Junctional adhesion molecule C (JAM-C) is an immunoglobulin superfamily protein expressed in epithelial cells, endothelial cells, and leukocytes. JAM-C has been implicated in leukocyte transendothelial migration, angiogenesis, cell adhesion, cell polarity, spermatogenesis, and metastasis. Here, we show that JAM-C undergoes S-palmitoylation on two juxtamembrane cysteine residues, Cys-264 and Cys-265. We have identified DHHC7 as a JAM-C palmitoylating enzyme by screening all known palmitoyltransferases (DHHCs). Ectopic expression of DHHC7, but not a DHHC7 catalytic mutant, enhances JAM-C S-palmitoylation. Moreover, DHHC7 knockdown decreases the S-palmitoylation level of JAM-C. Palmitoylation of JAM-C promotes its localization to tight junctions and inhibits transwell migration of A549 lung cancer cells. These results suggest that S-palmitoylation of JAM-C can be potentially targeted to control cancer metastasis.

Junctional adhesion molecule C (JAM-C) is a member of the JAM family and is localized at the cell-cell contact sites, in particular the tight junction (TJ) region of endothelial and epithelial cells (1–3). JAM-C is a type I transmembrane protein with two conserved immunoglobulin-like domains in the extracellular amino (N)-terminal region (Fig. 1A). At its intracellular carboxyl (C)-terminal region, JAM-C contains a PDZ-binding domain motif, which allows JAM-C to interact with other PDZ motif-containing proteins at the TJ, including ZO-1 and PAR-3 (1–3).

JAM-C has been found to regulate leukocyte adhesion and transmigration across endothelial cells through a heterophilic interaction between endothelial JAM-C and leukocyte integrin MAC-1 (αMβ2) (4). JAM-C is essential for polarized round spermatids, and JAM-C-deficient mice have defective spermatid differentiation (5). JAM-C deletion in mice leads to neuropathy, and some Jam-C mutations affect the integrity of the vascular system and brain (6, 7).

Recently, JAM-C has been shown to play a role in metastasis and development of certain cancer cells (8–11). In melanoma cells, the homophilic JAM-C/JAM-C trans-interaction between melanoma cells and endothelial cells promotes metastasis (12). Additionally, the JAM-C expression level in fibrosarcoma and lung cancer cells is reported to be positively correlated to metastasis. Knocking down JAM-C in highly metastatic lung cancer cells leads to a decrease in cell migration (8, 13). JAM-C, therefore, could be a therapeutic target for certain cancers.

Cys palmitoylation (S-palmitoylation), a reversible lipid post-translational modification, is the addition of a 16-carbon palmitoyl group onto cysteine residues of proteins via a labile thioester bond. S-Palmitoylation plays a crucial role in cell signaling, localization, and protein-protein interactions (14, 15). Palmitoyltransferases (DHHCs) catalyze S-palmitoylation. To date, 23 mammalian DHHCs have been identified (16, 17). Here we showed that JAM-C undergoes S-palmitoylation on two membrane-proximal cysteine residues (Cys-264 and Cys-265), and this modification can be catalyzed by DHHC7. We found that S-palmitoylation of JAM-C promotes its localization to the cell-cell contact region and regulates cell migration.

### Results

JAM-C Is S-Palmitoylated on Two Conserved Juxtamembrane Cysteine Residues, Cys-264 and Cys-265.—In addition to a PDZ-binding domain, the JAM-C intracellular C terminus contains two transmembrane proximal cysteine residues (Fig. 1A). These two cysteine residues are conserved across multiple mammalian species, suggesting these residues may have an important role (Fig. 1B). Moreover, JAM-C has been identified in palmitoyl-protein proteomics studies as a protein that potentially has Cys palmitoylation, but it was not validated (18). To test whether S-palmitoylation occurs on JAM-C, we used a metabolic labeling strategy using an alkyn-tagged palmitic acid analogue (Alk14) (19, 20). Jurkat cells and human umbilical vein endothelial cells were cultured in the presence of Alk14, and endogenous JAM-C was immunoprecipitated and conjugated to BODIPY-azide via click chemistry (Fig. 2A). JAM-C had fluorescent labeling, suggesting that it contains fatty acylation (Fig. 2B). Similar results were obtained from ectopically expressed FLAG-tagged JAM-C in human embryonic kidney 293T (HEK-293T) cells (Fig. 2C and supplemental Fig. S1).
To further confirm that this modification occurs on cysteine residues, we mutated Cys-264 and Cys-265 to serine. We observed a significant decrease in the fluorescence signal of the single cysteine mutants C264S and C265S. However, the C264S mutation had a more profound effect on the fluorescence signal. Furthermore, we observed no fluorescence signal for the double cysteine mutant C264S/C265S (CCSS for short). These results indicated that S-palmitoylation on JAM-C depends on both Cys-264 and Cys-265 (Fig. 2, D and E). After the samples were treated with 0.5 m hydroxylamine (pH 10.0), most of the fluorescence signal on FLAG-JAM-C was removed (Fig. 2F), further confirming that JAM-C has cysteine palmitoylation. However, there was some hydroxylamine-resistant signal, similar to what was observed with TNF-α, a protein with lysine myristoylation (21).

To ensure that the hydroxylamine treatment was sufficient, we compared the labeling of FLAG-JAM-C before and after hydroxylamine treatment with the labeling of the FLAG-TNFα mutant (K19R/K20R), a protein that has only cysteine palmitoylation (21), as well as other membrane proteins reported to have S-palmitoylation such as transferrin receptor 1 (22) and Syntaxin6 (18). After hydroxylamine treatment, the majority, if not all, of the labeling on the other proteins was removed. However, some FLAG-JAM-C fluorescence signal remained (supplemental Fig. S2, A and B). After hydroxylamine treatment, the fluorescence signal of the HA and FLAG pulldown experiments. Indeed, HA-tagged DHHC7 was able to pull down FLAG-tagged JAM-C and vice versa, suggesting that JAM-C physically interacts with DHHC7 (Fig. 4C). In contrast, FLAG-JAM-C and HA-DHHC15 did not exhibit a strong interaction as the signal is only slightly above the background on the Western blots (Fig. 4C).

To confirm that DHHC7 can directly catalyze the palmitoylation of JAM-C, we examined the S-palmitoylation of DHHC7 and a catalytic dead mutant, DHHS7, in which the conserved catalytic cysteine residue is mutated to serine. As expected, only overexpression of DHHC7, but not DHHS7, augmented the JAM-C fatty acylation level in HEK-293T cells (Fig. 4A). Similar results were also obtained in U87 cells (Fig. 4B). These results suggest that the catalytic activity of DHHC7 is required for the S-palmitoylation of JAM-C.

Additionally, we investigated whether DHHC7 could interact with JAM-C. HA-tagged DHHC7 was co-overexpressed with FLAG-tagged JAM-C in HEK-293T cells. We carried out HA and FLAG pulldown experiments. Indeed, HA-tagged DHHC7 was able to pull down FLAG-tagged JAM-C and vice versa, suggesting that JAM-C physically interacts with DHHC7 (Fig. 4C). In contrast, FLAG-JAM-C and HA-DHHC15 did not exhibit a strong interaction as the signal is only slightly above the background on the Western blots (Fig. 4C).

**JAM-C Is a DHHC7 Target**—We next wanted to identify palmitoyltransferases that can control JAM-C S-palmitoylation. In mammals, there are 23 DHHC enzymes known to act as palmitoyltransferases. We co-overexpressed HA-tagged DHHC1–23 with FLAG-tagged JAM-C in HEK-293T cells and examined the fatty acylation level using the Alk14 metabolic labeling approach. From the initial DHHC screening and subsequent comparison of JAM-C palmitoylation levels with DHHC7 and DHHC15 overexpressed (Fig. 3, A and B, and supplemental Fig. S3), we decided to further validate DHHC7 as a palmitoyltransferase of JAM-C. However, because the screening result might be complicated by DHHC and JAM-C expression levels, we could not rule out that other DHHCs may also palmitoylate JAM-C.

**Knockdown of DHHC7 Decreases JAM-C S-Palmitoylation**—Ectopic expression of the DHHCs might affect the selectivity of the enzyme and possibly lead to an increase in S-palmitoylation of nonspecific targets (23). To verify that JAM-C is a palmitoyltransferase of endogenous DHHC7, we generated DHHC7, DHHC12, and DHHC15 stable knockdown HEK-293T cells using shRNAs targeting DHHC7, DHHC12, and DHHC15 mRNA, respectively. We then examined the JAM-C palmitoylation level (Fig. 5, A–C). Knockdown of DHHC7 led to a
decrease in the JAM-C palmitoylation level compared with the control with scrambled shRNA, whereas no significant difference in JAM-C palmitoylation was observed in DHHC12 and DHHC15 knockdown cells. These results demonstrate that JAM-C is a palmitoylation target of endogenous DHHC7. Based on the BioGPS gene database, the DHHC7 gene expression level in the lung is relatively high compared with other tissues. Thus, we also looked at JAM-C palmitoylation in

**FIGURE 2. S-Palmitoylation of JAM-C depends on Cys-264 and Cys-265.** A, method for the detection of S-palmitoylation in JAM-C with Alk14, the palmitic acid analogue. Cells were cultured with Alk14 for metabolic labeling, JAM-C was immunoprecipitated from total lysate, and BODIPY-azide was then conjugated to the alkyne group using click chemistry. The fluorescence signal was imaged after SDS-PAGE. B, endogenous JAM-C in both Jurkat and human umbilical vein endothelial cells (HUVEC) was labeled by Alk14. C, overexpressed FLAG-tagged JAM-C in HEK-293T cells also contained fatty acylation. D, the C264S and C265S mutations of JAM-C decreased the Alk14 labeling signal. Compared with the wild type JAM-C, palmitoylation in the single cysteine mutants (C264S and C265S) was reduced, whereas it was abolished in the double cysteine mutant (CCSS). E, quantified fatty acylation level of the JAM-C mutants relative to WT (n = 3; error bars represent S.D.). The palmitoylation signal was quantified and normalized by the protein level on the Coomassie Blue gel using Quantity One software. ***, p < 0.001. F, the cytosolic lysine to arginine mutants 3KR and 4KR of JAM-C did not significantly reduce the Alk14 labeling signal. Representative results from two independent experiments are shown. IP, immunoprecipitation; WB, Western blotting; alk, Alk14.
JAM-C Palmitoylation

A

IP FLAG-JAM-C

| Ctrl | DHHC1 | DHHC2 | DHHC3 | DHHC4 | DHHC5 | DHHC6 | DHHC7 | DHHC8 | DHHC9 | DHHC10 | DHHC11 | DHHC12 | DHHC13 |
|------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|--------|--------|--------|
| Ctrl |       |       |       |       |       |       |       |       |       |        |        |        |        |
| Ctrl |       |       |       |       |       |       |       |       |       |        |        |        |        |
| Ctrl |       |       |       |       |       |       |       |       |       |        |        |        |        |

Fluorescence

Coomassie Blue

B

Fatty acylated JAM-C level
(relative to control)

C

IP FLAG-Jam3

| Ctrl (Res-Asx) | DHHC1 | DHHC7 | DHHC10 | DHHC15 |
|----------------|-------|-------|--------|--------|
| Ctrl           |       |       |        |        |
| Ctrl           |       |       |        |        |
| Ctrl           |       |       |        |        |

Fluorescence

Coomassie Blue

WB: HA

D

Fatty acylated JAM-C level
(relative to control)

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*
A549 lung cancer cells. DHHC7 stable knockdown in A549 cells decreased JAM-C palmitoylation (Fig. 5, D and E).

**S-Palmitoylation Promotes JAM-C Localization to the TJ**—To further understand the physiological function of JAM-C S-palmitoylation, we investigated whether this modification is required for the TJ localization of JAM-C. FLAG-tagged JAM-C WT and the palmitoylation-deficient CCSS mutant were ectopically expressed in A549 lung cancer cells. The cells were stained with anti-FLAG and anti-ZO-1 antibodies to visualize JAM-C and the TJ, respectively. Images were viewed and analyzed using ZEN 2012 imaging software (Zeiss). Both WT and the CCSS mutant were localized on plasma membrane or intracellular organelles. However, JAM-C WT was more concentrated at the TJ (white arrow) as indicated by the co-localization with ZO-1, whereas the CCSS mutant had very little co-localization with ZO-1 (Fig. 6). The co-localization between JAM-C and ZO-1 was quantified using the Coloc2 plug-in in Fiji software and is presented as the mean of Manders’ coefficient (M1 and M2) ± S.D. Thus, S-palmitoylation of JAM-C facilitates its localization to the TJ.

**JAM-C S-Palmitoylation Affects Cell Migration**—Because JAM-C S-palmitoylation affects its localization to the cell-cell contact TJ regions, we wondered whether it would also affect cell-cell adhesion. We therefore decided to check whether JAM-C S-palmitoylation could affect cell migration, which is influenced by cell-cell adhesion. A549 cells were transfected with FLAG-tagged JAM-C WT or the CCSS mutant for 24 h. We used a transwell migration assay, which was performed in a 24-well Transwell plate with 8-µm polycarbonate sterile membranes. Overexpression of JAM-C WT dramatically decreased cell migration compared with control cells that were transfected with an empty vector. In contrast, overexpression of the non-palmitoylatable CCSS mutant only slightly decreased the cell migration compared with control cells that were transfected with an empty vector (Fig. 7, A and B). The difference in cell migration was not due to the variation of cell proliferation.

| FIGURE 3. | DHHC7 overexpression enhances the palmitoylation of JAM-C. A, the palmitoylation levels of FLAG-tagged JAM-C co-overexpressed with different HA-tagged DHHC1–23 in HEK-293T cells. B, quantification of the results shown in A (mean ± S.D., n = 2). The palmitoylation signal was quantified and normalized with the protein levels on the Coomassie Blue gel using Quantity One software. The signal in control cells without DHHC overexpression was set to 1.00 and served as the reference point for all other samples. C, DHHC7 overexpression most significantly increased JAM-C palmitoylation. A representative result from two independent experiments is shown. D, the quantified fatty acylation level of JAM-C co-overexpressed with DHHC7, DHHC10, or DHHC15 relative to control (n = 2; error bars represent S.D.). *, p < 0.05; ***, p < 0.001. IP, immunoprecipitation; WB, Western blotting; Ctrl, control.

| FIGURE 4. | DHHC7 interacts with JAM-C, and its catalytic activity is required for JAM-C S-palmitoylation. A, overexpression of DHHC7, but not DHHS7, increased JAM-C S-palmitoylation in HEK-293T cells. HA-tagged DHHC7 or HA-tagged DHHS7 was co-overexpressed with FLAG-tagged JAM-C. FLAG-tagged JAM-C was pulled down for palmitoylation detection. B, the same experiment as in A was performed in U87 cells. The full FLAG JAM-C input Western blot can be found in supplemental Fig. S5. C, DHHC7 interacts with JAM-C. HA-tagged DHHC7 was co-overexpressed with FLAG-tagged JAM-C in HEK-293T cells. HA-tagged DHHC7 was pulled down, and FLAG-tagged JAM-C was detected by Western blotting and vice versa. In the two lanes labeled with mixed lysate, JAM-C and DHHC7 or DHHC15 were expressed in different cells, and then the cell lysate was mixed before IP. A representative result from two independent experiments is shown. IP, immunoprecipitation; WB, Western blotting; Ctrl, control.

| FIGURE 5. | DHHC7 overexpression enhances the palmitoylation of JAM-C. A, the palmitoylation levels of FLAG-tagged JAM-C co-overexpressed with different HA-tagged DHHC1–23 in HEK-293T cells. B, quantification of the results shown in A (mean ± S.D., n = 2). The palmitoylation signal was quantified and normalized with the protein levels on the Coomassie Blue gel using Quantity One software. The signal in control cells without DHHC overexpression was set to 1.00 and served as the reference point for all other samples. C, DHHC7 overexpression most significantly increased JAM-C palmitoylation. A representative result from two independent experiments is shown. D, the quantified fatty acylation level of JAM-C co-overexpressed with DHHC7, DHHC10, or DHHC15 relative to control (n = 2; error bars represent S.D.). *, p < 0.05; ***, p < 0.001. IP, immunoprecipitation; WB, Western blotting; Ctrl, control.
as there was no significant difference in cell proliferation when JAM-C WT and the CCSS mutant were overexpressed (data not shown). Thus, S-palmitoylation of JAM-C affects cell migration.

Discussion

Protein lipidation has become a more widely identified class of protein post-translational modifications and has been found to be involved in numerous biological pathways (15, 24). Here, using a bio-orthogonal palmitic acid probe (19, 20), we demonstrated that both endogenous and ectopically expressed JAM-C contain S-palmitoylation. S-Palmitoylation occurs on Cys-264 and Cys-265 of JAM-C. The S-palmitoylation on JAM-C is relatively resistant to hydroxylamine treatment for which the reason remains unclear. Because the hydroxylamine cleavage depends on the environment (25), one plausible explanation is the S-palmitoylation in the membrane-proximal region of JAM-C may be shielded from hydroxylamine attack. However, the decrease in labeling after hydroxylamine treatment supports that JAM-C has S-palmitoylation, which is further supported by mutagenesis studies.

The palmitoylacyltransferase family members share a conserved Asp-His-His-Cys (DHHC)-cysteine-rich domain as a catalytic domain of the enzyme (26, 27). DHHCs have broad substrate specificity, and how substrate specificity is determined remains unclear. By screening the 23 DHHCs, we have found that overexpression of DHHC7 can substantially increase the JAM-C palmitoylation level. Moreover, we showed that knockdown of DHHC7 decreased the S-palmitoyl level of JAM-C, supporting that JAM-C is a direct palmitoylation target of endogenous DHHC7. Nevertheless, other DHHCs may also be able to regulate JAM-C palmitoylation as some DHHCs have redundant functions and act on the same targets (28).

FIGURE 5. JAM-C palmitoylation level decreases in DHHC7 knockdown cells. A, HEK-293T cells were infected with lentiviruses containing scrambled shRNA (control) or DHHC7, DHHC12, or DHHC15 shRNAs. Puromycin-resistant cells were selected for stable DHHC knockdown cells and used for FLAG-tagged JAM-C overexpression. The FLAG-tagged JAM-C in the DHHC knockdown cells was then immunoprecipitated and detected for palmitoylation levels by fluorescence labeling. B, semiquantitative RT-PCR showing the mRNA levels of Dhhc7, Dhhc12, and Dhhc15 in the stable knockdown HEK-293T cells. C, quantified fatty acylation levels of FLAG-tagged JAM-C expressed in DHHC7, DHHC12, and DHHC15 knockdown HEK-293T cells relative to control (mean ± S.D., n = 2). The palmitoylation level from each group was quantified and normalized with the corresponding protein level on the Coomassie Blue gel using Quantity One software. The signal from FLAG-tagged JAM-C in the control knockdown cells was set to 1.00 and served as the reference point for the other samples. D, DHHC7 knockdown in A549 cells also decreased the palmitoylation of JAM-C. The experiments were carried out similar to that described in A. E, quantified fatty acylation levels of JAM-C in A549 cells (n = 2; error bars represent S.D.). *, p < 0.05; **, p < 0.01. IP, immunoprecipitation; WB, Western blotting; Ctrl, control.
lation of JAM-C (supplemental Fig. S4). We could not determine whether the CCSS JAM-C mutant can affect the phosphorylation on JAM-C due to the lack of proper antibodies to detect Ser-281 phosphorylation.

JAM-C has been reported to have homophilic interactions with JAM-C and heterophilic interactions with other PDZ domain-containing proteins such as ZO-1 and PAR3 at the cell tight junction. A possible explanation for the effect of palmitoylation on JAM-C localization is that S-palmitoylation facilitates the interaction of JAM-C with its interacting partners, leading to the enrichment of JAM-C at the cell-cell contact regions.

We demonstrated for the first time that JAM-C palmitoylation can affect cell migration. Overexpression of WT JAM-C decreased migration of A549 cells, but overexpression of the non-palmitoylatable JAM-C mutant did not affect the migration much. S-Palmitoylation of CD44, an adhesion protein, has previously been found to decrease the migration of breast cancer cells, and the invasiveness of cancer is negatively correlated with palmitoylation status of CD44 (31). This is similar to our finding that WT JAM-C decreases the cell migration of A549 cells, but the non-palmitoylatable mutant did not. The effect of JAM-C palmitoylation on cell migration is likely connected to
its effect on the cell-cell contact localization of JAM-C and cell tight junction integrity. However, exactly how JAM-C palmitoylation regulates cell migration remains elusive.

Currently, only a few substrate proteins for DHHC7 are known, including Fas (32), sex steroid receptors (33), phosphatidylinositol 4-kinase IIα (34), Gα (35), stress-regulated exon (28) and Scribble (36). Our findings expand the substrate scope of DHHC7, which eventually will help to understand the substrate specificity and function of different DHHCs.

Interestingly, a decrease in DHHC7 levels has been related to tumorigenesis (37), suggesting that DHHC7 may play a role in preventing tumorigenesis. Our findings that JAM-C palmitoylation affects cancer cell migration and that DHHC7 directly controls the palmitoylation level of JAM-C suggest that pharmacologically controlling DHHC7 could be a useful strategy to control cancer cell migration and possibly cancer metastasis. However, to fully take advantage of this, a more detailed understanding of the function of DHHC7 is needed.

Experimental Procedures

**JAM-C Cloning and Expression**—Human JAM-C cDNA was purchased from Transomic (clone ID BC012147). The full-length cDNA was PCR-amplified by Platinum® Pfx DNA polymerase (ThermoFisher) and subcloned into the pCMV-tag 4a vector using the BamHI and EcoRV restriction sites using the following primers: sense, 5′-AGTCAGGGATCCATGGCGCTGAGGCGGCCA-3′; antisense, 5′-AGTCAGGATATC-GATCACAAACGATGACTTTGTCG-3′. JAM-C mutants (C264S, C265S, and CCSS) were generated by QuikChange GATCACAAACGATGACTTGTGTCT-3′ amplified using Phusion high fidelity DNA polymerase and high fidelity DNA polymerase and the following mutagenic primers (the underlined nucleotide is the mutagenic codon): C264S: sense, 5′-AGTCAGGGATCCATGGCGCCTGAGGCGGCCA-3′; antisense, 5′-AGTCAGGATATC-GATCACAAACGATGACTTTGTCG-3′. JAM-C mutants (C264S, C265S, and CCSS) were generated by QuikChange mutagenesis. The JAM-C in pCMV-tag 4a vector was PCR-amplified using Phusion high fidelity DNA polymerase and the following mutagenic primers (the underlined nucleotide sequence code for the mutated amino acid): C264S: sense, 5′-CCCTGATACGCTTGCCATCAGCTTGATACAGACGTTGCTA-3′; antisense, 5′-GATGCCAACGTGATCCAGACGTTGCTA-3′; C265S: sense, 5′-TGATCACGTTGGGCATCAGCTGCAACGTGATCA-3′; antisense, 5′-GATCACGTTGGGCATCAGCTGCAACGTGATCA-3′. The plasmids of DHHC1–23 in pEF-BOS-HA master mixture contains the following: 3 M 1,2,3-triazol-4-yl)methyl] amine maleimide and scrambled primer (Sigma). Protein concentration was determined by Bradford assay (Pierce™ Coomassie Protein Assay kit). Protein samples were separated by 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad) for 90 min. The PVDF membrane was blocked with 5% bovine serum albumin (BSA; Santa Cruz Biotechnology) in TBST (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) and incubated with the primary antibody for 3 h at room temperature or overnight at 4 °C. After washing five times with TBST, the membrane was incubated with the secondary antibody for 1 h at room temperature. The membrane was washed five more times with TBST before it was developed in ECL-Plus Western blotting detection reagent (GE Healthcare). The signal was visualized using a Typhoon 9400 variable mode imager (GE Healthcare) with 457-nm excitation and 526-nm detection filters using a photomultiplier tube setting of 600 V (normal sensitivity). The signal was analyzed by ImageQuant TL v2005 and Quantity One (Bio-Rad).

**Metabolic Labeling of Palmitoylation on FLAG-tagged JAM-C**—Cells were transfected with FLAG-JAM-C in pCMV4a vector. After 24 h, the cells were incubated with 50 μM Alk14 in medium supplemented with 10% FBS for 6 h. Cells were collected and washed with 1× phosphate-buffered saline (PBS) three times. The cell pellets were lysed with 1% Nonidet P-40 lysis buffer. The protein concentration was determined by Bradford assay (Pierce Coomassie Protein Assay kit). The expression level of FLAG-JAM-C was confirmed by Western blotting. For FLAG-JAM-C immunoprecipitation, 25 μl of anti-FLAG M2 affinity gel was added into 600 μg of total cell lysate with a final volume of 1 ml in an Eppendorf tube. The samples were gently agitated at 4 °C for 2 h. The samples were then centrifuged at 1,000 × g for 2 min at 4 °C to remove the supernatant. The beads were washed three times with 0.1% Nonidet P-40 lysis buffer. The protein concentration was determined by Bradford assay (Pierce Coomassie Protein Assay kit). The click reaction reagent (Promega) was used for cell transfection according to the manufacturer’s instruction.

**Antibodies**—Anti-FLAG® M2-peroxidase (HRP) antibody (mouse monoclonal IgG) for Western blotting and anti-FLAG M2 affinity gel for immunoprecipitation were purchased from Sigma (catalogue numbers A8592 and A2220, respectively). Anti-FLAG antibody (mouse monoclonal IgG1) for immunofluorescence was purchased from Cell Signaling Technology (catalogue number 8146). Secondary antibody Alexa Fluor® 488 conjugate (mouse polyclonal IgG) was purchased from ThermoFisher (catalogue number A-11001). Anti-HA-peroxidase (rat IgG1) was purchased from Roche Applied Science (catalogue number 12013819001). The anti-human JAM-C antibody (mouse monoclonal IgG) was purchased from Enzo Life Sciences (catalogue number ALX-803-306), and anti-DHHC7 (rabbit IgG) antibody was purchased from Assay-Biotec (catalogue number R12-3691). All other peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology.

**Western Blotting**—Cells were collected and lysed with 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% (v/v) Nonidet P-40 (Igepal) containing protease inhibitor mixtures (Sigma)). Protein concentration was determined by Bradford assay (Pierce™ Coomassie Protein Assay kit). Protein samples were separated by 12% SDS-PAGE and transferred to PVDF membrane (Bio-Rad) for 90 min. The membrane was blocked with 5% bovine serum albumin (BSA; Santa Cruz Biotechnology) in TBST (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) and incubated with the primary antibody for 3 h at room temperature or overnight at 4 °C. After washing five times with TBST, the membrane was incubated with the secondary antibody for 1 h at room temperature. The membrane was washed five more times with TBST before it was developed in ECL-Plus Western blotting detection reagent (GE Healthcare). The signal was visualized using a Typhoon 9400 variable mode imager (GE Healthcare) with 457-nm excitation and 526-nm detection filters using a photomultiplier tube setting of 600 V (normal sensitivity). The signal was analyzed by ImageQuant TL v2005 and Quantity One (Bio-Rad).
DTT, 30% (v/v) glycerol, and 0.006% bromphenol blue) was added to each sample, and the samples were boiled at 95 °C for 5 min and centrifuged at 17,000 × g for 2 min. The supernatants were transferred into new Eppendorf tubes and loaded for 12% SDS-PAGE. The fluorescence signal was visualized with a Typhoon 9400 variable mode imager using 488-nm excitation and 520-nm detection filters and a photomultiplier tube setting of 550 V (normal sensitivity). The signal was analyzed by ImageQuantTL v2005 and quantified by Quantity One within a linear range of exposure.

Generation of DHHC7, DHHC12, and DHHC15 Stable Knockdown in HEK-293T Cell Line—Different DHHC shRNA lentiviral plasmids in pLKO.1-puro vector were purchased from Sigma (catalogue numbers DHHC7-SHCLNG-NM_017740, shRNA1-TRCN0000143647, shRNA2-TRCN0000275633; DHHC12-SHCLNG-NM_025428; and DHHC15-SHCLNG-NM_144969). To generate the lentiviruses, low passage number HEK-293T cells in a 10-cm² plates were transfected with 6 µg of each DHHC shRNA, 4 µg of pCMV-dR8.2, and 2 µg of pM2D.G mixed with 36 µl of FuGENE 6 (Promega). The cell media containing the lentiviruses were collected after 48 h by centrifugation and filtered through 0.45-µm syringe filter to remove cell debris. The day before infection, 3 × 10⁵ cells of low passage number HEK-293T cells were split into each well of a 6-well plate and grown overnight in 2 ml of DMEM with 10% FBS. The media were then removed and replaced with a mixture of 1.5 ml of the lentivirus medium collected above, 0.5 ml of fresh DMEM with 10% FBS, and 6 µg/ml Polybrene (Sigma). After 6 h, 3 ml of DMEM with 10% FBS was added to each well, and after 48 h, 1.5 µg/ml puromycin dihydrochloride (Santa Cruz Biotechnology) was added to the cells to select for stable DHHC knockdown cells.

Cell Migration Assay—A549 cells were transfected with FLAG-JAM-C WT in pCMV4a, the FLAG-tagged CCSS mutant in pCMV4a, or the empty pCMV4a vector (control) for 24 h and then cultured in RPMI 1640 serum-free medium for an additional 14 h. The cell migration assay was performed in a 24-well Transwell plate with 8-µm polycarbonate sterile membrane (Corning Inc.). A total of 3.5 × 10⁴ cells in 200 µl of RPMI 1640 serum-free medium were plated in each upper chamber and then placed in wells containing 600 µl of RPMI 1640 medium supplemented with 10% FBS. After 24 h, the cells on the upper surface were detached with a cotton swab, and the upper chambers were fixed. The cells in the lower filter were stained with 0.1% crystal violet for 15 min and then counted. The quantified results represent three random fields of migrated cells.

Immunofluorescence Microscopy—A total of 2 × 10⁵ A549 cells were split into each glass bottom culture dish (MatTek) and cultured overnight. The cells were then transfected with either the FLAG-JAM-C WT in pCMV4a vector or the CCSS mutant. After 24 h, cells were washed twice with 2 ml of 1× PBS (ThermoFisher), fixed with 4% paraformaldehyde in PBS on ice for 15 min, and then kept at room temperature for 5 min. Then cells were subsequently washed three times with 2 ml of 1× PBS and incubated with 0.1% saponin (TCI America) with 3% BSA in 1× PBS at room temperature. After 1 h, the blocking solution was removed, and the anti-FLAG antibody (rabbit monoclonal IgG1; 1:1,000; Cell Signaling Technology) and the anti-ZO1 antibody (mouse monoclonal IgG1; 1:800; BD Biosciences) in a saponin-BSA solution were added to the cells and incubated at 4 °C overnight. The cells were washed with 1 ml of saponin-BSA solution five times and then incubated with the secondary antibodies conjugated with Alexa Fluor 488 (goat anti-rabbit IgG polyclonal antibody; ThermoFisher; 1:1,000) or Texas Red® (goat anti-mouse IgG polyclonal antibody; ThermoFisher; 1:1,000) in the saponin-BSA solution at room temperature for 1 h. The cells were washed with 1 ml of saponin-BSA solution five times, then mounted with 200 µl of DAPI Fluoromount-G (Southern Biotech), and covered with a cover glass. After 24 h, the cells were visualized with a Zeiss LSM 710 confocal microscope with a 63×/1.4 oil immersion objective. Images were viewed and analyzed using ZEN 2012 imaging software. For JAM-C and ZO-1 co-localization analysis, the Manders’ overlap coefficient with automatic thresholds (M1 and M2) was calculated using the Coloc2 plug-in in Fiji (background subtraction; rolling ball radius, 50 pixels) (38).

Statistical Analysis—Data are shown as mean ± S.D. Differences were analyzed by two-tailed Student’s t test between two groups: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Author Contributions—P. A. and H. L. designed the experiments and wrote the paper. N. A. S. carried out the experiment shown in Figs. 3, A and B, and 4C, and supplemental Figs. S1–S3 and synthesized Alk14. J. C. performed and analyzed the experiment shown in Fig. 7. All authors reviewed and approved the final version of the manuscript.

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