CD81 promotes a migratory phenotype in neuronal-like cells

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Brief title: CD81 in neuronal migration

Abstract

Tetraspanins, such as CD81, can form lateral associations with each other and with other transmembrane proteins. These interactions may underlie CD81 functions in multiple cellular processes, such as adhesion, morphology, migration and differentiation. Since CD81 role in neuronal cells' migration has not been established, we here evaluated CD81 effects in the migratory phenotype of SH-SY5Y neuroblastoma cells. CD81 was enriched at SHSY-5Y cell's membrane, co-localizing with its interactor F-actin in migratory-relevant structures of the leading edge (filopodia, stress fibres and adhesion sites). CD81 overexpression increased the number of cells with a migratory phenotype, in a potentially PI3K-AKT mediated manner. Indeed, CD81 also co-localized with AKT, a CD81-interactor and actin remodel agent, at the inner leaflet of the plasma membrane. Pharmacologic inhibition of PI3K, the canonical AKT activator, led both to a decrease in the acquisition of a migratory phenotype and to a redistribution of intracellular CD81 and F-actin into cytoplasmic agglomerates. These findings suggest that in neuronal-like cells CD81 bridges active AKT and actin, promoting the actin remodelling that leads to a motile cell morphology. Further studies on this CD81-mediated mechanism will improve our knowledge on important physiological and pathological processes such as cell migration and differentiation, and tumour metastasis.

Keywords: CD81 tetraspanin; actin remodelling; Neuronal migration; PI3K-AKT signalling; SH-SY5Y neuroblastoma cells.

Introduction

CD81 is a 26 kDa integral membrane protein member of the tetraspanin family, an evolutionarily conserved family of membrane proteins containing 4 transmembrane domains and expressed in most human tissues (Hemler 2005). Tetraspanins have the striking ability to form lateral associations with each other and with other tissue type-specific proteins, at the cell membrane. These associations take the form of tetraspanin-enriched microdomains (TEM) that form a dynamic membrane network known as the 'tetraspanin web' (Shoham *et al.* 2006). Tetraspanins regulate a wide range of proteins such as integrins, cell surface receptors, and signalling molecules (Jiang *et al.* 2015). A major difficulty in the study of tetraspanins is to identify functions that are specific for a given tetraspanin, and to determine how this function relates to specific tetraspanin-associated proteins (Boucheix et al. 2001).

CD81 has been emerging as a regulator of a multitude of cellular processes, including adhesion, proliferation, differentiation, cell migration, and microvillus formation (Bari *et al.* 2011; Levy *et al.* 1998). Cell migration is usually driven by extracellular signals and involves an assemblage of protein-protein interactions. The proteins that play a role in this process include cadherins (involved in cell-cell adhesion), integrins (involved in cell-extracellular matrix (ECM) adhesion), Rac/Rho (for actin cytoskeletal remodelling, protrusion/contraction), and matrix metalloproteinases (for pericellular proteolysis/proteolytic ECM remodelling) (Jiang *et al.* 2015). Increasing evidence indicates that the CD81 tetraspanin may help to coordinate the cell migration process by regulating the function of key proteins involved in all aspects of this process. For example, CD81 supports maturation and surface expression of EWI-2, which modulates integrin-dependent cell motility and spreading. CD81 is also closely associated with the α4β1 integrin, regulating α4β1 adhesion under shear flow conditions. The association of CD81 with type II phosphatidylinositol 4-

kinase (PI4K) is also believed to play a role in cell migration and tumour cell proliferation (Boucheix & Rubinstein 2001; Hemler 2005; Jiang *et al.* 2015). In immune cells, CD81 regulates the dynamics and membrane localization of the small GTPase Rac1 during membrane protrusion, and promotes the formation of adhesion complexes (Tejera *et al.* 2013).

CD81 is mainly studied in the immune system, but a few studies have started to implicate CD81 in the nervous system physiology (Boucheix et al. 2001; Hemler et al. 2005). As various mechanisms of cell migration are similar in cells of various origins, a role for CD81 in neuronal migration is plausible. Neuronal migration is, along with axon guidance, a fundamental and critical mechanism underlying the development of the brain architecture, and also in neuroregeneration. Migrating neurons are highly polarized in the direction of their movement and undergo the extension of a leading process, the translocation of the nucleus into the leading process, and the elimination of the migrating neuron's trailing process, leading to the net movement of the cell (Marín *et al.* 2010). As all these involve actin remodelling and cell adhesion, we here evaluated, for the first time, if CD81 regulates actin remodelling and motility-related cell morphology in neuronal-like cells.

Materials and Methods

SH-SY5Y cell culture, transfection and pharmacologic modulation

The SH-SY5Y human neuroblastoma cell line is one of the best well-established in vitro neuronal model (da Rocha *et al.* 2015). Undifferentiated SH-SY5Y cells are morphologically described as neuroblast-like, non-polarized cell bodies with few truncated processes (Kovalevich & Langford 2013). These characteristics make them a suitable model to study the effects of CD81 in the migratory phenotype of neuronal-like cells. SH-SY5Y cells were maintained in Minimum Essential Media (MEM):F12 (1:1) supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Thermo Fisher) and 1% Antibiotic-Antimycotic solution (AA; Gibco, Thermo Fisher), in a 5% CO2 humidified incubator at 37ºC. Cells were sub-cultured when a cell density of 90% was achieved, and plated on sterilized coverslips inside six-well plates. When indicated, SH-SY5Y cells were transfected for 24 h with 1 µg of pCDM8 hCD81 cDNA (Addgene plasmid # 11588; a kind gift from Dr. Shoshana Levy) using the TurboFect™ reagent (Fermentas Life Science). For the inhibition of PI3K, CD81 transfected cells were treated with 10 µM of the phosphatidylinositol 3 kinase (PI3K) inhibitor LY294002 (Selleck Chemicals), for 18 hours before fixation.

Immunocytochemistry (ICC) and microscopy

Non-transfected and CD81 transfected SH-SY5Y cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 10 min. For immunocytochemistry (ICC) procedures, the following primary and secondary antibodies were incubated for 1-2h, following the manufacturer's instructions: mouse monoclonal anti-CD81 M38 (Abcam, Cat. No. ab79559); rabbit monoclonal anti-AKT (Cell Signalling Cat. No. 9272) and Alexa 488- and 594-conjugated IgGs (Molecular Probes). To stain F-actin, Alexa Fluor Phalloidin in 1%

bovine serum albumin (BSA) phosphate-buffered saline (PBS) was added 30 min in the dark. After three washes with PBS and a last one with distilled water, preparations were mounted with Vectashield® media (Vector Laboratories) containing or not the DAPI nuclear staining probe. Fixed cells were visualized by confocal microscopy [LSM 510 Meta confocal microscope (Carl Zeiss), with a 63x oil objective].

Cells morphological analysis

The morphology of the transfected and non-transfected cells was monitored to score the typical migratory phenotype of spatial asymmetry with a clear distinction between cell front and rear, stress fibres along the cell, lamellipodia and filopodia at the cell front, and actin filament rearrangements (Qian *et al.* 2005). The migratory phenotype was monitored in ~65 transfected and ~100 non-transfected cells, in five independent biological replicas (n=5). Cell count was performed using the 'cell counter' plugin of the ImageJ software (U.S. National Institute of Health), and data expressed as the percentage of cells with a migratory phenotype as a function of the total number of scored cells. In the PI3K inhibition assays, the decrease in the percentage of CD81 transfected migratory cells, resulting from the presence of the PI3K LY294002 inhibitor, was quantified in three independent biological replicas (n=3), where the migratory phenotype was monitored in 100-135 transfected cells per condition.

Data analysis and statistics

Data was expressed as mean values of at least triplicates, ± standard error of the mean. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software (version 19). The normality was tested with the Shapiro-Wilk test and all p values were > 0.05, so the null hypotheses that these data were sampled from normally distributed populations, were not rejected. Nevertheless, due to the small sample size, both

parametric and non-parametric tests were used for post hoc evaluations of differences among groups. The independent sample t-test and the Mann-Whitney U test were used for statistical significance analysis of the migratory phenotype data of Figure 1. The one-tail Student's t-test and the Wilcoxon test were used for statistical significance analysis of PI3K inhibition data (Figure 3). $p < 0.05$ in at least one of the tests was considered as statistically significant.

Results

CD81 co-localizes with F-actin in motile-related structures

To be able to migrate, cells have to acquire a polarized migratory phenotype that involves the remodelling of its cytoskeleton, with particular emphasis on the actin cytoskeleton (Qian *et al.* 2005; Xue & Hemmings 2013). CD81 is known to physically interact with actin, and to promote cytoskeleton remodelling in other cell types (Perez-Hernandez *et al.* 2013; Tejera *et al.* 2013). In order to better understand the influence of CD81 in neuronal cytoskeleton remodelling, and to access its co-localization with actin in SH-SY5Y cells, an ICC analysis was performed in SH-SY5Y cells. Results show that CD81 was highly located at the cells' membranes, including in structures also stained for filamentous actin (F-actin), when at endogenous levels (Figure 1A) but particularly when overexpressed (Figure 1B). Moreover, CD81 was particularly enriched in structures related to cell migration, such as filopodia (Figure 1B, open arrows in dashed rectangle), stress fibres (Figure 1B, full arrows, as in (Auer *et al.* 2017)) and stress fibres' terminals, most likely adhesion sites (Figure 1B, asterisks). These are subcellular regions where a high degree of co-localization between F-actin and transfected CD81 could be observed (Figure 1B, orange staining in 'Merge').

CD81 overexpression promotes the acquisition of a migratory phenotype

A first observation of the CD81 overexpressing neuroblastoma population denoted the presence of a high number of cells with a migratory phenotype. This includes a more triangular shape, F-actin concentration at the leading front and at the cell's rear, and a typical array distribution of the stress fibres (Bari *et al.* 2011; Qian *et al.* 2005). The number of CD81 overexpressing cells with this phenotype was scored, and compared to the number of migratory-like non-transfected cells in the neighbourhood. Data shows that CD81

overexpression doubled the number of cells with this migratory phenotype (Figure 1C). These results suggest a role for CD81 in promoting cellular mechanisms that underlie the acquisition of a migratory phenotype. Further, they suggest that actin, a known interactor of CD81 (Perez-Hernandez *et al.* 2013), is involved in the CD81 effects on cell morphological transformation.

CD81 co-localizes with AKT, in transfected and non-transfected SH-SY5Y cells

We further analysed if another CD81 interactor, AKT, could also be involved in CD81-promotion of neuronal-like cells migratory phenotype. AKT (also known as protein kinase B) is a serine/threonine protein kinase that regulates many processes, including metabolism, proliferation, cell survival, growth and angiogenesis (Yu & Cui 2016). When activated by PI3K, AKT phosphorylates various substrates involved in cytoskeleton remodelling, cell growth and cell survival in neurons. Moreover, the PI3K/AKT signalling pathway is essential for the modulation of the cytoskeleton during cell migration (Xue et al. 2013). As AKT is a CD81 interactor protein, and also the best-known target of PI3K, we investigated if the activation of the AKT/PI3K pathway is part of the mechanism by which CD81 promotes actin remodelling by blocking PI3K to prevent activation and phosphorylation of AKT.

Co-localization analyses, both in CD81 transfected and non-transfected cells (Figure 2), showed that AKT1 was present in the cytosol and at the plasma membrane of the SH-SY5Y cells. Moreover, it could be observed that AKT1 mainly co-localized with CD81 at cellular projections (Figure 2B, open arrows) and at the inner leaflet of the plasma membrane (Figure 2, full arrows). Scarcer co-localization between these proteins was observed in cytoplasmic zones further away from the PM. Since AKT is cytoplasmic and translocates to the plasma membrane when activated, this co-localization suggests that CD81 interacts particularly with

the active form of AKT, and that AKT signalling might be involved in CD81 functions in cell motility.

PI3K/AKT signalling is involved in CD81-mediated actin remodelling and migratory phenotype

PI3K is a canonical upstream activator of AKT, and a possible functional interaction between CD81 and the PI3K-AKT pathway was pursued by treating CD81 transfected cells with LY294002, a PI3K inhibitor. Inhibition of PI3K resulted in a ~40% decrease in the percentage of transfected cells with a migratory phenotype, when compared to non-treated control cells (Figure 3A). PI3K inhibition also inhibited the acquisition of a migratory phenotype in non-transfected cells, although only by ~20% (data not shown). These results strengthen the hypothesis that the PI3K-AKT pathway mediates the CD81 promotion of migration in these neuronal-like cells.

Further, when treated with LY294002 (Figures 3B and 4), CD81 still strongly co-localized with F-actin in CD81 overexpressing cells as it did in unexposed cells of Figure 1B. Nevertheless, contrary to untreated cells where stress fibres and normal F-actin distribution is observed (Figure 1B), the F-actin distribution was now altered. CD81 overexpressing cells exposed to the PI3K inhibitor presented less stress fibres (Figures 3B and 4, arrowheads) and a redistribution of the CD81 staining pattern (Figures 3B and 4 open arrows). A more thorough analysis of the CD81 and F-actin distribution in SH-SY5Y cells exposed to the PI3K (Figure 4) revealed that CD81 overexpressing cells had a high number of CD81- and F-actin positive filopodia (or less and longer filopodia), but very few stress fibres (arrowheads in Figures 3B and 4). Moreover, CD81 was less enriched at the cell periphery, and was redistributed from the cell membrane to the perinuclear region and to cytoplasmic agglomerates (Figure 4, arrowheads and zoom-in of the nuclear plane). The percentage of

CD81 transfected cells with a perinuclear ring-like enrichment triplicated when PI3K was inhibited (from ~10% to ~30%), and the number of smaller differentiated cells, with filopodia and cortical F-actin but no stress fibres, duplicated (data not shown). Taken together, these results indicate that the PI3K/AKT signalling pathway is part of the CD81 mechanism of F-actin remodelling into cell motility-related structures.

Discussion

CD81 is a tetraspanin protein that has been mainly studied in the immune system. Few functions have been attributed to it, but it has been implicated in processes such as cell adhesion and migration. Its expression levels, subcellular distribution and functions in other cells, such as neuronal cells, are still barely known. In the present work, we aimed to evaluate the role of CD81 in the migratory phenotype of these neuronal-like cells.

We first confirmed that CD81 is endogenously expressed in the nervous system-related SH-SY5Y cell line. This is not surprising since CD81 has been already implicated in the nervous system physiology, being required for the normal development of the brain (Geisert *et al.* 2002). Geisert *et al.* (2002) studied the effects of a CD81 -/- mutation on the CNS of mice and reported that these mice have extremely large brains. This was as a result of an increased number of astrocytes and microglia, with no apparent effect on the number of neurons and oligodendrocytes (Geisert *et al.* 2002). Accordingly, CD81 was reported to control astrocytes and microglia cell number by suppressing cell proliferation, in a cell-cell contact-dependent manner (Kelić *et al.* 2001). Potentially related to this, CD81 is found concentrated at regions of cell-cell contact in cultured astrocytes, and may play a central role in the process of CNS scar formation in spinal cord injury (Dijkstra *et al.* 2000).

In the work here presented we additionally observed that CD81 is mainly present at the plasma membrane of SH-SY5Y neuroblastoma cells, as expected for a protein of the tetraspanin family. CD81 was also found in some spot-like structures of the cytoplasm, with the smaller of these potentially being exosomes, since CD81 was recently observed to be enriched in exosomes-like vesicles (Andreu et al. 2014).

More importantly, CD81 was found to clearly promote the number of migrating neuronal-like cells, similarly to its role in immune cells. Cell migration is a highly coordinated cellular

event, key for various physiological and pathological major processes, including embryonic development, wound healing, immune response (Qian et al 2004). Migration is highly based on actin filament polymerization and remodelling, and motile cells have characteristic discrete actin structures at the cell periphery for attachment to the substratum: focal adhesion, stress fibres, lamellipodia, filopodia, and membrane ruffles (Hall 1998). Overexpression of CD81 alone altered the cells morphology, inducing a reorganization of the actin filaments into cell motility-related structures.

A previous bioinformatics analysis of CD81-interacting proteins performed by our group (unpublished data) showed that CD81 was linked to intracellular signalling components involved in cytoskeletal regulation. Included in this group were the following key proteins: AKT1, Rac and cytoskeleton-related proteins, including actin and tubulin. Actin itself is a CD81 interacting protein, as described by Perez-Hernandez et al. (2013). Our results demonstrated that CD81 perfectly co-localizes with F-actin in stress fibres and filopodia at the leading edge of migrating SH-SY5Y cells. We have also observed that CD81 is highly abundant in cytoplasmic cellular spots at the end of the stress fibres that transverse the cell, most probably mature focal adhesions. These are key subcellular locations for a protein with regulatory role on cell migration, and agree with such a role for CD81 on neuronal cells. The CD81 role in neuronal cell migration seems not only to involve F-actin, but also other actin regulators, such as AKT1. The AKT kinase plays a crucial role in neurogenesis by activating the proliferation, migration and differentiation of neural stem and other cells (Koh & Lo 2015; Qian *et al.* 2005). AKT1 is an AKT isoform involved in a variety of signalling pathways related to cell motility and cytoskeleton remodelling. When AKT1 is activated by phosphorylation in the cytoplasm, it is targeted to the inner leaflet of the plasma membrane and phosphorylates a number of subtracts, including actin (Xue et al. 2013). In the present work, we performed an ICC in SH-SY5Y cells to access the subcellular co-localization of

CD81 and AKT1. AKT1 presented the expected subcellular localization along the entire cytoplasm but, interestingly, CD81 and AKT1 mainly co-localized at the inner leaflet of the plasma membrane and at some cellular projections. In CD81 overexpressing migrating cells, AKT1 co-localized with CD81 at the leading edge. These suggest that CD81 co-localizes with active, membrane-recruited AKT1, and raises the hypothesis that a tri-complex of CD81/AKT/actin may exist and function in neuronal-like cell motility. Activated AKT at the leading edge of the cell is already known to participate in the regulation of cell polarity and in the reorganization of the cytoskeleton, mediating contraction of the cellular body that facilitates directed cell migration (Xue et al. 2013). AKT is one of the best-known targets of PI3K, and the PI3K/AKT pathway has role in neural migration by e.g. enhancing the secretion of matrix metalloproteinase (MMP)-2 and MMP-9 (Koh et al. 2015). In chicken embryo fibroblast (CEF) cells, the expression of active PI3K forms alone is enough to induce the remodelling of actin filaments towards the formation of cell's motility structures (Qian et al 2004). The authors further reported that either the inhibition of PI3K activity with LY294002, or the disruption of AKT activity in CEF cells inhibited both actin remodelling and PI3K-induced cell migration (Qian *et al.* 2004). In our work, further support to the existence of a CD81/AKT/actin tri-complex active in cell motility comes from our PI3K pharmacological inhibitor data. The blocking of this signalling pathway, and thus of AKT activation, partially impaired the CD81 positive effect on cell motile phenotype. Further, PI3K inhibition leads to altered CD81 and actin subcellular distribution in the transfected SH-SY5Y neuroblastoma population (Figures 3 and 4). This population comprises two types of cells: the larger S-type cells that are more neuroepithelial-related, and the smaller more neuronal-related N-type cells (da Rocha *et al.* 2015). In all CD81 overexpressing cells there was a decrease in F-actin stress fibres. Moreover, CD81 overexpressing larger cells (potentially S-type) presented and intracellular accumulation of CD81/F-actin into

perinuclear ring-like structures, cytoplasmic agglomerates and/or protruding filaments. Other authors have reported that CD81 overexpression promoted the formation of microvilli in B-cells, via reorganization of the cortical actin cytoskeleton (Bari *et al.* 2011). Further, CD81 overexpressing smaller (potential N-type) cells increased their neuronal-like differentiated phenotype.

The fact that F-actin co-localizes with several of the CD81 intracellular agglomerates agrees with the hypothesis that CD81-actin interaction is part of the mechanism by which CD81 regulates cell migration. Since impairing AKT activation resulted both in CD81 and actin re-location and in decreased CD81-induced motile phenotype (Figures 3 and 4), and since AKT is known to bind CD81 and to phosphorylate actin to promote it's remodelling, the hypothesis that a complex of active AKT-CD81-actin exists is very reasonable, and would partially explain the role of CD81 in cell migration. We hypothesize that if such a complex exists it is necessary for, at least, actin polymerization and the polarized distribution of F-actin into motile structures such as stress fibres and adhesion sites.

In addition to AKT, the molecular mechanism by which CD81 promotes neuronal-like cells motility may also involve other CD81 interactors and actin regulator such as Rac. This small G protein of the Rho family is known to promote actin polymerization and to be involved in cell migration. Rac may act downstream CD81, since a direct association between CD81 and Rac in TEM was reported, with CD81-Rac complexes being most prominent at motile cells' leading edge (Tejera *et al.* 2013). These authors hypothesize that CD81 regulates Rac1 dynamics and localization at the cell membrane during membrane protrusion and during the formation of adhesion complexes (Tejera *et al.* 2013). Noteworthy, besides activating AKT, PI3K can also activate Rac, and both molecules can be involved in PI3K-induced cell motile phenotype (Qian *et al.* 2004). Other authors have already hypothesized that CD81 may coordinate cell migration via the regulation of key migration-related proteins with which

CD81 interacts in the ‘tetraspanin web’(Boucheix & Rubinstein 2001; Hemler 2005; Jiang *et al.* 2015). Since Rac, AKT and actin are CD81-binding proteins, and CD81 is involved in tetraspanin microdomains that may ‘catalyse’ cellular processes, we propose a working scenario where, in TEM, CD81 serves as an anchor for proteins such as AKT, Rac and actin, to promote their interaction and the actin cytoskeleton remodelling that will lead to the acquisition of the polarized cell motile morphology. Although the SH-SY5Y cell line has been extensively used in the study of neuronal cell cultures, it is tumour-derived and cannot fully recapitulate the properties of the neuronal cells in vivo (Gordon *et al.* 2013). Thus our data should be validated using primary neuronal cells or human induced pluripotent stem cell (iPSC)-derived neurons. The confirmation of our hypothesis will improve our knowledge of the molecular mechanisms behind neuronal cell migration, an important event underlying various major neurological processes, such as neural development and neuroregeneration.

Conclusion

In neuronal-like cells, similarly to its role in immune cells, CD81 promotes cell transformation events that underlie the acquisition of a migratory phenotype. The mechanism behind CD81-enhancement of SH-SY5Y motility passes by the remodelling of the actin cytoskeleton. Moreover, our findings indicate that the PI3K-AKT signalling mediates this CD81 role in actin remodelling and subsequent polarization into a motile cell, and support the existence of a CD81-AKT-actin complex as a key molecular effector of this CD81 role.

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410

411 **Figures Captions**

412

413 **Figure 1. CD81 transfected neuronal-like cells have increased migratory phenotype. A.**
414 and **B.** Confocal micrographs of SH-SY5Y neuroblastoma cells non-transfected (**A.**) or
415 transfected (**B.**) with CD81 for 24h. Cells were fixed and subjected to ICC with an anti-CD81
416 antibody (labeled with AlexaFluor488, in green), red AlexaFluor568 Phalloidin to stain F-
417 actin, and DAPI to stain the cells' nuclei (in blue). Dashed rectangle in **B.** images – cell
418 leading edge. Full arrows – stress fibres. Open arrows – filopodia and similar cell protrusions.
419 Asterisks – potential focal adhesion sites. Endg, endogenous; Trf, transfected. **C.** Graphic
420 representation of the percentages of cells with a migratory phenotype, visually scored in SH-
421 SY5Y cells overexpressing or not CD81. *, $p < 0.05$, statistical significance determined by
422 either the independent sample t-test or the Mann-Whitney U test ($p = 0.012$ in both); n=5.

423

424 **Figure 2. Co-localization between CD81 and AKT1.** Confocal microscopy analysis of the
425 subcellular co-localization of CD81 (in green), either exogenous (**A.**, transfected cells) or
426 endogenous (**B.**, parental cells), with AKT1 (in red). ICC was performed using an anti-CD81
427 antibody (green, secondary antibody AlexaFluor488) and anti-AKT1 antibody (red,
428 secondary AlexaFluor594). Open arrows – filopodia and similar cell protrusions. Full arrows
429 – location at the cell membrane.

430

431 **Figure 3. PI3K inhibition decreases the percentage of migrating cells. A.** SH-SY5Y cells
432 transfected with CD81 cDNA were incubated with 10 μM LY294002, a PI3K inhibitor for
433 18h. The effect of PI3K inhibition on the number of CD81-transfected cells with migratory
434 phenotype was quantified, taking the number of CD81-transfected migratory cells in control

(untreated) cells as 100%. *, $p < 0.05$, statistical significance according to the one sample t-test; $p = 0.0504$ when using the non-parametric Wilcoxon test; n=3. **B.** Confocal microscopy images of transfected CD81 and F-actin staining, in control cells (untreated; upper panel) and in cells incubated with the LY294002 PI3K inhibitor (lower panel). Full arrows indicate F-actin stress fibres. Arrowheads indicate a decrease in stress fibres in LY294002 treated cells. Open arrow indicates cell with a CD81 'ring-like' distribution.

Figure 4. PI3K inhibition alters CD81 and F-actin subcellular distribution. CD81-transfected SH-SY5Y cells were incubated with 10 μM of the PI3K inhibitor LY294002, for 18h. Confocal microphotographs at the plasma membrane (**A.**) and nuclear (**B.**) focal planes show the abnormal concentration of CD81 internally, at cytoplasmic agglomerates and internal filaments, besides filopodia. CD81 and F-actin co-localize extensively in these structures (zoom-ins). Full arrows indicate F-actin stress fibres in non-transfected cells. Arrowheads indicate a decrease in stress fibres in transfected cells. Open arrows indicate the CD81 'ring-like' distribution.

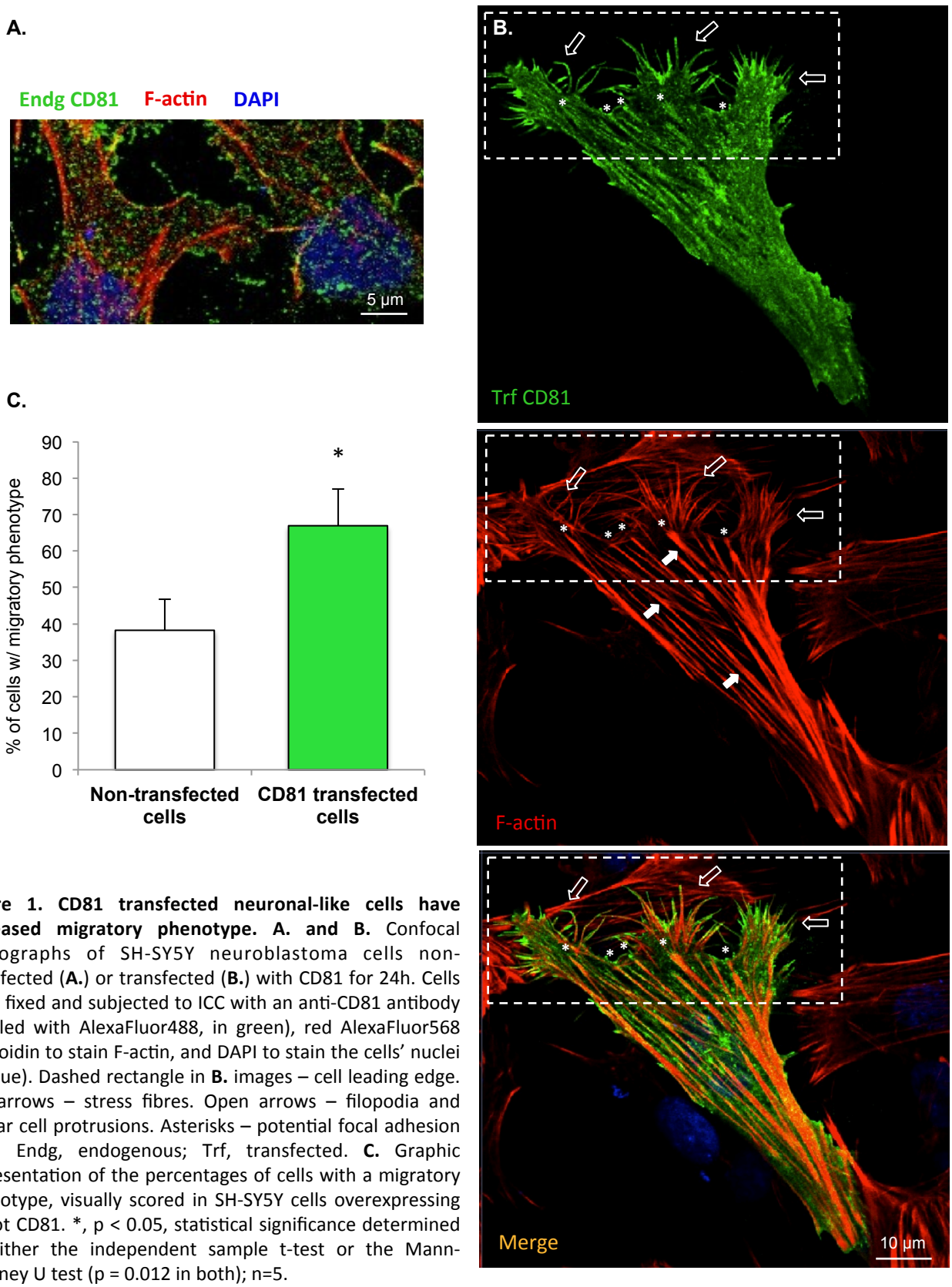


Figure 1. CD81 transfected neuronal-like cells have increased migratory phenotype. A. and B. Confocal micrographs of SH-SY5Y neuroblastoma cells non-transfected (**A.**) or transfected (**B.**) with CD81 for 24h. Cells were fixed and subjected to ICC with an anti-CD81 antibody (labeled with AlexaFluor488, in green), red AlexaFluor568 Phalloidin to stain F-actin, and DAPI to stain the cells' nuclei (in blue). Dashed rectangle in **B.** images – cell leading edge. Full arrows – stress fibres. Open arrows – filopodia and similar cell protrusions. Asterisks – potential focal adhesion sites. Endg, endogenous; Trf, transfected. **C.** Graphic representation of the percentages of cells with a migratory phenotype, visually scored in SH-SY5Y cells overexpressing or not CD81. *, $p < 0.05$, statistical significance determined by either the independent sample t-test or the Mann-Whitney U test ($p = 0.012$ in both); n=5.

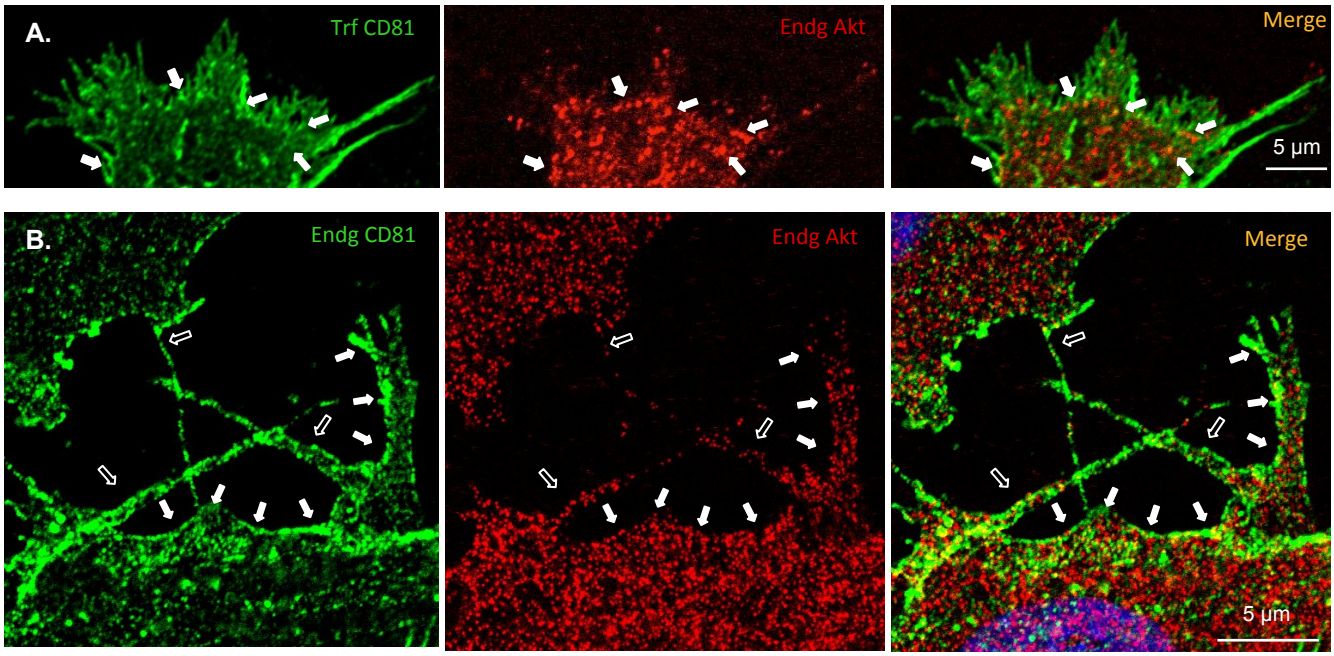


Figure 2. Co-localization between CD81 and AKT1. Confocal microscopy analysis of the subcellular co-localization of CD81 (in green), either exogenous (**A.**, transfected cells) or endogenous (**B.**, parental cells), with AKT1 (in red). ICC was performed using an anti-CD81 antibody (green, secondary antibody AlexaFluor488) and anti-AKT1 antibody (red, secondary AlexaFluor594). Open arrows – filopodia and similar cell protrusions. Full arrows – location at the cell membrane.

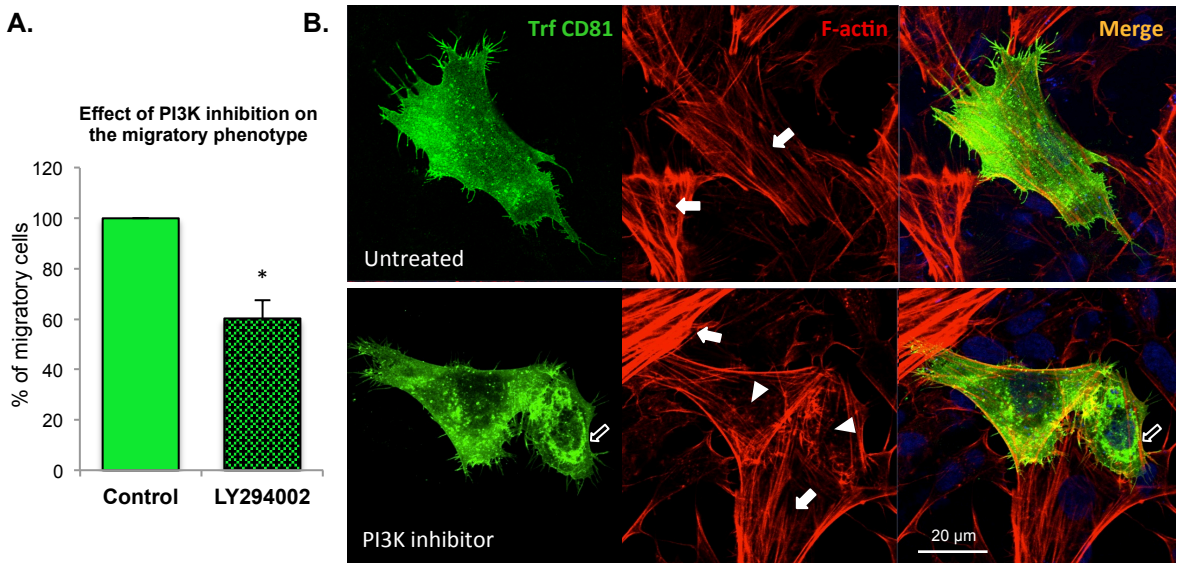
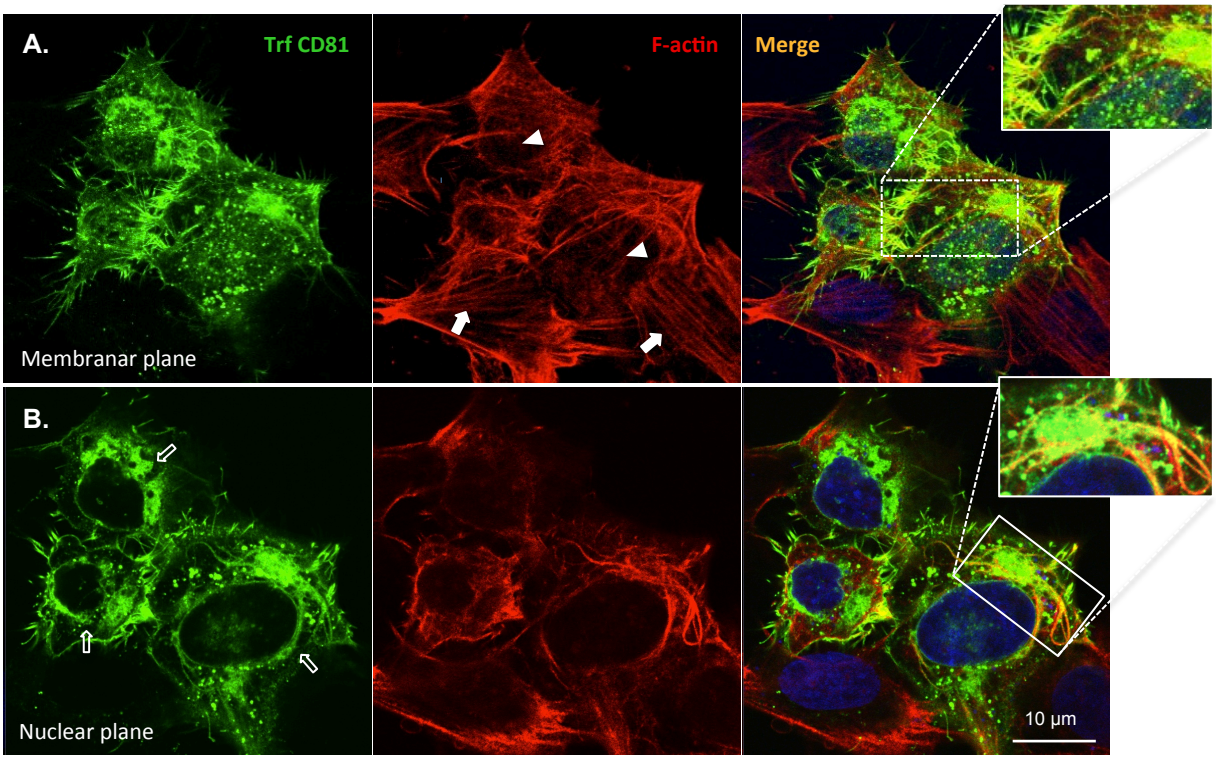


Figure 3. PI3K inhibition decreases the percentage of migrating cells. A. SH-SY5Y cells transfected with CD81 were incubated with 10 µM LY294002, a PI3K inhibitor, for 18h. The effect of PI3K inhibition on the number of CD81-transfected cells with migratory phenotype was quantified, taking the number of CD81-transfected migratory cells in control (untreated) cells as 100%. *, $p < 0.05$, statistical significance according to the one sample t-test; $p = 0.0504$ when using the non-parametric Wilcoxon test; n=3. **B.** Confocal microscopy images of transfected CD81 and F-actin staining, in control cells (untreated; upper panel) and in cells incubated with the LY294002 PI3K inhibitor (lower panel). Full arrows indicate F-actin stress fibres. Arrowheads indicate a decrease in stress fibres in LY294002 treated cells. Open arrow indicates cell with a CD81 'ring-like' distribution.



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