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## **Reticular adhesions are a distinct class of cell-matrix adhesions that mediate attachment during mitosis**

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## Abstract

Adhesion to the extracellular matrix (ECM) persists during mitosis in most cell types. However, while classical adhesion complexes (ACs), such as focal adhesions (FAs), do and must disassemble to enable mitotic rounding, the mechanisms of residual mitotic cell-ECM adhesion remain undefined. Here, we identify ‘reticular adhesions’, a class of AC that is mediated by integrin  $\alpha V\beta 5$ , formed during interphase, and preserved at cell-ECM attachment sites throughout cell division. Consistent with this role, integrin  $\beta 5$  depletion perturbs mitosis and disrupts spatial memory transmission between cell generations. Reticular adhesions (RAs) are morphologically and dynamically distinct from classical FAs. Mass spectrometry defines their unique composition, enriched in phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2)-binding proteins, but lacking virtually all consensus adhesome components. Indeed, RAs are promoted by PI-4,5-P2, and form independently of talin and F-actin. The distinct characteristics of RAs provide a solution to the problem of maintaining cell-ECM attachment during mitotic rounding and division.

## Introduction

Cell-ECM attachment occurs through a range of integrin-containing ACs, including focal complexes, FAs and fibrillar adhesions<sup>1,2</sup>, and modulates many processes including cell movement, proliferation and differentiation<sup>3</sup>. Though structurally and functionally varied, ACs overlap substantially in their composition, sharing a 60-protein consensus adhesome<sup>4</sup>. As one of the most abundant consensus adhesome proteins, talin-1 is viewed as an indispensable contributor to integrin activation<sup>5</sup> and AC organisation<sup>6</sup>. Adaptor proteins that couple integrins to F-actin, such as vinculin and paxillin, are also universally associated with ACs, reflecting the pivotal role of F-actin in AC function<sup>7</sup>.

Cell-ECM adhesion is also critical for mitotic progression and for the transmission of spatial memory between generations<sup>8–11</sup>, a key factor controlling differentiation and tissue development<sup>12</sup>. Paradoxically, the importance of cell-ECM attachment during mitosis conflicts with the observed disassembly of classical ACs at mitotic onset<sup>13</sup>, since failure of AC disassembly perturbs division<sup>14,15</sup>. Furthermore, integrins implicated in mitotic adhesion, such as  $\beta 1$ , function not at the adhesion plane but in the detached cell cortex<sup>16</sup>. Overall, the nature of mitotic ACs remains profoundly unclear<sup>17,18</sup>.

Here, we identify a class of ‘reticular’ AC with a unique adhesome, formed by integrin  $\alpha V\beta 5$  during interphase in the absence of both talin and F-actin. RAs persist throughout mitosis, providing the ECM anchoring that is necessary for efficient division. Thus, RAs provide a solution to the paradox of mitotic cell-ECM attachment.

## Results

### *$\alpha V\beta 5$ is the predominant integrin used by cells in long-term culture*

The integrin consensus adhesome was derived from cells plated on fibronectin<sup>4,19</sup>. To study the adhesome of cells that had assembled their own ECM, we performed mass spectrometry analysis of AC composition in U2OS cells following 72 h growth. Unexpectedly, the most abundant integrin subunits identified were  $\alpha V$  and  $\beta 5$ , with much lower levels of  $\beta 1$ ,  $\beta 3$ ,  $\beta 8$ ,  $\alpha 5$  and  $\alpha 3$  (Fig.1A). Subsequent immunofluorescence analysis confirmed that very distinct  $\alpha V\beta 5$ -positive ACs were visible in a range of cells in long-term culture, with little  $\alpha V\beta 3$  or  $\beta 1$  labelling detected in U2OS, A549 and A375 cells (Fig.1B and Supplementary Fig.1A). Notably,  $\alpha V\beta 5$  was simultaneously detected in classical FAs at the cell periphery and in reticular structures across the cell body, also

visible after 24 and 48 h of cell growth (Supplementary Fig.1B). U2OS cells were then plated on the integrin  $\alpha$ V ligand vitronectin (VN). Confocal imaging showed  $\beta$ 5 associated with two different structures following 3h of spreading: peripheral focal ACs containing talin and vinculin, and centrally-distributed, punctate or reticular structures lacking these components (Fig.1C-D).

Subsequently, similar  $\alpha$ V $\beta$ 5-positive, talin-negative structures were detected in each of nine cell lines assessed, including the cancer cell lines CS1-b5, HeLa, MCF7 and BT549 (Supplementary Fig.1C), immortalised non-transformed HME1 and RPE1 cells (Supplementary Fig.1D), and primary MAE and HDF cells (Supplementary Fig.1E), indicating that formation of  $\alpha$ V $\beta$ 5-positive, talin-negative structures is characteristic of a wide range of cell types. Integrin  $\alpha$ V and  $\beta$ 5 subunits colocalised (Supplementary Fig.2A), but co-labelling of  $\beta$ 5 with antibodies against various AC-related proteins, including consensus adhesome components,  $\alpha$ V $\beta$ 5 binding partners, cytoskeletal proteins (including F-actin), and phosphotyrosine, revealed no specific colocalisation (Supplementary Fig.1C and Supplementary Fig.2B-M). Integrin  $\beta$ 5 labelling intensity in both talin-1-positive and -negative structures correlated with VN concentrations (Fig.1E-G), while U2OS cells plated on laminin (not a ligand for  $\alpha$ V $\beta$ 5) only formed vinculin-positive ACs (Supplementary Fig.2N). In conclusion, formation of the reticular structures depends on  $\alpha$ V $\beta$ 5-ECM ligand binding.

We next labeled the integrin  $\beta$ 5 extracellular domain without prior cell permeabilisation (Supplementary Fig.2O). Strong colocalisation with integrin  $\beta$ 5-2GFP demonstrated  $\alpha$ V $\beta$ 5 plasma membrane embedding and antibody specificity. Moreover, total internal reflection (TIRF) imaging of live U2OS cells co-expressing  $\beta$ 5-2GFP and mCherry-vinculin (U2OS- $\beta$ 5V) revealed central,  $\alpha$ V $\beta$ 5-positive, vinculin-negative structures in the TIRF plane (Fig.1H). Dark intracellular regions in mCherry-vinculin signals indicated where tensioned ventral membranes arced out of the TIRF plane, leaving no cytoplasmic signal. These dark regions corresponded with large gaps between  $\alpha$ V $\beta$ 5-positive, vinculin-negative puncta, suggesting the latter to be attachment points that pin the ventral plasma membrane to the substrate. This hypothesis was supported by live cell interference reflection microscopy, where close cell-substrate proximity corresponded precisely with integrin  $\beta$ 5-2GFP signals in both vinculin-positive FAs and vinculin-negative structures (Fig.1I). Collectively, these data indicate that  $\alpha$ V $\beta$ 5-positive, consensus adhesome component-negative reticular structures are *bona fide* cell-ECM ACs. These are hereafter termed “reticular adhesions” (RAs).

### ***Reticular and focal ACs are morphologically and dynamically distinct***

RAs were more numerous than classical focal ACs at all sizes (Fig.2A), increased in size more frequently (Fig.2B) and were localised further from the cell periphery (Fig.2C). There was no correlation between RA size and integrin  $\beta$ 5 clustering density, unlike the increased integrin density observed in larger FAs (Fig.2D)<sup>20,21</sup>. This implies molecular-scale differences between the maturation of FAs and RAs, with the latter being more homogenous. RAs formed as small puncta, grew by net peripheral integrin recruitment, producing ring-like or reticular structures that ultimately fragmented and disassembled, all without recruiting vinculin (Fig.2E-H; Supplementary Movie 1 and cropped region from Fig.2H in Supplementary Movie 2). Thus, RAs form *de novo* as a distinct class of AC.

Quantitative tracking highlighted stark differences in dynamics between RAs and FAs (Fig.2I-N; Supplementary Movie 3): isotropic RA growth produced low displacement (Fig.2J), while FAs elongated anisotropically and slid at high velocities, reflecting F-actin-derived forces driving asymmetric component recruitment (Fig.2K)<sup>22,23</sup>. Isotropic growth and immobility in RAs suggests the absence of such directed mechanical cues<sup>24</sup> and complements the observed lack of F-actin. This conclusion was supported by locally disordered motion of RA trajectories (Fig.2L). In contrast, FAs moved co-linearly within different cell lobes (Fig.2M), reflecting aligned, centripetal F-actin-derived forces<sup>25</sup>. The relationship between average AC velocity and lifetime revealed that, for both FAs and

RAs, fast movement corresponded with short lifetime. Thus, fast-moving FAs existed for less than half the lifespan of RAs, which were relatively static and long-lived (Fig.2N).

Fluorescence recovery after photobleaching analysis revealed that, despite their increased lifetime as complexes,  $\beta 5$ -2GFP turnover in RAs was faster and more extensive than in FAs (Fig.3A-E; Supplementary Movie 4). Conversely, variability in  $\beta 5$ -2GFP fluorescence recovery was lower in RAs (Fig.3F), suggesting relative homogeneity in molecular organisation and dynamics across their lifespan, consistent with the homogeneity in integrin clustering densities (Fig.2D).

In stochastic optical reconstruction microscopy (STORM), both AC types displayed small internal clusters of integrin  $\beta 5$  (Fig.3G), consistent with the integrin  $\beta 1$  nanocluster organization within ACs that we recently reported<sup>26</sup>. Minimal differences were observed between the AC types in terms of nearest neighbour distances between nanoclusters and molecular localisation counts per nanocluster (Fig.3H-I). Thus, despite the absence of consensus adhesome components (including talin-1, thought to control nanoscale integrin organisation<sup>6</sup>) and differences in macromolecular dynamics, the molecular scale organisation of integrin  $\beta 5$  is virtually identical in FAs and RAs.

### ***RAs mediate cell attachment but form independently of F-actin and talin***

Disruption of actin polymerisation by cytochalasin D or latrunculin A prior to cell-ECM attachment inhibited FA, but not RA, formation (Fig.4A-B; Supplementary Fig.3A and Supplementary Movie 5; note the simultaneous formation of both RAs and FAs within 20-30min of control cell attachment). Cytochalasin D inhibited cell spreading, but not RA numbers relative to cell area, as evidenced by matched linear trends in cell area *versus* AC number within treated and control cells (Fig.4C). Notably, while cytochalasin D substantially reduced vinculin levels in surviving FAs,  $\beta 5$  densities increased in RAs (Fig.4D). Further, cytochalasin D treatment after attachment caused disassembly of FAs, but retention of RAs (Supplementary Fig 3A).

Integrin  $\beta 3$ - and  $\beta 5$ -negative CS1-wt cells did not attach to VN, while CS1 cells stably expressing  $\beta 5$  (CS1- $\beta 5$ ) attached strongly (Fig.4E and Supplementary Fig.3B) and formed both FAs and RAs. CS1- $\beta 5$  cells treated with cytochalasin D attached approximately half as strongly as unperturbed CS1- $\beta 5$  cells, demonstrating that RAs facilitate cell attachment in the absence of F-actin. This residual adhesion was blocked by competitive inhibition of  $\alpha V\beta 5$ -VN binding using cyclic RGD peptides, confirming  $\alpha V\beta 5$  specificity (Fig. 4E). Thus, RAs forming in the absence of F-actin facilitate attachment in the absence of FAs.

To assess the role of talin in RA formation, talin-1-null mouse embryonic stem cells (mES talin-1 -/-) were transfected with talin-2 siRNA. Reduction of talin limited cell spreading (Fig.4F-H; Supplementary Fig.3C)<sup>27</sup> and ablated FAs (Fig.4F,G); however, integrin  $\beta 5$  was more densely concentrated within RAs upon talin-2 knock-down (Fig.4F,G,I), similar to cells treated with cytochalasin D (Fig.4A-D). Thus, RAs can form independently of talin. Upon activation by manganese or the talin-1 head domain, integrin  $\alpha V\beta 3$  forms reticular-like clusters in the centre of the cell<sup>28,29</sup>. In contrast,  $\alpha V\beta 5$  clustered independent of talin or additional activation stimuli. Furthermore, mRFP-tagged talin-1 head or rod domains neither localised to RAs nor altered  $\alpha V\beta 5$ -containing RAs (Supplementary Fig.3D). Expression of EGFP-tagged integrin  $\beta 5$  extracellular domain fused to the integrin  $\beta 3$  tail domain also demonstrated localisation to RAs (Fig.4J,K), identifying the  $\beta 5$  extracellular domain as the key facilitator of  $\alpha V\beta 5$  clustering in RAs.

### ***RA composition is unique***

We next used mass spectrometry to define RA composition. U2OS cells were treated with cytochalasin D to deplete FAs, followed by ventral membrane AC isolation and processing. 199 proteins were identified in the control condition, 18 of which were consensus adhesome components (Fig.5A and Supplementary Table 2)<sup>4</sup>. Conversely, cytochalasin D-treated samples revealed 53

proteins selectively associated with RAs, only one of which was a consensus adhesome protein (tensin-3). Four proteins were discounted from further analysis due to exceptionally high representation in the CRAPome database<sup>30</sup>, leaving a reticular adhesome of 49 proteins. Of these, 41 formed a highly connected protein-protein interaction network (Fig.5B). Lower diversity in the reticular adhesome supports evidence of relative homogeneity in both integrin clustering density (Fig.2D) and dynamics (Fig.3F). Gene ontology analysis revealed enrichment of terms relating to membrane organisation and endocytosis (Fig.5C-D), in contrast to the control condition that was enriched for terms related to cell adhesion and regulation of actin cytoskeleton (Supplementary Fig.4A-B). Ontology analysis was consistent with mass spectrometric identification of a number of known endocytic adaptors in RAs (Fig. 5B). Six candidates were validated by immunofluorescence, including NUMB, DAB2 (Fig.5E-F), EPS15L1, HIP1R, WASL and ITSN1 (Supplementary Fig.4C-F). Despite the observation that RAs did not associate with F-actin and formed following disruption of F-actin, a number of actin-binding proteins were identified in the reticular adhesome and two of these (tensin-3 and talin-2) colocalised with  $\beta 5$  in cytochalasin-treated cells (Fig. 5G-H). In contrast, the Arp2/3 complex component Arp3 did not localise with  $\beta 5$ -positive structures (Supplementary Fig.4G) and inhibition of Arp2/3 did not abrogate RAs, despite reducing FA intensity (Supplementary Fig. 5A-C).

### ***The balance between reticular and focal ACs is shaped by PIP status***

The putative RA protein interaction network contained many components reported to bind PI-4,5-P2 (Fig.5B; Supplementary Table 2). In five out of six cases where siRNA-mediated PIP regulator depletion would be expected to reduce PI-4,5-P2 levels (PI4KA, PI4K2A, PIP5K1B, PIP5K1C and PTEN), a shift in  $\beta 5$ -2GFP intensity ratio was observed from RAs to FAs (Fig.6A-C; Supplementary Fig 6A). Correspondingly, depletion of PIK3C2A, which generates phosphatidylinositol-3,4,5-trisphosphate (PIP3) from PI-4,5-P2, caused a relative shift from FAs to RAs. Depletion of targets that produce PI-4,5-P2 reduced  $\beta 5$ -2GFP levels in both AC types (Fig.6C), yet because effects were more pronounced for RAs, the ratio to FAs decreased. In contrast, PIK3C2A depletion perturbed only FAs. Neomycin (a PI-4,5-P2 binding inhibitor) reduced  $\beta 5$ -2GFP intensities in RAs while increasing intensities in FAs (Fig.6D; Supplementary Fig.6B). Conversely, LY294002 (a PIP3 formation inhibitor) increased RA and reduced FA intensities. These findings indicate that FAs and RAs are in an equilibrium, with PI-4,5-P2 promoting RAs and PIP3 promoting FAs.

### ***RAs persist throughout division when FAs disassemble***

siRNA-mediated knock-down of integrin  $\beta 5$  reduced cell proliferation (Fig.7A) without affecting S-phase progression (Fig.7B). We therefore probed a potential role for  $\beta 5$  in mitosis. Unlike classical ACs, RAs persisted throughout division (Fig.6C-I, Supplementary Movie 6), remaining free of consensus adhesome components (Supplementary Fig.7A-E). In virtually all cells on purified laminin or fibronectin, where integrin  $\beta 1$  is preferentially engaged (Supplementary Fig.7F,G), we detected no  $\beta 1$ -labeled ACs during mitosis.  $\beta 1$ -containing ACs were detected during mitosis only in normal human fibroblasts on fibronectin. In other cells, mitotic cells retained adhesion by cell-cell association. These results suggest a selective role for  $\alpha V\beta 5$  in mitotic cell attachment.

The pre-mitotic footprint of the mother cell is transmitted with high precision to post-mitotic daughter cells (Fig.7C,D)<sup>31,32</sup>. During the rounding phase of mitosis, this footprint was demarcated by membrane dye-labeled retraction fibres and integrin  $\beta 5$ -2GFP-labeled RAs (Fig.7C,F,G). The exquisite correspondence between retraction fibres (Fig.7H) and RAs (Fig.7I) was highlighted by 3D-visualisation of a similarly staged mitotic cell (Fig.7J-L; Supplementary Movie 7). Here, retraction fibres angled down and attached precisely at sites decorated with  $\beta 5$ -labeled RAs. A role for RAs in directing post-division cell spreading was also exemplified by live cell imaging (Supplementary Movie 8). Quantitative comparison of FA and RAs during division confirmed that

the number and intensity of vinculin-positive FAs fell to virtually zero during mitosis, while  $\beta 5$ -positive RA numbers and  $\beta 5$  intensity were maintained (Fig.7M,N). As previously reported, mitotic retraction fibres contain dense actin filaments (Supplementary Fig.7H,I, Supplementary Movie 9). We detected weak F-actin signals in RAs at the tips of mitotic retraction fibres (Supplementary Fig.7H-K), with F-actin concentrations well below those within the retraction fibres, suggesting that RAs have limited coupling to F-actin following mitotic cell rounding or that retraction fibres function via membrane tension and mediate adhesion independent of F-actin.

### ***RAs are required for division and inter-generational spatial memory-transmission***

Detailed comparison of RA distributions before, during and after mitosis (Fig.8A-C; Supplementary Movie 10) indicated that the overall geometry of central RAs remained virtually unchanged between generations, providing a potential mechanism for spatial memory storage. In contrast, peripheral RAs (generally associated with mitotic retraction fibres) underwent significant remodeling characterised by both narrowing and intensification of the complex (Fig.8D). Nanoscale STORM imaging confirmed that central mitotic RAs were indistinguishable in nano-organisation from interphase RAs (Fig.2X-Z *versus* Fig.8D-F), while peripheral mitotic retraction fibre-associated RAs were linearised and condensed. This was confirmed by quantification of nanocluster nearest neighbour distances and molecular localisation counts per nanocluster (Fig.8E,F). Such molecular-scale remodelling functionally implicates RAs in the mechanical process of cell-ECM attachment during division.

Many cells exhibit a preference to divide along the major axis of the pre-mitotic mother cell, thus determining the spatial arrangement of daughter cells<sup>8-11</sup>. We therefore measured the residual angle between the pre-mitotic major axis and the mitotic division axis in HeLa cells (Fig. 8G,H), chosen for their expression of RAs (Supplementary Fig.1B) and their extensive mitotic characterisation<sup>33</sup>. Residual angle distributions were skewed towards zero (indicating spatial memory retention) in control cells and  $\beta 5$ -rescued cells. By contrast, mitotic axis orientation in integrin- $\beta 5$ -depleted cells was almost random relative to the pre-mitotic major axis, indicating a loss of spatial memory. Thus, RAs are required for inter-generational spatial memory transmission during division.

Only 20% of  $\beta 5$ -depleted cells underwent normal division, *versus* 75% for controls (Fig.8I). A range of defects were observed in  $\beta 5$ -depleted cells, including delayed mitosis (often with incomplete cytokinesis), repeated cell rounding and re-spreading without division, and failure of cytokinesis resulting in bi-nucleate daughter cells (Supplementary Fig.8A,B; Supplementary Movies 11-14). The frequency of these errors<sup>34</sup> was reduced by  $\beta 5$ -EGFP rescue (Fig 8I; Supplementary Fig.8C,D; Supplementary Movie 15). Together, these findings demonstrate that integrin  $\beta 5$ -mediated RAs are essential for normal progression of division in HeLa cells.

## **Discussion**

Here, we report the identification and characterization of a previously unrecognized cellular structure, the reticular adhesion, an AC mediating cell-ECM attachment during mitosis. RAs form in a diverse array of cell types and are characterised by both the presence of integrin  $\alpha V\beta 5$  and the absence of consensus adhesome components. Furthermore, in contrast to FAs, RAs can form independently of F-actin and talin.

RAs persist throughout mitosis and provide a solution to the paradox of mitotic cell-ECM adhesion, which endures despite the absence of all previously known ACs<sup>13,15,18</sup>. Cell-ECM attachment is essential for spatial memory transmission between cell generations, including defining the axis of division<sup>8-11</sup> and facilitating cytokinesis<sup>35-37</sup>. To date, it has been unclear how residual adhesion is maintained during mitosis, how mitotic retraction fibers<sup>38</sup> are tethered to the substratum, and how re-spreading is guided thereafter. RAs now provide mechanisms underpinning all these phenomena.

The unique characteristics of RAs appear suited to these roles in division. For instance, F-actin independence decouples RAs from large-scale cytoskeletal remodeling during cell rounding, whilst the ability to interact with membrane retraction fibers is maintained. This key role for RAs in division is confirmed by integrin  $\beta 5$  depletion, which causes multiple mitotic defects and disturbed spatial memory transmission.

While we find an important role for  $\alpha V\beta 5$  during division, cells can also proliferate on ECM ligands not engaging  $\alpha V\beta 5$ . This implies that cells can deploy alternate adhesion receptors for mitotic anchorage. For example, integrin  $\alpha 6\beta 4$ -positive hemidesmosomes persist through mitosis<sup>39,40</sup> and, despite disassembly of precursor FA complexes and loss of consensus adhesome components, residual clusters of integrin  $\beta 1$  continue to decorate the cell-ECM interface in mitotic retinal pigment epithelial cells<sup>41</sup>. These  $\beta 1$  integrin clusters differ from RAs since they are remnants of disassembled FAs, while RAs represent a distinct AC population during both interphase and mitosis. Nonetheless, investigation of mitotic adhesion roles for alternative integrins, and their relationship to RAs, is now merited. The limited phenotype of  $\beta 5$  knockout mice suggests redundancy of function amongst adhesion receptors and/or a specialized role for  $\alpha V\beta 5$  in regulating division within specific ECM environments. Both  $\beta 5$  knockout<sup>42</sup> and overexpression<sup>43</sup> in mice cause deficiencies in osteoblast/osteoclast function, potentially reflecting mitotic defects related to differentiation errors<sup>12</sup> in cells on rigid, RGD-rich substrates. Indeed, these environments may be analogous to long-term cell culture conditions, where we show that cells preferentially utilize integrin  $\alpha V\beta 5$ . As  $\alpha V\beta 5$  is expressed at high levels in a number of proliferative diseases, this raises the possibility that it promotes disease progression by enhancing division within specific ECM environments. It is now important to determine the role of  $\alpha V\beta 5$  and RAs *in vivo*, within physiological and disease settings. In this context, a focal adhesion-independent role for  $\alpha V\beta 5$  in 3D skin formation and tumour invasion has been reported<sup>44</sup>.

Remarkably, RAs have remained uncharacterized, although early studies reported similar reticular  $\alpha V\beta 5$  labeling patterns in cells spread on VN<sup>45</sup>. The experimental induction of morphologically comparable structures, such as through manganese or talin-head mediated activation of  $\alpha V\beta 3$ <sup>28,29</sup>, suggests the potential for other integrins to form similar structures given modulation of their activity state. RAs lack not only F-actin, but virtually all consensus adhesome components. Most notably, both talin-1 and kindlin are absent, despite being considered necessary and ubiquitous integrin activators<sup>46</sup>. Moreover, perturbations of talin and F-actin indicate that RAs can form independently of these proteins.

Proteomic analysis of RAs identified a distinct adhesome, highly enriched in PI-4,5-P2-binding proteins. These include clathrin-mediated endocytosis adaptors, such as Dab2 and Numb, previously shown to interact directly with the integrin  $\beta 5$  cytoplasmic tail *in vitro*<sup>47</sup>. These data are consistent with recent evidence of integrin-mediated ECM attachment via clathrin-coated structures (CCSs)<sup>48,49</sup>. Indeed, integrin  $\beta 5$  can localize within clathrin plaques<sup>48,50-52</sup> that are postulated to associate with areas of strong adhesion<sup>34,53-56</sup>. It will be important to determine whether RAs associate with clathrin lattices to facilitate this adhesion and whether clathrin structures remain associated with the substratum during mitosis. Given that both RAs and CCSs can form in the absence of talin, it follows that some integrins may not depend on talin for their activation<sup>49</sup>. In this context, it is also notable that we observe near identical nanoscale integrin  $\beta 5$  clustering between talin-1-positive and -negative ACs during interphase, despite previous suggestions that talin-1 determines nanoscale integrin organisation<sup>6</sup>. Thus, both in terms of integrin activation and organisation, it is possible that either alternative proteins can replace talin-1 functions in RAs, or that  $\beta 5$  ligand-binding and nanoscale organisation are independent of cytosolic regulators. Regardless, the composition, regulation and function of integrin-mediated ACs appear more diverse than previously recognized.



In conclusion, we have defined RAs, an overlooked cellular structure and AC class. Functionally, by mediating cell-ECM attachment during mitosis, RAs provide a distinctive solution to the paradox of mitotic cell attachment, where classical ACs must disassemble but cells must also remain adherent. These discoveries not only delineate a specific form of AC, but also highlight areas of adhesion biology that merit further attention, including the integrins and ACs employed *in vivo*.

### **Author contribution statement**

JL, MCJ, JAA, ML, MJH and SS conceived the project and devised experiments. JL and JAA performed live cell imaging and related image analyses. JL, MCJ, JAA, XG and HO performed fixed cell imaging. XG performed the siRNA screening for PIP regulators. MCJ and JAA undertook experiments relating to integrin  $\beta 5$  RNAi and mass spectrometry analyses. AO performed STORM imaging and related image analyses. HO and SG performed immunoblotting. JL performed image analyses, statistical analyses and data visualisation. JL, MCJ, JAA, XG, ML, MJH and SS contributed to writing of the manuscript.

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## Figure Legends (for main figures)

### **Figure 1. Integrin $\alpha V\beta 5$ forms novel talin and vinculin negative reticular adhesion structures**

(A) Mass spec analysis of integrin subunits detected in adhesions isolated (after cell removal) from U2OS cells grown in complete medium for 3 d on tissue culture plastic. Results are mean spectral counts from  $n = 3$  biologically independent experiments, where thin horizontal lines indicate median values. P-values reflect comparison via two-sided unpaired t-testing between integrin subunits  $\alpha v$  or  $\beta 5$  and the next highest expressed subunit,  $\beta 1$ . (B) U2OS cells were plated on glass coverslips for 72 h. Confocal images of immuno-fluorescently labeled integrins  $\alpha V\beta 3$  (LM609),  $\beta 1$  (9EG7) and  $\alpha V\beta 5$  (15F11). (C-F) U2OS cells plated for 3 h in serum-free media on surfaces coated with 10  $\mu\text{g/ml}$  vitronectin (VN), except where otherwise specified. Confocal images of talin (C) or vinculin (D) immunofluorescence with that of integrin  $\beta 5$ . Boxed areas shown at higher magnification to the right. (E,F) Co-labeling of talin (E) and  $\beta 5$  (F) in cells grown on glass coated with 1, 3, or 10  $\mu\text{g/ml}$  VN. (G) Quantified intensities of talin-positive (blue) or –negative (red)  $\beta 5$  structures. Data from 81 cells ( $>23$  per condition) and  $n = 6132$  adhesions derived from 3 biologically independent experiments. Boxplot centre and box edges indicate median and 25<sup>th</sup> or 75<sup>th</sup> percentiles, respectively. Boxplot notches approximate 95% confidence intervals (see methods for details). P-values reflect two-sided unpaired Mann-Whitney U testing. (H) TIRF images of an mCherry-vinculin- and  $\beta 5$ -2GFP-expressing U2OS cell (U2OS- $\beta 5V$ ). Arrows in magnified boxes highlight regions lacking vinculin signal, which fall between  $\beta 5$  -positive, vinculin-negative puncta. (I) Confocal and interference reflection microscopy (IRM) images of a U2OS- $\beta 5V$  cell exemplify correlations between  $\beta 5$ -positive, vinculin-negative structures and regions with close cell-substrate proximity. All images representative of results from at least 3 biologically independent experiments. Scale bars: 10  $\mu\text{m}$ . Source data for panels A and G are available in Supplementary Table 1.

### **Figure 2. Comparison of focal and reticular adhesion morphometry and dynamics**

(A) Histogram of focal (blue) and reticular adhesions (red) by area (error bars = 95% confidence intervals (CI)). (B) Frequency of reticular adhesions by area, represented as fold-change relative to focal adhesions. (C) Percentage of adhesions located at distances from cell border (error bars = 95% CI). (D) Adhesion area *versus* mean integrin  $\beta 5$  intensity relative to smallest focal adhesions (error bars = 95% CI). (E) Representative image from live imaging of mCherry-vinculin and (F)  $\beta 5$ -2GFP (merged in G; **Supplementary Movie 1**). (H) Zoomed regions of E-G at time points indicated (**Supplementary Movie 2**). Scale bars: E-G (10  $\mu\text{m}$ ); H (1  $\mu\text{m}$ ). (I-M, **Supplementary Movie 3**). (I) Merged image of  $\beta 5$ -2GFP and mCherry-vinculin at representative time point. Trajectories of reticular (J) and focal adhesions (K) colour-coded by mean velocity (green = slow; red = fast). Trajectories of reticular (L) and focal adhesions (M) colour-coded by net adhesion motion angle. Line thicknesses indicate instantaneous adhesion velocity. (N) Aggregate analysis of all trajectories of average adhesion velocity *versus* corresponding average adhesion lifetime (dashed lines indicate adhesion class average lifetimes; error bars = 95% CI). Data in A-N derive from live imaging and analysis of 14 U2OS- $\beta 5V$  cells (in 4 biologically independent experiments) over 12 h (10 min intervals), providing  $n = 30,123$  focal adhesion and  $n = 91,898$  reticular adhesion observations. Source data for panels A-D and N are available in Supplementary Table 1.

### **Figure 3. Comparison of focal and reticular adhesion integrin dynamics and nanoscale structure**

(A) Integrin  $\beta 5$ -2GFP and mCherry-vinculin pre-bleach, post-bleach and post recovery (30 min; **Supplementary Movie 4**). Circles indicate focal and reticular adhesion bleach regions, supported by

single channel images (B-C). Scale bars: A-C (10  $\mu\text{m}$ ). (D) Square regions corresponding to circles in A-C. 5th column: Colour-scaled images (low to high values = black, red, orange, yellow, white) of intensity recovery for focal and reticular adhesions. (E) Aggregate FRAP recovery curves for  $n = 63$  focal and  $n = 68$  reticular adhesions (from 15 cells across 3 biologically independent experiments). Recovery curves are displayed as mean per timepoint (circles)  $\pm$  95% CI. Loess regression defines a smoothed fit (lines)  $\pm$  a moving 95% CI envelope. P-values indicated reflect comparison of Loess fitted curves assessed via two-sided unpaired Kolmogorov-Smirnov testing. (F) Post-bleach recovery time versus standard deviation of recovery at each time point. (G-I) Comparison of integrin  $\beta 5$  nanoclustering. (G)  $\beta 5$  immuno-labeling and mCherry-vinculin in a U2OS cell plated on VN and imaged via confocal microscopy. Representative focal (1 and 2) and reticular adhesions (3 and 4) cropped from matched confocal and stochastic optical reconstruction microscopy (STORM) images ( $\beta 5$  only, 'royal' look-up table intensity-scaled as in legend). Scale bars 2  $\mu\text{m}$  (500 nm in cropped images).  $\beta 5$  nanocluster nearest neighbour distances (H) and molecular localization counts per nanocluster (I) based on STORM data. 216 focal and 162 reticular adhesions were assessed, including  $n = 5530$  nanoclusters across 4 biologically independent experiments. Boxplot centre and box edges indicate median and 25<sup>th</sup> or 75<sup>th</sup> percentiles, respectively, while whiskers indicate the median  $\pm$  1.5\*IQR or the most extreme observations within these limits. Boxplot notches approximate 95% CIs. Source data for panels E-F and H-I are available in Supplementary Table 1.

**Figure 4. Reticular adhesions form in the absence of F-actin and talin**

(A-B) Confocal images of integrin  $\beta 5$ -2GFP and mCherry-vinculin in cells pre-treated in suspension and during spreading on VN with DMSO (A) or 20  $\mu\text{M}$  cytochalasin D (CytoD) (B) (**Supplementary Movie 5**). (C) Cell area *versus* reticular (red) or focal (blue) adhesion number following indicated treatments (means  $\pm$  95% confidence intervals, black lines = linear regression, 12 cells per condition,  $n = 7018$  focal and  $n = 4570$  reticular adhesions across 3 biologically independent experiments). (D) Quantification of immuno-labeling intensities for vinculin and  $\beta 5$  per adhesion in U2OS cells attached to VN and treated with DMSO (blue) or CytoD (red). CytoD significantly reduces vinculin intensities but increases  $\beta 5$  (p-values reflect two-sided unpaired Mann-Whitney U testing, 2533 adhesions from 22 DMSO-treated cells; 1410 adhesions from 18 CytoD-treated cells across  $n = 3$  biologically independent experiments). (E) Boxplots summarizing  $n = 6$  biologically independent attachment assays using CS1-wt (lacking  $\alpha V\beta 5$ ) and CS1- $\beta 5$  (expressing  $\alpha V\beta 5$ ) cells in the presence or absence of: 20  $\mu\text{M}$  CytoD; and/or non-inhibitory cyclic RAD peptides; and/or  $\alpha V\beta 5$  inhibitory cyclic RGD peptides. Cell attachment relative to maximum (= 100) CS1- $\beta 5$  + cRAD. Boxplot centre and box edges indicate median and quartiles while whiskers indicate median  $\pm$  1.5\*IQR or the most extreme observations within these limits. P-values reflect two-sided unpaired t-testing with Holm-Bonferroni correction for multiple tests. (F-G) Representative confocal images of mES talin-1  $-/-$  cells transfected with control (F) or talin-2-specific siRNAs (G) plated on VN and immuno-labeled against  $\beta 5$  and talin-2. (H-I) Single cell (as in F-G)-based quantification of residual talin expression *versus* (H) cell spread area or (I) mean  $\beta 5$  intensity in segmented adhesions standardized as fold change relative to each internal control, summarised across  $n = 6$  independent experiments. Linear regression p-values: correspondence between residual talin levels and cell area or  $\beta 5$  adhesion intensity. (J-K) Confocal images (representative of 3 biologically independent experiments) of U2OS cells expressing integrin  $\beta 5$ -2GFP or integrin  $\beta 5$ - $\beta 3$ -tail-2GFP plated on VN and immuno-labeled against vinculin. Scale bars: 10  $\mu\text{m}$ . Source data for panels C-E and H-I are available in Supplementary Table 1.

**Figure 5. Mass spectrometry reveals the distinct reticular adhesome**

(A) U2OS cells were plated in complete medium for 3 d on tissue culture plastic then treated with either DMSO (steady state) or CytoD (reticular enriched) and mass spec analysis was performed of the remaining adhesions after cell removal (n = 3 biologically independent experiments). Venn diagrams summarise overlap between proteins identified by mass spec analysis of ventral membrane preparations isolated from each condition overlaid with the 60 consensus fibronectin-adhesome proteins<sup>5</sup>. (B) STRING interaction network of reticular adhesion enriched proteins with interaction confidence as indicated. PI4,5P2 binding (direct, green; indirect, yellow; absent, grey) as indicated (C,D) Gene-ontology analysis of reticular adhesion enriched proteins (CytoD-treated) showing terms from Biological Process (C) and KEGG pathway analysis (D) significantly enriched over whole cell proteome. P-values were derived from EASE scores calculated using a modified Fishers Exact test with Holm-Bonferroni correction from multiple tests using the DAVID annotation system. (E-H) Confocal images of U2OS cells cultured on glass coverslips for 72 h then treated with 20  $\mu$ M CytoD for 2 h and immuno-labeled against integrin  $\beta$ 5 and (E) NUMB; (F) DAB2; or in cells transfected with (G) EGFP-tensin3; or (F) EGFP-talin2; along with staining of F-actin. Images in E-H representative of 3 biologically independent experiments. Scale bars: 10  $\mu$ m, except in zoomed regions cropped in E-F, 5  $\mu$ m.

**Figure 6. Reticular versus focal adhesion balance is shaped by PIP status**

(A) Representative images illustrating DAPI (nuclei) and F-actin staining as well as localisation of mCherry-vinculin and integrin  $\beta$ 5-2GFP fluorescence following treatment with control siRNA or siRNAs targeting PI4KA or PIK3C2. Scale bars 50  $\mu$ m. (B) Boxplots summarizing single cell quantification (from images as shown in A; n = 3917 cells analysed, averaging 280 +/- 131 (SD) per condition) of integrin  $\beta$ 5 intensity ratios (represented as Z-scores) between reticular and focal adhesions following knockdown of various PIP2 and PIP3 regulators. Data derive from 2 biologically independent experiments. Boxplot centre and box edges indicate median and 25<sup>th</sup> or 75<sup>th</sup> percentiles, respectively, while whiskers indicate the median +/- 1.5\*IQR (inter-quartile range) or the most extreme observations within these limits. Boxplot notches approximate 95% confidence intervals (see methods for details). P-values reflect two-sided unpaired Mann Whitney U testing with Holm-Bonferroni correction from multiple tests. (C) Parallel coordinates plot displaying (as Z-scores) mean focal and reticular adhesion integrin  $\beta$ 5 intensities, as well as the ratio of reticular *versus* focal adhesion intensities, following knockdown of PIP2 and PIP3 regulators, based on n = 3917 cells analysed, averaging 280 +/- 131 (SD) per condition. Data derive from 2 biologically independent experiments. (D) Boxplots summarizing single cell quantification (from images as shown in **Supplementary Figure 6B**; n = 3018 cells analysed, averaging 1006 +/- 307 (SD) per condition) of mean focal and reticular adhesion integrin  $\beta$ 5 intensity and ratio following 30 min treatment with 10 mM Neomycin (PIP2 binding inhibition) or 25  $\mu$ M LY294002 (inhibition of PIP3 generation). Boxplot features as detailed above. P-values reflect two-sided unpaired Mann Whitney U testing with Holm-Bonferroni correction from multiple tests. Data in D derived from 3 biologically independent experiments. Source data for panels B-D are available in Supplementary Table 1.

**Figure 7. Reticular adhesions persist during mitosis and transmit spatial memory from pre-mitotic to post-mitotic daughter cells**

(A-B) Proliferation of control or integrin  $\beta$ 5 knockdown U2OS cells over 3 d post attachment. A: mean +/- SD of n = 14 replicates across 3 biologically independent experiments; p-values: two-sided unpaired t-testing relative to day zero); B: percentage of EdU-positive cells 3 d post attachment; n = 26 fields of view containing 55-217 cells each across 3 biologically independent experiments,



distribution of individual values in blue rings). Boxplot centre and box edges indicate median and 25<sup>th</sup> or 75<sup>th</sup> percentiles, respectively, whiskers indicate median +/- 1.5\*IQR (inter-quartile range) or the most extreme observations within these limits. Boxplot notches approximate 95% CI. P-values: two-sided unpaired t-testing for Control vs  $\beta$ 5-KD. U2OS cells labeled with far-red membrane dye (C) and expressing mCherry-vinculin (E) and integrin  $\beta$ 5-2GFP (F), replated on vitronectin and imaged every 10 min via spinning-disc confocal microscopy during mitosis (see **Supplementary Movie 6**). Images show a cell 120 min before, during and 120 min after mitosis. (D) An overlay of membrane labeling with cell boundaries outlined at -120 min = red, 0 min = green, +120 min = blue) highlights recovery of the pre-mitotic adhesion footprint by daughter cells. Membranous retraction filaments formed during mitosis (H; cropped from blue ROI in C) overlap exactly with integrin  $\beta$ 5-2GFP-positive adhesion complexes (I; cropped from yellow ROI in F). Scale bars C-F = 10  $\mu$ m, H and I = 5  $\mu$ m. (J-K; see **Supplementary Movie 7**) Three alternate views (above (J), beside (K) and below (L); orientation indicated by arrows in planar schematics) of a 3D confocal-reconstructed mitotic cell showing condensed DNA (white), cell membrane labelling (red; cut through to expose DNA) and integrin  $\beta$ 5-2GFP labelling of RAs (green). Images in C-L representative of 5 biologically independent experiments. (M-N) Quantification of vinculin-positive adhesion complex (AC) number (M, blue) and intensity (N, blue) vs  $\beta$ 5-2GFP-positive adhesion complex number (M, red) and intensity (N, red) during mitosis. Mean values from n = 5 cells shown +/- standard deviation, derived from 3 biologically independent experiments. Scale bars: 10  $\mu$ m except in H; 5  $\mu$ m. Source data for panels A, B, M and N are available in Supplementary Table 1.

### **Figure 8. Requirement of reticular adhesions for mitosis and post-mitotic re-spreading**

(A-C) Confocal images of integrin  $\beta$ 5-2GFP adhesions at three time points relative to mitosis (-50 min (pre); 0 min, +30 min (post)). (B) Overlay of pre- and post-mitosis adhesions, cropped and zoomed in C, confirm the persistence of reticular adhesions throughout mitosis (see **Supplementary Movie 9**). Images in A-C representative of at least n = 5 biologically independent experiments. (D, left) Integrin  $\beta$ 5 in a representative U2OS mitotic cell plated on VN and imaged via conventional TIRF microscopy. (D, right). Representative central (Non-retraction; orange box) and peripheral (Retraction; green box) reticular adhesions cropped from matched conventional and stochastic optical reconstruction microscopy (STORM, 'royal' look-up table intensity-scaled as in legend) images (right). (E,F) Quantification of integrin  $\beta$ 5 nanocluster nearest neighbor distances (E) and molecular localization counts per nanocluster (F) based on STORM data. In total, 95 retraction and 83 non-retraction mitotic reticular adhesions were quantified, including n = 3512 nanoclusters across 2 biologically independent experiments. Boxplot centre and box edges indicate median and 25<sup>th</sup> or 75<sup>th</sup> percentiles, respectively, while whiskers indicate the median +/- 1.5\*IQR (inter-quartile range) or the most extreme observations within these limits. Boxplot notches approximate 95% confidence intervals. Scale bars: (A-D, left) 10  $\mu$ m; (D, right) 500 nm. (G-I) Based on **Supplementary Figure 8** and **Supplementary Movies 10-14**. Comparison between control siRNA (Control; n = 297 cells) and integrin  $\beta$ 5 knock down ( $\beta$ 5 KD; n = 176 cells) or post-knockdown  $\beta$ 5 rescue (Rescue; n = 195 cells) effects on spatial memory transmission between HeLa cell generations, defined by residual angle measurement between the pre-mitotic cell major axis and the cell division axis (data derived from 2 biologically independent experiments). Box-plots (G; blue rings indicate individual cell measurements) and probability density plots (H) indicate the distribution of residual angles. Boxplot centre and box edges indicate median and 25<sup>th</sup> or 75<sup>th</sup> percentiles, respectively, while whiskers indicate the median +/- 1.5\*IQR (inter-quartile range) or the most extreme observations within these limits. Boxplot notches approximate 95% confidence intervals. P-values reflect two-sided unpaired Mann-Whitney U testing. (I) Plots showing the percentage of rounding cells that progressed through

normal cell division in each of  $n = 3$  biologically independent experiments. P-values reflect two-sided unpaired t-testing. Source data for panels E-I are available in Supplementary Table 1.

## Methods

### *Cell culture, plasmid generation, transfection and stable cell generation*

*Cell Culture:* U2OS human osteosarcoma cells (ATCC), HeLa human cervical carcinoma cells (ECACC), MCF-7 human breast carcinoma cells (ATCC), A549 human lung carcinoma cells (ECACC) and A375 human melanoma cells (ECACC) were maintained in DMEM (Gibco) supplemented with 10% FBS (Sigma) and 2mM L-glutamine (Gibco). U2OS- $\beta$ 5V cells stably expressing integrin  $\beta$ 5-2GFP and mCherry-vinculin were maintained with the addition of 600  $\mu$ g/ml Geneticin (G-418 sulphate; Gibco). H1299 human non-small lung cancer cells (gift from Benny Geiger, The Weizmann Institute of Science, Rehovot, Israel) and CS-1 wild-type hamster melanoma cells were cultured in RPMI-1,640 (Gibco) medium supplemented with 10% FBS and 5 mg/ml L-glutamine. CS-1 cells stably expressing integrin  $\beta$ 5 (CS1- $\beta$ 5) were maintained with the addition of 500  $\mu$ g/ml G-418. BT549 (ductal breast carcinoma, ATCC) cells were maintained in RPMI 1640 medium containing 10% FBS and 1 mM L-glutamine. Mouse aortic endothelial (MAE) cells (ATCC) were grown in RPMI 1640 medium with 5% FBS. Human hTERT immortalized retinal pigment epithelial ((hTERT-RPE1) cells (kind gift from Jorg Mansfeld, University of Dresden, Germany) were cultured in DMEM/F12 (GIBCO) supplemented with 10% FBS and 2mM L-glutamine. Normal human dermal fibroblasts (NHDF) (Biowhittaker) were grown in DMEM (Gibco) supplemented with 10% FBS (Sigma) and hTERT-human microvascular endothelial cells (HME1) (ATCC) were grown in MEGM (Lonza) supplemented with MEGM BulletKit (Lonza). All live cells were incubated and imaged in a humidified environment at 37°C with 5% CO<sub>2</sub>.

*DNA plasmid generation and sourcing:* For construction of integrin  $\beta$ 5-2GFP, EGFP was duplicated in a pEGFP-N1 backbone vector (gift of Dr Pat Caswell, University of Manchester, UK), then a full-length integrin  $\beta$ 5 cDNA (gift of Dr Errki Ruoslahti, Burnham Institute) was subcloned into the 2XEGFP-N1 vector using the EcoRI site of the original pEGFP-N1 vector. The mCherry-vinculin plasmid was kindly provided by Dr Vic Small (IMBA, Austria). Csk-GFP was kindly provided by Dr Akira Imamoto (University of Chicago, USA). GFP-Tensin3 was kindly provided by Dr Pat Caswell (University of Manchester, UK) and GFP-talin2 was kindly provided by Dr Ben Goult (University of Kent, UK). RFP-talin1 Head and Rod constructs were kindly provided by Professor Maddy Parsons (King's College, UK). LifeAct-RubyRed was kindly provided by Roland Wedlich-Soldner (Max-Planck Institute for Biochemistry, Germany).

*Transfection and stable cell line generation:* Cells were transfected at 70 – 90% confluence, 24 h after plating into 12 well culture plates (except where otherwise stated). For DNA plasmid transfection, 0.3-2  $\mu$ g of total DNA was mixed with 0.5-3  $\mu$ l of Lipofectamine Plus or Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. For RNA transfection, except where otherwise stated, 15-30 pmol of siRNA was transfected together with 0.5-3  $\mu$ l of RNAiMAX (Thermo Fisher Scientific). Cells were typically imaged 24 to 48 h after transfection. U2OS- $\beta$ 5V cells expressing integrin  $\beta$ 5-2GFP and mCherry-vinculin were established via manual single colony selection followed by selection with 600  $\mu$ g/ml G-418.

*ECM surface coating:* Cells were typically assayed in 96-well glass-bottomed plates (0.17 mm optical glass; Matrical Bioscience). Glass coating was performed at 37°C for 2 h after blocking with 1% heat-denatured bovine serum albumin (BSA; Sigma-Aldrich) for 2 h at 37°C. ECM ligand coating concentrations were 10  $\mu$ g/ml except where otherwise indicated.

Vitronectin and fibronectin were purified from human plasma as detailed previously<sup>57,58</sup>, while purified laminin was acquired commercially (Sigma-Aldrich).

### ***Antibodies, immunofluorescence labelling and immuno-blotting***

Primary antibodies used for immunofluorescence and/or immuno-blotting are summarized in Supplementary Table 4 and include: anti-integrin  $\alpha$ V $\beta$ 5 (15F11; MAB2019Z) (Millipore); anti-integrin  $\alpha$ V $\beta$ 5 (P1F6) (Abcam); polyclonal (rabbit) anti-integrin  $\beta$ 5 (ab15459) (Abcam); anti-integrin  $\beta$ 5 (4708S) (Cell Signalling Technology); anti-integrin  $\alpha$ V (LM142) (Merck Millipore); anti-talin2(53.8) (BioRad); anti-talin (8d4) (Sigma Aldrich); anti-talin 1 (TA205) (Santa Cruz); anti-talin 2 (68E7) (Abcam); anti-integrin  $\alpha$ V $\beta$ 3 (LM609) (Abcam); anti-integrin  $\beta$ 3 (AP3) (Abcam); anti-integrin  $\beta$ 1 (LM534) (Millipore); anti-vinculin (hVIN-1) (Sigma Aldrich); anti-vinculin (V9131) (Sigma-Aldrich); anti-intersectin 1 (HPA018007) (Atlas Antibodies, Sigma-Aldrich); anti-NUMB (2733) (Cell Signaling Technologies); anti-EPS15L1 (HPA055309) (Atlas Antibodies, Sigma-Aldrich); anti-HIP1 (HPA013606) (Atlas Antibodies, Sigma-Aldrich); anti-WASL (HPA005750) (Atlas Antibodies, Sigma-Aldrich); anti-DAB2 (12906) (Cell Signaling Technologies); anti-paxillin (5H11) (Sigma Aldrich); anti-FAK (BD Biosciences); anti-zyxin (H-200) (Santa Cruz); anti-kindlin 2 (ab74030) (Abcam); anti-ICAP1 (115228) (Abcam); anti-DOK1 (HPA048561) (Atlas Antibodies, Sigma-Aldrich); polyclonal (rabbit) anti-phosphotyrosine (1000) (Cell Signaling); anti-cytokeratin (27988) (Abcam); anti-alpha tubulin (DM1A) (Thermo Fisher Scientific); anti-vimentin (8978) (Abcam); anti-ARP3 (ab49671) (abcam). Anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa 488, 568 or 647 were used as appropriate (Thermo Fisher Scientific). For fixed F-actin labelling, phalloidin pre-conjugated with Alexa 488, 568 or 647 was used as appropriate (Thermo Fisher Scientific). DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride; Thermo Fisher Scientific) nucleic acid stain was used as a nuclear marker as appropriate.

Immunofluorescence labeling was performed either manually or using liquid-handling robotics (Freedom EVO, Tecan) to minimise experimental variability, as described previously<sup>59</sup>. In either case, standardised procedures were used except where otherwise stated. Cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 20 min, washed 3x with PBS and permeabilised using 0.1% TX-100 (Sigma-Aldrich) for 5 min at room temperature. Cells were then blocked for 15 min with 1% BSA in phosphate-buffered saline (PBS) (PBS/BSA). Primary antibody immuno-labelling then proceeded at room temperature for 30 min. After PBS/BSA washing, secondary antibodies conjugated with either Alexa 488, 568 or 647 fluorophores were applied for 30 min at room temperature. Finally, cells were washed 3x with PBS.

Immuno-blotting was performed on SDS-polacrylamide gels with proteins transferred to Immobilon-P-Membranes (Millipore). Membranes were probed with anti-talin 2 mouse monoclonal (68E7) (Abcam) at 1:500 dilution, anti-alpha tubulin (DM1A) (Thermo Fisher Scientific) at 1:500 dilution, or anti-integrin  $\beta$ 5 (4708S) (Cell Signalling Technology) at 1:1000 dilution. Proteins were detected using the enhanced chemiluminescence reagent (ECL, Amersham Pharmacia Biotech).

### ***Imaging***

Live and fixed cell imaging was primarily performed using either a Nikon Ti2-mounted A1R confocal microscope running NIS elements software (Nikon) with a PlanApo VC 60X / 1.4

NA oil-immersion objective or a Leica TCS SP5 Acousto-Optical Beam Splitter confocal microscope using a 63X objective (HCX Plan Achromat, NA 1.25) and LCS software (Leica). Live fluorescence imaging during cell division employed a Yokogawa CSU-X1 spinning-disk confocal and Andor EM-CCD. TIRF imaging employed a Nikon Ti2 inverted microscope configured for minimal (~90 nm) evanescent wave penetration. Live cell imaging intervals were 0.5 - 5 min over for 1 - 8 h, with pixel resolutions between 0.13 - 0.21  $\mu\text{m}$ . Live cells were maintained in normal culture medium, absent FCS / FBS, at 37°C and 5% CO<sub>2</sub>. Live cell interference reflection microscopy (IRM) employed a Zeiss LSM 510 confocal microscope and Plan-Apochromat 63X / 1.4 NA oil objective, with post-sample dichroic mirror displacement allowing reflected laser light (561 nm) detection.

Fluorescence recovery after photobleaching (FRAP) analyses were performed via confocal and analysed as described previously<sup>60</sup>. Briefly, three sequential images were acquired of integrin  $\beta$ 5-2GFP and mCherry-vinculin in U2OS- $\beta$ 5V cells prior to bleaching, enabling robust recovery standardization. Both reticular and focal adhesions (2-3 each per cell) were then bleached using 35% of maximal 488 nm laser power over 40 rapid iterations (< 3 s per cell). Recovery was monitored for a total of 1875 seconds, with intervals of 6 s for the first 120 s and intervals of 45 s thereafter.

Stochastic optical reconstruction microscopy (STORM) was performed in U2OS cells fixed during either interphase or mitosis. Cells were labeled using rabbit polyclonal anti-integrin  $\beta$ 5 antibody (ab15459) and with Alexa 405-Alexa 647 double labeled secondary. Secondary antibodies (Jackson ImmunoResearch) were labelled in-house, as previously described<sup>61</sup>. A Nikon N-STORM system with Apo internal reflection fluorescence 100X / 1.49 NA objective was used, with images acquired via EM-CCD camera. Prior to STORM imaging, TIRF images of integrin  $\beta$ 5 and mCherry-vinculin were acquired, enabling diffraction-limited definition of reticular and focal adhesions using the criteria detailed under Image Analysis. Thereafter, 647 nm laser light excited Alexa 647, with 405 nm light used for reactivation. Standard STORM imaging buffer was used, containing 100 mM Cysteamine MEA, 0.5 mg/ml glucose oxidase, 40  $\mu\text{g}/\text{ml}$  catalase, and 5% Glucose (all Sigma Aldrich).

### ***Image Analysis***

Patch Morphology Analysis Dynamic software (Digital Cell Imaging Laboratories, Belgium) was used for analysis of static (fixed) and dynamic (live) cell imaging data, except where otherwise specified. Analysis strategy and parameterisation are as described previously<sup>20,21,59,62</sup>. Briefly, both cells and intracellular adhesion cohorts were segmented according to pixel intensity gradient analysis. A variety of morphological, pixel intensity and dynamic properties were then extracted for each cell and for each adhesion<sup>21</sup>. Relationships between each adhesion and its (parent) cell were maintained. Minimal adhesion size was set to 0.3  $\mu\text{m}^2$ . For live cell data, adhesion tracking parameters included: linear motion interpolation over maximum 1 missing time point; 3  $\mu\text{m}$  maximum adhesion step-size per time point; 4 time point minimum track lifetime. When quantifying differences between reticular and focal adhesions, we used the absence or presence (respectively) of canonical adhesome components as a defining indicator. Specifically, we applied a threshold such that segmented adhesions (delineated by integrin  $\beta$ 5) were defined as reticular if they contained less than the mean of background fluorescence values (pixel intensities inside the cell boundary but outside segmented adhesions) plus two standard deviations for a canonical adhesion marker (vinculin or talin). Integrin  $\beta$ 5-positive adhesions with greater than this value of fluorescence (for the canonical adhesion marker) were classed as focal adhesions.

For FRAP analyses, PAD software was used to segment integrin  $\beta$ 5-2GFP-positive adhesions found in the last (3<sup>rd</sup>) pre-bleach image frame. Focal and reticular adhesions were distinguished based on mCherry-vinculin content, as described above. Identical adhesion boundaries (from pre-bleach frame 3) were then used as fluorescence recovery measurement locations for all subsequent image frames. Adhesions adjudged to move during this period were excluded from further analysis. Integrin  $\beta$ 5-2GFP fluorescence recovery curves were first standardised relative to intensity fluctuations (including non-specific photo-bleaching) in non-bleached areas of the cell. Thereafter, intensity values in bleached regions were standardised per adhesion as a percentage of the mean of the three pre-bleached images. The standard deviation of percentage recovery, per time point, was also recorded. Recovery curves are displayed as mean per timepoint (circles)  $\pm$  95% confidence intervals (per timepoint). Loess regression defines a smoothed fit (line)  $\pm$  a moving 95% confidence interval envelope. Statistical differences between Loess fitted curves were assessed via two-sided Kolmogorov-Smirnov testing.

STORM data were analysed using Insight3 software (developed by Bo Huang, University of California, San Francisco). First, localisation coordinates were precisely defined via Gaussian fitting. Next, reticular and focal adhesions were segmented and defined using conventional TIRF images of integrin  $\beta$ 5 and vinculin, based on the thresholding criteria detailed above. Clustering was then performed on integrin  $\beta$ 5 localisations within each adhesion type, revealing coordinate position and localisation counts for integrin nanoclusters found within each adhesion. DBSCAN was used for clustering<sup>63</sup>, with epsilon (search radius) set to 10 nm and minimum points (within epsilon radius) set to 3. Nearest neighbour distances between nanoclusters and localisation numbers per cluster were assessed for each adhesion type using R.

Three-dimensional rendering and animation of confocal images was performed using NIS elements software. Additional supplementary movies were prepared in FiJi software<sup>64</sup>.

### ***Mass Spectrometry analysis of the Reticular Adhesome***

Four 10 cm-diameter dishes per condition of U2OS cells were cultured for 48 h to 90% confluency then treated with either DMSO or 20  $\mu$ M Cytochalasin D (Sigma-Aldrich) for 2 hours. To isolate adhesion complexes, cells were incubated with the membrane permeable cross-linker dimethyl-3, 3'-dithiobispropionimidate (DTBP, Sigma Aldrich; 6 mM, 5 min). DTBP was then quenched using 1 M Tris (pH 8.5, 2 min), after which cells were again washed once using PBS and incubated in PBS at 4°C. Cell bodies were then removed by a combination of cell lysis in RIPA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% (w/v) TX-100, 1% (w/v) sodium deoxycholate (DOC), 0.5% (w/v) sodium dodecylsulfate (SDS); 3 min) and a high-pressure water wash (10 s). Protein complexes left bound to the tissue culture dish were washed twice using PBS, recovered by scraping in 200  $\mu$ l recovery solution (125 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 15mM DTT), and incubated at 70°C for 10 min. Each sample was subsequently precipitated from solution by addition of four volumes -20°C acetone, incubated for 16 h at -80°C, and resuspended in reducing sample buffer.

For mass spectrometric, samples were separated by SDS-PAGE on a 4-12% SDS Bis-Tris gel (Thermo Fisher), stained for 10 min with Instant Blue (Expedeon), and washed in water overnight at 4°C. Gel pieces were excised and processed by in-gel tryptic digestion as previously described<sup>4</sup>. Peptides were analysed by liquid chromatography (LC)-tandem MS using an UltiMate 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA,

USA) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher). Peptides were separated on a bridged ethyl hybrid C18 analytical column (250 mm x 75  $\mu$ m I.D., 1.7  $\mu$ m particle size, Waters) over a 1 h gradient from 8% to 33% (v/v) ACN in 0.1% (v/v) FA. LC-MS/MS analyses were operated in data-dependent mode to automatically select peptides for fragmentation by collision-induced dissociation (CID). Quantification was performed using Progenesis LC-MS software (Progenesis Q1, Nonlinear Dynamics, Newcastle, UK; <http://www.nonlinear.com/progenesis/q1-for-proteomics/>). In brief, automatic alignment was used, and the resulting aggregate spectrum filtered to include +1, +2, and +3 charge states only. An .mgf file representing the aggregate spectrum was exported and searched using Mascot (1 missed cleavage, fixed modification: carbamidomethyl [C]; variable modifications: oxidation [M]; peptide tolerance:  $\pm$  5 ppm; MS/MS tolerance:  $\pm$  0.5 Da), and the resulting .xml file was re-imported to assign peptides to features. Three separate experiments were performed and abundance values for proteins identified in analysis were used to determine which proteins were enriched over 2-fold following treatment with Cytochalasin D. While 53 proteins were detected in original mass spectrometry data, 4 were excluded in further analysis due to high representation in the CRAPome database<sup>30</sup>. The putative reticular adhesome interaction network was constructed using the online STRING protein-protein interaction database (v10)<sup>65</sup> including experimentally validated interactions only, with a 'medium' interaction confidence score ( $>$  0.4). Even at higher confidence (interaction confidence score  $>$  0.7), this interaction network is dense: 91 known interactions relative to 11 randomly expected (based on proteome-wide interaction frequencies). Biological process- and KEGG pathway-enrichment analyses were performed using the DAVID Bioinformatics resource<sup>66</sup>.

### ***PIP regulator siRNA screening and drug-based perturbation of PI4,5P2 and Arp2/3***

U2OS- $\beta$ 5V cells were treated with pooled siRNAs (4 siRNAs per target; ON-TARGET SMART Pool plus; Dharmacon) via reverse transfection in the inner 60 wells of 96-well optical glass plates. Each plate contained 5 negative (untreated; mock transfected; 3 non-targeting siRNA controls) and 3 positive targeting controls (against EGFP, integrin  $\alpha$ v or integrin  $\beta$ 5). The primary screen was repeated twice, with a secondary validation assay using 4 siRNAs individually, per target (Dharmacon) also repeated twice. siRNA sequences are displayed in Supplementary Table 5. To prepare the siRNA library, 1  $\mu$ l of each siRNA pool from 2  $\mu$ M stock was mixed with 30  $\mu$ l nuclease-free water and added to 96-well glass-bottom plate wells, before drying at RT. For reverse transfection, 30  $\mu$ l of RNAiMAX was first added to 9 ml of Opti-MEM (Thermo Fisher Scientific). 30  $\mu$ l of this mixture was added to each well, followed by a 30 min incubation. 90% confluent U2OS- $\beta$ 5V cells grown in 75 cm<sup>2</sup> flasks were trypsinized and resuspended with 30 ml of growth medium. 100  $\mu$ l of the resulting cell suspension was added to each well and pipetted 5 times to disperse cells. Final siRNA concentration was 15 nM. Cells were incubated for 48 h before fixation with 4% PFA (15 min) and subsequent permeabilization with 0.2% TX-100 in PBS. Finally, cells were incubated for 1 h with DAPI and Alexa 647-conjugated phalloidin before 3x PBS washing.

Cells were imaged with a Nikon A1R confocal microscope with PlanApo VC 60X / 1.4 NA oil-immersion objective. Image settings were identical for all samples and repeats. Montage images were acquired and stitched in NIS-elements software, enabling high-resolution acquisition of  $\sim$ 100 cells and  $\sim$ 5000 adhesions per condition, per experimental repeat. Image data were quantified and analysed using KNIME software. Individual cells were segmented using Voronoi tessellation based on DAPI (nuclei) and phalloidin (cell body) staining. Integrin  $\beta$ 5-positive adhesions were then segmented and split using spot detection and the

Wählby method<sup>67</sup>, respectively. Focal and reticular adhesions were defined based on mCherry-vinculin content as described above. Background-corrected intensity values were extracted per channel, for each adhesion, per cell. Mean integrin  $\beta$ 5 intensity values in reticular adhesions were divided by values in focal adhesions, to generate the relative intensity ratio. All values were Z-score standardized using robust statistics (median and median absolute deviation) relative to the combination of (3) non-targeting siRNA controls per 96-well plate. Resulting response distributions were plotted using R and RStudio software.

U2OS- $\beta$ 5V cells cultured and plated as described above, including 48 h incubation in 96-well optical glass plates, were treated for 30 min with either: DMSO (control); 10 mM Neomycin (PIP2 binding inhibition) or 25  $\mu$ M LY294002 (inhibition of PIP3 generation). For treatment with Arp2/3 inhibitor, U2OS cells plated onto glass coverslips and cultured for 48 hours were treated for 2 hours with 50  $\mu$ M CK-666 (Arp2/3 inhibitor), or 50  $\mu$ M CK-689 (Arp2/3 inhibitor control; inactive analogue of CK-666). Cells were then fixed, permeabilised and labeled as described above. Imaging and analysis was again performed using KNIME, as described above for siRNA screening.

### ***Talin knock-down and response analysis***

Talin 1-null mouse embryonic stem cells (mES talin 1 -/-; kind gift of David Critchley, University of Leicester) were transfected using RNAiMAX (Thermo Fisher Scientific) as per the manufacturer's instructions using either non-targeting control siRNA (ON-TARGETplus Non-targeting Control; 5'-UGGUUUACAUGUCGACUAA-3') or talin 2-specific siRNAs designated: talin-2 siRNA1 (5'-GCAGAAUGCUAUUAAGAAAUU-3'); talin-2 siRNA2 (5'-CCGCAAAGCUCUUGGCUGAUU-3'), or; talin-2 siRNA3 (5'-AAGUCAGUAUUACGUUGUUUU-3'). siRNAs were synthesized by GenePharma (Shanghai, China). Cells were incubated for 48 h then plated for 3 h on 10  $\mu$ g/ml vitronectin. Fixation and permeabilisation conditions were tuned to retain cytoplasmic talin 2, as described previously<sup>59</sup>. Briefly, labelling was performed using liquid-handling robotics (Freedom EVO, Tecan) to reduce experimental variability. Cells were fixed with 2% PFA for 10 min, washed with PBS and permeabilised using 0.1% TX-100 for 5 min at room temperature. Cells were then blocked for 15 min with 1% PBS/BSA. Immuno-labeling followed at room temperature for 30 min, targeting integrin  $\beta$ 5 (polyclonal Ab; ab15459, Abcam) and talin (pan-talin mouse monoclonal Ab '53.8', BioRad or; anti-talin 2 mouse monoclonal Ab '68E7', Abcam). After 1% PBS/BSA washing, Alexa 488 and 647 secondary antibodies were applied, targeting rabbit and mouse primary antibodies, respectively. Images of integrin  $\beta$ 5 and residual talin 2 were acquired with a Nikon A1R confocal and oil-immersion objective (PlanApo VC 60X / 1.4 NA). Image analysis was performed using PAD software to record residual talin (mean) intensities per cell, mean  $\beta$ 5 intensities per segmented adhesion (per cell), and cell area. Values were scaled as fold-change relative to control siRNA. 20-40 cells were imaged per condition in each of 4 experimental repeats with talin-2 siRNA1, or single confirmatory experiments with talin-2 siRNA2 and 3. Immunoblotting was performed as described above.

### ***Integrin $\beta$ 5 knock-down and mitotic analysis***

siRNA used for knockdown of  $\beta$ 5 targeted the sequence 5'-GGGAUGAGGUGAUCACAUG-3' and was obtained from Dharmacon. For rescue of  $\beta$ 5 expression, an siRNA-resistant WT  $\beta$ 5-EGFP clone was generated using the QuickChange



IIXL site-directed mutagenesis kit (Agilent Technologies) to introduce silent mutations in the siRNA target sequence. The primers were: forward 5'-AGCCTATGCAGGGACGAAGTTATTACCTGGGTGGACACC-3' and reverse 5'-GGTGTCCACCCAGGTAATAACTTCGTCCTGCATAGGCT-3' (Obtained from Eurofins Genomics).

Cells were transfected simultaneously with either non-targeting or  $\beta 5$  siRNA together with either EGFP alone (pEGFP-N1 empty vector; gift of Pat Caswell, University of Manchester) or WT  $\beta 5$ -GFP using Lipofectamine 2000 (ThermoFisher) according to the manufacturer's instructions. Six hours after transfection, cell cycle synchronisation was initiated by addition of 2mM thymidine (Sigma). After 18 h, cells were released by replating in fresh medium, and a second dose of thymidine added 8 h later. Medium was replaced the next morning and imaging started 5 h after the second release.

Images were acquired on an ASMDW live cell imaging system (Leica) equipped with a Cascade II EM CCD camera (*Photometrics*) and a 20x/ 0.50 Plan Fluotar air objective. Images were collected every 10 min using Image Pro 6.3 software (Media Cybernetics Ltd) and processed using ImageJ.

*Mitotic alignment analysis:* Prior to analysis of mitotic alignment, image files were computationally blinded by randomised file name encoding. Thereafter, Fiji software was used to measure the angular difference between the long axis of the mother cell prior to cell division, and the axis of cytokinesis. All observed cell division events were analysed. Where multiple attempts at cytokinesis were observed, the orientation of the first attempt was used for angular measurement. Data were summarised using R software.

### ***Adhesion Assay***

Cell adhesion assays were performed as described previously<sup>68</sup>. Briefly, non-tissue culture-treated, polystyrene 48-well cluster plates (Corning Costar Corporation) were coated with 10  $\mu$ g/ml vitronectin as detailed above, and blocked with 1% heat-denatured BSA.  $5 \times 10^4$  CS1-wt (negative control) or CS1- $\beta 5$  cells were seeded per well and allowed to attach for 30 min under incubation conditions. Cells were treated during attachment as indicated with combinations of cytochalasin D (20  $\mu$ M) and either cyclic RAD (non-inhibitory control) or cyclic RGD (competitive inhibitor of integrin  $\beta 5$ -vitronectin interaction) peptides (20  $\mu$ g/ml), kindly provided by Merck KGaA, Darmstadt, Germany, used as described previously<sup>69</sup>. After attachment, non-adherent cells were removed by repeated washing. Remaining cells were labeled with DAPI and imaged via Nikon A1R confocal and 10x air objective, enabling automated cell counting via NIS elements software.

### ***Statistics and reproducibility***

Except where otherwise stated, all data presented reflect at least three biologically independent experiments. For analyses based on per cell quantification and/or intracellular adhesion population analyses, exact cell and/or adhesion numbers are presented in figure legends.

Statistical analyses and graphical representation were predominantly performed using R software (v3.5.1) and RStudio (v.1.1.453), or in some cases within Excel. All raw quantitative data and R analysis code are provided as described under code availability. Image analyses were predominantly automated via either commercial PAD software (v6.3) or

open-source Knime software (v3.6.0), ensuring uniform treatment and reproducibility. A representative Knime image analyses workflow together with sample images and subsequent data integration workflows are provided as described under code availability. Manual image analyses were performed in imageJ following computational blinding of image data identity. All graphical data representations are described in legends, along with statistical significance testing and correction procedures. For data visualization, boxplot notches indicate  $\pm 1.58$  times the interquartile range divided by the square root of observation number, approximating the 95% confidence interval. Except where otherwise stated, error bars also represent 95% confidence intervals estimated as described above. Statistical significance tests were all unpaired and two-tailed, and included either t-testing (for small, parametric data sets) or Mann-Whitney U testing (for large, potentially non-parametric data sets). Holm-Bonferroni corrections were applied to correct for multiple hypothesis testing. P-values are presented numerically in each instance.

### ***Code availability***

Where associated with open-source software tailored for this study, code underpinning this study is available through an associated public GitHub repository: <https://github.com/locusJ/Lock-et-al-NCB-2018-Reticular-Adhesions-Data-and-Analysis-Repository>.

This includes a custom Knime image quantification workflow used in automated analysis of siRNA and drug perturbation screening, and an associated custom Knime data integration workflow. Sample images from the analysis of Arp2/3-inhibition effects are also included. An R markdown script coding for the majority of graphical outputs and statistical significance testing is included, as is the associated HTML notebook summarizing this process and results. This R code calls the multi-sheet excel file provided as Supplementary Table 1, which contains all presented quantitative data. In some cases, graphical analyses were generated directly in excel; these are embedded within relevant sheets of this file.

A file titled “Instructional Workflow for Data Exploration and Reproduction.pdf” is provided within this repository, outlining the use of included code and data.

### ***Data availability***

Mass spectrometry data have been deposited in ProteomeXchange Consortium via the PRIDE partner repository with the primary accession codes PXD008645 and PXD008680. Source data for Fig.4A-D and Supplementary Fig.4A-B have been provided as Supplementary Table 2. All other quantitative data are presented in Supplementary Table 1, while sample images from screening analyses are provided via the GitHub repository described under code availability. Additional data supporting the findings of this study are available from the corresponding authors on reasonable request.

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