Calreticulin: non-endoplasmic reticulum functions in physiology and disease

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ABSTRACT **Calreticulin (CRT), when localized to the endoplasmic reticulum (ER), has important functions in directing proper conformation of proteins and glycoproteins, as well as in homeostatic control of cytosolic and ER calcium levels. There is also steadily accumulating evidence for diverse roles for CRT localized outside the ER, including data suggesting important roles for CRT localized to the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix (ECM). Furthermore, the addition of exogenous CRT rescues numerous CRT-driven functions, such as adhesion, migration, phagocytosis, and immunoregulatory functions of CRT-null cells. Recent studies show that topically applied CRT has diverse and profound biological effects that enhance cutaneous wound healing in animal models. This evidence for extracellular bioactivities of CRT has provided new insights into this classically ER-resident protein, despite a lack of knowledge of how CRT exits from the ER to the cell surface or how it is released into the extracellular milieu. Nonetheless, it has become clear that CRT is a multicompartmental protein that regulates a wide array of cellular responses important in physiological and pathological processes, such as wound healing, the immune response, fibrosis, and cancer.—Gold, L. I., Eggleton, P., Sweetwyne, M. T., Van Duyn, L. B., Greives, M. R., Naylor, S.-M., Michalak, M., Murphy-Ullrich, J. E. Calreticulin: non-endoplamic reticulum functions in physiology and disease. *FASEB J.* 24, 665–683 (2010). www.fasebj.org**

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Calreticulin (CRT) is a 46-kDa chaperone protein consisting of three structurally and functionally distinct domains (1, 2). The middle P- and C-terminal domains contain a number of high- and low-affinity calcium-interacting sites, respectively. The N-terminal domain contains a signal sequence for targeting to the ER, and the C-terminal domain has a KDEL sequence for retrieval/retention in the ER. Within the lumen of the ER, CRT, in concert with other ER-resident chaperones, ensures proper folding of proteins and glycoproteins, mainly *via* its lectin-binding site; prevents protein aggregation; and is engaged in protein quality control through identifying and banning misfolded proteins from the ER for ubiquitin-mediated destruction. Another important function for CRT directed from the ER is in the regulation of calcium metabolism, which influences a variety of cellular functions, including cell signaling, particularly through integrins. The absence of the CRT gene is embryonically lethal (3).

It is now well recognized that CRT is localized to intracellular, cell surface, and extracellular compartments and that CRT regulates a variety of diverse and important biological processes from these non-ER compartments. For example, CRT has been shown to be required for antigen processing and presentation for the adaptive immune response (4, 5), the uptake of CRT-expressing cancer cells by dendritic cells (4), phagocytosis of apoptotic cells (6), cell adhesion, migration (7–13), cellular proliferation (7), thrombospondin 1 (TSP1)-mediated focal adhesion disassembly (for cell migration) (11–15), and resistance to anoikis (cell death induced by loss of cell adherence) (16). Because of CRT's role in these biological activities, this classic ER-resident protein is emerging as a critical mediator of physiological and pathological processes, such as wound healing, the immune response, fibrosis, and cancer. However, the regulation of cellular processes by CRT localized to a specific cellular compartment—from within the ER, the cytoplasm, by clas-

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sic cell surface receptor signaling, from the extracellular matrix (ECM), or any combination of these means—remains poorly understood. Cell surface CRT has not been shown to have direct signaling capacity, but only to transduce intracellular signaling through the low-density lipoprotein receptor-related protein 1 (LRP1) in certain functions (11, 16, 17). As exogenously added CRT promotes diverse functions (6, 7, 11, 15, 18, 19), it is likely that other signaling receptors that mediate CRT-driven processes will be identified. Moreover, the mechanisms involved in CRT signal transduction mediated by either extracellular or cell surface-bound CRT are especially unclear, since mechanisms regulating the exit of CRT from the cell are currently only beginning to be defined.

Heretofore, CRT has been regarded primarily as a molecule that performs diverse functions from the ER or by regulating cell signaling in conjunction with its control over ER calcium levels. A recent review focuses on CRT as a multiprocess protein, however, still with emphasis on ER dynamics and ER-associated signaling (2). This is the first detailed review addressing the role of non-ER CRT in cellular regulation and disease from the perspective of its multicompartment localization, as well as recent advances in our understanding of how CRT transits to the cell surface and extracellular milieu.

RECENT RESULTS

CRT functions on the cell surface and in the ECM

Precedence for CRT functioning outside the ER

Early findings of CRT on the surface of many mammalian cells, including platelets, fibroblasts, apoptotic cells, and endothelial cells, provided clues that this intracellular chaperone protein might function outside the ER (6, 12, 14, 20–23). The existence of an ER resident protein such as CRT on the cell surface was initially a difficult concept to accept. However, multiple lines of evidence using biochemical, immunohistochemical, and functional assays eventually provided credible evidence for the existence and, importantly, the functionality of cell surface forms of CRT (**Fig. 1**). Early studies showed that cell surface CRT was required to mediate the mitogenic activity of the Bβ chain of fibrinogen on fibroblasts (23). Cell surface CRT also mediates melanoma cell spreading (22) and serves as a cell surface receptor for the complement component, C1q (24, 25). More recent studies substantiate the function of CRT outside the ER, as described below.

Cell adhesion

Some of the earliest indications for the involvement of non-ER CRT in cell-adhesion regulation stemmed from work showing that CRT in the cytoplasm binds to the KxGFFFKR amino acid sequence in the cytoplasmic tails of α integrins, where it was presumed to stabilize

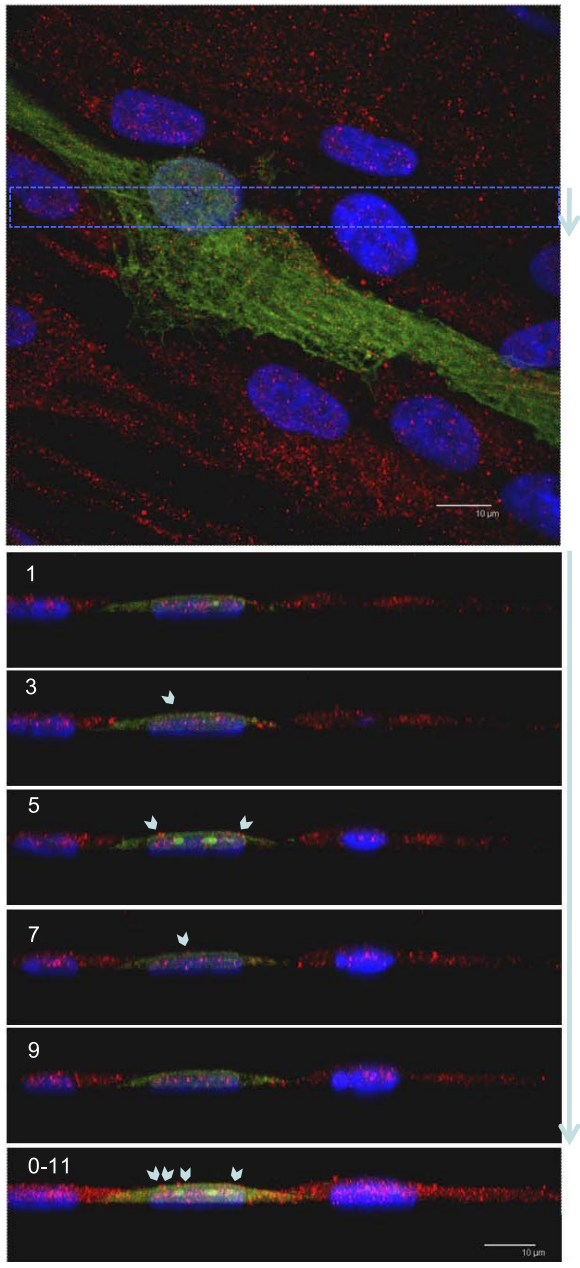

Figure 1. Cell surface staining of CRT in human foreskin fibroblasts. Cells were transiently transfected with enhanced green fluorescent protein (EGFP) to label all cellular compartments (green). Nonpermeabilized cells were fixed with ice-cold 3% buffered formalin and then probed with a rabbit antibody to CRT (antibody 62, provided by P.E.) and a secondary Texas Red-labeled goat anti-rabbit antibody (red). Confocal images were collected in both the *x-y* and the *y-z* planes. Top panel: cell imaged at a slice from the most apical *x-y* section superimposed on a single midsection slice to show both the cell surface staining and the cytoplasm/nuclei. CRT is distributed as punctae at the cell surface. Dashed line indicates the *y-z* region imaged in the bottom panels. Panels *1*, *3*, *5*, *7*, *9*: images of 5 of 12 slices imaged through the *y-z* plane from the apical to basal aspect of the cell. Small arrowheads indicate CRT punctae, which are apical to the cell membrane of the EGFP tagged cell. Panel *0–11*: compilation of the 12 *y-z* sections.

integrin-ligand binding and possibly activate focal adhesion kinase (FAK) (10, 26). Accordingly, CRT coimmunoprecipitates with integrins during cell adhesion to ECM (27) and is found in integrin-based adhesion complexes with LRP1 (28). The chaperone function of CRT is important for ensuring proper folding of integrins and, in certain cases, binds integrins. For example, CRT binds to the extracellular domains of the collagen binding integrin, $\alpha 2\beta 1$, on platelets (1, 29, 30). CRT on the cell surface was shown to bind to glycosylated (mannoside lectin), but not nonglycosylated forms of laminin to mediate spreading of melanoma cells, providing an important indication of the lectin function of CRT in cell adhesion (22).

Focal adhesion disassembly

Landmark studies showed that CRT existed on the cell surface of bovine aortic endothelial cells and fibroblasts and interacted with soluble TSP1 to mediate focal adhesion disassembly, an important component of the cell migratory process (14). Furthermore, it was shown that N-terminal aa 19–36 of CRT bind to the N-terminal domain of TSP1 (residues 17–35) in a coreceptor complex with the low-density LRP1 (LRP1/CD91/α2-macroglobulin receptor) (11, 15, 17). Engagement of cell surface CRT by either TSP1 or a peptide mimetic (hep I) of the CRT binding sequence stimulates CRT-LRP1 association at the cell membrane. This activates signaling through both Gαi-coupled and PI3K-dependent FAK and ERK activation in endothelial cells and fibroblasts, culminating in transient down-regulation of Rho activity to affect cytoskeletal reorganization and loss of focal adhesions (11–13, 15).

Cell surface CRT is sufficient to mediate focal adhesion disassembly through LRP1 signaling as lack of TSP1 responsiveness in K42 CRT-null mouse embryonic fibroblasts (MEFs) can be rescued by a short incubation with exogenous purified GST-CRT (15) but not, as expected, with GST-CRT lacking the TSP1 binding site. Moreover, forced expression of CRT in null K42 MEFs localized CRT to the cell surface and restores TSP1-mediated focal adhesion disassembly (16). In contrast, expression of a CRT lacking the TSP1 binding site, although localized to the cell surface, failed to rescue responsiveness to TSP1 stimulation of focal adhesion disassembly. Thus, localization of CRT to the cell surface is important in the regulation of focal adhesions *via* TSP1.

Cell migration

A role for CRT in cell migration is directly related to its regulation of focal adhesion formation and disassembly, integrin stabilization, and ECM assembly. CRT-null MEF K42 cells migrate poorly on fibronectin and laminin as compared to wild-type cells, although CRT-null MEFs do not have impaired migration in response to fetal bovine serum (FBS) on fibronectin/vitronectin substrates (11, 31). CRT interaction with TSP1 increases random and directed cell migration in bovine aortic endothelial cells and fibroblasts through its regulation of Rho, as described above (11, 13). Migration of endothelial cells in response to TSP1/hep I was inhibited by the LRP1 receptor-associated protein (RAP) (11), substantiating the requirement for LRP1 signaling in CRT/TSP1-mediated migration. Interestingly, whether the process of TSP1/CRT/LRP1-mediated focal adhesion disassembly enhanced or inhibited migration was dependent on the type of chemoattractant, such that chemotaxis was reduced by basic fibroblast growth factor (bFGF) but increased by acidic FGF.

Recent studies show that exogenously added CRT stimulates keratinocyte and fibroblast migration (7). The addition of CRT to primary human keratinocytes and fibroblasts in an *in vitro* scratch-plate assay of wound healing dose-dependently induces motility/migration of these cells to close the denuded area. Moreover, studies using migration chambers show that CRT stimulates concentration-dependent directed migration of human keratinocytes and fibroblasts and the human THP-1 (American Type Culture Collection TIB-202) monocyte and THP-1-derived macrophage cell lines (7). Evidence for the role for non-ER CRT in adhesion and focal adhesion disassembly is likely part of its important function in cell migration and the process of homing cells to sites of injury/repair, such as cutaneous wound healing.

Anoikis

The nature of cell interactions with the ECM and the organization of the cytoskeleton varies with different external stimuli and can be modulated over a dynamic spectrum. Stationary cells engaged in functions for growth and differentiation typically are strongly adherent and have focal adhesions and stress fibers. Cells with disrupted cell-matrix interactions usually round and detach to undergo anoikis (anchorage-independent cell death) (32, 33). In contrast, matricellular proteins, such as TSP1, SPARC, and tenascin-C, can stimulate a state of intermediate cell adhesion, characterized by a spread cell phenotype, which involves restructuring focal adhesion plaques with loss of vinculin and associated stress fibers, while maintaining integrin and talin associations (34). It has been proposed that this intermediate adhesive state is an adaptive condition in which there is cytoskeletal plasticity and increased motility under conditions that maintain cell survival signals. Such an intermediate adhesive state would support cell migration and survival during embryogenesis and wound healing. In support of this idea, a recent study shows that TSP1 signaling through the CRT/LRP1 complex in fibroblasts confers resistance to anoikis (anchorage-independent survival) in fibroblasts under nonadherent experimental conditions (16). Cells lacking LRP1 or expressing CRT lacking the TSP1 binding site (minus residues 19–36) cannot respond to TSP1 rescue from anoikis. This cell survival process utilizes the PI3K/Akt intermediate signaling pathway

and acts by inhibiting apoptotic signaling *via* caspase 3 and PARP1 in nonadherent fibroblasts. These studies purport additional mechanisms by which the CRT/TSP1/LRP1 receptor complex affects wound healing and fibrogenesis: the process of anoikis is important in tissue homeostasis and remodeling (35–37), and anoikis resistance aligns with prolonged survival of cells involved in fibroproliferative diseases, such as arthritis and atherosclerosis, and extended myofibroblast survival involved in lung fibrosis (38–41).

Phagocytosis

Another function in which non-ER CRT utilizes LRP1 is in the clearance of apoptotic cells, a process referred to as efferocytosis (by the phagocytic cell) (6). Cell surface CRT in association with phosphatidyl serine (PS) has been shown to provide the obligate recognition signal for the removal of dead cells by both professional (*e.g.*, macrophages, neutrophils) and nonprofessional phagocytes (*e.g.*, fibroblasts). However, different from the function of focal adhesion disassembly in which the CRT/TSP1/LRP1 signaling complex is on the same responding cell in a *cis* configuration, CRT and LRP1 are in a *trans* configuration such that the CRT is exposed on the surface of the apoptotic cell to be engulfed by the phagocyte expressing LRP1. In addition, this process requires the down-regulation or disruption of CD47/integrin-associated protein (IAP) on the apoptotic target cell, thereby blocking the ability of CD47 to engage cell surface SIRP-α (SHPS-1) on the phagocyte. Accordingly, CRT in the absence of CD47 permits engulfment of live cells (42). In *Drosophila*, cell surface CRT is necessary for phagocytosis of apoptotic cells by hemocyte-derived 1(2) mbn phagocytes, which is blocked by antibodies to CRT (43). Moreover, the extent of uptake varies directly with the level of CRT cell surface expression and blocking CRT transcription with siRNA inhibits the phagocytic uptake of the apoptotic cells. These studies underscore the significance of CRT in phagocytosis, as suggested by the evolutionary conservation of this function. Furthermore, the critical presence of CRT on the surface of apoptotic cells for uptake by phagocytes is reiterated by the lack of engulfment of dead CRT-null mouse embryo fibroblasts (K42 cells) unless rescued by exogenous addition of CRT (6).

Plasminogen activator inhibitor-1 (PAI-1) acts as an antiphagocytic signal by opposing CRT-LRP-1-mediated phagocytosis of apoptotic neutrophils, as shown by an increase in phagocytosis in PAI-1 deficiency (44). Moreover, phagocytosis signaled by the decrease in cell surface CD47 can be overridden by PAI-1. Accordingly, whereas CRT and PAI-1 colocalize on viable cells, PAI-1 is lost on apoptotic neutrophils. It appears that the levels of PAI-1 in conjunction with cell surface CRT might, therefore, tightly regulate the early phase of wound healing both through regulating clot formation and by controlling the net accumulation of neutrophils.

The consequence of lack of clearance of apoptotic/dead cells can trigger immunoreactivity as a basis of autoimmune disease (45–47). For example, studies show that antibodies against CRT are prevalent in autoimmune diseases, such as systemic lupus erythematosis (SLE) and celiac disease (48), thereby providing evidence that immunogenic forms exist. CRT also plays an important role as a bridging molecule in the recognition and clearance of apoptotic cells *via* the CD91/C1q collectin/ficolin pathway by binding directly to C1q, collectin, and ficolin proteins that recognize apoptotic debris (24, 49, 50). Impaired recognition of apoptotic neutrophils by a C1q/CRT/LRP1-dependent pathway is associated with SLE (51). In addition, CRT and its signaling partner LRP1 have been identified as surface molecules that bind to the "shared epitope" (SE), a 5-aa sequence that is associated with rheumatoid arthritis (19). Binding to SE ligand induces nitric oxide (NO)-mediated prooxidative signaling, which is blocked by antibodies to both LRP1 and CRT. Furthermore, exogenous CRT bound to the cell surface restored SE-triggered signaling in CRT- or LRP1-null mouse embryonic fibroblasts. It has been shown recently that CRT binds to amyloid-β peptide 1–42 in an ELISA, raising the possibility that CRT might play a role in Alzheimer's disease (52). Interestingly, the interaction of cell surface CRT on *Trypanosoma cruzi* (TcCRT) with C1q provided the apoptotic mimicry that allows the parasite to gain entry to host cells through LRP1 signaling as its mode of infectivity (53) and autoimmune disorders that follow, such as Chagas disease and related autoimmune cardiomyopathy (54).

CRT signal transduction and cell surface binding partners

Cell surface-associated CRT does not have an apparent mechanism for direct signaling since it lacks a transmembrane domain. Although early studies suggested the existence of differentially glycosylated and/or GPI-linked forms of cell surface CRT, referred to as "ectocalreticulin", confirmation of the existence of such forms has been elusive (55). To date, no other signaling receptor besides LRP1/CD91 has been shown to mediate extracellular signals from exogenously added or cell surface CRT (6, 11, 16–18, 56). It will be interesting to determine whether disparate functions, such as cell migration and proliferation that are clearly induced by exogenously added CRT during *in vivo* wound healing (7), are similarly dependent on LRP1 signaling or whether other yet to be discovered transmembrane cell surface binding partners, in conjunction with or without LRP1, participate in cell surface CRT signal transduction. It is also possible that CRT can signal through engagement of cell surface carbohydrate molecules, including the glycosaminoglycan chains of proteoglycans, through its lectin-binding activity. Similar to TSP1, the pluripotent signaling immunoregulatory cell surface protein CD69 binds to an N-terminal fragment of CRT on the surface of human peripheral blood

monocytes and might mediate functions including adhesion (57).

The possibility that cell surface CRT can signal on its own is unlikely but cannot be excluded. Nonetheless, considering the plethora of emerging functions of CRT, it is highly likely that this seemingly nonsignaling protein binds or engages by binding or modifying other transmembrane molecules on the cell surface to mediate signaling.

CRT in the ECM

There is only one report describing CRT as a component of the ECM. In this study, CRT was detected by immunohistochemical electron microscopy in the predentin matrix of the tooth (58). This finding is consistent with the recent observation that CRT protein is increased in fibrotic tissues (59), which suggests that CRT plays a role in enhancing ECM formation and fibroblast anoikis resistance, strongly associated with fibrogenesis (as discussed above). Immunohistochemical analysis of CRT localization in arteries from a rabbit model of atherosclerosis (**Fig. 2**) suggests that CRT is localized to the ECM following injury/damage during vascular remodeling. Studies now provide biochemical evidence for CRT localization to the detergent-insoluble ECM of human foreskin fibroblasts in culture **(Fig. 3** and unpublished results). CRT localization to the ECM is further induced by ascorbic acid treatment, which stimulates collagen expression. Given the known interactions of CRT with fibrillar collagens, laminin, and TSP1 (14, 22, 60, 61) and its ability to modulate matrix metalloproteinase activity (62), it is possible that CRT in the ECM might act to modulate ECM structure or turnover. In addition, CRT could act as a molecular link between the ECM and the cell surface to trigger events at the cell surface while tethered in the ECM. The release of CRT from injured or dying cells during stress conditions of hypoxia (48) and its incorporation into the ECM, as described above, might further modify ECM structure and cellular function in both wound repair and diverse responses to injury, including fibrosis. As CRT associated with the ECM is a new discovery and not well described, we anticipate that novel functions associated with or stimulated by CRT sequestered in the ECM will continue to unfold.

Non-ER CRT in physiological and pathological processes

Wound healing and supportive in vitro *studies*

With studies showing that CRT is involved in migration, phagocytosis, and fibrogenesis, it is not surprising that

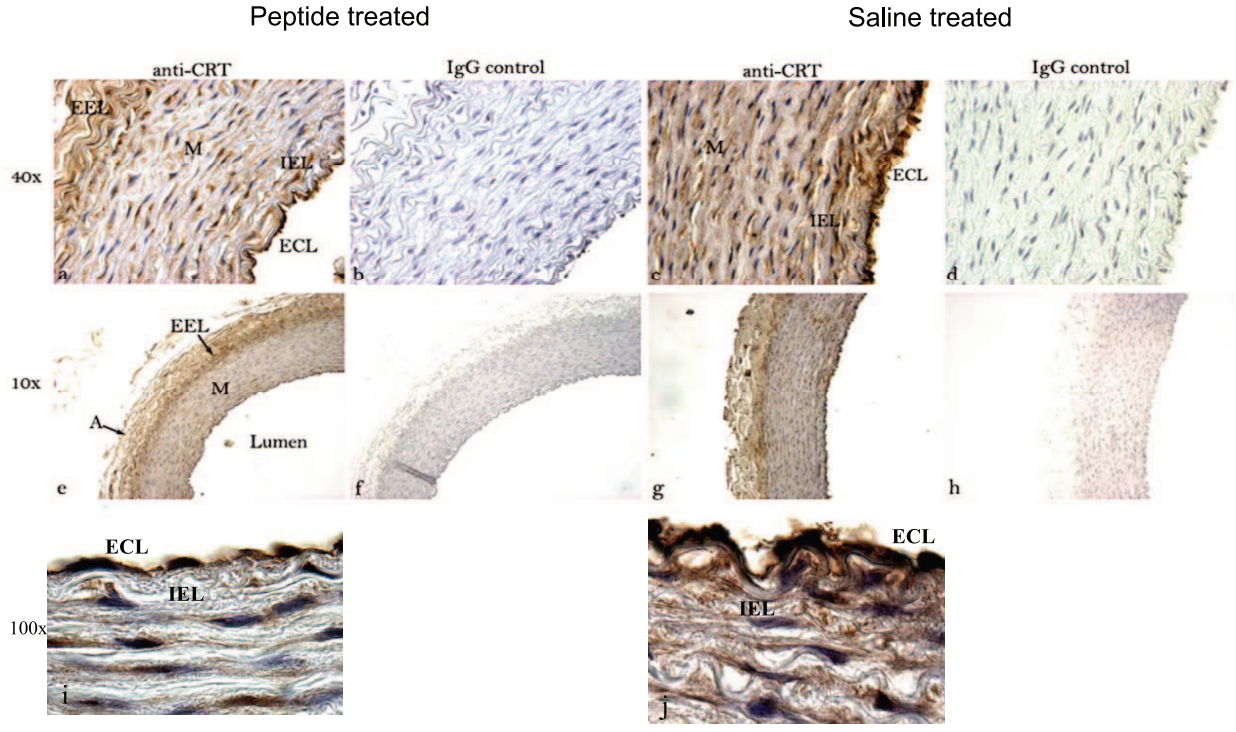

Figure 2. CRT expression is increased in the endothelium and media of atherosclerotic arteries. New Zealand White rabbits were fed a 0.25% high-fat diet. Rabbits were treated with saline or a lipid-lowering synthetic peptide, hE-18A (as described in ref. 155) for 2 mo. CRT expression was assessed by immunohistochemical analysis using an antibody from Thermo Scientific Pierce Antibodies (Rockford, IL, USA). Arteries of the saline-treated atherosclerotic rabbit (*c, d, g, h, j*) had increased staining for CRT in the endothelium and media of the coronary arteries as compared to the hE-18A-treated rabbits (*a, b, e, f, i*). ECL, endothelium; IEL, internal elastic lamina; M, media; EEL, external elastic lamina; A, adventitia. Arteries were provided by Dr. G. M. Anantharamaiah and Dr. C. Roger White (Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA).

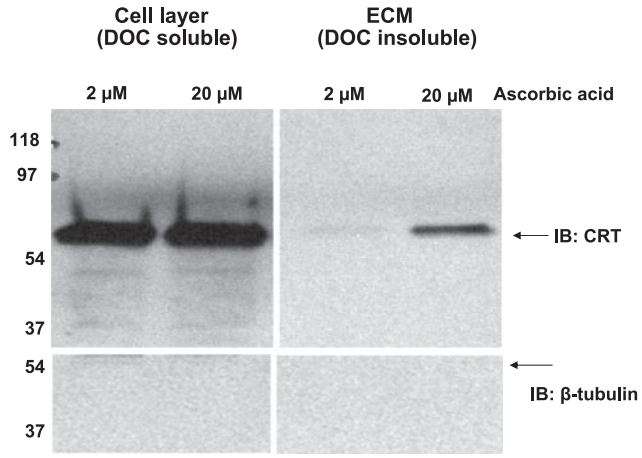

Figure 3. CRT is localized to the detergent-insoluble ECM of fibroblasts. Human foreskin fibroblasts were treated for 72 h with 2 or 20 μM ascorbic acid. Cells were removed by trypsin-EDTA treatment. Remaining cell layer was scraped into 4% deoxycholate (DOC) to separate soluble proteins from the insoluble ECM. Supernatant was collected as the soluble fraction. DOC-insoluble pellets were washed again with 4% DOC, and the pellets were collected as the ECM fraction. DOC-soluble and -insoluble proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes for immunoblotting for CRT using a rabbit anti-CRT antibody (SPA-600; Stressgen Biotechnologies, Ann Arbor, MI, USA). ECM from cells treated with 20 μM ascorbate have increased amounts of CRT in the ECM as compared ECM fractions obtained from cells treated with 2 μM ascorbate. Blots were stripped and reprobed with antibodies to β-tubulin and β-actin (not shown) to assess contamination of the ECM fraction with cellular components.

topically applied non-ER CRT increased the rate of wound closure with major histological differences in the CRT-treated wounds, which were identical in two animal models at the same doses (unpublished results; mouse wounds: **Fig. 4**) (7). Specifically, the quality of wound healing was improved by affecting both the epidermal and dermal aspects of the cutaneous repair process. The CRT-treated wounds show an increased rate of epithelialization by migrating keratinocytes and a strong induction of granulation tissue (neodermis) produced by greater numbers of fibroblasts compared to controls in porcine and murine animal models (**Fig. 5*A***).

The dense cellularity and abundant granulation tissue (neodermis) observed in the CRT-treated wounds (Fig. 5*A*), as previously discussed (CRT functions on the cell surface; migration), is explained by the ability of exogenous CRT to function *in vitro* in the induction of cellular migration and proliferation of primary human keratinocytes and fibroblasts, the most critical functions and cell types that enable wound healing **(Fig. 6)** (7). Moreover, CRT stimulated the migration of monocyte and macrophage cell lines *in vitro*, explaining the elevated tissue macrophages observed in the CRT-treated porcine wounds compared to controls. In addition, the expression of α5 and β1 integrins (a fibronectin receptor), the integrin chains expressed early in wound healing (63, 64), were shown to be up-regulated on keratinocytes and fibroblasts following treatment with CRT *in vitro* (unpublished results and Fig. 6). As wound granulation tissue is composed of ECM proteins, the ligands for integrins, particularly collagens and fibronectin, CRT appears to perform an essential role in both integrin chaperoning and in the expression of specific integrins, thus enabling cellular migration into the wound. Because CRT is the requisite mediator of the removal of apoptotic cells by phagocytic cells, CRT putatively affects two different processes related to monocytes that are important in tissue repair. First, CRT potentially acts as a chemotactic agent for diapedesis of monocytes into the wound bed, where the monocytes become activated into tissue macrophages. Second, CRT likely participates in the process of wound debridement of dead cells and tissue, obligatory for healing (6). Both the presence of necrotic tissue and infection are critically important deterrents of wound healing. Although an effect of CRT on ameliorating infections has not yet been shown, microbial phagocytosis by cell surface CRT might also contribute to wound resolution.

In *in vitro* studies, exogenous addition of CRT to human primary keratinocyes and fibroblasts stimulates proliferation more potently than either epidermal growth factor (EGF) or FGF, respectively (7). These results are consistent with the high number of basal regenerative keratinocytes and fibroblasts of the neodermis labeled with markers of cell proliferation in murine and porcine wounds topically treated with CRT. Stimulation of proliferation is a novel direct role for CRT. Moreover, keratinocytes have not been shown to be a target cell of CRT in any capacity thus far. The possible relevance of CRT to cell proliferation has only been reported in its ability to maintain nuclear localization and stabilization of p53 (a cell cycle protein that is increased during stress, such as DNA damage) (65) and the up-regulation of insulin receptor expression with associated downstream PI3K/Akt signaling (66). In addition, the presence of CRT in the nucleus (67) and its ability to interact with the cytoskeleton of murine ova for signal transduction related to cell cycle activities have been shown (68).

The growth of microcapillaries in the wound granulation tissue is essential for wound healing, and CRT has also been shown to stimulate new blood vessel growth (69) and to induce proliferation of human microvascular endothelial cells, an important aspect of angiogenesis (7). However, in multidomain, multifunctional extracellular proteins, especially those of the ECM, the function of isolated domains can differ from those of the intact protein. Similarly, CRT and its fragments appear to have similar differential functions with respect to angiogenesis regulation. In this context, the N-terminal domain of CRT (aa 1–80) has been shown to have antiangiogenic activity, particularly in tumor models (70–72). This fragment, also known as vasostatin, binds to laminin and is thought to inhibit angiogenesis by preventing endothelial cell adhesion to laminin in the capillary basement membrane (73, 74).

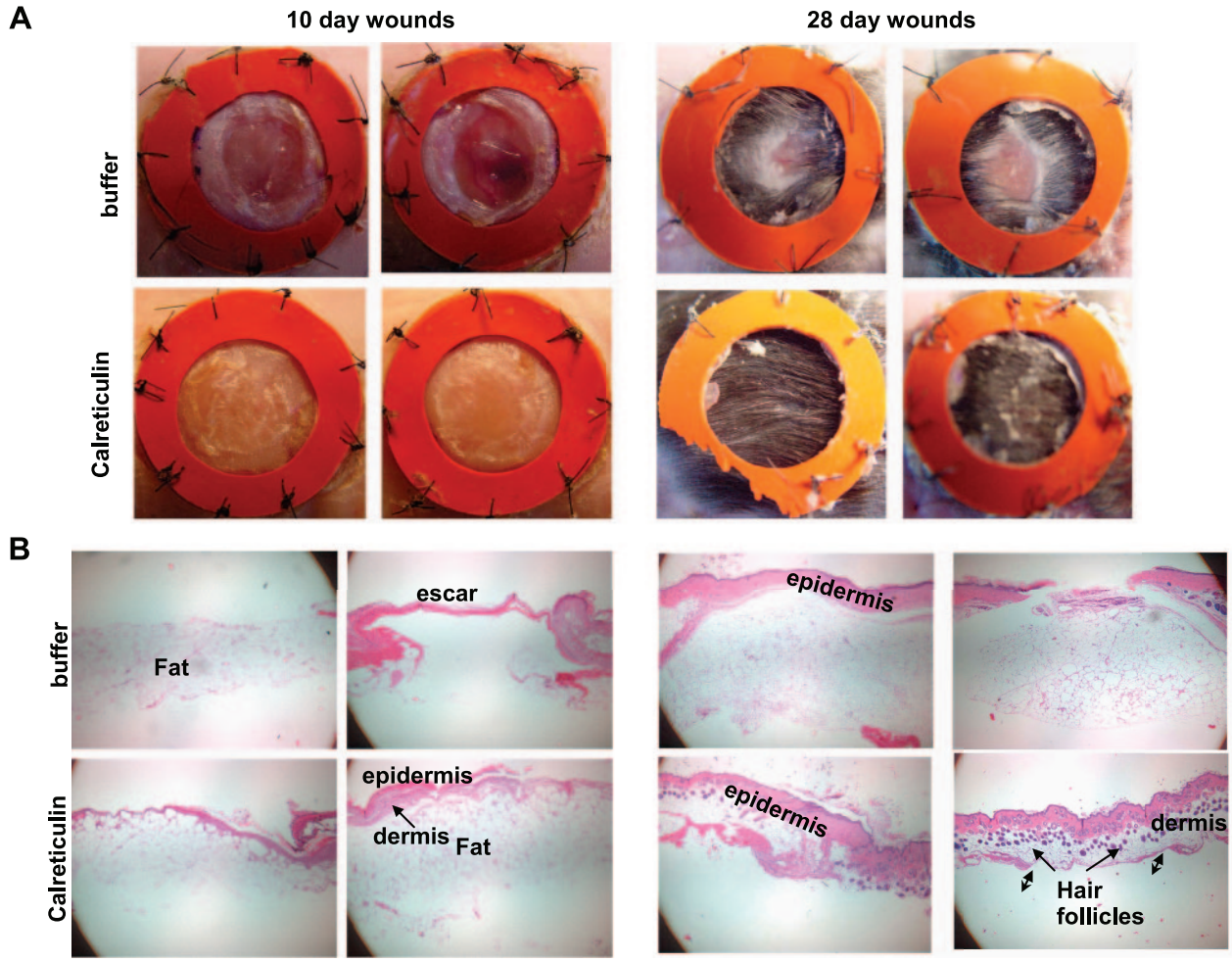

Figure 4. CRT enhances the rate and quality of wound closure in a murine diabetic wound model at 10 and 28 d postwounding. *A*) Gross wounds. Leptin receptor-deficient mice (*db/db*; BKS.Cg-m/lepr^{db}) 8 to 12 wk old were used as a model of human type 2 diabetes mellitus; these mice have impaired rate and quality of wound healing. Unlike humans and pigs, rodents are loose-skinned haired animals with a *panniculus carnosus* muscle layer directly below the dermis that contracts following wounding, thereby causing a short window for the measurement of epithelial migration over the wound. To facilitate the measure of rate of epithelial migration (by digital imaging) over the granulation tissue (neodermis), 5.0-mm full-thickness excisional wounds (2/animal) were created on the dorsum of each mouse, and a silicon (orange) splint was centered on the wound to prevent wound contraction (wound resurfacing) (156). CRT (200 μg/d for 4 d) was applied to the wounds, and the wound tissue was excised at 3, 7, 5, 10, 14, 21, and 28 d postwounding. CRT-treated wounds show a marked increase in reepithelialization starting at d 3 compared to the buffer-treated controls (10 mM Tris and 3 mM Ca, pH 7.0; n=6 mice/time point). Accelerated wound reepithelialization of the CRT-treated wounds compared to controls is illustrated at 10 and 28 d postwounding. It is notable that at 28 d postwounding, hair has grown back in the CRT-treated but not the buffer-treated wounds. *B*) Histology of wounds. Histology of each hematoxylin-and-eosin-stained corresponding wound shown in *A* illustrates that whereas buffer-treated wounds have little reepithelialization and a paucity of granulation tissue, CRT-treated wounds have reepithelialized and contain a layer of granulation tissue over the fat layer in the 10-d wounds. At 28 d postwounding, the CRT-treated wounds are remarkably more mature, and hair follicles are observed directly in the wounded area, which is marked by the disrupted *panniculus carnosus* (arrowheads). The presence of hair follicles is unusual. However, it has been shown that Wnt-dependent *de novo* hair follicle regeneration can occur in adult mouse skin from migrating stem cells generated by epidermal cells outside the wound (157). Original view ×10.

This might reflect differential regulation of angiogenesis induced by tumors as compared to physiological angiogenesis in wound healing, or alternatively, differences in dose dependence, as concentrations used in tumor angiogenesis studies did not impair wound healing (75). Whereas wound-related proteases may cleave CRT to release the N-terminal domain, the angiogenic effects of CRT in wound healing should be further investigated.

Exogenous addition of CRT to human primary dermal fibroblasts *in vitro* induces a dose-dependent increase in fibronectin, collagen type I, and TGF-β3, the major proteins directly related to the abundant granulation tissue observed in wounds topically treated with CRT (Fig. 5*B* and unpublished results). This is the first study showing induction of matrix proteins by exogenous CRT in cells critical to wound healing. Consistent with the *in vitro* effect, an increase in TGF-β3 but not TGF-β1 or TGF-β2 is also shown in the porcine and murine CRT-treated wounds by immunohistochemical analysis (unpublished results on murine studies and

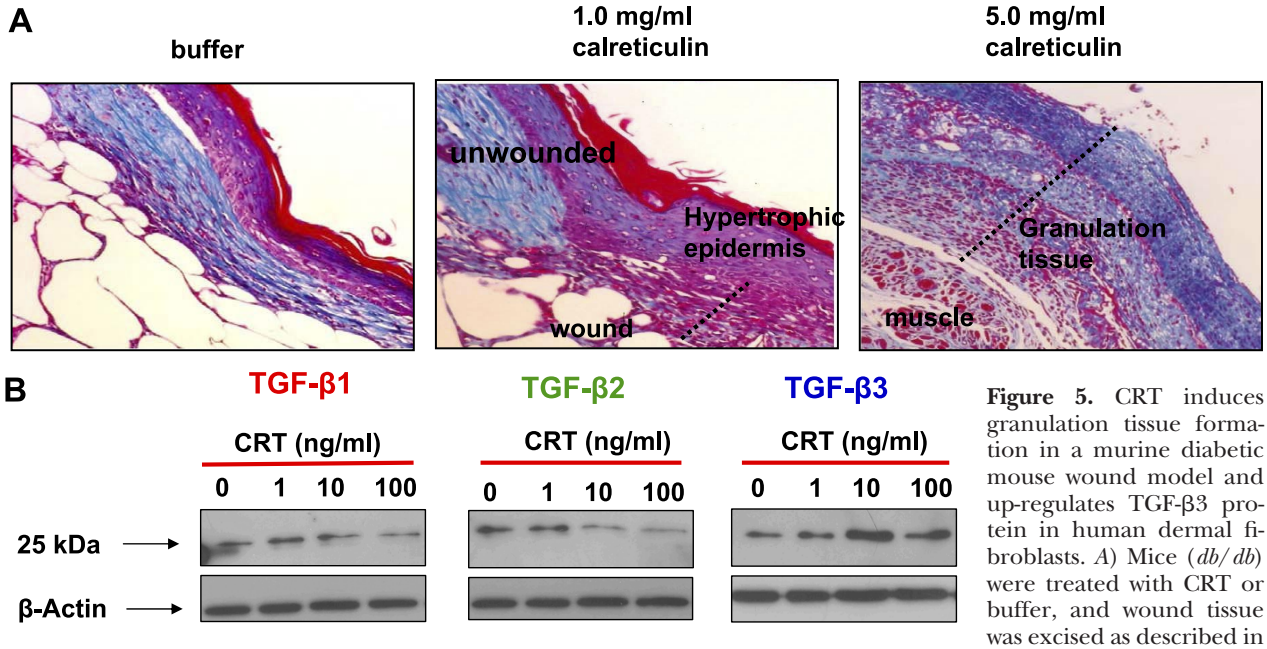

Figure 5. CRT induces granulation tissue formation in a murine diabetic mouse wound model and up-regulates TGF-β3 protein in human dermal fibroblasts. *A*) Mice (*db/db*) were treated with CRT or buffer, and wound tissue was excised as described in Fig. 4. Formalin-fixed, paraffin-embedded wound tissue (5.0 μm) was stained with hematoxylin and eosin and evaluated by light microscopy. Topical application of CRT increased granulation tissue volume in a dose-dependent manner (dotted line indicates depth of granulation tissue). *B*) Immunoblot for expression of TGF-β3 by human primary dermal fibroblasts *in vitro*. Fibroblasts were treated with increasing concentrations of CRT in serum-free medium. After 24 h, cell lysates were analyzed for TGF-β isoform expression by immunoblotting with TGF-β isoform-specific IgG prepared to peptides of TGF-β isoforms (prepared by L.I.G.). Exogenous CRT induces TGF-β3 protein but not TGF-β1 or TGF-β2 in a dose-dependent manner with a peak response at 10 ng/ml. This result is consistent with the increased expression of only the TGFβ3 isoform shown in models of porcine (7) and murine wound healing *in vivo* following topical application of 5.0 mg/ml CRT, observed at 5 d postwounding. Original view ×100.

ref. 7). It is interesting and perhaps important that CRT induced only the TGF-β3 isoform, as only this isoform is involved in collagen gel matrix contraction (to stimulate wound contraction) (76), acceleration of wound healing with decreased scarring (77, 78), cellular motility (79), and induction of hyaluronan, an important component of neodermal formation (80). Furthermore, TGF-β3 has been proposed as the master traffic controller of epidermal and dermal cell motility during repair (81), in which its concentration is highest in serum following clot formation. As TGF-β stimulates collagen and fibronectin synthesis (82), CRT might induce these matrix proteins both indirectly and directly.

Interestingly, there is new evidence for a direct effect of intracellular CRT on up-regulation of collagen: recent studies show that CRT has both transcriptional and posttranslational effects on fibrillar collagen expression by mouse fibroblasts mediated by CRT regulation of calcium signaling (unpublished results). Furthermore, collagen matrix deposition is modulated by CRT-dependent fibronectin assembly (unpublished results), and CRT expression correlates with fibronectin mRNA, protein, and assembly into the ECM (83, 84). Moreover, CRT stimulation of MMP-2, MMP-9, and MTI-MMP (62) suggests further roles for CRT in ECM remodeling during the final stages of wound healing. Finally, preliminary *in vitro* studies indicate that CRT might be involved in conversion of fibroblasts to myofibroblasts, which are important in wound contraction and ECM production by up-regulating α-smooth muscle actin (unpublished results).

The binding of cell surface CRT on platelets to α2β1 integrin to mediate collagen binding required for platelet activation also suggests a role for CRT in regulating platelet degranulation and clot formation (29, 30). As CRT, TGF-β and TSP1 are constituents of platelet α-granules and released following platelet degranulation, TGF-β and CRT would be available at the wound site to stimulate fibroblast proliferation, and both CRT and TSP1 could facilitate the migratory process (17, 85–87). Moreover, TSP1 activates latent TGF-β1 into its receptor-binding bioactive form important in matrix induction (88). Although difficult to test *in vivo*, CRT/TSP1/LRP1-mediated focal adhesion disassembly leading to cell migration is likely to be functionally and operationally integral to the wound-repair process.

Many proteins important in wound healing are up-regulated by the hypoxic environment, such as vascular endothelial growth factor (VEGF) (89) and TGF-β3, which has an HIF1-α response element in the promoter region of the gene (90). Therefore, CRT has likely evolved as an important physiological mediator of wound healing and other extracellular processes due to its release by natural cell death during injury. CRT is dynamically expressed during murine and porcine wound healing, providing support for its role in this process (7). However, the proportions of intracellular compared to extracellular CRT that are associated with

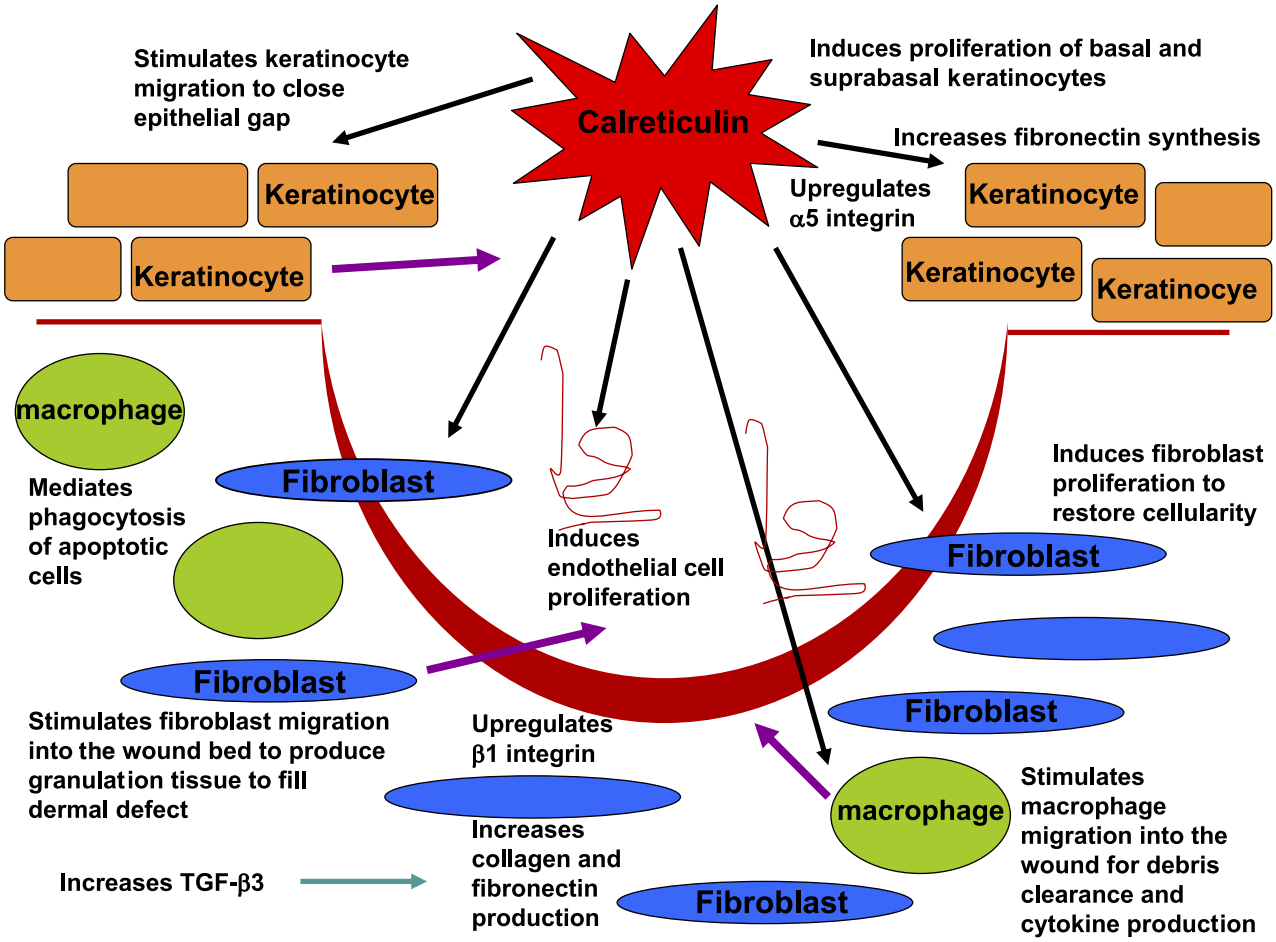

Figure 6. CRT exerts diverse biological effects on human keratinocytes, fibroblasts, monocytes, and macrophages *in vitro*, consistent with its role in wound healing *in vivo*.

specific wound-healing functions remains to be delineated.

CRT improves the rate and quality of wound healing in diabetic animal models (unpublished results and ref. 7). Furthermore, exogenous CRT augments *in vitro* functions shown to be most deficient in chronic diabetic wound healing, such as cell migration, proliferation, growth factor production, and the uptake of apoptotic cells by macrophages (91, 92). Therefore, CRT might have therapeutic potential for healing wounds of type 2 diabetic patients and other chronic nonhealing wounds. In this context, of interest is the protective effect of CRT on excessive glucose influx into cells (a stress-related condition also found in hyperglycermia associated with type 2 diabetes) through its ability to down-regulate glucose transporter-1 (GLUT-)1 by degradative means (93) and decrease insulin receptor β expression (66).

Cancer and the adaptive immune response

The process of immune-mediated destruction of cancer cells has more recently been shown to be dependent on the cell surface expression of CRT (4, 5, 56, 94–97). These studies show that anthracyclins, (*e.g.*, doxorubicin), or inhibitors of protein phosphatase 1/GADD34, involved in the dephosphorylation of eIF2α, a protein hyperphosphorylated during ER stress (98), induces rapid (within minutes) translocation of CRT to the surface of preapoptotic cells for their clearance by dendritic cells and primary tumor antigen-specific T lymphocytes (5, 94, 96, 99, 100). Conversely, lack of CRT on the cell surface renders dying cancer cells nonimmunogenic, and therefore, phagocytosis by dendritic cells is suppressed. Thus, the presence of CRT on the tumor cell surface is a crucial event that subsequently initiates the immune response against the tumor. Moreover, the addition of exogenous CRT or inhibitors of the protein phosphatase 1/GADD34 restore immunogenic cell death induced by mitomycin C. In addition, antitumor effects of CRT are also observed *in vivo*. Notably, adsorption of CRT to live tumor cells enhances phagocytosis by dendritic cells *in vitro* and *in vivo*, but this is contingent on agents that induce cell death. Therefore, cell surface exposure of CRT is required and sufficient for induction of proimmunogenic cell death in conjunction with chemotherapy (94, 99, 101). The potential application of CRT in cancer therapy is underscored by these studies and has been practiced using CRT as an adjuvant for chemotherapy.

This was achieved by administering a chimeric construct of CRT with tumor antigen peptides delivered by a viral vector or by using gene therapy, which enhanced immunogenicity more than tumor antigen alone (102–104).

Anthracyclins induce the rapid translocation of presynthesized CRT from the ER to the cancer cell surface by lowering calcium levels in the ER; agents that directly lower ER Ca^{2+} levels can also induce this effect (95). The translocation process involves the activity of the actin cytoskeleton (105) and the ER-resident protein, ERp57 (a disulfide isomerase) (18, 56). Through their direct interaction, ERp57 is an obligate partner in the translocation of CRT to the surface during anthracyclin treatment as knockdown of ERp57 by shRNA obviates CRT cell surface exposure, resistance to anthracyclin treatment *in vivo*, and lack of the antitumor response. These data also might implicate ERp57 as an obligate partner in cell surface exposure of CRT, in general. The addition of exogenous recombinant CRT rescues the antitumor immune response, thus implicating a defect in the translocation of CRT to the surface as a possible mechanism of patient's resistance to anthracyclins (18, 97).

The mechanism of the immune response to preapoptotic (dying) cancer cells expressing cell surface CRT induced by anthracyclins or γ irradiation is temporal, involving an early non-PS-dependent cell surface appearance and at later stages of apoptosis, cell surface exposure concomitant with PS (6, 106). Early cell surface exposure of CRT is followed by expression of heat-shock proteins, such as Hsp90 and Hsp70, their subsequent release and finally, a release of high-mobility group I (HMGB1) protein (4), which acts on toll-like receptor 4 (TLR4) expressed by dendritic cells. The paradigm further purports the processing and presentation of tumor antigens by dendritic cells, followed by T-cell activation and the generation of cytotoxic/cytolytic T cells ($CD8^+$) against the apoptotic cancer cells. However, the role of cell surface CRT in antigen processing is controversial. Attempts at showing that the essential role of CRT in major histocompatibility complex (MHC) class I peptide loading, for the biogenesis of MHC class I molecules and antigen presentation, is related to cell surface CRT have not been met, and therefore these specific critical immune functions of CRT appear to remain in the domain of the ER (107–109).

As the success of chemotherapy lies in the induction of cell death by apoptosis or necrosis that will elicit an adaptive immune response to the tumor, an intact immune system and cell surface CRT has also been shown to be essential in T-cell-deficient mice (96). The possibility that the induction of cell surface CRT can convert nonimmunogenic to immunogenic chemotherapy by involving dendritic and T cells to obviate tumors is being tested (94, 99, 110). In fact, following treatment with anthracyclin, a positive response associated with the up-regulation of cell surface CRT on circulating cancer cells was obtained in patients with acute myeloid leukemia (96). Finally, these studies have very important implications for prediction of the success of cancer therapies in that cell surface exposure of CRT on cancer cells would promote a positive response to anticancer therapy (111, 112).

Interestingly, CRT on the cell surface of dendritic cells has been shown to be a receptor for NY-ESO-1, a tumor-associated antigen, also with potential for involvement in the adaptive immune response against tumors by dendritic cell cross-presentation to $CD8^+$ T cells (113). Further, it has been proposed that intracellular pathogens, such as viruses and parasites, avoid host cell death by mechanisms that prevent cell surface exposure of CRT, thereby subverting the adaptive immune response to the pathogen (114). The presence of the N-domain cleavage product of CRT (ectocalreticulin) in serum has been shown to be part of the autoantibody signature for early detection of hepatic cancer and also is proposed to be responsible for induction of immune reactivity against tumor antigens (115). However, it is notable as a counterargument, that CRT and Hsps, as part of their antiapoptotic activity, would promote cancer cell survival, which is exactly the target of geldanomycin, a 17-AAG analog-inhibitor of Hsp90α now in phase I and II clinical trials for treatment of a number of human cancers (116–118).

CRT has other effects on the immune response. To facilitate T-cell immune behavior requiring adhesion *via* integrins, TSP1 at the plasma membrane interacts with integrins, LRP1, CRT, and integrin-associated protein (IAP/CD47) to mediate adhesion and migration of these cells (119). The function of T-lymphocyte adhesion and TSP1 turnover requires the interaction of CD47 with the C-terminus of TSP1 (120). In addition, cell surface exposure of CRT is essential for PS-independent macrophage recognition, tethering, and engulfment of cells undergoing autophagy prior to cell death (121, 122). This process is important in the induction and secretion of the proinflammatory cytokines, IL-6, IL-8, IL-10, and TNF-α, consistent with an immune response. The presence of CRT in cytotoxic granules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells has been shown to be required for CTL-target cell interaction and for death synapse formation for effective cell killing (123).

Interestingly, the presence of non-ER extracellular CRT is associated with a variety of largely immune-related diseases (**Table 1**), although its frank involvement in the pathogenesis of any of these disorders remains to be determined.

A recent study shows that inhibitor peptides of the PP1/GADD34 complex fuse to targeting molecules, which induces CRT exposure on the cell surface of a variety of tumor cell lines, increases the phagocytosis of anticancer-targeted proapoptotic peptide-treated tumor cells by dendritic cells, and reduces tumor growth in mouse models of colon, mammary, and fibrosarcoma tumors (124). These data suggest that targeted peptides can increase cell surface exposure of cancer

TABLE 1. *Detection of extracellular CRT and anti-CRT in clinical conditions*

Disease	CRT	Anti-CRT	References
Urothelial carcinoma	>2.9 ng/ml urine		158
Rheumatoid arthritis	In serum	Yes, IgG	159
Refractory coeliac disease		Yes, IgA	160
Preeclampsia	50% increase in serum compared to normal pregnancy		161
Inflammatory bowel disease		Yes	162
Bladder cancer	In urine		163
Alzheimer disease	Brain		164
SLE	Plasma/serum	Yes, IgG	50
Primary biliary cirrhoris		Yes, IgA	165

cells for a novel therapeutic approach to killing cancer cells.

CONTROVERSY AND UNANSWERED QUESTIONS

In order for CRT to function at or near the cell surface and in the extracellular environment, it must move from the ER *via* various subcellular compartments and then be released. With scant evidence to date, little is known about how CRT partitions into different cellular compartments and out of the cell to exert the array of functions described herein. However, because of the expanding roles of non-ER CRT in wound healing and tumor recognition (described in Physiological Processes), the mechanism of release of CRT from the cell has been an intense focus of research. Moreover, as CRT has a C-terminal KDEL retrieval amino acid sequence, it is even more difficult to envision how CRT retaining this sequence would be able to exit the ER to the cell surface and become a part of classic recycling mechanisms of the protein secretory pathway. Without evidence to support this idea, perhaps proteolytic cleavage of the KDEL amino acid sequence or conformational masking of this sequence due to interactions with other proteins or change in pH and/or calcium levels may be involved in the release of CRT from the ER (125). Clearly, the fact that RNA interference-mediated reduction of CRT on the cell surface of apoptotic cells inhibited the engulfment of dead cells by phagocytes provides support for autologous production and transport of CRT to the cell surface, which could be either derived from the ER or straight from the cytosol (43).

There are two classic ways in which predominantly intracellular proteins can be released from cells: active secretion or by cell death. There is evidence to suggest that CRT is released from dying cells by necrosis and, as stated above, we propose this is a physiological mechanism for CRT release during tissue injury prior to repair (48, 126). CRT, as an ER chaperone, and Hsps, as cytoplasmic chaperones, respond to inflammatory damage and can be released in an apparent nonspecific manner. This probably has physiological relevance in signaling and activating antigen presenting cells (APCs).

The mechanism of active secretion of CRT from cells has been more difficult to explain. Chaperone proteins do not have an N-terminal signal sequence, precluding their release through the classic mechanism of protein secretion, which involves transport from the ER to the Golgi, and then fusion with the cell membrane for localization to the cell membrane or for secretion into the extracellular space. Hsp-90α has been shown to be released through an exosomal pathway in keratinocytes induced by TGF-α through epidermal growth factor receptor (EGFR) signaling (127). Exosomes are a distinct population of 30- to 90-nm intracellular nanovesicles (128, 129) contained within larger multivesicular bodies (MVBs) that usually function as reservoirs for degradation of misfolded proteins endocytosed from the cell surface or by sorting from the *trans*-Golgi (130). However, exosomes have also been shown to fuse with the plasma membrane as a mechanism of release (131, 132) of proteins derived from the cytosol. Although exosomes do not contain proteins from the ER, such as CRT, it is still possible that CRT might exit the cell *via* this route, particularly if it retrotranslocates from the ER to the cytoplasm, as proposed (133).

As CRT is a stress-response protein (*e.g.*, hypoxia, heat, chemical and physical stress, and irradiation), attention has focused on the effect of cell oxidative stress on CRT translocation and extracellular release. The predominant location of CRT and other ER proteins is within the hyperoxic environment of the ER, causing their susceptibility to oxidative damage (134). Production of the antioxidant NO leads to overexpression of CRT, which in turn leads to increased ER Ca^{2+} concentrations (135). Similarly, hypoxic conditions, such as those found in wounds, can lead to overexpression of CRT by 2- to 3-fold *via* p38 MAPK signaling and oxidative stress during rat cardiomyocyte injury, causing a 7-fold increase in CRT expression (136). Oxidative damage or posttranslational modification of CRT can affect both its function and subcellular location. There is little information on the routing of CRT from the ER to the cytoplasm. Retrotranslocated proteins are usually marked as damaged and succumb to proteasomal degradation (133, 137). Interestingly, CRT is shown to be an inefficient substrate for the proteosome

and thus avoids the usual ubiquitylation and degradation following retrotranslocation from the ER to the cytoplasm, making it the only known mammalian protein that is retrotranslocated from the ER and not degraded. In this report (133), CRT previously inserted into the ER membrane is processed by a signal peptidase for retrotranslocation to the cytoplasm (and not by premature ribosome release from the ER membrane). These studies show that CRT is driven into the cytoplasm by an intraluminal decrease in calcium in the ER regulated by the C-terminal low-affinity, high-capacity Ca-binding C-domain of the molecule (133). It is also possible, however, that CRT avoids proteasomal degradation by gaining access to the cytoplasm due to a suboptimal signal peptide (138). Once in the cytosol, CRT can be posttranslationally modified by arginyl transferase, which attaches an arginine residue to the N-terminal aspartic acid residue of the protein, thereby localizing CRT to cytoplasmic stress granules (139). However, the influence of CRT arginylation on its cytosolic function remains unknown. Clearly, the release of CRT from the ER by retrotranslocation, avoidance of proteosome degradation, and its arginylation makes it a rather unconventional subcellular retrotranslocating protein.

The release of CRT from the cell is also unusual. Several divergent hypotheses have been postulated, but all agree that ER stress, possibly induced by oxidative stress by reactive oxygen species (ROS) or UV irradiation, followed by apoptotic processing, leads to extracellular release of CRT. Most glycosylated proteins in mammalian cells are exported to the cell surface by the ER/Golgi-dependent secretory pathway. A recent study demonstrated that cells exposed to stress triggered a number of preapoptotic cell regulatory proteins (caspase 8, Bap31, Bax activation), which were indispensable for CRT surface exposure in a complex with ERp57 (CRT/ERp57) (56). However, this study does not explain how CRT as a nonglycosylated protein (125) is secreted *via* the secretory pathway, but it did demonstrate the tracking of artificially glycosylated recombinant CRT (created *n*-glycosylation sites) straight from the ER to the Golgi with fusion of the plasma membrane and surface exposure by SNARE-(secretory vesicle-associated protein) dependent exocytosis (the secretory pathway) (56). The process was calcium dependent, occurring through actin-dependent anterograde and not microtubule-associated retrograde (from the ER). In addition, it was shown that stressing cells with UV irradiation leads to activation of caspase 1 and secretion of CRT and other leaderless sequence proteins in a caspase 1-dependent manner (140). Concordantly, preliminary studies show that oxidative/apoptotic stress leads to overexpression of CRT, which accumulates in the cytosol in association with PS (unpublished results), the lipid that is externalized on apoptotic cells by a membrane flip-flop mechanism on apoptotic cell death. There is precedent for this membrane inversion to facilitate transport of proteins from inside the cell to the outer membrane, as shown for annexin exposure

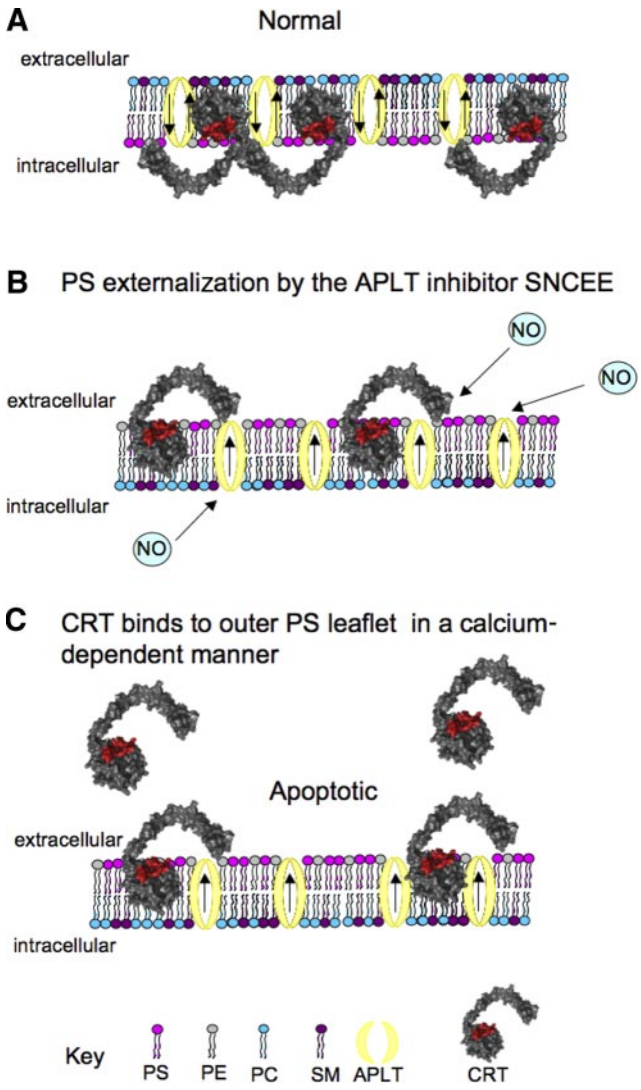

Figure 7. Schematic model of a potential means for CRT release during apoptotic stress. *A*) During oxidative stress, CRT expression increases. During such stress, CRT can retrotranslocate from the ER into the cytosol and associate with PS on the inner plasma membrane (PM) leaflet. *B*) Increased CRT production leads to activation of iNOS and NO production. Potentially, the increased NO production can nitrosylate the free cysteines on the flipase enzyme–aminophospholipid transferase (APLT). This blocks APLT, which retains PS on the inner leaflet of the PM. The majority of PS flips to the outer surface, and CRT is released at the same time. *C*) CRT has a lipophilic and hydrophobic region (red) that may allow CRT to associate with PS. Its interaction with PS is calcium dependent. Lowering the calcium concentration allows release of CRT from PS. SNCEE, *S*-nitroso-L-cysteine-ethyl ester.

during apoptosis (141, 142). The overexpression of CRT induces increased NO production (n.b., both NO induces CRT and CRT induces NO; refs. 19, 143), which has the ability to inhibit aminophospholipid translocase (APLT), which helps retain PS on the cytosolic side of cells, thereby allowing CRT to flip out of the cell in association with PS (**Fig. 7**). Another ER-resident protein, Hsp-70, is associated with PS on the surface of PC12 pheochromoctyoma cells (144). Similarly, as described here, CRT was shown to colocalize

with areas of cell surface exposure of PS on apoptotic cells prior to their uptake by phagocytes (6). In addition, in lower organisms, it was shown that during phagocytosis in *Dictyostelium*, GFP-tagged CRT and calnexin are directly linked between the ER and the phagocytic cup enclosing a particle (145).

Notably, since exogenous addition of CRT rescues CRT-null cells in numerous and varied CRT-dependent functions, as described above [*e.g.*, TSP1-stimulated focal adhesion disassembly, clearance of apoptotic cells, and immunogenic cell death (the uptake of cancer cells by dendritic cells) restored SE ligand signaling in arthritis] (5, 15, 19), it is possible that cell surface CRT is not only autologously derived by passive release or active secretion but may emanate from neighboring cells in a paracrine manner. There is also evidence showing that CRT is contained in maturing phagosomes and that during phagocytosis, the ER might fuse with the plasma membrane at sites of particle ingestion, allowing access to the ER lumen (146). In addition, CRT has been found in the Golgi (69) and might be routed to the cell surface by remaining associated with proteins that it chaperones, such as α-integrins and MHC class 1 molecules (10, 147). Certainly, secretory proteins containing transmembrane domains could potentially shuttle CRT to the cell surface. A compilation of proposed potential routes for intracellular transit and exit routes for CRT is presented in **Fig. 8**. Finally, the cell surface exposure of CRT on dying *Saccharomyces cerevisiae* underscores both the obligate and evolutionary significance of CRT, exposing itself on the cell surface despite the enigmatic nature of the transit mechanism (148).

PROSPECTS AND PREDICTIONS

In this review, we provide compelling evidence for CRT in the regulation of cellular processes from multiple cellular compartments (*i.e.*, nucleus, ER, cytosol, cytosolic vesicles, secretory granules, and the plasma membrane), as well as its presence in the ECM (**Table 2**). There are at least four major provocative unanswered areas open for discovery: *1*) what cellular mechanisms dictate compartmental transit of CRT from the ER to the cell surface and extracellular space (Fig. 8); *2*) which receptors other than the LRP1 transduce signaling for the varied functions shown for CRT; *3*) whether ER-CRT is required for the apparent extracellular receptor-driven varied non-ER functions observed; and *4*) what other functions and vital system processes involve a role for CRT.

CRT is not the only chaperone that increases under cell stress conditions and is likely released from injured

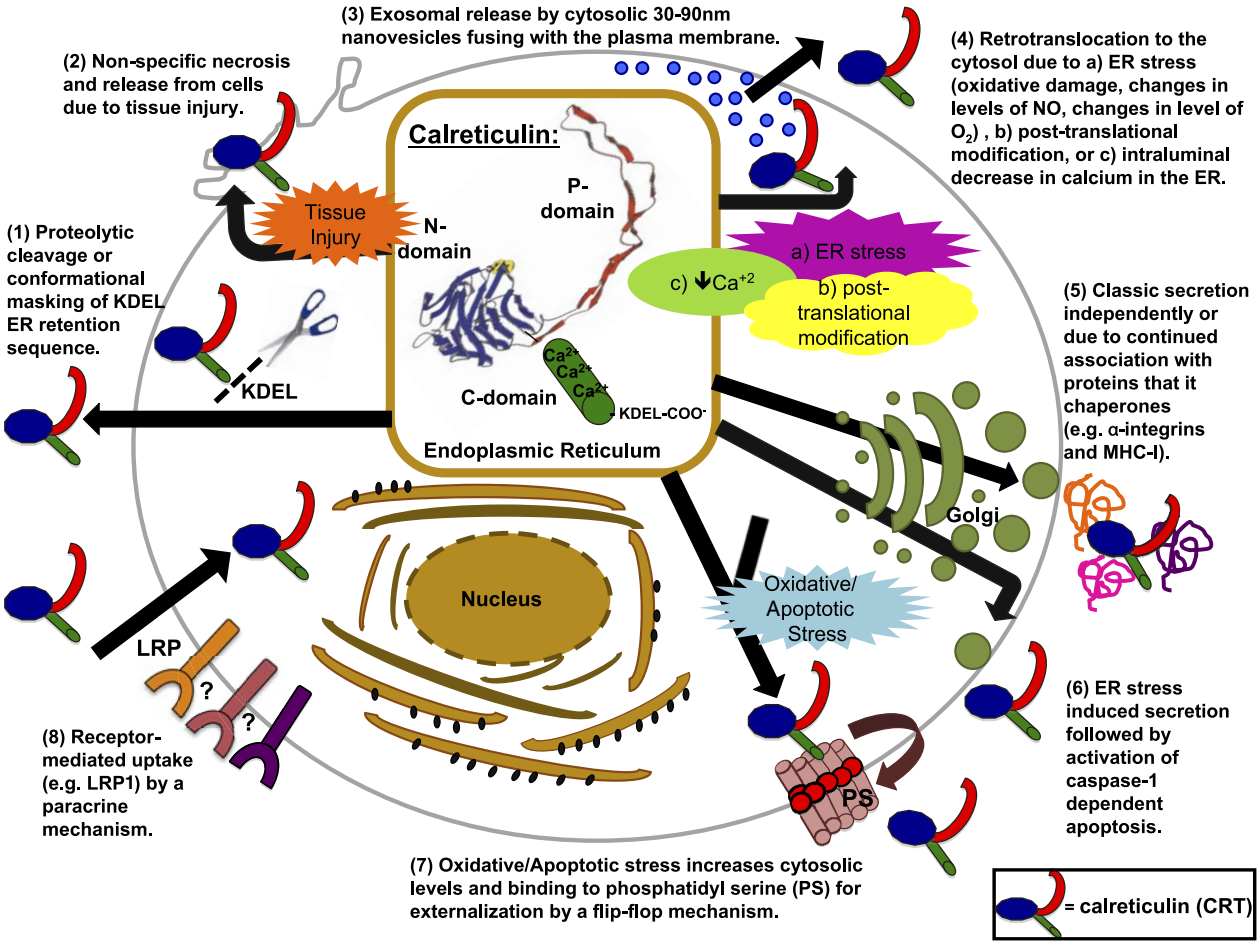

Figure 8. Potential exit routes for CRT from the ER to the cytoplasm, cell surface, and extracellular space.

TABLE 2. *Summary of cell surface and extracellular functions of CRT*

Location	Function	Cell type	References
Cell surface	Receptor for mitogenic activity of B beta chain of fibrinogen	HFL-1 and IMR-90 human fetal fibroblasts	23
	Initiates melanoma cell spreading on laminin	B16 mouse melanoma cells	22
	Binds C1q on apoptotic cell ingestion	Human monocyte-derived macrophages	24
	On the engulfing cell: enhances apoptotic cell ingestion	Human alveolar macrophages	166
	On the apoptotic cell: aids in apoptotic cell clearance	Apoptotic cells: Jurkat T cells, human neutrophils, mouse embryonic fibroblasts	6
	On the tumor cell surface: elicits an immune response against the tumor	CT26 colon carcinoma cell line and MCA205 fibrosarcoma cell line treated with anthracyclin, γ-irradiated, or exposed to UVC light	5, 97
	Forms aggregates on cell surface as a marker for phagocytosis of apoptotic cells in invertebrates	S2 cell line derived from *Drosophila* hemocytes	43
	Mediates focal adhesion disassembly in response to thrombospondin-1	Bovine aortic endothelial cells, mouse embryonic fibroblasts	12–15
	Increases migration in response to thrombospondin-1	Bovine aortic endothelial cells, mouse embryonic fibroblasts	11
	Mediates anoikis resistance by thrombospondin-1	Mouse embryonic fibroblasts	16
	Binds to the shared epitope associated with rheumatoid arthritis	Murine myeloid leukemia cell line, MI, that can be differentiated into macrophages	19
	Binds α2β1 integrin and glycoprotein VI to mediate platelet-collagen interactions	Human platelets	30
Exogenous or ECM	Not described	Found in odontoblasts and pre-dentin ECM	58
	Unknown	Found in fibroblast ECM; vascular wall of atherosclerotic rabbit arteries	Unpublished results (Figs. 2 and 3)
	Increases rate of wound healing, induces granulation tissue, increased closure of epithelial gap	Porcine normal and diabetic wound healing models, murine wound healing model	7
	Increases cell migration	Primary adult human epidermal keratinocytes: CCD 1070SK human foreskin fibroblasts; THP-1 human monocytes; THP-1 human derived differentiated macrophages	7
	Stimulates proliferation	Human primary keratinocytes; human foreskin fibroblasts; human dermal microvascular endothelial cells	7
	Reduced intimal hyperplasia, increased whole blood clotting time	Sites of balloon injury in Sprague-Dawley rats	167

and dying cells. Similar to CRT, heat-shock proteins (Hsps) have been shown to regulate wound healing, motility, migration, apoptosis, and the immune response and are found in various cellular compartments (149–153). For example, Hsp-90α, Hsp-60, Hsp-70, Hsp-47, and Gp96 demonstrate many of these functions, and both the Hsp cytosolic chaperones and the ER chaperones containing the KDEL ER-retention amino acid sequence are found in the external plasma membrane and in the extracellular space. As all chaperones like CRT lack a leader sequence for secretion, their exit routes are largely speculative. However, as discussed, Hsp70 and Hsp90 cytosolic chaperones gain exit from the cell by exosomal release (152, 153).

Interestingly, also similar to CRT, Hsp-60, Hsp-70, and Hsp-90 bind LRP1 for signal transduction. However, at least 6 other signaling receptors, including TLR2/4 and CD40, have been identified (153). Functionally similar to CRT, Hsp-90α binds CD91/LRP1 to trigger macrophages to secrete cytokines, stimulates dendritic cells to express antigen presenting costimulatory molecules, and is involved in wound healing (126, 151, 154).

Here, we present reports showing that CRT at the cell surface and ECM have specific functions distinct from

its intracellular activities. Moreover, that exogenously supplied CRT that might subsequently become surface bound or remain in the extracellular milieu, has significant potential for therapeutic application in both impaired wound healing and cancer therapy. With the more recent discoveries of non-ER functions of CRT playing a significant role in many physiological and pathological processes, mechanisms, pathways/means for the transport of CRT to the cell surface and extracellular space, newly identified signaling coreceptors, a web of downstream intracellular signaling pathways/cascades and crosstalk, and exposure of more non-ER functions, are certain to unfold for this classic ER chaperone. CRT could promote distinct functions depending on whether it signals from intracellular, cell surface, and/or extracellular compartments. Alternatively, certain processes might require signaling from CRT localized to multiple cellular compartments, particularly if CRT is required to chaperone proteins involved in its specific function. FJ

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REFERENCES

1. Bedard, K., Szabo, E., Michalak, M., and Opas, M. (2005) Cellular functions of endoplasmic reticulum chaperones calreticulin, calnexin, and ERp57. *Int. Rev. Cytol.* **245,** 91–121
2. Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I., and Opas, M. (2009) Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem. J.* **417,** 651–666
3. Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K. H., Opas, M., MacLennan, D. H., and Michalak, M. (1999) Calreticulin is essential for cardiac development. *J. Cell Biol.* **144,** 857–868
4. Tesniere, A., Apetoh, L., Ghiringhelli, F., Joza, N., Panaretakis, T., Kepp, O., Schlemmer, F., Zitvogel, L., and Kroemer, G. (2008) Immunogenic cancer cell death: a key-lock paradigm. *Curr. Opin. Immunol.* **20,** 504–511
5. Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J. L., Castedo, M., Mignot, G., Panaretakis, T., Casares, N., Metivier, D., Larochette, N., van Endert, P., Ciccosanti, F., Piacentini, M., Zitvogel, L., and Kroemer, G. (2007) Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat. Med.* **13,** 54–61
6. Gardai, S. J., McPhillips, K. A., Frasch, S. C., Janssen, W. J., Starefeldt, A., Murphy-Ullrich, J. E., Bratton, D. L., Oldenborg, P. A., Michalak, M., and Henson, P. M. (2005) Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* **123,** 321–334
7. Nanney, L. B., Woodrell, C. D., Greives, M. R., Cardwell, N. L., Pollins, A. C., Bancroft, T. A., Chesser, A., Michalak, M., Rahman, M., Siebert, J. W., and Gold, L. I. (2008) Calreticulin enhances porcine wound repair by diverse biological effects. *Am. J. Pathol.* **173,** 610–630
8. Coppolino, M. G., and Dedhar, S. (1999) Ligand-specific, transient interaction between integrins and calreticulin during cell adhesion to extracellular matrix proteins is dependent upon phosphorylation/dephosphorylation events. *Biochem. J.* **340**, 41–50
9. Coppolino, M. G., and Dedhar, S. (2000) Bi-directional signal transduction by integrin receptors. *Int. J. Biochem. Cell Biol.* **32,** 171–188
10. Coppolino, M. G., Woodside, M. J., Demaurex, N., Grinstein, S., St-Arnaud, R., and Dedhar, S. (1997) Calreticulin is essential for integrin-mediated calcium signalling and cell adhesion. *Nature* **386,** 843–847
11. Orr, A. W., Elzie, C. A., Kucik, D. F., and Murphy-Ullrich, J. E. (2003) Thrombospondin signaling through the calreticulin/LDL receptor-related protein co-complex stimulates random and directed cell migration. *J. Cell Sci.* **116,** 2917–2927
12. Orr, A. W., Pallero, M. A., and Murphy-Ullrich, J. E. (2002) Thrombospondin stimulates focal adhesion disassembly through Gi- and phosphoinositide 3-kinase-dependent ERK activation. *J. Biol. Chem.* **277,** 20453–20460
13. Orr, A. W., Pallero, M. A., Xiong, W. C., and Murphy-Ullrich, J. E. (2004) Thrombospondin induces RhoA inactivation through FAK-dependent signaling to stimulate focal adhesion disassembly. *J. Biol. Chem.* **279,** 48983–48992
14. Goicoechea, S., Orr, A. W., Pallero, M. A., Eggleton, P., and Murphy-Ullrich, J. E. (2000) Thrombospondin mediates focal adhesion disassembly through interactions with cell surface calreticulin. *J. Biol. Chem.* **275,** 36358–36368
15. Goicoechea, S., Pallero, M. A., Eggleton, P., Michalak, M., and Murphy-Ullrich, J. E. (2002) The anti-adhesive activity of thrombospondin is mediated by the N-terminal domain of cell surface calreticulin. *J. Biol. Chem.* **277,** 37219–37228
16. Pallero, M. A., Elzie, C. A., Chen, J., Mosher, D. F., and Murphy-Ullrich, J. E. (2008) Thrombospondin 1 binding to calreticulin-LRP1 signals resistance to anoikis. *FASEB J.* **22,** 3968–3979
17. Orr, A. W., Pedraza, C. E., Pallero, M. A., Elzie, C. A., Goicoechea, S., Strickland, D. K., and Murphy-Ullrich, J. E. (2003) Low density lipoprotein receptor-related protein is a calreticulin coreceptor that signals focal adhesion disassembly. *J. Cell Biol.* **161,** 1179–1189
18. Panaretakis, T., Joza, N., Modjtahedi, N., Tesniere, A., Vitale, I., Durchschlag, M., Fimia, G. M., Kepp, O., Piacentini, M., Froehlich, K. U., van Endert, P., Zitvogel, L., Madeo, F., and Kroemer, G. (2008) The co-translocation of ERp57 and calreticulin determines the immunogenicity of cell death. *Cell Death Differ.* **15,** 1499–1509
19. Ling, S., Pi, X., and Holoshitz, J. (2007) The rheumatoid arthritis shared epitope triggers innate immune signaling via cell surface calreticulin. *J. Immunol.* **179,** 6359–6367
20. Arosa, F. A., de Jesus, O., Porto, G., Carmo, A. M., and de Sousa, M. (1999) Calreticulin is expressed on the cell surface of activated human peripheral blood T lymphocytes in association with major histocompatibility complex class I molecules. *J. Biol. Chem.* **274,** 16917–16922
21. Sadasivan, B., Lehner, P. J., Ortmann, B., Spies, T., and Cresswell, P. (1996) Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP. *Immunity* **5,** 103–114
22. White, T. K., Zhu, Q., and Tanzer, M. L. (1995) Cell surface calreticulin is a putative mannoside lectin which triggers mouse melanoma cell spreading. *J. Biol. Chem.* **270,** 15926–15929
23. Gray, A. J., Park, P. W., Broekelmann, T. J., Laurent, G. J., Reeves, J. T., Stenmark, K. R., and Mecham, R. P. (1995) The mitogenic effects of the B beta chain of fibrinogen are mediated through cell surface calreticulin. *J. Biol. Chem.* **270,** 26602–26606
24. Ogden, C. A., deCathelineau, A., Hoffmann, P. R., Bratton, D., Ghebrehiwet, B., Fadok, V. A., and Henson, P. M. (2001) C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J. Exp. Med.* **194,** 781–795

25. Tarr, J., and Eggleton, P. (2005) Immune function of C1q and its modulators CD91 and CD93. *Crit. Rev. Immunol.* **25,** 305–330
26. Dedhar, S. (1994) Novel functions for calreticulin: interaction with integrins and modulation of gene expression? *Trends Biochem. Sci.* **19,** 269–271
27. Coppolino, M., Leung-Hagesteijn, C., Dedhar, S., and Wilkins, J. (1995) Inducible interaction of integrin alpha 2 beta 1 with calreticulin. Dependence on the activation state of the integrin. *J. Biol. Chem.* **270,** 23132–23138
28. Tran, H., Pankov, R., Tran, S. D., Hampton, B., Burgess, W. H., and Yamada, K. M. (2002) Integrin clustering induces kinectin accumulation. *J. Cell Sci.* **115,** 2031–2040
29. Kreis, S., Schonfeld, H. J., Melchior, C., Steiner, B., and Kieffer, N. (2005) The intermediate filament protein vimentin binds specifically to a recombinant integrin alpha2/beta1 cytoplasmic tail complex and co-localizes with native alpha2/beta1 in endothelial cell focal adhesions. *Exp. Cell. Res.* **305,** 110–121
30. Elton, C. M., Smethurst, P. A., Eggleton, P., and Farndale, R. W. (2002) Physical and functional interaction between cell-surface calreticulin and the collagen receptors integrin alpha2beta1 and glycoprotein VI in human platelets. *Thromb. Haemost.* **88,** 648–654
31. Rauch, F., Prud'homme, J., Arabian, A., Dedhar, S., and St-Arnaud, R. (2000) Heart, brain, and body wall defects in mice lacking calreticulin. *Exp. Cell. Res.* **256,** 105–111
32. Frisch, S. M., and Screaton, R. A. (2001) Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13,** 555–562
33. Frisch, S. M., and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J. Cell Biol.* **124,** 619–626
34. Murphy-Ullrich, J. E. (2001) The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptive state? *J. Clin. Invest.* **107,** 785–790
35. Zouq, N. K., Keeble, J. A., Lindsay, J., Valentijn, A. J., Zhang, L., Mills, D., Turner, C. E., Streuli, C. H., and Gilmore, A. P. (2009) FAK engages multiple pathways to maintain survival of fibroblasts and epithelia: differential roles for paxillin and p130Cas. *J. Cell Sci.* **122,** 357–367
36. Gilmore, A. P. (2005) Anoikis. *Cell Death Differ.* **12**(Suppl. 2), 1473–1477
37. Boudreau, N., Sympson, C. J., Werb, Z., and Bissell, M. J. (1995) Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. *Science* **267,** 891–893
38. Valentijn, A. J., Zouq, N., and Gilmore, A. P. (2004) Anoikis. *Biochem. Soc. Trans.* **32,** 421–425
39. Michel, J. B. (2003) Anoikis in the cardiovascular system: known and unknown extracellular mediators. *Arterioscler. Thromb. Vasc. Biol.* **23,** 2146–2154
40. Ortiz-Munoz, G., Houard, X., Martin-Ventura, J. L., Ishida, B. Y., Loyau, S., Rossignol, P., Moreno, J. A., Kane, J. P., Chalkley, R. J., Burlingame, A. L., Michel, J. B., and Meilhac, O. (2009) HDL antielastase activity prevents smooth muscle cell anoikis, a potential new antiatherogenic property. *FASEB J.* **23,** 3129–3139
41. Horowitz, J. C., Rogers, D. S., Sharma, V., Vittal, R., White, E. S., Cui, Z., and Thannickal, V. J. (2007) Combinatorial activation of FAK and AKT by transforming growth factor-beta1 confers an anoikis-resistant phenotype to myofibroblasts. *Cell. Signal.* **19,** 761–771
42. Subramanian, S., Tsai, R., and Discher, D. E. (2006) The 'metabolon,' CD47, and the 'phagocytic synapse': molecular co-localization and species divergence. *Transfus. Clin. Biol.* **13,** 31–38
43. Kuraishi, T., Manaka, J., Kono, M., Ishii, H., Yamamoto, N., Koizumi, K., Shiratsuchi, A., Lee, B. L., Higashida, H., and Nakanishi, Y. (2007) Identification of calreticulin as a marker for phagocytosis of apoptotic cells in *Drosophila*. *Exp. Cell. Res.* **313,** 500–510
44. Park, Y. J., Liu, G., Lorne, E. F., Zhao, X., Wang, J., Tsuruta, Y., Zmijewski, J., and Abraham, E. (2008) PAI-1 inhibits neutrophil efferocytosis. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 11784–11789
45. Gaipl, U. S., Sheriff, A., Franz, S., Munoz, L. E., Voll, R. E., Kalden, J. R., and Herrmann, M. (2006) Inefficient clearance of dying cells and autoreactivity. *Curr. Top. Microbiol. Immunol.* **305,** 161–176
46. Hanayama, R., Tanaka, M., Miyasaka, K., Aozasa, K., Koike, M., Uchiyama, Y., and Nagata, S. (2004) Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* **304,** 1147–1150
47. Henson, P. M., and Hume, D. A. (2006) Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol.* **27,** 244–250
48. Eggleton, P., and Llewellyn, D. H. (1999) Pathophysiological roles of calreticulin in autoimmune disease. *Scand. J. Immunol.* **49,** 466–473
49. Honore, C., Hummelshoj, T., Hansen, B. E., Madsen, H. O., Eggleton, P., and Garred, P. (2007) The innate immune component ficolin 3 (Hakata antigen) mediates the clearance of late apoptotic cells. *Arthritis Rheum.* **56,** 1598–1607
50. Eggleton, P., Lieu, T. S., Zappi, E. G., Sastry, K., Coburn, J., Zaner, K. S., Sontheimer, R. D., Capra, J. D., Ghebrehiwet, B., and Tauber, A. I. (1994) Calreticulin is released from activated neutrophils and binds to C1q and mannan-binding protein. *Clin. Immunol. Immunopathol.* **72,** 405–409
51. Donnelly, S., Roake, W., Brown, S., Young, P., Naik, H., Wordsworth, P., Isenberg, D. A., Reid, K. B., and Eggleton, P. (2006) Impaired recognition of apoptotic neutrophils by the C1q/calreticulin and CD91 pathway in systemic lupus erythematosus. *Arthritis Rheum.* **54,** 1543–1556
52. Duus, K., Hansen, P. R., and Houen, G. (2008) Interaction of calreticulin with amyloid beta peptide 1–42. *Protein Pept. Lett.* **15,** 103–107
53. Aguilar, L., Ramirez, G., Valck, C., Molina, M. C., Rojas, A., Schwaeble, W., Ferreira, V., and Ferreira, A. (2005) F(ab′)2 antibody fragments against *Trypanosoma cruzi* calreticulin inhibit its interaction with the first component of human complement. *Biol. Res.* **38,** 187–195
54. Ribeiro, C. H., Lopez, N. C., Ramirez, G. A., Valck, C. E., Molina, M. C., Aguilar, L., Rodriguez, M., Maldonado, I., Martinez, R., Gonzalez, C., Troncoso, R., Lavandero, S., Gingras, A. R., Schwaeble, W., and Ferreira, A. (2009) *Trypanosoma cruzi* calreticulin: a possible role in Chagas' disease autoimmunity. *Mol. Immunol.* **46,** 1092–1099
55. Zhu, Q., Zelinka, P., White, T., and Tanzer, M. L. (1997) Calreticulin-integrin bidirectional signaling complex. *Biochem. Biophys. Res. Commun.* **232,** 354–358
56. Panaretakis, T., Kepp, O., Brockmeier, U., Tesniere, A., Bjorklund, A. C., Chapman, D. C., Durchschlag, M., Joza, N., Pierron, G., van Endert, P., Yuan, J., Zitvogel, L., Madeo, F., Williams, D. B., and Kroemer, G. (2009) Mechanisms of pre-apoptotic calreticulin exposure in immunogenic cell death. *EMBO J.* **28,** 578–590
57. Vance, B. A., Harley, P. H., Backlund, P. S., Ward, Y., Phelps, T. L., and Gress, R. E. (2005) Human CD69 associates with an N-terminal fragment of calreticulin at the cell surface. *Arch. Biochem. Biophys.* **438,** 11–20
58. Somogyi, E., Petersson, U., Hultenby, K., and Wendel, M. (2003) Calreticulin—an endoplasmic reticulum protein with calcium-binding activity is also found in the extracellular matrix. *Matrix Biol.* **22,** 179–191
59. Kypreou, K. P., Kavvadas, P., Karamessinis, P., Peroulis, M., Alberti, A., Sideras, P., Psarras, S., Capetanaki, Y., Politis, P. K., and Charonis, A. S. (2008) Altered expression of calreticulin during the development of fibrosis. *Proteomics* **8,** 2407–2419
60. McDonnell, J. M., Jones, G. E., White, T. K., and Tanzer, M. L. (1996) Calreticulin binding affinity for glycosylated laminin. *J. Biol. Chem.* **271,** 7891–7894
61. Peerschke, E., and Ghebrehiwet, B. (1990) Platelet C1q receptor interactions with collagen- and C1q-coated surfaces. *J. Immunol.* **145,** 2984–2988
62. Wu, M., Massaeli, H., Durston, M., and Mesaeli, N. (2007) Differential expression and activity of matrix metalloproteinase-2 and -9 in the calreticulin deficient cells. *Matrix Biol.* **26,** 463–472
63. Gailit, J., and Clark, R. A. (1994) Wound repair in the context of extracellular matrix. *Curr. Opin. Cell Biol.* **6,** 717–725
64. Singer, A. J., and Clark, R. A. (1999) Cutaneous wound healing. *N. Engl. J. Med.* **341,** 738–746
65. Mesaeli, N., and Phillipson, C. (2004) Impaired p53 expression, function, and nuclear localization in calreticulin-deficient cells. *Mol. Biol. Cell* **15,** 1862–1870

66. Jalali, S., Aghasi, M., Yeganeh, B., and Mesaeli, N. (2008) Calreticulin regulates insulin receptor expression and its downstream PI3 Kinase/Akt signalling pathway. *Biochim. Biophys. Acta* **1783,** 2344–2351
67. Yoon, G. S., Lee, H., Jung, Y., Yu, E., Moon, H. B., Song, K., and Lee, I. (2000) Nuclear matrix of calreticulin in hepatocellular carcinoma. *Cancer Res.* **60,** 1117–1120
68. Tutuncu, L., Stein, P., Ord, T. S., Jorgez, C. J., and Williams, C. J. (2004) Calreticulin on the mouse egg surface mediates transmembrane signaling linked to cell cycle resumption. *Dev. Biol.* **270,** 246–260
69. Sezestakowska, D., Szabo, E., Eggleton, P., Opas, M., and Young, P. (2006) 7th International Workshop on Calreticulin, Niagara Falls, Canada: the complexities of calreticulin from folding to disease prevention and therapeutic application. *Calcium Binding Proteins* **1,** 135–139
70. Yao, L., Pike, S. E., Pittaluga, S., Cherney, B., Gupta, G., Jaffe, E. S., and Tosato, G. (2002) Anti-tumor activities of the angiogenesis inhibitors interferon-inducible protein-10 and the calreticulin fragment vasostatin. *Cancer Immunol. Immunother.* **51,** 358–366
71. Cai, K. X., Tse, L. Y., Leung, C., Tam, P. K., Xu, R., and Sham, M. H. (2008) Suppression of lung tumor growth and metastasis in mice by adeno-associated virus-mediated expression of vasostatin. *Clin. Cancer Res.* **14,** 939–949
72. Jazowiecka-Rakus, J., Jarosz, M., Kozlowska, D., Sochanik, A., and Szala, S. (2007) Combination of vasostatin and cyclophosphamide in the therapy of murine melanoma tumors. *Acta Biochim. Pol.* **54,** 125–133
73. Yao, L., Pike, S. E., and Tosato, G. (2002) Laminin binding to the calreticulin fragment vasostatin regulates endothelial cell function. *J. Leukoc. Biol.* **71,** 47–53
74. Pike, S. E., Yao, L., Jones, K. D., Cherney, B., Appella, E., Sakaguchi, K., Nakhasi, H., Teruya-Feldstein, J., Wirth, P., Gupta, G., and Tosato, G. (1998) Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. *J. Exp. Med.* **188,** 2349–2356
75. Lange-Asschenfeldt, B., Velasco, P., Streit, M., Hawighorst, T., Pike, S. E., Tosato, G., and Detmar, M. (2001) The angiogenesis inhibitor vasostatin does not impair wound healing at tumor-inhibiting doses. *J. Invest. Dermatol.* **117,** 1036–1041
76. Li, W. Y., Huang, E. Y., Dudas, M., Kaartinen, V., Warburton, D., and Tuan, T. L. (2006) Transforming growth factor-β3 affects plasminogen activator inhibitor-1 expression in fetal mice and modulates fibroblast-mediated collagen gel contraction. *Wound Repair Regen.* **14,** 516–525
77. Wu, L., Siddiqui, A., Morris, D. E., Cox, D. A., Roth, S. I., and Mustoe, T. A. (1997) Transforming growth factor β3 (TGF β3) accelerates wound healing without alteration of scar prominence. Histologic and competitive reverse-transcription-polymerase chain reaction studies. *Arch. Surg.* **132,** 753–760
78. Ferguson, M. W., Duncan, J., Bond, J., Bush, J., Durani, P., So, K., Taylor, L., Chantrey, J., Mason, T., James, G., Laverty, H., Occleston, N. L., Sattar, A., Ludlow, A., and O'Kane, S. (2009) Prophylactic administration of avotermin for improvement of skin scarring: three double-blind, placebo-controlled, phase I/II studies. *Lancet* **373,** 1264–1274
79. Schor, S. L., Ellis, I. R., Harada, K., Motegi, K., Anderson, A. R., Chaplain, M. A., Keatch, R. P., and Schor, A. M. (2006) A novel 'sandwich' assay for quantifying chemo-regulated cell migration within 3-dimensional matrices: wound healing cytokines exhibit distinct motogenic activities compared to the transmembrane assay. *Cell. Motil. Cytoskeleton* **63,** 287–300
80. Ellis, I. R., Banyard, J., and Schor, S. L. (1999) Motogenic and biosynthetic response of adult skin fibroblasts to TGF-β isoforms (-1, -2 and -3) determined by 'tissue response unit': role of cell density and substratum. *Cell Biol. Int.* **23,** 593–602
81. Bandyopadhyay, B., Fan, J., Guan, S., Li, Y., Chen, M., Woodley, D. T., and Li, W. (2006) A "traffic control" role for TGFβ3: orchestrating dermal and epidermal cell motility during wound healing. *J. Cell Biol.* **172,** 1093–1105
82. Leonard, C. M., Fuld, H. M., Frenz, D. A., Downie, S. A., Massague, J., and Newman, S. A. (1991) Role of transforming growth factor-beta in chondrogenic pattern formation in the embryonic limb: stimulation of mesenchymal condensation and fibronectin gene expression by exogenenous TGF-β and evidence for endogenous TGF-β-like activity. *Dev. Biol.* **145,** 99–109
83. Papp, S., Fadel, M. P., Kim, H., McCulloch, C. A., and Opas, M. (2007) Calreticulin affects fibronectin-based cell-substratum adhesion via the regulation of c-Src activity. *J. Biol. Chem.* **282,** 16585–16598
84. Papp, S., Szabo, E., Kim, H., McCulloch, C.A., and Opas, M. (2008) Kinase-dependent adhesion to fibronectin: regulation by calreticulin. *Exp. Cell Res.* **314,** 1313
85. Agah, A., Kyriakides, T. R., Letrondo, N., Bjorkblom, B., and Bornstein, P. (2004) Thrombospondin 2 levels are increased in aged mice: consequences for cutaneous wound healing and angiogenesis. *Matrix. Biol.* **22,** 539–547
86. Raugi, G. J., Olerud, J. E., and Gown, A. M. (1987) Thrombospondin in early human wound tissue. *J. Invest. Dermatol.* **89,** 551–554
87. Reilly, D., Larkin, D., Devocelle, M., Fitzgerald, D. J., and Moran, N. (2004) Calreticulin-independent regulation of the platelet integrin αIIbβ3 by the KVGFFKR αIIb-cytoplasmic motif. *Platelets* **15,** 43–54
88. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouck, N. (1998) Thrombospondin-1 is a major activator of TGF-β1 in vivo. *Cell* **93,** 1159–1170
89. Ahmed, Z., and Bicknell, R. (2009) Angiogenic signalling pathways. *Methods Mol. Biol.* **467,** 3–24
90. Nishi, H., Nakada, T., Hokamura, M., Osakabe, Y., Itokazu, O., Huang, L. E., and Isaka, K. (2004) Hypoxia-inducible factor-1 transactivates transforming growth factor-β3 in trophoblast. *Endocrinology* **145,** 4113–4118
91. Brem, H., and Tomic-Canic, M. (2007) Cellular and molecular basis of wound healing in diabetes. *J. Clin. Invest.* **117,** 1219–1222
92. Ochoa, O., Torres, F. M., and Shireman, P. K. (2007) Chemokines and diabetic wound healing. *Vascular* **15,** 350–355
93. Totary-Jain, H., Naveh-Many, T., Riahi, Y., Kaiser, N., Eckel, J., and Sasson, S. (2005) Calreticulin destabilizes glucose transporter-1 mRNA in vascular endothelial and smooth muscle cells under high-glucose conditions. *Circ. Res.* **97,** 1001–1008
94. Obeid, M., Panaretakis, T., Tesniere, A., Joza, N., Tufi, R., Apetoh, L., Ghiringhelli, F., Zitvogel, L., and Kroemer, G. (2007) Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from "silent" to immunogenic. *Cancer Res.* **67,** 7941–7944
95. Tufi, R., Panaretakis, T., Bianchi, K., Criollo, A., Fazi, B., Di Sano, F., Tesniere, A., Kepp, O., Paterlini-Brechot, P., Zitvogel, L., Piacentini, M., Szabadkai, G., and Kroemer, G. (2008) Reduction of endoplasmic reticulum Ca^{2+} levels favors plasma membrane surface exposure of calreticulin. *Cell Death Differ.* **15,** 274–282
96. Chaput, N., De Botton, S., Obeid, M., Apetoh, L., Ghiringhelli, F., Panaretakis, T., Flament, C., Zitvogel, L., and Kroemer, G. (2007) Molecular determinants of immunogenic cell death: surface exposure of calreticulin makes the difference. *J. Mol. Med.* **85,** 1069–1076
97. Obeid, M., Panaretakis, T., Joza, N., Tufi, R., Tesniere, A., van Endert, P., Zitvogel, L., and Kroemer, G. (2007) Calreticulin exposure is required for the immunogenicity of γ-irradiation and UVC light-induced apoptosis. *Cell Death Differ.* **14,** 1848–1850
98. Zhang, K., and Kaufman, R. J. (2008) Identification and characterization of endoplasmic reticulum stress-induced apoptosis in vivo. *Methods Enzymol.* **442,** 395–419
99. Obeid, M., Tesniere, A., Panaretakis, T., Tufi, R., Joza, N., van Endert, P., Ghiringhelli, F., Apetoh, L., Chaput, N., Flament, C., Ullrich, E., de Botton, S., Zitvogel, L., and Kroemer, G. (2007) Ecto-calreticulin in immunogenic chemotherapy. *Immunol. Rev.* **220,** 22–34
100. Viaud, S., Terme, M., Flament, C., Taieb, J., Andre, F., Novault, S., Escudier, B., Robert, C., Caillat-Zucman, S., Tursz, T., Zitvogel, L., and Chaput, N. (2009) Dendritic cell-derived exosomes promote natural killer cell activation and proliferation: a role for NKG2D ligands and IL-15Ralpha. *PLoS One* **4,** e4942
101. Zitvogel, L., Apetoh, L., Ghiringhelli, F., Andre, F., Tesniere, A., and Kroemer, G. (2008) The anticancer immune response: indispensable for therapeutic success? *J. Clin. Invest.* **118,** 1991–2001

102. Hsieh, C. J., Kim, T. W., Hung, C. F., Juang, J., Moniz, M., Boyd, D. A., He, L., Chen, P. J., Chen, C. H., and Wu, T. C. (2004) Enhancement of vaccinia vaccine potency by linkage of tumor antigen gene to gene encoding calreticulin. *Vaccine* **22,** 3993–4001
103. Cheng, W. F., Hung, C. F., Chen, C. A., Lee, C. N., Su, Y. N., Chai, C. Y., Boyd, D. A., Hsieh, C. Y., and Wu, T. C. (2005) Characterization of DNA vaccines encoding the domains of calreticulin for their ability to elicit tumor-specific immunity and antiangiogenesis. *Vaccine* **23,** 3864–3874
104. Cheng, W. F., Lee, C. N., Su, Y. N., Chai, C. Y., Chang, M. C., Polo, J. M., Hung, C. F., Wu, T. C., Hsieh, C. Y., and Chen, C. A. (2006) Sindbis virus replicon particles encoding calreticulin linked to a tumor antigen generate long-term tumor-specific immunity. *Cancer Gene Ther.* **13,** 873–885
105. Zitvogel, L., Casares, N., Pequignot, M. O., Chaput, N., Albert, M. L., and Kroemer, G. (2004) Immune response against dying tumor cells. *Adv. Immunol.* **84,** 131–179
106. Franz, S., Herrmann, K., Furnrohr, B. G., Sheriff, A., Frey, B., Gaipl, U. S., Voll, R. E., Kalden, J. R., Jack, H. M., and Herrmann, M. (2007) After shrinkage apoptotic cells expose internal membrane-derived epitopes on their plasma membranes. *Cell Death Differ.* **14,** 733–742
107. Wearsch, P. A., and Cresswell, P. (2008) The quality control of MHC class I peptide loading. *Curr. Opin. Cell Biol.* **20,** 624–631
108. Zhang, Y., Kozlov, G., Pocanschi, C. L., Brockmeier, U., Ireland, B. S., Maattanen, P., Howe, C., Elliott, T., Gehring, K., and Williams, D. B. (2009) ERp57 does not require interactions with calnexin and calreticulin to promote assembly of class I histocompatibility molecules, and it enhances peptide loading independently of its redox activity. *J. Biol. Chem.* **284,** 10160–10173
109. Raghavan, M., Del Cid, N., Rizvi, S. M., and Peters, L. R. (2008) MHC class I assembly: out and about. *Trends Immunol.* **29,** 436–443
110. Apetoh, L., Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Piacentini, M., Kroemer, G., and Zitvogel, L. (2007) Immunogenic chemotherapy: discovery of a critical protein through proteomic analyses of tumor cells. *Cancer Genomics Proteomics* **4,** 65–70
111. Kepp, O., Tesniere, A., Zitvogel, L., and Kroemer, G. (2009) The immunogenicity of tumor cell death. *Curr. Opin. Oncol.* **21,** 71–76
112. Kepp, O., Tesniere, A., Schlemmer, F., Michaud, M., Senovilla, L., Zitvogel, L., and Kroemer, G. (2009) Immunogenic cell death modalities and their impact on cancer treatment. *Apoptosis* **14,** 364–375
113. Zeng, G., Aldridge, M. E., Tian, X., Seiler, D., Zhang, X., Jin, Y., Rao, J., Li, W., Chen, D., Langford, M. P., Duggan, C., Belldegrun, A. S., and Dubinett, S. M. (2006) Dendritic cell surface calreticulin is a receptor for NY-ESO-1: direct interactions between tumor-associated antigen and the innate immune system. *J. Immunol.* **177,** 3582–3589
114. Kepp, O., Senovilla, L., Galluzzi, L., Panaretakis, T., Tesniere, A., Schlemmer, F., Madeo, F., Zitvogel, L., and Kroemer, G. (2009) Viral subversion of immunogenic cell death. *Cell Cycle* **8,** 860–869
115. Chignard, N., Shang, S., Wang, H., Marrero, J., Brechot, C., Hanash, S., and Beretta, L. (2006) Cleavage of endoplasmic reticulum proteins in hepatocellular carcinoma: Detection of generated fragments in patient sera. *Gastroenterology* **130,** 2010–2022
116. Neckers, L. (2006) Chaperoning oncogenes: Hsp90 as a target of geldanamycin. In *Molecular Chaperones in Health and Disease. Handbook in Experimental Pharmacology*, Vol. 172 (Gaestel, M., ed) pp. 259–277, Springer, Berlin
117. Calderwood, S. K., Khaleque, M. A., Sawyer, D. B., and Ciocca, D. R. (2006) Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem. Sci.* **31,** 164–172
118. Gehrmann, M., Radons, J., Molls, M., and Multhoff, G. (2008) The therapeutic implications of clinically applied modifiers of heat shock protein 70 (Hsp70) expression by tumor cells. *Cell Stress Chaperones* **13,** 1–10
119. Forslow, A., Liu, Z., and Sundqvist, K. G. (2007) Receptor communication within the lymphocyte plasma membrane: a role for the thrombospondin family of matricellular proteins. *Cell. Mol. Life Sci.* **64,** 66–76
120. Li, Z., He, L., Wilson, K., and Roberts, D. (2001) Thrombospondin-1 inhibits TCR-mediated T lymphocyte early activation. *J. Immunol.* **166,** 2427–2436
121. Petrovski, G., Zahuczky, G., Majai, G., and Fesus, L. (2007) Phagocytosis of cells dying through autophagy evokes a pro-inflammatory response in macrophages. *Autophagy* **3,** 509–511
122. Petrovski, G., Zahuczky, G., Katona, K., Vereb, G., Martinet, W., Nemes, Z., Bursch, W., and Fesus, L. (2007) Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. *Cell Death Differ.* **14,** 1117–1128
123. Sipione, S., Ewen, C., Shostak, I., Michalak, M., and Bleackley, R. C. (2005) Impaired cytolytic activity in calreticulin-deficient CTLs. *J. Immunol.* **174,** 3212–3219
124. Obeid, M. (2009) Anticancer activity of targeted proapoptotic peptides and chemotherapy is highly improved by targeted cell surface calreticulin-inducer peptides. *Mol. Cancer Ther.* **8,** 2693–2707
125. Corbett, E. F., Michalak, K. M., Oikawa, K., Johnson, S., Campbell, I. D., Eggleton, P., Kay, C., and Michalak, M. (2000) The conformation of calreticulin is influenced by the endoplasmic reticulum luminal environment. *J. Biol. Chem.* **275,** 27177–27185
126. Basu, S., Binder, R. J., Suto, R., Anderson, K. M., and Srivastava, P. K. (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-κB pathway. *Int. Immunol.* **12,** 1539–1546
127. Cheng, C. F., Fan, J., Fedesco, M., Guan, S., Li, Y., Bandyopadhyay, B., Bright, A. M., Yerushalmi, D., Liang, M., Chen, M., Han, Y. P., Woodley, D. T., and Li, W. (2008) Transforming growth factor alpha (TGFα)-stimulated secretion of HSP90α: using the receptor LRP-1/CD91 to promote human skin cell migration against a TGFβ-rich environment during wound healing. *Mol. Cell. Biol.* **28,** 3344–3358
128. Hegmans, J. P., Gerber, P. J., and Lambrecht, B. N. (2008) *Exosomes Methods Mol. Biol.* **484,** 97–109
129. Yu, X., Harris, S. L., and Levine, A. J. (2006) The regulation of exosome secretion: a novel function of the p53 protein. *Cancer Res.* **66,** 4795–4801
130. Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W., and Geuze, H. J. (2000) Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci.* **113,** 3365–3374
131. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., and Raposo, G. (2002) The biogenesis and functions of exosomes. *Traffic* **3,** 321–330
132. Fevrier, B., and Raposo, G. (2004) Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr. Opin. Cell Biol.* **16,** 415–421
133. Afshar, N., Black, B. E., and Paschal, B. M. (2005) Retrotranslocation of the chaperone calreticulin from the endoplasmic reticulum lumen to the cytosol. *Mol. Cell. Biol.* **25,** 8844–8853
134. Rabek, J. P., Boylston, W. H., 3rd, and Papaconstantinou, J. (2003) Carbonylation of ER chaperone proteins in aged mouse liver. *Biochem. Biophys. Res. Commun.* **305**, 566–572
135. Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E., and Mori, M. (2001) Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10845–10850
136. Xu, F. F., Liu, X. H., and Zhu, X. M. (2008) [Calreticulin upregulation induced by hypoxic preconditioning relieves oxidative stress injury in rat cardiomyocytes.] *Sheng Li Xue Bao* **60,** 29–37
137. Caramelo, J. J., and Parodi, A. J. (2008) Getting in and out from calnexin/calreticulin cycles. *J. Biol. Chem.* **283,** 10221–10225
138. Shaffer, K. L., Sharma, A., Snapp, E. L., and Hegde, R. S. (2005) Regulation of protein compartmentalization expands the diversity of protein function. *Dev. Cell.* **9,** 545–554
139. Decca, M. B., Carpio, M. A., Bosc, C., Galiano, M. R., Job, D., Andrieux, A., and Hallak, M. E. (2007) Post-translational arginylation of calreticulin: a new isospecies of calreticulin component of stress granules. *J. Biol. Chem.* **282,** 8237–8245
140. Keller, M., Ruegg, A., Werner, S., and Beer, H. D. (2008) Active caspase-1 is a regulator of unconventional protein secretion. *Cell* **132,** 818–831

141. Freikman, I., Amer, J., Ringel, I., and Fibach, E. (2009) A flow cytometry approach for quantitative analysis of cellular phosphatidylserine distribution and shedding. *Anal. Biochem.* **393,** 111–116
142. Mirnikjoo, B., Balasubramanian, K., and Schroit, A. J. (2009) Suicidal membrane repair regulates PS externalization during apoptosis. *J. Biol. Chem.* **284,** 22512–22516
143. Patel, J. M., Li, Y. D., Zhang, J., Gelband, C. H., Raizada, M. K., and Block, E. R. (1999) Increased expression of calreticulin is linked to ANG IV-mediated activation of lung endothelial NOS. *Am. J. Physiol. Lung Physiol.* **277,** L794–L801
144. Arispe, N., Doh, M., Simakova, O., Kurganov, B., and De Maio, A. (2004) Hsc70 and Hsp70 interact with phosphatidylserine on the surface of PC12 cells resulting in a decrease of viability. *FASEB J.* **18,** 1636–1645
145. Muller-Taubenberger, A., Lupas, A. N., Li, H., Ecke, M., Simmeth, E., and Gerisch, G. (2001) Calreticulin and calnexin in the endoplasmic reticulum are important for phagocytosis. *EMBO J.* **20,** 6772–6782
146. Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J., and Desjardins, M. (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. *Cell* **110,** 119–131
147. Elliott, T., and Williams, A. (2005) The optimization of peptide cargo bound to MHC class I molecules by the peptide-loading complex. *Immunol. Rev.* **207,** 89–99
148. Madeo, F., Durchschlag, M., Kepp, O., Panaretakis, T., Zitvogel, L., Frohlich, K. U., and Kroemer, G. (2009) Phylogenetic conservation of the preapoptotic calreticulin exposure pathway from yeast to mammals. *Cell Cycle* **8,** 639–642
149. Macario, A. J., and De Macario, E. C. (2007) Chaperonopathies by defect, excess, or mistake. *Ann. N. Y. Acad. Sci.* **1113,** 178–191
150. Tsutsumi, S., and Neckers, L. (2007) Extracellular heat shock protein 90: A role for a molecular chaperone in cell motility and cancer metastasis. *Cancer Sci.* **98,** 1536–1539
151. Li, W., Li, Y., Guan, S., Fan, J., Cheng, C. F., Bright, A. M., Chinn, C., Chen, M., and Woodley, D. T. (2007) Extracellular heat shock protein-90α: linking hypoxia to skin cell motility and wound healing. *EMBO J.* **26,** 1221–1233
152. Multhoff, G. (2007) Heat shock protein 70 (Hsp70): membrane location, export and immunological relevance. *Methods* **43,** 229–237
153. Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., and Garrido, C. (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. *J. Leukoc. Biol.* **81,** 15–27
154. Basu, S., Binder, R. J., Ramalingam, T., and Srivastava, P. K. (2001) CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* **14,** 303–313
155. Datta, G., Chaddha, M., Garber, D. W., Chung, B. H., Tytler, E. M., Dashti, N., Bradley, W. A., Gianturco, S. H., and Anantharamaiah, G. M. (2000) The receptor binding domain of apolipoprotein E, linked to a model class A amphipathic helix, enhances internalization and degradation of LDL by fibroblasts. *Biochemistry* **39,** 213–220
156. Galiano, R. D., Michaels, J., Dobryansky, M., Levine, J. P., and Gurtner, G. C. (2004) Quantitative and reproducible murine model of excisional wound healing. *Wound Repair Regen.* **12,** 485–492
157. Ito, M., Yang, Z., Andl, T., Cui, C., Kim, N., Millar, S. E., and Cotsarelis, G. (2007) Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature* **447,** 316–320
158. Kageyama, S., Isono, T., Matsuda, S., Ushio, Y., Terai, A., Arai, Y., Kawakita, M., Okada, Y., and Yoshiki, T. (2009) Urinary calreticulin in the diagnosis of bladder uroethelial carcinoma. *Int. J. Urol.* **16,** 481–486
159. Goeb, V., Thomas-L'Otellier, M., Daveau, R., Charlionet, R., Fardellone, P., Le Loet, X., Tron, F., Gilbert, D., and Vittecoq, O. (2009) Candidate autoantigens identified by mass spectrometry in early rheumatoid arthritis are chaperones and citrullinated glycolytic enzymes. *Arthritis Res. Ther.* **11,** R38
160. Sanchez, D., Palova-Jelinkova, L., Felsberg, J., Simsova, M., Pekarikova, A., Pecharova, B., Swoboda, I., Mothes, T., Mulder, C. J., Benes, Z., Tlasklova-Hogenova, H., and Tuckova, L. (2008) Anti-calreticulin immunoglobulin A (IgA) antibodies in refractory celiac disease. *Clin. Exp. Immunol.* **153,** 351–359
161. Gu, V. Y., Wong, M. H., Stevenson, J. L., Crawford, K. E., Brennecke, S. P., and Gude, N. M. (2008) Calreticulin in human pregnancy and pre-eclampsia. *Mol. Hum. Reprod.* **14,** 309–315
162. Watanabe, L., Ohira, H., Orikasa, H., Saito, K., Kanno, K., Shioya, Y., Obara, K., and Sato, Y. (2006) Anti-calreticulin antibodies in patients with inflammatory bowel disease. *Fukushima J. Med. Sci.* **52,** 1–11
163. Kageyama, S., Isono, H., Wakabayashi, Y., Okada, Y., Kontani, K., Yoshimura, K., Terai, A., Arai, Y., and Yoshiki, T. (2004) Identification by proteomic analysis of calreticulin as a marker for bladder cancer and evaluation of the diagnostic accuracy of its detection in urine. *Clin. Chem.* **50,** 857–866
164. Taguchi, J., Fujino, Y., Tsujioka, Y., Takahashi, M., Tsuboi, Y., Wada, I., and Yamada, T. (2000) Different expression of calreticulin and immunoglobulin binding protein in Alzheimer's disease brain. *Acta Neuropathol.* **100,** 153–160
165. Kreisel, W., Siegel, A., Bahler, A., Spamer, C., Schiltz, E., Kist, M., Seilnacht, G., Klein, R., Berg, P. A., and Heilmann, C. (1999) High prevalence of antibodies to calreticulin of the IgA class in primary biliary cirrhosis: a possible role of gut-derived bacterial antigens in its aetiology? *Scand. J. Gastroenterol.* **34,** 623–628
166. Vandivier, R. W., Ogden, C. A., Fadok, V. A., Hoffmann, P. R., Brown, K. K., Botto, M., Walport, M. J., Fisher, J. H., Henson, P. M., and Greene, K. E. (2002) Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J. Immunol.* **169,** 3978–3986
167. Dai, E., Stewart, M., Ritchie, B., Mesaeli, N., Raha, S., Kolodziejczyk, D., Hobman, M. L., Liu, L. Y., Etches, W., Nation, N., Michalak, M., and Lucas, A. (1997) Calreticulin, a potential vascular regulatory protein, reduces intimal hyperplasia after arterial injury. *Arterioscler. Thromb. Vasc. Biol.* **17,** 2359–2368

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