NOTE TO USERS

CHARACTERIZATION OF A NOVEL DOWNSTREAM TARGET OF GATA-4 IN THE HEART

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I dedicate my thesis to all of the tinmen, scarecrows, and lions in my life who have given me the heart, the wisdom, and the courage I need to walk on my own little yellow brick road.

ABSTRACT

Although cardiac hypertrophy is a compensatory mechanism of the stressed heart, it ultimately leads to cardiac dysfunction. GATA-4, a member of the GATA family, plays an essential role in this process, nevertheless, its mechanism of action is still largely unknown. We have isolated a new evolutionarily conserved protein, ENDO1, that belongs to a new subfamily of PHD finger proteins. In adult mouse, ENDO1 was highly expressed in the kidney, lung, spleen, heart and brain. In ventricular cardiomyocytes, endogenous ENDO1 was expressed in the nucleus and cytoplasm. Overexpression analysis of antisense and sense GATA-4 suggests that GATA-4 downregulates endogenous ENDO1 in cardiomyocytes at the mRNA and protein levels. The protein levels of ENDO1 were enhanced in cardiomyocytes stimulated with phenylephrine, a hypertrophic stimulus. Structure-function analysis revealed that ENDO1 inhibits the activity levels of the BNP promoter. Our results indicate that the ENDO1 protein may play a significant role in cardiac development and differentiation.

RÉSUMÉ

L'hypertrophie cardiaque est un mécanisme compensatoire qui conduit à la dysfonction cardiaque. GATA-4, un membre de la famille GATA, joue un rôle essentiel dans ce processus. Par contre, son mécanisme d'action demeure grandement inconnu. Nous avons isolé une nouvelle protéine, ENDO1, qui appartient à la nouvelle sous-famille de protéines à doigts PHD et qui est conservée dans l'évolution. Chez la souris adulte, ENDO1 était fortement exprimée dans les reins, les poumons, la rate, le cœur et le cerveau. Dans les cardiomyocytes ventriculaires, ENDO1 était exprimée de façon endogène dans le noyau et le cytoplasme. L'analyse de la surexpression de l'anti-sens et du sens de GATA-4 dans les cardiomyocytes suggère que GATA-4 diminue l'expression endogène d'ENDO1 au niveau de l'ARN et de la protéine. Le niveau protéique d'ENDO1 était également augmenté les cardiomyocytes traités avec la phényléphrine, un stimulus hypertrophique. Les analyses structurelles et fonctionnelles ont montré qu'ENDO1 inhibe le niveau d'activité du promoteur du BNP. Nos résultats suggèrent qu'ENDO1 joue un rôle important dans le développement et la différenciation cardiaques.

Table of Contents

ABBREVIATIONS

aa:	amino acid
ACE:	angiotensin converting enzyme
ANF:	atrial natriuretic factor
Ang-1:	angiopoietin 1
Ang-2:	angiopoietin 2
ARA267-α:	androgen receptor-associated protein 267
ATP:	adenosine triphosphate
bFGF:	basic fibroblast growth factor
BHC:	BRAF-histone deacetylase complex
bHLH:	basic helix-loop-helix
BMP:	bone morphogenetic protein
BNP:	brain natriuretic peptide
bp:	base pair
CARP:	cardiac ankyrin-repeated protein
CBP:	CREB binding protein
CD:	cardiac disease
cDNA:	complementary deoxyribonucleic acid
CHD:	congenital heart disease
cTNC:	cardiac troponin C
Da:	Dalton
DCM:	dilated cardiomyopathy
DIGs:	detergent-insoluble, glycolipid-enriched complexes
DNMT3L:	DNA methyltransferase 3L
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	dimethyl sulphoxide
EC:	endothelial cell
ECE-1:	endothelin converting enzyme-1
ECM:	extracellular matrix
EE:	endocardial endothelium

ENDO1:	endothelial clone 1
EPO:	erythropoietin
EPO-R:	erythropoietin receptor
ER:	endoplasmic reticulum
ERG:	ETS-related gene
ES:	embryonic stem cells
EST:	expressed sequence tag
ET-1:	endothelin-1
ETS-1:	E26 transformation specific-1
FBS:	fetal bovine serum
FGF:	fibroblast growth factor
FLI-1:	friend leukemia integration-site 1
FOG:	friend of GATA
HA:	hemagglutinin
HDAC:	histone deacetylase
HIFα:	hypoxia-inducible transcription factor
IRX:	iroquois homeobox gene
KSHV:	Kaposi's sarcoma associated-herpes virus
LB:	Luria Broth
LMO:	LIM-only bridging protein
LV:	left ventricle
c-MIR:	cellular MIR
MAP:	mitogen activated protein
MEF2:	myocyte enhancer factor 2
MEKK1:	mitogen activated protein kinase kinase kinase 1
MHC:	myosin heavy chain
MIR:	modulator of immune recognition
MMD1:	male meiocyte death1
MMP-1:	matrix metalloproteinase-1
mRNA:	messenger ribonucleic acid
MyoCapE:	myocardial capillaries

NERF-2:	new ETS-related factor 2
NF-κB1:	nuclear factor kappa B1
NLS:	nuclear localization sequence
NO:	nitric oxide
ORF:	open reading frame
PC:	proprotein convertase
PCR:	polymerase chain reaction
PDGFβ:	platelet-derived growth factor
PDGFRβ:	platelet-derived growth factor receptor
PECAM:	platelet-endothelial cell adhesion molecule
Pf1:	PHD factor 1
PHD:	plant homeodomain
PHF6:	PHD-finger gene 6
RA:	retinoic acid
RT:	reverse transcriptase
RV:	right ventricle
S1P or S2P:	site-1 protease or site-2 protease
SCAP:	SREBP-cleavage-activating protein
SREBP:	sterol regulatory element binding protein
SRF:	serum response factor
SUMO:	small ubiquitin-like modifier
SV40:	simian virus 40
Th:	T-helper cells
TGFβ:	transforming growth factor β
TM:	transmembrane
Ub:	ubiquitin
UCH:	ubiquitin carboxyl-terminal hydrolase
UTR:	untranslated region
VEGF:	vascular endothelial growth factor
Wg:	wingless
YY1:	yin yang1

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1. INTRODUCTION

1.1. Rationale for studying the heart

Cardiac Disease (CD) is one of the leading causes of death in North America, and in particular, congenital heart disease comprises approximately 30% of deaths associated with CD, (some of which are indicated in Figure 1). Little is known regarding the molecular mechanisms that are impaired due to genetic mutations or deletions. Further understanding regarding the fetal genetic program is necessary to comprehend how the heart develops and functions during embryonic development, and why there is reactivation of these mechanisms during pathologic conditions in the adult. Overall, this will help open the avenue towards developing additional preventative therapies against cardiac disease and for new pharmacologic targets.

1.2. Overview of the early stages in cardiac development

Heart development is largely a complex series of steps that requires the co-operation of various cell types. The early stages of heart development give some initial insight into the kinds of intercellular communications that are required for proper cardiac functioning. The proliferation and differentiation of cardiomyocytes are alone not sufficient to drive cardiogenesis, and as will be revealed in this section, studying other systems in parallel may be required to achieve a better understanding of not only development, but of pathologies as well.

1.2.1. Formation of the heart field

The heart is the first functional organ to form in the embryo and it arises through a complex network of interactions between different cell populations, which become apparent at the time of gastrulation. At gastrulation the embryo is comprised of two tissue layers: the epiblast, responsible for giving rise to all fetal tissues, and the visceral endoderm, which comprises the yolk sac endoderm (1). Mesoderm, which contains the precursors of vascular endothelium and the cardiac progenitor cells, is formed by an epithelial-mesenchymal transformation and ingression of epiblast cells at the primitive streak at 6.5 days after fertilization (E6.5) (1). Certain of these cells migrate anterolaterally to form the anterior lateral plate mesoderm, a pair of bilaterally symmetrical regions of the embryonic mesoderm termed the heart field. Each

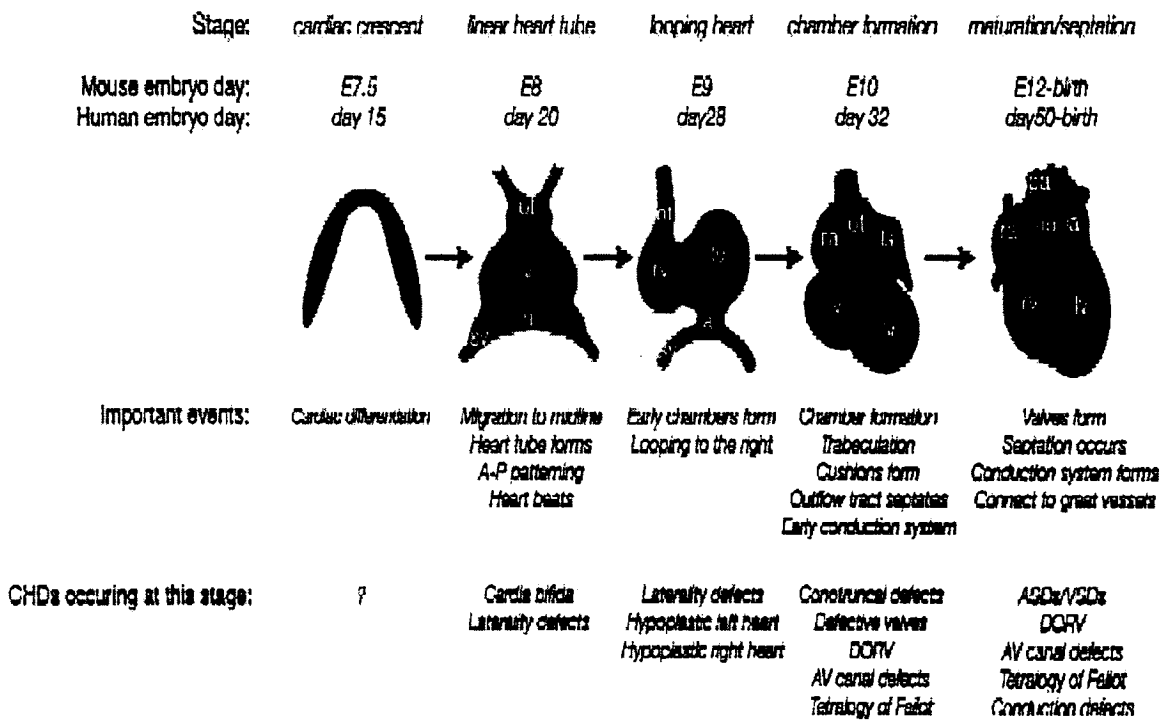

Figure 1. Representation of the main phases of early cardiac morphogenesis and the congenital defects associated with each phase. Ot, outflow tract; v, ventricle; a, atria; sv, sinus venosa; rv, right ventricle; lv, left ventricle; ra, right atrium; la, left atrium; pa, pulmonary artery; ao, aorta. (Figure adapted from (20))

independent bilateral region is termed the cardiac primordium (2). Following its formation, the lateral plate mesoderm splits into the somatic mesodermal layer, which contains the skeletal muscle progenitor cells, and the splanchnic mesodermal layer, which contains the precursors for the myocardium, endocardium, and pericardium. These layers are necessary for establishing the three layers of the heart tube. The next stage is commitment of these precursor cells to a cardiac fate. This is established via inducing signals, which are released from the anterior lateral endoderm, a region that is in contact with the cardiac precursors and migrates along with them during the early stages of development. This vital interaction between the endoderm and mesoderm leads to the differentiation of precursor cells to cardiomyocytes in the anterior splanchnic mesoderm to then form the cardiac crescent (E7.5). Candidate inducing factors in the endoderm responsible for initiating the cardiomyogenic program include the bone morphogenetic proteins (BMPs) and the fibroblast growth factors (FGFs) (3,4).

1.2.2. Generation of the cardiac tube and looping to form the mature heart

Formation of the cardiac crescents is followed by their fusion at the ventral midline of the embryo to form a primitive linear heart tube (E8.25). Remarkably, this heart tube is capable of beating as a peristaltic pump despite the absence of valves and a conduction system (5). The straight heart tube consists of an inner endocardium and an outer myocardium, which are separated by an extracellular matrix called the cardiac jelly. This primitive heart has an anteroposterior polarity, where the heart tube is already divided into segments destined to become the outflow tract, aortic sac, conotruncus, right ventricle, left ventricle, atria, sinus venosus and inflow tract in an anterior to posterior direction respectively (6). Proper localization of the four-chambers in the mature heart is the result of proper rightward looping of the heart tube (E10.5), indicating the presence of left-right asymmetry in the embryo. Comparison of this stage of heart development with other vertebrate species reveals that looping of the heart to the right is an evolutionarily conserved mechanism, and disruption of this process compromises embryonic survival, in turn suggesting the presence of evolutionarily conserved transcription factors that are responsible for regulating the looping stage of the heart. As indicated by Table 1, numerous other stages of heart development are common to all vertebrate species (7), and although we have gained a lot of knowledge regarding cardiogenesis in vertebrates, it

Table 1. Commonality in cardiac morphogenesis between various species

Stages in Cardiac Development	Mouse(257, 258)	Chick(259-261)	Human (262)	Frog(263, 264)	Zebrafish (265)
Migration of precardiac cells from epiblast	7dpc (primitive streak)	HH4 (definitive streak)	15-16 days	stage10	50% epiboly (5.5 hpf)
First evident assembly of myocardial plate	7dpc (late primitive streak; just presomite)	HH5 head process (19-22hrs)	18 days	~ stage 13	8-10 somites (~13hpf)
Generation of single heart tube initiated	8dpc (5-10 somites)	HH9 (7 somites)	22 days (4-10 somites)	stage 28	20 somites (~19 hpf)
Tubular heart starts contraction	8.5 dpc (8-10 somites)	HH10 (10 somites)	23 days	~ stage 33	26 somites (22 hpf)
Looping	8.5 dpc	HH11 (11-13 somites)	23 days	stages 33-36	33 hpf
Cushions form	9.5 dpc	HH17	28 days (30-38 somites)	~ stage 41	48 hpf

Abbreviations: dpc, days post-coitum; HH, Hamburger Hamilton; hpf, hours post fertilization. (Table adapted from(266))

must be emphasized that studying cardiac development alone is not sufficient enough to explain the phenotypes associated with cardiac congenital defects. For example, although trisomy 21 (or commonly referred to as Down's Syndrome) displays atrioventricular canal defects, it also displays deficiencies in cardiac cushion formation (8) which, in turn, are processes that require proper endothelial cell maturation (described later). Interestingly, during the maturation stages of the heart (beginning at around E10), several congenital heart defects are associated with disruption of development of the endothelium, as indicated in Figure 1. How the endothelium functions to ensure proper heart functioning during the remodeling and maturation stages of heart development will be discussed in later sections. However, it will become evident that the interaction between endothelial cells and cardiomyocytes is indispensable for survival, and thus in addition to cardiac development, an understanding of endothelial development is also very important.

1.3. Establishment of the endothelium

Similar to the heart, the development of the endothelium is a step-wise process that is comprised of commitment of stem cells to endothelial cells, vasculogenesis and angiogenesis. The major factors that play a role during these processes have also been shown to be crucial for proper heart development, and thus rationalizes the overall importance for studying the establishment of the endothelium.

1.3.1. The hemangioblast

The beginning of an avascular embryo to become vascular starts from the commitment of stem cells to become hemangioblasts, the precursors to endothelial and blood cells. Starting at E7.5, the earliest hematopoietic cells are detected in the blood islands (aggregations of mesenchymal cells) of the yolk sac (9), which will eventually give rise to the hematopoietic system. Surrounding these cells are a layer of angioblasts (endothelial precursors) which will give rise to differentiated endothelial cells. Due to the simultaneous development of these two lineages, and common expression of genes between blood and endothelial precursors such as VEGFR-2, CD34, Tie-2, GATA-2, LMO-2, and Scl/Tal1 (10), a hypothesis emerged suggesting that perhaps these two systems originate from a common precursor called the hemangioblast.

1.3.2. Vasculogenesis

As vasculature development begins at approximately E7.5, hemangioblasts will migrate to the paraxial mesoderm and assemble into cellular aggregates, or blood islands. The inner cell population differentiates into hematopoietic precursors and the outer cell population gives rise to angioblasts (11). It is around this time during embryonic development that vasculogenesis commences, which involves angioblast differentiation and proliferation, to form a plexus of endocardial tubes. This leads to the formation of the dorsal aorta, cardinal veins and the embryonic stems of yolk sac arteries and veins (12). Furthermore, in contrast to what was originally believed, angioblasts not only migrate intraembryonically, but also postnatally and are recruited from the circulation for *in situ* vessel growth. Thus, they may contribute to neovascularisation and to the development of pathologies (12).

1.3.3. Angiogenesis

Further maturation of this primitive vascular network during embryonic development is established through angiogenesis. Angiogenesis is a step-by-step process involving sprouting (enlargement of venules which then generates sprouts), division of intervascular pillars of periendothelial cells called intussusception, and formation of transendothelial cell bridges, which then separate into individual capillaries (13). Periendothelial cells are crucial in protecting the primitive vasculature from severe rupture and regression in two ways; first, by focusing on the production of extracellular matrix and formation of a basement membrane, and second, by inhibiting the proliferation and migration of endothelial cells (13), thus forming parent vessels.

The exact mechanisms by which angiogenesis executes further remodeling still remains to be determined, however, the sequence of events underlying angiogenesis is shown in Figure 2. First, endothelial cells are activated within pre-existing vessels and nitric-oxide mediated vasodilation of parent vessels takes place. The extracellular matrix and basement membranes then begin to breakdown, via for example, MMP-1 (matrix metalloproteinase-1). This leads to the migration of endothelial cells from the parent vessel to areas requiring angiogenesis, which in turn is directed by chemotactic factors released from fibroblasts, mast cells, monocytes, platelets, and neutrophils. Endothelial cells proliferate until there is lumen formation in the new vessels, and then redifferentiate to a quiescent phenotype. Recruitment of pericytes along the newly formed vascular

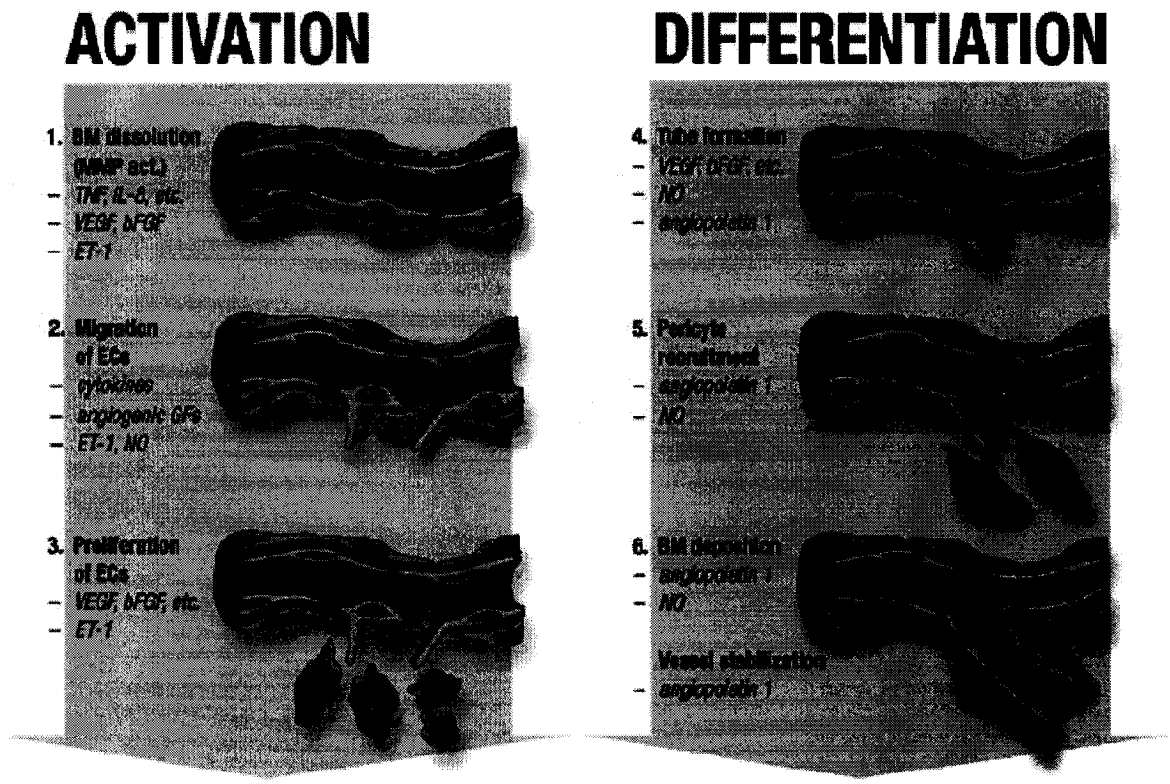

Figure 2. A representation of the phases involved in angiogenesis with names of some of the key factors involved in each phase. (Figure adapted from (256))

structures follows, and then there is formation of a new basement membrane by the newly organized endothelial cells and pericytes. Lastly, there is remodeling of the neovascular network, with maturation and stabilization of the blood vessels. This complex process is a major therapeutic target in the adult, since it can either be involved in the progression of a pathologic state (eg. tumors) or it can allow for physiologic adaptation to unfavorable conditions (eg. ischemia), thus emphasizing the importance of fully understanding the molecular mechanisms underlying blood vessel development (14,15).

1.3.4. Molecular regulation of blood vessel development

The tight regulation of blood vessel development is quite complex, particularly at the level of progenitor cell differentiation where there has been much debate. As will be discussed in later sections, the factors involved in blood vessel development have also been shown to play vital roles in signaling between myocardial and endocardial cells. Therefore, further knowledge regarding the roles that these factors play in vasculogenesis and angiogenesis will permit us to better understand how disruption of proper endothelial cell maturation can lead to cardiac defects.

Specific transcripts are expressed at distinct stages during blood vessel development, as depicted by Table 2 and Figure 3. For example, angioblasts, or endothelial precursors, are defined as being $Tal1^{+}/Flk1^{+}$ cells, where Tal1 (also called SCL or stem cell leukemia gene) is a bHLH (basic helix-loop-helix) protein, and Flk1 is a VEGF (vascular endothelial growth factor) receptor (VEGFR-2). Because Tal1 is more highly expressed in cells associated with vasculogenesis than angiogenesis (16), it suggests that endothelial cells of more mature blood vessels are $Tal^{-}/Flk1^{+}$, even though some studies suggest otherwise (10,16). The other VEGF receptor, Flt1 (VEGFR-1), plays a role later during blood vessel development. Expression of both receptors and sufficient levels of VEGF are essential for angioblast differentiation and survival (9,12,17), and moreover, disruption of VEGF leads to cardiac defects (18). In addition, VEGF is also an active player during sprouting, and acts in conjunction with tyrosine kinase receptors (Tie-1 and Tie-2) and its angiopoietin ligands (Ang-1 and Ang-2) to regulate vascular growth and maturation (19-21). Ang-1 has been demonstrated to activate Tie-2, whereas Ang-2, inhibits Tie-2 and is a natural antagonist for Ang-1. This

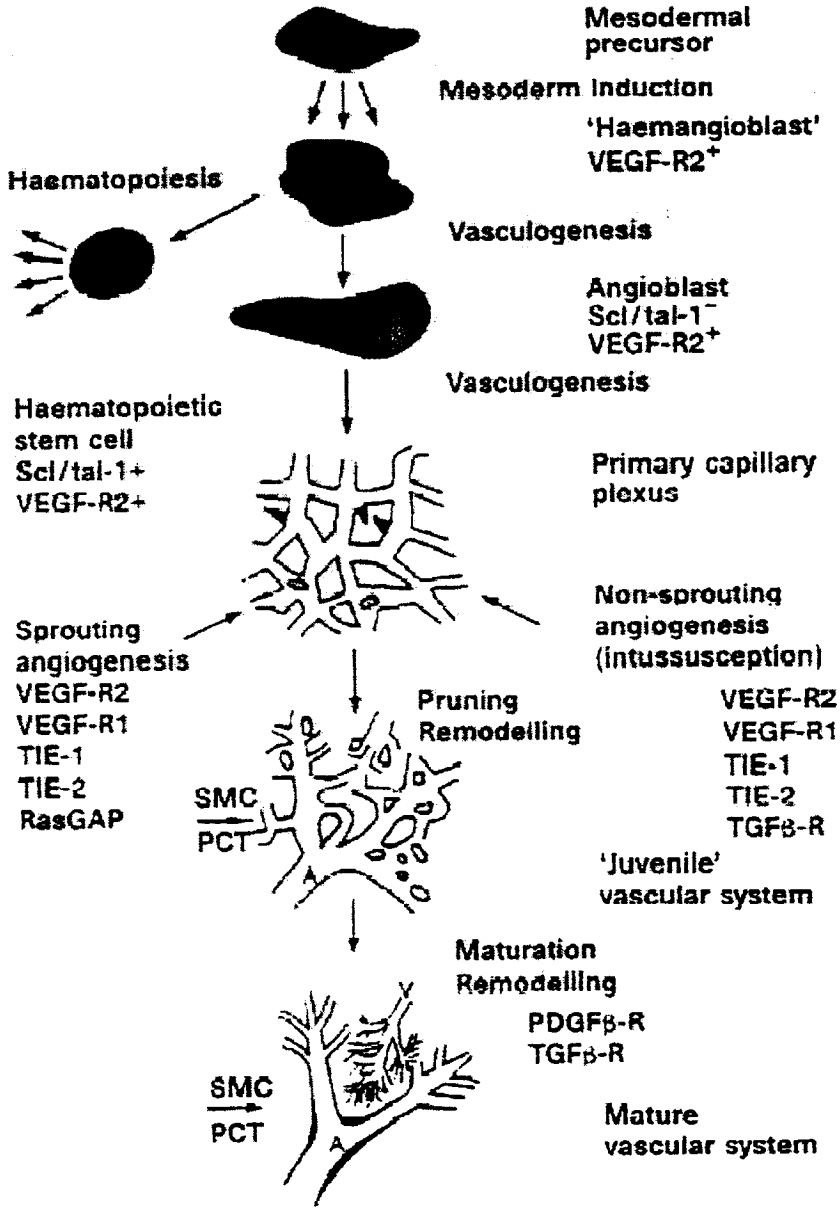

Figure 3. A graphic representation of the processes involved in blood vessel development along with a list of the key vasculogenic signalling molecules involved in each step. Abbreviations: SMC, smooth muscle cells; PCT, pericytes. (Figure adapted from (255))

Table 2. Phenotypes associated with signalling molecules that are expressed during specific stages of blood vessel development

Gene knockout	Time of Death	Stage of vessel development	Mutant phenotype
VEGF-A (+/-)(269)	E11.5	Vasculogenesis/ (angiogenesis)	Reduced red blood cells; defective heart and aorta formation; defective sprouting
VEGF-A (-/-)[a] (269)	E10.5	Vasculogenesis	Absent dorsal aorta; defective endothelial cell development
VEGFR-1(34)	E8.5	Vasculogenesis	Failure of endothelial cell formation
VEGFR-2(33,270)	E9.5	Vasculogenesis	Excess endothelial cells form abnormal vessel structures entering vessel lumens
Ang1(36,271)	E10.5	Angiogenesis	Defective vessel remodelling, organization, and sprouting; heart trabeculation defects
Ang2(272)	E12.5 – P1	Maturation of vasculature	Poor vessel integrity, edema, and hemorrhage
Tie1(19,37)	E13.5 – P2	Maturation of vasculature	Poor vessel integrity, edema, and hemorrhage
Tie2(37,273)	E10.5	Angiogenesis	Defective vessel remodelling, organization, and sprouting; heart trabeculation defects
EphB2/EphB3(274)	E10.5 (≈ 30%)	(Vasculogenesis)/ angiogenesis	Some defective vessel primordial; defective vessel remodelling organization, and sprouting; heart trabeculation defects
EphB4 (275)	E10.5	(Vasculogenesis)/ angiogenesis	Defective vessel remodelling organization, and sprouting; heart trabeculation defects
TGF-β_1 (276)	E10.5	Vasculogenesis	Defects in yolk sac hematopoiesis and vasculogenesis
TGF-β-RI (277)	E10.5	Vasculogenesis/ angiogenesis	Impaired circulation; lack of vessel sprouting
TGF-β-RII (278)	E10.5	Vasculogenesis	Impaired hematopoiesis; vascular abnormalities
PDGFβ (279)	E17 – P1	Vasculogenesis/ (angiogenesis)	Hemorrhage and edema; microvessel leakage
PDGFβ-R (280)	E18.5 – P1	Vasculogenesis/ (angiogenesis)	Hemorrhage and edema; microvessel leakage

[a] Here VEGF null ES cells were used. (Table adapted from (272))

suggests specific Tie-angiopoietin signalling pathways for specific events during vasculogenesis; for example, Ang-1 is required more for sprouting and Ang-2 is required more for extracellular matrix (ECM) destabilization (12). Furthermore, growth factors such as transforming growth factor (TGF-β) and platelet-derived growth factor (PDGF-β) and their receptors are also important for the proper development of blood vessels. For example, increased shear stress in blood vessels leads to greater secretion of PDGF from endothelial cells. This leads to activation of the PDGF receptor on neighbouring perivascular cells and permits further maturation of blood vessels (22,23). Moreover, this activates TGF-β, which can help inhibit endothelial cell proliferation and stabilize their phenotype (24). Figure 3 summarizes the role of these factors during various stages of vasculogenesis and angiogenesis. Given the extensive network of blood vessels, it is plausible that to ensure proper hemodynamic demands to the various organs of the body, communication between various cell types is essential. Therefore in order for the heart to function properly, proper endothelial-myocardial signaling is required.

1.4. Endothelial-myocardial signaling

Although the establishment of the heart and endothelium are described independently from one another above, these are not just parallel events in embryonic or postnatal development. Endothelial-myocardial signaling is vital for overall cardiac homeostasis. The organization of endothelial cells, and their interaction with cardiomyocytes during various stages of cardiac development, indicate how these intercellular interactions are indispensable for survival.

1.4.1. The contribution of the endothelium to overall cardiovascular homeostasis

The endothelium plays a vital role in regulating cardiovascular homeostasis by regulating vascular permeability, controlling blood vessel caliber according to the hemodynamic and hormonal demands, and maintaining blood fluidity. Several factors such as vasoconstricting and vasodilating factors, pro- and anticoagulant factors, pro-angiogenic factors and factors involved in inflammatory responses are credited for the proper execution of endothelial cell functions. This dynamic interaction between endothelial cells and the surrounding environment is vital to the proper functioning of the overall cardiovascular system, and this characteristic is common to all endothelial cells. However, the heart is composed of only 40% of cardiomyocytes while the rest consists of

fibroblasts, macrophages, circulating blood cells, vascular smooth muscle cells, and endothelial cells. Therefore, it is not surprising that various intercellular interactions would play an essential role in maintaining cardiac homeostasis (25).

1.4.2. Endothelial organization in the heart: cardiac endothelium versus coronary vascular endothelium

In order to understand how endothelial cells regulate cardiac function, understanding the significance of their location and their cellular morphology is very important. These characteristics can reveal a lot about why there is heterogeneity of endothelial cells in the heart, since differences in location and cellular morphology can explain why a subset of endothelial cells may function differently than another.

Endothelial cells can affect cardiac function from two locations: the cardiac endothelium and the coronary vascular endothelium. The latter contributes to cardiac function indirectly by controlling coronary blood supply to the myocardium (25). In contrast, the cardiac endothelium can directly influence cardiac function due to the proximity of cardiac endothelial cells in the myocardial capillaries (MyoCapE) and in the endocardial endothelium (EE). These cells are located adjacent to cardiomyocytes, and thus allows for direct intercellular communication and signalling as depicted in Figure 4. For example, due to the proximity of endocardial cells to cardiomyocytes, NO (nitric oxide), which has a short half life of 20 sec, can diffuse over a broad enough range to reach neighbouring cardiomyocytes and send signals. In addition, species differences in the intercellular distance and cell number ratio are also accountable for differences in endothelial signalling to cardiomyocytes (26), and may even be a source of discrepancy between data obtained from different species.

1.4.3. Interaction between endocardial cells and cardiomyocytes in the heart

In order to understand how various subtypes of endothelial cells are able to affect cardiomyocyte function, it is important to comprehend their involvement in vital processes such as cardiac growth, contractile performance, and rhythmicity, in order to permit further distinction between EE and MyoCapE cells.

1.4.3.1. Cardiac growth

Cardiac growth takes place during embryonic and postnatal stages of development. During embryonic growth, endothelial-myocardial signalling is essential

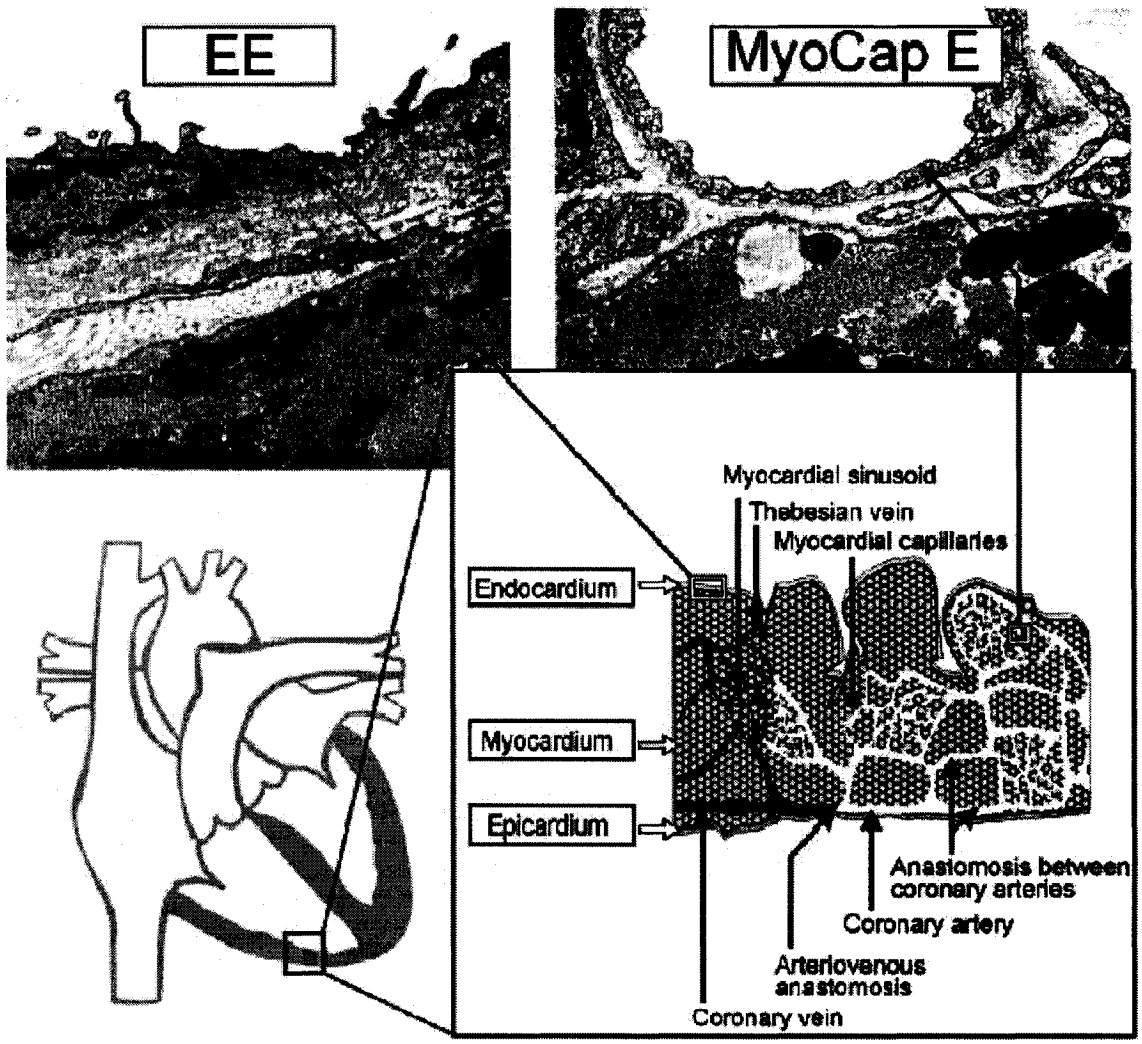

Figure 4. Location of the EE and MyoCapE cells in the developing heart (Figure adapted from (48))

for proper differentiation of myocardial cells and subsequent development of cardiac structures. Postnatally, cardiomyocytes are terminally differentiated, but retain the ability to increase in size. This hypertrophy may be a cause for several pathologic conditions and therefore understanding the molecular pathways underlying these processes will be therapeutically useful.

1.4.3.1.1. Embryonic cardiac development

The later stages of embryonic heart maturation are largely dependent on endothelial-myocardial signalling. Further *in vivo* analysis of the factors which are implicated in cardiac development demonstrate the indispensability of endothelial cells for heart maturation, and subsequent survival.

1.4.3.1.1.1. Overview of embryonic heart maturation

There are three vital consecutive steps for the maturation of the heart during embryonic development: myocardial trabeculation, endocardial cushion development, and myocardial compaction. Initially, endocardial endothelial cells migrate into the cardiac jelly and toward the myocardial tube. Soon after the endothelial cells migrate towards the myocardial tube, the EE lines most of the cardiomyocytes and forms a monolayer, and subsequently, the primitive spongy heart. At this double-walled stage, the earliest rhythmic contractions originate, and some beating cardiomyocytes migrate towards the endocardium, creating protrusions, or *trabeculae*, resulting in increased surface area of endocardial endothelial cells in the sponge-like heart. Following trabeculation, the cardiac jelly undergoes regional swellings which arise from mesenchymal outgrowths, also known as *cardiac cushions* (27) as shown in Figure 5. These lead to the division of the cardiac tube into the atria, ventricles, and outflow tract. Migration of endothelial cells in the cardiac cushions then submit themselves to endothelial-mesenchymal transformation. Proper expansion of these cushions are essential for the subsequent development of septal and valvular structures which will establish the boundaries of the developing chambers. Following cushion formation, the outer avascular myocardial layers become *compact*, and angioblasts from the epicardium begin forming primitive coronary vessels, leading to vascularization of the maturing heart and establishment of a coronary circulation (28). Overall, as will be described below, these final stages of heart maturation are a source of interactions between endocardial

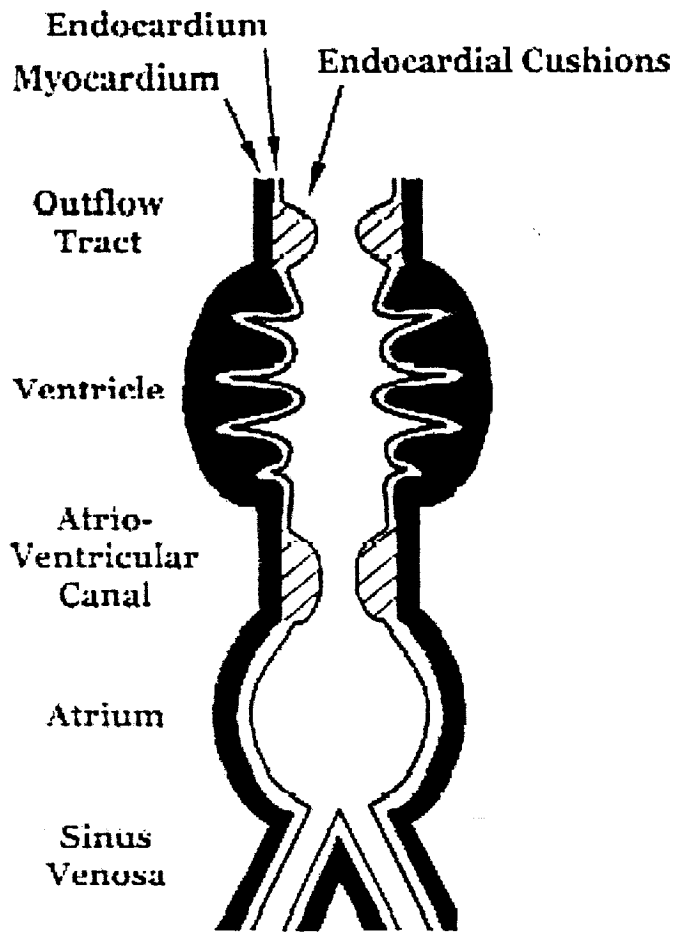

Figure 5. Graphic representation of the location of endocardial cushions in the linearized heart before looping. (Figure adapted from (254))

endothelial cells and cardiomyocytes and prove to be crucial for the development of the heart and survival of the embryo.

1.4.3.1.1.2. Molecular regulation of cardiac myocytes and endothelial cell interaction

The factors that were demonstrated to play essential roles in endothelial development have also been shown to be essential for cardiac development, particularly in the maturation stages of the heart. Insight into the molecular signalling mechanisms between endothelial cells and cardiomyocytes reveal the significant level of dependence that these two cell types have on one another.

1.4.3.1.1.2.1 Myocardial trabeculation

As previously mentioned, *myocardial trabeculation* constitutes one of the first steps of heart maturation, and is largely responsible for the maintenance of proper blood flow during the early stages of cardiac morphogenesis. Several knockout studies have demonstrated the importance of the endocardial endothelial cells for myocardial trabeculation. The *cloche* gene is, at present, the earliest gene known to be expressed in endocardial endothelial cells (29,30). Mutant zebrafish lacking the *cloche* gene were missing the inner endocardial endothelial tube and the developing myocardial layer remained somewhat smaller and dysmorphic and failed to develop the trabeculation within the ventricle (31). VEGF and its receptors, which were described previously as playing vital roles during blood vessel development, are also essential for the initiation and establishment of mature endocardial endothelial-myocardial interactions. Flk-1 belongs to the family of tyrosine kinase receptors, and although expressed later than the *cloche* gene, it is expressed in all endothelial cells (32). Binding with VEGF is necessary for initial proliferation and differentiation of endocardial endothelial cells. Mice which have the Flk-1 receptor mutated, like *cloche*, also were void of an endocardial endothelium, with failure of myocardial maturation leading to embryonic lethality (33). On the otherhand, in Flt-1 receptor knockout mice, endocardial endothelial cells did differentiate, but were disorganized leading to embryonic lethality (34). Similarly, mice lacking other factors essential for endothelial cell differentiation, such as Tie2 (35-37) and angiopoietin –1 (38), also display abnormal differentiation of the endothelial endocardial layer and myocardial trabeculation. These findings emphasize the important roles that these endocardial-myocardial interactions have for normal cardiogenesis. Taken

together, these results suggest that although cardiomyocytes do develop in the absence of an endocardial tube, endocardial endothelial cells remain a necessity for proper myocardial maturation and embryonic survival.

1.4.3.1.1.2.2. Endocardial cushion development

Endocardial cushion development is required for the development of the valves and septa in the heart and serves as a very good example of the importance of endocardial-myocardial signalling. For example, in order to induce endocardial endothelial-to-mesenchymal transformation, the myocardium secretes various molecules such as EDTA-soluble (ES) messenger protein complexes, eg., ES/130 protein (27,39). This induction, which is elicited initially by the myocardium, is amplified due to the endogenous presence of these complexes in the EE and in the endocardial cushion itself (39). At present, three members of these myocardium-derived induction protein complexes have been identified: fibronectin, KLAMP-1, and transferrin. For the former two members, the presence of retinoic acid is essential, which is an important fact considering that many congenital valvuloseptal defects have been due to impaired mesenchymal cell formation in the endocardial cushion, which in turn have been associated with abnormal levels of retinoic acid (the major active metabolite of Vitamin A) or Vitamin A (40-42).

1.4.3.1.1.2.3. Myocardial compaction

Later on in cardiac development, there is *myocardial compaction* of the outer myocardial layers. Initially, the compact zone is avascular and about as thick as two myocardial cells. When the thickness reaches about 3-4 cells through cardiomyocyte proliferation, angioblasts start forming vascular tubes from the epicardium (43). Briefly, the compact zone comprises the outermost layers of the ventricular cavities and a major portion of the septum. In birds, the septum arises simply by the coalescence of trabecular sheets, whereas in mammals, an additional step of compaction is required (44). The ventricular septum is thus formed by two distinct processes: 1) through cushion formation, and 2) through myocardial compaction which will form the major muscular portion of the septum (45).

Many genes are required for initiating the thickening of the outer compact ventricular myocardium and are expressed in the cardiomyocytes, but it is still unclear

what role the EE and MyoCapE play during this developmental process. Endothelin (ET-1) is a peptide protein that is highly expressed in the EE and has been shown to play critical roles in the development of the ventricular myocardium and septum. Around the time of cushion formation, ET-1 mRNA is expressed in the entire endocardial endothelium (46,47). In addition, ET-1 mRNA has also been shown to be expressed in rat cardiomyocytes, but the expression pattern still remains higher near the endocardium (48). Similarly, the mRNAs for ET-converting enzyme (ECE-1) and for the ET_A receptor are both present in the endocardial endothelium and subjacent myocardium. At the onset of compaction, mRNA of both ECE-1 and ET-1 are expressed in the endocardial cushion tissue and in the endocardial endothelium of the ventricular cavities (49), and they have been shown to contribute to the compaction process of the ventricular wall and the large portion of the muscular septum. Even though, ET-1 deficient mice displayed no abnormality in endothelial-to-mesenchymal transformation, at a later stage of development, ventricular septal defects were present (48,50,51), overall indicating how disruption of a particular endothelial-myocardial signaling pathway can alter heart maturation.

1.4.3.1.2. Postnatal cardiac development

It used to be a widely accepted dogma that in the postnatal and adult heart, cardiomyocytes are terminally differentiated, however, during the last decade or so, this has become a topic of hot debate (52-54). However, although it still remains to be determined whether postnatal cardiomyocytes still retain the ability to divide, it is known that cardiomyocytes do respond by hypertrophic growth to various stimuli such as mechanical and oxidative stress, as well as metabolic (hypoxia), neurohormonal, and growth factors. The hypertrophic growth may well involve endothelial-myocardial interactions and understanding the pathways implicated in this process are necessary since they may be implicated during adaptive hypertrophy and pathologic conditions.

1.4.3.1.2.1. Promyocardial growth

Experiments testing the indispensability of cardiac endothelial cells to cardiomyocyte differentiation, by, for example, growing cardiomyocytes cocultured with endothelial cells, demonstrated that only the endocardial endothelial cells could maintain the adult cardiomyocyte phenotype, whereas vascular endothelial cells led to

dedifferentiation of cardiomyocytes and the re-expression of fetal proteins, as is the case in cardiac disease (55). Promyocardial growth also results from endocardial endothelial release of factors such as ET-1 and AngII, whose individual pathways can subsequently cross-talk with each other in the endothelium to then act on the adjacent cardiomyocytes (56-58). Studies have also shown that endothelial cells stimulate the secretion of ANF from co-cultured atrial cardiomyocytes, likely reflecting the action of ET-1 originating from the endocardial cells that can alter gene expression in cardiomyocytes. Moreover, cardiomyocytes can also reciprocate signalling to the MyoCapE, evidence for which was provided for when MyoCapE cells were shown to have increased growth in the presence of cardiomyocytes (59). Taken together, the cardiac endothelial cells are indispensable during embryonic and postnatal conditions, and thus further emphasizes the need for a more complete molecular understanding of endothelial function.

1.4.3.1.2.2. Antimyocardial growth

In contrast to the promyocardial growth pathways described above, endothelial-myocardial interactions are also important for antimyocardial growth. For example, clinical observations support an antigrowth effect of NO on adult cardiomyocytes, which in part is regulated by bradykinin. Bradykinin is broken down by angiotensin converting enzyme (ACE) which stems from endocardial cells. When ACE inhibitors are present, bradykinin breakdown is inhibited and induces NO release (60). Interestingly, bradykinin has a promyocardial growth effect in a cardiomyocyte *monoculture*, but an antimyocardial growth effect in cardiomyocyte-endothelial *cocultures*, thus revealing how the effects of a single molecule can be drastically altered simply under the influence of endothelial cells.

1.4.3.2. Cardiac contractile performance

A few decades ago, researchers found that the vascular endothelium was able to control vascular smooth muscle contraction (61), and from this finding emerged the question of whether or not the endothelium was able to influence myocardial contraction in a similar manner. Selective damage of the EE in papillary muscle of cats showed altered twitch contraction patterns (62,63) which was further explained when removal of the EE (64) demonstrated reduced responsiveness of the contractile proteins to intracellular Ca^{2+} concentration. Subsequently, other groups have shown that in fact, not

only the EE, but the MyoCapE as well can regulate the contractility of the subjacent myocardium (59,65). Interestingly, this response has been credited to several known auto- and paracrine signalling events which were also implicated in partially regulating myocardial growth, such as NO, ET, PGI_2, and ANGII (59,66-70). In addition, cross-talk of other endothelium- and myocardium- derived signalling pathways is required for proper regulation of cardiac contractility (71,72) and overall demonstrates how the cardiac endothelial cells may tightly regulate indispensable processes via several molecular mechanisms.

1.4.3.3. Cardiac rhythmicity

A unique property of some specialized cardiomyocytes is their rhythmic and sequential contraction. Abnormalities in the excitability or conduction in the specialized conductive tissue or terminal Purkinje fibers can lead to arrhythmias. In the adult, Purkinje fibers are located in very close proximity to the endocardial endothelial surface, suggesting that the EE may play a major role in the proper development and functioning of these fibers.

1.4.3.3.1. Differentiation of terminal Purkinje fibers: role of cardiac endothelium

Although it is not understood why or how some cardiomyocytes differentiate into conducting Purkinje fibers, it is interesting to note that factors from the EE might be involved. $ErbB_2$ (HER-1 or Neu) and Erb4 (HER-4) are tyrosine kinase receptors that are required for trabeculation of the primitive spongy heart. In $ErbB_2$ or $ErbB_4$ null mice, there is the occurrence of irregular heartbeats which may be due to abnormalities in conduction as a result of deficient trabeculation (which is the potential origin of the conduction system). In this embryonic trabecular layer, there is approximately an equal number of cardiomyocytes and EE cells, and therefore it is plausible to infer that either individually or cooperatively, signalling pathways from these cells may contribute to Purkinje cell differentiation (73). It is worth mentioning that endothelium-derived ET signalling pathways, which were previously mentioned to be involved in myocardial compaction, have also been shown to induce conversion of some cardiomyocytes into Purkinje fibers (74). This demonstrates that due to the multifunctional roles that these endothelial signalling pathways have during various stages of cardiac development,

disruption of endothelial maturation can severely impair several stages of cardiac development.

1.5. Endothelial dysfunction: role in the pathogenesis of cardiac failure

Given the vital roles of endothelial cells in cardiac development and maintenance of cardiac function, many studies have been conducted to investigate the consequences of endothelial dysfunction on cardiac homeostasis.

1.5.1. Peripheral vascular endothelial dysfunction in cardiac failure

Many reports have found that endothelial dysfunction can lead to cardiac failure from regions of the body distal to the heart; for example, endothelial dysfunction in skeletal muscle can lead to early fatigue and excess intolerance during strenuous activity which can contribute to cardiac failure (75-81). This kind of peripheral dysfunction is an early mediator of cardiac failure (76,82). This emphasizes the importance of the multifunctional endothelium in not only directly, but also indirectly influencing cardiac function from several vascular systems (such as the renal and pulmonary vasculatures) (83,84), and shows us how disturbance of a single system can lead to detrimental effects on overall cardiovascular homeostasis.

1.5.2. Coronary endothelial dysfunction in cardiac failure

The coronary endothelium also has been shown to contribute indirectly to the development of cardiac disease. Studies of several animal models and humans suffering from cardiac disease (85,86), revealed that the coronary circulation has impaired dilatory response to either acetylcholine or bradykinin, which in turn inhibits the production of NO and is thus an early contributor to endothelial dysfunction. This functional impairment disrupts regulation of coronary vasoconstriction, smooth muscle cell proliferation and remodelling, and leads to lipid deposition in the vessels, thus contributing to the development of coronary artery disease which indirectly leads to cardiac failure due to, for example, impaired myocardial perfusion (86-89). This phenotype has actually been reported in mice that only express the $VEGF_{120}$ (90) isoform and in fact provides a strong link between coronary vascular endothelium and cardiac disease.

1.5.3. Cardiac endothelial dysfunction in cardiac failure

Further evaluation of the manner in which the MyoCapE contributes to cardiac disease has been carried out in various animal models. For example, in dogs and rats which were submitted to ventricular volume and pressure overload experiments respectively (91,92), cellular lesions in the EE were detected. Subsequently, studies followed discerning whether these lesions are an early event in the progression towards cardiac disease. These EE lesions were induced upon treatment with various agents such as catecholamines, angiotensin, and ANF, and researchers showed that following these treatments, the mechanical performance of the subjacent myocardium was compromised (93). Moreover, MyoCapE cells from biopsies of patients suffering from various forms of cardiomyopathy had reduced NO production when stimulated with NO-inducing agents such as bradykinin (85). Taken together, overall cardiac endothelial dysfunction seems to be a *cause* for progression towards cardiac disease, however the molecular network responsible for this transition to a pathologic state remains to be deciphered.

1.5.4. Rationale for studying endothelial cells

The endothelium is a system that requires the integration of several signalling pathways in order to ensure the proper functioning of not only itself, but consequently other organs and systems as well. In particular, all of the detrimental effects of the major cardiovascular risk factors such as hypertension, smoking, and diabetes can be further accentuated by endothelial dysfunction. Several conflicting data regarding the roles and functions of the endothelium, in the literature arise due to species differences, various cellular sources (eg. cardiomyocytes, fibroblasts, etc), technical issues, and so forth. This re-emphasizes the need to fully understand the network of pathways that are working together. In addition to the pursuit of better insight into known genes, the discovery of novel genes in endothelial cells or cardiomyocytes will also aid in bringing us closer to an overall understanding of endocardial-myocardial interactions and will push us further towards more beneficial therapeutic strategies in the years to come. One approach towards obtaining a better molecular understanding of these intercellular interactions is by analyzing the **transcription factors** that are largely responsible for regulating key signalling pathways during development.

1.6. Transcription factors implicated in endothelium establishment

Not very many transcription factors have been identified as playing a role in the establishment of the endothelium. Nevertheless, their roles have been demonstrated to be essential for endothelial development and thus demonstrate how the need to discover additional transcription factors is crucial for a better molecular understanding.

1.6.1. ETS family of transcription factors

The ETS family consists of approximately 30 mammalian family members which are defined by a conserved DNA-binding ETS domain, that binds to a purine-rich consensus sequence GGA(A/T) in the promoter region of target genes. Among these, ETS-1, ERG, Fli-1, TEL, and NERF-2 are expressed in endothelial cells during embryonic vascular development and angiogenesis in the adult (94).

1.6.1.1. ETS-1 (E26 transformation specific-1)

ETS-1 is the first member of this family, which has been shown to be expressed in the blood islands of the yolk sac, where the hemangioblasts are located (95,96). Later in development, ETS is found in areas such as the heart, dorsal aorta, and intersegmental arteries (97). Moreover, human fetal blood vessels also display high levels of ETS-1 (98). In adults, ETS-1 expression is downregulated in fully differentiated tissues, however, increased expression of ETS-1 is observed during wound healing and tumor angiogenesis (99-104). *In vitro* studies have demonstrated that ETS-1 promotes endothelial cell adhesion and organisation into capillary-like structures (105). This EC specific expression has been attributed to an EC-specific element in the first intron of the gene (106). During angiogenesis, VEGF and bFGF (basic fibroblast growth factor) induce ETS-1 expression in ECs via the MAP kinase-ERK1/2 pathway and is an example of a signalling cascade acting upstream of ETS-1 (107). Downstream targets of ETS-1 include MMP-1, which is required for the degradation of basement membranes (108), Flt-1 (109), Flk-1 (110) and Tie2 (111). Despite its expression in the embryonic blood vessels, ETS-1 is not a master regulator of blood vessel development, as demonstrated by the presence of normal blood vessels in ETS-1 knockout mice (112). This, in part, may be explained by the fact that other ETS family member transcription factors, such as Erg and Fli-1, display overlapping patterns of expression with ETS-1, suggesting functional redundancy between the different members.

1.6.1.2. Fli-1 (friend leukemia integration-site 1)

Fli-1 is expressed in angioblasts and is hypothesized to be essential for the function of ECs in decidual blood vessels (113). Later in embryonic development, Fli-1 is expressed in newly formed ECs which are involved in vasculogenesis (114). However, unlike ETS-1, Fli-1 is not expressed in adult endothelial cells. Interestingly, the expression pattern of zebrafish Fli-1 was examined and was shown to overlap with that of GATA-2 (a marker of hematopoiesis and a member of the GATA family of transcription factors) during its initial expression in posterior lateral mesoderm in a potential hemangioblast population (115). As the vasculature starts to form, the expression patterns of Fli-1 and GATA-2 diverge from one another in sites of the developing vasculature and blood formation respectively, thus implicating Fli-1 as an early marker of hemangioblast formation. Fli-1 knockout mice demonstrate that this factor is not essential for vasculogenesis and angiogenesis since these processes were able to develop normally. However, embryonic lethality was observed around E11.5-E12.5 due to loss of vascular integrity, and as a result, cerebral hemorrhaging (114). These mice also showed reduced Tie2 expression. which incidentally contains ETS binding sites, suggesting it to be a potential downstream target of Flil.

1.6.1.3. ERG (ETS-related gene)

ERG is another transcription factor belonging to the ETS family that contains the ETS domain. The partial overlap of expression of ERG and Fli-1 together with their homology suggest some functional redundancy between the two members. As embryonic blood vessel development progresses, ERG expression decreases with endothelial maturation. Later, ERG expression becomes restricted to just the developing capillaries in the vasculature, suggesting that ERG functions mainly during vasculogenesis (116). Further insight into the function of ERG was gained when the Xenopus homologue of ERG was ectopically expressed in the ventral part of the Xenopus embryo. Premature endothelial differentiation was observed and thus suggests ERG to be involved in endothelial differentiation, which in turn is consistent with its expression pattern (117). Furthermore, ERG has been shown to be a regulator of the extracellular matrix glycoprotein SPARC and thrombospondin in endothelial cells (118) which are essential for cellular adhesion and proliferation, and inhibition of angiogenesis, respectively. In addition, although it is plausible that there is some functional redundancy between ERG

and Fli-1, it seems that in Fli-1$^{-/-}$ mice, ERG was not able to compensate for the loss of vascular integrity observed, suggesting specialised functions for each protein.

1.6.1.4. TEL (translocated ETS leukemia)

TEL is a protein which has been shown to function as both a transcriptional activator and repressor (119). Interestingly, TEL binds to Fli-1, thus preventing Fli-1 from transactivating its target genes (120,121). In addition, stromelysin, an MMP, was also shown to be transcriptionally repressed by TEL (122). TEL plays an essential role in vascular structural maintenance in hematopoietic tissues in early embryonic development. This role for TEL is further supported by TEL$^{-/-}$ mice, where normal vasculogenesis was observed, but there was a failure to carry out yolk sac angiogenesis (123). In addition, by E10.5, vascular integrity was no longer maintained, resulting in breakdown of yolk sac angiogenesis end embryonic lethality at E12.5. In the adult, only mature vessels express TEL, and thus unlike ETS1, new vessel formation via angiogenesis does not result in increased TEL expression (124).

1.6.1.5. NERF2 (new ETS-related factor 2) and ELF-1

NERF and ELF-1 are closely related transcription factors which belong to the Ets family. NERF has three alternatively spliced isoforms: NERF1a, NERF1b, and NERF2, however only the latter can function as a transcriptional activator which is expressed preferentially in ECs. Although both NERF2 and ELF-1 are expressed in the vasculature, ELF-1 is expressed in very small as well as large extraembryonic vessels, whereas NERF2 is expressed in the larger vessels only. Later on in development, NERF2 and ELF-1 are both expressed during embryonic angiogenesis (125). The human homologues of NERF2 and ELF-1 as well have been shown to transactivate the Tie-1 and Tie-2 genes using transfection assays (111,126,127). In contrast, co-transfection assays in the same HEK 293 cell line reveal that the chicken homologue of NERF2, cNERF2, acts as a competitive inhibitor of cELF-1. Therefore, these transcription factors are able to function as both positive and negative regulators of the same gene in chicken, and suggests that change in function of these factors during evolution may indicate a need to conserve vital developmental pathways. Furthermore, their dual functions and early differences in expression pattern, may suggest differential regulation of Tie-1 and Tie-2 genes in order to ensure proper blood vessel development. Generation of mice with null

NERF2 and ELF-1 alleles will provide a better insight into their functions. Taken together, a substantial amount of evidence support a critical role for ETS factors in the establishment of the embryonic vasculature. It is noteworthy that these transcription factors are alone not sufficient to drive normal blood vessel development, and thus other factors are needed for commitment of embryonic stem cells to the endothelial cell fate.

1.7. Transcription factors implicated in heart development

The proper morphogenesis of the heart relies on highly regulated molecular pathways. The malfunctioning of these pathways can result in congenital heart disease (CHD). Insight into this crucial developmental program stems from a better understanding of the factors regulating the cardiac program, i.e., the trancription factors. A list of the key transcription factors which play vital roles during cardiac development is summarized in Figure 6.

1.7.1. Homeodomain transcription factors in the heart

1.7.1.1. Nk-2 homeobox genes and their role in cardiogeneis

Better understanding about the molecular blueprint of cardiac morphogenesis of vertebrates came from studies conducted in *Drosophila*. The isolation of the *Drosophila* gene *tinman* and characterization of its role in the formation of the dorsal vessel or heart led to many hypotheses regarding vertebrate heart development. The functions of the dorsal vessel of Drosophila are similar to that of the vertebrate heart, except that it has an "open circulation", where it pumps hemolymph through the interstices of tissues rather than through blood vessels (128). In the *Drosophila* heart, *tinman* is first expressed in the cardiogenic mesoderm where the cardiac progenitors arise. After gastrulation, *tinman* is expressed in the dorsal mesoderm which gives rise to the heart, the gut and some dorsal skeletal muscles. Later, *tinman* is expressed in part of the visceral mesoderm and maintained in the heart throughout adulthood (129). *tinman* mutants in *Drosophila* lack not only the heart, but also visceral and dorsal skeletal muscles, suggesting that *tinman* is a master regulator of cardiogenesis and skeletal myogenesis.

Given the crucial role that *tinman* plays, in the dorsal vessel, studies were then undertaken to find homologues of *tinman* in vertebrates. The first tinman homologue was a murine gene called Nkx2.5 (also called Csx). Nkx2.5 is initially expressed in the bilateral cardiac progenitors of the anterior lateral plate mesoderm and in part of the

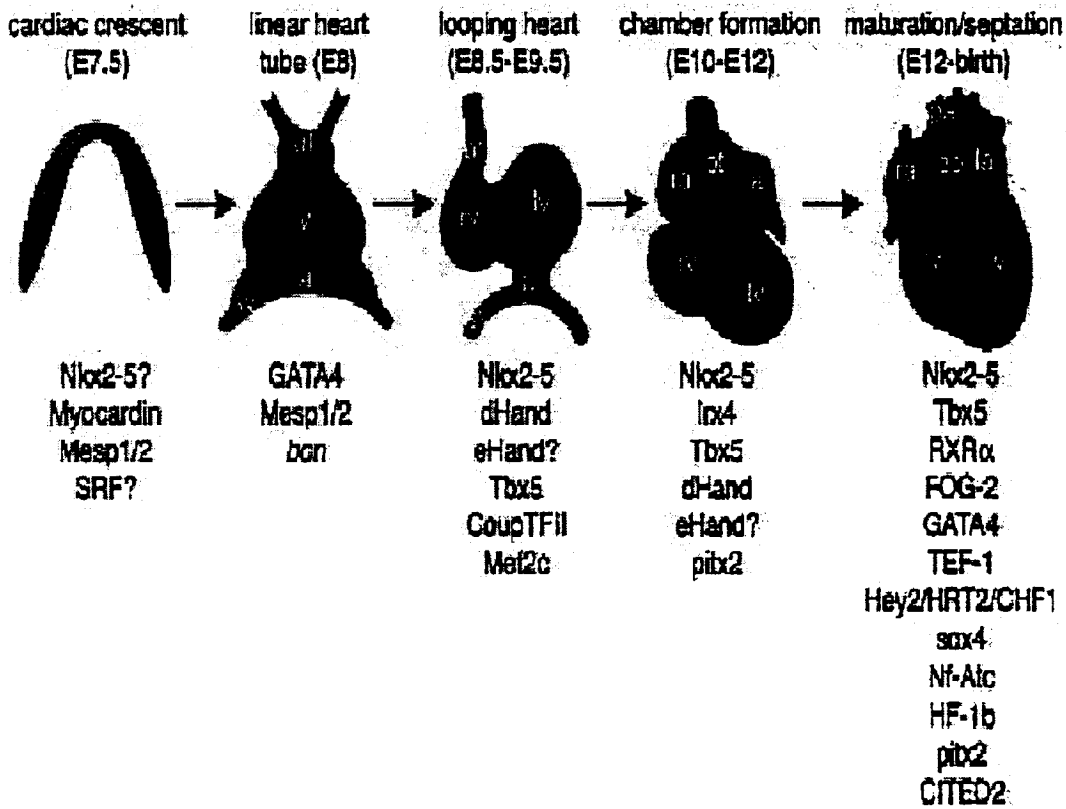

Figure 6. A list of the key cardiac transcription factors which play essential roles during vertebrate heart development. (Adapted from (156))

pharyngeal endoderm. It is the earliest known marker for the cardiac lineage in vertebrates. Murine Nkx2.5, is expressed in the cardiac mesoderm as early as the time of mesenchyme-epithelial transformation (130). Surprisingly, in homozygous Nkx2.5 knockout mouse, the early heart tube does form and most contractile proteins are expressed, except for a ventricular-specific myosin light chain gene. Later however, the heart tube fails to undergo normal looping and two transcription factors fail to be expressed in Nkx2.5 mutant hearts: the bHLH protein eHAND and the ankyrin-respeat protein CARP (131,132). Naturally, the following events of trabeculation and endocardial cushion formation were also blocked. Further studies pointed to Nkx2.5 having essential roles in cardiomyocyte terminal differentiation and maintenance of a ventricular gene expression program (133). Therefore, in contrast to its *Drosophila* orthologue, lack of Nkx2.5 in mice does not result in complete absence of heart formation and thus has a mutant phenotype that appears later than the *tinman* null phenotype, suggesting that murine Nkx2.5 gene may not function as a master regulator of cardiogenesis or that other Nkx genes partially compensate for Nkx2.5 (129). Indeed, several other *tinman* homologues have been isolated in a number of species, such as human (130), chick (134), frog (135) and fish (136), and were shown to be expressed during early heart development. So far only 2 members, Nkx2.3 and Nkx2.5 are reportedly present in multiple species, while Nkx2.6/Tix, Nkx2.7, Nxk2.8, and Nkx2.9 have been described in single species (137-140). Although no *tinman* homologues have been reported in *Drosophila*, expression of the different vertebrate *tinman* homologues is able to partially rescue the *tinman* mutant phenotype, suggesting possible redundancy between the different orthologues (141).

1.7.1.2. T-box

The importance of T-box genes in heart was initially raised by the finding that mutations in the Tbx5 gene were linked to Holt-Oram Syndrome. Later, this was confirmed in heterozygotes carrying mutations in Tbx1 or Tbx5, which displayed heart defects similar to those found in DiGeorge and Holt-Oram Syndromes respectively (142-147). Similar to Nkx2.5 and cardiac GATA genes (discussed below in later sections), Tbx5 is expressed in the bilateral cardiac primordia in various species such as mouse, Xenopus, chick and fish, suggesting important conservation of T-box function during this

stage of embryonic development. However, upon formation of the linear heart tube, cardiac Tbx5 expression becomes restricted only to the posterior areas of the subsequent atria, sinus venosa, and left ventricle (142,148-150). During left ventricular development, Tbx5 mRNA levels decrease and by late gestation and adulthood, Tbx5 can be detected, at low levels, equally in both the left and right ventricles in mice and humans (151). In order to decrease Tbx5 levels in frog and mice embryos, researchers used a dominant-negative approach, and Tbx5 homozygous null mutations, respectfully. The phenotype for both species displayed severe cardiac defects which affected the sinoatrial structures. Reductions in Nkx2.5 and GATA-4 expression were also present, perhaps suggesting that Tbx5 may, in part, regulate these indispensable transcription factors (142,150). Furthermore, ventricular differentiation is compromised in Tbx5 knockout mice, and there is reduced expression of ventricle specific genes such as Mlc2v, Irx4, and Hey2. These reduced expressions are a result of combinatorial interactions of Tbx5, Nkx2.5, and GATA-4, which act upstream of these ventricle-specific factors (152-154). Consistent with this, gain-of-function experiments show accelerated cardiac differentiation of P19C16 cell lines expressing Tbx5 and an up-regulation of Nkx2.5 mRNA levels (154). Transgenic Tbx5 overexpressing mice, chicken, or Xenopus hearts display a thin, hypoproliferative ventricular myocardium, which is not as dramatic a phenotype as that observed in knockout embryos (150,151,155). Thus it seems that particular threshold levels of Tbx5 during cardiac development needs to be maintained. Tbx1 studies also reveal similar dosage experiments (156). Other Tbox factors, like Tbx1, have been detected in the heart, such as zebrafish hrT, which has been shown to play a role in early cardiogenesis (157). In *Drosophila*, Tbx5 homologues are expressed in the dorsal vessel but their precise functions are not yet known. Extremely critical roles of Tbx5 orthologues in frogs and fish have been defined as it seems that lack of these factors results in complete absence of the heart, even though their expression is restricted to the single ventricle (158-161). Taken together, Tbx5 has been shown to have an early role in diversification of atrial and ventricular cell identities and displays functional cooperation with other vital transcription factors in the heart, which in turn, is essential for proper cardiogenesis and embryonic survival.

1.7.1.3. IRX4

Irx4, a member of the iroquois subgroup of homeobox genes, is specifically expressed in the prospective ventricular subarea of the early linear heart tube and expression persists in the ventricular chambers (162). The expression of Irx4 is downstream of Nkx2.5 (163). Knockouts of Irx4 in the mouse results in expansion of atrial and suppression of ventricular differentiation markers, whereas Irx4 overexpression in the chick heart has the opposite effect (162,164). Although it has been shown that Irx4 is involved in the up and down regulation of chamber-specific myosin heavy chain gene (165,166), it is not considered a master regulator of ventricle-specific gene expression, as demonstrated by only partial disturbance of ventricle-specific gene expression in Irx4 knockout mice (eg. decreased expression of eHAND in embryonic heart) (164). Other Irx genes, such as Irx6, Irx3, Irx5, Irk1, and Irx2, are also expressed in the heart, indicating perhaps some functional redundancy among the members. In addition, Irx4-deficient mice develop cardiomyopathy (164), and thus stresses the importance of correct chamber-specific gene expression for proper cardiac function. Moreover, Irx4 is thought to participate in specific pathways regulating gene expression in ventricles, as indicated by reduced expression of Irx4 in Nkx2.5 or dHAND knockouts (163) and no altered expression in MEF2C or RXRalpha null mice which also display ventricular defects (156). Given that Irx4 is also able to repress the quail homologue of atrium-specific slow myosin heavy chain 3 (164), it suggests that overall, Irx4 appears to play crucial roles in promoting ventricular and suppressing the atrial differentiation in the heart similar to Hey 2.

1.7.1.4. Pitx2

An important aspect of heart development is the establishment of the left-right axis, which is responsible for the rightward looping of the heart. In order to understand the molecular regulation of cardiac looping, the actual process of looping and the directionality of looping are important to distinguish, since the latter reflects the overall asymmetry of the embryo. Factors involved in the establishment of left-right asymmetry include nodal, sonic hedgehog, situs invertus, activin, lefty and Vg1 (167). Mutations in mice that affect the asymmetry of the embryo, such as lefty and situs invertus, disrupt the restricted expression pattern of these molecules. However these factors are not expressed

in progenitor cells, and thus more upstream factors in the laterality pathways need to be examined in order to understand how cardiac progenitors interpret signals related to asymmetry (129).

Pitx2, a bicoid-related homeodomain transcription factor, is an important regulatory laterality gene which is initially expressed in the left lateral plate mesoderm, and then later in the left side of the linear heart tube (168). This may suggest that Pitx2 may play a critical role in the left-right developmental program to direct rightward looping of the heart. Yu and colleagues demonstrated that it was the Pitx2C isoform that is expressed in the left heart field, and subsequently, left side of the heart tube in chick embryos, which regulates the directionality of looping (169). How this is executed remains undetermined since the downstream targets of Pitx2 are not known. However, Pitx2 does bind to the bicoid homeodomain site and has been shown to transactivate reporter genes containing this site *in vitro* (170). Overexpression of Pitx2C in the murine embryonic myocardium correlates with the double outlet right ventricle seen in mutants exhibiting defects in laterality. Loss-of-function of Pitx2 cause severe cardiovascular defects such as double inlet left ventricle and atrial isomerism (171,172). Moreover, mutation of Pitx2 displays a phenotype similar to that of Rieger's Syndrome, thus further emphasizing the need to understand these laterality pathways and the role they play in congenital heart diseases.

1.7.2. MADS box genes

1.7.2.1. MEF2C

Another class of transcription factors that play a role in cardiogenesis in both Drosophila and vertebrates are the MEF2 genes (myocyte enhancer factor-2), belonging to the MADS (indicating MCM1, agamous, deficiens, and SRF) box family. The four MEF2 genes (MEF2A, MEF2B, MEF2C, and MEF2D) in mice are expressed in precursors of cardiac, skeletal and smooth muscle lineages as well as in other cell types. MEF2C and MEF2B have the earliest expression in the heart field, which follows the onset of expression of GATA-4 and Nkx2.5, implying that MEF2 controls later stages of cardiomyocyte differentiation. MEF2A and MEF2D transcripts are expressed later during embryonic heart development (173). A targeted mutation in the MEF2C gene causes defects in heart morphogenesis (looping) and causes severe hypoplasticity in segments of

the heart corresponding to both ventricles, and leads to downregulation of cardiac-specific gene expression (such as ANF, alpha-MHC, MLC1A), similar to that observed in dHAND (see below) or Nkx2.5 mutants (156,174), thus suggesting that it is a direct activator of a subset of myocyte differentiation genes. It is worth noting that although MEF2C null mice display cardiac defects, it has not been determined whether these defects are a direct result of the lack of MEF2C expression in myocytes since these mice also have vascular malformations and impaired blood circulation. In addition, endocardial defects were also present which may be due to reduced expression of AngI and VEGF in the myocardium (175). This combination of vascular and cardiac defects are present in several knockout phenotypes as depicted in Table 3, and illustrates the complexity of interactions between the endothelium and myocardium.

1.7.2.2. Serum response factor (SRF)

Unlike MEF2, SRF does not have muscle-restricted expression. Binding sites for SRF (CarG boxes) are located in many cardiac genes (eg. ANF), and it has been postulated that SRF acts in concert with other myogenic factors. For example, myocardin, a trancription factor, which is largely expressed in cardiac and smooth-muscle cells, belongs to the SAP domain family of chromatin-remodelling proteins, and has been shown to activate cardiac muscle promoters by forming a ternary complex with SRF (176). Moreover, the role of myocardin has proven to be crucial for development since expression of a dominant negative mutant of myocardin in Xenopus embryos has been shown to interfere with myocardial cell differentiation (177). SRF null mutants have a severe gastrulation defect and no mesoderm formation (178). Further understanding of this phenotype partly comes from recent isolation of HOP, a small homeobox protein that is unable to bind to DNA, however is able to negatively regulate SRF (179). Mice homozygous for a Hop null allele display two different phenotypes: the first is embryonic lethality due to insufficient myocyte proliferation, and the second is a hyperplastic postnatal heart. Therefore, it seems that modulation of SRF activity in muscle cells plays a vital role in balancing the signals for growth and differentiation of the myocardium, which is also essential for embryonic survival (21).

1.7.3. bHLH (basic helix-loop-helix) proteins

1.7.3.1. dHAND and eHAND

Table 3. Knockouts or mutations in genes that generate cardiac defects

Protein	Species	Cardiac Defect
Signalling Molecule		
Neuregulin(267)	Mouse	Absent myocardial trabeculation
ErbB2, B4(267)	Mouse	Absent myocardial trabeculation
Endothelin-1(267)	Mouse	Outflow tract and arch defects
TGF-β(267)	Mouse	Cardiac valve defects
Transcription Factor		
GATA-4(267)	Mouse	Cardiac bifida
GATA-5(267)	Zebrafish	Cardiac bifida
FOG-2(267)	Mouse	Cardiac alignment, coronary artery defects
MEF2C(267)	Mouse	Hypoplastic right and left ventricles
dHAND(267)	Mouse, Zebrafish	Hypoplastic right ventricle, arch defects
Nkx2.5(267)	Mouse, Human	Looping, conduction defect, atrial septal defect
TBX5(267)	Human	Ventricular septal defect, atrial septal defect
NFATc(267)	Mouse	Absent valves
Pitx2 (268)	Mouse	Double inlet left ventricle, atrial isomerism, persistent truncus arteriosus

In purple are the factors which when mutated, generate a phenotype not only associated with cardiac defects but vascular defects as well. (Adapted from (267))

The MyoD family of basic-Helix-Loop-Helix proteins, which regulate skeletal myogenesis, are not expressed in the heart. Recently, two new bHLH genes, dHAND and eHAND, have been isolated that show prominent expression in the early cardiac progenitors, the looping heart tube, as well as in cardiac neural crest-derived cells (180). dHAND and eHAND have complementary patterns of expression in the embryonic murine heart. The expression of dHAND is more pronounced in the right ventricle (RV) than the left ventricle (LV), while eHAND is predominantly restricted to the LV (131,181-183). The precise role of eHAND in LV development is not clear because of early embryonic lethality caused by extraembryonic defects from the eHAND knockout mice (183,184). Clarification of its role in the heart will require tissue specific gene inactivation. In the dHAND-null embryos, the RV and outflow tract are initially formed, but the RV precursors undergo apoptosis, thus preventing further growth and maturation of this segment (185). In addition, there are defects in the morphology of the aortic sac and ventricular chambers, and in the looping morphogenesis. This chamber restricted expression pattern of the bHLH HAND genes is not conserved in the chicken heart (180). Nevertheless, its role remains essential, as demonstrated by experiments where chick embryos were incubated with antisense oligonucleotides to both of these genes simultaneously and showed cardiac defects at the looping stage. Because Nkx2.5, MEF2C, and dHAND knockout mice have similar phenotypes, it is suggested that perhaps these genes cooperate together in specifying the cardiac lineage and/or differentiation of the primitive heart tube. In *Drosophila*, no HAND-type bHLH factors are known (129), however, in lower vertebrates, such as frog and fish, a single cardiac gene does exist and is responsible for the development of the single ventricle found in these species (186,187).

1.7.3.2. MESP

The bHLH transcription factor MesP1 has been shown to be expressed in the early mesoderm around the onset of gastrulation. The function of Mesp1 appears to be very specific since its expression is rapidly decreased after E7.0, and only mesodermal cells which had ingressed through the primitive streak were $Mesp1^{+}$ (188). Mesp1 knockout mice displayed either complete or partial cardia bifida (inability of the lateral cardiac primordia to migrate and fuse), possibly due to the aberrant migration of

mesodermal cells (189). Interestingly, mice lacking both Mesp1 and Mesp2 (related to Mesp1), display complete block in migration of precursor cells (190). When chimeric mice were created with Mesp1/Mesp2 double-knockout embryonic stem cells, it was discovered that these transcription factors play an important role in ventricle formation, while they were insignificant for atrial formation. This reveals two things: first, Mesp1 and Mesp2 are perhaps required for ventricular patterning, and second, there is a very early lineage diversification of atrial and ventricular precursors. This was further supported by studies conducted in zebrafish (191) and chick embryos (192).

1.7.3.3. Hey2

Another class of bHLH proteins implicated in cardiogenesis is the Hairy family of transcription factors (193-196), which consists of 2 murine genes, Hey1 and Hey2 (also called HRT2, CHF1, HERP1, and Hesr1), the human HeyL gene, and the Drosophila dHey gene. While comparing the expression pattern of Hey1 and Hey2, an interesting finding was found. Although Hey1 and Hey2 are both expressed in developing somites and the heart, they exhibited complementary cardiac expression, where Hey1 was found in the developing inflow and outflow tracts and atrial precursors, and Hey2 was restricted to the part of the heart tube destined to be the ventricles (194). Even though Hey2 has been shown to function as a transcriptional repressor (196-198) its exact role in cardiogenesis is not known. Hey2 knockout mice showed isolated ventricular septal defects, suggesting that Hey2 may be an important downstream mediator of interventricular septum development (199). Due to this very specific phenotype, it is important to know in the future what are the upstream regulators and downstream targets of Hey2 in order to identify pathways which may not only shed light on molecular mechanisms controlling septation, but also on ventricular-specific expression, and possibly negative regulation of atrial-specific cardiac gene expression.

1.8. GATA factors

Members of the GATA family of transcription factors are zinc finger proteins which bind to the consensus DNA sequence (A/T)GATA(A/G). These factors have been shown to have indispensable roles in processes such as cell growth and differentiation during development. The founding member of this family is GATA-1 whose expression is largely restricted to the hematopoietic lineage. Two other members which were

subsequently cloned and found to be expressed in the hematopoietic lineage are GATA-2 and GATA-3. Altogether, these three members comprise the first subgroup of GATA factors. The second subgroup is comprised of GATA-4, -5, and –6, which are predominantly expressed in the heart and gut. Interestingly, GATA-5 is largely expressed in the endocardium, whereas GATA-4 and –6 are more expressed in the myocardium. Given the fact that these factors are not able to fully compensate for each other during development, and are necessary for embryonic survival (as demonstrated by gene targeting studies), understanding their individual functions are of great importance.

1.8.1. Hematopoietic GATA factors

1.8.1.1. GATA-1

The GATA-1 transcription factor is highly expressed during erythroid cell maturation and has been found to be essential for hematopoiesis. Chimeric mice containing GATA-1 null embryonic stem (ES) cells revealed that, although these GATA-1 null ES cells were able to differentiate into white blood cells, there was no contribution of these cells to the formation of mature red blood cells (200,201). In addition to erythroid lineage, lineage-specific GATA-1 knockdown mutant mice demonstrate that GATA-1 is also necessary for terminal megakaryocyte differentiation (202). Furthermore, although GATA-2 is expressed earlier than GATA-1 (200), studies in Xenopus have demonstrated that GATA-1, in conjunction with the bHLH SCL/Tal-1 and LMO-2 (LIM-only bridging protein) can induce the erythroid program in non-terminally differentiated cells in ectoderm, thus suggesting that GATA-1, at least in association with two other factors, can carry out some of the functions of GATA-2 (203). Gene targeting at the coding region of GATA-1 is embryonic lethal between E10.5-11.5 due to arrested erythropoiesis, and also lack of development of hematopoiesis in yolk sacs shows that GATA-1 is also required for differentiation of hematopoietic precursor cells. Moreover, rescue of GATA-1 deficient mouse embryos with different domains of GATA-1 indicate that the two zinc fingers may each play specific roles in the establishment of different blood lineages (204,205)

1.8.1.2. GATA-2

GATA-2 is expressed early on in development in hematopoietic stem cells and is first detected in extraembryonic and lateral plate mesoderm and even in cells of the early

yolk sac (206). GATA-2 knockout mice have drastic reduction in hematopoietic stem cells and are embryonic lethal around E10.5 (207). This is partially explained by the fact that SCL/Tal-1, a gene essential for all blood development, requires binding of GATA-2 in the progenitor enhancer region in order for it to be activated (208,209). Furthermore, unlike GATA-1 -/- ES cells, GATA-2 -/- ES cells in chimeric mice do not give rise to any hematopoietic lineages, thus suggesting that GATA-2 plays a role in definitive hematopoiesis. In addition to hematopoietic cells, GATA also appears to be involved in endothelial cell development (210).

1.8.1.3. GATA-3

GATA-3 is among the transcription factors required for development of T cell progenitors from the earliest stages of development. Overexpression of GATA-3 results in failure of T cell development, and this is associated with decrease in expression of PU.1 (211). In addition, GATA-3 has also been shown to play a critical role in the cell division of T-helper (Th) –2 cells, in order to ensure proper terminal differentiation. This was shown by a study where a potential upstream regulator of GATA-3 was knocked out in mice. Loss of this regulator, mel18 (a member of the Polycomb group), inhibited Th-2 cell differentiation, and displayed decreased GATA-3 expression. When cultures of the mel-$18^{-/-}$ cells were treated with a GATA-3-containing retrovirus vector, the cells were significantly rescued to the Th-2 cell differentiated phenotype (212). A mechanism by which GATA-3 carries out its functions is by interacting with the TGF-β signalling pathway. Activation of the TGF-β pathway in Th-2 cells, results in phosphorylation of Smad3. This phosphorylated protein then interacts with GATA-3, and aids in transactivating its target genes (213), and thus provides an example of the cooperative interaction between extracellular signals and tissue-specific transcription factors. Similar to GATA-1 and –2, GATA-3 has multiple regulatory units which aids in conferring specificity of expression (214,215,216)

1.8.2. Cardiac GATA transcription factors

1.8.2.1. GATA-5

1.8.2.1.1. Expression pattern and functional analysis of GATA-5

GATA-5, unlike any of the other cardiac GATA factors, is predominantly expressed in the endocardial layer of the heart, as indicated by in situ hybridization and

immunohistochemical studies performed in both Xenopus and rodent (217-220). The overall expression pattern of GATA-5 is quite conserved among species (217,218,221-223). During embryonic development, GATA-5 is expressed in the primitive endoderm and in the precardiac mesoderm. During heart development, GATA-5 is predominantly expressed in endocardial cells and becomes restricted to the atrial endocardium, which coincidentally gives rise to endocardial cushions (218), and suggests perhaps a role of GATA-5 in chamber-specific development. In regards to endocardium development, very few transcription factors have been found to play a role in endocardial endothelial cell differentiation. At present, NFATc and GATA-5 are the only transcription factors characterized thus far which have been shown to be essential for endocardial differentiation, since reduced levels of these factors results in blockage of endocardial differentiation (220). Subsequently, during mid-gestation and postnatal development, GATA-5 expression is turned off in the heart but not in other organs such as the lung or gut. GATA-5 knockout mice are not embryonic lethal, suggesting that it is the only GATA factor which is dispensable for vertebrate life. However, a phenotype corresponding to defects in genitourinary tract development (224) is present in females, which in turn, is in agreement with the expression pattern of GATA-5 in the urogenital ridge during embryonic development (225). In contrast, the phenotype of the GATA-5 mutant in zebrafish , *faust* (which can develop in the absence of endoderm), is embryonic lethal, and displays a reduced number of cardiomyocytes. Interestingly, this phenotype is similar to that observed in GATA-4 knockout mice (223).

1.8.2.1.2. Molecular regulation and mechanism of action of GATA-5 in cardiac development

A study done by Reiker and colleagues implicated GATA-5 as a master regulator of cardiogenesis in zebrafish when the role of GATA-5 was analyzed in swr (Bmp2b homologue), and Zoep (one-eyed pinkead homologue), mutant zebrafish (226). Members of the bone morphogenetic protein (Bmp) and EGF-CFC families have been shown to play vital roles during vertebrate myocardial development. Both swr and zoep mutants display severe defects in myocardial development as well as a reduced or absent expression of faust or Nkx2.5. Overexpression of *faust* in these mutants restores Nkx2.5 expression, and in addition, there is restoration of cmlc1, a myocardial sarcomeric gene,

in Zoep mutants, suggesting them to be downstream targets of GATA-5. These results indicate that swr and Zoep are, at least in part, able to regulate GATA-5, and that GATA-5 alone is sufficient to promote myocardial differentiation, via activation of Nkx2.5 (226). Other putative downstream targets include cardiac-specific cTnc (cardiac troponin C) (218), beta-myosin heavy chain promoter (227), and ET-1 (220), which interestingly is also activated by GATA-2 (228). Furthermore, GATA-5 can cooperative with other factors to regulate transcription; for example, recently it was shown that GATA-5 acts synergistically with NFATc and p300 to activate the ET-1 (220) and ANF promoters (229) respectively. It is also noteworthy that several other genes implicated in endocardial development, such as Msx1 (230), P-selectin (231), and TnX (232), have conserved GATA binding sites in their promoters and may be targets of GATA-5.

1.8.2.2. GATA-4 and GATA-6

1.8.2.2.1. Expression pattern during cardiac development

Similar to GATA-5, GATA-4 and GATA-6 are initially co-expressed in the precardiac mesoderm, however, as development continues, an apparent differential expression pattern is noticeable. After the formation of the linear heart tube at E8.5, in contrast to GATA-5, GATA-4 and –6 are expressed almost equally in both the atrial and ventricular myocardium (218). Following looping, cells in the endocardium which express GATA-5, also co-express GATA-4 suggesting perhaps they have common downstream targets (discussed below) (233). GATA-6 is expressed less than GATA-4 in the myocardium, endocardium, and the endocardial cushions, however they are broadly expressed in various cell types contributing to structural components of the heart such as the bulbis cordis and the venous valves (233). In spite of the similar expression patterns of GATA-4 and –6, they are not co-expressed in all cells. For example, although most of GATA-6 positive myocytes are also GATA-4 positive, the reverse is not true. This actually indicates that there are specific subsets of myocytes which carry out distinct functions, and further emphasizes the need to understand the molecular mechanism of action of these GATA factors in order to understand the specific cellular interactions of cardiac cells taking place during development. GATA-4 and GATA-6 continue to be expressed throughout the embryonic heart, and unlike GATA-5, they are co-expressed in the postnatal and adult heart. They share expression in other organs as well, such as the

small intestine, lung and liver. However, GATA-4 and GATA-6 are not co-expressed in organs such as the ovary, testis, stomach and bladder (233), thus implicating GATA-4 and –6 to have partially distinct roles.

1.8.2.2.2. Functional analysis

Several gain- and loss-of-function studies have been carried out in order to decipher the roles that GATA-4 and GATA-6 play in the heart. An embryonic pluripotent P19 carcinomal cell line was used to decipher the function of GATA-4 since this cell line provided a cellular model of inducible cardiac differentiation using DMSO. Following DMSO treatment, GATA-4 null lines failed to terminally differentiate and many of the precardiac cells underwent apoptosis (234,235), suggesting that GATA-4 plays an essential role in mediating cardiac survival, proliferation or differentiation (236). In addition, loss-of-function studies of GATA-4 reveal that it is necessary for cardiogenesis. Mice with null GATA-4 alleles die at E9.5 and exhibit the cardia bifida phenotype, and thus are unable to generate the primitive heart tube (237,238). Interestingly, these GATA-4 null mice have markedly upregulated levels of GATA-6 (and to a lesser extent GATA-5), which is not seen in the P19 model, suggesting that other cardiac GATA factors may be able to compensate for the lack of GATA-4 at earlier developmental stages. Complementary gain-of-function studies in P19 cells, where GATA-4 was stably overexpressed, displayed an earlier appearance of beating cardiac cells indicating that GATA-4 can really potentiate cardiogenesis (234). Similar roles for GATA-4 were detected in Xenopus and zebrafish which had overexpression of Nkx2.5 (166,239) and suggested that GATA-4 could potentiate cardiogenesis by recruiting more cells to the cardiogenic field. An additional role for GATA-4 in the zebrafish heart was identified when earlier expression of GATA-4 appeared to make precursor cells more competent towards cardiomyocyte differentiation (240), overall providing further evidence for the vital role of GATA-4 in heart development.

The available data regarding GATA-6 in the heart is a little more limited, however, overexpression of GATA-6 in Xenopus revealed its role in cardiogenesis (241). When GATA-6 mRNA was injected early on in development, cardiac precursor cells failed to differentiate and instead demonstrated enhanced proliferation. Following degradation of GATA-6 mRNA, cardiomyocytes resume differentiation, however due to

the greater number of precursors, a larger heart was formed. This plausible function of GATA-6 to regulate proliferation of cardiac progenitors is common to another GATA-factor, GATA-2 which regulates proliferation of hematopoietic progenitors (242). GATA-6 knockout mice are embryonic lethal around gastrulation due to extra embryonic endodermal defects (243), which precludes analysis of GATA-6 in the heart. However, other strategies have been developed in order to gain a better understanding regarding the role of GATA-4 and GATA-6 in the heart. Charron and colleagues developed a novel adenovirus-mediated antisense strategy to specifically inhibit either GATA-4 or GATA-6 in postnatal cardiomyocytes (244). Data from these experiments reveal that several cardiac genes are markedly downregulated and this study helped identify pathways that are differentially regulated by GATA-4 and GATA-6.

1.8.2.2.3. Molecular regulation and mechanism of action of GATA-4 and GATA-6 in cardiac development

As mentioned previously, gain-of-function studies of Nkx2.5, GATA-4, -5, and –6 in Xenopus demonstrate that although these factors are essential for critical processes of cardiogenesis, they alone are not sufficient to induce cardiomyocyte differentiation, suggesting that they need to interact with other proteins to ensure proper transcriptional regulation. Several GATA DNA binding elements have been identified in the promoters of various cardiac-specific genes such as ANF, BNP, alpha-myosin heavy chain, beta-myosin heavy chain, A1 adenosine receptor, cardiac troponin C, and angiotensin type1a receptor (245). As described in studies that carried out adenovirus-mediated downregulation of GATA-4 and GATA-6, these two GATA factors share many target genes such as ANF, BNP, α-MHC, β-MHC, cTnI, and platelet-derived growth factor receptor beta (PDGFRβ) (244). Further analysis revealed that some of these common targets had stronger affinities to GATA-4 such as α- and β-MHC. In addition, GATA factors are able to interact with numerous co-factors in order to regulate several pathways, such as, p300 (246), serum response factor (247), Nkx2.5 (153), myocyte enhancer factor 2 (MEF2) (248), nuclear factor of activated T-cells (NFAT) (249), the friend of GATA-2 (FOG-2) (250), and yin yang1 (YY1) (251). The GATA/Nkx2.5 interaction is particularly interesting due to the fact that this interaction is restricted only to GATA-4 and –5, and not to GATA-6 (252), thus providing evidence of signaling

pathways which are differentially regulated by GATA-4 and GATA-6. Remarkably, GATA-4 also interacts cooperatively with GATA-6 to regulate levels of ANF and BNP (244), which is in agreement with the fact that both of these factors are expressed in specific subsets of myocytes. In the adult heart, GATA-4 and –6 have been shown to play roles in cardiac hypertrophy which is generally characterized by an increase in ventricular and/or septal wall thickness. GATA-4 transgenic mice reveal activation of hypertrophy-associated genes such as ANF, BNP, beta-MHC, and ET-1, thus implicating GATA-4 in hypertrophic signaling. Although not much is known about the role of GATA-6 in hypertrophy, as mentioned before, GATA-6 does share regulation of hypertrophy-associated genes with GATA-4, and in addition, displays similar regulation under hypertrophic conditions, with for example phenylephrine (PE) (244), thus suggesting redundant regulation of hypertrophic gene expression with GATA-4. It is also noteworthy that some of the pathways regulated by GATA factors are conserved in Drosophila. The GATA factor homologue, *pannier*, has been shown to act as an upstream regulator and also a cofactor of tinman to synergistically activate downstream targets such as Dmef-2, a MEF2 homologue (253). Incidentally, some of the GATA target genes are also common to endothelial cells, such as ET-1, and suggests perhaps yet undiscovered correlation between GATA-4 and –6, and endothelial-myocardial signaling pathways. Overall, this implicates the GATA factors in playing essential roles in evolutionary conserved pathways that appear to be necessary for proper morphogenesis and survival.

1.9. Description of the project

1.9.1. Objective and rationale

Several of the transcription factors described thus far play crucial roles in cardiogenesis, but as mentioned previously, insight into their molecular mechanism of action is still lacking. Focusing on the vital transcription factors that are expressed in early precursors of the heart and learning more about their signalling pathways can reveal how key cardiac developmental stages are regulated, and furthermore, how these pathways can integrate with other developmental systems to achieve a proper functioning heart. Given the early and crucial functions that GATA-4 and GATA-6 play in the heart, particularly in cardiomyocytes, we wanted to gain a better insight into the distinct signalling pathways that accounts for the essential, non-redundant functions. Therefore

the objective of this project is to identify and functionally characterize differential downstream targets of GATA-4 and GATA-6.

1.9.2. Method

As depicted in Figure 7, an adenovirus-mediated strategy was used to downregulate the levels of GATA-4 or GATA-6 in cardiomyocytes (244) and then isolate, through an RT-PCR differential display, the transcripts which are upregulated or downregulated by GATA-4 and/or GATA-6. The advantage of using this strategy, is that in addition to known genes, novel genes can also be detected, and thus provide more insight into signalling pathways not yet known to exist. In addition to several previously known targets of GATA-4 and GATA-6 (such as ANF), numerous novel transcripts were also differentially regulated.

1.9.3. ENDO1

Among the novel genes detected was a transcript that contained a putative PHD finger, a domain which is implicated in chromatin-mediated transcriptional regulation and protein-protein interactions. This transcript, named ENDO1, was found to be selectively upregulated in cells overexpressing antisense GATA-4. Moreover, mRNA levels of this transcript were increased when TC13 endothelial precursor cells underwent differentiation via treatment with retinoic acid, thus suggesting it to play important roles in endothelial and myocardial development. ***Therefore, given the putative functions associated with PHD fingers, its expression in endothelial and myocardial cells, and its potential to be a downstream target of GATA-4, ENDO1 was further characterized to establish its role in cardiac development.***

2. MATERIALS AND METHODS

2.1. Screening of cDNA rat cardiomyocyte library

The cDNA rat cardiomyocyte library was previously made in our lab by Dr. Bruno DeLorme. In order to carry out the cDNA screening, MRA bacteria were grown in NZY broth (5g NaCl, 2g $MgSO4{\cdot}7H_2O$, 5g yeast extract, and 10g casein hydrolysate for 1L solution) containing 10mM $MgSO_4$. 200 µl of the bacteria were then added to 100 µl of SM Buffer (5.8g NaCl, 2.0g $MgSO4{\cdot}7H_2O$, 50mL Tris pH 7.5, and 5mL gelatin 2% for 1L solution) along with 1 µl of the cDNA library. After incubating the bacterial mix at 37°C, the mixture was poured on NZY agar plates. A nylon membrane was then put on top of the plate briefly, and then removed and incubated with denaturing (50mL NaOH 10N, and 300mL NaCl 5M for 1L solution), neutralizing (300mL NaCl 5M, and 500mL Tris pH 7.5 for 1L solution), and 2X SSC washing solutions in a series of steps. The membranes were left to dry and the cDNA was crosslinked by UV irradiation (stratalinker 2400 from Stratagene). The membranes were then incubated in a prehybridization solution (50mL SET 20x, 25mL $NaPyrPO_4$ 2%, 10mL SDS 10%, and 1mL Heparine 50mg/mL for 500mL solution) overnight at 65°C. For the hybridization step, the 1.20.1c cDNA fragment obtained from the RT-PCR differential display was radiolabeled with ^{32}P and used as a probe for screening. The probe was then incubated in the hybridization buffer (50mL SET 20x, 25mL $NaPyrPO_4$ 2%, 10mL SDS 10%, and 5mL Heparine 50mg/mL for 500mL solution) overnight at 65°C. Membranes were then developed using autoradiography and assessed for colonies containing the positive clones. The cDNA from the positive colonies was extracted by removing the piece of gel and vortexing it in SM buffer. Each sample is then re-exposed to the steps above until 100% of the colonies are found to be positive. This ensures that a very pure cDNA fragment has been isolated which is almost 100% homologous to 1.20.1c. Three clones were isolated from this process and were named 1A, 12B, and 9C1.

2.2. Transient transfection with HA-tagged ENDO1

A fusion protein expressing the influenza virus hemagglutinin (HA) epitope (amino acid sequence: SSYPYDVPDYASLGGPSR) was attached to the N-terminal part of the open

reading frame of ENDO1 in order to detect the cellular localization of recombinant ENDO1 protein. pCGN HA-ENDO1 vector was transfected into cells using the calcium-phosphate precipitation method (281). Two days after washing the DNA precipitate, cells were harvested for protein extraction and Western Blot analysis or fixed for immunofluorescence staining. All of the results shown were from experiments that used the murine ENDO1 cDNA in order to have the results comparable to any *in vitro* or *in vivo* analysis on mouse cell lines or animals.

2.3. Generation of the mutation/deletion constructs of ENDO1

The deletion constructs were prepared via PCR. The forward primer 5' GGGGTACCTAACGACAGGTGACTGTTGC 3' and the reverse primers used to construct ENDO1A and ENDO1C are 5' GGGGATCCTCACGGCCCT-GGGTCCTTTAGCC 3' and 5' GGGGATCCTCATGCAAACTCAGTGTGACACAGC 3' respectively. The mutation in ENDO1D was identified while sequencing different constructs of ENDO1C. ENDO1A stops at amino acid 131 and ENDO1C and ENDO1D stops at amino acid 114. The cDNA was cloned in the pCGN vector and transiently transfected in cells in a manner similar to pCGN HA-ENDO1.

2.4. High efficiency competent cells

A single colony of XL1 Blue cells was picked from a stock LB (Luria Broth Base) plate and inoculated in 2.4mL of LB supplemented with 50 μg/mL tetracycline. After culturing it overnight at 37°C with shaking, the culture was diluted in 1:100 in LB supplemented with tetracycline and grown until reaching the exponential phase (OD_{500}=0.4-0.6). The culture was then centrifuged at 5000 rpm at 4°C and the pellet was resuspended in ice cold TFB1 (30mM Potassium Acetate, 100mM RbCl, 10mM $CaCl_2$, 50M $McCl_2$ and 15% glycerol, pH 5.8). Cells were incubated on ice for 5 min before centrifugation at 5000 rpm at 4°C. Cells were then resuspended in 1/25 volume of ice cold sterile TFB2 (10mM MOPS, 75mM $CaCl_2$, 10mM RbCl, 15% glycerol, pH 6.5), and incubated on ice for 15 to 60 min. Finally, competent cells were quickly frozen on dry ice and were stored at –80°C.

2.5. Transformation and DNA purification

Routinely, transformations were carried out after an aliquot of competent cells was thawed out on ice and incubated with DNA for 10-15 minutes. Cells were subjected to heat shock for 45 seconds at 37°C followed by incubation on ice for at least 1 minute. Transformations were then plated on an LB plate containing the appropriate antibiotic for selection (100 μg/mL ampicillin) and incubated overnight at 37°C. Large-scale plasmid DNA preparation (maxi-prep) was carried out using the QIAGEN Plasmid Purification Protocol (QIAGEN corp.). Briefly, 500mL of the LB-Bacterial culture was centrifuged at 5000 rpm and resuspended in P1 Resuspension Buffer (50mM Tris-Cl pH 8.0, 10mM EDTA, and 100 μg/mL Rnase A). Cells were then lysed in the P2 Lysis Buffer (200mM NaOH and 1% SDS) and the genomic DNA, proteins, and cell debris were then precipitated using the P3 Neutralization Buffer (3.0M Potassium Acetate, pH 5.5). The supernatant was then removed after centrifugation. A QIAGEN-tip 500 was then equilibrated with the QBT Equilibration Buffer (750 mM NaCl, 50mM MOPS pH 7.0, 15% isopropanol, and 0.15% Triton X-100). Following equilibration, the plasmid-containing supernatant was loaded on the tip 500 and was allowed to enter the resin by gravity flow. The tip 500 was washed twice with QC Wash Buffer (1.0M NaCl, 50mM MOPS pH 7.0, and 15% isopropanol), and then the DNA was eluted with QF Elution Buffer (1.25M NaCl, 50mM Tris-Cl pH 8.5, and 15% isopropanol). Isopropanol was then added to the DNA sample and centrifuged at 5000 rpm. The pellet was washed with 70% ethanol and resuspended in 1 mL TE Buffer (pH 8.0). A smaller scale amplification of the plasmid can also be carried out by doing mini preps instead of maxi preps.

2.6. Single step method for RNA extraction

Total cellular RNAs were isolated by the thiocyanate-phenol-chloroform method (282). Briefly, frozen tissues (~ 200mg) or cells (1×10^7) were homogenized in 2 mL of guanidium solution (4M guanidium thiocyanate, 25mM Na Citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol). RNA was extracted with an equal volume of water-saturated phenol, 0.1 volume of 2M Na Acetate and 0.1 volume of chloroform/isoamyl alcohol 49/1 (v/v). The aqueous phase was separated by an incubation of 15 min on ice followed by a centrifugation at 5000 rpm at 4°C for 20min. The aqueous phase was collected and RNA

was precipitated with an equal volume of cold isopropanol for an hour on ice. The RNA pellet was recovered by a centrifugation at 5000 rpm for 20 min at 4°C. RNA was resuspended in a guanidium solution and reprecipitated as described above. Finally, the pellet was air-dried and RNA was dissolved in 100 µl DEPC-treated water. 1 µl of the RNA sample was resolved on a 1% agarose gel in order to verify the quality of the RNA isolated.

2.7. RT-PCR analysis

Total RNA was isolated from tissues or cell lines using the thiocyanate-phenol-chloroform method. 5 µg of total RNA was used for cDNA production using an oligonucleotide dT_{12-18} in the presence of reverse transcriptase (AMV-RT from Promega) for 2 hours at 42°C. Typically 100ng of cDNA (or about 5-50ng of plasmid DNA) was used to amplify ENDO1 with an RT-PCR mix consisting of 8.0 µl of $MgCl_2$ (25mM), 5.0 µl PCR Buffer, 0.8 µl of dNTP mix (25mM dATP, 25mM dGTP, 25mM dCTP, and 25mM dTTP), 0.5 µl Taq enzyme (Promega) and H_2O. The forward primer is 5' AGGACTCTGAGGTAGTGGAC 3', and the reverse primer is 5' TGCCTACTCCATCCAAAGC 3'. These primers can be used to amplify the mouse and rat ENDO1 homologues. A dose-response assay was carried out to determine the optimal amount of cDNA to be used for PCR amplification using the following: 5 min at 95°C, 30 sec at 95°C, 30 sec annealing temperature for each oligonucleotide pair, and 1min/kb at 72°C, repeated at the optimal number of cycles. The reaction for ENDO1 was carried out at an annealing temperature of 53.0°C for approximately 27 cycles. The amplification of GATA-4 was done using the conditions previously established in our lab. Amplification of GAPDH was used as an internal control. PCR products were resolved on 1.2% agarose gels. The PCR analysis was repeated three times to confirm the results.

2.8. cDNA probe synthesis for Northern and Southern Blot analysis

cDNA probes were ^{32}P labeled by random priming. 1 µg of $pd(N)_6$ and 20-50ng of cDNA were denatured at 100°C for 5min and allowed to re-anneal for 5 min on ice. cDNA fragments were synthesized using 2 units of DNA polymerase I Klenow fragment (Invitrogen). The reaction was performed in 50mM Tris-Cl pH 8.0, 5mM $MgCl_2$, 10mM

β-mercaptoethanol, 200mM HEPES pH 6.6, dATP (0.4mM), dGTP (0.4mM), dTTP (0.4mM), BSA 0.4 mg/mL, and ATP (0.6mM) for 2 hours at room temperature. The probes were precipitated by adding 1 µl tRNA, 30 µl 3M NH_4Oac pH 5.2, and 150 µl 95% ethanol. The cDNA was then centrifuged for 20 min at 10 000 rpm and the pellet was resuspended in 200 µl TE pH 8.0 Buffer. The radioactive count was determined for 1 µl of the probe using a scintillation vial. The probe was then boiled for 5 minutes and put in the hybridization buffer for the Northern or Southern Blots.

2.9. Northern Blot analysis

Total RNA (20 µg) from mice tissues or cell lines were denatured with formaldehyde and formamide and electrophoresis on a 1% agarose gel. This was then transferred to a nylon membrane (Maximum strength NYTRAN, Schleicher & Schuell Inc.) overnight. The membrane was UV crosslinked using the stratalinker 2400 (Stratagene). To ensure that no degradation of RNA occurred and that an equal amount of RNA was loaded, the Northern Blot was stained for approximately 30-60 min with methylene blue before hybridization. The membrane was then incubated with a prehybridization solution (0.2M $NaPO_4$ pH 7.2, 1mM EDTA, 1% BSA, 7% SDS, and 45% deionized formamide) for at least 2 hours at 42°C. The hybridization solution is identical to the prehybridization solution except that 1g of dextran sulfate was added to 10mL of the hybridization solution in order to optimize binding of the probe to the RNA. Hybridization was performed at 42°C overnight with an ENDO1 ^{32}P radioactively labeled probe corresponding to the open reading frame of ENDO1. After hybridization, the blot was washed at 65°C in washing buffer (40mM NaPO4 pH 7.2, 1mM EDTA, and 1% SDS) and analyzed by autoradiography. GAPDH was used as an internal control.

2.10. Immunofluorescence staining

The cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes. Cells were washed again with PBS and then blocked for one hour with TBS 1X 0.2% tween 5% BSA (bovine serum albumin), cells were incubated with the HA antibody (from Santa Cruz Biotechnology Inc., #sc-805) 1/500, or ENDO1 antibody (made previously in our lab) 1/500, overnight at 4°C. Following this step, cells were incubated

with anti-rabbit biotinylated antibody (from Vector Laboratories, #BA-1000) 1/250, and then one hour with avidin-fluorescein antibody (from Vector Laboratories, #A-2001) 1/250, with approximately 3-5 minutes of repeated washing with TBS 1X 0.2% tween in between. Every step after the last antibody was carried out in the dark. The Hoescht dye was then added to the cells for 10 minutes to stain the nuclei of the cells. The coverslips were mounted in DABCO and were examined under a fluorescence microscope.

2.11. Whole cell protein extracts

Once cells reached approximately 90% confluency, the cells were harvested into PBS in a microcentrifuge tube and centrifuged for 1 min to a pellet. This step was repeated twice in order to make sure that there was complete removal of the growth medium. The pellet was then resuspended in 100 µl of extraction buffer (20mM Hepes pH 7.8, 450mM NaCl, 0.4mM EDTA, 0.5mM DTT, 25% glycerol, 0.5mM PMSF). Cells were then momentarily incubated in dry ice and then a 37°C bath three times to lyse the cells. Samples were then centrifuged for 10 min at 4°C and the supernatant was collected.

2.12. Western Blot analysis

Whole cell extracts of transfected and non-transfected cells were prepared. 20 µg of protein extracts were boiled in Laemmli Buffer for 5 min, and then separated on a 12% polyacrylamide gel. The gel was then transferred on hybond-PVDF membrane (Immobilon transfer membranes from Millipore) and immunoblotted with an anti-HA antibody (from Santa Cruz Biotechnology Inc., #sc-805) 1/500, or an anti-ENDO1 antibody 1/1000, overnight at 4°C and then with an anti-rabbit peroxidase (from Sigma, #A 6154) at a dilution of 1/100 000. The revelation was carried out using the ECL plus western blotting detection system from Amersham Biosciences.

2.13. Cell cultures and transfections

NIH 3T3 cells were grown in 10% FBS (fetal bovine serum) DMEM (Dulbecco's Modified Eagle's Medium) solution, whereas C2C12 cells were grown in 20% FBS DMEM solution. Transfections were performed using the calcium phosphate precipitation method (281). Typically, 3 x 10^5 NIH 3T3 or C2C12 cells were plated in

35mm dishes 24 hours before transfection, and the medium was changed approximately 2-4 hours before transfection. In all experiments, the total amount of DNA was kept constant (usually about 2-2.5 μg per well) and DNA was pre-mixed in a 2X $CaCl_2$ solution. The $CaCl_2$ mixture was incorporated, drop-by-drop, in a 2X HBS solution, and gently mixed by air bubbling. The DNA precipitates were then added to the medium and the next morning, the medium changed. After 36-42 hours, cells were washed with PBS and lysed for 5-10 min. 100 μl was tested for luciferase activity with an LKB luminometer (283). The data was recorded automatically and expressed as the ratio of the results obtained with Sense ENDO1/Antisense ENDO1. For the deletion/mutation constructs of ENDO1, the results were also taken as a ratio, where the results obtained by ENDO1A, 1C, and 1D were taken over the results obtained by the Antisense ENDO1.

2.14. Phenylephrine stimulation of 4-day postnatal rat ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from 4-day old rats and 4 x 10^6 cells were plated on a dish in 10% FBS DMEM solution. The next day, the cells were washed and incubated with serum-free hormone-free media. Cells were stimulated with phenylephrine (0.1mM) beginning at 36 hours before whole cell protein extraction. The cells were stimulated every 12 hours with phenylephrine and the control cells were stimulated with the vehicle (0.1 mM ascetic acid). The media was swirled immediately after stimulation in order to allow equal exposure of the cells to the hypertrophic stimulating agent.

2.15. Induction of P19 cell differentiation

P19 cells were grown as aggregates (in bacteriology-grade plastic dishes) and exposed with differentiation agents for 4 days before being spread on culture dishes. Cardiac muscle differentiation was obtained by exposing P19 cells to 0.8% DMSO. During this process, the medium was changed every two days. The cardiac phenotype was visible after 5 days of differentiation in DMSO and aggregates started beating. Up to 30% of the aggregates are beating after 7 days of differentiation. Cells were extracted for RNA after approximately 12 days of differentiation.

3. RESULTS

3.1. Isolation of a novel transcript that is differentially regulated by GATA-4

In order to carry out the objective of identifying new downstream targets of GATA-4 or GATA-6, an RT-PCR differential display was carried out by another investigator in the lab. This approach utilizes adenoviruses that contain either a control vector containing LacZ, antisense GATA-4 or antisense GATA-6. Following infection of 4-day old postnatal rat cardiomyocytes with these adenoviruses, the mRNA is collected from the cells. RT-PCR is carried out using random oligos and autoradiography and gel electrophoresis is performed in order to identify differentially regulated transcripts which were further characterized by cloning and DNA sequencing. As depicted in Figure 1, a novel transcript, named ENDO1, was identified which appeared to be a downstream target of GATA-4 and not GATA-6. Based on the change of the expression level in this assay, ENDO1 is downregulated by GATA-4. Interestingly, ENDO1 was not the only novel transcript that was differentially regulated by GATA-4 or GATA-6. However, preliminary bioinformatic analysis revealed ENDO1 to belong to a hypothetical protein that contains a potential PHD (Plant homeodomain) finger. Proteins containing PHD fingers have been shown to be involved in chromatin mediated transcriptional regulation and/or protein-protein interactions (284). Table 1 lists some of the known PHD finger containing proteins and the multiple functions they play during development. Since many of the proteins play a role in transcriptional regulation, it was hypothesized that ENDO1 could be a novel transcriptional regulator in the heart. We thus decided to pursue further characterization of this gene. It is also noteworthy that, as a positive control for the RT-PCR differential display approach, several transcripts known to be targets for GATA-4 or GATA-6 were also identified in this screen, such as ANF (data not shown).

3.2. Isolation of the full open reading frame of ENDO1

The cDNA fragment obtained by the RT-PCR differential display, named 1.20.1c, was 346bp long. Using Clone Manager Version 7.0, an ORF was not detected with the 346bp sequence. Therefore, in order to obtain the full ORF, 1.20.1c was used as a probe to screen a cDNA rat cardiomyocyte library previously made in our lab. Three clones

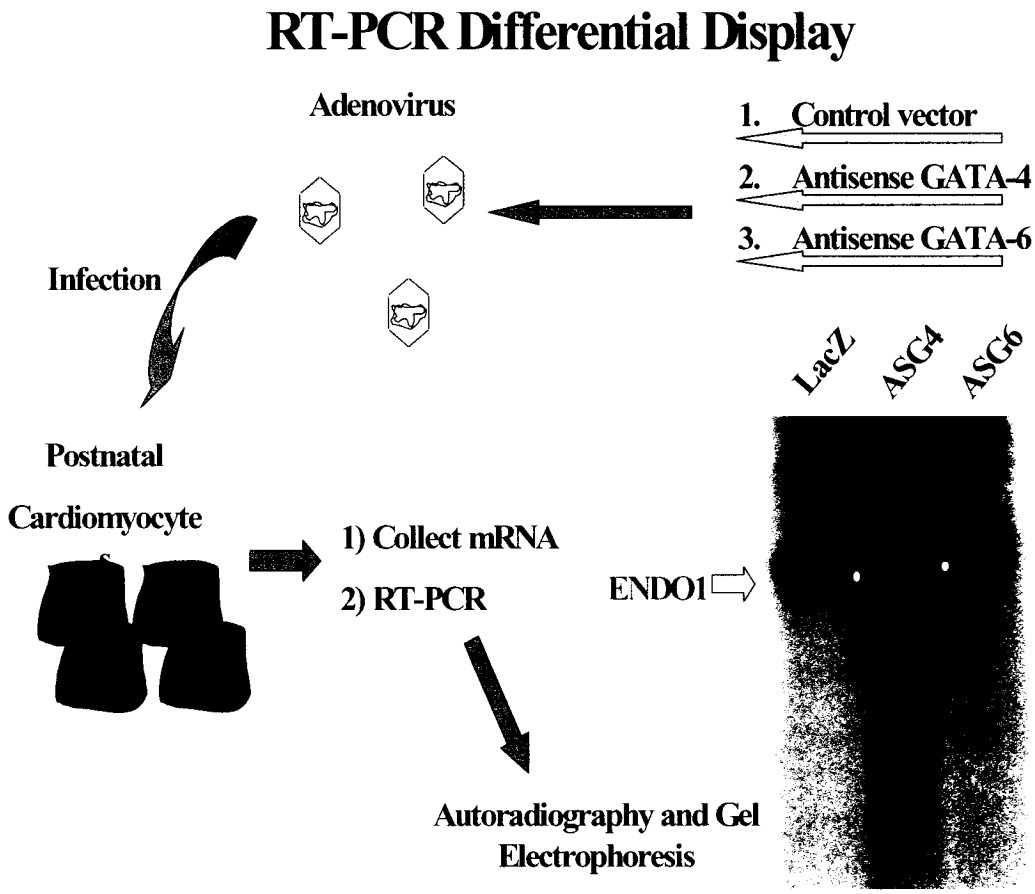

Figure 1. RT-PCR Differential Display Reveals A Novel Transcript, ENDO1, Which Is Differentially Regulated By GATA-4. Autoradiography analysis reveals a novel transcript, ENDO1, to be upregulated when cells are treated with Antisense GATA-4. There is no change in the levels of this transcript between cells with were treated with the adenovirus containing LacZ or Antisense GATA-6 (ASG6).

Protein:	Transcriptional Regulator	Transcriptional Co-regulator	E3 Ubiquitin Ligase	Histone Acetyltransferase	Histone Deacetylase	Cell Cycle	Other
K3			√				
K5			√				
c-MIR			√				
TRIP-Br1	√						
Pf1		√					
BHC	√				√		
CBP		√		√			
P300		√		√			
DNMT3L		√					
PHF6	(√)						
P47ING3	√					√	
ING2						√	√
ACF							√
Polycomblike	√						
Trithorax	√						
MEKK1			√				√
Pygopus		√					√
TIP5		√					
MMD1	√						
NIRF						√	
ARA267-α		√					

Table 1. PHD Finger Proteins Have Diverse Functions During Development. This chart lists some of the known PHD finger proteins and the multiple roles that most of them play. The checkmark in parentheses means that this is a predicted function of the protein and is not yet confirmed. (References for these proteins can be found in the discussion section.)

were isolated: 1A (1472bp), 12B (1074bp), 9C1 (1375bp). As indicated by Figure 2, the full sequence obtained from each of the clones is aligned with the 1.20.1c cDNA fragment. The boxed area shows 98.5% homology of the three clones with 1.20.1c. The differences detected in the sequence between the three clones may be due to errors in sequencing or naturally occurring mutations in the cDNA library. In bold print is the full open reading frame of ENDO1 that was obtained from clone 1A (determined by the Clone Manager program), and when blasted against the rat genome, it was found to be 100% homologous to rat chromosome 7 (7q12). As indicated by the sequence alignment, 1.20.1c appears to have mapped mostly at the 3' UTR (Untranslated Region). Furthermore, Northern Blot analysis using 1.20.1c as a probe indicated that the mRNA transcript is approximately 1.4kb (Figure 9), which is the approximate size of clone 1A. At present, only one rat EST (Expressed Sequence Tag) has been reported to contain part of the 3' UTR. This EST is listed in the Ensembl database (www.ensembl.org) and its transcript ID number is ENSRNOT00000010399. However, our 1A clone contains a 3' UTR region much greater than the EST listed in the database. Therefore, since the size of our clone is almost the same size as the mRNA detected in the Northern Blot, we feel that most, if not all, of the 5' and 3' UTR has also been isolated from the cDNA screening.

3.3. ENDO1 is an evolutionarily conserved transcript

A BLAST search performed at the NCBI website indicated ENDO1 to be homologous to human and murine hypothetical proteins as indicated in Figure 3A. Incidentally, a previous student in the lab had isolated the mouse homologue of ENDO1 when trying to look for new genes that are involved in endothelial cell differentiation. Hence, ENDO1 stands for endothelial clone 1. Amino acid comparison of the mouse and rat ENDO1 sequences revealed a difference of only 2 amino acids out of 246 amino acids, whereas in the human, there is a difference of 12 amino acids (Figure 3B). The Gene bank accession numbers are AAH02144.1 and AAH32624.1 for the mouse and human proteins respectively. Interestingly, between the three species, there is only one amino acid difference in the PHD finger.

Figure 4A looks more specifically at the PHD finger. BLAST searches revealed that the PHD consensus sequence (C_4HC_3), along with additional residues in ENDO1, is

```
1.20.1c         ------------------------------------------------------------
1A              AGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCG
12B             ------------------------------------------------------------
9C1             ------------------------------------------------------------

1.20.1c         ------------------------------------------------------------
1A              GCACGAGCGGAGCCGGAGCAAGCCGCTGCTGACGGGCCGGGCCGGGCCGGGACCCGGGCC
12B             ------------------------------------------------------------
9C1             ------------------------------------------------------------

1.20.1c         ------------------------------------------------------------
1A              AGGGAGAACCGAGGGGCCTGCGCGGCCGCCCGGCCCGGCCGCGGAACAGGCTCCTGGGAC
12B             ------------------------------------------------------------
9C1             ----------------------------CGGCACGAGCCGCGGAACAGGCTCCTGGGAC

1.20.1c         ------------------------------------------------------------
1A              CATGGGCCTCAGGCCCTGAGGACAGGAGGCTCCCTGTGGCCATGACGACAGGTGACTGTT
12B             ------------------------------------------------------------
9C1             CATGGGCCTCAGGCCCTGAGGACAGGAGGCTCCCTGTGGCCATGACGACAGGTGACTGTT

1.20.1c         ------------------------------------------------------------
1A              GCCACCTCCCTGGCTCCCTATGTGACTGCTCTAGCAGCCCTGCCTTTTCCAAGGTTGTGG
12B             ------------------------------------------------------------
9C1             GCCACCTCCCTGGCTCCCTATGTGACTGCTCTAGCAGCCCTGCCTTTTCCAAGGTTGTGG

1.20.1c         ------------------------------------------------------------
1A              AGGCCACAGGGCTTGGACCACCTCAGTATGTAGCTCAAGTGACTTCAAGGGATGGCCGGC
12B             ------------------------------------------------------------
9C1             AGGCCACAGGGCTTGGACCACCTCAGTATGTAGCTCAAGTGACTTCAAGGGATGGCCGGC

1.20.1c         ------------------------------------------------------------
1A              TGCTCTCAACTGTCATCCGGGCTTTGGATACACCGAGTGACTGTCCCTTCTGCCGAATCT
12B             ----------------------------------------CGGCACGAGCTGCCGAATCT
9C1             TGCTCTCAACTGTCATCCGGGCTTTGGATACACCGAGTGACTGTCCCTTCTGCCGAATCT

1.20.1c         ------------------------------------------------------------
1A              GCCATGAGGGAGCAAATGGGGAGAACTTGCTGTCCCCATGTGGCTGTACTGGCACCCTGG
12B             GCCATGAGGGAGCAAATGGGGAGAACTTGCTGTCCCCATGTGGCTGTACTGGCACCCTGG
9C1             GCCATGAGGGAGCAAATGGGGAGAACTTGCTGTCCCCATGTGGCTGTACTGGCACCCTGG

1.20.1c         ------------------------------------------------------------
1A              GAGCTGTGCACAAGAGCTGCCTGGAGAAATGGCTGTCTTCCTCCAACACCAGCTACTGTG
12B             GAGCTGTGCACAAGAGCTGCCTGGAGAAATGGCTGTCTTCCTCCAACACCAGCTACTGTG
9C1             GAGCTGTGCACAAGAGCTGCCTGGAGAAATGGCTGTCTTCCTCCAACACCAGCTACTGTG

1.20.1c         ------------------------------------------------------------
1A              AGCTGTGTCACACTGAGTTTGCAGTGGAAAAGCGGCCCCGACCTCTCACAGAGTGGCTAA
12B             AGCTGTGTCACACTGAGTTTGCAGTGGAAAAGCGGCCCCGACCTCTCACAGAGTGGCTAA
9C1             AGCTGTGTCACACTGAGTTTGCAGTGGAAAAGCGGCCCCGACCTCTCACAGAGTGGCTAA

1.20.1c         ------------------------------------------------------------
1A              AGGACCCAGGGCCGCGCACCGAGAAGCGGACACTGTGCTGTGACATGGTGTGCTTTGTGT
12B             AGGACCCAGGGCCGCGCACCGAGAAGCGGACACTGTGCTGTGACATGGTGTGCTTTGTGT
9C1             AGGACCCAGGGCCGCGCACCGAGAAGCGGACACTGTGCTGTGACATGGTGTGCTTTGTGT
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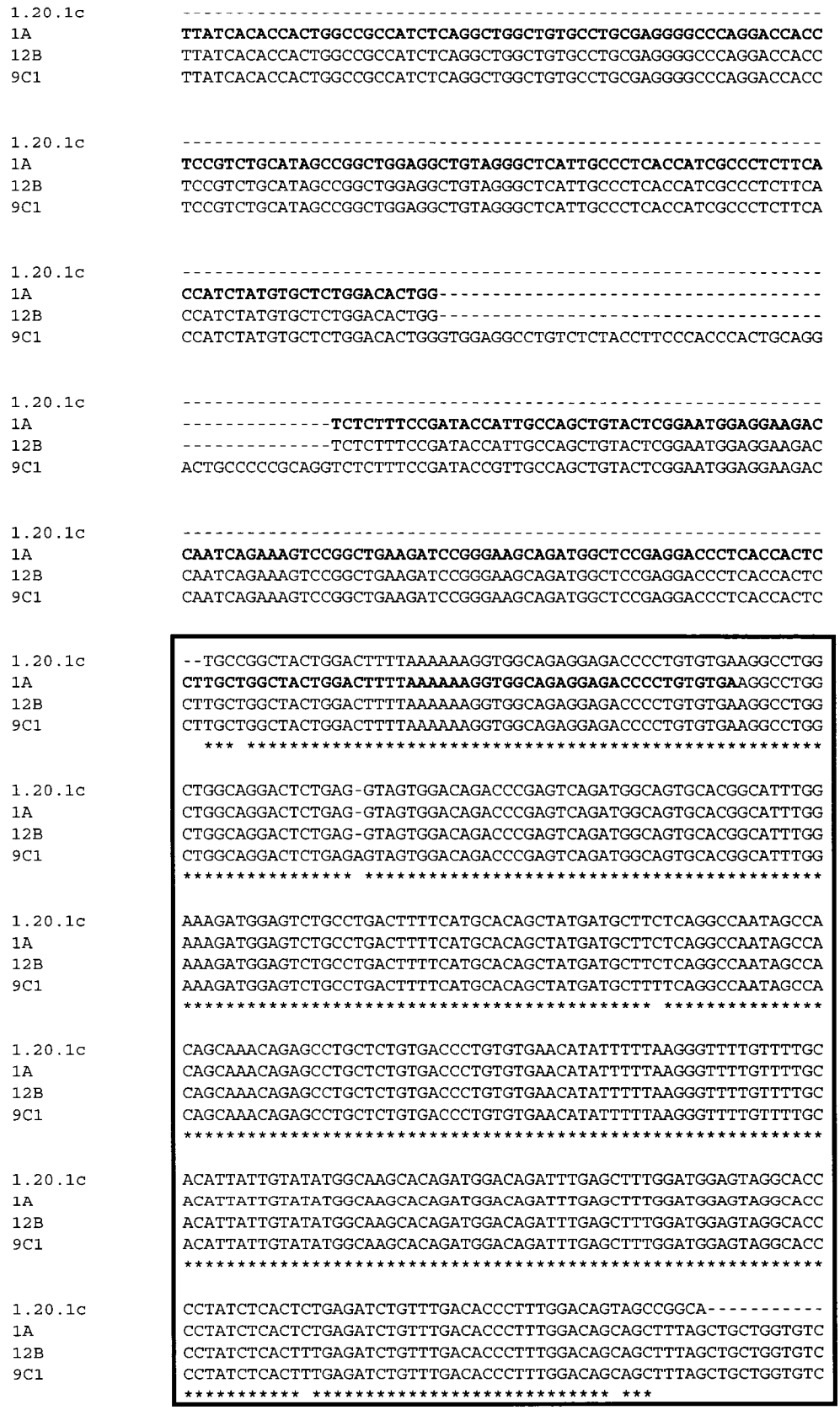
1.20.1c
1A
12B
9C1
TTATCACACCACTGGCCGCCATCTCAGGCTGGCTGTGCCTGCGAGGGGCCCAGGACCACC
TTATCACACCACTGGCCGCCATCTCAGGCTGGCTGTGCCTGCGAGGGGCCCAGGACCACC
TCCGTCTGCATAGCCGGCTGGAGGCTGTAGGGCTCATTGCCCTCACCATCGCCCTCTTCA
TCCGTCTGCATAGCCGGCTGGAGGCTGTAGGGCTCATTGCCCTCACCATCGCCCTCTTCA
CCATCTATGTGCTCTGGACACTGG
CCATCTATGTGCTCTGGACACTGG
CCATCTATGTGCTCTGGACACTGGGTGGAGGCCTGTCTCTACCTTCCCACCCACTGCAGG
TCTCTTTCCGATACCATTGCCAGCTGTACTCGGAATGGAGGAAGAC
TCTCTTTCCGATACCATTGCCAGCTGTACTCGGAATGGAGGAAGAC
ACTGCCCCCGCAGGTCTCTTTCCGATACCGTTGCCAGCTGTACTCGGAATGGAGGAAGAC
CAATCAGAAAGTCCGGCTGAAGATCCGGGAAGCAGATGGCTCCGAGGACCCTCACCACTC
CAATCAGAAAGTCCGGCTGAAGATCCGGGAAGCAGATGGCTCCGAGGACCCTCACCACTC
CAATCAGAAAGTCCGGCTGAAGATCCGGGAAGCAGATGGCTCCGAGGACCCTCACCACTC
--TGCCGGCTACTGGACTTTTAAAAAAGGTGGCAGAGGAGACCCCTGTGTGAAGGCCTGG
CTTGCTGGCTACTGGACTTTTAAAAAAGGTGGCAGAGGAGACCCCTGTGTGAAGGCCTGG
CTTGCTGGCTACTGGACTTTTAAAAAAGGTGGCAGAGGAGACCCCTGTGTGAAGGCCTGG
CTGGCAGGACTCTGAG-GTAGTGGACAGACCCGAGTCAGATGGCAGTGCACGGCATTTGG
CTGGCAGGACTCTGAG-GTAGTGGACAGACCCGAGTCAGATGGCAGTGCACGGCATTTGG
CTGGCAGGACTCTGAG-GTAGTGGACAGACCCGAGTCAGATGGCAGTGCACGGCATTTGG
CTGGCAGGACTCTGAGAGTAGTGGACAGACCCGAGTCAGATGGCAGTGCACGGCATTTGG
AAAGATGGAGTCTGCCTGACTTTTCATGCACAGCTATGATGCTTCTCAGGCCAATAGCCA
AAAGATGGAGTCTGCCTGACTTTTCATGCACAGCTATGATGCTTCTCAGGCCAATAGCCA
AAAGATGGAGTCTGCCTGACTTTTCATGCACAGCTATGATGCTTCTCAGGCCAATAGCCA
AAAGATGGAGTCTGCCTGACTTTTCATGCACAGCTATGATGCTTTTCAGGCCAATAGCCA
CAGCAAACAGAGCCTGCTCTGTGACCCTGTGTGAACATATTTTTAAGGGTTTTGTTTTGC
CAGCAAACAGAGCCTGCTCTGTGACCCTGTGTGAACATATTTTTAAGGGTTTTGTTTTGC
CAGCAAACAGAGCCTGCTCTGTGACCCTGTGTGAACATATTTTTAAGGGTTTTGTTTTGC
CAGCAAACAGAGCCTGCTCTGTGACCCTGTGTGAACATATTTTTAAGGGTTTTGTTTTGC
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ACATTATTGTATATGGCAAGCACAGATGGACAGATTTGAGCTTTGGATGGAGTAGGCACC
ACATTATTGTATATGGCAAGCACAGATGGACAGATTTGAGCTTTGGATGGAGTAGGCACC
ACATTATTGTATATGGCAAGCACAGATGGACAGATTTGAGCTTTGGATGGAGTAGGCACC
CCTATCTCACTCTGAGATCTGTTTGACACCCTTTGGACAGTAGCCGGCA
CCTATCTCACTCTGAGATCTGTTTGACACCCTTTGGACAGCAGCTTTAGCTGCTGGTGTC
CCTATCTCACTTTGAGATCTGTTTGACACCCTTTGGACAGCAGCTTTAGCTGCTGGTGTC
CCTATCTCACTTTGAGATCTGTTTGACACCCTTTGGACAGCAGCTTTAGCTGCTGGTGTC

```
1.20.1c         ------------------------------------------------------------
1A              AAGGGTGCGTGCCCATTGGGCTAGAAGGTTCCAGCAGGCTTCTTGCATTACTCTGCACCT
12B             AAGGGTGCGTGCCCATTGGGCTAGAAGGTTCCAGCAGGCTTCTTGCATTACTCTGCACCT
9C1             AAGGGTGCGTGCCCATTGGGCTAGAAGGTTCCAGCAGGCTTCTTGCATTACTCTGCACCT

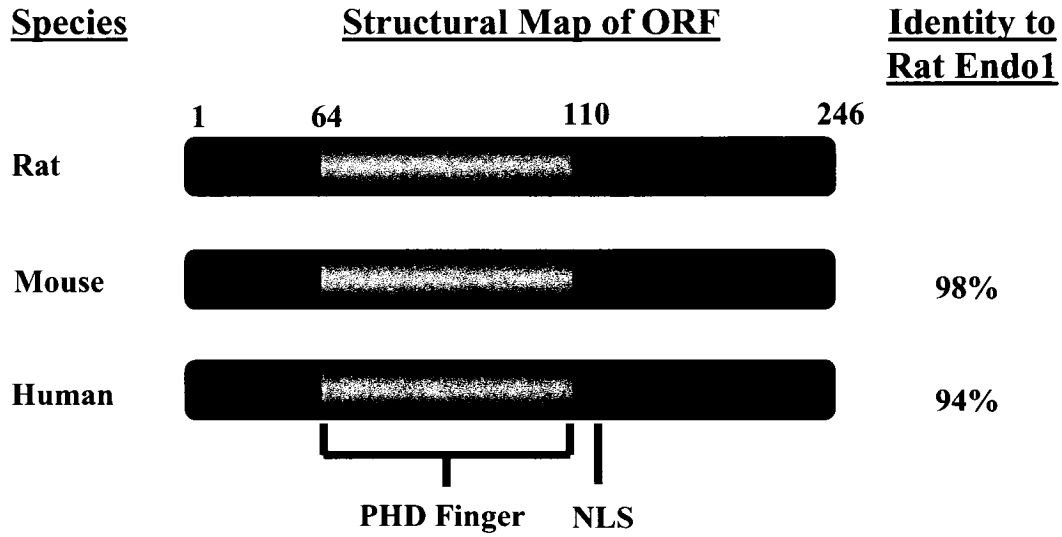

1.20.1c         ------------------------------------------------------------
1A              AGCTGGCTTGCTTTACTGGCACTCTTGACTTATAAACTTATAAAGTTGCACTGCATTTCA
12B             AGCTGGCTTGCTTTACTGGCACTCTTGACTTATAAACTTATAAAGTTGCACTGCATTTCA
9C1             AGCTGGCTTGCTTTACTGGCACTCTTGACTTATAAACTTATAAAGTTGCACTGCATTTCA


1.20.1c         ------------------------------------------------------------
1A              AAAACCTACTCCTAAATGAATAAAAGGAGCCCTTTGTTGGCTAAAAAAAAAAAAAAAAAA
12B             AAAACCTACTCCTAAATGAATAAAAGGAGCCCTTGTTGGCTAATATGGAAAAAAAAAAAA
9C1             AAAACCTACTCCTAAATGAATAAAAGGAGCCCTTGTTGGCTAAAAAAAAAAAAAAAAAAA


1.20.1c         ------------------------
1A              AAAAAAAAAAA-CTCGAGCCCGGT
12B             AAAAAAAAAAA-CTCAG-------
9C1             AAAAAAAAAAAACTCGAGCCCGGT
```

Figure 2. Sequence Alignment Of The Isolated cDNA Fragment With The Clones Obtained. The full nucleotide sequence of all the clones isolated (1A, 12B, and 9C1) and the sequence of the cDNA fragment (1.20.1c) that was obtained from the RT-PCR differential display are aligned above. The sequence alignment in the box shows the sequence homology between 1.20.1c and all of the clones. The sequence in 1A which is indicated in bold is the full 741 base pair open reading frame of ENDO1.

A.



B.

```
RAT       MTTGDCCHLPGSLCDCSSSPAFSKVVEATGLGPPQYVAQVTSRDGRLLSTVIRALDTPSD
MOUSE     MTTGDCCHLPGSLCDCSSSPAFSKVVEATGLGPPQYVAQVTSRDGRLLSTVIRALDSQSD
HUMAN     MTTGDCCHLPGSLCDCSGSPAFSKVVEATGLGPPQYVAQVTSRDGRLLSTVIRTLDTPSD
          ******************************************************.**. **

RAT       CPFCRICHEGANGENLLSPCGCTGTLGAVHKSCLEKWLSSSNTSYCELCHTEFAVEKRPR
MOUSE     CPFCRICHEGANGENLLSPCGCTGTLGAVHKSCLEKWLSSSNTSYCELCHTEFAVEKRPR
HUMAN     GPFCRICHEGANGECLLSPCGCTGTLGAVHKSCLEKWLSSSNTSYCELCHTEFAVEKRPR
          ************ ***********************************************

RAT       PLTEWLKDPGPRTEKRTLCCDMVCFVFITPLAAISGWLCLRGAQDHLRLHSRLEAVGLIA
MOUSE     PLTEWLKDPGPRTEKRTLCCDMVCFVFITPLAAISGWLCLRGAQDHLRLHSRLEAVGLIA
HUMAN     PLTEWLKDPGPRTEKRTLCCDMVCFLFITPLAAISGWLCLRGAQDHLRLHSQLEAVGLIA
          ************************.***************************.********

RAT       LTIALFTIYVLWTLVSFRYHCQLYSEWRKTNQKVRLKIREADGSEDPHHSLLATGLLKKV
MOUSE     LTIALFTIYVLWTLVSFRYHCQLYSEWRKTNQKVRLKIREADGSEDPHHSLLATGLLKKV
HUMAN     LTIALFTIYVLWTLVSFRYHCQLYSEWRKTNQKVRLKIREADSPEGPQHSPLAAGLLKKV
          ******************************************* * *.** **.*******

RAT       AEETPV
MOUSE     AEETPV
HUMAN     AEETPV
          ******
```

Figure 3. The ENDO1 Amino Acid Sequence Is Conserved Between Rats, Mice, And Humans. (A) The full rat ENDO1 protein transcript is highly conserved with that of mouse and humans. (B) The full 246 amino acid open reading frame is aligned with ENDO1 homologues from rats, mice and humans. The red boxes indicate amino acids that are conserved only between rats and mice. The blue boxes indicate the amino acids that are only conserved between rats and humans. The PHD finger is highlighted in green.

evolutionarily conserved. The Gene bank accession numbers are AF164113, NP_172878, AF130849, X76715, AF155739, NP_848545.1, and NP_611511.3 for STAT-B, putative phosphatase protein, PIT1, SSM4, axotrophin, the human putative protein, and the drosophila putative protein respectively. In addition, as depicted in Figure 4B, some PHD finger proteins that are members of the E3 ubiquitin ligase family were also found to be evolutionary conserved with ENDO1. Kaposi's sarcoma associated-herpes viruses (KSHV), MIR1 and MIR2 (modulator of immune recognition), along with a human homologue of MIR, c-MIR (cellular MIR), were found to be 53% conserved in the PHD finger domain, suggesting that ENDO1 may be a putative E3 ubiquitin ligase.

In particular, a subset of putative human and murine proteins were identified in the BLAST searches, which had approximately 50% of the PHD domain sequence homologous to ENDO1. The Gene bank accession numbers for these proteins are NP_082196.1 (mouse putative protein), NP_848545.1 (human putative protein), NP_060393.1 (human putative protein), and AAF36160.1 (human putative protein). This suggests that ENDO1 may belong to a novel subset of PHD finger proteins whose function is yet unknown. Indeed, as indicated in Figure 5, 12 other hypothetical proteins were found from the Ensembl database (www.ensembl.org), which were highly conserved in the PHD finger and throughout the transcript. Listed are the Ensembl translation codes of the hypothetical proteins. Each colored box around the name of the protein represents the species from which the proteins come from. Translation codes beginning with "ENSP", "SINFRUP", "ENSMUSP", "ENSCBRP", and "ENSRNOP", represent proteins from humans, *Danio rerio*, *Fugu rubripes*, mouse, *Caenorhabditis briggsae*, and rat respectively. Remarkably, almost all of these proteins are approximately 50% conserved throughout the whole transcript, therefore, elucidating the functions of ENDO1 may in turn determine the overall function of this subset of proteins.

3.4. Gene map of ENDO1

ENDO1 is localized to human chromosome 19, mouse chromosome 17, and rat chromosome 7. The coding region of ENDO1 in all three species is encoded in 4 exons. Using bioinformatics, the full 5' and 3' UTR (Untranslated region) of the human and mouse ENDO1 is known. The 5' UTR comprises an additional exon whereas the 3' UTR

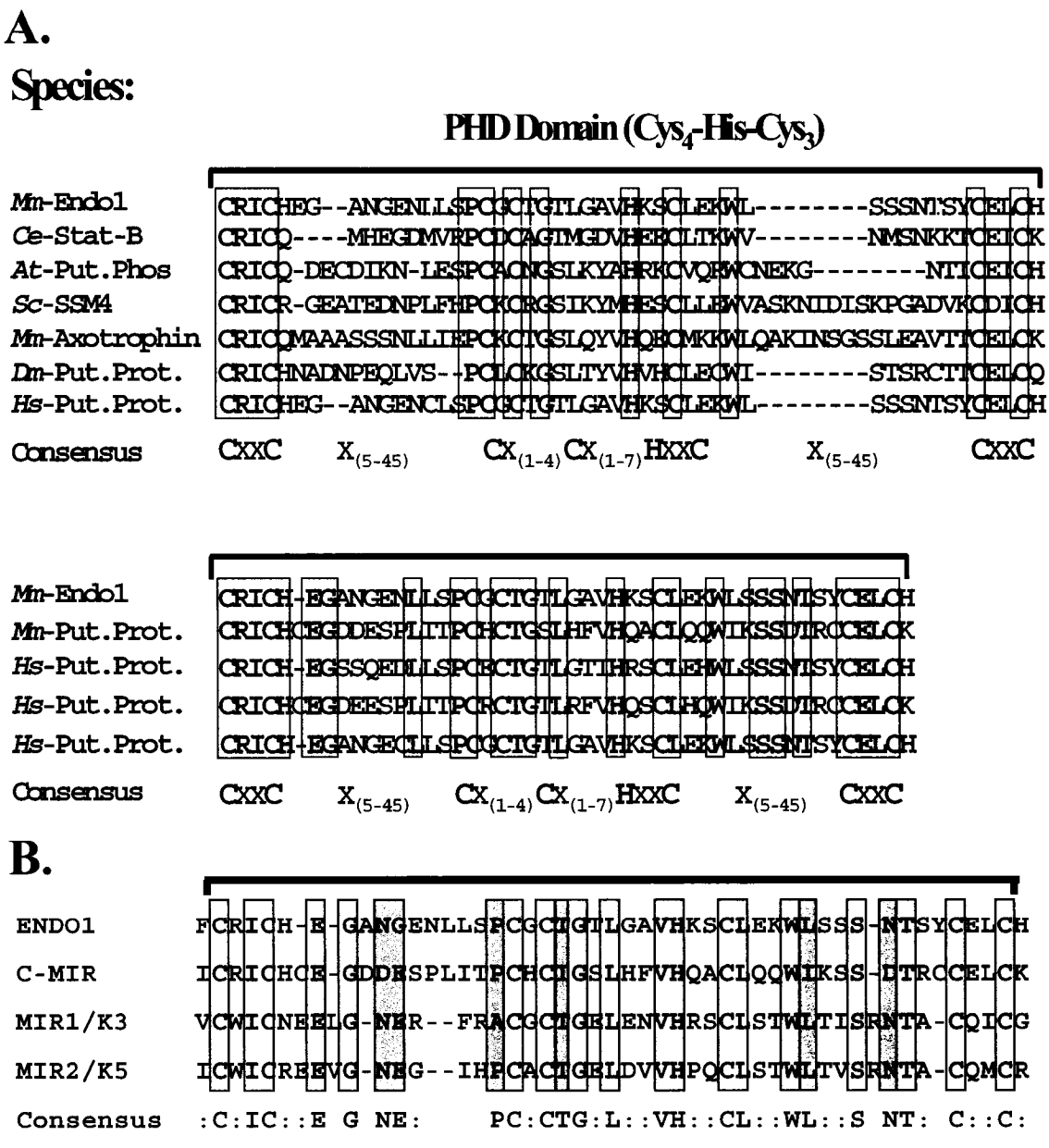
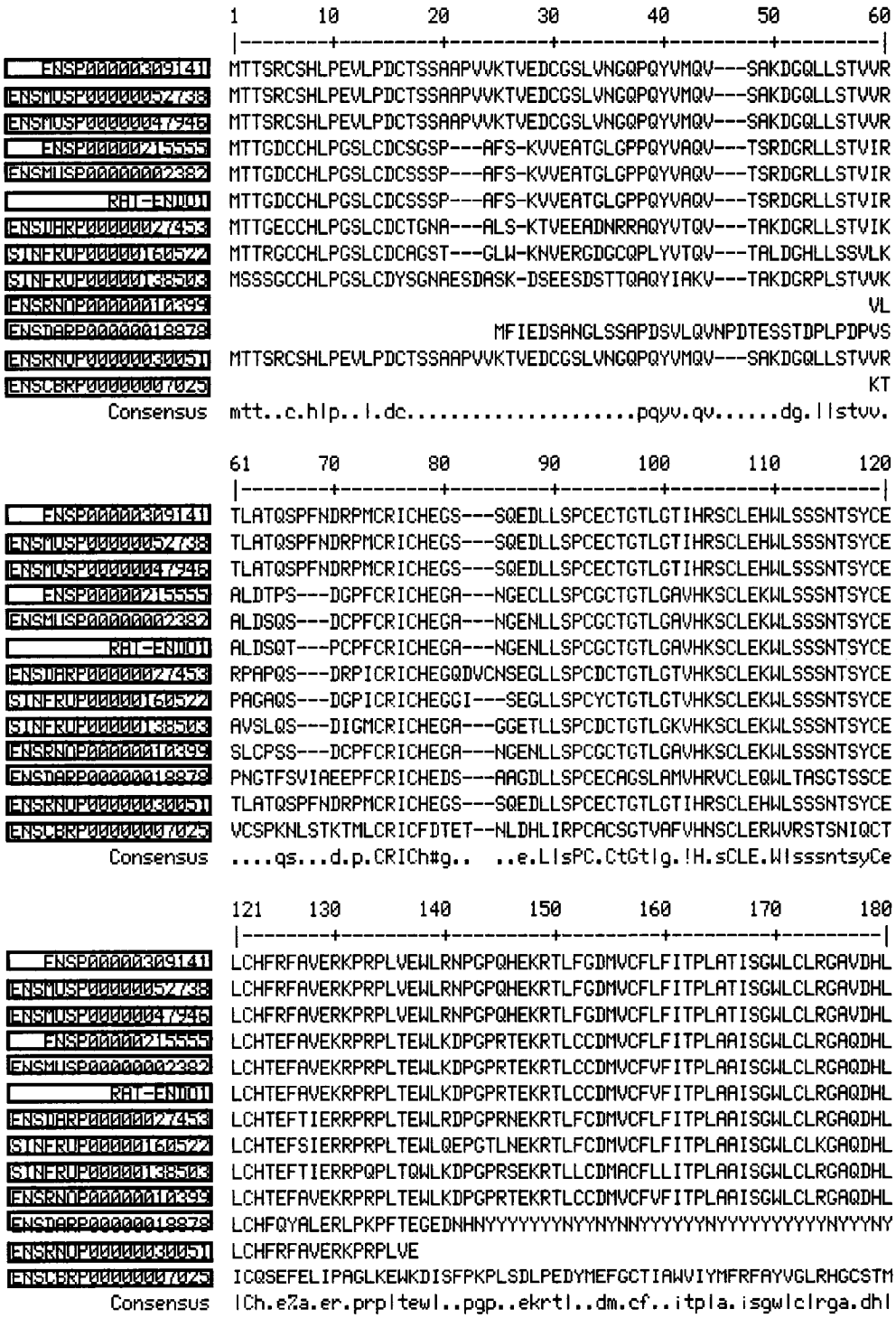


Figure 4. The ENDO1 PHD Domain Is Evolutionarily Conserved. (A) The PHD finger consensus sequence, along with other residues, are highly conserved between 6 different species, as indicated in the top half of the diagram. The bottom half shows an alignment of the mouse ENDO1 with other putative proteins whose function is yet unknown. (B) Amino acid sequence alignment of the PHD finger of ENDO1 with KSHV MIRs, and human c-MIR, reveals 53% homology with members of the E3 Ubiquitin Ligase family. The green boxes indicated 100% homology whereas the blue boxes indicate 75% homology with the different transcripts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ENSMUSP00000052738 MTTSRCSHLPEVLPDCTSSAAPVVKTVEDCGSLVNGQPQYVMQV---SAKDGQLLSTVVR
ENSMUSP00000047946 MTTSRCSHLPEVLPDCTSSAAPVVKTVEDCGSLVNGQPQYVMQV---SAKDGQLLSTVVR
ENSP00000215555 MTTGDCCHLPGSLCDCSGSP---AFS-KVVEATGLGPPQYVAQV---TSRDGRLLSTVIR
ENSMUSP00000002382 MTTGDCCHLPGSLCDCSSSP---AFS-KVVEATGLGPPQYVAQV---TSRDGRLLSTVIR
RAT-ENT001 MTTGDCCHLPGSLCDCSSSP---AFS-KVVEATGLGPPQYVAQV---TSRDGRLLSTVIR
ENSDARP00000027453 MTTGECCHLPGSLCDCTGNA---ALS-KTVEEADNRRAQYVTQV---TAKDGRLLSTVIK
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SINFRUP00000138503 MSSSGCCHLPGSLCDYSGNAESDASK-DSEESDSTTQAQYIAKV---TAKDGRPLSTVVK
ENSRNOP00000010399 VL
ENSDARP00000018878 MFIEDSANGLSSAPDSVLQVNPDTESSTDPLPDPVS
ENSRNOP00000030051 MTTSRCSHLPEVLPDCTSSAAPVVKTVEDCGSLVNGQPQYVMQV---SAKDGQLLSTVVR
ENSCBRP00000007025 KT
Consensus mtt..c.hlp..l.dc.....pqyv.qv.....dg.llstvv.
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RAT-ENT001 ALDSQT---PCPFCRICHEGA---NGENLLSPCGCTGTLGAVHKSCLEKWLSSSNTSYCE
ENSDARP00000027453 RPAPQS---DRPICRICHEGQDVCNSEGLLSPCDCTGTLGTVHKSCLEKWLSSSNTSYCE
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ENSRNOP00000010399 SLCPSS---DCPFCRICHEGA---NGENLLSPCGCTGTLGAVHKSCLEKWLSSSNTSYCE
ENSDARP00000018878 PNGTFSVIAEEPFCRICHEDS---AAGDLLSPCECAGSLAMVHRVCLEQWLTASGTSSCE
ENSRNOP00000030051 TLATQSPFNDRPMCRICHEGS---SQEDLLSPCECTGTLGTIHRSCLEHWLSSSNTSYCE
ENSCBRP00000007025 VCSPKNLSTKTMLCRICFDTET--NLDHLIRPCACSGTVAFVHNSCLERWVRSTSNIQCT
Consensus ....qs...d.p.CRICh#g.. ..e.LlsPC.CtGtlg.!H.sCLE.Wlsssntsyce
121 130 140 150 160 170 180
ENSP00000309141 LCHFRFAVERKPRPLVEWLRNPGPQHEKRTLFGDMVCFLFITPLATISGWLCLRGAVDHL
ENSMUSP00000052738 LCHFRFAVERKPRPLVEWLRNPGPQHEKRTLFGDMVCFLFITPLATISGWLCLRGAVDHL
ENSMUSP00000047946 LCHFRFAVERKPRPLVEWLRNPGPQHEKRTLFGDMVCFLFITPLATISGWLCLRGAVDHL
ENSP00000215555 LCHTEFAVEKRPRPLTEWLKDPGPRTEKRTLCCDMVCFLFITPLAAISGWLCLRGAQDHL
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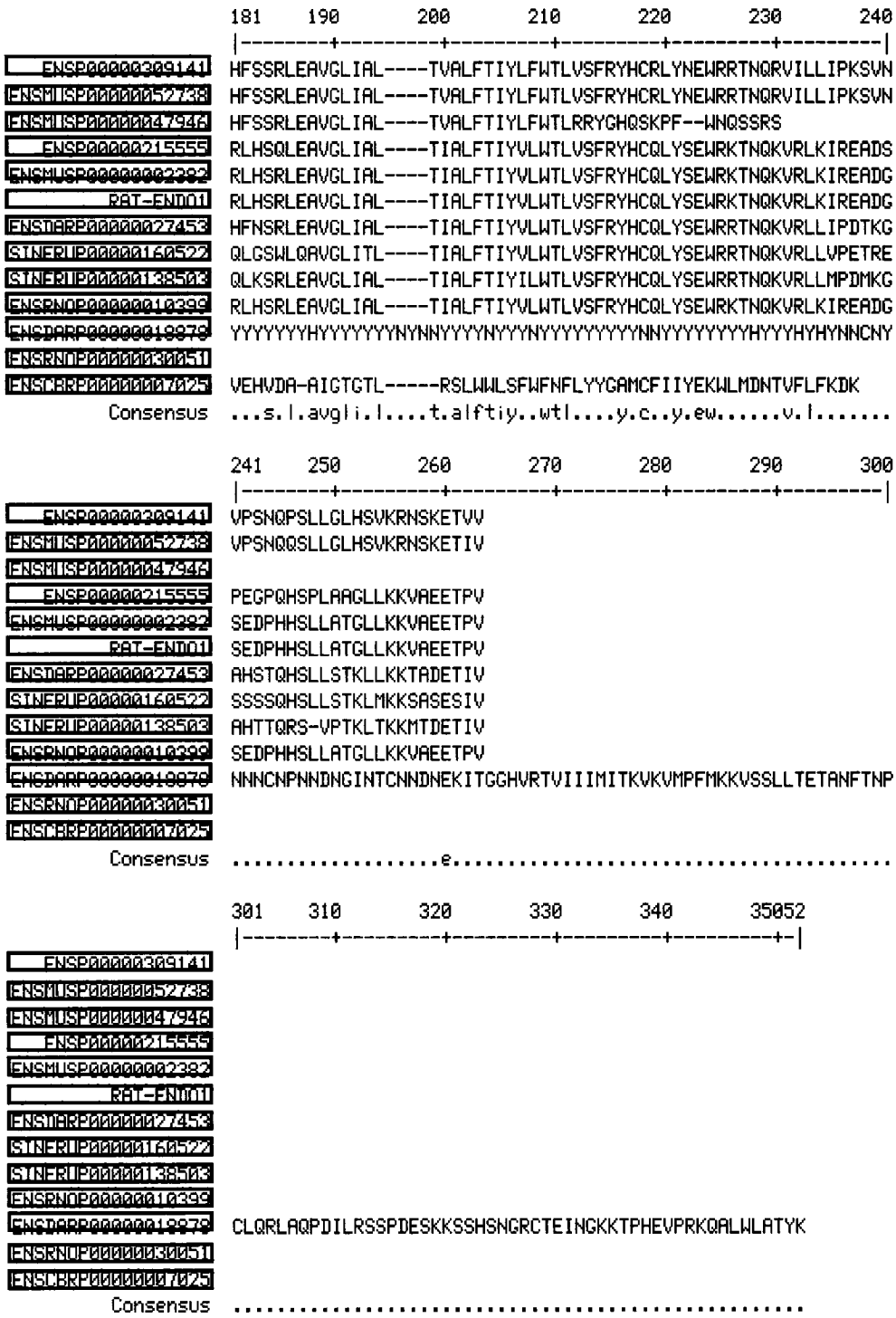


Figure 5. ENDO1 Belongs To A Family Of Unknown Proteins. The amino acid sequence of 12 other hypothetical proteins is aligned with rat ENDO1. The colored boxes indicate the different species from which the proteins come from. Translation codes beginning with "ENSP", "SINFRUP", "ENSMUSP", "ENSCBRP", and "ENSRNOP", represent proteins from humans, *Danio rerio*, *Fugu rubripes*, mouse, *Caenorhabditis briggsae*, and rat respectively.

is part of the last exon which contains the C-terminal coding region of ENDO1. Therefore in total there are 5 exons. For this reason, the gene structure map of the coding region of mouse ENDO1 begins at Exon2 and not at Exon1 as indicated in Figure 6A. Exon2 contains only the N-terminal region of ENDO1. The full PHD finger region including the putative nuclear localization sequence is encoded in Exon3. The two circles located at the ends of the PHD finger represent cytochrome c heme binding sites, which will be discussed later. Exon4 encodes the full transmembrane region and Exon5 includes the C-terminal end of ENDO1. Although we have isolated most of the 5' and 3' UTR, we cannot say for certain that the full 5' and 3' UTR region of the rat homologue has been isolated, since not many ESTs have been found to be homologous to this region. Figure 6B indicates the sequence of the exon/intron boundaries.

3.5. Spatial and temporal expression pattern of ENDO1

The expression pattern of ENDO1 in murine embryos was previously determined by another investigator in the lab using *in situ* hybridization (data not shown). ENDO1 was very abundant in regions such as the brain, liver, dorsal aorta, and blood vessels during mid-embryonic development. Furthermore, previous RT-PCR analysis shows that ENDO1 is differentially expressed in tissues during embryonic and postnatal development. RT-PCR conducted on embryonic tissues was consistent with *in situ* hybridization results. In postnatal tissues, ENDO1 is highly expressed in the brain, lung, liver, heart, and spleen. As shown in Figure 7, in the adult, high expression is retained in the brain, and is also present in ventricles, spleen, lung and kidneys, however expression in the liver is significantly reduced compared to embryonic and postnatal liver expression. There has not been extensive research on the expression pattern of GATA-4 in non-cardiac tissues. However, GATA-4 has been shown to be expressed in tissues that also express ENDO1 such as the liver (285). Future immunohistochemical analysis will be carried out in order to determine which cell types co-express ENDO1 and GATA-4 in cardiac and non-cardiac tissues.

3.6. ENDO1 is expressed in the differentiating murine heart

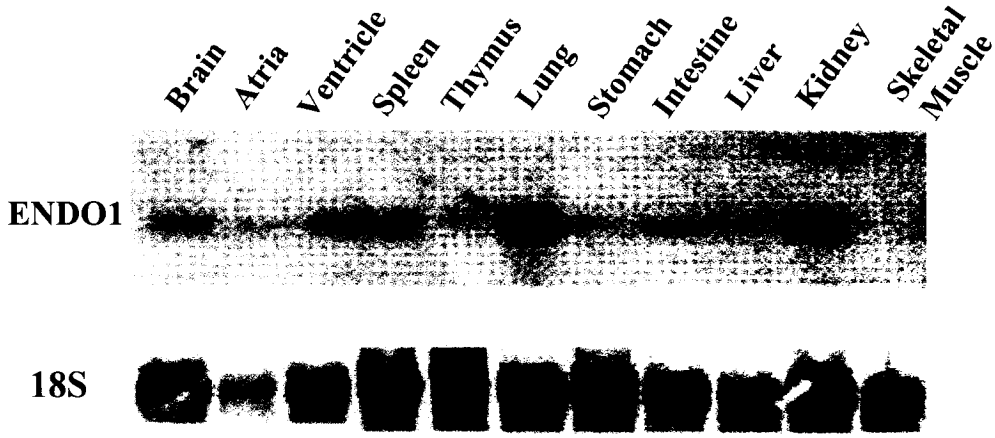


Figure 7. Differential Expression Pattern Of ENDO1 In Adult Mice. Northern blot analysis of the mRNA expression pattern of ENDO1 in adult mice. 18S subunit of the ribosome is used to control for the amount of mRNA loaded on gel.

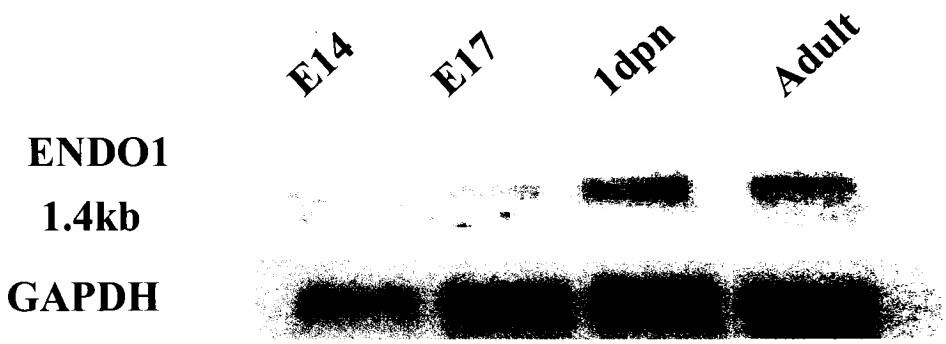
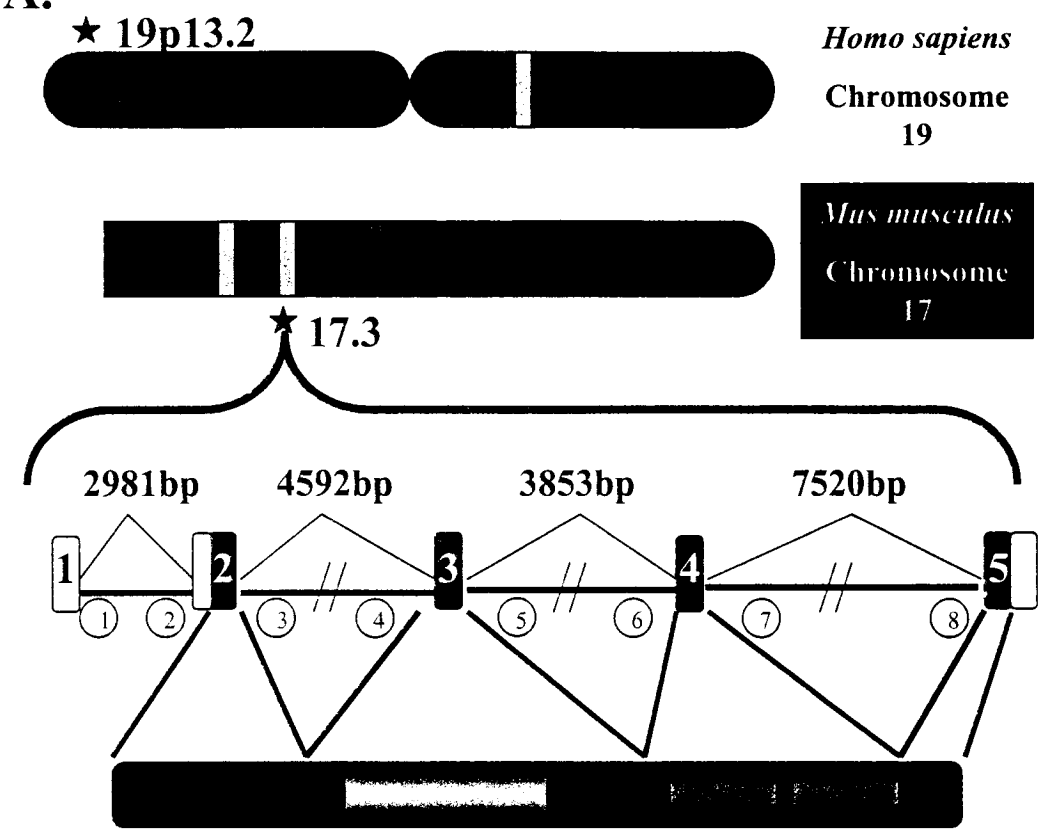


Figure 8. ENDO1 Is Expressed In The Differentiating Murine Heart. Northern Blot analysis reveals that the ENDO1 mRNA transcript is expressed in postnatal than in adult and embryonic hearts.

A.



B.

Exon/Intron Boundary	Sequence
1	**GGGATTACAG**/GTATGAACAA
2	CTGTTCCCAG/**GCTCCTGGAA**
3	**ACACACCGAG**/GTGAGTGGTG
4	CTGTGTCCAG/**TGATGGTCCT**
5	**CCTCACAGAG**/GTACCCTTAA
6	TCCCCTGCAG/**TGGCTGAAGG**
7	**CTGGACGCTG**/GTGAGTGGCT
8	TGCCCCACAG/**GTCTCCTTCC**

Figure 6. The Mouse Gene Structure For ENDO1. (A) The location of ENDO1 on its respective human and mouse chromosome is indicated with a star. Genomic organization of the murine ENDO1 gene is denoted by the black boxes, which represent the coding sequence, and by open boxes, which represent the 5' and 3' untranslated regions. The circled numbers indicate the exon/intron boundaries. (B) Indicate the boundary sequences with the exon sequences listed in bold.

In situ hybridization performed in 12.5 and 14-day old embryos did not reveal high level of expression of ENDO1. However, as shown in Figure 8, Northern Blot analysis reveals that ENDO1 expression appears to be highest in the postnatal heart and lowest in the embryonic heart. This is consistent with the abundant levels of ENDO1 found in 4-day old postnatal rat cardiomyocytes, indicated in Figure 9. This Northern blot also verifies the expression of ENDO1 in rat cardiomyocytes, from where it was isolated, and indicates an equal amount of expression of ENDO1 in atrial versus ventricular cardiomyocytes.

3.7. ENDO1 is expressed in hematopoietic cell lines

We looked at the expression of ENDO1 in hematopoietic cell lines since during development, ENDO1 was expressed in the fetal liver which is responsible for hematopoiesis. Figure 10 shows that in addition to ENDO1, GATA-4 was also found to be expressed in these cell lines, suggesting that GATA-4-ENDO1 pathways may be involved in hematopoietic cell development.

3.8. ENDO1 antibody generation

The secondary structure of ENDO1 was predicted using bioinformatics. ENDO1 is hypothesized to have two transmembrane domains and an N- and C-terminal region. To test the hypothesis of ENDO1 potentially being a transmembrane protein and to visualize the subcellular localization of the endogenous protein in cells, an antibody against ENDO1 was generated in our lab by a previous investigator. Figure 11A shows a schematic representation of the predicted secondary structure of ENDO1. The circled area defines the region of ENDO1 (amino acids 143 to 190) to which the antibody was targeted. Due to the high homology of the PHD finger of ENDO1 with other hypothetical proteins, as indicated in Figure 4, a more downstream C-terminal region was used as the epitope for generating the antibody.

3.9. Subcellular expression of ENDO1

C2C12 cells (adult mouse muscle myoblasts) were transfected with ENDO1 tagged with HA. Figure 11B indicates a C2C12 cell which is labeled with the HA

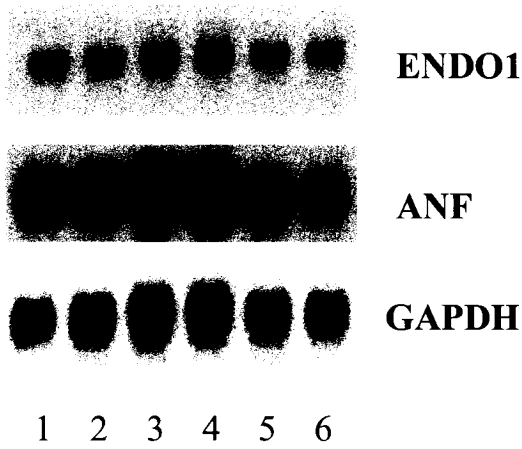


Figure 9. ENDO1 Expression In 4 Day Postnatal Rat Atrial And Ventricular Cardiomyocytes. Lanes 1-4 and 5-6 correspond to atrial and ventricular cardiomyocytes respectively. Northern Blot analysis reveals that the ENDO1 mRNA transcript is equally expressed in both ventricular and atrial cardiomyocytes, and is approximately 1.4kb. ANF expression, as expected, is higher in atrial cardiomyocytes than in ventricular cardiomyocytes. GAPDH is a housekeeping gene which accounts for variations in the amount of RNA loaded.

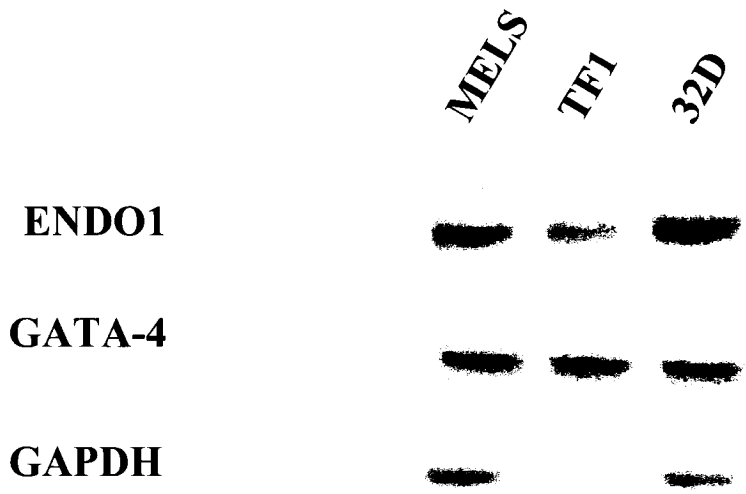


Figure 10. ENDO1 Is Expressed In Hematopoietic Cell Lines. RT-PCR analysis detects the presence of the ENDO1 mRNA transcript in MELS (mouse hematopoietic cells), TF1 (mouse erythroid/macrophage cells), and 32D (mouse myeloid cells).

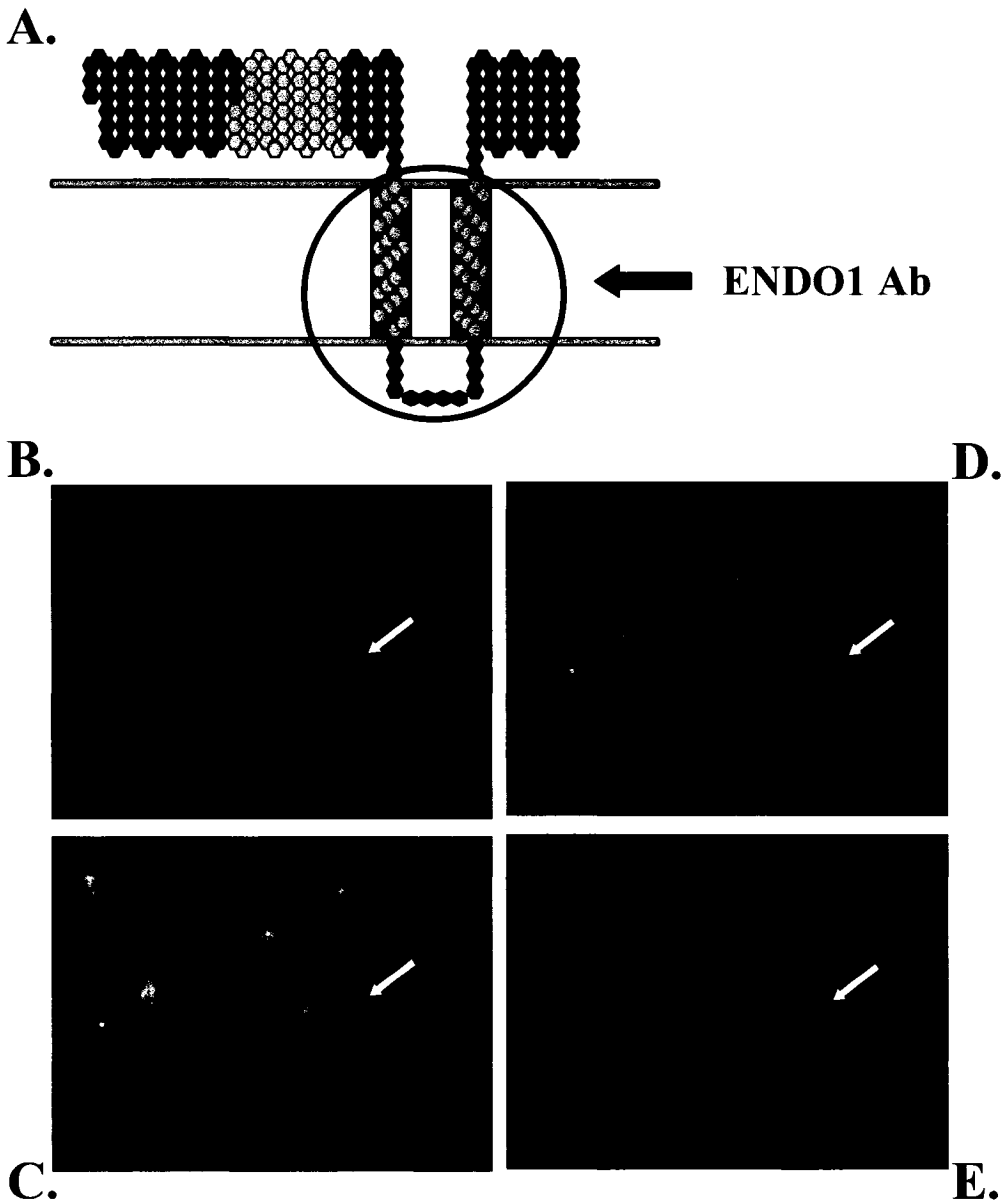


Figure 11. Generation Of The ENDO1 Antibody. (A) The predicted secondary structure of ENDO1 is drawn. The circled area indicates the region to which the ENDO1 antibody was targeted to. The area depicted in green in the N-terminal region is the PHD finger. (B) Immunocytochemisty was performed on C2C12 cells transfected with HA-ENDO1. The red staining indicates a cell overexpressing HA-ENDO1 which was detected with the HA antibody. (C) Double staining with the ENDO1 antibody is shown in green. The white arrow point to the cell overexpressing ENDO1. (D) Figures (B) and (C) are merged together. (E) Merged picture of Hoescht staining with ENDO1 shows that the exogenous ENDO1 is largely present around the nucleus.

antibody in red, indicating that there is overexpression of ENDO1. Figure 11C indicates C2C12 cells labeled with the ENDO1 antibody. The arrow indicates the cell that is overexpressing ENDO1. This is further verified by merging the pictures which is shown in Figure 11D. Figure 11E demonstrates a merged picture with cells stained with Hoescht and labeled with the HA antibody. This clearly demonstrates that the full length ENDO1 is concentrated in a particular region around the nucleus and is largely cytoplasmic. However some endogenous ENDO1 is found in the nucleus. Overall, the subcellular localization of ENDO1 seen with the HA and ENDO1 antibodies are identical, indicating that the antibody is functional in immunocytochemistry. It is noteworthy that, as depicted in Figure 11C, cells overexpressing ENDO1 did not show a difference in staining intensity with cells containing only endogenous ENDO1.

3.10. Overexpression of GATA-4 results in downregulation of ENDO1

Western Blot analysis of 293T (transformed human primary embryonal kidney cells) whole cell extracts reveals that proper overexpression of HA-ENDO1 and HA-GATA-4 was achieved as demonstrated by their detection with the HA antibody (Figure 12A). Overexpression of ENDO1 reveals the protein size is approximately 27 kDa. To verify if this size is identical to endogenous ENDO1, the Western Blot was reprobed with the ENDO1 antibody. The arrow indicates the band that corresponds to ENDO1. Similar to the immunocytochemistry result, there is no difference in intensity between the cells transfected with the empty vector, pCGN, and the vector containing HA-ENDO1. Cells overexpressing HA-GATA-4, as well as the full length antisense of ENDO1, resulted in decreased levels of ENDO1 in the 293T cells. This result demonstrates that GATA-4 is able to downregulate ENDO1 at the protein level. Overexpression of GATA-4 in 293T cells and adenovirus-mediated GATA-4 overexpression in ventricular cardiomyocytes (V.C.) demonstrate a decrease in ENDO1 protein levels in comparison to the untransfected 293T cells and the ventricular cardiomyocytes infected with the control plasmid containing LacZ (Figure 12B). Overall, this downregulation of ENDO1 was reconstituted in 2 different cell types, suggesting that ENDO1 is a *bona fide* target of GATA-4, and is consistent with the results obtained from the RT-PCR differential display. However, whether it is a direct or indirect target is yet to be determined.

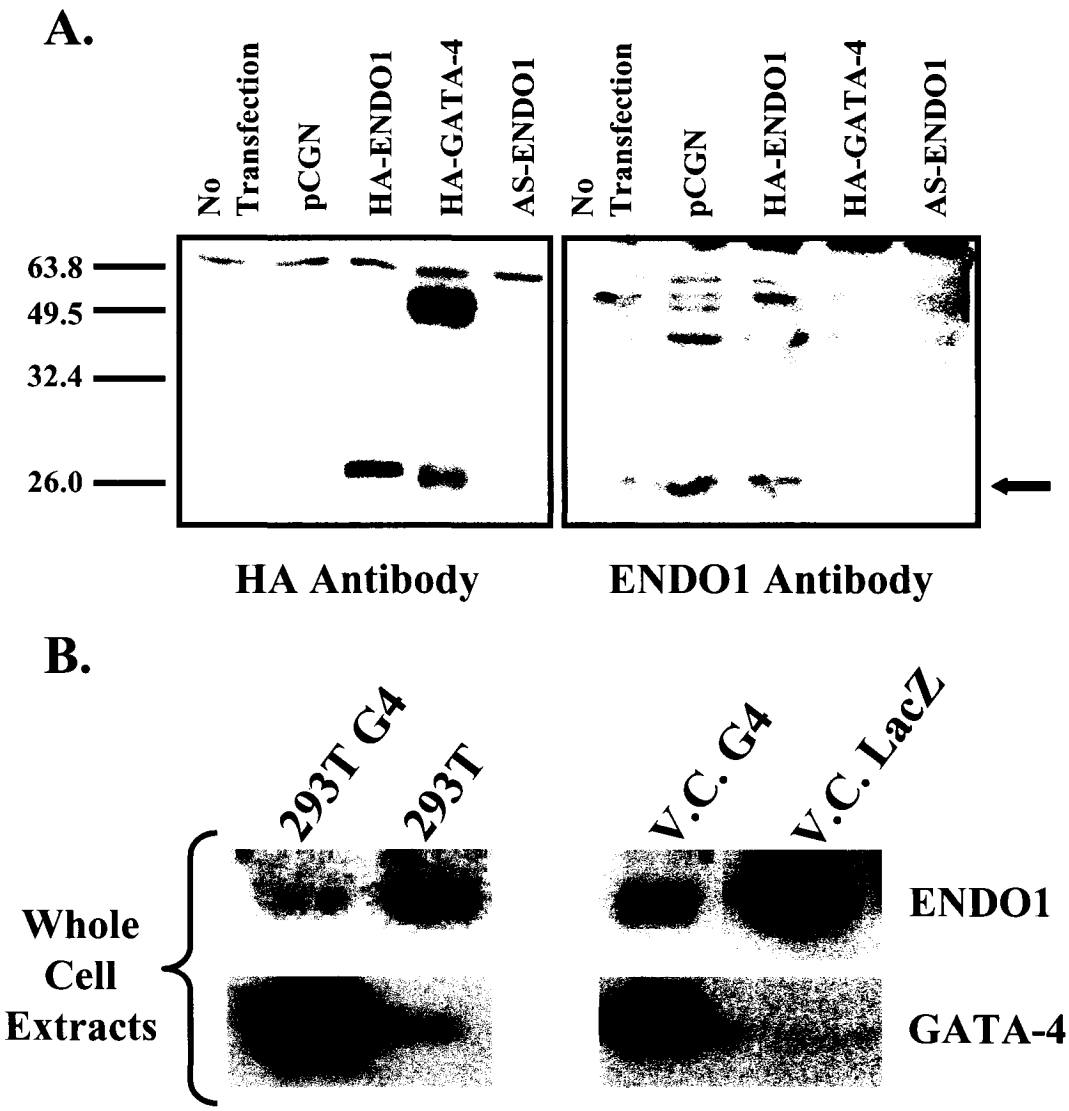


Figure 12. Preliminary Results Show That Negative Regulation Of ENDO1 By GATA-4 Can Be Reconstituted In Heterologous Cells. (A) ENDO1 and GATA-4 constructs were tagged with the HA epitope. Overexpression of these constructs, with the empty vector pCGN and Anstisense ENDO1, indicate that proper overexpression of these constructs was achieved in 293T cells. The same Western Blot, using whole cell extracts, was labeled with the ENDO1 antibody. The black arrow indicates the band that corresponds to ENDO1. ENDO1 protein levels are downregulated in cells overexpressing GATA-4 or the full Antisense ENDO1. (B) Similar results were seen in the Western Blot shown in the left figure. For the right figure, whole cell extracts were taken from ventricular cardiomyocytes (V.C.) overexpressing GATA-4 (G4) and LacZ using the adenovirus-mediated strategy. ENDO1 expression is inhibited in cells overexpressing GATA-4.

3.11. ENDO1 is upregulated in ventricular cardiomyocytes treated with a hypertrophic stimulating agent

GATA-4 has been previously demonstrated in our lab to play critical roles during cardiac hypertrophy (286). Cardiomyocytes treated with phenylephrine, a hypertrophic stimulus, for approximately 24-36 hours results in increase in cell size, reorganization of the cytoskeleton, and upregulation of fetal genes, all of which are signs of cardiac hypertrophy. GATA-4 levels were shown in our lab to be increased in cardiomyocytes upon stimulation with phenylephrine (data not shown). A time course of phenylephrine treatment was carried out in order to see if there was any change in ENDO1 expression (Figure 13). Western Blot analysis of whole cell extracts of cardiomyocytes reveals that ENDO1 expression increases with time and the expression peaks at approximately 24 hours. Since ENDO1 expression is highest around the time when cells are already hypertrophied, it suggests that ENDO1 probably plays a role in maintaining the hypertrophic phenotype, as opposed to inducing it.

3.12. Structure-function analysis of ENDO1

Since ENDO1 has transmembrane domains, we wanted to see what the subcellular localization of the soluble protein would be. Figure 14A depicts the wild-type ENDO1 protein. The PHD finger is between amino acids (aa) 64 and 110 and the NLS is between 117-121aa. The cytochrome c heme binding sites are located between 64-68aa and 106-110aa. The first transmembrane domain is between 139-161aa, and the second is between 174-196aa. In addition to these domains, the diagram indicates several potential regulation sites that may regulate the function and/or localization of ENDO1. As shown in Figure 14B three deletion/mutation constructs were generated where the transmembrane domains were deleted in order to visualize the distribution of soluble ENDO1 protein. ENDO1A does not contain the putative nuclear localization sequence. ENDO1C and ENDO1D both contain the putative NLS, however when sequencing the clones, one of the constructs had the second cysteine of the PHD consensus sequence mutated to an arginine. All of these constructs are in the pCGN vector, and therefore they have all been tagged with the HA epitope to allow for detection with the HA antibody.

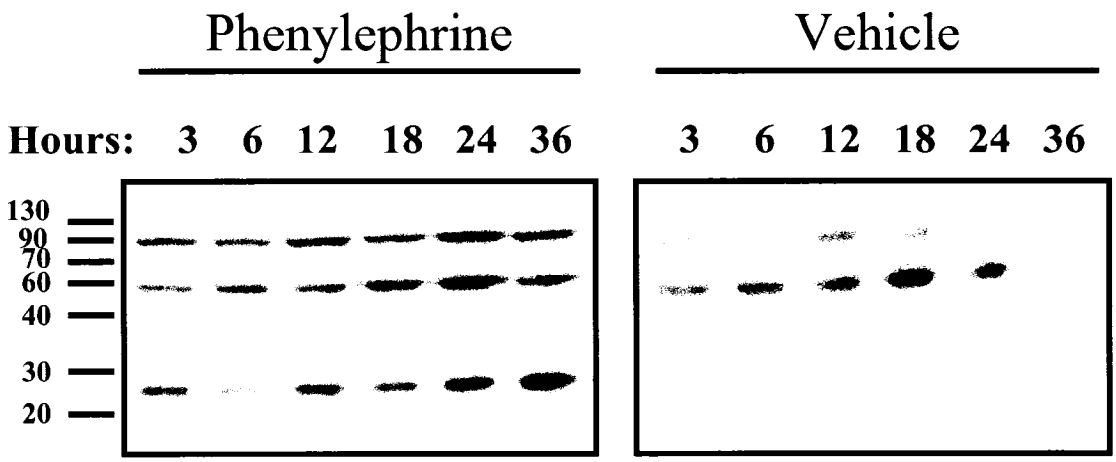
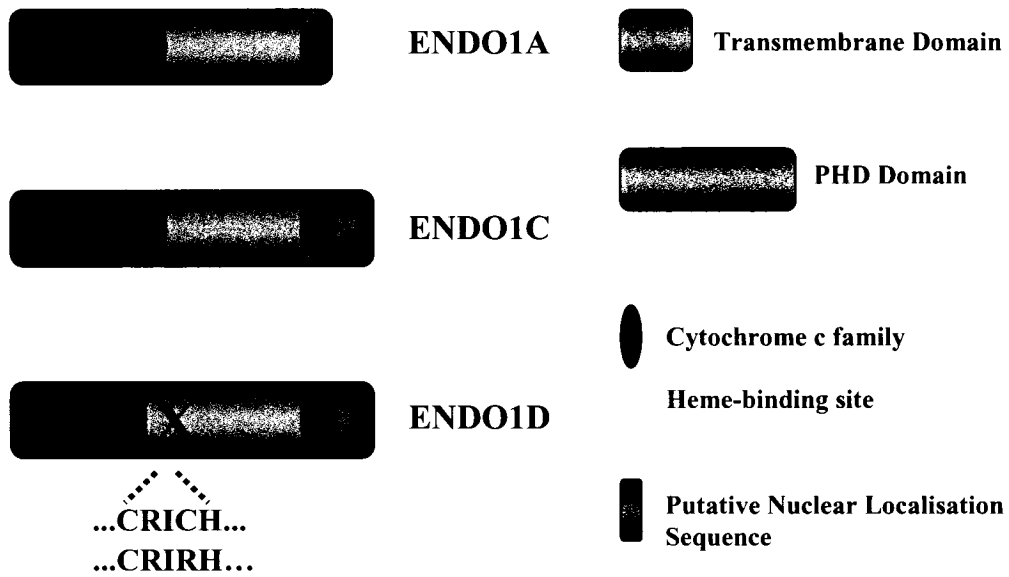


Figure 13. ENDO1 Is Involved In a Cellular Process That Requires GATA-4. Western Blot of whole cell extracts from ventricular cardiomyocytes treated with phenylephrine for varying lengths of time. ENDO1 proteins levels increase with prolonged stimulation with phenylephrine, compared with cells just treated with the vehicle.

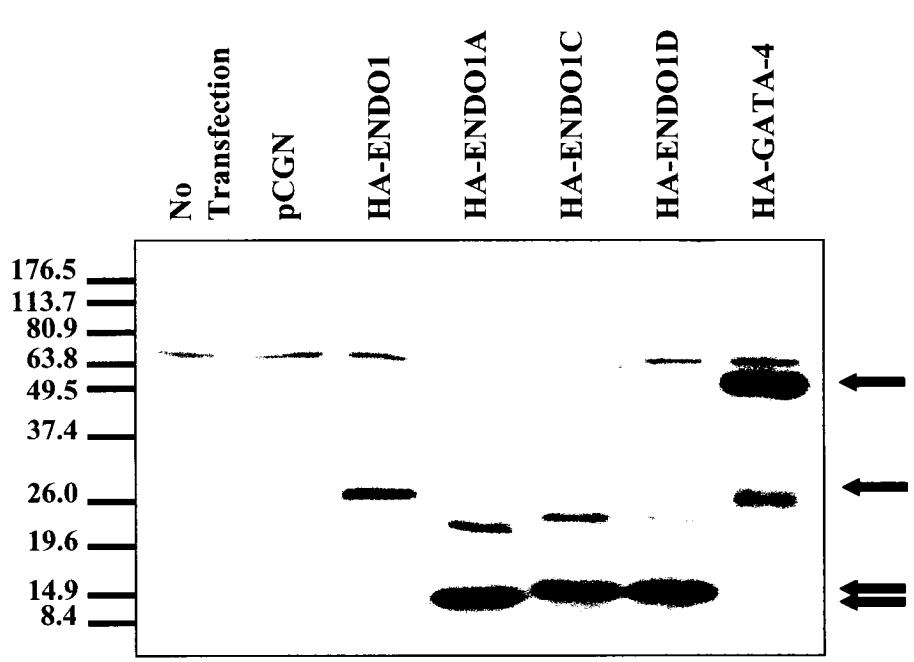
A.


Casein kinase II phosphorylation site
N-glycosylation site
Protein kinase C phosphorylation site
N-myristoylation site

B.


ENDO1A
ENDO1C
ENDO1D
...CRICH...
...CRIRH...
Transmembrane Domain
PHD Domain
Cytochrome c family
Heme-binding site
Putative Nuclear Localisation Sequence

C.


No Transfection
pCGN
HA-ENDO1
HA-ENDO1A
HA-ENDO1C
HA-ENDO1D
HA-GATA-4
176.5
113.7
80.9
63.8
49.5
37.4
26.0
19.6
14.9
8.4

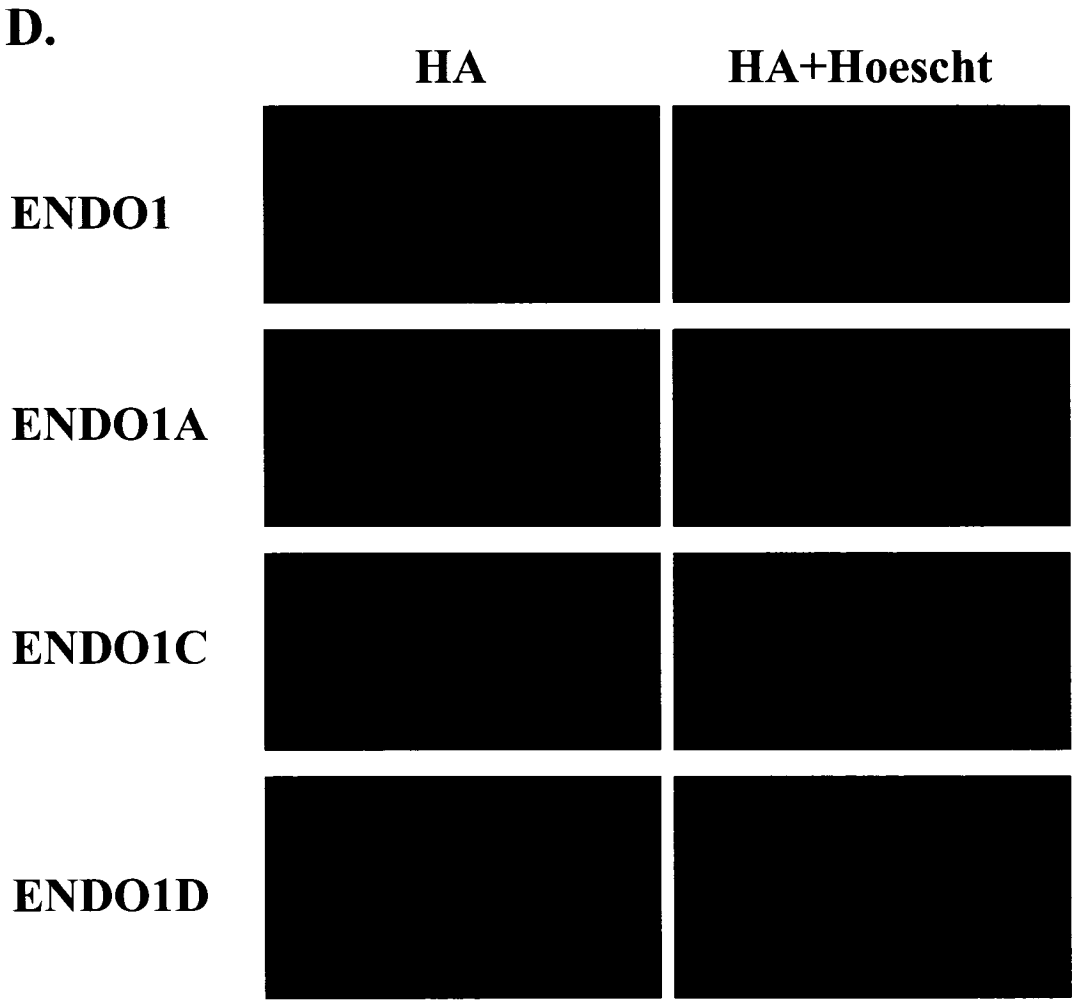


Figure 14. Structure-Function Analysis Strategy For ENDO1. (A) Depicts all of the potential domains and regulation sites in the full coding region of ENDO1. (B) Depicts the mutated constructs of ENDO1. Construct ENDO1A has the transmembrane region and the putative nuclear localization sequence (NLS) deleted. Constructs ENDO1C and ENDO1D retain the NLS, however, the second cysteine in the PHD consensus sequence is mutated in ENDO1D. All of these constructs have an HA epitope at the N-terminus. (C) Western Blot containing whole cell extracts from transfected 293T cells shows that these constructs are expressed at the right size, as indicated by the black arrows. (D) Immunocytochemistry was done on ventricular cardiomyocytes overexpressing these constructs using the HA antibody. The wild-type ENDO1 protein was found to be localized around the nucleus. ENDO1A appears to be retained in the cytoplasm possibly in the membranes of some organelles. The expression of ENDO1C is more reconstituted around the nucleus and ENDO1D is expressed in the nucleus.

Western Blot analysis of whole cell extracts taken from 293T cells shows that these constructs are expressed at the predicted size as indicated by the arrow (Figure 14C). Extracts from 293T cells overexpressing HA-GATA-4 was used as a positive control in order to ensure that the immunohistochemistry worked properly. Figure 14D displays the results from the immunocytochemistry performed in ventricular cardiomyocytes which were transfected with each of the ENDO1 constructs. HA staining reveals the wild-type ENDO1 protein to be localized around the nucleus as expected. ENDO1A is largely localized in the cytoplasm, however its distribution seems to be in the membrane of cytoplasmic organelles. This may be explained by the fact that the construct contains some N-myristolation sites in the PHD finger. These sites can allow the construct to bind to membranes and prevent it from being soluble. The expression of ENDO1C is more reconstituted around the nucleus compared to ENDO1A. It does however seem to be present in other organelles in the cytoplasm, possibly in the membrane. ENDO1D, unlike the other constructs, has its expression restricted to the nucleus. This expression pattern cannot be due to mere diffusion since ENDO1C is the same molecular weight as ENDO1D. Thus it seems that the second cysteine in the PHD consensus sequence plays an important role in regulating the distribution and mechanism of action of ENDO1.

3.13. Differential subcellular localization of ENDO1 in cardiac cells

The endogenous expression of ENDO1 was determined using the ENDO1 antibody on ventricular cardiomyocytes and cardiac fibroblasts (Figure 15). The cardiomyocyte expression of ENDO1 was largely localized around the nucleus and in the plasma membrane. In contrast, ENDO1 expression in cardiac fibroblasts is predominantly in the nucleus, plasma membrane, and perhaps in the cytoskeleton. This expression pattern is particularly interesting since it provides the first evidence, in cell culture, that endogenous ENDO1 can be present predominantly in the nucleus. The difference in subcellular localization suggests that ENDO1 is differentially regulated depending on the cell type. This differential regulation can occur via several pathways since ENDO1 is predicted to have numerous sites of regulation, as depicted in Figure 14A.

3.14. ENDO1 represses the activity of cardiac promoters

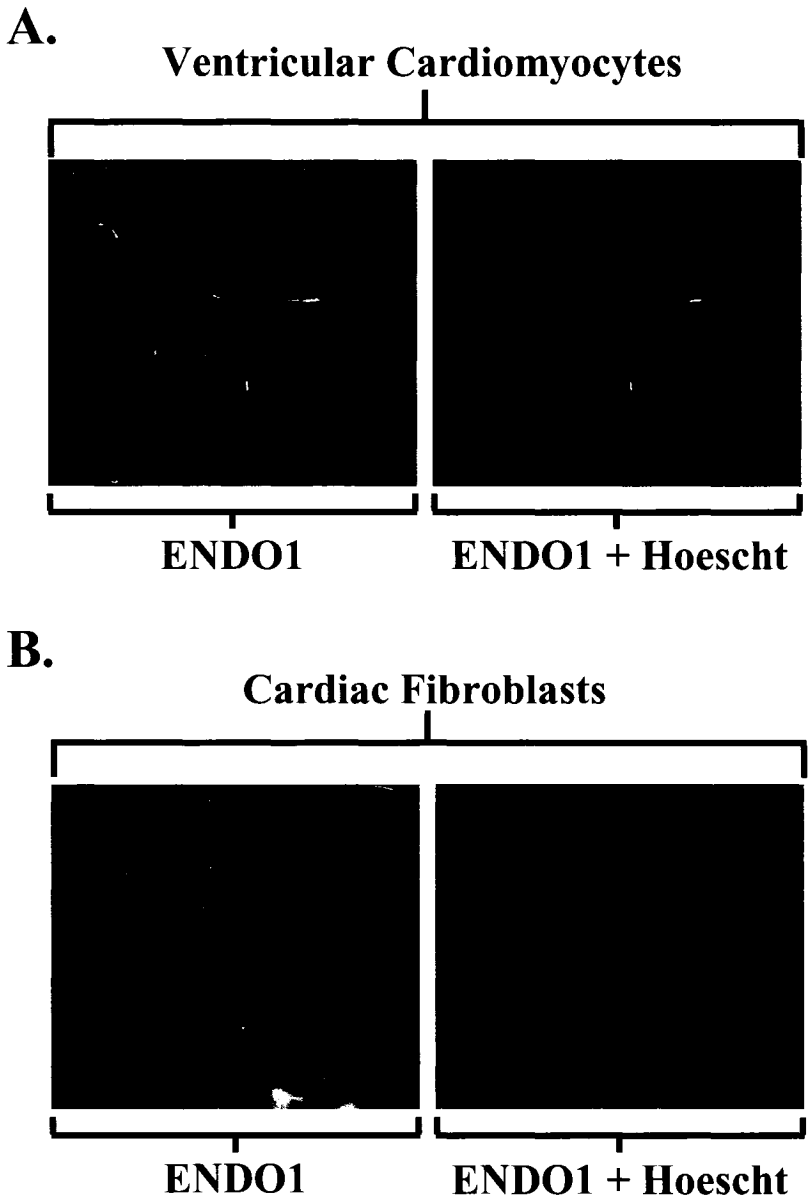
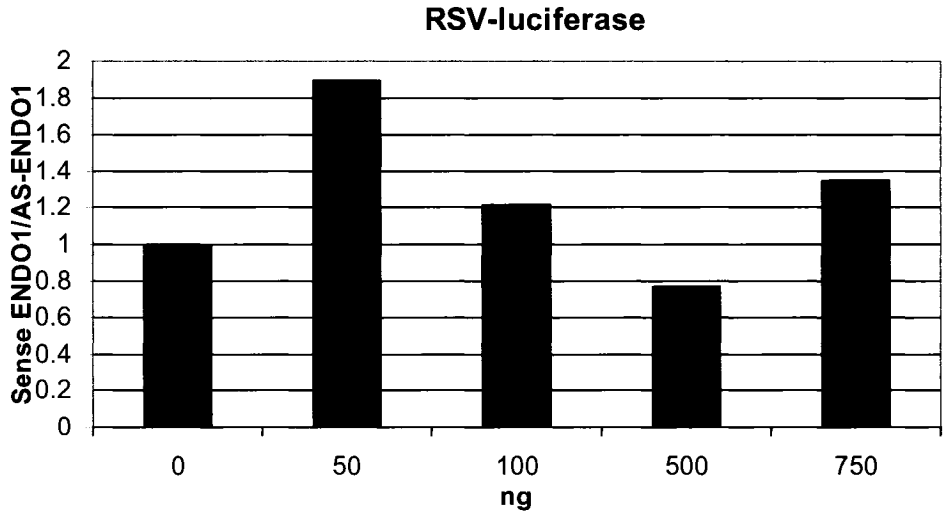


Figure 15. Endogenous Expression Of ENDO1 In Cardiac Cells. (A) Ventricular cardiomyocytes taken from 4-day old postnatal rats were labeled with the ENDO1 antibody. A merged picture of ENDO1 staining with Hoescht reveals that ENDO1 is largely localized around the nucleus. (B) Cardiac Fibroblasts taken from 4-day old postnatal rats were labeled with ENDO1 antibody. The merged picture also with Hoescht reveals ENDO1 staining largely in the nucleus and plasma membrane.

Given that it is possible for ENDO1 to be expressed in the nucleus and contains a PHD finger, we wanted to see whether ENDO1 was able to transcriptionally regulate any promoters. Since ENDO1 is highly expressed in 4-day old postnatal cardiomyocytes, we tested cardiac promoters as potential targets of ENDO1. NIH 3T3 cells (mouse fibroblast cell line) were transfected with either sense or antisense ENDO1 and the promoter of interest. Cells were transfected with antisense ENDO1 which would reduce any endogenous ENDO1. A ratio of the RLU readings from the luciferase assay of sense ENDO1 versus antisense ENDO1 was calculated in order to decipher the effect of ENDO1 on a promoter. Figure 16A displays the effect of ENDO1 on the RSV promoter. Overall ENDO1 had little effect on this promoter. Figure 16B displays the inhibitory effect of ENDO1 on the 2.2kbp rat BNP promoter. Consistently, a 2-4 fold inhibitory effect was observed in different cell types.

In order to map the region of the BNP promoter on which ENDO1 is acting, several deletion/mutation constructs were used, as shown in Figure 17A. Figures 17B-E shows that ENDO1 has an inhibitory effect on all of these promoters except for NP 80, which has two GATA sites mutated, and NP 160, which lacks the GATA sites. The two distal GATA sites on the NP 80 promoter were mutated from TCT**GATAA**ATCAG**AGATAA**CC to TCT**GGTAA**ATCAG**AGGTAA**CC, where the GATA sites are in bold and the mutations are underlined. It can be inferred that the region on which ENDO1 is acting is located between –116 and –79. Since GATA-4 can bind to DNA and other proteins, ENDO1 may bind to GATA-4 and inhibit its activity, or ENDO1 may inhibit the BNP promoter by directly binding to the GATA sites and acting as a competitive inhibitor of GATA-4. The activation on the NP 80 promoter may be explained by the fact that the mutated GATA sites may constitute a new binding site on which ENDO1 may act, causing transcriptional activation. Alternatively, ENDO1 may act either as a transcriptional repressor or activator depending on the promoter context. Nevertheless, the fact that the inhibition is eliminated when the GATA sites are either deleted or mutated supports the hypothesis that this region is a target of ENDO1. At present, experimental conditions are being optimized in order to test whether ENDO1 is acting directly or indirectly on the GATA binding sites.

A.



B.

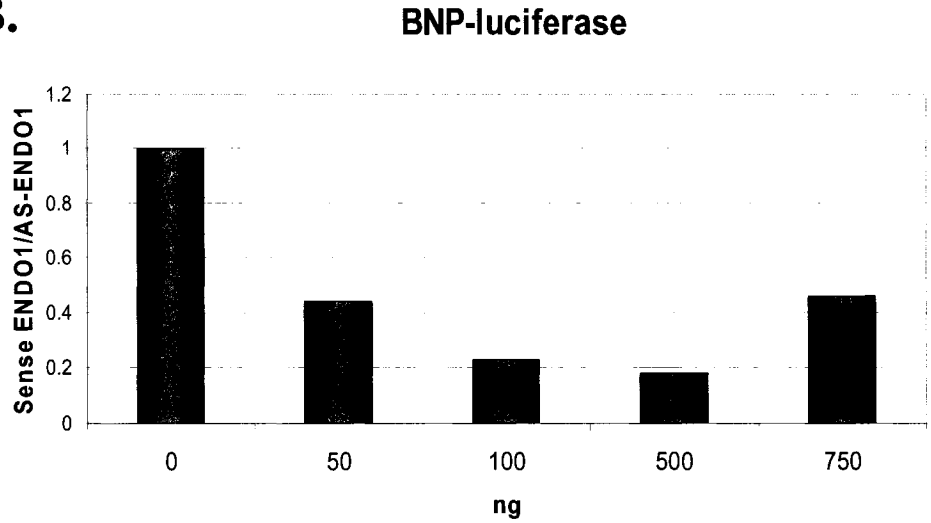
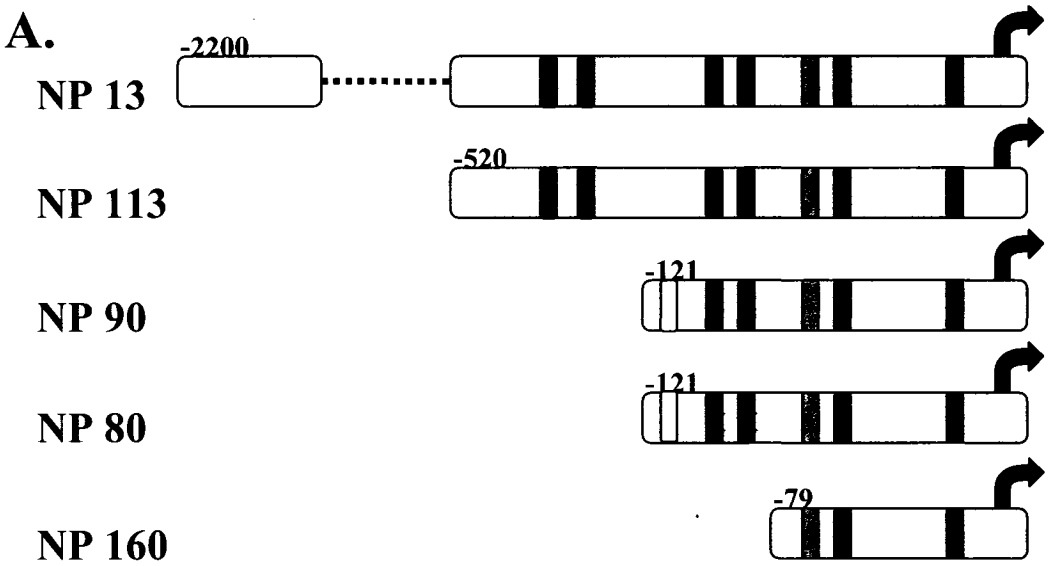
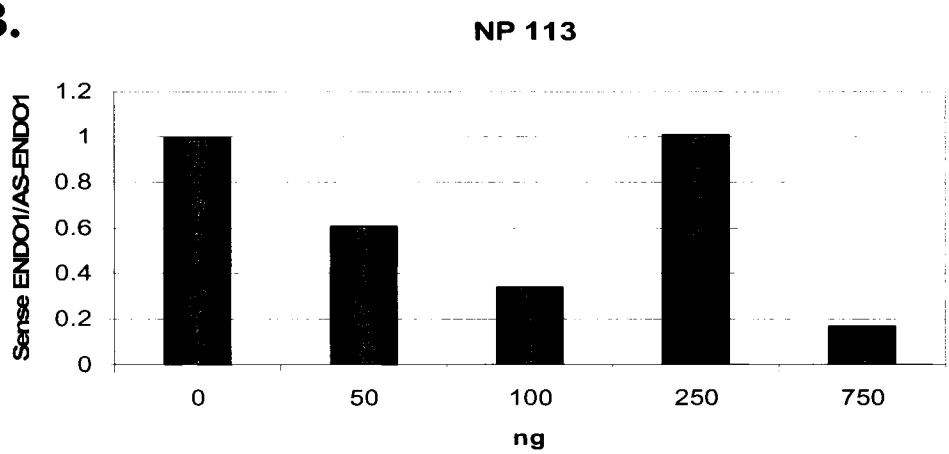
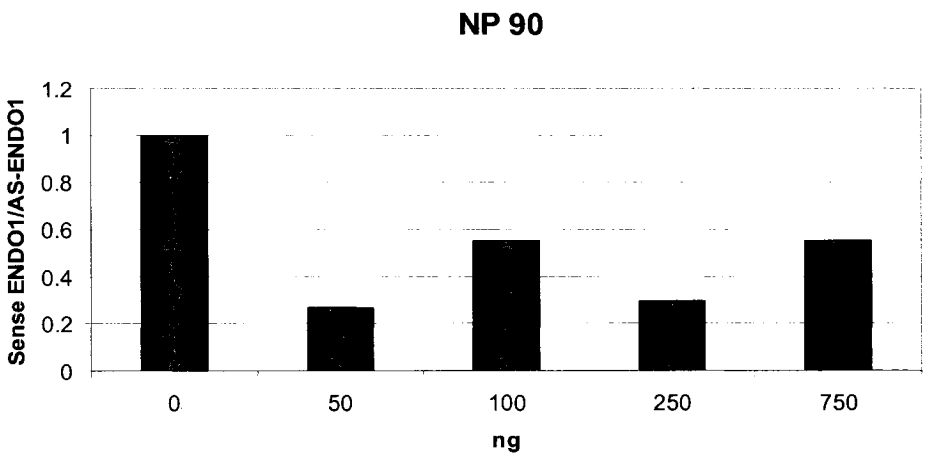


Figure 16. ENDO1 Represses The BNP Promoter. Either Sense or Antisense ENDO1 was co-transfected in NIH 3T3 cells with the promoter of interest. Transfection with the RSV promoter showed no effect on the RSV promoter. However, ENDO1 was found to have an inhibitory effect on the BNP promoter. The results presented here are representative of two transfection assays.


A.
-2200
NP 13
-520
NP 113
-121
NP 90
-121
NP 80
-79
NP 160


B.
NP 113
Sense ENDO1/AS-ENDO1
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
250
750
ng


C.
NP 90
Sense ENDO1/AS-ENDO1
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
250
750
ng

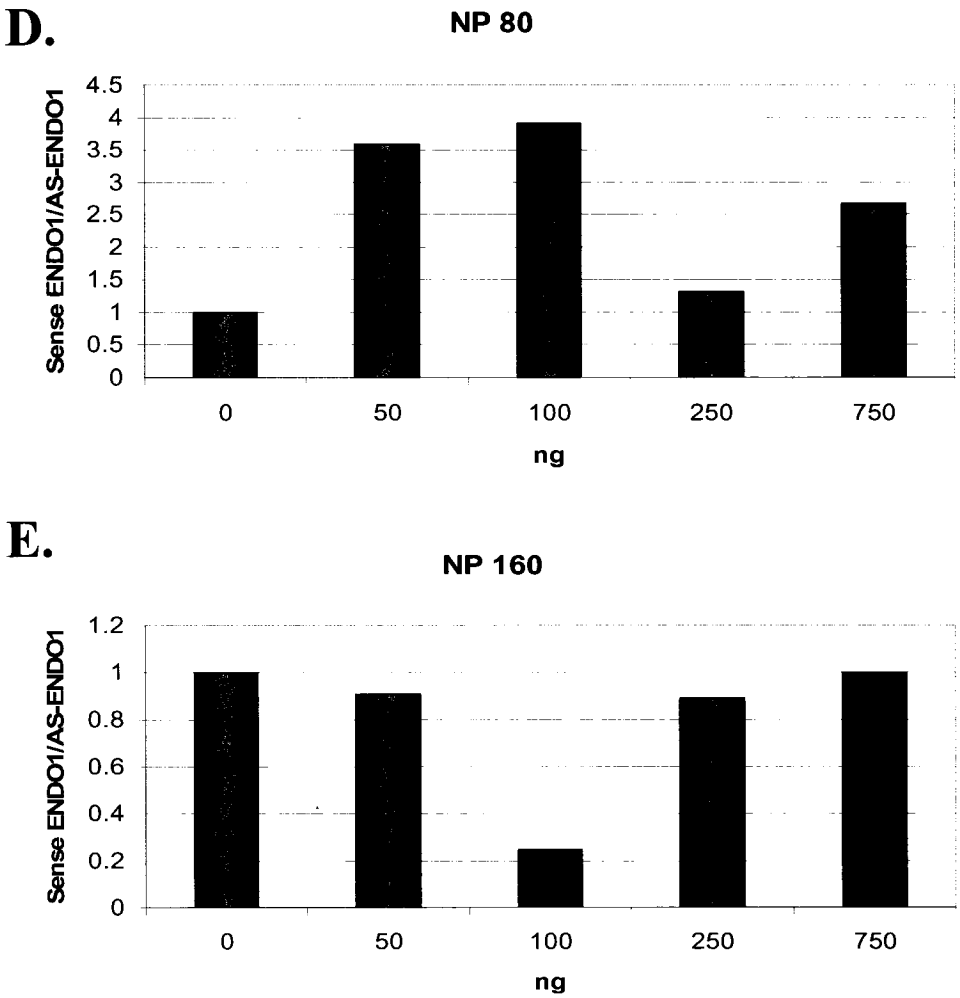


Figure 17. Mapping The Promoter Element That Is Responsible For The Inhibitory Effect Of ENDO1. (A) Different deletion/mutation reporter constructs were used in order to map the region to which ENDO1 is acting on. The red bars indicate GATA binding sites, the blue bars indicate YY1 binding sites, the orange bars indicate CACC binding sites, the white bars indicate MCAT binding sites, the black bars indicate NFE2 binding sites, and the green bars indicate T-MARE binding sites. The white X's represent mutations in the binding sites. (B-E) ENDO1 inhibited all the constructs except for NP 160 where there was no effect, and NP 80, where almost a 4-fold activation was attained. The results presented here are representative of two transfection assays.

3.15. Soluble ENDO1 may lead to transcriptional activation of the BNP promoter

In addition to NIH 3T3 cells, C2C12 cells are also widely used for transfection assays since the basal level of the BNP reporter constructs are high enough to allow analysis. Consistent with the results observed in Figure 16B, Figure 18 shows that transfection with the full length of ENDO1 results in repression of the BNP promoter. In contrast, preliminary results obtained with the ENDO1 deletion/mutation constructs show activation of the BNP promoter with increasing doses. This change may be attributed to either a different subcellular localization of the soluble form of ENDO1, the removal of a transcriptional repressor domain located in the C-terminal, or interaction with different proteins. Further experiments testing the various ENDO1 deletion/mutation constucts on the numerous BNP promoter constructs will provide more insight into the mechanism of action of ENDO1. In conjunction, parallel studies involving co-transfection with GATA-4 will help decipher the relationship of ENDO1 with GATA-4.

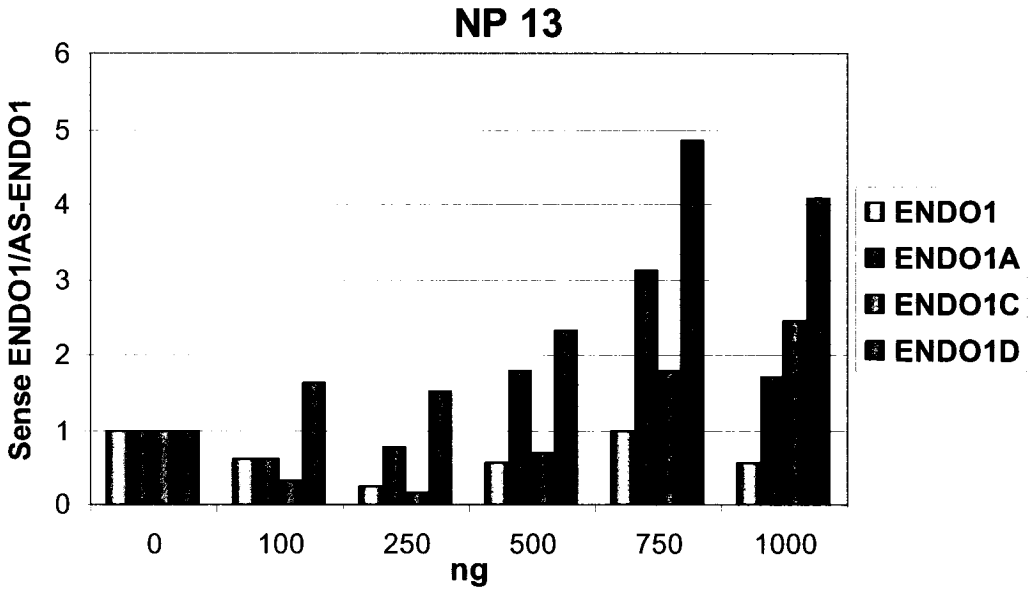


Figure 18. Effect Of The Soluble Form Of ENDO1 On BNP Promoter Activity. C2C12 cells were transfected with the different ENDO1 constructs. The full length ENDO1 is able to repress the BNP promoter. All three of the other constructs demonstrate activation on the BNP promoter. The highest activation was achieved by ENDO1D. It is important to note that this experiment was only carried out once and is a preliminary result.

4. DISCUSSION

4.1. Isolation of a new gene that is a downstream target of GATA-4 and implicated in endothelial cell differentiation

In our study, we have described the isolation of a novel transcript, ENDO1, which was isolated as a new GATA-4 target in cardiomyocytes. The RT-PCR differential display study revealed that ENDO1 is specifically targeted by GATA-4, and not GATA-6, at the RNA level. This result was later verified at the protein level in 293T cells and ventricular cardiomyocytes, where overexpression of GATA-4 resulted in downregulation of ENDO1. This finding is very significant since identification of downstream targets of GATA-4 will shed light into new pathways involved in embryonic and postnatal cardiac development. The distribution of ENDO1 in additional organs that also express GATA-4, such as the lung and liver, could implicate GATA-4-ENDO1 pathways in various developmental systems.

Although it was not shown in the results section, it is worth mentioning that ENDO1 was additionally implicated as a novel factor involved in endothelial cell differentiation based on microarray and Western Blot analysis (287). The microarray analysis was previously conducted in our lab using the TC13 cell line which can differentiate into endothelial cells following treatment with retinoic acid (RA). TC13 cells showed a marked upregulation of ENDO1 after just 3 hours of RA treatment by Western Blot analysis. Terminal differentiation of TC13 cells into endothelial cells requires approximately 4 days of RA treatment, therefore suggesting that ENDO1 is involved in the initial steps of differentiation.

In the literature review section, it was clearly emphasized that in order to understand the mechanisms behind the proper functioning of the heart, it was important to study the endothelial as well as the cardiac system. Several GATA factors have been shown to be expressed in endothelial cells such as GATA-2, GATA-5, and even GATA-4 (220). It is interesting to note that during endothelial differentiation of TC13, ENDO1 upregulation also coincides with downregulation of GATA-4 and activation of GATA-5. Thus, GATA-ENDO1 pathways may play dual roles during endothelial cell differentiation. It is also noteworthy that, although GATA-4 does downregulate the

expression of ENDO1, both proteins are detectable in the same cell types and overexpression of GATA-4 is not able to fully suppress the expression of ENDO1. Further evaluation of the relationship between GATA-4 and ENDO1 in endothelial cells and cardiomyocytes may shed light into the communication that takes place between these two cell types or unravel conserved pathways in the two closely related mesodermal cells.

4.2. Distribution of ENDO1 during embryonic and postnatal development

The expression pattern of ENDO1 gives a good insight into the developmental pathways that ENDO1 may be involved in. Although the onset of expression of ENDO1 is not yet known, the expression of ENDO1 can be detected in the brain, heart, vasculature and fetal liver during embryonic development as assessed from *in situ* hybridization and RT-PCR analysis (287). This is in agreement with our results, which show that ENDO1 is expressed in endothelial cells and cardiomyocytes. Furthermore, expression of ENDO1 in the fetal liver suggests that ENDO1 may be involved in hematopoiesis. Indeed, analysis of 4 different hematopoietic cell lines revealed that ENDO1 is expressed in these cells, along with GATA-4. Interestingly, co-expression of ENDO1 in hematopoietic and endothelial cells may suggest it to be expressed in hemangioblasts, and could represent a new marker for these stem cells. Furthermore, prominent expression of ENDO1 in the brain suggests it to play major developmental roles in neuronal cells as well, thus implicating the functions of ENDO1 to extend to various developmental systems.

The tissue-specific expression pattern of ENDO1 in the adult suggests other putative roles for ENDO1. For example, ENDO1 is highly expressed in the largest organ of the lymphatic system, the spleen. The spleen is a highly vascularized organ that functions as a "blood reservoir" (288). Vessels in the spleen are present in a vasodilated state, which allows them to store extra volumes of blood. Under situations where there is, for example hemorrhaging or anoxia, the vessels vasoconstrict and release some of the blood into the circulation. Since ENDO1 contains two cytochrome c heme binding sites (which may be involved in sensing cellular oxygen levels), and is expressed in endothelial cells, it may be part of the signal that stimulates the vessels to contract under

abnormal situations. In regards to other systems, further insight can be gained from immunohistochemical analysis on sections from the lung and kidneys, which will provide additional information on the specific cell types that ENDO1 is expressed in, and in turn, the kinds of processes in which ENDO1 is involved.

4.3. PHD finger proteins play important roles during development

High conservation in a specific domain may imply a certain function of a protein that is indispensable for survival. Interestingly, ENDO1 was shown to contain a plant homeodomain (PHD) finger that we show to be evolutionarily conserved in a total of 9 different species. PHD finger (C_4HC_3) proteins are part of the RING finger family of proteins and was initially identified from the *Arabidopsis* homeobox protein (289) nearly a decade ago. Since that time, hundreds of proteins have been identified containing this domain, however, the biological functions attributed to this domain are still largely unknown (284).

As indicated in Table 1, many PHD finger proteins have multiple functions during development. In general, PHD finger proteins are associated with chromatin remodeling and protein-protein interactions. For example, ACF (290) is implicated in chromatin remodeling. Histone acetyltransferases, such as CBP (CREB binding protein) and p300 (291), and histone deacetylases, such as HDAC1 (292), have been implicated in chromatin mediated transcriptional regulation. In general, active chromatin (euchromatin), in part, is associated with hyperacetylated histones, whereas in contrast, heterochromatin is associated with hypoacetylated histones (293,294). Overall, tight regulation of acetylation on histones is important for gene regulation. Previous investigators have demonstrated that the PHD finger of CBP is essential for not only its histone acetyltransferase function, but also its transcriptional activity (295). However, although the PHD finger of p300 is dispensable for these functions, like CBP, it is still essential for embryonic development (296-299). TIP5 and DNMT3L (DNA methyltransferase 3L) (300,301), as indicated in Table 1, associate with a histone deacetylase, and their PHD fingers are essential for protein interaction with HDAC1, which in turn is necessary for regulating gene repression. Similarly, Pf1 (PHD factor one) binds to the mSin3A-histone deacetylase to carry out transcriptional repression (302).

Pygopus (303) carries out transcriptional activation by binding with the *Drosophila* Armadillo protein which is involved in Wingless (Wg)/Wnt signaling. Overall these proteins indicate how the PHD finger can mediate transcriptional regulation indirectly through vital protein interactions with chromatin remodeling proteins.

In addition to being transcriptional co-regulators, PHD fingers can also directly activate or repress promoters. As indicated in Table 1, TRIP-Br1 (304), BHC (BRAF-histone deacetylase complex) (305), PHF6 (PHD-finger gene 6) (306), p47ING3 (307), polycomblike proteins (308), trithorax proteins (308), MMD1 (Male Meiocyte Death1) (309) and ARA267-α (androgen receptor-associated protein 267-alpha) (310), can mediate transcriptional regulation directly. The underlined transcription factors are transcriptional repressors, whereas the factors that are not underlined are transcriptional activators. Transcriptional targets include critical factors such as homeotic genes (308) and cell cycle regulators (307,309), which is consistent with the view that transcription factors play vital roles during development (311,312).

Various additional functions have been associated with PHD finger proteins. Some of the most tightly regulated pathways are those that are involved in cell cycle regulation. ING2, an evolutionarily conserved protein, has been shown to interact with phosphoinositides through its PHD finger (313). Phosphoinositides are involved in diverse cellular functions, including survival, growth and proliferation (314,315). ING2, in cooperation with chromatin remodeling proteins, is able to regulate the transcription of the tumor suppressor p53 in order to induce apoptosis. In addition, another ING family member, p47ING3, also induces apoptosis by activating the transcription of p53, and the promoters that are transactivated by p53, such as Bax(307), overall demonstrating how key regulators of cell survival are, in part, regulated by PHD finger proteins.

4.4. ENDO1 belongs to new subclass of PHD finger proteins that may function as E3 ubiquitin ligases

4.4.1. The ubiquitin system

Recently, several investigators have suggested that some PHD finger proteins act as E3 ubiquitin ligases (316,317). Ubiquitin (Ub) is one of the most conserved proteins in vertebrates, and it is probably due to the fact that it plays critical roles during

development. Ubiquitin-dependent proteolysis of proteins plays a role in several cellular processes such as cell cycle regulation, transcription, antigen presentation, receptor endocytosis, fate determination, and signal transduction (318,319). Ub is a 76 amino acid protein that participates in an enzymatic cascade of events that requires energy. Briefly, a Ub-activating enzyme, E1, is activated in order to form a UB-thiol ester bond. The Ub is then transferred to a Ub-conjugating enzyme, E2, and forms an E2-thiol ester bond. In the last step, Ub is transferred from an E2 to a target protein. This transfer is facilitated by an E3 Ub ligase. Upon conjugation of Ub with the new protein, there is the addition of Ub's on the C-terminal glycine residue, forming a polyubiquitin chain, which targets the protein for 26S proteosome-mediated degradation. Since the ubiquitination system has a profound effect on many cellular processes, it is thought that the specificity of targeting proteins is brought about by the E3 ligases. Just a couple of years ago, E3 Ub ligases were divided in only two categories: the catalytic HECT domain E3s and RING finger E3s. Now, many PHD finger proteins are being identified as novel E3 Ub ligases. Overall, the importance of the ubiquitination system during development must not be underestimated, since disruption of any of the genes that encode for the 20S proteosome core in yeast results in a lethal phenotype (320). As the awareness of the critical roles that the ubiquitin system plays during development increases, more target proteins are being identified. In particular, a recent study demonstrated that GATA-2, which plays critical roles in hematopoiesis and cardiovascular development, is targeted by a SUMO (small ubiquitin-like modifier) E3 ligase, PIASy (321). Interaction of PIASy with GATA-2 not only leads to GATA-2 SUMOylation, but also repression of the endothelin-1 promoter, which is normally activated by GATA-2. In general, the ubiquitin system seems to target important regulators of homeostasis, therefore, finding new targets or members of this system will provide a lot of insight into the molecular pathways governing development.

4.4.2. Viral PHD finger E3 ubiquitin ligases

Some controversy has arisen over whether a subclass of E3 Ub ligases are PHD finger proteins, since some researchers are arguing that they are not PHD fingers, but rather variations of the RING finger domain that is responsible for this activity (322). Strong evidence from both sides of the story has not been generated, and therefore at present, this speculation can be exercised as a new hypothesis regarding the putative

functions of PHD finger proteins. A recent review from Ganem and colleagues (316) identified two viral, PHD finger containing, proteins as E3 ubiquitin ligases. These KSHV MIRs, MIR1 and MIR2, are able to downregulate human MHC (major histocompatibility complex) class I molecules from the membrane. This is the method that viruses utilize in order to avoid T-cell mediated immunity, which involves cytotoxic T lymphocytes destroying the cells that are infected with viruses. Several experiments were performed in order to test whether these two viral genes possessed any E3 ubiquitin ligase activity. For example, some studies found that MIR1 and MIR2 were able to ubiquitinate the MHC (myosin heavy chain) class I chains, and that mutation of two specific lysine residues in the chains abrogated ubiquitination (323,324). In addition, experiments using a fused protein containing the MIR2 PHD domain was able to auto-ubiquitinate itself *in vitro*, suggesting that these viral genes are *bona fide* E3 ubiquitin ligases (323). Overall, Ganem and colleagues suggest that MIR1 and MIR2 comprise a new family of membrane-bound E3 ubiquitin ligases that belong to the group of PHD finger proteins.

4.4.3. Similarities of ENDO1 with PHD finger E3 Ub ligases

Although this new defined class of proteins is largely based on viral proteins, its classification can include host proteins. For example, MEKK1, a cellular mitogen-activated protein (MAP) kinase kinase kinase, contains a PHD domain that was also found to possess E3 ubiquitin ligase activity (317). A closer look at MIR1 and MIR2 shows many similarities with ENDO1. Ganem and colleagues describe a certain conservation of residues and structural organization that is common among the PHD finger proteins associated with E3 ubiquitin ligase activity. First, the PHD domain has to be located in the N-terminal region, which is separated from the C-terminal region by two transmembrane domains. This secondary structure requirement is in agreement with the predicted structure of ENDO1. Furthermore, like MIR1 and MIR2, ENDO1 may be a type III transmembrane protein, where its N- and C-terminal ends extend into the cytosol. There is also conservation in the spacing between the third and fourth cysteines in the PHD consensus sequence (not the case in MEKK1) and also of the tryptophan residue between the fifth and sixth cysteine residues. Sequence alignment of the PHD finger of ENDO1 with MEKK1 and several of the viral proteins, including a human homologue of

MIR1/2, c-MIR (325), indicate that the conservation of these residues are well respected. Recently, several new families of E3 ubiquitin ligases have been identified in several species (326-329). Interestingly, ENDO1 seems to belong to a new family containing at least 12 other members that share the same conserved residues in the PHD domain as other PHD E3 ubiquitin ligases. It is noteworthy that the functional significance of these conserved residues and structural domains still remains to be determined. Interestingly, SSM4, shown to be homologous to ENDO1, has also been reported to be an E3 ubiquitin ligase (330), demonstrating that this potentially new class of E3 ubiquitin ligases is evolutionarily conserved.

4.5. The role of ubiquitination in cardiac diseases

The dysregulation of the ubiquitin system has been associated with several human pathological states such as chronic degenerative diseases of the nervous system and muscle breakdown, tumour biology, cell atrophy, apoptosis, cancer and antigen presentation to the immune system (331). Recently, Dunn and colleagues showed that there is an increase in the number of hyperubiquitinated proteins in dilated cardiomyopathy (DCM) versus undiseased human hearts (331). Enzymes of the UCH (ubiquitin carboxyl-terminal hydrolase) family play a key role in the ubiquitin-proteosome pathway (332). UCH is abundantly expressed in the fetal human heart and its expression declines postnatally. The upregulation of UCH seen in DCM is in agreement with the paradigm that there is re-expression of fetal genes in diseased adult heart. Furthermore, all of the proteins that were ubiquitinated in Dunn's study had decreased levels of expression. Therefore, inappropriate ubiquitination of proteins does lead to proteolysis and depletion of proteins, which in turn can affect several cellular processes in the heart. Targeted proteins include mitochondrial ATP synthase, creatine kinase, haemoglobin, and myoglobin. Another recent study conducted by Schaper and colleagues show that not only is there an increase in ubiquitin conjugation, but in comparison there is also decreased levels of deubiquitinating enzymes and lysosomal proteolytic enzymes, such as cathepsin D in diseased hearts (333). This reduction in proteolytic activities leads to the accumulation of polyubiquitinated proteins, and in turn, cell death. Based on these studies, targeting the ubiquitin system, via for example E3 Ub ligases, may be a new

therapeutic intervention in heart disease, as is the case for cancer therapy (334). In this context, upregulation of ENDO1 in α1-induced hypertrophic cardiomyocytes (Figure 13) is noteworthy. Gain and loss experiments using adenovirus-mediated gene transfer into cardiomyocytes would help establish the role of ENDO1 in myocyte growth.

4.6. Genetic diseases associated with human chromosome 19p13.2-13.3

Thus far in the literature, the full length cDNA of ENDO1 has been isolated as a novel gene implicated in hematopoiesis (335). The only additional information available in the literature regarding ENDO1 is its chromosomal localization. In the human genome, ENDO1 has been localized to chromosome 19p13.2-13.3. This locus encodes many unknown transcripts, including several putative RING finger proteins, which makes it difficult to infer any putative roles of ENDO1. However, some genetic diseases have been associated with this locus such as familial hypercholesterolemia (336), ataxia (337), vacuolar neuromyopathy (338), polycystic liver disease (339), susceptibility to atherosclerosis (340) and thyroid cancer (341). In addition to ENDO1 being located in or near the locus related to these diseases, it is interesting that ENDO1 is abundantly expressed in the organs in which the disease is present, such as the liver, heart, and blood vessels. Furthermore, in regards to chromosomal location, a few known protein coding genes in close proximity to ENDO1, such as Rab11B and HNRPM, and are evolutionarily conserved between humans (chromosome 19), rats (chromosome 7), and mice (chromosome 17), suggesting the retainment of a certain set of signalling pathways. Conservation of pathways usually implies that they are indispensable for survival, and therefore, determination of the function of the other proximal putative proteins will be essential for understanding normal development and genetic diseases (342).

4.7. Subcellular localization of ENDO1

In order to decipher the mechanism of action of ENDO1, the subcellular localization of several ENDO1 deletion/mutation constructs was analyzed. The endogenous expression pattern of ENDO1 varies between cell types suggesting that ENDO1 undergoes different mechanisms of regulation. Different deletion/mutation constructs were made in order to decipher the domains of ENDO1 that may be

susceptible to cleavage and/or post-translational modifications. Structure-function analysis of the subcellular localization of the ENDO1 constructs reveals it to be localized not only in the cellular membranes (ENDO1A and ENDO1C) but also in the nucleus (ENDO1D). The difference between the expression of ENDO1C and ENDO1D is accounted for by a mutation in the second cysteine of the PHD consensus sequence to an arginine. Recently, Farrer and colleagues demonstrated that mutations in the RING finger 1 domain of Parkin (an E3 Ub ligase) results in a different localization of the protein (343). Several hypotheses can be generated regarding why ENDO1D translocates to the nucleus. Amino acid mutations may account for changes in the conformation of the protein, which in turn may allow it to interact with different binding partners and facilitate its transport to the nucleus. Alternatively, the second cysteine may also undergo a process called palmitoylation.

Palmitoylation is a post-translational modification process where a fatty acid, palmitate, is attached to a cysteine residue, making the protein more hydrophobic. The increase in hydrophobicity allows for the protein to interact with lipid bilayers, thus anchoring it to the membrane. The successful lipid-mediated anchoring of the protein to the membrane relies on several different factors, which are consistent with the predicted characteristics of ENDO1. For example, the palmitoylation of cystolic cysteine residues often occurs near the final transmembrane (TM) domain. Since ENDO1 only has two TM domains located close to each other, as long as the cysteine residue of interest is not located in between the TM domains, it is acceptable. There are certain structural requirements for palmitoylation. The distance between the TM domains and the cysteine residue must be certain residues apart (344,345). Furthermore, many proteins that undergo myristoylation or isoprenylation, are also found to undergo palmitoylation. In fact, these are other sequential events, with myristoylation/isoprenylation taking place first. The latter helps enhance subsequent palmitoylation (346,347). If a protein only undergoes palmitoylation, such as GAP43, there needs to be roughly 4-5 hydrophobic residues flanking the palmitoylated cysteine (348,349). Since this is not the case of ENDO1, the first hypothesis with myrisotylation is more relevant since ENDO1 does have two predicted N-myristoylation sites. This may explain the discrepancy in staining seen between the three mutant constructs. ENDO1A and ENDO1C seem to be attached to

membranes, which may be due to the presence of N-myristoylation and palmitoylation. In contrast, ENDO1D may be translocated to the nucleus, despite having two intact N-myrsitoylation sites, due to the instability gained from the loss of palmitoylation on the second cysteine of the PHD consensus sequence. Moreover, the presence of a basic residue, arginine, near the palmitoylated cysteine in ENDO1, may play a role in the distribution of ENDO1 (350). Overall, palmitoylation can play critical roles in regulating the activity of a protein, as shown for members of the RGS family of GTPase-activating proteins (351). Disruption of these regulatory mechanisms can compromise several critical processes (352,353). Moreover, the additional predicted N-glycosylation, Casein Kinase II and Protein Kinase C phosphorylation sites may play important roles in the ENDO1-signaling pathways. Differences in these residues among the family members of ENDO1 may suggest different regulatory mechanisms for each member.

Interestingly, a recent RING finger E3 Ub ligase, Sakura, was shown to be associated with membranes of neuronal cells through palmitoylation (354). Similar to Sakura setting the first example of a RING finger E3 Ub ligase to be palmitoylated in the brain, future studies may involve demonstrating that ENDO1 is the first palmitoylated PHD finger E3 Ub ligase shown to be present in the heart. Furthermore, the intracellular localization of ubiquitinated proteins helps determine their fate. Proteins ubiquitinated in the nucleus or nuclear membrane do not have the same outcome as those ubiquitinated in the cytosol or plasma membrane respectively (355). This may be one of the reasons why ENDO1 displays different subcellular expression patterns.

4.8. ENDO1 may be cleaved by members of the PC family

Analysis of the protein sequence of ENDO1 revealed that ENDO1 may be a target of the mammalian proprotein convertases (PC family). The PCs recognize the general consensus motif (K/R)-X_n-(K/R), where n = 0, 2, 4, 6, and X is any amino acid (356). This consensus sequence is similar to the RLHSR sequence present between amino acids 168-172 in ENDO1. There are 8 currently known members of the PC family: Furin, PC1/2, PC2, PC4, PACE4, PC5/6, PC7/LPC, and SKI-1/S1P. Although these proteins are ubiquitously expressed, Dr. Seidah's lab has previously shown that some of these family members are highly expressed in the heart and vasculature (357). Briefly, PC1 and PC2

are expressed in the intracardiac para-aortic ganglia. Furin and PACE4 are highly expressed in the atria and ventricles. Furin is also expressed in all cardiac tissues and cells, and incidentally, Furin knockout mice are embryonic lethal and show the *cardia bifida* phenotype (358). In contrast to PACE4, PC5 transcripts are expressed in the endothelial cells lining coronary vessels and the valve leaflets of the heart. Overall localization of these PC family members in the heart and cardiac blood vessels make them appealing candidates for upstream regulators of ENDO1.

Plasmids containing the cDNA of Furin and PC5 were generously obtained from Dr. Seidah's lab. Western Blot analysis of extracts overexpressing Furin or PC5 did not show any cleaved product of ENDO1 (data not shown). However, this result is not conclusive, since there may not only be other members which could cleave ENDO1, but also targets of PC members show the requirement of a stimulus in order for cleavage to take place. For example, SREBP (sterol regulatory element binding protein) is a membrane-bound bHLH transcription factor, which has a similar secondary structure like that of ENDO1. Its amino terminal has the bHLH domain required for transcriptional activation. In order for SREBP to regulate transcription, it needs to be cleaved so that it can be translocated to the nucleus. SREBP is anchored in the endoplasmic reticulum (ER) and nuclear envelope by its two TM domains. Two separate cleavage events are required in order to free SREBP. These are carried out by the PC family members, S1P (site-1 protease) and S2P (site-2 protease). S1P and S2P are located in the golgi whereas SREBP is located in the ER. In order for SREBP to travel to the golgi, it binds to SCAP (SREBP-cleavage-activating protein) which allows for its translocation to the golgi. However, in order for the SREBP-SCAP complex to migrate to the golgi, the cellular demand for sterols must be high (359,360). A similar proteolytic cascade may be required for ENDO1, where a binding partner and/or stimulus is required for proper migration and cleavage of ENDO1. One possible stimulus could be hypoxia, since ENDO1 contains two cytochrome c heme binding sites. Additional proteins that carry out transcriptional regulation such as Notch1 or huASH1 also undergo proteolysis to release their activation domains (361,362), and therefore cleavage of membrane-bound transcription factors may be a common way of regulating the expression of their target genes.

4.9. Cytoplasmic ENDO1 may be predominantly localized in the golgi

Based on the results obtained from the immunocytochemistry, a couple of speculations lead us to believe that ENDO1 is localized to the golgi, an organelle responsible for protein modification and cell sorting. First of all, members of the PC family, which are expressed in the golgi, have a subcellular expression pattern similar to ENDO1 (363,364). Secondly, it has been previously shown that palmitoylated proteins are not homogeneously distributed on the plasma membrane. Rather, they tend to concentrate on the golgi apparatus, or in plasma membrane subdomains that are enriched in cholesterol and sphingomyelin (365). This may allow for the lipids to aggregate into raft-like domains which are insoluble in non-ionic detergents, and are therefore called detergent-insoluble, glycolipid-enriched complexes (DIGs). In general, DIGs have been involved in facilitating signal transduction by targeting proteins to the rafts, and have been important in processes such as endocytosis and cholesterol trafficking (366,367). This may explain why in ventricular cardiomyocytes, ENDO1 is partially expressed in the plasma membrane. Lastly, if ENDO1 has its N- and C-terminal regions extending into the cytoplasm, like SREBP, then the region between its two TM domains makes it highly favorable for ENDO1 to be localized to the golgi. The golgi apparatus is an acidic compartment. The presence of basic amino acids in the intertransmembrane region of ENDO1 (amino acids 162-173), like histidine, allow for hydrogen ions to bind to this region, making it compatible with the environment in the lumen of the golgi. Taken together, confirmation of the golgi-specific expression of ENDO1 is important in order to allow for the pursuit of the hypotheses already generated regarding the mechanism of action of ENDO1. This could be achieved by specific co-labeling and confocal or immunogold electron microscopy.

4.10. ENDO1 may be involved in autoregulation

The results obtained from our immunocytochemistry and Western Blot analysis suggest that ENDO1 auto-regulates itself. Detection of endogenous ENDO1 in C2C12 cells overexpressing HA-ENDO1 indicate that the total level of ENDO1 is not different in cells overexpressing ENDO1 versus wild-type cells. Similar results were obtained in 293T cells transfected with either the empty vector, pCGN, or with HA-ENDO1. This

suggests that when ENDO1 levels are very high, there is a negative feedback mechanism on ENDO1 in order to decrease its levels to that which is present at normal conditions. To the best of our knowledge, only one E3 Ub ligase has been shown to ubiquitinate itself and subsequently degrade. BRCA1 (breast and ovarian cancer-specific tumor suppressor), a RING finger E3 Ub ligase, can bind with another RING finger protein, BARD1, and control cell signaling. Under certain conditions, it has been shown that BARD1 enhances autoubiquitination of BRCA1, which can either lead to BRCA1 degradation or involvement in DNA damage regulation depending upon which lysine is polyubiquitinated (368). Further analysis is required to establish whether ENDO1 autoregulates itself impart through autoubiquitination.

4.11. ENDO1 is upregulated in cardiomyocytes treated with phenylephrine

Soon after birth, cardiomyocytes lose the ability to divide and compensate instead by increasing in size, a process known as cardiac hypertrophy. Although originally meant as an adaptive mechanism, if prolonged, it could lead to cardiac disease, and eventually cardiac failure. As mentioned in the literature review, several lines of evidence have implicated GATA-4 in cardiac hypertrophy. Upon stimulation with phenylephrine, GATA-4 levels and activity are increased in ventricular cardiomyocytes. Time course experiments of ventricular cardiomyocytes treated with phenylephrine show that ENDO1 protein levels are increased around the time when cardiomyocytes are hypertrophied, suggesting that ENDO1 may not be involved in inducing cardiac hypertrophy, but rather maintaining the hypertrophied state. Although we would hypothesize ENDO1 levels to be downregulated in conditions where GATA-4 is increased, it may just be that ENDO1 is also regulated by other proteins and signaling pathways that are activated in hypertrophied myocytes. This is possible since cardiac hypertophy is partially defined by the upregulation of several fetal genes, and therefore, the cellular pathways present in normal cardiomyocytes may be different than those in hypertrophic cardiomyocytes.

4.12. ENDO1 is a novel transcriptional regulator of cardiac promoters

The presence of the PHD finger in ENDO1 led us to test the hypothesis of whether ENDO1 is involved in transcriptional regulation. Indeed, transfection of the full

length ENDO1 protein led to repression of the BNP promoter. Other promoters were tested in addition to the BNP promoter such as ANF and ET-1. Preliminary analysis of these latter two promoters suggest that they may also be downstream targets of ENDO1 (data not shown). This is not all that surprising since PHD fingers are largely transcriptional repressors or co-repressors. Deletional/mutational analysis of the BNP promoter suggests that ENDO1 acts on the GATA binding sites. Further analysis needs to be done in order to determine whether ENDO1 acts directly or indirectly on the BNP promoter. However, it is plausible, based on the data obtained thus far, that GATA-4 may downregulate ENDO1 in order to allow for the activation of its own target genes. At present, in order to obtain a better insight into the structural domains of ENDO1 which are required for transcriptional repression, studies using the deletion/mutation constructs of ENDO1 are being used. Since preliminary analysis suggests that removal of the C-terminal region results in activation of the promoter, it may be that proteolytic processing of ENDO1 plays an essential role in determining the effect that ENDO1 has on its target genes. Therefore, the post-translational modifications of ENDO1 may be very improtant for determining the effect ENDO1 has on overall cellular functions.

4.13. E3 ubiquitin ligases as regulators of gene expression

Since the discovery that there are only ~ 30 000 genes in the human genome, it was proposed that many of these proteins must play multifunctional roles during development and pathological conditions. As shown in the PHD finger chart in the results section (Table 1), several known PHD finger proteins have already been shown to play multiple roles. The hypothesis that ENDO1 may be an E3 Ub ligase as well as a transcription factor is not unusual, since E3 Ub ligases themselves have been shown to play a role in transcriptional regulation through various mechanisms. For example, cellular oxygen levels are able to regulate the gene expression level of several proteins through ubiquitination. In situations where there is plenty of oxygen, hypoxia-inducible transcription factor (HIF1α) is rapidly ubiquitinated. In contrast, when oxygen levels are low, HIF1α is not ubiquitinated and is able to activate transcription of oxygen or hypoxia regulated genes, such as VEGF (369). Similarly, the presence of cyctochrome c heme binding sites in ENDO1 could also implicate it in regulating genes under conditions of

hypoxia. E3 ligases can also mediate transcriptional events by ubiquitylating certain domains of a protein and then targeting it for proteolytic processing by the proteosome in order to activate their transcriptional domains. This is the case of nuclear factor kappa B1 (NF-κB1), where its p105 and p100 precursors are ubiquitinated and subsequently cleaved by the proteosome to generate the active p52 and p50 subunits (370). Furthermore, SPT23 and MGA2, two yeast transcription factors involved in the activation of genes required for the synthesis of polyunsaturated fatty acids, are initially synthesized as inactive precursors that are anchored to the endoplasmic reticulum membrane. When yeast cells are lacking polyunsaturated fatty acids, the inactive precursor proteins are ubiquitinated by the HECT domain of E3 Ub ligase Rsp5, and processed by the proteosome. This allows for their translocation to the nucleus where they are able to activate the necessary genes (371). Lastly, another mechanism by which E3 Ub ligases can regulate transcription is demonstrated by studies carried out on the yeast transcription factor, Met4 (372). Met4 binds to the promoters of methionine-regulated genes, and it can interact with other transcription factors, such as the basic helix-loop-helix factor Cbf1, in order to activate these genes in conditions were cells are deprived of methionine. In situations where there is an adequate amount of methionine, Met4 is ubiquitinated, however it is not targeted for proteolysis. Instead, the ubiquitinated Met4 still binds to its promoters, but since the interaction with Cbf1 is now disrupted, there is no activation of its target genes. Subsequent deubiquitination of Met4 activates transcription, overall suggesting that ubiquitination may indeed be an important mechanism by which the expression of genes is regulated.

5. CONCLUSION

In our lab, we have isolated a novel gene, ENDO1, which in addition to being inhibited specifically by GATA-4, has also previously been shown *in vitro* to be involved in endothelial cell differentiation. This novel transcript may be part of a novel subfamily of PHD finger proteins that may have E3 ubiquitin ligase activity as a common function. ENDO1 is expressed in several areas during embryonic development, such as blood vessels, the heart, brain and liver, suggesting ENDO1 to be a multifunctional protein that is involved in numerous cell types and developmental systems. ENDO1 is a 27kDa protein that has a differential subcellular expression pattern in cells. In cardiac fibroblasts, it is predominantly expressed in the plasma membrane and nucleus, whereas in other cell types tested, it seems to be in the membranes of the nucleus and organelles, such as the golgi.

Analysis of the functional roles of ENDO1 demonstrates that it participates in processes common to GATA-4, such as cell differentiation and cardiac hypertrophy. This suggests that it plays vital roles during development and possibly cardiac disease. In addition to its putative role as an E3 ubiquitin ligase, ENDO1 may be a multifunctional protein, where it acts as a transcriptional repressor of a cardiac promoter, BNP. Given that it can mediate the expression of at least one critical cardiac gene suggests other targets to be genes that play important roles during development. Further analysis at the *in vivo* level, using for example, transgenic mice overexpressing HA-ENDO1 in endothelial cells, may give us an insight into the kind of signaling pathways that ENDO1 is involved in between the cardiac and endothelial developmental systems. Overall, we have only started to shed some light into the putative biological functions of ENDO1, however, further studies need to be carried out in order to develop a more precise understanding of its mechanism of action.

6. PERSPECTIVES

6.1. Refine analysis of subcellular localization of ENDO1

Future studies will help us better comprehend the role of ENDO1 in cardiomyocytes and endothelial cells. To begin with, in order to obtain a more complete characterization of ENDO1, it is important to confirm the subcellular expression of ENDO1 using antibodies specific to organelles. This will give us further insight into the mechanism of action of ENDO1; for example, if ENDO1 is found to be localized to the golgi, then it may be plausible that ENDO1 may be targeted by proprotein convertases.

6.2. Determine under which conditions ENDO1 is cleaved

Studies dedicated towards finding a stimulus that triggers ENDO1 cleavage will be necessary in order to confirm whether members of the PC family are involved in proteolysis of ENDO1. Cleavage of ENDO1 could be tested under conditions of hypoxia, since ENDO1 does contain two cytochrome c heme binding sites which could implicate it as having an oxygen sensory role. This, in turn, could implicate it to have important roles during vasculogenesis/angiogenesis, since oxygen sensors have been found to play important roles during this developmental period (373).

6.3. Determine if ENDO1 is a *bona fide* E3 ubiquitin igase

Given the high homology of the PHD domain and secondary structure of ENDO1 to other E3 Ub ligases, it will be interesting to see whether ENDO1 can ubiquitinate proteins. Should ENDO1 be discovered to be a novel E3 Ub ligase in cardiomyocytes and endothelial cells, future studies would include finding new downstream targets of ENDO1. The mechanism by which ENDO1 regulates the activity of these proteins may give us new information on GATA-4-dependent signaling pathways.

6.4. Decipher how ENDO1 transcriptionally regulates its target genes

Since we have shown that ENDO1 can transcriptionally repress the BNP promoter, mapping the binding element on which ENDO1 is acting will open the doors towards additional studies that can give information as to whether ENDO1 is acting

directly or indirectly on the promoter. The pursuit of finding potential binding partners will aid in deciphering the mechanism of action of ENDO1's transcriptional regulation. The studies involving the deletion/mutation constructs need to be repeated with and without GATA-4 in order to get a better idea as to what structural domains of ENDO1 act on and interact with the BNP promoter and GATA-4 respectively. As mentioned in the discussion, ubiquitination of proteins themselves can be used as a tool for regulating activation or repression of events. Should ENDO1 be discovered to be a *bona fide* E3 Ub ligase, future studies will involve deciphering whether ENDO1's E3 Ub ligase activity and transcriptional regulation are mutually exclusive events.

Based on our transfection studies, we hypothesize ENDO1 to be acting directly or indirectly on the GATA binding sites of the BNP promoter. It would therefore be interesting to see how GATA-4 regulates the expression and/or function of ENDO1, and the impact that GATA-4-ENDO1 signaling has on cellular events. At present, co-expression of GATA-4 with the reporter constructs, and the wild-type and deletion/mutation constructs of ENDO1, in transfection assays, are being carried out to see if GATA-4 could be a potential binding partner of ENDO1. Further studies involve pulldown assays which will allow us to see if there is a physical interaction of ENDO1 with GATA-4.

6.5. *In vitro* loss- and gain-of-function analysis

In order to understand which cellular processes are affected by ENDO1, *in vitro* loss- and gain-of-function studies can be carried out in progenitor cells. For example, stable clones expressing either sense or antisense ENDO1 in TC13 and P19 cells will give us a better insight into their specific roles in endothelial cells and cardiomyocytes respectively. The gain-of-function experiments using these stable lines will allow us to decipher whether the overexpression of ENDO1 is sufficient for differentiation, or whether its absence blocks the differentiation or proliferation, of these progenitor cells.

6.6. *In vivo* gain-of-function analysis

At present, we are pursuing *in vivo* gain-of-function analysis using Tie2-HA-ENDO1 transgenic stable lines that we have recently produced. Further analysis of the

copy numbers of the transgene present in the lines of mice and confirmation of the protein expression of HA-ENDO1 in endothelial cells will allow us to assess the effect that ENDO1 has on the vasculature and potentially other systems. It will be particularly interesting to look at the effect of endothelial-specific overexpression on cardiac development in order to assess how ENDO1 plays a role in the communication events between these two systems.

6.7. *In vivo* loss-of-function analysis

Future *in vivo* loss-of-function studies will involve producing ENDO1 knockout mice. Since the PHD finger is entirely localized in Exon3, removal of this exon may abolish not only its function as a transcriptional regulator but also its potential E3 ligase activity. Given that it is a downstream target of an indispensable transcription factor, ENDO1 may play very critical roles during development. The phenotype may be embryonic lethal, in which case analysis may have to be predominantly done on heterozygotes. Overall, the combination of *in vitro* and *in vivo* studies will open the avenue towards discovering new signaling pathways that may be involved in the communication between cardiomyocytes and endothelial cells. This will lead to a better understanding of developmental systems and pathological conditions, which in turn, will lead to better therapeutic interventions in the future.

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8. APPENDIX FOR ETHICS CERTIFICATES

Canadian Nuclear Safety Commission | Commission canadienne de sûreté nucléaire

03055-1-08.0

NUCLEAR SUBSTANCES AND RADIATION DEVICES LICENCE | PERMIS PORTANT SUR LES SUBSTANCES NUCLÉAIRES ET LES APPAREILS À RAYONNEMENT

Licence Number / Numéro de permis

I) TITULAIRE DE PERMIS

Conformément à l'article 24 (2) de la Loi sur la sûreté et la réglementation nucléaires, le présent permis est délivré à:

Institut de recherches cliniques
de Montréal
110, avenue des Pins Ouest
Montréal (Québec)
H2W 1R7
Canada

Ci-après désigné sous le nom de «titulaire de permis»

II) PÉRIODE

Ce permis est valide du 1er février 2003 au 31 janvier 2008.

III) ACTIVITÉS AUTORISÉES

Le présent permis autorise le titulaire à posséder, transférer, importer, exporter, utiliser et entreposer les substances nucléaires et les équipements autorisés qui sont énumérés dans la section IV) du présent permis.

Le présent permis est délivré pour le type d'utilisation: études de laboratoire - 10 laboratoires ou plus où des radio-isotopes sont utilisés ou manutentionnés (836)

IV) SUBSTANCES NUCLÉAIRES ET ÉQUIPEMENT AUTORISÉ

ARTICLE	SUBSTANCE NUCLÉAIRE	SOURCE NON SCELLÉE QUANTITÉ MAXIMALE	ASSEMBLAGE DE LA SOURCE SCELLÉE QUANTITÉ MAXIMALE	ÉQUIPEMENT - FABRICANT ET MODÈLE
1	Calcium 45	100 MBq	s/o	s/o
2	Cobalt 57	40 MBq	s/o	s/o
3	Cobalt 59	40 MBq	s/o	s/o
4	Chrome 51	400 MBq	s/o	s/o
5	Fer 59	2 GBq	s/o	s/o
6	Hydrogène 3	3 GBq	s/o	s/o
7	Iode 123	500 MBq	s/o	s/o
8	Iode 125	3 GBq	s/o	s/o
9	Iode 131	500 MBq	s/o	s/o
10	Phosphore 32	30 GBq	s/o	s/o
11	Phosphore 33	100 MBq	s/o	s/o
12	Rubidium 86	40 MBq	s/o	s/o
13	Soufre 35	3 GBq	s/o	s/o
14	Zinc 65	40 MBq	s/o	s/o
15	Césium 137	s/o	400 kBq	s/o
16	Radium 226	s/o	400 kBq	s/o
17	Césium 137	s/o	1110 kBq	Beckman LS series
18	Radium 226	s/o	370 kBq	Wallac Série 1200

La quantité totale d'une substance nucléaire non scellée possédée ne doit pas excéder la quantité maximale qui est indiquée pour une source non scellée correspondante. La quantité de substance nucléaire par source scellée ne doit pas excéder la quantité maximale indiquée par source scellée correspondante. Les sources scellées doivent être utilisées seulement dans l'équipement indiqué corrrespondant.

V) ENDROIT(S) OÙ LES ACTIVITÉS AUTORISÉES PEUVENT ÊTRE EXERCÉES

utilisées ou entreposées à (aux)
endroit(s) suivant(s):

110, avenue des Pins Ouest

Copie de la commission

.../ 2

Montréal (Québec)

VII) CONDITIONS

1. Interdiction visant l'utilisation chez les humains
Le permis n'autorise pas l'utilisation des substances nucléaires dans ou sur le corps d'une personne.
(2696-0)

2. Classification des zones, pièces et enceintes
Le titulaire de permis désigne chaque zone, pièce ou enceinte où on utilise plus d'une quantité d'exemption d'une substance nucléaire non scellée à un moment donné selon la classification suivante :

(a) de niveau élementaire si la quantité ne dépasse pas 5 LAI,
(b) de niveau intermédiaire si la quantité utilisée ne dépasse pas 50 LAI,
(c) de niveau supérieur si la quantité ne dépasse pas 500 LAI,
(d) de confinement si la quantité dépasse 500 LAI;
(e) à vocation spéciale, avec l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.

À l'exception du niveau élementaire, le titulaire de permis n'utilise pas de substances nucléaires non scellées dans ces zones, pièces ou enceintes sans l'autorisation écrite de la Commission ou d'une personne autorisée par celle-ci.
(2108-1)

3. Liste des laboratoires
Le titulaire de permis tient à jour une liste de toutes les zones, salles et enceintes dans lesquelles plus d'une quantité d'exemption d'une substance nucléaire est utilisée ou stockée.
(2569-1)

4. Procédures de laboratoire
Le titulaire affiche en tout temps et bien en évidence dans les zones, les salles ou les enceintes où des substances nucléaires sont manipulées une affiche sur la radioprotection qui a été approuvée par la Commission ou une personne autorisée par la Commission et qui correspond à la classification de la zone, de la salle ou de l'enceinte.
(2570-1)

5. Surveillance thyroïdienne
La personne
a) qui utilise à un moment donné une quantité d'iode 125 ou d'iode 131 volatiles dépassant :
(i) 5 MBq dans une pièce ouverte,
(ii) 50 MBq dans une hotte,
(iii) 500 MBq dans une boîte à gants,
(iv) toute autre quantité dans une enceinte de confinement approuvée par écrit par la Commission ou une personne autorisée par celle-ci;
(b) qui est impliqué dans un déversement mettant en cause plus de 5 MBq d'iode 125 ou d'iode 131 volatiles;
(c) chez laquelle on détecte une contamination externe à l'iode 125 ou l'iode 131;
doit se prêter à un dépistage thyroïdien dans les cinq jours suivant l'exposition.
(2046-7)

6. Dépistage thyroïdien
Le dépistage de l'iode 125 et de l'iode 131 internes se fait :
(a) par mesure directe à l'aide d'un instrument capable de détecter 1 kBq d'iode 125 ou d'iode 131;
(b) par essai biologique approuvé par la Commission ou une personne autorisée par celle-ci.
(2600-2)

7. Essai biologique thyroïdien
Si la charge thyroïdienne dans une personne depasse 10 kBq d'iode 125 ou d'iode 131, le titulaire de permis doit présenter immédiatement un rapport préliminaire à la Commission ou à une personne autorisée par

celle-ci. Dans un délai de 24 heures, la personne en question doit subir des essais biologiques par une personne autorisée par la Commission à offrir un service de dosimétrie interne.
(2601-4)

8. Dosimétrie des extrémités
Le titulaire de permis veille à ce que toute personne qui manipule un contenant renfermant plus de 50 MBq de phosphore 32, de strontium 89, d'yttrium 90, de samarium 153 ou de rhénium 186 porte une bague dosimetre. Le dosimetre est fourni et lu par un service de dosimétrie autorisé par la Commission.
(2578-0)

9. Critères de contamination
En ce qui a trait aux substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis, le titulaire de permis veille à ce que :
(a) la contamination non fixée dans toutes les zones, pièces ou enceintes où on utilise ou stocke des substances nucléaires non scellées ne dépasse pas :
(i) 3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 30 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 300 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la contamination non fixée pour toutes les autres zones ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2.
(2642-2)

10. Déclassement
Avant le déclassement d'une zone, d'une pièce ou d'une enceinte où s'est déroulée l'activité autorisée, le titulaire de permis veille à ce que :
(a) la contamination non fixée pour les substances nucléaires figurant au tableau «Classification des radionucléides» du guide sur les demandes de permis ne dépasse pas :
(i) 0,3 Bq/cm2 pour tous les radionucléides de catégorie A,
(ii) 3 Bq/cm2 pour tous les radionucléides de catégorie B,
(iii) 30 Bq/cm2 pour tous les radionucléides de catégorie C,
selon une moyenne établie pour une surface ne dépassant pas 100 cm2;
(b) la mise en disponibilité de toute zone, pièce ou enceinte contenant une contamination fixée soit approuvée par la Commission ou une personne autorisée par celle-ci;
(c) toutes les substances nucléaires et tous les appareils à rayonnement ont été transférés conformément aux conditions du permis;
(d) tous les panneaux de mise en garde contre les rayonnements ont été retirés ou ont été rendus illisible.
(2571-2)

11. Stockage
Le titulaire :
a) veille à ce que seules les personnes autorisées par lui aient accès aux substances nucléaires radioactives ou aux appareils à rayonnement stockés;
b) veille à ce qu'à tout endroit occupé à l'extérieur de la zone, de la salle ou de l'enceinte de stockage le débit de dose provenant des substances ou appareils stockés ne dépasse pas 2,5 microSv/h;
c) a des mesures en place pour assurer que les limites de dose indiquées dans le Règlement sur la radioprotection ne sont pas dépassées en raison du stockage de ces substances ou appareils.
(2575-0)

12. Évacuation (laboratoires)
Lorsqu'il évacue des substances nucléaires non scellées dans une décharge municipale ou un réseau d'égouts, le titulaire de permis veille à ce que les limites suivantes ne soient pas dépassées :

COLONNE 1	COLONNE 2(a)	COLONNE 3(b)
-	LIMITES	LIMITES
Substance	solides à la	liquides

nucléaire -	décharge municipale (quantité au kg)	(hydrosolubles) l'égout municipal (quantité par an)
Carbone 14	3,7 MBq	10 000 MBq
Chrome 51	3,7 MBq	100 MBq
Cobalt 57	0,37 MBq	1000 MBq
Cobalt 58	0,37 MBq	100 MBq
Hydrogène 3	37 MBq	1 000 000 MBq
Iode 125	0,037 MBq	100 MBq
Iode 131	0,037 MBq	10 MBq
Phosphore 32	0,37 MBq	1 MBq
Phosphore 33	1 MBq	10 MBq
Soufre 35	0,37 MBq	1000 MBq
Technétium 99m	3,7 MBq	1000 MBq

(a) Les limites indiquées à la colonne 2 s'appliquent aux quantités de déchets solides de moins de trois tonnes par an. Les substances nucléaires évacuées dans la décharge municipale doivent être sous forme solide et distribuées uniformément dans les déchets; la concentration doit être inférieure aux limites indiquées à la colonne 2. Lorsqu'on évacue plus d'une substance nucléaire à la fois, le quotient obtenu en divisant la quantité de chaque substance par sa limite correspondante de la colonne 2 ne doit pas dépasser un.

(b) Les limites indiquées à la colonne 3 s'appliquent à la forme liquide (hydrosoluble) de chaque substance nucléaire qui peut être évacuée par an et par bâtiment.
(2161-3)

13. Exigences concernant les contaminamètres
Le titulaire de permis met en tout temps à la disposition des travailleurs sur les lieux de l'activité autorisée un contaminamètre portatif en bon état de fonctionnement.
(2572-1)

14. Rapport annuel de conformité
Deux mois avant l'anniversaire de la date d'expiration indiquée à la section II de son permis, le titulaire de permis soumet par écrit à la Commission, ou à une personne autorisée par celle-ci, un rapport annuel de conformité de l'activité autorisée qui contient des renseignements opérationnels suffisants et sous une forme que la Commission juge acceptable.
(2916-4)

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Fonctionnaire désigné en vertu du paragraphe 37(2)(c) de la Loi sur la sûreté et la réglementation nucléaires

Copie de la commission