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## Understanding the rules governing NCX1 palmitoylation

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## *Introduction*

The ability to identify sites of post-translational modification in proteins is central to understanding the molecular details of pathways regulating their activity. Through recent advances in mass spectrometry, many of the phosphorylation sites in the human proteome have been experimentally determined, and the kinases responsible predicted according to well-established consensus sequence motifs. However, what of other post-translational modifications? Can we predict these with as much certainty as we can phosphorylation? Although the palmitoyl proteomes of many different tissues have been determined,<sup>1</sup> reliable palmitoylation site prediction has thus far eluded us.

## *Palmitoylation of NCX1*

The ubiquitous sodium / calcium exchanger NCX1 mediates transmembrane calcium transport controlled by the sodium gradient and membrane potential in numerous tissues. In cardiac muscle calcium efflux via NCX1 controls diastolic function,<sup>2</sup> but abnormal NCX1 function contributes to cardiac arrhythmias and ischemia-reperfusion injury.<sup>3</sup> In the absence of PIP<sub>2</sub>, NCX1 inactivates when its autoinhibitory XIP domain (located at the N-terminal end of its large regulatory intracellular loop situated between transmembrane domains 5 and 6) interacts with a distal region of the same loop. NCX1 is palmitoylated at a single cysteine in this regulatory loop: unpalmitoylated exchangers are resistant to inactivation and retain substantial activity when wild type exchangers inactivate<sup>4, 5</sup>.

There are multiple cysteines in the NCX1 regulatory loop but only cysteine 739, located at the C terminal end of this loop close to transmembrane domain 6, becomes palmitoylated. In our recent paper<sup>6</sup> we asked why this particular cysteine gets palmitoylated when neighbouring ones do not. A fusion protein between the NCX1 regulatory loop and YFP is palmitoylated at C739<sup>4</sup>, unequivocally demonstrating that the exchanger's transmembrane domains are dispensable for palmitoylation. The 'instructions' to palmitoylate NCX1 must therefore lie within the regulatory loop.

Alanine scanning mutagenesis around C739 was almost entirely without effect on NCX1 palmitoylation. Indeed, one of the only point mutations we could identify as having an effect on NCX1 palmitoylation was the rare human polymorphism S738F, which enhanced NCX1 palmitoylation, possibly by impeding its depalmitoylation. In contrast, deletion of a small ~20 amino acid region at the very C terminal end of the regulatory loop completely abrogated NCX1 palmitoylation. The secondary structure predictor Jpred strongly suggests that this region forms an  $\alpha$ -helix which is largely hydrophobic, with a small hydrophilic face (Figure 1). We hypothesised that membrane association of the hydrophobic side of this helix and / or the amino acids on its hydrophilic face may be important for NCX1 palmitoylation. Breaking the helix with prolines, introducing negative charges to its hydrophobic face, or alanine mutagenesis of the hydrophilic face all impaired NCX1 palmitoylation, whereas alanine mutagenesis on the helix's hydrophobic face was without effect. The distance between the helix and C739 was not crucial: inserting alanine between the two did not impair NCX1 palmitoylation. When C739 and the amphipathic helix were added to the C terminus of YFP, the fusion protein became palmitoylated and, as a consequence, membrane localised. Our data are therefore consistent with a model in which the hydrophobic face of the

amphipathic helix interacts with the membrane, while the hydrophilic face is exposed to the cytosol where it engages with the cellular palmitoylation machinery, with enzyme selectivity dictated by the requirement for specific residues at particular positions on the hydrophilic face of the helix.

#### *Substrate recognition by palmitoylating enzymes*

So what does this tell us concerning NCX1 palmitoylation and more generally about the palmitoylation of ion transporters and integral membrane proteins by DHHC-PATs? The hydrophobic face of the recognition helix will be readily accommodated within the lipid bilayer, positioning C739 close to the membrane for palmitoylation (Figure 1). Notably though, despite its hydrophobicity the helix alone is not sufficient to act as a membrane anchor, as an unpalmitoylatable (C739A) YFP-NCX1 intracellular loop fusion protein is freely distributed in the cytosol<sup>4</sup>. By interacting with negatively charged phospholipid head groups, polybasic domains may also position cysteines adjacent to membranes facilitating their palmitoylation<sup>7</sup>. As for the hydrophilic face of the helix, D741, H745, T748 but not K752 all play a role in the recognition of NCX1 by its palmitoylating enzyme, with H745 being the most important residue. A protein-protein interaction between this hydrophilic face and the palmitoylating enzyme probably precedes NCX1 palmitoylation.

This relationship is notably different from that between the Na pump regulator phospholemman and its palmitoylating enzyme DHHC5, in which a disordered domain interaction between the intracellular regions of these proteins precedes palmitoylation<sup>8</sup>. This highlights the difficulties of predicting palmitoylation sites in proteins: the recognition rules that apply to one DHHC-PAT may apply to closely related family members, but not to all enzymes. From a therapeutic point of view this is good news, because it means we should be able to specifically target individual DHHC-PATs, or even individual substrates, but there is still a long way to go before palmitoylation sites can be accurately predicted. Once we have identified the DHHC-PAT for NCX1, we will have a set of recognition rules for this particular enzyme that can be tested with other substrates. There are 22 other DHHC-PATs in the human genome and likely many more recognition rules to discover.

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*Figure Legend*

An amphipathic  $\alpha$ -helix directs palmitoylation of NCX1. Palmitoylation of NCX1 at C739 requires an  $\alpha$ -helix at the C terminal end of the NCX1 regulatory intracellular loop. The hydrophobic face of the helix probably interacts with the membrane, while the cytosolic hydrophilic face is likely recognised by the cellular palmitoylation machinery.