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Interaction and localization of Necl-5 and PDGF receptor β at the leading edges of moving NIH3T3 cells: implications for directional cell movement

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Running title: Interaction of Necl-5 with PDGF receptor

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Abstract

It was previously shown that platelet-derived growth factor (PDGF) receptor physically and functionally interacts with integrin $\alpha_v\beta_3$, effectively inducing cell movement. We previously showed that Necl-5, originally identified as a poliovirus receptor, interacts with integrin $\alpha_v\beta_3$ and enhances its clustering and the formation of focal complexes at the leading edges of moving cells, resulting in an enhancement of cell movement. We showed here that Necl-5 additionally interacts with PDGF receptor in NIH3T3 cells and regulates the interaction between PDGF receptor and integrin $\alpha_v\beta_3$, effectively inducing directional cell movement. PDGF receptor co-localized with Necl-5 and integrin $\alpha_v\beta_3$ at peripheral ruffles over lamellipodia, which were formed at the leading edges of moving cells in response to PDGF, but not at the focal complexes under these ruffles, whereas Necl-5 and integrin $\alpha_v\beta_3$ co-localized at these focal complexes. The clustering of these three molecules at peripheral ruffles required the activation of integrin $\alpha_v\beta_3$ by vitronectin and the PDGF-induced activation of the small G protein Rac and subsequent reorganization of the actin cytoskeleton. These results indicate a key role of Necl-5 in directional cell movement by physically and functionally interacting with both integrin $\alpha_v\beta_3$ and PDGF receptor.

Introduction

Cells move toward chemotactic substances (chemoattractants), such as growth factors, chemokines, and cytokines (Gumbiner 1996; Lauffenburger & Horwitz 1996). These substances first bind to their cell surface receptors which then transduce signals for cell movement. It was previously believed that the receptors for chemoattractants localize at leading edges of moving cells, but their localizations and regulatory mechanisms remained undetermined. It was reported that T cells respond to chemokines, such as SDF1α and that chemokine receptors, such as CXCR4, localize at leading edges (Shimonaka *et al.* 2003). Contrary to T cells, it was reported that, in amoeba *Dictiostellium discoideum*, a cell surface receptor for cyclic AMP, a chemoattractant for this amoeba, is uniformly distributed along the plasma membrane, but that its downstream signaling molecules, such as PTEN and PI3-kinase, are unevenly distributed: PI3-kinase localizes at leading edges whereas PTEN localizes at rear edges (Comer & Parent 2002; Weiner 2002). Thus, the localization of each receptor varies and the mechanisms underlying their localizations remain unknown. Fibroblasts respond to platelet-derived growth factor (PDGF) as a chemoattractant, and polarize in the direction of higher concentrations of PDGF (Ronnstrand & Heldin 2001). However, the localization and behavior of PDGF receptor remain unknown.

At leading edges, special structures necessary for cell movement are dynamically formed: these structures include protrusions, such as filopodia and lamellipodia, peripheral ruffles, focal complexes, and focal adhesions (Hall 1998; Rottner *et al.* 1999; Zaidel-Bar *et al.* 2004). These structures are formed by reorganization of the actin cytoskeleton, which is regulated by the actions of the Rho family small G proteins: lamellipodia and ruffles are formed by the action of

Rac; filopodia are formed by the action of Cdc42; and focal complexes are formed by the action of Rac and Cdc42 (Rottner *et al.* 1999). The formation of these structures is inhibited by the action of Rho. Focal complexes are transformed to focal adhesions by inactivation of Cdc42 and Rac and activation of Rho (Rottner *et al.* 1999; Ballestrem *et al.* 2001). The activities of these small G proteins are cooperatively regulated by receptors and integrins.

Integrins are cell-matrix adhesion molecules that are essential for cell movement in cooperation with cell surface receptors for chemoattractants (van der Flier & Sonnenberg 2001; Comoglio *et al.* 2003). Integrins form cell-matrix junctions called focal complexes and focal adhesions (Cram & Schwarzbauer 2004). Focal complexes are smaller in size than focal adhesions, and are formed at contact sites between protrusions and extracellular matrix (ECM) proteins at leading edges (Rottner *et al.* 1999; Zaidel-Bar *et al.* 2004). Focal adhesions are formed at sites to the rear of focal complexes. Ruffles randomly attach to matrix and some of them form new focal complexes; pre-existing focal complexes are transformed to focal adhesions. Integrins have at least two forms: conformations with either low or high affinity for their ECM binding partners (van der Flier & Sonnenberg 2001; Takagi *et al.* 2002; Cram & Schwarzbauer 2004). When talin binds to the low-affinity form, this integrin is converted to the high-affinity form (Cram & Schwarzbauer 2004). Upon binding to ECM proteins, integrins transduce signals inside cells that then cause the reorganization of the actin cytoskeleton, eventually resulting in the clustering of integrins and the formation of focal complexes and focal adhesions (Cram & Schwarzbauer 2004). Of the many integrins, integrin $\alpha_v\beta_3$ forms focal complexes and this integrin is often up-regulated in cancer cells (Ballestrem *et al.* 2001; Guo & Giancotti 2004).

It was previously shown that PDGF receptor physically interacts with integrin $\alpha_v\beta_3$ (Schneller *et al.* 1997; Woodard *et al.* 1998) and that this interaction

synergistically enhances cell movement and proliferation (Woodard *et al.* 1998). However, the regulatory and molecular mechanisms of this interaction remain unknown.

We recently found that nectin-like molecule (Necl)-5 forms a complex with integrin $\alpha_v\beta_3$ and enhances its clustering and subsequent formation of focal complexes at the leading edges of moving NIH3T3 cells in response to PDGF (Minami *et al.* 2007). This action of Necl-5 requires both the activation of PDGF receptor by PDGF and the activation of integrin $\alpha_v\beta_3$ by vitronectin, an ECM protein that binds to integrin $\alpha_v\beta_3$ (Cheresh 1987). Necl-5 was originally identified as a poliovirus receptor (PVR)/CD155 in humans (Mendelsohn *et al.* 1989; Koike *et al.* 1990) and as the product of a gene, Tage4, which is overexpressed in colon carcinomas in rodents (Chadeneau *et al.* 1994). PVR/CD155 was also shown to be overexpressed in many human cancer cells (Gromeier *et al.* 2000; Masson *et al.* 2001; Bottino *et al.* 2003). This molecule, with four nomenclatures, was re-named Necl-5 (Takai *et al.* 2003). However, it remains unknown how the Necl-5-integrin complex is clustered at leading edges or how cells determine the direction of movement in response to PDGF.

In the present study, we attempted to address these issues and found that Necl-5 interacts not only with integrin $\alpha_v\beta_3$ but also with PDGF receptor and that the Necl-5-PDGF receptor complex also localizes at peripheral ruffles over lamellipodia at the leading edges of NIH3T3 cells, which were formed in response to PDGF in a vitronectin-dependent manner. We discuss the role of this interaction in directional movement. In this study, we studied concerning PDGF receptor β and it is simply referred to as PDGF receptor.

Results

Co-localization of PDGF receptor with Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles over lamellipodia of leading edges

We previously showed that when NIH3T3 cells are sparsely plated on μ-slide VI flow dishes pre-coated with vitronectin, starved of serum, and directionally stimulated by PDGF, most cells become polarized and form protrusive lamellipodia, peripheral ruffles over the lamellipodia, focal complexes under the ruffles, and focal adhesions at leading edges in the direction of higher concentrations of PDGF (Minami *et al.* 2007). The formation of these leading edge structures requires the activation of PDGF receptor by PDGF, the activation of integrin $\alpha_V\beta_3$ by vitronectin, and the expression of Necl-5. Necl-5 physically interacts with integrin $\alpha_V\beta_3$ and the Necl-5-integrin $\alpha_V\beta_3$ complex localizes at peripheral ruffles over the lamellipodia of leading edges and the focal complexes under the ruffles, but not at the focal adhesions. Consistent with these observations, the immunofluorescence signals for Necl-5 and integrin β_3 were concentrated and co-localized at peripheral ruffles over the lamellipodia **(Fig. 1Aa)**. In the basal sections of the cells, the signals for Necl-5 and integrin β_3 were observed at the focal complexes under the peripheral ruffles **(Fig. 1Aa; fuzzy dot-like structures and line-like focal complexes (FXs))**. The signal for integrin β_3, but not that for Necl-5, was observed at the focal adhesions at sites to the rear of leading edges. Essentially the same results were obtained when cells were stained for integrin α_V instead of integrin β_3 (data not shown). Indeed, unless otherwise specified, the same results were essentially obtained for both integrin α_V and integrin β_3 in the experiments that follow. The signal for PDGF receptor was faintly detected at the peripheral ruffles and co-localized with those for Necl-5 and integrin β_3 in the middle sections of the cells. In the basal sections

of the cells, the signal for PDGF receptor was detected as an indistinct signal under the peripheral ruffles. By co-localization analysis, PDGF receptor loosely co-localized with Necl-5, but weakly co-localized with integrin β_3. Therefore, PDGF receptor might not be concentrated at focal complexes.

In NIH3T3 cells stably overexpressing Necl-5 (Necl-5-NIH3T3 cells), the signals for Necl-5 and integrin β_3 were enhanced at the peripheral ruffles and the focal complexes of leading edges, as described previously (Minami *et al.* 2007) **(Fig. 1Ba; belt-like structures and belt-like FXs)**. The signal for PDGF receptor was also enhanced at the peripheral ruffles and co-localized with those for Necl-5 and integrin β_3 in the middle sections of the cells. In the basal sections of the cells, the signal for PDGF receptor was detected as an indistinct signal under the peripheral ruffles, similar to that in NIH3T3 cells. Taken together, these results indicate that PDGF receptor co-localizes with Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles over the lamellipodia of leading edges of NIH3T3 cells, but not at the focal complexes under these peripheral ruffles.

In NIH3T3 cells in which Necl-5 was knocked down by an siRNA vector (Necl-5-knockdown-NIH3T3 cells), definite leading edges were not formed, the signal for Necl-5 was negligible **(Fig. 1C, a and b)**, and the signal for integrin b_3 was not concentrated at any region, as described previously (Minami *et al.* 2007) (data not shown). The signal for PDGF receptor was not concentrated at any peripheral region, although dot-like signals for PDGF receptor were detected inside cells **(Fig. 1C, a and b)**. These signals might represent endocytosed PDGF receptor. When NIH3T3 or Necl-5-NIH3T3 cells were similarly cultured on dishes coated with laminin, an ECM protein that does not bind to integrin $\alpha_V\beta_3$ (Cheresh 1987), instead of vitronectin-coated ones, or were cultured on vitronectin-coated dishes but not stimulated by PDGF, they did not show polarized structures with protrusions, ruffles or focal complexes **(Fig. 1, Ab and**

Bb, and data not shown**)** (Minami *et al.* 2007). None of the signal for PDGF receptor, Necl-5, or integrin β_3 was concentrated at any peripheral region. These results are consistent with our earlier observations (Minami *et al.* 2007) and indicate that the formation of the leading edge structures and the localization of PDGF receptor as well as Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles require the activation of PDGF receptor by PDGF, the activation of integrin $\alpha_V\beta_3$ by vitronectin, and the expression of Necl-5.

Physical interaction of Necl-5 with PDGF receptor

We then examined whether Necl-5 physically interacts with PDGF receptor using a co-immunoprecipitation assay. We co-expressed HA-tagged PDGF receptor (PDGF receptor-HA) and either of FLAG-tagged Necl-5 (FLAG-Necl-5) or FLAG alone as a control, in HEK293 cells, and cultured the cells in suspension in the presence or absence of PDGF and AG1296. We used HEK293 cells because the expression of integrin $\alpha_V\beta_3$ was not significantly detected by Western blotting in this cell line (data not shown). The suspension culture enabled the detection of a possible *cis*-interaction between Necl-5 and PDGF receptor on the plasma membrane, rather than *trans*-interaction. The presence of PDGF predominantly made PDGF receptor the tyrosine phosphorylated form, whereas the presence of AG1296 made it the non-phosphorylated form (data not shown). AG1296 is an inhibitor of the kinase activity of PDGF receptor (Kovalenko *et al.* 1997). Because overexpression of PDGF receptor induces autophosphorylation of this protein to some degree even in the absence of PDGF (data not shown), this inhibitor was added to keep PDGF receptor from being auto-phosphorylated. When FLAG-Necl-5 was immunoprecipitated from cell lysates using the anti-FLAG monoclonal antibody (mAb), PDGF receptor-HA was co-immunoprecipitated with FLAG-Necl-5 irrespective of the presence or

absence of PDGF and AG1296 (**Fig. 2A)**. The amount of co-immunoprecipitated PDGF receptor-HA in the presence or absence of PDGF and AG1296 was not significantly different. PDGF receptor-HA was not co-immunoprecipitated with FLAG from cells expressing FLAG as a control, irrespective of the presence or absence of PDGF and AG1296. These results indicate that Necl-5 physically interacts in *cis* with both the tyrosine-phosphorylated and non-phosphorylated forms of PDGF receptor to similar extents.

We next examined whether the extracellular and/or cytoplasmic regions of Necl-5 are necessary for the interaction with PDGF receptor. We expressed FLAG-Necl-5 with either the cytoplasmic or extracellular region deleted (FLAG-Necl-5-ΔCP or FLAG-Necl-5-ΔEC, respectively) and PDGF receptor-HA, in HEK293 cells, and performed similar experiments to those described above. PDGF receptor was co-immunoprecipitated with FLAG-Necl-5-ΔCP, irrespective of the presence or absence of PDGF and AG1296, whereas PDGF receptor was not co-immunoprecipitated with FLAG-Necl-5-ΔEC in either condition **(Fig. 2A)**. The amount of PDGF receptor-HA that was co-immunoprecipitated with FLAG-Necl-5-ΔCP was about three times as much as that co-immunoprecipitated with the full-length FLAG-Necl-5. The amount of co-immunoprecipitated PDGF receptor-HA in the presence or absence of PDGF and AG1296 was not significantly different. These results indicate that Necl-5 physically interacts in *cis* with both the tyrosine-phosphorylated and non-phosphorylated forms of PDGF receptor, through its extracellular region, and that the cytoplasmic region of PDGF receptor is not essential for this interaction, but regulates it.

We then confirmed that endogenous Necl-5 physically interacts not only with endogenous integrin $\alpha_V\beta_3$ but also with endogenous PDGF receptor. NIH3T3 cells were cultured on vitronectin-coated dishes, treated with the chemical cross-linker DTSSP, and then endogenous integrin α_V was

immunoprecipitated from cell lysates. DTSSP was used as we previously showed an interaction between endogenous Necl-5 and endogenous integrin $\alpha_V\beta_3$ using this reagent in NIH3T3 cells (Minami *et al.* 2007). Necl-5 and PDGF receptor were co-immunoprecipitated with integrin α_V from cell lysates **(Fig. 2B)**. Taken together, these findings suggest that Necl-5 physically interacts with both integrin $\alpha_V\beta_3$ and PDGF receptor at peripheral ruffles over lamellipodia of leading edges of NIH3T3 cells which are formed in response to PDGF in a vitronectin-dependent manner.

It was previously shown by a co-immunoprecipitation assay using endogenous proteins from NIH3T3 and rat microvascular endothelial cells that PDGF receptor physically interacts with integrin $\alpha_V\beta_3$ (Schneller *et al.* 1997; Woodard *et al.* 1998), but it remains unknown whether this interaction is direct or mediated by Necl-5, because we previously showed that Necl-5 physically interacts with integrin $\alpha_V\beta_3$ (Minami *et al.* 2007) and we showed here that Necl-5 physically interacts with PDGF receptor. In addition, it was shown that integrin $\alpha_V\beta_3$ has two forms: low- and high-affinity forms (van der Flier & Sonnenberg 2001; Takagi *et al.* 2002; Cram & Schwarzbauer 2004). We previously showed that Necl-5 physically interacts with both forms to similar extents (Minami *et al.* 2007), but it remains unknown with which form of integrin $\alpha_V\beta_3$ PDGF receptor interacts. We next examined these two issues. We expressed PDGF receptor-HA, and either EGFP-tagged integrin β_3 (integrin β_3-EGFP) and integrin α_V, or EGFP alone as a control, in HEK293 cells, and cultured the cells in suspension in the presence or absence of PDGF and AG1296. Mn^{2+} and cyclo-RGDfV, an integrin-binding peptide, were also added to the medium to make predominantly the high-affinity form of integrin $\alpha_V\beta_3$ (Takagi *et al.* 2002). Alternatively, we co-expressed PDGF receptor-HA, and either EGFP-tagged integrin $\beta_3^{T329C/A347C}$ (integrin $\beta_3^{T329C/A347C}$-EGFP) and integrin α_V, or EGFP alone

as a control, in HEK293 cells, and cultured the cells in suspension in the presence or absence of PDGF and AG1296. Ca^{2+} and Mg^{2+} were added to the medium to make predominantly the low-affinity form of integrin $\alpha_V\beta_3$. Integrin $\beta_3^{T329C/A347C}$ does not make the high-affinity form with integrin α_V (Luo *et al.* 2004). We used HEK293 cells because the expression of Necl-5 was not significantly detected by Western blotting in this cell line (data not shown). When integrin β_3-EGFP was immunoprecipitated from cell lysates using the anti-GFP polyclonal antibody (pAb), integrin α_V and PDGF receptor-HA were co-immunoprecipitated with it, irrespective of the presence or absence of PDGF and AG1296 **(Fig. 2C)**. Essentially the same results were obtained when integrin $\beta_3^{T329C/A347C}$-EGFP was immunoprecipitated. None of these molecules was co-immunoprecipitated with EGFP from cells expressing EGFP as a control. These results are consistent with earlier observations (Schneller *et al.* 1997; Woodard *et al.* 1998) and indicate that PDGF receptor physically interacts with both the low- and high-affinity forms of integrin $\alpha_V\beta_3$ to similar extents and that these interactions are not mediated by Necl-5.

Taken together, the present and previous results indicate that Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$ can form any combinations of binary complex.

Actin cytoskeleton-dependent localization of PDGF receptor at peripheral ruffles

It was shown that the actin cytoskeleton is necessary for the clustering of integrin in general (Cram & Schwarzbauer 2004). Because PDGF is necessary for the formation of leading edge structures, including lamellipodia, peripheral ruffles over the lamellipodia, focal complexes under the ruffles, and focal adhesions, and the localization of PDGF receptor, Necl-5, and integrin $\alpha_V\beta_3$ at their specific sites, we examined whether the localization of PDGF receptor as well as Necl-5 and

integrin $\alpha_V\beta_3$ at the peripheral ruffles is dependent on the actin cytoskeleton. When NIH3T3 cells were sparsely plated on μ-slide VI flow dishes pre-coated with vitronectin, starved of serum, and directionally stimulated by PDGF in the presence of cytochalasin D, cells did not form protrusions, such as lamellipodia and ruffles **(Fig. 3A)**. The immunofluorescence signal for actin stress fibers was lost, but the signal for peripheral actin remained and that for actin patches appeared inside the cells. The signal for integrin β_3 was faintly detected at focal adhesions. Focal complexes were not observed at any region. The signal for Necl-5 was not concentrated at any peripheral region. The signal for PDGF receptor was also not concentrated at any peripheral region, but was detected as internalized dot-like structures inside the cells. Essentially the same results were obtained when this experiment was performed using Necl-5-NIH3T3 cells, except for a decrease in the intensity of the internalized dot-like signals for PDGF receptor **(Fig. 3B)**. These results indicate that the localization of PDGF receptor as well as Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles is dependent on the actin cytoskeleton.

Rac-dependent localization of PDGF receptor at peripheral ruffles

We previously showed that the PDGF-induced activation of Rac is involved in the formation of lamellipodia and peripheral ruffles, the Necl-5-dependent clustering of integrin $\alpha_V\beta_3$, and the formation of focal complexes at the leading edges of moving NIH3T3 cells (Minami *et al.* 2007). We examined whether the PDGF-induced activation of Rac is involved in the localization of PDGF receptor as well as Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles. When NIH3T3 cells expressing a dominant-negative mutant of Rac1 (Rac1DN) were sparsely plated on μ-slide VI flow dishes pre-coated with vitronectin, starved of serum, and directionally stimulated by PDGF, the extents of protrusions, ruffles, and focal

complexes were significantly reduced compared with those in NIH3T3 cells **(Fig. 4A and see Fig. 1Aa)**. Moreover, the immunofluorescence signal for Necl-5 or integrin β_3 was not concentrated at any peripheral region; consistent with our earlier observations (Minami *et al.* 2007). The signal for PDGF receptor was not concentrated at any peripheral region. Essentially the same results were obtained when this experiment was performed using Necl-5-NIH3T3 cells **(Fig. 4B)**. These results indicate that the PDGF-induced activation of Rac is involved in the localization of PDGF receptor as well as Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles.

Regulation of the interaction between PDGF receptor and integrin $\alpha_V\beta_3$ by Necl-5 and PDGF

We finally studied the role of the interaction between Necl-5 and PDGF receptor. We first examined whether this interaction affects the interaction between PDGF receptor and integrin $\alpha_V\beta_3$. Necl-5-NIH3T3 cells were cultured on vitronectin-coated dishes in the presence or absence of PDGF, treated with DTSSP, and then endogenous integrin α_V was immunoprecipitated from cell lysates. Necl-5 and PDGF receptor were co-immunoprecipitated with integrin α_V from cell lysates, irrespective of the presence or absence of PDGF **(Fig. 5A)**. The amount of Necl-5 co-immunoprecipitated with integrin α_V was not affected by PDGF, but the amount of PDGF receptor co-immunoprecipitated with integrin α_V was 10-20% reduced by PDGF. When wild-type NIH3T3 cells were similarly subjected to the same co-immunoprecipitation assay, Necl-5 and PDGF receptor were co-immunoprecipitated with integrin α_V from cell lysates, irrespective of the presence or absence of PDGF **(Fig. 5B, see also Fig. 2B)**. The amount of Necl-5 co-immunoprecipitated with integrin α_V was not affected by PDGF, but the amount of PDGF receptor co-immunoprecipitated with integrin α_V was 50-60%

reduced by PDGF. When Necl-5-knockdown-NIH3T3 cells were similarly subjected to the same co-immunoprecipitation assay, PDGF receptor, but not Necl-5, was co-immunoprecipitated with integrin α_V, irrespective of the presence or absence of PDGF **(Fig. 5C)**. The amount of PDGF receptor co-immunoprecipitated with integrin α_V was about 90% reduced by PDGF. These results are consistent with those of the immunofluorescence microscopic examination shown in **Fig. 1** and indicate that Necl-5 and PDGF regulate the interaction between PDGF receptor and integrin $\alpha_V\beta_3$: PDGF reduces the interaction and Necl-5 blocks this reduction.

It should be noted that the amount of Necl-5 that was co-immunoprecipitated with integrin α_V from wild-type NIH3T3 cells was slightly reduced by PDGF **(Fig. 5B)** (Minami *et al.* 2007). The exact reason for this reduction is not known, but may be due to the facts that Necl-5 co-localizes with integrin $\alpha_V\beta_3$ at focal complexes, but not at focal adhesions, and that PDGF induces the formation of focal complexes and the subsequent transformation of focal complexes to focal adhesions.

Enhancement of cell movement by Necl-5 and PDGF

We previously showed that Necl-5 enhances the movement of L and NIH3T3 cells in the presence of serum containing various growth factors and ECM proteins (Ikeda *et al.* 2004; Fujito *et al.* 2005). Using a Boyden chamber assay, we then examined whether overexpression or knockdown of Necl-5 affects directional cell movement. Necl-5-NIH3T3 and Necl-5-knockdown NIH3T3 cells were cultured on vitronectin-coated cell culture inserts and stimulated by PDGF. Necl-5-NIH3T3 cells moved more rapidly than wild-type NIH3T3 cells, whereas Necl-5-knockdown-NIH3T3 cells moved more slowly than control-NIH3T3 cells **(Fig. 6, A and B)**. Movement of these three types of cells was dependent on

vitronectin and they did not move on BSA- or laminin-coated cell culture inserts (data not shown) (Woodard *et al.* 1998; Ikeda *et al.* 2004). These results are consistent with our earlier observations (Ikeda *et al.* 2004; Fujito *et al.* 2005) and indicate that the expression level of Necl-5 regulates PDGF-induced directional cell movement.

Discussion

It is well known that fibroblasts respond to PDGF as a chemoattractant and form leading edge structures, including lamellipodia, peripheral ruffles over the lamellipodia, focal complexes under the ruffles, and focal adhesions, in the direction of higher concentrations of PDGF (Ronnstrand & Heldin 2001). However, the localization and behavior of PDGF receptor remained unknown. We showed here for the first time that PDGF receptor, like Necl-5 and integrin $\alpha_V\beta_3$, localizes at the peripheral ruffles over the lamellipodia, but not at the focal complexes or the focal adhesions. We previously showed that Necl-5 and integrin $\alpha_V\beta_3$ co-localize at the peripheral ruffles and the focal complexes, but not at the focal adhesions, and that integrin $\alpha_V\beta_3$ localizes additionally at the focal adhesions (Minami *et al.* 2007). This localization of PDGF receptor at the peripheral ruffles is dependent on the activation of PDGF receptor by PDGF, the activation of integrin $\alpha_V\beta_3$ by vitronectin, and the expression of Necl-5. The localization of PDGF receptor at the peripheral ruffles was faintly observed in wild-type NIH3T3 cells, but became prominent when Necl-5 was overexpressed. Thus, we succeeded in identifying the specific sites where PDGF receptor clusters and is concentrated in moving cells. The reason why the specific localization of PDGF receptor in moving fibroblasts was not identified might be that the involvement of Necl-5 in this localization was not known.

It has been shown that PDGF receptor physically interacts with integrin $\alpha_V\beta_3$ (Schneller *et al.* 1997; Woodard *et al.* 1998). We previously showed that Necl-5 physically interacts with integrin $\alpha_V\beta_3$ (Minami *et al.* 2007). In the present study, we confirmed these earlier observations and further showed that Necl-5 physically interacts with PDGF receptor through their extracellular regions. The cytoplasmic region of Necl-5 was not necessary for this interaction, but it may

regulate the interaction, because the amount of PDGF receptor-HA co-immunoprecipitated with FLAG-Necl-5-ΔCP (FLAG-Necl-5 with the cytoplasmic region deleted) was about three times as much as that co-immunoprecipitated with the full-length FLAG-Necl-5. There are theoretically *cis*- and *trans*-interactions between Necl-5 and PDGF receptor; however, the results of the immunoprecipitation assay using suspended HEK293 cells support an interaction in *cis*. Therefore, Necl-5 and PDGF receptor on the plasma membrane of the same cell physically interact with each other in *cis*.

PDGF receptor and integrin $\alpha_V\beta_3$ each have two forms: tyrosine-phosphorylated and non-phosphorylated forms and low- and high-affinity forms, respectively (Heldin *et al.* 1998; Takagi *et al.* 2002; Cram & Schwarzbauer 2004). It remained unknown which forms of PDGF receptor and integrin interact with each other, but we showed here that PDGF receptor and integrin $\alpha_V\beta_3$ have potencies to interact with any forms to similar extents. In addition, we showed here that Necl-5 interacts equally with both forms of PDGF receptor. PDGF receptor, Necl-5, and integrin $\alpha_V\beta_3$ all co-localize at peripheral ruffles over lamellipodia of leading edges of NIH3T3 cells. These three molecules might form a ternary complex there, but it is practically difficult to obtain definitive evidence for this conclusion because each of Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$ can form any combinations of binary complex. Therefore, at present we conclude that each of Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$ forms at least each binary complex and localizes at peripheral ruffles of lamellipodia of leading edges.

It was previously shown that cell movement requires the synergistic activity of PDGF- and integrin-induced signaling (Woodard *et al.* 1998). PDGF-induced signaling requires integrin $\alpha_V\beta_3$ activation by binding to its ECM proteins (Woodard *et al.* 1998). The binding of talin to integrin increases the

affinity of integrin for its specific ECM protein, and the binding of integrin to its specific ECM protein transduces signals inside cells leading to the reorganization of the actin cytoskeleton, which is necessary for integrin clustering (Cram & Schwarzbauer 2004). These inside-out and outside-in signaling pathways from the growth factor receptor and integrin bring about the formation of focal complexes and focal adhesions. Consistently, we previously showed that PDGF-induced, Necl-5- and integrin $\alpha_V\beta_3$-dependent formation of leading edge structures requires the PDGF-induced activation of Rac and subsequent reorganization of the actin cytoskeleton (Minami *et al.* 2007). In the present study, we confirmed these earlier observations and showed that the actin cytoskeleton and Rac activation are further necessary for the localization of PDGF receptor at peripheral ruffles over lamellipodia of leading edges of NIH3T3 cells. In addition, we showed here that Necl-5 and PDGF regulate the interaction between PDGF receptor and integrin $\alpha_V\beta_3$: PDGF reduces the interaction and Necl-5 blocks this reduction. Thus, Necl-5 plays a key role in this interaction. Taken together, these findings suggest that when PDGF reaches and binds to its receptor and induces Rac activation at specific regions; Rac activated in this way induces reorganization of the actin cytoskeleton, which induces formation of leading edge structures, formation of complexes of Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$, and concentration of these molecules at specific sites. The clustered complexes then enhance PDGF-induced Rac activation in a positive-feedback manner. Such a feedback amplification mechanism might efficiently form leading edge structures in the direction of higher concentrations of PDGF.

The present result that activated PDGF receptor is highly concentrated at peripheral ruffles over lamellipodia of leading edges, but not at focal complexes, suggests that PDGF-induced signaling, at least the PDGF-induced activation of Rac, is transduced from the peripheral ruffles, but not from the focal complexes,

the focal adhesions, or other areas. Receptors including PDGF receptor undergo internalization by endocytosis upon binding of their specific ligands (Heldin *et al.* 1998). After PDGF receptor is down-regulated from the cell surface of the peripheral ruffles, PDGF-induced signaling is supposed to be diminished. When such ruffles attach to ECM proteins, they form new protrusions and focal complexes. This interpretation is consistent with the fact that PDGF receptor was not concentrated at focal complexes where Necl-5 and integrin $\alpha_V\beta_3$ are concentrated. Thereafter, Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$ which remain diffusely on the plasma membrane, are recruited to peripheral ruffles to cluster in response to PDGF in a manner dependent on the activation of integrin $\alpha_V\beta_3$ and transduce the next cycle of signals. Thus, the dynamic clustering of Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$, the formation of peripheral ruffles, the dynamic clustering of Necl-5 and integrin $\alpha_V\beta_{3,}$ and the formation of focal complexes may determine the direction of cell movement toward higher concentrations of PDGF. However, further studies are necessary to elucidate the detailed mechanisms underlying these dynamic events, which are essential for efficient directional cell movement.

Experimental procedures

Constructions

pCAGIPuro-FLAG-Necl-5, pCAGIZeo-FLAG-Necl-5-ΔCP, and pCAGIZeo-FLAG-Necl-5-ΔEC were prepared as described previously (Ikeda *et al.* 2003; Ikeda *et al.* 2004). A small interfering RNA (siRNA) vector against Necl-5 (pBS-EGFP-H1-Necl-5) and a control siRNA vector against luciferase (pBS-EGFP-H1-control) were prepared as described previously (Fujito *et al.* 2005; Kajita *et al.* 2007). pBS-H1 vector was a gift from Dr. H. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan). Expression vectors were kindly provided as follows: wild-type human integrin α_V and human integrin β_3 (pcDNA3-α_V and -β_3) were from Dr. J. C. Norman (University of Leicester, Leicester, UK), and a dominant-negative mutant of human integrin β_3 (pcDNA3.1-Myc-His(+)-$\beta_3^{T329C/A347C}$) was from Dr. J. Takagi (Osaka University, Suita, Japan). To obtain EGFP-tagged integrin $\beta_3^{T329C/A347C}$ (pEGFP-N3-integrin $\beta_3^{T329C/A347C}$), a fragment of integrin $\beta_3^{T329C/A347C}$ from pcDNA3.1-Myc-His(+)-$\beta_3^{T329C/A347C}$ was cloned into pEGFP-N3 vector (Clontech). To obtain EGFP-tagged wild-type integrin β_3 (pEGFP-N3-integrin β_3), a fragment of the extracellular region of integrin $\beta_3^{T329C/A347C}$ in pEGFP-N3-integrin $\beta_3^{T329C/A347C}$ was replaced with the same region of wild-type integrin β_3 from pcDNA3-β_3. To obtain HA-tagged PDGF receptor β (pcDNA3.1Hyg-PDGFRβ-HA), a fragment of PDGF receptor β, the stop codon of which had been deleted by PCR cloning from human PDGF receptor β cDNA, and an oligonucleotide encoding an HA-tag and a stop codon, were cloned into pcDNA3.1Hyg (Invitrogen). Human PDGF receptor β cDNA was a gift from Dr. H. Matsui (Kobe University, Kobe, Japan). pEFBOS-myc-Rac1DN was prepared as described previously (Komuro *et al.* 1996).

Cell culture, transfection, and siRNA experiments

NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. HEK293 cells were maintained in DMEM supplemented with 10% fetal calf serum. Necl-5-NIH3T3 cells (NIH3T3 cells stably expressing FLAG-tagged Necl-5) were prepared as described previously (Kajita *et al.* 2007). For transient expression experiments, cells were transfected with various expression vectors using LipofectAMINE 2000 reagent (Invitrogen). Necl-5-knockdown-NIH3T3 cells (NIH3T3 cells in which Necl-5 was knocked down) were prepared as described previously (Fujito *et al.* 2005; Kajita *et al.* 2007). Briefly, NIH3T3 cells were transfected with pBS-EGFP-H1-Necl-5. As a control, pBS-EGFP-H1-control was used. For the co-immunoprecipitation assay with Necl-5-knocked down NIH3T3 cells, a double-stranded 25 nt RNA duplex, stealth RNAi, to Necl-5 (5'-AGCGCCAGGGCAAUAUGCUU-CUAAU-3') and a control stealth RNAi duplex were purchased from Invitrogen and NIH3T3 cells were transfected with stealth RNAi duplex using LipofectAMINE RNAiMAX reagent (Invitrogen).

Antibodies and reagents

A rat mAb against the extracellular region of Necl-5 (mAb-i, 1A8-8) was prepared as described previously (Ikeda *et al.* 2003). Rabbit anti-PDGF receptor β mAb (Y92) was purchased from Abcam. Hamster anti-integrin α_V and β_3 mAbs (H9.2B8 and 2C9.G2, respectively; for immunostaining) were purchased from BD Biosciences. The following mouse mAbs were purchased from commercial sources: anti-integrin α_V (for Western blotting; BD Biosciences), anti-FLAG M2 (for immunoprecipitation and Western blotting; Sigma), anti-actin (Chemicon), and anti-HA mAbs (Babco). The following rabbit pAbs were purchased from

commercial sources: anti-PDGF receptor β (Upstate), anti-FLAG (for Western blotting; Sigma), and anti-GFP pAbs (for immunoprecipitation and Western blotting; MBL). Hybridoma cells (9E10) expressing a mouse anti-Myc mAb were purchased from the American Type Culture Collection. Fatty acid-free BSA, laminin, ConA, cyclo-RGDfV, and cytochalasin D were purchased from Sigma. AG1296 was purchased from Calbiochem. HRP-conjugated secondary Abs were purchased from GE Healthcare. Fluorophore (FITC, Cy3, and Cy5)-conjugated secondary Abs were purchased from Jackson Immuno Research. Human recombinant PDGF-BB was purchased from PEPROTECH. Vitronectin was purified from human plasma (Kohjinbio) as described previously (Yatohgo *et al.* 1988). The chemical cross-linker 3,3′-dithiobis [sulfosuccinimidylpropionate] (DTSSP), which is membrane-insoluble and has a disulfide bond, was purchased from PIERCE.

Directional stimulation by PDGF

A concentration gradient of PDGF was generated as described previously (Minami *et al.* 2007). Briefly, a μ-Slide VI flow (uncoated; Ibidi) was used. The μ-Slide VI flow has six parallel channels, which were coated with 5 μg/ml vitronectin. Cells were seeded at a density of 5 x 10^3 cells per square centimeter, cultured for 16 h, and starved of serum by incubating in DMEM containing 0.5% BSA for 1 h. A concentration gradient of PDGF was generated using DMEM containing 0.5% BSA and 30 ng/ml PDGF, according to manufacturer's protocol. For the experiments using cytochalasin D, cells were treated with 1 mM cytochalasin D during serum starvation and PDGF stimulation. After 30 min of PDGF stimulation, cells were fixed with acetone/methanol (1:1) at –20˚C. Alternatively, cells were fixed with 4% paraformaldehyde/PBS and then incubated with 100% MetOH at –20˚C. Cells were incubated with 1% BSA in PBS, and then

with 20% BlockAce (Dainippon Pharmaceutical) in PBS, prior to immunofluorescence microscopy. Samples were analyzed using digital eclipse C1si-ready (NIKON) confocal laser-scanning microscope systems.

Co-immunoprecipitation assay using HEK293 cells

HEK293 cells were co-transfected with various combinations of plasmids, cultured for 24 h, detached with 0.05% trypsin and 0.53 mM EDTA, and treated with a trypsin inhibitor (Sigma). To make predominantly the high-affinity form of integrin $\alpha_V\beta_3$, cells were cultured in suspension with Ca^{2+}-free DMEM (Invitrogen) containing 0.5% BSA, 1 mM $MnCl_2$, 50 μg/ml cyclo-RGDfV and/or 30 ng/ml PDGF or 50 μM AG1296 for 30 min, collected by centrifugation, washed with Wash buffer (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 1 mM Na_3VO_4), and lysed with Buffer A (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM $MnCl_2$, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μM APMSF). To make predominantly the low-affinity form of integrin $\alpha_V\beta_3$, cells were cultured in suspension with DMEM containing 0.5% BSA and/or 30 ng/ml PDGF or 50 μM AG1296 for 30 min, collected by centrifugation, washed with Wash buffer and lysed with Buffer B (20 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 10 μM APMSF). The lysates were rotated for 30 min and subjected to centrifugation at 12,000 x *g* for 20 min. The supernatant was pre-cleared with protein G-Sepharose 4 Fast Flow beads (GE Healthcare) at 4˚C for 1 h, incubated with the anti-FLAG mAb at 4˚C for 4 h, and then incubated with protein G-Sepharose beads at 4˚C for 2 h. After the beads were extensively washed with Buffer A or B, bound proteins were eluted by boiling the beads in SDS Sample Buffer (60 mM Tris-HCl, pH 6.7, 3% SDS, 2% 2-mercaptoethanol, and 5% glycerol) for 5 min

and subjected to SDS-PAGE followed by Western blotting.

Co-immunoprecipitation assay using NIH3T3 cells

NIH3T3, Necl-5-NIH3T3, and Necl-5-knockdown-NIH3T3 cells were plated at a density of 5 x 10^3 cells per square centimeter on vitronectin-coated dishes, cultured for 16 h, starved of serum with DMEM containing 0.5% BSA for 1 h, and then stimulated by DMEM containing 0.5% BSA and/or 3 ng/ml PDGF. After 30 min, cells were washed with ice-cold PBS and incubated with 2 mM DTSSP in PBS at 4˚C for 2 h. To quench the cross-linking reaction, 1 M Tris-HCl (pH 7.5) was added at a final concentration of 20 mM. Cells were washed with PBS and lysed with Buffer B. The lysates were rotated for 30 min and subjected to centrifugation at 12,000 x *g* for 20 min. The supernatant was pre-cleared with protein A-Sepharose (GE Healthcare) at 4˚C for 1 h, incubated with the anti-integrin α_V pAb or rabbit serum, as a control, at 4˚C for 16 h, and then incubated with protein A-Sepharose beads at 4˚C for 4 h. After the beads were extensively washed with Buffer B, bound proteins were eluted by boiling the beads in SDS Sample Buffer for 5 min and subjected to SDS-PAGE followed by Western blotting.

Boyden chamber assay

Boyden chamber assay was performed as described previously (Ikeda *et al.* 2004). For the assay using NIH3T3 and Necl-5-NIH3T3 cells, PET membrane cell culture inserts (8.0-μm pores, BD Falcon) were used. For the assay using Necl-5-knockdown-NIH3T3 cells, NIH3T3 cells were transfected with pBS-EGFP-H1-Necl-5 or pBS-EGFP-H1-control and HTS fluoroBlock cell culture inserts (8.0-μm pores, BD Falcon) were used. Cell culture inserts were coated with 3 μg/ml of vitronectin and then blocked with 1% BSA/PBS. Cells that had

been serum starved with DMEM containing 0.5% BSA for 18 h were detached with 0.05% trypsin and 0.53 mM EDTA and then treated with a trypsin inhibitor. Cells were then resuspended in DMEM containing 0.5% BSA and seeded at a density of 4 x 10^4 cells per insert. Cells were incubated at 37°C for 14 h in the presence of 30 ng/ml PDGF-BB. PDGF-BB was added only to the bottom well in order to generate a concentration gradient. After incubation, the inserts were washed with PBS and the cells were fixed using 3.7% formaldehyde. Migrated cells were observed by Crystal Violet staining (for NIH3T3 and Necl-5-NIH3T3 cells) or EGFP fluorescence (for Necl-5-knockdown-NIH3T3 cells). The number of migrated cells in five randomly chosen fields per insert was counted by microscopic examination.

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Figure legends

Figure 1. Co-localization of PDGF receptor with Necl-5 and integrin $\alpha_V\beta_3$ at peripheral ruffles over lamellipodia of leading edges. (A-C) Immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated μ-slide dishes. Cells were double or triple stained with the anti-Necl-5, the anti-PDGF receptor β, and the anti-integrin β_3 mAbs. **(A)** Wild-type NIH3T3 cells; **(B)** Necl-5-NIH3T3 cells; **(C)** wild-type NIH3T3 cells transfected with siRNA vector; **(Ca)** Necl-5-knockdown-NIH3T3 cells; **(Cb)** control-NIH3T3 cells. **(Aa, Ba, Ca, Cb)** in the presence of PDGF; (**Ab, Bb)** in the absence of PDGF. **Arrowheads,** leading edges; **FXs,** focal complexes; **FA,** focal adhesion; **bars,** 10 μm. Inset boxes show the areas of high magnification images. The high magnification images are indicated as intensity ratio images. Analysis of the co-localization of the two indicated proteins and generation of the intensity ratio images were performed using ImageJ 1.34S software with an RG2B colocalization plug-in. The results shown are representative of three independent experiments.

Figure 2. Physical interaction of Necl-5 with PDGF receptor. (A) Physical interaction of Necl-5 with PDGF receptor. HEK293 cells were transfected with various combinations of indicated vectors and cultured in suspension under the indicated conditions. FLAG-tagged Necl-5, Necl-5-ΔCP, or Necl-5-ΔEC was immunoprecipitated using the anti-FLAG mAb and samples were assessed by Western blotting using the anti-FLAG and the anti-HA mAbs. **(B)** Physical interaction between Necl-5, PDGF receptor, and integrin $\alpha_V\beta_3$ at an endogenous level. NIH3T3 cells were cultured on vitronectin-coated dishes and starved of serum. Cells were treated with DTSSP and then subjected to a

co-immunoprecipitation assay using the anti-integrin α_V pAb. Samples were assessed by Western blotting using the anti-integrin α_V mAb, the anti-Necl-5 mAb, and the anti-PDGF receptor pAb. **(C)** Physical interaction between PDGF receptor and integrin $\alpha_V\beta_3$. EGFP-tagged integrin β_3 was immunoprecipitated using the anti-GFP pAb and samples were assessed by Western blotting using the anti-GFP pAb, the anti-integrin α_V mAb, and the anti-HA mAb. The results shown are representative of three independent experiments.

Figure 3. Actin cytoskeleton-dependent localization of PDGF receptor at peripheral ruffles. (A and B) Immunofluorescence images of PDGF-stimulated NIH3T3 cells cultured on vitronectin-coated μ-slide dishes in the presence of 1 μM cytochalasin D. Cells were triple stained with various combinations of the anti-Necl-5, the anti-integrin β_3, the anti-PDGF receptor β, and the anti-actin mAbs. **(A)** Wild-type NIH3T3 cells; **(B)** Necl-5-NIH3T3 cells. **Bars,** 10 μm. The results shown are representative of three independent experiments.

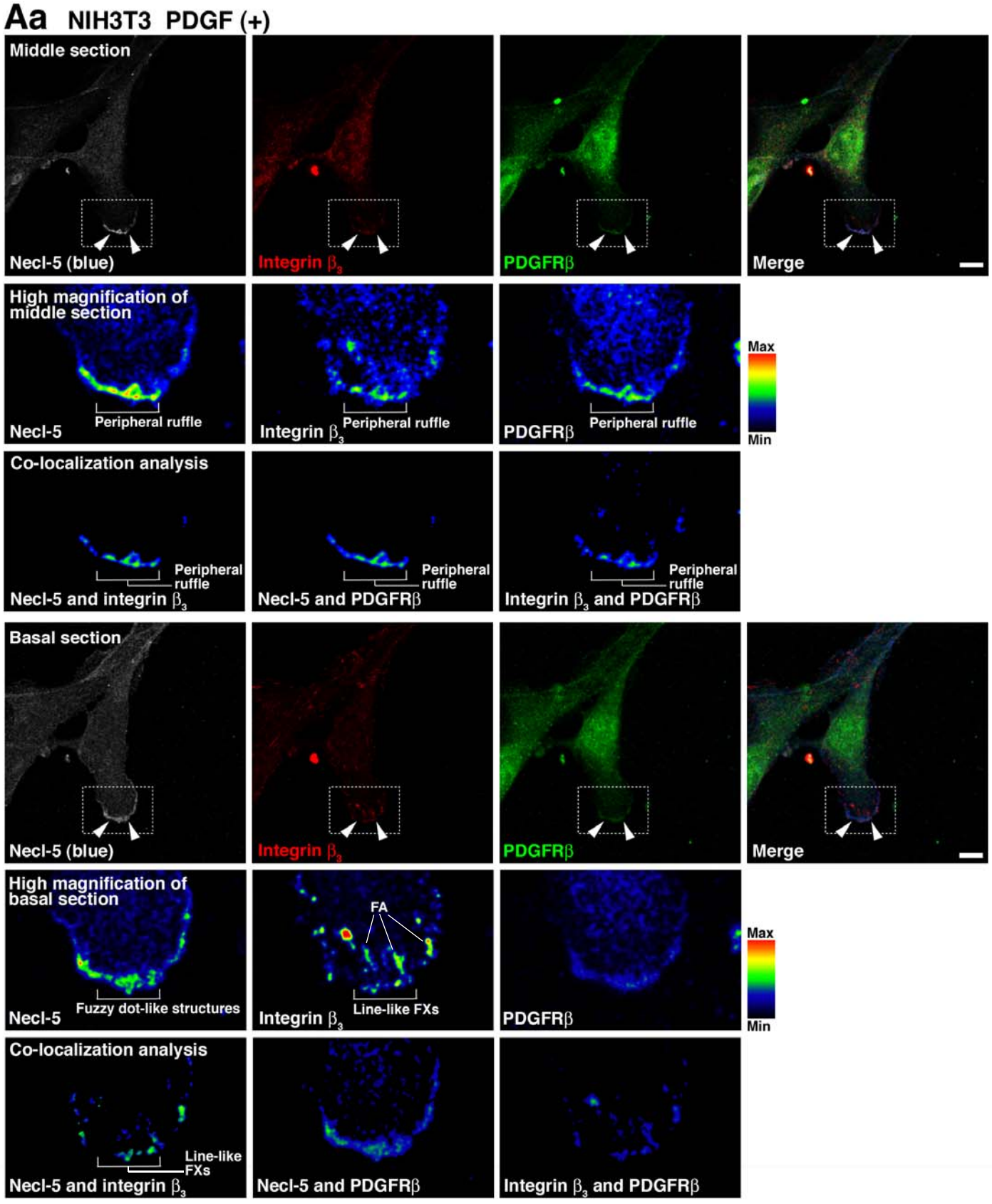
Figure 4. Rac-dependent localization of PDGF receptor at peripheral ruffles. (A and B) Immunofluorescence images of PDGF-stimulated NIH3T3 cells expressing Rac1DN, cultured on vitronectin-coated μ-slide dishes. Cells were transfected with myc-tagged Rac1DN and triple stained with various combinations of the anti-Necl-5, the anti-integrin β_3, the anti-PDGF receptor β, and the anti-myc mAbs. **(A)** Wild-type NIH3T3 cells; **(B)** Necl-5-NIH3T3 cells. **Bars,** 10 μm. The results shown are representative of three independent experiments.

Figure 5. Regulation of the interaction between PDGF receptor and integrin $\alpha_V\beta_3$ by Necl-5 and PDGF. NIH3T3 cells were cultured on

vitronectin-coated dishes, starved of serum, and cultured in the absence or presence of PDGF. Cells were treated with DTSSP and then subjected to a co-immunoprecipitation assay using the anti-integrin α_V pAb. Samples were assessed by Western blotting using the anti-integrin α_V mAb, the anti-Necl-5 mAb, and the anti-PDGF receptor pAb. **(A)** Necl-5-NIH3T3 cells, **(B)** NIH3T3 cells, **(C)** control-NIH3T3 and Necl-5-knockdown-NIH3T3 cells. **Asterisks**, the relative level of the amounts of co-immunoprecipitated PDGF receptor in the absence or presence of PDGF. Intensities were normalized to 1.0 for respective cells cultured in the absence of PDGF. The results shown are representative of three independent experiments.

Figure 6. Enhancement of cell movement by Necl-5 and PDGF. For measurement of cell movement with a Boyden chamber assay, NIH3T3 cells were starved of serum and incubated on cell culture inserts coated with vitronectin in the presence of PDGF in the bottom well for 16 h. **(A)** Wild-type NIH3T3 and Necl-5-NIH3T3 cells. The cells were stained by crystalviolet and the migrated cells were counted. **(B)** Control- and Necl-5-knockdown-NIH3T3 cells. Wild-type NIH3T3 cells were transfected with an siRNA vector, pBS-EGFP-H1-Necl-5 or pBS-EGFP-H1-control, and their transfection efficiencies were determined to be about 20%. The EGFP-positive migrated cells were counted. The number of control EGFP-positive migrated cells was consistent with that of control migrated cells in **(A)**, considering the transfection efficiency. **Asterisks**, $P<0.001$. The results shown are the means ± standard error of three independent experiments.

Fig. 1A. Amano et al.



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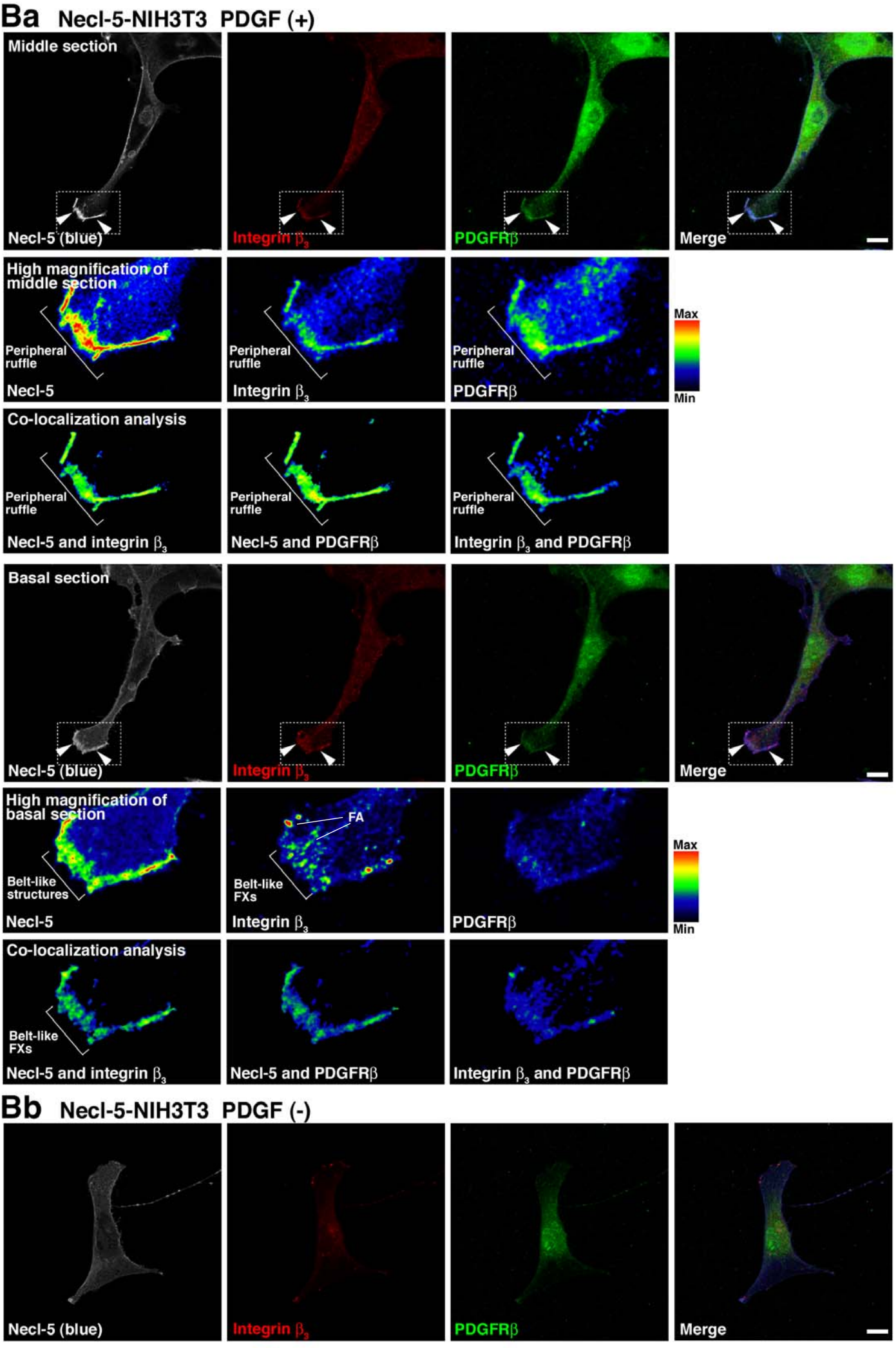

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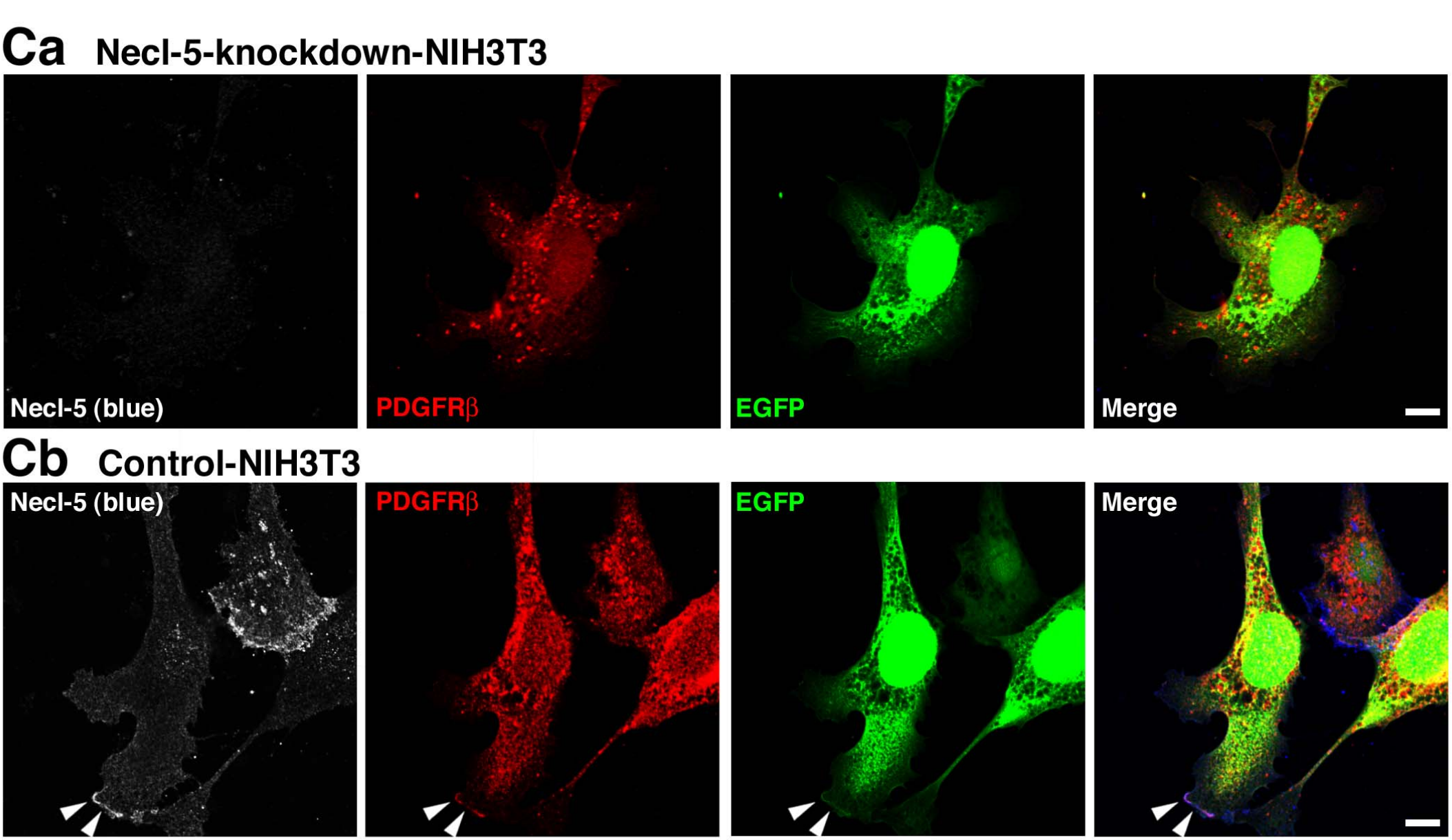

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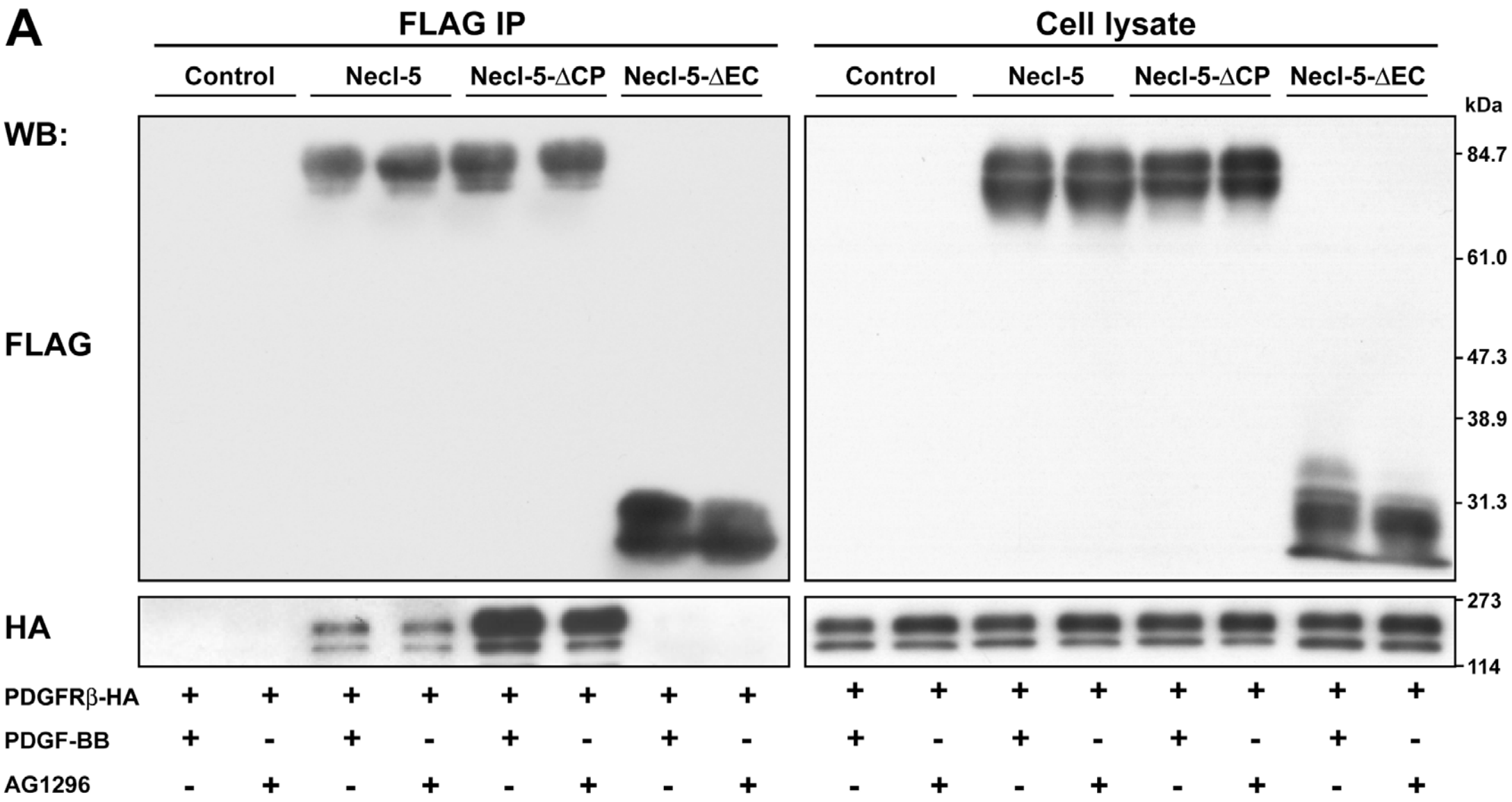

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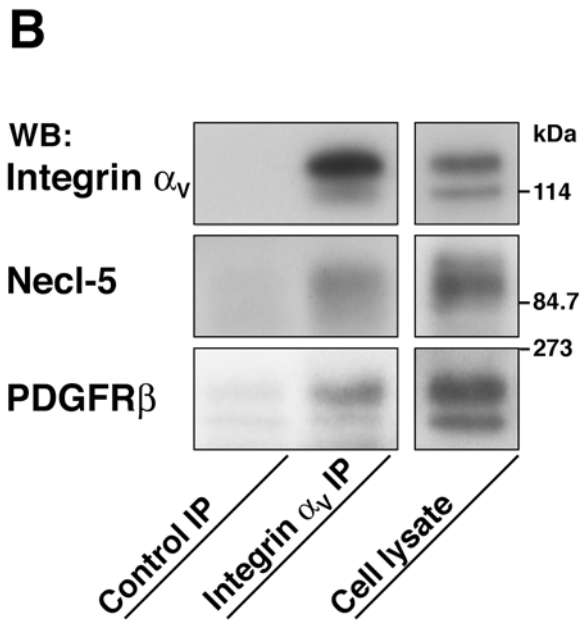

Fig. 2C. Amano et al.

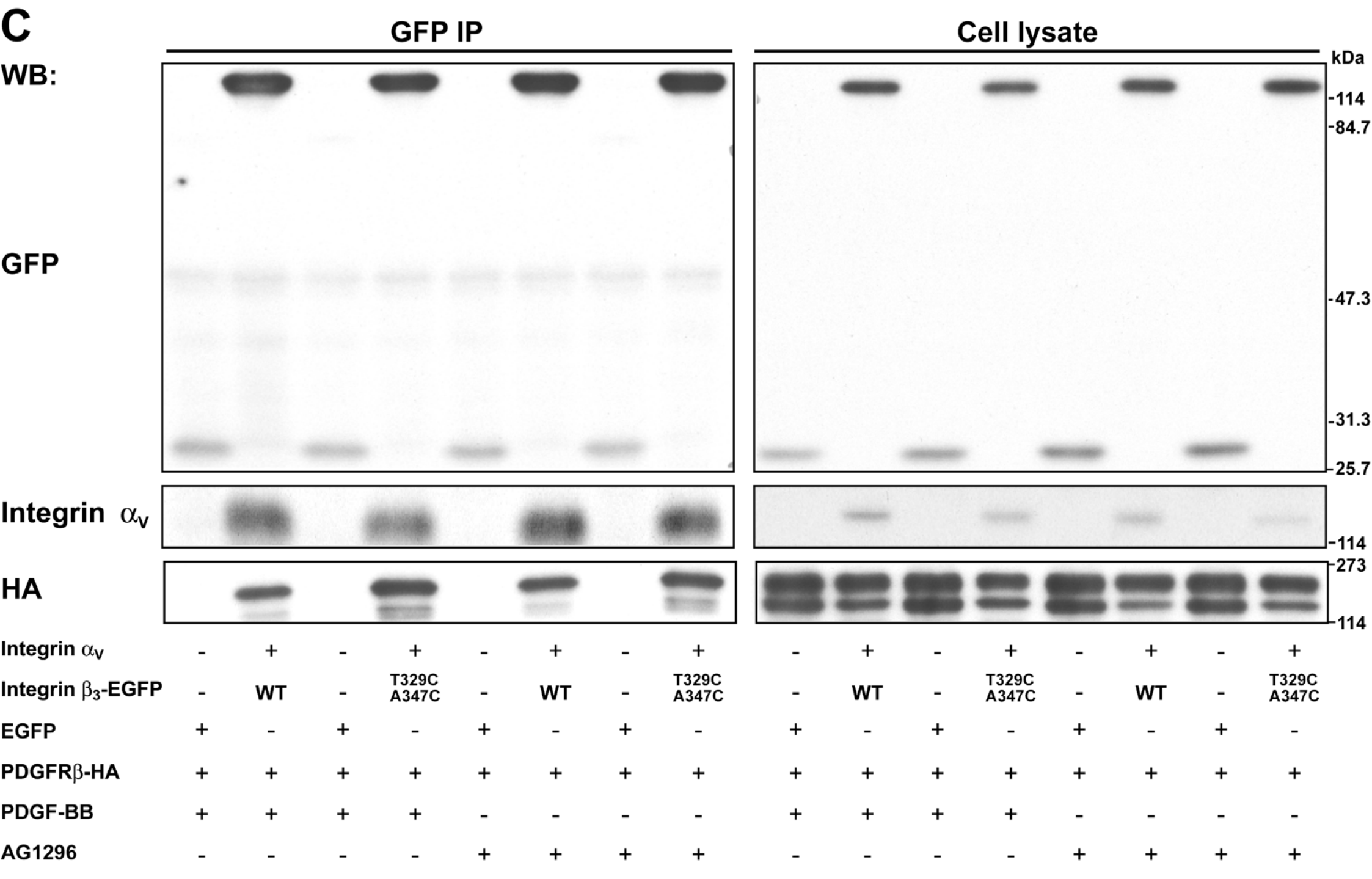

Fig. 3. Amano et al.

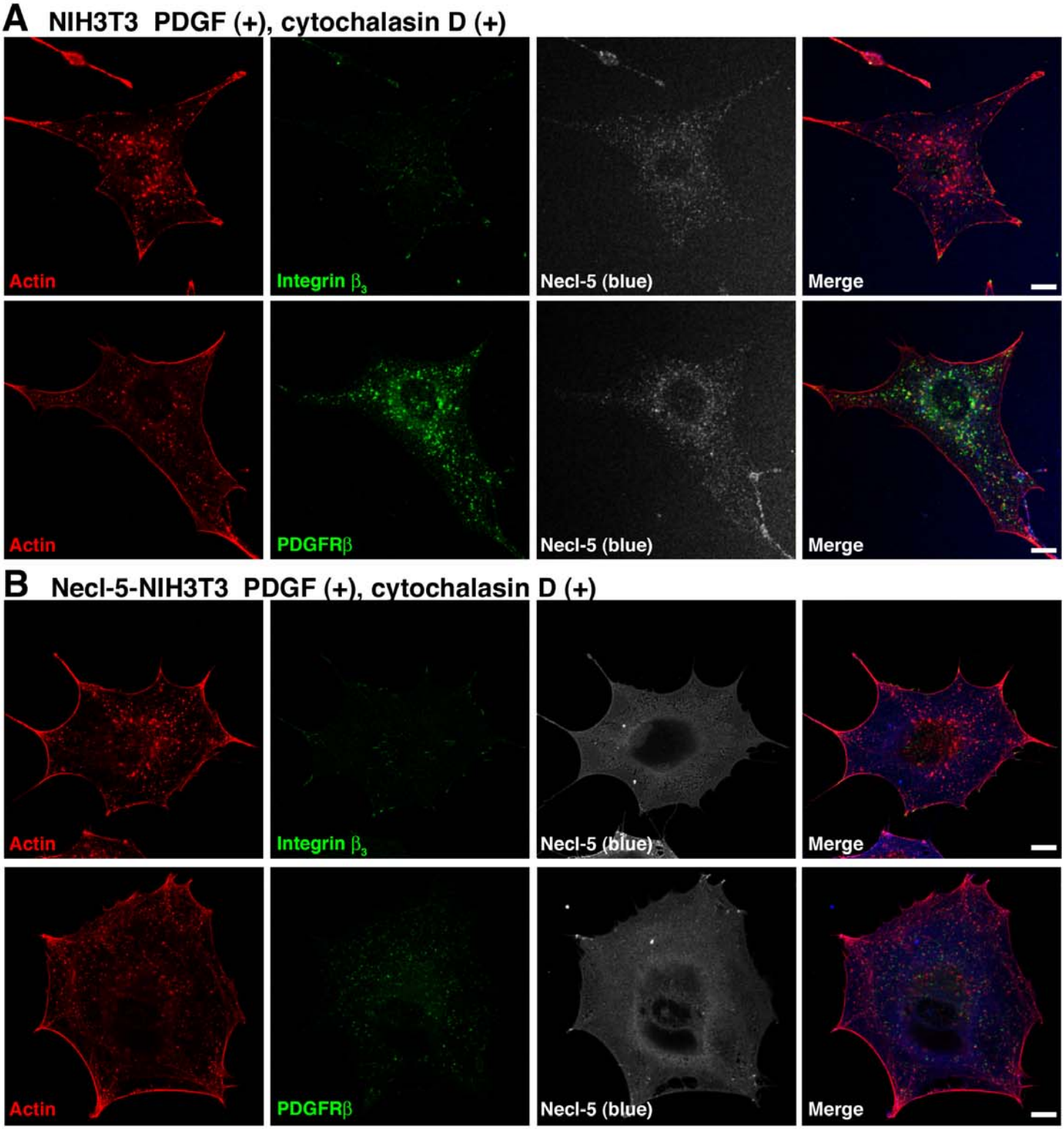

Fig. 4. Amano et al.

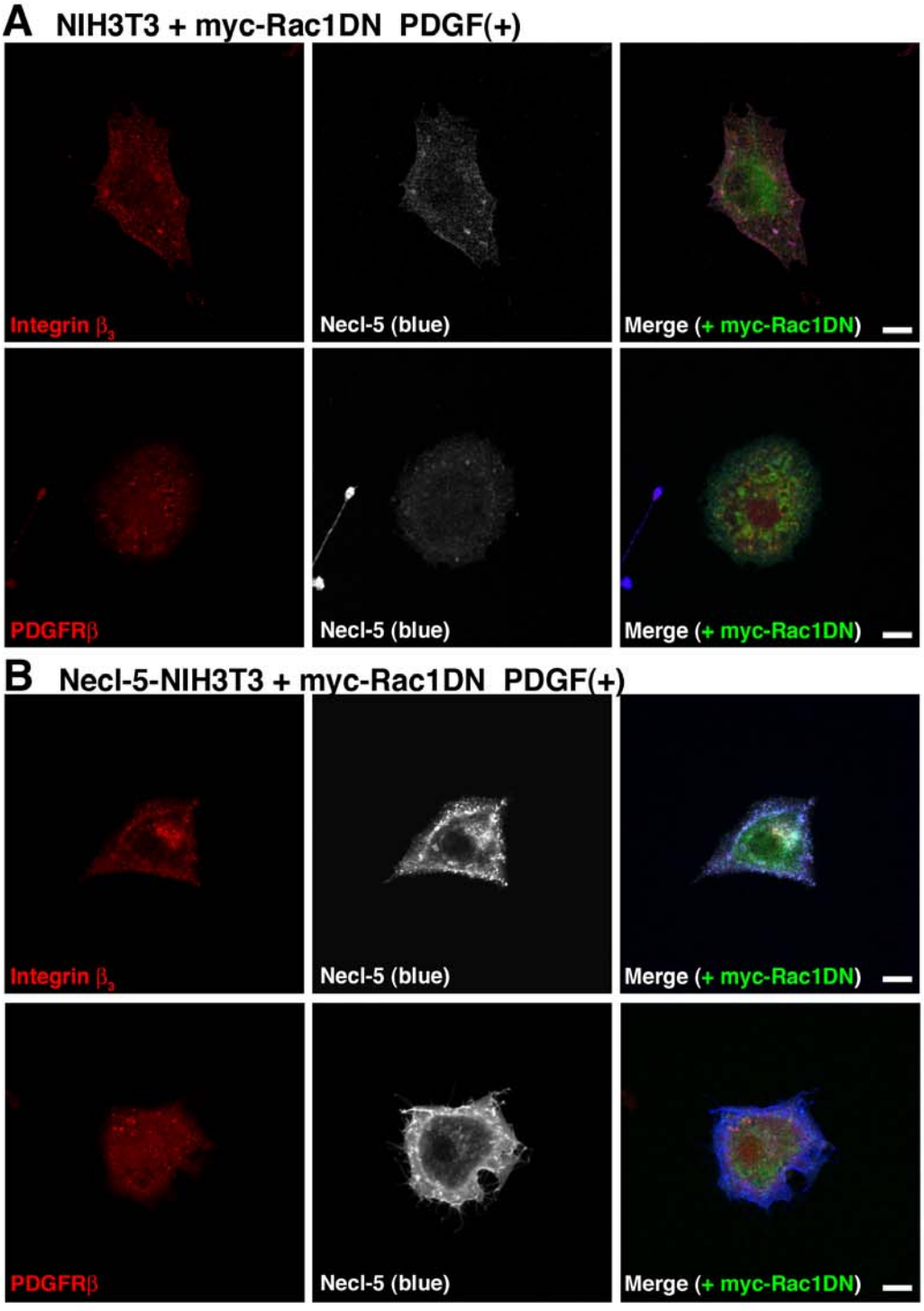

Fig. 5. Amano et al.

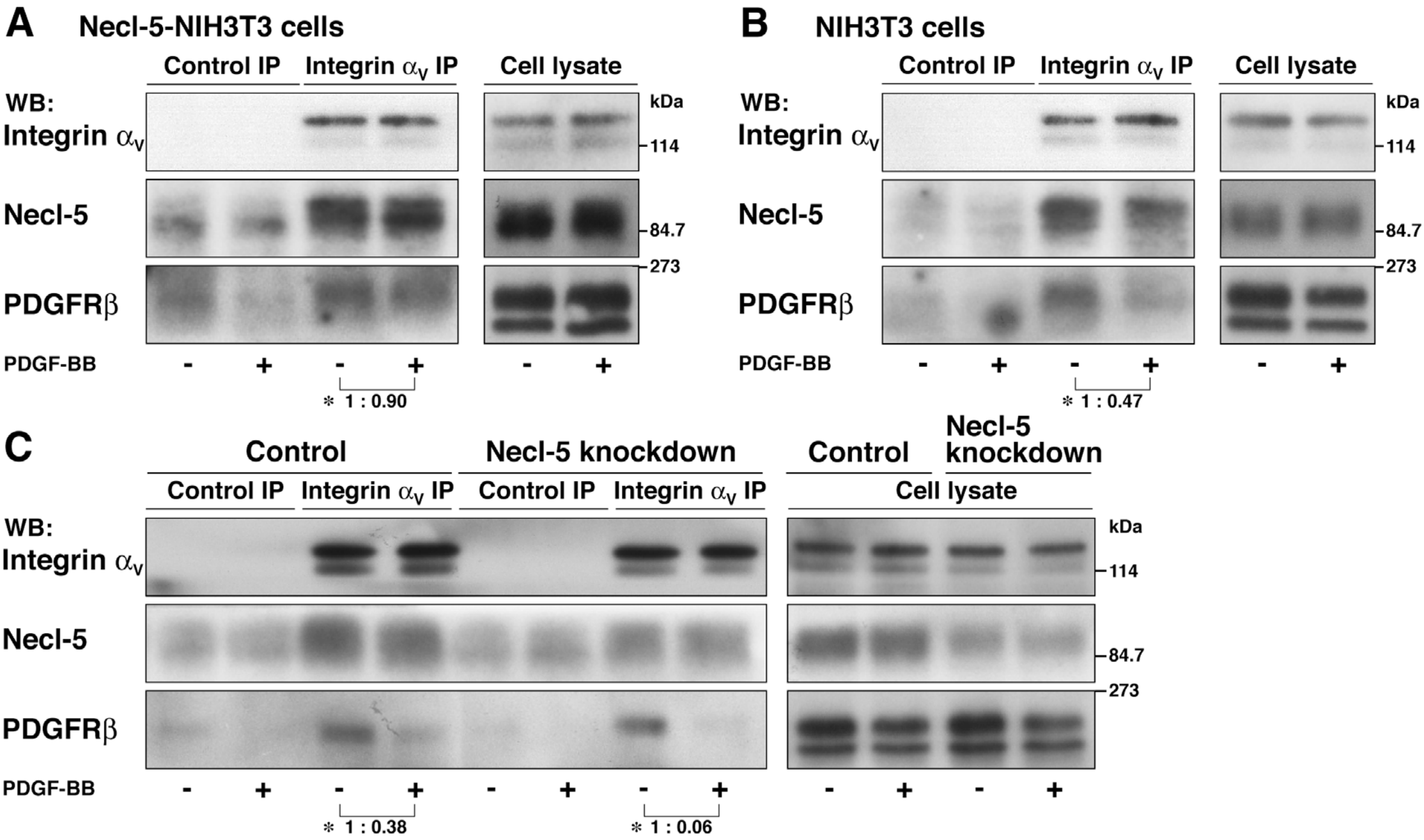

Fig. 6. Amano et al.