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A direct interaction of JAM-C with the tight junction scaffold protein ZO-2

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Abstract

Tight junctions are sites of cell-cell contacts at the apical region of epithelial intercellular junctions that are involved in barrier formation, cellular signaling and cell-cell adhesion. Tight Junctions are formed by integral membrane proteins associated with cytoplasmic scaffolding and adapter proteins through which they are linked to the underlying actomyosin and microtubule cytoskeletons. Here, we have addressed the interaction of the Junctional Adhesion Molecule (JAM)-C with the zonula adherens (ZO) protein ZO-2. Using a combination of cell-based recruitment assays and biochemical in vitro experiments, we find that JAM-C and ZO-2 directly interact in a PDZ domain-dependent manner. Notably, the interaction requires PDZ domain 3 as well as the SH3 domain of ZO-2 indicating that ZO-2 forms a functional supramodule to interact with JAM-C. We also find that JAM-C recruits cingulin to cell-cell contacts, and that JAM-C localization at tight junctions is significantly upregulated in the absence of JAM-A. Our findings have implications on important aspects of tight junction biology including mechanosensing and liquid-liquid phase separation.

Introduction

Tight junctions (TJ) are sites of direct contacts of adjacent epithelial cells at the apical region of intercellular junctions ¹. The direct cell-cell contact sites are established by claudins, a family of integral membrane proteins with four membrane-spanning domains ², which interact in cis and trans to form a mesh-like network of strands which prevents an uncontrolled passage of solutes and water along the paracellular pathway ³. Additional integral membrane proteins at the TJs include members of the TJ-associated Marvel protein (TAMP) family including occludin, tricellulin and MarvelD3, as well as members of the immunoglobulin superfamily (IgSF), such as the coxsackie- and adenovirus receptor (CAR) and the junctional adhesion molecules (JAMs) JAM-A, JAM-C and JAM4 ⁴.

All integral membrane proteins localized at the TJs interact through their cytoplasmic tails with peripheral membrane proteins which serve as scaffolds and adaptors ⁵. These proteins are characterized by various protein-protein interaction domains including PDZ domains, Src homology 3 (SH3) domains, guanylate kinase-like (GuK) domains, or proline-rich (PR) domains. The presence of multiple of such protein-protein interaction domains is the basis of the principal function of scaffolding proteins, i.e. the assembly of large protein complexes for intermolecular cross-talk, their localization at specific submembraneous sites, and their functional interaction with the microtubule network and the actomyosin cytoskeleton ⁵.

A family of scaffolding proteins that is particularly highly enriched at the TJs of epithelial cells and the postsynaptic density of neuronal cells is the membrane-associated guanylate kinase (MAGUK) family ^{6,7}. Members of this family are characterized by the presence of one or several PDZ domains, an SH3 domain, and a catalytically inactive GuK domain. Importantly, one of the PDZ domains often exists in a tandem organization with the SH3 and the GuK domains building up the PDZ – SH3 – GuK (PSG) core unit, a supramodule with distinct binding properties with regard to the isolated domains ⁸. MAGUK family members located at the TJs include the zonula occludens (ZO) proteins ZO-1, ZO-2 and ZO-3 ⁹, Protein associated with Lin-7 1 (Pals1) ¹⁰, and Membrane-associated guanylate kinase inverted 1 (MAGI-1) ¹¹. Other

scaffolding proteins at the TJs include the partitioning defective (PAR) polarity proteins PAR-3 and PAR-6, and two proteins with 10 or 13 PDZ domains, Pals1-associated tight junction protein (PATJ) and Multiple PDZ domain protein 1 (MUPP1), respectively ⁵. Most likely, additional scaffolding proteins localize either stably or transiently to TJs ¹² to regulate the TJ dynamics and plasticity ¹³.

One intriguing example of TJ plasticity regulated by scaffolding proteins is their response to mechanical stress. ZO-1 binds to integral membrane proteins like claudins and JAMs through the N-terminal PDZ domains and to the actin cytoskeleton through actin-binding regions at its C-terminus ⁵. ZO-1 exists in a closed conformation but can be unfolded in response to actomyosin-generated forces and upon binding to the cingulin (CGN) – non-muscle myosin IIB (NMIIB) complex via its C-terminal ZU5 domain ¹⁴⁻¹⁶, which results in an exposure of binding sites for ligands like the transcription factor DbpA and the integral membrane protein occludin ¹⁷. ZO proteins thus sense tensile forces at the TJs and integrate these forces into processes like gene expression and the paracellular barrier function ¹⁸. Another example of TJ plasticity and the role of scaffolding proteins is the ability to repair leaks that occur during cell elongation of cell junctions ¹³. A local activation of mechanosensitive Ca^{2+} channels as a result of local membrane tension triggers Ca^{2+} influx followed by activation of RhoA and increased actomyosin-based contractility at the sites of the breaches resulting in local accumulation of ZO-1 and occludin which reinforce the barrier function at those sites ^{19,20}. Finally, an intriguing property of scaffolding proteins at the TJs is their ability to self-organize into biomolecular condensates in a process called liquid-liquid phase separation (LLPS) ^{21,22}. Recombinant ZO-1 forms lipid droplet-like condensates in vitro, and both ZO-1 and ZO-2 undergo phase-separation in cultured cells and living organisms, a process that is strongly enhanced by their association cell-cell adhesion receptors including JAM-A ²³⁻²⁶.

In this study, we have addressed a putative interaction of the scaffolding protein ZO-2 with JAM-C, a member of the JAM family of adhesion receptors ^{27,28}. JAM-C localizes to TJs in various cultured cell lines of epithelial origin ²⁹⁻³¹ and of primary retinal pigment epithelial cells ^{32,33}. It interacts with the TJ-localized scaffolding proteins ZO-1 and Par-3 ³⁴. Using a

heterologous CHO cell system, we find that JAM-C is sufficient to recruit ZO-2 to cell-cell contacts. The recruitment of ZO-2 is mediated through the C-terminal PDZ binding motif (PBM) of JAM-C. Recruitment assays and biochemical experiments indicate that the interaction is direct and requires both PDZ domain 3 and the SH3 domain of ZO-2. We identify two hydrophobic amino acid residues in the ZO-2 SH3 domain that are predicted to interact with the PBM of JAM-C. We also find that JAM-C recruits the adaptor protein cingulin (CGN) to cell-cell contacts. Finally, we find that JAM-C expression at cell-cell junctions of polarized epithelial cells is upregulated in the absence of JAM-A. Our findings strongly suggest that JAM-C directly interacts with ZO-2 through the PSG core module which forms a functional supramodule, and that JAM-C is associated with several scaffolding and adapter proteins at TJs including ZO-1, ZO-2 and CGN. Our findings also have implications on various aspects of TJ biology including mechanosensation and LLPS.

Results

JAM-C promotes the recruitment of ZO-2 to cell-cell contacts

Given the important role of cell adhesion receptors and their associated scaffolding proteins in mechanosensing and phase separation, we addressed the question of whether JAM-C contributes to the localization of ZO-2 at cell-cell contacts. We used CHO cells since these cells lack expression of classical adhesion receptors including JAMs, cadherins or nectins. Scaffolding proteins that are normally recruited by cell adhesion receptors reside in the cytoplasm but are absent at cell-cell contacts. Importantly, ectopic expression of these cell adhesion receptors results in the recruitment of their cytoplasmic binding partners to cell-cell junctions. For example, ZO-1 and Par-3 are recruited by JAM-A, JAM-C and VE-cadherin ³⁴⁻³⁶, α -catenin, β -catenin and p120^{ctn} are recruited by E-cadherin and VE-cadherin ^{36,37}, and Par-3 is recruited by VE-cadherin and Nect-4 ^{36,38}. CHO cells are thus a suitable system to analyze the ability of a single adhesion receptor to recruit a specific scaffolding protein to intercellular junctions.

Ectopic expression of JAM-C in these cells resulted in a robust recruitment of endogenous ZO-2 to JAM-C-based cell-cell contacts (Fig. 1A). The recruitment of ZO-2 was comparable to that of ZO-1 and Par-3, two scaffolding proteins previously shown to interact with JAM-C³⁴ (Fig. 1B).

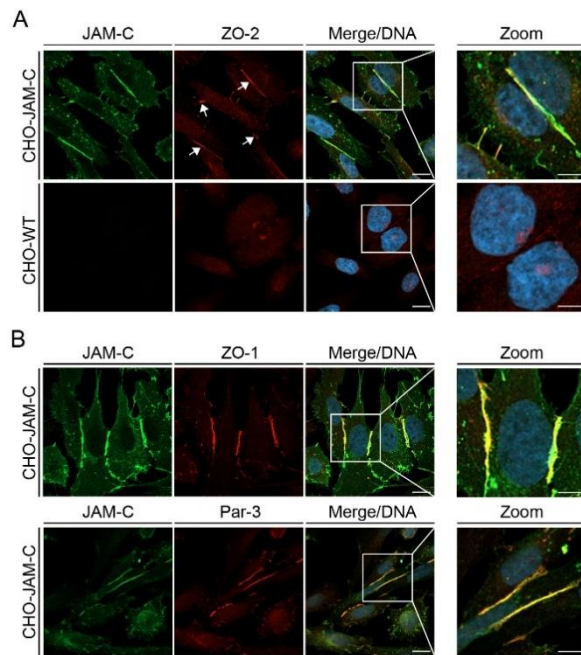
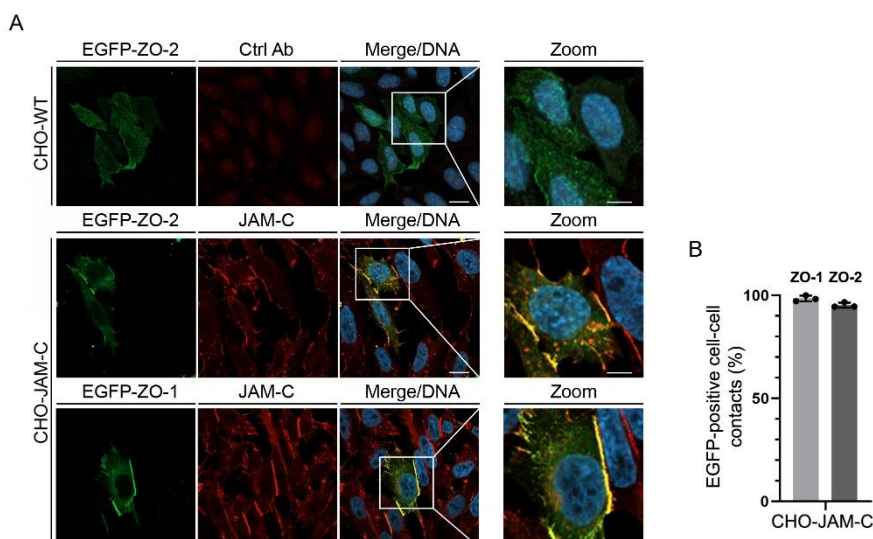


Fig. 1: ZO-2 is recruited to JAM-C-based cell-cell contacts in CHO cells. (A) CHO WT cells (CHO-WT) or CHO cells stably expressing mJAM-C (CHO-JAM-C) were stained with antibodies against JAM-C together with antibodies against ZO-2 as indicated. Arrows indicate ZO-2-positive cell-cell contacts. (B) CHO-JAM-C cells were stained with antibodies against JAM-C together with antibodies against ZO-1 or Par-3 as indicated. Right panels in (A) and (B) show magnifications of the insets marked by white squares in the Merge/DAPI images. Data is representative for 4 independent experiments. Scale bars: 10 μ m (regular images), 5 μ m (zoomed insets).

Similar to endogenous ZO-2, EGFP-ZO-2 was absent at cell-cell contacts of CHO-WT cells but was prominently localized at cell-cell contacts of CHO-JAM-C cells (Fig. 2A). The recruitment of EGFP-ZO-2 by JAM-C was similarly efficient as the recruitment of EGFP-ZO-1 (Fig. 2B) which was previously described to be recruited by JAM-A in CHO cells³⁵. These observations indicated that JAM-C can efficiently recruit ZO-2 to intercellular junctions.

Fig. 2: JAM-C recruits ZO-2 to cell-cell contacts as efficiently as ZO-1.

(A) CHO WT cells (CHO-WT) or CHO cells stably expressing mJAM-C (CHO-JAM-C) were transfected with EGFP-ZO-2 or EGFP-ZO-1 as indicated and stained with antibodies against JAM-C. Right panels (Zoom) show magnifications of the insets marked by white squares in the



Merge/DAPI images. Scale bars: 10 μ m (regular images), 5 μ m (zoomed insets). **(B)** Statistical evaluation of ZO-1 and ZO-2 recruitment by JAM-C. The bar graph shows the fraction of EGFP-ZO-1- or EGFP-ZO-2-positive cell-cell contacts in CHO-JAM-C cells. Data is derived from N = three independent experiments, each data point represents the mean value of one experiment. Statistical analysis was performed with unpaired Student's t-test. Data are presented as mean values $\pm$ SD. Number of analyzed cell-cell contacts: n(JAM-C-ZO-1): 195; n(JAM-C-ZO-2): 182.

JAM-C interacts with ZO-2 through its PDZ domain binding motif

The interaction of JAM-C with the TJ scaffold proteins ZO-1 and Par-3 is mediated by its C-terminal PBM (-SSFVLCOOH)³⁴. To test if the recruitment of ZO-2 involves the PBM of JAM-C, we expressed full length JAM-C (JAM-C/f.l.) or a PBM deletion mutant of JAM-C (JAM-C/ Δ 5) in CHO-WT cells. Endogenous ZO-2 was efficiently recruited by JAM-C/f.l. but not by JAM-C/ Δ 5 (Fig. 3A, B), strongly suggesting that JAM-C recruits ZO-2 through one (or several) of the three PDZ domains of ZO-2. As further support of this notion, pulldown experiments showed that EGFP-ZO-2 could be efficiently precipitated from HEK293T cell lysates with a GST fusion protein containing the intact cytoplasmic domain of JAM-C (GST-JAM-C/f.l.) but not with a GST JAM-C cytoplasmic domain fusion protein that lacks the PBM (GST-JAM-C/ Δ 5) (Fig. 3C). To further address the interaction of JAM-C and ZO-2 in cells we performed co-immunoprecipitation (CoIP) experiments from transfected HEK293T cells. ZO-2 immunoprecipitates contained JAM-C/f.l. but not JAM-C/ Δ 5 (Fig. 3D). Together, these observations strongly suggested that JAM-C and ZO-2 interact in a PDZ domain-dependent manner.

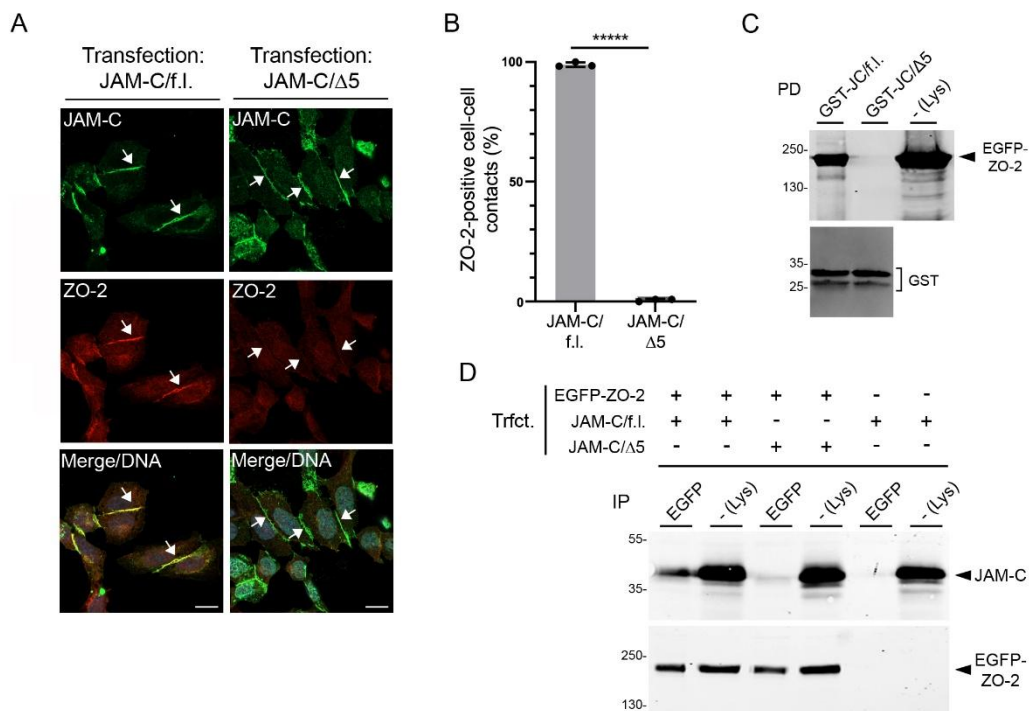


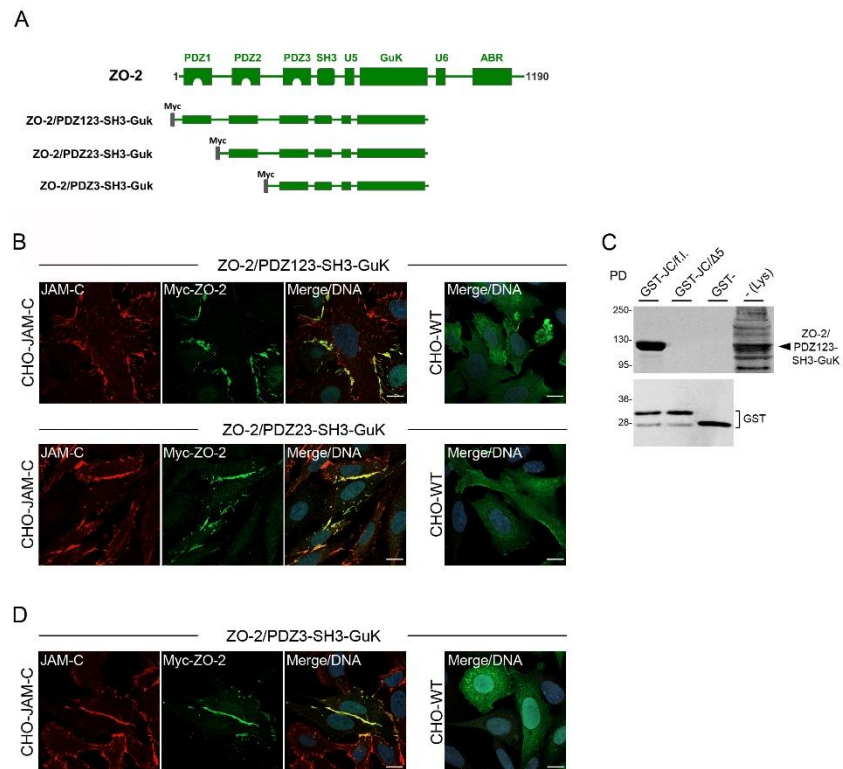
Fig. 3: JAM-C interacts with ZO-2 through a PDZ domain-mediated interaction. (A) CHO WT cells were transiently transfected with JAM-C full length (JAM-C/f.l.) or a JAM-C truncation mutant lacking the PDZ domain binding motif (JAM-C/Δ5). Cells were stained with antibodies against JAM-C and ZO-2. Scale bars: 10 μm. **(B)** Statistical analysis of ZO-2 recruitment by JAM-C/f.l. and JAM-C/Δ5. The bar graph shows the fraction of cell-cell contacts in which endogenous ZO-2 co-localizes with either JAM-C/f.l. or JAM-C/Δ5. Statistical analysis was performed with unpaired Student's t-test. Data is derived from N = three independent experiments, each data point represents the mean value of one experiment. Data are presented as mean values ± SD. Number of analyzed cell-cell contacts: n(JAM-C/f.l.): 360; n(JAM-C/Δ5): 317. ****P<0.00001. **(C)** GST-pulldown experiment. GST fusion proteins containing the entire cytoplasmic domain of JAM-C (GST-JC/f.l.) or a cytoplasmic domain deletion mutant lacking the PBM (GST-JC/Δ5) or GST alone (GST-) were incubated with lysates from HEK293T cells transfected with EGFP-tagged full length ZO-2 (EGFP-ZO-2). Bound proteins were analyzed by immunoblotting using anti-GFP antibodies. The lane labeled "- (Lys)" contains a postnuclear supernatant of transfected cells (25% of input). The amounts of GST fusion proteins used in the pulldown experiments were analyzed with anti-GST antibodies (bottom panel, 10% of input). Data is representative of N = three independent experiments. **(D)** Co-immunoprecipitation experiment. HEK293T cells were transfected with EGFP-ZO-2 together with either JAM-C/f.l. or JAM-C/Δ5 as indicated. Cells transfected with JAM-C alone (lanes 5 and 6) served as IP control to test for unspecific precipitation of JAM-C. Immunoprecipitates were blotted with antibodies against JAM-C (top panels, 90% of input) or with antibodies against EGFP (bottom panels, 10% of input). Lanes labeled "- (Lys)" contain postnuclear supernatants of transfected cells (1.25% of input). Data is representative of N = three independent experiments. Abbreviations: Trfct., transfection; IP, immunoprecipitation.

JAM-C interacts with ZO-2 through the PDZ – SH3 – GuK core module

To characterize the interaction between JAM-C and ZO-2 in cells in more detail, we expressed ZO-2 truncation constructs consisting of either the three PDZ domains (ZO-2/PDZ123), or the

SH3 and GuK domains (ZO-2/SH3-GuK), or the C-terminal region comprising the actin- and CGN-binding region (ZO-2/C-term) in CHO-JAM-C cells and analyzed their localization at JAM-C-based cell-cell contacts. Notably, none of the three constructs was recruited by JAM-C (Suppl. Fig. S1). Previous studies have shown that the interaction of JAM-A with ZO-1 through PDZ domain 3 of ZO-1 requires the SH3 domain of ZO-1 immediately adjacent to PDZ3³⁹. In addition, structural studies for ZO-1 have shown that its PDZ3 exists in a tandem organization with the SH3 and GuK domains to form a supramodule⁴⁰. Since this tandem domain organization is conserved in ZO-2^{5,8} we tested ZO-2 constructs that contained the SH3 and GuK domains in addition to the PDZ domains (Fig. 4A).

Fig. 4: JAM-C interacts with a ZO-2 construct containing the PDZ, SH3 and GuK domains of ZO-2. (A) Cartoon of ZO-2 deletion constructs. (B) CHO-JAM-C or CHO-WT cells were transfected with the ZO-2/PDZ123-SH3-GuK construct and stained with antibodies against JAM-C (red) and against the Myc tag (green). Note that ZO-2/PDZ123-SH3-GuK is enriched at JAM-C-positive cell junctions in CHO-JAM-C cells but is localized diffusely in the cytoplasm of CHO-WT cells. Data is representative of N = three independent experiments.



Scale bars: 10 μ m. (C) GST-pulldown experiment. Top panel: GST fusion proteins (GST-JC/f.l., GST-JC/ Δ 5, GST-) were incubated with lysates from HEK293T cells transfected with a Myc-tagged ZO-2 construct consisting of the PDZ, SH3 and GuK domains (ZO-2/PDZ123-SH3-GuK). Bound proteins were analyzed by immunoblotting using anti-Myc antibodies. The lane labeled “- (Lys)” contains a postnuclear supernatant of transfected cells (25% of input). Bottom panel: The amounts of GST fusion proteins used in the pulldown experiments were analyzed with anti-GST antibodies (10% of input). Data is representative of N = three independent experiments. (D) CHO-JAM-C or CHO-WT cells were transfected with ZO-2 constructs lacking PDZ1 (ZO-2/PDZ23-SH3-GuK) or PDZ1 and PDZ2 (ZO-2/PDZ3-SH3-GuK) and stained as described in panel (B). Note that both constructs are recruited by JAM-C. Data is representative of N = three independent experiments. Scale bars: 10 μ m.

ZO-2 constructs containing the SH3 and GuK domains adjacent to either all three PDZ domains (ZO-2/PDZ123-SH3-GuK) or PDZ domains 2 and 3 (ZO-2/PDZ23-SH3-GuK) were efficiently recruited to JAM-C-based cell-cell junctions (Fig. 4B). In addition, the ZO-2/PDZ123-SH3-GuK construct was precipitated from lysates of transfected HEK293T by GST-JAM-C but not by GST-JAM-C/Δ5 (Fig. 4C). These findings suggested that the interaction of JAM-C with ZO-2 is PDZ domain-dependent but requires the SH3 and GuK domains adjacent to the PDZ domains. In order to test whether the PDZ3 domain alone in concert with the SH3 and GuK domains is sufficient for recruitment by JAM-C, we analyzed the recruitment of the ZO-2 PSG core unit (ZO-2/PDZ3-SH3-GuK). This construct was efficiently recruited by JAM-C (Fig. 4D) strongly suggesting that JAM-C interacts with PDZ3 of ZO-2, provided that PDZ3 exists in a tandem organization with the SH3 and GuK domains. Since a construct comprising the three PDZ domains alone, or a construct comprising the SH3 - GuK domains alone, are not recruited by JAM-C, these findings also suggest that the PSG core unit of ZO-2 forms a functional supramodule ⁸.

To address the question if other PDZ domains contribute to the interaction with JAM-C we analyzed the recruitment of ZO-2 in which individual PDZ domains were inactivated by mutating two conserved residues within the canonical “GLGF” motif, which is a key feature of PDZ domains ⁴¹ (Fig. 5A). Constructs with inactivated PDZ1 or PDZ2 (ZO-2/PSG-P1M, ZO-2/PSG-P2M, respectively) were efficiently recruited by JAM-C to cell-cell contacts (Fig. 5B, C). Inactivating PDZ3 (ZO-2/PSG-P3M) resulted in a reduction albeit not complete inhibition of recruitment by JAM-C (Fig. 5B, C). Inactivating PDZ3 in combination with PDZ2 (ZO-2/PSG-P23M) almost completely abolished recruitment to JAM-C-based cell-cell contacts (Fig. 5B, C). These observations support the notion that JAM-C recruits ZO-2 predominantly through PDZ3 of ZO-2. However, they also suggested a possible contribution of PDZ2. To further verify these observations we performed GST pulldown experiments with lysates of HEK293T cells transfected with the ZO-2 PDZ mutant constructs. ZO-2/PSG-P1M and ZO-2/PSG-P2M were efficiently precipitated by GST-JAM-C in a PBM-dependent manner (Fig. 5D). In contrast, ZO-2/PSG-P3M as well as ZO-2/PSG-P23M were not precipitated by GST-JAM-C (Fig. 5D).

Together, these observations strongly suggested that the interaction of JAM-C and ZO-2 involves the PBM of JAM-C and the PSG core unit of ZO-2.

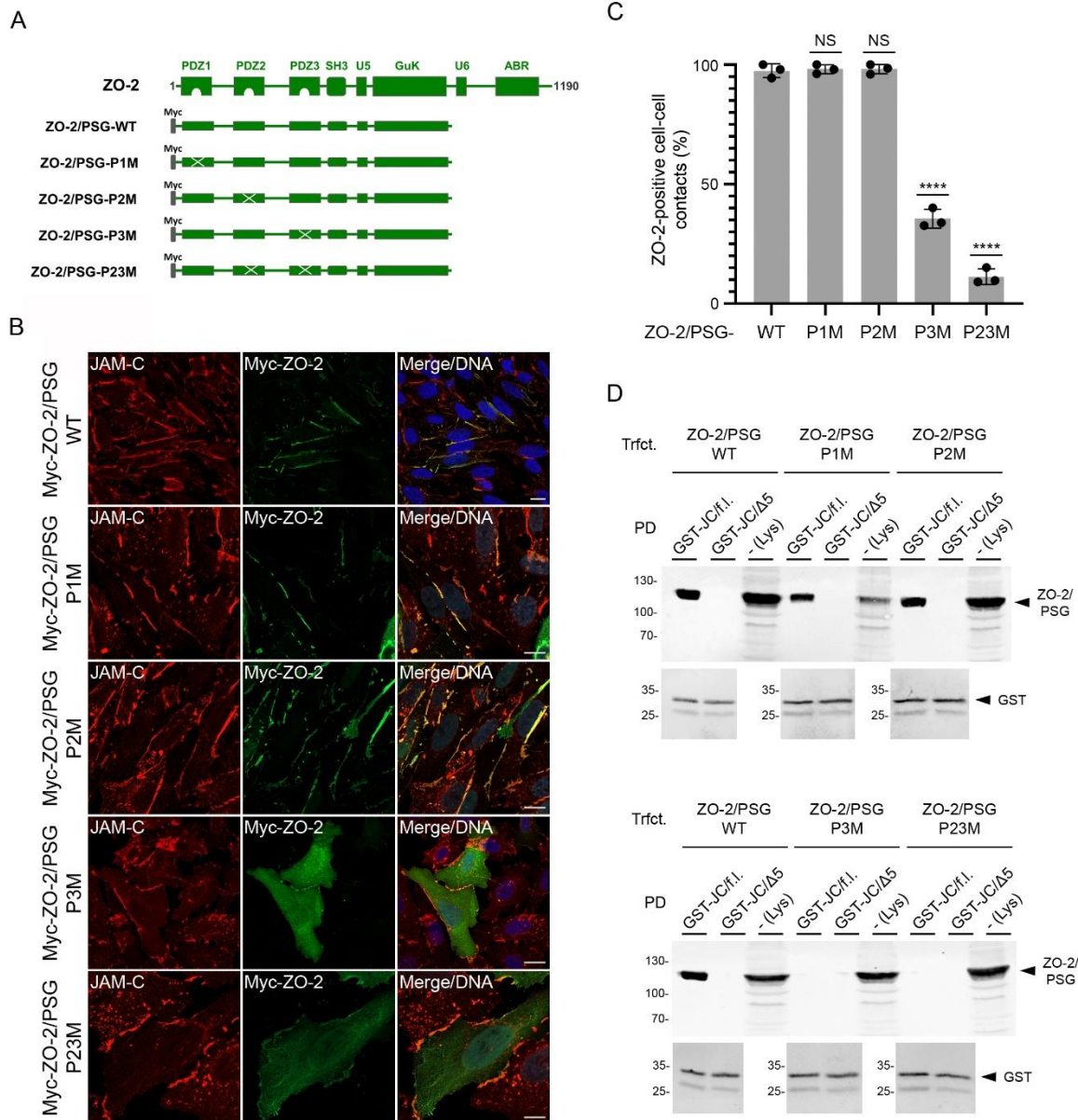


Fig. 5: JAM-C interacts with ZO-2 through PDZ domain 3 of ZO-2. (A) Cartoon of ZO-2 constructs used for mapping experiments. White crosses within the PDZ domains indicate function-inactivating mutations in the GLGF motif of the respective PDZ domain. (B) CHO cells stably expressing mJAM-C (CHO-JAM-C) were transfected with Myc-tagged ZO-2 constructs (depicted in A) and stained with antibodies against JAM-C (red) and Myc (green). Scale bars: 10 μ m. (C) Statistical analysis of ZO-2 constructs recruitment by JAM-C. The bar graph shows the fraction of cell-cell contacts in which Myc-ZO-2 co-localizes with JAM-C. Statistical analysis was performed with unpaired Student's t-test. Data is derived from N = three independent experiments, each data point represents the mean value of one experiment. Data are presented as mean values $\pm$ SD. Number of analyzed cell-cell contacts: n(ZO-2/PSG-WT): 154; n(ZO-2/PSG-P1M): 156; n(ZO-2/PSG-P2M): 175; n(ZO-2/PSG-P3M): 349; n(ZO-2/PSG-P23M): 207. ****P<0.0001. NS, not significant. (D) GST fusion proteins (described in the legend to Fig. 3) were incubated with lysates from HEK293T cells transfected Myc-tagged ZO-2/PSG constructs containing mutations in the GLGF motif in one or several PDZ domains (ZO-2/PSG-P1M, ZO-2/PSG-P2M, ZO-2/PSG-P3M, ZO-2/PSG-

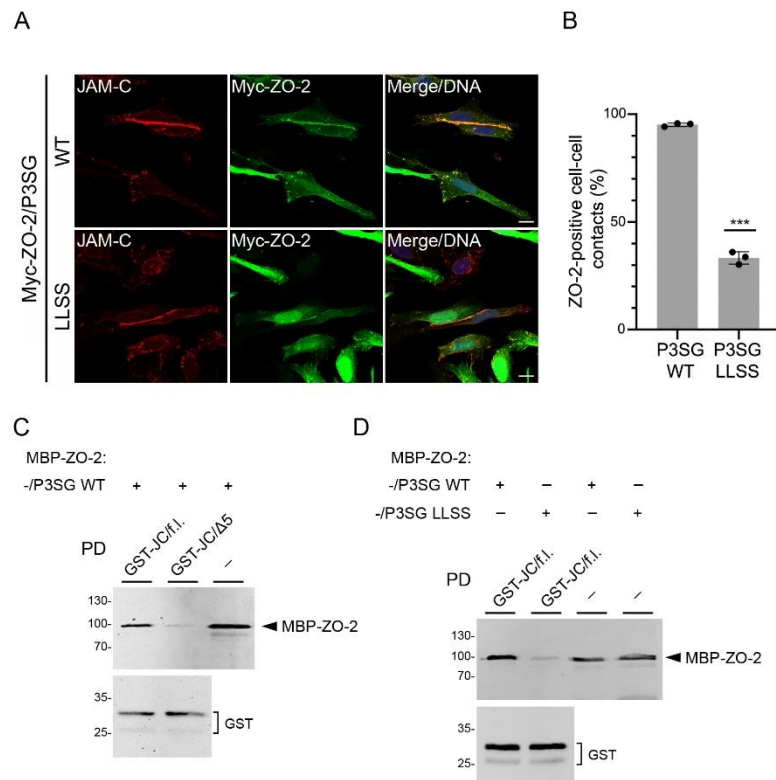
P23M). The unmutated construct (ZO-2/PSG-WT) served as positive control and is present in both gels. Bound proteins were analyzed by immunoblotting using anti-Myc antibodies. The lanes labeled “- (Lys)” contain postnuclear supernatants of transfected cells (25% of input). The amounts of GST fusion proteins used in the pulldown experiments were visualized with anti-GST antibodies (bottom panels, 10% of input). Data is representative of N = three independent experiments. Abbreviations: PD, pulldown; Trfct., transfection.

To further corroborate this notion, we mutated amino acid residues present in the SH3 domain of ZO-2 (L₆₃₇ and L₆₄₂). Structural studies with the ZO-1 PSG core unit complexed with a JAM-A peptide have shown that the homologous residues (L₅₄₉ and L₅₅₄ in ZO-1) interact with a residue in the PBM of JAM-A, i.e. F₂₉₈³⁹. Mutating L₆₃₇ and L₆₄₂ to serine in the ZO-2 PSG core unit impaired the recruitment to cell-cell contacts in CHO-JAM-C cells (Fig. 6A, B). These findings supported a contribution of the SH3 domain and suggested that the interaction of ZO-2 with JAM-C is direct. To test this, we performed in vitro binding assays with recombinant proteins. GST-JAM-C fusion proteins were immobilized on GSH sepharose beads and incubated with a recombinant MBP-ZO-2 fusion protein consisting of ZO-2 PSG core unit fused to MBP (MBP-ZO-2/P3SG). The ZO-2 fusion protein was detected in GST-JAM-C/f.l. but not in GST-JAM-C/ Δ 5 precipitates (Fig. 6C) confirming that the interaction is direct. Mutating L₆₃₇ and L₆₄₂ to serine in the MBP-ZO-2/P3SG construct (MBP-ZO-2/P3SG _LLSS) strongly reduced the interaction with GST-JAM-C (Fig. 6D) indicating that these two residues in the SH3 domain contribute to the direct interaction with JAM-C.

Together, these observations demonstrated that JAM-C directly interacts with ZO-2 in a manner which involves the PBM of JAM-C and both the PDZ3 and the SH3 domains of ZO-2, which are part of the ZO-2 PSG core unit. They also support the notion that the PSG core unit of ZO-2 forms a functional supramodule, as previously shown for the PSG core unit of ZO-1

Fig. 6: Hydrophobic residues in the SH3 domain of ZO-2 are involved in the interaction with JAM-C. (A) CHO cells stably expressing mJAM-C (CHO-JAM-C) were transfected with the Myc-tagged ZO-2 PSG core module, either wildtype (ZO-2/P3SG_WT) or with leucine residues Leu637 and Leu642 present in the SH3 domain mutated to serine residues (ZO-2/P3SG_LLSS).

Cells were stained with antibodies against JAM-C (red) and the Myc tag (green). Scale bars: 10 μ m. (B) Statistical analysis of ZO-2 constructs recruitment by JAM-C. The bar graph shows the fraction of cell-cell contacts in which Myc-ZO-2 co-localizes with JAM-C at cell-cell contacts. Statistical analysis was performed with unpaired Student's t-test. Data is derived from N = three independent experiments. Data are presented as mean values $\pm$ SD, each data point represents the mean value of one experiment. Number of analyzed cell-cell contacts: n(ZO-2/P3SG-WT): 208; n(ZO-2/P3SG-LLSS): 228. ***P<0.001. (C, D) GST-pulldown experiment with recombinant MBP-ZO-2 proteins purified from E.coli bacteria. GST-JAM-C fusion proteins (GST-JC/f.l., GST-JC/ Δ 5) were immobilized on GSH sepharose beads and incubated with recombinant, purified MBP fusion proteins consisting of the either wildtype or mutated ZO-2 PSG core unit fused to MBP (MBP-ZO-2/P3SG, MBP-ZO-2/P3SG_LLSS). Bound proteins were analyzed by immunoblotting using anti-MBP antibodies. The lanes labeled “-” contain 50 ng of purified MBP-ZO-2/P3SG or MBP-P3SG_LLSS fusion proteins (5% of input), as indicated. Application of equal amounts of GST fusion proteins used in the pulldown experiments was controlled with anti-GST antibodies (bottom panel, 10% of input). Data is representative of N = three independent experiments. Abbreviations: MBP, maltose-binding protein; PD, pulldown.

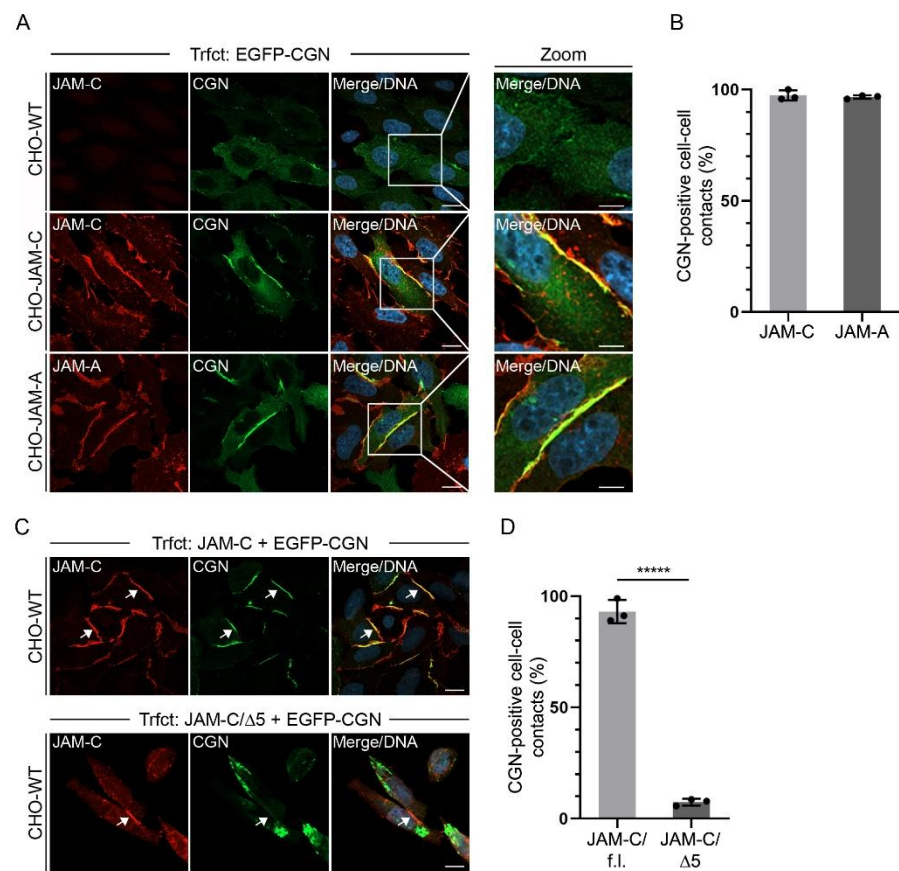


JAM-C recruits cingulin to cell-cell junctions

ZO-2 acts as a scaffold protein at the TJs which interacts with numerous proteins including integral membrane proteins like claudins and occludin, and other scaffolding and adaptor proteins like ZO-1, cingulin (CGN) and CGN-like 1 (CGNL1)^{5,42}. CGN acts as adaptor protein that binds various regulators of Rho family GTPases and recruits these regulators to TJs through its interaction with ZO proteins⁵. Given that CGN interacts with ZO-2 through a region in the C-terminus of ZO-2 that does not overlap with the PSG core module⁵, we hypothesized

that JAM-C recruits CGN to cell-cell junctions in CHO cells. CGN was efficiently recruited to cell-cell junctions in CHO cells expressing either JAM-C or JAM-A but not in CHO-WT cells (Fig. 7A, B). Deletion of the PBM in JAM-C abolished the recruitment of CGN (Fig. 7C, D) suggesting that its recruitment by JAM-C is indirect and mediated by a PDZ domain protein. These observations indicate that CGN exists in a complex with JAM-C most likely through an indirect association via a JAM-C-interacting scaffolding protein like ZO-1 or ZO-2.

Fig. 7: JAM-C recruits Cingulin to cell-cell contacts through its PDZ domain binding motif. (A) Wildtype CHO cells (CHO-WT) or CHO cells stably expressing either mJAM-C (CHO-JAM-C) or mJAM-A (CHO-JAM-A) were transfected with EGFP-tagged Cingulin (EGFP-CGN) and stained with antibodies against JAM-C or JAM-A as indicated. The right panels (Zoom) show magnifications of the insets marked by white squares in the



Merge/DNA images. Scale bars: 10 μ m (regular images), 5 μ m (zoomed insets). **(B)** Statistical evaluation of CGN recruitment by JAM-A and JAM-C. The bar graph shows the fraction of EGFP-CGN-positive cell-cell contacts in JAM-C- and JAM-A-expressing CHO cells. Data are derived from N = three independent experiments, each data point represents the mean value of one experiment. Data are presented as mean values $\pm$ SD. Number of analyzed cell-cell contacts: n(JAM-C–CGN): 202; n(JAM-A–CGN): 210; **(C)** CHO WT cells were transiently co-transfected with EGFP-CGN and either JAM-C full length (JAM-C/f.l.) or a JAM-C mutant lacking the PDZ domain binding motif (JAM-C/Δ5). Cells were stained with antibodies against JAM-C. Scale bars: 10 μ m. **(D)** Statistical analysis of CGN recruitment by JAM-C/f.l. and JAM-C/Δ5. The bar graph shows the fraction of cell-cell contacts in which EGFP-CGN co-localizes with either JAM-C/f.l. or JAM-C/Δ5. Statistical analysis was performed with unpaired Student's t-test. Data are derived from N = three independent experiments, each data point represents the mean value of one experiment. Data are presented as mean values $\pm$ SD. Number of analyzed cell-cell contacts: n(JAM-C/f.l.): 279; n(JAM-C/Δ5): 131. ****P<0.00001. Abbreviations: CGN, Cingulin; Trfct, transfection.

JAM-C expression is suppressed by JAM-A in MDCK cells

Several examples of cross-talk between adhesion receptors localized at cell-cell contacts exist. In endothelial cells, VE-cadherin regulates the expression of the adherens junction (AJ)-localized N-cadherin and, vice versa, N-cadherin regulates the expression of VE-cadherin ^{43,44}. A similar mutual regulation has been described for the integrin family of cell-matrix adhesion receptors ⁴⁵. To test a possible regulation of JAM-C by JAM-A in epithelial cells we used MDCKII cells with a CRISPR/Cas9-mediated inactivation of the JAM-A gene (MDCK/JAM-A KO) ^{46,47} (Suppl. Fig. 2) and analyzed the localization of endogenous JAM-C at cell-cell junctions using a polyclonal antibody generated against canine JAM-C (see Methods for details). In MDCKII wildtype cells, JAM-C was localized weakly at cell-cell contacts with a prominent variability in fluorescence intensity between individual cells (Fig. 8A). In MDCK/JAM-A KO cells the JAM-C fluorescence intensity at cell-cell contacts was strongly increased and more homogenously distributed among cells. Ectopic expression of mJAM-A suppressed junctional localization of JAM-C in MDCK/JAM-A KO cells (Fig. 8A, B). To test if the upregulation of JAM-C was dependent on the state of confluency MDCK cells were seeded at low density and fixed at subconfluency. As observed in confluent cells, JAM-C was significantly upregulated in JAM-A KO cells, and upregulation of JAM-C was suppressed by ectopic expression of mJAM-A (Suppl. Fig. 3). To test if JAM-C localized at TJs, MDCK/JAM-A KO cells were cultured on transwell filters to promote polarization. JAM-C immunofluorescence overlapped with ZO-1 at the apical region of cell-cell junction but was absent at E-cadherin-positive regions at the lateral membrane domain indicating a TJ-specific localization of JAM-C (Fig. 8C). JAM-C also colocalized with ZO-1 at the apical cell-cell contact region when cells were grown to three-dimensional cysts (Fig. 8D). Together, these findings indicated that JAM-C localization at cell-cell contacts is suppressed by JAM-A in MDCK cells and that endogenous JAM-C localizes to TJs in these cells.

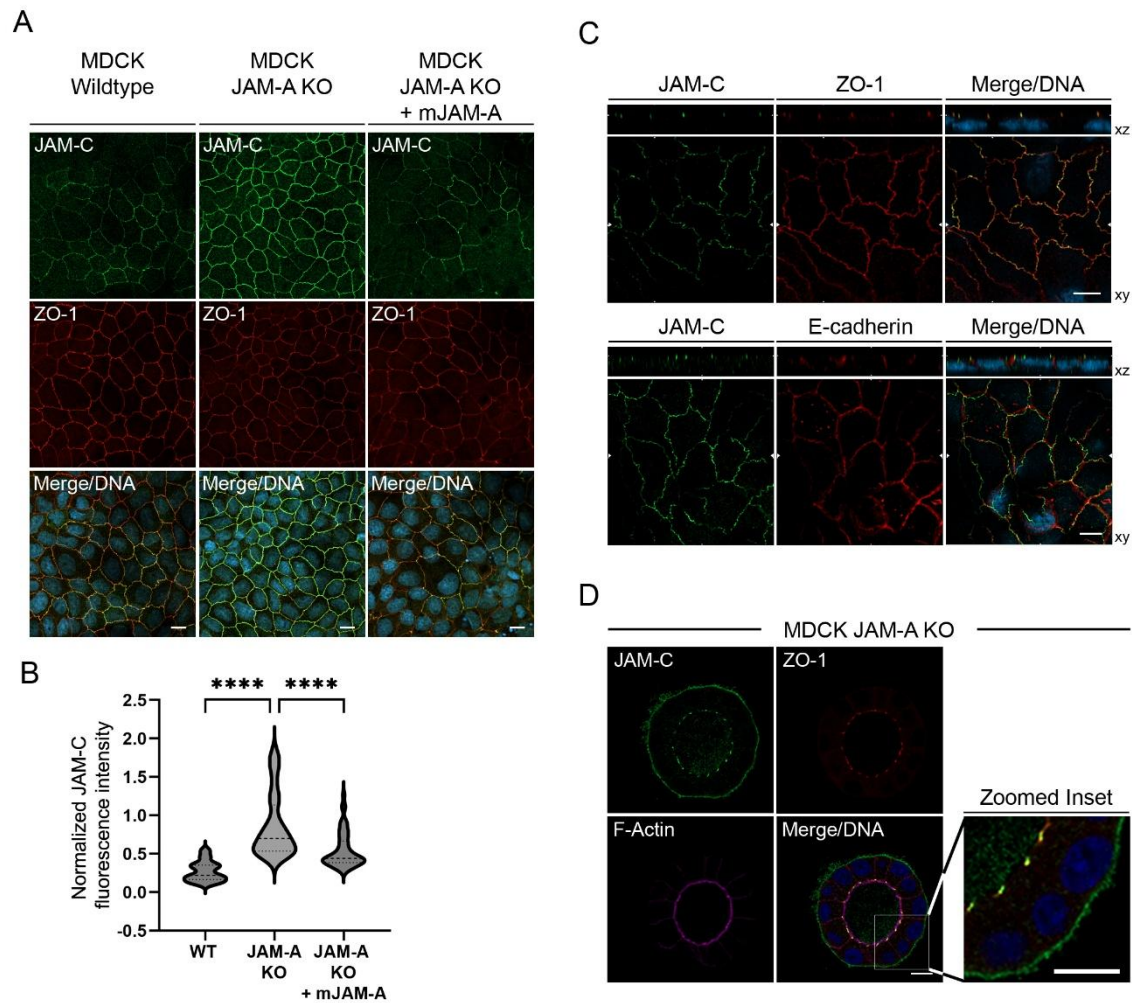


Fig. 8: JAM-C is upregulated and localizes to TJs in JAM-A knockout MDCK cells. (A) Wildtype MDCKII cells, JAM-A knockout MDCKII cells or JAM-A knockout MDCK cells ectopically expressing mJAM-A were grown to confluency and stained with antibodies against JAM-C and ZO-1. Scale bars: 10 μ m. (B) Statistical analysis of JAM-C localization at cell-cell contacts in confluent cells. The JAM-C and ZO-1 fluorescence intensities were analyzed using Imaris software. The data shows the ratios of JAM-C fluorescence cell-cell contacts to the ZO-1 fluorescence intensity and is depicted as normalized JAM-C fluorescence intensity. Statistical analysis was performed with Kruskal-Wallis test and Dunn's post hoc Multiple Comparisons test. Data were obtained and data points were pooled from at least 11 randomly chosen fields of view (FOV) per experiment derived from three independent experiments (WT cells: 59 FOV; JAM-A KO cells: 57 FOV; JAM-A KO cells expressing mJAM-A: 55 FOV). Data is presented as Violin plot. Broken lines indicate mean values, dotted lines indicate first and third quartiles. **** $p < 0.0001$. (C) JAM-A knockout MDCKII cells were grown on polycarbonate filters to induce polarization and stained with antibodies against JAM-C and ZO-1 (top panels) or against JAM-C and E-cadherin (bottom panels). Cells were analyzed by confocal microscopy. Rectangles depict XZ projections of the XY projections at positions indicated by small arrowheads. Scale bars: 5 μ m. (D) JAM-A knockout MDCKII cells were grown in 3D matrigel matrices for 4 – 6 days and stained as indicated. Scale bars: 10 μ m.

Discussion

In this study, we identify the TJ-associated transmembrane protein JAM-C as a direct binding partner of the scaffolding protein ZO-2. The interaction is mediated by the C-terminal PDZ-binding motif (PBM) of JAM-C and involves both the PDZ3 and SH3 domains of ZO-2. Two hydrophobic residues within the ZO-2 SH3 domain are critical for complex formation, defining a previously unrecognized structural determinant of this interaction. Functionally, JAM-C efficiently recruits ZO-2 to cell–cell contacts. JAM-C also promotes the accumulation of CGN at cell–cell junctions, most likely indirectly via ZO-2 or its homologue ZO-1. Moreover, JAM-C expression is negatively regulated by JAM-A in polarized epithelial cells, in which endogenous ZO-2 is strictly confined to tight junctions. Together, these findings position JAM-C as a regulatory component of the ZO protein scaffold at tight junctions.

A key finding of this study is that the PDZ domains of ZO-2 alone are insufficient to mediate JAM-C binding. Although recruitment of ZO-2 depends on the C-terminal PDZ-binding motif (PBM) of JAM-C, a construct comprising all three PDZ domains fails to localize to cell–cell contacts in response to JAM-C, indicating that additional structural elements are required. Insights from the interaction of JAM-A with ZO-1, which involves PDZ3 and residues within the adjacent SH3 domain ^{39,48}, prompted us to examine a similar contribution of the SH3 domain in ZO-2. Structural analysis of the ZO-1 PSG module in complex with the C-terminal peptide of JAM-A demonstrated that a conserved phenylalanine within the PBM engages two hydrophobic residues in the SH3 domain (L₅₄₉YNGKL₅₅₄) ^{39,49}, establishing the SH3 domain as a critical component of the binding interface. Notably, the corresponding residues are conserved in JAM-C and ZO-2 (L₆₃₇YDGKL₆₄₂). Consistent with this model, mutation of two hydrophobic residues within the ZO-2 SH3 domain to polar residues markedly impaired JAM-C-dependent recruitment in cells and strongly reduced complex formation in vitro, demonstrating that efficient binding requires cooperative engagement of PDZ3 and the adjacent SH3–GUK module. These findings support the concept that the PSG region of ZO-2 functions as an integrated supramodule. Together with the JAM-A–ZO-1 interaction ^{39,40} and

the interaction of Crumbs with Pals1⁵⁰, our data establish JAM-C–ZO-2 as a further example of an integral tight junction membrane protein engaging a MAGUK PSG supramodule.

Our recruitment assays in CHO cells indicate that, among the three PDZ domains of ZO-2, PDZ3 is not solely responsible for the interaction with JAM-C and that PDZ2 contributes to this association. This conclusion is based on analyses of PDZ domain mutants. Although inactivation of PDZ3 markedly reduced recruitment to JAM-C–based cell–cell contacts, residual localization was retained, in contrast to mutants with inactivated PDZ1 or PDZ2. Only simultaneous inactivation of PDZ2 and PDZ3 completely abolished junctional recruitment. One interpretation is that JAM-C engages not only PDZ3 within the PSG supramodule but also PDZ2. Consistent with this notion, multi-PDZ proteins can use more than one PDZ domain to bind the same ligand. For example, the JAM-related IgSF member TMIGD1 associates with PDZ1 and PDZ3 of the polarity protein Scribble⁵¹. Alternatively, the residual recruitment observed with the PDZ3 mutant may reflect indirect association via endogenous ZO-1 and/or ZO-2. The PDZ2 domains of both ZO-1 and ZO-2 are capable of homo- and heterodimerization through domain swapping^{52,53}. This mechanism involves reciprocal β -strand exchange between monomers, generating dimers with two functional PDZ domains^{52,54}. As the PDZ3 mutant construct used here retains an intact PDZ2 domain, it may interact with endogenous ZO proteins via PDZ2-mediated domain swapping, thereby enabling indirect recruitment to JAM-C–containing junctions. Importantly, several independent lines of evidence argue that PDZ3 within the PSG supramodule is the principal determinant of direct JAM-C binding: (i) the PSG core unit is sufficient for junctional localization; (ii) a ZO-2 construct with inactivated PDZ2, but not PDZ3, is precipitated by GST–JAM-C; and (iii) the recombinant PSG core unit directly binds GST–JAM-C in vitro. Together, these findings support a model in which PDZ3 of ZO-2 mediates the primary direct interaction with JAM-C.

A further finding of this study is that JAM-C expression is markedly upregulated in MDCKII cells lacking JAM-A, indicating that JAM-A suppresses JAM-C expression under these conditions. Reciprocal regulation among JAM family members has also been observed in other cellular contexts. In the outer limiting membrane of the retina—a specialized junctional region

in which photoreceptor cells and Müller glial cells are connected through heterotypic cell–cell contacts ⁵⁵—systemic inactivation of JAM-C results in a strong upregulation of JAM-A ³². These findings suggest negative regulation of JAM-A by JAM-C in Müller glial cells and/or photoreceptors. In contrast, in multiciliated cells of the airway epithelium, depletion of JAM-C does not alter the expression levels of JAM-A or JAM-B ⁵⁶. Taken together with our data, these observations indicate that cross-regulation within the JAM family is highly context dependent. The directionality and magnitude of this regulatory interplay are likely determined by tissue-specific functional requirements and by the distinct roles that individual JAM proteins fulfill within a given epithelial or neuroepithelial niche.

Our findings have important implications for TJ biology, particularly in the context of mechanoregulation and LLPS. TJs are subject to tensile stress ^{14,17}, and ZO proteins function as key mechanotransducers linking the junctional membrane to the actin cytoskeleton. Under tension, ZO-1 adopts an extended conformation that exposes binding sites for DbpA and occludin ¹⁷. This conformation is stabilized by heterodimerization with ZO-2 and by interaction with CGN ^{15,17}. Similar to ZO-1, ZO-2 can likewise undergo tension-dependent conformational extension ¹⁷. The ability of JAM-C to recruit both ZO-2 and CGN to cell–cell junctions therefore suggests that JAM-C may actively modulate TJ mechanoregulation.

As another important aspect of TJ biology, LLPS of ZO proteins has emerged as a key mechanism regulating TJ assembly ²³⁻²⁵. Although ZO condensates can form in the cytoplasm, their assembly at cell–cell contacts is markedly enhanced by membrane receptors that engage ZO proteins, including claudins and JAM-A ²⁶. Receptor oligomerization further promotes condensate formation ²⁶. Members of the JAM family form cis-dimers via a conserved motif within their first Ig-like domain, and these cis-dimers interact in trans to generate higher-order clusters at cell–cell junctions ^{28,57}. Through its ability to oligomerize at cell–cell contacts and to associate with ZO-1 ³⁴ and ZO-2 (this study), JAM-C is therefore well positioned to promote the nucleation and stabilization of ZO condensates at junctional membranes. Given that JAM-A similarly interacts with both ZO-1 and ZO-2 ^{34,39,48,58-60}, cooperative functions of JAM family members in driving ZO phase separation appear likely. In addition, homo- and

heterodimerization of ZO proteins via PDZ2 domain swapping is expected to further reinforce multivalency and thereby facilitate LLPS.

Beyond its proposed roles in mechanosensation and LLPS, JAM-C may also contribute to the mechanical resilience of the apical junctional complex. Mechanical tension generated during morphogenesis or cell division imposes pulling forces on cellular junctions, leading to transient local breaches that are rapidly sealed by actin polymerization and actomyosin contractility^{19,61}. Breach formation is more pronounced in cells lacking claudins and JAM-A, and is further exacerbated by the additional loss of Coxsackie and adenovirus receptor (CAR), a JAM-A/JAM-C-related IgSF protein at TJs that interacts with ZO-1⁶². These observations indicate that integral membrane components of tight junctions act cooperatively to confer resistance to mechanical stress and to limit breach formation.

Given its direct interaction with ZO-1 and ZO-2, JAM-C is well positioned to contribute to tensile stress resistance at tight junctions. Future studies will be required to delineate more precisely the role of JAM-C in mechanosensation, the regulation of ZO protein condensate dynamics, and the maintenance of junctional integrity under mechanical load.

Material and Methods

Cell culture and transfections

HEK293T cells (ATCC-CRL-2316) were obtained from Dietmar Vestweber (MPI Münster, Münster, Germany) and were grown in DMEM containing 10% FCS, 1% NEAA, 2 mM L-Glu, 100 U/ml Pen/Strep. CHO^{dhfr-} cells (ATCC-CRL-2316)⁶³ were obtained from Dietmar Vestweber (MPI Münster, Münster, Germany) and cultured in α MEM medium (Pan Biotch #P04-21500) containing 10% FCS, 2 mM glutamine, 100 U/ml Pen/Strep. CHO^{dhfr-} cells stably transfected with either mJAM-A or mJAM-C have been described previously^{34,35}. Briefly, cells were grown in α MEM medium supplemented with either blasticidin (7 μ g/ml, JAM-A CHO) or G418 (500 μ g/ml, JAM-C CHO). Note that JAM-C CHO cells were generated using a S281A mutant of JAM-C because phosphorylation of JAM-C at Ser281 prevents cell-cell contact

localization^{31,34}. MDCKII cells⁶⁴ were obtained from the European Collection of Cell Cultures (ECACC #00062107) or were provided by Masayuki Murata (University of Tokyo, Tokyo, Japan). MDCKII cells with CRISPR/Cas9-mediated inactivation of the JAM-A gene (JAM-A KO MDCKII)⁴⁶ were cultivated in DMEM high glucose medium (Sigma-Aldrich (SA) #D5671) containing 10% FCS, 2 mM glutamine, 100 U/ml Pen/Strep. JAM-A KO MDCKII with tetracycline-regulated expression of mouse JAM-A (mJAM-A)⁴⁷ were cultured in DMEM medium additionally containing 1 µg/ml puromycin. Expression of mJAM-A was induced by addition of 2 µg/ml doxycycline. To analyze JAM-C localization in polarized epithelial cells, MDCKII cells were grown on polycarbonate filters (0.45 µm pore size, Corning #3413) coated with growth factor reduced basement membrane Corning Matrigel matrix (Merck #CLS354230) in 24-well tissue culture dishes. Transient transfections of cDNAs were performed using X-Fect (Xfect™ Transfection Reagent, TaKaRa Bio Europe SAS, #631318, Saint-Germain-en-Laye, France), according to the manufacturer's instructions. All cell lines were routinely tested and found to be negative for mycoplasma contamination.

Expression vectors

Human ZO-2 constructs in pKE081myc (N-terminal myc tag,⁴⁸: ZO-2/PDZ1-3 (PDZ domains 1,2,3; AA1-606); ZO-2/SH3-GuK (SH3 and GuK domains, AA591-889); ZO-2/Cterm (C-terminus, AA877-1190); ZO-2/P123SG_WT (PDZ1,2,3, SH3, GuK domains, AA1-892); ZO-2/P23SG (PDZ2,3, SH3, GuK domains AA281-892); ZO-2/P3SG (PDZ3, SH3, GuK domains, AA489-892); ZO-2/P123SG_P1M (AA1-892, F₄₄A_G₄₅E); ZO-2/P123SG_P2M (AA1-892, Y₃₁₉A_G₃₂₀E); ZO-2/P123SG_P3M (AA1-892, V₅₂₁A_G₅₂₂E); ZO-2/P123SG_P23M (AA1-892, Y₃₁₉A_G₃₂₀E _V₅₂₁A_G₅₂₂E); ZO-2/P3SG_LLSS (AA489-892, L₆₃₇S_L₆₄₂S). Human ZO-2 in pMAL-c2X: ZO-2/P3SG (AA489-892); ZO-2/P3SG_LLSS (AA489-892, L₆₃₇S_L₆₄₂S). Human ZO-2 in pEGFP-C3 (Addgene plasmid #27422; <http://n2t.net/addgene:27422>; gift from Marius Sudol⁶⁵. Murine ZO-1 in pEGFP-C1 (kindly provided by Dr. Karl Matter (University College London, UK) and Dr. Junichi Ikenouchi (Kyushu University, Japan). Canine Cingulin in pcDNA3: EGFP-Cingulin (AA1196)-Myc. Murine JAM-C in pcDNA3: mJAM-C/f.l. (AA1-310,

S_{281A}), mJAM-C/ Δ 5 (AA1-305, S_{281A}). Murine JAM-C in pGEX plasmid vectors: mJAM-C-CP (cytoplasmic part, AA264-310) in pGEX-5X-2; mJAM-C-CP Δ 5 (AA264-305) in pGEX-6P-2.

Antibodies and reagents

The following antibodies were used in this study: mouse mAb anti-Myc 9E10⁶⁶ (IF 1:500, WB 1:500); goat pAb anti c-Myc (Santa Cruz #sc-789-G; WB 1:500); mouse mAb anti-GFP (Takara #632375, WB 1:1000); alpaca anti-GFP nanobody (Nano-Tag #N0310, used for IP); rabbit pAb anti-MBP (New England Biolabs (NEB) E8030S; WB 1:5000); mouse mAb anti-GST (ThermoFisher #13-6700; WB 1:500); mouse mAb anti-ZO-1 (Invitrogen #33-9100; 1:500 in regular ICC (IF), 1:250 in MDCK cyst assays (IF); rat mAb anti-ZO-1 R40.64 (IF 1:250); rabbit pAb anti-ZO-2 (Invitrogen #71-1400; IF 1:200); rabbit pAb anti-Par-3 (Millipore #07-330; IF 1:250); mouse mAb anti-E-cadherin (BD Biosciences #610181; 1:100 in regular ICC (IF), 1:50 in MDCK cyst assays (IF); rat mAb anti-mJAM-A⁶⁷; IF dil 1:300); rabbit anti-cJAM-A pAb Affi831 (IF 1:500) has been described before⁶⁸; goat anti-mJAM-C (R&D Systems #AF1213; WB 1:500, IF 1:333). Rabbit anti-mJAM-A pAb Affi1165, rabbit anti-mJAM-C Affi1166, rabbit anti-mJAM-C Affi1840, and rabbit anti-canine JAM-C pAb Affi1917 were generated by immunizing rabbits with fusion proteins consisting of the extracellular domain of the respective JAM protein (mouse JAM-A: Gene ID 23929; mouse JAM-C: Gene ID 83964; canine JAM-C: Gene ID 489271) fused to the Fc region of human IgG, as described previously⁶⁹. The antibodies were affinity-purified by adsorption at the antigen covalently coupled to cyanogen bromide (CNBr)-activated sepharose beads (Amersham Biosciences Europe, Freiburg, Germany). Antibodies directed against the Fc part were depleted by adsorption at human IgG coupled to CNBr-activated sepharose beads. Affinity-purified antibodies were dialyzed against PBS. Affi1165 was used at 1:500 (IF), Affi1166 was used at 1:500 (IF), Affi1917 was used at 1:200 in regular ICC (IF), 1:100 in MDCK cyst assays (IF). Secondary antibodies and fluorophore-conjugated antibodies: Fluorophore-conjugated antibodies for Western blotting: IRDye 800CW Donkey anti-Rabbit IgG (LI-COR Biosciences #926-32213, WB 1:10.000), IRDye 680CW Donkey anti-mouse IgG (LI-COR Biosciences #926-68072, WB 1:10.000).

Fluorophore-conjugated secondary antibodies for ICC: Donkey anti-Mouse IgG (H+L) Alexa Fluor 594 (ThermoFisher Scientific #A-21203); Donkey anti-Rabbit IgG (H+L) Alexa Fluor 594 (ThermoFisher Scientific #A-21207); Donkey anti-Rabbit IgG(H+L) Alexa Fluor 488 (ThermoFisher Scientific #A-21206); Donkey anti-Mouse IgG (H+L) Alexa Fluor 488 (Dianova/Jackson ImmunoResearch Europe Ltd #715-545-150); Donkey anti-Mouse IgG (H+L) Alexa Fluor 647 (Dianova/Jackson ImmunoResearch Europe Ltd #715-605-150). All Alexa Fluor-conjugated secondary antibodies used in IF experiments were used at a dilution of 1:800. Alexa Fluor 647-Phalloidin (Thermo Fisher Scientific, Invitrogen #A22287; dil 1:200).

Immunocytochemistry and immunofluorescence microscopy

For immunofluorescence microscopy, cells were grown on Cultrex Basement Membrane Extract (Cultrex BME, R&D Systems #3432-010-01)-coated glass slides. Cells were washed with PBS and fixed with either 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 min at RT. To detect intracellular proteins, PFA-fixed cells were incubated with PBS containing 0.5% Triton X-100 for 10 min. Cells were washed with 100 mM glycine in PBS (3 x 10 min, RT), blocked for 1 h in blocking buffer (PBS, 10% FCS, 0.2% Triton X-100, 0.05% Tween-20, 0.02% BSA) and then incubated with primary antibodies in blocking buffer for 1 h at room temperature (RT) or overnight at 4°C. After incubation, cells were washed with PBS (3 x 5 min, RT) and incubated with fluorochrome (AlexaFluor488, AlexaFluor568, AlexaFluor594, AlexaFluor647)-conjugated, highly cross-adsorbed secondary antibodies (Invitrogen) for 2 h at RT protected from light. DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, SA). Samples were washed three times with PBS and mounted in fluorescence mounting medium (Mowiol 4-88, SA). Immunofluorescence microscopy was performed using the confocal microscope LSM 800 Airyscan (Carl Zeiss, Jena, Germany) equipped with the objective Plan-Apochromat x 63/1.4 oil differential interference contrast (Carl Zeiss). Image processing and quantification was performed using ZEN 2012 (Carl Zeiss), ImageJ (National Institutes of Health, Bethesda, MD), and Imaris (Bitplane, Version 9.1.2) softwares.

Cell junction analysis

Analysis of construct recruitment by JAM-C or JAM-C/ Δ 5 was performed by visual identification of cell-cell contact sites. EGFP-construct- or Myc-construct-transfected cells were identified based on the cytoplasmic EGFP or Myc fluorescence signal. JAM-C-positive cell-cell contacts were counted positive for the transfected constructs when the EGFP- or Myc-based fluorescence signals co-localized with the JAM-C-based fluorescence along the cell-cell contact sites. For quantification of cell-cell contact localization of endogenous proteins and transfected constructs, at least 50 cell-cell contacts per condition and per experiment were analyzed. Data is represented as fraction of cell-cell contacts positive for the transfected construct. Data is derived from at least 3 independent experiments. Statistical analysis was performed using unpaired Student's t test, data is plotted as means $\pm$ standard deviation (SD). P-values: *P<0.05, ****P<0.001. Analysis of JAM-C localization at cell-cell contacts in JAM-A KO MDCKII cells was performed using Imaris software. Based on the ZO-1 fluorescence signal a surface mask for cell-cell contacts was generated. Background structures that were incorrectly identified during automated segmentation were manually removed. The mean fluorescence intensities of ZO-1 and JAM-C signals were measured in each surface. Data is presented as JAM-C : ZO-1 fluorescence intensity ratio. At least 11 fields of view per condition and per experiment were analyzed. Data is derived from N= 3 independent experiments. Data was analyzed using GraphPad Prism. To test for statistical significance, a Kruskal-Wallis-test and a Dunn's multiple comparisons test were performed.

Immunoprecipitation and Western blot analysis

For immunoprecipitations (IPs), cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40 (AppliChem, Darmstadt, Germany), 1% Halt Protease Inhibitor cocktail (Thermo Scientific #78429) for 1 h with overhead rotation at 4°C followed by centrifugation (15.000 rpm, 20 min at 4°C). Postnuclear supernatants were precleared by incubation with protein A– or protein G–Sepharose beads (GE Healthcare, Solingen, Germany), followed by incubation with 2 μ g of antibodies coupled to protein A– or protein G–

Sepharose beads overnight at 4°C. Beads were washed four times with lysis buffer, bound proteins were eluted by boiling in 1x SDS-sample buffer/150 mM DTT. Eluted proteins were separated by SDS-PAGE and analyzed by Western blotting with near-infrared fluorescence detection (Odyssey Infrared Imaging System Application Software Version 3.0 and IRDye 800CW-conjugated antibodies; LI-COR Biosciences, Bad Homburg, Germany). All IP and Western blot experiments shown in this study are representative for at least three independent experiments.

Generation of recombinant GST- and MBP-fusion proteins

The generation of recombinant of Glutathion-S-Transferase (GST) and Maltose-binding protein (MBP) fusion proteins was performed essentially as described previously ⁴⁸. Briefly, the transformed bacteria (*E.coli* BL21, GE-Healthcare) were grown at 30°C in LB medium/0.2% glucose to $A_{600} = 0.5$. Expression of recombinant proteins was induced with 0.25 mM isopropyl- β -D-thiogalactopyranoside (IPTG, Calbiochem #420322) for 2 h. The bacteria were harvested by centrifugation, resuspended in 25 ml lysis buffer (PBS for GST fusion proteins; column buffer, i.e. 20 mM Tris-HCl (pH7.4), 0.2 M NaCl, 1 mM EDTA, for MBP fusion proteins) supplemented with Halt protease inhibitors (ThermoScientific #78429), and lysed by passaging through a French pressure cell. The cell lysates were centrifuged (Sorvall JA 25.50 rotor, 30 min, 10.000 rpm) and filtrated through a 0.45 μ m filter. GST and MPB fusion proteins were isolated from the raw lysates by affinity chromatography on glutathione (GSH) sepharose resin (glutathione-Sepharose 4B beads, Cytiva #17-0756-0) and amylose resin (New England Biolabs (NEB) #E8021L), respectively. The protein solutions were dialyzed against PBS, adjusted to 50% (w/v) glycerol and stored at -20°C. Purified proteins were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining.

GST pulldown experiments

In vitro binding experiments were performed with recombinant GST-JAM-C fusion proteins purified from *E.coli* and immobilized on GSH-Sepharose 4B beads. For the analysis of direct

protein interactions in vitro, recombinant MBP-ZO-2 constructs were incubated with 3 µg of immobilized GST fusion proteins for 1 h to overnight at 4°C under constant agitation in buffer B (10 mM Hepes-NaOH (pH7.4), 100 mM KCl, 1 mM MgCl₂, 0.1% Triton X-100). After 4 washing steps in buffer B bound proteins were eluted by boiling for 5 min in SDS sample buffer, subjected to SDS-PAGE and analyzed by Western blotting with near-infrared fluorescence detection. To study interactions of recombinant proteins with proteins expressed in cells, cellular lysates were incubated with 3 µg of immobilized GST fusion proteins and incubated overnight at 4°C under constant agitation. After 4 washing steps in buffer B bound proteins were eluted by boiling for 5 min in SDS sample buffer, subjected to SDS-PAGE and analyzed by Western blotting with near-infrared fluorescence detection. All in vitro binding experiments shown in this study are representative of at least three independent experiments.

MDCK II cyst assays

MDCK II cells were adjusted to a single-cell suspension with a concentration of 6×10^4 cells/ml in serum-free, ice-cold DMEM. The cell suspension was mixed 1:1 with ice-cold Matrigel matrix and plated into glass bottom cell culture slides (Greiner #543078). The gels were allowed to polymerize for 20 min at 37 °C in a humidified incubator with 5% CO₂. After polymerization, 200 µl DMEM supplemented with 10% FCS was carefully added to the top of the gels and exchanged every two days. After four to six days MDCK cysts were fixed with 4% PFA for 1h at RT. After fixation, cells were washed twice with PBS, 3% BSA and permeabilized in PBS, 0.1% Triton X-100 for 20 min at RT. Blocking was performed in PBS, 5% BSA for 1h at RT. Antibody incubations were performed in PBS, 1% BSA, 0.05% Triton X-100. Primary antibodies were incubated overnight at 4°C, secondary antibodies were incubated for 2 to 3 h at RT. DNA was stained by incubating cysts in PBS, DAPI (1:400), 1% BSA, 0.05% Triton-X100. After three washes with PBS, 1% BSA, 0.05% Triton X-100, (10 min at RT each wash) and one wash with PBS, cysts were mounted by adding two drops of mounting medium (Ibidi #50001) per well. Cysts were analyzed using a confocal microscope as specified in the Immunocytochemistry and Immunofluorescence Microscopy section.

Statistics and reproducibility

Results are expressed either as arithmetic means $\pm$ SD as indicated. To test the normality of data sample distributions, the D'Agostino-Pearson normality test was used. Data were statistically compared using unpaired, two-tailed Student's *t*-test, or probed for being statistically different from a fixed value using One sample *t*-test. Alternatively, Kruskal-Wallis test followed by a Dunn's Multiple Comparisons test were used. 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. For each statistical analysis data derived from at least three independent experiments were used. Experiments were considered independent when they reflected biological replicates acc. to ⁷⁰. Sample sizes are indicated for all experiments in the respective figure legends.

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Ethics approval

NA

Author contributions

K.E. designed and conceived the study. A.S., V.T., F.B., and K.E. performed experiments. A.S., M.F.S., V.T., and K.E. analyzed the data. S.C., M.F. and M.A.-L. provided reagents. K.E. wrote the manuscript.

Abbreviations

AA, amino acid, IF, immunofluorescence; JAM, Junctional Adhesion Molecule; CGN, Cingulin; LLPS, Liquid-Liquid Phase Separation; PBM, PDZ domain-binding motif; PDZ, Postsynaptic density 95 - Discs Large - Zonula occludens-1; ZO, Zonula occludens.

Competing interests

The authors declare that they have no conflict of interest.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary information files.

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