Review

Rise of palmitoylation: A new trick to tune NCX1 activity

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ABSTRACT

The cardiac Na+/Ca2+ Exchanger (NCX1) controls transmembrane calcium flux in numerous tissues. The only reversible post-translational modification established to regulate NCX1 is palmitoylation, which alters the ability of the exchanger to inactivate. Palmitoylation creates a binding site for the endogenous XIP domain, a region of the NCX1 intracellular loop established to inactivate NCX1. The binding site created by NCX1 palmitoylation sensitizes the transporter to XIP. Herein we summarize our recent knowledge on NCX1 palmitoylation and its association with cardiac pathologies, and discuss these findings in the light of the recent cryo-EM structures of human NCX1.

1. Introduction

The Na$^+$/Ca^{2+} Exchanger (NCX) lies at *"the heart"* of Ca^{2+} homeostasis in many excitable and non-excitable cell types including cardiac, neuronal and kidney cells [1]. The NCX family is composed of three isoforms; NCX1, NCX2 and NCX3, encoded by distinct genes. The expression profile of these isoforms through the body varies: NCX1 is abundant in heart, kidney and brain, while NCX2 and NCX3 are predominantly expressed in brain and skeletal muscle [2].

Particularly well characterized in the heart, NCX1 broadly contributes to cardiac excitation-contraction coupling (ECC) through its ability to mediate bidirectional electrogenic Na+/Ca2+ counter-transport (Fig. 1). In healthy hearts, NCX1 is the main door through which Ca2+ leaves cells to balance intracellular Ca2+ ([Ca2+]i) levels meaning that it directly controls relaxation and consequently ventricular filling during diastole. NCX1 and Sarcoendoplasmic Reticulum Calcium ATPase (SERCA) work together to facilitate Ca2+ removal from the cytosol during ECC. NCX1 extrudes of ~28% of [Ca2+]i while SERCA removes ~70% of [Ca2+]i in rabbit ventricular myocytes, whose balances of Ca2+ fluxes closely mimic human [3]. This interplay between NCX1 and SERCA defines the size of the sarcoplasmic reticulum Ca2+ store, which controls the magnitude of the force generated by the heart muscle during the cardiac contraction, thus NCX1 indirectly controls systole. Elevated NCX1 activity is implicated in various cardiac pathologies [4]. Given the vital role of NCX1 to maintain a "healthy heart", an in-depth understanding of (1) NCX1 structure and its link to physiological characteristics of the exchanger and (2) the regulatory mechanism(s) of NCX1 function is important to devise target-specific

pharmacological strategies to tackle cardiac pathologies.

2. Understanding protein palmitoylation

Palmitoylation is a type of post-translational modification characterized by the reversible conjugation of palmitic-acid to target proteins via a thioester-bond. The change in protein structure induced by palmitoylation alters many aspects of protein function including cellular trafficking, sorting, membrane association, stability, and activity [5,6]. Palmitoylation is an enzymatically regulated process [4,6]: catalysed by protein-acyltransferases (PATs); which possess a unique Cys-rich Asp-His-His-Cys (DHHC) motif, and reversed with the removal of palmitate by specific depalmitoylating enzymes including acyl-protein thioesterases (APTs; APT1, APT2) and the lysosomal palmitoyl-protein thioesterases (PPTs; PPT1, PPT2). Proteomics analysis indicates that 10–20% of the proteome is palmitoylated [7–9] and that palmitoylation is estimated to be the fifth most abundant PTM in the proteome [10]. Recent cardiac-palmitoylome data reported 454 proteins palmitoylated in the heart [11]. Strikingly, the majority of cardiac proteins involved in excitation-contraction coupling and calcium cycling (and consequently control of cardiac output) are palmitoylated (Fig. 1: Nav1.5 [12], Cav1.2 [13,14], NCX1 [15], Kv1.5 [16], Phospholamban (PLB) [17]). Additionally, many Ca2+ transport proteins (i.e. TRPM7 [18], TRPM8 [19], ORAI1 [20,21], STIM1 [22], SERCA1 [23], IP3R [24], AMPA Receptor Subunits; GluR1, GluR2, GluR3, GluR4 [25], and more) across various tissue types which are pivotal for a wide range of physiological functions are palmitoylated [26].

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2.1. NCX1 palmitoylation: "Endogenous regulator of the exchanger activity"

Palmitoylation is the only protein post-translational modification that serves as an endogenous modulator of NCX1 activity. While NCX2 and NCX3 are dually palmitoylated [27], NCX1 accommodates one palmitoylatable Cys- at position 739 in its intracellular loop. This differential palmitoylation of the different NCX family members may partially explain the functional differences between them [28]. An amphipathic α-helix situated at 740-756aa (Fig. 2A) in the NCX1 regulatory loop controls the palmitoylation status of the exchanger [29]. Mutating Cys- at 739 to Ala- completely abolishes NCX1 palmitoylation; which leads to improper NCX1 inactivation [15]. *What does improper NCX1 inactivation mean?* NCX1 exhibits a distinctive biophysical behaviour characterized by current decay; called inactivation, following the peak current. Disruption of NCX1 palmitoylation disrupts this characteristic inactivation of the exchanger current [15]. When NCX1 palmitoylation is altered, the transition between activation and inactivation is disturbed, changing steady-state intracellular Ca^{2+} [30]. This finding opens the door for a further question: *How could palmitoylation determine the shift between "active" and "inactive" states of the exchanger?* NCX1 exists in dimeric and monomeric forms. Based on that, our initial thought was that palmitoylation could control NCX1 dimerization because we could measure palmitoylation-dependent changes in intermolecular FRET between NCX1 dimers [30]. However, it turned out that the palmitoylation of the exchanger initiates local conformational changes within the intracellular loop of the exchanger that control inactivation. Inactivation itself has long been known to rely on a polybasic region of the exchanger's large intracellular loop, called the XIP domain (Exchanger Inhibitory Peptide). We identified the site where XIP binds to inactivate NCX1 (amino acids 709–728): it is very close to the palmitoylation site, meaning that palmitoylation likely restructures this region of the protein to control XIP binding and NCX1 inactivation [30].

2.2. Cracking the code: molecular basis of NCX1 palmitoylation

The fact that cardiac pathologies are remarkably associated with imbalanced Ca2+ in the heart as a result of impaired NCX1 activity means that tuning NCX1 palmitoylation, therefore its inactivation, would be a promising approach to tackle improper Ca^{2+} handling in cardiac tissue. This requires a detailed understanding of the molecular identity of palmitoylation/depalmitoylation machinery of NCX1. It is known that palmitoylation of NCX1 is governed by an amphipathic helix (Fig. 2A) [29]. We have recently reported that this helix directly interacts with many members of zDHHC-PAT family; a group of enzymes catalysing palmitoylation [27] and APT1; the depalmitoylating enzyme of NCX1 [30]. Full length NCX1 with transmembrane (TM) domains localizes on the plasma membrane, however an NCX1 truncation containing the regulatory intracellular loop and the palmitoylation site is detected only in certain puncta in the cytoplasm, which enables the tracking of the palmitoylated NCX1 population within the cell. In short, following its palmitoylation, NCX1 (truncation) gets anchored to Golgi or ER membrane [27]. In a separate set of experiments, by Retention Using Selective Hook (RUSH) strategy, we found NCX1 was palmitoylated when it was "caged" in the Golgi or ER, supporting our findings that NCX1 interacts with Golgi, ER and Golgi/ER resident acyl-transferases. Taken all together, NCX1 is clearly a substrate for multiple zDHHC-PATs, and NCX1 palmitoylation appears to determine its subcellular localization (Fig. 2B). It is, thus, conceivable that the cellular palmitoylation machinery operates and navigates the shuttle between the plasma membrane and intracellular palmitoylating compartments; which could be the case for many other transmembrane proteins.
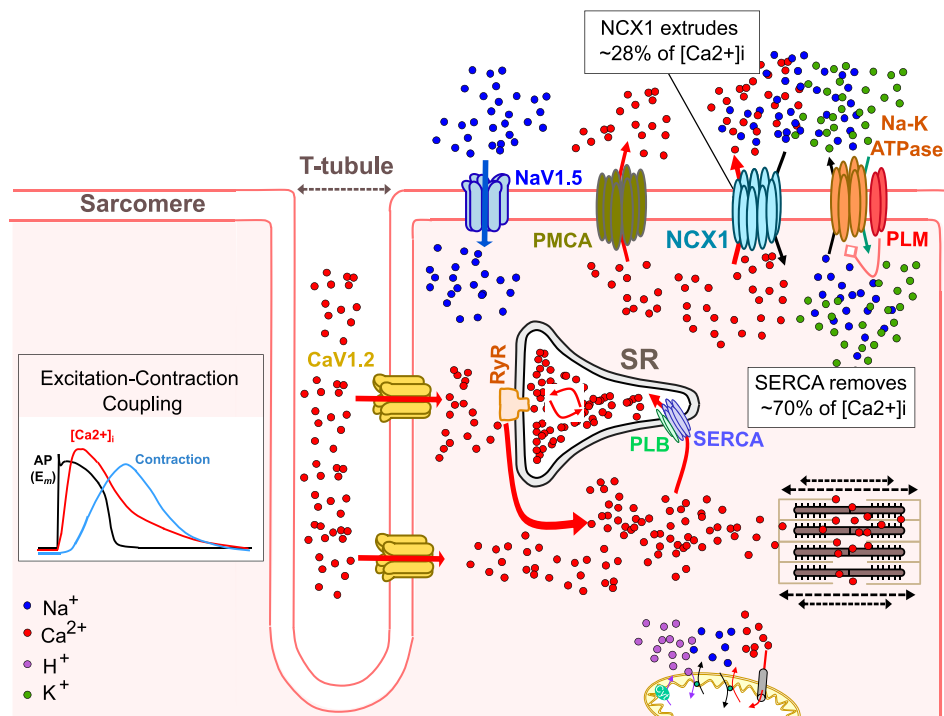
Fig. 1. Zooming into the cardiac Excitation-Contraction Coupling (ECC); The membrane depolarization; which is prompted by Na+ (blue circles) entry via NaV1.5 channels as the action potential (AP) is initiated, triggers Ca2+ (red circles) entry via CaV1.2; L-type Ca2+ channels, located at the T-tubules. This, then, activates Ca2+ induced Ca2+ release by Ryanodine (RyR) receptor resident on Sarcoplasmic Reticulum (SR) in a close proximity to CaV1.2 that elevates [Ca2+]i. Following, Ca2+ ions bind to myofilaments which inititates the contraction. At this stage, returning [Ca2+]i to baseline is promoted by Ca2+ removal from cytoplasm via both SERCA (~70%) and NCX1 (28%) that is required for muscle relaxation. The major players of the cardiac ECC are palmitoylated (i.e. Nav1.5; blue [12], Cav1.2; yellow [13,14], NCX; light blue [15]).
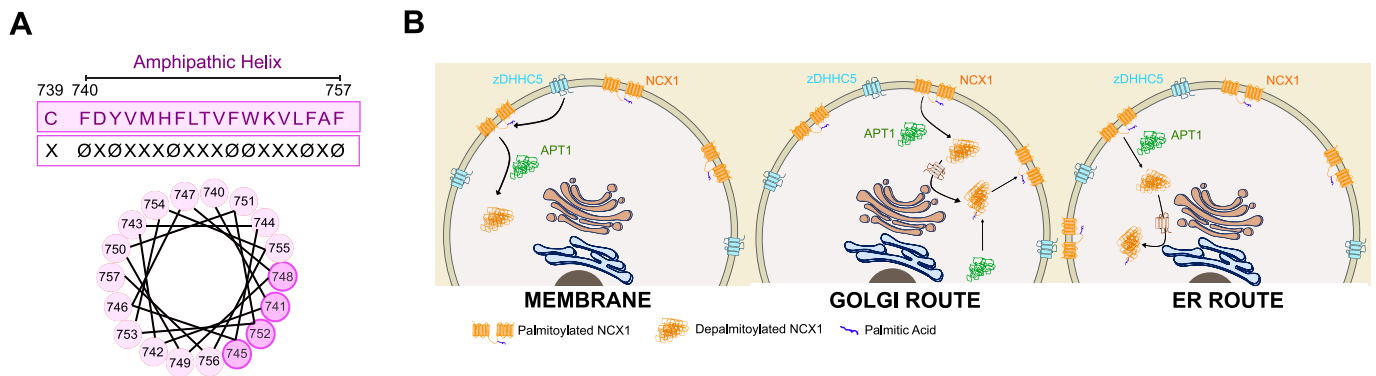
Fig. 2. Subcellular localization of NCX1 is ultimately controlled by the molecular identity of palmitoylation machinery. (A) Close look up at NCX1 palmitoylation site; an amphipathic helix structure (hydrophilic face formed by D741, H745, T748 and K752) controls NCX1 palmitoylation/depalmitoylation by interacting with palmitoylating and depalmitoylating enzymes of the exchanger. (B) Palmitoylation of the exchanger is catalysed by multiple zDHHC-PATs. Shift between palmitoylation and depalmitoylation of NCX1 governs its intracellular compartmentalization. NCX1 (orange) is palmitoylated at the plasma membrane (left), Golgi (middle) and ER (right). zDHHC5 (blue); plasma membrane localized zDHHC-PAT, palmitoylates NCX1. Moreover, APT1 (green) depalmitoylates NCX1 in the plasma membrane and Golgi but not in the ER [27].

2.3. Relevance of NCX1 palmitoylation to cardio-metabolic complications

Heart Failure (HF) is a globally concerning condition with a creeping increase in prevalence. Twenty six million individuals worldwide were living with HF in 2017 [31]. HF is the final stage in cardiac pathologies in which the heart can no longer match cardiac output to systemic demands, and can develop with either reduced ejection fraction (EF); HFrEF, or preserved ejection fraction; HFpEF [32]. The common observation in the failing heart is abnormal Ca^{2+} handling [33]. Typically, the balance between the amount of Ca^{2+} stored in the sarcoplasmic reticulum (SR) by SERCA and the amount of Ca^{2+} extruded across the sarcolemma by NCX1 is shifted. Not only does this impair Ca2+ handling and contractile function, but the enhanced inward currents generated by enhanced NCX1 removal of Ca2+ at the end of systole can be profoundly arrhythmogenic. Pogwizd and colleagues reported inappropriate NCX1 activity; elevated by 60%, in an arrhythmogenic rabbit HF model [34]. In a canine model of heart failure, NCX1 function is profoundly different for reasons that cannot be accounted for simply by changes in expression [35]. Most recently we showed that proper NCX1 inactivation is essential for balanced cytoplasmic Ca^{2+} [30]. Increased NCX1 activity comes with life-threatening consequences such as contractile dysfunction and arrhythmogenesis. Despite the ubiquitous role of NCX1 in heart, the underlying mechanism behind the inappropriate NCX1 activity in cardiac pathologies has not yet been fully understood.

Palmitoylation is the only post-translational modification identified as a modulatory mechanism of exchanger inactivation [15,30]. This covalent and reversible control of NCX1 inactivation serves as life-saving mechanism protecting cells from fatal Ca^{2+} overload. With this being said, Main and colleagues reported that palmitoylation level of the exchanger is altered in cardiac pathology in animal models and human: decreased in pig and rabbit model of Myocardial Infarction (MI)-induced HF, but increased in the patients with end-stage ischemic HF [36]. Elevated NCX1 palmitoylation, as observed in the patients with HF, would diminish Ca2+ removal from the cell due to the enhanced NCX1 inactivation which in turn would lead to diastolic dysfunction.

Cardiac function is significantly affected by metabolic factors such as insulin and glucose levels [37]. Insulin in particular is a well-established modulator of a broad range of cellular signalling pathways including palmitoylation [38]. We, most recently, identified a novel insulin dependent mechanism which modifies NCX1 palmitoylation and therefore its activity. Simply put, insulin acts on NCX1 palmitoylation in a three step process: (1) insulin activates fatty Acyl (FA)-CoA synthesis resulting in elevated free FA-CoA levels, (2) this, then, favours FA

loading to zDHHC5 at its catalytic site (C134, between TM2 and TM3 in this integral membrane enzyme), and (3) feeding zDHHC5 with more FA-CoA enhances increases NCX1 palmitoylation [39]. Considering that zDHHC5 possesses numerous substrates, including cardiac proteins with established roles in cardiac function, a paradigm in which insulin controls protein palmitoylation via zDHHC5 has important implications for cardiac physiology. The pathology of insulin resistance, a hallmark of metabolic disease, may be associated with aberrant protein palmitoylation.

3. Insights on NCX1 structure and how it relates to its function

3.1. What did we know about the exchanger structure and function?

Earlier studies leveraging electrophysiological and biochemical approaches to understand NCX1 structure and physiology have defined NCX1 architecture as a 10 transmembrane domains (TMs) protein with a large intracellular loop which harbours functionally key structural components; Exchanger Inhibitory Peptide (XIP), Calcium Binding Domains (CBDs: CBD1 and CBD2), XIP Binding Domain (XBD) and palmitoylation site (Fig. 3A) [40]. The CBDs; CBD1 and CBD2, sit at the centre of the intracellular loop, and act as a Ca2+ sensor which mediate allosteric activation of NCX1. The binding properties of CBD1 and CBD2 are markedly distinct [41–43]. CBD1 harbours four Ca2+ binding sites (Ca1, Ca2, Ca3 and Ca4): two Ca2+ ions bind to Ca3 and Ca4 sites with a high affinity (~200nM), and two Ca2+ ions bind to Ca1 and Ca2 with lower affinity (>5-10μM) [44]. The number of Ca2+ binding sites at CBD2 ranges from zero to three based on the ortholog, isoform and splice variant and the affinity to Ca2+ is lower than CBD1 (~5-200μM) [45–48]. Structurally each CBD is equipped with a β sandwich composed of seven antiparallel β strands (Fig. 3B) [49]. Calcium binding sites lie within the Loop C—D and Loop E-F in each domain [50,51]. In addition to the CBDs, the XIP domain; which is located at the start of the intracellular loop (residues 219 to 238), is another physiologically important component of the exchanger involved in Na+ dependent inactivation. The XIP domain physically interacts with the twenty aa segment between residues 709 and 728, and this interaction is controlled by residues 739–756 which contain a palmitoylatable Cysteine (Cys-739) and amphipathic α-helix [30].

3.2. Recent advances: cryo-EM structure of NCX1 vs AlphaFold

Previously the inward and outward-facing conformational structures of and the mechanism of ion exchange in the archaebacterial exchanger
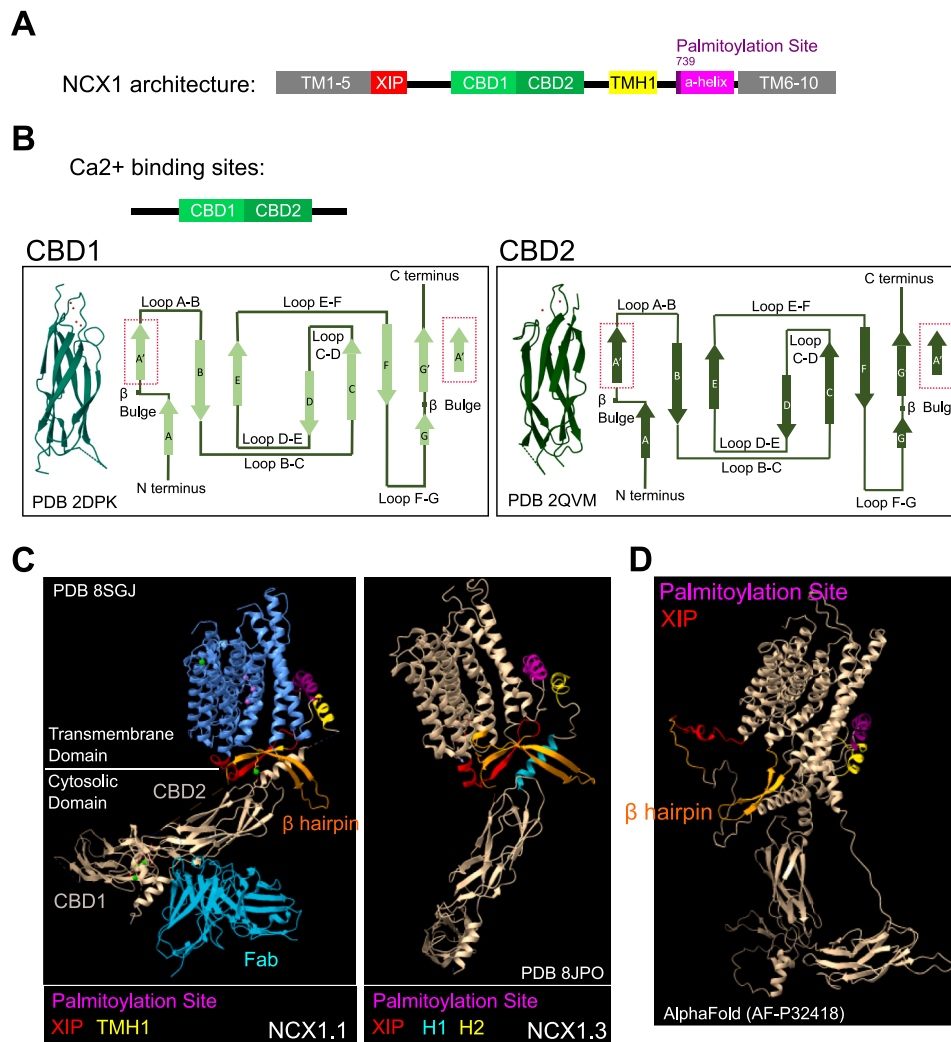
Fig. 3. Organization of NCX1 structure; (A) NCX1 is a 10TM domain protein with a large intracellular loops that accomodates Exchanger Inhibitory Peptide (XIP; red), Calcium Binding Domains (CBDs: CBD1; lighter green, and CBD2; darker green), TMH1(yellow) and palmitoylation site (purple). (B) Each CBD has β sandwich motif formed by seven antiparallel β sheets [50,51] (CBD1; left panel, and CBD2; right panel). The β bulge exists within the A and G strands creating A' and G' strands. The A' strand (inserted in red box) links G' strand at opposing site rather than B strand as A strand does. (C) The inward facing structure of human NCX1.1 [54] (PDB 8SGJ: XIP; red, TMH1; yellow, palmitoylation site (TMH2); purple, and β hairpin; orange) and the structure of NCX1.3 [55] (PDB 8JPO: XIP; red, H1; blue, H2; yellow, palmitoylation site (H3)) indicate the XIP domain is contained within a four stranded β hub (red and orange). (D) AlphaFold [56] prediction of NCX1 structure (AF- P32418: XIP; red, palmitoylation site; purple, and β hairpin; orange), Protein structures were built in ChimeraX [69].

NCX_Mj were identified with a high resolution [52,53]. Two recent studies uncovered the structure of mammalian NCX1.1 (cardiac splice variant of NCX1) and NCX1.3 (kidney splice variant of NCX1) providing critical insights on NCX1 folding [54,55]. The inward facing structure of NCX1.1 (inactivated state, in the presence of high (200mM) Na+ and low Ca2+) was resolved with a high resolution; 3.1Å (with the help of a monoclonal antibody raised against NCX1 which increases the stability of the protein) [54]. This structure revealed a tight binding between TM and cytosolic domains via a four stranded β hub consisting of a β hairpin composed of two cytoplasmic regions: the cytoplasmic loop between TM1 and 2 (β1–2), and the post TM5 region (β3–4; covering the XIP domain) (Protein Database (PDB) 8SGJ, Fig. 3C). Moreover two arch-shaped helices; TMH1 and TMH2, were observed between 708 and 757 aa residues. Interestingly, TMH2 domain is composed of the palmitoylated Cys- (C739) and amphipathic helix (740-757aa residues, Fig. 2A); which controls NCX1 palmitoylation. The C-terminal half of TMH2 (746–757 aa residues) makes hydrophobic contacts with the cytosolic ends of TM1 and TM6. Hence It is conceivable that TMH domains; TMH1 and TMH2, change their position together with TM1 and TM6 during the structural re-arrangements promoted by ion occlusion

and release.

Interestingly, AlphaFold; AI based 3D protein structure database [56] predicted an NCX1.1 structure slightly different from the cryo-EM structure presented by Xue and colleagues. For example, the cryo-EM structure indicates that the XIP domain forms a β strand rather than unstructured domain prediction by AlphaFold (Fig. 3D). Both cryo-EM structure and AlphaFold agree regarding the location of palmitoylation site which is adjacent to the membrane.

The following work from Dong and colleagues most recently mapped NCX1.3 structure with the resolution of 3.5Å, and detected structural similarities between NCX1.1 and NCX1.3 (Protein Database (PDB) 8JPO Fig. 3C). The very similar structures described for NCX1.1 and NCX1.3 are slightly surprising, since calcium binding to CBD2 relieves sodium dependent inactivation for NCX1.1, but not NCX1.3 [54,55]. As in the NCX1.1 structure, a four stranded β hub was detected in NCX1.3 structure composed of the intracellular loop between TM1 and TM2, and the cytosolic region right after TM5 [55]. In NCX1.3, CBD2 is connected to TM6 through three helices (H1, H2 and H3). The XIP domain makes multiple contacts within the hydrophobic groove created by TM1, β2, TM2, TM7, TM8, H1, and CBD2. Moreover H2 and H3 (which include

the palmitoylation site with amphipathic α-helix) are situated right before TM6 and correspond to TMH1 and TMH2 described in the NCX1.1 structure.

Although these studies bring breakthrough advances to the field and expand our knowledge regarding the structural mechanisms of NCX1, It should be noted that the currently available cryo-EM structures of both NCX1.1 and NCX1.3 falls short of explaining how palmitoylated NCX1 is structurally arranged, and how palmitoylation controls NCX1 inactivation. Indeed, the most important physical interactions identified for XIP in these structures take place outside the region of NCX1 that we identified as interacting with XIP (H2 in the NCX1.3 structure) [30,55]. Further understanding on palmitoylation dependent structural changes in conjunction with other regulatory elements of NCX1 (i.e. Na+ [57], Ca2+ [58] and PIP2 [59–62]) is critical to unpuzzle.

4. What's next in the journey?

Interest in palmitoylation is growing rapidly due to its versatile effect on numerous proteins. As summarized herein, palmitoylation serves as a unique endogenous regulatory mechanism to modify NCX1 activity reversibly and dynamically. Recent findings have broadened our knowledge on NCX1 palmitoylation: from the molecular basis of NCX1 recognition by the cell's palmitoylation machinery, to its cellular and physiological consequences. However we need to understand more about the pathways that control the activities of the key palmitoylating and depalmitoylating enzymes, and how these enzymes are remodelled in cardiovascular disease [36]. Having identified the molecular mechanism of NCX1 palmitoylation/depalmitoylation it may be possible to devise pharmacological strategies targeting exchanger palmitoylation to manipulate its activity, which in turn, may help to tackle improper Ca2+ handling in cardiac pathologies. Therapeutic strategies that target palmitoylating or depalmitoylating enzymes to control palmitoylation of their substrates to tune cellular physiology are yet to be developed, but may offer considerable promise. High quality selective inhibitors of thioesterases with in vivo activity have been described [63]. However, the active sites of the zDHHC family are highly conserved, and no inhibitors specific for one family member have yet been identified (although encouragingly compounds that can differentiate to some extent between zDHHC family members were recently reported) [64]. For instance, zDHHC5 is well-established to palmitoylate many substrates in various tissues. Active site inhibitors will therefore likely be of little value to target NCX1 palmitoylation by this enzyme, but targeting the ability of zDHHC5 to recruit an individual substrate (as has been described for its substrate phospholemman [65]) would be an effective way to specifically tune cellular physiology to cope with different pathologies. Last but not least, another point that NCX1 field could benefit from is to unravel the cryo-EM structure of palmitoylated NCX1 to deepen our knowledge of the structural mechanism behind palmitoylation dependent changes in NCX1 function and stability. Understanding how NCX1 is folded when it is palmitoylated could be particularly important as it would enable us to pharmacologically target those structural features that are unique to the palmitoylated form. The recently NCX1 cryo-EM structures were obtained from expression systems in which NCX1 is widely-reported to be palmitoylated, but fatty acylation of the NCX1 palmitoylated cysteine was not observed. Strategies to preserve thioester linked palmitate during protein preparation may be required (for example – application of potent and broad spectrum thioesterase inhibitors such as palmostatin B during protein preparation). However, since less than 100% of NCX1 is palmitoylated in cellular models this approach will likely not yield a homogenous population of protein. Reconsitution of purified active palmitoylating enzymes with their substrates has been reported by some labs [64,66,67], and may be possible for NCX1. It is perhaps pertinent that before the discovery of the zDHHC-PAT family, researchers found that incubating purified proteins with palmitoyl-CoA led to their spontaneous palmitoylation at the same cysteines that were palmitoylated in vivo [68]. As we seek a structure for palmitoylated NCX1, a simple, old-school approach may be the solution to a state-of-the art problem.

CRediT authorship contribution statement

Caglar Gök: Writing – original draft. **William Fuller:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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