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FHL2 switches MITF from activator to repressor of Erbin expression during cardiac hypertrophy

Inbal Rachmin [a,1,2,3,4], Eden Amsalem [a,2], Eliahu Golomb [b,3], Ronen Beeri [c], Dan Gilon [c,3], Pengfei Fang [d,2], Hovav Nechushtan [e,3], Gillian Kay [a,4], Min Guo [d,2,3], Peter Li Yiqing [f,2,3], Roger S.-Y. Foo [f,*,2], David E. Fisher [g,*,3], Ehud Razin [a,*,1], Sagi Tshori [h,1]

[a] Department of Biochemistry and Molecular Biology, The Institute for Medical Research Israel–Canada, Hebrew University Medical School, Jerusalem 91120, Israel
[b] Department of Pathology, Shaare Zedek Medical Center, Jerusalem 91031, Israel
[c] Heart Institute, Hadassah-Hebrew University Medical Center, P.O. Box 12000, Jerusalem 91120, Israel
[d] Department of Cancer Biology, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458, USA
[e] Sharett Institute of Oncology, Hadassah Hebrew University Medical center, P.O. Box 12000, Jerusalem 91120, Israel
[f] Cardiovascular Research institute, Center of Translational Medicine, National University of Singapore, 117599, Singapore
[g] Cutaneous Biology Research Center, Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Building 149, 13th Street Charlestown, Boston, MA 02129, USA
[h] Department of Nuclear Medicine, Hadassah-Hebrew University Medical Center, P.O. Box 12000, Jerusalem 91120, Israel

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ABSTRACT

Background: Congestive heart failure (CHF) is a significant health care burden in developed countries. However, the molecular events leading from cardiac hypertrophy to CHF are unclear and preventive therapeutic approaches are limited. We have previously described that microphthalmia-associated transcription factor (MITF) is a key regulator of cardiac hypertrophy, but its cardiac targets are still uncharacterized.
Methods and results: Gene array analysis of hearts from MITF-mutated mice indicated that ErbB2 interacting protein (Erbin) is a candidate target gene for MITF. We have recently demonstrated that Erbin is decreased in human heart failure and plays a role as a negative modulator of pathological cardiac hypertrophy. Here we show that Erbin expression is regulated by MITF. Under basal conditions MITF activates Erbin expression by direct binding to its promoter. However, under β-adrenergic stimulation Erbin expression is decreased only in wild type mice, but not in MITF-mutated mice. Yeast two-hybrid screening, using MITF as bait, identified an interaction with the cardiac-predominant four-and-a-half LIM domain protein 2 (FHL2), which was confirmed by co-immunoprecipitation in both mouse and human hearts. Upon β-adrenergic stimulation, FHL2 and MITF bind Erbin promoter as a complex and repress MITF-directed Erbin expression. Overexpression of FHL2 alone had no effect on Erbin expression, but in the presence of MITF, Erbin expression was decreased. FHL2–MITF association was also increased in biopsies of heart failure patients.
Conclusion: MITF unexpectedly regulates both the activation and the repression of Erbin expression. This ligand mediated fine tuning of its gene expression could be an important mechanism in the process of cardiac hypertrophy and heart failure.

1. Introduction

MITF is a basic helix–loop–helix leucine zipper (bHLH-Zip) DNA-binding protein [1]. Its gene resides at the *mi* locus in mice [2]. Mutations of this gene result in deafness, small eyes, and poorly pigmented eyes and skin [3]. In humans, heterozygous mutations in this gene cause Waardenburg Syndrome type II [4], resulting in hypopigmentation and deafness.

MITF regulates gene transcription by binding to E-box elements in the 5′-flanking regions or functional enhancers of MITF-responsive genes [5]. MITF functions as either a homodimer or heterodimer with transcription factors of the related MiT family [5,6].

We have previously demonstrated that the H isoform of MITF is highly expressed in cardiomyocytes [7], and that MITF-mutated mice have a diminished cardiac hypertrophic response to β-adrenergic stimulation, decreased cardiac function and a tendency for sudden death [8]. Moreover, we reported that middle-aged MITF-mutated mice have a much smaller heart mass and decreased cardiac function and output [8]. These observations indicate that MITF plays an essential role in the

* Corresponding authors.
E-mail addresses: mdcrfsy@nus.edu.sg (R.S.-Y. Foo), ehudr@ekmd.huji.ac.il (E. Razin).
[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[2] This author performed the experiments.
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[4] This author wrote the paper.

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development of cardiac hypertrophy [8]. In order to identify cardiac MITF target genes, we conducted a gene array analysis of mRNA from a pool of hearts derived from middle-aged MITF mutated mice (*ce*/*ce*) and compared it to that from their normal siblings (*sp*/*sp*).

One of the candidate target genes identified in this assay was the ErbB2 interacting protein (Erbin). Erbin is a member of the leucine-rich repeat and PDZ domain (LAP) proteins [9]. It was originally described as a binding partner of Her2/neu (ErbB2) [9]. We recently reported that Erbin is involved in cardiac hypertrophy. When cardiac hypertrophy was induced, *Erbin*$^{-/-}$ mice developed heart failure and following severe pressure overload all *Erbin*$^{-/-}$ mice died [10]. Little was known regarding the regulation of Erbin expression. The transcription factor c-Myb has been shown to directly regulate Erbin in HeLa cells [11], but no transcription factor regulating Erbin expression in the heart has been reported.

Here we used *in silico*, *in vitro* and *in vivo* approaches to demonstrate that Erbin expression in the heart is directly regulated by MITF. Under basal conditions MITF activates Erbin expression by binding two E-box elements in the Erbin promoter, whereas following β-adrenergic stimulation, MITF inhibits Erbin expression. We further found that this inhibition by MITF is mediated by its interaction with Four and a half LIM domain protein 2 (FHL2) while MITF is bound to its target gene. FHL2 is a LIM domain binding protein expressed predominately in the heart and in smooth muscle cells [12]. FHL2–MITF interaction is mediated by the LIM2 and LIM3 domains of FHL2 and the bHLH domain of MITF. Thus, activation/repression of Erbin expression in the heart is regulated by FHL2–MITF interaction.

2. Material and methods

2.1. Cell culture

HEK293T, NIH3T3 and H9c2 cells were maintained at 37 °C in growth medium, which was Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Biological Industries). Cells were serum-starved for 18 h in DMEM and treated with 10 μM isoproterenol (Sigma-Aldrich) overnight. Myocardial cells from ventricle fragments of hearts of 1 day old Sprague–Dawley rats were isolated by serial trypsinization as previously described [13]. Cells were suspended in F-10 medium containing 10% heat-inactivated FBS and 10% horse serum and penicillin–streptomycin antibiotic solution (Biological Industries). This medium was also used as the standard culture medium in the experiments. The cell suspensions were enriched for cardiomyocytes by pre-plating on tissue culture dishes for 30 min to allow attachment of fibroblasts. The cells were plated on 60 mm Petri dishes at a density of 10^6 cells/ml. For isoproterenol treatment cells were incubated with serum-free medium for 18 h and treated with 10 μM of isoproterenol (Sigma-Aldrich) for an additional 18 h.

2.2. Human left ventricular biopsies

Human left ventricular tissue was collected following a protocol approved by the Papworth (Cambridge) Hospital Tissue Bank Review Board and the Cambridgeshire Research Ethics Committee (United Kingdom). Written consent was obtained from every individual according to the Papworth Tissue Bank protocol. Left ventricular (LV) tissue was obtained from non-donor suitable healthy male individuals involved in road traffic accidents. At the time of transplantation or donor harvest, whole hearts were removed after preservation and transported in cold cardioplegic solution (cardioplegia formula and Hartmann's solution) similar to the procedure described before at Imperial College, London [14]. Following analysis by a cardiovascular pathologist, left ventricular segments were cut and stored immediately in RNAlater (Ambion).

2.3. Mice

All mouse lines were held and propagated in a specific pathogen-free environment. Both MITF *sp*/*sp* and MITF *sp*/*ce* mice were kindly provided by L. Lamoreux (College of Veterinary Medicine, Texas A&M University, and College Station, Texas, USA), were on a C57BL/6 background and were bred to produce *sp*/*sp* and *ce*/*ce* mice for experiments. MITF encoded by the mutated mouse allele (*ce*/*ce*) lacks the zip domain of MITF due to a stop codon between the HLH and zip domains while their normal littermates (*sp*/*sp*) express the full-length protein, apart from the six amino acids of exon 6a [15]. VGA-9tg/+ mice were kindly provided by H. Arnheiter (NIH, Bethesda, Maryland, USA). Mice carrying the *tg*/*tg* mutation have an insertion of approximately 50 copies of a transgene integrated inside the MITF promoter and are unable to express MITF. Mice aged 6–8 weeks were used for all the experiments apart from those represented in Fig. 1, for which 15 month old mice were used.

All experiments were performed in compliance with the Israeli Prevention of Cruelty to Animals Law and were approved by the Hebrew University Animal Care and Use Committee.

2.4. Administration of isoproterenol

For the induction of cardiac hypertrophy, 6 week old *sp*/*sp* and *ce*/*ce* MITF mutant mice were administered either 5 mg/kg isoproterenol (Sigma-Aldrich) or saline subcutaneously to the neck once a day for 7 days.

For the co-immunoprecipitation assay, 8 week old wild type (WT) mice were administered either 15 mg/kg isoproterenol (Sigma-Aldrich) or saline intraperitoneally (i.p) once a day for 5 days.

2.5. Gene array

Gene array membranes covering the known coding sequences of the entire mouse genome were printed using MicroGrid II Compact (BioRobotics) by the Interdepartmental Unit of the Medical School of the Hebrew University and Hadassah Medical Center. RNA was extracted from a pool of 15 month old normal (*sp*/*sp*) and *ce*/*ce* MITF mutated mice. The RNA was reversed transcribed, tagged by Cy3 and Cy5, and hybridized to the mouse custom-made mRNA array. Results were read using the GenePix 4000B system (Molecular Devices) and analyzed using Matlab written processing routines.

2.6. Antibodies

Anti-Erbin antibody (rabbit polyclonal sera) was kindly provided by Prof. Jean Borg (Marseille, France) and anti-MITF antibody (C5-mouse monoclonal) was kindly provided by Prof. David E. Fisher (Dana-Farber Cancer Institute and Children's Hospital, Boston, MA). Anti-β-actin antibody (Sigma-Aldrich), anti-tubulin antibody, anti-GAPDH (Santa Cruz) and anti-FHL2 (MBL) were purchased. These antibodies were used for EMSA, ChIP and Western blots.

2.7. Real-time quantitative PCR

Candidate MITF and Erbin responsive genes were measured using real-time quantitative PCR. Total RNA was extracted from the hearts of *ce*/*ce*, *sp*/*sp* and WT mice. mRNA levels of various genes were quantified by SYBR Green incorporation (ABgene SYBR green ROX Mix, ABgene). Real-time PCR was performed on the Rotor-Gene 3000 sequence detection system (Corbett).

The primers used for gene amplification for real-time PCR were as follows: β-actin sense, 5′-CCTGATCCACATCTGCTGGAA-3′; β-actin antisense, 5′-ATTGCCGACAGGATGCAGA A-3′; Erbin sense, 5′-GCATCCGCAG ACATCCAGTCCA-3′; Erbin antisense, 5′-GGCTGGC CCATTTGTCCATTA