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α-Actinin links LPP, but not zyxin, to cadherin-based junctions

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ABSTRACT

The actin regulator VASP localizes to cell–cell junctions and has been implicated in cell–cell adhesion. VASP is recruited to sites of actin dynamics by interactions with proline rich FPPPPP motifs. Zyxin and its relative LPP use FPPPPP motifs to recruit VASP to specific cellular locations, thus directing changes in actin dynamics. It has been proposed that zyxin and LPP localize to cell–cell junctions by binding α-actinin. However, the role of α-actinin in recruiting zyxin and LPP to cell–cell contacts has not been experimentally tested. Here we use zyxin and LPP fragments to demonstrate that the α-actinin binding site of both proteins independently targets to cell–cell junctions. While the α-actinin binding site is required for LPP localization and function at cell–cell contacts, zyxin localization and function at cell–cell contacts is independent of the α-actinin binding site. Perturbation of LPP function, but not that of zyxin, results in changes in anchoring of α-actinin to detergent-insoluble networks at cell–cell contacts.

The actin cytoskeleton plays an important role in cadherin-based adhesion [1]. Actin filaments have been thought to anchor cadherin complexes at cell–cell junctions by binding the cadherin complex component α-catenin [2]. Though actin anchoring of cadherin complexes cannot be reconstituted in vitro [3,4], it remains clear that cadherin-based adhesion does result in dramatic actin reorganization events at nascent cell–cell contacts [5–7]. In the possible absence of a direct link between cadherin complexes and actin, it is important to define how actin regulatory systems are recruited to cell–cell contacts.

VASP family members are actin regulatory proteins that localize to cell–cell junctions and have been implicated in cell–cell adhesion [8]. They are recruited to specific cellular sites through interactions with FPPPPP motifs [9]. The focal adhesion protein zyxin and its relative LPP contain arrays of FPPPPP motifs that bind VASP [10,11] and colocalize with VASP at epithelial cell–cell junctions [7,12]. FPPPPP peptides displace VASP from cell–cell contacts [7], demonstrating that FPPPPP motif containing proteins, such as zyxin and LPP, are required to localize VASP to cell–cell contacts. Zyxin/LPP mutants lacking LIM domains can either accelerate or reduce cell–cell adhesion, an effect that is determined by the presence of intact FPPPPP motifs [12]. Thus, zyxin and LPP likely alter cell–cell adhesion by binding VASP.

Bershadsky and Geiger [13] proposed that zyxin, and thus VASP, was recruited to cell–cell junctions through an interaction with α-actinin. In support of this, both zyxin and LPP contain functional α-actinin binding sites [14,15]. However, a role for α-actinin binding in zyxin or LPP localization or function has not been examined experimentally. Here we analyze recruitment of zyxin and LPP to cell–cell contacts in greater detail, focusing on the role of α-actinin. While the α-actinin binding region of both zyxin and LPP can independently localize to cell–cell contacts in MDCK cells, deletion of this region affects only the function of LPP, but not zyxin. LPP-VASP binding is required for α-actinin anchoring into detergent-insoluble cytoskeletal elements, suggesting that LPP may coordinate the actin regulatory activities of VASP and α-actinin at cell–cell contacts.

Materials and methods

Cell culture and cell aggregation assays. MDCK cells were cultured in Dulbecco's modified Eagle medium (DMEM)+10% FBS. Aggregation assays, as well as their statistical analysis, were performed as described [12]. Briefly, cells were grown in suspension at 2.5×10^5 cells per ml in 20 μl drops. Following 0–4 h incubation, cells were counted with or without trituration of aggregates. Cells were binned as part of small (1–10 cells), medium (11–50 cells), and large (>50 cells) aggregates. Average values of three experiments are presented. Log-odds scores were calculated from these values using Statistica 6.0.

Generation of cell lines. MDCK cells were transfected with pDSred2.C1 plasmids containing zyxin or LPP inserts using Effectene (Stratagene). Following 14 days of G418 selection, DSred2 fusion protein positive cells were isolated by FACS. Only those cells displaying DSred2 fluorescence within a specific range of fluorescence were collected and aggregation assays were performed within two passages of sorting.

Fluorescence. Cells grown on collagen-coated coverslips were washed with ice-cold Ringer's saline containing 1.8 mM $CaCl_2$. Cells were extracted with CSK buffer (10 mM PIPES pH6.8, 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1.8 mM $CaCl_2$,

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0.5% v/v Triton X-100) on ice for 10 min prior to fixation where appropriate. Cells were fixed on ice for 15 min with 4% paraformaldehyde in PBS and then processed for immunofluorescence with the appropriate antibodies. Anitbodies used were α-actinin (Sigma) and cadherin (E2). DSred2 fusion proteins were observed directly. All coverslips were mounted in VectaShield (Vector) and viewed under a Zeiss Axiophot microscope at room temperature. Pairs of extracted and whole cells were imaged with identical exposure settings.

Results and discussion

Zyxin and LPP share many key features, including an α-actinin binding site, an array of FPPPP motifs, and three C-terminal LIM domains (Fig. 1A). In both proteins, the LIM domains are separated from the FPPPPP motifs by a central region of over 250 amino acids. In a previous report, zyxin or LPP lacking a single one of these shared domains retained the ability to localize to cell–cell contacts [12]. However, zyxin and LPP are thought to act as scaffold proteins, binding multiple proteins simultaneously and complicating interpretation of how targeting to cellular sites might occur. In order to better define motifs in zyxin and/or LPP that can independently target to cell–cell junctions, DSred2 fusion proteins containing single zyxin or LPP domains were expressed in MDCK cells and their cell–cell junction localization examined. DSred2 alone does not display any specific localization to cell–cell contacts in MDCK cells (Fig. 1B). Fragments con-

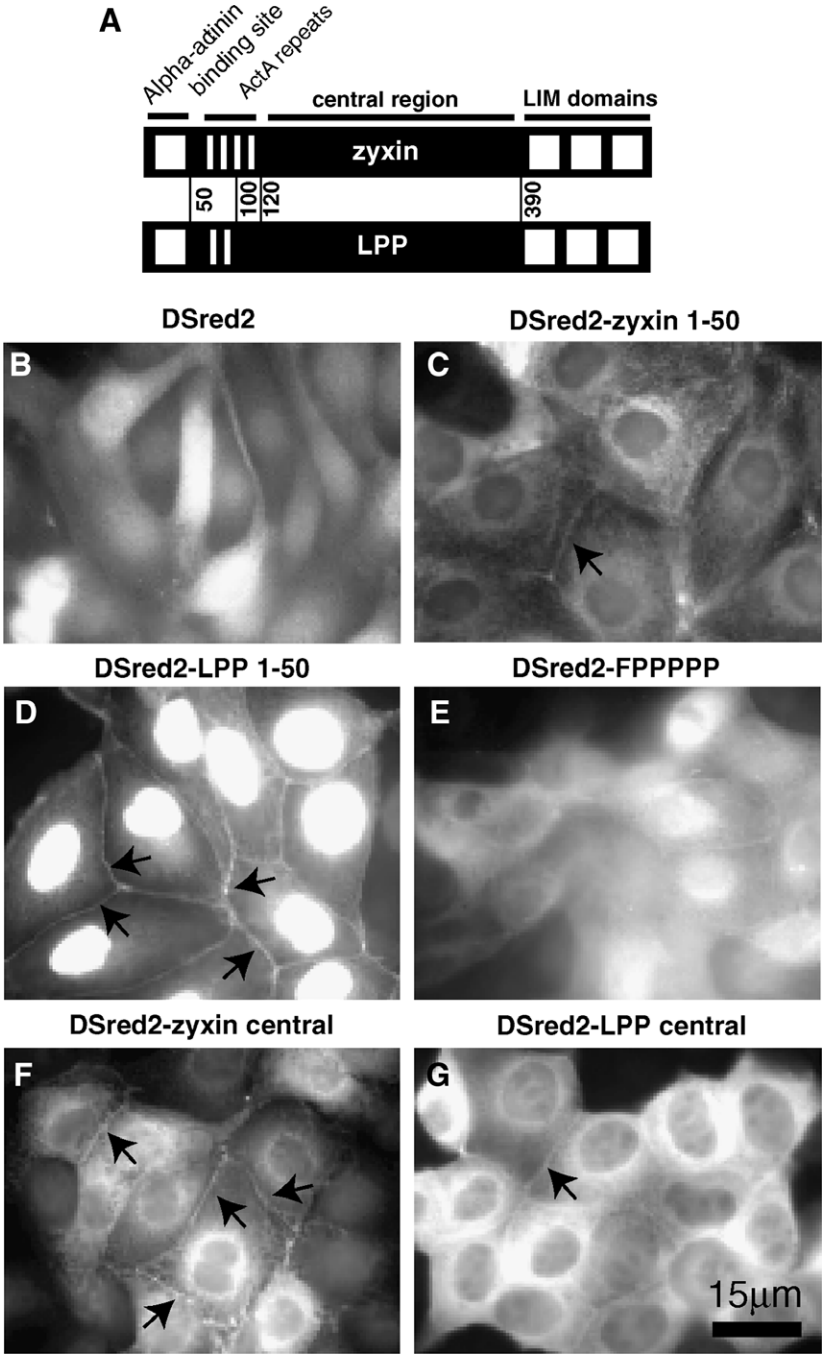

Fig. 1. Targeting of zyxin and LPP fragments to cell–cell junctions. (A) Molecular organization of zyxin and LPP, including the α-actinin binding site, VASP binding FPPPPP motifs, central region, and LIM domains. (B–G). Direct fluorescence of DSred2 (B), DSred2-zyxin 1–50 (C), DSred2-LPP 1–50 (D), DSred2-FPPPPP motif array (E), DSred2-zyxin central region (F), or DSred2-LPP central region (G) proteins expressed in MDCK cells. Arrows denote cell–cell junction localization.

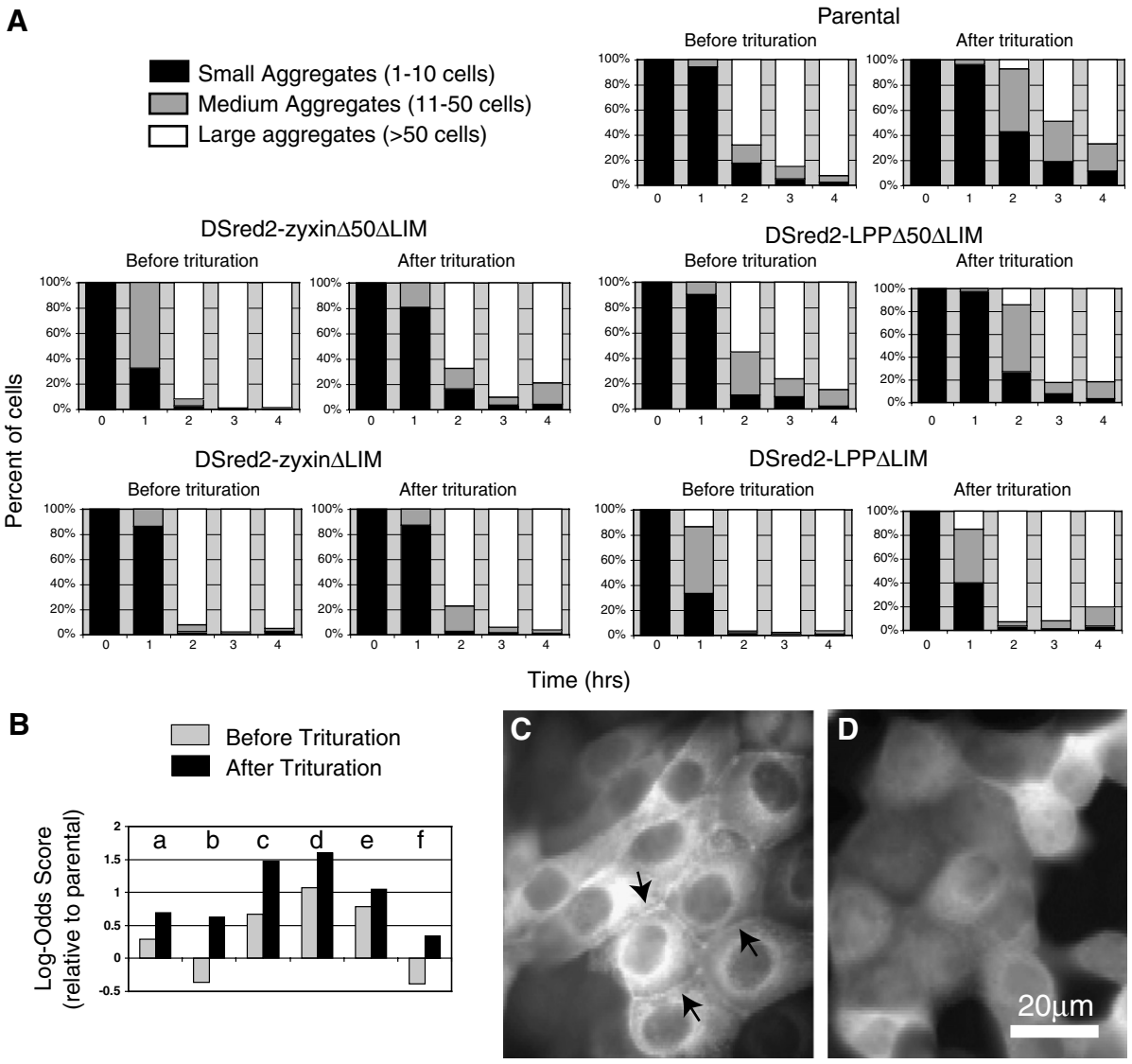

Fig. 2. LPP, but not zyxin, function in cell–cell adhesion requires α-actinin binding. (A) Rate of aggregation of parental MDCK cells or cells expressing DSred2 fused to the designated zyxin or LPP mutants. The ΔLIM mutant lacks the LIM domain region, while the Δ50ΔLIM mutant lacks both the LIM domain region and the first 50 amino acids containing the α-actinin binding site. (B) Statistical comparison of rates at which cell lines aggregate into large aggregates (>50 cells) in suspension as a function of time, before or after trituration. The log-odds scores were computed relative to the reference cell line, parental MDCK cells. The designation of the x axis is (a) DSred2-zyxin, (b) -LPP, (c) -zyxin$^{\Delta LIM}$, (d) -LPP$^{\Delta LIM}$, (e) -zyxin $^{\Delta 50\ \Delta LIM}$, and (f) -LPP$^{\Delta 50\ \Delta LIM}$. (C–D) Direct fluorescence of DSred2- zyxin$^{\Delta 50\Delta LIM}$ (C) or DSred2- LPP$^{\Delta 50\Delta LIM}$ (D) in MDCK cells.

sisting of the first 50 amino acids of zyxin or LPP, containing the α-actinin binding site, both localize to cell–cell junctions (Fig. 1C–D). While the N-terminal 50 amino acids of zyxin bind additional proteins, namely LIM-nebulette and Lasp-1 [16], neither protein was reported to localize to cell–cell contacts when expressed in HeLa cells [16]. Further, Lasp-1 is not observed at cell–cell contacts of gastric parietal epithelial cells [17]. Thus, the observed localization of this fragment is most consistent with α-actinin docking both zyxin and LPP to sites of cadherin-based adhesion. In contrast, a DSred2 fusion protein fused to an array of four VASP binding sites (FPPPP motifs) does not display significant localization at cell–cell contacts (Fig. 1E), consistent with the observation that introduction of an FPPPPP peptide into cells displaced VASP and not zyxin from cell–cell contacts [7]. Interestingly, DSred2 fusion proteins containing only the region of zyxin or LPP between FPPPPP motifs and LIM domains displayed different capabilities for targeting to cell–cell contacts when expressed in MDCK cells. While the zyxin fragment does target to cell–cell contacts, the LPP fragment shows minimal cell–cell contact localization (Fig. 1F–G). Given the low sequence homology between zyxin and LPP in this region, zyxin could bear additional cell–cell junction localization information that does not occur in the LPP sequence.

As the α-actinin binding sites from both zyxin and LPP can independently target to cell–cell junctions, we next sought to demonstrate that the α-actinin binding site was required for zyxin and LPP function in cell–cell adhesion. This was particularly important given the observation that zyxin may harbor additional cell–cell junction localization information that is not found in LPP. Zyxin or LPP mutants lacking LIM domains drive accelerated cell–cell junction formation and strengthening [12]. We reasoned that deletion of cell–cell junction targeting information from these mutants would diminish their activity. To test the role of the α-actinin binding site in targeting, the effect of expression DSred2-zyxin$^{\Delta 50\Delta LIM}$ and -LPP$^{\Delta 50\Delta LIM}$ on cell aggregation was assessed (Fig. 2A). The results were compared to those obtained for parental MDCK cells, whose junction formation is normal, as well as DSred2-zyxin$^{\Delta LIM}$ and -LPP$^{\Delta LIM}$ cells, which

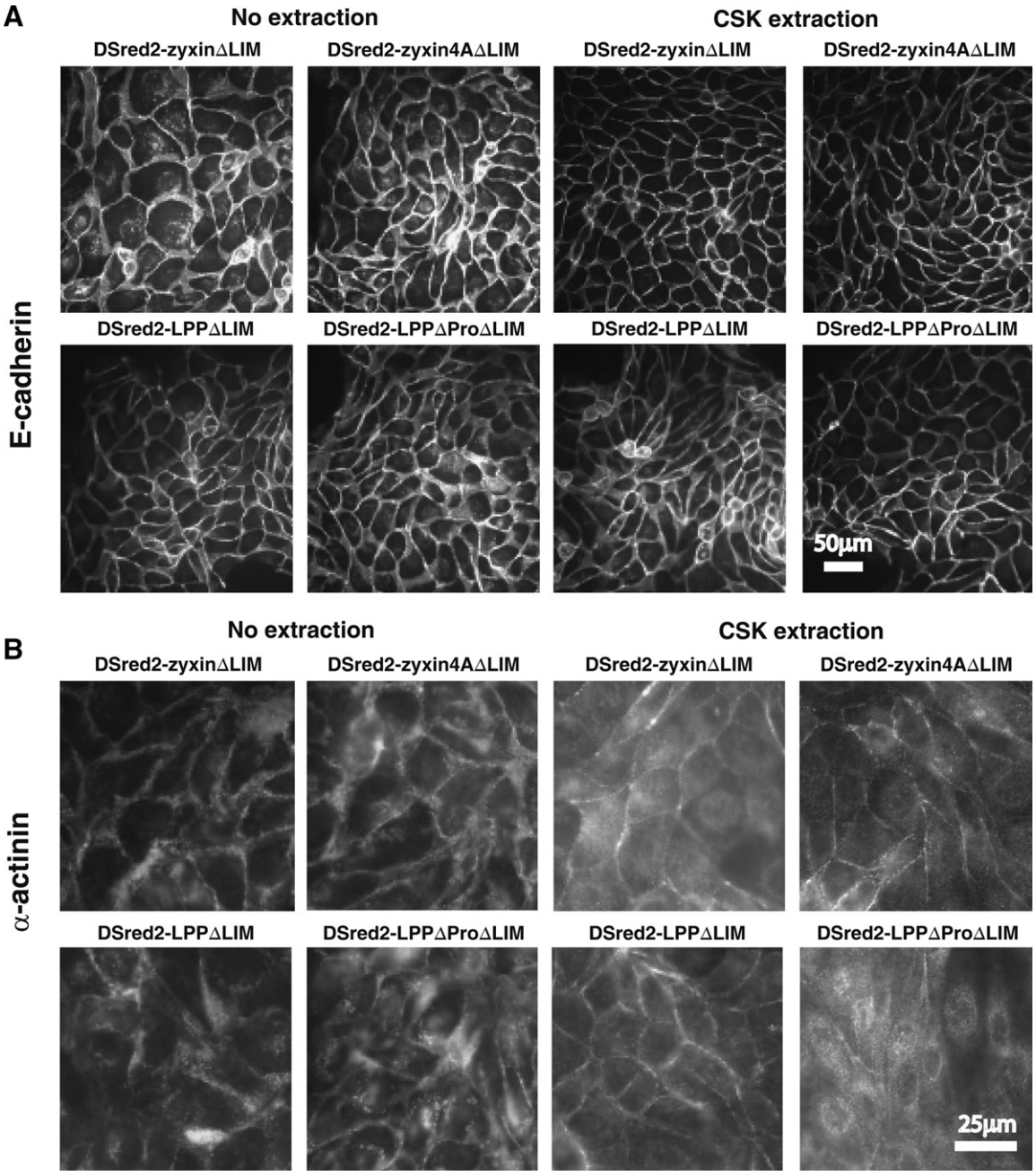

Fig. 3. Effect of zyxin or LPP activity on protein localization to cell–cell junctions. (A) Cadherin distribution in whole or detergent-extracted MDCK cells expressing the designated zyxin or LPP mutants, as determined with cadherin antibody staining. (B) α-Actinin distribution in whole or detergent-extracted MDCK cells expressing the designated zyxin or LPP mutants, as determined with cadherin antibody staining.

display accelerated cell–cell junction formation [12]. DSred2-zyxin$^{\Delta 50\Delta LIM}$ cells exhibited significantly accelerated cell–cell contact formation and strengthening that was similar to that for DSred2-zyxin$^{\Delta LIM}$ cells. After only 2 h of aggregation, large aggregates account for 91.9% of DSred2-zyxin$^{\Delta 50\Delta LIM}$ cells, compared to 92.2% of DSred2-zyxin$^{\Delta LIM}$ cells. Plateau phase of aggregation is reached after 2 h in suspension for both cell lines. Similar results are also obtained after trituration. Large aggregates account for 67.4% of DSred2-zyxin$^{\Delta 50\Delta LIM}$ cells after 2 h of aggregation, compared to 77.1% of DSred2-zyxin$^{\Delta LIM}$ cells. cell–cell junctions become strengthened sufficiently to withstand trituration forces at similar rates in these cell lines. Direct fluorescence reveals that DSred2-zyxin$^{\Delta 50\Delta LIM}$ still targets to cell–cell junction in these cells (Fig. 2C), consistent with the α-actinin binding site not being required for zyxin localization or function at cell–cell junctions.

In contrast, DSred2-LPP$^{\Delta 50\Delta LIM}$ cells exhibit a notably slower rate of cell–cell contact formation than those of DSred2-LPP$^{\Delta LIM}$ cells. Large aggregates account for 55.1% of DSred2-LPP$^{\Delta 50\Delta LIM}$ cells after 2 h in suspension (Fig. 2A), compared to 96.6% of DSred2-LPP$^{\Delta LIM}$ cells. Aggregation approaches plateau only by 3 h in suspension for DSred2-LPP$^{\Delta 50\Delta LIM}$ cells, but has already reached plateau phase by 2 h in DSred2-LPP$^{\Delta LIM}$ cells. Following trituration, 14.3% of DSred2-LPP$^{\Delta 50\Delta LIM}$ cells remain in large aggregates following 2 h in suspension, while 92.8 of DSred2-LPP$^{\Delta LIM}$ cells remain in large aggregates. The ability of DSred2-LPP$^{\Delta 50\Delta LIM}$ cells to form large aggregates over time is not blocked; large aggregates account for 76.3% and 84.7% of DSred2-LPP$^{\Delta 50\Delta LIM}$ cells prior to trituration

after 3 and 4 h in suspension, respectively. In fact, the rate of aggregation and cell–cell contact strengthening is similar to that of parental MDCK cells. Further demonstrating that the α-actinin binding site is required for cell–cell contact targeting and function of LPP, direct fluorescence shows that DSred2-LPP$^{\Delta 50\Delta LIM}$ does not target to cell–cell junctions in MDCK cells (Fig. 2D).

Multivariate logistical regression analysis was performed on the rate at which these cell lines formed large aggregates in order to statistically verify our interpretation (Fig. 3B). Based upon the rate at which cells aggregate into large groups, a log-odds score using parental MDCK cells as the reference cell line was calculated, both before and after trituration [12]. As statistical criteria for determining whether a cell line displayed enhanced or reduced cell–cell junction formation, we established that both log-odds scores (before and after trituration) must be greater or less than twice the standard error for all experiments (0.346763 and 0.358388 for before and after trituration, respectively). Based on these criteria, DSred2-zyxin$^{\Delta 50\Delta LIM}$ cells still display an accelerated rate of aggregation, while DSred2-LPP$^{\Delta 50\Delta LIM}$ cells display a rate of cell–cell junction formation that is not statistically different from either parental MDCK cells or DSred2-LPP cells.

We next sought to determine if zyxin or LPP were required for correct organization of cell–cell junctions. This was first tested by examining cadherin distribution in cells expressing zyxin or LPP mutants. Whether total cadherin staining in whole cells or only the detergent-resistant pool of cadherin in extracted cells were examined, DSred2-zyxin$^{\Delta LIM}$ or DSred2-LPP$^{\Delta LIM}$ cells showed similar cadherin distribution and signal intensity when compared to DSred2-zyxin$^{4A\Delta LIM}$ or DSred2-LPP$^{\Delta Pro\Delta LIM}$ cells, respectively (Fig. 3A). These results indicate that the morphology of mature cell–cell junctions, as well as the proper distribution of cadherin at the junction, is not affected by zyxin or LPP mutant expression. We then assessed whether perturbations in zyxin or LPP activity would alter the distribution of α-actinin in MDCK cells (Fig. 3B). All cell lines displayed a similar distribution of α-actinin staining when whole cell were examined. However, DSred2-LPP$^{\Delta Pro\Delta LIM}$ cells had little or no α-actinin staining at cell–cell contacts when detergent extraction was performed prior to fixation, demonstrating that LPP function is required to anchor α-actinin to detergent-insoluble elements at cell–cell contacts. Interestingly, LPP retains its ability to bind α-actinin in this experiment, demonstrating that LPP-VASP binding is required for the observed incorporation of α-actinin into detergent-insoluble networks at cell–cell contacts. In contrast, extracted MDCK cells expressing zyxin mutants had an indistinguishable α-actinin localization at cell–cell contacts, demonstrating that zyxin plays no role in α-actinin distribution at cell–cell junctions. Along with our observation that zyxin can target to cell–cell contacts independently of the α-actinin binding site, this suggests that α-actinin binding is not a primary mechanism of zyxin localization and is not required for zyxin function during establishment of cell–cell adhesions.

Zyxin and LPP function in recruiting VASP to cellular sites requires that zyxin and LPP be recruited by docking proteins. While it has been proposed that the already characterized α-actinin-zyxin and -LPP interaction could dock these proteins at cell–cell junctions, results presented here suggest that this is true for LPP only. Instead, zyxin must contain additional cell–cell junction localization information. LPPs FPPPPP motifs are required for the proper attachment of α-actinin to the detergent-resistant fraction of cell–cell junctions. Thus, LPP might coordinate actin regulatory activities of VASP with those of α-actinin at cell–cell contacts, as well as other sites.

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