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The role of SERCA2a/PLN complex, Ca^{2+} homeostasis, and anti-apoptotic proteins in determining cell fate

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Abstract Intracellular calcium is a major coordinator of numerous aspects of cellular physiology, including muscle contractility and cell survival. In cardiac muscle, aberrant Ca^{2+} cycling has been implicated in a range of pathological conditions including cardiomyopathies and heart failure. The sarco(endo)plasmic reticulum Ca^{2+} transport adenosine triphosphatase (SERCA2a) and its regulator phospholamban (PLN) have a central role in modulating Ca^{2+} homeostasis and, therefore, cardiac function. Herein, we discuss the mechanisms through which SERCA2a and PLN control cardiomyocyte function in health and disease. Emphasis is placed on our newly identified PLN-binding partner HS-1-associated protein X-1 (HAX-1), which has an anti-apoptotic function and presents with numerous similarities to Bcl-2. Recent evidence indicates that proteins of the Bcl-2 family can influence ER Ca^{2+} content, a critical determinant of cellular sensitivity to apoptosis. The discovery of the PLN/HAX-1 interaction therefore unveils an important new link between Ca^{2+} homeostasis and cell survival, with significant therapeutic potential.

Keywords Ca^{2+} regulation · Sarcoplasmic reticulum · Mitochondria · Cardiac function · Calcium ATPase · Apoptosis

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Introduction

In cardiac muscle, regulation of intracellular Ca^{2+} homeostasis is mediated by the sarcoplasmic reticulum (SR), an intracellular membranous network surrounding the contractile machinery. Through its direct involvement in Ca^{2+} cycling, the SR has a critical role in controlling contraction and relaxation in cardiac muscle. During excitation–contraction coupling, Ca^{2+} entry through the L-type Ca^{2+} channel triggers the release of Ca^{2+} from SR Ca^{2+} stores via the ryanodine receptor, resulting in increased cytosolic Ca^{2+} levels and initiation of contraction [1–4]. During relaxation, cytosolic Ca^{2+} is partly sequestered back into the SR lumen by the sarco(endo)plasmic reticulum Ca^{2+} adenosine triphosphatase (ATPase; SERCA) pump and partly extruded to the external medium through the action of plasma-membrane Ca^{2+} ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX; Fig. 1). In humans, about 70% of the cytosolic Ca^{2+} returns to the SR, while the rest is removed from the cell by the activities of NCX (28%) and PMCA (2%) [3]. As the SR represents the major source of Ca^{2+} store, the SR Ca^{2+} content and, therefore, the amount of Ca^{2+} available for release play a critical role in cardiac contractility.

Evidence from human and experimental studies has indicated that defects in SR Ca^{2+} handling are associated with attenuated contractility, which can progress to heart failure [5]. In particular, cardiac systolic dysfunction and impaired cardiac relaxation are common hallmarks of heart failure. At the cellular level, depressed relaxation reflects impaired removal of cytosolic Ca^{2+} and reduced cardiac SR loading. This could be due to decreased SERCA2 protein levels and/or increased inhibition by its regulatory protein PLN [6, 7]. Importantly, the progression of heart failure, which is marked by a decline in cardiac function, has been

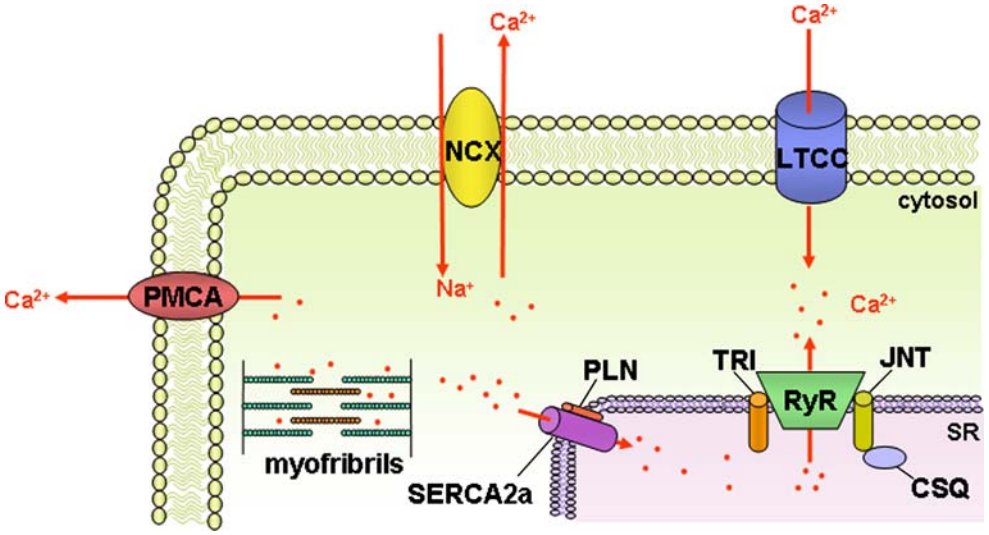

Fig. 1 Schematic representation of the major players implicated in excitation–contraction coupling of the heart. In response to membrane depolarization, Ca^{2+} entry through the L-type Ca^{2+} channel (LTCC) activates the ryanodine receptor (RyR) and triggers release of Ca^{2+} from sarcoplasmic reticulum (SR) Ca^{2+} stores. This results in increased cytosolic Ca^{2+} levels, which bind to myofibrils and initiate cardiac contraction. Reuptake of cytosolic Ca^{2+} to the SR lumen by the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA2a) pump and removal to the external medium through the action of plasma-membrane Ca^{2+} ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX) results in cardiac relaxation. *PLN* phospholamban, *JNT* junction, *TRI* triadin, *CSQ* calsequestrin

associated with cardiomyocyte loss through activation of the apoptotic pathways [8–10]. This occurs through a signaling cascade, which includes cytochrome c release from the mitochondria, activation of caspases, protein, and DNA degradation [8, 11, 12]. Loss of myocytes represents an important component of cardiac remodeling and contributes to the transition from an adaptive myocardial condition to end-stage heart failure [13].

This review focuses on the critical role of the SERCA2a/PLN complex in regulating SR Ca^{2+} cycling and cardiac contractility, and presents current knowledge on its detailed mode of function. An emphasis is given on the emerging role of HAX-1 in cardiac muscle, which through its interaction with PLN, could represent a link between Ca^{2+} homeostasis and cell survival. Deciphering the molecular mechanisms bridging Ca^{2+} cycling aberrations with apoptosis and, therefore, advanced stages of disease progression could unveil promising new therapeutic targets.

SERCA2a activity is critical in regulation of SR Ca^{2+} homeostasis

There are three different SERCA genes (human nomenclature *ATP2A1-3*), each encoding at least two different protein isoforms. Cardiac muscle expresses SERCA2a, a 110-kDa transmembrane protein that functions by transporting two Ca^{2+} from the cytosol to the SR lumen at the expense of adenosine triphosphate (ATP) hydrolysis [14–18]. As SERCA2a activity controls both the rate of cytosolic Ca^{2+} removal and the degree of SR Ca^{2+} load, it represents a fundamental determinant of both cardiac relaxation and contraction.

Transgenic animal models have been developed to define the functional role of the SERCA pump in Ca^{2+} homeostasis and cardiac physiology. Transgenic mice overexpressing SERCA2a by 1.2- or 1.5-fold exhibited increased SR Ca^{2+} transport and enhanced rates of cardiac contractility and relaxation [19–21]. No cardiac pathology was observed in these animals, suggesting that SERCA2a overexpression can be tolerated by the heart. On the other hand, absence of the SERCA2 gene is lethal, with the homozygous null (SERCA2−/−) mice dying early in development [22]. Heterozygous (SERCA2+/−) mice are viable, showing 35% decrease in SERCA2 protein levels as a result of the loss of one copy of SERCA2 allele. Although no cardiac pathology was exhibited at rest, reduction in SERCA2 levels in combination with an increased hemodynamic load resulted in an accelerated pathway to heart failure [23]. These mice show impaired intracellular Ca^{2+} homeostasis and decreased rates of cardiac contractile function, a finding that demonstrates the requirement for two functional copies of the SERCA2 gene for effective SR Ca^{2+} cycling and cardiac function [22, 24].

Mutation screening on specific SERCA2a genomic regions, corresponding to the PLN interacting region as well as the SERCA2a phosphorylation and nucleotide-binding domains, did not identify any genetic variants resulting in amino acid alterations in adult dilated cardiomyopathy patients [25]. This suggests that the SERCA2a sequence is highly conserved among individuals. Loss of function mutations in the SERCA2 gene are known to

cause Darier's disease, an autosomal dominant skin disorder [26–28]. Interestingly, however, these patients do not exhibit any cardiac pathology.

Although no SERCA2a mutations have been identified in heart disease patients to date, significant expression changes have been observed in failing hearts. Specifically, experimental models of myocardial failure were found to exhibit reduced SERCA2a messenger RNA (mRNA) and protein levels, suggesting an impairment in cytosolic Ca^{2+} removal, SR Ca^{2+} load and overall Ca^{2+} cycling [29–31]. Similarly, decreased SERCA2a expression and SR Ca^{2+} transport were observed in human failing hearts [32–37]. Taken together, these findings demonstrate a direct correlation between SERCA2a levels, SR Ca^{2+} transport, and heart failure.

SERCA2a-binding partners

While experimental evidence suggests that proteins involved in SR Ca^{2+} release, such as the ryanodine receptor, function as part of a macromolecular complex, the existence of such a protein complex in the regulation of SR Ca^{2+} uptake has only recently begun to emerge. In particular, SERCA2a has been found to interact with proteins of the SR lumen, such as histidine-rich calcium-binding protein [38] and calreticulin [39], while its cytosolic region has been shown to bind to S100A1 [40], acylphosphatase [41], and Bcl-2 (see section below) [42, 43]. Furthermore, PLN and sarcolipin have been found to bind to the cytosolic and/or transmembrane domains of SERCA2a, with accumulating evidence suggesting that these interactions lead to inhibition of the pump's affinity for Ca^{2+} [18, 44]. PLN has proven to be a major regulator of SERCA2a activity, and so far, it is the only SERCA2a-associated protein directly involved in cardiac disease development, including heart failure.

PLN is a major regulator of SERCA2a activity

PLN is a 52-amino-acid transmembrane protein of the SR that is expressed mainly in cardiac but also in smooth and slow-twitch skeletal muscles [45–47]. Based on its protein sequence, PLN appears to contain three domains, namely a largely helical cytosolic domain IA (amino acids 1–20), an unstructured domain IB (amino acids 21–30), and a domain II (amino acids 31–52) that forms a transmembrane helix [45, 48]. Detailed cross-linking and site-directed mutagenesis studies have demonstrated that residues in both the cytoplasmic (IA and IB domains) and the transmembrane portions of PLN can interact directly with SERCA2a [49–54]. PLN can be phosphorylated at serine 16 by the cAMP-dependent protein kinase (PKA) and threonine 17 by the Ca^{2+}–calmodulin-dependent protein kinase (CaMKII) [55, 56]. Moreover, it has been shown that PLN exists in both pentameric and monomeric forms, where the monomer is considered to be the functionally active unit, while the pentamer may act as a reservoir [57, 58].

PLN can interact with SERCA2a and inhibit its activity, thus preventing Ca^{2+} entry to the SR through SERCA2a [47]. Specifically, PLN acts by lowering the apparent affinity of SERCA2a for Ca^{2+} but has little or no effect on the maximal velocity rate (V_{max}) at saturating Ca^{2+} and ATP concentrations [59]. At low Ca^{2+} concentrations, PLN interacts and reversibly inhibits the affinity of SERCA2a for Ca^{2+}, while elevations in Ca^{2+} concentration lead to dissociation of the SERCA2a/PLN protein complex, an effect that may be due to Ca^{2+}-induced conformational changes of SERCA2a [18]. The phosphorylation state of PLN has also been shown to affect SERCA2a activity. In its dephosphorylated state, PLN interacts with SERCA2a and inhibits the enzyme's affinity for Ca^{2+}. However, upon β-adrenergic stimulation, phosphorylation of PLN relieves this inhibitory effect on SERCA2a, leading to enhanced SR Ca^{2+} transport [60, 61]. These findings indicate the crucial functional role of PLN in regulating SERCA2a activity in a Ca^{2+} or phosphorylation-dependent manner and further suggest its involvement as a key determinant of β-adrenergic stimulation in the heart, with potential applications in pharmacologic therapeutic approaches.

Lessons on PLN function from transgenic animal models

The importance of PLN in cardiac physiology is further emphasized by studies on genetically modified mouse models. Ablation of PLN was associated with an increased affinity of SERCA2a for Ca^{2+}, increased SR Ca^{2+} uptake, and increased contractile parameters, which resulted in an overall hypercontractile cardiac function that persisted throughout aging [62, 63]. Interestingly, the highly stimulated function of PLN null hearts could be minimally stimulated by β-adrenergic agonists [64, 65]. Similarly, heterozygous (PLN+/−) mice with 60% reduction in PLN levels exhibited significant increases in contractile parameters, although these increases were lower than those exhibited by the PLN null (PLN−/−) mice [66]. Conversely, two- and fourfold overexpression of PLN in mouse heart resulted in decreased SERCA2a affinity for Ca^{2+}, decreased contractile parameters, and depressed left ventricular function. Stimulation with β-adrenergic agonists restored the contractile parameters to levels similar to wild type by alleviating the inhibitory effects of PLN [6, 46]. Taken together, these animal model studies have demonstrated the fundamental role of PLN in SR Ca^{2+} cycling and

cardiac function under basal and β-adrenergic stimulated conditions.

In addition to PLN-deficient or PLN-overexpressing mouse models, transgenic mice overexpressing specific PLN mutants have allowed the in vivo examination of the importance of specific amino acid residues in PLN function and elucidation of their role in SR Ca^{2+} cycling. Chronic inhibition of SERCA2a was observed upon overexpression of superinhibitory PLN mutants (Asn27Ala, Leu37Ala, Ile40Ala, and Val49Gly), which have been shown to alter the ratio of PLN monomers and pentamers, thus affecting SERCA2a inhibition [58, 67–69]. All transgenic animals exhibited depressed SERCA2a function, decreased Ca^{2+} kinetics, and impaired contractility. As a result of SERCA2a chronic inhibition, some of these models developed significant left ventricular hypertrophy, which progressed to cardiac dysfunction and heart failure [58, 68, 69]. Therefore, these findings suggest that specific amino acids are critical for PLN function, as changes at these residues can cause alterations in SR Ca^{2+} handling and subsequently lead to cardiac remodeling and progression to heart failure.

Detailed evaluation of the functional significance of PLN dual-site phosphorylation at Ser16 and Thr17 was accomplished, following the generation of transgenic animals expressing phosphorylation-site-specific PLN mutants [70–73]. While Ser16 phosphorylation may be a prerequisite for Thr17 phosphorylation, it has been suggested that Ser16 phosphorylation may mediate the β-agonist response, and Thr17 may play an important role in frequency-dependent increase of cardiac contraction and relaxation.

Collectively, the findings from the numerous transgenic animal models have provided evidence for the pivotal role of PLN in the regulation of SR Ca^{2+} homeostasis and suggest its potential use as a promising therapeutic target for heart disease.

PLN mutations lead to cardiomyopathy and heart failure

Identification of PLN mutations in patients with cardiomyopathy and heart failure highlight the critical role of PLN and SR Ca^{2+} cycling in controlling cardiac function and provide clues on the molecular mechanisms underlying disease pathogenesis.

To date, six different PLN genetic variations have been reported [74–79]. Two of these mutations (R9C and R14Del) are characterized by gain-of-function, causing chronic inhibition of SERCA2a activity. Substitution of arginine by cysteine at amino acid position 9 (R9C) was linked with autosomal dominant inheritance of dilated cardiomyopathy and heart failure in a large American family [74]. The functional consequences of this mutation were evaluated by cellular and biochemical studies performed in a heterologous cell culture system, through the analysis of a generated transgenic mouse model and also by studies on human cardiac tissue obtained from explanted hearts. A significant reduction in the levels of PLN phosphorylation was observed, resulting in impaired cardiomyocyte Ca^{2+} handling and cardiac function. Overexpression of the R9C mutation prevented cAMP-dependent PKA phosphorylation of wild-type PLN by local trapping of PKA in a stable mutant PLN–PKA complex and thus minimizing the PKA-mediated relief of PLN inhibitory function on SERCA2a. While the R9C mutation itself was not a strong inhibitor of SERCA2a, its ability to block PLN phosphorylation caused a dominant effect, leading to chronic inhibition of SERCA2a [74]. The other gain-of-function mutation is a heterozygous deletion of arginine at amino acid residue 14 (R14del), which was associated with inherited dilated cardiomyopathy and premature death [75]. Transgenic mice overexpressing this PLN mutation recapitulated the human dilated cardiomyopathy phenotype and resulted in premature death. In vitro and in vivo assessment of the functional consequences of the mutation determined a dominant effect of R14del, which could not be reversed upon PKA phosphorylation, therefore resulting in chronic inhibition of SERCA2a activity. Through this chronic inhibition, both PLN mutations impair SR Ca^{2+} cycling, leading to cardiac dysfunction and heart failure.

A loss-of-function human PLN mutation, resulting in the substitution of a leucine residue at position 39 with a premature stop codon (L39X), was identified in two families with hereditary heart failure [76]. Adenoviral overexpression of PLN L39X mutation in rat cardiomyocytes did not have an effect on SERCA2a activity. Moreover, overexpression of PLN L39X mutant in HEK293 cells demonstrated that the mutant protein was unstable, a finding that was also verified in the explanted cardiac tissue of a heart failure patient where no PLN protein was detected. This indicates the existence of a naturally occurring "PLN null mutation" associated with lack of inhibition on SERCA2a activity. However, in contrast to the hypercontractile phenotype observed in the PLN null mouse, deficiency of PLN in human hearts results in significantly impaired cardiac function and leads to the development of heart failure. A possible explanation for this is that PLN may be of paramount importance in humans, as it is required to maintain a high Ca^{2+} reserve for proper cardiac function through human life [80]. The critical role of PLN in the human heart was further emphasized by the strong inhibitory function elicited through the expression of the "human PLN" in the mouse null heart, which led to cardiac remodeling [80].

In addition to the above changes, three genetic variations within the upstream noncoding region of the gene (A to G

at −77 bp, C to G at −42 bp, or A to C at −36 bp) have also been reported in patients with cardiomyopathy. Functional analysis of the effect of these changes in cultured cells determined alterations in the transcriptional activity of the PLN promoter, suggesting that they may lead to alterations in SR Ca^{2+} homeostasis and disease pathogenesis [77–79].

Taken together, these studies emphasize the importance of PLN in regulating SERCA2a activity and, in this way, SR Ca^{2+} homeostasis and cardiac function. Importantly, chronic inhibition or absence of PLN activity has been directly associated with heart failure.

PLN interacts with the anti-apoptotic protein HAX-1

The role of PLN in SR Ca^{2+} cycling has been carefully characterized. However, its function in other molecular pathways is only starting to come to light. Discovering these pathways would contribute toward the better understanding of heart failure pathogenesis and could reveal new therapeutic targets.

Recently, we reported the identification of HS-1-associated protein X-1 (HAX-1), a ~35-kDa ubiquitously expressed mitochondrial protein with anti-apoptotic function, as a PLN-binding partner [81]. The minimal binding region of HAX-1 was mapped to a C-terminal fragment, encoding amino acids 203–245, whereas the PLN-binding region contained amino acids 16–22, a region that includes both the Ser16 and Thr17 phosphorylation sites. This region of PLN contains residues Ile18, Glu19, Met20, and Pro21, which are suggested to form a turn connecting the two α-helical stretches of the protein. This conformation may provide the necessary flexibility to the protein that could be important in the kinetics of monomer–pentamer formation, in PLN phosphorylation and dephosphorylation, as well as in its association with SERCA2a. It was therefore proposed that binding of HAX-1 to this region of PLN may represent a regulatory mechanism on any of these reactions and could provide the means for controlling the conformation and activity of PLN [81]. Similarly to the SERCA2/PLN interaction, binding of HAX-1 to PLN was found to be diminished upon phosphorylation of PLN by cAMP-dependent protein kinase and increasing Ca^{2+} concentrations, thus indicating that HAX-1 may regulate the functional properties of PLN in the heart. Through this association, HAX-1 could therefore have an important role on SR Ca^{2+} cycling.

HAX-1 was originally identified to interact with HS1, a protein with proposed involvement in B cell signal transduction in hemopoietic cells [82]. Subsequently, HAX-1 has been found to interact with a number of cytoskeletal and viral proteins (Table 1). Although the biological significance of these interactions remains unclear, the existence of multiple interacting proteins for HAX-1 indicates its involvement in multiple cellular pathways. Immunofluorescence microscopy studies have localized HAX-1 to the mitochondria, endoplasmic reticulum, and the nuclear envelope [82, 85, 86, 88, 89, 95, 96]. We previously reported a preferential mitochondrial localization of HAX-1 after transient transfections in HEK293 cells. Interestingly, upon cotransfection with PLN, HAX-1 underwent cellular redistribution and colocalized with PLN at the ER [81]. A similar finding was also reported in the presence of the HAX-1 interacting protein Vpr. Specifically, coexpression of HAX-1 with the predominantly nuclear localized protein Vpr resulted in the codistribution of the two proteins in cytoplasmic bodies outside the nucleus and mitochondria [86]. It was therefore proposed that the subcellular localization and functional properties of HAX-1 may vary among different tissues, depending on which interacting partners are available [81].

Based on its weak sequence similarity to Nip3 and its homology to Bcl-2 domains BH1 and BH2, HAX-1 was initially proposed to be involved in promoting cell survival. Its anti-apoptotic function was supported by experimental evidence after the identification of its interaction with K15 Kaposis's sarcoma, Omi/HtrA2 protease, and Vpr [85, 86, 97]. Studies in Jurkat, HEK293, or Hela cell lines demonstrated that HAX-1 overexpression provides protection against Fas treatment, γ-irradiation, serum deprivation, Bax overexpression, or hypoxia/reoxygenation-induced cell death [81, 82, 85, 96]. Furthermore, HAX-1 was found to be overexpressed in psoriasis, a chronic inflammatory disease characterized by increased proliferation and diminished susceptibility to apoptosis, implicating HAX-1 in the regulation of cell viability [101].

Even though HAX-1 is highly expressed in skeletal and cardiac muscles, its role in muscle function has recently been unraveled through the identification of its interactions with caspase-9 [96] and PLN [81]. The PLN/HAX-1 interaction was suggested to play a role in modulating Ca^{2+} cycling in the regulation of cardiac contractility. Importantly, this complex may regulate SR/ER Ca^{2+} homeostasis, which could affect mitochondrial Ca^{2+} redistribution and in turn influence mitochondrial Ca^{2+} accumulation and initiation of the apoptotic cell death signaling cascade [102]. In the presence of PLN, HAX-1 exhibited an enhanced anti-apoptotic effect after hypoxia/reoxygenation-induced cell death [81]. This finding further emphasizes the critical functional role of the PLN/HAX-1 interaction, which represents a new link between Ca^{2+} handling and cell survival.

The identification of the HAX-1/caspase-9 interaction provided new evidence linking HAX-1 with the apoptotic pathway. Specifically, adenoviral overexpression of HAX-1 in rat cardiomyocytes resulted in significant attenuation of

Table 1 HAX-1 interacting proteins identified to date

Cellular localization	Method for interaction identification	Tissue or cell line studied	Proposed function of protein complex	Reference
Viral proteins				
Epstein–Bar virus nuclear antigen leader protein (EBNA-LP)	YTH; blood lymphocyte complementary DNA (cDNA) library	COS-7 cell line	Regulation in apoptosis of EBV-infected cells	[83, 84]
K15 protein of Kaposis's sarcoma–associated herpevirus	YTH; BCP-1 cDNA library	HeLa cell line	Proposed role in regulation of apoptosis, cell invasion and motility	[85]
Vpr protein of HIV	YTH; human bone marrow cDNA library	HeLa cell line	Regulation of apoptosis through suppression of Vpr proapoptotic activity	[86]
Rev protein of HIV	Immunoprecipitation in 293 T cell line	293 T, COS-1 cell lines	Regulation of stability/export of viral mRNA and target RNA for degradation	[87]
Epstein–Bar virus nuclear antigen 5 (EBNA5)	YTH; EBV transformed B-cell cDNA library	DG75 cell line	Possible regulation of B-cell receptor signaling and apoptosis	[88]
Cytoplasmic proteins				
Polycystic kidney disease 2 (PKD2)	YTH; mouse embryonic and human adult kidney libraries	Kidney, HeLa cell line	Involved in cell–matrix interactions	[89]
$G_{\alpha}13$, the α-subunit of the heterotrimeric G protein G13	YTH; HeLa cDNA library	COS-7, NIH3T3 cell lines	Regulation of Gα13 stimulated cell motility	[90]
β6 cytoplasmic domain of integrin $\alpha_v\beta6$	YTH; human keratinocyte cDNA library	VB6 and H400 oral squamous cell carcinoma cell lines	Regulation of clathrin-mediated endocytosis of $a_v\beta6$ integrins for cell migration and invasion	[91]
Interleukin-1α (IL-1α)	YTH; human T-lumphocyte cDNA library; Immunoprecipitation	Skin fibroblasts, HEK293 cell line	Possible contribution to IL-1α biological functions	[92, 93]
HS1	YTH; human B cell lymphoma cDNA library	COS-7 cell line	Possible regulation of B-cell signal transduction and survival	[82]
BSEP, MDR1, MDR2 ABC transporters	YTH; rat liver cDNA library	Liver, HEK293, MDCK II cell lines	Regulation of BSEP abundance in apical membrane for clathrin-mediated endocytosis during biliary transport	[94]
Mitochondrial proteins				
Prohibitin 2	Immunoprecipitation using mitochondrial extracts from HeLa cells	HeLa cell line	Maintenance of mitochondrial morphology	[95]
Caspase-9	YTH; adult human heart cDNA library	Adult rat cardiomyocytes	Regulation of mitochondria-mediated apoptosis through inhibition of caspase-9 activation	[96]
Omi/HtrA2 protease	YTH; human primary melanocyte and keratinocyte cDNA libraries	HEK293, HK-2 cell lines	Cleavage and inactivation of HAX-1 during apoptosis	[97]
ER proteins				
Phospholamban (PLN)	YTH; human heart cDNA library	Cardiac muscle, HEK293 cell line	Regulation of ER/SR Ca^{2+} homeostasis	[81]
Nuclear proteins				
DNA polymerase β	YTH; rat testis cDNA library	Testes	Control of mRNA stability, transport and/localized translation	[98]
Vimentin 3′UTR	YTH; HeLa cDNA library	HeLa cell line	Regulation of vimentin mRNA perinuclear localization for intermediate filament formation	[99]
Unknown				
KIAA0513	YTH; human fetal brain cDNA library	No further investigation	Unknown but proposed function in modulation of apoptosis	[100]

YTH yeast two-hybrid

hypoxia/reoxygenation-induced cell death. This was shown to be mediated through the direct interaction of HAX-1 with caspase-9, probably occurring within the mitochondria, which in turn led to inhibition of caspase-9 processing and subsequent inhibition of caspase-3 activation [96]. Based on its interaction with caspase-9, an initiator caspase of apoptosis, HAX-1 was proposed to act during early stages of apoptosis.

Collectively, these findings highlight the involvement of HAX-1 in SR/ER Ca^{2+} cycling, mediated through PLN, but also implicate HAX-1 in cell survival via inhibition of the caspase-9 apoptotic pathway. This critical new link between Ca^{2+} cycling and apoptosis could hold important clues for understanding the association of SERCA2a and PLN with heart failure.

HAX-1 and disease

HAX-1 deficiency was recently associated with autosomal recessive severe congenital neutropenia (Kostmann disease) [103]. Severe congenital neutropenia represents a primary immunodefiency syndrome comprising of a genetically heterogeneous group of disorders, which is characterized by low peripheral blood neutrophils and myeloid maturation arrest [104]. Three different nonsense HAX-1 mutations (W44X, R86X and Q190X) have been identified so far in different populations [103, 105, 106]. Analysis of the functional significance of the W44X mutation in cells from affected individuals determined a critical role for HAX-1 in the maintenance of the inner mitochondrial membrane potential. Specifically, deficiency of this protein caused destabilization of membrane potential, leading to increased apoptosis in myeloid cells and disease progression [103]. Given the recently identified involvement of HAX-1 in cardiac muscle, an important biological question yet to be answered is whether these patients present cardiac functional defects.

Although there have been no reports directly associating HAX-1 with heart disease, its potential involvement in Ca^{2+} homeostasis through PLN interaction makes it an interesting candidate for future genetic studies.

HAX-1 and the Bcl-2 family of proteins

Regulation of SR Ca^{2+} homeostasis is of vital importance for cardiac cell survival, as defects in SR Ca^{2+} cycling have been associated with cardiac dysfunction, heart failure, and death. The identification of HAX-1, a mitochondrial protein with anti-apoptotic function, as a binding partner of PLN indicates the direct association between a mitochondrial and SR protein, and provides new insights into key players and mechanisms modulating Ca^{2+} cycling in cardiac contractility and cell death. Accumulating evidence has unraveled a novel aspect of the Bcl-2 anti-apoptotic protein family in promoting cell survival through regulation of ER Ca^{2+} homeostasis. Interestingly, HAX-1 presents a number of structural and functional similarities to Bcl-2. Therefore, while the precise function of the anti-apoptotic protein HAX-1 in cardiac muscle is slowly beginning to emerge, its similarity to Bcl-2 implicates HAX-1 in the regulation of cell survival. This can give rise to novel hypotheses regarding the molecular mechanisms of HAX-1 function and potentially uncover new links between Ca^{2+} cycling and apoptosis, especially in cardiac disease.

In mammals, the Bcl-2 gene family consists of at least 12 members [107], encoding proteins with pro- or anti-apoptotic function that exert a key role in the regulation of apoptosis. These proteins are important in controlling the mitochondrial pathway of apoptosis and are critical in the decision toward initiation of the cellular death cascade, which leads to release of pro-apoptotic factors, caspase activation, and commitment to apoptosis. Bcl-2, the founding member of this family, is an anti-apoptotic protein that provides protection against various apoptosis-inducing agents by maintaining the outer mitochondrial membrane integrity and, in this way, preventing the release of pro-apoptotic factors. A characteristic feature of the Bcl-2 protein family is the presence of at least one of the four conserved Bcl-2 homology (BH) domains, which are thought to be important in mediating protein interactions between family members [108, 109]. Since HAX-1 presents structural similarity to Bcl-2 due to its sequence homology to the Bcl-2 domains BH1 and BH2, it becomes intriguing to speculate that these two mitochondrial proteins might participate in similar molecular pathways.

The Bcl-2 protein family and its role in ER Ca^{2+} homeostasis

Mitochondria are central players in the initiation of apoptosis, and numerous studies have focused on their role in regulating the molecular pathways leading to cell death. Recent experimental evidence has demonstrated the critical role of ER Ca^{2+} content in determining cellular sensitivity to apoptotic stimuli. In particular, increased ER Ca^{2+} load was associated with sensitization to apoptosis, while decreased ER Ca^{2+} levels are thought to provide protection against apoptotic stimuli [110, 111]. A communication between the ER and the mitochondria was proposed to represent a critical mechanism in determining cell fate, hence defining a functional role for the ER as a new gateway to apoptosis [110]. The spatial organization and proximity of ER and mitochondrial membranes has been

shown to result in the existence of close contact sites between the two organelles [102, 112–114]. This may facilitate Ca^{2+} movement between the two organelles, further pinpointing their critical relationship in defining cellular fate. The direct communication between ER and mitochondria could be of particular importance in tissues such as cardiac muscle, which require accurate and tight regulation of Ca^{2+} homeostasis coupled to bioenergetics on a beat-to-beat basis.

Over the past few years, it has become evident that anti-apoptotic proteins may be important in promoting cell survival by regulating homeostasis of additional organelles. Studies on Bcl-2 have determined that, in addition to its mitochondrial localization, it is also present in other membrane cellular compartments including the ER and the outer nuclear membrane [115–117]. Several other proteins of the Bcl-2 family, such as BAX, BAK, and Bcl-X_L, also localize to the ER [118–120]. While the function of Bcl-2 at these extra-mitochondrial sites remains unclear, experimental evidence suggests that it is most likely related to cell survival.

The use of specific organelle-targeted mutants showing restricted subcellular localization has been very important on deciphering the role of Bcl-2 at the ER membrane and thus further defining its contribution to regulation of cellular apoptosis. An ER-localized Bcl-2 mutant (Bcl-2-cb5), which was generated by exchanging the C-terminal tail of wild-type Bcl-2 with the corresponding sequence from an ER-specific isoform of cytochrome b_5, has been shown to protect against a variety of apoptotic-stimuli including serum starvation, ionizing radiation, brefeldin A, ceramide, and staurosporine [121–124]. Moreover, this ER-localized Bcl-2 mutant can inhibit the loss of mitochondrial membrane potential [124] and cytochrome c release [123], suggesting that Bcl-2-cb5 can indirectly protect the mitochondria. These studies indicate that Bcl-2 does not need to be associated with mitochondria to inhibit cytochrome c release and have implicated Bcl-2 in an apoptotic crosstalk between the ER and the mitochondria. Similarly to Bcl-2, HAX-1 can localize at the mitochondria or the ER, exhibiting anti-apoptotic properties in both sites [81]. Thus, HAX-1 could represent a new mediator of the ER–mitochondria crosstalk, with direct implications in cell death decisions.

Considering the critical function of ER in Ca^{2+} homeostasis, the role of Bcl-2 as a regulator of ER Ca^{2+} levels has been evaluated. In human breast epithelial cells and mouse lymphoma cells, Bcl-2 overexpression was shown to result in increased ER Ca^{2+} concentration, a finding which was proposed to correlate with maintenance of cell growth and viability [43, 125]. On the other hand, studies in HeLa cells [126], human prostate cancer cells [127], and HEK293 cells [128] identified decreased ER Ca^{2+} levels as the result of Bcl-2 overexpression. This decrease in ER Ca^{2+} concentration was proposed to enhance cell survival by reducing the amount of Ca^{2+} available for release and subsequent uptake by the mitochondria after apoptotic stimulus. The above discrepancies on the effect of Bcl-2 overexpression could be due to differences between cells lines, Bcl-2 expression levels, and Ca^{2+} measurement methodologies [108].

The anti-apoptotic protein Bcl-X_L has also been found to modulate ER Ca^{2+} levels and cell survival, further implicating anti-apoptotic proteins in the regulation of Ca^{2+} homeostasis. Specifically, Bcl-X_L overexpression in a murine T-cell line caused a dose-dependent decrease in the expression levels of type 1 inositol 1,4,5-triphosphate receptor (IP_3R) and thus in the amount of receptor-mediated Ca^{2+} released after IP_3 stimulation [129, 130]. The identification of an interaction between Bcl-X_L and IP_3R has provided additional evidence for the involvement of Bcl-X_L in the regulation of ER Ca^{2+} homeostasis, leading to enhanced cellular bioenergetics and preserved survival [131].

In addition to anti-apoptotic proteins, the pro-apoptotic proteins BAX and BAK have also been shown to modulate ER Ca^{2+} content, to affect the amount of Ca^{2+} taken up by the mitochondria and subsequently to induce apoptosis [119, 132]. Analysis of mouse embryonic fibroblasts (MEFs) from Bax and Bak double-knockout mice has provided valuable information on the role of these apoptotic proteins as regulators of ER Ca^{2+} homeostasis. A decreased ER Ca^{2+} content in Bax and Bak double-knockout MEF cells resulted in attenuated Ca^{2+} uptake by the mitochondria after ER Ca^{2+} release and consequent increased resistance to ceramide and arachidonic-acid-induced cell death [133]. Overexpression of BAX or SERCA corrected the ER Ca^{2+} imbalance in these cells and restored apoptotic response to cell death stimuli, thus demonstrating the involvement of BAX in modulating ER Ca^{2+} homeostasis and promoting cell death. The decreased levels of ER Ca^{2+} in Bax and Bak double-knockout MEF cells were associated with increased passive leak of Ca^{2+} and hyperphosphorylated state of inositol triphosphate receptor type 1 (IP3R-1) [134]. These findings suggest a role for BAX and BAK in both ER and mitochondria, with the two proteins potentially providing a Ca^{2+}-dependent crosstalk between the two organelles.

Taken together, these studies provide significant evidence on the involvement of Bcl-2 family proteins in the regulation of ER Ca^{2+} homeostasis, with direct effects on ER Ca^{2+} content and cell survival. In parallel to these proteins, HAX-1 is highly likely to play a similar role. Specifically, HAX-1 interacts with PLN, the major regulator of SERCA2a activity, and exhibits anti-apoptotic properties when localized to the ER, thus implicating it in the regulation of ER/SR Ca^{2+} homeostasis and cell survival (Fig. 2).

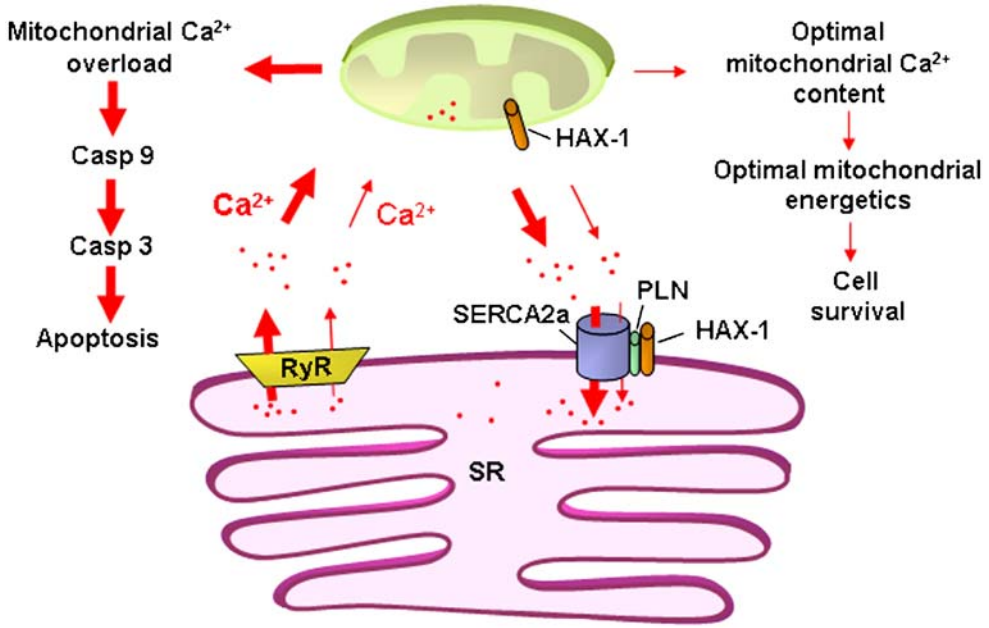

Fig. 2 HAX-1 is a promising new link between Ca^{2+} cycling and cell survival. During cardiac contraction, part of the SR Ca^{2+} content that is released from RyR channels can be taken up by closely positioned mitochondria. Sequestration of Ca^{2+} to the SR by SERCA2a results in refilling of the SR. PLN exerts inhibitory effects on SERCA2a activity and thus represents a critical regulator of SR Ca^{2+} homeostasis. The anti-apoptotic protein HAX-1 interacts with PLN, an association directly implicating HAX-1 in the regulation of SR Ca^{2+} uptake to promote cell survival. If SR Ca^{2+} uptake is tightly regulated, then SR Ca^{2+} content is maintained at optimal levels, and subsequently, mitochondria take up optimal amounts of Ca^{2+} (*thin arrows*). This can positively regulate mitochondrial bioenergetics to support cell function and promote cell survival. Conversely, deregulation of SR Ca^{2+} uptake, as seen in heart failure, can affect the amount of SR Ca^{2+} available for release and subsequently cause mitochondrial Ca^{2+} overload (*thick arrows*). This would result in caspase activation, initiation of the apoptotic signaling cascade, and cell death. HAX-1 is emerging as a critical player in the regulation of SR Ca^{2+} homeostasis, with direct effects on SR Ca^{2+} content and cell survival

Bcl-2 interacts with SERCA and modulates its activity

Although the precise mechanism through which Bcl-2 regulates ER Ca^{2+} homeostasis is still unclear, experimental evidence supports a number of different alternatives [42, 43, 128, 135–137]. Of particular interest is the effect of Bcl-2 on SERCA activity, mediated through their direct association that parallels the PLN/HAX-1 binding.

Overexpression of Bcl-2 in human breast epithelial cells and prostate cancer cells was shown to influence SERCA protein expression, resulting in an increase or decrease of SERCA2 or SERCA2b, respectively. Although the underlying cause of this discrepancy has not been clarified, these findings implicate Bcl-2 in SERCA expression regulation and provide a possible explanation for the observed alterations in ER Ca^{2+} levels [43, 127]. Immunoprecipitation studies in DHL-4 human lymphoma cell line, MCF10A breast epithelial cells and rat skeletal muscle identified a direct interaction between Bcl-2 and SERCA1 or SERCA2 [42, 43]. Further in vitro analysis of this interaction determined that Bcl-2 inhibits SERCA activity in a time- and dose-dependent manner, and causes a conformational transition of SERCA, leading to partial unfolding of the protein [42]. It was recently proposed that SERCA inactivation by Bcl-2 occurs through displacement of SERCA from caveolae-related domains of the SR into a different membrane environment [138]. Although the binding sites involved in the interaction between Bcl-2 and SERCA have not been determined, current findings suggest that the occurrence of a tight association with the ATP-binding or the Ca^{2+}-binding domains of SERCA is probably unlikely. It is possible, however, that a transient interaction of Bcl-2 with these domains may trigger transition of SERCA into an inactive conformation and may therefore release Bcl-2 from the protein complex [138].

Collectively, these findings reveal the critical role of Bcl-2 in modulating SERCA activity and ER Ca^{2+} levels. Furthermore, when considered jointly, the HAX-1 and Bcl-2 association with the SERCA/PLN complex may give rise to the hypothesis that there is a direct link between the SR Ca^{2+} uptake complex and apoptosis, with direct implications in cardiac function and disease development, such as heart failure.

Conclusion

Ca^{2+} cycling is a critical determinant of cardiomyocyte contractility and cardiac function. A major regulator of SR

Ca^{2+} uptake is the SERCA2a/PLN complex, with aberrations in its function being directly associated with heart failure. HAX-1, the novel binding partner of PLN, presents with numerous structural and functional similarities to the Bcl-2 family and is therefore emerging as an intriguing new link between Ca^{2+} homeostasis and cell survival. Over the past few years, it has become apparent that a crucial aspect in the function of anti-apoptotic proteins of the Bcl-2 family includes regulation of ER Ca^{2+} homeostasis and protection of mitochondria from Ca^{2+} overload. Similarly, HAX-1 could be implicated in the promotion of cell survival by indirectly or even directly affecting SERCA2a activity. This putative role of HAX-1 as a mediator between SR Ca^{2+} content and cell survival needs to be further evaluated. Elucidating the precise pathways implicated in this process will be invaluable in understanding the pathogenetic mechanisms of heart failure and importantly could unveil promising new therapeutic targets.

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