Published in final edited form as:

J Proteome Res. 2009 March ; 8(3): 1515–1526. doi:10.1021/pr800870d.

Transcription Factor TBX1 Overexpression Induces Downregulation of Proteins Involved in Retinoic Acid Metabolism: A Comparative Proteomic Analysis

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Abstract

TBX1 haploinsufficiency is considered a major contributor to the del22q11.2/DiGeorge syndrome (DGS) phenotype. We have used proteomic tools to look at all the major proteins involved in the TBX1-mediated pathways in an attempt to better understand the molecular interactions instrumental to its cellular functions. We found more than 90 proteins that could be targeted by TBX1 through different mechanisms. The most interesting observation is that overexpression of TBX1 results in down-regulation of two proteins involved in retinoic acid metabolism.

Keywords

DIGE; DiGeorge syndrome; proteomics; retinoic acid; T-box containing transcription factor

Introduction

The T-box genes are an evolutionarily conserved family of transcription factors that are expressed during embryogenesis. They play a key role in developmental mechanisms underlying the various processes of tissue differentiation.¹ The T-box gene TBX1 has been widely studied during the past decade, although its definitive function remains to be established. The human homologue of TBX1 lies within the 1.5 Mb del22q11.2 region. This region is associated with considerable phenotypic variability.²

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The DiGeorge syndrome (DGS) is a clinical example of a developmental disorder of the pharyngeal apparatus, and most cases are associated with heterozygous deletions in the long arm of chromosome 22. The finding of TBX1 point mutations in some patients with the typical DGS phenotype implicates this gene in the pathogenesis of DGS.³ More recently, TBX1 haploinsufficiency has been linked to behavioural disorders in mice and humans. Involvement of TBX1 haploinsufficiency in psychiatric diseases is supported by the identification of a family in which the phenotypic features of 22q11 deletion syndrome segregated with an inactivating mutation of TBX1.⁴ DGS is characterized by a wide variability in the severity and penetrance of the phenotype features even in patients with identical deletions.^{2,5} It is possible that genetic modifiers located outside del22q11.2 can affect phenotypic penetrance and expressivity.^{6,7}

The TBX1 gene is required for normal development of virtually all the structures and organs that derive from the pharyngeal apparatus.^{6,8} It is expressed in the pharyngeal endoderm, the core mesoderm of pharyngeal arches, head mesenchyme, sclerotome, the outflow tract of the heart, inner ear, and in part of the splanchnic mesoderm.^{1,9–12} Deletion of TBX1 in either the endoderm or mesoderm results in a phenotype reminiscent of TBX1-knockout mice, which indicates that TBX1 function is required in both tissues for normal brachial arch development.^{12,13}

Most studies devoted to TBX1 during development have focused on the cardiovascular system mainly in relation to DGS patients. TBX1 mouse mutants have two main types of cardiovascular defects, aortic arch patterning defects and cardiac outflow tract defects.^{8,14,15} The pharyngeal arch and cardiac abnormalities are associated with down-regulation of Fgf10 in the mesoderm.^{16,17} TBX1 controls deployment of Fgf10- expressing progenitor cells during heart tube extension. Although normal Fgf10 levels are dependent on TBX1, loss of Fgf10 alleles does not significantly modify the cardiac phenotype of TBX1 heterozygous or homozygous mutant embryos.¹⁸ Furthermore, tissue-specific deletion of TBX1, a hypomorphic allele, and fate mapping of TBX1-expressing cells revealed that the cardiac outflow tract abnormalities result from TBX1- mediated inhibition of cardiomyocyte precursor cells.¹⁹ In fact, in the absence of the gene, these cells proliferate at a lower rate and fail to contribute to the outflow tract in a sufficient number thereby resulting in severe morphogenetic defects.¹⁹

Guris et al.²⁰ suggested the intriguing possibility that a contiguous del22q11.2 syndrome, caused by interactions between the CRKL and TBX1 genes, a loss of retinoic homeostasis at local level and a subsequent retinoic acid (RA) aberrant signaling are critical steps in the pathogenesis of DGS associated with 22q11.2 deletions. Further evidence of interaction between TBX1 and RA signaling is the finding that this transcription factor can regulate Cyp26 genes, which are responsible for inactivation of RA.²¹

Although the TBX1 transcription factor has been well studied at the genetic level,²² several important aspects remain to be elucidated at the level of proteins, which are the transcriptional targets of TBX1. To this aim, we have therefore used proteomic tools. Over the past decade, proteomics has gained an instrumental role within biologic system studies, by enhancing our knowledge of the functions of biological networks through the generation of a tremendous amount of information. The study of the proteome can be divided into profiling, functional and structural proteomics.²³ The 2D DIGE (Differential Gel Electrophoresis) technology belongs to the “second generation” proteomic techniques, along with ICAT, iTRAQ and SILAC, that allow us to evaluate the relative abundance of protein species in two or more specific physiopathological conditions. Two-dimensional DIGE is based on fluorescence prelabeling of protein-mixtures before 2D gel electrophoresis. Protein samples are labeled with up to three spectrally distinct, charge and mass-matched fluorescent dyes known as CyDye DIGEfluors.^{24,}

²⁵ The labeled proteins are then mixed and separated simultaneously on the same 2D gel. The advantages of improved sensitivity and accuracy provided by the ability to separate more than one sample on a single gel make 2D DIGE a highly reproducible technique for identifying statistically significant differences. In fact, such a procedure has the ability to substantially reduce the effects of gel to gel variation on the quantitation of a protein spot on a gel. Therefore, the confidence that a difference in fluorescence intensity between two samples is due to biological rather than experimental variation has increased.^{24,25} The greater quantitative accuracy of 2D DIGE is enabled by three main factors: (1) the ability to run multiple samples on the same gel (multiplexing); (2) an internal standard (reference) sample which can be run on all gels; and (3) experimental designs unique to this technique. The linearity, sensitivity, and wide dynamic range (above 3.6 orders of magnitude) of these dyes have made 2D DIGE a quantitative technique that has been employed in several biological applications to examine the protein profiles of various tissues, cell lines and cell types including those from bacteria, yeast, plants, fruit fly, insect, mouse and rat liver, rat kidney, rat heart, rat lung, cat brain, mouse and rat brain, guinea pig brain, human brain and human cancer cells²⁶ and references therein.

We have used DIGE technology in the attempt to gain insight into the functional properties of TBX1. Specifically, we compared the P19CL6 mouse carcinoma cell line either expressing or not expressing TBX1. The comparative analysis revealed 92 differentially expressed proteins. The observed down-regulation of proteins involved in RA metabolism could be of particular relevance for TBX1 function and, eventually, may shed light on DGS pathophysiology.

Materials and Methods

Cell Culture

The TBX1-expressing cell line P19CL6_Tbx1-PA was obtained by transfecting P19CL6 cells with an expression vector containing the CMV promoter driving a mouse TBX1 cDNA fused with a TEV target site and a protein A-coding cDNA. The backbone vector is described elsewhere.³¹ Transfected cells were subjected to positive selection using G418 to obtain a stable transfectant. Using the same procedure but with an expression vector encoding the TEV site and protein A but not TBX1, we obtained a control cell line, P19CL6_PA. Cells were grown in Dulbecco-Modified Minimal Essential Medium supplemented with 10% fetal bovine serum (Gibco/Life Technologies, Rockville, MA), 2 mM L-glutamine (EuroClone, Paington, UK) and antibiotics (100 Units/mL penicillin and 100 mg/mL streptomycin) in a humidified chamber at 37 °C in 5% CO₂. Cells were maintained constantly under selection using G418, then trypsinized, washed with phosphate buffer saline (pH 7.4) and centrifuged.

Cultured cells were harvested and resuspended in a buffer containing 7 M urea, 2 M thiourea, 30 mM Tris-HCl pH 8.5, 4% CHAPS (w/v), 1×Complete EDTA free (Roche Applied Science, Indianapolis, IN). Cell debris was removed by centrifugation at 14,000 rpm at 4 °C for 30 min. The cell lysate supernatant was precipitated using a 2D clean up kit (GE Healthcare, Piscataway, NJ) and resuspended in 100 µL 7 M urea, 2 M thiourea, 30 mM Tris-HCl pH 8.5, 4% CHAPS (w/v).

Experimental Design of 2D DIGE Analysis

To prevent bias from sample heterogeneity, we grew 6 sets from the same clone of each cell type according to the experimental conditions described in the previous section. The six sample replicates were labeled with Cy2, Cy3 and Cy5, according to the protocols described in the Ettan DIGE User Manual (18–1173–17 GE Healthcare, Piscataway, NJ). To provide statistical significance, 6 independent gels were run as reported in Table 1. Usually, 50 µg of lysates from P19CL6_Tbx1-PA or P19CL6_PA were labeled with 400 pmol of Cy3 or Cy5. To prevent dye-specific protein labeling, the Cy3 or Cy5 was randomized between the lysates as shown

in Table 1. Therefore, ingels1–3, P19CL6_Tbx1- PA lysates were labeled with Cy3, and P19CL6_PA lysates were labeled with Cy5. In gels 4–6 we switched the dyes. Each Cy3/Cy5-labeled sample pair was mixed with a Cy2-labeled pooled standard sample containing an equal amount of all 12 samples analyzed. The Cy2/Cy3/Cy5 labeled samples were run together on the same gel (Gel 1–6 in Table 1). Labeling reactions were carried out in the dark on ice for 30 min before quenching with 1 μ L of 10 mM L-lysine for 10 min on ice. The labeled samples were then combined for the 2D DIGE analysis.

Samples were fractionated on 18 cm IPG strips with 3–11NL, 3–5.6, 4–7 and 6–11 pH ranges. IPG strips were rehydrated, in the absence of protein samples, with 350 μ L of rehydration buffer (350 μ L DeStreak rehydration solution, 0.5% Pharmalyte and 0.5% IPG buffer) overnight at room temperature. The strips were then transferred to the Ettan IPGphor system (GE Healthcare, Piscataway, NJ) for isoelectric focusing.

The samples were loaded on the strips with an equal volume of sample buffer containing 7M urea, 2M thiourea, 4% CHAPS, 1% DTT and 1% Pharmalyte. The sample was loaded with the anodic cup-loading method on pH 3–11NL, pH 4–7 and pH 6–11 IPG strips, and with the cathodic cup-loading method on pH 3–5.6 pH IPG strips. The IPG strips were focused for 18 h for a total of 60kV/h at 20 °C. Then, proteins were reduced with an equilibration buffer (6 M urea, 100 mM Tris pH 8.0, 30% glycerol (v/v), 2% SDS) containing 0.5% DTT for 15 min. Finally, proteins were alkylated for the same time with the buffer containing 4.5% IAA.

After the equilibration step, the strips were over layered onto 10% polyacrylamide gels (20 $\times$ 24 cm). The second dimension was carried out for 18 h at 2W per gel using an Ettan Dalt Twelve system (GE Healthcare, Piscataway, NJ). After electrophoresis, gels were scanned in a Typhoon 9400 scanner (GE Healthcare, Piscataway, NJ). The images labeled with Cy2, Cy3, and Cy5 were acquired at excitation/emission values of 488/520, 532/580, 633/670nm, respectively, with a band-pass of 30. Gels were scanned using parameters selected to prevent pixel saturation.

Image Analysis

Images were analyzed with the Decyder software version 5.2 (GE Healthcare, Piscataway, NJ) in batch processing mode. The maximum number of estimated spots per gel was fixed at 5000. Detection and quantification of protein spots were carried out by the differential in-gel (DIA) module, whereas protein-spot matching between different gels was obtained using the biological variation analysis (BVA) module. The DIA module was used for pairwise comparison of each sample (Cy3 and Cy5) with the Cy2 mixed standard present in each gel (see Table 1). In addition, DIA was used to detect spot boundaries and to calculate spot volume, normalized versus the volume of the corresponding spot present in the pool standard of the same gel. This analysis revealed the differentially expressed protein spots across six gels. The results from the intragel comparison (six DIA files) for all dyes were imported into the BVA module. The Cy2 image containing the highest number of spots was designated the “master image” and used as template. The protein spots belonging to the remaining internal standard images were automatically matched with “master image”.

Each spot intensity was then expressed as a mean value of the 6 gels, reducing intergel variation. Spot intensities were then compared in the two conditions: cell lines expressing TBX1 and control cells. Statistical significance of differences in spot intensity was determined with Student's *t* test. Only protein spots with a change in size of at least 1.20 fold (*t* test: $p \leq 0.05$) after normalization were considered significantly altered. We verified the validity of these changes and accuracy of spot matching by manual inspection of gels.

Protein Identification by Mass Spectrometry

Independent two-dimensional preparative gels, P19CL6_Tbx1-PA and P19CL6_PA were run in the three pH ranges indicated above to obtain sufficient amounts of protein for mass spectrometry (MS) analysis. Each preparative gel was run using 0.5 mg of protein extract. Gels were fixed in 40% methanol, 10% acetic acid solution overnight, fixed for a second time for at least 2 h, and then stained overnight in Sypro Ruby (Molecular Probes Inc., Eugene, OR) in the dark. Images were acquired using the Typhoon imager at an excitation/emission wavelength of 532/610 nm. Spots of interest were picked using an Ettan Spot Picker (GE Healthcare, Piscataway, NJ). After spot excision, gels were reacquired to verify successful gel plug removal. The gel pieces were first washed in 100% acetonitrile and 50 mM ammoniumbicarbonate. Enzymatic digestions were carried out with modified trypsin (Sigma) (10 ng/mL) in 50 mM ammonium bicarbonate, pH 8.5, at 4 °C for 45 min. The enzymatic solution was then removed. A new aliquot of the buffer solution was added to the gel particles and incubated at 37 °C for 18 h. A minimum reaction volume sufficient for complete rehydration of the gel was used. Peptides were extracted by washing gel particles in acetonitrile at 37 °C for 15 min, and lyophilized. The analysis was performed by μ LC-MS/MS with a Q-ToF hybrid mass spectrometer (Waters, Milford, MA) equipped with a Z-spray source and coupled online with a capLC chromatography system (Waters) or alternatively by using the LC/MSD Trap XCT Ultra (Agilent Technologies, Palo Alto, CA) equipped with a 1100 HPLC system and a chip cube (Agilent Technologies). After loading, the peptide mixture (7 μ L in 0.5% TFA solution) was first concentrated and washed at (i) at 1 μ L/min onto a C18 reverse-phase precolumn (Waters) or (ii) at 4 μ L/min in 40 nL enrichment column (Agilent Technologies Chip), with 0.1% formic acid as eluent. The sample was then fractionated on a C18 reverse-phase capillary column (75 μ m $\times$ 20 cm in the Waters system, 75 μ m $\times$ 43 mm in the Agilent Technologies Chip) at a flow rate of 200 nL/min, with a linear gradient of eluent B (0.1% formic acid in acetonitrile) in A (0.1% formic acid) from 5 to 60% in 50 min. Elution was monitored on the mass spectrometers without a splitting device. Peptides were analyzed using data-dependent acquisition of one MS scan (mass range from 400 to 2000 m/z) followed by MS/MS scans of the three most abundant ions. We used dynamic exclusion to acquire a more complete survey of the peptides. A permanent exclusion list of the most frequent peptide contaminants (keratins and trypsin doubly and triply charged peptides, 403.20, 517.00, 519.32, 525.00, 532.90, 559.32, 577.30, 587.86, 616.85, 618.23, 721.75, 745.90, 747.32, 758.43, 854.30, 858.43, 896.30, 1082.06) was included in the acquisition method to focus the analyses on significant data.

For data analysis, we used Mascot software (<http://www.matrixscience.com>) selecting NCBI database (www.ncbi.nlm.nih.gov) and *Mus musculus* as the taxonomic origin of the samples.

The protein search was governed by the following parameters: specificity of the proteolytic enzyme used for hydrolysis (trypsin); protein molecular weight was not considered; up to 1 missed cleavage; cysteines in form of *S*-carbamidomethylcysteines; unmodified N- and C-terminal ends; unmodified and oxidized methionines; putative pyroGlu formation by Gln; precursor peptide maximum mass tolerance of 300 ppm and a maximum fragment mass tolerance of 0.6 Da. According to the probability-based Mowse score,²⁸ the ion score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual scores >38 indicate identity or extensive homology ($p \leq 0.05$). In our experience, all MS/MS spectra with a Mascot score higher than 38 have a good signal/noise ratio leading to an unambiguous interpretation of the data. Individual MS/MS spectra for peptides with a Mascot score equal to 38 were inspected manually and included in the statistical analysis only if they contained a series of at least four continuous y or b ions.

Western Blot

P19CL6_Tbx1-PA and P19CL6_PA protein extracts (10 μ g) were resolved on a 10% SDS-PAGE gel, and the samples transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was blocked in 5% nonfat milk in PBS for 1 h, incubated with 1% milk/PBS 1 $\times$ containing peroxidase conjugated human IgG F(c) fragment (Rockland Immunochemicals, Inc., Gilbertsville, PA) or monoclonal antibodies anti-PCNA, anti-Nars and anti-VCL (Santa Cruz Biotechnology, Santa Cruz, CA), or polyclonal antibodies anti-ALDH1A2 (Upstate, Charlottesville, VA), anti-hnRNP F, anti-Atp5b, anti-Hsp90b1, anti-Hsp4, anti-Ppp2r1a (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-GAPDH antibody served as control. Peroxidase conjugated human IgG F(c) was used at a dilution of 1:5000. Monoclonal antibodies were used at a dilution of 1:1000, polyclonal antibodies were used at a dilution of 1:3000 and monoclonal anti-GAPDH at a dilution of 1:1000. Immunoblot detections were carried out using HRP-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). The resulting Western blot images were scanned and analyzed by Chemi Doc software (Biorad, Hercules, CA). Bands were defined, background was subtracted, and volumes were measured. The sizes of bands of proteins of interest were normalized by dividing by GAPDH band sizes visualized on the same membrane.

In Silico Analysis of Promoter Regions

We analyzed promoter regions of genes coding for differentially expressed proteins identified in this study by using the Genomatix software suite (www.genomatix.de). It combines several data mining sources, Bibliosphere, Gene2Promoter and GEMS-Launcher (MatInspector and FrameWorker) which integrates bioinformatics data in order to gain insight into promoter structure, transcription factor binding sites, gene interaction networks and signaling pathways. Particular attention was paid to transcription factor modules found in the promoter regions of the genes under investigation [Module Library Version 4.5, Vertebrate Modules (November 2007)].

Results

Stable TBX1-Expression

We evaluated the expression of TBX1 protein in cell lines stably expressing TBX1 (P19CL6_Tbx1-PA) and in control cells (P19CL6_PA) by Western blot analysis using human IgG that detects protein A (see construct in Figure 1). We obtained a band of about 70 kDa, which corresponds to the construct containing TBX1 in P19CL6_Tbx1-PA, and a single signal of about 20 kDa, which corresponds to TEV-protein A in P19CL6_PA. We therefore used the two cell lines for differential proteomic experiments.

Detection and Identification of Differentially Expressed Proteins

To identify proteins whose expression could be modified by the transcriptional factor TBX1, we carried out a comparative two-dimensional DIGE analysis of lysates of the control cell line (P19CL6_PA) and of the TBX1-expressing cell lines (P19CL6_Tbx1-PA). The experiments were performed on 6 sets from the same clone of each cell type thus ensuring analytical replication of a single biological replicate. The DIGE experimental design is reported in Table 1.

We first carried out differential proteomics experiments using a non linear 3–11 pH range in the first dimension. Using the statistical parameters reported in the Experimental Section we selected 150 differentially expressed spots. We then used a preparative gel to identify differentially expressed spots. Only protein spots on the Sypro Ruby-stained gels that matched the corresponding DIGE analytical gels were processed. 73 spots were excised from the

preparative Sypro Ruby stained gels and subjected to tryptic digestion. Tryptic peptides were then analyzed by μ LC-MS/MS and data were processed with the Mascot software using the parameters reported in the Materials and Methods. Only 32 spots out of 73 resulted in an unequivocal identification of protein. The remaining spots contained more than one protein species and therefore the fold change could not be directly assigned to a single protein species. Figure 2 shows the 3–11NL map where the identified spots are circled in red. Table 2 shows the proteins that were identified in the 3–11NL map shown in Figure 2. For each protein we indicate the gene symbol, the corresponding protein name, the gene accession number (reported also in Figure 2), the *p* value and the fold increase (measured as increase of spot volume) are reported. The cellular localization, the cellular process and the protein function are reported in the last three columns.

To improve separation and resolution, we did the comparative DIGE analysis and subsequent identifications using the pH 3–5.6, pH 4–7, and pH 6–11 IPG strips. The samples were analyzed as reported in Table 1. We found 70 differentially expressed spots in the 3–5.6-pH range, 115 in the 4–7 pH range gel and 92 in the 6–11 pH range. We then used preparative gels to identify differentially expressed proteins. A total of 25, 51 and 17 spots were excised from the gels in pH ranges 3–5.6, pH 4–7, and pH 6–11, respectively. These were subjected to tryptic digestion and identified by MS analysis. Figures 3, 4, and 5 show the 3–5.6, 4–7, and 6–11 maps where the identified spots are circled in red. This set of experiments led to the identification of 15 proteins from the 3–5.6 pH gel, 38 proteins from the 4–7 pH gel, and 7 proteins from the 6–11 pH gel. Table 3, 4, and 5 show the proteins that were present only in the 3–5.6, 4–7, and 6–11 maps shown in Figures 3, 4, and 5. For each protein we indicate the gene symbol, the corresponding protein name, the gene accession number (reported also in Tables 3, 4, and 5), the *p* value and the fold increase (measured as increase of spot volume). The cellular localization, the cellular process and the protein function are reported in the last three columns of each table. In summary, 69 proteins were up-regulated and 23 were down-regulated in cell lines expressing TBX1 versus control cell lines.

The Supporting Information Table 1S shows, for each protein entry, the identified peptide sequence, the MASCOT score for each peptide, the mass errors (ppm) on the precursor peptide, and the protein sequence coverage. Each protein is reported only with the gene symbol from the NCBI source to eliminate redundancy.

For technical validation of the two-dimensional DIGE expression pattern, we carried out Western blot analysis on proteins extracted from P19CL6_Tbx1-PA cells and P19CL6_PA cells using antibodies against PCNA, ALDH1A2, hnRNP F, Atp5b, Hsp90b1, Hsp4, Ppp2r1a, Nars, and VCL. The choice of these proteins was dictated by the use of commercially available antibodies. Figure 6 shows the results of the experiment which validate DIGE results. PCNA, ALDH1A2 and hnRNP F were found in fact under-expressed while the rest of the proteins were found overexpressed in P19CL6_Tbx1_PA. We used an anti-GAPDH antibody to normalize data.

As proof of concept, we performed a Western blot on 3–11 NL 2D gel validating the overexpression of Atp5b. Figure 1S (Supporting Information) shows the results of the experiment.

We are aware that the confidence in the interpretation of our results would have been improved by validation in an independent system. However, our data represent a good starting point for studies at protein level (see Discussion).

Classification of Identified Proteins

We classified the up- and down-regulated proteins according to Gene Ontology (www.ncbi.nlm.gov/sites/entrez) information concerning cellular processes and cellular localization (Figure 7). The proteins identified are related to metabolism, response to stress, signaling, structure, transcription, translation, cell redox homeostasis and RNA processing (Figure 7A). It is noteworthy that 37% of up-regulated proteins and 17% of down-regulated proteins are involved in processes related to metabolism (Figure 7A). Almost 30% of the up-regulated proteins either are present in the nucleus or shuttle between the cytoplasm and nucleus; 25% of the proteins are cytoplasmic, 3% are localized in the membrane and 11% in the endoplasmic reticulum; 11% are mitochondrial, 5% shuttle between mitochondrion and cytoplasm and the remainder do not have a known localization (Figure 7B). Almost 30% of the down-regulated proteins either are present in the nucleus or shuttle between the cytoplasm and nucleus; 5% of the proteins are localized in the mitochondrion, 33% of the proteins are cytoplasmic, 5% shuttle between nucleus, mitochondrion and cytoplasm and 24% do not have a known localization. This distribution shows that TBX1 plays a central role in the regulation of nuclear proteins and its activity affects all other cell compartments.

In-Silico Analysis of Promoter Regions

To evaluate whether transcription factor TBX1 exerts a direct or indirect effect on the differentially expressed proteins identified in this study, we performed an *in silico* analysis of the gene promoter regions using the Genomatix software suite. In this context, particular attention was paid to transcription factor modules identified in the promoter regions of the genes under investigation. We found several genes that share the NKXH-BRAC module, which is required for the synergistic effect exerted by TBX1 and NKX2.5 on activation of the PITX2 enhancer.²⁹ These genes are AHCY, CORO7, EEF2, HNRNPD, PPP2R1A that code for up-regulated proteins, and ALDH1A2 that codes for a down-regulated protein.

Discussion

Here we report the first use of two-dimensional DIGE analysis to identify proteins differentially expressed in cells overexpressing TBX1. We identified a set of 92 proteins that are related to a variety of cell processes.

Of the proteins down-regulated in our study, we focused on two (ALDH1A2 and PCNA) that may be involved in RA metabolism, which in turn has been implicated in TBX1 function. In fact, deficiency or excess of vitamin A and of its biologically active derivative, RA, are involved in various developmental and homeostatic processes in vertebrates, such as development of the pharyngeal arches which involves also TBX1.³⁰ In addition, RA deficiency or excess in the mouse recapitulates the typical phenotypes seen in human DGS syndrome,^{12,20} and perturbed RA signaling during embryogenesis causes a DGS phenotype in the mouse.^{31,32} Therefore, genes involved in RA metabolism or signaling are candidate modifiers of the DGS phenotype.

The ALDH1A2 enzyme catalyzes the oxidation of retinaldehyde into RA thereby providing most of the embryonic RA.^{33–35} ALDH1A2 determines cell fate. In fact, synthesis of RA by ALDH1A2 results in atrial cells, where lack of ALDH1A2 expression and RA signaling result in ventricular cells.³⁶ Inhibition of endogenous RA synthesis represses expression of atrial-specific markers and results in an oversized ventricle and an aplastic atria. Conversely, exogenous RA induces atrial-specific gene expression and produces a heart with a hyperplastic atria but lacking ventricles and outflow tract.³⁷ Our data suggest that an increase in TBX1 expression induces down-regulation of ALDH1A2 thereby resulting in a decrease of RA concentration. Very recently, gene expression profiling of the caudal pharyngeal region in

Tbx1^{-/-} and wild type embryos showed that genes required for cardiac morphogenesis such as ALDH1A2 were ectopically expressed.³⁸ In addition a conserved T-box consensus sequence was identified in the DNA sequence containing the first exon of ALDH1A2, which suggests that TBX1 might directly regulate ALDH1A2 expression levels.³⁸

A decrease in RA concentration could also explain PCNA down-regulation. PCNA is involved in cell proliferation during developmental processes. Stimulation with RA up-regulates PCNA expression.³⁹ Alternatively, PCNA down-regulation results directly from TBX1 overexpression.

Of the proteins up-regulated, we focused on aldolase 1 (the A isoform), pyruvate kinase and lactate dehydrogenase A. These are important glycolytic enzymes. Aldolase A is involved in glucose metabolism and catalyzes the reversible conversion of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Pyruvate kinase catalyzes the penultimate step in glycolysis, i.e., the conversion of phospho-enolpyruvate to pyruvate. The pyruvate kinase-catalyzed reaction is the second ATP-generating step of the glycolytic pathway and is particularly important in energy production during anaerobic glycolysis because it yields nearly 50% of total ATP. LDH is a tetramer of A and B subunits that catalyzes the conversion of pyruvate to lactate. This enzymatic reaction oxidizes NADH and replenishes NAD⁺, which is essential for the glycolytic conversion of glucose to pyruvate. Glycolytic enzymes have been implicated in cardiac metabolism and cardiomyocyte differentiation.^{40,41} Given the role of TBX1 in cardiovascular system development,¹ our finding that TBX1 overexpression up-regulates glycolytic enzymes may shed light on its function.

We also found increased levels of succinate dehydrogenase in our system. This enzyme is involved in the citric acid cycle, and its increase may imply some involvement of energy metabolism following TBX1 overexpression. Another interesting observation is the up-regulation of proteins of the proteasome complex, that is, Psmc3, Psmc4, and Psmb5. This complex contributes significantly to the regulation of cardiac function in normal and stressed myocardium.⁴² Therefore, delineation of a possible correlation between TBX1 and the regulation of the proteasome complex could cast light on the pathogenesis of cardiovascular diseases.

The last interesting finding to emerge from our study is that both CBX5 and TRIM28 were up-regulated following TBX1 overexpression. These two proteins are functionally strictly related. TRIM28, also known as transcriptional intermediary factor (TIF) 1b, is the corepressor for the large family of Kruppel-associated box-containing zinc finger (KRABZFP) proteins.⁴³ CBX5, also known as heterochromatin protein 1 alpha (HP1 α), belongs to the heterochromatin protein 1 (HP1) family of proteins (HP1 α , HP1 β , and HP1 γ) and participates in gene silencing by forming heterochromatic structures.⁴³ Interaction between CBX5 and TRIM28 plays a key role in cell differentiation which is characterized by progressive silencing of gene expression through a mechanism believed to involve heterochromatin. The correlation between TBX1 and these proteins has yet to be elucidated. Studies are under way in our laboratory to clarify this point.

Finally, the *in silico* analysis of promoter regions of genes coding for differentially expressed proteins identified in this study revealed that AH CY, CORO7, EEF2, HNRNPD, PPP2R1A, and ALDH1A2 share a common transcription factor module that is directly related to TBX1 function. TBX1 and NKX2.5 can synergistically activate the PITX2 enhancer. Specifically, TBX1 directly activates PITX2, and by interaction with NKX2.5, it is responsible for maintenance of PITX2 expression in the left secondary heart field.²⁹ Among our genes that are putative direct downstream targets of TBX1, all except ALDH1A2 are up-regulated in the experimental system we report. We hypothesize that TBX1 acts as an activator or repressor

factor depending on structural determinants of the specific promoter regions and/or on TBX1-interacting proteins. The finding that ALDH1A2 and the other genes display a similar TBX1-mediated regulation mechanism suggests a new functional activity for this transcription factor that warrants investigation.

In conclusion, we carried out a differential proteomic analysis to identify TBX1 targets. We show that various proteins may be targets of TBX1 function and that its activity could affect different cellular processes, among which pathways involved in retinoic acid metabolism. Our data represent a good starting point for further studies, conducted at protein level, aimed at elucidating TBX1 function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Centro Regionale di Competenza GEAR, Regione Campania for 2D-DIGE facility. We thank F. Talamo and L. Orsatti, IRBM-Pomezia for their technical assistance in the first experiments DIGE analysis. We thank Dr. S. Di Paola for his help in image analysis. We are grateful to Jean Ann Gilder for text editing. This work was supported by Ministero della Salute (Roma), Convenzione CEINGE-MIUR (2000) art 5.2, Convenzione CEINGE-Regione Campania, Progetto S. co. Pe, Prin 2006 to M.R.

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P19CL6_Tbx1_PA:

P19CL6_PA:

Figure 1.

Western blot analysis of P19CL6_Tbx1-PA cells and P19CL6_PA control cells. Proteins were separated on 10% SDSPAGE gel and immunoblotted with human IgG F(c). The constructs used in the study are shown at the bottom.

Figure 2.

Preparative 2D gel carried out using non linear pH 3–11 in the first dimension and 10% SDS PAGE in the second dimension. Red circles indicate the differentially expressed spots, picked-out and used for subsequent identification by mass spectrometry. Each protein is indicated with the gene accession number (gene ID) reported in Table 2.

Figure 3.

Preparative 2D gel carried out using pH 3–5.6 in the first dimension and 10% SDS PAGE in the second dimension. Red circles indicate the proteins identified only in this 2D map. Each protein is indicated with the gene accession number (gene ID) reported in Table 3.

Figure 4.

Preparative 2D gel carried out using pH 4–7 in the first dimension and 10% SDS PAGE in the second dimension. Red circles indicate the proteins identified only in this 2D map. Each protein is indicated with the gene accession number (gene ID) reported in Table 4.

Figure 5.

Preparative 2D gel carried out using pH 6–11 in the first dimension and 10% SDS PAGE in the second dimension. Red circles indicate the proteins identified only in this 2D map. Each protein is indicated with the gene accession number (gene ID) reported in Table 5.

Figure 6.

Western blot analyses of P19CL6_Tbx1-PA cells and P19CL6_PA control cells. Proteins were separated on 10% SDSPAGE gel and immunoblotted with anti-PCNA and anti-ALDH1A2, anti-hnRNP F, anti-Atp5b, anti-Hsp90b1, anti-Hsp4, anti-Ppp2r1a, anti-Nars, and anti-VCL. GAPDH was used as control of not differentially expressed protein.

Figure 7. Classification of the up-regulated ($n = 69$) and down-regulated ($n = 23$) proteins according to Gene Ontology Cellular Processes (A) and Cellular Localization (B).

Table 1

2D DIGE Experimental Design

gel	Cy3 (50 μ g)	Cy5 (50 μ g)	Cy2 (50 μ g)
1	P19CL6_Tbx1 replicate 1	P19CL6 replicate 1	Pool standard
2	P19CL6_Tbx1 replicate 2	P19CL6 replicate 2	Pool standard
3	P19CL6_Tbx1 replicate 3	P19CL6 replicate 3	Pool standard
4	P19CL6 replicate 4	P19CL6_Tbx1 replicate 4	Pool standard
5	P19CL6 replicate 5	P19CL6_Tbx1 replicate 5	Pool standard
6	P19CL6 replicate 6	P19CL6_Tbx1 replicate 6	Pool standard

Table 2

Up-Regulated and Down-Regulated Proteins Identified in 3–11 pH Gradient

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
Up-regulated proteins							
<i>mscly</i>	selenocysteine lyase	50880	1.60E-05	1.89	cytoplasm	Metabolism	catalytic activity
<i>mpaics</i>	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole, succinocarboxamide synthetase	67054	1.60E-05	1.89	unknown	Metabolism	catalytic activity
<i>mnars</i>	Asparaginyl tRNA synthase	70223	1.70E-03	1.76	nucleus/cytoplasm	Metabolism	aminoacyl-tRNA ligase activity
<i>mdk4</i>	cyclin-dependent Kinase 4	12567	7.20E-04	1.57	nucleus	Signaling	ligase activity
							nucleotide binding
							kinase activity
							nucleotide binding
<i>maldaa</i>	aldolase 1, A isoform	11674	2.90E-04	1.54	unknown	Metabolism	transferase activity
<i>mhmmpd</i>	heterogeneous nuclear ribonucleoprotein D	11991	2.90E-04	1.54	nucleus	Transcription	catalytic activity
							nucleic acid binding
<i>mef2</i>	eukaryotic translation elongation factor 2	13629	5.80E-04	1.44	cytoplasm	Translation	protein binding
							nucleotide binding
<i>2700060E02Rik</i>	RIKEN cDNA 2700060E02 (protein C14orf166 homologue)	68045	4.30E-05	1.41	nucleus/cytoplasm	Unknown	translation elongation factor activity
<i>mgliu1</i>	glutamate dehydrogenase 1	14661	2.60E-03	1.38	mitochondrion	Metabolism	catalytic activity
<i>mimph2</i>	Inosine 5'-phosphate dehydrogenase 2	23918	2.60E-03	1.38	unknown	Metabolism	catalytic activity
<i>mpkm2</i>	pyruvate kinase, muscle	18746	1.80E-04	1.35	mitochondrion	Metabolism	kinase activity
<i>mpsmb5</i>	proteasome (prosome, macropain) subunit, beta type 5	19173	3.60E-06	1.33	nucleus/cytoplasm	Metabolism	hydrolase activity
<i>mprdx1</i>	Peroxiredoxin 1	18477	3.60E-06	1.33	cytoplasm	Cell redox homeostasis	peroxidase activity
<i>mcacybp</i>	Calcyclin binding protein	12301	5.90E-05	1.3	nucleus/cytoplasm	Signaling	
<i>mstmn4</i>	stathmin-like 4	56471	5.90E-05	1.3	membrane	Signaling	
<i>mkpna2</i>	karyopherin (importin) alpha 2	16647	2.80E-04	1.28	nucleus/cytoplasm	Translation	protein binding
<i>mcndp2</i>	CNNP dipeptidase 2 (metalloprotease M20 family)	66054	2.80E-04	1.28	cytoplasm	Other	protein transporter activity
							hydrolase activity
<i>mshml2</i>	Serine hydroxymethyltransferase 2 (mitochondrial)	108037	1.40E-03	1.25	mitochondrion/cytoplasm	Metabolism	transferase activity
<i>npls3</i>	Pls3 plastin 3 (T-isoform)	102866	1.40E-03	1.25	cytoplasm	Structural component	protein binding

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
<i>mvat1</i>	vesicle amine transport protein 1 homologue (T californica)	26949	3.80E-04	1.22	unknown	Other	catalytic activity
<i>marbp</i>	acidic ribosomal phosphoprotein P0	11837	3.50E-07	Down-regulated proteins	unknown	Structural component	structural constituent of ribosome
<i>mcapzb</i>	capping protein (Actin filament) muscle Z-line, beta	12345	1.90E-03		cytoplasm/cytoskeleton	Unknown	protein binding
<i>macaca</i>	acetyl-CoA carboxylase	107476	9.20E-05		cytoplasm	Metabolism	catalytic activity
<i>mgnb3</i>	guanine nucleotide binding protein (G protein), beta 3	14695	2.60E-04	-1.32	unknown	Signaling	nucleotide binding
<i>mgnb2</i>	guanine nucleotide binding protein (G protein), beta 2	14693	2.70E-04	-1.32	unknown	Signaling	GTPase activity
<i>mcdc42bpa</i>	Cdc42 binding protein kinase alpha	226751	4.00E-03	-1.33	cytoplasm	Signaling	ATP binding
<i>mcops6</i>	COP9 (constitutive photomorphogenic) homologue, subunit 6 (<i>Arabidopsis thaliana</i>)	26893	3.90E-04	-1.34	nucleus/cytoplasm	Unknown	kinase activity
<i>mpark7</i>	DJ-1 protein (Parkinson disease (autosomal recessive, early onset)	57320	1.80E-04	-1.39	cytoplasm/nucleus/mitochondrion	Others	nucleotide binding
<i>macet1</i>	alpha cardiac Actin	11464	1.80E-04	-1.72	cytoplasm/cytoskeleton	Structural component	transferase activity
<i>mhmph1</i>	heterogeneous nuclear ribonucleoprotein H1	59013	1.80E-04	-1.76	nucleus	mRNA processing	protein binding
<i>mhmrf</i>	heterogeneous nuclear ribonucleoprotein F	98758	1.80E-04	-1.76	nucleus		structural constituent of muscle
<i>mvdac1</i>	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	22333	1.70E-04	-2.5	mitochondrion/membrane	Signaling	nucleic acid binding
							voltage-gated anion channel activity

Table 3

Up-Regulated Proteins in the pH 3–5.6 Map

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
<i>mhspp90b1</i>	Heat shock protein 90 kDa beta (Gp94), member 1	22027	8.10E-03	3.4	endoplasmic reticulum	Response to stress	nucleotide binding
<i>mpsmc3</i>	proteasome (prosome, macropain) 26Ssubunit, ATPase 3	19182	6.90E-03	2.12	nucleus/cytoplasm	Metabolism	protein binding hydrolase activity
<i>mpdia6</i>	Protein disulfide isomerase associated 6	71853	8.20E-03	2.1	endoplasmic reticulum	Cell redox homeostasis	nucleotide binding isomerase activity
<i>matp5b</i>	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	11947	2.20E-04	1.78	mitochondrion or membrane	Metabolism	transporter activity
<i>mcbx5</i>	Chromobox homologue 5 (Drosophila HPl1a)	12419	7.40E-04	1.74	nucleus	Other	hydrolase activity nucleotide binding chromatin binding
<i>mppp2r1a</i>	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), alpha isoform	51792	5.00E-02	1.71	cytoplasm	Signaling	protein binding protein binding
<i>mpsmc4</i>	proteasome (prosome, macropain) 26Ssubunit, ATPase 4	23996	6.70E-03	1.6	nucleus/cytoplasm	Metabolism	hydrolase activity
<i>mppp1r7</i>	protein phosphatase 1, regulatory (inhibitor) subunit 7	66385	5.20E-03	1.59	nucleus	Other	nucleotide binding protein binding
<i>mp4hb</i>	Prolyl 4-hydroxylase, beta polypeptide	18453	8.3E-03	1.55	endoplasmic reticulum or membrane	Cell redox homeostasis	isomerase activity
<i>mhspp90ab1</i>	Heat shock protein 90 kDa alpha (cytosolic), class B member 1	15516	2.00E-03	1.5	cytoplasm or mitochondrion	Response to stress	nucleotide binding
<i>meif1a</i>	Eukaryotic translation initiation factor 1A	13664	4.00E-02	1.5	membrane	Translation	protein binding nucleic acid binding

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
<i>mppm1g</i>	Protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	14208	2.90E-03	1.4	nucleus	Other	translation initiation factor activity transporter activity hydrolase activity
<i>mtxndc5</i>	Thioredoxin domain containing 5	105245	5.70E-03	1.32	endoplasmic reticulum	Cell redox homeostasis	isomerase activity
<i>mupm1</i>	Nucleophosmin 1	18148	1.90E-03	1.3	nucleus	Response to stress	nucleic acid binding protein binding
<i>mamp32a</i>	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	11737	3.80E-03	1.3	nucleus/cytoplasm	Transcription	protein binding

Up-Regulated and Down-Regulated Proteins Identified in 4–7 pH Gradient

Table 4

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
<i>mhylou1</i>	Hypoxia up-regulated 1	12282	4.10E–04	Up-regulated proteins	endoplasmic reticulum	Response to stress	nucleotide binding
<i>maco2</i>	acyl-CoA thioesterase 2	171210	7.00E–06		mitochondrion	Metabolism	hydrolase activity
<i>mhspa5</i>	heat shock protein 5	14828	1.60E–04		endoplasmic reticulum	Response to stress	nucleotide binding protein binding ribosome binding
<i>maars</i>	Alanyl-tRNA synthetase	234734	1.80E–03	3.98	cytoplasm	Metabolism	aminoacyl-tRNA ligase activity ligase activity nucleic acid binding nucleotide binding
<i>mgars</i>	glycyl-tRNA synthetase	353172	1.40E–03	3.28	cytoplasm	Metabolism	aminoacyl-tRNA ligase activity ligase activity nucleotide binding protein binding
<i>mhspa4</i>	Heat shock protein 4	15525	2.80E–03	3	cytoplasm	Response to stress	nucleotide binding
<i>mvcl</i>	Vinculin	22330	4.40E–03	2.92	cytoplasm/cytoskeleton	Structural component	protein binding structural molecule activity
<i>mlonp1</i>	Ion peptidase 1, mitochondrial	74142	3.80E–03	2.78	mitochondrion	Other	DNA binding hydrolase activity nucleotide binding catalytic activity protein binding protein binding
<i>mubal</i>	ubiquitin-like modifier activating enzyme 1	22201	1.10E–03	2.65	unknown	Metabolism	protein binding
<i>mpdcalsip</i>	Programmed cell death 6 interacting protein	18571	5.40E–03	2.65	cytoplasm	Signaling	protein binding
<i>mttrin28</i>	Tripartite motif protein 28	21849	8.40E–03	2.62	nucleus	Transcription	protein binding
<i>minmt</i>	Inner membrane protein, mitochondrial	76614	5.30E–03	2.55	mitochondrion	Unknown	
<i>mgdl2</i>	Guanosine diphosphate (GDP) dissociation inhibitor 2	14569	1.20E–03	2.46	cytoplasm/cytoskeleton/membrane	Other	GTPase activator activity
<i>msdha</i>	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	66945	2.60E–03	2.42	mitochondrion	Metabolism	oxidoreductase activity
<i>mcoro7</i>	Coronin 7	78885	2.10E–03	2.38	cytoplasm	Structural component	
<i>mmcl</i>	Nucleolin	17975	2.70E–03	2.36	nucleus	Other	nucleic acid binding nucleotide binding

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
<i>mehd3</i>	EH-domain containing 3	57440	7.10E-04	2.12	endocytic vesicle	Unknown	protein binding nucleotide binding
<i>mmhn</i>	Neurolysin (metallopeptidase M3 family)	75805	1.40E-05	2.11	cytoplasm/mitochondrion	Signaling	protein binding
<i>nganab</i>	Alpha glucosidase 2 alpha neutral subunit	14376	3.20E-03	2.09	endoplasmic reticulum or Golgi apparatus	Metabolism	hydrolase activity hydrolase activity
<i>msars</i>	seryl- aminoacyl- tRNA synthetase	20226	8.40E-04	2.04	cytoplasm	Metabolism	ligase activity
<i>mmthfd2</i>	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like	17768	7.40E-03	2.02	mitochondrion	Metabolism	nucleotide binding catalytic activity
<i>mslc25a24</i>	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 2	229731	1.60E-05	1.89	mitochondrion or membrane	Translation	binding
<i>EG245436</i>	predicted gene, EG245436	245436	4.20E-04	1.89	unknown	Unknown	
<i>mvil2</i>	Vfflin2	22350	8.90E-03	1.78	cytoplasm/cytoskeleton/membrane	Signaling	protein binding
<i>masns</i>	Asparagine synthetase	27053	5.00E-03	1.73	unknown	Metabolism	structural molecule activity ligase activity
<i>mpbgdh</i>	3-phosphoglycerate dehydrogenase	236539	9.90E-03	1.68	unknown	Metabolism	catalytic activity
<i>mfkbp4</i>	FK506 binding protein 4	14228	9.10E-04	1.64	nucleus	Signaling	protein binding isomerase activity
<i>mpdia3</i>	Protein disulfide isomerase associated 3	14827	4.90E-03	1.58	endoplasmic reticulum	Cell redox homeostasis	isomerase activity
<i>mdync11l1</i>	Dynein, cytoplasmic, fight intermediate chain 1	235661	2.90E-03	1.56	cytoplasm	Other	nucleotide binding
<i>mahcy</i>	S-adenosylhomocysteine hydrolase	269378	6.30E-03	1.46	cytoplasm	Metabolism	hydrolase activity
<i>mpgm2</i>	Phosphoglucumutase 2	72157	4.40E-03	1.43	unknown	Metabolism	phosphoglucumutase activity
<i>msept2</i>	Septin 2	18000	3.00E-04	Down-regulated proteins -1.53	unknown	Others	nucleotide binding
<i>mpcna</i>	Proliferating cell nuclear antigen	18538	7.50E-08	-1.67	nucleus	Others	protein binding
<i>madh1a2</i>	Aldehyde dehydrogenase family 1, subfamily A2	19378	1.80E-04	-1.76	cytoplasm	Signaling	protein binding oxidoreductase activity
<i>mpsme1</i>	Proteasome (prosome, macropain) 28 subunit, alpha	19186	2.60E-06	-2.18	cytoplasm	Unknown	proteasome activator activity
<i>madprh</i>	ADP-ribosylarginine hydrolase	11544	2.10E-04	-2.31	unknown	Unknown	protein binding hydrolase activity

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
<i>moat</i>	Oat protein (ornithine aminotransferase)	18242	4.90E-04	-2.34	mitochondrion	Metabolism	catalytic activity
<i>mlmgcs1</i>	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	208715	2.90E-04	-2.82	cytoplasm	Metabolism	transferase activity

Table 5
Up-Regulated and Down-Regulated Proteins Identified in 6–11 pH Gradient

gene	protein description	gene ID	p value	fold change	localization	cellular process	function
Up-regulated proteins							
mltdha	lactate dehydrogenase A	16828	4.90E-03	1.31	cytoplasm	Metabolism	catalytic activity
mtkt	transketolase	21881	1.90E-03	1.28	unknown	Metabolism	catalytic activity
mfubp1	far upstream element (FUSE) binding protein 1	51886	4.10E-02	1.21	nucleus	Transcription	nucleic acid binding
Down-regulated proteins							
mddx3x	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked	13205	9.30E-03	-1.25	nucleus/cytoplasm	Unknown	helicase/hydrolase activity
mvdac2	voltage-dependent anion channel 2	22334	3.10E-03	-1.38	mitochondrion/membrane	Signaling	voltage-gated anion channel activity
mpsnc6	protease (prosome, macropain) 26S subunit, ATPase 6	67089	6.40E-02	-1.38	nucleus/cytoplasm	Metabolism	hydrolase activity nucleotide binding
mhmmpa2b1	heterogeneous nuclear ribonucleoprotein A2/B1	53379	2.40E-03	-1.6	nucleus	RNA splicing mRNA processing	nucleic acid binding