Collective invasion of cancer: perspectives from pathology and development

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Short Running Title: Collective invasion of cancer

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Abstract

Clinical pathologists have long been aware that in many types of human malignant tumors, the cells are often connected and form groups of various sizes or "nests". In this way, they achieve "collective invasion" into the surrounding stroma, rather than spreading out individually. Such collective behavior is also a common feature of migration during embryonic and postnatal developmental stages, suggesting there are advantages gained by collective cell migration in the organisms. Recent studies have revealed the mechanisms underlying the collective invasion of cancer cells. These mechanisms differ from those observed in the migration of single cells in culture, including reliance on the epithelial-mesenchymal transition program. Whereas intercellular adhesion appears to be coordinated, cancer cell groups can be heterogenous, including cells that are leaders and those that are followers. There is also interaction with the tumor microenvironment that is a prerequisite for collective invasion of cancer. In this review, we describe recently emerging mechanisms underlying the collective migration of cells, with a particular focus on our studies on the actin-binding protein Girdin/GIV and the transcriptional regulator tripartite motif containing 27. These studies provide new perspectives on the mechanistic analogy between cancer and development.

Key words: cancer, collective migration, collective invasion, neuroblast, leading cell, Girdin, MRTF, Trim27

Many land animals, birds, fish and arthropods live and migrate in groups. This strategy protects them from predators and increase the likelihood of reaching their destination.[1] Analogously, during the development of multicellular organisms and the progression of human diseases, cells often form coordinated cohesive groups or nests to migrate through the interstitium toward their destination and to make and reshape tissues. During development, the cohesive cell behavior is termed "collective migration", and in the context of cancer, it is called "collective invasion".[2-4] Examples of collective migration during embryonic development include the migration of neural crest cells and neuroblasts, in which the cells organize and retain intercellular adhesion to migrate long distances to form discrete tissues and organs.[5, 6] Branching morphogenesis of epithelial ducts and glands as well as the reshaping of larger tissue structures are also supported by collective cell movements.[3, 4] Nascent endothelial cells in the developing vascular plexus in the retina and granulation tissues in wounds and inflammatory diseases also migrate in groups in angiogenesis.[3, 4] Intriguingly, it has long been argued that many types of human cancers with well or moderate differentiation form tightly connected groups of cells in order to undergo collective invasion into the neighboring stroma within the primary site as well as in metastatic regions rather than spread as single cells.[7-9] All of these observations suggest that the collective migration of cells is biologically significant and that it is more than the sum of their individual cells. This interpretation leads to the notion that the mechanisms underlying single cell migration cannot be applied to collective migration. Therefore, molecular mechanisms underlying cell migration that were analyzed utilizing single cells in culture must be reconsidered in the setting of collective migration. In this review, we describe our views and perspectives on the mechanisms of collective migration of cells during development and cancer, followed by a description of our recent studies that showed the involvement of the actin-binding protein Girdin (girders of actin, also termed Gα-interacting vesicle-associated protein; GIV) and the transcriptional regulator tripartite

motif-containing 27 (Trim27) in the collective migration of cells. Furthermore, we will briefly mention the possibility of developing new anticancer therapeutics to target collective invasion by cancer cells.

Cell migration: a prerequisite feature of cells in development and malignant diseases

In the adult body, migrating cells are not expected to travel long distances with the exception of some leukocytes, immune cells and stem cells such as mesenchymal stem cells. Most newly generated somatic cells (arising from their tissue stem cells or progenitors) reconstitute, reshape or remodel the local tissue structures to maintain their function and homeostasis, but they rarely migrate to other places. In contrast, in a malignant or neoplastic setting, we see cells moving from place to place, a process that interferes with the homeostasis and function of the involved tissues.

In contrast to adulthood, in embryonic and perinatal periods there are many types of cells that actively move around and migrate long distances through the body, a process that contributes to the development of tissues and organs.[4, 10] The numerous examples include the movements in gastrulation that result in the formation of three distinct germ layers; the migration of neural crest cells that generate the peripheral nervous system, the adrenal medulla, most of the bones and connective tissues in the head, and pigment cells of the skin (Fig. 1A); endothelial and epithelial cell migration that leads to vasculogenesis and branching morphogenesis in many organs;[3, 4] and neuroblast migration that contributes to the formation of the forebrain (Fig. 2).[10]

Cell biological and biochemical studies have revealed detailed molecular mechanisms of cell migration. Those include the remodeling and reorganization of the

cytoskeleton, including the actin filaments, microtubules and intermediate filaments, all of which are tightly regulated by multiple signaling pathways and chemical guidance cues provided by soluble growth factors, chemokines and cytokines. Physical forces secured by the attachment to the extracellular matrices (ECMs) and their dynamic remodeling are also crucial. Cell intrinsic and extrinsic transcriptional machineries that control gene expression and the mechanisms that control cell polarization and membrane trafficking are also crucial for cell migration. Readers can refer to a huge number of studies and reviews for the detailed mechanisms of cell migration.[11-13]

Collective cell migration: a major mode of cell migration in the body

As mentioned above, previous studies on developmental biology have shown that many types of moving cells preferentially form cohesive groups or nests during their collective migration toward their destinations.[3-5] This means that the cells need to be equipped with highly sophisticated and complex supracellular mechanisms to keep and remodel their cell-cell adhesion, maintain the net front-rear polarity within the groups, and define the distribution and separate roles of individual cells such as leading and following cells.[14] Integration of extracellular guidance cues and mechanosensory mechanisms are also crucial to guide the cellular groups along interfaces and paths of least resistance.[14] Accordingly, studies using imaging techniques over the last decade have begun to uncover the mechanisms supporting collective cell migration. These studies have demonstrated that the mechanism of collective cell migration is more complex that that seen for single cell migration.

Collective migration is also shared by many human malignant tumors.[2, 7-9] Histological observations of sections have long suggested that most human malignant tumor cells, particularly epithelial malignant tumor cells (except for those of poorly

differentiated cases) collectively proliferate and invade into the stroma, forming groups, nests and tubular architecture (Fig. 1B). Notably, even for poorly differentiated types of cancer, which are defined by their diffusely scattered morphology, the visualization of cancer cells by immunostaining reveals that they form loosely attached small nests or intertwined cords as they invade into the stroma (Fig. 1C). Such results suggest that collective migration is a fundamental property of cancer cells that is linked to their progression. This notion is further supported by observations of cohesive cancer cells in the blood or lymphatic vessels that indicate that cancer cells may penetrate in groups into the walls of those vessels followed by intravasation (Fig. 1Bf).

Such observations of cells migrating throughout the organisms demonstrate the importance of dissecting specific mechanisms for collective cell migration. However, most known mechanisms for cell migration have been analyzed on culture dishes or 3D matrices in which the focus was individual cells. Therefore, the findings gained from such studies need to be carefully examined before they can be appreciated in the setting of collective cell migration. For example, a number of studies have emphasized the importance of the epithelial-mesenchymal transition (EMT) program whereby cancer cells depolarize, lose their intercellular adhesion and acquire a mesenchymal morphology, accompanied by increased invasion potential.[15] Indeed, many studies have shown that proteins that are responsible for the EMT program are highly expressed in cancer tissues to provide a driving force behind invasion, making the EMT hypothesis attractive and plausible. A central issue here, however, is that cells in transition are rarely observed in human cancer tissues, leading to the notion that the EMT program is quickly activated in cancer cells over short periods of time or is observed only in certain limited contexts.[16] Further studies are clearly needed to look at the relationship between the EMT program and collective invasion of cancer in the framework of our understanding of human pathology. Of note, recent studies using genetically engineered

mouse models and lineage tracing techniques have questioned the contribution of the EMT program in cancer invasion and metastasis, and revealed instead that it contributes chemoresistance.[17, 18]

Girdin: a regulator for collective migration of neuroblasts

The above notions clearly indicate that one of the ways to address the mechanisms for collective migration is to assess key molecules that are involved in both embryonic development and cancer progression. One of the regions where one can observe collective cell migration in the body is the subventricular zone (SVZ), which is located between the parenchyma of the striatum and the lateral ventricle (Fig. 2A). In the SVZ, immature neuroblasts are continuously generated from neural stem cells throughout perinatal and adult periods, a process designated “adult neurogenesis” or just “neurogenesis”.[19] The newly born neuroblasts migrate along the pathway called the rostral migratory stream (RMS) toward the olfactory bulb (OB) where the neuroblasts give rise to local interneurons or contribute to the replacement and turnover of older cells (Fig. 2A). A characteristic mode of migration executed by the neuroblasts has been termed “chain migration”, where individual neuroblasts transiently form and resolve intercellular contacts and follow each other in single file to form a stream (Fig. 2C).[20] It is known that some types of malignant melanoma cell also exhibit chain migration when they undergo invasion and metastasis (Fig. 1Bd).[7] Chain migration of cells is regulated by complex mechanisms that include their interaction with the surrounding astrocytes or glial cells.[21] Those glial cells form the glial tube network that surrounds the tangentially migrating neuroblasts in the RMS, where intercellular communication between the glial cells and neuroblasts is crucial (Fig. 2C). One of the mechanisms is

the Slit-Robo signaling pathway that induces dynamic changes in the shapes of glial cells that drive the migration of neuroblasts through the RMS.[21]

Several cell-intrinsic mechanisms for collective migration of neuroblasts have been identified, and these include pathways that involve Girdin/GIV.[22, 23] Girdin, which was identified by several research groups including our own laboratory, is a substrate for the Akt kinase as well as an activator for Gα proteins (Fig. 3).[22, 24] Interestingly, one of the obvious phenotypes observed in Girdin knockout (KO) mice was a defect in chain migration of SVZ neuroblasts (Fig. 2A). In Girdin KO mice, individual neuroblasts were oriented randomly, with impaired polarization and cell-cell interaction.[23] Defects in the migration of SVZ neuroblasts have also been reported in other genetically modified mice, most of which, however, are the consequence of conditional deletion of particular genes from the neural lineage.[25] The defective development of the OB in Girdin whole-body conventional KO mice indicated important and specific roles for Girdin in collective migration of SVZ neuroblasts. In the following, we will further describe the functions of Girdin revealed by cell biological and biochemical approaches.

Girdin is a hub protein that controls multiple signaling pathways

Girdin was identified in our laboratory as a substrate for the Akt kinase and an actin-binding protein.[22, 26] The protein consists of 1871 amino acids (1870 and 1845 in isoforms) that has an N-terminal (NT) domain, a long coiled-coil domain and a C-terminal (CT) domain (Fig. 3B). The CT domain has the Akt phosphorylation site (Ser-1416), which is also responsible for the binding to the actin cytoskeleton. Girdin was also identified as an activator for trimeric Gα proteins, including Gαi and Gαs that have pivotal functions in membrane trafficking as well as downstream from G

protein-coupled receptors (GPCRs).[24, 27] Girdin is widely expressed in most types of immortalized cell lines, including cancer cells where it resides diffusely in the cytoplasm as well as near the actin stress fibers, cortical actin, focal adhesions, and lamellipodia during cell migration (Fig. 3A).[22, 28-31] RNA interference-mediated knock down of Girdin attenuated actin organization and cell migration in cultured cells and neuroblasts, leading to the notion that Girdin is a critical regulator of cell migration.[22, 27-29, 32, 33]

As indicated by the presence of its Akt phosphorylation site, Girdin is phosphorylated downstream of many kinases, including epidermal growth factor receptor (EGFR),[34] Src kinase,[34] cyclin-dependent kinase 5 (CDK5)[35] and focal adhesion kinase (FAK),[31] all of which are involved in the regulation of cell migration induced by growth factor stimulation and adhesion to the ECM (Fig. 3). Binding directly to receptor tyrosine kinases (RTKs), adaptor proteins and Gα proteins, Girdin also enhances and integrates RTK and GPCR signaling pathways, suggesting that Girdin is a multi-modular signal transducer.[34] Girdin is highly expressed in some types of human malignancies, including breast, colon, and esophageal cancers and brain glioma, where its expression levels correlate with the prognosis of the patients (Fig. 1B).[28, 33, 36, 37]

The remodeling of actin filaments and the regulation of RTK and GPCR signaling pathways are not the only functions of Girdin, all of which are also regulated by various other proteins. A proteomic approach expanded our understanding of Girdin's role in regulating directional migration of neuroblasts and gave us insight into its specific mode of action.[38] Girdin interacts with Par-3, which is a scaffold protein that is critical for cell polarization (Fig. 3). Par-3 binds to Girdin's CT domain and the binding is independent of growth factor stimulation. The expression of a Par-3 mutant incapable of binding with Girdin dysregulated polarization in migrating fibroblasts in culture dishes and

epithelial cells forming acinar structures in 3D collagen matrices.[38] These findings suggested that the Girdin/Par-3 protein complex participated in determining cell polarity. Indeed, our investigation of tissue sections from the RMS in the brain of Girdin KO mice showed that the localization of the Golgi apparatus, one of the hallmarks of cell polarization, was severely dysregulated in migrating neuroblasts, showing the *in vivo* significance of Girdin in the regulation of cell polarity.[38] Another study showed that Par-3 sits upstream from Girdin, regulating its transcription in collaboration with activator protein 2 (AP-2) transcription factor.[39] Thus, Girdin synergizes with Par-3 both at protein and transcriptional levels to regulate cell polarity. A recent study identified Girdin's interaction with Gem-interacting protein (Gmip), a RhoA-specific GTPase-activating protein. Gmip is essential for the speed control of migrating SVZ neuroblasts, supporting the view that Girdin is a regulator of neuroblast migration.[40]

Selective endocytosis in collective cell migration

Most of the aforementioned studies on Girdin did not revealed how Girdin controlled collective cell migration. We recently localized Girdin within endocytic vesicles and identified a dynamin GTPase protein that interacts with Girdin (Fig. 3).[41] Dynamin is a mechanochemical protein essential for endocytosis and is responsible for the membrane scission or pinch-off of clathrin-coated endocytic vesicles at the plasma membrane.[42] Biochemical studies showed that the Girdin NT domain directly bound to the GTPase and GTPase effector domain (GED) domains of dynamin and that interaction promotes GTPase activity in dynamin and the scission of membrane vesicles. Interestingly, Girdin depletion suppressed endocytosis of E-cadherin but not EGFR or integrin β1 in HeLa cervical cancer cells. Those data suggested that Girdin defined the specificity for the membrane proteins that undergo endocytosis.[41] Further biochemical details are

beyond the scope of this review, but the data showed that Girdin interacted with EGFR and integrin β1 and thereby competitively inhibited Girdin's interaction with dynamin and Girdin's ability to fine-tune dynamin activity. The study provides a new mechanism for selective endocytosis and also defines features of Girdin's functions.

It is clear that endocytosis, intracellular trafficking and the recycling of integrin receptors for the ECM are essential for cell motility. The endocytosis of EGFR is also important to coordinate the strength and duration of its signal. The question then becomes: what is the significance of Girdin-mediated selective endocytosis of E-cadherin but neither integrins nor EGFR?. In mice expressing a Girdin loss-of-function mutant (Basic-mut), immunofluorescent staining of SVZ neuroblasts showed dysregulated subcellular localization and expression of N-cadherin (Fig. 2B).[43] Polysialated neural cell adhesion molecule (PSA-NCAM) is another cell adhesion molecule essential for the migration of SVZ neuroblasts.[44] Both the localization and expression of PSA-NCAM were intact in Basic-mut mice, indicating that Girdin specifically functions in cadherin trafficking in migrating neuroblasts. This observation led to the hypothesis that Girdin mediated the trafficking of cadherin molecules and that the remodeling of adherens junctions of adjacent migrating cells has roles in the collective behavior of neuroblasts (Fig. 2C). Supporting this notion, electron microscopic observation localized Girdin at cell-cell contacts between neuroblasts (Fig. 2C).[23] It is also plausible to speculate that Girdin has a similar role in the collective invasion of cancer cell groups or nests, although direct evidence is not currently available. Further detailed analysis on cancer cells implanted in animals would be needed to prove the hypothesis. The significance of cadherin dynamics at adherens junctions has also been proven in collectively moving astrocytes where the retrograde flow of N-cadherin along lateral cell sides and following endocytosis at the cell rear play an important role.[45] We recently showed the involvement of Girdin in the recycling

of VE-cadherin in endothelial cells through its interaction with the small G protein R-Ras that controlled transendothelial permeability.[46] An obvious future goal of these studies will be to reveal mechanisms and precise modes of action of Girdin at adherence junctions in achieving collective cell migration.

Leaders and followers: an alliance for collective invasion of cancer

Previous studies have described the importance of spatial activation of particular proteins involved in collective cell migration. These include the small G proteins Rac and Rho, phosphatidyl inositol 3-kinase (PI3K) and extracellular-signal-regulated kinase (ERK), as described in other in-depth reviews and research articles.[4, 14, 47] The architecture and composition of extracellular stromal components (collagen scaffolds) and the gradients of growth factors and chemokines are also crucial for chemotaxis and haptotaxis of cell groups.[14] Importantly, cell-autonomous or intrinsic mechanisms such as the control of contact inhibition of locomotion (CIL) mediated by cadherins and the Wnt signaling pathway are also indispensable for proper collective polarity and movement of cells during development.[48] Here, we describe the importance of cancer cell heterogeneity and plasticity, particularly underlining the key role of "leading cells" in cancer cell groups and gene expression specific to these cells.

Cancer cell heterogeneity has been appreciated as an essential component of cancer progression. Although genetic and epigenetic alternations that drive the heterogeneity among cancer cells are widely appreciated, there are other mechanisms that are thought to play key roles in developing intratumor diversity. Close observations of tissue sections from human cancers, particularly squamous cell carcinomas, have sometimes underscored how chromatin organization and fine structure differ between leading cells at the front of invading cancer cell groups and those that are following,

indicating some differences in gene transcriptional activity (Fig. 4A). Several research groups (including ours) have noted high expression of integrin β1 specifically in the leading cells of cancer cell groups (Fig. 4B).[47, 49, 50] Podoplanin was also identified to be expressed by leading cells (Fig. 4B).[51] Importantly, those proteins are innately expressed by epithelial basal cells in normal tissues. These observations support a new concept that collective invasion of cancer requires gene expression that defines a conserved basal epithelial program.[52] Other proteins expressed by leading cells include integrin β3, α-catulin, fascin, cytokeratin-14 and p63. The combination of proteins depends on the type of cancer tissue and the contexts.[50-54]

It is not clear whether leading cells exist in all types of cancers. To our knowledge, the mechanisms that define leading cells or their equivalents in adenocarcinoma have not been identified. In colon cancers, most of which are adenocarcinomas with variable differentiation, the tumor "budding" that is defined by small clusters of cancer cells ahead of the invasive front of tumors represents a prognostic factor.[55] Although some studies have speculated that the tumor buds are the histomorphological correlate of cells to undergo EMT, another intriguing hypothesis is that the tumor buds also co-opt the mechanisms for collective migration.[56] Detailed analysis of gene expression in cells that constitute such budding and comparison to that of leading cells would be an intriguing issue.

Mechanisms defining leading cells

Integrin β1 and spatial activation of its downstream signaling play obvious roles in leading cells.[14] Nonetheless, the mechanism that specifically induces its expression in leading cells remains unknown. Possible mechanisms include signals derived from stromal components or the tumor microenvironment, including endothelial cells,

cancer-associated fibroblasts (CAF), and immune cells (Fig. 4C). We recently showed that lung cancer cells express high levels of integrin β1 when they are cultured with media conditioned by CAFs.[50] Given the fact that leading cells in cancer cell groups are exposed to stromal components, the finding suggests that factors produced from CAFs could modulate gene expression in leading cells (Fig. 4C). Another important feature of CAFs in promoting collective invasion of cancer is that they physically remodel the surrounding ECM to make paths or tunnels to subsequently provide routes that can be exploited by cancer cells.[57, 58]

There are also cell-intrinsic mechanisms that define leading cells. We recently analyzed the absence of cadherin-mediated cell-cell contacts, which constitutes the presence of a "free end" or "contact-free leading edge" in leading cells (Fig. 4C, D). We found that such a "free end" was crucial for integrin β1 expression by leading cells.[59] The presence of a free end activates small GTPases, including Rho, accompanied by changes in the dynamics of the actin cytoskeleton. The latter induces nuclear translocation of a transcriptional factor (myocardin-related transcription factor-B [MRTF-B]) in the leading cells (Fig. 4C, D). Cumulative evidence shows that MRTF-B interacts with monomeric globular actin (G-actin) to sense the dynamics of actin remodeling and transmit it to the nucleus to alter gene expression.[60] Although detailed mechanisms are beyond the scope of this review, we showed that the presence of a free end stimulates the formation of a nuclear protein complex composed of MRTF-B and tripartite motif containing 27 (Trim27), also termed ret finger protein.[59] The MRTF-B/Trim27 complex upregulates integrin β1 expression via control of the expression of integrin β1-specific miRNA-124 (Fig. 4D). Indeed, depletion of MRTF-B and Trim27 and the inhibition of integrin β1 by neutralizing antibody abrogated the formation of invading strands in cancer cell groups in an organotypic culture model and spheroid 3D invasion assays.[59] The in vivo significance of MRTF-B or Trim27 was

shown in a orthotopic mouse tumor model, where MRTF-B- or Trim27-depleted FaDu squamous cell carcinoma cells showed limited ability to undergo collective invasion and metastasis when they are transplanted into the tongues (Fig. 4E).[59] These findings indicate a new pathway in leading cells that defines their function in driving collective invasion.

We argue from the above that the significance of cadherin-mediated intercellular adhesion varies with its location within cancer cell groups. In the leading cells at the invasive forefront of cancer cell groups, the partial loss of cadherin-mediated adhesion, which leads to the generation of contact-free leading edges (free ends), is essential for specific gene expression. On the contrary, the dynamic flexible remodeling of cell-cell adhesion mediated by the endocytosis and recycling of cadherins along lateral cell sides is crucial for both leading and following cells to retain their configuration (Fig. 4D). The basic idea underlying the model is that cancer cell heterogeneity and plasticity are key determinants of cancer progression.

Angiogenesis is another setting in which we observe collective migration of cells, as vessel branching occurs in developmental, postnatal and adult stages. In this case, a few endothelial cells, the “tip cells” (corresponding to leading cells in cancer), take the lead to guide the following and proliferating “stalk cells” (corresponding to following cells in cancer).[4] Studies showed that endothelial cells compete for the tip cell position depending on their expression levels of vascular endothelial growth factor receptor. The competition leads to dynamic position shuffling of tip and stalk cells.[61] Therefore, it could be argued that, also in cancer, leading and following cell fates are not fixed but dynamically regulated by intrinsic and/or extrinsic signals. More sophisticated intravital imaging analyses of animal cancer models are required to resolve these issues.

Further questions and perspectives

This review concerns the mechanisms for collective migration of neuroblasts and cancer cells, mainly focusing on research on Girdin and the MRTF-B/Trim27 protein complex published by our laboratory. These analyses provide new insights into molecular mechanisms that define the mode of migration of those cells. A large number of biological questions, however, remained unsolved. For example, direct evidence showing the *in vivo* significance of Girdin in collective invasion of cancer is not available at present. Also, although it has been shown that Girdin undergoes phosphorylation by several kinases and interacts with many other proteins, the significance of those phosphorylations and interactions in collective cell migration is unknown (Fig. 3).[62, 63] One clue came from our study showing that Akt-mediated Girdin phosphorylation has roles in retinal angiogenesis and neovascularization in postnatal development and pathological contexts.[29, 64] The migration of SVZ neuroblasts, however, is not dependent on Akt-mediated Girdin phosphorylation, implicating context- and tissue-dependent roles of Girdin phosphorylation in cell migration.[23] Trim27 expression levels in many types of cancers correlate with their prognosis,[65] although the contribution of integrin β1-positive leading cells on patient prognosis remains to be unresolved. Furthermore, the functions of Girdin and the MRTF-B/Trim27 complex described here are not limited to the regulation of cell migration (Fig. 3B). A recent study uncovered the involvement of Girdin and its phosphorylation in synaptic plasticity in hippocampal neurons and memory.[66]

Another provocative question is whether the collective appearance of cancer cell groups simply reflects the localized expansion and deregulated proliferation of tumors. Uncontrolled expansion and proliferation frequently result in hypoxic necrosis of tumor tissue as well as the failures to coordinate development of the tumor microenvironment. From that perspective, the correlation between cell cycle control and cell position within

the cancer cell groups is an attractive issue to be investigated. Intriguingly, Girdin has been reported to be a regulator that orchestrates the "migration–proliferation dichotomy", a phenomenon that cell migration and proliferation do not occur simultaneously.[33, 35]

Although many studies have described the spatial and temporal activation of particular proteins in leading cells, the validity for targeting the leading cells in collectively invading cancer cell groups as an anticancer therapeutic approach is unknown.[14] As described above, the dynamic position shuffling of leading and following cells in cancer cell groups makes it difficult to specifically target the leading cells. Rather, data showing the roles of CAFs in promoting collective invasion of cancers as well as gene expression in the leading cells support the notion that the tumor microenvironment is a key to the development of new therapeutics.[50, 57, 58] The regulation of collective behavior of cancer cells by immune cells is also a relevant issue in this field. Finally, the spatial distribution of cancer stem cells in cancer cell groups and its relevance to cancer cell niche are also important unresolved issues, the resolution of which will have large implications for our understanding of the mechanisms for resistance to conventional anticancer therapies.

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Figure Legends

Figure 1. Collective cell migration observed in the body

(A) Developing adrenal gland in 13-week human embryo, showing the groups of neural crest-derived cells. Hematoxylin and eosin stain.

(B) Representative images for adenocarcinoma in the pancreas **(a)**, adenocarcinoma in the colon **(b)**, sebaceous carcinoma in the skin **(c)**, melanoma in the skin **(d)**, breast invasive ductal carcinoma **(e)**, and the lymphatic invasion of rectal adenocarcinoma **(f)**. In **(e)**, Girdin expression was visualized by immunohistochemical (IHC) staining (brown).

(C) Poorly differentiated adenocarcinoma of the stomach (upper panel) and the visualization of cancer cells by keratin immunostaining (lower panel). Note that individual cancer cells retain intercellular adhesions to form small groups during streaming.

Figure 2. Collective migration of neuroblasts and its regulation by Girdin

(A) Nissl-stained brain sections of wild-type and Girdin KO mice at postnatal day 15. Note significant deficits in the chain migration of neuroblasts in Girdin KO mice. SVZ, subventricular zone; OB, olfactory bulb; RMS, rostral migratory stream.

(B) Localization and expression of N-cadherin (upper panels) and PSA-NCAM (lower panels) in migrating SVZ neuroblasts in wild-type and Girdin Basic-mut mice (green). Nuclei were visualized by DAPI (4'6-diamidino-2-phenylindole) staining (blue). Reprinted from Biochemical and Biophysical Research Communications, Vol 463, Muramatsu et al., Potential involvement of kinesin-1 in the regulation of subcellular

localization of Girdin, pages 999-1005, Copyright (2015), with permission from Elsevier.

(C) A model for the role of remodeling of cell-cell adhesion in the chain migration of SVZ neuroblasts. Individual SVZ neuroblasts transiently form and resolve intercellular contacts and follow each other to form streams (dashed arrows). The formation of the path by glial tubes (green) and retrograde flow and endocytosis of cadherins (purple arrows) are crucial for chain migration. Girdin preferentially localizes to small zonula adherens-like contacts between neuroblasts (red arrowheads, far left panel). SVZ neuroblasts in Girdin KO or Basic-mut mice show defective collective polarity, leading to the defect in their migration and OB formation.

Figure 3. Girdin functions revealed by cell biology

(A) A model for the regulation of cell motility by Girdin. In quiescent cells (left panel), Girdin localizes on cortical actin filaments to cross-link them beneath the plasma membrane. Quick-freeze deep-etch electron microscopic observation and immunogold-labeling showed a clear dot surrounded by a halo of platinum-carbon coating (pseudo-colored red). Image revealed Girdin localization on actin filaments and clathrin-coated pits. In migrating cells stimulated by guidance cues such as growth factors or the ECM, phosphorylated Girdin localizes at the leading edge and focal adhesions (right panel). Girdin is phosphorylated by several kinases that have multiple roles, including the control of actin reorganization, Akt signaling and membrane trafficking. Girdin interaction with dynamin regulates selective endocytosis for cadherins, whereas that with Par-3 contributes to the determination of cell polarity.

(B) Schematic illustration of the structure of Girdin and its interacting proteins. The domains of Girdin that are responsible for binding to its interactants, and their functions are indicated. Phosphorylation sites by the indicated kinases are also shown. CLASP2, CLIP-associating protein 2; Dlg5, discs large homolog 5.

Figure 4. Leading cells in collective invasion of cancer

(A) Images taken from tissue sections of esophageal squamous cell carcinoma (left) and colon adenocarcinoma (right) show the alterations in chromatin density and organization in leading cells at the invasive front that faces surrounding stroma (arrowheads). Note that such findings are not frequently observed in sections that pathologists encounter in routine practice. It is also possible that the findings simply represent the reactivation of the basal epithelial program.[52]

(B) Expression of integrin β1 (left) and podoplanin (right) in the leading cells and collective invasive strand in squamous cell carcinomas that arise in the lung and oral cavity, respectively (arrowheads). Note that staining for α-smooth muscle actin (SMA) visualizes CAFs infiltrating in the stroma.

(C) Invasive front of cancer cell groups in skin squamous cell carcinoma is schematically illustrated to clearly depict distinct leading (pink) and following (blue) cells. Many studies argued that the heterogeneity of cancer cells, secreted factors and guidance cues (yellow) and traction forces provided by infiltrating CAFs (green), and physical interplay between the leading cells and CAFs are crucial for collective invasion of cancer cells.

(D) Proposed model for the differential expression of integrin β1 in leading cells (pink) but not following cells (blue). The MRTF-B/Trim27 protein complex transmits a signal

via Rho from the loss of intercellular adhesion or the presence of a free end (contact-free leading edge) to the nucleus, thereby regulating integrin β1 expression via *miR-124* in the leading cells (for details see Kato et al., 2014). The dynamic and flexible remodeling of cadherin-mediated adhesion along lateral cell sides also plays an important role in collective invasion of cancer.

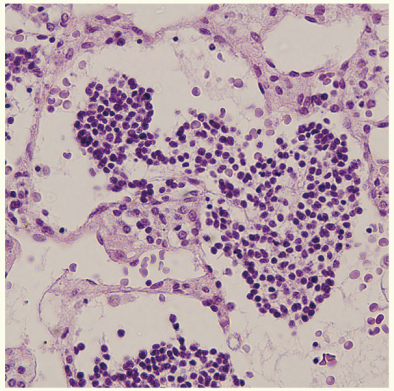
(E) Effects of MRTF-B or Trim27 depletion on collective invasion in a mouse tongue cancer model. The indicated FaDu cells were injected into mouse tongues, and representative images of tissue sections stained with H&E are shown. Note that control cells exhibited collective invasion into the surrounding stroma of the tongue, which were less frequent in MRTF-B- or Trim27-depleted cells (arrowheads).

Development

Human cancer

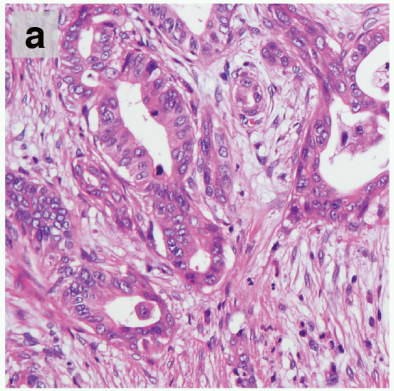
A

Adrenal gland (13 weeks, human)

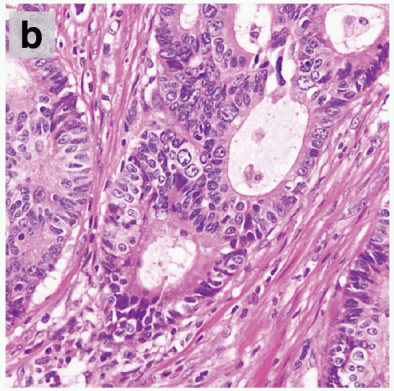


B

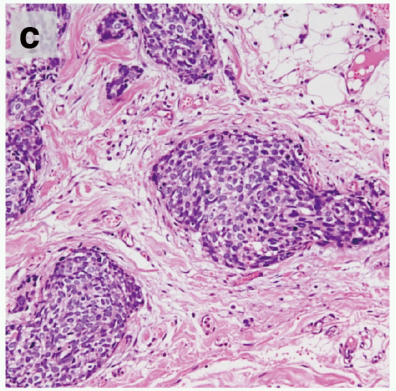
Pancreatic cancer



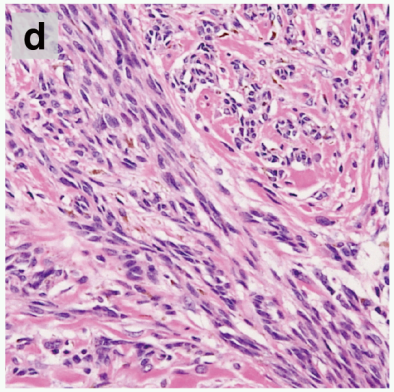
Colon cancer



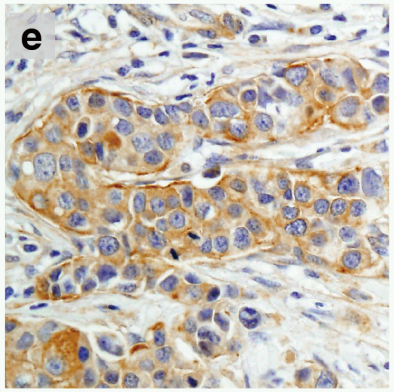
Sebaceous cancer



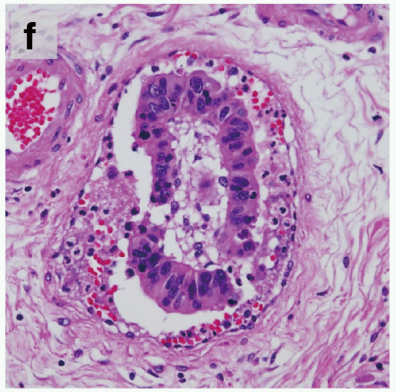
Melanoma



Breast cancer
(IHC: Girdin)

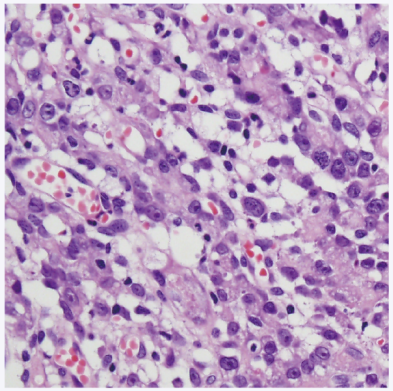


Lymph vessel invasion

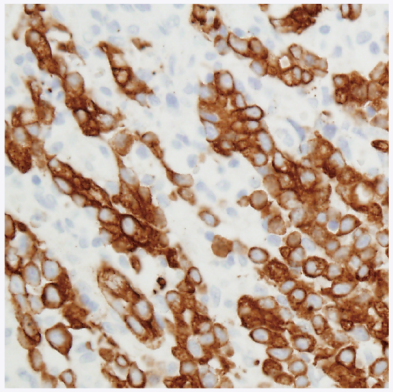


C

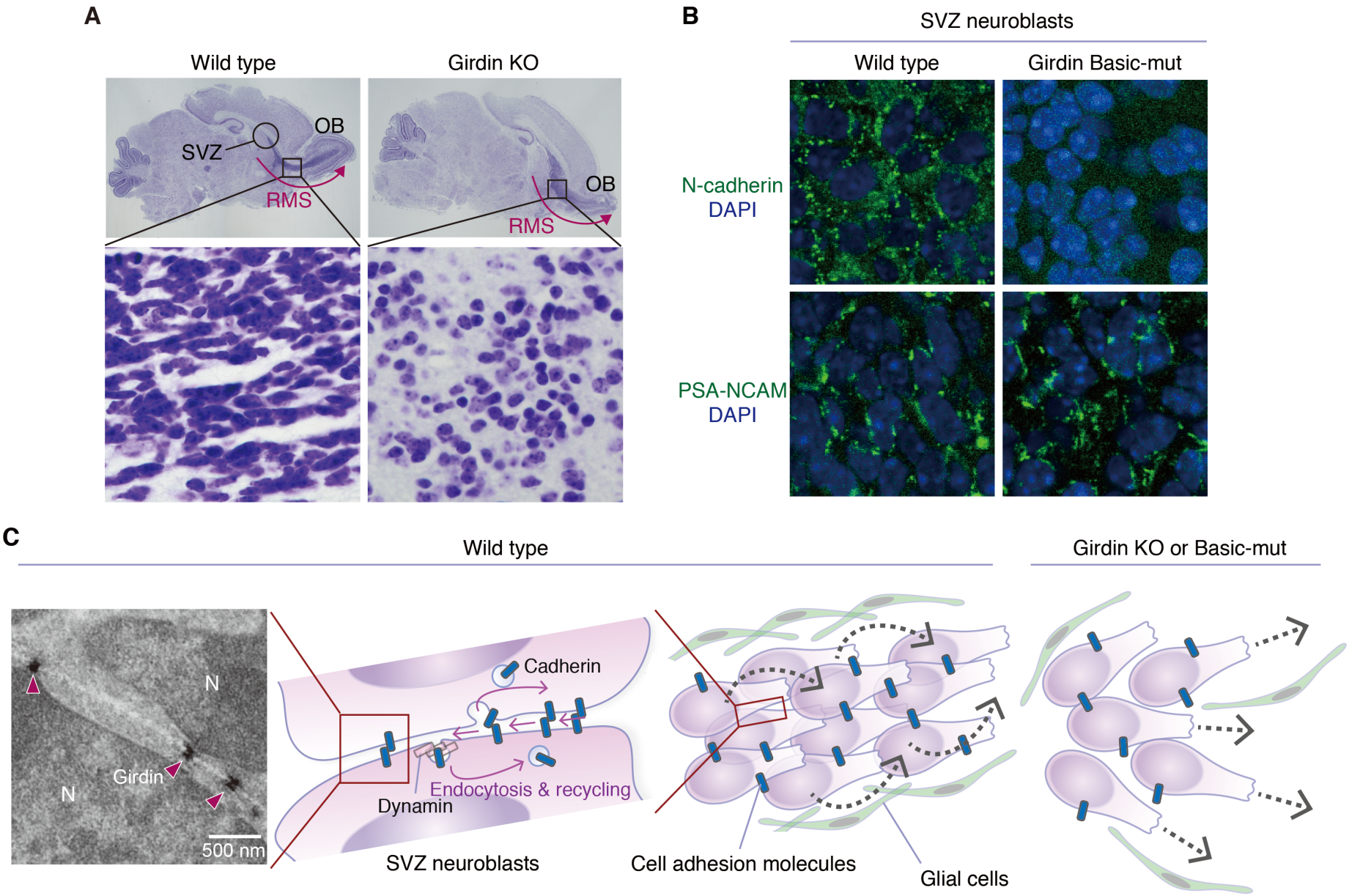
Gastric cancer
(Poorly differentiated type)



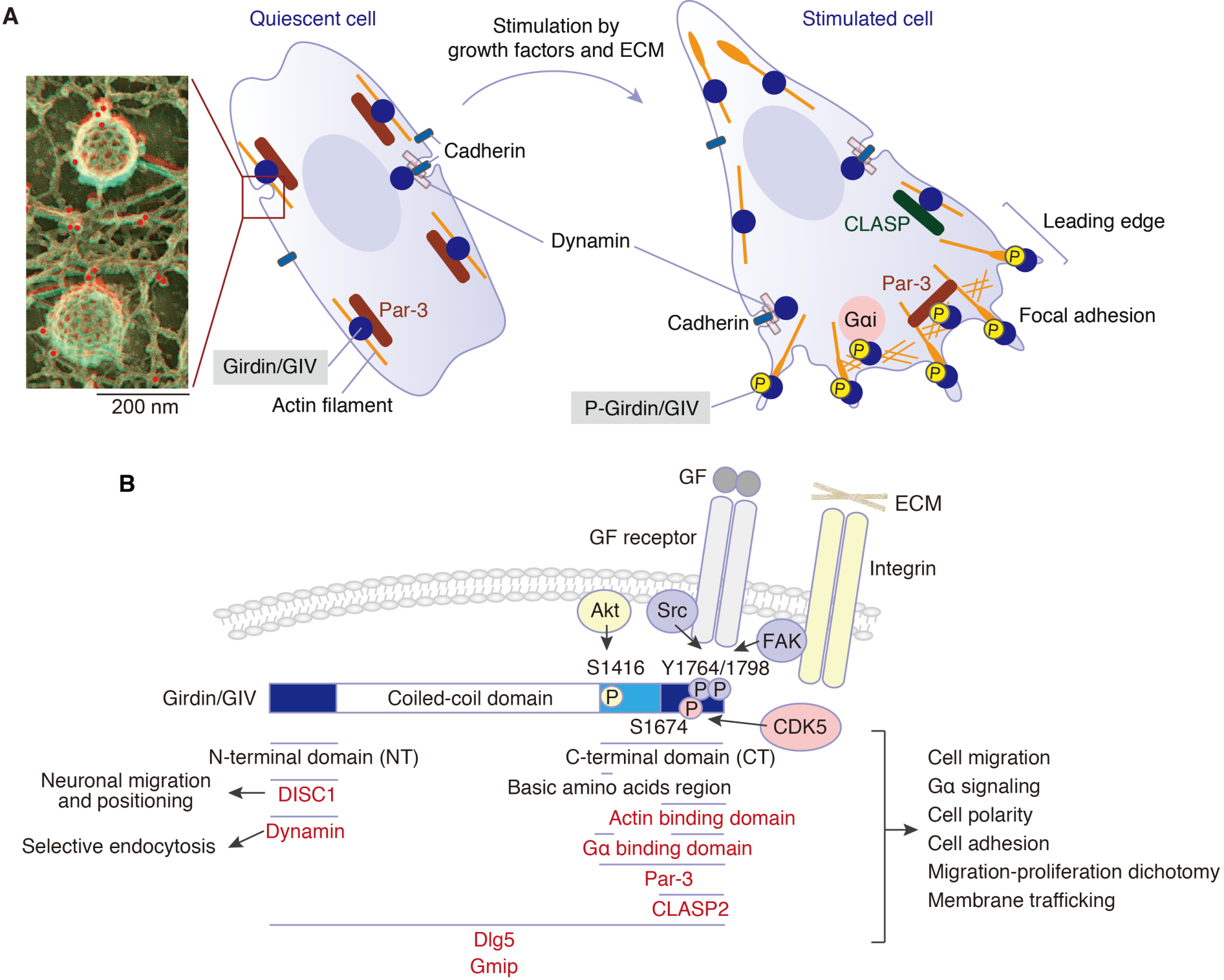
Keratin (AE1/AE3) staining



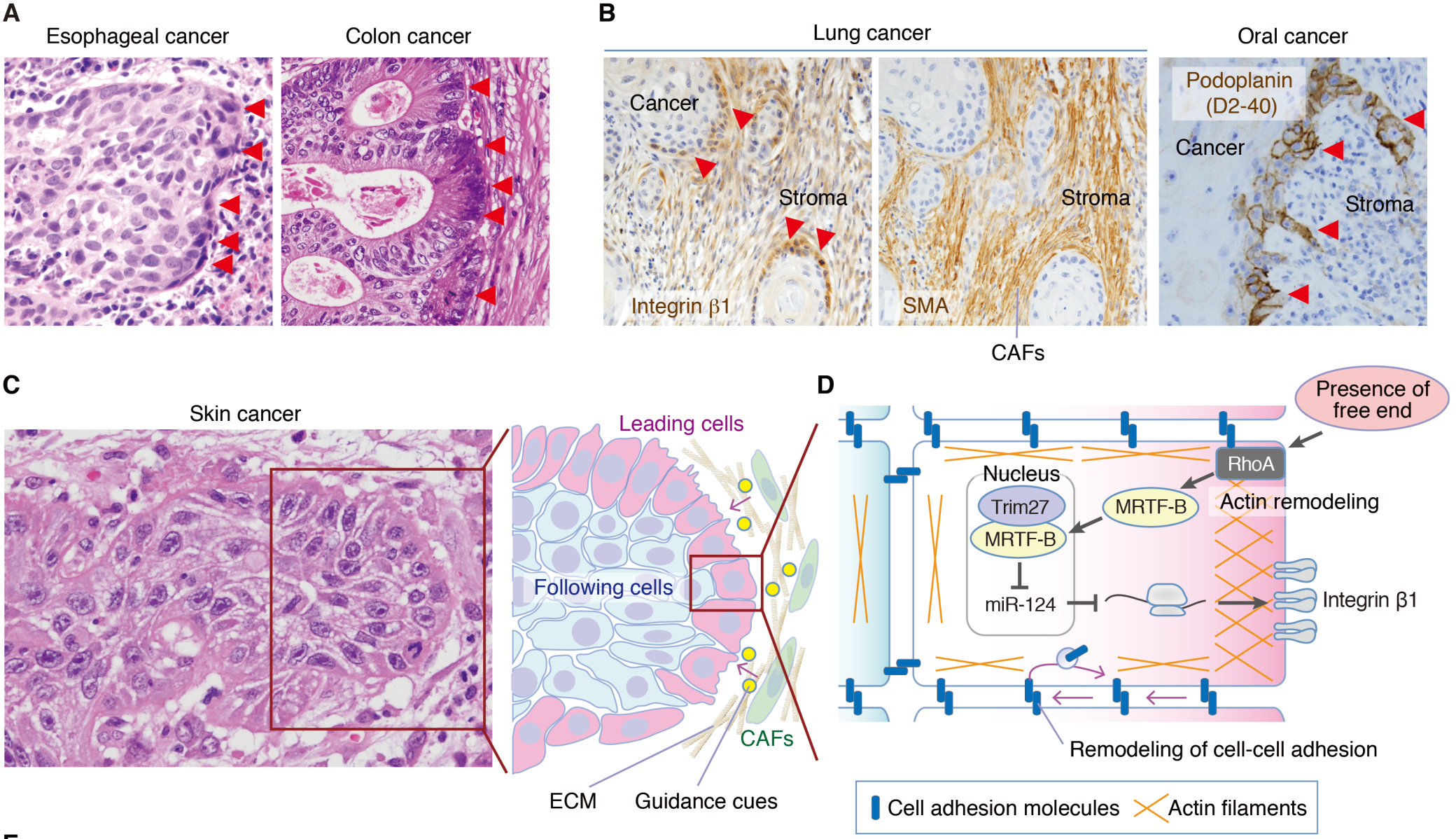
Wang et al., Figure 1



Wang et al., Figure 2



Wang et al., Figure 3



Wang et al., Figure 4