Cadherin-11 endocytosis through binding to clathrin promotes cadherin-11-mediated migration in prostate cancer cells

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Abstract
Cadherin-11 (Cad11) cell adhesion molecule plays a role in prostate cancer cells migration. Because disassembly of adhesion complexes through endocytosis of adhesion proteins has been shown to play a role in cell migration, we examined whether Cad11 endocytosis plays a role in Cad11-mediated migration. The mechanism by which Cad11 is internalized is unknown. Using GST pulldown assay, we found that clathrin binds to Cad11 but not E-cadherin cytoplasmic domain. Using deletion analysis, we identified a unique sequence motif, VFEEE, in Cad11 membrane proximal region (amino acids 11-15) that binds clathrin. Endocytosis assays using K⁺-depletion buffer showed that Cad11 internalization is clathrin-dependent. Proximity ligation assays showed that Cad11 co-localizes with clathrin. Immunofluorescence showed that Cad11 localizes in vesicles stained for early endosomal marker, Rab5. Deletion of VFEEE sequence from Cad11 cyto domain (Cad11-cle-Δ5) leads to inhibition of Cad11 internalization and reduces Cad11-mediated cell migration in C4-2B and PC3-mm2 prostate cancer cells. These observations suggest that clathrin-mediated internalization of Cad11 regulates surface trafficking of Cad11 and that dynamic turnover of Cad11 regulates the migratory function of Cad11 in prostate cancer cells.

KEY WORDS: Cadherin-11, Clathrin, Endocytosis, Migration, Prostate Cancer
INTRODUCTION
The cadherin family of adhesion molecules play important roles in cell-cell communication and cell sorting during development (Takeichi, 1990). In addition to mediating homophilic adhesion, cadherins have been shown to mediate cell polarity, junctional formation, and cell migration (Gumbiner, 1996). The dynamic regulation of the surface levels of cadherin proteins is critical for the coordination of these complex cellular functions.

Cadherins constitute a family of proteins (Hulpiau and van Roy, 2009). Different cadherins are expressed at various stages of development to mediate specific cellular activity (Angst et al., 2001). E-cadherin (E-Cad) is mainly expressed in epithelial cells and plays a role in maintaining cellular contact in polarized epithelial cells (Nelson et al., 2013). Cadherin-11 (Cad11) (also known as osteoblast cadherin), on the other hand, is a mesenchymal cadherin mainly expressed in osteoblasts (Cheng et al., 1998; Marie et al., 2014; Okazaki et al., 1994).

Aberrant expression of cell adhesion molecules occurs in cancer progression. While E-Cad, which plays a role in maintain cell polarity (Nelson et al., 2013), was found to be down-regulated in cancers, aberrant over-expression of mesenchymal cadherins, including N-cadherin (N-Cad) and Cad11 (Kosalkova et al., 2015; Padmanabhan and Taneyhill, 2015; Tamura et al., 2008), were observed in some cancers. Such a cadherin switch has been shown to occur during the progression of prostate (Chu et al., 2008; Huang et al., 2010; Lee et al., 2010), breast (Tamura et al., 2008) and pancreatic (Martinez-Contreras et al., 2015; Xiumin et al., 2015) cancers. We found that Cad11 expression in prostate cancer (PCa) plays a role in the metastasis to bone in part through increased adhesion of PCa cells with osteoblasts (Chu et al., 2008; Huang et al., 2010; Lee et al., 2013). A similar role of Cad11 was observed in breast cancer bone metastasis (Tamura et al., 2008). We also found that expression of Cad11 increased the migration and invasion of PCa cells (Huang et al., 2010). Interestingly, Cad11 expression was upregulated in castration-resistant PCa (Lee et al., 2010), which frequently leads to PCa metastasis in bone. These observations suggest that Cad11 may also be involved in PCa bone metastasis through increasing cell migration. Together, these studies suggest that Cad11 plays important roles during both normal development and pathological conditions.

In migrating cells, the assembly and disassembly of adhesion complex allow the cell to migrate (Broussard et al., 2008; Webb et al., 2002). Endocytosis of adhesion proteins has been shown to play a role in the disassembly of adhesion complex (Alarcos et al., 2015; Bockus et al., 2015; Jin et al., 2014; Kowalczyk and Nanes, 2012; Mosesson et al., 2008; Xiao et al., 2005). The mechanism by which Cad11 is internalized is unknown. In this study,
we identified proteins that interact with the cytoplasmic domain of Cad11 and found that clathrin was one of the Cad11 interacting proteins. We found that clathrin binds to the cytoplasmic domain of Cad11 but not that of E-Cad, suggesting differences in the regulatory mechanisms between these two cadherins. Our studies showed that clathrin plays a role in Cad11 endocytosis and that the dynamic turnover of Cad11 promotes Cad11-mediated migration in PCa cells.
RESULTS

Clathrin binds to the cytoplasmic domain of Cad11 but not E-Cad

To search for proteins that interact with the cytoplasmic (cyto) domain of Cad11, we used the GST fusion protein containing Cad11-cyto domain in a GST pulldown assay. We constructed GST-Cad11-cyto containing two copies of cyto domain in tandem to increase the likelihood of binding with interacting proteins (Fig. 1A, upper panel). To avoid the disruption of protein conformation of the individual cyto domain, four glycine residues were incorporated as a spacer in between the two copies of cyto domain (Fig. 1A, upper panel). Recombinant GST-Cad11-cyto protein was used to pull down proteins from the cytosol prepared from L-cells. Silver staining of SDS-PAGE gel revealed a prominent band at ~180 kDa in the GST-Cad11-cyto but not GST alone samples (Fig. 1A, lower left panel, lanes 4 vs 2). This protein was excised from the gel and identified to be clathrin by mass spectrometry. The low molecular weight proteins in the GST-Cad11-cyto pull down were found to be degradation products of GST-Cad11-cyto (data not shown). Immunoblotting using an anti-clathrin antibody further confirmed that the 180 kDa protein is clathrin (Fig 1A, right panel). These results suggest that clathrin is one of the proteins that interact with the cytoplasmic domain of Cad11. Next, we examined whether clathrin also binds to the cytoplasmic domain of E-Cad. The cytoplasmic domain of E-Cad was expressed as a GST-fusion protein GST-E-Cad-cyto using strategy similar to that of GST-Cad11-cyto (Fig. 1B, left panel). GST-E-Cad-cyto did not pull down clathrin from L-cell cytosol while GST-Cad11-cyto did (Fig. 1B, right panel). Immunoblotting using antibody against the cytoplasmic domain of E-Cad or Cad11 confirmed the specificity of the GST-E-Cad-cyto and GST-Cad11-cyto constructs used in the pulldown assays (Fig. 1B, right panel). These observations suggest that clathrin binds to the cyto domain of Cad11 but not E-Cad under our experimental condition. Similar results were obtained by using cell lysates prepared from C4-2B4 cells (Fig.1C), a cell line derived from LNCaP prostate cancer cell line (Thalmann et al., 2000).

Clathrin binds to the juxtamembrane region of Cad11

To map the clathrin binding site on Cad11, we generated Cad11 cyto-domain mutants that contained deletions in either the juxtamembrane domain (JMD) (GST-Cad11-ΔJMD) or the β-catenin binding subdomain (CBS) (GST-Cad11-ΔCBS) (Fig. 2A, upper panel), and used them in pulldown assays. We found that clathrin bound to Cad11-cyto and Cad11-ΔCBS but not to Cad11-ΔJMD and E-Cad-cyto, suggesting that the clathrin binding site is located...
within the JMD domain (Fig. 2A, lower right panel). It has previously been reported that p120-catenin binds to the JMD domain while β-catenin binds to the CBS domain of cadherins (Nanes et al., 2012). As expected, p120-catenin was found to bind to the cyto domains of both E-Cad and Cad11 and also Cad11-ΔCBS, but not ΔJMD (Fig. 2A, lower right panel). β-catenin was found to bind to the cyto domains of E-Cad and Cad11 and also Cad11-ΔJMD, but not Cad11-ΔCBS (Fig. 2A, lower right panel). These results show that the clathrin binding site is located within the JMD domain.

To further identify the clathrin binding sequence within the JMD domain, we first generated GST fusion proteins containing N-terminal 51, 40, 35, and 30 amino acids of the Cad11 cyto domain (Fig. 2B, upper panel). Clathrin was found to bind to all four deletion mutants, while p120-catenin only bound to the deletion mutant containing the first 51aa (Fig. 2B, lower left panel). These observations suggest that the sequence between 40-51 amino acids of Cad11 contains a motif that is critical for the binding of p120-catenin. This observation is consistent with previous report by Thoreson et al. (Thoreson et al., 2000) showing that p120-catenin binding to aa 756-774 of E-Cad cytoplasmic domain (Fig. 2B, upper panel, thick black lines). We further generated GST fusion proteins containing N-terminal 25, 20, 15, and 10 amino acids of the Cad11 cyto domain (Fig. 2B, upper panel). We found that clathrin bound to all of these proteins except GST-Cad11-10aa (Fig. 2B, lower right panel). The amino acid sequence between Cad11-10aa and Cad11-15aa is VFEEE. Interestingly, this sequence is similar to the clathrin-binding box (LxEx(D/E)) located at the C-terminal tail of arrestin (Krupnick et al., 1997; Macedo et al., 2014). Sequence alignment showed that the VFEEE sequence is unique to Cad11 and is absent in the corresponding region of E-Cad (Fig. 2C), consistent with the lack of clathrin binding to E-Cad cyto domain (Fig. 1B). Sequence alignment of the cadherin cyto domains showed that the LxEx(D/E) motif may also present in P-Cad and N-Cad, but whether clathrin binds to the cytoplasmic domain of P-Cad or N-Cad has not been determined.

**Cad11 is internalized through a clathrin-dependent pathway in C4-2B cells**

Clathrin is a major component of coated vesicles, which are known to play a role in endocytosis of membrane proteins. To examine whether clathrin is involved in Cad11 endocytosis, we employed C4-2B PCa cells that expressed low/undetectable endogenous Cad11. Cells were transfected with Cad11-WT and Cad11 internalization was analyzed by endocytosis assays. We found that at 4°C, mouse anti-Cad11 antibody (1A5) bound to Cad11
on the cell surface (Fig. 3A). When the temperature was shifted to 37°C to allow for endocytosis, the internalized Cad11-1A5 antibody complexes were detected as green dots in the cytosol (Fig. 3A). We next used a mild acid wash to remove antibodies associated with surface proteins but not those associated with internalized proteins. The loss of surface-bound Cad11 antibodies but not internalized Cad11 antibodies after acid wash confirmed Cad11 internalization at 37°C but not at 4°C (Fig. 3A). Potassium (K⁺) depletion has been shown to prevent the assembly of clathrin into coated pits at the plasma membrane (Salazar and Gonzalez, 2002; Wang et al., 1993; Xiao et al., 2005). To test whether Cad11 internalization is mediated by clathrin, we conducted endocytosis assays under K⁺-deprivation condition. As shown in Fig. 3A, under 37°C assay condition, depletion of intracellular K⁺ led to a decrease in the number of internalized Cad11 (green dots) by 70%. These observations suggest that Cad11 internalization is mediated through clathrin-dependent endocytosis.

The endocytic pathway is composed of a series of dynamic stages, including clathrin-coated vesicles to early endosomes, then late endosomes/multivesicular bodies, and finally lysosomes for degradation (Vanlandingham and Ceresa, 2009). We conducted immunofluorescence staining in C4-2B-Cad11 cells to determine whether internalized Cad11 co-localizes with clathrin in early endosomes or late endosomes. Immunofluorescence staining showed that internalized Cad11 was co-localized in some vesicles stained positive for Rab5, an early endosome marker (Nielsen et al., 1999) (Fig. 3B left and supplemental Fig. S1), but not in vesicles stained positive for Rab7 (Fig. 3B, right and supplemental Fig. S1), a late endosome marker (Vanlandingham and Ceresa, 2009). Similar results were found in PC3-mm2 cells (Supplemental Fig. S2). These results indicate that internalized Cad11 was retained in early endosomes, which represent the recycling compartment.

To examine whether interactions between Cad11 and clathrin through the clathrin binding motif VFEEE is involved in Cad11 internalization, we generated a mutant Cad11 that contains a deletion of the VFEEE sequence (Cad11-cla-Δ5) (Fig. 3C) and expressed the Cad11-cla-Δ5 in C4-2B cells (C4-2B-Cad11-cla-Δ5). Western blot showed that C4-2B-Cad11-WT and C4-2B-Cad11-cla-Δ5 cells expressed Cad11 protein at a similar level (Fig. 3C). Endocytosis assays at 37°C showed that more Cad11 was endocytosed in C4-2B-Cad11-WT cells than those in C4-2B-Cad11-cla-Δ5 cells (Fig. 3D). Quantification showed that Cad11 endocytosis was decreased by 40% in C4-2B-Cad11-cla-Δ5 cells compared to that in C4-2B-Cad11-WT cells (Fig. 3D). The C4-2B-vector cells were used as a negative control (Fig. 3D). These observations indicate that Cad11 and clathrin interaction through the clathrin binding motif VFEEE regulates Cad11 endocytosis.
Next, we examined whether Cad11 and clathrin are co-localized in endocytic vesicles. In C4-2B-Cad11-WT cells, internalized Cad11 were found to co-localize with clathrin in endocytic vesicles (Fig. 3E, see insets, and supplemental Fig. S3). Incubation of cells in K$^+$-depletion medium significantly reduced their co-localization in endocytic vesicles (Fig. 3E). In the C4-2B-Cad11-cla-Δ5 cells, only a few clathrin-coated endocytic vesicles were co-stained with Cad11. Incubation of C4-2B-Cad11-cla-Δ5 cells in K$^+$-depletion medium did not further reduce the number of co-stained vesicles (Fig. 3E).

The interaction of clathrin with its substrates is usually transient and of low affinity. As a result, attempts to pull down Cad11 with endogenous clathrin in a co-immunoprecipitation assay were not successful (data not shown). To further demonstrate an interaction between Cad11 and clathrin, we performed proximity ligation-based assays (PLA) (Egeland et al., 2015) on C4-2B-vector, C4-2B-Cad11 or C4-2B-Cad11-cla-Δ5 cells. Cells were first incubated with both mouse anti-Cad11 antibody and goat anti-clathrin antibody and then incubated with anti-mouse and anti-goat antibodies that have a unique short DNA strand attached to each antibody. If the two epitopes on Cad11 and clathrin are sufficiently close, the attached oligonucleotides on the respective antibodies will hybridize or become ligated, producing a template for a rolling circle DNA amplification, which can be probed efficiently with fluorescent oligonucleotide probes. The appearance of discrete fluorescent spots in the immunofluorescent images indicates that Cad11 and clathrin were present in close proximity. We found that the number of fluorescent spots in C4-2B-Cad11 cells was significantly higher than that in C4-2B-vector or C4-2B-Cad11-cla-Δ5 cells (Fig. 3F). These observations further support a direct interaction between Cad11 and clathrin in C4-2B4-Cad11 cells.

Internalization of Cad11 in PC3mm2 cells

We further examined whether clathrin is also involved in Cad11 endocytosis in PC3mm2 cells that endogenously express Cad11. To do so, we first depleted endogenous Cad11 and then reconstituted Cad11-deficient cells with either Cad11 WT or Cad11-cla-Δ5 that contains the deletion of the clathrin binding motif. Cad11 shRNA, which targets the 3’ untranslated region of Cad11 in the pLKO.1 vector, was used to knock down endogenous Cad11 (PC3mm2-shCad11). PC3mm2 transfected with empty vector (PC3mm2-pLKO) was used as a control. PC3mm2-shCad11 cells were then transfected with cDNA encoding Cad11-cla-Δ5 in the pBMN-I-GFP vector (PC3mm2-shCad11-cla-Δ5-GFP). PC3mm2 cells transfected with empty pBMN-I-GFP vector (PC3mm2-shCad11-GFP) or Cad11 (PC3-mm2-shCad11-Cad11-
GFP) were used as negative and positive controls, respectively. qRT-PCR and western blot showed that the message levels of Cad11-cla-Δ5 in PC3mm2-shCad11-cla-Δ5-GFP cells were higher than the endogenous levels of Cad11 in the vector transfected PC3mm2-pLKO cells, but the protein levels were comparable (Fig.4A, 4B), whereas the levels of Cad11 in PC3mm2-shCad11-Cad11-GFP cells were higher than those in PC3mm2-pLKO cells (Fig. 4A, 4B). The lower levels of Cad11-cla-Δ5 compared to Cad11 in the reconstituted shCad11 cells is possibly due to differences in protein folding or stability because we have found that introduction of mutation and/or deletion to membrane proteins frequently leads to decreased levels of mutant protein production compared to the wild type.

When cells were incubated with anti-Cad11 antibody at 4°C, Cad11 signals were readily detected at the cell surface in PC3mm2-pLKO, PC3mm2-shCad11-cla-Δ5-GFP and PC3mm2-shCad11-Cad11-GFP cells (Fig. 4C). After acid washing that removed cell surface-bound anti-Cad11 antibody, no Cad11 signals were detected in these cells (Fig. 4C, 4°C acid wash). These observations indicate that both Cad11 cl-Δ5 mutant and wild type Cad11 proteins were expressed at the cell surface. No Cad11 signals were observed in PC3mm2-shCad11 and PC3mm2-shCad11-GFP cells at 4°C, indicating efficient knockdown of endogenous Cad11.

We next conducted endocytosis assays on this panel of PC3mm2 cells. Upon shifting the temperature to 37°C, endocytic vesicles containing Cad11 (green dots) were detected in the cytosol of PC3mm2-pLKO and PC3mm2-shCad11-Cad11-Cad11-GFP cells (Fig. 4C, 37°C, no acid wash). Further, these cytosolic Cad11 punctate signals persisted after acid wash (Fig. 4C, 37°C, acid wash). However, the number of internalized green dots was decreased by 67% in PC3mm2-shCad11-cla-Δ5-GFP cells as compared to PC3mm2-pLKO (Fig. 4C). These observations suggest that clathrin is also involved in Cad11 endocytosis in PC3mm2 cells.

Immunostaining of Cad11 and clathrin in this panel of PC3mm2 cells showed colocalization of Cad11 and clathrin in the endocytic vesicles of PC3mm2-pLKO and PC3mm2-shCad11-Cad11 cells (Fig. 4D, see insets, supplemental Fig. S4), and the co-staining was diminished under the condition of K⁺-depletion (Fig. 4D). In PC3mm2-shCad11-cla-Δ5-GFP cells, there was much less co-staining of Cad11 with clathrin compared to that observed in PC3mm2-pLKO and PC3mm2-shCad11-Cad11 cells (Fig. 4D). We further conducted PLA assays to detect the interaction of Cad11 and clathrin on PC3mm2 cells. We observed that there were less red fluorescent spots in PC3mm2-shCad11, PC3mm2-shCad11-GFP and PC3mm2-shCad11-cla-Δ5 cells as compared to PC3mm2-pLKO.1 cells.
that endogenously express Cad11 (Fig. 4E). There were more red spots in PC3mm2-shCad11-Cad11 cells than PC3mm2-PLKO.1 cells (Fig. 4E), consistent with the overexpressed levels of wild-type Cad11 in the reconstituted shCad11 cells. Together, these observations indicate that the interaction of Cad11 and clathrin is one of the pathways responsible for Cad11 internalization in PCa cells.

Clathrin-dependent endocytosis on Cad11-mediated migration

Previous studies have shown that Cad11 plays a role in the migration of PCa cells (Huang et al., 2010; Lee et al., 2013; Lee et al., 2010). Since endocytosis plays a role in the turnover of Cad11 from the cell surface, which may have an effect on Cad11-mediated cellular activities, we examined whether clathrin-dependent endocytosis has an effect on Cad11-mediated migration. We performed wound healing migration assays on C4-2B-vector, C4-2B-Cad11-WT and C4-2B-Cad11-cla-Δ5 cells. Expression of Cad11-WT significantly increased C4-2B cell migration compared to C4-2B vector control cells (Fig. 5A). Expression of C4-2B-Cad11-cla-Δ5 also increased C4-2B cell migration, however, to a lesser extent than that in C4-2B-Cad11-WT cells (Fig. 5A). Moreover, we applied live-cell imaging to capture cell movement in ibidi migration chambers by time-lapse recording. As shown in Fig. 5B, the gaps remained visible in C4-2B-vector and C4-2B-Cad11-cla-Δ5 cells, while the gap was almost closed in C4-2B-Cad11 cells at 23 h. Quantification analysis revealed that the gap distance was significantly shorter in C4-2B-Cad11 cells than that in C4-2B-vector and C4-2B-Cad11-cla-Δ5 cells (Fig. 5B, upper right panel). The cell moving path was also longer in C4-2B-Cad11 cells compared to C4-2B-vector and C4-2B-Cad11-cla-Δ5 cells (Fig. 5B, bottom left panel). Quantification of the average cell moving speed also showed significantly faster movement in C4-2B-Cad11 cells than that in C4-2B-vector and C4-2B-Cad11-cla-Δ5 cells (Fig. 5B, lower right panel).

The possibility that changes in cell migration may be due to an effect of Cad11 or Cad11-cla-Δ5 expression on cell proliferation was examined. We found that there was no significant difference in the cell numbers amongst the different C4-2B cell lines used in the migration assays at 24 h (Fig. 5C, left panel). Similarly, no significant difference in cell viability, as measured by Presto blue assay, was observed amongst the C4-2B cell lines used in the migration assays at 24 h (Fig. 5C, right panel). In addition, no significant difference was observed in the number of apoptotic cells, as measured by Annexin V binding assay, amongst the C4-2B cell lines (Fig. 5D).
In addition to C4-2B cells, we also performed wound healing assays on the panel of PC3mm2 cells as described in Fig. 4. Knockdown of endogenous Cad11 in PC3-mm2 cells (PC3mm2-shCad11 and PC3mm2-shCad11-GFP) significantly reduced cell migration relative to that in vector control cells (PC3mm2-pLKO) (Fig. 6A). The migratory activity was recovered by re-expression of wild-type Cad11 (PC3mm2-shCad11-Cad11) but not by Cad11-cla-Δ5 (PC3mm2-shCad11-Cad11-cla-Δ5) in PC3mm2 cells (Fig. 6A). Similarly, faster gap closure (Fig. 6B, upper panels) and longer cell moving path (Fig. 6B, lower panels) was observed in PC3mm2-pLKO.1 cells and in PC3mm2-shCad11-Cad11 cells compared to PC3mm2-shCad11-Cad11-cla-Δ5 cells by live-cell imaging. No significant differences in the cell numbers (Fig. 6C, left panel), cell viability (Fig. 6C, right panel) and the number of apoptotic cells (Fig. 6D) were observed amongst the different PC3mm2 cell lines used in the migration assays at 24 h. These observations indicate that the Cad11-mediated effects on cell migration were not due to cell proliferation. Together, these results suggest that turnover of Cad11 from the cell surface through clathrin-mediated endocytosis plays a role in Cad11-mediated migration in PCa cells.

Discussion

We showed that clathrin directly interacts with Cad11 and is involved in Cad11 endocytosis. We further showed that clathrin-mediated Cad11 endocytosis regulates Cad11-mediated migration. Although clathrin has been shown to mediate E-Cad endocytosis, we found that E-Cad cyto domain does not contain the specific “clathrin binding motif” VFEEE found in Cad11 and clathrin does not bind to E-Cad cyto under our experimental condition, suggesting that clathrin-mediated endocytosis for E-Cad and Cad11 are distinct.

Cadherin adhesion complex has been shown to play critical roles during normal development as well as malignancy (Kowalczyk and Nanes, 2012). Cad11 is a mesenchymal cadherin mainly expressed in osteoblasts as well as in lung, testis, and brain tissues at low levels (Okazaki et al., 1994). It is likely that Cad11 endocytosis provides dynamic changes necessary for Cad11-mediated cell migration that plays a role in osteogenesis and neuronal cell migration during normal development. In Cad11-mediated neuronal migration in Xenopus development, it was shown that excessive Cad11 expression prevented cell migration in the embryo and cleavage of Cad11 extracellular domain to reduce Cad11-mediated adhesion is important for Cad11-mediated neuronal migration (Borchers et al., 2001). Peglion et al. (Peglion et al., 2014) showed that clathrin-mediated endocytosis of N-Cad plays a role in the cycling of adherens junction components for anterograde transport.
during collective cell movement. Gavard and Gutkin (Gavard and Gutkind, 2006) showed that VEGF stimulation promotes the rapid endocytosis of VE-cadherin, resulting in disruption of the endothelial barrier and increased vascular permeability. Recently, Padmanabhan and Taneyhill (Padmanabhan and Taneyhill, 2015) showed that clathrin-mediated endocytosis of cadherin-6B plays a role in neural crest cell EMT and migration. Thus, the dynamic changes in the surface levels of cadherin molecules through clathrin-mediated endocytosis affect cadherin-mediated adhesion and migratory activities.

Defects in recycling of adhesion complexes have been shown to be involved in malignant transformation (Mosesson et al., 2008). Cad11 has been shown to promote the metastasis of prostate or breast cancer cells to bone (Chu et al., 2008; Tamura et al., 2008). Because acquisition of migration property is a hallmark of metastatic cancer cells, it is possible that clathrin-mediated Cad11 endocytosis plays a role in the metastatic colonization of PCa cells in bone. We have recently shown that Cad11 plays a role in cell migration through interaction with p80-angiomotin (Ortiz et al., 2014). Whether clathrin-mediated Cad11 endocytosis regulates Cad11-angiomotin complex formation and dissociation is unknown.

During classical clathrin-mediated endocytosis, membrane receptors are clustered in clathrin-coated pits followed by membrane invagination and vesicle scission (Goldstein et al., 1979). In clathrin-coated pits, clathrin triskelia formed lattices recruit adaptor proteins, e.g. AP2, DAB and dynamin, to form endocytic vesicles (Hulpiau and van Roy, 2009). Thus, the binding of clathrin to cargo is typically indirect, and the adaptor protein interactions with cargo are also of low affinity. Although many reports described clathrin-mediated endocytosis of E-cadherin (Ivanov et al., 2004; Kon et al., 2008; Le et al., 1999; Miyashita and Ozawa, 2007), it is likely that the interaction of clathrin with E-cadherin tail is indirect. Thus, clathrin does not bind to E-cadherin cytoplasmic domain under the experimental conditions used in the present study. We were able to pull down clathrin using Cad11-cyto domain through the “clathrin binding motif” in Cad11. However, we did not find AP-2 or DAB in the Cad11-cyto pulldown by Western blot (data not shown). In addition, we did not detect clathrin in PC3 cells immunoprecipitated with anti-Cad11 antibody (data not shown). This may be due to the low affinity or the transient nature of Cad11-clathrin interaction in vivo. It is also possible that the detergents used in the solubilization of Cad11 from membranes interfered with Cad11-clathrin interaction.

While clathrin-mediated endocytosis is a common mechanism for the turnover of cadherin family proteins, the structural motifs utilized in this process appear to be distinct for
different cadherins. In VE-cadherin, Nanes et al. (Nanes et al., 2012) identified a dual-function motif consisting of three highly conserved acidic residues (DEE) that alternately serve as a p120-binding interface and an endocytic signal (Fig. 2C). In contrast, E-cad contains a dileucine endocytic signal that is not present in VE-cadherin, and mutation of the DEE sequence in E-Cad only modestly inhibited its internalization (Nanes et al., 2012). Our studies revealed yet a third motif, the “clathrin-binding motif” VFEEE, in Cad11 that is distinct from the dileucine endocytic signal in E-Cad and p120-binding site in VE-Cadherin.

In conclusion, we have identified a unique structural motif that mediates Cad11 endocytosis. Because dynamic turnover of Cad11 is required for proper adhesion and migration activity, our studies provide a mechanism by which clathrin-mediated Cad11 endocytosis regulates the migratory function of Cad11 in PCa cells.
MATERIALS AND METHODS

Cell lines and antibodies
Human kidney 293T (HEK293T), Phoenix cells, and L-cells were purchased from American Culture Type Collection and were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Human prostate cancer cell lines C4-2B and PC3mm2 (Huang et al., 2010; Lira et al., 2008) were grown in RPMI 1640 (Invitrogen) containing 10% FBS. Goat anti-clathrin and mouse anti-Cad11 cyto (5B2H5) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Mouse anti-Cad11 antibody mAb1A5 was generated as described previously (Lee et al., 2013). Rabbit polyclonal Rab5 antibody and rabbit recombinant monoclonal Rab7 antibody were purchased from ABCAM (Cambridge, MA, USA). Mouse anti-E-cad, anti-p120 catenin, and anti-β-catenin antibodies were purchased from BD Transduction Lab (San Jose, CA). The oligonucleotides used were purchased from Sigma-Aldrich and the sequences were listed in supplemental Table 1.

Construction and expression of GST-cyto fusion proteins
GST fusion proteins containing two copies of Cad11-cyto domain (GST-Cad11-cyto) or E-Cad-cyto domain (GST-E-Cad-cyto) were constructed as described in Ortiz et al. (Ortiz et al., 2014). The construction of GST fusion proteins containing one copy of cytoplasmic domains with deletions in the juxtamembrane domain (GST-ΔJMD-cyto) or β-catenin binding domain (GST-ΔCBS-cyto), respectively, were described in Ortiz et al.(Ortiz et al., 2014). GST fusion proteins containing N-terminal 51, 40, 35, 30, 25, 20, 15, and 10 amino acids of the Cad11 cyto domain were generated by PCR using the forward primer and reverse primers listed in supplemental Table 1. The GST-cyto fusion proteins were expressed in *Escherichia coli* and purified using glutathione-agarose beads (GST beads, GE Healthcare Life Sciences, Piscataway, NJ).

GST pulldown assay for Cad11-cyto associated proteins
L-cells that do not express any of the major cadherins were grown as monolayer on tissue culture plates. Cells were then scraped from plate in cold distilled water containing protease inhibitors and homogenized with a Dounce homogenizer. After centrifugation of the cell lysates for 15 min at 13,000 rpm, the supernatant fraction was collected. The supernatant fraction was mixed with GST or GST-Cad11-cyto protein immobilized on glutathione-
agarose beads on a rocker at 4°C overnight. The glutathione-beads were spun down, and were washed three times with cold lysis buffer (50 mM phosphate, and 100 mM NaCl, and 0.01% Triton-X100) containing protease inhibitors, and analyzed on a 4–12% gradient NuPage gels (Novex, San Diego, CA). The gel was stained with silver stain. The specific protein band at 180 kDa was cut from the gel and analyzed by mass spectrometry. For Western blots, the gel was transferred to a nitrocellulose membrane (Schleicher & Schnell), stained with Ponceau S, and followed with immunoblotting with specific antibodies as indicated. Signals were detected with a chemiluminescent detection kit (Pierce Biotechnology).

**Generation of cell lines expressing wild-type Cad11 or Cad11 mutants**

Cad11 with deletion of VFEEE in the clathrin binding site (Cad11-cla-Δ5) was generated using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and verified by DNA sequencing. The sequences of the primers are listed in supplemental Table 1. Wild-type Cad11 (Cad11-WT) and mutant Cad11 (Cad11-cla-Δ5) in bicistronic pBMN-I-neo vectors were transected into Phoenix cells and the culture media were collected for infection of C4-2B cells as described previously (Huang et al., 2010; Lira et al., 2008). C4-2B cells were infected with retrovirus followed with G418 selection to generate C4-2B-Cad11-WT and C4-2B-Cad11-cla-Δ5 cell lines. C4-2B cells infected with empty vector (C4-2B-Vector) were used as a control.

PC3mm2 is a bone metastasis–derived PCa cell line expressing endogenous Cad11 (Chu et al., 2008). To express mutant Cad11 in PC3mm2 cells, we first knocked down endogenous Cad11 in PC3 mm2 cells using lentiviral vector pLKO.1-puro containing Cad11 shRNA as we described previously (Ortiz et al., 2014). The lentiviral vector containing non-targeting shRNA (pLKO.1 puro, Sigma-Aldrich) was used as a control. Following the establishment of PC3mm2-shCad11 cells, we infected the cells with Cad11-cla-Δ5 or Cad11-WT retroviruses to generate PC3mm2-shCad11-Cad11-cla-Δ5-GFP, PC3mm2-shCad11-Cad11-GFP cells as described above for C4-2B cells. PC3mm2 cells infected with empty pBMN-I-GFP vector (PC3mm2-shCad11-GFP) were used as a control.

**Endocytosis assay and co-localization with clathrin**

Cells (5 × 10^4) were seeded on to cover slips in the well of 24-well plate the day before the endocytosis assay. Before starting internalization assay, cells were incubated with anti-Cad11 mAb 1A5 (Lee et al., 2013), which recognizes extracellular domain, at 4°C for 30 min to
allow for antibody binding to cell surface Cad11. After washing cells with ice-cold PBS buffer containing 3% (w/v) BSA to remove unbound antibody, cells were changed to either fresh RPMI medium containing 10% FBS (v/v) or to potassium (K⁺) depletion buffer (20 mM Hepes, 140 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂) and incubated at 37°C for 30 min to allow the internalization to occur. Then cells were washed with ice-cold 3% BSA/PBS wash buffer or washed with ice-cold acid wash buffer (0.5 M NaCl/0.2 M acetic acid) for 4 min to remove the surface-bound antibody followed by fixing cells with 100 % methanol at -20°C for 6 min. Cells were immunostained with Alexa488-labeled anti-mouse (m-AF488) secondary antibody (1:300) at room temperature (RT) for 1 h in the dark followed by counterstaining with 4’, 6-diamidino-2-phenylindole (DAPI, 1:500 in PBS) for 10 min. The number of internalized vesicles with Cad11 was calculated using Image J software.

**Co-localization by immunofluorescence**

For the co-localization of Cad11 and clathrin, cells were incubated with anti-Cad11 antibody mAb 1A5 and goat anti-clathrin antibody (1:100). For the co-localization of Cad11 with endosome markers, cells were incubated with mouse anti-Cad11 antibody and rabbit anti-Rab5 antibody or anti-Rab7 antibody. After incubation at 4°C overnight, cells were incubated with secondary antibody mouse-AF488 (1:400) and goat-AF594 (1:300) (Jackson ImmunoResearch) or rabbit-AF594 (1:400) (Jackson ImmunoResearch) at RT for 1 h. Cells were then counterstained with DAPI for 10 min and mounted with anti-fading mounting medium.

**Proximity ligation assay (PLA)**

Proximity ligation assay was performed using Duolink PLA In Situ Red Starter Kit (Mouse/Goat, Sigma) per the manufacture’s instruction. The primary antibodies were mouse anti-Cad11 antibody (Invitrogen, 1:150) and goat anti-clathrin antibody (1:100, Santa Cruz Biotechnology, Inc.). Images were acquired by FluoView 1000 IX2 confocal microscope (Olympus).

**Wound healing migration assay**

We employed a wound healing assay using both scratching and ibidi migration chamber. For scratching, cells were seeded onto 6-well plate and were grown to a confluent monolayer. Then “wounds” were generated by scratching lines through the monolayer using 200 µl tips. The cells that moved into the created open gaps were then imaged over 24 h by microscope at
4 × magnification. The filled area was quantified using image J software. When using μ-Slide 8-well ibiTreat microscopy chamber (Ibidi, Madison, Wisconsin), we applied live-cell imaging to capture cell movement by time-lapse on an Olympus IX81 DSU Spinning Disk Confocal Microscope. The rate of gap closure and the speed of cell movement were analyzed from the time-lapse movies using 3I’s Slidebook software.

**Cell proliferation, viability and apoptosis assay**

For cell proliferation assay, cells were seeded into 6-well plate (3 × 10^5 /well) and allowed to attach onto the plate overnight. The number of cells was counted using a hemocytometer after digestion. Cell number counted the following day was set as day 1 and at 24 h later was set as day 2. The 24 h’s proliferation rate was expressed as folds of day 1.

For cell viability measurement, 3 × 10^4 cells in 200 µl of culture medium were seeded into wells of 96-well plates. The cell viability was determined on the following day (day1) and another 24 h later (day2). On the day of assay, the medium was changed with 100 µl (for 96-well plate) of fresh medium. PrestoBlue reagent (Life Technologies, Grand Island, NY) was added to each well at 1: 10 ratio, and the cells were further incubated at 37°C for 2 h. The medium (100 µl) was used for measuring absorbance OD value at 570 nm and 600 nm respectively. The wells containing culture medium without cells were used as background.

To determine if cell apoptosis may affect migration, we detected the early stage of cell apoptosis using flow cytometric analysis of cells labeled with APC-conjugated annexin V (BioLegend, San Diego, CA) on a FACScan™ model flow cytometer (Beckman Coulter “Gallios”).
**Statistical Analysis**
Data from three or more independent experiments were used in analysis and values were expressed as mean ± s.e.m. Statistical significance was assessed by Student’s t test. The level of significance was set at $P < 0.05$.

**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
S.H.L., L.Y.Y.L., A.P.K. and R.L.S. conceived and designed the experiments. T.P., M.A.B., X.L., Y.C.L. and A.O. performed the experiments. R.L.S., T.P., L.Y.Y.L., S.H.L. analyzed the data. R.L.S., T.P., A.P.K., L.Y.Y.L., S.H.L. wrote the manuscript. All authors read and approved the final manuscript.

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References


Fig. 1. Clathrin binds to Cad11-cyto domain. (A) GST fusion protein containing two copies of Cad11-cyto domain separated by four glycines was used in a pulldown assay (upper panel). GST-Cad11-cyto-agarose or GST-agarose were incubated with or without L-cell cytosolic fraction and the proteins were separated by SDS-PAGE and visualized with silver stain (lower left panel). The prominent protein at 180 kDa was identified to be clathrin by mass spectrometry. Western blot showed that the 180 kDa protein pulled down by GST-Cad11-cyto agarose reacted with anti-clathrin antibody (right panel). (B) Clathrin binds to
Cad11 but not E-Cad cyto domain. GST fusion proteins containing two copies of E-cadherin (GST-E-Cad-cyto) or Cad11 (GST-Cad11-cyto) were incubated with L-cell cytosol in GST pulldown assays (left panel). The proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and visualized with Ponceau S (right panel). The membranes were immunoblotted with anti-clathrin, anti-Cad11 or anti-E-Cad antibodies (right panel). (C) Cad11 but not E-Cad cyto domain pulled down clathrin in C4-2B4 cells. GST fusion proteins containing two copies of E-cadherin (GST-E-Cad-cyto) or Cad11 (GST-Cad11-cyto) were incubated with C4-2B4 cell cytosol in GST pulldown assays. The proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and visualized with Ponceau S (left panel). The membranes were immunoblotted with anti-clathrin antibodies (right panel).
Fig. 2. Clathrin binds to the juxtamembrane region of Cad11-cyto domain. (A) Deletion constructs of Cad11-cyto domain (upper left). Ponceau S staining of GST and GST fusion proteins containing E-Cad-cyto, Cad11-cyto, or Cad11-cyto domain with deletions in juxtamembrane domain (Cad11-ΔJMD), or deletions in β-catenin binding domain (Cad11-ΔCBS) (lower left). Western blot of the GST pulldown products with anti-clathrin, anti-p120 catenin or anti-β-catenin antibodies (lower right). (B) Sequences of Cad11 JMD domain deletion mutants. Sequences corresponding to p120-catenin binding in E-Cad are marked with bold lines. GST pulldown assays using GST and GST-Cad11-cyto proteins containing N-terminal 51, 40, 35, 30, 20, 15, and 10 amino acids of the Cad11 cyto domain (Ponceau S) were analyzed by immunoblotting with anti-clathrin or p120 catenin antibodies. p120-catenin only binds to GST-Cad11-cyto 51aa fusion protein while clathrin binds to all GST-Cad11-cyto fusion proteins except GST-Cad11-cyto 10aa. (C) Sequence alignment of the cytoplasmic domains of human Cad11, E-Cad, P-Cad, N-Cad and VE-Cad. The amino acid sequences that are essential for Cad11-cyto to bind with clathrin or p120 are marked with thick lines.
Fig. 3. Clathrin mediates Cad11 endocytosis in C4-2B cells. (A) Endocytosis assay for Cad11. C4-2B-Cad11 cells were incubated with anti-Cad11 antibody mAb 1A5 at 4°C.
Cad11 internalization was initiated by shifting the temperature to 37°C. A mild acid wash, which removes mAb 1A5 associated with surface Cad11, was used to distinguish the internalized Cad11 from surface Cad11. Potassium (K⁺) depletion was used to block clathrin-mediated internalization of Cad11. After fixation, cells were incubated with fluorescence-labeled anti-mouse antibody, counterstained with DAPI for nuclei and imaged by confocal microscopy. The number of internalized Cad11 protein-containing particles were quantified and expressed as mean ± s.e.m. (n = 10). **: P < 0.01. Magnification: × 400. Scale bar, 20 µm. (B) Co-localization of Cad11 (green) and Rab5 (red) or Rab7 (red) by immunofluorescence staining. Boxed areas are enlarged. (C) Expression of wild-type Cad11 (Cad11-WT) and mutant Cad11 with deletion of VFEEE in the JMD domain (Cad11-cla-Δ5) in C4-2B cells. (D) Endocytosis assay of C4-2B cells expressing Cad11-WT, Cad11-cla-Δ5 or vector alone. The data were analyzed as in (A) and expressed as mean ± s.e.m. (n = 10). **: P < 0.01. Magnification: × 400. (E) Co-localization of Cad11 and clathrin (enlarged in inset) in C4-2B-Cad11 and C4-2B-Cad11-cla-Δ5 cells after internalization assay with or without K⁺-depletion. Cells were stained with anti-Cad11 mAb 1A5 and goat-anti-clathrin and counterstained with DAPI. (F) Proximity ligation assays (PLA) to determine the interaction of Cad11 and clathrin in C4-2B cells. Red fluorescent spots indicate co-localization of Cad11 and clathrin. In the enlarged insets, bright field images were superimposed on the fluorescence images to reveal the cell borders. Quantification of PLA spots per cell is shown in the right panel. **: P < 0.01. Images without primary antibody were used as a negative control.
Fig. 4. Cad11 endocytosis in PC3mm2 cells. (A) Knockdown of endogenous Cad11 in PC3mm2 cells followed by reconstitution with Cad11 cyto domain mutant. Cad11 RNA
levels relative to β-actin were determined by real time RT-PCR. (B) Cad11 protein levels in the cells indicated were determined by Western blot and quantified relative to β-actin. (C) Cad11 internalization in PC3mm2 cells was determined at 4°C, followed by shifting the temperature to 37°C, with or without acid wash, and analyzed by immunofluorescence and quantified as described in Fig. 3. **: $P < 0.01$. (D) Co-localization of Cad11 and clathrin in PC3mm2 cells. Cells were immunostained with anti-Cad11 mAb 1A5 or anti-clathrin antibodies after internalization assay with or without K⁺-depletion. Boxed areas are enlarged. Scale bars, 20 µm. (E) Proximity ligation assay (PLA) to determine the interaction of Cad11 and clathrin in PC3mm2 cells was performed as in Fig. 3F. Quantification of PLA spots per cell is shown in the right panel. *: $P < 0.05$; **: $P < 0.01$. 
Fig. 5. Effects of Cad11 and clathrin interaction on Cad11-mediated cell migration, proliferation, and apoptosis in C4-2B cells. (A) Cad11-mediated migration in C4-2B cells
measured by wound healing scratch assay (Left). Number of cells that migrated into the scratched area before and after the 24-hr incubation period was determined by Image J and expressed as percentage (%) of total area (Middle). Number of cells migrated into the scratch area after subtracting the background levels at 0 hr (Right). *: \( P < 0.05 \); **: \( P < 0.01 \). (B) Cell migration analyzed by live-cell imaging. Gap closure and cell moving path over the time period of 23 hr (Left). Boxed areas are enlarged. Quantification of gap distance (upper right panel) and cell moving speed (lower right panel). *: \( P < 0.05 \). (C) Effect of Cad11-WT and Cad11-cla-Δ5 on proliferation of C4-2B cells as measured by cell number (Left) and by cell viability assay (Right). (D) Flow cytometry analysis of C4-2B cells labeled with APC-conjugated annexin V.
Fig. 6. Effects of Cad11 and clathrin interaction on Cad11-mediated cell migration, proliferation, and apoptosis in PC3mm2 cells. (A) Wound healing scratch assay (Left). Quantification of scratch assay by Image J (middle and right). **: $P < 0.01$. (B) Cell migration analyzed by live-cell imaging. Gap closure and cell moving path over the time period of 23 hr (Left). Boxed areas are enlarged. Quantification of gap distance (upper right) and cell moving speed (lower right). (C) Effect of Cad11-WT and Cad11-cla-Δ5 on proliferation of PC3mm2 cells as measured by cell number (Left) and by cell viability assay (Right). (D) Flow cytometry analysis of PC3mm2 cells labeled with APC-conjugated annexin V.