2 Materials and methods

2.1 Materials

2.1.1 Plasmids

pGEM-T vector	Promega, Heidelberg, Germany
pGL3-promoter vector	Promega, Heidelberg, Germany
pRL-null vector	Promega, Heidelberg, Germany

2.1.2 Antibodies

Acetyl-H3 antibodies	Biomol, Hamburg, Germany
H3-trimethyl lysine 9 antibodies	Abcam, Cambridge, UK
Sp1 antibodies	Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA
XPA antibodies	Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA

2.1.3 Biological materials

Human MTC Panel I	Clontech Laboratories, Inc., USA
TOP 10F' E. coli competent cells	Clontech Laboratories, Inc., USA
Total RNA of normal mammary gland	BD Biosciences, Erembodegem, Belgium

2.1.4 Cell medium

DMEM 1x	Biochrom AG, Berlin, Germany
Fetal calf serum	Biochrom AG, Berlin, Germany
Epith-o-ser	C-C-Pro, Neustadt, Germany
Mammary epithelial cell growth medium	MEGM; PromoCell, Heidelberg, Germany
Opti-MeM I Reduced Serum Medium	Invitrogen, Groningen, Netherlands
RPMI 1640 with glutamine	Biochrom AG, Berlin, Germany

2.1.5 Enzymes

Alkaline phosphatase, *Shrimp*	Roche Diagnostic GmbH, Mannheim, Germany
Proteinase K	Promega, Heidelberg, Germany
RNAsin, RNAse inhibitor	Promega, Heidelberg, Germany
Restriction enzymes	New England BioLabs, Beverly, USA

SssI methylase	New England BioLabs, Beverly, USA
T4 DNA ligase	Promega, Heidelberg, Germany
T4 polynucleotide kinase	New England BioLabs, Beverly, USA
Taq$^{\alpha}$ *I*	Roche Diagnostic GmbH, Mannheim, Germany

2.1.6 Equipment

Ultrasound homogenizator, Sonicator, Bandelin Sonopuls HD2070	Bandelin Electronics, Berlin, Germany
UV spectrometer, GeneQuant pro RNA/DNA Calculator	Amersham Biosciences, Freiburg, Germany
Hybridizer HB-1D	Techne Inc., Duxford, Cambridge, USA
LightCycler "Rotor Gene 2000"	Corbett Research, Sydney, Australia
Gel Dryer, Model 583	BioRad, Muenchen, Germany
Model SA gel electrophoresis unit	Invitrogen, Groningen, Netherlands
Nylon membrane, Hybond N+	Amersham Biosciences, Freiburg, Germany
PCR cycler – Perkin Elmer DNA thermal cycler (for radioactive labelling)	Perkin Elmer, Norwalk, USA
Thermocycler, Mastercycler gradient	Eppendorf, Hamburg, Germany
Power supply, Powerpak 200	BioRad, Muenchen, Germany
Power supply, Powerpak 3000	BioRad, Muenchen, Germany
Electroblotter, the Panther Semidry Electroblotter HEP3	PeqLab- Owl Separation Systems, Biotechnologie GmbH, Erlangen, Germany
UV Stratalinker 1800	Stratagene, La Jolla, CA, USA
Vacuum concentrator, model 5301	Eppendorf, Hamburg, Germany
Phosphoimager, Storm 860	Molecular Dynamics, Inc., Sannyvale, CA, USA

2.1.7 Kits

Dual-Luciferase Reporter Assay system	Promega, Heidelberg, Germany
iScript cDNA Synthesis kit	Bio-Rad, Muenchen, Germany
QIAamp DNA kit	Qiagen, Hilden, Germany
QIAfilter plasmid Maxiprep kit	Qiagen, Hilden, Germany
QIAprep spin kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction kit	Qiagen, Hilden, Germany
QuickChange XL Site-Directed Mutagenesis kit	Stratagene, La Jolla, CA, USA

Wizard DNA Clean-Up system	Promega, Heidelberg, Germany

2.1.8 Polymerases

Exo⁻ Pfu DNA polymerase	Stratagene, La Jolla, CA, USA
Expand Long Template PCR system	Roche Diagnostic GmbH, Mannheim, Germany
Fast Taq polymerase	Roche Diagnostic GmbH, Mannheim, Germany
Taq polymerase, Invitaq	InViTek, Berlin, Germany

2.1.9 Reagents

[α-^{32}P CTP]	MP Biomedicals, Co., Irvine, Ca, USA
[γ-^{32} P ATP]	MP Biomedicals, Co., Irvine, Ca, USA
2-mercaptoethanol	Sigma, Deisenhofen, Germany
5-Aza-CdR	Sigma, Deisenhofen, Germany
Ammonium acetate	Merck, Darmstadt; Germany
ATP, lithium salt	Roche Diagnostic GmbH, Mannheim, Germany
Betain	Sigma, Deisenhofen, Germany
Boric acid	Roth, Karlsruhe, Germany
Bromphenol blue	Merck, Darmstadt; Germany
BSA	Roth, Karlsruhe, Germany
Chloroform	Roth, Karlsruhe, Germany
Deoxycholate	Sigma, Deisenhofen, Germany
Dimethyl sulfate	Fluka Biochemica, Ulm, Germany
$dNTP_S$	InViTek, Berlin, Germany
DTT	Roth, Karlsruhe, Germany
EDTA	Roth, Karlsruhe, Germany
Ethanol 96%	Merck, Darmstadt; Germany
Ficoll-Plague™ Plus	Amersham Pharmacia Biotech AG, Uppsala, Sweden
Formaldehyde 37%	Roth, Karlsruhe, Germany
Formamide 99%	Serva Electrophoresis GmbH, Heidelberg, Germany
Formic acid 95%	Sigma, Deisenhofen, Germany
Glycogen	Roche Diagnostic GmbH, Mannheim, Germany
Hydrazine (64%)	Sigma, Deisenhofen, Germany

Hydroquinone	Sigma, Deisenhofen, Germany
Interleukin-2 for cell cultures	Pharma Biotechnologie Hannover, Hannover, Germany
KCl	Merck, Darmstadt, Germany
LiCl	Sigma, Deisenhofen, Germany
Lipofectamine 2000	Invitrogen, Groningen, Netherlands
$MgCl_2$	InViTek, Berlin, Germany
Na-cacodylate	Sigma, Deisenhofen, Germany
NaOH	Merck, Darmstadt, Germany
NP-40	Fluka Biochemica, Ulm, Germany
PBS 1x	Invitrogen, Groningen, Netherlands
Phenol	Merck, Darmstadt; Germany
Phytohemagglutinin	Biochrom AG, Berlin, Germany
Penicillin/streptomycin	Biochrom AG, Berlin, Germany
Piperidine 99%	Sigma, Deisenhofen, Germany
PMSF	Sigma, Deisenhofen, Germany
Protease inhibitor coctail tablets, Complete Mini	Roche Diagnostic GmbH, Mannheim, Germany
S-adenosylmethionine	New England BioLabs, Beverly, USA
Salmon sperm DNA/protein A agarose	Upstate, Charlottesville, USA
Salmon Sperm DNA	Sigma, Deisenhofen, Germany
SDS	Roth, Karlsruhe, Germany
Sephadex G-50	Pharmacia Biotech AB, Uppsala, Sweden
Sodium acetate	Merck, Darmstadt; Germany
Sodium bisulfite	Sigma, Deisenhofen, Germany
Sucrose	Merck, Darmstadt; Germany
Sybr™ Green I	BioWhittaker, Belgium
TBE 1x	100 mM tris, 100 mM boric acid, 2 mM EDTA pH 8.0
Tris	Invitrogen, Groningen, Netherlands
Triton X-100	Roth, Karlsruhe, Germany
Trizol reagent	Invitrogen, Groningen, Netherlands
tRNA *E.coli*	Roche Diagnostic GmbH, Mannheim, Germany
Urea	Roth, Karlsruhe, Germany
Water, Ampuwa	Fresenius Kabi, Bad Homburg, Germany
Xylene cyanole	Merck, Darmstadt, Germany

2.1.10 Cell cultures

Four breast cancer cell lines (T47D, MDA-MB-231, MCF7 and ZR75-1), HeLa S3 and the A549 lung cancer cell line were obtained from American Type Culture Collection and cultured in the recommended medium. Human mammary epithelial cells (HMEC-184 and HMEC-48R) were obtained from reduction mammoplasty and provided by Martha Stampfer (Lawrence Berkeley Laboratories, Berkeley CA, USA). Additional mammary epithelial cells (HMEC-219 and HMEC-1001) were purchased from Clonetics (Clonetics, BioWhittaker, Verviers, Belgium) or isolated from normal mammary epithelium (NME), which was obtained from healthy women of the Universtätsfrauenklinik Halle by reduction mammoplasty and cultivated in epith-o-ser up to a passage 4 (HMEC-141). Clonetics cell lines (HMEC-219 and HMEC-1001) were available only at post-stasis stadium and sub-cultured until they reached agonescence. HMECs were cultivated in serum free mammary epithelial cell growth medium (Epith-o-ser) to no more than 80% confluence. Cells were grown at 37°C in 5% CO_2 and medium was changed every 3 days. To determine the population doublings, the cells were counted at each passage.

2.1.11 Cultivation of the peripheral blood mononuclear cells (PBMC)

For cultivation, mononuclear cells from blood were isolated from healthy person according to the following protocol. Blood was collected using syringe containing Li-heparin (Sarstedt AG & Co., Nümbrecht, Germany). Five ml of blood was diluted with 5 ml of RPMI medium. Further, 10 ml of blood mix was overlayered onto 3 ml Ficoll-Plague^{TM} Plus and spun without a brake for 30 min at 1400 rpm at 10°C. Interphase containing PBMC was collected and washed twice with PBS. Isolated cells were incubated for 5 h at 37°C in 5% CO_2 in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Further, the non-adherent cells were transferred into new flask and cultivated in RPMI medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 4.8 µg/ml phytohemagglutinin at 37°C in 5% CO_2. Separation of adherent cells from non-adherent cells was performed to remove monocytes; therefore cultivated cells were mainly lymphocytes. After 72 h, medium was changed to RPMI supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 25 units/ml interleukin-2. After 4 days of cell incubation at 37°C in

5% CO_2, cells were spun for 5 min at 1500 rpm at RT, washed with PBS and used for DNA and RNA isolations.

2.1.12 Oligonucleotides

All primers were generated by Oligo 4.0 software (National Bioscience, Inc. Plymouth, USA) and produced desalted by Invitrogen (Invitrogen, Groningen, Netherlands). Linker primers for LM-PCR were produced and purified by high pressure liquid chromatography by Qiagen (Qiagen, Hilden, Germany).

2.2 Methods

2.2.1 Treatment of cells with 5-aza-2'-deoxycytidine (5-Aza-CdR)

For expressional analysis by RT-PCR (see below chapters 2.2.8 and 2.2.9), cells of HMEC-184 passage 13 and the breast carcinomas (T47D, MDA-MB-231, MCF7 and ZR75-1) were grown for 4 days in the presence or absence of 10 µM 5-Aza-CdR.

2.2.2 DNA isolation from tissues and cultured cells

Genomic DNA was extracted according to Sambrook and colleagues (Sambrook *et al.*, 1989). Briefly, DNA was isolated by cell lysis with *Proteinase K* (0.375 mg/ml) digestion at 55°C for 6 – 8 h and by extraction with phenol/chloroform. After precipitation with EtOH, DNA was dissolved in H_2O and quantified by UV spectrometry.

2.2.3 DNA isolation from blood

DNA from blood was isolated using *QIAamp DNA* kit according to the manufacturer's instructions (Qiagen), eluated with water and quantified by UV spectrometry.

2.2.4 I*n vitro* methylation of the HeLa DNA

For *in vitro* methylation, 20 µg of the HeLa DNA was treated with 60 units of *SssI* methylase (New England BioLabs) at 37°C in 200 µl of reaction mix containing 160 µM S-adenosylmethionine. After 4 h of incubation, an S-adenosylmethionine was added to a final concentration of 320 µM and the incubation was continued overnight. Further, the DNA was purified with phenol/chloroform, precipitated and dissolved at 1 µg/µl in H_2O.

2.2.5 Bisulfite treatment of the DNA

Bisulfite treatment of the DNA was carried out according to the protocol of Clark and colleagues (Clark *et al.*, 1994). Two µg of genomic DNA was denatured by adding NaOH to a final concentration of 0.3 M and incubating at 37°C for 15 min. Sodium bisulfite, to a final concentration of 3.2 M, and hydroquinone, to a final concentration

of 0.5mM, were added to the denaturated DNA; samples were carefully mixed and incubated at 55°C for 16 h. The modified DNA was purified through the *Wizard DNA Clean-Up* system (Promega). NaOH, to a final concentration of 0.3 M, was added and DNA was incubated for 10 min at 37°C. After adding of 2 µg of glycogen and one volume of 7.5 M ammonium acetate, the bisulfite-treated DNA was precipitated and dissolved in 100 µl of H_2O.

2.2.6 Methylation specific PCR (MSP)

DNA methylation pattern of the *p16* CpG island was determined by MSP using primers pairs *p16*-M and *p16*-U (Table 2-1) and the conditions as described by Herman and colleagues (Herman *et al.*, 1996). Briefly, 100 ng of bisulfite-treated genomic DNA was amplified in 25 µl of reaction volume using the following final concentrations: 1x *Taq* buffer, 2 units of *Taq* polymerase (InViTek), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 4% formamide and 10 pmoles of specific primers to methylated or unmethylated DNA (Table 2-1). After an initial denaturation step at 95°C for 2 min, the cycling conditions were as follows: 92°C for 30 s, annealing temperature (Tan) (Table 2-1) for 30 s and 72°C for 30 s for 40 cycles. The last elongation step was performed at 72°C for 5 min. To prevent degradation of primers and template by 3'→5' exonuclease activity of *Taq* polymerase at low temperature (http://www1.qiagen.com/products/pcr/ proofstartsystem /default.aspx), the polymerase was added to PCR mix at 65°C (Hot Start). PCR products were resolved on a 2% TBE agarose gel.

Table 2-1. P16 gene: primers and PCR conditions for MSP

	Primers (5'→3')	T_{an}, ºC	Size of PCR product, bp
P16-M[1]	U: TTATTAGAGGGTGGGGCGGATCGC L: GACCCCGAACCGCGACCGTAA	65	150
P16-U[2]	U: TTATTAGAGGGTGGGGTGGATTGT L: CAACCCCAAACCACAACCATAA	60	148

[1]Primer pair for amplification of the methylated DNA. [2]Primer pair for amplification of the unmethylated DNA.

2.2.7 Methylation analysis of the *RASSF1* locus

2.2.7.1 Combined bisulfite restriction analysis (COBRA)

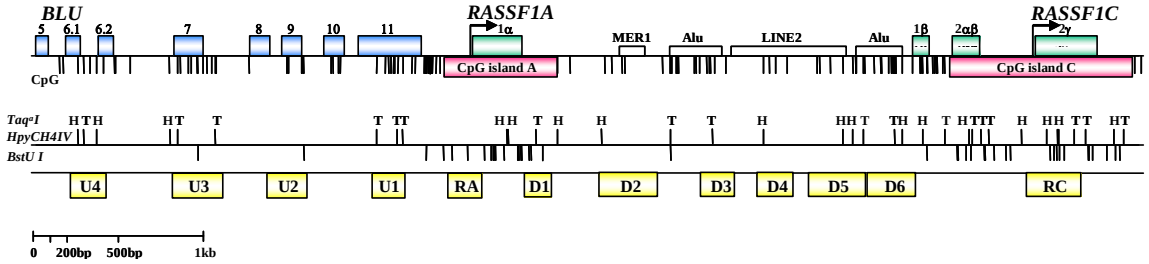

Figure 2-1. Map of the *RASSF1* locus. The arrows indicate the transcriptional start sites of the *RASSF1* isoforms. The *RASSF1* and *BLU* exons are marked by the green and blue boxes, respectively. Red boxes represent the *RASSF1A* and *RASSF1C* CpG islands. The localizations of the CpG islands were determined by CpGplot (http://www.ebi.ac.uk). Obs/Exp. sets the minimum average observed to expected ratio of C plus G to CpG in a set of 10 windows that are required before a CpG island is reported. Additional DNA elements (*Alu*, *MER1 and LINE2*) were located by RepeatMasker (ftp.genome.washington.edu/RM/RepeatMasker.html) and marked by white boxes. CpGs are marked by bars. The coding DNA strand was deaminated *in silicio*. The indicated 12 PCR fragments (yellow boxes) of the 7 kb locus were analyzed by COBRA. The restriction cutting sites of CpG containing sequence are shown (*HpyCH4IV*, *Taq*$^{\alpha}$*I* and *BstUI*).

The DNA methylation status of the *RASSF1* locus was determined by COBRA (Xiong and Laird, 1997). For this analysis, the primers for 12 fragments (U4, U3, U2, U1, RA, D1, D2, D3, D4, D5 and D6) of the *RASSF1* locus were generated (Figure 2-1 and Table 2-2).

For the first PCR of COBRA, 100 ng of bisulfite-treated genomic DNA was amplified in 25 µl of the reaction volume using the following final concentrations: 1x *Taq* buffer, 2 units of *Taq* polymerase (InViTek), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, formamide (Table 2-2) and 10 pmoles of each primer (Table 2-2). After an initial denaturation step at 95°C for 5 min, the cycling conditions were as follows: 95°C for 20 s, T_{an} (Table 2-2) for 30 s and 72°C for 50 s (number of cycles is shown in Table 2-2). The final elongation step was performed at 72°C for 5 min.

For the nested PCR of COBRA, 5 µl of the first PCR products was amplified in 50 µl of the reaction volume using the following final concentrations: 1x *Taq* buffer, 4 units of *Taq* polymerase (InViTek), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, formamide (Table 2-2) and 10 pmoles of each primer (Table 2-2). After an initial denaturation step at 95°C for 5 min, the cycling conditions were as follows: 95°C for 20 s, T_{an} (Table 2-2) for 30 s and 72°C for 40 s (number of cycles is shown in Table 2-2). The final elongation step was performed at 72°C for 5 min.

Twenty to fifty ng of the nested PCR products was digested with 2 units of restriction enzyme in 10 µl of reaction mix as described in Table 2-2. PCR product of *in vitro* methylated HeLa DNA was used as a control for complete digestion. The restriction products were resolved on a 2% TBE - agarose gel and analyzed by ImageJ 1.28V software (NIH, USA).

2.2.7.2 Bisulfite sequencing

Amplified bisulfite PCR products were subcloned into the *pGEM-T* vector according to the manufacturer's instructions (Promega). Briefly, 2 µl of PCR products was ligated with 25 ng of *pGEM-T* vector using 1.5 units of *T4 DNA* ligase (Promega) in 10 µl of reaction mix for 4 h at RT. After ligation, the DNA was transformed in *TOP 10F' E. coli* competent cells according to the manufacturer's instructions (Clontech). After "Blue/White" screening, the plasmid DNA from 5 white clones was isolated by the *QIAprep spin* kit (Qiagen) and sequenced by automated DNA sequencers (SeqLab, Göttingen, Germany) using T7B (5' TAATACGACTCACTATAGGG) and M13RL (5' GGAAACAGCTATGACCATGAT) primers.

2.2.8 RNA isolation and reverse transcription

Total RNA was extracted from cells using the Trizol reagent according to the manufacturer's instructions (Invitrogen), dissolved in water solution of *RNAsin* (1u/µl) and quantified by UV spectrometry.

cDNA was synthesized from 0.5 µg of RNA using the *iScript cDNA Synthesis* kit (BioRad) in a total volume of 20 µl which consisted of 4 µl of 5x iScript reaction mixture, 1 µl of reverse transcription mix and RNA in a nuclease-free water. cDNA synthesis conditions were as follows: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C. For real time PCR experiments, ready cDNA was diluted thrice in water.

For expression analysis in normal human mammary gland, total RNA from this tissue was obtained from Clontech. To analyze gene expression in different human tissues (heart, whole brain, placenta, lung, liver, skeletal muscle and kidney), ready cDNA from the Human MTC panel I was utilized (Clontech).

Table 2-2. COBRA: PCR and restriction conditions

	First PCR			Nested PCR				Restriction	
	Primers (5'→3')		T_{an}, ºC (cycles[1]; FA[2],%)	Primer (5'→3')		T_{an}, ºC (cycles[1]; FA[2],%)	Size[3], bp	Restriction enzyme	Product
RC	CU	GTTTTTTGTGGTAGGTGGGGTTTG	57 (25; 0)	CU2	GGTGGGGTTTGTGAGTGGAGTTT	57 (40; 0)	311	*Hpy CH4*	4, 66, 117, 124
	CL	AATCCRAATCCTCTTAACTACAATAACCAC		CL2	ACTACTCRTCRTACTACTCCAAATCATTTC				
D6	5U	GGGGTGAGAATGGAGAATGGAATAT	57 (25; 2)	5U	GGGGTGAGAATGGAGAATGGAATAT	57 (40; 2)	282	*Bstu I*	142, 184
	5L	AAAACCACAAACAAAAAAACCTACTCAAC		6L2	CCAAACTAATCTCAAACTCCTAATCTCA				
D5	5U	GGGGTGAGAATGGAGAATGGAATAT	57 (25; 2)	5U2	GGGTGGATTATTTGAGATTAGGAGTTT	55 (40; 2)	368	*Hpy CH4*	58, 106, 204
	5L	AAAACCACAAACAAAAAAACCTACTCAAC		5L	AAAACCACAAACAAAAAAACCTACTCAAC				
D4	4U	GTGAGGTTGAAGAAAAGGGAATTAAATTT	58 (36; 2)				245	*Hpy CH4*	49, 196
	4L	CCCCCTACAACTCCTACTCAACTCCTT							
D3	2U	TTTTTTTTGATTTAGTGAATTAGATGTTAAA	54 (20; 2)	3U2	GGGGGGAGTATAAAGTTGTGATAGAAT	57 (40; 2)	256	*Taq$^{\alpha}$ I*	48, 208
	2L	CTATATTCAAACAATTCTCCCACCTCA		2L	CTATATTCAAACAATTCTCCCACCTCA				
D2	2U	TTTTTTTTGATTTAGTGAATTAGATGTTAAA	54 (20; 2)	2U	TTTTTTTTGATTTAGTGAATTAGATGTTAAA	56 (40; 2)	380	*Hpy CH4*	39, 341
	2L	CTATATTCAAACAATTCTCCCACCTCA		2L2	CCCCCCAACTAAATTTATAATATCCTC				
D1	1U	GAGGGGAAGGGGTAGTTAAGGGGTA	57 (25; 2)	1U2	GGAAGGGGTAGTTAAGGGGTAG	54 (40; 2)	185	*Bstu I*	12, 32, 72, 79
	1L	TTCCCTTCACCCTAAAAATTCTAAAAAA		1L2	AACAACCACCTCTACTCATCTATAACCC				
RA	AU	GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT	55 (20; 0)	AU	GTTTTGGTAGTTTAATGAGTTTAGGTTTTTT	54 (40; 6)	184	*Taq$^{\alpha}$ I*	21, 82, 92
	AL	ACCCTCTTCCTCTAACACAATAAAACTAACC		AL2	CCCCACAATCCCTACACCCAAAT				
U1	u1U	TGGGAAAAGTATGGAAAGATTTGTGTT	57 (25; 2)	u12	TAAATGAGGGTTGTAGTTGTTGAGGGT	57 (35; 2)	237	*Taq$^{\alpha}$ I*	27, 55, 122
	u1L	TACTAAAAAAAAAAAAATCCCCACATCC		u1L2	TAAAACAACACACTTAACCTACCCACTAAA				
U2	u2U	TGGTTTATTTGTAGAGTTTTTTGGTTTATTTG	59 (25; 2)	u2U2	GAAGGATTTGGTGTTGGAATAGGTAGG	59 (40; 2)	254	*Bstu I*	33, 221
	u2L	CCACCCACATCCATACCTCCTCCTACA		u2L2	CCTCCCTACCATTTCCACAAACCT				
U3	u3U	GTGTGTTGGTTTTTTTTTTTTAGGTAAGTTG	58 (25; 2)	u3U	GTGTGTTGGTTTTTTTTTTTAGGTAAGTTG	57 (35; 2)	331	*Bstu I*	151, 180
	u3L	AAAATACCTATAAAAACCCATATCCCACTAA		u3L2	ATCACCTAAAACCCAAAAACTAAAAAAAA			*Taq$^{\alpha}$ I*	37, 79, 215
U4	u4U	GTGAATATTGTGTGATTTTTTAGGAGTTGTA	56 (25; 2)	u4U2	TTGATGGAATTTGAGATTGTATTGAAGG	57 (35; 2)	283	*Hpy CH4*	41, 132, 110
	u4L	AATAAAAAAAAACCCTACCTCCTTCCC		u4L	AATAAAAAAAAACCCTACCTCCTTCCC			*Taq$^{\alpha}$ I*	77, 206

[1]Number of cycles. [2]Formamide concentration in PCR mix. [3]Size of PCR product

2.2.9 Quantification of transcription level by real time RT-PCR

2.2.9.1 Real time PCR

Real time PCR was carried out in a LightCycler "Rotor Gene 2000" using *Sybr*^{TM} *green I* detection. Reactions were set up in 25 µl of volume using the following final concentrations: 1x *Taq* buffer (1.5 mM $MgCl_2$), 1 unit of *Fast Taq* polymerase (Roche), 0.25 mM dNTPs each, 10 pmoles of each primer (Table 2-3), 0.2x *Sybr*^{TM} *Green I* (BioWhittaker), formamide (Table 2-3) and 2 µl of cDNA. After an initial denaturation step at 95°C for 5 min, the cycling conditions were as follows: 95°C for 20 s, T_{an} (Table 2-3) for 30 s, 72°C for 30 s and a fluorescence measurement after 15 s of the appropriate measurement temperature (Tm) (Table 2-3) for 50 cycles. The final elongation step was performed at 72°C for 5 min. The melting temperature of the PCR products were analyzed by a fluorescence measurement at every 1°C step after 5 s from 70°C up to 99°C. All measurements were independently repeated three times with several cDNA preparations. The amplification of PCR products was verified using melting curve option and subsequent gel electrophoresis using 2% TBE agarose gel.

Table 2-3. RT-PCR: Primers and conditions.

	Primers (5'→3')	T_{an}, ºC	Tm, ºC	FA[1], %	Size of PCR product, bp
RASSF1A	U: GGCTGGGAACCCGCGGTG L: TCCTGCAAGGAGGGTGGCTTCT	60	83	2	239
RASSF1C	U: AGCTCGAGCAGTACTTCACCGC L: TCCTGCAAGGAGGGTGGCTTCT	64	83	2	261
p16	U: GCTGCCCAACGCACCGAATAGT L: CTCCCGGGCAGCGTCGTG	60	88	2	157

[1]Formamide concentration.

Data analysis was performed by Rotor Gene Software version 4.6 using comparative method (see chapter 2.2.9.3). In experiments with cDNA from different tissues, the *RASSF1A* and *RASSF1C* expression levels were plotted relative to the transcription levels in the pancreas (=100%). For analysis of the *RASSF1A* and *RASSF1C* expressions in PBMC, HeLa, HF, mammary gland, HMECs and breast cancer cell lines, the expression levels were plotted relative to transcription levels in HF (=100%). To verify the *RASSF1A* and *RASSF1C* expressions in HMEC-184 after 5-Aza-CdR

treatment, the expression levels were plotted relative to transcription level in untreated cells (=100%). For analysis of the *p16*^{INK4} transcription in HeLa, HF, HMECs, A549, T47D, ZR75-1, MCF7 and MDA-MB-231, the expression levels of *p16* ^{INK4} were plotted relative to expression levels in HeLa (=100%).

2.2.9.2 Analysis of melting curve

Real time PCR method is based on the quantification of DNA amount at every cycle. A special fluorophor *Sybr*^{TM} *green I* is utilized for this analysis. *Sybr*^{TM} *green I* is sensitive to low amount of DNA in contrast to ethidium bromide (Schneeberger *et al.*, 1995). The fluorescence of double stranded DNA and *Sybr*^{TM} *green I* is at least eleven folds higher than with single stranded DNA (Zipper *et al.*, 2004).

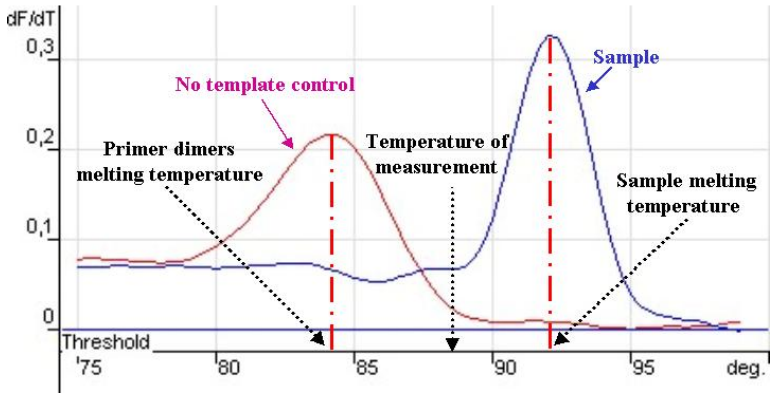

Figure 2-2. Melting data of double stranded DNA. Melting data of a sample (blue line) and of a non template control (pink line) are present as a first derivation of fluorescence level (dF/dT) versus temperature. The peaks of the graph represent the melting temperature of probes. The optimal temperature for the fluorescence measurement of the specific PCR product is indicated.

In real time PCR experiments with *Sybr*^{TM} *green I*, the measurement of DNA amount is performed after every elongation step at specific temperature for every primer pair. This temperature is determined using melting curve analysis (Figure 2-2). To perform this analysis, the fluorescence measurement of PCR products at every 1°C step after 5 s from 70°C up to 99°C takes place as a last step of real time PCR. Further, these raw data are presented as the first derivation of fluorescence level (dF/dT) versus temperature (Figure 2-2) (Rotor Gene Software version 4.6). The peaks of this derivation present temperature when maximal changing of fluorescence occurs during melting (Figure 2-2). Using this derivation, it is possible to identify the temperature

(Tm) when primer dimers are already melting and PCR products are double stranded (Figure 2-2). At this Tm, the amount of DNA is measured at every cycle of PCR.

2.2.9.3 Comparative method

Comparative quantification of gene expression was performed using the Rotor Gene Software version 4.6 in comparative quantification mode. This quantification is a real time PCR analysis technique, which allows the estimation of relative expressions of genes without requiring a standard curve (Herrmann and Corbett_Research, 2002). Comparative quantitation is used to compare a certain sample to any other in the same experiment (Rotor Gene Software version 4.6). The method evaluates the amplification of each sample, and then calculates an average with error coefficient. The average of the amplification is required to compare the reaction of samples by analysis the relative Take-Off points of each sample (Herrmann and Corbett_Research, 2002). To calculate the Take-off point, the second derivative of the raw data of fluorescence measurements at every cycle is taken (Rotor Gene Software version 4.6) (Figure 2-3). A peak of this derivative is a time point when the reaction increases most rapidly. The peak occurs shortly after Take-off of the reaction (Figure 2-3). The Take-Off is the last point before which the fluorescence signal emerges from the background (Herrmann and Corbett_Research, 2002). In different experiments, different probes were used as standard reaction and the DNA (cDNA) amount in these samples were defined as 100%. The comparative concentrations were calculated only for probes with amplification rate from 1.6 up to 2.0. Variabilities of reactions were about 5%.

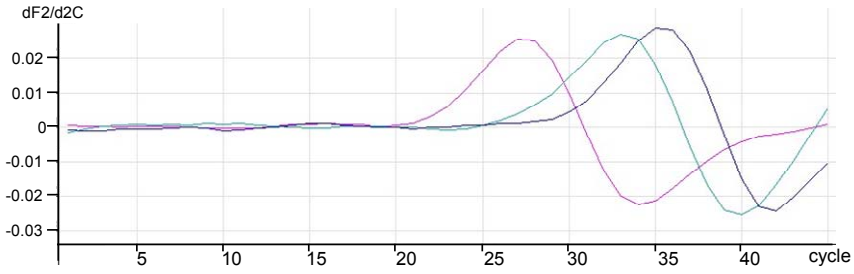

Figure 2-3. The second derivative of the raw data. Lines on the graph are the second derivative of the raw data of the reactions with cDNA of HF (pink), HMEC-48R p15 (green) and HMEC-48R p16 (violet). The peaks of this function determinate a time when reaction increases most rapidly. Take-off reactions are 23.2 for HF and 28.4 for 48R p16, 31.1 for 48R p17.

2.2.10 Luciferase assay

2.2.10.1 Amplification of the *RASSF1A* and *RASSF1C* promoter fragments

To clone fragments of the *RASSF1A* and *RASSF1C* promoters (Figure 2-4), 50 ng of the human fibroblasts genomic DNA was amplified in 50 µl of reaction volume using the following final concentrations: 1x *Taq* buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.5 M betain and 3.75 units of proof reading *Taq* polymerase from *Expand Long Template PCR* system (Roche) and 20 pmoles of each primers (Table 2-4). After an initial denaturation step at 94°C for 2 min, the cycling conditions were as follows: 94°C for 20 s, T_{an} (Table 2-4) for 30 s and 68°C for 2 min for 30 cycles. The final elongation step was at 68°C for 7 min. All primers harbored a new *EcoRI* site (5'GAATTC) (Table 2-4).

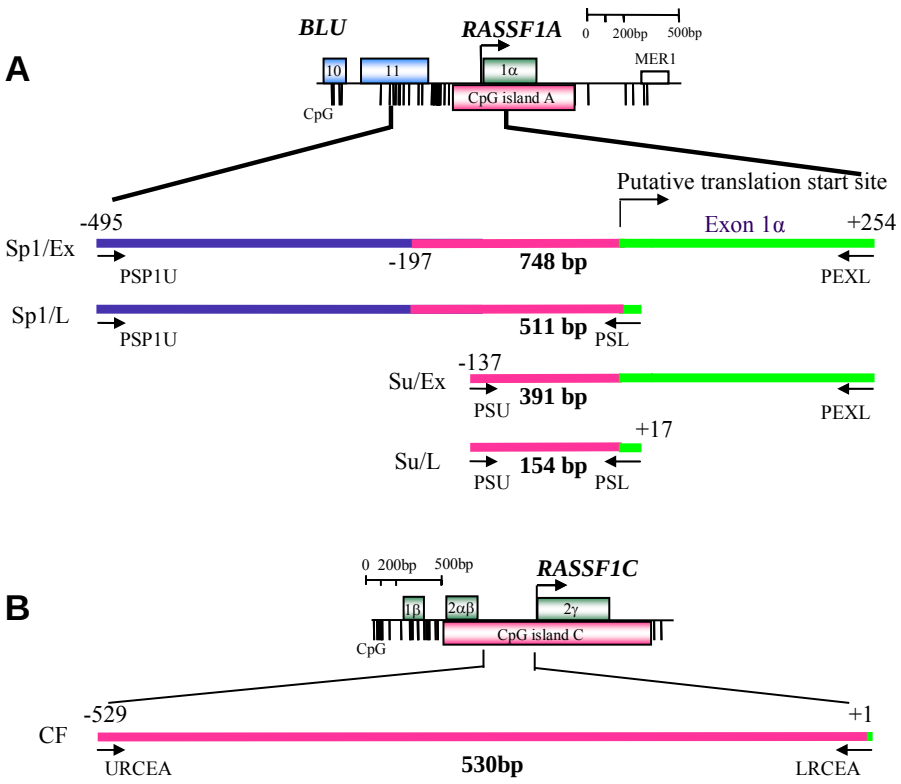

Figure 2-4. Amplification of fragments of the *RASSF1A* and *RASSF1C* promoters. A. A map of the *RASSF1A* promoter region is shown. For further details see Figure 2-1. The four DNA fragments of the *RASSF1A* CpG island were amplified using several primer combinations (Table 2-4). Green line indicates a sequence of the exon 1α. The red line represents a sequence of the *RASSF1A* CpG island fragment located upstream from the putative translation start. Blue line shows a sequence of the putative *RASSF1A* promoter fragment located upstream from the *RASSF1A* CpG island. **B.** A map of the *RASSF1C* promoter region is shown. DNA fragment of the *RASSF1C* CpG island was amplified using URCEA and LRCEA primers (Table 2-4). Green line indicates a sequence of the exon 2γ of *RASSF1C*. Red line represents a sequence the *RASSF1C* CpG island fragment located upstream from the putative *RASSF1C* translation start.

Table 2-4. Conditions for amplification of the *RASSF1A* and *RASSF1C* promoter fragments

	Fragment	Primers	T_{an}, ºC	PCR/fragment[1]	Primers (5'→3')
Prom.A	Sp1/L	PSP1U+ PSL	64	535/512 bp	**PSP1U:**GAATTC[2]ATTAATTGGAGAGCAGAGCGGGCGGTA **PSU:**GAATTC[2]ATTAATCGCGGCTCTCCTCAGCTCCTTC **PSL:**GAATTC[2]***ACCGGT***[3]TCAGGCTCCCCCGACATGGC **PEXL:**GAATTC[2]***ACCGGT***[3]TCACGCGCGCACTGCAGGC
	Sp1/Ex	PSP1U+ PEXL	64	772/749 bp	
	Su/L	PSU+ PEXL	64	178/154 bp	
	Su/Ex	PSU+ PSL	64	415/391 bp	
Prom.C	CF	URCEA+LRCEA	65	537/530 bp	**URCEA:** GGAATTC[2]TCGAGGGCTGCCTGGGTG **LRCEA:** GGAATTC[2]TAGCCGTACCCGCCCGTCCC

[1]Size of PCR product / size of the *RASSF1* fragment in PCR product. [2]*EcoRI* restriction site (5'GAATTC). [3]*AgeI* restriction site (5'ACCGGT).

2.2.10.2 Cloning of the *RASSF1* promoter fragments into the *pGEM-T* vector

PCR products were gel purified using the *QIAquick Gel Extraction* kit (Qiagen) and cloned into the *pGEM-T* vector (Promega) using for transformation *TOP 10F' E. coli* competent cells (Clontech). Ligation and transformation were performed according to the manufacturer's instructions (Promega, Clontech). After "Blue/White" screening, the plasmid DNA from 5 white clones was isolated by the *QIAprep spin* kit (Qiagen) and dissolved in 50 µl of elution buffer. To determine the presence of the PCR products in the *pGEM-T* vectors, 4 µl of plasmid DNA from each clone was analyzed by restriction analysis with 10 units of *EcoRI* (New England BioLabs) in 10 µl of reaction mix at 37ºC for 2 h. The restriction products were resolved on a 1% TBE agarose gel. The sequences of plasmids were verified (see chapter 2.2.10.3).

30 µg of the verified plasmid was treated with 80 units of *EcoRI* (New England BioLabs) in 100 µl of reaction mix at 37ºC for 4 h. After resolving the restriction products on a 1% TBE agarose gel, promoter fragments were isolated using the *QIAquick Gel Extraction* kit according to the manufacturer's instructions (Qiagen).

2.2.10.3 Sequencing

DNA sequence analysis was carried out by automated DNA sequencers (SeqLab, Göttingen, Germany) using T7B and M13RL primers (see chapter 2.2.7.2).

2.2.10.4 Cloning of the *RASSF1* promoter fragments in the *pRL-null* vector

Five µl of *pRL-null* vector (Promega) was treated with 30 units of *EcoRI* (New England BioLabs) in 100 µl of reaction mix at 37ºC for 4 h. The plasmid DNA was

precipitated and dissolved in 50 µl of H_20. Two µl of digested DNA was used as negative control for the dephosphorylation reaction; whereas 48 µl of DNA was treated at 37ºC for 15 min with 11 units of *Shrimp* alkaline phosphatase (Roche) in 100 µl of reaction mix. After precipitation DNA was dissolved in 45 µl of H_20 and then used for ligation.

One µl of the dephosphorylated *pRL-null* vector was ligated with 2 µl of the *EcoRI* digested *RASSF1* promoter fragment using 1.5 units of *T4 DNA* ligase (Promega) in a 10 µl reaction mix for 4 h at RT. After ligation, the DNA was transformed in *TOP 10F' E. coli* competent cells according to the manufacturer's instructions (Clontech). DNA of 5 clones was isolated by the *QIAprep spin* kit according to the manufacturer's instructions (Qiagen), treated with diagnostic restriction enzymes to determine the orientation of the insert (Table 2-5) and analyzed by sequencing (see chapter 2.2.10.3).

Table 2-5. Analysis of orientation of the *RASSF1* promoter fragments in the *pRL-null* vector

Construct	Restriction enzyme	Right orientation[1]	Wrong orientation[2]
CF-pRLnull	*XmaI*	110 bp, 278 bp, 3.5 kb	156 bp, 278 bp, 3.4 kb
	XhoI	53 bp, 3.8 kb	584 bp, 3.3 kb
Sp1/L-pRLnull	*AgeI, HindIII*	570 bp, 3.3 kb	49 bp, 3.8 kb
Sp1/Ex-pRLnull	*AgeI, HindIII*	807 bp, 3.3 kb	49 bp, 4 kb
Su/L-pRLnull	*AgeI, HindIII*	213 bp, 3.3 kb	49 bp, 3.5 kb
	BamHI	1.6 kb, 1.9 kb	1.5 kb, 2 kb
Su/Ex-pRLnull	*AgeI, HindIII*	450 bp, 3.3 kb	49 bp, 3.7 kb
	BamHI	1.8 kb, 1.9 kb	1.5 kb, 2.3 kb

[1]Sizes of restriction products of constructs containing the right orientated promoter fragment. [2]Sizes of restriction products of constructs containing the wrong orientated promoter fragment.

2.2.10.5 *In vitro* methylation of the Sp1/L-pRLnull construct

Twenty µg of Sp1/L-pRLnull DNA was treated with 60 units of *SssI* methylase (New England BioLabs) and 160 µM S-adenosylmethionine 37°C overnight in 200 µl of reaction mix. In parallel, a mock methylation was performed with 20 µg of Sp1/L-pRLnull plasmid DNA. After DNA purification with phenol/chloroform, 1 µg of glycogen was added. The DNA was precipitated and dissolved in H_2O at a concentration of 1µg/µl and quantified by UV spectrometry. In the luciferase assays, expression of the *in vitro* methylated Sp1/L-pRLnull plasmid was compared to the mock methylated Sp1/L-pRLnull.

2.2.10.6 Generation of constructs containing the mutated *RASSF1A* promoter

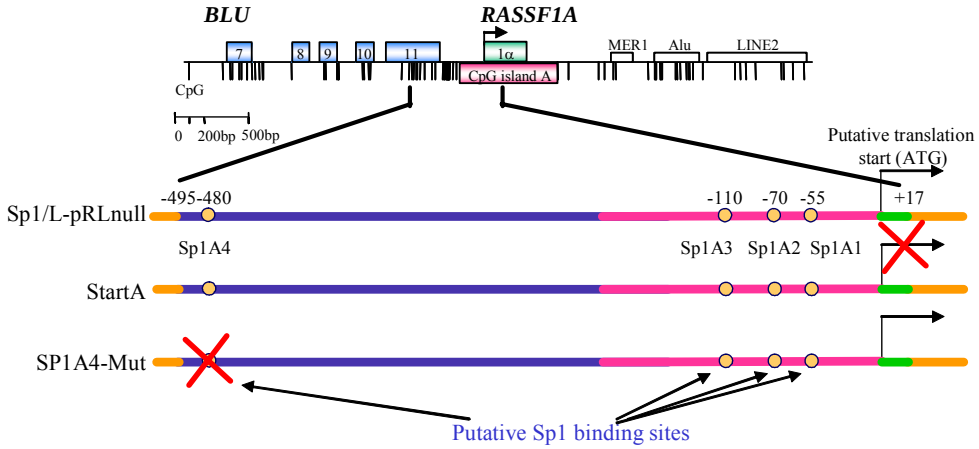

Figure 2-5. Mutations of the *Sp1* and translation start sites in the *RASSF1A* promoter. A map of the *RASSF1A* promoter region is shown. For further details see Figure 2-1. Constructs StartA and Sp1A4 were generated by site-directed mutagenesis of the Sp1/L-pRLnull plasmid. The crosses indicate new mutations in the putative *RASSF1A* translation start site and the putative *Sp1* site. Yellow and green lines represent sequences of the *pRL-null* vector and the exon 1α of *RASSF1A*, respectively. Red and blue line indicate sequences of the *RASSF1A* CpG island fragment located upstream from the putative translation start site and the putative *RASSF1A* promoter fragment located upstream from the *RASSF1A* CpG island, respectively.

Plasmids Sp1A4-Mut and StartA were generated by the *QuickChange XL Site-Directed Mutagenesis* kit using the Sp1/L-pRLnull vector (Figure 2-5) with primers listed in Table 2-6 according to the manufacturer's instructions (Stratagene). After transformation and "Blue/White" screening, the plasmid DNA was isolated from 5 white clones by the *QIAprep spin* kit and analyzed by sequencing.

Table 2-6. Primers used for site-directed mutagenesis of the *Sp1* and translation start sites in the *RASSF1A* promoter

Construct	Original sequence→ mutated sequence	Primers (5'→3')
StartA	ATG → CTG	CTGMTU:CCCAACCGGGCCCTGTCGGGGGAGCC CTGMTL:GGCTCCCCCGACAGGGCCCGGTTGGG
Sp1A4-Mut	GGGCGG→ AAGCGA	ASP1MTU:GAGAGCAGAGCAAGCGATAAAGCTGCTGAC ASP1MTL:GTCAGCAGCTTTATCGCTTGCTCTGCTCTC

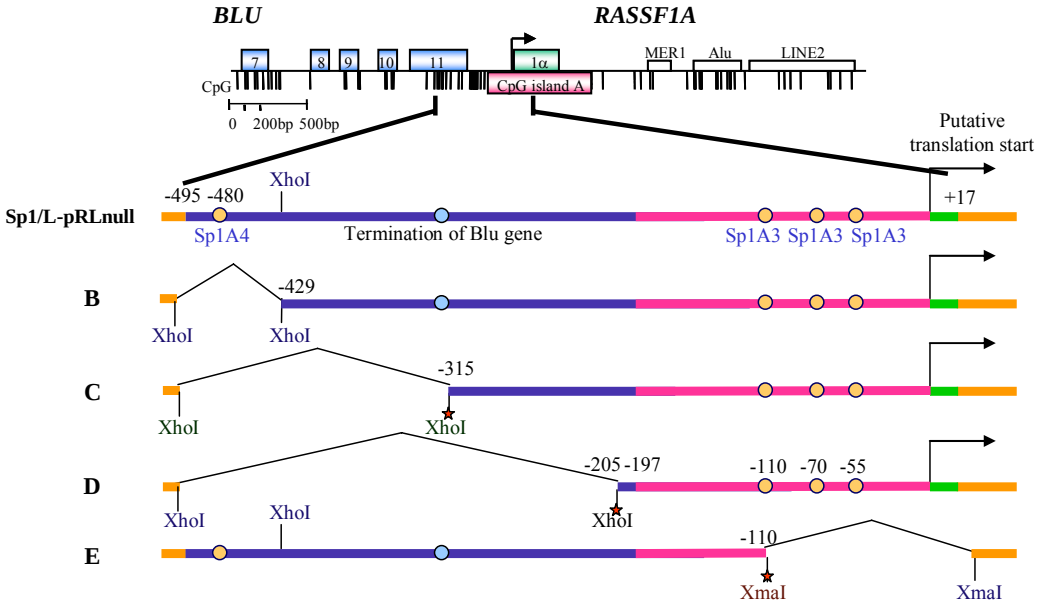

Figure 2-6. Deletions in the *RASSF1A* promoter. A map of the *RASSF1A* promoter region is shown. For further details see Figure 2-1. Plasmids B, C, D and E were generated by deletions in the *RASSF1A* promoter fragments of Sp1/L-pRLnull plasmid using *XhoI* and *XmaI* restriction sites. A red star symbols indicate the restriction sites generated by site-directed mutagenesis. Yellow and green lines represent sequences of the *pRL-null* vector and the exon 1 α of *RASSF1*A, respectively. Red and blue lines outline sequences of the *RASSF1A* CpG island fragment located upstream from the putative *RASSF1A* translation start site and the *RASSF1A* promoter fragment located upstream from CpG island, respectively.

To generate the B construct (Figure 2-6), 11 µg of Sp1/L-pRLnull plasmid was restricted with 30 units of *XhoI* enzyme (New England BioLabs) at 37ºC for 4 h. The digested DNA was resolved on a 1% TBE agarose gel and DNA fragments with 3.7 kb size were isolated from gel using the *QIAquick Gel Extraction* kit (Qiagen). The DNA fragments were selfligated using 1.5 units of *T4 DNA* ligase (Promega) in 10 µl of reaction mix for 4 h at RT. The ligated DNA was transformed in *TOP 10F' E. coli* competent cells according to the manufacturer's instructions (Clontech). Plasmid DNA from two clones was isolated by the *QIAprep spin* kit (Qiagen) and analyzed by sequencing. To generate C, D and E (Figure 2-6) constructs, new restriction sites in Sp1/L-pRLnull plasmid were created using the *QuickChange XL Site-Directed Mutagenesis* kit and primers listed in Table 2-7 according to the manufacturer's instructions (Stratagene). After DNA transformation and "Blue/White" screening, the plasmid DNA from two positive clones was isolated by the *QIAprep spin* kit (Qiagen). Further, 5 µg of plasmid DNA was treated with 60 units of *XhoI* (New England BioLabs) (for the C and D constructs) or 30 units of *XmaI* (New England BioLabs) (for the E construct) in 100 µl of reaction mix at 37ºC for 4 h. After resolving the

digested DNA on a 1% TBE agarose gel, the DNA fragments with 3.6 kb size were isolated using the *QIAquick Gel Extraction* kit (Qiagen). Further, the DNA fragments were selfligated using 1.5 units of *T4 DNA* ligase (Promega) in 10 µl of reaction mix for 4 h at RT and transformed in *TOP 10F' E. coli* competent cells according to the manufacturer's instructions (Clontech). Plasmid DNA from 5 clones was isolated by the *QIAprep spin* kit (Qiagen). Sizes of the *RASSF1A* inserts in the constructs were analyzed by restriction (see Table 2-8) and sequencing analysis.

Table 2-7. Primers used for site-directed mutagenesis of the *RASSF1A* promoter

Construct	Fragment size[1]	Restriction site [2]		Primers (5'→3')
		enzyme	position	
C	330 bp	*XhoI*	-315	UMXC1: GTAAAGCTGGCCTCGAG[3]AAACACGGGTATC LMXC1: GATACCCGTGTTTCTCGAG[3]GCCAGCTTTAC
D	220 bp	*XhoI*	-205	UMXD1: GCGGGGGGGGCTCTCGAG[3]AGCGCGCCCAG LMXD1: CTGGGCGCGCTCTCGAG[3]AGCCCCCCCCGC
E	393 bp	*XmaI*	-110	UMXE1: CAGCTCCTTCCCGGGCCC[4]AGTCTGGATCC LMXE1: GGATCCAGACTGGGCCC[4]GGGAAGGAGCTG

[1]Size of the *RASSF1A* fragment in construct after deletion. [2]New generated restriction site. [3]*XhoI* restriction site (5'CTCGAG). [4] *XmaI* restriction site (5'GGGCCC).

Table 2-8. Restriction analysis of the C, D and E constructs

Construct	Restriction enzyme	Construct with deletion[1]	Construct without deletion[2]
C-pRLnull	*XhoI, EcoRI*	330 bp, 3.3 kb	53 bp, 63 bp, 115 bp, 330 bp, 3.3 kb
D-pRLnull	*XhoI, EcoRI*	220 bp, 3.3 kb	53 bp , 63 bp, 220 bp, 225 bp, 3.3 kb
E-pRLnull	*PstI, EcoRI*	393 bp, 3.3 kb	19 bp, 535 bp, 3.3 kb

[1]Restriction products of plasmid with successfully deleted fragment. [2]Restriction products of the mutated Sp1/L-pRLnull vector without deletion.

2.2.10.7 Generation of the constructs containing the mutated *RASSF1C* promoter

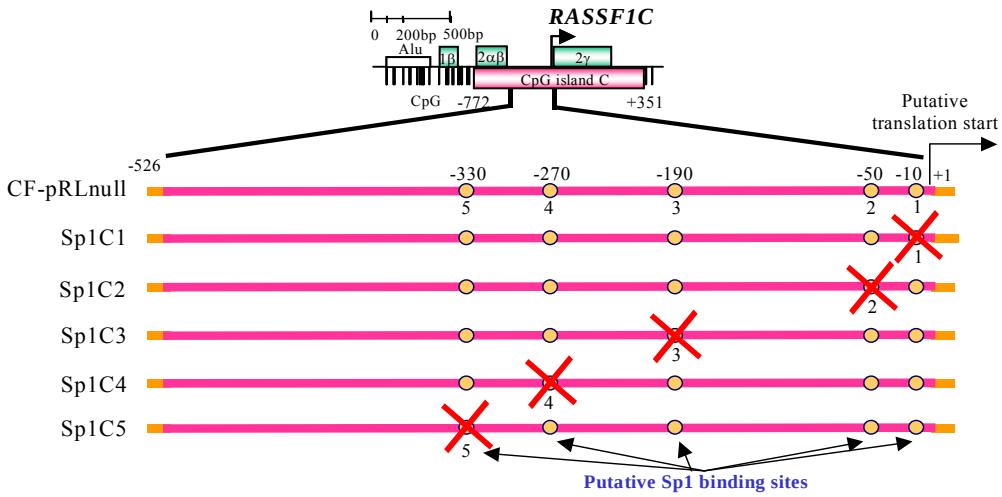

Figure 2-7. Mutations of the *Sp1* sites in the *RASSF1C* promoter. A map of the *RASSF1C* promoter region is shown. For further details see Figure 2-1. Plasmids Sp1C1, Sp1C2, Sp1C3, Sp1C4 and Sp1C5 were generated by site-directed mutagenesis of the putative *Sp1* sites in the *RASSF1C* promoter using the CF-pRLnull construct. The crosses indicate mutations in the putative *Sp1* sites. Yellow and pink lines indicate sequences of the *pRL-null* and the *RASSF1C* CpG island fragment, respectively. Green line represents a sequence of the exon 2γ of *RASSF1C*.

Plasmids Sp1C1, Sp1C2, Sp1C3, Sp1C4 and Sp1C5 were generated by the *QuickChange XL Site-Directed Mutagenesis* kit using the CF-pRLnull construct (Figure 2-7) with primers listed in Table 2-9 according to the manufacturer's instructions (Stratagene). After transformation and "Blue/White" screening, the plasmid DNA from 5 white clones were isolated by the *QIAprep spin* kit (Qiagen) and analyzed by sequencing.

Table 2-9. Primers used for site-directed mutagenesis of the *RASSF1C* promoter

Construct	Original sequence→ mutated sequence	Primers (5'→3')
Sp1C1	CCGCCC→TCGCTT	Sp1C1U: TCCCGCACCTTCTCGCTTTCGCCTCCGGCC Sp1C1L: GGCCGGAGGCGAAAGCGAGAAGGTGCGGGA
Sp1C2	CCGCCC→TCGCTT	Sp1C2U: GGACGCTGGCACTCGCTTCCGTTCCCTGTG Sp1C2L: CACAGGGAACGGAAGCGAGAGCCAGCGTCC
Sp1C3	CCGCCC→TCGCTT	Sp1C3U: GCGTGCGTGTCCTCGCTTCGGCGTTCCTGC Sp1C3L: GCAGGAACGCCGAAGCGAGGACACGCACGC
Sp1C4	GGGCGG→AAGCGA	Sp1C4U: CGCACGCGACCGAAGCGATGGTTGGCGGCT Sp1C4L: AGCCGCCAACCATCGCTTCGGTCGCGTGCG
Sp1C5	GGGCGG→AAGCGA	Sp1C5U: GGACTGGGGGACAAGCGAGTACGGCTATGG Sp1C5L: CCATAGCCGTACTCGCTTGTCCCCCAGTCC

2.2.10.8 Cell transfection and *Dual - Luciferase Reporter Assay* system

For transfection, the plasmid DNA was isolated by the *QIAfilter plasmid Maxiprep* kit (Qiagen) and quantified by UV spectrometry.

In 6-well plates, 3 µg of vector containing the *RASSF1* promoter and 120 ng of *pGL3-promoter* vector were co-transformed in HeLa S3 cells. To determine the background, 3 µg of *pRL-null* vector (Promega) and 120 ng *pGL3-promoter* vector (Promega) were co-transformed in cells grown in one of the wells. *Lipofectamine 2000* was used for transfection according to the manufacturer's instructions (Invitrogen). After 6 h of transfection, Opti-MeM I Reduced Serum was replaced by appropriate culture medium. After 18 h, cells were washed with PBS and rocked with passive lysis buffer (*Dual-Luciferase Reporter Assay* system) for 15 min at RT. Expression of constructs was analyzed by *Dual-Luciferase Reporter Assay* system according to the manufacturer's instructions (Promega).

2.2.10.9 Analysis of *Dual - Luciferase Reporter Assay* data

To determine the transfection efficiency, *pGL3-promoter* vector containing the *Firefly Luciferase* gene under the *SV40* promoter was used for co-transfection. In every experiment, transfection of the *pRL-null* vector was performed to determine the *Renilla Luciferase* expression in a vector without insert.

For every sample, reaction was performed with substrates for both luciferases, thus every sample had two raw data:

A - raw data with *Renilla Luciferase* substrate

B - raw data with *Firefly Luciferase* substrate = transfection efficiency

Reactions with *Renilla Luciferase* substrate were normalized for the reaction with *Firefly Luciferase* substrate in the same sample by formula: C = A / B. Normalized reaction with the *pRL-null* vector ($C_0 = A_0 / B_0$) was defined as a background and expression of vector containing the *RASSF1* promoter fragment was calculated by formula: $D = C - C_0$. Expression of one of the constructs containing the *RASSF1* (A or C) promoter fragments was defined as 100% ($S_{tandard}$= 100%) and expression of the *pRL-null* vector was defined as 0% ($D_{pRL\text{-}null}$=0%). On the basis of there formulas, D and the average of D with standard deviations were calculated for all samples.

2.2.11 The electro mobility-shift assay (EMSA).

2.2.11.1 Isolation of nuclear extract

Nuclear extract was isolated as described by Tommasi and Pfeifer with some modifications (Tommasi and Pfeifer, 1995). To isolate nuclei, HeLa S3 cells were washed twice with cold PBS and incubated in lysis buffer (10 mM hepes-KOH pH 7.9, 10 mM KCL, 0.3 M sucrose, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 2.0 mM 2-mercaptoethanol, 0.5 mM PMSF, 1% NP-40) on ice for 20 min. The nuclei were scraped, spun for 10 min at 4000 rpm at 4ºC, transferred into Eppendorf tube and pelleted. Further, the nuclei were resuspensed and gently extracted in 2.5 volume (volume of nuclei) of cold nuclei extraction buffer (20 mM Hepes-KOH pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 2.0 mM 2-mercaptoethanol, 0.5 mM PMSF, 20% Glycerol). After centrifugation for 30 min at 12000g at 4ºC, the supernatant was dialyzed against 50 volumes (volumes of supernatant) of dialysis buffer (20 mM Hepes-KOH pH 7.9, 100 mM KCL, 0.2 mM EDTA, 2.0 mM 2-mercaptoethanol, 0.5 mM PMSF, 20% glycerol) overnight at 4ºC. After measuring the protein concentration by Bradford method (Bradford, 1976), the nuclear extract was stored at -80ºC in aliquots.

2.2.11.2 Labelling of oligos

Labelling of oligos was carried out as previously described by Latchman with some modifications (Latchman, 1995). Two complementary single stranded oligonucleotides (Table 2-10) with concentrations 50 pmol/µl were mixed in equimolar amounts, annealed by heating for 5 min at 80ºC and gradually cooled to RT over a period of 5 h.

For labelling of double stranded oligos, reaction was set up in 20 µl of volume using the following final concentrations: 1x buffer *T4 polynucleotide* kinase, 100 pmol of double stranded annealed oligonucleotides, 20 µCi [γ-^{32} P ATP] and 10 units of *T4 polynucleotide* kinase (New England BioLabs). After incubation for 30 min at 37ºC and adding of 20 µg of glycogen, oligos were precipitated and dissolve in 20 µl of H_2O.

Table 2-10. Oligonucleotides for EMSA

Upper oligo (5'→3')	Lower oligo (3'→5')	ds oligo[1]
AGCAGAGCGGGCGGTAAAGCTG	TCGTCTCGCCCGCCATTTCGAC	Sp1A4
AGCAGAGCGttCGGTAAAGCTG	TCGTCTCGCaaGCCATTTCGAC	Sp1A4-m
CTCCTTCCCGCCGCCCAGTCTG	GAGGAAGGGCGGCGGGTCAGAC	Sp1A3
CTCCTTCCCGttGCCCAGTCTG	GAGGAAGGGCaaCGGGTCAGAC	Sp1A3-m
GTCGGGGCCCGCCCTGTGGCCC	CAGCCCCGGGCGGGACACCGGG	Sp1A2
GTCGGGGCCCGaaCTGTGGCCC	CAGCCCCGGGCttGACACCGGG	Sp1A2-m
CTGTGGCCCCGCCCGGCCCGCG	GACACCGGGGCGGGCCGGGCGC	Sp1A1
CTGTGGCCCCGaaCGGCCCGCG	GACACCGGGGCttGCCGGGCGC	Sp1A1-m

[1]Double stranded oligonucleotides

2.2.11.3 EMSA

The DNA-binding assays were carried out as described by Tommasi and Pfeifer with some modifications (Tommasi and Pfeifer, 1995). Binding reactions were set up on ice in 20 µl volume using the following final concentrations: 13 mM Hepes-KOH pH 7.9, 64 mM KCL, 0.5 mM $MgCl_2$, 0.13 mM EDTA, 0.3 mM PMSF, 13% glycerol, 1.5 µg of *Salmon* sperm DNA, 2 µg of antibodies (*Sp1* or *XPA*, both from Santa Cruz Biotechnology) and 5 µg of nuclear extract proteins. In some experiments, a 250 pmol (2500 pmol in case with SpA4) of the double stranded unlabelled oligonucleotides listed in Table 2-10 was included as competitor. After incubation of the binding mix for 10 min on ice, 2 µl of the radioactive labelled oligos was added and incubation was continued for 1 h. The DNA – protein complex was mixed with 2 µl of EMSA loading buffer (0.2% xylene cyanol, 0.2% bromophenol blue) and resolved on a 6% polyacrylamide gel in TBE 0.25x at 100 Volt for 4 h. The gel was dried under vacuum using gel dryer (BioRad) for 1 h at 80ºC and analyzed by a phosphoimaging.

2.2.12 Ligation-mediated PCR (LM-PCR)

LM-PCR is a method of genomic analysis to determine primary DNA nucleotide sequences, methylation patterns, DNA lesion formation and repair, and *in vivo* protein–DNA footprints (reviewed by Dai *et al.*, 2000). This technique is based on the ligation of an oligonucleotide linker onto the 5'-end of each DNA molecule, where 5'-end was generated by chemical cleavage of the strand. The presence of linker on all 5'-ends allows the exponential PCR, which results in amplification of the signal. The general LM-PCR steps are outlined in Figure 2-8. LM-PCR of the cleaved DNA was performed as previously described by Dammann and Pfeifer with some modification (Dammann and Pfeifer, 1997).

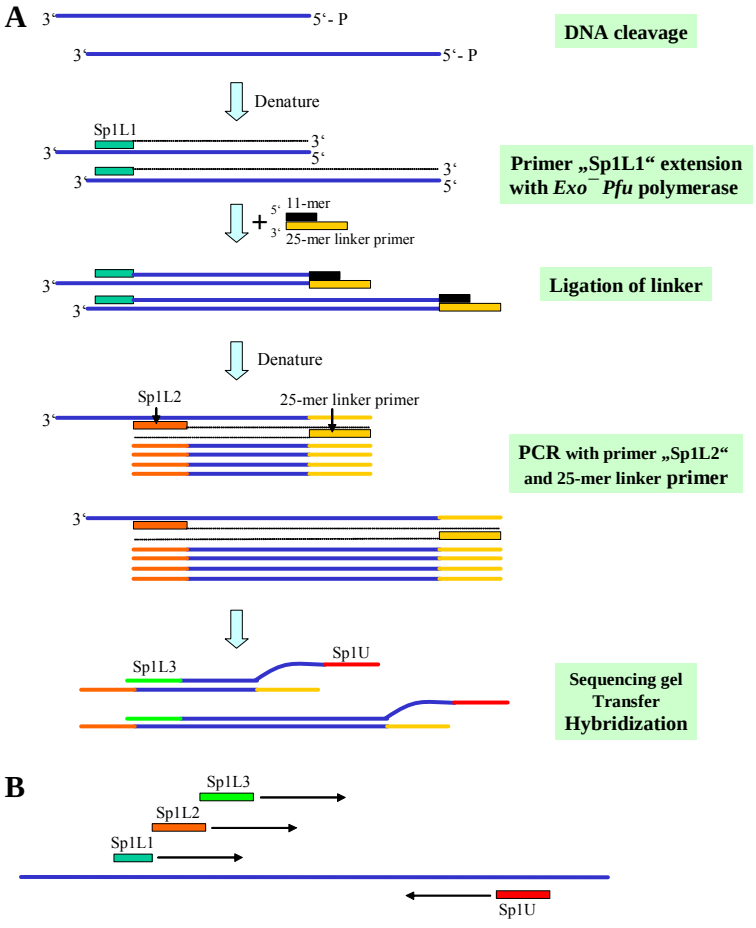

Figure 2-8. Outline of the ligation–mediated PCR procedure. A. LM-PCR procedure. The first step of the technique is a cleavage of the DNA. Next step is a generation of blunt end on one side using primer extension of a gene-specific oligonucleotide (primer Sp1L1). Third step is a ligation of linkers to the blunt ends. Next step is an expontial PCR amplification using the longer oligonucleotide of the linker (25-mer linker primer) and a second gene-specific primer (primer Sp1L2). After amplification, the DNA fragments were separated on the sequencing gel, electroblotted onto nylon membranes and hybridized with gene specific probe to visualize the sequence ladders. **B.** Arrangement of primers in a LM-PCR.

2.2.12.1 *In vivo* footprinting using dimethyl sulfate

For genomic footprinting experiments, HeLa S3 cells were treated with medium containing 0.2% dimethyl sulfate for 5 min at RT. Further, cells were washed with cold PBS, scraped, spun for 5 min at 1000 g at 4ºC and washed once more with cold PBS.

2.2.12.2 DNA isolation

The following DNA isolation method was used to prevent single and double stranded DNA breaks, which can be produced by the isolation procedure. The quality of DNA, which was obtained, allows high amplification efficiencies.

DNA from cells for chemical cleavage was isolated according the following procedure: $1x10^7$ to $1x10^8$ cells were washed with cold PBS, scraped and spun for 5 min at 1000 g at 4ºC. Cells were resuspensed in 4 ml of cold buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl and 2 mM EDTA pH 8.0). After adding of 4 ml of cold buffer A containing 1% NP-40, cells were incubated for 5 min on ice and spun for 5 min at 100 g at 4ºC. The nuclei pellet was washed in cold buffer A without nonindet P - 40 and resuspensed in 3 ml of buffer B (150 mM NaCl, 5 mM EDTA pH 7.8). After adding of 3 ml of buffer C (20mM tris pH 8.0, 20 mM NaCl, 20 mM EDTA pH 8.0, 1% SDS, 600 µg/ml *Proteinase K*), nuclei were incubated at 45ºC for 3 h. Further, DNA was extracted using phenol/chloroform, precipitated and dissolved in water.

2.2.12.3 Chemical cleavage of DNA

DNA was cleaved according to the Maxam Gilbert procedure (Maxam and Gilbert, 1977). Using vacuum concentrator, 50 µg of genomic HeLa S3 DNA were dried and dissolved in water volume according to the base-specific reaction protocol. All chemical cleavages were performed on ice.

G reaction: Five µl of DNA was dissolved in 200 µl of dimethyl sulfate buffer (50 mM Na-cacodylate, 1 mM EDTA pH 8.0). Further, 1 µl of dimethyl sulfate was added (99%) and DNA was incubated for 5 min at RT. The reaction was stopped by adding of 50 µl of dimethyl sulfate stop buffer (1.5 M Na-acetate pH 7.1, 1 M 2-mercaptoethanol). After adding of 100 µg of glycogen, DNA was precipitated by 750 µl of precooled 96% ethanol.

G+A reaction: 25 µl of 100% formic acid was added to 11 µl of DNA and incubated for 10 min at RT. After stopping the reaction by 50 µl of dimethyl sulfate stop buffer, 5 µl of 20 mg/ml glycogen was added. DNA was precipitated with 750 µl of precooled 96% ethanol.

T+C reaction: 47 µl of 64% hydrazine was added to 20 µl of DNA and incubated for 20 min at RT. After stopping the reaction by 200 µl of hydrazine stop buffer (0.3 M Na-acetate pH 7.5, 0.1 M EDTA pH 8.0), 5 µl of 20 mg/ml glycogen was added. DNA was precipitated with 750 µl of precooled 96% ethanol.

C reaction: 47 µl of 64% hydrazine and 15 µl of 5 M NaCl were added to 5 µl of DNA and incubated for 20 min at RT. After stopping the reaction by 200 µl of hydrazine stop buffer, 5 µl of 20 mg/m glycogen was added. DNA was precipitated with 750 µl of precooled 96% ethanol.

Samples from four reactions (G, G+A, C, T+C) were further treated together as described below. After incubation for 30 min at -70˚C, DNA was precipitated and dissolved in 225 µl of water. Further, DNA was precipitated once more and dissolved in 50 µl of water.

For piperidine treatment, 50 µl of 2 M piperidine was added to DNA and incubated for 30 min at 90˚C. After cooling on ice for 5 min and adding of 40 µg of glycogen, DNA was precipitated, dried overnight in vacuum concentrator and dissolved in 50 µl of water.

2.2.12.4 Primer extension

For primer extension *Exo⁻Pfu DNA* polymerase was used (Stratagene). Reactions were set up in 30 µl of volume using the following final concentrations: 1x cloned *Pfu* reaction buffer, 0.25 mM dNTPs each, 1 pmol Sp1L1 primer (5'GGAGGCCAGCTTTACTGTGCTA), 1.5 units of *Exo⁻Pfu DNA* polymerase, 2 µg of cleaved DNA. After an initial denaturation step at 95°C for 5 min and following annealing at 56°C for 2 min 30 s, the reaction mix was gradually heated from 57°C up to 74°C with 1°C step for 3 s. The final elongation step was done at 72°C for 15 min.

2.2.12.5 Linker preparation

Linker was prepared by annealing a 25-mer linker primer 20 pmol/µl (5'GCGGTGACCCGGGAGATCTGAATTC) to 11-mer linker primer 20 pmol/µl (5'GAATTCAGATC). For annealing, primer mix was heated at 95°C for 3 min and gradually cooled to RT over a period of 5 h.

2.2.12.6 Ligation

45 µl of a ligation mix (13.33 mM $MgCl_2$, 30 mM DTT, 1.1 mM ATP, 16.7 mg BSA, 100 pmol linker, 50 mM tris pH 7.7, 3.25 units of *T4 DNA* ligase (Promega)) was added to primer extension mix on ice. After 24 h of incubation at 16°C, the ligation was stopped by heating for 10 min at 70°C and adding of 30 µl of stop-mix (7.2 M ammonium acetate, 4 mM EDTA pH 8.0, 20 µg glycogen). Ligated fragments were precipitated and dissolved in 50 µl of H_20.

2.2.12.7 PCR amplification

For PCR amplification, 50 µl of PCR mix (2 x Fast *Taq* buffer, 3 mM $MgCl_2$, 0.5 mM dNTPs each, 10 pmol/µl 25-mer linker primer (5'GCGGTGACCCGGGAGATCTGAATTC), 10 pmol/µl Sp1L2 primer (5'TAGAGGAAGAGGGTCCCCACATCCG) and 4 units of *Fast Taq* polymerase (Roche)) was added to ligated fragments. After denaturation for 5 min at 95°C, the PCR cycling conditions were as follows: 95°C for 30 s, 66°C for 30 s and 72°C for 1 min for a total of 25 cycles. The last elongation step was performed for 10 min at 72°C. After amplification, 25 µl of PCR-stop mix (1.56 M sodium acetate, 60 mM EDTA pH 7.7, 10 mg tRNA) was added. The DNA was extracted using 250 µl of phenol/chloroform (92 µl phenol + 158 µl chloroform), precipitated and dissolved in 6 µl of formamide loading dye (62.6% formamide, 1.33 mM EDTA pH 7.7, 0.03% xylene cyanole, 0.03% bromphenol blue).

2.2.12.8 Gel electrophoresis and electroblotting.

Three µl of PCR products was denaturated at 95°C for 3 min and separated on 8% polyacrylamide gel containing 7 M urea in TBE 1x for 4 h with the following parameters: 3000 Volt, 75 Watt, 50°C (glass temperature). Further, DNA fragments

were electroblotted onto nylon membrane using electroblotter at 17 Volt and 2 Ampere for 40 min in TBE 1x.

2.2.12.9 Preparation of a single stranded PCR probe

PCR products were used as a template to synthesize a single strand probe. These PCR products were generated as follow: 100 ng of genomic HeLa S3 DNA was amplified in 25 µl of reaction mix (1x *Taq* buffer, 2 units of *Taq* polymerase (InViTek), 0.2 mM NTPs, 1.5 mM $MgCl_2$, 20 pmoles of primers Sp1L2 (5'TAGAGGAAGAGGGTCCCCACATCCG) and Sp1U (5'CTGCAGTTGCTGAGGGCCGACC)). After the first denaturation for 5 min at 95°C, DNA was amplified for 40 cycles with following conditions: 95°C for 30 s, 66°C for 30 s and 72°C for 30 s. The last elongation step was performed for 10 min at 72°C. After gel purification using the *QIAquick Gel Extraction* kit (Qiagen), PCR products were dissolved in 30 µl of water.

For probe labelling, 3 µl of PCR product was amplified in 100 µl of reaction mix (1x *Taq* buffer, 5 units of *Taq* polymerase (InViTek), 1 mM dATP, 1 mM dGTP, 1 mM dTTPs, 30 µCi [α-^{32}P CTP], 1.5 mM $MgCl_2$, 50 pmoles Sp1L3 primer (5'CCTGGCCCTCCTGGTCCGGTTT)). After the first denaturation for 5 min at 95°C, DNA was amplified for 30 cycles with following conditions: 95°C for 1 min, 64°C for 2 min and 72°C for 3 min. PCR products were clean from radioactive nucleotides using a Sephadex G-50 column, 400 µl of probe was used for hybridization.

2.2.12.10 UV cross linking, hybridization and exposure

DNA was UV cross linked to membrane with UV Stratalinker at 1.2 mjoules after electroblotting (see chapter 2.2.12.8). The membrane was prehybridizated at 64°C for 4 h in hybridization buffer (0.25 M Na_2HPO_4 pH 7.2, 1 mM EDTA pH 7.7, 7% SDS, 1% BSA) and hybridized with a single stranded gene specific PCR probe overnight at 64°C. The membrane was washed twice with a 64°C warm washing buffer 1 (20 mM Na_2HPO_4 pH 7.2, 1mM EDTA pH 7.7, 2.5% SDS, 0.25% BSA) and twice with a 64°C warm washing buffer 2 (20 mM Na_2HPO_4 pH 7.2, 1 mM EDTA pH 7.7, 1% SDS). Signals were analyzed by a phosphoimaging.

2.2.13 Chromatin Immunoprecipitation (ChIP)

2.2.13.1 Cell treatment and DNA shearing

ChIp was performed as described in the Upstate protocol for the *Chromatin Immunoprecipitation Assay* kit (http://www.upstate.com/img/coa/17-295-27400.pdf) with some modifications. In order to perform ChIP analysis, histones and other proteins were crosslinked to the DNA in cells by adding formaldehyde to a final concentration of 1% to the culture medium and by incubating for 10 min at 37°C. The cells were washed twice with cold washing buffer (PBS, 1 tablet protease inhibitor cocktail pro 50 ml, 1 mM PMSF), scraped into conical tube and spun for 4 min at 2000 g at 4˚C. The cell pellet was resuspensed in a SDS lysis buffer (1% SDS, 10 mM EDTA pH 8.0, 50 mM tris pH 8.1, 1 mM PMSF, 1 tablet protease inhibitor cocktail pro 10 ml) with cell concentration of $1x10^6$ cells pro 200 µl of SDS lysis buffer and incubated for 10 min on ice. Further, cell lysate was sonicated to shear DNA to lengths between 200 and 500 bp using ultrasound homogenizator and spun for 10 min at 13000 g at 4˚C. After centrifugation, 200 µl of supernatant fraction was transferred into a new tube and diluted in 1800 µl of cold ChIp dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA pH 8.0, 16.7 mM tris pH 8.1, 167 mM NaCl, 1 mM PMSF, 1 tablet protease inhibitor cocktail pro 10 ml). Twenty microliters (1/100 volume) of this solution was kept to quantitate the DNA amount in different lysates and this probe was considered to be the “input” control.

2.2.13.2 Immunoprecipitation

To reduce nonspecific background, the diluted cell supernatant was pre-cleared with 80 µl of *salmon sperm DNA/protein A agarose* using following protocol: 80 µl of *salmon sperm DNA/protein A agarose* was added to the 2 ml of cell lysate. After incubation for 1 h on ice, the cell lysate was spun for 1 min at 1000 rpm and supernatant fraction was transferred to a new tube for immunoprecipitation.

After adding of 2 µg of antibodies (*H3-trimethyl lysine 9* or a*cetyl-H3* or *Sp1*), cell lysate was incubated overnight at 4°C. Further, the antibody/chromatin complex was isolated by adding of 60 µl of *salmon sperm DNA/protein A agarose,* incubating for 1 h on ice and pelleting for 1 min at 1000 rpm. For a negative control, a no antibody immunoprecipitation was utilized.

The protein A agarose/antibody/protein complex was washed with 1 ml of low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM tris pH 8.1, 150 mM NaCl), 1 ml of high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8.0, 20 mM tris pH 8.1, 500 mM NaCl), 1 ml of LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA pH 8.0, 10 mM tris pH 8.1) and 2 ml of TE buffer (10 mM tris pH 8.0, 1 mM EDTA pH 8.0).

2.2.13.3 Extraction of immunoprecipitated DNA

For quantitative PCR analysis, the DNA was eluted from agarose beads as follows. The protein A agarose/antibody/protein complex was incubated in 250 µl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at RT. After pulse centrifugation, the eluate was collected. The elution step was performed twice.

Reverse protein-DNA crosslinking was performed by adding of 20 µl of 5 M NaCl to the combined eluates (500 µl) and incubating overnight at 65°C. Analogously, "Input" sample was treated in 0.2 M NaCl overnight at 65°C. After this treatment, the following solutions: 10 µl of 0.5 M EDTA pH 8.0, 20 µl of 1 M tris pH 7.0 and 2 µl of 10 mg/ml *Proteinase K* were added to DNA in "input" sample and to immunoprecipitated DNA. The DNA was incubated for 3 h at 45°C. After phenol extraction and precipitation, the DNA was dissolved in 50 µl of water. The histone modifications and *Sp1* binding were quantified by real time PCR using the primers listed in Table 2-11. "Input" sample and "no antibody" probe were used as positive (100%) and background (0%) controls, respectively.

2.2.13.4 Real time PCR of immunoprecipitated DNA

Real time PCR was carried out in the LightCycler "Rotor Gene 2000" using *Sybr™ green I* detection. Reactions were set up in 25 µl of volume with the following final concentrations: 1x *Taq* buffer (1.5 mM $MgCl_2$), 1 unit of *Fast Taq* polymerase (Roche), 0.25 mM dNTPs each, 10 pmoles of each primer (Table 2-11), 0.2 x *Sybr™ Green I*, formamide (Table 2-11) and 2 µl of DNA. After an initial denaturation step for 5 min at 95°C, the cycling conditions were as follows: 95°C for 20 s, T_{an} (Table 2-11) for 30 s 72°C for 30 s and a fluorescence measurement after 15 s of appropriate

Tm (Table 2-11) for a total of 50 cycles. The last elongation step was performed for 5 min at 72°C. Further, the melting temperatures of the PCR products were analyzed by a fluorescence measurement at every 1°C step after 5 s from 70°C up to 99°C. The amplification of PCR products was verified using the melting curve option (see chapter 2.2.9.2) and subsequence electrophoresis in 2% TBE agarose gel. All measurements were performed thrice. Data analysis was performed using the comparative method described in a chapter 2.2.9.3. (Rotor Gene Software version 4.6). The amount of DNA in analyzed samples was plotted relative to DNA amount in "input" sample (100%) using comparative method. A DNA amount in "no antibody" probe was defined as background control (0%).

Table 2-11. ChIp: Primers and conditions.

	Primers (5'→3')	T_{an}, ºC	Tm, ºC	FA[1], %	PCR product size, bp
A2	U: GATCACGGTCCAGCCTCTG L: CTCGAGCCTTCACTTGGGGT	62	85	2	109
A1	U: CTGGGGGAGGCGCTGAAGTC L: GCTCAGGCTCCCCCGACATG	62	85	4	115
C1	U: CGATTTCCCGGCGGCACA L: CCAGCGTCCGGGCAAGCG	60	85	4	200

[1]Formamide concentration in PCR mix.