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JAM-A interacts in cis with $\alpha 3\beta 1$ integrin through CD151 to regulate collective cell migration of polarized epithelial cells

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Abbreviations: CIL, contact inhibition of locomotion; CMA, cell-matrix adhesion; CNI, collagen type I; ECM, extracellular matrix; FN, fibronectin; JAM, junctional adhesion molecule; LA, LifeAct; LN, laminin; TEM, tetraspanin-enriched microdomain; VN, vitronectin;

Abstract

Junctional adhesion molecule (JAM)-A is a cell adhesion receptor localized at epithelial cell-cell contacts with enrichment at the tight junctions. Its role during cell-cell contact formation and epithelial barrier formation has intensively been studied. In contrast, its role during collective cell migration is largely unexplored. Here we show that JAM-A regulates collective cell migration of polarized epithelial cells. Depletion of JAM-A in MDCK cells enhances the motility of singly migrating cells but reduces cell motility of cells embedded in a collective by impairing the dynamics of cryptic lamellipodia formation. This activity of JAM-A is observed in cells grown on laminin and collagen I but not on fibronectin or vitronectin. Accordingly, we find that JAM-A exists in a complex with the laminin- and collagen I-binding $\alpha 3 \beta 1$ integrin. We also find that JAM-A interacts with CD151, a tetraspanin that forms a stoichiometric complex with $\alpha 3 \beta 1$ integrin and that regulates $\alpha 3 \beta 1$ integrin activity in different contexts. Mapping experiments indicate that JAM-A associates with both $\alpha 3 \beta 1$ integrin and CD151 through its extracellular domain. Similar to depletion of JAM-A, depletion of either $\alpha 3 \beta 1$ integrin or CD151 in MDCK cells slows down collective cell migration. Our findings suggest that JAM-A, $\alpha 3 \beta 1$ integrin and CD151 exist as a functional complex to regulate collective cell migration of epithelial cells.

Introduction

Collective cell migration is a process in which groups of cells migrate in a coordinated manner. Movements of cell collectives occur during development, repair and regeneration processes but also during pathophysiological processes like cancer invasion [1, 2]. In contrast to collectively migrating cancer cells, which adopt mesenchymal characteristics and which form loose and rather transient cell-cell junctions, collectively migrating epithelial cells maintain their apico-basal polarity and remain tightly connected through stable intercellular junctions [3].

The coordination of directed migration is enabled by the formation of polarized protrusions beneath the cell migrating in front of the cell, so-called cryptic lamellipodia [4, 5], while at the same time maintaining regular cell-cell junctions at the apical cell-cell junctions. The formation of oriented cryptic lamellipodia requires intact adherens junctions which act as scaffolds for the Arp2/3 complex and its regulator Wave [6]. A structure similar to cryptic lamellipodia has been observed in collectively migrating endothelial cells [7-9]. Similar to cryptic lamellipodia of migrating epithelial cells, their formation requires an intact cadherin/catenin complex indicating an active role of intercellular junctions in the formation of polarized protrusions during collective cell migration.

Recent evidence strongly suggests a role for intercellular junctions in mechanosensing and transduction. Leader cells at the forefront of a cellular collective respond to chemical cues by front-to-rear polarization, i.e. increased protrusive activity at their front and increased actomyosin-driven contractility at their rear end [2]. The forces generated by migrating leader cells are transmitted to the cells behind the front through intercellular junctions-mediated force-sensing and force-transducing mechanisms [10-15]. Molecular components localized at all structures at cell-cell junctions including tight junctions (TJs), adherens junctions (AJs), desmosomes and gap junctions contribute to the behaviour of collectives of migrating cells [16]. Interestingly, physical properties associated with forces such as strain energy or monolayer tension and those associated with kinematic behaviour such as monolayer velocity are differently influenced by adhesion molecules and their associated cytoplasmic proteins

[16], indicating that signalling inputs derived from multiple cell-cell adhesion receptor systems are integrated into coordinated cell behaviour during collective cell migration.

Junctional adhesion molecule A (JAM-A) is a member of the immunoglobulin superfamily (IgSF) with a variety of functions in different cells types [17]. In polarized epithelial cells, JAM-A is localized at intercellular junctions and is enriched at TJs where it is phosphorylated by the Par3 – aPKC – Par6 polarity protein complex [18]. Depletion of JAM-A in SK-CO15 epithelial cells results in reduced cell migration in scratch-wounding assays [19]. In different tumor cell lines, the levels of JAM-A were found to correlate both positively and negatively with cell migration [20-23] suggesting a context-dependent function of JAM-A during migration. In endothelial cells, JAM-A regulates cell migration in response to bFGF through its interaction with tetraspanin CD9 and $\alpha v\beta 3$ integrin [24-26].

In this study, we addressed the role of JAM-A during collective cell migration of polarized epithelial cells. Using MDCKII cells in a monolayer expansion model [16], we find that JAM-A is required for efficient migration of MDCKII cell collectives. Depletion of JAM-A increases the migration velocity of singly migrating MDCKII cells, but slows down the migration velocity of MDCKII cell collectives. We find that JAM-A interacts with $\alpha 3\beta 1$ integrin as well as with tetraspanin (Tspan) CD151, a major $\alpha 3\beta 1$ integrin-binding partner and regulator of $\alpha 3\beta 1$ integrin functions. Our findings suggest a tetraspanin-enriched microdomain (TEM) in which CD151 links JAM-A to $\alpha 3\beta 1$ integrin and identify JAM-A as a regulator of collective cell migration in polarized epithelial cells.

Methods

Cell culture and transfections

MDCKII cells with inducible expression of JAM-A shRNAs [27] or CRISP/Cas9-mediated inactivation of the JAM-A gene [28], and HEK293T cells (ATCC #CRL-3216) were cultivated in DMEM high glucose medium (Sigma-Aldrich #D5671) containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 U/ml streptomycin. For stable MDCKII cell lines with

inducible expression of JAM-A shRNAs, the growth media was additionally supplemented with 500 µg/ml G418 and 5 µg/ml blasticidin [29], and shRNA expression was induced by the addition of 2 µg/ml doxycycline. Stable LifeAct-EGFP (LA-EGFP) or LifeAct-mCherry (LA-mCherry) expressing MDCKII cells were generated by lentiviral transduction and selection in growth media containing 0.1 mg/ml zeocin or 1 µg/ml puromycin, respectively. MDCKII JAM-A KO cell lines expressing murine JAM-A (mJAM-A)/WT, mJAM-A/Y281F or mJAM-A/S285A under a tetracycline-regulated promotor were generated by lentiviral transduction and maintained in DMEM media containing 1 µg/ml puromycin and 2 µg/ml doxycycline (selectively added for induction of mJAM-A expression). These cell lines were additionally transfected with LA-EGFP or LA-mCherry, as described above.

Transient transfections of siRNAs were performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Transfections of expression vectors were performed using Lipofectamine 2000 (Thermo Fisher Scientific) or Xfect transfection reagent (TaKaRa Bio Europe SAS, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. Lentiviral particles for the generation of stably transfected cell lines expressing either shRNAs or cDNAs were generated by cotransfection of the lentiviral vector and the packaging vectors psPAX2 and pMD2.G (kindly provided by Dr. Didier Trono, Addgene plasmids 12260 and 12259) in a ratio of 3:2:1 into HEK293T cells. Lentiviral transduction of cells was performed as described [29].

RNA interference, plasmid vectors and constructs

The following siRNAs and shRNAs directed against canine (c) genes were used: cJAM-A shRNA 5'-CCAGTAAGAAGGTGATTTA-3' (in pLVTHM (Addgene #12247) and in pEmU6proT (provided by Dr. Karl Matter); cCD151 siRNA pool (NCBI Gene ID475992, siTOOLS Biotech, München), cCD9 siRNA (NCBI Gene ID611695, siTOOLS Biotech, München), cITGA3 (NCBI Gene ID491074, siTOOLS Biotech, München); negative control siRNA pool (neg. ctrl. siPOOL Neg D1, siTOOLS Biotech, München); negative control siRNA (Qiagen 1027280), OnTarget plus non-targeting pool (Dharmacon D-001810-10-05). Knockdown efficiencies of all shRNAs and siRNAs used in this study are depicted in Suppl. Fig. 1. The following constructs were

used: LifeAct-eGFP in pFUGW (provided by Dr. R. Wedlich-Söldner), LifeAct-mCherry in pLV-PGK-Puro (provided by Dr. H. Farin); hJAM-A/WT, hJAM-A/ Δ 3, hJAM-A/ Δ 40, Δ D1-hJAM-A, hJAM-A/MC, hJAM-A/N185Q in pFLAG-CMV-1 (Sigma-Aldrich (SA), Munich, Germany); Flag-mJAM-A/WT, Flag-mJAM-A/Y281F, Flag-mJAM-A/S285A in pInducer21-Puro (Addgene #46948) [30] (provided by Dr. T. Weide); hCD151 in pEGFP-C1 (TaKaRaBio).

Antibodies and reagents

The following antibodies were used in this study: mouse mAb anti- α -catenin (BD TL 610194, IF 1:600), mouse mAb anti- β -catenin (BD TL 610154, IF 1:600), mouse mAb anti-CD9 clone MM2-57 (Millipore CBL162, WB 1:1000), rabbit pAb anti-Flag (SA, F7425, IF 1:1000, WB 1:1000), rabbit pAb anti-GAPDH clone FL-335 (SCBT SC-25778, WB 1:3000), mouse mAb anti-Integrin α 3 / CD49c (BD TL 611044, WB 1:500, IF 1:500), mouse mAb anti-occludin (Invitrogen, OC-3F10, IF 1:800) mouse mAb anti- α -tubulin (SA, clone B-5-1-2, T5168, WB 1:10000), rat mAb anti-ZO-1 (kindly provided by Dr. B. Stevenson, University of Alberta, Edmonton, Canada), clone R40.76, IF 1:800). Isotype-specific control antibody: rabbit IgG (Thermo Scientific 02-6102). A polyclonal antibody against canine JAM-A (Affi831) was generated by immunizing rabbits with a fusion protein consisting of the extracellular part of canine JAM-A fused to the Fc-part of human IgG, as described previously [27]. Reagents: recombinant human vitronectin (VN, Peprotech 140-09), rat tail type I collagen (CN I, Advanced BioMatrix #5153), laminin (LN, isolated from human fibroblasts, SA #L4544), fibronectin (FN, isolated from human plasma, SA #F2006).

Quantitative Polymerase Chain Reaction (qPCR)

To analyze the mRNA level of CD9 and CD151, the total RNA was extracted from the cell lysate using the RNeasy Mini Kit following the instructions from Qiagen (Hilden, Germany) and transcribed in cDNA using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Waltham, Massachusetts, US). The following primer pairs were used (forward; reverse): CD9_1: GCTCTTGCTGGGATTGCAG; GAAACCCACCAGCATCATG, CD9_2:

CTGGGGTTGTTCTTTGGC; CGTAGTGGATGGCCTTCAG, CD9_3:
 GTGGAACAGTTTATCTCCGAC; GATAGCACAGCACAGGATC, CD151:
 GAGAAGACGACATGTGGCAC; GTAGGCTGTGGCCAGGTAG, CD151_2:
 GTTGTCGTCATGGTGA CTG; GTTCAGCTGCTGGTAGTAG, CD151_3:
 CAACCTGAAGGACACCATG; CACCCTCCACCTTGTAGATG, GAPDH: TCACC-
 ACCATGGAGAAGGC; GGCTAGAGGAGCCAAGCAG, HPRT: GCTTGCTGGTGAAAAG-
 GAC; TTATAGTCAAGGGCATATCC

Immunoprecipitation and western blot analysis

For immunoprecipitations, cells were lysed in lysis buffer (150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 0,02% (w/v) NaN₃, 10 mM TrisHCl pH 7.4, 1% (w/v) Brij98 (Merck, Darmstadt, Germany) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail; Roche, Indianapolis, IN) and phosphatase inhibitors (PhosSTOP™, Roche, Indianapolis) for 30 min on ice and then centrifuged for 20 min at 4°C. For one experiment (Figure 4A), 1% (v/v) Nonidet P-40 (NP-40, AppliChem, Darmstadt, Germany) was used as an alternative for Brij98. The resulting supernatants were incubated with 2.5 µg of antibodies coupled to protein A-Sepharose beads (GE Healthcare, Solingen, Germany) for 4 h at 4°C. Afterwards, the beads were washed five times with lysis buffer and bound proteins were eluted using SDS sample buffer. For Western blot analysis of the tetraspanin CD9 by immunoblotting, SDS sample buffer without DTT was added for elution, and 50 mM DTT was selectively added to the samples used for the detection of JAM-A. Proteins were separated by SDS-PAGE and analysed by Western blot with near-infrared fluorescence detection (Odyssey Infrared Imaging System Application Software Version 3.0 and IRDye 800CW- or IRDye 680CW-conjugated antibodies; LI-COR Biosciences, Bad Homburg, Germany). All immunoprecipitations shown in the figures are representatives for at least three experiments. For quantification of signal intensities, the same software as for data acquisition (Odyssey application software Version 3.0) was used.

Immunoprecipitation of GFP fusion proteins

GFP fusion proteins were precipitated by using the GFP Selector (NanoTag Biotechnologies, Göttingen, Germany). After cell lysis, the postnuclear supernatant was added to 20 μ l of GFP Selector slurry. After incubation for 1 h at 4°C, the GFP Selector beads were washed five times with lysis buffer. Bound proteins were eluted by boiling in SDS sample buffer analyzed by Western blotting as described above.

Immunofluorescence microscopy

For immunofluorescence microscopy, cells were grown on collagen-coated glass slides for 24 h and subsequently fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 min at room temperature (RT) or methanol (BioChemica, Billingham, England) for 5 min on ice. For PFA-fixed cells, permeabilization was performed by applying 0.5% Triton X-100 in PBS for 10 min at RT and the cells were washed three times with 100 mM glycine in PBS. For both fixation methods, unspecific binding sites were blocked for 1 h using blocking buffer (10% FCS, 0.2% Triton X-100, 0.05% Tween-20, 0.02% BSA in PBS) and the primary antibody was applied overnight at 4°C. After washing three times with PBS, the cells were incubated with fluorochrome (AlexaFluor488, AlexaFluor594 or AlexaFluor647)-conjugated, highly cross-adsorbed secondary antibodies (Invitrogen) and 2,4-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 2 h at RT. F-actin was visualized using AlexaFluor647-conjugated phalloidin. The samples were washed thoroughly with PBS and mounted in fluorescence mounting medium (Mowiol 4-88, Sigma-Aldrich). Immunofluorescence microscopy was performed using the confocal LSM800 Airyscan microscope (Carl Zeiss, Jena, Germany) equipped with a Plan-Apochromat x 63/1.4 oil differential interference contrast objective (Carl Zeiss). Image processing and quantification was performed using ZEN 2012, ImageJ (National Institutes of Health, Bethesda, MD) and Imaris (Bitplane, Version 9.1.2) software.

Single cell migration

For single cell migration assays, MDCK II cells transfected with either LA-EGFP or LA-mCherry were seeded on CNI-, VN-, FN-, or LN-coated microscope slides (Ibidi μ -Slide 8 well glass

bottom, Ibidi #80827) at a cell density of 2000 cells / cm². After overnight incubation, the cells were analyzed using the confocal LSM780 microscope (Carl Zeiss, Jena, Germany) with a Plan-Neofluar × 20/0.5 objective for 10 h with images taken every 10 min. The velocity of single cells was determined by tracking the cells using the TrackMate Plugin from ImageJ, which tracks the cell from frame to frame and thereby determines its average velocity.

Collective cell migration

For the analysis of collective cell migration epithelial cell monolayers, a monolayer expansion assay was used in which collective cell migration is triggered by a free surface [13, 31]. MDCK cells were seeded in different compartments of CNI-, VN-, FN-, or LN-coated microscope slides (Ibidi μ-Slide 2 well glass bottom, Ibidi #80287) separated by a removable silicone stamp (Ibidi Culture-Inserts 2 Well for self-insertion, Ibidi #80209). Cells were grown for 72 h to confluency before removal of the stamp to trigger sheet migration. Pictures were taken directly after removal of the stamp and 8 h later by using an EVOS digital inverted microscope. The collective cell migration speed was calculated as the mean distance of the front of the cell sheet from the initial position of the cell sheet's front at the end of the observation time using ImageJ. Briefly, the cell-free area measured at the end of the observation period ($t_1 = 8\text{h}$) was subtracted from the cell-free area at the beginning (t_0) resulting in the total area covered by migrated cells. This area was divided by the height of the gap resulting in the total distance that the cells had migrated. To take into account that the cells close the gap from both sides the total distance was divided by two resulting in the distance migrated by a single sheet. The migration speed of the cellular collective was calculated by dividing the distance of a single sheet by the observation time and is given in μm/min.

Analysis of single cell motility within cell collectives

Cell motility of single cells embedded in migrating cell collectives was analyzed by seeding co-cultures of LA-mCherry- and LA-EGFP-transfected MDCK cells (mixed at ratios of 1:5) on CNI-coated microscope slides used for collective cell migration (see above). After cells had reached

full confluency (72 h after seeding) stamps were removed to induce collective cell migration. 6 h after removal of the stamps, cell behaviour was recorded for 10 h with images taken every 3 min using the confocal LSM780 microscope (Carl Zeiss, Jena, Germany) with a Plan-Apochromat x 63/1.4 oil objective. For analysis, the LA-mCherry and LA-EGFP channels, a median filter was applied and cells from one channel were segmented as binary (black and white) images (BW) by manual thresholding.

For single cells movies, cell segmentation was performed acc. to [32]. Briefly, the center of the segmented cell was determined for each frame to calculate the average velocity and directionality using the TrackMate Plugin for ImageJ. To access the dynamic changes of the cell outline, the segmentations of a cell at two different time points was compared using the Jaccard Index J according to the following formula:

$$J(t) = \frac{|A \cap B|}{|A \cup B|}$$

Where A and B are the segmented cells at time point $(t - \Delta t)$ and $(t + \Delta t)$ respectively:

$$A = BW(t - \Delta t)$$

$$B = BW(t + \Delta t)$$

The Jaccard Index is equal to 1 if the cell outline is not changed between the two time points and tends to zero for higher differences, indicating higher protrusion/retraction activity at the cell border. Note, that this index can change, even if the center of the segmented cell does not move at all.

For movies with one or more cells, the protrusion and retraction activity along the segmented region was quantified. To estimate the protrusion activity, the area that was exclusively occupied by the segmented cells at the time point $(t + \Delta t)$ but not at time point $(t - \Delta t)$ was determined and normalized to the perimeter of the segmented area at time point t :

$$Area\ Increase(t) = \frac{|B \setminus A|}{Perimeter(BW(t))}$$

In analogy, the retraction activity was calculated:

$$Area\ Decrease(t) = \frac{|A \setminus B|}{Perimeter(BW(t))}$$

For these calculations Δt was set to 3 min.

All parameters are represented as mean values over the entire duration of the experiment. The scripts to calculate the Area Increase, the Area decrease and the Jaccard indices are freely available by the authors upon request. Jaccard indices we

Statistics

Results are expressed as arithmetic means $\pm$ SD as indicated. To test the normality of data, D'Agostino-Pearson normality test was used. Data with normal distributions were statistically compared by using unpaired, two-tailed student's t-test, whereas Mann-Whitney U test was applied for data without normal distribution. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, CA). P-values are indicated as follows: *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Results

JAM-A limits single cell migration in a cell-autonomous manner in polarized epithelial cells

We first analyzed single cell migration of MDCKII cells after depletion of JAM-A using a doxycycline (dox)-regulatable JAM-A shRNA expression system [27] (Suppl. Fig. 1). To address a possible contribution of the extracellular matrix (ECM), cells were plated on different ECM components including collagen type I (CNI), fibronectin (FN) and vitronectin (VN). Cell motility was analyzed by live cell microscopy over a period of 10 h. Surprisingly, JAM-A depletion resulted in a significant increase in migratory speed (Fig. 1A, Suppl. Movie 1, 2) suggesting that JAM-A limits motility at the single cell level. The increase in migration speed after JAM-A depletion was only observed in cells plated on CNI but not in cells plated on FN or on VN suggesting that its function in limiting migratory activity in single cells depends on its functional interaction with a CNI-binding integrin. To further confirm the increased motility of

MDCKII cells in the absence of JAM-A we used JAM-A knockout (KO) MDCKII cells with a CRISPR/Cas9-mediated inactivation of the JAM-A gene [28] (Suppl. Fig. 2). As observed after shRNA-mediated downregulation of JAM-A expression, JAM-A gene inactivation resulted in a significantly increased migration velocity which was restored to levels observed in control MDCKII cells by ectopic expression of murine JAM-A (mJAM-A) (Fig. 1B). Interestingly, ectopic expression of mJAM-A constructs with mutations at phosphorylation sites regulating JAM-A function, i.e. Tyr281 and Ser285 [18, 33, 34], did not restore the migratory activity to levels observed in MDCKII WT cells (Fig. 1C). Together, these findings indicated that JAM-A limits the migratory activity in polarized epithelial cells in the absence of cell-cell adhesion, suggesting a cell-autonomous function of JAM-A in cell migration. They also suggested that this function depends on a functional interaction of JAM-A with a CNI-binding integrin and involves phosphorylation of JAM-A at Tyr281 and at Ser285.

JAM-A positively regulates collective cell migration in polarized epithelial cells

We next analyzed the role of JAM-A in collectively migrating cells in a monolayer expansion model [13, 31]. To this, cells were grown to confluency on slides in which individual chambers are separated by removable silicon stamps. After reaching confluency, cell sheet migration was induced by removing the stamps. Migration of the monolayer was monitored over a period of 8 h. In contrast to single cells, depletion of JAM-A resulted in a reduced migration velocity of the cellular collective (Fig. 2A). This effect was only observed when cells migrated on CNI but not when cells migrated on FN or on LN (Fig. 2A). The reduction in migration velocity of cell collectives was also observed after CRISPR/Cas9-mediated inactivation of the JAM-A gene (Fig. 2B). Ectopic expression of mJAM-A in these cells almost completely restored the migration velocity to levels observed in control MDCKII cells (Fig. 2B), whereas ectopic expression of mJAM-A/Y281F or mJAM-A/S285A did not restore this function (Fig. 2C). These observations indicated that in contrast to its inhibitory function on cell migration in single cells, JAM-A promotes cell migration when cells migrate in a collective in a Tyr281- and Ser285-phosphorylation-dependent manner.

JAM-A regulates cryptic lamellipodia dynamics during collective cell migration

To further understand the role of JAM-A during collective cell migration we focussed on single cells embedded in an environment of collectively migrating cells. For this, we performed collective cell migration experiments with mixed populations of LA-mCherry and LA-EGFP-labelled cells. We first analyzed the formation of cryptic lamellipodia by quantifying the extent of cellular overlap between individual cells and their neighbours on the basis of the merged mCherry and EGFP fluorescence signals. We observed that the total area of overlap between individual cells did not change after JAM-A depletion (Fig. 3A; Suppl. Movie 3, 4). However, JAM-A-depleted cells showed reduced protrusion dynamics as indicated by smaller areas of positive and negative protrusion formation, i.e. overlapping areas that grow or shrink over time (Fig. 3B, Suppl. Movie 5, 6). We next analyzed the motility, average velocity and directionality of individual cells within a collective of migrating cells over a period of 10 h. We observed that cell motility as analyzed by the relative change in a cell's position (indicated by Jaccard indices closer to 1) was reduced after JAM-A depletion (Fig. 3C, Suppl. Movie 7, 8). Accordingly, the migration velocity as well as migration directionality were reduced after JAM-A depletion (Fig. 3C). These findings indicated that JAM-A regulates the motility of individual cells during collective cell migration, most likely by impacting on the dynamics of protrusion formation.

JAM-A interacts with $\alpha 3\beta 1$ integrin in MDCKII cells

The lack of efficient locomotion of cells during collective cell migration after depletion of JAM-A together with our observations that JAM-A-regulated single cell and collective cell migration depend on CNI (Fig. 1A, Fig. 2A) suggested that JAM-A might cooperate with a CNI-binding integrin in MDCKII cells. We focussed on $\alpha 3$ integrin, which forms a CNI- and LN-binding heterodimer with $\beta 1$ integrin [35, 36]. In co-immunoprecipitation (CoIP) experiments we found that JAM-A and $\alpha 3\beta 1$ integrin exist in a complex in MDCKII cells (Fig. 4A). This interaction was detectable in Brij98-containing lysis buffer but not in NP40-containing lysis buffer. Since Brij98 maintains interactions mediated by tetraspanins (Tspans) [37], a family of integral membrane

proteins with four membrane spanning domains [37-40], these findings suggested that the interaction between JAM-A and $\alpha 3\beta 1$ integrin is indirect and mediated by a member of the Tspan superfamily. To test if the interaction depends on integrin activation by ligand binding, as described for the interaction between JAM-A and $\alpha v\beta 3$ integrin in endothelial cells [26, 41], we performed CoIP experiments with MDCKII cells cultured on $\alpha 3\beta 1$ integrin-interacting and -non interacting ECM proteins, i.e. LN and VN, respectively. The interaction was detectable on all ECM proteins (Fig. 4B) suggesting that JAM-A is constitutively associated with $\alpha 3\beta 1$ integrin.

To further characterize the interaction of JAM-A with $\alpha 3\beta 1$ integrin we performed CoIP experiments with mutants of JAM-A which lacked either the cytoplasmic domain (JAM-A/ $\Delta 40$), or the membrane-distal Ig-like domain which contains the dimerization motif [42] (JAM-A/ $\Delta D1$), or the asparagine residue required for glycosylation present in the membrane-proximal Ig domain (JAM-A/N185Q), or the entire extracellular domain (JAM-A/MC) (Fig. 4C). The interaction of JAM-A with $\alpha 3\beta 1$ integrin was detectable in the absence of the cytoplasmic domain indicating that the interaction involves the extracellular domain of JAM-A. The interaction was also detectable in the absence of the membrane-distal Ig-like domain and in the absence of N-linked glycosylation but not when the entire extracellular domain of JAM-A was deleted (Fig. 4C). These findings indicated that JAM-A interacts with $\alpha 3\beta 1$ integrin through the membrane-proximal Ig-loop of its extracellular domain, and that this interaction is independent of JAM-A dimerization and of N-linked glycosylation.

To address the question of a functional interaction between JAM-A and $\alpha 3\beta 1$ integrin we analyzed collective cell migration of MDCKII cells after depletion of the $\alpha 3$ subunit by RNAi. Similar to the depletion of JAM-A, depletion of the $\alpha 3$ integrin chain resulted in a reduced migration velocity of cell collectives grown on CNI (Fig. 4D). Reduced migration velocity was also observed when cells were grown on LN (Fig. 4D), the second major ligand of $\alpha 3\beta 1$ integrin [36]. These observations suggested that JAM-A and $\alpha 3\beta 1$ integrin exist in a complex to co-operate in the regulation of collective cell migration in polarized epithelial cells.

JAM-A interacts with $\alpha 3\beta 1$ integrin-interacting tetraspanins CD151 and CD9

At least 12 different integrin heterodimers have been found in associations with Tspans [40, 43]. Tspans interact laterally with several binding partners, and these interactions are frequently sensitive to Triton-X100 or NP40-based lysis conditions [37, 39]. Our observation that the interaction of JAM-A with $\alpha 3\beta 1$ integrin is sensitive to NP40-based lysis but detectable in Brij98-based lysis buffers suggested that the JAM-A - $\alpha 3\beta 1$ integrin interaction is indirect and mediated by a member of the tetraspanin family. We focussed on CD151/Tspan24, which directly interacts with $\alpha 3\beta 1$ integrin through an association that involves the extracellular domains of the two proteins [44-46], and which regulates $\alpha 3\beta 1$ function in many cell types [47-53]. Due to the lack of antibodies that recognize canine CD151 in Western blot analyses, we ectopically expressed a EGFP-CD151 fusion protein in MDCKII cells and analyzed its interaction with JAM-A by CoIP. JAM-A readily co-immunoprecipitated with CD151 (Fig. 5A). Similar to the interaction of JAM-A with $\alpha 3\beta 1$ integrin, the interaction with CD151 was retained in the absence of the cytoplasmic domain as well as in the absence of the D1 Ig domain of JAM-A (Fig. 5B) indicating that JAM-A interacts with CD151 through the extracellular, membrane-proximal Ig domain of JAM-A. To test if CD151 is solely responsible for the JAM-A - $\alpha 3\beta 1$ integrin association, we performed CoIP experiments in CD151-depleted MDCKII cells. The JAM-A - $\alpha 3\beta 1$ integrin interaction was retained in the absence of CD151 (Fig. 5C), suggesting the presence of additional Tspans in a JAM-A – CD151 - $\alpha 3\beta 1$ integrin-containing tetraspanin-enriched microdomain (TEM).

CD9/Tspan29 has been described as a second $\alpha 3\beta 1$ integrin-interacting Tspan [40, 54]. Previous findings showed that JAM-A interacts with CD9 in platelets and endothelial cells [26, 55]. We therefore analyzed the association of JAM-A with CD9 in MDCKII cells. We found that CD9 coimmunoprecipitates with JAM-A (Fig. 5D). This interaction was retained in the absence of the cytoplasmic domain of JAM-A (Fig. 5E) suggesting that in contrast to endothelial cells, in which the interaction with CD9 is mediated through its PDZ domain binding motif [26], JAM-A interacts with CD9 in MDCK epithelial cells through its extracellular domain. Overall, these findings suggested that JAM-A exists with $\alpha 3\beta 1$ integrin in TEM and that the physical

interaction of JAM-A with $\alpha 3\beta 1$ integrin can be mediated by Tspan proteins CD151 and CD9 (Fig. 5F).

Depletion of CD151 attenuates collective cell migration

To address a possible role of the interaction of JAM-A with $\alpha 3\beta 1$ integrin through Tspan CD151 we depleted CD151 in MDCKII cells by RNAi and analyzed the migration velocity during collective cell migration. Knockdown of CD151 significantly attenuated migration velocities of collectively migrating cells (Fig. 6). Similar to what was observed after depletion of $\alpha 3\beta 1$, the depletion of both CD151 and JAM-A reduced the migratory speed of collectively migrating cells on both major $\alpha 3\beta 1$ integrin ligands, i.e. CNI and LN (Fig. 6). Overall, these findings suggested that JAM-A, CD151 and $\alpha 3\beta 1$ integrin co-exist in a TEM to form a functional unit which regulates collective cell migration in polarized epithelial cells.

Discussion

JAM-A is a cell-cell adhesion receptor that is localized at cell-cell junctions of polarized epithelial cells with enrichment at the TJs [18, 56, 57]. Its role in the regulation of the epithelial barrier function, which involves phosphorylation at two sites in the cytoplasmic tail, has been intensively studied [18, 27, 33, 57]. By addressing JAM-A's role in cell migration of polarized epithelial cells, we find that JAM-A limits cell motility when cells migrate as individuals but promotes cell migration when cells migrate as a cellular sheet. The regulation of collective cell migration is most likely mediated through its interaction with $\alpha 3\beta 1$ integrin, which is mediated by Tspan CD151 and perhaps CD9. We propose the existence of a TEM in which Tspan CD151 connects JAM-A to $\alpha 3\beta 1$ integrin to assemble a signalling complex that allows a functional crosstalk of JAM-A and $\alpha 3\beta 1$ integrin during collective cell migration.

Cell motility and collective cell migration

One observation in our study is that JAM-A depletion results in increased motility of single cells but reduced motility when cells are embedded in a collective of migrating cells. These observations thus indicated a cell-autonomous function of JAM-A in single epithelial cells. A similar, i.e. cell-autonomous and motility-limiting function of JAM-A has been observed in endothelial cells and dendritic cells (DCs) [58, 59]. In JAM-A-deficient endothelial cells the increased motility correlates with longer protrusions [59], suggesting that JAM-A limits protrusion formation in these cells. This activity has been proposed to be mediated through glycogen synthase kinase (GSK)-3 β and its upstream regulator atypical protein kinase C-zeta (α PKC ζ). In MDCK cells, GSK-3 β regulates membrane protrusions and migration through Rac1 [60] opening the possibility that JAM-A regulates single cell motility in polarized epithelial cells through a similar mechanism as in endothelial cells. Interestingly, JAM-A has also been found to regulate cell motility of neutrophils. In these cells, JAM-A co-clusters with α 5 β 1 integrin and regulates β 1 integrin internalization and recycling, and the absence of JAM-A results in increased β 1 integrin surface localization concomitant with reduced cell motility [61]. Our findings of an increased motility of singly migrating MDCKII cells adds to the growing evidence of a cell-autonomous function of JAM-A.

Surprisingly, despite an increased motility of singly migrating cells after JAM-A depletion, individual cells embedded in a collective of cells show an impaired motility, reduced migration velocity and reduced directionality after JAM-A depletion, which results in a reduced migration velocity of the cell collective. We found that the size of the cryptic protrusions does not change upon JAM-A depletion. However, the dynamics of cryptic lamellipodia formation is reduced, which is expected in more static cell-matrix interactions and thus reduced motility of single cells. We speculate that JAM-A depletion alters traction force generation. JAM-A depletion in MDCK cells has recently been found to enhance focal adhesion formation and to increase traction forces [62]. An enhanced formation of focal adhesions resulting in stronger cell-matrix adhesion and concurrent increased traction forces would explain why JAM-A-depleted MDCK cells show reduced migration velocities during collective cell migration [63]. Since we observed a reduced migration velocity upon JAM-A depletion when cells migrate as collective but not

when cells migrate as single cells, we speculate that the regulation of traction force formation by JAM-A depends on signalling events triggered by trans-homophilic interactions of JAM-A.

An intriguing possibility how JAM-A-generated signalling events influences cell-matrix adhesions and traction force generation during collective cell migration is that JAM-A acts as a sensor of mechanical forces during collective cell migration. Studies with collectives of migrating cells both *in vitro* and *in vivo* indicate that collective cell migration requires mechanical coordination between neighbouring cells, and that mechanical forces are transmitted through cell-cell contacts from leader cells at the edges of migrating colonies to the follower cells in their center over a long range [11-13, 15, 64], reviewed in [65, 66]. Tension on JAM-A applied through trans-homophilic JAM-A interaction activates RhoA in a Ser285 phosphorylation-dependent manner in CHO cells [67]. Importantly, in MDCK cells JAM-A regulates tensile stress on the TJ scaffolding protein ZO-1 through its interaction with PDZ domain 3 [62, 68]. These observations thus suggest the possibility that JAM-A localized at TJs contributes to the regulation of collective cell migration by transmitting tensile forces at the TJs to its directly associated scaffold protein ZO-1 which changes conformation in response to tensile forces [62, 69]. This possibility is supported by our observation that Ser285 phosphorylation of JAM-A, which is observed exclusively at the TJs [18], is required for the regulation of collective cell migration of JAM-A (Fig. 2). JAM-A might thus contribute to the regulation of collective cell migration by different mechanisms, one involving the transmission of mechanical strain through TJs and concurrent regulation of actomyosin contractility, traction forces and focal adhesion formation via RhoA [62, 69, 70], and one involving the regulation of integrin-dependent functions through its association with $\alpha 3\beta 1$ integrin (see below).

The JAM-A – CD151 – $\alpha 3\beta 1$ TEM

As the second major observation of our study, we find that JAM-A interacts with $\alpha 3\beta 1$ integrin in MDCK cells. This interaction involves the membrane-proximal extracellular Ig domain of JAM-A, identifying for the first time a cis-heterophilic interaction partner which is linked to the extracellular domain of JAM-A. This interaction is most likely indirect and mediated through a

Tspan since it is only detectable under lysis conditions that retain Tspan-mediated interactions, i.e. Brij98 [37, 40]. We identified Tspans CD151 and CD9 as possible mediators of this interaction. Both tetraspanins are binding partners of $\alpha\beta 1$ integrin [40]. We consider CD151 as more likely to link JAM-A and $\alpha\beta 1$ integrin during collective cell migration since CD151 has been found to regulate a number of $\alpha\beta 1$ integrin-dependent functions including cell-matrix adhesion, cell motility and migration on LN [46, 50, 53, 71], intercellular junction formation [48, 52, 72] or cell survival [73]. Our observations that depleting either $\alpha\beta 1$ integrin or CD151 results in a similar retardation of collective cell migration in MDCK cells like depleting JAM-A supports the view that JAM-A regulates collective cell migration of MDCK cells through its physical association with $\alpha\beta 1$ integrin mediated by CD151 and possibly CD9.

How JAM-A regulates collective cell migration through these associations remains unclear. Both $\alpha\beta 1$ integrin and CD151 have been identified at cell-cell junctions of various cell types including tumor cells [52, 72], keratinocytes [73, 74], and kidney epithelial cells derived from collecting ducts [48]. Therefore, it is likely that the CD151 (and possibly CD9)-based TEM containing JAM-A and $\alpha\beta 1$ integrin is localized at cell-cell junctions. We speculate that JAM-A regulates $\alpha\beta 1$ integrin-associated functions. Studies in platelets have shown that Tyr-phosphorylated JAM-A recruits the Src kinase Csk to inhibit $\alpha\text{IIb}\beta 3$ -associated Src during outside-in signalling [75]. We therefore envisage a scenario for MDCK cells in which the lateral association of JAM-A with $\alpha\beta 1$ integrin through a tetraspanin as linker protein might serve to generate spatial proximity between JAM-A and possibly a JAM-A-associated regulatory protein and an $\alpha\beta 1$ integrin-associated signalling molecule. Interestingly, we found that a JAM-A construct with a mutation in the known Tyr phosphorylation site (JAM-A/Y281F) [33] fails to restore single cell and collective cell migration in JAM-A null MDCK cells to levels observed after expression of JAM-A/WT (Fig.1, Fig. 2), strongly suggesting that Tyr281 phosphorylation is required to mediate JAM-A's function during single and collective cell migration. This observation is intriguing since phosphorylation/dephosphorylation allows for dynamic regulation of signalling events as they occur during cell migration. Recent observations in integrin-based cell-matrix adhesion complexes indicate that signal propagation within these

complexes does not involve gross changes in the composition of the adhesion complex, but is regulated by the relay of phosphotyrosine-dependent signals [76]. The incorporation of JAM-A and $\alpha 3\beta 1$ integrin in a single protein cluster by the activity of CD151 and possibly CD9 could thus serve to rapidly transmit phospho-JAM-A-dependent regulatory events on $\alpha 3\beta 1$ integrin-dependent signalling pathways.

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DECLARATIONS

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Conflict of interest

The authors declare that they have no competing financial interests.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files, or are available from the corresponding author upon reasonable request. The source data underlying Fig. 1, Fig. 2, Fig. 3, Fig. 4 and Fig. 6 are provided as a Source Data file.

Code availability

Software scripts are freely available from the authors upon request.

Author contributions

S.T. and K.E. designed and conceived the study. S.T. performed experiments. J.S. and H.S. generated and provided software used for data analysis. T.O., M.F., L.F. and V.G. provided reagents. S.T., J.S. and K.E. analysed the data. S.T. and K.E. wrote the manuscript.

Ethics approval

Studies involving humans or animals were not performed.

Consent to participate

N.A.

Consent for publication

All authors have seen the manuscript and agree with publication.

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Legends to Figures and Supplemental Movies

Figure legends

Fig. 1: JAM-A limits single cell migration. (A) Single cell migration of MDCKII cells with inducible expression of JAM-A shRNAs (pEmU6proT, -Dox: shRNA not induced, +Dox: shRNA induced). Control cells (-Dox) were stably transfected with LA-mCherry, JAM-A KD cells (+Dox) were stably transfected with LA-EGFP. Cells were cultured on collagen, vitronectin or fibronectin as indicated. Quantifications of single cell migration speed are shown in right panels. Number of cells analyzed: collagen: -Dox: 45, +Dox: 38, fibronectin: -Dox: 48, +Dox: 44; vitronectin: -Dox: 52, +Dox: 52, 5 independent experiments. **(B)** Quantifications of single cell migration speed of JAM-A KO MDCKII cells reconstituted with an inducible expression vector for murine JAM-A (pInducer21, -Dox: not induced, +Dox: induced). As control cells, MDCKII cells with inducible JAM-A shRNA expression in the absence of Dox were used. Number of cells analyzed: Ctrl MDCKII: 63; JAM-A KO, -Dox: 64; JAM-A KO, +Dox: 63; 3 independent experiments. **(C)** Quantifications of single cell migration speed of JAM-A KO MDCKII cells reconstituted with an inducible expression vector encoding phospho-deficient mJAM-A mutants (mJAM-A Y281F or mJAM-A S285A in pInducer21, -Dox: not induced, +Dox: induced). Number of cells analyzed: Ctrl MDCKII: 70; JAM-A KO + mJAM-A Y281F: -Dox: 67; +Dox: 65; JAM-A KO + mJAM-A S285A: -Dox: 62; +Dox: 65; 4 independent experiments). All statistical analysis shown in this figure was performed using Mann-Whitney-U test. Data are presented as mean values $\pm$ SD. NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Source data are provided as a Source Data file.

Fig. 2: JAM-A positively regulates collective cell migration. (A) Monolayer expansion assays of MDCKII cells with inducible expression of JAM-A shRNAs (described in the legend to Fig. 1). Cells were seeded on collagen-, fibronectin- or vitronectin-coated microscope slides separated by a silicone stamp. Collective migration was triggered by stamp removal. The migration velocity of the cell collective was quantified by measuring the cell-free area directly

after the removal of the stamp and 8 h later (see Methods section for details). Left panels: Representative images of monolayer expansion immediately after stamp removal (0 h) and after 10 h (10 h). Right panels: Quantifications of collective cell migration velocities on different ECM substrates. Number of images analyzed: collagen: $n = 12$ for each cell line (4 independent experiments), fibronectin: $n = 10$ for each cell line (3 independent experiments), vitronectin: $n = 9$ for each cell line (3 independent experiments). **(B)** Quantifications of collective cell migration speed of JAM-A KO MDCKII cells reconstituted with an inducible expression vector for murine JAM-A (see legend to Fig. 1B). Number of images analyzed: $n = 12$ for each condition, 3 independent experiments. **(C)** Quantifications of collective cell migration speed of JAM-A KO MDCKII cells reconstituted with an inducible expression vector encoding phospho-deficient mJAM-A mutants (see legend to Fig. 1C). Number of images analyzed: $n = 14$ for ctrl MDCKII cells, $n = 12$ for JAM-A KO MDCKII cells and mJAM-A expressing JAM-A KO MDCKII cells; 3 independent experiments. All statistical analysis shown in this figure was performed using two-tailed student's t-test. Data are presented as mean values $\pm$ SD. NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Source data are provided as a Source Data file.

Fig. 3: JAM-A regulates cryptic lamellipodia dynamics and cell motility in cellular collectives. **(A)** Formation of cryptic lamellipodia in collectively migrating cells. Homotypic suspensions of differently labelled (LA-mCherry and LA-EGFP, mixed at 1:5 ratio) MDCKII cells were observed by live cell microscopy over a period of 10 h. Left panel: Representative immunofluorescence signals of cryptic lamellipodia. Middle panels: Binary images of cryptic lamellipodia shown in the left panels. Right panel: Quantification of overlapping areas. Areas of overlapping regions were calculated on the basis of the merged LA-EGFP and LA-mCherry signals using an ImageJ Macro (see Methods for details). Data indicate the mean areas of overlap during the observation period. Number of videos analyzed: $n = 40$, 6 independent experiments. **(B)** Dynamics of cryptic lamellipodia during collective cell migration. Left panels: Representative immunofluorescence signals of overlapping areas. Middle panels:

Representative binary images illustrating increases (white areas) and decreases (black areas) of cellular overlaps over the observation period (8 h). Right panel: Quantification of changes in overlapping regions. Changes were calculated using an ImageJ Macro on the basis of the merged LA-EGFP and LA-mCherry signals (see Methods for details). Data indicate the mean increase or decrease of overlapping regions during the observation period. Number of videos analyzed: $n = 40$, 6 independent experiments. **(C)** Migration behavior of single cells embedded in a cell collective. Left panels: Representative immunofluorescence signals of LA-mCherry-positive cells embedded in a LA-EGFP-positive collective. Middle panel: Binary images of single LA-mCherry-positive cells. Right panels: Jaccard index, migration velocity and directionality of single cells. Note that high Jaccard index values reflect low motility (see Methods for details). Number of cells analyzed: $n = 50$ for ctrl MDCKII cells, $n = 47$ for JAM-A KD MDCKII cells, 6 independent experiments. Statistical analysis of migration velocities was performed using Mann-Whitney-U test and all other statistical analysis were performed using two-tailed student's t-test. Data are presented as mean values $\pm$ SD. NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Source data are provided as a Source Data file.

Fig. 4: JAM-A interacts with $\alpha 3\beta 1$ integrin in MDCKII cells. **(A)** CoIP of $\alpha 3\beta 1$ integrin with JAM-A. The interaction of JAM-A with $\alpha 3\beta 1$ integrin is detectable in Brij98-based lysates but not in NP40-based lysates. Note that the weak JAM-A signal in Brij98 lysed cells is due to lower solubility of JAM-A in Brij98. Abbreviations: IP, immunoprecipitation; Lys, lysate. **(B)** CoIP of $\alpha 3\beta 1$ integrin with JAM-A from cells cultured on collagen, laminin or vitronectin. JAM-A interacts with $\alpha 3\beta 1$ integrin independent of $\alpha 3\beta 1$ integrin ligation. **(C)** CoIPs of JAM-A deletion mutants with $\alpha 3\beta 1$ integrin. JAM-A constructs with deletions of the entire cytoplasmic domain ($\Delta 40$), the membrane-distal Ig-like domain ($\Delta D1$), the entire extracellular domain (MC), or with point mutations affecting the N-linked glycosylation site of JAM-A (N185Q) were transfected into JAM-A-deficient MDCKII cells and analyzed for interaction with $\alpha 3\beta 1$ integrin by CoIP. Note that deletion of the extracellular domain abolishes the interaction with $\alpha 3\beta 1$ integrin. Abbreviations: f.l., full length; MC, membrane and cytoplasmic. **(D)** Collective

cell migration velocity of control MDCKII cells (Ctrl siRNA pool) and $\alpha 3$ integrin KD MDCKII cells ($\alpha 3$ ITGN siRNA pool) on collagen and laminin. Left panels: Representative images of monolayer expansion immediately after stamp removal (0 h) and after 8 h (10 h). Right panels: Statistical evaluation of monolayer expansion on collagen and laminin. Data are derived from 3 independent experiments ($n = 12$ for each condition). Statistical analysis shown in this figure was performed using two-tailed student's t-test. Data are presented as mean values $\pm$ SD. **** $P < 0.0001$. Source data underlying figure part D are provided as a Source Data file.

Fig. 5: JAM-A interacts with tetraspanins CD151 and CD9 in MDCKII cells. (A) CoIP of CD151 with JAM-A. GFP-CD151 immunoprecipitates were immunoblotted for JAM-A (top panels) and GFP (bottom panels). (B) CoIPs of JAM-A deletion mutants with CD151. JAM-A deletion or mutant constructs (described in Fig. 4) were transfected into JAM-A-deficient MDCKII cells and analyzed for interaction with CD151 by CoIP. Note that the JAM-A – CD151 interaction is maintained after deletion of the entire cytoplasmic domain. (C) CoIP of $\alpha 3\beta 1$ integrin with JAM-A in CD151-depleted MDCKII cells. Note that the absence of CD151 does not abolish the interaction between JAM-A and $\alpha 3\beta 1$. Abbreviation: PNS, postnuclear supernatant. (D) CoIP of CD9 with JAM-A. JAM-A immunoprecipitates obtained from MDCKII cells were immunoblotted for CD9 (top panels) and JAM-A (bottom panels). (E) CoIPs of JAM-A deletion mutants with endogenous CD9. JAM-A deletion constructs lacking the PDZ domain-binding motif ($\Delta 3$) or the entire cytoplasmic domain ($\Delta 40$) were transfected into JAM-A-deficient MDCKII cells and analyzed for interaction with CD9 by CoIP. Note that the JAM-A – CD9 interaction is retained after deletion of the entire cytoplasmic domain. (F) Cartoon of JAM-A association with $\alpha 3\beta 1$ integrin through tetraspanins CD151 and CD9. Note that the interaction of JAM-A with both tetraspanins is mediated by the extracellular domain of JAM-A (red arrows), and that the interaction of CD151 with $\alpha 3\beta 1$ integrin is also mediated by the extracellular domains of the two proteins (green arrow) [44]. Western blot data shown in this figure are representatives of at least three independent experiments.

Fig. 6: Depletion of CD151 attenuates collective cell migration. Monolayer expansion assays of MDCKII cells after individual or simultaneous siRNA-mediated depletion of CD151. MDCKII cells were transiently transfected with siRNA pools directed against canine CD151 (CD151 KD). As control, the same MDCKII cells were transfected with a scrambled siRNA pool (Ctrl KD). JAM-A KD cells are LA-EGFP MDCKII cells with induced expression of JAM-A shRNAs, see legend to Fig.1). Collective cell migration was analyzed as described in the legend to Fig. 2. Top panels: Representative images of monolayer expansion immediately after stamp removal (0 h) and after 8 h (8 h). Bottom panels: Statistical evaluation of monolayer expansion on collagen and laminin. Number of images analyzed: $n = 12$ for each condition, 3 independent experiments. Statistical analysis was performed using two-tailed student's t-test. Data are presented as mean values $\pm$ SD. *** $P < 0.001$, **** $P < 0.0001$. Source data are provided as a Source Data file.

Suppl. Fig. 1: Efficiencies of siRNA- and shRNA-mediated knockdowns. (A) Left panel: JAM-A protein expression in MDCKII cells stably transfected with a conditional shRNA expression vector that expresses JAM-A shRNAs under a doxycycline-regulated promoter (pEmU6-proT, Tet-ON) without or with addition of doxycycline (-Dox or +Dox, respectively). Right panel: JAM-A protein expression in pEmU6-proT/JAM-A shRNA-transfected MDCKII cells stably transfected with LA-EGFP or LA-mCherry without or with addition of doxycycline (-Dox or +Dox, respectively). (B) Expression of the $\alpha 3$ integrin chain in LA-EGFP-expressing MDCKII cells after transient transfection with a control siRNA pool (ctrl) or a canine $\alpha 3$ integrin-specific siRNA pool ($\alpha 3$ ITGN). (C) Expression of CD151 mRNA in LA-EGFP-expressing MDCKII cells after transient transfection with a control siRNA pool (ctrl KD) or a canine CD151-specific siRNA pool (CD151 KD). CD151 mRNA expression levels were determined by qPCR (see Methods for details). Data are presented as mean values $\pm$ SD ($n = 3$ independent experiments).

Suppl. Fig. 2: Characterization of JAM-A KO MDCKII cells reconstituted with mJAM-A constructs. **(A)** JAM-A protein levels in control MDCKII cells (MDCKII cells transfected with pEmU6-proT/JAM-A shRNA, shRNA expression not induced, Ctrl MDCKII) and JAM-A KO MDCKII cells (CRISPR/Cas9-mediated JAM-A gene inactivation, JAM-A KO MDCKII). **(B)** Immunofluorescence analysis of JAM-A KO MDCKII cells. Cells were stained with antibodies as indicated. Note that markers for tight junctions (ZO-1) and adherens junctions (α -catenin, β -catenin) are unchanged after inactivation of the JAM-A gene. Scale bars: 10 μ m. **(C)** Expression levels of murine JAM-A (mJAM-A) constructs (mJAM-A/WT, mJAM-A/Y281F, mJAM-A/S285A) ectopically expressed in JAM-A KO MDCKII cells using a doxycycline-regulated expression vector (pInducer21, Tet-ON) without or with addition of doxycycline (-Dox or +Dox).

Suppl. Movies legends

Suppl. Movie 1 (SupplMovie1_single_cell_migration_ctrl.avi): **Single cell migration of a ctrl MDCKII cell.** Confocal laser scanning microscopy time-lapse movie of a LA-mCherry expressing MDCKII cell without induction of JAM-A shRNA expression (-Dox, ctrl). Z-stacks were taken every 10 minutes over a time-period of 10 h. Left: original video, right: analysis using the Trackmate plugin of ImageJ.

Suppl. Movie 2 (SupplMovie2_single_cell_migration_JAM-A_KD.avi): **Single cell migration of a JAM-A KD MDCKII cell.** Confocal laser scanning microscopy time-lapse movie of a LA-EGFP expressing MDCKII cell with induction of JAM-A shRNA expression (+Dox, JAM-A KD). Z-stacks were taken every 10 minutes over a time-period of 10 h. Left: original video, right: analysis using the Trackmate plugin of ImageJ.

Suppl. Movie 3 (SupplMovie3_cryptic_lamellipodia_ctrl.avi): **Cryptic lamellipodia in ctrl MDCKII cells.** Confocal laser scanning microscopy time-lapse movie of mixed LA-EGFP and LA-mCherry expressing MDCKII cells without induction of JAM-A shRNA expression (-Dox, ctrl). Z-stacks were taken every 3 minutes over a time-period of 10 h. Left: original video with overlapping areas highlighted in yellow, right: analysis of overlapping areas by ImageJ.

Suppl. Movie 4 (SupplMovie4_cryptic_lamellipodia_JAM-A_KD.avi): **Cryptic lamellipodia in JAM-A KD MDCKII cells.** Confocal laser scanning microscopy time-lapse movie of mixed LA-EGFP and LA-mCherry expressing MDCKII cells with induction of JAM-A shRNA expression (+Dox, JAM-A KD). Z-stacks were taken every 3 minutes over a time-period of 10 h. Left: original video with overlapping areas highlighted in yellow, right: analysis of overlapping areas by ImageJ.

Suppl. Movie 5 (SupplMovie5_cryptic_lamellipodia_ctrl.avi): **Dynamic of cryptic lamellipodia in collectively migrating ctrl MDCKII cells.** Confocal laser scanning microscopy time-lapse movie of mixed LA-EGFP and LA-mCherry expressing MDCKII cells without induction of JAM-A shRNA expression (-Dox, ctrl). Z-stacks were taken every 3 minutes over a time-period of 10 h. Left: original video with overlapping areas highlighted in yellow, right: analysis of the dynamic of overlapping areas by ImageJ (increase in area shown in white, decrease in black).

Suppl. Movie 6 (SupplMovie6_cryptic_lamellipodia_JAM-A_KD.avi): **Dynamic of cryptic lamellipodia in collectively migrating JAM-A KD MDCKII cells.** Confocal laser scanning microscopy time-lapse movie of mixed LA-EGFP and LA-mCherry expressing MDCKII cells with induction of JAM-A shRNA expression (+Dox, JAM-A KD). Z-stacks were taken every 3 minutes over a time-period of 10 h. Left: original video with overlapping areas highlighted in yellow, right: analysis of the dynamic of overlapping areas by ImageJ (increase in area shown in white, decrease in black).

Suppl. Movie 7 (SupplMovie7_single_cell_in_collective_ctrl.avi): **Migration behavior of single cells embedded in a cell collective (-Dox).** Confocal laser scanning microscopy time-lapse movie of mixed LA-EGFP and LA-mCherry expressing MDCKII cells without induction of JAM-A shRNA expression (-Dox, ctrl). Z-stacks were taken every 3 minutes over a time-period of 10 h. Left: original video, right: binary video of the single LA-mCherry expressing cell which were used to determine Jaccard indices, migration velocities and directionalities.

Suppl. Movie 8 (SupplMovie8_single_cell_in_collective_JAM-A_KD.avi): **Migration behavior of single cells embedded in a cell collective (+Dox).** Confocal laser scanning microscopy time-lapse movie of mixed LA-EGFP and LA-mCherry expressing MDCKII cells with induction of JAM-A shRNA expression (+Dox, JAM-A KD). Z-stacks were taken every 3 minutes over a time-period of 10 h. Left: original video, right: binary video of the single LA-mCherry expressing cell which were used to determine Jaccard indices, migration velocities and directionalities.

Figures

Figure 1

JAM-A limits single cell migration. (A) Single cell migration of MDCKII cells with inducible expression of JAM-A shRNAs (pEmU6proT, -Dox: shRNA not induced, +Dox: shRNA induced). Control cells (-Dox) were stably transfected with LA-mCherry, JAM-A KD cells (+Dox) were stably transfected with LA-EGFP. Cells were cultured on collagen, vitronectin or fibronectin as indicated. Quantifications of single cell migration speed are shown in right panels. Number of cells analyzed: collagen: -Dox: 45, +Dox: 38, fibronectin: -Dox: 48, +Dox: 44; vitronectin: -Dox: 52, +Dox: 52, 5 independent experiments. (B) Quantifications of single cell migration speed of JAM-A KO MDCKII cells reconstituted with an inducible expression vector for murine JAM-A (pInducer21, -Dox: not induced, +Dox: induced). As control cells, MDCKII cells with inducible JAM-A shRNA expression in the absence of Dox were used. Number of cells analyzed: Ctrl MDCKII: 63; JAM-A KO, -Dox: 64; JAM-A KO, +Dox: 63; 3 independent experiments. (C) Quantifications of single cell migration speed of JAM-A KO MDCKII cells reconstituted with an inducible expression vector encoding phospho-deficient mJAM-A mutants (mJAM-A Y281F or mJAM-A S285A in pInducer21, -Dox: not induced, +Dox: induced). Number of cells analyzed: Ctrl MDCKII: 70; JAM-A KO + mJAM-A Y281F: -Dox: 67; +Dox: 65; JAM-A KO + mJAM-A S285A: -Dox: 62; +Dox: 65; 4 independent experiments). All statistical analysis shown in this figure was performed using Mann-Whitney-U test. Data are presented as mean values $\pm$ SD.

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Supplementary Files

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