Calreticulin (CRT) regulates Transforming Growth Factor-β (TGF-β) stimulated extracellular matrix production

Kurt A. Zimmerman¹, Lauren V. Graham¹², Manuel A. Pallero¹, and Joanne E. Murphy-Ullrich¹

¹From the Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-0019
²present address: Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

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To whom correspondence should be addressed: Joanne E. Murphy-Ullrich, Department of Pathology, University of Alabama at Birmingham, 668B Volker Hall, 1720 2nd Ave. S, Birmingham, AL 35294-0019, Tel: 205-934-0415, FAX: 205-975-9340, E-mail: murphy@uab.edu

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Background: Endoplasmic reticulum (ER) stress is associated with fibrotic diseases although the mechanisms are not completely understood.

Results: The ER stress protein calreticulin regulates TGF-β stimulated extracellular matrix through control of intracellular calcium and NFAT signaling.

Conclusion: Calreticulin is necessary for TGF-β stimulated extracellular matrix production.

Significance: These findings identify calreticulin as a mechanistic link between ER stress and fibrosis.

SUMMARY
Endoplasmic reticulum (ER)¹ stress is an emerging factor in fibrotic disease, although precise mechanisms are not clear. Calreticulin (CRT) is an ER chaperone and regulator of Ca²⁺ 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 Ca²⁺ signaling is important for induction of ECM. CRT -/- MEFs failed to increase intracellular Ca²⁺ 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 Ca²⁺-dependent pathways as a critical molecular link between ER stress and TGF-β fibrotic signaling.
also inhibit TGF-β-induced type I collagen production independent of Smad reporter activity (14). 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 (1). Enhanced ER stress in alveolar epithelial cells facilitates epithelial to mesenchymal transition, a process which occurs in some forms of fibrosis (15). Despite growing evidence for ER stress as a 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 (1,6,15). However, ER stress is also associated with fibroproliferative remodeling in tissues such as the diabetic vasculature where apoptosis is not a significant initiating factor (3,4). 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 (16,17). 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) (17). 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 (18,19). 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 (20,21). 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 (20,22,23).

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 (24). CRT regulation of fibronectin is also thought to involve ER Ca\(^{2+}\) (25,26). 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 (2-4). Cardiac specific overexpression of CRT during development results in interstitial fibrosis, although the mechanisms have not been defined (27). 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 (2,3,28-30).

TGF-β is a major stimulus of ECM production in fibroproliferative diseases (31). 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. (32-36). Ca\(^{2+}\)-dependent pathways also regulate TGF-β stimulation of ECM (37,38). TGF-β treatment leads to increased cytosolic Ca\(^{2+}\), which induces calcineurin-mediated NFAT dephosphorylation and enhanced expression of fibronectin (37,38). 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 (38). TGF-β can increase IP3 levels, thereby causing release of ER Ca\(^{2+}\) (39). In addition, TGF-β can increase cytoplasmic Ca\(^{2+}\) through translocation of type III IP3 receptors to the cell surface, through stimulation of H\(_{2}\)O\(_{2}\) mediated Ca\(^{2+}\) release, or through a c-Jun dependent mechanism (40-43).

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 (20,23,24). 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 (GGAGGAAAAACTGTTCATAACAGAAGGC GT) and Smad (AGGCCAGACA) 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 (44). 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 (45). Rat lung fibroblasts (RFL6) stably expressing an empty vector (pcDNA3.1, Invitrogen) were generated as previously described (gift of Dr. James Hagood) (46). 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 (47). 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 (SI0449004) 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 Amaza Biosystems (Amaza GmbH, Lonza) in an Amaza 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 Amaza Biosystems (Amaza GmbH, Lonza) in an Amaza 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 (24). 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.

Ca²⁺ 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 1A,B). In contrast, TGF-β failed to stimulate an increase in either COL1A1 or fibronectin transcript in MEFs lacking CRT (Figure 1A,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 1C,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 1C,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 2A). 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 (48) shows increased fibronectin in the cellular fraction following TGF-β treatment (Figure 2B). Similarly, TGF-β failed to stimulate an increase in secreted soluble collagen in the conditioned media of CRT -/- MEFs (Figure 2C). 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 (24). Ascorbic acid increases collagen transcript stability and is a cofactor for proline hydroxylation of procollagen, which enhances translation and secretion efficiency (49,50).

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 (44). Similar to wild type cells, TGF-β induced a significant increase in COLIA1 and fibronectin transcript production in CRT /- MEFs stably transfected with HA-tagged CRT (Figure 3A).

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 3B) (44,47).

**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 fibrosis and rat thy-1 (-) fibroblasts have robust production of ECM in response to TGF-β (46,51.52). 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 4A-D). Similar results were obtained by siRNA knockdown of human CRT in lung fibroblasts isolated from IPF patients (Figure 4E). 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 (2). 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 (24). TGF-β treatment induced a greater increase in fibronectin and collagen I protein as compared to TGF-β-stimulated parental cells (Figure 5A-C).

**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 (1,14,53). 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 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 6A,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 6A,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 6A,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 (33,36,54). 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 7A). Since CRT -/- MEFs are defective in their ability to induce MEFC2 nuclear translocation (21), 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 phosphorylated Smad 3 (Figure 7B). 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 reporter activity in wild type and CRT -/- MEFs (Figure 7C). LY364947, an ALK5 TGF-βRI inhibitor, blocked TGF-β stimulation of Smad 2/3 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 7C). 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 Ca²⁺** TGF-β can stimulate the slow release of intracellular Ca²⁺, which activates the calcineurin/NFAT pathway to increase fibronectin expression in mesangial cells (37,41,55-57). Since CRT regulates both ER Ca²⁺ and calcineurin/NFAT activity (17,21,22), we asked whether TGF-β stimulation of Ca²⁺ release was altered in CRT -/- MEFs. Wild type and CRT -/- MEFs were treated with TGF-β or the Ca²⁺ ionophore, ionomycin, as a positive control (58). Ca²⁺ release was measured over time using the Ca²⁺ binding fluorescent dye Fluo-4 AM. With ionomycin, both wild type and CRT -/- MEFs induced a large increase in cytosolic Ca²⁺, although Ca²⁺ re-uptake occurred more slowly in CRT -/- MEFs (Figure 8A,B). In contrast, TGF-β increased cytoplasmic Ca²⁺ levels in wild type, but not in CRT -/- MEFs (Figure 8A,B), suggesting that CRT is required for TGF-β-dependent Ca²⁺ signaling.

To determine whether TGF-β stimulation of cytosolic Ca²⁺ 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 Ca²⁺ re-uptake to effectively deplete ER releasable Ca²⁺ (59). Thapsigargin blocked the ability of TGF-β to stimulate collagen I and fibronectin transcript (Figure 8C,D), suggesting that TGF-β stimulation of cytosolic Ca²⁺ release is required for induction of ECM proteins. Cyclopiazonic acid, a SERCA2b inhibitor which acts in a manner similar to thapsigargin (60), also inhibited TGF-β stimulated ECM production (data not shown). To determine if increased cytoplasmic Ca²⁺ 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 8E), 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 Ca²⁺-activated calcineurin and NFAT activity can regulate TGF-β stimulated ECM production in the presence of sustained increases in cytoplasmic Ca²⁺ (37,38). Given the importance of CRT for TGF-β stimulation of cytoplasmic Ca²⁺ levels and the deficient NFAT activation in CRT -/- mouse embryos (20,23), 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 9A) (61). Furthermore, TGF-β...
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 PxxIxxT sequence and prevents calcineurin-dependent NFAT dephosphorylation and nuclear translocation (61,62). 11R-VIVIT blocked TGF-β stimulation of ECM in wild type MEFs (Figure 9C,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 (63), 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 (1,2). 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 Ca2+ and NFAT activity is required for TGF-β stimulation of collagen type I and fibronectin transcript (Figure 10). 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 ((2,4) 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 (24). In addition, CRT regulation of Src-dependent fibronectin expression and matrix deposition impacts collagen matrix assembly (24,25). Our current studies now show that CRT regulation of Ca2+-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 (64-67). 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 Ca2+ and activate calcineurin, which dephosphorylates NFAT resulting in NFAT nuclear translocation (37,38). Despite these data, the mechanism by which TGF-β increases cytosolic Ca2+ remains unclear. Our data suggest that CRT is essential for TGF-β to increase cytoplasmic Ca2+ levels when treated with TGF-β. There are several mechanisms by which TGF-β increases cytoplasmic Ca2+ levels since cells lacking CRT were unable to increase cytoplasmic Ca2+ when treated with TGF-β. There are several mechanisms by which TGF-β increases cytoplasmic Ca2+ levels, including increasing IP3 levels to cause release of Ca2+ from thapsigargin sensitive stores in the ER, mediating translocation of type III IP3 receptors to the cell surface, stimulating H2O2 mediated Ca2+ release, and via a c-Jun dependent mechanism (39-43). Rises in cytoplasmic Ca2+ levels are typically regulated by ER mediated Ca2+ release or through store operated Ca2+ entry through channels present on the plasma membrane (68,69). CRT can regulate both ER mediated Ca2+ release and store operated Ca2+ entry (19,20,70-72). The ability of thapsigargin to block responses to TGF-β suggests that CRT regulation of ER Ca2+ stores might be involved, although TGF-β stimulation of wild type MEFs in media lacking extracellular Ca2+ failed to stimulate a rise in cytoplasmic Ca2+, suggesting that CRT might also regulate cytoplasmic Ca2+ levels through regulation of

stimulation NFAT reporter activity is also absent in CRT +/- MEFs (Figure 9B).
store operated Ca\(^{2+}\) entry (data not shown). In addition, treatment of CRT -/- MEFs with TGF-\(\beta\) in the presence of ionomycin failed to make CRT -/- cells responsive to TGF-\(\beta\), showing that TGF-\(\beta\) stimulation of ECM is not simply a function of increased calcium levels and suggesting that additional CRT-dependent functions are involved in regulating TGF-\(\beta\) stimulation of ECM.

TGF-\(\beta\) 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 (21,23). Furthermore, studies by Gooch et al., showed that TGF-\(\beta\) activates calcineurin in a time and dose dependent manner and that calcineurin is required for TGF-\(\beta\) stimulated ECM in glomerular mesangial cells (37). These studies showed that TGF-\(\beta\) 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-\(\beta\) (38). 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 (73,74).

CRT can regulate cell behavior, ECM production and wound healing from multiple cellular compartments, including at the cell surface and as an extracellular ligand (75,76). 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-\(\beta\) responses, since CRT -/- MEFs stably expressing CRT lacking the TSP1 binding sequence had normal responses to TGF-\(\beta\) (47).

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-\(\beta\) signaling (45,77), we also examined the ability of TGF-\(\beta\) to stimulate Smad reporter activity in CRT deficient cells. Although reporter activity in CRT -/- MEFs was reduced as compared to wild type cells, TGF-\(\beta\) 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 (78,79). We also investigated whether the lack of CRT impacted non-Smad pathways regulated by TGF-\(\beta\) (32-36). 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-\(\beta\) (data not shown). There was a trend towards increased phosphorylated p-38 MAPK in CRT -/- cells treated with TGF-\(\beta\), 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 (3-5,80,81). 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 (3-5). 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 (8,15). Although there is a clear role for ER stress induced apoptosis in some models of fibrosis (6), there is also evidence for ER stress involvement in processes important for fibroproliferative remodeling which are independent of apoptosis (82,83). Knockdown of GRP78, another ER stress response protein, also reduces TGF-\(\beta\) or tunicamycin stimulated collagen and \(\alpha\)-smooth muscle actin production in human lung fibroblasts (82). 150-kDa oxygen-regulated protein (ORP150) mediates TGF-\(\beta\) myofibroblast induction and collagen production in human lung fibroblasts (83). The mechanisms by which GRP78 and ORP150 mediate responsiveness to TGF-\(\beta\) were not addressed in these reports. Our data now suggest that CRT is an important ER stress factor in fibrotic remodeling through regulation TGF-\(\beta\) stimulated ECM production. It is interesting that CRT mediates TGF-\(\beta\)
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 (84). Nonetheless, the majority of evidence suggests that enhanced ER stress is associated with exacerbated fibrotic outcomes (1-7,10,14,15,85).

TGF-β has been shown to increase expression of ER stress response proteins, including CRT in the human IPF lung fibroblasts and rat vascular smooth muscle cells (data not shown) (82,83). 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 (86)] and reduced expression of multiple ECM proteins (24), 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.

References
Intracellular calreticulin regulates multiple steps in fibrillar collagen expression, trafficking, and deficient mouse.


12


**Acknowledgements**

The authors wish to thank Drs. Michal Opas (University of Toronto) and Dr. Marek Michalak (University of Alberta) for the generous gifts of L fibroblasts and CRT-/- MEFs and Dr. Victor Thannickal (UAB) for the gift of human IPF lung fibroblasts. We thank Abbott Labs for the gift of A285222. We thank Dr. Majd Zayzafoon and Dr. John Chatham, Department of Pathology, UAB for helpful discussions. We also thank Claire Gamlin for her work on signaling studies on non-canonical TGF-β pathways. We would also like to thank Shawn Williams of the UAB High Resolution Imaging Facility for assistance in acquiring confocal images. This work was supported by AHA Innovation grant 12IRG160008 to JMU and NIH T32 HL007918 to KAZ and NIH T32 GM008361 to LVG. This work was performed in facilities supported by NIH grant C06RR15490.

**Footnotes**

1 Abbreviations: TGF-β, transforming growth factor-beta; ER, endoplasmic reticulum; NFAT, nuclear factor of activated T cells; IPF, idiopathic pulmonary fibrosis; MEF2C, myocyte enhancer factor 2C; CRT, calreticulin; ECM, extracellular matrix; MEFs, mouse embryonic fibroblasts; Smad, small mothers against decapentaplegic; UUO, unilateral ureteral obstruction; PI3K, phosphatidylinositol-3-kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; IP3, inositol-3-phosphate; TSP1, thrombospondin 1; GRP78, glucose regulated protein 78; ALK5, activin like kinase 5; SERCA2b,
sarcoplasmic/endoplasmic reticulum calcium ATPase; JNK, c-jun N-terminal kinase; AKT; GRP94, glucose regulated protein 94; PDI, protein disulfide isomerase; ORP150, oxygen-regulated protein 150; IPF, idiopathic pulmonary fibrosis
Figure 1. 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 2. CRT is required for TGF-β stimulation of fibronectin and collagen I protein. (A and B) 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. (A) 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). (B) The 4% DOC soluble (cell fraction) and insoluble (extracellular matrix) fractions of cells treated as in (A) were 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. (C) 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.

Figure 3. 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.

Figure 4. Knockdown of CRT in Thy1 +/- rat lung fibroblasts and human 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. (E) 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 5. 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.

Figure 6. 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 7. 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.

Figure 8. TGF-β stimulates Ca²⁺ release and Ca²⁺ 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. (E) 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.
Figure 9. 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 COLIA1 (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.

Figure 10. Model of CRT regulation of TGF-β ECM transcription TGF-β binds to the heterotetrameric receptor complex (blue bars) to activate receptor type I kinase activity, which phosphorylates Smad2 and Smad 3. TGF-β also increases cytosolic Ca²⁺. The specific mechanism by which TGF-β regulates Ca²⁺ release and the involvement of Smad signaling in Ca²⁺ release are not yet clear (orange arrow). TGF-β stimulated Ca²⁺ release is dependent on CRT regulation of ER Ca²⁺ and possibly CRT regulated store operated Ca²⁺ entry (not depicted). Increased cytosolic Ca²⁺ leads to activation of calcineurin which dephosphorylates cytoplasmic NFAT, resulting in NFAT activation and nuclear translocation. NFAT can directly stimulate transcription of ECM proteins or partner with other known matrix inducing transcription factors such as AP-1 and SP-1. In the absence of CRT, there is a failure of TGF-β to increase cytoplasmic Ca²⁺, activate NFAT, and upregulate ECM transcription. In contrast, under pathological ER stress, CRT expression can be upregulated, resulting in increased TGF-β stimulated ECM.
Figure 1
Figure 2

A.

<table>
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<tr>
<th>Wild type MEFs</th>
<th>CRT -/- MEFs</th>
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<tbody>
<tr>
<td>Fibronectin</td>
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</tr>
<tr>
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<tr>
<td>β-tubulin</td>
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TGF-β (pM)  0  5  50  100  200  400

Fibronectin

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Collagen I

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<td>Collagen type I/β-tubulin</td>
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B. 

DOC soluble

DOC insoluble

Fibronectin (Light)

Fibronectin (Dark)

β-tubulin

TGF-β

-  +  -  +  -  +  -  +
WT MEFs  CRT -/- MEFs  WT MEFs  CRT -/- MEFs

C.

μg/ml collagen/10⁴ cells

0.0  0.2  0.4  0.6  0.8  1.0  1.2  1.4

-  +  -  +
Figure 3

A. TGF-β

B. RQ Value (Normalized to S9)
Figure 4

A. 

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<tr>
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<td>NT + TGF-β</td>
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C. 

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D. 

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Figure 4

E.

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Figure 5

A. 

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<tr>
<td>β-tubulin</td>
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TGF-β  

-  
+  
-  
+  

24 hour treatment

B. 

<table>
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TGF-β  

-  
+  
-  
+  

48 hour treatment

C. 

<table>
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<tr>
<td>Parental</td>
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<tr>
<td>CRT overs</td>
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</table>

- Fibronectin
- Collagen I

*
Figure 6

A. | Wild type MEFs | CRT -/- MEFs |
---|---|---|
Tunicamycin | - | - | + | + | - | - | + | + |
TGF-β | - | + | - | + | - | + | - | + |
Collagen I (dark blot) | | | | | | | |
Collagen I (light blot) | | | | | | | |
GRP 78 | | | | | | | |
β-tubulin | | | | | | | |

B. GRP78

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<td>1.0</td>
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C. Collagen

<table>
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* denotes significant difference compared to control.
Figure 7

A. Wild type MEFs vs. CRT -/- MEFs

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<tr>
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<tr>
<td><strong>Minutes</strong></td>
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B. CRT WT vs. CRT -/-(p-Smad 3)

C. Firefly luciferase

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<td>TGF-β</td>
<td>5.0</td>
<td>4.0</td>
</tr>
<tr>
<td>TGF-β + LY364947</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 8

A. Wild type MEFS

B. CRT-/- MEFS

C. 

D.
Figure 8

E.
Figure 9

A. Wild type MEFs vs. CRT -/- MEFs

TGF-β

0 min

5 min

15 min

NFATc3

B. Fold induction (firefly/Renilla)

Fold induction (firefly/Renilla) vs. TGF-β

wild type MEFs

CRT -/- MEFs

C. RQ Value (Fibronectin/S9)

RQ Value (Fibronectin/S9) vs. TGF-β

11R-VIVIT

D. RQ Value (COL1A1/S9)

RQ Value (COL1A1/S9) vs. TGF-β

11R-VIVIT
ER Stress

- CRT
- Ca$^{2+}$

TGF-β

- Smad2/3

Ca$^{2+}$

- NFAT-P

NFAT

Calcineurin

ECM

NFAT

CRT

Ca$^{2+}$

Ca$^{2+}$

Ca$^{2+}$

Ca$^{2+}$

Ca$^{2+}$

Ca$^{2+}$

Figure 10