Promoter cloning and characterization of the human programmed cell death protein 4 (pdcd4) gene: evidence for ZBP-89 and Sp-binding motifs as essential Pdcd4 regulators

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Synopsis
Pdcd4 (programmed cell death protein 4) is an important novel tumour suppressor inhibiting transformation, translation, invasion and intravasation, and its expression is down-regulated in several cancers. However, little is known about the transcriptional regulation and the promoter of this important tumour suppressor. So far the following is the first comprehensive study to describe the regulation of Pdcd4 transcription by ZBP-89 (zinc-finger-binding protein 89), besides characterizing the gene promoter. We identified the transcriptional start sites of the human pdcd4 promoter, a functional CCAAT-box, and the basal promoter region. Within this basal region, computer-based analysis revealed several potential binding sites for ZBPs, especially for Sp (specificity protein) family members and ZBP-89. We identified four Sp1/Sp3/Sp4-binding elements to be indispensable for basal promoter activity. However, overexpression of Sp1 and Sp3 was not sufficient to enhance Pdcd4 protein expression. Analysis in different solid cancer cell lines showed a significant correlation between pdcd4 and zbp-89 mRNA amounts. In contrast with Sp transcription factors, overexpression of ZBP-89 led to an enhanced expression of Pdcd4 mRNA and protein. Additionally, specific knockdown of ZBP-89 resulted in a decreased pdcd4 gene expression. Reporter gene analysis showed a significant up-regulation of basal promoter activity by co-transfection with ZBP-89, which could be abolished by mithramycin treatment. Predicted binding of ZBP-89 to the basal promoter was confirmed by EMSA (electrophoretic mobility-shift assay) data and super-shift analysis for ZBP-89. Taken together, data for the first time implicate ZBP-89 as a regulator of Pdcd4 by binding to the basal promoter either alone or by interacting with Sp family members.

Key words: programmed cell death 4 (Pdcd4), promoter, transcription, tumour suppressor, zinc-finger-binding protein 89 (ZBP-89), zinc finger protein

INTRODUCTION
Pdcd4 (programmed cell death 4) is an important novel tumour suppressor, inhibiting carcinogenesis, tumour progression, invasion and intravasation. Pdcd4 interacts with translation initiation factors, namely, the RNA helicase eIF4A (eukaryotic initiation factor 4A) and the scaffolding protein eIF4G, and is involved in the control of eukaryotic transcription and translation. The pdcd4 gene has originally been identified as a gene whose expression is strongly up-regulated during apoptosis in response to different inducers. Pdcd4 was cloned independently by different groups and the human gene is located at chromosome 10q24 [1]. In normal tissue, pdcd4 expression is ubiquitous and has been reported, for example, in small duct epithelial cells of the normal mammary gland [1,2], growth-suppressed human neuroendocrine pancreatic carcinoma cells [3], normal human lung tissue [4] and senescent human fibroblasts [5]. Pdcd4 levels are elevated in human IL-12 (interleukin 12)-induced NK (natural killer) and T-cells [6], in acute myeloid leukaemia cells during

Abbreviations used: AcH3, acetylated histone H3; 5-aza-dC, 5-aza-2-deoxycytidine; ChIP, chromatin immunoprecipitation; COX-2, cyclo-oxygenase 2; eIF, eukaryotic initiation factor; EMSA, electrophoretic mobility-shift assay; HDAC1, histone deacetylase 1; IL, interleukin; NF-Y, nuclear factor Y; Pdcd4, programmed cell death protein 4; RACK1, rapid amplification of cDNA ends RA, retinoic acid; RAR, retinoic acid receptor; RT–PCR, reverse transcription–PCR; RXR, retinoid X receptor; siRNA, small interfering RNA; Sp1, specificity protein 1; TSA, trichostatin A; ZBP-89, zinc-finger-binding protein 89

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granulocyte differentiation [7], and a high expression was found in the suprabasal layer of the epidermis and suprabulbar area of the hair follicle [8], indicating a role of Pdcd4 during differentiation. In several cancers, Pdcd4 expression is down-regulated, such as in lung- and renal-derived tumours [9], tongue tumours [10], hepatoma tissue [11], invasive ductal breast carcinoma [12], skin cancer [8], human glioma [13], nasopharyngeal carcinoma [14], colorectal cancer [15] and HCC (hepatocellular carcinoma) [16]. Furthermore, the expression of Pdcd4 is regulated by drugs with anticancer effects such as topoisomerase inhibitors, COX-2 (cyclo-oxygenase 2) inhibitors, Herceptin, and RAR (retinoic acid receptor) agonists. Moreover, recent studies by us and others identified Pdcd4 as a target of miR-21 (microRNA-21) and showed that the expression of Pdcd4 seems to be regulated in part at the translational level [17,18]. However, little is known about the gene promoter and cognate transcription factors that mediate pdcd4 gene expression. So far, only a few studies were undertaken to find potential cis-regulatory elements and investigate their influence on pdcd4 gene expression. In one initial study, the structure of the mouse gene was characterized. It was shown that the gene spanned approximately 21 kb, including 11 exons, and presented in a single copy [19]. The putative transcriptional start site was located 192 bp upstream from the translational start site, harbouring a TATA sequence and CAAT box at positions −21 and −81 respectively. Furthermore, putative binding sites for NF-κB (nuclear factor κB) at position −488 to −470 and two C/EBP (CCAAT/enhancer-binding protein)-binding sites (−424 to −416 and −254 to −246) were found. For the chicken pdcd4 promoter, it was demonstrated that the transcription factor v-Myb was able to induce pdcd4 transcription [20], and a knock-out of c-Myb reduced pdcd4 levels [21]. Recently, it was shown that epigenetic mechanisms, such as DNA methylation, might also contribute to pdcd4 regulation. By knocking down DNA methyltransferase 1 in a hepatocellular carcinoma cell line, it was verified that demethylation of DNA-induced pdcd4 expression [22], even through a demethylating agent resulted in no change in Pdcd4 amounts in breast cancer cell lines [12]. In addition, pdcd4 5′-CpG island methylation within the promoter was associated with reduced Pdcd4 mRNA in human glioma cell lines and tissue, and a restoration of pdcd4 was observed after blocking methylation in glioma cells [23].

Zinc finger transcription factors play key roles in cell differentiation, motility, proliferation and survival. ZBP-89 (zinc-finger-binding protein-89; also known as BFCOL1, BERF-1 and ZNF-148) is a Krüppel-type zinc-finger transcription factor that binds to the same GC-rich sequences as Sp1 or Sp3 [24]. It was cloned by screening of an expression library, with a GC-rich epidermal growth factor responsive element from the gastrin promoter [25]. Having bifunctional regulatory domains, ZBP-89 can activate or repress transcription of a variety of genes. It is known that ZBP-89 can repress the expression of vimentin [26], ENA-78 (epithelial neutrophil-activating peptide-78) [27], β2-integrin CD11b [28], gastrin [29], ODC (ornithine decarboxylase) [30], SOX18 [31], p16 [32], Pax7 [33] and bovine adenodoxin [24]. In contrast, it activates the expression of p21waf1 [34], the T-cell α- and β-receptor [35], the lymphocyte-specific protein-tyrosine kinase (Ick) [36], type 1 collagen [37], IAP (intestinal alkaline phosphatase) [38], GHR (growth hormone receptor) [39] and stromelysin [40]. So far, the exact mechanism as to how ZBP-89 exactly exercises these effects is still under investigation. It is widely believed that whether ZBP-89 binding stimulates or inhibits gene transcription depends on the specific promoter and perhaps other, as yet unidentified, factors. It can directly inhibit transcription, as in the case of the gastrin gene [29], indirectly inhibit transcription through competition with Sp1 for binding to overlapping binding sites [30], or through blocking the activity of Sp1 by protein–protein interaction [26]. Moreover, ZBP-89 is usually over-expressed in human cancer cells, where it can efficiently induce apoptosis through p53-dependent and -independent mechanisms [41,42]. Therefore, ZBP-89 may be interesting as a potential target in cancer therapy.

In consequence, the present study was undertaken (i) to for the first time characterize the core promoter of the human pdcd4 gene encoding this important tumour suppressor and (ii) to delineate major transcription factors and cis-elements by which transcriptional regulation is mediated. Taken together, we identified the transcriptional start sites of the human pdcd4 promoter, a functional CCAAT-box, and the basal promoter region spanning 548 bp upstream of the first exon. Furthermore, we show first evidence for Sp family transcription factors and especially ZBP-89 as important regulators for the transcription of the pdcd4 gene in solid cancers.

**MATERIALS AND METHODS**

**Materials, antibodies and cell lines**

Media/FBS (fetal bovine serum) were purchased from Invitrogen/Gibco and Sigma. All cell lines were obtained from the A.T.C.C. The following antibodies were from Santa Cruz: Sp1 (sc-59X), Sp2 (sc-643X), Sp3 (sc-644X), Sp4 (sc-654X), ZBP-89 (sc-48811X), trimethyl histone H4 (sc-134216), pan-acetyl (sc-8663), NF-YB (nuclear factor YB; sc-7711X) and unspecific IgG. The Anti-Xpress™ antibody was purchased from Invitrogen. Oligonucleotides were supplied by Metabion and are listed in and 2. Mithramycin and 5-aza-dC (5-aza-2-deoxycytidine) were from Sigma.

**Western blotting and immunoprecipitation**

Cell lysis, quantification of lysate protein and Western immunoblotting were performed as described previously [42a].

**Reporter assays, RNA interference and transfection**

Cells were transfected using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. Dual-luciferase assays were performed according to the manufacturer’s protocol (Promega). Cells were transfected with siRNAs (small interfering RNAs) specifically targeting the zbp-89 and sp1 genes (Nos. s515207, s515208, s13318 and s13319; Ambion),
or a non-silencing control (No. 4611G; Ambion). Experiments such as luciferase assays were performed at least in triplicate, after separate and independent transient transfections with at least 85% transfection efficiency. Luciferase assays were performed on 96 well plates, using 150 ng of pdcd4–luciferase constructs or pGL3-basic vector together with 50 ng of Renilla luciferase plasmid. Alternatively, 100 ng of pdcd4-luciferase constructs or pGL3-basic vector and 10 ng of Renilla luciferase plasmid was used together with 150 ng of expression plasmid. The primers used are shown in Supplementary Table S1 (at http://www.bioscirep.org/bsr/032/bsr0320281add.htm).

Nuclear extracts, EMSA (electrophoretic mobility-shift assay) and ChIP (chromatin immunoprecipitation)

Preparation of nuclear extracts and EMSAs was done as described in [43], using oligonucleotides spanning regions +2/+42, −223/−185, −170/−131 and −88/−51 of the pdcd4 promoter. ChIP assays were performed using the ChIP assay kit from Upstate. Protein–DNA complexes were immunoprecipitated with 2 μg of trimethyl histone H4, pan-acetyl, NF-Y (nuclear factor Y), Sp-1, Sp-2, Sp-3, Sp-4 and ZBP-89 antibodies. DNA was purified and eluted with 100 μl of elution buffer using the Qiagen reaction purification kit and quantitative PCR amplification was done using 2 μl of DNA sample with primers amplifying region −241/−42 of the pdcd4 promoter for 30 cycles using SYBR® Green PCR Mastermix (Applied Biosystems). As a negative control, a region within the pdcd4 promoter region −3495/−3294 was amplified for 35 cycles. Amplification of soluble chromatin prior to immunoprecipitation was used as an input control. The oligonucleotides used in the EMSA and ChIP assays are shown in Supplementary Table S2 (at http://www.bioscirep.org/bsr/032/bsr0320281add.htm).

Total RNA extraction, cDNA synthesis and real-time-PCR

Total RNA from cell lines was extracted using the RNeasy Mini Kit (Qiagen), and total RNA from tissue specimen using TRIzol® Reagent (Invitrogen). For real-time PCR, 500 ng of total RNA was reverse-transcribed in a total volume of 25 μl of elution buffer using the Qiagen reaction purification kit and quantitative PCR amplification was done using 2 μl of DNA sample with primers amplifying region −241/−42 of the pdcd4 promoter for 30 cycles using SYBR® Green PCR Mastermix (Applied Biosystems). As a negative control, a region within the pdcd4 promoter region −3495/−3294 was amplified for 35 cycles. Amplification of soluble chromatin prior to immunoprecipitation was used as an input control. The oligonucleotides used in the EMSA and ChIP assays are shown in Supplementary Table S2 (at http://www.bioscirep.org/bsr/032/bsr0320281add.htm).

RACE (rapid amplification of cDNA ends)

5’-RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech) as described by the manufacturer’s protocol, to identify the transcriptional start site of the pdcd4 gene. The 5’-RACE ready first-strand cDNA was synthesized using 1 μg of total RNA from MCF-7 cells and human placenta. The 5’-flanking sequence of pdcd4 was amplified using a universal primer (UMP, Clontech) and a pdcd4-specific primer located within exon 3 (see Supplementary Table S2). The resulting PCR products were gel-purified, cloned into the pGEM-T easy vector (Promega), and 20 different positive clones of each were sequenced.

5-Aza-dC treatment

A total of 4×10^5 cells were plated in a six-well plate in their respective medium supplemented with 10% FBS, and allowed to grow for 24 h. Cells were treated either with a final concentration of 10 μM 5-aza-dC for 72 h, changing the drug every 24 h, or with normal growth medium as a control. For expression analysis, cells were washed with ice-cold PBS, and total RNA or protein was isolated.

Preparation of reporter constructs

A 4042 bp regulatory region 3826 bp upstream from the major translational start site, including the first exon, of the pdcd4 gene (NCBI Reference Sequence: NT_030059.13) was amplified by PCR from human genomic DNA and cloned into pGL3-Basic (Promega) using KpnI and XhoI, to generate the pdcd4-luciferase reporter plasmid. A series of different 5’ truncated fragments were created by PCR amplification from the approximately 4 kb reporter plasmid, and inserted into pGL3-Basic as described above. Site-directed mutagenesis was performed with the QuikChange® kit (Stratagene) using the −345/+355 and −55/+355 reporter plasmids as templates. The site-specific mutations were confirmed by sequencing.

Expression vectors and construction of ZBP-89 expression plasmids

Expression vectors containing human Sp1 or Sp3 (pCMV-Sp1/Sp3) were gifts from Dr Guntram Suske (Institut für Molekularbiologie und Tumorforschung, Philipps-University Marburg, Marburg, Germany). cDNA for ZBP-89 was amplified by PCR (forward: 5'-GAACCTGAATGACGTCGGCAGCAACATGGAATCTAGATTAGCCAAAAGTC-3' and reverse: 5'-GAATCTAGATTGCGCAAACTGTTG-3'). The cDNA was ligated into pGL3-Basic vector using KpnI and XhoI, to generate the pdcd4-luciferase reporter plasmid. A series of different 5’ truncated fragments were created by PCR amplification from the approximately 4 kb reporter plasmid, and inserted into pGL3-Basic as described above. Site-directed mutagenesis was performed with the QuikChange® kit (Stratagene) using the −345/+355 and −55/+355 reporter plasmids as templates. The site-specific mutations were confirmed by sequencing.

Statistical analysis

Statistical analysis was performed using SPSS version 14.0. To determine correlations between variables, Spearman’s correlation coefficient was calculated; a P-value < 0.05 was considered significant.

RESULTS

Mapping of the pdcd4 transcriptional start site(s) and functional analysis of the promoter region

To identify the transcription initiation site(s) for pdcd4, 5’-RACE was performed using a pdcd4-specific primer anchored within
A. Total RNA from MCF-7 and normal human placenta was used for 5′-RACE PCR analysis. RACE was conducted as described in the Materials and methods section. A major band at approximately 570 bp was detected which was later cloned into the pGEMT-Easy vector and sequenced. M, 100 bp ladder DNA marker; C, MCF-7; P, placenta. 

B. The genomic sequence of the region containing the pdcd4 transcription initiation sites is shown. This sequence of 743 bp represents 388 bp upstream and 355 bp downstream of the pdcd4 gene. The numbers indicate the base location relative to the start site of the first exon indicated with a down arrow. Bold letters indicate the first exon. Letters with dots on top indicate the transcriptional start sites detected by RACE PCR. Underlined sequences represent transcription factor binding sites and a CCAAT box that were identified by TRANSFAC software (http://www.biobaseinternational.com). Deletion analysis of the pdcd4 promoter region. 

C. A series of the fragments of the 5′-flanking pdcd4 promoter region are schematically shown. The base positions relative to the start site of the first exon are indicated. 

D. Reporter assay showing pdcd4 promoter activity. Then 150 ng of pdcd4-luciferase constructs or pGL3-basic vector together with 50 ng of Renilla luciferase plasmid were transiently transfected into MCF-7 cells. The firefly luciferase activity was normalized with Renilla luciferase activity. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate.

Figure 1  RACE analysis for the transcriptional start site and nucleotide sequence of the 5′-flanking region of the human pdcd4 gene

(A) Total RNA from MCF-7 and normal human placenta was used for 5′-RACE PCR analysis. RACE was conducted as described in the Materials and methods section. A major band at approximately 570 bp was detected which was later cloned into the pGEMT-Easy vector and sequenced. M, 100 bp ladder DNA marker; C, MCF-7; P placenta. (B) The genomic sequence of the region containing the pdcd4 transcription initiation sites is shown. This sequence of 743 bp represents 388 bp upstream and 355 bp downstream of the pdcd4 gene. The numbers indicate the base location relative to the start site of the first exon indicated with a down arrow. Bold letters indicate the first exon. Letters with dots on top indicate the transcriptional start sites detected by RACE PCR. Underlined sequences represent transcription factor binding sites and a CCAAT box that were identified by TRANSFAC software (http://www.biobaseinternational.com). Deletion analysis of the pdcd4 promoter region. (C) A series of the fragments of the 5′-flanking pdcd4 promoter region are schematically shown. The base positions relative to the start site of the first exon are indicated. (D) Reporter assay showing pdcd4 promoter activity. Then 150 ng of pdcd4-luciferase constructs or pGL3-basic vector together with 50 ng of Renilla luciferase plasmid were transiently transfected into MCF-7 cells. The firefly luciferase activity was normalized with Renilla luciferase activity. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate.
Histone modifications associated with the basal pdcd4 promoter region

Promoters of transcribed genes are enriched with K4 trimethylation and hyperacetylation on the N-terminal tail of histone H3. Amounts of modified histones H3K4me3 (histone H3 Lys4 trimethylation) and AcH3 (acetylated histone H3) have been shown to correlate positively with the level of transcription. For pdcd4, it was recently shown that 5′ CpG island methylation blocks Pdcd4 expression at the mRNA level [23]. In silico analysis of the promoter revealed a GC-rich region (−241/−41) within the core promoter with 12 CpG sites within the region of the putative zinc finger transcription factor binding sites. To clarify the role of epigenetic suppression of the pdcd4 gene, we first examined pdcd4 expression upon treatment with the DNA methyltransferase inhibitor 5-aza-dC. As shown in Figure 2(A), the inhibitor led to an increase in Pdcd4 mRNA and protein. To further investigate local histone acetylation and H3 methylation in the chromatin associated with the basal pdcd4 promoter region, a ChIP assay was performed. The histone-associated DNAs, immunoprecipitated with antibodies against AcH3 and H3K4me3, were individually performed. The histone-associated DNAs, immunoprecipitated with antibodies against AcH3 and H3K4me3, were individually performed. The histone-associated DNAs, immunoprecipitated with antibodies against AcH3 and H3K4me3, were individually performed. The histone-associated DNAs, immunoprecipitated with antibodies against AcH3 and H3K4me3, were individually performed.

Mithramycin represses pdcd4 promoter activity and decreases pdcd4 mRNA and protein amounts

Because the initial computer-based screen for cis-elements within the active basal promoter region revealed different potential zinc-finger-binding motifs, we further investigated the functional relevance of zinc finger transcription factors for pdcd4-gene expression. Towards this end, cells were treated with mithramycin, which binds to GC-rich DNA sequences and impedes the binding of GC-specific transcription factors by steric hindrance. Cells were transfected with the reporter construct containing the active basal promoter region and treated with 50 nM mithramycin. Mithramycin decreased pdcd4 promoter activity by more than 50% (Figure 3A). To confirm an effect of mithramycin on pdcd4 gene expression, cells were treated with 50 nM mithramycin for 24 and 48 h. As shown in Figures 3(B) and 3(C), the inhibition of zinc finger transcription factor binding led to a significant decrease in Pdcd4 mRNA and protein in all three cell lines. These observations support our hypothesis that the GC-rich and zinc-finger-binding sequences identified within the basal promoter are required for Pdcd4 expression.

A CCAAT box is required for pdcd4 promoter activity and is competent to bind NF-Y

In order to confirm that the putative CCAAT box at +19 to +23 shows a specific binding, we generated oligonucleotide probes spanning this site, and the corresponding mutated or deleted probes, and performed EMSA analysis. Unlabelled probe abolished the shifting of all bands except for one unspecific band. In contrast, competition with unlabelled oligonucleotides, with either a mutation or deletion of the CCAAT sequence, showed a shift of three bands, indicating a specific binding at this cis-element (Figure 2F, left panel). The fact that NF-Y specifically binds CCAAT sequences in many eukaryotic genes prompted us to determine whether this element is also bound by NF-Y. Comparison of the pdcd4 gene CCAAT box and its flanking sequences with the NF-Y-consensus showed only one mismatch (C/G to A). Therefore, a supershift analysis was conducted to investigate whether NF-Y transcription factors can bind to that CCAAT-box using an antibody against the NF-YB subunit of the NF-Y trimeric complex. Addition of the antibody resulted in a single supershifted band demonstrating that NF-Y specifically binds the CCAAT box at +19 to +23 (Figure 2F, right panel). This was confirmed in a ChIP analysis for the same region, in which a clear increase in NF-Y binding to the natural pdcd4 promoter motif was observed (Figure 2G). To test the functional relevance of the CCAAT box, reporter constructs containing mutations or a deletion at this site were constructed. The wild-type sequence and the mutation or deletion are presented in Figure 2(D). Transient transfection of the different reporter constructs into MCF-7 cells revealed that mutagenesis of the site at +19/+23 resulted in a more than 60% loss in reporter activity. The deletion led to a loss of 50% (Figure 2E). Taken together, these data show functionality of the pdcd4-CCAAT-box which is bound by NF-Y.

Sp sites are functionally relevant for pdcd4 basal promoter activity

Sp family transcription factors are ubiquitously expressed and represent zinc finger-containing DNA-binding proteins. They bind GC-rich motifs with high affinity and can regulate the expression of TATA-containing and TATA-less genes via protein–protein interactions, or interplay with other transcription factors and/or components of the basal transcriptional machinery [45]. On the basis of the finding of different potential Sp-binding sites within the basal promoter region at positions −195, −162, −147, −86 and −76, we asked to what extent these factors are required for pdcd4 gene expression. To show that all Sp sites are relevant for basic pdcd4 promoter activity, we transiently transfected the wild-type reporter construct spanning the active basal promoter region (pGII3dei1_500), or a mutated reporter construct (pGII3dei1_500xmut) into RKO, HCT-116 and MCF-7 cells. The reporter assays revealed that mutation of four sites resulted in a 59% (RKO), 57% (HCT-116) or 50% (MCF-7) loss in reporter activity.
Figure 2  ChIP for AcH3 and H3K4me3 binding for the pdcd4 promoter upon 5-aza-dC treatment

RKO cells were treated with 5-aza-dC (Aza) at a final concentration of 10 μM for 72 h and subjected to ChIP and Western blotting. (A) Treatment with Aza increases Pdcd4 mRNA and protein. Total RNA was extracted, reverse-transcribed and quantified by quantitative-PCR using specific TaqMan primers and probes for Pdcd4. Samples were analysed in quadruplicates, and relative Pdcd4 expression normalized against β-actin. Pdcd4 amount is shown as a relative quantification, with untreated cells as a reference. Protein extracts were prepared and subjected to Western blotting with polyclonal anti-Pdcd4 antibody. Equal amounts of protein were confirmed using a monoclonal anti-β-actin antibody (AC-15). (B, C) Immunoprecipitated DNA with specific antibodies for acetylated and methylated histones was amplified by PCR using a primer set specific for the pdcd4 region (−241 to −41) encompassing the basal promoter region in front of the pdcd4 transcriptional start sites (left panel). As a negative control, immunoprecipitated DNA was amplified by a primer set specific to an off-target region 4 kb upstream of the Pdcd4 transcriptional start sites (−3495 to −3294 bp) (right panel). A CCAAT box is required for pdcd4 promoter activity. (D) Schematic representation of the site-directed mutagenesis and deletion performed on the CCAAT box motif within the basal promoter region. The base positions relative to the start site of the first exon are indicated. (E) Reporter assay showing pdcd4 promoter activity. The firefly luciferase activity was normalized with Renilla luciferase activity. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate. EMSA, supershift and ChIP demonstrated the in vitro and in vivo binding of NF-Y transcription factor to the pdcd4 promoter region. (F) Gel-shift and supershift analysis for the CCAAT box at +19/ +23. Equal protein amounts of nuclear extract from cells were incubated with an end-labelled oligonucleotide (+2/+42) in the presence or absence of a 100-fold excess of the unlabelled competitor (Comp.) sequences. Data are representative of three different experiments. (G) The in vivo association of NF-Y with the pdcd4 basal promoter was evaluated with ChIP assay in HCT-116 cells. Equal amounts of chromatin were incubated with the indicated antibodies and quantified by real-time PCR using a primer set specific for the basal region. As a negative control, immunoprecipitated DNA was amplified by a primer set specific to an off-target region in the pdcd4 promoter.
Regulation of Pdcd4 expression by ZBP-89

Figure 3 Pdcd4 promoter activity and expression is repressed by mithramycin

(A) Mithramycin represses the activity of the basal promoter region of pcd4. Then 150 ng of pcd4-luciferase constructs or pGL3-basic vector together with 50 ng of Renilla luciferase plasmid were transiently transfected into the cells, and 6 h post-transfection cells were mock-treated or treated with 50 nM mithramycin. The firefly luciferase activity was normalized with Renilla luciferase activity. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate. (B) Mithramycin treatment leads to a decreased Pdcd4 mRNA level. Total RNA was extracted, reverse-transcribed and quantified by real-time PCR using specific TaqMan primers and probes for Pdcd4. Samples were analysed in quadruplicate, and relative pdcd4 expression normalized against β-actin. Pdcd4 amounts are shown as a relative quantification, with cells that were either mock-treated or treated with 50 nM mithramycin. (C) Mithramycin treatment decreases Pdcd4 protein. Pdcd4 protein amounts were analysed by using anti-Pdcd4 antibody and equal amounts of protein were confirmed using an anti-β-actin antibody.

activity as compared with the wild-type construct (Figure 4A). Gel-shift and supershift analysis was conducted to investigate whether Sp transcription factors can bind to the basal promoter region, and to differentiate individual Sp family members bound to this region. Nuclear extracts of cells were incubated with three different probes (del713_6, del713_8 and del713_11) spanning the above described Sp-binding sites. As shown in Figure 4(B), a 100-fold excess of unlabelled probe abolished the shifting of all bands, indicating a specific binding of transcription factors within all three oligonucleotides (lanes 1, 2, 8, 9, 15 and 16). In supershift analysis, especially Sp1 (lanes 7, 19 and 17), in addition to Sp3 (lanes 12 and 19) and Sp4 (lane 20), was identified as the major Sp family protein bound to this region. To gain further evidence for Sp1 or Sp3 being essential for promoter activity, RKO cells were transiently co-transfected with the wild-type reporter construct spanning the active basal promoter region (pGl3del1_500), and either an expression plasmid for Sp1 or Sp3 (pCMV-Sp1 and pCMVSp3). Both Sp1 and Sp3 strongly induced pcd4 promoter activity [Sp1 176% (RKO), 17% (HCT-116), 45% (MCF-7); Sp3 193% (RKO), 84% (HCT-116), 59% (MCF-7)], suggesting that either of these transcription factors alone can positively drive pcd4 gene expression (Figure 4C). Since these data implicated Sp1 or Sp3 as possible transcription factors mediating pcd4 gene expression at these promoter motifs, cDNA and cell lysates from cells overexpressing Sp1 or Sp3 and vector transfected cells were compared for Pdcd4 mRNA and protein amounts. In Sp1 or Sp3 transfected RKO cells, only the amount of endogenous Pdcd4 mRNA was noticeably higher than in vector-transfected cells, whereas endogenous Pdcd4 mRNA remained unaffected in HCT-116 and MCF-7 cells (Figure 4D).
Figure 4  Sp sites are functionally relevant for pdcd4 basal promoter activity

(A) Effect of site-directed mutagenesis of Sp-transcription-factor-binding sites on pdcd4 promoter activity. The firefly luciferase activity was normalized with Renilla luciferase activity. Mock-vector-co-transfected luciferase activity was set to 100 %, and activity of the other constructs was calculated and plotted as a percentage of this value. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicates. EMSA and supershift analysis demonstrated the binding of Sp-transcription factors to the pdcd4 promoter region. (B) EMSA and supershift analysis show a specific binding of Sp1, 3 and 4 within the basal region spanning 295 bp. Equal amounts of nuclear extract from MCF-7 cells were incubated with the corresponding end-labelled oligonucleotide for the basal promoter region of the pdcd4 promoter in the presence or absence of a 100-fold excess of the unlabelled competitor sequence and the indicated specific antibodies against Sp family members. Sp complexes are indicated by an arrow. * Indicates supershifted bands, lower # indicates bands competed by the antibody. Overexpression of Sp1 and Sp3 induces Pdcd4 promoter activity and increases Pdcd4 mRNA, but is not sufficient to enhance Pdcd4 protein amounts. (C) The firefly luciferase activity was normalized with Renilla luciferase activity. Mock-vector-co-transfected luciferase activity was set to 100 %, and activity of the other constructs was calculated and plotted as a percentage of this value. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicates. (D) Total RNA from cells transfected with the Sp1-, Sp3- or empty-expression plasmid was extracted, reverse transcribed and quantified by real-time PCR using specific TaqMan primers and probes for Pdcd4. Samples were analysed in quadruplicate, and relative Pdcd4 expression levels normalized against β-actin. Pdcd4 amounts are shown as a relative quantification, with untreated cells as a reference. (E) Cells were transfected in a six-well plate with 4 μg of pCMV-Sp1, pCMV-Sp3, the empty vector (ev) or (F) with a siRNA targeting Sp1, with scRNA used as a control. After 48 h post-transfection, cells were harvested and cell lysates were immunoblotted. Sp1, Sp3 and Pdcd4 protein amounts were analysed by using anti-Sp1, Sp3 or Pdcd4 antibody and equal amounts of protein were confirmed using an anti β-actin antibody.

This observation was paralleled by the absence of an influence of Sp1 or Sp3 overexpression on the Pdcd4 protein level in all three cell lines (Figure 4E). Owing to the high endogenous Sp1 expression in all cell lines examined, we performed additional knockdown experiments. As shown in Figure 4(F), expression of a siRNA oligonucleotide against Sp1 decreased endogenous Sp1 protein to almost 55 % in RKO, to 55 % in HCT-116 and 51 % in MCF-7 cells respectively, but had little effect (~4 %) on Pdcd4 protein in these cells. This led us to the assumption that Sp1 or Sp3 alone are not sufficient to regulate Pdcd4.
Expression of Pdcd4 and ZBP-89 correlate in different cell lines and basal pdcd4 promoter activity is induced by ZBP-89

The zinc finger transcription factor ZBP-89 has been implicated in the induction of growth arrest and apoptosis [41]. Furthermore, it can interact with Sp family members and HDAC1 (histone deacetylase 1) to regulate gene expression, and we found several potential binding sites within the basal promoter region of the pdcd4 gene (Figure 1B). These observations and the low impact of Sp transcription factors on the Pdcd4 protein level raised the question whether ZBP-89 mainly regulates the expression of the pdcd4 gene. We first determined the expression of ZBP-89 and Pdcd4 mRNA in 17 different cancer cell lines (colorectal, gastric, breast and lung cancer) by QRT–PCR [quantitative RT–PCR (reverse transcription–PCR)] (Figure 5A). A significant positive correlation (c = 0.76) between pdcd4 mRNA and zbp-89-mRNA was observed in these 17 cell lines (P = 0.001) (Figure 5A). As shown by the TRANSFAC database search and the previous results, the basal pdcd4 promoter region contains different Sp and ZBP-89-binding sites. To explore the possibility that the promoter activity to a major extent is regulated by ZBP-89, the activity of the luciferase construct driven by the basal promoter region in which all four Sp sites were mutated (pGLdel15004xmt) was measured after transient overexpression of ZBP-89. As can be seen (Figure 5B), the activity of this promoter construct was still inducible by ZBP-89, leading us to the assumption that, additionally to Sp1 and Sp3, ZBP-89 is able to enhance pdcd4 gene expression. These observations were further supported by mitomycin inhibition of cells transiently transfected with the reporter construct containing the active promoter region, together with either the empty expression vector or the ZBP-89 expression construct (Figure 5C).

ZBP-89 binds to the pdcd4 basal promoter region

Based on the previous findings, we investigated whether ZBP-89 binds to the predicted sites within the pdcd4 promoter. Gel-shift and supershift analysis was conducted using radiolabelled oligonucleotides (del1713_6 and del1713_8) spanning the ZBP-89-binding sites. Gel-shift and supershift analysis conducted using radiolabelled oligonucleotides (del1713_6 and del1713_8) spanning the ZBP-89-binding sites at positions -208, -154 and -127, and nuclear extracts of the colon carcinoma cell line HCT-116. As shown in Figure 5(E), binding of specific proteins was considerably higher for both oligonucleotides in ZBP-89 transfecteds (lanes 1, 5, 9 and 13). Supershift analysis using a specific antibody recognizing the Xpress-Tag of the pcDNA6HisC construct specifically shifted (lanes 5 and 7) or blocked (lanes 13 and 15) a specific band as compared with the IgG control (lanes 8 and 16) after overexpression of ZBP-89. These results support the notion that ZBP-89-transcription factors are bound to the basal pdcd4 promoter region.

In vivo evidence for the physical interaction of ZBP-89 with the natural pdcd4 promoter

Gel-shift analysis had implicated different zinc-finger transcription factors bound to oligonucleotides corresponding to the basal region of the pdcd4 promoter. To determine which transcription factors bind to this region of the natural pdcd4 promoter, we performed ChIP analysis, using anti-Sp1, -Sp2, -Sp3, -Sp4 and -ZBP-89 antibodies. Quantitative PCR amplification of the immunoprecipitated DNA using the primers specific for region -241/-42, identified ZBP-89 as the main endogenous binding partner to this natural promoter region, as compared with Sp1, Sp2, Sp3 and Sp4 (Figure 5D). The relative quantity of Sp transcription factors bound to the natural promoter region reflects the data in previous gel-shift analysis, where mainly Sp1 and, to some extent, Sp3 was found (Figure 4B). These results confirm that, in vivo, Sp1, Sp3 and ZBP-89 bind to the pdcd4 promoter, supporting the role of ZBP-89 as the main regulator within this basal promoter region.

ZBP-89 is a potent regulator of pdcd4 promoter activity and gene expression

The results from EMSA, supershift and ChIP assay demonstrate that ZBP-89 predominantly binds to the pdcd4 promoter. To confirm a decisive function of ZBP-89 for the expression of Pdcd4, we performed overexpression and knockdown experiments with ZBP-89 in RKO, HCT-116 and MCF-7 cells. Overexpression of ZBP-89 led to a significant increase (100–150 %) in pdcd4-basal promoter activity compared with the vector control (Figure 6A). This observation was further supported by the fact that the mRNA level of Pdcd4 was increased 200–250 % after 24 h for RKO and HCT-116 and after 48 h for MCF-7 respectively (Figure 6B). To gain evidence that ZBP-89-driven pdcd4 gene expression is relevant at the protein level, Western blot analysis was performed for ZBP-89, Pdcd4 and β-actin as internal control. In contrast with Sp1 and Sp3 (Figures 4E and 4F), ZBP-89 increased endogenous Pdcd4 protein levels by 30–80 % after 48 h of overexpression, as compared with the control (Figure 6C). To corroborate these results, we performed knockdown experiments with ZBP-89 in RKO, HCT-116 and MCF-7 cells. Knockdown of ZBP-89 by siRNA was confirmed by semi-quantitative RT–PCR (results not shown) and Western blotting (Figure 6F). A decrease in Pdcd4 expression by approximately 40 % was observed at the protein level. As expected, ZBP-89 knockdown cells showed a clear decrease in pdcd4 promoter activity (RKO 30 %, HCT-116 30 % and MCF-7 45 %), endogenous Pdcd4 mRNA (RKO 25 %, HCT-116 50 % and MCF-7 45 %) and endogenous Pdcd4 protein (RKO 60 %, HCT-116 63 % and MCF-7 66 %) (Figures 6D–6F). Expression of a siRNA against ZBP-89 lowered the activity of the basal pdcd4 promoter by almost 60 % compared with scrambled siRNA (Figure 6D). In addition, we also observed a 30–50 % reduction in endogenous Pdcd4 mRNA in cells transfected with ZBP-89 siRNA, as compared with control cells transfected with scrambled siRNA (Figure 6E), suggesting that ZBP-89 significantly regulates pdcd4 gene expression in these cell lines. Additionally, we were interested to ascertain if the observed increase in basal pdcd4 promoter activity after ZBP-89 expression can be affected by NF-Y binding by the CCAAT-box. As can be seen (Figure 7A), the activity of the promoter construct mutated at +19/+23 was still inducible by ZBP-89 (>200 %) and enhanced compared with the wild-type CAAT box (>120 %), leading us to the assumption that ZBP-89 has no influence on the CCAAT-box activity. We further investigated the binding of acetylated histones to the
Figure 5  ZBP-89 and Pdcd4 mRNA correlate positively in different cancer cell lines

(A) Total RNA was extracted, reverse-transcribed and quantified by real-time PCR using specific TaqMan primers and probes for human ZBP-89 and Pdcd4. Samples were analysed in quadruplicate, and relative expression levels normalized against β-actin. ZBP-89 and Pdcd4 amounts are shown as a relative quantification, with corresponding expression of wild-type RKO cells as a reference. ZBP-89 sites are functionally relevant for the basal promoter activity and can be repressed by mithramycin. (B) Pdcd4-luciferase construct bearing the basal promoter region mutated at four Sp sites, or pGL3-basic vector together with Renilla luciferase plasmid and ZBP-89 expression plasmid were transiently transfected into HCT-116 cells. (C) Pdcd4-luciferase constructs or pGL3-basic vector together with Renilla luciferase plasmid and ZBP-89
Regulation of Pdcd4 expression by ZBP-89

Basal pdcd4 promoter region after ZBP-89 expression, because recent studies have shown that ZBP-89 can function as a repressor of the vimentin and p16 promoter by recruiting histone deacetylase [32,46]. To support our observations and to exclude the possibility that ZBP-89 is a suppressor related to our promoter, a ChIP assay was performed (Figure 7B). Based on the observation that overexpression of ZBP-89 resulted in a high amount of ZBP-89 protein in MCF-7 cells (Figure 6C), we overexpressed ZBP-89 in this cell line and immunoprecipitated with antibodies against AcH3 and ZBP-89. The results in Figure 7(B) show a higher binding of ZBP-89 after overexpression, accompanied by an increase (>200%) in the binding of acetylated histones, which is commonly seen in genes that are being actively transcribed into RNA and supporting the notion that ZBP-89 is indeed an activator of pdcd4 promoter activity. Finally, we investigated the effect of 5-aza-dC on ZBP-89-induced pdcd4 gene expression. Towards this end, we examined Pdcd4 mRNA expression upon treatment with the DNA methyltransferase inhibitor 5-aza-dC either with overexpression of ZBP-89, or the empty vector. In line with the previous results, as shown in Figure 6(C), the inhibitor led to an increase (230%) of Pdcd4 mRNA after treatment with 5-aza-dC in HCT-116 cells transfected with the empty vector. A similar increase (200%) was observed for the cells overexpressing ZBP-89. Interestingly, the combination of 5-aza-dC treatment upon ZBP-89 overexpression led to a significant increase (600%) compared with the untreated cells. These results support the notion that ZBP-89 indeed is bound to the pdcd4 promoter, leading to enhanced pdcd4 gene expression, and confirm the data that the histone modifications at the basal pdcd4 promoter region are strongly associated with the accessibility for transcription factors like ZBP-89.

**DISCUSSION**

Pdcd4 is expressed ubiquitously in normal tissue and was initially identified as a gene that is up-regulated during apoptosis after treatment of cells with different pro-apoptotic substances [47]. In addition, there is accumulating evidence that the relative abundance of Pdcd4 expression is critical in cancer development. Accordingly, down-regulation of Pdcd4 was discovered in a variety of tumours and was associated with poor prognosis [48]. The important role of Pdcd4 as a tumour suppressor is further supported by studies showing an induction in the expression of this protein by drugs with anticancer effects [49,50]. Since the microRNA miR-21 was discovered as a translational regulator of Pdcd4, and phosphorylation of Pdcd4 protein has been implicated to be accompanied by proteasomal degradation, we know how this molecule can be modulated at the translational and protein level [17,51]. However, the transcriptional regulation of Pdcd4 has not yet been described systematically. So far, only the mouse pdcd4 promoter was characterized in silico, and a potential transcriptional start site, TATA sequence and a CCAAT box have been described. Additionally, a region that participated in the response to camptothecin has been elucidated [19]. Furthermore, the transcription factor v-Myb has been shown to be important for the induction of the chicken promoter [20]. In our present study, for the first time, we identified and characterized the human pdcd4 promoter and its activity. Through a series of deletion studies, we identified a GC-rich core promoter, which is sufficient to confer high-level activity to a luciferase reporter gene, mapping in the 5′-flanking region between −345 and −55 upstream of the first exon. In addition, we revealed several putative binding sites for transcription factors of the Sp family and ZBP-89 within this region. Interestingly, a fragment spanning the regions −2253/−1999 showed the highest luciferase activity. We confirmed the existence of multiple transcriptional start sites within the first exon, and a functional CCAAT box at +19/+23. EMSA and ChIP analysis proved the binding of NF-Y, a transcription factor usually bound to active CCAAT boxes. Functionality of the CCAAT box was further supported by mutation or deletion of this specific element, leading to lower promoter activity.

In the present study, we also found that histone modifications within the basal promoter region contribute to the expression of the pdcd4 gene. Genes can be regulated at the transcriptional level as well as through epigenetic modifications. One common phenomenon in cancer cells is DNA methylation within the promoter of tumour suppressor genes. It results in transcriptional silencing of these genes and promotes tumour development. For pdcd4, it was recently shown that methylation modifies mRNA expression in a hepatocellular cell line and human glioma cells [22,23]. Beyond that, modifications to histone proteins, such as methylation and acetylation, are thought to help keeping genes active or silent. Histone modification alters the status of open chromatin domains and thus affects gene transcription [44]. Our findings, that in RKO colorectal cancer cells, treatment with a DNA methyltransferase inhibitor leads to an up-regulation of mRNA and protein by inducing histone hyperacetylation and hypomethylation within the basal promoter region, are important for understanding how this gene is regulated at the epigenetic level.

expression plasmid were transiently transfected into the cells and 6 h post transfection, cells were mock-treated or treated with 50 nM mithramycin. The firefly luciferase activity was normalized with Renilla luciferase activity. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate. EMSA, supershift and ChIP demonstrating the in vitro and in vivo binding of ZBP-89 transcription factor to the pdcd4 promoter region. (D) The in vivo association of Sp1, Sp2, Sp3, Sp4 and ZBP-89 with the pdcd4 basal promoter was evaluated with ChIP assay in HCT-116 cells. Equal amounts of chromatin were incubated with the indicated antibodies and quantified by real-time PCR, using a primer set specific for the basal region. As a negative control, immunoprecipitated DNA was amplified by a primer set specific to an off-target region in the pdcd4 promoter. (E) Equal amounts of nuclear extract from vector-control HCT-116 cells and ZBP-89-transfected HCT-116 cells were incubated with two end-labelled oligonucleotides (del713/6 and del713/8) in the presence or absence of a 100-fold excess of the unlabelled competitor (Comp.) sequence, and the indicated antibody. EMSA was carried out using 10 μg of nuclear extract, 1 μg of poly(dI-dC) (di-dC), 1×10^5 c.p.m. of 32P-labelled oligonucleotide and 1 μg of antibody. Complexes were subsequently analysed by gel electrophoresis. ZBP-89 complexes are indicated by an arrow. *Indicates supershifted bands. Data are representative of three different experiments.
Figure 6  **ZBP-89 significantly regulates *pdcd4* promoter activity and mRNA/protein expression**

(A) Cells were co-transfected with *pdcd4*-luciferase construct bearing the basal promoter region or pGL3-basic vector together with *Renilla* luciferase plasmid and ZBP-89 expression plasmid. The firefly luciferase activity was normalized with *Renilla* luciferase activity. Luciferase activity was set to 100 %, and activity of the other constructs was calculated and plotted as a percentage of this value. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate. Overexpression of Sp1 or Sp3 increases Pdcd4 mRNA and protein amounts. (B) Total RNA was extracted from vector- and transiently ZBP-89-transfected cells, reverse-transcribed and quantified by Q-PCR (quantitative PCR) using specific TaqMan primers and probes for human *Pdcd4*. Samples were analysed in quadruplicate, and relative Pdcd4 expression normalized against β-actin. ZBP-89 amounts are shown as a relative quantification (RQ), with corresponding expression of vector-transfected cells as a reference. (C) Additionally, cells were transfected in a six-well plate with 4 μg of ZBP-89 expression plasmid and empty vector (ev) alone as a mock control. After 24 or 48 h post transfection, cells were harvested and cell lysates were immunoblotted for ZBP-89, Pdcd4 and β-actin. (D) Knockdown of ZBP-89 decreases activity of the *pdcd4* promoter. Cells were first transfected with a siRNA targeting ZBP-89, or scRNA (scrambled RNA) as a control, and after 24 h co-transfected with *pdcd4*-luciferase construct bearing the basal promoter region or pGL3-basic vector together with *Renilla* luciferase plasmid. The firefly luciferase activity was normalized with *Renilla* luciferase activity. Luciferase activity was set to 100 %, and activity of the other constructs was calculated and plotted as a percentage of this value. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicates. (E) Knockdown of Sp1 or Sp3 decreases Pdcd4 mRNA and protein levels. Total RNA was extracted from siRNA targeting ZBP-89 or scRNA-transfected cells, reverse-transcribed and quantified by real-time PCR using specific TaqMan primers and probes for human Pdcd4. Samples were analysed in quadruplicate, and relative Pdcd4 expression normalized against β-actin. ZBP-89 amounts are shown as a relative quantification, with corresponding expression of vector-transfected cells as a reference. (F) Additionally, cells were transfected in a six-well plate with a siRNA targeting ZBP-89 or scRNA. After 48 h post-transfection, cells were harvested and cell lysates were immunoblotted for ZBP-89, Pdcd4 and β-actin.

Sp family proteins and other Krüppel-like factor proteins regulate basal/constitutive expression of genes involved in multiple functions in both normal and cancerous tissues [52]. Genes that regulate growth and cell cycle progression frequently contain proximal GC-rich promoter sequences, and their interactions with Sp proteins and other transcription factors are critical for their
Regulation of Pdcd4 expression by ZBP-89

Figure 7 ZBP-89 induces pdc4 promoter activity that is defective in NF-Y binding

(A) A pdc4 luciferase construct with a mutated CCAAT box or pGL3-basic vector together with Renilla luciferase plasmid were transiently transfected into RKO, HCT-116 and MCF-7 cells. The firefly luciferase activity was normalized with Renilla luciferase activity. The results are presented as the means ± S.D. from three independent experiments performed in quadruplicate. ZBP-89 overexpression increases histone acetylation status of the pdc4 promoter. (B) MCF-7 cells were transiently ZBP-89 transfected and subjected to ChIP. Equal amounts of chromatin were incubated with the indicated antibodies and quantified by real-time PCR, using a primer set specific for the basal region. As a negative control, immunoprecipitated DNA was amplified by a primer set specific to an off-target region in the pdc4 promoter 4 kb upstream from the Pdcd4 transcriptional start site (−3495 to −3294 bp). ZBP-89 induced pdc4 promoter activity is enhanced by 5-aza-dC treatment. (C) HCT-116 cells were transiently transfected with ZBP-89 and treated with 5-aza-dC at a final concentration of 10 μM for 24 h and subjected to Q-PCR. Total RNA was extracted, reverse-transcribed and quantified by quantitative-PCR using specific TaqMan primers and probes for Pdcd4. Samples were analysed in quadruplicates, and relative Pdcd4 expression was normalized against β-actin. Pdcd4 amount is shown as a relative quantification (RQ), with untreated cells as a reference.

Strategies for inhibiting such transcription factor-dependent pathways have focused on several approaches which include drugs that inactivate GC-rich DNA motifs. Mithramycin, a cell-permeable reagent, binds GC-rich motifs, and studies show that treatment of cells with this compound decreases DNA binding of transcription factors known to influence gene expression through interaction with GC-rich regions [52]. We therefore incubated cells with this drug and observed a reduced basal promoter activity and mRNA level leading to a completely abolished expression of Pdcd4 protein. Consistent with this observation, sequence analysis of the fragment −345 to −55 identified several consensus transcription factor binding sites for Sp family members at positions −195, −162, −147, −86 and −76. Furthermore, mutation of all Sp sites decreased the pdc4 promoter activity by 50% in two colorectal and one breast cancer cell line and led us to the assumption that pdc4 expression is influenced by Sp family members. This notion was supported by a study showing that pdc4 is up-regulated by IL-12 [6]. IL-12, which has shown significant antitumour activity in several preclinical animal tumour models, led to an enhanced sensitivity to Fas-induced cell apoptosis through regulating the binding of transcription factors towards an NF-κB–Sp1 element in the Fas promoter [53]. Besides, it is known that the expression of Pdcd4 is up-regulated in senescent fibroblasts, a situation in which Sp1 also plays a pivotal role [5]. P16 is a tumour suppressor protein that has been widely proposed to mediate entrance of the cells into the senescent stage, and it was shown that an increase in Sp1-binding activity acts as a positive transcription regulatory element of the induction of p16 expression during cell aging [54]. Furthermore, it was shown that senescence-associated phosphorylated extracellular-signal-regulated protein kinases 1 and 2 enhanced p21 (Sdi1) transcription by phosphorylating Sp1 [55].
However, even though we observed an endogenous binding of Sp1, Sp3 and to some extent Sp4 in EMSA and ChIP studies in parallel with an induction of pdc4d promoter activity in all cell lines and an increased Pdcd4 mRNA in RKO, we failed to observe a regulation of Pdc4d protein after overexpression or knockdown of Sp1 or Sp3. One possible explanation for this non-existent inducible protein expression could be the fact that Pdcd4 is tightly controlled at the translational level [17]. On the other hand, the activity of Sp1 and Sp3 at any specific promoter is governed by several variables, including the ratio of expression of Sp1 and Sp3, the expression and modification state of interacting proteins with which Sp1 or Sp3 form a complex, and variation in binding activities brought about by, for example post-translational modifications. Furthermore, the methylation around GC boxes is one way to regulate the DNA-binding activity of Sp1 and Sp3. Indeed we observed an induction of the pdc4d promoter activity following demethylation. In contrast with the situation in senescent cells, where Sp1 is activated through phosphorylation, a recent study has linked the anti-angiogenic effects of celecoxib to the modulation of Sp1 transcription factor activity followed by inactivation of this transcription factor by phosphorylation [56]. Furthermore, COX-2 inhibitors decreased vascular endothelial growth factor expression in colon cancer cells by enhanced degradation of Sp1 and Sp4 proteins [57], whereas it was shown by other groups that pdc4d expression is up-regulated through this inhibitor [50].

As forced expression of Sp transcription factors induced pdc4d promoter activity, but failed to regulate the Pdcd4 protein amounts, we investigated the importance of other transcription factors for full activation of the basal promoter region. Besides Sp-binding sites, within the basal promoter region at positions −208, −154 and −127, we found cis-elements for another Krüppel-type zinc-finger transcription factor, ZBP-89. This observation prompted us to suggest the importance of ZBP-89 binding to its respective site for the activation of the pdc4d gene. Originally, ZBP-89 was proposed to be ubiquitously expressed [25]. However, it is now apparent that ZBP-89 has been implicated in many cellular functions related to cancer, including cell growth, differentiation, transformation, myogenesis, haemopoiesis and senescence [58]. Furthermore, diverse studies have shown that ZBP-89 is elevated in some tumour tissues and cell lines, including gastric-, colorectal-, breast cancer, melanoma, HCC and in a variety of other gastrointestinal cancers [58]. This is important since Pdcd4 has been shown to act as an apoptotic factor [47,50], besides being a suppressor of malignant transformation [59,59a], tumorigenesis, tumour progression [60] and cellular senescence [5]. However, the potential role of ZBP-89 in the transcriptional regulation of Pdcd4 has not been determined so far. In our study, overexpression of ZBP-89 was able to significantly induce basal pdc4d promoter activity, even after mutation of all four Sp sites within the basal promoter region. Consistent with this observation, mithramycin treatment inhibited the DNA binding of ZBP-89 in all three cell lines examined. Moreover, EMSA and ChIP data showed the in vivo occupancy of the pdc4d promoter with ZBP-89 as clear indicators for a role of especially ZBP-89 in the transcription of the pdc4d gene. Most notably, ZBP-89 was the most prominent protein bound in ChIP compared to Sp1, 2, 3 or 4. It has been suggested that ZBP-89 can function through multiple mechanisms and can be either an activator or a repressor of a variety of genes. These mechanisms include the competition of ZBP-89 with transcription activators such as Sp1 for overlapping binding sites, thereby decreasing promoter activity and transcription intensity [61]. Others include the ability of ZBP-89 to recruit the co-activator p300 to the promoter of the target gene, resulting in an up-regulation of gene expression [41]. Finally, ZBP-89 is able to recruit a co-repressor to a specific promoter, and this co-repressor either negatively regulates other factors that are present, or alters the local chromatin structure [62]. Based on our results, the function of ZBP-89 towards the Pdcd4 promoter most likely is that of a transcriptional activator and potential tumour suppressor, potentially by interacting with other co-activators. This notion is supported by the fact that we found that histone modifications, such as the acetylation status of histone H3, within the basal promoter region contribute to the expression of the pdc4d gene. This suggests that an alteration of the local histone structure influenced through ZBP-89 might be able to drive the expression of pdc4d. Consistent with these results, another study has investigated the mode of action of the HDAC inhibitor TSA (trichostatin A) on vimentin gene expression. They documented an increase in the acetylation status of histone H3 at the endogenous vimentin gene after TSA treatment. Interestingly, they found that recruitment of HDAC1 to the respective promoter was brought about via ZBP-89. Even though in this study ZBP-89 acted as a transcriptional repressor, we actually observed a higher binding of acetylated histones after ZBP-89 expression. Based on that, one could speculate that, in an analogous situation but different cellular background and gene-specific manner, ZBP-89 acts as a transcriptional activator or repressor.

In contrast with Sp1 or Sp3, we observed a significant induction or reduction of Pdcd4 mRNA and protein after overexpression, or knock down, of ZBP-89. Previous studies reported a regulation of Pdcd4 and ZBP-89 under similar experimental conditions. It was shown that under inflammatory conditions that can also contribute to the generation of a tumour-promoting environment by stromal cells, Pdcd4 and ZBP-89 were down-regulated in macrophages or fibroblasts [63,63a]. Furthermore, stimulation by RA (retinoic acid) resulted in increased expression of these proteins in breast cancer cells and HeLa cells [49,64] and finally, Pdcd4 and ZBP-89 both seem to be enhancers of drug sensitivity [9,65]. Additionally, overexpression of ZBP-89 can inhibit the proliferation of human stomach adenocarcinoma cells [66]. Regarding RA, we have also observed an enhanced pdc4d promoter activity and elevated protein amounts of Pdcd4 in breast cancer cell lines after stimulation with all-trans RA (results not shown). This observation is supported by a study showing an enhanced Pdcd4 protein expression following RA treatment in breast cancer cell lines [67]. Consistent with those results, we found a cis-element downstream of the first exon of the pdc4d promoter, which showed an enhanced binding of complexes after stimulation with all-trans RA. However, no influence on the binding of any transcription factors, including the zinc finger transcription factors examined in our present study, was observed after RA.
treatment in EMSA or ChIP within the basal promoter region, and the inducible cis-element identified was not bound by RAR or RXR.

Finally, various studies have shown that ectopic overexpression of ZBP-89 can enhance apoptosis and that ZBP-89, like Pdcd4, is inducible by different pro-apoptotic stimuli [41,47,49,65,68,69]. However, the molecular mechanism leading to the inhibitory effect of ZBP-89 is largely unknown. Initial studies have shown that ZBP-89 overexpression stabilizes the p53 protein and enhances its transcriptional activity [42]. Later it was found that ZBP-89 also induces apoptosis through a p53-independent mechanism that requires JNK (c-Jun N-terminal kinase) [41]. Recently, it has been suggested that ZBP-89 can enhance tumour cell death through up-regulating the pro-apoptotic molecule Bak, a member of the Bcl-2 family in HCC cells [68]. These results supported an earlier study that demonstrated that ZBP-89 can significantly enhance the sensitivity of HCC cells to anti-tumour agents such as 5-fluorouracil, by increasing caspase-6 activity, G2/M-phase abrogation and S-phase arrest [65]. As mentioned, it has been suggested that Pdcd4 expression is increased during apoptosis in response to different inducers and it was shown for HCC that down-regulation of Pdcd4 by specific siRNAs weakened the sensitivity against 5-fluorouracil [70]. In addition, Pdcd4 was shown to be a pro-apoptotic molecule involved in TGFβ1 (transforming growth factor β1)-induced apoptosis in human HCC cells [69], supporting the conclusion that Pdcd4 and ZBP-89 are potentially directly related in their function as tumour suppressors. Our findings on ZBP-89-mediated regulation of the pdc4d promoter can also help us to understand how ZBP-89 accomplishes its pro-apoptotic role in many types of cancers.

In conclusion, this is the first study, to our knowledge, to characterize the human pdcd4 promoter. Our results clearly demonstrate that ZBP-89 is a potent trans-regulator, along with especially Sp1 and 3, for constitutive pdcd4 promoter activity. After further clarification of diverse translational and protein modifying mechanisms, our present results on the roles of Sp1, Sp3 and ZBP-89 in the regulation of the humane pdcd4 gene provide a basis for the understanding of the transcriptional regulation of this important tumour suppressor. Particularly since the role of ZBP-89 in cancer has been discussed ambivalently to date, our data support the role of ZBP-89 in inhibiting carcinogenesis. The observation that ZBP-89 as a regulator of Pdcd4 behaves as a tumour suppressor is in line with other studies investigating the role of ZBP-89 in cancer. Pdcd4 is, besides its well-characterized functions as a suppressor of transformation, translation and inducer of apoptosis, a potent suppressor of invasion and intravasation, the two decisive initial steps of the metastatic cascade. In consideration of these aspects, it is important to stress the role of ZBP-89 as a potent trans-regulator of Pdcd4.

**REFERENCES**


**AUTHOR CONTRIBUTION**

Jörg Hendrik Leupold made substantial contributions to the conception and design of the study, carried out the molecular genetic studies, performed the protein expression analysis and drafted the paper. Irfan Ahmed Asangani participated in the ChIP assays. Giridhar Mudduluru participated in molecular genetic studies. Heike Allgayer conceived the study, and participated in its design and co-ordination, revised it critically for important intellectual content and helped to draft the paper. All authors read and approved the final paper.

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SUPPLEMENTARY ONLINE DATA

Promoter cloning and characterization of the human programmed cell death protein 4 (pdcd4) gene: evidence for ZBP-89 and Sp-binding motifs as essential Pdcd4 regulators

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Table S1 Oligonucleotides used for luciferase reporter constructs and site-directed mutagenesis

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<td>CCCGGCCTCCGG----AAGGCAGCAGG</td>
<td>–4175/–4149</td>
</tr>
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<td>FWDCCAAATmut</td>
<td>ACCCGGCTCCGGCTGATAAGGCAGGCC</td>
<td>–4176/–4149</td>
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<tr>
<td>Del713_6mt1/2FWD</td>
<td>TCCCCATCCACGTTTCTTTCTCCGACTTCTTTCCAG</td>
<td>–4393/–4357</td>
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<tr>
<td>Del713_8mt3/4/5FWD</td>
<td>AAGCAGCTCTACCGCCACTCGGACATCCCTTCTCTCC</td>
<td>–4364/–4320</td>
</tr>
<tr>
<td>Del713_11mt3/4FWD</td>
<td>CATGCAGCTCCCGGCCCTTTCCTCAGACTCTTCTC</td>
<td>–4274/–4257</td>
</tr>
</tbody>
</table>

1 To whom correspondence should be addressed (email heike.allgayer@umm.de).
Table S2 Oligonucleotides used for EMSA, ChIP, RACE and ZBP-89 cloning

Oligonucleotides used for EMSA, ChIP, RACE and ZBP-89 cloning FWD, forward; REV; the numbers indicate the base location relative to the translational start site (set as +1) of pdcd4 gene NT_030059.13.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→3′)</th>
<th>Position (5′→3′)</th>
</tr>
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<tbody>
<tr>
<td>Primers for EMSA</td>
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<td></td>
</tr>
<tr>
<td>FWDCCAAttw</td>
<td>CGACCACCGGGCCCTCGGCCAATAAGCGCGCCCTCGCCC</td>
<td>−4181/ −4141</td>
</tr>
<tr>
<td>FWDCCAAttmt</td>
<td>CGACCACCGGGCCCTCGCGCTGAACGCGGCCCTCGCCC</td>
<td>−4181/ −4141</td>
</tr>
<tr>
<td>FWDdel713_6</td>
<td>GCTTCATCCTCGTCCCCATCCCCAGCAGCTCTTCCTTTTC</td>
<td>−4405/ −4367</td>
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<tr>
<td>FWDdel713_8</td>
<td>CGCCACGCGCTCCCCACATCCCCAGCAGCTCTTCCTTTTC</td>
<td>−3451/ −3403</td>
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<tr>
<td>FWDdel713_11</td>
<td>CGCCCTCCGGCGCCCTCCCCACGCTCTTCCTTTTCTGC</td>
<td>−4270/ −4232</td>
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<tr>
<td>Primers for ChIP</td>
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<td>FWD</td>
<td>GTGAGCTGGGTCGAGGAAGC</td>
<td>−4423/ −4404</td>
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<tr>
<td>REV</td>
<td>GGGAGAGGAGTGAAACGTGGA</td>
<td>−4244/ −4224</td>
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<td>FWDcontro</td>
<td>CAGCTGCAAAGGCACAGGTGA</td>
<td>−7677/ −7657</td>
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<td>REVcontro</td>
<td>GTGCCTTTAGTGTTGGGAG</td>
<td>−7497/ −7477</td>
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<tr>
<td>Primer for RACE pdcd4-specific</td>
<td>TCGCCTATCCAGCAACCTCCCTTT</td>
<td>+5448/ +5472</td>
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