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PhD THESIS WORK:

STUDY OF THE MECHANISMS REGULATING NA^+/CA^{2+} EXCHANGER 3 LOCALIZATION IN MITOCHONDRIA AND ITS PATHOPHYSIOLOGICAL IMPLICATIONS

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1. SUMMARY

Several proteins are localized in two cellular compartments at the same time with special targeting signals that are localized at the N-terminal domain. Among these protein, the isoform 3 of the Na^+/Ca^{2+} exchanger (NCX3) family has been found on both plasma membrane and outer mitochondrial membrane (OMM), where it plays an important role in the maintenance of cytosolic and mitochondrial Ca^{2+} homeostasis. In particular, mitochondrial NCX3 takes part in mitochondrial Ca^{2+} efflux from the OMM, promoting cell survival during hypoxia, through an AKAP121-anchored signaling complex. Interestingly, the amount of NCX3 localized on the OMM of neurons decreases during oxygen glucose deprivation and is restored after re-oxygenation (OGD-Rx), suggesting the presence of a regulatory mechanism the subcellular sorting of NCX3. The aim of the present study was to investigate the molecular determinants and the mechanism regulating the subcellular sorting of NCX3 on mitochondria.

Results obtained showed that the silencing of either translocase of outer mitochondrial membrane (TOM), TOM22 or TOM70, decreased the amount of NCX3 on mitochondria suggesting that these two mitochondrial import receptors are involved in the recognition and

transport of this antiporter on the OMM. By contrast, the silencing of the other mitochondrial import receptor, TOM20, did not show affect the subcellular localization of the antiporter.

By using a bioinformatics approach, we identified some putative regions of NCX3 that might contain a mitochondrial localization signal. On the basis of these data, we analyzed the mitochondrial fraction of baby hamster kidney (BHK) cells stably transfected with several chimeric proteins containing the sequences of NCX3 and NCX1 and NCX3 mutants. Mitochondrial extraction of these chimeric proteins revealed that NCX3 is provided with three independent regions containing the molecular determinants for its subcellular localization on OMM and that are localized in the C-terminal region of the cytosolic f-loop (718-756 aa), in the alpha1 (109-133 aa) and alpha2 (788-829 aa) sequences.

Furthermore, we generated several site-directed mutants of NCX1 in which single amino acids in the alpha1 or alpha2 region was substituted with the corresponding one present in NCX3. Results showed that, chimeric proteins provided with the backbone of NCX1 and amino acids regions 109-133, 718-787, 788-829 of NCX3 were localized on mitochondria, supporting the conclusion that there are multiple mitochondrial localization signals spread along the protein.

Another aspect that emerged from these results is the proximity of the mitochondrial localization signal and the N-terminal signal peptide for plasma membrane in NCX3 sequence as it occurs in other proteins with multiple subcellular localizations. In fact, post-translational modifications, i.e. PKA phosphorylation or cleavage of the N-terminus close to the mitochondrial localization sequence, can regulate the protein topology inside the cell. In this regards, we excluded the participation of PKA phosphorylation in NCX3 topology on mitochondria, since the specific inhibitor of PKA phosphorylation, KT5720, did not influence the distribution of the antiporter between the plasma membrane and mitochondria. On the other hand, the removal of the signal peptide at the N-terminus (NCX3Δ2-31), increased the amount of the exchanger on mitochondria, supporting the hypothesis that the cleavage of this signal peptide, occurring during the antiporter synthesis, can participate in the regulation of NCX3 topology under physiological or pathophysiological conditions.

2 INTRODUCTION

2.1 Mitochondrial Calcium Homeostasis

Mitochondria are highly specialized organelles, crucial for ensuring numerous fundamental physiological processes, such as energy conversion, redox balance, modulation of calcium (Ca^{2+}) signaling and important biosynthetic pathways. In eukaryotic cells, the ATP production is functionally coupled to metabolic demands and the cells are able to adapt oxidative respiration by activating of matrix dehydrogenases in response to changes in extracellular microenvironment and metabolic nutrient availability (1). The mitochondrial volume, number and distribution within the cells are strictly controlled according to local energy demands and in cells of highly metabolic tissues such as muscle, liver and central nervous system, the trafficking of mitochondria is critical. In particular, the brain uses over 95% of the ATP of the CNS (2) and the mitochondria distribution within neurons is concentrated in regions associated to a high metabolic activity, such as synapses, node of Ranvier and myelination/demyelination interfaces (3; 4; 5; 6). The 50-60% of total brain ATP is used to the generation, processing and transmission of

neural impulses that is conditional to the maintaining of ion gradients of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) across the plasma membrane.

Besides performing oxidative phosphorylation, mitochondria are able to sense and shape calcium transients, thus controlling cytosolic Ca^{2+} signals and Ca^{2+}-dependent proteins. Indeed, despite in the past was believed that mitochondria playing only a marginal role in physiological intracellular Ca^{2+} homeostasis, whereas the Ca^{2+} affinity of the mitochondrial transporters is low, it has been recently well established that these organelles have a huge capacity to accumulate calcium and operate a key role in the intracellular calcium signaling (7).

Calcium homeostasis is fundamental for cell; it carries information to virtually all processes important to cell metabolism, proliferation and differentiation but also transmits signals that promote the programmed demise of cells (8; 9). The maintenance of mitochondrial calcium homeostasis is an important requirement ensuring mitochondrial function. The energy demand and calcium homeostasis are strictly correlated each other, then the ATP-utilizing cellular processes that are triggered by calcium are regulated by energy suppliers under control of calcium itself (10). Indeed, Ca^{2+} sensitive dehydrogenases can regulate

oxidative phosphorylation and ATP synthesis during times of high cellular demand (11; 12). Moreover, the presence of high levels of Ca^{2+} in the mitochondrial matrix is necessary for the activity of mitochondrial enzymes (13). However, when the calcium into the mitochondria overcomes its storage capability, as happens in pathological conditions, such as in neuronal anoxia or neurodegenerative impairments, it results in a subsequent increase in reactive oxygen species free radical production (ROS) in order to face the rapid rise of $[Ca^{2+}]_i$. The result of this process is the damage of the inner mitochondrial membrane (IMM) and the oxidation of the proteins involved in oxidative phosphorylation, in proton pumping and in ATP production (14). Another consequence of the rise of mitochondrial calcium concentration is the increase of inner mitochondrial membrane permeability that occurs in the opening of mitochondrial permeability transition pore (mPTP). This event causes the release of cytocrome C, that in turn causes an impairment of cellular respiration and the activation of programmed cell death (15; 16). As a consequence of this cascade events, mitochondrial membrane permeability and ATP production are irreversibly affected and, moreover, there is an impairment of the mitochondrial capability to

regulate intracellular calcium concentration in response to stress stimuli (17), thus cells are intended to die.

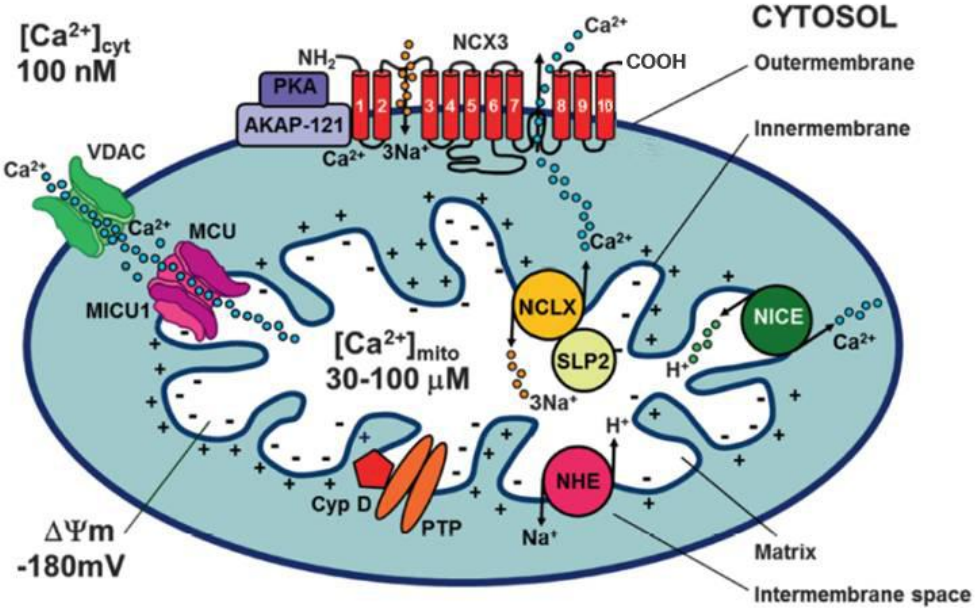

Fig. 1 Mitochondrial calcium homeostasis mechanisms

Ca^{2+} mitochondrial traffic across the mitochondrial inner membrane (IMM) takes place by two pathways. The Ca^{2+} influx pathway is represented by the mitochondrial calcium uniporter (MCU), a protein localized in the inner membrane, which markedly enhances mitochondrial Ca^{2+} uptake into the mitochondrial matrix. MCU activity is driven by an electrochemical potential across the inner mitochondrial

membrane, usually estimated at -200, -180 mV and generated either by the respiratory chain activity and by ATP hydrolysis (18; 19). The Ca^{2+} efflux pathway is catalyzed by antiporters that drive Ca^{2+} out of the mitochondrial matrix in exchange with either Na^{+} or H^{+} (20). In particular, two main antiporters have been characterized in the 1970s, the Na^{+}/Ca^{2+} exchanger (NCLX or NCXmit), mostly expressed in excitable cells (muscle and brain) (21), and a $H+/Ca^{2+}$ exchanger (HCX_{mit}), that represents the prevailing route in most other tissues (23).

NCLX was the first mitochondrial antiporter identified and localized on mitochondria cristae (24). It is the main mechanism responsible for the maintaining of Na^{+}-dependent Ca^{2+} efflux from the inner mitochondrial membrane. However, when the intramitochondrial Ca^{2+} rises or there is a mitochondrial membrane depolarization, the mPTP channel also participates in the Ca^{2+} efflux from the inner membrane of mitochondria. The physiological role of Ca^{2+}-induced permeability is still unclear, the opening of a large pore in the IMM would allow maximal Ca^{2+} release even for very small [Ca^{2+}] gradients, indeed PTP has also been proposed to represent a way of clearing the mitochondrial matrix of damaged or unneeded molecules, for removing damaged mitochondria or inducing apoptosis (21). Recently, OMM has also been recognized as a player in

the mitochondrial Ca^{2+} cycling. Indeed, it serves as an important permeability barrier to both Ca^{2+} uptake and efflux. Despite this, the voltage dependent anion channel (VDAC) located on the OMM, plays a relevant role in the regulation of Ca^{2+} permeability, thus favoring the activity of the specific transport systems of the inner membrane (25).

The maintenance of a healthy mitochondrial population is essential to avoid the impairment of the processes they regulate. The cellular Ca^{2+} deregulation, leading to mitochondrial calcium overload and cell death trough PTP opening, followed by mitochondrial swelling, has been described as final step for many neurodegenerative diseases. Thus, the escape of Ca^{2+} control is thought to be critically important for triggering the cell death after a traumatic brain injury as well as in several neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's diseases and Amyotrophic Lateral Sclerosis (ALS).

2.2 The Na^+/Ca^{2+} exchanger (NCX)

The Na^+/Ca^{2+} exchanger (NCX) is an ion transporter that plays a crucial role in in the function and regulation of several cellular activities. NCX

belongs to the Ca^{2+} cation antiporter superfamily (CaCA) and consists of 10 transmembrane (TM) segments (26). NCX was discovered and characterized in the late 1960s, when Baker et al. (27), Reuter and Seitz (28), and Martin and De Luca (29) realized the presence of counter transport mechanism that exchanged Na^+ and Ca^{2+} ions across the plasma membrane of different excitable and non excitable cells. However, the most crucial advancement in NCX research was made in 1988 (30) and 1990 (31) when Philipson and his colleagues purified and cloned the first isoform of this antiporter: NCX1. Remarkably, few years later, the same group cloned NCX2 (32) and NCX3 (33), two isoforms selectively expressed in the brain and in the skeletal muscle whereas, in 1999, it proposed a topological model of the NCX1 exchanger with 11 TM domains (34). Only recently, it was demonstrated by solving the crystal structure of NCX from the archaebacterium Methanococcus jannaschii (NCX_Mj) (35) the 10 TM domains structure of NCX and it appears that this membrane topology is the same in mammalian NCX proteins (26).

NCX is allosterically regulated by its substrates, Ca^{2+} and Na^+, and by H^+ (36; 37) and it can mediate Ca^{2+} and Na^+ fluxes across the plasma membrane with a stoichiometry of 1:3, respectively (38). Depending on

the electrochemical gradient of [Ca^{2+}], and [Na^{+}], NCX catalyzes the influx or the extrusion of Ca^{2+}, thus operating either in the *forward mode*, coupling the uphill extrusion of Ca^{2+} to the influx of Na^{+} ions, or in the *reverse mode*, mediating the extrusion of Na^{+} and the influx of the Ca^{2+} ions (36; 39). When $[Ca^{2+}]_i$ rise and the cells require to return in resting conditions NCX is able to work in *forward mode,* coupling both the extrusion of Ca^{2+} to the influx of Na^{+} (40). Otherwise, under other physiological circumstances, when $[Na^{+}]_i$ rise, or membrane depolarization occurs, the Na^{+}/Ca^{2+} exchanger operates in *reverse mode*, thus mediating the extrusion of $[Na^{+}]_i$ and the influx of Ca^{2+} ions (41; 42).

The NCX family by its participation to the regulation of intracellular concentration of Ca^{2+} and Na^{+} ions in excitable cells, has a relevant physiological function in the maintaining of cellular homeostasis. Indeed, the Na^{+}/Ca^{2+} exchanger, in parallel with selective ion channels and ATP-dependent pumps, maintains the physiological cytosolic concentrations of these ions. NCX expression in all cell types is involved in many physiological events throughout the body. In this regard, NCX in muscle tissues is mostly known for its role in Ca^{2+} extrusion during muscle relaxation. Moreover, NCX is important in the cardiac

development, and its loss of function causes heart failure on the midterm. NCX is also required in skeletal and smooth muscle where it could possibly act as a modulator of the sarcoplasmic reticulum (SR) content. NCX is also implicated during brain development and neurotransmitter release. In non-excitable tissues, NCX carries out very diverse roles such as the Ca^{2+} reabsorption in the distal convoluted tubules of the kidney and the Na^{+} extrusion in erythrocytes and participates to the control of insulin release in pancreatic β-cells (43).

2.3 Molecular Biology of NCX

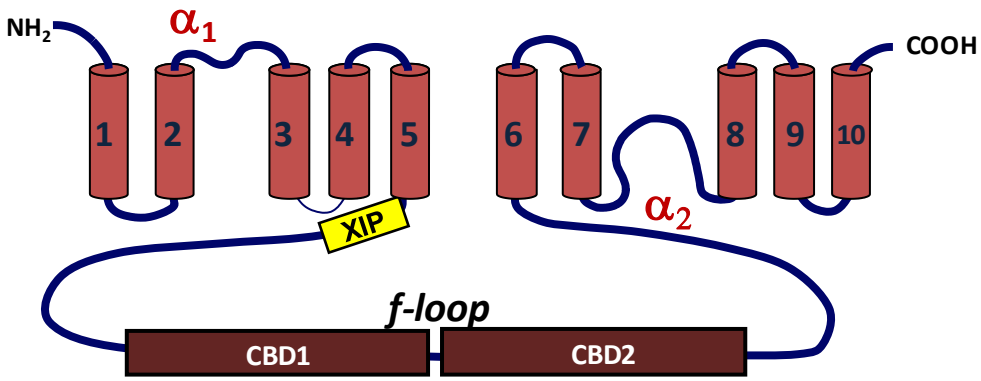

Fig. 2 Molecular topology of NCX

In mammals three genes coding for the three different isoforms have been found: NCX1, NCX2 and NCX3. NCX1 gene is expressed ubiquitously in almost all mammalian cells including neurons, cardiomyocytes, skeletal and smooth myocytes. At the post-transcriptional level, at least 17 NCX1 and 5 NCX3 proteins are generated through an alternative splicing of the primary nuclear transcripts (44; 45; 46; 47). These variants arise from a region of the large intracellular f loop and are encoded by six small exons defined A to F. These exons are used in different combinations in a tissue-specific manner (48), but to maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive (44). In the excitable tissues, such as those of the brain and heart, the variants are usually characterized by the presence of exon A, whereas kidney, stomach, and skeletal muscle tissues comprise NCX with exon B (48). The occurrence of two parallel genome duplications early in the vertebrate lineage gave rise to, most likely, four isoforms of NCX. The fourth isoform, NCX4, present in teleost, amphibian, and reptilian genomes, has been lost in birds and mammals during evolution (49). From this early duplication, NCX family conserved a strong homology of sequence of about 70% in mammals. The canine heart NCX1 is

composed of 938 amino acids and it has a theoretical molecular mass of 120 kDa. NCX2 have been found only in the central nervous system (CNS), whereas NCX3 have been found in both CNS and skeletal muscle. These two gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and 105 kDa, respectively. In addition, NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2 (50). From the structural point of view, NCX amino-terminus is located in the extracellular space as well as the carboxyl terminus. The 10 TM segments can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1-5), and into a C-terminal hydrophobic domain, composed of the last six TMS (6-10). These two hydrophobic domains are important for the binding and the transport of ions. The first (1-5) TMS are separated from the last six (6-10) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f loop (51). Although the f loop is not implicated in Na^+ and Ca^{2+} translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as Ca^{2+} and Na^+ ions, NO, phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC), protein kinase A (PKA),

and ATP. The f-loop has been reported to exert a Ca^{2+} regulatory function. Indeed, within this intracellular domain, two Ca^{2+} binding domain (CBD1 and CBD2) are found, that undergo conformational changes upon binding of Ca^{2+} and look very similar in the Ca^{2+} bound form, whereas in the absence of Ca^{2+}, the domains show dramatic structural differences. At the N-terminal end of the f loop near the membrane lipid interface, an autoinhibitory domain, rich in both basic and hydrophobic residues, named exchange inhibitory peptide (XIP) (52), has been identified. Therefore, NCX isoforms are characterized by several peculiarities, in particular, the presence of α-repeats in the TM segments. The NCX protein amino acid sequence found between TMS2 and TMS3 is called α_1 repeat, whereas the one found between TMS7 and TMS8 is named α_2 repeat. Both these regions are located on the opposite site of the membrane and include two segments composed of 12 and 9 highly conserved residues separated by a non conserved segment of 18 to 20 amino acids (53). The α-repeats allow for the ion translocation across the plasma membrane. These regions contain four cation-binding sites, that share several negatively charged ligands. Therefore, Ca^{2+} and Na^{+} binding sites cannot be occupied simultaneously, which confirms a ping-pong mechanism of translocation in which one Ca^{2+} and three Na^{+}

ions are translocated sequentially in separate steps rather than simultaneously across the membrane. This mechanism implies the alternating access mechanism of Na^+ and Ca^{2+} on the NCX ion binding sites in the inward (cytosolic) and outward (extracellular) conformations. Extracellular Na^+ and Ca^{2+} ions reach these binding sites by two passages specific for each cation. Less information is available on the inward-facing conformation of NCX.

2.4 Mitochondrial Na^+/Ca^{2+} Exchanger 3 (mNCX3)

By catalyzing Na^+-dependent Ca^{2+} efflux, mitochondrial NCX3 plays a fundamental role in regulating mitochondrial Ca^{2+} homeostasis, oxidative phosphorylation and Ca^{2+} crosstalk among mitochondria, cytoplasm and the endoplasmic reticulum (ER). Whereby, the mitochondrial activity of NCX3 exerts a pivotal role under both physiological and pathophysiological conditions. Indeed, among NCX gene products, NCX3, is not inhibited by the low levels of intracellular ATP. Indeed, this isoform, thanks to the ATP-independence (54; 55), exerts a neuroprotective role in stroke (56) and in the ischemic

preconditioning (57). On the other hand, NCX3 also plays a relevant role in the spatial learning and memory processes where it contributes to presynaptic Ca^{2+} clearance and thus enhances synaptic plasticity of hippocampal neurons (58). Recently, NCX3 was also found on the outer mitochondrial membrane where it contributes to the Ca^{2+} efflux in collaboration with NCLX. In particular, the activity of the mitochondrial NCX3 is dependent from the interaction with the anchoring protein, AKAP121 (59). NCX3 also performs an interesting role during ischemia. Indeed, hypoxic conditions downregulates, where reoxygenation increases, the amount of NCX3 on mitochondria (59), suggesting the presence of a regulation of the cellular sorting of NCX3. These data open a new perspective on NCX3 regulation that is based on it subcellular sorting under physiological or pathophysiological conditions.

2.5 Bimodal targeting of precursor proteins

Some proteins can be located in different subcellular compartments, such as plasma membrane and mitochondria, guided by activation of bimodal targeting signals that produce a dual distribution between the subcellular

compartments. (60; 61). They may be distinguished two main groups based on the number of translation products that are generated. The first group of proteins, includes isoproteins generated by different translation products can be obtained by different paths that are based on two genes, two mRNAs from a single gene or two translation products from the same mRNA. The examples of isoproteins described in all this cases differ by the presence or absence of a targeting signal (60; 61). A second group is represented by proteins result of the translation of a single protein product that hides two targeting signals or a chimeric signal that can target the protein to different organelles (60; 61). Indeed, in contrast to the canonical mitochondrial targeting signal, which is considered to be a structural element that binds to chaperones and receptors facilitating the targeting, the chimeric signal is more dynamic. It targets the protein to mitochondria or ER depending upon the physiologic demand of the cell (60; 61). Recent proteomic studies of mitochondrial proteins suggest that more than 1000 proteins, many of which may lack canonical cleavable targeting signals, may be translocated to mitochondria. Interestingly, dual localized mitochondrial proteins are generally characterized by a lower probability of mitochondrial targeting (MitoProtII score) and a lower net charge compared to exclusive

mitochondrial proteins, thus, not only the N-terminal targeting signal but also by the entire protein properties (such as its net charge), mediate dual targeting of mitochondrial proteins (62; 63). Another important finding demonstrated that nearly one-third of the yeast mitochondrial proteins are also localized in other cellular compartments (63). Examples of proteins that are bimodally targeted include the catalytic subunit of protein kinase A (PKA) (64), Alzheimer's amyloid precursor protein (APP) (65), α-synuclein (66), protein kinase C (PKC) isoforms, the protein kinase B (PKB), proteins involved in oncogenesis and apoptosis including tumor protein 53 (p53), the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), the B-cell lymphoma XL (Bcl XL) and (Bcl2) 2 factors (67; 68), nuclear receptors such as estrogen receptor (E2), Thyroid hormone receptor 3 (T3), the glucocorticoid receptor (GR) and others. The chimeric mitochondria targeting signal requires activation by post-translational modification, a sequence-specific processing by a cytosolic endoprotease or PKA-mediated phosphorylation (69; 70; 71). In the first mechanism, the nascent chain escapes the signal recognition particle (SRP) binding and it is cleaved by a cytosolic Serine protease in sequence-specific manner to activate the cryptic mitochondrial signal of the protein; this nascent chains that are

translated as membrane free proteins are translocated to mitochondria with the help of Hsp70 and Hsp90 chaperones **(Fig 3)**. The endoprotease responsible for the activation of the N-terminal targeting signal of CYP1A1 is a member of the Ser-protease family (72). The processing of CYP1A1 is sequence specific, indeed both the core cleavage site (+32/+33) and the flanking amino acids (30-VRVTRT-35) are required for processing (72). Interestingly, this protease was also found to process p53 (PAGSR), GR (ILLDFSK), RXR (ASFTK), and CYP1B1 (RLLRQR) which all carry Ser-protease consensus sites, shown in parentheses, and the processed form of each protein targets to mitochondria (72).

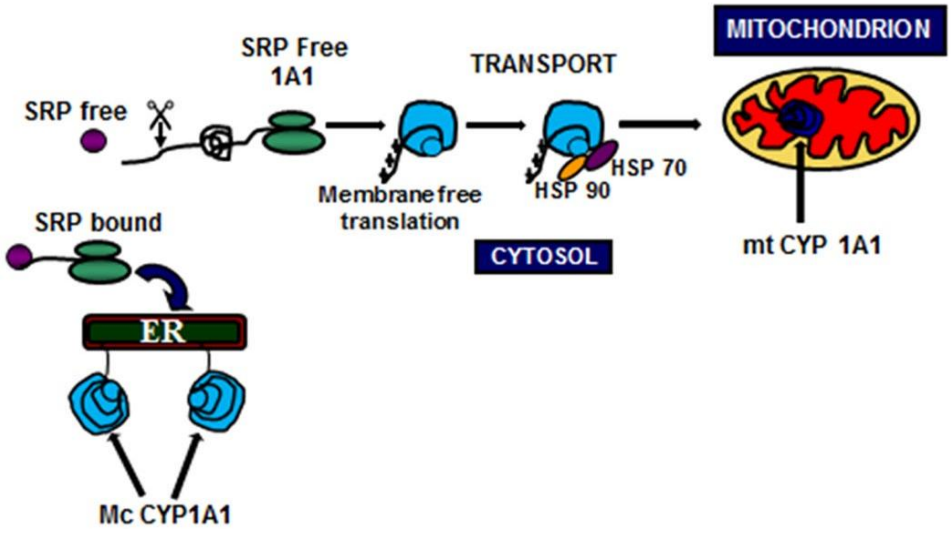

Fig. 3 N-terminal cleavage mechanism (Avadhani et al., 2011)

In the second mechanism of bimodal protein targeting, the nascent chain can be phosphorylated by PKA on specific Serin residues, in turn, produce a recognizable mitochondrial targeting signal. In particular, when the nascent chains are not phosphorylated, SRP bind and guide the unfolded protein to ER membrane, whereas, under high PKA conditions, the serine-phosphorylation reduces the affinity of nascent chains for SRP and the protein can be recognized by Hsp70 and Hsp90 chaperones and imported in mitochondria (**Fig. 4**). An example of this mechanism is represented by the monooxygenases CYP2E1 and 2B1 that contain single PKA phosphorylation sites at Ser129 and Ser128, respectively. CYP2D6, instead, contains a high consensus PKA target site at Ser135, and potential sites of lower consensus at Ser148 and Ser217 (73; 74). On the other hand, PKA phosphorylation activity also regulates the import efficiency of mitochondria suggesting multiple regulatory mechanisms of the subcellular sorting.

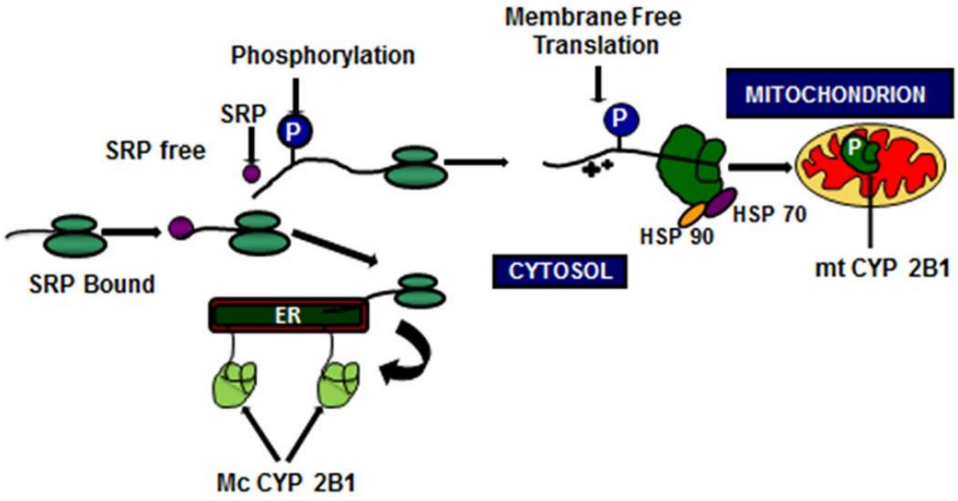

Fig. 4 PKA-mediated phosphorilation mechanism (Avadhani et al. ., 2011)

2.6 Protein Import Pathways into Mitochondria

Almost 99% of mitochondrial proteins are encoded by nuclear genes, including NCX3, thus they must be imported by several ATP-dependent translocases that recognize, translocate, and insert in the mitochondrial membrane the mitochondrial protein (**Fig 5**). In particular, mitochondrial proteins contain several mitochondrial localization signals and post-translational modifications that allow their recognition.

On the basis of the mitochondrial localization signals, mitochondrial proteins can be divided into two main classes. The first class of

precursor proteins carry an N-terminal cleavable extensions, termed pre-sequences, that are characterized by a stretch of hydrophobic and positively charged residues. The function of these cleavable extensions is to interact with the mitochondrial import receptors and direct the pre-protein across both outer and inner membranes (75; 76). The second class of precursor proteins carry different internal targeting signals that are not cleaved in the mature form. These precursors have the same primary sequence, but not the same conformation, of the mature protein (76; 77). In particular, it is known that many outer membrane proteins display mitochondrial localization signals with an α-helical conformation, that are often flanked by positively charged residues. However, the majority of these non-cleavable precursor proteins contain quite distinct targeting signals at various positions within their primary structure. Indeed, these targeting sequences can be found at the amino terminus (signal anchor sequence), at the carboxy terminus (tail anchor) and in the middle of the proteins (77; 78; 79; 80; 81). The exact nature and targeting mechanisms of these internal signals are not clear.

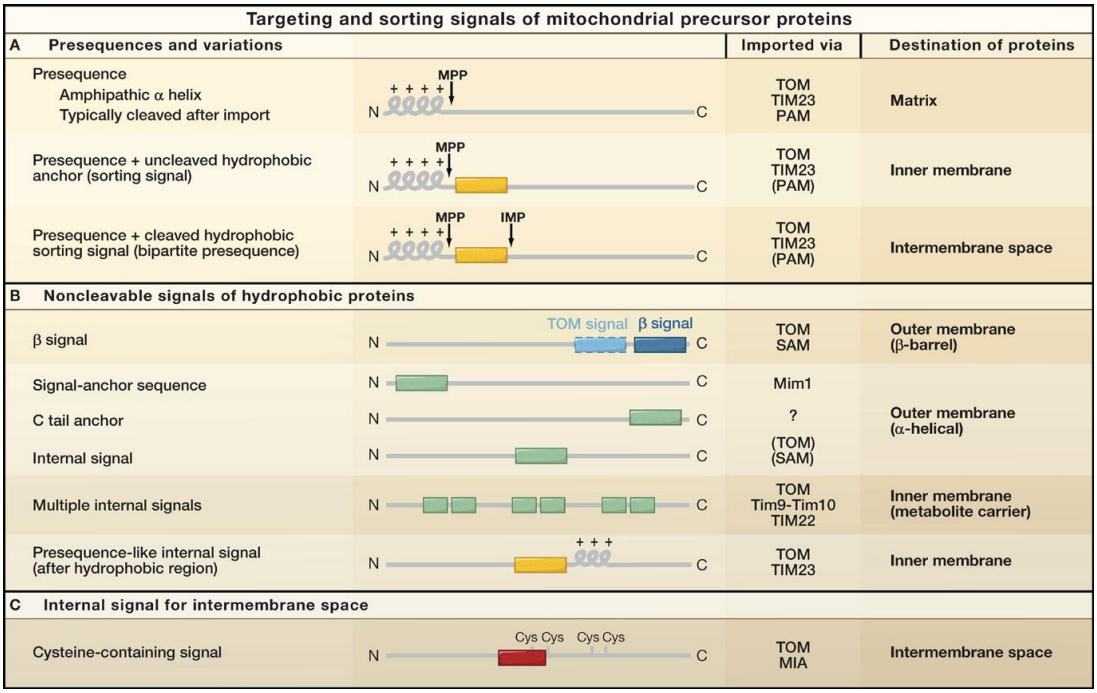

Fig. 5 Mitochondrial targeting signals

On the basis of the mitochondrial signals nature, mitochondrial proteins can follow multiple insertion pathway into the mitochondrial membranes (**Fig. 5**). The outer mitochondrial membrane (TOM) complex represents the central entry gate for practically all nuclear-encoded mitochondrial proteins (**Fig 6**).

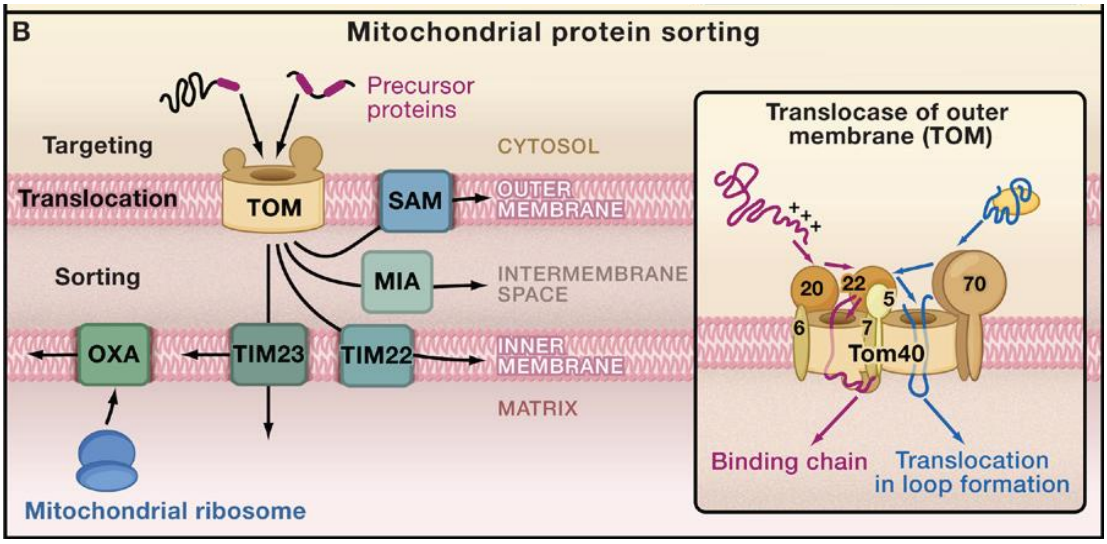

Fig. 6 Mitochondrial protein sorting (Chacinska et al., 2009)

TOM complex consists of seven different subunits that can be grouped into three categories: the receptors Tom20, Tom22, and Tom70; the channel-forming protein Tom40; and three small Tom proteins, Tom5, Tom6, and Tom7 (82; 83). Tom40 is an integral membrane protein with a β-barrel structure and is the central component of TOM. It forms the channel for pre-protein translocation across the outer membrane and is organized as an oligomer that forms two to three channels per TOM complex (84; 85). Three receptor proteins function as part of the TOM complex. Tom20 is the initial recognition site involved in recognizing the pre-sequence of a pre-protein (86) and transfers the pre-proteins to the central receptor Tom22. Tom70 forms the initial recognition site for

precursors of inner membrane metabolite carrier proteins, which carry multiple internal targeting signals; Tom70 transfers these precursor proteins to Tom22 (87; 83). From here, the precursors are inserted into the Tom40 channel. A typical pre-sequence has a length of about 10–30 amino acid residues and forms an amphipathic α-helix. One half of the helix possesses a hydrophobic surface that is recognized by a binding groove within Tom20 (88), whereas the other half is positively charged and recognized by the receptor Tom22 (89). With the help of the small protein Tom5, the pre-protein is then transported to the general import pore (90) Tom40 (82). After translocation through the Tom40 pore, the pre-sequence binds to the intermembrane space domain of the receptor Tom22 (91). Tom40 itself does not simply form a passive pore but rather interacts with the pre-proteins in transit.

Pre-proteins with cleavable pre-sequences, as NCX3, are thus guided across the outer membrane by a chain of binding sites, including the cytosolic receptors Tom20, Tom22, Tom5, the Tom40 translocation channel, and the intermembrane space domain of Tom22 (91; 92). The other small Tom proteins, Tom6 and Tom7, do not directly interact with precursor proteins but are required for the assembly and stability of the TOM complex (93). These precursors are usually synthesized without a

pre-sequence but contain multiple internal targeting signals (94; 95). Precursor proteins synthesized on cytosolic ribosomes are usually guided by cytosolic targeting factors or chaperones to the mitochondrial surface. Cytosolic chaperones, particularly heat shock proteins of the Hsp70 and the Hsp90 classes, bind these hydrophobic precursors and prevent their aggregation in the cytosol. The precursor chaperone complexes are recognized by the surface receptor Tom70, which possesses binding sites for both the precursor proteins and the chaperones (95; 96; 97; 98). Several Tom70 molecules simultaneously bind to one precursor molecule, most likely to prevent misfolding of the precursor, indeed carrier precursor proteins contain multiple internal targeting signals and induce Tom70 molecules to form oligomers (95). The binding of several Tom70 proteins is thought to prevent aggregation of the hydrophobic precursor. In an ATP dependent step, the chaperones are released and the precursor is then transferred, in a loop formation, into the TOM channel (95). The carrier precursors are then transferred to the import pore Tom40. In this transfer are involved also the other receptor proteins, Tom20 and Tom22, as well as Tom5. Even if the receptors Tom20 and Tom22 preferentially recognize pre-sequences, while Tom70 mainly interacts with hydrophobic precursor proteins carrying internal targeting

signals, the three mitochondrial import receptors show a partially overlapping specificity (99). Upon inactivation of one receptor, the other receptors can partially replace with its function. Interestingly, the precursors of outer membrane proteins that contain internal targeting signals mainly use the receptors Tom20 and Tom22. Based on the complexity of the protein structure, the interaction of the precursors proteins with TOM complex can be followed by participation of the sorting and assembly machinery (SAM) of the outer membrane. The precursors of outer membrane proteins that possess a complex topology with multiple β-strands (β-barrel proteins), such as porin and Tom40, are first imported via the TOM complex to the intermembrane space side (93) and, with the help of the small Tim proteins of the intermembrane space (100; 101), they are then passed on to the SAM (93) where they can be exported and integrated into the outer membrane (**Fig. 7 A**). Instead, outer membrane proteins with a relatively simple topology, such as with one transmembrane segment, only require the TOM machinery for insertion into the membrane (93). Another variation of outer membrane insertion was identified for proteins with several transmembrane α-helical segments. These precursors require the receptor

Tom70, that probably functions in a chaperone-like manner to transfer the precursors into the outer membrane (79) (**Fig. 7 B**).

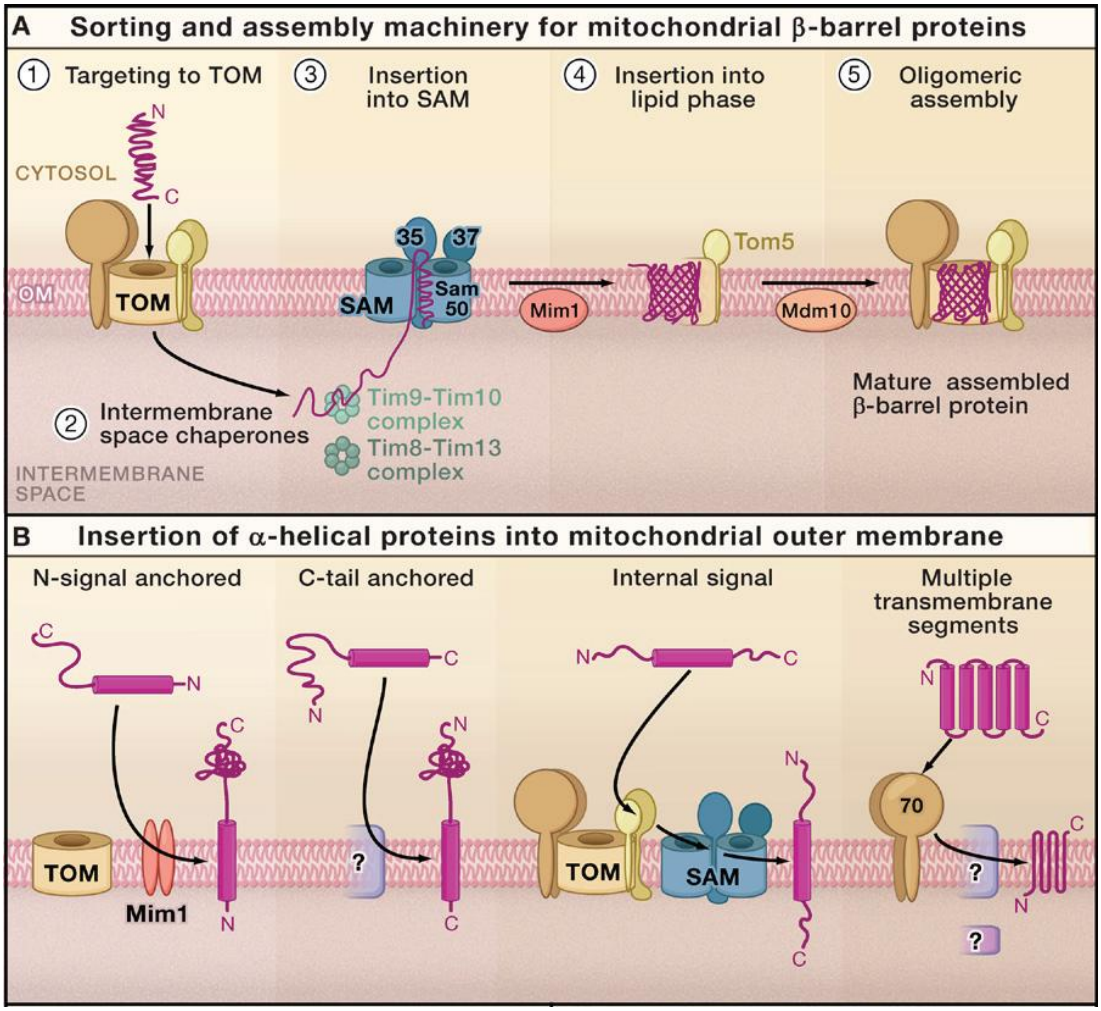

Fig. 7 Mitochondrial protein import of β-barrel (A) or α-helical proteins (Chacinska et al., 2009)

3. AIM OF THE STUDY

NCX3, besides its plasma membrane localization, is also localized on mitochondria, where it plays an important role in the maintenance of cytosolic and mitochondrial Ca^{2+} homeostasis (59). Recent studies demonstrated that a number of proteins, as well as NCX3, have been shown to be present in multiple cellular sites and that this bimodal localization is regulated in response to the cell physiological demand trough the activation of special targeting signals that are localized at the N-terminal domain (60). NCX3 shows several peculiarities that make its mitochondrial localization even more interesting respect to the role of the exchanger in the maintenance of $[Ca^{2+}]_i$ homeostasis and the prevention of cell death under oxygen-glucose deprivation conditions. Interestingly, results obtained in neurons exposed to oxygen-glucose deprivation (OGD) demonstrated that the amount of NCX3 localized on the OMM of neurons can change, suggesting the presence of a regulatory mechanism of the subcellular sorting of NCX3. Furthermore, since the hypothesis of a mechanism regulating the NCX3 localization could open a new perspective regards a possible connection between the role of NCX3 sorting within the cell and its neuroprotection function (59), the

aim of this study was to investigate the molecular determinants and the mechanism regulating the subcellular sorting of NCX3 on mitochondria.

4 MATERIALS AND METHODS

4.1 Plasmids and site directed mutagenesis

NCX1/NCX3 (193-445, 718-795, 788-829) or NCX3/NCX1 (143-167, 168-226, 707-776, 777-818) chimeras were kindly provided by Takahiro Iwamoto of Fukuoka University (Iwamoto et al., 2004).

NCX1/NCX3 718-780 chimera, the deletion mutant NCX3Δ2-31, and the fusion proteins including EGFP and CBD1 or CBD2 regions of NCX3, were obtained with QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies).

Briefly, $NCX3^{\Delta 2\text{-}31}$ was obtained by engineering the plasmid pKCRH-NCX3 and using primers pairs designed to remove 93 nucleotides sequence spanning from +3 to +96 referring to transcriptional start site (+1), of rat NCX3 cDNA (GenBank accession no. U53420). The fusion proteins EGFP-CBD1 and EGFP-CBD2, were obtained by subcloning part of rat NCX3 cDNA in a pEGFP-C2 vector (Clontech, USA). All mutants obtained were verified by sequencing both DNA strands (Primm, Milan, Italy).

4.2 Cell culture

Untransfected baby hamster kidney (BHK) cells and BHK cells stably transfected with canine cardiac NCX1.1 and NCX3.3 were a generous gift from Dr. Kenneth D. Philipson (University of California, Los Angeles, CA). BHK cells were all grown on plastic dishes in a mix of Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1) (Life technologies) supplemented with 5% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma). Cells were cultured in a humidified 5% CO2 atmosphere and maintained at 37°C.

4.3 DNA and siRNA transfection and pharmacological treatment

All plasmids were transiently or stably co-transfected with pEGFP (Clontech), to verify the transfection efficiency, by using Lipofectamine 2000 (Life Technologies, USA). For the selection of stable transfected BHK cells, 24 h after transfection 10µg/ml of G418 (Sigma) was added in the complete medium for 2 weeks, the medium was changed every two days.

For the silencing experiment, validated specific siRNAs from QIAGEN were used for the knockdown of Tom70 (Hs_TOMM70A_1), Tom20 (Hs_TOMM20_5) and Tom22 (Hs_TOMM22_9). The siRNAs were transiently transfected twice with a time interval of 24 h for TOMM70, or 48 h for TOMM20 and TOMM22, in BHK cells with a final concentration of 250 pmol/ml by using Lipofectamine 2000 (Life Technologies). NCX3 cDNA was transfected after 12h of siRNA treatment. Cells were processed for Western blot 16h after transfection. PKA phosphorylation in BHK cells was inhibited with 1μM of KT5720 in complete medium for 24 h.

4.4 Mitochondrial extracts

Mitochondrial extracts were obtained with differential centrifugations. Firstly, BHK cells were collected in a 15-ml falcon tube, after washing in phosphate-buffered saline medium (PBS), and incubated with 1 ml of trypsin solution per 10-cm plate. The trypsinization was stopped by adding 6 ml of growth medium, pelleted by centrifugation at 1000 rpm for 3 minutes and resuspended in 300 μl of ice cold homogenization buffer solution (Buffer A) containing the following (mM): 250 mannitol,

0.5 EGTA, 5 HEPES (pH 7.4), 1.5 MgCl2, 0.1% aprotinin, 0.7 mg/ml pepstatin, and 1 mg/ml leupeptin, and gently disrupted by passing ten times through a 26-gauge needle. The suspension was centrifuged at 2000 g for 5 minutes at 4°C. After the first centrifugation, pellets, corresponding to the fraction containing membranes but not intracellular organelles including mitochondria, were separated and lysed with lysis buffer containing (mM): 20 Tris-HCl (pH 7.5), 10 NaF, 150 NaCl, 1 PMSF, 1% Nonidet P-40, 1 Na_3VO_4, 0.1% aprotinin, 0.7 mg/ml pepstatin, and 1 ml/mg leupeptin, and kept on ice for 15 minutes. The supernatant, was centrifuged at 500 g for 5 minutes at 4°C. After this centrifugation the pellet was discarded, and the supernatant corresponding to the cytosolic fraction containing the organelles, was further centrifuged at 19,000 g for 10 minutes at 4°C in order to separate the mitochondrial from the cytosolic fraction. Supernatants (cytosol) were then removed and assessed for protein content. Next, the pellets containing mitochondria were lysed in 50 µl of the same lysis buffer mentioned above and kept on ice for 15 minutes. Finally, samples were purified again by centrifugation (18,000 g, 10 minutes) and supernatants (mitochondria or membranes) were assessed for protein content by Bradford's assay (Bradford, 1976). The three fractions obtained,

membranes, mitochondria and cytosol, were used for Western blotting. All the procedure was carried out at 4°C to minimize the action of proteases and phospholipases. The purity of the mitochondrial preparation was assessed by evaluating the expression of the proteins VDAC-1 and tubulin, or actin.

4.5 Western blotting

20 μg of protein samples were analyzed on 8% SDS-PAGE and electrotransferred to Hybond ECL nitrocellulose paper (Amersham). Membranes were first blocked with 5% non-fat dry milk in 0.1% Tween-20 (TBS-T; 2 mM Tris–HCl, 50 mM NaCl, pH 7.5) for 2 hours at room temperature. They were then incubated overnight at 4°C in the blocked buffer using the appropriate antibodies: 1:1000 anti-NCX1 (rabbit polyclonal antibody, Swant), 1:5000 anti-NCX3 (rabbit polyclonal antibody, Swant), 1:500 anti-GFP (mouse monoclonal antibody, Abcam), 1:1000 anti-TOMM70 (mouse monoclonal antibody, Santa Cruz BT), 1:500 anti-TOMM20 (mouse monoclonal antibody, Abcam), 1:500 anti-TOMM22 (mouse monoclonal, Abcam), 1:1000 CREB (mouse monoclonal antibody, Cell Signaling), 1:1000 p-CREB (rabbit

polyclonal, Millipore) and subsequently incubated with the secondary antibodies for 1 hour (1:5000; Amersham). Immunoreactive bands were detected with the ECL kit (Amersham). Discrimination among the distinct types of extracts was ensured by detection of the endogenous protein tubulin, or actin, (localized on plasma membrane), with respectively 1:5000 mouse polyclonal antibody anti-α-tubulin (Sigma) or 1:1000 mouse polyclonal antibody anti-β-actin (Sigma), and VDAC with 1:1000 mouse polyclonal antibody anti-VDAC (Santa Cruz BT) (localized on the outer mitochondrial membrane).

4.6 Statistical analysis

Data were generated from a minimum of three independent experiments. Data are expressed as mean ± SEM. Statistical analysis among means were determined by Student's t test or ANOVA followed by Student-Newman-Keuls test. The threshold for statistical significance data was set at $p<0.05$.

5 RESULTS

5.1 Analysis of subcellular localization of NCX3/NCX1 chimeras

Western blot experiments on mitochondrial and plasma membrane fractions obtained from BHK cells stably transfected with NCX1 or NCX3 were firstly performed to validate the differential protein extraction methodology. Results showed the localization of NCX1 on plasma membrane, and the dual localization of NCX3 on both plasma membrane and mitochondria (**Fig. 8**).

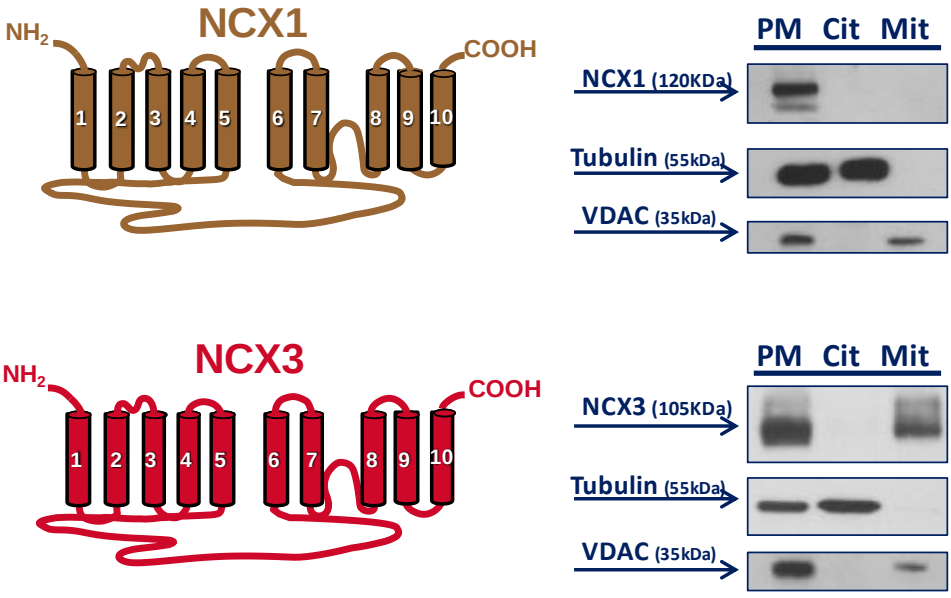

Fig. 8 Western blot of differential extraction of plasma membrane, cytosolic and mitochondrial proteins from BHK cells stably expressing NCX1 or NCX3. Tubulin and VDAC immunosignals were used to verify the purity of samples.

Plasmids expressing NCX3/NCX1 143-167, 168-226, 707-776, 777-818 chimeras (**Fig. 9**) were stably transfected in BHK cells and then analyzed for their subcellular localization. The results showed the presence of the proteins on both plasma membrane and mitochondria **(Fig. 10)**.

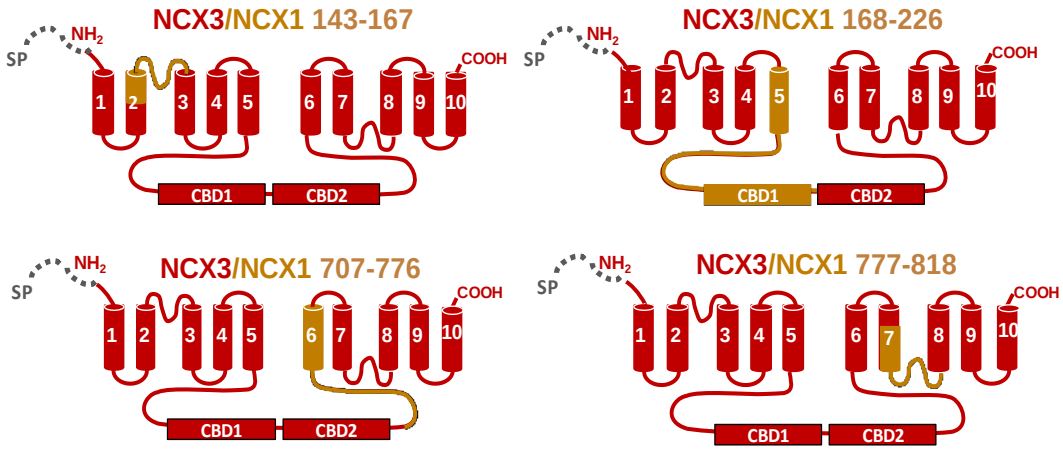

Fig.9 Topologies of chimeric proteins with NCX3 (in red) and NCX1 (in yellow) amino acid sequences. Signal peptide sequence is dashed (in grey)

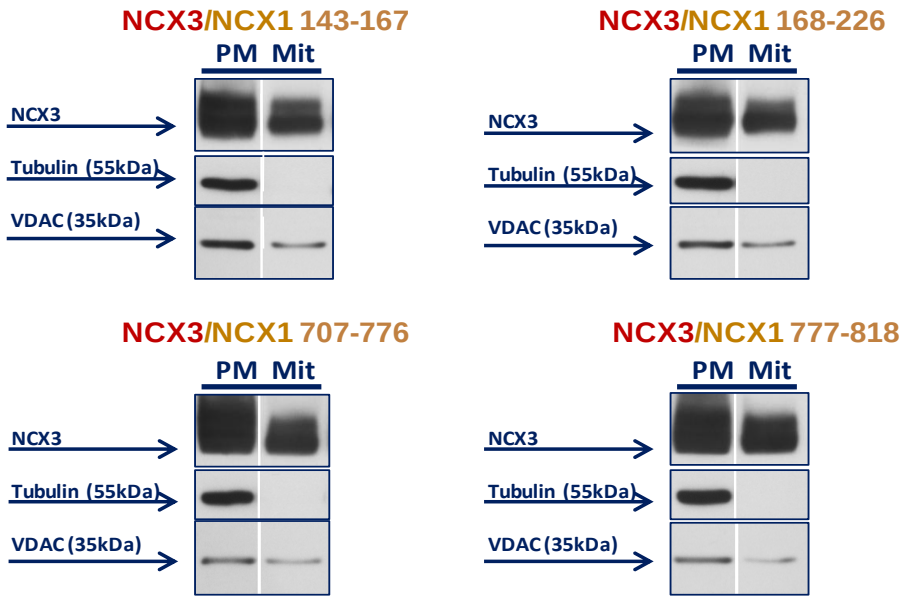

Fig. 10 Western blot of differential protein extracts from BHK cells stably expressing NCX3/NCX1 chimeric proteins. The purity of mitochondrial preparation was validated observing the absence of tubulin signal and the

5.2 Analysis of subcellular localization of NCX1/NCX3 chimeras

The bioinformatics analysis of NCX3 sequence showed the regions with low sequence homology between NCX1 and NCX3. These regions were used to calculate the relative probability to contain mitochondrial targeting peptides (mTPs) by using TargetP1.1 software (**Table 1**).

Domain	Region of NCX3	Sequence Length (aa)	Relative Probability (A.U.)
N-Terminal	1 – 109	109	58
	109 – 133	25	86
	133 – 192	59	63
	193 – 445	242	66
Cytosolic loop	446 – 717	235	51
C-Terminal	718 – 787	68	67
	788 – 829	42	61
	830 – 927	147	10

Table.1 Results of the relative probability (in arbitrary units A.U.) to contain mitochondrial targeting peptides (mTPs) in the N-terminal, C-terminal and cytosolic domains of NCX3.

On the basis of this information, we designed NCX1/NCX3 chimeras, where several regions of NCX1 (109-133, 193-445, 446-718, 718-787, 788-829) were replaced with the same corresponding regions of NCX3. Results showed that the substitution of the region 109-133 of NCX1 with the corresponding region of NCX3 localized the chimeric protein on mitochondria **(Fig. 11)**. By contrast, the substitution of the region 788-829 of NCX1 with the corresponding region of NCX3 did not localize the chimeric protein on mitochondria **(Fig. 11)**.

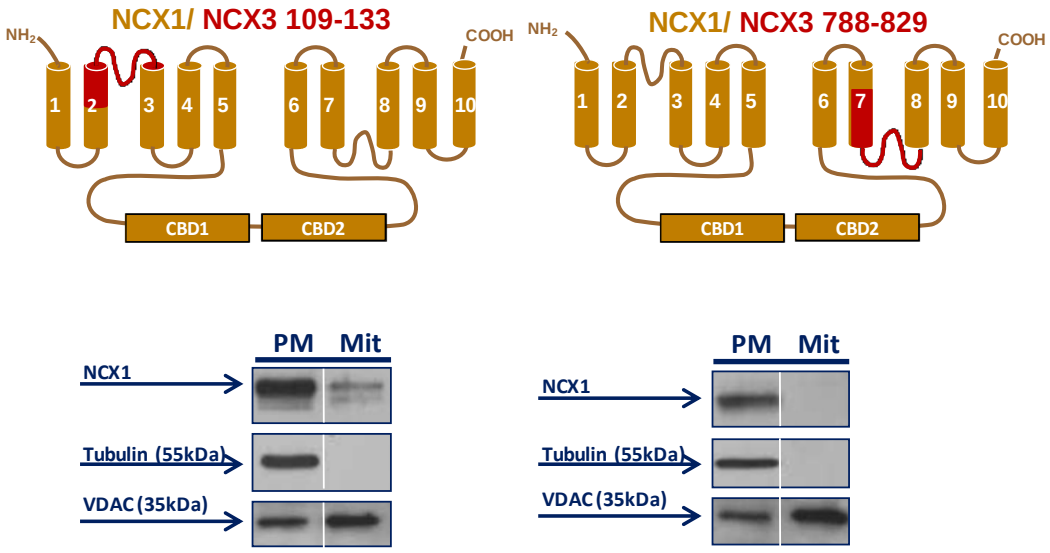

Fig. 11 Western blot of differential protein extracts from BHK expressing the chimeric proteins NCX1/NCX3 109-133 or NCX1/NCX3 193-445.

Furthermore, NCX1/NCX3 chimeras in which regions 718-795 or 788-829 of the C-terminal domain of NCX1 was substituted with the corresponding region of NCX3 displayed a subcellular localization forboth mitochondria and plasma membrane (**Fig. 12**).

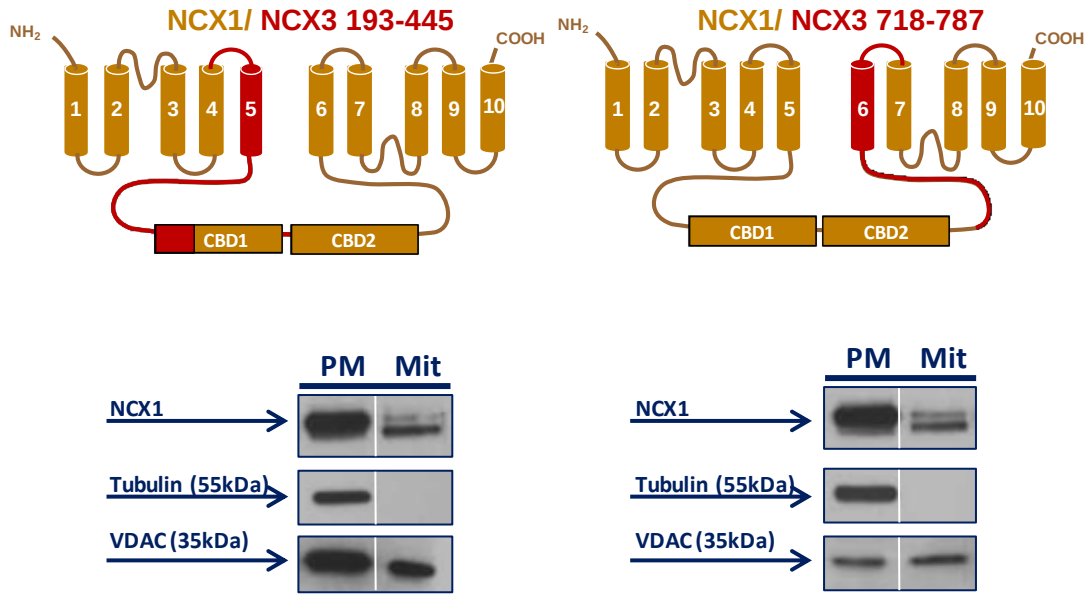

Fig. 12 Western blot of differential protein extracts from BHK expressing the chimeric proteins NCX1/NCX3 788-829 or NCX1/NCX3 718-795.

Since the region 718-795 was wide, another smaller region (718-780) corresponding to a portion of cytosolic domain was analyzed. Results showed that this chimera was also localized on both plasma membrane and mitochondria (**Fig. 13**).

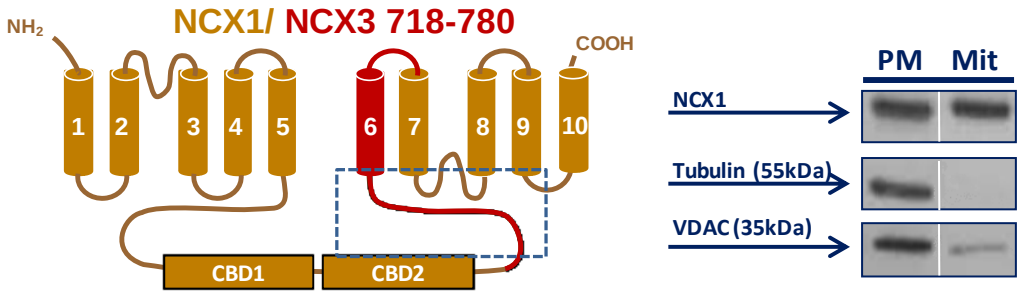

Fig. 13 Western blot of differential protein extracts from BHK expressing the chimeric protein NCX1/NCX3 718-780.

5.3 Analysis of subcellular localization of CBD1 or CBD2 domains of NCX3

Western blot experiment on subcellular fractions from BHK cells transfected with the plasmids expressing the NCX3 sub-domains CBD1 and CBD2 fused with EGFP showed no mitochondrial localization as well **(Fig. 14 A)**. By contrast, EGFP alone did not show mitochondrial localization **(Fig. 14 B).**

Results obtained excluded that these regions are involved in the mitochondrial localization of NCX3.

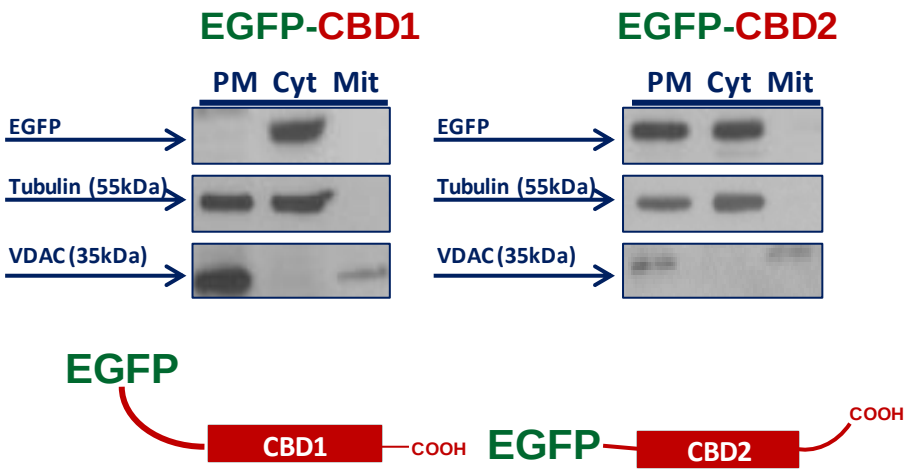

Fig. 14 A Western blot of differential protein extracts from BHK expressing the EGFP fused with the sub domains CBD1 or CBD2 of NCX3 protein.

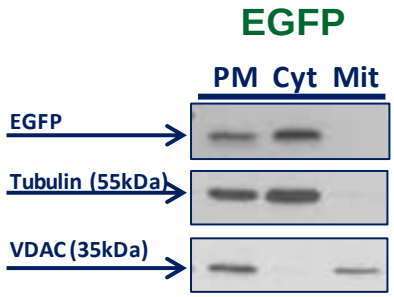

Fig. 14 B Western blot of differential protein extracts from BHK expressing the EGFP

5.4 Identification of mitochondrial chimeric targeting signals in NCX3

Western blot analysis on plasma membrane and mitochondrial fractions, obtained from BHK cells stably transfected with the wild type form of NCX3 and treated with the PKA inhibitor KT5720, was performed to evaluate the involvement of a PKA-mediated mechanism of activation of mitochondrial chimeric targeting signals in NCX3. The efficiency of the inhibitor was primary tested on BHK cells assessing the expression of the main molecular target of PKA, the cAMP response element-binding protein (CREB) and its phosphorylated form (pCREB). The results obtained demonstrated that, although the CREB/p-CREB phosphorylation rate was decreased (**Fig 15 A**), the PKA activity inhibition did not show interference with subcellular targeting of NCX3 in plasmamembrane and mitochondrial compartments, compared to the control cells (**Fig. 15 B**).

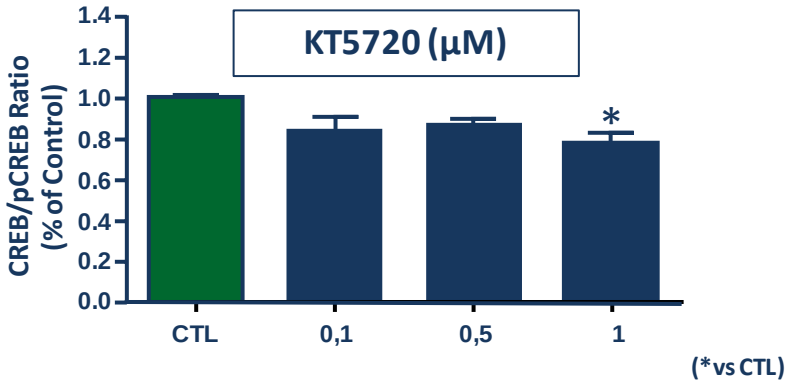

Fig. 15 A. Western blot analysis of CREB/pCREB ratio in total extracts from BHK cells treated with 1µM of KT5720 for 24h.

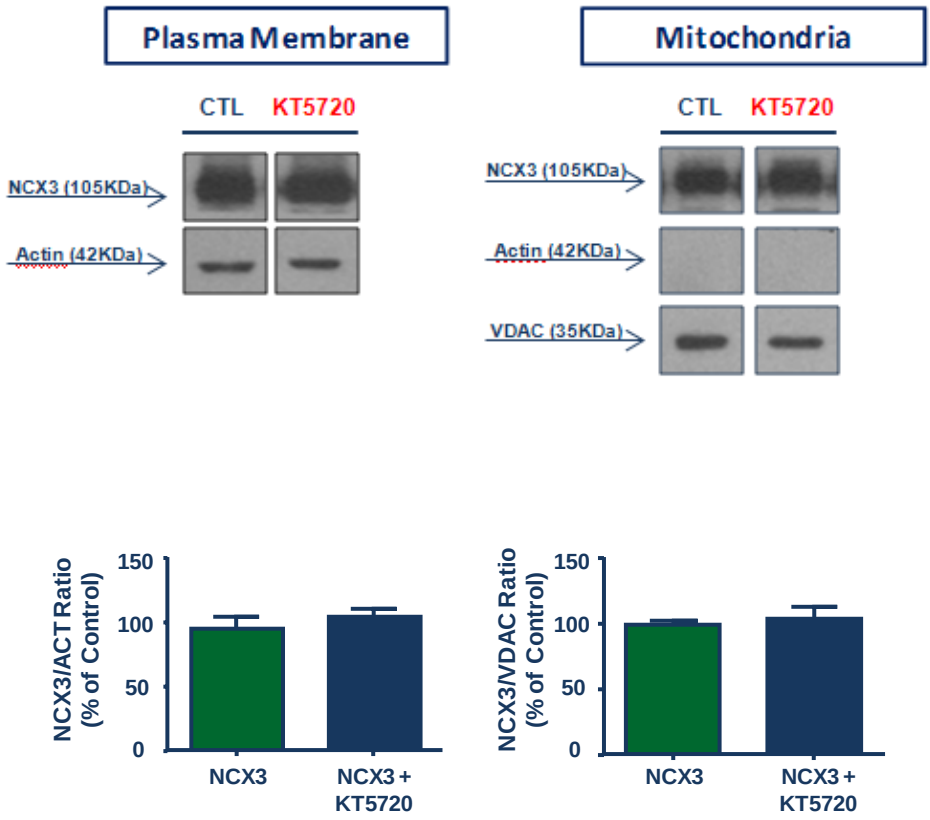

Fig. 15 B Western blot analysis of subcellular localization of NCX3 in BHK cells transiently expressing NCX3 (CTL) and treated with KT5720 1µM for 24h.

The mutant exchanger NCX3Δ2-31 displayed an increased ratio in mitochondrial localization normalized for VDAC, as compared to the control cells (**Fig. 16**).

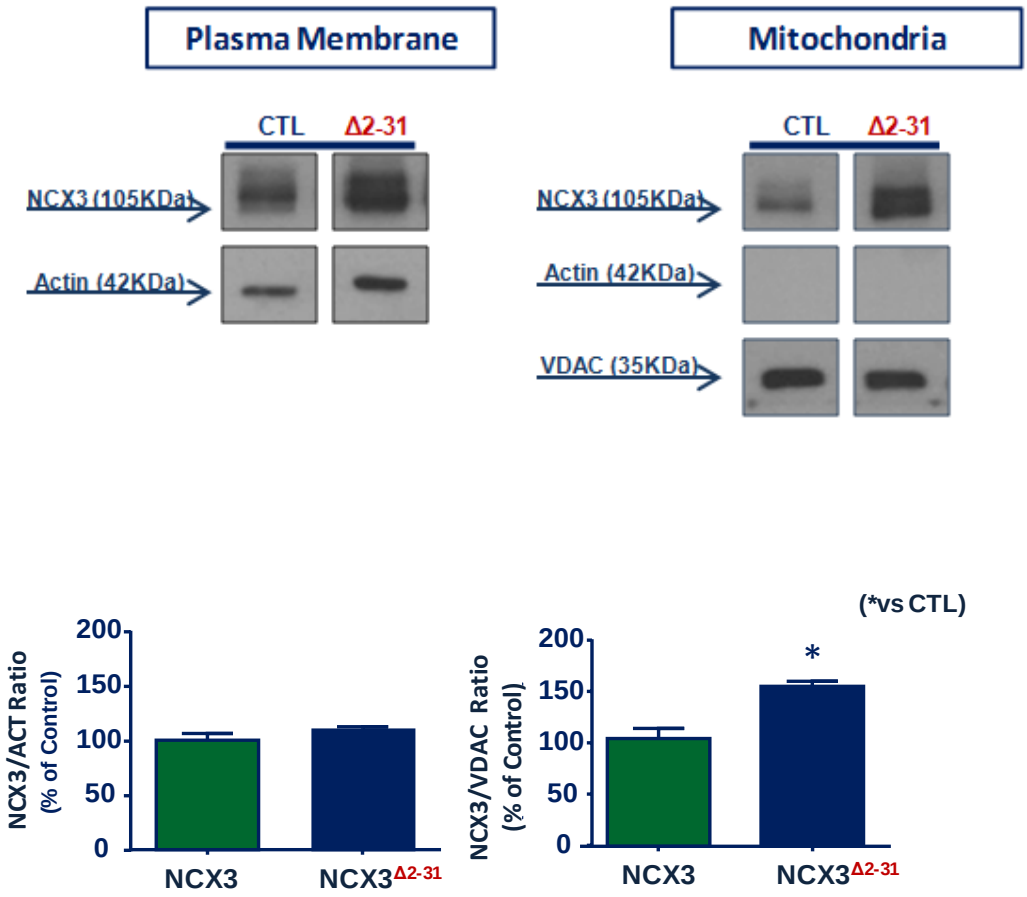

Fig. 16 Western blot analysis of NCX3 (CTL) and NCX3 lacking the first 30 amino acids (NCX3$^{\Delta 2\text{-}31}$) stably expressed in BHK cells.

5.5 Silencing experiment of the mitochondrial outer receptors

Western blot on BHK cells treated with siTOM20, siTOM22 or siTOM70 showed that these mitochondrial receptors significantly decreased at 48h from the siRNA transfection (**Fig. 17**).

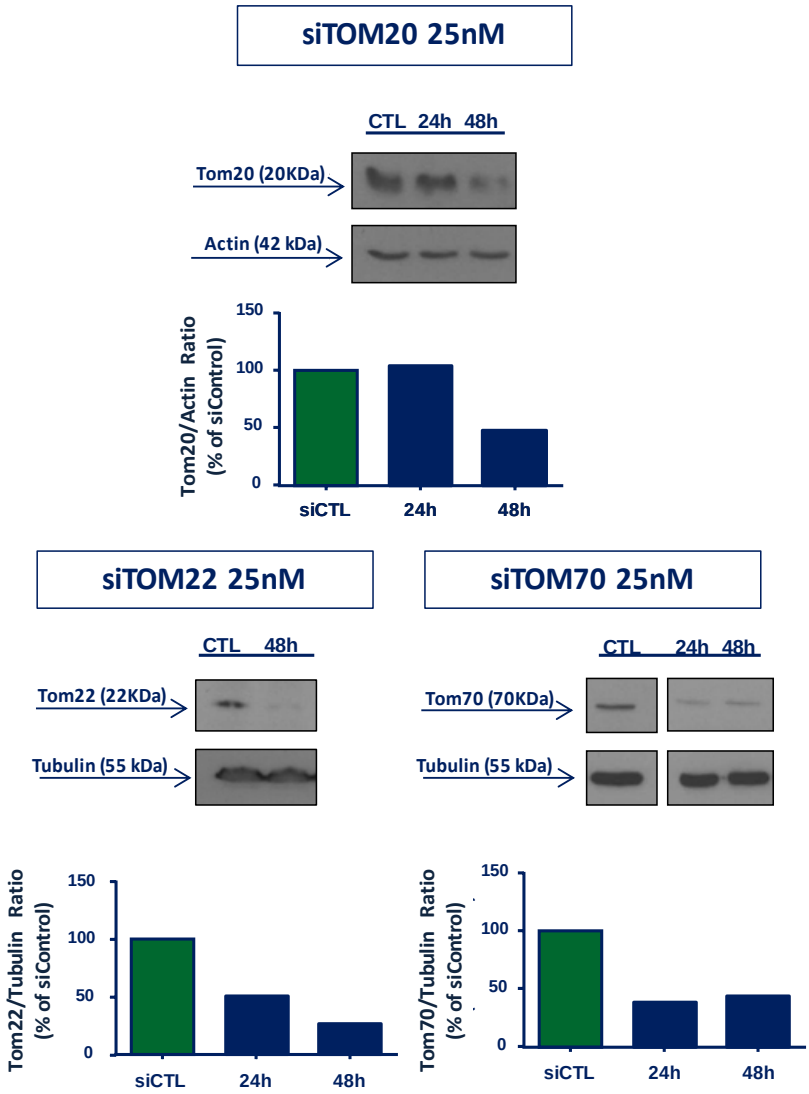

Fig. 17 Effect of the silencing by RNA interference of the mitochondrial outer membrane receptor TOM20, TOM22 or TOM70 in BHK cells.

The silencing of TOM22 or TOM70 was caused a decrease of NCX3 immunosignal on mitochondrial fraction as compared to BHK cells transfected with untargeted siRNA (**Fig. 18**). By contrast, the silencing of the mitochondrial receptor TOM20 did not affect the amount of NCX3 on mitochondrial fraction (Fig. 12).

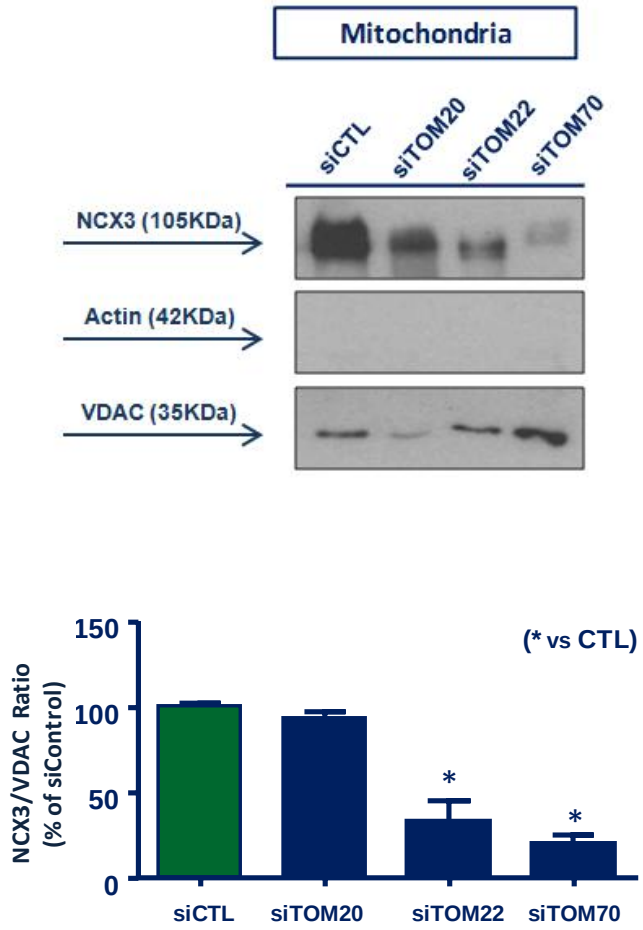

Fig. 18 NCX3 expression on mitochondria of BHK cells after RNA interference of TOM20, TOM22 or TOM70 receptors.

6. DISCUSSION

In this study, we found for the first time the mechanisms involved in the recognition and import of NCX3on mitochondria.

The most interesting results we obtained is the presence of multiple mitochondrial localization signals spread along the entire sequence of NCX3 antiporter as it occurs in other proteins present on the outer mitochondrial membrane (78; 79; 80; 81; 82). Our data showed that these signals are localized at the amino terminus, 109-133aa corresponding to the α_1 repeat, at the carboxy terminus, 788-829aa corresponding to the α_2 repeat, and at the C-terminal portion of the f-loop, 718-787aa. Interestingly, each identified amino acid regions was able to localize NCX1 on mitochondria suggesting that these mitochondrial targeting signals do not form a unique recognizable structure.

Another aspect that deserves consideration is that the first mitochondrial signal of NCX3,α_1, is close to a signal peptide that is cleaved during the protein maturation, as it occursin other proteins with multiple subcellular localizations. Furthermore, this signal peptide for the plasma membrane localization seemed to be competitive with mitochondrial localization

sequences present on NCX3. Indeed, the removal of the signal peptide, by using a deletion mutant of NCX3, $NCX3^{\Delta 2\text{-}31}$, increased the amount of the antiporter on mitochondria. These data strongly suggest that NCX3 is provided with a chimeric localization signal that might be controlled by regulating the endoproteolytic cleavage of its signal peptide as it occurs in other proteins with multiple subcellular localizations. It is possible to speculate that this might represent a mechanism for the regulation of the Ca^{2+} homeostasis under physiological and pathophysiological conditions, indeed, NCX3 can reorganize its subcellular localization depending on several physiological and pathological conditionshelping cells to recover basal conditionsas demonstrated in previous papers (59).

Another interesting finding emerging from our studies is the participation of both mitochondrial receptors Tom70 and Tom22 in the import of NCX3 on mitochondria. In fact, the silencing of either Tom70 or Tom22 causes a significant decrease in NCX3 localization on mitochondria. The requirement of both mitochondrial receptors is not surprising because Tom70 is mainly involved in the recognition of precursor proteins targeted to mitochondria provided with multiple transmembrane α-helical segments (81) as NCX3, whereas Tom22 is mainly involved in the transfer of the pre-protein on the outer

mitochondrial membrane (88; 84).In addition, our results showed that the recognition of NCX3 by the mitochondrial import machinery was not affected by a PKA-dependent phosphorylation as it occurs for several mitochondrial proteins.

Although further experiments are needed to characterize the regulation of the subcellular localization of NCX3, we can hypothesize this mechanism might affect the subcellular ionic homeostasis and thus the cellular function and vitality.

7. REFERENCES

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