

**The Wilms' Tumor Suppressor Protein WT1 acts as a key Transcriptional Repressor of the Human Thromboxane A<sub>2</sub> Receptor Gene in Megakaryocytes.**

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## **ABSTRACT**

In humans, TP $\alpha$  and TP $\beta$  isoforms of the thromboxane A<sub>2</sub> receptor are transcriptionally regulated by distinct promoters, designated Prm1 and Prm3. Previous investigations identified two upstream repressor regions (URR) 1 and URR2 within Prm1. Herein, it was sought to characterize Prm1, identifying the factor(s) regulating URR1 and URR2 in human erythroleukemia (HEL) 92.1.7 cells. Genetic reporter assays and 5' deletions confirmed the presence of URR1 and URR2 but also identified a third repressor, designated RR3, within the proximal "core" promoter. Bioinformatic analysis revealed several GC elements representing putative sites for Egr1/Sp1/Wilms tumor (WT)1 within URR1, URR2 and RR3. While mutation of three GC elements within URR1 and of an adjacent GC element suggested that repressor binding occurs through a cooperative mechanism, repressors binding to the single GC elements within URR2 and RR3 act independently to regulate Prm1. While EMSAs and supershift assays demonstrated that each of the GC elements can bind Egr1 and WT1 *in vitro*, chromatin immunoprecipitations established that WT1 is the factor predominantly bound to each of the repressor regions *in vivo*. Additionally, ectopic expression of -KTS isoforms of WT1 decreased Prm1-directed gene expression and TP $\alpha$  mRNA expression. Collectively, these data establish WT1 as a critical repressor of Prm1, suppressing TP $\alpha$  expression in the platelet progenitor megakaryoblastic HEL cells.

**Key Words:** thromboxane receptor, Wilms' Tumor, WT1, gene, transcription.

## INTRODUCTION

The Wilms' tumor 1 (WT1) gene encodes a zinc finger transcription factor that plays a central role in the development of the genitourinary, haematopoietic and central nervous systems [1,2]. The discovery that mutation of the WT1 gene is one of the main causes of the paediatric kidney cancer Wilms' tumor led to its identification as a tumor suppressor protein [3]. More recently, however, the finding that wild-type WT1 is abundantly expressed in a variety of cancers, including those of the breast [4], oesophagus [5] and pancreas [6] has suggested that it may play an oncogenic role in certain instances. Moreover, accumulating evidence implicates WT1 as an important factor associated with both normal and aberrant haematopoiesis (reviewed in [1]).

As well as playing an important role as a transcriptional regulator, the WT1 protein also appears to be involved in post-transcriptional regulation, with specific functions in RNA metabolism and splicing [7,8], and possibly in translation [9]. Its wide-ranging functions are attributed to the existence of multiple isoforms of the WT1 protein that arise due to alternative splicing or the use of multiple translational start codons within the WT1 gene (reviewed in [10]). The best characterized isoforms are the +/- exon 5 variants and the +/- KTS isoforms. Alternative splicing at these two sites gives rise to four different protein isoforms, specifically (+/+), (+/-), (-/+) and (-/-) with respect to the presence or absence of exon 5 and KTS sequences, respectively. In general, the -KTS isoforms may repress or activate transcription, while the +KTS isoforms have a reduced ability to bind DNA (reviewed in [1]).

WT1 has four C-terminal Kruppel-like C<sub>2</sub>H<sub>2</sub> fingers that share significant identity with those of early growth response (Egr)1, another zinc finger transcription factor. Moreover, the zinc finger domain of WT1 mediates binding to DNA at the Egr1 consensus sequence 5'GCG(G/T)GGGCG3', but its affinity for this site is somewhat less than that of Egr1 [11]. Additionally, WT1 has also been reported to bind to another motif, 5'GCGTGGGAGT3', termed the Wilms' Tumor Element (WTE) [12]. Depending on the cellular context and/or promoter, WT1 can act as either an activator or a repressor of transcription. For example, over-expression of WT1 in the human erythroleukemia K562 cell line or in human breast cancer cell lines activated the c-Myc promoter [13], whilst WT1 repressed the same promoter in HeLa cells [14]. WT1 can interact with other DNA-binding transcription factors, including p53 [15], as well as specific co-activators, such as CBP [16], or co-repressors, including BASP1 [17], to regulate transcription. It is thought that it is the nature of binding of these co-factor partners that largely determines whether WT1 functions as a transcriptional activator or repressor.

The prostanoid thromboxane (TX) A<sub>2</sub> plays a central role in haemostasis, acting as a potent mediator of platelet aggregation [18], but can induce other diverse cellular responses including constriction of vascular and bronchial smooth muscle cells [18,19]. Within the kidney, TXA<sub>2</sub> induces contraction of glomerular mesangial cells and intrarenal vascular tissue, decreasing glomerular filtration rates [20]. Moreover, in glomerulonephritis, a leading cause of end-stage renal failure, it is the most

abundant eicosanoid synthesized in the nephritic glomeruli [21].- Alterations in the levels of TXA<sub>2</sub> or the TXA<sub>2</sub> receptor (TP) are associated with a variety of vascular disorders [22,23], inflammatory renal diseases [24] and in renal failure [25].

In humans, TXA<sub>2</sub> signals through two isoforms of the TXA<sub>2</sub> receptor, termed TP $\alpha$  and TP $\beta$ , that are encoded by a single gene and arise by alternative splicing [26-28]. TP $\alpha$  and TP $\beta$  are identical for their N-terminal 328 amino acid residues but differ exclusively in their C-tail domains [27,28]. TP $\alpha$  and TP $\beta$  regulate both common and distinct signaling pathways [26,29-31] but are subject to entirely different modes of regulation, such as through both agonist-dependent (homologous) desensitization and through intra-molecular cross-talk between other signaling systems [32-36]. Hence, while the functional relevance for the existence of two TP receptors in primates is currently unknown, there is abundant evidence that they have distinct physiologic roles. Moreover, while TP $\alpha$  and TP $\beta$  mRNAs are differentially expressed in a range of cell types [37], platelets exclusively express TP $\alpha$  [38]. Consistent with this, TP $\alpha$  and TP $\beta$  are transcriptionally regulated by distinct promoters, termed Prm1 and Prm3, respectively, within the single TP gene [39,40]. A recent study aimed at characterizing Prm1 in HEL cells localized the proximal “core” promoter and identified two upstream activator regions (UAR) and two upstream repressor regions (URR) within Prm1 [41]. Therein, it was established that the “core” Prm1 is under the control of the general transcription factors Sp1 and Egr1, as well as the more haematopoietic-specific NF-E2, while GATA-1 and Ets-1 bind and regulate UAR1 (from -7962 to -7717). However, the *trans*-acting factors regulating UAR2 (from -7717 to -7504), as well as URR1 (from -8500 to -7962) and URR2 (from -6848 to -6648) remained unidentified.

Hence, the central aim of the current study was to identify the key *cis*-acting elements and *trans*-acting factors that mediate repression of Prm1 within the previously identified URR1 and URR2 sequences. Herein, through 5' deletion and genetic reporter assays, a third repressor region, designated RR3, was also unmasked within the proximal “core” Prm1. Amongst the transcription factor binding elements identified in URR1, URR2 and RR3 within Prm1 were multiple GC-rich elements predicted to represent putative WT1/Egr1/Sp1 binding sites. Data herein established that WT1, as opposed to Sp1 or Egr1, is the major transcription factor that binds the three repressor regions under basal conditions in the megakaryoblastic HEL cell line and WT1 binding correlates with transcriptional repression of Prm1 and TP $\alpha$  expression. Considering the reported role of WT1 as a transcriptional repressor of several genes, as well as its role in normal and aberrant haematopoiesis [42,43] and in renal (dys)function [1,2], the finding of its regulation of Prm1 and TP $\alpha$  expression may have important clinical implications.

## MATERIALS AND METHODS

### Materials

pGL3Basic, pRL-Thymidine Kinase (pRL-TK) and Dual Luciferase® Reporter Assay System were obtained from Promega Corporation. DMRIE-C®, RPMI 1640 culture media and fetal bovine serum (FBS) were from Invitrogen Life Technologies. *Anti-WT1* (sc-192 X), *anti-Sp1* (sc-59 X), *anti-Egr1* (sc-110 X), normal rabbit IgG (sc-2027) and goat *anti-rabbit* horseradish peroxidase (sc-2204) were obtained from Santa Cruz Biotechnology. All antibodies used for ChIP analysis were ChIP-validated by the supplier (Santa Cruz) and have been widely used in the literature for such analyses.

### Construction of luciferase-based genetic reporter plasmids

The plasmid pGL3b:Prm1, containing a Prm1 sequence (2605 bp) from the human TP gene in the pGL3Basic reporter vector, in addition to pGL3b:Prm1B, pGL3b:Prm1BΔGata/Ets, pGL3b:Prm1BΔ, pGL3b:Prm1C, pGL3b:Prm1D and pGL3b:Prm1E have been previously described [41]. Additional 5' deletion sub-fragments of Prm1 were amplified by the polymerase chain reaction (PCR) using pGL3b:Prm1 as template and subsequently sub-cloned into pGL3Basic. Specifically, for pGL3b:Prm1I, a PCR fragment was generated using the sense primer Kin358 (5'GAGAGGTACCTGAGAGACAGCGGGAGACAGAGAC3'; nucleotides (nu) -6258 to -6235, where the underlined sequence corresponds to a *Kpn1* cloning site) and the antisense primer Kin109 (5'AGAGACGCGTCTTCAGAGACCTCATCTGCGGGG3'; complementary to nu -5917 to -5895 of Prm1, where the underlined sequence corresponds to a *Mlu1* cloning site). For pGL3b:Prm1J, a fragment was generated using sense primer Kin391 (5'GAGAGGTACCCCTCCATCTGTGTGGGTCCTC 3'; nu -6122 to -6102) and the antisense primer Kin109. The identity and fidelity of all Prm1-derived sub-fragments in the corresponding recombinant pGL3Basic plasmids were verified through DNA sequencing.

### Site-directed Mutagenesis

Site-directed mutagenesis (SDM) was carried out using the QuikChange™ method (Stratagene). The identity of the Prm1 elements subjected to SDM and the corresponding plasmids generated, as well as the identity, sequence and corresponding nucleotides of the specific primers used are listed below. In each case, the – designation indicates nucleotides 5' of the translational ATG start codon (designated +1).

1. GC at -8345 changed from tgccccCGCCccccac to tgccccTGACccccac using template pGL3b:Prm1 to generate pGL3Basic:Prm1<sup>GC\*(-8345)</sup>. Primers used: Kin423 (5' TGGAAGCTGCCCTGACCCACCCAGCTTC 3') and the complementary oligonucleotide Kin424
2. GC at -8281 changed from gcccgGCCcggcggga to gcccgGTTcggcggga using template (a) pGL3b:Prm1 to generate pGL3Basic:Prm1<sup>GC\*(-8281)</sup> and (b) pGL3Basic:Prm1<sup>GC\*(-8345,-7831)</sup> to generate

- pGL3Basic:Prm1<sup>GC\*(-8345,-8281,-7831)</sup>. Primers used: Kin478 (5' CTCCCTGCCCCGGTTC<sup>CGCCGAAACC</sup> 3') and the complementary oligonucleotide Kin479
- GC at -8146 changed from cGGGGGGTgggGGGCGGGGGCgggcca to cGGGGGTcgtGGGTGGATGGCgggcca using template (a) pGL3b:Prm1 to generate pGL3Basic:Prm1<sup>GC\*(-8146)</sup> and (b) pGL3Basic:Prm1<sup>GC\*(-8345,-8281,-7831)</sup> to generate pGL3Basic:Prm1<sup>GC\*(-8345,-8281,-8146,-7831)</sup>. Primers used: Kin510 (5' GTGCTGGCGGGGGTTCGTGGGGCGGGGGGCG 3') and the complementary oligonucleotide Kin511, as well as Kin512 (5' GGGGTTCGTGGGGTGGATGGCGGGCCAAGAC 3') and the complementary Kin513
  - GC at -7831 changed from t<sup>actGCCcctcatct</sup> to t<sup>actGTCCtctcatct</sup> using template (a) pGL3b:Prm1 to generate pGL3Basic:Prm1<sup>GC\*(-7831)</sup>, (b) pGL3b:Prm1B to generate pGL3Basic:Prm1B<sup>GC\*(-7831)</sup> and (c) pGL3Basic:Prm1<sup>GC\*(-8345)</sup> to generate pGL3Basic:Prm1<sup>GC\*(-8345,-7831)</sup>. Primers used: Kin361 (5' TCCGTCTCTCACTGTCCTCTCATCTGGAGCCC 3') and the complementary oligonucleotide Kin362
  - GC at -6717 changed from tetgtct<sup>CCCAccca</sup> to tetgtct<sup>ATCAccca</sup> using template pGL3Basic:Prm1D to generate pGL3Basic:Prm1D<sup>GC\*(-6717)</sup>. Primers used: Kin502 (5' CATCCCTCTGTCCTATCACCCACCCCTGG 3') and the complementary oligonucleotide Kin503
  - GC at -6206 changed from cagcgcc<sup>CCCAccgt</sup> to cagcgcc<sup>TACAaccgt</sup> using template (a) pGL3Basic:Prm1I to generate pGL3Basic:Prm1I<sup>GC\*(-6206)</sup> and (b) pGL3Basic:Prm1D<sup>GC\*(-6717)</sup> to generate pGL3Basic:Prm1D<sup>GC\*(-6717,-6206)</sup>. Primers used: Kin506 (5' GCTGCCAGCGGCCTACACCCGTCCCAGC 3') and the complementary oligonucleotide Kin507

### Cell culture

Human erythroleukemia (HEL) 92.1.7 cells were obtained from the American Type Culture Collection (ATCC) and were grown at 37 °C in a humid environment with 5 % CO<sub>2</sub>. HEL cells were cultured in RPMI 1640, 10 % fetal bovine serum (FBS).

### Assay of luciferase activity

HEL cells were co-transfected with the various pGL3Basic-recombinant plasmids, encoding firefly luciferase, along with pRL-TK, encoding renilla luciferase, using DMRIE-C® transfection reagent and assayed for firefly and renilla luciferase 48 h later using the Dual-Luciferase Reporter Assay System™, as previously described [44]. Relative firefly to renilla luciferase activities (arbitrary units) were calculated as a ratio and were expressed in relative luciferase units (RLU).

To investigate the effect of over-expression of exon 5 (+ or -) or KTS (+ or -) isoforms of WT1 on Prm1-directed gene expression, HEL cells were co-transfected with either pGL3b:Prm1 or pGL3b:Prm1D (1.5 µg) plus 200 ng of pRL-TK along with either pcDNA3:WT1 (+/+), pcDNA3:WT1 (+/-),

pcDNA3:WT1 (-/+), pcDNA3:WT1 (-/-) (0.5 µg), or as a control, pcDNA3 (0.5 µg). Cells were harvested 48 h post-transfection and assayed for luciferase activity, as above, or subjected to western blot or RT-PCR analysis.

The plasmids pcDNA3:WT1 (+/+), pcDNA3:WT1 (+/-), pcDNA3:WT1 (-/+ and pcDNA3:WT1 (-/-) were generously donated by Dr. Charles T. Roberts JR, Oregon National Primate Research Center, Oregon, USA and have been previously described [45].

### **Western blot analysis**

The expression of WT1, Sp1 and Egr1 proteins in HEL cells was confirmed by western blot analysis. Briefly, whole cell protein was resolved by SDS-PAGE (10 % acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membrane according to standard methodology. Membranes were screened using *anti*-WT1, *anti*-Sp1 or *anti*-Egr-1 sera in 5 % non fat dried milk in 1x TBS (0.01 M Tris/HCl, 0.1 M NaCl) for 2 h at room temperature. Thereafter, membranes were washed and screened using goat *anti*-rabbit horseradish peroxidase (sc-2204), followed by chemiluminescence detection as previously described [36].

### **Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from HEL 92.1.7 cells ( $5 \times 10^6$  approximately) using TRIzol reagent (Invitrogen Life Technologies). RT-PCR was carried out with DNase 1-treated total RNA using oligonucleotide primers to specifically amplify TP $\alpha$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA sequences, as previously described [37]. The following primers were used:

1. Kin16: TP $\alpha$  forward 5' GAGATGATGGCTCAGCTCCT 3'
2. DT75: TP $\alpha$  reverse 5' CCAGCCCCTGAATCCTCA 3'
3. Kin291: GAPDH forward 5' CCACAGTCCATGCCATCAC 3'
4. DT92: GAPDH reverse 5' CATGTGGGCCATGAGGTC 3'

### **Electrophoretic mobility shift and supershift assays**

Nuclear extract was prepared from HEL cells as previously described [44]. Oligonucleotides corresponding to the sense (5' end-labeled with biotin) and antisense strands of each probe (90 µM) were annealed by heating at 95 °C for 2 min followed by slow cooling to room temperature. Electrophoretic mobility shift assays were performed as previously described [41]. The identities and sequences of the forward biotin-labeled oligonucleotide probes are listed below. Sequences of the corresponding non-labeled complementary oligonucleotides are omitted.

1. GC<sup>-8345,-8281</sup> probe (Kin733; 5'[Btn] GAAGCTGCCCCGCCCCACCCAGCTTCCTGACTTT GGCTGTGTCCAGAGCTAAGAATAGACGCTCCCTGCCCGGCCCCCGCCGGAAACCG 3', nu -8350 to -8260 of Prm1)
2. GC<sup>-8146</sup> probe (Kin737; 5'[Btn] GTGCTGGCGGGGGGTGGGGGGCGGGGGGCGGGCCAA GACCGG3', nu -8153 to -8118 of Prm1)
3. GC<sup>-7831</sup> probe (Kin739; 5'[Btn] TCCTCCGTCTCTCACTGCCCCCTCATCTGGAGCCCCAG 3', nu -7842 to -7805 of Prm1)
4. GC<sup>-6717</sup> probe (Kin762; 5'[Btn] CACCCCCATCCCTCTGTCTCCACCCCACCCCTGGA AG 3', nu -6730 to -6691 of Prm1)
5. GC<sup>-6206</sup> probe (Kin764; 5'[Btn] GCCGCGGGCTGCCAGCGGCCCCCACCCGTCCCAGCTC GGC3', nu -6218 to -6178 of Prm1)

The sequences of the competitor/non-competitor oligonucleotides used were as follows:

1. Prm1<sup>-8345</sup> competitor (Kin458; 5' CTGGAAGCTGCCCCGCCCCACCCAG 3', nu -8453 to -8327 of Prm1)
2. Prm1<sup>-8281</sup> competitor (Kin742; 5' CTCCCTGCCCGGCCCCCGCCGGAAACCGC 3', nu -8287 to -8259 of Prm1)
3. Prm1<sup>-8146</sup> competitor (Kin745; 5' GTGCTGGCGGGGGGTGGGGGGCGGGGGGCGGGCCA AGACCGG 3', nu -8153 to -8112 of Prm1)
4. Prm1<sup>-7831</sup> competitor (Kin798; 5' TCCTCCGTCTCTCACTGCCCCCTCATCTGGAGCCCCA G 3', nu -7842 to -7805 of Prm1)
5. Prm1<sup>-6717</sup> competitor (Kin779; 5' CACCCCCATCCCTCTGTCTCCACCCCACCCCTGG AAG 3', nu -6730 to -6691 of Prm1)
6. Prm1<sup>-6206</sup> competitor (Kin780; 5' GCCGCGGGCTGCCAGCGGCCCCCACCCGTCCCAGCT CGGC 3', nu -6218 to -6179 of Prm1)
7. WTE consensus (Kin748; 5' CGAGTGCGTGGGAGTAGAATT 3')
8. Sp1 consensus (Kin651; 5' ATTCGATCGGGGCGGGGCGAGC 3')
9. Egr1 consensus (Kin746; 5' GGATCCAGCGGGGGCGAGCGGGGGCGA 3')
10. non-specific (Kin484; 5' GGGCCGAGGACAGGTGAAGTGGGGACAG 3')

For supershift assays, nuclear extract (2.5 µg total protein) was pre-incubated with 4 µg of *anti-Sp1*, *anti-Egr1*, *anti-WT-1* or *anti-cJun* sera for 2 h at room temperature. Thereafter, the nuclear extract-antibody mixtures were incubated for 20 min at room temperature with the appropriate biotin-labelled double-stranded probe, as previously described [41].

### Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) assays were performed using HEL 92.1.7 cells, as previously described [41]. Chromatin samples were immunoprecipitated with 10 µg of *anti-WT1*, *anti-Sp1*, *anti-Egr1*, normal rabbit IgG control antibody, or a “no antibody” control. The identities of the primers used for the ChIP PCR reactions, as well as their sequences and corresponding nucleotides within Prm1 are listed below.

1. Kin462 (5' CGAGACCCTGCAGGCAGACTGGAG 3'; -8460 to -8437)
2. Kin463 (5' GAGATGGGGAAACTGAGGCACAAAG 3'; -8030 to -8006)
3. Kin468 (5' GCCTTGCAGAGATGTGGTGAGGC 3'; -7978 to -7973)
4. Kin467 (5' GAGGTGAGCTAGGAAGACATCTTG 3'; -7630 to -7607)
5. Kin233 (5' GAGAGGTACCGCTCCAAAGCCACCTCCG 3'; -6848 to -6831)
6. Kin144 (5' AGAGACGCGTCGCTTCCTCGGGAGCCTCA 3'; -6455 to -6437)
7. Kin456 (5' CTTCCCCAGAAGGCTGTAGGGTGTC 3'; -6368 to -6344)
8. Kin109 (5' AGAGACGCGTCTTCAGAGACCTCATCTGCGGGG 3'; -5917 to -5895)
9. Kin364 (5' TTGGGTCCAGAAGGTCGAGGC 3'; -1081 to -1061)
10. Kin365 (5' GCGAACCAGGGCGAGGC 3'; -711 to -695)

### **Statistical analysis**

Statistical analyses of differences were analyzed using the two-tailed Student's unpaired *t*-test. All values are expressed as mean ± standard error of the mean (SEM). *P*-values < 0.05 were considered to indicate statistically significant differences and \*, \*\*, \*\*\*, \*\*\*\* indicate *p* < 0.05, *p* < 0.01, *p* < 0.001 and *p* < 0.0001, respectively.

## RESULTS

### Identification of three distinct repressor regions within Prm1 of the human TP Gene

The TP $\alpha$  and TP $\beta$  isoforms of the TXA<sub>2</sub> receptor (TP) are under the transcriptional regulation of Prm1 and Prm3, respectively, within the human TP gene [39]. Prm1 is defined as nucleotides -8500 to -5895 located 5' of the translation initiation codon within the human TP gene [39]. In a recent study aimed at characterizing Prm1 within the megakaryoblastic HEL 92.1.7 cell line, two upstream repressor regions (URR) were identified (**Figure 1A** & [41]). However, the factors regulating URR1 (-8500 to -7962) and URR2 (-6848 to -6648) remained to be identified (**Figure 1A** & [41]). The aim of the current study was to further characterize Prm1 and to identify the *cis*-acting elements and *trans*-acting factors mediating its repression through the aforementioned URR1 and URR2 in HEL cells.

Initially, and consistent with previous findings [41], genetic reporter assays and progressive 5' deletion of nucleotides from -8500 to -6648 to yield the core promoter (Prm1E; **Figure 1A**) confirmed the presence of two upstream regions of repression, namely URR1 and URR2. Specifically, deletion of nucleotides from Prm1 (-8500) to generate Prm1B (-7962) yielded a 2.3-fold increase in luciferase expression ( $p < 0.0001$ ), while 5' deletion of Prm1D (-6848) to generate Prm1E (-6648) resulted in a 1.5-fold increase in luciferase activity ( $p = 0.0032$ ). Further 5' deletion of Prm1E (-6648) to generate Prm1I (-6258) resulted in a 1.6-fold decrease in luciferase expression ( $p = 0.0002$ ), thereby uncovering an activator region within the "core" Prm1. Consistent with this observation, two functional overlapping Sp1/Egr1 elements have been previously identified within this region, specifically at -6294 and -6278, that mediate activation of Prm1 [41]. However, herein, further 5' deletion of Prm1I (-6258) to generate Prm1J (-6123) yielded a 2-fold increase in luciferase activity ( $p < 0.0001$ ) to reveal a third, previously unidentified, repressor region (-6258 to -6123) also located within the "core" Prm1. The luciferase expression directed by Prm1J was substantially higher than that of the empty pGL3Basic vector (**Figure 1A**). Consistent with this observation, two functional overlapping Sp1/Egr1 elements within Prm1J, specifically at -6022 and -6007, in addition to an NF-E2 element at -6080 were previously identified, and these elements mediate activation of Prm1 within this core proximal region [41]. Hence, collectively, through these and previous studies, three distinct regions of repression have been identified within Prm1; namely, the two previously identified URR1 (-8500 to -7962) and URR2 (-6848 to -6648) and an additional repressor region, designated RR3, located between -6258 and -6123 within the proximal core promoter.

### Identification of multiple GC-enriched elements in the -8500 to -7962 repressor region of Prm1

Bioinformatic analysis [46] to identify transcription factor elements within URR1 revealed three putative GC elements representing putative overlapping WT1/Egr1/Sp1 sites at -8345, -8281 and -8146, where the 5' nucleotide of each element is indicated (**Table I** and **Figure 1B**). Site-directed mutagenesis (SDM) of

each of these GC elements led to substantial increases in luciferase activity directed by Prm1 (2.0-fold, 1.9-fold, and 2.5-fold, respectively;  $p < 0.0001$  in each case; **Figure 1B**). A fourth GC element was identified somewhat adjacent to URR1, specifically at -7831 within Prm1B. Mutation of this element also substantially increased luciferase activity directed by Prm1 (2.1-fold;  $p < 0.0001$ ). These data suggest that the GC elements at -8345, -8281, -8146 and -7831 mediate repression of Prm1.

To investigate the combined contribution of GC elements in directing Prm1 activity, the effect of collectively mutating the sites within Prm1 (-8500) was examined (**Figure 1C**). Disruption of the GC<sup>-7831</sup> element within Prm1<sup>GC\*(-8345)</sup> to generate Prm1<sup>GC\*(-8345,-7831)</sup> did not significantly affect luciferase expression directed by Prm1<sup>GC\*(-8345)</sup> ( $p = 0.3781$ ). However, disruption of the GC<sup>-8281</sup> element in Prm1<sup>GC\*(-8345,-7831)</sup>, generating Prm1<sup>GC\*(-8345,-8281,-7831)</sup>, yielded a 1.6-fold decrease in luciferase expression compared to that of Prm1<sup>GC\*(-8345,-7831)</sup> ( $p = 0.0043$ ). Luciferase expression of Prm1<sup>GC\*(-8345,-8281,-8146,-7831)</sup> was not significantly different than that of Prm1<sup>GC\*(-8345,-8281,-7831)</sup> ( $p = 0.7499$ ). Therefore, generation of Prm1<sup>GC\*(-8345,-8281,-8146,-7831)</sup> from Prm1<sup>GC\*(-8345)</sup> led to an overall 1.7-fold decrease in luciferase expression ( $p = 0.0024$ ), suggesting that repressor factors rely on a cooperative mechanism of binding to multiple neighbouring GC elements. It is likely that disruption of cooperative binding upon SDM shifts the overall affinity of intact GC elements for activator, rather than for repressor factors. Consistent with this suggestion, disruption of GC<sup>-7831</sup> in the Prm1B (-7962) sub-fragment, which does not contain any of the other three GC elements, actually decreased the luciferase activity directed by Prm1B (1.4-fold;  $p = 0.0004$ ; **Figure 1D**). This effect is in contrast to the substantial increase (2.1-fold;  $p < 0.0001$ ; **Figure 1B**) in luciferase expression that occurred upon disruption of the same GC element within Prm1 where the other three GC elements at -8345, -8281 and -8146 were intact (compare **Figures 1B** and **1D**).

The expression of WT1, Sp1 and Egr1 in the HEL 92.1.7 cell line was confirmed by immunoblot analysis (**Figure 2**). Consistent with previous studies in K562 cells [47], a doublet of WT1 protein at 52/54 kDa was detected herein in HEL cells (**Figure 2A**). Also consistent with studies in K562 cells [48], an immunoreactive Egr1 band of approximately 82 kDa was detected in HEL cells (**Figure 2B**), while abundant expression of the ubiquitous Sp1 protein was also confirmed (**Figure 2C**). Thereafter, electrophoretic mobility shift assays (EMSAs) were carried out to investigate the presence and identity of nuclear factors capable of binding to the overlapping WT1/Egr1/Sp1 elements at -8345, -8281, -8146 and -7831 *in vitro* (**Figure 3**). It has been well documented that both Egr1 and WT1 proteins can bind to the Egr1 consensus sequence [11], while it has also been suggested that the WTE may act as a specific binding element for WT1 [12]. Moreover, overlapping sites for Sp1 and Egr1/WT1 are frequently found in promoter sequences due to the similarity in their consensus elements [49].

Incubation of biotin-labeled oligonucleotide probes encoding GC<sup>-8345</sup> and GC<sup>-8281</sup> (**Figure 3A & 3B**), GC<sup>-8146</sup> (**Figure 3C & 3D**) and GC<sup>-7831</sup> (**Figure 3E & 3F**) with nuclear extract prepared from HEL cells resulted in the appearance of a number of protein-DNA complexes. Specifically, incubation of the

probe encoding both GC<sup>-8345</sup> and GC<sup>-8281</sup> elements with nuclear extract generated three main complexes, C1-C3 (**Figure 3A**). C1 and C2 were partially competed by non-labeled competitors containing either the specific GC<sup>-8345</sup>, GC<sup>-8281</sup>, consensus Sp1 or Egr1 sequences (**Figure 3A**, lanes 3 – 6, respectively). C3 was efficiently competed by consensus Sp1 or Egr1 sequences but not by GC<sup>-8345</sup> or GC<sup>-8281</sup> sequences. The WTE sequence or a non-specific competitor based on a random sequence within the TP gene failed to compete with C1, C2 or C3 complexes (**Figure 3A**, lanes 7 and 8, respectively). Therefore, it seems that complexes C1 and C2 consist of Sp1, Egr1 and/or WT1 proteins bound to the GC<sup>-8345</sup> and GC<sup>-8281</sup> elements *in vitro*, whilst C3 may consist of Sp1, Egr1 and/or WT1 bound to an unidentified element within the probe (**Figure 3A**). Thereafter, in order to investigate the specific nature of the factors that bind to GC<sup>-8345</sup> or GC<sup>-8281</sup> *in vitro*, supershift assays were carried out. While no supershifted complex was observed upon addition of an *anti*-WT1 antibody, both C2 and C3 were substantially reduced following its addition. The reduction in C2 and C3 despite the absence of a supershifted complex is thought to be due to aggregation of specific antibody-protein complexes and failure of these large complexes to enter the gel. Moreover, addition of *anti*-Sp1 or *anti*-Egr1 antibodies led to reductions in all three complexes but without the appearance of supershifted complexes *per se*. Addition of an *anti*-cJun antibody, which acted as a negative control, did not have any substantial effects on binding patterns to the probe. Therefore, these data indicate a specific role for WT1, Sp1 and Egr1 binding to GC<sup>-8345</sup> and/or GC<sup>-8281</sup> *in vitro* (**Figure 3B**).

Incubation of the GC<sup>-8146</sup> probe with nuclear extract generated four main complexes, C1-C4 (**Figure 3C**). All four complexes were efficiently competed by non-labeled competitors containing the GC<sup>-8146</sup> or consensus Egr1 sequences, while none of the four complexes were competed by the consensus Sp1, WTE or non-specific oligonucleotide sequences. Despite the fact that none of the four complexes were competed by the WTE sequence, addition of an *anti*-WT1 antibody substantially reduced C2, C3 and C4, indicating a role for WT1 binding to the probe. Moreover, addition of an *anti*-Egr1 antibody appeared to increase formation of higher complexes, suggesting that Egr1 may also possibly play a role in binding to GC<sup>-8146</sup> *in vitro*. However, addition of an *anti*-Sp1 antibody or an *anti*-cJun antibody, which acted as a negative control, did not have any substantial effects on binding patterns to the probe (**Figure 3D**). Thus, it is indicated that WT1, and possibly Egr1, bind to the GC<sup>-8146</sup> element *in vitro*.

Incubation of the GC<sup>-7831</sup> probe with nuclear extract generated a single complex C1 and a diffuse faster migrating complex (**Figure 3E**) that were competed by a non-labeled competitor containing the GC<sup>-7831</sup> element, or by consensus Sp1 or Egr1 sequences. C1 was competed to a much lesser extent by the WTE sequence, but was not competed by the non-specific oligonucleotide. Therefore, C1 is likely to consist of Sp1, Egr1 and/or WT1 proteins complexed to the GC<sup>-7831</sup> element. It was notable, however, that a second slower-migrating complex appeared where the main complex C1 was competed by non-labeled competitors. Thus, it is indicated that an increase in binding of unidentified protein(s) to an

element within the probe may occur when the protein(s) involved in the formation of C1 are unavailable for binding to the probe. Thereafter, pre-incubation of nuclear extract with an *anti*-WT1 antibody prevented the generation of C1, suggesting a role for WT1 binding to GC<sup>-7831</sup> despite the fact that no supershift complex was observed. Pre-incubation of nuclear extract with an *anti*-Egr1 antibody reduced C1 to a lesser extent, while addition of an *anti*-Sp1 antibody or an anti-cJun antibody, which acted as a negative control, did not have any substantial effect on formation of C1 (**Figure 3F**). Overall, these EMSA data (**Figure 3**) indicate that GC<sup>-8345</sup>, GC<sup>-8281</sup>, GC<sup>-8146</sup> and GC<sup>-7831</sup> elements have a sequence capacity to bind Egr1 and WT1 isoform(s), while GC<sup>-8345</sup> and GC<sup>-8281</sup> also have a capacity to bind Sp1.

Thereafter, to investigate whether endogenous Sp1, Egr1 and/or WT1 can actually directly bind to chromatin encoding Prm1 *in vivo*, chromatin immunoprecipitation (ChIP) assays were carried out on chromatin extracted from HEL cells (**Figure 4**). PCR analysis using primers to amplify the 5' Prm1 repressor region, specifically from -8460 to -8006 and containing GC elements at -8345, -8281 and -8146, resulted in amplification of DNA recovered from both the input chromatin and from an *anti*-WT1 immunoprecipitate, but not from *anti*-Sp1, *anti*-Egr1 or control IgG precipitates (**Figure 4A**). Furthermore, PCR analysis using primers specific to the adjacent Prm1 region, specifically from -7978 to -7607 and containing the GC element at -7831, also resulted in amplification of DNA recovered from the input chromatin, *anti*-WT1 and, to a much lesser extent, from *anti*-Egr1 immunoprecipitates. However, these primers failed to amplify DNA from *anti*-Sp1 or control IgG precipitates (**Figure 4B**). These data provide evidence that WT1, but not Sp1, occupies element(s) within the Prm1 region between -8460 and -7607 of chromatin in HEL cells *in vivo*, while Egr1 occupies elements within this region to a much lesser extent than WT1. Conversely, as controls, primers specific to the -1081 to -695 region of Prm3 of the TP gene resulted in generation of an amplicon from the input chromatin, but not from Sp1, Egr1 or control IgG precipitates (**Figure 4C**).

Hence, to expand these studies, the effect of ectopic expression of WT1 on Prm1-directed reporter gene expression and TP $\alpha$  mRNA was investigated (**Figure 4D – 4G**). The four main isoforms of WT1, specifically (+/+), (+/-), (-/+), and (-/-) with respect to the presence or absence of exon 5 and KTS sequences, respectively, were over-expressed in HEL cells (**Figure 4E**) and the effects on Prm1-directed luciferase activity were investigated. While immunoblot analysis confirmed over-expression of WT1 (**Figure 4E**), the (+/-) and (-/-) isoforms led to 1.4-fold ( $p = 0.0013$ ) and 1.7-fold ( $p = 0.0007$ ) reductions in Prm1-directed luciferase expression, respectively, while neither the (+/+) nor (-/+) isoforms had any significant effect ( $p = 0.4817$  and  $p = 0.8881$ ; **Figure 4D**). Consistent with this, RT-PCR confirmed that ectopic expression of the transcriptionally active (+/-) and (-/-) isoforms both reduced TP $\alpha$  mRNA expression with no substantial changes in GAPDH expression (**Figure 4F**). Moreover, densitometric analysis revealed that ectopic expression of (+/-) and (-/-) isoforms led to significant decreases in TP $\alpha$  mRNA expression relative to GAPDH mRNA expression, compared to HEL cells transfected with a

control plasmid ( $p = 0.0167$ ,  $p = 0.0080$ ; **Figure 4G**). Taken together, these data indicate that -KTS isoforms of WT1 mediate repression of Prm1 and TP $\alpha$  expression and considering the data from mutational, EMSA and ChIP analyses, it appears that WT1 exerts this repression by binding to GC elements at -8345, -8281, -8146 and -7831.

### Identification of GC elements in the -6848 to -6648 and -6258 to -6123 repressor regions of Prm1

Amongst the transcription factor binding elements identified within URR2 located between -6848 and -6648 (**Figure 1A**), was a putative GC element at -6717 predicted to represent a putative overlapping site for WT1/Egr1/Sp1. Additionally, bioinformatic analysis of the “core” repressor region, referred to as RR3 and located between -6258 and -6123 of Prm1, also revealed a GC element, specifically at -6206 (**Table I**). Hence, SDM was used to disrupt the putative GC<sup>-6717</sup> and GC<sup>-6206</sup> elements within either Prm1D (-6848) or Prm1I (-6258; **Figure 5**). Mutation of the GC<sup>-6717</sup> element within Prm1D resulted in a 4.8-fold increase in luciferase expression compared to that of the wild-type Prm1D ( $p < 0.0001$ ). Mutation of the GC<sup>-6206</sup> element within Prm1I led to a 1.3-fold increase in luciferase expression ( $p = 0.0083$ ; **Figure 5A**). Mutation of the same GC<sup>-6206</sup> element within the Prm1D sub-fragment led to a 3-fold increase in luciferase activity compared to that of the wild-type Prm1D ( $p < 0.0001$ ; **Figure 5B**). To investigate the combined contribution of GC<sup>-6717</sup> and GC<sup>-6206</sup> elements in directing Prm1 activity, the effect of collectively mutating these elements within Prm1D was examined (**Figure 5B**). The luciferase activity directed by Prm1D<sup>GC\*(-6717,-6206)</sup>, in which both GC elements at -6717 and -6206 were mutated, was significantly higher than that of either Prm1D<sup>GC\*(-6717)</sup>, in which the -6717 element alone was mutated, or Prm1D<sup>GC\*(-6206)</sup>, in which the -6206 element alone was mutated ( $p < 0.0001$  in each case). Hence, collectively, these data indicate that GC elements at -6717 and -6206 bind factors that act independently to mediate repression of Prm1.

Thereafter, EMSAs were employed to confirm the presence of nuclear factors capable of binding to the GC<sup>-6717</sup> element *in vitro*. Incubation of a GC<sup>-6717</sup> probe with nuclear extract prepared from HEL cells generated a single DNA - protein complex, C1 (**Figure 6A**). C1 was efficiently competed by a non-labeled competitor containing the GC<sup>-6717</sup> sequence, and by consensus Sp1 or Egr1 sequences, but was not competed by WTE or non-specific sequences. Despite the fact that C1 was not competed by the WTE sequence, pre-incubation of nuclear extract with *anti*-WT1 appeared to lead to the formation of a weak supershift complex, as well as preventing formation of C1. This suggested a role for WT1 binding to the GC<sup>-6717</sup> probe. Moreover, addition of an *anti*-Egr1 antibody reduced C1 to a lesser extent, indicating that Egr1 can also bind to the GC<sup>-6717</sup> element *in vitro*. However, addition of an *anti*-Sp1 antibody or an *anti*-cJun antibody did not have any substantial effects on binding to the probe (**Figure 6B**). Thus, it is suggested that C1 consists of complexes of Egr1 and WT1 proteins bound to the GC<sup>-6717</sup> probe. To investigate whether Sp1, Egr1 and/or WT1 can bind to chromatin encoding -6848 to -6648 region of

Prm1 *in vivo*, ChIP assays were carried out using chromatin extracted from HEL cells (**Figure 6C**). PCR analysis using primers specific to this region of Prm1 and containing the GC element at -6717 generated amplicons from input chromatin, *anti*-WT1 and, to a much lesser extent, from *anti*-Egr1 immunoprecipitates, but not from *anti*-Sp1 or the control IgG precipitates. These data provide evidence that WT1, and to a lesser extent, Egr1 occupy element(s) within the -6848 to -6648 region of Prm1 *in vivo*.

EMSAs were also employed to investigate the presence of nuclear factors capable of binding to the GC<sup>-6206</sup> element *in vitro*. Incubation of a GC<sup>-6206</sup> probe with nuclear extract generated a single diffuse complex C1 that was efficiently competed by a non-labeled competitor containing the GC<sup>-6206</sup> sequence, or by consensus Sp1 or Egr1 sequences, but not by WTE or non-specific sequences (**Figure 6D**). Thereafter, pre-incubation of nuclear extract with an *anti*-WT1 antibody prevented formation of C1, indicating that WT1 binds to the GC<sup>-6206</sup> element. Moreover, addition of an *anti*-Egr1 antibody also reduced C1, indicating that it may also bind to the probe. However, addition of an *anti*-Sp1 antibody or an *anti*-cJun antibody did not have any substantial effects on binding to the probe (**Figure 6E**). Thus, C1 is likely to consist of Egr1 and WT1 proteins bound to the GC<sup>-6206</sup> probe. To investigate whether Sp1, Egr1 and/or WT1 can bind to the proximal Prm1 (from -6320 to -5895) *in vivo*, ChIP assays were carried out (**Figure 6F**). PCR generated amplicons consisting of Prm1 sequences between -6368 and -5895 from input chromatin, *anti*-WT1, *anti*-Sp1 and *anti*-Egr1 immunoprecipitates but not from the control IgG precipitate. Consistent with the latter findings, it has previously been established that both Sp1 and Egr1 bind to this region (-6368 to -5895) of Prm1 *in vivo*, where binding occurs at overlapping Sp1/Egr1 elements at -6294, -6278, -6022 and -6007 within the proximal Prm1 [41]. Herein, evidence is also presented that WT1 binds to the proximal Prm1 *in vivo*, and that binding of WT1 is likely to occur at the GC element at -6206, while Egr1 may also bind to this element. Conversely, as controls, primers specific to the -1081 to -695 region of Prm3 of the TP gene resulted in generation of an amplicon from the input chromatin, but not from Sp1, Egr1 or control IgG precipitates (**Figure 6G**).

Thereafter, the effects of ectopic expression of WT1 on luciferase activity directed by Prm1D (-6848) and Prm1I (-6258) were investigated. The (+/+), (+/-), (-/+) and (-/-) isoforms of WT1 were over-expressed in HEL cells. The (+/-) and (-/-) isoforms reduced Prm1D-directed luciferase activity by 1.5-fold ( $p = 0.0074$ ) and 1.6-fold ( $p = 0.0082$ ), respectively. However, neither the exon 5(+)/ KTS(+) nor exon 5(-)/ KTS(+) isoforms had a significant effect on luciferase activity directed by Prm1D ( $p = 0.4525$  and  $p = 0.8236$ , respectively; **Figure 6H**). None of the four isoforms of WT1 had a significant effect on Prm1I-directed luciferase expression ( $p = 0.8833$ ,  $p = 0.1722$ ,  $p = 0.4767$  and  $p = 0.1437$ ; **Figure 6I**). Collectively, data generated from mutational analysis, EMSAs, ChIP analysis and over-expression studies indicate that -KTS isoforms of WT1 bind to elements within URR2 (from -6848 to -6648) and RR3 (from -6258 to -6123) and act independently to repress Prm1 activity.

## DISCUSSION

In humans, TXA<sub>2</sub> signals through two isoforms of its cognate GPCR, termed TP $\alpha$  and TP $\beta$  [26-28]. Imbalances in the levels of TXA<sub>2</sub> and TP have been implicated in a number of vascular and pulmonary disorders [22,23], as well as in inflammatory renal diseases and in renal failure [24,25]. Since distinct promoters control transcription of TP $\alpha$  and TP $\beta$  [39,40], identification of the factors regulating Prm1 and Prm3 may lead to a greater understanding of the relative extent to which TP $\alpha$  and TP $\beta$  contribute to such vascular and renal pathologies. Previous investigations established that AP1 and Oct-2 are the key factors regulating basal expression of Prm3 in HEL cells [44]. Moreover, specific peroxisome proliferator-activated receptor (PPAR) $\gamma$  ligands [50,51] suppressed Prm3 but had no effect on Prm1-directed luciferase expression. Recently, a study aimed at characterizing Prm1 identified the transcription factors Sp1, Egr1 and NF-E2 as having a central role in regulation of the Prm1 “core” promoter under basal conditions in HEL cells. That study also identified two upstream activator regions (UAR1 and UAR2) and two upstream repressor regions (URR1 and URR2) within Prm1 [41]. Characterization of UAR1, from -7962 to -7717, revealed central roles for GATA-1 and Ets-1 in the upstream activation of Prm1 in HEL cells. However, the *trans*-acting factors regulating UAR2 (from -7717 to -7504) as well as URR1 (from -8500 to -7962) and URR2 (from -6848 to -6648) remained to be identified. In the current study, genetic reporter and 5' deletion analyses revealed a third, previously unidentified repressor region, herein designated RR3 (from -6258 to -6123), within the core Prm1. Hence, the central aim of the current study was to identify the key factors that mediate repression of Prm1 within HEL cells, focusing on the identification of those elements and factors regulating URR1, URR2 and RR3 sequences.

Amongst the transcription factor elements identified by bioinformatic analysis of URR1 [46] were multiple GC-rich elements containing putative WT1 binding sites. WT1 mediates repression of several gene promoters, including the Egr1 promoter [15,52], IGF1 receptor [53], IGFII [54] and PDGF-A [55], as well as mediating auto-repression of its own gene [56]. Additionally, WT1 is thought to be an important factor in the regulation of haematopoiesis. Whilst it is highly expressed in a subset of CD34+ progenitors, it is down-regulated early in the course of differentiation of these cells [43]. Additionally, WT1 mRNA is down-regulated during induction of erythroid and megakaryocytic differentiation of the K562 cell line [57]. Recently, -KTS isoforms of WT1 have been confirmed to act as transcriptional regulators during haematopoiesis, where they activate transcription of the erythropoietin receptor [58]. Moreover, increased expression of WT1 has been reported to occur in acute human leukemias [59].

Considering the function of WT1 as a transcriptional repressor in many cases, as well as its role in haematopoietic differentiation, it was sought to determine whether WT1 can act as a repressor of Prm1 in HEL cells. Mutation of GC-rich elements containing putative overlapping WT1/Egr1/Sp1 binding sites, specifically at -8345, -8281, -8146 and -7831, alleviated repression of Prm1. Despite the indication that these GC elements mediate repression of Prm1, collective mutation of the sites resulted in de-activation of the promoter. As outlined in the model presented in **Figure 7**, these mutational analyses suggest that repressor factor(s) normally bind to neighboring GC elements at -8345, -8281, -8146 and -7831 in a cooperative manner (**Figure 7B**) and it is suggested that mutation of any of these GC elements by SDM disrupts cooperative binding, thereby alleviating repression of Prm1. In the absence of repressor binding to the remaining intact sites, it is proposed that these elements may now have an increased affinity for factors, such as Egr1 or Sp1, that mediate activation, as opposed to WT1-mediated repression of Prm1 (**Figure 7C**). Therefore, it is suggested that disruption of remaining elements results in de-activation of the promoter (**Figure 7D**), leading to the overall decrease in luciferase expression upon generation of Prm1<sup>GC\*(-8345,-8281,-8146,-7831)</sup> from Prm1<sup>GC\*(-8345)</sup> (**Figure 1C**). Evidence for this proposed model of cooperative binding comes from further studies whereby disruption of GC<sup>-7831</sup> in the Prm1B (-7962) sub-fragment, which does not contain any of the other four GC elements, actually decreased the luciferase activity directed by Prm1B (**Figure 1D**). This effect is in contrast to the substantial increase in luciferase expression that occurred upon disruption of the same GC element within Prm1, where the other three GC elements at -8345, -8281 and -8146 were intact (**Figure 1B**). The contrasting outcomes of disrupting the same element in two distinct Prm1 fragments with different 5' sequences highlights the influence of cooperation among specific factors on binding to local promoter elements within Prm1.

EMSA using the Egr1 consensus sequence as a non-labeled competitor suggested that each of the four aforementioned GC elements has a sequence capacity to bind Egr1 and/or WT1 (**Table II**), since both Egr1 and WT1 proteins have been widely reported to bind to the consensus Egr1 sequence [11]. However, none of the complexes that bind to the four GC elements at -8345, -8281, -8146 and -7831 were competed by the WTE sequence, an element reported to be selectively bound by WT1 [12]. Despite this finding, supershift assays strongly indicated that WT1 binds to GC<sup>-8345</sup>, GC<sup>-8281</sup>, GC<sup>-8146</sup> and GC<sup>-7831</sup>, as well as indicating a role for Egr1 binding to these elements *in vitro* (**Table II**). Collectively, these data suggested that the latter four GC elements may have a binding affinity for WT1 isoforms that bind to Egr1 consensus elements but not to the specific WTE sequence. With respect to the latter WTE, while it has been reported to act as an effective competitor for WT1 binding within certain promoters, in the case of the aforementioned GC elements within URR1, or indeed within URR2 and RR3, it is evident that it does not act as an effective competitor. The reason for this apparent discrepancy is unclear but may, for example, reflect differences in WT1 isoform binding specificity and/or other cell-type specific effects. Not surprisingly, EMSAs and supershift assays indicated that GC<sup>-8345</sup> and GC<sup>-8281</sup> also have a sequence

capacity to bind Sp1 (**Table II**), since overlapping sites for Sp1 and Egr1/WT1 are frequently found in promoter sequences due to the similarity in their consensus elements [49]. Interestingly, it has previously been reported that Sp1 binding to the GC element at -8345 mediates increased Prm1 activity in response to phorbol 12-myristate 13-acetate (PMA) in K562 cells [60]. Therefore, it is suggested that this element may play a diverse role in Prm1 regulation. Herein, ChIP analysis indicated that endogenous WT1, and to a lesser extent Egr1, but not Sp1, are bound *in vivo* to the Prm1 region (from -8460 to -7607) of chromatin extracted from HEL cells. Moreover, ectopic over-expression of -KTS isoforms of WT1 led to modest, but significant, decreases in Prm1-directed luciferase expression and in TP $\alpha$  mRNA expression. It is likely that only modest reductions in Prm1-directed luciferase expression were seen due to the already abundant endogenous expression of WT1 in HEL cells, and it is likely that over-expression of -KTS isoforms may have led to greater reductions in Prm1-directed gene expression if the total amount of transfected DNA herein was not limited by the luciferase-based reporter assay itself. Moreover, since the transcriptional effects of WT1 may be dependent on synergistic activity of more than one isoform of the protein, data from over-expression studies may not reflect the true extent of repression of Prm1 activity by WT1 [61]. Collectively, these data indicate that WT1 is the repressor factor that binds to the GC elements at -8345, -8281, -8146 and -7831. It is proposed that WT1 overcomes competition from other factors, such as Egr1 and/or Sp1, by a cooperative method of binding that relies on multiple neighboring GC elements within Prm1 to exert its repression (**Figure 7 & Table II**). Similar models have been reported for the IGFII [54] and PDGF-A [55] gene promoters, where maximal repression by WT1 was dependent upon multiple WT1 binding sites. Other transcription factors, including the glucocorticoid receptor [62] and RUNX1 [63], have also been reported to employ a similar model for regulation of promoters that contain multiple binding elements for the specific transcription factor. It is proposed that formation of homodimers or multimers of these proteins bring together distant chromatin sites by looping to facilitate changes in transcriptional activity.

Bioinformatic analysis [46] of the two remaining repressor regions within Prm1, namely URR2, located between -6848 and -6648, and RR3, located between -6258 and -6123 within the proximal “core” promoter, also revealed putative GC elements in both cases. Mutational analysis of the GC elements in both regions, specifically at -6717 and -6206, indicated that they both mediate repression of Prm1. EMSAs and supershift assays to analyze WT1/Egr1/Sp1 binding to the -6717 and -6206 elements *in vitro* revealed that these elements had a sequence capacity to be bound by Egr1 and WT1 (**Table II**). Moreover, ChIP analysis revealed WT1 as the predominant protein bound to the -6848 to -6648 region *in vivo*, as well as indicating that Egr1 can bind to this region to a much lesser extent. ChIP analysis also revealed a role for WT1 binding to the “core” Prm1. We have previously reported binding of both Sp1 and Egr1 to this “core” region *in vivo*, specifically at overlapping Sp1/Egr1 elements at -6294, -6278, -6022 and -6007 [41]. Data herein suggest that WT1 also binds to the proximal Prm1, and it is likely that

binding of WT1 occurs at the GC<sup>-6206</sup> element in RR3. Moreover, over-expression of the -KTS isoforms, specifically (+ exon 5/ - KTS) and (- exon 5/ - KTS), repressed luciferase activity directed by Prm1D (-6848). In contrast to the cooperative and co-dependent manner in which WT1 binds to GC<sup>-8345</sup>, GC<sup>-8281</sup>, GC<sup>-8146</sup> and GC<sup>-7831</sup>, it seems that WT1 binds to the -6717 and -6206 elements independently to mediate repression of Prm1 (**Figure 7**).

In the current study, it was sought to identify the key *cis*-acting elements and *trans*-acting factors of the three distinct repressor regions, URR1 (from -8500 to -7962), URR2 (from -6848 to -6648) and RR3 (from -6258 to -6123). Herein, it is reported that the repression exerted within each of the three regions in the megakaryoblastic HEL 92.1.7 cell line is largely attributable to the zinc finger transcription factor WT1. Considering the importance of TXA<sub>2</sub> and TP within the kidney (reviewed in [20]), together with this novel role for WT1 as a repressor of Prm1, it is possible that WT1 may play a role in regulation of Prm1 and TP $\alpha$  expression in the renal system. Whilst further studies are required to investigate this hypothesis, it is suggested that abnormal Prm1 activity and TP $\alpha$  expression due to aberrant transcriptional regulation by WT1 may contribute to pathologies of several diseases, including Wilms' tumor, inflammatory renal diseases and renal failure. Moreover, WT1 triggers lineage-specific differentiation of human primary haematopoietic progenitor cells [64] and WT1 mRNA is down-regulated during induction of erythroid and megakaryocytic differentiation of the K562 cell line [57]. Therefore, down-regulation of WT1 may act to increase Prm1 activity, thereby increasing TP $\alpha$  expression, during megakaryocytic differentiation of HEL cells. Interestingly, WT1 is a downstream target of nitric oxide (NO) signaling and WT1 represses matrix metalloproteinase-9 through a NO-sensitive pathway [65]. More specifically, it is thought that NO promotes translocation of WT1 from the nucleus to the cytoplasm, thereby interfering with its transcriptional activity [65]. Notably, it has been previously established that TP $\alpha$  also undergoes NO-mediated desensitization involving direct NO/cGMP-dependent protein kinase G phosphorylation at Ser<sup>331</sup> within its unique C-tail domain [35]. Collectively, those latter studies and findings herein suggest that NO may regulate TP $\alpha$  expression and signaling by a complex mechanism involving its regulation of Prm1-directed TP $\alpha$  transcription by WT1 and its regulation of TP $\alpha$ -mediated intracellular signaling by NO/cGMP-dependent protein kinase G. NF-E2, GATA-1 and Ets-1 were previously identified as key regulators of Prm1 during megakaryocytic differentiation [41]. Collectively, the data from this and previous studies suggest that combinatorial gene regulation by WT1, GATA-1, Ets-1 and NF-E2 may be critical for regulation of TP $\alpha$  expression during different stages of megakaryocytic differentiation.

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**TABLES**

**Table I:** Consensus sequences for Egr1, WTE and Sp1, as well as sequences of GC elements within Prm1.

Element*	Sequence**
Egr1 consensus [12]	5' GcG <u>GGGG</u> C <u>G</u> 3'
WTE [12]	5' gtgcG <u>TGGG</u> aGtagaat 3'
Sp1 consensus [66]	5' g <u>GGGC</u> GGGgc 3'
Prm1 <sup>-8345</sup> (-)	5' ctggG <u>TGGGGGCGGG</u> gGcagctt 3'
Prm1 <sup>-8281</sup> (-)	5' tccgGcG <u>GGGG</u> CCGGgcag 3'
Prm1 <sup>-8146</sup> (+)	5' ggc <u>GGGGGGTGGGGGGCGGGGGGCGGG</u> ccaa 3'
Prm1 <sup>-7831</sup> (-)	5' agatGaG <u>GGGGC</u> Agtga 3'
Prm1 <sup>-6717</sup> (-)	5' ccagG <u>GGTGGGGTGGG</u> aGgacaga 3'
Prm1 <sup>-6206</sup> (-)	5' acggG <u>TGGG</u> gGccgctg 3'

\*The + and – designation indicates that elements are found on the sense or antisense strands of Prm1, respectively. \*\*Base pairs underlined denote the core sequences of the elements, while base pairs in capital letters are in positions that exhibit a high conservation profile [46].

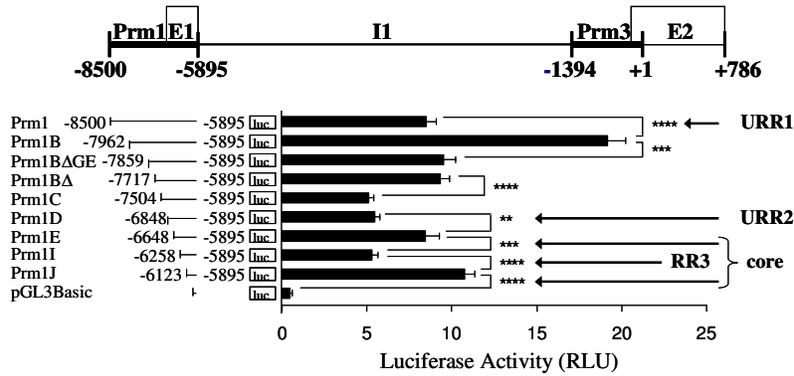
**Table II: Factors that may compete for binding to specific GC elements within Prm1**

<b>Element</b>	<b>Factors that bind to GC elements within Prm1*</b>
GC <sup>-8345</sup>	Sp1, Egr1 and WT1
GC <sup>-8281</sup>	Sp1, Egr1 and WT1
GC <sup>-8146</sup>	Egr1 and WT1
GC <sup>-7831</sup>	Egr1 and WT1
GC <sup>-6717</sup>	Egr1 and WT1
GC <sup>-6206</sup>	Egr1 and WT1

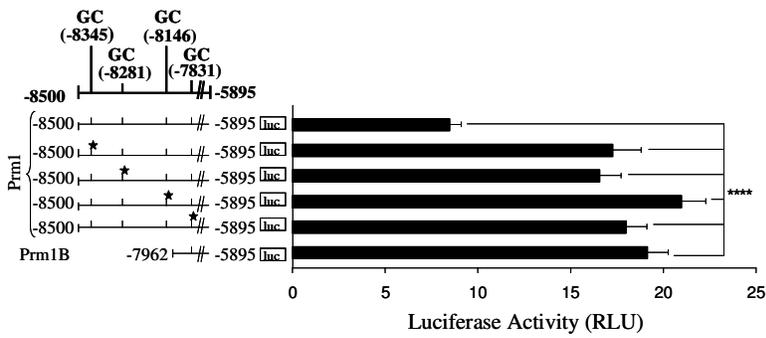
\*Determined from EMSA, antibody supershift assays and ChIPs.

**FIGURES**

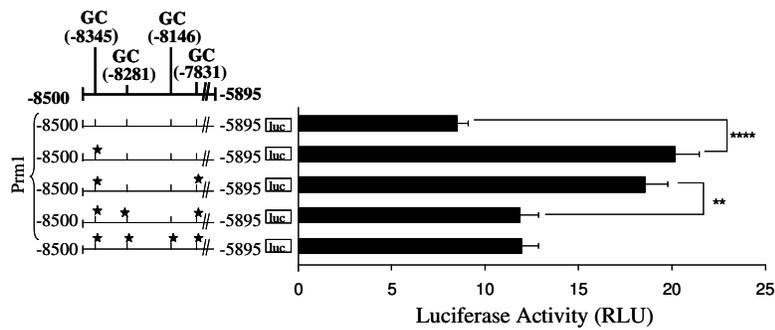
**(A)**



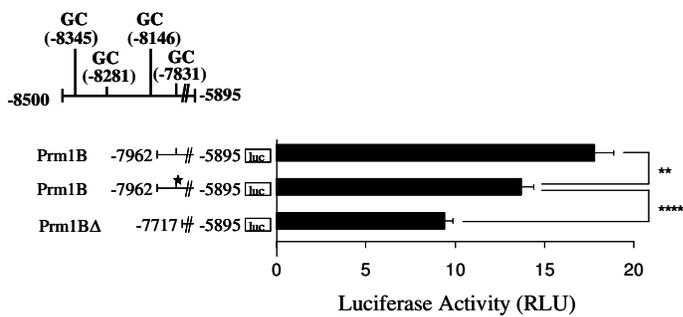
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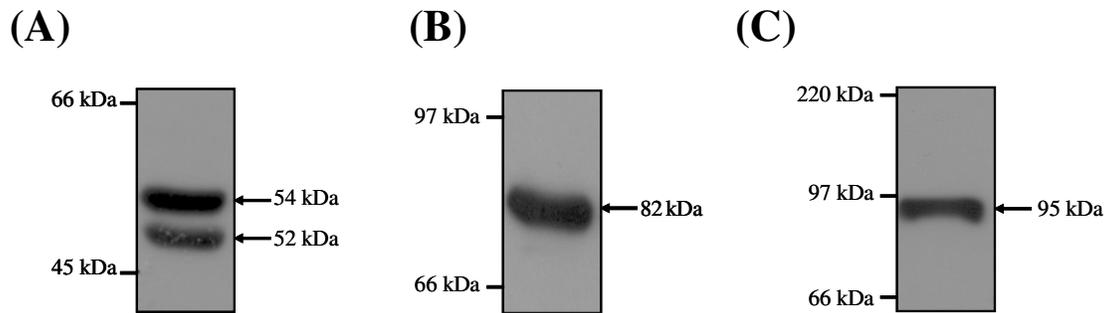
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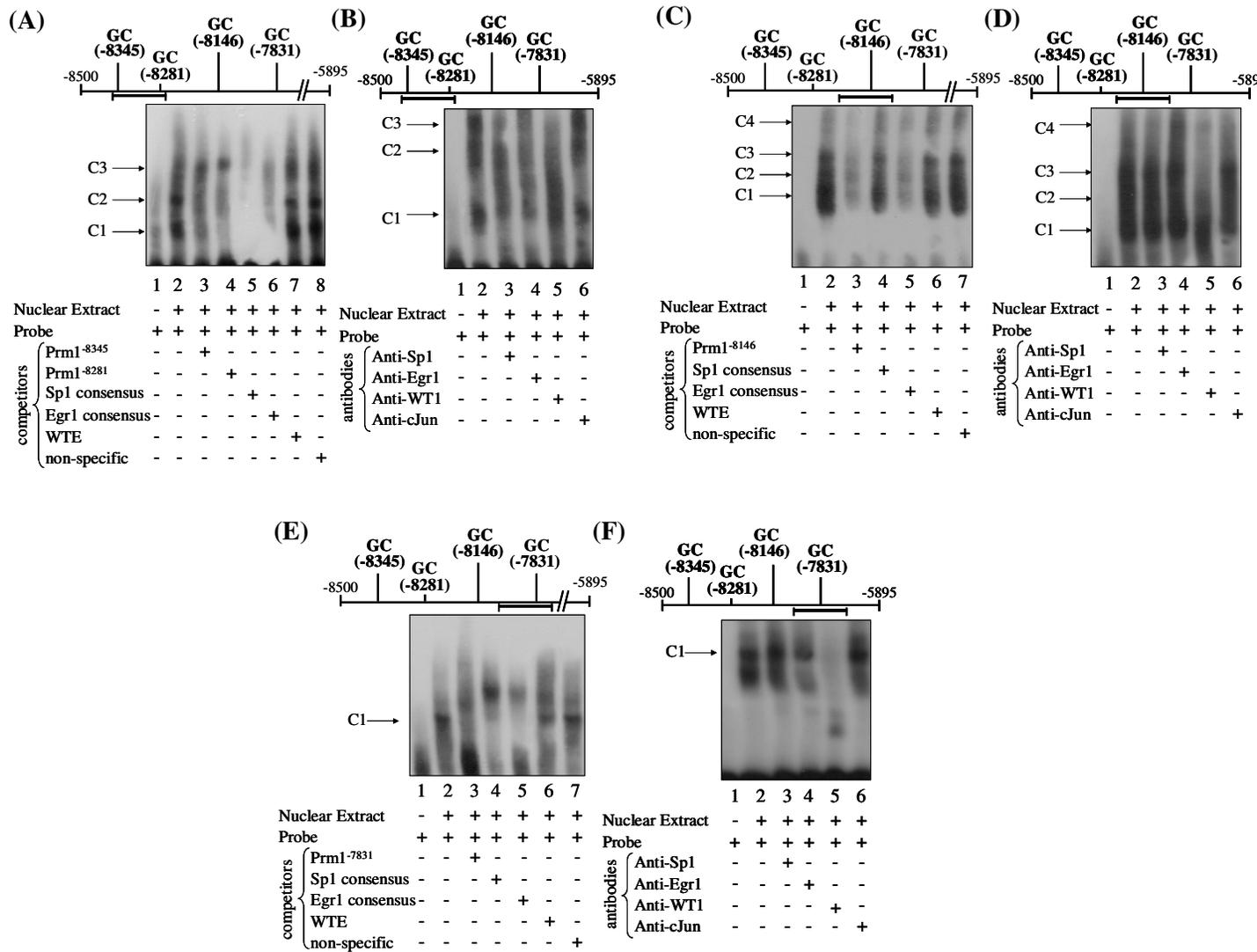
**(D)**



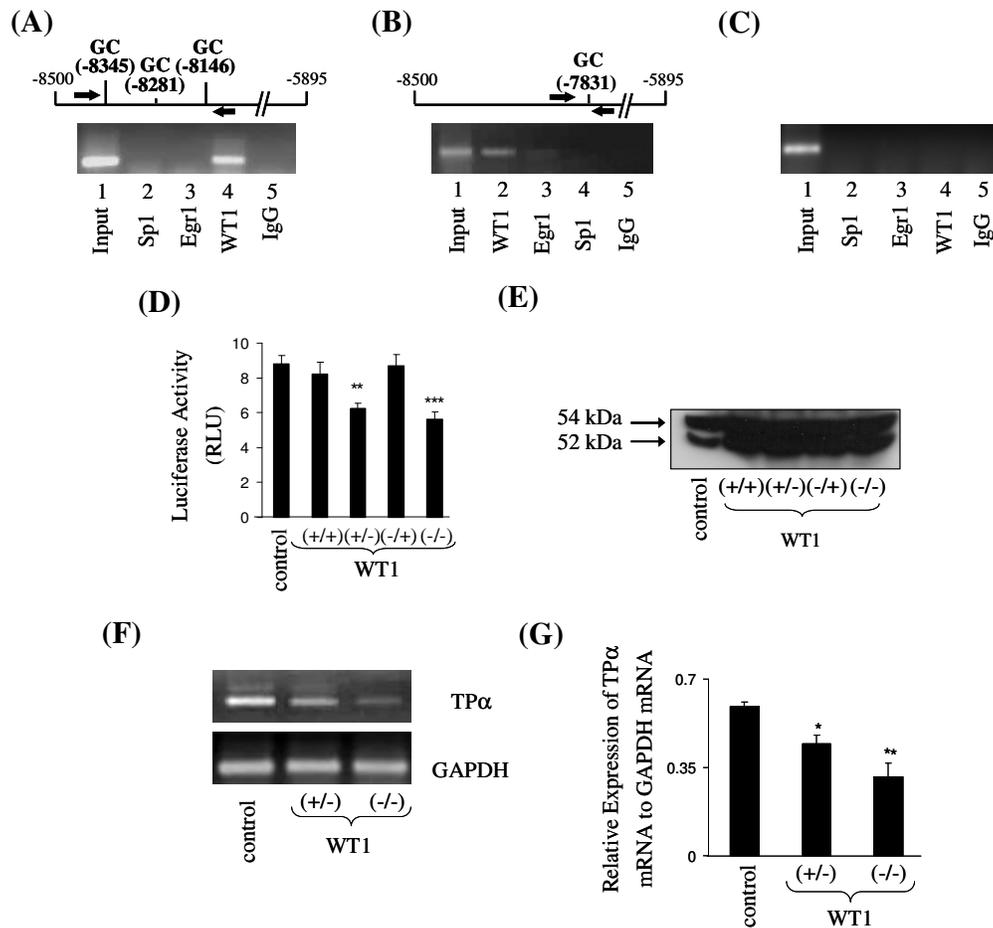
**Figure 1: Effect of 5' deletions on Prm1-directed gene expression and identification of GC elements within the -8500 to -7962 region of Prm1. Panel A:** Schematic of the human TP gene spanning nucleotides -8500 to +786 encoding Prm1 (-8500 to -5895), Prm3, exon (E)1, intron (I)1 and E2, where nucleotide +1 represents the translational start site (ATG). pGL3Basic plasmids (2 µg) encoding Prm1 (-8500 to -5895) or its 5' deletion fragments Prm1B (-7962), Prm1BΔGata/Ets (-7859), Prm1BΔ (-7717), Prm1C (-7504), Prm1D (-6848), Prm1E (-6648), Prm1I (-6258), Prm1J (-6123) or, as a control, pGL3Basic were co-transfected with pRL-TK into HEL 92.1.7 cells. Mean firefly relative to renilla luciferase activity was expressed in arbitrary relative luciferase units (RLU ± SEM; n = 6). **Panels B, C and D:** GC elements containing putative overlapping WT1/Egr1/Sp1 binding sites within Prm1, where the 5' nucleotide is indicated and the star symbol signifies mutated elements. pGL3Basic plasmids (2 µg) encoding: **(B)** Prm1, Prm1<sup>GC\*(-8345)</sup>, Prm1<sup>GC\*(-8281)</sup>, Prm1<sup>GC\*(-8146)</sup>, Prm1<sup>GC\*(-7831)</sup> and Prm1B; **(C)** Prm1, Prm1<sup>GC\*(-8345)</sup>, Prm1<sup>GC\*(-8345,-7831)</sup>, Prm1<sup>GC\*(-8345,-8281,-7831)</sup> and Prm1<sup>GC\*(-8345,-8281,-8146,-7831)</sup>; or **(D)** Prm1B, Prm1B<sup>GC\*(-7831)</sup> and Prm1BΔ were co-transfected with pRL-TK into HEL cells. Luciferase activity was expressed as mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 8).



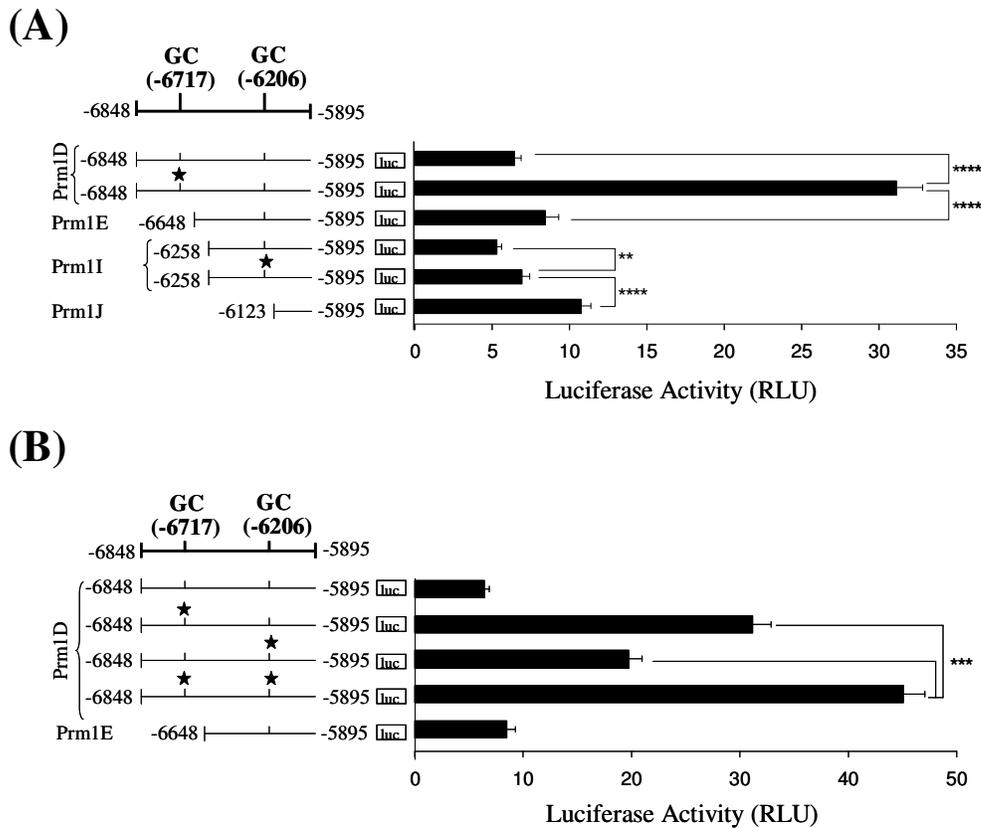
**Figure 2: Expression of WT1, Sp1 and Egr1 proteins in HEL 92.1.7 cells.** Immunoblot analysis of WT1 (**Panel A**), Egr1 (**Panel B**) and Sp1 (**Panel C**) expression in HEL cells (60  $\mu$ g whole cell protein per lane). The positions of the molecular size markers (kDa) are indicated to the left, while the sizes of WT1, Sp1 and Egr1 are indicated to the right of the panels, respectively.



**Figure 3: Nuclear factor binding to 5' GC elements within Prm1 *in vitro*.** EMSAs (**Panels A, C, E**) and supershift assays (**Panels B, D, F**) using nuclear extract from HEL cells and biotin-labeled double-stranded probes encoding GC<sup>-8345</sup>, GC<sup>-8281</sup> (**Panels A & B**), GC<sup>-8146</sup> (**Panel C & D**) and GC<sup>-7831</sup> (**Panel E & F**). In each case, the horizontal bar indicates the relative position of the probe within Prm1. **Panels A, C & E:** Nuclear extract was pre-incubated with the vehicle (-) or with excess non-labeled competitor oligonucleotides (+) prior to addition of the relevant probe. **Panels B, D & F:** Nuclear extract was pre-incubated with *anti-Sp1*, *anti-Egr1*, *anti-WT1* or, as a negative control, *anti-cJun* prior to addition of the relevant probe. Images are representative of three independent experiments.

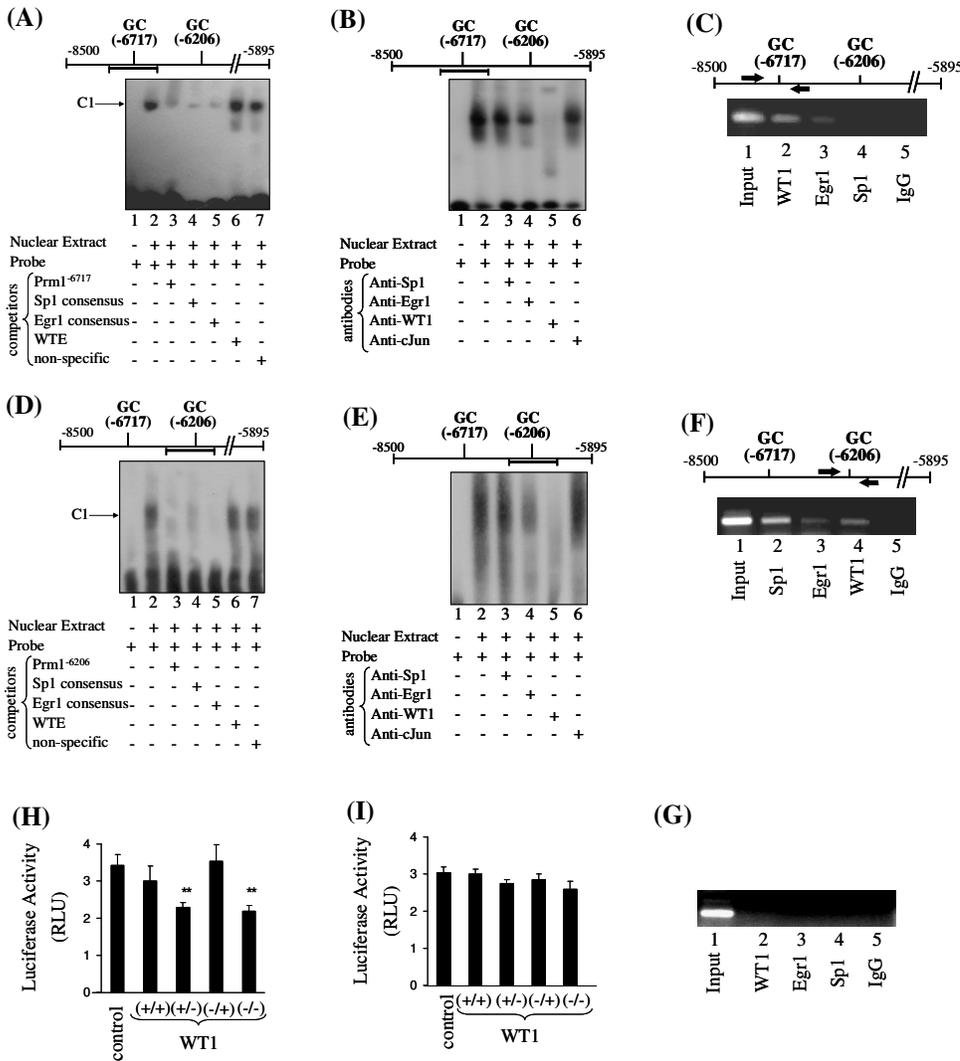


**Figure 4: ChIP analysis of WT1, Sp1 and/or Egr1 binding to the 5' region of Prm1 and effect of over-expression of WT1 on Prm1-directed luciferase expression. Panels A - C:** ChIP analysis of WT1, Sp1 and/or Egr1 binding to Prm1 in HEL 92.1.7 cells. Schematic of Prm1 and primers (arrows) used in the PCR to amplify the -8460 to -8006 (**Panel A**) or the -7978 to -7607 (**Panel B**) regions of Prm1 using either input chromatin or chromatin extracted from *anti*-WT1, *anti*-Egr1, *anti*-Sp1 or, as a control, normal rabbit IgG immunoprecipitates. Primers to detect a region of Prm3 (-1081 to -695; **Panel C**) from input chromatin, *anti*-Sp1, *anti*-Egr1, *anti*-WT1 or normal rabbit IgG precipitates were used as a negative control. Images are representative of three independent experiments. **Panels D & E:** HEL cells were transiently co-transfected with either 0.5  $\mu$ g of pcDNA3 (control) or 0.5  $\mu$ g of recombinant pcDNA3 encoding (+/+), (+/-), (-/+) or (-/-) isoforms of WT1, along with pGL3b:Prm1 (1.5  $\mu$ g) plus pRL-TK (200 ng). Cells were either assayed 48 h post-transfection for mean luciferase activity (RLU  $\pm$  SEM; n = 9) or subjected to western blot analysis (40  $\mu$ g whole cell protein per lane). The size of WT1 isoforms are indicated to the left of the panel. **Panels F & G:** RT-PCR analysis using primers to amplify TP $\alpha$  and GAPDH mRNA sequences from total RNA isolated from HEL cells following transfection with 0.5  $\mu$ g of pcDNA3 (control) or 0.5  $\mu$ g of recombinant pcDNA3 encoding either the (+/-) or (-/-) isoform of WT1. Densitometric analysis (**Panel G**) was carried out to assess the relative expression of TP $\alpha$  mRNA to GAPDH mRNA in HEL cells transfected with pcDNA3 (control) or recombinant pcDNA3 encoding either the (+/-) or (-/-) isoform of WT1.



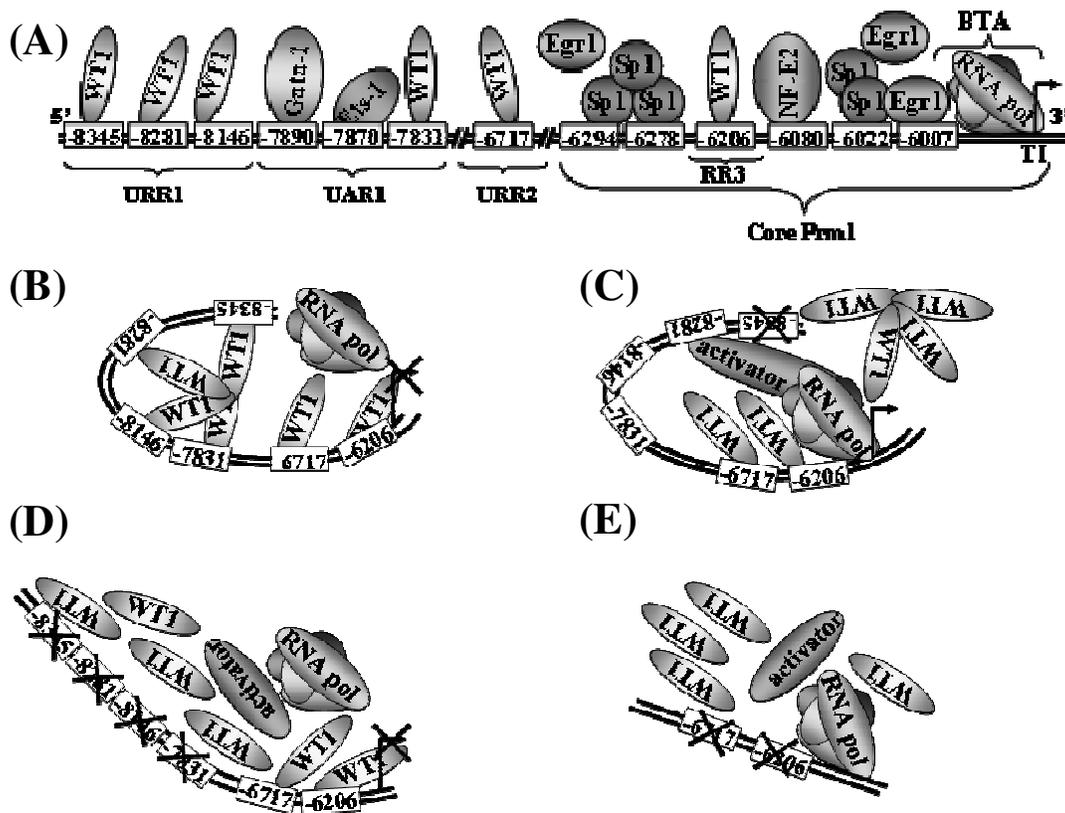
**Figure 5: Identification of GC elements within Prm1 regions from -6848 to -6648 and -6258 to -6123. Panels A and B:** Schematic of the Prm1 region from -6848 to -5895 and the relative positions of GC elements containing putative overlapping WT1/Egr1/Sp1 binding sites, where the 5' nucleotide is indicated and the star symbol signifies mutated elements. Recombinant pGL3Basic encoding: **(A)** Prm1D (-6848), Prm1D<sup>GC\*(-6717)</sup>, Prm1E (-6648), Prm1I (-6258), Prm1I<sup>GC\*(-6206)</sup> or Prm1J (-6123); or **(B)** Prm1D, Prm1D<sup>GC\*(-6717)</sup>, Prm1D<sup>GC\*(-6206)</sup>, Prm1D<sup>GC\*(-6717,-6206)</sup> or Prm1E were co-transfected with pRL-TK into HEL 92.1.7 cells. Luciferase activity was expressed as mean firefly relative to renilla luciferase activity (RLU ± SEM; n = 9).

**Figure 6: Nuclear factor binding to GC<sup>-6717</sup> and GC<sup>-6206</sup> elements within Prm1 *in vitro* and *in vivo* and effect of over-expression of WT1 on Prm1D and Prm1I-directed luciferase expression.**



**Panels A, B, D, E:** EMSAs (**Panels A & D**) and supershift assays (**Panels B & E**) using nuclear extract from HEL cells and a biotinylated double-stranded probe encoding (**Panels A & B**) the Prm1 GC<sup>-6717</sup> element and (**Panels D & E**) the Prm1 GC<sup>-6206</sup> element, where the location of the specific probe within Prm1 is indicated by the horizontal bar. **Panels A & D:** Nuclear extract was pre-incubated with vehicle (-) or excess non-labeled competitor oligonucleotides (+) before addition of the specific probe. One complex, C1, was observed in each case. **Panels B, & E:** Nuclear extract was pre-incubated with *anti-Sp1*, *anti-Egr1*, *anti-WT1* or, as a negative control, *anti-cJun* prior to addition of the relevant probe. The images are representative of three

independent experiments. **Panels C, F & G:** ChIP analysis and schematic of Prm1 and primers (arrows) used in the PCR to amplify the -6848 to -6437 (**Panel C**) and the -6368 to -5895 (**Panel F**) regions of Prm1 from input chromatin or from chromatin extracted from *anti-WT1*, *anti-Egr1*, *anti-Sp1* or, as a control, normal rabbit IgG immunoprecipitates, as indicated. Primers to detect a region of Prm3 (-1081 to -695; **Panel G**) from input chromatin, *anti-WT1*, *anti-Egr1*, *anti-Sp1* or normal rabbit IgG precipitates were used as a negative control. The images are representative of three independent experiments. **Panels H & I:** HEL cells were transiently co-transfected with either 0.5 µg of pcDNA3 (control) or 0.5 µg of recombinant pcDNA3 encoding (+/+), (+/-), (-/+) or (-/-) isoforms of WT1, along with pRL-TK (200 ng) and 1.5 µg of either pGL3b:Prm1D (**Panel H**) or pGL3b:Prm1I (**Panel I**). Cells were either assayed 48 h post-transfection for mean luciferase activity (RLU ± SEM; n = 9). The asterisks (\*) indicate that over-expression of WT1 significantly reduced Prm1D-directed luciferase expression in HEL cells, where \*\* indicates *p* < 0.01.



**Figure 7: Proposed model for WT1-mediated repression of Prm1 in HEL 92.1.7 cells.** **Panel A:** Schematic representation of the relative positions of functional binding elements within Prm1 (not drawn to scale), as well as binding of the basal transcription apparatus (BTA) to the transcription initiation (TI) site. Overlapping Sp1/Egr1 elements at -6294, -6278, -6022 and -6007, as well as an NF-E2 element at -6080, located within the “core” proximal promoter, direct efficient basal activity of Prm1 in megakaryoblastic HEL cells. Additionally, GATA-1 and Ets-1 bind elements at -7890 and -7870, respectively, within UAR1 to increase Prm1 activity in HEL cells [41]. The data herein indicate that WT1 binds to GC elements within URR1, specifically at -8345, -8281 and -8146, as well as elements at -7831 within UAR1, -6717 within URR2 and -6206 within RR3, to repress Prm1 activity. **Panels B, C, D and E:** Proposed model for WT1- mediated repression of Prm1 in HEL cells. It is suggested that WT1 overcomes competition from other factors, such as Egr1 and Sp1, by binding cooperatively to neighboring GC elements at -8345, -8281, -8146 and -7831 and independently to GC elements at -6717 and -6206 to mediate repression of Prm1-directed transcription by the basal transcription apparatus in HEL cells (**Panel B**). Mutation of any of the upstream GC elements at -8345, -8281, -8146 and -7831 by SDM interferes with cooperation among WT1 proteins binding to these elements, thereby inhibiting WT1 binding and alleviating repression of Prm1. In the absence of repressor binding to the remaining intact sites, these elements may now have a higher affinity for activating factors (**Panel C**). Disruption of remaining upstream GC elements blocks the binding of activators and results in de-activation of the promoter (**Panel D**). Furthermore, mutation of GC elements at -6717 and -6206 in Prm1D (-6848) alleviates repression of Prm1 (**Panel E**).