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Genomewide analysis of gene expression associated with Tcof1 in mouse neuroblastoma

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Abstract

Mutations in the Treacher Collins syndrome gene, TCOF1, cause a disorder of craniofacial development. We manipulated the levels of Tcof1 and its protein treacle in a murine neuroblastoma cell line to identify downstream changes in gene expression using a microarray platform. We identified a set of genes that have similar expression with Tcof1 as well as a set of genes that are negatively correlated with Tcof1 expression. We also showed that the level of Tcof1 and treacle expression is downregulated during differentiation of neuroblastoma cells into neuronal cells. Inhibition of Tcof1 expression by siRNA induced morphological changes in neuroblastoma cells that mimic differentiation. Thus, expression of Tcof1 and treacle synthesis play an important role in the proliferation of neuroblastoma cells and we have identified genes that may be important in this pathway.

Keywords: Treacher Collins syndrome; Neuroblastoma; Microarray

Treacher Collins syndrome (TCS) is an autosomal dominant disorder of craniofacial development. The main features of TCS include abnormalities of the external and middle ear, hypoplasia of the mandible and zygomatic bones, downward slanting of the eyes, coloboma, and cleft palate [1]. The TCS gene, TCOF1, has been localized on human chromosome 5q32–33.1 [2]. TCOF1 encodes for a protein that has been named treacle and consists of mainly a central repeated element, which shows homology to the rat nucleolar phosphoprotein Nopp140 [3–5]. Treacle was found to complex with human Nop56p, a component of the box C/D small nucleolar ribonucleoprotein which plays an important role in the early steps of pre-rRNA processing [6]. The direct association with human Nop56p and its localization within the nucleolus [7,8] suggest that treacle is involved in ribosome biogenesis [6], and may specifically affect ribosomal DNA transcription [9]. Inhibition of treacle expression using siRNA leads to inhibition of ribosomal DNA transcription [9]. The thorough characterization of ribosomal nucleolar proteins has become very important with the identification of their novel nuclear functions that range from RNA biogenesis to control of tumor suppression [10,11]. In addition, cell growth and proliferation are associated with changes in the rate of ribosomal production [11].

The human and mouse Tcof1 genes display 74.3% identity at the nucleotide level [3]. In the Tcof1 knockout mouse, immuno-staining of E11.5 mice with anti-microfilament antibody indicated that the neural crest cell derived organs were underdeveloped and may be due to a high level of apoptosis [12]. The study suggests that treacle synthesis is required for rapidly proliferating cells such as neural crest cells. Neuroblastoma (NB) cells are neural crest derived cells [13] and growth factor withdrawal or treatment with retinoic acid leads NB cells

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to differentiate [14]. The differentiated cells also exit from the cell cycle [15]. Gene and protein expression profiling during differentiation of NB cells triggered by 13-*cis* retinoic acid showed the increased expression of many genes that are known to be involved in the differentiation of NB cells [16]. This suggests that NB cells are suitable for gene expression profiling.

Double stranded RNA interference (RNAi) is a technological advance that enables researchers to reduce gene expression at the post-transcriptional level. RNAi is a RNA mediated sequence specific gene silencing mechanism [17–19]. Until recently, existing approaches to suppress specific gene expression in mammalian cells such as antisense and dominant negative strategies have proven inefficient and inconsistent. Therefore, RNAi with its characteristic efficacy at very low concentrations holds great promise for exploring gene function in mammalian cell cultures [20]. Application of RNAi to post-mitotic primary neuronal cultures effectively inhibited the expression of endogenous and transfected genes [20]. This suggests the usefulness of RNAi approaches in NB cells as a model for silencing Tcof1.

Our hypothesis is that expression of Tcof1 as well as those genes that are associated with Tcof1 expression and its protein treacle is required for the proliferation of NB cells. The research objectives of this study were first to determine the expression of Tcof1 in the NB cell line N1E-115. The second objective was to determine the pattern of expression of Tcof1 and its protein treacle during NB cell proliferation and differentiation, and to correlate these changes to the morphological changes of the NB cells and lastly, to perform gene expression profiling to determine genes that are associated with the overexpression of Tcof1 by stable transfection of Tcof1 and also with siRNA knock down of Tcof1 translation in the same cell line.

Materials and methods

Cell culture. The NB cell line N1E-115 was a gift from Dr. Elliot Richelson (was obtained from Dr. John Bigbee). The cell culture media used are the same as described [21]. Briefly the cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin. For differentiation NB cells were cultured with serum for 24 h and subsequently cultured for 6 days without serum.

Antibody production. A full-length Tcof1 cDNA clone, pmTcof1, was constructed using a 3.5 kb *Eco*RI/*Sfi*I fragment of a partial mouse Tcof1 cDNA clone λ4. This fragment contains the 5′ end of the gene and was inserted into a 3′ RACE Tcof1 clone in Bluescript (Stratagene, La Jolla, CA) [3]. The 5′ fragment (nucleotides 1–1331) of the Tcof1 cDNA was amplified from the pmTcof1 clone using HiFi Taq polymerase (Invitrogen, Carlsbad, CA) at 55 °C annealing temperature with the forward primer that included an *Eco*RI linker (TTAG AATTCTATGGCCGAGGCCAGGAAGCG) and the reverse primer with a *Not*I linker (AAGCGGCCGCGTTGGCACCTTTTCCCGAT GA). The product was TA cloned into the PCR2.1 TOPO vector (Invitrogen) and sequenced to confirm the insert was the correct fragment. The insert was subsequently subcloned into the pET-43.1b[+] expression vector using *Eco*RI/*Sal*I restriction enzyme sites (5′Tcof1/pET-43.1b[+]). The pET-43.1b[+] vector has a His tag and the bacterial NUS protein tag for high expression levels in bacteria. For protein isolation 5′Tcof1/pET-43.1b[+] vector or vector alone was cultured in BL21DE3 cells and proteins were induced by the addition of 100 mg/L of IPTG. Cells were lysed using an Auestin Emulsiflex at ~20,000 psi and the proteins were isolated over a Ni–NTA agarose column. Polyclonal antibodies were produced commercially at Bethyl Laboratories. Briefly the fusion protein was injected into rabbits as an immunogen. The antibodies were affinity purified and the NUS specific antibodies were removed when immunosorbed over the NUS protein. Specificity of the antibody was shown when the antibody detected an in vitro transcribed and translated Tcof1 protein product (data not shown). When Western and immunoprecipitation experiments were performed only a single band of the correct size (205 kDa) was observed.

Creation of stable transfectants in NB cells for overexpression of Tcof1. The full-length mouse Tcof1 gene was cloned into the pDONR201 vector (Invitrogen) by using PCR to generate a full-length clone with attached *att* B sites using primers mTcof1B1nativeF GGGGA CAAGTTTGTACAAAAAAGCAGGCTTAGAAGGAGATAGAA CCATGGCCGAGGCCAGGAAGCG and mTcof1B2nativeR GGG GACCACTTTGTACAAGAAAGCTGGGTCTCACACGGCAGG CTCGGCTG using the full-length clone pmTcof1 as a template. The insert was recombined into the vector as described by the manufacturer (Invitrogen). After the reaction the clone (N2) was converted to a Gateway ENTR vector with *att* L recombination sites. The full-length Tcof1 cDNA N2 clone was subcloned into the expression vector, pDEST12.2 (Invitrogen), which has a CMV promoter. The empty expression vector was prepared by digesting with NotI and SalI to remove the stuffer fragment, the ends Klenow-filled, and ligated.

NB cells were transfected either with clone pDEST12.2-N2 or the empty vector. The transfection of NB cells and selection of the cells were as described [21] and performed at the Molecular Biology Core Facility, Massey Cancer Center. Briefly, in six-well plates, 2×10^5 cells were grown in 2 ml DMEM supplemented with 10% FBS, 1% penicillin/streptomycin until cells reached 40–60% confluence. The cells were transfected with 5 μg N2 or empty vector using lipofectin (Invitrogen). The cells were incubated for 24 h in the presence of serum free DMEM. The media were changed to DMEM plus serum and incubated further for 48 h. After 48 h the cells were replated into 100 mm tissue culture dishes in the presence of selection media (geneticin G418 500 μg/μl). Clonal populations were isolated and maintained in selection media.

Real-time quantitative RT-PCR. Total RNA was isolated using RNAqueous-4PCR isolation kit (Ambion, Austin, TX), according to the manufacturer. RNA concentration was determined by measuring UV absorbance at 260 nm. The quality of RNA was assessed by absorbance ratio of 260/280 nm. The ratio was approximately 2.0. First strand cDNA was reverse-transcribed from 200 ng of total RNA using M-MLV reverse transcriptase and oligo(dT) primer according to manufacturer's instructions (Invitrogen). Real-time quantitative RT PCR was performed in triplicate using Applied Biosystems (ABI) PRISM 7900HT with standard temperature protocol. The primers were designed using Primer Express program version 2.0 (ABI). The sequences of the primers are shown in Table 1. Gapdh is used as endogenous control. Reactions were run with 1.5 μl cDNA, 0.1 pmol/μl primers, and iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA) in a total volume of 15 μl. Acquired data were analyzed using SDS software version 2.2. Gene expression levels were normalized with GAPDH.

Western blot analysis. Lysates were equally loaded into each well and separated on 10% SDS–PAGE gels. The protein was transferred to PDVF membrane according to the manufacturer (Bio-Rad). The membrane was incubated with the primary antibody, rabbit 5′

Table 1
Primers used for quantitative real-time RT-PCR

Gene	Primer	Sequence	Product size (bp)
Ndrg1	Forward	5′ AGTTCGATGTTCAGGAGCAGGA 3′	101
	Reverse	5′ ATACGTGAGGATGACAGGACGG 3′	
Tbx2	Forward	5′ TTCATCGCTGTCACTGCCTA 3′	116
	Reverse	5′ TGCTTCCTTTTCTCCCGAC 3′	
Mapk14	Forward	5′ AGTCCATCATTCACGCCAAAAG 3′	118
	Reverse	5′ AATTCCTCCAGTGACCTTGCG 3′	
Cnbp	Forward	5′ CCTTCTTGGTTCTTCGTCCGA 3′	106
	Reverse	5′ CAGATCGTCCACACTTGAAGCA 3′	
Ddx42	Forward	5′ GGTTTCGGAAATCTCGCTTCA 3′	114
	Reverse	5′ TTCCTCGGTCCGAATTCTCAG 3′	
Nolc1	Forward	5′ CGGAGGTGGCCAGTAAATTTG 3′	101
	Reverse	5′ GGTGGACTTGAGCCAGAAGCTA 3′	
Tcof1	Forward	5′ CAGGCTGTGAACACCACAAAGA 3′	132
	Reverse	5′ CGTCACACTGGTTCTGAGAGCA 3′	
Gapdh	Forward	5′ AGCCTCGTCCCGTAGACAAAA 3′	111
	Reverse	5′ TGGCAACAATCTCCACTTTGC 3′	

anti-treacle, at a concentration of 0.1 μg/ml and at a dilution of 1:25,000 for the secondary antibody (Donkey anti-rabbit, Amersham Biosciences, Piscataway, NJ). The immune-complex was detected using ECL-plus chemiluminescence (Amersham Biosciences) and exposed to X-ray film following the manufacturer's protocol. The same membrane was stripped and then probed with a mouse monoclonal anti-alpha tubulin antibody at 1 μg/ml (Sigma–Aldrich, St. Louis, MO).

siRNA knockdown. Two target siRNA sequences were selected from the open reading frame of mouse Tcof1 using Qiagen's Target Finder design tool (Valencia, CA). The siRNA sequences targeting Tcof1 are from position 1081 to 1101, target 1, and 606 to 626 target 2, relative to the start codon (GenBank Accession No. U81030). Each siRNA sequence was submitted to a BLAST search against the mouse genome sequence [22]. Then siRNAs were chemically synthesized (Qiagen, Valencia, CA) and resuspended in RNase free water. A fluorescence labeled non-silencing siRNA that is known not to have sequence homology to mouse DNA was obtained from Qiagen. This control was used to determine transfection efficiency, as well as a negative control to determine the specificity of Tcof1 siRNA. The gene silencing effect of the Tcof1 siRNA was determined by Western blot analysis.

Transfection of siRNA for gene targeting was carried out with RNAiFect transfection reagent (Qiagen). We monitored transfection with fluorescent-labeled negative control. N1E-115 cells were transfected and cultured for 24 h in six-well plates with fluorescent labeled non-silencing, negative control siRNA and then examined under a fluorescence microscope. We localized siRNA in more than 50% of the cells after 4–6 h of incubation with labeled siRNA. Experimental transfections were carried out in 24-well plates after optimization of siRNA transfection. We used 1.5 μg siRNA in a 1:3 ratio of siRNA to transfection reagent. The effective cell density at transfection was 40,000 cells per well. Gene silencing was monitored after 48 and 72 h at the protein level by Western blotting. There was complete inhibition of treacle expression at 72 h and knockdowns were subsequently performed at 72 h.

Microarray experiments. To identify genes that are associated with Tcof1 overexpression or associated with downregulation of Tcof1 microarray analysis was performed in triplicate. Cell lines and technical replications were randomly processed at each major microarray stage (RNA extraction, labeling, and hybridization) in an effort to reduce effects attributable to biases in measurement error. Total RNA was isolated from parental (wildtype), siRNA treated (knockdown), and Tcof1 overexpressing NB cells using the RNeasy mini kit from Qiagen. Synthesis of cDNA was performed using the superscript choice system (Invitrogen). cDNA synthesis and microarray hybridization were performed in the Nucleic Acid Research facilities. For first strand cDNA synthesis, a T7$(dT)_{24}$ primer containing a T7 promoter site was used. Then double stranded cDNA was purified with the Gene Chip Sample Cleanup Module from Affymetrix (Santa Clara, CA). The biotinylated cRNA was made using microarray high yield RNA transcript labeling kit (Enzo Life Science, Famingdale, NY). The cRNA was then purified with Gene Chip Sample cleanup Module. After quantitation, at $A_{260,}$ the labeled cRNA was fragmented to 35–200 base fragments using metal induced hydrolysis in a fragmentation buffer (Tris–acetate, pH 8.1, potassium acetate, and magnesium acetate). The fragmented cRNAs were then hybridized to Affymetrix mouse A340 oligonucleotide arrays (Affymetrix) for 16 h, at 45 °C and using the GeneChip hybridization oven. In addition, the hybridization mix was spiked with biotinylated cRNA for control sequences spotted into the arrays. Spiked hybridzation controls included labeled transcripts from *Escherichia coli* bioB, bioC, bioD, and cre from bacterophage PI. Following hybridization, the arrays were washed and then stained in GeneChip fluidics station 4000, according to the Affymetrix protocol. Staining was done in a three-step procedure starting with a streptavidin–phycoerythrin staining solution, followed by incubation with biotinylated anti-streptavidin and finally a second staining with streptavidin–phycoerythrin. Stained arrays were scanned with an Agilant Gene-Array Scanner (Affymetrix).

Microarray data analysis.Microarray data were initially processed using the Microarray Suite Software version 5.0 (Affymetrix). A median total hybridization intensity (TGT = 500) was used to normalize microarrays. Scaling factors for microarrays were between 1.9 and 2.6, and 3′ versus 5′ β-actin ratios were below 2 for all microarrays. Probeset summaries were calculated using the robust multi-array average method [23] implemented in the "Affy" package available through the Bioconductor project (www.bioconductor.org) and the R statistical language [24]. Genes with significantly different changes in expression levels between cell lines were identified using the Significance Analysis of Microarray (SAM) program [25]. This method utilizes a rank order based permutation scheme to estimate the false discovery rate (FDR) which is defined as the percent of genes identified by chance. We used SAM to calculate two-sample *t*-statistics for comparisons of gene expression between the different cell lines. Hierarchical clustering [26] was applied to the resulting set of genes with significant changes to identify those genes with similar expression profiles to Tcof1 and to examine possible functional relationships.

Results and discussion

Characterization of Tcof1 in N1E-115 cells

We determined that there is differential expression of Tcof1 in undifferentiated and differentiated N1E-115 cells. Tcof1 was down regulated in NB cells that were induced to differentiate (Fig. 1). This difference was also determined by examining the level of treacle expression between the two cell types (Fig. 2A). It has been previously shown that there are differences in cell morphology between undifferentiated and differentiated NB cells which we also observed [27]. The differentiated NB cells produce neurites while non-differentiated cells had no or very few neurites (Figs. 2B and C). It is also known that once neuroblastoma cells differentiate into neurons they are much more adherent and cease to divide [15,28,29].

We identified stably transfected N1E-115 clones that express higher levels of Tcof1 when compared with clones transfected with empty vector or with the parental cells (Fig. 1). Overexpression at the protein level was also observed (Fig. 3A). Overexpressed cells tended to proliferate faster when compared to the controls (data not shown).

To determine the effect of Tcof1 inhibition on proliferation and differentiation of NB cells and to identify genes that are changed as a result of downregulation of Tcof1 translation, we inhibited translation of Tcof1 in N1E-115 cells using siRNA. Comparison of treacle in siRNA treated N1E-115 cells using Western blot analysis with the parental cells or cells treated with siRNA transfection reagent or a negative control siRNA showed that there was silencing of Tcof1 translation (Figs. 3B and C). The most complete silencing was observed using 1.5 μg/ml siRNA at 72 h. When we compared the morphology of NB cells treated with siRNA for 72 h with non-treated cells, there were morphological differences. The cells treated with siRNA tended to behave like cells cultured without serum to induce differentiation. They had neurites and tended to stick more to the culture dish. We needed more volume of trypsin to lift the cells off the tissue culture wells. Inhibition of Tcof1 expression also led to reduction of proliferation by 40–50% when compared to the siRNA untreated parental control cells (data not shown).

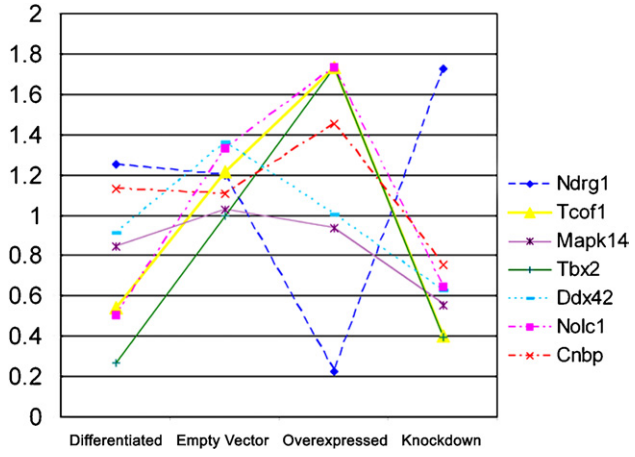

Fig. 1. Relative gene expression levels using real-time quantitative RT-PCR analysis. The *x*-axis shows different treatment groups of N1E-115 cells and the *y*-axis shows relative gene expression levels when compared to undifferentiated NB cells. Gene expression levels were normalized with GAPDH expression. Tcof1 is shown to be downregulated in differentiated and knockdown cells, and overexpressed in stably transfected cells. Tcof1 expression is slightly elevated in the vector only control. Tbx2, Nolc1, and Cnbp expression was identified to be positively correlated with Tcof1 expression while Ndrg1 expression was negatively correlated with Tcof1 expression. Mapk14 and Ddx42 expression was not confirmed to increase with Tcof1 overexpression.

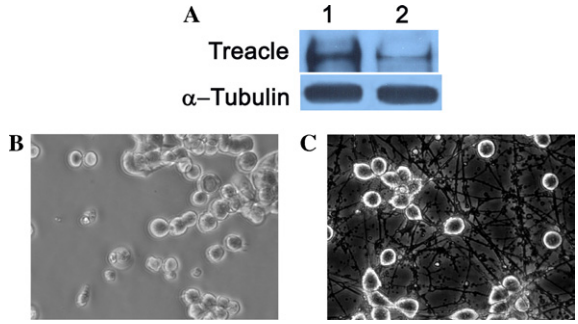

Fig. 2. (A) Western blots using anti 5′ Tcof1 antibody or α-tubulin antibody in undifferentiated (lane 1) and differentiated (lane 2) N1E-115 NB cells. The morphology of undifferentiated (B) and differentiated (C) N1E-115 NB cells.

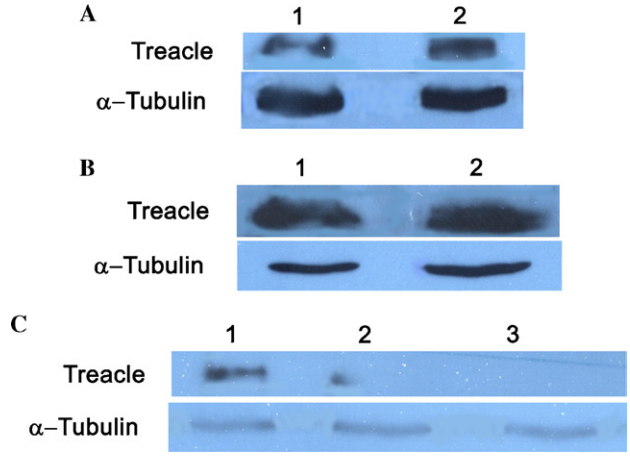

Fig. 3. Western blots using anti 5′ Tcof1 antibody or α-tubulin antibody. (A) Lane 1 shows wildtype N1E-115 cells and lane 2 shows stably transfected cells that overexpress Tcof1. (B) Lane 1 shows wildtype N1E-115 cells and lane 2 shows N1E-115 cells transfected with negative control siRNA. (C) Lane 1 shows wildtype N1E-115 cells, and lanes 2 and 3 show N1E-115 cells transfected with 1.0 and 1.5 μg/ml Tcof1 specific siRNA, respectively.

Our results strongly suggest that treacle is required for the proliferation of N1E-115 cells. Higher levels of expression correlated with faster proliferation of the cells and lower levels of Tcof1 expression correlate with adhesion to the plastic and early imitation of neurite outgrowth or differentiation of the NB cells.

Identification of genes coordinately or negatively regulated with Tcof1

To identify novel genes that have a similar pattern of expression as Tcof1, we used the mouse NB N1E-115 cell line as a model. Wildtype, overexpressed or knockdown Tcof1 expression cell lines were used in microarray studies. Three comparisons were initially made and a large number of genes were significantly changed when Tcof1 was overexpressed when compared with the parental cells or the knockdown cells and a much smaller number were observed when comparing knockdown and wildtype (data not shown). Clustering gene expression intensity across the three cell types identified a set of candidate genes whose expression profiles either positively or negatively correlated with Tcof1 expression (Fig. 4). Fifty-four transcripts were identified to be positively correlated with Tcof1 expression (group 1) while 18 transcripts were identified to be negatively correlated when compared to the levels of Tcof1 expression (group 2) (Tables 2 and 3).

Expression of five genes from group 1 and one from group 2 was validated using quantitative real-time RT-PCR (Fig. 1). Changes of expression in the correct direction as indicated by the microarray of Cnbp, Nolc1, Tbx2, and Ndrg1 were confirmed by this method. Two genes Mapk14 and Ddx42 were found to be downregulated with knock down of Tcof1 but the overexpression of these genes was not confirmed, thus indicating the importance of independent validation of microarray data. We also measured the expression of these genes in the vector only control by quantitative real-time RT-PCR to confirm that changes in gene expression were not the result of nonspecific cellular changes (Fig. 1).

Genes that would promote proliferation are likely to have similar expression patterns with that of Tcof1. Expression of genes that are negatively correlated with Tcof1 expression may repress proliferation or possibly promote cell death. Genes identified in both categories support this hypothesis. Genes involved in cell cycle regulation and metabolism were identified in both groups. Included in the first group were also involved in RNA processing, protein transport, proteosome activity, and cell morphology in addition to other nucleolar genes. A cluster of developmental genes are also found in the first group of genes, one of which, Acvr2, has also been found to be important in craniofacial development [30].

Another developmentally important gene, Tbx2, is the most significantly changed gene in group 1 ($\log_2$ fold change of 1.56) [31]. TBX2 is a known transcriptional repressor and specifically regulates $p19^{ARF}$ in mouse embryo fibroblasts [32]. Activation of Cdkn2a ($p19^{ARF}$ in mouse) stabilizes p53 and leads to growth arrest. Its expression should lead to the suppression of p53 function and aid in the cells' ability to divide. Interestingly, Cdkn2a is represented in the microarray and the expression of this gene is significantly repressed ($\log_2$ fold change of -0.63) as Tbx2 expression increases though its expression does not increase as Tbx2 expression is reduced in the knockdown cells. There may be other factors that are needed to activate Cdkn2a expression under these conditions.

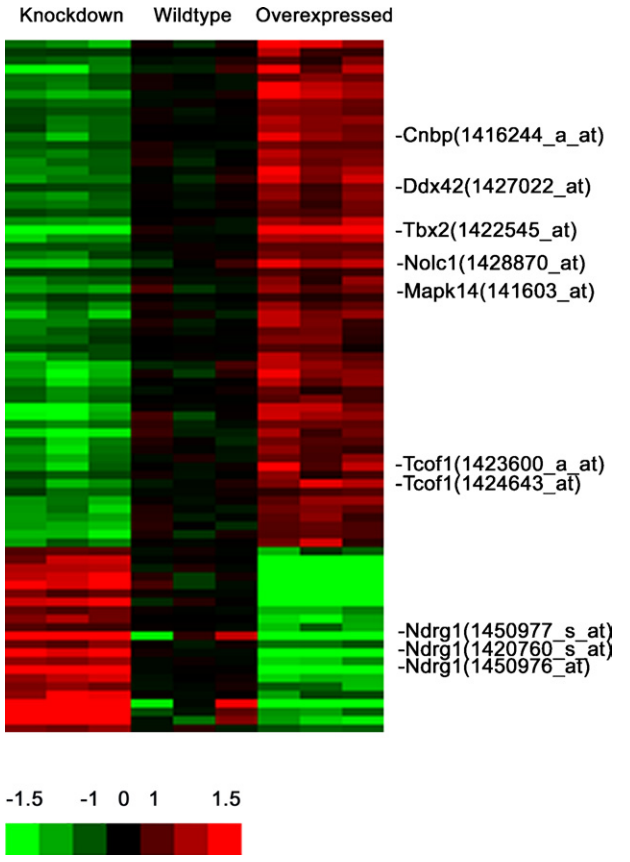

Fig. 4. Hierarchical clustering of gene expression data. Listed are the 54 transcripts that follow the same expression pattern as Tcof1 and the 17 transcripts that follow the opposite pattern. Each row represents an individual transcript and each column is an individual microarray that has been labeled by cell type. In this figure, transcript intensity levels have been standardized by the mean wildtype intensity level. The scale is in log base 2 and extends from -1.5 to 1.5 units.

Other genes that follow the expression of Tcof1 in the three growth conditions include three involved in the ubiquitin pathway. These include Ube1c, an E1 ubiquitin-activating protein, and two proteins, Arih1 and Rnf37, that associate with the ubiquitin-conjugating E2 protein, Ube2l3 (formerly UbcM4). The human homologue has been shown to ubiquitinate p53 in vitro

Table 2
Genes coordinately regulated with Tcof1

Gene symbol	Description
	Cell cycle
Bclaf1	BCL2-associated transcription factor 1
Gpiap1	GPI-anchored membrane protein 1
Mapk14	Mitogen activated protein kinase 14
Rasa1	RAS p21 protein activator 1
Tbrg4	Transforming growth factor beta regulated gene 4
Txnrd1	Thioredoxin reductase 1
1110004D19Rik	RIKEN cDNA 1110004D19 gene
	Cytoskeleton
Ddef1	Development and differentiation enhancing
Iqgap1	IQ motif containing GTPase activating protein 1
Stmn3	Stathmin-like 3
	Developmental
Acvr2	Activin receptor IIA
Cnbp	Cellular nucleic acid binding protein
Tbx2	T-box 2
	Metabolism
Akr1b3	Aldo-keto reductase family 1, member B3 (aldose reductase)
Oazin	Ornithine decarboxylase antizyme inhibitor
Pprc1	Peroxisome proliferator activated receptor γ, coactivator-related 1
	Nuclear transport
Nup155	Nucleoporin 155
Ranbp2	RAN-binding protein 2
Thoc1	THO complex 1
	Nucleolar proteins
Arl5	ADP-ribosylation factor-like 5
Nolc1	Nucleolar and coiled-body phosphoprotein 1
Tcof1	Treacher Collins Franceschetti syndrome 1, homolog
	Proteosome activity
Arih1	Ariadne ubiquitin-conjugating enzyme E2 binding protein homolog 1 (*Drosophila*)
Psmc6	Proteasome (prosome, macropain) 26S subunit, ATPase, 6
Rnf37-pending	Ring finger protein 37
Ube1c	Ubiquitin-activating enzyme E1C
	RNA processing
Ddx42	DEAD (Asp-Glu-Ala-Asp) box polypeptide 42
Fnbp3	Formin binding protein 3
Hnrpdl	Heterogeneous nuclear ribonucleoprotein D-like
Rbm10	RNA binding motif protein 10
Upf2	UPF2 regulator of nonsense transcripts homolog (yeast)
	Other or unknown function
Ankrd10	Ankyrin repeat domain 10
BC024322	cDNA sequence BC024322
BC053071	cDNA BC053071
BC053440	cDNA BC053440
Cebpz	CCAAT/enhancer binding protein zeta
Dnajc11	DnaJ (Hsp40) homolog, subfamily C, member 11
D8Ertd594e	DNA segment, chr 8, ERATO Doi 594, expressed
Epb4.1l1	Erythrocyte protein band 4.1-like 1
Gtf3c4	General transcription factor IIIC, polypeptide 4
Mfap1	Microfibrillar-associated protein 1
Pparbp	Peroxisome proliferator activated receptor binding protein
Ppig	Peptidyl-prolyl isomerase G (cyclophilin G)
Ppil4	Peptidyl-prolyl isomerase (cyclophilin)-like 4
Safb2	Scaffold attachment factor B2
Sdfr1	Stromal cell derived factor receptor 1
Tce4	T-complex expressed gene 4
Terf2	Telomeric repeat binding factor 2
Tex292	Testis expressed gene 292
Tulp4	Tubby like protein 4
1700034P14Rik	RIKEN cDNA 1700034P14 gene
2310037I24Rik	RIKEN cDNA 2310037I24 gene
2610028H07Rik	RIKEN cDNA 2610028H07 gene
4932432N11Rik	RIKEN cDNA 4932432N11 gene

and a mouse hypomorph of Ube2l3 showed growth retardation due to impairment of placental growth [33,34]. Knockout animals of Ube1c also show important developmental regulation with selective apoptosis of the inner cell mass but not of trophoblastic cells [35]. Rasa1 (RAS p21 protein activator 1), a known cell cycle protein, is also coordinately regulated with Tcof1. Rasa1 is known to reduce p38 MapK (gene product of Mapk14) activity through the promotion of GDP-Ras. Mapk14 is known to activate p53 by directly phosphorylating the protein [36,37].

Another interesting gene that has coordinate expression with Tcof1 is Nolc1. Tcof1 has been postulated to be important in ribosome biogenesis by its nucleolar localization and the fact that it has weak but significant homology to Nopp140, the rat homologue of Nolc1 [4].

Table 3
Genes negatively regulated with Tcof1

Gene symbol	Description
	Cell cycle
Bnip3	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3
Bnip3l	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like
Egln3	EGL nine homolog 3 (*Caenorhabditis elegans*)
Sncb	Synuclein, β
	Metabolism
Ak4	Adenylate kinase 4
Fabp3	Fatty acid binding protein 3, muscle, and heart
Galk1	Galactokinase 1
Grhpr	Glyoxylate reductase/hydroxypyruvate reductase
Nudt14	Nudix (nucleoside diphosphate linked moiety X)-type motif 14
Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1
	Other or unknown
Adm	Adrenomedullin
Amhr2	Anti-Mullerian hormone type 2 receptor
Anxa2	Annexin A2
Ndrg1	N-myc downstream regulated 1
4933428I03Rik	RIKEN cDNA 4933428I03 gene
2010012D11Rik	RIKEN cDNA 2010012D11 gene
2300002L21Rik	RIKEN cDNA 2300002L21 gene
2810049G06Rik	RIKEN cDNA 2810049G06 gene

Nopp140 has been hypothesized to act as a chaperone that mediates pre-rRNP export from the nucleus as well as ribosomal protein import from the cytoplasm during ribosome biogenesis. Both TCOF1 and NOLC1 proteins are found in the hNop56p pre-rRNP complex though TCOF1 was shown to directly interact with hNop56p and NOLC1 was not [6]. As TCOF1 is likely to affect ribosome biogenesis by playing a role in ribosomal DNA expression [9], a gene with homology to TCOF1, NOLC1. a gene associated with snoRNPs, has been shown to be coordinately expressed with TCOF1 and thought to have distinct but overlapping functions [38].

The most significant gene to be negatively correlated with Tcof1 is the N-myc downstream-regulated gene 1 (Ndrg1). It has been shown to be upregulated in growth-arrested differentiated cells and under conditions of cellular stress [39–41]. It has also been shown to be repressed in cell transformation [40,42]. Agents that induce the expression of Ndrg1 include p53, retinoic acid, vitamin D, and increased levels of intracellular calcium and forskolin [39,42,43]. In humans NDRG1 has been shown to be especially important in the maintenance of the peripheral nervous system. A mutation identified in NDRG1 causes hereditary motor and sensory neuropathy-Lom (HMSNL) an autosomal recessive form of Charcot-Marie-Tooth disease [44]. Since knock down of Tcof1 in N1E-115 cells is also associated with growth arrest and differentiation the upregulation of Ndrg1, a gene known to be associated with these cellular phenomenon, is not surprising.

Two other genes that are negatively correlated with Tcof1 expression are Bnip3 and Bnip3l. They are both known to bind the anti-apoptotic proteins E1B 19K and Bcl-2 but both are pro-apoptotic proteins [45,46]. Thus, the reduction of Tcof1 may reduce the ability of a cell to synthesize protein could activate an apoptotic response through Bnip3 and Bnip3l. In mice heterozygous for a Tcof1 null allele, an increase in apoptosis in prefusion neural folds was observed [12].

It is interesting to note that a third of genes that are negatively correlated with Tcof1 are involved in cellular metabolism. This includes a gene (Fabp3) involved in intracellular metabolism and/or transport of long-chain fatty acids and has been shown to arrest growth of mammary epithelial cells [47]. Other genes are involved in different metabolic pathways such as galactose (Galk1) and pyruvate (Grhpr) metabolism (Nudt14), purine homeostasis (Ak4), and the catabolism of nucleotide sugars (Nudt14) [48,49]. This may be a general cellular stress response when the cell senses that protein synthesis is impaired and the cell tries to generate additional energy.

Genes that are changed when Tcof1 expression is knocked down may not be the same set of genes that change when N1E-115 cells are made to differentiate through serum starvation. In the real-time validation of the microarray data we also looked at expression of these genes during differentiation. We had previously shown that Tcof1 is downregulated under these circumstances as are Tbx2 and Nolc1. These may be genes involved in general pathways of cellular quiescence. Differences in gene expression between these two cellular states may distinguish between specific pathways that cells can use to control proliferation. Surprisingly, Ndrg1 was not up regulated in differentiated neuroblastoma cells as this gene has been shown to be upregulated in a number of different cell types during differentiation [39–41].

Treacle was found to complex with components that play an important role in ribosomal RNA processing [6]. Cell growth and proliferation are associated with ribosomal production [11]. Thus, previous studies and our study suggest that Tcof1 is involved in the proliferation of NB cells by regulating the rate of ribosome synthesis. Though we have not determined the specific mechanism(s) on how treacle controls the proliferation of NB cells we have identified possible pathways in which ribosome biogenesis may regulate the cell cycle. Further studies are needed to determine if p53 is important in this control. In conclusion, this study shows that the increased expression of Tcof1 and the high rate of treacle synthesis play an important role in the proliferation N1E-115 cells.

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