# VDAC2 as a novel target for heart failure: Ca2+ at the sarcomere, mitochondria and SR
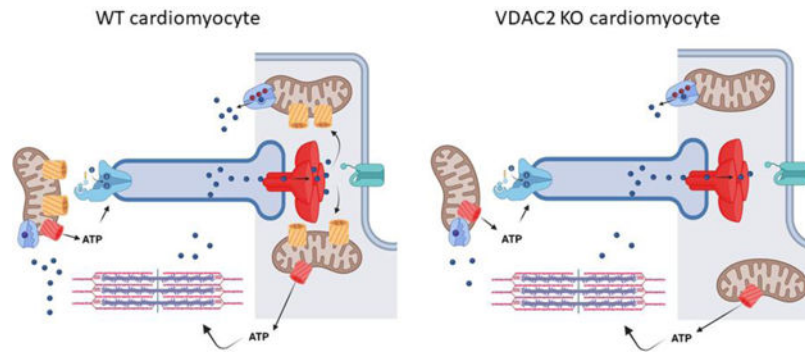
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## Abstract

Despite a growing number of successful therapies, heart failure remains the most common cause of death and disability worldwide. Thus, new and novel therapeutic strategies are urgently needed. Mitochondria of cardiomyocytes generate ATP that is needed to power cardiac contraction. Mitochondrial-derived ATP activate myosin ATPase at the sarcomere and the sarcoplasmic reticular (SR) ATPase $Ca^{2+}$ pump, both which intersect with $Ca^{2+}$ during contraction. Failure to maintain the relationship between mitochondria and SR can lead to cardiomyocyte dysfunction and heart failure. Here, we discuss recent discoveries that reveal $Ca^{2+}$ transport via the voltage dependent anion channel (VDAC) into the mitochondria can favorably impact cardiac contraction and prevent cardiac arrhythmias. In a broader view, discussion of the opening of a new era for HF therapeutics that will address the sarcomere, SR and mitochondria as a functional unit.

## Graphical Abstract

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Conflict of interest

The author has declared that no conflict of interest exists.

## Keywords

Voltage dependent anion channels; Calcium; Contractility; Mitochondria; Heart failure; Excitation Contraction Coupling; Mitochondrial $Ca^{2+}$ uptake transporter

## 1. Introduction

With each heartbeat, blood is ejected from the left ventricle and delivered to the body. The amount of blood ejected changes based on the metabolic demands of the heart. As such, intensity of cardiac contraction, or contractility, must be regulated over a range of workloads such as during sleep or intense exercise and requires adjustments in oxygen consumption [1] [2]. A balance of the demand for myocardial oxygen consumption is met by the energy produced (i.e. ATP) by mitochondrial metabolism in order to ensure adequate contractility[3]. When oxygen consumption exceeds supply, contractility is impaired and cardiac function declines[4]. In addition to this energy balance for ATP, contractility also requires the transient flux of cytosolic $Ca^{2+}$ on a beat-to-beat basis in order to activate key $Ca^{2+}$ dependent steps of actin-myosin crossbridge shortening[5]. Indeed, the height of the $Ca^{2+}$ transient and ATP availability are key determinants of the force of contraction of individual cardiomyocytes which involves communication between the sarcomere, sarcoplasmic reticulum (SR) and mitochondria[6, 7].

$Ca^{2+}$ transients in cardiomyocytes are triggered by the opening of the L-type $Ca^{2+}$ channels in the T-tubule membrane and then the emptying of the ryanodine receptor (RYR2) $Ca^{2+}$ stores by $Ca^{2+}$ induced $Ca^{2+}$ release[8, 9]. Dispersal of $Ca^{2+}$ throughout the cytosol enables $Ca^{2+}$ to interact with troponin C to relieve inhibition of the actomyosin complex and shorten the cardiomyocyte in a mechanism involving ATP hydrolysis by myosin. ATP hydrolysis is also required for $Ca^{2+}$ re-sequestration in the sarcoplasmic reticulum (SR) by the $Ca^{2+}$ ATPase, SERCA2a. Thus, ATP and $Ca^{2+}$ must both be available to ensure proper systolic and diastolic function of the heart[10]. The ATP levels must remain constant in the cardiomyocyte and require matching of the ATP production and degradation. In this case, ATP must be regenerated and ADP and Pi removed from the sarcomere[11]. Much like $Ca^{2+}$, sufficient supply of ATP must be available to the myosin-ATPase to power contraction[12]. Importantly, ADP, the reaction product of ATP hydrolysis, must be removed from the sarcomere or cardiomyocytes will fail to relax resulting in damage[11, 13]. On the other hand, cardiomyocytes rapidly move $Ca^{2+}$ into and out of the cytosol in order to prevent cell damage from $Ca^{2+}$ overload[14]. Often this involves sequestration of $Ca^{2+}$ in the SR or mitochondria and also involves extrusion of $Ca^{2+}$ across the T-tubule membrane. Failure to remove $Ca^{2+}$ from the cytosol during diastole prevents the relaxation of the cardiomyocyte and limits contractility. It is clear that cardiomyocytes must regulate the production and storage of both ATP and $Ca^{2+}$ in order to maintain function and this involves collaboration between mitochondria and the SR.

The heart must function continuously to ensure survival and operates with limited energy reserve. In fact, reserves are sufficient to provide ATP for one minute of cardiac contractions until cardiac standstill ensues. To meet this challenge, an efficient metabolic system has

evolved in the heart to produce and consume only the exact amount of ATP required to maintain homeostasis. Too little ATP resulting from energy depletion of the heart would limit contractility and trigger arrhythmias resulting in heart failure. Too great a demand for ATP can result in excessive redox damage that impairs cardiomyocytes and even triggers apoptosis inducing heart failure. ATP concentration remains constant over a range of workloads. To achieve balance, cardiomyocytes utilize different metabolism pathways. The phosphocreatine shuttle is an energy transfer system in cardiomyocytes that provides communication about ATP-ADP-Pi status between the cytosol and mitochondria whereby ATP is regenerated and delivered to the ATPases at the sarcomere (myosin) and the SR (SERCA2a). Muscle specific isoforms of creatine kinase enzymes localize to the sarcomere or the SR whereas mitochondrial isoforms can be found in the mitochondrial free space. ATP/ADP are then channeled from the cytosolic locations into the mitochondria where it is phosphorylated to ATP [15, 16]. In this way the phosphocreatine shuttle connects sites of consumption with ATP re-supply sites in the mitochondria. In addition, ADP is rapidly removed from the sarcomere and SR in order limit product-inhibition of the myosin-ATPase or SERCA2a-ATPase. The phosphocreatine transfer system ensures the rapid flux of energy equivalents to accommodate excitation contraction coupling (ECC).

Mitochondria adjust oxidative metabolism in order to maintain bioenergetics and ATP homeostasis for cardiac contraction[17]. Key metabolic enzymes sense the changes in mitochondrial $Ca^{2+}$ that accompany $Ca^{2+}$ transients and alter activity for those dehydrogenases involved in the TCA cycle[18, 19]. Fuel substrates derived from many carbon sources including oxidation of glucose, ketones, fatty acids and amino acids are available to the cardiomyocytes. During heart failure, substrate metabolism is limited and metabolic inflexibility develops when failing cardiomyocytes utilize glucose metabolism whereas healthy cardiomyocytes prefer fatty acid oxidation [20, 21]. $Ca^{2+}$ binds to the solute carrier family members (SLC2512 and SLC25A13) via EF-binding domains that reside in the intermembranous space[22]. $Ca^{2+}$ bound to SLC25A12 activates the transport of cytosolic glutamate across the IMM in exchange for mitochondrial aspartate. These $Ca^{2+}$-dependent transporters are part of the cytosolic-mitochondrial aspartate/malate shuttle and supply reducing equivalents that are available to the electron transport chain (ETC) in a $Ca^{2+}$ dependent manner[23]. Matrix $Ca^{2+}$ can bind and activate the pyruvate dehydrogenase complex (PDH), the NAD-linked isocitrate dehydrogenase (ICDH) and the 2-alpha ketoglutaraldyde (KDH). Pyruvate is the end product of glycolysis and is converted to acetyl-CoA by the pyruvate dehydrogenase enzyme complex[24]. Importantly, the PDH-phosphatase (PTP1a) requires $Ca^{2+}$ binding, and an increase in $[Ca^{2+}]_M$ can accelerate acetyl-coA production which provides two carbons to the enzymes of the TCA-cycle located in the inner mitochondrial membrane (IMM)[25]. $Ca^{2+}$ may also regulate the ATP-synthase via post-translational mechanism or through other indirect mechanism. What is clear from these studies is that mitochondrial $Ca^{2+}$ influences energy production at several levels in the mitochondria and acts in a feed forward mechanism enabling energy demand to match efficiently with ATP supply[26].

## 2. Mitochondrial Calcium Signaling

Mitochondria occupy 30–40% of the cell volume of cardiomyocytes, have critical roles in the generation of ATP, protect cells from redox damage, and confer metabolic flexibility by which the cell ensures energetic adaptability[27]. How mitochondria coordinate these cellular functions is likely to require changes to the level of mitochondrial $Ca^{2+}$ levels. $Ca^{2+}$ signaling has emerged over the last several decades as a critical factor in cell homeostasis where it can interact with $Ca^{2+}$ sensitive enzymes localized in the mitochondrial IMM and matrix in order to modulate oxidative metabolism. As such, mitochondria require an elaborate signaling system to control mitochondrial $Ca^{2+}$ levels involving its uptake, efflux and buffering. Mitochondrial $Ca^{2+}$ levels range from 100 nM in resting cells to 1μM following cellular stimulation and even levels around 1M under pathologic conditions[28]. $Ca^{2+}$ influx to mitochondria can also influence cell $Ca^{2+}$ signaling that protects the cardiomyocyte and provides optimal energy production. Mitochondria can functionally influence $[Ca^{2+}]_c$ as ion channel modulators, as firewalls to separate vulnerable regions of the cell from very high $[Ca^{2+}]_c$ levels, and in the formation of nanotunnels to promote intermitochondrial connections (Figure 1)[29–31]. Common to each of these interactions is the close proximity of the mitochondria to the cytosolic $Ca^{2+}$ source that highlights the importance of the relationship of mitochondria with the architecture of the cardiomyocyte. For example, does the spatial relationship between the mitochondria and the source of cytosolic $Ca^{2+}$ flux matter? The mitochondria have a low affinity mechanism for $Ca^{2+}$ uptake and therefore require very high $Ca^{2+}$ levels to operate these transport mechanisms. Often this requires mitochondria to have close contact with RYR2 or the inositol trisphosphate receptor (IP3R). In comparison to global $Ca^{2+}$ signals, these local $Ca^{2+}$ release events can deliver intense amounts of $Ca^{2+}$ and rapidly fill mitochondria. In contrast, mitochondria in close apposition to the $Ca^{2+}$ pumps (SERCA2a) are not exposed to very high levels of $Ca^{2+}$ as $Ca^{2+}$ is diverted into the SR. It is clear these spatial and temporal parameters can lead to heterogenous $Ca^{2+}$ signals among different mitochondria.

Like the rest of the cell, individual mitochondria require a separate repertoire of ion channels, transporters and exchangers that direct $Ca^{2+}$ uptake into the matrix (Figure 2). $Ca^{2+}$ collects in two spaces of the mitochondria that include the intermembranous space and the interior matrix. $Ca^{2+}$ must therefore pass through two separate membranes to reach the mitochondrial matrix where it is heavily buffered by phosphate. First, cytosolic $Ca^{2+}$ crosses the outer mitochondrial membrane (OMM) via the voltage dependent anion channel (VDAC) into the intramitochondrial space[32]. $Ca^{2+}$ then is transported across the inner mitochondrial membrane (IMM) into the mitochondrial matrix via the mitochondrial $Ca^{2+}$ uptake (MCU) transporter. A second mechanism to alter mitochondrial $Ca^{2+}$ levels is the mitochondrial permeability transition pore (mPTP). Under unusual cellular conditions that involve matrix $Ca^{2+}$ overload (e.g. ischemia), the ATP synthase can act in reverse to hydrolyze ATP and form a mega-$Ca^{2+}$ channel that shares features with the mPTP. The mPTP can then uncouple oxidative phosphorylation when the IMM potential dissipates, resulting in increased permeability to $Ca^{2+}$. $Ca^{2+}$ extrusion mechanisms for removal of $Ca^{2+}$ across the IMM and mitochondria matrix include the sodium $Ca^{2+}$ exchanger (NCLx) and $H^+/Ca^{2+}$ antiporter assumed to be the Leucine Zipper and EF handing containing

transmembrane protein 1 (LETM1)[33–35]. Mitochondrial $Ca^{2+}$ levels are set by a number of factors including the actions of these transporters, mitochondria size and fusion-fission capacity, membrane potential and through interactions with other organelles including ER, lysosomes, surface membrane and nucleus[29, 36, 37]. Mitochondrial $Ca^{2+}$ signals differ not only between cell types but also vary among different mitochondria within the same cell[38, 39]. In atrial cardiomyocytes, mitochondria sequester $Ca^{2+}$ released during excitation contraction coupling (ECC). As shown by the Bootman lab, cytosolic $Ca^{2+}$ signals originate from the periphery of atrial cell, unlike ventricular cardiomyocytes, and do not spread toward the interior of the cell. Rather mitochondria sequester $Ca^{2+}$ released from the RYR2 stores. In fact, inhibiting mitochondrial $Ca^{2+}$ signaling led to globalized $Ca^{2+}$ transients in atrial cells following pacing[30]. These results highlight the importance of mitochondria in the shaping and control of cytosolic $Ca^{2+}$ signals. Given the clinical importance of atrial fibrillation worldwide, targeting mitochondrial $Ca^{2+}$ signaling in atrial cell might represent an important novel strategy.

Mitochondria are dispersed throughout the cardiomyocyte at various locations including the perinuclear region (PNM), subsarcolemmal domains (SLM) and intramyofibrillar (IFM) regions[40]. These populations of mitochondria exhibit differences in $Ca^{2+}$ handing, redox balance, motility and morphology. These differences may reflect the spatial relationship of these mitochondria to its neighboring source of ATP consumption. The clearest example of this is that IFM, compared to PNM, have more robust $Ca^{2+}$ handling, are more resistant to redox challenge and are static without much migration. The IFM mitochondria are closest to the sarcomere and have the greatest ATP demands. This has raised the hypothesis that subpopulations of mitochondria must adapt to their immediate environment. Notably, IFM reside in the rigid architecture of the myofibrils and are subject to rhythmic contractions. The restricted environment of the sarcomeric lattice and the mechanical signals ensure IFM mitochondria do not migrate along microtubules and do not undergo extensive changes in shape. In contrast, PNM are more mobile and seems to have different functional roles through interactions with the nucleus and lysosomes. One explanation for the difference in $Ca^{2+}$ handling for these different mitochondria is that $Ca^{2+}$ handling proteins are located in specific domains of the mitochondria themselves and therefore create a heterogynous mitochondrial $Ca^{2+}$ response. For example, it has also been shown that differential localization of mitochondrial $Ca^{2+}$ uptake transporter (MCU) near the junctional SR ensures exposure to high $Ca^{2+}$ levels to activate MCU whereas NCLx is expressed away from the junctional SR[41]. The mechanism underlying these different populations is presently unknown but may involve interaction with different $Ca^{2+}$ signals (e.g. RYR2 or IP3R), connections with the cytoskeleton (desmin, tubulin or a-synuclein) or other factors[42, 43]. Most studies of mitochondrial function have been performed over the years using isolated mitochondrial preparations where spatial considerations are lost as are connections to the cytoskeleton. Given that different phenotypes have been assigned to these distinct populations, it will be important to understand how these regions of mitochondria behave in isolated mitochondrial preparation.

Sub-populations of mitochondria differ in mitochondrial $Ca^{2+}$ uptake capacity, buffering capacity and $Ca^{2+}$ release. In particular, IFM take up the greatest amount of $Ca^{2+}$ compared to the SLM and PNM. Lu et al used the genetically encoded $Ca^{2+}$ indicator (mitycam)

to measure $[Ca^{2+}]_M$ in isolated and intact cardiomyocytes[40]. Detailed measurements of $Ca^{2+}$ enabled comparisons of the different fractions that led to the conclusion that mitochondria located at the Z-line exhibited markedly elevated amplitude of $[Ca^{2+}]_M$ that rise more rapidly than $Ca^{2+}$ uptake for mitochondria located at the M-line. These different populations of mitochondria may account for the spatial and temporal $Ca^{2+}$ signals in cardiomyocytes. Cardiomyocytes must adapt their energetic need to events at the sarcomere and the membrane and local control of mitochondrial $Ca^{2+}$ signals can confer adaptability. Similarly, mitochondria are subject to fission-fusion events and migrate via microtubules throughout the cell. Another key parameter for mitochondrial $Ca^{2+}$ uptake is the source of the cytosolic $Ca^{2+}$ flux. Global changes in $Ca^{2+}$ transients offer much less $Ca^{2+}$ than microdomains of the mitochondria that involve close proximity of OMM to ER membranes or lysosomal membranes[40, 44]. How mitochondrial $Ca^{2+}$ contributes to these events is increasingly been recognized and will likely lead to a greater understanding of the physiologic role of mitochondria $Ca^{2+}$ signaling confers on the cardiomyocyte.

The interdependence of mitochondrial and cytosolic $Ca^{2+}$ signaling has been recognized with increasing frequency and recent studies have established the pathogenic implications as well as potential therapeutic possibilities[11, 42, 45]. Disordered $Ca^{2+}$ release from the SR of cardiomyocytes is a well-established pathogenic mechanism that contributes to cardiomyocyte death for patients subjected to acute cardiac injury such as ischemia or in patients with genetic mutations in key $Ca^{2+}$ handling proteins. Leaky RYR2 channels, faulty SERCA2a pumping and inefficient buffering by calsequestrin are all associated with arrhythmia and depressed contractility[46–48]. Here inhomogeneous $Ca^{2+}$ gradients, spontaneous sparks, elevated diastolic $Ca^{2+}$ levels can give rise to dysfunctional $Ca^{2+}$ transients[49]. For example, mutations in the RYR2 $Ca^{2+}$ release channel cause catecholaminergic polymorphic ventricular tachycardia (CPVT), a rare genetic cause of sudden cardiac death. Dysregulated $Ca^{2+}$ release can be sequestered into mitochondria at high levels.

Mitochondrial $Ca^{2+}$ overload compromises energetic efficiency by impairing oxidative phosphorylation resulting in reduced contractility, greater production of reactive oxygen species (ROS) and even trigger cardiomyocyte apoptosis. The increased ROS produced by faulty RYR2 $Ca^{2+}$ release oxidize the RYR2 channel and amplify greater $Ca^{2+}$ leak. Cardiomyocytes from mice bearing the RYR2 mutant exhibited $Ca^{2+}$ signaling and mitochondrial defects, including the presence of nanotunnels that develop between adjacent mitochondria[29]. These nanotunnels facilitate communication between mitochondria and protect myocardial energy reserves. Excessive $Ca^{2+}$ leak causes impaired ATP production by mechanism that involve inhibition of complex I or TCA cycle enzymes pyruvate dehydrogenase (PDH) and $\alpha$-ketoglurate dehydrogenase ($\alpha$KGDH) and the adenine nucleotide translocase (ATN). The emerging view of mitochondria $Ca^{2+}$ signaling is that key relationships between the mitochondria and surrounding organelles modulate ATP production to promote optimal energetic efficiency.

## 3.  Cardiac VDAC channels

The outer mitochondrial membrane (OMM) is a key barrier for mitochondria and provides insulation from events occurring in the cytosol. As a consequence, OMM permeability which is mediated by the VDACs is the primary means of communication between mitochondria and cytosol. VDAC1–3 comprise a family of channels that assemble into tetramers with a beta-barrel structure[50, 51]. Functionally, VDAC porins open and close in order to exchange nucleotides, metabolites and ions across the OMM, but recent work has revisited the role of VDACs in mitochondrial $Ca^{2+}$ handling[52, 53]. The gating mechanism for VDACs, as established from lipid bilayer studies, show they exist in high and low conductance closed states. In the high conductance state, VDACs are permeable to metabolites and nucleotides and less permeable to $Ca^{2+}$. In contrast, the closed state of VDAC are less permeable to metabolites and yet exhibit much greater permeability to $Ca^{2+}$[32]. Importantly, voltage changes across the bilayer govern the transitions of VDAC from the high conductance states at neutral voltages to the closed states at more negative membrane potentials. Because the OMM potential does not ordinarily vary in resting cells, it has been difficult to extrapolate the relevance of the required membrane change to gating properties of VDACs. Recent evidence has accrued to indicate VDAC gating is influenced by lipid content of the bilayer, post translation modification of VDAC (ubiquitination, phosphorylation, and acetylation), $Ca^{2+}$ binding or even interaction with protein partners (e.g. mono-tubulin, Bcl-2 or other factors)[54–57]. These different post-translational modifications to VDACs support the idea that VDACs are subject to regulation by different signaling pathways that are located on both sides of the OMM. It is therefore likely that VDACs must interpret signals emanating from different cellular context. Consequently, VDACs have now emerged as a key therapeutic target for a range of illnesses from cardiac arrhythmias to cancers. Notably, VDACs can impact $Ca^{2+}$ signaling, oxidative metabolism and generation of reactive oxygen species[32]. The different VDAC isoforms are highly homologous and are expressed ubiquitously in most tissues and cells types, with the exception of VDAC3 channels which are most often found in the testis. While VDAC1 and VDAC3 appear to have overlapping function based on mitochondrial studies from MEFs lacking either or both isoforms, it appears VDAC2 has non-overlapping functions since VDAC2 KO mice are embryonically lethal[58]. These studies also show that VDAC2 has a key role in BCl-2 apoptotic signaling. It remains unknown whether cardiogenesis was impacted by the deletion of VDAC2 as has been described for adenine nucleotide translocator (ANT) KO mice[59, 60].

The role of VDAC2 in cardiac rhythmicity emerged from a landmark study by Shimizu et al[61]. These investigators used the zebrafish mutant called temblor as a model of cardiac arrythmia. Tremblor cardiomyocytes lack Na/$Ca^{2+}$ exchanger (NCX1) and display dysfunctional $Ca^{2+}$ transients. A suppressor screen for compounds that could restore proper cardiac rhythmicity identified Efsevin as a hit. Unexpectanly, biochemical studies showed that the drug bound to VDAC2 and promoted $Ca^{2+}$ uptake into the mitochondria. Additionally, VDAC2 overexpression in the tremblor cardiomyocytes was also able to restore cardiac rhythmicity. These studies establish the idea that VDAC2 mediated $Ca^{2+}$ uptake could correct abnormal $Ca^{2+}$ events occurring in the cytosol. While

mitochondrial $Ca^{2+}$ microdomains have been described for VDACs and RYR2 or IP3R, no such associations have been demonstrated for NCX1. Enhancing mitochondria $Ca^{2+}$ uptake can rescue cardiomyocytes lacking NCX1 and implies that NCX1 may interact with mitochondria in a microdomain which is separate from that described for ECC. Spontaneous $Ca^{2+}$ release events in embryonic cardiomyocytes trigger NCX1 and triggers contraction as part of the $Ca^{2+}$ clock mechanism in order to create regenerative $Ca^{2+}$ transients rather than ECC that focuses mostly on the cav1.2-RYR2 mechanism. In the case of $Ca^{2+}$ clock, NCX1 depolarizes cells to the threshold potential for cav1.2 that can trigger an action potential and contraction. It has also been proposed that $Ca^{2+}$ clock mechanism re-emerging in the failing heart to cause arrhythmias and is likely the mechanism underlying the Tremblor mutant. The notion that increasing $Ca^{2+}$ uptake into the mitochondria under conditions of faulty NCX1 function and elevated diastolic $Ca^{2+}$ implies that mitochondria may function as a popoff valve for elevated diastolic $Ca^{2+}$ levels. However, it is equally likely that greater VDAC2-$Ca^{2+}$ uptake leads to greater ATP production that can fuel SERCA2 pumping and myosin-ATPase activity to match demand. Other possibilities include the altered association of VDAC2 with CPT1a on the OMM that would alter acetyl-carnitine and fatty acid oxidation. Similarly, VDAC2 has an important role in the removal of ADP from the contractile apparatus. Increased sarcomeric ADP levels is known to inhibit the myosin-ATPase activity as is described in failing cardiomyocytes. Thus, agents that might increase VDAC2 permeability can increase ADP mitochondrial transport and thereby promote myofibril relaxation and contractility.

How mitochondria interact with surrounding organelles, such as the S/ER, lysosomes and plasma membrane, provides insight into how mitochondria can shape cytosolic $Ca^{2+}$ signaling as well as cellular processes such as apoptosis and ROS generation[62]. Several different protein partners exist for different VDACs and these interactions can regulate OMM permeability and therefore influence mitochondrial $Ca^{2+}$ levels, energy transfer and apoptosis. VDAC partners with IP3R and RYR2 located in the S/ER membrane to form $Ca^{2+}$ microdomains. Here, these channels govern local $Ca^{2+}$ release, a fraction of which can be transferred to the mitochondria. Increased matrix $Ca^{2+}$ can bind PTP1a and activate pyruvate dehydrogenase. VDACs have also been shown to interact with enzymes linked to glycolytic metabolism. Hexokinases (HK) are part of the glycolytic cascade and localize to the OMM. HKI and HKII can phosphorylate glucose to form G-6P which commits glucose to mitochondrial oxidation. HKI/II both interact with VDAC1 and can reduce VDAC conductance[63]. VDAC has been shown to interact with the OMM cholesterol transport protein TSPO[64]. TSPO can inhibit VDAC mediated $Ca^{2+}$ transport and has been implicated in mitophagy[65]. Along these same lines, other studies established that carnitine palmitoyltransferase (CPT1a) is an OMM protein that mediates the transfer an acyl groups from long chain fatty acid to carnitine. Acylcarnitine can that transport to the matrix for fatty acid oxidation. Reports that CPT1a interacts with VDAC are intriguing and suggest a cross talk between fatty acid oxidation and OMM permeability[66]. It is clear from these studies that VDAC can interact with several metabolic pathways in a bidirectional manner to link events in the cytosol with mitochondrial function whether involving fuel metabolism, ROS signaling, ATP production or apoptosis.

VDACs have also been shown to interact with cytoskeletal elements that have important functional impact on VDAC permeability[67]. Tubulin polymerizes to assemble microtubules that are used by mitochondria to migrate throughout the cell. Tubulins can also influence fusion and fission events that are important to mitochondrial quality control[55, 68, 69]. Importantly, monomeric tubulin can interact with VDAC where it can block the channel function by plugging the pore. Another cytoskeletal element desmin was identified as a VDAC partner where it may regulate $Ca^{2+}$ transfer to the mitochondrial matrix[70]. Desmin is expressed in all muscle types and contributes to the structural support of these contractile cells. DES mutant mice develop dilated cardiomyopathy and heart failure that models human disease. Interestingly, dysmorphic mitochondria and defects in respiration are a part of the phenotype of DES mutant mice that can be rescued by expressing the heat shock protein α-crystalin beta (CRYAB). Alpha-synnuclein (αSyn) is a Parkinson's disease gene that is a cytoskeletal element that can also interact with VDAC and mediate $Ca^{2+}$ transport[71, 72]. Collectively, these studies demonstrate that VDAC interacts with key cytosolic proteins that can regulate OMM permeability and therefore have impact of $Ca^{2+}$ signaling, ATP production, ROS production and apoptosis.

## 4. VDAC2 and Dilated Cardiomyopathy

Given the recent evidence that VDAC2 has a key role in cardiac rhythmicity, focus on the function of this VDAC in the heart has been intensely pursued. Germline VDAC2 deletion from mice has hampered studies on the role of VDAC2 in the adult heart because of the embryonically lethality that occurred at early stages such as embryonic day 10. Whether this embryonic lethality results from defects cardiac formation or absence of rhythmically beating cells was not addressed in these early studies. MEFs derived from the VDAC2 KO mice exhibited increased sensitivity to apoptosis and Bcl-2 signaling that involved greater release of cytochrome C. Informed by these early studies, Shankar et al. generated a cardiac restricted VDAC2 KO mouse mice[73]. These mice develop features consistent with a dilated cardiomyopathy (DCM) including reduced ejection fraction, increased cardiac fibrosis, and elevations of the serum biomarker brain natriuretic peptide (BNP). These mice exhibit reduced survival that is often seen in patients with DCM. Mechanistic studies show that cardiomyocytes from VDAC2 KO mice exhibit severely reduced mitochondrial $Ca^{2+}$ transfer. The functional consequence of this reduced $Ca^{2+}$ transfer is complex and impacts cytosolic events including contractility. Is matrix $Ca^{2+}$ reduced in the VDAC2 KO mice? Is there an impairment in PDH activity or other TCA cycle enzymes that are $Ca^{2+}$ dependent? Answers to these questions may explain why VDAC KO mice have a clear cardiac phenotype when compared with the MCU KO mice[74, 75]. While deletion of MCU subunits like EMRE and MICU1/2 result in decompensated cardiac function MCU-$Ca^{2+}$ uptake is not necessary for adequate contractility[76–78].

Shankar and colleagues found that VDAC2-mediated $Ca^{2+}$ uptake regulates cytosolic $Ca^{2+}$ signaling including ECC and the diastolic oscillations of the $Ca^{2+}$ clock. Cardiomyocytes from VDAC2 KO mice show $Ca^{2+}$ transients with depressed amplitudes and prolonged tau compared to WT. Extensive electrical remodeling occurs in the VDAC2 KO cardiomyocytes that change properties of the action potential (AP) including a significantly prolonged AP50 duration. These results are rather surprising given prior reports showing that

reduced mitochondria $Ca^{2+}$ uptake actually had the opposite effect. Cardiomyocytes (HL-1 cells) treated with VDAC2 silencing lentiviruses revealed no change in cytosolic $Ca^{2+}$ transients in response to action potentials or store depletion with caffeine, but the cardiomyocytes with VDAC2 depletion revealed increased the intensity, width and duration of $Ca^{2+}$ sparks. Similar approaches to alter MCU-mediated $Ca^{2+}$ uptake in cardiomyocytes increased contractility and reduced RYR2 leak[79]. It is likely that these differences reflect bidirectional $Ca^{2+}$ signaling between the SR and mitochondria. Shankar and colleagues actually hypothesize that deletion of VDAC2 altered the connection between OMM and the SR membrane and therefore reduced a critical $Ca^{2+}$ microdomain. Specifically, VDAC2 was shown to co-localize and co-precipitate with NCX1 and SERCA2a. VDAC is known to interact with RYR1 but the notion that VDAC localize together with SERCA2 and NCX1 implies a different mechanism for VDAC2. NCX1 is localized in the t-tubule membrane whereas SERCA2 lines the longitudinal SR which is quite a distance from the terminal cisternae and dyads. Certainly, these findings seem to resolve a controversy in the role mitochondria play in failing cardiomyocytes. Studies for years have shown differences in the connections between mitochondria and the SR in the failing cardiomyocyte. Reduced mitochondria $Ca^{2+}$ transfer was thought to impose energetic starvation on the cardiomyocyte. In contrast, others have suggested that loss of the mito-SR connections are detrimental. In fact, actions to augment mito-$Ca^{2+}$, such as overexpressing MCU, can mitigate the contractile defects imposed in heart failure[79]. Thus, many questions can be raised from these studies: is there a hierarchy for the VDAC2 interaction and these different microdomains? A greater understanding of how these different microdomains are established by VDAC2 can help understand how VDAC2 and Efsevin affect SR $Ca^{2+}$ content and action potentials of cardiomyocytes. Mitochondria may enhance $Ca^{2+}$ uptake under conditions of elevated diastolic $Ca^{2+}$ and thereby function as a popoff valve for elevated diastolic $Ca^{2+}$ levels. Here the interaction of VDAC2 and SERCA2 could mediate such a connection. However, it is equally likely that reduced VDAC2-$Ca^{2+}$ uptake leads to impaired ATP production that must fuel SERCA2 pumping and myosin-ATPase activity. Similarly, VDAC2 has an important role in the removal of ADP from the contractile apparatus. Deletion of VDAC2 would prevent ADP transfer to the mitochondria and would inhibit the myosin-ATPase activity leading to impaired cardiac relaxation. An interesting observation made in this work is that VDAC2 depletion from the heart did not alter mitochondrial ROS production or respiration. This is rather unexpected but is line with the notion that VDAC2 specifically regulates mitochondrial $Ca^{2+}$, whereas cardiac VDAC1 has been shown to increase ROS production and promote apoptosis[32]. It is well established that SR-$Ca^{2+}$ release is delivered to the outer mitochondrial membranes where $Ca^{2+}$ can then concentrate in the mitochondrial matrix in order to facilitate metabolism and energetics. In contrast, VDAC2 deletion from cardiomyocyte did alter profiles of gene expression for enzymes involved in glucose metabolism.

## 5. Mitochondria as a therapeutic target for heart failure

Heart failure is a disease syndrome with very high mortality resulting from progressive pump failure and sudden cardiac death from arrhythmias. Available treatments have focused on modifying actions of neurohormones such β-adrenergic receptor antagonists

and inhibitors of renin-angiotensin-II cascade that are activated deleteriously in the HF patients or by use of implanted defibrillators for treat life threatening arrhythmias. Targeting mitochondrial metabolism in the failing heart has long been pursued as therapeutic strategy but has never proven to be clinically effective. Most often approaches are designed to enhanced mitochondrial biogenesis, enhance electron transport chain induced ATP generation, target substrate utilization and/or prevent oxidative damage. These approaches invariably alter the bioenergetic balance in the mitochondria without regard for the SR. As a result, cardiomyocytes with increased mitochondrial mass and greater ATP generation will invariably have greater myocardial oxygen consumption which contributes to greater mortality in HF. Review of the clinic trails.gov found 42 trials linked to heart failure and mitochondria. Table 1 identifies many of these drugs and nutrients being tested. Given the growing number of small molecules that appear to modify mitochondrial $Ca^{2+}$, like the gliflozins and Efsevin, it is likely that a new era for treating HF has arrived and will seek to target the mitochondria to enhance cardiac performance. Iron replacement therapy in HF patients has emerged as an important target. While the O2 carrying capacity of hemoglobin is definitely important in HF patient with anemia, the expectation is that iron replacement also enhances ETC as cytochromes require iron. Along this same line, protocols are testing nutrients to improve mitochondrial function in HF. For example, beetroot juice and epicatechin (dark chocolate) are being tested as enhancers of mitochondrial function in HF. MitoQ is an antioxidant that consists of ubiquinol attached to a lipophilic cation. MitoQ has been tested in a small group of aging subjects with vascular disease and shown to improve aortic stiffness and as well as cholesterol metabolism. MitoQ is being tested in humans with HF to see in the reduced oxidative stress and improve mitochondria function and heart failure (Table 1).

## 6.  VDAC activators

Efsevin was recently identified as potential antiarrhythmic agent as it suppressed ventricular dysrhythmias in a Zebrafish deficient in NCX1[61, 80]. Efsevin is a dihydropyrrole carboxylic ester compound that has a short half-life. Lipid bilayer experiments with VDAC2 show that Efsevin directly binds VDAC2 to reduce open probability and shifts to a closed state. This shift leads to greater cation permeability and exclusion of anion and metabolites. Recent studies have demonstrated that Efsevin binding site on VDAC2 occurs between the inner channel wall and the N-terminal helix associated with pore which is important for the $Ca^{2+}$ transfer from the SR into the mitochondria[81]. Efsevin is unstable with a short half-life which is limiting its clinical applications but recent studies have shown that Efsevin can reduce arrhythmia in cardiomyocytes from CPVT mice.

While it is established that Efsevin can restore rhythmic $Ca^{2+}$ oscillation in cardiomyocytes, it is unclear how it can alter contractility[61, 80]. To address this issue the Shankar et al. utilized an ex vivo assay for force production from cardiac slices. WT and VDAC2 KO mice were subjected to pressure overload by transaortic constriction (TAC) as a model of heart failure. Efsevin was superfused over cardiac slices and contractile force was measured. Acute drug delivery in this model of cardiac hypertrophy demonstrated that Efsevin augmented contractility. These preliminary studies broaden the mechanism by which Efsevin works but will await future chronic and in vivo studies to provide insight

to the preclinical application of this drug. It will be important to test a more bioavailable analogue of Efsevin in other models of heart failure. Additional agents that activate VDAC2 that were also identified in a suppressor screen that include ezetimibe and disulfiram, both of which are FDA approved drugs[82]. Ezetimide limits cholesterol absorption from the gastrointestinal track but at present no connection to mitochondria has been established. Disulfiram is an inhibitor of the mitochondrial enzyme aldehyde dehydrogenase 2 (ALDH2) that is known to detoxify reactive aldehydes from cardiomyocytes. These agents require urgent testing in arrhythmia *in vivo* models and, if successful, transition to human trials.

### 3.3   SGLT2 inhibitors.

In the last several years, new medications that inhibit the sodium-glucose transporter type II (SGLT2) (e.g. empagliflozin, canaglifozin and dapagliflozin) were found to significantly reduce HF mortality and hospitalization and improve quality of life[83–85]. Interestingly, while these medications act by preventing reabsorption of glucose in the kidney, their benefits seem mostly related to improvement in heart failure outcomes, independent of the effect on blood glucose. How these agents confer cardioprotection has led to some speculation that these agents may influence mitochondrial function and thereby improve cardiac function[86]. Specifically, by altering total glucose load in the body and a reduction in the insulin/glucagon ratio create conditions that favor a shift in metabolism towards ketogenesis. Ketone production may well be protective in the heart as it is the ideal fuel source in heart failure[87]. As a result, mitochondrial efficiency improves and promotes greater contractility and less arrhythmias. Some gliflozins might also inhibit SGLT1 that is highly enriched in the heart. Molecular docking studies have indicated that Empagliflozin binds glut-4 as well as SGLT1 and NHE1[88]. In this model, limiting glucose uptake in cardiomyocytes activates AMPK and inhibits mTor signaling which would be cardioprotective. The beneficial effects of empagliflozin may be related to LV remodeling that is favorable and protective. Finally, studies have directly addressed the role of empagliflozin on mitochondrial $Ca^{2+}$ content[89, 90]. Studies show that altered Na in the mitochondria reduced NCLX activity leading to higher mitochondrial $Ca^{2+}$ content. As a class the gliflozins offer novel ways to impact mitochondrial function and $Ca^{2+}$ signaling in cardiomyocytes and offer new therapies to treat patients with heart failure.

## 7.   MCU activation

$Ca^{2+}$ crosses the IMM through the actions of the MCU complex that include MICU1, MICU2, ERME. Matrix $Ca^{2+}$ content plays a key role in oxidative phosphorylation, ROS production and cell death pathways. Loss of function studies in mice for MCU have been challenging as MCU KO mice in certain genetic background have no particular phenotype, whereas in the C57/B6 background this mutation is embryonically lethal. Moreover, loss of function and gain of function studies with MCU and its components of the complex demonstrate a relative role for MCU in different cardiac conditions. Enhancing $Ca^{2+}$ transfer to the matrix may well be an approach to enhance cardiomyocyte survival during heart, ischemia and even arrhythmias[79]. Thus, the therapeutic targeting of the MCU complex is logical. High throughput screens for small molecules that act as directly on the MCU complex have been characterized[91, 92]. Using a clever approach, Arduino et

al reconstituted human MCU complex in yeast mitochondria. They exploited the D-lactate shunt in yeast to bypass ETC and TCA cycling on the $Ca^{2+}$ transfer. They identified the chemotherapeutic agent mitoanxtrone as an MCU inhibitor. Interestingly, MTX is known to cause cardiotoxicity in patients[93]. Another agent, Kaempferol, is a plant flavonoid with anti-oxidant properties. Kaempferol can directly activate MCU and enhance the $Ca^{2+}$ transfer from the S/ER into the mitochondria. In cardiomyocyte, MCU complex activators might enhance mitochondrial $Ca^{2+}$ uptake and offer favorable energetic conditions that can be cardioprotective.

## 8. Elamipretide for mitoprotection

Elamipretide (SS-31) is an aromatic cationic tetrapeptide that was identified as a modulator of damaged mitochondria. Specifically, SS-31 concentrates in the mitochondrial inner membrane where it interacts with cardiolipin to stabilize components of the cytochrome system as well as MCUs. In this way, Elamipretide could target damaged mitochondria to enhance bioenergetic efficiency while leaving healthy mitochondria intact. Elamipretide is currently being tested in a phase III trial for patients with the inherited mitochondrial disorder Barth's syndrome. Many preclinical studies using animal models of heart failure show that SS-31 can repair mitochondrial dysfunction that can improve contractility. Elamipretide has been tested as a single dose infusion in patient with heart failure with reduced ejection fraction (HFrEF). This early phase trial established the safety of the single infusion of this peptide in HF patients. Mechanistic insight to the actions of Elamipretide in the human heart was obtained from studies using heart explanted at the time of cardiac transplantation. Here heart from patient with heart failure and control hearts (not used for transplant) represented the non-failing control were studied using a cardiac splice approach. Importantly Elamipretide did not alter mitochondrial respiration of the non-failing heart slices. In contrast, Elamipretide improved mitochondrial respiration that was supported by specific substrates including pyruvate and malate or glutamate whereas succinate respiration was not different in Elamipretide treated slices. Indeed, oxygen flux improved in HF-heart-section treated with Elamipretide that involved supercomplexes of the electron transport chain. Based on the success of these preclinical and safety studies, a phase II study with Elamipretide was designed to test a longer treatment course (i.e. 28 days) in a similar population of HFrEF patients who were already on guideline directed medical therapy. Results confirmed the safety of the drug but did not identify improvement in the primary end point of LV function as assessed by cardiac magnetic resonance imaging (cMRI). Elamipretide did not improve cardiac volumes or ejection fraction in these patients. There was a trend for improved quality of life assessments in these patients but this was not significant. Based on this study, despite many positive preclinical studies, the use of Elamipreptide in heart failure cannot be supported for the treatment HFrEF. It should be noted that treatment for 28 days might not be sufficient time to remodel mitochondria in cardiomyocytes and subsequently improve LV function, the primary end point of the trial. Repair of the mitochondria would then need to be translated to reverse remodeling of the heart which might be expected to take much longer as it does for other therapies including β-blockers and biventricular pacing. Thus, discarding Elamipreptide at this point might be premature. Finally, it is possible that Elamipreptide induced improvement in

mitochondrial respiration and fitness might reduce arrhythmias, as occurs with Efsevin, but was not assessed in any trial to date.

## 9. Conclusion

Despite tremendous progress over the last several decades, treatments for heart failure have offered only modest benefit for reducing mortality and deferring hospitalization in patients with compromised cardiac function. Optimism has been raised by novel strategies designed to improve mitochondrial fitness in the failing cardiomyocyte[94]. Previous drug design strategies for mitochondria have aimed to improve oxidative metabolism, stimulate mitochondrial biogenesis and ATP availability which would alter the energy balance and potentially improve contractility. However, several obstacles led to failure for these approaches and include unwanted ROS generation, increased oxygen consumption and damage to healthy mitochondria. Recent evidence establishes VDAC in the OMM of cardiomyocyte mitochondria as an important target. In fact, unbiased screens, discussed here, have identified drugs that target opening of VDAC2 in the improvement of cardiac function and automaticity. Promoting the flux of $Ca^{2+}$ from the cytosol (and SR) into the mitochondria seems to active metabolic enzymes needed to power ATP production. Depletion of VDAC2 in mice clearly reproduced many features of dilated cardiomyocyte and targeting VDAC2 activation reduces arrhythmias. This strategy will likely open new lines of thinking involving the connection between mitochondria and the energy deficits in the failing heart.

## Acknowledgements

## Abbreviations

| | |
|---|---|
| $Ca^{2+}$ | Calcium |
| CM | cardiomyocyte |
| $[Ca^{2+}]_i$ | Cytoplasmic calcium concentration |
| GoF | Gain of function |
| HF | Heart Failure |
| LoF | Loss of function |
| RYR2 | Ryanodine Receptor type 2 |
| S/ER | Sarco(endo)plasmic reticulum |
| SOCE | Store-operated calcium entry |
| TG | Thapsigargin |
| VSM | Vascular smooth muscle |

| Cav1.2 | Voltage-dependent calcium channels |
|---|---|
| ATP | Adenine Trisphosphate |
| ETC | Electron Transport Chain |
| SGLT2 | sodium-glucose transporter type II |
| ECC | Excitation Contraction Coupling |

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## Highlights

- VDAC2 deletion from cardiomyocytes cause dilated cardiomyopathy and increased mortality.

- Cytosolic Ca2+ transients are reduced as a cause of impaired contractility.

- VDAC2 inteacts with RYR2, SERCA2a and NCX1 in cardiomyocytes.

- VDAC2-enhanced $Ca^{2+}$ uptake in CM prevents heart failure.

- Enhanced Ca2+ uptake in mitochondria is a novel strategy to treat heart failure.
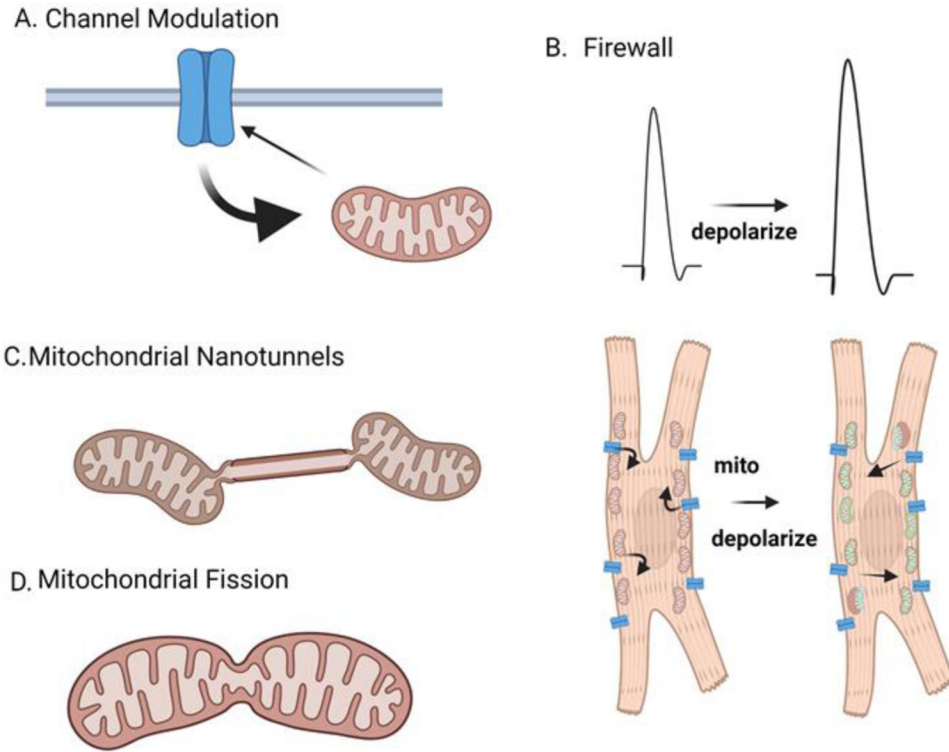
**Figure 1:**

Mitochondria can influence $Ca^{2+}$ transients of cardiomyocytes. The cartoon depicts different mechanisms by which mitochondria respond to altered cytosolic $Ca^{2+}$ levels. A) Mitochondria take up $Ca^{2+}$ from store operated $Ca^{2+}$ channels and prevent $Ca^{2+}$ dependent inactivation of the channel. B) Mitochondria can serve as a firewall to prevent global increases in $Ca^{2+}$. In atrial cardiomyocytes, $Ca^{2+}$ entry across the sarcolemma (via cav1.2) is loaded into mitochondria that are present underneath the surface membrane. The amplitude of the $Ca^{2+}$ transients becomes larger when cells are pretreated with agent that depolarize the mitochondrial membrane. C) Nanotunnels are specialized membrane extensions that connect two mitochondria. This phenomenon develops in cardiomyocytes with leaky $Ca^{2+}$ release channels (RYR2). D) Elevated $Ca^{2+}$ levels in the cytosol can trigger morphologic change to mitochondria called fission.
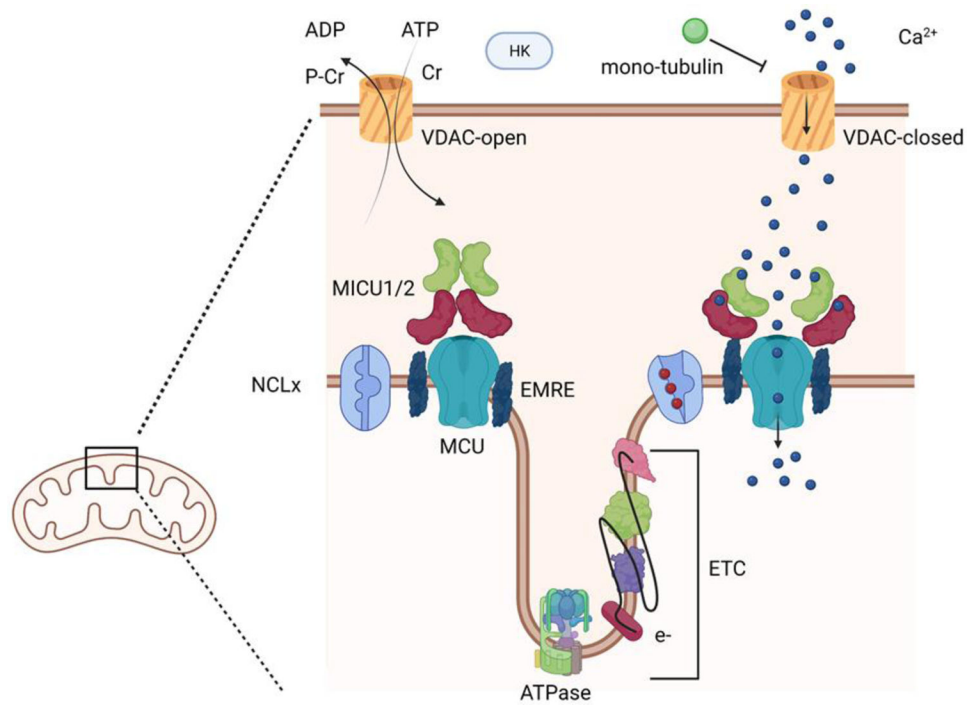
**Figure 2:**

Mitochondrial Ca2+ signaling toolkit. The schematic demonstrates the outer and inner mitochondrial membranes. VDAC is present in the OMM where it exists in open and closed conductance states. $Ca^{2+}$ flows through the closed VDAC channels. At the IMM, mitochondrial uptake transporter (MCU) exits in a complex with MICU1/2 and EMRE. MICU1/2 act as $Ca^{2+}$ sensor in the mitochondrial outer space. EF hands bind $Ca^{2+}$ and stimulate conformation changes that activate MCU-dependent $Ca^{2+}$ transport.
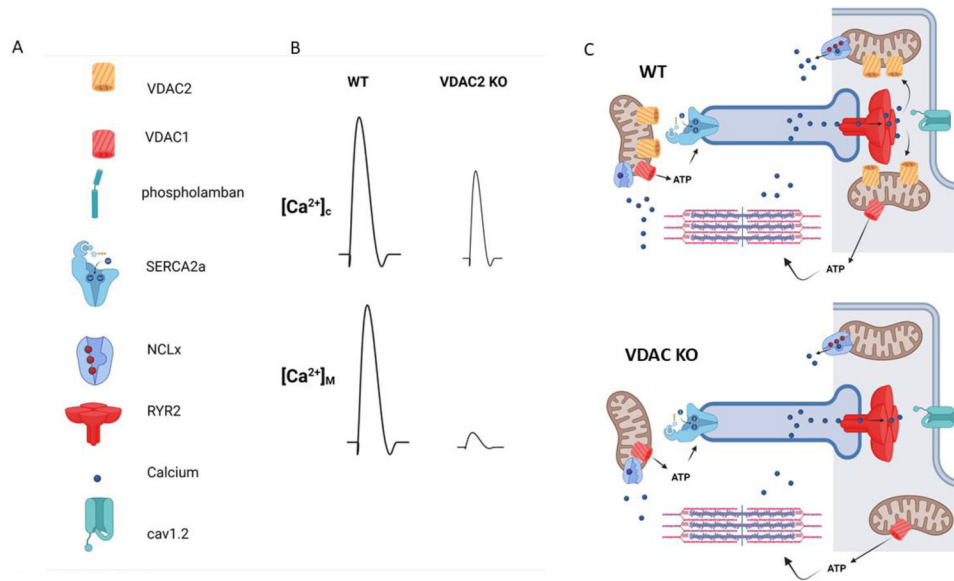
**Figure 3:**

VDAC2 deletion in cardiomyocytes causes defects in ECC. A) legend for $Ca^{2+}$ handling proteins in C. B) Cytosolic and Mitochondria Ca2+ responses in WT and VDAC2 KO cells. Deletion of VDAC2 leads to a reduction in $Ca^{2+}$ release and reduced $Ca^{2+}$ transfer into mitochondria. VDAC1 may have more a role in regulating redox signaling and apoptosis, whereas VDAC2 has a dominant role in $Ca^{2+}$ signaling.

**Table 1:**

Clinical Trials registered in ClinicTrials.gov targeting mitochondria function in patient with heart failure.

| Intervention | Target | Citations |
|---|---|---|
| Mito Q | Mitochondrial Antioxidant | [95] |
| Epicatechin | ETC | [96] |
| Ferric Carboxymaltose | ETC- Cytochome-Fe binding | [97] |
| 14N Sodium Nitrate | NO-CGAMP signaling | [98] |
| Nicotinamide ribose | SIRT/NAD+ signaling | [99, 100] |
| 3-hydroxybutyrate | Ketone oxidation | [101, 102] |
| Clonidine | a-adrenergic blocker | [103] |
| Empagliflozin | Sodium Glucose Transporter 2 inhibitor | [104] |
| Beet Root Juice | Mitochondrial efficiency; NO availability | [105] |
| KNO3 + L-carnitine | Enhances oxidative phos | [106] |
| Dapagliflozin | Sodium Glucose Transporter 2 inhibitor | [107, 108] |
| Sodium Valproate | FAO | [109, 110] |