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

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Synopsis

Axl is a receptor tyrosine kinase which promotes anti-apoptosis, 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, *cis*-acting elements and promoter methylation driving expression of *Axl*. The 2.4 kb sequence upstream of the translational start site, and sequential 5′-deletions were cloned and revealed a minimal GC-rich region (−556 to +7) to be sufficient for basal Axl promoter activity in Rko, HCT116 and HeLa cells. Within this minimal region, five Sp (specificity protein)-binding sites were identified. Two sites (Sp a and Sp b) proximal to the translation start site were indispensable for Axl promoter activity, whereas mutation of three additional upstream motifs (Sp c, Sp d and Sp e) was of additional relevance. Gel-shift assays and chromatin immunoprecipitation identified that Sp1 and Sp3 bound to all five motifs, and mutation of all motifs abolished binding. Mithramycin, which inhibits binding of Sp factors to GC-rich sites, dramatically reduced Axl promoter activity and Axl, Sp1 and Sp3 expression. In *Drosophila* Schneider SL2-cells, exogenous expression of Sp1/Sp3 increased Axl promoter activity. Use of Sp1/Sp3 siRNAs (small interfering RNAs) significantly reduced Axl promoter activity and protein levels in Rko and HeLa cells. Methylation-bisulfite sequencing detected methylated CpG sites within three Sp motifs (Sp a, Sp b and Sp c) and GC-rich flanking sequences, and demethylation by 5-aza-2′-deoxycytidine up-regulated Axl and Sp3 expression in low-Axl-expressing Colo206f/WiDr cells, but not in high-Axl-expressing Rko cells. The results of the present study suggest that *Axl* gene expression in cancer cells is (1) constitutively driven by Sp1/Sp3 bound to five core promoter motifs, and (2) restricted by methylation within/around Sp-binding sites. This might enhance the understanding and treatment of essential mechanisms associated with cancer and other diseases.

Key words: Axl promoter, Axl receptor tyrosine kinase (RTK), 5-aza-2′-deoxycytidine (5-aza-dC), CpG methylation, specificity protein (Sp), transcription

INTRODUCTION

Axl (from the Greek word ‘anexelekto’ or uncontrolled) belongs to the Tyro 3 family of RTKs (receptor tyrosine kinases) which includes Mer and Sky, and was originally identified as a transforming gene in human leukaemias [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 extracellular structure, resembling neural cell-adhesion molecules), and a distinctive intracellular kinase domain [1]. Human Axl is a 140 kDa protein with a 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 Tyro 3 family members with different affinities. Gas6 was first discovered as a gene which is up-regulated in growth-arrested

Abbreviations used: AP-1, activator protein 1; 5-aza-dC, 5-aza-2′-deoxycytidine; BTEB1, basis-transcription-element-binding protein 1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility-shift assay; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MZF1, myeloid zinc finger 1; NF-κB, nuclear factor κB; RT–PCR, reverse transcription–PCR; RTK, receptor tyrosine kinase; siRNA, small interfering RNA; Sp1 etc., specificity protein 1 etc; TIEG1 etc., transforming growth factor β-inducible early protein 1 etc; TTBS, Tris-buffered saline with 0.1% Tween 20; uPAR, urokinase plasminogen activator receptor.

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cells [5], suggesting a role in the prevention of cellular stresses, such as apoptosis. Subsequent studies have suggested Gas6 has a 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]. Overexpression 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 PI3K (phosphoinositide 3-kinase), Akt, Src, ERK (extracellular signal-regulated kinase) and NF-κB (nuclear factor κB) [15,16]. Gas6/Axl signalling is well studied in NIH 3T3 mouse fibroblasts, where it is known to transduce anti-apoptotic signals via NF-κB 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 and certain types of breast cancers, and in sarcomas [18].

The *Axl* gene is located on 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 have been found for these isoforms. In one initial study, the *Axl* 5′-upstream region was shown to be GC-rich, lack TATA and CAAT boxes, and harbour multiple transcription start sites. These transcription start sites include the major start site at −169, and a second major start site at −460, 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 characterization of its promoter have been published so far.

Therefore in the present study we have characterized the Axl promoter and its methylation status in different colorectal cell lines. We identified binding sites for various transcription factors within the −556 to −182 core region upstream to the *Axl* translational start codon, among them five binding sites for Sp1 (specificity protein 1)/Sp3, one for MZF1 (myeloid zinc finger 1) and one for AP-1 (activator protein 1). Furthermore, we show that the Sp-binding sites, or adjacent CpG sites, are partially methylated in low-Axl-expressing cells, in contrast with 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 cells.

EXPERIMENTAL

Cell culture, reagents and treatments

All human cells were obtained from the A.T.C.C. (Manassas, VA, U.S.A.), and grown at 37°C in DMEM (Dulbecco's modified Eagle's medium), MEM (minimal essential medium), McCoy's or RPMI 1640 medium supplemented with 10% (v/v) FCS (fetal calf serum). Mock-treated control cells were handled in an identical manner with 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% (v/v) FCS at 27°C. Mithramycin was purchased from Sigma–Aldrich.

Preparation of protein extracts and immunoblotting

Cells were washed with PBS and lysed in extraction buffer (Biosource). The protein concentration was determined using the BCA (bicinchoninic acid) kit (Pierce). For immunoblotting, samples (40 μg/lane) were boiled for 5 min, separated via SDS/PAGE (10% gels), and transferred on to PVDF membranes. After transfer, the membranes were blocked with 5% (w/v) non-fat dried skimmed milk powder in TTBS (Tris-buffered saline with 0.1% Tween 20) for 3 h at room temperature (25°C), and then probed with the indicated primary antibodies (all from Santa Cruz Biotechnology) against Sp1 (sc-59x, 1:2500 dilution) Sp3 (sc-644x, 1:5000 dilution), Axl (sc-1096, 1:1500 dilution) and β-actin (sc-1616, 1:2000 dilution) for 2 h at room temperature. After three washes with TTBS, the blots were incubated at 25°C with either rabbit (Sp1, Sp3 and β-actin) or goat (Ax1) (1:5000 dilution) horseradish-peroxidase-conjugated secondary antibodies for 30 min. After final washes with TTBS, the membranes were exposed to film after use of ECL® (enhanced chemiluminescence) (Amersham Biosciences).

RNA isolation and RT–PCR (reverse transcription–PCR)

Total RNA was extracted from tissue-culture cells using the RNeasy Midi kit (Qiagen) following the manufacturer's instructions. The RNA samples were quantified using a spectrophotometer and visualized on a Mops/formaldehyde gel (1% gel) for quality assurance. Total RNA (1 μg) was reverse-transcribed by random hexamer primers using SuperScript II reverse transcriptase (Invitrogen). The single strand cDNA was amplified by PCR using specific primer pairs. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. The PCR products were analysed by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide. The sequences of the sense and antisense primers used for RT–PCR were: GAPDH (sense, 5′-GTCTTCACCACCATGGAGAA-3′ and antisense, 5′-ATCCACAGTCTTCTGGGTGG-3′) and Axl (sense, 5′-GGTGGCTGTGAAGACGATGA-3′ and antisense, 5′-CTCAGATACTCCATGCCACT-3′).

Preparation of nuclear extract and EMSA (electrophoretic mobility-shift assay)

Preparation of nuclear extracts and EMSAs were essentially performed as described previously [20]. In brief, nuclear extracts (5 μg) were incubated in a buffer [25 mM Hepes (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT (dithiothreitol), 0.05 M NaCl and 4% (v/v) glycerol] with 500000 c.p.m. of [γ-^{33}P]ATP (PerkinElmer) (phage T4 polynucleotide kinase, ICN #151935)

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Table 1 Oligonucleotides used for EMSA and site-directed mutagenesis
Where appropriate, the mutated nucleotides are indicated by underlining. For, forward; M, mutant oligonucleotide; N, normal oligonucleotide.

Number	Name	Sequence (5′ → 3′)	Position (5′ to 3′)
1	Sp_a_For_N	CAAAGGGGGAGCCAGGGGCGGAGAAAGGGTTGCCCAAG	−239 to −202
2	Sp_a_For_M	CAAAGGGGGAGCCAGGTTTAGAGAAAGGGTTGCCCAAG	
3	Sp_b_For_N	GGCTCTGGCCCTGGTGGGCGGAGGCAAAGGGGGAGC	−263 to −228
4	Sp_b_For_M	GGCTCTGGCCCTGGTGGTTTAAGGCAAAGGGGGAGC	
5	Sp_c_For_N	GGCTGGGGGTGGAGGCGGGGAGAGGGGCGTCACG	−524 to −491
6	Sp_c_For_M	GGCTGGGGGTGGAGTATTGGAGAGGGGCGTCACG	
7	Sp_d_For_N	CCTGGCTGGGGCTGGGCTGGGGGTGGAGGCGGGGAGAGG	−538 to −500
8	Sp_d_For_M	CCTGGCTGGGGCTGGGTCTTTGGTGGAGGCGGGGAGAGG	
9	Sp_e_For_N	GGAGGCCTGGCTGGGGCTGGGCTGGGGGTGGAGGCG	−543 to −508
10	Sp_e_For_M	GGAGGCCTGGCTGGGTATTAGCTGGGGGTGGAGGCG	
11	Sp_cde_For_M	CTGGCTGGGTATTAGTCTTTGGTGGAGTATTGGAGAGG	−537 to −500

end-labelled oligonucleotide containing regions −239 to −202 (Sp a), −263 to −228 (Sp b), −524 to −491 (Sp c), −538 to −500 (Sp d) and −543 to −508 (Sp e) of the Axl promoter (Table 1) for 20 min in the absence or presence of a 50-fold excess of unlabelled oligonucleotide to demonstrate the specificity of binding at room temperature. A total of 0.5 μg of dI-dC (polydeoxyinosinic deoxycytidylic acid) was present in each reaction to block non-specific binding. The reactions were subjected to PAGE (5% gel) containing 5% (w/v) glycerol in 0.25 × Tris/Borate/EDTA buffer. For supershift analysis, 1 μg of antibody [anti-Sp1 (sc-59x), anti-Sp2 (sc-643x), anti-Sp3 (sc-644x), anti-Sp4 (sc-645x) and rabbit anti-IgG (sc-2338x) antibodies (all purchased from Santa Cruz Biotechnology)] was added to the reactions 20 min after the 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 oligonucleotides was processed in each EMSA.

Generation of luciferase reporter constructs

Approx. 2400 bp of the upstream sequence from the translation start of the *Axl* gene was amplified by PCR from human genomic DNA and cloned into the pGL3-Basic vector (Promega) using KpnI and XhoI restriction sites. A series of different truncated fragments of the Axl promoter were created by PCR amplification from this 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 [Institut für Molekularbiologie und Tumorforschung (IMT), Philipps-Universität Marburg, Marburg, Germany].

Table 2 Primers used for delineation constructs
F, forward; R, reverse.

Number	Name	Sequence (5′ → 3′)	5′ position
1	AxlF1	GAAGGTACCATGACAACCCAGGCAAAGTG	−2376
2	AxlF2	GAAGGTACCACAAAGCAAGACTGGGTCTC	−1759
3	AxlF3	GAAGGTACCCAGTCCCACCAGAAGGAGAG	−1276
4	AxlF4	GAAGGTACCTGCCCGGCCAACAACTATTC	−1010
5	AxlF5	GAAGGTACCTGTCTGTGGGCCAGTAGC	−727
6	AxlF6	GAAGGTACCGTGCGTGTGTGTGTGTGTG	−614
7	AxlF7	GAAGGTACCAATGAAGGGCCAAGGAGGC	−556
8	AxlF8	GAAGGTACCAGGGGAGTGGAGTTCTGG	−478
9	AxlF9	GAAGGTACCGGCAGGGGTGCTGAGAAG	−181
10	AxlR1	GAACTCGAGACGCCATGGGTGCCAAAC	+7
11	AxlR2	GAACTCGAGGAGCTGAGGAGGGGGAAG	−338

Site-directed mutagenesis analysis

Mutant constructs were generated using the QuikChange® XL site-directed mutagenesis kit (Stratagene) using AxlP3 (−1276 to +7) as the DNA template. The sequences of the mutant oligonucleotides used are detailed in Table 1. Successful incorporation of the mutations was confirmed by automated DNA sequencing.

Transfection and luciferase assays in mammalian cell lines

For luciferase assays, 1.3×10^5 Rko, 5×10^4 HeLa or 2×10^5 HCT116 cells were plated in 24-well plates. Luciferase reporter plasmids (1 μg each) were transfected in Optimem medium (Gibco) 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 h 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 μl of passive lysis buffer (Promega) for 20 min. Luciferase activity (20 μl of cell lysate) was measured via the Dual-Luciferase reporter assay system (Promega) following the manufacturer's instructions. Assays for all samples were performed in triplicate, and the means of the results were calculated.

Luciferase assays in *Drosophila melanogaster* SL2 insect cells

For luciferase assays, at the time of transfection 5×10^5 cells were plated into a 24-well plate. AxlP3 (250 ng) was co-transfected with 150 ng of either Sp1 or Sp3 along with *Renilla* luciferase as an internal control for transfection efficiency, using Effectene transfection reagent (Qiagen). After 48 h of transfection, cells were collected in Eppendorf tubes and lysed with 100 μl of passive lysis buffer (Promega). Luciferase activity (50 μl of cell lysate) was measured via the Dual-Luciferase reporter assay system (Promega) following the manufacturer's instructions. Assays for all samples were performed in triplicate, and the means of the results were calculated.

ChIP (chromatin immunoprecipitation) assay

ChIP assays were performed via a commercially purchased ChIP kit (Upstate Biotechnology) 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 by the addition of formaldehyde directly to tissue-culture medium to a final concentration of 1% and incubating for 10 min at room temperature. This was then neutralized by incubation with 125 mM glycine for 10 min. Cells were then washed twice with ice-cold PBS (with protease inhibitors), scraped, pelleted, resuspended in SDS lysis buffer [50 mM Tris/HCl (pH 8.1), 1% SDS and 10 mM EDTA] and incubated for 10 min on ice. The lysates were then sonicated ten times (15 s at 30% amplitude) and samples were rested on ice for 1 min between sonication steps. After sonication, the samples were centrifuged (18 360 ***g***, 10 min, 4 °C), the supernatants were diluted 10-fold in ChIP dilution buffer [16.7 mM Tris/HCl (pH 8.1), 0.01% SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA and 167 mM NaCl] with protease inhibitors, and pre-cleared with 80 μl of salmon sperm DNA/protein A–agarose for 30 min at 4°C. Cross-linked chromatin was incubated overnight at 4°C with 3 μg of anti-Sp1, 3 μg of anti-Sp3 or 3 μg of control anti-IgG antibodies in a total volume of 1 ml. Antibody–protein–DNA complexes were isolated by immunoprecipitation with 60 μl of salmon sperm DNA/protein A. After extensive washing, pellets were eluted by freshly prepared elution buffer (1% SDS and 0.1 M $NaHCO_3$). Formaldehyde cross-linking was reversed by 6 h incubation at 65°C. Samples were purified through PCR purification kit columns (Qiagen) and used as templates for PCR. The primers used for ChIP were as follows: region 1, sense 5′-CCTGGCCCCTTTAAGAAAG-3′ and antisense 5′-CTGCCTCCTTCCCTCACT-3′; and region 2, sense 5′-GTGTGTGTGTGTCCTTGTCC-3′ and antisense 5′ GGGCTCTGTGTCTGGTAAAC 3′. These were used to amplify a 159 bp and 168 bp fragment corresponding to the core Axl promoter. As a negative control, a region in the uPAR (urokinase plasminogen activator receptor) promoter region (from −937 to −745) was amplified with the following primers (sense 5′-GCAGACATCAATAACATTCACC-3′ and 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

siRNAs (small interfering RNAs) designed to target human Sp1 and Sp3 mRNAs were purchased from Ambion. Briefly, 1.3×10^5 Rko or 5×10^4 HeLa cells were plated into either a 24-well plate (luciferase assay), or 7.5×10^5 and 3×10^5 cells into six-well plates (Western blot analysis) the day before transfection. AxlP3 reporter plasmids were co-transfected with 25 or 50 nM Sp1/Sp3 siRNAs or with 50 nM scRNA (scrambled RNA), and luciferase assays were performed 48 h after transfection. Rko or HeLa cells were transfected with either 50 nM or 100 nM Sp1, Sp3 or combinations of Sp1 and Sp3 siRNA (50 nM or 75 nM Sp1 and Sp3 siRNAs) using Lipofectamine™ 2000 (Invitrogen). After 48 h post-transfection, cells were washed with PBS and harvested for protein extraction.

5-aza-dC (5-aza-2′-deoxycytidine) treatment

Cells were plated in a six-well plate in their respective medium supplemented with 10% (v/v) FCS, and allowed to grow for 24 h. Cells were treated either with a final concentration of 10, 20 or 40 μM 5-aza-dC for 5 days, changing the drug every 24 h, or with 10% (v/v) FCS-supplemented medium as a control. On the fifth day, cells were washed with PBS and grown without treatment for 24 h. Finally, cells were washed with ice-cold PBS and total RNA was isolated using the Qiagen RNeasy mini kit.

Sodium bisulfite conversion of DNA

WiDr or Rko genomic DNA (1 μg) was used for bisulfite conversion using the EpiTect Bisulfite Kit (Qiagen) following the manufacturer's instructions. Bisulfite-treated DNA was amplified with two sets of methylation-specific primers. Axl 5′-flanking region-specific primers were: set one (amplified product −376 to −98) sense 5′-GGGTATAGTTATTAGGGTTTTTTTT-3′ and antisense 5′-TTTAACACTACCTACCTAACACAAC-3′, and set two (amplified product −669 to −357) sense 5′-TGTTTTAGT-TTGTGTGTGTTAGTGA-3′ and antisense 5′-AAACCCTAA-TAACTATACCCCCTATC-3′. PCR was performed as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 45 s, and final extension at 72°C for 5 min. Purified PCR products were used for pGMT-easy cloning and ten clones were examined by DNA sequencing from each cell line.

Statistical analysis

Statistical analyses were performed using SPSS statistical software (SPSS, Chicago, IL, U.S.A.). Differences between groups were calculated with the Student's *t* test, and *P* values of $\leqslant 0.05$ were considered to be statistically significant.

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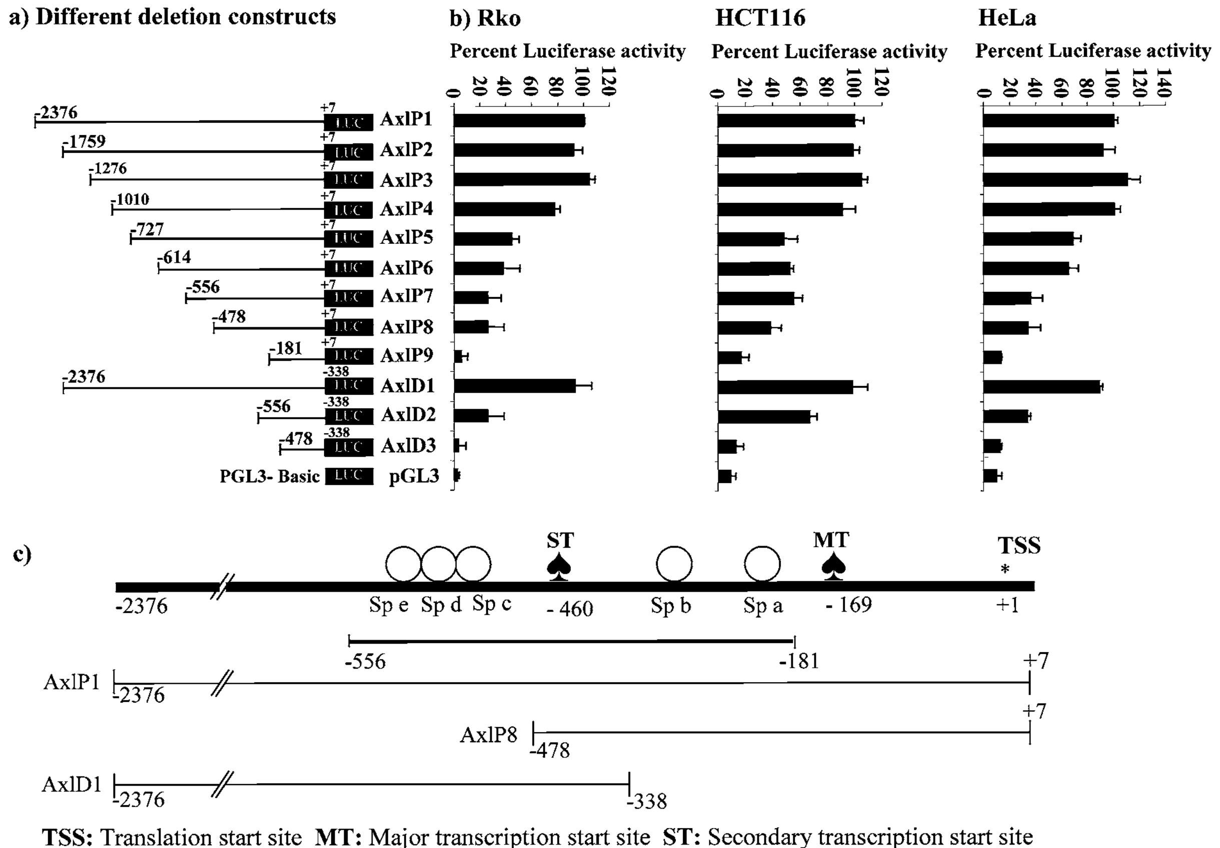

Figure 1 Identification of the core promoter region of Axl

(**a**) Schematic representation showing the design of pGL3 reporters to test the relative contribution of different regions of the Axl promoter. LUC, luciferase. (**b**) Luciferase reporter assay showing Axl promoter activity in Rko, HCT116 and HeLa cells. Axl constructs (1 μg) were co-transfected with a *Renilla* luciferase plasmid, serving as an internal control for transfection efficiency, into Rko, HCT116 and HeLa cells, and luciferase activity was measured 48 h post-transfection. Activity of AxlP1 (−2376 to +7), the largest deletion construct, was set to 100%, and the activity of the other constructs was calculated and plotted as a percentage of this value. Results are means ± S.D. ($n = 3$). (**c**) Schematic representation of promoter, showing promoter region AxlP1 (−2376 to +7) with different transcription start sites and putative Sp-family-binding sites.

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. The sequence does not contain typical TATA and CAAT boxes or an initiator sequence (see Supplementary Figure S1a at http://www.bioscirep.org/bsr/028/bsr0280161add.htm). As published previously, the promoter has different transcriptional start sites, including two major sites located at −169 and −460 upstream of the translation start site [19]. A TRANSFAC database search (GenBank® accession number BC032229) revealed several putative-binding sites for transcription factors including MZF1 and AP-1, and multiple Sp-binding sites. On the basis of potential *cis*-elements and major transcriptional start sites, we generated a series of 5′-promoter deletion constructs as outlined in Figure 1(a). Rko, HCT116 and HeLa cell lines, characterized by high-basal mRNA levels of *Axl* (Supplementary Figure S1b), were used for the present study. In the rest of the present study, 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 the *cis*-elements regulating basal *Axl* gene expression, we transfected a series of deletion luciferase constructs (Figure 1a) into Rko, HCT116 and HeLa cells. The AxlP3 construct (−1276 to +7) exhibited a maximum percentage

of luciferase activity compared with the promoterless luciferase vector pGL3-basic (Figure 1b). Further deletion constructs, AxlP4 (−1010 to +7), AxlP5 (−727 to +7), AxlP6 (−614 to +7), AxlP7 (−556 to +7) and AxlP8 (−478 to +7), showed a gradual decrease in luciferase activity in Rko, HCT116 and HeLa cells, with the activity of the last two constructs being comparable (Figure 1b). AxlP1 (−2376 to +7) and AxlP2 (−1759 to +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 to +7. AxlD1 (−2376 to −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 to −338), which also contains the second major transcription start site, showed similar luciferase activity to AxlP7 (−556 to +7), which contains both major transcription start sites. AxlP9 (−181 to +7) and AxlD3 (−478 to −338) do not contain any GC-rich regions and show a complete loss of luciferase activity, comparable with pGL3-Basic control vector activity (Figure 1b). Taken together, although maximum promoter activity lies within −1272 to +7, our results suggest that the 5′-flanking region of *Axl* located between −556 to −182 includes the minimally required core promoter, since activity of AxlP7 (−556 to +7) and AxlP8 (−478 to +7) are similar, and luciferase activity is completely absent downstream of −478. The region −556 to −182 of the promoter is GC-rich, and in a TRANSFAC database search is predicted to bind zinc-finger proteins, such as Sp family transcription factors (Figure 1c), but also AP-1 and MZF1. Overall, our observations with all of the deletion constructs were similar in the Rko, HCT116 and HeLa cell lines, 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 to −181. Sp motifs are defined as Sp a (−225 to −219), Sp b (−250 to −242), Sp c (−512 to −505), Sp d (−524 to −516) and Sp e (−529 to −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 mutant constructs were generated, with mutations either in single regions (Sp a, Sp b, Sp c, Sp d and Sp e), or in combinations of two or more regions (Sp ab, Sp cde and Sp abcde) (Figure 2a and Table 1). Mutant and wild-type constructs were transfected into Rko and HeLa cell lines and compared for reporter activity. We observed that all single mutants and combination mutants significantly reduced promoter activity compared with the wild-type construct in both cell lines, with the exception of Sp c activity in Rko cells (Figure 2b). The single mutant constructs Sp a, Sp b, Sp d and Sp e showed 40%, 60%, 30% and 30% reduced activity respectively compared with wild-type in Rko cells, and 40%, 30%, 60% and 60% reduced activity respectively in HeLa cells. Out of the combined mutant constructs, Sp ab (Sp a and Sp b both mutated) and Sp abcde (all five motifs mutated) showed the most dramatic (70%) reduction in promoter activity in both of the cell lines examined. Even though all motifs were mutated in Sp abcde, it did not reduce promoter activity further when compared with the Sp ab mutant construct. However, the Sp cde combination mutant construct alone reduced reporter activity by 50% in both of the cell lines, implying the existence of a co-operative function of these Sp motifs. In addition to Sp mutations, other mutant constructs were generated based on MZF1 and AP-1 consensus-binding domains at −564 to −556 and −297 to −292, yet neither of these affected basal promoter activity (results not shown). Taken together, the results suggests that all Sp-binding sites are relevant for Axl core promoter activity, with Sp a and Sp b being essential.

Nuclear proteins of the Sp family bind different 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, EMSAs were performed using Rko cell nuclear extracts, and radiolabelled wild-type and mutant probes (Table 1). Sp c, Sp d and Sp e are in close proximity to each other; therefore, a competition experiment was conducted with increasing concentrations of 25× and 50× of either individual mutant (Sp c, Sp d or Sp e) or combination mutant (Sp cde) oligonucleotides (using the oligonucleotides synthesized for site-directed mutagenesis), with the Sp d wild-type oligonucleotide being used for labelling, and 50× unlabelled oligonucleotide for non-radioactive competition (Figure 3a). As shown in Figure 3(a), competition with mutant Sp c and Sp d completely or partially abolished binding of Sp transcription-factor complexes, suggesting that the binding of some of the factors stayed intact, whereas when mutant Sp e was used as a competitor, most of the Sp transcription-factors complexes stayed intact. Competition with an oligonucleotide mutated for Sp cde did not change the binding complexes, even when used at increasing concentrations. These results 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 Sp b sites with their respective labelled wild-type oligonucleotides. We observed intact binding complexes even after the addition of increasing concentrations of Sp a and Sp b mutant oligonucleotides (Figure 3a). The wild-type competition specifically abolished binding to Sp a and Sp b, suggesting 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, Sp b and Sp cde motifs using antibodies against Sp1, Sp2, Sp3, Sp4, and IgG as a control (Figure 3b). For all three motifs, the incubation with Sp1, Sp2 and Sp3 antibodies led to a shift of the major bands, implicating the presence of these Sp family members. Individual supershift analysis of Sp c and Sp e also showed binding of Sp1 and Sp3 (results not shown).

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

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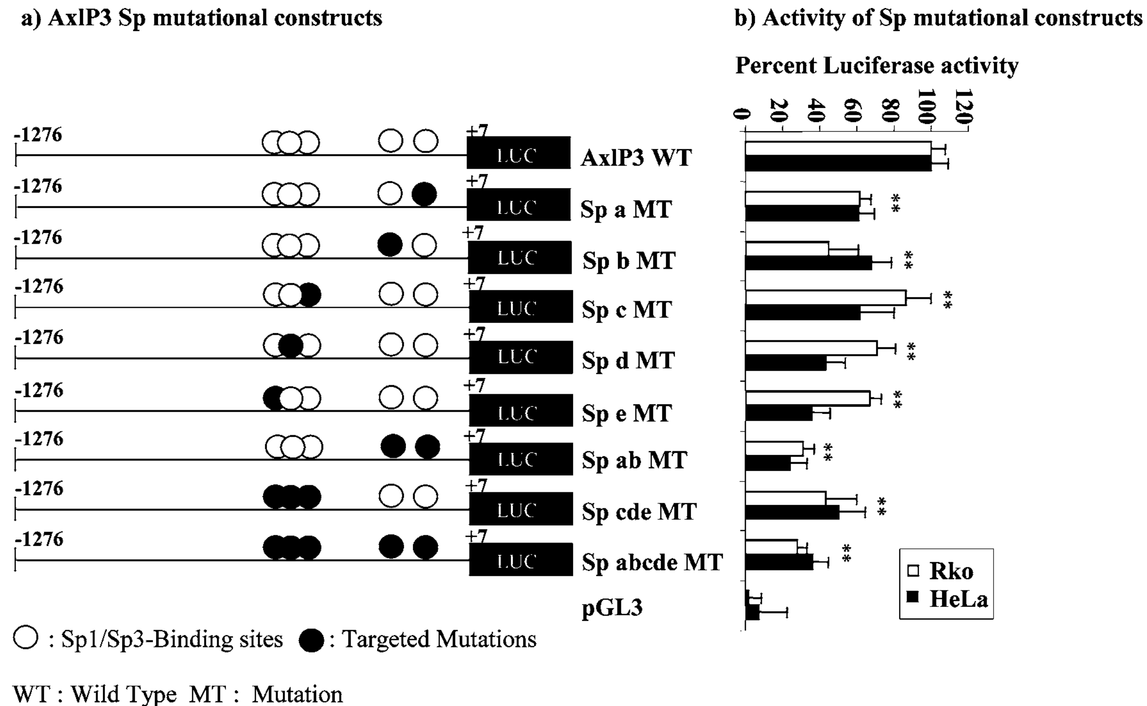

Figure 2 Effect of site-directed mutagenesis of Sp transcription-factor-binding sites on Axl promoter activity
(**a**) Schematic representation of the site-directed mutagenesis performed on the Sp-family-binding motifs within the core promoter region. Luc, luciferase. (**b**) AxlP3 (−1276 to +7), the promoter construct which demonstrated maximum activity, was mutated at the sites indicated in (**a**), either singly or as combinations of mutations. Wild-type/mutant Axl promoter construct (1 μg) and *Renilla* luciferase plasmid were co-transfected into Rko and HeLa cells. The activity of AxlP3 wild-type was set to 100%, and the activity of the mutant constructs was calculated and plotted as a percentage of this value. Results are means ± S.D ($n = 3$). The statistical difference of the activity of the mutated constructs in all panels is shown against AxlP3 wild-type activity, ** $P \leqslant 0.05$.

PCR amplification of Sp1/Sp3 immunoprecipitated DNA using primers against region 1 (covering Sp a and Sp b, 159 bp), region 2 (covering Sp c, Sp d and Sp e, 168 bp) (Figure 3) and a non-specific region of the uPAR promoter without Sp-binding sites as a negative control, resulted in single bands of sizes consistent with the expected size for the respective regions of the Axl promoter, with no specific band being amplified in the non-specific control (Figure 3c). These results confirmed that Sp1 and Sp3 are indeed binding to the Sp a, Sp b, Sp c, Sp d and Sp e 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 h post-transfection, cells were treated with either medium alone, or medium containing various concentrations of mithramycin (25, 50, 100 and 200 nM) and incubated for 24 h. Increasing concentrations of mithramycin gradually and significantly decreased Axl promoter activity (Figure 4a). For example, the 200 nM-treated sample showed a 50% reduction in promoter activity compared with the medium-treated control (Figure 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 (50–400 nM concentrations). Pre-incubation with mithramycin inhibited binding of the Sp family transcription factors to all identified Sp-binding regions of the Axl promoter (Figure 4b). These observations confirm that the GC-rich and Sp-binding sequences identified contain Axl core promoter activity and this is 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 or 24 h and compared with a control treated with medium only for 24 h. We found that treatment of Rko cells with mithramycin led to an almost 90% decrease in expression of *Axl* mRNA and protein after 16 h (Figures 4c and 4d). 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 levels were decreased 4 h after treatment, and completely undetectable 16 h after treatment, which was paralleled by a decrease in Sp1/Sp3 protein amounts (Figures 4c and 4d). These results suggests that mithramycin not only inhibits the binding of Sp1 and Sp3

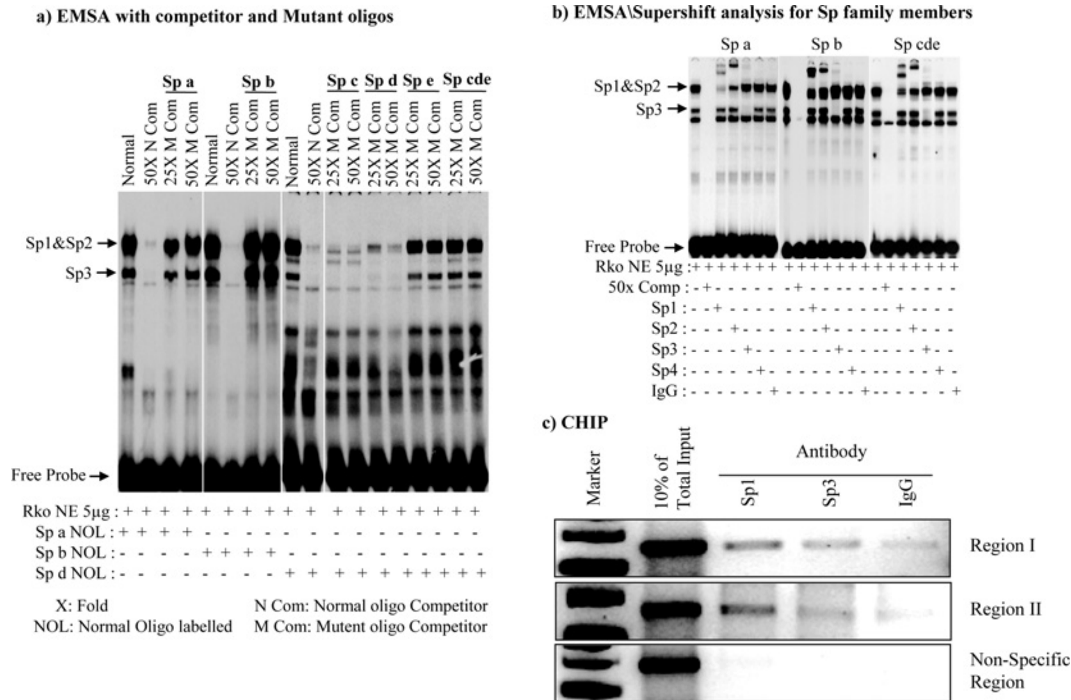

Figure 3 EMSA, supershift and ChIP assays of Sp-family members

(**a**) EMSA was performed with Rko nuclear extract (NE) and a [γ-^{33}P]-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 oligonucleotides were used (Sp a, Sp b, Sp c, Sp d, Sp e and Sp cde). Nuclear extracts (5 μg) were incubated with unlabelled wild-type or mutant oligonucleotides for 20 min prior to the addition of labelled oligonucleotides, and incubated for an additional 20 min. Samples were electrophoresed on a Tris/Borate/EDTA polyacrylamide gel (4% gel) at 220 V for 3 h, dried and exposed to X-ray film overnight. (**b**) Supershift analysis performed for Sp-family members (Sp1, Sp2, Sp3 and Sp4) binding to motifs Sp a (−239 to −202), Sp b (−263 to −228) and Sp cde (−538 to −500). Nuclear extract (5 μg; NE) was incubated for 20 min with unlabelled oligonucleotides for competition (Comp), followed by the γ-^{33}P-labelled probe for 20 min. Complexes were incubated with 2 μg of various antibodies (anti-IgG antibody as negative control) as indicated for 1 h at 4°C. Specific supershifted complexes are indicated (arrows). (**c**) The association of Sp1 and Sp3 with the endogenous Axl promoter was assayed by ChIP in Rko cells. Sp1/Sp3 immunoprecipitated DNA was amplified by PCR using two sets of specific primers [regions 1 (Region I) and 2 (Region II), see the Experimental section]. As a negative control, off-target primers were used. Total input (10%) was used as a positive control as indicated.

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 that 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 to +7) construct, or the mutant constructs for Sp a, Sp b, Sp cde and Sp abcde respectively, and increasing concentrations of either Sp1 or Sp3 expression vectors. Overexpression of Sp1 and Sp3 led to a significant increase in Axl wild-type promoter activity in a dose-dependent manner (Figure 5a). In contrast, the mutant constructs Sp a, Sp b, Sp cde and Sp abcde showed a significant reduction in responsiveness to Sp1 or Sp3. Specifically, mutant Sp a and Sp b did not show any response to Sp3 overexpression compared with their vector controls, and the Sp cde mutant showed a 50% reduction in response to Sp3 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 compared with AxlP3 wild-type (Figure 5b). The Sp abcde mutant did not show a significant response to Sp1 or Sp3 overexpression (Figure 5b). The activity of the Sp abcde mutant following Sp1 or Sp3 overexpression was dramatically reduced compared with AxlP3 wild-type (Figure 5b). These results confirmed that Sp a and Sp b are mediating Axl promoter activity following Sp1/Sp3 overexpression, with an additional role for the Sp cde site.

The Axl promoter is especially regulated by Sp1 or Sp3, both of which are often expressed ubiquitously 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 co-transfected with AxlP3 and either PacSp1 or PacSp3 (insect-expression vectors encoding human Sp1 or Sp3 respectively). Both Sp1 and Sp3 strongly induced AxlP3 promoter activity (150% activity),

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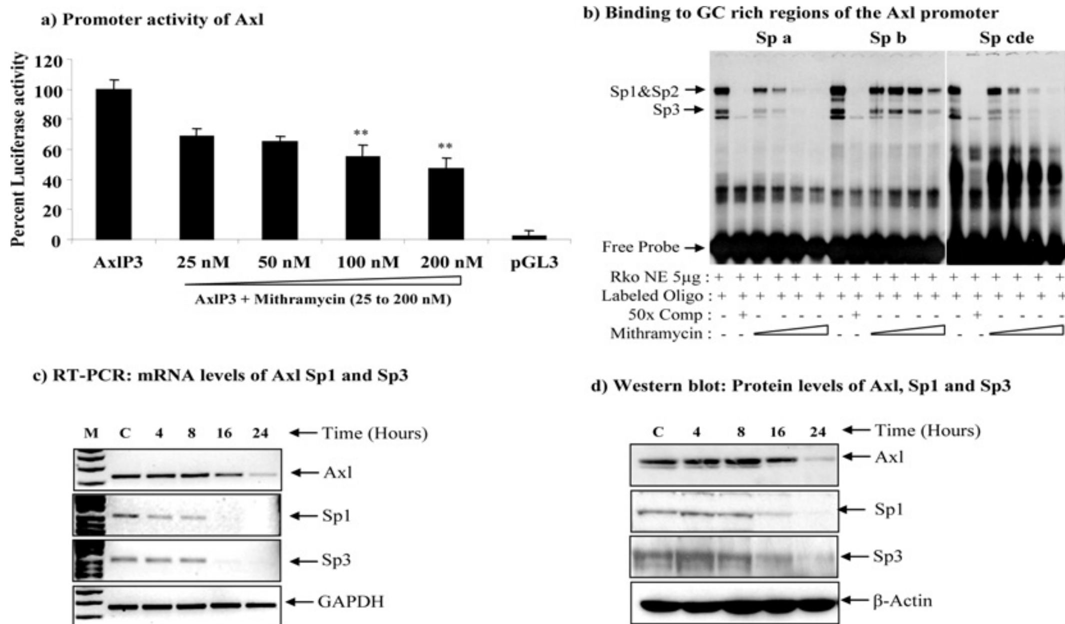

Figure 4 Axl promoter activity and expression is repressed by mithramycin

(**a**) pGL3-Basic and AxlP3 were transfected into Rko cells and 12 h post-transfection cells were mock-treated or treated with 25, 50, 100 or 200 nM mithramycin. After 24 h treatment, all plates were harvested and the resulting luciferase activity was measured. Equal amounts of DNA (1 μg) were transfected for each sample. Luciferase activity of cells transfected with AxlP3 and unexposed to mithramycin was determined to be 100%, and the activities of all of the 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. Results are means ± S.D. ($n = 3$). The statistical difference of the promoter activity is shown against AxlP3 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 to −202), Sp b (−263 to −228) and Sp cde (538–500) with mithramycin incubation. γ-^{33}P-labelled oligonucleotides were pre-incubated for 1 h with increasing concentrations of mithramycin (50, 100, 200 and 400 nM) and 50× excess wild-type oligonucleotides were pre-incubated for 20 min before the addition of labelled oligonucleotides. Sp-transcription-factor-binding efficiency was reduced for all regions in a dose-dependent manner on incubation with increasing concentrations of mithramycin. NE, nuclear extract. (**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, C) 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 a control. The final products were separated by agarose gel electrophoresis, stained with ethidium bromide 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 in (**c**), and cell lysates were separated by SDS/PAGE, transferred on to PVDF membranes and immunoblotted for Axl, Sp1, Sp3 or β-actin (as a loading control).

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

To gain evidence that Sp1 and Sp3-driven *Axl* gene expression is relevant at the protein level, Rko cells were transfected with 4 μg of Sp1, Sp3 or a control (mock, vector only). Both Sp1 and Sp3 increased Axl protein levels after 48 h of overexpression (Figure 5c) compared with the control. Taken together, it is clear that Sp1 or Sp3 positively regulate *Axl* gene expression.

siRNA strategies against Sp1 and Sp3 down-regulate 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 knockdown experiments were performed. Either Rko or HeLa cells were transfected with either Sp1 or Sp3-specific siRNA in a dose-dependent manner. The knockdown of Sp1 and Sp3 was confirmed by Western blotting (Figures 6c and 6d). Expression of an siRNA oligonucleotide against Sp1 or Sp3 decreased Axl promoter activity to almost 80% and 70% in Rko and to 50% and 40% in HeLa cells respectively (Figures 6a and 6b). This was paralleled by a significant reduction in the quantity of Axl protein in the presence of increasing concentrations of siRNAs against either Sp1 or Sp3; the quantity of Axl protein being even further reduced when incubated with a combination of Sp1 and Sp3 siRNA oligonucleotides (Figure 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.

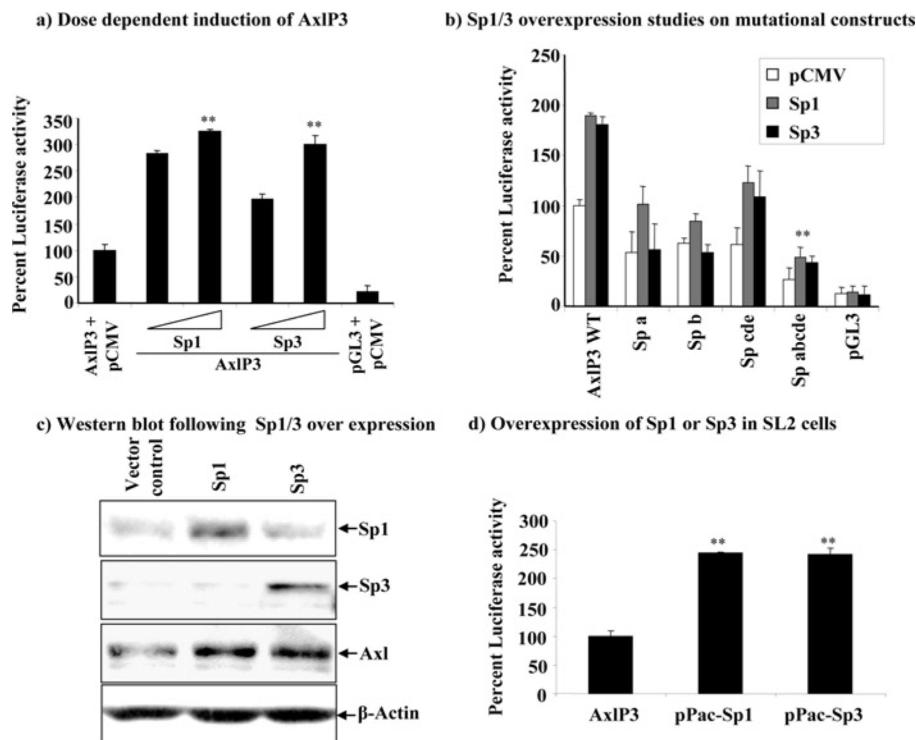

Figure 5 Overexpression of Sp1 or Sp3 induces Axl promoter activity and protein expression
(**a**) Overexpression of Sp1 or Sp3 in Rko cells increases activity of the Axl promoter. Rko cells were co-transfected with 1 μg of AxlP3 (−1276 to +7) and 0.5 μg and 1 μg of the Sp1 or the Sp3 expression vector, 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. Results are means ± S.D. ($n = 3$) (**b**) Rko cells were co-transfected with 1 μg of AxlP3 wild-type or mutated luciferase reporter vectors and 1 μg of either Sp1- or Sp3-expressing vectors. AxlP3 mock-treated activity was set to 100%, and the activity of the mutated constructs were calculated and plotted as a percentage of this value. Results are means ± S.D. ($n = 3$). (**c**) Overexpression of Sp1 or Sp3 increases Axl protein levels. Rko cells were transfected in a six-well plate with 4 μg of pCMV-Sp1, pCMV-Sp3 and vector alone as a mock control. After 48 h post-transfection, cells were harvested and cell lysates were immunoblotted for Sp1, Sp3, Axl and β-actin (used as a 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, pPacSp1 or pPacSp3, along with *Renilla* luciferase (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* luciferase served as an internal control for transfection efficiency. All results are expressed as a percentage of the activity resulting from the AxlP3 promoter and pPac (which was set at 100%). Results are means ± S.D ($n = 3$). For all panels (**a–c**), the statistical difference of the promoter activity is shown against AxlP3 mock-transfected cells. $^{**}P < 0.05$.

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 and Axl protein levels with Sp1/Sp3 expression (Figures 7a and 7b), Rko, HCT116, SW480 (colorectal cancer) and HeLa (derived from cervical cancer) cell lines showed high endogenous expression of Axl, which was paralleled by high Sp1 and Sp3 mRNA and protein levels. However, another group of colorectal cancer cell lines (HCT15, HT29, Geo, WiDr and Colo206f) showed almost undetectable basal *Axl* gene expression despite moderate expression levels of Sp1 and Sp3 mRNA and protein. This observation prompted 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 with regards to the methylation of CpG sites, the colorectal cell lines Colo206f and WiDr, characterized by low constitutive Axl expression, were treated with increasing concentrations of a selective inhibitor of DNA methyltransferases (5-aza-dC). 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 (Figure 8a). To identify methylated regions of the Axl promoter and to compare potential methylation between high- and low-Axl expressing colorectal cancer cells, 1 μg of genomic DNA from WiDr (low-Axl expressing) and Rko (high-Axl expressing) cells was subjected to bisulfite conversion. DNA sequencing revealed that Sp a, Sp b and Sp c were partially methylated in low-Axl-expressing

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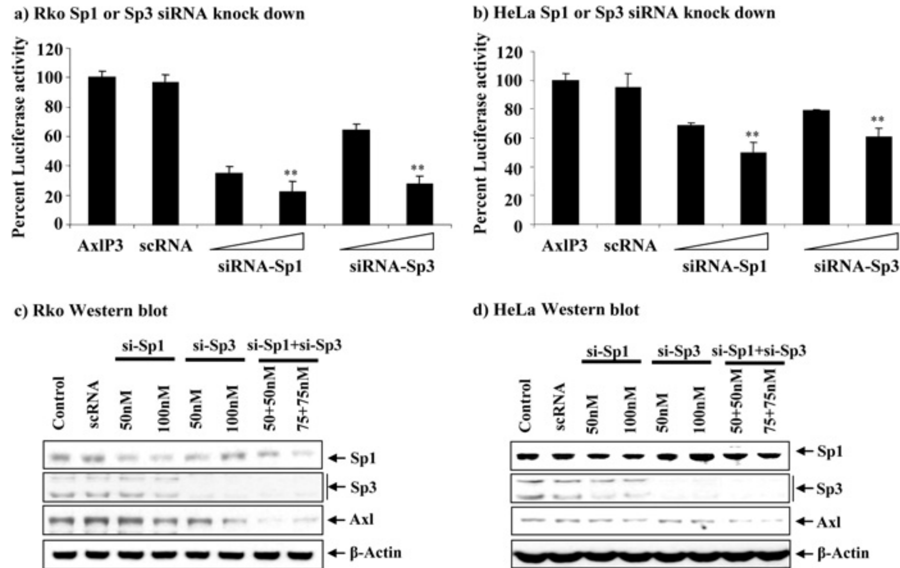


Figure 6 Knockdown of either Sp1 or Sp3 decreased Axl promoter activity and protein amounts
(**a** and **b**) Rko or HeLa cells were transfected with control scRNA (scrambled RNA) or a specific siRNA targeting either Sp1 or Sp3 in a dose-dependent manner. After 48 h post-transfection, all cells were harvested for the 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%, and the activity of the other constructs was calculated and plotted as a percentage of this value. Results are means ± S.D. ($n = 3$). The statistical difference of the promoter activity is shown against AxlP3 scRNA-transfected cells. ** $P < 0.05$. (**c** and **d**) Either Rko or HeLa cells were transfected with siRNA targeting Sp1 or Sp3, and/or both Sp1 and Sp3 in a dose-dependent manner, with scRNA used as a control. After 48 h post-transfection, cells were harvested and cell lysates were immunoblotted for Sp1, Sp3, Axl and β-actin (used as a loading control). scRNA has no effect on either Sp1 or Sp3.

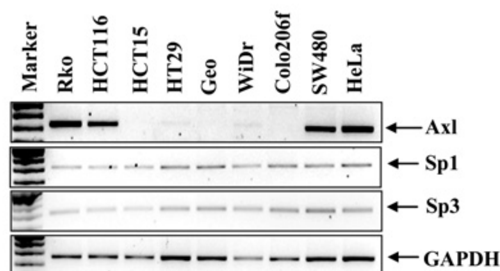
WiDr cells (Figures 8b and 8c, positions 4, 5 and 8), but not in high-Axl-expressing Rko cells. Furthermore, in WiDr cells, CG-rich flanking regions of GC-rich areas around the Sp sites identified were also partially methylated (Figure 8c, positions 6, 7 and 9). These results demonstrate that methylated CpG sites, in part within identified Sp-binding motifs, in part with in GC-rich flanking regions, influence constitutive *Axl* gene expression in addition to the binding of Sp1- and Sp3-transcription factors.

DISCUSSION

Axl is a member of the mammalian Tyro 3 RTKs, which are widely overexpressed in different cancers [18], and an increasing number of reports have shown an essential functional relevance of Axl in tumour progression, invasion, metastasis, anti-apoptosis, angiogenesis and immunological escape [12,22–25]. In the present study, we have systematically characterized the promoter of this highly important gene in diverse colorectal cell lines and also in the HeLa cell line for the first time. In the present paper, we have focused on delineating the minimally required basal Axl promoter to drive endogenous gene expression. In this context, we have clearly identified five different Sp-binding regions within the minimally required basal promoter region −556 to −183, in which Sp1 and Sp3 transcription factors bound to. 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 treatment, which showed a decrease in promoter activity, mRNA and protein levels, and also in Sp1 and Sp3 expression, brought about by this GC-rich region binding molecule. In addition to Sp1 and Sp3 acting as essential regulators of basal *Axl* gene expression, we show evidence that the methylation of CpG sites within specific Sp1 motifs, and also in promoter regions flanking these motifs, modulates *Axl* gene expression.

Sp1 and Sp3 are zinc-finger proteins that belong to the Sp family of transcriptional factors, regulating the transcription of many housekeeping 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_2H_2 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

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



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

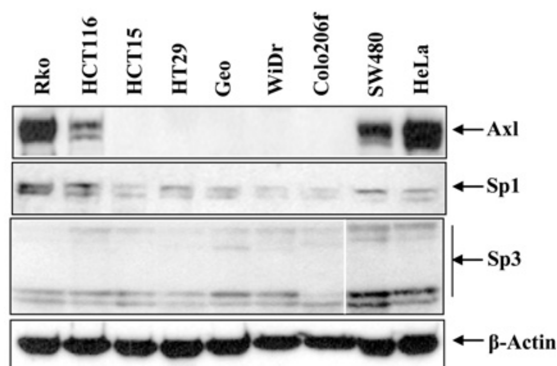


Figure 7 Screening for Axl, Sp1 and Sp3 expression in nine different cell lines

(**a** and **b**) All of the cell lines were grown and treated under identical conditions: cells were harvested in their exponential phase either for RNA by screening transcript levels of Axl, Sp1, Sp3 and GAPDH (used as a control) by RT–PCR (**a**) or for protein lysates for analysis by Western blotting (**b**). The final products of RT-PCR were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed under UV illumination. The protein samples were resolved by SDS/PAGE, transferred on to PVDF membranes and immunoblotted for Axl, Sp1, Sp3 and β-actin (loading control).

in the brain. In the present study, we observed binding of Sp2, in addition to Sp1 and Sp3, to some of the five Sp-binding motifs identified within the Axl promoter, and so it is certainly possible that, besides Sp1 and Sp3, Sp2 will also transactivate the Axl promoter via these sites. However, due to the limited availability of suitable constructs and antibodies, 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 [29]. Sp3 has been shown to be able to act as either a 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 similarity 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 level of endogenous *Axl* mRNA or Axl protein (results not shown), therefore it is unlikely that a similar situation is relevant for the expression of Axl.

Within the −556 to −181 region minimally required for Axl promoter activity, we identified five Sp consensus-binding sites within a generally GC-rich region. With EMSA and ChIP studies, we clearly showed that Sp1 and Sp3 bound strongly to oligonucleotides corresponding to all of the Sp-binding motifs, and also to the endogenous GC-rich region. Functional mutagenesis studies targeting these Sp-binding sites revealed that the Sp a and Sp b motifs are especially indispensable for basal Axl promoter activity, whereas the Sp c, Sp d and Sp e sites show an additional modulating relevance for basal promoter activity. However, since a construct mutated for all five Sp-binding sites still exerted a minimal reporter activity compared with an empty vector control, we cannot rule out the possibility that additional parameters besides Sp factors binding to the five identified Sp sites might 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 (transforming growth factor β-inducible early protein 1) or TIEG2 [36–38], might bind to the GC-rich Axl core promoter. In addition, an AP-1 and an MZF1 motif also identified within the core promoter might contribute to minimal activation; however, this is unlikely, since mutations of either of the motifs did not change reporter activity in our luciferase experiments (results not shown). Still, our co-transfection experiments of Sp1 or Sp3 with the Axl promoter showed a significant increase in promoter activity in the SL2 cell line (which does not endogenously express human Sp1 and Sp3) [39,40], and our observation that Sp1 or Sp3 significantly increase *Axl* gene expression (whereas siRNA knockdown of either Sp1 or Sp3 dramatically reduced Axl promoter activity and expression) clearly imply that both transcription factors are essential for Axl promoter transactivation and endogenous gene expression [40]. In this context, it is of 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 have been conducted mainly in the hepatocytic cell line HepG2, it is interesting to speculate that, in gastrointestinal tumours, Sp1 and Sp3 may regulate the constitutive expression of a RTK which is highly relevant for tumour progression, invasion and angiogenesis, in addition to up-regulating one of its specific ligands, to enhance tumour progression.

Mithramycin, having been shown to reduce the activity of the Sp1 promoter as well as inhibiting Sp1 binding [41], in our present study also reduced Axl promoter activity in a dose-dependent manner and almost abolished the expression of Axl, Sp1 and Sp3 mRNA and protein within 24 h [40]. Mithramycin is a cell-permeable reagent frequently used to explore the sequence dependence of DNA-binding factors known to bind to GC-rich

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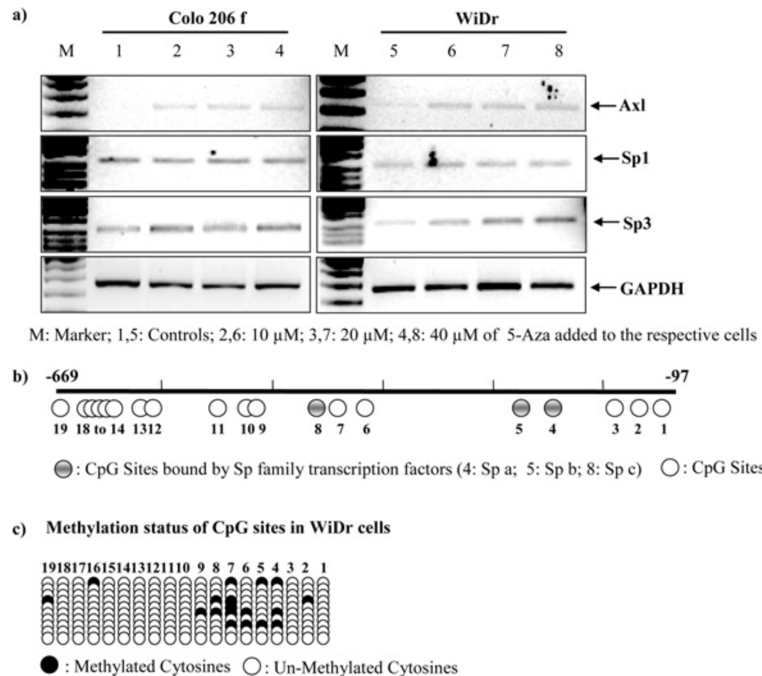

Figure 8 Effects of 5-aza-dC treatment on the expression of Axl, Sp1 and Sp3 mRNA in colorectal cancer cell lines, and bisulfite sequencing of CpG sites within the Axl promoter

(**a**) RT–PCR analysis of Axl, Sp1 and Sp3 mRNA expression in Colo206f and WiDr cells treated with 10, 20 and 40 μM 5-aza-dC (5-Aza) for 5 days, 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 and −97 relative to the translation start site and includes 19 CpGs. There are three CpG-containing Sp-binding sites within this sequence (grey circles), corresponding to Sp a, Sp b and Sp c respectively (positions 4, 5 and 8). (**c**) DNA from WiDr cells was treated with bisulfite, and the Axl promoter was amplified by PCR. The PCR product was ligated into the pGMT-easy vector using the TA cloning system. Ten subclones were picked and sequenced. As a result, some of the CpG sites, some within the Sp a, Sp b and Sp c motifs, others within GC-rich flanking regions, are partially methylated in WiDr cells.

regions by steric hindrance. In our present study, overexpression or knockdown of Sp1 or Sp3 had a significant, but not 100%, impact on Axl promoter activity, whereas mithramycin treatment completely abolished the expression of *Axl* mRNA or Axl 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 as BTEB1, TIEG1 or TIEG2, might add to Axl promoter transactivation to a small extent and might have also been blocked by mithramycin. At present, we cannot rule out the possibility of a remote additional impact of other such 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 cells (a cervical cancer cell line) for mRNA transcript and protein levels of human Axl, Sp1 and Sp3. In Rko, HCT116, SW480 and HeLa cells, a high endogenous Axl expression 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, the chromatin remodelling factors p300 or PCAF [p300/CREB (cAMP-response-element-binding protein)-binding protein-associated factor] and other DNA-binding proteins [26,42,44,45]. Furthermore, as stated previously, the ratio of Sp1 to Sp3 in certain cellular contexts may determine the degree of activation [46,47]. Such mechanisms can certainly explain the discrepancy between Axl, Sp1 and Sp3 expression levels found in some of the cell lines investigated.

Our finding that 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, tumour suppression, embryonic development, differentiation and other phenomena [49]. Specifically, there have been many interesting reports showing that the methylation of CpG sites within Sp-transcription-factor-binding motifs, but also of their flanking regions, might affect the binding and 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 to −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 competition with other factors, such as MeCP2 (methyl CpG-binding protein), 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 Sp3 expression was also 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 the 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 will elucidate the function of further upstream transcriptional elements in the constitutive and inducible expression of this RTK, which is essential for many functions associated with cancer.

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