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CADM1 suppresses c-Src activation by binding with Cbp on membrane lipid rafts and intervenes colon carcinogenesis

Yumi Tsuboi [a], Masaaki Oyama [b], Hiroko Kozuka-Hata [b], Akihiko Ito [c], Daisuke Matsubara [d], Yoshinori Murakami [a, *]

[a] Division of Molecular Pathology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[b] Medical Proteomics Laboratory, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[c] Department of Pathology, Kindai University Faculty of Medicine, Osaka, Japan
[d] Division of Integrative Pathology, Jichii Medical University, Shimotsuke, Japan

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ABSTRACT

Cell adhesion molecules act as tumor suppressors primarily by cell attachment activity, but additional mechanisms modifying signal transduction are suggested in some cases. Cell adhesion molecule 1 (CADM1), a membrane-spanning immunoglobulin superfamily, mediates intercellular adhesion by trans-homophilic interaction and acts as a tumor suppressor. Here, we investigated CADM1-associated proteins comprehensively using proteomic analysis of immune-precipitates of CADM1 by mass spectrometry and identified a transmembrane adaptor protein, Csk-binding protein (Cbp), known to suppress Src-mediated transformation, as a binding partner of CADM1. CADM1 localizes to detergent-resistant membrane fractions and co-immunoprecipitated with Cbp and c-Src. Suppression of CADM1 expression using siRNA reduces the amount of co-immunoprecipitated c-Src with Cbp and activates c-Src in colon cancer cells expressing both CADM1 and Cbp. On the other hand, co-replacement of CADM1 and Cbp in colon cancer cells lacking CADM1 and Cbp expression suppresses c-Src activation, wound healing and tumorigenicity in nude mice. Furthermore, expression of Cbp and CADM1 was lost in 55% and 83% of human colon cancer, respectively, preferentially in tumors with larger size and/or lymph node metastasis. CADM1 would act as a colon tumor suppressor by intervening oncogenic c-Src signaling through binding with Cbp besides its authentic cell adhesion activity.

1. Introduction

Several cell adhesion molecules, such as E-cadherin, act as tumor suppressors by mediating cell-to-cell adhesion and arraying a sheet of differentiated epithelia cells to protect against abnormal growth, invasion, or metastasis of malignant cells. Furthermore, E-cadherin is shown to be involved in suppressing Wnt signaling by sequestering α- and β-catenins to plasma membranes [1]. These findings suggest that cell adhesion molecules other than E-cadherin could also play roles in modifying oncogenic signaling to achieve tumor suppression in addition to their original functions in cell adhesion.

CADM1 is an immunoglobulin superfamily cell adhesion molecule (CAM) which we have previously identified as a tumor suppressor in non-small cell lung cancer (NSCLC) [2]. CADM1 is expressed in the brain, mast cell, testis and various epithelial tissues including lung and colon [3]. CADM1 expression is often lost in various cancers, including NSCLC and colon cancer [4–6]. The cytoplasmic domain of CADM1 harbors two protein-binding motifs, a protein 4.1-binding motif and a class II PDZ-binding motif. Through these motifs, CADM1 associates with 4.1B/DAL1 and PDZ-domain containing membrane-associated guanylate kinases (MAGuKs), including MPP1-3, CASK, and Pals2 [7–10]. A CADM1 mutant lacking the cytoplasmic domain of CADM1 lost suppressor activity of tumor formation in nude mice [11], indicating that the cytoplasmic domain of CADM1 is critical to its tumor suppressor function. We have previously shown that depletion of CADM1 by

Abbreviations: CADM1, Cell adhesion molecule 1; Cbp, Csk-binding protein; Csk, C-terminal Src kinase; SH2, Src-homology domain 2; BMMC, bone marrow-derived cultured mast cells; TfnR, transferrin receptor; Flot1, Flottilin-1; DRM, detergent resistant membrane.

* Corresponding author. Division of Molecular Pathology, Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan.
E-mail address: ymurakam@ims.u-tokyo.ac.jp (Y. Murakami).

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siRNA induces mislocalization of its binding proteins and abrogates epithelia-like structure [8], suggesting that CADM1 is involved in membrane localization of its binding proteins and maintenance of epithelial cell structure.

A non-receptor tyrosine kinase, c-Src is overexpressed and activated in various human cancers, especially colon and breast cancers, and induces malignant transformation of cancer cells [12]. The activation of c-Src is regulated by phosphorylation of its tyrosine residues, Tyr418 (Y418) and Tyr529 (Y529). Autophosphorylation of Y418 in kinase domain locks the catalytic domain into the active conformation and facilitates activation of c-Src. In contrast, phosphorylation of Y529 in its C-terminal tail, which is catalyzed by a cytoplasmic protein, C-terminal Src kinase (Csk), allows intramolecular interaction with SH2 domain of c-Src, resulting in its inactivation. Csk-binding protein (Cbp) was previously identified as a transmembrane adaptor protein which localizes to membrane microdomains, lipid rafts, and recruits Csk to plasma membranes. When Cbp binds and recruits Csk and activated-c-Src to lipid rafts, Csk phosphorylates Y529 of c-Src to inactivate the catalytic activity of c-Src. Thus, Cbp suppresses c-Src-mediated cell transformation and tumorigenesis [13]. A Cbp mutant lacking the lipid raft-anchoring sites failed to suppress c-Src-mediated cell transformation and to accumulate c-Src in lipid rafts, suggesting that lipid raft localization is important for Cbp-mediated c-Src inactivation. Interestingly, even in Csk-deficient fibroblasts, Cbp suppresses c-Src-mediated transformation, implying that Cbp also suppresses c-Src in a Csk-independent manner [14].

Here, we identified Cbp as a novel CADM1-associated protein by proteomic approach and suggest that CADM1 inactivates c-Src in a Cbp-dependent manner by accumulating Cbp-Src complex in lipid raft fractions. Intervention of tyrosine kinase activity on the lipid rafts could provide a unique mechanism of a cell adhesion molecule, CADM1 to suppress tumorigenesis in addition to its classical cell adhesion activity.

2. Materials and methods

2.1. Mice, cells and antibodies

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). *Cadm1*$^{-/-}$ mice were generated and maintained in our laboratory [15]. Bone marrow-derived cultured mast cells (BMMCs) from C57BL/6 mice and *Cadm1* knockout mice were obtained as described previously [16]. All animal experiments were carried out according to the institutional guidelines with the approved number, PA13-73, in the Institute of Medical Science, the University of Tokyo, Japan. Eight human colon cancer cells and HEK293 cells were from the American Type Culture Collection (Manassas, VA, USA) and the Health Science Research Resources Bank (Osaka, Japan), respectively. The anti-CADM1 polyclonal antibody (C18) was generated against a synthetic peptide of 18 amino acids at the carboxyl terminus of human CADM1 [8]. Other antibodies used are listed in Table S1.

2.2. Expression vectors and transfection

A vector expressing a full-length of human CADM1 was previously described [2]. To obtain a vector expressing human Cbp or FLAG-tagged Cbp, a full length Cbp cDNA was amplified by reverse transcription-PCR and cloned into p3xFLAG-CMV-14 vector (Sigma, St. Louis, MO, USA). To obtain HA-tagged CADM1 expressing vectors, wild-type (FL) or its truncated mutant lacking the cytoplasmic domain (ΔCT) of CADM1 cDNA were amplified by PCR and cloned into pSecTag2/Hygro (Invitrogen, Carlsbad, CA, USA). Plasmids were transfected using Lipofectamine 2000 or Lipofectamine LTX (Invitrogen). To obtain stable clones, cells were selected against 400 μg/mL of hygromycin or G418 sulfate.

2.3. siRNAs

The siRNA sequences targeting human CADM1 (siCADM1) and human Cbp (siCbp) are described in Table S2. A control siRNA (siControl), ON-TARGET plus Non-targeting pool, was from Dharmacon. The siRNA was transfected into cells using Lipofectamine 2000 (Invirtogen) for 72 h.

2.4. Mass spectrometry analysis

BMMCs were treated with a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitors) for 30 min on ice and centrifuged. Protein extracts were incubated overnight at 4 °C with anti-CADM1 polyclonal antibody (C18) cross-linked to the Protein A sepharose beads using dimethyl pimelimidate (DMP). The beads were washed with a wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA), and the protein complexes were eluted with 0.1 M Glycine (pH 2). The sample was digested with trypsin at 37 °C overnight and desalted using ZipTip (C_{18}; Millipore). The digested peptide mixture was then analyzed on a nanoflow LC-MS/MS system. The acquired MS/MS spectra were processed against the RefSeq (National Center for Biotechnology Information (NCBI)) protein database [17], using the Mascot algorithm (Matrix Science, London, United Kingdom) [18].

2.5. Detection of phosphorylated c-Src

Cells were suspended in a TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate), homogenized using a Dounce homogenizer, and centrifuged at 1,000xg for 10 min. The resulting supernatant was then centrifuged at 10,000xg for 50 min and the pellet (membrane fraction) was resuspended in 1x Laemmli sample buffer. SDS-PAGE and immunoblotting were carried out as described previously [19].

2.6. Preparation of detergent-resistant membrane fraction

Cells were suspended in TNE buffer, homogenized using a Dounce homogenizer, and centrifuged at 1,000xg for 10 min 1% Triton X-100 was added to the supernatant. After 30 min on ice, lysates (1 ml) were mixed with 1 ml of 80% sucrose in TNE buffer and overlaid with 6 ml of 35% sucrose and 3 ml of 5% sucrose. After centrifugation at 180,000xg for 20-24 h, eleven fractions were collected from the top of gradient. For the immunoprecipitation assay, DRMs were solubilized with TNE buffer containing 2% octyl-D-glucoside and 1% Nonidet P-40.

2.7. Wound-healing assay

A suspension of 4×10^4 cells was applied into each well of Culture-Insert in μ-Dish (ibidi GmbH, Martinsried, Germany) and incubated for 48 h. The wounded areas were photographed and measured at 0 and 20 h after Culture-Inserts were removed, and the relative speed of wound closure in each clone was calculated. Data are shown as means ± SD of at least four independent experiments.

2.8. Tumorigenicity assay

A suspension of 8×10^5 cells in serum-free McCOY's 5A medium (200 μl) was injected subcutaneously into 6-week-old female BALB/c Ajcl-nu/nu mice (CREA Japan, Inc., Tokyo, Japan). Tumor volume was measured every 3 or 4 days.

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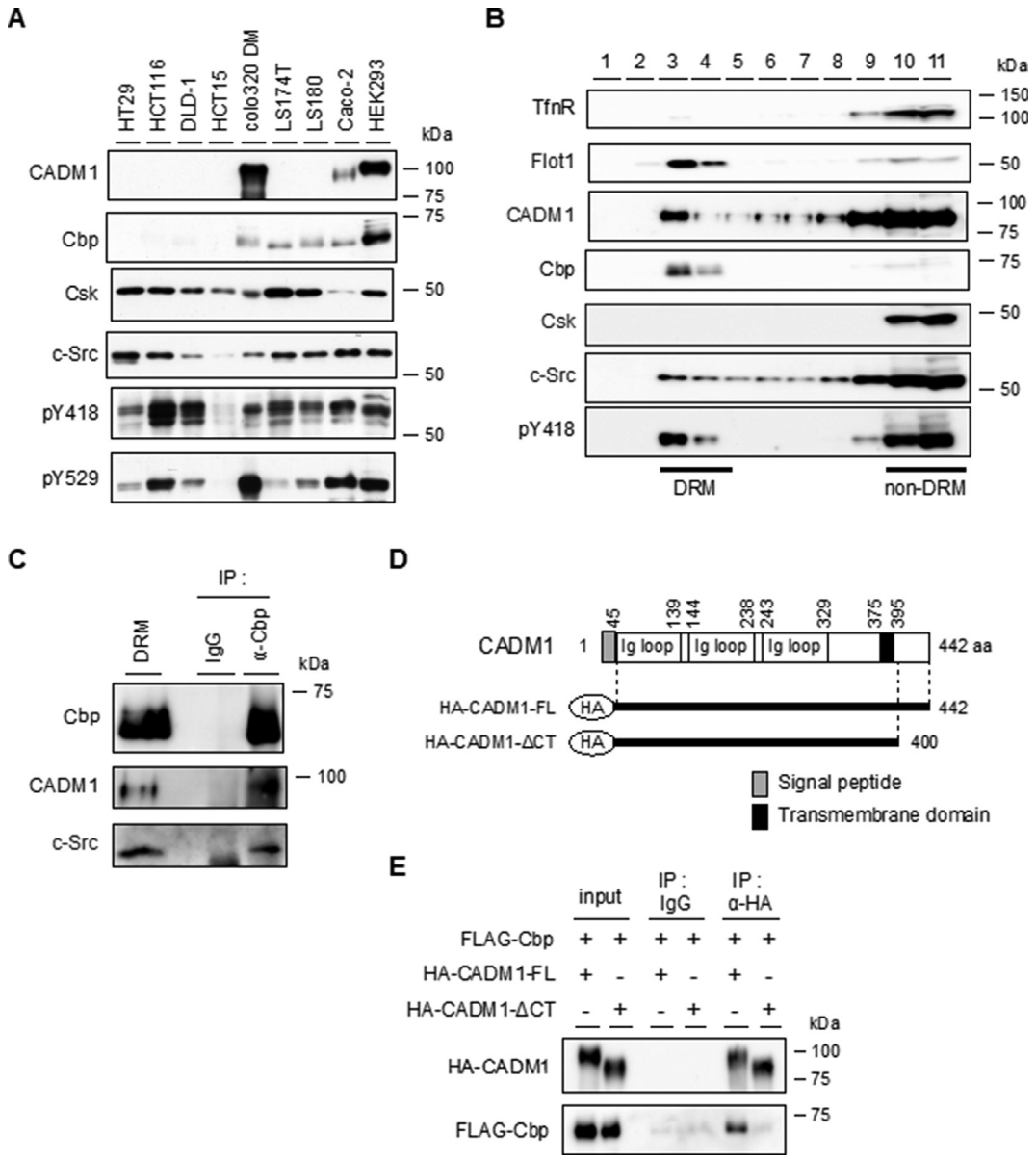

Fig. 1. Association of CADM1, Cbp and c-Src in detergent-resistant membrane (DRM) fraction. (A) Immunoblotting of CADM1 and related proteins in membrane fractions from 8 colon cancer and HEK293 cells. (B) Immunoblotting of CADM1 and related proteins in a series of fractions (1-11) from HEK293 cells separated on sucrose density gradients. (C) Immunoprecipitation of solubilized DRM fractions from HEK293 cells with anti-Cbp antibody. The broad signal detected by anti-c-Src antibody in the middle lane is derived from non-specific reaction with IgG. (D) Schematic model of CADM1 protein. (E) Immunoprecipitation of full-length CADM1 (HA-CADM1-FL) and its cytoplasmic deletion mutant (HA-CADM1- ΔCT) with Cbp in HEK293 cells.

2.9. Immunohistochemistry (IHC)

Tissue microarray of 39 colon cancer was purchased from US BIOMAX (Rockville, MD, USA). The study was approved by the Institutional Ethics Review Committee. IHC staining for CADM1 and Cbp was performed using C-18 and anti-Cbp (MEM-255) antibody, respectively as previously described [20]. Tumors were defined with scores of 1 (11-30% cells with membrane expression), 2 (31-60%), or 3 (61-100%) as positive staining for CADM1 expression and tumors with a score of 0 (0-10%) as negative staining.

2.10. Statistical analysis

The statistical difference was determined by the two-tailed Student's *t*-test or Pearson's chi-square test. A *p*-value of <0.05 was considered as statistically significant.

3. Results

3.1. Csk-binding protein (Cbp) is identified as a CADM1-associated protein

To identify novel CADM1-associated molecules, proteins were extracted from bone marrow-derived cultured mast cells from C57BL/6 mice (BMMCs-B6) and from *Cadm1* knockout mice (BBMCs- *Cadm1*$^{-/-}$) as a control and immunoprecipitants with anti-CADM1 antibody were analyzed by mass spectrometry (MS). Among proteins detected only in cell extracts from BMMCs-B6 but not from BMMCs-*Cadm1*$^{-/-}$, we picked up membrane proteins including CADM1 and focused on Csk-binding protein, Cbp, as a

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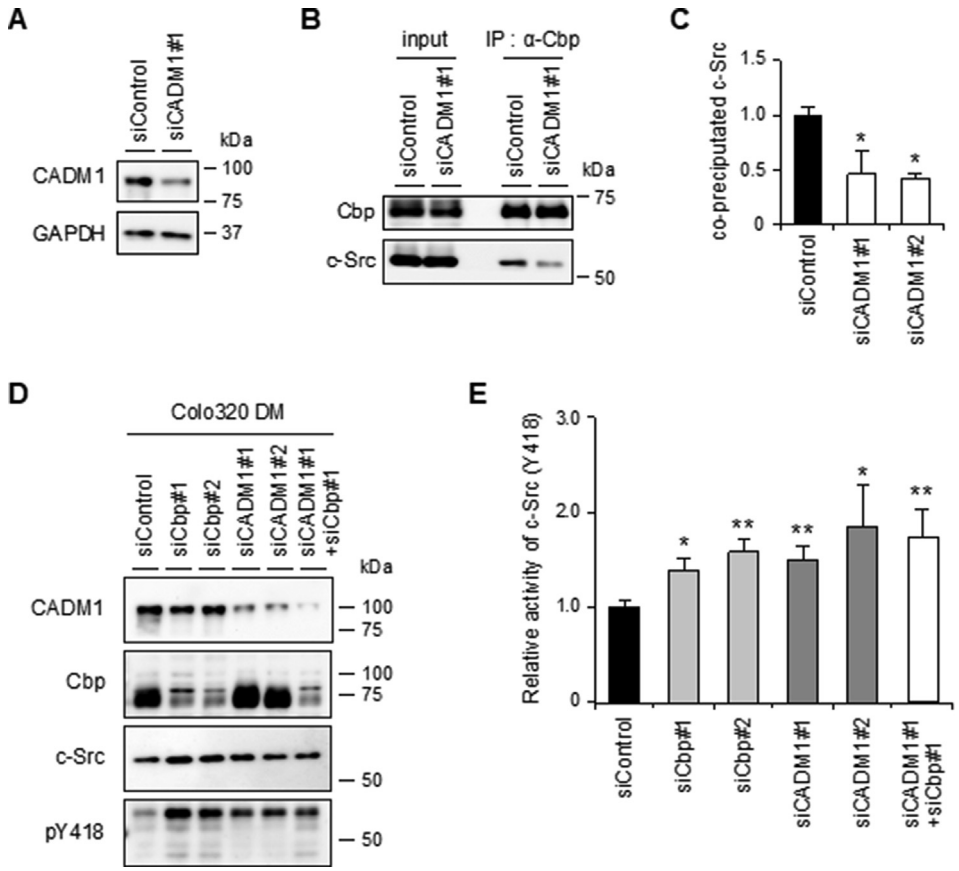

Fig. 2. Down-regulation of CADM1 expression reduced the association of Cbp and c-Src and enhanced c-Src activation. (A) Down-regulation of CADM1 by siRNA in HEK293 cells. (B, C) Reduced amounts of co-immunoprecipitated c-Src with Cbp by down-regulation of CADM1. The relative intensity of co-precipitated c-Src with Cbp (C) calculated from the signal intensity of co-precipitated c-Src (B). n ≥ 3, *, $P < 0.01$. (D) Expression of CADM1 and related proteins in membrane fraction in Colo320DM cells after transfection of various siRNA. (E) The relative activity of c-Src (Y418) calculated from the signal intensity of total c-Src and phosho-Src (pY418). N ≥ 3, *, $P < 0.05$, **, $P < 0.01$.

CADM1-associated protein candidate.

3.2. CADM1 enhances Cbp-Src association by forming a complex in lipid raft

Protein expression of CADM1 and Cbp was markedly down-regulated in 6 (75%) and 4 (50%) out of 8 colon cancer cells, respectively, whereas c-Src and Csk were expressed in all 8 cells. Since Cbp-mediated inactivation of c-Src was dependent on its localization in lipid rafts [14], we examined the membrane distribution of CADM1. We separated DRM fractions, including lipid rafts, using sucrose gradient centrifugation [21]. Flot1, a marker of DRM fractions, was detected in fractions 3 and 4, whereas TfnR, a marker of non-DRM fractions, was in fractions 9-11, indicating that fractions 3 and 4 and 9-11 were DRM and non-DRM fractions, respectively (Fig. 1B). Notably, Cbp was detected only in DRM fractions, whereas CADM1 and c-Src were detected in both DRM and non-DRM fractions. Immunoprecipitation of solubilized DRM fractions with anti-Cbp antibody demonstrated that CADM1 and c-Src were co-precipitated with Cbp, indicating that CADM1, Cbp and c-Src form a protein complex in lipid rafts (Fig. 1C).

To investigate the region of CADM1 responsible for forming a complex with Cbp, we constructed a deletion mutant lacking a cytoplasmic domain of CADM1 (CADM1-ΔCT) (Fig. 1D). The expression vector of FLAG-tagged Cbp (FLAG-Cbp) and either HA-tagged full-length of CADM1 (HA-CADM1-FL) or mutant CADM1 (HA-CADM1-ΔCT) were co-transfected into HEK293 cells, and protein extracts were immunoprecipitated with anti-HA antibody (Fig. 1E). Co-precipitated Cbp with CADM1-ΔCT was markedly reduced in comparison with that with CADM1-FL, suggesting that the cytoplasmic domain of CADM1 is important for forming a complex with Cbp.

Next, we examined whether CADM1 is involved in association of Cbp and c-Src in lipid rafts. Since Cbp is localized only in DRM fractions (Fig. 1B), cell lysates from HEK293 were immunoprecipitated with anti-Cbp antibody to examine the association of Cbp and c-Src in lipid rafts. When CADM1 expression was down-regulated using siRNA against CADM1 (Fig. 2A), co-immunoprecipitated c-Src with Cbp was reduced in comparison with that in cells treated with control siRNA (Fig. 2B and 2C). These findings suggest that CADM1 forms a protein complex with Cbp and c-Src, and promotes Cbp-Src association in lipid rafts.

3.3. CADM1-knockdown increased c-Src activation in colon cancer cell lines

To examine whether CADM1 regulates c-Src activity, siRNA against Cbp (siCbp) or CADM1 (siCADM1) were transfected into a colon cancer cell line, Colo320 DM, which expresses both CADM1 and Cbp (Fig. 2D) and the membrane fractions were examined by western blotting. Kock-down of Cbp by siCbp did not affect the expression level of CADM1, whereas knock-down of CADM1 by siCADM1 did not affect Cbp expression, *vice versa*. On the other hand, activated c-Src of Cbp-knockdown cells (siCbp#1, #2) detected by phosphorylation of Y418 significantly increased compared with that of siControl in Colo320 DM cells (Fig. 2D and

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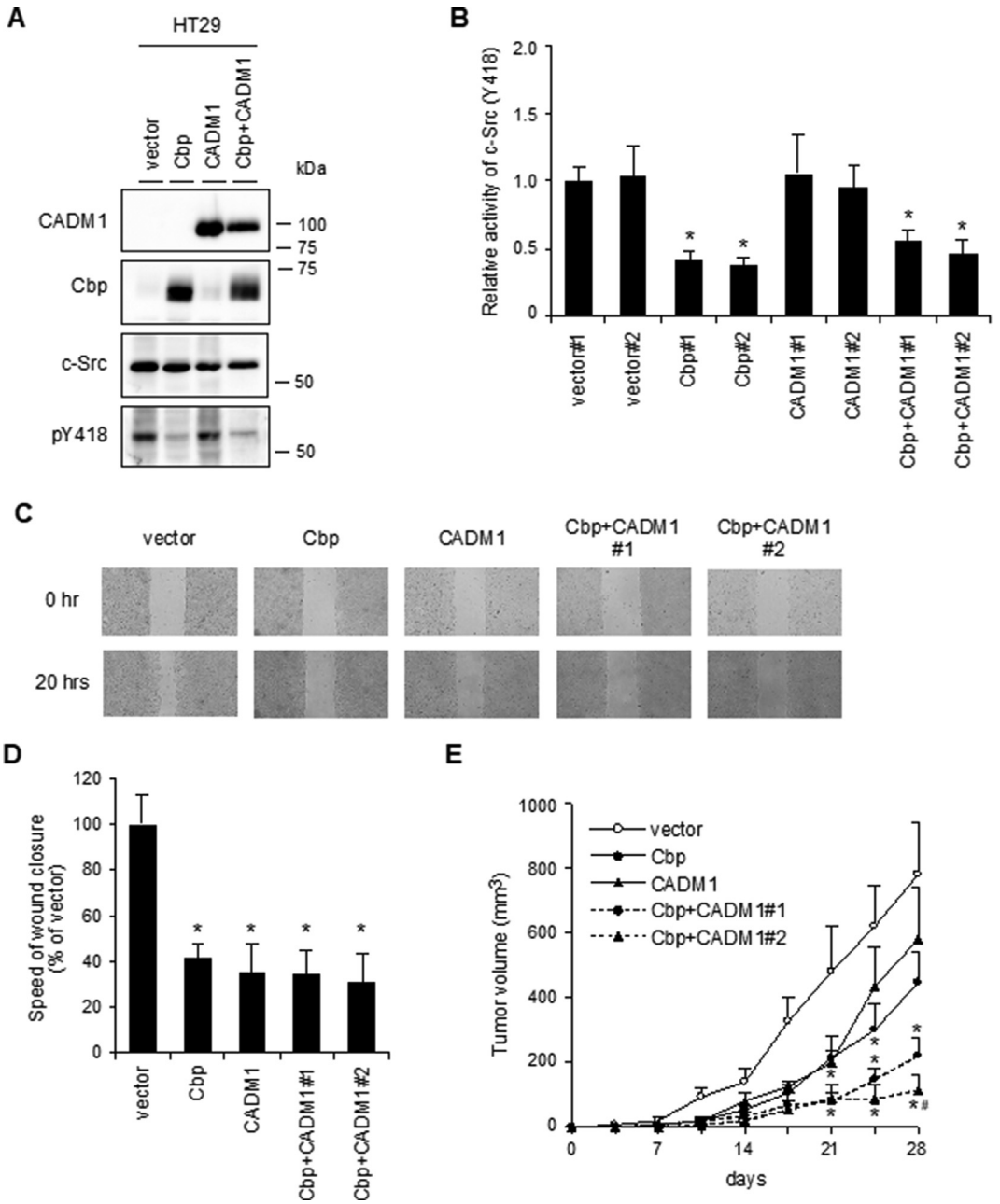

Fig. 3. Expression of CADM1 and Cbp suppressed c-Src activation, wound healing, and tumorigenesis in HT29 cells. (A) Immunoblotting of CADM1 and related proteins in membrane fractions from HT29 derivatives. (B) The relative activity of c-Src (Y418) calculated from the signal intensity of total c-Src and phosho-Src (pY418). N ≥ 3, *, $P < 0.05$. (C) Pictures of wound healing assay in H29 derivatives at 0 and 20 h after Culture-Inserts were removed. (D) The relative speed of wound closure in HT29 derivatives calculated from wounded area measured in (C). n ≥ 4, *, $P < 0.001$. (E) Subcutaneous tumor formation of HT29 derivatives into BALB/c nu/nu mice. N ≥ 3, *, $P < 0.05$ (vs. vector).

2E). The amount of activated c-Src of CADM1-knockdown cells (siCADM1#1, #2) also increased significantly, indicating that CADM1 suppresses c-Src activation. No synergistic effect on c-Src activation was observed when both CADM1 and Cbp were knocked down (siCADM1#1 + siCbp#1), suggesting that CADM1 and Cbp suppresses c-Src activation in the identical pathway.

3.4. CADM1 suppresses activation of c-Src pathway in colon cancer cells

Next, expression of Cbp and/or CADM1 were introduced in colon cancer cell, HT29, lacking both CADM1 and Cbp expression (Fig. 3A). Expression of Cbp (Cbp #1, #2) markedly suppressed activation of c-Src, whereas expression of CADM1 (CADM1 #1, #2) did not suppress c-Src activation (Fig. 3A and 3B). Co-expression of CADM1 with Cbp in two independent clones (CADM1+Cbp #1, #2) also suppressed activation of c-Src significantly without any synergistic effect. Similar results were obtained when Cbp and/or CADM1 were expressed in another colon cancer cell HCT116, lacking both CADM1 and Cbp expression (Figure S1A). These findings indicate that CADM1 suppresses c-Src activation in Cbp-dependent manner. On the other hand, the wound-healing assay showed that cell motility of HT29 was suppressed not only by single replacement of Cbp but also by that of CADM1 (Fig. 3C and 3D), implying that CADM1 suppresses cell motility in Cbp-Src-independent manner. Following this, we examined the subcutaneous tumor

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Table 1
Expression of Cbp and/or CADM1 and clinicopathological factors in 39 cases of primary colon cancer.

	Expression of Cbp/CADM1			*P*-value
	(+)/(+)	(+)(-) or (-)(+)	(-)/(-)	
Grade				
Low	4	13	14	0.56
high	0	4	4	
Stage				
I-II	4	11	11	0.32
III-IV	0	6	7	
pT				
T1	0	0	0	0.038*
T2	1	4	1	
T3	3	9	5	
T4	0	4	12	
pN**				
N0	6	6	10	0.11
N1-4	0	6	6	

*$P = 0.0087$ in T1-T3 vs T4.
**pN was unknown in 5 cases.

formation of HT29 cells in nude mice (Fig. 3E). Tumor volumes of HT29 cells expressing either Cbp or CADM1 decreased, whereas those of HT29 cells expressing both Cbp and CADM1 more prominently decreased. Similar results were obtained when Cbp and/or CADM1were expressed in another colon cancer cell line, HCT116 (Figure S1B). These findings suggest that CADM1 would suppress colon tumorigenicity in nude mice in Cbp-Src-dependent and independent manner.

3.5. Loss of Cbp and CADM1 in primary human colon cancer

Finally, we examined the expression of CADM1 and Cbp in 39 human colon cancer by immunohistochemistry (IHC). Both proteins were expressed along the cell membrane in normal colonic epithelia, whereas expression of CADM1 and Cbp was lost in 33 (83%) and 22 (55%) tumors, respectively (Figure S2). Loss of CADM1 was significantly correlated with stages (I, II vs III, IV: $P = 0.039$), pT (T2 vs T3 vs T4: $p = 0.018$), and pN (N0 vs N1-N4: $P = 0.028$), whereas loss of Cbp was with pT (T1-T3 vs T4: $P = 0.027$) (Table S3). Interestingly, expression of both, either, and neither of CADM1 and/or Cbp was correlated with pT of tumors (T2 vs T3 vs T4: $P = 0.037$) (Table 1).

4. Discussion

Recently, accumulating evidence suggests that the CAMs regulate signaling pathways, including growth factor receptor signaling and tyrosine kinase signaling, in addition to mediating authentic cell-cell interaction. For instance, it has been reported that NCAM and N-cadherin interact with FGFR and stimulate FGFR signaling in the absence of FGF in neural cells [22]. In endothelial cells, VE-cadherin forms a protein complex with PECAM and VEGFR2 and activates VEGFR2 cascade in the absence of VEGF [23].

In this study, to investigate the signaling pathways regulated by CADM1, CADM1-associated proteins were identified using immunoprecipitation and subsequent MS analysis. Comparison of possible binding molecules from cells from C57BL/6 mice with those from *Cadm1*$^{-/-}$ mice narrowed down the candidate proteins specifically bind to CADM1. Since mast cells, but not fibroblasts or peripheral blood cells, express CADM1 [4,24], we used bone marrow-derived cultured mast cells from wild mice or *Cadm1*$^{-/-}$ mice and successfully identified Cbp as a novel CADM1-associated protein expressed in colon epithelia.

Then, we demonstrated that CADM1 formed a protein complex with Cbp and c-Src in lipid rafts and suggested that CADM1 would promote association of Cbp-Src complex leading to suppression of c-Src signaling. Suppression of either CADM1 or Cbp expression by siRNA activated c-Src without any synergistic effect (Fig. 2D and E), whereas replacement of Cbp, but not CADM1, into cells lacking both CADM1 and Cbp suppressed Src activity (Fig. 3A and B). These results suggest that CADM1 intervenes Src-signaling in Cbp-dependent manner. On the other hand, replacement of only CADM1 did suppress wound healing activity and tumorigenicity in nude mice, assumingly through a unique activity of CADM1 that is independent of Cbp-Src (Fig. 3C-3E). Src-dependent and independent effects of CADM1 appeared to be more drastically demonstrated in tumorigenicity in mice, because CADM1 and Cbp showed synergistic activity in tumor suppression.

IHC of CADM1 and Cbp in human colon cancer tissues confirmed the previous results showing high incidence of loss of expression in CADM1 and Cbp (83% and 55% in this study, respectively) in colon cancer [2,14] (Table 1). More importantly, IHC demonstrated significant correlation between larger size of tumors in pT and loss of neither, either and both of CADM1 and/or Cbp, supporting the synergistic effect of CADM1 and Cbp in human colon cancer as observed in tumor suppressor activity in nude mice (Fig. 3E).

As to the Cbp-Src independent activity of CADM1 in tumor suppression, we have previously reported that CADM1 associates with an actin binding protein, 4.1B/DAL-1 [25], which is also shown to be a tumor suppressor in a variety of cancer [26]. Loss of CADM1 expression induces mislocalization of 4.1B and abrogation of epithelia-like structure, which could cause morphological transformation of cancer cells [8,27]. Therefore, formation of the epithelial cell structure by CADM1-4.1B complex might be one of the Src-independent activities of CADM1 in tumor suppression.

Loss of CADM1 and Cbp was also reported in advanced NSCLC [2,28], suggesting that CADM1 may be involved in lung tumorigenesis through Cbp-Src dependent and independent pathways. In NSCLC, it is also demonstrated that CADM1/Necl-2 associates with ErbB3/ErbB2 on the membrane and suppresses ErbB3/ErbB2 signaling as a tumor suppressor by recruiting a tyrosine phosphatase, PTPN13 [29]. These findings suggest that CADM1 may cross-talk with various tyrosine kinase signaling depending on each tissue by interacting with tyrosine kinases on the cell membrane, where the lipid rafts could provide an interacting field of CADM1 with tyrosine kinases. Further studies would be required to verify this hypothesis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.05.103.

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