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Characterization of TBX20 isoforms and protein interactions in heart development

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CHARACTERIZATION OF TBX20 ISOFORMS AND PROTEIN INTERACTIONS IN HEART DEVELOPMENT

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ABSTRACT

Proper cardiogenesis is critical for the development of vertebrates. Abnormalities in cardiogenesis can lead to congenital heart defects (CHDs), which occur in approximately 1% of live births. The cardiac transcription factor network contains different transcription factor families which direct the expression of critical cardiac genes. Determining how the cardiac transcription factors are regulated will provide insight in the mechanisms of cardiogenesis and CHDs. The T-box (TBX) transcription factor family is an ancient gene family important for development. Several TBX genes are expressed within the developing heart and play critical roles in differentiation, proliferation, and morphogenesis. One important TBX protein is TBX20, which is critical for cardiogenesis in mice. In humans, missense mutations in TBX20 have been found in patients with congenital heart defects.

Characterization of modifiers of TBX20 activity will help elucidate the genetic mechanisms of heart development and CHDs. A yeast two-hybrid screen using an embryonic mouse heart cDNA library and TBX20b as bait was used to identify protein interactions. This led to the identification of an interaction with muskelin (MKLN1), a primarily cytoplasmic protein with potential roles in scaffolding of signal transduction machinery and nucleocytoplasmic protein shuttling. MKLN1 directly binds to the T-box DNA-binding domain of only the TBX20b isoform by its kelch repeats domain. Immunostaining of transfected cells revealed colocalization within the cytoplasm;

however, there was no change in subcellular localization of TBX20b by overexpression of MKLN. Immunohistochemistry staining of embryonic mouse hearts indicated coexpression in the endocardial valvular and myocardial interventricular cells. This study identified and characterized a novel protein interaction with the potential to regulate TBX20 activity.

The TBX transcription factor family includes many important regulators of vertebrate development. Currently, three TBX genes are alternatively spliced into different isoforms. This chapter reviews the importance of the cardiac transcription factor network and emphasizes the need for isoform-specific expression and functional analyses. Overall, this dissertation helps to define the cardiac transcription factor network and highlights the importance of isoforms and interacting proteins.

Keywords: heart development, T-box transcription factors, TBX20, MKLN1, protein-protein interaction

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LIST OF ABBREVIATIONS

AA	amino acids
ACTH	adrenocorticotropic hormone
AVC	atrioventricular cushions
AV	atrioventricular
BMP	bone morphogenetic protein
CHDs	congenital heart defects
E	embryonic day
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
FHF	first heart field
IFT	inflow tract
MKLN1	muskelin
OFT	outflow tract
POMC	pituitary-derived pro-opiomelanocortin
RV	right ventricle
SHF	second heart field
TBX	T-box
TOF	tetrology of Fallot
VSD	ventricular septal defects

INTRODUCTION

Transcriptional Regulation of Cardiogenesis

In vertebrate embryogenesis, the heart is the first organ to form and is essential for continued development. Mice are commonly used as a model for vertebrate cardiogenesis and are used to describe the developmental stages discussed below (Figures 1 and 2) [1,2,3,4]. Heart development begins with the specification of cardiac progenitor cells in the lateral plate mesoderm which form a cardiac crescent by embryonic day (E) 7.5. These cardiac progenitor cells are organized into the first heart field (FHF) and the second heart field (SHF). The FHF is located on the ventral side of the cardiac precursors and is the first to migrate providing a framework for future development. At E8.0, the FHF fuses at the midline to form a linear heart tube consisting of a layer of myocardial cells which surround an inner layer of endocardial cells separated by an extracellular matrix (ECM) referred to as the "cardiac jelly." At E9.0, the heart tube undergoes looping, which orients the ventricles, atria, outflow tract (OFT), and inflow tract (IFT), through coordinated elongation and migration. This coincides with the recruitment of cells from the SHF which originate from the dorsal side of the cardiac precursors to form the future atria, right ventricle (RV), and OFT. At E10.5, the looped heart undergoes morphological changes to form four chambers. The chamber myocardium in the future ventricles forms muscular projections called trabeculae. The non-chamber myocardium develops into the atrioventricular canal (AVC) and OFT. In the non-chamber myocardium, the nearby endocardium receives signals from the myocardium to undergo epithelial-

to-mesenchymal transition (EMT). The EMT of endocardial regions will form cellularized cushions to provide the framework for the valves and septa. The septation of the ventricles, atria, and OFT begins after E11.5. The specification and morphological changes necessary for cardiogenesis are controlled by different transcription factor families which compete and coordinate the expression of critical cardiac genes.

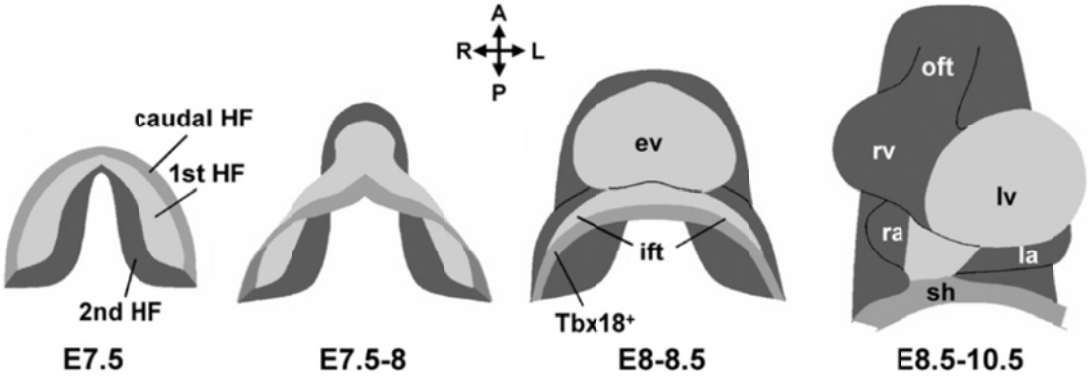

Figure 1: Schematic of early cardiogenesis in mice. The left ventricle develops from the FHF (light gray). The atria, right ventricle, and outflow tract develop from the SHF (dark gray). The caudal heart field will form the sinus horns (medium gray). A: anterior, P: posterior: L: left, R: right.

Image from "T-box factors determine cardiac design" by W. Hoogaars, P. Barnett, A. Moorman, and V. Christoffels, 2007. *Cell. Mol. Life Sci.*, 64, p647. Reprinted with permission from Springer Publishers.

The transcriptional networks controlling heart development are highly conserved throughout vertebrate development [5]. Important transcription factor families include the homeodomain transcription factor NKX2-5, the family of T-box (TBX) proteins, the HAND family of basic helix-loop-helix proteins, and the GATA family of zinc finger proteins. NKX2-5 is crucial for the initiation and maintenance of early cardiac progenitor cells. GATA4, GATA5, and GATA6 are expressed in the developing heart. GATA4 induces differentiation in early cardiac cells. GATA4 may also play a role in the cell migration needed for heart looping, although GATA4 null mice also exhibit abnormalities of endoderm migration indicating that the altered heart looping may be a secondary defect

[6,7,8]. Several T-box genes are expressed during heart development and work as activators and/or repressors to coordinate proliferation, elongation, and myocardium differentiation [9]. The transcription factors form a network that stabilizes and enhances the cardiac gene program by controlling cell fate, expression of contractile proteins, and morphogenesis [10].

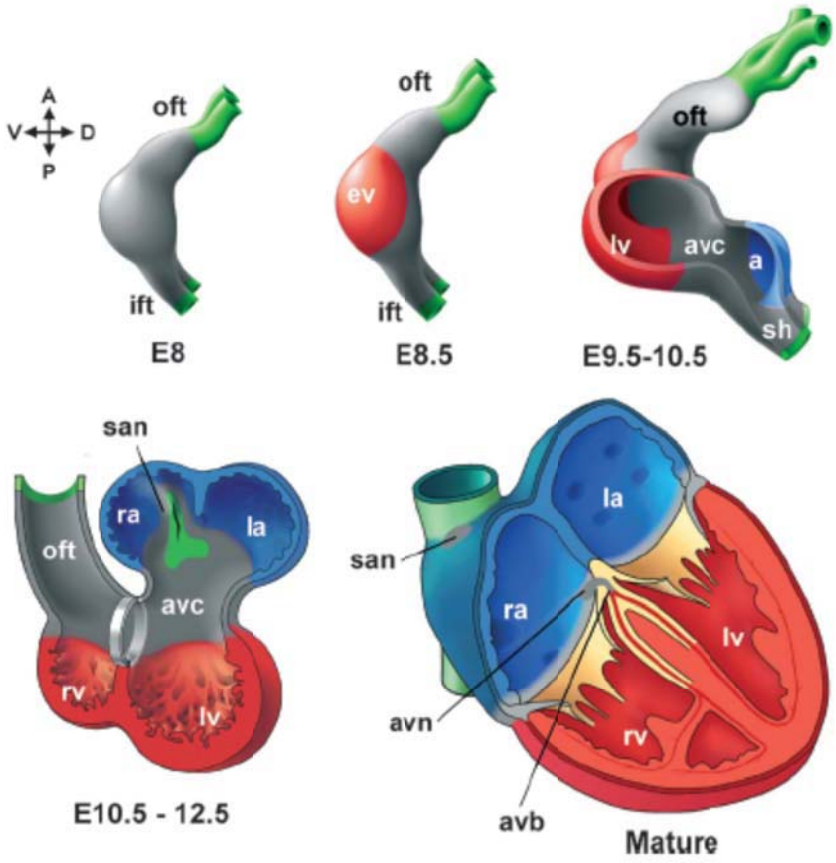

Figure 2: Later stages of heart development in mice. The linear heart tube expands and loops to place the ventricles (red) and the atria (blue) in proper alignment with the non-chamber myocardium (gray) which form the valves and septa at the outflow tract (oft), inflow tract (ift), and atrioventricular canal (avc). The sinus horn (sh) myocardium, developed from the caudal heart field, form the sinoatrial node (san) (green). ev: embryonic ventricle, lv: left ventricle, a: atria, ra: right atria, la: left atria, rv: right ventricle, avn: atrioventricular node, avb: atrioventricular bundle.

Image from "T-box factors determine cardiac design" by W. Hoogaars, P. Barnett, A. Moorman, and V. Christoffels, 2007. *Cell. Mol. Life Sci.*, 64, p648.

Failure of proper cardiogenesis can lead to congenital heart defects (CHDs), which is the leading cause of infant mortality presenting in approximately 1% of live births [11]. Mutations in or the absence of cardiogenic transcription factors are responsible for many CHDs thus revealing the critical need for proper transcription factor expression and function. NKX2-5 is expressed early and maintains expression throughout development. Mutations in NKX2-5 have been mapped to families with CHDs, specifically atrial septal defects (ASD) and atrioventricular (AV) block [12]. Later studies revealed a larger spectrum of abnormalities including ventricular septal defects (VSD), Ebstein's anomaly of the tricuspid valve, and tetrology of fallot (TOF) [13]. Mutations in GATA4 lead to septation defects [14]. Mutations in several TBX genes lead to CHDs. Mutations in *TBX5*, the causative gene in Holt-Oram syndrome, are responsible for a spectrum of CHDs including ASD, VSD, and conduction system defects. Missense mutations or microdeletions of *TBX1* lead to TOF, persistent truncus arteriosus, and interrupted aortic arch which are CHDs found in DiGeorge syndrome. Missense and nonsense mutations of *TBX20* have been found in patients with a spectrum of CHD phenotypes [15,16]. The variability of malformations from the same or similar mutations indicates a requirement for precise dosage of transcription factor quantity. Similarities of CHD phenotypes between different transcription factors support the model of a core cardiogenic transcription network. Similar CHDs resulting from different mutations indicate impaired protein and/or genetic interactions.

T-box Transcription Factor Family

The TBX genes are an ancient family of transcription factors important for the development of many tissues and organs. Characterization of the tailless, or T, mice led

to the discovery of a novel transcription factor, Brachyury [17]. Brachyury is a transcription factor that binds DNA through a novel domain designated the T-box [18,19,20]. The T-box DNA-binding domain is approximately 180-200 amino acids (aa) and has a novel DNA-binding motif that can contact the DNA on both minor and major grooves [21]. The T-box DNA binding element (TBE) was identified in Brachyury to be a partially palindromic sequence (5'-T(G/C)ACACCTAGGTGAAATT-3'). The T/2 site refers to half of the sequence. TBX proteins bind as monomers or dimers to the T or T/2 site which appears in different orientations and numbers [20,21,22]. Searching genomes for other genes with similar T-box domains revealed numerous highly related transcription factor genes [23]. All TBX genes share the T-box DNA-binding domain with high homology while the other domains share little to no homology. The T-box family is thought to have evolved from tandem duplication and cluster dispersion resulting in TBX genes that are closely related and within close proximity. For example, TBX4 and TBX5 are transcriptional activators responsible for hindlimb and forelimb development, respectively. In addition, TBX2 and TBX3 are unique in that they are primarily transcriptional repressors [24].

In general, TBX transcription factors are expressed during embryogenesis and are required for the development of several tissues/organs such as limbs, heart, and mammary glands. Human disorders have been discovered to be caused by mutations or deletions in TBX1, TBX3, TBX4, TBX5, TBX19, TBX20, and TBX22 [25]. TBX genes without a currently known human disorder typically reveal developmental disorders in homozygous null mice. *TBX1* is in the critical region of 22q11 deletion syndrome and is most likely responsible for the cardiac phenotype seen in DiGeorge Syndrome (OMIM: 188400)

[26,27]. *TBX3* is the causative gene in Ulnar-Mammary Syndrome (OMIM: 181450) which is characterized by defects in limb, mammary gland, dental, and genital development [28]. Holt-Oram syndrome (OMIM: 1429000), an autosomal dominant disorder involving defects in heart and forelimb development, is caused by haploinsufficiency of *TBX5* [29,30]. Mutations in *TBX19* cause recessive isolated pituitary-derived pro-opiomelancortin (POMC)-derived adrenocorticotropic hormone (ACTH) deficiency, and indicate a role in regulating POMC gene expression [31]. TBX22 is important for palatogenesis, and mutations can cause X-linked cleft palate with ankyloglossia [32]. In mouse and chicken studies, TBX4 is important for hindlimb development [33,34]. Mutations in human *TBX4* lead to malformations of lower limbs and pelvis [35]. While the TBX genes regulate the development of different tissues and organs, several are involved in different aspects cardiogenesis.

TBX Transcription Factor Family Role in Cardiogenesis

Identification of mutations in *TBX5* and *TBX1* as the cause of two common human disorders involving CHDs uncovered the importance of other TBX transcription factors in heart development. TBX1-5, TBX18, and TBX20 are all expressed in the heart during development (Figure 3) [36]. TBX1 is expressed in the cardiac progenitor cells and is involved in proliferation of the SHF, specifically with OFT development. TBX2 and TBX3 are transcriptional repressors that specify the non-chamber myocardium development which is important for septation and valvulogenesis [37]. *TBX5* is an important cardiac gene expressed early in the linear heart tube where the gradient of TBX5 levels leads to chamber specification and morphogenesis. TBX18 is associated with the "third heart field" (caudal heart field) which develops into the sinus venosus, an embry-

onically important structure within the atria [38]. TBX20 is expressed in the myocardium and endocardium of the developing heart and later exhibits enhanced expression in the developing AVC and OFT.

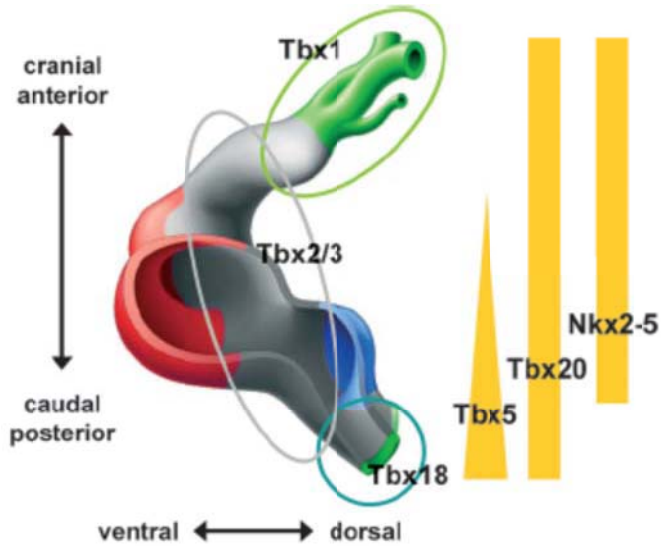

Figure 3: The T-box transcription factor family expression during heart development. TBX1 is expressed in the outflow tract (green). TBX18 is expressed in the sinus horns (green). TBX2/3 are expressed in the non-chamber myocardium (grey). TBX5 and TBX20 are expressed throughout the myocardium. The gradient expression of TBX5 is highest in the ventricle myocardium (red).
Image from "T-box factors determine cardiac design" by W. Hoogaars, P. Barnett, A. Moorman, and V. Christoffels, 2007. *Cell. Mol. Life Sci.*, 64, p654.
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The TBX family is critical to the cardiac transcription factor network that drives chamber versus non-chamber morphogenesis. As the linear heart tube forms, the outer layer of cells is composed of primary myocardium, which is less proliferative and maintains a more embryonic cell phenotype. After heart looping, the outer curvature of the future ventricle regions balloons out and undergoes morphological changes to become chamber myocardium, which is more proliferative and differentiated into a working muscle. Specific areas of the looped heart maintain the primary, or non-chamber, myocardial state as this is important for the development of the AVC, OFT, and IFT. TBX5 and

TBX20 are known drivers of chamber myocardium morphogenesis. TBX5 and TBX20 both interact with NKX2-5 and GATA4 to activate chamber-specific genes *Nppa*, *Cx40*, *Cx43*, and *Mycn* which are important for differentiation and proliferation [39,40,41,42]. The chamber myocardium must proliferate to expand and form the trabeculae, muscular projections within the ventricles. Non-chamber myocardium development depends on TBX2 and TBX3-mediated repression of *Nppa* and *Cx40* [43,44,45,46,47].

One intersection in the transcriptional control between chamber and non-chamber myocardium development is the coexpression of TBX20 and TBX2 within non-chamber myocardium cells. TBX20 controls chamber development through inhibition of TBX2 within the chamber myocardium. However, this control is removed in the non-chamber myocardium (Figure 4). Currently, there are two different models of TBX20 repression of TBX2 activity. TBX20 has been shown to directly inhibit *Tbx2* expression by a chromatin immunoprecipitation assay where TBX20 bound to the TBE in the 5' region of *Tbx2*. In addition, cell transfection studies using luciferase reporters resulted in TBX20-specific inhibition of the *Tbx2* promoter expression [48]. In contrast, TBX20 has been shown to inhibit *Tbx2* through interference of BMP/Smad signaling. *Tbx2* expression is activated by BMP/Smad signaling, and TBX20 can inhibit Smad-activated *Tbx2* expression even when critical residues for DNA-binding are mutated. This led to the discovery that TBX20 interacts with phospho-Smad1/5 and inhibits interaction with Smad4, thus providing a DNA-independent mechanism for the inhibition of TBX2 expression [49].

While these two studies conclude that TBX20 inhibits TBX2 activity, the mechanism for how TBX20 suppression is relieved in the non-chamber myocardium to allow for TBX2 control has not been fully defined. Models have hypothesized the existence of

a TBX20-inhibitory protein that acts specifically in the non-chamber myocardium [37]. In addition, the upstream regulation of the TBX proteins is important to prevent premature or ectopic expression of the TBX2/3 repressors. The BMP signaling pathway has been shown to activate the expression of several TBX proteins. The timeline and quantity of BMP signaling factors may distinguish which TBX target drives the developmental context.

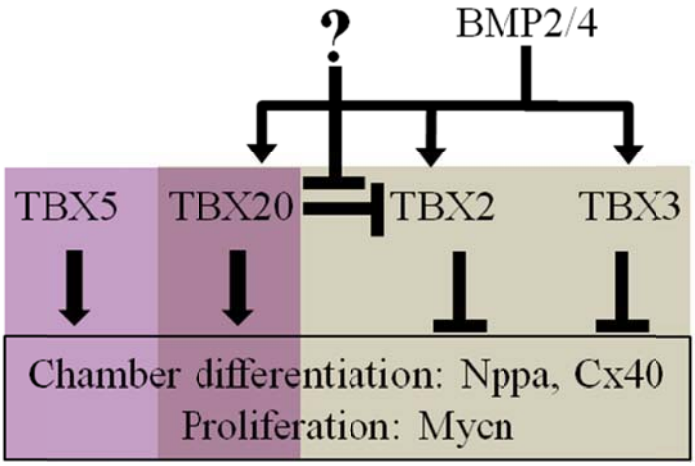

Figure 4: Model of TBX control of chamber differentiation. TBX5 and TBX20 are expressed in chamber myocardium (purple) and drive the expression of genes important for chamber differentiation and proliferation. TBX2 and TBX3 are expressed in non-chamber myocardium (grey) and inhibit the same chamber-specific genes. TBX20 is expressed in both chamber and non-chamber myocardium and prevents TBX2-controlled inhibition of chamber-specific genes. An unknown mechanism releases TBX2 from TBX20 control in the non-chamber myocardium.

TBX transcription factors coordinate the expression of cardiac-specific genes throughout cardiogenesis. While the T-box domain of TBX transcription factors is highly conserved throughout the family, the domains outside of the T-box increase the opportunity for interactions with cooperative or competitive cofactors including other TBX factors. This culminates into a regulation network where different TBX factors can interact with each other as well as other cofactors to coordinate the expression of TBX target

genes. Understanding how this transcriptional network is regulated will lead to a better understanding of how the heart develops.

The Role of TBX20 in Cardiogenesis

This study focuses on the role of TBX20 in heart development. Genome-wide screens searching for novel T-box genes identified TBX20 in several species [50,51,52,53,54,55]. TBX20 is located on chromosome 7p14.3 in humans and chromosome 9 in mice. TBX20 is expressed in the developing heart in several species including humans, mice, chicken, frog, and zebrafish. Other common tissues include the developing eye and central nervous system [50,51,53,54,55,56,57]. In mice and humans, TBX20 is alternatively transcribed into different isoforms, primarily the full length TBX20a isoform (445 aa) and the TBX20b isoform (297 aa) (Figure 5). Both transcripts contain exons 1-6, encoding the N-terminal portion (aa 1-100) and the T-box DNA-binding domain (aa 101-289). TBX20a also contains exons 9-10, encoding for a 148 aa extended C-terminal portion with characterized activation and repression domains. TBX20b is transcribed with exon 7 which leads to termination at aa 297 [41]. Both isoforms are expressed in the developing and adult hearts of mice and humans.

Figure 5: Schematic of TBX20. The TBX20 isoforms are identical through the T-box DNA-binding domain (yellow) where TBX20a continues with activation (green) and repression (red) domains. Non-synonymous mutations found in patients with CHDs are marked with black bars. The asterisk indicates a non-synonymous mutation that is not predicted to alter structure or function. The X indicates amino acid 121 where two missense mutations have been identified, one of which is a gain-of-function alteration.

CHDs and Adult Cardiomyopathy

TBX20 has critical functions in human heart development and adult cardiomyopathies. Missense and nonsense mutations within TBX20 have been identified in patients with CHDs including atrial septal defects (ASD), ventricular septal defects (VSD), and various other malformations (Figure 5). The first study sequenced 352 people with CHDs and found one missense and one nonsense mutation that segregated with the family CHDs. The I152M mutation is at a highly conserved amino acid position in the T-box DNA-binding domain region and biophysical studies determined that the amino acid change affects the protein structure, stability, and DNA-binding activity. The Q195X mutation resulted in a protein too unstable for DNA-binding ability assays. The mutant TBX20 proteins revealed reduced transcriptional ability by transfection studies using the *Nppa* reporter construct. Two individuals with the Q195X mutation also presented with dilated cardiomyopathy, which is the first look at adult heart disease due to mutations in TBX20. The underlying CHDs could sensitize the patients to develop cardiomyopathy and more studies will help dissect any potential role of TBX20 that would indicate future counseling for patients [15]. A second study analyzed the *TBX20* T-box genomic region of over 200 Chinese children presenting with CHDs. One non-synonymous mutation of A63T was not predicted to alter protein structure or function. The two missense mutations I121F and T262M were recognized to be in highly conserved positions predicted to affect protein structure and function [58]. A third screening of children with CHDs for *TBX20* mutations identified two non-synonymous mutations of H186D and L197P [59]. Future mutation analysis of the full *TBX20* genomic region may reveal more mutations affecting TBX20 function and thus CHDs.

The dosage sensitivity of TBX20 is apparent in CHDs and adult cardiomyopathies. Mutation analysis of *TBX20* in patients with ASD revealed a gain-of-function mutation I121M [60]. The I121M mutation is slightly unstable and has no significant change in DNA-binding ability. However, the mutant protein greatly induced *Nppa* and *Gja5* enhancers as determined by a luciferase reporter assay with further induction by the addition of NKX2-5 or GATA4/5 cofactors. In addition to the CHDs caused by TBX20 mutations, patients with adult cardiomyopathies have been found to express TBX20a and TBX20b at increased levels compared to normal controls [61]. While TBX20 is critical for vertebrate heart development, the dosage must also be tightly regulated.

TBX20 Expression in Heart Development

TBX20 expression has been detailed in the developing mouse heart. At E7.5, TBX20 is expressed in the cardiac mesoderm and migrates with the formation of the cardiac crescent. At E8.0-8.5, TBX20 is expressed in the looping heart with increased expression in the myocardium compared to the endocardium. From E9.5-11.5, there is an enhancement of expression in the developing OFT and AVC. This includes the endocardial/endothelial cells that have delaminated and migrated into the cellularized cushion regions. In addition, TBX20 expression is enhanced in the compact myocardial wall of the ventricles and is attenuated in the trabecular projections. While TBX20 is enhanced in these regions, expression throughout the myocardium of all four chambers is maintained [41,51,53,54].

Knockout Mouse Models of TBX20

Several studies have explored the function of TBX20 through knockout and knockdown experiments in mice. Three knockout mouse models have been developed

and all show embryonic lethality by E10.5 due to cardiac insufficiency. The linear heart tube forms but does not undergo looping, elongation, or chamber differentiation. Cardiomyocyte differentiation and anterior/posterior axis formation are unaffected. Proliferation is decreased but apoptosis is unaffected at E8.5 [48,62]. The SHF develops normally as indicated by the usual expression of *Isl1*, *Fgf8*, *Foxh1*, *Fgf10*, and *Mef2c* [62,63]. While the specification of the SHF develops normally, the later recruitment from the SHF for elongation is affected as indicated by the absence of *Wnt11* and *Dhand* expression [62]. Chamber versus non-chamber myocardium development is affected. Chamber myocardium is not evident due to the decreased or absent expression of *Nppa*, *Smpx*, *Hand1*, and *Cx40* [62,63]. The cause of this is most likely the ectopic expression of TBX2. *In vivo* RNAi knockdown of TBX20 allowed for further embryonic development and revealed a role for TBX20 in the development of the RV and OFT modeling [64]. Overall, TBX20 is important for the proliferation and recruitment of SHF progenitors needed in heart elongation and looping as well as chamber myocardium differentiation and growth.

Transcriptional Activities of TBX20

As a transcription factor, TBX20 can regulate the expression of cardiac genes by direct DNA-binding of promoter elements, indirect regulation by cofactor binding, and DNA-independent regulation through interactions with Smad signaling proteins. Reporter assays have discovered that TBX20 can activate the promoter sequences of *Nppa*, *Nkx2-5*, *Pitx2*, *Fgf10*, *Myh7* and *Mycn* [48,64]. The expression of *Nppa* and *Mycn* is important for the differentiation and proliferation of the chamber myocardium. Regulation of NKX2-5 expression strengthens the cardiac transcription network as NKX2-5 is an important cardiac transcription factor. PITX2 is a transcription factor important for guiding

left/right identity in the development of the atria and ventricles [65]. Expression of FGF10 is important for the proper development of the OFT and RV [66]. *Myh7* encodes for beta-myosin heavy chain which is specifically expressed during embryogenesis and is important for cardiac muscle formation.

TBX20 is unique among TBX proteins because of its characterized activation and repression abilities. As demonstrated by the TBX20 knockout mice, TBX20 inhibits the expression of TBX2 in the chamber myocardium. Another target for repression is *Isl1* which is evident by TBX20's ability to bind and repress *Isl1* promoter regions as indicated by luciferase reporter assays [48]. TBX20 is expressed within the endocardium and avian endocardial cell studies have revealed an important role for TBX20 control of specific extracellular matrix (ECM) components. Gain-of-function and loss-of-function analysis of TBX20 revealed inhibited expression of chondroitan surface proteoglycan genes *versican* and *aggrecan* as well as activated expression of matrix metalloproteinase genes *mmp9* and *mmp13*. Control of these genes indicates a repressive role of TBX20 in controlling ECM remodeling that is critical for the valvulogenesis in the mesenchymal cells in the AVC [67]. These gene targets of TBX20 suggest roles in proliferation, elongation, chamber differentiation, and valvulogenesis. It is important to note that the precise cell type, timing, and presence of cofactors influence the overall results of TBX20-medicated activation or repression of target genes.

Protein-protein interactions are critical within the cardiogenic transcriptional network. As stated previously, TBX20 also functions as a cofactor to modulate the transcriptional activity of other transcription factors. In different cellular contexts, TBX20 can bind and synergize transcription with NKX2-5, GATA4, and GATA5 [41]. These cofac-

tors are important for reinforcing the cardiac transcription network and promoting cardiac differentiation. Interaction with other transcription factors can also influence the regulation of different target genes as the presence of multiple transcription factors can enhance DNA-binding and strengthen the transcriptional protein complex. TBX20 can interact with GATA4 and ISL1 to synergistically activate the expression of NKX2-5 which reinforces the SHF differentiation transcriptional program important for OFT development. TBX20 also interacts with ISL1 itself to activate *Mef2c* expression, which is important for SHF differentiation [64]. In *Xenopus laevis*, TBX20 and TBX5 can physically interact as shown by a GST-pulldown assay [56]. While the T-box domain is critical for DNA-binding, it is also the interaction domain of all the protein interactions mapped thus far.

A unique function of TBX transcription factors is that currently TBX1, TBX6, and TBX20 have been recently discovered to interact with proteins involved in the BMP/Smad signaling pathways. In exploring TBX20's role of inhibiting *Tbx2* expression, the promoter region of *Tbx2* was fully defined and characterized [49]. With TBX20 control over *Tbx2* expression, TBE binding sites were expected but not found within the minimal regulatory region that controls endogenous *Tbx2* expression. Instead, Smad-binding sites were discovered and *in vitro* luciferase reporter assays revealed activation of the *Tbx2* promoter upon coexpression with Smad5 and the constitutively active BMP receptor, ALK3. This *Tbx2* promoter fragment was ectopically expressed in TBX20 null mice, but the mechanism of TBX20 control on *Tbx2* expression was in question. As indicated by an *in vitro* luciferase reporter assay, TBX20 coexpressed with Smad5/ALK3 can diminish the expression of the *Tbx2* promoter. To determine if this was DNA-independent, the assay was repeated with TBX20 mutated at the critical residues for

DNA-binding and the expression of the *Tbx2* promoter was still decreased. Coimmunoprecipitation assays revealed that TBX20 can bind to Smad1/5 and effectively compete with Smad4 for activation of TBX2. This study introduces a novel aspect of TBX20 transcriptional regulation and suggests further complexity to chamber development.

Purpose of Research

The heart is a complex organ that must form correctly for the ensured survival and health of all developing vertebrates. The cardiogenic transcription factor network is crucial for the timing and specificity of critical cardiac gene expression. While the major players within the cardiogenic transcription factor network have been identified, there are major gaps in the identification of interacting proteins. The purpose of this study is to identify novel interacting partners to TBX20 to further elucidate the regulatory mechanisms of the cardiogenic transcription factor network. A yeast two-hybrid library of murine cDNA from E9.5-11.5 hearts was screened for potential interactions with TBX20b. MKLN1 was identified as a novel interacting partner to TBX20 and the TBX20b-MKLN1 interaction is characterized in Chapter 3. One unique aspect of MKLN1 is that it only interacts with the TBX20b isoform. Alternative splicing of TBX proteins is reviewed in Chapter 4. Overall, these studies introduce a novel protein that interacts with a critical transcription factor within the cardiac transcription factor network and suggests that TBX isoforms have an underestimated role within vertebrate development.

Summary of Dissertation

The studies herein are comprised mostly from my work with Dr. Kai Jiao involving the identification of novel proteins interacting with TBX20. However, prior to joining Dr. Jiao's laboratory, I previously worked with Dr. Michele P. Marron from the Depart-

ment of Pediatrics. The overall goal in Dr. Marron's work was to identify autoantigens involved in the initiation of Type 1 Diabetes. My project was to create a "humanized" mouse model with the murine major histocompatibility complex (MHC) genes knocked out and the human diabetogenic MHC alleles transgenically expressed. Dr. Marron closed her laboratory after my qualifying exam, and I immediately transferred into Dr. Jiao's laboratory. While the introduction and conclusions focus on my work with TBX20 in cardiogenesis, my work in Type 1 Diabetes is included as its own chapter. This dissertation includes all of my graduate work performed at UAB.

CREATION OF A HUMANIZED MHC MOUSE MODEL WITH TRANSGENIC NON-OBESE DIABETIC MICE

Paige DeBenedittis and Michele P. Marron, Ph.D.

Abstract

Type 1 diabetes (T1D) is an autoimmune disease where T cells target and destroy the insulin-producing β cells of the pancreas resulting in insulin-dependency. The overall goal of this study was to identify autoantigens early in the progression of T1D using non-obese diabetic (NOD) mice as a model. The major genetic susceptibility to T1D in both humans and NOD mice is the major histocompatibility complex (MHC) alleles. The MHC allele most strongly associated with T1D in humans is the HLA-DQ8 class II allele. The common HLA-A2 class I allele introduces risk for T1D when coexpressed with high-risk class II alleles. NOD mice that transgenically express HLA-DQ8 in the absence of murine MHC class II (NOD.*Aβ*$^{-/-}$.*DQ8*) do not develop diabetes and instead develop autoimmune myocarditis [1, 2]. However, the Aβ$^{-/-}$ mutation is on the H2^{b} MHC haplotype and encodes for a different K^{b} MHC class I molecule. The H2-K^{b} expression may confer a protective effect against the development of diabetes. NOD mice which do not express MHC class I molecules due to a targeted mutation of the β-2 microglobulin subunit (NOD.*β2m*$^{-/-}$) are completely resistant to T1D [3]. NOD.*β2m*$^{-/-}$ mice that transgenically express the HHD construct, containing the HLA-A2 heavy chain covalently linked to human β2-microglobulin (NOD.*β2m*$^{-/-}$.*HHD*), develop T1D [4]. Thus, the HHD construct restores T1D development to the NOD.*β2m*$^{-/-}$ mice and can

select for pathogenic CD8+ T cells necessary for T1D initiation. The hypothesis of this study is that the T1D resistance found in NOD mice transgenically expressing HLA-DQ8 in the absence of murine MHC class II (NOD.*Aβ*$^{-/-}$.*DQ8*) is due to the absence of autoreactive CD8+ T cells capable of initiating the disease. Introducing HHD expression to the NOD.*Aβ*$^{-/-}$.*DQ8* mice will provide CD8+ T cells known to be capable of inducing T1D. This mouse like will create an opportunity to study the roles of autoreactive CD4+ and CD8+ T cells in T1D and allow for the identification of antigens with relevance to human patients.

Abbreviations

β2m, β-2 microglobulin; DMK, dystrophia myotonica-protein kinase; GAD, glutamic acid decaroxylase; H/E, hematoxylin and eosin; IGRP, islet-specific glucose-6-phosphastase catalytic subunit-related protein; MHC, major histocompatibility complex; NOD, non-obese diabetic; Prkdc, protein kinase, DNA activated, catalytic polypeptide; scid, severe combined immunodeficiency; T1D, type 1 diabetes.

Introduction

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease where T cells destroy the insulin-producing β cells of the pancreas. This autoimmune process can initiate early in life and persist unnoticed until insulin production has diminished to levels where the patient develops hyperglycemia and a life-long dependence on insulin therapy. The pathogenesis of T1D is of great interest as there is little known about the initiating events of this complex disease. With the destruction of the β cell mass occurring in the

preclinical stage, it is important to discover methods of identifying people at risk for developing T1D as well as therapeutics to better treat the disease.

Type 1 diabetes has many proposed genetic and environmental factors. Twin studies have shown approximately 50% concordance rate for T1D, indicating a significant environmental component in addition to genetic factors [8]. The main genetic susceptibility lies in the major histocompatibility complex (MHC) alleles which encode MHC class I and II molecules, among other genes [9]. The role of MHC class I and II molecules is to present peptides for recognition by T cells. MHC class I molecules present intracellular peptides to CD8+ T cells while MHC class II molecules present extracellular peptides to CD4+ T cells. CD4+ and CD8+ T cell development is dependent upon the expression and function of the MHC class II and I molecules, respectively. Studies in non-obese diabetic (NOD) mice have shown that both CD4+ and CD8+ T cells are major players in the pathogenesis of T1D, with the cytotoxic CD8+ T cells initiating β cell death and then continuing to play a role in the development of the immune response [10]. CD4+ T cells play an important role in the progression of the immune response as CD4+ T cells from diabetic mice are capable of transferring the disease.

The Role of MHC Class II Molecules in T1D in Both Mice and Humans

The majority of T1D patients express the MHC class II allele HLA-DQ8 [11]. The NOD mouse expresses a unique MHC class II allele of I-A^{g7} in the absence of I-E. This I-A^{g7} allele shares a high sequence homology with the human HLA-DQ8 allele, both of which contain a non-aspartic acid in position 57 of the beta chain [12, 13]. Peptide binding experiments have shown that both HLA-DQ8 and I-A^{g7} MHC molecules can

bind and present the same peptides [14]. Also, studies have shown that human HLA-DQ8 can substitute for the murine I-A^{g7} for selecting diabetogenic CD4+ T cells [15].

Three laboratories have created lines of NOD mice transgenically expressing human MHC class II HLA-DQ8 allele in the absence of murine MHC class II expression (NOD.*Aβ*$^{-/-}$.*DQ8*).The C. David laboratory created HLA-DQ8 transgenic mice on the B10.M (H-2f/f) background and then backcrossed onto NOD.*Aβ*$^{-/-}$ [18]. Histological sections of the pancreata show peri-insulitis in approximately 70% of these mice [19]. The J. Elliott laboratory injected the DQ8 construct directly into NOD mice, then crossed to NOD.*Aβ*$^{-/-}$. These mice developed autoimmune myocarditis, but not T1D [2]. Finally, the Lipes laboratory developed three lines of transgenic HLA-DQ8 on the C57BL/6 background. These were then backcrossed onto the NOD.*Aβ*$^{-/-}$ background [1]. None of these lines developed T1D; however, myocarditis was observed and correlated with the level of DQ8 transgene expression, indicating that the myocarditis is a direct result of the DQ8 transgene and not due to insertional mutagenesis. Following these reports of myocarditis, the transgenic mice from C. David's laboratory were further backcrossed to NOD. At the N15 generation, myocarditis was observed in the NOD.*Aβ*$^{-/-}$.*DQ8* mice [20]. While NOD mice have a propensity to developing autoimmune disease, they do not normally develop myocarditis. Nonetheless, results from these three laboratories show that NOD.*Aβ*$^{-/-}$.*DQ8* mice develop peri-insulitis, but not overt T1D, and are susceptible to myocarditis.

The Role of MHC Class I Molecules in T1D in Both Mice and Humans

The HLA-A2 class I allele is expressed in about 30% of Caucasians and is associated with T1D susceptibility [16]. NOD mice transgenically expressing the human HLA-A2 allele (designated NOD.*HHD*) were created [17]. The HHD construct encodes for a chimeric molecule containing α1 and α2 domains from HLA-A2, α3 domain from murine D^b for more efficient interaction with murine CD8, and a covalently linked human β2-microglobulin (β2m) subunit. These NOD.*HHD* mice develop T1D at a significantly increased rate, signifying the importance of the HLA-A2 allele in the susceptibility of T1D [4]. In order to study the role of CD8+ T cells in the pathogenesis of T1D, the β2m locus, expression of which is required for MHC class I cell surface expression and thus CD8+ T cell development, has been inactivated in a line of NOD mice (designated NOD.$\beta 2m^{-/-}$). These mice were completely protected from developing T1D while their β2m-expressing littermates developed T1D at the expected rates, indicating the significance of CD8+ T cells in the initiation of T1D [3]. The NOD.*HHD* mice were crossed with the NOD.$\beta 2m^{-/-}$ mice to create a line of NOD mice transgenically expressing the human HLA-A2 class I allele in the absence of murine MHC class I. This NOD.$\beta 2m^{-/-}$.*HHD* mouse line develops T1D, with 55% of HHD transgenic female mice developing T1D by 30 weeks of age. These results demonstrated that the MHC class I molecule encoded by the HHD construct is capable of selecting diabetogenic CD8+ T cells [4].

Generating a Humanized Mouse Model of T1D

The roles of MHC molecules in the development of T1D are difficult to directly study in human patients. Pancreatic lymphocytes are difficult to detect and access in humans, particularly since the autoimmune process can proceed asymptomatically for many years. The NOD mouse model allows for easier access to the β cells and

diabetogenic T cells in the pancreas for further study. Furthermore, NOD mice transgenically expressing human MHC alleles allow for the study of specific human immune molecular interactions. The NOD mice important for this study are characterized in Table 1. The lack of spontaneous diabetes development in the NOD.*Aβ*$^{-/-}$.*DQ8* mice is possibly due to the potential protective effect of the MHC class I molecules that are in linkage with the MHC class II knockout. The K^b class I molecules may not select autoreactive CD8+ T cells or may select CD8+ T cells which confer protection. As stated above, NOD mice transgenically expressing the human class I HLA-A2 alleles in the absence of murine MHC class I (NOD.*β2m*$^{-/-}$.*HHD*) develop diabetogenic CD8+ T cells. Thus, introducing HHD expression in the absence of murine MHC class I and II expression will provide pathogenic CD8+ T cells to initiate the disease, and will eliminate expression of K^b as well as any protective CD8+ T cells which might develop.

Using a breeding strategy to intercross the NOD.*β2m*$^{-/-}$.*HHD* and NOD.*Aβ*$^{-/-}$.*DQ8* mice will create a line of mice that transgenically express the human MHC class I and II molecules in the absence of murine MHC molecules (NOD.*Aβ*$^{-/-}$.*DQ8*.*β2m*$^{-/-}$.*HHD* mice). The NOD.*Aβ*$^{-/-}$.*DQ8*.*β2m*$^{-/-}$.*HHD* mouse line will allow for the study of a common human MHC class I molecule in addition to the most diabetogenic human MHC class II molecule without the contribution of the murine MHC class I and II molecules. NOD mice are a useful model for T1D which has been enhanced with the use of transgenic and targeted knockout genetic strategies. The NOD mouse allows for multiple time points in the disease process to be examined which will be helpful for the elucidation of T1D pathogenesis, much of which remains unclear [21].

Materials and Methods

Breeding Strategy and Genotyping

The stocks of NOD.*β2m*$^{-/-}$.*HHD* [4] and NOD.*scid.Aβ*$^{-/-}$.*DQ8* [20] mice were maintained at UAB. The NOD.*β2m*$^{-/-}$.*HHD* mice were intercrossed with NOD.*scid.Aβ*$^{-/-}$.*DQ8* mice to obtain an F1 generation. The F1 mice were brother-sister mated and their litters were genotyped by PCR using DNA obtained by tail tip digests.

Histology

Pancreata and hearts were fixed in Bouins, rinsed in water, and sent to the UAB Comparative Pathology histology core for processing. Hematoxylin and eosin (H/E) staining was performed.

Flow Cytometry Analysis

Lymphocyte subsets were enumerated by flow cytometry. Single-cell suspensions of splenocytes were stained with CD4, CD8, B220, and Ig antibodies to identify cell populations. Cells were also stained for expression of transgenic HLA molecules (DQ8 and A2) and murine MHC Class I (K^d) and II (A^{g7}) molecules.

Results

Generating NOD.Aβ$^{-/-}$*.DQ8.β2m*$^{-/-}$*.HHD Mice*

The stocks of NOD.*β2m*$^{-/-}$.*HHD* and NOD.*scid.Aβ*$^{-/-}$.*DQ8* mice were maintained at UAB. The NOD.*scid.Aβ*$^{-/-}$.*DQ8* line was created from the NOD.*Aβ*$^{-/-}$.*DQ8* mice developed by C. David. They were originally shown to develop insulitis but not

autoimmune myocarditis until further backcrossing onto the NOD background led to spontaneous development of autoimmune myocarditis development [20]. The NOD.*Aβ*$^{-/-}$.*DQ8* mice were originally crossed onto the NOD.*scid* background which has a mutation resulting in the inactivation of the protein kinase, DNA activated, catalytic polypeptide gene (*Prkdc*^{scid}).This protein is involved in DNA double-strand break repair and V(D)J recombination. Mice homozygous for the scid (severe combined immunodeficiency) mutation do not develop B or T lymphocytes due to the failure of V(D)J recombination. The NOD.*β2m*$^{-/-}$.*HHD* mice were intercrossed with NOD.*scid.Aβ*$^{-/-}$.*DQ8* mice to obtain an F1 generation. The F1 mice were brother-sister mated and their litters were genotyped by PCR using DNA obtained by tail tip digests. Mice were further intercrossed for multiple generations to eliminate the scid mutation, are homozygous for the β2m and Aβ null alleles, and are at least heterozygous for the HHD and DQ8 transgene constructs. These mice are designated as NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD*. Control lines of NOD.*scid.Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* and single transgenic lines of NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$ and NOD.*Aβ*$^{-/-}$.*β2m*$^{-/-}$.*HHD* were generated.

Histological Examination of NOD.Aβ$^{-/-}$*.DQ8.β2m*$^{-/-}$*.HHD and Related Mice Reveals Insulitis and Myocarditis*

Preliminary hematoxylin/eosin (H/E) histological examination shows infiltrating lymphocytes in the hearts and pancreata of some NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* mice. As shown in Figure 1, the NOD mouse has no infiltrating lymphocytes in the heart but shows the expected insulitis in the pancreas. The NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* mouse has lymphocytic infiltrates in the heart and in the pancreas. In another preliminary study (summarized in Table 2), H/E histology was performed on NOD, NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$

.*HHD*, and NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{+/-}$.*HHD* mice. As expected, infiltrating lymphocytes were observed within the islet mass of the NOD mouse. Lymphocyte-deficient NOD.*scid.Aβ*$^{-/-}$.*DQ8* mice were used as negative controls for histological analyses. Histological sections of the pancreata in both NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* and *NOD.Aβ*$^{-/-}$.*DQ8.β2m*$^{+/-}$.*HHD* mice revealed infiltrating lymphocytes concentrated near ducts or vasculature. Analysis of the heart sections showed no infiltrating lymphocytes in the NOD and NOD.*scid.Aβ*$^{-/-}$.*DQ8* mice, as expected. However, analysis of the NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* and NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{+/-}$.*HHD* mice revealed infiltrating lymphocytes in the heart muscle.

Analysis of Splenic Lymophocyte Populations in NOD.Aβ$^{-/-}$*.DQ8.β2m*$^{-/-}$*.HHD and Related Mice*

Flow cytometry assessments of B and T cell development have been performed on the initial NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* and related mice (shown in the Table 3 and Figure 2). Overall, the spleens were generally enlarged in the NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* and related mice. The increase in B cell proportion seen in the NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* and related mice when compared to NOD and NOD.*β2m*$^{-/-}$.*HHD* controls is due to the decreased T cell populations in these mice. While the expression of the human transgenes allows for CD4+ and CD8+ T cell development, this occurs at decreased levels, most likely due to the inefficient binding between the transgenic MHC molecules and the murine CD4 or CD8 coreceptors. Decreased efficiency of selection can also be seen with the MHC class I molecules in the NOD.*β2m*$^{-/-}$.*HHD* control mice compared to the NOD, which is consistent with previously reported data [4]. It is noteworthy that the mice were not age-matched with controls. The NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$

.*HHD* and related mice were over 26 weeks in age while the NOD mice were between 12-16 weeks. The age of the NOD mice was necessary as the NOD mice would become diabetic and require euthanization before reaching 26 weeks of age. Overall, these assessments show that the B and T cells develop as expected in the transgenic mice.

Discussion

Generating the first NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* mice has allowed for preliminary studies including enumeration of the splenocytes by flow cytometry and observation of lymphocyte infiltration by routine H/E histological studies. Additional backcrossing to NOD during the generation of the NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* revealed a myocarditis phenotype not originally reported for this line, but which was recently discovered by the original laboratory following an additional five backcrosses to NOD [20]. In addition to myocarditis, there is peri-insulitis found in NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* mice. The establishment of the NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* mice has shown that the addition of the diabetogenic class I MHC molecule allows for pancreatic lymphocyte infiltration to develop. To further characterize these mice, spontaneous and induced diabetes incidence will need to be assessed. These investigations will compare the percent and rate of T1D development the transgenic mice versus the NOD mice. If there is no spontaneous T1D, then splenocyte transfer studies can be employed to determine if there are T cells present capable of inducing T1D.

The infiltrating lymphocytes shown in NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD* mice can be further characterized by determining if they are activated by known autoantigens. Once the CD4+ and CD8+ T cells are isolated from the pancreata and hearts of the NOD.*Aβ*$^{-/-}$

.DQ8.β2m$^{-/-}$.HHD mice, they can be used in an ELISPOT assay to detect T cell recognition of known autoantigens of interest, including T1D autoantigens of insulin, IGRP (islet-specific glucose-6-phosphatase catalytic subunit-related protein), DMK (dystrophia myotonica-protein kinase), GAD (glutamic acid decarboxylase), and myocarditis autoantigens such as myosin.

The overall goal of this study was to identify early autoantigens recognized by human MHC molecules for the study of T1D. While the NOD.*Aβ$^{-/-}$.DQ8.β2m$^{-/-}$.HHD* mice created develop peri-insulitis and autoimmune myocarditis, it is still a humanized mouse model allowing for the study of autoantigens recognized by human MHC class I and II molecules. The infiltrating lymphocytes of both the heart and the pancreas can be further characterized to discover novel autoantigens required for the initiation of human autoimmune diseases.

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Figure 1: Preliminary histology of pancreas and heart.

Top: H/E staining of hearts. Boxed section labeled **a** shows lymphocyte infiltration in the myocardium. Bottom: H/E staining of pancreata. Insulitis and peri-insulitis are labeled as **b**.

Figure 2: Flow cytometry analysis of preliminary studies of NOD and NOD.*Aβ-/-*.*DQ8.ß2m-/-.HHD* mice.

B cell populations were enumerated by staining with anti-B220 (RA3-6B2) and anti-Ig. CD4+ and CD8+ T cell populations were stained with anti-CD4 (GK1.5) and anti-CD8 (53-6.7). MHC class II expression was determined by staining with anti-I-A^{g7} (AMS32.1) for the murine molecule and anti-DQ (Leu10/SK10) for the transgenic DQ8 molecule.

Table 1: Characteristics of NOD mice used in this study				
Strain	MHC class I	MHC class II	Diabetes Incidence (% at 30 wks)*	Ref.
NOD	K^dD^b	A^{g7} E^{null}	90	[4]
NOD.*β2m*$^{-/-}$	null	A^{g7} E^{null}	0	[4]
NOD.*HHD*	K^dD^b Human A2	A^{g7} E^{null}	100	[4]
NOD.*β2m*$^{-/-}$.*HHD*	Human A2	A^{g7} E^{null}	55	[4]
NOD.*Aβ*$^{-/-}$.*DQ8*	K^bD^b	Human DQ8	0	[1,2,19]
NOD.*Aβ*$^{-/-}$.*DQ8*.*β2m*$^{-/-}$.*HHD*	Human A2	Human DQ8	?	
* Females only				

Table 2: Results of preliminary histological examination		
	Lymphocyte Infiltration	
Genotypes	Pancreas	Heart
NOD	+	-
NOD.*scid*.*Aβ*$^{-/-}$.*DQ8*	-	-
NOD.*Aβ*$^{-/-}$.*DQ8*.*β2m*$^{-/-}$.*HHD*	+	+
NOD.*Aβ*$^{-/-}$.*DQ8*.*β2m*$^{+/-}$.*HHD*	+	+

Table 3: Summary of splenic lymphyocyte populations by flow cytometry								
		Total Cell	B cells		CD4+ T cells		CD8+ T cells	
	n	#	%	#	%	#	%	#
NOD	7	1.11E+08 ±1.4E+07	37.91 ±2.33	4.29E+07 ±6.73E+06	26.71 ±1.8	2.96E+07 ±4.22E+06	11.16 ±1.17	1.23E+07 ±2.69E+06
NOD.*β2m*$^{-/-}$.*HHD*	5	1.64E+08 ±1.96E+07	37.34 ±2.41	6.11E+07 ±8.8E+06	38.16 ±2.26	6.30E+07 ±9.76E+06	2.69 ±0.36	4.63E+06 ±1.15E+06
NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$.*HHD*	12	2.89E+07 ±5.03E+07	54.00 ±1.57	1.71E+08 ±3.48E+07	17.17 ±1.06	4.94E+07 ±8.22E+06	2.35 ±0.19	6.64E+06 ±1.08E+06
NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{+/-}$.*HHD*	4	2.02E+08 ±5.66E+07	48.00 ±3.29	9.60E+07 ±2.50E+07	15.19 ±1.02	2.94E+07 ±6.54E+07	13.53 ±1.67	2.64E+07 ±7.47E+06
NOD.*Aβ*$^{-/-}$.*β2m*$^{-/-}$.*HHD*	6	2.01E+08 ±4.84E+07	64.07 ±2.94	1.24E+08 ±2.37E+07	2.48 ±0.72	6.07E+06 ±3.44E+06	2.64 ±0.36	5.37E+06 ±1.59E+06
NOD.*Aβ*$^{-/-}$.*DQ8.β2m*$^{-/-}$	7	2.79E+08 ±2.1E+08	54.77 ±1.94	1.54E+08 ±3.43E+07	17.58 ±1.17	4.84E+07 ±9.89E+06	0.69 ±0.06	2.09E+06 ±6.35E+05

Figure 1

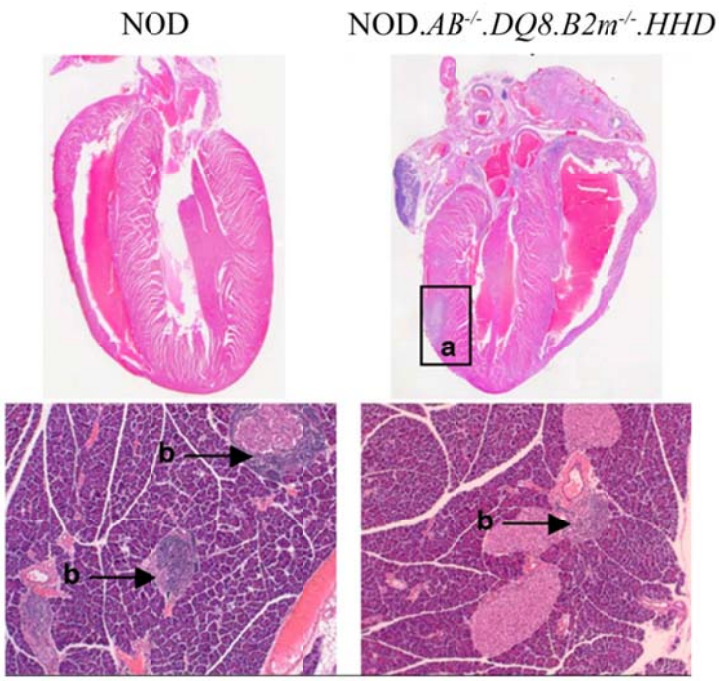
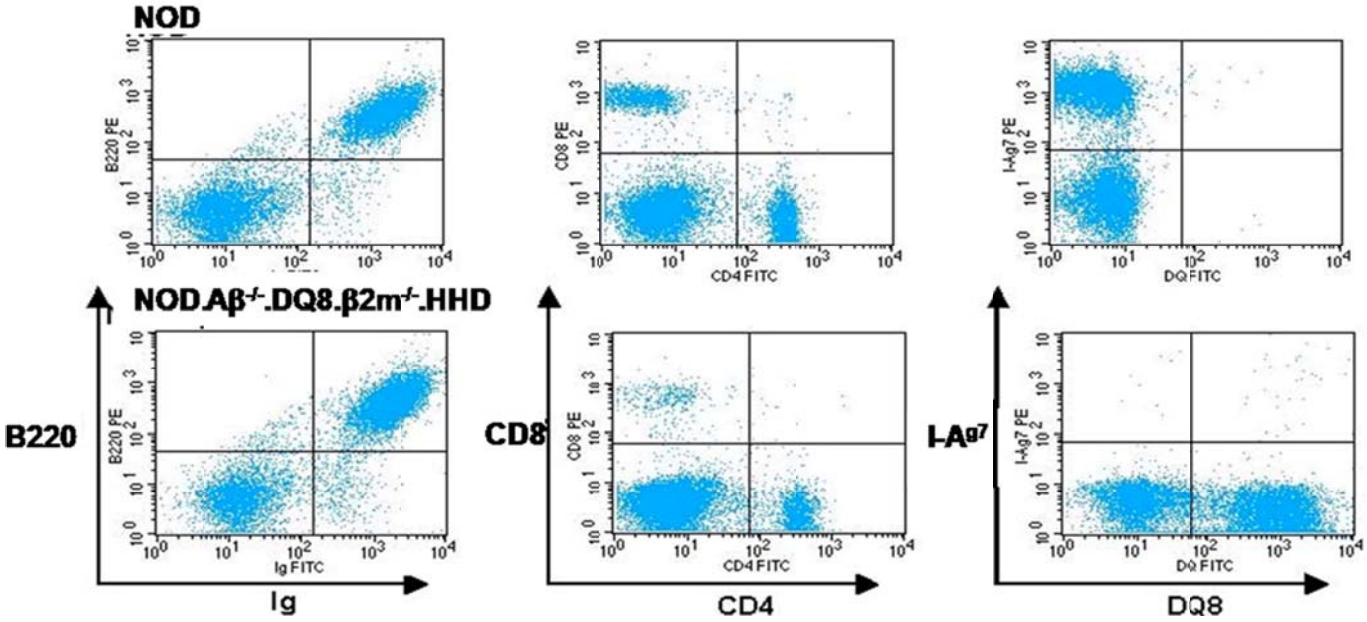


Figure 2


NOD
NOD.Aβ-/-.DQ8.β2m-/-.HHD
B220
CD8
I-Ag7
B220 PE
CD8 PE
I-Ag7 PE
Ig FITC
CD4 FITC
DQ FITC
Ig
CD4
DQ8

CHARACTERIZATION OF THE NOVEL INTERACTION BETWEEN MUSKELIN AND TBX20, A CRITICAL CARDIOGENIC TRANSCRIPTION FACTOR

by

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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.

Keywords

Yeast two-hybrid; heart development; TBX20; MKLN1; transcription factor; protein-protein interaction

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.

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 TBX1, 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 co-expression 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 [6,7,8,9]. Consistent with mouse models, missense mutations within human *TBX20* have been identified in patients with congenital heart defects (CHD) [10,11,12]. 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 [6,7,8,9]. In later heart development, TBX20 functions in chamber differentiation in part through repression of TBX2 activity [6,7,8]. 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. 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.

Materials and Methods

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 $5x10^6$ 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).

Copurification Studies

For the *in vitro* copurification studies, TBX20b was cloned into pGEX-2T (GE-Healthcare). 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 hours, lysates were collected and copurified with glutathione sepharose beads rotating at 4°C for 4-6 hours. 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).

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 7um 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 hours, 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).

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.

Results

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 x 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 con-

tained 3 unique clones of MKLN1. The interaction of TBX20b and MKLN1 was confirmed with a yeast cotransformation assay as shown in Figure 1a. A single colony from each cotransformation plate was re-plated 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 (Figure 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. The copurification assay resulted in the whole cell lysate and GST-bound elution fractions which were analyzed by SDS-PAGE and western blot analysis. HA-MKLN1 was found in both whole cell lysates, but

it only copurified with GST-TBX20b, confirming an interaction between TBX20b and MKLN1 within mammalian cells (Figure 1c).

Characterization of the Interaction Domains between TBX20 and MKLN1

While the TBX20b isoform was used in the yeast two-hybrid screening, the TBX20a isoform is also of interest in studying the genetic regulation of cardiogenesis and was included in the following experiment. A copurification assay using transfected mammalian cells was done as previously stated. COSM6 cells were cotransfected with HA-MKLN1 in addition to GST, GST-TBX20b, or GST-TBX20a. Interestingly, HA-MKLN1 did not copurify with the GST-TBX20a isoform (Figure 2a). To identify the domain of interaction, truncated TBX20b constructs were cloned into the GST vector to separate the N-terminal portion and the T-box domain. The copurification assay revealed that the T-box domain of TBX20 (aa 102-297) is responsible for the interaction with HA-MKLN1, whereas the N-terminal portion of TBX20 does not bind to HA-MKLN1. (Figure 2a).

The MKLN1 domain required for interaction with TBX20b was determined by creating HA-tagged truncated constructs of MKLN1 and using the same copurification assay with GST-TBX20b. MKLN1 was truncated from N- and C-terminal ends deleting domains listed in Figure 2b. The first 259 amino acids of MKLN1 consist of a discoidin-like domain and a LiSH/CTLH domain. The C-terminal 476 amino acids of MKLN1 consist of a kelch motif with 6 kelch repeats followed by a C-terminal tail with no functional characteristics. The removal of the kelch motif and C-terminal tail abolished the interac-

tion with GST-TBX20b. The interaction was narrowed to the kelch repeats domain with the removal of the last 3 repeats resulting in no interaction (Figure 2b).

TBX20b and MKLN1 colocalize in the perinuclear cytoplasm

To further explore the TBX20b-MKLN1 interaction, the subcellular localizations of the proteins were determined. As a transcription factor, the primary subcellular localization for TBX20 is in the nucleus. However, with previous studies placing the majority of MKLN1 in the cytoplasm [17,18,19,20], it was of interest to determine where the TBX20b-MKLN1 interaction was localized. COSM6 cells were cotransfected with myc-TBX20b and HA-MKLN1 and analyzed by immunofluorescent staining. HA-MKLN1 was localized primarily in the cytoplasm and myc-TBX20b was localized primarily in the nucleus with some cytoplasm protein. Colocalization was seen in the cytoplasm with perinuclear intensity (Figure 3a). These localization results were verified by using a nuclear/cytoplasmic fractionation kit on transfected cell lysates with western blot analysis confirming the immunostaining result (Figure 3b). MKLN1 has been identified within protein complexes involved in nucleocytoplasmic shuttling and an interaction with a transcription factor, such as TBX20b, could indicate a negative regulation by sequestering the transcription factor from the nucleus. However, the overexpression of HA-MKLN1 in the transfected COSM6 cells did not result in any identifiable changes in subcellular localization of TBX20.

Expression Studies of TBX20 and MKLN1 using Immunohistochemistry

To determine where and when the TBX20b-MKLN1 interaction takes place within cardiogenesis, the expression patterns of TBX20 and MKLN1 were analyzed in devel-

oping mice from E9.5-12.5 by immunohistochemistry. TBX20 expression analysis confirmed previously published data with expression in the ventricular myocardium wall, atrial myocardium, and endocardial cushions [14,21,22]. TBX20 expression correlates to its functions necessary for the development of the chamber myocardium in the ventricles and the valvuloseptal structures that develop from the endocardial cushion regions. Expression patterns of MKLN1 have not been examined throughout mouse heart development. From E9.5-10.5, MKLN1 expression is seen in numerous embryonic tissues including the heart. Within the heart, MKLN1 expression is found throughout the developing myocardium. From E11.5-12.5, MKLN1 expression is restricted and enriched within the endocardial cells in the AVC and in the myocardial cells of the IVS (Figure 4). Regions of overlapping expression between TBX20 and MKLN1 can be seen in the endocardial cushion cells of the developing atrioventricular region as well as in the myocardial cells in the interventricular region. Development of these regions is critical for proper cardiogenesis, failure of which leads to valvuloseptal defects

Discussion

Identifying novel regulators of critical cardiac transcription factors is crucial to better define the regulatory networks involved in cardiogenesis and provide insights into the mechanisms of CHD. In this study, a novel interaction between TBX20b and MKLN1 was discovered. Interestingly, this is an isoform-specific interaction with MKLN1 only interacting with the shorter TBX20b isoform. The TBX20 locus is alternatively transcribed into multiple TBX20 isoforms, mainly TBX20a and TBX20b [14]. Both isoforms are expressed in the heart throughout development and into adulthood with the TBX20a being the major transcript in both mice and humans [14,23]. Both isoforms can interact

with cardiac transcription cofactors and activate transcription of critical cardiac genes and, in some cases, TBX20b can activate transcription more effectively than the longer isoform [14]. Both isoforms have identical T-box DNA-binding domains which suggest that they activate the same target genes. An isoform-specific protein interaction adds further complexity to the activities of TBX20 transcription factors.

TBX transcription factors are characterized by a highly conserved DNA-binding domain designated the T-box, which is typically 180 amino acids and recognizes a 24 base pair sequence called the T-site [3,14,24]. TBX20 binds to the T/2-site (half of the typical T-site) as a monomer in a molten globule state at 37°C, which increases conformation flexibility and potentially allows for more protein-protein interactions [24]. The T-box of TBX20 is also important for the binding of cofactors such as NKX2-5, GATA4 and GATA5 [14]. The binding of these proteins to the T-box implies cooperative or competitive binding with DNA and/or cofactors. Since MKLN1 binds to TBX20b at the T-box domain, determining if MKLN1 can alter DNA-binding or cofactor binding abilities of TBX20b would be of interest in future studies.

MKLN1 is a 735 aa protein with discoidin-like and LisH/CTLH domains in the N-terminal half. The C-terminal portion contains a kelch motif with 6 repeats [19,25]. The kelch motif typically contains 5-7 repeats that each form a 4-stranded β sheet, or blade, in a β-propeller and is most often involved in protein-protein interactions within protein complexes [25,26]. Proteins containing a kelch motif have a variety of functions, ranging from cytoskeleton organization to gene regulation. For instance, the kelch motif-containing protein KEAP1 regulates gene expression by sequestering the transcription factor NRF2 [26]. The binding of TBX20b to the kelch motif of MKLN1 suggests the

presence of a protein complex with other binding partners in the cytoplasm. Identification of other protein partners in the TBX20b-MKLN1 interaction complex would be of interest to further explore regulatory roles of MKLN1 on TBX20b activity.

To better understand the localization of the TBX20b-MKLN1 interaction throughout heart development, the expression patterns of TBX20 and MKLN1 were determined by immunohistochemistry. In this study, TBX20 expression supports previous reports [14,22,27,28]. MKLN1 is expressed in a variety of tissues in developing and adult mice including the skeletal muscle, liver, heart, and parts of the central nervous system [17,20,29]. In this study, MKLN1 expression was detailed in heart development of E9.5-12.5 mice. While MKLN1 expression is broadly expressed in E9.5-10.5 hearts, in later stages there is strong expression in the endocardial cells of the developing AVC and the myocardial cells in the IVS. The coexpression of TBX20 and MKLN1 correlate to regions important for the developing valves and septa.

While the functions of MKLN1 have been largely unexplored, the colocalization between TBX20b and MKLN1 at the perinuclear region suggests that the potential nucleocytoplasmic shuttling roles of MKLN1 regulate TBX20b activity [30]. However, any nuclear function of MKLN1 has yet to be determined and future studies could reveal new methods of transcription factor regulation. In addition, TBX20 does have a significant cytoplasmic localization which opens the possibility of a cytoplasmic, DNA-independent role for the transcription factor. Instead of a decrease of TBX20 activity in the nucleus due to sequestering, perhaps MKLN1 and TBX20b play a DNA-independent role in heart development. Recent publications have spotlighted DNA-independent functions of TBX20. TBX20 reduced the BMP/Smad signaling through the binding and inhibiting the

Smad1/5 signaling that is required to activate *Tbx2* expression [31]. This DNA-independent regulation of critical signaling pathways is a relatively unexplored function of transcription factors. Another potential regulatory role for the TBX20b-MKLN1 interaction involves the development of the AVC which will form the heart valves. Valvulogenesis requires changes in cellular proliferation and ECM properties. TBX20 has been identified as regulating proliferation and the expression of different ECM components [6,7,8,9,13]. MKLN1 was first identified having a role in the regulation of cell attachment through ECM signaling [17]. Perhaps TBX20 and MKLN1 regulate the changes in the ECM during valvuloseptal development. Understanding how TBX20 function is regulated in different developmental processes throughout cardiogenesis would provide key information in how CHD occur and are identified.

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Fig. 1: Identification of MKLN1 as a novel interacting partner to TBX20b.

(A) AH109 yeast cells were cotransformed with pGBTK-T7 and pGAD-T7 vector controls, pGBTK-TBX20b with the pGAD-T7 control, pGAD-MKLN1 with the pGBTK-T7 control, and pGBTK-TBX20b with pGAD-MKLN1. Each cotransformation was replated as a patch on a Trp/Leu plate, and replica-plated onto a Q plate. Growth with pGBTK-TBX20b and pGAD-MKLN1 indicates protein interaction. (B) GST and GST-TBX20b proteins were isolated from bacterial lysates and copurified with *in vitro* radiolabeled MKLN1. The bottom panel shows the coomassie-stained SDS-PAGE gel with radioactive MKLN1 input (lane 1), and the GST-pulldown elution fractions of GST (lane 2) and GST-TBX20 (lane 3). The top panel is the radiography film revealing presence of MKLN1. (C) COSM6 cells were transfected with HA-MKLN1 and GST or GST-TBX20b as indicated. Interacting proteins were isolated by copurification. Whole cell lysate (WCL) and elution fractions (EF) were analyzed by SDS-PAGE and western blot analysis probing for GST and HA1.1.

Fig. 2: Identification of the interaction domains of TBX20b and MKLN1.

(A) Top: Schematic of TBX20 isoforms. Bottom: COSM6 cells were transfected with HA-MKLN1 and GST, GST-TBX20a, GST-TBX20b, and truncations of GST-TBX20b. Copurification assays and western blot analyses compared whole cell lysate (WCL) and the elution fraction (EF). The blots were probed with GST and HA1.1 antibodies as before. (B) Top: Schematic of MKLN1 protein. Bottom: Similar copurification analyses were performed with truncated HA-tagged MKLN1 constructs. Gels were loaded as in panel (A) comparing WCL and EF with the GST and the GST-TBX20b transfected cells.

Western blot analysis of the truncated HA-MKLN1 constructs (top-to-bottom) are listed left-to-right.

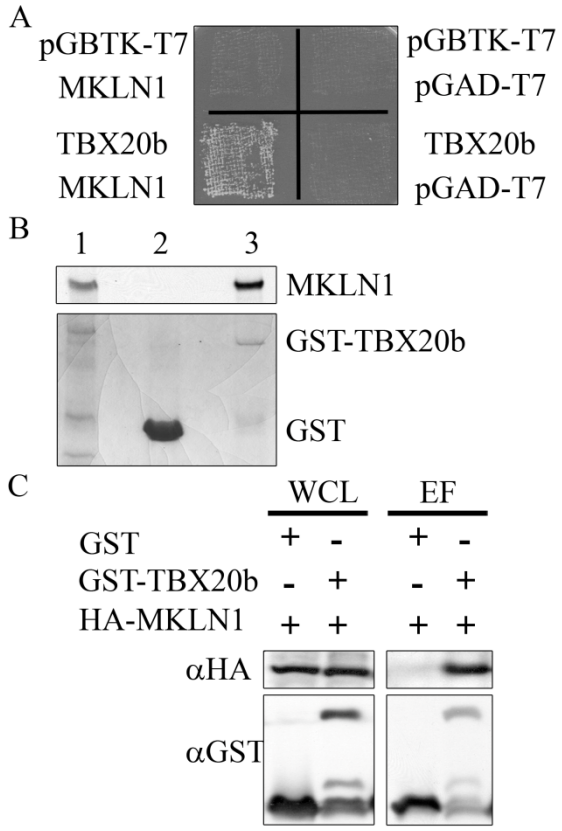
Fig. 3: Colocalization of TBX20b and MKLN1.

(A) COSM6 cells were transfected with myc-TBX20b and HA-MKLN1. Fluorescent immunocytochemistry was performed with antibodies for TBX20, HA1.1, and DAPI for counterstain. (B) COSM6 cells were transfected with 1. myc-TBX20b, 2. HA-MKLN1, and 3. myc-TBX20b and HA-MKLN1. Cells were lysed and fractionated into cytoplasmic (C) and nuclear (N) fractions and analyzed by western blotting. MEK1/2 and LSD1 are cytoplasmic and nuclear controls, respectively.

Fig. 4: Immunohistochemistry in the developing mouse heart.

ICR mice matings were timed to collect embryos at the stated time points. Embryos were dissected and processed for immunohistochemistry on sagittal sections (E9.5-11.5) and frontal sections (E12.5). Anti-TBX20 and anti-MKLN1 were visualized with the DAB chromogen system. Slides were counterstained with hematoxylin. Boxed areas of developing endocardial cushion region indicate higher magnification panel below. (v), ventricles; (a), atrium; (IVS), interventricular septum; (AVC), atrioventricular cushions.

Figure 1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2
3
MKLN1
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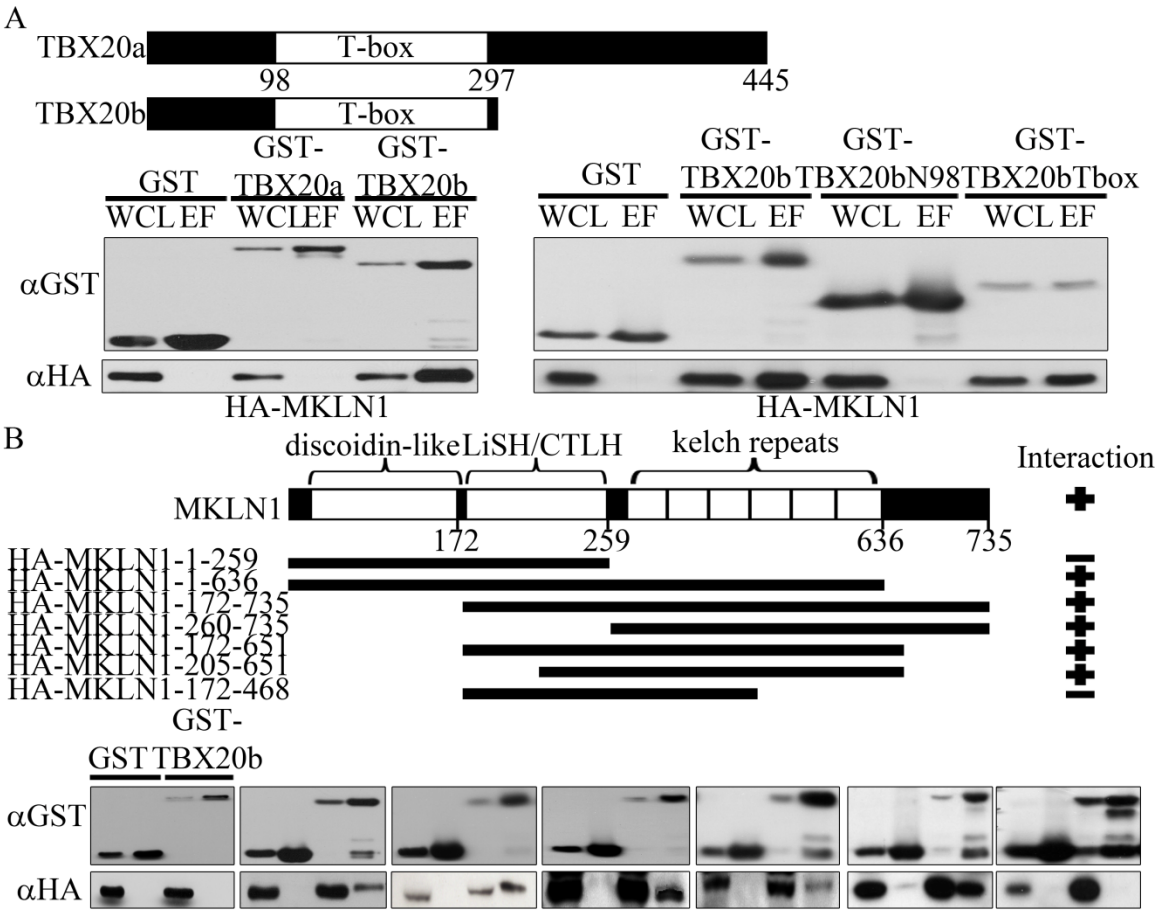
Figure 2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αHA
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TBXTbox
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B
discoidin-like
LiSH/CTLH
kelch repeats
Interaction
MKLN1
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259
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735
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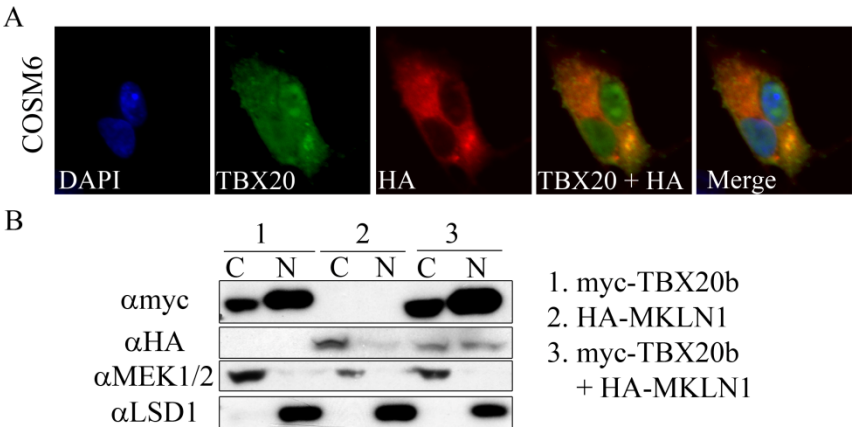
Figure 3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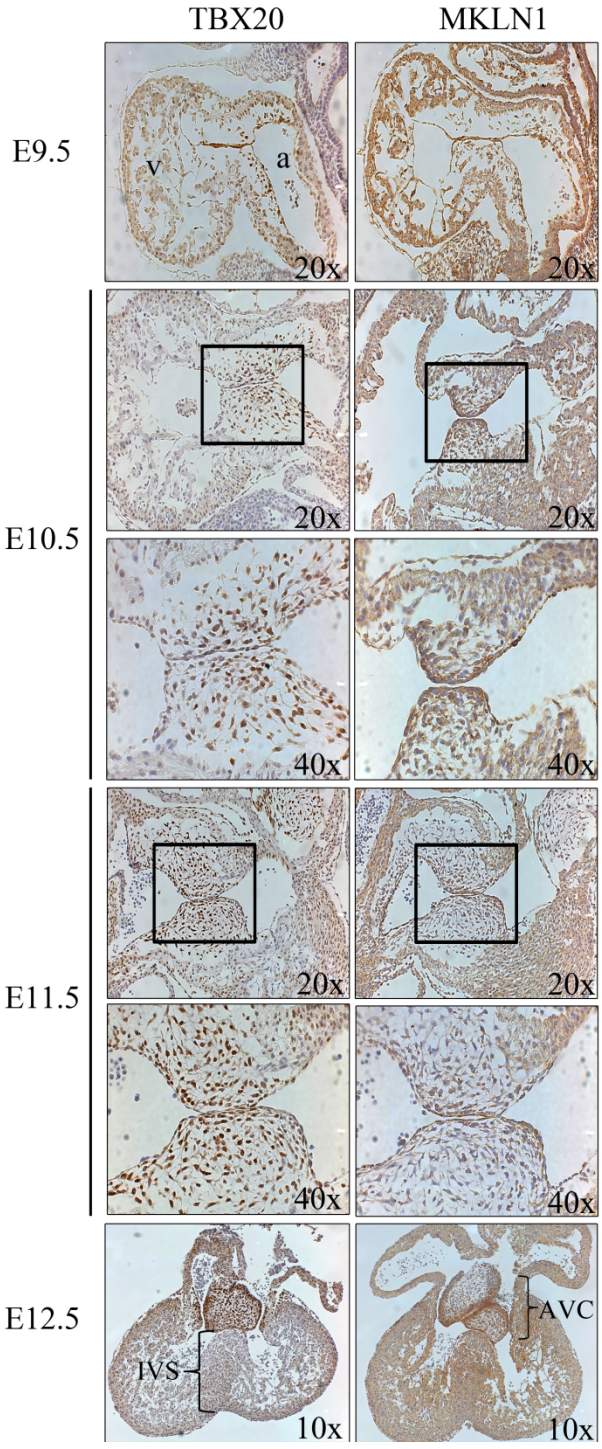
2. HA-MKLN1
3. myc-TBX20b
+ HA-MKLN1

Figure 4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40x
E12.5
IVS
AVC
10x
10x

ALTERNATIVE SPLICING OF T-BOX TRANSCRIPTION FACTOR GENES

by

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Abstract

T-box (TBX) transcription factors are an ancient gene family with critical roles in embryogenesis. Currently, *TBX3*, *TBX5*, and *TBX20* are TBX genes defined to have multiple protein isoforms created by alternative splicing and characterized by expression and functional studies. These proteins are important for development as mutations lead to severe developmental disorders in humans and mice. Cumulative studies suggest that alternative splicing of these genes can regulate TBX activities during multiple biological processes including cardiogenesis, limb development, and cancer mechanisms. This mini-review focuses on how alternative splicing adds complexity to transcriptional regulation of target genes controlled by TBX transcription factors.

Abbreviations

aa, amino acids; EMSA, electrophorectic mobility shift assay; hADSC, human adipocyte stromal cells; HOS, Holt-Oram syndrome; mouse embryonic fibroblast, MEF; nt, nucleotide; TBE, T-box binding element; TBX, T-box; UMS, ulnar-mammary syndrome

Introduction

T-box (TBX) transcription factors are an ancient gene family important for organogenesis and embryogenesis. Genome searches for genes sharing the T-box DNA-binding domain revealed numerous highly-related transcription factor genes [1]. TBX transcription factors recognize a 20-24 nucleotide palindromic sequence called the T-site or half of the sequence, the T/2 site [2]. The T-box family is thought to have evolved from a primordial gene that gained diversity through tandem duplication and then cluster dispersion resulting in TBX genes maintaining similar functions and loci [3]. While the TBX transcription factors can be expressed in similar developmental times and tissues,

they provide distinct functions through control of expression levels, timing and localization of expression, and interaction with different cofactors. Isoforms of the same TBX gene can have different subcellular localizations, expression levels, and functional characteristics. Cumulative studies have shown that alternative splicing provides a potential critical role in the regulating TBX protein activities.

Alternative splicing creates different protein products from a single gene and is estimated to affect approximately 90% of the genes expressed in humans [4,5,6]. Multiple isoforms can increase the function and the complexity of the genome. There are multiple types of alternative splicing, with the most common being exon skipping [7,8]. The control of alternative splicing is defined by the pre-mRNA sequence and the availability of spliceosome proteins [4,9]. Alternative splicing is regulated in a developmental stage-specific or tissue-specific manner depending on the localization, timing, and type of factors expressed.

This mini-review highlights the alternatively spliced isoforms found within the TBX transcription factor family. Alternatively spliced isoforms have been described in expression and function analyses in three TBX transcription factor genes: *TBX3*, *TBX5* and *TBX20* (Figure 1). All three genes are critical for development as mutations or deletions lead to severe developmental disorders in humans and mice. Determining how these transcription factors are regulated is important to fully understand complex processes such as cardiogenesis, limb development, and cancer mechanisms. From recent studies, expression and functional analyses have revealed a potential regulatory role of protein isoforms on TBX transcription factor functions.

TBX3 and TBX3+2a

TBX3 is a transcriptional repressor important for the development of multiple tissues. Haploinsufficiency of TBX3 causes ulnar-mammary syndrome (UMS, OMIM ID: 181450), an autosomal dominant disorder affecting 1 in 100,000 live births [10,11]. UMS is characterized by defects in upper limb, apocrine gland, mammary gland, dental, and genital development and the defects are highly variable within families. TBX3 is expressed primarily in the tissues affected in UMS. In addition, TBX3 is overexpressed in different cancer cells including malignant primary breast cancer tumors and immortalized cancer cell lines of breast cancer, bladder carcinoma, and melanoma [12,13,14]. In most studies, TBX3 functions as inhibiting senescence and thereby promoting cell proliferation and immortalization. Overexpression of TBX3 in primary cells leads to immortalization [12,15,16]. Ablation of TBX3 expression leads to a decrease in proliferation with a cell type-dependent increase in apoptosis [13,17,18]. TBX3 regulation of proliferation is cell-type dependent as TBX3 inhibits proliferation during cardiogenesis [14,19,20,21]. Other functions of TBX3 include potential roles in bone development, where a decrease in TBX3 expression reduced the differentiation of osteoblasts and the osteogenic-differentiation of human adipose stromal cells (hADSC) [13,17]. These studies place TBX3 function as important for the proliferation and specification of cells and tissues critical for development.

TBX3 isoforms are designated TBX3 and TBX3+2a [22]. Alternative splicing of exon 2a results in a 60 nucleotide (nt) insertion incorporating 20 amino acids (aa) within the T-box DNA-binding domain (Figure 1) [11,12]. The transcripts of TBX3 and TBX3+2a are found in multiple tissues in mice and humans. This insertion is highly

conserved within other TBX3 mammalian orthologs and is absent in the avian genome. In other vertebrate and invertebrate genomes, insertions at the exon 2a site are observed, however they do not exhibit similarity to the mammalian transcripts. Currently, there are no other insertions of this nature within the TBX family [14]. The ratio of the transcripts varies between tissues and species indicating tissue-specific and species-specific regulation of expression. Both isoforms are expressed in breast cancer cell lines, and the ratio between isoforms is altered between cell lines, indicating a potential role in cancer mechanisms [12]. In hADSC, TBX3 is the predominant isoform and upon differentiation with osteogenic medium, the isoform ratio is altered with increasing amounts of TBX3+2a expressed, indicating a potential role in differentiation [17]. With the altered isoform levels, future studies should concentrate on determining the importance of isoform amounts. It will be important to identify is isoform-specific target genes exist as a subset of these genes can be alternatively regulated depending on isoform protein levels.

With the 20 aa insertion within the T-box DNA-binding domain, it was important to determine whether both isoforms could bind to DNA. Two separate studies offered conflicting results. In the first study, analysis by electrophoretic mobility shift assay (EMSA) was used to determine if the amount of T-box binding element (TBE) DNA would increase when incubated with nuclear extracts of mouse embryonic fibroblast (MEF) cells overexpressing TBX3 or TBX3+2a. The addition of TBX3 resulted in an increase of bound DNA, indicating DNA-binding ability. However, the addition of TBX3+2a did not result in any discernable increase of bound DNA [12]. While TBX3+2a was not able to bind DNA in this study, another EMSA study was modified to strictly test

the DNA-binding capabilities of both isoforms. TBX3 and TBX3+2a proteins were purified from bacterial cultures and incubated with the consensus TBE and the TBE within the *Nppa* promoter region. This assay resulted in TBX3 and TBX3+2a binding to DNA. Through comparison to TBX3/DNA structure [23], they propose that the 20 aa insert does not contact the DNA and most likely does not affect DNA-binding [14]. The direct DNA-binding experiments and structural analysis more conclusively show that both isoforms can bind to DNA. In future studies, DNA-binding affinities and confirmation of DNA-binding within relevant cell types should be determined for both isoforms.

TBX3 and TBX+2a share common transcriptional targets that function in inhibiting senescence. In one study, overexpression of TBX3 in MEF cells resulted in continuous and increased growth, while TBX3+2a resulted in decreased lifespan. However, it was later noticed that the TBX3+2a isoform was unknowingly used in a similar functional study resulting in increased proliferation [14,24]. In luciferase reporter assays, TBX3 and TBX3+2a inhibited the expression of $p21^{CIP1}$, a tumor suppressor gene involved in p53-mediated senescence [14]. Future studies should focus on identifying additional target genes with roles in proliferation and senescence. Analyzing target gene expression between primary and immortalized cell lines can establish a connection between TBX3 isoform protein levels and target gene expression.

In vivo analysis of TBX3 isoform function has been analyzed in the developing hearts of mice. Transgenic mice were created with TBX3 or TBX3+2a overexpressed in the myocardium of the developing heart. Embryos overexpressing TBX3 or TBX3+2a resulted in abnormalities or failure in chamber formation and heart looping. In addition,

the ectopic expression resulted in a severe decrease in expression of the chamber-specific markers of *Cx40* and *Nppa* [14]. In luciferase reporter assays, TBX3 and TBX3+2a decreased the NKX2-5/TBX5-driven activation of the *Nppa* promoter. In addition, both isoforms physically interact with NKX2-5 via the T-box DNA-binding domain [14]. While these studies show no isoform-specific function during heart development, more relevant cell types will need to be tested. Since TBX3 is critical for mammary and limb development, future studies should include functional analysis within these developmental systems.

TBX5a and TBX5b

TBX5 is critical for forelimb development and cardiogenesis [25,26,27,28,29]. Haploinsufficiency of TBX5 causes Holt-Oram Syndrome (HOS, OMIM ID: 142900), an autosomal dominant disorder characterized by upper limb malformations and cardiac septation defects, which occurs in 1 in 100,000 live births [30,31]. Analysis of TBX5 expression identified an alternatively spliced isoform [32]. The longer TBX5 isoform (518 aa) is designated TBX5a. Alternative splicing of exon 8 inserts 40 nt after the T-box DNA-binding domain creating 4 aa and a stop codon resulting in a 255 aa protein designated TBX5b (Figure 1). Both isoforms are expressed in different tissues and cell lines. During heart development, the ratio is altered with TBX5a being more prominent in embryonic hearts and TBX5b in adult hearts. In transfected cell studies, TBX5a is strictly localized to the nucleus and TBX5b in both the nucleus and cytoplasm. These localizations are seen in various cell types including cardiac, skeletal muscle, and fibroblast cells [32].

Both isoforms can bind to DNA with TBX5a showing a stronger binding affinity. TBX5a can bind to the *Nppa* promoter and activate transcription. However, due to the lack of the C-terminal transcriptional activation domain on the TBX5b isoform, TBX5b cannot activate the *Nppa* promoter in luciferase reporter assays in cell lines or cardiomyocytes. It is noteworthy that increasing amounts of TBX5b does not attenuate TBX5a-driven activation of *Nppa* transcription. Both TBX5 isoforms physically interact with GATA4 and can collaborate to activate transcription of GATA4 target genes. However, TBX5a has a stronger binding affinity for GATA4 and is the only isoform to interact with NKX2-5, resulting in isoform-specific activation of *Nppa* [32].

Examining TBX5 isoform amounts throughout heart development revealed that TBX5a is more prominent in proliferative developing cells while TBX5b is more prominent in terminally differentiated cells. To identify potential isoform-specific functions, TBX5a was overexpressed in adult hearts using tamoxifen-inducible α-myosin heavy chain-driven *Cre* transgenic mice [32]. Treatment increased TBX5a expression in the ventricles with a concomitant upregulation of *Nppa*. Overexpression of TBX5a resulted in cardiac hypertrophy with immunohistochemical analysis showing an increase in myocyte growth without an increase in proliferation. To study TBX5b function, the proliferative myoblast cell line, C2C12, transfected with TBX5b resulted in cell morphology changes and significant cell death due to increased apoptosis. Similar overexpression studies with TBX5a did not result in changes of cell morphology or apoptosis. These studies support the roles of TBX5a regulating cardiomyocyte growth and TBX5b regulating cardiomyocyte growth arrest. Future studies should include

conditional isoform-specific knockout mouse model strategies to determine developmental time point and cell type requirements for each isoform.

TBX20a and TBX20b

TBX20 is a critical cardiogenic transcription factor important for proliferation, chamber specification, and valvulogenesis. Missense mutations have been identified in human patients with congenital heart defects and adult cardiomyopathies [33,34,35]. TBX20 physically interacts with NKX2-5, GATA4, and GATA5 and synergizes with these cofactors to regulate target genes [36]. TBX20 can activate or repress cardiac target genes important for cardiac chamber specification and extracellular matrix formation within endocardial cushions [37,38,39].

Characterization of TBX20 identified multiple isoforms expressed in several species. One study comprehensively characterized TBX20 isoforms designated TBX20a-d [36]. The two most-studied isoforms are TBX20a and TBX20b (Figure 1). In mice, both transcripts contain exons 1-6 which encode for the entire T-box DNA-binding domain. TBX20a is the full length protein of 445 aa encoded by exons 1-6, 9, and 10. TBX20b is truncated after the T-box DNA-binding domain at 297 aa due to alternative splicing of exon 7 which contains a termination codon [36].

The TBX20 isoforms are coexpressed during heart development with TBX20a expressed at higher levels in both mice and humans [36,40]. One study examined TBX20a-specific expression in E9.0-12.0 mouse hearts. While general TBX20 expression is seen throughout the myocardium, TBX20a is restricted to the developing outflow tract with less myocardial expression [37]. In our laboratory, we determined the presence of TBX20 isoforms by western blot analysis of protein lysates collected from

the head, heart, and body of E12.5 mice (Figure 2a). TBX20a was only expressed in the heart while TBX20b was expressed in all three samples. While this was a brief look into TBX20 expression during development, it does reveal the need for careful isoform-specific expression analysis. We also examined the subcellular localization of myc-tagged TBX20 isoforms by immunofluorescent staining of transfected COSM6 cells. Similar to TBX5, TBX20a is localized exclusively in the nucleus while TBX20b has cytoplasmic and nuclear localization (Figure 2b). Future studies of heart development will need to define isoform-specific role within the heart, specifically in determining cell-type specific expression of TBX20 isoforms. Conditional ablation of each TBX20 isoform in knockout mouse models will help define cell type-specific roles. In addition, future studies involving the development of other embryonic structures should concentrate on the functions of TBX20b.

The TBX20 isoforms have similar and dynamic protein interactions and transcriptional activities. Both isoforms can bind to DNA, interact with cofactors NKX2-5, GATA4, and GATA5, and activate transcription. TBX20b can synergistically increase activation of the NKX2-5-binding sites and the *Nppa* promoter when coexpressed with cofactors NKX2-5, GATA4, and GATA5. While TBX20a can activate transcription with the same cofactors, the effect is additive instead of synergistic, most likely due to the C-terminal repression domain [36]. A recently published study from our laboratory identified muskelin as a novel interacting partner to only the TBX20b isoform [41]. Muskelin is an intracellular protein involved in protein complexes of nucleocytoplasmic shuttling and signal transduction machinery [42,43,44,45,46,47]. As protein interactions are critical for maintaining a cardiac transcription factor network, future studies should

identify more isoform-specific protein interactions and test their role in regulating TBX20 activities.

To determine potential functional differences between the TBX20 isoforms, *Xenopus* embryos were injected with TBX20a or TBX20b mRNA [36]. Ectopic overexpression of TBX20a resulted in changes in cell migration of the anterior/posterior axis. The formation of a protrusion resembling a secondary anterior/posterior axis or tail was observed and analysis revealed induction of lateral mesoderm and endoderm. Injections of TBX20b resulted in no change. This study suggests that within the *Xenopus* system, the activities of TBX20a cannot be replaced by TBX20b.

Conclusions

The TBX genes are important for proper embryogenesis with mutations or deletions causing developmental disorders in humans and mice. Regulation of TBX transcription factor activity has been characterized through protein interactions and DNA binding affinities. An emerging mechanism of regulation is the production of different protein isoforms by alternative splicing. This mini-review highlights the role of alternative splicing within the TBX genes, TBX3, TBX5, and TBX20, and how alternative splicing adds another level of complexity in TBX transcriptional regulation. Future studies should determine the prevalence of alternative splicing of the other TBX genes. Most expression analyses show general expression without defining isoform specificity. Determining isoform-specific expression throughout development will help define cell-type specific functions. The expression analyses can then be supported by the use of isoform-specific knockout mouse model strategies to further define isoform-specific functional significance. In addition, with tissue-specific expression evident with

these TBX genes, it will be important to determine the upstream regulators of alternative splicing, potentially tissue-specific expression of splicing proteins. Finally, these TBX proteins have significant roles in human development and determining whether the pathogenic mutations affect alternative splicing or isoform-specific function could have clinical significance.

Acknowledgments

We regret that we couldn't reference all studies in this area due to space limitations. The work in KJ's lab is supported by a UAB faculty development grant and a NHLBI R01 grant (HL095783-01A1) awarded to KJ.

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Fig. 1: Schematic of TBX protein isoforms.

The protein structures of the murine TBX3, TBX5, and TBX20 protein isoforms are depicted with the T-box DNA-binding domain in white. The 20 aa insertion within the T-box of TBX3+2a is shown in grey.

Fig. 2: TBX20 isoforms show distinct tissue expression and subcellular localizations.

(A) Tissue from E12.5 ICR mice was dissected into head, heart, and body lysates. Lysates were analyzed by SDS-PAGE and western blot analysis. Antibody used was TBX20 (Sigma, HPA008192). TBX20a is 55 kDa. TBX20b is 33 kDa.

(B) COSM6 cells were plated on glass coverslips in a 24 well plate and transfected with 0.8 ug of DNA using Lipofectamine 2000 according to manufacturer's protocol (Invitrogen). TBX20a and TBX20b were cloned into the pCMV-Tag3 construct to create a myc-tagged fusion protein (Stratagene). Cells were processed for immunocytochemistry using antibodies for myc (Cell Signaling). Coverslips were mounted onto glass microscope slides with DAPI counterstaining mounting solution (Vectashield).

Figure 1

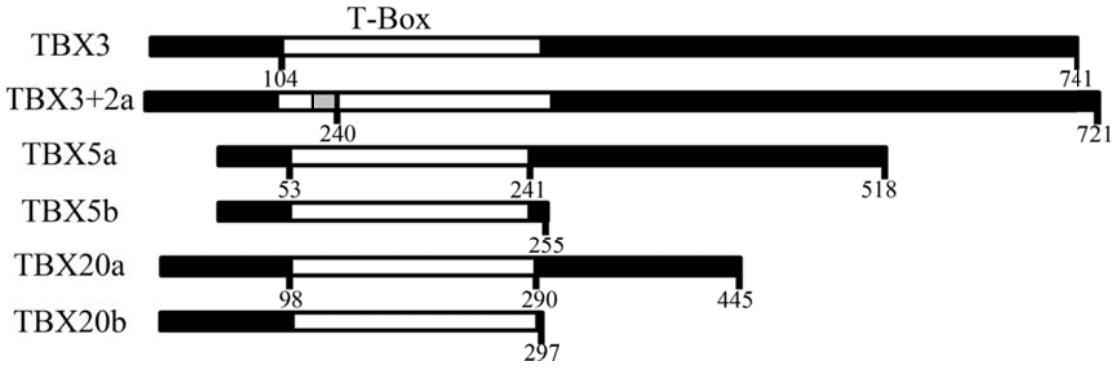
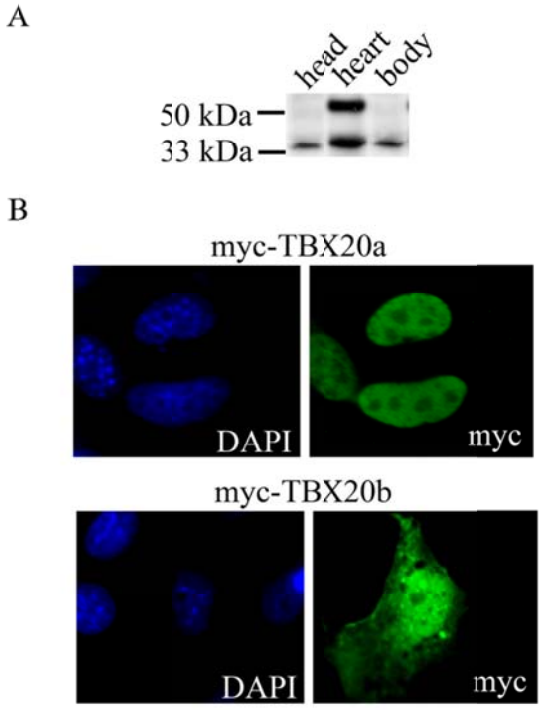

T-Box
TBX3
104
741
TBX3+2a
240
721
TBX5a
53
241
518
TBX5b
255
TBX20a
98
290
445
TBX20b
297

Figure 2


A
head
heart
body
50 kDa
33 kDa
B
myc-TBX20a
DAPI
myc
myc-TBX20b
DAPI
myc

CONCLUSIONS

Characterization of a Novel Interaction between MKLN1 and TBX20b

The major goal of this study was to identify proteins expressed during heart development that physically interacted with TBX20. While TBX20 is a key component in the cardiogenic transcription factor network, the currently known interacting proteins are limited to the cofactors which further reinforce the cardiogenic transcriptional network. Identifying novel interacting proteins is important to determine how TBX20 activity is regulated or modified. This study identified a novel interaction with muskelin (MKLN1). MKLN1 is a primarily cytoplasmic protein found within protein complexes with potential roles in nucleocytoplasmic shuttling and signal transduction machinery. While the TBX20b-MKLN1 interaction has been confirmed by multiple methods, the potential functional significance can be surmised by exploring the specifics of the interaction.

Isoform Specificity

One important feature of the TBX20-MKLN1 interaction is that MKLN1 only interacts with the TBX20b isoform. A few studies have been conducted to explore the potential functional differences between the TBX20 isoforms which are described in Chapter 4. Expression analysis is difficult due to the inability to create TBX20b-specific antibodies or *in situ* probes. However, TBX20a-specific expression was compared to TBX20 general expression by *in situ* probing of whole mount E9.0-E12.0 mouse hearts [64]. TBX20 general expression is seen in the myocardium of all chambers and the de-

veloping OFT through E9.0-12.0. TBX20a-specific expression is at lower levels in the myocardium with enhanced expression in the developing OFT. Specifying the spatiotemporal expression patterns of the TBX20 isoforms will be crucial to determine any functional significance to protein interactions. Because TBX20 is alternatively spliced through exon-skipping, isoform-specific exons can be targeted to create isoform-specific knockout mice. In combination with tissue-specific expression of *Cre* recombinase, specific TBX20 isoform expression can be removed from endocardial or myocardial cells which would determine any isoform-specific function.

Subcellular Localizations

The importance of subcellular localization of TBX proteins is defined through studies of TBX5. The subcellular localization of TBX5 has been found to be either nuclear or cytoplasmic depending on cell type and differentiation state [68,69]. One mechanism of the dynamic localization changes of TBX5 is due to interaction with LMP4, a cytoplasmic, actin-binding PDZ-LIM protein [70]. TBX5 and LMP4 are coexpressed in the developing limb buds and heart in chickens [70,71,72]. Transfected cell studies show TBX5 localizing strictly to the nucleus unless coexpressed with LMP4 which alters TBX5 localization to include the actin stress fibers in the cytoplasm [68,70]. This relocalization of TBX5 affects the TBX5-dependent transcriptional activation of *Nppa* and *Fgf10* with increasing amounts of LMP4 decreasing the luciferase output induced by TBX5 [68]. In developing chickens, TBX5 is expressed in the AVC. However, as with TBX20, the activation of chamber-specific genes is downregulated in the region. In addition, LMP4 is coexpressed with TBX5 in both the AVC and OFT and thus the subcellular localization of TBX5 is broadened to the cytoplasm [71]. The TBX5-LMP4 interaction

may indicate a negative regulation by LMP4 of TBX5 activity in non-chamber myocardium. However, the role of isoforms has not been determined within this TBX5-LMP4 interaction.

Both TBX5 and TBX20 have similar alternatively spliced isoforms in which there is a shorter isoform that is truncated after the T-box DNA-binding domain. While the TBX20b-MKLN1 interaction has been determined to be isoform-specific, the TBX5-LMP4 interaction has not been studied in an isoform manner and the interaction domain has not been identified. The subcellular localization of the TBX5 isoforms differ with the TBX5a isoform located exclusively in the nucleus and the TBX5b isoform exhibiting expanded cytoplasm localization in addition to the nucleus [73]. A dominant-negative function of the shorter isoforms is unlikely as demonstrated by reporter experiments where the overexpression of the shorter isoform does not lead to a decrease in the longer isoform function.

While the subcellular localizations of the TBX20 isoforms have not been published, experiments from this study examined TBX20 isoform localizations in transfected COSM6 cells (Appendix Figure 1). Immunofluorescent staining revealed that TBX20a is almost exclusively located within the nucleus. TBX20b is primarily localized in the nucleus with substantial cytoplasmic localization. While MKLN1 has a small nuclear protein fraction, the conclusion that MKLN1 only interacts with TBX20b could be due to lack of opportunity to interact with TBX20a. TBX20a and TBX20b are assumed to have similar, perhaps identical, transcriptional targets. If MKLN1 can alter transcriptional activities in an isoform-specific manner, then the target genes between the TBX20 isoforms could differ significantly. Reporter assays and quantitative real time PCR (qRT-PCR)

analyses were attempted to determine whether overexpression of MKLN1 altered TBX20b transcriptional activity; however, predicted gene targets were unresponsive to TBX20b induction. Future studies should include microarray analysis to identify genes that are specific targets of TBX20a or TBX20b. Broadening the functional capabilities of the TBX transcription factors to include cytoplasmic roles and interactions with signaling factors will be worth further investigation. More studies are needed to define the role of TBX isoforms throughout heart development and the role of cytoplasmic interacting proteins in altering TBX functions.

Cardiomyocyte Expression

To provide a more developmentally relevant cell study, endogenous expression of TBX20 and MKLN1 was determined in primary mouse embryonic cardiomyocytes (Appendix Figure 2). Examination of E12.5 cardiomyocytes revealed that TBX20 is localized throughout the cell with slightly increased amounts in the nucleus and cell periphery (Appendix Figure 2a). In contrast, the subcellular localization of MKLN1 is different than expected from previous studies with more nuclear protein than cytoplasmic protein visible (Appendix Figure 2b). Nuclear MKLN1 has been identified in neuronal cell types, specifically in the hippocampus of the developing central nervous system [74]. This experiment concluded that both proteins are expressed within primary cardiomyocytes and suggests that the subcellular localization of MKLN1 is cell type dependent.

To examine the TBX20-MKLN1 interaction within cardiomyocytes, NkL-TAg cells, a myocardium cardiomyocyte cell line with embryonic and adult characteristics [75], were transfected with HA-MKLN1 and immunostained for endogenous TBX20 and HA expression (Appendix Figure 3). The localization of endogenous TBX20 is similar to

the primary expression with protein localized throughout the cell. HA-MKLN1 is localized primarily in the cytoplasm with less protein in the nucleus than in the primary cells. These studies place the TBX20-MKLN1 interaction primarily within the cytoplasm with potential nuclear interaction. Resolving the impact of subcellular localization upon the TBX20b-MKLN1 interaction will be important to determine any functional significance within cardiogenesis.

Model of Chamber Differentiation

A unique feature of TBX20 is its expression in both chamber and non-chamber myocardium (Introduction Figure 4). As a driver of chamber myocardium differentiation, the mechanism of removal of TBX20 control in non-chamber myocardium regions has yet to be defined. Potential control mechanisms could be the presence of a TBX20 inhibitory factor or a spatiotemporal-specific increase of a TBX2 inducer. The TBX20 inhibitory factor must be expressed specifically in the non-chamber myocardial regions. Understanding how TBX20 inhibits TBX2 is important in order to determine how the inhibition is removed in non-chamber myocardium. As discussed in the Introduction, both direct and indirect mechanisms of TBX2 inhibition by TBX20 have been established. As determined by chromatin immunoprecipitation analysis, TBX20 directly binds to *Tbx2* promoter elements and inhibits expression in luciferase reporter assays [48]. In contrast, TBX20 proteins that have been mutated to remove the DNA-binding capabilities still inhibit *Tbx2* expression through inhibition of Smad1/5 activation [49]. The mechanism of removal of TBX20 activity within non-chamber myocardium is hindered by the complexities of defining how TBX20 inhibits TBX2 originally. Until more studies are conducted,

both mechanisms of TBX20 inhibition of TBX2 are plausible and will need to be unraveled.

Potential mechanisms for the removal of TBX20 control on *Tbx2* expression include the presence of a TBX20 inhibitor, a TBX2 inducer, or TBX20 isoform-specific expression and function. MKLN1 is expressed within the non-chamber myocardium making it a candidate for a negative regulator of TBX20 function. MKLN1 may inhibit TBX20 activity by blocking DNA binding or Smad binding/activation. A potential induction mechanism for TBX2 is the BMP/Smad signaling pathway. While BMP2 induces the expression of TBX20 and TBX2 [76,77], there could be distinct induction of the TBX target due to BMP2 levels. For example, with larger amounts of BMP2, TBX2 could be induced beyond TBX20's inhibitory control. The levels of BMP2 or other similar signaling factors will need to be defined throughout tissue-specific and developmental stage-specific assays. The mechanism of TBX20 control of *Tbx2* expression could be TBX20-isoform specific. With the characterized repression domain present only in TBX20a, the inhibition of *Tbx2* expression is most likely mediated through TBX20a. Isoform-specific expression patterns of TBX20 have not been detailed through different structures within the heart. It is possible that the TBX20 expression seen in the non-chamber myocardium could be TBX20b, which may not inhibit *Tbx2* expression. This highlights the need for isoform-specific expression analysis as well as determining the upstream regulators of alternative splicing.

Extracellular Matrix Remodeling and Valvulogenesis

Valvulogenesis is a crucial process in heart development. Signals from the non-chamber myocardium induce the nearby endocardium to undergo epithelial-to-

mesenchymal transition (EMT). The endocardial cells detach, invade the ECM between the cell layers, and expand into cellularized mesenchymal cushions. These cushions will form the primitive valve and septal structures of the AVC, OFT, and IFT. EMT requires coordinated changes in cell adhesion, cell migration, and proliferation. With TBX20 and MKLN1 being coexpressed in the endocardial cushions in the AVC, a potential functional significance to the TBX20-MKLN1 interaction can be hypothesized to be related to EMT and valvulogenesis. MKLN1 was identified as an important regulator in cell attachment, and the functions of MKLN1 have been inferred through protein interaction studies. It is hypothesized that MKLN1 plays a role in the regulation of cytoskeletal organization and cell adhesion response due to its responsiveness to thrombospondin-1 (TSP-1) and its interaction with p39, an activator of CDK5 [78,79]. MKLN1 is also a part of the CTLH complex of proteins including the Ran-binding protein of the microtubule organizing center (RanBPM)/RanBMP9. The CTLH complex involves nucleocytoplasmic shuttling and is important for MKLN1-regulated cell spreading and morphology changes [80,81,82]. While the primary hypothesis for MKLN1 regulation of TBX20 is by control of transcriptional activities, perhaps there are unknown cytoplasmic functions of TBX20 that are connected more directly to the regulation of cytoskeletal and cell adhesion responses needed for EMT and valvulogenesis.

Significance and Future Directions

The cardiogenic transcription factor network is complex and dynamic with many players interacting through cooperation and competition. While different transcription factor families are critical to cardiogenesis, many members of the TBX family play major roles. TBX20 is of particular interest due to its broad expression with specific functions

that are spatiotemporally dependent on many developmental factors. This study identified a novel interaction between TBX20b and MKLN1. Future studies will need to confirm any potential regulatory role MKLN1 provides over TBX20 function. While this study did not observe any changes in subcellular localization of TBX20b due to overexpression of MKLN1, transcriptional regulation can still occur. Determining TBX20-isoform specific gene targets would provide vital information for the importance of isoform function in cardiogenesis.

This study reveals new information of potential functions for both MKLN1 and TBX20. MKLN1, a primarily cytoplasmic protein, has not been previously identified to interact with a transcription factor. While the TBX20b-MKLN1 interaction predominantly takes place in the cytoplasm, there is a possibility for a nuclear-dependent role of MKLN1. DNA-binding capabilities and changes in gene expression due to MKLN1 would provide further clues to potential function. TBX20 has a cytoplasmic fraction consisting largely of the TBX20b isoform. Recent studies have explored a DNA-independent function of TBX transcription factors through the BMP/Smad signaling pathway. Future experiments to determine potential cytoplasmic functions of TBX20 and other TBX proteins would provide a new layer of complexity to the cardiogenic transcription factor network. Recently, a MKLN1 knockout mouse model was created and used to specifically determine the regulation of $GABA_A$ receptor transport within neurons [83]. MKLN1 interacts with both actin filaments and microtubule protein trafficking systems within neurons. If MKLN1 has similar functions within cardiomyocytes, it would be important to determine if and why the TBX20b isoform is exclusively transported.

This study also reviewed the alternatively spliced isoforms present in the TBX family. Isoforms of TBX5 and TBX20 are of particular interest with regards to CHDs and adult cardiomyopathies. There needs to be a more complete investigation of the pathogenic mutations discovered in the TBX genes and how they affect the functions of the alternatively spliced isoforms. Most of the pathogenic mutations discovered in TBX20 reside in the T-box DNA-binding domain which is identical to both isoforms, and thus the pathogenic mutation would affect the functions of both isoforms. It is important to dissect the roles of individual isoforms in order to more comprehensively diagnose patients.

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APPENDIX A

ISOFORM SPECIFICITY OF TBX20-MKLN1 INTERACTION AND EXPRESSION IN CARDIOMYOCYTES

In the copurification analysis of the TBX20-MKLN1 interaction, MKLN1 interacted exclusively with the TBX20b isoform. While this experiment may not recapitulate the cellular context needed for interaction, the subcellular localizations of TBX20a and MKLN1 may provide more insight into the lack of interaction. COSM6 cells were transfected with myc-TBX20a and HA-MKLN1 and then immunostained for myc and HA expression which revealed that TBX20a and MKLN1 do not colocalize within the cell (Appendix Figure 1). TBX20a holds an exclusively nuclear localization while MKLN1 is primarily within the cytoplasm. In contrast, the colocalization between myc-TBX20b and HA-MKLN1 (Appendix Figure 1) remains in the cytoplasm even though there is still myc-TBX20b present in the nucleus. This evidence provides further support for the isoform-specificity of the TBX20b-MKLN1 interaction.

With the exception of the expression analysis by immunohistochemistry, these studies were primarily done in cell types irrelevant to heart development. To further explore the potential developmental significance of the TBX20-MKLN1 interaction, an endogenous expression analysis was conducted on cultured primary embryonic cardiomyocytes. Hearts from E12.5 mice were dissected, cultured, and processed for immunostaining. The TBX20 antibody revealed the localization of both isoforms and confirmed previous data (Appendix Figure 2a). TBX20 is localized throughout the cell with slightly more protein within the nucleus. There is also significant localization along the cell periphery. Staining for endogenous MKLN1 was more difficult and required the use of a tyramide signal amplification (TSA) kit. MKLN1 staining revealed different localization compared to the transfected cell studies (Appendix Figure 2b). In primary embryonic cardiomyocytes, MKLN1 is expressed throughout the cell with enhancement in the nu-

cleus. The +TSA amplified control without the MKLN1 primary antibody revealed no staining (Appendix Figure 2c). While these results cannot support any isoform-specificity, they do indicate the importance of the cell type and developmental age of the cells in understanding the significance of the interaction.

Due to poor MKLN1 antibody staining, an immortalized cell line was transfected with HA-MKLN1 to determine colocalization between TBX20 and MKLN1. The NkL-TAg cell line is an immortalized ventricular myocardium cell line with both embryonic and adult cellular characteristics [81]. Immunostaining for TBX20 and HA revealed that TBX20 maintains similar localization as primary cells and MKLN1 maintains a more cytoplasmic localization. There is more nuclear MKLN1 present as compared to the COSM6 transfected cell studies but less than in primary cardiomyocytes. This suggests that while MKLN1 localization is cell type dependent, the interaction between TBX20 and MKLN1 can occur throughout the cell.

Figure 1: TBX20a does not colocalize with MKLN1 in COSM6 cells.

COSM6 cells were plated on glass coverslips in a 24 well plate and transfected with 0.8 ug of DNA using Lipofectamine 2000 according to manufacturer's protocol (Invitrogen). pCMV-Tag3 construct to create a myc-tagged fusion proteins for (A) TBX20a and (B) TBX20b (Stratagene). MKLN1 was cloned into the pCMV-HA expression vector (Clontech). Cells were processed for immunocytochemistry. Antibodies included myc (Cell Signaling) and HA (HA1.1 16B12). Coverslips were mounted onto glass microscope slides with DAPI counterstaining mounting solution (Vectashield).

Figure 2: Endogenous expression of TBX20 and MKLN1 in primary embryonic cardiomyocytes.

Hearts were dissected from E12.5 ICR mice, digested, and cultured on glass coverslips. The cells were then processed for immunocytochemistry. Antibodies included (A) TBX20 (Abcam ab42468) and (B) MKLN1 (Abcam ab56135). A TSA kit was used for the MKLN1 staining (Perkin Elmer). Coverslips were then mounted onto glass microscope glass using DAPI mounting medium (Vectashield). (C) A negative control with TSA amplification without primary antibody is shown.

Figure 3: Colocalization of HA-MKLN1 and TBX20 in NkL-TAg cells.

NkL-TAg cells were placed on glass coverslips in a 24 well plate. MKLN1 was cloned into pCMV-HA expression vector (Clontech). Cells were transfected with HA-MKLN1 at 0.8 ug per well according to Lipofectamine 2000 protocols (Invitrogen). Immunostaining included antibodies for TBX20 (Abcam ab42468) and HA (HA1.1 16B12). Co-

verslips were mounted onto glass microscope slides with DAPI counterstaining mounting solution (Vectashield).

Figure 1

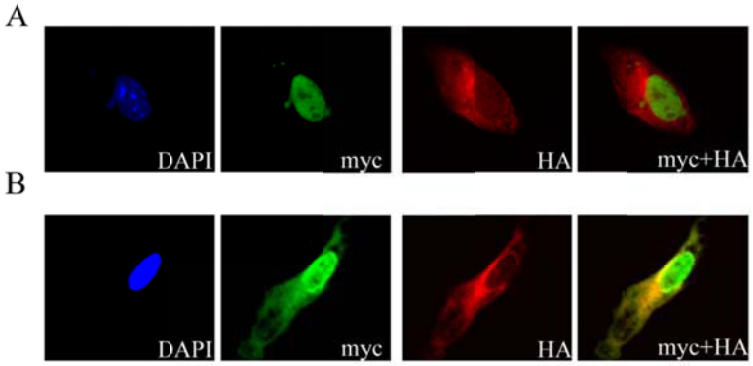


Figure 2

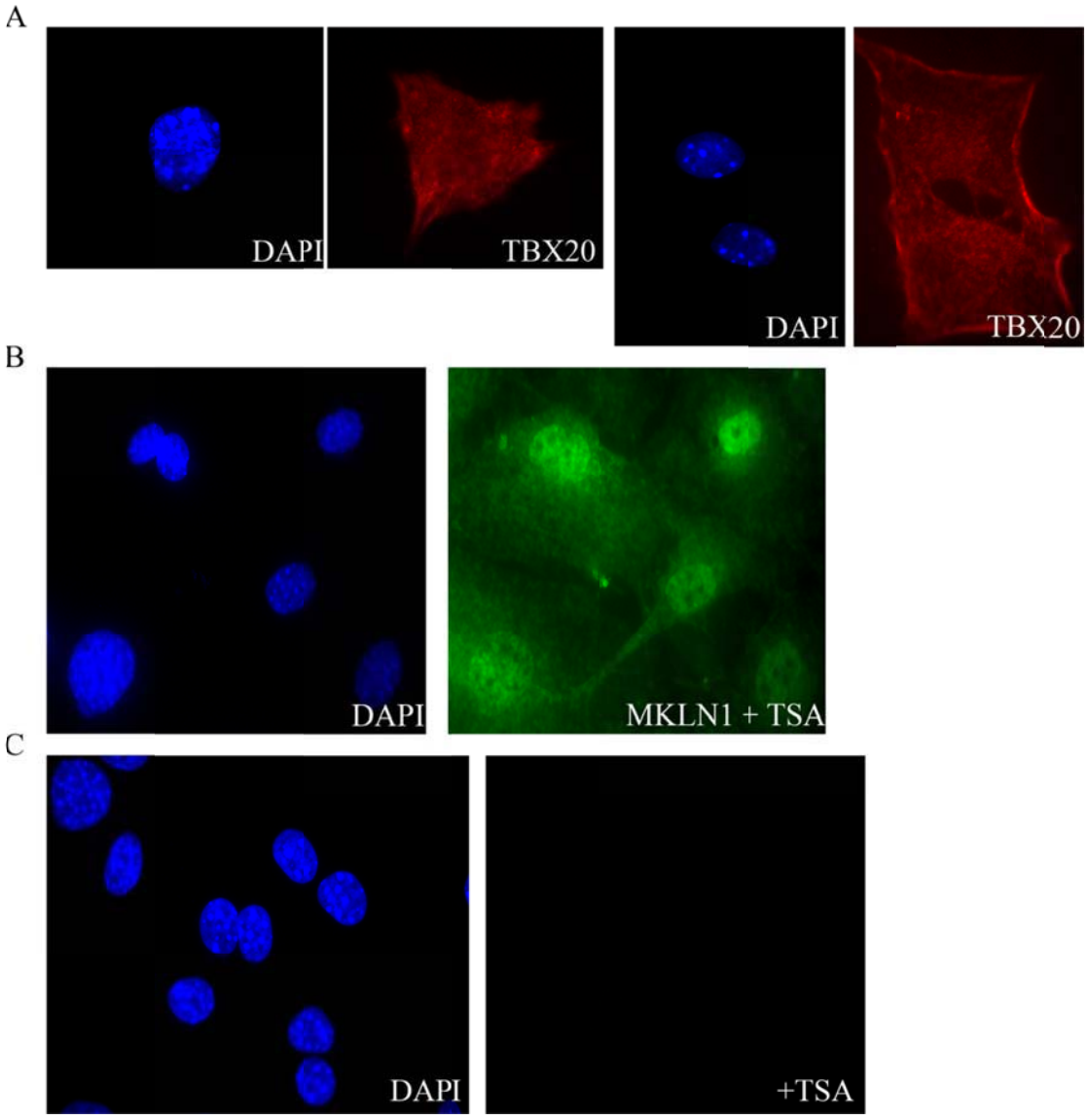
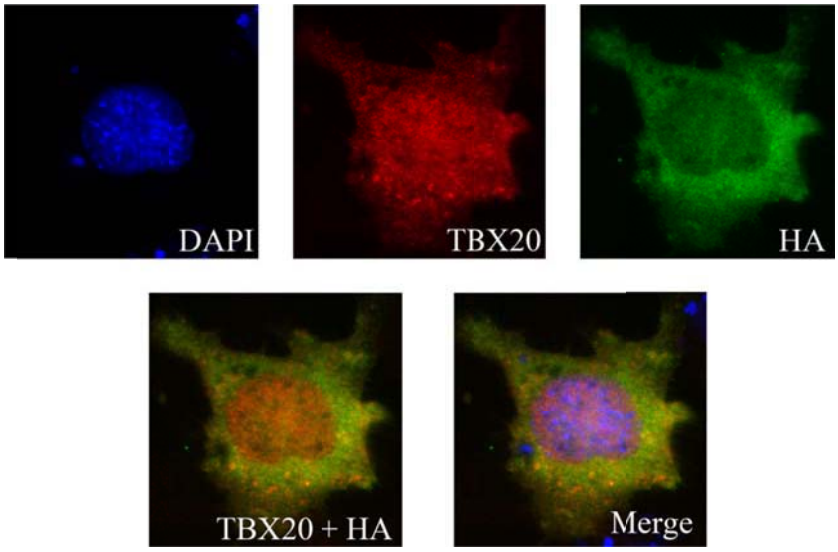


Figure 3



APPENDIX B

IACUC FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: May 7, 2007

TO: Michele P. Marron, Ph.D.
KAUL-740 0024
FAX: 975-5689

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Modeling T1D in Humanized NOD Mice
Sponsor: Juvenile Diabetes Foundation
Animal Project Number: 070507519

On May 7, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	20
Mice	A	1580

Animal use is scheduled for review one year from May 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070507519 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
1670 University Boulevard
205.934.7692
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35294-0019

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: September 20, 2010

TO: Jiao, Kai
KAUL-768 0024
996-4198

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: The Roles of Semaphorin Signaling During Mouse Valvuloseptal Development
Sponsor: NIH
Animal Project Number: 100908908

On September 20, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	230

Animal use is scheduled for review one year from September 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100908908 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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