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Distinct global binding patterns of the Wilms tumor gene 1 (WT1) -KTS and +KTS isoforms in leukemic cells

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ABSTRACT

The zinc finger transcription factor Wilms tumor gene 1 (*WT1*) acts as an oncogene in acute myeloid leukemia. A naturally occurring alternative splice event between zinc fingers three and four, removing or retaining three amino acids (±KTS), is believed to change the DNA binding affinity of WT1, although there are conflicting data regarding the binding affinity and motifs of the different isoforms. Increased expression of the WT1 -KTS isoform at the expense of the WT1 +KTS isoform is associated with poor prognosis in acute myeloid leukemia. We determined the genome-wide binding pattern of WT1 -KTS and WT1 +KTS in leukemic K562 cells by chromatin immunoprecipitation and deep sequencing. We discovered that the WT1 -KTS isoform predominantly binds close to transcription start sites and to enhancers, in a similar fashion to other transcription factors, whereas WT1 +KTS binding is enriched within gene bodies. We observed a significant overlap between WT1 -KTS and WT1 +KTS target genes, despite the binding sites being distinct. Motif discovery revealed distinct binding motifs for the isoforms, some of which have been previously reported as WT1 binding sites. Additional analyses showed that both WT1 -KTS and WT1 +KTS target genes are more likely to be transcribed than non-targets, and are involved in cell proliferation, cell death, and development. Our study provides evidence that WT1 -KTS and WT1 +KTS share target genes yet still bind distinct locations, indicating isoform-specific regulation in transcription of genes related to cell proliferation and differentiation, consistent with the involvement of *WT1* in acute myeloid leukemia.

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Introduction

The Wilms tumor gene 1 (*WT1*) was discovered as a tumor suppressor in the pediatric kidney malignancy Wilms tumor,[1] and later identified as an oncogene in many solid tumors and in leukemia.[2] *WT1* mutations occur in 10% of cases of acute myeloid leukemia (AML), and carry a poor prognosis.[3-6] Wild-type WT1 is expressed in acute leukemia cells,[2] and high expression correlates with a poor outcome in AML.[7-9] The ubiquitous overexpression in leukemia has spurred the development of vaccines aimed at raising a T-cell response against WT1 for therapeutic purposes.[10] In mice, overexpression of *WT1* induces myeloproliferation, while overexpression of *WT1* plus the *RUNX1/RUNX1T1* (*AML1-ETO*) fusion gene induces AML.[11]

Four major WT1 isoforms have been identified. These result from two alternative splicing events, leading to inclusion/exclusion of 17 amino acids (17AA) from exon five, and inclusion/exclusion of the three amino acids KTS between zinc fingers three and four. The KTS insertion is thought to hamper DNA binding, as it might displace zinc finger four and thereby decrease DNA binding affinity.[12] A perturbed ratio between isoforms may contribute to pathological function; increased expression of

WT1 +17AA/ -KTS relative to WT1 +17AA/+KTS correlates with resistance to therapy in AML.[13] Additionally, high expression of WT1 +17AA/-KTS has been linked to a higher risk of relapse.[14] Despite these observations, the functional differences between WT1 isoforms remain unknown. Several DNA binding motifs have been identified for WT1 (listed in *Online Supplementary Table S1*). No motif has been conclusively shown to bind only WT1 -KTS or only WT1 +KTS protein, and there are conflicting results regarding the binding affinity and the ability to influence target gene transcription of the different isoforms.

In the present study, we carried out the first analysis of the global DNA binding patterns of WT1 -KTS and WT1 +KTS isoforms in leukemic cells. Our analysis shows that the two isoforms bind to many shared target genes and that binding of either isoform is correlated with active transcription, yet the binding sites of the isoforms are distinct.

Methods

Chromatin immunoprecipitation and streptavidin capture

For establishment of K562 clones expressing biotinylated WT1 -KTS or WT1 +KTS, we used a previously published protocol[15] with some modifications as described in detail in the *Online Supplementary Methods*. Briefly, cells were transfected with *E. coli* biotin protein ligase (BirA) enzyme and the respective isoform of WT1. Two clones were then chosen for subsequent chromatin immunoprecipitation (ChIP) analysis, aiming for expression levels of biotinylated WT1 that were comparable with those of wild-type WT1. ChIP and streptavidin capture were performed with nuclear extracts from the K562 clones expressing BirA and tagged WT1 +17AA/-KTS or WT1 +17AA/+KTS, and from the K562 clones expressing BirA only as background control. Further methodological details are given in the *Online Supplementary Methods*.

Library preparation and sequencing

From immunoprecipitated DNA, libraries were prepared using the ThruPlex DNA-seq kit (Rubicon Genomics, Ann Arbor, Michigan, USA). Library purification removed the fragments above 700 bp, and below 200 bp. The sequencing was performed on a NextSeq 500 sequencer (Illumina, San Diego, CA, USA) using Illumina's NextSeq 500/550 High Output Kit v2 (75 cycles). Some samples were sequenced on an Illumina platform at the Science for Life Laboratory core facility in Uppsala, Sweden. Further methodological details are given in the *Online Supplementary Methods*. Data for all samples are available in the Gene Expression Omnibus (GEO) under accession number GSE81009.

Data analysis

Data were analyzed as described by Sanden *et al.*[16] with some modifications and extensions as detailed in the *Online Supplementary Methods*.

Results

WT1 -KTS peaks are enriched close to transcription start sites, and WT1 +KTS peaks inside gene bodies

To characterize the genome-wide binding of WT1 -KTS and WT1 +KTS we performed streptavidin capture of the biotinylated proteins with attached chromatin followed by deep sequencing (ChIP-Seq). Two independent experiments yielded 2,009 overlapping peaks of WT1 binding for WT1 -KTS, and three independent experiments yielded 21,831 overlapping binding peaks for WT1 +KTS. According to ENCODE-recommendations for background control in ChIP-Seq experiments with tagged proteins,[17] the clone containing BirA only and no tagged protein, from which both clones expressing tagged WT1 were made, was sequenced after streptavidin capture to the same depth as the other samples. Three independent experiments yielded only five recurrent peaks, none of which matched peaks from our WT1 samples. From this background analysis we conclude that background binding is negligible.

Analysis of the distance from the center of each WT1 -KTS peak to the closest transcription start site (TSS) revealed that the WT1 -KTS binding sites are primarily located around the TSS of genes, with as many as 26% of all peaks being located within 500 bp, and 42% within 2,000 bp, up- or down-stream of a TSS (Figure 1A).

The preferential localization of WT1 -KTS peaks close to the TSS was mirrored by the relationship of the WT1 -KTS peaks to distinct gene elements. This analysis showed that the peaks are found primarily within or immediately adjacent to genes, with a strong overrepresentation of binding to the 2 kb just upstream of the TSS, to exons (particularly first exons), and to first introns and junctions between exons and introns (Figure 1C). Fewer intergenic peaks were found than could be expected from a comparison with a randomly generated set of 30,000 control genomic locations (Figure 1C). Taken together, our data demonstrate preferential binding of WT1 -KTS close to TSS, consistent with a transcription factor role for WT1 -KTS. All differences, as compared to the randomized genomic positions, with the exception of that for the 2-30 kb upstream of the TSS and the 2-5 kb downstream of the transcription end, were statistically significant ($P<0.001$).

For WT1 +KTS, a minor enrichment around the TSS could also be seen (Figure 1C), but it was not as prominent as for WT1 -KTS. WT1 +KTS peaks were enriched, as compared to the 30,000 random locations, within the exons of target genes, the intron-exon junctions, and to a lesser extent in introns, up to 30 kb upstream of the TSS, and up to 5 kb downstream of the transcription end (Figure 1c). All differences, as compared to the random positions, were statistically significant ($P<0.001$).

To investigate whether or not WT1 binds enhancer regions, we utilized a set of 43,011 enhancer regions defined by the FANTOM consortium after cap analysis of gene expression (CAGE) analyses.[18] Upon comparison of our WT1 peaks with the defined enhancer regions, we found that WT1 -KTS peaks clearly co-localize with enhancers (7.3% of the WT1 -KTS peaks coincided with these enhancer regions), but that only 0.7% of the WT1 +KTS peaks did so (Figure 1D). The small WT1 +KTS enrichment was, however, statistically significant. We conclude that WT1 -KTS binds enhancer regions to a much larger extent than does WT1 +KTS.

WT1 -KTS peaks show more similarity than WT1 +KTS peaks to other transcription factor tracks

We compared our WT1 peaks to tracks of peaks from other factors in the ENCODE database.[19,20] The similarity

of our peaks and the ENCODE track under comparison is based on nucleotide bases present in both peak tracks and as such describes a strong similarity between the binding patterns.[16] Table 1 lists the 20 tracks that were most alike the WT1 -KTS and WT1 +KTS peaks. All tracks used in the comparison were derived from the K562 cell line and are available in the ENCODE database (*Online Supplementary Table S5A* provides a complete list of all ENCODE tracks showing similarity to those of WT1 –KTS, while *Online Supplementary Table S5B* lists those for WT1 +KTS).

For WT1 -KTS, the most similar track was the one of EGR-1, which is not surprising considering that the two proteins can bind the same DNA sequences.[21] The 20 tracks that most resembled the WT1 -KTS peaks included other transcription factors, consistent with our observation that WT1 -KTS preferentially binds close to TSS and in enhancer regions.

Interestingly, no track in the ENCODE database showed a high degree of similarity to the WT1 +KTS peaks. There were, however, several tracks for which the similarity, though small, was significant. The transcription factor track most resembling that of WT1 +KTS, was Zinc Finger Protein 263 (ZNF263), which, like WT1 +KTS, was found to bind to a large extent within its target genes rather than to promoter regions.[22]

Methylation and acetylation of lysine residues on histones can contribute to activation or repression of gene transcription. In some cases, the modifications appear to be secondary to transcription rather than contributing to it. Thus, the histone modifications can be used in ChIP-Seq experiments as markers not only for chromatin states, but also for transcriptional activity. Trimethylation of histone H3 at lysine 27 (H3K27me3) is a well-established mark of transcriptional repression and heterochromatin that is regulated by the Polycomb complex 2.[23] On the top 20 list of the tracks that most resemble WT1 +KTS, five tracks are for this repressive histone mark. There are two more histone marks on the WT1 +KTS list, one track for trimethylation of histone H3 on

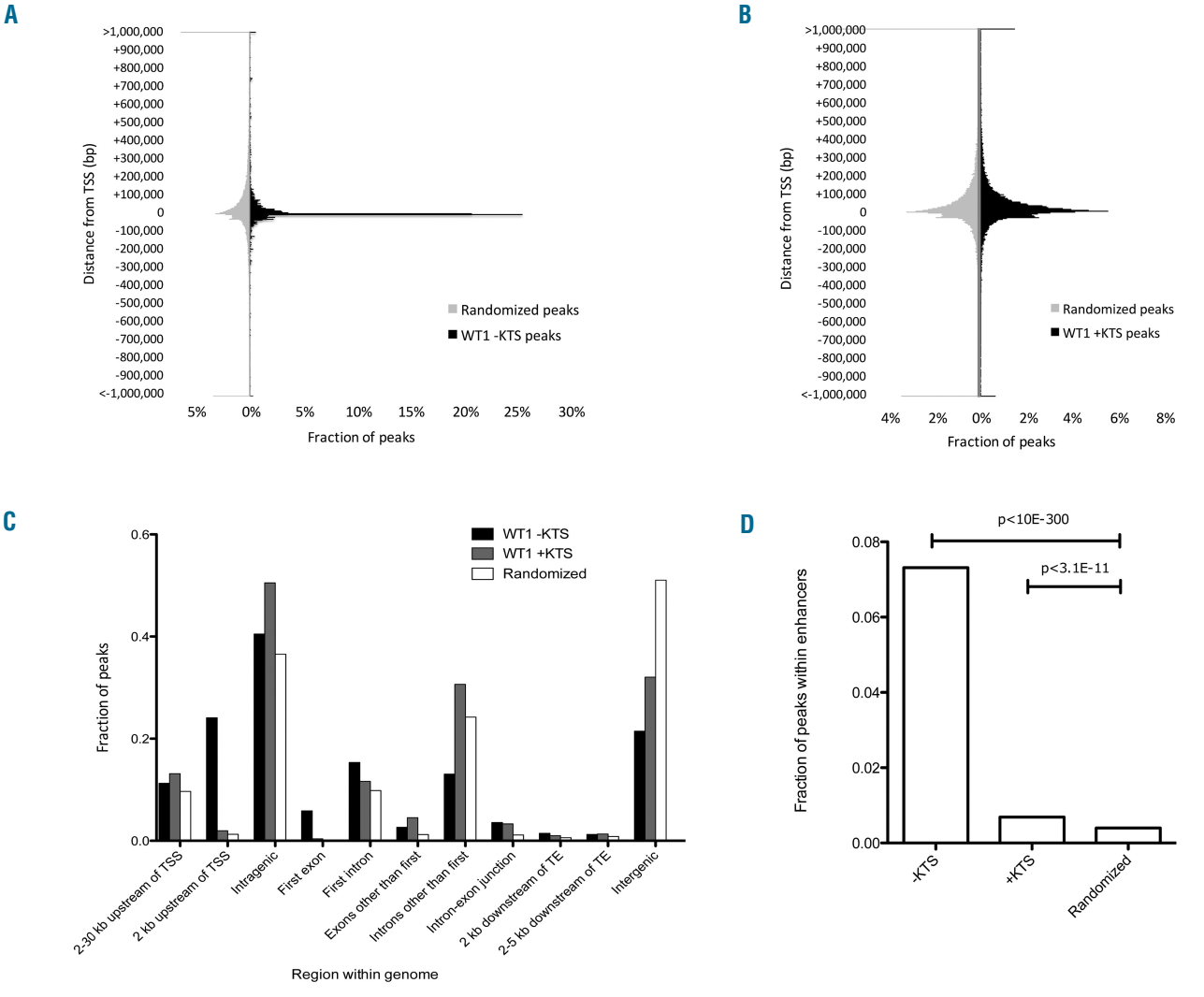

Figure 1. WT1 -KTS, and to a lesser extent WT1 +KTS, peaks are enriched around the transcription start sites of target genes and in enhancers. After chromatin immunoprecipitation, sequencing and peak calling, the peak centers were annotated in reference to the closest transcription start site in the genome. The graphs depict the distribution of (A) WT1 -KTS peaks and (B) WT1 +KTS peaks, (distance from the closest TSS in base pairs). In (C) all peaks are shown in reference to gene bodies in the genome. (D) Fraction of peaks that correspond to enhancer areas, as defined by CAGE data.[18] "Randomized" refers to 30,000 positions randomly distributed across the genome. (TSS: transcription start site; TE: transcription end).

lysine 36 (H3K36me3), which is considered a marker for the gene body of actively transcribed genes[23] and three tracks for monomethylation of histone H3 on lysine 4 (H3K4me1), a histone mark of active enhancers and gene bodies of actively transcribed genes.[23] Furthermore, an open chromatin track comes first on the list of most similar tracks. Together, these histone marks reveal WT1 +KTS as a factor that co-localizes with both repressive and activating histone marks, perhaps indicating a potential for both repression and activation of target genes.

Table 1. Similarity between WT1 peaks and ENCODE ChIP-Seq tracks.

WT1 -KTS ENCODE tracks	Track description	Similarity score	*P* value
wgEncodeHaibTfbsK562Egr1V0416101PkRep1.strack	Egr1 transcription factor	0.108274	9.99E-04
wgEncodeHaibTfbsK562Cbx3sc101004V0422111PkRep1.strack	Cbx3 heterochromatin component	0.058714	9.99E-04
wgEncodeHaibTfbsK562Zbtb7asc34508V0416101PkRep2.strack	Zbtb7 transcription factor	0.056838	9.99E-04
wgEncodeOpenChromDnaseK562G1phasePk.strack	Open chromatin DNaseI G1 phase cells	0.04972	9.99E-04
wgEncodeOpenChromDnaseK562G2mphasePk.strack	Open chromatin DNase I G2/M phase cells	0.049081	9.99E-04
wgEncodeOpenChromDnaseK562PkV2.strack	Open chromatin DNase I	0.048757	9.99E-04
wgEncodeHaibTfbsK562Hey1Pcr1xPkRep1.strack	Hey1 transcription factor	0.048251	9.99E-04
wgEncodeHaibTfbsK562MaxV0416102PkRep2.strack	Max transcription factor	0.047713	9.99E-04
wgEncodeOpenChromDnaseK562SahactrlPk.strack	Open chromatin DNase I SAHA control (only DMSO) treatment	0.047693	9.99E-04
wgEncodeOpenChromDnaseK562Saha1u72hrPk.strack	Open chromatin DNase I SAHA (Vorinostat) 1 uM 72 hours	0.047583	9.99E-04
wgEncodeOpenChromDnaseK562NabutPk.strack	Open chromatin DNase I sodium butyrate 72 hours	0.045986	9.99E-04
wgEncodeOpenChromDnaseK562Pk.strack	Open Chromatin DNase I	0.044686	9.99E-04
wgEncodeHaibTfbsK562MaxV0416102PkRep1.strack	Max transcription factor	0.042228	9.99E-04
wgEncodeHaibTfbsK562E2f6sc22823V0416102PkRep2.strack	E2f6 transcription factor	0.041029	9.99E-04
wgEncodeHaibTfbsK562E2f6V0416102PkRep2.strack	E2f6 transcription factor	0.041029	9.99E-04
wgEncodeOpenChromChipK562CmycPk.strack	Cmyc transcription factor	0.040484	9.99E-04
wgEncodeHaibTfbsK562Hey1Pcr1xPkRep2.strack	Hey1 transcription factor	0.037512	9.99E-04
wgEncodeOpenChromSynthK562Pk.strack	Open chromatin (synthesis of FAIRE, ChIP tracks and DNase I)	0.035282	9.99E-04
wgEncodeOpenChromFaireK562Pk.strack	Open chromatin (formaldehyde assisted isolation of regulatory elements)	0.034536	9.99E-04
wgEncodeBroadHistoneK562H2azStdPk.strack	H2az (histone 2 variant)	0.028038	9.99E-04

WT1 +KTS ENCODE tracks	Track description	Similarity score	*P* value
wgEncodeOpenChromSynthK562Pk.strack	Open chromatin (synthesis of FAIRE, ChIP tracks and DNase I)	0.003739	9.99E-04
wgEncodeHaibMethyl450K562SitesRep1.strack	Methylated DNA	0.003115	9.99E-04
wgEncodeSydhTfbsK562Znf263UcdPk.strack	Znf263 transcription factor	0.002099	9.99E-04
wgEncodeBroadHistoneK562H3k4me1StdPk.strack	H3K4me1 histone modification	0.001946	2.50E-02
wgEncodeBroadHistoneK562Chd1a301218aStdPk.strack	Chd1 chromatin remodeller	0.001739	7.99E-03
wgEncodeBroadHistoneK562CtcfStdPk.strack	Ctcf	0.001564	9.99E-03
wgEncodeSydhHistoneK562bH3k4me1UcdPk.strack	H3K4me1 histone modification	0.001531	9.99E-04
wgEncodeSydhHistoneK562H3k4me1UcdPk.strack	H3K4me1 histone modification	0.001531	9.99E-04
wgEncodeUwHistoneK562H3k27me3StdPkRep1.strack	H3K27me3 histone modification	0.001092	9.99E-04
wgEncodeUwHistoneK562H3k27me3StdPkRep2.strack	H3K27me3 histone modification	0.001065	9.99E-04
wgEncodeUwHistoneK562H3k27me3StdPkRep2.strack	H3K27me3 histone modification	0.001065	9.99E-04
wgEncodeSydhHistoneK562bH3k27me3bUcdPk.strack	H3K27me3 histone modification	0.00094	8.99E-03
wgEncodeSydhHistoneK562H3k27me3bUcdPk.strack	H3K27me3 histone modification	0.00094	8.99E-03
wgEncodeBroadHistoneK562Cbx3sc101004Pk.strack	Cbx3 heterochromatin component	0.000936	9.99E-04
wgEncodeHaibTfbsK562Mef2aV0416101PkRep1.strack	Mef2a	0.000692	9.99E-04
wgEncodeSydhTfbsK562Znf143IggrabPk.strack	Znf143	0.000608	2.00E-03
wgEncodeHaibTfbsK562Pu1Pcr1xPkRep1.strack	Pu1	0.000549	2.00E-03
wgEncodeSydhTfbsK562Atf3StdPk.strack	Atf3	0.000548	3.80E-02
wgEncodeUwHistoneK562H3k36me3StdPkRep1.strack	H3K36me3 histone modification	0.000535	9.99E-04
wgEncodeSydhTfbsK562Gata2UcdPk.strack	Gata2	0.000502	2.00E-03

The score for similarity between our peaks and those of ENCODE K562 tracks, and the *P* values, were calculated as described in the Methods section.

Another indication that this could be the case is that WT1 +KTS, but not WT1 -KTS, significantly co-localizes with a track for methylated DNA in Table 1. The positive correlations for WT1 -KTS to DNA methylation are far down the list of similar tracks, and the similarity score for the top hit is better for WT1 +KTS. In contrast to the WT1 +KTS isoform, the WT1 -KTS track does not match the repressive H3K27me3 mark (where five H3K27me3 tracks are positive for WT1 +KTS, only one is positive for WT1 -KTS and it scores at position 207 in similarity to WT1 –KTS). WT1 -KTS is also a worse match for H3K36me3 (only 1 track is positive for WT1 –KTS as compared to 2 for WT1 +KTS, and it scores at position 213). Rather, WT1 -KTS shows greater similarity with multiple open chromatin tracks as well as the H3K4me1 mark (3 tracks for H3K4me1 are positive for each isoform, but WT1 –KTS has a higher similarity score). The H3K4me3 mark has five tracks positive for co-localization with WT1 –KTS and two very low-scoring positives for WT1 +KTS, consistent with this mark and WT1 –KTS both appearing mainly around the TSS, which is not the case for WT1 +KTS. *Online Supplementary Table S5A* gives a complete list of all tracks showing similarity to those of WT1 –KTS and *Online Supplementary Table S5B* to those of WT1 +KTS.

In summary, both transcription factors co-localize with histone marks for active transcription, but only WT1 +KTS peaks co-localize with a transcriptional repression mark. The binding pattern of WT1 -KTS is similar to those of other transcription factors, whereas the binding pattern of WT1 +KTS is not. This observation is consistent with our previous finding, that WT1 +KTS localizes only to a small extent near the TSS and to enhancer regions.

WT1 -KTS and WT1 +KTS bind different locations within the same genes

To avoid uncertain gene annotations we limited our investigation of target genes to the genes in which the peak was situated within the promoter area, defined as the 2,000 bp upstream of the TSS, or within the gene body. For WT1 -KTS, 1,141 unique target genes could be identified in this way, and for WT1 +KTS 5,775 genes. Of these, 508 genes were found to be shared by the two isoforms, a highly significant overlap (Figure 2). Not surprisingly, considering the differences in general binding pattern, the peaks of the WT1 -KTS and WT1 +KTS isoforms on these shared target genes rarely coincided. Only for eight (1.6%) of the genes, did the binding sites overlap, and only for a further 23 (4.5%) of the genes, was there a WT1 -KTS and a WT1 +KTS peak within 1 kb of each other.

Upon closer inspection of the target genes that we identified to be common to both isoforms, we found that they include several previously known WT1 targets, including BCL2-Like 1 (*BCL2L1/BCLXL*),[24] DNA (Cytosine-5)-Methyltransferase 3 Alpha (*DNMT3A*),[25] Platelet-Derived Growth Factor Alpha Polypeptide (*PDGFA*),[26] SMAD Family Member 3 (*SMAD3*),[27] and Vitamin D Receptor (*VDR*).[28]

In conclusion, among our identified target genes that include previously known targets, WT1 -KTS and WT1 +KTS isoforms bind, to a large extent, to the same target genes, but in distinct locations, suggesting at least partially different molecular mechanisms.

Known motifs of WT1 binding are found within peaks

To investigate the binding pattern of the WT1 isoforms further, we searched the peaks for predicted WT1 binding sites, using the FIMO tool and the two WT1 motif position weight matrices from the TRANSFAC database.[37,38] The first motif, called GCGGGGGGGT, with a matrix for a total of 17 bases, was found at least once within 44% of WT1 -KTS peaks, while the second motif, called GGGGshort, consisting of a matrix for 12 bases, was even more frequent and was present in 78% of WT1 -KTS peaks (Figure 3). For WT1 +KTS, the motif frequencies were much lower, 17% and 21%, respectively. When the motif search was performed on the entire genome both motifs proved so scarce that less than 1% of the WT1 peaks would be expected to have motif occurrence, making both WT1 -KTS and WT1 +KTS peaks highly enriched for the TRANSFAC WT1 motifs (Figure 3).

To search for enrichment of other motifs as well, we used the DREME software that performs unbiased searches for nucleotide patterns within the peaks. Table 2 lists the top ten identified motifs ranked according to significance of enrichment among WT1 -KTS peaks (all significantly enriched motifs are listed in *Online Supplementary Table S2*, and a list of previously published WT1 motifs is given in *Online Supplementary Table S1*). Motifs number 1, 2, 4, 8 and 11 found for WT1 -KTS are matches for four different previously published motifs[21,29-31] (see alignment in *Online Supplementary Figure S3*). Several of the enriched motifs in Table 2 are annotated to EGR-1, which is not surprising since the three zinc fingers of EGR-1 are homologous to number two through four or WT1's four zinc fingers,[21] and most WT1 motifs defined so far are EGR-1-like. The TOMTOM database did not contain a WT1 motif, explaining why no WT1 annotations can be seen in Table 2. Taken together, these motif analyses reveal enrichment in the WT1 -KTS peaks of several different motifs previously found to bind the WT1 protein.

For WT1 +KTS, only one among the enriched motifs listed in Table 3, motif number 3, is a match for a core of a previously known motif[31] (the complete list of significantly enriched motifs is given in *Online Supplementary*

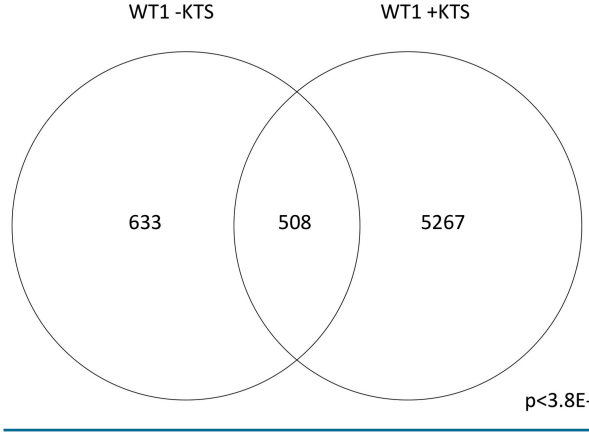

Figure 2. WT1 -KTS and WT1 +KTS have highly significant overlap in target genes. Taking into account only genes with at least one peak within the promoter area or the gene body, 44.5% of the WT1 -KTS target genes are also target genes of WT1 +KTS.

Table S2, the core sequence in *Online Supplementary Table S1*, and alignment in *Online Supplementary Figure S3*). This core motif was found in 50% of our WT1 +KTS peaks. In contrast to WT1 -KTS, EGR-1 binding sites were not found among the WT1 +KTS peaks, consistent with a recurrent hypothesis of WT1 +KTS not binding the EGR-1 motif. None of the enriched motifs for WT1 +KTS was identified as the binding motif for any other transcription factor, but further investigations are required in order to determine whether the enriched motifs bind directly to WT1 +KTS, or might instead bind a protein partner.

We conclude that the two TRANSFAC WT1 motifs are highly enriched in our WT1 peaks. These motif occurrences were more numerous for WT1 -KTS than for WT1 +KTS. Apart from the TRANSFAC motifs, we also found four previously published motifs enriched within our WT1 -KTS peaks, and one previously published motif within our WT1 +KTS peaks.

WT1 binds to genes related to development and oncogenic processes

To understand the function of the target genes better, we performed a Gene Ontology (GO) analysis with a GO Slim Mapper tool. Results from the analysis mode "process" are presented in *Online Supplementary Table S3* and those for "function" in *Online Supplementary Table S4*. Again, we limited the analysis to genes with peaks within the promoter or gene body areas. The GO groups found for the two isoforms are very similar.

Several categories found by the GO analysis describe processes previously known to be influenced by WT1. WT1's role in embryonic development and in the transitions between mesenchymal and epithelial states necessary for embryonic development[32] is consistent with enrichment of genes in groups such as anatomical structure development, but also groups such as cell motility, cell junction organization and cytoskeleton organization. WT1 is indispensable for the embryonic development of reproductive organs,[33] and the genes in the GO reproduction group were also enriched in our peaks.

Several gene groups are consistent with a role in oncogenesis, including cell division, proliferation, response to stress, cell-cell signaling, and cell death. This enrichment indicates that WT1 has the ability to influence many of the properties of malignancy through transcriptional regulation of target genes, consistent with the well-documented role for WT1 in tumor development.[2,32,34]

Another interesting group is nucleocytoplasmic transport. Accumulating evidence suggests that transport from the nucleus to cytoplasm is tightly regulated and at least in some instances selective for certain transcripts,[35] potentially providing WT1 with further possibilities to influence protein production. Enrichment of groups such as transcription factor activity, transcription factor binding, and histone binding indicates that WT1, through its target genes, might also have the ability to further influence transcription downstream, and possibly also splicing.

In summary, the target genes of both WT1 isoforms are found in GO groups for embryonic development and homeostasis, as well as functions central to oncogenesis such as cell death, cell proliferation and differentiation.

WT1 -KTS and WT1 +KTS target genes are more likely to be transcribed than non-target genes

Making use of FANTOM consortium CAGE data on gene expression (*http://fantom.gsc.riken.jp/5*; accessed 27 January 2016),[36] we compared the expression pattern of all genes in K562 cells with that of our identified target genes of the WT1 isoforms, as described in the *Online Supplementary Methods*. Both WT1 -KTS and WT1 +KTS target genes were enriched among the intermediately expressed genes, at the expense of the non-expressed gene group (Figure 4). For WT1 -KTS there was also an enrichment in the highly expressed group. These data are consistent with the notion of WT1 as an activator of transcription.

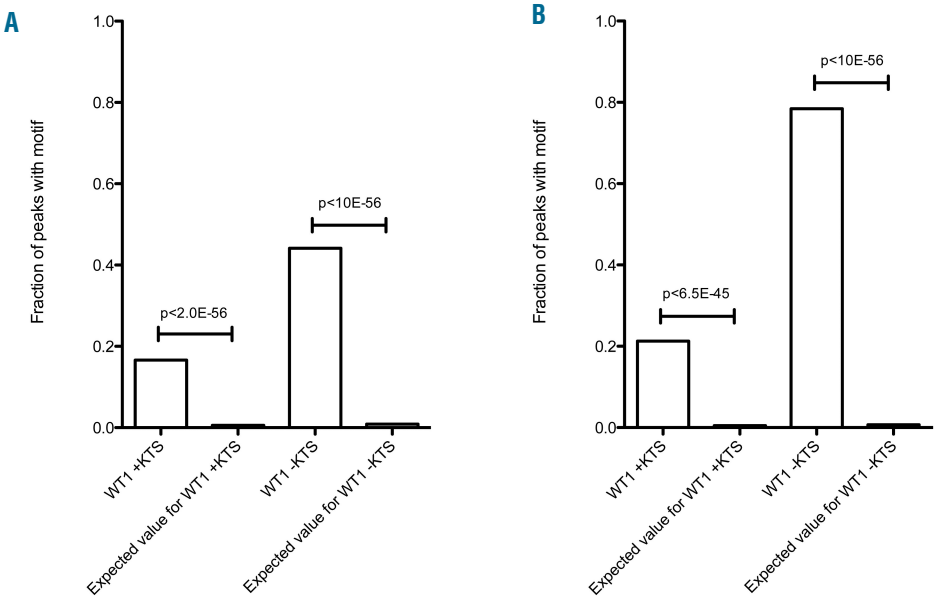

Figure 3. WT1 motif occurrence differs between isoforms. We performed a search within our WT1 -KTS and WT1 +KTS peaks for two known WT1 motif matrices from the TRANSFAC database.[37,38] (A) The fraction of peaks with at least one occurrence of the GCGGGGGGGT motif. (B) The fraction of peaks with at least one occurrence of the GGGG short motif.

Table 2. Unbiased motif search within the WT1 -KTS peaks identified several WT1 motifs.

	Motif	E-value	Peaks with motif	TOMTOM matches
1	GYGKGGGM	1.30E-267	89.9%	EGR1, EGR2
2	CCTCCYMC	9.50E-38	44.1%	SP1, ZNF263, EGR1, SP2
3	ARATA	4.30E-31	26.5%	-
4	ACTCCCAC	4.30E-31	4.2%	-
5	GGAARY	3.80E-10	58.4%	-
6	TGATWA	1.70E-09	7.7%	-
7	ABAAA	5.00E-08	47.3%	-
8	CCCCGCCC	2.50E-05	14.5%	EGR1, SP1, KLF5, SP2, KLF4, EGR2, E2F4, E2F6, E2F3, E2F1, KLF1
9	CCGCCKCC	8.40E-04	16.0%	-
10	GCYGGGA	3.40E-03	23.0%	-

Using DREME software, the WT1 -KTS peaks were analyzed to find short nucleotide sequences enriched in our material as compared to the entire genome. The top ten most enriched motifs of a total of 13 are listed. The enriched sequences were then investigated using TOMTOM software to identify proteins whose DNA binding motif matched the sequences. Sequences number 1 (Nakagama 1995),[29] 2 (Wang 1990, Bickmore 1992),[30,31] 4 (Nakagama 1995)[29] and 8 (Rauscher 1990)[21] match previously published WT1 motifs (for alignment see *Online Supplementary Figure S3*).

Trimethylation of histone H3 at lysine 4 (H3K4me3) is a good marker for actively transcribed genes, having the ability to recruit parts of the transcription complex directly.[23] We, therefore, conducted a ChIP-Seq analysis of the H3K4me3 pattern and compared it to that of our WT1 target genes. In our investigation, 60% of the WT1 -KTS target genes in the K562 cells had a H3K4me3 peak center within 1 kb of the TSS, as compared to 41% of all genes in the tagged WT1 -KTS/BirA K562 cells (Figure 5). For WT1 +KTS, 69% of the target genes were positive for H3K4 trimethylation around the TSS, as compared to 59% of all genes in the tagged WT1 +KTS/BirA K562 cells (Figure 5). Both differences were statistically significant.

We conclude that the target genes of both WT1 -KTS and WT1 +KTS are more highly expressed than genes not bound by WT1, as shown by CAGE transcription data from K562 cells and by the H3K4me3 mark of active transcription.

Table 3. Unbiased motif search within the WT1 +KTS peaks identified one known WT1 motif.

	Motif	E-value	Peaks with motif	TOMTOM matches
1	TAWTTTTW	1.4e-2917	35.5%	-
2	TARTCCCA	1.5e-2706	40.0%	-
3	GAGGCBGA	1.0e-2550	50.3%	-
4	CAGGAGAW	2.4e-2412	42.5%	-
5	GGKTTCAY	1.4e-2463	42.0%	-
6	GTKAGCCR	6.7e-2119	42.2%	-
7	GTAGAGAY	1.7e-2064	31.9%	-
8	CGCCCGSC	2.2e-1871	35.3%	-
9	GCTACTY	1.8e-1663	38.9%	-
10	TGCAGTGR	8.4e-1946	36.2%	-

Using DREME software, the WT1 +KTS peaks were analyzed to find short nucleotide sequences enriched in our material as compared to the genome. The top ten most enriched motifs of a total of 26 are listed. The enriched sequences were then investigated using TOMTOM software to identify proteins whose DNA binding motif matched the sequences. For neither of the sequences was a matching motif found within the TOMTOM database. Sequence number 3 matches a previously published WT1 motif (Bickmore 1992)[31] (for alignment see *Online Supplementary Figure S3*).

Discussion

WT1 is an oncogene recurrently mutated and overexpressed in AML. Here, we report the findings of the first ChIP-Seq of WT1 performed in human cells, the very first in hematopoietic cells, as well as the first that is isoform specific. Our investigation revealed that WT1 -KTS and WT1 +KTS bind specifically to partly overlapping, partly unique, sets of target genes in leukemic cells. Both isoforms, but WT1 -KTS much more than WT1 +KTS, bind the TRANSFAC[37,38] motifs; however, the alternative motifs identified are not shared. The ability of WT1 +KTS to bind WT1 -KTS target sequences has been the subject of much research and discussion, and our results indicate that the two proteins bind quite separately. This almost complete lack of shared peaks cannot be fully accounted for by the motif analysis, and might instead be due to smaller differences between motifs still fitting the same motif matrix, to differences in protein partners, and/or to differences in flanking sequences. One ChIP-chip (chromatin immunoprecipitation coupled with DNA microarrays) and three ChIP-Seq investigations into the target genes of WT1, although not distinguishing WT1 -KTS and WT1 +KTS from each other, have been published.[39-42] All studies were performed on mouse kidney cells. The motifs found to be most enriched for the WT1 -KTS isoform in our investigation are similar to those found in the four previous studies, closely resembling the established EGR-1 and WTE motifs. Our distribution of -KTS peaks, centering around the TSS, resembles the results of Dong *et al.*[41] and Kann *et al.*,[42] with comparable concentration in the promoter area (23.1% in our investigation as compared to 21% found by Kann *et al.* and 16.9% by Dong *et al.*) although due to differences in the analysis software exact comparisons are not possible. Motamedi *et al.*[40] found a different distribution of peaks, with only 20% of peaks located within 5 kb of a TSS, whereas we found 46.5% of all -KTS peaks in this area. Moreover, several of the GO groups enriched in our investigation were also found to be enriched in the earlier ChIP-Seqs and ChIP-chip investigations into WT1, such as cytoskeleton and adhesion,[39,41,42] cell cycle,[39,42] and development.[39]

We found that the WT1 -KTS isoform binds as a traditional transcription factor around the TSS and to known

enhancer elements, whereas the WT1 +KTS binding pattern is enriched predominantly within gene bodies, with no other transcription factors closely resembling its binding pattern. With the exception of EGR-1, which binds some of WT1's target sequences, the proteins whose binding most closely resemble that of WT1 -KTS are ZBTB7, HEY1 and MYC and its binding partner MAX. However, binding motifs annotated for these transcription factors did not appear in our analysis (Table 2 and *Online Supplementary Table S2*). The reason for this is unclear. One explanation could be that while peaks of different tracks overlap (and thus score as similar tracks), the overlap may not necessarily contain the binding motifs (and thus not be detected in the motif-analysis). Another possibility is indirect binding to DNA, suggesting that they are possible protein partners of WT1, inviting further investigation. Interestingly, *MYC* is a known target gene of WT1,[43] although we did not find any peaks in our material that can be safely assigned to the *MYC* gene. *HEY1* has also been found to be a WT1 target gene in murine cells.[39] The combination of a binding partner and target gene of WT1 has been described for Zinc Finger 224 (*ZNF224*).[44,45]

Intriguingly, we found ten times more peaks for the WT1 +KTS isoform, as compared to the number of WT1 -KTS peaks. During peak calling, we noticed that the fewer peaks obtained with WT1 -KTS were higher, as compared to the larger number of peaks obtained with WT1 +KTS, suggesting that the occupancy of WT1 was higher at WT1 -KTS binding sites than at the binding sites of WT1 +KTS. This could indicate that WT1 -KTS isoforms are more concentrated on fewer high affinity sites, while WT1 +KTS isoforms are more dispersed on a larger number of binding sites with lower affinity. This speculative notion would be consistent with some published data showing lower affinity to DNA for the WT1 +KTS isoform, but further experiments are needed to draw definitive conclusions.

For our identified WT1 –KTS and WT1 +KTS target genes, both CAGE data[36] from K562 cells, and the H3K4me3 pattern around TSS, indicate that the target genes are more likely to be transcribed than non-target genes. This pattern suggests a mostly activating function for both isoforms, consistent with data from two of the earlier ChIP-Seq investigations[41,42] in which WT1 was found to be predominantly activating in mouse kidney glomeruli cells. When, instead, we analyzed the localization of all the WT1 peaks from our investigation, and compared the localizations with those of peaks from ChIP-Seq investigations present in the ENCODE database, we found that tracks for methylated DNA and the repressive histone mark H3K27me3 overlapped with our WT1 +KTS peaks to the extent of becoming top similarity hits. This was not the case for our WT1 –KTS peaks. Both isoforms are known from previous reports to be capable of both activation and repression.[43,46-50] Our finding could indicate that, even though the dominant influence of both isoforms on the target genes seems to be activating, in our cellular context WT1 +KTS co-localizes more often than WT1 –KTS with repressive complexes. Importantly, it should be pointed out that our investigation was restricted to WT1 +17AA isoforms. The 17AA isoform could significantly modify the transactivating properties of WT1, and although an influence of the 17AA isoform domain on DNA binding is not supposed, it cannot be totally excluded.

How could WT1 +KTS, binding within gene bodies, affect transcription? Our own ChIP-Seq analysis shows that WT1 +KTS binding is only weakly enriched within enhancers (enhancers as defined by CAGE), compared to

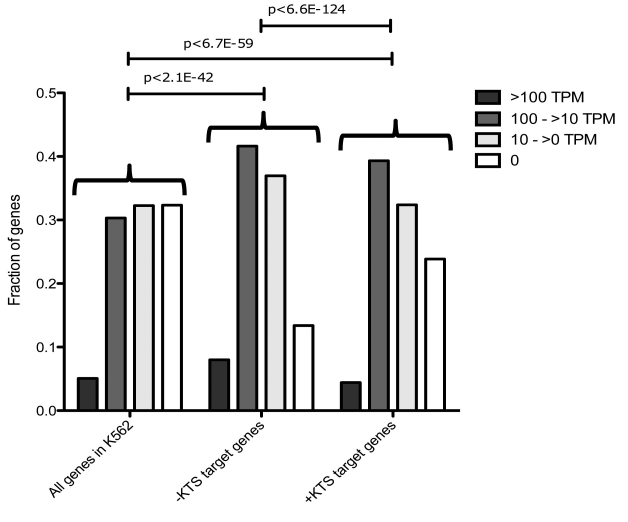

Figure 4. WT1 target genes are enriched among the expressed genes in K562 cells. Comparing CAGE data in tags per million (TPM), from the publicly available FANTOM5 consortium datasets,[36] of all genes in K562 cells with the genes bound by WT1 -KTS and WT1 +KTS, the WT1 target genes were significantly enriched among the expressed genes at the expense of the silenced genes. Expression levels of the target genes of the two isoforms also differed significantly from each other. The *P*-values refer to differences of gene distribution in expression bins for all genes in K562 cells, -KTS target genes, and +KTS target genes, respectively.

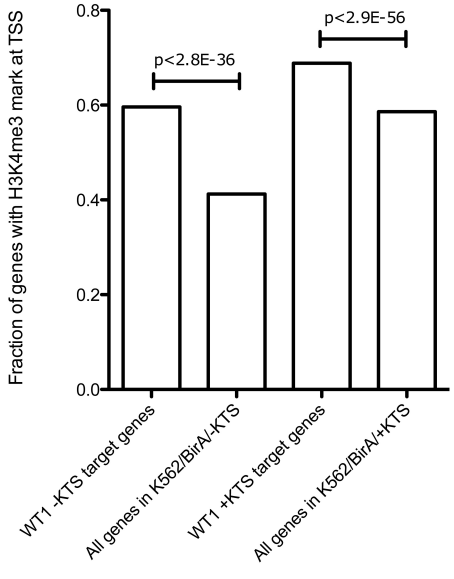

Figure 5. Genes with a H3K4me3 peak around the transcription start site (TSS) are enriched among the WT1 target genes for both -KTS and +KTS. Compared to all genes, a larger proportion of WT1 -KTS and +KTS target genes had a H3K4me3 ChIP-Seq peak, a mark of active transcription, within 1 kb of the TSS.

the strong enrichment of WT1 -KTS peaks (Figure 1D), arguing against WT1 +KTS binding to enhancers (as defined by CAGE). Nevertheless, target genes of WT1 +KTS are highly enriched for H3K4me3 around TSS (Figure 5), although WT1 +KTS peaks and H3K4me3 peaks do not overlap. Moreover, H3K4me1 and H3K36me3 score high in the list of tracks similar to WT1 +KTS (Table 1). These observations possibly indicate that WT1 +KTS binds within the gene body in an enhancer-like fashion, affecting chromatin modification also around the TSS to enhance transcription.

Many of the GO groups enriched in our study indicate functions central to oncogenesis, such as cell proliferation, cell death, locomotion, cell adhesion, and cell-cell signaling. The GO analysis also showed enrichment in groups involved in formation of anatomical structures and embryo development. The key to WT1's role in embryogenesis lies in its effect on transitions between the epithelial and mesenchymal states. These transitions have a well-established role in metastasis of solid tumors and also a role in the generation of cancer stem cells.[32] Not much is known about their function in leukemia, but a recent study found that epithelial-mesenchymal transition genes were upregulated in promyelocytic leukemia, and that one of the genes found to be overexpressed in the network was *WT1*.[51]

In conclusion, our ChIP-Seq investigation into the binding pattern of WT1 -KTS and WT1 +KTS in leukemic cells reveals that the binding of both WT1 isoforms is correlated to active transcription, and that WT1 -KTS and WT1 +KTS share target genes, although the binding motifs are mostly different and the peaks of the two isoforms with very few exceptions do not overlap. The target genes for both isoforms reveal functions central to oncogenesis, consistent with an effector role in leukemia.

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