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Methods paper

TFII-I regulates target genes in the PI-3K and TGF-β signaling pathways through a novel DNA binding motif

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ABSTRACT

General transcription factor (TFII-I) is a multi-functional protein involved in the transcriptional regulation of critical developmental genes, encoded by the *GTF2I* gene located on chromosome 7q11.23. Haploinsufficiency at *GTF2I* has been shown to play a major role in the neurodevelopmental features of Williams–Beuren syndrome (WBS). Identification of genes regulated by TFII-I is thus critical to detect molecular determinants of WBS as well as to identify potential new targets for specific pharmacological interventions, which are currently absent. We performed a microarray screening for transcriptional targets of TFII-I in cortex and embryonic cells from *Gtf2i* mutant and wild-type mice. Candidate genes with altered expression were verified using real-time PCR. A novel motif shared by deregulated genes was found and chromatin immunoprecipitation assays in embryonic fibroblasts were used to document *in vitro* TFII-I binding to this motif in the promoter regions of deregulated genes. Interestingly, the PI3K and TGFβ signaling pathways were over-represented among TFII-I-modulated genes. In this study we have found a highly conserved DNA element, common to a set of genes regulated by TFII-I, and identified and validated novel *in vivo* neuronal targets of this protein affecting the PI3K and TGFβ signaling pathways. Overall, our data further contribute to unravel the complexity and variability of the different genetic programs orchestrated by TFII-I.

1. Introduction

General transcription factor (TFII-I) is member of an ubiquitously expressed, multifunctional transcription factor family which operate as a molecular switch to convey signals from multiple pathways and mediate cellular response (Roy, 2001). TFII-I was originally identified as a protein that binds to the initiator (Inr) core promoter element and was later shown to bind to various upstream elements that include the E box (Roy et al., 1997), the downstream immunoglobulin control element (DICE) sequence (Tantin et al., 2004) and the consensus BRGATTRBR sequence (Chimge et al., 2008). Structurally, TFII-I consists of multiple I-repeats, each of which contains a putative helix–loop–helix motif that is potentially important for protein–protein interactions (Roy, 2007). Growth factor signaling leads to rapid tyrosine phosphorylation, followed by nuclear translocation of TFII-I, and subsequent activation of target genes (Misra et al., 2009; Parker et al., 2001; Roy, 2007). In addition to its function as a transcription factor, cytosolic TFII-I regulates calcium homeostasis by modulating agonist-induced extracellular Ca^{2+} entry (Caraveo et al., 2006; Patterson et al., 2002). In the other hand, recent data suggest that other member of the family *GTF2IRD2* has evolved as an inhibitor of TFII-I (Palmer et al., 2012).

The gene encoding TFII-I, *GTF2I* (Entrez Gene ID 2969), has been mapped to an interval of the human chromosome 7q11.23, a region commonly deleted in Williams–Beuren syndrome (WBS; OMIM no. 194050). WBS is a rare developmental disorder with sporadic occurrence (1/7000-1/20000), characterized by mild intellectual disabilities with a cognitive profile including relatively preserved verbal skills, very deficient visuospatial abilities and a characteristic personality showing high sociability with strangers and increased anxiety (Morris, 2010). Additional features include craniofacial dysmorphism, growth retardation, odynoacusis, as well as cardiovascular, endocrine and connective tissue abnormalities (Morris et al., 1988; Tassabehji, 2003). The implication of *GTF2I* haploinsufficiency in the origin of this disorder is extensively documented but the concrete pathways and pathogenic mechanisms remain unclear (Antonell et al., 2010a; Ferrero et al., 2010; Sakurai et al., 2011). In mouse, the *Gtf2i* gene (Entrez Gene ID 14886, MGI 1202722) is located on chromosome 5G2. *Gtf2i* expression is important for embryonic development, as heterozygous knock-out mice featured anomalies similar to those observed in WBS: retarded growth, microcephaly, craniofacial and skeletal defects,

Abbreviations: WBS, Williams–Beuren syndrome; TFII-I, general transcription factor II-I; ES, embryonic stem cells; MEFs, mouse embryonic fibroblast; aCGH, microarray comparative genome hybridization; qRT-PCR, quantitative real-time polymerase chain reaction; ChIP, chromatine immunoprecipitation; CNVs, copy number variants; DEG, differentially expressed genes; NGF, nerve growth factor.

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odynacusis and hypersociability (Enkhmandakh et al., 2009; Lucena et al., 2010; Sakurai et al., 2011).

The mechanisms whereby decreased TFII-I can promote anomalies similar to those observed in WBS have been subject to exhaustive cellular and biochemical studies (Antonell et al., 2010a; Dai et al., 2009; Roy, 2007; Stasyk et al., 2005). Transcriptomic profiles resulting from changes in TFII-I dosage have been already defined in cell lines and tissues (Chimge et al., 2007b; Enkhmandakh et al., 2009). So far, involvement of TFII-I in gene regulation has been validated for 20 direct target genes involved in transcriptional regulation, chromatin remodeling, cell cycle, muscle development and neurogenesis (Chimge et al., 2008; Lazebnik et al., 2009; Makeyev et al., 2010, 2013). Herein, we have extended these observations by comparative transcriptome analysis of brain tissues and ES cells from mutants of TFII-I. Our data unveil the existence of a new consensus binding domain that increases the number of distinct transcriptional networks that depend on the TFII-I signals further endorsing the concept of the function domains as key regulator of the biochemical, genetic and biological outcomes of TFII-I.

2. Materials and methods

2.1. Animals and cell culture maintenance

Animals were maintained under standard animal housing conditions in a 12-h dark-light cycle with free access to food and water. Animal care was in accordance with ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by the Local Ethical Committee (IMAS-IMIM/UPF). Animals used were already described (Lucena et al., 2010). After behavioral phenotyping, cortex from *Gtf2i*$^{+/\Delta exon2}$ and WT animals were immediately dissected from fresh brains and quickly frozen by liquid nitrogen until RNA extractions. The age of mice vary between 20 and 25 weeks.

Clone XS0353 was obtained from the Sanger Institute Gene Trap Resource (SIGTR) mouse ES cell line collection (http://www.sanger.ac.uk/PostGenomics/genetrap/). aCGH analysis between XS0353 and wild-type cell lines did not shown neither major genomic alterations (deletions or duplications) in chromosome 5 nor in the rest of chromosomes. We found a total of 11 CNVs containing 24 genes (Table S1, supplementary information). None of them were found deregulated in the expression array analysis, in concordance with previous findings (Henrichsen et al., 2009). XS0353 cell line was cultured in standard conditions, Knockout DMEM medium (Invitrogen), supplemented with 20% Knockout Serum Replacement for ES cells (Invitrogen), LIF, β-mercaptoethanol, no essential aminoacids and penicillin/streptomycin. Cells were always cultured in a monolayer of feeder cells and maintained at 37 °C in a humidified 5% CO_2 chamber. We performed RT-PCR, qRT-PCR and western analysis to ensure the loss-of-function of the gene-trap allele (Fig. S1, supplementary information).

2.2. RNA isolation and quantification

Total RNA was extracted, from XS0353 cell line and cortex, by using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), followed by a second extraction using RNeasy (Qiagen) in both cases according to the manufacturer's instructions. Quality of all RNA samples was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). Only those samples with an RNA Integrity Number (RIN) >7 were used for hybridization.

2.3. Microarray and data analysis

Cortex tissues were hybridized to the Agilent 4x44K v1 Mouse Whole Genome chips. Fluorescent images were obtained with the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and TIFF images were quantified with the use of the Spot program (http://experimental.act.cmis.csiro.au/Spot/index.php) under the R environment (http://www.r-project.org). The resulting raw values were filtered and an intensity cut-off was applied, selecting those points with a foreground median/background median >3 in at least one channel. A series of programs, collectively packaged as Array File Maker 4.0 (AFM), were use to manipulate and manage DNA microarray data (Breitkreutz et al., 2001). AFM 4.0, Quantarray Data Handler 3.0, and Array Database 1.0 can be downloaded at the Tyers Lab Home Page [http://www.mshri.on.ca/tyers/] and are copyrighted against commercial gain.

ES cells were hybridized using duplicates to the GeneChip Mouse Genome 430 2.0. Array (Affymetrix) microarray images were processed with Microarray Analysis Suite 5.0 (Affymetrix).

All samples demonstrated characteristics of high-quality cRNA (3′/5′ ratio of probe sets for glyceraldehyde-3-phosphate dehydrogenase and β-actin of <1.5) and were subjected to subsequent analysis. Raw expression values obtained directly from CEL files were preprocessed using the RMA method (Irizarry et al., 2003), a three-step process which integrates background correction, normalization and summarization of probe values. These normalized values were the basis for all the analysis. Previous to any analysis, data were submitted to non-specific filtering to remove low signal genes (those genes whose mean signal in each group did not exceed a minimum threshold) and low variability genes (those genes whose standard deviation between all samples did not exceed a minimum threshold).

2.4. Statistical analysis

The selection of differentially expressed genes was performed using a linear model approach following the methodology ("Empirical Bayes and Linear Models for Microarrays") (Smyth, 2004) and implemented in the limma Bioconductor package.

Briefly, the method consists of fitting a linear model (similar to standard ANOVA) for each gene followed by variance regularization based on empirical Bayes modelling. The empirical Bayes analysis implemented yields several performance measures which help to decide which genes appear to be differentially express. We called differentially expressed those genes having a B statistic (it roughly indicates the logarithm of the odds that a gene is differentially expressed versus that it is not) greater than 0.

2.5. cDNA obtention, qRT-PCR experiments and data analysis

To validate the expression results obtained in the array analysis, 1 μg of the same mRNA used for the array hybridization was used for first-strand cDNA synthesis with Superscript II (Invitrogen). Primers and probes were designed to span an intron in all cases using the Primer3 software Version 0.4.0 (Rozen et al., 2000) (Table S2, supplementary information). Real-time PCR was performed using the SYBR Green Ready Master Mix according to the manufacturer's instructions on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The standard curve method was used for the analysis. The results were normalized respect to a housekeeping gene selected for its stable expression among the different tissues and cell lines. A reagent-only (no DNA) negative control sample was always included in each run. Experiments were performed a minimum of 3 times in 384-well plates with three replicates per sample. Raw data were obtained using SDS 2.4 software (Applied Biosystems).

2.6. Chromatin immunoprecipitation

ChIP experiments were performed using home-made MEF cell lines with endogenous TFII-I expression for mouse genes and Hela cell line was used in human analysis. We selected three wild-type cells lines and two homozygous (*Gtf2i*$^{\Delta ex2/\Delta ex2}$) and two heterozygous (*Gtf2i*$^{+/\Delta ex2}$) cell lines derived from embryos of *Gtf2i*$^{+/\Delta ex2}$ intercrosses. MEFs were