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## Regulation of *Ncx1* Gene Expression in the Normal and Hypertrophic Heart

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### Abstract

The Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX1) is crucial in the regulation of [Ca<sup>2+</sup>]<sub>i</sub> in the cardiac myocyte. The exchanger is upregulated in cardiac hypertrophy, ischemia, and failure. This upregulation can have an effect on Ca<sup>2+</sup> transients and possibly contribute to diastolic dysfunction and an increased risk of arrhythmias. Studies from both *in vivo* and *in vitro* model systems have provided an initial skeleton of the potential signaling pathways that regulate the exchanger during development, growth, and hypertrophy. The *Ncx1* gene is upregulated in response to  $\alpha$ -adrenergic stimulation. We have shown that this is via p38 $\alpha$  activation of transcription factors binding to the *Ncx1* promoter at the -80 CARG element. Interestingly, most of the elements, including the CARG element, which we have demonstrated to be important for regulation of *Ncx1* expression are in the proximal 184 bp of the promoter. Using a transgenic mouse, we have shown that the proximal 184 bp is sufficient for expression of reporter genes in adult cardiomyocytes and for the correct spatiotemporal pattern of *Ncx1* expression in development but not for upregulation in response to pressure overload.

### Keywords

Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; NCX1; cardiac hypertrophy; regulation of gene expression; signal transduction; transgenic mice

## INTRODUCTION

The heart is very dynamic and adapts to changes in hemodynamic demands by the enlargement of the ventricle via individual cardiomyocyte hypertrophy. The cardiac hypertrophic response is characterized by an increase in cardiac mass, protein content, and size of the individual cardiocyte. Initially, this results in increased myofilaments and improved contractile function. But, if the pathological stimulus is prolonged or sufficiently severe, and the increase in mass is insufficient to normalize ventricular wall stress, decompensated hypertrophy or heart failure will occur. These stimuli are often initiated by

chronic hypertension, myocardial infarction, or ischemia associated with coronary artery disease. One of the features of the failing heart is a prolonged action potential and depressed contractility. In many models of heart disease and failure, the expression and activity of the  $\text{Ca}^{2+}$  sequestering sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$ -ATPase is decreased, and/or the activity and protein level of  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX1) is increased.<sup>1-4</sup> The exchanger catalyzes the electrogenic exchange of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  across the plasma membrane in either the  $\text{Ca}^{2+}$ -influx or  $\text{Ca}^{2+}$ -efflux mode. NCX1 is one of the essential regulators of  $\text{Ca}^{2+}$  homeostasis within cardiomyocytes and changes in its activity can affect contractility. The exchanger is upregulated at the transcriptional level in cardiac hypertrophy and failure.<sup>1,5-8</sup> Increased exchanger activity leads to increased  $\text{Ca}^{2+}$  extrusion and acts to preserve low diastolic  $\text{Ca}^{2+}$  levels, which may compensate in part for depressed SR  $\text{Ca}^{2+}$ -ATPase function. However, this adaptation has been demonstrated to have a number of deleterious consequences. First, in the failing heart, peak systolic  $\text{Ca}^{2+}$  is decreased because a higher percentage of  $[\text{Ca}^{2+}]_i$  is being removed by NCX1, rather than returning to the SR.<sup>9</sup> In addition to the increase in NCX1 expression and activity, and reduced SR  $\text{Ca}^{2+}$ -ATPase function, Marx *et al.*<sup>10</sup> have shown that the ryanodine receptor is hyperphosphorylated in the failing heart. This results in increased overall  $\text{Ca}^{2+}$  release from the channel and has also been shown to contribute to a persistent unloading of SR  $\text{Ca}^{2+}$  stores.

Second, changes in *Ncx1* expression and/or activity can contribute to aberrant contractile function. Because the exchanger is an electrogenic transporter, the increase in  $\text{Ca}^{2+}$  translocation across the plasma membrane results in greater risk of an inappropriately triggered depolarization (delayed after depolarization, DAD) before the relaxation cycle has completed.<sup>3,11</sup> This can cause arrhythmia and sudden death, a common cause of death from heart disease. Another potential problem arises from the lower levels of cytosolic  $\text{Ca}^{2+}$  in combination with prolonged action potential duration, which can promote reverse mode,  $\text{Ca}^{2+}$  in  $\text{Na}^+$  out, activity. This would not only slow the rate of decay of the  $\text{Ca}^{2+}$  transient and relaxation, but also contribute to possible arrhythmogenesis and diastolic dysfunction.<sup>12</sup> Finally, in hearts that have been exposed to ischemia or hypoxia, reoxygenation can result in serious injury due to hyper-contracture caused by spontaneous  $\text{Ca}^{2+}$  oscillations.<sup>13</sup> Several studies have shown that these oscillations are primarily elicited by reverse mode activity of the NCX1.<sup>14-16</sup> There is still a great deal more to be understood, but clearly, changes in exchanger expression are important to cardiac function. Therefore, we have begun to identify the molecular pathways and transcription factors and elements that are responsible for the upregulation of the exchanger gene in cardiac hypertrophy and failure.

There are multiple tissue-specific variants of *Ncx1* resulting from alternative promotor usage (H1, K1, and Br1) and alternative splicing.<sup>17-19</sup> Using transgenic models we demonstrated that the *1831Ncx1* H1 promotor was sufficient for driving the normal spatiotemporal pattern of NCX1 expression in cardiac development.<sup>17</sup> Importantly, at later fetal stages (E14 and older), and in the adult the *1831Ncx1* H1 promotor-driven reporter gene expression remains heart specific when the normal heart-restricted pattern of the endogenous NCX1 is no longer present.<sup>20</sup> Koban *et al.* showed cardiac-specific expression of reporter genes using either 2.1 kb or 2.6 kb rat *Ncx1* H1 promotor.<sup>21</sup> Expression was first detected at E7.5 and remained cardiac specific in the adult animal. Interestingly, here reporter gene expression was diminished with age. Importantly, we demonstrated that the feline *1831Ncx1* H1 promotor is sufficient for the upregulation of *Ncx1* in response to pressure overload in transgenic mice.<sup>17</sup>

### **p38 ACTIVITY IS IMPORTANT FOR MEDIATING *Ncx1* EXPRESSION**

Several signaling pathways, including those activated by G protein-coupled receptor (GPCR) agonists, integrins, nonreceptor protein tyrosine kinases, protein kinase C, intracellular  $\text{Ca}^{2+}$ , and calcineurin are implicated in the initiation and maintenance of

hypertrophy. The mitogen-activated protein kinases (MAPK), which transduce extracellular signals into the nucleus, are induced by hypertrophic stimuli. Importantly, their activation can initiate a hypertrophic response. p38 MAPK is of distinct interest since it has been shown to play a possible role in cardiocyte growth,<sup>22</sup> proapoptotic<sup>23</sup> or antiapoptotic<sup>24</sup> pathways. *In vitro* studies in neonatal cardiomyocytes show that activation of p38 induces expression of atrial natriuretic peptide<sup>22,23,25</sup> and inhibition of p38 reduced agonist-induced brain natriuretic peptide promoter activity.<sup>26,27</sup> Activation of p38 has been shown to mediate a negative inotropic effect, contribute to loss of contractility, and increase myocardial stiffness.<sup>28</sup> Andrews *et al.* have also shown that p38 can mediate the downregulation of SERCA2, which resulted in altered  $[Ca^{2+}]_i$  regulation.<sup>29</sup> In addition to regulating SERCA, p38 also plays a role in mediating *Ncx1* expression.<sup>30-32</sup> We showed that the p38  $\alpha/\beta$  inhibitor SB202190 inhibits 50% of *Ncx1* upregulation mediated by  $\alpha$ -adrenergic stimulation.<sup>32</sup> Overexpression of a dn p38 in adult cardiomyocytes also leads to a 50% decrease in  $\alpha$ -adrenergic stimulation of *Ncx1*-promotor-driven reporter gene as well as endogenous *Ncx1* transcript. Our results clearly showed that p38 contributed to the  $\alpha$ -adrenergic-mediated *Ncx1* upregulation. The MEK-1 and -2 inhibitor U0126 inhibits approximately 35% of the PE-stimulated *Ncx1* upregulation. Therefore, p38 and ERK-1 and -2 together mediate the majority of the  $\alpha$ -adrenergic stimulation of the *Ncx1* promoter in adult cardiomyocytes (FIG. 1). In order to determine if activated p38 is sufficient for induction of the *Ncx1* promoter, we overexpressed the constitutively active forms of the p38 upstream kinases, MKK3 and MKK6. Overexpression of either MKK3 or MKK6 resulted in upregulation of the *Ncx1* promoter-driven reporter gene.<sup>32</sup> p38 $\alpha$ , p38 $\delta$ , and p38 $\gamma$  have been detected in the heart with p38 $\alpha$  as the predominant isoform.<sup>33</sup> p38 $\beta$  has not been detected at the mRNA or protein level in human,<sup>33</sup> rat,<sup>34</sup> or mouse<sup>28</sup> heart. SB202190 completely prevented NCX1 upregulation in response to overexpression of MKK3bE or MKK6bE. p38 $\alpha$  is the only p38 isoform activated by MKK3bE, which is also inhibited by SB202190. Therefore, activation of p38 is not only critical for a portion of the  $\alpha$ -adrenergic-stimulated *Ncx1* upregulation, it is sufficient for *Ncx1* upregulation and it appears that this is mediated chiefly via p38 $\alpha$ .

## THE *Ncx1*-80 CARG ELEMENT MEDIATES p38-STIMULATED UPREGULATION OF THE EXCHANGER

The *Ncx1* promoter contains several consensus sequences for a number of potential DNA-binding factors, which we have shown to be important in regulating *Ncx1* expression in neonatal<sup>6</sup> and adult cardiomyocytes. These include two GATA elements, two MEF2 elements, two E-Box elements, a CARG element, and a binding site for Nkx 2.5 (FIG. 1). These elements are also present in the human and rat and interestingly, the first 184 bp of the human<sup>35</sup> and feline<sup>19</sup> *Ncx1* H1 promoters have a 97% identity and the rat<sup>36</sup> and feline sequences 92%. In isolated adult cardiocytes the -123 GATA and -50 GATA element resulted in 35% and 29% of wild-type promoter activity respectively (FIG. 2). A point mutation disrupting the -80 CARG element resulted in luciferase activity of less than 20% of the control level.<sup>37</sup> In addition to the CARG and GATA elements being important to basal *Ncx1* expression in adult cardiomyocytes, the -80 CARG element is required for *Ncx1* upregulation in response to p38 activation (see FIG. 1).<sup>32</sup> Our study also showed that the -80 CARG element is responsible for part of the  $\alpha$ -adrenergic-stimulated upregulation.

## THE PROXIMAL 184 bp OF THE PROMOTOR IS SUFFICIENT FOR EXPRESSION BUT NOT FOR UPREGULATION IN RESPONSE TO PRESSURE OVERLOAD

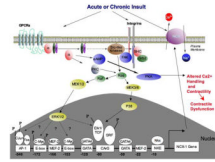
The majority of the consensus sequences for DNA-binding factors, which are important in regulating *Ncx1* expression, are found in the proximal 184 bp of the promoter. Our early work demonstrated that the *184Ncx1* promoter is sufficient for driving reporter expression in both neonatal and adult cardiomyocytes. Interestingly, the *184Ncx1* promoter is upregulated in response to  $\alpha$ -adrenergic stimulation in neonatal cardiomyocytes but is recalcitrant to  $\alpha$ -adrenergic stimulation in adult cardiomyocytes.<sup>6,37</sup> Subsequently, Xu *et al.* demonstrated that the *184Ncx1* promoter fragment could direct *LacZ* expression to the embryonic heart, with no expression in extracardiac tissues.<sup>37</sup> Expression is restricted to cardiomyocytes and is not observed in endothelial or endocardial cushion cells. The penetrance of expression varies between transgenic lines (FIG. 3). But even in lines where most cardiomyocytes express the reporter at 9.5 days (see line 1 FIG. 3) the expression pattern becomes patchy by E14.5 and diminished overall in the newborn<sup>37</sup> and in the adult (FIG. 4 A). In addition to the patchy cardiac myocyte expression in the adult, *184Ncx1*- $\beta$ -gal reporter activity was detected in the sinus venosus, and the pulmonary and caval veins, which was never observed in any of the *1831Ncx1* transgenic mouse lines.<sup>17</sup> Koban *et al.* showed for the rat *Ncx1* promoters, that expression of the transgene could be detected in the conduction system.<sup>21</sup> Interestingly, even though reporter gene expression is extremely patchy in the adult the *184Ncx1*- $\beta$ -gal transgene expression is consistently found within the cardiac myocytes that appeared to be part of the adult conduction system (FIG. 4 B). Positive *lacZ* staining could be detected in cells surrounding papillary muscles and in the wall of the left ventricle (FIG. 4 B, arrowheads). These data indicate that the *184Ncx1*- $\beta$ -gal transgene contains the enhancer elements sufficient to drive the correct cardiac myocyte-specific expression early in development but that it is missing some of the elements that contribute to the persistence of ubiquitous cardiomyocyte expression and extracardiac expression in the neonate and adult. What appears to be affected is the probability of the expression of the reporter gene in any particular cardiocyte not the level of expression. This probability of expression may be highest in specialized cardiomyocytes that make up the conduction system, though we have not yet confirmed this using a specific marker against specialized conduction system myocytes. Additionally, most of the cells that continue to express the transgene in late development and in the adult appear to be subendocardial rather than subepicardial.

Although the minimal promoter is sufficient to direct the cardiomyocyte-restricted expression at the proper time in development, it is not sufficient to mediate upregulation in response to pressure overload.<sup>37</sup> Clearly, these data indicate that although elements within the proximal 184 bases may be required for its expression there are additional distal elements necessary for *Ncx1* regulation. We are currently analyzing the role of distal elements to determine which are required for upregulation and to maintain levels of expression in all cardiocytes. In spite of the loss of regulation by pressure overload and persistence of ubiquitous cardiocyte expression in the adult, it is remarkable that such a small region of the *Ncx1* promoter is capable of driving the correct temporal- and tissue-specific expression.

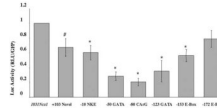
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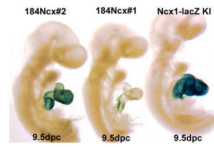


**FIGURE 1.** Diagram of the signaling pathways and transcription elements on the proximal *Ncx1* promoter important for the regulation of exchanger expression in the adult cardiocyte.



**FIGURE 2.**

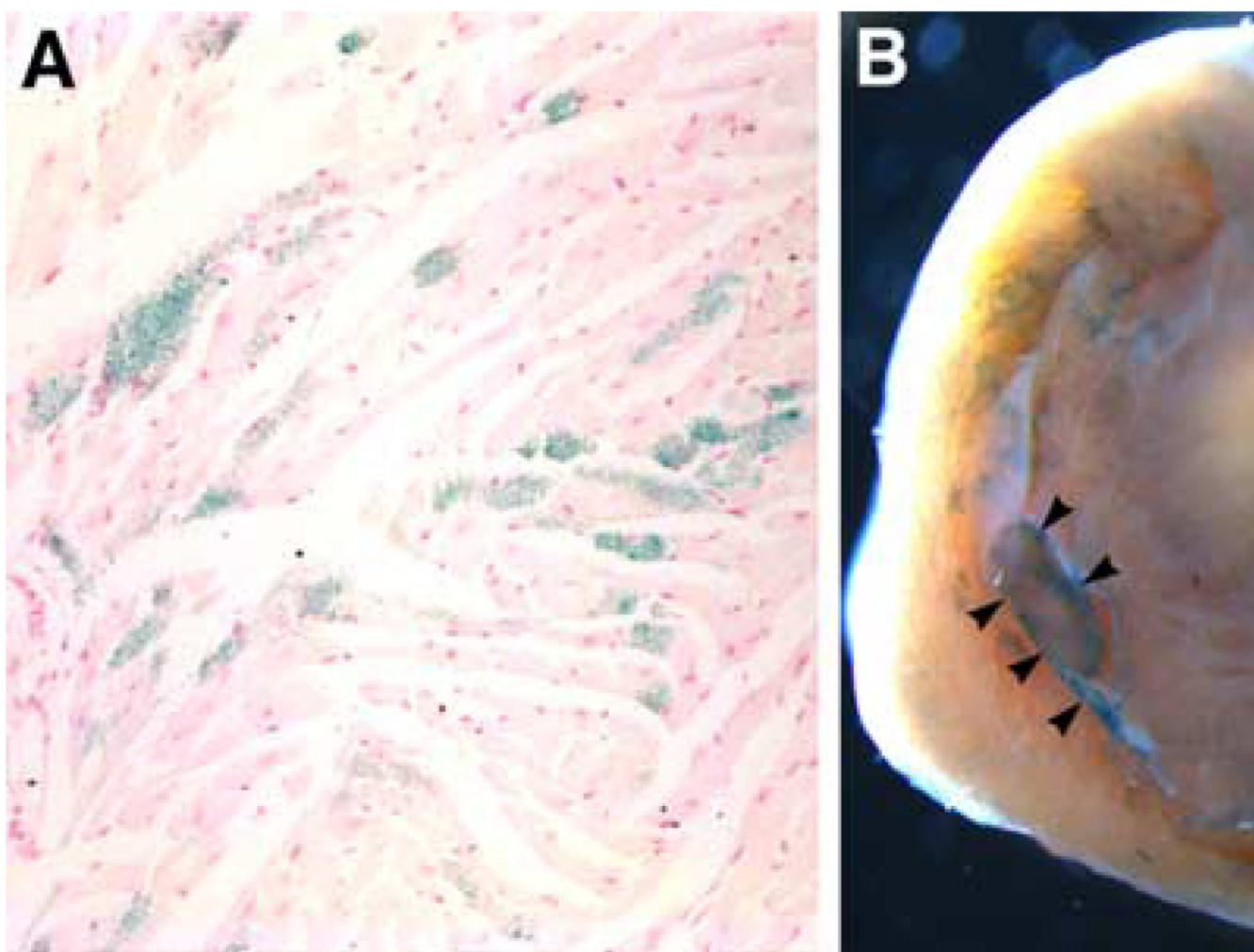
Effect of *Ncx1* promoter transcriptional element mutations on basal gene expression in adult feline cardiomyocytes. Adult feline cardiomyocytes were infected with the wild-type or mutant *Ncx1* promoter luciferase reporter adenoviruses (MOI 1.5). Cells were then incubated for 48 h and lysed in reporter buffer. Luciferase activity was determined relative to green fluorescent protein. All values are averages from four separate cell isolation experiments performed in triplicate. \* $P < 0.0002$  when compared to wild-type promoter and #  $P < 0.007$  when compared to wild-type promoter. (Figure taken with permission from Ref. 37.)



**FIGURE 3.**

Temporal-spatial detection of endogenous *Ncx1<sup>lacZ</sup>* and *184Ncx1*  $\beta$ -galactosidase reporter activity within the mouse cardiovascular system. Both *184Ncx1* and endogenous *Ncx1<sup>lacZ</sup>* expression are present *in utero* throughout the E9.5 embryonic heart, and absent from the rest of the embryo and extraembryonic tissues. Reporter activity is restricted to the common ventricle and atria, but is absent from the aortic sac/truncus arteriosus and sinus venosus.





**FIGURE 4.** Expression of the *184Ncx1- $\beta$ -gal* transgene in young adult hearts. **(A)** Transverse histological section of the left ventricular free wall showing mosaic *LacZ* staining in cardiomyocytes. **(B)** Whole mount *LacZ* staining of a central region of a heart showing *LacZ*-positive cells in and around a papillary muscle as well as the right ventricular free wall (arrowheads).