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Characterization of the novel interaction between muskelin and TBX20, a critical cardiogenic transcription factor

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ABSTRACT

The genetic regulation necessary for the formation of a four-chambered heart is tightly regulated by transcription factors such as TBX20, a member of the T-box (TBX) transcription factor family. TBX20 is critical for proper cardiogenesis and is expressed in the heart throughout development. Missense mutations in TBX20 have been found in patients with congenital heart defects (CHD). Characterization of modifiers of TBX20 activity will help elucidate the genetic mechanisms of heart development and CHD. A yeast two-hybrid assay screening an embryonic mouse heart cDNA library with TBX20b as bait was used to identify potential modifiers of TBX20 activity and identified an interaction with muskelin (MKLN1), a primarily cytoplasmic protein with potential roles in signal transduction machinery scaffolding and nucleocytoplasmic protein shuttling. In cellular studies, MKLN1 directly binds to the T-box DNA-binding domain of only the TBX20b isoform by its kelch repeats domain. Immunostaining of mammalian cells transfected with tagged TBX20b and MKLN1 revealed colocalization primarily in the cytoplasm. Immunohistochemistry analysis of embryonic mouse hearts reveals coexpression in the developing endocardial valvular and myocardial interventricular cells. This novel interaction between TBX20b and MKLN1 may help elucidate new regulatory mechanisms within heart development.

1. Introduction

Cardiogenesis is tightly regulated by networks of different transcription factors. The T-box (TBX) transcription factor family is important for the development of many mesoderm-derived structures, including the heart [1,2]. All TBX transcription factors share a highly conserved DNA-binding domain, designated the T-box [3]. Several TBX factors are directly involved in cardiogenesis including TBX2, TBX3, TBX5, TBX18, and TBX20 [4,5]. Each of these factors can be characterized as activators, repressors, or as having both properties depending on the developmental context. The coexpression of TBX transcription factors, each with different activities, targets, and cofactor binding abilities, adds complexities within the transcriptional networks driving cardiogenesis. Identifying proteins involved in regulating TBX transcription factor activity would provide important information in understanding the transcriptional regulation required for cardiogenesis.

TBX20 is critical for proper cardiogenesis and is expressed throughout heart development. Homozygous *Tbx20* null mice die by embryonic day (E) 10.5 due to cardiac insufficiency from an unlooped, un-elongated heart tube that has not differentiated into chamber myocardium [6–9]. Consistent with mouse models, missense mutations within human *TBX20* have been identified in patients with congenital heart defects (CHD) [10–12]. TBX20 functions in the early cardiac determination processes, regulates proliferation, and regulates elongation of the heart tube through cell recruitment from the secondary heart field [6–9]. In later heart development, TBX20 functions in chamber differentiation in part through repression of TBX2 activity [6–8]. TBX20 is also important in valvuloseptal formation through regulation of extracellular matrix (ECM) components and epithelial-to-mesenchymal transition (EMT) in the developing atrioventricular canal (AVC) and outflow tract (OFT) regions [13]. Multiple isoforms of TBX20 have been identified with the two major isoforms being TBX20a at 445 amino acids (aa) and TBX20b at 297 aa. Both are identical through the length of TBX20b with TBX20a containing an extended C-terminal region with characterized activation and repression domains [14].

To discover regulators of TBX20 during heart development, a yeast two-hybrid assay was employed to identify novel interacting partners using the human TBX20b isoform as bait. The yeast two-hybrid assay screened a library of cDNAs from E9.5–11.5 mouse hearts and identified muskelin (MKLN1) as an interacting protein.

Abbreviations: aa, amino acids; AVC, atrioventricular canal; CHD, congenital heart defects; E, embryonic day; ECM, extracellular matrix; EF, elution fraction; EMT, epithelial-to-mesenchymal transition; GST, glutathione S-transferase; HA, hemagglutinin; MKLN1, muskelin1; OFT, outflow tract; Q, quadruple; TBX, T-box; Trp/Leu, tryptophan and leucine; WCL, whole cell lysate.

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In this study, the TBX20b–MKLN1 interaction was confirmed as a novel isoform-specific interaction occurring in the perinuclear region of mammalian cells. Expression analysis determined that TBX20 and MKLN1 are coexpressed in regions involved in development of the interventricular septum and the valvuloseptal regions of the AVC.

2. Materials and methods

2.1. Plasmid construction and yeast two-hybrid library screening

The human *TBX20b* isoform cDNA (BC120946.1) was obtained from Open Biosystems and was cloned into the DNA-binding domain vector, pGBTK-T7 (Clontech). To create the yeast two-hybrid screening library, cDNA fragments were fused with the Gal4 activation domain in the pGAD-T7 vector (Clontech). First, mRNA was isolated from embryonic hearts of ICR mice between E9.5 and E11.5, and primed with the NotI-oligo-d(T) primer to generate cDNAs using the SuperScript Plasmid System for cDNA Synthesis and Cloning (Invitrogen). pGAD-T7 was modified by digestion with PvuII and self-ligation to remove the NotI site. Then, SalI and NotI sites were inserted into the polylinker region. Finally, the cDNA fragments were linked with a SalI adaptor, digested with NotI, and directionally cloned into the SalI and NotI sites of the modified pGAD-T7 vector to generate the library. At least 5×10^6 independent clones were included in the primary library and all 16 randomly picked clones contained inserts with an average size of 1.9 kilobases (data not shown). The yeast two-hybrid screening assay was done in AH109 cells following Clontech's Matchmaker protocol. One of the resultant clones was the full ORF of *Mkln1* (RefSeq. NM_013791.2).

2.2. Copurification studies

For the *in vitro* copurification studies, TBX20b was cloned into pGEX-2T (GEHealthcare). BL21 cells were transformed with either GST or GST-TBX20b and protein production was induced with IPTG treatment. GST and GST-TBX20b proteins were purified with glutathione Sepharose beads (Invitrogen) and incubated with *in vitro* translated radiolabeled MKLN1 generated with the TNT-coupled transcription/translation system (Promega). Protein complexes were isolated by centrifugation, eluted, and analyzed by SDS–PAGE and radiography.

For mammalian cell overexpression copurification studies, MKLN1 was cloned into the pCMV-HA expression vector (Clontech) and TBX20b was cloned into the pCMV-GST vector [15]. The copurification studies were done as previously described [16]. Briefly, COSM6 cells were transfected according to the Lipofectamine 2000 protocol (Invitrogen). After 48 h, lysates were collected and copurified with glutathione Sepharose beads rotating at 4 °C for 4–6 h. The beads were collected by centrifugation and the interacting proteins were eluted. Whole cell lysate and copurification elution fractions were analyzed by SDS–PAGE and Western blot analysis. Antibodies included GST (GEHealthcare, 27457701V) and HA (HA1.1 16B12). HRP-conjugated secondary antibodies were used for visualization of the antibody complexes with enzyme-linked chemiluminescence (Millipore).

2.3. Immunohistochemistry and immunocytochemistry

ICR mice matings were timed to collect E9.5–12.5 embryos. After dissection, the whole embryo or embryonic hearts were fixed in 4% paraformaldehyde, dehydrated with ethanol washes, cleared with Histo-clear (National Diagnostics) and embedded in paraffin wax. The tissues were sectioned at 7 μm and processed for immunohistochemistry. Antibodies included TBX20 at 1:1000 (Sigma, HPA008192) and MKLN1 at 1:50 (Sigma, HPA022817). Signals were visualized through a DAB chromogen system (Dako) and slides were counterstained with hematoxylin.

For cellular immunostaining, cells were plated on glass coverslips in a 24-well plate overnight. Cells were transfected using Lipofectamine 2000. After 48 h, cells were stained overnight with primary antibody at 4 °C. The cells were washed, stained with fluorophore-conjugated secondary antibody and mounted onto glass microscope slides with DAPI counterstaining mounting solution (Vectashield).

2.4. Subcellular localization analysis

Subcellular localization of TBX20 and MKLN1 was determined with the nuclear/cytoplasmic fractionation kit from ThermoScientific. Cells were transfected as described previously. TBX20b was cloned into the pCMV-Tag3 construct to create a myc-tagged fusion protein (Stratagene). Protein lysates were quantified and analyzed by SDS–PAGE and Western analysis. Antibodies for cytoplasmic and nuclear fraction controls are MEK1/2 (Cell Signaling, L38C12) and LSD1 (Cell Signaling, C69G12), respectively.

3. Results

3.1. Yeast two-hybrid assay identified MKLN1 as a novel interacting partner to TBX20b

A novel yeast two-hybrid library was constructed to facilitate the identification of protein interactions relevant to mouse heart development. The library contains cDNAs isolated from the hearts of E9.5–11.5 mice. To avoid interference from the activation/repression domains of the longer human TBX20a isoform [14], the shorter human TBX20b isoform was used as bait. In this study, the yeast two-hybrid assay yielded 1.5×10^5 transformants as determined by growth on tryptophan/leucine (Trp/Leu) double dropout selection plates. After selection for protein interaction on quadruple (Q) dropout plates, fifteen colonies were established. Sequence analysis showed that 13 colonies contained three unique clones of MKLN1. The interaction of TBX20b and MKLN1 was confirmed with a yeast cotransformation assay as shown in Fig. 1A. A single colony from each cotransformation plate was replated as a patch on a Trp/Leu dropout plate, with growth confirming cotransformation. The yeast patches were replica-plated into a Q dropout plate. Growth of yeast cotransformed with pGBTK-TBX20b and pGAD-MKLN1 on the Q plate confirmed the protein–protein interaction.

An *in vitro* copurification assay using a GST pull-down method with a GST-TBX20b fusion protein and radiolabeled MKLN1 confirmed direct interaction. *In vitro* translated, radiolabeled MKLN1 was generated and incubated with GST or GST-TBX20b proteins isolated from bacterial cultures. Protein complexes were isolated by GST pull-down copurification and analyzed by SDS–PAGE. The presence of the GST and GST-TBX20b proteins are shown on the coomassie-stained gel, which was then exposed on radiography film to reveal the presence of MKLN1. MKLN1 was found in the input lane and the GST-TBX20b elution lane, confirming the direct interaction between the radiolabeled MKLN1 and GST-TBX20b (Fig. 1B).

To confirm the TBX20b–MKLN1 interaction within mammalian cells, a copurification assay using transfected cell lysates was done. COSM6 cells were cotransfected with HA-MKLN1 and GST or GST-TBX20b. Lysates were collected and whole cell lysates were saved. The remaining lysates were incubated with GST-binding beads to isolate the GST fusion proteins and interacting proteins.