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Thy-1 interaction with Fas in lipid rafts regulates fibroblast apoptosis and lung injury resolution

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Abstract

Thy-1-negative lung fibroblasts are resistant to apoptosis. The mechanisms governing this process and its relevance to fibrotic remodeling remain poorly understood. By using either sorted or transfected lung fibroblasts, we found that Thy-1 expression is associated with downregulation of anti-apoptotic molecules Bcl-2 and Bcl-xL, as well as increased levels of cleaved-caspase 9. Addition of rhFasL and staurosporine, well-known apoptosis inducers, caused significantly increased cleaved caspase 3, 8, and PARP in Thy-1-transfected cells. Furthermore, rhFasL induced Fas translocation into lipid rafts and its colocalization with Thy-1. These *in vitro* results indicate that Thy-1, in a manner dependent upon its GPI anchor and lipid raft localization, regulates apoptosis in lung fibroblasts via Fas-, Bcl-, and caspase-dependent pathways. *In vivo*, Thy-1 deficient (*Thy1*^{-/-}) mice displayed persistence of myofibroblasts in the resolution phase of bleomycin-induced fibrosis, associated with accumulation of collagen and failure of lung fibrosis

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DISCLOSURE/CONFLICT OF INTEREST

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resolution. Apoptosis of myofibroblasts is decreased in *Thy1*^{-/-} mice in the resolution phase. Collectively, these findings provide new evidence regarding the role and mechanisms of Thy-1 in initiating myofibroblast apoptosis that heralds the termination of the reparative response to bleomycin-induced lung injury. Understanding the mechanisms regulating fibroblast survival/apoptosis should lead to novel therapeutic interventions for lung fibrosis.

Keywords

Thy-1; fibroblasts; apoptosis; caspases; Fas; FasL; lipid raft; fibrosis

INTRODUCTION

Myofibroblast apoptosis is a fundamental mechanism during normal wound healing. During the resolution phase, this cell-specific apoptosis is triggered mainly by the extrinsic pathway via Fas receptor (Apo-1/CD95) trimerization, Bcl protein downregulation, and subsequent caspase cascade activation.¹ Programmed cell death is characterized by marked changes in cellular morphology, including the appearance of membrane-bound apoptotic bodies, internucleosomal DNA fragmentation, as well as by cleavage of poly(ADP-ribose) polymerase (PARP).² However, fibroblasts insensitive to apoptotic stimuli can induce accumulation of apoptosis-resistant myofibroblasts, a hallmark of pathological fibrosis.^{3-5,6}

Mechanisms underlying the resolution of pulmonary fibrosis have been recently reviewed.⁷ In recent years, increasing attention has been paid to understanding the apoptotic alterations leading to non-resolving and progressive fibrotic remodeling with the intention of developing targeted therapies for lung fibrosis. Thus, it was found that increased expression of Fas, a cell-surface death receptor in the tumor necrosis factor (TNF) receptor superfamily, is necessary to directly sensitize apoptosis-resistant fibroblasts.⁸ Furthermore, a recent study showed that TNF- α is an inducer of apoptosis that mediates lung fibrosis resolution.⁹ In addition, it has been described that functional FasL(+) immune cells could promote resolution of lung fibrosis,¹⁰ indicating that factors that interfere with the Fas/FasL interaction between T cells and myofibroblasts could abrogate immune surveillance during fibrosis. Taking together, these recent emerging studies strongly indicate that both preservation of apoptosis susceptibility in fibroblasts, and apoptosis induction in apoptosis-resistant myofibroblasts have significant therapeutic potential.

Thy-1/CD90 is a glycosylphosphatidylinositol (GPI) anchored cell surface glycoprotein that modulates apoptosis signaling pathways in a variety of cell types including hepatic cells,¹¹ glomerular mesangial cells,¹² and dermal fibroblasts.¹³ It has become evident that Thy-1-mediated apoptotic mechanisms are cell type-dependent.¹⁴ For example, Thy-1 must aggregate in order to signal thymocyte apoptosis, whereas apoptosis of Thy-1(+) thymoma cells occurs through a mechanism requiring new transcription. We have found that Thy-1(-) myofibroblasts are resistant to apoptosis in response to collagen gel contraction.^{15,16} Furthermore, Thy-1 deficient (*Thy1*^{-/-}) mice develop more severe lung fibrosis than wild-type (Wt) mice, associated with the accumulation of lung myofibroblasts.¹⁷ However, the

molecular mechanisms by which Thy-1 modulates fibroblast apoptosis and Thy-1's importance during lung tissue remodeling remain to be characterized.

In this study, we evaluated the mechanisms whereby Thy-1 modulates apoptosis-related intracellular signaling pathways, and the role of membrane microdomain localization via the Thy-1-specific GPI anchor in this process. We also studied the *in vivo* effect of Thy-1 during myofibroblast apoptosis by using *Thy1* null mice. Our data demonstrate that Thy-1 surface expression is necessary and sufficient to promote apoptosis in lung myofibroblasts.

MATERIALS AND METHODS

Animals and Bleomycin-Induced Fibrosis

Adult Thy-1 knock-out (*Thy1*^{-/-})¹⁸, a kind gift of Kevin Kelley, Mt. Sinai School of Medicine, and Wt mice on C57BL/6 background (both genders) were utilized for this study. All mice were housed in the Association for Accreditation and Assessment of Laboratory Animal Care (AAALAC)-approved animal facility at the University of California San Diego, School of Medicine. To generate pulmonary injury and fibrosis, mice anesthetized (i.p.) with ketamine (80mg/kg)/xylazine (10 mg/kg) were orotracheally instilled with bleomycin (5 U/kg BW, Tecoland Corporation) dissolved in sterile saline (100 μ L) via MicroSprayer (MS-IA-1C, Penn-Century) or sterile saline for controls.

Histopathological Evaluation

At each time-point after bleomycin instillation, lungs were fixed (10% formalin at a constant pressure of 20 cm H₂O), and paraffin embedded. Five μ m-thick sagittal sections of all lobes of fixed lungs were cut and subsequently stained for H&E and Masson's trichrome staining to evaluate the lung fibrotic response.

Detection of Myofibroblast Apoptosis *in vivo*

Co-localization of cleaved caspase-3, an apoptosis marker, and α -SMA, a myofibroblast marker, was conducted to confirm myofibroblast apoptosis by immunofluorescence. Five μ m-thick OTC-embedded samples were cut and then fixed using 4% formaldehyde, permeabilized in 0.2% Triton X-100 for 5 minutes, and blocked with 10% normal goat serum. Primary antibodies for alpha smooth-muscle actin (aSMA; 1:200; Ab21027, Abcam), cleaved-Caspase 3 (1:200; #9661, Cell Signaling) along with AlexaFluor-labeled secondary antibodies (Life Technologies) were used. Images were acquired at 20x (Plan-fluor, 0.5 N.A.) or 60x (Plan-apochromat, 1.4 N.A.) magnification with a fluorescence microscope. Myofibroblasts expressing high levels of aSMA and/or cleaved caspase-3 were counted in six different random high-powered fields of the lung in each mouse, 5–6 mice per group.

Cell Culture

Rat fetal lung fibroblasts, which are entirely Thy-1-negative (RFL6; American Type Culture Collection, Manassas, VA), were cultured in Ham's F12 K nutrient mixture (F-12K) media containing 10% FBS and 1% penicillin/streptomycin. Mouse embryonic fibroblasts (MEFs) were isolated from C57BL/6 mice and sorted for Thy-1 expression using FITC-labeled Thy-1.2-specific antibodies as previously described.¹⁹ Sorted MEFs were cultured in

Dulbecco's modified Eagle's medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% FBS, in a humidified incubator with 5% CO₂. Two different batches of sorted Thy-1 subpopulations of MEFs were used in this study and yielded similar results.

Cell Transfection

RFL-6 cells were stably transfected with the mammalian expression vector pcDNA3.1 Zeo⁺ containing the full-length murine Thy-1.2 (CD90.2) cDNA (RFL-6 Thy-1⁺) or empty vector (EV) (RFL-6 Thy-1⁻) as previously described.²⁰ The expression of Thy-1 at levels similar to those in naturally occurring Thy-1(+) fibroblasts was confirmed by FACS analysis.^{19,21} For Thy-1 GPI anchor studies, RFL-6 cells were transfected with wild type-human Thy-1 (hThy-1) or human Thy-1-hinge TR3, a chimeric construct in which the GPI anchor of hThy-1 was replaced with the GPI addition sequence of TRAIL receptor 3.²² Absence of Thy-1 protein in untransfected and EV-transfected RFL-6, and appropriate levels of expression in Thy-1- and Thy-1 mutant-transfected cells have been demonstrated previously.^{22, 23}

Antibodies and Reagents for *in vitro* Assays

Soluble human recombinant FasL (rhFasL) (Kamiya Biomedical Company, Seattle, WA); staurosporine (ACROS Organics, Pittsburgh, PA); caspase 8 inhibitor II (EMD Millipore, Billerica, MA); protein G-agarose (Roche Diagnostics, Indianapolis, IN); Z-FA-FMK (negative control for caspase inhibitors), FITC Rat anti-mouse CD90.2, FITC Rat IgG1, κ Isotype, Annexin V apoptosis detection kit and APO-Direct kit (BD Pharmingen, San Jose, CA); rabbit anti-PARP antibody, rabbit anti-caspase 3 antibody, rabbit anti-cleaved caspase 3 antibody, rabbit anti-cleaved caspase 8 antibody and rabbit anti-cleaved caspase 9 antibody (Cell Signaling Technologies, Danvers, MA); anti-mouse Thy-1 (AbD Serotec, Oxfordshire, UK), and anti- α actin polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX).

Cell Treatments

Cells were grown to 80% confluence in culture dishes and made quiescent in culture media supplemented with 0.4% FBS for 24 hours. Fresh 0.4% FBS culture media was added before stimulation with indicated concentrations of rhFasL or Staurosporine for 16 hours. For caspase 8 inhibition experiments, Thy-1 (+) RFL-6 cells were pretreated with or without caspase 8 specific inhibitor for 30 minutes followed by treatment with rhFasL or staurosporine.

Apoptosis Assay

RFL-6 Thy-1 (+) and RFL-6 Thy-1 (-) cells were rendered quiescent in cultured media supplemented with 0.4% FBS for 24 hours, then treated with the indicated concentrations of rhFasL for 16 hours. After treatment, adherent and non-adherent cells were harvested by centrifugation and stained with Annexin V and PI, resuspended in 500 μ l binding buffer and analyzed by flow cytometry.

DNA Fragmentation and TUNEL Assays

The APO-Direct assay kit was used as per the manufacturer's protocol. Briefly, cells were cultured at a density of 0.4×10^6 cells in six-well dishes and treated with 50 ng/mL of rhFasL for 16 hours. Labeled cells were counted in a flow cytometer and analyzed using Cell Quest software (San Jose, CA).

Immunoblotting

At the end of respective cell treatments, cells were washed with cold PBS twice and lysed with 1X SDS reducing sample buffer containing protease inhibitors. Cell lysates were collected in siliconized tubes and sonicated for 20 seconds three times. After centrifugation at $4,000 \times g$ for 1 minute at 4°C , cell lysates were stored at -80°C in aliquots until use. Equal volumes of cell lysate were loaded on SDS-PAGE gels under reducing conditions. After electrophoresis, proteins were transferred to PVDF membranes at 100V for 1 hour at 4°C . To block nonspecific protein binding sites, the membranes were incubated with 5% non-fat milk in Tris-buffered saline/Tween-20 (0.1%) for 1 hour at room temperature. Membranes were incubated with primary antibodies in Tris-buffered saline/Tween-20 (0.1%) overnight. Membranes were washed extensively before being incubated with appropriate peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Immunodetection was performed by chemiluminescence.

Isolation of Lipid Rafts and Immunoprecipitation

Membrane fractionation and immunoprecipitation were performed as previously described.²⁴ Proteins from membrane fractionation were analyzed by immunoblotting. For immunoprecipitation, 10-cm plates of RFL-6 Thy-1 (+) and RFL-6 Thy-1 (-) cells were washed twice with cold PBS, scraped in 1 ml of modified Lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM 150 mM NaCl, 1 mM EGTA, plus mammalian protease inhibitor cocktail) and homogenized in a dounce homogenizer. Precleared samples were incubated with primary antibody for Fas for 1 to 3 hours then precipitated by incubating with protein G-agarose overnight. Pellets were washed once in lysis buffer followed by washes in buffers 1 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.2% Triton X-100) and 2 (10 mM Tris-HCl, pH 7.5, and 0.2% Triton X-100). Immunoprecipitated proteins were analyzed by immunoblotting.

Immunofluorescence Staining

RFL-6 Thy-1 (+) cells were cultured on coverslips in 12 well plates, grown to 70% confluence, made quiescent with culture media supplemented with 0.4% FBS for 24 h, and stimulated with 50ng/mL rhFasL for 16 hours. Cells were washed with 2X serum free medium and incubated with FITC-Rat anti-Thy-1.2 (1:20) or Rat IgG1 κ isotype as control. Then cells were fixed with 3.7% formaldehyde for 15 minutes, washed with sterile PBS, blocked in 5% normal goat serum, and incubated with mouse IgM or Mouse anti-Fas (1:50) followed by Texas Red X-conjugated secondary antibody (1:40). Coverslips were washed and mounted using Gelvatol mounting medium²⁵ on glass microscope slides and examined with an Olympus FV 1000 Spectral Confocal fluorescence microscope. Images were acquired with a digital camera and analyzed with Velocity analysis software (PerkinElmer, Waltham, MA).

Data Analysis and Statistics

Data are presented as the mean \pm SEM and statistical differences in the mean values for animal (n=10) and cell culture studies (n=3) were determined by using a one-way ANOVA test with posthoc analysis using Tukey's multiple comparison test using GraphPad Prism 4.0.

RESULTS

Thy-1 Expression Promotes Fibroblast Susceptibility to Apoptosis

The ability of Thy-1 to promote apoptosis was assessed in RFL-6 Thy-1 (+) and RFL-6 Thy-1 (-) cells. At 10 ng/ml, rhFasL was insufficient to induce apoptosis in either cell type. However, an increased number of apoptotic cells was observed in RFL-6 Thy-1 (+) cells in response to 50 and 100 ng/ml rhFasL (from ~15% to 35% as demonstrated by Annexin V/PI assay). The number of apoptotic cells remained unchanged in RFL-6 Thy-1 (-) cells at the same concentrations of rhFasL (Figure 1A). To verify Annexin V/PI data, we measured apoptosis using a terminal nucleotidyl transferase-mediated nick end labeling assay for DNA fragmentation, an irreversible event that commits the cell to die, using the APO-DIRECT Kit, in identically treated cells. Findings are similar to those from the Annexin V assay (Figure 1B). These results indicate that Thy-1 can promote apoptosis in fibroblasts.

Thy-1 Expression Increases the Activation of Caspase 3 and 8

In order to understand the mechanisms by which Thy-1 promotes apoptosis in fibroblasts, RFL-6 Thy-1 (+) and RFL-6 Thy-1 (-) cells were treated with the indicated concentrations of rhFasL for 16h, and then processed for immunoblotting. As shown in Figure 2A, cleavage of caspase 3 was not detected in untreated and 50 ng/mL rhFasL-treated RFL-6 Thy-1 (-) cells. In RFL-6 Thy-1 (+) cells, there was a significant increase in cleavage of caspase 3 (two small ~ 19 kDa and 17 kDa fragments) in response to 50 and 100 ng/mL rhFasL. Treatment with staurosporine resulted in increased cleavage of caspase 3 in Thy-1 (+) cells (Figure 2A lower panel). To further confirm this result, sorted Thy-1 (+) and Thy-1 (-) MEF cells were treated with rhFasL or staurosporine, and cleavage of caspase 3 and 8 was assessed. We found that Thy-1 expression was associated with increased levels of cleaved caspase 3 and 8 in Thy-1(+) MEFs (Figure 2B). Our results suggest that Thy-1 promotes the activation of the apoptotic extrinsic pathway.

Inhibition of Caspase-8 Decreases the Cleavage of Caspase 3 and Decreases the Susceptibility to Apoptosis Induced by rhFasL in RFL-6 Thy-1 (+) Cells

Thy-1 expression at the cell surface seems to be enough to promote apoptosis susceptibility in fibroblasts via the extrinsic pathway. To further confirm the participation of caspase 8 activation in the apoptotic process, RFL-6 Thy-1 (+) cells were pretreated with different concentrations of a specific caspase-8 inhibitor for 30min followed by 50ng/mL rhFasL treatment for 16h (Figure 3A). Pretreatment with caspase 8 inhibitor inhibited rhFasL-induced activation of caspase 3 in RFL-6 Thy-1 (+) cells in a dose-dependent manner. In addition, RFL-6 Thy-1 (+) cells were pretreated with 20 μ M of caspase 8 inhibitor negative control or caspase 8 inhibitor for 30 min followed by 50ng/mL rhFasL treatment for 16h.

Caspase 8 inhibitor effectively reduced the basal level of apoptosis in untreated cells, and abolished rhFasL-induced apoptosis (Figure 3B).

Thy-1 Inhibits Expression of the Anti-apoptotic Proteins Bcl-2 and Bcl-xL and Promotes Mitochondrial Pathway Mediated Apoptosis Indicated by Cleavage of Caspase 9

We next wanted to assess whether Thy-1 promotes apoptosis via the intrinsic pathway as well. As showed in Figure 4A, Thy-1 expression results in significant decrease in the basal expression levels of Bcl-2 and Bcl-xL. In response to staurosporine treatment, Thy-1 expression leads to significantly greater cleaved caspase 9 than in untreated control samples (Figure 4B). To define whether the activation of caspase 9 is caspase 8-dependent, Thy-1 (+) MEF cells were pretreated with caspase 8 inhibitor, and cell lysates were analyzed for cleaved caspase 9 as shown in Fig 4C. The results showed that caspase 8 inhibition does not alter caspase 9 activation. All together, these data imply that Thy-1 is able to independently activate the extrinsic and intrinsic apoptotic pathways in fibroblasts.

PARP Cleavage in Response to rhFasL and Staurosporine-Induced Apoptosis

PARP is a well-known substrate for cleaved caspase-3 during apoptosis. In order to prove the activation of caspase-3 in cells expressing Thy-1, we determined the extent of PARP cleavage in relation to Thy-1 expression and apoptotic stimuli. We showed that Thy-1 expression slightly increased the basal level of cleaved PARP (Figure 5A). Treatment with rhFasL at the indicated concentrations caused increased PARP cleavage in Thy-1 (+) RFL-6 cells in a dose-dependent manner, whereas Thy-1 (-) RFL-6 cells did not show induction of cleaved PARP under the same conditions. Likewise, treatment with staurosporine (1 μ M) increased the expression of cleaved PARP only in Thy-1 (+) MEF fibroblasts similarly to the response to rhFasL (Figure 5B). These results indicate that expression of Thy-1 is sufficient to sustain caspase-3 activity leading to PARP cleavage.

Thy-1-Mediated Susceptibility to Apoptosis Requires GPI Anchorage

Many of the signaling effects of Thy-1, such as modulation of Src family kinases and focal adhesion kinase phosphorylation depend upon its glycosylphosphatidylinositol (GPI) anchor and lipid raft localization⁽²⁰⁾ We wanted to determine whether Thy-1's GPI anchor was needed to induce susceptibility to apoptosis in lung fibroblasts. Transfection of RFL-6 cells, which are Thy-1 negative, with a human Thy-1 construct resulted in increased susceptibility to apoptosis similar to that seen with the murine construct (Figure 6, gray-shaded bars). To determine whether the Thy-1-specific GPI anchor is required, we transfected RFL-6 cells with a human Thy-1-hinge TR3 vector, a chimeric construct in which the GPI anchor of hThy-1 was replaced with the GPI anchor addition sequence of TRAIL receptor 3, which has previously been shown to segregate to distinct membrane microdomains.²⁶ The expression of this chimeric Thy-1 construct failed to induce FasL-mediated apoptosis (Figure 6, black filled bars). Similar findings were seen in response to staurosporine (Data not shown). These results indicate that Thy-1 requires its specific GPI anchor in order to induce susceptibility to apoptosis in lung fibroblasts.

Thy-1 Colocalizes with Fas

Based on our data so far, Thy-1 and Fas seems to work together to induce apoptosis in lung fibroblasts. To gain insights into the mechanisms of their interaction, we examined the transcriptional effect of rhFasL on Fas protein expression in lipid rafts isolated from RFL-6 Thy-1 (+) cells. We found that Thy-1 does not affect basal expression of Fas, but there is a slight increase in Fas in response to FasL stimulation in both Thy-1- and Thy-1+ fibroblasts (Figure 7A). A specific CD95 Fas band clearly co-immunoprecipitates with mThy-1.2 in response to FasL stimulation (Figure 7B). Confocal microscopy demonstrates colocalization of Thy-1 with Fas receptor after treatment with rhFasL in RFL-6 Thy-1 (+) cells (Figure 7C). Thus, our results indicate that the close interaction between Thy-1 and Fas in lipid rafts regulates fibroblast apoptosis.

Thy1^{-/-} Mice Display Persistent Fibrotic Remodeling and Compromised Injury Resolution

To test the relevance of the *in vitro* results in lung fibrosis, we examined fibrotic remodeling in *Thy1*^{-/-} and Wt mice following single-dose intratracheal bleomycin instillation (a self-limited fibrotic response). Histopathological examination (H&E staining) of tissue slides indicated persistent, non-resolving fibrosis in *Thy1*^{-/-} mice, whereas Wt mice demonstrated restoration of normal appearing histology in most observed areas, at day 56 after Bleomycin instillation. *Thy1*^{-/-} mice were characterized by an obvious fibroproliferative response, enhanced cellularity, alveolar septal thickening, architectural distortion, and severe alveolar destruction (data not shown). There was a highly significant increase in lung collagen deposition, as determined by increased trichrome staining (Figure 8A&B) and Western blotting (Figure 8C) in *Thy1*^{-/-} lungs relative to Wt lungs. These results demonstrated that mice lacking Thy-1 failed to resolve lung fibrosis, suggesting that Thy-1 is extremely important for normal tissue remodeling following bleomycin-induced lung injury.

Decreased Fibroblast Apoptosis Associated with Myofibroblast Accumulation in Mice Lacking Thy-1

Immunohistochemical detection of cleaved caspase-3 and aSMA (Figure 9A) was performed to assess myofibroblast apoptosis in *Thy1*^{-/-} vs. Wt mice after bleomycin instillation. At 28 days after bleomycin, myofibroblasts appeared throughout the lungs of *Thy1*^{-/-} and Wt mice. Quantitative data showed that $36.8 \pm 5.3\%$ vs $30.4 \pm 6.3\%$ cells/hpf stained positive for aSMA in *Thy1*^{-/-} and Wt mice, respectively. However, the number of aSMA-stained cells positive for cleaved caspase-3 [CL-caspase3(+)/aSMA(+)] apoptotic myofibroblasts] was significantly less in *Thy1*^{-/-} mice than in Wt mice (Figure 9B). At Day 56, there remained aSMA(+) myofibroblasts along with persistently abnormal lung architecture in *Thy1*^{-/-} mice, whereas Wt mice demonstrated disappearance of aSMA staining in most observed areas.

DISCUSSION

This study provides new evidence that expression of Thy-1 in lipid rafts and colocalization with Fas promote lung fibroblast susceptibility to apoptosis, whereas fibroblasts lacking Thy-1 are resistant to apoptosis, via caspase 9- and caspase 8-dependent pathways. Activation of caspases for apoptosis requires the Thy-1-specific GPI anchor. These findings

suggest that localization of Thy-1 to specific lipid raft microdomains places Thy-1 in proximity to the downstream signaling molecules that regulate apoptosis. Our *in vivo* study demonstrates that Thy-1 is essential for disappearance of α SMA(+) fibroblasts and resolution of fibrosis following bleomycin-induced lung injury in the single-dose model, indicating that absence of Thy-1 was associated with an apoptosis-resistant myofibroblast phenotype, and persistence/progression of lung fibrosis.

Apoptosis is typically executed by caspase activation and regulated by the Bcl-2 protein family. It is thought that Bcl-2 and Bcl-xL inhibit FasL-induced apoptosis because they prevent the release of cytochrome *c* from mitochondria and subsequent activation of caspase-9; however, inhibition of apoptosis by Bcl-2 affects other pathways in addition to cytochrome *c*, caspase-9 and its activator Apaf-1.²⁷ The association of between Bcl-2 family members and development of pulmonary fibrosis has been reviewed elsewhere.²⁸ In this study, the expression of Bcl-2 and Bcl-xL was decreased in response to Thy-1 expression, and could lead to caspase cascade activation of apoptosis pathways from caspase 9 to effector caspase 3 and PARP. Considering the possibility of crosstalk between the extrinsic and intrinsic pathways in response to Fas,^{29–31} we also measured the activation of caspase 8, a downstream effector of the death receptor-mediated extrinsic apoptotic pathway. Notably, cleavage of caspase-8 and 3 were significantly increased after rhFasL and staurosporine treatment in Thy-1(+) cells. Caspase 8 inhibition reduced rhFasL-mediated apoptosis, confirming that caspase 8 cleavage is upstream of caspase 3 cleavage, indicating activation of the extrinsic pathway dependent on Thy-1. However, caspase 8 inhibition did not alter the cleavage of caspase 9, suggesting that the caspase 9 activation is likely to be independent of the caspase 8-mediated extrinsic apoptotic pathway. Therefore, the data here demonstrate that Thy-1 mediates apoptotic signaling via both caspase 9- and caspase 8-dependent pathways.

The presence of the GPI anchor affects the conformation and subcellular localization of Thy-1. To determine whether the GPI anchor is required for Thy-1-mediated apoptosis, we used a construct in which the GPI anchor of CD90.2 was replaced with a sequence from the transmembrane domain of CD8 (Thy-1-CD8), which is known to lack the inhibitory effects of wild type Thy-1 on neurite outgrowth.^{20, 32} Thy-1 lacking the GPI anchor does not localize to the same membrane microdomains as wild-type Thy-1, suggesting the GPI anchor is necessary for localization of Thy-1 to specific lipid raft microdomains,³² however, mutant chimeric Thy-1 proteins are insufficiently specific to rule out other structural effects unrelated to specific GPI function. Expression of Thy-1-CD8 in Thy-1 (–) cells did not increase rhFasL-mediated apoptosis, unlike expression of Wt Thy-1, suggesting that the GPI anchor and lipid raft localization of Thy-1 is necessary for Thy-1-mediated apoptotic signaling.

To determine whether the GPI anchor specific to Thy-1 is required, we replaced the GPI anchor of human Thy-1 with the GPI addition sequence of TRAIL receptor 3 and we transfected RFL-6 cells with human Thy-1-hinge-TR3.²² The specific GPI sequences for Thy-1 and TRAILR3 have previously been shown to segregate to distinct membrane microdomains.³³ The results showed that the expression of Thy-1-hinge-TR3 rendered cells insensitive to rhFasL-mediated apoptosis compared with wild-type hThy-1 expression,

confirming that the Thy-1-specific GPI anchor and its specific membrane subdomain localization is required for its pro-apoptotic effects.

Lipid rafts are tightly packed, liquid-ordered phase microdomains in plasma membranes and participate in various cellular functions, including cell survival and cell death.^{34,35} Thy-1 is localized in specific lipid raft subdomains, and its localization is important to its biological functions. Fas-mediated apoptosis involves translocation of Fas and downstream signaling molecules into lipid rafts, a process that can be pharmacologically modulated. Engagement of FasL leads to trimerization of Fas and clustering of its intracellular death domain, which in turn leads to the recruitment of the cytoplasmic Fas-associated death domain. The cascade of caspases is then set off and this ultimately leads to cell death. In Thy-1(+) cells rhFasL treatment induced the colocalization of Thy-1 with Fas as demonstrated by confocal microscopy.

The role of Thy-1 for fibroblast apoptosis in tissue remodeling was examined in *Thy1^{-/-}* mice vs. Wt mice after bleomycin instillation. *In vivo* data revealed persistence of both myofibroblast and collagen accumulation with abnormal lung architecture in *Thy1^{-/-}* mice, whereas Wt mice showed normal or near-normal alveolar structure at day 56. Thy-1 loss could contribute, at least in part, to fibroblast resistance to Fas-induced apoptosis seen in pathological fibrosis and aging lungs.^{21,36,37} Several recent studies of organ fibrosis strongly support this role for Thy-1 in myofibroblast apoptosis and tissue remodeling. Thy-1/ β 3 integrin-induced apoptosis of dermal fibroblasts is mediated by up-regulation of FasL expression.¹³ Another study has reported that Thy-1 up-regulates FasL expression via Src family kinases.³⁸ Thy-1/ β 3 integrin interaction also triggers tyrosine phosphorylation of focal adhesion proteins promoting focal adhesion formation and cell attachment.³⁹ Relevance of this process to human fibrotic lung disease was highlighted by a recent study describing Thy-1's role as a molecular mechanosensor. In that study, normal human fibroblasts were shown to undergo apoptosis when shifted from a physiologically stiff (18.7 kPa) to a soft (1.8 kPa) extracellular matrix substrate; however Thy-1(-) myofibroblasts from lungs of individuals with Idiopathic pulmonary fibrosis (IPF) were resistant to apoptosis induced by decreased matrix stiffness.⁴⁰ These studies indicate that Thy-1 functions via its subcellular localization or molecular interactions to facilitate cellular susceptibility to apoptosis. Moreover, there is the possibility that additional molecules may modify Thy-1/ β 3 integrin interaction.⁴¹

An additional important finding of this study is that *Thy1^{-/-}* mice have persistent, non-resolving fibrosis following single-dose intratracheal bleomycin, unlike the spontaneously resolving fibrosis seen in Wt mice. The myofibroblast resistance to apoptosis associated with progressive fibrosis is more characteristic of progressive fibrotic disorders in humans, such as IPF. Therefore the *Thy1^{-/-}* model may be a more relevant preclinical model for testing antifibrotic therapeutics.

In summary, this study characterized the mechanism of Thy-1 mediated apoptosis in lung (myo)fibroblasts, critical effector cells in fibrosis resolution. This modulation of Thy-1 was dependent on lipid raft integrity and the Thy-1 specific GPI anchor. The *in vivo* results strongly support a regulatory role for Thy-1 in initiation of (myo)fibroblasts apoptosis that

heralds the termination of the reparative response to lung injury. Thy-1-mediated pro-apoptotic effects could either halt the progression or speed the resolution of lung fibrosis via the pro-apoptotic effects. Therefore, it is possible that Thy-1 re-expression through targeted gene therapy or molecular mimicry could restore apoptosis susceptibility in apoptosis-resistant lesional fibroblasts in pulmonary fibrosis and other fibrotic diseases.

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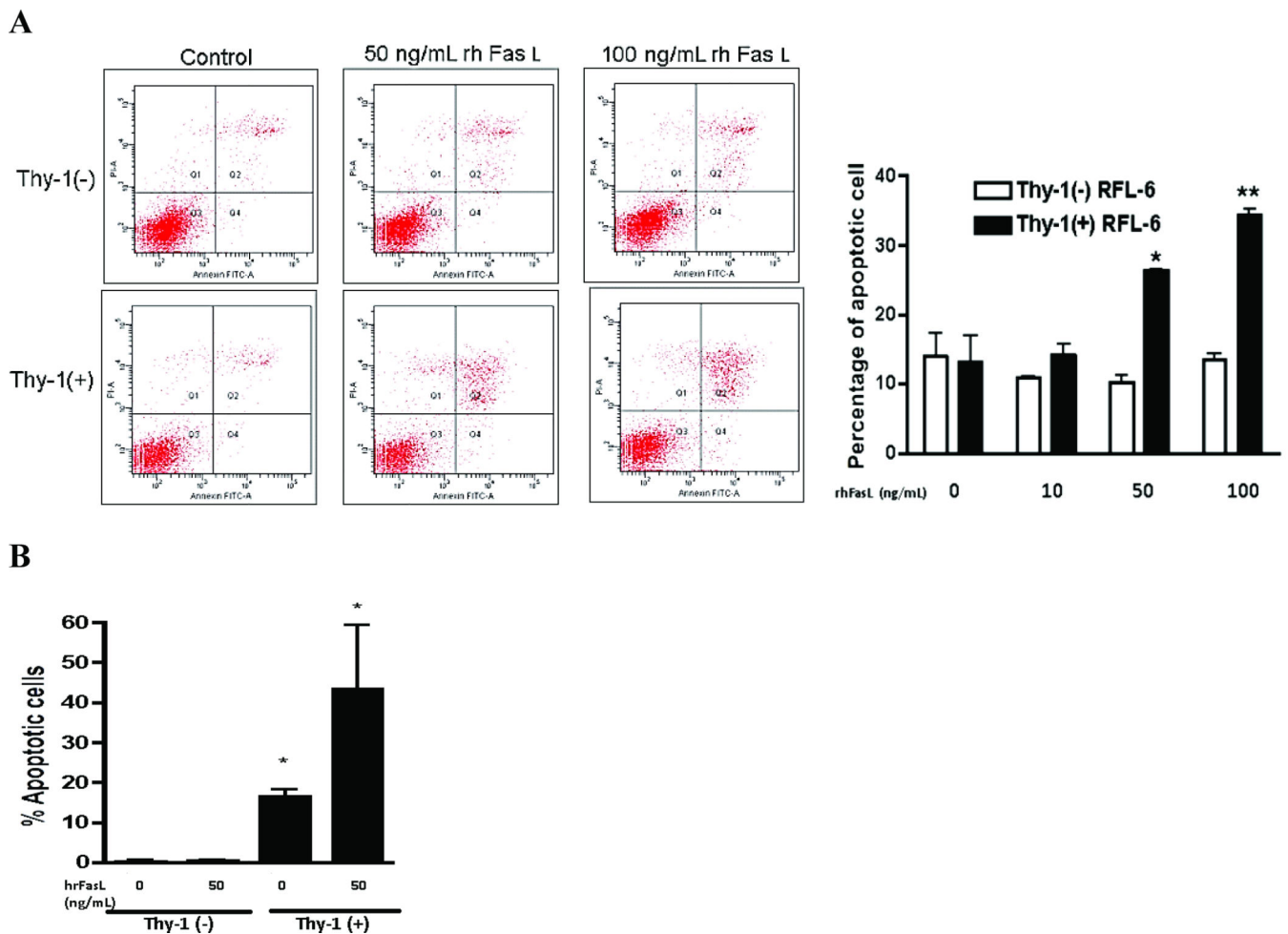


Figure 1. Thy-1 expression promotes fibroblast susceptibility to apoptosis

A. RFL-6 Thy-1 (–) and Thy-1 (+) fibroblasts were treated with the indicated concentrations of rhFasL for 16h, and then cells were harvested and stained with Annexin V and PI and apoptosis quantified by flow cytometry. Results are representative of three independent experiments. Upper panels: Dot plots of FACS analysis with FITC-Annexin V and PI. The lower-left quadrants of the dot plot panels represent live cells (Annexin V–/PI–); the lower-right quadrants of the panels quadrants represent early apoptotic cells (Annexin V+/PI–); the upper-right quadrants of the panels quadrants represent later apoptotic cells (Annexin V+/PI+). Lower panel shows a quantitative representation of the flow cytograms. Each bar represents the mean±SEM of early + late apoptosis for at least three experiments. *denotes $p < 0.0001$ compared with controls. B. Effect of Thy-1 on apoptosis in Thy-1 (–) and Thy-1 (+) fibroblasts. Cell treatments are the same as above. After treatment, cells were stained with FITC-dUTP, terminal nucleotidyl transferase–mediated nick end labeling assay. The growing cells were treated with rhFasL for 16 h and the extent of apoptosis was assessed by APO-Direct assay kit. The analyses were done using Cell Quest software; bar represents the mean±SEM of at least three experiments. * $p < 0.0001$ compared with controls.

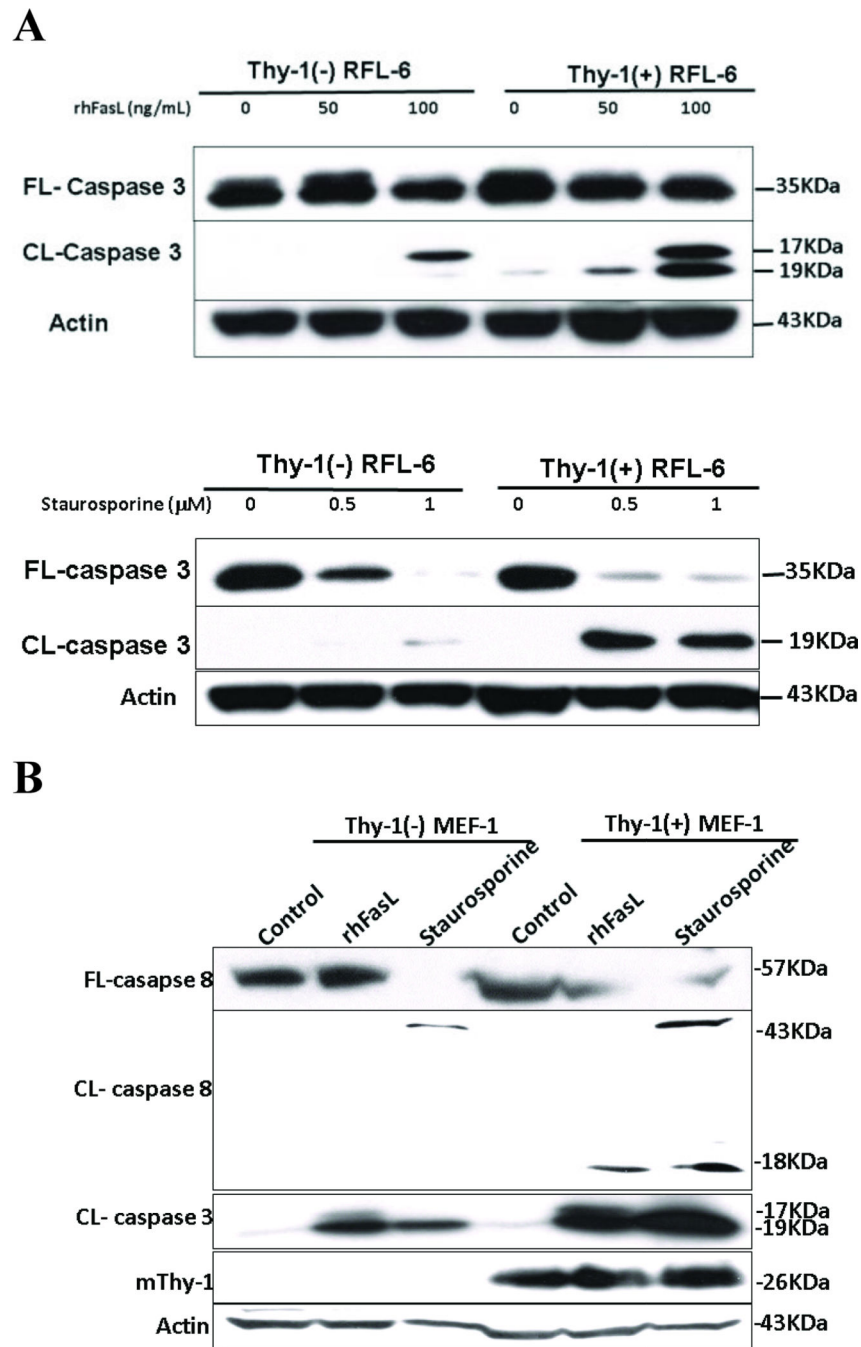


Figure 2. The effect of Thy-1 expression on the activation of caspase 3 and 8 in response to apoptosis inducers

A. Upper panel: Thy-1 (-) and (+) RFL6 cells were treated with the indicated concentrations of rhFasL for 16h. Lower panel: Thy-1 (-) and (+) RFL6 cells were treated with the indicated concentrations of staurosporine. Cell lysates were subjected to Western blot analysis using cleaved caspase-3 and caspase-8 antibody. Procaspase 3 (full length or FL-caspase 3) and cleaved caspase 3 (CL-caspase 3) expressions are representative of three independent experiments. The cleaved Caspase-3 Rabbit mAb detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3. B. Sorted Thy-1 (-) and Thy-1 (+)

MEF were rendered quiescent and then treated with the 50 ng/mL rhFasL and 1 μ M staurosporine for 16h, and then cells were harvested and analyzed by Western blots for caspase activation. Pictures are representative of three independent experiments. β -actin is used as internal control.

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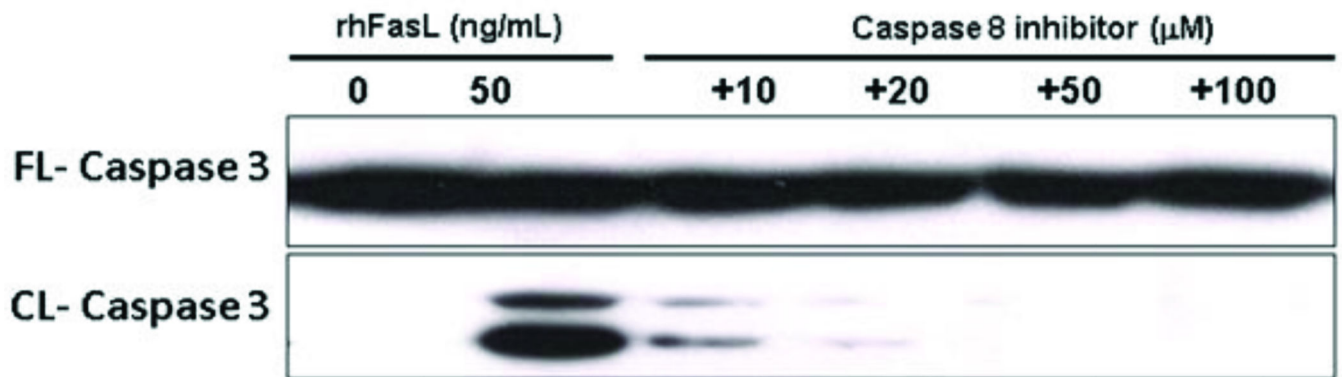
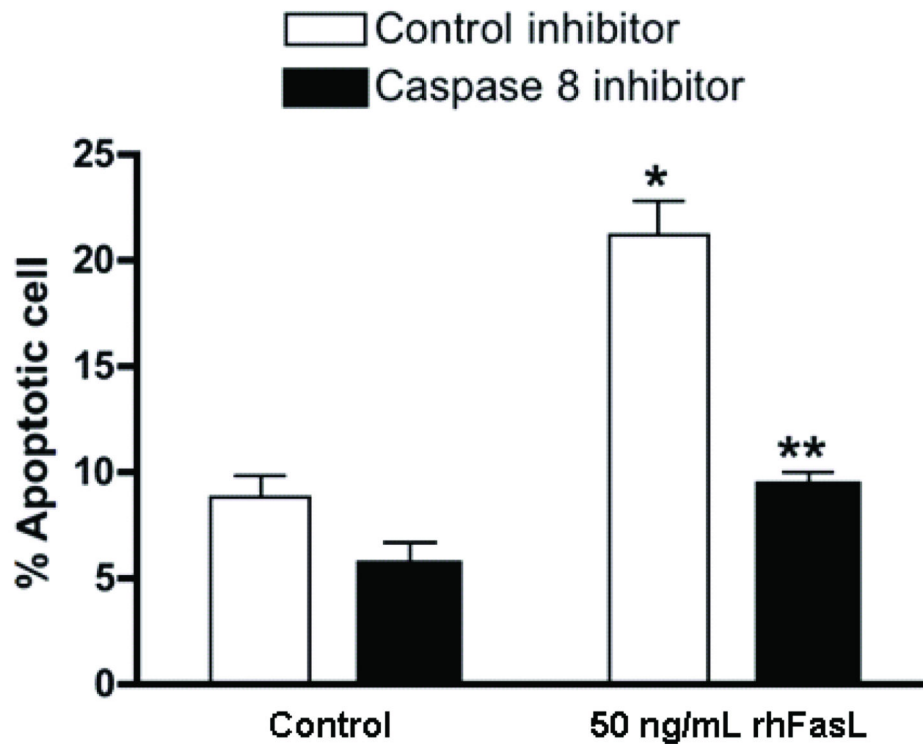
A**B**

Figure 3. Inhibition of caspase-8 decreases cleaved caspase 3 and abolishes the induction of apoptosis by rhFasL in Thy-1 (+) cells

A. Caspase-8 inhibitor was added to a final concentrations indicated for 30 min and then Thy-1 (+) RFL-6 were treated with 50ng/mL rhFasL for 16h. Immunoblotting with anti-cleaved caspase-3 identifies caspase-3 cleavage products in Thy-1 (+) RFL-6. B. Thy-1 (+) cells were pretreated with 20 μ M of caspase 8 inhibitor negative control or caspase 8 specific inhibitor for 30 min followed by 50ng/mL rhFasL for 16h, and then cells were collected and stained with Annexin V and PI followed by flow cytometry as described in Methods. Bar graph shows average of three independent experiments of FACS analysis with FITC-

Annexin V and PI. * $p < 0.0001$ compared with control inhibitor-treated control. ** $p < 0.0001$ compared with hrFasL-treated cells pretreated with caspase 8 inhibitor. FL denotes full length, CL is cleaved caspase.

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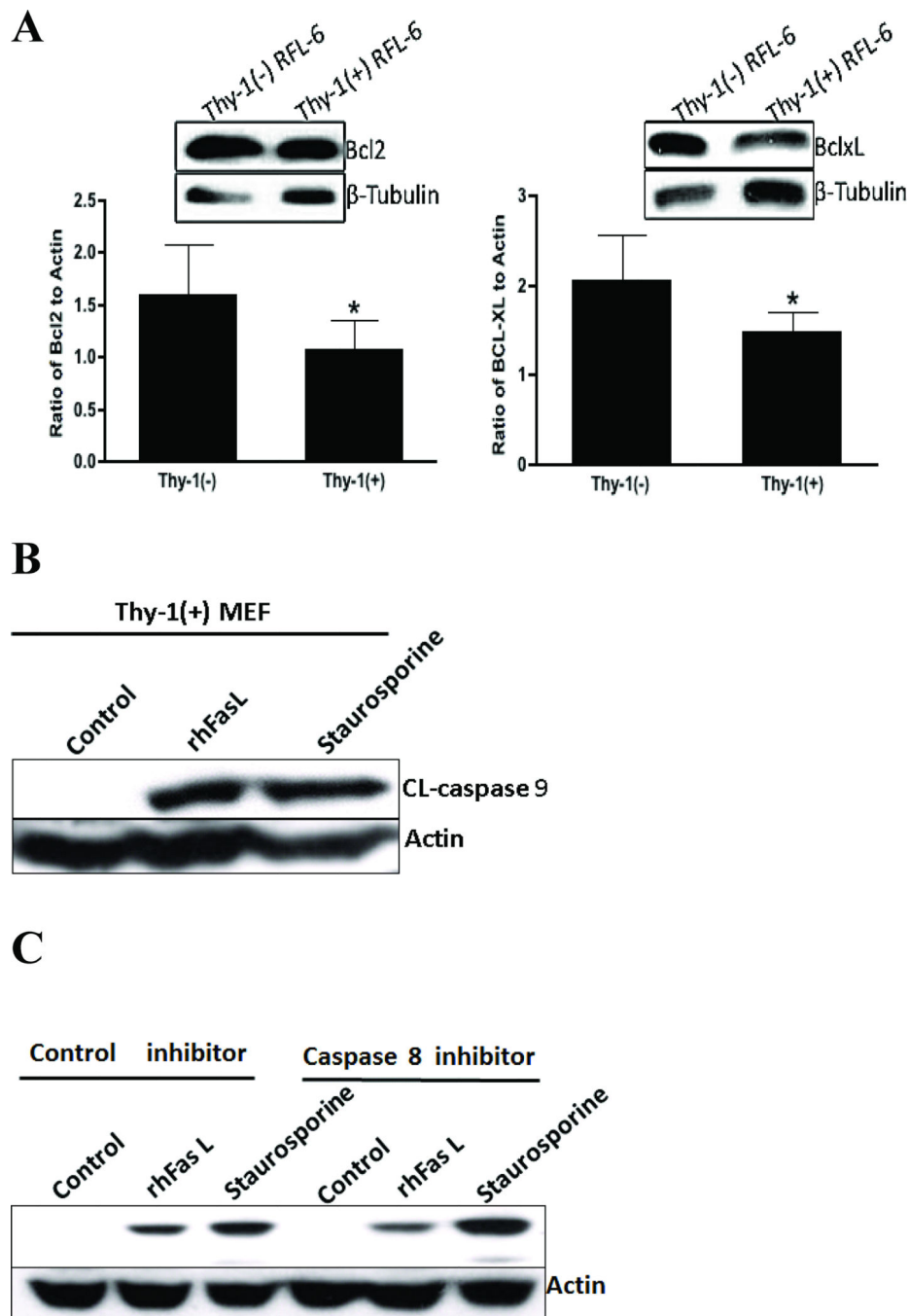


Figure 4. The effect of Thy-1 expression on the expression of anti-apoptotic proteins, Bcl2 and Bcl-xL and the activation of caspase 9

A. Basal level of Bcl2 and Bcl-xL expression by immunoblotting in Thy-1 (-) and (+) RFL6 cells. * $p < 0.05$ compared with Thy-1 (-) RFL-6. Thy-1 (-) and (+) RFL-6 cells were treated with 50ng/mL rhFasL and 1 μ M Staurosporine and immunoblotted using anti-cleaved caspase-9. B. Thy-1.2 (+) MEFs were treated with 50ng/mL rhFasL and staurosporine for 16 hours, cell lysates were collected for analysis of cleaved caspase 9. C. Thy-1.2 (+) MEFs were pretreated with 20 μ M negative control or caspase 8 specific inhibitor for 30 min followed by 50ng/mL rhFasL and staurosporine for 16 hours, cell lysates were collected for

analysis of cleaved caspase 9. Results are representative of three independent experiments.
FL is full length CL is cleaved caspase.

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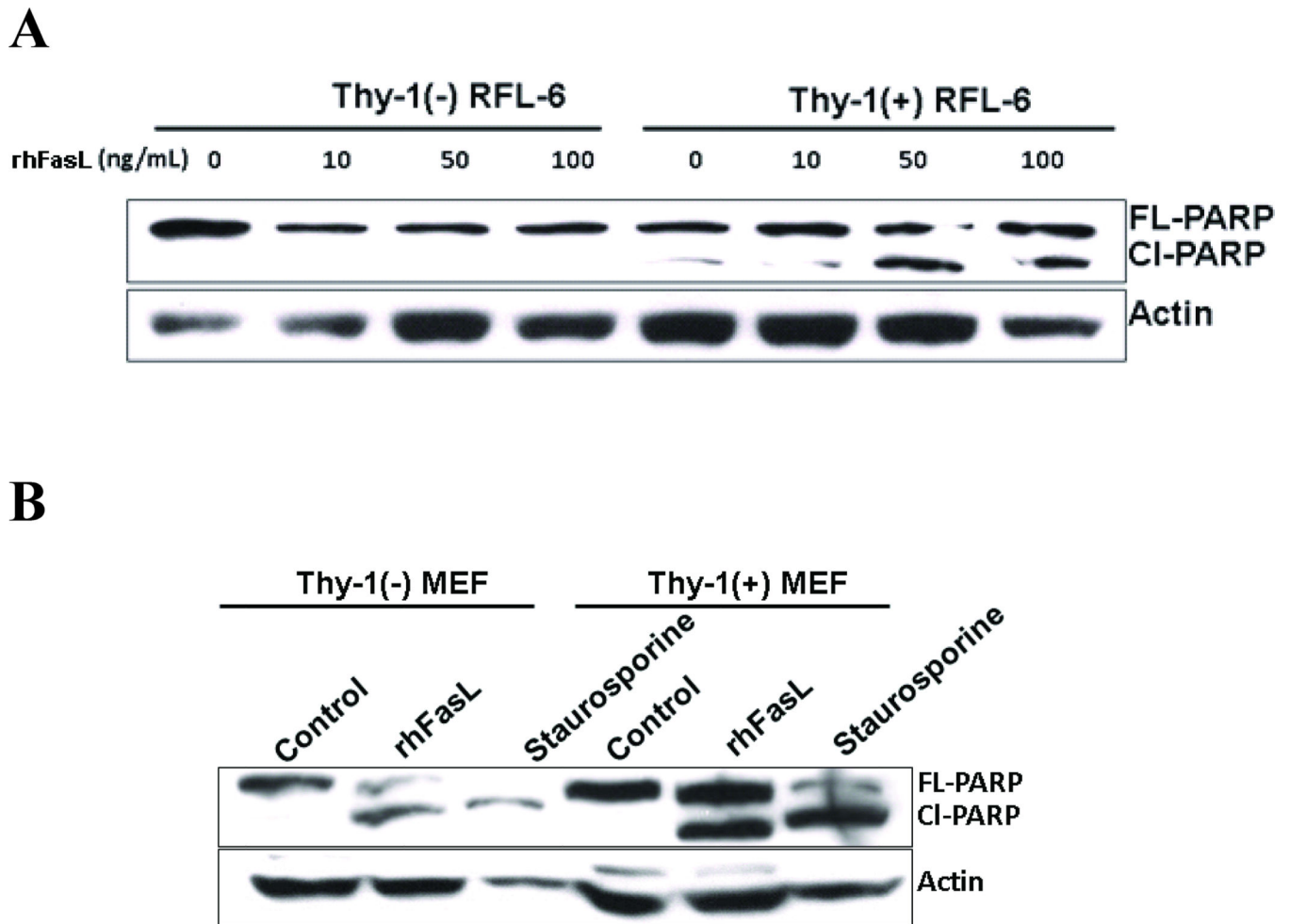


Figure 5. The effect of Thy-1 expression on PARP cleavage

A. Thy-1 (-) and (+) RFL6 cells were treated with the indicated concentration of rhFasL followed by immunoblotting using antibody against PARP. B. Thy-1 (+) MEFs were rendered quiescent and then treated with the 50ng/mL rhFasL and 1 μ M staurosporine for 16 hours. PARP cleavage was analyzed by anti-PARP. Three independent experiments were performed with similar results.

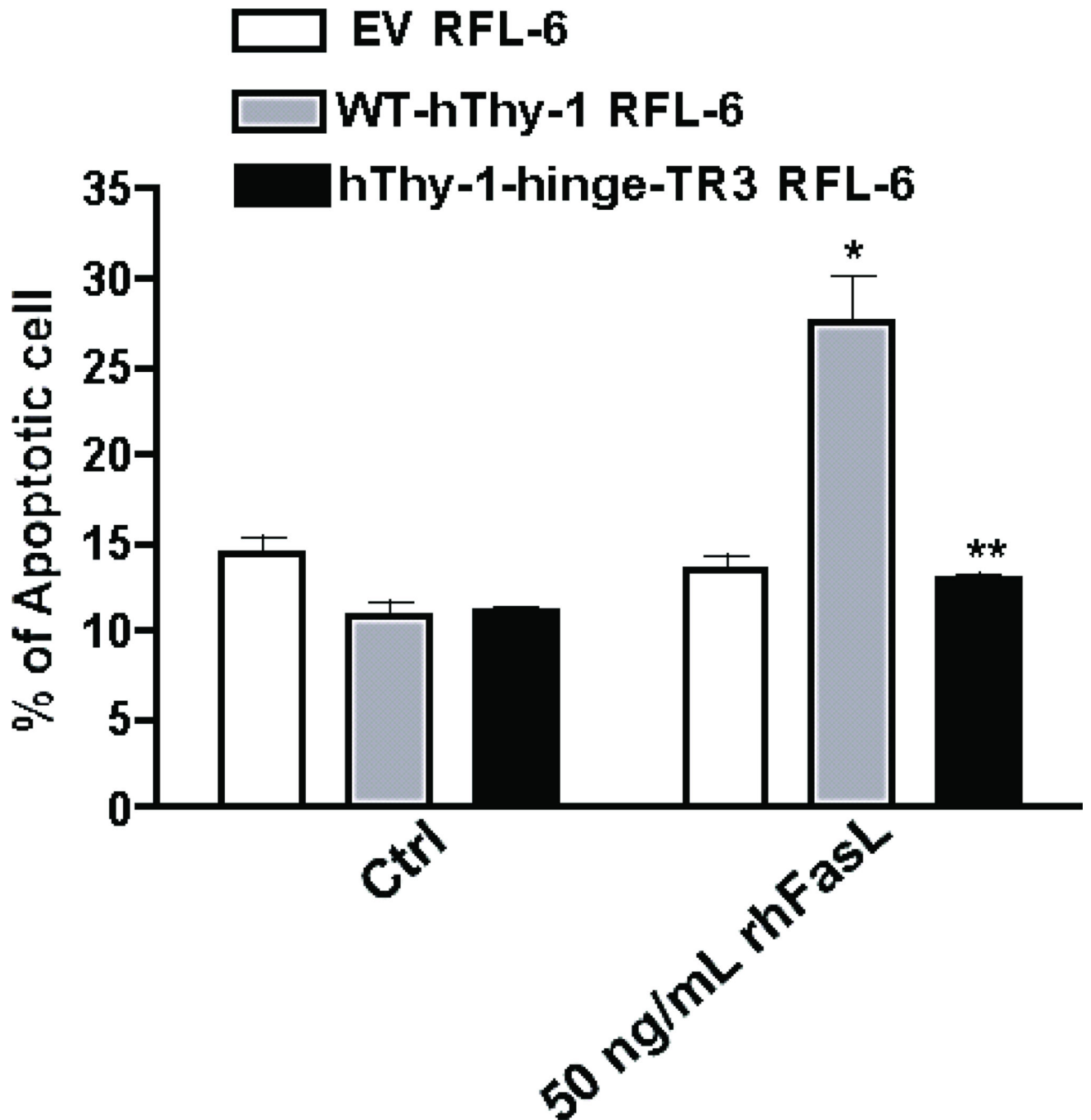


Figure 6. The GPI anchor of mouseThy-1.2 and the Thy-1-specific GPI anchor of human Thy-1 are required for Thy-1-mediated apoptosis

Three RFL-6 cell lines (EV, hThy-1 and hThy-1-TR3 RFL-6) were used in this experiment were treated with 50 ng/mL rhFasL for 16 hours. Bar graphs show average of three independent experiments. Prism two-tailed t test was used for statistical analysis and * $p < 0.05$ compared with untreated control. ** $p < 0.01$ compared with rhFasL-treated Thy-1 RFL-6. FL is full length CL is cleaved caspase.

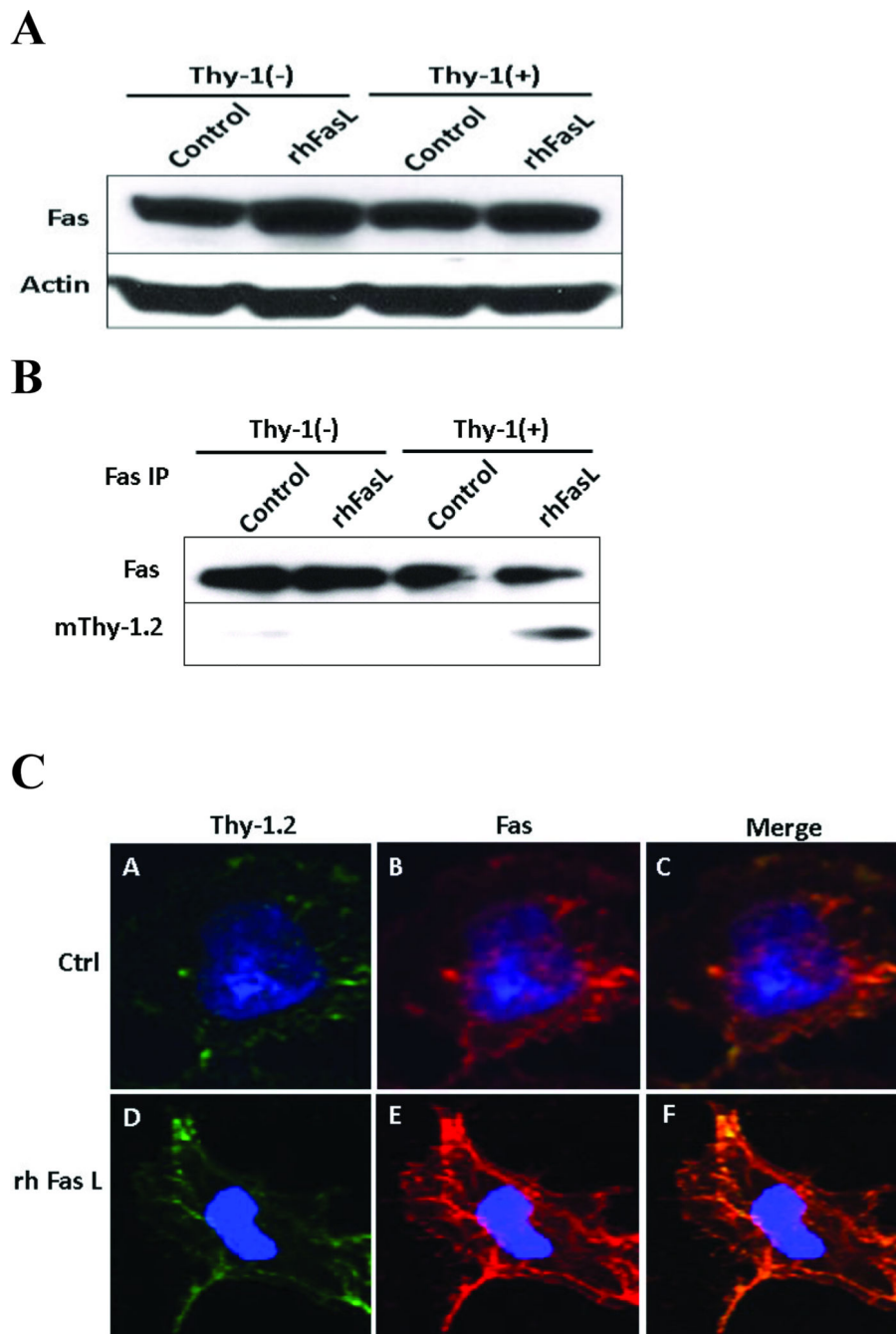


Figure 7. Interaction of Thy-1 with Fas-mediated signaling pathway and lipid raft integrity are required for the activation of caspase 3

A. Membrane fractionation and immunoprecipitation of lipid rafts was performed as described in Methods. Total cell lysates prepared from Thy-1.2 (+) RFL-6 were used for IP with anti-Fas or normal mouse IgM as described experimental procedures. Proteins from the immunoprecipitates were detected by Western blotting using anti-mouse Thy-1.2 antibody (upper panel) or anti-Fas antibody (lower panel). B. Cell lysates were collected from Thy-1.2 (-) and Thy-1.2 (+) RFL-6 followed by immunoblotting. C. Cells were cultured on coverslips as shown in Materials and Methods and colocalization of Thy-1 with Fas in Thy-1

(+) RFL-6 was performed. Staining shows Thy-1.2 (A and D, green) and Fas (B and E, red) associated with the plasma membrane. Yellow color indicates colocalization of Thy-1.2 and Fas after apoptosis induction by 50 ng/mL rhFasL (C and F).

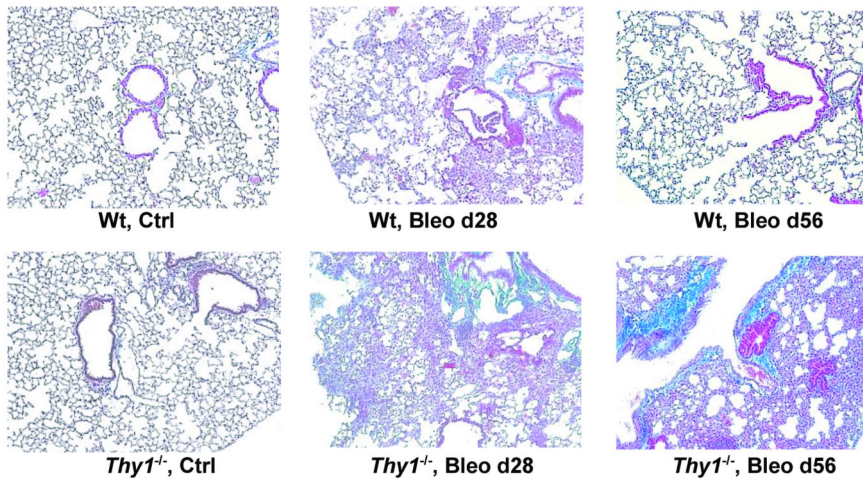
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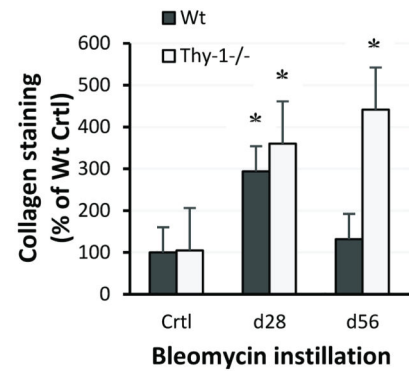
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A



B



C

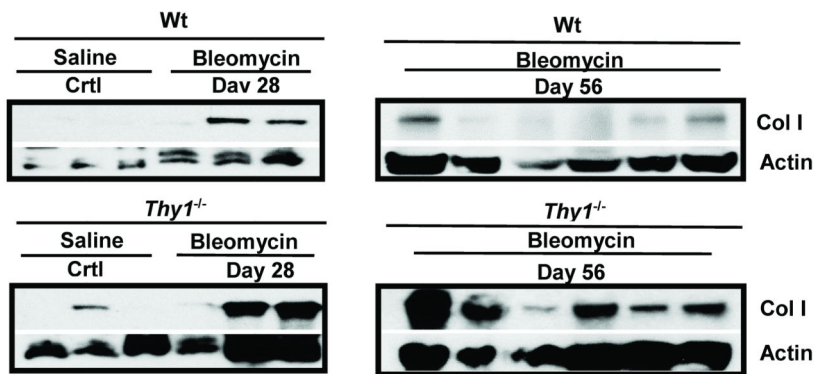
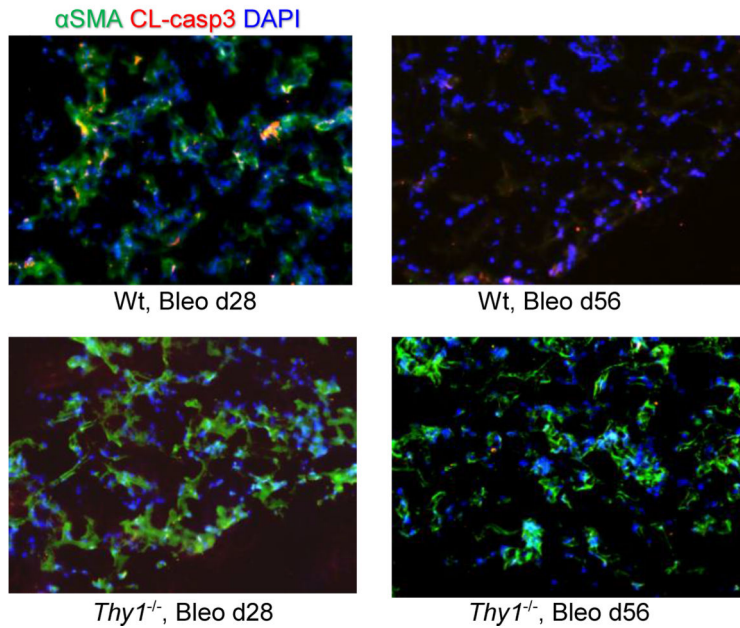


Figure 8. Failure of resolution of bleomycin-induced lung fibrosis in *Thy1*^{-/-} mice, leading to persistent extracellular matrix accumulation

Mice were instilled with bleomycin (5 U/kg) by orotracheal MicroSprayer MS-IA-1C (Penn-Century). The lung tissue was collected at each time-point, fixed (10% formalin), embedded (paraffin), and stained with Masson's Trichrome after intratracheal instillation of bleomycin, revealing collagen accumulation with persistently abnormal lung architecture in *Thy1*^{-/-} mice at day 56, whereas Wt mice demonstrated restoration of normal appearing histology in most observed areas. A. Masson's Trichrome stain, 10X. B. Quantification of collagen staining (% increase over Wt saline). C. Immunoblots of Col I, in Wt and *Thy1*^{-/-} mice over time after bleomycin instillation. * $p < 0.05$ when compared to Wt Ctrl group.

A



B

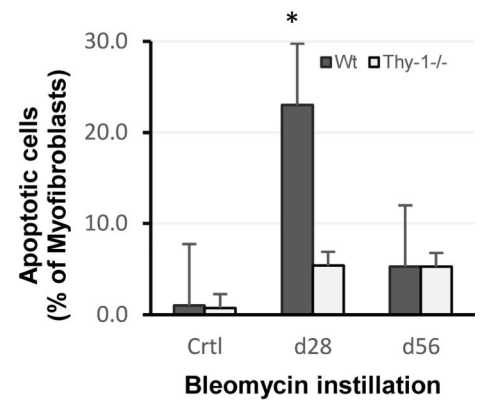


Figure 9. Decreased apoptosis of lung myofibroblasts in *Thy1*^{-/-} mice and accumulation in the fibrotic resolution phase

Lung tissue was collected at indicated time points. 5 μ m OCT-embedded frozen tissue sections were fixed with 4% PFA and processed for double immunostaining of α SMA (myofibroblasts, green) and CL-Casp3 (apoptosis, red). Nuclei were counterstained with DAPI (blue). Wt mice displayed strong apoptotic signaling in α SMA (+) cells at day 28. *Thy1*^{-/-} mice had persistent α SMA (+) cells throughout the lungs. A. double immunostaining of α SMA (green) and CL-Casp3 (red). B. Apoptotic cells as % of total α SMA(+) cells. One-way ANOVA and Newman-Keuls multiple comparisons tests were used to calculate statistical significance. * p <0.05 when compared to Wt Ctrl group.

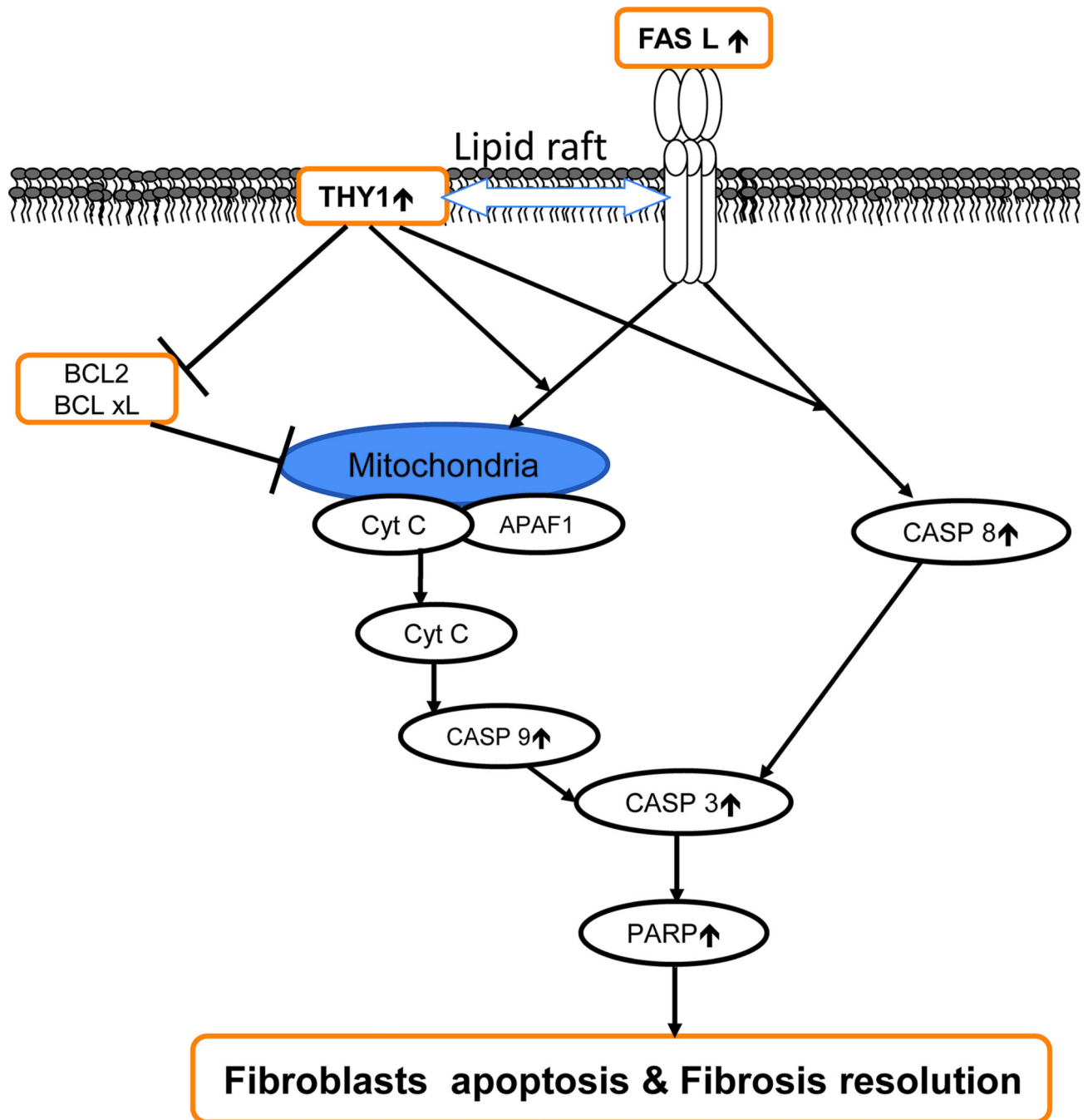


Figure 10. The model of Thy-1 mediated apoptosis pathway induced by FasL
 Stimulated Thy-1 increases in the activation of caspase 8, 3 and 9 followed by PARP activation. The action of caspase 9 activation is likely to be independent of caspase 8- and 3-mediated apoptotic pathway as indicated in the figure. Caspase 8 inhibition can reduce caspase 9 activation mediated by FasL but not by staurosporine, indicating that the anti-apoptotic proteins such as Bcl-2 and Bcl-xL are involved. The expression of Bcl-2 and Bcl-

xL is decreased Thy-1 (+) cells. Decreased levels of Bcl-2 and Bcl-xL will induce activation of the mitochondrial pathway.

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