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Collective directional memory controls the range of epithelial cell migration

Helena Canever*, Hugo Lachuer*, Quentin Delaunay, François Sipieter, Nicolas Audugé, Philippe P Girard, Nicolas Borghi
Université Paris-Cité, CNRS, Institut Jacques Monod, Paris, France

For correspondence:
nicolas.borghi@ijm.fr

* These authors contributed equally

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eLife Assessment

The authors make the **valuable** observation that directional memory during epithelial cell migration is enhanced compared to single-cell migration. They attribute this effect to adherens junctions and vinculin dimerization. In the work, central measures should be defined more precisely, and the support for their claims about the roles of adherens junctions and vinculin dimerization in memory enhancement remains **incomplete**.

[Editors' note: this paper was previously reviewed by another journal.]

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Abstract

Cell migration is a fundamental behavior in multicellular development, regeneration, and homeostasis, which is deregulated in cancer. Epithelial cells migrate individually when isolated and collectively within a tissue. However, how interactions between cells affect their ability to explore space and their sensitivity to guidance signals is poorly understood. We show that isolated cells that are persistent random walkers adopt a superdiffusive behavior in an epithelium. The effect is stronger than external guidance cues and enables cells to reach greater distances than when isolated. This directional memory emerges from fractional Brownian motion that relies on velocity coordination between neighboring cells with intact intercellular adhesion. Furthermore, we show how the stability and mechanosensitivity of adhesion complexes ultimately regulate the speed of collective migration and the sensitivity to guidance signals via dimerization of the adhesion protein vinculin. Together, our results show how cell speed, persistence, and directionality define the efficiency of spatial cell exploration on short, intermediate, and long time scales, respectively.

Introduction

In multicellular organisms, cell migration is an essential process in tissue morphogenesis, homeostasis, and repair. Deregulation of cell migration is also involved in the metastatic invasion of cancers (1). Cell migration occurs in different ways, depending on the intrinsic properties of cells and how they interact with each other and their environment. As a result, cells may migrate individually or collectively, guided by local or global signals (2).

For these reasons, how cells explore their environment and how cell interactions and guidance cues affect this exploration are fundamental questions that have motivated abundant research for over 70 years (3, 4). Since then, various models have proven useful in describing the exploration of space by individually migrating cells. Directional persistence has emerged as a consistent, though highly variable, feature depending on the type of cell or the context, and could arise from the non-Gaussian distribution of cell displacements, temporal velocity correlations, or both (5–8). When migrating collectively, neighbor cells typically exhibit correlated velocities that depend on their interactions (9–11). This coordination and the diverse mechanisms underlying it have been the subject of intense scrutiny for the past decade (12). In comparison, how each cell in the collective explores its environment has received much less attention, with a few exceptions (13–15).

Remarkably, it remains unclear whether neighbor interactions actually help or hinder the exploratory capacity or sensitivity to guidance cues of each cell in the collective, much less whether and how they quantitatively or qualitatively affect the underlying cellular and molecular mechanisms of migration.

Adherent cells form Focal Adhesions (FAs) on their sub-strate, which they continuously assemble at their front and dissassemble at their back during migration (16). There, mechanosensory proteins, such as vinculin, provide force transmission from the contractile cytoskeleton (17). In cohesive tissues, cells also adhere to each other through cadherin-based Adherens Junctions (AJs) where α-catenin and vinculin enable dynamic connections with the cytoskeleton and force transmission (18). Vinculin comprises a head and a tail domain whose intramolecular interaction competes with intermolecular dimerization and binding to the actin cytoskeleton of its tail, and with binding of its head to adapter proteins in FAs and AJs (19). In AJs, α-catenin is this adapter, which also binds actin by its own tail (20). Vinculin deficiencies impact the migration of cells as individuals, in a collective, and with guidance cues, although in a cell type- and mutation-dependent manner (11, 21–32). Alpha-catenin deficiencies have an impact on guided and coordinated collective migration, involving the interaction of α-catenin with vinculin in AJs (11, 26, 33, 34). However, the lack of comparable metrics, scales and molecular, cellular and migratory contexts between studies makes it difficult for a general picture to emerge, where the functions of proteins in regulating the stability and mechanosensitivity of adhesions could explain how they control the intrinsic speed of cells, their persistence, and their responses to interactions with neighbors and guidance cues.

Here, we address this issue by comparing the exploratory ability of epithelial cells as individuals, in the collective and exposed to a guidance cue, while genetically disrupting the interactions of cells with their substrate and neighbors and monitoring the associated effects on the dynamics and mechanics of adhesion complexes. We show that individual epithelial cells migrate with transient directional memory and that vinculin regulates their speed by synergizing FA stability and cytoskeletal tension transmission through its dimerization and actin-binding abilities, respectively. In contrast, cells that migrate collectively acquire virtually infinite directional memory, which specifically requires α-catenin in AJs and re-sults from the coordination of velocities between neighboring cells. In this context, vinculin additionally controls the speed of cells from their AJs through its impact on their mobility, but in a manner specifically dependent on its dimerization. Overall, this results in a spatial exploration that is less efficient than that of individual cells on time scales less than $\sim$ 3hrs but more efficient beyond that. Finally, cell guidance by an epithelial wound also requires α-catenin, but the directional bias is too small to significantly affect the efficiency of spatial exploration for hours. In this context of guided collective cell migration, vinculin dimerization further regulates cell speed from AJs by providing mechanosensitivity to the entire epithelium of the wound.

Results

Single epithelial cells are persistent random walkers

To characterize the motion of single epithelial cells, we tracked nucleus-stained MDCK cells overnight, sparsely plated on collagen-coated glass coverslips, which offer a reasonable approximation of the basement membrane(35–37) (see Materials and Methods)(Fig. 1A, movie 1). We found that a persistent random walk model (38, 39) was more likely than a pure or superdiffusion model to describe the average Mean Square Displacement (MSD) as function of time interval Δt: the MSD transitioned from ballistic (MSD $\sim \Delta t^2$, $\Delta t \to 0$) to pure diffusion (MSD $\sim \Delta t$, $\Delta t \to \infty$) with a persistence time of the order of 10min (Fig. 1B; Table S1). To confirm the underlying walk model, we examined the step size R for the time intervals Δt = 10min and Δt = 3hrs. In both cases, R followed a Rayleigh distribution, in agreement with a Gaussian walk regardless of temporal resolution (Fig. 1C, S1A). Finally, we examined the angular memory, which we define as the deviation of each elementary 10min step from the past direction over an increasingly longer past (Δt, see Materials and Methods). We found that its distribution was

skewed towards small angles at small Δt and uniform at large Δt (Fig. S1B). In other words, the angular memory decreased with increasing Δt (Fig. 1D). Overall, these results show that single epithelial cells migrate as persistent random walkers, displaying transient directional persistence overcome by pure diffusion on longer time scales.

To investigate the robustness of this behavior, we examined the effects of overexpressing full-length (FL) vinculin or vinculin mutants, also equipped with a tension sensor module (40, 41). Specifically, we considered the T12 mutant with charge-to-alanine mutations in the tail that impair the headtail interaction (42), the Y1065E (YE) mutant with a phsophomimetic mutation in the tail that impairs the tail-tail interaction (43) and the Tailless mutant (TL) lacking amino acids beyond 883 that are required for all tail interactions (44) (Fig. S2A). All constructs localized to FAs in stable cell lines (Fig. S2B,C), which exhibited an MSD better described by a persistent random walk model than by a pure or superdiffusion model (Fig. 2A, Table S1). The effects of mutant expression on persistence times and diffusion coefficients were variable and did not show a correlation (Fig. S2D). In addition, the diffusion coefficient scaled well with the squared apparent speed with a common persistence time for all cell lines (Fig. S2E), showing that cell lines differed primarily in apparent speed rather than persistence. Specifically, TL and YE mutants with impaired actin-tail or tail-tail interactions decreased cell speed, while the T12 mutant with altered head-tail interaction increased cell speed relative to FL vinculin (Fig. 2B). This could not be explained by differences in expression levels between cell lines since the effects of YE and TL were stronger than those of FL despite an equal or lower expression, and those of T12 completely opposite despite a higher expression (Fig. S2F). Rather, this indicated a dominant effect, consistent with previous reports (44). Over-all, these results show that the persistence, but not the speed of single migrating cells is robust to mutations in the FA protein vinculin.

The combination of Focal Adhesion stability and force transmission from the cytoskeleton predicts the migration speed of single cells

The transmission of cytoskeletal forces through FAs is essentially based on vinculin (44–46). Thus, to identify the underlying cellular mechanisms and molecular determinants of speed regulation by FAs, we took advantage of the tension sensor module in vinculin and its mutants to assess their mechanical state by Molecular Tension Microscopy (Fig. 2C) (see Materials and Methods). We found that alteration of actin-tail and headtail interactions (TL and T12) decreased and increased vinculin tension relative to FL vinculin, respectively (Fig. 2D), as seen in other cell types (40, 41). In contrast, alteration of the tail-tail interaction (YE) did not substantially affect vinculin tension, meaning that vinculin dimerization is unnecessary for force transmission (Fig. 2D). Next, we investigated a potential relationship between vinculin tension and single cell speed across mutants, but found no significant correlation (Fig. S2G), indicating that single cell speed is not simply a result of vinculin force transmission.

The stability of the core cytoskeletal linkers of FAs is based on vinculin (30, 47). Therefore, we investigated the effects of mutations on vinculin turnover by Fluorescence Recovery After Photobleaching (FRAP, Fig. S2H, movie 2) (see Materials and Methods). We found that TL and T12 mutants, both of which impair head-tail interaction, had a significantly higher immobile fraction than FL vinculin (Fig. 2E), consistent with previous studies in other cell types (30, 44, 47–50). This shows that impaired head-tail interaction, regardless of tension, is sufficient for vinculin stability. In contrast, the YE mutant that impairs the tail-tail interaction showed a significantly smaller immobile fraction (Fig. 2E). This shows that vinculin dimerization promotes its stability. Additionally, this shows that cell migration speed does not simply result from vinculin stability since T12 and TL induce opposite effects on migration speed while both stabilize vinculin, and T12 and YE have antagonist effects on vinculin stability while both promote cell speed.

Together, this supports that migration speed decreases upon loss of force transmission or FA stability (TL and YE) and increases upon strengthening of both (T12). We therefore propose that single cell migration speed is the product of FA stability and force transmission, the former

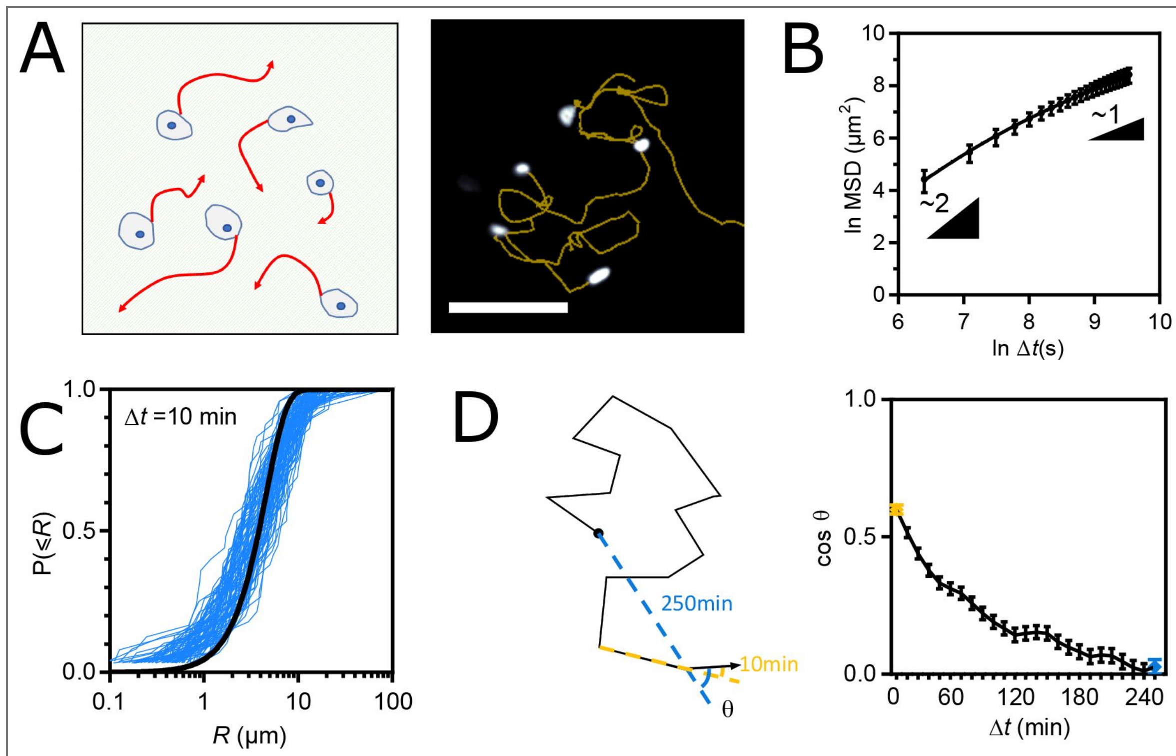

Fig. 1.

(A) Left: Schematic representation of the single cell migration assay. Right: fluorescence image of nucleus-stained cells and their migration tracks in yellow. Scale bar = 100μm. (B) MSD(Δt) of WT cells fit with the Fürth model. (C) Cumulative distributions of the step length R for Δt = 10min. One blue curve per frame pair along a movie. The black line is a global fit of all blue curves with a cumulative Rayleigh distribution function. (D) Left: Schematic migration track and angular memory (cos $\theta(\Delta t)$, see Materials and Methods) at Δt = 10min (yellow) and Δt = 250min (blue). Right: Angular memory (cos $\theta(\Delta t)$) between Δt = 10min (yellow dot) and Δt = 250min (blue dot). Data of (B-D) from 94 cell tracks of 2 independent experiments. Mean ± SEM.

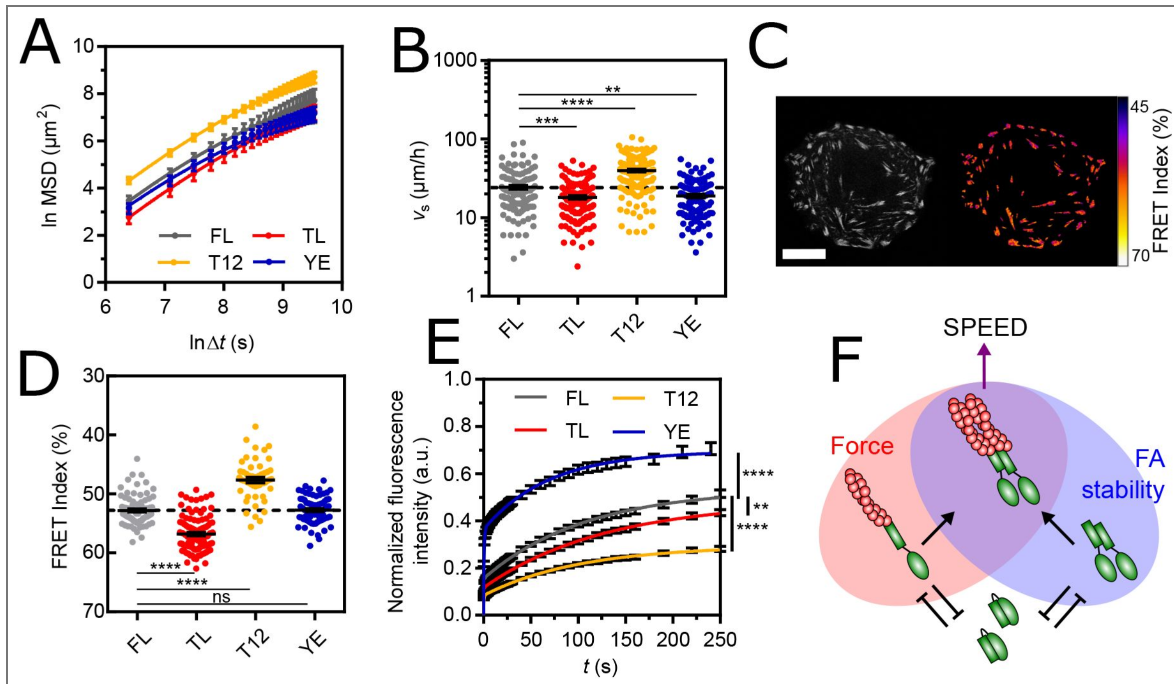

Fig. 2.

(A) MSD(Δt) of vinculin FL and mutant cells fit with the Fürth model. (B) Apparent speed of single, vinculin FL and mutant cells. (C) Left: direct fluorescence of vinculin TSMod in a single cell. Right: FRET Index map (see Materials and Methods). Scale bar = 20 μm. (D) FRET Index of TSMod in the FL, TL, T12 and YE constructs in FAs. (E) Fluorescence recovery after photobleaching of FL, TL, T12 and YE constructs in FAs fit with a 2-phases association model. (F) Proposed model: at FAs, the head-tail interaction of vinculin (green) competes with both tail dimerization and actin filaments (red) binding, which provide FA stability and force transmission, respectively, together promoting cell speed. Data of (A,B) from 125 (FL), 129 (TL), 193 (T12) and 110 (YE) cell tracks of 3 independent experiments. Data of (D) from 81 (FL), 102 (TL), 52 (T12) and 84 (YE) cells of 4 (FL) and 3 (TL, T12, YE) independent experiments. Data of (E) from 105 (FL), 112 (TL), 118 (T12) and 65 (YE) cells of 2 independent experiments. Mean ± SEM. Two-tailed Mann-Whitney tests between each mutant and FL (B,D). Extra-sum-of-squares F test with shared immobile fraction between each mutant and FL as the null hypothesis (E).

enabled by vinculin tail-tail interaction and the latter by vinculin-actin binding, and both competed for by its head-tail interaction (Fig.2F).

Collectively migrating epithelial cells are superdiffusive, fractional Brownian walkers

To assess how intercellular adhesion affects the persistent random walk of cells, we tracked MDCK cells plated at confluence on collagencoated glass coverslips (see Materials and Methods) (Fig. 3A, movie 3). In these conditions, the cell shape index (~ 4.2) was well above the rigidity threshold ($q \simeq 3.8$, Fig. S3A) (see Materials and Methods), which supports that the tissue behaved as a fluid with negligible resistance arising from cortical tension against cell rearrangements (51). We found that within the same time frame as in single cell migration experiments, cells no longer migrated as persistent random walkers but adopted superdiffusive behavior with an anomalous exponent of approximately 1.7 (Fig. 3B, Table S2). Remarkably, this superdiffusive behavior allowed cells to eventually reach a greater distance than single cells in the same amount of time, even though confluence also lowered their apparent speed (Fig. S3B).

To distinguish between the various walk models that could produce such a superdiffusive behavior (Lévy flight, fractional Brownian motion, or their combinations (7, 52)), we examined the step size distributions and found that they were consistent with a Gaussian walk on any time scale up to Δt = 3hrs (Fig. 3C, S3C). However, we found that the angular deviation of any 10min-step from the direction of the previous step was skewed toward small angles and invariant with the time scale of the previous step up to Δt = 250min (Fig. S3D). In other words, the angular memory never disappeared within our experimental time frame, unlike that of single cells (Fig. 3D compared to Fig. 1D). Gaussianity and virtually infinite directional memory are characteristics of a pure fractional Brownian motion and thus rule out Lévy walks or other combination. Cell superdiffusion thus emerges from a fractional Brownian motion.

The transition in directional memory emerges from Adherens Junction-mediated intercellular adhesion

To assess whether these effects resulted from simple steric hindrance due to crowding by neighboring cells or from genuine AJ-mediated intercellular adhesion, we examined the collective migration of cells depleted of α-catenin, without which cells cannot form AJs (53) (see Materials and Methods). Remarkably, the cells lost most of their superdiffusive behavior (with an anomalous exponent α dropping from 1.7 to ~ 1.2) and recovered a higher cell speed, despite being as crowded as WT cells (Fig. 3E). Consistently, angular memory vanished in the experimental time frame (Fig. 3F), as for single migrating cells. Next, to test whether cadherin subtypes could compensate for each other, we used cells depleted of E-cadherin but that retained cadherin-6 expression (53) (see Materials and Methods). These cells exhibited a slightly higher anomalous exponent than WT cells (Fig. 3E) and, consistently, infinite angular memory (Fig. 3F). This shows that E-cadherin specifically is largely dispensable, if not slightly detrimental, for cells to migrate in a superdiffusive manner, just as it is for the maintenance of AJs (53). Similarly, different cadherin subtypes can compensate for the defficiency of E-cadherin in other contexts (54–56). Therefore, the drop in cell speed and the transition in directional memory observed at confluence are the results of *bona fide* AJ-mediated intercellular adhesion, but independently of the cadherin subtype.

Velocity coordination between neighbors suffices to produce superdiffusion

Because velocity coordination is lost upon disruption of AJs by α-catenin depletion (Fig. S3E) (11), we sought to determine whether this effect of intercellular adhesion sufficed to confer a superdiffusive behavior to confluent cells regardless of other intercelullar adhesion features. To do so, we turned to *in silico* simulations of interacting self-propelled particles (SPP, see Materials and Methods). First, we modeled individual cells as persistent random walkers with the same

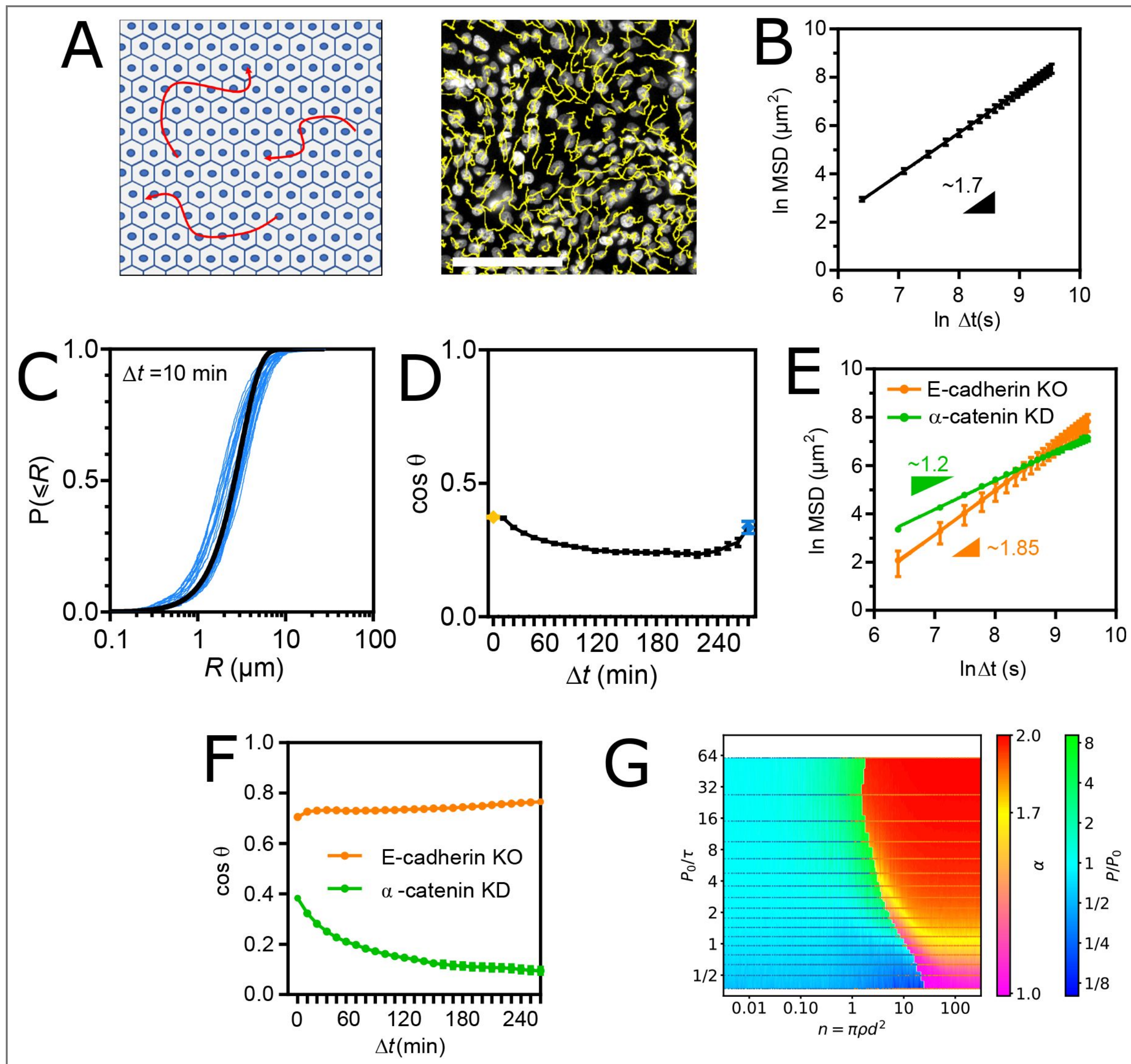

Fig. 3.

(A) Left: Schematic representation of the collective cell migration assay. Right: fluorescence image of nucleus-stained cells and their migration tracks in yellow. Scale bar = 100μm. (B) MSD(Δt) of WT cells fit with a straight line in ln-ln scale. (C) Cumulative distributions of the step length R for Δt = 10min. One blue curve per frame pair along a movie. The black line is a global fit of all blue curves with a cumulative Rayleigh distribution function. (D) Angular memory (cos $\theta(\Delta t)$) between Δt = 10min (yellow dot) and Δt = 250min (blue dot). (E) MSD(Δt) of E-cadherin KO and α-catenin KD cells fit with a straight line in ln-ln scale. (F) Angular memory (cos $\theta(\Delta t)$) of E-cadherin KO and α-catenin KD cells. Data of (B-D) from 11 Fields Of View (FOV, 500-1000 tracks each) of 3 independent experiments. Data of (E-F) from 3 (E-cadherin KO) and 4 (α-catenin KD) FOV (500-100 tracks each) of 2 (E-cadherin KO) and 3 (α-catenin KD) independent experiments. Mean ± SEM. (G) Migration behavior of Self-Propelled Particles with Vicsek interactions as a function of P_0/τ and number of interacting neighbors $n = \pi\rho d^2$. Blue and orange points are simulation coordinates that resulted in persistent and super-diffusive behavior, respectively. Cold and warm color backgrounds are interpolations from points above of P/P_0 and α values, respectively.

apparent speed and intrinsic persistence P_0 in isolation for all cells, in compliance with single cell migration results. Accordingly, P_0 is directly proportional to the time τ needed to make a step and a function of the intrinsic directional freedom of the cell between each step. Then, we modeled velocity coordination with a classic Vicsek interaction within a cell-scale distance d (57), and explored the MSD of particles at densities ρ covering isolated cells up to beyond confluence. We found that as the number of interacting neighbors $n = \pi \rho d^2$ increased, the effective persistence P of the particles increasingly diverged from P_0 before the migration behavior transitioned to superdiffusion (Fig. 3G). Strikingly, for a range of realistic values of interaction distance $d \sim 10 - 30 \mu m$, confluent density $\rho \sim 2000 - 4000$ cells/mm^2 and various degrees of directional freedom affecting $1 \lesssim P_0/\tau \lesssim 10$, the anomalous exponent fell around $\alpha \sim 1.7$ (yellow region), consistent with experimental observations (see Fig. 3).

Furthermore, this model predicted that provided a minimal number of interacting neighbors was reached, persistent random walkers would always transition to superdiffusers regardless of their intrinsic persistence. To test this, we tracked the single and collective cell migration of RPE-1 cells, another epithelial cell line (Fig. S3F, movies 4, 5). We found that single RPE-1 cell migration was also much better described by a persistent random walk than by a diffusion model, although with a persistence around 1 hr, making the difference hard to notice by eye within the experimental time window (Fig. S3E). Moreover, RPE-1 cell migration at confluence was again much better described by a superdiffusion model with an anomalous exponent around 1.6, as expected (Fig. S3E).

We conclude that velocity coordination is a feature of intercellular adhesion that suffices to produce superdiffusion at confluence in a manner that is very robust to the intrinsic persistence of cells, as predicted by our model.

Stabilization of Adherens Junctions by vinculin dimerization lowers cell migration speed

To further investigate the regulation of collective migration by intercellular adhesions, we examined the effects of confluence on vinculin mutant cell lines. All cell lines behaved as fluid tissues and their cells remained superdiffusive regardless of the expressed mutant (Fig. S4A, Fig. 4A, Table S3), showing that vinculin plays little or no role in the regulation of directional memory by intercellular adhesions.

Therefore, we examined cell speed. We reasoned that if vinculin neither plays a role in the regulation of cell speed by AJs, cell speed at confluence can be entirely predicted from single cell speed for each vinculin construct. We indeed found that TL and T12 mutants remained slower and faster, respectively, than FL (Fig. 4B). Moreover, the three cell lines exhibited the same $\sim 10 \mu m/h$ decrease in speed as WT cells at confluence compared to single cells (Fig. S4C). This indicates that AJs decrease cell speed independently of the effects of these mutants, which therefore have no other role in cell migration at confluence than those at FAs as in single cells. In contrast, the YE mutant was faster than FL at confluence (Fig. 4B), while it was slightly slower in single cells (see Fig. 2B). In fact, confluence induced a $\sim 10 \mu m/h$ increase, instead of a decrease in cell speed for this mutant (Fig. S4C). Altogether, this supports that functions impaired in YE, and not TL or T12, are key for the AJ-dependent decrease in cell speed at confluence.

At confluence, cortical actomyosin contractility promotes the recruitment and tension of vinculin in AJs (58). Here, all vinculin constructs colocalized with α-catenin and actin in intercellular contacts (Fig. S4D,E). To assess what distinguishes the YE mutant from the other constructs, we compared their relative recruitment and tension. In AJs, we found that YE showed a lower recruitment and tension than FL and T12, similar to TL, although to a milder extent (Fig. S4F, 4D). In FAs, in contrast, YE experienced a higher tension than FL, similar to T12, although to a milder extent (Fig. 4E). Altogether, the effect of YE on tension was neither different nor even stronger than that of the other mutants (Fig. S4G). Furthermore, there was no correlation between tension and cell migration speed among cell lines (Fig. S4H). This rules out tension as a regulator of collective cell migration speed.

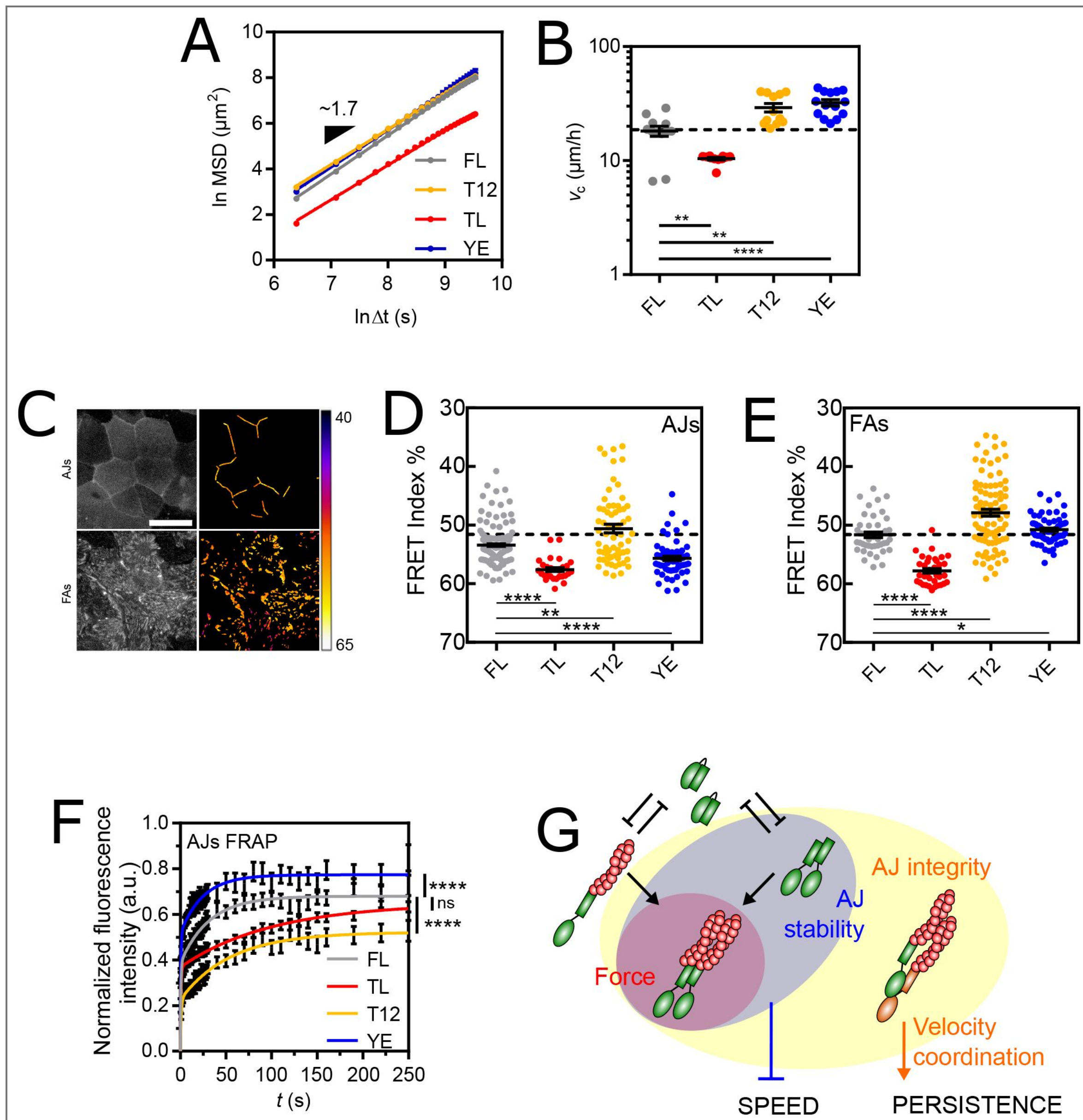

Fig. 4.

(A) MSD(Δt) of vinculin FL and mutant cells fit with a straight line in ln-ln scale. (B) Apparent speed of vinculin FL and mutant cells at confluence. (C) Left: direct fluorescence of vinculin TSMod in a confluent monolayer. Right: FRET Index map (see Materials and Methods). Top: in the AJs plane. Bottom: in the FAs plane. Scale bar = 20μm. (D) FRET Index of TSMod in the FL, TL, T12 and YE constructs in AJs at confluence. (E) FRET Index of TSMod in the FL, TL, T12 and YE constructs in FAs at confluence. (F) Fluorescence recovery after photobleaching of FL, TL, T12 and YE constructs in AJs fit with a 2-phases association model. (G) Proposed model: at AJs, α-catenin (orange) provides AJ integrity (connection to the cytoskeleton), which promotes persistence. The head-tail interaction of vinculin (green) competes with both tail dimerization and actin filaments (red) binding. Dimerization provides AJ stability, which slows cell down. Data of (A-B) from 12 (FL), 11 (TL, T12) and 14 (YE) FOV (500-1000 tracks each) of 3 independent experiments. Data of (D) from 169 (FL), 30 (TL) and 64 (T12, YE) contacts of 5 (FL) and 3 (TL, T12, YE) independent experiments, and (E) from 45 (FL), 35 (TL), 104 (T12) and 67 (YE) cells of 4 (FL) and 3 (TL, T12, YE) independent experiments. Data of (F) from 75 (FL), 137 (TL), 78 (T12) and 36 (YE) contacts of 4 (FL, TL, T12) and 3 (YE) independent experiments. Mean ± SEM. Two-tailed Mann-Whitney tests between each mutant and FL (B,D,E). Extra-sum-of-squares F test with shared immobile fraction between each mutant and FL as the null hypothesis (F).

Thus, we examined the stability of vinculin and its mutants by FRAP in AJs (Fig. S4I, movie 6), the stability of which is based on that of vinculin(18). FRAP experiments showed that the YE mutant had a significantly smaller immobile fraction than FL vinculin, while the TL and T12 mutants had larger immobile fractions, just as in FAs, although only that of the T12 mutant was significantly different (Fig. 4F). Therefore, the lack of stability in AJs is the unique difference between YE and the other mutants that appears at confluence. Overall, these results support that the stabilization of AJs (with TL, FL or T12), regardless of the tension they transmit through vinculin, equally lowers the speed of cell migration initially determined by FAs. In contrast, when AJs are unstable (YE), cells collectively migrate proportionally faster than single cells. Therefore, we propose that vinculin tail dimerization ensures the stability of AJs, which adapts the collective cell migration speed to the single cell migration speed (Fig. 4G).

Superdiffusion overcomes the weak directional bias caused by adhesion-dependent sensitivity to external guidance cues

While cells adopt a superdiffusive behavior at confluence, they do so in any possible direction. Thus, we sought to evaluate how the addition of an external guidance cue would affect the spatial exploration efficiency of collectively migrating cells. In principle, a directional bias eventually overcomes any diffusion behavior on a sufficiently long time scale, resulting in a ballistic motion (MSD $\sim \Delta t^2$, $\Delta t \rightarrow \infty$). To assess when it would occur, we wounded a confluent epithelial monolayer and tracked nucleus-stained cells as they invaded the free surface to close the wound (Fig. 5A, movie 7). Approximately 3 hours after wounding, the apparent speed of cells stabilized (Fig. S5A), consistent with previous reports (59), and was independent of the distance to the wound (Fig. S5B). Thus, we examined the MSD from 3hrs after wounding. Surprisingly, we found that the migration behavior remained superdiffusive with an anomalous exponent around 1.6 (Fig. 5B). Furthermore, as in the case of unbiased collective migration, the anomalous exponent decreased to around 1.2 for cells depleted of α-catenin, whereas cells depleted of E-cadherin remained superdiffusive, (Fig. 5C). Similarly, vinculin mutants did not affect superdiffusive behavior (Fig. 5D). Overall, these results show that space exploration efficiency remains dominated by the superdiffusive behavior of cells up to a few hours after exposure to an external guidance cue.

This raised the question whether cell velocity was biased at all or if cells merely filled the wounded area by isotropic superdiffusion. To assess this, we examined the angular distribution of cell velocities. In the steady state speed regime (at 6hrs), a directional bias was clearly visible (Fig. 5E). The bias appeared within 3hrs post-wound and remained stable throughout (Fig. 5F). This shows that a directional bias of such magnitude maintained from 3hrs onward is too weak to substantially improve the spatial reach of collectively migrating cells in the time range of the experiment. Finally, we asked whether disrupted AJs would alter the sensitivity of cells to directional bias. Remarkably, cells depleted of α-catenin exhibited very little directional bias compared to WT cells (Fig. 5F). Together, this shows that AJ-mediated intercellular adhesion is required not only for cells to acquire virtually infinite directional memory (see Fig. 3E, 5C), but also for them to perceive and migrate toward external guidance cues.

Vinculin dimerization scales wound healing rate to the collective cell migration speed through the regulation of Adherens Junction mechanosensitivity

Next, we wondered whether AJs were involved in the regulation of directed collective cell migration through additional mechanisms distinct from the regulation of directional memory (see Fig. 3E, 5C) and sensitivity to guidance cues by α-catenin (see Fig. 5F), and of speed by vinculin dimerization and stabilization of AJs at confluence (see Fig. S4C). To do so, we assessed whether the rate of wound healing, at which the wounded epithelium covers the free surface (see Materials and Methods), could be predicted from the speed of collective cell migration at confluence in vinculin mutant cell lines (see Fig. 4B). We expected a correlation if no additional regulation depended on vinculin. We found that this was mostly the case, with the exception of the

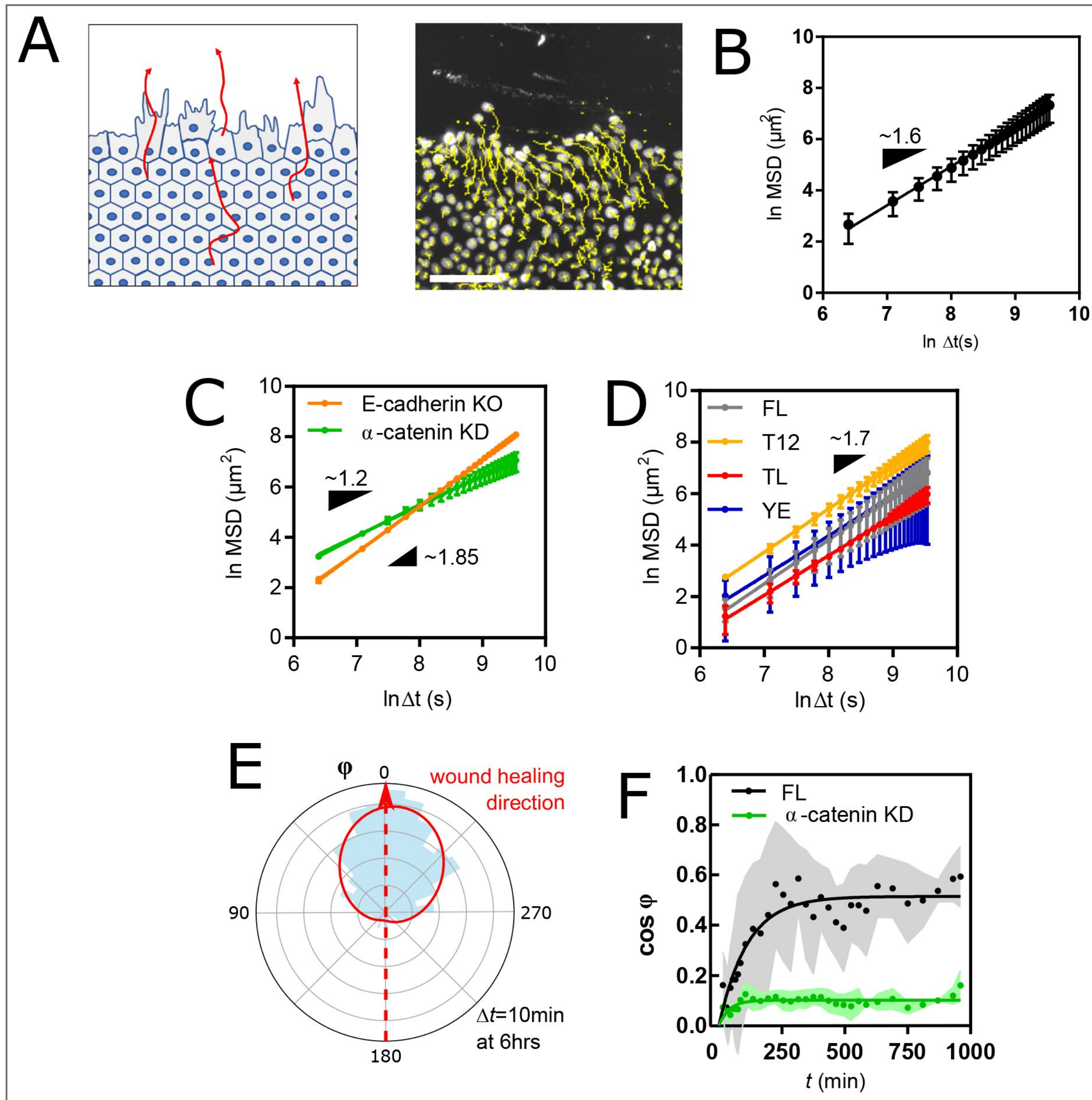

Fig. 5.

(A) Left: Schematic representation of the guided collective cell migration assay. Right: fluorescence image of nucleus-stained cells and their migration tracks in yellow. Scale bar = 100μm. (B) MSD(Δt) of WT cells fit with a straight line in ln-ln scale. (C) MSD(Δt) of E-cadherin KO and α-catenin KD cells fit with a straight line in ln-ln scale. (D) MSD(Δt) of vinculin FL and mutant cells fit with a straight line in ln-ln scale. (E) Angular distribution of apparent velocities of vinculin FL cells at 6hrs post-wound fitted with a Von Mises distribution giving a concentration $\kappa \sim 1.3$ (solid red line). (F) Directionality (cos $\Phi(t)$, see Materials and Methods) of vinculin FL and α-catenin KD cells. All data from 4 (all but YE) and 3 (YE) FOV (500-1000 tracks each) of 4 (all but YE and α-catenin KD) and 3 (YE, α-catenin KD) independent experiments. Mean ± SEM.

YE mutant. Indeed, while vinculin FL, T12 and TL cells closed the wound at a rate proportional to their migration speed at confluence, YE mutant cells were less efficient in wound healing than expected (Fig. 6A). This supports that vinculin dimerization, in contrast to other vinculin tail interactions, promotes wound healing in a manner that cannot be explained from its effects on collective migration at confluence, nor on single cell migration.

To determine how such an effect might emerge from this mutant, we therefore excluded its impact on adhesion stability. Rather, we evaluated how vinculin tension perceived biased collective cell migration (Fig. 6B-E). Indeed, we had previously found that biased collective migration of epithelial cells imprints a transcellular gradient of molecular tension in E-cadherins, which are under higher tension away from the migration front (60). Consistently, we found that FL and T12 vinculin, both capable of dimerization and binding to actin, were increasingly recruited and under higher tension in AJs away from the migrating front (Fig. 6B, C). As expected, the TL mutant, incapable of dimerization or actin binding, did not show mechanosensitivity and, remarkably, neither did the YE mutant, only incapable of dimerization (Fig. 6C). Therefore, actin binding is not sufficient for vinculin to exhibit a transcellular tension gradient in AJs in response to a directional cue, dimerization is necessary.

In FAs (Fig. 6D, E), the TL mutant, which is insensitive to cytoskeletal tension by design, showed a lower FRET at the migration front. This suggests a cytoskeleton-independent increase in vinculin compression in back cells, as previously seen within single fibroblasts on adhesive patterns (61). The T12 mutant did not show a FRET gradient. Compared to TL, this suggests that an increase in cytoskeleton-dependent tension from front to back cells compensates for the opposite gradient of cytoskeleton-independent compression. FL vinculin showed a higher FRET at the migration fornt. This supports that the increase in tension from front to back cells overcompensates for the opposite gradient of cytoskeleton-independent compression. Remarkably, the YE mutant exhibited a mechanosensitivity similar to that of FL vinculin. This shows dimerization is not required for vinculin to exhibit a transcellular gradient in FAs in response to a directional cue.

Together, this shows that vinculin dimerization is involved in its mechanical sensitivity to external guidance cues only in AJs and not in FAs, while head-tail interaction is involved in none, and acting binding in both, expectedly. Overall, this supports that the wound healing rate scales with collective migration speed provided that both AJs and FAs are mechanosensitive to external guidance cues, a feature that depends on the dimerization of vinculin in AJs (Fig. 6F).

Discussion

In this work, we studied the exploratory capacity of epithelial cells, the influence of cell interactions and guidance cues on this exploration, and the dynamics and mechanics of the adhesion complexes that cells use in this process. Remarkably, we show that intercellular adhesion with neighbor cells within the epithelium provides virtually infinite directional memory for migration, as a result of velocity coordination. For this, proper interactions of AJs with the cytoskeleton through α-catenin are instrumental, but the type of cadherin involved is irrelevant. Additionally, cell speed depends on FAs stability and force transmission and is impeded by AJs stability, both controlled by vinculin. Finally, we show a specific role for vinculin dimerization in AJs, which regulates the mechanosensitivity of the epithelium as a whole to the guidance cue of a wound, and the wound healing rate of the epithelium.

Locomotion patterns of single cells have been observed and quantified for decades and can take many forms. When endowed with directional persistence arising from angular correlations between consecutive unit steps, cells adopt a socalled persistent random walk that eventually converges to a purely diffusive behavior, as initially observed in fibroblasts or single endothelial cells (4, 5). In contrast, in T cells and some metastatic cells that behave like Lévy walkers, the fattailed power-law distribution of step sizes results in superdiffusive behavior, even in the absence of temporal correlation in direction (7, 8). Here, our results show that single epithelial cells undergo a persistent random walk, with persistence on the order of several minutes to an hour depending on the cell line, beyond which cells diffuse purely (Fig. 1). Thus, the persistence

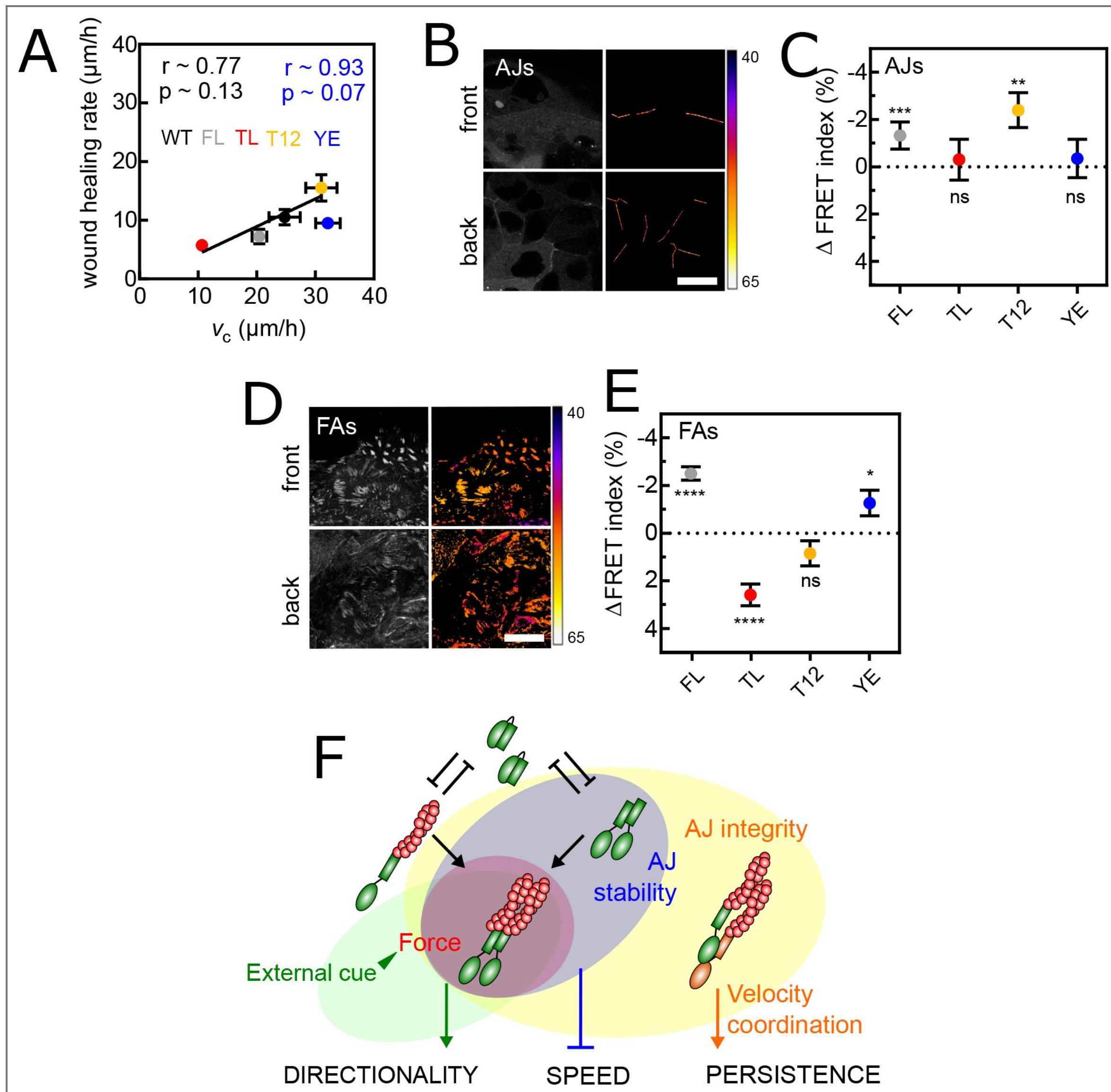

Fig. 6.

(A) Wound healing rate (see Materials and Methods) as a function of cell speed at confluence for WT, vinculin FL and mutant cells. Pearson coefficients r and p-values in black for all data, in blue minus YE. (B) Left: direct fluorescence of vinculin TSMod in the AJs plane of cells at the wound front (top) and back (bottom). Right: corresponding FRET Index maps. (C) FRET Index difference between front and back cells at AJs of TSMod in vinculin FL and mutants. (D) Left: direct fluorescence of vinculin TSMod in the FAs plane of cells at the wound front (top) and back (bottom). Right: corresponding FRET Index maps. (E) FRET Index difference between front and back cells at FAs of TSMod in vinculin FL and mutants. (F) Proposed model: at AJs, vinculin dimerization and actin binding provide mechanosensitivity to stable AJs, which promotes wound healing. Data of (A) from 4 (all but YE) and 3 (YE) FOV of 4 (all but YE) and 3 (YE) independent experiments. Data of (C) from 55 (FL), 16 (TL), 56 (T12) and 26 (YE) front cells and 122 (FL), 30 (TL), 71 (T12) and 48 (YE) back cells of 4 (FL, T12) and 3 (TL, YE) independent experiments. Data of (E) from 144 (FL), 63 (TL), 124 (T12) and 46 (YE) front cells and 155 (FL), 84 (TL), 128 (T12) and 51 (YE) back cells of 7 (FL, T12), 4 (TL) and 3 (YE) independent experiments. Mean ± SEM. Two-tailed Mann-Whitney test between front and back cells for each construct (C,E). Scale bars = 50μm.

of epithelial cells is in the low range compared to endothelial cells, whose persistence time can reach several hours (5). Furthermore, comparing cell lines that express various forms of vinculin mutants, we observe that persistence does not correlate with cell speed, consistent with endothelial cells exposed to growth factors (5). Mechanistically, both cell speed and persistence are under the control of actin retrograde flow, via connections with cell-matrix adhesions for the former, and control of polarity factors for the latter (62). Our data are thus consistent with the idea that the abnormal connection of cell-matrix adhesions to the cytoskeleton via vinculin uncouples speed from persistence.

Previously, vinculin-KO fibroblasts or epithelial cells have been shown to migrate faster than their WT counterparts (22, 24, 29). Here, we show that the migration speed of epithelial cells decreases with the expression of FL vinculin even at low levels above endogeneous (Fig. 1). Overall, this is consistent with the idea that vinculin acts primarily as a migration moderator, and this supports that insertion of the sensor module weakens to some extent the auto-inhibition of vinculin, thereby contributing to the dominance of all exogenous constructs over endogenous vinculin, in addition to the effects of their mutations. Mechanistically, our results support that the head-tail interaction of vinculin challenges both force transmission and stability of vinculin in FAs, as has been observed in other cell types (30, 44, 47–50), and is countered by the actin-vinculin tail interaction, which provides force transmission but is unnecessary for stability, and dimerization of the tail, which promotes stability and is dispensable for force transmission (Fig. 2D,E). Overall, the combination of maximum force transmission and stability predicts maximum cell speed (Fig. 2B). Therefore, we propose that the speed of single cells is the product of force transmission and stability in FAs (Fig. 2F, S5C).

At confluence, we show that epithelial cells adopt a super-diffusive behavior that a persistent random walk cannot explain (Fig. 2). This behavior has previously been observed in single cells or in a collective for a variety of cell types (6– 8, 13–15). However, proposed models involved non-Gausian displacement distributions, which are sufficient to support superdiffusion without long-term memory. Moreover, whether superdiffusion in the collective emerged from intercellular interactions or from cells that already walk alone in this manner remained to be demonstrated. Here, we show that superdiffusion can be explained by a pure fractional Brownian motion with Gaussian distribution of displacement and virtually infinite directional memory, which emerges from intercellular interactions (Fig. 2A-D).

Previously, subdiffusive fractional Brownian motion has explained subcellular particle mobility in the crowded cytoplasm through interactions with the viscoelastic medium (63). In contrast, we show here that a strong superdiffusive fractional Brownian motion emerges from intercellular adhesion mediated by AJs bound to the cytoskeleton but independent of cadherin type, and not just steric hindrance due to the mere presence of neighboring cells (Fig. 3E,F). Remarkably, the same AJ-based mechanism also allows for velocity coordination between neighboring cells (Fig. S3E) (11). The interplay between persistent migration and elastic coupling provided by AJs was previously shown to be sufficient for velocity coordination (64). We further show with our minimal agent-based model that such coordination is sufficient to explain the transition to superdiffusion (Fig. 3G). Remarkably, the transition occurs in a region where $\alpha \sim 1.7$ for a number of interacting neighbors $n \sim 6$, about the number of direct neighbors at confluence, and $P_0/\tau \sim 3$. This allows us to determine the decision time τ, which is intrinsic to persistent random walks, but cannot be experimentally determined from single cell migration experiments that only reveal P_0. For MDCK cells with persistence $P_0 \sim 10$min, this corresponds to a decision time τ of a couple minutes. For RPE-1 cells with persistence $P_0 \sim 1$hr, τ would rather be several tens of minutes to an hour. This suggests that RPE-1 cells are more persistent due to their longer decision time, rather than narrower directional freedom. Discovering the respective molecular determinants of decision time and directional freedom appears as an exciting future research direction. In the same modeling context, others have shown that velocity coordination also results in spontaneous fluctuations in particle density (65). We thus propose that at confluence, such spatiotemporal fluctuations in the number of interacting neighbors generate variations in instantaneous persistence of cells along their trajectories, ultimately resulting in a distribution of

persistence times across time scales. Consistently, the MSD of a persistent random walk at time P scales as P^{e-1}, the anomalous exponent being about that of the superdiffusive MSD, as if superdiffusing cells experienced a distribution of persistence times P that spanned all our experimentally accessible time scales.

In addition to inducing a transition from finite to infinite directional memory, we show that AJs have a second role: they globally decrease the apparent speed of cells, provided that vinculin is stable at AJs. Indeed, perturbation of vinculin dimerization leads to its low stability at AJs and faster than expected cell migration (Fig. 4B,F, S4C). Remarkably, this increase in cell speed is not the result of relaxed constraints on cortical deformations, as seen previously in unjamming transitions (66, 67), since vinculin mutants exhibit similar shapes indicative of a fluid phase (Fig. S4A). Alternatively, we speculate that the destabilization of AJs results in deeper intracellular changes within the cell's bulk, possibly due to the impaired ability to bundle actin of the vinculin mutant (43). This could therefore decrease the cell's own resistance to the bulk deformation required for migration but in a collective context only, and thus facilitate migration despite a less efficient FA machinery. Future studies may address this hypothesis.

Overall, intercellular adhesion normally impairs apparent cell speed but induces long-term directional persistence. As a result, while cells are collectively less efficient than single cells in exploring space at time scales of less than 3hrs, they can become more efficient beyond that (Fig. S3B). Thus, this emergence of long-term collective persistence may have a profound impact on tissue morphogenesis, regeneration, and homeostasis in a variety of contexts. In the case of wound healing, cells are further exposed to an external guidance cue. Here we show that this signal is sufficient to bias the velocity of each cell at the smallest experimental time scale (Fig. 5E). Detection of this signal is an emergent property of the collective, as disrupting intercellular adhesion renders cells nearly blind to the free space to invade (Fig. 5F). This is reminiscent of collective durotaxis and chemotaxis (68, 69). However, we show that this directional bias remains too small to drive ballistic motion in less than the longer experimental time range, such that cells remain superdiffusive throughout (Fig. 5A-D). We expect the directional bias to lead to more ballistic cell motion at longer time scales, with a lower limit of at least 3hrs.

This third role of AJs in directing collective cell migration is also dependent on vinculin. Specifically, wound healing rates scale with collective cell migtration speeds in a manner that depends on vinculin tail dimerization (Fig. 6A). In this context, vinculin tail dimerization is required to provide mechanosensitivity to AJs, so that both AJs and FAs can transmit molecular tension gradients across the tissue (Fig. 6B-E). This is again reminiscent of the requirements for supracellular durotaxis, which arises from intercellular force transmission (68). We speculate that the ability of cells to stiffen under force in a way that depends on vinculin dimerization may be involved (43).

In summary, we conclude that the spatial exploration efficiency of guided collective migration depends on the scale considered: at short time scales (<3hrs for MDCK cells), the intrinsic migration speed of individual cells is the limit, which can be overcome by collective directional memory at intermediate time scales (>3hrs for MDCK cells), a tissue-level property that guidance cues may additionally enhance, but at much longer time scales (»3hrs) (Fig. S5D). We speculate that this generic framework applies qualitatively to guided collective migration in general and hope that future studies will test this prediction.

Materials and Methods

Live MDCK type II G cells stably expressing fluorescently tagged proteins were monitored on a wide-field epifluorescence inverted microscope for migration experiments, on a scanning spectral confocal microscope for FRET experiments, or on a spinning disk confocal microscope for FRAP experiments. Image analyses were performed with Imaris and ImageJ, simulations with R, data analyses with Matlab and R and statistics with Prism and R software. Complete materials and methods are available in Supplementary Materials and Methods. New materials, reagents, and simulation and analysis scripts are available upon request.

Data availability

All data generated or analyzed during this study are included in the manuscript and supporting files; source data files have been provided for all figures.

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Author ORCID iDs

Hugo Lachuer: https://orcid.org/0000-0001-9090-7893
François Sipieter: https://orcid.org/0000-0002-1332-400X

Philippe P Girard: https://orcid.org/0000-0001-7202-6910

Nicolas Borghi: https://orcid.org/0000-0002-1558-5246

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Peer reviews

Reviewer #1 (Public review):

Summary:

In this work, the authors study the migration of isolated cells and of cells in ensembles. They quantify several aspects of the corresponding migration patterns and investigate how these quantities depend on molecules that are known to play an important role in migration. Furthermore, they study the effect of external cues on these migration processes.

Strengths:

The authors provide a clean and uniform setting for comparing the migration of isolated cells and of cells in an ensemble in control and mutant conditions, and in the presence and absence of external cues. This allows for a meaningful comparison between different conditions. In this way, the authors obtain useful data that link the migration of isolated cells to that of cells in collectives.

Weaknesses:

A major weakness of the manuscript is that the authors do not properly introduce the quantities and concepts they are working with. In this way, it is hardly accessible for a reader who does not have a thorough background in cell migration and anomalous transport. In addition, the manuscript uses some notions that are not standard, for example, vinculin or FA stability, which are not properly introduced. Most strikingly, "collective directional memory" is not defined.

The authors infer relationships between different quantities, but they remain qualitative, even though the authors use a language that suggests otherwise. For example, "The combination of Focal Adhesion stability and force transmission from the cytoskeleton predicts the migration speed of single cells" (p 2). I am not sure what is meant by prediction, but this heading suggests that knowledge of FA stability and force transmission yields the migration speed. Reading this line, I expect that if I give you values for FA stability and force transmission, you would give me a value for the migration speed. Such a quantitative mapping is not provided. In fact, it cannot be provided, because - as mentioned before - these quantities are not properly defined, so I would not know how to measure them. I do not even know their units.

Furthermore, the authors do interpret some of their results without explaining or justifying the basis for their interpretation. For example, they use the FRET index of vinculin - another notion that is not properly introduced - to make statements about mechanical stress.

It also seems that the figures could be improved. Some of the sketches are, in my opinion, not helpful. Examples are Figure 3A (how could a cell move while the hexagonal arrangement of the cells is maintained?) or Figures 2F, 4F, and 6F (what do the colored ellipses indicate?). In Figures 1B, 1D, 2A, 2E, 3B, 3D-F, 4A, 4F, 5B-D, it is not clear which lines merely connect data points and which lines are fits to the data.

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Reviewer #2 (Public review):

Summary:

The manuscript by Canever et al was assessed by three Referees at another journal, who brought up a range of critical points. I will not repeat a summary of the work; this can be found in the first-round reviews.

Strengths:

In their revised manuscript, the authors include substantial changes and additional reasoning. Along with their rebuttal letter, I think they make a very convincing case. While the claims are well supported by the analysis, I do not see that the findings need to be universal to be relevant. It might be rather surprising to me if there existed such a universality, in fact. I think that the findings are solid and interesting in their own right and are worthy of publication, especially with the amended discussion in this revision.

Weaknesses:

However, while the more bio-oriented parts are not fully accessible to me, I do have a few points from the data analysis point of view that need amendment.

(1) The used mathematical models need to be specified more precisely. First, the authors confuse Levy flights and walks. These are distinct processes in the sense that a Levy flight does not have a finite variance and thus no finite speed. The proper model here would be Levy walks. As in a big body of the literature, both notions are used interchangeably here, while they are distinct processes. Then the authors speak about a "superdiffusive model", for which I do not find a proper definition. There exists an entire range of superdiffusive models, each with a different physical background, so this needs more clarity. The authors may consult one of the standard reviews for more details, e.g., Soft Matter 8, 9043 (2012) or Phys Chem Chem Phys 16, 24128

(2014). Overall, a few equations (maybe in the Supplement) would help to be more specific.

(2) For fractional Brownian motion, the authors should check the displacement correlation function; it should show slowly decaying, positive correlations. More details on the practical analysis of FBM can be found, e.g., in Phys Chem Chem Phys 27, 14350 (2025). These correlations should decay as a function of the bin time, e.g., as discussed for the opposite case of subdiffusion in Phys Rev E 88, 010101(R) (2013) [cf Fig 3b]. In general, FBM was determined to be a highly relevant process for a number of systems, including amoeba cells at shorter times, see the detailed analysis in Phys Rev Res 4, 033055 (2022). In this paper, there are also different ways to characterise the motion in terms of scaling. Exponents are detailed.

(3) Some relevant approaches discussed in literature that should be discussed in the context of this work: eLife 9, e52224 (2020); Rep Prog Phys 86, 126601 (2023); Chaos 35, 023145 (2025). In the context of non-Gaussianity for active particles: Phys Rev E 104, 064615 (2021); New J Phys 25, 013010 (2023).

(4) In the abstract, I am having some issues with the formulation in the sentence: "This directional memory emerges from fractional Brownian motion". It sounds as if FBM were a fully clarified phenomenon. I would prefer some statement along the lines that the data are consistent with such a mathematical modelling approach.

After fixing these points, I think the manuscript will clearly warrant being shared.

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Reviewer #3 (Public review):

This manuscript focuses on the presence/origin of directional memory during epithelial cell migration. It starts by analyzing single cells and then moves to more complex systems (confluent layers and scratch assays). The paper first demonstrates that the migration in all of these systems is well-described by persistent random walks, which likely emerge from fractional Brownian motion. This is an important demonstration, as it implies orientation memory in the systems. Then the paper proceeds to attempt to discern the origin of this memory and claims to establish key roles for adherens junctions and vinculin dimerization. While for the most part the manuscript is well-written, there are some significant overinterpretations in experimental results. The largest issue is demonstrating the role of vinculin dimerization, which is not a well-studied phenomenon inside living cells, as all data is reliant on a single point mutation (Y1065E). Additionally, the authors seem to be over-interpreting several of the assays; the statistical analysis does not seem to encompass all comparisons made, and the molecular model proposed does not clearly explain the observed results. The discussion could also be strengthened by considering other aspects of vinculin behavior (e.g., vinculin catch bonding) as well as discussing some other recent similar papers.

(1) Likely the most significant issue with the manuscript is the interpretation of the vinculin Y1065E variant and the assumption that the only defect the mutations cause is a lack of dimerization. Vinculin dimerization is mediated by a conformational change in the vinculin tail domain induced by F-actin binding (Thompson, FEBS Letters, 2013). Dimerization of the vinculin tail domain has been clearly demonstrated in in vitro systems involving purified proteins, as the authors point out in the manuscript. However, the dimerization of full-length vinculin has not been well characterised in living cells. There are several reasons to suspect dimerization is potentially not prevalent in cells. For instance, in the presence of other actin-binding proteins, there may not be sufficient binding sites available on neighboring actin filaments to facilitate dimerization. Additionally, pY1065 vinculin and vinculin Y1065E have been associated with increased vinculin activation (Huang, JBC, 2014), so other effects seem possible. While the Y1065E variant clearly has an effect on the tension sensor readout and vinculin dynamics, further experimental evidence is needed to show that these effects are due to a lack of dimerization in living cells. To justify the definitive claims made in the manuscript, the authors likely need to develop, or employ, an assay for detecting vinculin dimerization in living cells. The authors could choose between intermolecular FRET, proximity labeling assays (i.e., antibodies with DNA for signal amplification), bimolecular fluorescent complementation (i.e., split GFP) based approaches, or some other approach. It should be noted that working with full-length vinculin, not just Vt, and designing an assay that can incorporate vinculin Y1065 variants (Y1065E and potentially Y1065A/F) would strengthen results. Also, the authors should be aware that the observation of strong dimerization may invalidate the use of FRET-based tension sensors in this system or at least necessitate intermolecular FRET control experiments.

(2) The authors have seemed to assume that FRAP and adhesion stability are interchangeable. To this reviewer's knowledge, this is not the standard in the field. FRAP informs about molecular dynamics. Stability assays, which probe the spatial position of an entire focal adhesion over time (Zaidel-Bar, JCS, 2007, although other approaches are equally suitable), are typically used for assessing adhesion stability. If the authors wish to make strong claims about the stability of the adhesions, non-FRAP-based assays should be employed. Alternatively, the authors could interpret the FRAP data simply in terms of vinculin dynamics.

(3) A major conclusion in the manuscript is that in response to overexpression of a specific vinculin construct, focal adhesions behave the same in single cells, confluent cells, and collectively migrating cells for all the mutants but Y1065E. However, outside of the FRET

measurements, there is not much evidence to support this claim. The authors should perform a greater comparison of the focal adhesions between the systems used in the manuscript (single cell, confluent cells, collectively migrating cells). Key measurements would include focal adhesion number per cell, focal adhesion size, focal adhesion orientation, vinculin dynamics (e.g., FRAP), focal adhesion stability, and some indicators of focal adhesion composition. For the last aspect, focusing on focal adhesion components that also have roles in adherens junctions, such as VASP, seems appropriate. Without such characterization, it is an overinterpretation to assume that focal adhesions are the same in each system and, therefore, effects are due to vinculin behavior in the adherens junctions.

(4) What is shown in Figure 3G is not clear. How are P/Po and alpha shown on different areas of the same plot?

(5) It seems that an insufficient statistical test was used in many experiments. There are comparisons being made between systems (cell migration speed, FRET index...) that are not directly compared in a statistical test. Statistical tests are limited to differences from control (over-expression of full-length vinculin), and consistent increases or decreases (not quantitative values) are taken as evidence of similarity across systems. It seems that a more rigorous and standard approach would be to use an ANOVA/MANOVA with a suitable post-hoc test to perform all of these.

(6) It is unclear how a lack of vinculin dimerization at adherences junctions perturbs epithelial migration, but the complete lack of vinculin tail, which can also not dimerize, does not. In other words, how can TL "have no other role in cell migration at confluence than those at FAs as in single cells." Notably, the authors do not include the tailless variation in the schematic model figures. These results should be included and explained.

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Author response:

[Editors' note: The authors included an author response to reviews from another journal]

> ***Reviewer #1 (Comments to the Authors):***
>
> *In this manuscript the authors describe that cells in collective movements adopt a superdiffusive behavior to out pace individual cells. This behavior is regulated by cell-cell junctional stability and force transmission. The authors state that speed is regulated by vinculin through mechanosensitivity.*
>
> *While is makes intuitive sense that cells may move more efficiently collectively as it reduces their exploratory space and therefore increases their efficiency of movement,*

We agree that this is an intuitive explanation. However, previous literature had shown that confluent cells may or may not migrate depending on conditions that do not solely depend on the space available per cell, but also involve the intrinsic activity of the cell, its cortical tension, and its adhesion with its neighbors, with sometimes counterintuitive effects (doi: 10.1016/J.CEB.2021.07.011). This was the reason that motivated us to investigate how these various ingredients affected space exploration efficiency on different time scales.

Our results indeed refute the intuition that cells move more efficiently when their exploratory space is reduced by showing that the outcome depends on the time scale considered (Fig. S3B). Specifically, on short time scales (less than 3 hours), the area explored by individual MDCK cells is larger than that explored by MDCK cells at confluence. On a longer time scale (greater than 3 hours), however, the area explored by confluent MDCK cells is larger. This switch is a direct consequence of the change in migratory behavior from persistent random walk to superdiffusion, Moreover, its position in time depends on the cell

line: extrapolation of our results on RPE-1 cells suggests that it should theoretically occur after approximately 300hrs, if this time scale was experimentally accessible (Fig. S3F).

> *...the role of junctions specifically is less clear.*

We are sorry that we were not able to clearly convey the roles of junctions. We have substantially rewritten our text to address this and all the changes are highlighted in orange. As summarized in Fig. 6F, junctions have three roles. The first role is on persistence, through velocity coordination between neighbors, the second is on speed, through the stability of junctions, and the third role is on directionality, through the sensitivity of the monolayer to the wound edge.

The first role is evidenced thanks to the comparison of the MSD between single cell and confluent migration assays and the use of the alpha-catenin KD cell line. Alpha-catenin depletion is known to be the most potent disruptor of adherens junctions (DOI:10.1091/mbc.e06-05-0471, , DOI:10.1126/science.aaf7119, (DOI:10.1073/pnas.1002662107, DOI:10.1073/pnas.1119313109), and we show that it significantly alters the superdiffusive behavior that emerges in the confluent migration assay (Fig. 3E,F, 5C). Therefore, junction integrity is critical for the control of cell persistence.

Moreover, alpha-catenin depletion induces a loss of velocity coordination between neighbors (Fig. S3E), which we show through numerical simulations to induce superdiffusion (Fig. 3G). By contrast, E-cadherin KO and vinculin mutants have no effect on the superdiffusion of confluent cells (Fig. 3E, 4A). Therefore, the critical molecular ingredient is the link provided by alpha-catenin to the cytoskeleton that provides junction integrity.

The second role of junctions is evidenced thanks to the comparison of cell speeds between single and confluent migration assays with the vinculin mutants (Fig. S4A). Results show that cell speed is reduced of about 10µm/h by confluence, regardless of the mutant except for YE, whose only difference with other mutants is its lower stability (Fig. 4F). This supports that junction stability, and not the other effects of mutants, controls cell speed (we provide a detailed demonstration in the response to the following question). As expected, junction integrity is required as well, as seen from the higher cell speed of the alpha-catenin KD cell line compared to WT (first MSD point in Fig. 3B, E).

The third role of junctions is evidenced thanks to the comparison between confluent and directed migration assays (Fig. 6A). Results show that the wound healing rate is proportional to cell speed at confluence, regardless of the mutant except for YE, which displays no tension gradient in junctions from front to back cells (Fig. 6C). This supports that such gradient is required for cells to identify on which side is the wound edge. As expected, junction integrity is required as well, as seen from the loss of directional bias of the alpha-catenin KD cell line (Fig. 5F).

> *The authors chose vinculin as the basis by which to manipulate tensions at cell-cell junctions, but this comes with considerable drawbacks. Namely, since vinculin appears at both cell-cell and cell-matrix junctions, its role and the role of its mutations is not clear here. The authors state that the collective migration speed is related to junctional stability, but because vinculin is also at FA, how can this be concluded?*

We apologize for the lack of clarity. We hope that the highlighted changes in the revised manuscript will improve this point. As exemplified above, comparing cell migration between isolated cells and confluent cells is essential to enable us to distinguish between the contributions of AJs and FAs. Indeed, since isolated cells lack AJs, the impact of vinculin mutants on single cell migration can only be explained by their effects on FAs. This is how we first determine the effects of vinculin mutants on migration that depend on FAs. Because confluent cells also have FAs, we expect that the effects of vinculin mutants on the migration

of isolated cells will still be present in confluent cells, to which will be added the effects of these mutants on AJs and their consequences on migration, if any.

Therefore, when compared to WT cells, if a given mutant decreases or increases migration speed in individual cells, and does so in confluent cells in the same proportion, then its effects at confluence can be entirely explained by its effects in individual cells, and there are no additional effects of that mutant from AJs. This is indeed what we observe for all mutants except the YE mutant (Fig. S4C), leading us to conclude that none of the vinculin mutants, except the YE mutant, have an effect on migration at confluence that results from AJs. In contrast, the YE mutant has effects on migration at confluence that cannot be explained by its effect on individual cell migration. Therefore, the effects of YE at confluence depend on AJs, whether they result from alterations in AJs, FAs, or both. To distinguish between these scenarios, we proceed by elimination, comparing the effects of YE to those of other mutants on force transmission and adhesion stability, and how these two factors associate with migration speed, as explained below. In FAs, YE alters force transmission differently in individual cells and at confluence, but we already know from Fig. 2 that force transmission in FAs cannot alone explain the speed of migration. This result rules out an indirect effect of AJs on cell migration at confluence through FAs. Furthermore, in AJs, YE affects stability and force transmission, but TL has the same effect on force transmission as YE and we already know that none of the effects of TL on migration depend on AJs (Fig. 3, S4C). This result rules out an effect of force transmission in AJs on migration speed at confluence. We conclude that stability at the AJ level, which is the remaining property specifically impaired by YE, is what regulates migration speed at confluence.

> *The manuscript's logic and flow are not clear in some places, making the story hard to follow. As one example, the FRAP data, which the authors suggest is used to investigate vinculin's combined role does not help in this capacity as the interpretation and its connection to the bigger story are not clear.*

We are sorry again for the lack of clarity. We used FRAP data to evaluate the effects of vinculin mutants on adhesion stability. Indeed, mutants have different effects on adhesion stability (Fig. 2E, 4F). In addition, they also have different effects on force transmission (Fig. 2D, 4D,E). The partial overlap in functional alterations caused by the mutants allows us to test the involvement of the overlapping function (here stability) in the overall migration outcome. For example, if two mutants have a similar effect on adhesion stability but different effects on migration speed (such as TL and T12), we can then rule out that speed results from adhesion stability. Similarly, if two mutants have different effects on stability but a similar effect on speed (such as TL and YE), we can also rule out that speed results from stability. We applied the same reasoning to force transmission to conclude that neither adhesion stability nor force transmission alone is sufficient for cells to migrate rapidly. However, the combination of the two enables rapid migration.

> *As another example, the information derived from the use of the mutants is not clear in the context of the message in the manuscript since they affect cell-cell and cell-matrix junctions and in some places show results that are counter intuitive and not well-explained, to which the authors admit they are surprising but then do not explain their meaning.*
>
> *As such, it is very hard to follow the logic with regard to the information resulting from the mutant experiments.*

We provide above a detailed break-down of our strategy to analyze the results. We regret that our manuscript did not adequately convey our conclusions and we hope that the new version of the manuscript improves this point.

> *Proliferation has been shown to play a role in wound healing. Does proliferation change in the various conditions?*

This is an important point. The average speed of cells at confluence is approximately 20 µm/h (Fig. 4B), which means that each cell moves approximately its own size in one hour. During this time, assuming a 16-hour cell cycle, 6% of the cells would have divided, each of them likely pushing one of its neighbors a distance equivalent to the size of a cell. Therefore, cell proliferation accounts for at most a few percent of the total cell movement. For this reason, we can assume that growth does not account for a large part of the movement we observe. This is consistent with previous work showing that proliferation does not contribute significantly to wound healing (DOI: 10.1073/pnas.0705062104, DOI: 10.1083/jcb.201207148).

> *Minor comments:*
>
> *The authors should provide a better description of the mutants: what does a tailless mutant not bind, or bind differently? More context is needed to help interpret the results. While the mutants have all been published on before, it would be helpful to have more information here so that the manuscript is easier to follow.*

We are sorry that the information we provided was insufficient. We have now detailed the mutations to help the reader understand how interactions are altered.

> *Figure 1A is not necessary. Figure 1 overall is fairly predictable as there have been many papers using the persistent random walk as the best model to single cell migration (dating back to the early 1990's). The authors define a new term, angular memory, which they show decreases with increasing delta t as one would predict.*

We acknowledge that persistent random walks have already been observed for individual cells, as in references 3-4 cited in the introduction. Nevertheless, we believe that Figure 1 is important because not all cells migrate as persistent random walkers when isolated. Some migrate in a more exotic manner, resulting in superdiffusive behavior, as in references 5-8 cited in the introduction. Since we observe superdiffusive behavior at confluence (Figure 2), it was therefore necessary to show whether or not single cells were superdiffusive too. We also use this figure to introduce angular memory, a measure that, to our knowledge, has never been used before. According to intuition, it decreases to 0 for persistent random walkers, just as another resembling measure, velocity autocorrelation, would do. However, the angular memory of fractional Brownian walkers does not vanish with increasing delta t (Fig. 3D), while velocity correlation would, just as that of persistent random walkers. This difference makes angular memory much more appropriate for distinguishing between the two migration behaviors, and prompted us to introduce it in the first figure as a reference.

> *In the wound healing assay, which cells were measured? Leading edge or interior, and does it matter?*

Figure 5A shows that cells behave differently depending on their distance from the wound. This is because the traces shown correspond to the first few hours of the movie, during which the cells at the front begin to move first. Figure S5A shows the speed of the cells over time after the wound and indicates that the cells reach a stable speed after approximately 3 to 4 hours. Figure S5B shows the speed of the cells as a function of distance from the wound at steady state. These results show that the speed of the cells no longer depends on the distance from the wound at this stage. As indicated in the “Materials and Methods” section, we only considered time points beyond this stage for subsequent analyses of population-averaged MSD and velocity presented in Figure 5, so the location of cells at the front or rear was irrelevant.

> ***Reviewer #2 (Comments to the Authors):***

> *To migrate cells must spatially explore their environments, a process that is guided by intrinsic signals (adhesive and mechanical properties, etc) and extrinsic (gradient cues) signals. This exploration can occur on the single or multicellular level. In this study, the authors examine the effect of cell-cell interactions, guidance cues, and cell mechanics in the exploratory capacity of MDCK cells. The authors show that cell-cell adhesion provides a "infinite directional memory for migration" and cell speed is dependent upon the focal adhesion stability, cell mechanics, and the mobility of adherens junctions-these processes are modulated by vinculin.*
>
> *My three major concerns with the manuscript are as follows:*
>
> *(1) While there is potential new information about the role cell-cell junctions and guidance cues play in cell migration, there is not enough NEW insight presented. Rather the role of vinculin in these processes is expected given what is already known about its ability to control focal adhesion stability, mechanics, and adherens junctions.*

We agree that our cell migration results make sense based on the effects of vinculin mutants on the stability and force transmission of adhesions. Nevertheless, we argue that this was not the only possible scenario. Indeed, we find that none of the effects of vinculin mutants on AJs (except YE) have an impact on cell migration (Fig. S4C). One might have expected that the increased stability provided by the TL and T12 mutants would reduce the speed of collective cell migration, just as the YE mutant increased cell speed due to its altered stability. This is not what we found, and this reveals a nonlinear relationship between AJ stability and migration speed that could be investigated more thoroughly in future studies. Another example is that the effects of the mutants on force transmission in AJs do not impact migration speed at confluence but do impact directed collective migration (Fig. 6). One might have expected that vinculin-mediated force transmission in AJs would impact collective migration, whether directed or not.

More importantly, we show that the role of intercellular adhesion in cell migration is more complex than expected. Indeed, it depends on the timescale considered: intercellular adhesion is detrimental to short-term spatial exploration and beneficial in the long term (Fig. S3B). Such a timescale-dependent behavior is impossible to predict from previously known effects of the mutants or other molecular considerations. Furthermore, we show that this behavior can be fully explained by the coordination of velocities between neighbors, which depends on intact connections between AJs and the cytoskeleton via alpha-catenin, but is independent of vinculin mutants that connect AJs to the cytoskeleton in parallel with alpha-catenin. One might have expected these connections to also have an impact on velocity coordination, and thus on spatial exploration, but we show that this is not the case (Fig. 3). Finally, we show that directed collective migration has a negligible impact on cell exploration at our experimental timescale (Fig. 5), whereas we initially expected the wound to make migration more ballistic. This reveals that such a directional signal affects spatial exploration at much longer timescales than expected.

Overall, our results quantify the outcome of competing effects and provide timescales at which one effect outweighs the other in influencing cell migration. We believe this is an original approach that provides substantial new insights into collective cell migration.

> *(2) The phenotypes of the cells expressing the mutant vinculins varying greatly. These phenotypes are not addressed despite the fact that they could potentially complicate the analyses. For example, there are dramatic differences between focal adhesion numbers and sizes in the cells expressing the different vinculin mutants; cell spreading is also dramatically altered. Likewise, the T12 mutant vinculin has previously been reported to have increased adhesive strength, increased traction forces, and cell spreading. How does this knowledge change the interpretation?*

We agree that vinculin mutants may have effects on the size and number of FAs, cell spreading, and traction forces that we do not examine here. These consequences can be explained by the effects of these mutants on force transmission in FAs and on their stability, which we report in our work. They do not affect our interpretations. Here, we provide a predictive model of migration speed based on the combination of two consequences of vinculin function, namely stability and force transmission. An interesting avenue for future research would be to assess whether these combinations can be reduced to a single higherlevel effect of vinculin on the cellular phenotype that would be sufficient to predict migration speed. This work remains to be done, as neither FA size and number, cell spreading, adhesion force, nor traction forces alone are sufficient to predict migration speed.

> *Along the same lines, it has previously been established that tagged version of vinculin do not efficiently integrate into adherens junctions. Published work from the Nelson laboratory suggests that GFP-vinculins do not localize to cell-cell junctions and work from other laboratories suggests localization occurs only when the endogenous vinculin is silenced.*

We are aware that some GFP-vinculin constructs may not localize as well as the endogenous protein at AJs. This is due to the localization of the GFP tag on the head of vinculin and depends on the length of the linker between GFP and the head of vinculin. The longer the linker, the easier the interaction with AJ partners. Unlike these constructs, the vinculinTSMod sensors we use in our work do not carry a GFP on the head and do not suffer from the same limitations.

Furthermore, vinculin recruitment to AJs depends on force, with little or no recruitment when tension on the AJs is relaxed (DOI: 10.1038/ncb2055). Vinculin recruitment has in fact already been used as an indicator of AJ tension in Drosophila (DOI: 10.1038/s41467-01807448-8). Consequently, the amount of vinculin visible at the AJs varies depending on the tension exerted on the AJs, which our results confirm: vinculin is more difficult to detect at the AJs in cells located at the front of a wound than in those located at the back (Fig. 6B), which is consistent with the difference in vinculin tension between front and back cells (Fig. 6C) and to the E-cadherin tension gradient between front and back cells (DOI: 10.1083/jcb.201706013). Overall, these results show that vinculin is not always easy to detect at AJs, but this is due to the properties of vinculin, which the constructs we use reproduce better than previous constructs (see also below).

> *The images in figure S2 and the prebleach images in figure S4 do not show convincing localization of the mutant vinculins to cell-cell adhesions. This is of special concern given that YE mutant protein hardly has any discernable localization to cell-cell junctions; additionally, none of the mutant proteins were tested for their ability to co-localize with adherens junction components. This raises the question if the parameters being examined and the conclusions drawn from them are affected by a difference in localization.*

We agree that the recruitment of vinculin at intercellular contacts may be difficult to see.

Besides force-dependent effects mentioned above, other factors are involved. The images shown in Figures S2 and S4 are from live cells in which cytoplasmic vinculin is still present, and its level proportional to the mobility of vinculin. Indeed, the TL and T12 mutants show a more marked contrast between intercellular contacts and the cytoplasm, which is consistent with their greater stability at AJs (Fig. 4F). Conversely, YE shows lower contrast, which is consistent with the lower stability of this construct at AJs (Fig. 4F). The FL construct lies between the two. As a result, the cytoplasmic content can variably mask vinculin recruitment at the AJs depending on the mutant.

We have now performed additional quantifications of mutant recruitment at intercellular contacts as a function of distance from the basal surface of the cells and relative to their recruitment in FAs, in live cells. Results are shown in the new Fig. S4F. We find that all the constructs are recruited to intercellular contacts with a density that is at most half of that in FAs and that varies along the height. FL shows the highest density, localized more apically, consistent with the localization of an AJ-bound actin belt. The mutants appear to be more homogenously distributed along the height of the lateral surface, which may be explained by their impaired autoinhibition (TL, T12), or mechanosensitivity (YE). This variability also contributes to the difficulty in seeing vinculin recruitment in all cells in a single z-slice.

To confirm the proper recruitment of vinculin constructs to AJs we have performed immunofluorescence against alpha-catenin and phalloidin on each of the stable cell lines. Results are shown in the new Fig. S4D and E. In these experiments, cell permeabilization allows for the release of some of the cytoplasmic pool of vinculin, which highlights the recruitment of all vinculin constructs to intercellular contacts. There, all vinculin constructs colocalize with alpha-catenin and F-actin, as expected. Additionally, images displayed are maximum intensity projections to mitigate recruitment variability along the height.

Overall, our results clearly support the localization of vinculin at intercellular contacts, and the differences between the constructs are consistent with the effects of their mutations.

> *(3) There is a lack of new mechanistic insight. Conclusions are made about a role of vinculin dimerization. This conclusion appears to be based upon the usage of the mutant version of vinculin Y1065. Did the authors directly measure the ability of this mutant protein to dimerize? Is actin binding also affected.*

The binding properties of the Y1065E mutant, including its dimerization and binding to actin, have already been characterized by other researchers (ref. 40 in our manuscript, as well as DOI:10.1111/j.1432-1033. 1997.01136.x or DOI: 10.1016/j.febslet.2013.02.042). We assumed that these properties are now well established and can be used to explain higher-level phenotypes that we show for the first time, to our knowledge.

> ***Reviewer #3 (Comments to the Authors):***
>
> *Canever et al. tracked two epithelial cell lines on collagen coated glass and showed that isolated cells (non confluent) move as persistent random walkers, whereas confluent monolayers migrate super diffusive, with long range directional memory. By systematically perturbing adhesion machinery they found that focal adhesion mutations mainly tune the speed of single cell tracks, but cannot create long range memory, while force bearing adherens junctions are essential for the super diffusive regime-genetically perturbing them collapses collective memory. These interesting results identify junctional tension as important to switch epithelial cells/sheets between individual and collective search modes - an important quantitative insight that is of clear relevance to cell biologists.*
>
> *- The presented data is nicely quantitative and convincing, but I have subtle concerns about the generality of the findings. While the authors show that the differential behavior, they describe is not cell-line specific (MDCK, RPE), there are no experiments evaluating the generality of their conclusions across different matrix conditions. How are the measured migration parameters affected by matrix stiffness? Cell migration on collagen coated glass coverslips is a relatively narrow and artificial condition. How is the collective directional memory expected to behave on softer substrates? The generality of the conclusions could be strengthened by repeating measurements using hydrogels of varying stiffness. Further, it should be discussed to which tissues in the body the selected matrix conditions and migration modes plausibly apply.*

We agree that the generality of our results and the relevance of glass-rigid substrates is an important point. In vivo, epithelial cells rest on a basement membrane with a typical stiffness of approximately 10 MPa, as demonstrated by experimental evaluations on various tissue explants, including renal glomeruli and Bruch's membrane, which are relevant to MDCK and RPE-1 cells (DOI: 10.1111/j.1742-4658.2007.05823.x, DOI: 10.1172/JCI106898, DOI:10.1038/eye.1987.35), we have added these references in the manuscript to support our experimental strategy. In vitro, the most significant effects of substrate stiffness on FAs and cell migration generally occur at much lower stiffnesses, between 0.2 and 100 kPa, and cell phenotypes generally plateau at levels comparable to those observed on glass, even below 100 kPa (DOI: 10.1242/jcs.133645, DOI: 10.1038/ncb3268, DOI:10.1039/c5ib00307e, DOI: 10.1039/c9sm01893j). Furthermore, substrate stiffness has much more moderate effects on confluent cells than on isolated cells. For example, it has been previously demonstrated that confluent layers of MCF10A epithelium showed no change in velocity coordination in the range of 3 to 65 kPa (DOI: 10.1083/jcb.201207148). Therefore, collagen-coated glass appears to be a reasonable model for the basement membrane. Overall, we believe that we have conducted our experiments under physiological conditions, and that our results apply to a wide range of substrate stiffnesses.

> *- It would be nice to see how long it takes confluent cell layers to close rectangular wounds of defined size when cells migrate as individual (adherens junctions perturbation) versus collective (wt) (on substrates of different stiffness). Presumably, there should be faster wound closure under the collective regime, at least for simple shaped wounds.*

This is an interesting question, which our results indirectly address. In our study, we measured the wound healing speed of the WT MDCK cell line as well as lines expressing mutant vinculin constructs (Fig. 6A). These results show that this speed ranges from 5 to 15 µm/h depending on the construct expressed (and for reasons that we explain in the manuscript). These values make it easy to estimate the time required to close a wound based on its width. For example, it would take 5 hours to close a 100 µm wide wound for the WT cell line, which has a rate of 10 µm/h (on both sides of the wound).

Wound closure for cells with disrupted adhesive junctions has already been documented (DOI: 10.1083/jcb.200910041). The results show that wound closure is indeed slower than with WT cells. Although this previous study does not reveal the underlying causes, our work now shows that there are two factors: weaker directional memory due to impaired intercellular coordination and, in the longer term, an additional lack of sensitivity to the guidance signal provided by the wound.

> *- Akin to substrate stiffness variation, I am missing experiments that test the effect of cytoskeletal tension on these migration modes. Experiments with Rho kinase or myosin inhibitors could meaningfully broaden the scope of this study.*

Rho kinase or myosin inhibitors applied to cells during the time required to assess migration patterns (a movie recorded overnight is necessary to obtain a statistically reliable calculation of MSD over 3 to 4 hours) are likely to affect many other cellular processes in addition to the cytoskeletal tension directly involved in migration. We believe that the accumulation of these effects will make interpretation of the results very difficult. For example, it has been shown that inhibition of ROCK by Y27 promotes healing of corneal endothelial lesions by affecting proliferation through cyclin D and p27 (DOI: 10.1167/iovs.13-12225), or by improving respiration, which would provide the energy necessary for migration (DOI: 10.1096/fj.202101442RR). Consistently, another study on HaCaT epidermal cells confirms that myosin phosphatase accelerates wound healing through proliferation (DOI: 10.1016/j.bbadis.2018.07.013). In contrast, in HUVEC cells, ROCK inhibition significantly

impaired the proliferation and migration of vascular endothelial cells in vitro in a dose-dependent manner (DOI: 10.1097/ICO.0000000000000493).

Furthermore, previous studies have highlighted that differential contractility at the subcellular level is important for collective migration (DOI: 10.1038/ncb2133, DOI: 10.1083/jcb.201706013), which is not possible to examine with global activation or inhibition of contractility. This prompts the development of more refined and specific measurement and disruption strategies to assess the respective impact of cytoskeletal tension on cell-cell and cell-matrix adhesion mechanisms. Our work, which uses biosensors to assess how this tension differentially affects cell-cell and cell-matrix adhesions, is a step in this direction. The localized spatio-temporal activation or inhibition of myosin subtypes or Rho GTPase regulators specific to these adhesion structures will likely answer these questions in the future, but we believe that the development and application of these approaches will require a substantial amount of work that goes beyond the scope of our study.

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