REVIEW ARTICLE

Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum

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Calreticulin is an ER (endoplasmic reticulum) luminal Ca^{2+}-buffering chaperone. The protein is involved in regulation of intracellular Ca^{2+} homoeostasis and ER Ca^{2+} capacity. The protein impacts on store-operated Ca^{2+} influx and influences Ca^{2+}-dependent transcriptional pathways during embryonic development. Calreticulin is also involved in the folding of newly synthesized proteins and glycoproteins and, together with calnexin (an integral ER membrane chaperone similar to calreticulin) and ERp57 [ER protein of 57 kDa; a PDI (protein disulfide-isomerase)-like ER-resident protein], constitutes the 'calreticulin/calnexin cycle' that is responsible for folding and quality control of newly synthesized glycoproteins. In recent years, calreticulin has been implicated to play a role in many biological systems, including functions inside and outside the ER, indicating that the protein is a multi-process molecule. Regulation of Ca^{2+} homoeostasis and ER Ca^{2+} buffering by calreticulin might be the key to explain its multi-process property.

Key words: calcium homoeostasis, calreticulin, endoplasmic reticulum (ER), protein folding, quality control.

INTRODUCTION

The ER (endoplasmic reticulum) plays a vital role in many cellular processes, including Ca^{2+} storage and release, lipid and protein synthesis, folding and post-translational modification. The ER is also involved in cellular signalling and organelle–organelle communication, including ER stress-dependent activation of transcriptional processes, Ca^{2+} signalling and communication to the plasma membrane Ca^{2+} channels and ERAD (ER-associated degradation). Furthermore, continuous fluctuation of the ER luminal Ca^{2+} concentration may function as signalling for many ER functions, including protein and lipid synthesis [1]. Taken together, it is apparent that the ER may be defined as a multifunctional organelle that is able to detect and integrate incoming signals, modulate its own luminal dynamics and generate output signals in response to environmental changes [1].

To carry out these diverse cellular functions, the ER contains many luminal and integral membrane proteins. Calreticulin is a unique ER luminal Ca^{2+}-binding chaperone implicated to play a role in many cellular functions, including lectin-like chaperoning, Ca^{2+} storage and signalling, regulation of gene expression, cell adhesion, wound healing, cancer and autoimmunity, to name a few. The aim of the present review is to focus on the latest developments in the calreticulin field, with major emphasis on the structure and function of the mammalian protein and its role in ER dynamics and ER-associated signalling.

STRUCTURAL AND FUNCTIONAL DOMAINS OF CALRETICULIN

Calreticulin is a 46 kDa ER luminal Ca^{2+}-binding protein and molecular chaperone. The protein contains an N-terminal cleavable signal sequence that directs it to the ER, and an ER KDEL (Lys-Asp-Glu-Leu) retention/retrieval signal. Calreticulin, together with calnexin and ERp57 (ER protein of 57 kDa), is involved in the chaperoning of nascent polypeptides that traverse through the ER [2]. Calnexin is a 90 kDa ER integral membrane protein and homologue of calreticulin [3]. Calnexin also has a cleavable N-terminal amino acid signal sequence that directs it to the ER, a transmembrane domain and a cytoplasmic C-terminal RKPRRE (Arg-Lys-Pro-Arg-Arg-Glu) ER-retention signal [3]. ERp57 is an oxidoreductase that forms functional complexes with calreticulin and calnexin [2]. Calreticulin and calnexin show a high degree of structural and functional similarities, including domain-like structure. For example, the two proteins exhibit 40 % amino acid sequence similarity and 30 % amino acid sequence identity [3–5]. Functionally, both proteins efficiently suppress the aggregation of glycosylated and non-glycosylated proteins via protein–oligosaccharide and protein–protein interactions [2].

Abbreviations used: BiP, immunoglobulin heavy-chain-binding protein; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; C/EBP, CCAAT/enhancer-binding protein; COUP-TF1, chicken ovalbumin upstream promoter-transcription factor 1; CTL, cytotoxic T-lymphocyte; EDEM, ER degradation-enhancing 1,2-mannosidase-like protein; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; ERp57, ER protein of 57 kDa; Evi-1, ecotropic viral integration site 1; GATA6, GATA-binding protein 6; Grp, glucose-regulated protein; Hsp, heat-shock protein; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; LRP, low-density lipoprotein receptor-related protein; MEF2C, myocyte enhancer factor 2C; MMP, matrix metalloproteinase; NFAT, nuclear factor of activated T-cells; Nkx2.5, NK2 transcription factor related, locus 5; PACS-2, phosphofurin acidic cluster sorting protein 2; PDI, protein disulfide-isomerase; PPAR, peroxisome-proliferator-activated receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; SLE, systemic lupus erythematosus; SOCI, store-operated Ca^{2+} influx; SSA, Smith surface antigen; Stim1, stromal cell-surface molecule 1; TAP, transporter associated with antigen processing; TGF, transforming growth factor; TSP-1, thrombospondin 1; UGGT, UDP-glucose:glycoprotein transferase.

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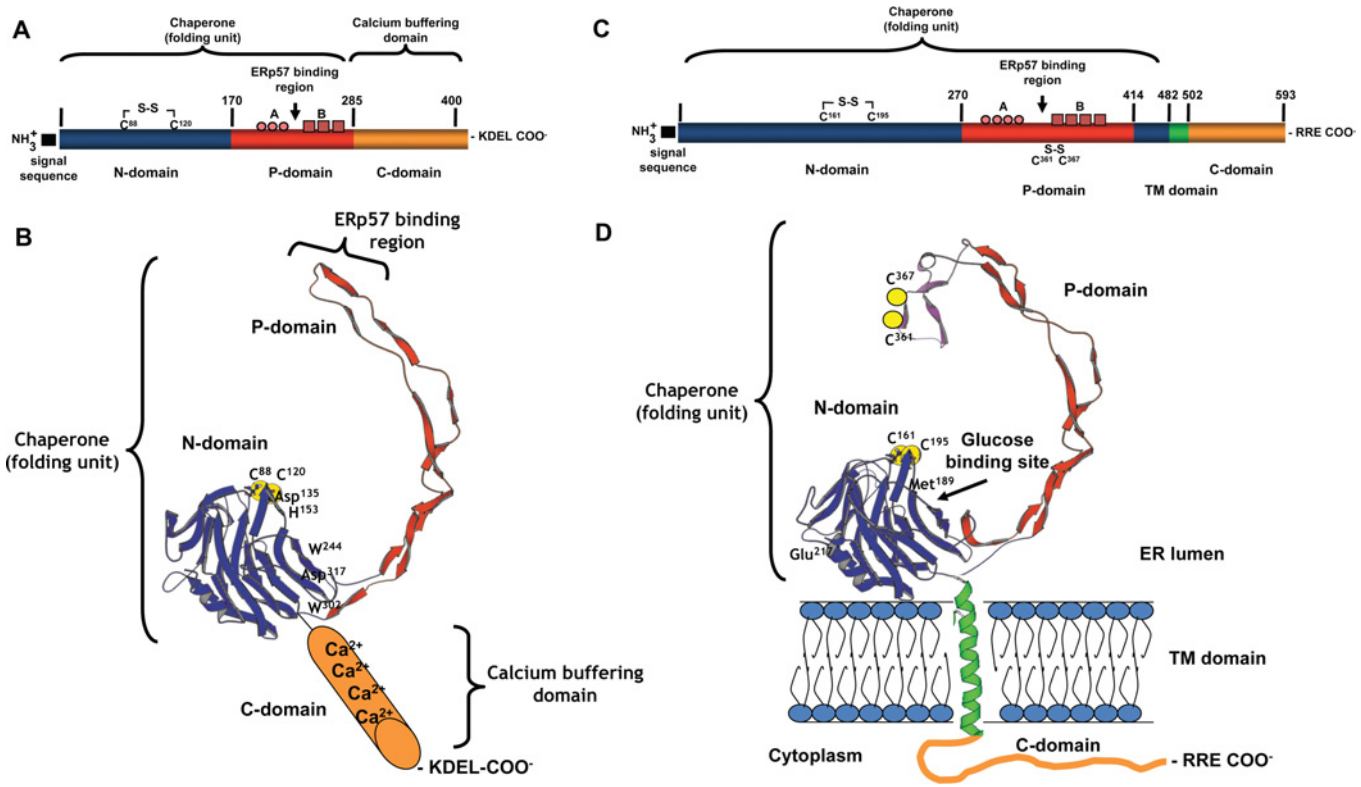


Figure 1 Model of the structure of calreticulin

(**A**) Linear representation of calreticulin domains is shown. The protein contains an N-terminal amino acid signal sequence (black box), N-domain (blue box), P-domain (red box), C-domain (orange box) and a C-terminal KDEL ER retrieval signal. Repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX) are indicated by pink circles and squares respectively. The amino acids involved in the thiol linkage are indicated and connected with an S–S, and the numbers delineate the amino acid residues at the transition between the various domains. (**B**) Three-dimensional model of the N- and P-domains of calreticulin based on the NMR studies of the P-domain of calreticulin [23] and crystallographic studies of calnexin (PDB code 1JHN) [6]. Calreticulin contains a globular N-domain (in blue) and central proline-rich P-domain (in red) which forms a characteristic loop. The N- and P-domains of calreticulin are responsible for the chaperone function of the protein. The C-terminal C-domain contains a large number of negatively charged amino acids and is involved in high-capacity Ca^{2+} storage. Yellow balls represent the cysteine residues (Cys^{88} and Cys^{120}), which form a thiol bridge in calreticulin. The locations of an essential His^{153} and a putative carbohydrate-binding pocket are indicated. Trp^{244} and Trp^{302} are located in the globular domain. The ERp57-binding site is indicated. (**C**) Linear representation of calnexin domains. The protein contains an N-terminal amino acid signal sequence (black box), N-domain (blue boxes), P-domain (red), transmembrane domain (green box), C-domain (orange box) and a C-terminal RKPRRE ER retrieval signal. Repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX) are indicated by pink circles and squares respectively. The amino acids involved in the thiol linkages are connected with an S–S, with the numbers delineating the amino acid residues at the transition between the various domains. (**D**) The three-dimensional structure of the N- and P-domains of calnexin is based on crystallographic studies of calnexin (PDB code 1JHN) [6]. The N-domain (in blue), central proline-rich P-domain (in red) which forms a characteristic loop and contains a disulfide bond (C^{361}–C^{367}), is followed by a transmembrane helix (in green) and a cytoplasmic C-terminal C-domain. Yellow balls represent the cysteine residues (Cys^{161} and Cys^{195}, and Cys^{364} and Cys^{367}) which form thiol linkages in calnexin. TM, transmembrane.

Calreticulin and calnexin are composed of three distinct structural and functional domains: a globular N-domain, an extended P-domain and an acidic C-domain (Figure 1).

The N-domain

X-ray crystallography has identified the N-terminus of calnexin as a Ca^{2+}-binding globular β-sandwich, similar to legume lectin, exhibiting an interaction with a glucose moiety (Figure 1D) [6]. Prediction of the secondary structure of calreticulin, based on that of calnexin, suggests that the N-terminal region forms a globular domain composed of eight antiparallel β-strands (Figure 1B) [7]. The N-domain of calreticulin includes the polypeptide- and carbohydrate-binding sites [8,9], a Zn^{2+}-binding site [10] and a disulfide-linkage site [11]. The N-domain of calreticulin forms a stable core that is resistant to proteolysis in the presence of Ca^{2+} [12].

In vitro studies indicate that the polypeptide- and oligosaccharide-binding regions are located in the N- and P-domain of calreticulin [8]. Oligosaccharide binding by this region induces conformational change in the chaperone, thereby influencing polypeptide binding [13]. There is a requirement for both the N-domain, with the oligosaccharide- and polypeptide-binding regions, as well as the P-domain, containing the secondary binding sites, to generate full chaperone function of calreticulin [8]. Polypeptide binding is favoured under conditions that induce unfolding in calnexin, while oligosaccharide binding occurs under conditions that enhance the structural stability of calnexin [14]. These two interactions may be responsible for the chaperone function of calreticulin and calnexin. There is only limited information available about molecular features of substrate binding to calreticulin. Kapoor et al. [9] identified two amino acid residues in calreticulin's globular N-domain (Tyr^{109} and Asp^{135}) that abolish interaction of the protein with oligosaccharides. A number of additional residues might also be involved in sugar binding to the protein, including Lys^{111}, Tyr^{128} and Asp^{317} [15,16]. The disulfide linkage between Cys^{88} and Cys^{120}, as well as Trp^{244} and Trp^{302}, located in the globular domain, are also critical for chaperone function of calreticulin [17]. In calnexin, six amino

acid residues in the globular N-domain have been identified as being important for oligosaccharide binding (Tyr^{156}, Lys^{167}, Tyr^{186}, Met^{189}, Glu^{217} and Glu^{426}) [18]. Mutation of a distinct histidine residue in calreticulin, His^{153}, results in conformational changes of the protein, leading to loss of its chaperone function [19]. Interestingly, calreticulin with ablated lectin function is still able to chaperone polypeptides, specifically involved in loading of peptide on to MHC class I [20]. Importantly, functional studies indicate that the N-domain, in conjunction with the P-domain, may form a functionally important 'folding unit' responsible for chaperone function of calreticulin [21] and probably calnexin.

The P-domain

The middle portion of calreticulin and calnexin, named the P-domain, is proline-rich, suggesting potential flexibility of this region in these proteins (Figure 1). The P-domains in these two proteins contain pairs of repeats (repeat A, IXDPXA/DXKPEDWDX, and repeat B, GXWXPPXIXNPXYX) (Figure 1). There are three sets of AB repeats in calreticulin [4,5] and four in calnexin [3]. These repeat amino acid sequences form an important structural backbone of the P-domain and may be involved in the lectin-like function of the proteins [22]. NMR studies reveal that the structure of the P-domain of calreticulin contains an extended region stabilized by three antiparallel β-sheets [23] that interacts with ERp57 (Figure 1B) [17,24,25]. *In vitro* analysis indicate that this region of calreticulin binds Ca^{2+} with a relatively high affinity ($K_d = 1\ \mu M$), but low capacity (1 mol of Ca^{2+} per mol of protein) [26,27]. X-ray studies showed that the P-domain of calnexin also forms a similar extended arm structure (Figure 1D) [6]. Both calreticulin and calnexin facilitate protein folding in conjunction with ERp57 [8,28]. Using NMR spectroscopy techniques, ERp57-binding sites have been identified at the tip of the P-domain in calreticulin and calnexin [25,29]. The interaction of calreticulin with ERp57 is disrupted by mutations of Glu^{239}, Asp^{241}, Glu^{243} and Trp^{244} in the tip of the P-domain [17].

The C-domain

The C-domain of calreticulin is of special interest because it contains a large number of negatively charged residues that are responsible for the Ca^{2+}-buffering function of the protein. It binds over 50% of ER luminal Ca^{2+} [21] with high capacity (25 mol of Ca^{2+} per mol of protein) and low affinity ($K_d = 2$ mM) (Figures 1A and 1B). The C-domain of calnexin is also an interesting region. It extends from the transmembrane α-helix, contains an ER-retention amino acid sequence, and stretches of negatively charged amino acid residues that bind Ca^{2+} with moderate affinity [3,27]. The physiological significance of a low-affinity Ca^{2+}-binding site in the cytoplasmic tail of calnexin remains a mystery, considering low Ca^{2+} concentrations in the cytoplasm. This region of calnexin may play an important role in the regulation of protein–protein interactions via specific phosphorylation [30–32]. Phosphorylation of Ser^{562} may act as a molecular switch regulating the interaction of calnexin with SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase) 2b and affecting SERCA2b function [33], thereby coupling Ca^{2+} signalling and Ca^{2+}-sensitive chaperone functions in the ER. More recently, calnexin was shown to perform an important role during rhodopsin maturation and photoreceptor cell survival [34]. Mutations in *Drosophila* calnexin result in severe disruption of rhodopsin expression, as well as aberrant cytoplasmic Ca^{2+} levels, indicating a role for calnexin during Ca^{2+} homoeostasis, via Ca^{2+}-binding sites, the P-domain site and the acidic cytoplasmic tail [34]. Calnexin interacts with a cytoplasmic sorting protein, PACS-2 (phosphofurin acidic cluster sorting protein 2), located at the ER–mitochondrial junctions or mitochondria-associated membrane. This interaction is regulated by phosphorylation of two cytoplasmic serine residues, Ser^{554} and Ser^{564}, with PACS-2 sorting localization of calnexin between the ER, the mitochondria-associated membrane and the plasma membrane [35].

CALRETICULIN, A MOLECULAR CHAPERONE

The ER is the first compartment in the secretory pathway. Approx. 30% of all cellular proteins that are synthesized into the ER, where they interact with molecular chaperones and are transported as cargo through the ER to different intracellular destinations as well as to the extracellular environment. Within the ER, nascent unfolded proteins interact with molecular chaperones and enzymes including BiP (immunoglobulin heavy-chain-binding protein)/Grp (glucose-regulated protein) 78, calreticulin, calnexin, Grp94 and the thiol oxidoreductases PDI (protein disulfide-isomerase) and ERp57, all involved in generating conformationally competent and functional proteins [36]. Each of these chaperones or folding factors has their own unique mechanism to prevent the transport of misfolded proteins out of the ER. BiP/Grp78 and Grp94 have the ability to recognize exposed hydrophobic regions common to nascent unfolded proteins, assisting in their folding and assembly, while calreticulin and calnexin interact with nascent glycoproteins via polypeptide and lectin binding [2]. PDI and ERp57, both thiol oxidoreductase folding factors, utilize the oxidizing environment of the ER to generate disulfide linkages, with the formation of intra- and inter-chain disulfide bonds an integral part of the maturation of most secretory and membrane-bound proteins in the ER. If the protein is unable to fold properly, it is targeted for degradation by the proteasomal pathway [2]. Build-up of these misfolded or unfolded proteins triggers a variety of signalling pathways that control subsequent ER stress. Chaperone activity is necessary for a protein to obtain functional shape, but has been observed to be redundant in numerous tissues and may not play an essential function in organism viability. For example, both Grp94- and calreticulin-deficient mouse models, although lethal, demonstrate disruption in heart development as the cause of lethality, but have normal development of other embryonic organs [37,38]. Calnexin deficiency is not embryonic lethal and $Cnx^{-/-}$ mice are viable, but have neurological problems [39].

Synthesis of the N-linked oligosaccharide starts on the cytoplasmic side of the ER by the addition of the sugars to a lipid anchor, dolichyl phosphate. Initially, two *N*-acetylglucosamine residues and five mannose residues are added to dolichyl phosphate, with the oligosaccharide then flipped into the lumen of the ER. In the ER lumen, four mannose residues and three glucose residues are added. This oligosaccharide is composed of $Glc_3Man_9GlcNAc_2$. As the nascent protein traverses the translocon as an extended chain and emerges into the lumen of the ER, an enzyme, oligosaccharyltransferase, closely associated with the translocon recognizes a specific sequence in the protein, NXS/T (Asn-Xaa-Ser/Thr), and attaches the oligosaccharide to the asparagine residue via an amide linkage. This close association is very important, as proteins are co-translationally translocated into the ER lumen, while being co- and post-translationally modified by N-linked glycosylation. Two ER luminal enzymes then modify the oligosaccharide by cleaving the terminal glucose residues. Glucosidase I, an ER luminal enzyme, removes an initial glucose residue, whereas glucosidase II cleaves two further glucose residues. Before glucosidase II cleaves the third glucose residue, the glycoprotein is recognized by the quality-control cycle

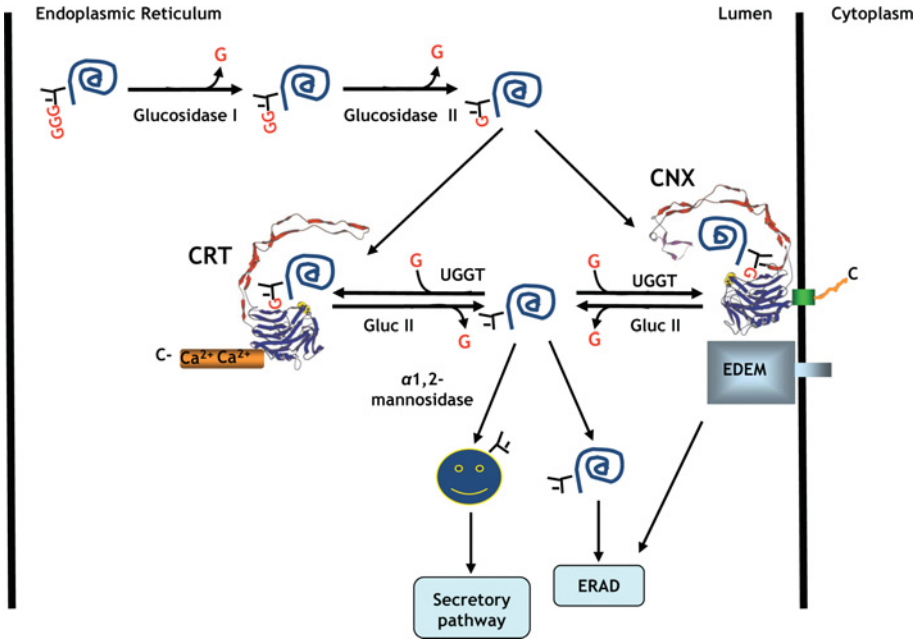


Figure 2 Calreticulin/calnexin cycle

Calreticulin and calnexin, together with ERp57, constitute the calreticulin/calnexin cycle that is responsible for the folding and quality control of newly synthesized glycoproteins. In the ER lumen, the oligosaccharide of newly synthesized glycoproteins is composed of $Glc_3Man_9GlcNAc_2$. Glucose residues are sequentially removed from this oligosaccharide by glucosidases I and II. Calreticulin and calnexin bind monoglucosylated carbohydrate on newly synthesized glycoproteins. UGGT, which can re-glucosylate chains that have been glucose-trimmed, in conjunction with the action of glucosidase II, establishes a cycle of de-glucosylation and re-glucosylation. This de-glucosylation–glucosylation cycle may be repeated several times before a newly synthesized glycoprotein is properly folded and enters the secretory pathway or sent is for degradation (ERAD). Prolonged interaction with calnexin also targets a protein for degradation via an interaction with EDEM. CNX, calnexin; CRT, calreticulin; G, glucose residue; Gluc II, glucosidase II. An animated version of this Figure can be seen at http://www.BiochemJ.org/bj/417/0651/bj4170651add.htm.

proteins, calreticulin and calnexin (Figure 2) [2]. Once the protein has been properly folded, the third glucose is removed by glucosidase II, the protein is released from the quality-control cycle and it is transported out of the ER. Misfolded proteins, with the third glucose removed, are recognized by UGGT (UDP-glucose:glycoprotein glucosyltransferase), and this enzyme carries out a re-glucosylation reaction to create a folding substrate recognized by calreticulin or calnexin (Figure 2). This forces incompletely folded glycoproteins to remain in the calreticulin/calnexin cycle until they have attained their proper conformation and are no longer recognized by UGGT (Figure 2). Prolonged interaction with calnexin targets a protein for degradation via an interaction with the EDEM (ER degradation-enhancing 1,2-mannosidase-like protein) [2]. The misfolded protein is recognized by α1,2-mannosidase I, which specifically cleaves mannose residues, allowing recognition by the ERAD (ER-associated degradation) machinery (Figure 2) [2].

The chaperone function and the Ca^{2+}-binding capacity of calreticulin are involved in a variety of cellular systems (Figure 3). MHC class I assembly provided a convenient model for studying the chaperone function of calreticulin and calnexin. MHC class I heterotrimeric complex is assembled in the ER with the assistance of a number of chaperones and folding factors and consists of a polymorphic glycosylated heavy chain, a non-polymorphic β_2-microglobulin and a peptide. The peptide-loading complex consists of the peptide transporter [TAP (transporter associated with antigen processing)], ERp57, calreticulin, calnexin and tapasin [40,41]. Both calreticulin and calnexin promote the assembly of MHC class I, as well as retaining incompletely assembled complexes in the ER [41]. Initially, TAP and tapasin associate with each other and are recognized by calnexin and ERp57 in a glycan-independent manner to form an intermediate complex with the heavy chain. This intermediate complex binds the MHC class I–β_2-microglobulin dimers, with calnexin released followed by calreticulin interaction, generating the MHC class I-loading complex. The complex is ready for peptide loading, and, once bound, the MHC class I–β_2-microglobulin dimer is dissociated and the MHC–peptide complex is transported to the cell surface [41]. One member of the loading complex, ERp57, forming a functional dimer with tapasin, is responsible for facilitating peptide binding as well as editing the bound peptides to maximize their affinity [42]. ERp57-deficient mice are not viable, and have disrupted assembly of MHC class I complexes, specifically in B-cells, identifying ERp57 as an integral component of the peptide-loading complex [43]. In calreticulin-deficient fibroblasts, MHC class I molecules display unusually rapid export from the ER, inefficient peptide loading and impaired T-cell recognition at the cell surface [44]. Expression of the ER luminal chaperone domains of calnexin does not rescue these defects, demonstrating that MHC class I assembly specifically depends on calreticulin [44]. Recently, Ireland et al. [20] demonstrated that lectin-deficient point mutations in calreticulin can fully rescue all of the MHC class I defects observed in the calreticulin-deficient fibroblasts. In addition, chaperone-deficient calnexin is able to form a complex with the heavy chains of the MHC class I molecule [18]. This indicates that calreticulin and calnexin utilize peptide-based interactions to assist in the assembly of MHC class I molecules and that assembly may not be dependent on oligosaccharide interaction.

Calreticulin and calnexin are homologous lectin molecular chaperones, with calreticulin-deficient cells having accelerated folding with an accumulation of misfolded proteins, whereas, in calnexin-deficient cells, folding is significantly impaired [45]. When both chaperones are prevented from interacting with

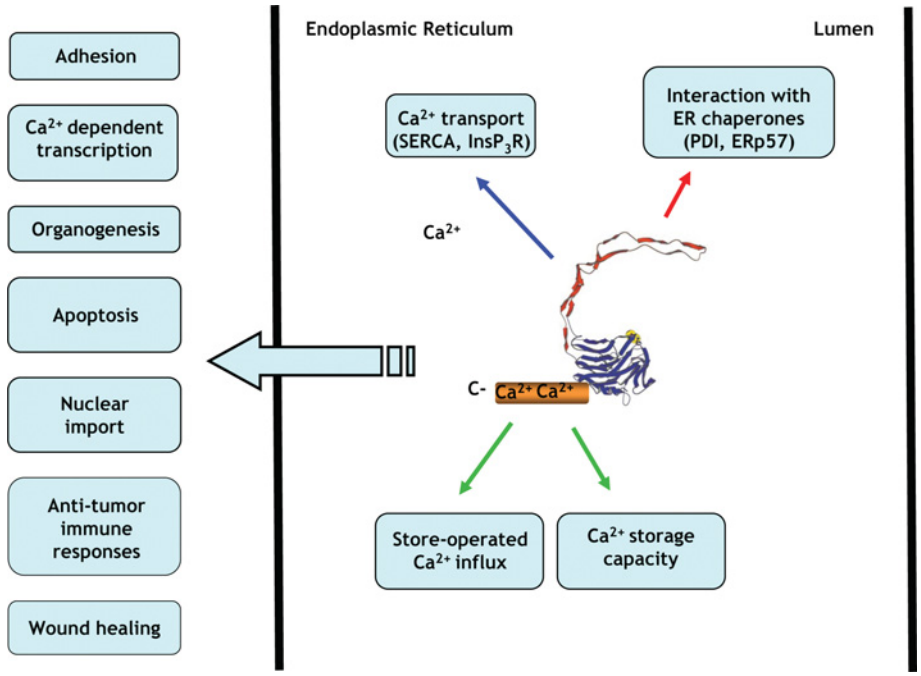


Figure 3 Calreticulin, a multi-process ER luminal Ca^{2+}-buffering chaperone

A model of calreticulin-dependent events occurring inside and outside the ER lumen. Calreticulin, in the lumen of the ER, plays a role as a Ca^{2+}-binding/storage chaperone. The protein affects the Ca^{2+} capacity of the ER stores. Calreticulin, in the ER lumen, interacts with other chaperones, specific substrates and other ER proteins including SERCA2b and $InsP_3R$ (blue arrow). Changes in the expression of calreticulin influence SOCI (green arrow). Calreticulin (and calnexin) form complexes with the ER chaperones, including PDI and ERp57 and binds monoglucosylated carbohydrate on newly synthesized glycoproteins (red arrow). From the lumen of the ER, calreticulin may affect many cellular functions, including cell adhesion, apoptosis, nuclear transport and anti-tumour immune responses.

substrate, a complete disruption in ER quality control is observed [45]. Calnexin binding to oligosaccharides requires the structural stability of calnexin [14] and oligosaccharide binding can be lost upon Ca^{2+} depletion [22], while polypeptide recognition requires a specific partially unfolded conformation of calnexin as a result of transient Ca^{2+} depletion [14]. In conjunction with studies carried out on calreticulin-deficient cells, calnexin deficiency results in compromised quality control, with accumulation of misfolded protein and up-regulation of ER stress [45,46]. Calnexin deficiency results in an increased rate of folding, a reduction in the folding efficiency and retention of misfolded protein in the ER [45,46].

CALRETICULIN, A REGULATOR OF Ca^{2+} HOMOEOSTASIS

Ca^{2+} performs an important role in the cell as a universal signalling molecule influencing various developmental and cellular processes. The majority of intracellular Ca^{2+} is stored in the lumen of the ER. Fluctuations of the ER luminal Ca^{2+} concentration result in impaired ER–Golgi trafficking [47], impeded transport of molecules across the nuclear pore [48] and disrupted chaperone function [49]. It appears that any disruption in Ca^{2+} stores within the ER as well as obstruction of Ca^{2+} released from the ER has the potential to activate transcriptional and translational cascades. These cascades ultimately regulate chaperones that are responsible for protein folding within the ER, proteins responsible for ER stress and ERAD, as well as proteins involved in the apoptotic pathway. Extracellular Ca^{2+} concentration is in excess of 2 mM, free cytoplasmic Ca^{2+} concentration is approx. 100 nM, total ER Ca^{2+} concentration is up to 1 mM and the free ER Ca^{2+} concentration is approx. 200 μM. ER Ca^{2+} homoeostasis and signalling are maintained by controlling Ca^{2+} release from the ER by the $InsP_3R$ (inositol 1,4,5-trisphosphate receptor) and ryanodine receptor, whereas the ER stores are refilled by the SERCA. The Ca^{2+} present in the ER stores serves as a source of easily releasable Ca^{2+}, but is also important as a regulator of a number of ER enzymes and proteins, including feedback regulation of the $InsP_3R$, the ryanodine receptor and SERCA.

Within the lumen of the ER are a number of Ca^{2+}-buffering proteins that are responsible for binding ER luminal Ca^{2+} as well as being involved in numerous aspects of ER function. Ca^{2+}-buffering proteins are critical, as the total Ca^{2+} concentration of the ER is in the micromolar to millimolar range. Many of these Ca^{2+}-buffering proteins display high Ca^{2+}-binding capacity (10 mol of Ca^{2+} per mol of protein or higher) and low affinity ($K_d = 1$ mM or higher), whereas others have a low capacity (1–2 mol of Ca^{2+} per mol of protein), but high affinity ($K_d = 1$ μM). Calreticulin utilizes an acidic region as the high-capacity Ca^{2+}-binding site, with 43 acidic amino acid residues out of the last 82 amino acids of the protein, to bind 25 mol of Ca^{2+} per mol of protein with low affinity ($K_d = 2$ mM) [26]. Calreticulin also contains a high-affinity ($K_d = 10$ μM) low-capacity (1 mol of Ca^{2+} per mol of protein) binding site contained in the proline-rich arm domain with a potential EF-hand-like helix–loop–helix motif [26]. Over 50 % of Ca^{2+} stored in the ER lumen is bound to calreticulin [21]. Not surprisingly, overexpression of calreticulin leads to increased amounts of Ca^{2+} in intracellular stores [50–52], whereas calreticulin-deficient cells have reduced Ca^{2+}-storage capacity in the ER and delayed agonist-mediated Ca^{2+} release [38,53].

Release of Ca^{2+} from the ER during Ca^{2+} signalling triggers a distinct event at the plasma membrane, termed SOCI (store-operated Ca^{2+} influx), which is responsible for providing Ca^{2+}

Table 1 Consequences of calreticulin loss-of-function or gain-of-function on Ca^{2+} homoeostasis

Cause	Impact on Ca^{2+} homoeostasis
Calreticulin deficiency (loss-of-function)	Inhibition of agonist-induced Ca^{2+} release Reduced Ca^{2+} capacity of the ER Reduced free Ca^{2+} concentration in the ER lumen Delayed SOCI
Calreticulin up-regulation (gain-of-function)	Increased agonist-induced Ca^{2+} release Increased Ca^{2+} capacity of the ER Increased free Ca^{2+} concentration in the ER lumen Decreased SOCI

necessary for refilling the ER stores after Ca^{2+} signalling. A protein located at the membrane of the ER, Stim1 (stromal cell-surface molecule 1), has been identified that senses ER luminal Ca^{2+} levels and transmits this to a protein at the plasma membrane, Orai1, a Ca^{2+} transporter that regulates SOCI [54]. Calreticulin-overexpressing fibroblasts demonstrate disrupted SOCI [50–52], owing to a decrease in ER Ca^{2+} release, demonstrating the involvement of calreticulin in the regulation of SOCI (Table 1). With overexpression of calreticulin, there may be reduced level of free Ca^{2+} available to bind to the EF-hand of Stim1. Furthermore, total Ca^{2+} ER concentration could be significantly increased to the high millimolar range, much higher than the affinity of the EF hand of Stim1. It remains to be seen whether calreticulin and/or other ER luminal Ca^{2+} buffers regulate Stim1's Ca^{2+}-sensing function and consequently SOCI.

Analysis of chaperone and Ca^{2+}-buffering properties of calreticulin provided important clues to the function of the protein. Interestingly, studies using animal models and cells overexpressing or deficient in calreticulin indicate that calreticulin-dependent Ca^{2+} buffering and regulation of Ca^{2+} homoeostasis may be one of the (if not the most) important functions of the protein. Calreticulin-deficient cells have impaired Ca^{2+} homoeostasis, yet only a modest decrease in protein folding [45]. Increased expression of calreticulin has no impact on protein folding, but significantly affects Ca^{2+} homoeostasis (Table 1) [50–52]. Calreticulin-deficient mice, with the exception of the cardiac tissue, develop normally during early stages of embryogenesis, suggesting that calreticulin's chaperone function might not be essential during embryogenesis [38]. The development of the cardiac tissue in calreticulin-deficient mice is impaired owing to the Ca^{2+}-buffering role of calreticulin, but not its chaperone function [55,56]. Further studies with calreticulin and calnexin gene-knockout mice indicate that these proteins are unable to compensate for the loss of each other, suggesting that, during embryonic development, they must have unique functions [21,38,39]. One function of calreticulin that cannot be compensated for by calnexin is its role in modulation of Ca^{2+} homoeostasis [21,52]. Several viable $Crt^{-/-}$ cell lines have been created, indicating that, in mammalian cell culture, calreticulin and the calreticulin/calnexin cycle are not essential for cell survival [38]. These findings support further the importance of calreticulin in ER Ca^{2+} buffering and in modulation of Ca^{2+} homoeostasis over its function as a molecular chaperone.

TRANSCRIPTIONAL REGULATION OF THE CALRETICULIN GENE

Calreticulin is differentially expressed under variety of physiological and pathological conditions. For example, reduced levels of calreticulin are found in differentiated tissues such as the heart and the brain [38,53,57,58]. In contrast, calreticulin is up-regulated in highly differentiated tissues or upon induction of ER stress [59]. Considering a differential expression of calreticulin in many tissues and cell types, it is not surprising that the calreticulin gene is under tight control of several specific transcription factors.

The calreticulin promoter contains many potential transcription-factor-binding sites [59]. However, only a few have been tested experimentally and shown to be potent activators or repressors of the gene. Nkx2.5 (NK2 transcription factor related, locus 5), COUP-TF1 (chicken ovalbumin upstream promoter-transcription factor 1), GATA6 (GATA-binding protein 6), Evi-1 (ecotropic viral integration site 1) and MEF2C (myocyte enhancer factor 2C) play important roles in the regulation of expression of calreticulin during cardiogenesis and have been identified as important transcription factors regulating the calreticulin gene in general (Table 2) [53,55,60]. Nkx2.5 is a critical regulator of the developing heart [61] and it activates the calreticulin gene during cardiac differentiation. COUP-TF1 is highly expressed during embryonic development and it is a potent repressor of the gene. GATA6 is responsible for activation of the calreticulin gene in the embryonic heart, whereas Evi-1 may contribute to the decline of transcriptional activity of the calreticulin gene in the postnatal heart [60]. Interestingly, the calreticulin gene is also a target of PPAR (peroxisome-proliferator-activated receptor) γ, a member of the PPAR transcription factor family, predominantly expressed in the adipose tissue, and is critical for adipogenesis [62]. PPARγ activates the calreticulin gene; however, increased levels of calreticulin have an inhibitory effect on PPARγ and adipogenesis, suggesting that elevated calreticulin may inhibit the activity of PPARγ [62]. Similarly, the transcription factor MEF2C, an important activator of the calreticulin gene during cardiogenesis, has been shown to be influenced by calreticulin [55]. In the absence of calreticulin, nuclear translocation of MEF2C is compromised, resulting in inhibition of MEF2C transcriptional activity [55]. On the other hand, MEF2C activates expression of calreticulin, which in turn enhances the transcriptional activity of MEF2C.

Transcription factors identified so far as the regulators of the calreticulin gene appear to be critical during embryonic development or under pathological conditions. They may be responsible for up-regulation of the calreticulin gene because of the role of calreticulin in modulation of ER Ca^{2+}. This is likely because of the essential role that Ca^{2+} plays during development and in many pathologies.

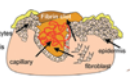
CALRETICULIN, A MULTI-COMPARTMENT PROTEIN

Calreticulin is a multifunctional Ca^{2+}-binding protein that mainly functions in the ER as a Ca^{2+} buffer and molecular chaperone. Immunogold labelling studies indicate that calreticulin is localized to the ER and to the nuclear envelope [63]. The protein has an N-terminal cleavable amino acid signal sequence and a C-terminal KDEL (Lys-Asp-Glu-Leu) ER retrieval signal. These specific amino acid sequences are responsible for targeting and retrieval of calreticulin to the ER lumen. Yet, over the last 20 years, calreticulin has also been implicated in a variety of processes that occur outside the ER lumen, including at the cell surface, in the cytoplasm and within the nucleus [64–78].

Cell-surface calreticulin has been suggested to function in both antigen presentation and complement activation [44,79,80], clearance of apoptotic cells [78], immunogenicity of cancer cell death [72], wound healing [82,83] and thrombospondin signalling [75,84–86]. More specifically, calreticulin acts as a second general recognition ligand at the cell surface upon phagocytosis,

Table 2 Multi-process functions of calreticulin

Calreticulin affects many transcriptional and signalling pathways and, as a consequence, has an impact on embryonic development, animal physiology and pathology. ATF6, activating transcription factor 6; CRT, calreticulin; eIF2α, α subunit of eukaryotic translation initiation factor 2; ERK, extracellular-signal-regulated kinase; CREB, cAMP-response-element-binding protein; LPL, lipoprotein lipase; PKC, protein kinase C; Xbp1, X-box-binding protein 1.

	 CRT knockout mouse	 CRT and cardiac development	 CRT and CNS development	 CRT and stem cell differentiation	 CRT and wound healing
In-vivo and *in-vitro* studies	• Embryonic lethal at E14.5 due to defects in the developing heart and exencephaly • Lethality can be rescued by cardiac-specific expression of constitutively active calcineurin	• CRT-deficient heart has defects in myofibrillogenesis and thinner ventricular walls compared to wild-type hearts • Over-expression of calreticulin in the heart leads to arrhythmias or sudden heart block following birth	• CRT deficiency leads to failed cranial neural tube closure, thus exencephaly in 16% of the mice • CRT expression is higher in the embryonic brain and retina compared to the adult counterparts	• CRT-deficient embryonic stem cells show impaired cardiomyogenesis • CRT-deficient embryonic stem cells and stem cells containing only the N+P domain of calreticulin show enhanced adipogenesis and reduced osteogenesis compared to wild type stem cells	• Accelerates wound re-epithelialization, increases granulation tissue in mice and pigs • Stimulates proliferation *in vivo* and *in vitro* • Chemoattractant for keratinocytes, fibroblasts, monocytes, macrophages •Induces extracellular matrix proteins, integrins
Pathway involved	• Calcium homeostatic pathway • Calcineurin pathway	• PKC and ERK pathways •Calcineurin pathway • Apoptotic pathways • Nkx2.5 pathway	• Calcium signaling pathways • Unfolded Protein response (UPR) pathways • Caspase 7 and caspase 12 apoptotic pathways	• CaMKII signaling pathways • GATA4/NF-AT pathway • Calcineurin pathway	• unproved, probably related to intracellular pathways involved in proliferation and migration
Proteins and transcription factors	• Calcineurin; NFAT; GATA4	• Nkx2.5; NF-AT; GATA4; • MEF2C, Evi-1 • Akt; ERK • calcineurin • Caspase 8; Bax; p53; Bcl	• ATF6α; Xbp-1; eIF2α • Caspase 7; caspase 12	• GATA4; NF-AT; calcineurin • CaMKII; calmodulin; PPARγ; C/EBPα; aP2; LPL	• α5,β1 integrin • Fibronectin, collagen α1, TGF-β3, α-smooth muscle actin

stimulating the LRP (low-density lipoprotein receptor-related protein) on the surface of the engulfing cell [78,84]. Calreticulin is found on the extracellular surface of platelets, where it interacts with integrin $\alpha 2\beta 1$ and glycoprotein VI, demonstrating a role for calreticulin in the modulation of the platelet–collagen interaction [87]. The protein is localized to the extracellular plasma membrane surface of many cell types, where it may play a role in antigen-processing events [88] and serve as a mediator of adhesion [89]. Finally, calreticulin localizes to the extracellular matrix in teeth, where it may be involved in mineralization [90]. The molecular mechanisms responsible for targeting of calreticulin to the plasma membrane remain a mystery. A likely mechanism involves ER-stress-induced up-regulation of expression of calreticulin overwhelming the KDEL retrieval system and components of the secretory pathway resulting in the movement of the protein to the cell surface. Alternatively, an ER-specific protease may remove calreticulin's KDEL retrieval signal, promoting translocation of the protein to the Golgi and beyond. Calreticulin may also reach the cell surface from the cytoplasm via vesicular transport and exocytosis. These hypotheses need to be tested experimentally, as the cell-surface targeting of the protein is becoming an important aspect of calreticulin biology and ER function.

More difficult to explain are the activities of calreticulin reported to occur in the cytoplasm. For example, calreticulin binds to the sequence KXGFFKR found in the cytoplasmic tail of α-integrins [91]. As a result, calreticulin has been suggested to serve as a cytoplasmic activator of integrins and a signal transducer between integrins and Ca^{2+} channels in the plasma membrane, with calreticulin mediating the coupling between Ca^{2+} release and Ca^{2+} influx [92]. Another study established that calreticulin could interact directly with hormone receptors, such as the glucocorticoid and androgen receptor, and could inhibit steroid-sensitive gene transcription [66,93]. Independently, calreticulin was also suggested to function as a nuclear import protein [71,94]. Additionally, a few studies have linked calreticulin to the nucleus by showing that calreticulin is a component of the nuclear matrix in hepatocellular carcinomas [95,96], and that it binds to core histones [97]. The cytoplasmic and nuclear functions of calreticulin that depend on physical interactions between calreticulin and substrates cannot be explained by indirect effects [68]. What is the mechanism of calreticulin distribution to the cytoplasm? Co-translational insertion of calreticulin into the ER lumen involves the same machinery used for the biosynthesis of luminal, secreted and membrane proteins. In the course of experiments addressing calreticulin biosynthesis, Afshar et al. [68] found that calreticulin is fully inserted into the ER, but it subsequently undergoes retrotranslocation into the cytoplasm. This could fit with the proposed functions of calreticulin outside the ER [68]. Shaffer et al. [67] have shown that a minor pool of calreticulin must be localized to the cytoplasm along with the ER pools in order for glucocorticoid-mediated gene expression to be affected. Thus cytoplasmic calreticulin may perhaps be necessary to fine-tune the signalling from the ER lumen. The above suggest that, if

there is calreticulin in the cytoplasm, there are at least three possible mechanisms leading to the cytoplasmic localization of the protein: (i) calreticulin is not efficiently targeted to the ER, leading to accumulation of the precursor calreticulin in the cytoplasm; (ii) calreticulin is retrieved after processing (removal of the signal peptide) from the ER to the cytoplasm by reverse movement, possibly through the translocon/Derlin; and (iii) that calreticulin may be redistributed to the cytoplasm as a result of leaking out of the ER.

CALRETICULIN, A MULTI-PROCESS PROTEIN

Since the molecular cloning of calreticulin's cDNA 20 years ago [4,5], the protein has been implicated to play a role in an incredible number of pathways and biological systems. Many of these functions remain difficult to explain, considering what we know about Ca^{2+} buffering and chaperone function of the protein. However, these findings do indicate that the protein has the capacity to influence many processes at the cellular, organ and/or animal level and may therefore be considered a multi-process molecule.

Immunity

Calreticulin has been identified as an antigen in sera from patients suffering from several autoimmune diseases, including SLE (systemic lupus erythematosus) [98,99], coeliac disease [100–102], rheumatic disease [103] and various parasitic diseases [104,105], which implies a pathological role for calreticulin in autoimmune diseases. Parasite calreticulin also affects parasite infectivity by modulating the host's complement system to help the parasite to evade the immune response of the host [105].

Autoimmune disease is characterized by the presence of auto-antibodies, which are targeted for attack by the innate immune system. Auto-antibodies may recognize double-stranded DNA, Ro–La complexes and calreticulin [106]. Calreticulin associates with ribonucleoprotein complex Ro–SSA (Smith surface antigen), which acts as an auto-antigen in most of patients with Sjogren's syndrome and SLE disorders [107]. Calreticulin binds to the Ro–SSA component of hYRNAs (human cytoplasmic RNAs), Ro52 and Ro60, resulting in epitope spreading [108]. Moreover, when calreticulin is localized on the cell surface, it interacts with C1q, the first component of complement, and activates the classical complement pathway [109,110]. C1q plays an important role in uptake of apoptotic cells by macrophages through indirect interaction with α_2-macroglobulin receptor CD91 [111]. Calreticulin may mediate these interactions [112]. In SLE patients, apoptotic neutrophils exhibit reduced ability to be identified and removed by the C1q/calreticulin/CD91 pathway [113]. Auto-antibodies to calreticulin have also been identified in polychondritis, a systemic inflammatory disease, where the body generates autoimmunity to cartilage-related components [114]. This may be explained by the presentation of calreticulin at the cell surface, resulting in immunogenicity. All these observations indicate that calreticulin may be an important auto-antigen in some individuals and it may also play a role in the pathology of autoimmune disease through association with other complexes. Linkage of calreticulin with a target antigen significantly enhances the antigen-specific cell-mediated and humoral immune responses in vaccinated mice [115–117]. Mice vaccinated with DNA vaccines encoding calreticulin linked to a target antigen have demonstrated significant protective anti-tumour effects [117].

Another member of the immune system, CTLs (cytotoxic T-lymphocytes), are cytolytic cells that release specific factors, Ca^{2+}-activated perforin and granzymes (proteases), upon interaction with target cells, with the final result being that the targeted cell undergoes lysis and apoptosis. Calreticulin co-localizes with perforin in the secretory granules (lysosome-like vesicles) of the CTL [118]. It appears that the Ca^{2+}-binding capacity of calreticulin is involved in the protection of CTLs, whereby chelation of Ca^{2+} by calreticulin inactivates perforin involved in penetrating the plasma membrane of a target cell [119]. Porcellini et al. [120] demonstrated how the Ca^{2+} signalling responsible for modulating the T-cell adaptive immune response is dependent on calreticulin, with the induction of auto-immune diseases. Sipione et al. [118] used calreticulin-deficient cells to demonstrate that calreticulin is not critical for the cytolytic activity of the granzymes and perforin, but that it is required for efficient targeting and contact of the CTL to the target cell.

Cancer

A significant role for calreticulin in combating cancer has been shown in a series of more recent studies with major implications for novel cancer therapy that essentially involves arming the immune system for removal of cancer cells [121–123]. Pre-apoptotic translocation of calreticulin to the cell surface is critical for immunogenic cell death, with presentation at the cell surface upon treatment with anthracyclines [121]. Calreticulin co-translocates to the cell surface with ERp57, allowing presentation to T-cells, triggering the initiation of the immune response and subsequent apoptosis of the immunogenic cell, preventing organism damage. Disruption of the interaction of calreticulin with ERp57 prevents surface exposure of calreticulin and renders the cell resistant to T-cell attack and subsequent immune response [124]. Depletion of calreticulin also renders resistance to T-cell attack [121]. Exogenous application of calreticulin was able to overcome this resistance, demonstrating the importance of calreticulin in the immunogenic response to tumour cells [121,124]. Interestingly, a decrease in ER luminal Ca^{2+} also induced calreticulin exposure on the cell surface, with ER Ca^{2+} potentially regulating cell-surface exposure of calreticulin [122]. As calreticulin is responsible for buffering Ca^{2+} in the ER as well as a plasma membrane signal for targeted killing, calreticulin-dependent Ca^{2+} signalling could shape the immune response [120]. The potential application of calreticulin in cancer therapy is underscored by these studies and has been practised using calreticulin as an adjuvant for chemotherapy. This was achieved by administering a chimaeric construct of calreticulin with tumour antigen peptides delivered by a viral vector or using gene therapy, with results indicating enhanced immunogenicity more than by the tumour antigen alone [125,126]. In addition, anthracycline-treatment of patients with acute myeloid leukaemia caused cell-surface expression of calreticulin on circulating cancer cells [127]. These are fascinating findings which may translate into medically targeted immune response towards tumour cells, in conjunction with chemotherapy or radiotherapy [123].

Apoptosis

Apoptosis performs a necessary function in the maintenance of an organism as well as defending against foreign pathogens. Any breakdown during apoptosis results in human disease, caused by gene suppression, activation or mutation. ER Ca^{2+} release is necessary for activation of transcriptional cascades, as well as direct regulation of apoptotic proteins responsible for cellular death [128]. Ca^{2+} release from the ER is intrinsically involved in the triggering of both cytoplasmic and mitochondrial

membrane-mediated apoptosis [129]. Calreticulin has been implicated in the cellular response to apoptosis. Overexpression of calreticulin results in increased sensitivity to apoptosis induced by either thapsigargin or staurosporine, with a concomitant increase in cytochrome *c* released from the mitochondria. In contrast, calreticulin-deficient cells are resistant to apoptosis, owing to decreased Ca^{2+} stores in the ER [130]. This was one of the first observations directly linking calreticulin and ER luminal Ca^{2+} to cell sensitivity to apoptosis. Recent studies have shown that calreticulin, intraluminal Ca^{2+} and a disruption in Ca^{2+} regulation influence apoptotic events in cardiomyocytes [131]. Cell-surface calreticulin may also play a role in apoptosis [76]. It appears that a variety of ER-dependent events may have an impact on apoptotic pathways including interaction between the ER and the mitochondria, with changes in the flux of Ca^{2+} [52] or changes in the ER luminal Ca^{2+} concentration [132,133].

Calreticulin has been detected on the surface of many mammalian cells, including platelets, fibroblasts, apoptotic cells and endothelial cells [84,86,134], and it appears on the cell surface of calreticulin-null mouse embryo fibroblasts following enforced expression by transfection [135]. On the cell surface, the calreticulin molecule interacts with the Hep I domain of TSP-1 (thrombospondin 1) in a co-receptor complex with the LRP (LRP–CD91–α_2-macroglobulin receptor), for signalling through both G_i-coupled and PI3K (phosphoinositide 3-kinase)-dependent ERK (extracellular-signal-regulated kinase) activation in endothelial cells and fibroblasts to mediate focal adhesion disassembly important in the process of migration [78,84,86]. Recent studies indicate that the binding of calreticulin to CD91 leads to the induction of phagocytosis and pro-inflammatory responses [136]. Cell-surface calreticulin in association with phosphatidylserine provides the obligate recognition signal for the removal of dead cells by both professional (e.g. macrophages, neutrophils) and non-professional phagocytes (e.g. fibroblasts). However, different from the function of focal adhesion disassembly in which the LRP–calreticulin–TSP-1 signalling complex is on the same responding cell, in a *cis* configuration, the calreticulin is exposed on the surface of the apoptotic cell to be engulfed by the phagocyte expressing LRP, in a *trans* configuration. In addition, this process requires the down-regulation or disruption of both CD47/IAP (integrin-associated protein), on the apoptotic target cell, and of SIRP (signal regulatory protein)-α [SHPS-1 (Src homology 2 domain-containing protein tyrosine phosphatase substrate 1)] on the engulfing cell as the absence of CD47, but the presence of calreticulin permits engulfment of live cells. The critical presence of calreticulin on apoptotic cells for uptake by phagocytes is underscored by the lack of engulfment of calreticulin-null mouse embryo fibroblasts unless phagocytosis is rescued by exogenous addition of calreticulin.

Vasostatin and angiogenesis

The N-terminal fragment of calreticulin, named vasostatin (referred to as calreticulin/vasostatin), has been isolated from the supernatant of an Epstein–Barr immortalized cell line [137]. This is different from vasostatin-1, a naturally occurring N-terminal fragment, amino acids 1–76, of chromogranin A [138]. Calreticulin/vasostatin inhibits the proliferation of endothelial cells [137]. Full-length calreticulin and calreticulin/vasostatin also inhibit angiogenesis *in vivo* [139,140]. Yao et al. [141] implicated laminin on the surface of the endothelial cells as the target of extracellular calreticulin. Calreticulin/vasostatin blocks the interaction of endothelial cells with laminin, thus reducing their ability for extracellular matrix attachment and subsequent growth [141]. Since solid tumours are dependent on an adequate blood supply, angiogenesis is vital in maintaining their growth. Inhibition of angiogenesis has been a key in minimizing tumour growth and effective anti-angiogenic agents have been widely sought. Calreticulin/vasostatin has emerged as potentially ideal angiogenesis inhibitor, as it suppresses endothelial cell proliferation and it is small, soluble, stable and easy to produce and deliver [137]. Indeed, several reports have indicated the efficacy of calreticulin/vasostatin treatment [142–146]. Unfortunately, some cell/tissue types appear to produce enhanced malignancy after calreticulin/vasostatin treatment [146], decreasing the enthusiasm for use of calreticulin/vasostatin as an angiogenesis inhibitor.

Wound healing

Wound healing is a dynamic process that involves a temporal orchestration of multifunctional events and numerous cells. In a cutaneous wound, both the injured epithelium and dermis are the targets of the repair process, which involves three major overlapping phases. First, the inflammatory phase involves fibrin/fibronectin clot formation and platelet aggregation providing a scaffold for the migration of inflammatory cells, which phagocytose bacteria, remove dead tissue and cells, and secrete growth and angiogenic factors into the wound. In the next proliferative phase, epithelial cells at the wound margins proliferate, express integrins and migrate to resurface the wound. During the final remodelling phase, proteases [collagenases and gelatinases, such as MMP (matrix metalloproteinase)-2 and MMP-9] aid in remodelling the wound. Calreticulin is temporally and spatially expressed during wound healing [82,83], particularly by fibroblasts in the injured dermis, but it is unclear whether this expression is intracellular and/or extracellular. Calreticulin has diverse and identical biological effects on all phases of the wound healing process [83] in murine and porcine models of normal and impaired repair (N.B. porcine cutaneous wounds heal most similarly to humans) [147]. Significantly, using human cells important in wound healing in *in vitro* assays explained how calreticulin healed the wounds observed in the animal models.

Calreticulin, topically applied to cutaneous full-thickness excisional wounds (extending through to the muscle layer below the dermis) in diabetic mice (leptin-deficient) [148] and both normal and cortisone-impaired pigs, causes the wounds to resurface more rapidly and to contain more abundant and cellular granulation tissue to aid in filling in the wound defect compared with controls [83]. In addition, a more rapidly stratified epithelium and more condensed neodermis appears at an earlier time point, indicating faster maturing well-healed wounds [83]. This is the first time any agent used in an effort to heal wounds has been shown to exert positive effects on both the epidermal and dermal aspects of cutaneous wound repair.

Furthermore, topical/extracellular/exogenous calreticulin treatment of both the murine and porcine wounds induced cellular proliferation of specific cells, as depicted by Ki67-immunoreactive basal and suprabasal keratinocytes and fibroblasts of the dermis. Importantly, this proliferative effect of calreticulin was recapitulated *in vitro*, as calreticulin stimulates proliferation of human primary keratinocytes and human dermal fibroblasts more than 2-fold with pg/ml and ng/ml concentrations respectively, and greater than EGF (epidermal growth factor)- and FGF (fibroblast growth factor)-positive controls [83]. In addition, calreticulin stimulates proliferation of microvascular endothelial cells, implying a potential effect on angiogenesis. Calreticulin has been shown to mediate proliferation of fibroblasts, albeit not directly, as the binding to the Bb chain of fibrinogen is required [149]. Therefore, as calreticulin binds directly to the collagen integrin receptor, $\alpha 2\beta 1$, on the platelet surface and is contained in platelet

granules [150,151], upon platelet de-granulation, the calreticulin released would be available and important in stimulating fibroblast proliferation.

Calreticulin induced migration/motility of human primary keratinocytes and fibroblasts with peak concentrations at the pg/ml and ng/ml level respectively in a scratch plate assay, the classic *in vitro* model for wound healing [83]. Furthermore, calreticulin induced directed concentration-dependent migration of keratinocytes, fibroblasts, monocytes and macrophages, each cell type with a unique critical function in wound repair [83]. Interestingly, calreticulin maintained the same sensitivity (peak responses) in both the motility and migration assays, with keratinoctyes being 1000-fold more sensitive than fibroblasts. This supports the concept of temporally and spatially regulated local concentrations of factors in directing the wound healing process. In addition, exogenous calreticulin up-regulates $\alpha 5$ and $\beta 1$ integrins on keratinocytes and fibroblasts (L. I. Gold, unpublished work). Importantly, the effect of calreticulin (at ng/ml level) on migration of monocytes and macrophages (at ng/ml levels) was corroborated *in vivo* in calreticulin-treated porcine wounds in which a 3-fold increase in macrophages compared with a PDGF (platelet-derived growth factor)-BB positive wound healing controls was observed in the wounds. Thus it appears that calreticulin has an important role in attracting peripheral blood monocytes to the wound where they are activated into tissue macrophages that keep the wound free of bacteria and debris. Once these cells arrive at the wound, the essential role for calreticulin in phagocytosis of apoptotic cells is an important function in wound healing.

Consistent with the abundant granulation of tissue/neodermis observed in the calreticulin-treated porcine and murine wounds, calreticulin induces extracellular matrix proteins *in vitro* in both keratinocytes and fibroblasts. Calreticulin treatment of fibroblasts dose-dependently increases the expression of TGF (transforming growth factor)-$\beta 3$, but not TGF-$\beta 1$ or TGF-$\beta 2$ isoforms. The TGF-$\beta 3$ isoform was also specifically up-regulated during repair in the murine and porcine wounds [83]. Importantly, only the TGF-$\beta 3$ isoform is involved in specific functions important to wound healing, such as collagen gel matrix contraction (to stimulate wound contraction) [152], acceleration of wound healing with decreased scarring [153], as well as other functions [154]. The expression of fibronectin in keratinocytes and fibroblasts and collagen type I in fibroblasts was also increased by exogenous calreticulin *in vitro*, with peak responses similar to calreticulin induction of proliferation and migration for each cell type (L. I. Gold, unpublished work). Since TGF-$\beta 3$ induces collagen and fibronectin synthesis, calreticulin may induce these proteins both directly and/or via its effect on TGF-$\beta 3$. Enforced expression of calreticulin affects not only mRNA and protein levels of fibronectin, but also fibronecitn matrix assembly, by clustering of fibronectin receptors and influencing the formation and stability of focal contacts and fibrillar adhesions [155,156]. In addition, calreticulin increases the levels MMP-2, MMP-9 and MTI-MMP (membrane type 1 matrix metalloproteinase) [157], proteins that are important in extracellular matrix remodelling required for the final phases of the healing process. In addition, calreticulin may be a component of the extracellular matrix of wounds and stimulate certain functions from this location, as it was shown to be present in tooth predentin [90]. One of the most important functions in wound healing is for cells to migrate into the injured tissue, proliferate and produce growth factors and extracellular matrix proteins. Calreticulin exerts profound effects on these functions both *in vivo* and *in vitro*.

The hypoxic environment of the wound would naturally up-regulate calreticulin, shown to be increased under hypoxic stress [158]. The extracellular function of calreticulin as an important physiological mediator of wound healing has probably evolved from the release of intracellular proteins into the extracellular space by injured and dying cells. Interestingly, other ER-resident stress-response proteins, such as Hsp (heat-shock protein) 90, Hsp70 and Hsp47, have been shown to play a role in migration and wound healing [159–163]. The dual nature of ER proteins as physiological mediators of both intracellular and extracellular function appears to be just beginning to unfold.

Cardiogenesis and embryonic development

Calreticulin deficiency (loss-of-function) is embryonic lethal between 12.5 and 14.5 days post-coitum, and the embryos showed significantly decreased ventricular wall thickness and deep intertrabecular recesses in the ventricular walls [38,64], indicating that calreticulin may be involved in the pathology of cardiovascular diseases. Studies using calreticulin-deficient mice and embryonic stem cells revealed that calreticulin deficiency leads to impaired myofibrillogenesis [164,165]. Electron microscopic analysis revealed 'wavy' and thin myofibrils in the ventricles of calreticulin-null embryos, compared with wild-type [165]. These *in vivo* observations parallel those *in vitro* of myofibrillar organization of cardiomyocytes differentiated from calreticulin-null embryonic stem cells [164]. There is also deficient intercalated disc formation in the hearts of calreticulin-null mice [165]. Intercalated discs are adherens-type junctions of cardiac muscle and contain vinculin, N-cadherin and catenins [166]. N-cadherin–β-catenin interactions are crucial for cardiomyocyte differentiation and myofibrillogenesis [167]. Accordingly, myofibrils in $Crt^{-/-}$ myocytes lack the degree of ordering typical for wild-type cells at this developmental stage [165]. Could it be that it is a 'community' behaviour of $Crt^{-/-}$ cells that is responsible for cardiac defects? Indeed, in fibroblasts underexpressing calreticulin, N-cadherin is down-regulated [168] and this is also observed in calreticulin-null mice [165]. This is supported further by the notion of the defective 'community' behaviour of $Crt^{-/-}$ cells being involved in cardiac defects given by the observation that the calreticulin-null cardiac phenotype appears to be similar to a milder form of either the N-cadherin- or vinculin-knockout phenotypes [169]. Calreticulin does affect expression of N-cadherin and vinculin and their mRNAs and, consequently, cell adhesion [168,170–172]. Changes in expression of adhesion molecules in calreticulin-deficient heart might contribute to the developmental abnormalities caused by the absence of calreticulin.

At first, these findings were unexpected, as calreticulin abundance in adult cardiac tissue is very low. However, examination of the expression of the calreticulin gene in the developing embryo shows little expression in most tissues, but strong expression in the heart, liver and in some central nervous system tissues during the stages of development when calreticulin deficiency is lethal [38]. Importantly, a negligible level of calreticulin was detected in the heart of 3-week-old transgenic mice [38]. These findings showed that the calreticulin gene is down-regulated during the late stages of development and after birth, which is in agreement with earlier observations that mature hearts express a relatively low level of calreticulin [173,174]. Overexpression of calreticulin (gain-of-function) in the heart causes bradycardia, complete heart block and sudden death in mice, characterized by dilated ventricular chamber and atria, thinner ventricular walls and disarray of cardiomyocytes [175,176]. Calreticulin auto-antibody has been identified from patients suffering from congenital heart block [177], a disorder of cardiac electrical conduction. Interestingly, mice overexpressing calreticulin in the heart have a similar phenotype to the complete congenital heart block seen in children [175,176], suggesting that calreticulin

may play a role in the pathogenesis of this disease. Importantly, analysis of transgenic animals revealed that overexpression of calreticulin in the heart results in disruptive cardiac signalling including connexin43, a component of gap junctions, and MEF2C [176]. Together, all these findings indicate that calreticulin is critical for normal heart development and function. Interestingly, skeletal muscle development is grossly unaffected by calreticulin [56], indicating that calreticulin may be critical for the proper development of the heart only, even though the protein is found in striated muscle.

What might be the molecular mechanisms responsible for calreticulin-dependent cardiac development and pathology? In the early stages of cardiac development, calreticulin is required to ensure normal Ca^{2+} release from the ER and thus proper activation of Ca^{2+}-dependent transcriptional pathways, including those dependent on the phosphatase activity of calcineurin. The activation of calcineurin depends on the sustained release of Ca^{2+} from ER stores, and nuclear translocation of NFAT (nuclear factor of activated T-cells) is regulated by dephosphorylation by calcineurin [178], which depends on calreticulin abundance [7]. Thus calreticulin may indirectly regulate transcriptional activity of the GATA-4–NFAT complex by affecting calcineurin activity. Nuclear import of NFAT3 is impaired in calreticulin-null cells, and re-expression of calreticulin restores NFAT3 nuclear translocation [38]. Another transcription factor that is essential for cardiomyogenesis is the MEF2C [179]. Like NFAT, its nuclear translocation is impaired in the absence of calreticulin; however, an increase in cytoplasmic Ca^{2+} concentration or expression of constitutively activated calcineurin restores the transcriptional activity of MEF2C (Table 2) [55]. Furthermore, MEF2C activates expression of calreticulin, which in turn enhances the transcriptional activity of MEF2C [55]. Collectively, these data demonstrate that transcription factors involved in cardiac development (NFAT and MEF2C) are dependent on calreticulin. This hypothesis was originally tested using a transgenic mouse model that expressed an activated calcineurin (truncated recombinant calcineurin with reduced Ca^{2+}-sensitivity). Cardiac-specific overexpression of activated calcineurin is sufficient to rescue calreticulin-deficient embryonic lethality, producing viable calreticulin-deficient mice [56]. Calreticulin-deficient embryos and newborn mice expressing activated calcineurin in the heart do not show any significant defects in cardiac development [56]. Instead, they exhibit normal development of the ventricular wall, with signs of early hypertrophy which probably result from the expression of activated calcineurin [56]. The remarkable reversal of the embryonic lethality that results from calreticulin deficiency, by the expression of only one protein in the heart (activated calcineurin), highlights the importance of both calreticulin and calcineurin in Ca^{2+}-dependent signalling cascades during early cardiac development. Studies on calreticulin-deficient stem cells provide further evidence for this hypothesis [164].

Adipogenesis

Mesenchymal stem cells have the potential to differentiate into myocytes, adipocytes, osteocytes and chondrocytes. Calreticulin influences cardiogenesis, thus the question remains as to how might calreticulin affect mesenchymal stem cell commitment towards the other lineages, such as the adipocyte lineage? In the absence of calreticulin, stem cells differentiate into adipocytes, as indicated by the increased adipogenic marker expression [PPARγ2, C/EBPα (CCAAT/enhancer-binding protein α) and aP2 (adaptor protein 2)], whereas the opposite is found when cells express calreticulin [62]. Previously, it has been shown that calreticulin can act as a transcriptional regulator of PPAR, whereby it inhibits the binding of PPAR/RXR (retinoid X receptor) to the PPRE (PPAR-responsive element), thus inhibiting transcription activation by peroxisome proliferators and by fatty acids [66,93,180]. PPARγ is a transcriptional activator of the calreticulin gene [62]. Thus PPARγ can up-regulate calreticulin and, conversely, calreticulin can inhibit PPARγ activity. Furthermore, calreticulin has been shown to have RNA-binding activity that inhibits C/EBP mRNA translation [181]. In terms of embryonic stem cell differentiation, there is an inverse relationship between calreticulin and expression of PPARγ2 upon induction of adipogenesis with retinoic acid [62]. Retinoic acid induces the expression of calreticulin, but reduces the expression of PPARγ2, indicating that calreticulin exerts its effect within the commitment and/or initial stages of adipogenesis. It appears that calreticulin is involved in a hierarchical process, whereby transcriptional activation of the calreticulin gene by PPARγ is the early event, followed by calreticulin modulation of PPARγ transcriptional activity. Calreticulin may act as a Ca^{2+}-dependent molecular switch that negatively regulates commitment to adipocyte differentiation by down-regulating the expression and transcriptional activation of pro-adipogenic transcription factors.

By which additional mechanism could calreticulin be exerting its anti-adipogenic effects? Previous studies using 3T3-L1 pre-adipocytes indicate that adipogenesis might be affected by internal and external Ca^{2+} levels [182–184]. Increasing cytoplasmic Ca^{2+} levels using the SERCA inhibitor thapsigargin inhibits early stages of adipogenesis, indicating that ER Ca^{2+} plays a role during adipogenesis [184]. Thus adipogenesis is a Ca^{2+}-sensitive process and may be regulated by calreticulin's Ca^{2+}-buffering function. Indeed, in 3T3-L1 pre-adipocytes and embryonic stem cells, increased cytosolic Ca^{2+} concentration leads to a decrease in adipocyte differentiation [62]. Most importantly, expression of the Ca^{2+}-handling P- and C-domain of calreticulin inhibits adipogenesis, whereas the expression of the chaperoning N- and P-domain does not have any effect on adipogenesis [62].

It is conceivable that the observed inhibitory effect of calreticulin on adipogenesis is not only due to the increased Ca^{2+}-storage capacity, but is partially due to its role in regulating Ca^{2+}-dependent signalling pathways. For example, the Ca^{2+}- and calmodulin-dependent protein phosphatase calcineurin inhibits adipogenesis [183,185]. Calcineurin is a downstream target of calmodulin, and calmodulin can activate calcineurin and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) pathways simultaneously. Thus it is plausible that, while the calcineurin pathway inhibits adipogenesis, the calmodulin/CaMKII pathway may promote adipogenesis in the absence of calreticulin. Upon inhibition of calmodulin, the calreticulin-containing cells exhibited increased adipogenesis, whereas inhibition of CaMKII attenuates adipogenesis in calreticulin-null cells [62]. Thus this suggests that calreticulin and CaMKII-dependent pathway(s) are important during the adipocyte differentiation process.

Skeletogenesis

Both osteoblast and chondroblast development are marked by commitment and differentiation of stem cells and primitive progenitors through proliferative/relatively undifferentiated precursor stages to mature matrix-synthesizing cells. It is thought that the two lineages derive from a common multipotential mesenchymal stem cell and may also share a common bipotential osteochondroprogenitor.

There are limited data available on calreticulin expression or function in osteogenic cells *in vivo* or *in vitro*. St-Arnaud et al. [186] reported that the expression of calreticulin was down-regulated during the first 14 days of osteoblast differentiation in the

MC3T3-E1 cell model. Using a gain-of-function experimental strategy, they also showed that overexpression of calreticulin, in this cell model, inhibits both basal and vitamin D-induced expression of the osteocalcin, accumulation of Ca^{2+} into the extracellular matrix and mineralization of bone nodules in long-term cultures [186]. These effects were attributed to loss of binding of the vitamin D receptor to the vitamin D-responsive elements in the osteocalcin gene and/or to modulation of cell adhesiveness. Alternatively, it is well established that calcineurin plays an important role in chondrogenesis [187] and osteogenesis [188], as it has been shown that calreticulin affects calcineurin activation [55,56]. Hence, calreticulin may be affecting osteocyte and chondrocyte differentiation via the calcineurin pathway.

Neuronal development

The abundance of calreticulin is high in mouse embryonic cerebral cortex, cerebellar cortex and retina, compared with the adult counterparts, which suggest calreticulin's importance in the central nervous system development [189]. Calreticulin ablation-related developmental defects include problems related to closure of the neural tube (exencephaly) and umbilical hernia (omphalocele), which may both comprise alterations in cell adhesion and motility [38,190]. In a study by Rauch et al. [190], 16% of calreticulin-deficient embryos exhibited exencephaly, which may be a result of defective neural tube closure. Exencephaly may be caused by defective formation of actin cytoskeleton and impaired adhesion to fibronectin. Interestingly, it has been reported that inhibition of actin microfilaments with cytochalasin D inhibits closure of the cranial neural folds; however, spinal neural tube closure is unaffected [191]. This is in accordance with the results found by Rauch et al. [190], where they observed exencephaly, but no defects in spinal neural tube closure in calreticulin-null embryos.

CONCLUSIONS

Since the discovery of calreticulin 35 years ago [192], the protein continues to be implicated in an amazing number of biological systems, including protein folding, regulation of Ca^{2+} homoeostasis, modulation of transcriptional pathways, cell adhesion, apoptosis and embryonic development, to name a few. Extensive physiological, cell biological, biochemical and molecular biological studies on the function of calreticulin indicate that the protein is indeed a multi-process molecule affecting many cellular functions inside and outside of the ER environment. Recently, cell-surface functions of calreticulin received particular consideration, as they may have an impact on processes relevant to embryonic development and human pathology, such as cancer and wound healing. It appears that the regulation of Ca^{2+} homoeostasis by calreticulin, but not its chaperone function, might be the key to explain its multi-process nature.

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