AMERICAN SOCIETY FOR MICROBIOLOGY | Molecular and Cellular Biology

Dissection of Thrombospondin-4 Domains Involved in Intracellular Adaptive Endoplasmic Reticulum Stress-Responsive Signaling

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Thrombospondins are a family of stress-inducible secreted glycoproteins that underlie tissue remodeling. We recently reported that thrombospondin-4 (Thbs4) has a critical intracellular function, regulating the adaptive endoplasmic reticulum (ER) stress pathway through activating transcription factor 6α (Atf6α). In the present study, we dissected the domains of Thbs4 that mediate interactions with ER proteins, such as BiP (Grp78) and Atf6α, and the domains mediating activation of the ER stress response. Functionally, Thbs4 localized to the ER and post-ER vesicles and was actively secreted from cardiomyocytes, as were the type III repeat (T3R) and TSP-C domains, while the LamG domain localized to the Golgi apparatus. We also mutated the major calcium-binding motifs within the T3R domain of full-length Thbs4, causing ER retention and secretion blockade. The T3R and TSP-C domains as well as wild-type Thbs4 and the calcium-binding mutant interacted with Atf6α, induced an adaptive ER stress response, and caused expansion of intracellular vesicles. In contrast, overexpression of a related secreted oligomeric glycoprotein, Nell2, which lacks only the T3R and TSP-C domains, did not cause these effects. Finally, deletion of Atf6α abrogated Thbs4-induced vesicular expansion. Taken together, these data identify the critical intracellular functional domains of Thbs4, which was formerly thought to have only extracellular functions.

Thrombospondins (Thbs) are secreted Ca^{2+}-binding glycoproteins that have fundamental roles in wound healing and tissue repair (1, 2). The thrombospondin family consists of two subgroups based on conservation and oligomeric structure. Thbs3, Thbs4, and Thbs5/cartilage oligomeric matrix protein (COMP) form pentamers and are the most evolutionarily conserved among the five mammalian thrombospondins. Thbs1 and Thbs2 form trimers and have evolved additional domains, such as a type 1 repeat important for transforming growth factor beta (TGFβ) signaling (1, 3–5). While traditionally characterized as extracellular matrix (ECM) or matricellular proteins, thrombospondins have also been ascribed an intracellular function (6–8), mediating an adaptive endoplasmic reticulum (ER) stress response (8). Thbs proteins traverse the secretory pathway from the ER to the Golgi apparatus and then on to post-Golgi vesicles, where they likely facilitate secretion of ECM proteins (1, 9, 10). Once secreted, Thbs proteins transiently reside in the ECM, where they interact with fibronectin, integrins, and collagens (11–16) and also participate in collagen fibril assembly (1, 11, 13). Thbs proteins are also recycled and taken back into the cell through the low-density receptor-related protein (LRP) (17).

All thrombospondin family members contain a coiled-coil domain responsible for oligomerization within the ER (5, 18, 19), as well as an epidermal growth factor (EGF)-like repeat domain, a type III repeat (T3R) domain, and an L-type lectin (TSP-C) domain that comprise the C-terminal Ca^{2+}-binding "signature domain" (1, 4, 20). Thbs1, -2, -3, and -4 also contain an N-terminal laminin G-like (LamG) domain that facilitates interactions with ECM proteins and glycoproteins (1, 4, 21). However, the roles of the various domains of thrombospondins in the intracellular and secretory compartments remain unknown.

Human mutations in *THBS5*/*COMP* result in skeletal dysplasias, including pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED), with most of the disease-causing mutations occurring in the T3R domain (22–27). THBS5 mutant proteins that cause PSACH or MED exhibit a spectrum of molecular defects, including reduced Ca^{2+} binding and accumulation in the ER, with delayed secretion. This results in chondrocytes that have a dilated rough ER and inclusions containing THBS5 and other ECM proteins, ultimately leading to an altered ECM composition and chondrocyte death (24–31). A majority of the Ca^{2+} bound by thrombospondins interacts with the T3R domain (20, 32), suggesting that T3R-mediated Ca^{2+} binding may be critical for proper trafficking and/or secretion of thrombospondins.

All thrombospondin family members are expressed at low levels in the heart but highly induced in response to cardiac stress, including myocardial infarction or pressure overload (8–10, 33, 34). Thbs4 is one of the most evolutionarily and structurally conserved family members (1, 35), and its mRNA expression appears to be restricted mostly to heart and skeletal muscles (36, 37). Thbs4 overexpression in the heart protects from pressure overload and myocardial infarction injury, in part by modulating ECM remodeling (8, 9). Thbs4 also protects the heart through an intracellular functionality that includes mediating a cardioprotective ER stress response through a direct mechanism involving activating transcription factor 6α (Atf6α) (8). Cardiomyocyte-specific overexpression of Thbs4 results in a dramatic expansion of intracellular vesicles *in vivo* (8), although the mechanism for this vesicular expansion mediated by Thbs4 is unknown. Thbs4 over-

Received 17 June 2015 **Returned for modification** 8 July 2015
Accepted 1 October 2015

Accepted manuscript posted online 12 October 2015

Citation Brody MJ, Schips TG, Vanhoutte D, Kanisicak O, Karch J, Maliken BD, Blair NS, Sargent MA, Prasad V, Molkentin JD. 2016. Dissection of thrombospondin-4 domains involved in intracellular adaptive endoplasmic reticulum stress-responsive signaling. Mol Cell Biol 36:2–12. doi:10.1128/MCB.00607-15.

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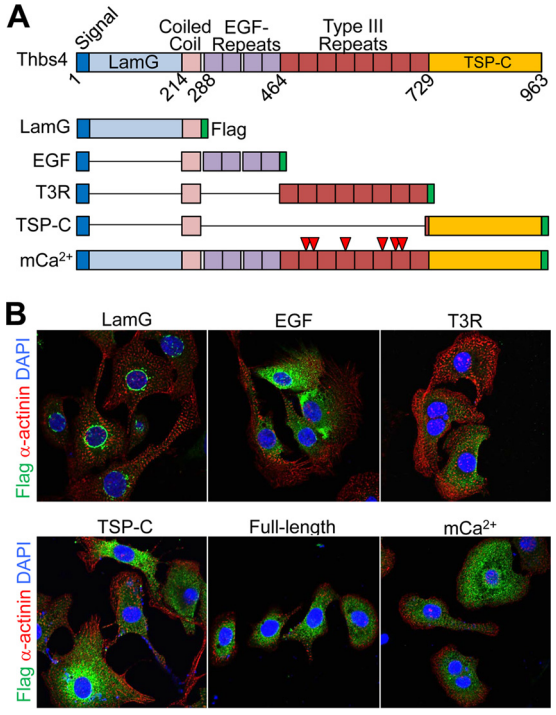

FIG 1 Generation and overexpression of Thbs4 domains and a calcium-binding mutant in neonatal cardiomyocytes. (A) Schematic diagram depicting the Thbs4 domains used and the calcium-binding mutant (mCa^{2+}) adenoviral construct generated. All constructs contained the N-terminal signal peptide (blue) and the coiled-coil domain (pink). Flag-tagged Thbs4 constructs containing the N-terminal laminin G domain (LamG), the epidermal growth factor-like repeat domain (EGF), the type III repeat domain (T3R), or the C-terminal L-type lectin domain (TSP-C) were generated along with a wild-type Thbs4 (full-length) adenovirus. Arrowheads in the T3R domain of Thbs4 indicate sites of mutagenesis of DXDXDG Ca^{2+}-binding motifs to AXAXAG. (B) Neonatal rat cardiomyocytes transduced with the indicated Thbs4 adenoviruses and immunostained for Thbs4 (Flag; green), α-actinin (cardiomyocyte marker; red), and DNA in the nucleus (DAPI; blue).

expression in the heart also reduces protein aggregate formation and ameliorates cardiac dysfunction in aggregation cardiomyopathy models (8), indicating prominent roles in regulating ER stress-responsive signaling, protein quality control, and intracellular trafficking during cardiac disease.

MATERIALS AND METHODS

Animals. Cardiomyocyte-specific Thbs4 transgenic (TG) mice were described previously (8). Neural EGF-like 2 (Nell2) cardiomyocyte-specific transgenic mice were generated on the FVB/N background by subcloning the human Nell2 cDNA (Open Biosystems) into the α-myosin heavy chain promoter expression vector (38), followed by NotI digestion and gel purification to remove the vector backbone for oocyte injection of the appropriate DNA fragment at the Cincinnati Children's Hospital Transgenic Animal and Genome Editing Core Facility. *Atf6α*$^{-/-}$ mice have been described previously (39). All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (40) and approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Cell culture and adenoviral transduction. The various Thbs4 domains were cloned into the p3xFlag-CMV vector (Sigma) as depicted in Fig. 1A, using the In-Fusion HD cloning system (Clontech). A Thbs4 Ca^{2+}-binding mutant containing mutations in six DXDXDG calcium-binding sites (D499/501/503A, D512/514/516A, D571/573/575A, D632/634/636A, D655/557/659A, and D668/670/672A) within the T3R domain of mouse Thbs4 was generated by gene synthesis (BioBasic) and subcloned into p3xFlag-CMV. In order to obtain a functional TSP-C mutant adenovirus with efficient overexpression in cells, we included a short (27 amino acid) C-terminal portion of the T3R domain for added stability (Fig. 1A), likely because part of the T3R domain structure is required for proper folding and assembly of the TSP-C domain (20, 27, 32). The Flag-tagged Thbs4 domains and the Ca^{2+}-binding mutant were then subcloned into an adenoviral vector (pAdenoX-CMV; Clontech) and transfected into HEK293 cells to generate recombinant adenoviruses. The β-galactosidase (βgal) adenovirus, Cn-Atf6α adenovirus, and Atf6α-ER adenovirus, containing the ER luminal domain of Atf6α with a C-terminal KDEL ER retention signal, were described previously (8). The cDNAs for human Nell2 and mouse BiP were also cloned into pAdenoX to generate recombinant adenoviruses as described above. All constructs were confirmed by DNA sequencing.

Primary neonatal rat cardiomyocytes were isolated from 1- to 2-day-old Sprague-Dawley rat pups, cultured on gelatin-coated dishes as previously described (8), and maintained in M199 medium (Corning) supplemented with 1% bovine growth serum (BGS) (HyClone). Wild-type (WT) mouse embryonic fibroblasts (MEFs) and *Atf6α*$^{-/-}$ MEFs were described previously (8) and were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone) supplemented with 10% BGS. Cells were transduced with adenovirus for 4 h and then given fresh medium. For ER stress studies, cardiomyocytes were cultured on laminin-coated (Invitrogen) dishes in serum-free M199 medium supplemented with 1 mM pyruvate (Invitrogen) and harvested 48 h after adenoviral transduction. For electron microscopy, rat neonatal cardiomyocytes were cultured in M199 medium supplemented with 5% BGS, MEFs were cultured in DMEM supplemented with 10% BGS, and cells were harvested 4 and 3 days, respectively, after adenoviral transduction.

Purified adenoviruses were injected into the left and right gastrocnemius muscles of 1-day-old Sprague-Dawley rat pups (Harlan), followed by an additional injection 48 h later (10^8 viral particles for each injection). Rat pups were sacrificed at 8 days of age, and the muscles were harvested for Western blotting.

Luciferase assays. Luciferase assays were performed as described previously (41). Briefly, COS cells were cotransfected with plasmids encoding the 5×Atf6-Luc reporter (Addgene) (42), CMV-βgal, and the various Thbs4 domain mutants or the empty Flag vector by use of Lipofectamine 3000 (Invitrogen). The pCGN-Atf6α plasmid, encoding a constitutively nuclear mutant of Atf6α (Cn-Atf6α), was used as a positive control (Addgene) (42). Luciferase assays were performed with a luciferase assay system (Promega), and luminescence emitted from cell lysates was quantified on a Victor X Light luminometer (Perkin-Elmer). Results were normalized to β-galactosidase activity to control for transfection efficiency.

Immunoprecipitation and Western blotting. For analysis of secreted proteins, cardiomyocytes grown in 10-cm dishes were washed several times in serum-free medium and kept in 4 ml of fresh serum-free M199 medium for 4 h, and then the medium was collected and concentrated in Amicon Ultra centrifugal filters (Millipore) by centrifugation at 5,000 × *g* for 30 min at 4°C. Laemmli buffer was added to 30-μl aliquots of concentrated medium, and samples were loaded directly onto SDS-PAGE gels without boiling. Staining of nonspecific bands on polyvinylidene difluoride (PVDF) membranes with Ponceau S (Sigma) was used as a loading control for concentrated medium. Secreted proteins were deglycosylated using a protein deglycosylation mix (New England BioLabs). For immunoprecipitation experiments, cardiomyocytes were harvested in TBS (20 mM Tris, pH 7.5, 137 mM NaCl) with 1% Triton X-100 and protease inhibitors (Roche) and immunoprecipitated with anti-Flag M2 magnetic beads (Sigma) or with anti-myc (Cell Signaling Technology) or anti-BiP (Abcam) antibodies coupled to protein G Dynabeads (Invitrogen). Proteins were resolved by SDS-PAGE and silver stained according to the manufacturer's instructions (Sigma) or immunoblotted as described pre-

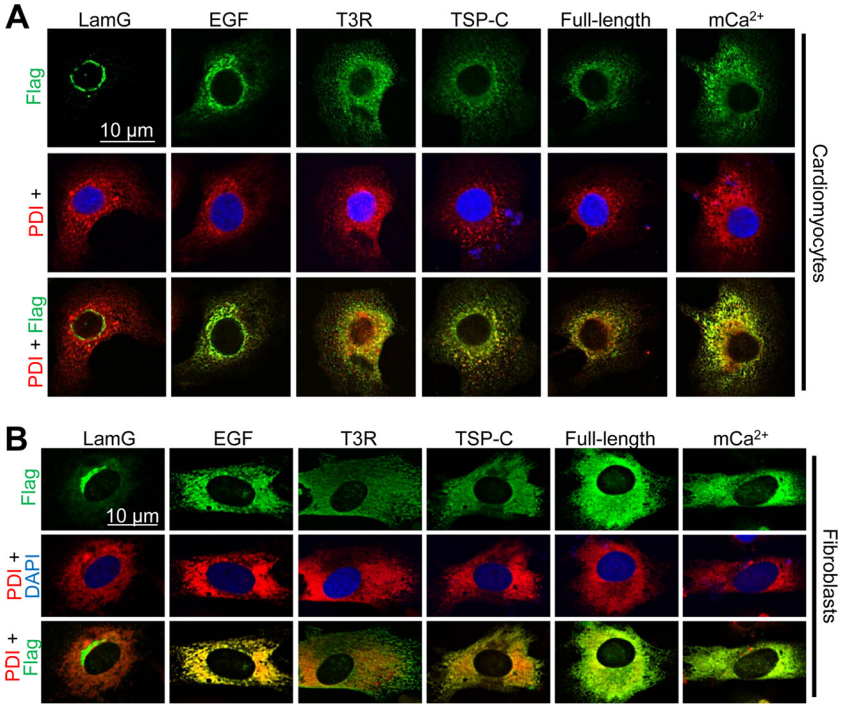

FIG 2 Localization of Thbs4 within the ER. Neonatal rat cardiomyocytes (A) and neonatal rat cardiac fibroblasts (B) were transduced with the indicated Thbs4 adenoviral constructs and coimmunostained for Thbs4 (Flag; green) and PDI (red), used to mark the ER. Nuclei were stained with DAPI (blue).

viously (43). For determination of novel interacting proteins, immunoprecipitated bands were cut from silver-stained gels, digested with trypsin, and identified by matrix-assisted laser desorption ionization–tandem time of flight (MALDI-TOF/TOF) mass spectrometry at the University of Cincinnati Proteomics Laboratory. Antibodies used for immunoblotting were against Flag, myc, calreticulin, inositol-requiring enzyme 1α (IRE1α), PKR-like ER kinase (PERK), and protein disulfide isomerase (PDI; Cell Signaling Technology), against BiP and sarcomeric α-actin (Sigma), against Gapdh (Fitzgerald), and against Thbs4 (Santa Cruz).

Immunocytochemistry and electron microscopy. For immunostaining experiments, cardiomyocytes were fixed in 4% paraformaldehyde and blocked and stained in blocking solution (phosphate-buffered saline [PBS], 5% goat serum, 1% bovine serum albumin [BSA], 1% glycine, 0.2% Triton X-100) (43). Primary antibodies used were against Flag (Cell Signaling Technology) (1:500), PDI (Abcam) (1:200), GM130 (BD Transduction Laboratories) (1:100), Godz (Abcam) (1:100), KDEL (Novus Biologicals) (1:100), and α-actinin (Sigma) (1:1,000) and were followed by Alexa Fluor-conjugated secondary antibodies (Invitrogen) and DAPI (4′,6-diamidino-2-phenylindole; Invitrogen) nuclear DNA stain. Imaging was performed on a Nikon A1 confocal microscope.

Transmission electron microscopy (TEM) was performed as described previously (44). Briefly, neonatal cardiomyocytes or mouse hearts were fixed in glutaraldehyde and cacodylate, embedded in epoxy resin, sectioned, counterstained with lead citrate and uranyl acetate, and then imaged.

Statistical analysis. Statistical analysis was performed using Student's *t* test. Standard errors of the means are shown for averaged data.

RESULTS

To dissect the functional roles of the different domains within Thbs4, we generated adenoviruses to express the LamG, EGF, T3R, and TSP-C domains (Fig. 1A). All constructs were engineered to contain an N-terminal signal peptide for proper trafficking to the secretory pathway, the coiled-coil domain to ensure proper assembly and oligomerization in the ER (5, 18, 19), and a C-terminal Flag tag to facilitate detection (Fig. 1A). We also generated a full-length Thbs4 Ca^{2+}-binding mutant (mCa^{2+}) containing aspartate-to-alanine mutations in six of the DXDXDG Ca^{2+}-binding sites within the T3R domain (Fig. 1A, red arrowheads). Four of these six mutated Ca^{2+}-binding sites are homologous to mutations in THBS5/COMP that cause human skeletal disorders (20, 22–24, 26, 31, 45). All adenoviral constructs had high-efficiency transduction (>95%) in cultured neonatal cardiomyocytes and exhibited a vesicular immunostaining pattern, with the exception of the LamG mutant, which showed perinuclear, Golgi apparatus-like immunolocalization (Fig. 1B).

One of the more recently proposed functions for Thbs4 is within the ER and secretory vesicles, where it participates in the protective ER stress response and imparts enhanced secretion and better resolution of unfolded protein accumulation (8). In the present study, we analyzed each of the Thbs4 domain constructs by immunostaining of neonatal rat cardiomyocytes or primary rat heart neonatal fibroblasts for localization of Thbs4 versus the known ER marker protein PDI. As expected, full-length Thbs4 was localized predominantly to the ER in both cell types (Fig. 2). Some colocalization at the ER was also observed for the EGF domain (Fig. 2). The T3R and TSP-C domain mutants exhibited robust colocalization with PDI at the ER, while the LamG domain appeared to be mostly excluded from the ER (Fig. 2). The mCa^{2+} mutant was also localized predominantly to the ER (Fig. 2), consistent with the increased retention of skeletal disease-causing THBS5/COMP Ca^{2+}-binding mutants in the ER (1, 25, 27, 31). The various Thbs4 mutants were also costained with anti-KDEL as

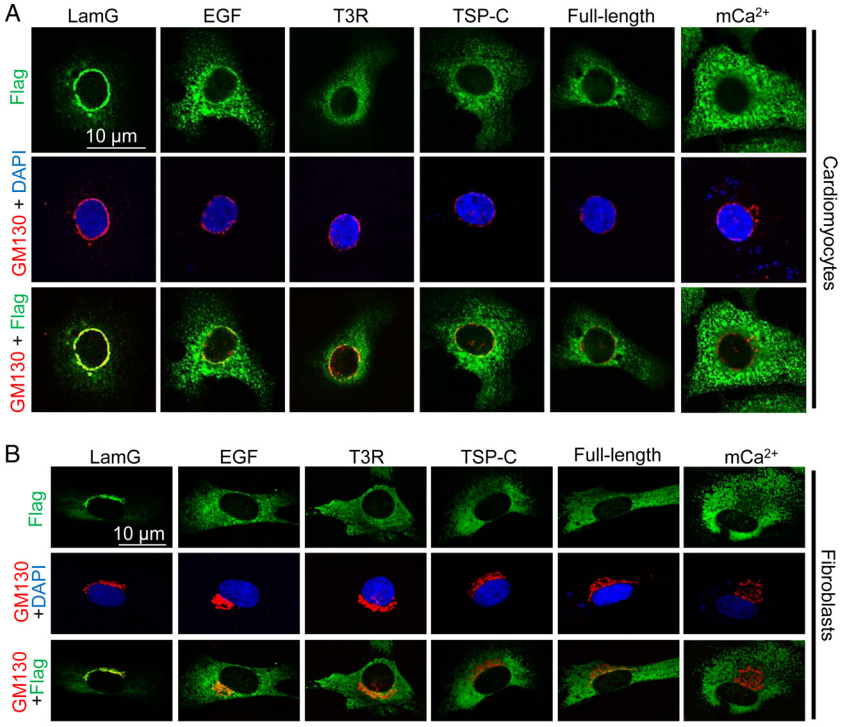

FIG 3 Localization of Thbs4 at the Golgi apparatus. Neonatal rat cardiomyocytes (A) and neonatal rat cardiac fibroblasts (B) were transduced with the indicated Thbs4 adenoviral constructs and coimmunostained for Thbs4 (Flag; green) and GM130 (red), used to mark the Golgi apparatus. DNA was stained with DAPI (blue) to mark the nuclei.

an additional ER marker, revealing identical intracellular immunolocalization patterns (data not shown).

Thbs4 also localizes to the Golgi apparatus, where it regulates the transit of Atf6α to the nucleus after cleavage in the ER and the Golgi apparatus (8, 46, 47). To determine which domains of Thbs4 localize to the Golgi apparatus, neonatal rat cardiomyocytes and rat heart neonatal fibroblasts transduced with the various Thbs4 constructs were coimmunostained for Thbs4 and GM130 (48). As previously reported (8), full-length Thbs4 also localized to the Golgi apparatus (Fig. 3). The EGF, T3R, and TSP-C domain mutants exhibited some colocalization at the Golgi apparatus, similar to full-length Thbs4 (Fig. 3), although the LamG domain mutant displayed an intracellular immunolocalization pattern almost exclusively at the Golgi apparatus in cardiomyocytes and fibroblasts (Fig. 3). However, the Thbs4 mCa^{2+} mutant appeared to be mostly excluded from the Golgi apparatus compared to wild-type full-length Thbs4 (Fig. 3), suggesting enhanced retention of this mutant in the ER. Identical results were obtained when neonatal cardiomyocytes were costained with another Golgi apparatus-specific marker, the palmitoyltransferase Godz (Golgi apparatus-specific DHHC zinc finger protein) (49; data not shown).

Thbs4 is generated in the ER, after which it traverses the Golgi apparatus and post-Golgi vesicles on its way to being secreted. Each of the Thbs4 domain mutants and the mCa^{2+} mutant were examined for the ability to be secreted into the medium from overexpressing cardiomyocyte cultures. All of the proteins encoded by the various Thbs4 constructs showed good overexpression at the predicted molecular weights in whole-cell protein lysates (Fig. 4A). Full-length Thbs4 was secreted into the medium as previously reported (1, 7, 8); however, the mCa^{2+} mutant was not secreted (Fig. 4B and Table 1), consistent with defects observed with human THBS5/COMP mutations (24, 25, 45). The T3R and TSP-C domains were both secreted from neonatal rat cardiomyocytes (Fig. 4B), proving that a portion of the domains are capable of exiting the ER and traversing the secretory pathway. In contrast, the EGF domain showed minimal secretion by cardiomyocytes, while the LamG domain was robustly secreted (Fig. 4B). The secreted forms of the Thbs4 domain mutants ran as multiple bands and at slightly higher molecular weights due to their glycosylation (data not shown).

To further examine the intracellular functionality of Thbs4, an unbiased assessment of Thbs4-interacting proteins was performed with Thbs4-overexpressing neonatal rat cardiomyocytes by immunoprecipitation and mass spectrometry protein identification. The most abundant immunoprecipitated band was cut out, sequenced, and identified as BiP (Grp78) (Fig. 5A), an ER-resident chaperone that is critical for regulating the ER stress response. This result was confirmed by immunoblotting for BiP in Thbs4 immunoprecipitates from cardiomyocytes (Fig. 5B) or after immunoprecipitation of BiP followed by immunoblotting for Thbs4 (Fig. 5C). To determine if BiP and Thbs4 interact *in vivo*, heart extracts from cardiomyocyte-specific Thbs4-overexpressing transgenic (TG) mice (8) were immunoprecipitated with a BiP antibody and immunoblotted for Thbs4, and an interaction was observed (Fig. 5D). The Thbs4 domain-containing constructs were employed to further examine the region in Thbs4 that interacted with BiP. The results showed that the Ca^{2+}-binding mutant retained the interaction with BiP, suggesting that BiP interacts with Thbs4 within the ER (Fig. 5E). More importantly, the TSP-C

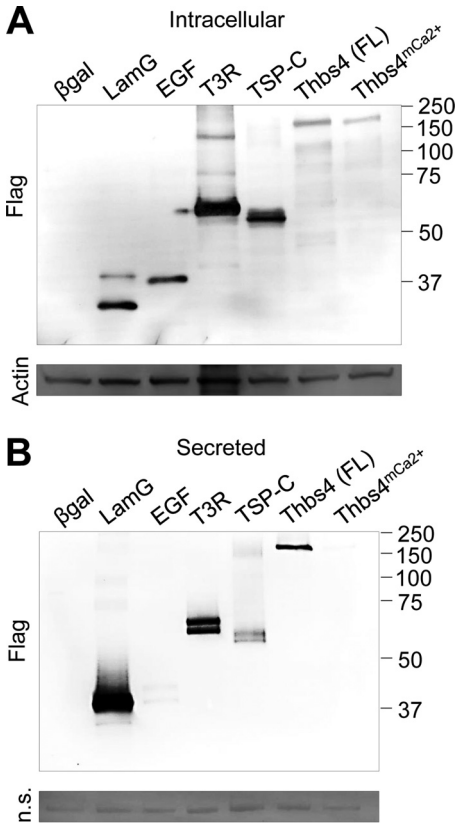

FIG 4 Intracellular expression and secretion of Thbs4 constructs. (A) Western blot to show intracellular expression of Thbs4 mutants. Neonatal rat cardiomyocytes were transduced with adenoviruses encoding full-length (FL) wild-type Thbs4, the calcium-binding mutant (mCa^{2+}), or Thbs4 domains. A βgal-expressing virus was used as a control virus, and sarcomeric α-actin was used as a loading control. (B) Western blot to show secretion of Thbs4 mutants and domains. Media from cardiomyocytes overexpressing the various Thbs4 constructs were concentrated and immunoblotted for detection of secretion. Ponceau staining of a nonspecific band was used as a loading control (n.s.).

domain strongly associated with BiP, while the LamG, EGF, and T3R domains had no ability to interact (Fig. 5E and Table 1). These results suggest that the TSP-C domain is likely critical for the intracellular function of Thbs4 within the ER compartment, possibly in conjunction with BiP and the sensing and initiation of the ER stress response.

To determine if BiP affects the Thbs4-dependent ER stress response, cardiomyocytes were transduced with a Thbs4 adenovirus, with or without a BiP-expressing adenovirus (AdBiP). While overexpression of BiP alone did not affect the basal levels of ER stress-responsive proteins in cardiomyocytes, cooverexpression with Thbs4 resulted in blunting of the Thbs4-mediated induction of the ER stress proteins calreticulin and PDI (Fig. 5F and G). These results suggest that the interaction of BiP antagonizes the ability of Thbs4 to activate the adaptive ER stress pathway.

We previously demonstrated that Thbs4 mediates the ER stress response in part by enhancing Atf6α activation (8). BiP is also known to interact with Atf6α to retain it within the ER membrane in the absence of ER stress, but during the unfolded protein response, BiP releases Atf6α, allowing it to translocate to the Golgi apparatus for proteolytic activation (47, 50, 51). In the present study, neonatal rat cardiomyocytes were first transduced with adenoviruses encoding an ER-retained mutant of Atf6α (Atf6α-ER) (8) and the various Thbs4 domain mutants, followed by immunoprecipitation of Atf6α and immunoblotting for Thbs4. Full-length Thbs4 interacted with Atf6α, as did the mCa^{2+} mutant (Fig. 6A and Table 1). Importantly, the T3R and TSP-C domains also interacted with Atf6α (Fig. 6A and Table 1). We previously reported that the T3R domain of Thbs4 interacts directly with Atf6α in glutathione *S*-transferase (GST) pulldown assays (8), and here we confirmed this interaction in cultured rat cardiomyocytes and identified the TSP-C domain as a binding domain for both Atf6α (Fig. 6A) and BiP (Fig. 5E). Consistent with the interaction data, full-length Thbs4, the mCa^{2+} mutant, and the TSP-C domain each induced Atf6-dependent transcription, as observed using a luciferase-Atf6 reporter construct, upon cotransfection in COS cells (Fig. 6B). These results suggest that the TSP-C domain functions in the ER as a critical regulator of Thbs4-mediated activation of Atf6α-dependent transcription.

To examine the domains of Thbs4 that function to activate the ER stress pathway, neonatal rat cardiomyocytes were transduced with the various Thbs4 domains, and protein extracts were generated and immunoblotted for induction of ER stress-responsive factors. Full-length Thbs4 induced the expression of BiP, calreticulin, and PDI (Fig. 6C and D), as previously reported (8). Importantly, the TSP-C domain alone induced the same ER stress-responsive factors, although the other Thbs4 domains and the mCa^{2+} binding mutant had only a mild inductive effect on the adaptive ER stress response (Fig. 6C and D). The level of activation of the Thbs4-dependent ER stress pathway by each of the various Thbs4 domains was similar to the ability of each domain to induce Atf6 transcriptional activity (Fig. 6B to D). Full-length Thbs4 and the mCa^{2+} mutant also induced modest but significant increases in the total protein levels of PERK but did not alter the levels of IRE1α or phosphorylated PERK or IRE1α (data not shown). Therefore, Thbs4 has the most profound effects on the Atf6α

TABLE 1 Summary of Thbs4 protein domain-regulated biologic activities[a]

Thbs4 construct	Intracellular localization		Secretion	ER stress response	BiP interaction	Atf6α interaction	ER expansion
	ER	Golgi apparatus					
LamG domain	−	++++	++++	+	−	−	−
EGF domain	++	+	±	+	−	−	−
T3R domain	+++	+	++	+	−	+	+++
TSP-C domain	+++	+	+	+++	+	+	+++
Full-length protein	++	+	++	+++	+	+	+++
mCa^{2+} mutant	+++	−	−	+	+	+	+++

[a] −, absent; ±, minor secretion detected; +, present; ++, substantial; +++, very robust; ++++, exclusive Golgi localization/secretion.

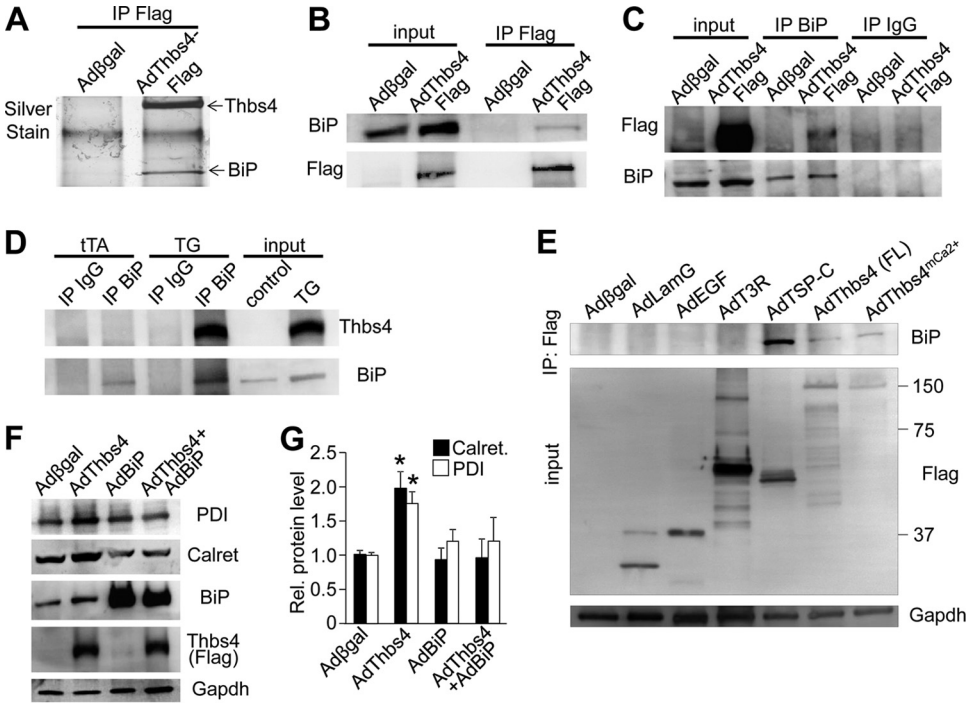

FIG 5 Thbs4 interacts with BiP in the heart. (A) Silver-stained SDS-PAGE gels with Thbs4-interacting proteins from neonatal rat cardiomyocytes transduced with the Thbs4-Flag adenovirus or Adβgal virus, as a negative control. A Flag antibody was used for immunoprecipitation (IP). BiP was identified as a Thbs4-interacting protein by mass spectrometry sequencing. (B) Western blots for BiP and Flag-Thbs4 from neonatal cardiomyocyte protein extracts that were immunoprecipitated with a Flag antibody. Adβgal was used as a control infection. (C) Western blots for BiP and Flag-Thbs4 from neonatal cardiomyocyte protein extracts that were immunoprecipitated with a BiP antibody and immunoblotted for Thbs4 (Flag). Adβgal was used as a control infection. (D) Western blots for Thbs4 and BiP from heart extracts from cardiomyocyte-specific Thbs4 transgenic mice (TG) and littermate controls. Extracts were immunoprecipitated for detection of BiP and immunoblotted for Thbs4. tTA tetracycline transactivator single transgenic littermate control mice. (E) Western blots for BiP and the various Thbs4 domains or the calcium-binding mutant (mCa^{2+}) from adenovirus-transduced cardiomyocytes. Wild-type full-length (FL) Thbs4 and βgal (as a control) viruses were also used. (F) Western blotting for the indicated proteins from neonatal cardiomyocytes infected with recombinant adenoviruses encoding Thbs4 and/or BiP. Calret, calreticulin. (G) Quantitation of the data shown in panel F, but including data from 2 additional experiments. *, $P < 0.05$ versus Adβgal.

branch of the ER stress pathway, by interacting with and facilitating Atf6α activation (8).

To further evaluate the ability of the various Thbs4 domains to activate the adaptive ER stress response in cardiomyocytes *in vivo*, we injected purified adenoviruses encoding the Thbs4 constructs into the gastrocnemius muscles of early postnatal rat pups and harvested muscle tissue for Western blotting analyses 5 days later (Fig. 7A). The results again revealed induction of the ER stress-responsive factors BiP, PDI, and calreticulin by full-length Thbs4 and the TSP-C domain, with some induction also observed in muscles overexpressing the mCa^{2+} mutant and the T3R domain. Only minor activation of the ER stress response was observed in muscles overexpressing the LamG or EGF domain (Fig. 7B).

We showed previously that overexpression of Thbs4 in the heart results in a profound and unprecedented expansion of the ER and post-ER vesicular compartments (8). To understand the potential mechanistic basis for how Thbs4 might mediate such a dramatic expansion of intracellular vesicles, we analyzed the activities of the various Thbs4 domains, overexpressed in neonatal rat cardiomyocytes, by transmission electron microscopy. The results demonstrate that similar to full-length wild-type Thbs4, the T3R and TSP-C domains induced robust vesicular expansion in cultured neonatal cardiomyocytes, as did the mCa^{2+} mutant (Fig. 8A). In contrast, expansion of vesicles was not observed in cardiomyocytes transduced with the β-galactosidase (βgal)-expressing control adenovirus or the LamG or EGF domain (Fig. 8A and Table 1). These results suggest that the mechanism whereby Thbs4 leads to vesicular expansion is dependent on the T3R and TSP-C domains, which also interact directly with Atf6α. The observation that the mCa^{2+} mutant also causes vesicular expansion suggests that the signal for this expansion is from the ER compartment, as this mutant appears to be retained exclusively within the ER and is defective in secretion. Mechanistically, Atf6α has been shown to expand the ER (52), and here we demonstrated that overexpression of constitutively nuclear Atf6α (Cn-Atf6α) similarly induced a robust expansion of intracellular vesicles in cultured neonatal cardiomyocytes (Fig. 8A).

To determine if Atf6α is required for Thbs4-induced vesicular expansion, we performed electron microscopy on wild-type (WT) and *Atf6α*$^{-/-}$ MEFs transduced with a Thbs4 adenovirus or a βgal-expressing control adenovirus. Overexpression of Thbs4 resulted in vesicular expansion compared to that with βgal infection in WT MEFs but not in *Atf6α*$^{-/-}$ MEFs (Fig. 8B), demonstrating that Thbs4-induced vesicular expansion requires Atf6α.

To ensure that the observed effects of full-length Thbs4 and the T3R and TSP-C domains on induction of the ER stress pathway and vesicular expansion are specific to the molecular function of

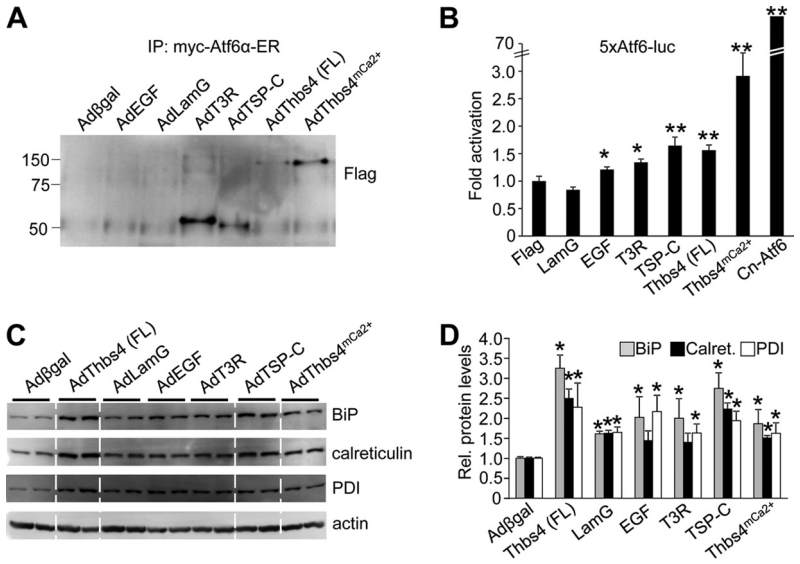

FIG 6 Domains of Thbs4 that interact with Atf6α and activate the ER stress pathway. (A) Western blot showing that Thbs4 interacts with Atf6α via the T3R and TSP-C domains. The blot shows immunoprecipitation of proteins from neonatal rat cardiomyocytes infected with adenoviruses encoding the indicated Thbs4 domains, the full-length protein, or the mCa^{2+} mutant protein along with an ER-retained form of Atf6α. (B) Luciferase activities driven by an Atf6 reporter construct transfected into COS cells with the indicated Thbs4 constructs. Constitutively nuclear (Cn) Atf6α was used as a positive control. *, $P < 0.05$; **, $P < 0.01$ compared to the Flag vector ($n = 4$). (C) Western blot for the indicated ER stress pathway proteins from neonatal cardiomyocytes infected with recombinant adenoviruses expressing the indicated Thbs4 proteins or domains. The areas within the dashed lines show the two more prominent constructs that induced ER stress response protein expression. (D) Quantitation of protein expression levels from panel C, along with those of two additional samples run separately and not shown. *, $P < 0.05$ versus Adβgal.

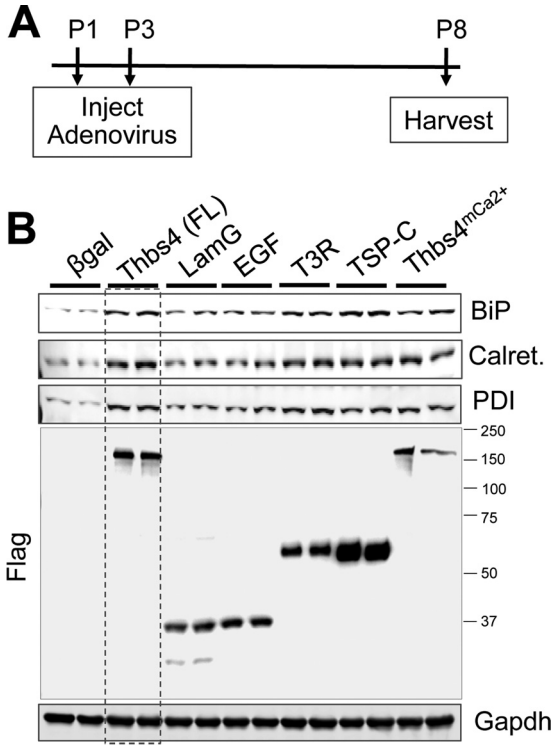

FIG 7 Domains of Thbs4 that activate the adaptive ER stress pathway *in vivo*. (A) Schematic of the experimental protocol. Purified adenoviral constructs were injected into the gastrocnemius muscles of postnatal day 1 rat pups, followed by an additional injection 48 h later (10^8 viral particles per injection), and muscle tissue was harvested at postnatal day 8 for analyses. (B) Western blotting for the adaptive ER stress response factors BiP, PDI, and calreticulin from muscles infected with the indicated Thbs4-based constructs or Adβgal, as a control. The lower Flag Western blot confirms the expression of each of the constructs in the virus-injected muscles. Gapdh was used as a processing and loading control.

Thbs4 and not an artifact due to protein overexpression, we also evaluated these parameters in neonatal rat cardiomyocytes overexpressing Nell2, a related secretory glycoprotein that, similar to Thbs4, oligomerizes in the ER and contains some of the same functional domains (53). Nell2 contains an N-terminal LamG domain, an EGF-like domain, and a coiled-coiled domain responsible for its oligomerization, but it lacks the T3R and TSP-C domains seen in Thbs4 (Fig. 9A). We generated a recombinant adenovirus to overexpress Nell2 in cultured neonatal cardiomyocytes, demonstrating that, like Thbs4, Nell2 localized to the ER (Fig. 9B) and was secreted (Fig. 9C). However, in contrast to that of Thbs4, overexpression of Nell2 did not activate the adaptive ER stress pathway (Fig. 9D). Moreover, to further evaluate the effects of Nell2 in comparison to those of Thbs4, we generated cardiomyocyte-specific transgenic mice overexpressing Nell2 (Fig. 9E) and performed immunoblotting on cardiac lysates from Nell2- or Thbs4-overexpressing hearts. The data showed that, in contrast to Thbs4 overexpression, Nell2 overexpression did not induce the adaptive ER stress response *in vivo* (Fig. 9E). Moreover, we performed TEM on Thbs4 and Nell2 transgenic hearts, demonstrating a normal ultrastructure in the Nell2 transgenic hearts, without the vesicular expansion phenotype observed in Thbs4 transgenic hearts (Fig. 9F). Taken together, these data demonstrate that Thbs4-induced adaptive ER stress signaling is not an artifact associated with protein overexpression. Moreover, we never observed intracellular protein aggregates in any of our cells overexpressing Thbs4 or any of the domain constructs or in hearts of Thbs4 TG mice (data not shown). Indeed, our past data actually showed that Thbs4 overexpression in the heart helped to resolve cardiomyopathy in two mouse models of protein aggregation-based disease (8). Collectively, our data suggest that Thbs4 has a specific molec-

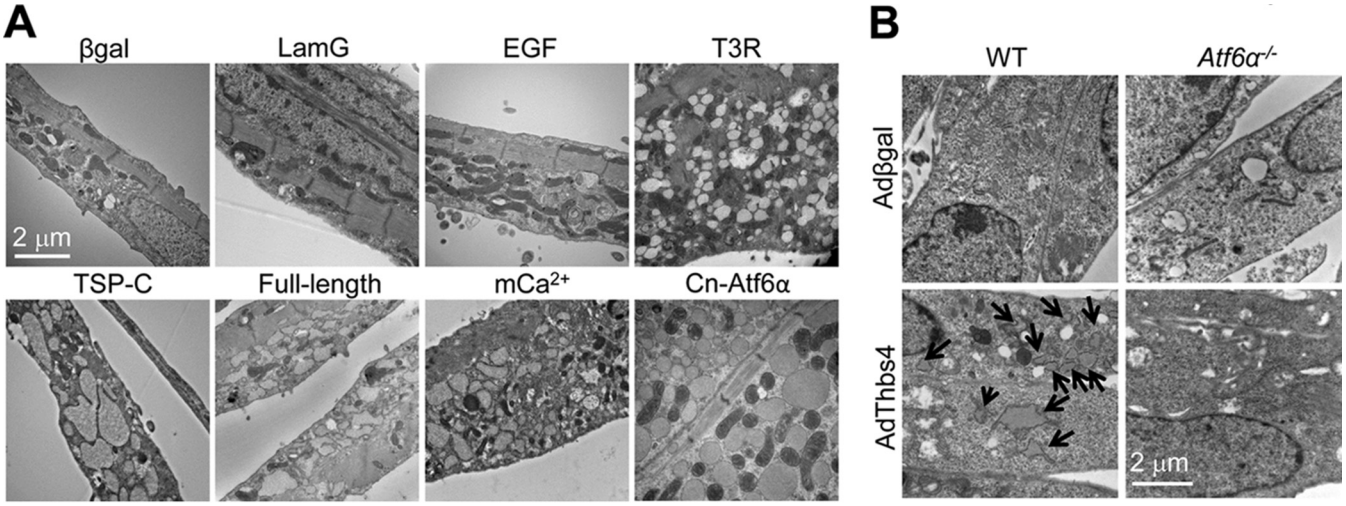

FIG 8 Thbs4 induces Atf6α-dependent vesicular expansion. (A) Transmission electron microscopy (TEM) was performed on neonatal rat cardiomyocytes transduced with the indicated Thbs4 adenoviral constructs or Adβgal, as a control. Overexpression of the T3R and TSP-C domains, as well as the calcium-binding mutant and full-length Thbs4 proteins, resulted in expansion of vesicles similar to that of a constitutively nuclear (Cn) Atf6α mutant protein. (B) TEM was also performed on MEFs transduced with full-length Thbs4, revealing vesicular expansion in WT MEFs but not *Atf6*α-deleted MEFs. Adβgal was used as a control. Black arrows show the expanded vesicles.

ular function within the ER and the secretory pathway, in part by regulating ATF6α. Indeed, Nell2 overexpression had no effect, and because it lacks only the ATF6-interacting T3R and TSP-C domains, this suggests molecular specificity for the proposed mechanism of Thbs4 functionality within the cell.

DISCUSSION

We recently reported that thrombospondins can function intracellularly within the ER, where they mediate an adaptive ER stress response, in part by regulating Atf6α translocation to the Golgi apparatus and then the nucleus (8). Here we generated Thbs4 domain-expressing constructs, in addition to a Ca^{2+}-binding mutant within the T3R domain, to dissect the roles of the different domains that putatively underlie the multifaceted functions of thrombospondin proteins. We observed that the Thbs4 LamG domain exhibited intracellular localization almost entirely at the Golgi apparatus, from which it was then rapidly secreted, while the mCa^{2+} mutant protein exhibited retention within the ER, reduced Golgi apparatus translocation, and defective secretion.

The mCa^{2+} mutant may also serve as a dominant negative mutant for Thbs5/COMP, since Thbs4 and Thbs5/COMP can assemble as heteropentamers *in vivo* (54). Regardless, the lack of secretion by the mCa^{2+} mutant suggests that Ca^{2+} binding or the sensing of ER Ca^{2+} is required for proper folding and/or trafficking of Thbs4. Indeed, Ca^{2+}-binding sites have been shown to be important for proper folding of thrombospondins (20, 27, 32), and reduced Ca^{2+} levels cause impaired secretion and retention of Thbs1 in the ER of renal cell carcinoma cells (55). However, the mCa^{2+} mutant of Thbs4 still induced vesicular expansion, suggesting that while this mutant did not traverse the secretory pathway, it still functioned properly within the ER to facilitate Atf6α activation and downstream vesicular expansion. This effect may be due to its interaction with BiP, as releasing BiP from binding to Atf6α permits the cleavage and translocation of this transcription factor and its regulation of expression of other ER stress response factor genes (see below). Indeed, activated Atf6α is sufficient to induce membrane biosynthesis and ER expansion (52), and we demonstrated that activated Atf6α can drive substantial vesicular expansion in cultured cardiomyocytes and that Atf6α is required for Thbs4-mediated expansion of intracellular vesicles.

We previously demonstrated that Thbs4 activates an adaptive ER stress pathway that protects the heart from pressure overload, myocardial infarction, and aggregation cardiomyopathy, in part by inducing activation of Atf6α (8). Atf6α signaling is adaptive in the heart and protects it from myocardial infarction (56) and ischemia reperfusion (57). However, it is not clear if the protective aspect of Atf6α induction is due to ER expansion and the greater capacity of the ER compartment or to some other action that has yet to be identified. Importantly, overexpression of Nell2, a thrombospondin-like oligomeric secretory protein that contains LamG and EGF domains but lacks the Atf6α-interacting T3R and TSP-C domains (53), does not induce the adaptive ER stress pathway or expansion of intracellular vesicles. Moreover, the absence of Atf6α abolishes the ability of Thbs4 to induce vesicular expansion. Thus, these data genetically demonstrate that vesicular expansion by Thbs4 requires Atf6α and that this interaction may be a major mechanism whereby the Thbs proteins function intracellularly.

We also identified the ER chaperone BiP as an additional binding partner of Thbs4 that affects its functionality from within the cell. BiP is a nexus ER stress response protein that directly senses unfolded proteins to initiate activation of Atf6α, PERK, and IRE1α signaling (50, 51, 58). Both BiP and Thbs4 interact with the ER luminal domain of Atf6α (8, 50), suggesting that BiP and Thbs4 may compete to determine whether Atf6α remains embedded in the ER membrane or shuttles to the Golgi apparatus and nucleus. Importantly, the TSP-C domain alone, which binds BiP and Atf6α, was sufficient to induce vesicular expansion, Atf6 transcriptional activity, and induction of ER stress proteins. Indeed, we demonstrated that overexpression of BiP can blunt the Thbs4-mediated induction of the ER stress pathway, suggesting compe-

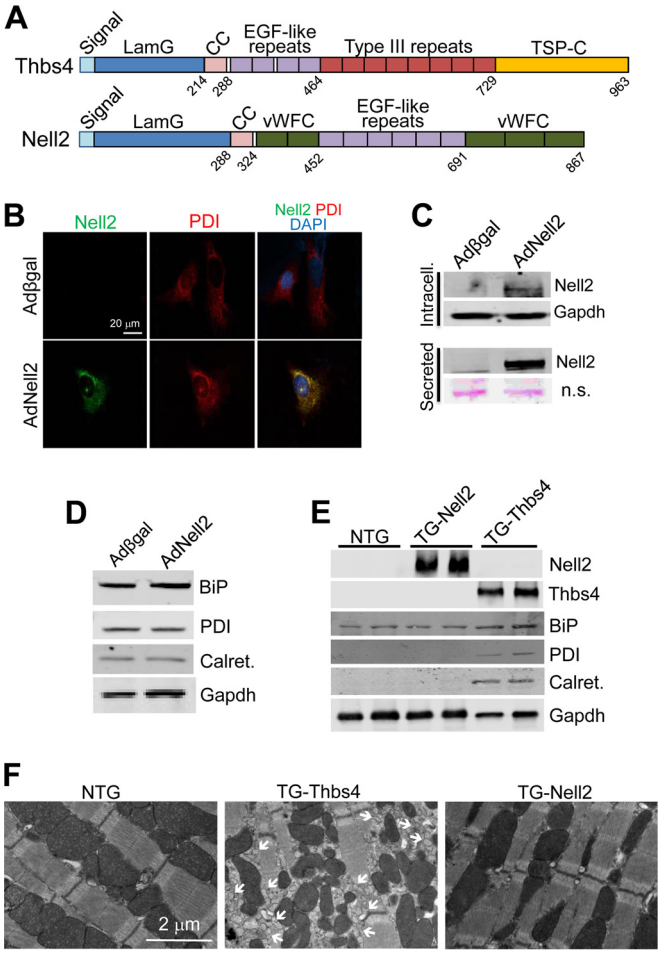

FIG 9 Induction of the ER stress pathway and vesicular expansion are specific to Thbs4. (A) Schematic of the domain structure of Thbs4 and the related secreted glycoprotein Nell2. Abbreviations: CC, coiled coil; vWFC, von Willebrand factor C. (B) Immunostaining of neonatal cardiomyocytes transduced with a Nell2 adenovirus or a βgal control virus, showing colocalization of Nell2 with PDI at the ER. (C) Immunoblotting of lysates from Nell2-overexpressing neonatal cardiomyocytes shows intracellular expression and secretion of Nell2 into the medium. (D) Immunoblotting of extracts of Nell2-overexpressing cultured cardiomyocytes reveals no induction of the adaptive ER stress response. (E) Overexpression of Nell2 in transgenic mouse hearts does not induce the adaptive ER stress response (TG-Nell2) as observed in Thbs4 TG hearts. (F) Nell2 overexpression in the TG mouse heart does not induce expansion of intracellular vesicles as observed with Thbs4 overexpression in the heart. White arrows show the expanded vesicles.

tition with Thbs4 for binding to Atf6α. We cannot rule out, however, the possibility that BiP alleviates the Thbs4-mediated induction of the ER stress pathway through its classical roles as an ER chaperone and repressor of Atf6α, PERK, and IRE1α activation.

Thbs4 is strongly induced by disease or stress stimuli in the heart and skeletal muscles, while other Thbs family members are induced in other tissues and cell types with injury or stress. This suggests a mechanism whereby the ER is appropriately enhanced during injury in an attempt to repair or remodel the ECM by enhanced secretory function. This concept further suggests that Thbs proteins are bifunctional with respect to cellular location, with equally important intracellular and extracellular functions. However, in the heart and skeletal muscles, it has been very difficult to observe Thbs4 localization within the ECM or extracellular space between cells, even with high levels of overexpression in a transgenic approach (8). Essentially all of the Thbs4 produced by transgene-mediated expression or with induction of endogenous Thbs4 after injury to the heart shows intracellular localization to the ER and other vesicular compartments (8). The same observation was also made with Thbs1 overexpression in the heart and skeletal muscles, where protein localization was intracellular, with very little observable outside the cell (8). Thus, the intracellular functionality of Thbs proteins may be predominant in heart and skeletal muscles.

ACKNOWLEDGMENTS

We thank Zeenat Jamal for technical assistance.

We have no conflicts of interest to declare.

FUNDING INFORMATION

HHS | NIH | National Heart, Lung, and Blood Institute (NHBLI) provided funding to Jeffery D. Molkentin under grant number R01HL105924. HHS | NIH provided funding to Matthew J. Brody under grant number F32HL124698 (National Research Service Award). The Howard Hughes Medical Institute provided funding to Jeffery D. Molkentin.

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