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► **To cite this version:**

Giridhar Mudduluru, Heike Allgayer. The human receptor tyrosine kinase Axl gene - promoter characterization and regulation of constitutive expression by Sp1, Sp3, and CpG methylation. *Bioscience Reports*, 2008, 28 (3), pp.161-176. 10.1042/BSR20080046 . hal-00479299

**HAL Id: hal-00479299**

**<https://hal.science/hal-00479299>**

Submitted on 30 Apr 2010

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## The human receptor tyrosine kinase *Axl* gene - promoter characterization and regulation of constitutive expression by Sp1, Sp3, and CpG methylation

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**Running title:** Sp1/Sp3 regulates *Axl* gene expression

### Synopsis

*Axl*-receptor-tyrosine-kinase promotes antiapoptosis, mitogenesis, invasion, angiogenesis, and metastasis, and is highly expressed in cancers. However, the transcriptional regulation of this important gene has never been characterized. The present study was initiated to characterize the promoter, first cis-acting elements, and promoter methylation driving expression of *Axl*. The 2.4kb sequence upstream of the translational start site, and sequential 5'-deletions were cloned and revealed a minimal GC-rich region (-556/+7) to be sufficient for basal *Axl*-promoter activity in Rko, HCT116, and HeLa cells. Within this minimal region, five Sp-binding sites were identified. Two sites (Sp-a, Sp-b) most proximal to the translation start site were indispensable for *Axl*-promoter activity, whereas mutation of three additional upstream (Sp-c, Sp-d, Sp-e) motifs was of additional relevance. Gelshift and chromatin-immunoprecipitation identified especially Sp1 and Sp3 bound to all 5 motifs, mutations of all motifs abolishing binding. Mithramycin, inhibiting binding of Sp-factors to GC-rich sites, dramatically reduced *Axl*-promoter activity, *Axl*-, Sp1-, and Sp3-expression. In *Drosophila* Schneider SL2-cells, exogenous expression of Sp1/Sp3 increased *Axl*-promoter activity. Sp1/Sp3-siRNAs significantly reduced *Axl*-promoter activity and protein in Rko and HeLa cells. Methylation-bisulfite sequencing detected methylated CpG-sites within three Sp-motifs (Sp-a, -b, -c) and GC-rich flanking sequences, demethylation by 5-aza-2'-deoxycytidine upregulating *Axl*- and Sp3-expression in low *Axl*-expressing Colo206f/WiDr, but not high *Axl*-expressing Rko-cells. These data suggest that *Axl* gene expression in cancer-cells 1) is constitutively driven by Sp1/Sp3 bound to 5 core-promoter motifs, 2) is restricted by methylation within/around Sp-binding sites. This might enhance understanding and treatment of essential mechanisms associated with cancer and other diseases.

Key words: *Axl* receptor tyrosine kinase, *Axl* promoter, Methylation, 5-aza-dC, Sp1, Sp3

## Introduction

*Axl* (from the Greek word 'anexelekto,' or uncontrolled) belongs to the Tyro 3 family of receptor tyrosine kinases (RTKs) which includes Mer and Sky, and was originally identified as a transforming gene in human leukemias [1]. These RTKs are characterized by the presence of two immunoglobulin-like domains and two fibronectin-type III domains in the extracellular region (a unique extra-cellular structure, resembling neural cell adhesion molecules), and a distinctive intracellular kinase domain [1]. Human *Axl* is a 140 kDa protein with roughly equal distribution of amino acids on either side of the plasma membrane [1,2]. The main ligand for *Axl* RTK family members is Gas6, the product of growth-arrest-specific gene 6 [3], a soluble member of the vitamin K-dependent protein family [4], which binds to Tyro3 family members with different affinities. Gas6 was first discovered as an up-regulated gene in growth arrested cells [5], suggesting its role in preventing cellular stresses, such as apoptosis. Subsequent studies have suggested its role in cell survival [6,7], proliferation [8,9], stimulation of cell migration [10], and cell-cell adhesion via *Axl* [11]. Intracellular signalling of *Axl* is also activated by homophilic and heterophilic interactions [12], mediated mainly by a multi-substrate docking site [13]. Over-expression of *Axl* can transform fibroblasts even in the absence of a ligand [14]. Different signalling molecules are activated by *Axl* in different cell types, among them being phosphatidylinositol 3-kinase (PI3K), Akt, Src, extracellular signal-regulated kinase (ERK), and nuclear factor kappaB (NF- $\kappa$ B) [15,16]. Gas6/*Axl* signaling is well studied in NIH3T3 mouse fibroblasts, where it is known to transduce antiapoptotic signals via NF- $\kappa$ B activation, and increased protein levels of Bcl-xl [17]. An increased expression of *Axl* is associated with invasion, metastasis, and angiogenesis in various cancer cells, and is found in metastatic colon and prostate carcinoma, gastric, endometrial, certain types of breast cancers, and sarcomas [18].

The *Axl* gene is located at human chromosome 19, encoded by 20 exons, and distributed over a region of 44 kb. Different isoforms of *Axl* mRNA are generated by alternative splicing of exon 10 [19]. Until now, no functional differences were found for these isoforms. In one initial study, the *Axl* 5' upstream region has been shown to be GC-rich, lack TATA and CAAT boxes, and harbour multiple transcriptional start sites. These transcription start sites include the major start site at -169, and a second major start site at -460 bp upstream of the translation start site [19]. However, although *Axl* has been shown to influence different major cell physiological properties, and to be associated with diverse cancers and cancer-specific properties, surprisingly little is known about its transcriptional regulation, and no further studies on the characterisation of its promoter have been published so far.

Therefore, in this first and comprehensive study, we characterize the *Axl* promoter and its methylation status in different colorectal cell lines. We identify binding sites for various transcription factors within the -556 to -182 bp core region upstream to the *Axl* translational start codon, among them five binding sites for Sp1/Sp3, one for MZF1, and one for AP1. Furthermore, we show that the Sp binding sites, or adjacent CpG sites, are partially methylated in low *Axl*-expressing in contrast to high *Axl*-expressing cells. The results presented here are the first to show that Sp1 and Sp3 transcription factors have an essential role in the basal expression of the *Axl* gene, and that the methylation status of CpG sites in, or in close proximity to, Sp binding motifs modulates *Axl* gene expression in cultured colon cancer.

## Experimental procedures

### Cell culture, reagents and treatments

All human cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and grown at 37°C in DMEM, MEM, McCoy's or RPMI media supplemented with 10% fetal calf serum (FCS). Mock-treated control cells were handled identically to drug-treated cells with the exception that medium only was added. *Drosophila melanogaster* SL-2 cells were maintained in Schneider's insect medium supplemented with 10% FCS at 27°C.

### Preparation of protein extracts and Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed in extraction buffer (Biosource, CA). Protein concentration was determined by BCA kit (Pierce, Rockford, IL, USA). For immunoblotting, samples (40  $\mu$ g/lane) were boiled for 5 min, separated via SDS-PAGE, and

transferred to PVDF membranes. After transfer, the membranes were blocked with 5% nonfat milk containing Tris-buffered saline with 0.1% Tween (TTBS) for 3 h at room temperature, and then probed with the indicated primary antibodies Sp1 (sc-59x, 1:2500) Sp3 (sc-644x, 1:5000), Axl (sc-1096, 1:1500) and  $\beta$ -Actin (sc-1616, 1:2000) for 2 h at room temperature (all antibodies Santa Cruz Biotechnology, Santa Cruz (sc)). After three washes with TTBS blots, were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase. After final washes with TTBS, the membranes were exposed to film after enhanced chemiluminescence (ECL) (Amersham Biosciences).

### RNA isolation and Reverse Transcriptase-PCR

Total RNA was extracted from tissue culture cells by the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. The RNA samples were quantified using a spectrophotometer and visualized on a MOPS-formaldehyde gel for quality assurance. 1  $\mu$ g of total RNA was reversely transcribed by random hexamer primer using SuperScript II reverse transcriptase (Invitrogen). The single strand cDNA was amplified by PCR using specific primer pairs. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The PCR products were analysed by electrophoresis on a 2% ethidium bromide-stained agarose gel. The sequences of the sense (S) and antisense (AS) primers used for RT-PCR were: GAPDH (S, 5'-GTCTTCACCACCATGGAGAA-3', and AS, 5'-ATCCACAGTCTTCTGGGTGG-3') Axl (S, 5'-GGTGGCTGTGAAGACGATGA-3', and AS, 5'-CTCAGATACTCCATGCCACT-3').

### Preparation of Nuclear extract and Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts and EMSAs were essentially done as described previously [20]. In Brief, nuclear extracts (5  $\mu$ g) were incubated in a buffer [25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 0.05M NaCl, 4% (v/v) glycerol] with 50,000 cpm of the [<sup>33</sup>P]ATP-end-labeled (phage T4 polynucleotide kinase, ICN #151935) oligonucleotide containing regions -239/-202 (Sp a), -264/-228 (Sp b), -524/-491 (Sp c), -538/-500 (Sp d) and -543 /-508 (Sp e) of the *Axl* promoter (listed in table 1) for 20 min in the absence, or presence, of a 50-fold excess of unlabelled oligonucleotide to show specificity of the binding at room temperature. A total of 0.5  $\mu$ g of poly deoxyinosinic-deoxycytidylic acid (d-IdC) was present in each reaction to block unspecific binding. The reactions were subjected to gel electrophoresis on a 5% polyacrylamide gel containing 5% glycerol in 0.25x Tris borate-EDTA buffer. For supershift analysis, 1  $\mu$ g of antibody (anti-Sp1, anti-Sp2, anti-Sp3, anti-Sp4, and rabbit IgG control, Santa Cruz Biotechnology; #. sc-59 X, sc- 643 X, sc-644 X, sc-645 X, sc-2338 X, respectively) was added to the reactions 20 min after nuclear extracts and oligonucleotide had been incubated. Supershift reactions were incubated for 60 min at 4°C and electrophoresed at 4°C to ensure complex stability. The gels were dried and analysed by autoradiography. As a negative control, a lane without nuclear extract and oligos was processed in each EMSA.

### Generation of luciferase reporter constructs

Approximately 2400 bp upstream sequence from translation start of the *Axl* gene was amplified by PCR from human genomic DNA and cloned into the pGL3-basic vector (Promega) using *Kpn* I and *Xho* I restriction sites. A series of different truncated fragments of the *Axl* promoter was created by PCR amplification from the 2.4 kb fragment with the primers listed in table 2, and inserted into pGL3-basic using the same restriction sites.

### Expression vectors

Expression vectors containing human Sp1/Sp3 (pCMV-Sp) and Drosophila Sp1/Sp3 (pPac-Sp) transcription factors were gifts from Dr. Guntram Suske, Philipps-Universität Marburg, Germany.

### Site-directed mutation analysis

Mutant constructs were generated by QuikChange XL site-directed mutagenesis kit from Stratagene (La Jolla, CA) using AxlP3 (-1276 to +7) as a template. The sequence of the mutant oligonucleotides is depicted in Table 1. Successful incorporation of the mutations was confirmed by automated sequencing.

### Transfection and Luciferase Assays in Mammalian cell lines

For luciferase assays,  $0.13 \times 10^6$  Rko,  $0.05 \times 10^6$  HeLa or  $0.2 \times 10^6$  HCT116 cells were plated in 24 well plates. Luciferase reporter plasmids (1  $\mu$ g of each) were transfected in optimum medium using Lipofectamine 2000 (Invitrogen). For all luciferase reporter assays, pRL-TK (50 ng, Renilla Luciferase; Promega) was also co-transfected and measured to normalize transfection efficiency. After 6 hours of transfection, optimum medium was replaced with regular medium, and the cells were incubated at 37°C for 48 h. Cells were washed twice with PBS and lysed with 100  $\mu$ l passive lysis buffer (Promega) for 20 min. Luciferase activity of 20  $\mu$ l of cell lysate was measured via the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Assays for all samples were performed in triplicate, and the results were averaged.

### Luciferase Assays in *Drosophila melanogaster* SL2 Insect Cells

For luciferase assays, at the time of transfection  $0.5 \times 10^6$  cells were plated in a 24 well plate. 250 ng of AxIP3 were co-transfected with 150 ng of either Sp1 or Sp3 along with Renilla (Internal control for transfection efficiency) with effectin transfection reagent (Quiagen). After 48 h of transfection, cells were collected in Eppendorf tubes and lysed with 100  $\mu$ l of passive lysis buffer (Promega). Luciferase activity of 50  $\mu$ l of cell lysate was measured via the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. Assays for all samples were performed in triplicate, and the results were averaged.

### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays were performed via a commercially purchased chromatin immunoprecipitation kit (Upstate Biotechnology, Lake Placid, NY), using either anti-Sp1 (sc-59 X), anti-Sp3 (sc-644 X) or anti-IgG (as control, sc-2338 X) antibodies. Rko cells were first cross-linked for 10 min by adding formaldehyde directly to tissue culture medium to a final concentration of 1% at room temperature, and then neutralized with 125mM Glycine for 10 min. Cells were then washed twice with cold PBS (with protease inhibitors), scraped, pelleted, resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), and incubated for 10 min on ice. The lysates were then sonicated 10 times, each sonication for 15 sec at 30% amplitude, resting on ice for 1 min between sonication. After sonication, the samples were centrifuged, the supernatants diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris-HCl (pH 8.1)) with protease inhibitors, and precleared with 80  $\mu$ l salmon sperm DNA/protein A Agarose-50% for 30 min at 4°C. Cross-linked chromatin was incubated overnight with 3  $\mu$ g Sp1, 3  $\mu$ g Sp3, or control IgG in a total volume of 1 ml at 4°C. Antibody-protein-DNA complexes were isolated by immunoprecipitation with 60  $\mu$ l salmon sperm DNA/protein A. After extensive washing, pellets were eluted by freshly prepared elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). Formaldehyde cross-linking was reversed by 6h incubation at 65°C. Samples were purified through PCR purification kit columns (Qiagen) and used as a template in PCR. ChIP primers: Region1: sense 5'- CCTGGCCCTTTAAGAAAG-3'; antisense 5'-CTGCCTCCTTCCCTCACT-3' and Region2: sense 5'-GTGTGTGTGTGTCCTTGCC-3'; antisense 5'-GGGCTCTGTGTCTGGTAAAC 3' were used to amplify a 159 bp and 168 bp fragment corresponding to the core *Axl* promoter, respectively. As negative control, a region in the urokinase receptor (uPAR) promoter region -937/-745 was amplified with the following set of primers, sense 5'-GCAGACATCAATAACATTCACC-3'; antisense 5'-GCCTGGCAACAGAACAAGAC-3'). Amplification of soluble chromatin prior to immunoprecipitation was used as an input control.

### Knockdown of Sp1 or Sp3 via RNA Interference

Short interfering RNAs (siRNAs), designed to target human Sp1 and Sp3 mRNAs, were from Ambion. Briefly,  $0.13 \times 10^6$  of Rko or  $0.05 \times 10^6$  of HeLa cells were plated in either a 24 well plate for Luciferase assay, or  $0.75 \times 10^6$  and  $0.3 \times 10^6$  cells in 6 well plates for Western blot analysis the day before transfection. AxIP3 reporter plasmids were co-transfected with 25 or 50 nM of Sp1/Sp3 or 50 nM of scrambled RNA (scrRNA), and luciferase assays were performed 48h after transfection. Rko or HeLa cells were transfected with either 50 nM, 100 nM of Sp1, Sp3, or combinations of Sp1 and Sp3 with different concentrations (50 nM or 75 nM Sp1 and Sp3) with Lipofectamin (Invitrogen). After 48 h, cells were washed with PBS and harvested for protein lysate.

### 5-aza-2'-deoxycytidine (5-aza-dC) treatment

Cells were plated in a 6 well plate on Day 0 in respective medium supplemented with 10% FCS, and allowed to grow stably for 24 hours. Cells were treated either with a final concentration of 10, 20 or 40  $\mu$ M of 5-aza-dC for 5 days, changing the drug for every 24 hours, or with 10% FCS medium as a control. On the fifth day, cells were washed with PBS and grow without drug for 24 hours. Finally, cells were washed with ice cold PBS and total RNA isolated using the Qiagen Mini R-Neasy Kit.

### Sodium bisulfite conversion of DNA

1  $\mu$ g of WiDr or Rko genomic DNA was used for bisulfite conversion, using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer's instructions. Bisulfite treated DNA was amplified with two sets of methylated specific primers: Axl 5'-flanking region-specific primers Set one (amplified product -376bp to -98bp) 5'-GGGTATAGTTATTAGGGTTTTTTTT-3' (sense) and 5'-TTTAACACTACCTACCTAACACAAC-3' (antisense), and Set two (amplified product -669bp to -357bp) 5'-TGTTTTAGTTTGTGTGTGTTAGTGA-3' (sense) and 5'-AAACCCTAATAACTATACCCCTATC-3' (antisense). The PCR reaction was carried out at 94°C for 2 minutes, 30 cycles at 94 °C for 30 seconds, annealing at 62 °C for 30 seconds, extension at 72 °C for 45 seconds, and final extension at 72 °C for 5 minutes. Purified PCR products were used for pGMT – easy cloning. 10 clones were sent for sequencing from each cell line.

### Statistical analysis

Statistical analyses were performed using the SPSS statistical software (statistical package for social sciences, SPSS, Inc., Chicago, IL). Differences between groups were calculated with Student's t-test, and P-values of  $\leq 0.05$  considered statistically significant.

## Results

### Cloning and putative regulatory elements of the Axl 5'-flanking region

To define the Axl promoter and identify potential cis-elements involved in the transcriptional regulation of Axl, we isolated the 5' flanking region of 2376 bp upstream of the translation start site (TSS). The sequence does not contain typical TATA and CAAT boxes or an initiator sequence (Supplementary Fig. a). As published by others, the promoter has different transcriptional start sites including two major sites located at -169 bp and -460 bp upstream of the TSS, respectively [19]. A TRANSFAC database (GenBank accession no: BC032229) search revealed several putative binding sites for transcription factors including MZF1, AP1, and multiple Sp binding sites. Based on potential cis-elements and major transcriptional start sites, we generated a series of 5' promoter deletion constructs as outlined in Fig 1a. Rko, HCT116 and HeLa cell lines, characterised by high basal mRNA amounts of Axl (Supplementary Fig. b), were used for this study. In the following, we will concentrate on characterizing the minimally required core promoter for constitutive expression of the Axl gene.

### Functional delineation of the basal Axl promoter region

To determine the minimal Axl promoter region and narrow down cis-elements regulating basal Axl gene expression, we transfected a series of deletion luciferase constructs (Fig. 1a) into Rko, HCT116, and HeLa cells. The AxlP3 construct (-1276/+7) exhibited a maximum percent luciferase activity as compared to the promoterless luciferase vector pGL3 Basic (Fig. 1b). Further deletion constructs, AxlP4 (-1010/+7), AxlP5 (-727/+7), AxlP6 (-614/+7), AxlP7 (-556 /+7), and AxlP8 (-478 /+7), showed a gradual decrease in luciferase activity in Rko, HCT116, and HeLa cells, the activity of the last two constructs being comparable (Fig 1b). AxlP1 (-2376 /+7) and AxlP2 (-1759/ +7) contain more of the 5' flanking promoter region than AxlP3, however, these constructs do not show more promoter activity than AxlP3, indicating that the maximum promoter activity lies within -1272 /+7 bp. AxlD1 (-2376/ -338), which lacks the major transcriptional start site, showed similar luciferase activity when compared with AxlP1, suggesting that both of the major transcription start sites are important. Correspondingly, AxlD2 (-556/-338), also including the second major transcription start site, showed similar luciferase activity as AxlP7 (-556 /+7), which include both major transcription start sites. AxlP9 (-181/+7) and AxlD3 (-478/-338) do not contain any GC-rich regions and show a complete loss of luciferase activity, comparable to the pGL3-Basic control vector activity (Fig. 1b). Taken together, whereas maximum promoter activity lies within -1272 /+7 bp, data suggests that the 5' flanking region

of *Axl* located between -556/-182 bp includes the minimally required core promoter, since activity of *AxlP7* (-556/+7) and *AxlP8* (-478/+7) are similar, and luciferase activity is lost completely downstream of -478. Region -556/-182 of the promoter is GC-rich, and by TRANSFAC database search is predicted to bind zinc-finger proteins such as Sp family transcription factors (Fig. 1c), but also AP-1 and MZF-1. Overall, our observations generated with all deletion constructs were similar in the Rko, HCT116, and HeLa cell line, excluding cell-line specific biases.

### Five Sp family transcription factor binding sites regulate *Axl* core promoter activity

Deletion constructs and *in-silico* analysis of the promoter revealed putative Sp binding GC-rich regions within a core promoter region of -556/-181. Sp-motifs are defined as Sp a (-225/-219), Sp b (-250/-242), Sp c (-512/-505), Sp d (-524/-516), and Sp e (-529/-523). To analyse their functional relevance, each binding motif was mutated at four bases by site-directed mutagenesis within the *AxlP3* (-1276 to +7) reporter plasmid, which showed maximum promoter activity. Different mutational constructs were generated, with mutations either in single regions (Sp a, Sp b, Sp c, Sp d, Sp e), or combinations of two or more regions (Sp ab, Sp cde, and Sp abcde) (Fig. 2a, Table 1). Mutated and wild type constructs were transfected into Rko and HeLa cell lines and compared for reporter activity. We observed that all single mutations and their combinations significantly reduced promoter activity as compared to the wild type in both cell lines, with the exception of Sp c activity in Rko cells (Fig. 2b). The single mutational constructs Sp a, Sp b, Sp d and Sp e showed 40%, 60%, 30%, and 30% reduced activity as compared to the wild type in Rko cells, and 40%, 30%, 60% and 60% in HeLa cells, respectively. Out of the combined mutational constructs, Sp ab (mutated for Sp a, and Sp b) and Sp abcde (mutated for all 5 motifs) showed the most dramatic (70%) reduction in promoter activity in both of the cell lines. Even though all motifs were mutated in Sp abcde, it didn't reduce promoter activity further when compared with the Sp ab mutational construct. However, the Sp cde combinational construct alone reduced reporter activity by 50% in both of the cell lines, implicating a cooperative function of these Sp-motifs. In addition to Sp mutations, other mutational constructs were generated based on MZF1 and AP1 consensus binding domains at bp (-564/-556, -297/-292), yet neither of them could affect basal promoter activity (data not shown). Taken together, data suggests that all Sp binding sites are relevant for *Axl* core promoter activity, among them Sp a, and b being essential.

### Nuclear proteins of the Sp family bind different binding motifs of the *Axl* core promoter

To demonstrate the binding of Sp family member transcription factors to their respective binding sites in the core promoter of *Axl*, gel shift analysis was performed using Rko cell nuclear extracts, and radio-labelled wild type and mutant probes (Table 1). Sp c, d and e are in close proximity, therefore, a competition experiment was conducted with increasing concentrations of 25x and 50x of either individual (Sp c, Sp d, Sp e) or combination mutant (Sp cde) oligos (using the oligos of site directed mutagenesis), the Sp d wild type oligo being used for labelling, and 50x unlabelled oligos for cold competition (Fig. 3a). As shown in Fig. 3a, competition with mutated Sp c and d completely or partially abolished binding of Sp transcription factor complexes, implicating that the binding of some of the factors stayed intact, whereas with mutant Sp e as a competitor, most of the Sp transcription factors complexes stayed intact. Competition with an oligo mutated for Sp cde did not change the binding complexes, even with increasing concentrations. These data show that transcription factors are binding to this region, however, they are binding predominately to the Sp e site. The same kind of competition experiment was performed for the Sp a, and b sites with their respective labelled wild type oligos. We observed intact binding complexes even after adding increased concentrations of mutated oligos Sp a and Sp b (Fig. 3a). The wild type competition specifically abolished binding to Sp a and Sp b, implicating sequence specific binding of nuclear proteins. To confirm that the specific complexes bound in the EMSA are indeed composed of Sp family members, we performed supershift assays for the Sp a, b, and cde motifs using antibodies against Sp1, Sp2, Sp3, Sp4, and IgG as a control (Fig. 3b). For all three motifs, the incubation with Sp 1, 2 and 3 antibodies led to shift of the major bands, implicating the presence of these Sp family members. Individual super shift analysis of Sp c and e also showed binding of Sp 1 and 3 (data not shown).

In order to analyze the binding of Sp family members to the endogenous *Axl* promoter, we used functionally well characterised antibodies against Sp1 or Sp3 in ChIP analysis in Rko cells. PCR

amplification of the Sp 1/3 immunoprecipitated DNA using primers for Region I (covering Sp a and Sp b, 159bp), Region II (covering Sp cde, 168 bp) (Fig 3) and a non-specific region of the u-PAR promoter without Sp binding sites as a negative control, resulted in single bands of sizes consistent with the respective expected regions of the *Axl* promoter, no specific band being amplified for the non-specific control (Fig. 3c). These results confirmed that Sp1 and Sp3 are indeed binding to the Sp a, b, and cde motifs of the endogenous *Axl* promoter.

### **Mithramycin represses *Axl* promoter activity, and decreases *Axl* mRNA and protein levels**

To further assess the functional relevance of Sp transcription factors on *Axl* gene expression, cells were treated with mithramycin which binds to GC-rich DNA sequences and impedes the binding of GC-specific transcription factors by steric hindrance [21]. Rko cells were transfected with *AxlP3*. After 12 hours of transfection, cells were treated with either medium alone, or various concentrations of mithramycin (25, 50, 100 and 200 nM) for 24 hours. Increasing concentrations of mithramycin gradually and significantly decreased *Axl* promoter activity (Fig. 4a). For example, the 200 nM treated sample showed a 50% reduction of promoter activity when compared with the medium treated control (Fig. 4a). EMSA analysis was performed to assess the ability of mithramycin to prevent Sp-binding to the GC-rich region of the *Axl* promoter in a dose dependent manner (50 to 400 nM concentrations). Pre-incubation with mithramycin inhibited the binding of Sp family transcription factors to all identified Sp binding regions of the *Axl* promoter (Fig. 4b). These observations confirm that the GC-rich and Sp-binding sequences identified contain *Axl* core promoter activity, this being effectively blocked by mithramycin.

To investigate the mithramycin effect on *Axl* gene expression, Rko cells were treated with 100 nM mithramycin for 4, 8, 16, 24 h, as compared to 24 h of medium control. We found that treatment of Rko cells with mithramycin led to an almost 90% decrease in expression of *Axl* mRNA and protein after 16 hours (Fig. 4c, d). Since Sp1 and Sp3 genes also contain GC rich promoters driven by Sp family members, we additionally screened Sp1 and Sp3 mRNA and protein levels following mithramycin treatment. Sp1 and Sp3 mRNA amounts were decreased 4 hours after treatment, and completely un-detectable after 16 hours, which was paralleled by a decrease of Sp1/Sp3 protein amounts (Fig. 4c, d). This data suggests that mithramycin not only inhibits the binding of Sp1 and Sp3 transcription factors to the *Axl* promoter, but also represses the expression of Sp1 and Sp3 by binding to their GC rich promoter regions. The results further confirm Sp1 and Sp3 as essential regulators of *Axl* gene expression.

### **Sp1 or Sp3 increase *Axl* promoter activity and gene expression**

Next, the effect of Sp1 and Sp3 overexpression on the activity of the *Axl* promoter in Rko cells was investigated. Rko cells were co-transfected with the *AxlP3* (-1276/+7) construct, or the mutational constructs for Sp a, Sp b, Sp cde and Sp abcde, respectively, and either Sp1 or Sp3 expression vectors in increasing concentrations. Overexpression of Sp1 and Sp3 led to a significant increase of *Axl* wild type promoter activity in a dose dependent manner (Fig. 5a). In contrast, the mutants Sp a, Sp b, Sp cde, and Sp abcde showed a significant reduction of responsiveness to Sp1 or Sp3. Specifically, mutant Sp a and Sp b did not show any response to Sp3 over-expression as compared to their vector control, and the Sp cde mutant showed a 50% reduction of response to Sp3 when compared with *AxlP3* wild type. The Sp1 response was reduced by 80%, 100%, and 50% for mutant Sp a, Sp b, and Sp cde, respectively, when compared with the *AxlP3* wild type (Fig. 5b). The Sp abcde mutant did not show a significant response to Sp1 or Sp3 overexpression (Fig. 5b, panel Sp abcde). The activity of the Sp abcde mutant following Sp1/3 overexpression was dramatically reduced as compared to the *AxlP3* wild type (Fig 5b, panel *AxlP3* WT). These results confirmed that especially Sp a and b are mediating *Axl* promoter activity following Sp1/Sp3 overexpression, with an additional role for the Sp cde site.

The *Axl* promoter is regulated by especially Sp1 or Sp3, both of which often being ubiquitously expressed in mammalian cells [21]. To gain further evidence for Sp1 or Sp3 being essential for promoter activity, the *Drosophila* SL2 cell line which is devoid of endogenous human Sp family members was cotransfected with *AxlP3*, and either PacSp1, or PacSp3 (insect expression vectors encoding human Sp1 or Sp3). Both Sp1 and Sp3 strongly induced (150%) *AxlP3* promoter activity,



suggesting that either of these transcription factors alone can positively drive *Axl* gene expression (Fig. 5d).

To gain evidence that Sp1 and Sp3-driven *Axl* gene expression is relevant at the protein level, Rko cells were transfected with 4  $\mu$ g of Sp1, Sp3, or a mock control (Vector alone). Both Sp1 and Sp3 induced *Axl* protein amounts after 48 hours of overexpression (Fig. 5c) when compared with the control. Taken together, it is clear that Sp1 or Sp3 positively regulate *Axl* gene expression.

#### **siRNA strategies against Sp1 and Sp3 downregulate *Axl* promoter activity and *Axl* gene expression**

To provide evidence that inhibition of endogenous Sp1 or Sp3 transcription factors can inhibit *Axl* promoter activity and expression, siRNA knock-down experiments were performed. Either Rko or HeLa cells were transfected with either Sp1 or Sp3-specific small interfering oligonucleotides (siRNA) in a dose dependent manner. The knock down of Sp1 and Sp3 was confirmed by Western blotting (Fig. 6 c, d). Expression of a siRNA against Sp1 or Sp3 decreased *Axl* promoter activity to almost 80% and 70% in Rko, and to 50% and 40% in HeLa cells, respectively (Fig. 6 a, b). This was paralleled by a significant reduction of *Axl* protein amounts with increasing concentrations of siRNA for either Sp1 or Sp3, the protein amounts of *Axl* being even further reduced with a combination of si-Sp1 and si-Sp3 (Fig. 6). This confirms that the knockdown of endogenous Sp1 and Sp3 represses transactivation of the *Axl* promoter, and basal *Axl* gene expression in Rko and HeLa cells.

#### ***Axl* gene expression is also dependent on DNA methylation in cultured colorectal cancer cells**

In assessing human cancer cell lines for the concordance of *Axl* mRNA/protein and Sp1/3 expression (Fig. 7a, b), Rko, HCT116, SW480 (colorectal cancer) and HeLa (derived from cervical cancer) cells showed a high endogenous expression of *Axl* which was paralleled by high levels of Sp1 and Sp3 mRNA and protein. However, another group of colorectal cancer cell lines (HCT15, HT29, Geo, WiDr, colo206f) showed an almost undetectable basal *Axl* gene expression despite of moderate expression levels of Sp1 and Sp3 mRNA and protein. This observation promoted us to speculate that, apart from Sp1 and Sp3, other, potentially epigenetic mechanisms, could be influencing basal *Axl* gene expression.

In order to investigate one potential epigenetic mechanism in terms of methylation of CpG sites, the colorectal cell lines Colo206f and WiDr characterized by a low constitutive *Axl* expression were treated with a selective inhibitor of DNA methyltransferases (5-aza-dC) with increasing concentrations. In 5-aza-dC treated cells, *Axl* and Sp3 expression was significantly induced in a dose dependent manner, whereas no significant differences were observed in Sp1 mRNA expression (Fig. 8a). To define methylated regions of the *Axl* promoter and to compare a potential methylation between high- and low *Axl* expressing colorectal cancer cells, 1  $\mu$ g of genomic DNA from WiDr (low *Axl*) and Rko (high *Axl*) cells was subjected to bisulfite conversion. Sequencing revealed that the Sp sites a, b, and c were partially methylated in low *Axl*-expressing WiDr cells (Fig., 8b,c areas 4, 5 and 8), but not in high *Axl*-expressing Rko cells. Furthermore, in WiDr cells, CG-rich flanking regions of GC-rich areas around the Sp-sites identified were also partially methylated (Reg. F, areas 6, 7, 9) (Fig. 8b, c). These data demonstrate that methylated CpG sites, in part within identified Sp-binding motifs, in part in GC-rich flanking regions, influence constitutive *Axl* gene expression apart from the binding of Sp1 and Sp3 transcription factors.

#### **Discussion**

*Axl* as a member of the mammalian Tyro 3 receptor tyrosine kinase family, which is widely overexpressed in different cancers [18], and an increasing number of reports has shown an essential functional relevance of *Axl* in tumor progression, invasion, metastasis, antiapoptosis, angiogenesis, and immunological escape [12,22-25]. In our present work, we have systematically characterized the promoter of this highly important gene in diverse colorectal and also the HeLa cell line for the first time. In the present paper, we have focused on delineating the minimally required basal *Axl* promoter driving endogenous gene expression. In this context, we have clearly identified five different Sp-binding regions within the minimally required basal promoter region -556/-183, bound especially with Sp1 and Sp3 transcription factors. The dominant role of Sp1 and Sp3 in controlling *Axl* gene

expression was solidified by either overexpression or siRNA knockdown of Sp1 and Sp3, which led to decreased or increased promoter activity and Axl protein levels, respectively. This is supported by mithramycin experiments, showing a decrease of promoter activity, mRNA, and protein, and also Sp1 and Sp3 expression, brought about by this GC-rich region binding molecule. In addition to Sp1 and Sp3 as essential regulators of basal Axl gene expression, we show first evidence that the methylation of CpG sites within specific Sp1 motifs, and also in promoter regions flanking these motifs, is modulating *Axl* gene expression.

Sp1, and Sp3 are zinc finger proteins that belong to the Specificity Factor (Sp) family of transcriptional factors, regulating the transcription of many house-keeping genes, but also tissue-specific, viral, and inducible genes by binding and acting through the GC box motif “GGGCGG” [26,27]. Sp family members are characterized by a motif of three conserved C<sub>2</sub>H<sub>2</sub> zinc fingers, which form the DNA-binding domain [27]. Among Sp family transcription factors, very little functional activities are known for Sp2, and Sp4. Sp2 is characterized by the glutamine-rich activation domain, indicating that it can act as a potential activator [21], whereas Sp4 is a tissue specific Sp family member predominately found in the brain. Since, in our present study, we found binding of Sp2 in addition to Sp1 and Sp3 to some of the five Sp-binding motifs identified within the Axl promoter, it is certainly possible that, besides Sp1 and Sp3, also Sp2 will transactivate the Axl promoter via these sites. Due also to a limited availability of suitable constructs and antibodies, however, our study, focused on Sp1 and Sp3, Sp1 being characterized by glutamine rich regions which act as strong activation domains [28], and a short C-terminal domain D, which is required for synergistic activation [21]. Sp3 has been shown to be able to act as either transcriptional activator [30,31] or transcriptional repressor [32] by binding to GC rich regions. It has been suggested that the functional activation of Sp3 also depends on the presence of binding sites within target promoters, since it has been observed that promoters with a single binding site were strongly activated, whereas promoters with multiple binding sites were either inactive or weakly activated by Sp3 [33]. Sp1 and Sp3 share more than 90% sequence homology in the DNA-binding domain and bind to the same GC-rich elements. Therefore, it has been hypothesized that the ratio between Sp1 and Sp3, or the cellular background may lead to either activation or repression of gene expression [34,35]. For the cell lines investigated in our present paper, we did not find a significant correlation between the ratio of expression of Sp1 and Sp3 with the amounts of endogenous Axl mRNA or protein (data not shown), therefore, it is unlikely that a similar situation is relevant for the expression of Axl.

Within the -556/-181 base pair region minimally required for Axl promoter activity, we identified five Sp consensus binding sites within a generally GC rich region. With EMSA and chromatin IP studies, we clearly showed that especially Sp1 and Sp3 are strongly bound to oligonucleotides corresponding to all of the Sp binding motifs, and also to the endogenous GC-rich region. Functional mutagenesis studies for these Sp binding sites revealed that especially the Sp a and Sp b motif are indispensable for basal Axl promoter activity, whereas the Sp cde sites show an additional and modulating relevance for basal promoter activity. Still, since a construct mutated for all five Sp binding sites still exerted a minimal reporter activity as compared to an empty vector control, we cannot rule out the possibility that additional parameters besides Sp factors bound to the five Sp sites identified remotely co-regulate the Axl promoter. For example, Sp family members might bind to GC rich regions other than the Sp consensus motifs identified within the Axl core promoter. Moreover, other zinc finger proteins with a high affinity towards GC rich regions, such as BTEB1 (basis transcription element binding protein 1), TIEG1 or TIEG2 ( TGFβ- inducible early protein 1 and 2) [36-38] might bind to the GC rich Axl core promoter. In addition, an AP-1 and an MZF-1 motif also identified within the core promoter might contribute to a minimal activation, however, this is unlikely since mutations of either of the motifs did not change reporter activity in our luciferase experiments (data not shown). Still, our cotransfection experiments of Sp1 or Sp3 with the Axl promoter, showing a significant increase in promoter activity in the SL2 cell line (null for endogenous expression of human Sp1 and Sp3) [39,40], and our observation that Sp1 or Sp3 significantly increase Axl gene expression, whereas an siRNA knock down for either Sp1 or Sp3 dramatically reduced Axl promoter activity and expression, clearly imply the notion that both transcription factors are essential for Axl promoter transactivation, and endogenous gene expression [40]. In this context, it is interesting to note that a second ligand of the Axl receptor, protein S, has also been shown to be constitutively regulated by Sp1 and Sp3

transcription factors [39]. Although these experiments had been conducted mainly in the hepatocytic cell line HepG2, it is interesting to speculate that in gastrointestinal tumors, Sp1 and Sp3 may regulate the constitutive expression of a tyrosine kinase receptor highly relevant for tumor progression, invasion, and angiogenesis, in addition to upregulating one of its specific ligands, to enhance tumor progression.

Mithramycin, having been shown to reduce the activity of the Sp1 promoter as well as to inhibit Sp1 binding [41], in our present study reduced Axl promoter activity in a dose dependent manner, and almost abolished the expression of mRNA and protein of Axl, Sp1, and Sp3 within 24 hours [40]. Mithramycin is a cell-permeable reagent frequently used to explore the sequence dependence of DNA-binding factors known to bind to GC-rich regions by steric hindrance. In our present study, overexpression or knock down of Sp1 or Sp3 had a significant, however not 100% impact on Axl promoter activity, whereas mithramycin treatment completely abolished the expression of Axl mRNA or protein. One explanation for this small discrepancy might be that all GC rich regions of the Axl promoter were completely blocked by mithramycin. Moreover, as already stated above, other transcription factors known to bind to GC rich regions and/or exhibit similar binding specificity as Sp1, such BTEB1, TIEG1 or TIEG2, might add to Axl promoter transactivation to a small extent, and might also have been blocked by mithramycin. At the present time, we cannot rule out the possibility of a remote additional impact of suchlike transcription factors apart from Sp1 or Sp3 in *in vivo* conditions.

In the present paper, we also provide the first systematic screening of a number of human colorectal cancer cell lines in addition to HeLa (cervical cancer cell line) for transcript and protein amounts of human Axl, Sp1, and Sp3. In Rko, HCT116, SW480 and HeLa cells, a high endogenous Axl expression was correlated with high amounts of endogenous Sp1 and Sp3. On the other hand, HCT15, HT29, Geo, WiDr and Colo206f cells did not show significant endogenous Axl gene expression, although they have detectable transcript and protein levels of Sp1 or Sp3. This certainly can be explained by the fact that Sp1 and Sp3 regulate many more genes that might also be of relevance for the latter cell lines, besides Axl. Moreover, the activity of Sp family transcription factors towards specific promoter motifs is certainly not only defined by their expression, but also by post-translational modifications and protein-protein interactions. It has been well described that post-translational modifications such as phosphorylation, glycosylation, or sumoylation modify the transactivating activity of, for example, Sp1 [42,43]. Also, Sp1 can exert indirect effects on transcription via its interaction with, for example, chromatin remodelling factors (p300 or PCAF), and other DNA-binding proteins [26,42,44,45]. Furthermore, as stated before, the ratio of Sp1 to Sp3 in certain cellular contexts may determine the degree of activation [46,47]. Suchlike mechanisms certainly can explain the discrepancy between Axl, Sp1, and Sp3 expression levels found in some of the cell lines investigated.

Our finding that the methylation of some of the Sp binding sites, and their flanking regions, within the basal Axl promoter may modulate Axl gene expression, certainly merits discussion. CpG methylation is well known to be important for gene imprinting [48], cell cycle control, tumor suppression, embryonic development, differentiation, and other phenomena [49]. Specifically, there have been many interesting reports showing that the methylation of CG sites within Sp transcription factor binding motifs, but also of their flanking regions, might affect binding and the transactivating potential of Sp1 and Sp3 [50-52]. In the present study, we found that the basal Axl promoter is extremely GC rich, with 19 CpG sites within the region -669/-97, and harbours many putative CpG methylation sites. CpG methylation can inhibit Sp1 binding by two mechanisms, either by directly inhibiting the binding of Sp1, or by contributing to a competition with other factors like MeCP2 for binding sites [53,54]. In our present study, we found that some of the CpG sites within Sp family binding motifs, or within adjacent CG rich regions, were partially methylated in WiDr cells, which are characterized by an almost undetectable endogenous Axl gene expression. In contrast, in high Axl expressing Rko cells, no methylation of such sites was found. This observation suggests that CpG methylation of the basal Axl promoter is related to endogenous Axl gene expression. To substantiate this hypothesis, we found that a demethylating agent (5-aza-dC) was able to activate Axl gene expression in the low Axl expressing WiDr cell line. Moreover, not only Axl, but also Sp3 expression was induced by 5-aza-dC

in Colo206f and WiDr cells in a dose dependent manner. This supports the hypothesis that in low Axl expressing colon cancer cells, transcription of the Axl gene is kept silent by partial methylation of CpG islands, within the basal promoter. The additional interesting observation that 5-aza-dC directly affected the expression of Axl and Sp3, but not Sp1, prompts us to speculate that the demethylating agent interferes with the ability of Sp1 and Sp3 to transactivate Axl gene expression in two different ways: in case of Sp3, it may directly interfere with Sp3 gene expression by demethylating sequences within its gene promoter. In contrast, it may directly modulate Sp1 binding to the Axl gene promoter by demethylating target sites within the Axl promoter sequence. Thus, the effect of 5-aza-dC on Sp1/Sp3 regulated Axl promoter activity might be both direct and indirect.

Taken together, this is the first study to characterize the Axl gene promoter, and to establish a major role for Sp1, Sp3, and also methylation of the core promoter, as key regulators of constitutive Axl gene expression in diverse colorectal and HeLa cancer cells. Further ongoing studies of our group will elucidate the function of further upstream transcriptional elements in the constitutive and inducible expression of this tyrosine kinase receptor, which is essential for many functions associated with cancer.

### Acknowledgements

HA was supported by Alfried Krupp von Bohlen und Halbach Foundation (Award for Young Full Professors), Essen, Wilhelm Sander Stiftung, Munich, Auguste-Schaedel-Dantscher-Stiftung, Garmisch, Hella-Bühler-Foundation, Heidelberg, Dr. Ingrid zu Solms Foundation, Frankfurt, B. Braun Foundation, Melsungen, and the Hector Foundation, Weinheim, Germany. This publication contains parts of the dissertation of Giridhar Mudduluru performed in partial fulfillment of the requirements for the PhD at the Medical Faculty Mannheim, University Heidelberg, and the DKFZ (German Cancer Research Center) Heidelberg. We thank Erika Hillerich for excellent help with the manuscript. We thank Dr. Guntram Suske, Philipps-Universität Marburg, Germany, for the generous gift of Sp1 and Sp3 human and insect expressing vectors.

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## Footnotes

The abbreviations used are: ChIP, chromatin immunoprecipitation; RT-PCR, Reverse transcription PCR; siRNA, small interfering RNA; scRNA, scrambled RNA;

## Tables

**Table 1. Oligos used for EMSA and Mutagenesis**

S. No	Name	Sequence of normal(N) and mutated(M) oligos (5'→3')	Position (5'to3')
1	Sp_a_For_N	CAAAGGGGAGCCAGGGGCGGAGAAAGGGTTGCCCAAG	-239 to -202
2	Sp_a_For_M	CAAAGGGGAGCCAGG tt ta GAGAAAGGGTTGCCCAAG	
3	Sp_b_For_N	GGCTCTGGCCCTGGTGGGCGGAGGCAAAGGGGGAGC	-263 to -228
4	Sp_b_For_M	GGCTCTGGCCCTGGTGG tt ta AGGCAAAGGGGGAGC	
5	Sp_c_For_N	GGCTGGGGGTGGAGGGCGGGGAGAGGGGCGTCACG	-524 to -491
6	Sp_c_For_M	GGCTGGGGGTGGAG ta t tGGAGAGGGGCGTCACG	
7	Sp_d_For_N	CCTGGCTGGGGCTGGGCTGGGGGTGGAGGGGGAGAGG	-538 to -500
8	Sp_d_For_M	CCTGGCTGGGGCTGGG t e t t tGGTGGAGGGGGGAGAGG	
9	Sp_e_For_N	GGAGGCTGGCTGGGGCTGGGCTGGGGGTGGAGGGCG	-543 to -508
10	Sp_e_For_M	GGAGGCTGGCTGGG ta T taGCTGGGGGTGGAGGGCG	
11	Sp_cde_For_M	CTGGCTGGG ta t taGt c t tGGTGGAG ta t tGGAGAGG	-537 to -500

Sp\_x\_For\_N : Forward normal oligo; Sp\_x\_For\_M : Forward Mutated oligo

**Table 2. Primers used for delineation constructs**

S.No	Name	Sequence (5'-----3')	5' position
1	AxIF1	GAAGGTACCATGACAACCCAGGCAAAGTG	-2376
2	AxIF2	GAAGGTACCACAAAGCAAGACTGGGTCTC	-1759
3	AxIF3	GAAGGTACCCAGTCCACCAGAAGGAGAG	-1276
4	AxIF4	GAAGGTACCTGCCCGCCAACAATTC	-1010
5	AxIF5	GAAGGTACCTGTCTGTGGCCAGTAGC	-727
6	AxIF6	GAAGGTACCGTGGTGTGTGTGTGTGTG	-614
7	AxIF7	GAAGGTACCAATGAAGGGCCAAGGAGGC	-556
8	AxIF8	GAAGGTACCAGGGGAGTGGAGTCTGG	-478
9	AxIF9	GAAGGTACCGGCAGGGGTGCTGAGAAG	-181
10	AxIR1	GAACTCGAGACGCCATGGGTGCCAAAC	+7
11	AxIR2	GAACTCGAGGAGCTGAGGAGGGGGAAG	-338

AxIF: Axl Forward; AxIR: Axl Reverse

## Figure legends

### Fig 1 Identification of the core promoter region of *Axl*.

a) The schematic representation shows the design of pGL3 reporters to test the relative contribution of different portions of the *Axl* promoter. b) Luciferase reporter assay showing *Axl* promoter activity in Rko, HCT116 and HeLa. 1  $\mu$ g of *Axl* constructs were co-transfected with a Renilla luciferase plasmid, serving as internal control for transfection efficiency, into Rko, HCT116 and HeLa, and luciferase activity was measured 48 h post transfection. Activity of AxIP1 (-2376/+7), the largest deletion construct, was set to 100% (first bar), and activity of the other constructs was calculated and plotted as a percentage of this value. All samples were performed in triplicate, the bars representing the mean. Error bars, ( $\pm$  S.D). c) Schematic representation of maximum promoter activity showing promoter region AxIP3 (-1276/+7) with different transcription start sites, and putative Sp family binding sites.

### Fig 2 Effect of site directed mutagenesis for Sp transcription factor binding sites on *Axl* promoter activity.

a) Schematic representation of site directed mutagenesis performed for Sp family binding motifs within the core promoter region. b) AxIP3 (-1276/+7), the promoter construct showing maximum activity, was mutated at the different sites indicated, either single or combinations. 1  $\mu$ g of wild type/mutant *Axl* promoter construct along with Renilla luciferase plasmid, were transfected into Rko and HeLa cells. AxIP3, wild type activity was set to 100% (first bar), and activity of the mutated constructs was calculated and plotted as a percentage of this value. All samples were performed in triplicate and the results were averaged and plotted as shown. Error bars, ( $\pm$  S.D). The statistical difference of the activity of the mutated constructs in all panels is shown against AxIP3 WT activity. \*\*,  $p \leq 0.05$ .

### Fig 3 EMSA, super shift and chromatin immunoprecipitation assays of Sp family members

a) EMSA was performed with Rko nuclear extract and a  $\gamma$ -p33 labelled oligonucleotide corresponding to Sp a, Sp b and Sp d of the *Axl* promoter. For competition, a 25 and/or 50-fold excess of unlabelled wild type or mutant oligos was used (Sp a, Sp b, Sp c, Sp d, Sp e and Sp cde). 5  $\mu$ g of nuclear extract were incubated with unlabelled wild type or mutant oligos for 20 min prior to the addition of labelled oligonucleotides, and incubated for additional 20 min. Samples were electrophoresed on a 4% tris borate-EDTA polyacrylamide gel at 220 volts for 3 hours, dried and exposed on x-ray film over night. b) Super shift analysis performed for Sp family members (Sp1, Sp2, Sp3 and Sp4) binding to motifs Sp a (-239/-202 bp), Sp b (-263/-228 bp), and Sp cde (538/-500 bp). 5  $\mu$ g of nuclear extract was incubated for 20 min with unlabelled oligos for competition, followed by the  $\gamma$ -p33 labelled probe for 20 min. Complexes were incubated with 2  $\mu$ g of antibody/IgG as indicated for 1 hour at 4 degrees. IgG served as negative control. Specific supershifted complexes are indicated with arrows. c) The association of Sp1 and Sp3 with the endogenous *Axl* promoter was assayed with ChIP in Rko cells. Sp1/Sp3 immunoprecipitated DNA was amplified by PCR using two sets of specific primers (Region I, Region II, see methods). As a negative control, off-target primers were used. 10% of the total input served as positive control.

### Fig 4 *Axl* promoter activity and expression is repressed by mithramycin.

a) pGL3-basic and AxIP3 were transfected into Rko cells. 12 hours after transfection, cells were mock-treated or treated with 25, 50, 100, or 200 nM mithramycin. Another 24 h after treatment, all plates were harvested and the resulting luciferase activity was measured. Equal amounts of DNA (1  $\mu$ g) were transfected for each sample. Luciferase activity of cells transfected with AxIP3 and unexposed to mithramycin was set to 100% (first bar), and the activities of all other samples were calculated and plotted as a percentage of this value. Relative luciferase activity of each sample was normalized to the activity of a co-transfected Renilla luciferase plasmid. All assays were performed in triplicate, and the results were averaged. Error bars, ( $\pm$  S.D). The statistical difference of the promoter activity is shown against AxIP3 untreated transfected cells. \*\*,  $p < 0.05$ . b) Mithramycin binding to GC-rich regions inhibits the binding efficiency of Sp family members. EMSA analysis was performed for the regions containing Sp a (-239/-202 bp), Sp b (-263/-228 bp) and Sp cde (538/-500 bp) with mithramycin incubation.  $\gamma$ -p33 labelled oligos were pre-incubated for 1 hour with mithramycin with increasing concentrations (50, 100, 200 and 400 nM), and 50x excess wild type oligos were pre-incubated for 20 min before adding labelled oligos. Sp transcription factor binding efficiency was reduced for all



regions with dose dependent incubations of mithramycin. c) Mithramycin treatment leads to decreased *Axl*, *Sp1* and *Sp3* mRNA levels. RT-PCR was performed on total mRNA isolated from Rko cells which were either mock-treated (control) or treated with 100 nM mithramycin, and harvested at the indicated times after exposure. Reactions were performed in parallel under identical conditions, using primer pairs targeting *Axl*, *Sp1*, *Sp3*, or GAPDH as control. The final products were separated via electrophoresis in ethidium bromide-labeled agarose and photographed under UV illumination. d) Mithramycin treatment decreases *Axl*, *Sp1* and *Sp3* protein levels in a time dependent manner. Rko cells were treated as for RT-PCR analysis, and cell lysates were separated via SDS-PAGE, transferred to PVDF membranes, and immunoblotted for *Axl*, *Sp1* and *Sp3*, or actin (as a loading control).

**Fig 5 Overexpression of Sp1 or Sp3 induces *Axl* promoter activity and protein expression.**

a) Overexpression of *Sp1* or *Sp3* in Rko cells increases activity of the *Axl* promoter. Rko cells were cotransfected with 1  $\mu$ g of *AxlP3* (-1276/+7) and 0.5  $\mu$ g and 1  $\mu$ g of the *Sp1* or the *Sp3* expression vector, along with 50 ng of renilla luciferase plasmid. *Sp1* and *Sp3* increased *Axl* promoter activity in a dose dependent manner. Mock vector co-transfected *AxlP3* luciferase activity was set to 100%, and activity of the other constructs was calculated and plotted as a percentage of this value. b) Rko cells were cotransfected with 1  $\mu$ g of *AxlP3* wild type or mutated luciferase reporter vectors and 1  $\mu$ g of either *Sp1* or *Sp3* expressing vectors. *AxlP3*, mock treated activity was set to 100% (first bar), and activity of the mutated constructs activity were calculated and plotted as a percentage of this value. All samples were performed in triplicate and the results were averaged and plotted as shown. Error bars, ( $\pm$  S.D). c) Over-expression of *Sp1* or *Sp3* increases *Axl* protein amounts. Rko cells were transfected in a 6 well plate with 4  $\mu$ g of pCMV-*Sp1*, pCMV-*Sp3*, and vector alone as a mock control. After 48 hours of transfection, cells were harvested and cell lysates immunoblotted for *Sp1*, *Sp3*, *Axl*, and actin (Loading control). Either *Sp1* or *Sp3* overexpression led to increased amounts of *Axl* protein. d) Either *Sp1* or *Sp3* can drive *Axl* promoter activity in SL2 cells. SL2 insect cells were tested for reporter activity under different conditions. Cells were plated in a 24 well plate and transfected with the *AxlP3* luciferase reporter either with pPac, pPac*Sp1*, or pPac*Sp3*, along with Renilla (which drives expression of *Sp1* and *Sp3* via an insect-specific promoter). As shown, *Sp1* and *Sp3* induces *Axl* promoter activity in the SL2 cell line. Renilla served as internal control for transfection efficiency. All samples were performed in triplicate and the results averaged and plotted as shown, with all results expressed as a percentage of the activity resulting from the *AxlP3* promoter and pPac (which was set at 100%). Error bars, ( $\pm$  S.D). The statistical difference of the promoter activity in all panels is shown against *AxlP3* mock transfected cells. \*\*,  $p < 0.05$ .

**Fig 6 Knockdown of either Sp1 or Sp3 decreased *Axl* promoter activity and protein amounts**

a,b) Rko or HeLa cells were transfected with control scRNA (scrambled RNA) or a specific siRNA targeting either *Sp1* or *Sp3* in a dose dependent manner. Forty-eight hours after transfection, all cells were harvested for luciferase assay. siRNAs targeting either *Sp1* or *Sp3* led to decreased *Axl* promoter activity. In contrast, scRNA had no effect on *Axl* promoter activity. *AxlP3* luciferase activity was set to 100% (first bar), and activity of the other constructs was calculated and plotted as a percentage of this value. All samples were performed in triplicate, and the results were averaged and plotted as shown. Error bars, ( $\pm$  S.D). The statistical difference of the promoter activity is shown against *AxlP3* scRNA transfected cells. \*\*,  $p < 0.05$ . c,d) Either Rko or HeLa cells were transfected with siRNA targeting *Sp1* or *Sp3*, and/or both of them, in a dose dependent manner, scRNA as a control. After 48 hours, cells were harvested and cell lysates immunoblotted for the proteins indicated. scRNA has no effect on either *Sp1* or *Sp3*.

**Fig 7 Screening for *Axl*, *Sp1* and *Sp3* expression in nine different cell lines**

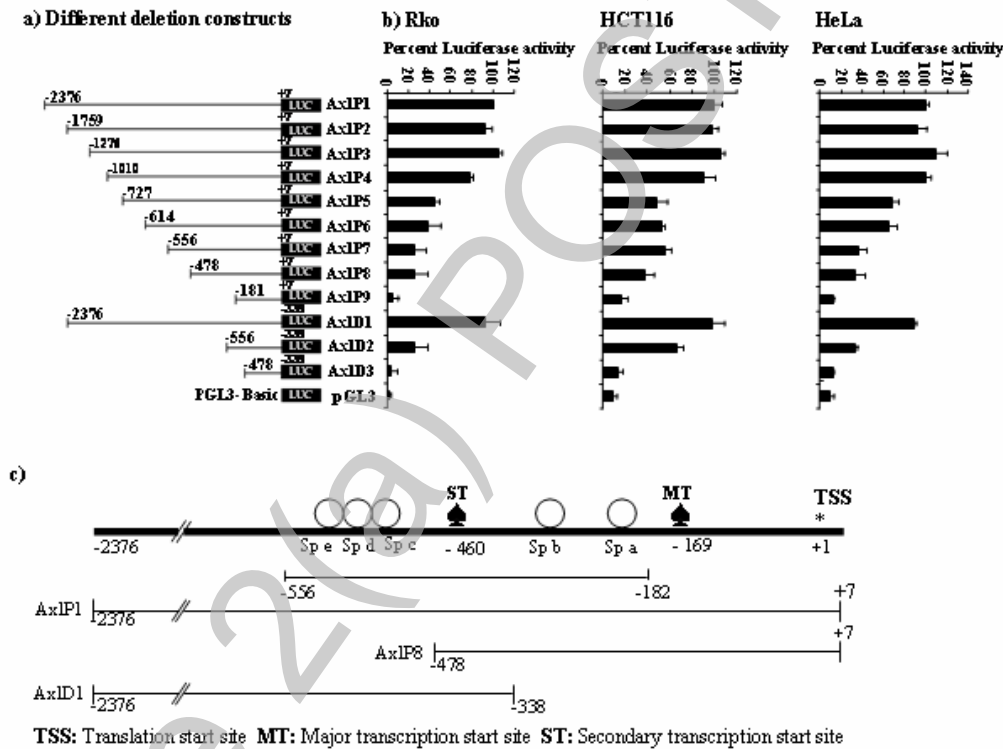
a,b) All the cells were grown and treated under identical conditions: Cells were harvested in their exponential phase for either for RNA or protein lysate. Expression transcript levels of *Axl*, *Sp1*, *Sp3* and GAPDH (Control) were screened through RT-PCR, and the lysates separated via SDS-PAGE, transferred to PVDF, and immunoblotted for *Axl*, *Sp1*, *Sp3*, and actin (loading control).

**Fig 8 Effects of 5-Aza-dC treatment on the expression of Axl, Sp1 and Sp3 mRNA in colorectal cancer cell lines, and Bisulfite sequencing of CpG sites within the Axl promoter.**

a) RT-PCR analysis for Axl, Sp1 and Sp3 mRNA expression in Colo 206 f and WiDr cells treated with 10, 20, 40  $\mu$ M 5-Aza-dC for 6 d, showing that the demethylation reagent induces *Axl* and *Sp3* gene expression but not *Sp1*. b) Schematic representation of the CpG sites of Axl. This sequence spans 572 bp between positions -669 to -97 relative to the translation start site, including 19 CpGs. There are three CpG-containing Sp binding sites within this sequence that are indicated with shaded circles, corresponding to Sp a,b, and Sp c, respectively (position 4, 5 and 8). c) DNA from WiDr cells was treated with bisulfite, and the Axl promoter was PCR amplified. The PCR product was ligated into the pGMT easy vector by using the TA cloning system. Ten subclones were picked and sequenced. As a result, some of the CpG sites, some within the Sp a,b and c motif, others within GC-rich flanking regions, are partially methylated in WiDr cells.

**Figures**

**Fig 1**



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BSR20080046

Fig 2

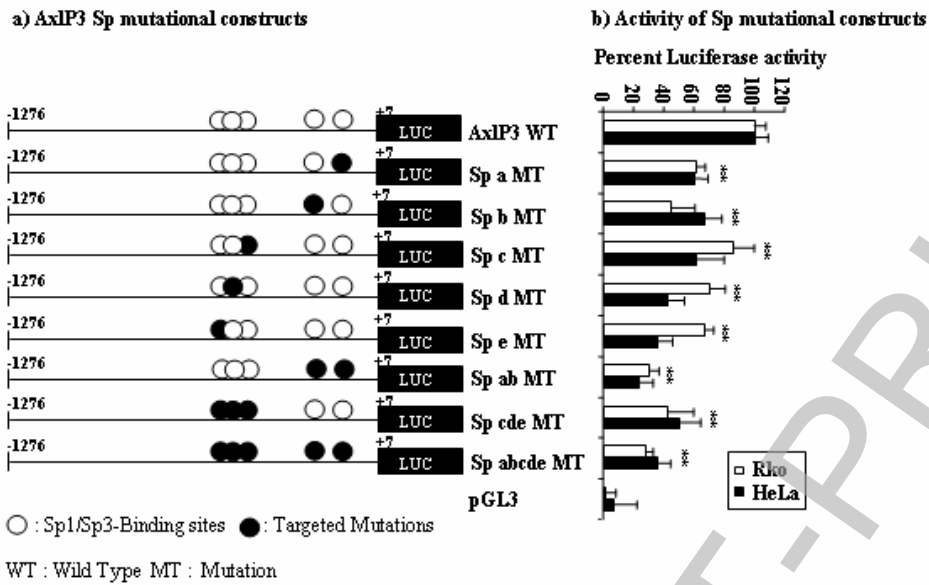
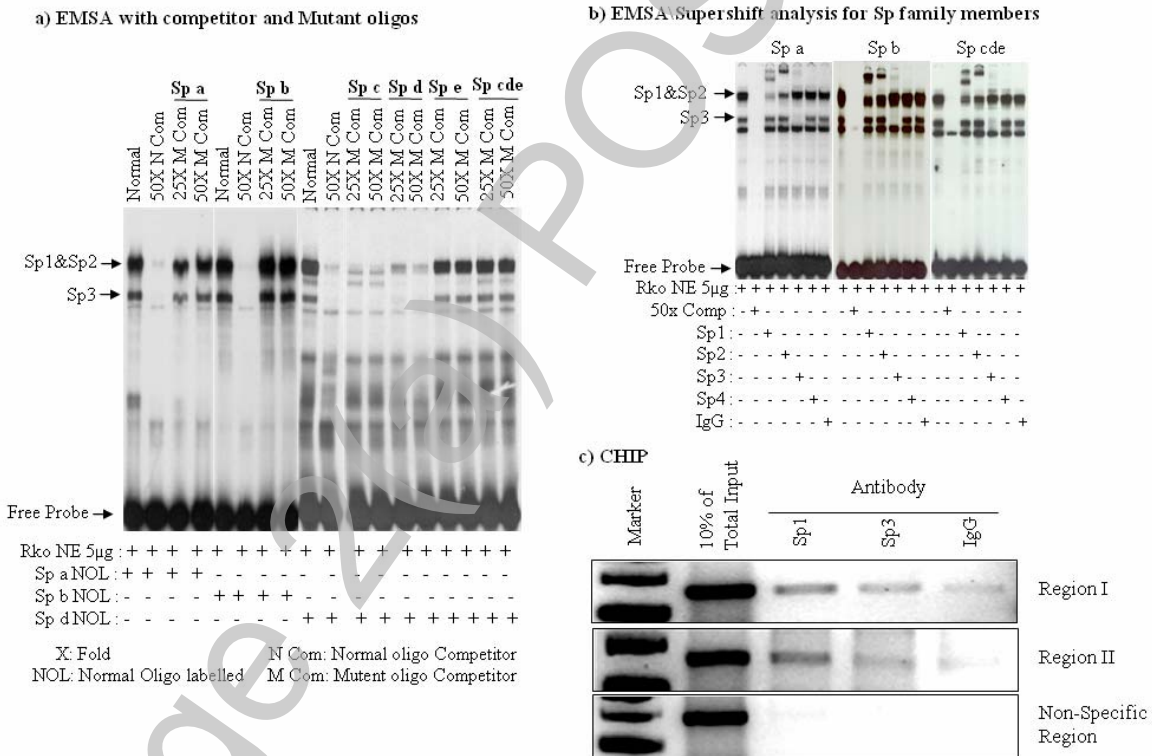


Fig 3



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BSR20080046

Fig 4

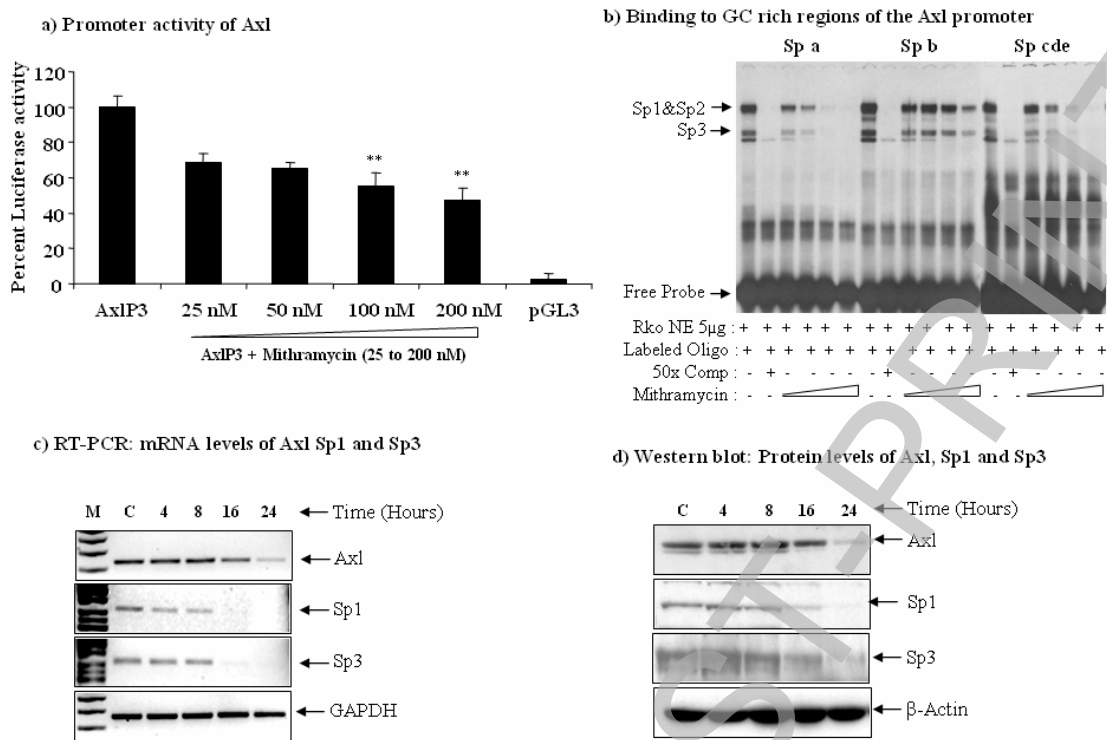
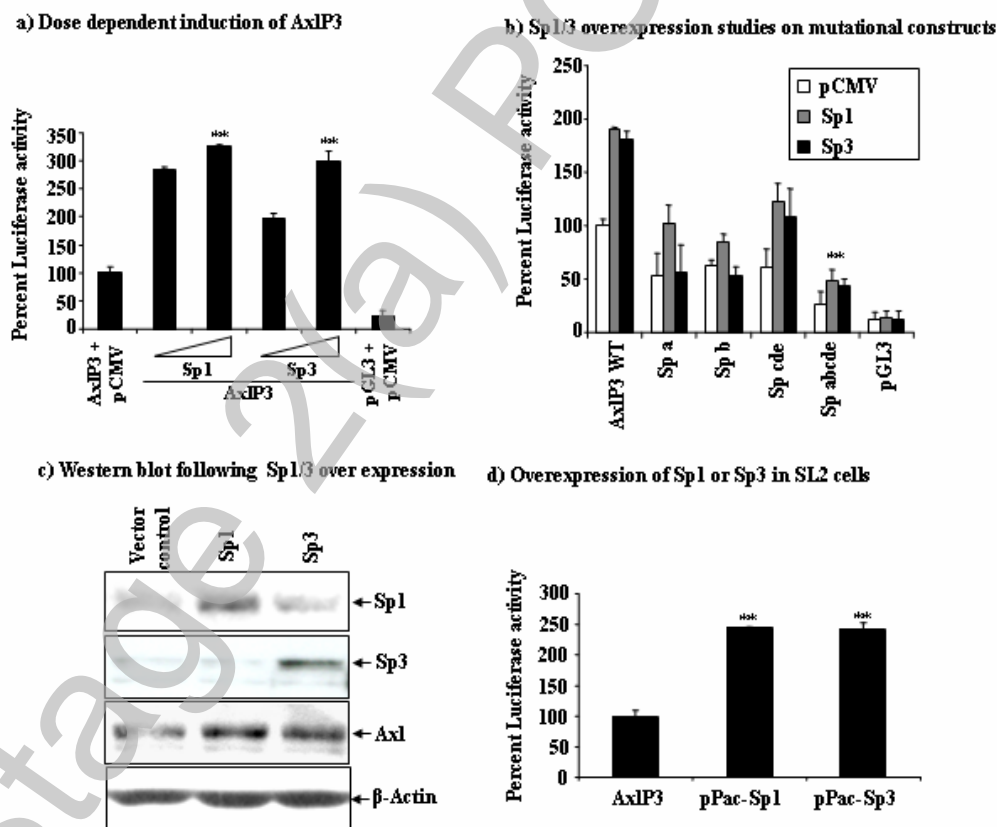


Fig 5



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BSR20080046

Fig 6

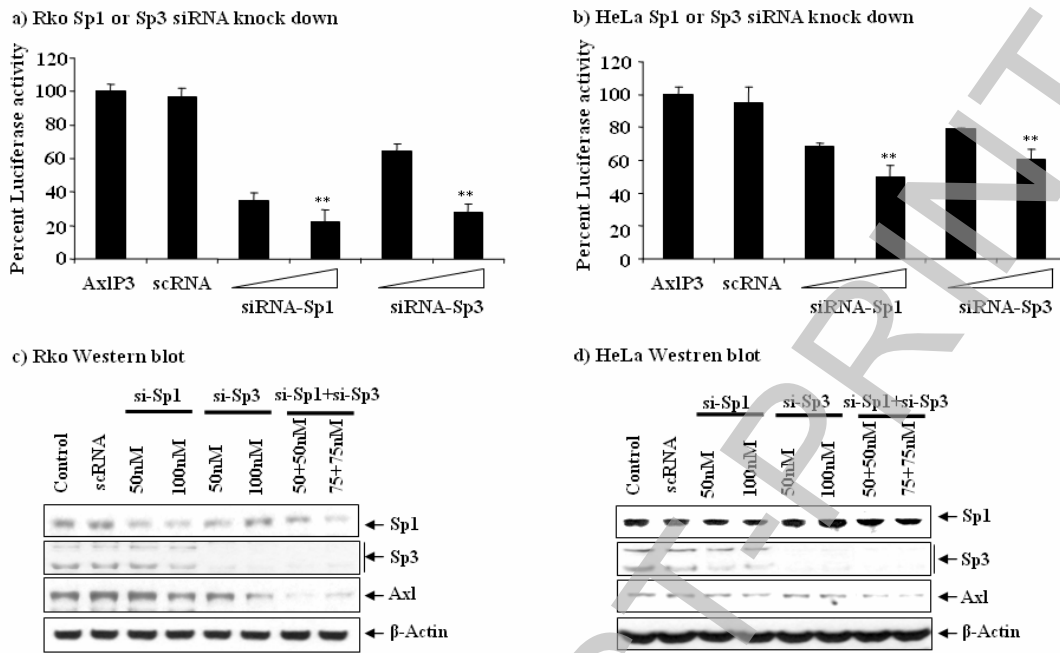
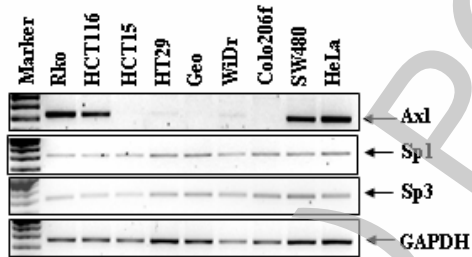


Fig 7

**a) RT-PCR: mRNA levels of AXL, Sp1 and Sp3**



**b) Western blot: Protein levels of Axl, Sp1 and Sp3**

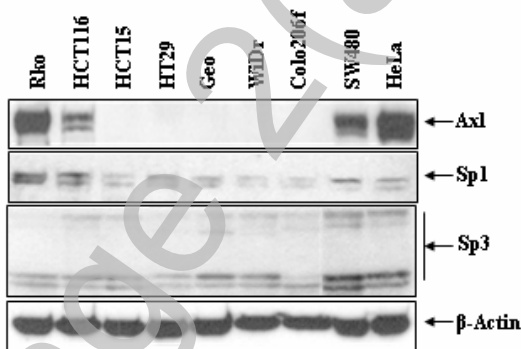
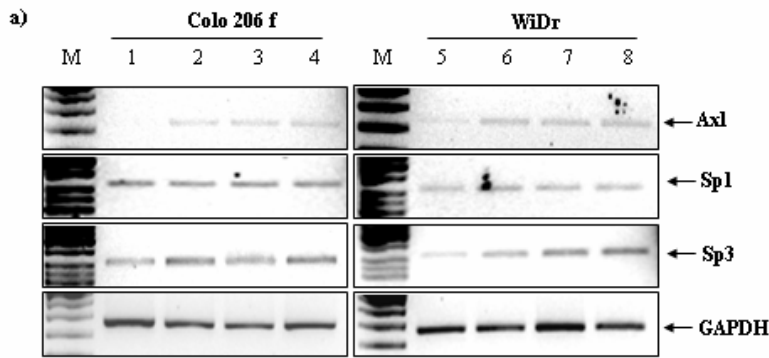
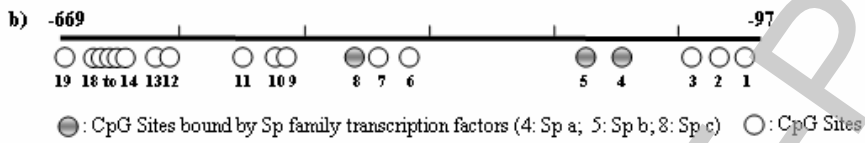


Fig. 8



M: Marker; 1,5: Controls; 2,6: 10  $\mu$ M; 3,7: 20  $\mu$ M; 4,8: 40  $\mu$ M of 5-Aza added to the respective cells



c) Methylation status of CpG sites in WiDr cells

