THE SODIUM-CALCIUM EXCHANGER (NCX): TEMPERATURE ADAPTATION AND EVOLUTIONARY HISTORY

by

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ABSTRACT

The sodium-calcium exchanger (NCX) is an important regulator of intracellular calcium and is highly conserved across species. NCX among different species that live in diverse environments demonstrate adaptation to different conditions while maintaining a relatively high degree of identity. For further understanding of NCX temperature sensitivity, we characterized NCX gene sequences from a wide variety of genomes for analyses. However, these analyses did not lead to specific predictions of temperature sensitivity among the various homologs of NCX.

By comparing NCX orthologs with different temperature dependencies yet with high genotype conservation, ten amino acids were predicted as being primarily responsible for the variation in phenotype. Mutation of these ten amino acids and activity measurement over a range of temperatures resulted in a significant change in its temperature sensitivity. Further work to elucidate the changes in NCX function at different temperatures is required to establish the specific mechanisms underlying NCX temperature dependence.

Keywords: Temperature dependence; Gene evolution; Mutagenesis; Transporter electrophysiology; Thermostability; Orthologs

Subject Terms: Protein temperature adaptation; Comparative physiology; Molecular evolution; Evolutionary genetics; Calcium transporter

DEDICATION

For my Dad

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AP	Action Potential
ATP	Adenosine 5'TriPhosphate
[cation$^+$]	cation concentrations, $_o$ and $_i$ refer to extracellular and cytosolic concentrations, respectively
Ca^{2+}	Calcium
CICR	Ca^{2+} Induced Ca^{2+} Release
EC	Excitation-Contraction
E_m	membrane potential
ER	Endoplasmic Reticulum
EST	Expressed Sequenced Tags
$I_{Na/Ca}$	Na^+/Ca^{2+} exchange current
NCX	Na^+/Ca^{2+} Exchanger
SERCA2a	Sarcoplasmic Reticulum Ca^{2+}-ATPase (2a) pump
SL	Sarcolemma
SR	Sarcoplasmic Reticulum
XIP	eXchange Inhibitor Peptide

CHAPTER 1:
GENERAL INTRODUCTION

1.1 Introduction

The human heart typically beats up to 3 billion times throughout one's lifetime and one-quarter of the energy consumed for the contraction-relaxation cycle is devoted to calcium (Ca^{2+}) transport across the membranes (1). Ca^{2+} is an essential intracellular messenger ion responsible for the regulation of diverse biological events across all kingdoms (2). The wide variety of processes involving Ca^{2+} include: cell division, transport, motility, gene transcription, cell cycle, secretion and muscle contraction (3-6). Normally cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is kept low ($\leq 10^{-7}$ M) by membrane transport proteins in the sarcoplasmic or endoplasmic reticulum (SR/ER), mitochondria and plasma membrane, meanwhile the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) is about four orders of magnitude higher ($\sim 10^{-3}$ M) (7). Due to this steep electrochemical gradient a strict control in Ca^{2+} levels prevent irreversible cellular damage. Rapid increases and decreases of $[Ca^{2+}]_i$ provide transient signals and modulation for $[Ca^{2+}]_i$ steady state alterations according to the cell's needs. Efficient and rapid removal of Ca^{2+} from the cytosol on a beat-to-beat basis maintains elevated $[Ca^{2+}]_i$ for only brief periods of time (8).

Ca^{2+} from the extracellular fluid can be transported through voltage-gated or receptor-operated Ca^{2+}-specific and non-specific channels on the plasma membrane or from intracellular stores (i.e.: ER/SR). Strong buffering by the SR/ER and mitochondria

(9), and cytoplasmic proteins such as parvalbumin, calretinin and calbindin (10) regulate $[Ca^{2+}]_i$. As a result, localized $[Ca^{2+}]_i$ signalling can be generated without a global $[Ca^{2+}]_i$ increase. This local signal may be mediated by Ca^{2+}-dependent proteins such as calmodulin and troponin C that are structurally related in that they contain 'EF-hand' Ca^{2+} binding motif(s). Relatively small disturbances in Ca^{2+} homeostasis in the cellular cascades can have fatal consequences since Ca^{2+} is a crucial intracellular messenger controlling both cell survival and death.

Important features in skeletal and cardiac muscle include the striated structure and organized arrangement of contractile elements that reflect their ability for fast contraction and relaxation in contrast with smooth muscle. Ca^{2+} ions switch on or off the striated muscles by moving into the cytosol from the outside of the cell and various intracellular stores (Figure 1-1). The main mechanisms that remove Ca^{2+} from the cytosol involve Ca^{2+} ATPase pumps in the ER or SR and Na^+/Ca^{2+} exchanger (NCX) in the plasma membrane. Other minor safeguarding means include the mitochondrial NCX and plasma membrane Ca^{2+}-ATPase (PMCA).

In cardiac myocytes the main Ca^{2+} extrusion across the plasma membrane is mediated by NCX, and it is responsible for removing most of the Ca^{2+} that enters on a beat-to-beat basis. The exchange current generated through this transporter is vital to cardiac myocytes as well as many other cells. NCX functions as the main mechanism of Ca^{2+} extrusion from the cardiomyocytes to permit relaxation after every excitation-contraction (E-C) coupling with the forward mode; in which Ca^{2+} is transported out of the cell in exchange of Na^+ influx. Also, NCX is capable of changing net Ca^{2+} movement directionality in a manner driven by the electrochemical gradients for Na^+ and Ca^{2+} and

the membrane potential. While in reverse mode, it has been suggested that the influx of Ca^{2+} through NCX contributes to the initiation of contraction (11-13). This dual functionality that may be altered during different stages of the action potential (AP) and with varying $[Na^{+}]_i$ and $[Ca^{2+}]_i$, renders NCX as an important plasma membrane Ca^{2+}-specific transport system.

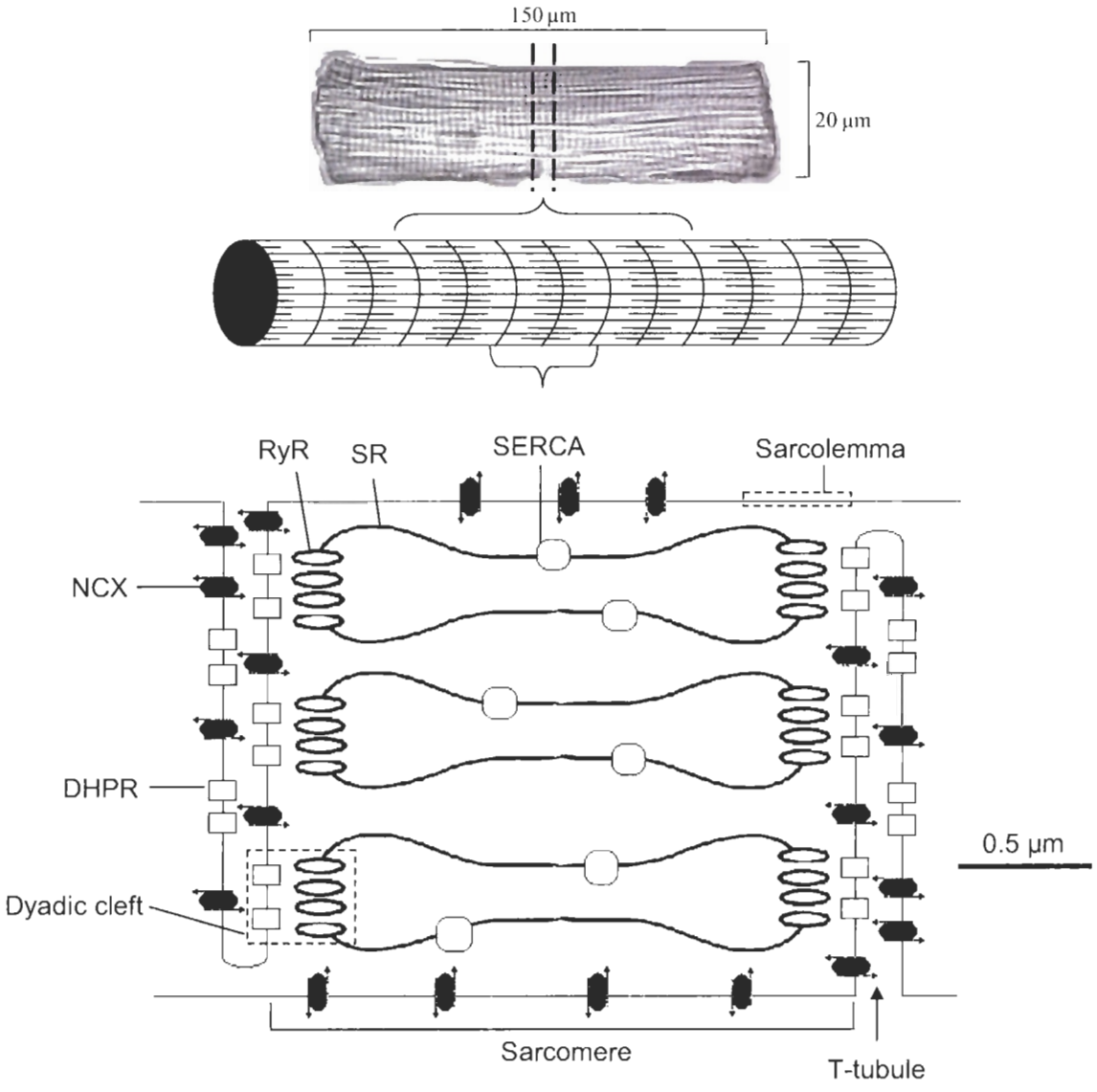

Figure 1-1 Cardiac myocyte architecture

A typical adult mammalian heart cell measures ~ 150 x 20 µm and its contractile machinery is composed of sarcomeres in tandem that make up a myofibril. The sarcoplasmic reticulum (SR) stores, releases and sequesters the free Ca^{2+} involved in contraction. Invaginations of the outer cell membrane (T-tubules) aid in carrying the electrical depolarization of the membrane and other transporters such as NCX and DHPR toward the SR and RyR to effectively trigger Ca^{2+} release. SERCA and NCX are the main transporters involved in Ca^{2+} removal.

NCX expression is ubiquitous in other tissue types, with the highest levels found in cardiac tissue plasma membrane or sarcolemma (SL) (14-16). Expression is also high in other excitable (e.g. brain and nerve cells) and osmoregulatory tissues (e.g. kidney) (17,18) whereas lower expression levels are found in non-excitable cells (e.g. liver) (19).

NCX protein sequence is also highly conserved across a phylogenetically diverse group of organisms from invertebrates to vertebrates (20). The widespread presence of NCX in diverse tissues and conservation across species highlights its importance as a Ca^{2+} transporter.

Our knowledge of NCX structure and function has advanced dramatically due to three key advances. First, the canine heart NCX cloning by Nicoll *et al.* in 1990 facilitated the analysis and manipulation of this protein for functional and biochemical analyses (21). Second, the giant excised patch clamp technique, developed by Hilgemann (22), has granted an assay sensitive enough for NCX current detection. Third, the extensive use of ion-selective fluorescent dyes and antibody probes with confocal imaging methods allow the study of Ca^{2+} and NCX distribution and function (23). These factors contributed to the significant advances of the physiological study of NCX including cardiac muscle relaxation (24,25), control and refilling of the SR Ca^{2+} in the heart (26) and in the ER of neuronal and non-excitable cells (27), control of neurosecretion (28) and excitation-contraction (E-C) coupling (11,29). Our focus on the cardiac NCX has been due to its high density and importance in this tissue.

Although most studies focus on the cardiac NCX in mammals, our consideration of lower vertebrate cardiac NCX is due to its different sensitivity to temperature while maintaining relatively high protein sequence identity (~ 78 %) with mammalian cardiac NCX. The cardiac NCX of rainbow trout (*Oncorhynchus mykiss*) maintains full activity under hypothermic conditions of 4 – 8 °C that are cardioplegic to mammals (30). The primary structural differences between the dog and trout cardiac NCX, expansion of the NCX sequence library, understanding of NCX evolutionary processes, mutational

experiments, and other poikilotherm phenotype analyses can be used to decipher the specific amino acids involved in the thermodynamic nature of these orthologs.

1.2 NCX Role in Excitation-Contraction Coupling in the Mammalian Heart

1.2.1 Excitation-Contraction Coupling Mechanisms

During the cardiac AP, Ca^{2+} enters the cell via SL L-type Ca^{2+} channels (dihydropyridine receptor or DHPR) located primarily within the transverse tubular system (or T-tubules). This Ca^{2+} can contribute directly to the activation of the myofilaments or more probably bind the ryanodine receptor (RyR) and triggering Ca^{2+} release from the SR (31) in a process known as Ca^{2+}-induced Ca^{2+}-release (CICR). As the $[Ca^{2+}]_i$ rises it binds troponin C (TnC) thereby initiating a series of events that end in the interaction of actin with myosin (myofilaments) to produce contraction, as shown in Figure 1-2.

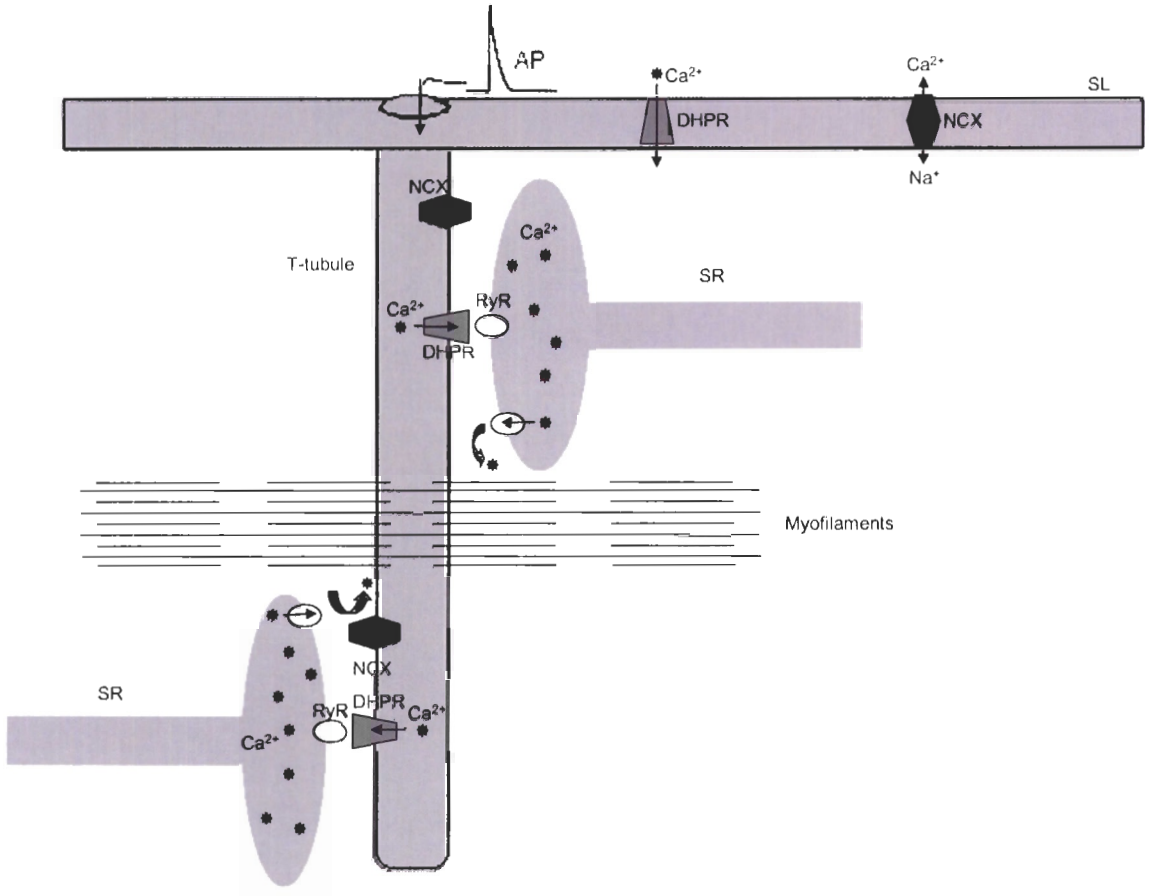

Figure 1-2 Adult cardiac excitation-contraction coupling

Ca^{2+} entrance through the voltage sensitive DHPR, after membrane depolarization by an action potential (AP), triggers RyR to release Ca^{2+} from the SR. Ca^{2+} released from the SR is the predominant Ca^{2+} to trigger contraction through the myofilaments. Ca^{2+} induced Ca^{2+} released (CICR) is facilitated by T-tubule structures by allowing closer DHPR and RyR proximity.

For relaxation to occur, Ca^{2+} must be removed from the cytosol, lowering $[Ca^{2+}]_i$ back to ~ 100 nM such that Ca^{2+} will dissociate from troponin C. The Ca^{2+} transport processes involved in removing Ca^{2+} from the cytoplasm include: a) re-uptake into the SR via the SR Ca^{2+}-ATPase pump (SERCA2a); b) transport across the plasma membrane via the plasma membrane Ca^{2+}-ATPase (PMCA), c) uptake by mitochondria via the mitochondrial Ca^{2+} uniporter, and d) the SL NCX. These Ca^{2+} transport systems are all in direct competition for cytosolic Ca^{2+} that maintain its resting cytosolic concentration ~

10^{-7} M, that is four orders of magnitude lower than the extracellular environment. Typically in mammalian myocytes, SERCA2a is the chief cytosolic Ca^{2+} extrusion system (highly species dependent at 60 - 90 %); while NCX is the primary Ca^{2+} efflux system in the cell. The PMCA and mitochondria uniporter are less significant for relaxation, together contributing 1 – 2 % of the Ca^{2+} removal (32). However, it has been suggested that in lower vertebrates (frogs and rainbow trout) (33) and neonate mammals (34) NCX accounts for a significantly greater percentage of Ca^{2+} removal for relaxation (up to ~ 55 %), in part due to the lack of T-tubules (Figure 1-3), higher NCX density, lower SR quantity and lower ryanodine sensitivity than the mammalian myocardium (30).

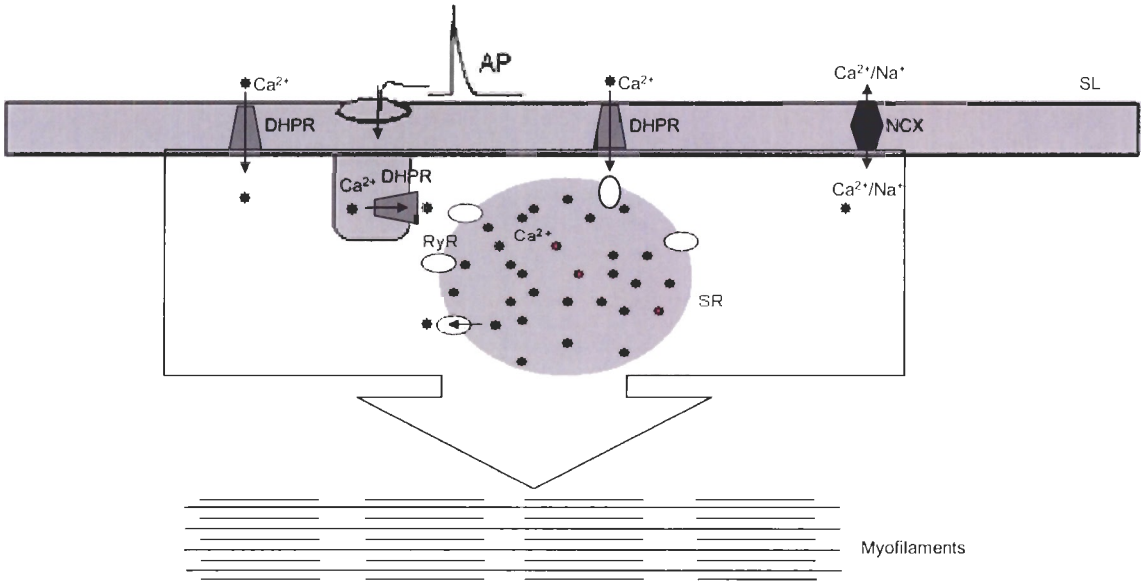

Figure 1-3 Neonate heart

Lack of or underdeveloped T-tubules in neonate myocytes decreases DHPR – RyR proximity to induce CICR. Excitation coupling mechanism in neonate may rely predominantly on Ca^{2+} transported through the SL.

In the steady state, the amount of Ca^{2+} leaving the cell via the exchanger equals the amount of Ca^{2+} that enters through the voltage gated Ca^{2+} channels (DHPR). It has been suggested that NCX may be contributing to repolarization of cardiomyocytes. However, the NCX reverse mode contribution to Ca^{2+} influx may be only important in lower vertebrates and during early development stages of mammals, leading NCX to play a more prominent role in E-C coupling (Figure 1-3). The importance of NCX can be seen in its key role in heart relaxation. Therefore, in the next section, the literature review will focus on creating a more complete picture of NCX physiological roles, molecular structure and evolutionary history that served as a guide to the goals and hypotheses of this thesis.

1.3 Historical Background

Ringer (36) demonstrated experimentally the fact that extracellular Ca^{2+} is a prerequisite for cardiac muscle contraction and its inverse relationship to $[Na^+]_o$ in frog heart muscle. This was later linked with Ca^{2+} competition with external Na^+ for negatively charged substances (ie: cardiac contractile proteins) exposed at the cell surface (37). The existence of a Na^+/Ca^{2+} exchange mechanism was first postulated when investigations of guinea pig auricles (38) and squid giant axons (39) indicated specific coupling between an inward movement of Ca^{2+} with an outward Na^+ movement and vice-versa. After these preliminary studies a more thorough understanding of NCX thermodynamics and kinetic mechanisms was unveiled. It was not until after 1990, when Nicoll *et al.* (21) cloned the canine cardiac NCX1, that more significant data on the structure and function of NCX emerged . The literature review in this chapter will concentrate on cardiac NCX.

1.4 Molecular Structure of the Na^+/Ca^{2+} Exchanger

Preparation of the cardiac SL vesicles initiated protein purification that led to the identification of NCX molecular masses at 70 kDa (fragmented), 120 kDa (glycosylated mature protein) and 160 kDa (non-reduced conditions). Then antibodies were raised and used to screen a cardiac expression library to clone the full-length NCX, in which Na^+/Ca^{2+} exchange functionality and identity of this clone was confirmed by expressing it in *Xenopus* eggs.

1.4.1 NCX Topology

The cloned canine NCX1.1 protein is comprised of ~ 980 amino acids with a predicted molecular mass of 108 kDa without the signal peptide (first 32 amino acids in the N-terminus). The discrepancy between the deduced (108 kDa) and apparent (120 kDa) NCX molecular mass on SDS-PAGE is probably due to N-linked glycosylation at asparagine N-9 (40). Under non-reducing protein gel solution conditions, NCX also has a band at 160 kDa that is theorized to arise by a parachute effect from disulfide bonding, causing NCX to run slower.

Mature NCX secondary topology was initially deduced by hydropathy analysis to have 11 putative transmembrane segments (TMSs). Later, two putative TMSs were experimentally shown to be part of the intracellular or reentrant loops with the use of membrane-impermeable sulfhydryl reagents on NCX mutants (with sequential re-introduced cysteines) and epitope-specific antibodies (41-43). Hence, the most recent topology of the mature NCX contains 9 TMSs organized with the first 5 TMSs in the N-terminus and the latter 4 TMSs in the C-terminus, separated by a large intracellular loop-f

that comprises more than half the NCX protein located in between TMSs 5 and 6 (Figure 1-4).

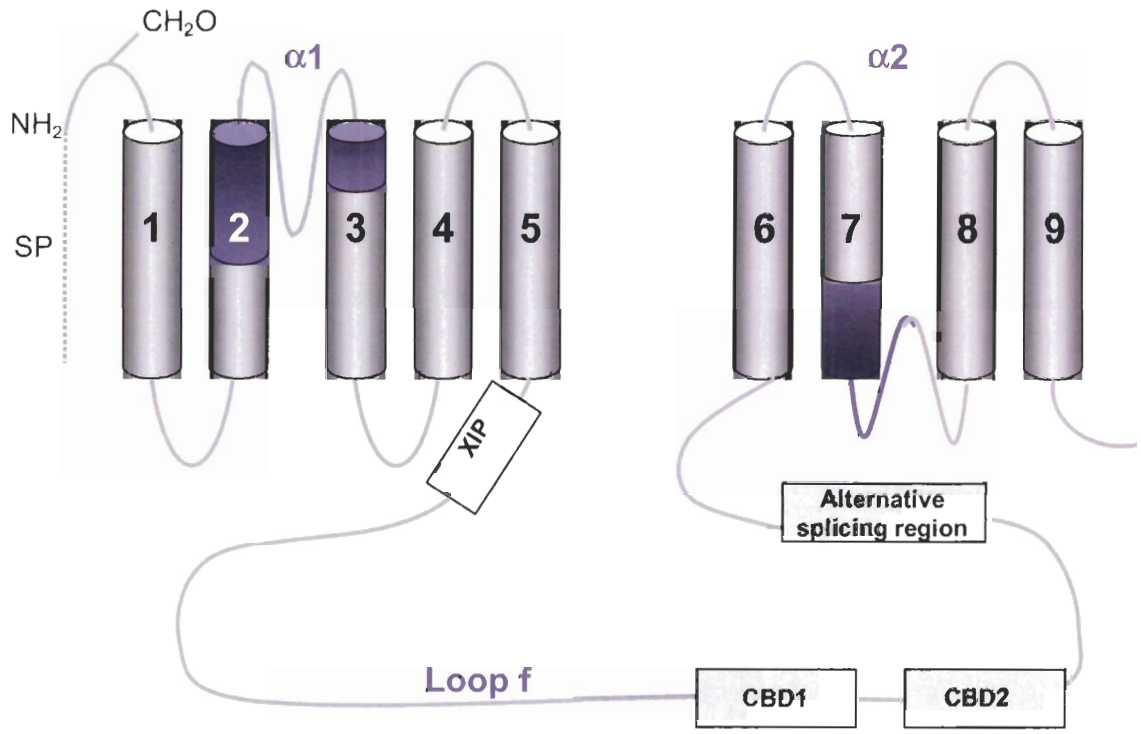

Figure 1-4 NCX topology

The nine transmembrane segments (TMS) are separated into two groups, N- and C-terminus, by the large f loop that contains regulatory sites such as the exchange inhibitor peptide (XIP), Ca^{2+} binding domains (CBD1 and CBD2) and the alternative splice region. Regions α-1 and α-2 repeats, important for ion translocation, are located from TMS2 to TMS3 and TMS7 plus a section of the re-entrant segment (darker regions). The dotted line indicates the location for the signal peptide (SP) at the N-terminus and CH_2O represent known glycosylation sites in NCX.

The α-1 and α-2 repeats are highly conserved amino acid sequences and mutagenesis experiments suggest they make a significant contribution to ion binding and translocation (44). This explains the preservation of NCX ion exchange even after a large portion of the cytoplasmic loop-f is truncated (45). The large intracellular loop-f mainly contains regulatory sites involving Na^+-dependent exchange inhibitor peptide (XIP), Ca^{2+} binding sites and alternatively spliced region (Figure 1-4). The highly basic

XIP region was found to have an autoinhibitory effect: the addition of the synthetic XIP peptide inhibited NCX currents in excised vesicle patches (46). Since NCX's Ca^{2+} binding sites do not resemble EF-hands, they were identified using nested deletions, measuring Ca^{2+} affinity in fusion proteins and sequence analysis (47). Meanwhile the alternative splice site was discovered with PCR and Northern assays from various tissues that revealed this variable region of 110 amino acids (48). The signal peptide in the N-terminus is suspected of targeting and inserting NCX into the plasma membrane; however, proven insufficient when NCX was expressed lacking the large portions of the cytoplasmic loop-f. Only coexpression of TMSs domains with loop-f resulted in plasma membrane localization in oocytes indicating cooperation from both TMSs domains and regulatory loop-f presence for proper NCX trafficking (45).

1.4.2 NCX Structure

Crystallization of highly hydrophobic proteins (ie: transporters) has proven to be difficult and thus the organization of NCX TMSs had not been spatially defined. However, alternative molecular biology techniques have provided important information on NCX helix packing. One such method, the electrophoretic mobility shift assay (EMSA), takes advantage of NCX differential mobility under reducing and non-reducing conditions due to disulfide bond formation. A modified cysteine-less NCX with selected reintroduction of cysteine pairs would form disulfide cross-links and infer structural information about the proximity of TMSs (49,50). These experiments demonstrated proximity and possible interaction among residues within TMSs 2, 3, 7, and 8 that contain both α1 and α2 repeats forming part of the ion translocation pathway (49). More recently (50), TMSs 1, 2 and 6 have been found in proximity with thiol-specific cross-

linkers broadening the NCX helix packing model. The positioning and amphipathic faces of TMSs 2, 3 and 7 is suggestive of an exchanger pore, but without a high resolution structure revealing the loops and α-repeat arrangement at this proposed pore, the mechanism of exchange is still vague. Recently, part of the regulatory loop-f structure has been determined. The canine NCX1.1 Ca^{2+} binding domain1 or CBD1 (371-500) has been resolved by NMR (51) and x-ray diffraction of crystals (52). CBD1 crystal structure revealed an immunoglobulin fold (Figure 1-5) (52) that binds four Ca^{2+} ions with stronger affinity than that in CBD2 (51). The different affinities and anti-parallel position of these domains suggest NCX dual Ca^{2+} sensitivity thresholds for flexibility over a wide range of $[Ca^{2+}]_i$ (51). Also, the finding of a second Ca^{2+} binding site (CBD2) that includes the alternative exons A or B may explain the specificity of these mutually exclusive exons expression in NCX1. Exon A is expressed only in excitable cells (ie: cardiac and skeletal tissues) and exon B in non-excitable cells (ie: kidney tissue). Dunn *et al.* (53) demonstrated that a couple of substitutions (R610D and C617K within exon B) in NCX1.3 (kidney) can completely change its regulatory phenotype similar to NCX1.4 (brain) containing exon A and vice versa. These partial structural findings provide some useful information of NCX function but a more complete knowledge of NCX structure is still required understand its molecular mechanism of ion exchange.

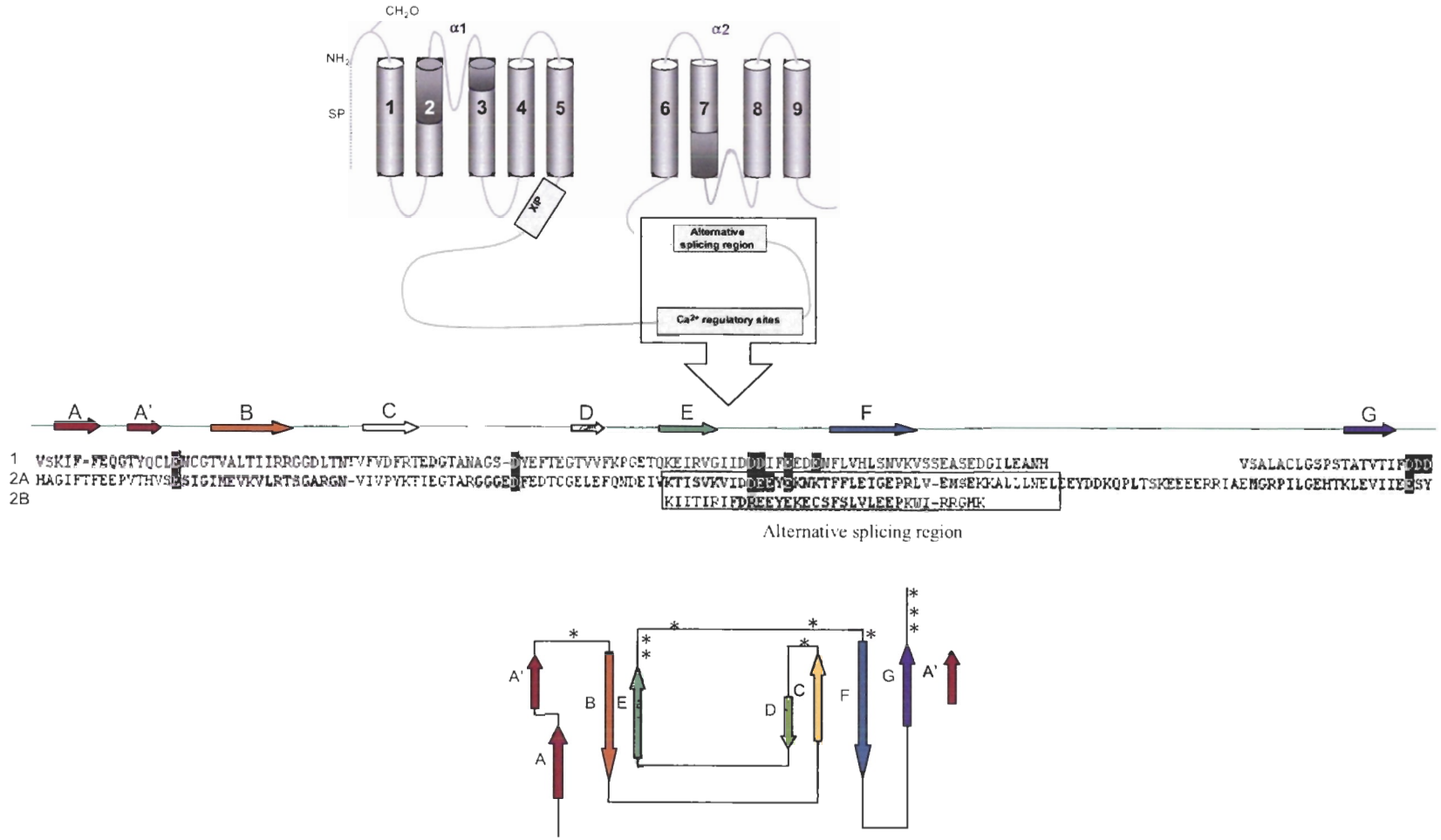

Figure 1-5 Ca^{2+} binding domains (CBD)

Boxed area on the NCX topology figure contains the CBDs and the alternative splice site. Both CBD1 and CBD2 (with A and B alternative spliced exons, boxed) sequences are shown with highlighted residues that interact with Ca^{2+} ions. CBD1 secondary structure is represented by arrows as beta sheets and each beta sheet is labelled from A to G. CBD1 immunoglobulin folds are shown with asterisks (*) representing the location of residues responsible for coordinating the Ca^{2+} ions (52).

1.5 Na^+/Ca^{2+} Countertransport

A basic demonstration of Na^+-Ca^{2+} exchange was provided by experiments by Reeves and Sutko (54) in which isolated rabbit ventricular SL vesicles pre-loaded with Na^+, accumulated Ca^{2+} when an outwardly directed Na^+ gradient was formed across the vesicle membrane. The proposed function of Na^+-Ca^{2+} exchange in most tissues was to extrude Ca^{2+} from the cell, utilizing the energy of the inwardly directed Na^+ gradient (38). NCX performs a particularly important activity in regulating cardiac contractility and electrical activity. Na^+/Ca^{2+} exchange does not require ATP but depends on cell membrane potential (E_m) and concentration of [Na^+] and [Ca^{2+}] on either side of the SL. NCX in various experimental animals produced a net Ca^{2+} flux either into or out of the cells (38,39). NCX electrogenicity arises from the unequal exchange of electric charge resulting in a membrane current. Conventionally, Ca^{2+} movements in or out of the cell have been assigned as reverse or forward mode directionality, respectively. The main function of NCX is to extrude Ca^{2+} in most types of cells, although some studies indicate NCX removal of Na^+ as its main function in red blood cells (55-57).

1.5.1 NCX Electrogenicity

This electrogenic membrane transporter exchanges 1 Ca^{2+} for 3 Na^+ to either side of the plasma membrane, producing a current ($I_{Na/Ca}$) (58). The reversal potential for Na^+/Ca^{2+} exchange ($E_{Na/Ca}$) is derived from the coupling ratio (considered to be 3 Na^+ for 1 Ca^{2+}) and the equilibrium potentials for Na^+ (E_{Na}) and Ca^{2+} (E_{Ca}) as described by the equation $E_{Na/Ca} = 3\ E_{Na} - 2\ E_{Ca}$.

$E_{Na/Ca}$ relative to E_m determines the net direction of Ca^{2+} transport. Ca^{2+} outward/forward movement is identified as a Na_o^+-dependent inward current attributed to

Na^+/Ca^{2+} exchange ($I_{Na/Ca}$) and Ca_i^{2+}-dependent Na^+ influx, activated by nontransported $[Ca^{2+}]_i$ and inactivated by high $[Na^+]_i$. When $E_m < E_{Na/Ca}$, Ca^{2+} extrusion and Na^+ influx is favoured (inward I_{NCX}). However, when $E_m > E_{Na/Ca}$, a net Ca^{2+} influx would be observed as a Na_i^+-dependent Ca^{2+} influx/reverse mode and Ca_o^{2+}-dependent Na^+ efflux (outward I_{NCX}). The exchanger typically works in forward mode (Ca^{2+} efflux / Na^+ influx) in resting cardiomyocytes. However, there is the potential for the reverse mode to be observed during the AP which allows for Ca^{2+} entry when $E_m > E_{Na/Ca}$ (59).

Although some studies suggest lower (38) and higher (60,61) stoichiometries, the majority of experiments are consistent with a stoichiometry of 3 Na^+ : 1 Ca^{2+} (62-67). The first indication of electrogenicity from NCX transport in guinea pig cardiomyocytes inferred an NCX coupling ratio > 2 : 1 (68). A coupling ratio of 3 Na^+ to 1 Ca^{2+} was first calculated with a null approach which assumes that the net Ca^{2+} movement via NCX do not occur when the driving force is zero in cardiac SL vesicles by measuring ion fluxes, reversal potentials Na^+ and Ca^{2+} concentrations (64).

1.6 Regulation

Various extracellular and intracellular factors regulate the mammalian cardiac NCX1. Among the activators include Ca^{2+}, PKC activators, PIP_2, phosphorylation and monovalent cations. Meanwhile NCX activity can be inhibited by Na^+, XIP, protons, PKC inhibitors and drugs such as KB-R7943 and SEA0400. Three main factors that affect NCX1 activity will be discussed below.

1.6.1 Sodium Regulation

A large $[Na^+]$ electrochemical gradient is maintained across the plasma membrane of intact cells with $[Na^+]_o \sim 140$ mM and $[Na^+]_i \sim 8$ mM. Ca^{2+} uptake rates are steeply dependent on $[Na^+]_i$ in the range of 8 and 12 mM, and therefore within physiological variation. Hence small changes in intracellular Na^+ levels would have substantial effect on Ca^{2+} influx and contractility (69). A functional competition between Ca^{2+} and Na^+ at both sides has been measured under different experimental conditions. $[Na^+]_i$ and $[Ca^{2+}]_i$ interact competitively with respect to outward current and in a mixed competitive-noncompetitive fashion with respect to the inward current suggested by Matsuoka and Hilgemann, in which Na_i half-maximum concentration (K_m) is 20 – 30 mM and Na_o K_m is at 50 – 70 mM (67). However, $[Ca^{2+}]$ K_m exhibit a wider range than that for $[Na^+]$ and is complicated by other coexisting conditions such as [Na], E_m, pH and Ca_i-dependent allosteric regulation (29). More direct evidence of Na^+-dependent inactivation was demonstrated by the existence of an endogenous self-inhibitory site in the cytoplasmic loop-f known as the XIP site (46). Na^+-dependent inactivation can be prevented by mutagenesis within the XIP region (70) or with chymotrypsin treatment that truncates NCX regulatory regions of the large intracellular loop-f (71). Immediately distal to TMS 5, the XIP site is composed of 20 amino acids (251 – 270 in canine NCX 1) and no evidence of direct interaction of Na^+ ions with this site has been demonstrated , but the presence of high levels above 30 mM $[Na^+]_i$ (29) is required to cause substantial $I_{Na/Ca}$ inactivation. Hence, possible XIP interaction with NCX itself could trigger or directly induce mechanical inactivation of Na^+/Ca^{2+} exchange. Also, the fact that the XIP site lacks conservation among orthologs would seem to indicate differences in inactivation processes and/or recognition for binding in NCX across species.

1.6.2 Calcium Regulation

Extracellular and intracellular Ca^{2+} play important roles in modulating cardiac NCX function. NCX inactivation contributed by either Na^{+}-dependent inactivation or complete removal of $[Ca^{2+}]_i$ can be reversed by increasing $[Ca^{2+}]_i$, respectively (72). Regulatory $[Ca^{2+}]_i$ required to increase the peak outward $I_{Na/Ca}$ flux exhibits a $K_m \sim 0.3$ μM (29). The K_m for Ca^{2+} in the attenuation of Na^{+}-dependent inactivation is higher at ~ 2 μM (73). Large deletions of the cytoplasmic loop in NCX1 do not prevent $I_{Na/Ca}$ flux but deregulate the exchanger in response to changes in $[Na^{+}]_i$, $[Ca^{2+}]_i$ and synthetic [XIP].

The two Ca^{2+}-binding sites are located in the loop-f (amino acid number 371 – 508) (74) CBD 1 and 2 (52), correspond to domains in the homologous NCX from fruitfly CALX named Calx-β1 and β2 (52) but regulate the exchanger in an opposite manner by decreasing CALX activity in the presence of $Ca^{2+}{}_i$ (75). Due to the lack of information in the intramolecular interactions of these Calx-β domains with the rest of the NCX molecule, it is not understood how these homologous sequences execute opposite regulatory responses. Interspecies high sequence identity of these domains support the theory that these Ca^{2+} binding domains act as allosteric regulators (76).

1.6.3 Lipid Bilayer Composition

Various experiments have revealed that cardiac NCX function is highly sensitive to membrane phospholipid composition including asymmetry of distribution (77-80). To understand control of NCX transport, NCX was reconstituted into vesicles of various compositions (81). Reconstitution of these experimental vesicles with acidic phospholipids and 20 % cholesterol gave optimal NCX transport performance (77). Also, Philipson (82) demonstrated that anionic amphiphiles and phospholipids stimulate NCX

activity and cationic amphiphiles inhibit NCX. In sarcolemmal vesicles, NCX stimulation or inhibition can be influenced by exposure of amphiphiles that associate with the sarcolemmal membrane, in which the length of the hydrophobic chain (or hydrophobicity level) was correlated to the stimulation or inhibition potency on Na^+/Ca^{2+} exchange (82).

1.7 Pathophysiology: Role of NCX

Increased Ca^{2+} influx without subsequent removal can lead to cytosolic Ca^{2+} overload. If Ca^{2+} reaches high enough levels it may activate Ca^{2+}-dependent hydrolytic enzymes or proteases and/or apoptotic pathways, which can potentially lead to cell death. Such pathology can be directly linked to mutations in the SERCA pump (83) or RyR (84) that may result in Ca^{2+} leakage or transporter dysfunction. Although no NCX1 defects have been associated with failing hearts, elevated $[Na^+]_i$ and longer AP duration are commonly observed along with Ca^{2+} flux imbalance. In heart failure, enhanced Ca^{2+} entry due to increased NCX expression, higher $[Na^+]_i$ and prolonged AP may provide inotropic support for failing myocytes (85). Other pathological conditions, such as cardiac ischemia/reperfusion or heart failure, can lead to Ca^{2+} overloading of the SR and hence mechanical and electrical dysfunction of myocytes due to increased NCX-mediated Ca^{2+} entry or decreased Ca^{2+} efflux due to a rise in $[Na^+]_i$ (86).

1.7.1 Ischemia/Reperfusion

During ischemic conditions, the reduction in oxygen availability reduces oxidative phosphorylation while anaerobic glycolysis is accelerated, producing lactate and protons that induce acidosis (87). The relation between intracellular acidification and

the excess Ca^{2+} accumulation in cardiac myocytes has been explained by the phenomenon referred to as the 'pH paradox' (88). Ions H^+, Na^+ and Ca^{2+} play important roles manipulated by both Na^+/H^+ exchanger (NHE) and NCX in the heart during ischemia/reperfusion. The elevated $[H^+]$ due to acidosis causes Na^+ influx into the cell to expulse the excess H^+ by NHE, which in turn drives NCX into reverse mode and therefore brings $[Ca^{2+}]_i$ levels higher risking cell damage (89).

Therapeutic agents, such as ouabain, used to increase cardiac contraction force in patients presenting with hypocontractile states such as heart failure, often induce cardiac arrhythmia (90). Inhibition of the Na^+/K^+-ATPase by ouabain leads to the rise of $[Na^+]_i$ accompanied by Ca^{2+} overload by NCX. The exact roles of the NCX are difficult to study because of the multiplicity of Ca^{2+} flux pathways in cardiomyocytes and the lack of specific inhibitors.

1.7.2 NCX Inhibitors

The clinical benefit of developing selective NCX activity inhibitors has been pharmacologically attempted to avoid $Ca^{2+}{}_i$ accumulation during myocardial ischemia-reperfusion. So far, two reasonably selective inhibitors have been identified. Developed in 1996, the drug KB-R 7943 (2- [2- [4 (4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulfonate) inhibited NCX outward currents in a concentration dependent manner up to 10 μM (64). The effect was the same for NCX inward current, although a greater concentration (50 μM) was needed for maximal inhibition (91). Studies have shown KB-R7943 inhibited ouabain-induced arrhythmias in isolated myocytes, but high doses are known to also inhibit voltage-gated Na^+ channels and Ca^{2+} channels (92) seriously complicating the therapeutic role of this compound (93). KB-R7943 was the most

selective drug for NCX until 2001 in which SEA0400 (2- {4- [(2, 5-difluorophenyl) methoxy] phenoxy}5 – ethoxy aniline) was developed and was shown to be > 100 fold more potent than KB-R7943 in inhibiting NCX and had negligible affinity for Na^+ and Ca^{2+} channels (94).

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CHAPTER 2:
NCX GENETICS & TEMPERATURE DEPENDENCE OVERVIEW

Calcium is used as a second messenger and in signalling across a phylogenetically diverse group of organisms; thus, precise control of concentration and movement is essential to cellular processes. Not surprisingly, NCX is expressed ubiquitously and found in species from all kingdoms. NCX is a member of the cation : Ca^{2+} antiporter (CaCA) superfamily and belongs to the 2.A.19 family of the Transport Classification Database (TCDB) or solute carrier 8A (SLC8A) (1). Since the cloning of the first NCX in 1990 (2), the molecular study of NCX expanded rapidly by allowing sequence manipulation and expression in controlled conditions. The following sections will review NCX known gene structure, expression profiles, phylogeny, and temperature dependence; to facilitate a background to this thesis hypotheses and objectives.

2.1 Expression Regulation

Distinct Ca^{2+} regulatory mechanisms are found in different tissues. For example, in synaptic boutons the $[Ca^{2+}]_i$ during a nerve AP can rise within a few hundred microseconds (μsec) to a peak of 100 μM or more and then return to submicromolar levels with similar speed (3). $[Ca^{2+}]_i$ in cardiac myocytes can reach ~ 1 μM within 10 – 20 msec and takes longer than a synapse to decay, while in renal epithelial cells $[Ca^{2+}]_i$ is maintained at relatively constant levels (4). All three tissues cited above (nerve, heart and kidney) contain large amounts of NCX protein but exhibit distinct Ca^{2+} transients

signalling pathways. Because Ca^{2+} handling is so different in these tissues, NCX transcript expression is differentially regulated in such an array of tissues to meet specific physiological demands.

2.1.1 NCX Paralogs

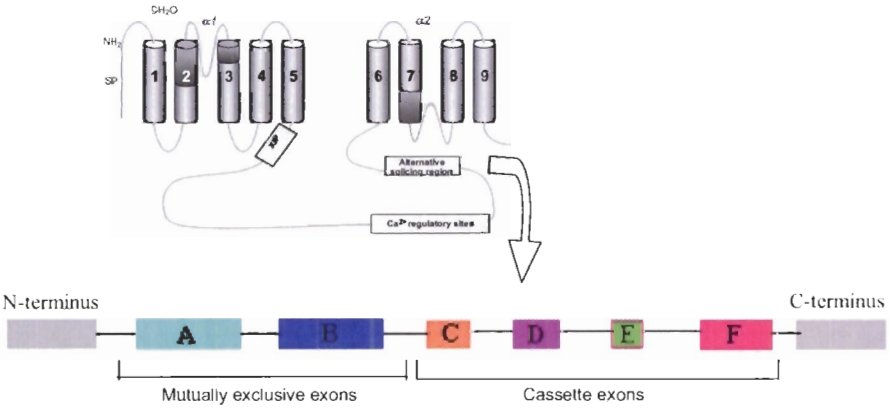
NCX belongs to a superfamily characterized by the presence of α repeats with three dominant genes coding for three exchangers (NCX1, NCX2 and NCX3) in mammals. NCX1 was cloned from canine heart (2), NCX2 (5) and NCX3 (6) were later cloned from rat brain and skeletal muscle cDNA libraries, respectively.

NCX1 expression is the most widely distributed paralog and is found in tissues such as cardiac and skeletal muscles, placenta, brain, lung, pancreas, kidney, liver, neurons and astrocytes (7,8). In contrast NCX2 (5) and NCX3 (6) are restricted to skeletal muscle and neural tissues. On the basis of sequence similarity and hydropathy analysis, all three NCX proteins are modelled to share similar topology (9). The ubiquitous expression of different NCX1 splice variants accommodates a diverse Ca^{2+} management with the same gene in a variety of tissues. Analysis of cDNAs from rabbit cardiac and kidney tissues showed a restricted region within the regulatory intracellular loop to be variable in size and sequence and is referred to as the alternative splice region (10).

2.1.2 Alternative Splicing

An alternative splice region found in the large intracellular loop between the CBD1 and TMS 6 is conserved across homologs and among species. Differential exon expression is tissue specific for all three NCX paralogs. The alternative splice region

may play a role in altering NCX Ca^{2+} affinity using different combinations of exons to meet tissue specific demands of Ca^{2+} exchange. For mammalian NCX1 a total of six exons (A-F), found within CBD2 and before TMS6, make up tissue-specific isoforms with exons A or B being mutually exclusive (Figure 2-1). Exon A is commonly expressed in excitable cells such as cardiac, skeletal muscle and neuronal cells, while exon B is usually found in all other tissues including stomach, kidney, testes, liver, and lung (9).




```
                          Exon A or B                          Exon C    Exon D  Exon E          Exon F
NCX1 : KTISVKVIDDEEYEKNKTFFIEIGEPRLVEMSEKK ALLLNEL GGETLT GKKMY GQPVFRKVHARDHPIPSTVISIS
       KIITIRIFDREEYEKECSFSLVLEEPKWIRR-GLK
NCX2 : KTLQVKIVDDEEYEKKDNFFIELGQPQWLKR-GIS ALLLNQG
NCX3 : KTIHIKVIDDKAYEKNKNYVIEMMGPRMVDMSVQK ALLLSPE
       KTIRVKIVDEEEYERQENFFLALGEPKWMER-GIS
```

Figure 2-1 Canine NCX alternative spliced regions

Alternative splice site is located in the C-terminus of loop-f, before TMS6. There are two mutually exclusive exons A and B and four cassette exons C – F. All cassette exons are present in the mammalian NCX1 genes, while only exon C can be found in NCX2 and NCX3.

The other four exons (C, D, E and F) are cassette exons, which are also expressed in a tissue-specific manner. The functional diversity of NCX1 in different tissues and cell types may be formulated by specific combinations of these cassette exons and the mutually exclusive exons. A total of 32 splice variants are possible for NCX1 but only 16 splice combinations (Table 2-1) have been found to date in mammalian tissue (9).

Table 2-1 Alternative spliced exon combinations found in specific tissues in rat (9).

Exons	Terminology	Tissue
ACDEF	NCX1.1	Heart, skeletal muscle
ACDE	NCX1.8	Heart
ACD	NCX1.6	Brain
ADF	NCX1.5	Eye
AD	NCX1.4	Brain, eye
BCD	NCX1.2	Kidney, skleletal muscle
BDF	NCX1.7	Thymus, kidney, skeletal muscle, small and large intestine, aorta, lung, adrenal gland, spleen, stomach, pancreas, testes, VSMC, endothelial cells
BD	NCX1.3	Thymus, kidney, skeletal muscle, small and large intestine, aorta, lung, adrenal gland, spleen, stomach, pancreas, testes, VSM, endothelial cells, eye, liver
BDE	NCX1.9	Skeletal muscle
BDEF	NCX1.10	Skeletal muscle
BCDEF	NCX1.11	
ADEF	NCX1.12	
AC	NCX2.1	Brain, skeletal muscle
AC	NCX3.1	Skeletal muscle
B	NCX3.2	Brain, skeletal muscle
BC	NCX3.3	Brain

The variety of these alternative splice exons are not as well documented in non-mammalian species. Whether these tissue-specific exon combinations correlate with species other than mammals is not known, except for the ACDF combination expressed in rainbow trout (NCX-TR1.0) (11) and tilapia (NCX-TL1.0) (12) hearts while mammalian cardiac tissue has the extra cassette exon E (ACDEF). A full length clone of an additional rainbow trout NCX1 with exon E has been analyzed *in situ* and exhibited no significant differences with the NCX-TR1.0 phenotype (Marshall, C.R. *et al.* unpublished). Plus, NCX2 and NCX3 with a smaller number in splice combinations than NCX1, found with exons A and C in NCX2 and A/B and C in NCX3 (9) may indicate that the later NCX paralogs lack alternative splicing exons or other combinations have not been found due to low expression. Although many alternative splice isoforms have

been found, the NCX alternative splicing link to functional consequences have not been explored in detail.

Based on NMR analysis of NCX Ca^{2+} binding sites (K_d ~140 – 400 nM), Hilge *et al.* (13) suggest that the cassette exons C-F could modulate Ca^{2+} binding effects thereby fine-tuning overall NCX activity. The ability of different splice variants to affect Ca^{2+} affinity has been hypothesized previously (14,15). The mutually exclusive exons A and B have been identified to be phenotypically different by one amino acid in rat NCX1 D578 in exon A and R578 in exon B (16). It is not known whether this alternative site is conserved in non-mammalian species or not due to the small number of available sequences. The presence of only two exons in the alternative splice site of CALX (fruitfly *Drosophila* NCX version) compared to six exons in the mammalian NCX gene suggests the alternative splice site has evolved to expand and perhaps "fine tune" its exchanger activity. Unlike the mammalian NCX, both CALX isoforms show negative regulation induced by Ca^{2+}_i (17).

2.1.3 Alternative Promoters and Tissue-Specific Expression

Three tissue-specific promoters involved in NCX1 expression have been suggested to play a role in determining the splice variations within the large cytoplasmic loop (18). Selective transcription binding sites have been suggested as one of the various NCX tissue specific transcript selections. Three alternative promoters, spread over a genomic region of ~ 20 kb from rat NCX1 coding region, have been identified to take part in tissue specificity and abundance in expression in heart, kidney cortex and brain (19). Although the regulation of NCX2 and NCX3 through promoters is not well understood, both are exclusively found in brain and skeletal muscle (9). Studies mostly

emphasized on the cardiac NCX1 specific promoter, Ht, demonstrating a minimum promoter of 137 bp upstream of the transcription start site (19). Cardiac-specific promoter GATA elements may drive the normal spatiotemporal pattern while additional elements have been found to enhance the recognition of pressure overload and hypertrophy to signal changes in NCX expression. Xu *et al.*, reported that both CArG ($CC(A/T)_6GG$) and GATA elements are required for cardiomyocyte transgenic lines for pressure overload detection to up-regulate NCX expression (20). The serum response factor (SRF), which binds to the CArG box, is involved in proliferation and myogenesis accompanied by reactivation of fetal gene programs triggered by changes in $[Ca^{2+}]$ and mechanical stretch (21). Consequently, during cardiac hypertrophy SRF-dependent genes such as NCX are normally upregulated (22). Differential NCX gene expression is one of several critical differences between neonate and adult hearts. Other aspects of NCX regulation are crucial for cellular function, volume, structure, and organelle re-arrangements in which mammalian myocytes go through during development.

2.1.4 Development and Pathophysiological Conditions

As mentioned in the previous chapter, the presence of t-tubules is essential in the process of CICR and regulation of $[Ca^{2+}]_i$ for cardiomyocyte function. However, in mammalian neonates and lower vertebrates such as teleosts, heart cells have underdeveloped or lack t-tubules. Without the tight coupling between the SR Ca^{2+} release channels and DHPR, NCX is has been shown to play a larger role in Ca^{2+} handling. Several lines of evidence show a significantly higher density of NCX expression during the earliest stages of development suggesting NCX is the main mechanism of Ca^{2+} flux in cardiac myocytes. Further to this point the growth of t-tubules

correlates to a decline in NCX expression. Hence, high expression in late fetal and neonatal rat hearts decreases 6.5 fold to adult levels by 20 days after birth and increases again in senescent rats or during heart failure (23). Hypertrophy, at the molecular level, is characterized by the activation of gene expression patterns that are similar to fetal stages but often down-regulated or silent at adult stages (24). Numerous factors lead to abnormal Ca^{2+} cycling: for instance, the decrease in expression of SERCA2a and phospholamban with the concomitant stimulation of NCX expression during heart dysfunction at the transcriptional level due to increased β-adrenergic stimulation (25). Alterations in cellular signalling that provoke changes in gene transcription and functionality of NCX (and other ionic transport proteins) demonstrates a complex mechanism of NCX regulation involved in heart development and disease.

2.1.5 Localization and Density

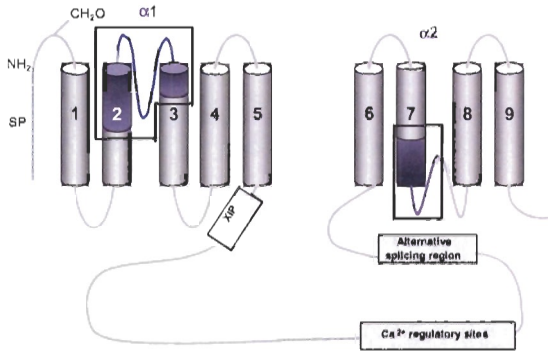
Localization of a protein within any cell is important for optimization of its function (26). NCX location and spatial relationship to other key proteins and organelles in cardiomyocytes can dictate its role in E-C coupling. NCX positioning in the t-tubules and more specifically in close proximity to the dyad (DHPR and RyR) would suggest NCX contribution in triggering SR Ca^{2+} release (27); but so far, no significant colocalization of NCX with DHPR or RyR has been shown. Functionality and significance of NCX has also been correlated to its density levels at different developmental stages. Huang et al. (28), demonstrated that in rabbit cardiac myocytes I_{NCX} density is negatively correlated with developmental age. Consistent with other reports (29,30), higher NCX activity may be directly correlated with the elevated protein expression and density in neonate cardiomyocytes in comparison to adult hearts.

Neonate specific isoforms of cardiac NCX have not been found as an alternative to explain differences in neonate NCX activity levels in contrast to that of adults.

2.2 Evolution

The first observation regarding NCX molecular evolution was the identification of intramolecular homology, including the similarities of the α-repeats, TMS and regulatory sites in all identified exchangers (31). NCX belongs to the cation : Ca^{2+} antiporter superfamily that includes five families: bacterial and archaeal exchanger (YrbG), H^{+}/Ca^{2+} exchanger (CAX), K^{+}-dependent Na^{+}/Ca^{2+} exchanger (NCKX), Na^{+}/Ca^{2+} exchanger (NCX) and a newly recognized family found only in *C. elegans* and *D. melanogaster* designated cation/Ca^{2+} exchanger (CCX) (1). The superfamily is defined by a similar topology composed of α-repeat regions and two homologous N- and C- terminal TMSs (1).

Most cloned NCX genes are mammalian which limits NCX gene evolutionary origin studies. In the mammalian clones and genomes, a consistent number of three NCX paralogs have been identified. These three paralogs, *NCX1*, *NCX2* and *NCX3*, are from separate genes with sequence identity between 68 – 75 % with the highest identity at a signature motif in the α-1 repeat G(S/G)SAPE and α-2 repeat GTS(I/V)PD (1) and TMSs with > 80 % identity (Figure 2-2). Na^{+}/Ca^{2+} exchange appears to occur in virtually all mammalian cells.




```
NCX1α1  108 GSSAPEILLSVIEVCGHNFTAGDLGPSTIVGSAAFNMFIII--A 149
NCX1α2  809 .T.V.DTFA.KVAA--TQDQYA.ASIGNVT..N.V.V.LG.GV. 850
```

Figure 2-2 Sequence alignment of α-1 and α-2 repeats

Repeats α-1 and α-2 (boxed) are located within TMS2 and 3, and TMS7, respectively, facing opposing sides of the membrane. Sequence alignment of α-1 and α-2 repeats demonstrates some sequence conservation.

2.2.1 NCX Gene Family of Non-mammalian Species

Studies on non-mammalian NCX such as cockroach salivary ducts (32), fruitfly (33), crayfish presynaptic terminals (34), muscle cells in barnacles (35) and lobster (36), gills of shore crab (37), heart of rainbow trout (38), shark (39) and zebrafish (40,41), rectal gland of dogfish (42), tilapia intestines (43) and others (44,45) illustrate the importance of NCX in an extensive variety of species. The observation that NCX plays an essential function in numerous non-mammalian species indicates early NCX presence from an evolutionary perspective.

2.2.2 NCX Phylogeny

As reported recently by Marshall *et al.* (46), the wide variety of NCX homologs resulted from two early sequential gene replications indicating that all metazoans should express some exchanger homolog. NCX2 and NCX3 homologs have been cloned only from mammals, while only a few NCX1 have been cloned from other vertebrates and invertebrates. However, the explosive growth in the number of sequenced genomes in

the past few years has permitted more extensive phylogenetic analyses and different hypotheses on the timeline of NCX divergence. The finding of NCX2 and NCX3 in organisms other than mammals (46) refuted the idea that non-mammalian vertebrate exchangers diverged before mammalian NCX split into three branches (1). Also the identification of a putative fourth NCX in the teleost genomes was suggested to result from NCX duplication after teleost divergence from mammalian species and providing two serial mammalian NCX gene duplications (46).

The versatility offered by electrogenic transporters, that use both chemical and electrical gradients as primary drivers of ion exchange, enables organisms to survive inconsistent extracellular environments (47). Therefore, tissue and species wide distribution, functional and evolutionary conservation of the NCX family may represent an ancestral condition and requirement. However at the same time mutations in NCX can also allow for organisms to adapt to changes in the environment and provide advantageous features without compromising NCX main functionality and identity. NCX cold temperature adaptation has been distinguished in the rainbow trout from the mammal form. In neonatal and lower vertebrate cardiomyocytes, significantly higher NCX expression and function are thought to play an essential role in normal cardiac function in these organisms.

2.3 Temperature Dependence

The stability of proteins is fundamental in biochemistry. The study of enzymes under extreme environmental conditions is an increasingly important field in both industrial and medical applications. Several archaea and bacteria are capable of withstanding temperatures higher than 100 °C. The fact that orthologous proteins can

perform the same function over a large temperature range demonstrates the incredible plasticity of the different permutations in protein structure with the usual 20 amino acids used to build all proteins. Microbes that succeed in extremes of life have been used as models for the study of general defence strategies evolved by all organisms that provide some general mechanisms for both extremes of adaptation. Although vertebrates do not survive in these extreme temperatures, they can be found to exist over the range - 1.8 to + 50 °C (48,49). The vertebrates existing in both temperature extremes correspond to ectotherms (or poikilotherms), in which body temperatures depends on absorption of heat energy from their habitat and in general conform to environmental conditions. In the study of cardiac activity, most mammals cannot maintain circulation under hypothermic conditions as indicated by reports of cardiac arrest or severe ventricular fibrillation in dogs during hypothermia at ~ 20 °C body temperature (50). In contrast to mammals, poikilotherms, such as the rainbow trout, sustain normal cardiac contractility that allows them to swim against strong currents at temperatures that can approach 4°C. Such resistance to low temperatures has been attributed mainly to conformational stability of proteins (51). The rapid sequencing of extremophile genomes (mostly thermophiles) has rendered many amino acid composition analyses, among thermo-, psychro- and meso-thermophiles, to determine the link between protein sequence and protein structural stability at extreme temperatures. Hence analysis of these vertebrate protein adaptations to environmental temperature is important to the study of the specific molecular mechanisms of this phenomenon.

2.3.1 Mechanisms for Adaptation to High Temperature

Extreme thermophilic microorganisms, which include only bacterial and archaeal species, with an optimum growth temperature above 70 °C express enzymes that are very resistant to heat denaturation. At room temperature, these thermophilic enzymes lose activity due to their conformational rigidity designated to stabilize and protect them from heat denaturation (52). A prominent signature of hyperthermophilic proteins is a bias towards charged (D, E, K, R) residues at the expense of non-charged polar (N, Q, S, T) residues or Charged versus Polar uncharged (CvP)-bias composition (53). Replacement of polar non-charged residues by those with charge normally confers significant protein conformational rigidity in hyperthermophilic species (54). Significant increases in the residues E, V, I, and Y and decreases in N (increased rate of deamination at high temperatures), A, T, and H have also been reported in thermophiles (55). A structural comparison of the adenylate kinases among extremophiles suggested that high temperature adaptation involves the use of electrostatic interactions of ion pairs to increase stability (56). However, no general rules have been suggested to set apart cold-adapted proteins from others due to their unique means of adaptation.

2.3.2 Mechanisms for Adaptation to Low Temperature

Psychrophilic organisms, consisting of microorganisms, thrive at temperatures in the range of - 10 to + 20 °C. Most biochemical reactions required in cell metabolism are highly temperature dependent (57); therefore, these extremophiles express proteins that must be highly active at low temperatures to provide optimal catalytic power. Amino acids changes in soluble cold-adapted enzymes have been suggested to contribute to optimum enzymatic activity due to enhancement of local flexibility that allow for

conformational changes at low temperatures (51). These heat-labile proteins with high specific activity and catalytic efficiency at low temperatures have been suggested to contain certain amino acid compositional patterns. Psychrophilic soluble proteins have been found with more apolar surface area than mesophilic and thermophilic counterparts to avoid cold denaturation implying more strategic design selection for cold-adapted proteins than the heat-resistant proteins (58). While others report that the changes in frequency of particular residues may involve more polar and G residues, low R/K ratio (59) or greater number of the negatively charged E residues in the surface of the psychrophilic protein and higher ratio of I/L residues (60). The variability in cold-resistant protein characteristics seems to reflect the individual protein's form of adaptation and stability that may depend on their structure, inter- and intramolecular interactions and function. This delicate balance of thermal stability, flexibility and kinetic efficiency of the cold-adapted proteins requires further and more extensive analyses.

2.4 Salmonid vs. Mammalian NCX

Mammalian NCX has been reported to have a temperature coefficient (Q_{10} or fold change in rate per 10°C change) of ~ 2.0 between 25°C and 37°C, but greater than 2.0 between 7°C and 20°C as seen in Figure 2-3 (61). However, in poikilothermic species such as some teleosts, cardiac NCX activity is relatively insensitive to temperature with Q_{10} of ~ 1.2 (11). Using a series of chimeras it was found that differences in wild-type canine NCX1.1 and trout NCX - TR1.0 temperature dependences can be attributed to sequence differences in the N-terminal transmembrane segments (TMSs) (62). Protein alignments indicate ~ 86 % identity between NCX1.1 and NCX-TR1.0 in the N-terminal

TMS, which consists of 280 residues. This leaves ~ 35 residue differences that likely confer the differential temperature sensitivity between these orthologs.

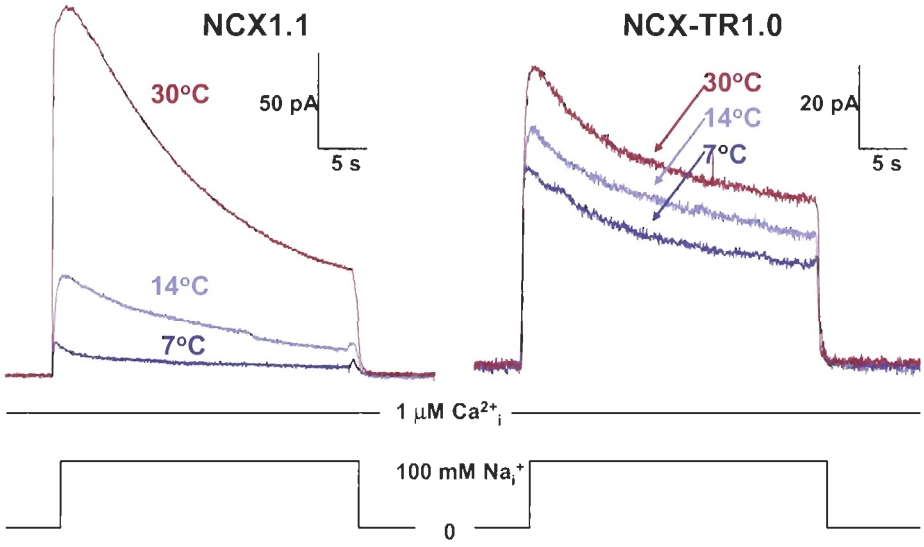


Figure 2-3 Wild-type dog and trout cardiac NCX outward currents
Outward currents were measured at three temperatures (30, 14 and 7 °C) for both wild-type dog and trout cardiac NCX in a giant excised patch clamp at 100mM Na_i^+ and 1 μM Ca_i^{2+}. From laboratory data files, copyright Dr. Larry Hryshko, published in part in *American Journal of Physiology*.

Important information on the specific amino acids responsible for trout NCX cold adaptation was derived by the cloning of NCX from the tropical fish tilapia (*Oreochromis mossambicus*). As rainbow trout, tilapia also lives in freshwater habitats and is a popular cultured seafood delicacy. However, both teleost species' habitat temperature ranges differ significantly. Rainbow trout can live in temperatures down to 0 °C (63) and up to 23 °C, whereas tilapia can tolerate temperatures as high as 42 °C but not lower than 14 °C (12). The salmonid (trout) fish arose approximately 100 mya (64) while the cichlids (tilapia) may have been present for ~ 260 mya (65). During evolution temperature pressures to one or both may have selected for multiple mutations that resulted in NCX activities with Q_{10} values of 2.2 and 1.2 in tilapia and trout (12), respectively (Figure

2-4). Although the divergence is large, both teleosts belong within the same lineage (actinopterygian) whereas mammals belong to the sarcopterygian lineage. The addition of the tilapia NCX clone can contribute to the separation of amino acid changes due to species divergence from temperature adaptation mutations. The most obvious residues that should be tested first are the ones common between the mammalian and tilapia N-terminus and, at the same time, different in trout NCX. As mentioned previously (12), the protein sequence alignment among dog, tilapia and trout suggests ten amino acids within the N-terminus to be involved in the differential NCX temperature dependences.

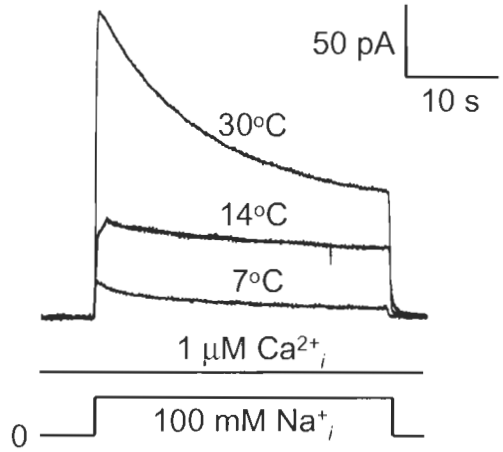


Figure 2-4 Tilapia cardiac NCX outward currents

Outward currents of the tilapia cardiac NCX at 30, 14 and 7 °C demonstrate tilapia's NCX similarity to mammalian temperature sensitivity. From laboratory data files, copyright Dr. Larry Hryshko, published in part in *Journal of Biological Chemistry*.

Uncovering the molecular basis of cold adaptation is of considerable interest for it may provide the nature of enzymatic catalysis and potential biotechnology applications. It is central to understand the relationship between the primary and tertiary structure of proteins that ultimately may provide the prediction of the three-dimensional structure from its amino acid sequence.

2.5 Hypotheses and Objectives

Despite the many differences observed between mesophilic and psychrophilic proteins, single amino acid substitutions may be capable of conferring most psychrophilic characteristics (66). Based on previous observations (12), substitution of specific amino acids in the N-terminus of the canine NCX with amino acids homologous in the rainbow trout could potentially provide a mammalian mesotherm NCX with a poikilotherm temperature resistance. Nevertheless, defining the specific amino acids involved in temperature resistance of NCX activity has proven to be challenging. By examining in detail NCX gene evolutionary history and using comparative physiology, we hypothesize that amino acids affecting temperature dependence can be revealed.

The specific preservation of activity at low temperatures brings about interest in the factors within the primary structure of NCX that link activity with temperature. Therefore, examination of NCX evolutionary history may provide clues to the specific amino acids in NCX N-terminus that modulate temperature sensitivity on this transporter as a whole.

Various high identity NCX genes in vertebrates and single invertebrate homologs may indicate NCX duplicated after the emergence of primitive chordate organisms at the same time as genome duplication occurrence (2R). In separate genome duplication of teleost species, the number of NCX genes should be doubled that of any other vertebrate. Hence, further analysis and search of more NCX genes to strengthen this family's phylogenetic tree may provide a new and precise history of NCX evolution.

In this study, the expansion of our NCX library with new public genomes available will aid in the exploration of the evolutionary relations among members of the

NCX family. Gene structure examination will be indicative of the evolution of the various NCX homologs specific to tissue and species. By developing a more complete NCX phylogeny and gene structure analysis, we hope to decipher the specific residues within NCX that determine how it will function at specific temperatures.

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CHAPTER 3:
NCX GENE EVOLUTION FINDINGS

NCX has been shown to be an important transporter of Ca^{2+} across a wide variety of species, but little is known of NCX phylogeny and molecular evolution. Previous work has shown the existence of at least three NCX paralogs in all vertebrates indicating two serial NCX gene duplications occurred after vertebrates diverged from invertebrates (1); however the lack of representative sequences makes the more accurate timing of these duplications difficult to determine. Hence, the study of evolution of NCX genes may allow further understanding of proteins with high degree of conservation, adaptation and distribution to different cells due to their essential role in the viability of higher life forms.

3.1 Methods in NCX Gene Evolution Analyses

3.1.1 Data Mining of NCX Sequences

tBLASTn searches were performed using known NCX protein sequences in available genome sequences in ENSEMBL database. Predicted and mRNA sequences of interest have yielded over 100 candidate genes for NCXs from GenBank and Ensembl. Species names, gene names and location are listed in Table 3-1. The available genomic sequences of invertebrate and vertebrate species were analyzed to identify homologous NCX genes. Due to some inaccuracies and missed annotations of NCX genes in Ensembl, tBLASTn search method was utilized to find/correct predicted exons and

complete segmented NCX protein sequences with cloned mammalian NCX protein sequences. Identification of candidate NCX sequences was based on conservation within the 9 TMSs, α-repeats and regulatory sites, originally established by known sequences. Hence, we have been able to categorize and/or complete over 35 NCX protein sequences in 10 different species genomes.

Table 3-1 Eukaryote NCX homologs list

Scientific	Common	NCX	Locus/Scaffold/ Chromosome
Mammalian Species Name			
Bos taurus	cow	1,2,3	11, 18, Scf5732
Canis familiaris	dog	1,2,3	17, 1, 8
Cavia porcellus	guinea pig	1	CPU04955
Felis catus	cat	1	NM_001009848
Homo sapiens	human	1,2,3	2, 19, 14
Macaca mulatta	macaque	1,2,3	13, 19, 7
Monodelphis domestica	opossum	1,2,3	1, Scf14576, 1
Mus musculus	mouse	1,2,3	17, 7, 12
Ornithorhynchus anatinus	platypus	1	Cntg424
Oryctolagus cuniculus	rabbit	1	OCU52665
Pan troglodytes	chimpanzee	1,2,3	2, 19, 14
Rattus norvegicus	rat	1,2,3	6, 1, 6
Spermophilus tridecemlineatus	squirrel	1,3	Scf4432, 15412
Avian Species Name			
Gallus gallus	chicken	1,3	3, 5
Amphibian and Teleost Species Name			
Danio rerio	zebrafish	1,1d,2,2d,3,4,4d	17,11,15,5,13,21,7
Gasterosteus aculeatus	stickleback	1d,2,2d,3,4,4d	Scf87,7,1,10,1,23
Oncorhynchus mykiss	rainbow trout	1d	AF175313
Oreochromis mossambicus	tilapia	1d	AY283779
Oryzias latipes	medaka	1,1d,2,2d,3,4,4d	15,Scf1982,14,1s3,22,14, 18
Squalus acanthias	spiny dogfish	1	DQ068478
Takifugu rubripes	fugu	1,1d,2,2d,3,4,4d	Scf131,3897,306,162,3,8, 1080
Tetraodon nigroviridis	green pufferfish	1,1d,2,2d,3,4,4d	17,Un47,16,Un126,10,7, Un21
Xenopus tropicalis	clawed frog	1,2,3,4	Scf41,572,218,684

Scientific Invertebrate Species Name	Common	NCX	Locus/Scaffold/ Chromosome
Aedes aegypti	mosquito	1	Cntg1.920
Anopheles gambiae	mosquito	1	2R
Apis mellifera	honeybee	1	Group5
Caenorhabditis elegans	roundworm	1,2	V,V
Caenorhabditis briggsae	roundworm	1,2	CBG12917,CBG06769
Ciona intestinalis	sea squirt	1	10q
Ciona savignyi	sea squirt	1	reftig 37
Drosophila melanogaster	fruitfly	1	3R
Loligo opalescens	squid	1	LOU93214
Strongylocentrotus purpuratus	sea urchin	1	XM_001184049

Targeted databases included: the non-redundant protein and nucleotide databases at NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ensembl Genome Browser v31-v34 and v39 (http://www.ensembl.org/) as seen in Table 3-1. The NCX ESTs (expressed sequenced tags) and clones were obtained from NCBI Nucleotide database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).

3.1.2 Phylogenetic Analyses

To understand the origins of the eukaryotic NCX family, we performed a phylogenetic study of all NCX sequences from all species. Multiple sequence alignments and rooted trees were prepared using ClustalX (v. 1.83); alignments were manually edited with Genedoc to remove the alternative spliced exons and visualized with TreeView32. Bootstrapping was performed (1000 replicates), and most nodes demonstrated high confidence values. Protein sequence names were assigned according to species scientific name and NCX homolog number (i.e .= human NCX1 is labelled as H.sapiens1). Incomplete NCX sequences missing over 10% amino acids were not included in the phylogenetic tree although some are listed in Table 3-1. A related NCX from the

bacterium pirellula (*Rhodopirellula baltica*) was used as the outgroup to root the phylogenetic tree.

3.1.3 Synteny Alignment

The Ensembl database and Evolutionarily Conserved Regions (ECR) browsers (http://ecrbrowser.dcode.org/) (2) were used to locate syntenic regions among teleost, avian, and mammalian genomes. In the ECR browser, scaffold section known to contain the NCX4 gene flanked by a minimum of 30,000 basepairs was used to align with all other available genomes to find synteny patterns. The Ensembl database was used to find the predicted orthologs in the genomic regions found in the ECR browser.

3.2 Origins of Vertebrate NCX Gene Duplication

The protein products of NCX homologs in all species range in size from ~ 800 – 990 residues and show overall identity of ~ 66 %. The conservation of NCX protein sequence is remarkably high considering its diversity in physiological roles, depending on NCX tissue localization and cellular environment, as mentioned in the previous chapter. NCX has been shown to be an important transporter of Ca^{2+} across a wide variety of species, but little is known of NCX phylogeny and molecular evolution. Previous work has shown the existence of at least three NCX paralogs in all vertebrates suggesting two serial NCX gene duplications occurred after vertebrates diverged from invertebrates (1); however the lack of representative sequences makes a more accurate timing of these duplications difficult to determine. Hence, the study of evolution of NCX genes may allow further understanding of proteins with high degree of conservation,

adaptation and distribution to different cells due to their essential role in the viability of higher life forms.

Most invertebrate species such as squid, fly, honeybee and mosquito only have one NCX gene, whereas vertebrate species have at least three NCX genes with high protein sequence conservation among all organisms. To better determine the timing of NCX duplication events, we searched the protochordate *C. intestinalis* genome for the presence of NCX genes. The *Ciona's* distinctive evolutionary position as an invertebrate chordate has been suggested to contain an approximation to the ancestral match of nonduplicated chordate genes, hence it may provide insights into vertebrate evolutionary origins (3).

3.2.1 Alignment and Phylogeny

A few invertebrate genomes (ie: sea urchin and sea squirt) contain several genes belonging to the CaCA transporter family, but only one gene appears ancestral to vertebrate NCX genes with at least 40% identity to mammalian NCX protein sequence. The *Ciona* NCX gene is positioned in the phylogenetic tree in a manner that provides a clear separation of the invertebrate and vertebrate NCX sequences as seen in Figure 3-1. The presence of a single copy of an NCX gene shared among vertebrates in *Ciona* is consistent with previous findings that indicate that every gene that the *Ciona* genome contains corresponds to a paralogous family in vertebrates for many signalling molecules, transcription factors and channels (3,4). The *C. intestinalis* NCX displays ~ 56 % overall identity among vertebrate NCX homologs. Even though other genes annotated as NCX were found in *C. intestinalis* genome, it is hypothesized that these genes evolved after the divergence of *Ciona* from other vertebrates as evidenced by their lower sequence identity

to vertebrate NCX sequences and the *Ciona* NCX-like (~ 33 % identity) gene. *C. elegans* have been found to have various NCX genes, (C10G8.5 and Y113G7A.4) two of which contain ~ 45 % identity (labelled as *C.elegans*1 and *C.elegans*2 in Figure 3-1 according to NCBI annotation but does not correlate to our vertebrate NCX annotation) and the rest with 33 - 15 % protein identity to mammalian NCX sequences. As in the sea urchin (*S. purpuratus*), three NCX sequences were found but only one (XM_001184049) was prominent with ~ 55 % identity with the vertebrate NCX, while the others had less than 35 % identity. The phylogenetic tree in Figure 3-1 shows that the duplication events giving rise to NCX paralogs in vertebrates occurred after the divergence of *C. intestinalis*, which is estimated to be 486 mya (5). Around this time, there were at least two gene duplications before the emergence of teleost species. Therefore, from the existence of one NCX gene in organisms lower than chordates, duplication events brought about four NCX paralogs in vertebrates.

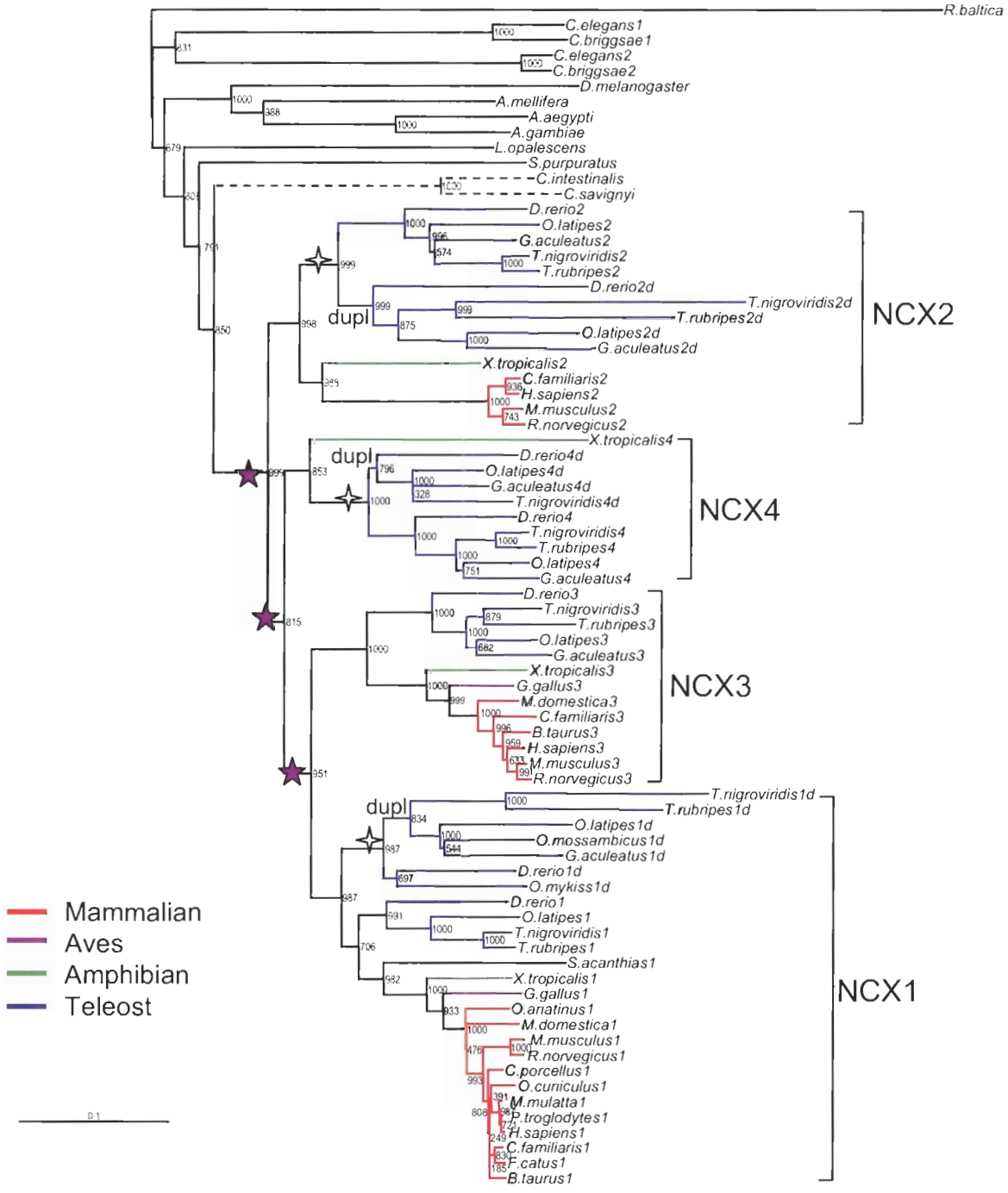


Figure 3-1 NCX phylogenetic tree

Phylogenetic tree composed of all the known and postulated NCX protein sequences. Pirellula bacterial strain (*Rhodopirellula baltica*) was used to root this tree. The five-point stars (★) indicate NCX duplication after *Ciona intestinalis* divergence and the four-point stars (✧) indicate teleost NCX duplication. Branches are colour coded for the vertebrate species, in which red designates mammals, purple indicates birds/aves, green are amphibians, and blue are teleosts/fish. The dashed branch (---) indicates the division between invertebrate and vertebrate NCX. A non-scaled, simplified representation of NCX evolution, rooted with the *Ciona* is at the right side. The scientific names of species are shown on the phylogenetic tree and their common names are in Table 3-1.

3.3 NCX4

3.3.1 NCX4 Expression in Teleosts

NCX4 has been suggested to be the product of gene duplication after teleost divergence from tetrapods because it was not found in any other species (1). More recently, NCX4 was also found in greenpuffer fish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*), and stickleback fish (*Gasterosteus aculeatus*) strengthening the idea that NCX4 is consistently present in fish species. Evidence of possible expression of NCX4 was found in various teleost species. Zebrafish (*Danio rerio*) NCX4 ESTs (Accession #) BQ263135 and BQ284787 were found to have the highest nucleotide and protein sequence identity of 98.8 % and 99.6 %, respectively, to 880 nucleotides (~ 1/3 of the whole cDNA or 290 amino acids) of the predicted zebrafish NCX4, which were found in chromosome 7. A third EST, BI875890 contains an overlapping sequence with both previous ESTs and has 98.6 % nucleotide identity to the corresponding segment (514 nucleotides). Another recently available genome from the stickleback fish, which is similar to all other teleost genomes, contains NCX genes and their duplicates (except for NCX3 and an incomplete *NCX1* gene duplicate). EST DT993818 contains 846 nucleotides (282 amino acids) that match 100% to the genomic stickleback NCX4 found through tBLASTn in ENSEMBL. Also, EST DT978254 contains 876 nucleotides (292 amino acids) from the alternative splice site to the partial last exon. Without the rainbow trout genome available to search for NCX4 gene, NCX ESTs sequences of 267 amino acids (802 bp) in length of the N-terminus (accession # AJ130842) from Kraev, A., Bertaggia, D. and Carafoli, E (unpublished) has 71.3 % identity with our TR-NCX1.1 clone. Another section of the C-terminus (accession # CA366341) with 159 amino acids

(478 bp) (6) were found in Genbank for rainbow trout. These partial sequences have the highest identity with NCX4 sequences in all teleosts species (85-92%), which indicates the possibility of NCX4 gene expression in the rainbow trout. In spite of the presence of NCX4 ESTs and strong protein sequence conservation, since protein expression and function have not been validated; there is still a possibility that NCX4 could be a pseudogene (7).

3.3.2 Evolutionary History of NCX4

Interestingly, mining of the *Xenopus tropicalis* genome also yielded more than three NCX genes. In the Ensembl browser (v31 and v32), an NCX tBLASTn search was performed in the *X. tropicalis* genome in which four scaffolds contained sequences with the highest identity to the sample NCX protein sequence. The NCX genes found in scaffolds 684, 572, and 218 were identified as NCX1, 2 and 3, respectively in Ensembl v31. However, when a second search in a later Ensembl version (v32) resulted in another NCX sequence in scaffold 41, we recognized that NCX1 is actually located in scaffold 41 meanwhile scaffold 684 contains NCX4 based on protein sequence alignment. The presence of NCX4 in the frog genome indicates that the gene is the product of an ancient gene duplication event and not that of a teleost-specific duplication event as previously thought (1). This also gives rise to a new hypothesis that the NCX4 gene may be present in avian and mammalian genomes.

3.3.3 Synteny Alignment

Finding a putative NCX4 gene in amphibians prompted the re-examination of other vertebrate genomes. In our search for NCX genes in the available chicken genome

(Ensembl v34), only NCX1 and NCX3 were found in their entirety. Partial high identity NCX exons were found in three separate unidentified chromosomes in which they may correspond to NCX2 or to more than one other NCX gene. Therefore, it is possible that in the chicken genome the NCX4 gene may have been lost due to the lack of selective pressure for this specific NCX homolog; but the possibilities that the quality of the genomic partial sequences are too poor to determine their specific homolog identity or that duplication and loss of NCX4 is specific to the avian genome. Synteny analysis of the complete chicken NCX genes confirmed their identity as NCX1 (in chromosome 3) and NCX3 (in chromosome 5). Meanwhile, synteny analysis for the partial chicken NCX genes located in an unknown chromosome (13,972,500-13,977,183 bp region) resulted in alignment with segments corresponding to NCX2 genes in other organisms. However, the other partials of chicken NCX gene resulted in insignificant synteny alignments probably due to the extensive sequence gaps. Therefore, the result is inconclusive and refinement of the chicken genome will be required to confirm the presence or absence of NCX4.

After extensive searches in mammalian genomes for the NCX4 gene, it was concluded that this gene is not present in the available mammalian genomes. To verify this, the Evolutionary Conserved Regions (ECR) Browser was used to find syntenic links among the teleost and amphibian NCX4 loci with mammalian counterparts. Syntenic alignments were consistent among the teleost and amphibian NCX4 surrounding genes, but high syntenic alignment with the mammalian genomic DNA confirmed that NCX4 was completely eliminated in mammals (Figure 3-2).

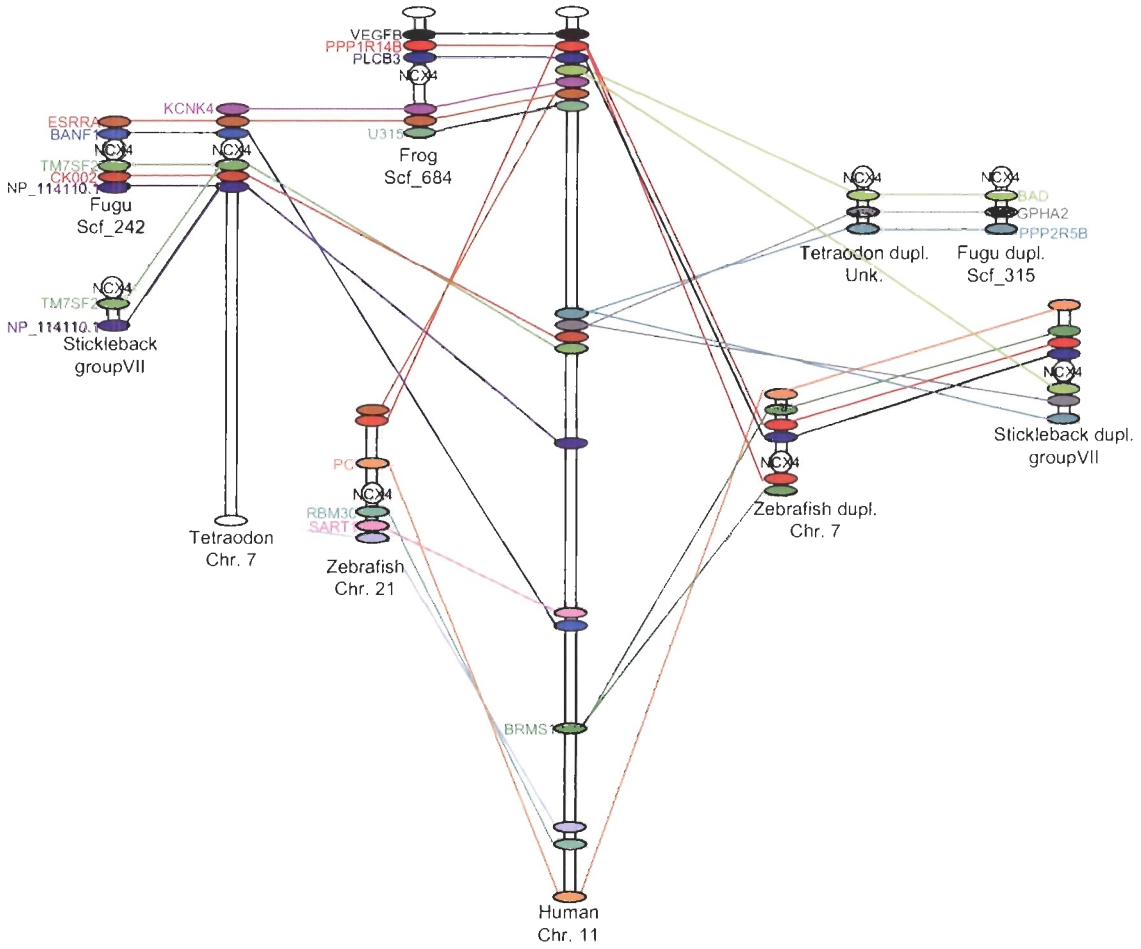


Figure 3-2 Synteny alignment diagram at NCX4 loci.

Synteny diagram among zebrafish, fugu, frog, tetraodon and human. NCX4 gene is represented as a circle and all other surrounding genes are represented as ovals and orthologs are associated with a line.

3.4 Teleost NCX Phylogeny

Previously more than three NCX genes have been found only in teleost species leading to the hypothesis that a separate round of gene duplication occurred only in fish species. Examining the genomes of fish species more closely, NCX duplicates for each NCX paralog were expected based on the whole genome duplication event; suggested to have occurred after the divergence of teleosts from other vertebrates (8). Indeed, duplicates were found for all NCX genes except for NCX3 duplicate gene, bringing the

number of potential NCX genes in teleost species to seven overall (Figure 3-3 and Table 3-2). These duplicates show high conservation patterns in known functional regions including α-1 and α-2 repeats, XIP site, Ca^{2+} binding sites and exon-intron boundaries. In this thesis, distinction between the duplicate and the original genes has been defined based on sequence identity with mammalian single NCX paralogs; duplicate forms being the most distant from the mammalian sequences. Hence, the original genes in fish genomes are simply called NCX while the lower sequence identity copies are referred as NCX duplicates.

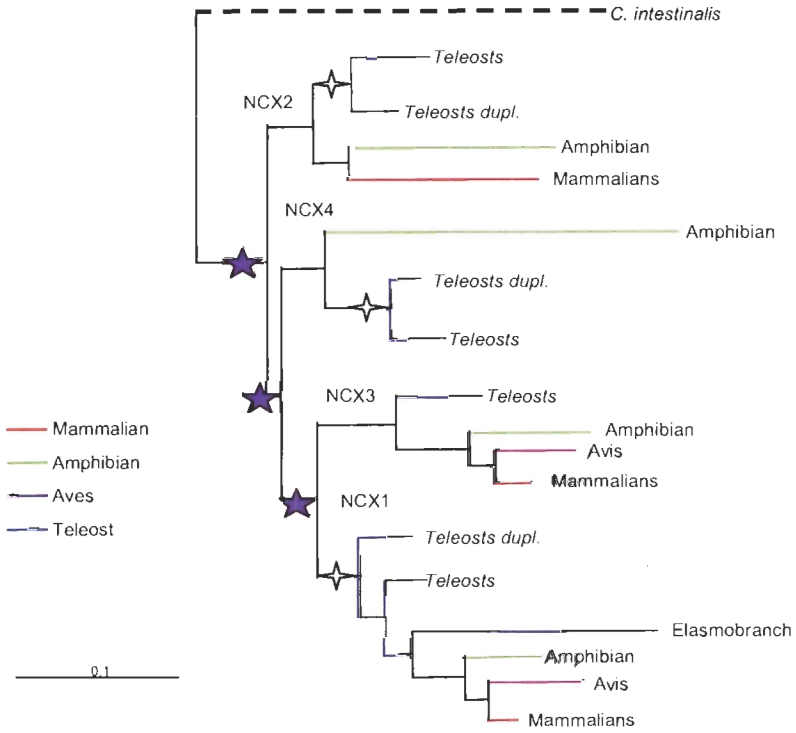
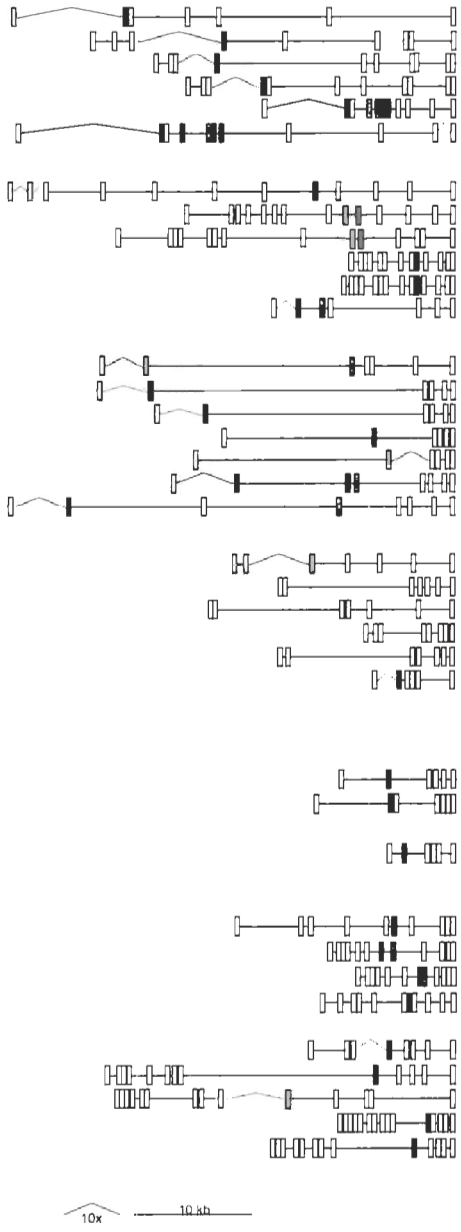


Figure 3-3 Summarized vertebrate NCX phylogenetic tree
Vertebrate NCX phylogenetic tree derived from the more detailed phylogenetic tree in Figure 3-1.

Table 3-2 Teleost NCX gene homologs and localization



Gene	Organism	Chr/Sc	Area	Version	Source
NCX1	*D.rerio*	Chr.17	18,740,417 - 18,859,925	V34	Ensembl
NCX1	*O.latipes*	Sc.272	14,381 - 128,226	200406	Medakasite
NCX1	*T.nigroviridis*	Chr.17	10,412,883 - 10,354,154	V34	Ensembl
NCX1	*T.rubripes*	Sc.131	209,799 - 268,614	V32	Ensembl
NCX1	*G.gallus*	Chr.14	15,921,826 - 15,841,283	V31	Ensembl
NCX1	*X.tropicalis*	Sc.41	1,789 - 164,247	V32	Ensembl
NCX2	*D.rerio*	Chr.15	46,306,625 - 46,367,990	V34	Ensembl
NCX2	*G.aculeatus*	Sc.7	15,645,456 - 15,670,007	V39	Ensembl
NCX2	*O.latipes*	Sc.4	95,320 - 68,267	200506	Medakasite
NCX2	*T.nigroviridis*	Chr.16	6,792,073 - 6,801,368	V34	Ensembl
NCX2	*T.rubripes*	Sc.306	21,040 - 31,209	V32	Ensembl
NCX2	*X.tropicalis*	Sc.572	258,233 - 300,405	V31	Ensembl
NCX3	*D.rerio*	Chr.13	30,655,514 - 30,723,113	V31	Ensembl
NCX3	*G.aculeatus*	Sc.10	12,007,219 - 12,088,179	V39	Ensembl
NCX3	*O.latipes*	Sc.223	673,561 - 601,197	200506	Medakasite
NCX3	*T.nigroviridis*	Chr.10	11,431,554 - 11,453,500	V32	Ensembl
NCX3	*T.rubripes*	Sc.3	671,667 - 717,064	V31	Ensembl
NCX3	*G.gallus*	Chr.5	24,529,908 - 24,615,239	V31	Ensembl
NCX3	*X.tropicalis*	Sc.218	578,642 - 485,421	V31	Ensembl
NCX4	*D.rerio*	Chr.21	22,338,760 - 22,412,421	V34	Ensembl
NCX4	*G.aculeatus*	Sc.1	15,488,059 - 15,600,582	V39	Ensembl
NCX4	*O.latipes*	Sc.539	17,830 - 41,537	200406	Medakasite
NCX4	*T.nigroviridis*	Chr.7	6,997,753 - 6,987,941	V31	Ensembl
NCX4	*T.rubripes*	Sc.8	42,468 - 53,188	V34	Ensembl
NCX4	*X.tropicalis*	Sc.684	565,270 - 536,979	V31	Ensembl

Dupl. Gene	Organism	Chr/Sc	Area	Version	Source
NCX1	*G.aculeatus*	Sc.87	19,917,572 - 19,941,015	V39	Ensembl
NCX1	*O.latipes*	Sc.2214	11,440 - 2,474	200406	Medakasite
		Sc.2330	17,499 - 16,198	200406	Medakasite
NCX1	*T.rubripes*	Sc.3897	9,694 - 11,292	V31	Ensembl
		Sc.4834	7,802 - 3,740	V31	Ensembl
NCX2	*D.rerio*	Chr.5	38,347,485 - 38,367,266	V31	Ensembl
NCX2	*G.aculeatus*	Sc.1	12,617,360 - 12,630,893	V39	Ensembl
NCX2	*O.latipes*	Sc.478	922,000 - 101,393	200506	Medakasite
NCX2	*T.rubripes*	Sc.162	198,251 - 211,861	V31	Ensembl
NCX4	*D.rerio*	Chr.7	45,085,145 - 45,041,516	V34	Ensembl
NCX4	*G.aculeatus*	Sc.23	7,574,625 - 7,679,026	V39	Ensembl
NCX4	*O.latipes*	Sc.47	1,826,796 - 1,751,879	200506	Medakasite
NCX4	*T.nigroviridis*	Unk.	21,236,474 - 21,245,679	V31	Ensembl
NCX4	*T.rubripes*	Sc.1080	80,209 - 65,614	V34	Ensembl

3.4.1 Teleost NCX Family Duplication

ESTs from the rainbow trout were found to belong to NCX1 (CX255655 and CX255654) and NCX3 (CA352444). Even though our lab cloned the complete rainbow trout NCX1, the putative NCX1 EST does not have a high enough protein sequence identity (71 %) for it to belong to the same gene. However, this EST still has the highest percentage alignment with NCX1 genes in comparison to all other three paralogs from other species. This indicates that this is another NCX1 gene that belongs to the rainbow trout genome. As seen in Figure 3-1, the cloned NCX1 from cardiac tissue groups with the other teleost duplicate NCX1 sequences. The functionality of both duplicated genes is supported by Langenbacher *et al.* (9) finding of two NCX1 genes in the zebrafish and demonstrated distinct tissue-specificity and effect. NCX1h in the heart was found to play a role in rhythmic contraction, hence the NCX1h knockout has been shown to cause cardiac fibrillation; while NCX1n was predominantly found in the brain and neural tube (9). NCX1h was also placed with the other teleost duplicate NCX1 sequences in the phylogenetic tree as the cloned trout cardiac NCX1.

3.5 Gene Structure

In this study, we undertook a more detailed examination of NCX gene structure. Through examination of parameters such as number of exons, intron density range, gene length, sequence alignment and alternative splice exons, we hoped to clearly define the NCX phylogenetic tree. The alignment of all ortholog and paralog NCX proteins demonstrated high sequence conservation in most regions, especially in the α1 and α2 repeat regions, and the Ca^{2+}-dependent activation sites. Certain features not taken into account in the bootstrapping of the phylogenetic tree (e.g. the alternative splice site and

intron density) will be examined in the following sections. Further analysis of these genes may allow a more comprehensible view of the NCX gene evolution.

3.5.1 Alternative Splice Site

In NCX1 the C-terminal portion of the loop-f is subject to extensive alternative splicing where differential combinations of six exons (A, B, C, D, E and F) produces sixteen tissue specific splice variants (10). Although Schulze *et al.* (11) observed distinct voltage dependence and Hurtado *et al.* (12) described differences in Na^+-dependent inactivation induced by increase in $[Ca^{2+}]_i$ in NCX1.1 versus NCX1.3; the physiological significance of NCX alternative splicing remains unclear. A comprehensive evolutionary analysis of NCX alternative splicing has not been conducted to date.

Exons known to correspond to the alternatively spliced area have been found in all NCX genes. Although it is not known if the alternative splice site section of NCX in teleosts is spliced as it is in mammals due to their limited number of exons (Table 3-3). The cloned rainbow trout NCX1 (13) contained all known alternative splice exons except for exon E, and hence was named TR NCX1.0. However, a second clone from the same library yielded an identical gene with exon E that was named TR-NCX1.1 (unpublished). Due to their complete identity, except for an alternative exon, both of these transcripts are most likely from the same gene with no known functional difference. An increasing number of exons in the alternative splice site, in which NCX4 has the least with only exon A and NCX1 has the most with all known six exons (A-F) (Table 3-3), demonstrate a pattern to an important point in NCX evolution. The increasing number of exons correlates with NCX gene isoform evolution seen in the phylogenetic tree (Figure 3-1). For instance, NCX4 with only exon A in teleosts and amphibians indicates the lack of

alternative splice site in this gene. Hence, referring to some teleost NCX as having alternative splice regions may be inappropriate due to the lack of exon alternatives to assemble different combinations.

Table 3-3 General alternative splice exons from all NCX homologs

Species	NCX	A	B	C	D	E	F
Teleost	4all	KLLNVKIIDDEEYEKNKTFTIVLEEPILLEVGQKH					
Amphibian	4	RFIEIQVIDDEEYEKNKNFYVELGEPEMQRSGKKS					
Teleost	2all		QSFQVRIIDDEEYEKHENFFIVLEEPRWLKRGIS	ALLLNQE			
Amphibian	2		KTLQVKIVDDEEYEKQENFFIILEEPRWMKRGIS	ALLLNQG			
Mammalian	2		KTLQVKIVDDEEYEKKDNFFIELGQPQWLKRGIS	ALLLNQG			
Teleost	3	KFIHVKIIDDEEYEKNKNFFLELAEPRMVDMSLQK					
Amphibian	3	KTIQIKIFDDEEYEKNKTFFIELREPHLVDLSVQK	KTIRVKIIDEEEYERQESFFIALGEPKWMKRGIS	ALLLNQG			
Aves	3	KTIHIKVIDDEEYEKNKSFFIELMSPRMVDMSLQK					
Mammalian	3	KTIHIKVIDDEAYEKNKNYFIEMMGPRMVDMSFQK	KTIRVKIVDEEEYERQENFFIALGEPKWMERGIS	ALLLSPG		GKTSY	
Teleost	1	KTIQINIIDDEEYEKNKNFFLEIGEPQLLEMSERK	KTIKVKILDHEEYDKHANFFIELQEPEWRRRGWT				
Amphibian	1	KIISVKIIDDEEYEKNKTFFLEVGEPRLVEMSEKK	KCITLKILDREEYDKESNFFLVLQEPIWIRRGMK	ALLLNEL	GDFTIT	GKILY	GKPVLRKVQVRDHPIPSTVIILT
Aves	1	KTISIKVIDDEEYEKNKTFYLEIGEPRLVEMSEKK	KFITLRILDREEYEKECSFFLVLGDPVWLRRGVK	ALLLNEL	GGFTIT	GKLWK	GKPVFRKVQARERPLPCTVVTIR
Mammalian	1	KTISVKVIDDEEYEKNKTFFLEIGEPRLVEMSEKK	KIITIRIFDREEYEKECSFSLVLEEPKWIRRGMK	ALLLNEL	GGFTIT	GKYLF	GQPVFRKVHAREHPILSTVITIA
Teleost exceptions							
Pufferfish	1d	KTIQVNIIDDEEYEKNKNFFIELGDPRLLEMSERK		ARLLQEV			GRDMYRKVQEWHPSAAMINIPGM
Medaka	1d	KTIRINIIDDEEYEKNKNFFLEMGEPLLLEMSERK	KTIALRIMDREEYDKKASFCVELQEPFWNSRWTG	AVLLQEV			
Green pufferfish	1d		KSLHIRIVDDGEFGQDKNFLLELGEPRLLDPSQS				DRDVYRKLQGWNLPDAVINITGM
Trout	1d	KSIQINIIDDEEYEKNKNFFLEMGEPQLLEMSERK		AVLLQEI	GGFVKT		GRDVYRKVQGRDNPVPATIISLA
Tilapia	1d	KTIRINIIDDEEYEKNKNFFLEIGEPRLLEMSERK		AVLLQEV	GGFVKT		GRDIYRKVQGREHPVPSNIISIA
Dogfish	1	KTIEIKVIDDEEYEKNKNFFIEIGEPRLVEMSEKK		ALLLNEL	GPFTKT	AKYFN	GHAIYRKVHFRDNPIPSTVICIA
Zebrafish	1d	KTIQINIIDDEEYEKNKNFFLEIGEPQLVEMSERK		AMLLHEC	GGFVKT	DKQLY	GRDVYRKVQGRDKPIPSTIISIS
Stickleback	1d	KTIQINIIDDEEYEKNKNFFLEIGEPQLLEMSERK	KTIAVRVIDRDEYDKQASFYIELQEPYRNQRRWT	AVLLQEV	GGFVKT		GRDVYRKVQGRDHPAPSAVISIT

As seen in the phylogenetic tree (Figure 3-3), NCX2 and NCX4 are the first genes to branch out from the ancestral NCX form and have the least number of exons in the alternative splice region. Meanwhile, NCX1 and NCX3 in all species have the most number of exons and are the last to branch out in the phylogenetic tree. Teleosts NCX1 genes from the dogfish, stickleback, rainbow trout and tilapia, have a variety of exons (D-F) not seen in NCX2 and NCX4 (Table 3-3), which brings up the possibility of alternative spliced exons' appearance as this gene evolved. Other supporting evidence that exon A started as a 'normal' exon (instead of an alternative exon) can be seen in the invertebrate versions of NCX. Multiple alignments of vertebrate and invertebrate NCX protein sequences demonstrate high identity in the TMSs and exon A. Most invertebrates do not have known NCX variants. Except for *D. melanogaster* (CALX1.1 and 1.2) and *C. elegans* (NM_072013 and NM_072014), that have two variants each, their alternative exons have no similarity to the known vertebrate alternative exons located after exon A. Exon A duplication must have resulted in exon B based on their sequence similarity and their mutual exclusivity (where only one of them is expressed and required in the protein). First, signs of both exons A and B are apparent in the sea urchin (*S. purpuratus*), the only invertebrate; whether alternative expression of A and B occurs is not known. Meanwhile, there are no clues as to where the other smaller exons (C, D, E and F) within the alternative splice site came about. The spiny dogfish (*S. acanthias*) NCX1 clone DQ068478 is seen with all alternative exons (with the exception of B due to mutual exclusivity with A) and being part of the shark family, it is not suspected to have duplicates of any NCX paralog in which only the ray-finned fish has been theorized to go through whole genome replication (14).

3.5.2 Exon Splicing Patterns

As examined previously by Li et al. (15), exon boundaries of NCX1 and NCX3 are identical with 5 exons excluding the alternatively spliced exons. However, the first exon in NCX1 and 3 is divided into three exons in NCX2 in mammals. As for the amphibian, all four NCX genes N-termini TMSs plus the majority of the large cytoplasmic loop are maintained as one exon. In teleosts, the only similar exon splicing patterns to mammalian include NCX1 duplicates and NCX3 with one exon that includes the N-terminus plus the large loop-f; while, NCX1 has 3 exons, NCX2 has 4 to 8 exons with no specific differences between duplicates, NCX4 has 3 exons and NCX4 duplicates have 9 to 10 exons, except for *Danio* NCX4 with 3 exons in both (Table 3-3). Without accounting for the alternative splice exons, the C-terminus exon splicing is generally well conserved to 4 exons in all NCX genes. NCX2 and NCX4 are similar in the increased exon number in the N-terminus of the gene, whereas NCX1 and NCX3 exon splicing is more conserved and minimal. Both NCX2 and NCX4 genes have more exons in comparison to NCX1 and NCX3, without accounting for the alternatively spliced exons. Exon number in both NCX2 and NCX4 genes varies between 12 and 14. Meanwhile, NCX1 and NCX3 genes are consistent with 9 and 5 exons, respectively. Invertebrates, such as the *D. melanogaster*, *A. gambiae* and *A. mellifera* have 8 exons but the *C. intestinalis* and *C. elegans* have 10 and 14 exons, respectively. Similar to the alternative splice site, in which evolution seems to be taking place in an isolated manner, the N-terminus may have a higher evolutionary rate than the rest of the gene. It is due to the N-terminus inconsistent exon-intron boundaries that the number of exons varies among homologs. Interestingly, the expansion of the number of NCX sequences, grouping NCX genes based on exon boundaries resulted in a similar arrangement as NCKX genes

grouping, in which two pairs (NCKX 1 and 2 vs. NCKX 3 and 4) resulted in distinct exon arrangements (15).

3.5.3 Gene Lengths and Intron Density

Mammalian NCX1 and NCX3 genes average 290 and 135 kb in length, respectively, and represent the longest NCX genes across species, while mammalian NCX2 average 30 kb. However, the lower vertebrates exhibit the same length patterns but in smaller scale as expected due to their smaller genome in comparison to mammalian genomes. The teleost and amphibian NCX2 gene and duplicates, which are not much lower than the mammalian version, averaged at 22 kb, close to NCX4 gene and duplicates at 30 kb. However the avian, amphibian and teleosts NCX1 and NCX3 were greatly reduced in length averaging 103 and 65 kb, respectively, in comparison to mammalian versions. Only NCX1 duplicates demonstrate a shorter gene length pattern in comparison to NCX1 across teleost species with an average of 10 kb. Invertebrate NCX gene lengths ranged from 7 to 90 kb.

To quantify the evolution of introns, the intron density equation (number of introns per kb of coding sequence) was used to measure the number of introns per NCX gene (16). As shown in Table 3-4, NCX2 and NCX4 in all species demonstrate a wide range in intron density, 2-4 and 2-5 introns per kb, respectively. Meanwhile, intron density among all mammals, bird, amphibian, and zebrafish NCX1 maintain a consistent value of 1 intron per kb and the rest of the teleost NCX1 intron density is consistent at 2-3 introns per kb. Also, NCX3 gene intron density is preserved among all species at 1 intron per kb.

Table 3-4 NCX intron density chart

Organisms	NCX1	NCX1 duplicate	NCX2	NCX2 duplicate	NCX3	NCX4	NCX4 duplicate
H. sapiens	1.32		2.17		1.38		
C. familiaris	1.36		2.67		1.38		
M. musculus	1.33		2.53		1.38		
R. novergicus	1.33		2.17		1.38		
G. gallus	1.30				1.44		
X. tropicalis	1.33		1.56		1.39	1.48	
D. rerio	1.36		3.10	3.14	1.42	1.77	2.16
T. nigroviiridis	2.82	1.66	3.42		1.45	2.15	4.66
T. rubripes	2.72	1.55	3.86	3.32	1.45	1.81	4.76
O. latipes	2.84	1.40	3.75	3.35	1.45	1.76	4.43
G. aculaeatus		1.45	4.12	3.33	1.45	1.76	4.35

3.6 Discussion

3.6.1 NCX Origin

The evolutionary history of NCX among all available vertebrate genomes, including *Ciona*, was revealed with various points of comparative analyses in gene structure. First, NCX duplication is initiated after primitive chordates (ie: *Ciona*), probably due to genome duplication after cephalochordate (amphioxus) divergence from hagfishes and lampreys. Meanwhile, a second genome duplication has been suggested to occur after the divergence of hagfishes and lampreys from gnathostomes (jawed fishes) (14,17). Hence, the finding of one NCX gene in *Ciona's* genome and its positioning in the phylogenetic tree, dividing vertebrate from invertebrate branches, correlate with previous evolutionary studies (1). The structure in the phylogenetic tree (Figure 3-1) indicates three serial duplications, but this does not correlate to the theory of dual genome duplication (2R) early in the vertebrate lineage. If double genome duplication occurred, the phylogenetic tree would show a symmetric topology, in which two parallel duplications would result in four orthologs. However, the serial duplication in Figure 3-1

could also be a result of two genome duplications followed by independent gene duplication and loss (18). For instance, the Hox gene family is evident of tetraploidization event that follows the "4:1 rule" in the number of vertebrate to invertebrate genes (3). Availability of both hagfish and lamprey genomes, may provide two high identity NCX genes if the genome duplication theory which is postulated to occur before the jawless vertebrates emergence about 700-450 mya (19), holds true. Presently, four NCX genes would be expected due to a second genome duplication after the jawless vertebrates branched off or before the appearance of cartilaginous fish about 450 mya (19).

3.6.2 NCX4

Marshall *et al.* (1) identified a fourth putative NCX gene common among fish genomes and hypothesized to have emerged after tetrapod divergence from teleost; but no clones of this NCX4 have been reported to date. NCX4 expression has not been proven but its high sequence conservation and ESTs found in NCBI indicate at least transcription of NCX4. Discovery of NCX4 in the amphibian genome (*X. tropicalis*) has completely altered the previous theory on the origin of NCX4 (1). Now, we indicate that NCX4 arose before tetrapod emergence rendering avian and mammalian organisms with a possible fourth NCX gene. However, searches with tBLASTn and synteny alignments did not yield any avian or mammalian NCX4 genes probably due to NCX4 gene loss in birds and mammals; while strong selective pressure in teleost and amphibian retained this gene. Gene duplication has long been recognized as an important factor in the evolution of new genes. On the other hand, NCX4 gene loss in most tetrapods indicates how evolution would also eradicate genes due to selection. One possible reasoning of the

greater variety of exchanger proteins required by teleosts and amphibians may be the usage of gills or moist skin in comparison to air breathing creatures with lungs. Hence, it is likely that birds and aquatic animals that do not use gills would not preserve nor select for NCX4. Further studies of NCX4 expression and tissue specificity would aid in understanding teleost retention of this gene.

3.6.3 Teleost NCX Duplication

Our analysis has confirmed the existence of seven NCX paralogs in the teleost, four in the amphibian and three in the mammalian genomes. Duplicates of all NCX genes, except for NCX3, have been found in fish genomes which associates with a fish-specific genome duplication (20). ESTs have been found to correspond to these duplicate genes but the most compelling proof that these duplicate genes are expressed and functional are the NCX1 clones from rainbow trout (13) and zebrafish cardiac tissues that belong to the duplicate NCX1 gene group; while the other NCX1 clone found in zebrafish belongs to neural tissue (9). NCX emergence from primitive nervous systems, adaptation to other cellular environments with addition of exons and domains has been suggested (21). Brain tissue in adult rats have been found to contain all three paralogs but with different alternative splicing (Table 2-1), NCX1.4 (AD), NCX1.5 (ADF), NCX2.1 (AC), NCX3.2 (B) and NCX3.3 (BC) (10). As for the zebrafish, NCX3 only has exon A, NCX2 only has exon B while NCX1 from chromosome 17 corresponding to the neural tissues contains both exons A and B. The zebrafish neural NCX1 is expressed with only exon B. Therefore, correlation on alternative splicing exons with neural tissue specificity is not likely to be comparable among different species (i.e.: mammals vs. teleosts). When comparing mammalian tissue specific alternative splicing isoforms, heart

tissue in rats only has one alternative splicing isoform NCX1.1 (ACDEF) which is the exact same for the zebrafish cardiac version. However, in zebrafish, the lack of possible isoforms seems to limit the teleost to usage and dependence on their duplicate genes as seen in the neural zebrafish NCX1. The teleosts' greater number of genes might allow expression in different tissues that may counter balance their smaller number of alternative splicing exons in comparison to their mammalian counterparts.

3.6.4 Gene Structure Analyses

Analyzing the alternative splice site, intron density, exon/intron boundaries, sequence alignments and gene lengths, all together lead one to conclude that NCX2 and NCX4 have many gene structural similarities, while NCX1 and NCX3 have their own gene features. Exon-intron boundary and intron density patterns could indicate that NCX2 and NCX4 are genes with a higher rate of mutation in comparison to NCX1 and NCX3. The inverse correlation to these data, gene length comparison among homologs demonstrated that NCX2 and NCX4 are more compact in comparison to NCX1 and NCX3. Gene lengths of both NCX2 and NCX4 are comparable to the sea squirt and invertebrate NCX genes. These differences noted in NCX gene lengths, intron density and exon number may hint about their relation to each other and evolutionary pattern. The distinct numbers of alternative splice exons among species and NCX genes suggest an interesting pattern of NCX gene evolution and adaptation. However, these features do not seem to contain a pattern when used to compare NCX1 N-termini in organisms with distinct body temperatures.

3.7 References

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CHAPTER 4:
NCX TEMPERATURE DEPENDENCE RESULTS

Despite exhaustive phylogenetic and gene structure analyses of the collected NCX genes, we could not determine a genotype-phenotype correlation with respect to temperature sensitivity mainly due to our restricted knowledge of NCX phenotypes. Therefore, it was hypothesized that the cloning of NCX from a warm-adapted teleost would lead to further genotype-phenotype association and tilapia (*Oreochromis mossambicus*) was chosen for this purpose (1). The tilapia cardiac NCX-TL1.0 clone was then expressed in *Xenopus* oocytes to study its electrophysiology to confirm its similarity to mammalian phenotype correlating with its warm living environment. Alignment of the protein sequence of the N-terminus of tilapia NCX-TL1.0, rainbow trout NCX-TR1.0 and canine NCX1.1, and focusing on the amino acids identical between the canine NCX1.1 and tilapia NCX-TR1.0 but mismatched with the trout NCX-TR1.0 should separate residues specific for temperature sensitivity from residues with neutral or temperature-independent effects. The section encompassing residues 68 to 275 in the NCX1.1 was considered to contain the significant residues involved in temperature sensitivity (2). Residues corresponding to the signal peptide (1- 32 amino acids) are known to be cleaved in the mature NCX with no direct involvement in NCX activity and the first extracellular loop a (amino acids 33 – 67) lack sequence conservation; therefore residues 1 to 67 were ignored for potential mutagenesis. Ten amino acids were targeted as potential contributors to temperature dependence.

4.1 Methods in Temperature Dependence Analyses

4.1.1 Single-Site Mutagenesis and cRNA Synthesis

After assessment of the dog NCX1.1 sequence, minimal nucleotide changes were made to obtain changes in the amino acid sequence. Forward and reverse complement primers were ordered from Invitrogen and listed in Table 4-1 with the mismatch nucleotides underlined.

Table 4-1 Mutagenesis primers

Mutation #	Forward (5' to 3')	Reverse complement (5' to 3')
1- P34T	GGAGCCCCAAAACACTTCCTTTGAG	CTCCAAAGGAAGTGTTTTGGGGCTCCC
2- V151F	GTGTATTTTGTGGCCATGTTCTACATGTTTCTCGGAG	CTCCGAGAAACATGTAGAACATGGCCACAAAATACAC
3- T172D	GTATGTGGCCATAACTTCGATGCAGGAGACCTAGGTCCC	GGGACCTAGGTCTCCTGCATCGAAGTTATGGCCACATAC
4- L151F	GTTATCATCATCGCGTTTTGTGTTTATGTGGTCCC	GGGACCACATAAACACAAAACGCGATGATGATGAAC
5- T161H	GTGGTCCCAGATGGAGAGCACAGGAAGATTAAGCATTTGCG	CGCAAATGCTTAATCTTCCTGTGCTCTCCATCTGGGACCAC
6- A174T	CTTCGTGACAGCAACCTGGAGCATCTTTGC	GCAAAGATGCTCCAGGTTGCTGTCACGAAG
7- V194I	CATCTCTCCTGGGATCGTGGAAGTCTG	CAGACTTCCACGATCCCAGGAGAGATG
8- L202V	GAAGTCTGGGAAGGTTTGGTTACTTTCTTCTTCTTTCC	GGAAAGAAGAAGAAAGTAACCAAACCTTCCCAGACTTC
9- F213M	CCTATCTGTGTTGTGATGGCTTGGGTGGCAGATAGG	CCTATCTGCCACCCAAGCCATCACAACACAGATAGG
10-F223V	CTTGTAGACATACTTGTAAACCAGCAGCCTCCTATCTGC	GCAGATAGGAGGCTGCTGGTTTACAAGTATGTCTACAAG

Initial wild-type dog NCX1.1 in the pBlueScript vector was amplified with PCR with the first pair of primers. PCR settings were 18 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 12 min. The PCR product was then transformed into *E. coli* and grown in LB broth with ampicillin for selection of bacteria containing the transformed plasmid. Then, the DNA plasmid was extracted and purified with a miniprep kit (QIAGEN). Mutagenesis was confirmed with DNA sequencing and subsequently used for the next set of primers. Mutated constructs were then used as a template and the latter steps were repeated with each pair of the listed primers for subsequent mutations (Figure 4-1). All mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) in canine NCX1.1 (3) and confirmed by sequencing.

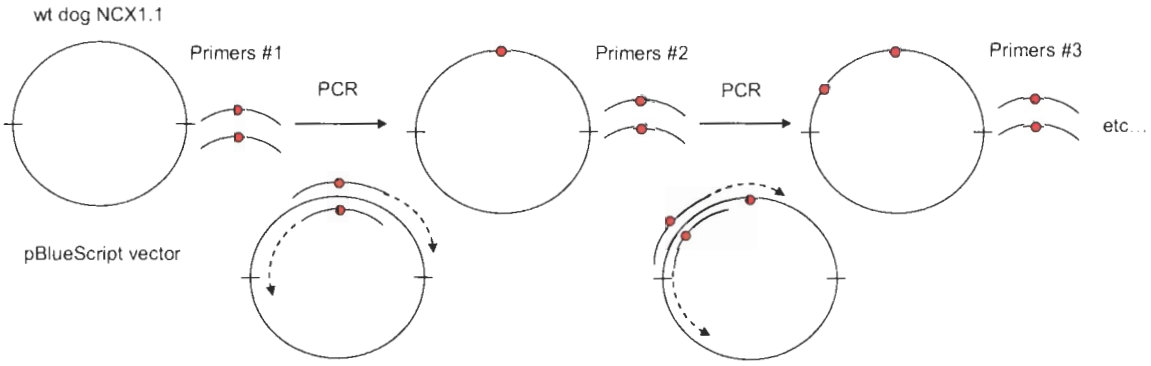

Figure 4-1 NCX mutagenesis technique

Initially wild type (wt) dog NCX1.1 construct was used as the template and amplified (PCR) with the first set of primers #1 to introduce the first mutation (filled circle). The product, confirmed with sequencing, was subsequently used for the next round of PCR with the second set of primers #2 and this was repeated until all ten mutations were generated in one construct.

Mutagenesis was performed in the canine NCX1.1 by substituting 10 amino acids of the trout NCX-TR1.0 residues at homologous sites: 1) P34T, 2) V51F, 3) T127D, 4) L150F, 5) T160H, 6) A174T, 7) V194I, 8) L202V, 9) F213M and 10) F223V (Figure 4-2). The same method was utilized to mutate the wild-type trout NCX1.0 construct, in

which trout amino acids at the selected sites were substituted with dog amino acids creating a reverse mutant to verify the results on the mutant with the dog NCX background.

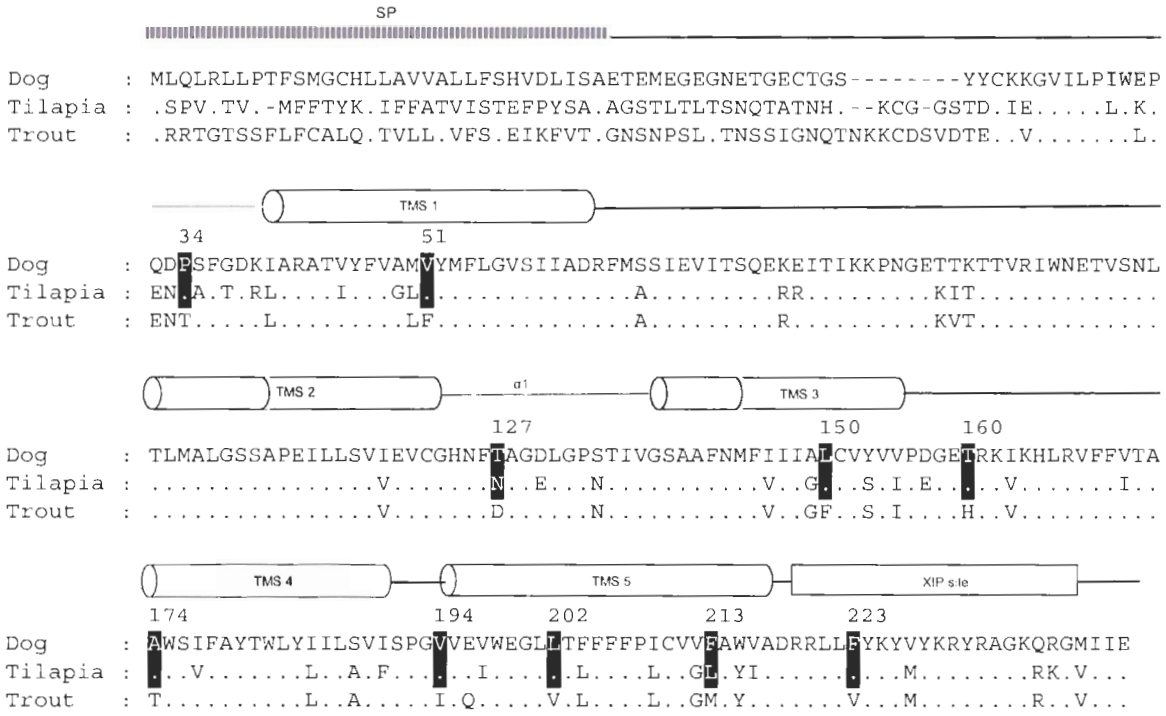

Figure 4-2 Alignment of NCX1 N-termini from dog, tilapia and trout sequences and proposed topology

Protein sequence alignment from the signal peptide (SP) to the XIP site (inclusive). TMS are labelled and represented as cylinders, while lines indicate intra- or extracellular loops. The α-1 repeat is indicated in grey. Highlighted amino acids in black indicate 1) possible amino acids involved in temperature dependence and 2) differences between warm- and cold-adapted organisms. Numbers above the highlighted residues and NCX topology refer to their position in the dog NCX1 mature protein sequence.

cDNAs were linearized with *HindIII* to synthesize cRNA at 37 °C with T3 mMessage mMachine™ In Vitro Transcription Kit (Ambion Inc., Austin, TX), precipitated with LiCl, washed with 70 % ethanol, and resuspended in nuclease-free

water. The cRNA concentration was assessed spectroscopically, while cRNA purity was determined with a 1 % agarose gel.

4.1.2 Preparation of *Xenopus* Oocytes

As previously mentioned (4), *Xenopus laevis* were normally anesthetized with benzocaine. Buffered isotonic solution was used in oocyte harvesting and preparation and then incubation with collagenase aided in the dispersal of the eggs. At mature stages (V-VI) eggs were washed and selected for injection with ~ 23 ng of cRNA and incubated at 18 °C. Exchange activity was assessed 3-7 days after injection using the giant excised patch technique (see below).

4.1.3 Electrophysiology, Analysis and Statistics

Na^+/Ca^{2+} exchange currents were measured with the giant excised patch-clamp technique (5). As mentioned previously (4), pure inward Na^+/Ca^{2+} exchange current measurements with pipettes containing (in mM): 100 Na-MES, 20 CsOH, 20 TEA-OH, 10 EGTA, 10 HEPES, 8 sulfamic acid, 4 $Mg(OH)_2$, 0.25 ouabain, 0.1 niflumic acid, and 0.1 flufenamic acid (pH 7.0 at room temperature with MES). Eliciting currents by rapidly switching between Ca^{2+}-free and 30 μM Ca^{2+}-containing Li^+- based bath solutions containing (in mM) 100 Li^+-aspartate, 20 CsOH, 20 MOPS, 20 TEA-OH, 10 EGTA, 0-9.91 $CaCO_3$, and 1.0-1.5 $Mg(OH)_2$ (pH 7.0 at 30 °C with MES or LiOH) to generate free $[Ca^{2+}]_i$ of 0 and 30 μM. Thus, the perfusing solutions included: 1) 1.5 mM Mg^{2+} to provide 0 free Ca^{2+} plus 1.0 mM free Mg^{2+}; 2) 9.91 mM Ca^{2+} plus 1.01 mM Mg^{2+} to provide 30 μM free Ca^{2+} plus 1.0 mM free Mg^{2+}. Raw current versus temperature data were baseline-corrected by subtracting currents generated in Ca^{2+}- free

from 30 μM Ca^{2+}- containing, Mg^{2+} - based solutions. Typical outward and inward current traces are shown below to demonstrate their differences and key solution requirements to activate these current traces.

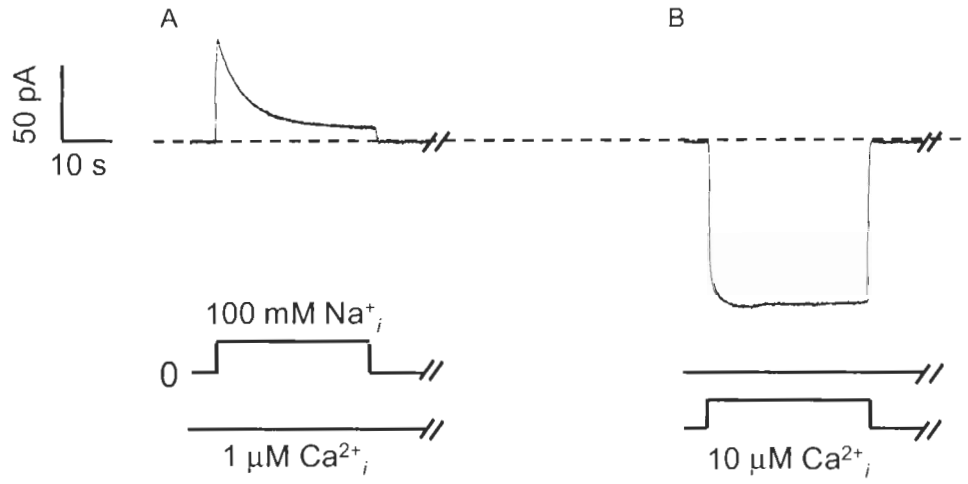

Figure 4-3 Typical NCX1.1 outward and inward currents
In a giant excised patch clamp, NCX outward current (A) is generated mainly with 1 μM Ca^{2+}_i and 100 mM Na^+_i while the inward current (B) does not require Na^+_i but high [Ca^{2+}_i] at 0 mV.

Previously, the measurement of outward currents (Figure 4-3A) were limited to one temperature per current trace and three temperatures (7, 14 and 30 °C) per patch due to its short lifespan and setup limitations on temperature control (Figure 2-3 and Figure 2-4) (2,6). Outward currents were measured to include Na^+- and Ca^{2+}-dependent regulation studies at these three temperatures (from low to high) before current decline. Energy of Activation was estimated from the slope of an Arrhenius plot using these three temperatures, in which the raw data was transformed into a logarithm curve versus 1/temperature. A new setup with an improved solenoid and additional temperature probe allowed for more accurate and faster temperature switching and measurement. With this setup it was possible to continuously monitor inward current and temperature thus

generating thousands of data points per experiment. However, with these new measures the linear correlation between temperature and activity in an Arrhenius plot was lost. This new data led to view the relative differences among constructs with an alternative formula, the Hill equation that allowed the computation of the mid-point temperature (T_{50}) on each curve from a normalized – temperature graphs and offered comparative differences among the tested constructs.

The inward currents (Figure 4-3B), at a holding potential of 0 mV, were measured every 8 msec with an average of 10,000 - 25,000 points per trace. Also a 0.35 mm temperature probe was located ~1 mm from the patch to measure the temperature as it was ramped up by ~ 8 °C / min. Raw currents (pA) versus temperature (K) data were baseline-corrected by a linear function, in which Ca^{2+}-free traces were subtracted from those generated with Ca^{2+}-containing solutions. Statistical significance was determined with one-way ANOVA using EXCEL software. To analyze the data, the Hill equation (ORIGIN software) was used.

$$I = I_{max}\left(\frac{T^h}{T_{50}{}^h + T^h}\right)$$

In which I = current (pA), T = temperature (K), T_{50} = temperature at half of the maximal current (I_{max}) in K and h = slope of current versus temperature (pA/K). Unless indicated otherwise, a $p < 0.05$ was considered significantly different.

4.2 Mutagenesis Effect on Canine NCX1.1

The deca-mutant, in which dog NCX1.1 has ten specific mutations as mentioned previously, resulted in a temperature dependence that was intermediate between the wild-type dog and trout phenotypes (Figure 4-4). The effect of temperature is often expressed in terms of the fold change in activity that results from a 10 °C change in temperature or Q_{10}. With a $Q_{10} \sim 1.7$, the deca-mutant in a dog NCX background demonstrated a temperature sensitivity that was intermediate between dog and trout NCX. From the Hill equation, in which a proportional current was plotted versus temperature, one could determine the temperature at the midpoint (assigned as T_{50}) of the maximal inward current (I_{max}) of the different NCX constructs (Figure 4-4). A T_{50} of 12.9 ± 1.4 °C exhibited by the trout NCX - TR1.0 is low in contrast to a T_{50} of 23.5 ± 0.4 °C observed in the mammalian NCX1.1. Meanwhile, the T_{50} from the mutant (99% dog NCX and 1% trout NCX in genotype) was intermediate at 18.8 ± 0.4 °C. The ten mutations in the dog NCX resulted in significant alteration in its temperature sensitivity. Considerable difference between the mutant dog NCX and the wild type trout NCX temperature dependence indicated that the modifications were not sufficient to recapitulate trout phenotype. Unfortunately, phenotype measurements on the mutant of the trout NCX construct were not obtained due to oocyte lack of viability after cRNA injections.

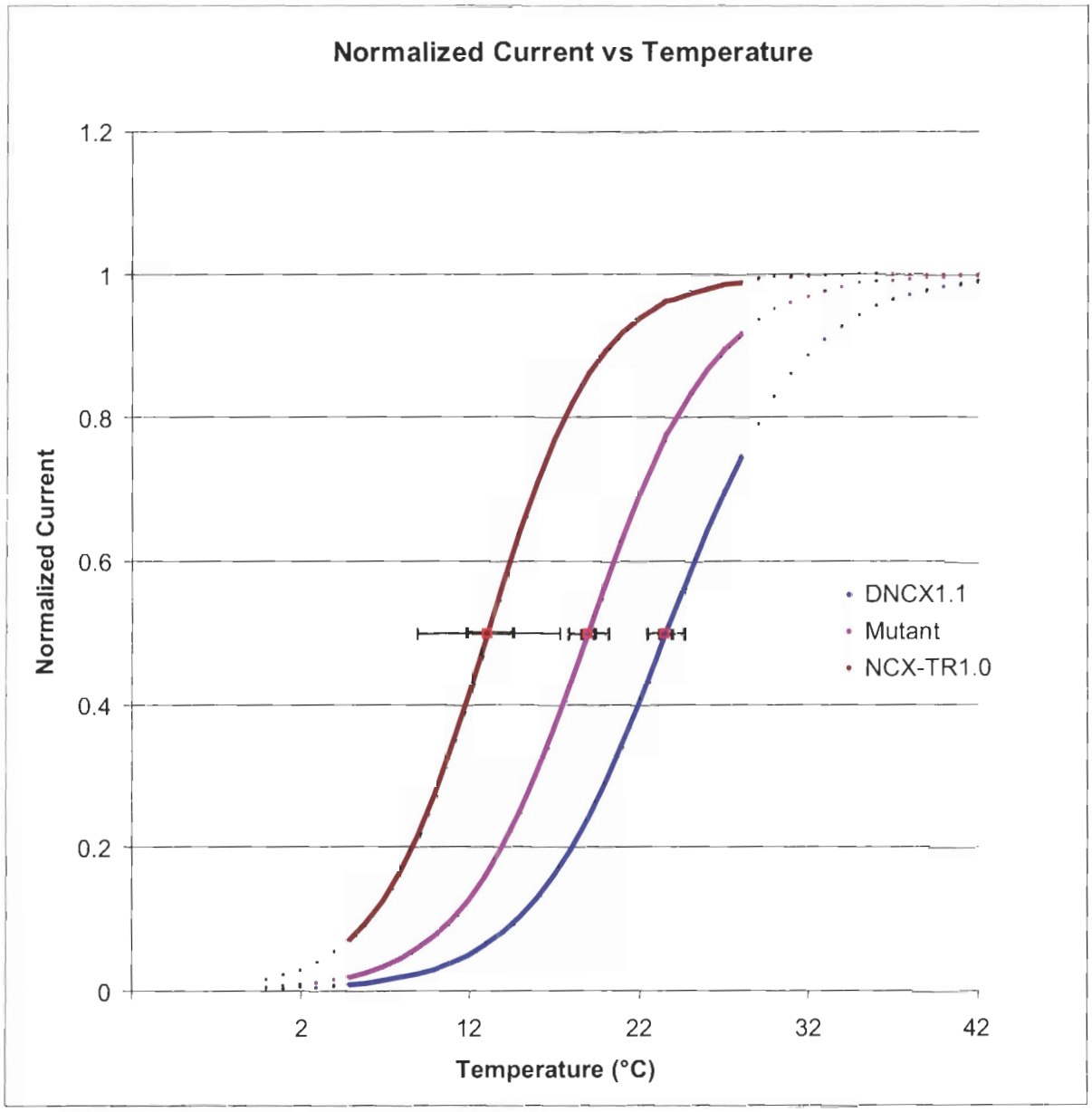

Figure 4-4 NCX normalized current versus temperature graph

Inward I_{NCX}/I_{max} versus temperature graph, in which the y-axis is the normalized NCX current and the x-axis indicate temperature (°C). Wild-type NCX1 from trout (n = 9) and dog (n = 9) are represented in brown and blue, respectively and the pink curve indicates ten amino acid mutant (n = 10). Dotted sections of the curves indicate extrapolation, whereas solid lines represent the temperature window within which data were acquired from 5 – 28 °C. Inner brackets indicate standard error and outer brackets standard deviation.

4.3 NCX N-Terminus Alignments

Alignments among available teleost NCX1 N-termini, without known NCX phenotype, from organisms with varying environmental temperature limits were also analyzed. The known high and low temperature limits are shown in the following Table 4-3.

Table 4-2 Teleost organism viability temperature limits

Teleost		Low (°C)	High (°C)	Optimal (°C)	Source
Oreochromis mossambicus	Tilapia	12	38	25-30	(7)
Tetraodon nigroviridis	Green pufferfish	24	28		a
Takifugu rubripes	Fugu	15	30	25	(8)
Oryzias latipes	Medaka	4	37		(9)
Gasterosteus aculeatus	Stickleback	8	30	25	(10)
Danio rerio	Zebrafish	23	31	27	(11)
Oncorhyncus mykiss	Rainbow trout	4	25	17	(12)

a http://www.thetropicaltank.co.uk/Fishindx/puf-nigr.htm

Based on findings (13) in zebrafish, in which the duplicate NCX1 gene expresses in the heart tissue while the other (NCX1n) was found in neuronal tissue, we examined the other available teleost NCX1 duplicates with trout, tilapia and dog sequences. Protein sequence alignment of zebrafish (NCX1h) with trout, tilapia and dog demonstrated that the zebrafish contains half of the targeted amino acids for temperature dependence identical to dog/tilapia amino acids with the other half identical to trout amino acids (Figure 4-5). In the stickleback, the number of amino acids identical to the dog/tilapia is

greater than the number of residues identical to the trout NCX, 7 vs. 3, respectively. Both medaka and fugu contain 9 identical residues to the warm-adapted NCX with either one (in fugu) or no identical residue (in medaka) to the trout sequence as seen in Figure 4-5. As for the green pufferfish all suggested temperature related amino acids were identical to the dog/tilapia residues. These observations suggest that ectothermal species exposure to a variety of environmental temperatures may not confer NCX temperature sensitivity and activity level. Therefore, a variety of species from different habitat conditions may yield a more complete understanding of NCX phenotype and body temperature relationship.

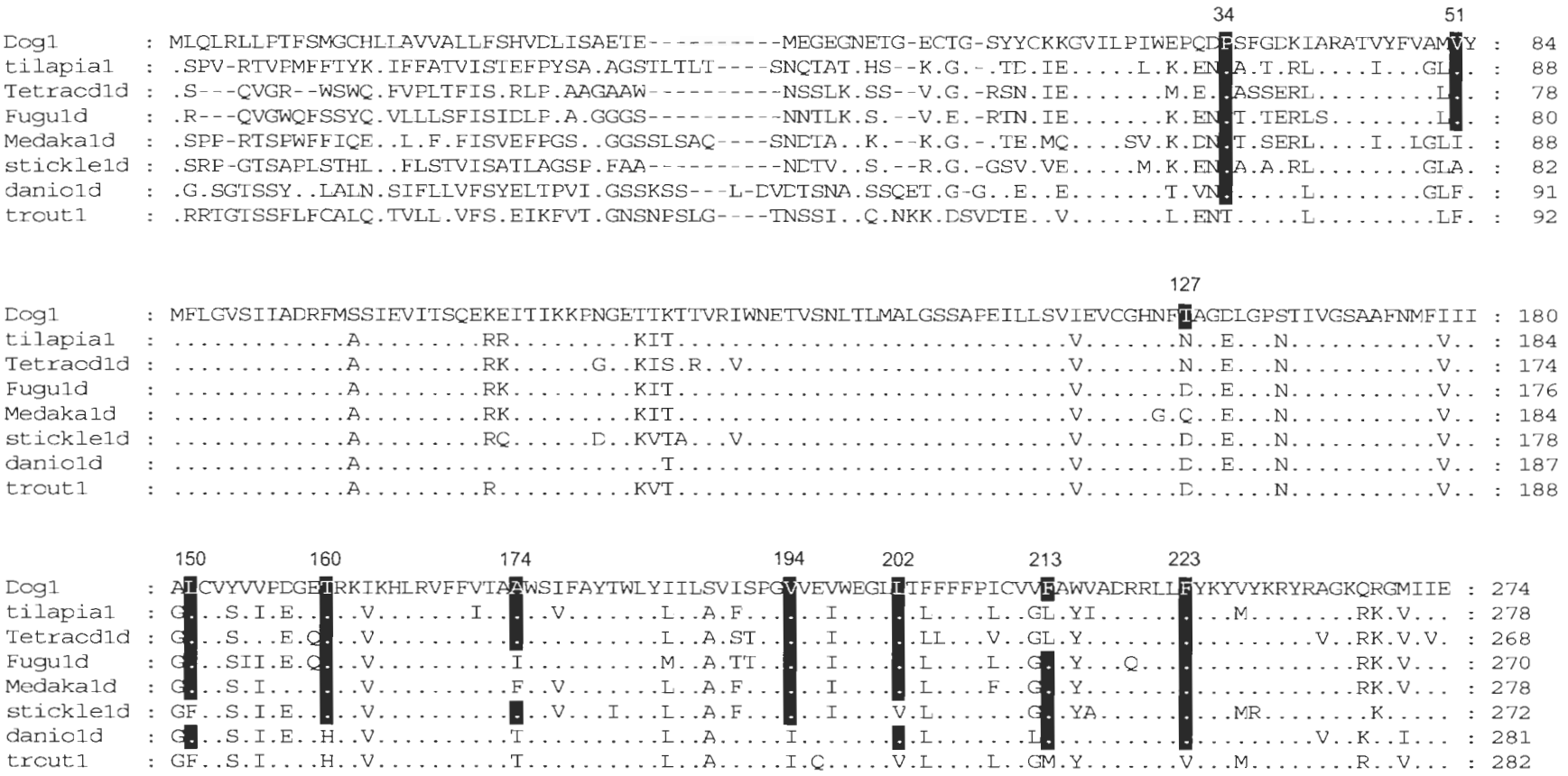

Figure 4-5 Teleost and mammalian NCX1 N-termini sequence alignment.

NCX N-termini sequence alignment in which dots indicate identity to the top sequence (canine NCX). Conserved amino acids predicted for temperature dependence are highlighted in black among species.

Recent availability of a large variety of amphibian NCX1 N-termini sequences used for amphibian diversification studies provided an opportunity to further examine ectotherm vertebrates that are more closely related to mammals. Although NCX N-termini sequences of the dog and the tilapia differ by ~ 31 residues, the amphibian sequences only differed by 10 amino acids maximum at the same sequence location (71 – 242). All available NCX1 N-termini of amphibian (caecilians, salamanders and frogs) sequences (14) were missing two sites (34 and 51) suspected of playing a role in temperature dependence. Protein sequence alignments resulted in four specific amino acids suspected of involvement in temperature dependence. The pattern within these sites includes a Q/E in position 127, I in position 150, V/I at 160 and/or I at 194 in amphibians with large variety of body temperature limits and ranges obtained from Amphibiaweb (http://amphibiaweb.org/lists/index.shtml). The restricted availability of amphibian active body temperature ranges challenges the determination of genotype-phenotype correlation with respect to temperature dependence. Even though some amphibians habitat temperatures are known (Table 4-3), it does not translate to the temperature range in which they are at an active state with full cardiac activity. Therefore, more NCX phenotypes and evidences on amphibian species active body temperature ranges are required to understand the significance of these amino acids in relation to NCX temperature sensitivity and habitat temperature among ectothermal species.

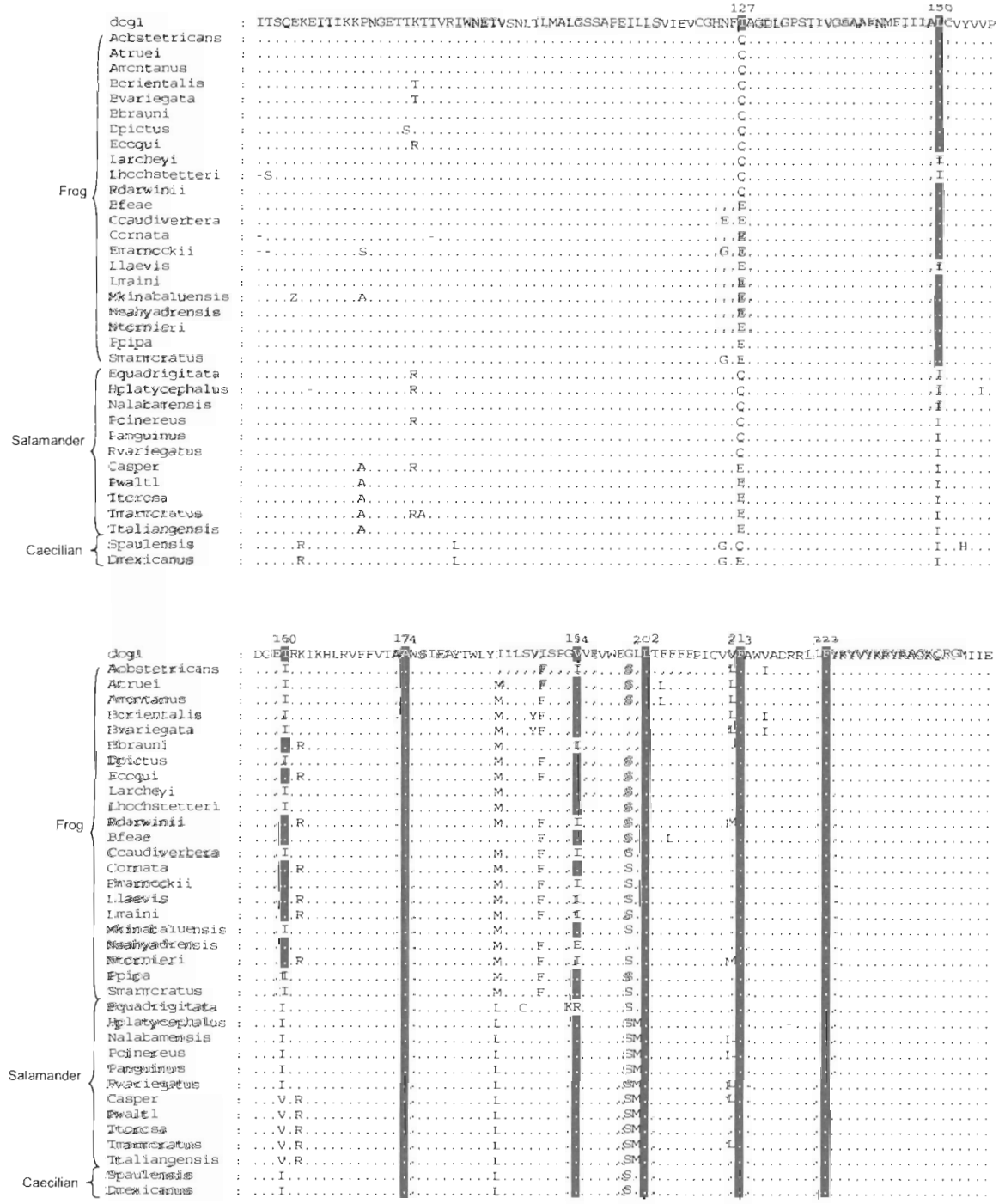

Figure 4-6 Temperature dependent residue alignment of mammalian vs. amphibians

NCX N-termini sequence alignment in which dots indicate identity to the top sequence (dog NCX). The mammalian and conserved amino acids suspected of playing a role in temperature dependence are highlighted in grey among species. For complete names of amphibian species refer to the following Table 4-3.

Table 4-3 Amphibian temperature dependent residue variation in comparison to dog NCX and body temperature ranges

Amphibians	127T	150L	160L	194V	Temp (°C)
Frog					
Alytes obstetricans	Q		I		9 - 30
Ascaphus truei	Q		I		< 16
Ascaphus montanus	Q		I		< 16
Bombina orientalis	Q		I		10 - 30
Bombina variegata	Q		I		10 - 30
Bufo brauni	Q			I	10 - 30
Discoglossus pictus	Q		I		9 - 30
Eleutherodactylus coqui	Q				N/A
Leiopelma archeyi	Q	I	I		8 - 14
Leiopelma hochstetteri	Q	I	I		8 - 14
Rhinoderma darwinii	Q			I	5 - 21
Brachytarsophrys feae	E				N/A
Caudiverbera caudiverbera	E		I	I	N/A
Ceratophrys ornata	E				N/A
Eleutherodactylus marnockii	E			I	30
Lepidobatrachus laevis	E	I		I	N/A
Litoria maini	E			I	N/A
Meristogenys kinabaluensis	E		I		N/A
Nasikabatrachus sahyadrensis	E		I	E	N/A
Nectophrynoides tornieri	E			I	N/A
Pipa pipa	E		I		N/A
Scaphiophryne marmoratus	E		I		N/A
Salamander	Q	I	I	R	19 - 22
Eurycea quadrigitata	Q	I	I		-2 - 11
Hydromantes platycephalus	Q	I	I		0 – 18
Necturus alabamensis	Q	I	I		10 - 39
Plethodon cinereus	Q	I	I		6 - 12
Proteus anguinus	Q	I	I		6 - 15
Rhyacotriton variegates	Q	I	I		18 - 28
Calotriton asper	E	I	V		13 - 17
Pleurodeles waltl	E	I	V		18 - 30
Taricha torosa	E	I	V		N/A
Triturus marmoratus	E	I	V		N/A
Tylototriton taliangensis	E	I	V		N/A
Caecilian					
Siphonops paulensis	Q	I	I		18 -28
Dermophis mexicanus	E	I	I		22 -27

4.4 Discussion

4.4.1 Comparative Physiology

The unique NCX temperature phenotypes shown to be associated with the NCX N-terminus (2), leaving the regulatory cytoplasmic loop and C-terminus as negligible segments in this phenomenon. Hence, NCX N-terminus has been the main target for deciphering the specific regions or residues that allow NCX stability in cold-adapted organisms. Our goal of determining the specific combinations of amino acids involving cold adaptation in NCX has been difficult due to the great number of permutations possible with 35 different residues between the mature dog NCX1.1 and trout NCX - TR1.0 within the conserved areas of their N-termini (Figure 4-2). The number of possible combinations of these species differencing residues would translate into unnecessary and tedious mutagenesis and patching. However, limited knowledge of NCX N-terminus tertiary structure and multiple molecular mechanisms of cold adaptation, a simpler approach was required to minimize mutational analysis.

The expressed clone of the tilapia NCX - TL1.0 (1) that has a similar temperature phenotype as the mammalian NCX (1) aided in the identification of residues that are unique to the temperature phenotype. The tilapia NCX - TL1.0, unlike the trout NCX - TR1.0, is cold sensitive. Alignment of the dog, trout and tilapia NCX protein sequences has suggested ten possible amino acids as shown in Figure 4-2 (1). These residues were the same between dog and tilapia, the warm-adapted organisms, and different from cold-adapted trout. Therefore, these ten residues were mutated in the dog NCX1.1 into amino acids found in the trout at homologous positions. Consequently, we hypothesized that the dog NCX1.1 mutant with ten residue changes would exhibit a phenotype close to that of

the trout NCX even though ~ 99% of the protein sequence was derived from dog NCX. However, the phenotype of this new NCX construct resulted in a temperature phenotype that was intermediate between that of trout and dog wild-type NCX. Despite this, the intermediate temperature dependence indicated significant involvement of the ten residues (out of 981) that were mutated in the dog NCX1.1. Such results would indicate that either: 1) residues selected plus other unknown residues may be required to be mutated to obtain a complete trout phenotype or 2) a few of the ten mutated residues could be interfering with the other significant mutations to prevent achieving cold tolerance. To test the latter hypothesis, sequential revisions back to the dog sequence could be used to identify amino acids that are not determinants of the NCX temperature sensitivity.

To understand strategies of the trout NCX-TR1.0 that maintain activity with large temperature deviations, we need to analyze the adaptation at the protein level by mutative exchanges and forces created by selected residues that determine the final protein structure and resistance to temperature changes.

4.4.2 Thermodynamics and Structure of NCX

Conservation of both the topology and functional efficiency provided stability of proteins determined by intramolecular forces during evolution under corresponding physiological conditions. Maintenance of activity at low temperatures by psychrophilic enzymes have been demonstrated to unfold at moderate temperatures due to their decreased stability (15). Moreover, these weak intramolecular forces have been suggested to originate from either global or localized flexibility (15). From our results with NCX experiments, only a few amino acids may be contributing to the stabilization

of NCX through intramolecular interactions. The elucidation of the main molecular force that contributes to the kinetic thermal stability has been shifting among hydrogen bonding, charge effects and hydrophobic interactions. Low energy hydrogen bonds are strengthened at low temperature (16) due to the lowered molecular motion and increased density. Whereas hydrophobic interactions are commonly assumed to be weakened because of their entropic origin (17), whereas hydrophobic interactions are commonly assumed to be weakened because of their entropic origin in which order in the surrounding water is induced by hydrophobic units (18). Trout maintenance of a steady Q_{10} could result from the appropriate combination of amino acids that can compensate each other to maintain activity over a large temperature range. Therefore, extremes of temperature are expected to cause divergent effects depending on the relative weight of the various intermolecular forces contributing to the stabilization of the molecule and general predictions are very difficult to make.

The predicted topological model of NCX provides an idea to where the residues, studied in this thesis, are located (i.e. : loop/TMS and intracellular/extracellular) (Figure 4-7).

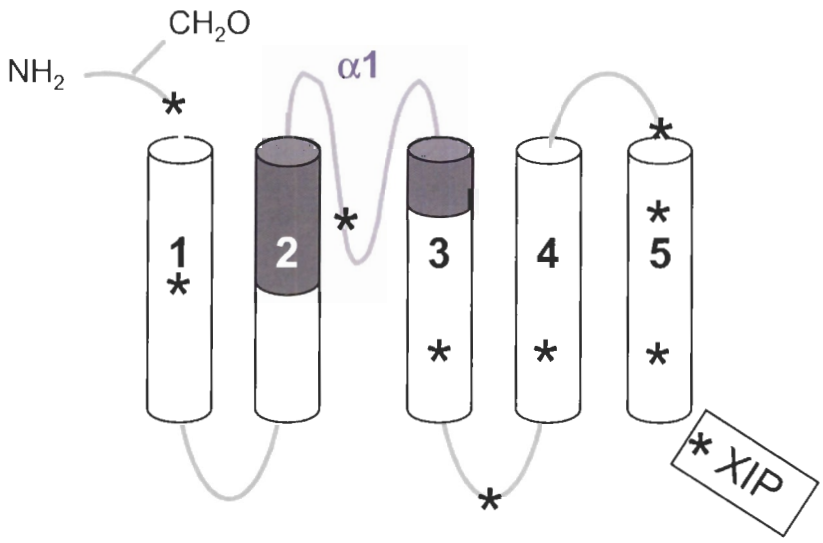

Figure 4-7 Canine NCX1 N-terminus topology and location of mutations
NCX N-terminus topology with cylinders representing TMS and lines as extra- or intra-cellular loops. Dark grey highlights the α -1 repeat and light gray is the XIP site. Black asterisks (*) indicate mutation locations significant for temperature dependence.

However, without knowledge of the tertiary structure of NCX, it is difficult to predict the possible temperature dependent intramolecular interactions. Recently suggested helix packing arrangements, involving a few TMS from both N- and C-termini of NCX, can be utilized to predict possible interactions of our suspected residues involved in NCX temperature dependence. The suggested helix packing, by Ren *et al.* (19), combines interactions of TMS6 and TMS1 with a previous helix packing model composed of TMS2, 3, 7 and 8 (20). As seen in Figure 4-8, few amino acids that we have proposed to have significant roles in temperature sensitivity interactions are located within this TMS packing model. Hence, possible interactions by these suggested residues can be better visualized with helical wheels (axial projections of a regular α-helix). With the exception of residues 34, 127, 160 and 223 that are located in the NCX loops; our suspected amino acids positioned within TMSs are seen in Figure 4-8.

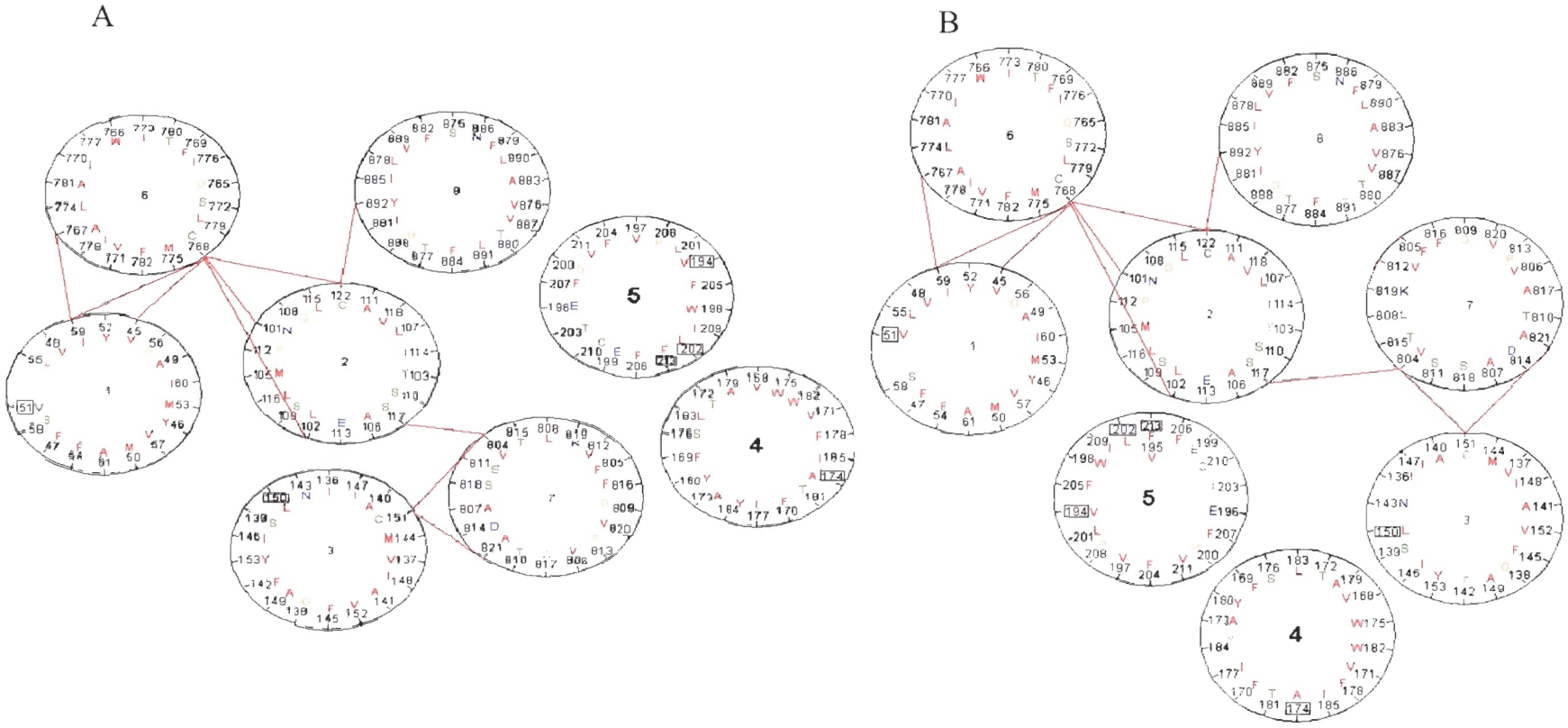

Figure 4-8 Two possible NCX packing models and interactions among TMSs 1, 2, 3, 4, 5, 6, 7, and 8.

Shown axial projection for assumed normal α-helixes of NCX are 18 residues or less due to drawing program limitations. TMSs 1, 2, 3, 6, 7, and 8 (circles) interactions (red lines) have been demonstrated experimentally (13,14) while TMSs 4 and 5 (bold) relative locations are predicted for the purpose of our studies. In figure A, TMS5 and 4 locate between TMS7 and 8 while in figure B, they are between TMS2 and 3. Numbers close to the perimeter of the helical wheels represent the amino acid sites in the dog NCX1 and the boxed numbers correspond to the residues mutated in our studies. Amino acids are colour coded according to the Benner *et al.* (21) hydrophobic scale.

Since most of our TMS-located residues reside in the two TMS not included in the NCX helical packing models, we provide possible locations for TMS4 and 5 relative to the present NCX packing model. Based on the helical packing model and TMS5 characteristic amphiphatic helix, TMS5 could be located between TMS7 and 8 or TMS2 and 3 as seen in Figure 4-8 A and B, respectively. The amphiphatic feature of TMS5 could place it as part of the pore, in which the polar half of TMS5 faces the soluble side and the opposite half, with the amino acids (shown in red), interacts with the hydrophobic side. The loop that joins TMS5 with TMS4, contains only three amino acids (one being a proline) and may be limiting TMS4 distance from TMS5 (Figure 4-9).

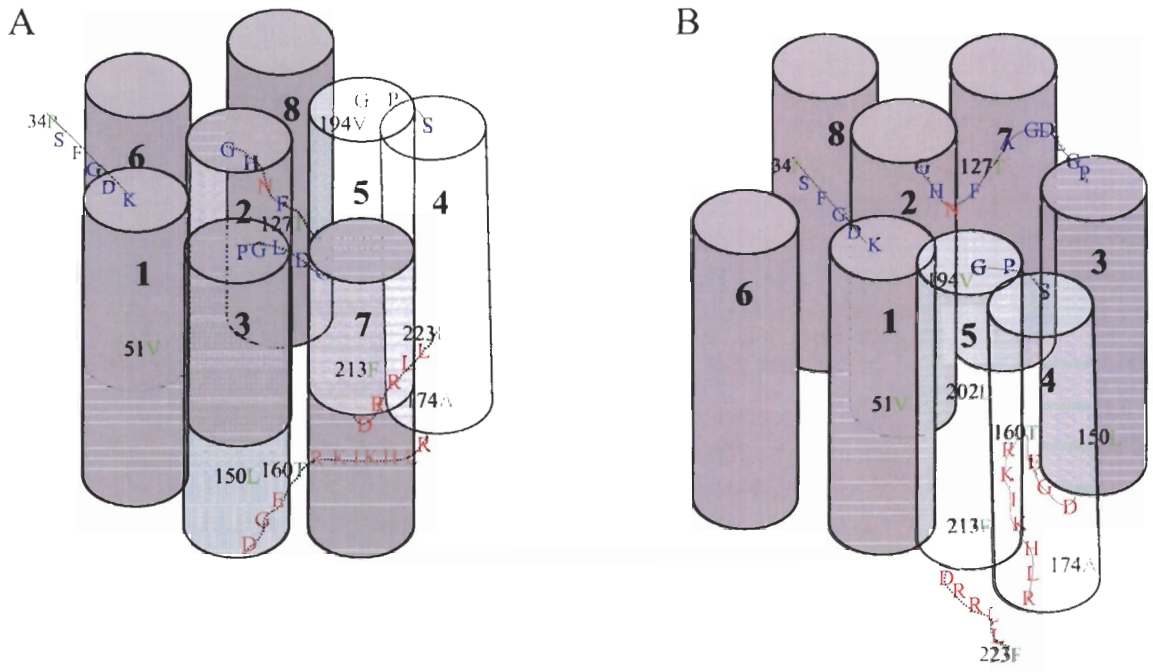

Figure 4-9 NCX helix alternative packing models side view

Different view of Figure 4-8 with the alternative positioning of TMS4 and 5. Additional loops containing target residues are shown in green, while amino acids accessible to the extracellular and intracellular sides are shown in blue and red, respectively. Figures A and B relate to A and B of Figure 4-8.

The side view of the NCX helix packing including the intra- and extracellular loops provides a clearer view of the amino acids chosen; positioning mainly on the TMSs and loops that may be part of the pore. Therefore, further studies on NCX temperature dependency may also reveal translocation mechanics.

4.4.3 Comparative Genetics and Habitat Temperatures Among Ectotherms

The fruitfly can survive over a similar range of temperature conditions (from 12 to 31 °C) (22) as to the rainbow trout but with limits 10 °C or higher, in which CalX1.1 shows a Q_{10} (~ 1.8), that is reflective of its sensitivity to low temperatures. Even though, squid (*Loligo opalescens*) NCX Q_{10} of ~ 1.2 (similar to that of rainbow trout NCX Q_{10}), its smaller temperature range and possible sensitivity to low temperatures of less than 10 °C, squid does not show habitat and Q_{10} correlation as seen with trout habitat and NCX Q_{10}. Therefore our attempt to correlate the variety of ectotherms active body temperatures with NCX temperature adaptation may show inaccuracies. Indeed, NCX N-termini alignments of frogs and salamanders with mammalian demonstrated higher identity (> 90%) than tilapia vs. mammalian (~ 84%). These amphibians, as teleosts, are certainly exposed to extreme body temperatures but only differ from mammalian NCX N-terminus by a maximum and minimum of 10 and 4 residues, respectively. Among the suspected amino acids only four differed in amphibian from mammalian sequences possibly suggesting that NCX may require less than ten amino acid changes to allow ectotherm organisms to have a normal cardiac function at high and low temperature exposure.

Although the selected residues did not confer total phenotype transformation, results demonstrated great significance in these mutations in relation to temperature

dependence. Various species temperature dependence phenotype and expansion of NCX sequences will provide additional bioinformatic and experimental data required to discover the specific residues responsible. The molecular interactions determining NCX temperature dependence are complex and multifaceted in nature.

4.5 References

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CHAPTER 5:
GENERAL OVERVIEW

5.1 Summary

How can ectotherms adapt to such a wide spectrum of habitat temperatures? The basic biochemical components, which include enzymatic and structural proteins, nucleic acids and cellular membranes, are responsive to temperature changes (among many other environmental factors). These biochemical elements require proper stability to maintain activity and sustain life when exposed to environmental challenges. It is through adaptation that molecular stability can be sustained or even enhanced to cope with environmental change. Important insights to molecular evolution and temperature-resistant selective advantages can be directed to characterize influences in protein stability. Therefore, extensive evolutionary history is required to differentiate phylogenetic divergence from temperature sensitivity trait variations. In combination with evolutionary studies, comparative physiology of closely related organisms with homologous target proteins, but with distinctive phenotypes, would provide significant inference of genetic influence on protein activity. However, such ideal conditions are rare; orthologous proteins only from distant organisms with differing physiology were accessible for our studies.

The rainbow trout NCX stability and activity through a broad temperature range prompted our interest in how the amino acid sequence and structure of this protein allow it to perform and maintain function at low temperatures. Strategies that have allowed it

to adapt to changing environmental temperatures may be as simple as a few specific mutations that can modify the structure and the functionality over the range of its physiological temperatures. Without the complete tertiary structure of NCX, as with many other membrane embedded proteins, it is hard to pinpoint the structural background of these sequence modifications; it might follow from a more precisely designed active site or a local increase in the flexibility of functionally important regions. This adaptation strategy cannot be deduced by extrapolating the amino acid changes seen between endotherm and ectotherm due to the large number of differences in which most are independent of temperature dependence.

Because of the presence of a well conserved NCX in vertebrates and invertebrates, NCX origin is not recent (> 450 mya) making its phylogeny easily correlated to animal phylogeny. Although genetic structure analysis did not reveal definitive temperature-related characteristics, it elucidated NCX gene evolutionary history; in which, protein sequence analysis alone would have not portrayed. In the determination of a genetic basis for differences in temperature resistance in proteins of high identity, a small number of amino acids responsible for adaptation traits were highlighted. Mammalian NCX exhibit minimal activity at lower temperatures relative to their orthologs adapted to colder conditions. In this study, we demonstrated that specific mutations within the N-terminus of the mammalian NCX can allow for activity to be maintained at much lower temperatures in comparison to the wild-type version. *In vitro* mutation that enhances mammalian NCX activity at low temperatures can provide an approach to molecular adaptation of temperature tolerance.

5.2 NCX Evolution

Rapid growth in the number of sequenced genomes has allowed for the constant expansion of the NCX gene library. Although most NCX expression and function has been characterized from mammalian organisms, NCX gene presence and conservation is seen among a great variety of animal sequenced genomes. Gathering NCX sequences among species adapted to a variety of body temperatures and applying these to evolutionary analyses, we hypothesized temperature dependence traits to be revealed by finding correlations in NCX gene features with species' body temperature range.

NCX presence in all species' sequenced genomes has allowed the construction of a phylogenetic tree that correlated to animal evolution and revealed that the origin of NCX duplication was initiated at the emergence of vertebrate organisms. At least four paralogs are hypothesized to have resulted and found in teleost and amphibian organisms but only three endured in mammalian genomes. Also, duplicate forms of these NCX genes have been found only in teleost genomes that correlate with the isolated genome duplication in ray-finned fish (1).

The presence of the NCX4 gene, found only in teleost and amphibian genomes, indicates that NCX duplicated at least three times after primitive chordates (i.e.: *Ciona*) and before tetrapods emergence. It has not been demonstrated unequivocally that NCX4 is expressed in these species despite the evidence of ESTs in GenBank. As a consequence, there is no information as to its function and possible selective pressure in teleosts and amphibians. As for the duplicate NCX genes in teleosts, only NCX1 duplicates have been cloned and demonstrated to be functional in cardiac tissue in zebrafish (2), trout (3) and tilapia (4). Zebrafish is the only teleost in which both NCX1

have been cloned from cardiac (NCX1h) and neural tissues (NCX1n) (2). The NCX gene redundancies in teleost may indicate subfunctionality and substitute usage of alternative splicing exons as seen in mammalian organisms that have only three NCX genes.

When NCX sequences are compared among all species, the main areas of conservation (i.e.: TMS and regulatory sites) can be observed along with large areas that are not conserved. These phylogeny studies can provide an overall evolution of the NCX gene family but the lack of data on closer relatives to the trout, in which NCX has been phenotypically characterized to differ in temperature sensitivity, temperature adaptation traits are difficult to separate from 'divergence baggage'.

5.3 NCX Temperature Adaptation

Significant insight into the molecular mechanisms of these temperature adaptation traits came with the cloning and temperature phenotyping of the tilapia tropical fish NCX. Tilapia NCX exhibits a mammalian-like (cold sensitive) phenotype, even as a member of the teleosts tilapia NCX1 protein sequence still demonstrated large differences. However, the combination of mammalian NCX1 similar phenotype and sequence alignments, tilapia NCX1 proved useful in deducing the residues that could be responsible for temperature adaptation and ignoring residues resulting from divergent evolution. Based on previous studies (5) the N-terminus of NCX1 was targeted for analysis and ten amino acids were suspected of being implicated in temperature sensitivity in NCX activity. By selecting the few amino acids within the N-terminus that match in mammals with the tilapia but at the same time differ in the trout sequence; we intended to derive the teleost specific amino acids from temperature adaptation residues and manipulate these residues to improve canine NCX1.1 temperature resistance.

Mammalian, teleost and mammalian mutated forms showed phenotypes with different temperature ranges, indicating that the selected mutations produced changes in the protein conformation rendering a significant change in temperature-dependence. Although significant modification of the mammalian NCX1 phenotype was observed, complete phenotype transformation to that of trout NCX was not achieved. The similarity of tilapia NCX - TL1.0 protein phenotype to mammalian has been attributed to genotype, but the mechanism of exchange may be divergent and thus temperature adaptation traits may have varied. Minor differences in tertiary interactions could result in different residues responsible for temperature dependence in different species. Utilizing the NCX helix packing model (6), we were able to roughly visualize our target residues spatial arrangement relative to the rest of the protein. This model involving both TMS and loop structures showed that the majority of the target residues locate in TMSs that surround the proposed NCX pore. Therefore, further sequence comparison among the species with known temperature phenotypes could yield more comprehensive changes required to achieve complete trout NCX temperature phenotype and also provide insight on NCX tertiary structure stability.

Species geographical distribution drives these organisms to adapt to temperature variation, especially those organisms in which environmental conditions determine their metabolism and activity state. Consequently, the amphibian (ectotherms) species with the closest protein sequence to mammalian NCX1 available seemed to have potential in deducing temperature dependence residues. However, lack of reports on most amphibian active body temperature ranges, makes temperature adaptation traits in NCX difficult to correlate with body temperature. Plus, amphibian cardiac NCX temperature dependence

phenotypes are also limited. The collection of NCX1 N-terminus from amphibians highlighted fewer candidates for NCX1 temperature-related phenotype. Even with such models (with high protein sequence identity and differing body temperatures) it has been difficult to elucidate specific amino acids that could confer temperature resistance seen in ectothermal NCX1.

5.4 Critique

This thesis focuses chiefly on comparisons of the ectotherm NCX1 protein with its mesophilic counterparts. Such studies are complicated by the large evolutionary distances that separate natural orthologs adapted to different temperatures. Therefore, comparative studies of evolutionarily related proteins are limited, in which a large fraction of the amino acid substitution may be neutral due to divergent evolution; while others may reflect adaptation to other environmental conditions such as high salt concentrations. Ignorance of the selective pressures under which the protein evolved further obscures attempts to identify specific adaptive mechanisms. Changing one residue may affect the energetics of all coupled residues obscuring the interpretation of site-directed mutagenesis experiments and complicating our ability to understand protein function. Therefore, previous single mutations and small chimera changes to the N-terminus of NCX1 rendered inconclusive results. The group of amino acid mutations performed in this project rendered the most straightforward results in comparison to previous attempts.

Other caveats include the differential protein expression in the *Xenopus* oocytes. In particular, the trout NCX1 typically exhibits relatively low expression levels in this heterologous expression system which results in a reduced signal-to-noise ratio and

additional difficulties in the already challenging giant excised patch technique. Also, our intentions of obtaining phenotypes of the reverse mutant, in which the ten targeted residues from dog NCX1 were inserted in homologous sites in the trout NCX1, resulted in nonviable oocytes. Whether protein expression levels affect protein stability and translocation properties is not known. Since there is no compelling evidence for NCX dimerization it is unlikely that expression level differences have significant effects ion translocation.

Although the patch clamp setup has been improved by introducing a system that can provide a greater range, measure, and control of temperature in the solution bath, previous constructs (i.e.: chimeras and other single site mutations) may have to be revisited to allow data comparison and consistent data conclusions. Resolving our data with T_{50}s, from normalized current vs. temperature plots, is useful in comparing the differences among the exchangers regarding to temperature; yet our understanding of NCX kinetics without calculating the energy of activation is limited.

5.5 Conclusion

This thesis focuses on two main points, the evolution of the NCX family among all species with available genome sequences and the amino acids responsible for temperature dependence within the cardiac NCX1. This project started with the knowledge that the NCX1 N-terminus (from the TMS domains to the XIP site) is the sole variable determinate of the unique temperature phenotypes. The residues which were selected based on alignment comparisons and phenotype experiments, were demonstrated to play a major role in temperature dependence when these selected canine amino acids were exchanged to amino acids found in the trout NCX1. It is assumed that NCX1 N-

terminus contains three groups of amino acids: 1) the first group is conserved throughout evolution to maintain core function of ion exchange and regulation; 2) the second group is not conserved due to its lack of stability and function involvement, and 3) the third group include key amino acids that may not have a direct role in NCX function, but are involved in overall NCX stability, that are modified only when perturbations, such as change in temperature, drive selection pressure to overcome stress. Using comparative and evolutionary approaches, we were able to present the latter group and demonstrate basic biochemical design and diversity in all species in terms of temperature adaptation. In addition, expansion of the NCX family will provide a useful background and guide to future NCX phenotypical data.

5.6 Future Directions

This project has provided more detailed information with regards to the specific amino acids involved in temperature dependence and has expanded the NCX evolutionary history. Despite this, further studies are required to provide more definitive results.

In conjunction with the evolutionary studies of NCX, the newly discovered NCX4 gene will be cloned (or synthesized) to analyze its functionality *in vitro*. Although its transcription has been detected through the presence of ESTs, cloning of NCX4 may be complicated if this gene exhibits low and/or tissue-specific expression. Confirming functionality of NCX4 and tissue specific expression patterns could demonstrate gene and functional conservation driven by selective pressures in teleosts and amphibians.

In terms of NCX temperature dependence, further mutations and even mutation reversals are required to determine the molecular mechanisms of the unique temperature phenotypes. Based on the most recent teleost and amphibian NCX1 alignments, one can make greater inferences as to the specific amino acids involved in NCX stability and be more specific in comparison with the information derived from with the previous sequence alignments using trout, tilapia and dog cDNA sequences.

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