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DNA-binding and regulatory properties of the transcription factor and putative tumor suppressor p150^{Sal2}

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Abstract

The product of the SALL2 protein p150^{Sal2} is a multi-zinc finger transcription factor with growth-arrest and pro-apoptotic functions that overlap those of p53. Its DNA binding properties are unknown. We have used a modified SELEX procedure with purified p150^{Sal2} and a pool of oligonucleotides of random sequence to identify those that are bound preferentially by p150^{Sal2}. The consensus sequence for optimal binding *in vitro* is GGG(T/C)GGG, placing p150^{Sal2} among a large group of GC box-binding proteins including the Sp1 family of transcription factors. A triple zinc finger motif in p150^{Sal2} similar to that in Sp1 is required for DNA binding. p150^{Sal2} and Sp1 show evidence of co-operative binding *in vitro* and of interaction *in vivo*. p150^{Sal2}, a known activator of the CDK inhibitor p21$^{Cip1/Waf1}$ (p21), binds to regions of the human p21 promoter that contain variations of the consensus sequence in multiple copies. p150^{Sal2} is also shown to bind to the BAX promoter with similar elements and to activate its expression following an apoptotic stimulus. These results demonstrate binding of p150^{Sal2} to two natural promoters with GC elements related to the optimal binding sequence defined *in vitro* and whose regulation is important for suppression of tumor growth.

Keywords

SALL2 transcription factor; DNA binding; growth arrest; apoptosis; polyoma virus

1 INTRODUCTION

p150^{Sal2} is the product of the *Sall2* gene, a member of the SALL (‘spalt-like’) gene family which encodes multi-zinc finger transcription factors. Orthologues of the homeotic gene *Spalt* in Drosophila [1], SALL genes are conserved from flies to man. SALL genes function in embryonic development in vertebrate as well as invertebrate species [2]. *Sall4* is an important transcriptional regulator in mouse embryonic stem cells [3-5] and is essential for maintaining a pluripotent state ([6]. *Sall4* also regulates growth and survival in human leukemic cells [7]. *Sall2* is the only member of the family suggested to act as a tumor suppressor [8, 9]. *Sall2* plays a role in neuronal development, affecting neurite outgrowth [10] and, along with *Sall1* and *Sall4*, neural tube closure in mice [11]. Naturally occurring

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mutations in these genes give rise to developmental abnormalities in man [12-15]. The roles of SALL genes in development are not well understood at a molecular level.

$p150^{Sal2}$ was first identified in the mouse as a binding target of the polyoma virus large T (tumor) antigen. This was done using a procedure designed to identify tumor suppressors or other cellular factors with which the virus must interact in order to replicate efficiently and which may undergo spontaneous loss or alteration in certain cancer cells [8]. $p150^{Sal2}$ acts in some manner to inhibit DNA replication by the virus and the binding of $p150^{Sal2}$ by large T overcomes this inhibition. A virus mutant unable to bind $p150^{Sal2}$ is unable to replicate or induce tumors broadly in the mouse [8].

Though strongly expressed in the normal ovary, $p150^{Sal2}$ is not expressed in some human ovarian carcinoma-derived cell lines. Restoration of expression in such tumor cells results in suppression of growth in SCID mice. Tumor suppression is accompanied by both a rise in apoptotic index and a fall in mitotic index. $p150^{Sal2}$ binds within the extended promoter of $p21^{Cip1/Waf1}$ (p21) and transactivates p21 in the absence of p53 [16]. Independent studies have shown that *SALL2* expression is required for human fibroblasts to exit the cell cycle and to maintain a quiescent state under conditions of serum deprivation. Downregulation of *SALL2* prevents G1 arrest in serum-deprived cells [17]. These observations establish $p150^{Sal2}$ as a negative regulator of cell growth with functions in postnatal as well as embryonic tissues. They also suggest that it may act as a tumor suppressor based on its ability to regulate cell growth and survival.

Knowledge of the DNA-binding properties of $p150^{Sal2}$ is presently lacking but essential to understanding its regulatory functions. A triple zinc finger motif in $p150^{Sal2}$ is required for binding and transactivation of the p21 promoter [16] but the sequence specificity of binding is not known. The current investigation was undertaken to determine the DNA binding specificity of $p150^{Sal2}$ by identifying the optimal DNA sequence for binding *in vitro* and to demonstrate that related sequence elements are present in natural promoters regulated by the endogenous protein. A modified SELEX procedure has been used to establish $p150^{Sal2}$ as a GC-box-binding protein. Evidence is presented that $p150^{Sal2}$ binds to GC-rich elements in the human p21 and BAX promoters consistent with its growth arrest and pro-apoptotic functions and ability to transactivate these genes.

2 MATERIAL AND METHODS

2. 1 Cell lines and plasmids

293 (human embryonic kidney cells) and HOSE (established human ovarian surface epithelial cells) [16] were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. $p150^{Sal2}$ and deletion mutants Z3D (deletion of amino acids of 631-711) and Z4D (deletion of amino acids of 911-956) have been reported previously [8]. Z1D (amino acids 35-55) and Z2D (amino acids 373-421) were made using QuikChange site directed mutagenesis kit (Stratagene) and cloning into the pEBG GST expression vector [18]. The G6TI luciferase vector containing 6 GC-boxes [19, 20] was a gift from Dr. G. Gill (Tufts University). The renilla luciferase vector (pTL-Renilla) was constructed by replacing the firefly luciferase gene in pTL (Panomics) with renilla luciferase from phRluc/SV40 (Promega) using the Hind III and Xba I sites.

2. 2 GST fusion protein purification

GST, GST-tagged $p150^{Sal2}$ or zinc finger deletion protein expression vector was transfected into 293 cells using Lipofectamine 2000 (Invitrogen) and GST-fusion protein purification was performed using GST beads (Amersham). After elution, proteins for EMSA were

dialyzed in HEPES (pH 7.9) buffer [21] and concentrated using Ultracel YM-10 centricon (Millipore). Proteins were quantified using the Bio-Rad Protein Assay and by SDS-PAGE electrophoresis and Coomassie Blue staining.

2. 3 Random sequence DNA library synthesis

A random pool of DNA [5'-ATGTGGATCCACTGACGG$(N)_{20}$GCTACGCCTCGAGATTG- 3'] and two PCR primer sequences corresponding to the first 18 nucleotides (SELEX primer 1, 5'-ATGTGGATCCACTGACGG-3') and complementary to the last 17 bases (SELEX primer 2, 5'-CAATCTCGAGGCGTAGC-3') were purchased from Integrated DNA Technologies (IDT). The random DNA library was generated using 10 units Klenow fragment (New England Biolabs) in a 40 μl reaction containing 100 pmol DNA pool and 1 nM SELEX primer 2, as per the manufacturer's protocol. Following a 30 min incubation at 25 °C, the reaction was stopped by heating at 75 °C for 20 min.

2. 4 SELEX procedure

The SELEX protocol was modified from [22]. Briefly, 4 pmol random DNA library and 1 pmol glutathione bead-bound GST-$p150^{Sal2}$ were incubated for 30 min at room temperature in 1x binding buffer (20mM HEPES, pH. 7.5, 50mM KCl, 1mM dithiothreitol, 3 mM MgCl, 1mM EDTA, 5% glycerol and 0.5% NP-40). The beads were pelleted (5 min, 2000g) and washed four times with 1 ml of 1x binding buffer. The final pellet was suspended in 45 μl of Platinum PCR SuperMix (Invitrogen) and subjected to 20 cycles of PCR amplification (as per manufacturer's protocol) using 125 ng each of SELEX primer 1 and 2. The resulting PCR products were purified using MicroSpin G-50 columns (Amersham biosciences) and subjected to the next cycle of SELEX. Seven additional cycles of SELEX were then performed and the final DNA fragments cloned into pGEM-T Easy vector (Promega) following the manufacturer's protocol. Twenty-four individual clones were sequenced.

The random DNA library and PCR product from the last SELEX cycle were end-labeled with [α-^{32}P]-dATP using T4 Polynucleotide Kinase (Invitrogen) as per the manufacturer's protocol. Single strand oligonucleotides containing the Consensus Sequence (CS) 5'-GGATCACTGGGTGGGAATCACGCT-3' Sp1 binding site [23] (5'-ATTCGATCGGGGCGGGGCGAGC-3'), Oct1 binding site [24] (5'-TGTCGAATGCAAATCACTAGAA-3', appropriate mutants, and their complimentary sequences, were purchased from IDT. Double stranded DNA was generated by annealing complimentary oligonucleotides (buffer: 10 mM Tris pH. 8.0, 1mM EDTA and 50 mM NaCl). The DNA was radiolabeled either by T4 PNK as described above, or obtained from IDT with two additional Gs at their 5' termini to form an overhang, thus allowing Klenow Fragment (Invitrogen) fill-in reaction with [α-^{32}P] dCTP. All the probes were purified using MicroSpin G-50 columns (Amersham) following the manufacturer's instructions

2. 5 EMSA procedure

Electrophoretic mobility shift assays were carried out using purified GT-$p150^{Sal2}$ as described [21] with modification. Briefly, 100 ng purified GST or GST-$p150^{sal2}$ and 200,000 cpm radiolabeled probe were incubated in 20 μl reaction (10 mM HEPES pH 7.5, 25 nM KCl, 2.5 mM MgCl, 5 μl ZnCl, 3% glycerol, 2 μg BSA and 200 ng poly dA-dT). For competition studies, 50 or 100 molar excess unlabeled oligonucleotide was added to the mixture. Recombinant human Sp1(rhSp1) protein was purchased from Promega. Supershift was performed by adding 300ng polyclonal antibody to Sp1 (PEP2, Santa Cruz). After 20 min incubation at room temperature, the mixtures were loaded onto 4% acrylamide (60:1 acrylamide: bisacrylamide gel). The oligonucleotides-protein complex was separated by running gels in 0.5X TBE buffer at 180 volts for 2.5 hours. Dried gels were exposed to a

PhosphorImager screen (Molecular Dynamics) and individual bands were quantitated with ImageQuant. p21 promoter binding reactions were performed using nuclear extracts [25].

2. 6 Luciferase assays

Hela cells at 90% confluence were cotransfacted with Flag-Sp1 [20] and/or GST-p150^{Sal2} expression vectors as indicated, and 0.3 μg G6Tl-Luc construct and 20 ng pTL-Renilla control vector. For dosage response experiments empty expression vector was included to normalize the quantity of DNA transfected. Cells were harvested 36 hours after transfection, and 10 μl of lysate was measured for luciferase activity using Promega luciferase assay kit.

2. 7 Immunoprecipitation and pulldown assays

HOSE cells grown to 85 % confluence were harvested by scraping, washed with ice cold PBS, and lysed using 1 ml of NP-40 lysing buffer [26]. 50 μl of 50% Protein A-Sepharose slurry was used to precipitate proteins bound by 3 μg Sp1 polyclonal antibody (Santa Cruz Biotechnology). Protein complexes were washed five times using the lysis buffer. Immnuoprecipitates were collected and analyzed by western blot with antibody against p150^{Sal2}. Hose cells were also transfected with constructs expressing GST-p150^{Sal2} or GST alone using Lipofectamine 2000. Cells were harvested 36 hours later and protein complexes collected on glutathione beads. Complexes were analyzed by western blot with antibody against Sp1. A flag-tagged Sp1 clone was also used in GST pulldown assays. HOSE cells were transfected with Flag-Sp1 and pEBG or GST-p150^{Sal2} vectors. GST pull-down and western blot were performed both in the presence and absence of Ethidium Bromide (20μg/ml) or Benzonase nuclease (25 U/ml; Novagen).

2. 8 ChIP assays

ChIP assay was perfomed as described [27] with slight modification. For interaction of p150^{sal2} with the BAX promoter, HOSE cells were first transfected with pcDNA or pcDNA-p150^{Sal2} for 48 hours. Interaction of the endogenous protein was also examined in HOSE cells following treatment with 25 ug/ml etoposide for 22 hours to induce apoptosis. Cross-linking was done with 1.4% formaldehyde. Cells were lysed with IP buffer [0.5% Triton X-100, 20 mM Tris, pH 7.5, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 1 mM Na vanadate, 100 μg/ml PMSF, and protease inhibitor cocktail (Roche; Indianapolis, IN, USA)]. Chromatin was sheared by sonication and incubated with rabbit polyclonal antibody to an N-terminal fragment of p150^{Sal} or normal rabbit IgG. Sheared chromatin was incubated with protein A/G beads (Santa Cruz; CA, USA) for 2 hours, washed five times with IP buffer. Chelex 100 slurry was added to the washed beads. Beads were boiled and incubated with Proteinase K (Invitrogen; Carlsbad, CA, USA) at 55°C for 30 min. Samples were boiled again, cleared by centrifugation and the supernatants taken for real-time PCR. The bound chromatin fraction was amplified with human BAX promoter-specific primers flanking GC boxes at −117 for 40 cycles. Forward primer - 5'-GGCGCCACTGCTGGCACTTA-3'; reverse primer - 5'-CTCCCCGGACCCGTCCATCA-3'. As a control, human Aldolase A gene was amplified with 5'-CGCAGAAGGGGTCCTGGTGA-3' and 5'-CAGCTCCTTCTTCTGCTCCGGGGT-3'. Real time PCR was carried out on a Roche LightCycler 480 using SYBR Green Master Mix and quantitated as described [27]. The data was analyzed by the comparative C_T ($\Delta\Delta C_T$) method and quantitated relative to the aldolase A gene and normalized to the control.

2. 9 Quantitative RT-PCR

Total RNA was isolated using RNeasy® kit (Qiagen), reversed transcribed using QuantiTect Reverse Transcription Kit (Qiagen) and quantitated by RT-PCR using specific primers:

BAX - F: 5'-GGAGATGAACTGGATAGCAA-3' and R: 5'-AGCCACAAAGATGGTCACT-3', p21 – F: 5'-TCCCGTGGACAGTGAGCAGTTG-3' and R: 5'- GACACACAGAGTGAGGGCTAAG-3', and aldolase A the same as above.

3 RESULTS

3. 1 p150^{Sal2} binds selectively to DNA oligomers containing -GGG(T/C)GGG-

A modified SELEX procedure was used to identify DNA oligonucleotides with highest affinity binding to p150^{Sal2} (Figure 1A). A GST-p150^{Sal2} fusion protein containing full length (1005 aa) human p150^{Sal2} protein with alternative exon E1A [8, 16] was expressed and purified from 293 cells. Purified GST-p150^{Sal2} (Figure 1B; denoted hereafter in the text as p150^{Sal2}) was incubated with double-stranded deoxyribonucleotides carrying inserts of random sequence twenty nucleotides long. A sufficient quantity of DNA from the library was used initially to allow theoretical representation of all possible 20-mers (4 pmoles, ~2.2X coverage). Bound oligonucleotides were separated from unbound DNA using glutathione beads. Bound DNA was amplified by PCR and subjected to seven additional rounds of selection. Results were analyzed by electrophoretic mobility gel shift assay (EMSA). Selective enrichment of bound sequences after eight rounds is evident from the level of binding (compare Figure 1C, lanes 1 and 3) and from the effects of unlabelled oligonucleotides used as competitor DNAs (Figure 1C, lanes 4, 5 and 6).

Following the final round of selection, twenty-four clones were chosen at random and sequenced. Nineteen unique sequences were represented; five clones were represented twice (Figure 1D). Inspection of these oligonucleotides revealed a heptanucleotide -GGG(T/C)GGG- as a consensus sequence for binding to p150^{Sal2}. The selected oligonucleotides contained at least one and more often multiple heptamer sequences that conformed either to the consensus itself (-GGGNGGG-) or to sequences differing at a single position (solid and dotted underlines, respectively, Figure 1D). In the majority of clones, the GG dinucleotide in the vector immediately 5' to the inserts contributed to possible binding sequences.

DNA binding specificity was analyzed further using a set of twenty-one oligonucleotides with defined sequences. These contained internal heptanucleotide sequences representing three substitutions at each position of the consensus binding site. Flanking sequences in this set of oligonucleotides lacked Gs immediately adjacent to the heptanucleotides. Oligonucleotides were radiolabeled and evaluated for binding by EMSA and densitometry (Figure 2A). Binding strengths were assessed relative to the consensus sequence –GGGTGGG- (Figure 2B). Although T at position 4 was found most frequently in the set defined by SELEX (Figure 1D), oligonucleotides containing C at this position appeared to bind somewhat more strongly in the gel shift assay. Substitutions for G at the 5' positions generally showed somewhat greater inhibitory effects than substitutions at the 3' positions.

3. 2 p150^{Sal2} and Sp1 share DNA binding properties and show co-operative interactions

The consensus binding sequence for p150^{Sal2} is essentially the same as that for Sp1 [28]. p150^{Sal2} binds as expected to an oligonucleotide carrying a 12 bp GC-rich element known to serve as an Sp1 binding site (5'-ATTCGATCGGGGCGGGGCGAGC-3') ([23] (Figure 3A, lane 1). Binding was inhibited by an excess of cold oligonucleotide carrying either the Sp1 site or the p150^{Sal2} consensus site but not by a control oligonucleotide (Oct1) (Figure 3A, lanes 2 thru 4). p150^{Sal2} carries a putative C2HC zinc finger motif near its N-terminus followed by clusters of two, three and two C2H2 fingers. Plasmids carrying deletions of each set of zinc fingers were constructed as GST fusions and the purified proteins tested for binding to the labeled Sp1 oligonucleotide (Figure 3B). Removal of the triple zinc finger motif greatly reduced binding while removal of the other regions had little or no effect

(Figure 3C). This result extends earlier findings on the requirement for the triple zinc finger in p150^{Sal2} for binding to the p21 promoter ([16]. It also points to similarities between this motif in p150^{Sal2} and the triple zinc finger in Sp1 required for DNA binding [29, 30] with respect to spacing and linker sequences in the two proteins (Figure 3D). p150^{Sal2} and Sp1 share little homology outside of the triple zinc finger clusters.

When incubated together with the labeled Sp1 oligonucleotide, p150^{Sal2} and Sp1 formed DNA-protein complexes efficiently, suggesting possible synergism in binding (Figure 4A). Complexes formed in the presence of both proteins were 'supershifted' with antibody to Sp1. Whether both factors make direct contact with a single dodecamer or bind in 'piggyback' fashion with only one of the factors making direct contact with the DNA is unclear. To investigate whether p150^{Sal2} and Sp1 interact functionally, HeLa cells were transfected with an Sp1-luciferase reporter containing 6 'GC box' repeats (G6Tl) [20] to which both factors are expected to bind. Increasing amounts of a p150^{Sal2} expression vector resulted in a dose-dependent activation of this reporter (Figure 4B – left panel). Introduction of Sp1 and p150^{Sal2} together led to additive or possibly synergistic activation (Figure 4B - right panel).

The apparent synergy between p150^{Sal2} and Sp1 acting on an artificial promoter raises the possibility of protein-protein interaction and co-operative action *in vivo*. This possibility was explored using established human ovarian surface epithelial (HOSE) cells which express both factors endogenously. Cell extracts were immunoprecipitated with anti-Sp1 and blotted with anti-p150^{Sal2} antibody. Results showed evidence consistent with direct interaction between the endogenous proteins (Figure 4C). This result could also have arisen by virtue of both proteins binding to DNA fragments carrying two or more copies of shared or overlapping binding sequences. To test this possibility, HOSE cells were transfected with expression vectors for GST-p150^{Sal2} and Flag-tagged Sp1 or empty vector controls. Extracts prepared 36 hours post-transfection were treated with benzonase nuclease which has preference for GC-rich sequences [31] or with ethidium bromide to partially unwind DNA [32]. Treated extracts were then analyzed by GST pulldown followed by western blot. No evidence for interaction was seen following these treatments that either digest DNA (Figure 4D) or disrupt protein-protein interaction on DNA (Figure 4E). We conclude that p150^{Sal2} and Sp1 associate *in vivo* by binding to DNA with multiple copies of their common or overlapping binding sites. Protein-protein interaction may occur once assembled on DNA. Weak or transient interaction may also occur in the absence of DNA.

3. 3 p150^{Sal2} binds to regions of the p21 promoter containing GC boxes

p150^{Sal2} was previously shown to bind to two regions of the long p21 promoter and to transactivate p21 in the absence of p53 [16]. To identify more precisely the locations of p150^{Sal2} binding sites within the ~2.7 kb p21 promoter and to verify the presence of GC boxes at those sites, the larger fragments previously shown to bind were digested to produce a series of subfragments approximately 140-220 base pairs long. End-labeled subfragments were incubated with nuclear extracts from 293 cells expressing endogenous p150^{Sal2} and the products analyzed by EMSA (Figure 5). A variable fraction of the subfragments showed evidence of protein binding. The shifts generally gave rise to diffuse bands suggesting binding by multiple factors in the extract. When antibody to p150^{Sal2} was added, mobility shifts resulted in more focused bands with three of the six subfragments, viz., F1.2, F1.3 and F2.1 (compare lanes 2 with lanes 4 and 5 in Figure 5B and C). The optimal consensus sequence defined *in vitro* is not found in the p21 promoter. However, each of the subfragments that underwent shift with anti-p150^{Sal2} contains a GC-box related to the consensus sequence. Specifically, F1.2 contains GGAGGG, F1.3 site GGTCGGG, and F2.1 site GGAGGGG. These results confirm the presence of p150^{Sal2} binding sequences in a naturally regulated promoter. Similar sequences present elsewhere in the promoter

fragments showed no evidence of binding in this assay, presumably due to occupancy by other factors in the nuclear extract.

3. 4 p150^{Sal2} binds to and transactivates the BAX promoter

Restoration of p150^{Sal2} in an ovarian carcinoma-derived cell line was previously shown to result in elevated expression of the proapoptotic protein BAX as well as of p21. Suppression of tumor growth by p150^{Sal2} in these cells was accompanied by a roughly 3 fold increase in the apoptotic index along with a 3 fold reduction in mitoses [16]. The possibility that p150^{Sal2} binds and directly transactivates the BAX promoter was not examined. The human BAX promoter has two potential p150^{Sal2} binding sites located at positions −117 and -729 from the translational start site (Figure 6A). To investigate whether p150^{Sal2} binds to the BAX promoter, HOSE cells were first transfected with p150^{Sal2} and assayed by ChIP with anti-p150^{Sal2} antibody. Using primers flanking the proximal GC box at -117, binding of p150^{Sal2} to the BAX promoter increased 6-fold with no change in the level of binding to the aldolase promoter as control (Figure 6B). qRT-PCR was used to measure levels of BAX mRNA following transfection. Levels were elevated roughly 2.5 fold in transfected versus empty vector control (Figure 6C). Levels of BAX protein increased roughly 1.5 fold (Figure 6D).

To investigate the effect of endogenous p150^{Sal2} on the BAX promoter, HOSE cells were first treated for 22 hours with etoposide to induce apoptosis. ChIP assay with anti-p150^{Sal2} antibody showed that binding of endogenous p150^{Sal2} to the BAX promoter increased 3-fold compared to the levels of binding to the aldolase promoter (Figure 6E). This treatment also resulted in increased levels of both p150^{Sal2} and BAX mRNAs (Figure 6F) and proteins (Figure 6G). These results demonstrate direct binding and regulation of BAX by endogenous p150^{Sal2} following an apoptotic stimulus. They also suggest that the effect of restoration of p150^{Sal2} on apoptosis in ovarian carcinoma cells is due to direct interaction [16].

4 DISCUSSION

We have investigated the sequence specificity of DNA binding by the human SALL2 transcription factor p150^{Sal2}. The optimal sequence for binding *in vitro* is the heptanucleotide GGG(T/C)GGG. The binding specificity of p150^{Sal2} overlaps that of the Sp1 family of transcription factors. Though unrelated in overall amino acid sequence, p150^{Sal2} and Sp1 show similarity in their DNA binding domains, each marked by a cluster of three zinc fingers. Evidence for cooperative interaction between p150^{Sal2} and Sp1 comes from results of *in vitro* binding and synergistic action on an artificial promoter. Results of *in vivo* experiments suggest the likelihood of interaction at a distance by binding to separate GC elements found in natural promoters. Binding of p150^{Sal2} to two such promoters, BAX and p21, was analyzed further.

p150^{Sal2} was previously shown to bind and transactivate the p21 promoter [16]. Gel shift and ‘supershift’ experiments were carried out using short restriction fragments derived from the ~ 2.7 kb promoter to identify regions of binding and to confirm the expected presence of GC boxes. Three subfragments roughly 200 bp long were found to bind p150^{Sal2}. Each contains a GC element related to the consensus sequence. Evidence implicating p150^{Sal2} as a possible direct regulator of BAX was inferred from elevated expression and increased apoptosis following restoration of p150^{Sal2} to ovarian carcinoma cells [16]. Chromatin immunoprecipitation, qRT-PCR and immunoblotting were used to demonstrate recruitment of endogenous p150^{Sal2} to the BAX promoter and activation following delivery of an apoptotic stimulus.

GC elements related to the optimal binding sequence defined *in vitro* are present in the promoters of p21 and BAX regulated by $p150^{Sal2}$. Sp1 also plays roles in the regulation of p21 [33, 34] and BAX [35-37], consistent with the possibility of co-regulation by Sp1 and $p150^{Sal2}$ in a conditional and cell type-dependent manner. p21 and BAX are critical targets in the context of actions of transcription factors as tumor suppressors. They are also potentially important as targets of inhibition by DNA viruses whose replication is dependent on cell cycle progression and blocking of apoptosis. Knowledge of the DNA binding specificity together with genome-wide approaches to identify downstream targets and pathways should lead to a better understanding of the regulatory functions of $p150^{Sal2}$ in development, as a potential tumor suppressor, and as an inhibitor of replication of an oncogenic virus.

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Abbreviations used

ChIP	chromatin immunoprecipitation
CS	optimal consensus sequence for DNA binding *in vitro*
EMSA	electrophoretic mobility shift assay
GST	glutathione S-transferase
HOSE cells	established human ovarian surface epithelial cells
p21	the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$
SALL2	the Spalt-like gene 2
SELEX	systematic evolution of ligands by exponential enrichment

Research Highlights

- DNA binding properties of $p150^{Sal2}$ have been defined *in vitro* and *in vivo*.
- $p150^{Sal2}$ binds to GC-rich elements in the $p21^{Cip1/Waf1}$ and BAX promoters.
- $p150^{Sal2}$ activates the BAX promoter following an apoptotic stimulus.
- These DNA binding properties of $p150^{Sal2}$ underlie its tumor suppressor-like functions.

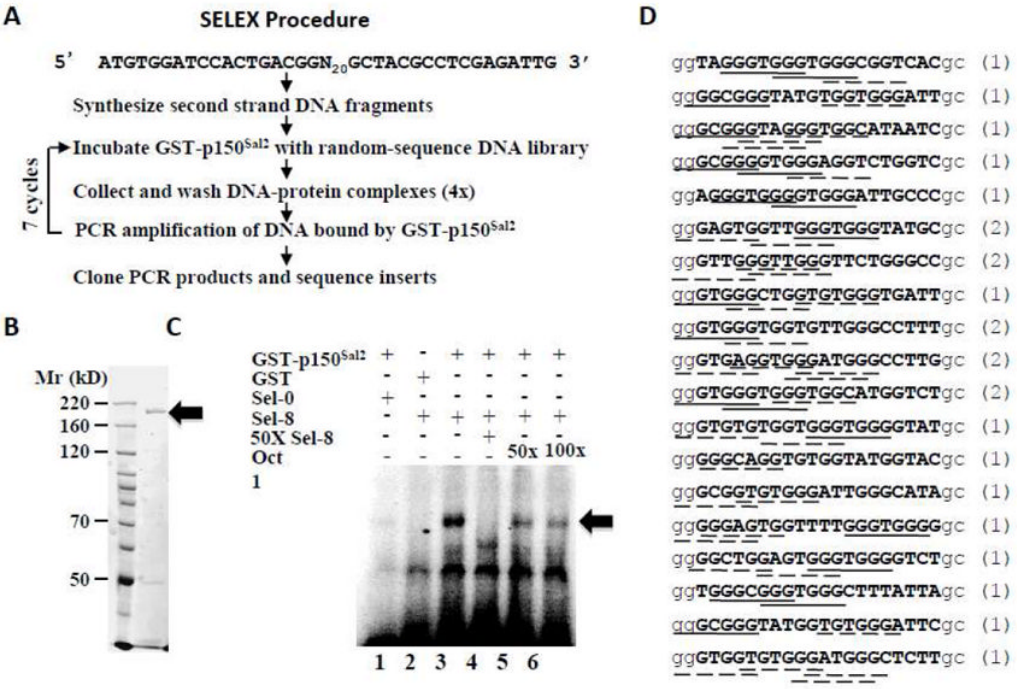
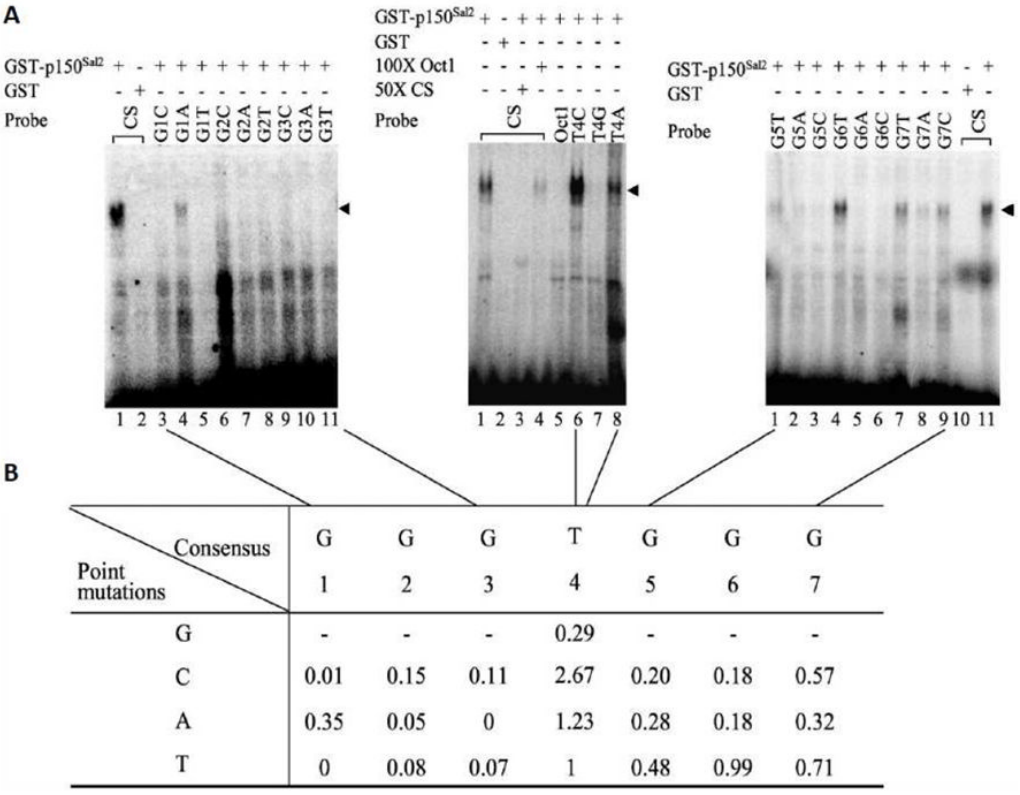


Figure 1. p150^{Sal2} binds selectively to DNA oligomers containing GGG(T/C)GGG

A - Outline of the SELEX protocol. **B** - Purified GST-p150^{Sal2} (◂) shown by SDS-PAGE stained with Coomassie Blue. **C** – EMSA (electromobility shift assay) was performed using 100ng purified GST-p150^{Sal2} or GST alone. Labeled oligonucleotide libraries before selection (Sel-0, lane 1) and after 8 cycles of selection (Sel-8, lane 2-6) were incubated with either GST (lane 2) or GST-p150^{Sal2} (lane 1, 3-6) for 20 min. Specific competition was conducted by adding 50 molar excess of unlabeled oligonucleotides from 8 cycles of selection (lane 4). Non-specific competition was performed with 50 and 100 -fold molar excess of unlabeled oligonucleotides containing Oct-1 binding site (lanes 5, 6). GST-p150^{Sal2} -DNA complexes are indicated (◂). **D** – Aligned sequences of 24 independent clones after 8 cycles of selection of GST-p150^{Sal2} binding. Insert sequences are in bold capital letters and vector sequences in small unbolded letters. Numbers in parentheses are the number of occurrences of each sequence. The consensus sequence (-GGGNGGG-) and sequences differing at a single position are indicated by solid and dotted lines, respectively.



Point mutations \ Consensus	G	G	G	T	G	G	G
	1	2	3	4	5	6	7
G	-	-	-	0.29	-	-	-
C	0.01	0.15	0.11	2.67	0.20	0.18	0.57
A	0.35	0.05	0	1.23	0.28	0.18	0.32
T	0	0.08	0.07	1	0.48	0.99	0.71

Figure 2. Determination of optimal binding sequence for p150^{Sal2}

A - EMSA was performed to test the binding efficiency of GST-p150^{Sal2} towards [α-32P]dCTP-labeled consensus sequence (CS) from SELEX and single base substitutions at each position (left panel, lanes 3-11; middle panel, lanes 6-8; right panel, lanes 1-9). Equal amount (100ng) of GST-p150^{Sal2} or GST vector was used in the binding reaction. Competition was performed using 50 or 100 fold excess of unlabeled oligonucleotides as specific or non-specific competitor (middle panel, lanes 3 and 4, respectively). **B** - Summary of binding efficiencies of GST-p150^{Sal2} to oligonucleotides. Band densities of oligonuleotide-GST-p150^{Sal2} complexes were quantified using a PhosphorImager and normalized to the SELEX consensus sequence set as 1. GST-p150^{Sal2}-DNA complexes are indicated (◂).

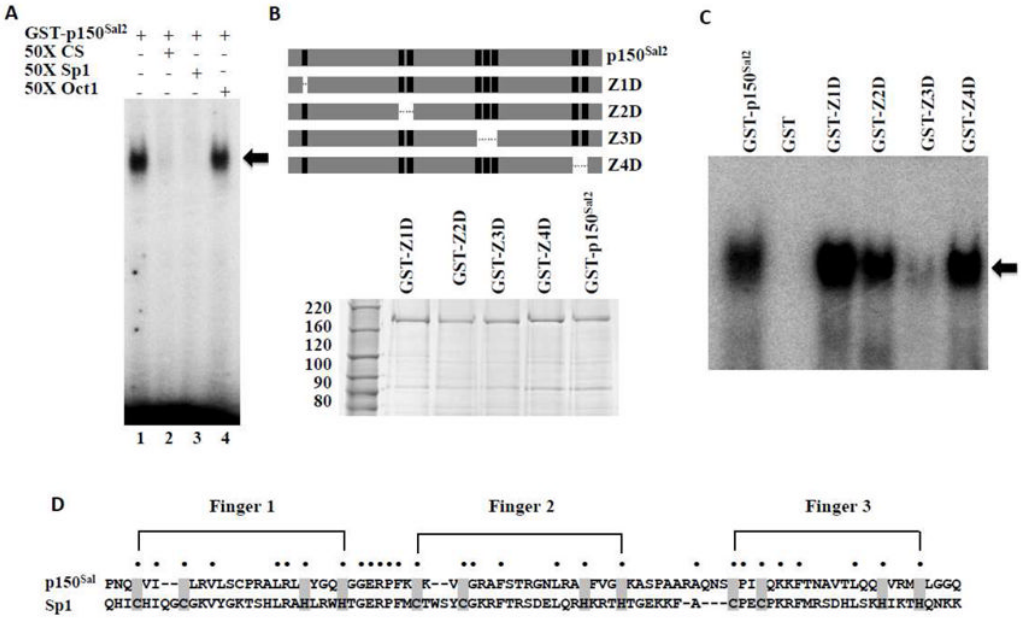


Figure 3. The triple zinc finger cluster in p150^{Sal2} is essential for DNA binding

A - Binding of p150^{Sal2} to an oligonucleotide carrying a confirmed Sp1 binding site (5'-ATTCGATCGGGGCGGGGCGAGC-3'). Competition was conducted by adding 50 fold molar excess of unlabelled oligonucleotides containing the p150^{Sal2} consensus sequence (5'-GGATCACTGGGTGGGAATCACGCT-3') (lane 2), Sp1 binding site (lane 3) or Oct 1 binding site (5'-TGTCGAATGCAAATCACTAGAA-3') (lane 4) in the reaction. DNA-GST-p150^{Sal2} complexes are indicated (◂). **B** - Top: Schematic of p150^{Sal2} illustrating zinc finger deletion mutants. Bottom: Purified GST-p150^{Sal2} and zinc finger deletion mutants. **C** - Equal amounts of purified GST-p150^{Sal2} or mutant proteins were incubated with labelled Sp1 oligonucleotide and complexes analyzed by EMSA (◂). **D** - Alignment of the triple zinc finger motif in p150^{Sal2} and Sp1 illustrating similarities of spacing of the C2H2 fingers and linking sequences.

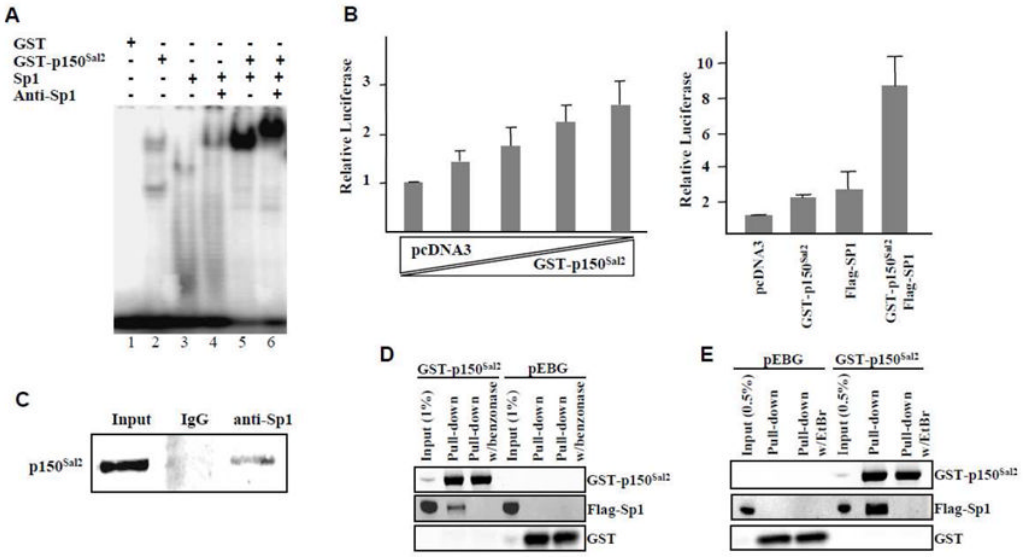


Figure 4. DNA binding and activation by p150^{Sal2} and Sp1

A – Binding of p150^{Sal2} and Sp1 to end-labeled Sp1 oligonucleotide by EMSA and supershift with anti-Sp1. **B** – Left: Relative activity of Sp1-luciferase reporter in HeLa cells transfected with increasing amounts of GST- p150^{Sal2} and decreasing amounts of empty vector. Right: Relative luciferase activity with empty vector, GST-p150^{Sal2}, Flag-Sp1 or both. Vector DNA was added as needed to equalize total amounts of DNA. pTL-Renilla was used to normalize for transfection efficiencies. Results are mean ± .S.D. of three experiments measured 36 hours after transfection. **C** – Co-immunoprecipitation of endogenous p150^{Sal2} with anti-Sp1 in HOSE cells and blotting with anti-p150^{Sal2}. **D** and **E** – HOSE cells were transfected with Flag-Sp1 and empty GST vector or GST-p150^{Sal2}. Extracts prepared 36 hours post-transfection were analyzed by GST pull-downs either directly (**C**), after benzonase treatment to digest DNA (**D**), or after incubation with ethidium bromide (EtBr) to unwind DNA (**E**).

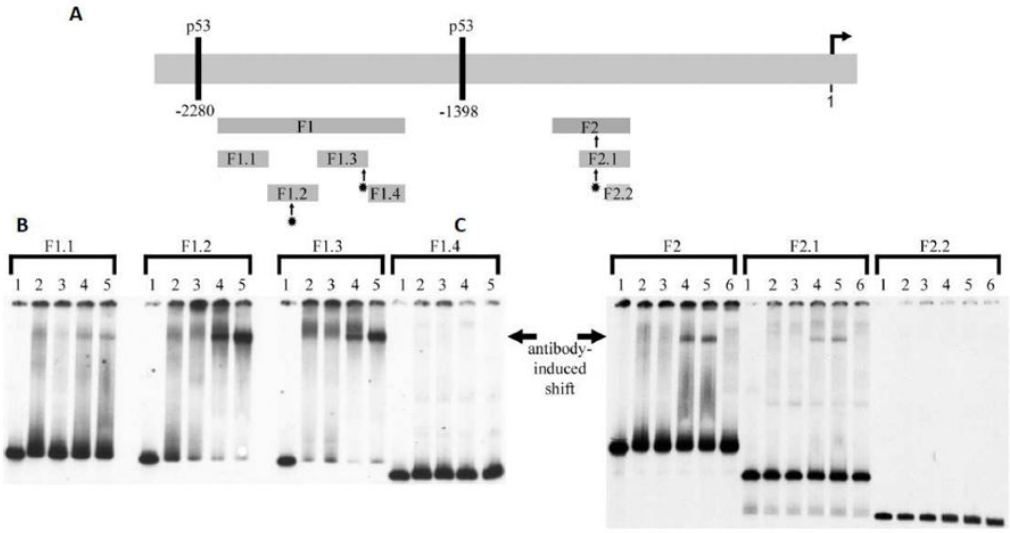


Figure 5. p150^{Sal2} binds *in vitro* to fragments of the p21$^{Cip1/Waf1}$ promoter containing GC boxes

A – Schematic of the ~ 2.6 kb promoter showing fragments F1 and F2 and subfragments generated by Xho and HindIII analyzed by EMSA. **B and C** – EMSA results showing binding of p150^{Sal2} to subfragments F1.2, F1.3 and F2.1 as indicated (-). Radioactively labeled subfragments were incubated with nuclear extracts of 293 cells and the products analyzed by EMSA. Antibody to p150^{Sal2} was used to induce supershifts in subfragments F1.2, F1.3 and F2.1. Lanes 1: DNA probe alone. Lanes 2: probe + nuclear extract. Lanes 3: probe + nuclear extract + antibody to Sp1 (control). Lanes 4: probe + nuclear extract + antibody to N-terminus of p150^{Sal2}. Lanes 5: probe + nuclear extract + antibody to C-terminus of p150^{Sal2}. Lanes 6: probe + nuclear extract + antibody buffer control. Antibody-induced shifts in mobility and focusing of bands are indicated (¢¡). Subfragments F1.2, F1.3 and F2.1 contain GC boxes. [See text].

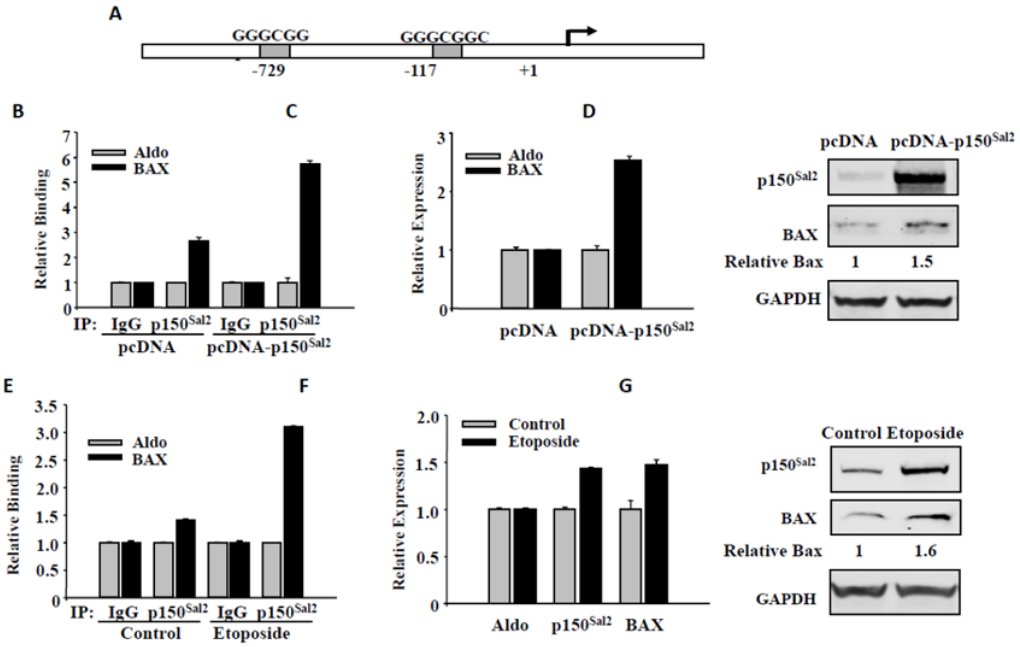


Figure 6. p150^{Sal2} binds to the BAX promoter and upregulates its expression

A - Schematic of the human BAX promoter showing GC boxes. **B** thru **D** - HOSE cells transfected with pcDNA-p150^{Sal2} or empty vector for 48 hours. **B** – ChIP assays for p150^{Sal2} binding to the BAX promoter. Assays were performed on chromatin fragments using antibody to p150^{Sal2} and normalized to preimmune rabbit IgG. Immunoprecipitated fractions were assayed by real time PCR for binding to the BAX and Aldolase promoters. Primers for BAX flanked the GC box at -117. **C** – Real time PCR on total cell RNA for BAX and Aldolase mRNAs. Results are normalized to Aldolase. **D** –Immunblots showing increased level of BAX protein in response to p150^{Sal2}. **E** thru **G** - HOSE cells were treated with 25 ug/ml etoposide for 22 hours to induce apoptosis. **E** - ChIP assay showing increased binding of endogenous p150^{Sal2} to the BAX promoter in treated cells. **F** - Real time PCR on total cell RNA showing elevated levels of p150^{Sal2} and BAX mRNAs in apoptotic cells. **G** – Immunoblots showing elevated BAX protein in treated cells.