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Overexpression of Transcription Factor Sp1 Leads to Gene Expression Perturbations and Cell Cycle Inhibition

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Abstract

Background: The ubiquitous transcription factor Sp1 regulates the expression of a vast number of genes involved in many cellular functions ranging from differentiation to proliferation and apoptosis. Sp1 expression levels show a dramatic increase during transformation and this could play a critical role for tumour development or maintenance. Although Sp1 deregulation might be beneficial for tumour cells, its overexpression induces apoptosis of untransformed cells. Here we further characterised the functional and transcriptional responses of untransformed cells following Sp1 overexpression.

Methodology and Principal Findings: We made use of wild-type and DNA-binding-deficient Sp1 to demonstrate that the induction of apoptosis by Sp1 is dependent on its capacity to bind DNA. Genome-wide expression profiling identified genes involved in cancer, cell death and cell cycle as being enriched among differentially expressed genes following Sp1 overexpression. In silico search to determine the presence of Sp1 binding sites in the promoter region of modulated genes was conducted. Genes that contained Sp1 binding sites in their promoters were enriched among down-regulated genes. The endogenous sp1 gene is one of the most down-regulated suggesting a negative feedback loop induced by overexpressed Sp1. In contrast, genes containing Sp1 binding sites in their promoters were not enriched among up-regulated genes. These results suggest that the transcriptional response involves both direct Sp1-driven transcription and indirect mechanisms. Finally, we show that Sp1 overexpression led to a modified expression of G1/S transition regulatory genes such as the down-regulation of cyclin D2 and the up-regulation of cyclin G2 and cdkn2c/p18 expression. The biological significance of these modifications was confirmed by showing that the cells accumulated in the G1 phase of the cell cycle before the onset of apoptosis.

Conclusion: This study shows that the binding to DNA of overexpressed Sp1 induces an inhibition of cell cycle progression that precedes apoptosis and a transcriptional response targeting genes containing Sp1 binding sites in their promoter or not suggesting both direct Sp1-driven transcription and indirect mechanisms.

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Introduction

Transcription factor Sp1 was the first identified member of the Sp/XKLF (Specificity protein/Kruppel-like factor) family. Sp1 protein comprises several domains of which the DNA binding domain is the most conserved among Sp family. The DNA binding domain of Sp1 consists of three contiguous Cys2His2 Zinc (Zn) fingers and mutational analysis has revealed that Zn fingers 2 and 3 are essential for Sp1 DNA binding activity [1]. Sp1 binds GC-rich elements [2] that are common regulatory elements in promoters of numerous genes. Sp1 binds individual Sp1 binding sites as a multimer and is capable of synergic activation on promoters containing multiple binding sites [3]. Sp1 regulates transcription by dynamically recruiting and forming complexes with many factors associated with transcription [4]. Although Sp1 has been described as a transcriptional activator it can also act as a repressor. Activation or repression of transcription by Sp1 depends on the promoter it binds to and on the co-regulators it interacts with [5].

An unbiased mapping of in vivo occupied Sp1 binding sites by combining chromatin immunoprecipitation and oligonucleotides arrays has led to the estimation that the human genome contains at least 12,000 Sp1 binding sites [6]. Therefore it is not surprising that Sp1 has been implicated in the expression of numerous genes.
involved in many aspects of cellular life such as metabolism, cell growth, differentiation, angiogenesis and apoptosis regulation. Although Sp1 is widely expressed and binds the promoters of a large number of genes, it is involved in tissue specific gene expression, its activity being finely modulated by a variety of stimuli through multiple post-translational modifications [7].

Sp1 expression levels are also regulated, changes in its expression levels being observed during marine development and during transformation. Indeed, differences in the levels of Sp1 of up to 100 times were observed during the development and the differentiation of mouse organs [8]. Importantly, Sp1 expression is increased in a number of tumour cells and this could be a critical factor for tumour development or maintenance. Indeed, Sp1 levels and/or activities are increased in gastric cancer, breast carcinoma and pancreatic carcinoma compared with normal tissues [1,9,10]. This elevated Sp1 expression is inversely correlated with the survival of patients with gastric cancer [9]. In primary pancreatic adenocarcinoma Sp1 overexpression identifies advanced stage tumours and predicts a poor clinical outcome [11]. Moreover, Sp1 levels slowly increase during mice skin tumour progression [12] and Sp1 accumulates in N-methyl-N-nitrosourea-induced mammary tumour cells compared to normal mammary cells [13]. Sp1 levels also increase during the process of transformation in a fibrosarcoma transformation model and reducing Sp1 expression in those human transformed fibroblasts inhibits their tumorigenicity [14]. Moreover reduction of Sp1 expression in pancreatic cancer cells inhibit their growth and metastasis in mouse models [15]. Sp1 could contribute to transformation via the regulation of expression of genes regulating cell growth (e-jun, Raf, cyclins, cyclin inhibitors, E2F1, TGF-β, IEX-1 and TCLI), apoptosis (Bcl-2) or angiogenesis (VEGF, FGF) [16,17]. Altogether, these findings show that Sp1 is overexpressed or overactivated in a number of cancers and that its activity plays a role in late stage of carcinogenesis.

Although Sp1 deregulation might be beneficial for tumour cells, we and others have previously shown that deregulation of Sp1 expression on its own induces apoptosis of several untransformed cell lines [18,19]. The aim of the study was to further characterise the functional and transcriptional responses of untransformed cells following Sp1 overexpression. We used wild-type and DNA-binding-deficient Sp1 to demonstrate that Sp1-induction of apoptosis in untransformed Baf-3 cells requires its binding to DNA. Genome-wide expression profiling showed that Sp1 overexpression induces a transcriptional response that is enriched for genes regulating cell death and cell cycle. Sp1 overexpression leads to the down-regulation of cyclin D2 expression and the up-regulation of cyclin G2 and cdk2c expression. Moreover, we show that this deregulation of cell cycle regulating genes is associated with the accumulation of cells in the G1 phase of the cell cycle. Finally, microarray data combined with promoter analysis revealed that only a fraction of the promoters of deregulated genes are enriched in Sp1-binding sites. This suggests that the transcriptional response induced by overexpressed Sp1 involves direct Sp1-driven transcription but also indirect mechanisms.

Materials and Methods

Cell culture

The murine IL-3-dependent Baf-3 cell line was maintained in DMEM (Gibco) containing 6% FBS (Pan Biotech GmbH) and 5% IL-3 as described [20]. Drosophila Schneider’s SL2 cells were cultured in Drosophila medium (Invitrogen) containing 10% FBS at 28°C [21]. Baf-3 clones expressing inducible Sp1 and GFP were obtained as described previously [18]. To repress ectopic Sp1 expression, cells were grown in presence of doxycycline (30 ng/ml, Sigma). To induce Sp1 and GFP expression, cells were washed 3 times and cultured without doxycycline. Among the inducible clones generated, one expressed a truncated form of Sp1 (Sp1Δ2,3) (Figure 1C) due to the integration into the genome of a retrovirus coding for a truncated Sp1 (data not shown). Sp1 is composed of the first 418 amino acids and is devoid of DNA binding domain and nuclear localisation sequences (Figure S1).

Retroviral constructions, transfections and transductions

The retroviruses coding for full-length human Sp1 protein followed by an internal ribosomal entry site and a truncated cell surface marker (CD2) to identify transduced cells were described previously [18]. Sp1 carrying mutations of the second and third Zn fingers (Sp1Δ2,3) (Figure S1) was obtained by PCR-mediated site-directed mutagenesis using pCMX-Gal4Nsp1ZF2M2 vector (a kind gift of Dr Hiro MW, Seoul, Korea) encoding the DNA binding domain (amino acids 622–720) mutated for the second Zn Finger. Mutation of the third Zn finger was obtained using the following oligonucleotides 5’-CCTCATGAAGCGCTAACCTGCAG-GGCTGGAATTTTCTTCTCACGTGTGGTCTAGTT-GTCGGTGTAGCTGACGTGAGAAGAATTCTTCATGCATGGC-CCCCTGATGCTACTAAG-3’, BamHI site: 5’-CCGCGGATCT-CTGCGAAAAAGAGAAGC-3’. The BamHI/EcoRI IRES-CD2. Sp1, Sp1Δ2,3 or EGFP cDNA PCR fragments were subcloned into pBSK-Sp1 to give pBSK-Sp1Δ2,3. The EcoRI-Xhol fragment was sequenced before subcloning into pMX-IRES-CD2. Sp1, Sp1Δ2,3 or EGFP cDNA were cloned under the Drosophila actin AC5 promoter into pAc5.1/V5-HisA vector (Invitrogen). The pGL2 derivative carries the tata-Sp1 promoter [21]. Transfections, transductions, magnetic selection of CD2-expressing cells and western blot analysis were performed as described [18].

Luciferase assay

SL2 cells were transfected with 5 μg of tata-Sp1 reporter plasmid and 0.5 μg of Drosophila expression vectors pPac, pPacSp1 or pPacSp1Δ2,3 and 0.5 μg of pPac-EGFP to quantify transfection efficiency. 48 hrs after transfection cells were lysed in 100 μl of Reporter Lysis Buffer (Promega) for 10 min. Samples were frozen at −80°C for at least 1 h. Luciferase assay was performed with Luciferase Assay System (Promega). Luciferase activity was normalized based on protein concentration (Bradford method) and on transfection efficiency determined as percentage of EGFP positive cells counted on a FACSscalibur (Becton-Dickinson) and analysed using the CellQuest software.

Electrophoretic mobility shift assay

Proteins Sp1 or Sp1Δ2,3 were synthesised in vitro using a reticulocyte coupled transcription/translation system (Promega). The oligonucleotide probe carrying the Sp1 consensus sequence (upper strand sequence: 5’-ATTGGATCCGGGGCCGGGG- GAGC-3’) was 32P end-labelled with T4 DNA polynucleotide kinase (Promega). The binding reaction was performed in a 15 μl volume of binding buffer containing 2.5 μl of in vitro protein, 1.5 μg poly dl-dC and oligonucleotide probe for 30 min at room temperature and run on a gel.

Cell viability and cell cycle analysis

Viability was measured using Fluorescein-5-isothiocyanate (FITC)-Annexin V staining (BD Biosciences Pharmingen) or Propidium Iodide staining (2 μg/ml, Sigma) by flow cytometry. Cell cycle was analysed using the Flow Cytometry Kit (BD Biosciences Pharmingen). Cells (2×106 cells/ml) were grown for 16 hrs and then labelled with BrdU (32 μM, 20 min). Cells were
fixed and permeabilised with Cytofix/Cytoperm and treated with DNase (1 hr, 37°C). Cells were stained with Allophycocyanin-conjugated anti-BrdU and 7-amino-actinomycin D (7-AAD). When required, transduced cells were stained with Phycoerythrin (PE)-coupled anti-CD2 antibody (30 min, 4 μg/ml, BD Biosciences Pharmingen). BrdU incorporation (FL4-H), 7-AAD (FL3-H) and CD2 expression (FL2-H) were recorded using a FACScalibur.

Immunofluorescence microscopy

Cells seeded on 12-mm glass coverslips coated with 0.01% Poly-L-lysine (Sigma) were fixed with 4% paraformaldehyde (BDH) in PBS for 5 min then permeabilised with 0.25% Triton X-100 (BDH) in PBS for 5 min. Sp1 was detected using anti-Sp1 polyclonal rabbit antibody (1:100, 120 min) followed by biotin-conjugated goat anti-rabbit antibody (5 μg/ml, Jackson ImmunoResearch, 30 min) and either FITC-conjugated streptavidin (1 μg/ml, BD Biosciences Pharmingen, 30 min) or TetramethylRhodamine- Iso-Thio-Cyanate (TRITC)-conjugated streptavidin (3 μg/ml, Jackson ImmunoResearch, 30 min). Actin was labelled with rhodamine-conjugated phalloidin (0.2 μg/ml, Sigma, 30 min).

Detergent Triton X-100 was used to remove soluble proteins while leaving more tightly bound protein [22]. Thirty hrs after transduction, 10⁶ cells were stained for 45 min with 0.5 μg biotinylated anti-CD2 antibody and with 0.75 μg TRITC-conjugated streptavidin for 20 min (Jackson ImmunoResearch). Cells were then washed in PBS containing 0.5 mM MgCl₂ and 0.5 mM CaCl₂, incubated for 5 min in CSK buffer (10 mM Pipes-KOH, pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂) and incubated for 3 min in CSK buffer containing 0.02% Triton X-100. A 10 times excess of CSK buffer was added and cells were loaded on Poly-L-lysine-coated coverslips and fixed immediately with 4% paraformaldehyde. Slides were mounted with ProLong anti-fade (Molecular Probes) to counterstain DNA. Images of cells (single sections) were obtained using a Zeiss LSM 510 confocal laser-scanning microscope. Sp1 staining in CD2-positive cells was quantified using Image J software. The ratio of Sp1 signal in transduced CD2 positive cells versus non transduced neighbouring cells was calculated.
Quantification by Real Time PCR

RNA extraction and reverse transcription were performed as previously described [18]. Real Time PCR analysis was carried out using Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen) on an ABI Prism 7700 (Perkin Elmer) [23]. Relative level of the target sequence against the HPRT reference sequence was calculated using the ΔΔCt method with calculated real efficiencies. The sequences of primers used were:

Mouse Sp1: F-TCATGGATCTGGTGGTGATGGG, R-G-TTGGATCCAGTGAGTGACCTC;

Human Sp1: F-GCCTCCAGACCATTAACCTCAGT, R-G-TCGCCCATTTTCTCGGC;

Cdkn2c: F-GTCCTTCTGTCAGCCTCCGATG, R-TCGGCAACCACACAGCACTTG;

Ccnd2: F- TTCATTGAGCACATCCTTCGC, R-AAGTCGCTAGCTATGAGATTTAC;

Gsn: F-AGGTCAAAGGACGCCGTGTAG, R-GCCAGAGCAGCACCCAGACTG;

Neo: Forward-ACCAAGGAAGGGGTGGCTA, Reverse-GTTTTGAGCTCCGCTATTTGGG;

Jun: F-AAGAACTGACGTGACCTAATTCG, R-GTTAAGGACACTACAGAAGCAATTCTAC;

Gm3F-AGGTCAAAAGGAGGCGBTGAG, R-GCCAGAGCAGCACCCAGACTG;

Ccdn2F- TTTATTGAGACATCCCTTGCC, R-AAGTCGCTAGCTATGAGATTTAC;

Ccn2G-GGGAGGGTGTTCTTCTCCCAAGC, R-AAGTGAGACATCCCTTGCC;

Cdkn2cG-GTCCTTCTGTCAGCCTCCGATG, R-TCGGCAACCACACAGCACTTG;

Human Sp1G-GCCTCCAGACCATTAACCTCAGT, R-G-TCGCCCATTTTCTCGGC;

Mouse Sp1G-TCACTGGACGTGAGTGATTTGGG, R-GGCTCCAGACCATTAACCTCAGT;

Hprt1F-TCATTATGCGAGGATTGGA, R-CAGAGGAGCACCCAGACTG.

Microarray analysis of gene expression

Inducible Baf-3-Sp1 clone 1 was grown in presence or absence of doxycycline for 28 hrs. Three independent experiments were performed. RNA extraction, reverse transcription and microarray hybridization on Affymetrix mouse GeneChip Mouse Genome 430 2.0 (Affymetrix) were carried out by ProfileXpert (Bron, France). Absolute expression transcripts levels were normalized for each chip by globally scaling all probesets to a target signal intensity of 500. Normalized data were analysed using GeneSpring GX 7.3 expression analysis software (Agilent Technology Inc). Differential probesets expression following Sp1 overexpression relative to control was determined using GeneSpring GX 7.3 statistical analysis module ANOVA (1 way test, assuming variance not equal, FDR 6% with multiple testing correction Benjamini and Hochberg). 1610 probesets corresponding to 1294 genes with unique gene symbol identifiers were differentially expressed with a fold change of expression of 1.5. To identify among deregulated genes the functional categories linked with Sp1 overexpression, differentially expressed probesets were imported into Ingenuity Pathway Analysis (Ingenuity Systems Inc, Mountain View, CA). The microarray data have been deposited and described in ArrayExpress under accession number E-MEXP-1702 (http://www.ebi.ac.uk/microarray-as/ae) in accordance with MIAME guidelines.

Computational analysis of promoters regions

Promoter sequences (2000 nucleotides upstream of the transcriptional start site) were retrieved using Biomart service of the Ensembl project. Elkon et al. have shown that 80% of Transcription Factor-binding sites are located within 1200 bases upstream of the transcription start site (TSS) [24]. Since there is no clear consensus for the length of a promoter, we have performed our analysis studying the 2000 bases upstream of the TSS. Sp1 binding motifs GGGCGGGG (VSSP1_Q6 as defined by TRANFAC 7.0) and GG[CC][T][G][CC][T][G][GG] [6] and STAT6 binding motif (TTTCN,GAA) [25] were searched in promoters using PATTERNn service (http://bioinfo.hku.hk/services/analyseq/cgi-bin/patternn_in.pl). We have also used the DiRE server (http://dire.dcode.org) to identify transcription factor binding sites within conserved Regulatory Elements (RE) of deregulated genes. A control list of 1700 probesets was generated randomly that fulfills two criteria: no change in their expression levels following Sp1 overexpression and an overall distribution of expression of the control list similar to the overall distribution of expression of the differentially expressed list (Figure S2). Two sided binomial tests were conducted in R software to determine whether there were significant differences in the percentage of promoters containing transcription factor binding site between the different gene lists.

Results

Induction of apoptosis by overexpressed Sp1 requires its binding to DNA

The overexpression of transcription factor Sp1 was achieved using an inducible Sp1 expression system in untransformed murine haematopoietic Baf-3 cell line [18]. In Baf-3-Sp1 clone 1 the expression of ectopic full-length wild-type Sp1 was rapidly induced following doxycycline removal (Figure 1A). The first cells entering apoptosis were detected 20 hrs after doxycycline removal. Similar results were obtained when studying a second inducible clone (Baf-3-Sp1 clone 2) in which induction of Sp1 expression and apoptosis induction following doxycycline removal are slower (Figure 1B). In contrast a truncated Sp1 (tSp1, Figure S1), that accumulates in the cytoplasm does not affect cell viability (Figure 1C).

We then assessed whether Sp1 requires nuclear localisation and/or DNA binding to induce apoptosis. Therefore, we have constructed a retrovirus coding for Sp1 carrying point mutations on the Zn fingers two and three (Sp1Zn2,3) that are essential for DNA binding [1]. In Baf-3-Sp1 clone 1 the expression of the ectopic full-length wild-type Sp1 was rapidly induced following doxycycline removal (Figure 1A). Similar results were obtained when studying a second inducible clone (Baf-3-Sp1 clone 2) in which induction of Sp1 expression and apoptosis induction following doxycycline removal are slower (Figure 1B). In contrast a truncated Sp1 (tSp1, Figure S1), that accumulates in the cytoplasm does not affect cell viability (Figure 1C).

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Transcriptional response associated with overexpression of Sp1

To have a better insight into the molecular perturbations induced by Sp1 overexpression, we performed a genome-wide expression profiling to identify a set of genes that are affected by overexpression of Sp1. Expression profiling was conducted comparing Baf-3-Sp1 clone 1 grown in the presence of doxycycline for 28 hrs as control and the same clone grown 28 hrs without doxycycline. This time corresponds to a point where cells already expressed high levels of Sp1 protein without any detectable cell death (Figure 1A). Three independent experiments were performed and analysed using the Affymetrix genechip Mouse genome 430 2.0 arrays (data deposited in ArrayExpress http://www.ebi.ac.uk/microarray-as/ae, accession number E-MEXP-1702). Sp1 overexpression was associated with a substantial modification of transcription profiles (Figure S4).

Differential probesets expression following Sp1 overexpression relative to control was determined using statistical analysis in GeneSpring: 1294 genes with unique gene symbol identifiers were found to be differentially expressed with a fold change of expression of 1.3. Among them, the expression of 766 genes was increased whereas the expression of 528 genes was decreased. With a two-fold change of expression, 172 genes with unique gene symbol identifiers were found to be differentially expressed (162 up-regulated genes, 10 down-regulated genes). Table 1 shows the most up-regulated and down-regulated genes. Sp1 overexpression has an effect on the expression of many genes involved in metabolism, ubiquitination and transcription.
### Table 1. Genes affected by Sp1 overexpression.

| Functional category | Symbol | Full name | Fold Change | UniGene | Ensembl | Affymetrix ID |
|---------------------|--------|-----------|-------------|---------|----------|---------------|
| Metabolism          | Upp1   | uridine phosphorylase 1 | 5.0 | Mm.4610 | ENSMUSG00000020407 | 1448562_at |
|                     | St8sia4| ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 | 3.6 | Mm.306228 | ENSMUSG00000040710 | 140391_a_at |
|                     | Galnt3 | UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 | 3.4 | Mm.38441 | ENSMUSG00000026994 | 1417588_at |
|                     | Slc39a8| solute carrier family 39 (metal ion transporter), member 8 | 3.3 | Mm.30239 | ENSMUSG00000053897 | 1416832_at |
|                     | Tgm2   | transglutaminase 2 | 3.1 | Mm.330731 | ENSMUSG00000037820 | 1433428_x_at |
|                     | Mtap   | methythioadenosine phosphorylase | −2.1 | Mm.28500 | ENSMUSG00000062937 | 1424426_at |
| Ubiquitin           | Arih1  | ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 | 5.5 | Mm.305925 | ENSMUSG00000025498 | 1442730_at |
|                     | Fbxw2  | F-box and WD-40 domain protein 2 | 3.2 | Mm.41711 | ENSMUSG00000024083 | 1427148_at |
|                     | Pja2   | praja 2 | −2.0 | Mm.21109 | ENSMUSG00000026879 | 1437171_x_at |
| Transcription       | Jun    | Jun | 3.8 | Mm.275071 | ENSMUSG00000025498 | 1417409_at |
|                     | Irf7   | interferon regulatory factor 7 | 3.5 | Mm.3233 | ENSMUSG00000025498 | 1417244_a_at |
|                     | Bcl9   | B-cell CLL/lymphoma 9 | −2.0 | Mm.87600 | ENSMUSG00000038256 | 1451574_at |
|                     | Sp1    | trans-acting transcription factor 1 | −2.3 | Mm.4618 | ENSMUSG00000062937 | 1445852_at |
|                     | Mecp2  | methyl CpG binding protein 2 | −2.5 | Mm.131408 | ENSMUSG00000031393 | 1438930_s_at |
| Adhesion/Migration  | Gsn    | Gelsolin | 6.2 | Mm.21109 | ENSMUSG00000026879 | 1437171_x_at |
|                     | Arl6ip1| ADP-ribosylation factor-like 6 interacting protein 1 | 5.3 | Mm.29924 | ENSMUSG00000030654 | 1423819_s_at |
|                     | Prickle1| prickle like 1 | 3.4 | Mm.150314 | ENSMUSG00000026879 | 1452249_at |
| Receptors/transmembrane | Neo1  | neogenin | 10.1 | Mm.42249 | ENSMUSG000000032340 | 1447693_s_at |
|                     | Tmem51 | transmembrane protein 51 | 6.5 | Mm.27587 | ENSMUSG00000040616 | 1424383_at |
|                     | Tmem71 | transmembrane protein 71 | 6 | Mm.132299 | ENSMUSG00000036944 | 1436212_at |
|                     | Tnfrsf22| tumor necrosis factor receptor superfamily, member 22 | 3.9 | Mm.261384 | ENSMUSG00000010751 | 1447621_s_at |
|                     | Tnfrsf9| tumor necrosis factor receptor superfamily, member 9 | 3.4 | Mm.244187 | ENSMUSG00000028965 | 1428034_a_at |
|                     | Tnfrsf12| tumor necrosis factor receptor superfamily, member 12 | 3.7 | Mm.45995 | ENSMUSG00000024349 | 1447621_s_at |
|                     | Clec2d | C-type lectin domain family 2, member d | −2.3 | Mm.197536 | ENSMUSG00000030157 | 1419477_at |
| Apoptosis           | Bmf    | Bcl2 modifying factor | 4.0 | Mm.210125 | ENSMUSG00000040093 | 1454880_s_at |
| Cell cycle          | Lats2  | large tumor suppressor 2 | 3.7 | Mm.347899 | ENSMUSG00000021959 | 1439441_x_at |
| Exocytosis/Trafficking | Stxbp6| syntaxin binding protein 6 (amisyn) | 7.4 | Mm.285400 | ENSMUSG00000021959 | 1439441_x_at |
|                     | Cd74   | class II antigen-associated, II | 3.9 | Mm.276499 | ENSMUSG00000026410 | 1425519_a_at |
|                     | Tcte1l | dynein light chain Tctex-type 3 | −2.0 | Mm.29150 | ENSMUSG00000031176 | 1449929_at |
| Chemokine           | P4f4   | chemokine (C-X-C motif) ligand 4 | 8.4 | Mm.332490 | ENSMUSG00000029373 | 1448995_at |
| Inflammation        | Gbp1   | guanylate nucleotide binding protein 1 | 7.8 | Mm.250 | ENSMUSG00000028269 | 1420549_at |
|                     | Gbp2   | guanylate nucleotide binding protein 2 | 5.1 | Mm.24038 | ENSMUSG00000028270 | 1418240_at |
but also many genes with unknown functions. The kinetic of expression of five of the most up-regulated genes (Neo1, Cca1, Gsa, Slc39a8, and Jun) was precisely monitored using quantitative real-time PCR (Figure 3, left panels). Their up-regulation starts to be detected 16 hrs following doxycycline removal and is also observed in Baf-3 cells transduced with wild-type Sp1 but not Sp1Zn2,3 indicating that the binding of Sp1 to DNA is necessary for their induction (Figure 3, right panels).

Endogenous Sp1 is down-regulated immediately following Sp1 overexpression

One of the most down-regulated gene following overexpression of human Sp1 is the endogenous murine sp1 gene (2.3-fold decrease, Table 1). Sp1 promoter contains Sp1 binding sites and the Sp1 protein has been shown to autoregulates its own expression [26]. As Baf-3-Sp1 clone 1 expresses human Sp1 as well as GFP under the same inducible promoter we could monitor, in parallel, GFP protein expression by flow cytometry and both endogenous murine and ectopic human Sp1 were detected 8 hrs after doxycycline removal (Figure 4A). This correlates with the expression of ectopic Sp1 at the mRNA and protein levels (Figure 4B and data not shown). Importantly, down-regulation of endogenous sp1 gene transcription starts to be observed as early as 8 hrs after doxycycline removal in the whole cell population where only 20% of the cells are GFP positive (Figure 4C). This down-regulation requires the binding of Sp1 to the DNA. Altogether these results indicate that an excess of DNA-bound Sp1 is sensed by cells and induces a rapid repression of endogenous sp1 gene expression, revealing a transcriptional negative feedback loop.

Genes with Sp1 consensus binding site in their promoters are enriched among down-regulated genes but not among up-regulated genes

We next determined whether the set of Sp1-modified genes was enriched for the presence of Sp1 binding motif in their promoters (consensus Sp1 binding motif GGGGCGGGG as defined by TRANFAC 7.0 [V$SP1_Q6] and its variant GG[G/T][G/T][G/T][GGG [6]]. As a control, we also estimated the degree of enrichment for STAT6 transcription factor binding site TTGCN,GAA [25]. The presence of Sp1 and STAT6 binding sites was searched using a computational approach in the promoter regions consisting of 2000 bp upstream from the transcription start site. As a control, we analysed the promoters of a control list generated randomly that fulfils two criteria: no change in the gene expression levels following Sp1 overexpression and an overall distribution of expression that is similar to the one of the differentially expressed list (Figure S2). There was no significant difference in the percentage of promoters containing STAT6 site among up or down-regulated and control genes. In contrast, Sp1 sites were enriched among the promoters of genes whose expression was repressed (Table 2). Moreover, down-regulated genes contain on average more Sp1 sites in their promoters than genes from the control list. Surprisingly, there was no enrichment for the presence of Sp1 sites among the genes whose expression was increased by Sp1.

| Functional category | Symbol | Full name                                      | Fold Change | UniGene   | Ensembl                | Affymetrix ID     |
|---------------------|--------|------------------------------------------------|-------------|-----------|------------------------|-------------------|
| Bioconduction       | Hba-a1 | hemoglobin alpha, adult chain 1                | −2,3        | Mm.196110 | ENSMUSG00000069917     | 1452757_s_at      |
|                     | Scoc   | short coiled-coil protein                      | −2,0        | Mm.379079 | ENSMUSG00000063253     | 1416267_at        |
|                     | Morc3  | microchordia 3                                | −2,1        | Mm.287329 | ENSMUSG00000039456     | 1452224_at        |
|                     | Calml4 | calmodulin-like 4                             | 3,4         | Mm.28623  | ENSMUSG00000033224     | 1424713_at        |
|                     | 49305131N10Rik | 49305131N10Rik | 3,2   | Mm.386996 | ENSMUSG00000039456     | 1457415_a_at      |
|                     | 4933411B09Rik | 4933411B09Rik | 4,7   | Mm.359303 | ENSMUSG00000028698     | 1456482_at        |
|                     | 3110040M04Rik | 3110040M04Rik | 3,5   | Mm.75540  | ENSMUSG00000028698     | 1456482_at        |
| Signal transduction | Pik3r3 | phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55) | 5,9 | Mm.253819 | ENSMUSG00000028698     | 1456482_at        |
|                     | Eps8   | epidermal growth factor receptor pathway substrate 8 | 3,4 | Mm.235346 | ENSMUSG00000015766     | 1422824_s_at      |
|                     | Serpinf1 | serine (or cysteine) peptidase inhibitor, clade F, member 1 | 4,3 | Mm.2044  | ENSMUSG0000000753      | 1416168_at        |
| Miscellaneous       | Hist2h2aa2 | histone cluster 2, H2aa2 | 11,0  | Mm.359303 | ENSMUSG00000028698     | 1456482_at        |
|                     | E230032D23Rik | E230032D23Rik | 6,5   | Mm.359303 | ENSMUSG00000028698     | 1456482_at        |
|                     | 4933411B09Rik | 4933411B09Rik | 4,7   | Mm.359303 | ENSMUSG00000028698     | 1456482_at        |

Genes identified by microarray analysis (Affymetrix GeneChip Microarray 430.2) as most up-regulated or down-regulated following ectopic Sp1 expression in Baf-Sp1 clone 1 (3 independent experiments). Differential gene expression following Sp1 deregulation relative to control was determined using GeneSpring GX 7.3 statistical analysis module ANOVA. The full list of deregulated genes following Sp1 overexpression with informations on each gene (ID, Gene Ontology, Kegg pathway, probeset quality, mean expression, mean deregulation) can be found at ftp://pbil.univ-lyon1.fr/pub/datasets/DeniaudE2009/.

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Table 1. Cont.

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The *in silico* analysis was extended using comparative genomic tool DiRE that predicts regulatory elements (RE) within the promoters of a set of genes based on their inter-species conservation and their transcription factor binding sites content. Again the percentage of candidate regulatory elements containing a Sp1 binding site was higher within down-regulated genes compared to a control list (Figure S5) and Sp1 was predicted to be the most important transcription factor within the promoters of down-regulated genes. Altogether, these analysis indicate Sp1-binding sites are enriched only in down-regulated genes but not in up-regulated genes. This suggests that down-regulation of gene expression could be directly mediated by Sp1 whereas up-regulation of gene expression does not seem to involve the association of Sp1 with proximal promoters. As Sp1 DNA-binding is necessary for deregulation of gene expression, up-regulation of gene expression could represent an indirect event of Sp1 binding to genomic DNA.

Deregulated expression of cell cycle regulating genes following Sp1 overexpression is associated with accumulation of cells in the G1 phase of the cell cycle

Among the genes found to be differentially expressed, using the Ingenuity Pathway Analysis software, we observed the highest enrichments for the following categories: cancer, cell death and cell cycle (Figure S6). The list of genes belonging to these 3 categories being very large, we focused our attention on the cell cycle category as only a very small number of genes involved in the core cell cycle machinery was found to be affected by Sp1 overexpression (Figure 5). As a matter of fact, microarray data indicated that among the genes associated with the progression through the cell cycle such as cyclins, cyclin-dependent protein kinases and cyclin-dependent kinase inhibitors, only three showed a significant modification of expression levels: *Ccnd2* (cyclin D2, 1.7-fold decrease), *Ccng2* (cyclin G2, 1.9-fold increase) and *Cdkn2c* (cyclin-dependent kinase inhibitor 2c/p18, 2.0-fold increase). The down-regulation of *Ccnd2* expression and the up-regulation of *Ccng2* and *Cdkn2c* expression can be observed 16 hrs after doxycycline removal in inducible Baf-3-Sp1 clone (Figure 6A) and require Sp1 binding to DNA (Figure 6B). These genes have all been involved in the regulation of the G1-S transition. Therefore, we next investigated whether Sp1-induced overexpression of these genes could impair cell proliferation. Baf-3-Sp1 clone 1 was grown in the absence or in the presence of doxycycline and the distribution of the cells through the different phases of the cell cycle was studied by flow cytometry after a pulse with BrdU. As soon as 22 hrs after Sp1 induction the percentage of cells in the G1 phase started to increase whereas the percentage of cells in the S phase decreased in comparison to control condition (Figure 7A). A more pronounced effect on cell cycle was observed 28 hrs after

![Figure 3. Changes in expression profile is dependent on Sp1 binding to DNA. Kinetic of expression levels of five differentially expressed genes (Neogenin, Slc39a8, Gelsolin, Cxcl4, Jun) following doxycycline removal in Baf-3-Sp1 clone 1 (left panel). At the indicated times, cells were harvested and mRNA levels were measured by real-time PCR and normalized to HPRT mRNA levels. Results show the ratio of the mRNA levels measured in the absence of doxycycline relative to mRNA levels measured in the presence of doxycycline at each time point. Results show the mean ± sd of at least 2 independent experiments. Expression levels in Baf-3 cells transduced with either control, Sp1 or Sp1Zn2,3 retroviruses purified by magnetic selection with anti-CD2 antibody 30 hrs post-infection (right panel). Results show the mean ± sd of at least 2 independent experiments. doi:10.1371/journal.pone.0007035.g003](http://www.plosone.org)
Cell Cycle Inhibition by Sp1

Discussion

This study extends previous findings that have shown that Sp1 overexpression is detrimental to untransformed cells [18, 19]. Our study shows that overexpressed Sp1 induces an inhibition of the cell cycle through the cell cycle of Baf-3 cells that precedes apoptosis. Both processes require the binding of Sp1 to DNA and are not observed following the expression of a cytoplasmic truncated form of Sp1 or a mutated Sp1 unable to bind DNA. Microarray analysis shows that among the genes found to be differentially expressed following Sp1 overexpression there is an enrichment in genes involved in cancer, cell cycle and cell death.

Indeed, analysis of Sp1-induced deregulated genes with Ingenuity Pathways Analysis software revealed that 223 of them are linked with apoptosis. This includes the up-regulation of proteins that induce apoptosis when overexpressed such as pro-apoptotic Bcl-2 family members (Bmf, Bcl2L11/Bim, Bcl2L13/Rambo) [27], caspase 6 [28], cell death inducing factors (CIDEB, DAP) [29,30], dependence receptor neogenin [31] (Table 1 and microarray data available in Array express). Therefore Sp1 overexpression could trigger apoptosis through one or a combination of these pathways.

A number of data support a link between Sp1 and cell cycle progression [16,32]. A computational approach by Elkon et al. comparing the promoters of a set of cell cycle regulated genes revealed that Sp1 binding sequences are significantly enriched in promoters of genes that are expressed in G1/S phase [24]. Although Sp1 has been linked with cell growth, both positive and negative effects of Sp1 on cell growth have been reported. This is consistent with the regulation of both growth promoting genes as well as growth inhibitory genes by Sp1 [16]. Although down-regulation of cell cycle inhibitor cdk1a/p21 has been implicated in Sp1-induced inhibition of proliferation in vascular smooth muscle cells [19], we did not find a significant modification of cdk1a gene expression upon Sp1 induction in Baf-3 cells (Figure 5). However, we identified cyclin D2, cyclin G2 and cdk2/p12 as being deregulated among cell cycle regulating genes following the binding to DNA of overexpressed Sp1. The products of these genes could be responsible for Sp1-mediated cell cycle inhibition that we observed as they are all involved in regulating G1/S progression. D-cyclins are believed to serve as a link between the extracellular environment and the cell cycle machinery, driving progression through the G1/S phase of the cell cycle. In Baf-3 cells, Interleukin-3 removal induces a decrease of cyclin D2 mRNA expression and a cell cycle arrest that can be abolished by ectopic expression of cyclin D2 [33]. Cdkn2c is a member of the INK4 family that block cell cycle progression by binding to cyclin dependent kinase Cdk4 or Cdk6 and inhibiting the action of cyclin D. Finally, cyclin G2 is an unconventional cyclin that is up-regulated in B cells responding to growth inhibitory signals and its overexpression promotes a cell cycle arrest in G1 phase [34,35]. These 3 genes have previously been shown to possess Sp1-binding sites in their promoters and/or to be regulated by Sp1 [36–38]. In our experimental system, regulation of these cell cycle genes is likely a direct consequence of Sp1 on transcription.

The genome-wide expression profiling in Baf-3 cells indicates that a substantial proportion of expressed genes shows a modification of their transcription following Sp1 deregulation. Using real-time PCR, we have confirmed the impact of Sp1 on the expression of 18 out of 18 of these genes and shown that it requires
Table 2. Frequency of Sp1 consensus binding sites in the promoters of genes deregulated by Sp1.

| Sp1 site: GGGGCGGGGG | Percentage of genes with binding sites | p-value | Number of binding sites per gene | p-value |
|----------------------|---------------------------------------|---------|---------------------------------|---------|
| control list (a)     | 15.1%                                 |         | 1.3                             |         |
| up-regulated genes (b) | 12.4%                              | 0.0715  | 1.2                             | 0.2585  |
| down-regulated genes (c) | 20.3%                              | 0.0031  | 1.7                             | 0.0001  |

Variants of Sp1 site: GG(G/T)(C/T)GGG

| Percentage of genes with binding sites | p-value | Number of binding sites per gene | p-value |
|---------------------------------------|---------|---------------------------------|---------|
| control list (d)                     | 39.4%   |                                 | 1.7     |
| up-regulated genes (e)               | 41.3%   | 0.3591                          | 1.7     | 0.82    |
| down-regulated genes (f)             | 47.5%   | 0.0005                          | 2       | 0.0004  |

STAT6 site: TTCNNGAA

| Percentage of genes with binding sites | p-value | Number of binding sites per gene | p-value |
|---------------------------------------|---------|---------------------------------|---------|
| control list (a)                     | 41.4%   |                                 | 1.4     |
| up-regulated genes (b)               | 40.0%   | 0.0528                          | 1.3     | 1       |
| down-regulated genes (c)             | 36.9%   | 0.0549                          | 1.3     | 0.1694  |

Transcription factor binding sites were searched among the first 2000 nucleotides of the promoter region of differentially expressed genes and non-regulated genes using PATTERN service. Sp1 binding motifs: GGGGCGGGGG and GG(G/T)(G/T)GGG. STAT6 binding motif: TTCNNGAA.

(a) Control list of non-regulated genes generated randomly as described in the experimental procedures.
(b) Up-regulated genes from differentially expressed gene list (>1.3 fold).
(c) Down-regulated genes from differentially expressed gene list (<1.3 fold).
(d) Statistical significance calculated as described in the experimental procedures. P-values are the comparison of up-regulated vs control and down-regulated vs control.

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Figure 5. Effect of Sp1 deregulation on the expression of cell cycle regulating genes. Fold change of Cyclins, Cyclin-dependent kinases and Cyclin-dependent kinases inhibitors expression in Sp1 overexpressing cells compared to non expressing cells. Expression ratios are from microarray data (GeneSpring GX).
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Sp1 binding to DNA (this study and data not shown). To determine the proportion of genes that were potentially transcribed via binding of Sp1 to their promoter, we have search for Sp1-binding sites in the promoter of regulated genes. We observe that only down-regulated genes show a significant enrichment for Sp1 sites in their promoters. It is unlikely that this inhibition is due to the squelching of factors required for transcription as there is no global shut-down of transcription following Sp1 overexpression. Moreover expression of high levels of mutant nuclear form of Sp1 does not lead to transcription modulation. Therefore our results suggest that overexpressed DNA-bound Sp1 exerts a specific inhibitory effect on Sp1-driven transcription in Baf-3 cells. Contrasting with Sp1, the genes induced by c-Rel deregulation show an enrichment for genes containing consensus NF-kB/Rel sites in their proximal promoter region [39]. However, the up-regulation of a large number of genes following Sp1 overexpression could result from an indirect process that requires the binding of Sp1 to DNA. Indeed, DNA-binding of Sp1 could trigger transcription through multiple mechanisms. First, Sp1 could induce the expression of transcription factors that would then activate transcription. This seems unlikely since promoters of up-regulated genes did not exhibit an enrichment for binding sites for the few transcription factors (c-jun, Fos, E2F2) that were up-regulated by Sp1 (data not shown). Moreover, the DiRE analysis does not reveal an enrichment of putative regulatory elements in the non-coding sequence of deregulated genes (Figure S3). Second, Sp1 might affect gene transcription through the control of non-coding RNA as it was observed that many Sp1 binding sites are localised next to non-coding RNA genes [6]. Finally, the human genome contains more than 12,000 occupied Sp1 binding sites [6] and Sp1 has also been shown to localize within subnuclear foci that infrequently overlap with sites of transcription [40]. Therefore, the association of deregulated Sp1 with DNA might perturb chromatin structure. Further experiments will be necessary to determine the precise mechanisms involved in the gene transcription up-regulation induced by overexpressed Sp1.

Deregulated expression of Sp1 has been associated with the late steps of cell transformation [14–16]. Analysis of the categories of genes enriched following Sp1 deregulation revealed that the highest enrichment was observed for genes belonging to the Cancer category (Figure S6). The expression of genes known to have important role in migration/metastasis were up-regulated following Sp1 overexpression. This includes actin-binding protein gelsolin a protein involved in cell motility and required for cancer cell invasion [41], matrix metalloproteases MMP9 which degrade extracellular matrix and promotes invasion [42], metastasis-associated proteins S100A4/metastasin [43] and cathepsin B [44] (data not shown). Altogether these results show that Sp1 overexpression is detrimental for untransformed cells. However, in cells having acquired resistance to cell cycle inhibition and cell death, expression of these genes involved in migration/metastasis could confer a selective advantage and contribute to their further transformation.

Supporting Information

Figure S1 Sp1 constructs used in the study. Wild-type of Sp1 (Sp1) consists of four transactivation domains (A to D) and a DNA-binding domain (DBD) composed of three Zinc fingers. The mutant form Sp1Zn2,3 carries point mutation on the second and third Zinc fingers of the DBD. The truncated Sp1 tSp1 consists of the first 418 amino acids.

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Figure S2 Distribution of the expression of the genes differentially expressed following Sp1 deregulation (1610 probesets) (A) and the non-regulated genes from a control list (1700 probesets) (B) generated as indicated in the experimental procedures.

Found at: doi:10.1371/journal.pone.0007035.s002 (0.38 MB EPS)

Figure S3 Zn finger mutations abolish Sp1 ability to induce apoptosis. Baf-3 cells were transduced with retroviruses encoding wild-type Sp1 (Sp1) or Sp1 mutants carrying mutations in their DNA binding domain and analysed 30 hrs later. (A) Cells were costained for actin (red), Sp1 (green) and DNA (blue) and analysed by confocal microscopy. Arrowheads indicate overexpressing cells. Scale bar, 10 μm. (B) Left Western blot analysis of Sp1 protein levels in Baf-3 cells transduced with either control, Sp1 or various Sp1Zn mutants retroviruses purified with anti-CD2 antibody 30 hrs post-infection. Right The percentage of apoptotic cells (Annexin V positive) was measured at various time points among infected cells (CD2 positive) by flow cytometry. Results show the mean ± sd of at least 2 independent experiments.

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Figure S4 Gene expression profiles following Sp1 overexpression. Scatter plots of the mean signal intensity values of three independent microarray experiments (Affymetrix Mouse Genome
430.2). Data were analysed using GeneSpring GX 7.3 expression analysis software (Agilent Technology Inc). (A) All probesets. (B) Probesets differentially expressed following Sp1 overexpression relative to control. Differential expression was determined using GeneSpring GX 7.3 statistical analysis module ANOVA 1 way test, assuming variance not equal, choosing a false discovery rate of 6% with multiple testing correction (Benjamini and Hochberg). This gave a list of 1610 differentially expressed probesets with a fold change of expression of 1.3.

Figure S5 Analysis of transcription factor binding sites within Regulatory Elements (RE), predicted using inter-species conservation, of (A) down-regulated genes, (B) up-regulated genes and (C) the control list (expressed but non-regulated). Search was performed via the DiRE server which uses RE located within as well outside promoters (http://dire.dcode.org, Gotea, V. and I. Ovcharenko (2008) DiRE: identifying distant regulatory elements of co-expressed genes. Nucleic Acids Research, doi:10.1093/nar/gkn300). The results show the occurrence of transcription factor binding sites within RE and the Importance of each transcription factor that is the product of its occurrence and its weight (i.e. its association with the input gene set). The number of genes eligible for DiRE analysis are indicated.

Figure 7. Cell cycle inhibition induced by Sp1 requires its binding to DNA. (A) Baf-3-Sp1 clone 1 was grown in presence of doxycycline (+dox) or in absence of doxycycline for the indicated times (-dox) and pulsed with BrdU for 20 min. Cells were harvested and processed for BrdU labelling and DNA content staining. Cell cycle distribution was analysed by flow cytometry. Representative cell cycle profile with or without doxycycline at 28 hrs (left panel). Percentage of cells in the different phases of the cell cycle 22 or 28 hrs after treatment (right panel). (B) Cell cycle distribution of Baf-3-Sp1 clone 2 at 72 hrs. (C) Baf-3 cells were transduced with control or Sp1 encoding retrovirus. Cell cycle distribution among transduced (CD2 positive) cells 30 hrs post-infection. (D) Cell cycle distribution of Baf-3-Sp1 clone analysed after 72 hrs. (E) Baf-3 cells transduced with control or Sp1\(^{2\text{Zn}2,3}\) encoding retroviruses were analysed 30 hrs later. Percentage of cells in the different phases of the cell cycle assessed among CD2 positive cells. Results show the means of ± sd of 2 to 3 independent experiments.

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Author Contributions

Conceived and designed the experiments: ED JM YL. Performed the experiments: ED JB RC MCM YL. Analyzed the data: ED JB RC BB LB JM MCM AL CAS AW JM YL. Contributed reagents/materials/analysis tools: JM MC JL CG. Wrote the paper: ED JM YL.

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