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Functional interrelationship between TFII-I and E2F transcription factors at specific cell cycle gene loci

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Abstract

Transcription factor TFII-I is a multifunctional protein implicated in the regulation of cell cycle and stress-response genes. Previous studies have shown that a subset of TFII-I associated genomic sites contained DNA-binding motifs for E2F family transcription factors. We analyzed the co-association of TFII-I and E2Fs in more detail using bioinformatics, chromatin immunoprecipitation, and co-immunoprecipitation experiments. The data show that TFII-I interacts with E2F transcription factors. Furthermore, TFII-I, E2F4, and E2F6 interact with DNA-regulatory elements of several genes implicated in the regulation of the cell cycle, including *DNMT1*, *HDAC1*, *CDKN1C*, and *CDC27*. Inhibition of TFII-I expression led to a decrease in gene expression and in the association of E2F4 and E2F6 with these gene loci in human erythroleukemia K562 cells. Finally, TFII-I deficiency reduced the proliferation of K562 cells and increased the sensitivity toward doxorubicin toxicity. The results uncover novel interactions between TFII-I and E2Fs and suggest that TFII-I mediates E2F function at specific cell cycle genes.

KEYWORDS
cell cycle, E2F, gene regulation, TFII-I

1 | INTRODUCTION

Transcription factor TFII-I (gene symbols are *GTF2I* in human and *Gtf2i* in mouse) is a multifunctional protein involved in activation and repression of gene expression.[1,2] TFII-I consists of multiple protein/protein interaction domains including six R-repeats and a leucine zipper.[3] The R-repeats resemble helix-loop-helix (HLH) domains and several studies have shown that TFII-I interacts with the HLH proteins USF and c-Myc.[4–6] TFII-I belongs to a family of related proteins including GTF2IRD1 (also known as Ben) and GTF2IRD2.[1,7,8] The genes encoding this family are located close together on chromosome 7 and haploinsufficiency of this region causes Williams Beuren (WB) syndrome, which is characterized by craniofacial and neurological defects.[7] Disruption of either the *Gtf2i* or the *Gtf2ird1* gene in mice recapitulates some of the phenotypes seen in WB syndrome.[8,9]

TFII-I was originally identified as an initiator binding protein that is able to recruit RNA polymerase II (Pol II) transcription complexes to promoters lacking a TATA-box.[10] However, TFII-I is not a general transcription factor of Pol II but rather positively or negatively modulates expression of

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genes involved in cell cycle regulation, DNA repair, cellular differentiation, or genes induced upon stress signals.[1,2] TFII-I has also been shown to exert functions outside the nucleus by facilitating nuclear import of transcription factors (eg, c-Rel) or by inhibiting agonist induced calcium entry through interactions with the transient receptor potential cation channel subfamily 3 member (TRPC-3).[11,12] There are four isoforms of TFII-I that are generated by alternative splicing.[1,2] The β-isoform appears to be located primarily in the nucleus and represses transcription through interactions with histone deacetylases (HDACs) and other co-repressor.[13,14] The Δ-isoform shuttles between cytoplasm and nucleus and is mostly involved in transcription activation.[1,2] The γ-isoform is expressed primarily in neuronal tissues and the α-isoform is not expressed in mice.

There is increasing evidence showing that TFII-I plays an important role in the cellular response to stress and DNA damage. In conjunction with transcription factor ATF6, TFII-I activates expression of the glucose-regulated protein (Grp) upon induction of endoplasmic reticulum (ER) stress.[15] TFII-I also interacts with the gene locus encoding the stress-response transcription factor ATF3.[16] Upon ER stress ATF3 expression is induced and depletion of TFII-I reduced expression levels of ATF3. A proteomic analysis revealed that TFII-I interacts with TAF15 and Elongin A, two co-regulators previously implicated in the stress-response.[16–18]

TFII-I is phosphorylated in response to mitogenic and growth factor stimuli and activates genes involved in cell cycle regulation and proliferation, including the cyclin D1 gene.[1,19] Previous genome-wide analysis revealed that TFII-I peaks are frequently associated with binding sites for E2F transcription factors, specifically E2F4 and E2F6.[16] E2Fs are major regulators of genes driving the cell cycle during cellular proliferation.[20] The E2Fs can be divided into several groups depending on the presence or absence of specific protein domains. E2F1, E2F2, and E2F3 are referred to as activating E2Fs. They interact with the retinoblastoma (RB) protein, which keeps them in an inactive configuration. Phosphorylation leads to the dissociation of RB and co-repressors and converts these E2Fs into transcription activators. Major targets of the activator E2Fs are genes encoding cyclin-dependent kinases.[20] E2F4 and E2F5 are known as repressor E2Fs that interact with RB related pocket proteins. E2F6 does not contain a pocket domain and represses transcription in a pocket-independent manner. The typical E2Fs bind DNA as heterodimers with the related dimerization partner (DP) proteins 1 and 2.[20] E2Fs, like TFII-I, contain leucine zipper motifs with which they interact with the DPs and perhaps other proteins.[20] Like TFII-I, E2Fs have been implicated in the regulation of the cell cycle as well as the stress- and DNA damage-response.[21–23] Several studies have shown that E2Fs are involved in the regulation of hematopoiesis mostly through their known effect on proliferation.[24,25] However, E2F2 and E2F4 have also been implicated in the regulation of erythroid maturation and both are up-regulated during differentiation of erythroid cells but not in other hematopoietic cell lineages.[26,27] Moreover, E2F2 is required for nuclear condensation during the final stages of erythroid maturation.[28,29]

In the current study we analyzed interactions between E2F transcription factors and TFII-I. The data show that TFII-I interacts with E2F1 and E2F6. E2F4, E2F6, and TFII-I associate with several cell cycle genes in K562 cells. Reduced TFII-I expression led to reduced transcription and E2F4/6 occupancy at these genes. Furthermore, diminished TFII-I expression resulted in impaired proliferation and increased sensitivity toward doxorubicin. The data thus suggests that TFII-I is an important mediator of the function of E2F transcription factors.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics analysis

USF1, Pol II, E2F4, E2F6, RNA-seq, and DNA-seq datasets from K562 cells were downloaded from the ENCODE project using the following accession numbers: GSM803441, GSM803410, GSM935600, GSM935597, GSM958729, and GSM816655.[30] The raw SRA dataset of TFII-I was downloaded from GSE51065 and aligned to the human genome hg19 by Bowtie 2 with default settings.[31] A bigwig file was generated using "BAM to BigWig" under the Galaxy project online tools.[32] Snapshots of the genome features were visualized in Integrative Genomics Viewer (IGV), hg19 by extracting the datasets for histone modifications (histone H3K4 monomethylation, H3K4me1; histone H3K4 trimethylation, H3K4me3; histone H3K27 acetylation, H3K27ac), USF1, Pol II, E2F4, E2F6, RNA-seq, and DNA-seq from the ENCODE project and uploading the TFII-I dataset.[33] The binding peaks of TFII-I, E2F4, E2F6, and USF1 were defined as overlapping if they occurred within a 500 bp window size (findPeaks.pl, HOMER 4.6).[34] ChIP-seq analysis, including overlapping peaks annotations (annotatePeaks.pl), consensus motif finding (findMotifsGenome.pl), and profiling of transcription factors at Pol II peaks (annotatePeaks.pl) was performed using HOMER 4.6 with default settings.[34]

2.2 | Cell culture and transfection

Human erythroleukemia K562 cells were grown in RPMI (Corning/Cellgro, Corning, NY) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were grown at 37°C under 5% CO_2 at a density between 1×10^5 and 2×10^6 cells/mL. To reduce expression of TFII-I (GTF2i), K562 cells were transfected with the plasmid pGIPZ-shTFII-I (Thermo Fisher Scientific, Waltham, MA). Control cells were

transfected with a plasmid expressing a scrambled control shRNA (pGIPZ-shSc). Transfections were performed using lipofectamin 2000 (Invitrogen, Carlsbad, CA) according to the procedure provided by the manufacturer. Briefly, 5×10^5 cells were transfected with 5 μg DNA in 2 mL of RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin. Stably transfected cells were selected using 2 μg/μL puromycin and subsequently subjected to single cell selection. Single cell clones were expanded in the presence of 1 ng/μL puromycin. The following antisense sequences were used to construct the plasmids: shRNA TFII-I, 5′-TTCATACACTGCAATGC AG-3′. shRNA SC, 5′-TCTCGCTTGGGCGAGAGTAAG-3′. The proliferation assay was performed by seeding 1×10^4 SC or TFII-I KD cells per well in six well plates with 2 mL media. Viable cells were counted every 24 h for up to 5 days by staining 10 μL of cells with tryphan blue. Doxorubicin-mediated toxicity was assessed using the MTS cytotoxicity assay from Promega (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI). Briefly, 2×10^4 SC or TFII-I KD cells were seeded per well in 96 well plates with 100 μL media and incubated with different concentrations of doxorubicin (0.19, 0.37, 0.75, 1.5, 3, 6.25, 12.5, 25, 50, 100, and 200 μM) for 24 h. Then 20 μL of MTS reagent was added and incubation proceeded for an additional 4 h in the 37°C incubator (5% CO_2). The absorbance density of each well was determined using an automated microplate reader (GloMax®-Multi Microplate Reader, Promega) at a wavelength of 49 nm. The IC50 value was determined using the GraphPad Prism 7.02 software (GraphPad software, La Jolla, CA) and represents the concentration of drug required to achieve a 50% inhibition of cell viability.

2.3 | Chromatin immunoprecipitation (ChIP)

ChIP was carried out as described previously.[35] Briefly, 2×10^7 control cells (SC, expressing the scrambled control shRNA) or TFII-I depleted K562 cells (transfected with TFII-I shRNA expression construct) were crosslinked in 1% formaldehyde for 10 min at room temperature (RT) and the reaction was quenched with glycine (125 mM). The chromatin was subjected to sonication to obtain 200–600 bp fragments. Cells were lysed and lysates were subjected to a pre-clearing step by incubation with IgG (Santa Cruz Biotechnology, Santa Cruz, CA, sc-2025) for 2 h at 4°C, and incubation with Dynabeads Protein A/G (Life Technologies, AS. Oslo Norway). After a 5 min placement on a magnetic rack the supernatant was incubated with specific antibody on a rotating wheel at 4°C overnight (Information with regard to antibodies is provided in the supplement). After several washes in low-salt, high-salt, and LiCl washes, crosslinking was reversed at 65°C overnight, and the DNA was purified by phenol, chloroform, isoamylalcohol, and chloroform extractions and precipitated with 2.5× (v/v) 100% ethanol. The DNA pellet was then washed in 70% ethanol and resuspended in 10 mM Tris-Cl (pH 8.5) and analyzed by qPCR as described previously.[35] qPCR was carried out using the primers shown in Supplementary Table S1.

2.4 | Co-immunoprecipitation and immunoblotting

Co-immunoprecipitation was essentially carried out as described previously,[36] with some minor modifications as outlined by Fan et al.[16] Briefly, antibodies (6–8 μg per 500 μg protein) were added to magnetic protein G beads (50 μg per 500 μg protein, Dynabeads, Protein G, Life Technologies AS), previously washed three times in PBS and twice in 100 mM sodium citrate (pH 5.0), in 1 mL HENG buffer (10 mM HEPES-KOH, pH 9.0, 1.5 mM MgCl2, 0.25 mM EDTA, 20% glycerol, 1 mM PMSF, and 1 mM DTT). After incubation for 2 h at RT the beads were rinsed twice with 100 mM sodium citrate (pH 5.0) and once with 200 mM triethanolamine (pH 8.2) and incubated in 20 mM dimethyl pimelimidate dihydrochloride (Sigma Aldrich, St. Louis, MO, D8388, in 200 mM triethanolamine, pH 8.2) for 45 min at RT while rotating. After washing the beads once with 50 mM Tris, pH 7.5, and three times with PBS containing 0.01% Tween 20, the IgG and antibody beads were blocked with 200 μg/mL chicken egg albumin (CEA) at RT for 1 h while rotating. Nuclear protein extracts were treated with 2.5 μL benzonase (Novagen/Thermo Fisher Scientific) per 500 μg protein for 30 min at RT and then diluted with HENG buffer to bring the KCl concentration to 125 mM. After preclearing the nuclear extracts with the IgG beads for 1 h at 4°C they were incubated with the antibody beads overnight at 4°C while rotating. The beads were washed 5 times for 5 min with HENG wash buffer (HENG buffer plus 300 mM KCl) and rinsed twice in PBS at 4°C before the proteins were eluted off the beads by incubation with 30 μL 1 × Laemmli buffer for 10 min at 80°C. Immunoblotting was carried out as described by Barrow et al.[35] Briefly, 10–20 μg protein was electrophoresed in 4–15% (wt/vol) TGX-Tris–HCl gels (Bio-Rad, Hercules, CA), transferred to Polyvinylidene fluoride (PVDF) membrane) and incubated with antibodies: The following antibodies were used in the experiments: αTFII-I (sc-9943), αE2F1 (sc-251), αE2F6 (sc-390022), rabbit αIgG (sc-2027), mouse αIgG (sc-2025).

2.5 | RNA extraction, cDNA synthesis, and quantitative PCR

RNA was isolated from K562 cells expressing TFII-I- or SC-shRNA using the RNeasy kit (Qiagen, Hilden, Germany) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was carried out as described by Barrow et al,[35] using the primers listed in the supplementary information.

3 | RESULTS

The previous analysis of the genome-wide occupancy of TFII-I in K562 cells revealed that TFII-I peaks were frequently associated with binding sites for the E2F family of transcription factors.[16] We analyzed E2F4 and E2F6 ChIP-seq data from K562 cells using the ENCODE database (The ENCODE Project[37]; The ENCODE Project[38]). There were 1015 chromosomal loci at which we detected association of TFII-I with E2F4 and E2F6 (Fig. 1A). The sites of overlap of TFII-I and E2F binding were distributed equally at promoters, introns, or intergenic regions.

The observation that 805 of the TFII-I/E2F peaks (about 80%) also revealed binding peaks for transcription factor USF1 (Fig. 1B) is consistent with previous findings showing that TFII-I and USF interact with each other.[4,6] Frequently, ChIP-seq tags of USF, TFII-I, and E2F4 distribute surrounding RNA polymerase II (Pol II) occupancy genome-wide (Fig. 1C), consistent with the notion that these proteins are primarily involved in transcription regulation. We next analyzed the presence of specific transcription factor binding motifs in TFII-I/E2F4/E2F6/USF1 binding peaks. About half of promoter associated peaks contained binding sites for E2F4 and E2F6 while about a quarter contained E-box motifs, which are binding sites for USF transcription factors (Table 1). The situation was different at non-promoter associated regions at which 43% of TFII-I/E2F4/E2F6/USF1 overlapping peaks contained a binding site for the erythroid specific transcription factor GATA1 and only about a quarter contained binding sites for E2F4, E2F6, or USF (Table 1). Our previous analysis of TFII-I only peaks identified the E-Box motif as well as binding sites for transcription factors Hbp1, NFATC, and GATA1/2 as the most common motifs.[16]

TFII-I and E2F transcription factors have been shown to regulate genes implicated in cell cycle regulation. We used the KEGG pathway (hsa04110) and the GO set (GO: 0007049), to identify TFII-I and E2F occupied loci near cell cycle genes. We identified 20 genes implicated in cell cycle regulation that had overlapping peaks for TFII-I and E2F4/6 nearby (Fig. 1D and Table 2). The list of genes include *CDC27* (cell division cycle 27), *CDKN1C* (cyclin dependent kinase inhibitor 1C), *E2F2*, and *HDAC1* (histone deacetylase 1). Taking advantage of the ENCODE database we provide a few examples for the association of TFII-I and E2Fs at selected gene loci in K562 cells. As shown in Fig. 2, TFII-I bound in close proximity to E2F4 and E2F6 at sites

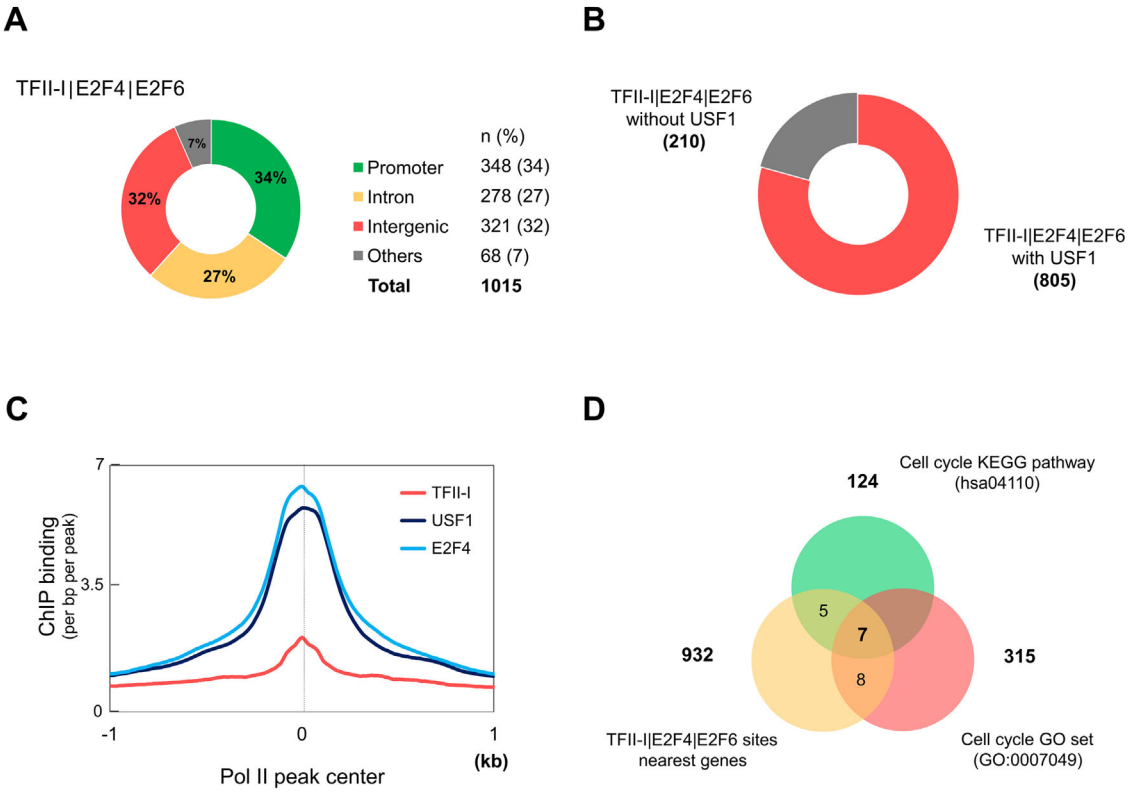

FIGURE 1 Bioinformatics analysis of TFII-I, USF1, and E2F transcription factor DNA-occupancy in K562 cells. (A) Genomic location annotation of chromosomal loci occupied by TFII-I, E2F4, and E2F6. (B) Number of TFII-I/E2F4/E2F6 occupied sites in the genome with and without USF1. (C) Density plot of TFII-I, E2F4, and USF1 ChIP-seq tags with respect to Pol II peaks in the genome. (D) Fraction of TFII-I/E2F4/E2F6 occupied sites close to genes implicated in cell cycle regulation

TABLE 1 TFII-I/E2F4/E2F6/USF1 overlapping peaks motif analysis

	Promoter (254)				Non-promoter regions (493)		
	Motif	% of targets sequences with motif	Fold enrichment relative to background sequences		Motif	% of targets sequences with motif	Fold enrichment relative to background sequences
E2F4		46.1	1.32	Gatal		43.4	3.12
E2F6		47.2	1.22	E2F4		21.5	1.49
USF1		24.0	1.48	E2F6		26.4	1.44
				USF1		26.4	1.89

TABLE 2 Cell cycle genes associated with TFII-I & E2Fs overlapping peaks

Gene name	Distance to TSS (bp)	Gene description
ABL1	25 557	ABL proto-oncogenel, non-receptor tyrosine kinase
ANAPC1	−287	Anaphase promoting complex subunit 1
ARAP1	−6805	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1
CCND3	17 624	Cyclin D3
	31 548	
CDC27	159	Cell division cycle27
	52 107	
	52 736	
CDKN1C	−4929	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CUL5	187	Cullin5
E2F2	−188	E2F transcription factor 2
GADD45A	49 047	Growth arrest and DNA-damage-inducible, alpha
GFI1B	−303	Growth factor independent 1B transcription repressor
	−90	
HDAC1	−77	Histone deacetylase 1
HUS1	310	HUS1 checkpoint clamp component
ORC5	70	Origin recognition complex subunit 5
PKMYT1	−394	Protein kinase, membrane associated tyrosine threonine 1
SERTAD1	−8185	SERTA domain containing 1
SPHK1	20 863	Sphingosine kinase 1
TIPIN	−137	TIMELESS interacting protein
UPF1	−174	UPF1 regulator of nonsense transcripts homolog (yeast)

near the *CDC27* and *CDKN1C* genes. There were multiple sites of overlap of TFII-I and E2F4/6 in the *CDC27* gene locus. Several of those sites mapped to gene internal regions while one was associated with the promoter (green triangles), which was associated with a Pol II peak and increased levels of H3K4me3. The gene internal sites also harbored histone marks typically associated with enhancer elements, H3K4me1 and H3K27ac.[39] A single site of transcription factor overlap was found about 5 Kb upstream of the *CDKN1C* gene. Again, this site coincided with histone marks associated with enhancer function. A single site of TFII-I/E2F4/E2F6 occupancy was found in the promoter of the *HDAC1* gene, which was associated with high levels of H3K4me3. Interestingly, we also found overlapping association of TFII-I and the repressor E2Fs at the *DNA-Methyltransferase 1* (*DNMT1*) gene, which maintains DNA methylation patterns during DNA synthesis.[40] Again, a single site of overlap was associated with the TSS. All of the sites occupied by TFII-I, E2F4, and E2F6 shown here contained binding peaks for USF1.

To examine interactions between TFII-I and E2Fs we performed co-immunoprecipitation experiments (Fig. 3A). The TFII-I antibody efficiently pulled-down TFII-I protein. Importantly, we found E2F1 and E2F6 in protein complexes precipitated with TFII-I specific antibodies. It should be mentioned that we used stringent conditions in the Co-IP experiments including the treatment of the protein extracts with benzonase which excludes the possibility that the interactions between TFII-I and E2F6 are mediated by DNA or RNA. Co-IP experiments with E2F4 antibodies did not yield conclusive results.

We previously demonstrated that siRNA mediated knockdown of TFII-I expression led to a reduction in expression of the *DNMT1* gene.[16] We generated stable K562 cell lines that express either shRNA directed against TFII-I (TFII-I KD) or a scrambled control (SC) shRNA. TFII-I expression was significantly suppressed in the TFII-I KD cells (Fig. 3B,C). We confirmed that *DNMT1* expression was reduced in the TFII-I KD cells compared to the SC control

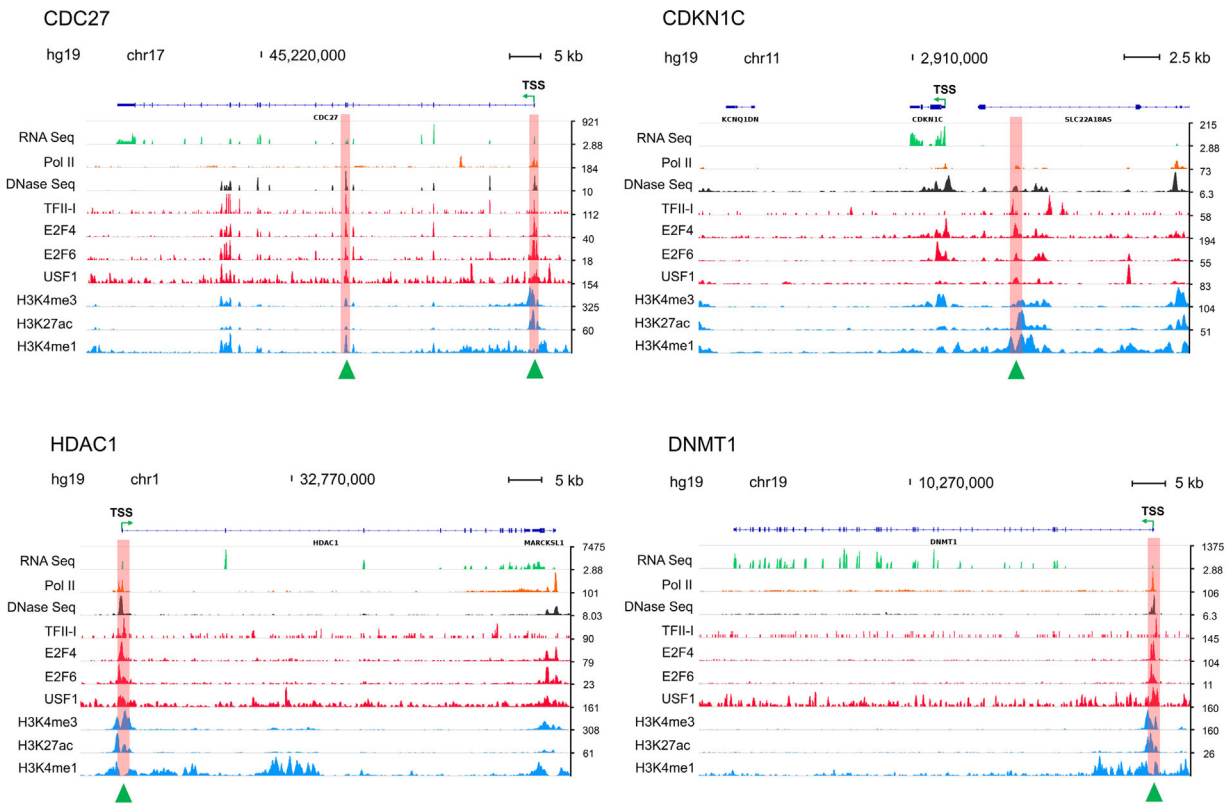

FIGURE 2 Occupancy of Pol II, TFII-I, E2F4, E2F6, and USF1 and histone modifications at the *CDC27*, *CDKN1C*, *DNMT1*, and *HDAC1* gene loci in K562 cells. Graphic representation of transcription (RNA Seq), DNase I sensitivity (DNase Seq), transcription factors occupancy, and enrichment of histone modifications (trimethylation of H3K4, H3K4me3; monomethylation of H3K4, H3K4me1; and acetylation of H3K27, H3K27ac) at the four different gene loci. Green arrows indicate the direction of transcription. The green triangles point to the site of overlap of the transcription factors

cells (Fig. 3C). Interestingly, we also detected a significant decrease in expression of the E2F4 and E2F6 genes, which was confirmed by western blot analysis (Fig. 3B,C). Consistent with the occupancy profile of TFII-I we observed decreased expression of the *DNMT1*, *HDAC1*, *E2F2*, *CDC27*, and *CDKN1C* genes (Fig. 3C). Reduction in HDAC1 expression in TFII-I KD cells did not quite reach statistical significance ($P = 0.054$). Expression of the control PGK1, CTCF, and GAPDH genes was not affected by diminished expression of TFII-I (Fig. 3B,C). Despite interactions of TFII-I with the *Cul5*, *GFI1B*, and *GADD45A* gene loci (Table 2) we did not detect a statistically significant reduction in the expression of these genes in TFII-I KD cells.

We next performed chromatin immunoprecipitation (ChIP) experiments to determine the occupancy of TFII-I, E2F4, E2F6, and Pol II, at the TFII-I peaks in the *CDC27*, *CDKN1C*, *DNMT1*, and *HDAC1* gene loci in K562 cells expressing shRNA directed against TFII-I (TFII-I KD) or in cells expressing the SC-shRNA (SC) (Fig. 4). As a negative control, we examined binding of these proteins to the promoter of the neuronal *necdin* gene. We observed efficient binding of all the proteins to most of the TFII-I peaks shown in Fig. 2 with the exception of the CDC27 promoter region which revealed background levels of binding that was similar to what was detected in the *necdin* gene. In TFII-I KD cells the binding of TFII-I and E2Fs at the *CDC27*, *CDKN1C*, *DNMT1*, and *HDAC1* gene loci is reduced compared to occupancy in SC cells demonstrating that TFII-I is required for the efficient interaction of E2F4 and E2F6 to these gene loci. Pol II binding to the HDAC1 gene locus was not as much affected as binding at the DNMT1 promoter in TFII-I KD cells consistent with our observation that expression of DNMT1 was reduced to lower levels compared to expression of HDAC1. Together the data demonstrate that TFII-I is required for E2F4/6 occupancy at several genes involved in cell cycle regulation.

TFII-I has been implicated in the process of cellular proliferation likely due to its involvement in the regulation of cell cycle genes.[1,2] We analyzed proliferation of K562 cells expressing the scrambled shRNA (SC) or the shRNA directed against TFII-I (TFII-I KD). The results demonstrate that reduced expression of TFII-I caused a two- to three-fold decrease in the proliferation rate of K562 cells (Fig. 5A). Doxorubicin is a DNA intercalating and cytotoxic drug used

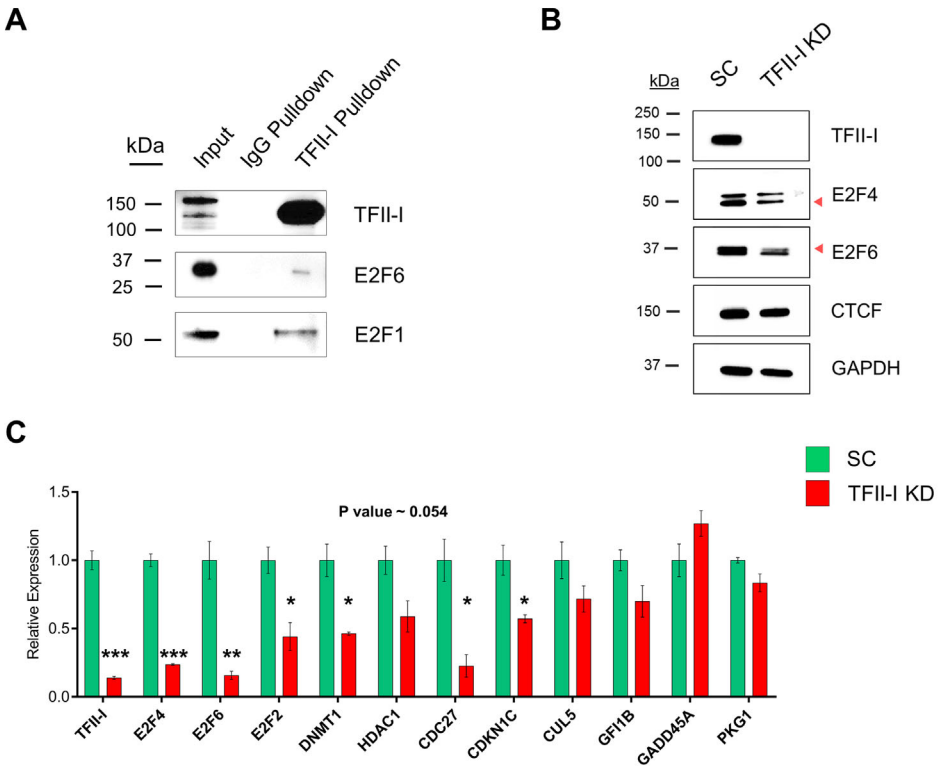

FIGURE 3 Interaction of TFII-I with E2F1 and E2F6 and the role of TFII-I in transcription of specific cell cycle and control genes. (A) Co-immunoprecipitation (Co-IP) analysis of TFII-I, E2F6, and E2F1 with whole cell extracts from K562 cells. Protein extracts were precipitated with TFII-I specific antibodies and analyzed by western blotting using TFII-I, E2F6, or E2F1 specific antibodies. (B) Western blot analysis of TFII-I, E2F4, E2F6, CTCF, and GAPDH in TFII-I KD and SC control cells. (C) Reverse transcriptase-qPCR analysis of gene expression in SC and TFII-I KD cells. RNA was isolated from the K562 cells expressing TFII-I specific or scrambled shRNA and reverse transcribed. The cDNA was analyzed by qPCR using primers specific for the genes as indicated. The experiment has been repeated three times ($n = 3$) and the error bars reflect the standard-error of the mean (***$P < 0.001$. **$P < 0.01$; *$P < 0.05$)

in chemotherapy.[41] We investigated if diminished expression of TFII-I changed the sensitivity toward doxorubicin toxicity. Indeed, we found that reduced expression of TFII-I increased the sensitivity toward doxorubicin mediated cell death at lower doxorubicin concentrations (Fig. 5B). The IC50 of doxorubicin was fourfold higher in SC cells compared to the TFII-I KD cells.

4 | DISCUSSION

Transcription factor TFII-I is unusual in terms of its structure and function.[1,2] It consists of multiple protein/protein interaction domains and functions both inside and outside the nucleus. In the nucleus TFII-I associates with co-regulatory proteins to activate or repress transcription of genes.[1,2] Recent studies have shown that TFII-I is also involved in DNA-translesion repair.[42] Our previous analysis of genome-wide occupancy of TFII-I revealed that it binds to both active and repressed genes.[16] We observed that at several stress-induced genes, TFII-I associated immediately downstream of Pol II peaks suggesting that it keeps the genes in a poised but repressed configuration and that it may assist Pol II in transitioning from the paused to the elongation active state. In agreement with this hypothesis we found that TFII-I interacts with transcription elongation factors, including Elongin A, that ER-stress induced transcription downstream of a TFII-I/Pol II associated peak in the ATF3 gene locus, and that reducing TFII-I activity led to a reduction in expression of the stress response gene ATF3.[16]

In the previous study, we noticed that TFII-I peaks are often associated with binding sites for E2F transcription factors.[16] Indeed, we now identified 1015 genomic sites at which TFII-I peaks are also associated with E2F4 and E2F6. 805 of the sites occupied by TFII-I and E2Fs are also associated with transcription factor USF1. This is consistent with previous data demonstrating physical interactions between TFII-I and the USF proteins.[4,6] Overlapping peaks for TFII-I, E2F4 and E2F6 frequently harbor binding motifs for E2F transcription factors or E-box motifs. The E-box found in the TFII-I/E2F bound loci at gene proximal or distal regulatory elements could associate with USF or other E-box

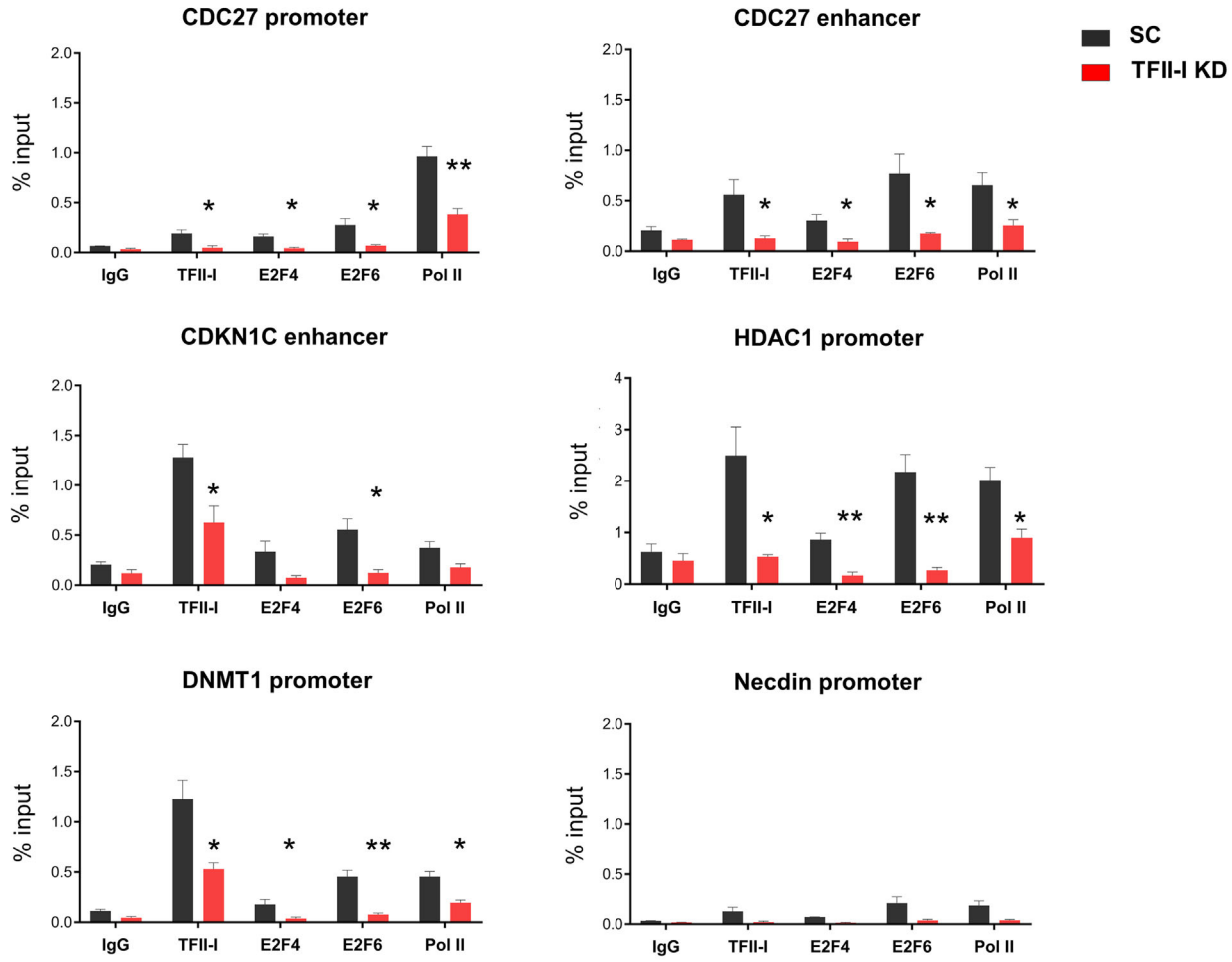

FIGURE 4 TFII-I mediated interactions of E2F4, E2F6, and Pol II with specific cell cycle gene genes in K562 cells. ChIP analysis of TFII-I, E2F4, E2F6, and Pol II at the *CDC27* (putative enhancer and promoter), *CDKN1C*, *DNMT1*, and *HDAC1* gene loci as well as at the negative control gene, *necdin*. Chromatin from TFII-I KD and SC control cells was crosslinked, fragmented, and precipitated with control IgG antibodies or with antibodies specific for TFII-I, E2F4, E2F6, and Pol II as indicated. The purified DNA was analyzed by qPCR using primers specific for the indicated genes. The ChIP experiments have been performed three times and PCRs were performed in triplicate. The error bars reflect the standard-error of the mean (**$P < 0.01$; *$P < 0.05$)

binding proteins, for example, Tal1.[43,44] Interestingly, distal sites occupied by TFII-I and E2Fs often harbor GATA-binding sites. In K562 cells GATA sites are bound by either GATA1 or GATA2 which play important roles in the differentiation and specification of erythroid cells.[45]

We found that TFII-I and the so-called repressor E2Fs occupy regulatory elements associated with genes encoding for cell-cycle regulators, including the *CDC27*, *CDKN1C*, and *HDAC1* genes. Previous studies showed that TFII-I regulates expression of the cyclinD1 gene in NIH3T3 cells.[19] We did not observe occupancy of TFII-I at the cyclin D1 gene in K562 cells, which may be due to the specific isoform of TFII-I tagged for the pull-down experiments or due to cell-type specific differences. TFII-I, E2F4, E2F6, and USF1 were also located at the TSS of the *DNMT1* gene, which also plays an important role in the cell cycle by maintaining DNA methylation patterns. Interestingly, a previous study found interactions between HDAC1, DNMT1, and E2F transcription factors and demonstrated that this protein complex repressed E2F target genes.[46] Reduced expression of TFII-I caused a reduction in the association of E2F transcription factors as well as Pol II and also resulted in a decrease in transcription of the *DNMT1*, *HDAC1*, *CDC27*, and *CDKN1C* genes. We demonstrated that TFII-I interacts with E2F6 and E2F1 using Co-IP experiments. This suggests that TFII-I directly assists in the recruitment of E2F transcription factors to co-occupied cell cycle genes. On the other hand, expression of E2F transcription factors was also reduced in cells with diminished TFII-I levels. The gene loci encoding the E2F4 and E2F6 transcription factors were not associated with peaks for TFII-I. Thus, it is possible that TFII-I regulates E2F chromatin associations directly and indirectly. Previous studies have shown that TFII-I mediates the association of the insulator protein CTCF with chromatin at specific loci.[47]

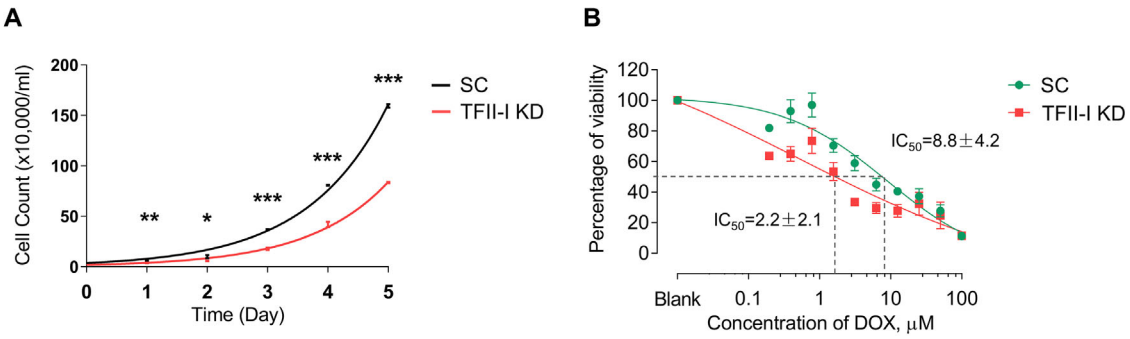

FIGURE 5 Reduced proliferation and increased sensitivity toward doxorubicin in TFII-I deficient K562 cells. (A) For the proliferation assay SC or TFII-I KD cells were seeded at a density of 1×10^4 cells per well in a 96 well plate. Cells (10 μL) were removed, stained with tryphan blue, and unstained cells were counted as viable. The experiment has been repeated three times ($n = 3$) and the error bars reflect the standard-error of the mean (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$). (B) Doxorubicin sensitivity assay. SC or TFII-I KD cells were incubated with different concentrations of doxorubicin and subjected to the MTS cytotoxicity assay. Viable cells were plotted against the doxorubicin concentration. The IC50 indicates the concentration of doxorubicin at which half of the cells are apoptosed. The experiments have been repeated four times ($n = 4$). The IC50 is represented as the best-fit value ± standard error

Thus, assisting transcription factor binding to chromatin may be a major aspect of TFII-I function in the nucleus.

We do not yet know if interactions of TFII-I with E2F transcription factors are direct or mediated by other proteins. We used stringent conditions in the Co-IP experiments including treatment of protein extracts with nucleases. This excludes the possibility that interactions between TFII-I and E2Fs are mediated by DNA or RNA. Both E2Fs and TFII-I contain leucine zipper motifs and thus direct interactions are feasible.[1,2,20] E2F4 lacks a nuclear localization sequence (NLS) and has been shown to shuttle between cytoplasm and nucleus.[20] As nuclear localization of TFII-I has been shown to be regulated by phosphorylation it is possible that TFII-I may assist E2F4 translocation to the nucleus.[1,2] There is precedence for the involvement of TFII-I in the nuclear transportation of transcription factors.[11,48]

The observation that reduced association of the repressor E2Fs, due to TFII-I deficiency, leads to decreased transcription may be counterintuitive. However, recent evidence demonstrated that E2F4 can function as a transcription activator.[49] It contains a transcription activation domain that is masked in the E2F/Rb complex. Several studies have shown that E2F4 can directly activate the transcription of genes in an Rb-independent manner.[50,51] Furthermore, it is possible that the reduction of E2F4/6 occupancy is also accompanied by a reduction in activator E2Fs (eg, E2F1 or E2F2) and that the association of these proteins with the target genes is also mediated by TFII-I. Consistent with this hypothesis is our observation that TFII-I interacts with E2F1. Furthermore, *E2F2* is among the genes occupied by TFII-I and thus, the repressor E2Fs could be regulated by TFII-I indirectly (Table 1). Involvement of TFII-I in the repression and activation of genes occupied by E2F transcription factors is consistent with previous observations showing that TFII-I is associated with active and repressed genes.[16] We previously demonstrated that TFII-I interacts with Brg1 containing chromatin remodeling complexes as well as with topoisomerases and Elongin A.[16] It is possible that TFII-I recruits Brg1, HDAC, and repressor E2Fs to regulatory elements in cell cycle as well as stress response genes to keep these elements in a poised but open configuration. Upon activation of these genes, HDACs and repressor E2Fs dissociate and TFII-I mediates the recruitment of activator E2Fs together with transcription elongation factors and topoisomerases. The scenario described here may be similar to one described for the immunoglobulin (*Ig*) heavy chain gene.[52] At this *Ig* gene locus TFII-I has previously been shown to switch from repressor to activator during activation of transcription. The switch is mediated by the transcription co-activator OCA-B which displaces HDACs and mediates interactions between the promoter and enhancers.

TFII-I and E2Fs may regulate genes in a coordinated fashion. The target genes may not only be involved in response to cell cycle progression but may also involve genes activated by cellular stress or genes involved in the DNA damage response. Both TFII-I and E2Fs have been implicated in these processes.[1,2,20]

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CONFLICT OF INTEREST

The authors declare that they do not have a conflict of interest with the contents of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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