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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 towards 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

TFII-I; E2F; Gene Regulation; Cell Cycle

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 [Roy, 2001;

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Roy, 2012]. TFII-I consists of multiple protein/protein interaction domains including 6 R-repeats and a leucine zipper [Doi-Katayama et al., 2007]. 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 [Crusselle-Davis et al., 2006; Du et al., 1993; Roy et al., 1993]. TFII-I belongs to a family of related proteins including GTF2IRD1 (also known as Ben) and GTF2IRD2 [Pober, 2010; Roy, 2001; Tassabehji et al., 2005]. 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 [Pober, 2010]. Disruption of either the *Gtf2i* or the *Gtf2ird1* gene in mice recapitulates some of the phenotypes seen in WB syndrome [Enkhmandakh et al., 2009; Tassabehji et al., 2005].

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 [Roy et al., 1991]. However, TFII-I is not a general transcription factor of Pol II but rather positively or negatively modulates expression of genes involved in cell cycle regulation, DNA repair, cellular differentiation, or genes induced upon stress signals [Roy, 2001; Roy, 2012]. TFII-I has also been shown to exert functions outside the nucleus by facilitating nuclear import of transcription factors (e.g. c-Rel) or by inhibiting agonist induced calcium entry through interactions with the transient receptor potential cation channel subfamily 3 member (TRPC-3) [Caraveo et al., 2006; Montano et al., 1996]. There are four isoforms of TFII-I that are generated by alternative splicing [Roy, 2001; Roy, 2012]. The β -isoform appears to be located primarily in the nucleus and represses transcription through interactions with histone deacetylases (HDACs) and other co-repressors [Crusselle-Davis et al., 2007; Tussié-Luna et al., 2002]. The δ -isoform shuttles between cytoplasm and nucleus and is mostly involved in transcription activation [Roy, 2001; Roy, 2012]. 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 [Parker et al., 2001]. TFII-I also interacts with the gene locus encoding the stress-response transcription factor ATF3 [Fan et al., 2014]. 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 [Andersson et al., 2008; Fan et al., 2014; Weems et al., 2015].

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 [Desgranges et al., 2005; Roy, 2001]. Previous genome-wide analysis revealed that TFII-I peaks are frequently associated with binding sites for E2F transcription factors, specifically E2F4 and E2F6 [Fan et al., 2014]. E2Fs are major regulators of genes driving the cell cycle during cellular proliferation [Trimarchi and Lees, 2002]. 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 [Trimarchi and Lees, 2002]. 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 [Trimarchi and Lees, 2002]. E2Fs, like TFII-I, contain leucine zipper motifs with which they interact with the DPs and perhaps other proteins [Trimarchi and Lees, 2002]. Like TFII-I, E2Fs have been implicated in the regulation of the cell cycle as well as the stress- and DNA damage-response [Dominguez-Brauer et al., 2009; Grant et al., 2013; Polager and Ginsberg, 2003]. Several studies have shown that E2Fs are involved in the regulation of hematopoiesis mostly through their known effect on proliferation [Kinross et al., 2006; Li et al., 2003]. 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 [Yang et al., 2016; Zhang et al., 2010]. Moreover, E2F2 is required for nuclear condensation during the final stages of erythroid maturation [Swartz et al., 2017; Tallack et al., 2009].

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 towards doxorubicin. The data thus suggests that TFII-I is an important mediator of the function of E2F transcription factors.

Materials and Methods

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 [Gerstein et al., 2012]. The raw SRA dataset of TFII-I was downloaded from GSE51065 and aligned to the human genome hg19 by Bowtie 2 with default settings [Langmead and Salzberg, 2012]. A bigwig file was generated using 'BAM to BigWig' under the Galaxy project online tools [Goecks et al., 2010]. 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 [Thorvaldsdóttir et al., 2013]. The binding peaks of TFII-I, E2F4, E2F6, and USF1 were defined as overlapping if they occurred within a 500bp window size (findPeaks.pl, HOMER 4.6) [Heinz et al., 2010]. 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 [Heinz et al., 2010].

Cell Culture and Transfection

Human erythroleukemia K562 cells were grown in RPMI (Corning, Cellgro) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were grown at 37°C under 5% CO₂ at a density between 1×10⁵ and 2×10⁶ cells/ml. To reduce expression of TFII-I (GTF2i), K562 cells were transfected with the plasmid pGIPZ-shTFII-I (Thermo Scientific). Control cells were transfected with a plasmid expressing a scrambled control shRNA (pGIPZ-shSc). Transfections were performed using lipofectamin 2000 (Invitrogen) according to the procedure provided by the manufacturer. Briefly, 5×10⁵ cells were transfected with 5 µg DNA in 2ml 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'-TTCATACACTGCAATGCAG-3'. shRNA SC, 5'-TCTCGCTTGGGCGAGAGTAAG-3'. The proliferation assay was performed by seeding 1×10⁴ SC or TFII-I KD cells per well in 6 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 trypan blue. Doxorubicin-mediated toxicity was assessed using the MTS cytotoxicity assay from Promega (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA). Briefly, 2×10⁴ 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% CO₂). 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) and represents the concentration of drug required to achieve a 50% inhibition of cell viability.

Chromatin Immunoprecipitation (ChIP)

ChIP was carried out as described by previously [Barrow et al., 2012]. Briefly, 2×10⁷ 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 to 600 bp fragments. Cells were lysed and lysates were subjected to a pre-clearing step by incubation with IgG (Santa Cruz, 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

[Barrow et al., 2012]. qPCR was carried out using the primers shown in supplementary table S1.

Co-Immunoprecipitation and Immunoblotting

Co-immunoprecipitation was essentially carried out as described previously [Rodriguez et al., 2005], with some minor modifications as outlined by Fan *et al.* [Fan et al., 2014]. Briefly, antibodies (6 to 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., Oslo, Norway), previously washed 3 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, 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) 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 10min at 80°C. Immunoblotting was carried out as described by Barrow *et al.* [Barrow et al., 2012]. Briefly, 10 to 20 µg protein was electrophoresed in 4-15% (wt/vol) TGX-Tris-HCl gels (Bio-Rad), 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).

RNA extraction, cDNA synthesis, and quantitative PCR

RNA was isolated from K562 cells expressing TFII-I- or SC- shRNA using the RNeasy kit (Qiagen) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was carried out as described by Barrow *et al.* [Barrow et al., 2012], using the primers listed in the supplementary information.

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 [Fan et al., 2014]. We analyzed E2F4 and E2F6 ChIP-seq data from K562 cells using the ENCODE database (The ENCODE Project Consortium, 2011; The ENCODE Project Consortium, 2012) [Consortium, 2011; Consortium, 2012]. There were 1015 chromosomal loci at which we detected association of TFII-I with E2F4 and E2F6 (Figure 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 [Crusselle-Davis et al., 2006; Du et al., 1993]. 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 [Fan et al., 2014].

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 figure 2, TFII-I bound in close proximity to E2F4 and E2F6 at sites 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 [Kim and Shiekhhattar, 2015]. 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 [Jeltsch and Jurkowska, 2016]. 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 (Figure 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 [Fan et al., 2014]. 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 and C). We confirmed that *DNMT1* expression was reduced in the TFII-I KD cells compared to the SC control 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 and C). Consistent with the occupancy profile of TFII-I we observed decreased expression of the *DNMT1*, *HDAC1*, *E2F2*, *CDC27*, and *CDKN1C* genes (Figure 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 and 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 figure 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 [Roy, 2001; Roy, 2012]. 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 in chemotherapy [Cappetta et al., 2017]. We investigated if diminished expression of TFII-I changed the sensitivity towards doxorubicin toxicity. Indeed, we found that reduced expression of TFII-I increased the sensitivity towards doxorubicin mediated cell death at lower doxorubicin concentrations (Fig 5B). The IC50 of doxorubicin was four-fold higher in SC cells compared to the TFII-I KD cells.

Discussion

Transcription factor TFII-I is unusual in terms of its structure and function [Roy, 2001; Roy, 2012]. 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 [Roy, 2001; Roy, 2012]. Recent studies have shown that TFII-I is also involved in DNA-translesion repair [Fattah et al., 2014]. Our previous analysis of genome-wide occupancy of TFII-I revealed that it binds to both active and repressed genes [Fan et al., 2014]. 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 [Fan et al., 2014].

In the previous study we noticed that TFII-I peaks are often associated with binding sites for E2F transcription factors [Fan et al., 2014]. 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 [Crusselle-Davis et al., 2006; Du et al., 1993]. 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 binding proteins, e.g. Tal1 [Anantharaman et al., 2011; Rojas-Sutterlin et al., 2014]. 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 [Bresnick et al., 2012].

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 [Desgranges et al., 2005]. 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 [Robertson et al., 2000]. 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 [Peña-Hernández et al., 2015]. 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 [Roy, 2001; Roy, 2012; Trimarchi and Lees, 2002]. E2F4 lacks a nuclear localization sequence (NLS) and has been shown to shuttle between cytoplasm and nucleus [Trimarchi and Lees, 2002]. 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 [Roy, 2001; Roy, 2012]. There is precedence for the involvement of TFII-I in the nuclear transportation of transcription factors [Montano et al., 1996; Tussié-Luna et al., 2001].

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 [Hsu and Sage, 2016]. 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 [Lee et al., 2011; Ma et al., 2014]. Furthermore, it is possible that the reduction of E2F4/6 occupancy is also accompanied by a reduction in activator E2Fs (e.g. 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 [Fan et al., 2014]. We previously demonstrated that TFII-I interacts with Brg1 containing chromatin remodeling complexes as well as with topoisomerases and Elongin A [Fan et al., 2014]. 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 [Ren et al., 2011]. 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 [Roy, 2001; Roy, 2012; Trimarchi and Lees, 2002].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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.

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.

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 (***, $p < 0.001$. ** $p < 0.01$; * $p < 0.05$)

Figure 4. TFII-I mediated interactions of E2F4, E2F6, and Pol II with specific cell cycle gene loci 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).

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 μ l) were removed, stained with trypan 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 IC_{50} indicates the concentration of doxorubicin at which half of the cells are apoptosed. The experiments have been repeated four times ($n=4$). The IC_{50} is represented as the best-fit value $\pm$ standard error.

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
46.1%	1.32		43.4%	3.12
47.2%	1.22		21.5%	1.49
24.0%	1.48		26.4%	1.44
			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	25557	ABL proto-oncogene 1, non-receptor tyrosine kinase
ANAPC1	-287	anaphase promoting complex subunit 1
ARAP1	-6805	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1
CCND3	17624	cyclin D3
	31548	
	52107	
CDC27	159	cell division cycle 27
	52736	
	52736	
CDKN1C	-4929	cyclin-dependent kinase inhibitor 1C (p57, Kip2)
CUL5	187	cullin 5
E2F2	-188	E2F transcription factor 2
GADD45A	49047	growth arrest and DNA-damage-inducible, alpha
GF11B	-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	20863	sphingosine kinase 1
TIPIN	-137	TIMELESS interacting protein
UPF1	-174	UPF1 regulator of nonsense transcripts homolog (yeast)
WEE1	-21557	WEE1 G2 checkpoint kinase
ZBTB17	23222	zinc finger and BTB domain containing 17