**Regulation of NCX1 by palmitoylation**

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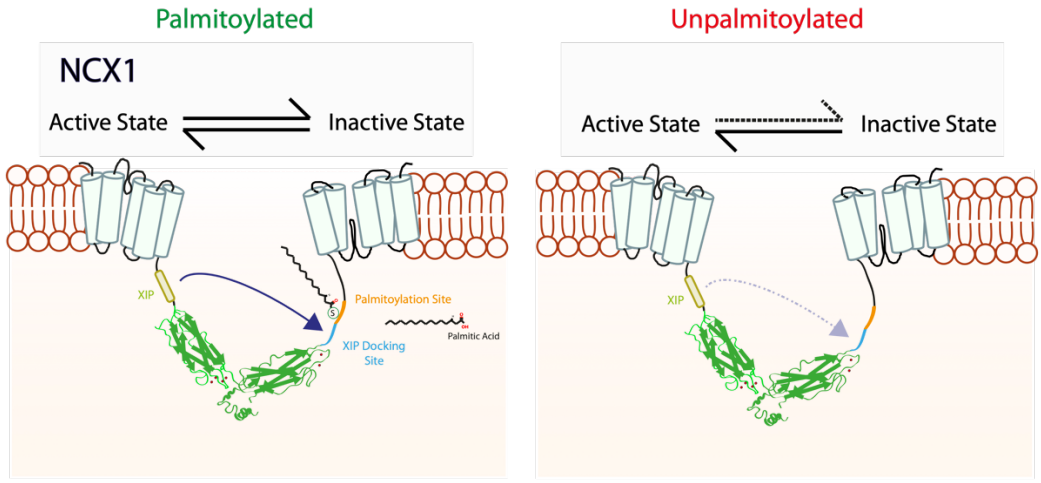
**Abstract**

Palmitoylation (S-acylation) is the reversible conjugation of a fatty acid (usually C16 palmitate) to intracellular cysteine residues of proteins via a thioester linkage. Palmitoylation anchors intracellular regions of proteins to membranes because the palmitoylated cysteine is recruited to the lipid bilayer. NCX1 is palmitoylated at a single cysteine in its large regulatory intracellular loop. The presence of an amphipathic $\alpha$-helix immediately adjacent to the NCX1 palmitoylation site is required for NCX1 palmitoylation. The NCX1 palmitoylation site is conserved through most metazoan phlya. Although palmitoylation does not regulate the normal forward or reverse ion transport modes of NCX1, NCX1 palmitoylation is required for its inactivation: sodium-dependent inactivation and inactivation by PIP2 depletion are significantly impaired for unpalmitoylatable NCX1. Here we review the role of palmitoylation in regulating NCX1 activity, and highlight future questions that must be addressed to fully understand the importance of this regulatory mechanism for sodium and calcium transport in cardiac muscle.

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## 1. Roles of NCX1 in cardiac tissue

### *1.1 NCX1 and cardiac physiology*

Cardiac diastole (and consequently ventricular filling) relies on the effective removal of Ca from the cytoplasm of ventricular myocytes to allow relaxation. This Ca removal occurs as a balanced competition governed by the activities of the intracellular Ca ATPase (SERCA 2a) and the surface membrane Na/Ca exchanger (NCX1). SERCA 2a stores Ca in the sarcoplasmic reticulum and NCX1 is the primary means to extrude Ca into the extracellular space [1]. NCX1 splice variants are widely expressed in both excitable and non-excitable tissues. NCX1.1 is the major Na/Ca exchanger in cardiac muscle [2].

In large mammals, SERCA mediates ~70% and NCX ~30% of Ca removal for a single Ca transient [3]. NCX can operate in either forward (Ca extrusion) or reverse (Ca influx) modes, depending on membrane potential, the prevailing Na gradient, and the subsarcolemmal free Ca concentration. For example at the start of the cardiac action potential (shortly after opening of voltage sensitive Na channels) when the membrane potential is positive but before substantial accumulation of intracellular Ca, NCX1 briefly operates in reverse mode and contributes to the initial Ca influx, triggering Ca release from the sarcoplasmic reticulum [4, 5]. As intracellular Ca accumulates later in the action potential, NCX1 operates in forward mode and removes intracellular Ca [6]. Balanced Ca flux is an important principal of excitation-contraction coupling: at steady state Ca entry via voltage gated Ca channels and reverse mode NCX1 at the start of the action potential must be balanced by Ca removal by forward mode NCX1 activity at the end of the action potential [7].

Beyond its role in the ventricles, NCX1.1 is also an integral part of the calcium clock, a rhythmic generator of action potentials in the sino atrial node (SAN) which relies on the functional coupling of surface-localised NCX1 to sarcoplasmic reticulum-localised ryanodine receptors (RyR) in SAN cells [8]. This coupling facilitates SAN depolarisation as Ca release following synchronised RyR openings generates an inward current through NCX1. Although the relative importance of this calcium clock and the membrane clock (generated by the funny current, predominantly via HCN4) is hotly debated, they are generally regarded as a coupled system: knockout of HCN4 induces bradycardia but does not abolish SAN activity [9], while NCX1 knockout disturbs but does not silence the node [10].

### *1.2 NCX1 and cardiac pathologies*

Contractile abnormalities that occur in left ventricular hypertrophy and heart failure can be caused by an imbalance between SERCA and NCX1. Most commonly, depletion of intracellular Ca stores (and consequent contractile dysfunction) occurs because either SERCA activity is decreased and/or relative NCX1 activity is increased [11, 12]. Whether increased NCX1 activity directly contributes to abnormal Ca handling remains uncertain. Genetic

overexpression of NCX1 does not overtly deplete the SR Ca store [13, 14], and hence does not appear to be a direct cause of contractile dysfunction in the absence of haemodynamic stress. Nevertheless excess NCX1 activity caused by genetic overexpression does impair the ability of L-type Ca channels to evoke SR Ca release, probably as a result of Ca depletion from the dyadic cleft by NCX1 [15]. Hence tight regulation of NCX1 activity in this domain is clearly key for Ca homeostasis and contractile function.

Ca removal by NCX1 in forward mode generates a net inward current. These inward currents generated by NCX1, especially when its expression / activity is promoted by cardiac pathologies, can cause arrhythmogenic delayed afterdepolarizations [11, 16-18]. Indeed, the downregulation of SERCA leading to reliance on a calcium handling mechanism that generates inward currents late in the cardiac action potential can be regarded as something of a 'perfect storm' for generation and propagation of arrhythmias. Genetically reducing NCX1 expression in the heart reduces the incidence of such arrhythmias [19]. Again, this highlights the importance of understanding NCX1 regulatory mechanisms and how they may be manipulated to minimise the contribution of NCX1 currents to arrhythmias.

In a setting of ischemia and reoxygenation of ischemic tissue, intracellular Na accumulation as a result of Na pump inhibition during ischemia [20, 21] generates a driving force for Ca entry via NCX1. Although NCX1 is inhibited by sodium accumulation during ischemia [22], it reactivates rapidly during reperfusion, when reverse mode NCX1 activity drives substantial intracellular Ca overload, leading to necrotic myocyte death [23]. Cardiac-specific deletion of NCX1 protects against ischemia-reperfusion injury [24]. The potential of NCX as a therapeutic target is therefore relevant to numerous pathologies, and it has long been recognised as a drug target in cardiovascular diseases [24-34].

**2. Protein Palmitoylation/Depalmitoylation**

Cellular proteins undergo a variety of post-translational modifications that can be either static or dynamic. These modifications can remarkably extend and modulate the function of the proteins by altering their protein and membrane interactions, stability and sorting. Palmitoylation (Figure 1) is one of these protein modifications and characterized with a reversible attachment of palmitate; 16C saturated fatty acid, onto cysteine(s) (Cys-, C) of a target protein via a thioester link [35]. This dynamic biochemical change with the attachment of palmitate tunes protein sorting by altering the recruitment of proteins to membranes via the palmitoylated cysteine(s) [36]. It is also evident that palmitoylation has a regulatory role in protein trafficking at ER - Golgi or protein cycling at endosomes/lysosomes [35-37]. The mechanistic process underlying the regulatory effect of palmitoylation on protein sorting could be explained as; (1) a simple attachment of palmitate to a target protein enables and facilitates

portioning of proteins into cholesterol rich lipid rafts [38] and (2) alters the orientation of the protein at the plasma membrane, hence affecting the protein-protein interactions [39, 40].

*2.1 Protein-Acyltransferases*

Palmitoylation of proteins is catalysed by protein-acyltransferases (PATs) which were first discovered in yeast [41, 42] and subsequently in mammals [43-48]. PATs possess a unique Cys-rich Asp-His-His-Cys (DHHC) motif, which is conserved from yeast to mammals and key for PAT activity both *in vitro* and *in vivo*. Twenty-three isoforms of zDHHC-PATs have been identified in humans [49]. Their structure usually accommodates 4 transmembrane (TM) domains with a ~50 amino acid conserved cytosolic core between TM2 and TM3 where the zDHHC-motif sits. N- and C- terminal domains of zDHHC-PATs, which are diverse, determine the substrate selectivity of zDHHC isoforms [49-51]. Furthermore, two members of zDHHC-PAT family; zDHHC17 and zDHHC13, contain ankyrin repeat (AR) domains at the N terminus. These DHHC-PATs are also known as Huntingtin interacting protein 14 (HIP14) and -like 14 (HIP14L), respectively due to the fact that they interact with and palmitoylate Huntingtin (HTT), the protein whose mutation causes Huntington Disease. HIP14/zDHHC17 knockout mice develop a range of the neuropathological, progressive behavioural and cognitive deficits characteristic of Huntington Disease. [52-55]. The ankyrin repeat domain enables zDHHC17 and zDHHC13 to interact with proteins possessing a [VIAP][VIT]XXQP consensus, including HTT, microtubule associated protein-6, SNAP23 and SNAP25 [56]. To date, various substrate/DHHC-PAT pairs have been identified to be critical in neuronal development and synaptic plasticity [57]. However, our knowledge on substrate recruitment for zDHHC-PATs in the cardiovascular system is limited to the zDHHC5/phospholemman (PLM) interaction. zDHHC5 promotes PLM-mediated inhibition of Na pump by palmitoylating PLM at 2 juxtamembrane cysteines; C40 and C42, in cardiac tissue [51].

*2.2 Depalmitoylating Enzymes*

Palmitoylation is a dynamic mechanism and reversed with the removal of palmitate by specific depalmitoylating enzymes. In contrast to the large family of palmitoylating enzymes determined, there is a small number of depalmitoylating enzymes reported including acyl-protein thioesterases (APTs; APT1, APT2) and the lysosomal palmitoyl-protein thioesterases (PPTs; PPT1, PPT2). Depalmitoylation of membrane bound proteins; HRAS, $G\alpha_i$, GAP-43., is promoted by APT1 and APT2 [58-62]. APT1 and APT2 vary in their substrate-selectivity. For instance, APT1 but not APT2 was found to depalmitoylate cysteine domains localized in S0-S1 loop of large conductance potassium (BK) channels [63]. APT1 was implicated in axon survival by facilitating membrane disassociation of axon survival factor Nicotinamide Mononucleotide Adenylyltransferase 2 (NMNAT2) [64]. As a result of functional screens to

uncover neuronal enriched microRNAs (miRNAs), miR-138 was singled out to negatively regulate dendritic spine size; however, more strikingly miR-138 targets APT1 Supporting this, APT1 knockdown experiments and pharmacological approaches inhibiting APT1 decreased the spine volume [65]. Most recently, using activity profiling, 7 additional depalmitoylating enzymes; FASN, PNPLA6, ABHD6, ABHD16A ABHD17A/B/C, which belong to serine hydrolase group have been identified [66]. Among these novel depalmitoylating enzymes, ABHD17, in particular, were reported to control palmitate turnover on post-synaptic density 95 (PSD95) [66] and diminish the abundance of palmitoylated PSD95 population in neurons, therefore, it affects synaptic function by altering synaptic clustering of PSD95 and AMPA receptors [67].

PPT1, a member of PPTs, was first purified from bovine brain as a depalmitoylating enzyme of HRAS [61]. Second member of PPTs; PPT2, shares 18% homology with PPT1 [68]. Importantly disruption of PPT1 and PPT2 is associated with severe lysosomal storage disorder, Neuronal Ceroid Lipofuscinosis (NCL) [69]. PPT1 is also detected in presynaptic compartments, suggesting a potential role for PPT1 in retaining the synaptic vesicle pool [70, 71]. On the other hand, the role of depalmitoylating enzymes in cardiovascular physiology and cardiac pathologies remains shrouded in mystery.

## 3. NCX1 Palmitoylation

Cardiac sodium/calcium exchangers; NCX1, is a key player in calcium homeostasis by mediating bidirectional $Na^{+}$-$Ca^{2+}$ exchange in an electrogenic manner. NCX1 structure consists of 10 TM domains with a large intracellular loop between TM5 and TM6 (Figure 2A). Within the TMDs there are two α repeats that sit between TM2 and TM3 and between TM7 and TM8 [72, 73]. The large intracellular loop of NCX1 is composed of an Exchanger Inhibitory Peptide (XIP) region [74] and Calcium Binding Domains (CBD; CBD1 and CBD2) [75, 76] which regulate its function. Two CBDs share a similar structure and interact with regulatory $Ca^{2+}$. CBD1 facilitates $Ca^{2+}$ dependent activation of NCX1 and coordinates four $Ca^{2+}$ while CBD2 promotes $Ca^{2+}$ dependent alleviation of $Na^{+}$ induced inactivation and harbour 2 $Ca^{2+}$ [75, 77]. The intracellular loop of NCX1 also accommodates XIP domain, which corresponds to 20 amino acids (219-238aa) and exerts an auto-inhibitory mechanism [74, 78]. Besides these insights into structure of NCX1 and its relation to the function, there are other cellular signalling pathways which modulate physiology of NCX1. Palmitoylation but no other posttranslational modifications have been found to directly regulate NCX1 activity [79, 80]. The role of phosphorylation in NCX1 function remains controversial [81-83].

### *3.1 Insights into the Mechanism of NCX1 Palmitoylation*

NCX1 contains multiple cysteine residues in its large intracellular loop but surprisingly only 1 of those, at position 739 at the C-terminal end of the loop, is palmitoylated (Figure 2B). A single mutation of this palmitoylation site from Cys- to Alanine (Ala-, A) leads to complete loss of palmitoylation of NCX1 [79, 80].

Mutagenesis scanning around this site by introducing Ala- at following positions; 735, 736, 737, 738, 740, 741, 742 and 743, are almost entirely without effect on NCX1 palmitoylation. Notably, a validated single nucleotide polymorphism (SNP) of NCX1; rs373510583 that generates mutation S738F, enhances NCX1 palmitoylation, likely by interfering with its depalmitoylation [84, 85]. Near the palmitoylation site, between 740 and 756, an α-helix structure with a large hydrophobic face and a small hydrophilic face resides (Figure 2C). This amphipathic α-helix, originally thought to form one of the NCX1 transmembrane domains, governs NCX1 palmitoylation but is not required its trafficking. Removing the helix entirely (Figure 2D) or disrupting either the hydrophobic face by adding negative charges or on the hydrophilic face by introducing Ala- abolished or impaired NCX1 palmitoylation (but not trafficking) while Ala- mutagenesis on hydrophobic face did not cause any changes in NCX1 palmitoylation [84, 85]. These data presented by Plain and colleagues clearly suggest that NCX1 palmitoylation is controlled by this element of secondary rather than a primary structure. Indeed, introduction of this short α-helix to non-palmitoylated proteins causes their palmitoylation. The hydrophilic face of this amphipathic α-helix is probably recognised by the NCX1 palmitoylating enzyme.

No other members of the Ca / cation exchanger superfamily possess a cysteine homologous to C739 in their intracellular loops, but the NCX1 palmitoylation site and the amphipathic α-helix required for NCX1 palmitoylation are conserved amongst most metazoan NCX1 orthologs (Table 1). NCX1 in bilaterians (with the possible exception of echinoderms, in which a cysteine is present within rather than adjacent to the amphipathic α-helix) is therefore likely regulated by palmitoylation. The one metazoan phylum to lack the NCX1 palmitoylated cysteine, the cnidaria, retains the amphipathic α-helix. Within this helix the histidine and lysine on the hydrophilic face (respectively in positions +6 and +13 relative to the palmitoylated cysteine, when present) are 100% conserved among NCX1 orthologs. We suggest that during metazoan evolution the palmitoylated cysteine was acquired after the amphipathic α-helix. Interestingly, NCX2 and NCX3, which have analogous palmitoylation sites to C739 in NCX1, also possess the same amphipathic helix but with a cysteine at the C terminal end as well and the N terminal end.

*3.2 Palmitoylation and NCX1 trafficking and subcellular distribution*

A fusion protein between YFP and the NCX1 intracellular loop (excluding the XIP domain but including the palmitoylation site) is localised to the Golgi apparatus in transiently transfected

cells, but adopts a cytosolic distribution if the palmitoylation site is removed [79, 80]. This strongly implicates Golgi-localised zDHHC-PATs as being responsible for palmitoylating and confining this fusion protein to the Golgi apparatus. Since the Golgi is a cellular 'hub' of palmitoylation [37, 86] containing high-activity, low-specificity zDHHC-PATs such as zDHHCs 3 and 7 [87], we do not rule out that other low-activity, high-specificity zDHHC-PATs can palmitoylate NCX1 in other cellular compartments. Importantly palmitoylation is not required for passage of NCX1 through the secretory pathway. Wild type and unpalmitoylatable NCX1 reach the cell surface equally [79], which strongly implies that palmitoylation acts as a modulator of NCX1 activity upon its arrival at the cell surface. Palmitoylation controls the affinity of cytoplasmic and single pass membrane domain proteins for lipid rafts [88, 89], but caveolae prepared for ventricular myocyte lysates contain both palmitoylated and non-palmitoylated NCX1 [79]. This suggests that palmitoylation does not sort NCX1 to cardiac caveolae but does not rule out more subtle effects on NCX1 membrane distribution.

## 4. NCX1 Inactivation

$Na^+$, $Ca^{2+}$ and $H^+$ are major allosteric regulators of NCX1 [90, 91]. Each regulator prompts a different physiological state in NCX1; (1) Increase in cytosolic $Na^+$ under conditions such as ischemia [92] and metabolic stress [93] drives NCX1 to "inactive state", a process termed $Na^+$ dependent inactivation, which limits $Ca^{2+}$ influx. (2) Binding of $Ca^{2+}$ to CBDs; with a high affinity to CBD1 ($K_d$ ~ 140-400nM) and a lower affinity to CBD2, evokes $Ca^{2+}$ dependent activation [94]. (3) $H^+$ acts as an additional regulator of NCX1 in cardiac tissue [94-97] possibly by modulating $Ca^{2+}$ affinity of CBD1 [91], but also through a direct effect on histidine residues outside the NCX1 large intracellular regulatory loop [98].

### *4.1 Mechanisms Underlying "Inactive States" of NCX1*

The typical NCX1 current observed in excised patch clamp experiments in response to application of $Na^+$ reaches the peak amplitude then decays over 10-20s to a steady-state which is 10%-15% of peak amplitude [99]. NCX1 inactivation triggered by $Na^+$ requires either an interaction with CBDs [91, 100] or XIP region [78]. Moreover $Na^+$ dependent inactivation can be manipulated by; (1) chymotrypsin application, which proteolyses the regulatory loop, (2) high cytosolic $Ca^{2+}$ (above 5μM), (3) ATP (mM concentration range). Once the inactivation decay of the NCX1 current has been initiated by $Na^+$, introduction of ATP to cytosolic side stimulates an "active state" of the exchanger. This positive regulatory effect of ATP on NCX1 current was eliminated by phosopholipase C (PLC) treatment but the presence of additional phosphatidylinositol 4,5-biphosphate (PIP2) rescued the positive regulatory effect of ATP [99]. This is a strong evidence that PIP2 positively increases NCX1 (reverse mode) activity by

abolishing NCX1 inactivation. Later, it has been also demonstrated that PIP2 binds to NCX1 at the XIP domain [101].

In order to inactivate NCX1 the XIP domain interacts with a binding site which also resides within the NCX1 intracellular loop. Mutagenesis and electrophysiological studies have identified residues 562-679aa [102, 103] as being required for XIP to inactivate NCX1. This relatively large region of the NCX1 intracellular loop includes part of CBD2: it is likely that 'minimal' XIP binding site is smaller, but it is yet to be identified, The interaction between the XIP domain and this binding site elicits the NCX1 "inactive state" as an auto-regulatory mechanism. Mutagenesis strategies targeting the XIP domain to understand the molecular nature of inactive NCX1-phenotype determined that point mutation at position 229 from Lysine (Lys-, K) to Glutamine (Gln-, Q); K229Q, leaves NCX1 insensitive to inactivation. In contrast, Phenylalanine (Phe-, F) to Glutamic acid (Glu-, E) mutation at position 223; F223E, causes NCX1 to be more sensitive to $Na^+$ depended inactivation [78]. Auto-inhibition by XIP can be mediated by changes in cellular PIP2 [99, 101, 104]. Tuning NCX1 inactivation by PIP2 relies on a physical interaction with the XIP domain [101]. A second lipid species, Acyl CoA esters, have also been identified as endogenous regulators of NCX1 inactivation [105].

*4.2 Palmitoylation and NCX1 Inactivation*

Besides the modulation of NCX1 inactivation by anionic lipid molecules, palmitoylation was found to be critical for NCX1 to achieve a complete inactivation [79, 80]. In cellular models expressing wild type and unpalmitoylatable NCX1, inactivation could be triggered by targeting either CBDs by $Ca^{2+}$ depletion (Figure 3A) or XIP by manipulating cellular PIP2 levels (Figure 3B). Complete chelation of cytosolic $Ca^{2+}$ completely inhibited NCX1 current over 4 min in wild type (WT) exchanger expressing tetracycline inducible stable cell lines, however, there was a still leftover current about 22% of amplitude of the initial NCX1 current in unpalmitoylatable (C739A) NCX1. Similar to this finding, in the absence of ATP in the cytoplasmic side PLC activation via a Ca transient whilst patch clamp recording caused 90% inhibition of WT-NCX1 current in a subsequent activation cycle while inhibition was about 30% in C739A-NCX1 current. Furthermore masking anionic PIP2 phospholipid heads using either heptalysine or EGTA/$Al^{3+}$ conjugates led remarkable inhibition of WT-NCX1 current but not in unpalmitoylatable [79, 80]. In the light of these findings, palmitoylation can be concluded as a key element for NCX1 inactivation.

The molecular events underlying Na dependent inactivation of NCX1 remain incompletely understood. This form of NCX1 inactivation requires the large intracellular loop (because it is abolished by chymotrypsin application [106] or the mutation K229Q in the XIP domain [78]), but does not directly involve Na interacting with the CBDs. Indeed, histidine residues in the linkers between transmembrane domains 2 & 3 and 3 & 4 appear to be involved in the Na-

dependent inactivation process [98, 107]. Notably, palmitoylation facilitates NCX1 inactivation whether it is induced by depletion of calcium or PIP2 [79, 80]. This finding suggests that palmitoylation facilitates the 'endpoint' of NCX1 inactivation – that is, the interaction of XIP with its binding site within the NCX1 large intracellular loop, rather than tuning the sensitivity of NCX1 to any one particular type of inactivation.

Ca regulation of NCX1 is a complex process. Occupancy of the Ca binding sites on CBD1 activates NCX1, whereas Ca binding to CBD2 relieves Na-dependent inactivation [75]. The fact that intracellular Ca activates wild type and unpalmitoylatable NCX1 with equal potency [79] suggests that palmitoylation at C739 does not influence the regulatory effect of CBD1. Indeed the NCX1 palmitoylation site is positioned such that the effect of palmitoylation will be to modify conformational flexibility between CBD2 and the sixth transmembrane domain (TMD6). By pinning a region of the CBD2-TMD6 linker to the membrane, palmitoylation likely changes the ability of CBD2 to influence the movement of this transmembrane domain, and ultimately therefore the ability of CBD2 to influence activity. This is consistent with the principal functional effect being on NCX1 inactivation. Given the extensive alternative splicing around CBD2 in NCX1 [108], it remains to be seen whether the influence of palmitoylation varies for different NCX1 splice variants. The palmitoylation site is retained in all NCX1 splice variants, but all functional work to date has focussed on the cardiac splice variant.

The extent of $Na^+$ dependent inactivation of NCX1 differs based on the cell type examined [22, 106]. Interestingly, NCX1 was also found to be palmitoylated at different levels in various cell types. Approximately 60% of total NCX1 population in rat ventricular myocytes was palmitoylated at most compare to of those in either stably- or transiently- NCX1 transfected HEK293, BHK and HeLa cells [79, 80]. Hence, these differences in NCX1 inactivation could be possibly due to the diversity of NCX1 palmitoylation in different cellular models.

*4.3 Cargo-dependent endocytosis and NCX1 palmitoylation*

Independent of its effect on NCX1 inactivation, a second phenotype is evident connected to NCX1 palmitoylation: the internalisation of membrane domains driven by either Ca overload or G protein activation is significantly accelerated in the presence of palmitoylated NCX1 [79]. This enigmatic endocytic mechanism was first described as 'massive endocytosis' in cells following Ca overload, and leads to internalisation of >50% of the surface membrane in only a few minutes [109-111]. The signalling pathways underlying this phenomenon remain incompletely understood. The release of CoA from mitochondria, the formation of palmitoyl CoA in the cytoplasm, and the activity of the surface membrane acyl transferase zDHHC5 to palmitoylate integral membrane proteins are all important events in the process [80, 112-115]. Ultimately this process is driven not by the presence of one particular palmitoylated cargo, but by an abundance of *any* palmitoylated integral membrane protein. Together the high affinity

of palmitoylated proteins for curved membranes [116], the ability of palmitoylated proteins to induce membrane curvature [117], and phase separation of the membrane ultimately leads to clustering and internalisation of proteins - particularly those possessing (or interacting with proteins possessing) bulky intracellular regions such as NCX1. When NCX1 is not palmitoylated the rate and extent of surface membrane internalisation is significantly slower [79], strongly implicating the presence of a palmitoylated 'cargo', but not the activity of this cargo in the endocytic process.

The physiological importance of these endocytic events is not entirely clear. Simply preventing the release of CoA from mitochondria increases cell size [112], suggesting a role for CoA-driven endocytosis in 'resting' membrane turnover – albeit not entirely in keeping with the enormous capacity of this pathway to remodel the surface membrane. Cellular injury induced by Ca overload is reduced when the massive endocytosis pathway is inhibited [113], suggesting the preferential loss of pumps and transporters from the surface membrane is responsible for cell death. However, whether palmitoylated NCX1 contributes significantly to membrane turnover or cellular injury in the heart is a question that can only be answered with genetic knockin models targeting the NCX1 palmitoylation site.

## 5. Future Perspectives

It is, now, evident that the palmitoylation status of NCX1 is a determinant factor for its physiology and controlled by the secondary structure. Indeed, this is only a small piece of a bigger puzzle and requires more questions to be addressed for a broad mechanistic insight into the molecular nature of NCX1 palmitoylation and its physiological consequences.

*What is more to do?*

*(1) Palmitoylation/Depalmitoylation Mechanism(s)*

Regarding the mechanism of NCX1 palmitoylation, all that we know so far is that an amphipathic α-helix near the palmitoylation site is a structural element controlling NCX1 palmitoylation. On the other hand, how NCX1 is palmitoylated and depalmitoylated, whether its palmitoylation is merely linked to its passage through the secretory pathway or occurs dynamically at the cell surface, or which enzymes catalyse palmitoylation/depalmitoylation of NCX1 remain unclear. Hence, next focus needs to be dissecting palmitoylating and depalmitoylating enzymes interacting with palmitoylation site within intracellular loop of the exchanger.

*(2) How Palmitoylation Affects Spatial Organization of NCX1*

Palmitoylation/Depalmitoylation processes tune protein trafficking: while dynamic palmitoylation modulates compartmentalization of transmembrane proteins, depalmitoylation of soluble proteins impacts their membrane release and their diffusion to cytosol. To date,

there is no clear evidence on how palmitoylation/depalmitoylation affects spatial organization of NCX1 in the cell.

*(3) Molecular Basis of Palmitoylation Dependent-Inactivation of NCX1*

Non-palmitoylated NCX1 does not inactivate when wild type NCX1 does. That could be speculated as palmitoylation may have an impact on XIP binding to its interacting domain which resides near the palmitoylation site, as it is well established that the interaction between XIP domain and its interacting region controls NCX1 inactivation. However, this requires a direct evidence on such a tuning mechanism of palmitoylation on interaction of XIP and its binding site.

*(4) Palmitoylation Status of NCX1 and Cardiac Pathologies*

Abnormal NCX1 function contributes to both contractile abnormalities and arrhythmogenesis in cardiac muscle. As palmitoylation is proved to have a remarkable impact NCX1 function by modulating its inactivation, this might contribute to the emergence of cardiac pathologies. Given the importance of NCX1 inactivation in reducing Ca overload during ischemia-reperfusion, the palmitoylation status of NCX1 may regulate cellular injury during myocardial infarction. Hence investigating the palmitoylation of NCX1 in models of contractile dysfunction and ischemia-reperfusion would be indeed helpful to understand the molecular mechanism behind the disease phenotype and tailor pharmacological strategies targeting NCX1 palmitoylation to cope with misregulation of $Ca^{2+}$ homeostasis and inappropriate contractility.

*(5) Palmitoylation of other NCX isoforms*

Significant functional differences exist between NCX1, NCX2 and NCX3 – including in properties established to be regulated by palmitoylation. All are regulated by an endogenous XIP domain but for example only NCX1 and NCX3 are susceptible to Na-dependent inactivation, and Ca binding to CBD2 therefore only alleviates Na-dependent inactivation of NCX1 and NCX3 [118]. The palmitoylation site and amphipathic $\alpha$-helix required for NCX palmitoylation are 100% identical between human NCX isoforms, meaning it is highly likely that all are regulated by palmitoylation. Indeed both NCX2 and NCX3 have cysteines at both ends of the amphipathic $\alpha$-helix, which may mean that they can be doubly rather than singly palmitoylated. If a single palmitoylation event reduces the conformational flexibility of the CBD2-TMD6 linker of NCX1 it is likely that double palmitoylation of the same region of NCX2 and NCX3 would induce even greater conformational inflexibility – effectively pinning both ends of the amphipathic $\alpha$-helix to the membrane. Since the effect of a single palmitoylation event is to accelerate NCX1 inactivation by XIP, we speculate that double palmitoylation in

the same region may have more profound effects on NCX behaviour. Any influence on the tilt and/or mobility of TMD6, for example, is likely to directly modify NCX activity, since this transmembrane domain is proposed to move substantially during the transitions between inward and outward facing conformations of NCX [72, 119].

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| Phylum | Common Name | Species | NCBI/Uniprot ID | Sequence |
|---|---|---|---|---|
| Ecdysozoa: Arthropoda | | | | |
| | Fruit fly | D. melanogaster | NP_001247215.1 | VPSCFSYVSHFVCLFWKVLFAF |
| | Mosquito | C. tarsalis | JAV31432.1 | SPSCADYIMHFLTLFWKIIFAF |
| Ecdysozoa: Nematoda | | | | |
| | Roundworm | C. elegans | NP_504415.2 | EPGCMDYVMHVLTVPWKLTFAT |
| | Hookworm | A. ceylanicum | ANCCEY_06143 | PPTCCDYIMHFMTMPWKLLFAT |
| Lophotrochozoa: Annelida | | | | |
| | Worm | C. teleta | [120] | LPSCMDYVMHFLTLFWKVLFAF |
| Lophotrochozoa: Mollusca | | | | |
| | Squid | D. opalescens | AAB52920.1 | LPSCMDYIMHFVCLFWKVLFAF |
| | Owl Limpet | L. gigantea | [120] | LPSCLDYVMHFLTLFWKLLFAF |
| Lophotrochozoa: Platyhelminthes | | | | |
| | Fluke | C. sinensis | G7YEK8 | LPSCMDYVMHFLTVFWKVLFAF |
| | Flatworm | S. mediterranea | A0A0H3YIW4 | MPSCTQYIFHYLSLFWKILFAF |
| Deuterostomia: Chordata | | | | |
| | Human | H. sapiens | NP_001106271.1 | LPSCFDYVMHFLTVFWKVLFAF |
| | Mouse | M. musculus | NP_001106269.1 | LPSCFDYVMHFLTVFWKVLFAF |
| | Chicken | G. gallus | NP_001072941.1 | LPSCFDYVMHFLTVFWKVLFAF |
| | Zebrafish | D. rerio | NP_001032179.1 | MPSCFDYVMHFLTVFWKVLFAF |
| | Pike-Perch | S. lucioperca | XP_031171840.1 | LPSCFDYVMHFLTVFWKVMFAF |
| | Lizard | P. muralis | XP_028580782.1 | LPSCFDYVMHFLTVFWKVLFAF |
| | Clawed frog | X. laevis | XP_018120632.1 | LPSCFDYVMHFLTVFWKVFFAF |
| Deuterostomia: Echinodermata | | | | |
| | Sea Urchin | S. purpuratus | W4XTQ8 | PPSKMDCIMHFITFFWKVIFAM |
| Radiata: Cnidaria | | | | |
| | Anenome | E. pallida | XP_020915137.1 | PPTYGDYMMHFLTVFWKILFAF |
| | Coral | S. pistillata | XP_022792788.1 | TPTYGDYMMHYLTVFWKLLFAI |

**Table 1: Conservation of the NCX1 palmitoylation site in metazoans.** The palmitoylated cysteine (highlighted in red) in NCX1 is present in all vertebrates and most invertebrates. The amphipathic $\alpha$-helix required for NCX1 palmitoylation is universally conserved (underlined: the amino acids comprising the hydrophilic face of this helix), with the possible exception of *D. melanogaster*, in which a serine is located on the hydrophobic face of the helix. Alignments show the NCX isoforms designated as NCX1 for each species with the exception of *C. elegans*. *C, teleta* and *L. gigantea* NCX1 sequences obtained from [120].

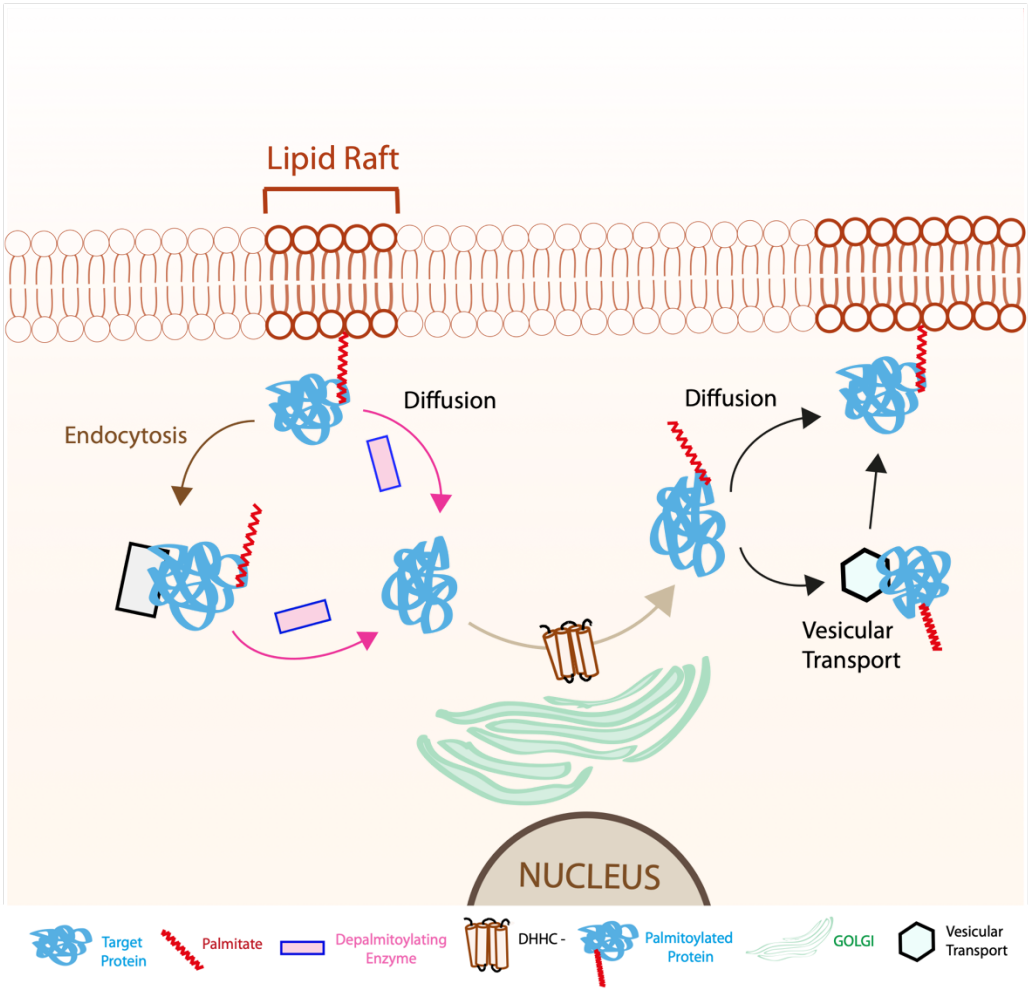


FIGURE 1

**Figure 1: Palmitoylation regulates cellular trafficking of proteins.** Palmitoylation of a Cys- provides a mechanism for continuous shuttling of target proteins between the plasma membrane and Golgi through palmitoylation/depalmitoylation cycles. Trafficking route depends on which palmitoylating/depalmitoylating enzyme interacts with the target protein.

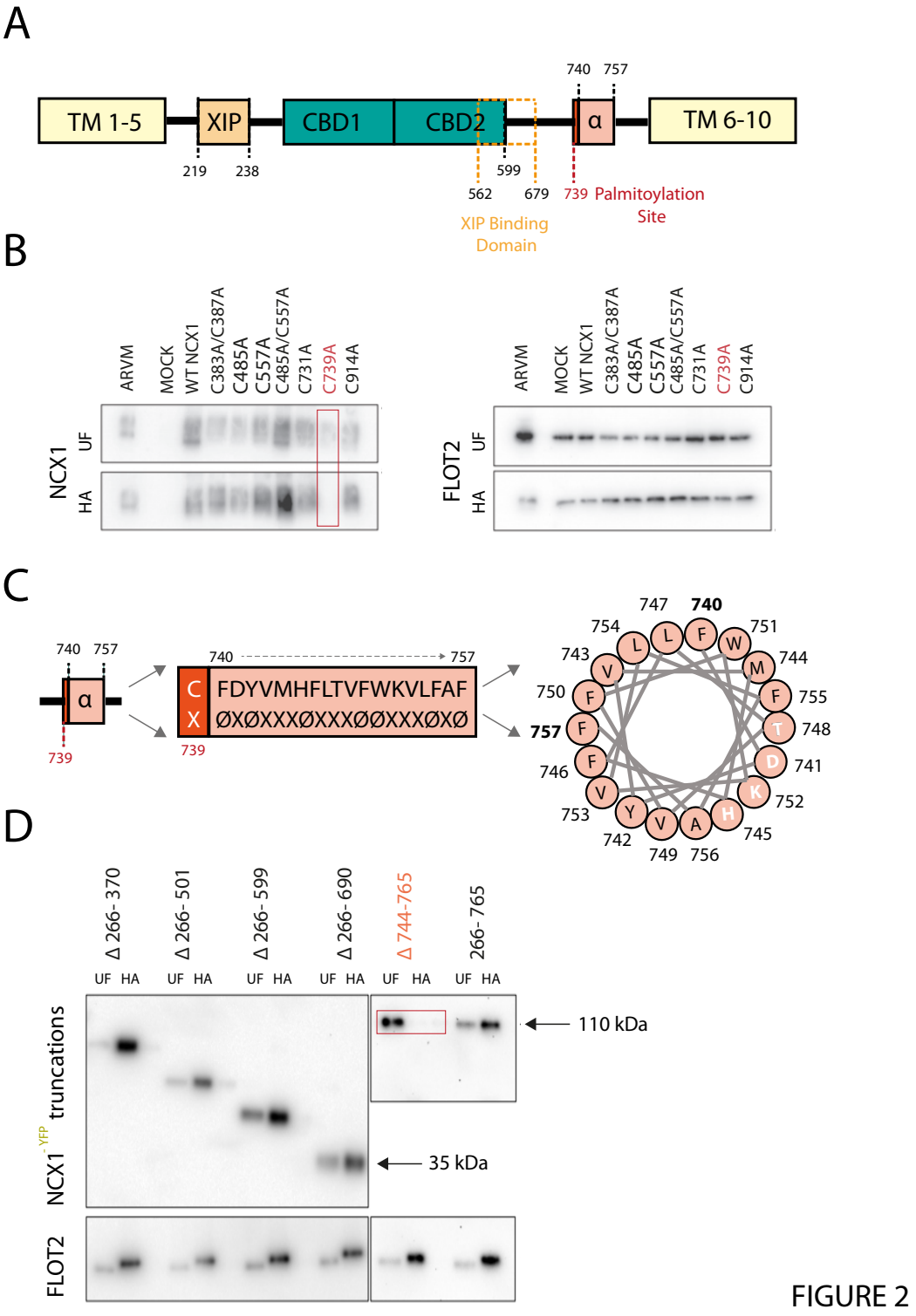


FIGURE 2

**Figure 2: NCX1 structure and palmitoylation site within large intracellular loop. (A)** Schematic of the exchanger structure shows the position of transmembrane domains and components of intracellular loop. The intracellular loop harbours XIP and its interacting domain, CBDs and palmitoylation site. **(B)** Mutagenesis screen on cysteines to search for palmitoylation site was followed by purification of palmitoylated NCX1 by resin-assisted

capture (adapted from [79], UF: unfractionated lysate, HA: purified palmitoylated proteins). NCX1 is palmitoylated at a single Cys- at position 739 and a mutation from Cys- to Ala- at this position leads complete loss of palmitoylation. **(C)** Schematic illustration provides a closer look at the NCX1 palmitoylation site. Palmitoylation of NCX1 at position 739 is controlled by an amphipathic α-helix residing between residues 740 and 756. **(D)** Deletion of the α-helix abolishes palmitoylation of the NCX1 intracellular loop (adapted from [85], UF: unfractionated lysate, HA: purified palmitoylated proteins).

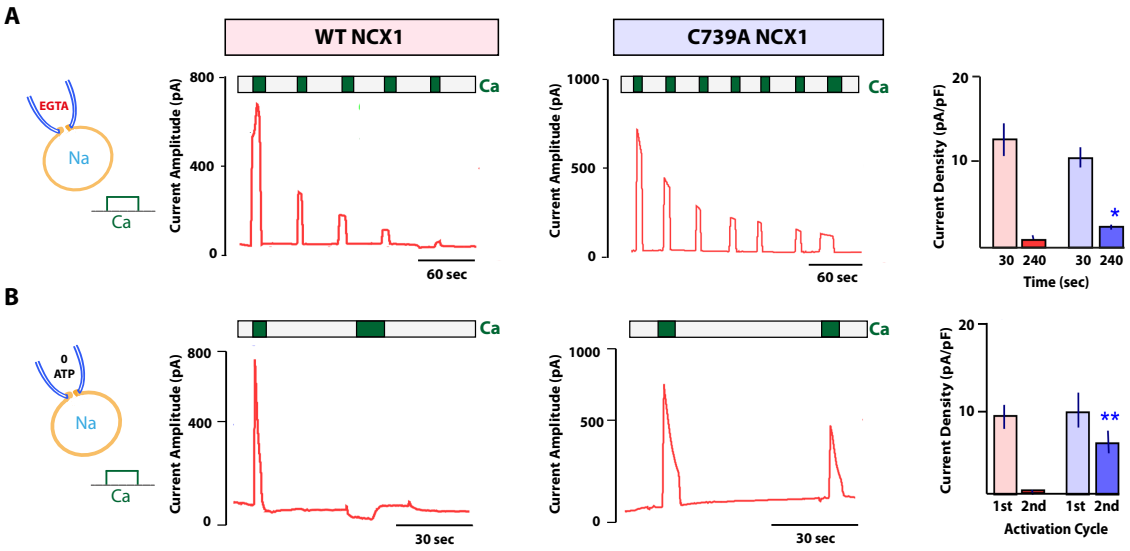


FIGURE 3

**Figure 3: Palmitoylation regulates NCX1 inactivation in voltage clamped engineered cell lines. (A)** Depletion of intracellular Ca2+ inactivates NCX1, however when it is unpalmitoylatable (C739), NCX1 inactivation is considerably slower. **(B)** In the absence of intracellular ATP NCX1-mediated Ca influx depletes PIP2 and promotes NCX1 inactivation. Unpalmitoylatable NCX1 (C739) remains activate (evidenced by substantial NCX1 mediated current upon application of extracellular Ca), while wild type NCX1 was completely inactivated by the same protocol (adapted from [79]).