

# Tumour follower cells: A novel driver of leader cells in collective invasion (Review)

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**Abstract.** Collective cellular invasion in malignant tumours is typically characterized by the cooperative migration of multiple cells in close proximity to each other. Follower cells are led away from the tumour by specialized leader cells, and both cell populations play a crucial role in collective invasion. Follower cells form the main body of the migration system and depend on intercellular contact for migration, whereas leader cells indicate the direction for the entire cell population. Although collective invasion can occur in epithelial and non-epithelial malignant neoplasms, such as medulloblastoma and rhabdomyosarcoma, the present review mainly provided an extensive analysis of epithelial tumours. In the present review, the cooperative mechanisms of contact inhibition locomotion between follower and leader cells, where follower cells coordinate and direct collective movement through physical (mechanical) and chemical (signalling) interactions, is summarised. In addition, the molecular mechanisms of follower cell invasion and metastasis during remodelling and degradation of the extracellular matrix and how chemotaxis and lateral inhibition mediate follower cell behaviour were analysed. It was also demonstrated that follower cells exhibit genetic and metabolic heterogeneity during invasion, unlike leader cells.

## Contents

1. Introduction
2. Cooperative mechanisms of CIL
3. Coordination and guidance of movement through biomechanics
4. ECM degradation and/or remodelling
5. Chemotaxis and invasion assays
6. Lateral inhibition
7. Genetic heterogeneity
8. Metabolic heterogeneity
9. Conclusions and perspectives

## 1. Introduction

Cancer cells include epithelial cells and complex stromal cells (1,2). To maintain motility, cancer cells can modify their morphology and migration patterns according to the environmental conditions they are exposed to. The initial stage of tumour metastasis is invasion, during which cancer cells move away from their original location. Collective cell migration may confer a more powerful invasive capacity on cancer cells than individual cell migration (3). Collective invasion is accomplished by both leader and follower cells moving together in cooperation. The leader cell directs the group, while the follower cells follow closely behind (4-6). The morphology of leader and follower cells differs significantly, with leader cells being polarized and spindle-shaped, while follower cells are tightly packed and maintain epithelial sheet-like properties (7). In addition, epithelial tumours have a predominant retention of epithelial characteristics and intercellular adhesion. Conversely, mesenchymal tumours typically invade individually, with only certain subtypes displaying collective invasion tendencies through epithelial-mesenchymal transition (EMT) (8). As an example, histopathological analyses frequently reveal that squamous cell carcinoma (SCC) invades in cluster forms (9). In organotypic coculture invasion models, cancer-associated fibroblasts (CAFs) act as leader cells to coordinate cancer cell migration (10). Follower cells in lung cancer exhibit an epithelium-like morphology when cultured in two dimensions, whereas leader cells appear mesenchyme-like (11). Therefore, further study of epithelial

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tumours is essential to gain a comprehensive understanding of follower cells.

Numerous studies have been conducted recently on the characteristics of leader cells *in vivo* and *in vitro*, demonstrating their important role in collective invasion (11-13). Furthermore, follower cells, which comprise the majority of the cell groups involved in collective invasion, have received increasing attention. Optimized spatiotemporal genomic and cellular analysis (SaGA) can specifically target, extract, separate and amplify leader and follower cells from a 3D microenvironment (11). The separated follower cells proliferate rapidly and divide frequently but are not highly invasive, whereas the separated leader cells are highly invasive, and divide and proliferate slowly (12,13). At the leading edge, specialized leader cells exert traction force on the follower cells (14). In addition to being pulled along by leader cells, follower cells are actively involved in selecting a specific direction of travel by extending protrusions underneath leader cells; these protrusions are known as cryptic lamellipodia (c-lamellipodia) (15). Since they typically possess c-lamellipodia, which are typically smaller and exhibit fewer adhesions with the extracellular matrix (ECM), the follower cells have less interactions with the ECM and exert less traction (16). The biomechanics generated by follower cells through c-lamellipodia facilitates collective invasion, allowing follower cells to invade as a group. Death of the leader cell causes the follower cells to cease migrating in the same direction, leading to random, slow movement of cells and an end to collective migration (17). Additionally, follower cells help the leader cells initiate appropriate polarization, strengthen leadership of the cells and ensure that sufficient leader cells are available to lead the invasion. This is achieved through the utilization of a multitude of signalling molecules, including chemotactic factors and physical contacts, whereby follower cells migrate in the wake of the leader cells and constitute a significant portion of the multicellular cluster. A variety of strategies are employed by follower cells to increase their invasive capacity, including contact inhibition locomotion (CIL), biomechanics, matrix remodelling, chemotaxis, lateral inhibition and exhibiting genetic and metabolic heterogeneity (18-21) (Fig. 1). Elucidation of the role of follower cells in collective invasion may identify new molecular targets for cancer treatment and intervention.

## 2. Cooperative mechanisms of CIL

*Homotypic CIL between leader and follower cells.* CIL is a multifaceted procedure that changes cell movement when one cell collides with another cell, and inappropriate regulation of CIL may promote the spread of cancerous cells. Normal cells exhibit strong CIL when adjacent cells come into contact with each other, helping to maintain proper tissue architecture and prevent overgrowth. This process is achieved through the activation of proteins involved in cell adhesion, cytoskeletal rearrangement and signalling pathways that suppress cell proliferation and migration (22,23). However, cancerous cells are defective in these pathways and display weaker or disrupted CIL, which may be caused by mutations or alterations in genes that regulate the aforementioned processes (22,23). One of the key differences in CIL mechanisms between normal cells and cancerous cells is the loss of stable cell-cell

adhesions in cancerous cells (24). Cancer cells decrease Ras homolog family member A (RhoA) activation at the front of the cell cluster, resulting in weaker intercellular adhesion and increased cell migration (10). As a result, cancerous cells can exhibit uncontrolled cell motility and tissue invasion.

CIL between cells of the same type is known as homotypic CIL, exhibited by both normal and tumour cells. By contrast, CIL between cells of different types is known as heterotypic CIL, which is often lost by tumour cells when they encounter normal cells. Homotypic CIL is established between leader and follower cells for collective migration. Initial contact followed by varying degrees of protrusion inhibition at the contact site enables the leader and follower cells to form protrusions towards the basement membrane in the direction of the movement, thus facilitating the directed migration of the cell cluster (23) (Fig. 2A). Certain types of sarcoma, such as the S180 and BAS56 cell lines as well as melanoma cells, with inappropriately regulated CIL acquire the capacity for collective motility in the cancer cell population, and they invade areas occupied by other types of cells (25). These CIL properties can promote tumour aggressiveness by preferentially directing cancer cells into the stromal environment in the form of clusters. During this process, leader-follower intercellular CIL is induced by intracellular signalling and mechanical coupling.

### *Establishing contact between leader and follower cells.*

Contacts between leader and follower cells are dynamic and continuous during collective invasion, which is the foundation of CIL. The follower cells follow the leader cells very precisely through cell-cell contact, exhibiting the characteristics of CIL (26-28). A different distribution of adhesion proteins has been observed between leader and follower cells when exposed to different levels of extracellular signals (26-28). The membrane proteins involved in the interaction between cells are E-cadherin and N-cadherin-coordinated adherens junctions (AJs), Ephrins/Eph receptors, Ig-superfamily proteins and planar cell polarity (PCP) members (Fig. 2B) (29). In addition to forming mechanical bonds between cells, membrane proteins also function as ligands/receptors that regulate intracellular signals, such as for determining cell polarity and cytoskeletal dynamics. Moreover, intercellular AJs combined with CIL prevent follower cell perimeter integrins from contacting the ECM, resulting in common adhesion structures and protrusions in any direction (30). As a result, this process significantly increases the efficiency of collective invasion.

*Activities of the cadherin family in CIL.* CIL involves the establishment of transient adhesion sites between cells via cadherins. Leader cells exhibit asymmetrical AJs, with integrin-based focal adhesions (FAs) at their extending fronts and cadherin-based AJs at the intercellular junctions on the trailing edges (17). However, follower cells possess symmetrical cadherin-based AJs that inhibit protrusion formation throughout their periphery (17,31). During the CIL of different cell types, different types of cadherins are found at the cell-cell contacts, generally at AJs, such as E-cadherin, N-cadherin and cadherin11 (23,32). The importance of E-cadherin for CIL in particular has been demonstrated. A high level of E-cadherin expression within follower cells is related to an epithelial phenotype and maintains intercellular

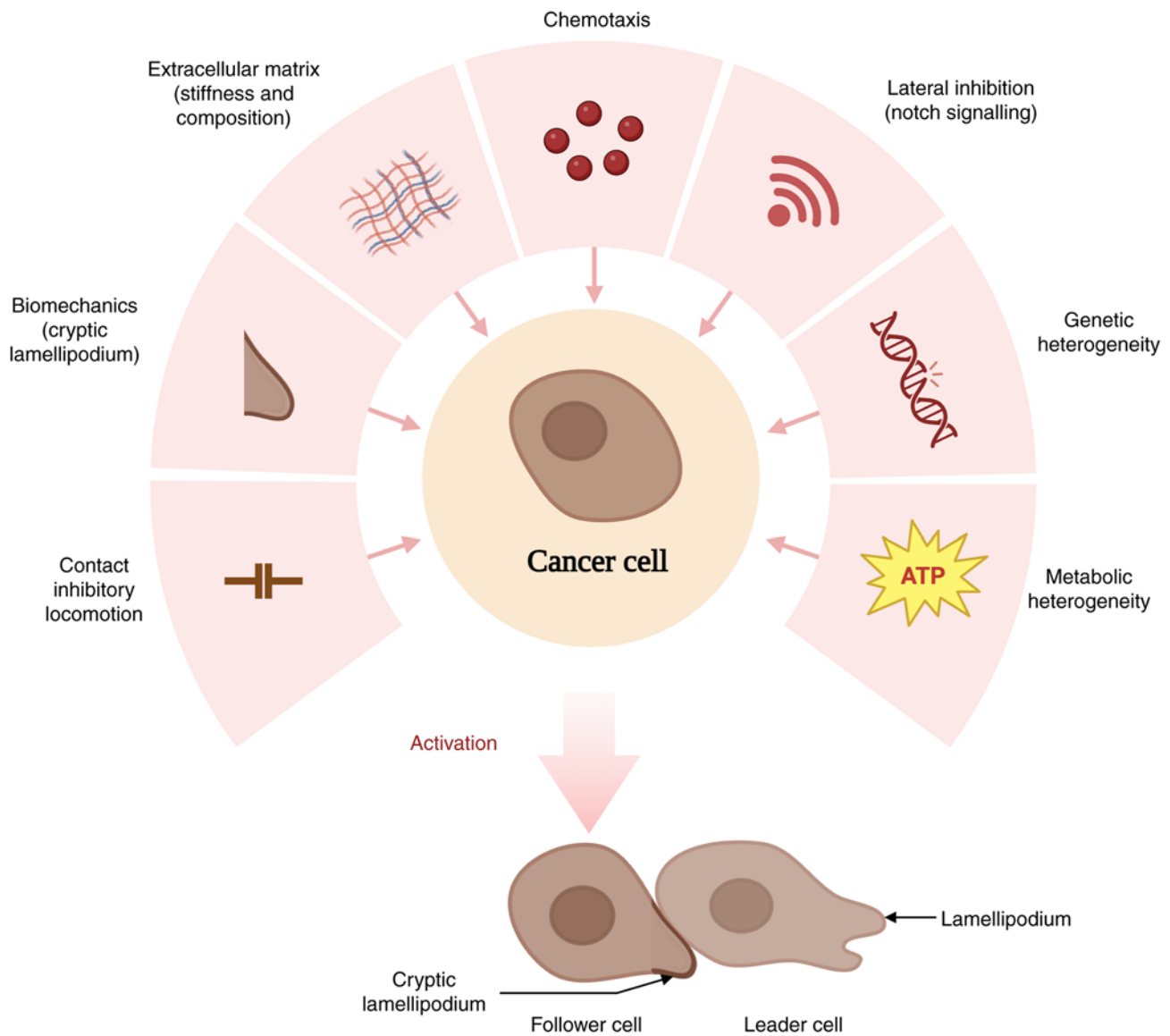


Figure 1. Influencing follower cell behaviour. During collective invasion, the invasive behaviour of follower cells is influenced by intercellular CIL, biomechanics, chemotaxis, lateral inhibition, genetic and metabolic heterogeneity and interaction with the extracellular matrix in the tumour microenvironment. i) CIL facilitates the inhibition of protrusions by establishing contact between the follower cell and the leader cell. ii) The cytoskeleton of follower cells forms c-lamellipodia under the regulation of biological signals. The traction and stress generated by follower cell movement is transmitted between cells to promote and limit the formation of leader cells. iii) Chemical signals released by follower cells cause collagen fibres to rearrange around cancer cells, and the mechanical transmission generated during movement alters matrix stiffness to facilitate the onset of collective invasive behaviour. iv) Follower cells move directionally towards the appropriate chemical gradient to promote their invasive behaviour. v) Lateral inhibition affects the cellular state during collective invasion through intercellular Notch-Delta-Jagged signalling; follower cells with upregulated Notch expression suppress their Delta expression and promote their Jagged expression, thereby consolidating the leader cell position. vi) Leader and follower cells differ in gene expression and jointly influence invasive behaviour with epigenetics. vii) The follower cells are more dependent on glucose transporter 1-mediated aerobic glycolysis. When the leader cells cannot provide enough energy to lead the collective invasion movement, the follower cells switch positions with the leader cells. The figure was adapted from 'Mechanisms of Cancer-associated Fibroblast Activation', by BioRender.com (2023). Retrieved from <https://app.biorender.com/biorender-templates>. CIL, contact inhibition locomotion.

CIL (33). E-cadherin is therefore generally considered an influential molecule in maintaining epithelial differentiation and counteracting cancer invasion (34). This finding may explain why the loss of E-cadherin contributes to migration *in vitro*, and its loss may adversely affect breast cancer metastasis *in vivo* (35).

The differential cadherin expression in leader and follower cells indicates that cadherin conversion might be associated with CIL acquisition (36,37). Through this cadherin modulation, cells can establish distinct adhesion properties that enable

the coordination of collective cell migration (36). The ideal ratio between E-cadherin and N-cadherin in follower and leader cell populations remains unknown and this ratio may vary depending on cell type and context. In general, E-cadherin is the cell-cell adhesion molecule most abundant in adherent cells. As such, in leader cells, which are highly mobile, the ratio of E-cadherin to N-cadherin is low (36,37). Conversely, in a follower cell, where adhesion activity is needed to maintain and guide its migration, the ratio of E-cadherin to N-cadherin is higher (38). Moreover, the ideal ratio between follower and

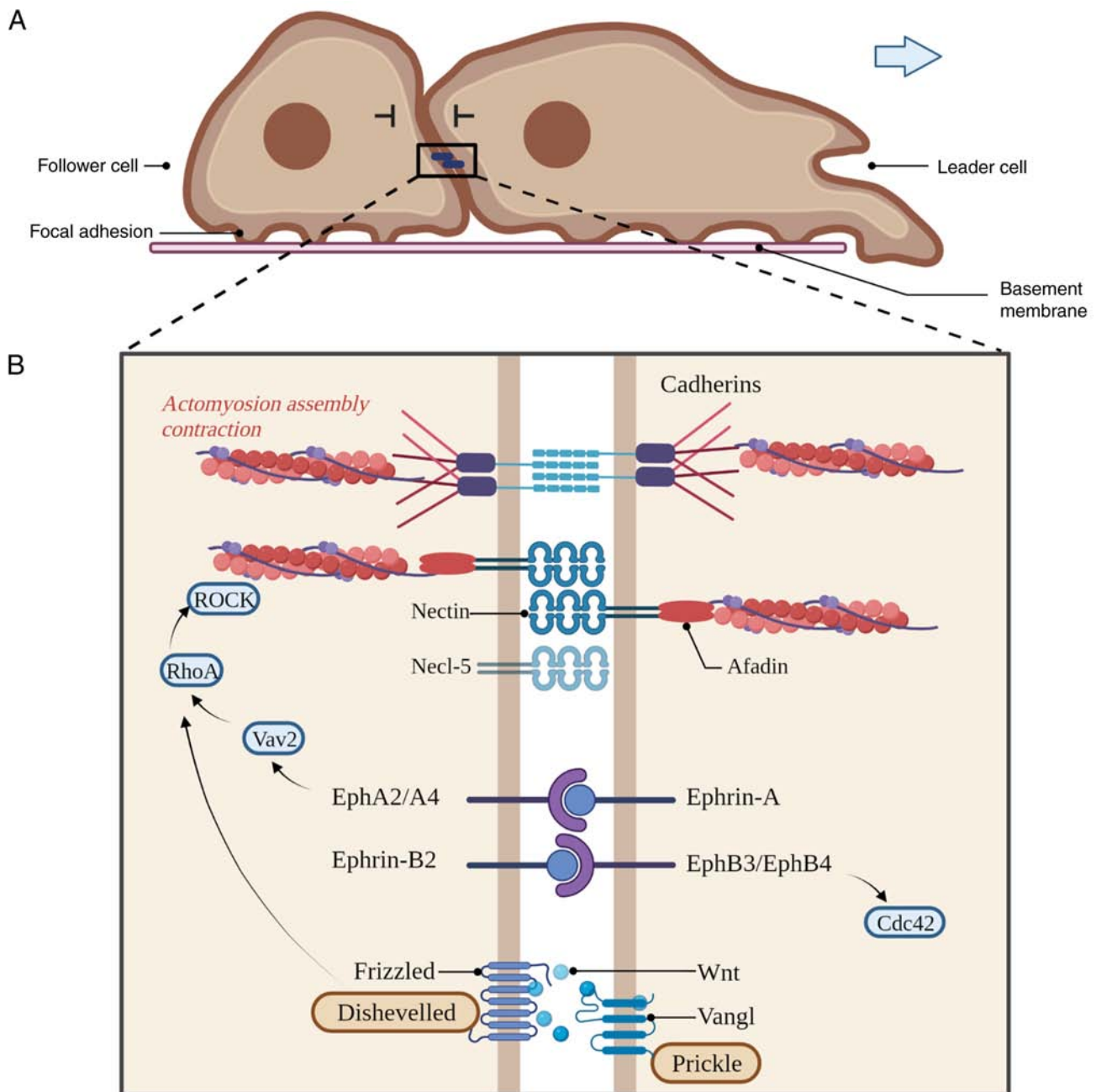


Figure 2. Intercellular surface receptors for homotypic CIL between leader and follower cells. (A) Leader and follower cells are the different invasive states of cancer cells during collective invasion. Leader and follower cells come into contact with each other to induce homotypic CIL (black inhibitory arrows), inhibit protrusion at the cell interface, alter cell polarity and promote collective directed movement of cancer cells across the basement membrane through focal adhesions. (B) After trans-interaction between nectin-3 and Necl-5, endocytosis removes Necl-5 from the cell surface. Next, trans-interaction between nectins occurs, which are linked to the cytoskeleton by afadin. Cell-cell adhesion sites based on nectin recruit cadherins. The proportion of ephrin-A/ephrin-B2 can affect the degree of RhoA activation in follower cells. The core planar cell polarity complexes demonstrate mutually exclusive localizations of Frizzled-Dishevelled and Vangl-Prickle. The leading edge of follower cells has Frizzled-Dishevelled, whereas the trailing edge of leader cells has Vangl/Prickle. The aforementioned cell surface proteins regulate the contact between leader and follower cells and are involved in CIL by regulating Rho GTPase. The figure was created with BioRender.com. CIL, contact inhibition locomotion; Necl-5, nectin-like molecule-5; RhoA, Ras homolog family member A; ROCK, EphA-Rho-Rho kinase.

leader cells depends on the tumour environment. For example, a higher ratio of N-cadherin has been reported to enhance cell migration, a process that involves expansive migration along tissues, environments and individual cells (36,37). By contrast, in developing epithelial layers, a higher ratio of E-cadherin to N-cadherin has been found to assist strong cell-cell adhesion, allowing cells to remain closely packed and form an epithelium (38).

When N-cadherin or E-cadherin expression is knocked down, the number of multicellular invasion chains are greatly decreased due to disrupted cell-cell junctions (38). Furthermore, in collective invasions of cancer cells, e-cadherin is diminished as a result of partial EMT, which results in the reprogramming that leads to the destabilization of cell-cell junctions and leads to increased number of invasive and metastatic cancer cells (24). As neural crest (NC) and cancer

cells undergo EMT, the switch from E-cadherin to N-cadherin contributes to the invasiveness of CIL by promoting cell detachment and enhancing migratory capacity (23,36,39). CAFs and mesenchymal leader cells are capable of guiding follower cells via heterotypic N- and E-cadherin interactions *in vitro* and *in vivo* (40,41). In addition, M2 macrophage leader cells isolated from an infection and inflammation mouse model were found to express E-cadherin (42). This observation suggests that E-cadherin may be involved in homotypic (between M2 macrophages) and heterotypic (between different cell types) interactions, possibly mediated by IL-4 and polyamine-induced E-cadherin/catenin complexes (42). However, it is unclear whether E-cadherin plays a role in collective tumour invasion dominated by tumour-associated macrophages through CIL.

**Other receptor families involved in CIL.** In addition to cadherin-based AJs, other receptor families are involved in establishing initial cell contact during CIL. Studies have found that nectin forms AJs before cadherin forms intercellular adhesion between leader and follower cells in many cultured cell lines, such as Madin-Darby Canine Kidney and MCF-7 cells (43,44). In addition, the removal of Necl-5 from the cell surface through endocytosis inhibits Ras-mediated cell proliferation signalling and contributes to the induction of CIL via the phosphorylation of sprouty2 by Src, as leader cells and follower cells establish intercellular contact (45). Notably, a major function of nectin-like molecule-5 (Necl-5) is to extend protrusions at the leading edge and generate traction in the direction of collective motility, which ultimately promotes cell invasion (46). Invasion and metastasis of cancer cells may also be influenced by the upregulation of Necl-5 (47,48).

An Eph receptor is a tyrosine kinase receptor that binds transmembrane ligands on adjacent cells, and bidirectional signalling resulting from Eph-ephrin interactions can lead to adhesion (49). The binding of ephrin-A ligands to EphA2 and EphA4 receptors leads to RhoA activation, contributing to homotypic CIL. As a result of RhoA activation at cell contact, membrane protrusion collapses and cell polarity is altered, resulting in directional migration (50). The failure of CIL depends on the activation of EphB3 and EphB4 by ephrin-B2 at the contact site in follower cells (50). However, the cell migration response is regulated by the ratio of ephrin-A/ephrin-B2 in follower cells, which determines whether cancer cells exhibit CIL (Fig. 2B) (50,51). Moreover, homotypic CIL is triggered by EphA-Rho-Rho kinase (ROCK) signalling in prostate cancer cells when two cells come into contact (50).

The contact between leader and follower cells triggers the transmission of CIL to the migrating population through Wnt/PCP signalling, which in turn activates RhoA (52-54). The leader and follower cell populations of the Wnt/PCP core component complex are asymmetrically localized, which regulates the collective invasion of cancer, at least in part (55).

**Cytoskeletal dynamics of protrusion inhibition and polarization.** In addition to the leader-follower cell contact, the actin cytoskeleton also plays a critical role in regulating CIL, cell morphology and polarity. In CIL, nectin-based cell-cell contact reorganizes the actin cytoskeleton and modulates cell polarization (28). Protrusions are typically inhibited through actin-mediated contraction at the contact site. This sudden

contraction also causes polarization of cancer cells at the leading edge. In CIL, the initial contact of cell surface proteins may regulate Rho GTPases. Specifically, cadherin activates intracellular signals, such as RhoA and Ena/vasodilator-stimulated phosphoprotein (VASP), which regulate cell polarity and cytoskeleton dynamics (56). RhoA is located at the cell contact site and induces stress fibre formation (57). In addition to inhibiting protrusions at the site of contact, cadherin junctions also exert local control over the expression and activity of Rac and actin-related protein-2/3 (ARP2/3) (58,59). Leader cells contain Rac, integrin  $\beta$ 1 and PI3K proteins at their leading edge, whereas the follower cells lack these proteins at their leading edge, and blocking these proteins impairs the movement of both types of cells (17). By binding and inhibiting RhoA, p120 regulates the cadherin-actin cytoskeleton through indirect activation of Rac1 and Cdc42 via Vav2, promoting the formation of protrusions and polarization (60-62). Rac1 and RhoA participate in the process of establishing front-rear polarity through mutually antagonistic interactions (63). Activated RhoA suppresses Rac1 activity, preventing excessive protrusion at the leading edge and ensuring proper cell adhesion. Cdc42 controls actomyosin arrangement to generate the force necessary for follower cells to follow the tracks of leader cells (63). Furthermore, Cdc42 is able to activate Rac1, thereby contributing to the generation and maintenance of cellular protrusions at the leading edge (63). The leading edge involves the engagement of all three GTPases (Rac1, RhoA and Cdc42), working together to control and regulate key aspects of cell migration (64). At the rear of leader cells and among follower cells, Rho and related proteins control actin contractility, resulting in the collapse of protrusions in response to cell contact (Fig. 3A) (65).

In addition, Rho GTPases regulate actomyosin contractility through F-actin polymerization and myosin light chain phosphorylation in both follower and leader cells (66). The carboxy terminus of discoidin domain receptor 1 (DDR1) is suggested to regulate cell polarity by recognizing the PDZ domains of Par3/Par6 (67). The DDR1/Par3/Par6 complex between follower cells inhibits ROCK-mediated actomyosin contraction by controlling RhoE recruitment to cell-cell interfaces. After depletion of DDR1, Par3 or Par6, actomyosin contractility increases, cohesion is lost and collective cell invasion is defective (68). By contrast, cancerous cells invade collectively due to a reduction in actomyosin contractility that is controlled by DDR1 at the intercellular junction between follower cells and leader cells (54,68). After knockdown of either Cdc42 or both Cdc42-binding protein kinases related to myotonic dystrophy kinase isoforms (MRCK $\alpha$  and MRCK $\beta$ ), the localisation and phosphorylation of myosin light chains at the cortex is significantly disrupted, affecting the invasion ability of follower cells (10). As a result, signalling events can be activated in a context-specific manner at specific subcellular sites with precise kinetics (69).

Actin cytoskeletons between leader and follower cells are connected by tight junctions, such as junctional adhesion molecules-a (JAM-A) (70). The downstream effects of Src are activated when JAM-A is deleted. This activation leads to the activation of various proteins, including extracellular signal-regulated kinase 1/2 (ERK1/2), Abi1, and paxillin. Additionally, the activity of Rac1 is also increased at the



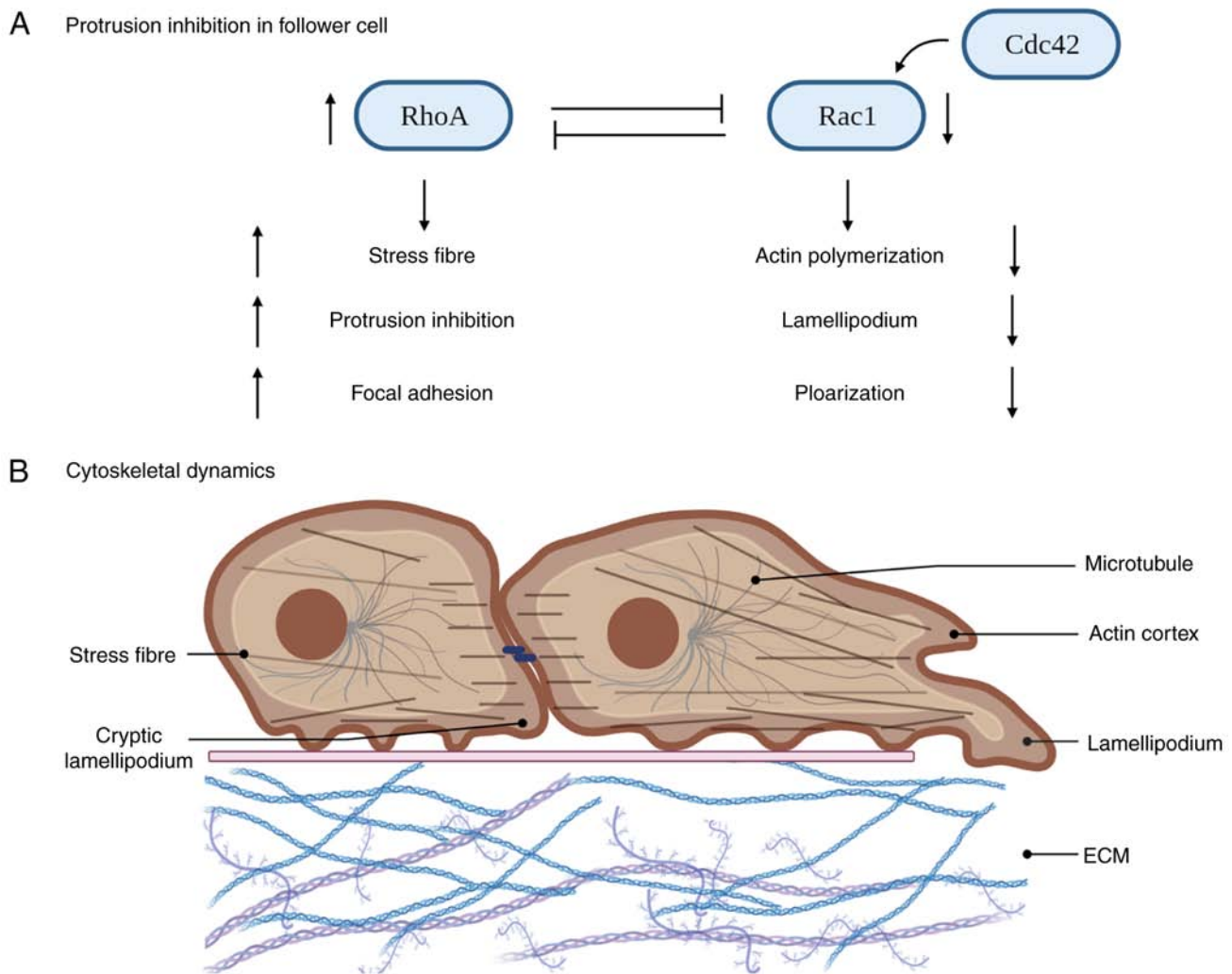


Figure 3. Protrusion inhibition and cytoskeleton dynamics in follower cells. (A) Intercellular contacts are involved in regulating Rho GTPase-mediated protrusion inhibition in follower cells. All three GTPases (RhoA, Rac1 and Cdc42) are activated in the anterior region of migrating cells. Protrusion collapse and cell polarity are controlled by RhoA, which is located at cell contact sites and induces stress fibre formation and actin-mediated contraction. When intracellular mechanical tension increases, it induces increased focal adhesion formation and stress fibre formation in follower cells. A mutually antagonistic interaction between Rac1 and RhoA establishes anterior-posterior polarity, and Cdc42 can activate Rac1. (B) CIL leads to changes in cell morphology, and this change is maintained by the actin cortex. Through microtubule capture at the leading edge, Rac and Cdc42 are activated to stabilize microtubules and to maintain the direction of polarization and migration during CIL. Rac1 polarization induces the polymerization of F-actin and the formation of c-lamellipodium. The figure was created with BioRender.com. CIL, contact inhibition locomotion; ECM, extracellular matrix; RhoA, Ras homolog family member A.

cell-cell contact site when JAM-A is deleted (71). As demonstrated in a study, a follower cell lacking JAM-A also migrated more quickly and was incapable of stopping when it collided with other cells (71). Consequently, CIL is severely impaired in the absence of JAM-A.

Follower cells undergo morphological changes as well as changes in their actin cytoskeleton (Fig. 3B) (72), which is maintained by the actin cortex, located on the side and back of the plasma membrane. Myosin II-dependent contractions and the formation of stress fibres coordinate the CIL response of colliding cells (73). A tight collaboration between actin and microtubule dynamics is observed *in vivo* during cell polarity and CIL (73,74). Migration of follower and leader cells may be initiated by retraction at the back of the cell (22). A major motor of the actin cytoskeleton is myosin II, which is activated within the cell body and at the rear of the cell prior to a spatial bias in actin polymerization at the leading edge of the cell (22). In contrast to normal cells, malignant cells have a reduction in

the absolute amount of F-actin (75). Flowing actin networks act as mechanotransmitters, providing tactile communication of CIL between cells, and transient stress fibres are formed as a result of the coupling of colliding actin networks (73). Transient stress fibres are also formed when protrusion tension increases, which facilitates cell migration. The tubulin of normal cells is extensively tyrosinated, whereas the tubulin of cancer cells is often de-tyrosinated, as observed in breast cancer tissues with poor prognosis (76). Microtubules can be linked to stress fibres by an actin-microtubule crosslinker. These structures align themselves between colliding cells during CIL based on the path of least resistance within the actin network (73). Rac and Cdc42 can stabilize microtubules, which inhibit the activity of stathmin (77). During CIL, Rac and Cdc42 activation maintain the direction of polarization and migration by controlling microtubule capture at the leading edge of the cell. Par3 can prevent the activation of Rac1 at the contact between cells and contribute to microtubule collapse (78). Migration

may disrupt cell-cell adhesion by releasing tension generated from engagement of the actin-clutch, and tension may cause damage to microtubule bundles or actin stress fibres (79,80). Overall, the investigation of microtubule dynamics and their relationship with actin stress fibres and signalling molecules provides valuable insights into the mechanisms driving collective invasion of tumor cells.

### 3. Coordination and guidance of movement through biomechanics

*c-lamellipodia formation.* Follower cells migrate through a dynamic cell-autonomous process by sending actin-dependent c-lamellipodia underneath the cells in front of them (81). c-lamellipodia are formed by adhesion proteins, such as wave and ARP2/3 complexes (82). These c-lamellipodia sporadically grow around E-cadherin-based AJs in adenocarcinoma-derived epithelial cells and tend to grow at junctions with mechanically weak surfaces (82). AJs also facilitate c-lamellipodia formation by recruiting actin regulators, which allows follower cells to migrate in an orderly fashion. C-lamellipodia growth can be uncontrolled when AJs are disrupted by the removal of  $\alpha$ E-catenin, which further results in myosin II activation and contraction of actomyosin cables associated with AJs (82). CIL and c-lamellipodia formation at cell-cell contacts appear to contradict each other. This contradiction arises due to the fact that c-lamellipodia formation is commonly associated with cell migration, while CIL involves the suppression of protrusion. A detailed examination of follower cells, however, revealed that not all follower cells strictly follow the CIL restrictions (82). In other words, follower cells located away from the leading edge may form c-lamellipodia on their basal surface, which is thought to exert small traction forces (29). Overall, traction and c-lamellipodia are less pronounced in follower cells. As WAVE and ARP2/3 complexes are distributed along the AJs, inhibiting them has the double effect of preventing the emergence of c-lamellipodia and preventing follower cells from trailing leader cells (82).

When leader cells die, the c-lamellipodia of nearby follower cells expand and exert substantial force, converting them into new leader cells and continuing collective cell migration (83). Breast cancer cell invasion, endothelial cell budding and epithelial tracheal branching are all instances in which leader cells appear transiently and are replaced by follower cells (84-89). To ensure that sufficiently qualified leader cells are present at the front of the collective invasion, follower cells gradually acquire the phenotype of leader cells when invading the complex microenvironment. The emerging actin cable joins the follower cell and two neighbouring leader cells together. After the follower cell has advanced to the leader cell via the contractile force along the actin cable, the original cable between them is interrupted and a thin extension from the new leader cell stretches to a tear (90). This distinct reconnection of the actomyosin cable between the follower cell and the neighbouring leader cell provides further insight into the mechanism of collective invasion.

*Follower cells pull on candidate leader cells.* A previous study using monolayer stress microscopy experiments demonstrated that mechanical interactions between follower cells determine

the emergence of leader cells (91). Generally, follower cells decide who becomes leader cells, not vice versa (92). Before collective invasion, follower cells generate local forces that can be transmitted to future leader cells and can be used to pull on them. This traction occurs before leader cells are formed. In a migrating follower cell, an actin network assembles into a branch at the leading edge and protrudes, extends and guides the cell, while an actin bundle near the trailing edge provides the force that constricts the cell posteriorly and propels it forwards (93). In response to this force, future leader cells polarize and form protrusions. After the leader cell forms, the leader cell pulls on the follower cell via actin contraction, and this cell continues to migrate, causing the next cell to be pulled and to transmit further directional signals (93). In addition, leader cells produce higher contractile capacity than follower cells (19,94). Depending on the length to which forces can be transmitted, the size of a cell group following leader cells may vary (91).

By interacting with its neighbours, a follower cell experiences a more substantial cumulative force. As a result of cell-cell adhesion proteins being distributed differently, actomyosin cytoskeleton contraction is heterogeneous at cell-cell junctions (27,95,96). Cellular stresses equilibrate traction forces, which are transmitted mainly by the cytoskeleton and intercellular junctions. Stress builds up throughout the migrating tissues and becomes more intense as the distance from the leading edge increases. It is also produced spontaneously in possible follower cells by the formation of c-lamellipodia (97). The stress field of a cohesive cell monolayer can also be estimated from traction force data. A substantial amount of tension builds during collective cell migration in expanding monolayers, as cell-cell junction stresses increase from edge to centre (98,99).

*Conversion of mechanical forces into biochemical reactions.* Two actin skeletons between cells are connected by cadherin, which form an intercellular force chain. Changes in actin contractility therefore affect cadherin tension (27). Mechanical forces may be converted into biochemical responses through molecular effects (100). Actin-regulating proteins, such as vinculin, formins, affadin, VASP, Zyxin and Testin, are recruited to cadherin junctions when tension is increased (41,101-103). P-cadherin, which forms associations with catenins, mediates force transmission between cells (104). The magnitude of intercellular tension is predicted by P-cadherin, whereas its build-up rate is predicted by E-cadherin. There is a competitive relationship between P-cadherin and E-cadherin during the response to a mechanotransduction pathway involving vinculin. When the level of E-cadherin is decreased, P-cadherin replaces its function to regulate tension, preventing a decrease in intercellular tension (104). P-cadherin-dependent collective cell movement is induced by Cdc42 through the regulation of intercellular stresses and traction force polarization (105). An acute stimulus by RhoA or an exogenous pulling force stimulates rapid AJ growth (106). E-cadherin expression in follower cells regulates intercellular mechanics through AJs, and these junctions stiffen cell aggregates and facilitate the transmission of traction forces (107-109). Via mechanical stimulation of E-cadherin, EGFR activates PI3K, which contributes to integrin adhesion and improves myosin

II-dependent cell contractility in breast carcinoma (110). In conjunction with these force responses, cytoskeletal remodeling and cortical stiffening are affected in junction-proximal regions, which may influence membrane protrusions and their extent and orientation (111). Increasing tension on E-cadherin enhances phosphorylation of  $\beta$ -catenin, lowering its stability at E-cadherin junctions and driving transcription to promote mesenchymal and basal fates (112–114).

The waves of ERK activation propagate through the leader and follower cells, linking them together and coordinating their behaviours (115,116). The pulling forces from leader cells can stretch the follower cells and activate the EGFR-ERK pathway, which then generates contractile forces on the rear side of the follower cells via ROCK signalling (115). ERK activation suppresses traction forces while activating contractile forces through the accumulation of F-actin in cell-cell contacts (116). It is possible that the impaired traction caused by the activation of ERK is due to the breakdown of actin stress fibres associated with FA, leading to the turnover of FA (116). The cells, via FA turnover, can contract efficiently upon contractile force generation, and the traction force gradually decreases between the follower cells. Consequently, collective invasion occurs through sustained traction forces, cell polarity and ERK activation.

Additionally, the negative feedback loop of Merlin-Rac interaction contributes to collective movement (117). Merlin, a tumour suppressor protein located at cell junctions in static monolayers, can translate the intercellular tension between leader and follower cells into molecular signals (117). Merlin is mainly restricted to cell junctions in follower cells by cell-cell tension but accumulates in the cytoplasm of leader cells due to high traction forces. Cell-cell tension induces Merlin to delocalize from cell junctions to the cytoplasm, where it coordinates Rac1 polarization. Rac1 polarization induces the growth of branching actin filaments and the formation of c-lamellipodium, which facilitates the migration of follower cells towards leader cells (117). The polarization of Rac1 activation in leader cells may stabilize merlin at the intercellular junctions by reducing Rac1 activity at the rear of leader cells and between follower cells (117). Aside from strengthening adhesion, Merlin expression in follower cells also enables these cells to pull and attract neighbours (118). Therefore, Merlin-Rac maintains both leader and follower cell functions in collective cell invasion.

#### 4. ECM degradation and/or remodelling

The ECM, offering both a biochemical and biomechanical context, plays a crucial role in cancer progression by regulating the ability of cancer cells to cross its barrier. By secreting diverse profibrotic growth factors and inflammatory factors, follower cells are primarily responsible for recruiting and activating stromal cells within the tumour microenvironment (119). In response to tumour-derived activation factors, stromal cells differentiate into CAFs, and CAFs act as myofibroblasts to reconstruct the ECM to facilitate tumour invasion (120–122). Follower cells themselves have also been shown to express altered ECM components, such as collagen I and III, and ECM-modifying enzymes (123–125). Lysyl oxidases are secreted by leader and

follower cells to crosslink collagen fibres (126). Crosslinked collagen stiffens the ECM, leading to integrin-dependent invasive behaviour. Due to increased ECM stiffness, force-loading rates at FAs change, resulting in the stretching of talin1 and vinculin recruitment (127). FA kinase (FAK), RhoA and Src are activated as a result of this, increasing the contractility of follower cells.

To regulate follower cell behaviour, integrins transduce signals from the ECM by assembling FAs, thereby stimulating follower cell cytoskeletal remodelling (128). Integrins act as major receptors for ECM molecules during cancer metastasis (129). The activation of integrins and downstream mechanotransduction adapters, such as p130CAS, occurs when mechanical tension is increased (130). As a result, there is an increase in FA and actin stress fibre formation in follower cells (130,131). In cancer, actin-rich protrusions attach to ECM molecules bound to integrins and to contractile structures within follower cells, causing the basement membrane to breach without protein hydrolysis (132). Moreover, follower cells can exert force on ECM networks, affect the ECM architecture reversibly or permanently and enhance stiffness and ligand density locally (132–134). A growing body of evidence indicates that the ECM is significantly stiffer in this state, compared with its normal state, indicating abnormalities in its composition and structure (135,136). The ECM is undoubtedly an important component of the tumour microenvironment, not only promoting malignancy but also regulating tumour invasion (20,135,136).

Follower cells may exhibit lower levels of durotaxis and matrix metalloproteinase (MMP) secretion than leader cells, but still play a critical role in the collective invasive process (137,138). Durotaxis is the directed migration of cells towards regions of high mechanical resistance. Moreover, cells migrate towards the ‘optimal matrix stiffness’ region, where they can generate the greatest traction, and when in a region above the optimal matrix stiffness, cells show an apparent tendency to migrate towards softer regions (138,139). By undergoing durotaxis, the follower cells are able to move towards regions of high mechanical resistance and utilize secreted MMPs to degrade the ECM and facilitate cell invasion. Follower cells are typically more dependent on the behaviour of leader cells and the signals they produce. Follower cells often follow the path carved out by the leader cells and can contribute to the spreading of the tumour (137,140–142). By traversing a larger space created by the leader cells, follower cells encounter less mechanical resistance and can spend less energy remodelling the ECM (143). As a result of increased crosslinking and force-mediated ECM remodelling, the tumour-surrounding interstitial matrix becomes linearized. For efficient cell migration, follower cells migrate along densely aligned collagen fibres (126). Membrane-type-1-MMP (MT1-MMP) is typically localized to invasive actin-rich cell structures (144). The delivery of MT1-MMP and other proteinases by exosomes outside the cell can also facilitate invasive lamellipodia maturation and degradation of the ECM (145). By inhibiting MMP activity in CAFs before adding SCC follower cells, the invasion of follower cells was effectively halted (10). MMP function is not required by follower cells once the matrix has been remodelled by CAFs (10). In addition, as follower cells tend



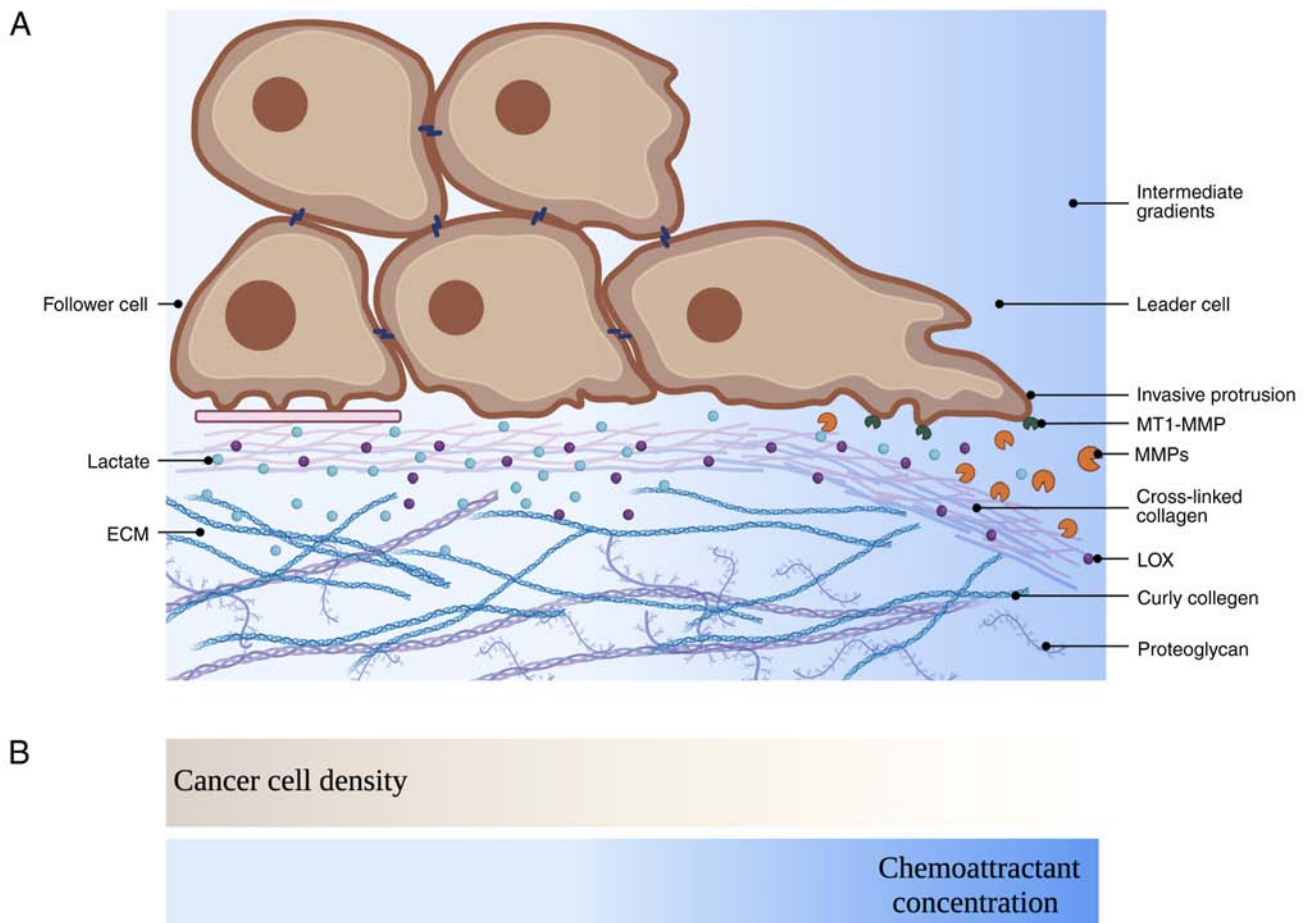


Figure 4. ECM remodelling and chemotaxis. (A) There are polymeric proteins and accessory molecules in the ECM that form a meshwork of non-cellular components. Follower cells sense the biochemical and mechanical properties of the ECM via integrin-based focal adhesions. The ECM is remodelled and sclerosed by the traction forces generated by the follower cells. ECM collagen is crosslinked by LOX and LOX-like proteins and degraded by MMPs, allowing the follower cells to disrupt basement membrane migration and invade the surrounding parenchyma. (B) After the chemoattractant has been broken down by follower cells, an intermediate gradient is formed that drives the cell population forwards for invasion. The highest follower cell density areas have the lowest local chemoattractant concentrations but maintain a very steep gradient at the invasion front where cell density is low, constantly directing follower cells to move directionally to areas they have not been. The figure was created with BioRender.com. ECM, extracellular matrix; LOX, Lysyl oxidase; MMPs, matrix metalloproteinases; MT1-MMP, Membrane-type-1-MMP.

to undergo glycolysis, increased lactate generation and excretion favour protease-mediated matrix remodelling and thus enhance the invasion of cancer cells (146-148) (Fig. 4A). Lactate-induced acidity has also been found to enhance the activity of certain proteases, namely MMP-2, MMP-9, cathepsin B and cathepsin L (149).

In glioblastoma, follower cells remodel the extracellular hyaluronic acid (HA) through a combination of synthesis, by hyaluronan synthases (HASs), and degradation, by hyaluronidases and MMPs (150,151). *In vitro* models have indicated that incorporating HA into gelatin matrices enhances the invasiveness of the follower cells (150). The production of HA by HASs (particularly HAS2) is significantly activated during invasion into the HA-rich ECM (151). The CD44 gene, which is a representative HA receptor, shares a close relationship with HAS, suggesting that there may be crosstalk between these two genes that stimulates signal cascades for glioblastoma follower cell invasion (152). Therefore, the invasion of glioblastoma follower cells is primarily influenced by the HA-rich ECM environments. This is mainly due to the high involvement of CD44 receptors and HASs (151,153,154).

## 5. Chemotaxis and invasion assays

According to extensive data, chemotactic cell migration guided by soluble signalling molecules facilitates invasion and metastasis (155,156). Chemoattractants are soluble proteins secreted into the extracellular space. These molecules are believed to be retained in this space by binding to glycosaminoglycans in the ECM, thereby establishing immobilized concentration gradients. Chemotactic gradients tend to be local and transient in nature. Furthermore, cells can navigate through complex topologies with self-generated chemotaxis (156). In self-generated gradients, follower cells produce an outwards-facing gradient by breaking down an attractive chemical, which serves as a guiding cue for leader cells. Leader cells need chemical gradients generated by follower cells in order to respond to external signals (157). As leader cells interact with their surroundings, their local gradient moves with them, resulting in directed movement that is exceptionally robust and capable of operating over long distances. However, if the attractant level is too high, then the follower cells cannot migrate but instead break down the molecule until the concentration is

low enough for a gradient to be observed (157). Additionally, if the cell leading wave does not have enough leader cells, the attractant is left behind, attracting more follower cells. In summary, cells show chemotaxis over long distances by moving in waves, with saturating chemoattractants in front and low levels behind (158). Without physically visiting their environment, follower cells acquire information about their surroundings (156,159) (Fig. 4B). Chemotaxis is a crucial concept for the understanding of the physiology of cells since it helps elucidate matrix composition.

It is believed that chemotaxis promotes the invasion and metastasis of follower cells. When a follower cell migrates, it senses a gradient of external chemoattractants, of which chemokines, chemotactic growth factors and lysophosphatidic acid (LPA) are major families (118,156,160). For example, as zebrafish posterior lateral line primordium collectively migrate, follower cells sense the attractant, CXCL12a, to migrate efficiently (118). The CXCL12/CXCR4 pathway also influences breast cancer cell metastasis to the lungs (161). Follower cells are CXCR4<sup>+</sup> and invade along CXCL12 gradients into organs expressing CXCL12 (for example, the lymph nodes and lungs). Moreover, when malignant lymphocytes are exposed to CCL19 gradients, their surface is stripped of CCR7, resulting in the retraction of protrusions and loss of polarity, which indicates follower cell behaviour. On the basis of intermediate gradients of CCL19, follower cells migrate towards the invasion front in cell migration (162). In addition, chemical gradients formed by EGF uptake can guide the movement of malignant epithelial cells within confined spaces (156). However, Muinonen-Martin *et al.* (160) noted that rather than acting as chemoattractants that could guide melanoma follower cells, growth factors acted as accessory factors that increased cell speed and chemotaxis efficiency, regulating melanoma cell behaviour. As such, the chemoattractant relay is rearranged to generate positional information using positive feedback. Furthermore, soluble chemicals are either degraded by enzymes or scavenged by decoy receptors (via endocytic internalization). In addition, follower cells are strongly induced to invade outwards by the LPA gradient (160). It has been demonstrated that follower cells can degrade LPA during melanoma metastasis, resulting in a gradient that the cells then respond to by migrating (160). Furthermore, expression of the LPA receptor (LPAR) in follower cells promotes metastasis and cell growth in metastatic breast cancer xenografts (163,164). An instrumental factor in the metastasis of pancreatic ductal adenocarcinoma cells from primary tumours is the neural Wiskott-Aldrich syndrome protein, which controls the recognition of LPA gradients by controlling LPAR1 (165). LPAR signalling mediates actin-myosin contractility and control of cell orientation, as well as matrix remodelling.

According to a mathematical analysis of the tracks of cells, follower cells are chemotactic and are attracted directionally by attractants (166). As follower cells respond to chemotactic gradients, they can alter them, resulting in robust chemotactic gradients. Furthermore, follower cells can reduce attractant concentrations when the attractant concentrations are too high. Depending on their needs follower cells alter themselves accordingly by inducing enzymes or cell division (166). However, the role of follower cells in generating chemokine gradients and their potential regulation by endocytosis have not yet, to the best of our knowledge, been studied.

## 6. Lateral inhibition

Among neighbouring cells, Notch signalling can coordinate divergent cell fate, which is termed lateral inhibition (167). Typically, follower cells upregulate Notch1 and Jagged1 (Jag1) expression, while leader cells upregulate Delta-like 4 (Dll4) expression to promote collective invasion (13,92,168). Notch also coordinates the adoption of similar follower cell fates by modulating lateral induction, a process through which it promotes the acquisition of comparable cell fates in adjacent cells (11,169). Through lateral inhibition, Notch-Delta signalling suppresses leader cell behaviour in follower cells. A ligand can bind to the Notch receptor in one of two ways: Delta-like or Jagged-like. In response to ligand-receptor binding and forces originating from endocytosis, the Notch receptor undergoes a specific conformational change, releasing the Notch intracellular domain (NICD) into the cytoplasm, leading to lateral inhibition and induction, which regulate collective invasion (170). NICD translocates to the nucleus and forms an active transcriptional complex with the DNA-binding protein, Rbpj, and the coactivator, Mastermind-like (Maml). Moreover, the NICD-Rbpj-Maml complex regulates the transcription of the Notch receptor and its ligands, thereby promoting the transcription of Hey/Hes1, an inhibitor of Delta but an activator of Jagged and Notch target genes (171,172). Therefore, lateral inhibition is observed between follower and leader cells when Notch-Delta signalling is dominant compared with Notch-Jagged signalling, while lateral induction takes place amongst follower cells when Notch-Jagged signalling is dominant (173). Moreover, Notch1 exhibits a higher affinity to Dll4 than to Jag1 following Fringe-mediated glycosylation of Notch1 (174). Based on the results of a study using mathematical modelling of Notch-Delta-Jagged signalling, Fringe proteins glycosylate the Notch receptor, resulting in a conformational change in the extracellular domain (175). Fringe can stabilize leader and follower cells by inhibiting Notch-Jagged binding, whereas its absence may shift the balance towards Notch-Jagged signalling (176).

VEGF stimulates leader cells by binding VEGFR-2 or VEGFR-3 in the microenvironment (177). Leader cells express Dll4 following VEGFR signalling, which activates intercellular Notch signalling to inhibit adjacent follower cells from turning into leader cells by targeting Notch1 (178-180). In follower cells, Notch signalling inhibits VEGFR function. Conversely, leader cells increase VEGFA secretion, which induces follower cell motility and invasion (89). In *Drosophila* oogenesis, upregulating Rac expression or PDGF/VEGF receptor expression can make follower cells switch positions from posterior to anterior and maintain their position as precursor cells, controlling migration throughout clusters (89). A landscape perspective on how Notch signalling affects leader and follower cell stability and transition may be a useful future direction of study.

## 7. Genetic heterogeneity

Compared with follower cells, leader cells display a variety of mitotic defects, the most prominent of which is cytokinetic instability (11). Follower cells are proliferative and may be able to rescue defective leader cells during collective movement.

A study of NSCLC cells isolated using SaGA technology found that gene expression in follower and leader cells was different (11). In follower cells, lysine demethylase 5B (KDM5B) mutations lead to phenotypic heterogeneity, and expression of ARP3 K240R promotes invasion, even in less invasive follower cells, and confers leader cell behaviour (12).

In a previous study, through a SaGA-based capture and amplification procedure, wild-type KDM5B was selectively enriched in leader cells, while mutant KDM5B L685W was selectively enriched in follower cells (12). KDM5B is a lysine demethylase enzyme responsible for catalysing the elimination of di- and trimethylation from histone H3 molecules that have been methylated at K4 (known as H3K4me2 and H3K4me3, respectively). KDM5B, functioning as a transcriptional repressor, promotes the leader cell phenotype by restricting follower cell behaviour (12). The mutation of KDM5B directly impacts invasive behaviour, serving as a unique epigenetic regulator that affects multiple pathways (181,182). Follower cells contain mutations in L685W near the zinc finger structural domain of KDM5B, which is essential for demethylase activity (183). Overexpression of KDM5B L685W, however, may enhance collective migration behaviour by enhancing heterogeneity and resulting in the emergence of cells with follower characteristics (184). Luminal breast cancer cells with upregulated KDM5B expression possess enhanced phenotypic stability, while cells with KDM5B depletion or repression exhibit enhanced transcriptional plasticity and can overcome therapeutic resistance (12).

The K240R mutation in ARP3, an essential component of the ARP2/3 complex, may interfere with its ubiquitylation at K240, thus resulting in either reduced ARP3 turnover or augmented activity (185). This mutation has been associated with enhanced leader cell behaviour, since it amplifies directional cellular protrusion events and accelerates the speed of cell motility, allowing the cell to detect signals that direct migration more easily. Further experiments are necessary to investigate the conjecture that ARP3 K240R is resistant to ubiquitylation, and if so, ascertain its effect on leader cell behaviour. Genetic heterogeneity can be identified in an invasion model using multiple cell lines and cancer types. Collective invasion may be influenced by a combination of multiple genetic and epigenetic changes rather than by isolated changes alone. Finally, identifying these key changes may support clinical judgement by monitoring relevant predictive biomarkers.

## 8. Metabolic heterogeneity

Leader and follower cells have been shown to exhibit metabolic heterogeneity (186,187), and these cells alter their metabolic activity to sustain their growth. As described by Warburg (188), cancer cells can reprogram their glucose metabolism so that glycolysis is their primary energy metabolism, even under aerobic conditions. Collective cellular invasion is accompanied by a metabolic shift towards glycolysis (94). Compared with leader cells, follower cells exhibit higher glucose uptake and glycolysis, as well as less dependence on oxidative phosphorylation (OXPHOS) (189,190). Although migrating cell populations do not share the same metabolic pathways, they do maintain a highly coordinated energetic state. Moreover,

migrating cells have appropriate mechanisms to adjust their metabolism accordingly.

Glycolysis is sustained by glucose transporter 1 (GLUT1) expression in follower cells, while mitochondrial respiration is sustained by active pyruvate dehydrogenase (PDH) expression in leader cells (184). Specifically, follower cells exhibit a higher level of GLUT1 expression and glucose uptake than leader cells during collective NSCLC invasion *in vitro* (190). GLUT1 is ubiquitous in all tumour types that have poor patient prognosis (191) and maintains glucose uptake by cancer cells (192-194). Follower cells may divert glucose uptake to the pentose phosphate pathway (PPP), which supports ribulobiogenesis and proliferation without altering citric acid cycle flux. The PPP begins with glucose-6-phosphate dehydrogenase, which is also more highly expressed in follower cells (190). In response to a decrease in glycolytic intermediates entering glycolysis and the PPP, follower cells switch from proliferation to invasion (190). In leader cells, GLUT1 expression is reduced by regulators, such as tumour suppressor p53 and hypoxia-inducible factor-1 (195,196). By contrast, GLUT1 upregulation in leader cells hinders their collective invasive ability (195-198). In follower cells, mitochondria are primarily found around the nucleus, whereas in leader cells, they are more common at the edge of the cytoplasm (190). The presence of mitochondria at the edge of the cytoplasm suggests a higher energy demand in the leading edge, where cellular protrusions and migratory activity occur. As PDH activity increases, mitochondrial distribution to the periphery of the cell increases (190). Since dichloroacetate (DCA) inhibits PDH kinases, DCA-treated follower cells have lower levels of phosphorylated PDH at position S293 and increase invasion, particularly in chains, similar to leader cells (190).

However, Zhang *et al* (19) found that leader cells rely more on glucose and have higher cellular energy levels than follower cells in breast cancer invasion models, which are used to drive collective invasion, which in turn gradually depletes energy levels. The metabolic profile of invasive cells can be affected by many factors, such as molecular heterogeneity, the microenvironment and the mode of migration (187). For example, it is possible that the metabolic pathway dictated by the density or sparsity of the microenvironment determines whether tumour cells increase glycolysis and decrease mitochondrial oxidative respiration during invasion. As a result of OXPHOS at the invasion front, ATP could be produced more efficiently to meet the energy demands of collective invasion. This process requires sufficient cellular energy levels in leader cells, as opposed to follower cells that produce less energy through glycolysis (199). As soon as a leader cell exhausts its available ATP, it exchanges its position with a follower cell, and the frequency of this exchange increases in denser collagen matrices (19). Hence, cancer invasion is facilitated by metabolic shifts between leader cells and follower cells. Cancer cells are reprogrammed to maintain proliferation and invasion in a dysregulated environment. Whether collective migration is proliferative or invasive is determined by two distinct metabolic preferences, and therefore, two distinct phenotypes should be taken into account to prevent the metabolic plasticity that can drive invading cells: Glycolysis and OXPHOS.

## 9. Conclusions and perspectives

In summary, CIL, biomechanics, remodelling of the ECM, chemotaxis, lateral inhibition and the genetic and metabolic heterogeneity associated with follower cells were discussed in the present review. These mechanisms determine movement polarity by identifying leader and follower cells and guiding cancer cells to acquire follower-like or leader-like morphology, function and behaviour during collective invasion. The follower cells receive signals from the leader cells and the microenvironment due to intracellular and intercellular signalling cascades as well as mechanotransduction. The signals are then transmitted to the entire mass of cells. When collective movements occur, follower cells maintain their phenotype and consolidate the status of the leader cell. Additionally, cell behaviour and fate can be changed by biomechanics and the microenvironment, resulting in follower cells adopting a leadership phenotype, which ultimately leads to genetic changes. In addition, follower cells may take over the positions of leader cells when their energy level drops below a certain level. For cells to move collectively, follower and leader cells must coordinate their movements to be controlled by physical (mechanical) and chemical (signalling) interactions.

However, the factors that drive follower cell formation and the mechanisms that regulate follower cell migration remain unknown. Currently, the impact of the molecular mechanism of cadherin mechanotransduction on the behaviour of leader-follower cells remains mostly unclear. To improve understanding of the interaction between cadherin junctions and cell mechanics, researchers should focus on the interaction between cadherin junctions and Rho-GTPases. *In vivo*, mechanocoupling is spatially controlled in a number of ways, such as by cell polarity and ECM remodelling. The more complex and physiologically relevant collective migration within 3D matrices is being replicated in a growing number of *in silico* models. The use of computational models can provide insights into the migration of follower cells and overcome limitations associated with experimental research (200,201). Furthermore, it is unclear how bioenergetic status affects the emergence of follower cells when taking over from failing leader cells. The metabolic, morphological and migration functions of cells are closely intertwined, so targeting cellular metabolism may provide a novel strategy for treating cancer by inhibiting the production of cellular energy. It is necessary to conduct additional experimental research to investigate the function of follower cells in collective movement and to analyse the mechanism of coordinated invasion.

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## Authors' contributions

XW conceived the outline of the manuscript by searching the literature, wrote a draft of the manuscript and created the figures. XL and YT reviewed and edited the manuscript. Data authentication is not applicable. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

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## Competing interests

The authors declare that they have no competing interests.

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