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The glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*) is regulated by myeloid zinc finger 1 (MZF-1) and is induced by calcitriol

Richard T. Piszczatowski [a,b], Brian J. Rafferty [c], Andre Rozado [a], Szilvia Tobak [a], Nathan H. Lents [a,*]

[a] Department of Sciences, John Jay College, The City University of New York, United States
[b] Albert Einstein College of Medicine, Yeshiva University, United States
[c] Department of Science, Borough of Manhattan Community College, The City University of New York, New York, NY 10019, United States

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ABSTRACT

Recently, new tissue-specific functions for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) have been discovered, aside from its archetypal function in glycolysis. This casts doubt on the legitimacy of using *GAPDH* as a normalization control for gene expression analysis. We report the binding of the myeloid zinc finger-1 (MZF-1) transcription factor to the human *GAPDH* promoter. Furthermore, we show that up-regulation of MZF-1 by 1,25-dihydroxyvitamin D_3 (calcitriol) induces *GAPDH* in HS-5 stromal fibroblasts, while knockdown of *MZF1* by shRNA leads to a concomitant reduction in *GAPDH* expression. This argues that MZF-1 regulates *GAPDH*, indicating a role for GAPDH in calcitriol-mediated signaling.

1. Introduction

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a 37 kDa enzyme responsible for the sixth step of glycolysis by catalyzing glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate in the presence of NAD+and inorganic phosphate[1]. Often regarded as a housekeeping gene, GAPDH serves as a common loading control for protein levels, an unaffected steady-state marker for gene expression analysis, and a negative control for transcription factor binding experiments such as chromatin immunoprecipitation (ChIP).

Until recently, GAPDH has been viewed as an abundant protein in cells, with a simplistic and well understood function, largely impervious to pharmacological and genetic manipulation. However, recent work has revealed novel and diverse roles for the enzyme, and has slowly brought it beyond the scope of a mere housekeeping protein[2]. Aside from its role in apoptosis, new nuclear functions of GAPDH have been discovered, including nuclear membrane fusion, regulation of histone gene expression, telomere structure, and nuclear RNA export [2,3]. Consequently, discoveries of new functions for GAPDH have raised questions as to the propriety of employing it as a reliable loading control and baseline for comparison during gene expression and protein analysis.

During our recent study of the regulation of connective tissue growth factor (CTGF) by myeloid zinc finger-1 (MZF-1), we attempted to utilize GAPDH as a negative control for chromatin immunoprecipitation experiments and a normalization standard during semi-quantitative RT-PCR, per commonly accepted protocol. MZF-1 is a transcription factor belonging to the Kruppel family of Zinc fingers that was isolated from a cDNA library of a patient with chronic myelogenous leukemia[4]. MZF-1 plays crucial roles in hematopoiesis and myeloid cell differentiation. The *MZF-1* gene is up-regulated in response to both retinoic acid and calcitriol, and is involved in the regulation of CD11b and CD14[4,5]. MZF-1 has a very narrow tissue distribution, and is localized to hematopoietic progenitors and cells of the myeloid lineage.

To our knowledge, a specific role for GAPDH within the hematopoietic compartment has not been elucidated, leading us to select GAPDH as a negative control for ChIP analysis, and as internal and loading controls for RT-PCR and protein analysis, respectively. Surprisingly, we observed that MZF-1 binds to the promoter region of GAPDH and additionally, regulates its expression. This suggests a possible new role for GAPDH in hematopoiesis and bone marrow function.

2. Materials and methods

2.1. Reagents

Calcitriol (1α-25 dihydroxyvitamin D_3), was purchased from Enzo Life Sciences. LY 294002 PI3-Kinase Inhibitor was purchased

* Corresponding author. Address: 524 W. 59th Street, New York, NY 10019, USA.
E-mail address: nlents@jjay.cuny.edu (N.H. Lents).

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from Cell Signaling Technologies. DMEM was purchased from MediaTech. The RevertAid Premium first-strand DNA synthesis kit was purchased from Fermentas. Taq DNA Polymerase and ThermoPol buffer were purchased from New England Biolabs. Trizol, Protein A and Protein G DynaBeads were purchased from Invitrogen, Life Technologies. Deoxy-NTPs and were purchased from Promega. All custom-synthesized primers were from Integrated DNA Technologies. SuperSignal West Femto Chemiluminescent Substrate, EZ-Run Protein Ladder, FBS, M-PER, antibiotics, buffers, and anti-GAPDH antibody were purchased from Thermo Fisher Scientific. Rabbit serum was purchased from MP Biomedicals. Anti-Ac-H3 antibody was purchased from Upstate Biotechnology. Anti-MZF-1 antibody, anti-tubulin antibody, and goat anti-rabbit IgG-HRP secondary antibody were purchased from Santa Cruz Biotechnology. The human epithelial carcinoma cell line HeLa, human megakaryoblast cell line MEG-01, human chondrosarcoma cell line SW1353, and the HPV-16 E6/E7 transformed human stromal fibroblast cell line HS-5 were all purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in DMEM with non-essential amino acids, 10% FBS, and penicillin–streptomycin. Cells were washed with 37 °C PBS during subculture.

2.2. Chromatin immunoprecipitation (ChIP)

ChIP was carried out as previously described[6]. Briefly, cells were cultured in 15 cm^2 dishes and cross-linked with an 11% formaldehyde solution. Nuclei were harvested, and immunoprecipitation was performed with sonicated chromatin overnight at 4 °C, using an equal amount of protein A & G Dynabeads for antibody capture. Beads were then washed 12 times with RIPA buffer at room temperature, and cross-links were reversed at 65 °C overnight. DNA was then extracted using Phenol Chloroform Isoamyl Alcohol, and ethanol precipitated. Semi-quantitative polymerase chain reaction (PCR) was then performed with gene-specific primers, followed by gel electrophoresis. Primer sequences are as follows (all sequences 5′–3′ with forward primer listed first): ROCK1, TCACCTTTCCTCACACCACA and GTGTCAGGGGAGCCATA AAA; GAPDH, CGGCTACTAGCGGTTTTACG and AAGAAGATGCGGC TGACTGT; GAPDH-3′-UTR, AACAAGGCCTTTTCCTCTCC and AGGCT GAGCTCCACTAACCA; FGF2, TCCCCATCCTCATCTTTCTG and CGTTT CCACCCCAAAGTAGA; FUT7, AAGATCGCACCACTTGATCC and AAC CAACACCCCACTCTCAG.

2.3. RT-PCR analysis

Cells for calcitriol/LY294002 treatment were grown in 35 × 10 mm dishes (protein) or 6-well dishes (mRNA) for one day prior to treatment. Calcitriol treatment was performed at a final concentration of 100 nM, and LY294002 inhibitor was used at a final concentration of 10 μM. Samples designated for mRNA analysis were harvested 24 h post-treatment while samples for Western blot analysis were harvested 72 h post-treatment. After agonist/inhibitor treatment, samples for mRNA analysis were harvested using 1 mL TRIzol™, and RNA was isolated using chloroform extraction, precipitated with isopropanol, and washed with 70% ethanol, all as described previously[7]. For first-strand cDNA synthesis, 1 μg of RNA was used, and reverse-transcriptase PCR was performed using the Fermentas RevertAid™ Premium First Strand cDNA Synthesis kit following manufacturer's protocol. Sample cDNA levels were normalized using a tubulin control primer, and levels of genes of interest were assessed through PCR. Primers available upon request. Primer sequences are as follows (all sequences 5′–3′ with forward primer listed first): ACTA, TACT CCTGCTTGCTGATCCA and GATGCAGAAGGAGATCACTGC; GAPDH, ACAGTCAGCCGCATCTTCTT and GACAAGCTTCCCGTTCTCAG; MZF1, AGTGTAAGCCCTCACCTCCA and CTCACCCGTGTGGATCTTCT; TUBB, TCTGTTCGCTCAGGTCCTTT and GAAGGCACCACACTGAAGGT.

2.4. Western blots

Cell pellets were harvested and lysed using Mammalian Protein Extraction Reagent (M-PER™), and protein concentration was determined by Bradford Assay. Samples were denatured in 1× Laemmli buffer and boiled for 10 min; an equal amount of protein for each sample was loaded and separated on pre-cast 8–16% gradient SDS–polyacrylamide gels (Pierce Biotechnology), then transferred to a PVDF membrane. Membranes were blocked in 5% milk in TBS-T, and probed with α-MZF-1 (rabbit IgG) or α-GAPDH (rabbit IgG) or anti-tubulin (rabbit IgG) antibody in 2.5% milk in TBS-T at a concentration of 1:1000 at room temperature for 2 h. Following washes with TBS-T, blots were incubated in HRP-conjugated secondary antibody at 1:10,000 in 2.5% milk in TBS-T for 1 h, and developed using the Supersignal™ West Femto chemiluminescence kit.

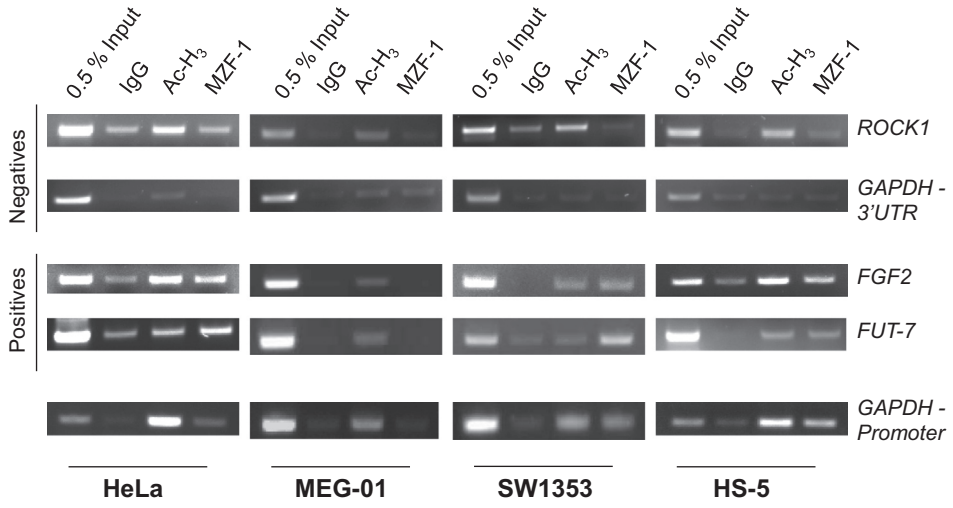


Fig. 1. Chromatin Immunoprecipitation assays of HeLa, MEG-01, SW1353, and HS-5 cells. Asynchronously growing populations of the indicated cell types were formaldehyde fixed and analyzed by ChIP assay. Each Immunoprecipitation contained 20 μg of chromatin and 2 μg of the indicated antibody (top of data panel), while input samples contained 0.1 μg of chromatin (0.5%). Immunoprecipitates were washed, DNA was purified, and PCR reactions were performed with primers specific to the promoter regions indicated (right of data panel).

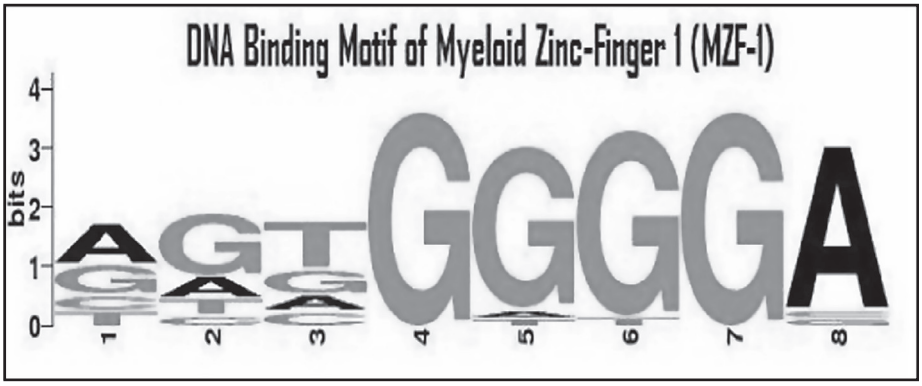
2.5. shRNA knockdown

shRNA-mediated knockdown of *MZF1* was performed using the HUSH-plasmid system (OriGene, Inc.) precisely according to provided protocol, briefly summarized here. MZF1-HUSH plasmids were purchased and introduced into asynchronously growing pre-confluent HS-5 cells via electroporation. Electroporation was performed using an AMAXA nucleofector II device (Lonza) using 2.5×10^6 cells and 1.5 mg of plasmid per cuvette, precisely according to manufacturer's recommended protocol for Nucleofector Kit V (Lonza) and electroporation program T-030. Following electroporation, cells were recovered in pre-equilibrated culture medium, seeded into 12-well plates, and grown for four days. Upon replating, 1.0 mg/mL puromycin was added to the culture medium and cells were grown for 2 weeks. Cells were harvested and prepared for cDNA synthesis as described above.

3. Results

During the course of investigating the binding of MZF-1 to the *CTGF* promoter, we selected GAPDH as a negative control for the ChIP experiment. MEG-01 cells were selected because they do not express MZF-1, thus useful for showing specificity of the MZF-1 antibody. Contrary to our expectation, the MZF-1 antibody precipitated the *GAPDH* promoter in 3 cell types that express MZF-1 (HeLa, HS-5, and SW1353 cells), but not in MEG-01 cells (Fig. 1). That the MZF-1 transcription factor specifically binds the *GAPDH* promoter indicates a potential regulatory function thereof.

To search for an MZF-1 site within the *GAPDH* gene, we scanned the *GAPDH* promoter for candidate MZF-1 binding sites. We first input the promoter region of the human *GAPDH* gene, designated as 1000 base pairs upstream of the transcription start site (TSS), into the TFSearch program (http://www.cbrc.jp/research/db/TFSEARCH.html)



-700 gcccggagagagccgctggtgcacacagggccgggattgtctgccctaat
-650 tatcaggtccaggctacagggctgcaggacatcgtgaccttccgtgcaga
-600 aacctccccctcccctcaagccgcctcccgagcctccttcctctccagg
-550 cccccagtgcccagtgcccagtgcccagcccaggcctcggtcccagagat
-500 gccaggagccagga**gatgggga**ggggggaagtgggggctgggaaggaacca
-450 cgggcccccgcccgaggcccatgggcccctcctaggcctttgcctgagca
-400 gtccggtgtcactaccgcagagcctcgaggagaagttccccaactttccc
-350 gcctctcagcctttgaaagaaagaaaggggaggggcaggccgcgtgcag
-300 ccgcgagcggtgctgggctccggctccaattccccatctcagtcgttccc
-250 aaagtcctcctgtttcatccaagcgtgtaagggtccccgtccttgactcc
-200 ctagtgtcctgctgcccacagtccagtcctgggaaccagcaccgatcacc
-150 tcccatcgggccaatctcagtcccttcccccctacgtcggggcccacacg
-100 ctcggtgcgtgcccagttgaaccaggcggctgcggaaaaaaaaa**agcggg**
-50 **ga**gaaagtagggcccggctactagcggttttacgggcgcacgtagctcag
1 GCCTCAAGACCTTGGGCTGGGACTGGCTGAGCCTGGCGGGAGGCGGGGTC
51 CGAGTCACCGCCTGCCGCCGCGCCCCCGGTTTCTATAAATTGAGCCCGCA

Fig. 2. The *GAPDH* gene contains binding sites for MZF-1. Upper panel: the DNA binding logo for MZF-1 based on TRANSFAC matrix table #M00083, created using WebLogo™. Lower panel: −700 to +100 of the human *GAPDH* promoter, returned from the UCSC Genome Browser Gateway, December 2013 assembly. Sequences that match to the MZF-1 binding motif are bold and underlined or italicized and boxed.

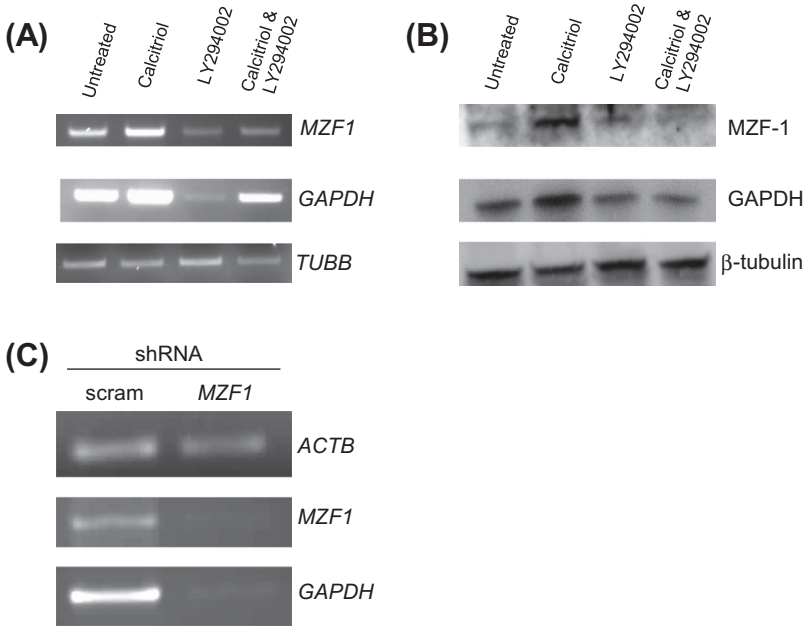


Fig. 3. *GAPDH* expression is affected by induction or knockdown of MZF1. Asynchronously growing populations of HS-5 cells were incubated in the presence or absence of 100 nM calcitriol and/or 10 μM LY294002 for 24 (panel A) or 72 (panel B) hours. (A) Cells were harvested in TRIzol, mRNA was extracted and cDNA synthesized, and PCR reactions performed with primers corresponding to the genes indicated on the right of the data panels. (B) Cell pellets were harvested, protein lysates prepared, and Western blots performed with antibodies indicated to the right of the data panels. (C) Cells were electroporated with the indicated shRNA plasmid, selected with puromycin for 2 weeks, and harvested for mRNA analysis.

[8]. Three candidate TF binding sites with at least 85% sequence identity to an established MZF-1 binding site position-weight matrix (PWM) found in TRANSFAC [9] (http://www.gwene-regulation.com/cgi-bin/pub/databases/transfac/getTF.cgi?AC=M00083) were identified. Two of these candidate binding sites overlap, with a shift of six base pairs, which is not altogether uncommon for TF binding sites (Fig. 2). Importantly, all three putative binding sites align well to the established PWM for MZF-1, a binding logo for which is also shown in Fig. 2.

In order to investigate whether the promoter occupancy of *GAPDH* by MZF-1 results in physiological regulation, we stimulated HS-5 cells with 1,25-dihydroxyvitamin D_3 (calcitriol) with or without the PI-3 kinase inhibitor LY294002. It has been shown that calcitriol increases MZF-1 production, an effect that is blocked by the PI3-kinase inhibitor LY294002 [5]. Upon stimulation with calcitriol, levels of both *MZF-1* and *GAPDH* mRNA increase, an effect that is inhibited by LY294002, as compared to β-tubulin (Fig. 3A). Similar increases in MZF-1 and GAPDH expression were observed on the protein level via Western blot analysis. Up-regulation of MZF-1 with calcitriol treatment induces GAPDH protein expression, and induction of both proteins was blocked upon LY294002 treatment (Fig. 3B). β-Tubulin levels were observed to be constant upon the loading of equal amounts of protein.

In order to confirm that the induction of *GAPDH* observed upon treatment of calcitriol is indeed mediated by MZF-1, we performed the opposite perturbation of knocking down *MZF1* using an shRNA plasmid. As shown in Fig. 3C, transfection of the MZF1-shRNA plasmid leads to a marked decrease in the steady-state level of *MZF1* mRNA. In addition, this affect is accompanied by a concomitant decrease in the expression of *GAPDH* (Fig. 3C). This further argues that the MZF-1 transcription factor binds to the *GAPDH* promoter and acts as a trans-activating factor for gene transcription.

4. Discussion

Contrary to our expectations, we have observed that the MZF-1 transcription factor binds to the *GAPDH* promoter in HeLa, HS-5, and SW1353 cell lines, as seen through chromatin immunoprecipitation (ChIP). The human *GAPDH* promoter harbors two elements that match the PWM of MZF-1 binding preference, one of which contains two overlapping binding sequences, and we have confirmed the presence of MZF-1 binding sites in the promoter region of the *GAPDH* gene in several species (data not shown). Treatment with vitamin D_3 and *MZF1*-shRNA confirmed that MZF-1 functionally regulates the *GAPDH* gene.

GAPDH has long been considered a housekeeping gene with little context-dependent regulation, and has largely been accepted as a control for steady-state gene expression in a wide variety of contexts. However, recent studies have uncovered novel tissue- and disease-specific functions for the enzyme that have brought heightened attention to the use of *GAPDH* as a housekeeping gene and loading control. Neurodegenerative diseases such as Alzheimer's and Huntington's have been shown to alter *GAPDH* expression, suggesting specialized roles for GAPDH in the brain[10]. Though no specific function for GAPDH in the bone marrow has yet been demonstrated, our discovery of the regulation of *GAPDH* by MZF-1 suggests an undiscovered role in hematopoiesis. Furthermore, our results demonstrate that GAPDH ought not be used as a loading control for gene expression and protein analysis in studies concerning the MZF-1 transcription factor or vitamin D signaling. Further, retinoic acid (vitamin A) has also been shown to induce *MZF1* [11]. Indeed, *GAPDH* is well suited as a *positive* control for MZF-1 function in cells that express this transcription factor. This discovery also invites close scrutiny toward experiments that utilize GAPDH as a "housekeeping control" for gene expression comparisons in any context.

Statement of financial interest

The authors declare that they have no competing interests, financial or otherwise, in any of the contents of this work.

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