occupancy was sufficient to stimulate transcription by RNA polymerase III (Tse et al. 1998). However RNA polymerase II binding is clearly increased after TsA treatment and spreads to the more distal promoter parts, including the negatively regulated promoter area present in the reporter gene construct N9. The acetylation status of H3 remains to be investigated.

Since the 5-LO promoter lacks TATAA or CCAAT boxes and only contains an initiator-like sequence, Sp1 is likely the driving force in recruiting the basal transcription machinery in this promoter context (Dishart et al. 2005). Increased in vivo binding of Sp1 would subsequently explain the increased RNA polymerase II binding and the observed rise in promoter activity in reporter gene assays as well as in 5-LO mRNA levels. So far it is not clear, if Sp3 is also able to attract the basal transcription machinery, but surely Sp3 could recruit coactivators such as HATs. Also the cotransfection studies, though performed in HeLa cells, suggest only activating functions of both Sp1 and Sp3. Sadly these ChIP data cannot discriminate protein binding to the single GC boxes. ChIP assays with stably transfected mutated promoter constructs could help to identify specific transcription factor binding (Banchio et al. 2004).
The high number and proximity of the different Sp1 binding sites just upstream of the major transcriptional start site of the 5-LO promoter suggests an interaction between the factors binding to these sites. Especially for Sp1 synergistic activation by oligomerization has been described (Mastrangelo et al. 1991). Sp1 may not only provide a platform for the basal transcription machinery, but also for corepressor/coactivator complexes until different stimuli cause transcriptional initiation. Only recently, the interaction between the zinc-finger domain of Sp1 and the corepressors NCoR, SMRT and BCoR has been described (Lee et al. 2005). Sp1 sites have also been found to be involved in tissue-specific gene expression. For example the expression of CD14 in monocyte/macrophages is mediated by Sp1 (Zhang et al. 1994). Sp1 also regulates the myeloid-restricted expression of the human haematopoietic cell kinase (Hauses et al. 1998). Interestingly Sp1 can loop DNA between distal and proximal promoter sites (Pascal and Tjian 1991; Yu et al. 2003). In MM6 cells 5-LO gene expression is strongly induced after differentiation of the cells to macrophages with $1,25(OH)_2D_3$/TGFβ (mRNA levels increase 43-fold after 24h treatment). In reporter gene assays though, $1,25(OH)_2D_3$/TGFβ failed to induce 5-LO promoter activity (Klan et al. 2003). Only the incorporation of the 5-LO coding sequence into the reporter gene constructs alone (pGL-ba-cdsInJM) or together with the promoter (pN10-cdsInJM; the effect is even enhanced when introns J and M are included) led to a significant stimulation by $1,25(OH)_2D_3$ and TGFβ, 28-fold and 14-fold respectively (unpublished data from our lab). Interestingly the addition of TsA to $1,25(OH)_2D_3$/TGFβ reduced the stimulation in the reporter gene assays (2.3-fold and 5.5-fold respectively for pN10-cdsInJM and pGl3-ba-cdsInJM) as well as at the mRNA level (16-fold). On the other hand the strong induction of the promoter activity by TsA is abolished by the presence of the cds. However, Seuter et al. could also show that Sp1 cotransfection, but not Sp3 cotransfection reestablished the TsA induced activity in reporter

gene assays (3.2- versus 8.9-fold induction for pN10-cdsInJM without/with Sp1 cotransfection). ChIP assays further revealed simultaneous in vivo binding of the VDR and RNA polymerase II to the proximal promoter area (-220 bp to +142 bp) of the 5-LO gene, lacking any VDRE surrounding the TIS, after $1,25(OH)_2D_3$ stimulation (Seuter et al. 2006). Other studies have shown that $1,25(OH)_2D_3$ induces complex formation between Sp1 and VDR at the p27 promoter and stimulates transactivation via a Sp1 consensus site within the promoter area (Huang et al. 2004; Cheng et al. 2006). Nevertheless the p27 promoter alone is $1,25(OH)_2D_3$ inducible in reporter gene studies. In experiments with the mutated proximal GC boxes GC4 and GC5 of the 5-LO promoter in pN10-cdsInJM did not reduce the $1,25(OH)_2D_3$/TGFβ induction (data not shown), indicating no interaction between the Sp1 sites and a possible downstream enhancer in the 5-LO cds. The possible competitive activation of 5-LO by TsA and $1,25(OH)_2D_3$/TGFβ cannot simply be explained by changes in chromatin structure, since histone protein H4 is already heavily acetylated in untreated MM6 cells, which do not express 5-LO (see Fig. 37 page 77). Both agents $1,25(OH)_2D_3$ and TsA stimulate RNA polymerase II binding to the proximal promoter area, but this effect does not explain, why TsA decreases $1,25(OH)_2D_3$/TGFβ induced 5-LO gene expression (Seuter et al. 2006).

A possible link between the TsA and TGFβ/$1,25(OH)_2D_3$ mediated induction of 5-LO expression could be the regulation of Smad 7 by acetylation/deacetylation. TGFβ signaling is mediated through the Smad family of transcription factors. Acetylation of the inhibitory Smad 7 by p300 leads to increased protein stability by preventing the ubiquitination of overlapping lysines and subsequent protein degradation (Gronroos et al. 2002). After TGFβ stimulation Smad 7 translocates to the activated TGFβ receptors at the plasma membrane together with the Smurf E3-ubiquitin ligase. The Smurf-Smad 7 complex blocks the interaction between the receptor-activated Smads and the receptor, thus blocking TGFβ induction. HDAC1 and HDAC3 strongly bind to Smad 7, HDAC2 weakly (Simonsson et al. 2005). Deacetylation by HDACs increases the degradation of Smad 7. Hence HDACi treatment would possibly counteract TGFβ induction, explaining why the combination of TsA and $1,25(OH)_2D_3$/TGFβ results in a lower 5-LO gene expression than $1,25(OH)_2D_3$/TGFβ alone.

HDACi as well as $1,25(OH)_2D_3$ induce cell differentiation. Differentiation of monocytes to macrophages is accompanied by an upregulation of 5-LO activity and protein expression. Also the platelet-activating factor acetylhydrolase gene is upregulated during the maturation of primary human monocytes into macrophages, a process, which is associated with an

increase of Sp1 and Sp3 DNA-binding activity to the promoter (Wu et al. 2003). Sp1 and Sp3 also activate the expression of the myeloid-restricted integrin genes CD11b and CD11c in differentiating monocytes (Chen et al. 1993; Noti et al. 1996; Noti 1997).

In HL-60 and MM6 cells, a differentiation inducer, such as $1,25(OH)_2D_3$, is necessary to upregulate 5-LO expression (Brungs et al. 1994; 1995). In MM6 cells the maximal upregulation of 5-LO primary transcripts already occurs after 8 h of treatment. For this, both agents are required and protein expression induced by TGFβ is involved (Harle et al. 1998). In order to achieve increased 5-LO protein activity at least 48 h treatment is necessary. In vivo binding of Sp1 and Sp3 to the 5-LO promoter is already induced after 6 h of TsA treatment in MM6 cells. Also the reporter gene data suggest an early activation of the promoter (compare Fig. 29 page 68). After 4 h of TsA treatment N10 is activated 3.9-fold. However TsA does not change the protein levels of Sp1, Sp3, HDAC1 or HDAC2 (Fig. 31 page 71). If $1,25(OH)_2D_3$/TGFβ would exert their activating effect simply by increasing Sp1/Sp3 binding to the proximal 5-LO promoter, the treatment would also increase promoter activity in reporter gene assays, which is not the case. In a study performed by Dishart, Sp1/Sp3 protein levels and their DNA binding affinity in MM6 cells did not alter after $1,25(OH)_2D_3$/TGFβ treatment (Dishart et al. 2005).

Factors binding to GC4 and GC5, likely Sp1 as well as Sp3, recruit HDAC activity towards the 5-LO promoter as demonstrated in reporter gene assays. In order to identify the HDACs recruited to the promoter, the effects of another HDACi were investigated. VPA preferentially inhibits class I HDACs, at least HDAC2 is 5-fold more efficiently inhibited than HDAC5 and HDAC6 (Gottlicher et al. 2001). In contrast to TsA, VPA also induces the proteasomal degradation of HDAC2 (Kramer et al. 2003). In the reporter gene study with the 5-LO construct N10 VPA evoked similar effects as TsA. Maybe due to the lower specificity of VPA in comparison to TsA (IC50 ~1 mM versus 37 nM, unpublished data and (Klan et al. 2003)) the promoter activation was less pronounced. Clearly nicotinamide, a class III HDAC inihibitor did not influence 5-LO promoter activity.

Most likely rather HDAC1, 2 or 3 are recruited to the 5-LO promoter than class II HDACs. All class I HDACs, except for HDAC8 are involved in transcriptional regulation. Several reports describe the interaction between Sp1 and HDAC1 or HDAC2 and their recruitment to different promoters (Sowa et al. 1997; Sowa et al. 1999; Xiao et al. 1999; Hou et al. 2002). HDAC1 association with the p21 (Gui et al. 2004) and metallothionein I promoter (Ghoshal et al. 2002) is inhibited by HDACi. A new study could show that in human colon cancer cells the p21 promoter is regulated by multiple HDACs including HDAC1, 2 and 3 (Wilson et al.

2006). The TsA inducible gdf11 promoter on the other hand is exclusivley regulated by HDAC3 (Zhang et al. 2004). For catalytic activity HDAC3 exists in a complex with the nuclear receptor corepressors NCoR/SMRT (Guenther et al. 2001). HDAC1 and HDAC2 together form the catalytic core of mSin3a, Mi2/NuRD/NRD and CoREST complexes (Cress and Seto 2000; Ng and Bird 2000; Grozinger and Schreiber 2002).

In cotransfection studies with the different class I HDACs, HDAC1, 2, 3 and 8, the potential repressors rather activated the promoter construct N13 (Fig. 38 page 79). Similarly in cotransfection studies with the human gdf11 promoter, also performed in HeLa cells, all cotransfected histone deacetylases induced promoter activity, except for HDAC3, later identified as the recruited corepressor of gdf11 promoter activity (Zhang et al. 2004). There are cases, where HDACs appear to be required for gene activation. For example, TsA decreases CD9 expression in macrophages (Wang et al. 2002) or expression of Hmga2 protein in HeLa, NIH3T3 and F9 cells (Ferguson et al. 2003). Thus indirect effects may account for the activating function of HDACs in these cotransfection experiments.

TsA treatment relieved the activition by HDAC1, 2 and 3. As in the experiments before, HDACi treatment induced transactivation by Sp1. Interestingly cotransfection of HDAC2 and HDAC8 enhanced this effect. It is hard to draw any conclusion from this experiment. The expression pattern of HDAC8, which was so far only detected in smooth muscle cells, and its potential role in regulation of smooth muscle cytoskeletal proteins rather excludes this protein as a potential regulator of 5-LO promoter activity (Waltregny et al. 2004; Waltregny et al. 2005). In concomitant Western blot analysis HDAC8 could not be detected in HeLa cells, whereas all other class I HDACs were expressed.

Sadly HDAC1 recruitment could not be demonstrated in DNA affinity purification assays, neither with HeLa nor MM6 cell extracts. Though HDAC2 binding to GC4 and GC5 was detected, the conditions used to screen for Sp1/Sp3 binding were not strict enough to show distinct association of HDAC2 to the separate probes. Instead HDAC2 already showed affinity to the beads only. HDAC3 and HDAC8 could not be detected at all. siRNA in combination with reporter gene assays would be a nice approach to investigate the recruitment of HDACs to the proximal Sp1 binding sites. Subsequently in ChIP experiments in vivo binding should be examined.

Histone acetyltransferases and histone deacetylases usually function in an equilibrium. Often HATs and HDACs are recruited simultaneously. HDACi treatment in these cases shifts the balance towards enhanced HAT activity. As known for HDACs, HATs not only target histone proteins but also transcription factors, including Sp1 and Sp3. Since TsA induced in vivo

binding of Sp1 and Sp3, posttranslational modifications might play a role in the increased binding and/or transactivation activity. Acetylation of Sp1/Sp3 or phosphorylation of Sp1 after TsA treatment has been described. In case of the IGFBP-3 promoter TsA treament released Sp3 and HDAC1 from a protein complex consisting of Sp1/Sp3/HDAC1/p300. TsA treatment furthermore induced phosphorylation of Sp1 and Sp1 binding was enhanced in gel shifts (Choi et al. 2002). However it was not shown, that phospho-Sp1 binding itself is increased or how TsA triggers phosphorylation. At the TGFβ type II receptor, TsA leads to the release of HDAC1 from a NF-Y/Sp1/HDAC1 protein complex with subsequent recruitment of p300 and PCAF with concomitant acetylation of Sp1 depending on PCAF acetyltransferase activity (Huang et al. 2005). HDAC1 is also released from the p21 and metallothionein I promoter after TsA treatment (Ghoshal et al. 2002; Gui et al. 2004). HDAC1 directly interacts with the zinc-finger DNA binding domains of Sp1 and interferes with the recognition of GC-boxes (Kang et al. 2005). Similarly p300 interaction with the DNA binding domain of the transcription factor Sp1 has been described. Despite acetylation of Sp1 by the acetyltransferase domain of p300 the pure physical interaction with p300 stimulated the DNA binding activity of Sp1 (Suzuki et al. 2000). DNA binding of Sp1 on the other hand inhibited the p300 interaction. Also the interaction between the inhibitory domain and DNA binding domain of Sp1 with the nuclear corepressors SMRT, NCoR and BCoR has previously been described. The same group also suggests a modulation of this interaction by MEK (Lee et al. 2005), but by phosphorylation of the corepressor molecules.

Many hematopoietic transcription factors are reversible acetylated. Acetylation can influence DNA binding, transcription activation, repressor activity, protein-protein interactions and protein stability (reviewed in (Glozak et al. 2005). The regulation of transcription factors by acetylation/deacetylation may influence tissue-specific and lineage-specific gene expression. Sadly the binding of acetylated Sp1 or Sp3 in DNA affinity purification assays could not be demonstrated. Possibly the affected protein amounts are not detectable in this assay.

Immunoprecipitation studies performed in our lab to identify increased acetylation of Sp1 or Sp3 did not produce repeatable results. This may be due to too low specificity of the acetyl-lysine antibody. Furthermore the antibody may just not recognize the acetylated lysine in our context.

In summary, TsA treatment induces 5-LO promoter activity and 5-LO mRNA levels. The data so far available suggest that increased in vivo binding of Sp1 and Sp3 to the proximal promoter with subsequent or concomitant RNA polymerase II binding are responsible for the upregulated gene transcription. Recruitment of HDACs or HATs via the Sp1 binding sites

GC4 or GC5 could not be demonstrated. Therefore it was not possible to show TsA induced variances in recruited protein complexes. We could not demonstrate TsA induced post-translational modifications of Sp1 or Sp3 either, such as acetylation. However increased acetylation of histone H4 protein, resulting in a transcriptional more accessible chromatin structure at least in MM6 cells does not seem the reason for increased Sp1/Sp3 binding as indicated by the already high basal acetylation status of H4 protein surrounding the 5-LO promoter. TsA does not alter the expression levels of Sp1/Sp3 either.

In future studies HDAC and HAT recruitment, as well as the association of corepressor complexes, such as NCoR, mSin3A, SMRT and BCoR should be studied. It would be of interest to improve the DAPA conditions in order to show specific HDAC2 recruitment. With re-ChIP analysis also the in-vivo interaction between Sp1 and i.e. HDACs or possible VDR and Smads could be investigated. $1,25(OH)_2D_3$/TGFβ effects on Sp1 and Sp3 in vivo binding should be investigated, to rule out or proof interacting pathways in 5-LO regulation.

5.2 Regulation of the 5-LO promoter by DNA methylation

As already outlined in the first part of the discussion 5-LO gene transcription is regulated by DNA methylation and the recruitment of histone deacetylases. Uhl and Klan could show that treatment of the 5-LO negative cell lines HL-60TB and U937 with the DNA methyltransferase inhibitor 5-aza-2`-deoxcytidine (AdC) demethylated the 5-LO promoter and induced 5-LO gene expression (Uhl et al. 2002). Concurrently in vitro methylation of 5-LO reporter gene plasmids strongly reduced the promoter activity in MM6 and HeLa cells (Klan et al. 2003). TsA treatment partially reversed the methylation dependent promoter repression, but the promoter activity never reached the level of the unmethylated plasmids.

These first experiments demonstrated a role of DNA methylation in the regulation of 5-LO, but did not further investigate the DNA sequences or proteins involved in this mechanism, such as methyl-CpG-binding proteins (MBDs), DNA methyltransferases (DNMTs) or histone modifying enzymes such as HDACs.

In the further study we focused on identifying relevant DNA sequences in the regulation by DNA methylation within the core promoter, comprising the sequence -778 bp to +53 bp in relation to the major TIS, present in the most active reporter gene construct N10, and its successive deletion variants N11 to N16. The data indicate that the more sites become methylated, the lower is the promoter activity of the reporter gene constructs. Complete methylation by in vitro methylation with *Sss*I methylase abolishes promoter activity, partial methylation by M. *Hpa*II or M. *Hha*I recognizing more distinct methylation sites, –CCGG-

and -CGCG- respectively, strongly reduced promoter activity (about 12-fold, 5-fold after *Hpa*II and *Hha*I methylation, respectively and 51-fold after *Sss*I methylation). It seems, that in the shorter deletion variants N14 to N16 the different methylation patterns have similar impacts on promoter activity, either due to the adjusting ratio of methylation sites or the special relevance of particular sites within this proximal promoter area. However, since the promoter activity was only significantly decreased in the larger deletion variants N10 to N13, it remains speculative to assume special sites within the promoter region of N14. Also the plasmid backbone may influence the promoter activity, indicated by the methylation effects of M. *Hpa*II on the control plasmid pGl3Prom, lacking any recognition site for this methylase within the sequence of the SV40 promoter, but showing a strong reduction in promoter activity (about 17-fold). MBDs binding to distal plasmid parts outside of the promoter area could influence promoter activity. Regulation from about 3 kb far distal sites have been described for MBD1 (Fujita et al. 2003). Also mutation of a putative MBD1 binding site in an FGF2-luciferase reporter construct did not relieve the repressing effect (Jorgensen et al. 2004), suggesting that other CpGs may instead attract the MBD1 protein.

Since DNA methylation often involves HDAC recruitment, we tested TsA treatment on in vitro methylated plasmids. Clearly, the treatment cannot reverse the repressing effect of the methylation on the promoter activity, indicating additional repressing mechanisms besides HDAC recruitment, i.e subsequent chromatin condensation, histone H3K9 methylation and HP1 binding (compare Fig. 45 C page 87). Studies with transiently and stably transfected promoter constructs did not show differences in HDAC recruitment, suggesting the formation of some kind of minichromatin of the transfected plasmids (Ishizuka and Lazar 2003; Zhang et al. 2004). However after *Hpa*II and *Hha*I methylation the induction by TsA is enhanced compared to the unmethylated plasmids (for example 17-fold versus 68–fold for N10 after *Hpa*II methylation), suggesting increased HDAC recruitment. The complete methylation by M. *Sss*I abolishes promoter activity and inducibility by TsA. DNA methylation at this stage dominates over histone acetylation/deacetylation.

In a cotransfection study with murine Mbd1, Mbd2, Mbd3 and rat MeCP2, only Mbd1 further reduced 5-LO promoter activity, most effectively after *Hpa*II methylation, both in N10 and the shorter deletion variant N13. TsA did not relieve the repressing effects, indicating other repressive mechanisms involved besides histone deacetylation. Repression by the TRD of MBD proteins is variably sensitive to TsA, indicating that HDAC activity is not consistently involved in the regulation by MBDs (Ng et al. 2000; Fujita et al. 2003). Whereas MBD2/MBD3, as well as MeCP2 are associated with HDAC activity in the corepressor

complexes mSin3a and Mi2-NuRD (Nan et al. 1998; Ng et al. 1999), MBD1 does not coimmunoprecipitate with HDAC1. MBD1 is capable of binding to both methylated and unmethylated promoters (Fujita et al. 2000; Ng et al. 2000). In gel shifts MBD1 binds to a single symmetrically methylated CpG site, containing a –CCGG- site, equivalent to the motif recognized by *Hpa*II methylase (Hendrich and Bird 1998). An other study demonstrated that MBD1 preferentially binds to densely methylated DNA fragments (Fujita et al. 1999). The interaction with non-methylated DNA depends on a third zinc-coordinating CXXC domain, which is not present in all MBD1 splice variants (Fujita et al. 1999; Jorgensen et al. 2004), whereas interaction with methylated DNA is mediated by the methyl-CpG-binding domain alone. Interestingly MBD1 can repress promoter activity from far distances (Fujita et al. 2003), eventually making it difficult to identify specific DNA sequences within in vitro methylated plasmids. Recently the interaction between MBD1 and MCAF1 has been described (Fujita et al. 2003). In the absence of MBD1 MCAF1 acts as an transcriptional activator, which interacts with Sp1. MBD1 inhibits this interaction and interferes with the Sp1-mediated transactivation. Furthermore the MBD1-MCAF1 complex associates with SETDB1, a histone methyltransferase, facilitating the formation of heterochromatin (Sarraf and Stancheva 2004; Ichimura et al. 2005). Sp1-activated transcription of methylated p16 and SNRPN promoters was inhibited by all MBD1 isoforms. The isoforms MBD1v1 and MBD1v2 carrying the CXXC3 domain also reduced Sp1-activated transcription from the unmethylated promoters (Fujita et al. 1999).

According to these reports MBD1 seems as the ideal candidate to initiate transcriptional repression of the 5-LO promoter. Interacting with MCAF1, MBD1 would interfere with Sp1 induced promoter activity. Subsequent recruitment of the histone methyltransferase SETDB1 may induce DNA methylation and heterochromatin formation by HP1 binding (Ichimura et al. 2005). Also the presence of the MCAF1-Sp1 complex in HeLa cells has been demonstrated by Fujita (Fujita et al. 2003).

Mbd1 reduces the promoter activity in both reporter gene constructs N10 and N13, especially after in vitro methylation. Only in combination with TsA treatment the unmethylated constructs are influenced by Mbd1. However, also the murine isoform Mbd1a, which was cotransfected, binds to unmethylated DNA (Jorgensen et al. 2004). Maybe HDAC recruitment by Sp1 via GC box 4 interferes with Mbd1 binding. The strong reduction in promoter activity by Mbd1 cotransfection after *Hpa*II methylation suggests a special relevance of the recognition motif –CCGG-. Since the effect occurred within the shorter deletion variant N13, further mutational study of the potential CpG sites of this promoter part was performed. If

possible, CpGs were mutated to TpG, thus preventing methylation of the cytosine (see Tab. 1 at page 47 for the exact primer sequences).

Unexpectedly the point mutation within the inverted repeat structure beside the 5-fold GC-box (N13MutInvRep1), indeed containing a –CCGG- methylation site, strongly reduced the promoter activity of N13 already in an unmethylated state. Apart from this mutation, only the elimination of the GC box 5 flanking methylation sites in N13MutMethII, including a *Hha*I methylation site, significantly reduced the promoter activity of the unmethylated construct. In the context of increased Sp1 binding in gel shifts after methylation of exactly these adjacent CpG sites (compare Fig. 56 page 105), it is reasonable that the same sites are of relevance for promoter activity in an unmethylated state. The impact of the point mutation in N13Rep1 remains unexplainable. So far, the role of the inverted repeat structure in the 5-LO promoter is unknown and the sequence does not contain a known putative response element for any transcription factor. However the intact structure of this element seems to be important for full promoter activity. None of the mutations changed the TsA response in the unmethylated state, underlining the role of GC4 in recruiting HDAC activity (compare Fig. 50 page 92).

Other *Hpa*II methylation sites were mutated within construct N13MutMethIII and N13MutMethInvRep2. After in vitro methylation with M. *Hpa*II the methylation triggered reduction of promoter activity was relieved in construct N13Rep1 and N13MMII. Since both constructs already displayed impaired promoter activity in an unmethylated state, the reduced impact of DNA methylation on their activity may just reflect their already low basal activity, but does not completely exclude a potential role in DNA methylation. Only the mutation of the *Hpa*II methylation sites in N13MMIII significantly relieved the methylation triggered repression, but only after *Hha*I methylation, which should not be affected by the mutation of an *Hp*aII site (compare Fig. 51 B page 93).

The additional cotransfection of Mbd1 further reduced the promoter activity of the mutants, but was not significantly changed by any mutation. Possibly Mbd1 alternatively from sites outside the promoter region represses the promoter activity, as described by Fujita (Fujita et al. 2003). Under these circumstances it is hard to draw any conclusion from the mutational analysis. Also the endogenous expression of MBD proteins may interfere with the overexpression of Mbd1 and conceal cotransfection effects.

N13 also contains a potential MIZF (MBD2-interacting zinc finger) recognition sequence -CGGAC- (at position +7 to +11 bp in relation to TIS), which is mutated in construct N13MMI (Sekimata et al. 2001; Sekimata and Homma 2004). MIZF recruits MBD2 and

potentially the HDAC containing Mi2-NuRD complex to specific target genes and allows sequence-dependent repression by MBD2. However, the mutation of this motif to -TGGAC- has no impact on the methylation effect, nor on the TsA response. The two so far known recognition sites for Kaiso, -CGCGCCCAAACG- serves as a recognition motif in a methylated state and –CTGCNA- in an unmethylated state, are not present in N13. However the minimal recognition motif -CTGCNA- is present in the 5-LO promoter sequence at -418 bp to –423 bp in relation to the TIS (also comprised in construct N10) and has to be considered in further studies. Apart from Kaiso, ZBTB4 and ZBTB38, two other members of the BTB/POZ transcription factor family, have been identified as CpG binding proteins. Both zinc-finger containing proteins can bind to a single methylated CpG residue. ZBTB4 recognizes the extended Kaiso binding site –TCCTGCNA-, whereas ZBTB38 binds to the E-box -CACCTG- (Filion et al. 2006). The ZBTB4 motif is not detectable within the 5-LO promoter region, but the E-box element is found at several positions (+26 to +31, -3031 to -3026, -3777 to -3772 on the direct strand and on the complementary strand at –3174 to -3169, -1039 to –1034 in relation to the 5-LO TIS).

The cotransfection study and mutational analysis present some evidence that MBDs, possibly MBD1, could be recruited to the proximal 5-LO promoter. However, no direct interaction in gel shifts or even in ChIP assays has so far been presented. The gel shifts performed with in vitro methylated probes (discussed below) clearly indicate additional protein binding to the methylated probes, especially when *Hpa*II recognition sites are methylated. Unfortunately supershift studies have not been performed yet to eventually identify MBD1 recruitment. Also DNA affinity purification assays failed to proof any MBD recruitment to the in vitro methylated promoter sequence of N13, probably due to the experimental conditions and low MBD protein abundance in HeLa cells. Even with nuclear extracts Western blot analysis revealed only faint protein bands.

Since the basal and TsA induced promoter activity depends on Sp1/Sp3 binding, the influence of DNA methylation on Sp1 binding was investigated. The experiments as in the methylation study focused on the proximal promoter area, which contains three functional Sp1 binding sites. At least in vitro Sp1 binding to the 5-fold GC box, GC box 4 and GC box 5 has been shown in gel shifts and/or DNase I footprints (In et al. 1997; Silverman et al. 1998; Dishart et al. 2005). In vivo binding of Sp1 and Sp3 covering the same promoter area was demonstrated in ChIP assays (compare Fig. 37 page 77).

Concerning Sp1-binding controversial studies exist, describing either impaired association with DNA after methylation or no influence of the DNA modification (Holler et al. 1988;

Clark et al. 1997; Fujita et al. 2000; Zhu et al. 2003). For example, in reporter gene assays with p16 and SNRPN promoter constructs the cotransfection of Sp1 even enhanced the promoter activity after in vitro methylation (Fujita et al. 2000). However complete methylation of 5-LO promoter constructs with *Sss*I methylase abolishes promoter activity (Uhl et al. 2002; Klan et al. 2003), suggesting that Sp1/Sp3 binding and transactivation is inhibited.

The gel shifts performed with nuclear extracts from HeLa and MM6 cells and the three different 5-LO promoter probes indicate differential binding characteristics of Sp1 depending on the surrounding promoter context and the complexity of the GC box. Clearly Sp1 binding to the 5-fold GC box and the consensus motif GC4 is impaired, when surrounding methylation sites are methylated, and almost abolished, when the GC box itself becomes methylated (compare Fig. 54 lanes 6, 10 and 14 and Fig. 58 lane 8 and 15). The slowest migrating band appears to be formed by Sp1 binding, since recombinant Sp1 protein shows the same migration height (compare Fig. 54 lane 3 and lane 6). Sadly supershift experiments did not work, thus it was not possible to identify the two faster migrating bands. Presumably these bands consist of the different Sp3 isoforms, since in other studies with HeLa cells the same gel shift patterns are produced and the bands disappear, when specific competitor is added (Zhu et al. 2003). Also Sp1 and Sp3 binding to all three sites has been demonstrated in DAPA. Both, methylation around GC4 (equivalent to in vitro *Hpa*II methylation) and methylation of the site itself, lead to recruitment of an unidentified protein or protein complex. Mutation of the same sites in the reporter gene study (compare Fig. 51, construct N13MMIII) did not alter promoter activity after in vitro methylation.

Looking at the 5-fold GC box, the recombinant Sp1 shift completely disappears when the probe is methylated (compare lane 8 and lane 15 in Fig. 58 page 101). However the Sp1 band produced with nuclear extract is hardly recognizable. The specific competitor does not completely prevent protein binding to this site, indicating some kind of competition between DNA binding proteins. In this case it is reasonable that Egr1 competes with Sp1 binding. Egr1 binding to the 5-fold GC box has been demonstrated before (Silverman et al. 1998). No other Egr1 binding site within the proximal 5-LO promoter was identified in the same study. Since the consensus binding site for Egr1 is –GCGGGGGCG- the specific competitor used in our gel shift experiments would not reduce Egr1 binding. The addition of the specific competitor even produces a more distinct band, when the probe is methylated. However only a supershift would help to identify the new band. So far it is unclear if Egr1 binding is influenced by DNA

methylation. If the slower migrating bands are really produced by Sp3 binding, Sp3 seems to bind better to the 5-fold GC box than to the consensus motif GC4.

Interestingly, Sp1 binding to the non-consensus motif is increased after methylation of the surrounding CpG sites (see Fig. 56 and Fig. 57). However to both the unmethylated and methylated probe rather Sp3 seems to bind than Sp1. Also other than Sp protein binding to this site is demonstrated, since the competitor does not relieve the signal of the faster migrating bands.

From these data it is clear, that DNA methylation, depending on its degree, at least reduces (*Hpa*II and *Hha*I methylation) if not abolishes promoter activity (*Sss*I methylation). At least during the establishment of complete DNA methylation HDAC activity is involved, indicated by the increased effects of the histone deacetylase inhibitor TsA on promoter activity after partial methylation by *Hpa*II and *Hha*I. Later or after complete methylation TsA does not reverse the repressing effect of DNA methylation anymore, being in line with the idea, that DNA methylation is the final step in transcriptional shut down.

The gel shifts demonstrate, that DNA methylation interferes with Sp1 binding to the activating binding sites GC4 and the 5-fold GC box. Additionally so far unidentified protein binding is enhanced after methylation most prominently to GC4. The methylation around GC5 induces Sp1 binding to this site, but Sp1 does not seem to be the major factor binding to this site.

The cotransfection study suggests an involvement of MBD1 in the regulation of the 5-LO promoter. However the data do not provide clear evidence of a specific binding site. Supershift studies in combination with ChIP assays also proving in vivo binding should be performed in the future.

6 Summary

5-LO is the key enzyme in the biosynthesis of proinflammatory leukotrienes, converting arachidonic acid to 5-HPETE, and in a second step 5-HPETE to leukotriene A4. Apart from the multiple regulation mechanisms on the enzyme level, also regulation of 5-LO gene expression is far more complex than originally expected. Although the 5-LO promoter possesses characteristics of so-called housekeeping genes, such as lack of TATA/CCAAT boxes and existence of several Sp1 binding sites, the 5-LO gene is tissue-specifically expressed in primarily immune competent cells of myeloid origin including granulocytes, monocytes, macrophages, mast cells and B-lymphocytes. So far two major mechanisms of 5-LO gene regulation have been identified, but are far from understood. 5-LO gene expression in MM6 and HL-60 cells is strongly induced after differentiation of the cells with TGFβ and $1,25(OH)_2D_3$. Up to now, it is unclear how both agents exactly stimulate expression. It seems that improved transcript elongation and maturation are involved. In some monocytic cancer cell lines, such as HL-60-TB and U937, TGFβ and $1,25(OH)_2D_3$ treatment are not able to activate 5-LO gene transcription. It was demonstrated, that in these cell lines the 5-LO core promoter is heavily methylated and that only demethylation by the DNA methyltransferase inhibitor 5-aza-2`-deoxycytidine (Adc) upregulated the 5-LO mRNA levels. It was also shown that the histone deacetylase inhibitor TsA could induce 5-LO mRNA levels, but only in $1,25(OH)_2D_3$/TGFβ inducible MM6 cells. Interestingly the $1,25(OH)_2D_3$/TGFβ effect on 5-LO expression is reduced, when combined with TsA. Already from these previous data one can assume multiple regulatory mechanisms on the transcriptional level of 5-LO, from the complete shut down of 5-LO gene expression by DNA methylation to facultative and to induced 5-LO gene transcription involving epigenetic mechanisms and differentiation-induced upregulation by $1,25(OH)_2D_3$/TGFβ. The work presented here focused on the regulation of the 5-LO promoter by DNA methylation and histone deacetylation.

In the first part of my studies, the upregulation of 5-LO mRNA levels by the histone deacetylase inhibitor TsA was investigated. Reporter gene assays revealed that 5-LO promoter activity is strongly induced after 24 h treatment with 330 nM TsA (construct N10 up to 35-fold in HeLa cells). The effect is dependent on the presence of the proximal Sp1 binding site GC4 (-53 bp to –48 bp in relation to the major TIS) in both HeLa and MM6 cells. In vitro binding of the transcription factor Sp1 to this site has been demonstrated in gel shift assays and DNase I footprints. Mutation of the binding site resulted in a loss of basal promoter activity in both 5-LO negative HeLa cells and in 5-LO positive MM6 cells, as well as in the

loss of TsA inducibility. The mutational study of different Sp1 binding sites in a larger promoter context revealed the interaction or respectively the additive effect of the multiple Sp1 binding sites of the 5-LO promoter on basal as well as on TsA upregulated promoter activity. However, GC4 seems to be of special relevance for both the basal promoter activity, possibly recruiting the basal transcription machinery, as well as for the TsA induced upregulation of 5-LO promoter activity. TsA does not alter the protein expression levels of Sp1 and Sp3 as investigated in Western blot analysis, neither in HeLa nor in MM6 cells. DNA affinity purification assays revealed that TsA had no effect on the DNA affinity of Sp1 or Sp3. In vitro binding of both Sp1 and Sp3 to the 5-fold GC box, GC4 and GC5 was demonstrated by DAPA analysis, but histone deacetylase inhibition did not change the associated protein amounts. Finally, in vivo binding of Sp1 and Sp3 was investigated in chromatin immunoprecipitation assay (ChIP) in MM6 cells. TsA clearly induced the association of both proteins to the promoter area surrounding the TIS. Upon TsA treatment also RNA polymerase II binding to the area surrounding the TIS (-318 to +52 bp) was increased and even initiated in the more distal promoter parts –1049 to –292 bp, which are negatively regulated in reporter gene assays. Interestingly histone H4 is already highly acetylated without TsA treatment and the acetylation status of H4 remains unchanged after histone deacetylase inhibition, indicating an open chromatin structure of the 5-LO gene in MM6 cells. In a cotransfection study with Sp1 and Sp3, the transactivating potential of factors was investigated and in accordance with the ChIP data, Sp1 and Sp3 increased the promoter activity, but only after TsA treatment.

Since alterations in chromatin structure do not seem to be the reason for increased Sp1/Sp3 binding to the 5-LO promoter, we assumed that TsA might trigger post-translational modifications of the transcription factors, such as acetylation, which in some cases facilitated DNA binding or transactivation. However in DNA affinity purification assays, I could not show increased binding of acetylated Sp1 or Sp3, not even identify the acetylation at all. Immunoprecipitation studies in our lab so far could not demonstrate an increase in Sp1 or Sp3 acetylation either.

In order to identify the HDAC activity recruited by the Sp1/Sp3 binding sites, we also performed DAPA, HDAC2 association was detected, but unspecifically. No other HDAC recruitment could be demonstrated, nor association of the histone acetyltransferases p300 or PCAF. At least in immunoprecipitation studies performed in our lab interaction between Sp1 and HDAC1 as well with HDAC2 was demonstrated. TsA did not alter the association though.

In gel shift assays, the influence of DNA methylation on Sp1 binding was investigated. The results indicate different roles for the three proximal promoter sites. Whereas Sp1 binding to the 5-fold GC box and GC4 is impaired by DNA methylation, binding to GC5 is even increased. The data also indicate, that possibly rather Sp3 than Sp1 is recruited to this non-consensus binding site independent of the methylation state. However, so far no supershift experiments were performed to prove Sp3 binding. Interestingly, DNA methylation not only decreases Sp1 binding to GC4 and the tandemized GC box, but also leads to prominent association of so far unidentified protein to thess promoter regions. A cotransfection study with methylated 5-LO promoter constructs and the murine methyl-CpG-binding proteins suggest MBD1 involvement in the regulation of the 5-LO promoter. A following mutational analysis of the different methylation sites within the proximal promoter area (-78 bp to +53 bp in relation to TIS) could not relieve the repressive effect of Mbd1 cotransfection. Thus it was not possible to identify a specific binding site for Mbd1. The reporter gene assays with in vitro methylated promoter constructs demonstrated increased HDAC recruitment after partial promoter methylation and complete loss of promoter activtiy after complete methylation. Since in gel shifts Sp1 binding is inhibited by DNA methylation, at least to the 5-fold GC box and the activating element GC4, and similarly the mutation/deletion of the same sites strongly reduces or inhibits promoter activity, it is likely to assume, that the loss of promoter activity after in vitro methylation is in the first place due to impaired Sp1/Sp3 binding. In future studies it would be of strong interest to investigate Sp1/Sp3 in vivo binding in a methylated 5-LO promoter area. Together the data underline the importance and complexity of Sp1/Sp3 binding to the GC-rich sites in the regulation of 5-LO promoter activity in response to the histone deacetylase inhibitor TsA as well as in respect to DNA methylation.

7 Zusammenfassung

Die 5-Lipoxygenase ist das Schlüsselenzym in der Leukotrienbiosynthese. Sie katalysiert den Einbau von molekularem Sauerstoff in Arachidonsäure zur 5-HPETE (5(S)-Hydroxyperoxy-6-trans-8, 11, 14-cis-eicosatetraensäure) und in einer weiteren Reaktion den Umbau der 5-HPETE zum instabilen Epoxid Leukotrien A4 (LTA4). Neben den vielfältigen Regulationswegen auf Enzymebene, gestaltet sich auch die Regulation der 5-LO-Genexpression weit komplexer als zunächst erwartet. Obwohl der 5-LO-Promotor einige Eigenschaften von sogenannten Haushaltsgenen aufweist, wie das Fehlen einer TATA- oder CCAAT-Box und das Vorhandensein zahlreicher Sp1-Bindungsstellen, wird die 5-Lipoxygenase zelltyp-spezifisch exprimiert, vorwiegend in immunkompetenten Zellen wie Granulozyten, Monozyten, Makrophagen, Mastzellen und B-Lymphozyten. Bisher konnten zwei wichtige Mechanismen der 5-LO-Genregulation beschrieben werden, die induzierte Genexpression nach Vitamin D3- und TGFβ-Behandlung, sowie die epigenetische Regulation durch DNA-Methylierung.

In den beiden Krebszelllinien, MM6 und HL-60, nimmt die 5-LO-mRNA-Menge nach Differenzierung der Zellen mit $1,25(OH)_2D_3$ (Vitamin D3) und TGFβ dramatisch zu (43-fach nach 24-stündiger Behandlung in MM6-Zellen). Bisher ist allerdings unklar, wie die beiden Agenzien die Expression stimulieren. Zumindest scheinen sowohl die Transkriptions-elongation als auch die mRNA-Reifung involviert zu sein.

In anderen monozytären Krebszelllinien dagegen, U937 und HL-60TB, konnten TGFβ und $1,25(OH)_2D_3$ die 5-LO-Transkription nicht aktivieren. Im gleichen Zusammenhang konnte gezeigt werden, dass die DNA des 5-LO-Kernpromotors in diesen 5-LO negativen Zelllinien stark methyliert vorliegt. Nur nach einer Demethylierung des Promotors mit 5-Azadeoxycytidin konnte die 5-LO-mRNA-Menge durch $1,25(OH)_2D_3$/TGFβ gesteigert werden.

Auch die Behandlung mit dem Histondeacetylase-Hemmer Trichostatin A (TsA) erhöht die 5-LO-mRNA-Menge (11-fach), allerdings nur in $1,25(OH)_2D_3$/TGFβ-induzierbaren MM6-Zellen. Unerwarteter weise führt die Kombination von TsA und $1,25(OH)_2D_3$/TGFβ im Vergleich zu $1,25(OH)_2D_3$/TGFβ alleine zu einer verringerten 5-LO-Expression (17-fach versus 43-fach).

Diese Daten zeigen, wie vielfältig die 5-LO-Transkription reguliert wird, ausgehend von der völligen Stilllegung des Gens, über eine fakultative bis hin zur induzierbaren Genaktivierung, die sowohl epigenetische Mechanismen als auch eine $1,25(OH)_2D_3$/TGFβ-abhängige

Induktion einschließen. Die hier präsentierte Arbeit legt ihren Schwerpunkt auf die Untersuchung der Regulation des 5-LO-Promotors durch DNA-Methylierung und Histondeacetylierung.

Der erste Teil befasst sich mit dem Einfluss des Histondeacetylase-Hemmers TsA auf die 5-LO-Genexpression. In Reportergenstudien zeigte sich, dass die 5-LO-Promotoraktivität nach einer 24-stündigen Behandlung mit TsA stark induziert wurde, 35-fach für das Promotorkonstrukt N10 (-778 bp bis +53 bp in Bezug auf den Haupttranskriptionsstart der 5-LO) in 5-LO-negativen HeLa-Zellen, 6-fach in 5-LO-exprimierenden MM6-Zellen. Auch die 5´-Promotordeletionsvarianten N11, N12 und N13 zeigen eine erhöhte Promotoraktivität im Reportergenassay nach TsA-Behandlung. Erst das Minimalkonstrukt N14 (-31 bp bis +53 bp in Relation zum Transkriptionsstart) reagiert nicht mehr auf eine Hemmung der Histondeacetylierung. Im Unterschied zum Reportergenplasmid N14, enthält N13 (-78 bp bis 53 bp) eine weitere Sp1-Bindungsstelle, die GC-Box 4 (-53 bp bis –48 bp in Relation zum Transkriptionsstart).

In-vitro wurde die Bindung des Transkriptionsfaktors Sp1 an dieses Element bereits in EMSAs und DNase I-Footprints gezeigt. Die Mutation der Bindungsstelle im Promotorkonstrukt N13 führt sowohl zum Verlust der basalen Promoteraktivität als auch der TsA-Induzierbarkeit sowohl in 5-LO-negativen HeLa-Zellen als auch in 5-LO-positiven MM6-Zellen. Eine Mutationsanalyse in einem größeren Promotorkontext verdeutlicht sowohl bei der Regulation der basalen als auch der TsA-induzierten Aktivität das Zusammenspiel bzw. den additiven Effekt der einzelnen Sp1-Bindungsstellen im 5-LO-Promotor. Außer der GC-Box 4 enthält der 5-LO-Promotor im Bereich –294 bp bis +53 bp weitere acht Sp1-Bindungsmotive, darunter eine 5-fach GC-Box. Die additive Mutation/Deletion dieser proximalen GC-Boxen GC1, GC2, GC4, GC5 und der 5-fach GC-Box im sonst aktivsten Promotorkonstrukt N10 heben die Promotoraktivität völlig auf. Allerdings haben die einzelnen Mutationen der Sp1-Bindungsstellen bei intakter 5-fach GC-Box im Konstrukt N10 deutlich weniger Einfluss auf die basale Promotoraktivität und Induzierbarkeit durch TsA, als bei Deletion der 5-fach GC-box in dem Konstrukt GC0, was auf ein Zusammenspiel der einzelnen Elemente hindeutet. Trotzdem zeigt gerade die GC-Box 4 auch im größeren Promotorkontext einen besonderen Stellenwert sowohl für die basale Aktivität, möglicherweise durch die Rekrutierung der basalen Transkriptionsmaschinerie, als auch für die TsA-induzierte Hochregulation der 5-LO Promotoraktivität.

TsA zeigt weder in HeLa- noch in MM6-Zellen einen Einfluss auf die Proteinexpression von Sp1 und Sp3. Mittels einem „DNA Affinitäts- und Aufreinigungsassay" (DAPA) wurde die

in-vitro Bindung von Sp1 und Sp3 an die verschiedenen proximalen Sp1-Bindungsstellen im 5-LO Promotor, die 5-fach GC-Box, die GC-Box 4 und GC-Box 5 nachgewiesen. TsA zeigte in diesen Versuchen keinen Einfluss auf die Bindungsaffinitäten von Sp1 und Sp3.

Wenn Sp1 und Sp3 mit den N13-GC-Mutanten im Reportergenassay kotransfiziert wurden, zeigte sich nur unter TsA-Behandlung eine Steigerung der 5-LO-Promotoraktivität, besonders wenn GC-Box 4 oder GC-Box 5 alleine mutiert waren (jeweils 11,7-fach und 4,5-fach), was auf eine Interaktion der beiden Bindungsstellen hindeutet. Der unmutierte Minimalpromotor in N13 wird durch Sp1 nur 3-fach induziert. Die Sp3-Expression zeigte keine nennenswerten Effekte, führte aber auch zu keiner Erniedrigung der Sp1-induzierten Promotoraktivität durch eine mögliche Kompetition, wenn beide Expressionsplasmide kombiniert wurden.

Schließlich wurde die in-vivo Bindung von Sp1 und Sp3 in Chromatin-Immunopräzipitationsstudien in Abhängigkeit von TsA untersucht. TsA induzierte deutlich die Sp1- und die Sp3-Bindung in den untersuchten MM6-Zellen, besonders im proximalen Promotorbereich von –318 bp bis +52 bp, der alle proximalen GC-Boxen und den Transkriptionsstart umfasst. Neben einer Zunahme der Sp1 und Sp3- Bindung induziert TsA auch die Rekrutierung der RNA-Polymerase II, auffälligerweise auch in den distalen Promoterarealen, die sich im Reportergenassay als negativ regulierte Bereiche erwiesen haben (-1049 bp bis –292 bp). Interessanterweise zeigt TsA keinen Einfluss auf den Histonacetylierungsgrad des 5-LO-Promotors. Selbst in unbehandelten MM6-Zellen zeigt sich bereits eine prägnante Acetylierung von Histon H4.

Es ist daher anzunehmen, dass in dieser Zelllinie die Chromatinstruktur auch ohne TsA-Behandlung weitgehend offen und daher leicht zugänglich für Transkriptionsfaktoren und die RNA-Polymerase II ist. Im Reportergenassay aktivieren Sp1 und Sp3 den 5-LO-Promotor, allerdings nur nach einer TsA-Behandlung der transfizierten Zellen. Da im ChIP-Versuch die Sp1/Sp3-Bindung unter TsA zunimmt, liegt die Vermutung nahe, dass TsA die Promoter-Aktivität durch eine Verbesserung der Sp1/Sp3-Bindung induziert.

Da Veränderungen der Chromatinstruktur, zumindest bzgl. der Histonacetylierung, nicht der Grund für eine verstärkte Sp1/Sp3-Bindung zu sein scheinen, nahmen wir mögliche posttranslationale Veränderungen von Sp1 und/oder Sp3, wie z. B. eine Acetylierung an, die in einigen Fällen die DNA-Affinität oder die Transaktivierung dieser Transkriptionsfaktoren erhöht. Leider konnte diese Annahme nicht bestätigt werden. Im DAPA ließ sich keine Bindung von acetyliertem Sp1 oder Sp3 nachweisen. Mit Hilfe der Immunpräzipitation konnte zwar eine Acetylierung von beiden Transkriptionsfaktoren gezeigt werden, aber keine Zunahme von acetyliertem Sp1 oder Sp3 nach TsA-Behandlung der Zellen.

Die TsA-vermittelte Induktion der 5-LO-Promotoraktivität und Genexpression deutet auf eine Rekrutierung von Histondeacetylasen hin. Die Daten der Mutationsanalyse im Reportergenassay zeigen, dass TsA seinen Effekt vorwiegend über die GC-Box 4 ausübt. Mittels DAPA sollte festgestellt werden, welche Histondeacetylase über diese Sp1-Bindungsstelle rekrutiert wird. Außerdem wurde eine mögliche Bindung an die 5-fach GC-Box und an GC-Box 5 untersucht. Sp1 und Sp3 binden an alle drei proximalen GC-Boxen etwa gleich stark, unabhängig von einer TsA-Behandlung. Leider konnte keine spezifische Rekrutierung von Klasse I-HDACs (HDAC1, 2, 3 und 8) gezeigt werden. HDAC1, 3 und 8 ließen sich nicht nachweisen und HDAC2 assoziert mit allen Proben, auch der Negativkontrolle ohne DNA-Sonde. Auch die Detektion der Histonacetyltransferasen p300 und PCAF scheiterte in diesen Versuchen. Leider zeigte auch die Kotransfektion von HDAC1, 2, 3 und 8 nicht die erwartete Abnahme der 5-LO-Promotoraktivität im Reportergenassay unabhängig von einer TsA-Behandlung sowie einer Sp1-Kotransfektion.

Aus den bisherigen Daten lässt sich zusammenfassend sagen, dass die TsA-vermittelte Erhöhung der 5-LO-mRNA Menge sowie die gesteigerte Promotoraktivität in MM6-Zellen wohl durch eine verbesserte in-vivo Bindung des Transkriptionsfaktors Sp1, sowie eine vermehrte Rekrutierung der RNA Polymerase II vermittelt wird. Leider konnte bisher nicht direkt nachgewiesen werden, ob und welche Histondeacetylasen oder auch Histoneacetyltransferasen vom 5-LO-Promotor rekrutiert werden.

Im zweiten Teil der Arbeit sollten Promotorsequenzen im 5-LO-Promotor ausfindig gemacht werden, die eine Rolle in der Regulation der 5-LO-Transkription durch DNA-Methylierung spielen. Die mögliche Rekrutierung von Methyl-CpG-bindenden Proteinen, sowie der Einfluss der DNA-Methylierung auf die Sp1-Bindung sollten näher beleuchtet werden.

In Reportergenstudien wurde zunächst der Effekt einer in-vitro Methylierung auf die Promotoraktivität verschiedener 5-LO-Reportergenplasmide untersucht. Dabei zeigte sich, dass eine Methylierung aller CpG-Stellen des hochaktiven Reportergenkonstruktes N10 zu einer totalen Aufhebung der Promotoraktivität führt (51-fache Reduktion der Promotoraktivität). Auch eine partielle Methylierung des Promotors durch Methylasen mit spezifischerem Erkennungsmotiv senkt die Promotoraktivität signifikant (12-fach bzw. 5-fach). Die Reduktion der Promotoraktivität korreliert mit der Anzahl der methylierten CpG-Stellen, wie sich auch bei einer Methylierung der Deletionsvarianten N11, N12 und N13 zeigt.

Die Reportergenassays zeigten auch eine verstärkte Rekrutierung von HDAC-Aktivität zumindest bei partieller Methylierung des Promotors und einen totalen Verlust an

Induzierbarkeit durch den Histonedeacetylase-Inhibitor TsA bei Methylierung aller CpG-Dinukleotide. Da DNA-Methylierung und Histondeacetylierung in der Regel Hand in Hand gehen, ist eine verstärkte Rekrutierung von HDACs wahrscheinlich. Nach vollständiger Methylierung des Promotors kann TsA durch eine Änderung des Histonacetylierungsgrades aber anscheinend die Promotoraktivität nicht mehr induzieren.

In EMSAs wurde der Einfluss der DNA-Methylierung auf die Sp1-Bindung untersucht. Die Ergebnisse deuten auf verschiedene Funktionen der drei proximalen Bindungsstellen im Zusammenhang mit einer DNA-Methylierung. Während die Sp1-Bindung an die 5-fach GC-Box und GC-Box 4 infolge der DNA-Methylierung abnimmt, stimuliert die Methylierung benachbarter CpG-Stellen die Bindung von Sp1 an GC-Box 5. Interessanterweise verhindert die DNA-Methylierung nicht nur die Sp1-Bindung an GC4 und die Tandem-GC-Box, sondern fördert deutlich die Proteinbindung eines bisher unidentifizierten Proteins. Die Kotransfektionsstudie mit in-vitro methylierten 5-LO-Promotorkonstrukten und den murinen Methyl-CpG-Bindungsproteinen weist auf eine Rekrutierung von MBD1 hin. Allerdings konnte in Mutationsstudien keine direkte Bindungsstelle für MBD1 ausgemacht werden und die durch die Mbd1-Kotransfektion vermittelte Reduktion der Promoteraktivität nicht aufgehoben werden. Durch Supershiftexperimente im EMSA und ChIP-Versuche sollte die mögliche MBD1-Bindung nachgeprüft werden.

Da in den EMSAs die Sp1-Bindung durch DNA-Methylierung verhindert wird, zumindest an die aktivierenden Elemente, die 5-fach-GC-Box und die GC-Box-4, und eine Mutation bzw. Deletion der gleichen Sp1-Bindungsstellen im Reportergenassay zu einem Verlust der Promotoraktivität führt, ist es wahrscheinlich, dass die in-vitro Methylierung schon durch die verhinderte Sp1/Sp3-Bindung zu einer Senkung der Promotoraktivität führt. Es wäre von Interesse in der Zukunft die in-vivo-Bindung von Sp1/Sp3 am methylierten 5-LO-Promotor zu untersuchen.

Zusammenfassend unterstreichen die Daten, wie wichtig und komplex die Sp1/Sp3-Bindung an die verschiedenen GC-reichen Sequenzen des 5-LO Promotors ist, sowohl in Bezug auf die Regulation durch den Histondeacetylase-Hemmer TsA, als auch in Bezug auf die Regulation durch DNA-Methylierung.

8 Abbreviations

14-3-3	14-3-3 protein recognition site
1,25(OH)$_2$D$_3$	1,25-dihydroxyvitamin D$_3$ (calcitriol/1,25-dihydroxycholecalciferol)
AA	arachidonic acid
aa	amino acid
AD	activation domain
AdC	5-aza-2`-deoxycytidine
AIA	aspirin-induced asthma
ALXR	lipoxin A$_4$ receptor
AP-1/2	activating protein-1/2
AR	androgen receptor
ATA	aspirin-tolerant asthma
ATF	activating transcription factor, also CREB
ATG	translational start codon
ATL	aspirin-triggered lipoxins
ATP	adenosine triphosphate
B	beads
bp	base pair(s)
BCL6	B-cell CLL/lymphoma 6 zinc finger protein
BLT	leukotriene B$_4$ receptor
BTB	broad complex, tramtrack and bric-a-bric domain, also POZ domain
BxPC-3	pancreatic carcinoma cell line
Caco-2	human Caucasian colon adenocarcinoma cells
CAT	chloramphenicol acetyl transferase assay
CBP	CREB binding protein
cDNA	copy DNA
cds	coding sequence
ChIP	chromatin immunoprecipitation assay
CK2	casein kinase 2
CLP	coactosin-like protein
CMV	cytomegalie virus
COX	cyclooxygenase
COUP-TFII	chicken ovalbumin upstream promoter transcription factor-II
cPLA$_2$	cytosolic phospholipase A$_2$
CREB	cAMP regulatory element-binding protein/cAMP-regulated enhancer binding protein
CRSP	cofactors required for Sp1 activation
CtBP	C-terminal binding protein
CTCF	CCCTC-binding factor, zinc finger protein
Cys	cysteine
CysLT	cysteinyl leukotriene
DAPA	DNA affinity purification assay
DBD	DNA-binding domain
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase I	desoxyribonuclease I
DNMT	DNA methyltransferase
DRIP	vitamin D receptor interacting proteins
DTT	dithiothreitol
EGFR	epidermal growth factor receptor

Egr1	early-growth response factor-1
EKLF	erythroid Kruppel-like factor
EMSA	electrophoretic mobility shift assays
ER	estrogen receptor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FEV	forced expiratory volume
FLAP	5-lipoxygenase activating protein
GATA	transcription factor binding to (A/T)GATA(A/G)
GCN5	histone acetyltransferase, general control of amino-acid synthesis 5
GM-CSF	granulocyte macrophage colony stimulating factor
GPCR	G protein coupled receptor
GPx	glutathione peroxidase
GR	glucocorticoid receptor
Grb-2	growth factor receptor-bound protein 2
GRIP-1	glucocorticoid receptor-interacting protein-1
GSH	glutathione
GST	glutathione S-transferase
H	histone
H3K9	histone 3 lysine 9
HAT	histone acetyl transferase
HCT116	human colon carcinoma cell line
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HeLa cells	epithelial cells derived from a cervix carcinoma
HepG2	human hepatoma cell line
Hep3B	human hepatoma cell line
His	histidine
HL-60 cells	human promyeloic leukemic cell line
HL-60TB	HL-60 cells negative for 5-LO
hLHR	human lueinizing hormone receptor
HMT	histone methyltransferase
HNF-4	hepatocyte nuclear factor 4
HP1	heterochromatin-associated protein 1
H(P)ETE	hydro(peroxy)eicosatetraenoic acid
HpODE	hydroperoxyoctadecaenoic acid
Hsp	heat shock protein
HTERT	human telomerase reverse transcriptase
HUB	HDAC6-, USP3-, BRAP2-related finger
HUVEC	human umbilical vein endothelial cells
IC50	half maximal inhibitory concentration
ID	inhibitory domain
IGFBP3	Insulin-like growth factor binding protein-3
IL	interleukin
Ile	isoleucine
InvRep	inverted repeat
JNK	c-jun NH_2-terminal kinase
K	lysine
kb	kilobase
kDa	kilo Dalton

KLF Kruppel like factor
KO knock out
L leucine
LBD ligand binding domain
LDL low-density binding protein
LO lipoxygenase
LRD ligand recognition domain
LT(R) leukotriene (receptor)
LTA4H leukotriene A_4 hydrolase
LTC4S leukotriene C_4 synthase
LX(R) lipoxin (receptor)
Lys lysine
MAPK mitogen-activated protein kinase
MBD(P) methyl-cytosine-binding domain (protein)
MCAF MBD1-containing chromatin-associated factor
MCF-7 human mammary gland epithelial cells
mcs multiple cloning site
MeCP methyl-CpG-binding protein
MEF2 myocyte enhancer factor-2
MEK MAPK kinase
MG63 human osteoblast-like cell line
Mi2 SWI2/SNF2 type helicase
MIA PaCa-2 pancreatic carcinoma cell line
MIZF MBD2-interacting zinc finger
MM6 cells Mono Mac 6 cells
mRNA messenger RNA
NA nicotinamide
NCoR nuclear receptor corepressor
NE nuclear extract
NES nuclear export signal
NF-1 nuclear factor 1
NFAT nuclear factor of activated T-cells
NF-Y nuclear factor Y or CCAAT-binding protien
NFκB nuclear factor of κ-light polypeptide gene enhancer in B cells
NIH3T3 mouse fibroblast cell line
NLS nuclear localization sequence
NO nitric oxide
NR nuclear receptor
NSAID non-steroidal anti-inflammatory drug
NuRD nucleosome remodeling histone deacetylase complex
Oct-1 octamer binding transcription factor
oxoETE oxoeicosatetraenoic acid
PAF platelet-activating factor
PBS phosphate buffered saline
PC phosphatidylcholine
PCAF p300/CBP-associated factor
PCNA proliferating cell nuclear antigen
PCR polymerase chain reaction
PG prostaglandin
PI 3-K phosphatidylinositol 3-kinase
PK protein kinase

PL	phospholipase
PLAT	Polycystin-1, Lipoxygenase, α-Toxin
PMA	phorbol-12-myristate-13-acetate
PML	promyelocytic leukemia
PMT	posttranslational histone modification
PMNL	polymorphonuclear leukocyte
PMSF	phenylmethansulfonylfluoride
POZ	poxvirus and zinc finger domain, also BTB domain
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
pre-mRNA	precursor-mRNA
RA	retinoic acid
RAR	retinoic acid receptor
Rb	retinoblastoma protein
RBCE	retinoblastoma control element
RE	response element
REST	repressor element 1 silencing transcription factor
RLU	relative light unit
RNA	ribonucleic acid
RNAi	RNA interference
ROR	retinoid orphan receptor
ROS	reactive oxygen species
RT-PCR	reverse transcription-polymerase chain reaction
RXRα	retinoid X receptor alpha
RZR	retinoid Z receptor
S	serine
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBE	Smad binding element
SE	standard error
SE14	Ser-Glu-containing tetradecapeptide repeat
SEAP	secreted alkaline phosphatase
Ser	serine
SF-1	steroidgenic factor-1
SH3	src-homology
Sin3	SWI independent
SIR	silent information regulator
siRNA	small interfering RNA
Ski	Sloan-Kettering Institute oncoprotein
SKIP	Ski-interacting protein
SL2	macrophage-like drosophila melanogaster cells
Smad	Sma and Mad related protein
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SMURF	Smad ubiquitylation regulatory factor
SNF	sucrose non-fermenter
SNRNP	small nuclear ribonucleoprotein polypetide N
Sp1	serum protein1/specific protein1/selective promoter factor1
sPLA$_2$	secretory phospholipase A$_2$
SRC-1	steroid receptor coactivator-1
STAT	signal transducers and activators of transcription
STI	soybean trypsin inhibitor

SUMO	sumoylation signal
SV-40	simian virus-40
SW620	human colon carcinoma cell line
SWI	mating type switch
TAFII	TATA-binding protein associated factor II
TBP	TATA-binding protein
TF	transcription factor
TFIIB	transcription factor IIB
TGFβ	transforming growth factor beta
TGFβR	TGFβ receptor
TNFα	tumor necrosis factor alpha
TGFβRE	TGFβ responsive element
TIS	transcription initiation site
TRD	transcriptional repression domain
Trp	tryptophane
TsA	Trichostatin A
TSS	translation start site
U937 cells	human lymphoma cell line
UK Pan-1	pancreatic adenocarcinoma cell line
UTR	untranslated region
VDR	vitamin D receptor
VDRE	vitamin D response element
VPA	valproic acid
w/o	without
YY1	Yin-Yan-1
ZBZB	zinc finger and BTB containing factor
ZE	cell extract
ZF	zinc finger

9 References

Aagaard, L., G. Laible, et al. (1999). "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31." Embo J **18**(7): 1923-38.

Abramovitz, M., E. Wong, et al. (1993). "5-lipoxygenase-activating protein stimulates the utilization of arachidonic acid by 5-lipoxygenase." Eur J Biochem **215**(1): 105-11.

Adamietz, P. and A. Rudolph (1984). "ADP-ribosylation of nuclear proteins in vivo. Identification of histone H2B as a major acceptor for mono- and poly(ADP-ribose) in dimethyl sulfate-treated hepatoma AH 7974 cells." J Biol Chem **259**(11): 6841-6.

Aiello, R. J., P. A. Bourassa, et al. (2002). "Leukotriene B4 receptor antagonism reduces monocytic foam cells in mice." Arterioscler Thromb Vasc Biol **22**(3): 443-9.

Albagli, O., P. Dhordain, et al. (1995). "The BTB/POZ domain: a new protein-protein interaction motif common to DNA- and actin-binding proteins." Cell Growth Differ **6**(9): 1193-8.

Allen, S. P., A. H. Chester, et al. (1992). "Effects of leukotrienes C4 and D4 on human isolated saphenous veins." Br J Clin Pharmacol **34**(5): 409-14.

Alroy, I., L. Soussan, et al. (1999). "Neu differentiation factor stimulates phosphorylation and activation of the Sp1 transcription factor." Mol Cell Biol **19**(3): 1961-72.

Amir, R. E., I. B. Van den Veyver, et al. (1999). "Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2." Nat Genet **23**(2): 185-8.

Ammanamanchi, S. and M. G. Brattain (2001). "Sp3 is a transcriptional repressor of transforming growth factor-beta receptors." J Biol Chem **276**(5): 3348-52.

Ammanamanchi, S., J. W. Freeman, et al. (2003). "Acetylated sp3 is a transcriptional activator." J Biol Chem **278**(37): 35775-80.

Anderson, R. M., K. J. Bitterman, et al. (2003). "Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae." Nature **423**(6936): 181-5.

Antequera, F. and A. Bird (1993). "Number of CpG islands and genes in human and mouse." Proc Natl Acad Sci U S A **90**(24): 11995-9.

Bachman, K. E., B. H. Park, et al. (2003). "Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene." Cancer Cell **3**(1): 89-95.

Badr, K. F., D. K. DeBoer, et al. (1989). "Lipoxin A4 antagonizes cellular and in vivo actions of leukotriene D4 in rat glomerular mesangial cells: evidence for competition at a common receptor." Proc Natl Acad Sci U S A **86**(9): 3438-42.

Bali, P., M. Pranpat, et al. (2005). "Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors." J Biol Chem **280**(29): 26729-34.

Banchio, C., L. M. Schang, et al. (2004). "Phosphorylation of Sp1 by cyclin-dependent kinase 2 modulates the role of Sp1 in CTP:phosphocholine cytidylyltransferase alpha regulation during the S phase of the cell cycle." J Biol Chem **279**(38): 40220-6.

Bandeira-Melo, C., M. F. Serra, et al. (2000). "Cyclooxygenase-2-derived prostaglandin E2 and lipoxin A4 accelerate resolution of allergic edema in Angiostrongylus costaricensis-infected rats: relationship with concurrent eosinophilia." J Immunol **164**(2): 1029-36.

Bannister, A. J., P. Zegerman, et al. (2001). "Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain." Nature **410**(6824): 120-4.

Bardwell, V. J. and R. Treisman (1994). "The POZ domain: a conserved protein-protein interaction motif." Genes Dev **8**(14): 1664-77.

Bateman, A. and R. Sandford (1999). "The PLAT domain: a new piece in the PKD1 puzzle." Curr Biol **9**(16): R588-90.

Battaglioli, E., M. E. Andres, et al. (2002). "REST repression of neuronal genes requires components of the hSWI.SNF complex." J Biol Chem **277**(43): 41038-45.

Bennett, C. F., M. Y. Chiang, et al. (1993). "Regulation of 5-lipoxygenase and 5-lipoxygenase-activating protein expression in HL-60 cells." Biochem J **289 (Pt 1)**: 33-9.

Bertos, N. R., A. H. Wang, et al. (2001). "Class II histone deacetylases: structure, function, and regulation." Biochem Cell Biol **79**(3): 243-52.

Bitterman, K. J., R. M. Anderson, et al. (2002). "Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1." J Biol Chem **277**(47): 45099-107.

Black, A. R., J. D. Black, et al. (2001). "Sp1 and kruppel-like factor family of transcription factors in cell growth regulation and cancer." J Cell Physiol **188**(2): 143-60.

Black, A. R., D. Jensen, et al. (1999). "Growth/cell cycle regulation of Sp1 phosphorylation." J Biol Chem **274**(3): 1207-15.

Boado, R. J., W. M. Pardridge, et al. (1992). "Differential expression of arachidonate 5-lipoxygenase transcripts in human brain tumors: evidence for the expression of a multitranscript family." Proc Natl Acad Sci U S A **89**(19): 9044-8.

Boden, S. E., T. Bertsche, et al. (2000). "MEK-1/2 inhibition prevents 5-lipoxygenase translocation in N-formylpeptide-challenged human neutrophils." Int J Biochem Cell Biol **32**(10): 1069-74.

Boeglin, W. E., R. B. Kim, et al. (1998). "A 12R-lipoxygenase in human skin: mechanistic evidence, molecular cloning, and expression." Proc Natl Acad Sci U S A **95**(12): 6744-9.

Bonello, M. R. and L. M. Khachigian (2004). "Fibroblast growth factor-2 represses platelet-derived growth factor receptor-alpha (PDGFR-alpha) transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical cis-acting element in the proximal PDGFR-alpha promoter." J Biol Chem **279**(4): 2377-82.

Bonventre, J. V., Z. Huang, et al. (1997). "Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A2." Nature **390**(6660): 622-5.

Borgeat, P., M. Hamberg, et al. (1976). "Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases." J Biol Chem **251**(24): 7816-20.

Boyes, J. and A. Bird (1991). "DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein." Cell **64**(6): 1123-34.

Brady, H. R., U. Persson, et al. (1990). "Leukotrienes stimulate neutrophil adhesion to mesangial cells: modulation with lipoxins." Am J Physiol **259**(5 Pt 2): F809-15.

Brash, A. R., W. E. Boeglin, et al. (1997). "Discovery of a second 15S-lipoxygenase in humans." Proc Natl Acad Sci U S A **94**(12): 6148-52.

Braun, H., R. Koop, et al. (2001). "Transcription factor Sp3 is regulated by acetylation." Nucleic Acids Res **29**(24): 4994-5000.

Brehm, A., E. A. Miska, et al. (1998). "Retinoblastoma protein recruits histone deacetylase to repress transcription." Nature **391**(6667): 597-601.

Brenner, C., R. Deplus, et al. (2005). "Myc represses transcription through recruitment of DNA methyltransferase corepressor." Embo J **24**(2): 336-46.

Brungs, M., O. Radmark, et al. (1994). "On the induction of 5-lipoxygenase expression and activity in HL-60 cells: effects of vitamin D3, retinoic acid, DMSO and TGF beta." Biochem Biophys Res Commun **205**(3): 1572-80.

Brungs, M., O. Radmark, et al. (1995). "Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25-dihydroxyvitamin D3." Proc Natl Acad Sci U S A **92**(1): 107-11.

Buhrmester, H., J. P. von Kries, et al. (1995). "Nuclear matrix protein ARBP recognizes a novel DNA sequence motif with high affinity." Biochemistry **34**(12): 4108-117.

Burgers, W. A., F. Fuks, et al. (2002). "DNA methyltransferases get connected to chromatin." Trends Genet **18**(6): 275-7.

Burke, L. J. and A. Baniahmad (2000). "Co-repressors 2000." Faseb J **14**(13): 1876-88.

Burleigh, M. E., V. R. Babaev, et al. (2002). "Cyclooxygenase-2 promotes early atherosclerotic lesion formation in LDL receptor-deficient mice." Circulation **105**(15): 1816-23.

Byrum, R. S., J. L. Goulet, et al. (1997). "Role of the 5-lipoxygenase-activating protein (FLAP) in murine acute inflammatory responses." J Exp Med **185**(6): 1065-75.

Cao, R., L. Wang, et al. (2002). "Role of histone H3 lysine 27 methylation in Polycomb-group silencing." Science **298**(5595): 1039-43.

Carlberg, C. (1995). "Mechanisms of nuclear signalling by vitamin D3. Interplay with retinoid and thyroid hormone signalling." Eur J Biochem **231**(3): 517-27.

Carmen, A. A., P. R. Griffin, et al. (1999). "Yeast HOS3 forms a novel trichostatin A-insensitive homodimer with intrinsic histone deacetylase activity." Proc Natl Acad Sci U S A **96**(22): 12356-61.

Celotti, F. and T. Durand (2003). "The metabolic effects of inhibitors of 5-lipoxygenase and of cyclooxygenase 1 and 2 are an advancement in the efficacy and safety of anti-inflammatory therapy." Prostaglandins Other Lipid Mediat **71**(3-4): 147-62.

Chahinian, H., B. Sias, et al. (2000). "The C-terminal domain of pancreatic lipase: functional and structural analogies with c2 domains." Curr Protein Pept Sci **1**(1): 91-103.

Chang, S., T. A. McKinsey, et al. (2004). "Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development." Mol Cell Biol **24**(19): 8467-76.

Charleson, S., J. F. Evans, et al. (1994). "Structural requirements for the binding of fatty acids to 5-lipoxygenase-activating protein." Eur J Pharmacol **267**(3): 275-80.

Charlier, C. and C. Michaux (2003). "Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs." Eur J Med Chem **38**(7-8): 645-59.

Chen, H. M., H. L. Pahl, et al. (1993). "The Sp1 transcription factor binds the CD11b promoter specifically in myeloid cells in vivo and is essential for myeloid-specific promoter activity." J Biol Chem **268**(11): 8230-9.

Chen, L. I., T. Nishinaka, et al. (1994). "The retinoblastoma gene product RB stimulates Sp1-mediated transcription by liberating Sp1 from a negative regulator." Mol Cell Biol **14**(7): 4380-9.

Chen, X. S. and C. D. Funk (2001). "The N-terminal "beta-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation." J Biol Chem **276**(1): 811-8.

Cheng, H. T., J. Y. Chen, et al. (2006). "Functional role of VDR in the activation of p27(Kip1) by the VDR/Sp1 complex." J Cell Biochem.

Choi, H. S., J. H. Lee, et al. (2002). "Trichostatin A, a histone deacetylase inhibitor, activates the IGFBP-3 promoter by upregulating Sp1 activity in hepatoma cells: alteration of the Sp1/Sp3/HDAC1 multiprotein complex." Biochem Biophys Res Commun **296**(4): 1005-12.

Chu, S. and T. J. Ferro (2005). "Sp1: regulation of gene expression by phosphorylation." Gene **348**: 1-11.

Chun, R. F., O. J. Semmes, et al. (1998). "Modulation of Sp1 phosphorylation by human immunodeficiency virus type 1 Tat." J Virol **72**(4): 2615-29.

Claesson, H. E. and S. E. Dahlen (1999). "Asthma and leukotrienes: antileukotrienes as novel anti-asthmatic drugs." J Intern Med **245**(3): 205-27.

Claria, J. and C. N. Serhan (1995). "Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions." Proc Natl Acad Sci U S A **92**(21): 9475-9.

Clark, J. D., L. L. Lin, et al. (1991). "A novel arachidonic acid-selective cytosolic PLA2 contains a Ca(2+)-dependent translocation domain with homology to PKC and GAP." Cell **65**(6): 1043-51.

Clark, S. J., J. Harrison, et al. (1997). "Sp1 binding is inhibited by (m)Cp(m)CpG methylation." Gene **195**(1): 67-71.

Colombo, R. (2002). "Re: sexuality preserving cystectomy and neobladder: initial results." J Urol **167**(4): 1803.

Courey, A. J., D. A. Holtzman, et al. (1989). "Synergistic activation by the glutamine-rich domains of human transcription factor Sp1." Cell **59**(5): 827-36.

Courey, A. J. and R. Tjian (1988). "Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif." Cell **55**(5): 887-98.

Crawley, G. C., R. I. Dowell, et al. (1992). "Methoxytetrahydropyrans. A new series of selective and orally potent 5-lipoxygenase inhibitors." J Med Chem **35**(14): 2600-9.

Cress, W. D. and E. Seto (2000). "Histone deacetylases, transcriptional control, and cancer." J Cell Physiol **184**(1): 1-16.

Cyrus, T., D. Pratico, et al. (2001). "Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein e-deficient mice." Circulation **103**(18): 2277-82.

Czermin, B., G. Schotta, et al. (2001). "Physical and functional association of SU(VAR)3-9 and HDAC1 in Drosophila." EMBO Rep **2**(10): 915-9.

Dahlen, S. E., J. Bjork, et al. (1981). "Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: in vivo effects with relevance to the acute inflammatory response." Proc Natl Acad Sci U S A **78**(6): 3887-91.

Daniel, J. M. and A. B. Reynolds (1999). "The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor." Mol Cell Biol **19**(5): 3614-23.

Daniel, J. M., C. M. Spring, et al. (2002). "The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides." Nucleic Acids Res **30**(13): 2911-9.

David, G., M. A. Neptune, et al. (2002). "SUMO-1 modification of histone deacetylase 1 (HDAC1) modulates its biological activities." J Biol Chem **277**(26): 23658-63.

Davie, J. R. (1997). "Nuclear matrix, dynamic histone acetylation and transcriptionally active chromatin." Mol Biol Rep **24**(3): 197-207.

Davie, J. R. and L. C. Murphy (1990). "Level of ubiquitinated histone H2B in chromatin is coupled to ongoing transcription." Biochemistry **29**(20): 4752-7.

De Luca, P., B. Majello, et al. (1996). "Sp3 represses transcription when tethered to promoter DNA or targeted to promoter proximal RNA." J Biol Chem **271**(15): 8533-6.

Defossez, P. A., K. F. Kelly, et al. (2005). "The human enhancer blocker CTC-binding factor interacts with the transcription factor Kaiso." J Biol Chem **280**(52): 43017-23.

Denis, D., J. P. Falgueyret, et al. (1991). "Characterization of the activity of purified recombinant human 5-lipoxygenase in the absence and presence of leukocyte factors." J Biol Chem **266**(8): 5072-9.

Dennig, J., M. Beato, et al. (1996). "An inhibitor domain in Sp3 regulates its glutamine-rich activation domains." Embo J **15**(20): 5659-67.

Dennis, E. A. (1997). "The growing phospholipase A2 superfamily of signal transduction enzymes." Trends Biochem Sci **22**(1): 1-2.

Detich, N., V. Bovenzi, et al. (2003). "Valproate induces replication-independent active DNA demethylation." J Biol Chem **278**(30): 27586-92.

Devillier, P., N. Baccard, et al. (1999). "Leukotrienes, leukotriene receptor antagonists and leukotriene synthesis inhibitors in asthma: an update. Part I: synthesis, receptors and role of leukotrienes in asthma." Pharmacol Res **40**(1): 3-13.

Di Croce, L., V. A. Raker, et al. (2002). "Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor." Science **295**(5557): 1079-82.

Dishart, D., N. Schnur, et al. (2005). "GC-rich sequences in the 5-lipoxygenase gene promoter are required for expression in Mono Mac 6 cells, characterization of a novel Sp1 binding site." Biochim Biophys Acta **1738**(1-3): 37-47.

Dixon, R. A., R. E. Diehl, et al. (1990). "Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis." Nature **343**(6255): 282-4.

Dixon, R. A., R. E. Jones, et al. (1988). "Cloning of the cDNA for human 5-lipoxygenase." Proc Natl Acad Sci U S A **85**(2): 416-20.

Doetzlhofer, A., H. Rotheneder, et al. (1999). "Histone deacetylase 1 can repress transcription by binding to Sp1." Mol Cell Biol **19**(8): 5504-11.

Dokmanovic, M. and P. A. Marks (2005). "Prospects: histone deacetylase inhibitors." J Cell Biochem **96**(2): 293-304.

Drazen, J. M., E. Israel, et al. (1999). "Treatment of asthma with drugs modifying the leukotriene pathway." N Engl J Med **340**(3): 197-206.

Drazen, J. M., C. M. Lilly, et al. (1994). "Role of cysteinyl leukotrienes in spontaneous asthmatic responses." Adv Prostaglandin Thromboxane Leukot Res **22**: 251-62.

Drazen, J. M., C. N. Yandava, et al. (1999). "Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment." Nat Genet **22**(2): 168-70.

Dwyer, J. H., H. Allayee, et al. (2004). "Arachidonate 5-lipoxygenase promoter genotype, dietary arachidonic acid, and atherosclerosis." N Engl J Med **350**(1): 29-37.

Ehrlich, M., M. A. Gama-Sosa, et al. (1982). "Amount and distribution of 5-methylcytosine in human DNA from different types of tissues of cells." Nucleic Acids Res **10**(8): 2709-21.

Emami, K. H., T. W. Burke, et al. (1998). "Sp1 activation of a TATA-less promoter requires a species-specific interaction involving transcription factor IID." Nucleic Acids Res **26**(3): 839-46.

Falgueyret, J. P., D. Denis, et al. (1995). "Characterization of the arachidonate and ATP binding sites of human 5-lipoxygenase using photoaffinity labeling and enzyme immobilization." Biochemistry **34**(41): 13603-11.

Feng, Q. and Y. Zhang (2001). "The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes." Genes Dev **15**(7): 827-32.

Ferguson, M., P. A. Henry, et al. (2003). "Histone deacetylase inhibition is associated with transcriptional repression of the Hmga2 gene." Nucleic Acids Res **31**(12): 3123-33.

Filion, G. J., S. Zhenilo, et al. (2006). "A family of human zinc finger proteins that bind methylated DNA and repress transcription." Mol Cell Biol **26**(1): 169-81.

Filippova, G. N., C. F. Qi, et al. (2002). "Tumor-associated zinc finger mutations in the CTCF transcription factor selectively alter tts DNA-binding specificity." Cancer Res **62**(1): 48-52.

Finnin, M. S., J. R. Donigian, et al. (1999). "Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors." Nature **401**(6749): 188-93.

Fischer, L., D. Szellas, et al. (2003). "Phosphorylation- and stimulus-dependent inhibition of cellular 5-lipoxygenase activity by nonredox-type inhibitors." Faseb J **17**(8): 949-51.

Fischle, W., F. Dequiedt, et al. (2001). "Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo." J Biol Chem **276**(38): 35826-35.

Fischle, W., F. Dequiedt, et al. (2002). "Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR." Mol Cell **9**(1): 45-57.

Fischle, W., V. Kiermer, et al. (2001). "The emerging role of class II histone deacetylases." Biochem Cell Biol **79**(3): 337-48.

Fischle, W., Y. Wang, et al. (2003). "Histone and chromatin cross-talk." Curr Opin Cell Biol **15**(2): 172-83.

Fojas de Borja, P., N. K. Collins, et al. (2001). "Cyclin A-CDK phosphorylates Sp1 and enhances Sp1-mediated transcription." Embo J **20**(20): 5737-47.

Ford-Hutchinson, A. W. (1994). "Leukotriene C4 synthase and 5-lipoxygenase activating protein. Regulators of the biosynthesis of sulfido-leukotrienes." Ann N Y Acad Sci **744**: 78-83.

Ford-Hutchinson, A. W., M. Gresser, et al. (1994). "5-Lipoxygenase." Annu Rev Biochem **63**: 383-417.

Frye, R. A. (2000). "Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins." Biochem Biophys Res Commun **273**(2): 793-8.

Fujita, N., N. Shimotake, et al. (2000). "Mechanism of transcriptional regulation by methyl-CpG binding protein MBD1." Mol Cell Biol **20**(14): 5107-18.

Fujita, N., S. Takebayashi, et al. (1999). "Methylation-mediated transcriptional silencing in euchromatin by methyl-CpG binding protein MBD1 isoforms." Mol Cell Biol **19**(9): 6415-26.

Fujita, N., S. Watanabe, et al. (2003). "MCAF mediates MBD1-dependent transcriptional repression." Mol Cell Biol **23**(8): 2834-43.

Fujita, N., S. Watanabe, et al. (2003). "Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression." J Biol Chem **278**(26): 24132-8.

Fuks, F., W. A. Burgers, et al. (2001). "Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription." Embo J **20**(10): 2536-44.

Fuks, F., P. J. Hurd, et al. (2003). "The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase." Nucleic Acids Res **31**(9): 2305-12.

Fuks, F., P. J. Hurd, et al. (2003). "The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation." J Biol Chem **278**(6): 4035-40.

Funk, C. D., X. S. Chen, et al. (2002). "Lipoxygenase genes and their targeted disruption." Prostaglandins Other Lipid Mediat **68-69**: 303-12.

Funk, C. D., S. Hoshiko, et al. (1989). "Characterization of the human 5-lipoxygenase gene." Proc Natl Acad Sci U S A **86**(8): 2587-91.

Furumai, R., Y. Komatsu, et al. (2001). "Potent histone deacetylase inhibitors built from trichostatin A and cyclic tetrapeptide antibiotics including trapoxin." Proc Natl Acad Sci U S A **98**(1): 87-92.

Gao, L., M. A. Cueto, et al. (2002). "Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family." J Biol Chem **277**(28): 25748-55.

Gartel, A. L., E. Goufman, et al. (2000). "Sp1 and Sp3 activate p21 (WAF1/CIP1) gene transcription in the Caco-2 colon adenocarcinoma cell line." Oncogene **19**(45): 5182-8.

Ge, Y. Z., M. T. Pu, et al. (2004). "Chromatin targeting of de novo DNA methyltransferases by the PWWP domain." J Biol Chem **279**(24): 25447-54.

Georgel, P. T., R. A. Horowitz-Scherer, et al. (2003). "Chromatin compaction by human MeCP2. Assembly of novel secondary chromatin structures in the absence of DNA methylation." J Biol Chem **278**(34): 32181-8.

Ghoshal, K., J. Datta, et al. (2002). "Inhibitors of histone deacetylase and DNA methyltransferase synergistically activate the methylated metallothionein I promoter by activating the transcription factor MTF-1 and forming an open chromatin structure." Mol Cell Biol **22**(23): 8302-19.

Gillmor, S. A., A. Villasenor, et al. (1997). "The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity." Nat Struct Biol **4**(12): 1003-9.

Glaser, K. B., M. J. Staver, et al. (2003). "Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines." Mol Cancer Ther **2**(2): 151-63.

Glozak, M. A., N. Sengupta, et al. (2005). "Acetylation and deacetylation of non-histone proteins." Gene **363**: 15-23.

Gottlicher, M., S. Minucci, et al. (2001). "Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells." Embo J **20**(24): 6969-78.

Gregoire, S. and X. J. Yang (2005). "Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors." Mol Cell Biol **25**(6): 2273-87.

Griffiths, R. J., E. R. Pettipher, et al. (1995). "Leukotriene B4 plays a critical role in the progression of collagen-induced arthritis." Proc Natl Acad Sci U S A **92**(2): 517-21.

Gronert, K., A. Gewirtz, et al. (1998). "Identification of a human enterocyte lipoxin A4 receptor that is regulated by interleukin (IL)-13 and interferon gamma and inhibits tumor necrosis factor alpha-induced IL-8 release." J Exp Med **187**(8): 1285-94.

Gronert, K., T. Martinsson-Niskanen, et al. (2001). "Selectivity of recombinant human leukotriene D(4), leukotriene B(4), and lipoxin A(4) receptors with aspirin-triggered 15-epi-LXA(4) and regulation of vascular and inflammatory responses." Am J Pathol **158**(1): 3-9.

Gronroos, E., U. Hellman, et al. (2002). "Control of Smad7 stability by competition between acetylation and ubiquitination." Mol Cell **10**(3): 483-93.

Grozinger, C. M., C. A. Hassig, et al. (1999). "Three proteins define a class of human histone deacetylases related to yeast Hda1p." Proc Natl Acad Sci U S A **96**(9): 4868-73.

Grozinger, C. M. and S. L. Schreiber (2000). "Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization." Proc Natl Acad Sci U S A **97**(14): 7835-40.

Grozinger, C. M. and S. L. Schreiber (2002). "Deacetylase enzymes: biological functions and the use of small-molecule inhibitors." Chem Biol **9**(1): 3-16.

Grunstein, M. (1997). "Histone acetylation in chromatin structure and transcription." Nature **389**(6649): 349-52.

Guenther, M. G., O. Barak, et al. (2001). "The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3." Mol Cell Biol **21**(18): 6091-101.

Gui, C. Y., L. Ngo, et al. (2004). "Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1." Proc Natl Acad Sci U S A **101**(5): 1241-6.

Gurvich, N., O. M. Tsygankova, et al. (2004). "Histone deacetylase is a target of valproic acid-mediated cellular differentiation." Cancer Res **64**(3): 1079-86.

Habenicht, A. J., M. Goerig, et al. (1989). "Early reversible induction of leukotriene synthesis in chicken myelomonocytic cells transformed by a temperature-sensitive mutant of avian leukemia virus E26." Proc Natl Acad Sci U S A **86**(3): 921-4.

Hagen, G., S. Muller, et al. (1994). "Sp1-mediated transcriptional activation is repressed by Sp3." Embo J **13**(16): 3843-51.

Haggarty, S. J., K. M. Koeller, et al. (2003). "Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation." Proc Natl Acad Sci U S A **100**(8): 4389-94.

Haidweger, E., M. Novy, et al. (2001). "Modulation of Sp1 activity by a cyclin A/CDK complex." J Mol Biol **306**(2): 201-12.

Hake, S. B. and C. D. Allis (2006). "Histone H3 variants and their potential role in indexing mammalian genomes: The "H3 barcode hypothesis"." Proc Natl Acad Sci U S A **103**(17): 6428-35.

Hake, S. B., B. A. Garcia, et al. (2006). "Expression patterns and post-translational modifications associated with mammalian histone H3 variants." J Biol Chem **281**(1): 559-68.

Hamberg, M. and B. Samuelsson (1974). "Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets." Proc Natl Acad Sci U S A **71**(9): 3400-4.

Hammarberg, T., S. Kuprin, et al. (2001). "EPR investigation of the active site of recombinant human 5-lipoxygenase: inhibition by selenide." Biochemistry **40**(21): 6371-8.

Hammarberg, T., P. Provost, et al. (2000). "The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity." J Biol Chem **275**(49): 38787-93.

Hammarberg, T., Y. Y. Zhang, et al. (1995). "Mutations at the C-terminal isoleucine and other potential iron ligands of 5-lipoxygenase." Eur J Biochem **230**(2): 401-7.

Han, I. and J. E. Kudlow (1997). "Reduced O glycosylation of Sp1 is associated with increased proteasome susceptibility." Mol Cell Biol **17**(5): 2550-8.

Harle, D., O. Radmark, et al. (1998). "Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation." Eur J Biochem **254**(2): 275-81.

Harle, D., O. Radmark, et al. (1999). "Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-beta and 1,25-dihydroxyvitamin D3." Adv Exp Med Biol **469**: 105-11.

Hauses, M., R. R. Tonjes, et al. (1998). "The transcription factor Sp1 regulates the myeloid-specific expression of the human hematopoietic cell kinase (HCK) gene through binding to two adjacent GC boxes within the HCK promoter-proximal region." J Biol Chem **273**(48): 31844-52.

Heise, C. E., B. F. O'Dowd, et al. (2000). "Characterization of the human cysteinyl leukotriene 2 receptor." J Biol Chem **275**(39): 30531-6.

Hemak, J., D. Gale, et al. (2002). "Structural characterization of the catalytic domain of the human 5-lipoxygenase enzyme." J Mol Model (Online) **8**(4): 102-12.

Hendrich, B. and A. Bird (1998). "Identification and characterization of a family of mammalian methyl-CpG binding proteins." Mol Cell Biol **18**(11): 6538-47.

Hendrich, B., J. Guy, et al. (2001). "Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development." Genes Dev **15**(6): 710-23.

Hendrich, B., U. Hardeland, et al. (1999). "The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites." Nature **401**(6750): 301-4.

Holler, M., G. Westin, et al. (1988). "Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated." Genes Dev **2**(9): 1127-35.

Hook, S. S., A. Orian, et al. (2002). "Histone deacetylase 6 binds polyubiquitin through its zinc finger (PAZ domain) and copurifies with deubiquitinating enzymes." Proc Natl Acad Sci U S A **99**(21): 13425-30.

Horike, S., S. Cai, et al. (2005). "Loss of silent-chromatin looping and impaired imprinting of DLX5 in Rett syndrome." Nat Genet **37**(1): 31-40.

Hoshiko, S., O. Radmark, et al. (1990). "Characterization of the human 5-lipoxygenase gene promoter." Proc Natl Acad Sci U S A **87**(23): 9073-7.

Hou, M., X. Wang, et al. (2002). "The histone deacetylase inhibitor trichostatin A derepresses the telomerase reverse transcriptase (hTERT) gene in human cells." Exp Cell Res **274**(1): 25-34.

Hu, E., Z. Chen, et al. (2000). "Cloning and characterization of a novel human class I histone deacetylase that functions as a transcription repressor." J Biol Chem **275**(20): 15254-64.

Hu, E., E. Dul, et al. (2003). "Identification of novel isoform-selective inhibitors within class I histone deacetylases." J Pharmacol Exp Ther **307**(2): 720-8.

Huang, W., S. Zhao, et al. (2005). "Trichostatin A induces transforming growth factor beta type II receptor promoter activity and acetylation of Sp1 by recruitment of PCAF/p300 to a Sp1.NF-Y complex." J Biol Chem **280**(11): 10047-54.

Huang, Y. C., J. Y. Chen, et al. (2004). "Vitamin D3 receptor/Sp1 complex is required for the induction of p27Kip1 expression by vitamin D3." Oncogene **23**(28): 4856-61.

Hubbert, C., A. Guardiola, et al. (2002). "HDAC6 is a microtubule-associated deacetylase." Nature **417**(6887): 455-8.

Hymes, J., K. Fleischhauer, et al. (1995). "Biotinylation of histones by human serum biotinidase: assessment of biotinyl-transferase activity in sera from normal individuals and children with biotinidase deficiency." Biochem Mol Med **56**(1): 76-83.

Ichimura, T., S. Watanabe, et al. (2005). "Transcriptional repression and heterochromatin formation by MBD1 and MCAF/AM family proteins." J Biol Chem **280**(14): 13928-35.

Imagawa, M., R. Chiu, et al. (1987). "Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP." Cell **51**(2): 251-60.

In, K. H., K. Asano, et al. (1997). "Naturally occurring mutations in the human 5-lipoxygenase gene promoter that modify transcription factor binding and reporter gene transcription." J Clin Invest **99**(5): 1130-7.

Ishizuka, T. and M. A. Lazar (2003). "The N-CoR/histone deacetylase 3 complex is required for repression by thyroid hormone receptor." Mol Cell Biol **23**(15): 5122-31.

Iversen, L., K. Kragballe, et al. (1997). "Significance of leukotriene-A4 hydrolase in the pathogenesis of psoriasis." Skin Pharmacol **10**(4): 169-77.

Izumi, T., S. Hoshiko, et al. (1990). "Cloning of the cDNA for human 12-lipoxygenase." Proc Natl Acad Sci U S A **87**(19): 7477-81.

Jackson, J. P., A. M. Lindroth, et al. (2002). "Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase." Nature **416**(6880): 556-60.

Jackson, S. P., J. J. MacDonald, et al. (1990). "GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase." Cell **63**(1): 155-65.

Jacobs, S. A. and S. Khorasanizadeh (2002). "Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail." Science **295**(5562): 2080-3.

Jang, S. I. and P. M. Steinert (2002). "Loricrin expression in cultured human keratinocytes is controlled by a complex interplay between transcription factors of the Sp1, CREB, AP1, and AP2 families." J Biol Chem **277**(44): 42268-79.

Janssen-Timmen, U., P. J. Vickers, et al. (1995). "Expression of 5-lipoxygenase in differentiating human skin keratinocytes." Proc Natl Acad Sci U S A **92**(15): 6966-70.

Johnson, C. A., D. A. White, et al. (2002). "Human class I histone deacetylase complexes show enhanced catalytic activity in the presence of ATP and co-immunoprecipitate with the ATP-dependent chaperone protein Hsp70." J Biol Chem **277**(11): 9590-7.

Johnstone, R. W. (2002). "Histone-deacetylase inhibitors: novel drugs for the treatment of cancer." Nat Rev Drug Discov **1**(4): 287-99.
Jones, P. L., G. J. Veenstra, et al. (1998). "Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription." Nat Genet **19**(2): 187-91.
Jones, S. M., M. Luo, et al. (2002). "Structural and functional criteria reveal a new nuclear import sequence on the 5-lipoxygenase protein." J Biol Chem **277**(41): 38550-6.
Jones, S. M., M. Luo, et al. (2003). "Identification of two novel nuclear import sequences on the 5-lipoxygenase protein." J Biol Chem **278**(12): 10257-63.
Jorgensen, H. F., I. Ben-Porath, et al. (2004). "Mbd1 is recruited to both methylated and nonmethylated CpGs via distinct DNA binding domains." Mol Cell Biol **24**(8): 3387-95.
Juan, L. J., W. J. Shia, et al. (2000). "Histone deacetylases specifically down-regulate p53-dependent gene activation." J Biol Chem **275**(27): 20436-43.
Kaludov, N. K. and A. P. Wolffe (2000). "MeCP2 driven transcriptional repression in vitro: selectivity for methylated DNA, action at a distance and contacts with the basal transcription machinery." Nucleic Acids Res **28**(9): 1921-8.
Kang, J. E., M. H. Kim, et al. (2005). "Histone deacetylase-1 represses transcription by interacting with zinc-fingers and interfering with the DNA binding activity of Sp1." Cell Physiol Biochem **16**(1-3): 23-30.
Kardassis, D., P. Papakosta, et al. (1999). "c-Jun transactivates the promoter of the human p21(WAF1/Cip1) gene by acting as a superactivator of the ubiquitous transcription factor Sp1." J Biol Chem **274**(41): 29572-81.
Kawagishi, Y., H. Mita, et al. (2002). "Leukotriene C4 synthase promoter polymorphism in Japanese patients with aspirin-induced asthma." J Allergy Clin Immunol **109**(6): 936-42.
Kawaguchi, Y., J. J. Kovacs, et al. (2003). "The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress." Cell **115**(6): 727-38.
Kawasaki, H. and K. Taira (2004). "Induction of DNA methylation and gene silencing by short interfering RNAs in human cells." Nature **431**(7005): 211-7.
Kennett, S. B., K. S. Moorefield, et al. (2002). "Sp3 represses gene expression via the titration of promoter-specific transcription factors." J Biol Chem **277**(12): 9780-9.
Kennett, S. B., A. J. Udvadia, et al. (1997). "Sp3 encodes multiple proteins that differ in their capacity to stimulate or repress transcription." Nucleic Acids Res **25**(15): 3110-7.
Khochbin, S., A. Verdel, et al. (2001). "Functional significance of histone deacetylase diversity." Curr Opin Genet Dev **11**(2): 162-6.
Kim, D. B. and N. A. DeLuca (2002). "Phosphorylation of transcription factor Sp1 during herpes simplex virus type 1 infection." J Virol **76**(13): 6473-9.
Kim, S. H., J. S. Bae, et al. (2005). "Polymorphism of tandem repeat in promoter of 5-lipoxygenase in ASA-intolerant asthma: a positive association with airway hyperresponsiveness." Allergy **60**(6): 760-5.
Kim, S. J., U. S. Onwuta, et al. (1992). "The retinoblastoma gene product regulates Sp1-mediated transcription." Mol Cell Biol **12**(6): 2455-63.
Kimura, H. and K. Shiota (2003). "Methyl-CpG-binding protein, MeCP2, is a target molecule for maintenance DNA methyltransferase, Dnmt1." J Biol Chem **278**(7): 4806-12.
Kirsh, O., J. S. Seeler, et al. (2002). "The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase." Embo J **21**(11): 2682-91.
Klan, N., S. Seuter, et al. (2003). "Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity." Biol Chem **384**(5): 777-85.
Klan, N. and D. Steinhilber (2003). "Transient transfection of the human myeloid cell line Mono Mac 6 using electroporation." Biotechniques **34**(1): 142-7.

Klose, R. J. and A. P. Bird (2006). "Genomic DNA methylation: the mark and its mediators." Trends Biochem Sci **31**(2): 89-97.

Klose, R. J., S. A. Sarraf, et al. (2005). "DNA binding selectivity of MeCP2 due to a requirement for A/T sequences adjacent to methyl-CpG." Mol Cell **19**(5): 667-78.

Kondo, E., Z. Gu, et al. (2005). "The thymine DNA glycosylase MBD4 represses transcription and is associated with methylated p16(INK4a) and hMLH1 genes." Mol Cell Biol **25**(11): 4388-96.

Koutsodontis, G., I. Tentes, et al. (2001). "Sp1 plays a critical role in the transcriptional activation of the human cyclin-dependent kinase inhibitor p21(WAF1/Cip1) gene by the p53 tumor suppressor protein." J Biol Chem **276**(31): 29116-25.

Kovacs, J. J., P. J. Murphy, et al. (2005). "HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor." Mol Cell **18**(5): 601-7.

Kramer, O. H., P. Zhu, et al. (2003). "The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2." Embo J **22**(13): 3411-20.

Kramer, R. M., E. F. Roberts, et al. (1996). "p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2." J Biol Chem **271**(44): 27723-9.

Kulkarni, S., S. Das, et al. (2002). "Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase." J Biol Chem **277**(15): 13167-74.

Kumar, A. P. and A. P. Butler (1997). "Transcription factor Sp3 antagonizes activation of the ornithine decarboxylase promoter by Sp1." Nucleic Acids Res **25**(10): 2012-9.

Kusner, E. J., C. K. Buckner, et al. (1994). "The 5-lipoxygenase inhibitors ZD2138 and ZM230487 are potent and selective inhibitors of several antigen-induced guinea-pig pulmonary responses." Eur J Pharmacol **257**(3): 285-92.

Kuwabara, K., K. Yasui, et al. (2000). "Effects of the second-generation leukotriene B(4) receptor antagonist, LY293111Na, on leukocyte infiltration and collagen-induced arthritis in mice." Eur J Pharmacol **402**(3): 275-85.

Kuzmichev, A., Y. Zhang, et al. (2002). "Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1)." Mol Cell Biol **22**(3): 835-48.

Lachner, M. and T. Jenuwein (2002). "The many faces of histone lysine methylation." Curr Opin Cell Biol **14**(3): 286-98.

Lachner, M., D. O'Carroll, et al. (2001). "Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins." Nature **410**(6824): 116-20.

Lee, H., N. Rezai-Zadeh, et al. (2004). "Negative regulation of histone deacetylase 8 activity by cyclic AMP-dependent protein kinase A." Mol Cell Biol **24**(2): 765-73.

Lee, J. A., D. C. Suh, et al. (2005). "Transcriptional activity of Sp1 is regulated by molecular interactions between the zinc finger DNA binding domain and the inhibitory domain with corepressors, and this interaction is modulated by MEK." J Biol Chem **280**(30): 28061-71.

Lee, T. H., H. C. Chang, et al. (2003). "Involvement of PKA and Sp1 in the induction of p27(Kip1) by tamoxifen." Biochem Pharmacol **66**(3): 371-7.

Leggett, R. W., S. A. Armstrong, et al. (1995). "Sp1 is phosphorylated and its DNA binding activity down-regulated upon terminal differentiation of the liver." J Biol Chem **270**(43): 25879-84.

Lehnertz, B., Y. Ueda, et al. (2003). "Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin." Curr Biol **13**(14): 1192-200.

Lemercier, C., M. P. Brocard, et al. (2002). "Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor." J Biol Chem **277**(24): 22045-52.

Lenardo, M. J. and D. Baltimore (1989). "NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control." Cell **58**(2): 227-9.
Lepley, R. A. and F. A. Fitzpatrick (1994). "5-Lipoxygenase contains a functional Src homology 3-binding motif that interacts with the Src homology 3 domain of Grb2 and cytoskeletal proteins." J Biol Chem **269**(39): 24163-8.
Lepley, R. A. and F. A. Fitzpatrick (1996). "Inhibition of mitogen-activated protein kinase kinase blocks activation and redistribution of 5-lipoxygenase in HL-60 cells." Arch Biochem Biophys **331**(1): 141-4.
Leslie, C. C. (1997). "Properties and regulation of cytosolic phospholipase A2." J Biol Chem **272**(27): 16709-12.
Lewis, J. D., R. R. Meehan, et al. (1992). "Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA." Cell **69**(6): 905-14.
Li, B., C. C. Adams, et al. (1994). "Nucleosome binding by the constitutive transcription factor Sp1." J Biol Chem **269**(10): 7756-63.
Li, H., T. Rauch, et al. (2006). "The Histone Methyltransferase SETDB1 and the DNA Methyltransferase DNMT3A Interact Directly and Localize to Promoters Silenced in Cancer Cells." J Biol Chem **281**(28): 19489-500.
Li, L., S. He, et al. (2004). "Gene regulation by Sp1 and Sp3." Biochem Cell Biol **82**(4): 460-71.
Liebich, H. M., E. Gesele, et al. (1993). "Non-enzymatic glycation of histones." Biol Mass Spectrom **22**(2): 121-3.
Lin, L. L., M. Wartmann, et al. (1993). "cPLA2 is phosphorylated and activated by MAP kinase." Cell **72**(2): 269-78.
Lin, M. C., F. Almus-Jacobs, et al. (1997). "Shear stress induction of the tissue factor gene." J Clin Invest **99**(4): 737-44.
Liu, T., S. Kuljaca, et al. (2006). "Histone deacetylase inhibitors: multifunctional anticancer agents." Cancer Treat Rev **32**(3): 157-65.
Lu, J., T. A. McKinsey, et al. (2000). "Signal-dependent activation of the MEF2 transcription factor by dissociation from histone deacetylases." Proc Natl Acad Sci U S A **97**(8): 4070-5.
Lu, J., T. A. McKinsey, et al. (2000). "Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases." Mol Cell **6**(2): 233-44.
Luger, K. (2003). "Structure and dynamic behavior of nucleosomes." Curr Opin Genet Dev **13**(2): 127-35.
Luo, J., A. Y. Nikolaev, et al. (2001). "Negative control of p53 by Sir2alpha promotes cell survival under stress." Cell **107**(2): 137-48.
Luo, M., S. M. Jones, et al. (2003). "Nuclear localization of 5-lipoxygenase as a determinant of leukotriene B4 synthetic capacity." Proc Natl Acad Sci U S A **100**(21): 12165-70.
Lupo, R., A. Breiling, et al. (2001). "Drosophila chromosome condensation proteins Topoisomerase II and Barren colocalize with Polycomb and maintain Fab-7 PRE silencing." Mol Cell **7**(1): 127-36.
Lynch, K. R., G. P. O'Neill, et al. (1999). "Characterization of the human cysteinyl leukotriene CysLT1 receptor." Nature **399**(6738): 789-93.
Maddox, J. F., M. Hachicha, et al. (1997). "Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor." J Biol Chem **272**(11): 6972-8.
Majello, B., P. De Luca, et al. (1997). "Sp3 is a bifunctional transcription regulator with modular independent activation and repression domains." J Biol Chem **272**(7): 4021-6.
Mancini, D. N., S. M. Singh, et al. (1999). "Site-specific DNA methylation in the neurofibromatosis (NF1) promoter interferes with binding of CREB and SP1 transcription factors." Oncogene **18**(28): 4108-19.

Mancini, J. A., M. Abramovitz, et al. (1993). "5-lipoxygenase-activating protein is an arachidonate binding protein." FEBS Lett **318**(3): 277-81.
Manev, H. and T. Uz (2002). "DNA hypomethylating agents 5-aza-2'-deoxycytidine and valproate increase neuronal 5-lipoxygenase mRNA." Eur J Pharmacol **445**(1-2): 149-50.
Margueron, R., P. Trojer, et al. (2005). "The key to development: interpreting the histone code?" Curr Opin Genet Dev **15**(2): 163-76.
Mariadason, J. M., G. A. Corner, et al. (2000). "Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids: comparison with trichostatin A, sulindac, and curcumin and implications for chemoprevention of colon cancer." Cancer Res **60**(16): 4561-72.
Marigo, V., D. Volpin, et al. (1994). "Identification of a TGF-beta responsive element in the human elastin promoter." Biochem Biophys Res Commun **199**(2): 1049-56.
Marks, P., R. A. Rifkind, et al. (2001). "Histone deacetylases and cancer: causes and therapies." Nat Rev Cancer **1**(3): 194-202.
Marks, P. A., V. M. Richon, et al. (2004). "Histone deacetylase inhibitors: development as cancer therapy." Novartis Found Symp **259**: 269-81; discussion 281-8.
Marmorstein, R. (2001). "Structure of histone deacetylases: insights into substrate recognition and catalysis." Structure **9**(12): 1127-33.
Marom, Z., J. H. Shelhamer, et al. (1982). "Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro." Am Rev Respir Dis **126**(3): 449-51.
Mastrangelo, I. A., A. J. Courey, et al. (1991). "DNA looping and Sp1 multimer links: a mechanism for transcriptional synergism and enhancement." Proc Natl Acad Sci U S A **88**(13): 5670-4.
Matsumoto, T., C. D. Funk, et al. (1988). "Molecular cloning and amino acid sequence of human 5-lipoxygenase." Proc Natl Acad Sci U S A **85**(1): 26-30.
Matsuyama, A., T. Shimazu, et al. (2002). "In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation." Embo J **21**(24): 6820-31.
McKinsey, T. A., C. L. Zhang, et al. (2000). "Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5." Proc Natl Acad Sci U S A **97**(26): 14400-5.
McKinsey, T. A., C. L. Zhang, et al. (2002). "MEF2: a calcium-dependent regulator of cell division, differentiation and death." Trends Biochem Sci **27**(1): 40-7.
McMahon, B. and C. Godson (2004). "Lipoxins: endogenous regulators of inflammation." Am J Physiol Renal Physiol **286**(2): F189-201.
McMahon, B., D. Mitchell, et al. (2002). "Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation." Faseb J **16**(13): 1817-9.
McMahon, B., C. Stenson, et al. (2000). "Lipoxin A4 antagonizes the mitogenic effects of leukotriene D4 in human renal mesangial cells. Differential activation of MAP kinases through distinct receptors." J Biol Chem **275**(36): 27566-75.
McMillan, R. M. and E. R. Walker (1992). "Designing therapeutically effective 5-lipoxygenase inhibitors." Trends Pharmacol Sci **13**(8): 323-30.
Meehan, R. R., J. D. Lewis, et al. (1992). "Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA." Nucleic Acids Res **20**(19): 5085-92.
Meehan, R. R., J. D. Lewis, et al. (1989). "Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs." Cell **58**(3): 499-507.

Michelassi, F., L. Landa, et al. (1982). "Leukotriene D4: a potent coronary artery vasoconstrictor associated with impaired ventricular contraction." Science **217**(4562): 841-3.
Milanini-Mongiat, J., J. Pouyssegur, et al. (2002). "Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription." J Biol Chem **277**(23): 20631-9.
Miller, D. K., J. W. Gillard, et al. (1990). "Identification and isolation of a membrane protein necessary for leukotriene production." Nature **343**(6255): 278-81.
Miska, E. A., C. Karlsson, et al. (1999). "HDAC4 deacetylase associates with and represses the MEF2 transcription factor." Embo J **18**(18): 5099-107.
Mitsiades, C. S., N. S. Mitsiades, et al. (2004). "Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications." Proc Natl Acad Sci U S A **101**(2): 540-5.
Morris, K. V., S. W. Chan, et al. (2004). "Small interfering RNA-induced transcriptional gene silencing in human cells." Science **305**(5688): 1289-92.
Mortensen, E. R., P. A. Marks, et al. (1997). "Epidermal growth factor and okadaic acid stimulate Sp1 proteolysis." J Biol Chem **272**(26): 16540-7.
Murphy, P. J., Y. Morishima, et al. (2005). "Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone." J Biol Chem **280**(40): 33792-9.
Muth, V., S. Nadaud, et al. (2001). "Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription." Embo J **20**(6): 1353-62.
Mutskov, V. and G. Felsenfeld (2004). "Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9." Embo J **23**(1): 138-49.
Nakayama, J., J. C. Rice, et al. (2001). "Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly." Science **292**(5514): 110-3.
Nan, X., F. J. Campoy, et al. (1997). "MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin." Cell **88**(4): 471-81.
Nan, X., H. H. Ng, et al. (1998). "Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex." Nature **393**(6683): 386-9.
Nathan, D., D. E. Sterner, et al. (2003). "Histone modifications: Now summoning sumoylation." Proc Natl Acad Sci U S A **100**(23): 13118-20.
Nathan, R. A. and J. P. Kemp (2001). "Efficacy of antileukotriene agents in asthma management." Ann Allergy Asthma Immunol **86**(6 Suppl 1): 9-17.
Naveh-Many, T. and H. Cedar (1982). "Topographical distribution of 5-methylcytosine in animal and plant DNA." Mol Cell Biol **2**(7): 758-62.
Ng, H. H. and A. Bird (2000). "Histone deacetylases: silencers for hire." Trends Biochem Sci **25**(3): 121-6.
Ng, H. H., P. Jeppesen, et al. (2000). "Active repression of methylated genes by the chromosomal protein MBD1." Mol Cell Biol **20**(4): 1394-406.
Ng, H. H., Y. Zhang, et al. (1999). "MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex." Nat Genet **23**(1): 58-61.
Noguchi, M., M. Miyano, et al. (1996). "Physiochemical characterization of ATP binding to human 5-lipoxygenase." Lipids **31**(4): 367-71.
Noiri, E., T. Yokomizo, et al. (2000). "An in vivo approach showing the chemotactic activity of leukotriene B(4) in acute renal ischemic-reperfusion injury." Proc Natl Acad Sci U S A **97**(2): 823-8.
Noti, J. D. (1997). "Sp3 mediates transcriptional activation of the leukocyte integrin genes CD11C and CD11B and cooperates with c-Jun to activate CD11C." J Biol Chem **272**(38): 24038-45.

Noti, J. D., B. C. Reinemann, et al. (1996). "Sp1 binds two sites in the CD11c promoter in vivo specifically in myeloid cells and cooperates with AP1 to activate transcription." Mol Cell Biol **16**(6): 2940-50.

Nowak, S. J. and V. G. Corces (2004). "Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation." Trends Genet **20**(4): 214-20.

Ochi, K., T. Yoshimoto, et al. (1983). "Arachidonate 5-lipoxygenase of guinea pig peritoneal polymorphonuclear leukocytes. Activation by adenosine 5'-triphosphate." J Biol Chem **258**(9): 5754-8.

Onishi, T., K. Yamakawa, et al. (2001). "Mitogen-activated protein kinase pathway is involved in alpha6 integrin gene expression in androgen-independent prostate cancer cells: role of proximal Sp1 consensus sequence." Biochim Biophys Acta **1538**(2-3): 218-27.

Pahl, H. L., R. J. Scheibe, et al. (1993). "The proto-oncogene PU.1 regulates expression of the myeloid-specific CD11b promoter." J Biol Chem **268**(7): 5014-20.

Palazzo, A., B. Ackerman, et al. (2003). "Cell biology: Tubulin acetylation and cell motility." Nature **421**(6920): 230.

Pan, M. R. and W. C. Hung (2002). "Nonsteroidal anti-inflammatory drugs inhibit matrix metalloproteinase-2 via suppression of the ERK/Sp1-mediated transcription." J Biol Chem **277**(36): 32775-80.

Pang, R. T., L. T. Lee, et al. (2004). "CpG methylation and transcription factors Sp1 and Sp3 regulate the expression of the human secretin receptor gene." Mol Endocrinol **18**(2): 471-83.

Park, J. H., Y. Jung, et al. (2004). "Class I histone deacetylase-selective novel synthetic inhibitors potently inhibit human tumor proliferation." Clin Cancer Res **10**(15): 5271-81.

Pascal, E. and R. Tjian (1991). "Different activation domains of Sp1 govern formation of multimers and mediate transcriptional synergism." Genes Dev **5**(9): 1646-56.

Peart, M. J., G. K. Smyth, et al. (2005). "Identification and functional significance of genes regulated by structurally different histone deacetylase inhibitors." Proc Natl Acad Sci U S A **102**(10): 3697-702.

Peters-Golden, M. and T. G. Brock (2001). "Intracellular compartmentalization of leukotriene synthesis: unexpected nuclear secrets." FEBS Lett **487**(3): 323-6.

Pflum, M. K., J. K. Tong, et al. (2001). "Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation." J Biol Chem **276**(50): 47733-41.

Phiel, C. J., F. Zhang, et al. (2001). "Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen." J Biol Chem **276**(39): 36734-41.

Piechele, G., S. Colli, et al. (1993). "5-Lipoxygenase gene expression in HL60 cells during differentiation with DMSO." Pharmacol Res **27**(1): 53-60.

Ponton, A., J. P. Thirion, et al. (1997). "Repression of the 5-lipoxygenase gene by c-myb overexpression in differentiated HL-60 cells." Prostaglandins **53**(1): 49-58.

Pore, N., S. Liu, et al. (2004). "Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism." Mol Biol Cell **15**(11): 4841-53.

Prokhortchouk, A., B. Hendrich, et al. (2001). "The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor." Genes Dev **15**(13): 1613-8.

Pugh, B. F. and R. Tjian (1990). "Mechanism of transcriptional activation by Sp1: evidence for coactivators." Cell **61**(7): 1187-97.

Qiu, C., K. Sawada, et al. (2002). "The PWWP domain of mammalian DNA methyltransferase Dnmt3b defines a new family of DNA-binding folds." Nat Struct Biol **9**(3): 217-24.
Rachelefsky, G. (1997). "Childhood asthma and allergic rhinitis: the role of leukotrienes." J Pediatr **131**(3): 348-55.
Rachez, C. and L. P. Freedman (2001). "Mediator complexes and transcription." Curr Opin Cell Biol **13**(3): 274-80.
Rafty, L. A. and L. M. Khachigian (2001). "Sp1 phosphorylation regulates inducible expression of platelet-derived growth factor B-chain gene via atypical protein kinase C-zeta." Nucleic Acids Res **29**(5): 1027-33.
Ragvin, A., H. Valvatne, et al. (2004). "Nucleosome binding by the bromodomain and PHD finger of the transcriptional cofactor p300." J Mol Biol **337**(4): 773-88.
Ray, A., H. Schatten, et al. (1999). "Activation of Sp1 and its functional co-operation with serum amyloid A-activating sequence binding factor in synoviocyte cells trigger synergistic action of interleukin-1 and interleukin-6 in serum amyloid A gene expression." J Biol Chem **274**(7): 4300-8.
Rea, S., F. Eisenhaber, et al. (2000). "Regulation of chromatin structure by site-specific histone H3 methyltransferases." Nature **406**(6796): 593-9.
Reisinger, K., R. Kaufmann, et al. (2003). "Increased Sp1 phosphorylation as a mechanism of hepatocyte growth factor (HGF/SF)-induced vascular endothelial growth factor (VEGF/VPF) transcription." J Cell Sci **116**(Pt 2): 225-38.
Riendeau, D., D. Denis, et al. (1989). "Stimulation of 5-lipoxygenase activity under conditions which promote lipid peroxidation." Biochem J **263**(2): 565-72.
Robertson, K. D., S. Ait-Si-Ali, et al. (2000). "DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters." Nat Genet **25**(3): 338-42.
Roos, M. D., K. Su, et al. (1997). "O glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions." Mol Cell Biol **17**(11): 6472-80.
Ross, S., J. L. Best, et al. (2002). "SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization." Mol Cell **10**(4): 831-42.
Rountree, M. R., K. E. Bachman, et al. (2000). "DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci." Nat Genet **25**(3): 269-77.
Rouzer, C. A., E. Rands, et al. (1988). "Characterization of cloned human leukocyte 5-lipoxygenase expressed in mammalian cells." J Biol Chem **263**(21): 10135-40.
Rouzer, C. A. and B. Samuelsson (1986). "The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase." FEBS Lett **204**(2): 293-6.
Ryu, S. and R. Tjian (1999). "Purification of transcription cofactor complex CRSP." Proc Natl Acad Sci U S A **96**(13): 7137-42.
Ryu, S., S. Zhou, et al. (1999). "The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1." Nature **397**(6718): 446-50.
Safe, S. and K. Kim (2004). "Nuclear receptor-mediated transactivation through interaction with Sp proteins." Prog Nucleic Acid Res Mol Biol **77**: 1-36.
Sala, A., M. Bolla, et al. (1996). "Release of leukotriene A4 versus leukotriene B4 from human polymorphonuclear leukocytes." J Biol Chem **271**(30): 17944-8.
Samuelsson, B. (1983). "Leukotrienes: a new class of mediators of immediate hypersensitivity reactions and inflammation." Adv Prostaglandin Thromboxane Leukot Res **11**: 1-13.
Samuelsson, B. (1983). "Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation." Science **220**(4597): 568-75.
Santini, M. P., C. Talora, et al. (2001). "Cross talk among calcineurin, Sp1/Sp3, and NFAT in control of p21(WAF1/CIP1) expression in keratinocyte differentiation." Proc Natl Acad Sci U S A **98**(17): 9575-80.

Sapetschnig, A., F. Koch, et al. (2004). "Complexity of translationally controlled transcription factor Sp3 isoform expression." J Biol Chem **279**(40): 42095-105.
Sapetschnig, A., G. Rischitor, et al. (2002). "Transcription factor Sp3 is silenced through SUMO modification by PIAS1." Embo J **21**(19): 5206-15.
Sarraf, S. A. and I. Stancheva (2004). "Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly." Mol Cell **15**(4): 595-605.
Sayers, I., S. Barton, et al. (2003). "Promoter polymorphism in the 5-lipoxygenase (ALOX5) and 5-lipoxygenase-activating protein (ALOX5AP) genes and asthma susceptibility in a Caucasian population." Clin Exp Allergy **33**(8): 1103-10.
Seigneurin-Berny, D., A. Verdel, et al. (2001). "Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways." Mol Cell Biol **21**(23): 8035-44.
Sekimata, M. and Y. Homma (2004). "Regulation of Rb gene expression by an MBD2-interacting zinc finger protein MIZF during myogenic differentiation." Biochem Biophys Res Commun **325**(3): 653-9.
Sekimata, M. and Y. Homma (2004). "Sequence-specific transcriptional repression by an MBD2-interacting zinc finger protein MIZF." Nucleic Acids Res **32**(2): 590-7.
Sekimata, M., A. Takahashi, et al. (2001). "Involvement of a novel zinc finger protein, MIZF, in transcriptional repression by interacting with a methyl-CpG-binding protein, MBD2." J Biol Chem **276**(46): 42632-8.
Sengupta, N. and E. Seto (2004). "Regulation of histone deacetylase activities." J Cell Biochem **93**(1): 57-67.
Serhan, C. N. (2002). "Lipoxins and aspirin-triggered 15-epi-lipoxin biosynthesis: an update and role in anti-inflammation and pro-resolution." Prostaglandins Other Lipid Mediat **68-69**: 433-55.
Serhan, C. N., M. Hamberg, et al. (1984). "Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes." Proc Natl Acad Sci U S A **81**(17): 5335-9.
Serhan, C. N. and M. Romano (1995). "Lipoxin biosynthesis and actions: role of the human platelet LX-synthase." J Lipid Mediat Cell Signal **12**(2-3): 293-306.
Serhan, C. N. and K. A. Sheppard (1990). "Lipoxin formation during human neutrophil-platelet interactions. Evidence for the transformation of leukotriene A4 by platelet 12-lipoxygenase in vitro." J Clin Invest **85**(3): 772-80.
Serhan, C. N., T. Takano, et al. (1999). "Lipoxin and aspirin-triggered 15-epi-lipoxin cellular interactions anti-inflammatory lipid mediators." Clin Chem Lab Med **37**(3): 299-309.
Shapiro, D. J., P. A. Sharp, et al. (1988). "A high-efficiency HeLa cell nuclear transcription extract." Dna **7**(1): 47-55.
Shirohzu, H., T. Kubota, et al. (2002). "Three novel DNMT3B mutations in Japanese patients with ICF syndrome." Am J Med Genet **112**(1): 31-7.
Sigal, E., C. S. Craik, et al. (1988). "Molecular cloning and primary structure of human 15-lipoxygenase." Biochem Biophys Res Commun **157**(2): 457-64.
Silverman, E. S. and J. M. Drazen (1999). "The biology of 5-lipoxygenase: function, structure, and regulatory mechanisms." Proc Assoc Am Physicians **111**(6): 525-36.
Silverman, E. S., J. Du, et al. (1998). "Egr-1 and Sp1 interact functionally with the 5-lipoxygenase promoter and its naturally occurring mutants." Am J Respir Cell Mol Biol **19**(2): 316-23.
Silverman, E. S., L. Le, et al. (2002). "Cloning and functional analysis of the mouse 5-lipoxygenase promoter." Am J Respir Cell Mol Biol **26**(4): 475-83.
Simonsson, M., C. H. Heldin, et al. (2005). "The balance between acetylation and deacetylation controls Smad7 stability." J Biol Chem **280**(23): 21797-803.

Singal, R. and G. D. Ginder (1999). "DNA methylation." Blood **93**(12): 4059-70.
Smale, S. T., M. C. Schmidt, et al. (1990). "Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID." Proc Natl Acad Sci U S A **87**(12): 4509-13.
Smith, W. G., A. F. Shaffer, et al. (1995). "Characterization of 5-lipoxygenase inhibitors in biochemical and functional in vivo assays." J Pharmacol Exp Ther **275**(3): 1332-8.
Song, J., H. Ugai, et al. (2001). "Independent repression of a GC-rich housekeeping gene by Sp1 and MAZ involves the same cis-elements." J Biol Chem **276**(23): 19897-904.
Song, J., H. Ugai, et al. (2001). "Two consecutive zinc fingers in Sp1 and in MAZ are essential for interactions with cis-elements." J Biol Chem **276**(32): 30429-34.
Sorg, B. L., N. Klan, et al. (2006). "Analysis of the 5-lipoxygenase promoter and characterization of a vitamin D receptor binding site." Biochim Biophys Acta.
Sowa, Y., T. Orita, et al. (1997). "Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites." Biochem Biophys Res Commun **241**(1): 142-50.
Sowa, Y., T. Orita, et al. (1999). "Sp3, but not Sp1, mediates the transcriptional activation of the p21/WAF1/Cip1 gene promoter by histone deacetylase inhibitor." Cancer Res **59**(17): 4266-70.
Spanbroek, R., R. Grabner, et al. (2003). "Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis." Proc Natl Acad Sci U S A **100**(3): 1238-43.
Spanbroek, R., H. J. Stark, et al. (1998). "5-Lipoxygenase expression in Langerhans cells of normal human epidermis." Proc Natl Acad Sci U S A **95**(2): 663-8.
Sparrow, D. B., E. A. Miska, et al. (1999). "MEF-2 function is modified by a novel co-repressor, MITR." Embo J **18**(18): 5085-98.
Spengler, M. L. and M. G. Brattain (2006). "Sumoylation inhibits cleavage of Sp1 N-terminal negative regulatory domain and inhibits Sp1-dependent transcription." J Biol Chem **281**(9): 5567-74.
Spengler, M. L., S. B. Kennett, et al. (2005). "Sumoylation of internally initiated Sp3 isoforms regulates transcriptional repression via a Trichostatin A-insensitive mechanism." Cell Signal **17**(2): 153-66.
Steinhilber, D. (1999). "5-Lipoxygenase: a target for antiinflammatory drugs revisited." Curr Med Chem **6**(1): 71-85.
Steinhilber, D., M. Brungs, et al. (1995). "The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes." J Biol Chem **270**(13): 7037-40.
Steinhilber, D., S. Hoshiko, et al. (1993). "Serum factors regulate 5-lipoxygenase activity in maturating HL60 cells." Biochim Biophys Acta **1178**(1): 1-8.
Sterner, D. E. and S. L. Berger (2000). "Acetylation of histones and transcription-related factors." Microbiol Mol Biol Rev **64**(2): 435-59.
Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-5.
Strunnikova, M., U. Schagdarsurengin, et al. (2005). "Chromatin inactivation precedes de novo DNA methylation during the progressive epigenetic silencing of the RASSF1A promoter." Mol Cell Biol **25**(10): 3923-33.
Su, K., M. D. Roos, et al. (1999). "An N-terminal region of Sp1 targets its proteasome-dependent degradation in vitro." J Biol Chem **274**(21): 15194-202.
Su, W., S. Jackson, et al. (1991). "DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1." Genes Dev **5**(5): 820-6.
Sun, J. M., H. Y. Chen, et al. (2002). "The transcriptional repressor Sp3 is associated with CK2-phosphorylated histone deacetylase 2." J Biol Chem **277**(39): 35783-6.

Suske, G. (1999). "The Sp-family of transcription factors." Gene **238**(2): 291-300.
Suzuki, S., A. Kumatori, et al. (1998). "PU.1 as an essential activator for the expression of gp91(phox) gene in human peripheral neutrophils, monocytes, and B lymphocytes." Proc Natl Acad Sci U S A **95**(11): 6085-90.
Suzuki, T., A. Kimura, et al. (2000). "Regulation of interaction of the acetyltransferase region of p300 and the DNA-binding domain of Sp1 on and through DNA binding." Genes Cells **5**(1): 29-41.
Taatjes, D. J., A. M. Naar, et al. (2002). "Structure, function, and activator-induced conformations of the CRSP coactivator." Science **295**(5557): 1058-62.
Taatjes, D. J., T. Schneider-Poetsch, et al. (2004). "Distinct conformational states of nuclear receptor-bound CRSP-Med complexes." Nat Struct Mol Biol **11**(7): 664-71.
Taatjes, D. J. and R. Tjian (2004). "Structure and function of CRSP/Med2; a promoter-selective transcriptional coactivator complex." Mol Cell **14**(5): 675-83.
Tager, A. M. and A. D. Luster (2003). "BLT1 and BLT2: the leukotriene B(4) receptors." Prostaglandins Leukot Essent Fatty Acids **69**(2-3): 123-34.
Tamaru, H. and E. U. Selker (2001). "A histone H3 methyltransferase controls DNA methylation in Neurospora crassa." Nature **414**(6861): 277-83.
Tamaru, H., X. Zhang, et al. (2003). "Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa." Nat Genet **34**(1): 75-9.
Tanese, N., B. F. Pugh, et al. (1991). "Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex." Genes Dev **5**(12A): 2212-24.
Tang, S., B. Bhatia, et al. (2004). "Evidence that Sp1 positively and Sp3 negatively regulate and androgen does not directly regulate functional tumor suppressor 15-lipoxygenase 2 (15-LOX2) gene expression in normal human prostate epithelial cells." Oncogene **23**(41): 6942-53.
Tie, F., T. Furuyama, et al. (2001). "The Drosophila Polycomb Group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3." Development **128**(2): 275-86.
Torigoe, T., H. Izumi, et al. (2005). "DNA topoisomerase II poison TAS-103 transactivates GC-box-dependent transcription via acetylation of Sp1." J Biol Chem **280**(2): 1179-85.
Tsai, S. C. and E. Seto (2002). "Regulation of histone deacetylase 2 by protein kinase CK2." J Biol Chem **277**(35): 31826-33.
Tsai, S. C., N. Valkov, et al. (2000). "Histone deacetylase interacts directly with DNA topoisomerase II." Nat Genet **26**(3): 349-53.
Tse, C., T. Sera, et al. (1998). "Disruption of higher-order folding by core histone acetylation dramatically enhances transcription of nucleosomal arrays by RNA polymerase III." Mol Cell Biol **18**(8): 4629-38.
Uhl, J., N. Klan, et al. (2002). "The 5-lipoxygenase promoter is regulated by DNA methylation." J Biol Chem **277**(6): 4374-9.
Underhill, C., M. S. Qutob, et al. (2000). "A novel nuclear receptor corepressor complex, N-CoR, contains components of the mammalian SWI/SNF complex and the corepressor KAP-1." J Biol Chem **275**(51): 40463-70.
Underwood, D. C., R. R. Osborn, et al. (1996). "Persistent airway eosinophilia after leukotriene (LT) D4 administration in the guinea pig: modulation by the LTD4 receptor antagonist, pranlukast, or an interleukin-5 monoclonal antibody." Am J Respir Crit Care Med **154**(4 Pt 1): 850-7.
Uozumi, N., K. Kume, et al. (1997). "Role of cytosolic phospholipase A2 in allergic response and parturition." Nature **390**(6660): 618-22.

Vaisanen, S., T. W. Dunlop, et al. (2005). "Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1alpha,25-Dihydroxyvitamin D3." J Mol Biol **350**(1): 65-77.

Valenzuela-Fernandez, A., S. Alvarez, et al. (2005). "Histone deacetylase 6 regulates human immunodeficiency virus type 1 infection." Mol Biol Cell **16**(11): 5445-54.

van der Vlag, J. and A. P. Otte (1999). "Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation." Nat Genet **23**(4): 474-8.

Van Lint, C., S. Emiliani, et al. (1996). "The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation." Gene Expr **5**(4-5): 245-53.

Vaziri, H., S. K. Dessain, et al. (2001). "hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase." Cell **107**(2): 149-59.

von Kries, J. P., H. Buhrmester, et al. (1991). "A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding." Cell **64**(1): 123-35.

Voo, K. S. and D. G. Skalnik (1999). "Elf-1 and PU.1 induce expression of gp91(phox) via a promoter element mutated in a subset of chronic granulomatous disease patients." Blood **93**(10): 3512-20.

Wachtershauser, A., D. Steinhilber, et al. (2000). "Expression of 5-lipoxygenase by human colorectal carcinoma Caco-2 cells during butyrate-induced cell differentiation." Biochem Biophys Res Commun **268**(3): 778-83.

Wade, P. A. (2001). "Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin." Hum Mol Genet **10**(7): 693-8.

Wade, P. A., A. Gegonne, et al. (1999). "Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation." Nat Genet **23**(1): 62-6.

Wallace, J. L., L. Carter, et al. (1994). "ML 3000 reduces gastric prostaglandin synthesis without causing mucosal injury." Eur J Pharmacol **271**(2-3): 525-31.

Waltregny, D., L. De Leval, et al. (2004). "Expression of histone deacetylase 8, a class I histone deacetylase, is restricted to cells showing smooth muscle differentiation in normal human tissues." Am J Pathol **165**(2): 553-64.

Waltregny, D., W. Glenisson, et al. (2005). "Histone deacetylase HDAC8 associates with smooth muscle alpha-actin and is essential for smooth muscle cell contractility." Faseb J **19**(8): 966-8.

Wang, C. H., Y. P. Tsao, et al. (2000). "Transcriptional repression of p21((Waf1/Cip1/Sdi1)) gene by c-jun through Sp1 site." Biochem Biophys Res Commun **270**(1): 303-10.

Wang, H., R. Cao, et al. (2001). "Purification and functional characterization of a histone H3-lysine 4-specific methyltransferase." Mol Cell **8**(6): 1207-17.

Wang, L., J. L. Brown, et al. (2004). "Hierarchical recruitment of polycomb group silencing complexes." Mol Cell **14**(5): 637-46.

Wang, L., C. Fan, et al. (2003). "Mutation of MEF2A in an inherited disorder with features of coronary artery disease." Science **302**(5650): 1578-81.

Wang, X. Q., M. L. Alfaro, et al. (2002). "Histone deacetylase inhibition results in decreased macrophage CD9 expression." Biochem Biophys Res Commun **294**(3): 660-6.

Watt, F. and P. L. Molloy (1988). "Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter." Genes Dev **2**(9): 1136-43.

Weitzel, F. and A. Wendel (1993). "Selenoenzymes regulate the activity of leukocyte 5-lipoxygenase via the peroxide tone." J Biol Chem **268**(9): 6288-92.

Wells, L., K. Vosseller, et al. (2001). "Glycosylation of nucleocytoplasmic proteins: signal transduction and O-GlcNAc." Science **291**(5512): 2376-8.

Werz, O. (2002). "5-lipoxygenase: cellular biology and molecular pharmacology." Curr Drug Targets Inflamm Allergy **1**(1): 23-44.
Werz, O., M. Brungs, et al. (1996). "Purification of transforming growth factor beta 1 from human platelets." Pharmazie **51**(11): 893-6.
Werz, O., E. Burkert, et al. (2002). "Extracellular signal-regulated kinases phosphorylate 5-lipoxygenase and stimulate 5-lipoxygenase product formation in leukocytes." Faseb J **16**(11): 1441-3.
Werz, O., J. Klemm, et al. (2000). "5-lipoxygenase is phosphorylated by p38 kinase-dependent MAPKAP kinases." Proc Natl Acad Sci U S A **97**(10): 5261-6.
Werz, O., N. Schneider, et al. (1997). "A test system for leukotriene synthesis inhibitors based on the in-vitro differentiation of the human leukemic cell lines HL-60 and Mono Mac 6." Naunyn Schmiedebergs Arch Pharmacol **356**(4): 441-5.
Werz, O. and D. Steinhilber (1996). "Selenium-dependent peroxidases suppress 5-lipoxygenase activity in B-lymphocytes and immature myeloid cells. The presence of peroxidase-insensitive 5-lipoxygenase activity in differentiated myeloid cells." Eur J Biochem **242**(1): 90-7.
Werz, O., D. Szellas, et al. (1998). "Nonredox 5-lipoxygenase inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity." Mol Pharmacol **54**(2): 445-51.
Wilborn, J., M. Bailie, et al. (1996). "Constitutive activation of 5-lipoxygenase in the lungs of patients with idiopathic pulmonary fibrosis." J Clin Invest **97**(8): 1827-36.
Wilson, A. J., D. S. Byun, et al. (2006). "Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer." J Biol Chem **281**(19): 13548-58.
Wiseman, J. S. (1989). "Alpha-secondary isotope effects in the lipoxygenase reaction." Biochemistry **28**(5): 2106-11.
Wolffe, A. P. (1999). "Architectural regulations and Hmg1." Nat Genet **22**(3): 215-7.
Wondrak, G. T., D. Cervantes-Laurean, et al. (2000). "Histone carbonylation in vivo and in vitro." Biochem J **351 Pt 3**: 769-77.
Wong, J., D. Patterton, et al. (1998). "Distinct requirements for chromatin assembly in transcriptional repression by thyroid hormone receptor and histone deacetylase." Embo J **17**(2): 520-34.
Wong, W. K., K. Chen, et al. (2003). "Decreased methylation and transcription repressor Sp3 up-regulated human monoamine oxidase (MAO) B expression during Caco-2 differentiation." J Biol Chem **278**(38): 36227-35.
Wu, X., T. M. McIntyre, et al. (2003). "Molecular characterization of the constitutive expression of the plasma platelet-activating factor acetylhydrolase gene in macrophages." Biochem J **375**(Pt 2): 351-63.
Xiao, H., T. Hasegawa, et al. (1999). "Both Sp1 and Sp3 are responsible for p21waf1 promoter activity induced by histone deacetylase inhibitor in NIH3T3 cells." J Cell Biochem **73**(3): 291-302.
Yamamoto, S., H. Suzuki, et al. (1997). "Arachidonate 12-lipoxygenases." Prog Lipid Res **36**(1): 23-41.
Yang, L., Q. Mei, et al. (2003). "An ERG (ets-related gene)-associated histone methyltransferase interacts with histone deacetylases 1/2 and transcription co-repressors mSin3A/B." Biochem J **369**(Pt 3): 651-7.
Yang, W. M., C. Inouye, et al. (1996). "Transcriptional repression by YY1 is mediated by interaction with a mammalian homolog of the yeast global regulator RPD3." Proc Natl Acad Sci U S A **93**(23): 12845-50.
Yang, W. M., S. C. Tsai, et al. (2002). "Functional domains of histone deacetylase-3." J Biol Chem **277**(11): 9447-54.

Yang, X., K. Su, et al. (2001). "O-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability." Proc Natl Acad Sci U S A **98**(12): 6611-6.

Yildirim, E., Z. Zhang, et al. (2003). "Valproate administration to mice increases histone acetylation and 5-lipoxygenase content in the hippocampus." Neurosci Lett **345**(2): 141-3.

Yokomizo, T., K. Kato, et al. (2001). "Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2." J Biol Chem **276**(15): 12454-9.

Yokomizo, T., K. Kato, et al. (2000). "A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders." J Exp Med **192**(3): 421-32.

Yoon, H. G., D. W. Chan, et al. (2003). "N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso." Mol Cell **12**(3): 723-34.

Yoshida, M., R. Furumai, et al. (2001). "Histone deacetylase as a new target for cancer chemotherapy." Cancer Chemother Pharmacol **48 Suppl 1**: S20-6.

Yoshida, M., M. Kijima, et al. (1990). "Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A." J Biol Chem **265**(28): 17174-9.

Youn, H. D., C. M. Grozinger, et al. (2000). "Calcium regulates transcriptional repression of myocyte enhancer factor 2 by histone deacetylase 4." J Biol Chem **275**(29): 22563-7.

Young, J. I., E. P. Hong, et al. (2005). "Regulation of RNA splicing by the methylation-dependent transcriptional repressor methyl-CpG binding protein 2." Proc Natl Acad Sci U S A **102**(49): 17551-8.

Yu, B., P. K. Datta, et al. (2003). "Stability of the Sp3-DNA complex is promoter-specific: Sp3 efficiently competes with Sp1 for binding to promoters containing multiple Sp-sites." Nucleic Acids Res **31**(18): 5368-76.

Zhang, C. L., T. A. McKinsey, et al. (2002). "Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy." Cell **110**(4): 479-88.

Zhang, D. E., C. J. Hetherington, et al. (1994). "Sp1 is a critical factor for the monocytic specific expression of human CD14." J Biol Chem **269**(15): 11425-34.

Zhang, W., D. E. Geiman, et al. (2000). "The gut-enriched Kruppel-like factor (Kruppel-like factor 4) mediates the transactivating effect of p53 on the p21WAF1/Cip1 promoter." J Biol Chem **275**(24): 18391-8.

Zhang, X., W. Wharton, et al. (2004). "Activation of the growth-differentiation factor 11 gene by the histone deacetylase (HDAC) inhibitor trichostatin A and repression by HDAC3." Mol Cell Biol **24**(12): 5106-18.

Zhang, Y. and M. L. Dufau (2002). "Silencing of transcription of the human luteinizing hormone receptor gene by histone deacetylase-mSin3A complex." J Biol Chem **277**(36): 33431-8.

Zhang, Y. and M. L. Dufau (2003). "Dual mechanisms of regulation of transcription of luteinizing hormone receptor gene by nuclear orphan receptors and histone deacetylase complexes." J Steroid Biochem Mol Biol **85**(2-5): 401-14.

Zhang, Y., N. Li, et al. (2003). "HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo." Embo J **22**(5): 1168-79.

Zhang, Y., H. H. Ng, et al. (1999). "Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation." Genes Dev **13**(15): 1924-35.

Zhang, Y. and D. Reinberg (2001). "Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails." Genes Dev **15**(18): 2343-60.

Zhang, Y. Y., T. Hammarberg, et al. (2000). "Analysis of a nucleotide-binding site of 5-lipoxygenase by affinity labelling: binding characteristics and amino acid sequences." Biochem J **351 Pt 3**: 697-707.

Zhang, Y. Y., B. Lind, et al. (1993). "Iron content of human 5-lipoxygenase, effects of mutations regarding conserved histidine residues." J Biol Chem **268**(4): 2535-41.

Zhang, Z., C. Q. Chen, et al. (2004). "DNA methylation as an epigenetic regulator of neural 5-lipoxygenase expression: evidence in human NT2 and NT2-N cells." J Neurochem **88**(6): 1424-30.

Zhao, S., K. Venkatasubbarao, et al. (2003). "Requirement of a specific Sp1 site for histone deacetylase-mediated repression of transforming growth factor beta Type II receptor expression in human pancreatic cancer cells." Cancer Res **63**(10): 2624-30.

Zhu, W. G., K. Srinivasan, et al. (2003). "Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter." Mol Cell Biol **23**(12): 4056-65.

10 Curriculum Vitae

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Studium

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Dishart, D., Schnur, N., Klan, N., Werz, O., Steinhilber, D., Samuelsson, B., Rådmark, O. (2005). "GC-rich sequences in the 5-lipoxygenase gene promoter are required for expression in Mono Mac 6 cells, characterization of a novel Sp1 binding site." Biochim Biophys Acta 1738(1-3): 37-47.

Schnur, N., Seuter, S., Katryniok, C., Rådmark, O., Steinhilber, D. (2006). "Proximal GC sites mediate upregulation of the 5-lipoxygenase promoter activity by the histone deacetylase inhibitor TsA." manuscript in preparation.

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“Nach dem Spiel ist vor dem Spiel“

Sepp Herberger, 1954