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Roles of cell adhesion molecules nectin and nectin-like molecule-5 in the regulation of cell movement and proliferation

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Summary

In response to chemoattractants, migrating cells form protrusions, such as lamellipodia and filopodia, and structures, such as ruffles over lamellipodia, focal complexes, and focal adhesions at leading edges. The formation of these leading edge structures is essential for directional cell movement. Nectin-like molecule-5 (Necl-5) interacts in *cis* with PDGF receptor and integrin $\alpha_v\beta_3$, and enhances the activation of signaling molecules associated with these transmembrane proteins, which results in the formation of leading edge structures and enhancement of directional cell movement. When migrating cells come into contact with each other, cell-cell adhesion is initiated, resulting in reduced cell velocity. Necl-5 first interacts in *trans* with nectin. This interaction is transient and induces down-regulation of Necl-5 expression at the cell surface, resulting in reduced cell movement. Cell proliferation is also suppressed by the down-regulation of Necl-5, because the inhibitory effect of Necl-5 on Sprouty2, a negative regulator of the Ras signaling, is diminished. PDGF receptor and integrin $\alpha_v\beta_3$, which have interacted with Necl-5, then form a complex with nectin, which initiates cell-cell adhesion and recruits cadherin to the nectin-based cell-cell adhesion sites to form stable adherens junctions. The formation of adherens junctions stops cell movement, in part through inactivation of integrin $\alpha_v\beta_3$ caused by the *trans*-interaction of nectin. Thus, nectin and Necl-5 play key roles in the regulation of cell movement and proliferation.

Introduction

Normal cells, when cultured *in vitro,* migrate and proliferate until they become confluent and form cell-cell junctions, after which cell migration and proliferation cease. This phenomenon was identified as 'contact inhibition of cell movement' and 'contact inhibition of cell division' (Bell, 1978; Fisher & Yeh, 1967). 'Contact inhibition', originally described in fibroblasts, is phenomenon of a cell ceasing to migrate in the same direction after contact with another cell (Abercrombie & Heaysman, 1953; Abercrombie & Heaysman, 1954). Since then, the concept of contact inhibition has included the observation that cells become immobilized and are unable to continue moving, once they form cell-cell adhesions, which has been demonstrated in epithelial wound healing (Abercrombie, 1970; Abercrombie & Ambrose, 1962). Nowadays the term 'contact inhibition of cell movement' is used broadly (Huttenlocher *et al.*, 1998; Zegers *et al.*, 2003), as is the case for this review article as well. Although the term 'contact inhibition of cell division' was taken from 'contact inhibition of cell movement', there is no clear evidence that this phenomenon is dependent on cell contact; in fact, there is compelling evidence that it is not (Dunn & Ireland, 1984; Martz & Steinberg, 1972). To avoid confusion between terminologies, this review describes the phenomenon of confluent cells down-regulating mitosis as 'density dependent inhibition of mitosis' (DDIM) (Stoker & Rubin, 1967).

In contrast to normal cells, transformed cells continue to migrate and proliferate, even after they come into contact with each other. Thus contact inhibition of cell movement and DDIM in transformed cells is disrupted, resulting in invasion into neighboring tissues and metastasis to other organs. Cell adhesion molecules (CAMs) and growth factor receptors play a role in cell movement and proliferation, and although their roles have been extensively studied (Lauffenburger & Horwitz, 1996; Benito & Lorenzo, 1993), the molecular mechanism for contact

inhibition of cell movement and DDIM has been poorly understood to date.

During the formation of cell-cell junctions from individually migrating cells, primordial cell-cell contact first occurs by a collision of moving cells. Mature cell-cell junctions in epithelial cells are then formed with the specialized cell-cell junction machinery, such as adherens junctions (AJs) and tight junctions (TJs), and with the apico-basal cell polarization at the cell-cell adhesion sites. These processes are called mesenchymal-epithelial transition (MET). AJs are coated with bundles of actin filaments and serve as a mechanically adhesive apparatus between neighboring cells. TJs are found especially at the apical side of AJs in epithelial cells and act as barriers to prevent leakage of soluble molecules through gaps between cells, and they act as fences to keep cell surface molecules at the basolateral region separate from those at the apical region (Tsukita *et al.*, 1999; Tsukita & Furuse, 2002). In contrast, certain physiological and pathological conditions, such as embryonic development and cancer progression, preferentially cause the disruption of cell-cell junctions. The cells lose their connection to neighboring cells and become free, which increases the likelihood for cell migration and proliferation. This phenomenon is called epithelial-mesenchymal transition (EMT), which is opposite to MET (Gumbiner, 2005).

The phenomena of contact inhibition of cell movement, DDIM, and EMT strongly correlate to the dynamic regulation between the formation and disruption of cell-cell junctions. Our recent studies on an immunoglobulin (Ig)-like cell-cell adhesion molecule, nectin, and its structurally related molecule nectin-like molecules (Necls), especially Necl-5, revealed the importance of nectin and Necl-5 for these phenomena (Sakisaka *et al.*, 2007; Ogita & Takai, 2006; Takai *et al.*, 2003; Takai & Nakanishi, 2003). This review focuses on the roles of nectin and Necl-5 in fundamental cellular functions, such as cell migration, proliferation, and cell-cell junctions, and the implications of contact inhibition of cell movement, DDIM, and

EMT.

Nectin: A molecule that plays a central role in the formation of cell-cell junctions

Molecular characteristics of nectin

Nectin is a Ca^{2+}-independent cell-cell adhesion molecule, localized strictly at AJs. It possesses three Ig-like loops within its extracellular region, a single transmembrane segment, and one cytoplasmic tail (Takai & Nakanishi, 2003; Takai et al., 2003). The nectin family consists of four members: nectin-1, nectin-2, nectin-3, and nectin-4. Nectin-1, nectin-2, and nectin-3 are widely expressed in adult tissues as well as various cells, including fibroblasts, epithelial cells, and neurons. Nectin-2 and nectin-3 are also expressed in cells that lacks cadherin, such as hematopoietic cells and spermatids, respectively (Lopez *et al.*, 1998; Ozaki-Kuroda *et al.*, 2002). In humans, the expression of nectin-4 is highly limited to the placenta, but is dramatically up-regulated in breast cancer (Reymond *et al.*, 2001; Fabre-Lafay *et al.*, 2005).

Each nectin forms first homo-*cis*-dimers, similar to cadherin. However, in contrast to cadherin, which homophilically *trans*-interacts, nectin induces the formation of homophilic or heterophilic *trans*-dimers (*trans*-interaction). Nectin-1 heterophilically *trans*-interacts with nectin-3 and nectin-4, and nectin-2 also *trans*-interacts with nectin-3. These heterophilic *trans*-interactions exhibit significantly higher affinity than the homophilic *trans*-interactions (Satoh-Horikawa *et al.*, 2000; Ikeda *et al.*, 2003). The first Ig-like loop at the extracellular region of nectin is necessary for the formation of *trans*-dimers, but not for *cis*-dimers, whereas the second Ig-like loop contributes to the formation of *cis*-dimers (Momose *et al.*, 2002; Yasumi *et al.*, 2003). The function of the third Ig-like loop is currently unknown.

All nectin family members directly bind afadin, which links nectin to the actin

cytoskeleton, similar to how catenins connect cadherin to the actin cytoskeleton (Takahashi *et al.*, 1999). The C-terminal PDZ-binding motif of nectin and the PDZ domain of afadin mediate this binding. The C-terminal motif of nectin-1, nectin-2, and nectin-3 is conserved (Glu/Ala-X-Tyr-Val; X is any amino acid), while that of nectin-4 is different (Gly-His-Leu-Val). However, all nectin family members interact directly with afadin.

Roles of nectin in the formation of cell-cell junctions

In epithelial cells, the primary CAMs in AJs and TJs are E-cadherin and claudin, respectively, and it is obvious that they play important roles in the formation of these junctions (Takeichi, 1991; Tsukita et al., 1999). Previous work from our laboratory has demonstrated the crucial role that nectin has in the formation of not only AJs, but also TJs, especially in the initial step of cell-cell junction formation (Takai & Nakanishi, 2003; Takai et al., 2003; Shimizu & Takai, 2003; Nakanishi & Takai, 2004; Sakisaka & Takai, 2004; Irie *et al.*, 2004). The *trans*-interaction of nectin first occurs at the primordial cell-cell contact sites, and is subsequently involved in the recruitment of cadherin and claudin to AJs and TJs, respectively.

Our results demonstrated that the intracellular signaling pathways are related to the nectin-induced formation of AJs **(Fig. 1)**. The *trans*-interaction of nectin induces the activation of c-Src. This is followed by the activation of Rap1 small G protein through the Crk-C3G complex and tyrosine-phosphorylation of FRG (a GDP/GTP exchange factor (GEF) for Cdc42) and Vav2 (a GEF for Rac) (Fukuhara *et al.*, 2004; Fukuyama *et al.*, 2005; Kawakatsu *et al.*, 2005). Both the activation of Rap1 and phosphorylation of FRG are necessary for further activation of FRG, which is followed by the activation of Cdc42. Similarly, both the activation of Cdc42 and phosphorylation of Vav2 are necessary for further activation of Vav2, which is followed by the activation of Rac. On one hand, activated Cdc42 and Rac

small G proteins facilitate the formation of filopodia and lamellipodia, respectively, at the primordial cell-cell contact sites, as well as increase the area of contact sites between adjacent cells. On the other hand, these small G proteins reorganize the actin cytoskeleton and promote the recruitment of the cadherin-catenin complex to the nectin-based cell-cell adhesion sites (Fukuhara *et al.*, 2003; Sato *et al.*, 2006).

The cross-talk exists between cell-cell and cell-matrix junctions in epithelial cells and non-epithelial cells, and integrin affects the formation of cell-cell junctions (Siu & Cheng, 2004; Monier-Gavelle & Duband, 1997; Schreider *et al.*, 2002). Consistently, the intracellular signals induced by *trans*-interacting nectin are dependent on the activation of integrin $\alpha_v\beta_3$ (Sakamoto *et al.*, 2006). Nectin and integrin $\alpha_v\beta_3$ physically interact at the cell-cell adhesion site. Activated integrin $\alpha_v\beta_3$ and its downstream signaling molecules, protein kinase C (PKC) and focal adhesion kinase (FAK), support the activation of c-Src (Sakamoto et al., 2006; Ozaki *et al.*, 2007). Conversely, integrin $\alpha_v\beta_3$ becomes inactivated after the establishment of cell-cell junctions. This is one of the underlying mechanisms of contact inhibition of cell movement and DDIM, because the activation/inactivation of integrin is important for the control of cell movement and proliferation. We also revealed that this inactivation is mediated by nectin (Sakamoto *et al.*, 2007), as described later in detail.

In epithelial cells, once AJs are created, TJs are then formed at the apical side of AJs, in parallel with or accompanied by, cell polarization. The following results have suggested that cadherin plays a key regulatory role in the formation and maintenance of TJs: (1) the formation and maintenance of TJs are dependent on extracellular Ca^{2+}, which is necessary for the cell-cell adhesion activity of cadherin (Gonzalez-Mariscal *et al.*, 1985); (2) E-cadherin-blocking antibodies inhibit the formation of TJs, as demonstrated by electron microscopy and barrier assay (Gumbiner *et al.*, 1988); (3) AJs and TJs are not formed in PC9 carcinoma cells,

which do not express α-catenin (Watabe *et al.*, 1994). We recently discover, however, that the *trans*-interaction of E-cadherin is not absolutely necessary for the formation of TJs, but rather the nectin-afadin system is indispensable. The recombinant protein of Nef, which is the extracellular fragment of nectin fused to the IgG Fc portion, inhibits intercellular interactions of nectin and blocks the formation of TJs (Fukuhara *et al.*, 2002a; Fukuhara *et al.*, 2002b). The treatment with tumor-promoting phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate, at low Ca^{2+} concentrations, or the knockdown of annexin II, a protein factor necessary for the formation of AJs, still results in the formation of TJs (Yamada *et al.*, 2006; Okamoto *et al.*, 2005). Similar results have also been reported using MDCK cells or *Drosophila* models (Capaldo & Macara, 2007; Harris & Peifer, 2004). Furthermore, nectin also binds Par-3 (Takekuni *et al.*, 2003), a member of the Par complex that regulates cell polarization (Suzuki & Ohno, 2006). Both the nectin-afadin complex and Par-3 are cooperatively involved in the formation of TJs (Ooshio *et al.*, 2007).

Although normal cells *in vitro* cease to migrate and proliferate once they have reached confluency and have formed mature cell-cell junctions, the cells must maintain cell-cell junctions to keep multicellular organisms alive. Growth factor receptors, including platelet-derived growth factor (PDGF) receptor, play a crucial role in cell survival (Franke *et al.*, 1995). In addition, we have newly identified an interaction between nectin and PDGF receptor at the cell-cell adhesion sites (unpublished data). This interaction as well as the function of afadin is necessary for PDGF-induced cell survival signals, such as the phosphatidylinositol 3-kinase/Akt pathway. This has been shown in not only NIH3T3 fibroblasts, but also embryoid bodies generated from embryonic stem cells, in which the depletion of afadin greatly increased apoptosis. These data are likely to provide additional confirmation that the tightly regulated cell survival by the nectin-afadin system is crucial for normal embryonic development.

Disruption of cell-cell junctions and EMT

Cell-cell junction disruptions are related to EMT and are necessary for the normal development of embryos and physiological processes of tissue turnover (Gumbiner, 2005). In pathological situations, such as cancer, disruption of E-cadherin-based AJs enhances tumor development, invasion, and metastasis. The disassembly of AJs and TJs also causes local inflammation by increasing the paracellular permeability of the epithelium and endothelium as well as the extravasation of monocytes and macrophages. The establishment of cell-cell junctions is mainly dependent on the formation of AJs. The molecular mechanism of CAMs, which help to maintain AJs at their own site, is important to prevent cell-cell junction disruption and to understand the phenomenon of EMT.

In response to growth factors, such as hepatocyte growth factor (HGF), E-cadherin at AJs is prepared to internalize through endocytosis. Increased internalization of E-cadherin causes cell-cell junction disruption, cell scattering, and finally EMT (Kamei *et al.*, 1999; Lu *et al.*, 2003; Bryant *et al.*, 2005). We have previously shown that Rab5 small G protein and a Ras-activated Rab5-GEF, RIN2, are positive regulators for the HGF-induced E-cadherin endocytosis and cell scattering (Imamura *et al.*, 1998; Kamei et al., 1999; Kimura *et al.*, 2006). In contrast, the activation of Rac induced by *trans*-interacting E-cadherin at AJs negatively regulates endocytosis of E-cadherin through a IQGAP-dependent reorganization of the actin cytoskeleton, and thereby stabilizes E-cadherin on the plasma membrane (Izumi *et al.*, 2004). The positive and negative regulation systems of E-cadherin endocytosis contribute to a balanced cycle of E-cadherin traffic between the plasma membrane and the cytoplasm, which helps to maintain AJs.

Given that the high-affinity form of integrin $\alpha_v\beta_3$ up-regulates cell migration

and proliferation, which tends to disrupt cell-cell adhesion, it is necessary to inactivate integrin $\alpha_v\beta_3$, following completion of cell-cell junctions. This helps to maintain the formation of cell-cell junctions for an extended period of time. The mature *trans*-interaction of nectin is likely to be involved in this integrin inactivation **(Fig. 2)**. Nectin associates with, and induces the activation of, a protein tyrosine phosphatase, PTPμ, at the cell-cell adhesion sites (Sakamoto et al., 2007). Following formation of cell-cell junctions, activated PTPμ inhibits phosphatidylinositol phosphate kinase type Iγ (PIPKIγ). PIPKIγ has been shown to be involved in the activation of integrin by increasing the generation of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and promoting the binding of talin to integrin (Martel *et al.*, 2001; Di Paolo *et al.*, 2002; Ling *et al.*, 2002). Thus, apart from functions during the formation of cell-cell adhesion, nectin negatively regulates PIPKIγ through PTPμ and inhibits integrin $\alpha_v\beta_3$ after the formation of cell-cell junctions.

Necl-5: A molecule that critically regulates cell movement and proliferation

Molecular properties of Necl-5

Necl is genetically and structurally similar to nectin, but does not bind afadin at its C-terminal cytoplasmic tail (Takai et al., 2003). Although the Necl family comprises five members, this review focuses specifically on Necl-5. Necl-5 was originally identified as a poliovirus receptor (PVR)/CD155 in humans (Mendelsohn *et al.*, 1989; Koike *et al.*, 1990) and as a gene product, Tage4, which is over-expressed in rat colon carcinoma (Chadeneau *et al.*, 1994). PVR/CD155 was subsequently shown to be over-expressed in many human cancer cells (Masson *et al.*, 2001; Sloan *et al.*, 2004). This molecule had four nomenclatures (Necl-5/PVR/CD155/Tage4), but was re-named Necl-5 (Takai et al., 2003). In contrast to the nectin family members, Necl-5 does not homophilically *trans*-interact but heterophilically *trans*-interacts with nectin-3 and other Ig-like molecules, such as CD96/Tactile and CD226/DNAM-1 (Bottino *et al.*, 2003; Fuchs *et al.*, 2004; Ikeda et al., 2003; Mueller & Wimmer, 2003). CD226/DNAM-1, which is a single membrane-spanning molecule with two Ig-like loops at its extracellular region, is mainly expressed in T cells and supports the differentiation and proliferation of T cells (Chen *et al.*, 2003; Shibuya *et al.*, 2003). CD96/Tactile is also a member of the Ig superfamily and promotes adhesion of T cells to target cells expressing Necl-5, which triggers T cell activation (Wang *et al.*, 1992; Fuchs et al., 2004). The physiological role of Necl-5 has been unclear for some time; however, its various cellular functions were recently clarified from a series of experiments in our laboratory.

Necl-5-mediated enhancement of cell movement

Necl-5 localizes at the leading edge of the migrating cells together with integrin $\alpha_v\beta_3$ and PDGF receptor (Ikeda *et al.*, 2004; Amano *et al.*, 2007). Necl-5 interacts

directly in *cis* with integrin $\alpha_v\beta_3$ through extracellular regions, resulting in clustering of integrin $\alpha_v\beta_3$ at the leading edge (Minami *et al.*, 2007a). Similarly, Necl-5 physically interacts with PDGF receptor and induces the accumulation of it at the leading edge (Amano et al., 2007). In addition, Necl-5 stabilizes the binary complex of integrin $\alpha_v\beta_3$ and PDGF receptor. Thus, the dynamic formation of the leading edge, which is necessary for cell migration, is dependent on Necl-5.

The leading edge is formed in the direction of cell migration and comprises specialized cellular structures, such as lamellipodia, peripheral ruffles, focal complexes, and focal adhesions (Hall, 1998; Rottner *et al.*, 1999; Zaidel-Bar *et al.*, 2004). Peripheral ruffles and focal complexes are formed above and beneath lamellipodia, respectively, and focal adhesions are formed at the backside of focal complexes. These structures are formed by re-organization of the actin cytoskeleton, which is regulated by the Rho family small G proteins. Lamellipodia and ruffles are formed by Rac signals, filopodia by Cdc42 signals, focal complexes by Rac and Cdc42 signals, and focal adhesions by Rho signals (Hall, 1998). Focal complexes are transformed into focal adhesions by inactivation of Cdc42 and Rac and activation of Rho (Rottner et al., 1999; Ballestrem *et al.*, 2001). Therefore, the regulation of these small G proteins is critical for the formation of leading edges and cell movement. A detailed observation of leading edge structures revealed that Necl-5, PDGF receptor, and integrin $\alpha_v\beta_3$ are all concentrated at peripheral ruffles; however, only two molecules, Necl-5 and integrin $\alpha_v\beta_3$, are concentrated at focal complexes. The transformation of focal complexes to focal adhesions results in the dissociation of Necl-5 from integrin $\alpha_v\beta_3$ **(Fig. 3)**.

A series of Necl-5 studies in our laboratory revealed the molecular mechanism of the regulation of small G proteins, which is involved in the PDGF-induced formation of leading edges. Upon stimulation with PDGF, Necl-5, PDGF receptor, and integrin $\alpha_v\beta_3$ cluster and are recruited to leading edges. Rap1

is activated through Crk and C3G (unpublished data). Crk is an adaptor protein that binds both C3G, a GEF for Rap1, and phosphorylated PDGF receptor. In addition, Crk also regulates the GEF activity of C3G (Ichiba *et al.*, 1997; Matsumoto *et al.*, 2000). In this way, activated Rap1 exerts three functions on the formation of leading edge structures **(Fig. 3)**. First, it interacts with Vav2, a GEF for Rac (Arthur *et al.*, 2004), and induces the activation of Rac at the leading edge, which increases the formation of peripheral ruffles and focal complexes (unpublished data). Second, activated Rap1 also binds ARAP1, a GTPase activating protein (GAP) for RhoA (Miura *et al.*, 2002), and inactivates RhoA. This results in the inhibition of the transition of focal complexes to focal adhesions, followed by increased focal complexes at the leading edge (unpublished data). Third, activated Rap1 also forms a complex with afadin. This complex recruits SPA-1, a GAP for Rap1, which in turn inactivates Rap1 (Su *et al.*, 2003) (unpublished data). If the GTP-bound active form of Rap1, which is likely to bind to and activate ARAP1, is inactivated by SAP-1, the activation of RhoA is induced at the leading edge.

Following stimulation with PDGF, PDGF receptor is down-regulated at the leading edge by endocytosis, and the activation of Rap1 is thereby gradually reduced. On one hand, the activation of Rac through Rap1 is decreased, followed by the impairment of ruffle formation. On the other hand, the activation of RhoA is increased by the inhibition of Rap1-ARAP1 signaling as described above. Activated RhoA consequently induces the activation of Rho-associated kinase (ROCK) to promote the transformation of focal complexes to focal adhesions (unpublished data). During this transformation, ROCK is probably involved in the dissociation of Necl-5 from integrin $\alpha_v\beta_3$ by regulating the actomyosin-driven contractility. The dynamic regulations of small G protein signals locally at the leading edge seem to be important for efficient cell migration.

Necl-5 also binds the dynein light chain, Tctex-1, in the cytoplasmic region of

Necl-5 (Mueller *et al.*, 2002; Ohka *et al.*, 2004). Dynein is a member of the microtubule (MT) plus-end-binding proteins (+TIPs) that participates in search and capture of MTs as well as intracellular retrograde transport of molecules (Mimori-Kiyosue & Tsukita, 2003). In directional cell migration, reorientation of the MT network is necessary for the search of membrane cues toward cell migration. The +TIPs are involved in the search and determination of the direction of cell movement. The direct interaction of Necl-5 with Tctex-1 targets the dynein/dynactin complex and plus ends of MTs to the leading edge of moving cells (unpublished data). Necl-5 also regulates the localization of MT-stabilizing proteins, such as LL5β, at the rear portion of the leading edge. Therefore, Necl-5 plays essential roles in capturing growing (pioneering) MTs, the reorientation of MT networks, and directional cell migration through MT-related proteins.

Necl-5-mediated enhancement of cell proliferation

PDGF-initiated intracellular signaling of cell proliferation is regulated by Necl-5 at a step downstream of PDGF receptor and upstream of Ras small G protein (Kakunaga *et al.*, 2004). In NIH3T3 cells, Necl-5 enhances activation of the Ras-Raf-MEK-ERK signaling cascade and causes up- and down-regulation of cell cycle regulators, including cyclins D2 and E and cyclin-dependent kinase inhibitor $p27^{Kip1}$. These effects contribute to shortening the G_1 phase of the cell cycle and enhance PDGF-induced cell proliferation.

We recently demonstrated that PDGF-induced activation of Ras-mediated cell proliferation signaling is contradictorily regulated by Necl-5 and Sprouty (Kajita *et al.*, 2007). Sprouty was originally identified as an antagonist of FGF, which contributes to apical branching of the *Drosophila* airways (Hacohen *et al.*, 1998). c-Src-catalyzed tyrosine-phosphorylated Sprouty inhibited the growth factor-induced activation of Ras and subsequent activation of the Raf-MEK-ERK

signaling at a site upstream of Ras and downstream of growth factor receptors (Gross *et al.*, 2001; Hanafusa *et al.*, 2002; Li *et al.*, 2004; Mason *et al.*, 2004). Thus, Sprouty has been shown to be a negative regulator of growth factor-induced intracellular signaling (Christofori, 2003; Kim & Bar-Sagi, 2004). Although Necl-5 and Sprouty exhibit opposite effects in growth factor-induced cell proliferation signaling, these molecules function at the same step of the signaling cascade.

When cells migrate individually, Necl-5 sequesters Sprouty2 and thus reduces the inhibitory effect of Sprouty2 on the PDGF-induced Ras signaling (Kajita et al., 2007). At the leading edge, where Necl-5 exists in abundance, Sprouty2 does not inhibit PDGF-induced Ras signaling. Therefore, cell proliferation takes place through the Raf-MEK-ERK signaling pathway. In contrast, when Necl-5 is down-regulated at the cell surface of confluent cultured cells, Sprouty2 is released by Necl-5 and inhibits Ras signaling. Thus, this down-regulation of Necl-5 is at least partly involved in DDIM, because the decrease in Necl-5 on the cell surface causes the suppression of cell proliferation in a cell density-dependent manner (Fujito *et al.*, 2005). A detailed molecular mechanism of how Necl-5 is down-regulated in confluent cells is addressed below.

Involvement of Necl-5 in contact inhibition of cell movement

Among the nectin and Necl family members, Necl-5 does not homophilically *trans*-interact, but heterophilically *trans*-interacts with nectin-3 (Takai et al., 2003). When individually migrating cells collide with each other, the initial cell-cell contacts takes place by the heterophilic *trans*-interaction of Necl-5 at the leading edge with nectin-3 on the adjacent cell surface (Ikeda et al., 2003). This *trans*-interaction induces the activation of Cdc42 and Rac (Sato *et al.*, 2005), both of which rearrange the actin cytoskeleton and increase the number of cell-cell adhesion sites. However, the *trans*-interaction of Necl-5 with nectin-3 is tentative, and

down-regulation of Necl-5 from the cell surface occurs by endocytosis in a clathrin-dependent manner (Fujito et al., 2005). The down-regulation of Necl-5 leads to a reduction in cell movement and proliferation, because signals initiated by integrin $\alpha_v\beta_3$ and growth factor receptors are not effectively transmitted into cells in the absence of Necl-5. Next, nectin-3 dissociated from Necl-5 is not internalized, but retained at the cell surface, and subsequently *trans*-interacts with nectin-1, which most likely further *trans*-interacts with nectin-3 among the nectin family members (Ikeda et al., 2003). This *trans*-interaction of nectins induces the recruitment of cadherin to the nectin-based adhesion sites, eventually establishing AJs, as described above. In some transformed cells, such as NIH3T3 cells that ectopically over-express the oncogene V12-Ki-Ras, the expression of Necl-5 is markedly up-regulated, and is not down-regulated, even following the formation of cell-cell junctions (Minami *et al.*, 2007b). In such cells, the suppression of cell movement and proliferation after the formation of cell-cell junctions is disrupted, and thus, contact inhibition of cell movement and DDIM are lost. Collectively, Necl-5 seems to essentially contribute to the underlying mechanisms of contact inhibition of cell movement and DDIM.

Conclusions

We have shown that nectin and Necl-5 are at least partly involved in the underlying mechanisms of both contact inhibition of cell movement and DDIM. Although Necl-5 enhances cell migration and proliferation cooperatively with integrin $\alpha_v\beta_3$ and PDGF receptor in migrating cells, such functions are suppressed when Necl-5 is down-regulated at the cell surface by the formation of nectin-based cell-cell adhesion. Following the establishment of cell-cell junctions, nectin, in turn, inhibits the activation of integrin $\alpha_v\beta_3$ through PTPμ and PIPKIγ. Therefore, both the decrease in Necl-5 and the increase in nectin at the cell-cell adhesion sites seem to be important for contact inhibition and DDIM by suppressing the function of integrin $\alpha_v\beta_3$. Although these phenomena have different molecular mechanisms, it is interesting that they are regulated by the same cell adhesion molecules, nectin and Necl-5. Moreover, controlled cell migration and proliferation are critical for physiological organization of tissues. If this process is disrupted, cells grow in an unlimited fashion and easily invade neighboring tissues, leading to pathogenesis, such as cancer and atherosclerosis. Finally, understanding the underlying molecular mechanisms of contact inhibition and DDIM would also largely develop the diagnosis and treatment of such diseases. Further studies in this field are needed to reach this goal.

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Figure legends

Fig. 1. Nectin-induced and integrin $\alpha_v\beta_3$-dependent intracellular signaling in the formation of cadherin-based AJs. Nectin-induced activation of c-Src is supported by integrin $\alpha_v\beta_3$-PKC-FAK signaling. Activated c-Src phosphorylates FRG, a Cdc42-GEF, and Vav2, a Rac-GEF, and contributes to the activation of Rap1 through the Crk-C3G complex. Rap1 then fully activates tyrosine-phosphorylated FRG, resulting in the activation of Cdc42. Similarly, Cdc42 and Vav2 activate Rac. The activated small G proteins reorganize the actin cytoskeleton and promote recruitment of the cadherin-catenin complex to the nectin-based cell-cell adhesion sites.

Fig. 2. Inactivation of integrin $\alpha_v\beta_3$ after formation of cell-cell junctions. The activation of integrin $\alpha_v\beta_3$ is induced by binding to vitronectin, an ECM protein, at its extracellular region, and to talin at its cytoplasmic tail. Activated integrin $\alpha_v\beta_3$ up-regulates kinase activity of c-Src and FAK, both of which are involved in the phosphorylation and activation of PIPKIγ, resulting in increased PI(4,5)P_2 production. PI(4,5)P_2 promotes the interaction of talin with integrin $\alpha_v\beta_3$. Thus, there is a positive feedback loop for the activation of integrin $\alpha_v\beta_3$. In contrast, when the nectin-induced cell-cell junctions are formed, the phosphatase activity of PTPμ is enhanced by *trans*-interacting nectin to dephosphorylate and inactivate PIPKIγ, which results in the disruption of the positive feedback loop and the inactivation of integrin $\alpha_v\beta_3$.

Fig. 3. Three roles of Rap1 in the formation of leading edge structures in migrating cells. (A) First role of Rap1. Upon PDGF stimulation, Rap1 becomes activated at the leading edge, interacts with Vav2, and induces the activation of Rac, which leads to the formation of peripheral ruffles and focal complexes. **(B)** Second

role of Rap1. Activated Rap1 binds to ARAP1 and inactivates RhoA. An inactive form of RhoA is incapable of transforming focal complexes to focal adhesions, so that focal complexes accumulate at the leading edge. **(C)** Third role of Rap1. Activated Rap1 forms a complex with afadin and this complex recruits SPA-1, which in turn inactivates Rap1. When Rap1 is inactivated, the function of ARAP is impaired and the activation of RhoA occurs at the leading edge, followed by the activation of ROCK and the transition of focal complexes to focal adhesions.

Fig. 1

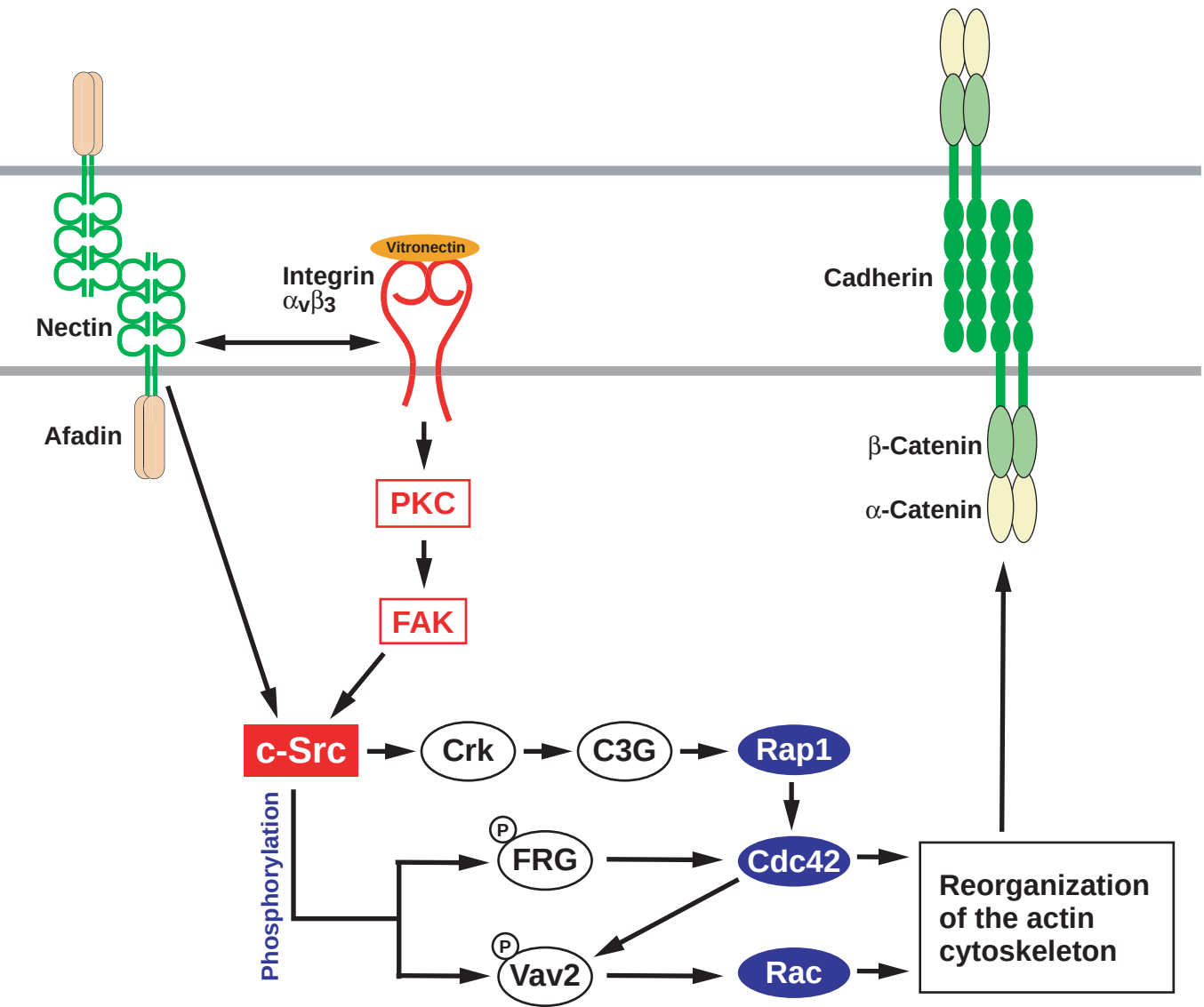

Nectin
Afadin
Integrin
αvβ3
Vitronectin
Cadherin
β-Catenin
α-Catenin
PKC
FAK
c-Src
Crk
C3G
Rap1
Phosphorylation
P
FRG
Cdc42
P
Vav2
Rac
Reorganization of the actin cytoskeleton

Fig. 2

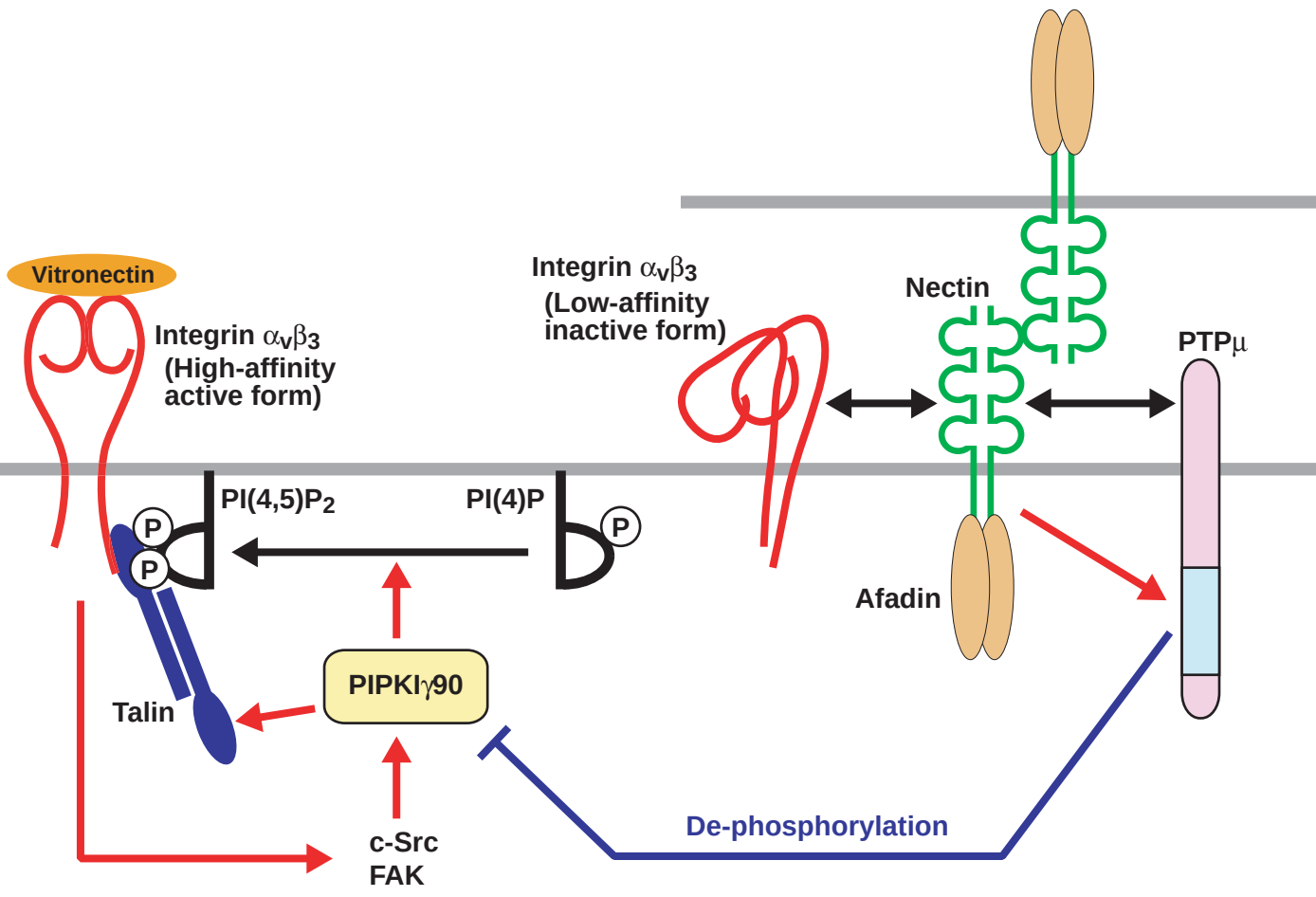
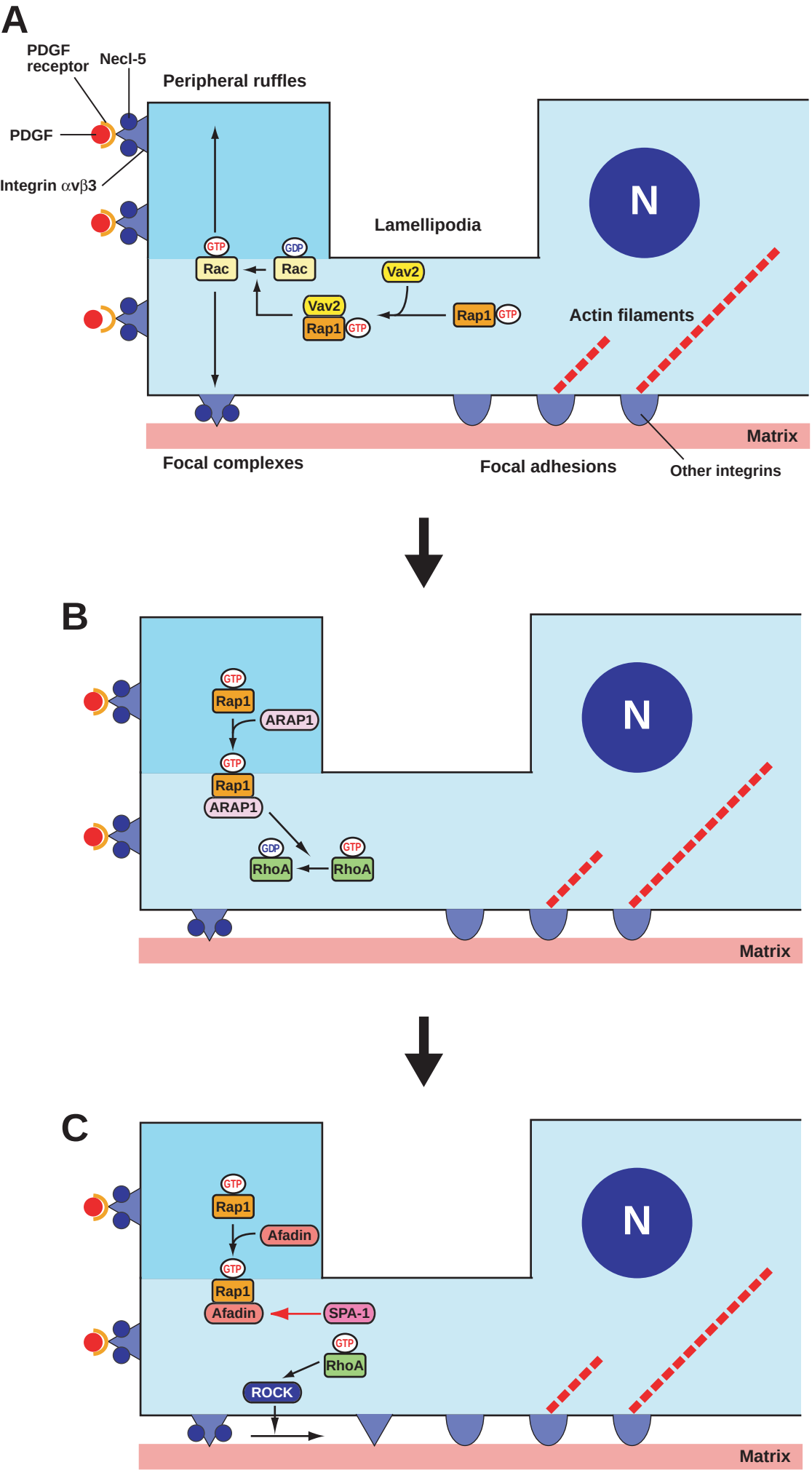

Vitronectin
Integrin αvβ3
(High-affinity
active form)
Integrin αvβ3
(Low-affinity
inactive form)
Nectin
PTPμ
PI(4,5)P2
PI(4)P
P
P
P
Afadin
Talin
PIPKIγ90
c-Src
FAK
De-phosphorylation

Fig. 3


A
PDGF receptor
Necl-5
Peripheral ruffles
PDGF
Integrin αvβ3
Lamellipodia
N
GTP
Rac
GDP
Rac
Vav2
Vav2
Rap1
GTP
Rap1
GTP
Actin filaments
Matrix
Focal complexes
Focal adhesions
Other integrins
B
GTP
Rap1
ARAP1
GTP
Rap1
ARAP1
N
GDP
RhoA
GTP
RhoA
Matrix
C
GTP
Rap1
Afadin
GTP
Rap1
Afadin
SPA-1
N
GTP
RhoA
ROCK
Matrix