N-glycosylation controls the function of junctional adhesion molecule-A

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ABSTRACT Junctional adhesion molecule-A (JAM-A) is an adherens and tight junction protein expressed by endothelial and epithelial cells. JAM-A serves many roles and contributes to barrier function and cell migration and motility, and it also acts as a ligand for the leukocyte receptor LFA-1. JAM-A is reported to contain N-glycans, but the extent of this modification and its contribution to the protein's functions are unknown. We show that human JAM-A contains a single N-glycan at N185 and that this residue is conserved across multiple mammalian species. A glycomutant lacking all N-glycans, N185Q, is able to reach the cell surface but exhibits decreased protein half-life compared with the wild- type protein. N-glycosylation of JAM-A is required for the protein's ability to reinforce barrier function and contributes to Rap1 activity. We further show that glycosylation of N185 is required for JAM-A–mediated reduction of cell migration. Finally, we show that N-glycosylation of JAM-A regulates leukocyte adhesion and LFA-1 binding. These findings identify N-glycosylation as critical for JAM-A's many functions.

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INTRODUCTION

Junctional adhesion molecule-A (JAM-A) was originally described as a platelet receptor (Naik *et al*., 1995) and later as an adherens and tight junction protein (Martin-Padura *et al*., 1998). JAM-A belongs to the immunoglobulin (Ig) superfamily of cell adhesion receptors and is characterized by two extracellular N-terminal Ig-like domains, a transmembrane domain, and a short C-terminal cytoplasmic tail that terminates with a PDZ-binding domain (Severson and Parkos, 2009). The first extracellular Ig-like domain regulates *cis*- and *trans*-homophilic interactions (Severson *et al*., 2008; Monteiro *et al*., 2014), and the second Ig-like domain serves as a ligand for the leukocyte adhesion receptor LFA-1 (Shaw *et al*., 2004) and stabilizes the aforementioned homophilic interactions (Wojcikiewicz *et al*., 2009). The C-terminus of JAM-A interacts with numerous proteins including zona occludens-1, AF-6 (Ebnet *et al*., 2000), Par3 (Ebnet *et al*., 2001), and CD9 (Peddibhotla *et al*., 2013). JAM-A has been implicated in a number of cellular functions, including leukocyte diapedesis (Martin-Padura *et al*., 1998; Ostermann *et al*., 2002; Shaw *et al*., 2004), platelet activation (Naik *et al*., 1995), angiogenesis (Naik and Naik, 2006), and barrier function (Aurrand-Lions *et al*., 2001; Laukoetter *et al*., 2007), and is a receptor for reovirus (Barton *et al*., 2001). The protein has been implicated in a number of pathologies, including vascular diseases (Babinska *et al*., 2007; Schmitt *et al*., 2014), inflammatory bowel disease (Vetrano and Danese, 2009), and cancers of the breast (Naik *et al*., 2008; McSherry *et al*., 2011; Murakami *et al*., 2011), lung (Zhang *et al*., 2013), and pancreas (Fong *et al*., 2012).

JAM-A, like most immunoglobulin superfamily proteins, is predicted to carry N-glycans on its extracellular domain. Protein N-glycosylation is an enzyme-driven co/posttranslational modification by which a core saccharide is covalently attached to the amide residue of asparagine in an N-X-S/T/C motif (where X cannot be P; Stanley *et al*., 2009). Subsequent processing in the endoplasmic reticulum and Golgi complex generates an array of N-glycan structures, and thus numerous "glycoforms" of any given glycoprotein exist on an individual cell (Moremen *et al*., 2012; Breitling and Aebi, 2013; Scott *et al*., 2013). The importance of N-glycosylation in regulating the

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Abbreviations used: EV, empty vector; FN, fibronectin; HUVEC, human umbilical vein endothelial cell; JAM-A, junctional adhesion molecule-A; N185Q, JAM-A point mutant with asparagine 185 mutated to glutamine; wt, wild-type human JAM-A.
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function of adhesion molecules such as E-cadherin (Pinho *et al.*, 2011; Langer *et al.*, 2012), PECAM-1 (Kitazume *et al.*, 2010), VE-cadherin (Geyer *et al.*, 1999), and ICAM-1 (Diamond *et al.*, 1991; Scott *et al.*, 2013; He *et al.*, 2014) has been reported. No studies examined the extent to which human JAM-A is N-glycosylated or whether this modification is important for the protein's function. In a proteome-wide screen of N-glycosylated proteins, it was suggested that human JAM-A is N-glycosylated at two positions, N185 and N191 (Chen *et al.*, 2009). However, the corresponding sequon at N191 is –NPT- and is in conflict with the consensus motif, suggesting that this site is unlikely to carry an N-linked oligosaccharide. Intriguingly, N185 is the site of a somatic mutation in breast cancer reported in the COSMIC database (Forbes *et al.*, 2008).

Our studies were undertaken to determine whether human JAM-A is N-glycosylated and whether this modification is required for JAM-A function. We show that human JAM-A carries a single N-glycan at N185. This modification is not required for protein expression or transport to the membrane. However, removal of the N-glycan abrogates the effect of JAM-A on barrier function, Rap1 activity, and collective and single-cell migration and prevents leukocyte binding and interaction with LFA-1. Collectively these data demonstrate that N-glycosylation is required for JAM-A function.

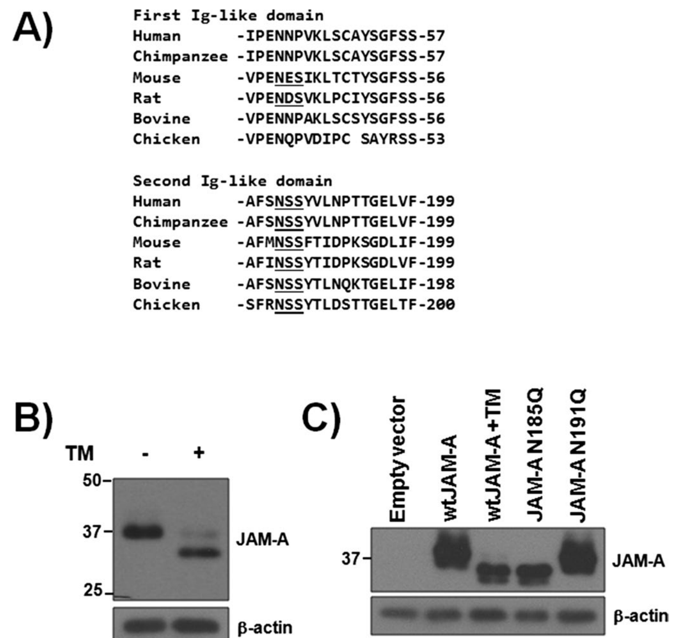

FIGURE 1: Human JAM-A contains a single N-glycan at N185. (A) Multispecies alignment of potential N-glycan sites. N-X-S/T consensus motifs are underlined. (B) Western blot analysis of JAM-A expression in HUVECs with or without tunicamycin (TM) treatment for 36 h. (C) Western blot analysis of CHO cells stably transfected with empty vector control or vectors expressing wild-type, N185Q, or N191Q human JAM-A. Some cells were pretreated with TM for 48 h. Data are representative of at least three separate experiments.

RESULTS

Human JAM-A contains one N-glycan

It was suggested that JAM-A carries N-glycans (Naik *et al.*, 1995; Martin-Padura *et al.*, 1998), but these studies failed to determine precisely how many N-glycans are on human JAM-A. It is well established that N-glycan site occupancy varies across species (Scott and Patel, 2013), so we first wanted to determine how many N-glycans are on human JAM-A, as well as JAM-A expressed by other vertebrates. As can be seen in Figure 1A, multispecies alignment demonstrates the potential for an N-glycan in the first Ig-like domain of rodent JAM-A that is absent in other vertebrates. A consensus N-glycan sequon –NSS- is found in the second Ig-like domain of all species analyzed and corresponds to N185 of human JAM-A. There are reports of a potential N-glycan on N191 of human JAM-A (Chen *et al.*, 2009), but the corresponding sequon is –NPT-, which is in conflict with the consensus motif. Indeed, none of the species analyzed expresses a consensus N-glycan motif at the residues corresponding to N191 in humans, suggesting that this site is not likely to be N-glycosylated. To determine the extent to which human JAM-A is N-glycosylated, we cultured human umbilical vein endothelial cells (HUVECs), which are widely reported to express JAM-A (Martin-Padura *et al.*, 1998; Naik and Naik, 2006), with or without tunicamycin, an inhibitor of N-glycosylation, for 36 h and assessed JAM-A expression by Western blot analysis. As seen in Figure 1B, tunicamycin treatment resulted in a lower–molecular weight (~4 kDa) form of JAM-A, indicating that the protein contains N-linked sugars. We next wanted to determine how many N-glycans human JAM-A supported and performed site-directed mutagenesis of N185 and N191. CHO cells were transfected with empty vector pCDNA3.1 (EV), wild-type human JAM-A (wt), JAM-A point mutant with asparagine 185 mutated to glutamine (JAM-A N185Q), or JAM-A N191Q. As can be seen in Figure 1C, JAM-A N185Q ran at a reduced molecular weight as compared with wt JAM-A. This reduced molecular weight was identical to wt JAM-A protein produced in the presence of tunicamycin. On the other hand, the N191Q mutant migrated at the same molecular weight as wt JAM-A, indicating that there is no modification at this residue. These data demonstrate that human JAM-A carries a single N-glycan at N185.

N-glycosylation regulates JAM-A dimerization and protein half-life

In some instances, N-glycosylation is required for protein folding and is critical for transport of proteins to the plasma membrane (Stanley *et al.*, 2009). We next determined whether the N185Q mutant was able to reach the cell surface. CHO cells expressing wt and N185Q were incubated with the cell-impermeable sulfo-NHS-biotin tag, which allows for the detection of surface-exposed proteins when precipitated against streptavidin. As can be seen in Figure 2A, both wt and N185Q JAM-A were labeled with sulfo-NHS-biotin, indicating that N-glycosylation is not required for surface transport. As a separate readout, surface JAM-A expression was assessed by flow cytometric analysis. Both wt and N185Q were able to reach the cell surface in CHO cells, HUVECs, and MDA-MB-231 cells (Supplemental Figure S1).

JAM-A forms homodimers, which are critical to the protein's function (Severson *et al.*, 2008; Monteiro *et al.*, 2014). Moreover, the second Ig-like domain, where N185 resides, has been implicated in stabilizing these dimers (Wojcikiewicz *et al.*, 2009). Thus we next determined whether N-glycosylation was important for JAM-A dimerization. As seen in Figure 2B, wt JAM-A dimerized in the presence of the cell-impermeable cross-linker BS^3, whereas the previously described dimerization-deficient mutant, 6163 (Mandell *et al.*, 2004), showed no evidence of dimerization. In the presence of BS^3, N185Q was able to form homodimers but at a reduced level compared with wt protein. To confirm this finding, we tested the ability of CHO cells expressing EV or wt or N185Q JAM-A to adhere and spread on JAM-A/fc, as well as the ability of wt to adhere and spread on JAM-A/fc treated with PNGaseF to remove N-glycans. The wt protein, but not N185Q, was able to adhere and spread to

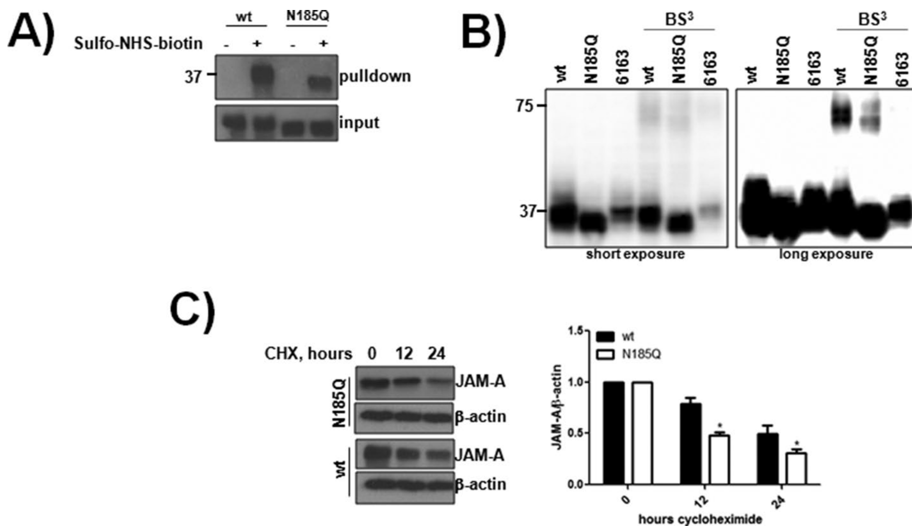

FIGURE 2: N-glycosylation is not required for JAM-A transport but regulates homodimerization and stability. (A) CHO cells expressing wt or N185Q human JAM-A were incubated with sulfo-NHS-biotin to label surface-exposed proteins. Biotinylated proteins were precipitated using streptavidin–agarose and detected by Western blot analysis. The data are representative of three separate experiments. (B) CHO cells expressing wt, N185Q, or 6163 JAM-A and some cells were incubated with BS^3 to cross-link proteins. Cells were processed for Western blot analysis. (C) CHO cells expressing wt or N185Q human JAM-A were incubated with 100 μM cycloheximide (CHX) for up to 24 h. Lysates were subjected to Western blot analysis for JAM-A and β-actin and densitometric analysis conducted using ImageJ. Statistical comparisons were assessed between the samples at each time point using a two-tailed Student's *t* test. $*p < 0.05$ between the samples from four separate experiments.

JAM-A/fc (Supplemental Figure S2A) only when N-glycans were present on the chimeric protein (Supplemental Figure S2B). Finally, junctional accumulation between wt and N185Q was assessed by confocal microscopy. Junctional accumulation was seen only in cells expressing both wt and N185Q JAM-A (Supplemental Figure S1D). Collectively these data demonstrate that N-glycans regulate JAM-A homophilic interactions.

Protein dimerization and N-glycans can both increase a protein's half-life by prolonging surface residency through interactions with other saccharides on the cell's surface. To determine whether N-glycosylation was a regulator of JAM-A half-life, we incubated cells with the translation inhibitor cycloheximide and monitored JAM-A depletion within whole-cell lysates. As can be seen in Figure 2C, the decay of N185Q was significantly increased compared with wt protein. Collectively these data show that N-glycosylation is not required for JAM-A transport but is a critical regulator of dimerization and protein stability.

N-glycosylation is required for JAM-A's effects on barrier function

Previously it was reported that expression of JAM-A in CHO cells reduces paracellular passage of fluorescein isothiocyanate–dextran (Martin-Padura *et al.*, 1998; Aurrand-Lions *et al.*, 2001). To determine whether glycosylation of JAM-A is required to support this function, we measured FITC-dextran flux across CHO cell monolayers expressing EV or wt or N185Q JAM-A. As seen in Figure 3A, expression of wt JAM-A reduced paracellular leak of FITC-dextran compared with EV control. However, N185Q JAM-A demonstrated a similar level of flux as EV cells, indicating that the N-glycan residue was critical for this increase in barrier function. As a separate readout of barrier function, cell impedance was determined. CHO cells were grown on gold-plated microtiter wells, and monolayer integrity was assessed using a real-time cell analyzer (RTCA; Wittchen *et al.*, 2011). As can be seen in Figure 3B, expression of wt JAM-A caused a small but significant increase in cell index, an arbitrary index of impedance, above both EV and N185Q JAM-A after 18 h, indicating an increase in barrier function.

It has been reported that JAM-A mediates barrier function by controlling Rap1 activity. We next determined Rap1 activity in CHO cells expressing EV or wt or N185Q human JAM-A that had been confluent for 24 h. As seen in Figure 3, C and D, expression of wt JAM-A significantly increased Rap1 activity above EV levels. N185Q JAM-A increased Rap1 activity compared with EV levels but to a lesser extent than wt JAM-A. Collectively these data show that N-glycosylation of JAM-A is required for the protein's ability to increase barrier function.

N-glycosylation controls JAM-A's effects on cell migration

There are numerous reports that JAM-A expression controls cell spreading, single-cell motility, and collective cell migration, with the effects being cell-type specific (Bazzoni *et al.*, 2005; Naik and Naik, 2006; McSherry *et al.*, 2009; Severson *et al.*, 2009). We first determined whether expression of wt or N185Q altered cell spreading. As seen in Figure 4, A and B, expression of wt or N185Q JAM-A had no effect on CHO cell spreading on fibronectin as assessed by total cell area or cell index by RTCA. Overexpression of wt caused an increase in HUVEC spreading but a decrease in MDA-MB-231 cell spreading (Supplemental Figure S3), highlighting the cell type–specific effects of the protein.

We next determined whether wt or N185 altered cell motility. Expression of wt JAM-A caused a significant decrease in single-cell velocity of CHO cells (Figure 4C; Supplemental Videos 1–3), as well as of HUVECs and MDA-MB-231 cells (Supplemental Figure S4), as compared with EV and N185Q. However, there was no effect on persistence of migration (Figure 4D).

Because expression of wt JAM-A reduced single-cell motility and this effect was glycosylation dependent, we examined whether a similar phenomenon occurred in collective migration of cells. As seen in Figure 5, expression of wt JAM-A significantly decreased wound closure compared with EV and N185Q. There are reports that overexpression of JAM-A increases rates of directed migration in HUVEC but only on vitronectin (Naik and Naik, 2006). We next determined whether this effect was controlled by N-glycosylation of JAM-A. As previously reported, overexpression of wt JAM-A increased the rate of haptotaxis of HUVECs to vitronectin but not fibronectin (Supplemental Figure S5). In contrast, N185Q migrated at the same rate as EV control toward both matrix proteins. Taken together, these data demonstrate that N-glycosylation controls JAM-A–mediated cell motility and migration. There are reports that JAM-A regulates β1 integrin (CD29) expression in some lines (McSherry *et al.*, 2009; Severson *et al.*, 2009) but not others (Huang *et al.*, 2006; Cera *et al.*, 2009), which could explain some of the foregoing observations. Knockdown or overexpression of JAM-A with siRNA did not alter CD29 expression in CHO cells, HUVECs, or MDA-MB-231 cells (Supplemental Figure S6).

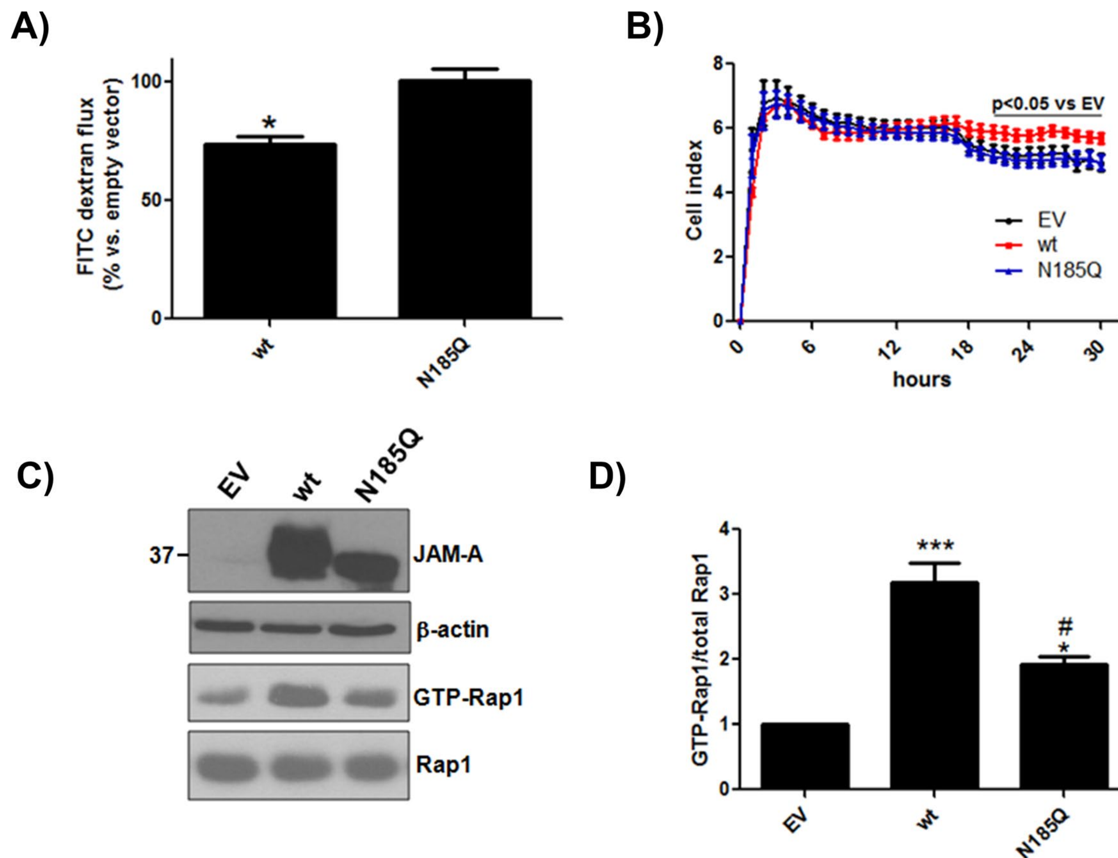

FIGURE 3: N-glycosylation regulates JAM-A–mediated increases in barrier integrity. (A) CHO cells expressing empty vector or wt or N185Q JAM-A were grown on 0.4-μm Transwell inserts for 48 h. FITC-dextran (10 kDa) was added to the upper chamber and measured from the lower chamber after 2 h of incubation. Data shown are representative of three separate experiments run in triplicate. Statistical differences were determined by one-way analysis of variance (ANOVA) with Tukey's posttest. **p* < 0.05 vs. empty vector and N185Q. (B) The same cells as in A were grown on RTCA plates, and impedance was assessed for 30 h. Data shown are representative of four separate experiments run in quadruplicate. Statistical differences were determined by two-way ANOVA with Bonferroni posttest against empty vector. (C) CHO cells transfected with empty vector or wt or N185Q human JAM-A were assayed for Rap1 activity by pull down using GST-RalGDS-RBD. (D) Quantification. **p* < 0.05 vs. EV; ****p* < 0.01 vs. EV; #*p* < 0.05 vs. wt by one-way ANOVA with Tukey's posttest from four separate experiments.

JAM-A N-glycosylation controls leukocyte binding

JAM-A supports leukocyte adhesion (Ostermann *et al.*, 2002). Specifically, the second Ig-like domain, where N185 is located, interacts with LFA-1 (Fraemohs *et al.*, 2004). As can be seen in Figure 6A, CHO cells expressing wt JAM-A supported significantly increased leukocyte adhesion compared with EV control cells. Strikingly, N185Q-expressing cells did not support leukocyte binding above EV control, suggesting that glycosylation of this residue is involved in LFA-1 binding.

To confirm this interaction, we tested the ability of CHO cells with or without JAM-A proteins to bind to LFA-1. CHO cells will not bind to LFA-1/fc chimeras unless they express JAM-A (Fraemohs *et al.*, 2004). As can be seen in Figure 6, B and C, cells expressing wt JAM-A demonstrated significantly more adhesion to LFA-1/fc than did EV cells. In contrast, cells expressing N185Q failed to adhere at levels above EV, indicating that N-glycosylation of JAM-A is required for LFA-1 binding. To confirm the specificity of LFA-1 binding, we assessed CHO cells expressing EV, JAM-A, or ICAM-1 for their ability to bind LFA-1/fc–coated beads. Cells expressing JAM-A and ICAM-1 bound significantly more beads than EV control (Supplemental Figure S7), confirming specificity of the ligand.

JAM-A glycosylation patterns are cell-type specific

Having confirmed that JAM-A contains N-glycans that regulate the protein's function, and in light of both our findings and previously published data demonstrating that JAM-A's effects are cell-type specific, we investigated whether the glycoprotein's saccharide content varied across a panel of endothelial and epithelial cells, as well as when the protein was exogenously expressed, using lectin affinity pull down (lectin specificity outline in Table 1). To test for sialic acid, a common capping sugar on N-glycan structures, we incubated human cells lines with *Sambucus nigra* lectin (SNA), a lectin that recognizes α-2,6-linked sialic acid, which is added to cells via the enzyme ST6GAL1. CHO cells express low levels of ST6GAL1 and were thus also tested with *Maackia amurensis* lectin (MAA), a lectin that recognizes α-2,3 sialic acid, the predominant sialic acid structure in these cells (Lee *et al.*, 1989; Xu *et al.*, 2011). As seen in Figure 7, N-glycans from all cell lines tested contained bianternary (LCA), trianternary (PHA-E), and sialyated structures (SNA or MAA). Of interest, UEA-1, a lectin specific for fucose, interacted with JAM-A produced in epithelial but not endothelial cells or cell expressing exogenous JAM-A. These data demonstrate a conserved N-glycan profile of JAM-A, including the global

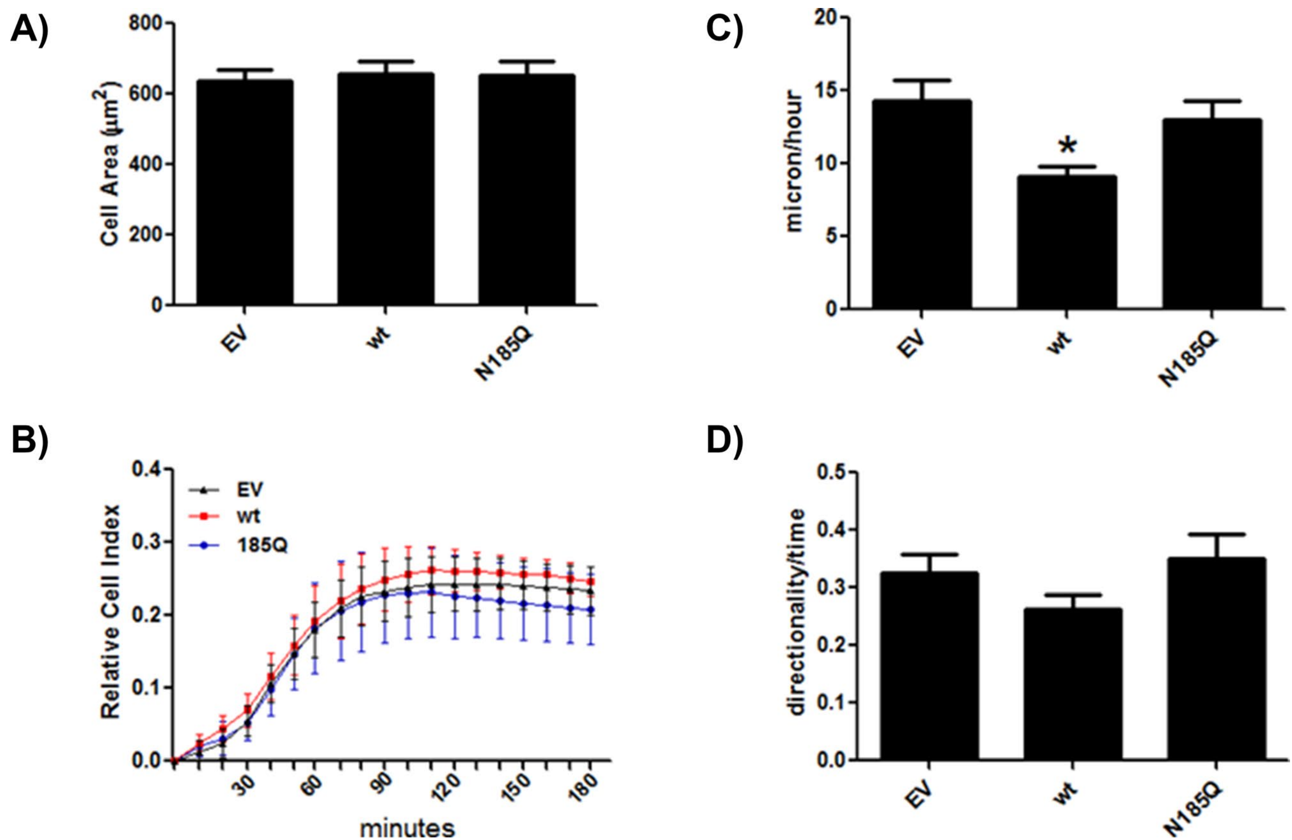

FIGURE 4: N-glycosylation controls JAM-A–mediated cell motility. Cell spreading was assessed by determining total cell area (A) or measuring cell impedance using RTCA (B). The total number of cells used in A was as follows: EV, 47; wt, 49; N185Q, 46. RTCA data are representative of three separate experiments run in quadruplicate. CHO cells expressing empty vector or wt or N185 JAM-A were plated on FN-coated glass-bottom dishes, and single-cell motility was assessed as velocity (C) and persistence (directionality/time) (D) using time-lapse images. The data are averages ± SEM from three different experiments. The total number of cells was as follows: EV, 52; wt, 53; N185Q, 46. Statistical differences were determined by one-way ANOVA with Tukey's posttest. *$p < 0.05$ vs. EV and N185Q.

presence of sialic acid and epithelial cell–specific presence of fucose.

DISCUSSION

Before this study, the extent of N-glycosylation on JAM-A and its involvement in the protein's functions were unknown. We have shown that human JAM-A carries a single N-glycan at residue N185 but not on the previously implicated residue N191 (Figure 1). Glycosylation of N185 is not required for surface transport but regulates dimerization and protein half-life (Figure 2). N-glycosylation of JAM-A at N185 is also a critical regulator of barrier function (Figure 3) and cell migration (Figures 4 and 5). Our data also indicate that N-glycans at N185 of JAM-A are required for leukocyte binding via LFA-1 (Figure 6). Finally, we report cell-specific JAM-A N-glycan signatures (Figure 7). Taken together, these data indicate that N-glycosylation of JAM-A is critical for the protein's function.

JAM-A is known to control barrier function in CHO cells (Martin-Padura *et al.*, 1998; Aurrand-Lions *et al.*, 2001), as well as in some epithelial cells (Monteiro and Parkos, 2012; Monteiro *et al.*, 2014). The reported mechanism is believed to be by controlling Rap1/2 activity, which is also dependent on JAM-A homodimerization (Severson *et al.*, 2008; Monteiro *et al.*, 2014). In the present study, we also found that JAM-A is able to increase barrier function and increase Rap1 activity in CHO cells. The N-glycan deficient JAM-A N185Q mutant does not increase barrier function and only slightly increases Rap1 activity. Dimerization of JAM-A is reported to occur through key residues in the first Ig-like domain (Ostermann *et al.*, 2002; Mandell *et al.*, 2004). Atomic force microscopy experiments elegantly demonstrated, however, that these dimers are stabilized by the second Ig-like domain, the region where N185 is located (Wojcikiewicz *et al.*, 2009), although it was unclear what portion of the second Ig-like domain was involved. We now identify N-glycans bound at N185 as regulators of dimerization. There are numerous reports of N-glycans regulating homodimers to control protein function, such as E-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1), and N-cadherin (Guo *et al.*, 2009; Pinho *et al.*, 2011; Langer *et al.*, 2012). For example, expression of PECAM-1 is reduced in cells that are missing ST6GAL1, a gene responsible for the addition of α-2,6 sialic acid on N-glycans. Loss of this enzyme results in decreased PECAM-1 surface residency and a loss of antiapoptotic signaling associated with the protein (Kitazume *et al.*, 2014). In addition, it has been reported that these sialic acid residues, which are similar to the ones on JAM-A, regulate dimerization of PECAM-1 (Kitazume *et al.*, 2010).

Several reports suggested that JAM-A controls cell motility and migration (Bazzoni *et al.*, 2005; Naik *et al.*, 2008; McSherry *et al.*, 2009; Gotte *et al.*, 2010; Murakami *et al.*, 2011; Cao *et al.*, 2014). However, the results from these studies have been conflicting. For example, it was reported that JAM-A expression decreases migration of murine endothelial and MDA-MB-231 cells but increases the

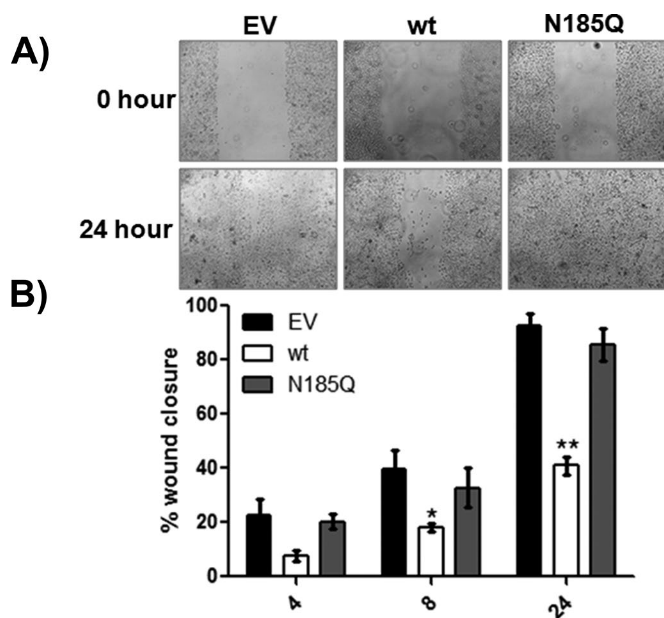

FIGURE 5: N-glycosylation controls JAM-A–mediated decrease in wound closure. CHO cells expressing EV or wt or N185Q JAM-A were grown to confluence on FN-coated glass-bottom dishes. A wound was made across the entire length of the glass insert using the tip of a 200-µl pipette tip, and closure was assessed over a 24-h period. (A) Representative micrographs at the beginning (0 h) and end (24 h) of an experiment. (B) Quantitation of wound closure. Five separate regions from each dish were monitored for closure. Data presented are the average ± SEM of the five regions from one dish and are representative of four separate experiments. Statistical differences were determined by one-way ANOVA with Tukey's posttest. *$p < 0.05$ vs. EV; **$p < 0.05$ vs. EV and N185Q.

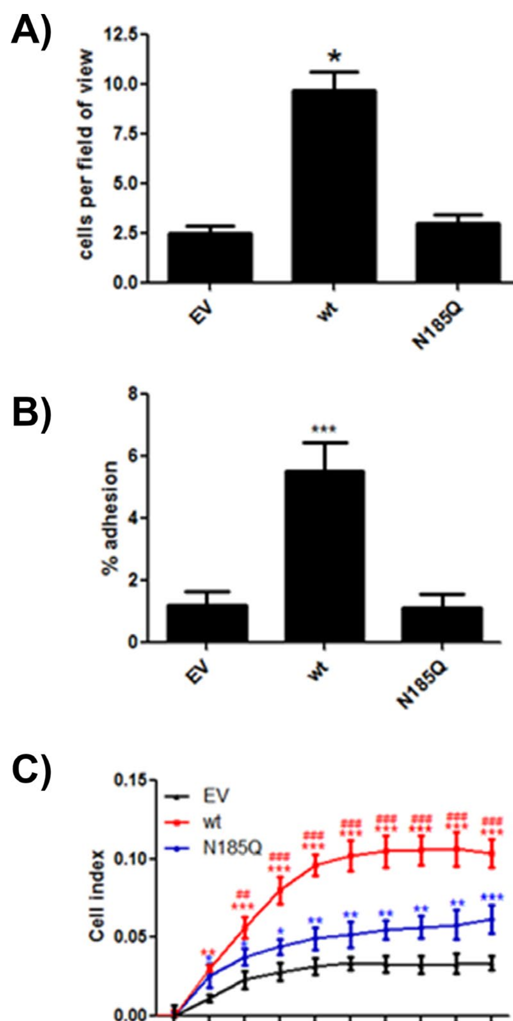

FIGURE 6: N-glycosylation of JAM-A is required for interactions with LFA-1. (A) Equal numbers of HL-60 cells were added to glass coverslips covered with confluent CHO cells expressing empty vector or wt or N185Q JAM-A in the presence of 100 µg/ml PMA for 30 min. Adherent HL-60 cells were counted after washing. Data are representative of three separate experiments run in triplicate. Statistical differences were determined by one-way ANOVA with Tukey's posttest. *$p < 0.05$ vs. EV and N185Q. (B) CHO cells labeled with CellTracker Green and expressing empty vector or wt or N185Q human JAM-A were allowed to adhere to microtiter plates coated with LFA-1/fc chimera (20 µg/ml). After washing, adherent cells were assessed on a fluorometer. Data are representative of three separate experiments. *$p < 0.05$ vs. EV and N185Q. (C) CHO cells expressing empty vector or wt or N185Q JAM-A were allowed to adhere and spread on RTCA plates coated with LFA-1/fc chimera (20 µg/ml) for 90 min. Data are representative of two independent experiments run in quadruplicate. Statistical differences were assessed by two-way ANOVA with Bonferroni posttest against EV and N185Q. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ vs. EV. $^{\#\#}p < 0.05$ and $^{\#\#\#}p < 0.01$ vs. N185Q.

migration of murine neutrophils, HUVECs, and MCF7 cells (Bazzoni *et al.*, 2005; Naik and Naik, 2006; Naik *et al.*, 2008; Cera *et al.*, 2009; McSherry *et al.*, 2009). In Figure 4 and Supplemental Figure S3, we show that JAM-A regulates cell spreading of HUVECs and MDA-MB-231 but not CHO cells. In addition, Figure 4 and Supplemental Figure S4 demonstrate that expression of exogenous wt JAM-A reduces random motility of all cells tested. One possible explanation for these cell type–specific effects could be unique N-glycan profiles associated with the different cells. Figure 7 demonstrates that cell-specific N-glycan profiles of JAM-A do exist; if and how these specific modifications regulate protein function are worthy of additional investigation.

Changes in N-glycosylation are often associated with cellular transformation and inflammation and could influence JAM-A function (Couldrey and Green, 2000; Chacko *et al.*, 2011; Guo *et al.*, 2012; Scott *et al.*, 2012; Liu *et al.*, 2013). It is intriguing to speculate that changes in JAM-A N-glycosylation as a result of disease progression could result in a loss-of-function state, such as is reported her earlier with the N185Q mutant. In fact, N185S is reported as a somatic mutation in breast cancer in the COSMIC database (Bamford *et al.*, 2004). This mutation would likely result in a loss of JAM-A function and lead to a more aggressive cell phenotype, which has been described in breast cancer cells lacking JAM-A (Naik *et al.*, 2008). Additional studies are needed to determine whether N185S behaves as a dominant negative when compared with the results obtained with wt JAM-A, which restored a normal breast epithelial phenotype (Naik *et al.*, 2008).

One of the first reported functions of JAM-A was as a leukocyte receptor and regulator of diapedesis (Martin-Padura *et al.*, 1998). In support of this, JAM-A$^{-/-}$ mice have been shown to have defects in leukocyte trafficking in disparate models of disease (Azari *et al.*, 2010; Lakshmi *et al.*, 2012; Schmitt *et al.*, 2014). JAM-A controls diapedesis by interacting with LFA-1 expressed on leukocytes (Fraemohs *et al.*, 2004). It has been known for some time that this

Lectin	Specificity
Lens culinaris agglutinin (LCA)	Biantennary N-glycan
Phaseolus vulgaris erythroagglutinin (PHA-E)	Triantennary N-glycan
P. vulgaris leucoagglutinin (PHA-L)	Tetraantennary N-glycan
Ulex europaeus agglutinin I (UEA-1)	α-Linked fucose
S. nigra lectin (SNA)	α-2,6-Linked sialic acid
M. amurensis lectin II (MAA)	α-2,3-Linked sialic acid

TABLE 1: Lectin specificity.

interaction involves the second Ig-like domain of JAM-A, but the precise region of binding was unknown. The present data suggest that the N-glycan at N185 is required for this function. At least two possible scenarios exist by which this N-glycan could control LFA-1 binding. First, it is possible that this N-glycan residue is required for positioning the amino acids within JAM-A that serve as a ligand for LFA-1. The second possibility is that the N-glycans themselves directly interact with LFA-1 or with an associated molecule on the surface of the leukocyte that controls LFA-1 to mediate the interaction. As examples, N-glycans of intercellular adhesion molecule 1 (ICAM-1) regulate the binding of both LFA-1 and MAC-1. In the case of MAC-1, complex N-glycans at positions N240 and N269 block the

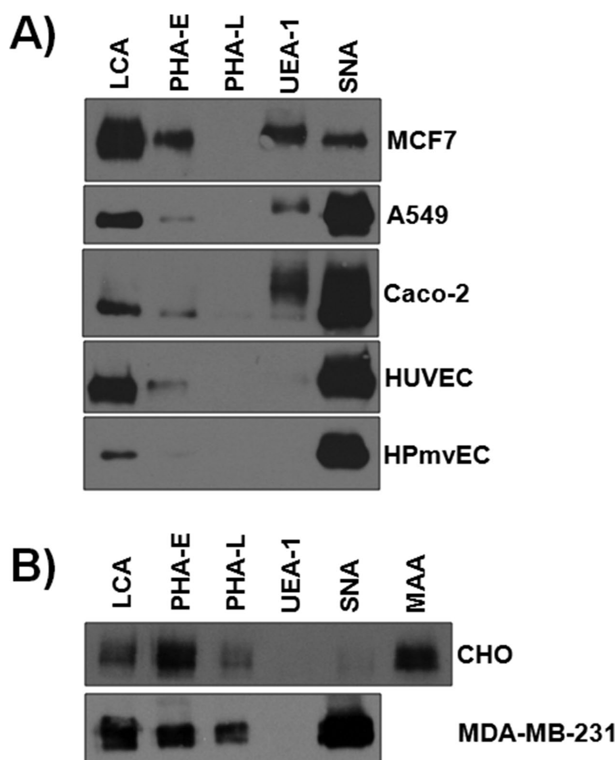

FIGURE 7: JAM-A N-glycan content differs between epithelial and endothelial cells. Endogenous expression of JAM-A from MCF7, A549, Caco-2, HUVEC, and pulmonary microvascular endothelial cells (HPmvEC) (A) and exogenous expression of the protein in CHO and MDA-MB-231 (B) was analyzed for N-glycan content after lysis and incubation with agarose-bound lectins as described in the *Materials and Methods*. Bound proteins were subjected to Western blot analysis for detection of JAM-A. Data are representative of two to four experiments per cell type.

binding motif on the third Ig-like domain of ICAM-1, which can be abolished by site-directed mutagenesis or production of the protein under conditions in which N-glycan complexity is limited. (Diamond *et al.*, 1991; Scott *et al.*, 2013). Further, the CD18 integrin, which is shared by LFA-1 and Mac1, binds ICAM-1 in a glycosylation-dependent manner (Sriramarao *et al.*, 1993). N-glycosylation at a single residue of ICAM-1, N103, is required for the proper positioning of the binding domain that interacts with LFA-1 (Diamond *et al.*, 1991).

This report has demonstrated that N-glycosylation of human JAM-A at N185 is required for the protein's known functions. These findings identify a novel regulator of JAM-A function and one that is altered during disease. It will be interesting to determine whether changes in glycosylation of JAM-A could affect JAM-A function during disease, leading to compromised barrier function and altered junctional stability. Future studies are needed to determine the N-glycan composition of JAM-A and understand how this changes during inflammation and disease.

MATERIALS AND METHODS

Cell culture and transfections

CHO-K1, HL-60, MDA-MB-231, A549, Caco-2, and MCF7 cells were purchased from the American Type Culture Collection (Manassas, VA). CHO-K1 and MDA-MB-231 were propagated in high-glucose DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO) and antibiotic/antimycotic solution (Invitrogen). MCF7 and HL-60 cells were propagated in RPMI-1640 containing 10% FBS and antibiotic/antimycotic solution. Caco-2 cells were propagated in MEM containing 20% FBS and antibiotic/antimycotic solution. HUVECs and human pulmonary microvascular endothelial cells were purchased from Lonza and cultured in EGM-2 + bullet kit (Lonza, Walkersville, MD). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. To generate stable lines, transfected cells were grown in the foregoing media supplemented with 1000 μg/ml G418 (Invitrogen) for 3 wk, at which time, individual colonies were selected and expanded. For knockdown of JAM-A, cells were transfected with SMARTpool ON-TARGET PLUS siRNA against human f11r (Dharmacon, Lafayette, CO) at a final concentration of 10 nM.

Plasmid and site-directed mutagenesis

The pCDNA3.1 HA-JAM-A plasmid was previously described (Naik *et al.*, 2001) and generously provided by Ulhas Naik (University of Delaware, Newark, DE). The pCMV ICAM-1 plasmid was previously described (Scott *et al.*, 2013). Site-directed mutagenesis to generate N185Q, N191Q, and 6163 was achieved by overlap PCR and confirmed by sequencing using the following primers. N185Q: forward, 5′-cacccgtgccttcagccagtcttcctatgtcctga-3′, and reverse, 5′-tcaggacataggaagactggctgaaggcacgggtg-3′. N191Q: forward, 5′-aactcttcctatgtcctgcagcccacaacaggagagctg-3′, and reverse, 5′-cagctctcctgttgtgggctgcaggacataggaagagtt-3′. 6163: forward, 5′-gtctccttggtcaaacgcccacgccacacggggagaaga-3′, and reverse, 5′-tcttctccccgtgtggcgtgggcgtttgaccaaggagac-3′.

Rap1 activity assay

Rap1 activity was determined as previously described (Wittchen *et al.*, 2005). Briefly, cells were lysed (50 mm Tris, pH 7.4, 75 mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS containing protease inhibitors and 1 μM sodium vanadate) and clarified by centrifugation. Active Rap1 was captured by incubation with 50 μg of glutathione *S*-transferase (GST)–RalGDS-RBD immobilized on glutathione–Sepharose beads. Active Rap1 bound to

beads was compared with total levels (from reserved input) by Western blot.

Western blot analysis

Lysates were resolved on SDS–PAGE gels and proteins transferred to nitrocellulose membranes. Blots were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline containing Tween-20 (TBST; 25 mM Tris, pH 7.6, 150 mM NaCl, 0.2% Tween-20). Blots were probed with primary antibodies against JAM-A (612120; BD Biosciences, San Jose, CA), Rap1 (2399; Cell Signaling, Danvers, MA), CD29 (9699, for human cell lines; Cell Signaling), CD29 (MAB2405, CHO cells; R&D Biosystems, Minneapolis, MN), ICAM-1 (4915; Cell Signaling), and β-actin (14-1116; Millipore, Billerica, MA). After washing with TBST, blots were incubated with species-appropriate horseradish peroxidase–conjugated secondary antibodies (Jackson Laboratories, West Grove, PA), and signals were detected using enhanced chemiluminescence substrate with x-ray film or a Bio-Rad Chemidoc system. Densitometric analysis was conducted on scanned films using ImageJ (National Institutes of Health, Bethesda, MD).

RTCA and cell spreading

Before plating, cells were incubated in DMEM containing 0.5% delipidated BSA and antibiotic-antimycotic solution for 1 h. Cells were then resuspended in the same medium for 1 h with gentle rotation. For the RTCA xCELLigence System (Roche Applied Sciences), 2000 cells/well were added to an E-plate 16 coated with fibronectin (FN; 25 μg/ml). Attachment and spreading were monitored as cell index, an arbitrary unit of cell impedance. For analysis of total spread area, cells were prepared as described and added to glass coverslips coated with 25 μg/ml FN. Cells were fixed with ice-cold ethanol and stained with Alexa 488–phalloidin (Invitrogen). Micrographs were captured using a Zeiss Axiovert 200M microscope equipped with a 63× objective lens and a Hamamatsu ORCA-ERAG digital camera. Area was calculated using ImageJ.

Wound-healing assay

Cells were plated onto FN-coated (25 μg/ml) glass-bottom dishes at a confluent density. After a 24-h incubation, a wound was created using a 200-μl pipette tip. Images were acquired at 0, 4, 8, 12, and 24 h after wounding. Each cell type was tested three times, with five regions per wound assessed. Percentage wound closure was calculated using ImageJ.

Lectin affinity pull down

Cells were lysed (50 mM Tris, 150 mM NaCl, 1% SDS, 5 μM 2-mercaptoethanol) and boiled for 10 min. Lysates were diluted 1:20 in TBS and incubated with 50 μl of lectin-conjugated agarose (Vector Laboratories, Burlingame, CA) for 4 h at room temperature. For MAA pull downs, 50 μg of biotin-tagged MAA (Vector Laboratories) was incubated with 50 μl of streptavidin agarose for 1 h at room temperature before addition of the lysates. Beads were washed five times with TBS, and bound proteins were released by boiling in sample buffer and processed for Western blot analysis as described.

Flow cyotometric analysis

Cells were trypsinized and incubated with Alexa Fluor 488–conjugated anti–JAM-A (FAB1103G; R&D Biosystems). Cells were washed three times in PBS, resuspended in sorting medium (2% FBS in PBS) containing 10 μg/ml DNase I and 5 mM EDTA, and passed through a 30-μm filter (04-004-2326; Partec). Cell populations were profiled for Alexa Fluor 488 signal with 488/525 ± 20 nm (excitation/emission) settings on a Bio-Rad S3 cell sorter. Cytometry data were analyzed with FlowJo software.

Haptotaxis assay

The bottoms of 8-μm-pore Transwell inserts (Corning, Corning, NY) were coated with FN (20 μg/ml) or vitronectin (20 μg/ml; Advanced Biomatrix, San Diego, CA). Cells were serum starved for 2 h before being added to the wells and given 4 h to migrate. Cells were fixed in ice-cold ethanol, cells from the upper chamber were gently removed with a cotton swab, and the migrated cells on the lower chamber were stained with Alexa 488–conjugated phalloidin. Three fields of view from three Transwells per condition were captured and averaged per experiment.

Immunostaining

Cells plated onto glass coverslips were fixed with ice-cold methanol before being blocked with 5% BSA in PBS. Cells were stained with antibody against JAM-A (sc-53624; Santa Cruz Biotechnology) and counterstained with Alexa Fluor 488–conjugated secondary and Hoechst 33342 (Invitrogen). Samples were mounted with Fluoro-Gel (17985-11; Electron Microscopy Sciences, Hatfield, PA) and imaged on an Olympus FV1000 confocal microscope using a 40× (numerical aperture 1.3) objective with 1.7× digital magnification. Image channels were pseudocolored and merged using ImageJ.

Single-cell tracking of motility

Cells were plated onto FN-coated (25 μg/ml) glass-bottom culture dishes 4–6 h before imaging. Time-lapse microscopy was performed on an incubator-housed microscope (20× objective; VivaView FL) with a camera (Orca ER/AG type c4742-80-12AG) with image acquisition every 15 min for 12 h. Cell velocity and persistence were measured with ImageJ using the Manual Tracking plug-in.

Monocyte adhesion assay

CHO cells were grown on FN-coated (10 μg/ml) 12-mm glass coverslips in 24-well plates and switched to serum-free medium 2 h before the experiment. Just before the experiment, the CHO cells were washed and then incubated in Hanks balanced salt solution (HBSS) containing CellTracker Green (Life Technologies)–labeled HL-60 cells (1.5×10^6 cells/dish) in the presence of 100 ng/ml phorbol myristate acetate (PMA) for 30 min. Cells were gently washed in warm PBS containing $CaCl_2$ and $MgCl_2$ to remove any unbound cells before being fixed with 3.7% formaldehyde in the same buffer for 20 min. Five random fields of view from three separate coverslips per condition were analyzed and quantified as cells per field of view. Images were captured using a Zeiss Axiovert 200M microscope equipped with a 20× objective lens and a Hamamatsu ORCA-ERAG digital camera. The experiment was conducted three times, and representative results from one experiment are shown.

CHO cell adhesion to fc-chimeric proteins

Black-walled 96-well microtiter (Corning) or E-16 RTCA plates were coated with LFA-1/fc chimera (20 μg/ml; R&D Biosystems) or JAM-A/fc (20 μg/ml) in 10 mM Tris, pH 9.0, at 4°C overnight. For some experiments JAM-A/fc was digested with 0.1 U of PNGaseF (Sigma-Aldrich) for 1 h at 37°C to remove N-glycans. Wells were then blocked with 0.5% heat- denatured BSA in PBS for 1 h at room temperature. CHO cells were labeled with CellTracker Green (and

allowed to adhere for 30 min at 37°C in HBSS. Wells were gently washed with warm PBS with 1 mM $CaCl_2$ and $MgCl_2$, and fluorescence of input and adherent cells was determined with a fluorescence plate reader (Tecan). Background binding to BSA-coated wells was negligible and subtracted.

Bead adhesion assay

Assays were performed as previously described (Lessey-Morillon *et al.*, 2014). Briefly, 4.5-µm tosylactivated paramagnetic beads (Invitrogen) were coated with LFA-1/fc according to the manufacturer. Beads were added to confluent cells in a 1:1 ratio and allowed to adhere for 15 min. Cells were washed five times with warm PBS to remove any unattached beads. Five fields of view from two separate experiments were acquired, and bound beads were determined.

Surface biotinylation assay

CHO cells were grown to confluence and washed three times with warm PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ before being incubated with the same buffer containing 0.5 mM sulfo-NHSbiotin (Pierce). After a 15-min incubation at room temperature, the reaction was quenched by the addition of 25 mM glycine for 10 min. Cells were again washed with PBS and lysed (25 mM Tris, pH 7.6, 150 mM NaCl, 0.1% SDS, 0.5% NP-40, protease inhibitors). Biotin-tagged proteins were enriched on streptavidin agarose for 1 h and released by boiling in sample buffer. Precipitated proteins and reserved input were analyzed for JAM-A by Western blot analysis.

BS^3 cross-linking: detection of JAM-A dimerization

Cells were washed three times with ice-cold PBS containing $CaCl_2$ and $MgCl_2$ (1 mM each) before being incubated with 5 µM BS^3 (Pierce) for 30 min at 4°C. The reaction was quenched by the addition of 25 µM glycine for 10 min at room temperature. Cells were washed three times and lysed directly in sample buffer for Western blot analysis.

Barrier function analysis

For FITC-dextran flux, cells were seeded (5×10^4 cells/well) onto FN-coated (10 µg/ml) 0.4-µm polycarbonate Transwell membranes (Corning) and cultured for 48 h. FITC-dextran (10 kDa) (Sigma-Aldrich) at a final concentration of 1 mg/ml was added to the upper chamber, and after 2 h, medium from the bottom chamber was collected and transferred to a black-walled, 96-well microtiter plate (Corning) for analysis. Fluorescence intensity was analyzed using a plate reader (Tecan; excitation 485 nm, emission 520 nm). For RTCA experiments, 5×10^4 cells were plated in each well of an E-Plate 16 (Roche Applied Science), and cell index was assessed for 30 h.

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