

Exosome release of β -catenin: a novel mechanism that antagonizes Wnt signaling

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CD82 and CD9 are tetraspanin membrane proteins that can function as suppressors of tumor metastasis. Expression of CD9 and CD82 in transfected cells strongly suppresses β -catenin-mediated Wnt signaling activity and induces a significant decrease in β -catenin protein levels. Inhibition of Wnt/ β -catenin signaling is independent of glycogen synthase kinase-3 β and of the proteasome- and lysosome-mediated protein degradation pathways. CD82 and CD9 expression induces β -catenin export via exosomes, which is blocked by a sphingomyelinase

inhibitor, GW4869. CD82 fails to induce exosome release of β -catenin in cells that express low levels of E-cadherin. Exosome release from dendritic cells generated from CD9 knockout mice is reduced compared with that from wild-type dendritic cells. These results suggest that CD82 and CD9 down-regulate the Wnt signaling pathway through the exosomal discharge of β -catenin. Thus, exosomal packaging and release of cytosolic proteins can modulate the activity of cellular signaling pathways.

Introduction

The Wnt signaling pathway plays crucial roles both in normal development and in diseases of cell proliferation, including cancer. The Wnt pathway exerts its effects largely by modulating gene transcription (Polakis, 2000; Logan and Nusse, 2004). Cytosolic β -catenin is the principal mediator of canonical Wnt signaling. In the absence of an extracellular Wnt ligand, cytosolic β -catenin is incorporated into a cytosolic protein complex containing Axin, the adenomatous polyposis coli gene product (APC), and glycogen synthase kinase-3 β (GSK-3 β). Axin and APC serve as scaffolding proteins that enable GSK-3 β to phosphorylate β -catenin at residues 33, 37, and 41 (Liu et al., 2002), thereby targeting it for ubiquitination by β -TrCP (β -transducin repeat-containing homologue protein) and subsequent degradation in the proteasome. Cytosolic β -catenin protein levels are thus kept low in the absence of Wnt ligand stimulation. Binding of a Wnt ligand to its coreceptors Frizzled (Fz) and low-density lipoprotein (LDL) receptor-related protein (LRP) 5/6 results in the activation of the Dishevelled (Dvl) protein, which then inhibits GSK-3 β -mediated phosphorylation of β -catenin. Cytosolic β -catenin is thus stabilized and is able to accumulate. This pool of β -catenin translocates to the nucleus, where binding to the T cell factor

(TCF)/lymphoid enhancer factor (LEF) transcription factors results in the activation of target gene expression (Logan and Nusse, 2004). In addition to its role in Wnt signaling, β -catenin is a component of the cadherin-based adherens junction complexes formed at cell–cell adhesion sites. β -Catenin binds the cytoplasmic domain of cadherin and acts as a structural protein by linking cell surface cadherins to the actin cytoskeleton (Daugherty and Gottardi, 2007). By sequestering β -catenin at the membrane, cadherins modulate the signaling properties of cytosolic β -catenin, creating a finely tuned balance between Wnt signaling and cell–cell adhesion (Heasman et al., 1994; Cox et al., 1996; Fagotto et al., 1996).

The tetraspanin transmembrane proteins possess four membrane-spanning domains. Tetraspanins engage in a very wide range of specific molecular interactions that result in the formation in the plane of the membrane of tetraspanin-enriched microdomains (TEM). Tetraspanins have been implicated in a multitude of biological processes, such as cell adhesion, migration, cell fusion, and signal transduction through their associated partner molecules (Hemler, 2005; Levy and Shoham, 2005). Several members of this family influence tumor growth and

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Abbreviations used in this paper: BMDC, bone marrow dendritic cell; ESCRT, endosomal sorting complex required for transport; GSK-3 β , glycogen synthase kinase-3 β ; MVB, multivesicular body.

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progression. Indeed, the tetraspan CD82 was first identified as a tumor suppressor gene. Down-regulation of CD82 expression has been strongly associated with poor prognosis in patients with several types of cancer (Tonoli and Barrett, 2005). In addition, a microarray analysis showed that CD9 expression in tumor cells correlated with down-regulation of several Wnt family genes and their targets, suggesting that CD9 may act as an upstream negative regulator in the Wnt signaling pathway (Huang et al., 2004). Previous immunoelectron microscopic studies have shown that several members of the tetraspanin family, including CD37, CD53, CD63, CD81, and CD82, are enriched in exosomes (Escola et al., 1998). However, the functional role of tetraspanins in exosomes remains unknown.

Exosomes are small membrane vesicles (30–100 nm in diameter) with a density of 1.13 g/ml that are secreted by various cells. Exosomes originate from the inward budding of an endosome's limiting membrane into its lumen, resulting in the formation of a multivesicular body (MVB). The outer membranes of MVBs can fuse with the plasma membrane and release their intraluminal vesicles to the extracellular space as exosomes (Théry et al., 2002; Lakkaraju and Rodriguez-Boulan, 2008; Schorey and Bhatnagar, 2008). Exosome composition varies depending on the cell type of origin. Exosome functions also depend on the cell types from which they are derived and their composition. As mentioned above, tetraspanins are enriched in the membranes of exosomes. In addition, exosomes also contain a number of tetraspan-interacting proteins, such as the major histocompatibility complexes (MHC) class I and II (Escola et al., 1998; Schorey and Bhatnagar, 2008).

In present study, we show that CD82 and CD9 expression profoundly inhibits Wnt signaling. CD82 and CD9 expression reduces the cellular pool of β -catenin by enhancing the exosome-associated export of β -catenin from the cell. The exosome-associated release of β -catenin requires the expression of E-cadherin, and CD82 was coimmunoprecipitated with the E-cadherin- β -catenin complex. Using tissue from CD9 knockout animals, we present data suggesting that tetraspans regulate exosome biogenesis in vivo.

Results

Tetraspanins suppress canonical Wnt/ β -catenin signaling by decreasing levels of β -catenin

To address the molecular mechanisms through which CD82 may regulate Wnt/ β -catenin signaling, we performed a TOP-flash luciferase assay in HEK 293T cells. The data were normalized by cotransfecting with a *Renilla* luciferase-encoding plasmid, which was used to control for variations in transfection efficiency. In this cell line, β -catenin expression led to up-regulation of the canonical Wnt/ β -catenin activity as indicated by increased luciferase activity (Fig. 1 A). When CD82 was cotransfected with β -catenin, we found that the luciferase activity was substantially reduced. We next tested whether CD82 inhibited the canonical Wnt/ β -catenin signaling pathway by regulating the level of β -catenin expression. We found that CD82 expression induced a significant decrease in β -catenin

protein levels in both cytosolic and nuclear fractions in HEK 293T cells (Fig. 1 B).

In addition to CD82, loss of expression of tetraspanin molecules CD9 or CD63 in tumor cells also correlates with poor prognosis and increased metastasis (Higashiyama et al., 1995; Radford et al., 1995; Boucheix et al., 2001). We therefore examined whether CD9 and CD63 also regulate Wnt/ β -catenin signaling. Similar to the behavior of CD82, expression of CD9 suppressed TCF promoter-driven luciferase activity and reduced the expression levels of β -catenin protein in both cytosolic and nuclear fractions (Fig. 1, A and B). However, CD63 did not suppress the luciferase activity stimulated by β -catenin in HEK 293T cells (Fig. 1 A). Consistent with the luciferase assay result, the expression levels of β -catenin were unchanged in CD63-expressing cells (Fig. 1 B). This was not due to low levels of CD63 expression because exogenous CD63 protein was abundantly expressed in these cells (unpublished data). CD9 and CD82 expression also inhibits Wnt/ β -catenin signaling activated by Wnt3a conditioned medium (Fig. S1).

To exclude any influence of variable plasmid transfection efficiencies, we used pNRTIS-21-CD9 and pNRTIS-21-CD82 constructs, in which the expression of the tetraspan is under the control of the Tet-Off promoter (Tenev et al., 2000). The expression levels of CD9 and CD82 were high when the cells were cultured in the absence of doxycycline, but were markedly reduced when cells were cultured in the presence of 100 ng/ml of the drug (Fig. 1, D and F). The Wnt/ β -catenin activity was substantially inhibited in cells cultured in the absence of doxycycline and was partially rescued when cells were treated with doxycycline (Fig. 1, C and E). Consistent with these Wnt/ β -catenin activity measurements, the expression levels of three different forms of β -catenin, including total β -catenin, dephosphorylated β -catenin (anti-ABC), and phosphorylated β -catenin, were almost identical in untransfected cells and in doxycycline-treated CD9- or CD82-expressing cells. These data demonstrate that tetraspan expression alters the size of the total β -catenin population rather than simply affecting its phosphorylation status. The quantity of β -catenin mRNA was not affected by expression of CD9 or CD82 (unpublished data). The inhibition of Wnt/ β -catenin signaling by CD9 and CD82 was further confirmed by immunofluorescence in CHO cells. As shown in Fig. 2 A, β -catenin immunostaining was predominantly localized in the nucleus when cells were cotransfected with either empty vector or with a plasmid expressing CD63. In contrast, upon coexpression with CD9 or CD82, the β -catenin staining was markedly reduced, particularly in nucleus, and manifest instead a punctuate peri-plasma membrane pattern. Collectively, these results suggest that CD9 and CD82 antagonize the canonical Wnt/ β -catenin signaling pathway by promoting a reduction in cellular levels of the β -catenin protein.

Knockdown of endogenous CD9 activates Wnt/ β -catenin signaling

To assess the role of endogenous CD9 expression in suppressing basal levels of Wnt signaling, two shRNA constructs targeting the human CD9 sequence and a scrambled control sequence were used as described in Materials and methods. As shown

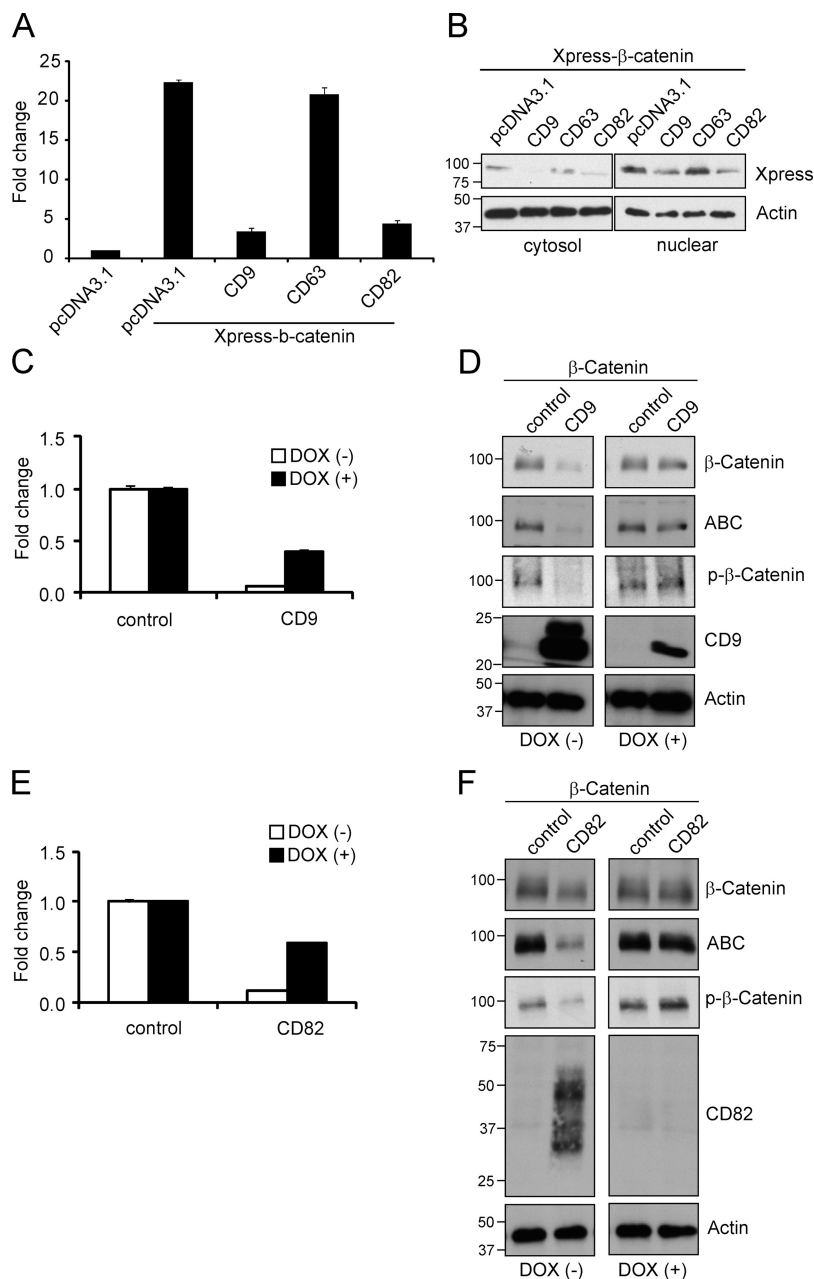


Figure 1. Effects of tetraspanins on TCF/LEF reporter activity and levels of β -catenin. (A) CD9 and CD82 expression inhibits β -catenin signaling. HEK 293T cells were transiently cotransfected with TOPflash, Xpress-tagged β -catenin, the indicated tetraspanins, and the *Renilla* luciferase vector. Activity of the β -catenin signaling pathway was quantified by measuring relative firefly luciferase activity units (RLUs) normalized to *Renilla* luciferase. Data are presented as the fold change as compared with the control condition (cells transfected with pcDNA3.1 empty vector alone). (B) CD9 and CD82 expression reduces cytosolic and nuclear pools of β -catenin. HEK 293T cells were harvested 24 h after transient transfection with the indicated plasmids. Cell lysates were analyzed by Western blotting using anti-Xpress (β -catenin) and anti- β -actin antibodies. (C–F) Inducible expression of tetraspanins inhibits the activity of β -catenin-mediated signaling. HEK 293T cells were transiently cotransfected with a cDNA encoding CD9 or CD82 under the control of a Tet-Off promoter, TOPflash, β -catenin, and *Renilla* luciferase vector. After culture in the presence (DOX (+)) and absence (DOX (-)) of 100 ng/ml of doxycycline for 48 h, cells were harvested for luciferase-based TOPflash assay of Wnt signaling (C and E) and immunoblotted with the indicated antibodies (D and F). Luciferase data are presented as the fold change as compared with the control condition (untransfected cells).

in Fig. 2 B, the expression level of endogenous CD9 was reduced when HeLa cells were transfected with shRNA targeted to CD9. In contrast, scrambled control shRNA did not reduce the expression level of endogenous CD9 (Fig. 2 B). Knockdown of endogenous CD9 resulted in an increase in the level of activated Wnt/ β -catenin activity (Fig. 2 B). These results further confirm the inhibitory effects of native CD9 on endogenous Wnt/ β -catenin signaling.

CD9 and CD82 induce GSK-3 β -, proteasome-, and lysosome-independent antagonism of canonical Wnt signaling

Phosphorylation of β -catenin by GSK-3 β leads to β -TrCP binding, which results in β -catenin degradation. Thus, we next tested whether CD9 and CD82 inhibition of Wnt/ β -catenin signaling involves GSK-3 β . Toward this end, we used two constitutively

active β -catenin mutants, Δ S45 and S33Y, which are insensitive to CK-1 α and GSK-3 β -mediated phosphorylation, respectively, and thus are not substrates for proteasome-induced protein degradation (Liu et al., 2002). Our data indicated that Wnt/ β -catenin signaling stimulated by these stabilized β -catenin proteins was susceptible to inhibition induced by the overexpression of CD9 and CD82 (Fig. 3 A; Fig. S2 A). This repression was associated with a decrease in levels of β -catenin protein in both the cytosolic and nuclear fractions (Fig. 3 B; Fig. S2 B). We also used LiCl and SB216763 to specifically inhibit GSK-3 β activity. LiCl and SB216763 treatment resulted in the activation of the TOPflash luciferase reporter assay and led to the accumulation of β -catenin in HEK 293T cells. However, CD9 and CD82 were still able to inhibit Wnt/ β -catenin activity and to reduce the cellular levels of the β -catenin protein in cells treated with these GSK-3 β inhibitors (Fig. 3 C; Fig. S2, C and D). In contrast,

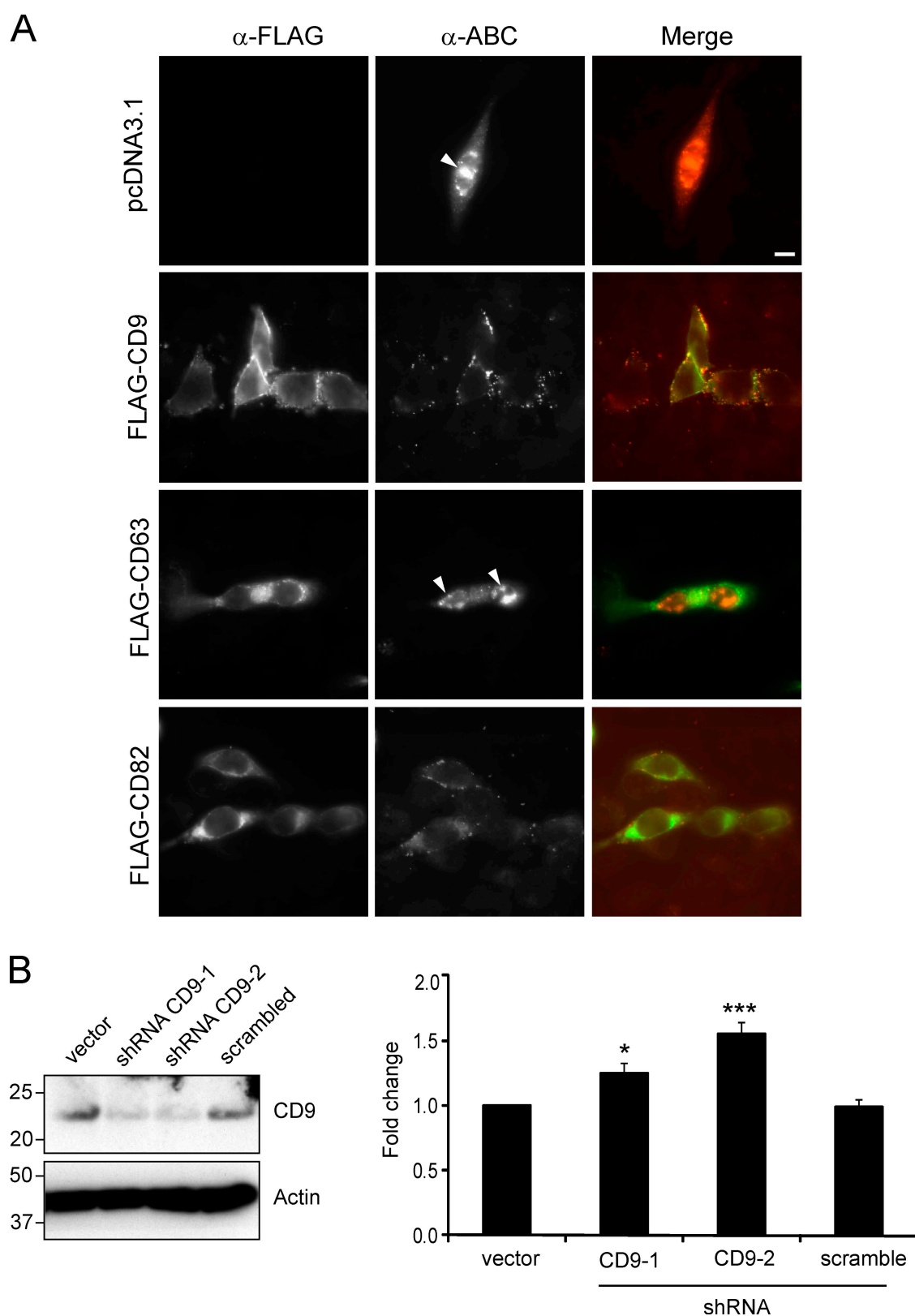


Figure 2. Localization of β -catenin in CHO cells. (A) CHO cells were transfected with the indicated plasmids and immunostained for tetraspanins and β -catenin using an anti-FLAG and anti-phosphorylated β -catenin (anti-ABC), respectively. In control and CD63-transfected cells, β -catenin immunostaining was predominantly localized in the nucleus (arrowhead). Upon coexpression with CD9 and CD82, β -catenin staining was decreased in the nucleus and was associated instead with a punctate plasma membrane pattern. Bar, 10 μ m. (B) Knockdown of CD9 stimulates Wnt/ β -catenin signaling. HeLa cells were cotransfected with shRNA targeted for CD9 sequences or with a scrambled control sequence as described in Materials and methods, as well as with plasmids encoding β -catenin, TOPflash, and *Renilla* luciferase. After 48 h cells were harvested and analyzed by Western blotting using anti-phosphorylated β -catenin (anti-ABC) and anti-CD9. β -Actin was used as a loading control (left). Corresponding lysates were used for luciferase assay (right). Data are shown as the fold change compared with control (cells transfected with empty vector). Data are presented as mean \pm SEM. *, $P < 0.05$; ***, $P < 0.005$ compared with control (t test).

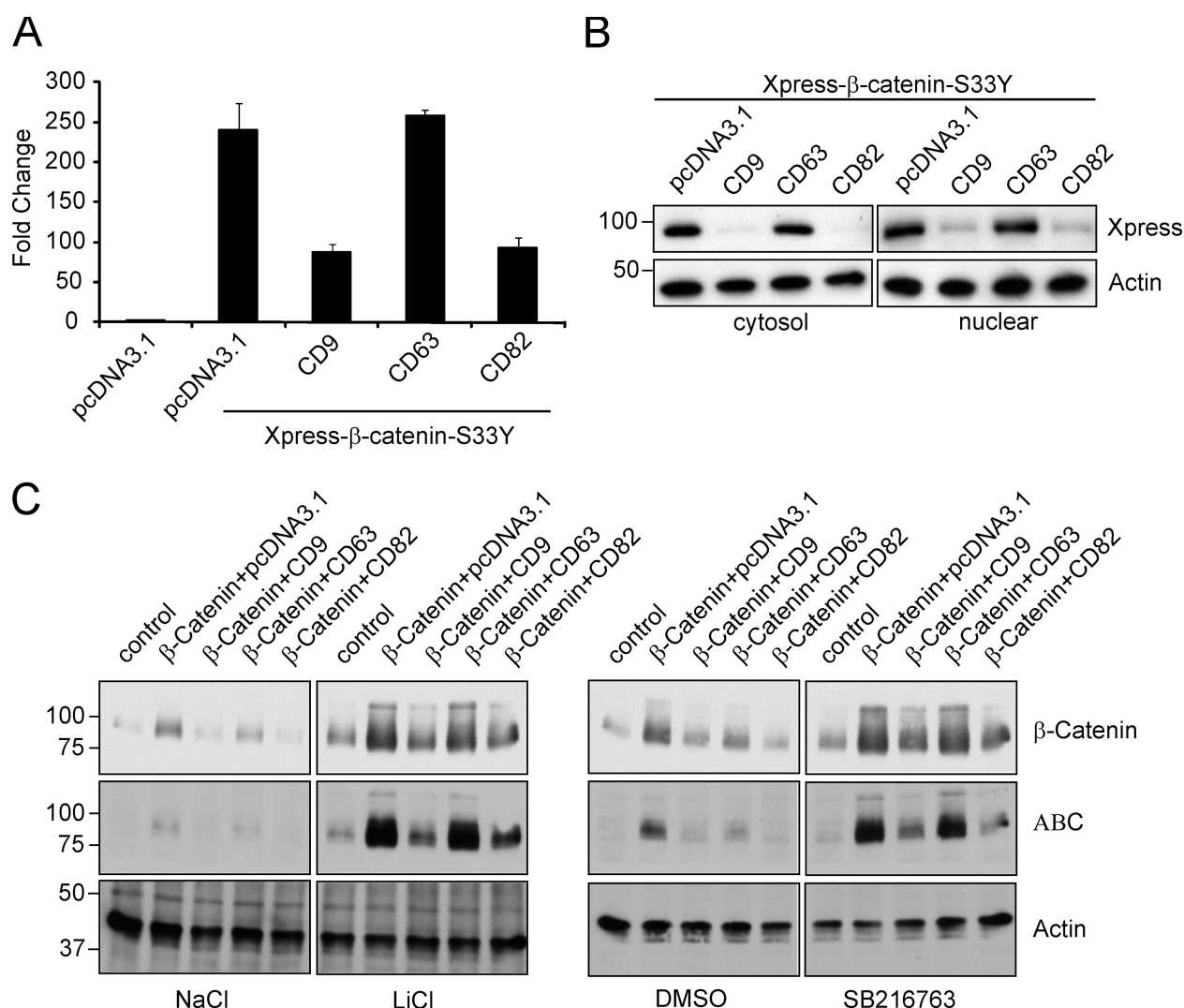


Figure 3. CD9 and CD82 inhibition of Wnt/β-catenin signaling is GSK-3β independent. (A) CD9 and CD82 but not CD63 inhibit TOPflash activity stimulated by mutant S33Y-β-catenin. 24 h after transfection with the indicated plasmids, cells were harvested for TOPflash luciferase assay. Data are presented as the fold change as compared with the control condition (cells transfected with pcDNA3.1 empty vector alone). (B) CD9 and CD82 expression reduced cytosolic and nuclear pools of the mutant S33Y-β-catenin protein. Cell fractions prepared from HEK 293T cells transfected with the indicated plasmids were immunoblotted using anti-Xpress (β-catenin) and anti-β-actin antibodies. (C) Effects of GSK-3β inhibitors on β-catenin levels. 12 h after transfection with the indicated plasmids, HEK 293T cells were treated with 50 mM NaCl, 50 mM LiCl, 20 μM SB216763, or DMSO (control) for 12 h. Lysates were blotted with anti-β-catenin and anti-phosphorylated β-catenin (anti-ABC). β-Actin was used as a loading control.

CD63 did not reduce either the Wnt/β-catenin activity or the level of β-catenin protein under these circumstances.

The data presented in Fig. 3 demonstrate that inhibitory effects of CD9 and CD82 on Wnt/β-catenin signaling do not involve the activity of GSK-3β. We thus wished to determine whether CD9- and CD82-mediated destabilization of β-catenin proceeds through an alternative GSK-3β-independent proteasome-associated protein degradation pathway and, as such, would be relieved in the presence of the proteasome inhibitor *clasto-lactacystin*. As shown in Fig. 4 A, HEK 293T cells were cotransfected with β-catenin and the indicated plasmids and then treated with 10 μM *clasto-lactacystin* for 16 h. Treatment with the proteasome inhibitor increased the basal levels of β-catenin. However, CD9 and CD82 markedly suppressed this level of β-catenin expression. We next wondered whether CD9 and/or CD82 would mediate β-catenin degradation through a pathway involving the

lysosome. We thus treated cells with the lysosome inhibitor bafilomycin A (250 nM) for 16 h. The reductions in the levels of β-catenin present in the cells expressing CD9 and CD82 were not reversed after treatment with this lysosome acidification inhibitor (Fig. 4 B). These results suggest that the degradation of β-catenin was not mediated through the lysosome-associated protein degradation pathway. Collectively, these results suggest that CD9 and CD82 inhibit the canonical Wnt/β-catenin signaling and reduce cellular β-catenin levels through a mechanism that is independent of GSK-3β and of either the proteasome- or lysosome-mediated protein degradation pathways.

Exosomes purified from CD9- and CD82-transfected cells are enriched for β-catenin
Exosomes are small (30–100 nm in diameter) membrane-bound vesicles released by a variety of cell types. As mentioned above,

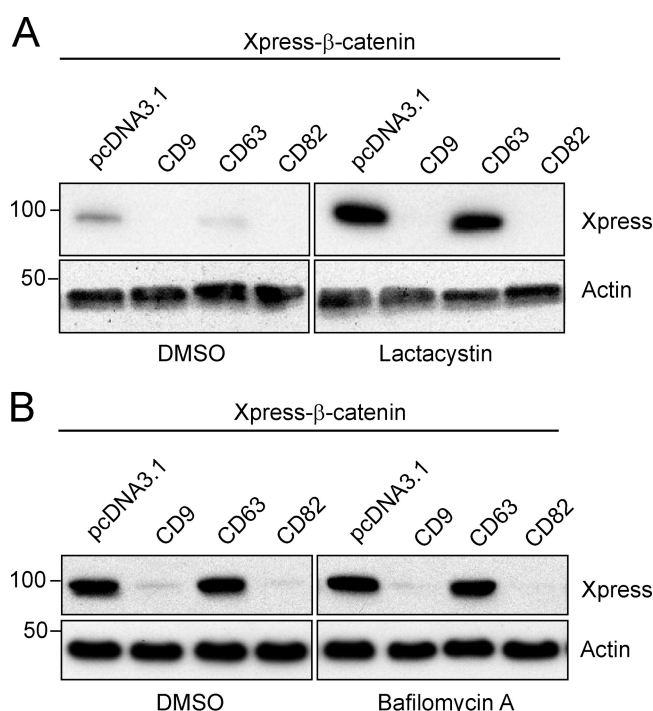


Figure 4. CD9- and CD82-mediated β -catenin destabilization is independent from the proteasome- and lysosome-associated protein degradation pathways. HEK 293T cells cotransfected with the indicated plasmids were treated with 10 μ M *clasto*-lactacystin (A), 250 nM bafilomycin A (B), or DMSO vehicle alone for 16 h before being harvested for Western blotting using anti-Xpress antibody (β -catenin). β -Actin was used as a loading control. CD9 and CD82 were still able to reduce β -catenin protein levels in the presence of both inhibitors.

tetraspanins are enriched in the membranes of exosomes. To test the possibility that tetraspanins reduce cellular levels of β -catenin by facilitating its export via exosomes, a number of experiments were performed. Standard protocols were used to isolate exosomes from media that was bathing cultured cells (Thery et al., 2006) throughout 48 h after their transfection. After several centrifugation steps as outlined in Materials and methods, exosomes were pelleted and analyzed by electron microscopy and Western blotting. Electron microscopy of a negatively stained exosome preparation revealed “cup-shaped” membrane vesicles with diameters of 30–100 nm (Fig. 5 A), which corresponds to the previously characterized ultrastructural morphology of exosomes (Février and Raposo, 2004). Interestingly, vesicles were detected in culture medium harvested from all conditions. However, more exosomes were purified from CD9- and CD82-transfected HEK 293T cells. These results suggest that CD9 and CD82 expression enhances exosome production by these cells. Aliquots of protein extracted from cells (10 μ g) and of protein extracted from pelleted exosomes (2 μ g) were subjected to Western blot analysis using antibodies specific for the known exosomal proteins HSP70 and flotillin-1, or for markers specific to other subcellular compartments, including early endosomes (EEA1), endoplasmic reticulum (calnexin), TGN (γ -adaptin), and a marker of apoptotic cells (cytochrome *c*). Western blot analysis (Fig. 5 B) shows the enrichment of tetraspanins in exosome fractions. In addition, ER, TGN, and early endosomal proteins were not detected in

the exosome fraction, which did contain the two exosomal proteins, HSP70 and flotillin. The exosome fraction also did not contain apoptotic vesicles, because the apoptotic marker cytochrome *c* was not detected in the exosome fractions. These results suggest that the contamination of the exosome fraction with material derived from these compartments or from apoptotic cells is minimal.

Interestingly, the results clearly demonstrate the presence of β -catenin in exosomes isolated from CD9- and CD82-transfected HEK 293T cells, whereas the exosomes isolated from empty vector and CD63-transfected cells contain far lower amounts β -catenin (Fig. 5 B). Approximately 60% of the total cellular β -catenin pool was detected in exosomes isolated from CD9 (57.25 ± 4.25 , $n = 3$)- and CD82 (56.91 ± 5.71 , $n = 3$)-transfected HEK 293T cells. Exosome content in preparations from both the tetraspan-transfected and vector alone cells was confirmed by the presence of flotillin, a lipid raft protein known to be localized in exosomes. To further confirm the roles of CD9 and CD82 in facilitating the transport of β -catenin into exosomes, exosome fractions isolated from HEK 293T cells transiently transfected with cDNAs encoding CD9 and CD82 under the control of a Tet-Off promoter were analyzed using Western blotting. Cells were cultured in the presence (DOX; +) and absence (DOX; –) of 100 ng/ml doxycycline for 48 h. As expected, in the presence of doxycycline CD9 and CD82 were not detected in exosomes, consistent with the very low levels of CD9 and CD82 expression in these cells. In contrast, there was dramatic enrichment of β -catenin in exosomes produced by CD9- and CD82-expressing cells that were grown in the absence of doxycycline (Fig. 5 C). To determine whether the pool of β -catenin that is recovered with the exosome fraction is contained within the lumens of vesicular structures, we treated the exosome fraction with trypsin in the presence or absence of detergent and assessed the quantity of intact β -catenin associated with this material by Western blotting. In the absence of detergent, the exosome-associated β -catenin was fully protected from trypsin degradation, whereas β -catenin was completely degraded when detergent was added (Fig. S3 A). These data demonstrate that the β -catenin protein recovered with the enriched exosome fraction is contained within the lumens of the exosomal vesicles.

Several signaling and trafficking pathways have been shown to influence exosome production. A p53-regulated gene product, TSAP6, has been shown to increase exosome production in cells subjected to DNA damage in a p53-dependent manner (Yu et al., 2006). However, expression of TSAP6 appears to have no effect on CD82-mediated exosome production and secretion (Fig. S3 B). The ESCRT complex (endosomal sorting complex required for transport) can play a role in exosome release (Géminard et al., 2004). Expression of dominant-negative forms of obligate ESCRT components Vps4 and Tsg101, however, did not prevent CD82-induced β -catenin release into exosomes (Fig. S3 C). Purified exosomes are enriched in ceramide. Inhibition of ceramide formation through treatment of cells with the sphingomyelinase (nSMase) inhibitor GW4869 markedly reduces exosome release (Trajkovic et al., 2008). Thus, we tested the effects of the nSMase inhibitor on the exosomal

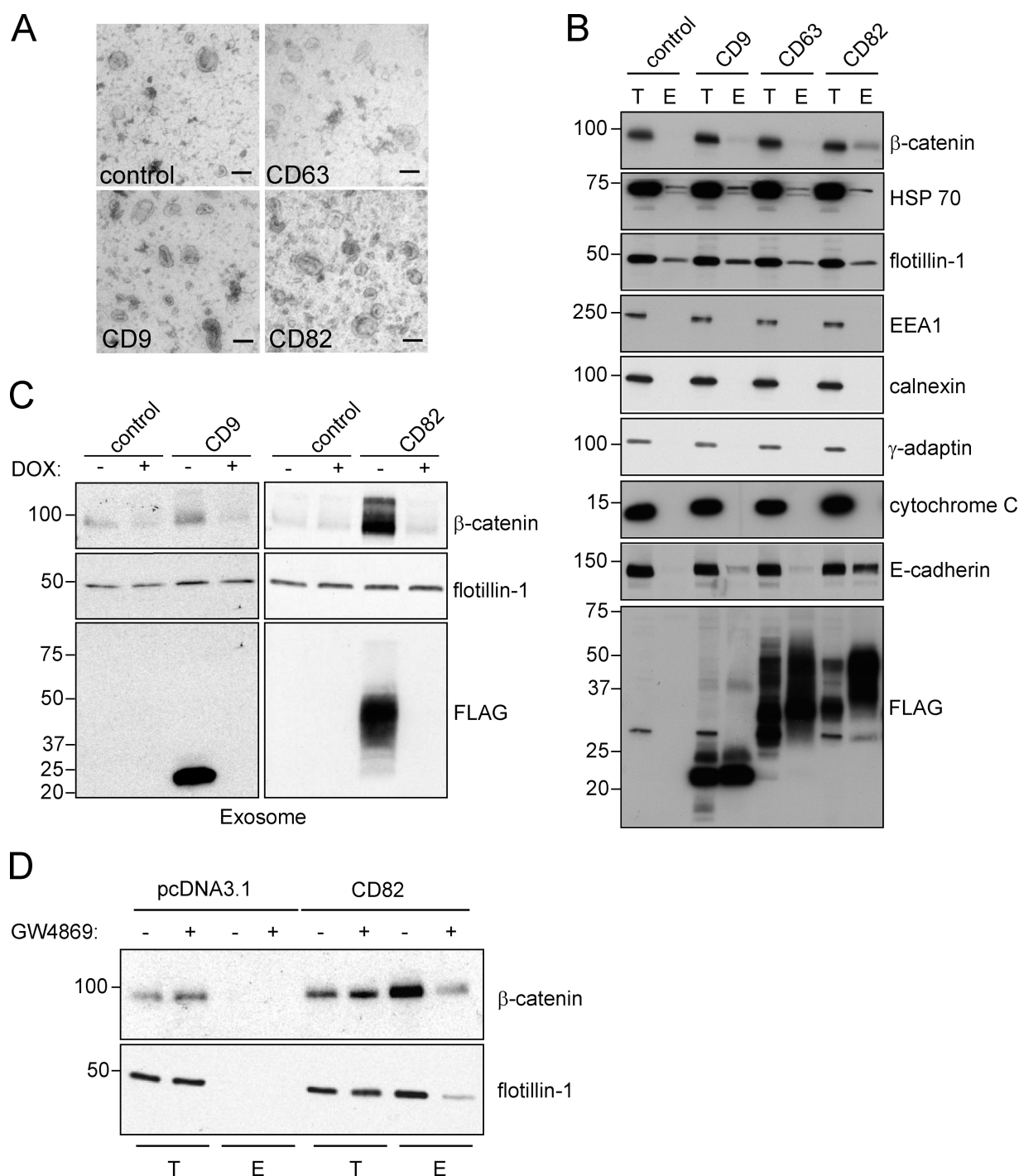


Figure 5. β-catenin is secreted into exosomes. (A) Electron microscopy of extracellular exosomes secreted by HEK 293T cells. Exosomes were isolated by sequential centrifugation steps as described in Materials and Methods from HEK 293T cells transfected with the indicated plasmids. Control cells were transfected with empty vector. Bar, 200 nm. (B) β-Catenin, E-cadherin, and tetraspanins are secreted into exosomes. Total lysate (T) and exosome (E) fractions purified from HEK 293T cells transfected with the indicated plasmids were analyzed by Western blotting using the indicated antibodies. EEA1, calnexin, and γ-adaptin are membrane markers for early endosomes, ER, and TGN, respectively. Cytochrome c was used as a marker for apoptotic cell fragments. (C) Exosome release of β-catenin is dependent on CD9 and CD82. HEK 293T cells were transiently transfected with a cDNA encoding CD9 and CD82 under control of the Tet-Off promoter or empty vector as control. After being cultured in the presence (DOX (+)) and absence (DOX (-)) of 100 ng/ml of doxycycline for 48 h, total lysate (T) and exosome (E) fractions were analyzed by Western blotting using the indicated antibodies. The exosomal protein, flotillin, was used as a loading control. (D) Exosome release of β-catenin is reduced after treatment with a sphingomyelinase (nSMase) inhibitor. HEK 293T cells were transfected with CD82 or with empty vector as control and then treated with 5 μM GW4869 or DMSO for 16 h. Total lysate (T) and exosome (E) fractions were blotted with anti-β-catenin and anti-flotillin-1 antibodies.

export of β-catenin. After treatment with GW4869, total exosome release was reduced, as indicated by the reduction of flotillin recovered in the exosome fraction. Consistent with

the reduction in exosome release, a marked reduction in exosome release of β-catenin was also observed (Fig. 5 D). Collectively, these results indicate that CD9 and CD82 reduce

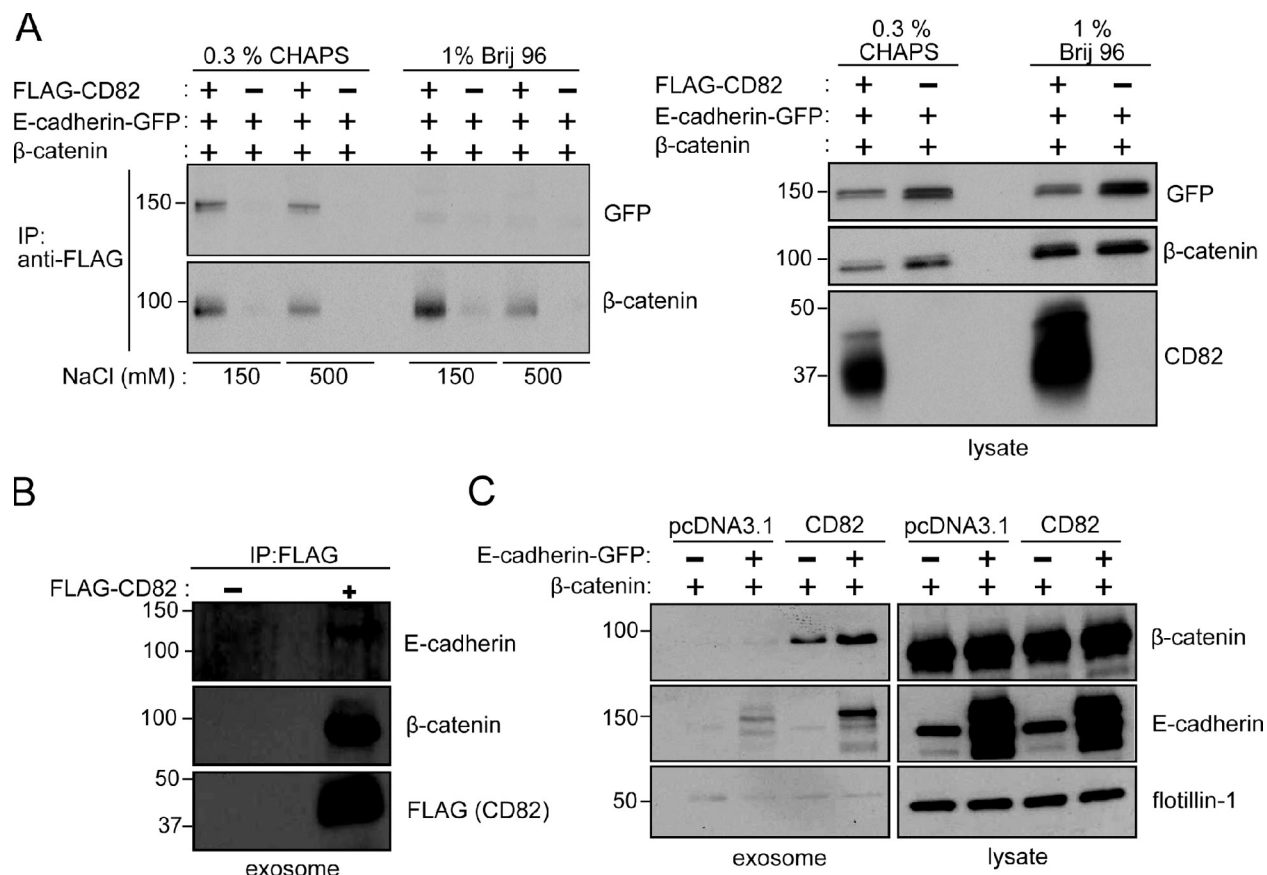


Figure 6. E-cadherin-dependent exosome release of β-catenin. (A) CD82 interacts with the E-cadherin-β-catenin complex. HEK 293T cells stably expressing CD82 or empty vector were transfected with E-cadherin-GFP and β-catenin. 24 h after transfection, cells were lysed with the indicated lysis buffer and immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitated samples were washed with the indicated concentration of NaCl, followed by Western blotting with anti-GFP and anti-β-catenin antibodies (left). Total cell lysates were subjected to Western blotting with the indicated antibodies (right). (B) CD82 interacts with the E-cadherin-β-catenin complex in exosomes. Exosomes were purified from HEK 293T cells stably expressing CD82 or empty vector. Exosome fractions were lysed with 0.3% CHAPS lysis buffer and immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitated samples were washed with lysis buffer, followed by Western blotting with the indicated antibodies. (C) E-cadherin is required for CD82-induced exosome release of β-catenin. A431D cells were cotransfected with the indicated plasmids. After 48 h, total lysate and exosome fractions were purified and analyzed by Western blotting using the indicated antibodies.

the cytoplasmic pool of β-catenin by facilitating this protein's transport into exosomes.

Exosome release of β-catenin requires E-cadherin

As shown in Fig. 5 B, E-cadherin was detected in exosome fractions purified from cells transfected with CD9 and CD82. E-cadherin is a type I, single-pass transmembrane glycoprotein that mediates Ca²⁺-dependent intercellular adhesion. The cytoplasmic region of E-cadherin is linked to the actin cytoskeleton through α- and β-catenin (Daugherty and Gottardi, 2007). A number of CD82- and CD9-interacting proteins have been detected in exosomes (Escola et al., 1998; Schorey and Bhatnagar, 2008). To investigate the possibility of an interaction between tetraspanins and the E-cadherin complex, HEK 293T cells stably expressing CD82 were generated. As shown in Fig. 6 A, E-cadherin and β-catenin could be coimmunoprecipitated with CD82 from lysates of cells dissolved in either 0.3% CHAPS or 1% Brij 96. The total quantity of E-cadherin, β-catenin, and CD82 present in the cell lysate is shown in Fig. 6 A (right). β-Catenin was not detected in immunoprecipitates from control

cells stably transfected with empty vector rather than vector encoding CD82 (Fig. S4 A). In addition, β-catenin was not detected in immunoprecipitates from HEK 293T cells transiently transfected with a vector encoding CD63 (Fig. S4 B). E-cadherin and β-catenin could also be coimmunoprecipitated with CD82 from purified exosomes dissolved in 0.3% CHAPS lysis buffer (Fig. 6 B).

To determine whether E-cadherin is required for the transport of β-catenin into exosomes, we examined the exosome-associated release of β-catenin in A431D cells, in which expression of endogenous E-cadherin is absent (Lewis et al., 1997). Both β-catenin and E-cadherin were readily detected in exosomes purified from culture medium of cells cotransfected with CD82 and E-cadherin (Fig. 6 C). The β-catenin protein was barely detected in exosomes derived from A431D cells cotransfected with β-catenin and either CD82 or E-cadherin alone. The quantity of β-catenin detectable in cell lysates is not different in cells cotransfected with both CD82 and E-cadherin, as compared with that found in cells expressing CD82 alone (Fig. 6 C, lanes 3 and 4). Like A431D cells, CHO cells express little or no E-cadherin (Chausovsky et al., 2000) and similar

results were obtained when CHO cells were induced to express wild-type β -catenin in the presence or absence of E-cadherin (Fig. S5 A). Approximately 70% of the cellular pool of β -catenin was detected in exosomes isolated from E-cadherin and CD82-transfected CHO cells (68.90 ± 3.08 , $n = 4$). E-cadherin-dependent discharge of β -catenin-S33Y from CHO cells was also observed (Fig. S5 B), further confirming that exosome release of β -catenin is independent of any need for GSK-3 β phosphorylation. Collectively, these results indicate that E-cadherin can stabilize cytosolic β -catenin and is required to mediate β -catenin's release into exosomes.

Reduction of exosome release in CD9 knockout mice

To investigate the role of tetraspanins in exosome release in vivo, bone marrow dendritic cells (BMDCs) were generated from wild-type and CD9 knockout mice as described in Materials and methods. BMDC-derived exosomes were purified from culture medium on d 7 and analyzed by Western blotting using anti-flotillin antibody. As shown in Fig. 7 A, BMDCs generated from CD9 knockout mice secreted a smaller quantity of exosomes as compared with BMDCs generated from wild-type mice. Quantitation of transmission electron micrographs of these exosome fractions confirmed these results, demonstrating that the quantity of morphologically recognizable exosomes released from the CD9 null cells is one third of that released from wild-type cells (Fig. S5 C). These data support the concept that tetraspanins play a central role in exosome release.

Discussion

Our work demonstrates a novel role for tetraspanins in inhibiting Wnt/ β -catenin signaling through the reduction of the cellular pool of β -catenin. The reduction of the cellular pool of β -catenin that is mediated by CD9 and CD82 expression occurred through a mechanism that is independent of GSK-3 β and either the proteasome- or lysosome-induced protein degradation pathways. It has been demonstrated that cellular β -catenin levels are regulated by two different mechanisms. In addition to the GSK-3 β -dependent pathway, recent studies suggest that β -catenin levels are regulated by a phosphorylation-independent pathway, which is initiated by an increase in Siah-family protein expression (Liu et al., 2001; Matsuzawa and Reed, 2001). However, we found that the endogenous level of the Siah-1 transcript did not change after expression of CD82 (unpublished data). Nuclear import/export of β -catenin is a crucial step in regulating Wnt signaling (Gordon and Nusse, 2006). We find that CD82 and CD9 expression reduced the level of β -catenin detectable in the nucleus. This is likely due to the reduction of total β -catenin rather than to any effect on the nuclear import/export mechanism. CD82 and CD9 did not affect the level of β -catenin transcripts or the rate of β -catenin synthesis, as assessed by pulse-chase analysis (unpublished data). Instead, CD82 and CD9 reduced the levels of both dephosphorylated and phosphorylated β -catenin, suggesting that CD82 and CD9 act through a novel mechanism rather than through any of the known kinase-dependent mechanisms, to regulate the level of β -catenin.

In the present study we have demonstrated a novel pathway for the cellular export of β -catenin. β -Catenin is released in association with exosomes and this export is enhanced by CD82 and CD9 expression. Exosomes are small (30–100 nm in diameter), membrane-bound vesicles released by variety of cells. Exosomes are now thought to play key roles in cell-to-cell communication, antigen presentation, and in the pathogenesis of retroviral infections and prion diseases. Interestingly, tetraspanin proteins are highly enriched in exosomes (Escola et al., 1998; Théry et al., 2002; Schorey and Bhatnagar, 2008). Consistent with previous studies, our purified exosomes are enriched for all of the tetraspan proteins tested. In addition, although ER, TGN, and early endosomal marker proteins were not detected in purified exosomes, this fraction did contain two well-characterized exosomal proteins, flotillin 1 and HSP70. Furthermore, β -catenin has been identified in exosomes from immature DCs and mature DCs by proteomic analysis (Segura et al., 2005). However, the mechanisms through which tetraspanins influence the production, content, and release of exosomes are largely unknown. The pathways for endosome formation and release may be either ESCRT dependent (de Gassart et al., 2004; Simons and Raposo, 2009) or independent. We found that the expression of dominant-negative forms of the ESCRT components Vps4 and Tsg101 (Sun et al., 1999; Garrus et al., 2001) had no effect on CD82-mediated exosome secretion of β -catenin. A recent study has demonstrated that budding of exosome vesicles into the lumen of the endosome requires the sphingolipid ceramide. The release of exosomes was reduced after the inhibition of ceramide synthesis (Trajkovic et al., 2008). Consistent with these data, the exosome-associated release of β -catenin was reduced after inhibition of neutral sphingomyelinase (nSMase). Thus, our results strongly suggest that CD82 and CD9 stimulate β -catenin secretion via the ceramide-dependent exosomal pathway.

These results raise fundamental questions regarding the mechanism by which β -catenin is targeted to exosomes. CD82- and CD9-interacting proteins, such as the major histocompatibility complex (MHC) class I and II molecules, among others (Escola et al., 1998; Schorey and Bhatnagar, 2008), have been identified in exosomes (Escola et al., 1998). These observations suggest that the trafficking of β -catenin to exosomes may require its direct or indirect interaction with the tetraspanins. Interestingly, E-cadherin was also detected in exosomes in cells that overexpressed either CD82 or CD9. Indeed, cadherin has been identified in immature DC- and mature DC-derived exosomes by proteomic analysis (Segura et al., 2005). β -Catenin binds to the cytoplasmic domain of E-cadherin and links cadherin to the actin cytoskeleton through α -catenin (Daugherty and Gottardi, 2007). It has been shown that E-cadherin can sequester the signaling pool of β -catenin and thus blunt its capacity to participate in the Wnt pathway. Overexpression of cadherin in *Xenopus* embryos reduced the level of β -catenin in the cytoplasm and nucleus by recruiting it to the plasma membrane (Fagotto et al., 1996), demonstrating that cadherin can act as a negative regulator of Wnt signaling. Our data demonstrate that E-cadherin and β -catenin were coimmunoprecipitated with CD82 and that E-cadherin is required in order for CD82 expression to drive the exosomal discharge of β -catenin. These observations suggest an

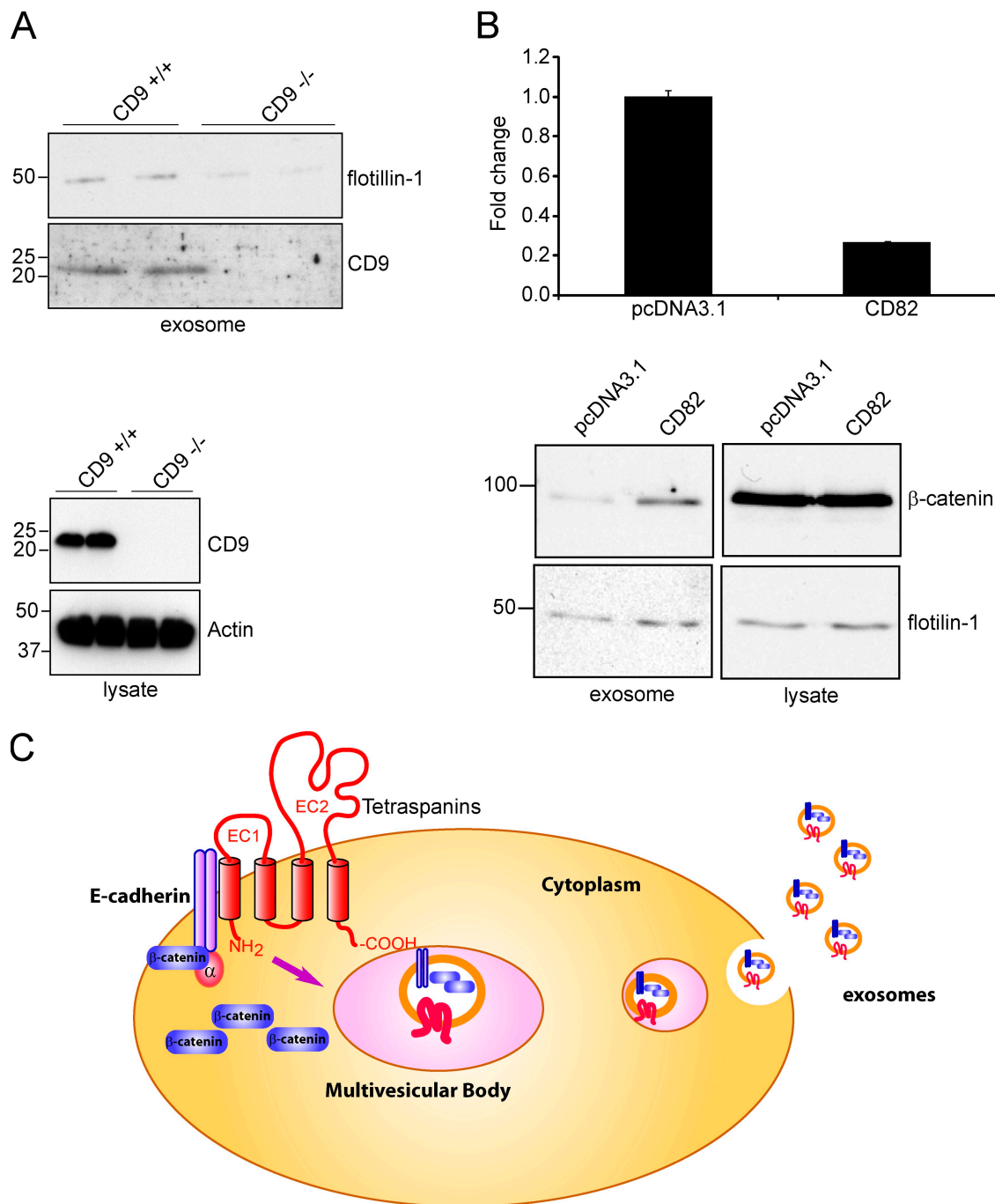


Figure 7. Effects of CD9 on Wnt/ β -catenin signaling and exosome release in vivo. (A) Reduction of BMDC-derived exosomes from CD9 knockout mice. Exosomes were purified from BMDCs derived from CD9 wild-type and knockout mice as described in Materials and methods and analyzed by Western blotting using the indicated antibodies. BMDC lysates were immunoblotted with anti-CD9 as shown in the bottom panel. (B) CD82 expression inhibits β -catenin signaling and facilitates the exosome release of β -catenin in PC3 cells. PC3 cells were transiently cotransfected with the indicated plasmids and with the TOPflash and the *Renilla* luciferase vectors. Activity of the β -catenin signaling pathway was quantified by measuring relative firefly luciferase activity units (RLUs) normalized to *Renilla* luciferase and expressed as fold change in the luciferase signal (top panel). In the bottom panel, Western blots of total lysates and exosome fractions were purified from PC3 cells transiently transfected with indicated plasmids. CD82 expression increased the exosome release of β -catenin by a factor of 3. (C) A novel role for tetraspanins in the regulation of Wnt/ β -catenin signaling. At steady state, tetraspanins are organized in a signaling complex with E-cadherin at the plasma membrane. This signaling complex, including tetraspanins, E-cadherin, and β -catenin, is internalized and delivered to early endosomes. Exosome biogenesis begins with outward vesicle budding at the limiting membrane of endosomes, generating intraluminal vesicles (ILVs). These exosome-containing endosomes eventually mature into late endosomes, also known as multivesicular bodies (MVBs). These MVBs then fuse with plasma membrane and release their intraluminal vesicles, referred to as exosomes, which contain β -catenin. The exosome targeting of β -catenin causes a reduction in the intracellular pool of β -catenin and therefore reduces Wnt/ β -catenin signaling.

entirely new pathway through which E-cadherin can act as a negative regulator of canonical Wnt signaling. It remains to be determined whether and how both the interaction between

tetraspanins and the E-cadherin- β -catenin complex and the consequent targeting of this complex to exosomes are regulated at the molecular level. It should be noted that the relative concentration

of β -catenin and E-cadherin was lower in exosomes produced by CD9-transfected cells as compared with the concentrations of these proteins detected in exosomes prepared from CD82-transfected cells (Fig. 5, B and C). Because the suppression of Wnt signaling induced by CD9 expression is quantitatively similar to that induced by CD82 expression, it is possible that CD9 also inhibits Wnt signaling through additional exosome-independent pathways.

In general, proteins are targeted to multivesicular bodies after they are endocytosed from the plasma membrane (Lakkaraju and Rodriguez-Boulan, 2008). The best-characterized mechanisms for protein sorting to MVBs involves ubiquitination of the target protein and its subsequent oligomerization (Fang et al., 2007; Zuccato et al., 2007; Lakkaraju and Rodriguez-Boulan, 2008). Regulatory phosphorylation has also been demonstrated to drive the sorting of target proteins into multivesicular lysosomes (Zuccato et al., 2007). The E-cadherin complex is endocytosed after tyrosine phosphorylation and ubiquitination (Fujita et al., 2002). Activation of a tyrosine kinase facilitates the interaction of E-cadherin with Hakai, an E3 ubiquitin ligase. Hakai mediates the ubiquitination of the E-cadherin complex and induces its endocytosis. Indeed, we find that CD82 expression induced the tyrosine phosphorylation of E-cadherin (unpublished data). However, we could not detect a consequent increase in the ubiquitination of E-cadherin. This result might indicate that CD82 induces the tyrosine phosphorylation on an E-cadherin tyrosine residue unrelated to the Hakai association.

A growing body of data suggests that interactions between ESCRT machinery and sorting motifs on target proteins can direct proteins into exosomes. A recent study showed that an interaction between the tyrosine-based motif of the transferrin receptor and the ESCRT component Alix induced TfR sorting into exosomes (Géminard et al., 2004). The C-terminal tail of CD82 contains potential tyrosine-based sorting sequences (Berdichevski and Odintsova, 2007). However, mutation of these motifs has no effect on its capacity to initiate exosome targeting of β -catenin (unpublished data). Further experiments will be required to completely understand the precise mechanisms through which exosome sorting is mediated by CD82.

It has been shown that CD9-containing exosome-like vesicles released from egg cells facilitate sperm-egg fusion. However, purified vesicles from CD9^{-/-} eggs impaired sperm-egg fusion (Miyado et al., 2008). We find that dendritic cells isolated from CD9 knockout mice exhibited reduced exosome release. This result suggests the important role that tetraspanins play in exosome biogenesis in vivo. Thus, our results demonstrate a novel mechanism through which the β -catenin pool is regulated by an export pathway. A model depicting this pathway is shown in Fig. 7 C. At steady state, tetraspanins are organized as signaling complexes with E-cadherin at the plasma membrane. This signaling complex, including tetraspanins, E-cadherin, and β -catenin, can be internalized and delivered to early endosomes. Exosome biogenesis begins with outward vesicle budding at the limiting membrane of endosomes, generating intraluminal vesicles (ILVs). This compartment eventually matures into late endosomes, also known as MVBs. These MVBs then fuse with the plasma membrane and release their intraluminal vesicles, referred to as exosomes, which contain β -catenin. The exosome

targeting of β -catenin causes a reduction in the intracellular pool of β -catenin, which therefore reduces Wnt/ β -catenin signaling. Consistent with roles of CD82 and CD9 as suppressors of tumor metastasis, CD82 is frequently down-regulated in advanced stages of cancer, and loss of CD82 has been strongly associated with poor prognosis in several human cancers (Dong et al., 1995; Tonoli and Barrett, 2005). The PC3 line of metastatic prostate cancer cells lack CD82 expression (Sridhar and Miranti, 2006). We found that restoration of CD82 expression in PC3 cells inhibited Wnt/ β -catenin signaling and induced the exosome release of β -catenin (Fig. 7 B). Indeed, CD82 was identified in a genome-wide siRNA screen to be a modulator of the Wnt/ β -catenin signaling pathway (Major et al., 2008). Down-regulation of CD9 also correlates with tumor progression in several types of cancer (Zöller, 2009). In addition, CD9 expression induces down-regulation of several Wnt family genes and their targets, as revealed by microarray analysis (Huang et al., 2004). Our results suggest that CD82 and CD9 might suppress tumor metastasis through down-regulation of the Wnt signaling pathway via a novel mechanism that leads ultimately to the exosomal discharge of β -catenin.

Materials and methods

Plasmids, antibodies, and reagents

The sequences encoding human CD9, CD63, and CD82 were amplified by PCR and inserted by subcloning into pcDNA3.1. FLAG tags were introduced at the N termini of these constructs. Xpress-tagged β -catenin was generated by PCR and cloned into the BamHI and NotI sites of pcDNA4/HisMax-A vector in the correct reading frame. To generate the β -catenin-pcDNA3.1 expression vector, Xpress-tagged β -catenin was digested with BamHI and NotI and cloned into the BamHI and NotI sites of the pcDNA3.1(+) vector. To generate the CD9 and CD82 bicistronic tetracycline-regulated expression vectors, CD9 and CD82 cDNA fragments were amplified by PCR, digested with EcoRI-NotI, and the purified cDNA fragments were ligated into EcoRI-NotI-digested pNRTIS-21 (provided by Frank D. Bohmer, Friedrich Schiller University, Germany). E-cadherin-GFP was a gift from Jennifer L. Stow (The University of Queensland, Australia). All constructs were verified by sequencing. PCR primer sequence and conditions are available upon request. The following antibodies were used: anti-FLAG antibodies from Sigma-Aldrich; anti-GFP from MBL; anti-dephosphorylated β -catenin (anti-ABC) clone 8E7 monoclonal antibody and anti-phosphotyrosine 4G10 platinum from Millipore; β -catenin (H-102) from Santa Cruz Biotechnology, Inc.; anti-HSP70 from Assay Design; anti-phospho- β -catenin (Ser33/37/Thr41) from Cell Signaling Technology; anti-cytochrome c, anti-flotillin-1, anti-E-cadherin clone 36, anti-mouse CD9, anti-adaptin γ , and anti-EEA1 from BD. The GW4869 compound was from Sigma-Aldrich.

Cell culture and transfection

HEK 293T, CHO cells, and PC3 cells were cultured in DME, MEM, Alpha medium, and RPMI 1640 medium supplemented with 10% fetal bovine serum, respectively. For transient transfection, HEK 293T and CHO cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. PC3 cells were transfected with FuGENE HD (Roche).

Exosome purification

Exosomes were isolated according to a procedure previously described, with slight modifications (Thery et al., 2006). Cells were cultured in medium supplemented with 10% depleted FBS (FBS was predepleted of bovine exosomes by ultracentrifugation at 100,000 g for 16 h at 4°C) for 24 h. The culture media was collected on ice and centrifuged at 2,000 g for 10 min to remove dead cells and then centrifuged at 10,000 g for 30 min to pellet any cellular debris. The resulting supernatant was then subjected to one 0.1- μ m filtration step using 0.1 μ m Supor Membrane (Pall Corporation). Exosomes that passed through this filtration step were pelleted by ultracentrifugation at 100,000 g for 70 min at 4°C. The exosome pellet was washed once in a large volume of PBS and resuspended in PBS for further analysis. Protein concentration was determined by a modified Bradford Assay (Bio-Rad Laboratories).

Generation of BMDC-derived exosomes

Bone marrow dendritic cells (BMDCs) were generated from CD9 WT vs. knock-out mice (gift from Ira Mellman, Yale University, New Haven, CT). BM cells were cultured with RPMI 1640 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, and 1% granulocyte/macrophage colony-stimulating factor (GM-CSF). Cells were counted, replated at the same number of cells per condition, and grown in fresh medium with 10% exosome-free FBS obtained by overnight ultracentrifugation (100,000 g) on d 6. DC supernatants were collected on d 7 and exosome purification was performed as described above.

Luciferase reporter assay

HEK 293T cells grown in 24-well culture plates were transiently transfected at 60–70% confluence with 0.2 µg of TOPflash TCF reporter plasmid, 0.02 µg of *Renilla* luciferase reporter (Promega; which was used to evaluate the efficiency of transfection), and various expression plasmids as indicated for individual experiments. The total quantity of DNA in the transfection mixes was adjusted to equal amounts with empty vector. 24 h after transfection, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer according to the manufacturer's specifications. The firefly luciferase activity was normalized to *Renilla* luciferase activity.

Electron microscopy of exosomes

Electron microscopic analysis of exosomes was based on a previously described protocol (Mears et al., 2004). The exosomes purified as described above were fixed by suspension in 2% wt/vol paraformaldehyde in 200 mM phosphate buffer (pH 7.4) and the suspended exosomes were dropped onto a formvar-carbon-coated grid and left to dry at room temperature for 20 min. After washes in PBS, the exosomes were fixed in 1% glutaraldehyde for 5 min. After water washes, the exosome samples were stained with saturated aqueous uranyl oxalate for 5 min. Samples were then embedded in 0.4% wt/vol uranyl acetate, 1.8% wt/vol methylcellulose on ice for 10 min. The excess liquid was removed. The grid was dried at room temperature for 10 min and viewed with an electron microscope (model 910; Carl Zeiss, Inc.) at 20,000x and 50,000x. Images were recorded on film and subsequently scanned into TIFF format. Images were not subjected to any post-acquisition processing.

Western blot analysis and immunoprecipitation

Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na₃VO₄, and complete protease inhibitor tablet) and the indicated detergents. Cell lysates were cleared by centrifugation at 14,000 g for 20 min. Supernatant fractions were used for Western blot or immunoprecipitation. For Western blot analysis, cell lysates and exosome fractions were resolved by SDS-PAGE and probed with the indicated antibodies. For immunoprecipitation analysis, lysates were incubated overnight with the indicated antibody, followed by Western blotting. To detect ubiquitination and tyrosine phosphorylation of E-cadherin, cells were lysed for 20 min in 1% Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 10 mM N-ethylmaleimide, 1 mM PMSF, 1 mM NaF, and complete protease inhibitor tablet). After centrifugation at 14,000 g for 20 min, the supernatants were immunoprecipitated for 1 h with rabbit polyclonal anti-GFP antibody and immobilized protein G (Thermo Fisher Scientific), and the recovered proteins were analyzed by SDS-PAGE followed by Western blotting with indicated antibodies.

Knockdown of CD9 expression using shRNA in HeLa cells

Two shRNAs encoding CD9-specific sequences and one shRNA encoding a control scrambled sequence were generated using the pSUPER.gfp/neo expression vector (OligoEngine) according to the manufacturer's instructions. Oligonucleotides for short hairpin RNA (shRNA) are as follows: CD9 target1 (CD9-1) sense strand, 5'-GATCCCCGCACCAAGTGCATCAAA-TACCTCAAGAGAGGTATTGATGCACCTGGTCTTTTAA-3' and antisense strand, 5'-AGCTTAAAAAGCACCAAGTGCATCAAAATACCTCTCTTGAAG-GTATTGATGCACCTGGTGCAGG-3'; CD9 target2 (CD9-2) sense strand, 5'-GATCCCCACAGGAGTCTATATTCTGATCTTCAAGAGAGATCAGAATA-TAGACTCTGTTTITTTAA-3' and antisense strand, 5'-AGCTTAAAAACAGG-AGTCTATATTCTGATCTTCAAGAGAGATCAGAATATAGACTCTCTGTGGG-3'; Control sense strand, 5'-GATCCCCGGTAAGCATTATCTTCAAGTCTTCA-AGAGAGACTTGAAGATAATGCTTACCTTTTAA-3' and antisense strand, 5'-AGCTTAAAAAGGTAAGCATTATCTTCAAGTCTCTCTTGAAGACTTGAA-GATAATGCTTACCGGG-3'. CD9 target2 and control scrambled sequences have been described previously (Mitsuzuka et al., 2005).

Immunofluorescence microscopy

Transfected CHO cells were grown on glass coverslips, fixed with cold methanol, permeabilized with 0.3% Triton X-100, and then blocked with

goat serum dilution buffer (GSDB; 16% goat serum, 0.3% Triton X-100, 20 mM NaPi, pH 7.4, and 150 mM NaCl). FLAG-tagged tetraspanins were detected using rabbit anti-FLAG antibodies (Sigma-Aldrich) followed by FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich). β-Catenin was detected with anti-dephosphorylated β-catenin (anti-ABC) clone 8E7 monoclonal antibody followed by goat anti-mouse IgG (H+L) Alexa Fluor 594 (Invitrogen), and stained coverslips were mounted in Aquamount (Lerner Laboratories) and visualized at room temperature with a microscope (Axio-phot; Carl Zeiss, Inc.) using a 63x NA 1.4 apochromatic objective. Images were acquired with a digital camera (Axiocam HRm; Carl Zeiss, Inc.) and Axiovision software (Carl Zeiss, Inc.). Images were exported in TIFF format and were not subjected to any post-acquisition processing.

Online supplemental material

The online supplemental material includes five supplemental figures as noted in the text and titled as follows: Fig. S1 shows that expression of CD9 and CD82 inhibits Wnt/β-catenin signaling activated by Wnt3a condition medium. Fig. S2 shows that CD9 and CD82 inhibition of Wnt/β-catenin signaling is GSK-3β independent. Fig. S3 shows that exosome-associated β-catenin resides within the lumens of exosome vesicles, and the TSAP6 and ESCRT pathways are not involved in CD82-induced exosome release of β-catenin. Fig. S4 shows that CD82 interacts with β-catenin. Fig. S5 shows that exosome release of β-catenin is E-cadherin dependent, and disruption of CD9 expression reduces exosome release in vivo. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201002049/DC1>.

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