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Calreticulin Regulates Tgf-ß Stimulated Extracellular Matrix Production

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CALRETICULIN REGULATES TGF-β STIMULATED EXTRACELLULAR MATRIX PRODUCTION

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MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Calreticulin (CRT) is an ER chaperone and regulator of Ca^{2+} signaling which is increased following ER stress and in fibrotic and vascular fibroproliferative disorders. Previously, we demonstrated that ER CRT regulates type I collagen transcript, trafficking, secretion, and processing into the extracellular matrix (ECM). These studies investigated the role of CRT in regulating ECM production through control TGF-β dependent signaling pathways. Our studies show that CRT -/- mouse embryonic fibroblasts (MEFs), rat lung fibroblasts, and human IPF lung fibroblasts with siRNA knockdown of CRT had impaired production of type I collagen and fibronectin when stimulated with TGF-β. Similarly, knockdown of CRT in CRT floxed vascular smooth muscle cells (VSMCs) with Cre-recombinase-IRES-GFP significantly impaired TGF-β stimulated type I collagen production. We showed that CRT regulates TGF-β stimulated ECM production through control of ER Ca^{2+} release and downstream calcineurin dependent NFAT signaling as pretreatment of MEFs or VSMCs with 11R-VIVIT significantly attenuated TGF-β stimulated ECM production. Artificial induction of ER stress by tunicamycin could not rescue the inability of CRT -/- MEFs to induce ECM production upon TGF-β treatment, suggesting that CRT may be a critical component of ER stress induced fibrotic and vascular fibroproliferative disease. To address the role of CRT in vascular fibroproliferative disease, we used a carotid artery ligation model of neointimal hyperplasia in CRT floxed mice. We showed that CRT is strongly upregulated following

carotid artery ligation induced vascular injury and that we were able to knockdown CRT to 50% of control levels using ultrasound (US) mediated delivery of Cre-recombinase-IRES-GFP plasmid with microbubbles (MB). Furthermore, knockdown of CRT significantly reduced neointimal area and the neointima-to-media ratio as compared to mice receiving GFP with US/MB or mice receiving Cre-recombinase-IRES-GFP with MB but lacking US. Cre-recombinase-IRES-GFP mediated knockdown of neointimal CRT reduced collagen production by approximately 25%. Together, these studies establish that CRT is a critical component of ER stress induced fibrotic and vascular fibroproliferative disease. In vitro studies show that CRT regulates TGF-β stimulated ECM production through control of ER Ca^{2+} release and downstream calcineurin/NFAT signaling.

Keywords: Calreticulin, TGF-β, ER stress, Extracellular matrix, fibrosis, neointimal hyperplasia

DEDICATION

This dissertation is dedicated to my mom, dad, girlfriend, family, and friends. Their unwavering support during my time in graduate school has been invaluable. To my mom, thank you for listening to me when things weren't going well and always providing encouraging words of support. To my dad, thank you for instilling a good work ethic in me and always encouraging me to work hard and do my best. To my girlfriend, Chelsea, thank your for your constant love and support. It means the world to me and I look forward to many more happy times with you! Finally, to my friends and family, thank you for the constant support and many good times throughout my time in grad school. I have made many memories here that I will never forget.

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LIST OF ABBREVIATIONS

4-PBA	4-phenylbuterate
AEC	alveolar epithelial cell
AngII	angiontensin II
AT	angiotensin
ATF-6	activating transcription factor-6
BMP	bone morphogenetic protein
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CRT	calreticulin
CsA	cyclosporine A
CTF1	collagen transcription factor-1
ECM	extracellular matrix
EDEM	ER degradation-enhancing 1,2 mannosidase-like protein
EMT	epithelial to mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal-related kinase
ESC	embryonic stem cells
FAK	focal adhesion kinase
FIP	familial interstitial pneumonia
FKBP12	FK506 binding protein 12

GLUT1	glucose transporter 1
GRP78	glucose regulated protein
HDAC	histone deacetylase
Hsp	heat shock proteins
IP_3	inositol triphosphate
IPF	idiopathic pulmonary fibrosis
IRE-1	inositol requiring protein-1
LAP	latency associated peptide
LTBP	latent TGF-β-binding protein
MAP3K	MAP kinase kinase kinase
MAPK	MAP kinase
MEF2C	myocyte-enhancer factor 2
MEFs	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MKK	MAP kinase kinase
MLCK	myosin light chain kinase
mTOR	mammalian target of rapamycin
NAC	N-acetylcysteine
NFAT	nuclear factor of activated T cell
OPN	osteopontin
PAI-1	plaminogen activator inhibitor-1
PDGF	platelet-derived growth factor
PDI	protein disulfide isomerase

PERK	protein kinase RNA (PKR)-like ER kinase
PI3K	phosphoinositide 3-kinase
PP1c	protein phosphatase 1c
PS	phosphatidylserine
RBL-1	rat basophilic leukemia
SARA	Smad anchor for receptor activation
SERCA	sarco-endoplasmic reticulum Ca^{2+} ATPase
SFTPC	surfactant protein C
SOCE	store operated Ca^{2+} entry
STZ	streptozotocin
TAC	transverse aortic constriction
TAK1	TGF-β-activated kinase
TSP-1	thrombospondin 1
TTF-1	thyroid transcription factor-1
TUDCA	taurine-conjugated ursodeoxycholic acid
UPR	unfolded protein response
UTR	untranslated region
UUO	unilateral ureteral obstruction
VSMC	vascular smooth muscle cells

CHAPTER 1

INTRODUCTION

Calreticulin

Calreticulin (CRT) is a 46 kDa protein originally identified in the sarcoplasmic reticulum of muscle in 1974 (1). It was initially believed that CRT was localized exclusively in the endoplasmic reticulum (ER) where it served as a protein chaperone and Ca^{2+} binding protein (2,3). However, since its discovery, our understanding of the localization and functions of calreticulin have greatly expanded. CRT is found on the cell surface, in the nucleus, and in the extracellular matrix and is involved in regulation of cell adhesion, focal adhesion disassembly, cell migration, anoikis resistance, apoptosis, and phagocytosis as well as its canonical functions (4). Furthermore, recent work from our lab demonstrates that thrombospondin 1 (TSP-1) binding to cell surface CRT stimulates collagen production and that CRT regulates collagen production through control of ER Ca^{2+} (5,6). Recently, a role for CRT in ER stress and injury has emerged, illustrating the diverse biological effects of CRT (7-9).

Structure

Calreticulin is composed of 9 exons and spans roughly 3.6 kb of human or 4.6 kb of mouse genomic DNA (10,11). It is localized to chromosome 19 in humans and chromosome 8 in mice, respectively (10,12). The complete amino acid sequence of calreticulin was originally described in 1989 and includes a 17 amino acid ER signal sequence and a C-terminal KDEL ER retention sequence (13). Despite increasing

knowledge regarding calreticulin, determination of its 3-D structure has been difficult due to an inability to crystallize the protein. However, it is possible to ascertain information regarding its conformation using the X-ray structure of its homologue, calnexin, and the NMR structure of the P-domain of calreticulin (14,15). Calreticulin is composed of three distinct domains, a globular β-sandwich N domain, a proline rich P domain, and an acidic C-terminal domain (Figure 1) (16). In addition, the stability of CRT is greatly dependent on its ability to bind ions as alteration in levels of Ca^{2+}, Zn^{2+} and Mg^{2+}-ATP alter susceptibility to enzymatic digestion in the presence of trypsin (17).

Based on the crystal structure for the N and P domains of calnexin, it is proposed that the N domain of CRT is globular (Figure 1) (18). The N domain binds lectin whereas the P arm contains tandem repeats of two proline rich sequence motifs which interact with one another in a head-to-tail fashion (Figure 1) (18). The structural homology between the P-domain of calnexin and CRT was confirmed using NMR reconstruction. Ellgaard et al. demonstrated that the P-arm of CRT exists in a hairpin fold that involves the entire polypeptide chain with the two structures in close proximity (14). Further evidence using small angle X-ray scattering confirms that the P-domain β-hairpin extends from the globular N-domain in spiral like fashion (16). Furthermore, the N domain of calreticulin contains the primary polypeptide and carbohydrate binding site and, along with the P-domain, is responsible for the chaperone function of CRT (Figure 1) (19,20). While little structural data regarding the C-domain of CRT exists, it is well established that this region contains several acidic residues and is characterized by its ability to bind Ca^{2+} with high capacity and low affinity (21). The C-domain is also highly susceptible to proteolytic cleavage due to lack of complex tertiary structure (22). It is also appreciated

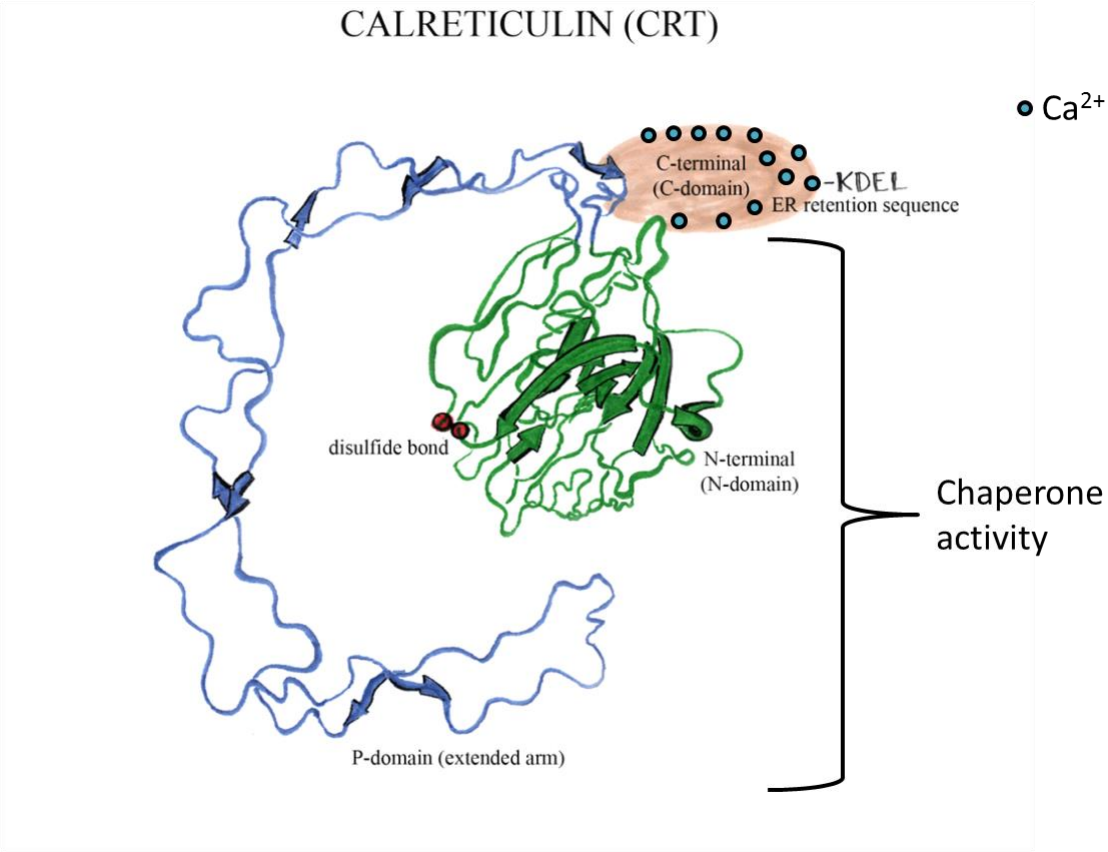

Figure 1. *Schematic of CRT structure*. CRT is comprised of three distinct domains, an N, P, and C-terminal domain. The N and P domain of CRT combine to provide the chaperone function of CRT whereas the C domain is responsible for binding Ca^{2+} with a low affinity but high capacity. The C-terminal domain of CRT contains a KDEL sequence which is responsible for maintaining CRT in the ER. (Figure created by Mariya T. Sweetwyne, used with permission).

that CRT dimerizes/oligomerizes at low pH or by addition of physical or chemical stressors such as urea or high temperatures (23,24). CRT can also undergo post-translational modifications. CRT is arginylated by arginyl-tRNA protein transferase and Lopez et al. demonstrated that arginylated CRT at the cell surface increases susceptibility to apoptosis (25,26). CRT is phosphorylated on serine/threonine residues and phosphorylated CRT regulates mRNA stability (to be discussed later) (27). Collectively, these data demonstrate the complexity of CRT structure and illustrate how calreticulin is able to serve multiple functions within a cell.

Function

CRT regulates ER Ca^{2+} levels

ER dependent Ca^{2+} signaling is involved in fertilization, proliferation, induction of ER stress, development, transcription factor activation, learning and memory, contraction and secretion, and regulation of apoptosis and necrotic cell death (28,29). Therefore, it is imperative that ER and cytoplasmic Ca^{2+} are well controlled: cytoplasmic Ca^{2+} concentration typically resides around 100 nM whereas ER Ca^{2+} concentration is approximately 1 mM and levels of extracellular Ca^{2+} approach 2 mM (30). The concentration differences are maintained by on/off stimuli which induce increased cytoplasmic Ca^{2+} or promote uptake of released Ca^{2+} present in the cytoplasm thereby maintaining cellular homeostasis. Increases in cytoplasmic Ca^{2+} are regulated by voltage-operated channels, receptor operated channels, store operated channels, or inositol triphosphate (IP_3) mediated ER Ca^{2+} release (28). Once released, Ca^{2+} signaling is regulated by speed, amplitude, and spatio-temporal patterning. After Ca^{2+} has carried out its signaling function, it is rapidly removed from the cytoplasm by pumps and

exchangers present on the ER and plasma membrane. The plasma membrane Ca^{2+}-ATPase pump and Na^{+}/Ca^{2+} exchangers remove Ca^{2+} from the cytoplasm to the extracellular environment whereas the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps Ca^{2+} from the cytoplasm into the ER (28). In addition, mitochondria can take up Ca^{2+} released from ER stores thereby adding to the complexity of Ca^{2+} regulation in the cell (31).

Following reuptake of Ca^{2+} from the cytoplasm to the ER by the SERCA pump, ER Ca^{2+} either remains in an unbound form or becomes bound by ER-Ca^{2+} binding proteins such as CRT, calnexin, and calsequestrin. The amount of unbound ER Ca^{2+} varies, but often ranges from 5 to several hundred μM (30,32). CRT is one of the main ER Ca^{2+} binding proteins and can bind 20 mol of Ca^{2+} per mol of protein with a low affinity (K_d = 2mM) through its acidic rich C-terminal domain (21). CRT can also bind 1 mol of Ca^{2+} per mol of protein with a high affinity (K_d = 10 μM) through its proline rich P-domain (21). Furthermore, CRT -/- mouse embryonic fibroblasts (MEFs) show a 1.8 fold reduction in levels of cellular Ca^{2+} indicating the CRT binds around 50% of cellular Ca^{2+} (33). Nakamura and colleagues demonstrated that the reduced Ca^{2+} levels in CRT -/- MEFs could be rescued by transfection with the P and C domain of CRT leading them to conclude that the C domain plays a critical role in maintaining ER Ca^{2+} levels (33). CRT -/- MEFs also demonstrate impaired bradykinin induced ER Ca^{2+} release, although the reason may be due to impaired folding of the bradykinin receptor rather than impaired IP_3 mediated ER Ca^{2+} release (33,34).

Since lack of CRT greatly reduces the Ca^{2+} storage capacity of cells, it can be postulated that overexpression of CRT would increase the amount of Ca^{2+} within the cell.

Indeed, several studies have demonstrated that overexpression of ER CRT increased the amount of Ca^{2+} stored in the ER without having a significant effect on cytoplasmic Ca^{2+} levels (35-37). Mery and colleagues eloquently demonstrated that overexpression of CRT in an L fibroblast cell line increased total cellular Ca^{2+} content by 2.1 fold versus parental cells (36). Furthermore, this group showed that the increased Ca^{2+} was found within thapsigargin sensitive stores and that cytoplasmic Ca^{2+} levels were increased when cells were stimulated with ATP or thapsigargin and ionomycin (36). Similarly, Arnaudeau illustrated that overexpression of CRT led to increased ER luminal Ca^{2+} and that these increased levels were due to enhanced Ca^{2+} bound to the C domain of CRT (37). Several groups demonstrated that overexpression of CRT affects agonist and store operated Ca^{2+} influx (36). Overexpression of CRT inhibited IP_3 mediated rises in intracellular Ca^{2+} in two separate studies and was dependent on the presence of the high-capacity Ca^{2+} binding C domain (38,39). It also appears that overexpression of CRT impairs store operated Ca^{2+} influx both in L-fibroblasts and in a rat basophilic leukemia cell line (RBL-1) (36,40). In addition, overexpression of ER CRT actually decreased mitochondrial Ca^{2+} content and membrane potential, suggesting a possible connection between the ER and mitochondria in disease states (37).

The first evidence demonstrating the importance of CRT *in vivo* came from a ground-breaking paper by Mesaeli and colleagues who demonstrated that CRT knockout was embryonic lethal due to defects in heart development and function (34). Despite low levels of CRT in the adult heart, Mesaeli et al. demonstrated that CRT is abundantly expressed during cardiac development (34). Furthermore, CRT -/- cells showed impaired nuclear import of nuclear factor of activated T cell (NFAT) leading this group to

conclude that CRT may act as an upstream regulator of the Ca^{2+}/calcineurin/NFAT transcription factor pathway (34). Calcineurin is a serine/threonine phosphatase 2B which is activated in response to a sustained elevation in cytoplasmic Ca^{2+} and is responsible for dephosphorylation of specific transcription factors leading to their nuclear translocation (41-43). CRT, through control of Ca^{2+}-dependent calcineurin activation, is an important regulator of myocyte-enhancer factor 2 (MEF2C) nuclear localization (44). This group concluded that CRT works upstream of calcineurin and MEF2C in a Ca^{2+} dependent manner (44,45). In addition, overexpression of constitutively active calcineurin can rescue CRT -/- mice from embryonic lethality demonstrating the importance of Ca^{2+}/CRT/calcineurin regulated pathways in embryonic development (46). Although these mice were viable, they suffered from abnormalities including hypoglycemia, growth retardation, elevated triglycerides and cholesterol, and died at 3-5 weeks post birth (46). Remarkably, CRT -/- mice expressing constitutively active calcineurin displayed normal ventricular wall thickness, although the overall size of the heart was increased. CRT regulated, Ca^{2+}-dependent calcineurin activation is a critical component of normal heart development. CRT downregulation following birth is critical as CRT overexpression in adult mice leads to complete heart block and sudden death (47). These data demonstrate the importance CRT during embryonic and post-natal development.

CRT regulates several cellular processes through modulation of ER and ER-mediated Ca^{2+} release. CRT -/- cells have increased potency for adipogenesis, which is rescued by artificially increasing levels of ER luminal Ca^{2+} (48). The authors showed that CRT -/- cells have increased levels of phosphorylated Ca^{2+}/calmodulin-dependent protein kinase

II (CaMKII) which, when inhibited, greatly reduced adipogenesis (48). CRT -/- embryonic stem cells (ESC), characterized by increased CaMKII activity, had reduced focal adhesions and lower levels of focal adhesion related proteins including vinculin, paxillin and phosphorylated focal adhesion kinase (FAK) (49). CRT, through a c-SRC pathway, induces fibronectin expression leading to alterations in focal adhesion and cell spreading (50,51). Similar to CRT -/- ESCs, CRT -/- MEFs exhibit increased c-SRC and CaMKII activity which correlated with decreased focal contacts, cell spreading, and fibronectin production (52). The increased activity of c-SRC and CaMKII was due to reduced levels of ER Ca^{2+} and artificially increasing levels of cytosolic Ca^{2+} with ionomycin significantly augmented fibronectin production (52). The correlation between CRT levels and fibronectin production was confirmed utilizing CRT underexpressing and CRT overexpressing fibroblasts. Overexpression of CRT lead to increased fibronectin production and incorporation into the ECM, an increase in cell spreading, and increased vinculin positive focal adhesions (50).

CRT control of cellular Ca^{2+} regulates focal adhesions, cell spreading, and fibronectin production; furthermore, a recent publication from our lab demonstrates that CRT regulates collagen expression, processing, secretion and deposition into the ECM (6). CRT -/- MEFs have reduced collagen type I and III transcript whereas CRT overexpressing L fibroblasts have increased collagen I transcript and protein (6). The reduced collagen levels in CRT -/- MEFs versus wild type MEFs was partially due to CRT-controlled, thapsigargin sensitive, ER Ca^{2+} since treatment of wild type cells with thapsigargin significantly reduced collagen expression (6). Together, these data illustrate

the importance of CRT-regulated Ca^{2+} levels in maintenance and control of cell adhesion, spreading, and ECM production.

CRT is a critical ER chaperone

The ER is the first compartment to encounter nascent polypeptides following translation. Once inside the ER, the nascent polypeptides interact with molecular chaperones and thiol oxidoreductases. Chaperones within the ER are grouped based on function: 1) the classical ER chaperones consist of the heat shock proteins (Hsp) including glucose regulated protein (Grp78)/Bip and Grp94; 2) the lectin chaperones consisting of CRT and calnexin, which recognize and fold proteins containing a specific pattern of sugar moieties; 3) the protein disulfide isomerase (PDI) family of thiol oxidoreductases that form disulfide bonds between neighboring cysteine residues; and 4) the substrate specific chaperones such as Hsp47 which functions as a collagen chaperone (30,53,54). Following entry into the ER, polypeptides containing the $Glc_1Man_9GlcNAc_2$ sugar moiety are specifically recognized by CRT and calnexin and enter into the CRT/calnexin cycle (55,56). Together, these chaperones ensure that the nascent polypeptide is folded properly; however, if prolonged interaction with calnexin occurs, the protein becomes targeted for degradation via interaction with ER degradation-enhancing 1,2 mannosidase-like protein (EDEM) (53). If the protein is properly folded, it is transported out of the ER to the golgi for further processing and secretion. CRT can also bind to nascent polypeptides lacking this sugar moiety. Saito and colleagues demonstrated that CRT can form stable complexes with unfolded, non-glycosylated substrates and prevent them from forming improper aggregation (57).

Significant insight into CRT folding properties has been obtained through study of the major histocompatibility complex (MHC) class I assembly in wild type and CRT -/- cells. MHC class I is composed of a polymorphic glycosylated heavy chain, a non-polymorphic β_2-microglobulin and is assembled with the assistance of the peptide loading complex composed of CRT and calnexin among others (58). CRT -/- MEFs display rapid MHC class I export from the ER, a 50-80% reduction in peptide loading, and impaired T-cell antigen recognition which can be rescued by transfection with CRT but not calnexin (59). Remarkably, lectin deficient point mutations in CRT can fully rescue the defects in MHC class I processing in CRT -/- MEFs (60). In addition, lectin deficient CRT bound a similar range of proteins with nearly identical kinetics as wild type CRT indicating that CRT can interact with proteins in the ER independent of its lectin binding properties (60).

The roles of CRT and calnexin in the CRT/calnexin cycle is quite complex. While cells deficient in CRT have accelerated maturation of cellular and viral proteins and a slight decrease in folding efficiency, calnexin deficiency completely prevents folding of some proteins, such as hemagglutinin, while having virtually no effect on other proteins (61). The differential effects on protein folding and secretion may be caused by different lectin-binding preferences between the two proteins. This hypothesis was confirmed by an eloquent study by Pipe and colleagues who demonstrated that two coagulation factors, Factor VIII and Factor V, had differential chaperone binding preferences in the ER despite similar structure and glycosylation patterns (62). They illustrated that Factor VIII only interacted with calnexin while Factor V coimmunoprecipitated with both CRT and calnexin (62). While both ER chaperones have similar structure and function and may be able to compensate for one another *in vitro*, loss of CRT or calnexin *in vivo* leads to

embryonic death or severe malignancy, respectively (34,63). Furthermore, CRT -/- cells have increased calnexin while calnexin deficient cells have increased CRT levels (61,64). Interestingly, both CRT and calnexin knockout cells have increased levels of GRP78, suggesting that other ER chaperones may be compensating for the loss of CRT or calnexin, at least *in vitro* (61,64).

Within the ER, CRT regulates Ca^{2+} homeostasis and protein folding through the CRT/calnexin cycle. Although these processes appear functionally distinct, there is evidence that cross-talk exists. Understanding of this cross-talk was obtained by altering ER Ca^{2+} content and measuring changes in CRT's ability to bind to PDI or ERp57 (65). Using this technique, it was demonstrated that CRT could interact with PDI at low Ca^{2+} concentrations (below 100 μM), but rapidly disassociated at higher Ca^{2+} levels (greater than 400 μM) (65). Alterations in the CRT-ERp57 complex occurred only at higher Ca^{2+} concentrations (65). This group concluded that the protein-protein interactions they observed were due to alterations in Ca^{2+} bound to the C domain of CRT and that CRT may serve as a Ca^{2+} sensor for ER chaperone proteins (65).

CRT and apoptosis

CRT mediates apoptosis and apoptotic cell clearance through control of ER Ca^{2+} release and its function on the cell surface (Figure 2). CRT is a major Ca^{2+} binding protein of the ER and Ca^{2+} release from the ER activates transcriptional cascades regulating apoptosis (15,66,67). A direct link between ER CRT and apoptosis was first reported by Nakamura et al. who demonstrated that cells deficient in CRT were resistant to thapsigargin and staurosporine induced apoptosis (68). The reduced sensitivity to

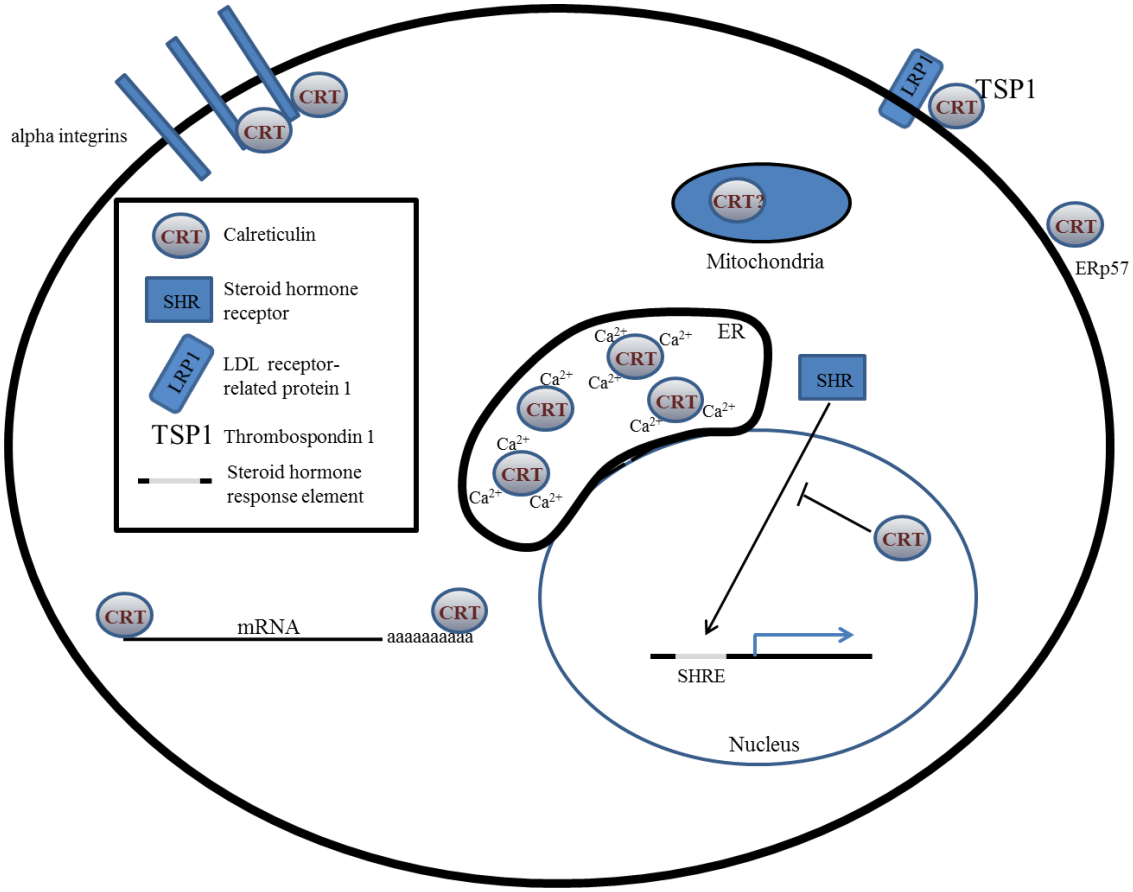

Figure 2. Localization and functions of CRT in the cell. In addition to its Ca^{2+} binding and ER chaperone function in the ER, CRT is found in other cellular compartments including the nucleus, cell surface, and cytoplasm. CRT regulates apoptotic cell clearance and focal adhesion disassembly on the cell surface. In the cytoplasm, CRT binds to the tail of alpha integrins and mRNA. CRT in the nucleus binds to steroid hormone receptors and inhibits steroid hormone receptor driven gene transcription. CRT may also be present in the mitochondria.

apoptotic stimuli was attributed to reduced cytochrome c release from the mitochondria and reduced caspase 3 activity (68). In accordance, overexpression of CRT sensitized cells to stress induced apoptosis in a cytochrome c, caspase 3 dependent manner (68). This report is in agreement with data from Arnaudeau et al. and Kageyama et al. who demonstrated that CRT overexpression sensitized cells to apoptotic stress in human embryonic kidney cells and rat cardiomyoblasts (37,69). Overexpression of CRT in cardiomyocytes correlates with enhanced levels of intracellular Ca^{2+}, a decrease in anti-apoptotic proteins, and increased levels of Bax, p53 and caspase 8, leading to cardiomyocyte apoptosis (70). Oxidative stress-induced apoptosis was significantly increased in cells overexpressing CRT due to enhanced H_2O_2 induced Ca^{2+} release and subsequent caspase-12 and calpain activation (71). Recently, Shi et al. illustrated that enhanced levels of CRT in a JEG-3 human choriocarcinoma cell line correlated with increased apoptosis although the mechanism responsible was not described (72). It is postulated that the increased sensitivity to apoptotic stimuli is caused by variations in the availability of Ca^{2+} for release and subsequent apoptotic activation (43).

The involvement of CRT in phagocytosis and removal of apoptotic cells is well established. It was first believed that CRT functioned at the cell surface of phagocytic cells where it bound to a plethora of opsonins, including C1q, present on apoptotic cells (73,74). Binding of CRT on the phagocytic cell to C1q on the apoptotic cell induces activation of the classical complement pathway (75,76). This activation occurs through C1q interaction with a CRT/CD91 (also known as LRP1) complex (77). CRT can also regulate apoptotic cell clearance in a trans manner through binding to CD91on the engulfing cell (78). This study showed that phagocytic uptake through CRT binding to

CD91 required disruption of CD47, a "don't eat-me" signal, on the target cell (78). Collectively, it is appreciated that cell-surface CRT is a critical regulator of apoptotic cell uptake.

Recent data demonstrate that in response to specific lethal stimuli such as anthracycline, CRT is transported to the cell surface in conjunction with ERp57 where it elicits an antitumor immune response (Figure 2) (79). These authors demonstrated that exposure of CRT on the cell surface preceded an induction of apoptotic signaling and was required for phagocytosis of tumor cells by dendritic cells (79). The immunogenicity of anthracycline-treated tumor cells is dependent on cell surface CRT as pretreatment with a CRT neutralizing antibodies prevented dendritic cell uptake (79). Remarkably, treatment of tumor cells with etoposide, mitomycin C or cisplatin was unable to induce CRT to the cell surface and cells failed to undergo imunnogenic cell death (79). These results have identified CRT as an "eat-me" signal that is exposed upon treatment with specific stimuli on dying cells undergoing immunogenic cell death. Recent data have provided insight into the mechanism through which CRT translocates to the cell surface following apoptotic stimuli. Tarr and colleagues demonstrated that CRT can directly associate with phosphatidylserine (PS) in a Ca^{2+}-dependent manner and that externalization of CRT is dependent on aminophospholipid translocase, which is responsible for flipping PS and CRT to the extracellular environment (80). The ability of cells to undergo apoptosis may also be dependent on modifications of CRT. Exogenously applied arginylated CRT increased apoptosis in wild type cells and overcame apoptosis resistance in cells lacking arginylated CRT on the cell surface (26).

CRT functions at the cell surface and extracellular environment

In addition to cell surface CRT and its involvement in apoptotic cell clearance, many other functions of cell surface and extracellular CRT have emerged. CRT is present on the cell surface of several mammalian cells including platelets, fibroblasts, and endothelial cells (78,81-83). Early studies on cell surface CRT demonstrated that CRT was required for the mitogenic activity of the Bβ chain of fibrinogen in fibroblasts (83). Furthermore, early indications of non-ER functions for CRT stemmed from work demonstrating that CRT could bind a specific amino acid sequence present in the cytoplasmic tails of α integrins (Figure 2) (84). CRT coimmunoprecipitated with integrins during cell adhesion to ECM and is found in complex with LRP1associated integrin-adhesion complexes (85,86). In addition, cell surface CRT interacts with the collagen receptor, integrin α2β1, on the surface of human platelets (87). The function of cell surface CRT in focal adhesions is believed to be mediated through its lectin binding properties as CRT on the cell surface bound to glycosylated, but not nonglycosylated forms of laminin, to mediate cell spreading of melanoma cells (88).

Evidence for the involvement of CRT in regulating focal adhesions comes from our lab and others who demonstrated that cell surface CRT was required for thrombospondin 1 (TSP1) mediated focal adhesion disassembly (Figure 2) (89). TSP1/CRT mediated focal adhesion disassembly is dependent on amino acids 17-35 of the N-terminal domain of TSP, a region termed hep1, and on amino acids 1-41of CRT (89,90). Further data narrowed the region of CRT required for TSP induced focal adhesion disassembly to amino acids 19-36 (90). TSP1 induces focal adhesion disassembly through a CRT/LRP1 receptor co-complex which signals through the phosphoinositide 3-kinase (PI3K) and extracellular signal-related kinase (ERK) pathways (81,82,91). PI3K and ERK activation

by TSP1 or hep1 culminated in transient down-regulation of Rho activity, cytoskeletal reorganization, loss of focal adhesions, and increased cell migration (81,82,92). Addition of exogenous CRT stimulated keratinocyte and fibroblast migration both *in vitro* and *in vivo* although the exact mechanism is unknown (93). TSP1 mediates anoikis resistance through binding to CRT/LRP1 on the cell surface in fibroblasts (94). Once again, TSP1 binding to cell surface CRT/LRP1 stimulates activation of PI3K and Akt, decreases caspase 3 activation and PARP1 cleavage, and prevents cell death (94).

Evidence for CRT in the ECM has been limited; however, a report by Somogyi and colleagues demonstrated that CRT was present in odontoblasts and predentin matrix in the tooth (95). This finding was verified using western blotting, immunohistochemistry, ultrastructural immunocytochemistry and in situ hybridization in developing rat teeth (95). The authors speculated that CRT is involved in regulating mineralization; however, no direct evidence for this exists. Immunohistochemical data from our lab suggest that CRT is localized to the ECM following injury/damage during vascular remodeling (4). Despite these initial observations, conclusive data demonstrating CRT in the ECM remains elusive; furthermore, the function of CRT in the ECM is unknown, but may be involved in modulating ECM structure or turnover.

CRT regulates gene expression and mRNA stability

Evidence demonstrating that CRT is involved in regulating gene expression came from studies showing that CRT can bind the amino acid sequence KXFFKR, which is similar to the DNA binding sequence of nuclear receptors (96,97). Indeed, CRT binds to the glucocorticoid receptor, androgen receptor, retinoic acid receptor, and vitamin D receptor, which all contain the KXFFKR amino acid sequence (Figure 2) (97-100).

Overexpression of CRT prevents glucocorticoid-induced transcriptional activation of a glucocorticoid response gene and this inhibition is dependent on the N domain of CRT (97). Dedhar and colleagues demonstrated that CRT could inhibit the binding of the androgen receptor to its hormone responsive DNA element in a KXFFKR sequence specific manner (98). In addition, this group demonstrated by immunoprecipitation studies that CRT can directly bind to the androgen receptor, further supporting the claim that CRT is involved in regulation of hormone mediated gene transcription (98). CRT is responsible for preventing the vitamin-D receptor from binding to DNA binding elements in the nucleus (99). This inhibition is specific for CRT binding to the KGFFRR sequence as calreticulin was unable to block DNA binding by the transcription factor ATF-a delta which lacks a KVFFKR sequence in its DNA binding domain (99). CRT prevents the binding of vitamin D receptor-retinoid X receptor-beta to the vitamin D response element located in the parathyroid hormone promoter and inhibits the transcriptional effect of vitamin D on the parathyroid hormone gene (101). Recently, addition of recombinant CRT to murine macrophages led to induction of TNF-α and IL-6 transcription and protein expression without affecting intracellular mRNA stability, indicating that extracellular CRT may also be able to regulate gene transcription (102). CRT can bind to transcription factors and enhance their transcriptional activity. Perrone and colleagues demonstrated that CRT can bind to thyroid transcription factor-1(TTF-1) and promote its folding, thereby leading to increased transcriptional activation of the TTF-dependent promoters (103).

The Ca^{2+} binding function of CRT is important in regulating gene transcription. Mesaeli et al. demonstrated that CRT -/- was embryonic lethal and that CRT -/- cells had

impaired nuclear translocation of Ca^{2+}/calcineurin activated NFAT3 (34). Embryonic lethality of CRT -/- mice could be rescued by introduction of a constitutively active calcineurin, indicating that CRT mediated control of calcineurin specific transcription factors was critical for embryonic development (46). These data were supported by Lynch and colleagues who illustrated that CRT was working upstream of Ca^{2+}, calcineurin, and the cardiac specific transcription factor, MEF2C (44). The inability of CRT -/- cells to induce NFAT or MEF2C nuclear translocation could be rescued by treatment with a Ca^{2+} ionophore, demonstrating that CRT-regulated Ca^{2+} release was essential for gene transcription (44). In addition to NFAT and MEF2C, Papp et al. demonstrated that cells overexpressing CRT have increased fibronectin mRNA levels and protein expression as compared to parental cells (50). Our lab demonstrated that cells lacking CRT have reduced transcript levels for type I collagen, type III collagen and fibronectin (6,104). Despite the decrease in transcript levels for these three ECM proteins, levels of TSP1 were actually increased in CRT -/- cells indicating that lack of CRT does not coincide with overall transcriptional defects (6). Together, these data indicate that CRT controls gene transcription through ER CRT-bound Ca^{2+} and through direct binding with steroid hormone receptors.

CRT is an mRNA binding protein that alters gene expression through control of mRNA stability and turnover (Figure 2). The first evidence of CRT binding to RNA was reported by Singh et al. who demonstrated that CRT could bind to the 3' stem-loop complex of rubella virus genomic RNA (105). This group also reported that phosphorylation of CRT was required for binding to the 3' stem-loop (105). Following

this initial discovery, several groups have demonstrated the RNA binding ability of CRT and that binding affects mRNA stability through post-transcriptional mechanisms.

Nickenig et al. demonstrated that CRT is involved in regulation of angiotensin (AT) 1 receptor mRNA stability (106). Treatment of vascular smooth muscle cells (VSMC) with angiotensin II (Ang II) decreased expression of AT (1) receptor mRNA which was dependent on CRT binding to base pairs 2175-2195 in the 3' untranslated region (UTR) (106). Similar to Singh et al., Nickenig and colleagues illustrated that CRT dependent mRNA destabilization was dependent on CRT phosphorylation (106). In addition, this group identified Src as the key enzyme responsible for phosphorylation of CRT and showed that following treatment of VSMCs with Ang II, CRT showed enhanced phosphorylation of tyrosine residues and decreased phosphorylation of serine/threonine residues (27). In particular, CRT had increased tyrosine phosphorylation of amino acid 285 in the region between the P and C domains and reduced serine phosphorylation in the N and P domains (27). This led the authors to predict that CRT mRNA binding properties were dependent on Src-mediated tyrosine phosphorylation and dephosphorylation. Further evidence implicating CRT in regulating mRNA levels comes from Totary-Jain and colleagues who demonstrated that CRT destabilizes glucose transporter 1 (GLUT1) mRNA in vascular cells (107). Using RNA mobility shift, UV cross-linking, and *in vitro* degradation assays, followed by mass-spectrometric analysis, this group illustrated that CRT bound to a 10 nucleotide cis-acting element in the 3' UTR of GLUT1 mRNA (107). Unfortunately, this group did not address the phosphorylation status of CRT in these studies; however, these data suggest that phosphorylation of CRT is important in regulating stability of mRNA through binding of the 3' UTR.

CRT can bind to the 5' region of mRNAs and affect there stability (Figure 2). Timchenko et al. showed that CRT could interact with the 5' region of C/EBPbeta mRNA and that interaction of CRT with stem loop structure of C/EBPbeta and C/EBPalpha mRNA led to inhibition of translation *in vitro* and *in vivo* (108). They also demonstrated that CRT can interact with GCU repeats in myotonin protein kinase and with GCN repeats within C/EBPbeta and C/EBPalpha mRNA (108). The ability of CRT to inhibit C/EBP protein translation is dependent on the GC rich nature of the stem loop region (108). Another report by this group illustrates that CRT can block translation of the cyclin-dependent kinase p21 through binding and stabilization of 5' stem loop structure of p21 mRNA (109). CRT and the RNA-binding protein CUBPG1 compete for the 5' binding site of the stem loop structure and CUBPG1 mediated displacement of CRT alleviates the translational repression of CRT (109). In addition, replacement of CRT from the 5' stem loop structure of C/EBPbeta mRNA by CUBPG1 following partial hepatectomy increases expression of a dominant negative 20-kDa isoform of C/EBPbeta (110). Collectively, these data implicate CRT in regulating stability of mRNA through binding to the 5' end and 3' UTR of mRNA.

CRT in stress conditions and fibrotic disease

CRT is increased by cellular stressors

CRT is an essential protein involved in ER Ca^{2+} binding, protein chaperoning, apoptosis, focal adhesions, and mRNA stability and turnover. In addition, it is appreciated that cellular stress induces CRT expression. CRT protein is increased following amino acid deprivation, ER Ca^{2+} depletion, oxidative stress, heat shock, and high glucose treatment (7,111-117). CRT was initially discovered to be upregulated

following amino acid deprivation by Plakidou-Dymock who identified CRT through N-terminal amino acid sequencing (117). Heal and McGivan used Chinese hamster ovary cells to demonstrate that depletion of amino acids caused a 4-fold increase in CRT levels and an overall increase in glycosylation of CRT, although this glycosylation was not required for the increase in CRT protein (112).

Late in the 1990's, two separate investigators identified that CRT was increased following alterations in ER-Ca^{2+} homeostasis. Llewellyn and colleagues showed that CRT and GRP78 were increased following treatment of HeLa cells with the SERCA inhibitor, thapsigargin, which depletes ER Ca^{2+} stores (115,118). Another report demonstrated that CRT was increased following ionomycin treatment in human epidermoid squamous carcinoma cells (113). The effects of ER-Ca^{2+} depletion on CRT levels and chaperone ability have been revisited several times since its original discovery. Rizvi et al. showed that Ca^{2+} depletion increased CRT polypeptide binding ability, chaperone activity, and capacity to oligomerize (119).

CRT is increased following treatment with a variety of chemicals and compounds. Oxidative stress induced increase in CRT was first reported by Nunez and colleagues who elegantly demonstrated that CRT was elevated following treatment with iron, which was used to induce oxidative stress (120). Furthermore, treatment with increasing iron concentrations increased oxidative stress and CRT, and pretreatment of cells with antioxidants prevented this increase (120). In support of CRT being upregulated following oxidative injury, two groups demonstrated that CRT was dramatically increased following either hypoxia preconditioning, hypoxia preconditioning plus myocardial infarction, or hypoxia preconditioning plus reoxygenation (121,122). In

addition, the authors showed that the hypoxia/reoxygenation injury induced increases in CRT occurred through a p38 MAP kinase (MAPK) pathway (121,122). Total and cell-surface CRT were increased in melanocytes following exposure to H_2O_2 leading to enhanced H_2O_2-mediated apoptosis (123). Following treatment with nitric oxide, J774 macrophages showed enhanced levels of CRT and GRP78 (124). CRT is increased both in vitro and in vivo following treatment with valproate. Chronic treatment with sodium valproate significantly increased levels of CRT in the rat cerebral cortex and hippocampus (125). This study was followed up by *in vitro* work which showed that acute and chronic treatment of rat glioma cells with sodium valproate increased CRT levels (126). These studies also reported an increase in GRP78 and GRP94 following treatment with sodium valproate. Other chemicals can increase CRT as treatment of NIH/3T3 cells with the anti-neoplastic agent bleomycin increased the expression of CRT (127).

ER stress and fibrotic disease

The ER is a specialized organelle that is involved in Ca^{2+} homeostasis and protein chaperoning. Within the ER lumen are several chaperone proteins including GRP78, GRP94, PDI, CRT and calnexin (128). Under normal physiological conditions, equilibrium exists between the number of nascent polypeptides in the ER and the number of ER chaperones available to assist in folding. Increased protein translation, accumulation of misfolded proteins, or altered Ca^{2+} homeostasis in the ER lead to the condition known as ER stress (129). In response to perturbation in ER equilibrium and induction of ER stress, eukaryotic cells have adapted a mechanism to cope with these disturbances termed the “unfolded protein response” (UPR). The objective of the UPR is

to reduce the protein load in the ER through a combination of inhibiting new protein synthesis and increasing levels of ER chaperones, including CRT and GFP78 (130). However, if ER stress persists, the cell eventually overrides its coping mechanisms and triggers apoptosis and removal of the cell (131). Indeed, ER stress and ER-stress-induced apoptosis are involved in a wide range of diseases including cancer, type 2 diabetes, neurodegeneration, and atherosclerosis (128). The induction of ER chaperones, inhibition of protein translation, and the induction of apoptosis is carried out by three ER stress response pathways: 1) activating transcription factor-6 (ATF-6); 2) inositol requiring protein-1 (IRE-1) and; 3) protein kinase RNA (PKR)-like ER kinase (PERK) (129). Activation of these ER stress response pathways is regulated through binding to GRP78 (132). Under basal conditions, ATF-6, IRE-1, and PERK are bound by GRP78 and maintained in an inactive state; however, following a build-up of unfolded proteins in the ER, GRP78 binds to the unfolded proteins leading to activation of the UPR stress-response pathways (132). In particular, ATF-6 is believed to be responsible for induction of CRT following ER stress. Schardt and colleagues demonstrated that acute myeloid lukemia cells transfected with ATF-6 could induce activation of a CRT reporter construct and that mutation of the ATF-6 putative binding site in the CRT promoter prevented this induction (133). Nevertheless, it is possible that other arms of the UPR can induce CRT activation although none have been identified to date. Together, these pathways work to maintain ER equilibrium and reduce levels of unfolded proteins following disruptions in homeostasis.

Recently, the involvement of ER stress and fibrotic disease has become appreciated. ER stress is involved in kidney fibrosis, cardiac fibrosis, liver fibrosis, lung fibrosis, and

pulmonary-arterial hypertension (130). The earliest evidence suggesting the involvement of ER stress in fibrotic disease came from patients with familial interstitial pneumonia (FIP) carrying a mutation in surfactant protein C (SFTPC) (134). These initial observations were followed up by Lawson and colleagues who evaluated biopsies of patients with SFTPC mutations, sporadic idiopathic pulmonary fibrosis (IPF), or normal lung histology. They identified prominent alveolar epithelial cell (AEC) staining for unfolded protein response (UPR) markers such as GRP78 in patients with SFTPC mutation and sporadic IPF, particularly in regions of fibrotic remodeling (135). Furthermore, two reports from the Blackwell laboratory investigated the mechanism of ER stress induced pulmonary fibrosis. Treatment of AECs with tunicamycin or expression of mutant SFTPC led to induction of ER stress and the UPR (136). Induction of ER stress in AEC decreased expression of E-cadherin and increased expression of mesenchymal markers such as N-cadherin, vimentin, and smooth muscle actin suggesting that ER stress promotes epithelial to mesenchymal transition (EMT) (136). The involvement of ER stress in pulmonary fibrosis was confirmed by experiments demonstrating that mice containing a mutant SFTPC or treated with tunicamycin developed enhanced fibrosis following bleomycin treatment (137). Surprisingly, mice treated with tunicamycin or expressing mutant SFTPC failed to show increased pulmonary fibrosis in the absence of a second fibrosis inducing stimuli (137). Collectively, these data provide strong evidence that ER stress is involved in pulmonary fibrosis, although the exact mechanism is unknown (138). Initial insight into the mechanism by which ER stress is regulating fibrotic disease was provided by Baek et al. who investigated the role of GRP78 in regulating TGF-β stimulated matrix production in

fibroblasts (139). Treatment of human or mouse fibroblasts with TGF-β increased expression of ER stress proteins and type I collagen (139). Furthermore, knockdown of GRP78 significantly attenuated TGF-β or tunicamycin induced collagen and smooth muscle actin production suggesting that GRP78 may be involved in regulating fibrosis through control of TGF-β stimulated collagen production (139). However, the authors failed to provide a mechanism which explains how GRP78 regulates TGF-β stimulated matrix production.

ER stress is enhanced in the unilateral ureteral obstruction (UUO) model of renal fibrosis as indicated by increased GRP78 and KDEL staining (140). Furthermore, attenuation of ER stress inhibited UUO induced fibrosis (140). Induction of diabetes in rats or mice increased levels of ER stress and treatment of mice with 4-phenylbuterate (4-PBA), a chemical chaperone, significantly reduced levels of ER stress and diabetic nephropathy (141,142). ER stress is associated with the pathogenesis of cardiac hypertrophy. Transverse aortic constriction (TAC), an animal model to produce pressure overload, is characterized by an increase in ER stress response proteins including GRP78 and an increase in the pro-fibrotic genes, collagen type I, III, and TGF-β (143). Treatment with 4-PBA significantly reduced both TAC-induced ER stress proteins and induction of pro-fibrotic genes (143). Likewise, treatment of mice with isoproterenol to induce cardiac fibrosis resulted in increased ER stress and enhanced collagen deposition in damaged areas of the endocardium (144). Treatment with 4-PBA reduced ER stress and decreased collagen deposition, leading the authors to conclude that ER stress is involved in the pathogenesis of cardiac fibrosis (144). These findings were supported by Kassan et al. who showed that collagen content, TGF-β activity, and ER stress markers

were all greatly upregulated following angiotensin II treatment (145). Inhibition of ER stress using taurine-conjugated ursodeoxycholic acid (TUDCA) and 4-PBA led to reduced collagen levels, TGF-β activity, and ER stress (145).

CRT and fibrotic disease

CRT is increased following physical and chemical stimuli; however, the involvement of CRT in disease was not fully appreciated until recently. It is now established that CRT expression is altered in human diseases including obesity and insulin resistance, Crohn's disease, rheumatoid arthritis, infiltrating ductal breast carcinomas, cystic fibrosis, colon cancer and many others (4,146-154). More recently, an appreciation for CRT in fibrotic and fibroproliferative diseases has been described: 1) CRT is increased in obese and insulin resistant patients; 2) APA hamsters receiving streptozotocin show enhanced CRT in the aorta; 3) Glucosamine overexpression enhances CRT levels; 4) Addition of exogenous CRT enhances porcine wound healing; 5) CRT levels are increased in a UUO model of renal fibrosis and; 6) CRT promotes epithelial to mesenchymal transition and fibrosis (7-9,152,155-157).

Kypreou and colleagues demonstrated that CRT was upregulated 3 days post UUO induced injury and further increased at 7 days post injury (8). Collagen levels, as measured by Sirius red staining, were significantly increased 7 days post injury demonstrating that upregulation of CRT precedes increased collagen production (8). Furthermore, this group illustrated that TGF-β, a major profibrotic stimulus, induced CRT production in HK-2 cells providing a potential mechanism through which CRT is involved in fibrotic disease (8). Surprisingly, a desmin -/- model of cardiac fibrosis failed to show an increase in CRT levels despite an increase in collagen production (8). CRT is

also involved in diabetic atherosclerosis as treatment of APA hamsters with streptozotocin (STZ) resulted in an increase in CRT staining in the medial layer 6 weeks following treatment (7). Furthermore, an increase in CRT mRNA was noted in the aorta of mice receiving STZ treatment compared to saline and treatment of mice with 4-phenylbutyrate reduced CRT mRNA in the aorta (7). More recently, the involvement of CRT in epithelial to mesenchymal transition in renal cells has been highlighted. Overexpression of CRT in kidney epithelial cells decreases expression of E-cadherin, while increasing expression of mesenchymal markers such as N-cadherin and fibronectin (157). This group showed that CRT-mediated down regulation of E-cadherin was occurring through induction of Slug, a repressor of the E-cadherin (157). Induction of Slug was dependent on Ca^{2+} as cells overexpressing CRT had a greater reduction in E-cadherin following thapsigargin or ionomycin treatment as compared to control cells (157). This report is in accordance with a recent publication by Prakoura and colleagues who showed that upregulation of epithelial CRT promoted tubulointerstitial fibrosis in the rat UUO model of renal fibrosis (9). They illustrated that overexpression of CRT promotes EMT and that overexpression of CRT *in vitro* resulted in increased collagen type I and fibronectin production (9). In addition, CRT heterozygous mice displayed reduced Sirius red staining for collagen at 17 days post ligation compared to wild type mice (9). Remarkably, CRT heterozygous mice demonstrated a notable attenuation in a majority of pro-fibrotic genes investigated including collagen type I, type III, type IV, fibronectin and TGF-β (9). Collectively, these data demonstrate that overexpression of CRT is directly involved in regulating fibrotic and fibroproliferative disease.

TGF-β

TGF-β structure and activation

The TGF-β superfamily of cytokines consists of TGF-β, activins, inhibins, Nodal, bone morphogenetic protein (BMP) and anti-Mullerian hormone (158). TGF-β is involved in cellular processes including growth inhibition, cell migration, invasion, EMT, ECM production and remodeling, and immune suppression (158). Within the TGF-β family, three highly homologous isoforms exist, TGF-β1, TGF-β2, and TGF-β3. While all three isoforms of TGF-β signal through a similar mechanism, their expression levels and functions differ greatly as demonstrated by knockout mice of each isoform (159-162). TGF-β is synthesized as a homo-dimeric pro-protein which undergoes proteolytic cleavage by furin-like enzymes in the trans golgi network, giving rise to a mature TGF-β dimer and an N-terminal latency associated peptide (LAP). TGF-β is secreted from cells in association with LAP and a latent TGF-β-binding protein (LTBP) to form the large latent complex. Activation of latent TGF-β occurs through interaction with TSP-1, integrins, matrix metalloproteinases, and plasmin and biophysical mechanisms including heat activation, acidic or alkali conditions, and shear stress (163-167). Our lab demonstrated that TSP-1 binds to and activates latent TGF-β through a specialized RFK amino acid sequence present in the type 1 repeats of TSP-1 (163,168-170). Once activated, TGF-β binds to its cell surface receptors and induces downstream signaling cascades.

Upon activation, TGF-β can to bind to its receptors at the cell surface. TGF-β signals through a heterotetrameric complex consisting of two related transmembrane serine/threonine kinase receptors, the type I and type II TGF-β receptors (TβRI and TβRII). These receptor kinases are composed of 12 members, 7 type I receptors and 5

type II receptors (171). Both receptors consist of 500 amino acids and contain an N-terminal extracellular ligand binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain (172). At the cell surface, TβRII exists as a homodimer in the absence and presence of a ligand and is responsible for initial binding of the TGF-β (173-175). Upon binding of TGF-β to its receptors, a heterotetrameric complex is formed followed by intramolecular autophosphorylation of TβRII at Ser213 and Ser409 (176). Luo and colleagues demonstrated that phosphorylation at these residues is required for activation of TβRII kinase activity and its ability to phosphorylate the TβRI (176). Active TβRII phosphorylates TβRI at serine-threonine residues in its GS domain, which contains a SGSGSG sequence, and induces its activation (173-175). Active TβRI signals through several pathways including the canonical Smad pathway to alter gene transcription and cellular function.

TGF-β signaling through Smad

Smad was the first intracellular mediator of TGF-β signaling identified and is broken into three distinct classes, the receptor Smads (R-Smads), the Co-Smad, and the inhibitory Smads (I-Smads) (172,177). The R-Smads and Co-Smad are proteins comprised of around 500 amino acids with two conserved domains, an N-terminal MH1 domain and a C-terminal MH2 domain. In addition, the R-Smads contain a characteristic SXS motif in the MH2 domain at their C-termini which can be phosphorylated by TβRI. The MH2 domain of Smad is highly conserved and responsible for receptor interaction, formation of Smad complexes, and contacting the nuclear pore complex for nuclear shuttling (172). Binding of the R-Smad to the phosphorylated TβRI is a complex process. Phosphorylation and activation of TβRI in the GS domain leads to enhanced

binding affinity for the R-Smad, in particular, for a positively charged surface patch in Smad2 (178). Recruitment and recognition of R-Smads by the receptors is facilitated by the Smad anchor for receptor activation (SARA) which immobilizes Smad 2 and Smad 3 near the cell surface (179). It is also recognized that SARA is targeted to the membrane of early endosomes, a characteristic which enhances the recruitment of Smad2 and Smad3 to the receptors for phosphorylation (179).

R-smads are directly phosphorylated at two C-terminal serine residues, 465 and 467, in the SSXS motif by activated TβRI (180,181). Phosphorylation of the R-Smads at these residues destabilizes their interaction with SARA and exposes the nuclear import sequence in the MH2 domain (182). In addition, data suggest that phosphorylation at these sites is required for interaction between the R-Smads and the Co-Smad, Smad 4 (180,181). Wu and colleagues demonstrated that phosphorylated Smad 2 or Smad 3 forms a stable homotrimer with Smad 4 and that this interaction is largely dependent on interaction between adjacent phosphorylated SSXS motifs (183). The exact makeup of the R-Smad/Co-Smad interaction is debatable as data suggests that Smad 4 can form a heterodimeric or heterotrimeric complex with Smads 2 and 3. Chacko et al. suggested that Smad 4 forms a heterotrimeric complex with two Smad 3 proteins whereas Wu and colleagues demonstrated that Smad 4 could form a heterodimeric complex with Smad 2 (183-185). Nevertheless, Inman and Hill suggested that Smad 4 could form a heterotrimeric complex with both Smad 2 and Smad 3 and that the composition of the heterotrimeric complex was dependent on the gene promoter (186). Independent of the exact composition of the complex, it is clear that following activation of the TβRI, R-

Smads become phosphorylated, form a complex with Smad 4, and translocate to the nucleus (187).

R-Smads typically reside in the cytoplasm; however, they are rapidly translocated into the nucleus following phosphorylation (Figure 3). The exact mechanism by which phosphorylated R-Smads enter the nucleus has been debated. Xiao and colleagues demonstrated that exposure of a C-terminal portion of the H2 helix in the MH1 domain of Smad, which contains the KKLKK sequence, was a critical nuclear localization sequence (187-189). The ability of this sequence to target Smad for nuclear translocation is dependent on phosphorylation by TβRI (188,190). Two groups have demonstrated that nuclear import of R-Smads is dependent on Smad3 binding to importin beta and that mutations in the MH1 domain prevented nuclear translocation of a Smad 3/4 complex but had no effect on phosphorylation, complex formation, or DNA binding affinity (187). In contrast, Xu et al. demonstrated that nuclear import of Smad 2/3 was dependent on binding of the MH2 domain to the nucleoporins CAN/Nup214 and Nup153 (191). This group illustrated that phosphorylation of Smad2 modifies its affinity for SARA but not CAN/Nup214 and Nup153 (191). This allows CAN/Nup214 and Nup153 to preferentially bind to a hydrophobic corridor in the MH2 domain of Smad2 and be translocated to the nucleus (191). This led the authors to conclude that by direct interaction with the nuclear pore complex, Smad undergoes constant nuclear/cytoplasmic shuttling in the absence of importins and the general export factor Crm-l (191). This constant nuclear/cytoplasmic shuttling is critical for maintaining sensitivity of TGF-β signaling. Despite contrasting evidence for which domain of Smad is responsible for nuclear translocation, it is clear that following phosphorylation, Smad can rapidly

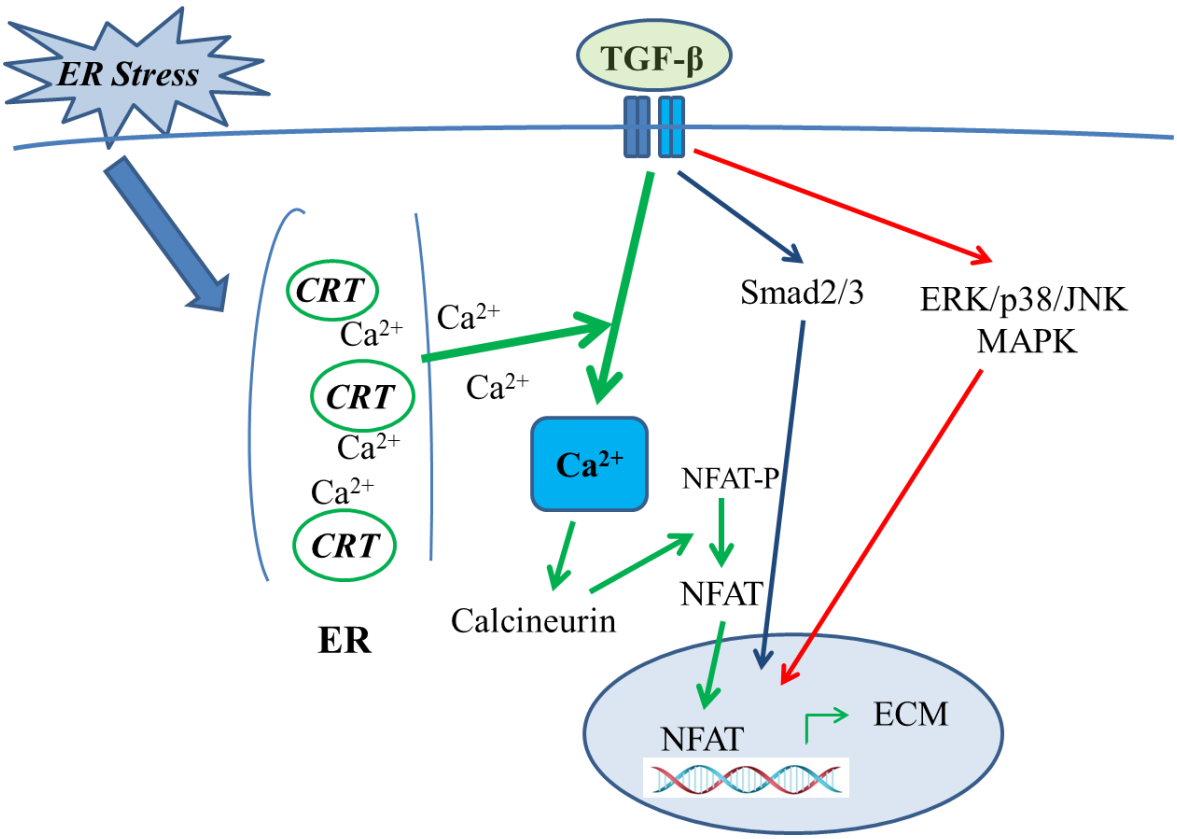

Figure 3. TGF-β signaling pathways involved in ECM production. Active TGF-β binding to its receptors induces signaling through several pathways. TGF-β is a potent inducer of Smad phosphorylation and nuclear translocation. TGF-β signals through the MAPK pathways including JNK, P38, and ERK MAP kinases. TGF-β induces rises in cytoplasmic Ca^{2+} levels in a CRT dependent manner leading to NFAT dephosphorylation and nuclear translocation.

translocate to the nucleus. Importantly, Smad 4 can also undergo nuclear/cytoplasmic shuttling independent of TGF-β signaling and that this shuttling is once again crucial for maintaining sensitivity of TGF-β signaling (192).

Smad-dependent gene transcription relies on Smad 3 and Smad 4 directly binding to the DNA. The optimal DNA binding sequence of Smad 3/4 contains only 4 base pairs, AGAC, although most naturally occurring DNA sequences are comprised of CAGAC(193,194). Dennler and colleagues demonstrated that a CAGA sequence within the plasminogen activator inhibitor-1 (PAI-1) promoter could drive TGF-β or activin dependent gene transcription (193). Despite the structural similarity between Smad 2 and 3, Smad 2 is unable to directly bind to DNA because of a 30 residue insertion which results in poor DNA binding ability (195). Despite the ability of Smad 3 and 4 to directly bind to DNA, their relative binding affinities for DNA is low (172). Rather, Smads function by interacting with several other DNA binding proteins and coactivators. This interaction is dependent on the MH2 domain of R-Smads which can bind and interact with transcriptional coactivators CBP, p300, ARC105 and Smif among others (196-199). R-Smads can also interact with corepressors to down-regulate gene transcription. Two transcriptional corepressors, c-Ski and SnoN can antagonize TGF-β signaling through direct binding with Smad 4 and the R-Smads (200). The c-Ski mediated inhibition of TGF-β signaling was primarily due to recruitment of histone deacetylase (HDAC) and interference of Smad-mediated binding to the transcriptional coactivator p300/CBP (200). TGF-β modulates corepressor inhibition of Smad driven gene transcription through other signaling cascades including ubiquitination and the Ras-MEK-MAPK pathways (172).

Termination of Smad signaling is controlled through ubiquitin-mediated degradation and phosphatase-mediated dephosphorylation of R-Smads. In 2006, Lin et al. demonstrated that PPM1A/PPC2α could dephosphorylate R-Smads in the SSXS motif (201). Overexpression of PPM1A/PPC2α abrogated Smad 2/3 phosphorylation mediated by constitutively active TβRI and that PPM1A/PPC2α could directly bind Smad 2/3 leading to nuclear export (201). Smad 1, 3 and 5 can be targeted for ubiquitination leading to proteasome-mediated degradation, although the E3 ligase responsible for ubiquitination appears to differ between R-Smads (202,203). In addition to direct action on R-Smads, modulation of Smad signaling can also occur at the type I and II TGF-β receptors. Chen et al. demonstrated that FKBP12 can directly bind to TβRI and inhibit TGF-β signaling (204). In addition, Smad 7 can inhibit TGF-β signaling through recruitment of protein phosphatase 1c (PP1c) to TβRI where it dephosphorylates key residues in the GS domain (205).

TGF-β signaling through non-Smad pathways

TGF-β is also capable of signaling through non-canonical pathways (Figure 3). TGF-β activates various branches of the MAPK pathway, Rho-like GTPase pathway, and PI3K/AKT pathway (206). TGF-β activates the ERK/MAPK pathway in epithelial cells, breast cancer cells and fibroblasts with a peak phosphorylation typically occurring several hours post stimulation (207-209). Although TGF-β receptors are well known to be serine/threonine kinases, evidence suggests that these receptors can also undergo tyrosine phosphorylation, thereby providing a rationale for their ability to activate MAPK pathways (206,210). Following tyrosine phosphorylation of the TGF-β receptors, a ShcA/Grb2/Sos complex is formed leading to Ras activation and downstream induction

of c-Raf, MEK and ERK (211). TGF-β induced activation of ERK may activate Smad as demonstrated by Hayashida et al. (212). In addition to ERK/MAPK signaling, TGF-β induces activation of the other MAPK pathways, predominantly JNK and p38. TGF-β induces p38 and JNK signaling through the MAP kinase kinase kinase (MAP3K), TGF-β-activated kinase 1 (TAK1), which induces activation of the MAP kinase kinase (MKK) (206). TGF-β activates JNK through MKK4 and p38 through MKK3/6 (213,214). The activation of these pathways is independent of Smad activity as cells expressing a mutant TβRI incapable of activating Smad but still retaining its kinase activity is sufficient to induce p38 MAPK activation (215). TGF-β activates RhoA-dependent signaling pathways in epithelial cells and primary keratinocytes (216,217). The activation of RhoA by TGF-β in epithelial cells resulted in EMT characterized by increased expression of N-cadherin and a down regulation of E-cadherin (216). TGF-β induces PI3K/Akt activation independent of Smad2/3 in a fibroblast cell line (218). TGF-β induces EMT in epithelial cells through phosphorylation of Akt at Ser473 (219). The ability of TGF-β to induce EMT through the PI3K/Akt pathway may be due to downstream effectors such as mammalian target of rapamycin (mTOR), which activate S6 kinase and eukaryotic initiation factor 4E-binding protein 1 (220). Other data suggest that TGF-β induced PI3K/Akt pathway engages in cross-talk with the Smad pathway. Remy et al. demonstrated that PI3K/Akt could inhibit Smad3 phosphorylation and nuclear translocation through direct physical interaction (221).

TGF-β stimulated Ca^{2+} release

TGF-β signaling through Ca^{2+} dependent pathways is now appreciated (Figure 3). The first evidence that TGF-β influenced cytoplasmic Ca^{2+} levels came from Muldoon

and colleagues who demonstrated that treatment of fibroblasts with TGF-β increased cytoplasmic Ca^{2+} (222). Treatment with TGF-β induced IP_3, which can bind to the IP_3 receptor on the ER membrane and induce Ca^{2+} release from the ER (222,223). The increase in cytoplasmic Ca^{2+} following TGF-β treatment could not be blocked by inhibitors of the L-type Ca^{2+} channels indicating that TGF-β was increasing cytosolic Ca^{2+} primarily through IP_3-mediated ER Ca^{2+} release (222). A study by McGowan and colleagues showed that TGF-β increases cytoplasmic Ca^{2+} levels in glomerular mesangial cells and that pharmacological inhibitors of the IP_3 receptor blocked this increase (224). Translocation of the type III IP_3 receptor, but not the type I IP_3 receptor, to the cell surface was required for TGF-β stimulated Ca^{2+} influx (224). The increase in cytoplasmic Ca^{2+} began approximately 5 minutes post TGF-β treatment and persisted 25 minutes following treatment (224). Maximal induction of TGF-β stimulated Ca^{2+} release, which was approximately 2-fold, occurred 30 minutes post treatment (224). TGF-β increased cytosolic Ca^{2+} in NIH3T3 cells which was dependent on ER Ca^{2+} as pretreatment with thapsigargin to deplete ER Ca^{2+} stores prevented this increase (225). The rise in cytoplasmic Ca^{2+} levels occurred rapidly following TGF-β treatment, with maximal induction occurring 7-12 minutes post treatment (225). Janowski et al. demonstrated that TGF-β stimulated Ca^{2+} release in MEFs was independent of extracellular Ca^{2+}, but required endogenous Ca^{2+} as inhibitors of IP_3 generation prevented TGF-β stimulated Ca^{2+} release (226). The exact mechanism by which TGF-β increased Ca^{2+} levels wasn't fully explored in this study, although it was shown to be dependent on c-Jun (226).

TGF-β can increase cytoplasmic Ca^{2+} independent of IP_3 dependent mechanisms. In MIN6 insulinoma cells, TGF-β stimulated an increase in cytoplasmic Ca^{2+} which was dependent on extracellular Ca^{2+} entry as nifedipine or nickel chloride inhibited this increase (227). In this study, TGF-β did not increase IP_3 or induce IP_3 mediated Ca^{2+} entry into the cytoplasm (227). Junn and colleagues demonstrated that TGF-β can increase cytosolic Ca^{2+} in a H_2O_2 dependent manner in human lung fibroblasts (228). Pretreatment of cells with N-acetylcysteine (NAC) or catalase, two antioxidants capable of reducing levels of H_2O_2, prevented TGF-β induced increases in cytoplasmic Ca^{2+} (228). In addition, inhibition of H_2O_2 production or Ca^{2+} increase with the Ca^{2+} chelator EGTA prevented TGF-β stimulated IL-6 production (228).

Calcium signaling

Ca^{2+} dependent signaling pathways

Ca^{2+} is an important second messenger involved in apoptosis, cell cycle regulation, and gene expression among others (229). Upon increased cytoplasmic Ca^{2+}, two main Ca^{2+}-dependent signaling pathways become activated, calcineurin/NFAT and CaMK. Several cytokines, hormones, neurotransmitters, and extracellular stimuli increase cytoplasmic Ca^{2+}concentrations (230). Multiple environmental stimuli, such as temperature fluctuations and mechanical stress, can induce Ca^{2+} oscillations within a cell (231). Depending on the stimuli, different mechanisms are responsible for increased intracellular Ca^{2+} leading to unique patterns of Ca^{2+} release (232). Tyrosine kinases and G-protein coupled receptors typically increase Ca^{2+} through IP_3 mediated ER Ca^{2+} release whereas voltage-gated and ligand-gated ion channels allow for influx of Ca^{2+} from the extracellular environment (229,233). Following increased intracellular Ca^{2+}, endogenous

Ca^{2+} stores are replenished through STIM-Orai mediated store operated Ca^{2+} entry (SOCE) from the extracellular environment (234). Therefore, it is imperative that the cell recognize and interpret signals in the proper manner. After extensive work, it has become appreciated that Ca^{2+} signals are decoded based on the frequency, amplitude and duration of the signal (232). Indeed, several studies have demonstrated that calcineurin/NFAT activation is a result of low frequency, low amplitude, sustained elevations in cytoplasmic Ca^{2+}, whereas CaMK activation occurs following high frequency, high amplitude, transient elevations in Ca^{2+} levels (232,235-239). Several other Ca^{2+}-dependent pathways are controlled by the frequency, amplitude and duration of Ca^{2+} release, including MAPK pathways, calpain, PKC and NF-κB (232). Importantly, TGF-β typically induces slow sustained increases in cytosolic Ca^{2+} suggesting preferential activation of the calcineurin/NFAT pathway by this cytokine.

Calmodulin

Propagation of Ca^{2+} signals within a cell depends on the frequency, amplitude and duration of Ca^{2+} in cytoplasm. Nevertheless, due to its rapid reuptake, it is imperative that Ca^{2+} interacts with other proteins to propagate its signal. Two groups of Ca^{2+}-associated proteins exist: the Ca^{2+} binding proteins and the Ca^{2+} sensing proteins (240). One group, including parvalbumin and calbindin, serve primarily as Ca^{2+} binding proteins and function as Ca^{2+} buffers or transporters (240). The most intensively studied Ca^{2+}-sensing protein is calmodulin, which is composed of 148 amino acids and contains 4 EF hand domains (229). Each EF hand domain binds to one Ca^{2+} ion with a relatively high affinity (μM range) (240). Two Ca^{2+} binding sites are found in the N and C terminal domains respectively, although the two C-terminal Ca^{2+} binding sites have 10 times

higher affinity for Ca^{2+} than the N-terminal sites (241). Following the initial binding of Ca^{2+} to the C-terminal domain of calmodulin, Ca^{2+} binds to the N-terminal domain causing a conformational change in calmodulin thereby exposing a hydrophobic pocket capable of binding other proteins (242,243). In addition, following Ca^{2+} binding to calmodulin, there is an increase in the α-helical content of the protein thereby facilitating interactions with calmodulin-binding proteins (244,245). Two of the most well studied calmodulin-binding proteins are CaMK and calcineurin.

Ca^{2+}-dependent CaMK activation

One target of calmodulin bound Ca^{2+} is the CaMK family of kinases consisting of myosin light chain kinase (MLCK) and the CaMK isoforms (I, II, III, IV) (229). CaMK are composed of an N-terminal kinase domain, an autoinhibitory and calmodulin binding domain, and, depending on the kinase, a C-terminal multimerization domain (229). Binding of calmodulin to CaMK occurs between amino acids 306-321 in CaMKII whereas the autoinhibitory domain resides between residues 295-299 (246-248). Binding of calmodulin to CaMKII in the regulatory domain causes a rearrangement of the catalytic domain allowing entry of ATP and substrate into the active site (249,250). In addition, binding of calmodulin to CaMK induces autophosphorylation in its kinase domain at Thr286 (251). Putkey and colleagues demonstrated that following autophosphorylation at thr286, calmodulin's affinity for CaMK is approximately 1000 times stronger, which they termed "calmodulin trapping" (252). This phenomenon allows calmodulin to tightly bind to CaMKs even if cytoplasmic levels of Ca^{2+} are at baseline levels (238). Eventually Ca^{2+} concentrations return to resting state and calmodulin disassociates from CaMK leading to autophosphorylation on residues 305 and

306 which causes optimal enzyme activity (253). Inactivation of CaMK is mediated by dephosphorylation of the critical residues involved in kinase activity (250).

Ca^{2+}-dependent calcineurin activation

Calcineurin is a serine/threonine phosphatase and is composed subunits A and B (41,43,254). It is found in virtually every tissue and cell where it is localized predominantly in the cytoplasm (254). Calcineurin was first identified and purified in 1978 by Klee and Krinks and is comprised of a Ca^{2+} binding calcineurin B subunit and a catalytically active calcineurin A subunit that also contains a calmodulin binding site and an autoinhibitory domain (254). Specifically, the N-terminal region of calcineurin A can interact with calcineurin B and calmodulin via amino acids 390-414 (43). Calcineurin B is comprised of 170 amino acids, shares 35% structural identity with calmodulin, and contains 4 Ca^{2+}-binding EF hand domains (255-257). Hashimoto et al. first identified a 25 amino acid sequence in the C terminus of calcineurin A that could completely inhibit the phosphatase activity the enzyme (258). Importantly, the authors concluded that this sequence was responsible for maintaining the low baseline phosphatase activity of calcineurin (258).

Activation of calcineurin occurs in response to sustained elevations in cytosolic Ca^{2+} and is dependent on Ca^{2+} binding to calmodulin and calcineurin B (41,42,259). Stemmer and Klee demonstrated that maximal activation of calcineurin occurs following addition of Ca^{2+} bound calmodulin; furthermore, they showed that calcineurin activity in the presence of calcineurin B was only 10% of calmodulin-bound calcineurin (259). The same group went on to show that binding of calcineurin B caused a conformational change in calcineurin A which facilitated optimal calmodulin binding and enzyme

activity (260). This study led the authors to conclude that optimal activation of calcineurin occurs when both calmodulin and calcineurin B are bound to calcineurin A (260). Calmodulin binding to its binding site on calcineurin A results in displacement of the autoinhibitory domain from the active site and optimal activity of calcineurin (261). Additional mechanisms of activating calcineurin include phospholipids and redox modifications of the catalytically active site (254).

Following activation, calcineurin can interact with and dephosphorylate a variety of targets including members of the AP-1 family of transcription factors, NF-κB, and NFAT (42). NFAT comprises five proteins related to the Rel/NF-κB family of transcription factors (262). NFAT contains 4 separate domains: the activation domain, the regulatory domain, the DNA binding domain, and the C-terminal domain (262). Within the regulatory domain of NFAT are two calcineurin docking sites, 13 phosphorylated serine residues responsible for maintaining calcineurin in the cytoplasm, and multiple transcription factor interaction sites (262). Of the 5 NFAT family members, four are regulated by calcineurin mediated dephosphorylation (262). NFAT proteins are phosphorylated and reside in the cytoplasm in resting cells; however, after calcineurin activation, they become dephosphorylated and migrate into the nucleus where they bind to DNA and regulate transcription. NFAT localization is also controlled by kinase phosphorylation while in the nucleus which promotes its return to the cytoplasm illustrating the tight regulatory control of this transcription factor.

Efficient dephosphorylation of NFAT by calcineurin requires a docking site which is present at the N terminus of the NFAT regulatory domain and has the consensus sequence PxIxIT (263,264). Upon calcineurin binding, NFAT becomes

dephosphorylated at 13 residues within the regulatory region leading to exposure of the nuclear localization sequence and masking of the nuclear export sequence (265,266). It is not known what order these phosphate groups are removed although Okamura and colleagues illustrated that the SRR1 region immediately adjacent to the PxIxIT docking site is preferentially dephosphorylated (265). In addition, following dephosphorylation in the cytoplasm, calcineurin translocates with NFAT into the nucleus where it maintains NFAT in a dephosphorylated state (267). However, if calcineurin activity is inhibited or Ca^{2+} levels reduced, NFAT becomes rephosphorylated by a variety of NFAT kinases and rapidly leaves the nucleus within 15 minutes (268-270). Porter et al. demonstrated that JNK, ERK, p38, and casein kinase II are all involved in regulating NFAT localization (266). JNK, ERK, and p38 can directly bind to and phosphorylate serine residues present in the regulatory region and overexpression of these kinases can successfully block ionomycin induced nuclear localization of NFAT (266). In addition, Beals and colleagues demonstrated that glycogen synthase kinase can phosphorylate NFAT and promote its nuclear export (271). The nuclear import and export proteins involved in shuttling of NFAT into and out of the nucleus are believed to be Rch1 and Crm1, respectively (272,273). Tight regulation of phosphorylation within the NFAT regulatory domain is critical for determining its subcellular localization.

Once NFAT is dephosphorylated and in the nucleus, it can either dimerize and directly bind DNA or form complexes with other transcription factors to bind DNA (262). NFAT homodimers can bind to the TNFα promoter through a κB-like site (274). In 2003, Giffin et al. demonstrated that NFAT homodimers could bind to the IL-8 promoter once again demonstrating the direct DNA binding ability of NFAT (275). In

addition to its ability to directly bind DNA, NFAT is often found in complex with other transcription factors, particularly members of the AP1 family. NFAT-AP1 complexes are known to contact a ~15 base pair stretch in which the two transcription factors are in direct contact with one another (276). Kel et al. demonstrated that NFAT-AP1 complexes bind at sites containing weak NFAT and strong AP1 binding sequences or weak AP1 and strong NFAT binding sequences suggesting strong cooperativity between these two transcription factors (277). The most well-known NFAT-AP1 complex is found on the IL-2 promoter where NFAT and AP1 partner to drive IL-2 dependent gene transcription (278). Likewise, NFAT-AP1 complexes can drive transcription of several other genes including GM-CSF and IL-4 (279). In addition to AP-1, NFAT can interact with several other transcription factors including Maf, ICER, GATA, Oct, HNF, MEF2, osterix, and PPAR-γ among others (262).

Ca^{2+} and NFAT in fibrosis

Importantly, NFAT has been implicated in regulation of collagen and other ECM proteins. Koga and colleagues demonstrated that NFAT can partner with osterix in osteoblasts to regulate type I collagen production (280). They showed that osteoblasts overexpressing NFAT had increased osterix-dependent activation of the Col1a1 promoter (280). Recently, Li et al. showed that downregulation of calcineurin and NFAT resulted in reduced mRNA levels of fibronectin, type IV collagen, and TGF-β in HK-2 epithelial cells (281). In cardiac fibroblasts, inhibition of calcineurin/NFAT signaling with either cyclosporine A or A285222 reduced myofibroblast formation and production of smooth muscle actin, collagen type I and collagen type III (282). Overexpression of NFAT resulted in an increase in type III, but not type I collagen (282). NFAT can also partner

with Smad to regulate gene transcription, although regulation of matrix proteins has not been shown to date (283). In addition, NFAT and calcineurin are involved in matrix production in response to TGF-β in vitro and in diabetic conditions in vivo (to be discussed in the next section).

Clinically, inhibition of calcineurin/NFAT signaling is important in preventing immune response leading to transplant rejection; therefore, several natural and synthetic inhibitors of this pathway have been developed (284). Cyclosporine (CsA) and FK506 were the first described inhibitors of calcineurin/NFAT signaling (285). However, inhibition of calcineurin/NFAT signaling only occurred when these compounds were in complex with their respective binding partner (286). Following association of CsA and FK506 with cyclophilin A and FK506 binding protein 12 (FKBP12) respectively, these complexes act as non-competitive inhibitors of calcineurin thereby limiting substrate access and masking the docking site for NFAT (284,287). In addition, several small peptide inhibitors of calcineurin/NFAT signaling have been developed. One of the more specific inhibitors is the PxIxIT family of peptides. As described earlier, this is the specific sequence on NFAT which is recognized and bound by calcineurin (288). This peptide is specific for calcineurin mediated NFAT dephosphorylation and not dephosphorylation of other phosphorylated proteins (263). Overexpression of VIVIT in Jurkat T cells prevented dephosphorylation of NFAT but not NF-κB demonstrating the selectivity of this peptide (264). Addition of 11 arginine residues to VIVIT enhances cell permeability and efficacy (289). Several additional inhibitors of calcineurin/NFAT signaling exist including A-285222, AKAP79, and CABIN (284).

TGF-β induction of ECM

TGF-β stimulation of ECM through Smad

TGF-β is a major regulator of ECM genes including collagen and fibronectin. Type I collagen is the major component of extracellular matrix and consists of two αI chains and one α2 chain. Following transcription, each chain is sent to the ER where it becomes hydroxylated allowing for formation of a triple helical procollagen protein (290). Procollagen is secreted from the cell and the N and C terminal residues removed to form mature type I collagen, which can combine with other processed type I collagens to form large collagen fibers (290). Ignotz and Massague provided initial data demonstrating that TGF-β induces production of collagen and fibronectin protein in multiple cell lines (291). Not only did TGF-β increase protein levels of collagen and fibronectin, but it also increased ECM protein secretion and incorporation into the ECM (291). The involvement of Smads in TGF-β stimulated matrix production was first recognized by Chen et al. in 1999 (292). This group illustrated that overexpression of Smad 3 or 4 caused trans-activation of a CAT reporter driven by a 772 basepair promoter region of type I collagen (292). In addition, they identified a CAGACA sequence, which binds to Smad in the PAI-1 promoter, in the proximal region of the type I collagen promoter (292). This landmark study also demonstrated that Smad bound to the CAGACA sequence following TGF-β treatment by the electromobility shift assay (292). Inhibition of Smad signaling with Smad 7 abrogated TGF-β stimulated activation of the collagen promoter providing clear evidence that TGF-β signals through Smad to induce type I collagen promoter activity (292). Following this groundbreaking discovery, several groups have confirmed the importance of Smads in regulating TGF-β signaling in a variety of cells and tissue contexts (293).

In addition to the direct DNA binding effects of Smad 3 and 4, the Smad proteins can form complexes with other transcription factors to induce ECM genes. Poncelet and colleagues demonstrated that Smad, in cooperation with Sp1, was responsible for driving activity of the type I collagen promoter (294). Furthermore, they demonstrated that inhibition of Smad or Sp1 was sufficient to inhibit TGF-β induced stimulation of the collagen promoter (294). Chung et al. demonstrated that AP-1 is also required for TGF-β induced trans-activation of a collagen reporter assay in NIH3T3 fibroblasts (295). This group showed that mutation of the AP-1 binding site in the collagen promoter reduced baseline activity and was required for TGF-β driven activation of the type I collagen reporter assay (295).

TGF-β stimulation of ECM through other pathways

TGF-β signals through the MAPK pathways to induce expression of ECM proteins. Sato et al. showed that p38 MAPK is activated following TGF-β treatment and that inhibition of p38 MAPK abrogated TGF-β induced type I collagen mRNA and promoter activity (296). Likewise, Hayashida and colleagues demonstrated that ERK MAPK was involved in regulating TGF-β stimulated collagen production in human mesangial cells (297). Blockade of the ERK pathway abrogated TGF-β induced type I collagen gene expression (297).

TGF-β induction of collagen transcription factor-1 (CTF1) in NIH3T3 cells is dependent on cytosolic Ca^{2+} increase and control of the Ca^{2+} dependent signaling pathways calcineurin and CaMK (225). Gooch and colleagues demonstrated that calcineurin is required for insulin growth factor-1 induced type IV collagen and fibronectin production in glomerular mesangial cells (298). In this study, calcineurin

activation led to nuclear translocation of NFAT suggesting that a calcineurin/NFAT pathway regulated production of ECM (298). Inhibition of calcineurin with CsA reduced STZ-mediated glomerular matrix production in a diabetic mouse model of nephropathy (299). In particular, mice receiving STZ and CsA had significantly reduced mRNA for both fibronectin and TGF-β in the glomeruli compared to mice receiving STZ only (299). In addition, treatment of mice with STZ resulted in increased calcineurin mediated NFAT nuclear translocation (299).

Direct evidence for calcineurin/NFAT involvement in TGF-β stimulated matrix production came from Gooch et al. in 2004. This group demonstrated that calcineurin is activated in a time dependent manner by TGF-β and that TGF-β stimulates matrix production 48 hours post treatment in glomerular mesangial cells (300). Pretreatment of cells with CsA reduced TGF-β stimulated NFAT nuclear translocation and prevented TGF-β stimulated fibronectin and collagen production (300). Furthermore, this group showed that TGF-β induced calcineurin activation is dependent on a slow steady increase in cytoplasmic Ca^{2+} levels following TGF-β treatment (300). The increase in Ca^{2+} levels is believed to be dependent on ROS production as pretreatment with an antioxidant prevented TGF-β stimulated Ca^{2+} release, calcineurin activation, and ECM production (300). Additional data demonstrate that TGF-β can induce direct binding of NFAT, but not AP-1, to the fibronectin promoter (301). This binding is inhibited by pretreatment with CsA or introduction of a dominant negative NFAT (301). TGF-β treatment induces NFAT nuclear translocation and DNA binding to a NFAT luciferase reporter assay which is inhibited by dominant negative NFAT (301). These data provide strong evidence for calcineurin/NFAT in regulating TGF-β stimulated ECM production.

TGF-β, VSMCs, and intimal hyperplasia

TGF-β is a profibrotic cytokine involved in the development of diabetic nephropathy, hepatic fibrosis and intimal thickening following arterial injury (302). Importantly, restenosis following arterial injury is primarily due to neointimal hyperplasia which involves the proliferation and migration of medial smooth muscle cells into the vascular lumen (303,304). The involvement of TGF-β in neointimal hyperplasia was first identified by Majesky et al. in 1991 who demonstrated increased TGF-β staining 24 hours post vascular injury (305). Since then, significant work has illustrated an important role for TGF-β in regulating VSMC proliferation, migration and ECM production in neointimal hyperplasia and restenosis. Introduction of a plasmid encoding TGF-β or recombinant TGF-β led to enhanced ECM production and intimal thickening (306,307). Furthermore, blockade of TGF-β with antibodies or a soluble TGF-β decoy receptor significantly attenuated neointimal formation clearly demonstrating an important role for TGF-β in regulating matrix production during neointima formation (308,309). Blockade of Smad signaling using adenoviral delivery of inhibitory Smad 7 attenuates neointimal formation and collagen production suggesting that targeting downstream TGF-β signaling may be an effective in treatment of neointimal hyperplasia in patients with restenosis (310).

The enhanced production of ECM *in vivo* in response to TGF-β is primarily due to its effects on the VSMCs. The effect of TGF-β on VSMCs appears to differ depending on the cell confluency, presence of additional growth factors, and concentration of TGF-β (302). Majack demonstrated that TGF-β inhibited platelet-derived growth factor (PDGF) induced VSMC proliferation in a dose dependent manner (311). The author also found

that inhibition of proliferation by TGF-β was dependent on the plating confluency as the growth of cells plated in monolayer was potentiated by TGF-β (311). Treatment of VSMCs with low doses of TGF-β (0.025 ng/ml) stimulated proliferation whereas treatment with higher doses of TGF-β (0.1 ng/ml) resulted in growth inhibition (312). TGF-β is also responsible for upregulating ECM proteins in VSMCs (313). Amento and colleagues provided initial data that TGF-β could induce type I and III collagen in VSMC culture (314). The critical region for TGF-β stimulated collagen production was localized to a 150 base pair fragment in the proximal region of the collagen promoter (315). Furthermore, smooth muscle cells isolated from injured rat aortas contain increased amounts of TGF-β and fibronectin mRNA as compared to uninjured aortic VSMCs (316). In addition to direct upregulation of ECM, TGF-β can also increase proteins associated with vascular injury. TGF-β induces expression of PAI-1 in endothelial cells as well as CTGF, indicating the multiple facets of TGF-β in vascular injury induced neointimal hyperplasia (302,317).

Angiotensin II (Ang II) regulates TGF-β expression and activation and can induce ECM production in VSMCs through TGF-β dependent and independent pathways (318). Rodriguez-Vita demonstrated that Ang II increased Smad 2 phosphorylation, nuclear translocation and induction of collagen and fibronectin (319). Furthermore, transfection with Smad 7 prevented Ang II induced collagen and fibronectin expression (319). Treatment with a p38 MAPK inhibitor prevented Smad 2 phosphorylation suggesting that Ang II activates the Smad pathway via MAPK activation and independent of TGF-β (319). Several animal models treated with Ang II show enhanced Smad pathway activation which can be blocked with type I Ang II receptor inhibitors (320).

Summary

Following injury, TGF-β and other cytokines are produced creating a unique milieu capable of inducing ECM production and fibrosis. It has recently become appreciated that ER stress is directly involved in promoting fibrotic and vascular fibroproliferative disease outcome. Several ER stress markers, including CRT, are increased in fibrotic and vascular fibroproliferative disease. Furthermore, evidence from our lab suggests that CRT regulates production of type I and III collagen and fibronectin transcript and protein. Based on these data, we hypothesize that the ER stress response protein, CRT, is a key component regulating TGF-β stimulated matrix production in fibrotic and vascular disease. The goal of these studies is to determine if CRT is regulating TGF-β stimulated matrix production *in vitro* and identify the potential mechanism through which this is occurring. In addition, we seek to determine whether CRT is involved in disease progression *in vivo* using a carotid artery ligation model of intimal hyperplasia. Our results demonstrate that CRT is required for TGF-β stimulated matrix production in fibroblasts and VSMCs. Furthermore, we show that CRT regulates this process through control of ER Ca^{2+} release and downstream calcineurin/NFAT activation. In addition, we show that CRT is important in vascular fibroproliferative disease as knockdown of CRT using US mediated Cre-recombinase-IRES-GFP delivery to CRT floxed mice significantly reduced neointimal hyperplasia and neointimal collagen production as compared to controls. Together, these studies show that CRT is a critical component of ER stress induced fibrotic and fibroproliferative disease and that CRT control of calcineurin/NFAT signaling may be involved in regulating ER stress induced disease.

Calreticulin regulates transforming growth factor-β-stimulated extracellular matrix production.

by

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CHAPTER 2

CRT REGUALTES TGF-β STIMULATED ECM PRODUCTION

Abstract

Endoplasmic reticulum (ER) stress is an emerging factor in fibrotic disease, although precise mechanisms are not clear. Calreticulin (CRT) is an ER chaperone and regulator of Ca^{2+} signaling upregulated by ER stress and in fibrotic tissues. Previously, we showed that ER CRT regulates type I collagen transcript, trafficking, secretion, and processing into the extracellular matrix (ECM). To determine the role of CRT in ECM regulation under fibrotic conditions, we asked whether CRT modified cellular responses to the pro-fibrotic cytokine, TGF-β. These studies show that CRT -/- mouse embryonic fibroblasts (MEFs) and rat and human idiopathic pulmonary fibrosis (IPF) lung fibroblasts with siRNA CRT knockdown had impaired TGF-β stimulation of type I collagen and fibronectin. In contrast, fibroblasts with increased CRT expression had enhanced responses to TGF-β. The lack of CRT does not impact canonical TGF-β signaling as TGF-β was able to stimulate Smad reporter activity in CRT -/- MEFs. CRT regulation of TGF-β stimulated Ca^{2+} signaling is important for induction of ECM. CRT -/- MEFs failed to increase intracellular Ca^{2+} levels in response to TGF-β. NFAT activity is required for ECM stimulation by TGF-β. In CRT -/- MEFs, TGF-β stimulation of NFAT nuclear translocation and reporter activity is impaired. Importantly, CRT is required for TGF-β stimulation of ECM under conditions of ER stress, since tunicamycin-induced ER stress was insufficient to induce ECM production in TGF-β

stimulated CRT -/- MEFs. Together, these data identify CRT-regulated Ca^{2+}-dependent pathways as a critical molecular link between ER stress and TGF-β fibrotic signaling.

Introduction

Endoplasmic reticulum (ER) stress is emerging as a factor in fibroproliferative diseases including pulmonary fibrosis, diabetic nephropathy, hypertension-associated cardiac fibrosis, and atherosclerosis (7,8,130,137,142,145,321,322). ER stress can be induced by glucose, glucosamine, and oxidative stress, factors known to induce fibroproliferative remodeling in multiple tissues (142,323,324). Chemical chaperones which reduce ER stress, such as valproate and 4-phenylbutyrate, reduce atherosclerosis and fibrosis (144,321,325,326) and also inhibit TGF-β-induced type I collagen production independent of Smad reporter activity (327). In addition, induction of ER stress by tunicamycin exacerbates lung fibrosis in the bleomycin model of pulmonary fibrosis, although tunicamycin alone did not induce fibrosis (137). Enhanced ER stress in alveolar epithelial cells facilitates epithelial to mesenchymal transition, a process which occurs in some forms of fibrosis (136). Despite growing evidence for ER stress as factor in fibrosis, the mechanisms by which ER stress predisposes to or exacerbates fibrosis are not clear. In the lung, ER stress induced alveolar epithelial cell apoptosis is thought to be a significant factor in the development of fibrosis (136,137,322). However, ER stress is also associated with fibroproliferative remodeling in tissues such as the diabetic vasculature where apoptosis is not a significant initiating factor (7,321). This suggests that ER stress can drive pathways that promote fibrosis through additional mechanisms. Calreticulin (CRT) is a 46 kDa ER protein that regulates cellular responses to stress through its roles in the unfolded protein response and its chaperone activity (30,328). In

addition, CRT also is important in ER Ca^{2+} buffering and regulation of downstream Ca^{2+}-dependent signaling pathways such as calcineurin and NFAT (nuclear factor of activated T cells) (30). Calreticulin -/- MEFs have decreased ER Ca^{2+} stores and impaired agonist induced Ca^{2+} release from the ER, whereas cells overexpressing CRT have enhanced Ca^{2+} binding depots within thapsigargin sensitive ER stores (36,43). Impaired ER Ca^{2+} release in the absence of CRT leads to defects in downstream Ca^{2+}/calcineurin signaling with reduced NFAT and MEF2C (myocyte enhancer factor 2c) nuclear translocation (34,44). CRT -/- mice can be rescued from embryonic lethality by constitutively active calcineurin, which induces MEF2C and NFAT translocation to the nucleus providing evidence that CRT is an upstream modulator of calcineurin signaling (34,45,46).

Our lab recently demonstrated that CRT regulates transcription of multiple extracellular matrix (ECM) proteins in a Ca^{2+}-dependent manner and that it has post-transcriptional effects on collagen trafficking and matrix assembly (6). CRT regulation of fibronectin is also thought to involve ER Ca^{2+} (50,52). Interestingly, CRT expression is increased in multiple models of fibrosis including bleomycin-induced pulmonary fibrosis, the UUO model of renal fibrosis, and in chronic diseases of fibroproliferative remodeling, such as atherosclerosis (7,8,321). Cardiac specific overexpression of CRT during development results in interstitial fibrosis, although the mechanisms have not been defined (329). CRT expression is upregulated by factors which are known to induce both ER stress and fibrosis, including glucose, oxidative stress, cigarette smoke, hypoxia, and TGF-β (8,321,330-332).

TGF-β is a major stimulus of ECM production in fibroproliferative diseases (333). TGF-β stimulation of fibrotic pathways occurs primarily through Smad 2/3

dependent pathways, although the importance of other TGF-β stimulated pathways, including PI3K, ERK, and p38 MAPK, is now recognized. (212,214,334-336). Ca^{2+}-dependent pathways also regulate TGF-β stimulation of ECM (300,301). TGF-β treatment leads to increased cytosolic Ca^{2+}, which induces calcineurin-mediated NFAT dephosphorylation and enhanced expression of fibronectin (300,301). Furthermore, constitutively active calcineurin or NFAT increases fibronectin promoter activity in mesangial cells, suggesting a role for Ca^{2+}-regulated NFAT in control of TGF-β driven matrix production (301). TGF-β can increase IP3 levels, thereby causing release of ER Ca^{2+} (222). In addition, TGF-β can increase cytoplasmic Ca^{2+} through translocation of type III IP3 receptors to the cell surface, through stimulation of H_2O_2-mediated Ca^{2+} release, or through a c-Jun dependent mechanism (224,226-228).

Given our previous findings that CRT regulates fibronectin and type I collagen transcription and the known role of CRT in modulating calcineurin-NFAT activity, we asked whether CRT might play a role in regulating TGF-β stimulation of ECM proteins (6,34,46). These studies show that CRT is required for cellular responsiveness to TGF-β. CRT mediates TGF-β responsiveness through regulation of TGF-β stimulated Ca^{2+} release and NFAT activity. Furthermore, CRT is required for TGF-β stimulation of ECM in tunicamycin-treated cells. Together, these data provide evidence that CRT is a critical regulator of TGF-β mediated ECM production and establish a new mechanism by which ER stress contributes to fibrosis.

Experimental procedures

Materials Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose was purchased from Invitrogen (Madison, WI). LY364947, SB203580,

tunicamycin, L-ascorbic acid, protease inhibitor cocktail, phosphatase inhibitor and ionomycin were purchased from Sigma (St. Louis, MO). DMEM and D-PBS were purchased from Cellgro (Manassas, VA). 11R-VIVIT and Hoechst (cat # 382061) were purchased from CalBiochem (Billerica, MA). A285222 was a gift of Abbott labs. Fluo-4 AM was purchased from Life Technologies (Carlsbad, CA). NFAT (GGAGGAAAAACTGTTTCATACAGAAGGCGT) and Smad (AGCCAGACA) Cignal reporter assay kits were purchased from SA Biosciences (Valencia, CA). TGF-β was purchased from R&D Systems (Minneapolis, MN). Goat anti-fibronectin (cat # 6952), rabbit anti-β-tubulin (cat # 9104), mouse anti-NFATc3 (cat # 8405) and rabbit anti-GRP78 (cat # 13968) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-Smad3 (cat # 9520S) and rabbit anti-phospho-Smad2 (cat # 3101) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit anti-β-actin IgG (cat # IMG-5142A) was purchased from IMGENEX (San Diego, CA). Rabbit anti-collagen type I IgG (cat # 203002) was purchased from MDbioproducts (St. Paul, MN). Rabbit anti-collagen Iα2 (cat # ab96723) was purchased from Abcam (Cambridge, MA). Mouse anti-Smad 2/3 (cat # 610842) was purchased from BD Transduction Laboratories. AlexaFluor 488 goat anti-rabbit IgG and AlexaFluor 488 goat anti-mouse IgG were purchased from Invitrogen (Madison, WI). Peroxidase conjugated AffiniPure rabbit anti-goat IgG (Cat # 305-035-003), goat anti-rabbit IgG (111-035-003) and goat anti-mouse IgG (115-035-146) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Western Lightning Chemiluminescence Reagent Plus was purchased from PERKinElmer Life Sciences (Waltham, MA) and Re-Blot strong stripping solution was purchased from Chemicon (Temecula, CA).

Cells Wild type mouse embryonic fibroblasts (MEFs), CRT -/- MEFs, calreticulin -/- MEFs stably transfected with the pcDNA3 expression vector to express rabbit HA-tagged CRT were gifts from Dr. Marek Michalak (University of Alberta, Edmonton, Alberta, Canada). Calreticulin -/- MEFs stably transfected with HA-tagged CRT lacking the TSP1 binding domain (aa19-36) were generated as described previously (94). Mouse L fibroblasts (parental cells and CRT overexpressors) were provided by Dr. Michal Opas (University of Toronto). These cell lines were engineered to overexpress (CRT overexpressors) CRT by 1.6 fold compared to parental cells as described previously (97). Rat lung fibroblasts (RFL6) stably expressing an empty vector (pcDNA3.1, Invitrogen) were generated as previously described (gift of Dr. James Hagood) (337). Human IPF lung fibroblasts were provided by Dr. Victor Thannickal (UAB). L fibroblasts were maintained in high (4.5 g/L) glucose DMEM with 10% FBS in the presence of 100 μg/ml G418 sulfate (Cellgro).

Quantitative Real Time PCR Cells were grown overnight in complete media containing 10% FBS, starved in low (0.5%) serum media overnight and treated with TGF-β or other compounds. After treatment, RNA was harvested with TRIZOL reagent and isolated according to manufacturer's specification. Quantitative real time PCR was performed using standard protocols with an Opticon instrument (MJ Research, model CFD-3200). Primers for mouse type I collagen (*Col1A1*) (Cat # PPM03845F-200), fibronectin (Cat # QT00135758), CRT (Cat # QT00101206) and S9 (Cat # PPM03695A) were obtained from Qiagen and verified by melt curve analysis. Transcript levels were assayed using SYBR green from Qiagen. Results were calculated using the delta delta CT method and are expressed as the mean +/- S.D. of three samples each assayed in triplicate

as indicated in figure legends. Results are representative of at least 2-3 separate experiments.

Immunoblotting for ECM proteins Following treatment, cells were harvested using 1X Laemmli lysis buffer (Bio-Rad, Hercules, CA) containing 1X protease inhibitor cocktail (Sigma cat # p8340). Following lysis, cells were sonicated for 7 seconds, 5% final β-mercaptoethanol was added and samples were boiled at 100 °C for 7 minutes. Samples were centrifuged and equal volumes were loaded in to 4-15% or 10% SDS-polyacrylamide gels. After separation by SDS-PAGE, samples were transferred onto a PVDF membrane at 100 volts for 100 minutes. Following transfer, membranes were blocked with 1% casein followed by application of the primary antibody. Membranes were washed in TBS-T and secondary antibody was applied for 1 hour at room temperature. Membranes were washed with TBS-T and developed using Western Lightning Chemiluminescence Reagent Plus (PERKinElmer Life Sciences). Membranes were stripped and reprobed with rabbit anti-β-tubulin or rabbit anti-β-actin IgG to normalize for cell protein. Densitometric analysis of immunoblots was performed using the NIH Image J program. Data are expressed as the mean band density normalized for cell protein from at least three separate experiments.

Deoxycholate extraction of the extracellular matrix fraction Deoxycholate (DOC) extractions of detergent soluble and insoluble fractions were performed similarly to previous reports (5). Briefly, wild type and CRT -/- MEFs were plated in full serum (10%) DMEM for 24 hours, switched to low serum (0.5%) DMEM with 20 μM ascorbic acid and treated with 100 pM TGF-β for 24 hours. After 24 hours, wells were rinsed with PBS and harvested by scraping with 300 μl of 4% DOC (4% DOC in 20 mM Tris-HCL,

pH 8.8 with 1x protease inhibitor). Lysates were homogenized with a 27 ½ gauge needle and tumbled overnight at 4°C. Precipitates containing the DOC insoluble portion were pelleted at 13,500 x *g* and washed 3 times with 4% DOC solution. The supernatant containing the DOC soluble fraction was removed, placed into a new tube and centrifuged twice as above. The DOC insoluble pellet was resuspended in 30 µl Laemmli buffer.

Soluble collagen assays Wild type and CRT -/- MEFs were cultured for 48 hours in DMEM with 10% FBS, switched to DMEM with 0.5% FBS and 20 µM ascorbic acid and treated with or without 10 pM TGF-β for 72 hours. Cells were dosed daily with TGF-β and ascorbic acid in 0.5% FBS. Conditioned medium was collected in the presence of protease inhibitor cocktail (Sigma) and centrifuged at 15,000 x g for 5 minutes to remove cellular debris. Soluble collagen in the media was measured using the Sircol assay (Biocolor, Ireland) as described by the manufacturer.

siRNA transfection of Thy-1 (-) rat lung fibroblasts and human IPF lung fibroblasts Non-targeting siRNA (SI03650325) or rat CRT siRNA (SI04449004) were purchased from Qiagen and resuspended in RNase free water. One million Thy-1 (-) rat lung fibroblasts were transfected via nucleofection using the MEF1 Nucleofector Kit from Amaxa Biosystems (Amaxa GmbH, Lonza) in an Amaxa Nucleofector II using program A-023. Transfected cells were cultured in DMEM with 10% FBS for 24 hours. Medium was switched to low serum (0.5% FBS) media for two hours followed by treatment with 100 pM TGF-β for 24 hours. Cells were washed with Dulbecco's phosphate buffered saline (D-PBS) (Cellgro) and lysed with 1X Laemmli buffer containing 1X protease inhibitor.

Human IPF lung fibroblasts were transfected with non-targeting siRNA or human CRT siRNA (SI02654589) resuspended in RNase free water. One million human IPF lung fibroblasts were transfected via nucleofection using the primary fibroblasts Nucleofector Kit from Amaxa Biosystems (Amaxa GmbH, Lonza) in an Amaxa Nucleofector II using program A-023. Transfected cells were cultured in DMEM with 10% FBS for 48 hours. Medium was switched to low serum (0.5% FBS) DMEM for 6 hours followed by treatment with 2 μM ascorbic acid and 100 pM TGF-β for 24 hours. Cells were washed with D-PBS and lysed with 1X Laemmli buffer containing 1X protease inhibitor.

Tunicamycin experiment Wild type and CRT -/- MEFs were plated with or without 0.01 μg/ml tunicamycin in DMEM with 10% FBS containing 20 μM ascorbic acid for 24 hours as previously described (6). Cells were treated with or without 100 pM TGF-β for 24 hours. Cells were washed with D-PBS and lysed with 1x Laemmli lysis buffer containing 1x protease inhibitor.

Immunofluorescence Wild type and CRT -/- MEFs were plated on glass coverslips in a 24 well plate in DMEM with 10% FBS for 24 hours and then switched to low serum (0.5% FBS) medium overnight. The next day, cells were treated with TGF-β, fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X for 3 minutes. Cells were washed with D-PBS and blocked for one hour with filtered, sterile 1% casein. Primary antibody was added as follows: rabbit anti-phospho-Smad 2 or 3 at a 1:150 dilution; mouse anti-NFATc3 at a 1:100 dilution in 1% casein solution overnight at 4°C. Cells were washed with PBS and the appropriate secondary AlexaFluor488 antibody (1:500 dilution) was added for 1 hour at room temperature.

Following washing, cells were incubated with 4 μg/ml Hoechst for 5 minutes. Images were obtained using a Nikon Eclipse TE2000-U inverted microscope equipped for epifluorescence with a Nikon camera or a Zeiss LSM 710 confocal microscope. Non-immune IgG and secondary antibody alone were used as negative controls. Images in a particular experiment were obtained using a uniform exposure time and images adjusted uniformly.

Ca^{2+} release assay Cells were plated in 24 wells plates in complete (10% FBS) media overnight. The next day, low serum (0.5%) FBS media containing 5 μM Fluo-4 AM was added to the cells. Dye was loaded into the cells for 20 minutes at 37°C. The plate was allowed to equilibrate at 37°C for 5 minutes. Following equilibration, cells were treated with either TGF-β (100 pM) or ionomycin (1 μM) as indicated. Cells were excited at 485 nm and emission measured at 520 nm every ten seconds for thirty minutes.

Reporter assays Wild type and CRT -/- MEFs were transfected with 2 μg of NFAT or Smad firefly luciferase reporter constructs with control renilla luciferase purchased from SABiosciences. Cells were transfected using the MEF 1 Nucleofector Kit from Amaxa Biosystems (Amaxa GmbH, Lonza) in Amaxa Nucleofector II using program A-023. Transfected cells were cultured in complete (10% FBS) media overnight and GFP expression was confirmed the next morning. Cells were starved in low serum (0.5% FBS) media and treated as indicated. Following treatment, cells were lysed with 1X lysis buffer (Promega) and firefly and renilla luciferase activity measured using a Dual Glo Luciferase kit from Promega according to the manufacturer's instructions. Luciferase reporter activity is normalized to the renilla luciferase control. Luciferase

reporter construct data are representative of at least three individual experiments each performed in triplicate.

Statistics Data were analyzed for statistical significance using one-way analysis of variance with Holm-Sidak post-hoc analyses (Sigma Stat). $p < 0.05$ was considered significant.

Results

Calreticulin is required for TGF-β mediated stimulation of collagen and fibronectin transcript and protein Wild type and CRT -/- MEFs were treated with TGF-β and levels of transcript compared by RTQ-PCR. TGF-β stimulated a significant increase in *fibronectin* and *COL1A1* transcript in wild type MEFs (Figure 4A,B). In contrast, TGF-β failed to stimulate an increase in either *COL1A1* or *fibronectin* transcript in MEFs lacking CRT (Figure 4A,B). Levels of *COL1A1* and *fibronectin* transcript were significantly increased 4 hours post stimulation and were maintained for up to 24 hours in wild type cells (Figure 4C,D). The lack of response in CRT -/- MEFs is not due to a delay in response to TGF-β, since no increase is observed over a 24 hour period with *COL1A1* or 12 hours for *fibronectin* (Figure 4C,D).

The failure of TGF-β to stimulate type I collagen and fibronectin transcription in CRT -/- MEFs correlates with a lack of protein stimulation as measured in cell lysates 24 hours after TGF-β treatment (Figure 5). In addition, the increased ECM expression in TGF-β-treated wild type cells is due to increased protein synthesis and not incorporation of serum fibronectin into the extracellular matrix as differential extraction of the DOC cell soluble from DOC insoluble extracellular matrix (338) shows increased fibronectin in the cellular fraction following TGF-β treatment (Figure 6A). Similarly, TGF-β failed

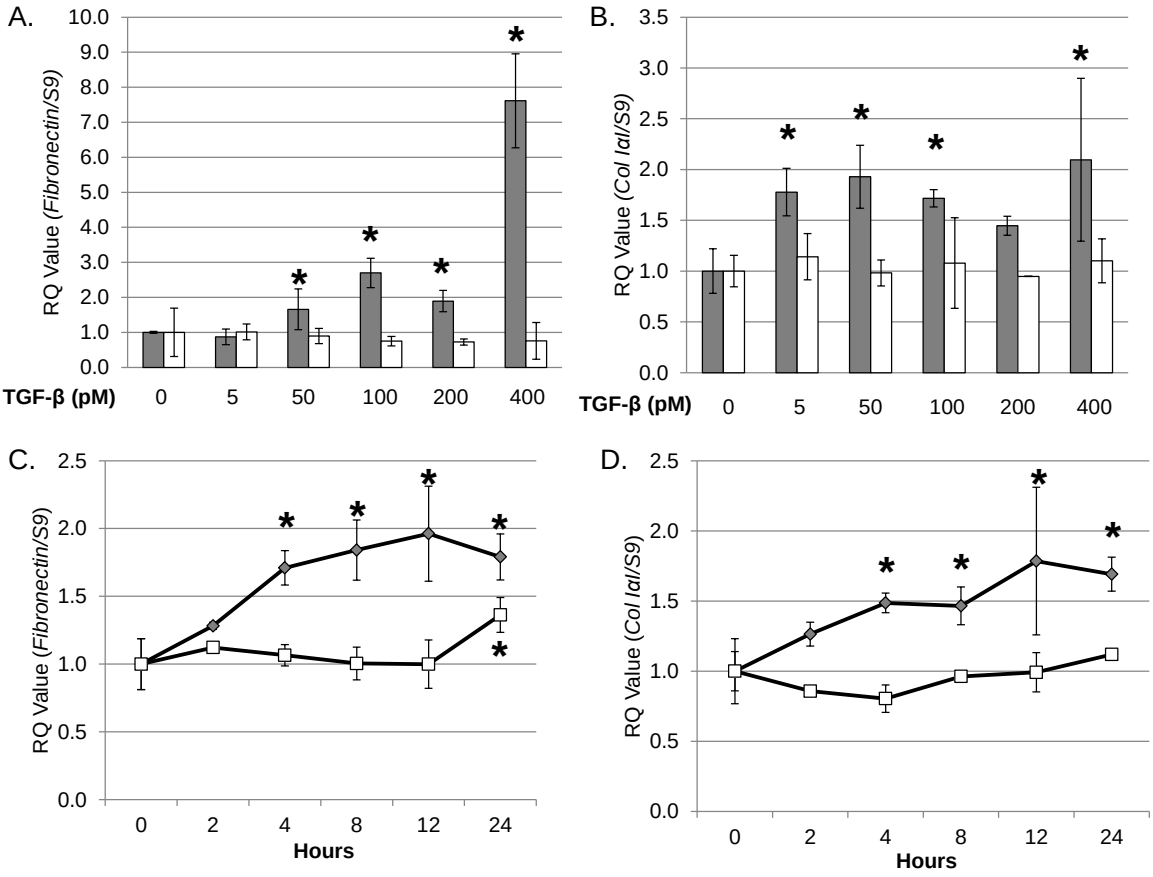

Figure 4. TGF-β induces fibronectin and COL1A1 transcript in wild type but not CRT -/- MEFs. Wild type (grey bars, grey symbols) and CRT -/- MEFs (open bars, open symbols) were grown overnight in media with 10% FBS, starved for 12 hours in low serum (0.5% FBS) media and treated with increasing concentrations of TGF-β for 4 hrs (A, B). Cells were also treated with 100 pM TGF-β over a 24 hour time period (C,D). RNA was harvested by TRIZOL and transcript levels of *fibronectin* (A, C) and *COL1A1* (B, D) determined by quantitative real time PCR. Values represent mean levels normalized to S9 +/- S.D. of triplicate samples each performed in technical triplicates. Values for untreated wild type and CRT -/- cells were set to 1. Each assay shown is representative of three separate experiments with similar results. * $p<0.05$ vs non-treated or time zero cells.

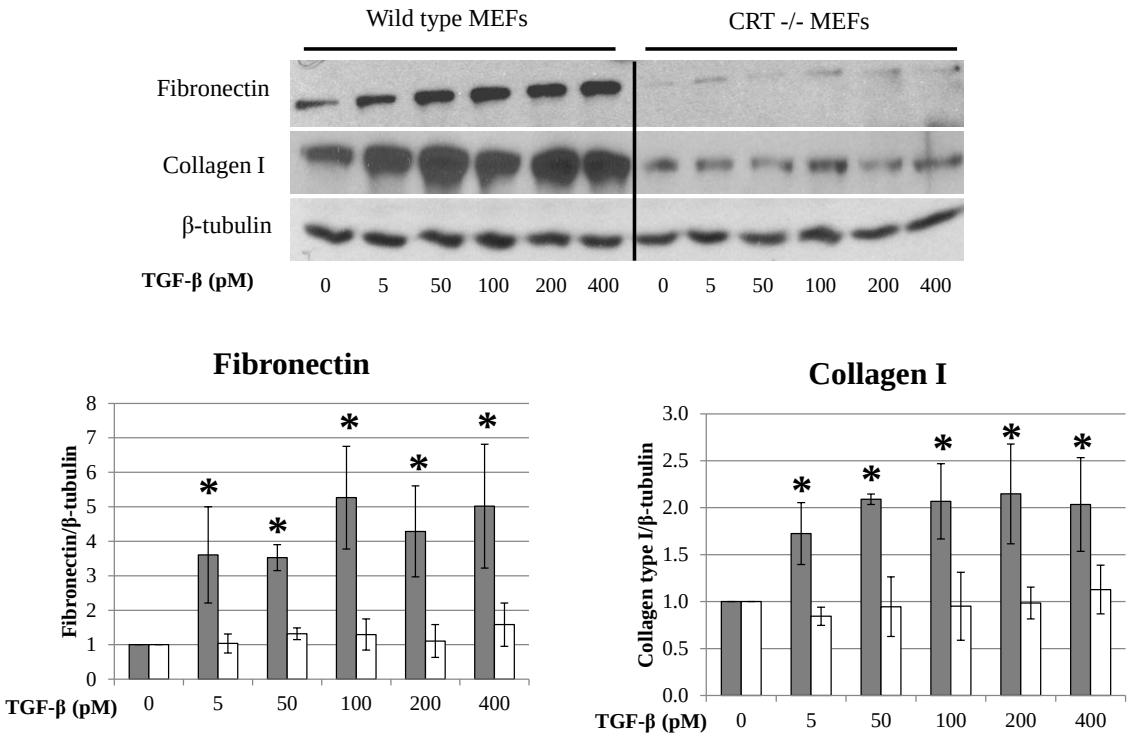

Figure 5. CRT is required for TGF-β stimulation of fibronectin and collagen I protein. Wild type (grey bars) and CRT -/- MEFs (open bars) were grown overnight in media with 10% FBS, starved for 2 hours in low serum (0.5% FBS) media and then treated with 100 pM TGF-β with 20 μM ascorbic acid for 24 hours. Laemmli cell lysates were immunoblotted for fibronectin and collagen. Results of a representative blot are shown. Results are the mean density of bands normalized to β-tubulin +/- S.D. (n=4 separate experiments).

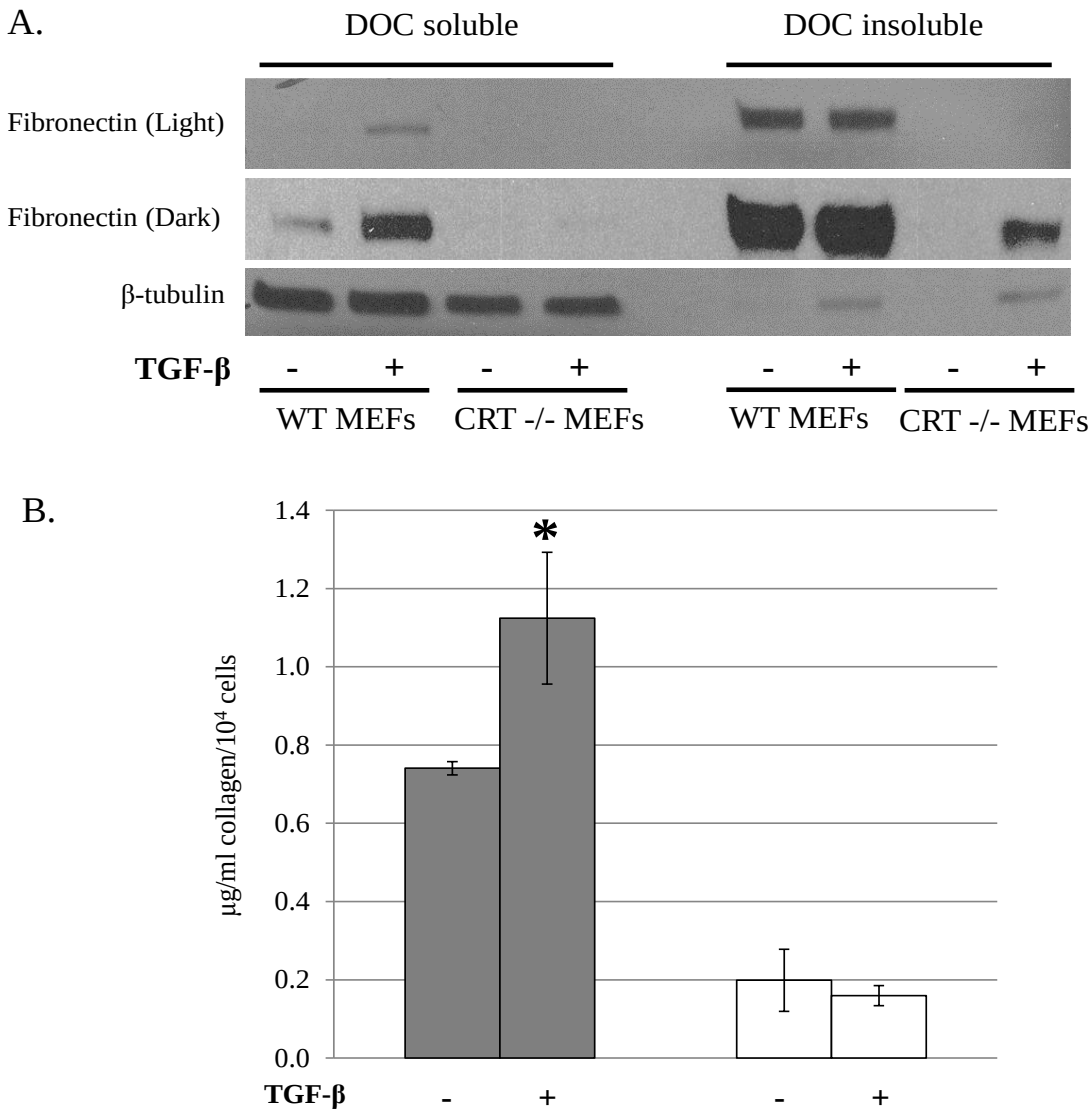

Figure 6. CRT is required for TGF-β stimulation of fibronectin and collagen I protein into the ECM. **(**A) Wild type and CRT -/- MEFs were grown overnight in media with 10% FBS, starved for 2 hours in low serum (0.5% FBS) media and then treated with 100 pM TGF-β with 20 µM ascorbic acid for 24 hours. (A) The 4% DOC soluble (cell fraction) and insoluble (extracellular matrix) fractions of treated cells were harvested with laemmli buffer, separated by SDS-PAGE, and immunoblotted for fibronectin. Membranes were re-probed with antibody to β-tubulin to determine loading and efficacy of fractionation of the cellular and extracellular matrix fractions. (B) Wild type (gray bars) and CRT -/- MEFs (white bars) were plated in media with 10% FBS overnight, switched to low serum media with 20 µM ascorbic acid and treated with 10 pM TGF-β for 72 hours, re-dosing TGF-β and ascorbic acid every 24 hours. After 72 hours, conditioned media from triplicate samples were pooled and levels of secreted soluble collagen measured by Sircol assay according to manufacturer's specifications. Results are means +/- S.D. from three separate experiments. *$p<0.05$ vs non-treated cells.

to stimulate an increase in secreted soluble collagen in the conditioned media of CRT -/- MEFs (Figure 6B). In these studies, both wild type and CRT -/- MEFs were stimulated with TGF-β in the presence of 20 μM ascorbic acid. We showed previously that CRT is a collagen chaperone and that CRT -/- MEFs have reduced ER to golgi trafficking and secretion of collagen, which is corrected by ascorbic acid (6). Ascorbic acid increases collagen transcript stability and is a cofactor for proline hydroxylation of procollagen, which enhances translation and secretion efficiency (339,340).

To test whether re-expression of CRT in the CRT -/- cells can rescue responsiveness to TGF-β, CRT -/- MEFs stably transfected with HA-tagged CRT were stimulated with TGF-β and transcript measured 4 hours post stimulation (94). Similar to wild type cells, TGF-β induced a significant increase in COL1A1 and fibronectin transcript production in CRT -/- MEFs stably transfected with HA-tagged CRT (Figure 7A).

Since cell surface CRT can act as a receptor for the matricellular protein thrombospondin 1 (TSP1) and stimulate collagen production, we examined whether TSP1 binding to cell surface CRT might be involved in regulating cellular responsiveness to TGF-β. TSP1 binding to cell surface CRT does not appear to be important for cellular responsiveness to TGF-β, since CRT -/- MEFs stably expressing CRT lacking the TSP1 binding site were able to respond to TGF-β (Figure 7B) (5,94).

Knockdown of CRT in fibrogenic lung fibroblasts inhibits the ability of TGF-β to stimulate ECM We next determined whether CRT expression is important for TGF-β-induced ECM production in fibroblasts known to be highly responsive to TGF-β. Thy-1 (-) lung fibroblasts predominate in the fibrotic foci of lungs with idiopathic pulmonary

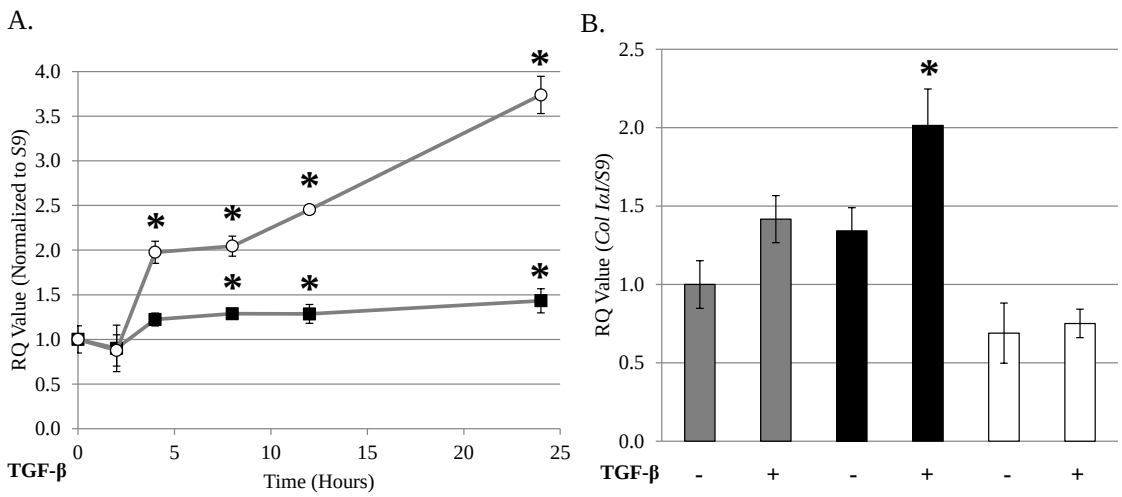

Figure 7. Impaired responsiveness to TGF-β in the CRT -/- MEFs can be rescued by transfection with CRT plasmid or CRT plasmid lacking the TSP1 binding site. (A) CRT -/- MEFs stably transfected with rabbit HA-tagged CRT were grown overnight in media with 10% FBS, starved overnight in low (0.5%) serum media, and treated with 100 pM TGF-β for 4 hours. RNA was harvested by TRIZOL and transcript levels of *fibronectin* (open circles) and *COL1A1* (closed squares) were determined by quantitative real time PCR. (B) Wild type MEFS (gray bars), CRT -/- MEFs stably expressing rabbit CRT lacking the TSP1 binding site (black bars), and CRT -/- MEFs (white bars) were treated as in (A), RNA, harvested and levels of *COL1A1* determined by RTQ-PCR. Results are the means normalized to S9 levels of triplicate samples +/- S.D. from one representative experiment. Experiments were repeated on three (A) or two (B) separate occasions. Means of untreated cells were set to 1. *$p<0.05$ vs untreated cells.

fibrosis and rat thy-1 (-) fibroblasts have robust production of ECM in response to TGF-β (337,341,342). SiRNA knockdown of CRT to 60% of control levels in the Thy-1 (-) rat lung fibroblasts blocked the ability of TGF-β to stimulate type I collagen and fibronectin protein (Figure 8A-D). Similar results were obtained by siRNA knockdown of human CRT in lung fibroblasts isolated from IPF patients (Figure 9). In these studies, knockdown of CRT to 35% of control levels reduced baseline ECM levels and attenuated TGF-β stimulated collagen I and fibronectin protein as compared to cells transfected with NT siRNA.

TGF-β induces a greater stimulation of ECM in cells overexpressing CRT CRT and other ER stress response proteins are increased in several models of fibrosis, including bleomycin–induced lung fibrosis and unilateral ureteral obstruction renal fibrosis (8). Therefore, we asked whether overexpression of CRT correlates with enhanced ECM production in response to TGF-β. L-fibroblasts overexpressing CRT (~1.6 fold increase in CRT) have increased collagen I and fibronectin transcript as compared to parental L-fibroblasts at baseline (6). TGF-β treatment induced a greater increase in fibronectin and collagen I protein as compared to TGF-β-stimulated parental cells (Figure 10).

ER stress in the absence of CRT is not sufficient to stimulate ECM production in response to TGF-β Increased ER stress exacerbates response to fibrogenic stimuli in vivo and in vitro (137,327,343). Since our data indicate that CRT is required for TGF-β stimulated ECM production, we asked whether increased ER stress in the absence of CRT is sufficient to stimulate ECM production or whether CRT is a critical component of ER stress-induced ECM production. Wild type and CRT -/- MEFs were treated with

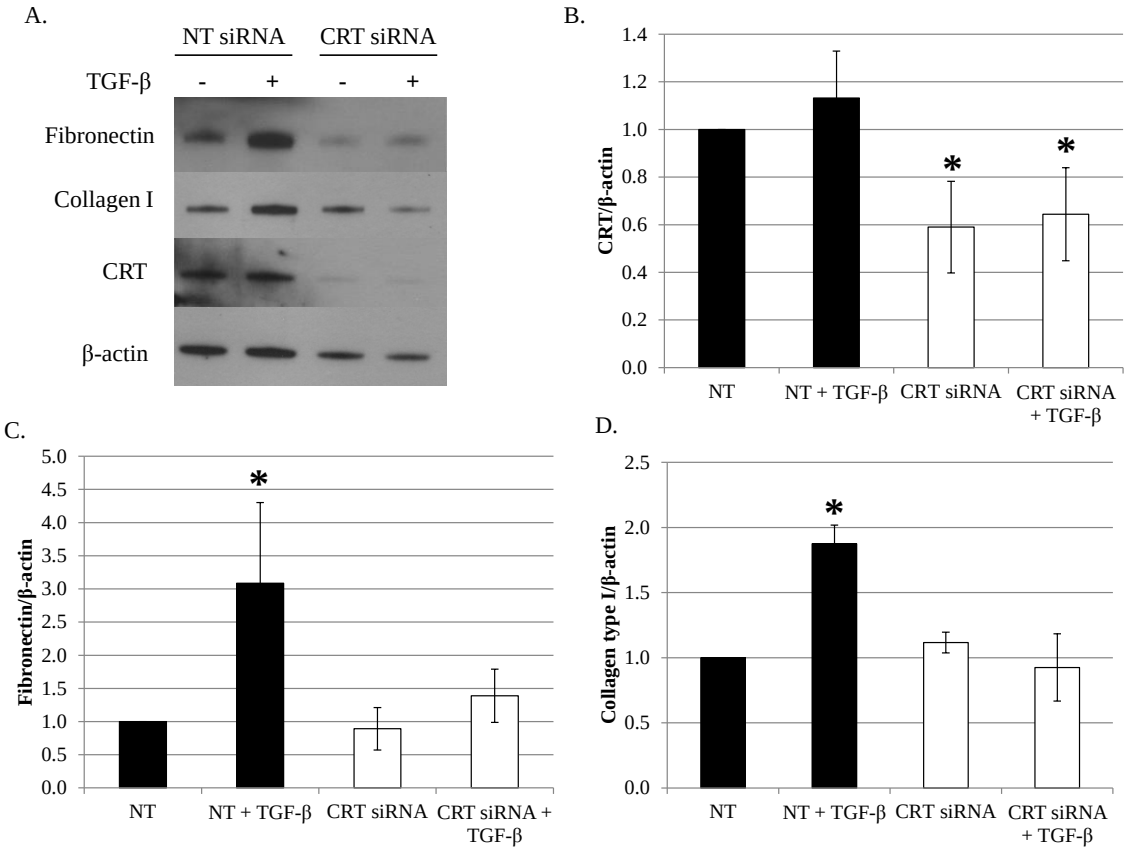

Figure 8. Knockdown of CRT in Thy1 -/- rat lung fibroblasts significantly inhibits TGF-β stimulated matrix production. Thy1 -/- rat lung fibroblasts were transfected with 100 nM non-targeting (NT) or CRT siRNA in media with 10% FBS and maintained for 24 hours. Cells were switched to low (0.5%) serum media and stimulated with 100 pM TGF-β for 24 hrs. Laemmli cell lysates were immunoblotted for (B) CRT, (C) fibronectin, and (D) collagen type I. A representative blot is shown in (A). Results are the mean density normalized to β-actin +/- S.D from three separate experiments.

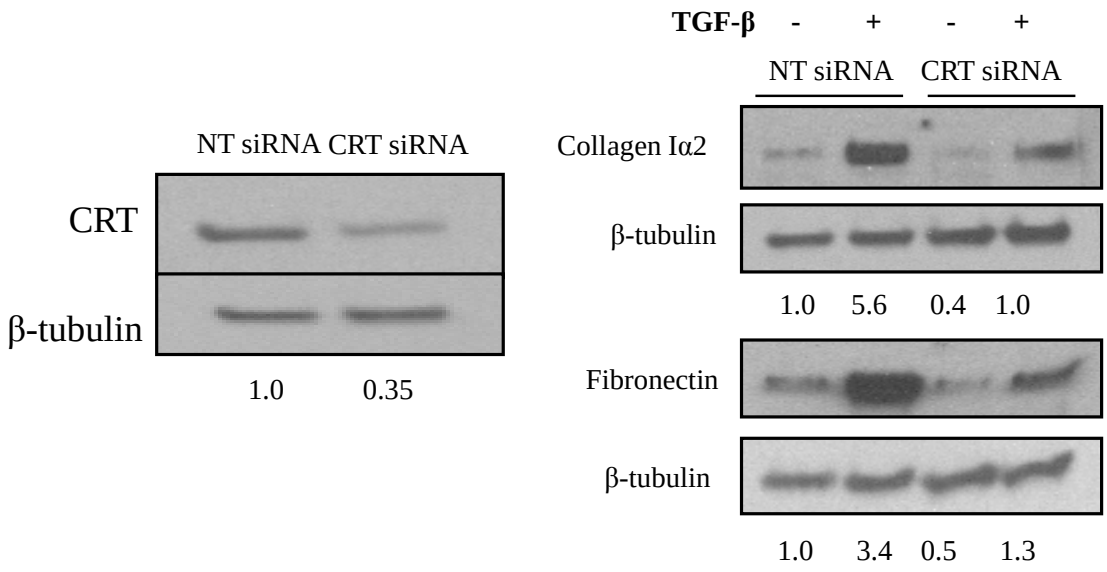

Figure 9. Knockdown of CRT in Human IPF fibroblasts significantly inhibits TGF-β stimulated matrix production. Human IPF lung fibroblasts were transfected with 200 nM non-targeting (NT) or CRT siRNA and maintained in media with 10% FBS for 48 hours. Cells were switched to low FBS media for 6 hours and then treated with 100 pM TGF-β with 2 μM ascorbic acid for 24 hours. Laemmli cell lysates were separated by SDS-PAGE and immunoblotted for CRT, fibronectin, collagen type Iα2, and β-tubulin. Densitometric analysis of bands normalized to β-tubulin are indicated below each band. Results are representative of 3 separate experiments. *$p<0.05$ vs untreated cells.

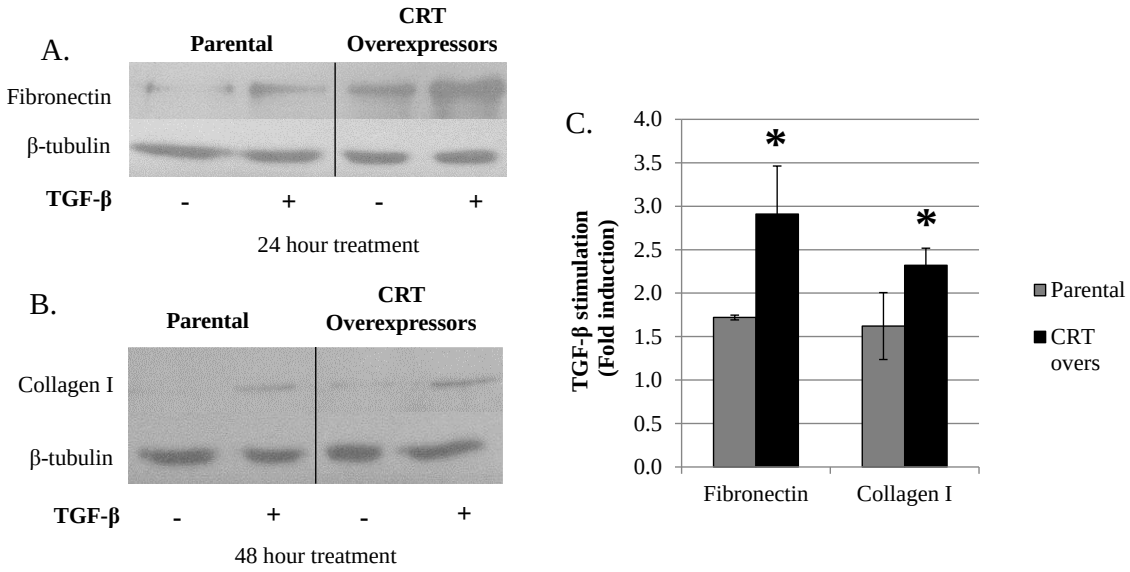

Figure 10. Overexpression of CRT increases TGF-β stimulation of ECM. Parental and CRT overexpressing L fibroblasts were grown overnight in media with 10% FBS, starved for 2 hours in media with low (0.5%) serum, and treated with 100 pM TGF-β for 24 hr for (A) fibronectin determinations or (B) 48 hours for collagen I determinations. Cells were harvested with Laemmli buffer, separated by SDS-PAGE and immunoblotted for fibronectin, collagen I, and β-tubulin. (C) Bands were analyzed by densitometry (n=3 separate experiments) and normalized to β-tubulin +/- S.D. Untreated cells were set to 1.0. *$p<0.05$ vs parental cells.

TGF-β in the presence or absence of the ER stress inducer tunicamycin. In the presence of tunicamycin, both wild type and CRT -/- MEFs showed increased levels of the ER stress response protein GRP78, although tunicamycin increased GRP78 to a greater extent in CRT -/- MEFs as compared to wild type cells (Figure 11A,B). In the presence and absence of tunicamycin, wild type MEFs increased collagen I levels when treated with TGF-β, although TGF-β stimulation of collagen I was not enhanced in tunicamycin treated cells as compared to cells treated with TGF-β alone (Figure 11A,C). However, enhanced ER stress due to tunicamycin treatment was not able to overcome the failure of CRT -/- MEFs to stimulate ECM in response to TGF-β (Figure 11A,C). Interestingly, tunicamycin did not increase CRT expression in wild type cells (data not shown). These data show that tunicamycin-induced ER stress is not sufficient for induction of ECM by TGF-β in the absence of CRT.

TGF-β-dependent Smad signaling is active in wild type and CRT -/- MEFs TGF-β signals ECM production primarily through Smad dependent pathways, although other pathways can mediate TGF-β signaling (335,336,344). To determine if Smad signaling is impaired in the CRT -/- MEFs, we examined wild type and CRT -/- MEFS for Smad 3 phosphorylation following TGF-β stimulation. Phosphorylated Smad 3 was detected in both TGF-β treated wild type and CRT -/- MEFs, suggesting that TGF-β engagement of its signaling receptors and receptor Smad phosphorylation are not defective in CRT -/- MEFs (Figure 12A). Since CRT -/- MEFs are defective in their ability to induce MEFC2 nuclear translocation (44), we asked whether phosphorylated Smad2/3 can be imported into the nucleus in CRT -/- MEFs treated with TGF-β. Immunofluorescence staining of TGF-β stimulated wild type and CRT -/- cells showed similar nuclear translocation of

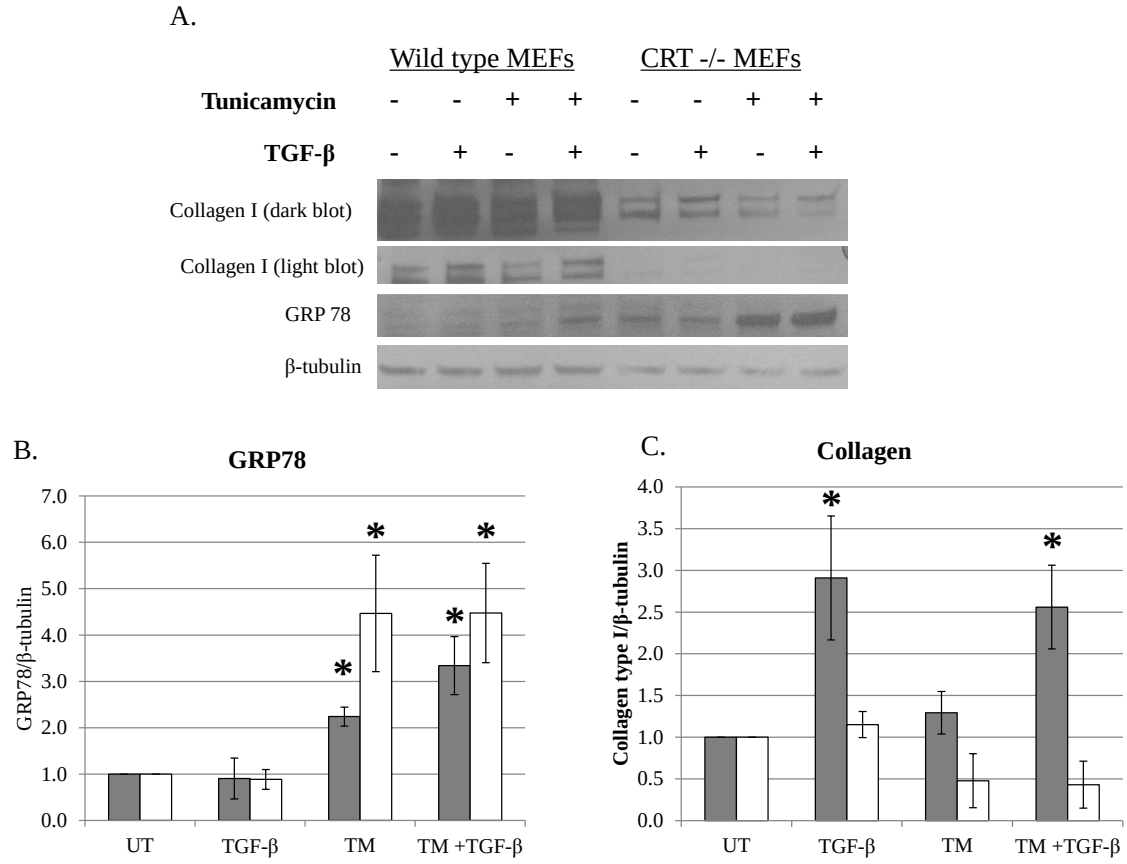

Figure 11. ER stress is insufficient to drive TGF-β stimulation of ECM in CRT -/- MEFs. (A) Wild type (gray bars) and CRT -/- MEFs (white bars) were grown overnight in DMEM with 10% FBS and 20 μM ascorbic acid with or without 0.01 μg/ml tunicamycin. Cells were treated with 100 pM TGF-β in low (0.5%) serum media containing 20 μM ascorbic acid for 24 hours. Laemmli cell lysates were immunoblotted for collagen I, GRP78 or β-tubulin. (A-C) Bands were analyzed by densitometry and normalized to β-tubulin. Results are mean densities normalized to β-tubulin +/- S.D from three separate experiments. *$p<0.05$ vs untreated cells.

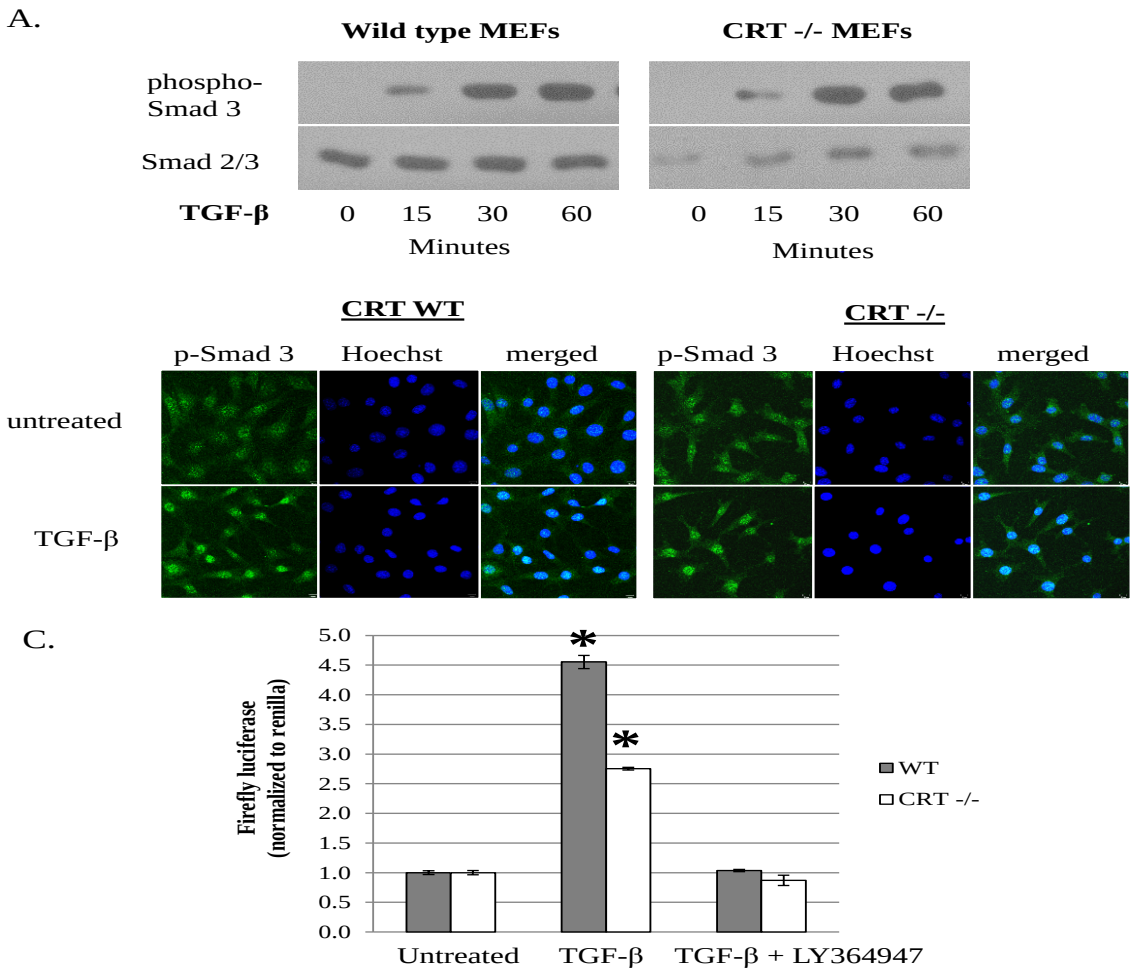

Figure 12. TGF-β stimulates Smad activity in wild type and CRT -/- MEFs. (A) Wild type and CRT -/- MEFs were grown overnight in media with 10% FBS, starved overnight in low (0.5%) serum media and treated with 100 pM TGF-β for 15, 30, or 60 minutes. Laemmli cell lysates containing phosphatase inhibitor were immunoblotted for phospho-Smad 3. Membranes were reprobed with antibody to Smad 2/3 or β-tubulin (data not shown) to normalize cell protein. The blot is representative of 4 separate experiments using 10-400 pM TGF-β. (B) Wild type and CRT -/- MEFs were grown overnight on glass coverslips in media with 10% FBS, starved in low serum media overnight, and treated with 100 pM TGF-β for 30 minutes. Cells were fixed, permeabilized, and incubated with antibody to phospho-Smad 3 followed by fluorescein conjugated secondary antibody. Nuclei were stained with Hoechst. Results are representative of 3 separate experiments. Confocal images were obtained at an original magnification of 600X. (C) Wild type and CRT -/- MEFs were transfected with the Smad 2/3/4 firefly luciferase reporter construct and the control renilla luciferase construct and kept in media with 10% FBS overnight, switched to low serum DMEM, and treated with 100 pM TGF-β for 8 hours. Some cells were treated with 100 pM TGF-β in the presence of 3 μM LY364947. Cells were lysed with 1X lysis buffer (Promega) and triplicate samples combined. Luciferase reporter activity is normalized to the renilla luciferase control. Data represent the means of samples from three separate experiments +/- S.D. * $p<0.05$ vs untreated control.

phosphorylated Smad 3 (Figure 12B). Smad 2 phosphorylation and nuclear translocation were also similarly stimulated by TGF-β in CRT -/- MEFs (data not shown). Furthermore, Smad binding to Smad-binding DNA elements is not deficient in the absence of CRT as TGF-β is able to induce Smad 2/3/4 reporter activity in wild type and CRT -/- MEFs (Figure 12C). LY364947, an ALK5 TGF-βR1 inhibitor, blocked TGF-β stimulation of Smad 2/3/4 reporter activity by both wild type and CRT -/- MEFs in this assay, suggesting that TGF-β receptor signaling is not deficient in CRT -/- MEFs (Figure 12C). We also confirmed that Smad signaling is important for TGF-β stimulation of ECM in wild type MEFs, since treatment of wild type MEFs with LY364947 significantly impaired TGF-β induced collagen and fibronectin transcript (data not shown). Finally, levels of active and total TGF-β were not decreased in the conditioned media of CRT -/- MEFs (data not shown).

CRT is required for TGF-β induction of cytosolic Ca^{2+} TGF-β can stimulate the slow release of intracellular Ca^{2+}, which activates the calcineurin/NFAT pathway to increase fibronectin expression in mesangial cells (227,235,270,300,345). Since CRT regulates both ER Ca^{2+} and calcineurin/NFAT activity (30,44,45), we asked whether TGF-β stimulation of Ca^{2+} release was altered in CRT -/- MEFs. Wild type and CRT -/- MEFs were treated with TGF-β or the Ca^{2+} ionophore, ionomycin, as a positive control (346). Ca^{2+} release was measured over time using the Ca^{2+} binding fluorescent dye Fluo-4 AM. With ionomycin, both wild type and CRT -/- MEFS induced a large increase in cytosolic Ca^{2+}, although Ca^{2+} re-uptake occurred more slowly in CRT -/- MEFs (Figure 13A,B). In contrast, TGF-β increased cytoplasmic Ca^{2+} levels in wild type, but not in CRT -/- MEFs (Figure 13A,B), suggesting that CRT is required for TGF-β-dependent

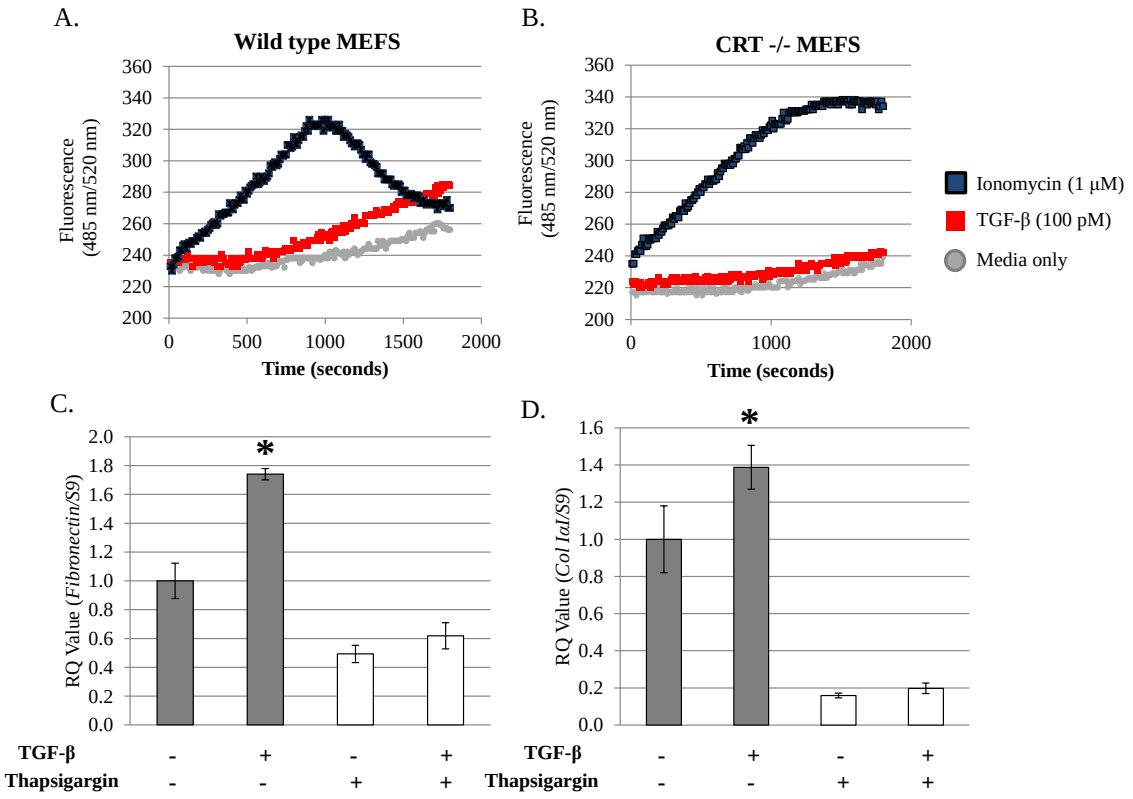

Figure 13. TGF-β stimulates Ca^{2+} release and Ca^{2+} dependent fibronectin and COL1A1 transcript are impaired in the CRT -/- MEFs. (A) Wild type and (B) CRT -/- MEFs were plated overnight in DMEM with 10% FBS, washed with low (0.5%) serum media and loaded with 5 µM Fluo-4 AM in low serum media with 10 mM HEPES. Cells were loaded with dye for 20 minutes at 37° C. After a 5 min equilibration, cells were stimulated with 100 pM TGF-β (red squares), 1 µM ionomycin (blue squares), or low serum media (grey circle). Cells were excited at 485 nM and emission read at 520 nM. Results are representative of a typical experiment repeated in quadruplicate on at least 4 different occasions. (C and D) Wild type MEFs were plated overnight in media with 10% FBS and starved overnight in low (0.5%) serum medium. Cells were pre-treated with thapsigargin (0.5 µM) for 30 minutes, and washed with low serum media to remove the thapsigargin. Cells were treated with or without 100 pM TGF-β for 4 hours. RNA was harvested with TRIZOL and transcript levels of (C) *fibronectin* and (D) *COL1A1* were determined by RTQ-PCR. Values represent the mean expression levels normalized to S9 +/- S.D. of triplicate samples from a single representative experiment. Experiments were repeated three times with similar results.

Ca^{2+} signaling.

To determine whether TGF-β stimulation of cytosolic Ca^{2+} release is important for stimulation of ECM, wild type MEFs were treated with TGF-β in the presence or absence of thapsigargin, a SERCA2b inhibitor which blocks ER Ca^{2+} re-uptake to effectively deplete ER releasable Ca^{2+} (347). Thapsigargin blocked the ability of TGF-β to stimulate collagen I and fibronectin transcript (Figure 13C,D), suggesting that TGF-β stimulation of cytosolic Ca^{2+} release is required for induction of ECM proteins. Cyclopiazonic acid, a SERCA2b inhibitor which acts in a manner similar to thapsigargin (348), also inhibited TGF-β stimulated ECM production (data not shown). To determine if increased cytoplasmic Ca^{2+} is sufficient to support TGF-β driven ECM expression in the absence of CRT, CRT -/- MEFs were treated with TGF-β in the presence of ionomycin. Despite an increase in cytoplasmic calcium with ionomycin, TGF-β was still unable to increase ECM transcript in CRT deficient cells (Figure 14), suggesting that while CRT-mediated calcium regulation is critical for TGF-β-driven ECM stimulation, calcium alone is not sufficient and other CRT-dependent factors are likely important.

CRT is required for TGF-β stimulation of NFAT activity The activity of the transcription factor NFAT is regulated by Ca^{2+}-activated calcineurin and NFAT activity can regulate TGF-β stimulated ECM production in the presence of sustained increases in cytoplasmic Ca^{2+} (300,301). Given the importance of CRT for TGF-β stimulation of cytoplasmic Ca^{2+} levels and the deficient NFAT activation in CRT -/- mouse embryos (34,46), we asked whether TGF-β stimulation of NFAT activity is defective in CRT -/- MEFs. TGF-β induces NFATc3 isoform nuclear translocation in wild type cells, whereas NFATc3 remained in the cytoplasm following TGF-β treatment of CRT -/- MEFs (Figure

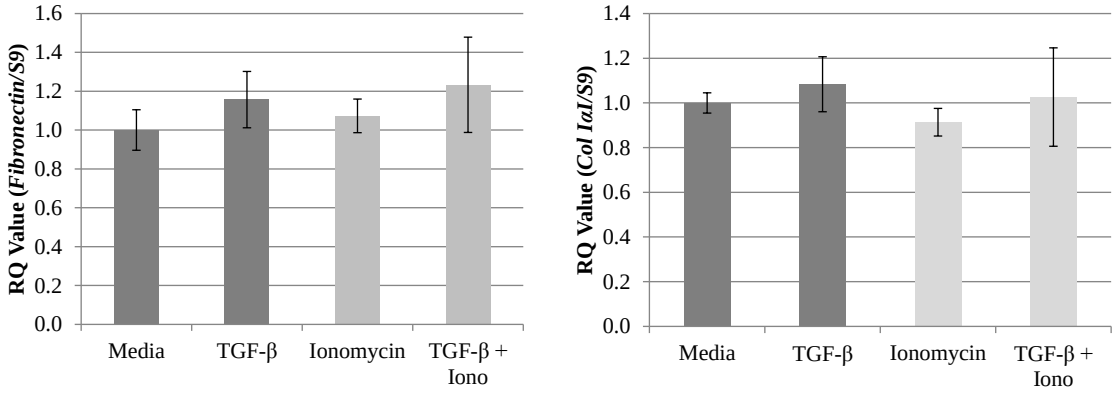

Figure 14. Increasing cytoplasmic Ca^{2+} with ionomycin is insufficient to induce fibronectin and COL1A1 transcript following TGF-β treatment in CRT -/- MEFs. CRT -/- MEFs were plated overnight in media with 10% FBS, starved overnight in low serum medium and treated with TGF-β (100 pM), ionomycin (1μM), or both for 4 hours. RNA was harvested with TRIZOL and transcript levels of *fibronectin* and *COL1A1* were determined by RTQ-PCR. * $p<0.05$ vs untreated control.

15A) (349). Furthermore, TGF-β stimulation NFAT reporter activity is also absent in CRT -/- MEFs (Figure 15B).

NFAT activity is required for TGF-β stimulated ECM production To determine if TGF-β stimulation of NFAT activity is important for production of type I collagen and fibronectin, we asked whether inhibition of NFAT activity blocked TGF-β stimulation of fibronectin and collagen transcription. We used a cell permeable peptide, 11R-VIVIT, which specifically blocks calcineurin binding to the NFAT PxIxIT sequence and prevents calcineurin-dependent NFAT dephosphorylation and nuclear translocation (264,349). 11R-VIVIT blocked TGF-β stimulation of ECM in wild type MEFs (Figure 15C,D) and in human lung fibroblasts (data not shown). As expected, 11R-VIVIT had no effect on CRT -/- MEF ECM production (data not shown). Similarly, another NFAT inhibitor, A285222, which maintains NFAT in the cytoplasm regardless of calcineurin activity (350), showed a dose dependent inhibition of TGF-β stimulated collagen and fibronectin transcript (data not shown). These results show that NFAT activity is necessary for TGF-β stimulation of ECM transcription in wild type MEFs and that CRT is required for TGF-β stimulation of NFAT activity.

Discussion

ER stress is emerging as a significant factor in fibrotic disorders (8,137). Although ER stress induced apoptosis is a contributing factor in fibrosis, a complete understanding of the mechanistic roles of ER stress in fibrosis remains to be defined. In our present studies, we have shown that the ER stress induced protein, CRT, is a critical regulator of fibrogenic responses to TGF-β. We show that CRT regulation of cytosolic Ca^{2+} and NFAT activity is required for TGF-β stimulation of collagen type I and

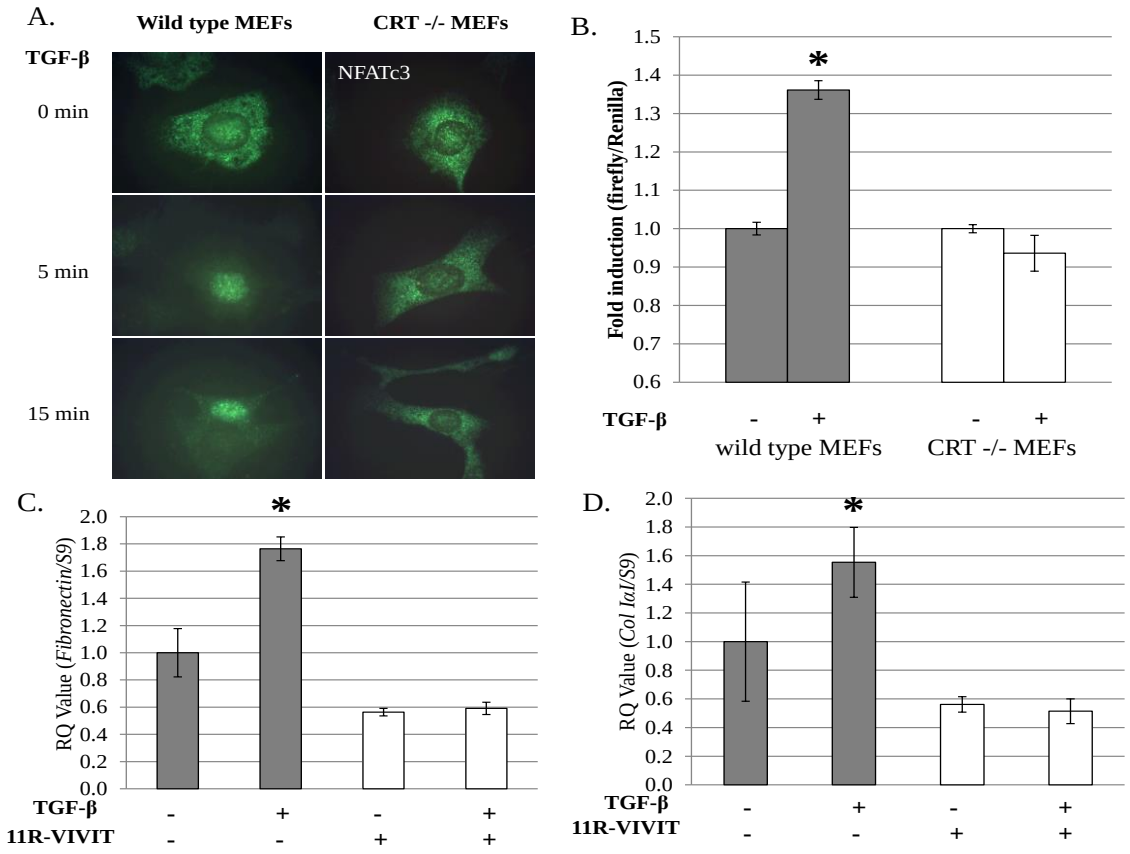

Figure 15. CRT -/- MEFs do not stimulate NFAT activity in response to TGF-β (A) Wild type and CRT -/- MEFs were grown overnight on glass coverslips in media with 10% FBS, starved overnight in low (0.5%) serum media and stimulated with 400 pM TGF-β for 5 or 15 minutes. Following treatment, cells were fixed, permeabilized, and incubated with anti-NFATc3 antibody. Cells were washed with PBS and incubated with a fluorescein labeled secondary antibody. Results are representative of one experiment performed on at least three separate occasions. Original magnification = 1000X (B) Wild type (gray bars) and CRT -/- MEFs (open bars) were transfected with an inducible NFAT reporter firefly luciferase reporter construct and a renilla luciferase control construct overnight in media with 10% FBS. Cells were starved for 2 hours in low serum media and then treated every 2 hrs with 100 pM TGF-β over an 8 hour span. After 8 hours, cells were lysed and triplicate samples combined. Luciferase reporter activity is normalized to the renilla luciferase control. Data represent the mean normalized luciferase activity +/- S.D. of one representative experiment performed in triplicate. The experiment was performed on three separate occasions with similar results. *p <0.05 vs untreated control. (C and D) Wild type MEFs were grown overnight in media with 10% FBS, starved overnight in low serum media, and pretreated with 2 μM 11R-VIVIT for 30 minutes (open bars). Cells were treated with or without 100 pM TGF-β +/- 11R-VIVIT peptide for 4 hours and RNA was harvested with TRIZOL. Transcript levels of *fibronectin* (C) or *COL1A1* (D) were determined by RTQ-PCR and normalized to S9 levels +/- S.D. Results are from one experiment of triplicate samples. Similar results were obtained in three separate experiments. *p<0.05 vs untreated control.

fibronectin transcript. Knockout or knockdown of CRT abrogates cellular responsiveness to TGF-β even in the presence of active Smad 2/3 signaling. Consistent with these observations, cells with increased CRT expression exhibit a relative increase in responsiveness to TGF-β. Importantly, these studies show that tunicamycin-induced ER stress is not sufficient to support TGF-β stimulation of ECM in the absence of CRT. These studies identify a critical mechanistic link between ER stress and fibrosis. CRT expression is increased in a number of different fibrotic tissues, including lung, kidney, and diabetic atherosclerotic vasculature ((7,8) and unpublished data), suggesting an involvement in fibrotic processes. CRT acts as a collagen chaperone to mediate collagen ER-golgi trafficking, processing and incorporation into the ECM (6). In addition, CRT regulation of Src-dependent fibronectin expression and matrix deposition impacts collagen matrix assembly (6,50). Our current studies illustrate that CRT regulation of Ca^{2+}-dependent NFAT activity is required for the ability of TGF-β to stimulate ECM transcription.

TGF-β is a pro-fibrotic cytokine that drives expression of ECM proteins and integrin ECM receptors (291,351-353). TGF-β signals through both the canonical Smad pathway and other non-Smad pathways (20-24). Although not well studied, there is evidence that TGF-β can increase cytoplasmic Ca^{2+} and activate calcineurin, which dephosphorylates NFAT resulting in NFAT nuclear translocation (300,301). Despite these data, the mechanism by which TGF-β increases cytosolic Ca^{2+} remains unclear. Our data suggest that CRT is essential for TGF-β to increase cytoplasmic Ca^{2+} levels since cells lacking CRT were unable to increase cytoplasmic Ca^{2+} when treated with TGF-β. There are several mechanisms by which TGF-β increases intracellular Ca^{2+} levels ,

including increasing IP3 levels to cause release of Ca^{2+} from thapsigargin sensitive stores in the ER, mediating translocation of type III IP_3 receptors to the cell surface, stimulating H_2O_2 mediated Ca^{2+} release, and via a c-Jun dependent mechanism (222,224,226-228). Rises in cytoplasmic Ca^{2+} levels are typically regulated by ER mediated Ca^{2+} release or through store operated Ca^{2+} entry through channels present on the plasma membrane (21,354). CRT can regulate both ER mediated Ca^{2+} release and store operated Ca^{2+} entry (33-36,38). The ability of thapsigargin to block responses to TGF-β suggests that CRT regulation of ER Ca^{2+} stores might be involved, although TGF-β stimulation of wild type MEFs in media lacking extracellular Ca^{2+} failed to stimulate a rise in cytoplasmic Ca^{2+}, suggesting that CRT might also regulate cytoplasmic Ca^{2+} levels through regulation of store operated Ca^{2+} entry (data not shown). In addition, treatment of CRT -/- MEFs with TGF-β in the presence of ionomycin failed to make CRT -/- cells responsive to TGF-β, showing that TGF-β stimulation of ECM is not simply a function of increased calcium levels and suggesting that additional CRT-dependent functions are involved in regulating TGF-β stimulation of ECM.

TGF-β stimulation of NFAT activation and nuclear translocation were impaired in CRT -/- MEFs suggesting that CRT mediated calcineurin activity is likely impaired in CRT -/- MEFs. Our data are consistent with prior reports demonstrating that CRT is needed for efficient NFAT nuclear translocation (44,46). Furthermore, studies by Gooch et al., showed that TGF-β activates calcineurin in a time and dose dependent manner and that calcineurin is required for TGF-β stimulated ECM in glomerular mesangial cells (300). These studies showed that TGF-β stimulation of fibronectin is dependent on NFAT binding to the fibronectin promoter as cells expressing a dominant negative NFATc

mutant are unable to induce ECM in response to TGF-β (301). NFAT signaling has also been shown to be important for myofibroblast induction and expression of collagens I and III by cardiac fibroblasts in response to mechanical stress and osteopontin by vascular smooth muscle cells in high glucose (282,355).

CRT can regulate cell behavior, ECM production and wound healing from multiple cellular compartments, including at the cell surface and as an extracellular ligand (4,93). However, cell surface CRT, which can act as a co-receptor for TSP1 to stimulate collagen production, does not appear to be involved in mediating these TGF-β responses, since CRT -/- MEFs stably expressing CRT lacking the TSP1 binding sequence had normal responses to TGF-β (5).

The absence of CRT does not appear to negatively impact Smad 2/3 signaling as Smad 2/3 phosphorylation and nuclear translocation are unaffected. Since nuclear CRT can affect DNA binding of some nuclear hormones such as vitamin D, a nuclear receptor known to antagonize TGF-β signaling (97,98), we also examined the ability of TGF-β to stimulate Smad reporter activity in CRT deficient cells. Although reporter activity in CRT -/- MEFs was reduced as compared to wild type cells, TGF-β was still able to stimulate Smad reporter activity 2.5-fold in CRT -/- MEFs, levels which have been shown to be sufficient for Smad-induced gene transcription in other systems (356,357). We also investigated whether the lack of CRT impacted non-Smad pathways regulated by TGF-β (212,214,334-336). In contrast to the negative effects of CRT deficiency on the calcium-NFAT pathway, the CRT -/- MEFs did not show alterations in levels of p-AKT, p-JNK, and p-ERK induced by TGF-β (data not shown). There was a trend towards

increased phosphorylated p-38 MAPK in CRT -/- cells treated with TGF-β, although these data did not reach statistical significance and the importance remains unclear. Recent literature suggests that ER stress is associated with fibrotic disorders such as diabetic atherosclerosis, pulmonary fibrosis, and diabetic nephropathy (1-7). Chronic stimuli such as high glucose, glucosamine or oxidative stress can up regulate ER stress proteins and are associated with enhanced matrix production and fibrotic disease (7,142,155,321,358). Chemical chaperones such as 4 phenylbutyric (4-PBA) reduce the expression of ER stress proteins such as CRT and GRP78 and also reduce fibrotic remodeling due to high glucose in animal models of atherosclerosis and nephropathy (7,142,321). Despite the growing appreciation for a role for ER stress in fibrotic disease, knowledge regarding the mechanisms by which ER stress regulates fibrosis is limited (130,136). Although there is a clear role for ER stress induced apoptosis in some models of fibrosis (322), there is also evidence for ER stress involvement in processes important for fibroproliferative remodeling which are independent of apoptosis (139,359). Knockdown of GRP78, another ER stress response protein, also reduces TGF-β or tunicamycin stimulated collagen and α-smooth muscle actin production in human lung fibroblasts (139). 150-kDa oxygen-regulated protein (ORP150) mediates TGF-β myofibroblast induction and collagen production in human lung fibroblasts (359). The mechanisms by which GRP78 and ORP150 mediate responsiveness to TGF-β were not addressed in these reports. Our data now suggest that CRT is an important ER stress factor in fibrotic remodeling through regulation TGF-β stimulated ECM production. It is interesting that CRT mediates TGF-β responsiveness through its role as a regulator of calcium signaling, rather than through its chaperone function, suggesting a unique role

for CRT in the ER stress response. In contrast to these reports, Vonk et al. showed that enhanced ER stress due to glucose and nutrient deprivation reduces collagen production in chondrocyte and dermal fibroblasts (360). Nonetheless, the majority of evidence suggests that enhanced ER stress is associated with exacerbated fibrotic outcomes (7,8,136,137,142,145,321,322,324,327,361).

TGF-β has been shown to increase expression of ER stress response proteins, including in the human IPF lung fibroblasts (data not shown) (139,359). However we did not observe TGF-β stimulation of either GRP78 or CRT in MEFs or Thy-1 -/- rat lung fibroblasts, suggesting that basal levels of CRT expression are sufficient to mediate responses to TGF-β. Furthermore, knockdown of CRT in Thy1 -/- rat lung fibroblasts to 60% of control levels was able to attenuate TGF-β stimulated matrix production, although baseline levels of matrix proteins were unaffected. The lack of effect on baseline matrix production might reflect a lower threshold of CRT required to maintain homeostatic levels of ECM expression in these cells. This would be consistent with our observations that knockdown of CRT to 35% of control levels does reduce basal ECM expression in human lung fibroblasts. Interestingly, CRT -/- MEFs have increased basal levels of the ER stress proteins GRP78, GRP94, calnexin, and PDI [data not shown and (64)] and reduced expression of multiple ECM proteins (6), suggesting that increased expression of other ER stress proteins cannot compensate for the lack of CRT in mediating ECM production in response to TGF-β (1). This conclusion is supported by our observation that TGF-β treatment of CRT -/- MEFs in the presence tunicamycin did not increase collagen I protein. These data suggest that enhanced levels of ER stress with TGF-β in the absence of CRT are not sufficient to drive fibrosis, implicating CRT as an

important link between enhanced levels of ER stress and fibrotic disease. Furthermore, these data suggest that ER CRT might be a novel therapeutic target to attenuate fibrosis. In conclusion, these studies demonstrate that CRT is required for TGF-β stimulated ECM production and provide a link between enhanced ER stress and TGF-β stimulation of ECM.

CALRETICULIN IS IMPORTANT FOR NEOINTIMAL HYPERPLASIA AND TGF-β STIMULATED ECM PRODUCTION IN VASCULAR SMOOTH MUSCLE CELLS

by

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CALRETICULIN IS IMPORTANT FOR NEOINTIMAL HYPERPLASIA AND TGF-β STIMULATED ECM PRODUCTION IN VASCULAR SMOOTH MUSCLE CELLS

Abstract

Objective- Endoplasmic reticulum (ER) stress is associated with vascular disease. Previously, we demonstrated that calreticulin (CRT), an ER stress response protein, is required for TGF-β stimulated extracellular matrix (ECM) production in fibroblasts. Since TGF-β regulates vascular fibroproliferative responses, we investigated whether CRT contributes to injury induced neointimal hyperplasia through regulation of TGF-β signaling in vascular smooth muscle cells (VSMCs).

Approach and Results- We used the carotid artery ligation model of vascular injury in CRT floxed mice to determine the role of CRT in vascular responses to injury. Cre-recombinase-IRES-GFP plasmid was delivered to CRT floxed mice using microbubbles (MB) with ultrasound (US) targeted to the carotid artery to reduce expression of CRT in the vasculature. VSMCs were also isolated from the floxed mice and used for *in vitro* studies. Cre-recombinase mediated knockdown of CRT in floxed VSMCs significantly decreased CRT transcript and protein and attenuated collagen type I protein expression in response to TGF-β. Furthermore, pretreatment of floxed VSMCs with the calcineurin/NFAT inhibitor, 11R-VIVIT, significantly inhibited TGF-β stimulated collagen production. CRT floxed mice treated with cre-recombinase plasmid had significantly reduced CRT expression in the neointima as compared to controls. In

addition, MB/US delivery of cre-recombinase to the carotid significantly reduced neointima formation and collagen production as measured by Masson's Trichrome staining. However, knockdown of CRT did not affect VSMC proliferation *in vitro* or *in vivo*.

Conclusions- CRT plays an important role in mediating VSMC responses to injury, potentially through CRT regulated TGF-β dependent signaling. This work establishes a novel role for the ER stress protein CRT in vascular disease.

Introduction

Neointimal hyperplasia is a hallmark of the vascular response to injury, both from acute injury due to restenosis following percutaneous coronary intervention and in response to chronic vascular injury in atherosclerosis. Neointimal hyperplasia is characterized by medial VSMC migration and proliferation from the subendothelial space to the lumen, followed by acquisition of the synthetic phenotype with the production of high levels of matrix proteins, including type I collagen, leading to vessel occlusion (362-364).

TGF-β is increased following vascular injury and plays a role in neointimal hyperplasia (305). Introduction of a plasmid encoding TGF-β or recombinant TGF-β enhances ECM production and intimal thickening (306,307). Furthermore, blockade of TGF-β with antibodies or a soluble TGF-β decoy receptor significantly attenuated neointimal formation (308,309). Blockade of TGF-β regulated Smad signaling using adenoviral delivery of inhibitory Smad 7 attenuates neointimal formation and collagen production, suggesting that targeting downstream TGF-β signaling may be an effective

therapeutic target for treatment of neointimal hyperplasia (310). Despite overwhelming evidence that TGF-β promotes neointimal hyperplasia, the role of TGF-β in VSMC proliferation is more controversial. Halloran and colleagues demonstrated that TGF-β inhibits human arterial SMC proliferation (365), whereas two separate reports have demonstrated that TGF-β stimulates VSMC proliferation through Smad3 and ERK dependent pathways (366,367).

ER stress is becoming appreciated as an important factor in multiple fibroproliferative diseases, including vascular disease (130). CRT is a calcium binding protein in the ER lumen, which has dual roles in regulating ER calcium stores and downstream calcium-dependent signaling pathways such as calcineurin and NFAT and in acting as a chaperone to regulate protein folding (30,34,43). CRT is upregulated in the aortic arch of streptozotocin treated hamsters and in the endothelium and media of atherosclerotic arteries from New Zealand White rabbits fed a high fat diet (4,7). CRT is critical for the development of fibrotic disease in multiple organs. CRT is upregulated following unilateral ureteral obstruction (UUO) induced renal injury and this increase proceeded collagen deposition (8). Mice heterozygous for CRT have reduced collagen levels following UUO induced injury as compared to wild type mice (9). CRT can also regulate SMC proliferation as siRNA knockdown of CRT inhibited PDGF-bb stimulated bronchiolar SMC proliferation (368). Furthermore, our lab has established that CRT is a critical regulator of ECM production in response to TGF-β, establishing a key mechanistic link between ER stress and fibrosis (6,104). CRT -/- MEFs have reduced type I and type III collagen and fibronectin transcript and protein (6). In addition, cells deficient in CRT are unable to induce ECM production in response to TGF-β even in the

presence of enhanced ER stress and active Smad signaling (104). CRT is required for TGF-β stimulated ECM production through control of ER calcium release and downstream activation of calcineurin/NFAT signaling (104). Inhibition of calcineurin/NFAT signaling prevented TGF-β stimulated ECM production in several fibroblast cell lines, illustrating the importance of CRT mediated calcineurin/NFAT signaling in TGF-β induced ECM production (104).

Because genetic deletion of the CRT gene (*calr*) results in embryonic lethality, it has been difficult to experimentally determine the role of CRT in disease in adult animals. In our current studies, we used the carotid artery ligation mouse model of acute vascular injury in newly available CRT floxed mice to determine whether CRT is involved in regulating vascular responses to injury and neointimal hyperplasia (369-371). We used a targeted ultrasound/microbubble approach to deliver Cre-recombinase plasmid to the carotid arteries of CRT floxed mice to knockdown CRT expression specifically in this tissue (372-377). Our results demonstrate that knockdown of CRT in vivo significantly reduces neointima formation and total collagen content as measured by Trichrome stain (370). This study demonstrates that CRT has an important role in regulating VSMC responses to acute injury and suggests the potential utility of targeting CRT for treatment of vascular disease.

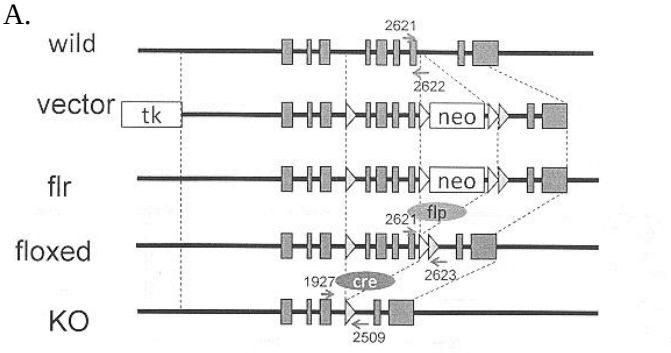
Experimental procedures

Chemicals and reagents Dulbecco's modified Eagle's medium (DMEM) with 1 g/liter glucose was purchased from Invitrogen (Madison, WI). Protease inhibitor cocktail was purchased from Sigma (St. Louis, MO). D-PBS was purchased from Cellgro (Manassas, VA). 11R-VIVIT was purchased from CalBiochem (Billerica, MA). TGF-β

was purchased from R&D Systems (Minneapolis, MN). Rabbit anti-β-tubulin (cat # 9104) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-collagen type I IgG (cat # 203002) was purchased from MDbioproducts (St. Paul, MN). Goat anti-calreticulin (cat# MBS222424) was purchased from MYBioSource (San Diego, CA). Rabbit anti-Ki67 (cat # ab15580) was purchased from abcam (Cambridge, MA). Peroxidase conjugated AffiniPure rabbit anti-goat IgG (Cat # 305-035-003) and goat anti-rabbit IgG (111-035-003) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Biotinylated rabbit anti-goat IgG (cat # BA-5000) and goat anti-rabbit (cat # BA-1000) secondary antibodies, hematoxylin QS (ca t# H-3404), VectaMount permanent mounting medium (cat # H-5000), Vectastain ABC kit (cat # PK-6100), and DAB peroxidase substrate kit (cat # SK-4100) were purchased from Vector Laboratories (Burlingame, CA). Western Lightning Chemiluminescence Reagent Plus was purchased from PerkinElmer Life Sciences (Waltham, MA). pEGFP-N1 (cat # 6086-1) and pCAG-Cre-recombinase-IRES2-GFP (Plasmid # 26646) were purchased from Clonetech and Addgene respectively. GFP (Invitrogen) or Cre-recombinase-IRES-GFP (Addgene, Woodhead, 2006) plasmids were purchased, grown in *E. coli*, and purified using a Qiagen Gigaprep kit. OPTISON microbubbles (product # NDC 0407-2707-03) were purchased from GE Healthcare (Buckinghamshire, United Kingdom). Celltiter 96 AQ_{ueous} cell proliferation assay was purchased from Promega (cat # G3581).

Generation of CRT floxed animals A thymidine kinase (tk) neomycin targeting vector was generated and used to perform homologous recombination on stem cells isolated from C57Bl/6 mice (Figure 16a). Embryonic stem cells were transfected with

A.



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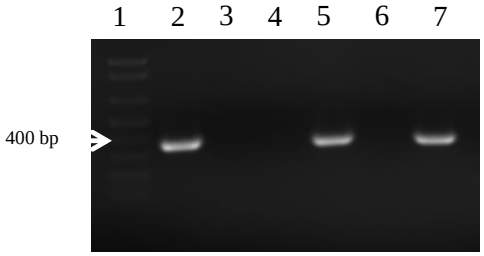


Lane 1. 100 bp DNA ladder
Lane 2. WT mice; WT primers
Lane 3. WT mice; floxed primers
Lane 4. Male floxed mouse; WT primers
Lane 5.Male floxed mouse; floxed primers
Lane 6.Female floxed mouse; WT primers
Lane 7. Female floxed mouse; floxed primers

Figure 16. Targeting strategy for generation of CRT floxed mice. (A) Schematic of method through which CRT floxed mice were generated. LoxP sites were genetically engineered to surround exons 4-7 of CRT. Breeding of heterozygous CRT floxed mice with transgenic mice expressing FLP recombinase was performed to remove the neo cassette. (B) Heterozygous floxed mice were crossed and mice containing homozygous floxed CRT alleles were confirmed by PCR.

the targeting vector and cultured in media containing gancyclvir for negative selection of non-homologous recombinants and neomycin for positive selection of homologous recombinants. Surviving clones were amplified and injected into blastocysts isolated from C57Bl/6 mice. Offspring containing the homolgous recombinant DNA with neomycin cassette were identified using the PCR (2621; 5'-gagtggaaaccacgtcaaattgacaacc -3', 2622; 5'- cttctctgataagttttcctctgacctc -3', 2623; 5'-agggttccggatccgatgaagttcc -3'). Wild type offspring produce a band when using primers 2621 and 2622 whereas CRT floxed offspring only produce a band when using primers 2621 and 2623. Heterozygous CRT floxed mice on C57Bl/6 background were mated with DBA/2J transgenic mice expressing FLP recombinase to remove the neo cassette. Mice heterozygous for the CRT floxed allele were mated and offspring containing homozygous floxed CRT alleles identified by PCR. Upon receipt of the mice, the presence of loxP sites in male and female breeders was confirmed by PCR (Figure 16b). Functionality of loxP sites were confirmed by using Cre-recombinase-IRES-GFP to knockout CRT in cells receiving the plasmid (Figure 17a,b).

Experimental animals Ten to 14-week-old CRT floxed mice on a mixed C57Bl/6 and DBA/2J (B6D2F1) background were provided as a generous gift by Dr. Marek Michalak (University of Alberta) and maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (6:00 A.M. to 6:00 P.M.). Mice were fed a standard rat pellet diet ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health.

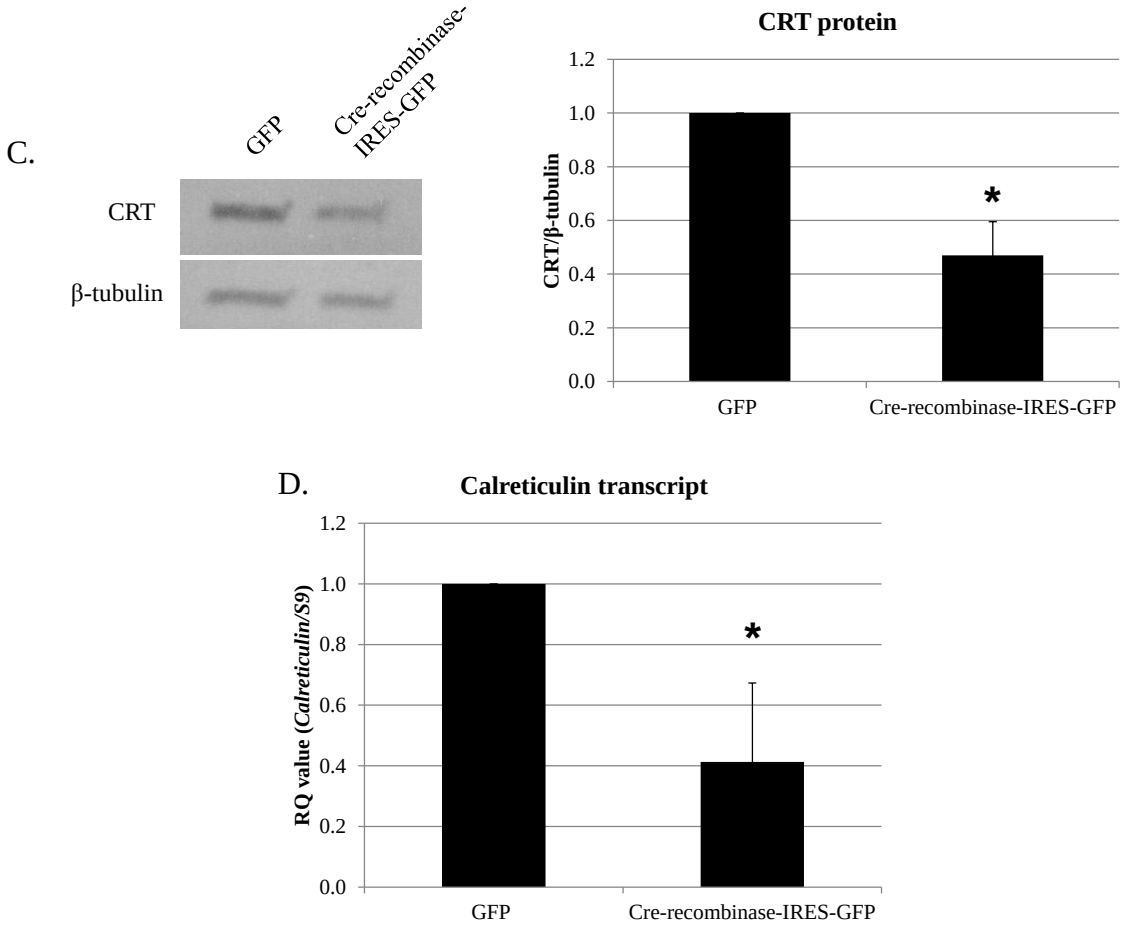


Figure 17. Verification of CRT floxed allele by western blot and RTQ-PCR in CRT floxed VSMCs (A,B) CRT floxed VSMCs were isolated from CRT floxed mice, transfected with 1 μg GFP or Cre-recombinase-IRES-GFP, and grown overnight in media with 10% FBS. Cells were switched to serum free DMEM for the remainder of the experiment. (A) Laemmli cell lysates were immunoblotted for CRT and normalized to β-tubulin. A representative blot is shown in (A). (B) After 72 hours, RNA was harvested by TRIZOL and transcript levels of *calreticulin* and *S9* were determined by quantitative real time PCR.

Plasmid injection and ultrasound Four weeks prior to harvest, CRT floxed mice were tail vein injected with a solution containing 200 μL OPTISON (GE Healthcare) and either 300 μg GFP plasmid (n=7 mice) or 300 μg Cre-recombinase-IRES-GFP (n=7 mice) plasmid in a total volume of 250 μL. Three mice injected with Cre-recombinase-IRES-GFP with MB but in the absence of US treatment served as a technical control for the specificity of US transfection. Immediately following tail vein injection, ultrasound was performed on the carotid artery. As previously detailed (378-382), the custom experimental ultrasound (US) setup involved single element (0.75 inch) immersion transducer (Olympus, Waltham, MA) in series with a signal generator (AFG3022B, Tektronix, Beaverton, OR) and power amplifier (A075, Electronics and Innovation, Rochester, NY). This study was completed using the following acoustic parameters: 1.0 MHz ultrasound frequency, 0.7 MPa peak negative pressure, 30 sec pulse repetition period, and 2 min duration of exposure. All animals received a 200 μL dose (approximately 1.2×10^8 microbubbles, MBs) of activated contrast media (Optison, Vendor, City). One week following US and/or MB delivery, mice were subjected to carotid artery injury as described below.

Carotid artery injury Three weeks prior to harvest, mice were anesthetized with1.5% isoflurane in oxygen and the right common carotid artery was exposed through a midline cervical incision and ligated with a 6-0 silk suture just proximal to the bifurcation.

Harvesting and Morphometric Analysis Twenty-eight days after injury, mice were anesthetized with ketamine (80 mg/kg IP; Abbott Laboratories) and xylazine (5 mg/kg IP; Rompun, Bayer Corp). The vasculature was immediately flushed with 0.01M sodium

phosphate buffer (pH 7.4) and perfused with 10% formalin. Both carotid arteries were excised, fixed in 10% formalin, embedded in paraffin, and sectioned. Representative serial sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope to locate the ligation site, then additional sections of the artery taken 350, 500, and 700 μm proximal to the ligation site were identified and treated with Verhoeff's elastin stain to enhance the elastic laminae.

Computer-assisted morphometric analysis of digitized images captured from each arterial section was performed with Image J analysis software. Measurements of the 3 sections obtained for each vessel were averaged for statistical purposes. The cross-sectional area of the media, i.e. the zone bounded by the external elastic lamina and the internal elastic lamina, and the area of the intima, i.e. the zone between the internal elastic lamina and the lumen, were calculated. All measurements were performed by a single examiner blinded to the treatment group.

Isolation of CRT floxed mouse VSMCs Mouse VSMCs were isolated from calreticulin-floxed mice as previously described (383). Briefly, mouse aortas were harvested, adventitia removed, and cells were digested with 1X collagenase for 30 minutes. The aorta was cut in to 1 mm pieces and cells were allowed to grow out via explant culture in low (1 g/L) glucose DMEM with 10% FBS for two weeks. Cells were removed and passaged using trypsin. For all experiments, cells were used between passage 3-7.

Knockdown of CRT in CRT-floxed VSMCs 500,000 CRT floxed VSMCs were transfected via nucleofection using the primary VSMC Nucleofector Kit from Amaxa Biosystems (Amaxa GmbH, Lonza) in an Amaxa Nucleofector II using program P-024.

Transfected cells were cultured in DMEM with 10% FBS for 24 hours. After 24 hours, media was switched to serum free media for 24 hours followed by treatment with 100 pM TGF-β for 48 hours. Cells were washed with Dulbecco's phosphate buffered saline (D-PBS) (Cellgro) and lysed with 1X Laemmli buffer containing 1X protease inhibitor.

Immunoblotting for ECM proteins Following treatment, cells were harvested using 1X Laemmli lysis buffer (Bio-Rad, Hercules, CA) containing 1X protease inhibitor cocktail (Sigma cat # p8340). Cells were sonicated for 7 seconds, 5% final β-mercaptoethanol was added and samples were boiled at 100 °C for 7 minutes. Samples were centrifuged and equal volumes were loaded in to 4-15% SDS-polyacrylamide gels. After separation by SDS-PAGE, samples were transferred onto a PVDF membrane at 100 volts for 100 minutes. Following transfer, membranes were blocked with 1% casein followed by application of the primary antibody. Membranes were washed in TBS-T and secondary antibody was applied for 1 hour at room temperature. Membranes were washed with TBS-T and developed using Western Lightning Chemiluminescence Reagent Plus. Membranes were probed with rabbit anti-β-tubulin IgG to normalize for cell protein. Densitometric analysis of immunoblots was performed using the NIH Image J program. Data are expressed as the mean band density normalized for cell protein from at least three separate experiments +/- S.D..

Quantitative Real Time PCR Cells were grown overnight in complete media containing 10% FBS and grown in serum free media for 72 hours. After treatment, RNA was harvested with TRIZOL reagent and isolated according to manufacturer's specification. Quantitative real time PCR was performed using standard protocols with an Opticon instrument (MJ Research, model CFD-3200). Primers for CRT (Cat #

QT00101206) and S9 (Cat # PPM03695A) were obtained from Qiagen and verified by melt curve analysis. Transcript levels were assayed using SYBR green from Qiagen. Results were calculated using the delta delta CT method and are expressed as the mean +/- S.D. of three samples each assayed in triplicate as indicated in figure legends. Results are representative of at least 3 separate experiments.

Immunohistochemistry Immunohistochemistry was performed on formalin fixed paraffin embedded sections according to procedure outlined by Cell Signaling. Briefly, slides were rehydrated in ethanol and antigen retrieval performed using 10 mM sodium citrate at 100 °C for 10 minutes. Slides were quenched in 3% H_2O_2 and blocked for 1 hour in 1% casein. Slides were incubated with goat anti-CRT IgG (1:1000) or rabbit anti-Ki67 IgG (1:1000) overnight. The next day, slides were incubated with biotinylated rabbit anti-goat IgG (1:250) or goat anti-rabbit IgG (1:250) for 1 hour, washed and developed using DAB substrate. All slides were exposed to DAB for an equal amount of time. Counterstain with hematoxylin was applied for 20 seconds. Coverslips were mounted using VectaMount permanent mounting medium. Images of CRT stained sections were taken using a Zeiss Axiovert 10 inverted microscope and positive stain determined using Metamorph Imaging Software.

Trichrome Stain Trichrome stain and Verhoeff's elastin stain was performed by the Comparative Pathology Laboratory core facility according to standard procedures.

Statistics All tests involving multiple groups were compared using one way ANOVA of rank sums and were considered significant if $p < 0.05$. All tests involving two groups were performed using Student's T Test and were considered significant if $P < 0.05$.

Results

Delivery of Cre-recombinase-IRES-GFP plasmid to CRT floxed mouse VSMCs reduces CRT transcript and protein Mice with the *calr* gene flanked by LoxP sites at exons 4 and 7 were generated as described by the Michalak lab (Figure 16a). PCR was performed on DNA from wild type and CRT floxed mice using primers specifically designed to detect the presence of LoxP sites (Figure 16b). Wild type mice produce a band only when wild type primers were used (lane 1), whereas CRT floxed male and female mice only produce a band with primers to detect presence of the LoxP sites (Lanes 5 and 7) (Figure 16b) Next, we confirmed that the Cre-recombinase-IRES-GFP plasmid could knockdown CRT in VSMCs isolated from CRT floxed mice. CRT floxed VSMCs were transfected with GFP or Cre-recombinase-IRES-GFP plasmid and levels of CRT transcript or protein determined 72 or 96 hours post transfection, respectively. Transfection with Cre-recombinase-IRES-GFP significantly reduced CRT transcript and protein (Figure 17a,b) demonstrating the feasibility of using this plasmid *in vivo*.

CRT regulates TGF-β stimulated collagen production, possibly through control of calcineurin mediated NFAT dephosphorylation CRT is a critical regulator of TGF-β stimulated ECM production including collagen and fibronectin in fibroblasts (104); therefore, we investigated the effect of CRT knockdown on TGF-β induced collagen production in CRT floxed VSMCs. Delivery of Cre-recombinase-IRES-GFP significantly down-regulated CRT protein (Figure 18a,b**)**. Furthermore, knockdown of CRT significantly attenuated TGF-β stimulated collagen production (Figure 18a,c). Despite the reduced production of collagen in response to TGF-β upon CRT knockdown, knockdown of CRT did not affect TGF-β stimulated fibronectin production (data not

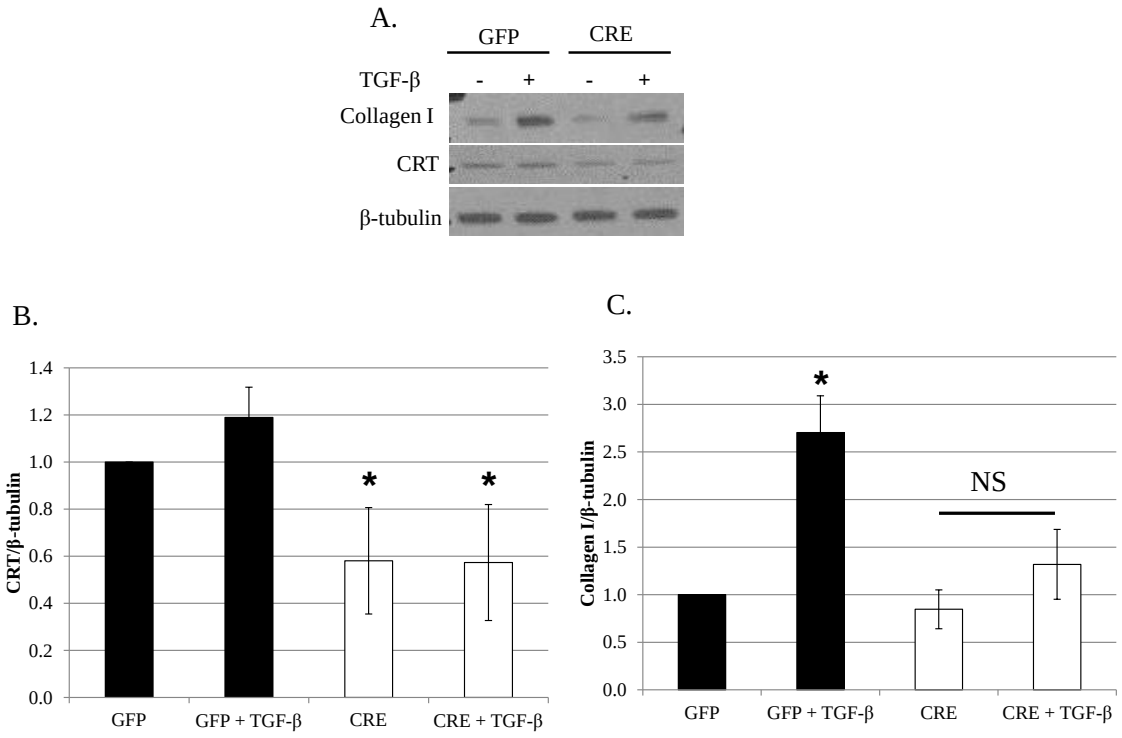


Figure 18. CRT knockdown inhibits TGF-β stimulated collagen production. (A-C) CRT floxed VSMCs were transfected with 1 μg GFP or Cre-recombinase-IRES-GFP plasmid and grown overnight in DMEM with 10% FBS. Cells were switched to serum free DMEM for 24 hours followed by treatment with 100 pM TGF-β for 48 hours. Laemmli cell lysates were immunoblotted for CRT and type I collagen. Results of a representative blot are shown. Results are the mean density of bands normalized to β-tubulin +/- S.D. (n=3 separate experiments). *$p<0.05$ vs GFP transfected cells.

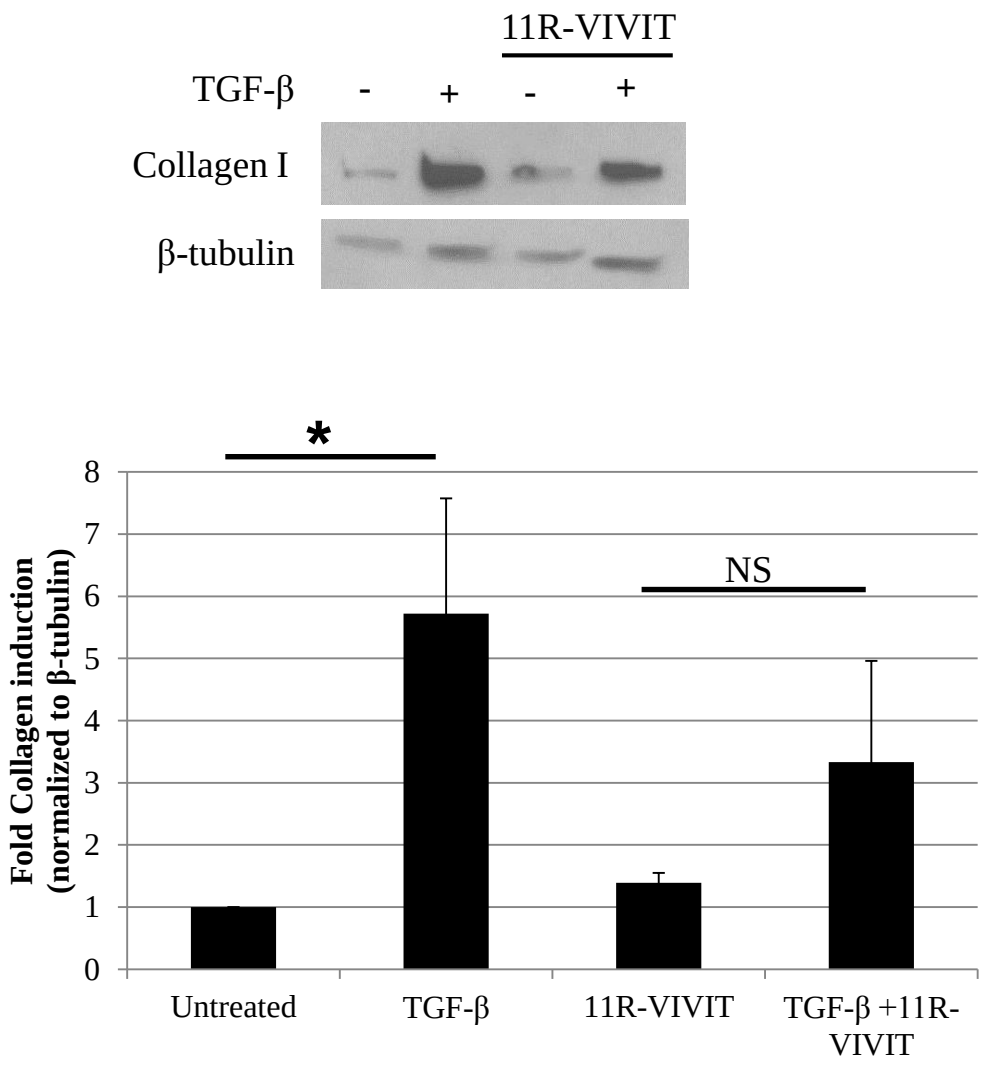


Figure 19. Inhibition of calcineurin/NFAT signaling with 11R-VIVIT impairs TGF-β stimulated collagen production. CRT floxed VSMCs were grown overnight in DMEM with 10% FBS, starved overnight in serum free media, and pretreated with 1 μM 11R-VIVIT for 60 minutes. Cells were treated with or without 100 pM TGF-β +/- 11R-VIVIT peptide for 48 hours and laemmli cell lysates immunoblotted for type I collagen. Results of a representative blot are shown. Bands were analyzed by densitometry and normalized to β-tubulin. Results are mean densities normalized to β-tubulin +/- S.D from three separate experiments. *$p<0.05$ vs untreated cells.

shown). We previously showed that CRT regulates TGF-β stimulated ECM production through control of calcineurin/NFAT signaling (104). Inhibition of calcineurin/NFAT signaling with 11R-VIVIT, a cell permeable peptide which blocks calcineurin binding to NFAT, significantly impaired TGF-β stimulated collagen and fibronectin transcript in fibroblasts (289). Therefore, we treated CRT floxed VSMCs with 11R-VIVIT and examined the ability of TGF-β to stimulate type I collagen production. Similar to our observations in fibroblasts, 11R-VIVIT significantly inhibited TGF-β stimulated collagen production in VSMCs (Figure 19). Inhibition of calcineurin/NFAT signaling with 11R-VIVIT also inhibited TGF-β stimulated collagen production in rat VSMCs (data not shown).

CRT expression is increased in the neointima following carotid artery ligation and microbubble/ultrasound delivery of cre-recombinase-IRES-GFP plasmid reduces neointimal CRT level Three weeks following carotid artery ligation induced vascular injury, strong immunostaining for CRT was observed in the neointima of mice receiving control GFP plasmid or Cre-recombinase-IRES-GFP with no US, demonstrating that vascular injury induces CRT expression (Figure 20a,b). Interestingly, immunostaining for CRT was primarily localized to the intimal endothelial cells, the neointimal smooth muscle cells, and to cells in the adventitia following carotid artery ligation (Figure 20a,b). To confirm the efficiency of US/MB mediated CRT knockdown using Cre-recombinase-IRES-GFP, immunohistochemistry for CRT was performed on mice receiving Cre-recombinase-IRES-GFP with MB/US. CRT immunostaining normalized to neointimal area was significantly decreased by approximately 50% in CRT floxed mice receiving Cre-recombinase-IRES-GFP plasmid with MB/US as compared to mice treated with

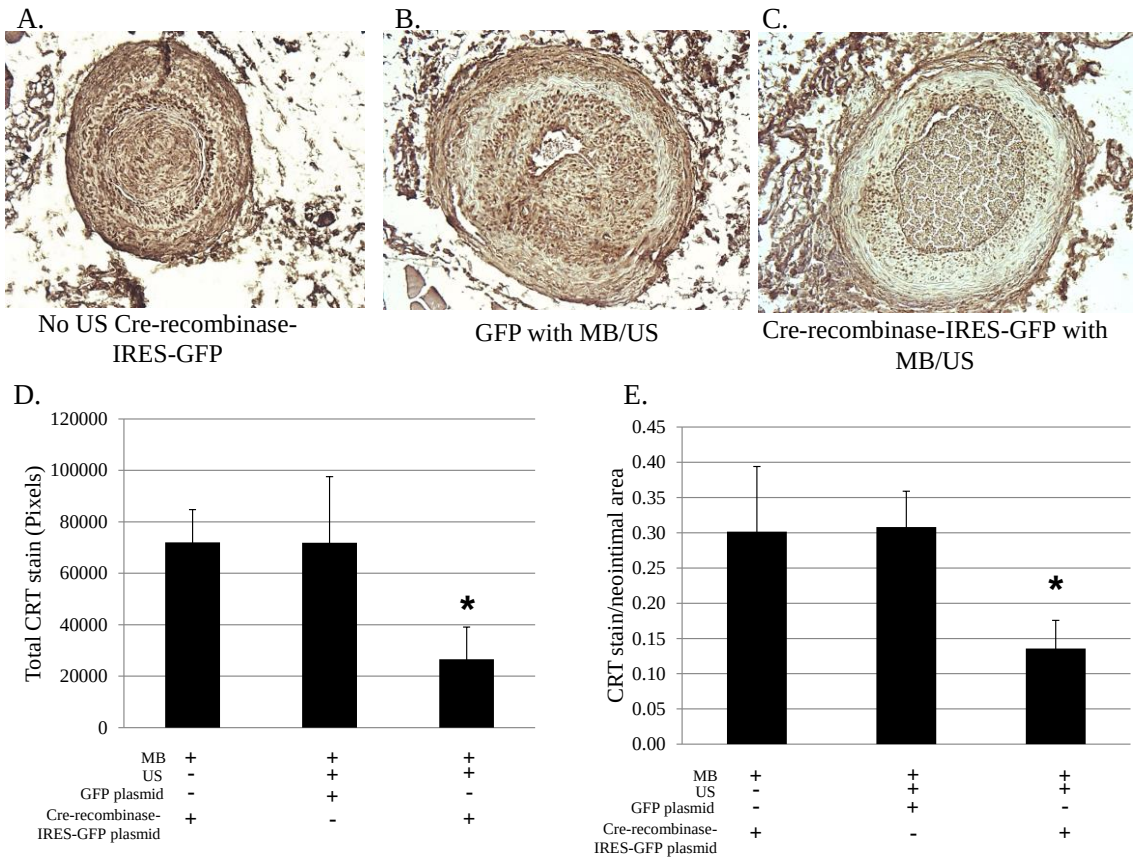


Figure 20. Delivery of Cre-recombinase-IRES-GFP reduces CRT stain in neointimal VSMCs. (A-E) Three weeks following carotid artery ligation, carotid arteries were perfusion fixed, embedded in paraffin, and sectioned. (A-C) Immunostaining for CRT was performed using a goat anti-CRT antibody. Representative images demonstrate strong CRT staining in the neointima of mice receiving GFP plasmid or Cre-recombinase-IRES-GFP without ultrasound. (D,E) Bar graphs quantifying positive CRT stain measured using Metamorph Imaging Software. (D) Quantification of total CRT stain present in the neointima. (E) Quantification of CRT stain normalized to neointima area. Images were obtained at 200X total magnification. Results are expressed as mean values +/- SEM of n=3 (No US Cre-recombinase-IRES-GFP), n=7 (GFP) or n=7 (Cre-recombinase-IRES-GFP). *$p<0.05$ vs MB with Cre-recombinase-IRES-GFP without US and GFP

control GFP plasmid with MB/US or the animals treated with Cre-recombinase-IRES-GFP plasmid with MB, but lacking US (Figure 20a-c,e). Total CRT stain in the neointima was also significantly reduced by treatment with Cre-recombinase-IRES-GFP with MB/US (Figure 20d). The importance of ultrasound for effective transfection of the plasmid is demonstrated by the failure of Cre-reombinase-IRES-GFP plasmid with MB to reduce CRT staining in the absence of US treatment. Medial and adventitial CRT staining was not reduced by Cre-recombinase-IRES-GFP plasmid with MB/US (data not shown).

Knockdown of CRT in CRT floxed mice reduces neointimal hyperplasia following vascular injury Previous reports demonstrate that US/MB mediated delivery of drugs, plasmid DNA, or siRNA significantly attenuate neointimal thickening and matrix production following vascular injury (372-376). To determine the effect of CRT knockdown on neointima formation, morphometric analysis was performed on carotid arteries from mice treated with Cre-recombinase-IRES-GFP with MB but no US, GFP control plasmid with MB/US, or Cre-recombinase-IRES-GFP with MB/US three weeks following carotid artery ligation. Morphometric analyses demonstrated that the neointima-to-media ratio of injured vessels was reduced by approximately 50% in the mice receiving the Cre-recombinase-IRES-GFP plasmid with MB/US as compared to control mice (Figure 21,22). In addition, treatment with Cre-recombinase-IRES-GFP significantly reduced neointimal area as compared to mice receiving the GFP plasmid with MB/US (Figure 22,b). Medial area did not differ between groups (Figure 22,a).

Knockdown of CRT with Cre-recombinase-IRES-GFP reduces collagen production Since knockdown of CRT inhibited TGF-β stimulated matrix production in

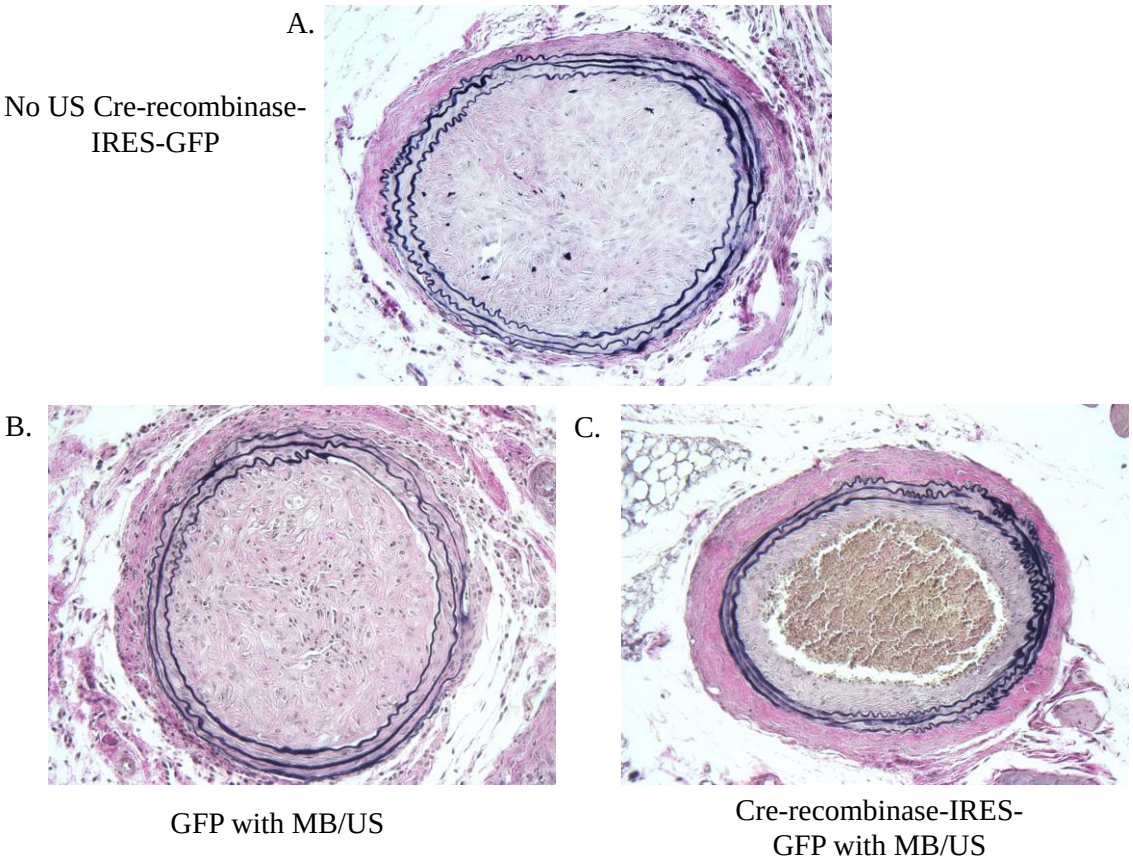


Figure 21. Delivery of Cre-recombinase-IRES-GFP attenuates neointimal hyperplasia following carotid artery ligation. (A-C) Representative elastin stained cross sections of right carotid arteries three weeks following carotid artery ligation. Delivery of Cre-recombinase-IRES-GFP without US was used as a technical control to assess specificity of US mediated delivery.

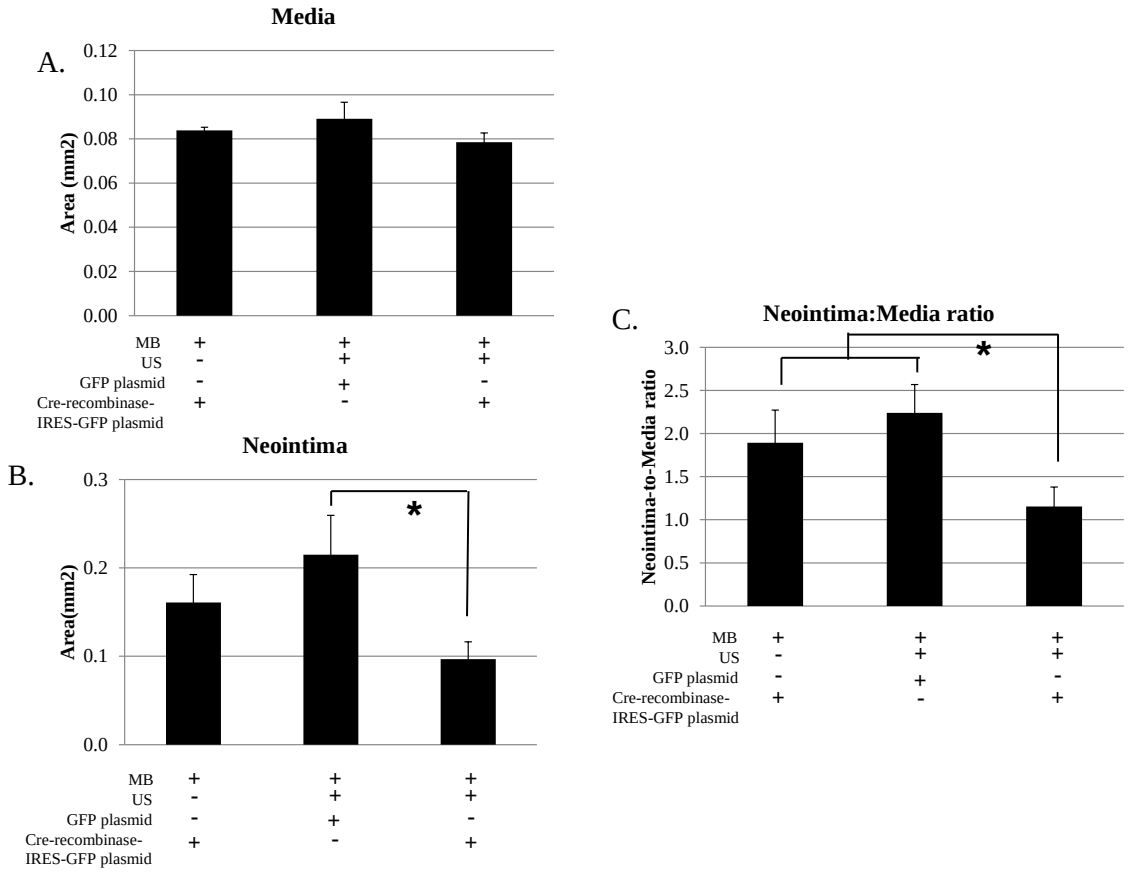


Figure 22. Delivery of Cre-recombinase-IRES-GFP attenuates neointimal area and neointima-to-media ratio. (A-C) Quantification of medial area, neointimal area, and neointima-to media ratio three weeks following carotid artery ligation are shown. Images were obtained at 200X total magnification and quantified using NIH Image J software. Results are expressed as means +/- SEM of n=3 (No US Cre-recombinase-IRES-GFP), n=7 (GFP) or n=7 (Cre-recombinase-IRES-GFP). *$p<0.05$ as indicated

CRT floxed VSMCs, we investigated the effect of CRT knockdown on collagen production in neointimal hyperplasia. Analysis of Masson's trichrome stained carotid artery sections showed that mice treated with Cre-recombinase-IRES-GFP with MB/US displayed significantly reduced total collagen content and a reduction in collagen content per neointimal area as compared to mice treated with GFP plasmid with MB/US or Cre-recombinase-IRES-GFP with MB but no US (Figure 23). Similar to CRT staining, the amount of collagen staining in the adventitia and media appeared similar between groups (data not shown).

Knockdown of CRT with Cre-recombinase-IRES-GFP does not affect VSMC proliferation or cell number in vitro or in vivo Miglino and colleagues demonstrated that knockdown of CRT significantly reduced PDGF-bb stimulated SMC proliferation (368). Furthermore, knockdown of CRT with Cre-recombinase-IRES-GFP significantly reduces neointimal hyperplasia. Therefore, we addressed what effect CRT knockdown had on VSMC proliferation. Knockdown of CRT with Cre-recombinase-IRES-GFP in CRT floxed VSMCs did not affect FBS or TGF-β stimulated proliferation following treatment (Figure 24a). In addition, knockdown of CRT *in vivo* with Cre-recombinase-IRES-GFP did not significantly affect cell proliferation as measured by Ki67 stain or the number of cells in the neointimal region 21 days post carotid artery ligation (Figure 24,b,c).

Discussion

ER stress is an emerging factor in fibrotic disease. Previously, our lab demonstrated that the ER stress response protein, CRT, is a critical regulator of TGF-β

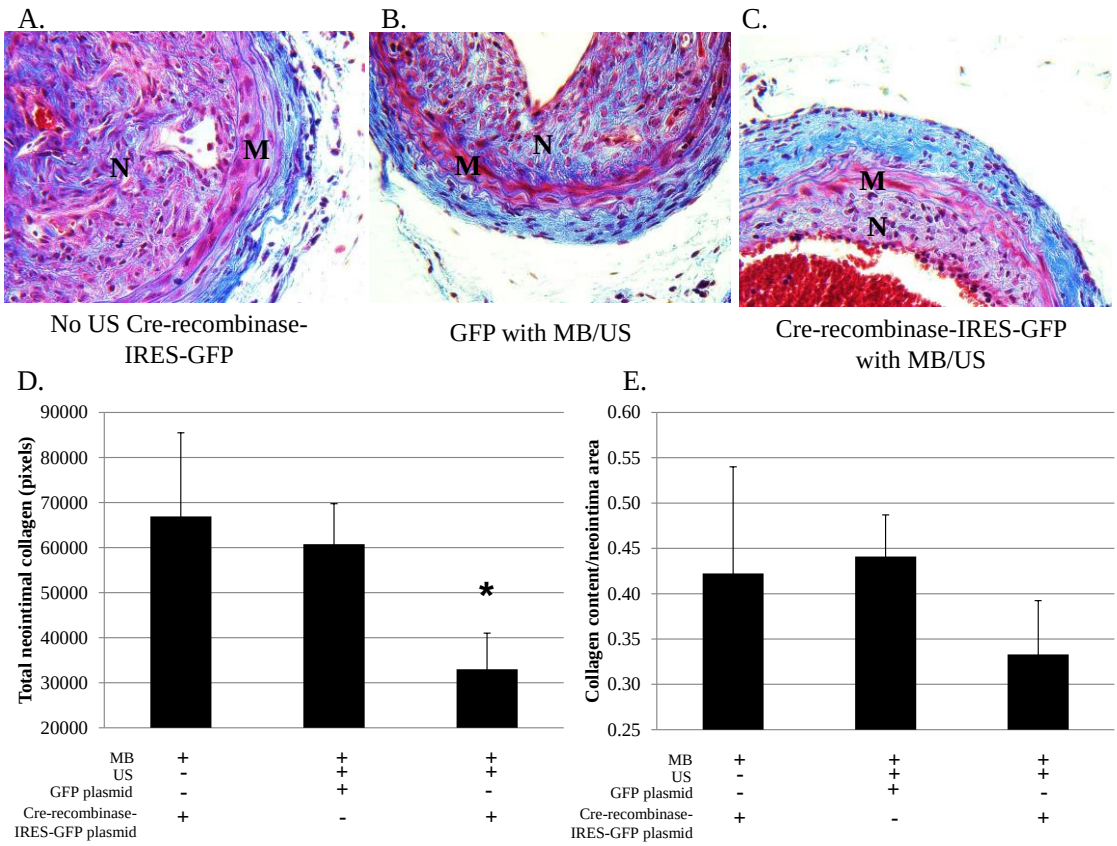


Figure 23. Knockdown of CRT in neointimal VSMCs reduces collagen staining as measured by Trichrome stain. (A-C) Representative Trichrome stained cross sections of right carotid arteries three weeks following carotid artery ligation. Delivery of Cre-recombinase-IRES-GFP without US was used as a technical control to assess specificity of US mediated delivery. (D-E) Quantification of total neointimal collagen content (D) and collagen content normalized to neointimal area (E) three weeks following carotid artery ligation was performed using Metamorph Imaging Software. Images were obtained at 400X total magnification. "M" represents medial region and "N" represents neointimal region. Results are expressed as means +/- SEM of n=3 (No US Cre-recombinase-IRES-GFP), n=7 (GFP) or n=7 (Cre-recombinase-IRES-GFP). *$p<0.05$ vs MB with Cre-recombinase-IRES-GFP without US and GFP

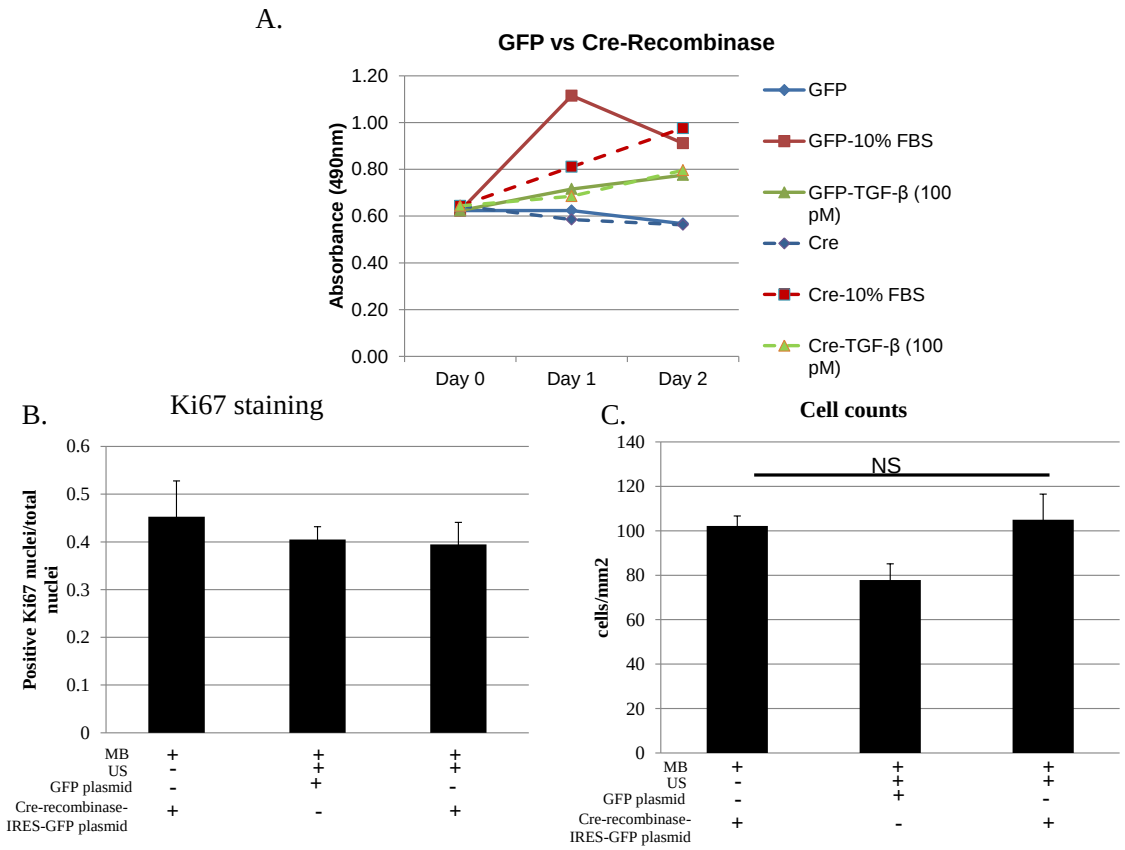


Figure 24. Knockdown of CRT with Cre-recombinase-IRES-GFP does not affect proliferation or total cell number. (A) CRT floxed VSMCs were transfected with 1 μg GFP or Cre-recombinase-IRES-GFP plasmid and grown overnight in DMEM with 10% FBS. Cells were switched to serum free DMEM for 24 hours followed by treatment with 10% FBS or 100 pM TGF-β. After the indicated number of days, cell number was determined using a MTS based proliferation assay. (B,C) (B) Quantification of Ki67 positive nuclei divided by total nuclei counted. For each animal, 3 representative sections were imaged and positive stained nuclei per total nuclei counted. (C) Quantification of cell numbers normalized to neointimal area. For each animal, 3 representative images from each section were taken and total number of hematoxylin stained nuclei counted.

stimulated ECM production in fibroblasts providing initial mechanistic data into how ER stress exacerbates fibrotic disease. Here, we demonstrate that knockdown of CRT reduces carotid artery ligation induced neointimal hyperplasia and collagen content in the novel CRT floxed mice. To address the mechanism by which CRT is regulating neointimal hyperplasia and collagen production *in vivo*, we isolated VSMCs from CRT floxed mice. We demonstrate that knockdown of CRT in floxed VSMCs significantly inhibits TGF-β stimulated collagen production. In addition, CRT may be regulating TGF-β induced collagen production in VSMCs through control of calcineurin/NFAT signaling as pretreatment of cells with the calcineurin/NFAT inhibitor, 11R-VIVIT, impaired TGF-β stimulated collagen production. These studies report the first *in vivo* data utilizing the novel CRT floxed mice and provide further evidence that the ER stress response protein CRT is required for progression of fibroproliferative disease *in vivo*. Furthermore, we provide *in vitro* data suggesting that CRT might be regulating neointimal hyperplasia through control of TGF-β stimulated ECM production in VSMCs.

ER stress is associated with fibrotic and vascular fibroproliferative disease including diabetic atherosclerosis, pulmonary fibrosis, and diabetic nephropathy (8,130,137,321). However, the mechanisms through which enhanced ER stress promotes vascular disease are not well understood. ER stress induced apoptosis is important (322), although other non-apoptosis dependent pathways are involved (139,359). CRT, an ER stress response protein, is involved in fibrotic and vascular fibroproliferative disease. Kypreou and colleagues demonstrated that CRT is upregulated following unilateral ureteral obstruction (UUO) induced injury and that increased CRT correlates with increased collagen deposition (8). Furthermore, Prakoura et al. showed that CRT

heterozygous mice have improved kidney function and reduced collagen levels following UUO induced injury compared to wild type mice (9). Mice heterozygous for CRT displayed decreased levels of type I and III collagen, fibronectin, and TGF-β (9). Treatment of APA hamsters with streptozotocin significantly increased CRT and atherosclerotic lesions suggesting that CRT is involved in atherosclerotic lesion formation (7). We also observed strong CRT immunostaining in lesions from apoE null mice (data not shown) and in the neointima following carotid artery ligation (Figure 20a,b). Our lab provided initial insight into the mechanism through which CRT regulates matrix production in fibrotic disease. We demonstrated that cells deficient in CRT had impaired TGF-β stimulated collagen and fibronectin production, despite active Smad 2/3 signaling. In addition, we showed that CRT -/- MEFs have impaired TGF-β stimulated calcium release and NFAT nuclear translocation *in vitro*. Our data now demonstrate that knockdown of CRT *in vivo* significantly inhibits carotid artery ligation induced neointimal hyperplasia and collagen production suggesting that CRT is a critical component of ER stress induced vascular disease.

Knockdown of CRT significantly inhibits neointimal hyperplasia and ECM production, possibly through control of TGF-β stimulated ECM production in VSMCs. However, the exact mechanism through which CRT knockdown impairs intimal hyperplasia is unknown. Miglino and colleagues showed that knockdown of CRT significantly impairs PDGF stimulated smooth muscle cell proliferation. Knockdown of CRT with Cre-recombinase-IRES-GFP did not affect proliferation levels *in vivo* 3 weeks following injury or in CRT floxed VSMCs treated with FBS or TGF-β. Furthermore, the number of cells present per neointima area was not significantly different between

groups, suggesting that CRT is not regulating neointimal hyperplasia through control of VSMC proliferation. It has been reported that CRT -/- MEFs, in addition to having reduced ECM proteins, migrate poorly compared to wild type cells (82). Furthermore, Nelson and colleagues demonstrated that type I collagen and fibronectin induce strong migration in VSMCs (384). Therefore, it is conceivable that reduction in ECM genes following knockdown of CRT *in vivo* may lead to alterations in SMC cell motility and migration into the intima in response to injury.

Remarkably, high levels of CRT staining are present in neointima following carotid artery ligation suggesting an important role for CRT in response to vascular injury. To our knowledge, this is the first evidence that CRT is increased in response to acute vascular injury and suggests that CRT may be a critical link between enhanced ER stress and vascular injury. Specifically, CRT might be enhancing neointimal hyperplasia through control of calcium mediated calcineurin/NFAT signaling. Consistent with this idea are observations that inhibition of NFAT activation with cyclosporine or GFP-VIVIT, a competitive inhibitor of calcineurin/NFAT binding, significantly attenuated balloon injury-induced neointima formation in rats (385). Here, we demonstrate that pretreatment of VSMCs with 11R-VIVIT significantly attenuates TGF-β stimulated collagen production. Together these observations support the idea that increased levels of neointimal CRT are promoting collagen deposition through control of TGF-β dependent calcineurin/NFAT signaling.

Genetic deletion of the *calr* gene results in embryonic lethality due impaired cardiac development and embryonic fibroblasts isolated from these embryos display impaired agonist induced calcium release and NFAT nuclear translocation (34).

Overexpression of constitutively active calcineurin rescues CRT -/- mice from embryonic lethality, although these mice typically die a few weeks following birth, possibly due to cardiac hypertrophy (46). Furthermore, mice engineered to overexpress CRT in the heart die around two weeks following birth due to complete heart block (47). Therefore, it has been difficult to experimentally determine the role of CRT in disease in adult animals because of the embryonic lethality, although heterozygous mice are viable to adulthood (9). Mice with double floxed CRT alleles can now be used to determine cell and tissue specific roles of CRT in adult mice. Indeed, these current studies are the first to specifically target the *calr* gene in one tissue. Ultrasound targeted microbubble delivery of Cre-recombinase-IRES-GFP was used to specifically target knockdown of CRT in the carotid artery. Importantly, knockdown to 50% of wild type levels appears to be sufficient to alter biologic responses to vascular injury.

Delivery of siRNA, therapeutic drugs, or plasmid DNA can be achieved using focused ultrasound microbubble mediated delivery (372-377). OPTISON microbubbles are albumin encapsulated perfluoropropane spheres that oscillate following application of ultrasound (386). A mixture of plasmid and microbubbles is often used for plasmid delivery/transfection as microbubbles exposed to high acoustic pressures and low frequencies undergo cavitation, leading to transient pore formation in cell membranes and intracellular plasmid uptake (386). Delivery of p53 plasmid DNA using MB/US delivery significantly attenuated balloon injury induced neointima formation in a rat (376). Delivery of ICAM-1 siRNA using MB/US delivery significantly prevented neointimal formation in a mouse wire injury model of neointimal hyperplasia (374). Despite the consensus that delivery of plasmid, drugs, or siRNA via MB/US can be achieved

successfully in larger mammals, delivery to the carotid artery in a mouse is more difficult. Delivery of US targeted plasmid to the carotid artery in mice has several technical challenges including the high flow rate within the vessels, the rapid clearance of microbubbles in mice, and the difficulty in precise delivery of the US pulse to a specific region of the carotid. Nonetheless, our results clearly demonstrate that this approach resulted in sufficient CRT knockdown (~50%) *in vivo* to observe a biological effect. In addition, treatment of CRT floxed VSMCs with Cre-recombinase-IRES-GFP *in vitro* led to 40% knockdown in CRT, which significantly inhibited TGF-β stimulated collagen production, providing further evidence that moderate alterations in CRT levels significantly impact VSMC responses to injury. This is consistent with observations showing that mice heterozygous for CRT have reduced fibrosis in renal models of injury (9).

In conclusion, we demonstrate that CRT is a critical regulator of neointimal hyperplasia and collagen production *in vivo*. Knockdown of CRT with Cre-recombinase-IRES-GFP significantly reduces neointima-to-media ratio and collagen content without affecting VSMC proliferation. In vitro studies suggest that in VSMCs, CRT regulates TGF-β mediated collagen production through calcineurin/NFAT signaling.

CHAPTER 4

DISCUSSION

Analysis of work and future directions

Previous work from our lab demonstrated that CRT was a critical regulator of collagen production, secretion, and incorporation into the extracellular matrix. In addition, those studies provided initial evidence that CRT may be required for TGF-β stimulated ECM production. The novel work provided in this thesis clearly demonstrates that CRT is required for TGF-β stimulated matrix production. Treatment of wild type, but not CRT -/- MEFS, with TGF-β increased collagen and fibronectin mRNA and protein. Knockdown of CRT in rat Thy1 (-) lung fibroblasts or human lung fibroblasts isolated from patients with idiopathic pulmonary fibrosis showed impaired responsiveness to TGF-β stimulated ECM production. Similarly, downregulation of CRT using Cre-recombinase-IRES-GFP in CRT floxed VSMCs significantly impaired TGF-β stimulated type I collagen production. In addition, overexpression of CRT led to enhanced responsiveness to TGF-β stimulated collagen and fibronectin. Surprisingly, induction of ER stress in the absence of CRT failed to rescue the inability of CRT -/- cells to induce ECM production upon TGF-β treatment. The ability of CRT to regulate TGF-β stimulated ECM production is due to CRT mediated control of intracellular Ca^{2+} levels. Cells deficient in CRT failed to increase cytoplasmic Ca^{2+} upon treatment with TGF-β and CRT -/- MEFs were unable to induce calcineurin mediated NFAT

dephosphorylation following TGF-β treatment. Inhibition of calcineurin/NFAT signaling with 11R-VIVIT significantly impaired TGF-β stimulated ECM production in MEFs and VSMCs, indicating that the inability of TGF-β to stimulate ECM in CRT -/- cells is due to impaired CRT mediated Ca^{2+} release and downstream NFAT nuclear translocation. Finally, we demonstrate the importance of CRT in vascular fibroproliferative disease. Knockdown of CRT in CRT floxed mice with US mediated delivery of Cre-recombinase-IRES-GFP plasmid significantly reduced neointimal hyperplasia and collagen production, establishing a novel role of CRT in regulating vascular responses to injury.

Overall, the fact that cells deficient in CRT are unable to induce ECM upon TGF-β treatment is not surprising. Since CRT is an ER chaperone, the inability of CRT -/- cells to increase ECM following TGF-β treatment may simply be due to impaired ECM protein folding or trafficking. However, the fact that TGF-β increases ECM transcript in wild type, but not in CRT -/- cells, points towards a transcriptional defect in the CRT -/- MEFs. Pretreatment of wild type MEFs with actinomycin D to inhibit transcription prevented TGF-β stimulated ECM production further supporting this hypothesis.

Remarkably, knockdown of CRT by 40% in VSMCs or Thy 1 (-) rat lung fibroblasts significantly inhibits the ability of TGF-β to stimulate ECM. These data demonstrate that moderate alterations of CRT have a significant impact on the ability of TGF-β to induce ECM gene expression. While moderate downregulation of CRT in VSMCs or Thy 1 (-) rat lung fibroblasts impaired TGF-β stimulated ECM, reduction of CRT in L-fibroblasts had no impact on TGF-β stimulated ECM. This implies that CRT regulated ECM production is cell line specific and may hint that CRT plays a greater role in regulating matrix production in more pro-fibrotic cell lines. Also, these data suggest

that slight alterations in CRT levels may have a profound impact on matrix production *in vivo,* although this needs to be explored further.

The involvement of CRT in fibrotic and vascular fibroproliferative disease has remained unknown until recently. Kypreou and colleagues provided data demonstrating that upregulation of CRT precedes collagen deposition in the UUO model of renal injury (8). However, data on the effect of CRT knockdown on fibrotic disease have remained elusive as CRT -/- mice are embryonic lethal (34). Some insight into the involvement of CRT in fibrotic disease has been obtained using mice heterozygous for CRT. Prakoura et al. showed that mice heterozygous for CRT display reduced collagen deposition following UUO induced injury and decreased levels of type I and III collagen, fibronectin, and TGF-β (9). For the first time, we report the effect of CRT knockdown on carotid artery ligation induced neointimal hyperplasia in developmentally normal adult CRT floxed mice. In addition, we utilize tissue targeted US to specifically deliver the Cre-recombinase-IRES-GFP plasmid to the carotid artery.

Following vascular injury, there is significant staining for CRT in neointimal VSMCs suggesting that CRT is potentially involved in regulating progression of neointimal hyperplasia. To our knowledge, this is the first report showing CRT upregulation following acute vascular injury. In addition, knockdown of CRT in CRT floxed mice using Cre-recombinase-IRES-GFP significantly attenuates neointimal hyperplasia and collagen production. These data are in agreement with prior reports demonstrating that CRT heterozygous mice have reduced collagen production following UUO induced renal injury (9). Importantly, we obtain significant down regulation of CRT in the highly proliferative neointimal VSMCs, whereas CRT staining in medial

VSMCs appears unaltered following vascular injury. While delivery of Cre-recombinase-IRES-GFP with US technology successfully achieved approximately 50% knockdown of CRT, it would be interesting to determine the effect of complete CRT knockout in VSMCs on carotid artery ligation induced neointimal hyperplasia using cell type specific genetic approaches. Based on studies in MEFs and VSMCs, it is expected that complete loss of CRT in VSMCs *in vivo* may lead to more dramatic effects on neointimal hyperplasia and collagen production. However, more studies need to be done to confirm this hypothesis.

While it is apparent that CRT -/- cells are unable to induce matrix production following TGF-β treatment, the exact mechanism needs further clarification. Our data provide clear evidence that CRT is required for TGF-β stimulated Ca^{2+} release and downstream NFAT nuclear translocation. Nevertheless, several experimental results suggest that CRT may serve as a regulator of transcription factor nuclear import (387). This idea is supported by data demonstrating that, following TGF-β treatment, cells deficient in CRT have reduced nuclear translocation and promoter driven gene expression of some transcription factors. Following transfection with NFAT, AP1, or SP1 luciferase reporter assays, CRT -/- MEFs have significantly reduced luciferase reporter activity following TGF-β treatment as compared to wild type cells (data not shown). These results imply a broader role for CRT in regulating either transcription factor nuclear import, DNA binding, or the ability to activate gene transcription. One preliminary experiment provided some initial evidence that this may be the case. Following treatment of wild type MEFs with TGF-β, CRT appeared to translocate to the nucleus in conjunction with Smad 3 (data not shown). This observation suggests that the inability of

CRT -/- cells to stimulate ECM gene transcript following TGF-β treatment may be a result of impaired or reduced shuttling of transcription factors into the nucleus. Although this is speculative, further investigation into this idea may prove fruitful.

A study by Baek et al. demonstrated that knockdown of GRP78 in lung fibroblasts inhibited TGF-β or tunicamycin induced collagen production. Unfortunately, levels of CRT were not addressed in this study. This study parallels the work described in this thesis and suggests that alterations in ER chaperone proteins may affect TGF-β stimulated matrix production. A key to understanding ER stress and fibrotic disease lies in the balance between these ER chaperones. Our data suggest that in the absence of CRT, elevated levels of GRP78 are unable to rescue defects in TGF-β stimulated matrix production. While the alternative has not been addressed, it would be interesting to alter levels of both ER chaperones simultaneously and determine what effect this has on matrix production. In particular, overexpression of both proteins may be required for the enhanced levels of matrix production seen in fibrotic disease associated with ER stress. In particular, transgenic overexpression of these proteins followed by a fibrotic stimulus would be of interest.

Insight into the involvement of the chaperone and calcium binding functions of CRT in ER-stress induced disease must also be addressed. It would be particularly interesting to facilitate overexpression of CRT, induce fibrosis, and treat animals with a chemical chaperone to reduce ER stress such as 4-PBA. If treatment of mice overexpressing CRT and treated with 4-PBA prevents a fibrotic outcome, it could be assumed that the chaperone function of CRT is not a critical player in fibrosis *in vivo*. However, if high levels of fibrosis persisted, two separate conclusions might be made: (1)

CRT is not critically involved in fibrotic progression, although this appears unlikely due to our studies and those of others (8,9,104) or (2) the calcium binding function of CRT, and not the chaperone function is required for fibrotic disease progression. Alternatively, insight into the role of CRT's Ca^{2+} binding function could initially be obtained using CRT -/- cells transfected with a vector expressing the N and P domain of CRT, but lacking the high Ca^{2+} binding C domain. Several other experiments with a similar rationale could also help distinguish the role of CRT and other ER chaperones in in ER stress induced fibrosis.

TGF-β induces an increase in cytoplasmic Ca^{2+} levels as measured by the cytoplasmic Ca^{2+} binding dye, Fluo-4. However, the exact mechanism by which TGF-β induced this increase is unclear. Evidence from literature suggests that several pathways might be involved, including IP_3 and ROS mediated Ca^{2+} release. Investigation into the mechanism by which TGF-β increases cytoplasmic Ca^{2+} has been complex. We have initial data suggesting that inhibition of mitochondrial ROS with mitoquinone (MitoQ) can inhibit TGF-β stimulated collagen mRNA (Figure 24**)**. MitoQ is a mitochondrial targeted compound that is reduced to an active antioxidant at complex II of the respiratory chain (388). Despite the initial data suggesting that TGF-β may be increasing Ca^{2+} and ECM genes through an ROS dependent mechanism, this idea has not been fully explored. It would be interesting to treat wild type MEFs with TGF-β in the presence of MitoQ and look for increases in cytoplasmic Ca^{2+}. If no increase is observed, it would further suggest that TGF-β is working through ROS to induce cytoplasmic Ca^{2+} and ECM proteins. The exact role of CRT in ROS induced Ca^{2+} release is also unknown. However, since CRT is in the ER, which is a redox sensitive environment, it could be speculated

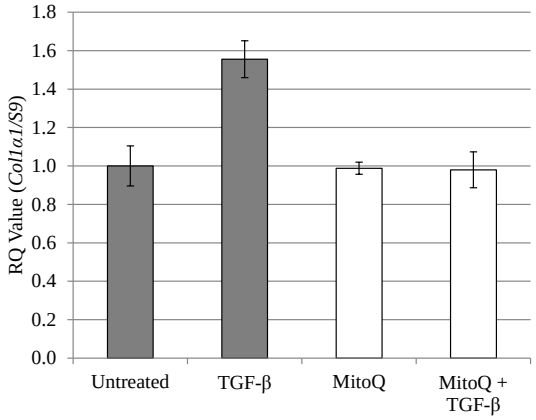


Figure 25. MitoQ inhibits TGF-β stimulated collagen transcript. Wild type MEFs were grown overnight in media with 10% FBS, starved overnight in low serum (0.5% FBS) media, and pretreated with vehicle only (grey bars) or 50 nM MitoQ for one hour (open bars). Cells were treated with or without 100 pM TGF-β for 4 hours and RNA was harvested with TRIZOL. Transcript levels of *COL1A1* were determined by RTQ-PCR and normalized to S9 levels +/- S.D. Results are from one experiment of triplicate samples.

that CRT may serve as an ROS inducible Ca^{2+} release protein. Much work would need to be done to confirm this hypothesis however.

TGF-β might increase cytoplasmic Ca^{2+} through several mechanisms. We performed a preliminary experiment investigating the role of extracellular Ca^{2+} in TGF-β stimulated cytoplasmic Ca^{2+} increase. Treatment of wild type MEFs with TGF-β in the absence of extracellular Ca^{2+} completely prevented increases in cytosolic Ca^{2+} (Figure 25). These data suggest that the inability of CRT -/- MEFs to stimulate increased cytoplasmic Ca^{2+} might be due to impaired extracellular Ca^{2+} influx. The effect of CRT on this process could be direct or indirect since CRT is present at the cell surface. CRT might also be impacting TGF-β stimulated extracellular Ca^{2+} influx through regulation of protein folding of Ca^{2+} channels. This speculation is in accordance with data by Nakamura et al. who showed that CRT -/- MEFs failed to increase cytoplasmic calcium following bradykinin treatment due to impaired folding of the bradykinin receptor (33). Nevertheless, these hypotheses need further investigation in order to clarify the exact mechanism by which TGF-β is increasing cytoplasmic Ca^{2+}.

Evidence for CRT in the extracellular matrix has been limited to date. Somogyi and colleagues demonstrated that CRT was present in the matrix of predentin and odontoblasts and work from our lab suggests that CRT is present in the detergent-insoluble ECM of fibroblasts (4,95). Furthermore, it has been demonstrated that cellular CRT co-immunoprecipitates with type I collagen in MEFs (6). Despite a lack of biochemical evidence, these data suggest that CRT is indeed present in the ECM. CRT might also be involved in organization of type I collagen in the extracellular matrix, although direct evidence for this is lacking. Although difficult, it would be interesting to

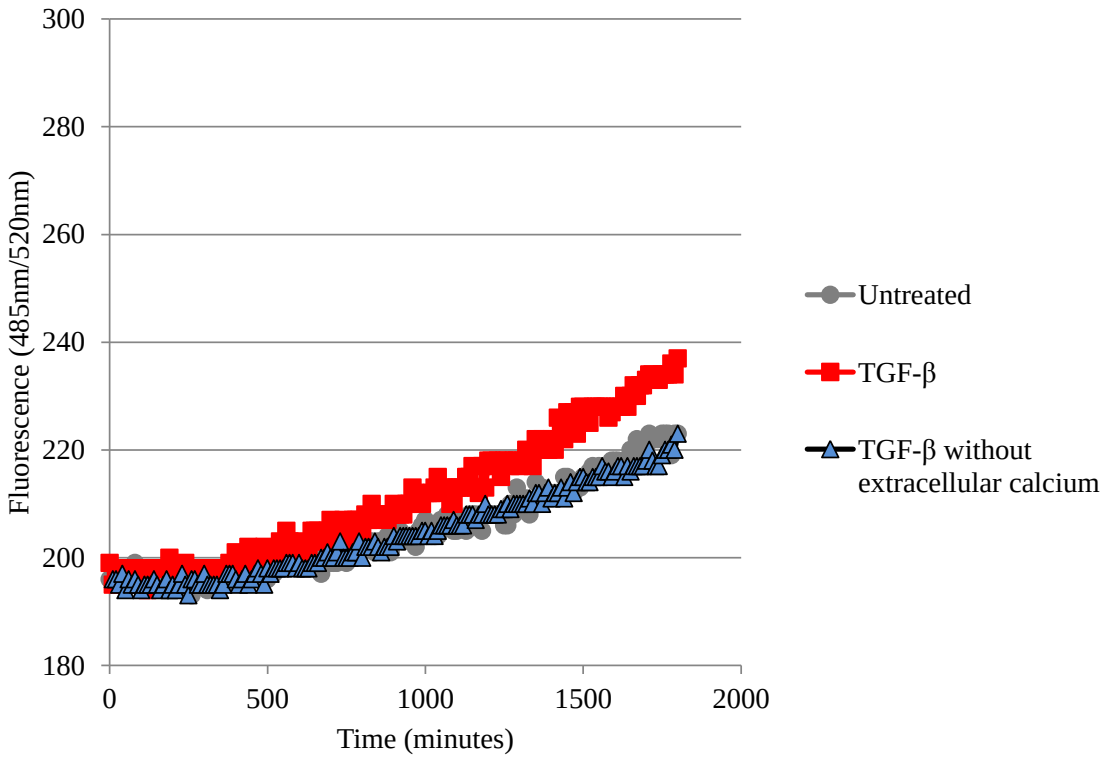


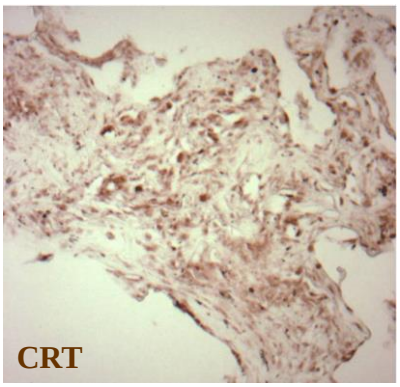
Figure 26. Removal of extracellular Ca^{2+} prevents TGF-β stimulated increases in intracellular Ca^{2+}. Wild type MEFs were plated overnight in DMEM with 10% FBS, washed with low (0.5%) serum media and loaded with 5 μM Fluo-4 AM in D-PBS lacking Ca^{2+} and magnesium. Cells were loaded with dye for 20 minutes at 37° C. After a 5 min equilibration, cells were stimulated with 100 pM TGF-β (red squares), 100 pM TGF-β without extracellular Ca^{2+} (blue triangles), or vehicle (grey circle). Cells were excited at 485 nM and emission read at 520 nM. Results are representative of a typical experiment repeated in quadruplicate on at least 4 different occasions.

investigate the organization of collagen fibrils by high resolution microscopy in wild type and CRT -/- MEFs.

The work described in this thesis has demonstrated that CRT is an essential component of TGF-β stimulated ECM production in fibroblasts and other matrix producing cell lines. Furthermore, our data illustrate that CRT is regulating TGF-β stimulated matrix production through control of Ca^{2+} release and activation of the calcineurin/NFAT transcription factor pathway. These data suggest that regulation of CRT *in vivo* may alter ECM production in response to injury. It would be interesting to investigate what effect CRT knockdown or overexpression would have on bleomycin induced pulmonary fibrosis. Based on our results in fibroblasts, one could hypothesize that overexpression of CRT *in vivo* would cause worsened fibrotic disease compared to control following bleomycin treatment. Furthermore, one would expect that knockdown of CRT in the lung would reduce bleomycin induced pulmonary fibrosis. It would be of particular interest to drive CRT overexpression or CRT knockdown specifically in pulmonary fibroblasts since a majority of data investigating ER stress and fibrosis centers around AEC ER stress. We hypothesize that upregulation of ER stress, particularly CRT, in pulmonary fibroblasts is an essential component to pulmonary fibrosis. Indeed, CRT levels are high in fibrotic foci of patients with idiopathic pulmonary fibrosis and in mice with bleomycin induced pulmonary fibrosis (Figure 26). Therefore, driving overexpression of CRT in pulmonary fibroblasts, but not AEC, would provide interesting insight into ER stress exacerbated pulmonary fibrosis.

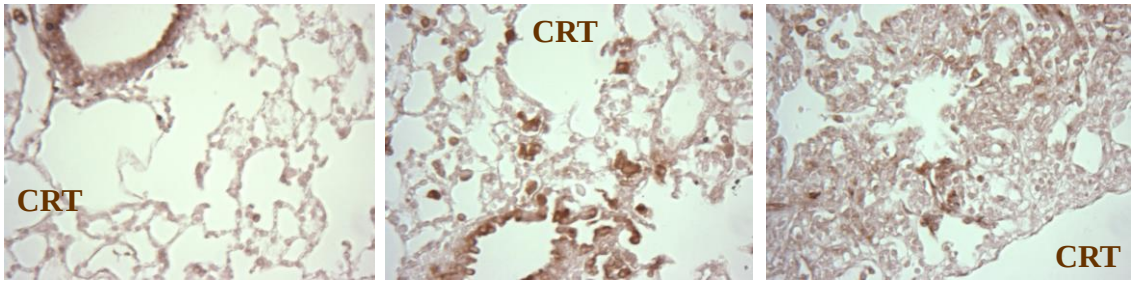
CRT regulates TGF-β stimulated matrix production through control of Ca^{2+} release and downstream calcineurin/NFAT signaling in fibroblasts. One experiment that

A.



CRT in human lung with IPF

B.



saline bleomycin bleomycin

Figure 27. CRT is present in patients with IPF and is increased in mice following treatment with the fibrotic inducing agent, bleomycin. (A) Human lung tissue from a patient with IPF stained with CRT. (B) Mice were treated with bleomycin (100 mg/kg) or saline via mini-osmotic pump delivery for 7 days. Mice were sacrificed 28 days post bleomycin delivery, lungs perfused with PBS, and harvested in OTC. Sections were stained using CRT antibody and pictures taken at 400X magnification.

would provide *in vivo* confirmation of this observation would involve pretreatment of mice with 11R-VIVIT followed by induction of pulmonary fibrosis with bleomycin. One would expect that pretreatment with the calcineurin/NFAT inhibitor should reduce or prevent matrix accumulation following injury. However, there are some caveats to this experiment. A majority of patients with pulmonary fibrosis don't present clinically until fibrosis is well developed; therefore, pretreatment of mice with the 11R-VIVIT compound may not be clinically relevant. A more clinically focused experiment would be to induce fibrosis with bleomycin, and deliver 11R-VIVIT 7 days following injury. The 7 day delay allows for resolution of the innate immune response and is typically considered the beginning of the pro-fibrotic phase (389). The results obtained from these experiments would provide critical information regarding the effect of 11R-VIVIT and calcineurin mediated NFAT dephosphorylation on bleomycin induced pulmonary fibrosis and provide *in vivo* conformation of the previously described *in vitro* results.

Conclusions and Summary

Although much progress has been made in the CRT field in recent years, several questions still remain. These studies demonstrate that CRT is a critical regulator of TGF-β stimulated ECM production through control of Ca^{2+} release and NFAT nuclear translocation. We show that in the absence of CRT, enhanced ER stress is not sufficient to drive TGF-β stimulated ECM production suggesting that CRT is a critical mediator of ER stress induced fibrosis. Knockdown of CRT using US mediated Cre-recombinase-IRES-GFP delivery to CRT floxed mice significantly reduced neointimal hyperplasia and collagen production. Together, these data show that CRT is playing a central role in ER stress induced fibrosis and vascular fibroproliferative disease and that targeting CRT

might be an effective therapeutic target for preventing morbidity and mortality associated with fibrotic disease. Further *in vivo* data demonstrating that alterations in CRT levels affect fibrotic outcome will be essential for progression of CRT-targeted therapeutics to the clinic.

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APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM

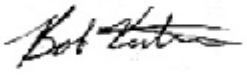
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: December 11, 2013

TO: JOANNE MURPHY-ULLRICH, Ph.D.
VH -G001
(205) 934-0415

FROM: 

Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Calreticulin in Diabetic Vascular Disease
Sponsor: American Heart Association
Animal Project_Number: 131209558

As of December 29, 2013 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

Species	Use Category	Number In Category
Mice	A	100
Mice	B	42

Animal use must be renewed by December 28, 2014. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 131209558 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-0019

APPENDIX B

INSTITUTIONAL REVIEW BOARD APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Review Board for Human Use

DATE: June 24, 2013

MEMORANDUM

TO: Kurt A Zimmerman
Principal Investigator

FROM: Cari Oliver, CIP
Assistant Director
Office of the Institutional Review Board (OIRB)

RE: Request for Determination – Human Subjects Research
IRB Protocol #N130429001 – Calreticulin is Required for TGF-B Stimulated Extracellular Matrix Production

A member of the Office of the IRB has reviewed your application for Designation of Not Human Subjects Research for above referenced proposal.

The reviewer has determined that this proposal is **not** subject to FDA regulations and is **not** Human Subjects Research. Note that any changes to the project should be resubmitted to the Office of the IRB for determination.

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