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Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein

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This study identified CD63, a member of the tetraspanin family, as a TIMP-1 interacting protein by yeast two-hybrid screening. Immunoprecipitation and confocal microscopic analysis confirmed CD63 interactions with TIMP-1, integrin β1, and their co-localizations on the cell surface of human breast epithelial MCF10A cells. TIMP-1 expression correlated with the level of active integrin β1 on the cell surface independent of cell adhesion. While MCF10A cells within a three-dimensional (3D) matrigel matrix form polarized acinar-like structures, TIMP-1 overexpression disrupted breast epithelial cell polarization and inhibited caspase-mediated apoptosis in centrally located cells, necessary for the formation and maintenance of the hollow acinar-like structures. Small hairpin RNA (shRNA)-mediated CD63 downregulation effectively reduced TIMP-1 binding to the cell surface, TIMP-1 co-localization with integrin β1, and consequently reversed TIMP-1-mediated integrin β1 activation, cell survival signaling and apoptosis inhibition. CD63 downregulation also restored polarization and apoptosis of TIMP-1 overexpressing MCF10A cells within a 3D-matrigel matrix. Taken together, the present study identified CD63 as a cell surface binding partner for TIMP-1, regulating cell survival and polarization via TIMP-1 modulation of tetraspanin/integrin signaling complex.

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Introduction

The family of tissue inhibitors of metalloproteinases (TIMPs), consisting of four members (TIMP-1 to -4), regulates dynamic processes of extracellular matrix (ECM) turnover and remodeling as well as activities of growth factors and their cell surface receptors, in part through inhibition of matrix metalloproteinases (MMPs). However, mounting evidence suggests that TIMPs can regulate angiogenesis, cell survival, proliferation and apoptosis independent of their MMP inhibitory activity via interactions with cell adhesion molecules or growth factor receptors (Guedez *et al*, 1998; Airola *et al*, 1999; Slee *et al*, 1999; Liu *et al*, 2003, 2005; Qi *et al*, 2003). TIMP-2 was shown to interact with α3β1 integrin, dissociating protein tyrosine phosphatase (PTP) and the SH2-containing PTP (SHP-1) from the β1 integrin complex, which in turn negatively regulates tyrosine kinase receptor signal transduction pathways critical for cell proliferation and angiogenesis (Slee *et al*, 1999). MMP-independent TIMP-3 inhibition of angiogenesis was also suggested as shown by its interaction with vascular endothelial growth factor (VEGF) receptor-2, blocking the binding of VEGF to its receptor (Qi *et al*, 2003).

TIMP-1 was discovered almost two decades ago as an MMP inhibitor with its identity to the erythroid-potentiating activity protein, a humoral factor that enhances the proliferation of human erythroid progenitors and certain cancer cells (Avalos *et al*, 1988; Nguyen *et al*, 1994). Since then, it has been suggested that TIMP-1 has pleiotropic activities in the regulation of proliferation, cell survival, and differentiation independent of its MMP inhibitory activity. Consistently, recent studies show that administration of TIMP-1 can suppress both intrinsic and extrinsic cell death pathways via an MMP-independent mechanism in lymphocytes and breast epithelial cells (Guedez *et al*, 1998; Airola *et al*, 1999; Liu *et al*, 2003, 2005). However, a cell surface binding protein responsible for mediating TIMP-1 survival signaling has not been identified.

In this study, we identified CD63 as a TIMP-1 interacting protein by yeast two-hybrid screening. CD63 is a member of the tetraspanins, a group of hydrophobic proteins containing four transmembrane α-helices, two extracellular loops, and a short cytoplasmic tail. CD63 is present in late endosomes, lysosomes, secretory vesicles, and in the plasma membrane. At the plasma membrane, the members of the tetraspanin family including CD63 are known to interact with cell adhesion molecules such as integrins, regulating intracellular signal transduction pathways including cell adhesion, motility, and survival (Berditchevski, 2001; Hemler, 2001; Yunta and Lazo, 2003). Here, we demonstrated a CD63-dependent TIMP-1 association with integrin β1 on the cell surface of human breast epithelial MCF10A cells. TIMP-1 maintained the activated conformation of integrin β1 (one of the main tetraspanin-interacting integrins) in a CD63-dependent manner regardless of cell anchorage, resulting in activation of cell survival signaling and inhibition of apoptosis. Within a three-dimensional (3D) matrigel matrix, MCF10A cells formed polarized acinar-like structures that resembled normal breast

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glandular epithelial structure, while TIMP-1 overexpression disrupted breast epithelial cell polarization and inhibited caspase-mediated apoptosis in centrally located cells in a CD63-dependent manner. Taken together, the present study identified CD63 as a cell surface binding partner for TIMP-1, addressing a new paradigm of cell signaling critical for breast epithelial cell polarization and survival via TIMP-1 modulation of a tetraspanin/integrin complex, in addition to extracellular TIMP-1's regulation of ECM turnover and remodeling through MMP inhibition.

Results

To identify TIMP-1 binding proteins, we performed yeast two-hybrid (Y2H) screening of a human placenta cDNA library using the bait plasmid encoding the full-length mature form of the human TIMP-1 protein lacking the signal peptide sequences. DNA sequencing analysis revealed that, out of 1.2×10^7 diploid colony-forming units screened, six positive colonies contained prey plasmids encoding for the CD63 protein, a member of the tetraspanin family of proteins. The purified CD63 prey plasmid was introduced back into yeast cells and tested for interactions with full-length TIMP-1, the N-terminal MMP-inhibitory domain of TIMP-1, the C-terminal domain of TIMP-1, or TIMP-2 bait by Y2H interaction mating assays. As shown in Figure 1A, CD63 interacted with TIMP-1 but not with TIMP-2, although TIMP-1 and TIMP-2 are highly homologous in their protein structures and their MMP inhibitory activities are mostly interchangeable. Importantly, CD63 interacted with the C-terminal, but not with the N-terminal domain of TIMP-1, suggesting TIMP-1 interaction with CD63 is independent of its MMP-inhibitory activity. In addition, incubating MCF10A lysates with biotinylated rTIMPs demonstrated that anti-CD63 antibody co-immunoprecipitated endogenous CD63 with recombinant TIMP-1 (rTIMP-1) but not with rTIMP-2 (Figure 1B). An immuno-complex of CD63 with endogenous TIMP-1 was also detected from lysates of T29 cells, a previously established MCF10A cell clone engineered to overexpress TIMP-1 (Airola *et al*, 1999; Figure 1C). The CD63 protein with 30–70% of its size (30–60 kDa) being heavily glycosylated (Stipp *et al*, 2003) exhibited a diffuse distribution on SDS–PAGE under a nonreducing condition (Figure 1C, left panel). CD63 interaction with TIMP-1 on the cell surface was assessed by confocal microscopic analysis. Immunofluorescence staining of nonpermeabilized parental MCF10A cells with antibodies to CD63 and TIMP-1 showed a punctate co-staining pattern on the cell periphery consistent with cell surface co-localization (Figure 1D).

To evaluate the significance of CD63 for TIMP-1 binding on the cell surface, we established CD63-knockdown MCF10A cells using a vector-based small hairpin RNA (shRNA) strategy. These studies were conducted in MCF10A cells in which the endogenous TIMP-1 expression was previously downregulated by antisense technology to increase the sensitivity of the cells to the exogenous rTIMP-1 (Liu *et al*, 2003). The antisense TIMP-1-MCF10A cells (referred to as AS TIMP-1-MCF10A) were stably transfected with the pSilencer vector containing CD63 target sequences or scrambled sequences. Immunoblot analyses confirmed significant downregulation of CD63 expression in cells receiving specific CD63 shRNA target sequences (Figure 1E). To examine the role of CD63 in mediating the surface binding of TIMP-1, CD63-knockdown and control AS-TIMP-1-MCF10A cells were incubated at 37°C with biotinylated rTIMP-1 protein. After extensive washes, the bound rTIMP-1 was detected by immunoblot analysis of cell lysates. As shown in Figure 1F, TIMP-1 was readily detected in control cells, but far less bound TIMP-1 was detected in CD63-knockdown AS-TIMP-1-MCF10A cells especially at 60 min postincubation, supporting a notion that CD63 is a cell surface interacting protein for exogenous TIMP-1.

At the plasma membrane, the members of the tetraspanin family, including CD63, are known to interact with cell adhesion molecules such as integrins and regulate intracellular signal transduction pathways. We previously showed that TIMP-1 is a potent inhibitor of apoptosis induced by a variety of apoptotic stimuli including growth factor withdrawal, staurosporine, tumor necrosis factor-related apoptosis-inducing ligand and anoikis, and the antiapoptotic activity of TIMP-1 is mediated by activation of the cell survival pathways including focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) pathway (Airola *et al*, 1999; Liu *et al*, 2003, 2005). Here, we asked whether TIMP-1 interacts with CD63/integrin complex, and if so, whether these interactions are critical for TIMP-1 regulation of cell survival. Since integrin β1 is thought to be one of the main tetraspanin-interacting integrins (Berditchevski *et al*, 1995, 1996, 1997), we first examined CD63 interactions with integrin β1. As shown in Figure 2A, anti-CD63 antibodies co-immunoprecipitated integrin β1 and endogenous TIMP-1 from lysates of MCF10A cells, suggesting that these proteins are forming a complex. Using a confocal microscopic analysis of nonpermeabilized cells, we further examined TIMP-1/CD63/integrin β1 interactions on the cell surface (Figure 2B). While a minimal level of endogenous TIMP-1 staining was detected on the cell surface of control AS-TIMP-1-MCF10A cells as expected from TIMP-1 downregulated cells, incubation of rTIMP-1 with these cells resulted in an enhanced signal consistent with binding of TIMP-1 protein to the cell surface (Figure 2B). Importantly, exogenously added rTIMP-1 protein was co-localized with integrin β1 on the cell surface of AS-shCont#5. When CD63 expression is downregulated by shRNA, cell surface localization of endogenous TIMP-1 was further reduced and these CD63-knockdown cells failed to bind exogenously administered rTIMP-1 (Figure 2B). Consequently, CD63 downregulation abrogated the co-localization of TIMP-1 with integrin β1. Taken together, these results demonstrate a critical role for CD63 in mediating TIMP-1 association with the cell surface and TIMP-1 co-localization with integrin β1.

Next, we investigated whether CD63 is required for TIMP-1-mediated ERK activation using exogenous administration of rTIMP-1. As shown in Figure 3A, administration of rTIMP-1 to AS-shCont#5 cells resulted in significant ERK activation in agreement with our previous reports (Liu *et al*, 2003, 2005). In contrast, downregulation of CD63 expression inhibited this effect of rTIMP-1. Consistently, downregulation of CD63 expression in MCF10A cells inhibited the ability of rTIMP-1 to enhance cell survival following serum starvation when compared to control cells (Figure 3B). In accordance, rTIMP-1-mediated inhibition of caspase-3 like activity was reduced in CD63-knockdown cells (Figure 3C). These results strongly suggest that CD63 plays a critical role

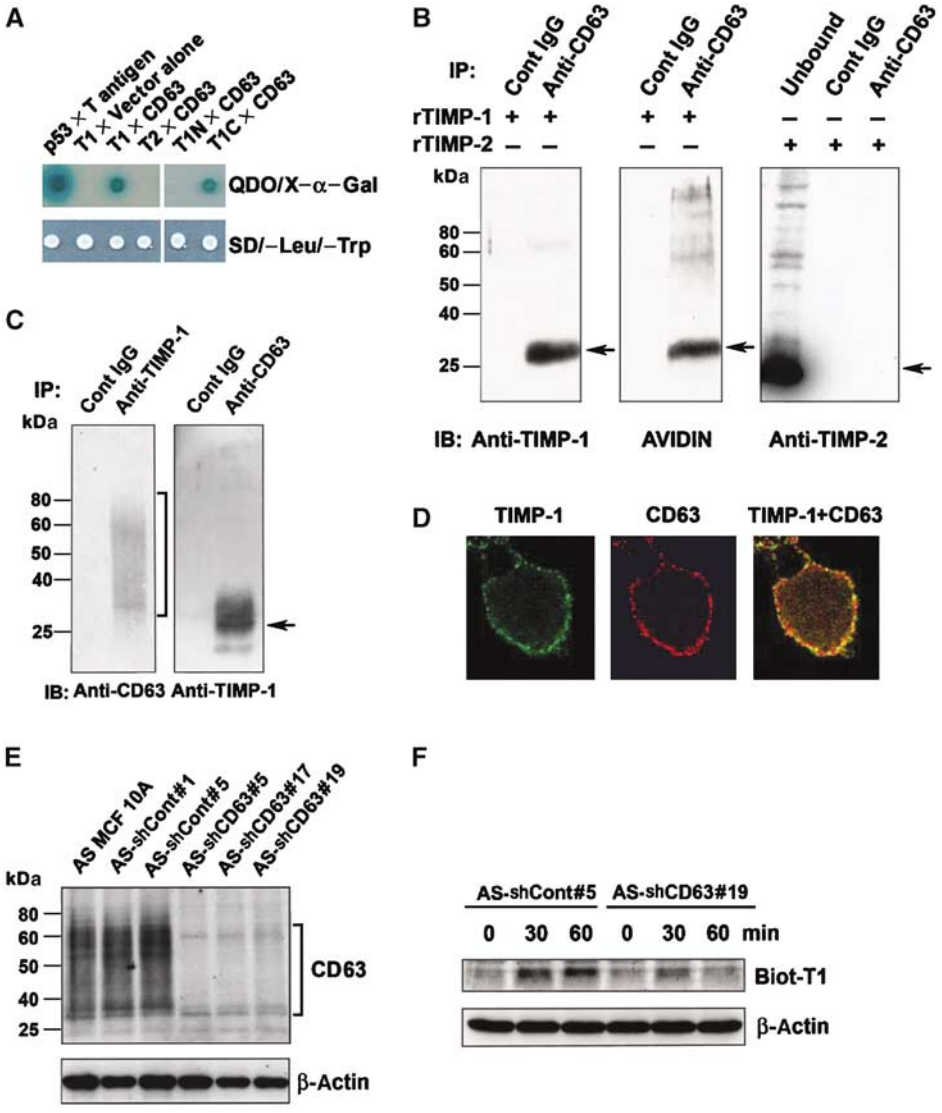

Figure 1 TIMP-1-specific interaction with CD63. (**A**) Yeast AH109 expressing p53 bait (as a positive control), full-length TIMP-1 (amino acids 1–184) lacking signal peptide (T1), full-length TIMP-2 (T2), N-terminal domain (amino acids 1–125) of TIMP-1 (T1N), or C-terminal domain (amino acids 126–184) of TIMP-1 (T1C) was mated with Y187 expressing large T antigen prey (as a positive control), vector alone (as a negative control), or full-length CD63. Diploids grow on SD/−Leu/−Trp selective media. The specific interaction between bait and prey proteins was detected on quadruple dropout (QDO) media SD/−Leu/−Trp/−His/−Ade containing X-α-Gal. (**B**) Anti-CD63 immunoprecipitates of MCF10A cell lysates in the presence of 500 ng/ml biotinylated rTIMP-1 (left and middle panel) or rTIMP-2 (right panel) were analyzed by Western blot analysis with anti-TIMP-1 monoclonal antibody, avidin-HRP, and anti-TIMP-2 polyclonal antibody. (**C**) TIMP-1 overexpressing MCF10A (T29) cell lysates were immunoprecipitated with anti-TIMP-1 polyclonal antibody or anti-CD63 monoclonal antibody, followed by immunoblot analysis using anti-CD63 under nonreducing condition or anti-TIMP-1 antibody under reducing condition, respectively. (**D**) MCF10A cells were grown on the coverslips overnight, blocked with PBS containing 10% horse serum 1% BSA, and co-stained with anti-CD63 Ab/Texas red conjugated secondary Ab (red staining), or with anti-TIMP-1 Ab/FITC conjugated secondary Ab (green staining). Co-localization of TIMP-1 and CD63 is shown as yellow staining (live cell staining). (**E**) Cell lysates of AS MCF10A clones stably transfected with control vector (AS-shCont#1 and AS-shCont#5) or with shRNA vector targeting CD63 (AS-shCD63#5, AS-shCD63#17 and AS-shCD63#19) were subjected to immunoblot analysis with an anti-CD63 antibody in a nonreducing condition. The bottom panel shows the β-actin levels of the same blot reprobed with an anti-human β-actin antibody. (**F**) AS-shCont#5 and AS-shCD63#19 cells were incubated with biotinylated TIMP-1 (500 ng/ml) for 0, 30, and 60 min at 37°C. For the 0 min time point, cells were washed right after the addition of biotinylated TIMP-1. Cells lysates (20 μg/lane) were subjected to immunoblot analysis with avidin-HRP.

in TIMP-1-mediated cell survival signaling and apoptosis inhibition.

To examine whether CD63 is also required for ectopically expressed TIMP-1-mediated cell survival, we downregulated CD63 in TIMP-1 overexpressing MCF10A cells (referred to as T29 cells) using an shRNA approach. Immunoblot analysis confirmed significant downregulation of CD63 expression in the pooled population of T29 cells transfected with CD63 shRNA vector compared to the control vector transfected cells (Figure 4A). The level of CD63 had little effect on TIMP-1 secretion as shown by comparable levels of extracellular TIMP-1 between control and CD63-knockdown T29 cells, while the cellular TIMP-1 levels decreased, presumably due to lack of TIMP-1 association with the cell surface of CD63-knockdown cells compared to the control cells (Figure 4B). Similar to the results obtained with MCF10A cells treated with

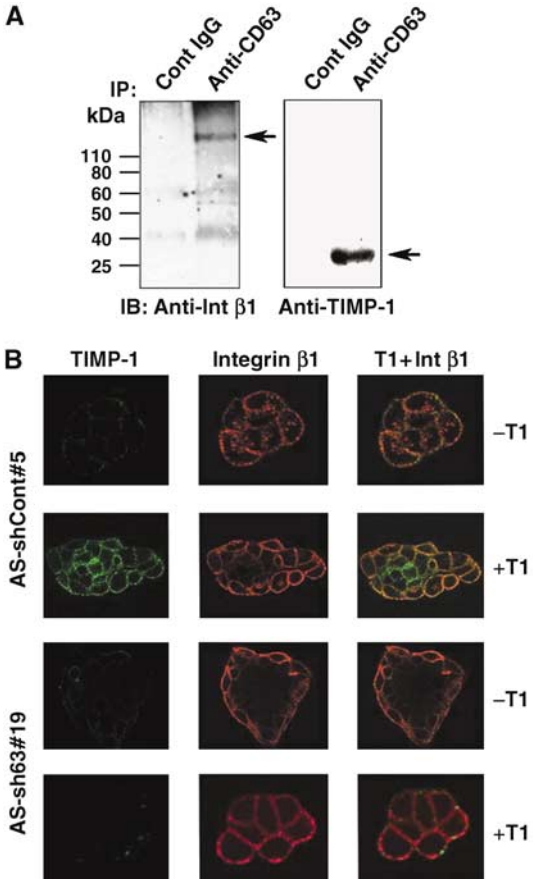

Figure 2 CD63 mediates TIMP-1 binding to cell surface and TIMP-1 co-localization with integrin β1 in human breast epithelial cells. (**A**) MCF10A cell lysates were immunoprecipitated with anti-CD63 antibody, followed by immunoblotting with anti-integrin β1 or anti-TIMP-1 monoclonal antibody. (**B**) AS-shCont#5 and AS-shCD63#19 cells were cultured on the coverslips overnight and incubated with or without 500 ng/ml TIMP-1 for 30 min. Live cells were co-stained with anti-integrin β1 Ab/Texas red conjugated secondary Ab (red staining) and anti-TIMP-1 Ab/FITC conjugated secondary Ab (green staining). Colocalization of TIMP-1 and integrin β1 is shown as yellow staining.

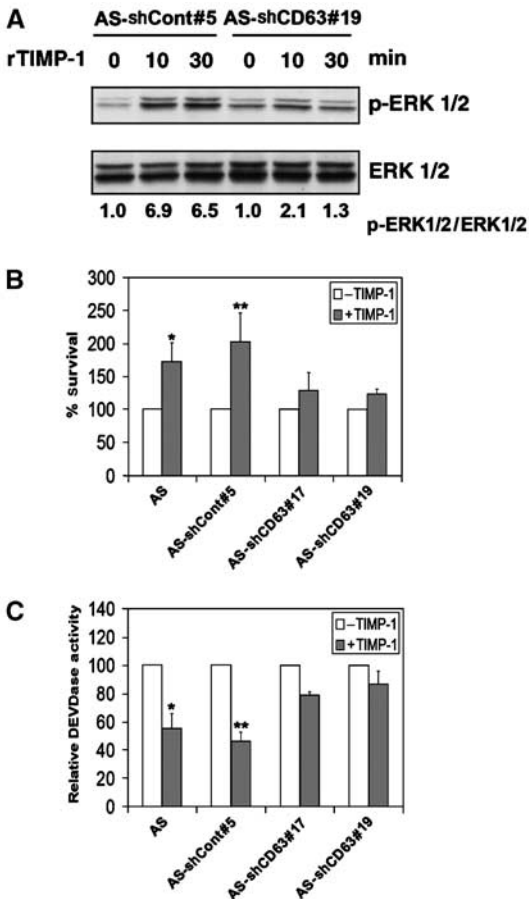

Figure 3 CD63 is required for exogenous TIMP-1-mediated ERKs activation and inhibition of apoptosis. (**A**) AS-shCont#5 and AS-shCD63#19 cells were cultured in serum-free medium for 48 h and then incubated with 500 ng/ml TIMP-1 for 10 and 30 min. Cell lysates (20 μg/lane) were subjected to immunoblot analysis with anti-active ERKs (pERK1/2) and anti-ERK1/2 antibodies. Densitometry analysis of the signals between pERK1/2 and ERK1/2 were presented by normalizing to the signal ratio in AS-shCont#5 or AS-shCD63#19 cells at 0 min. (**B**) AS MCF10A, AS-shCont#5, AS-shCD63#17, and AS-shCD63#19 were cultured in serum-free medium for 48 h in the presence and absence of 500 ng/ml TIMP-1. The percentage of cell survival was determined by WST-1 assay and normalized to the respective cells cultured in serum containing medium. Shown are the means ± s.e. of the sextuple experiments. $^{*}P<0.05$ versus AS MCF10A cells without TIMP-1 treatment (*t*-test); $^{**}P<0.05$ versus AS-shCont#5 cells without TIMP-1 treatment (*t*-test). (**C**) AS MCF10A, AS-shCont#5, AS-shCD63#17, and AS-shCD63#19 were cultured in serum-free medium for 24 h in the presence and absence of 500 ng/ml TIMP-1. The cells were then subjected to DEVDase activity assay. Shown are the means ± s.e. of the sextuple experiments. $^{*}P<0.05$ versus AS cells without TIMP-1 treatment (*t*-test); $^{**}P<0.001$ versus AS-shCont#5 cells without TIMP-1 treatment (*t*-test).

exogenous rTIMP-1 shown above, downregulation of CD63 reduced TIMP-1-mediated constitutive phosphorylation of ERK and FAK (~60 and 50% inhibition, respectively, over control cells) upon growth factor withdrawal (Figure 4C and D). Also, TIMP-1-induced cell survival significantly decreased and caspase-3 like activity was partially recovered in T29 cells with downregulated CD63 expression upon growth factor withdrawal (Figure 4E and F). Similarly, TIMP-1 failed to prevent staurosporine- and anoikis-induced caspase-3 like activity when CD63 expression was downregulated (Figure 4G and H), further demonstrating a critical role for CD63 in TIMP-1 inhibition of apoptosis in human breast epithelial cells.

Our study indicates that TIMP-1 binding to CD63 on the cell surface regulates cell survival signaling pathways likely involving the tetraspanin/integrin complex. Next, we asked whether TIMP-1/CD63/integrin β1 interactions modulate the integrin activity independent of cell adhesion. When the activation states of integrins were examined in suspension culture using antibodies that recognize the activated form of integrins (Ginsberg *et al*, 1990), TIMP-1 levels correlated with the levels of active integrins on the cell surface (compare among Neo, AS, and T29 cells in Figure 5). This is consistent with our previous finding that TIMP-1 constitutively activates FAK-mediated cell survival signaling pathway, independent of cell anchorage (Airola *et al*, 1999; Liu *et al*, 2003, 2005). Importantly, CD63 downregulation abolished TIMP-1-mediated integrin activation in T29 cells (T29-shCD63 in Figure 5).

Integrins regulate diverse cellular processes including cell adhesion, motility, proliferation, and survival (Schwartz and Baron, 1999). In addition, the distribution of cell surface integrins and activation of intracellular signaling is thought

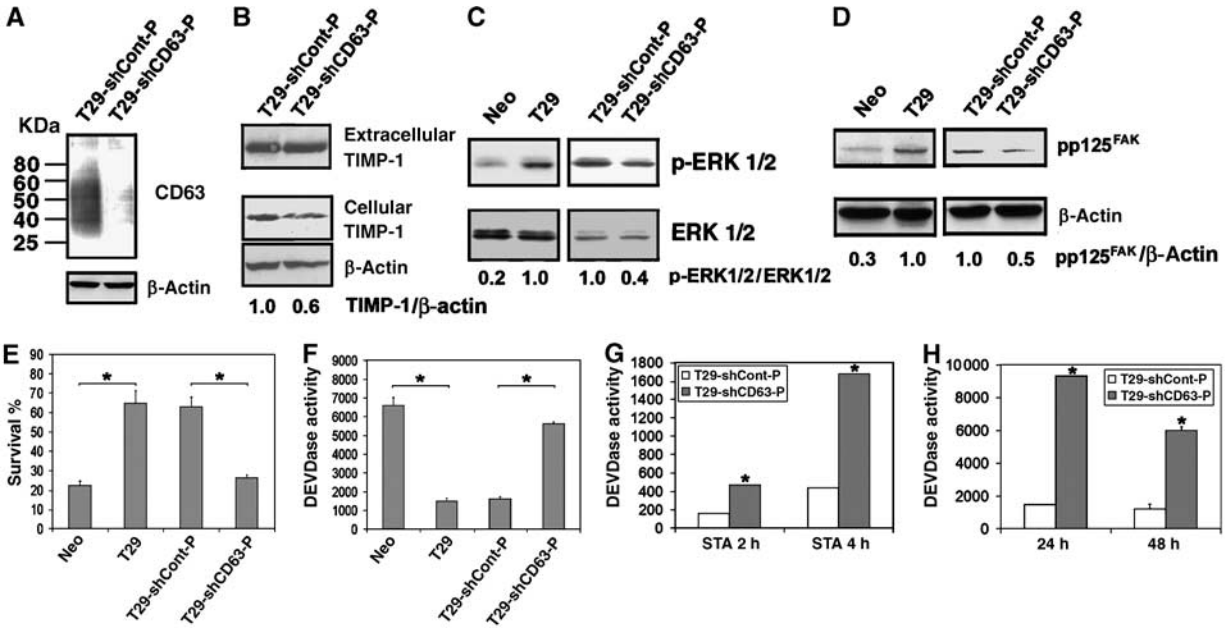

Figure 4 Downregulation of CD63 expression reverses TIMP-1mediated antiapoptotic activity. (**A**) T29-shCont-P and T29-shCD63-P cells were subjected to immunoblot analysis with an anti-CD63 antibody in a nonreducing condition. The bottom panel shows the β-actin levels of the same blot reprobed with an anti-human β-actin antibody. (**B**) Conditioned medium and cell lysates of T29-shCont-P and T29-shCD63-P cells were subjected to immunoblot analysis with an anti-TIMP-1 antibody. The bottom panel shows the β-actin levels of the same blot reprobed with an anti-human β-actin antibody. Densitometry analysis of the signals between cellular TIMP-1 and β-actin was presented by normalizing to the signal ratio in T29-ShCont-P cells. (**C**) Cell lysates (40 μg/lane) of 48 h serum-starved MCF10Aneo (Neo), T29, T29-shCont-P, and T29-shCD63-P cells were subjected to immunoblot analysis with anti-active ERKs (pERK1/2) and anti-ERK1/2 antibodies. Densitometry analysis of the signals between pERK1/2 and ERK1/2 were presented by normalizing to the signal ratio in T29 (left panel) or T29-shCont-P cells (right panel). (**D**) Cell lysates (40 μg/lane) of 48 h serum-starved Neo, T29, T29-shCont-P, and T29-shCD63-P cells were subjected to immunoblot analysis with anti-FAK (pY397) and anti-FAK antibodies. Densitometry analysis of the signals between anti-FAK (pY397) and anti-FAK were presented by normalizing to the signal ratio in T29 (left panel) or T29-shCont-P cells (right panel). (**E**) MCF10Aneo (Neo), T29, T29-shCont-P, and T29-shCD63-P cells were cultured in serum-free medium for 48 h. The percentage of cell survival was determined by WST-1 assay, and normalized to the respective cells cultured in serum containing medium. Three independent experiments were performed and the error bars represent standard deviation of the mean of sextuplicates. Asterisks depict statistically significant differences between Neo and T29 cells and between T29-shCont-P and T29-shCD63-P cells, by an unpaired, unequal *t*-test ($^*P<0.001$). (**F**) MCF10Aneo (Neo), T29, T29-shCont-P, and T29-shCD63-P cells were cultured in serum-free medium for 48 h. The cells were then washed with PBS and lysed with 200 μl caspase lysis buffer as previously described (Liu *et al*, 2003, 2005). After lysates were centrifuged at 16 000 *g* for 10 min, DEVDase activity in 50 μl cytosol was assayed and the activity was normalized per μg protein. Three independent experiments were performed and the error bars represent standard deviation of the mean of sextuplicates. Asterisks depict statistically significant differences between Neo and T29 cells and between T29-shCont-P and T29-shCD63-P cells, by an unpaired, unequal *t*-test ($^*P<0.001$). (**G, H**) Apoptosis was induced in T29-shCont-P and T29-shCD63-P cells by treatment with 0.5 μM staurosporine (G) or by culturing on polyHEMA-coated dishes (H). At indicated time points, the cells were washed with PBS and lysed with 200 μl caspase lysis buffer and DEVDase activity was measured. Three independent experiments were performed and the error bars represent standard deviation of the mean of triplicates. $^*P<0.05$ *versus* T29-shCont-P cells at the respective time points (*t*-test).

to regulate cell polarity and differentiation. In nonmalignant breast epithelium, integrin α6β4 is localized at contact sites with the basement membrane and is associated with establishment of epithelial cell polarity and survival, which is critical for the formation and maintenance of the hollow glandular architecture. When these cells become transformed, their tissue polarity is lost with a greater expression of total and cell surface integrin β1 (Fish and Molitoris, 1994; Reichmann, 1994; Lelievre *et al*, 1996). The significance of integrin β1 and its downstream signaling involving the FAK/PI3-K pathway in this process was demonstrated when treatment with an integrin β1 functional blocking antibody or pharmacological inhibitors of the FAK/PI3-K pathway restored polarization and apoptosis of transformed cells (Lelievre *et al*, 1996; Boulday *et al*, 2004; Xia *et al*, 2004). *In vitro* 3D culture of MCF10A cells has shown that the outer layer of polarized MCF10A cells in contact with the basement membrane survives due to activation of integrin-mediated cell adhesion survival signaling pathways, whereas the centrally located cells undergo apoptotic cell death involving caspase-3 activation (Debnath *et al*, 2002; Mills *et al*, 2004). When the role for TIMP-1 in forming a hollow acini-like structure was examined in 3D culture, TIMP-1 overexpression prevented both polarization and apoptosis of MCF10A cells growing within and consequently inhibited formation of acini-like structure (Figure 6A), in agreement with our previous report (Liu *et al*, 2005). Since CD63 mediates TIMP-1 binding to the cell surface, co-localization with integrin β1 and activation of integrin β1/FAK pathway, we asked whether TIMP-1 interaction with CD63 is responsible for its modulation of MCF10A cell polarization and/or inhibition of apoptosis in a 3D culture. Control shRNA transfected TIMP-1 overexpressing T29 cells failed to form a luminal structure as evidenced by weak and disorganized staining of integrin α6, as well as the absence of active caspase-3 in the center layer (Figure 6B). Interestingly, MCF10A T29 cells with CD63 downregulation by shRNA were capable of generating acini-like structures similar to those formed by the parental MCF10A cells (Figure 6B), as demonstrated by a single outer layer of integrin α6 staining and polarized epithelial

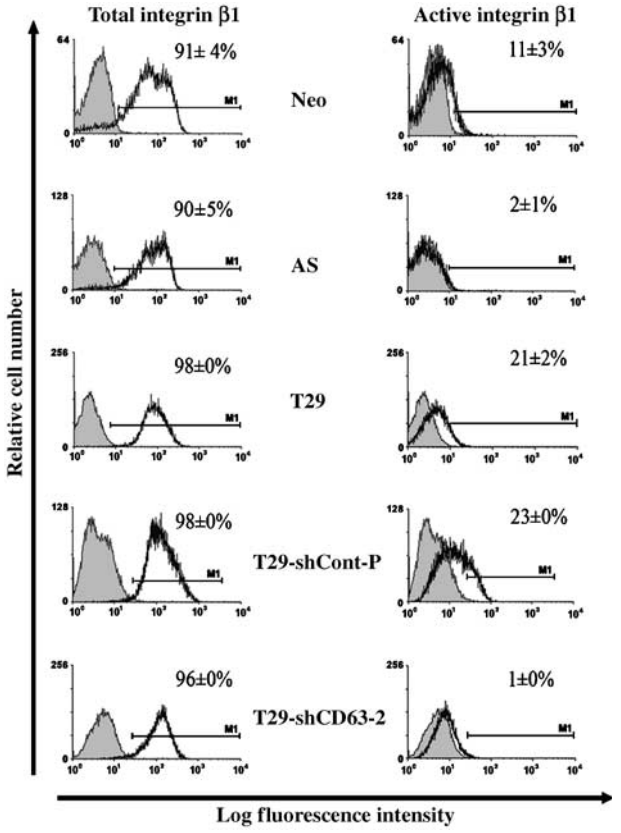

Figure 5 TIMP-1 enhances the level of integrin β1 in activated conformation on the cell surface. MCF10Aneo (Neo), AS TIMP-1 MCF10A (AS), TIMP-1 overexpressing MCF10A (T29), T29-shCont-P and T29-shCD63 cells were plated on polyHEMA coated tissue culture plates for 24 h and stained indirectly with either anti integrin β1 antibody (left panels) or an LIBS antibody recognizing only the active integrin β1 (right panels) followed by incubation with a FITC conjugated secondary antibody. Fluorescence was measured using a FACSCalibur machine. The percentage of gated cells stained for active or total integrin β1 (solid black line) was normalized to the percentage of gated cells stained with the FITC-secondary antibody only (negative control, shaded area). About 20 000 cells per experimental condition were analyzed in duplicates and the means ± s.d. were shown.

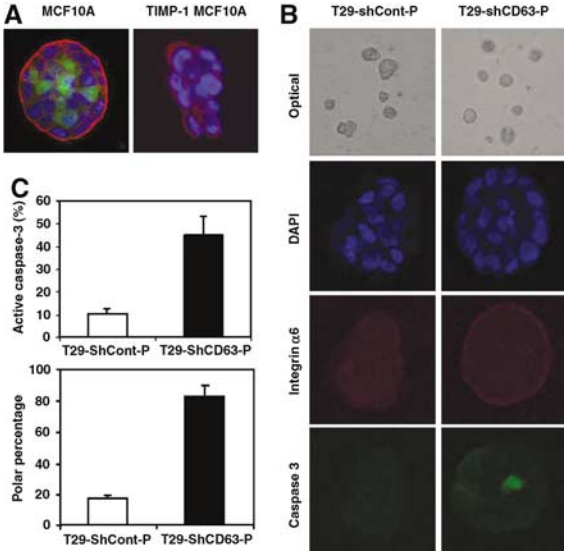

Figure 6 Downregulation of CD63 expression results in phenotypic reversion of TIMP-1 overexpressing MCF10A cells to the parental MCF10A cells in 3D culture. (A, B) MCF10A and TIMP-1 overexpressing MCF10A (T29) cells (**A**) and T29-shCont-P and T29-shCD63-P cells (**B**) were cultured in GFR matrigel as described in the Materials and methods (see Supplemental data). At 8 days, confocal microscopic images of cross-sections through the middle of developing acini are shown. Cells were stained with anti-integrin α6 Ab/Texas red conjugated secondary Ab (red staining) to delineate the basement membrane, anti-active caspase-3 Ab/FITC conjugated secondary Ab (green staining) to detect apoptotic cells, and with DAPI (blue) to counterstain the nuclei. (**C**) Quantitative analysis of the percentage of spheroids including active caspase-3 and polarity are shown in the right panel. (About 30 spheroids were analyzed for each condition from three independent experiments and the means ± s.d. were shown).

cells with apoptotic cells in the center of the acini. These studies show that CD63 downregulation reverses the non-polarization and cell survival induced by TIMP-1 overexpression in breast epithelial MCF10A cells. Approximately half of the T29-shCD63-P acini contained active caspase-3, whereas only ~10% of T29-shCont-P had detectable levels of active caspase-3. Similarly, when cell polarization was quantitated, more than 80% of T29-shCD63-P acini displayed well-organized DAPI staining and polarized distribution of basal integrin α6, while less than 20% of T29-shCont-P acini formed polarized spheroids (Figure 6C). It should be noted that modulation of CD63 expression had little effect on TIMP-1 secretion, as shown by immunoblot analysis of TIMP-1 using conditioned medium collected from T29-shCont-P and T29-shCD63-P cells (Figure 4B). This demonstrates that TIMP-1 regulation of cell survival and polarization of non-malignant breast epithelial cells is mediated, at least in part, by its interaction with CD63 on the cell surface leading to modulation of cell signaling, rather than extracellular MMP inhibitory activity of soluble TIMP-1.

Discussion

The present study identified CD63 as a cell surface binding partner for TIMP-1 in nonmalignant human breast epithelial cells, regulating cell survival, and polarization of the breast epithelial architecture. CD63 is an established component of the late endosomal and lysosomal membranes, also known as lysosomal associated membrane protein (LAMP-3) (Metzelaar and Nieuwenhuis, 1991). It was first identified as a 40-kDa platelet-activating glycoprotein (Pltgp40) (Hildreth *et al*, 1991) and was found to be identical to ME491, an antigen associated with human malignant melanoma (Hotta *et al*, 1988). A recent study reported increased CD63 expression on activated lymphocytes, especially on the cell surface where CD63 was suggested to function as a costimulatory signal to activate T cells and transmit cell survival signals when stimulated by anti-CD63 antibody (Pfistershammer *et al*, 2004). Our finding raises a question as to whether TIMP-1 is a physiological ligand for CD63 in hematopoietic cells for the activation of specific cell lineage, differentiation, and/or cell survival.

TIMP-1 regulates the integrin signaling complex via its interaction with CD63, a member of the tetraspanins, whereas TIMP-2 directly interacts with α3β1 integrin. Tetraspanins, which consist of 28 members in humans, can modulate integrin signaling pathways in both positive and negative manners. CD82 was shown to function as a metastasis suppressor gene involving downregulation of integrin signaling, whereas CD151 enhances the ligand-binding activity of integrins by maintaining their activated

conformation of the integrins (Hemler, 2001; Kovalenko *et al*, 2005; Nishiuchi *et al*, 2005; Tonoli and Barrett, 2005). It remains to be fully investigated as to how CD63 regulates the integrin activity at the molecular level, and how TIMP-1 affects CD63's regulation of integrin signaling in a cell-type specific manner. TIMP-1 interaction with the tetraspanin member CD63 implies potentially diverse effects of TIMP-1 signaling on many cellular processes. Tetraspanins not only interact with integrins but also interact with many Ig superfamily proteins, complement regulatory proteins, growth factors, growth factor receptors, and signaling enzymes (Hemler, 2001). Although the potential significance and complexity of tetraspanin-regulated signal transduction and protein trafficking is well appreciated, their molecular actions are poorly defined at present. The intracellular cytoplasmic tail of CD63 interacts with signaling molecules including phosphatidylinositol 4-kinases (PI4-k) and Src (Berditchevski *et al*, 1997; Yauch and Hemler, 2000; Heijnen *et al*, 2003). Increasing evidence suggests that CD63 regulates FAK, Src, Gab2, PI3K, Akt, and PI4K signaling pathways as shown by modulation of these pathways upon anti-CD63 antibody binding to CD63 in breast carcinoma cells and immune cells (Berditchevski and Odintsova, 1999; Sugiura and Berditchevski, 1999; Pfistershammer *et al*, 2004; Kraft *et al*, 2005). By binding to CD63, TIMP-1 may alter CD63 interactions with integrins within the tetraspanin microdomain and in turn influence integrin signaling. Alternatively, TIMP-1 binding to CD63 may modulate recycling and redistribution of the integrin complex and/or regulate their activity, resulting in changes in signal transduction pathways.

CD63 was shown to interact with integrin β1, but not with the integrin β4 subunit, complexed with its integrin α partner α3, α4, α5, or α6 (Yunta and Lazo, 2003). A TIMP-1/CD63/integrin β1 complex appears to provide constitutively activated survival signaling involving FAK and ERK pathways regardless of cell contacts with the basement membrane, mimicking transformed cell survival signaling and disrupting normal breast epithelial architecture. Interestingly, we noticed that TIMP-1 overexpression reduces the rate of MCF10A cell proliferation, which is associated with a prolonged G1 phase (Taube *et al*, 2006). Thus, TIMP-1-mediated potential 'oncogenic' activity appears to differ from other oncogenes that dysregulate signaling such as ras, cyclin D1, and ErbB2 (Debnath *et al*, 2002, 2003), which also disrupt normal breast epithelial structure, but are accompanied with cell growth stimulation (Boulday *et al*, 2004). The present study provides insights as to how extracellular TIMP-1 regulates intracellular signaling pathways critical for cell survival and polarization through its binding to CD63, a member of the tetraspanin family of proteins. Further elucidation of TIMP-1 actions at the molecular levels warrants continued investigation, especially in the light of the clinical studies that TIMP-1 overexpression is associated with poor prognosis of many human cancers (Kossakowska *et al*, 1991; Zeng *et al*, 1995; Fong *et al*, 1996; Yoshiji *et al*, 1996; Caterina *et al*, 1997; McCarthy *et al*, 1999; Schrohl *et al*, 2004), an unexpected finding considering its MMP inhibitory activity. Since loss of cell polarization and filling of the luminal space in the glandular epithelial structure is one of the hallmarks of breast cancer development/early progression, TIMP-1 regulation of cell signaling in this regard may be of particular importance in understanding breast cancer development and progression.

The functions of TIMP-1 during embryogenesis and branching morphogenesis have been extensively studied using animal models (reviewed in Fata *et al*, 2001). Downregulation of TIMP-1 expression by transgenic antisense expression resulted in excessive ductal branches with increased mammary epithelial cell proliferation. Conversely, slow-release pellet of rTIMP-1 protein lead to inhibition of ductal elongation with decreased mammary epithelial cell proliferation (Fata *et al*, 2001). In contrast, overexpression of TIMP-1 enhanced bifurcation in the salivary gland and increased the number of branches in lung development (Nakanishi *et al*, 1986; Ganser *et al*, 1991). Although branching morphogenesis was not greatly affected in TIMP-1 null mice, TIMP-1 deficiency resulted in changes in estrous cycles, uterine morphology, and systemic steroid concentrations suggesting a role for TIMP-1 in reproductive processes (Nothnick, 2000). These, sometimes conflicting, results of TIMP-1 upregulation or downregulation were mostly interpreted by its regulation of MMP-mediated ECM turnover and MMP-processing of growth factors, growth factor receptors, and cell surface adhesion molecules. In light of our finding, multifunctions of TIMP-1 may need to be re-evaluated in its relation to cellular microenvironment, the levels of MMPs, and availability of TIMP-1's cell surface binding protein CD63.

Materials and methods

Yeast two-hybrid screening

The TIMP-1 cDNA was inserted into pGBKT7 and transformed into the bait strain AH109 (BD Biosciences Clonetech). The correct orientation and in-frame fusion were confirmed by DNA sequencing. Expression of the fusion proteins was confirmed by immunoblot analysis using anti-TIMP-1 and anti-cMyc 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. TIMP-1 bait strain AH109 was mated with the Y187/pGADT7 human placenta cDNA library as instructed by the Clontech protocol. After three rounds of screening of 1.2×10^7 diploid colony-forming units, 88 colonies were grown on SD/−Ade/−His/−Leu/−Trp (QDO) plates and showed positive phenotypes 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 the T7 sequencing primer (5′-TAATACGACTCACTATAGGGC-3′) and 3′AD sequencing primer (5′-CTGTGCATGGTGCACCATCT-3′) to amplify the inserts. Sequencing analysis of the amplified inserts and BLAST search revealed that 13 independent gene products were reproducibly shown positive. Among those, CD63, protein-tyrosine phosphatase and solute carrier protein 2 were TIMP-1 interacting membrane proteins. Protein-tyrosine phosphatase was eliminated for further study, since the insert encoded the cytoplasmic domain.

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.

Establishing CD63-knockdown cell lines

Plasmids carrying shRNA targeted to CD63 were constructed following Ambion's web-based protocol. CD63 ShRNA 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 for 24 h. 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, followed by incubation for 30 min on ice with fluorescein isothiocyanate-conjugated (FITC) donkey anti-mouse IgG secondary antibody (Jackson Laboratories) in Flow PBS (1 × PBS, 2% horse serum, 0.1% sodium azide). Fluorescence was measured using a FACSCalibur machine (Becton Dickinson, San Jose, CA) and the percentage of gated cells (approximately 20 000 cells) stained with active or total integrin β1 (solid black lines) were normalized to the percentage of gated cells stained with FITC- secondary antibody only (shaded area).

See Supplemental data for Materials and methods in detail.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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