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Published in:
Biochemical and Biophysical Research Communications

DOI:
10.1016/j.bbrc.2016.11.114

2017

Document Version:
Peer reviewed version (aka post-print)

Anti-apoptotic quinolinate phosphoribosyltransferase (*QPRT*) is a target gene of Wilms' tumor gene 1 (WT1) protein in leukemic cells

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Key-words: WT1; QPRT; promoter; transcription; acute myeloid leukemia; NAD+.

Word count: 4,098.

ABSTRACT

Wilms' tumor gene 1 (*WT1*) is a zinc finger transcription factor that has been implicated as an oncogene in leukemia and several other malignancies. When investigating possible gene expression network partners of *WT1* in a large acute myeloid leukemia (AML) patient cohort, one of the genes with the highest correlation to *WT1* was quinolinate phosphoribosyltransferase (*QPRT*), a key enzyme in the *de novo* nicotinamide adenine dinucleotide (NAD+) synthesis pathway. To investigate the possible relationship between *WT1* and *QPRT*, we overexpressed *WT1* in hematopoietic progenitor cells and cell lines, resulting in an increase of *QPRT* expression. WT1 knock-down gave a corresponding decrease in *QPRT* gene and protein expression. Chromatin-immunoprecipitation revealed WT1 binding to a conserved site in the first intron of the *QPRT* gene. Upon overexpression in leukemic K562 cells, QPRT conferred partial resistance to the anti-leukemic drug imatinib, indicating possible anti-apoptotic functions, consistent with previous reports on glioma cells. Interestingly, the rescue effect of QPRT overexpression was not correlated to increased NAD+ levels, suggesting NAD+ independent mechanisms. We conclude that *QPRT*, encoding a protein with anti-apoptotic properties, is a novel and direct target gene of WT1 in leukemic cells.

INTRODUCTION

The WT1 zinc-finger transcription factor has critical functions during development (1), but has also a role in normal and malignant hematopoiesis. A small subset of hematopoietic progenitor cells express WT1 (2-4) and progenitors lacking WT1 are less competitive during hematopoietic reconstitution *in vivo* (5), suggesting positive effects on proliferation and/or survival. Inducible deletion of *WT1 in vivo* in adult mice leads to rapid failure of erythrocyte formation (6). In hematopoietic malignancy, WT1 is overexpressed in a majority of acute myeloid leukemia (AML) and acute lymphoid leukemia (ALL), correlated to poor clinical outcome (7-10). WT1 interferes with differentiation of leukemic cell lines (11-13) and in mice, WT1 cooperates with the leukemia fusion protein AML1-ETO *(RUNX1/RUNX1T1)* to rapidly induce leukemia (14). Also various types of solid cancers show over-expression of WT1 (15). Thus, clinical and experimental data indicate the importance of *WT1* as an oncogene in several forms of malignancy. The molecular mechanisms for WT1 are, however, incompletely understood.

Quinolinate phosphoribosyltransferase (QPRT) is a critical enzyme for *de novo* synthesis of nicotinamide adenine dinucleotide (NAD+) from tryptophan. NAD+ is an essential co-factor in the glycolysis, citric acid cycle and mitochondrial respiratory chain. However, NAD+ is also critical for certain signaling pathways in the cell (16), including poly(ADP-ribose) polymerase 1 (PARP1) -mediated ribosylation in DNA-repair (17, 18). NAD+ is also a critical cofactor for NAD-dependent protein deacetylases (sirtuins) in antioxidant pathways and DNA-repair mechanisms (19, 20). Both deacetylation catalyzed by sirtuins and ADP-ribosylation are NAD+ consuming processes (19, 20, 21), making cells dependent on NAD+ synthesis through the salvage pathway and through the *de novo* pathway, in which QPRT plays a key role. In line with this, QPRT was reported to increase resistance of glioma brain cancer cells to radiation and oxidative stress, by activating an NAD+ salvage pathway (22).

QPRT in leukemia is an area not previously studied. QPRT activity has been reported in normal platelets and erythrocytes (23) and in monocytes, but not in lymphocytes (24), suggesting a role for QPRT within several lineages of normal hematopoiesis. In this work we report *QPRT* as a novel and direct target gene of WT1 in leukemic cells.

MATERIALS AND METHODS

Gene expression partial correlations

We investigated a compendium of publically available microarray results from the tumor cells of 3,844 acute myeloid leukemia (AML) patients (GSE accession numbers GSE6891, GSE7757, GSE10358, GSE12417, GSE12662, GSE13159, GSE14468, GSE15061, GSE15434, GSE17855, GSE21261, and GSE22056) using the Ultranet tool (25) with the lambda 0.1 setting to find possible gene network partners of *WT1*. Ultranet computes partial correlation, minimizing indirect correlation exerted through other variables in the correlation matrix.

Cells and cell culture

Normal $CD34^+$ progenitor cells were enriched with the $CD34^+$ Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) from cord blood after full-term deliveries, donated after informed consent. $CD34^+$ cells were maintained in StemSpan SFEM medium supplemented with 20% FCS and StemSpan CC100 (Stemcell Technologies, Vancouver, Canada). K562 and U937 cells were from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured in RPMI-1640 with 10% FCS.

Vectors

Retroviral vectors for the *WT1*+17AA/-KTS isoform and the *WT1*+17AA(delZ) construct were as described (26). *QPRT* OmicsLink expression vector was purchased from GeneCopoeia (Rockville, MD, USA). WT1 shRNA lentiviral vector (27) was obtained through Addgene (https://www.addgene.org). Scrambled control lentiviral shRNA was purchased from Sigma-Aldrich (St Louis, Missouri, USA).

Transfection and stable expression of QPRT

K562 cells were electroporated at 260 V, 960 μF in a Biorad GenePulser II electroporator (Biorad, Hercules, California, USA) using the OmicsLink-*QPRT* expression vector or empty pcDNA3 vector as control. Cultures were allowed to expand in the continuous presence of 1.5 mg/ml G418.

Viral transduction and RNA extraction

Retroviral particles were produced as previously described (26). Lentiviral particles were produced at the core facility Vector Unit at the Faculty of Medicine, Lund University. Transduction was performed as described (26). Retrovirally transduced cells, expressing GFP from an IRES, were sorted, while lentivirally transduced cells were selected for by adding 1 μg/ml puromycin to the culture 48 hours post-transduction, followed by incubation for 72 hours. Total RNA was extracted from the cells with the RNeasy Mini Kit (QIAGEN Gmbh, Hilden, Germany).

Western blot

Antibodies used were: mouse anti-QPRT (clone 5D11; Abnova, Taipei City, Taiwan), rabbit anti-WT1 (C-19; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-WT1 (F-6; Santa Cruz), mouse anti-GAPDH (clone 7-B; Santa Cruz), and rabbit anti-histone H3 (ab 5103; Abcam, Cambridge, UK) antibodies.

Reverse transcription and qPCR

Reverse transcription PCR was performed using the High Capacity cDNA kit (ThermoFisher Scientific) according to manufacturer's instructions. Quantitative PCR (qPCR) was done using TaqMan Gene Expression Assays, *GAPDH* (Hs99999905_m1), *WT1* (Hs00240913_m1) and *QPRT* (Hs00204757_m1), all purchased from Applied Biosystems (Foster City, CA, USA) as assays-on-demand, on a StepOne Plus Real-Time PCR system (Life Technologies, Carlsbad, CA, USA).

Chromatin immunoprecipitation (ChIP)-PCR

ChIP-PCR was performed as described (28). Briefly, ChIP was made using the ChIP Assay Kit from Millipore (Billerica, MA, USA) with two distinct WT1 antibodies, WT1 (C-19 clone) or WT1 (F-6 clone), and with anti-HA (hemagglutinin) as negative control (all antibodies from Santa Cruz Biotechnology). PCR was performed with primers flanking an evolutionally conserved potential WT1 site in the *QPRT* first intron (forward primer: 5'-ATGGACTTCTGCTCTCGCTGGTAG-3', reverse primer:

5'-TCATTCCTTTGTTGCCACCG-3'). As a negative control, PCR of the *GAPDH* gene was performed as described (28).

NAD+/NADH assay

NAD+/NADH levels were determined using the NAD/NADH-Glo Assay (Promega, Madison, WI, USA) according to the manufacturers' instructions. Luciferase levels were quantified with a Glomax 20/20 Luminometer (Promega) using default settings.

Statistics

All calculations of significance were made using Student's t-test.

RESULTS

Expression of *WT1* and *QPRT* is highly correlated in AML

High expression of *WT1* is found in leukemic blasts from most AML patients (29). With the aim to identify putative novel target genes for WT1 in leukemia, we took advantage of the Ultranet tool (25) to identify partial correlations in gene expression between *WT1* and other genes in a large cohort of 3,844 AML patients. Partial correlation is a measure of the correlation between two variables, after the influence of other observed variables has been minimized (25). We found that *WIT-1* (Wilms' Tumor Upstream Neighbour 1) gene expression was most highly correlated to that of *WT1* (partial correlation 0.244) which can be explained by shared promoter area (30). Besides *WIT-1*, *QPRT* was the gene whose transcription showed the highest correlation with that of *WT1* (partial correlation 0.087). As for *QPRT*, no gene expression correlated better with its gene expression than that of *WT1*. This finding led us to investigate the functional relationship between *WT1* and *QPRT* expression.

WT1 overexpression in WT1 negative backgrounds increases *QPRT* levels

To evaluate the correlation between *WT1* and *QPRT*, we overexpressed WT1(+17AA/-KTS isoform) in $CD34^+$ progenitor cells. This isoform of WT1 was chosen since the 17AA domain can affect transcription and since the -KTS forms are known as the most efficient DNA-binders. Only a very small subset of $CD34^+$ cells has been found to express WT1 (2-4), making $CD34^+$

cells an essentially WT1-negative background. As a result of WT1 overexpression, the *QPRT* mRNA expression was doubled (Fig 1A). A truncated form of WT1, (WT1delZ), lacking all zinc fingers (26) had no effect, indicating a mechanism requiring intact DNA binding of WT1. To further investigate the WT1-QPRT relationship, we overexpressed WT1 in the U937 leukemia cell line, which is also WT1-negative. In this leukemic background, *QPRT* levels were increased more than two-fold as a result of WT1 expression (data not shown).

WT1 knock-down in K562 cells decreases *QPRT* expression

The K562 leukemic cell line shows, in contrast to U937, high levels of endogenous WT1. To find out whether *QPRT* levels in K562 cells are dependent on WT1, we knocked down WT1 through shRNA interference. Upon knock-down of WT1 (Fig 1B), *QPRT* mRNA levels decreased by 50% (Fig 2C). Comparable knock-downs were seen on the protein level (Fig 1D, E). Thus, QPRT expression in this WT1-positive environment was found to be partly dependent on WT1 expression.

WT1 binds to a conserved site on the *QPRT* promoter

To further investigate the transcriptional control by WT1 on *QPRT* expression, we searched for potential WT1 binding sites in the *QPRT* gene. First, we used the ENCODE Genome Browser (ENCODE Project Consortium 2012; Hg 19; https://genome.ucsc.edu/ENCODE/ (31)) to define a region likely to contain binding sites for transcriptional regulators, using the existing tracks from other transcription factors [Transcription Factor ChIP-seq (161 factors) from ENCODE with Factorbook Motifs (KDM5B)] (37), and the H3K4me3 [K562 H3K4me3 Histone Modifications by ChIP-Seq Signal from ENCODE/SYDH; data from the labs of Peggy Farnham (USC/Norris Cancer Center; previously at UC Davis) and Michael Snyder at Stanford University)] and H3K27ac [H3K27Ac Mark (Often Found Near Active Regulatory Elements) on 7 cell lines from ENCODE; data from the Bernstein Lab at the Broad Institute] histone modification tracks. With the aid of this information, we defined a 2.2 kb-region (the area chr16:29690000-29692200 (Hg 19)), -440 to +1760 bp relative to the transcription start site (TSS) (NM_014298.3) as the putative functional *QPRT* promoter. Next, we used the matrix based Matinspector tool from Genomatix (https://www.genomatix.de (38)) to predict binding sites in our defined region. This investigation yielded four potential WT1 binding sites in the

beginning of the first intron, of which two were overlapping, resulting in three distinct WT1 binding sites (#1, #2/3, and #4) (Fig 2).

Making further use of the ENCODE Genome Browser, we investigated the evolutionary conservation of the three sites using the Vertebrate Multiz Alignment & Conservation (100 Species) track with the PhastCons setting, that shows basewise conservation based on 100 vertebrate species (39). Site #1 showed no conservation, whereas #2/3 showed conservation of part of the motif, including the Matinspector defined core sequence TGGG. Site #4 was conserved to a lesser degree than #2/3, and the conserved bases did not include the core sequence (Supplementary Fig S1). From this evaluation, we selected site #2/3 for further investigation.

To investigate whether WT1 binds to this region *in vivo*, we performed chromatin immunoprecipitation (ChIP) from K562 cells. We designed primers flanking the selected sites (Fig 2), and performed PCR on chromatin immunoprecipitated with two different WT1 antibodies, and with anti-HA as control. ChIP with either WT1 antibody resulted in enrichment of the WT1 binding site, while an irrelevant DNA region (*GAPDH*) was not enriched (Fig 3). Although the exact binding site cannot be defined from this ChIP, we conclude that WT1 binds to the *QPRT* gene *in vivo*, most likely at the conserved #2/3 site.

Overexpression of QPRT in K562 cells increases resistance to imatinib

QPRT has been reported to have anti-apoptotic properties (22). K562 cells are dependent on the BCR-ABL1 oncogene, and thus sensitive to the clinically used tyrosine kinase inhibitor imatinib (40). After overexpression of QPRT in K562 cells (2.6-fold as determined by Western Blot, data not shown), we incubated cells with imatinib. Proliferation and viability of non-treated control cells and QPRT-overexpressing cells did not significantly differ from each other (Fig 4A, B). However, imatinib-treated QPRT-overexpressing cells had a significantly higher rate of proliferation (Fig 4A), as well as a significantly increased ratio of living to dead cells, as compared to imatinib-treated control cells (Fig 4B), indicating an increased resistance to imatinib treatment, due to high QPRT expression levels.

To investigate whether the QPRT-mediated resistance to imatinib was correlated to increased NAD+ levels, NAD+/NADH measurements were made. As shown in Fig 4C, D, both QPRT-overexpressing and control cells reacted with reduction of NAD levels. However, there was no indication of any rescuing effect of QPRT overexpression on the NAD reduction. As a positive

control for reduction of NAD levels, K562 wild-type cells were treated with the NAMPT enzyme inhibitor FK866 (41).

DISCUSSION

The transcription factor WT1 is an oncogene in leukemia. Here, we show that expression of *WT1* and *QPRT* in acute myeloid leukemia samples is highly correlated, that over-expression of WT1 induces expression of QPRT, that knock down of WT1 decreases QPRT levels, and that WT1 binds to a conserved site in the *QPRT* promoter. We conclude that *QPRT* is a target gene of WT1 in leukemia.

The major known function of QPRT is in the *de novo* pathway of NAD+ synthesis. In a study on QPRT in glioma brain cancer cells, QPRT was found to increase resistance to radiation and oxidative stress (22). QPRT expression increases with a higher degree of malignancy in glioblastoma and levels of QPRT correlated to an unfavorable prognosis (22). Interestingly, also WT1 expression correlates to malignancy (42) and glioma cell lines revealed an increased sensitivity of tumor cells to radiation treatment as a result of WT1 knock-down (43). However, with present data we can only speculate on whether the WT1 effect on radiation resistance was mediated via QPRT.

In our study, we find that QPRT overexpression in K562 cells increases resistance to imatinib. Earlier reports on QPRT in monocytic cells and neurons have indicated QPRT as a rate-limiting enzyme for NAD-synthesis (24, 44). Perhaps surprisingly therefore, we found no indication that the resistance to imatinib correlated to NAD+ levels. The lack of effect on NAD+ levels is, however, consistent with a recent report with heterozygous and homozygous QPRT knock-out mice, indicating that QPRT may not be rate-limiting for NAD-synthesis (45). Quite apart from its enzymatic effect on NAD+ production, QPRT may have additional pro-survival mechanisms. QPRT protein has been found to bind and sequester active caspase 3, without being itself a substrate for degradation. The interaction decreased the caspase 3-activity both in the absence and presence of apoptotic inducers, and siRNA against *QPRT* made the cells more prone to spontaneous cell death (46).

In summary, we demonstrate that *QPRT* is a novel and direct target gene of WT1, positively regulated by WT1 binding to the *QPRT* promoter. This is the first reported regulatory mechanism for QPRT, a protein with documented effects on cell death, both through sequestration of active caspase 3, and through *de novo* synthesis of NAD+, the latter found to

be an important mechanism in the prognosis of glioma patients. In accordance with these reports of QPRT acting as an oncogenic protein, we found that K562 cells with an increased level of QPRT were less vulnerable to imatinib treatment, suggesting an oncogenic potential in a leukemic setting.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors report no potential conflicts of interest.

ACKNOWLEDGEMENTS:

This work was supported by research grants from the Swedish Cancer Foundation and Children Cancer Foundation, the Gunnar Nilssons Cancer Foundation, the Fysiographic Society of Lund, the Swedish Foundation for Strategic Research (ICA08-0057), the Marianne and Marcus Wallenberg Foundation (2010.0112), and the Knut and Alice Wallenberg Foundation (2012.0193). The funding bodies had no role in designing, carrying out or reporting the study.

FIGURE LEGENDS

Fig 1. Overexpression of *WT1* induces increase of *QPRT* and knock-down of *WT1* induces reduction of *QPRT*. $CD34^+$ progenitor cells were retrovirally transduced with a vector encoding full length WT1+/- isoform (WT1), WT1 lacking zinc-fingers (WT1delZ), or with empty vector (control). Levels of *QPRT* mRNA were analyzed 48 hours after sorting **(A)**. K562 cells were transduced with a lentiviral vector expressing an shRNA directed against WT1 or against GFP as control. After transduction and selection, RNA and protein were analyzed. Shown are levels of WT1 mRNA **(B)**, QPRT mRNA **(C)**, WT1 protein **(D)** and QPRT protein (**E**). Mean values, bars ±S.E.M., n=3. Stars indicate statistical significance (**: $p<0.01$; ***: $p<0.001$)**.** Representative immunoblots are shown.

Fig 2. WT1 binds to the QPRT promoter. Chromatin immunoprecipitations (ChIP) using two distinct antibodies against WT1: F6, Santa Cruz (**A**), or C-19, Santa Cruz (**B**). ChIP with anti-HA antibody as negative control. PCR amplification of precipitated DNA was done with primers specific for the QPRT-promoter or with primers for the GAPDH promoter as negative control. n=2 for each antibody.

Fig 3. The QPRT-promoter. Solid arrow indicates transcription start site (TSS (NM_014298.3)) with exon 1 in bold italics. Boxes indicate four putative WT1-binding elements (#2 and 3 overlapping). Dashed arrows indicate the primers used for ChIP experiments. Start and end numbering are relative to the TSS.

Fig 4. QPRT overexpression in K562 cells reduces sensitivity to imatinib, but does not rescue cells from NAD-depletion. K562 cells stably overexpressing QPRT protein, and K562 transfected with empty pcDNA3 vector as control, were treated for 96 hours with 3 μM imatinib. Cell number, viability and NAD-levels were determined at indicated time points. Shown is number of viable cells **(A)**, viable fraction **(B)**, and NAD-levels at 24 hours **(C)** or 48 hours **(D)**. For positive control, cells were treated with NAMPT enzyme inhibitor FK866 for 48 h. Stars indicate statistical significance (*: $p<0.05$; **: $p<0.01$, n=3), n.s: not significant.

Fig 1.

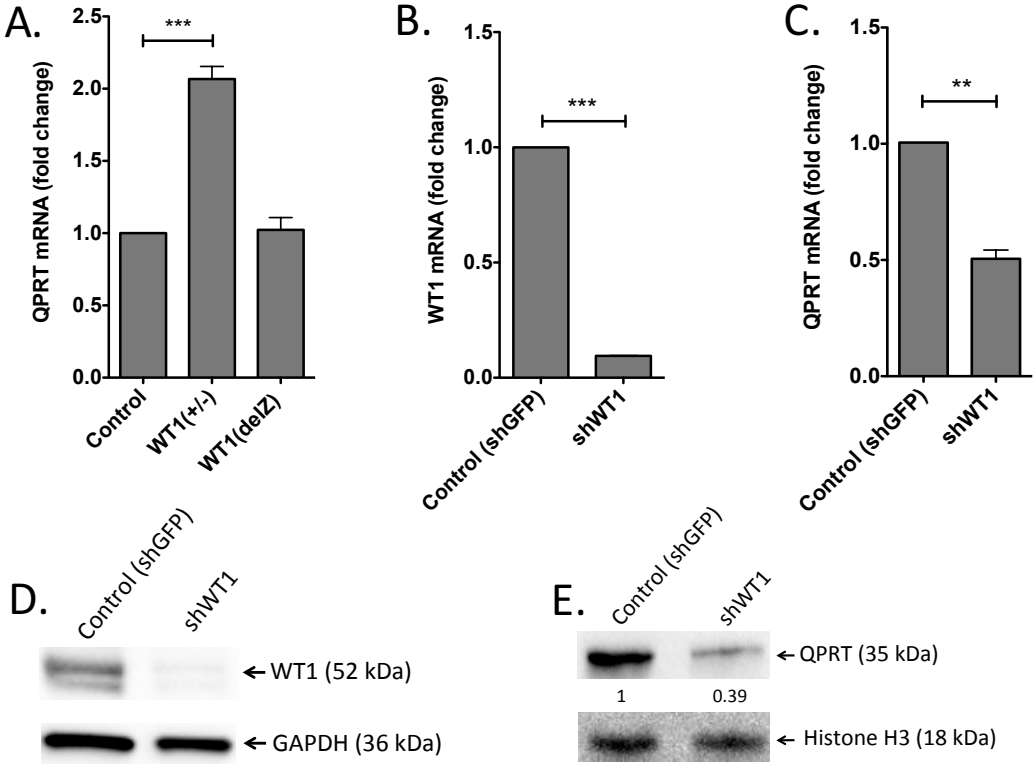

A.
QPRT mRNA (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
***
Control
WT1(+/-)
WT1(delZ)
B.
WT1 mRNA (fold change)
1.5
1.0
0.5
0.0
***
Control (shGFP)
shWT1
C.
QPRT mRNA (fold change)
1.5
1.0
0.5
0.0
**
Control (shGFP)
shWT1
D.
Control (shGFP)
shWT1
WT1 (52 kDa)
GAPDH (36 kDa)
E.
Control (shGFP)
shWT1
QPRT (35 kDa)
1
0.39
Histone H3 (18 kDa)

Fig 2.

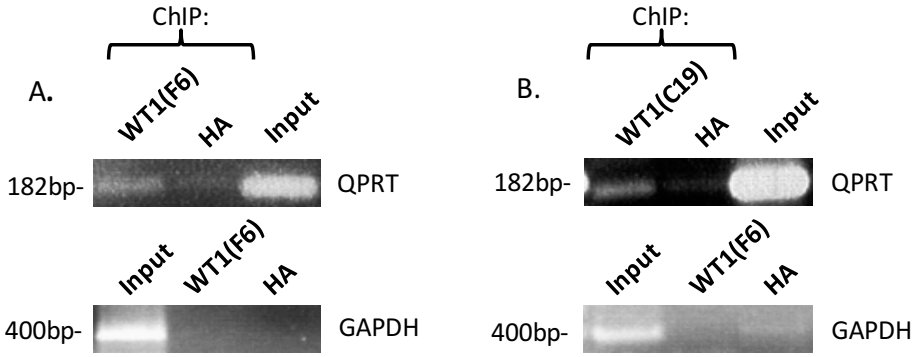
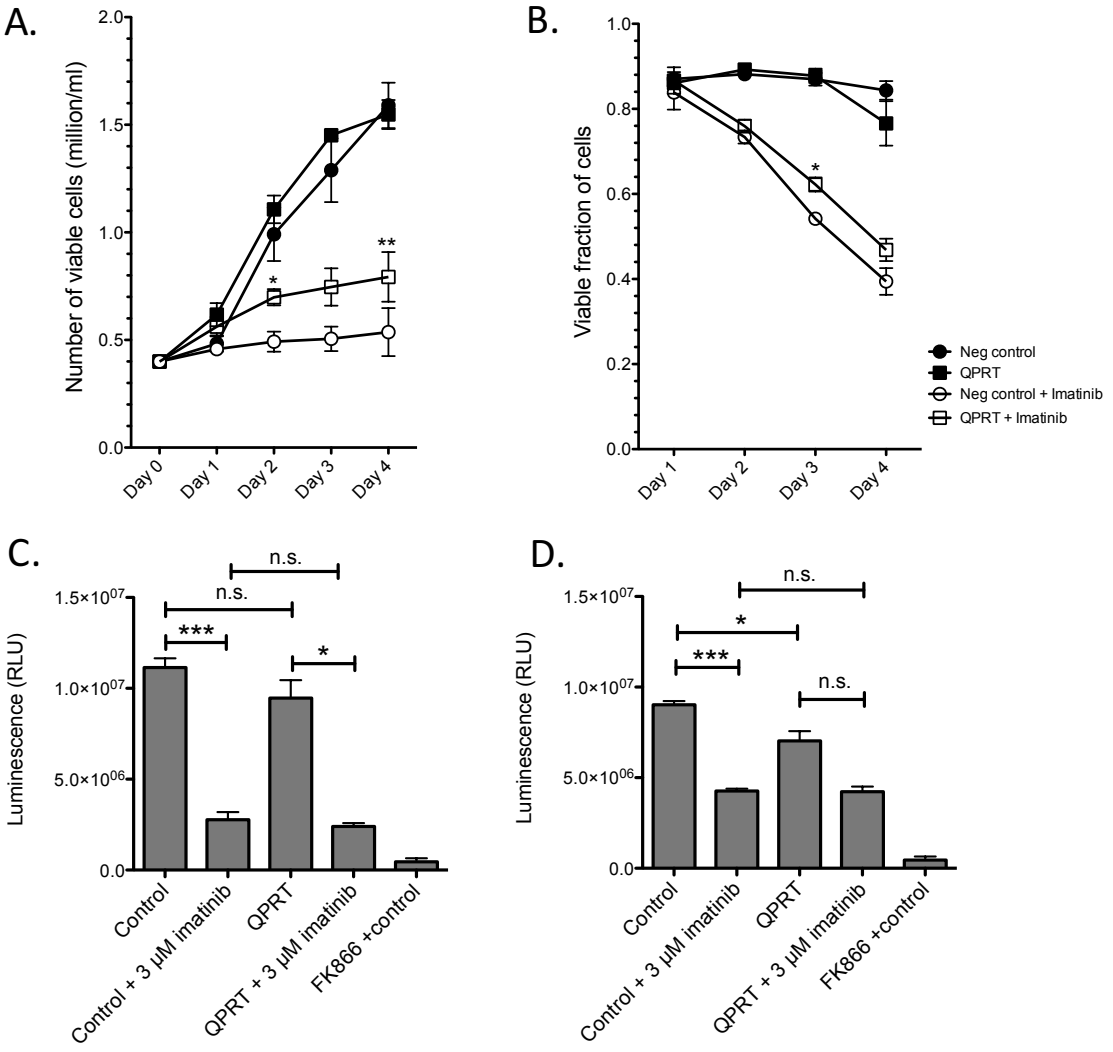

ChIP:
A.
WT1(F6)
HA
Input
182bp-
QPRT
Input
WT1(F6)
HA
400bp-
GAPDH
ChIP:
B.
WT1(C19)
HA
Input
182bp-
QPRT
Input
WT1(F6)
HA
400bp-
GAPDH

Fig 3.


-174 GCCTCGGGCTCCTTCACTGGCTGGGGCAGGGCTTCTGAGTTCCCCATCAGAGCCCCTCCTCTGACTTTCAGGGCCTGCTGAG
GAATCTGCAGGCCTGGCAGAGGACAGGAGGGAGGCTTGGGGAGCCTGGGAAGGGCCGGCTGACAGCTTGATGGGCCCTCCCTCCCTCCACA
GTCCCACCCCCAGCCTGGGGCCTCTGGGAGCCTTGGTCCTGAGCAGCCAACACACCAGCCCAGACAGCTGCAAGTCACCATGGACGCTGAA
GGTAAAGGGACACTCTCTCTGCCATGTCCCTGCACCCATCCCCCCACTGCCTACCCTGCCCCCACCCTGGCTTGTCTCAGCCCCTTGTTG
CCTCCTCTGGAGTCATCTCTCCTCTGGTCCCGCCATCGACTCCCTTACCTACCCCATGTCCCTGCAGCCTTGCTCAACCCCCAGAGGCCCC
TTCTCACTCTTCTTTCTCCTGTGTTGAGTCCCCAAGGAGCTCTGGGGAAGGAAGGGGCTTAAGCCCTAGAAAAGGGCAGGGTGGCCTGGAG
GAGGTGGCGTCTCCTGGGGGCAGTGGGGGCCGGAAGTGGGAGGCTGGTTGGGGGAGCAGCATGGACTTCTGCTCTCGCTGGTAGCTCGGGA
GAAGCAGAGACAAAGTCCTGTCCCTTCTGCAGCTGGGTGGGGGAGGGGGCTTGAGTCCCGTGATTCCCTGCCTGGAGGGAGCTGTATGCAA
ACTGTTGGGGTGATCAGTCCCAACCCCCAACCATGAAGTCCGGTGGCAACAAAGGAATGAGAAAAGACAAGTTAAGATTTAAAGCAGCCCC
AGGGGGCCAGTTGCTAAAGTGAAGGCTGCAGAGCCCTGGTTGCCTGATAATTTATTGAGTACAATCTTTTGATCTAAGAAGCAGATGGTAC
GGGGTGAAACGGTGAAGGTGGGGGTGTGCGCGTCATTTGAAAGATGTATAGCCGTGGTGGTTTATGTGAATTTCCTTTAAGCTCAAAGCAT
ATGTCTAACTACTAAGATAATCTTTTTTTTTTTTTTTTTGAGACGGAGTCTCGCTTTCCCCCAGGCTGGAGTGCA +894
#1
#2,3
#4

Fig 4.


A.
Number of viable cells (million/ml)
2.0
1.5
1.0
0.5
0.0
Day 0
Day 1
Day 2
Day 3
Day 4
*
**
B.
Viable fraction of cells
1.0
0.8
0.6
0.4
0.2
0.0
Day 1
Day 2
Day 3
Day 4
*
Neg control
QPRT
Neg control + Imatinib
QPRT + Imatinib
C.
Luminescence (RLU)
1.5×10^07
1.0×10^07
5.0×10^06
0.0
n.s.
n.s.
***
*
Control
Control + 3 µM imatinib
QPRT
QPRT + 3 µM imatinib
FK866 +control
D.
Luminescence (RLU)
1.5×10^07
1.0×10^07
5.0×10^06
0.0
n.s.
*
***
n.s.
Control
Control + 3 µM imatinib
QPRT
QPRT + 3 µM imatinib
FK866 +control

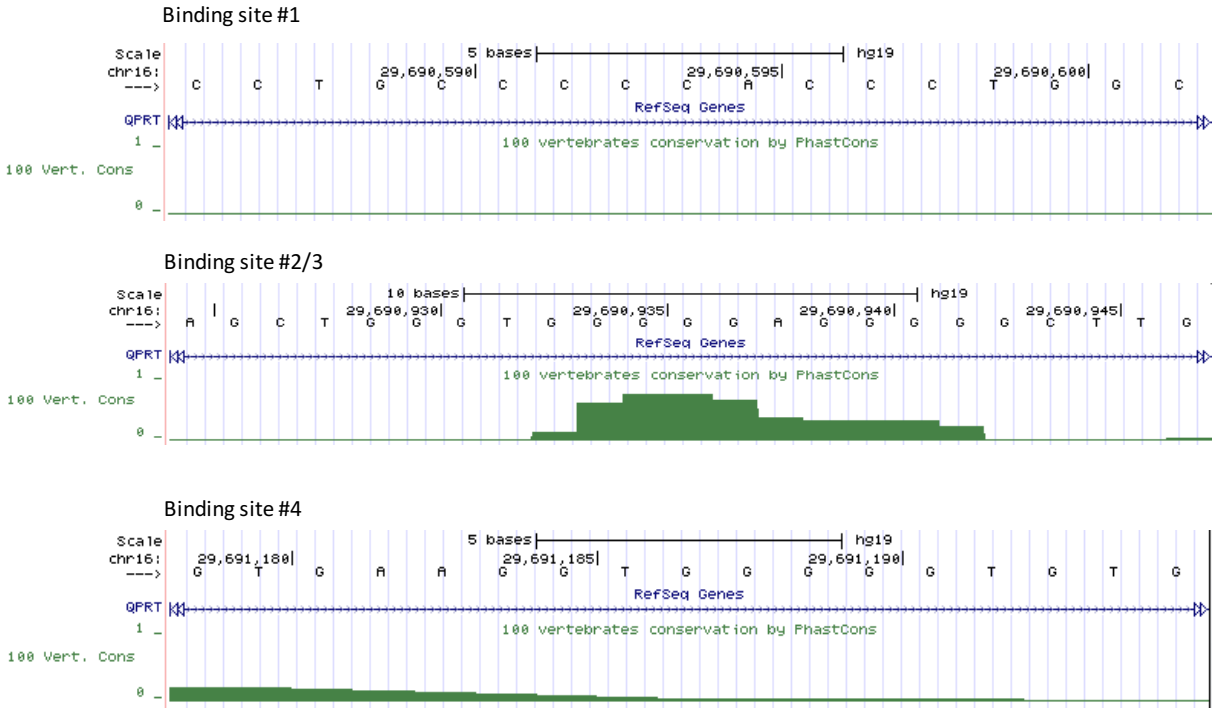


Fig S1. Evolutionary conservation of putative WT1 binding sites in the functional QPRT promoter. Analysis made using the Vertebrate Multiz Alignment & Conservation (100 Species) track with the PhastCons setting (1) in the ENCODE Genome Browser (ENCODE Project Consortium 2012; Hg 19; https://genome.ucsc.edu/ENCODE/; (2)).

1. J. Felsenstein, G.A. Churchill. A Hidden Markov Model approach to variation among sites in rate of evolution. Mol Biol Evol., 13 (1996) 93-104.
2. W.J. Kent, C.W. Sugnet, T.S. Furey, K.M. Roskin, T.H. Pringle, A.M. Zahler, D. Haussler. The human genome browser at UCSC. Genome Res. 12 (2002) 996-1006.