SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies. Anti-TIMP-1 Ab-2 (Clone 102 D1), anti-integrin β1, anti-integrin α6 and anti-CD63 monoclonal antibody (mAb) were purchased from Chemicon. (Temecula, CA), anti-active caspase-3, anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) and p44/42 MAP kinase polyclonal antibody (pAb) from Cell Signaling Technology (Beverly, MA), anti-human β-actin mAb, anti-mouse IgG peroxidase conjugate and anti-rabbit IgG peroxidase conjugate antibodies from Sigma (St. Louis, MO). FITC-conjugated rabbit IgG, Texas Red conjugated mouse IgG antibodies, and normal donkey serum were purchased from Jackson ImmunoResearch Laboratories Inc (West Grove, PA). Yeast strains and all media for yeast two-hybrid screening were purchased from BD Biosciences Clonetech (Palo Alto, CA). Growth factor-reduced basement membrane matrix (GFR matrigel) was purchased from BD Biosciences Discovery Labwares (Palo Alto, CA).

Human rTIMP-1 and TIMP-2 were produced using a vaccinia mammalian cell expression system as described previously (Li et al., 1999; Liu et al., 2003; Liu et al., 2005). For biotinylation of rTIMP-1, the purified recombinant TIMP-1 proteins were first buffer exchanged into PBS buffer using the Quick Spin Protein Columns (Roche Diagnostics Corp., Indianapolis, IN). Biotinylation of the TIMP-1 was then performed using NHS-PEO_4-Biotin for 2 hr at room temperature and quenched by adding NH_4Cl to a final concentration of 50 mM according to the manufacturer's directions (Pierce Chemical Co., Rockford, IL).

Cell Culture. Generation of TIMP-1 overexpressing MCF10A #29 (T29) and anti-sense TIMP-1 construct-transfected MCF10A clone (AS TIMP-1 MCF10A) was previously described (Li et al., 1999; Liu et al., 2003; Liu et al., 2005). Cells were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera toxin, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 0.5 μg/ml Fungizone in a 95% air and 5% CO_2 incubator at 37 °C.

Yeast Two-Hybrid Screening. Minimal dropout media (SD) contained 2% glucose or 2% galactose plus 1% raffinose. The dropout media lacked adenine, histidine, tryptophan, or leucine (designated as –Ade, -His, -Trp or –Leu respectively). Minimal medium containing 80mg/L X-α-gal was used to test LacZ reporter gene expression. YPD medium contained yeast extract, peptone, and 2% dextrose.

The yeast expression vector pGBKT7, which contains the coding sequences for the GAL4 DNA binding domain (amino acid 1-147), c-Myc tag and four reporter genes, HIS3, ADE2, MEL1, and LacZ, was used to express the fusion proteins. The TIMP-1 cDNA was inserted into pGBKT7 and the correct orientation and in-frame fusion were confirmed by DNA sequencing. The bait plasmid pGBKT7 was introduced into the yeast bait strain AH109 by LiOAc-mediated transformation. Transformants were selected by growth on SD/-Trp dropout media and expression of the fusion proteins was confirmed by immunoblot analysis using anti-TIMP-1 and anti-c-Myc antibodies. Before screening, the toxicity of the bait protein on the host strain was tested by comparing the growth rate of cells transformed with the empty DNA-BD vector with the rate of cells transformed with DNA-BD/bait plasmid. We also tested whether the bait alone would activate

reporter genes by growth of bait transformants on SD/-Trp/ X-α-gal, SD/-His/-Trp/ X-α-gal, and SD/-Trp/-Ade/ X-α-gal. For yeast two-hybrid screening, the yeast strain Y187 pretransformed with human placenta cDNA library in the pGADT7 plasmids was obtained from BD Biosciences Clontech (Palo Alto, CA). TIMP-1 bait strain AH109 was mated with the Y187/pGADT7 cDNA library as instructed by the Clontech protocol. The mating mixtures were plated on SD/-Ade/-His/-Leu/-Trp(QDO) plates. After three rounds of screening, the colonies that showed QDO/ X-α-gal positive phenotypes were replated on QDO/X-α-gal plates. Plasmids were rescued from the QDO/X-α-gal positive colonies by yeast mini-prep method. PCR amplification was performed using primers T7 sequencing primer (5'-TAATACGACTCACTATAGGGC-3') and 3'AD sequencing primer (5'-CTGTGCATGGTGCACCATCT-3') to amplify the inserts. The amplified inserts were sequenced and BLAST search was carried out.

To confirm CD63 interaction with TIMP-1, the purified CD63 prey plasmid was transformed back into the Y187 yeast strain and mated with TIMP-1 or TIMP-2 bait in the AH109 strain.

Immunoprecipitation. Cell lysates were obtained by lysing the cell monolayer with 1% Brij 96 lysis buffer (1% Brij 96, 25 mM Hepes, pH 7.5, 150 mM NaCl 5 mM $MgCl_2$, 2 mM PMSF, 1x protease inhibitor cocktail). Insoluble materials were pelleted at 12,000 rpm for 20 min and the cell lysates were precleared by incubation with agarose beads conjugated with goat anti-mouse or rabbit control antibodies overnight at 4°C. The precleared lysates were incubated with 500 ng/ml rTIMP-1 or rTIMP-2 at 4°C for 4 hrs. Immune complexes were precipitated with the agarose beads that were prebound with appropriate antibodies at 4°C overnight, followed by four washes with the

immunoprecipitation buffer. Immune complexes were then eluted from the beads with 2x SDS sample buffer and subjected to SDS-PAGE.

Immunoblot Analysis. Cell lysates were obtained by lysing the cell monolayer with SDS lysis buffer [2% SDS, 125 mM Tris-HCl (pH 6.8), and 20% glycerol]. The lysates were boiled for 5 min and then clarified by a 20 min centrifugation at 4°C. Protein concentration was measured using the BCA protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein samples in SDS sample buffer [1% SDS, 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, and 0.05% bromophenol blue] were boiled for 5 min and subjected to reducing SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.02% NaN_3 and 0.2% Tween 20 (T-TBS) for 1 h at room temperature. The membranes were incubated with T-TBS containing 5% milk and the primary antibodies. After three washes with T-TBS, the blot was incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies. The antigen was detected using the Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Inc., Boston, MA), according to the manufacturer's instruction. Intensity of the bands was quantified using UN-SCAN-IT V5.1 software (Silk Scientific, Inc., Orem, Utah, USA) and normalized to the intensity of the respective ß-actin band.

Immunofluorescent live cell staining. For immunofluorescence analysis, cells were plated on coverslips with 50% confluency overnight. Coverslips were washed three times with 1x PBS and blocked with PBS containing 10% horse serum and 1% BSA for 1 h. Cells were then stained with primary antibodies diluted in 1% BSA for 1 h at room

temperature, followed by three washes with PBS. After incubating with FITC-conjugated or Texas Red-conjugated secondary antibody for 1h, the coverslips were washed with PBS three times, and fixed with 4% paraformaldehyde and then mounted with anti-fade solution. Immunofluorescence microscopic analysis was performed using the Zeiss LSM310 confocal microscopy system equipped with krypton-argon (488 and 568 lines) and ultraviolet (364 line) lasers.

***Caspase Activity Assay*.** Cells were lysed in cell extract buffer (150 mM NaCl, 50 mM Tris-HCL (pH 7.5), 0.5 mM EDTA, and 0.5% Nonidet P-40). Lysates were kept on ice for 30 min and centrifuged at 15,000 × *g* for 10 min. Fifty microliters of the cytosolic fraction was incubated for 60 min at 37 °C in a total volume of 200 µl of caspase buffer {20 mM HEPES (pH 7.5), 50 mM NaCl, and 2.5 mM dithiothreitol} containing 25 µM Ac-DEVD-AMC for caspase-3-like activity (BioSource International, Inc., Camarillo, CA). 7-amino-4-methylcoumarin (AMC) fluorescence, released by caspase activity was measured at 460 nm using 360 nm excitation wavelength on a Spectra Maxi Germini fluorescence plate reader (Molecular Devices, Menlo Park, CA**)**. Caspase activity was normalized per microgram of protein as determined with a BCA protein assay kit (Pierce, Rockford, IL).

Cell Survival (WST-1) Assay. Cells were plated in a 96-well culture plate overnight followed by continuous culture in serum-free medium for 48 hr, cell viability was determined by WST-1 assay as specified by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The WST-1 colorimetric assay is based on the cleavage of the tetrazolium salt, WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene di-sulfonate), by mitochondrial dehydrogenases in viable cells to

form highly water-soluble formazan without an additional solubilization step. The absorbance of the converted dye was measured at a wavelength of 450 nm, with background subtraction at 650 nm, using a Bio-Rad Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA).

Establishing CD63-knockdown cell lines. Plasmids carrying shRNA targeted to CD63 were constructed following Ambion's web-based protocol. Briefly, four 19-mer hairpin sequences of the CD63 gene was inserted in the sense and antisense directions into the pSilencer 3.1-H1 hygro vector (Ambion, TX). Sequence specificity was confirmed in a BLAST search. Mixtures of target (1-4) vectors or control vector were transfected into AS TIMP-1 MCF10A cells using Lipofectamine 2000 (Invitrogen, CA), and the hygromycin-resistant stable clones were individually isolated (AS-shCD63#5, AS-shCD63#17, AS-shCD63#19, AS-shCont#1, and AS-shCont#5). TIMP-1 overexpressing MCF10A clone (T29) was transfected with either target 2 or control vector and the pooled populations of stable clones (T29-shCD63-P and T29-shCont-P, respectively) were utilized for the present study. The sequence information of the CD63 ShRNA targets are described below. Target 1: 5'-GGAGAACTATTGTCTTATG-3'; Target 2: 5'-AATCCCTTCCATGTCGAAG-3'; Target 3: 5'-TTTCAACGAGAAGGCGATC-3'; Target 4: 5'-TTGCTTTTGTCGAGGTTTT-3'.

Detection of active integrin β1 in suspension culture. Cells were cultured on polyHEMA (polyhydroxyethylmethacrylate) coated tissue culture plates as previously described (Li et al., 1999). After 24 hrs, the cells were collected, and washed twice with Hepes/NaCl buffer (20mM Hepes, 150 mM NaCl, 2 mg/ml D-glucose, pH 7.4) prior to the cell staining procedure. Cells were incubated with anti-integrin β1 antibody (Clone

P5D2, Chemicon, MAB1959) or with anti-active integrin β1 antibody (Clone HUTS-4, Chemicon, MAB2079Z) in Hepes/NaCl buffer for 30 min on ice. Cells were washed twice with Flow PBS (1X PBS, 2% horse serum, 0.1% sodium azide) and incubated for an additional period of 30 min on ice with fluorescein isothiocyanate-conjugated (FITC) donkey anti-mouse IgG secondary antibody (Jackson Laboratories) in Flow PBS. After two washes with Flow PBS, fluorescence was measured using a FACSCalibur machine (Becton Dickinson, San Jose, CA). The machine parameters were set up using cells alone with no staining, and approximately 20,000 cells were analyzed in each experimental condition. The WinMDI Version 2.8 (Joseph Trotter 1993-1998) software program was then applied to the data to permit gating of each of the cell lines. The percentage of gated cells stained with active or total integrin β1 (solid black line) were normalized to the percentage of gated cells stained with FITC- secondary antibody only (shaded area).

MCF10A morphogenesis assay in three dimensional (3D) culture. Three-dimensional culture was carried out as previously developed by Dr. Brugge's laboratory (Debnath et al., 2002; Mills et al., 2004). Assay medium (DMEM/F12 supplemented with 2% donor horse serum, 10μ g/ml insulin, 1 ng/ml cholera toxin, 100 μg/ml hydrocortisone, 50 U/ml penicillin, and 50 μg/ml streptomycin) containing 5 ng/ml EGF and 2% growth factor-reduced MATRIGEL (GFR matrigel) (BD Biosciences, Bedford, MA) was replaced every four days. At indicated time points, cells were washed four times with PBS containing 1 mM Ca^{2+} and Mg^{2+}, fixed in 4% paraformaldehyde at room temperature for 20 min, and permeabilized in 0.5% Triton X-100 in PBS for 10 min at 4°C. Cells were washed three times with PBS:Glycine buffer (130 mM NaCL, 7 mM Na_2HPO_4, 3.5 mM

NaH_2PO_4, 100 mM glycine) for 10 min, followed by incubation with IF buffer (130 mM NaCL, 7 mM Na_2HPO_4, 3.5 mM NaH_2PO_4, 7.7mM NaN_3, 0.1% BSA, 0.2% triton X-100, 0.05% Tween 20) for 1 h. After three washes with IF buffer, cells were incubated with a rat anti-integrin α6 monoclonal antibody (Chemicon, Temecula, CA), or with a rabbit anti-active caspase-3 polyclonal antibody (Cell Signaling Technology, Beverly, MA) at 4°C overnight. After three washes with IF buffer for 10 min, the coverslips were incubated with FITC-conjugated anti-rabbit and Texas Red-conjugated anti-rat secondary antibodies for 1h. After three washes with IF buffer for 20 min, the coverslips were counterstained with DAPI (Roche applied Science, Indianapolis, IN) and mounted with anti-fade solution. Confocal immunofluorescence microscopic analysis was performed using the Zeiss LSM510 confocal microscopy system equipped with krypton-argon (488 and 568 lines) and ultraviolet (364 line) lasers.

Statistical analysis. Statistical calculation was done using Microsoft Excel. For statistical significance, student's *t*-test values were calculated.