The role of β2-syntrophin in cell migration and proliferation

(Ο ρόλος της β2-syntrophin στην κυτταρική μετανάστευση και πολλαπλασιασμό)

MSc thesis

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Abstract

The accelerated cell proliferation and the initiation of cell migration are two key components of malignant transformation. Cells have to first disrupt the integrity of the epithelial monolayer and then start migrating. Cell-cell adhesion and apicobasal polarity are essential for proper epithelial function and consist two aspects regulated by the adaptor protein β2-syntrophin. Specifically, β2-syntrophin interacts with Tiam1, a Rac1-specific GEF, and thus controls Rac1 activity through which β2-syntrophin exerts some of its functions. Polarity proteins, in general, can either promote or impede cell migration leading to the malignant progression or inhibition of tumor metastasis, respectively. They are also implicated in the modulation of cell proliferation that is deregulated in cancer cells.

In this study, it is shown that depletion of β2-syntrophin leads to a reduction of cell migration. In wound healing assays, wild type cells display a complete closure of the wound, whereas cells with β2-syntrophin knockdown do not reach that point within 24 hours. It is also shown that knocking-down β2-syntrophin results in decreased cell proliferation that is observed 24 hours post-plating and thereafter. In search for a mechanism by which β2-syntrophin regulates cell migration, it is observed that the absence of β2-syntrophin slightly changes the size of focal adhesions at the leading edge cells. Further investigation is needed in order to define this mechanism.

Keywords: β2-syntrophin, Rho GTPases, Tiam1, migration, proliferation

Περίληψη

Ο αυξανόμενος κυτταρικός πολλαπλασιασμός και η έναρξη της κυτταρικής μετανάστευσης είναι δύο στοιχεία-κλειδιά του κακοήθους μετασχηματισμού. Τα κύτταρα πρέπει πρώτα να διαταράξουν την ακεραιότητα της επιθηλιακής στοιβάδας και στη συνέχεια να ξεκινήσουν να μεταναστεύουν. Η προσκόλληση κυττάρου με κύτταρο και η κορυφαιο-βασική πολικότητα είναι σημαντικές για την σωστή επιθηλιακή λειτουργία και αποτελούν δύο στοιχεία που ρυθμίζονται από την πρωτεΐνη προσαρμογής β2-syntrophin. Συγκεκριμένα, η β2-syntrophin αλληλεπιδρά με τον Tiam1, έναν Rac1-ειδικό GEF, ελέγχοντας έτσι την ενεργότητα της Rac1 μέσω της οποίας η β2-syntrophin εκτελεί κάποιες από τις λειτουργίες της. Οι πρωτεΐνες πολικότητας, γενικά, μπορούν είτε να προωθήσουν είτε να εμποδίσουν την κυτταρική μετανάστευση οδηγώντας σε κακοήθη εξέλιξη ή αναστολή της μετάστασης του όγκου, αντιστοίχως. Οι πρωτεΐνες αυτές εμπλέκονται επίσης στην ρύθμιση του κυτταρικού πολλαπλασιασμού, ο οποίος απορρυθμίζεται στα καρκινικά κύτταρα.

Σε αυτή τη μελέτη, αποδεικνύεται ότι η έλλειψη της β2-syntrophin οδηγεί σε μείωση της κυτταρικής μετανάστευσης. Σε δοκιμασίες wound healing, κύτταρα αγρίου τύπου παρουσιάζουν πλήρες κλείσιμο της «πληγής», ενώ κύτταρα με κατεσταλμένη τη β2-syntrophin δεν φτάνουν αυτό το σημείο μέσα σε 24 ώρες. Προκύπτει επίσης ότι η καταστολή της β2-syntrophin συντελεί σε μειωμένο κυτταρικό πολλαπλασιασμό που παρατηρείται 24 ώρες από το στρώσιμο των κυττάρων και μετά. Σε αναζήτηση ενός μηχανισμού ρύθμισης της κυτταρικής μετανάστευσης από την β2-syntrophin, παρατηρείται ότι η απουσία της β2-syntrophin αλλάζει σε μικρό βαθμό το μέγεθος των εστιακών συμφύσεων των κύτταρων στα άκρο της «πληγής». Περαιτέρω διερεύνηση απαιτείται με σκοπό να καθοριστεί αυτός ο μηχανισμός.

Λέξεις-κλειδιά: β2-syntrophin, Rho GTPases, Tiam1, μετανάστευση, πολλαπλασιασμός

1 Introduction

1.1 The Rho family of GTPases

The Rho family of GTPases constitutes part of a larger family; the Ras superfamily of small GTPases. Its protein members are found either in an active or inactive state, binding to either GTP or GDP, respectively. This cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs replace the bound GDP with GTP changing the conformation of the protein and making it able to interact with downstream effector molecules. On the contrary, GAPs stimulate the activity of GTPases to hydrolyze GTP to GDP, eventually making them inactive. In addition to this cycling state of GTPases there are a number of regulatory mechanisms that tightly control their active/inactive state that will be described further below.

1.1.1 Introduction

Rho proteins are implicated in a wide array of cellular functions. They play central roles in the regulation of the actin cytoskeleton, cell proliferation and migration, cell-cell and cell-matrix adhesions, as well as epithelial apicobasal polarity and transcription [1]. This functional multiplicity renders Rho signaling vital for the maintenance of cells in a non-cancerous state: a fact supported by a growing amount of *in vivo* evidence showing the involvement of disrupted Rho signaling in tumorigenesis. As we gain greater insight into the mechanisms that underlie Rho signaling, we learn more about its modulation during carcinogenesis. The best characterized members of Rho family are Rho, Rac1 and Cdc42 each one of them having multiple targets. This along with their vast number of regulatory proteins attributes an enormous complexity for Rho GTPase signaling but also an explanation for their multi-functionality.

1.1.2 Regulation of Rho GTPases

The Rho family of GTPases constitutes part of a larger family, the Ras superfamily of small GTPases. Rho proteins are consisted of five subfamilies: Rho-like, Rac-like, Cdc42-like, RhoBTB and Rnd as well as RhoD, Rif and RhoH/TTF that are not part of any of these

subfamilies [2]. Being GTPases, Rho protein members are found either in an active or inactive state, bound to either GTP or GDP, respectively (Figure 1). This cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs replace the bound GDP with GTP changing the conformation of the protein and making it able to interact with downstream effector molecules. GEFs constitute the activators of Rho signaling by the dissociation of GDP and the subsequent binding of GTP to the active site of Rho GTPases [3]. While some GEFs specifically activate only one GTPase, other GEFs activate a certain number of GTPases. For instance, the GEF Fgd1 activates Cdc42, and p115RhoGEF activates Rho, whereas Vav1 activates Rho, Cdc42 and Rac1, and Dbl activates Rho and Cdc42 [4]. GEFs are classified into two families; the Dbl homology-Pleckstrin homology (DH-PH)-containing family and the Dock180-related protein family containing a Dock homology region (DHR) domain [5]. Both domains specifically bind to the nucleotide-free state of Rho GTPases and are necessary for the catalytic activity of GEFs [5]. GEFs own additional domains which regulate their subcellular localization, activity and interactions with other proteins. As far as the latter is concerned, the structure of GEFs define the effector molecules that each Rho will bind to, thus regulating the specificity of downstream signaling of Rho GTPases. For example, Tiam1 specifically interacts with other proteins which are brought near Rac1 leading subsequently to their activation [6]. In contrast to GEFs, GAPs activate the GTPase activity of Rho proteins leading to the hydrolysis of bound GTP to GDP thus switching the GTPase to an inactive conformation [7].

The abundance of both GEFs (at least 80) [5] and GAPs (over 70) [8] indicates the importance for tightly controlling the Rho GTPase signaling. Both of them might also provide specificity regarding the downstream targets of GTPases as well as control their subcellular localization. In addition to this cycling state of GTPases, there are a number of regulatory mechanisms that tightly control their active/inactive state.

GDIs (guanine nucleotide dissociation inhibitors) comprise a third main regulator of Rho proteins that stabilize the inactive GDP-bound state. They work by sequestering inactive GTPases in the cytoplasm, thus preventing them from either stimulating downstream signaling or from degrading [9]. This is done by masking C-terminal lipid moieties that normally signal plasma membrane localization through their lipid-based membrane targeting sequence, thus inhibiting replacement of GDP with GTP. In this way, GDIs also control the membrane association/dissociation cycle of Rho proteins. Only three Rho GDIs have been identified so far; RhoGDI1 (being the best characterized) 2 and 3 [10, 11].

PTMs standing for post-translational modifications are another way of modulating Rho signaling [12]. These include phosphorylation, ubiquitylation, ADP-ribosylation, glucosylation, adenylylation, transglutamination/deamidation that affect different residues of Rho GTPases and can either activate or inactivate them.

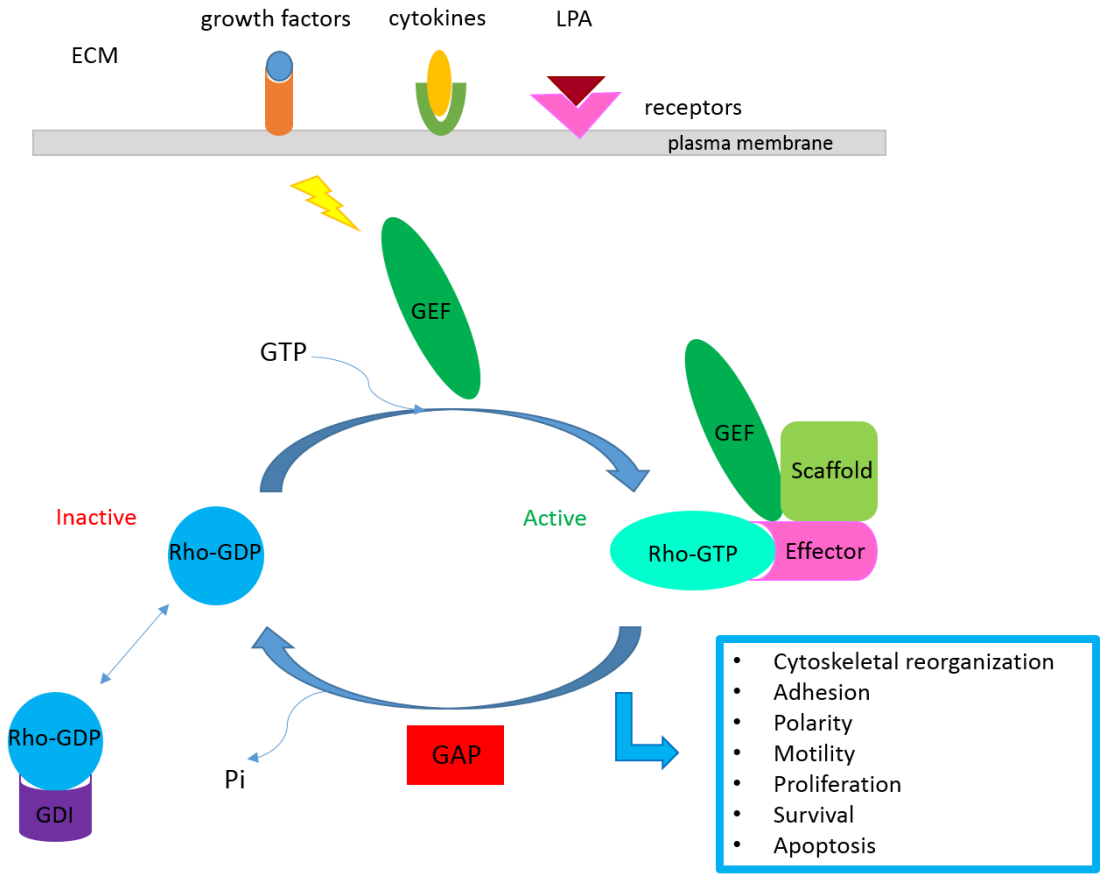


Figure 1: The Rho-GTPase cycle. Various stimuli including ECM, growth factors, cytokines and LPA bind to receptors and activate GEFs. These in turn exchange GDP with GTP in Rho GTPases making them active and able to associate with multiple downstream effectors leading to a wide variety of cellular changes. GAPs, on the contrary, promote the hydrolysis of GTP to GDP thus inactivating Rho GTPases. Additionally, GDIs sequestrate Rho proteins by stabilizing the inactive GDP-bound state.

1.1.3 Rho GTPases regulate

a) The actin cytoskeleton

Rho family proteins are well known for their roles regulating the actin cytoskeleton [13] and the bundles and networks of actin filaments which maintain cell shape and polarity. The dynamic capacity of these filaments enables the cytoskeleton to be rapidly reorganized

when required to change cell shape or size, especially important during migration and division. In 1992 it was firstly documented that active Rho was necessary for inducing assembly of stress fibers and focal adhesions in fibroblasts in response to the growth factors LPA and PDGF or insulin [14]. The same group in the same year showed that Rac1 induces actin reorganization to form lamellipodia and membrane ruffles and pinocytosis in fibroblasts again in response to growth factors [15]. Later it was shown that along with Rho and Rac, Cdc42 also regulates the assembly of multimolecular focal complexes leading to the formation of actin stress fibers, lamellipodia and filopodia (actin-rich membrane protrusions) [16]. In addition to this, Cdc42 promotes the formation of peripheral actin microspikes in fibroblasts [17]. Further contrasting roles of Rac1 and Rho began to emerge with evidence showing that Rac1 causes rapid actin polymerization in membrane ruffles, while Rho induces stress fiber formation via the bundling of actin filaments [18]. Rho, Rac1 and Cdc42 have the conserved ability to stimulate the formation of different actin-based structures in multiple metazoan organisms and cell types, like neurons and macrophages [19]. One pathway behind the regulation of the actin cytoskeleton by Rho is its interaction with mDia, a ligand of profilin, which results in actin polymerization into linear filaments [20]. mDia together with ROCK (Rho-associated kinase) mediate actin reorganization as well as regulate myosin phosphatase and thus myosin II suggesting a mechanism for regulating contractile actomyosin filaments [21, 22]. A key way Rac regulates the actin cytoskeleton is through its interaction with WAVE, an activator of Arp2/3 complex which subsequently elongates actin filaments [23, 24]. Meanwhile Cdc42 directly interacts with another Arp2/3 activator, N-WASP, thus stimulating the action of Arp2/3 complex, which along with formins is further required for the initiation of filopodia formation [25, 26]. Moreover, the recently identified presence of actin and actin-binding proteins, which are downstream effectors of Rho GTPases, in the nucleus suggests the role of Rho proteins in the regulation of both cytoplasmic and nuclear actin dynamics [27]. Deeper complexity regarding the regulation of the actin cytoskeleton by Rho GTPases is constantly emerging (for a more thorough review on this topic see “Rho GTPases: Masters of cell migration” by Sadok and Marshall, 2014 [28]).

b) Cell proliferation

Studies have shown that Rho GTPases control cell cycle progression, particularly the G1/S transition stage [29]. In quiescent non-transformed fibroblasts, expression of constitutively active forms of RhoA, Rac1 and Cdc42 starts the cell cycle by activating

progression through the G1/S phase and DNA synthesis [30]. Along with this evidence, expression of dominant-negative forms of Rac1 and Cdc42, or treatment with a Rho inhibitor C3 transferase, blocked serum-induced DNA synthesis [30]. Furthermore, RhoA has been reported to control G1/S transition in mammary epithelial cells, as it is needed for EGF and Ras-induced activation of Cyclin D1 and repression of $p21^{CIP1}$ [31, 32].

RhoA is involved in p27 (Kip1) degradation, another regulatory step in G1/S transition [33], through regulating its downstream effectors mDia, ROCK and Skp2 [34]. RhoA is also involved in the precise timing of Cyclin D1 expression during G1 [35, 36] that seems to also be induced by Rac1 and Cdc42 [37, 38], while Rac1 it directly lead to cyclin D1 expression [39, 40], and act along with ERK to allow the transition to S phase [41]. Cdc42 promotes G1 progression through p70 S6 kinase that induces cyclin E expression [42]. More recently, an inhibitor of Rac1 and Cdc42 was observed to suppress growth of primary human prostate cancer xenografts further supporting their role in cell proliferation [43].

Another Rho GTPase member, RhoC has been reported to regulate the proliferation of gastric cancer cells by interacting with IQGAP1 [44]. Overexpression of RhoC induces malignant transformation of hepatocytes *in vitro*, through increasing the expression of cell cycle-related genes while downregulating p27 (Kip1), as well as in nude mouse xenografts [45]. RhoD can regulate cell cycle progression through its effector Diaph1 as well as centrosome duplication [46]. In contrast, RhoE [47] and RhoBTB2 [48] halt cell cycle progression with the latter to also induce apoptosis.

Rho GTPases are also involved in mitosis and cytokinesis. For instance, Citron kinase, a Rho effector, is required for the transition from G2 phase to mitosis in hepatocytes [49] and p160ROCK, a Rho-associated kinase is needed for centrosome positioning [50]. Moreover, dominant-negative Rac1 expression in Rat2 fibroblast cells caused cell cycle arrest within the G2/M phase, indicating a requirement for active Rac1 in G2/M progression [51]. Additionally, overexpression of active Rac1 or Rac1 depletion halted MEF cell proliferation and leaded to defects at both the G2/M and G1/S transitions [52]. It was also shown that Rac1 cycles between the cytoplasm and the nucleus during the cell cycle, gathering in the nucleus in late G2 phase, and that expression of constitutively nuclear Rac1 promotes mitotic progression [53].

During mitosis, Cdc42 and mDia3 regulate microtubule attachment to kinetochores [54]. Cdc42 is activated at that stage by Ect2 and MgcRacGAP [55]. Later during cytokinesis, an anillin-Ect2 complex stabilizes central spindle microtubules at the cortex and it controls cleavage furrow formation [56, 57]. Further regarding cytokinesis, RhoA activates the

formins mDia1 and mDia2 leading to the formation of actin filaments to promote the assembly and tightening of the contractile ring [58, 59]. To restrain RhoA in a narrow equatorial zone, necessary for this contractile ring formation, a conserved MP-GAP (M phase-GAP) takes action [60]. ROCK is implicated in cytokinesis via the phosphorylation of myosin light chain at the cleavage furrow [61], while Citron kinase controls a molecular network involving KIF14 that is required for midbody formation [62]. Detailed analysis of the roles of Rho GTPases in mitosis and cytokinesis have been reviewed elsewhere [63]. What is more, in foci formation assays, simulating the growth factor and anchorage-independent growth of tumor cells, Rho GTPases synergize with active Ras indicating their essentiality in promoting proliferation of transformed cells [64].

c) Cell migration

Cell migration is required for embryonic development, during wound healing as well as for immune surveillance [65]. It is necessary for tumor metastasis, hence metastasis is a major therapeutic target. Reformation of the actin cytoskeleton to produce protrusions and contractions is required for active migration which in turn requires coordination Rho GTPases signalling. Correct regulation of Rho proteins is also vital for the transition between modes of cell migration, such as mesenchymal to amoeboid, depending on external stimuli and the microenvironment (reviewed in [66]). Particularly, in mesenchymal migration active Rac1 is needed for lamellipodia formation at the front of migrating cells driving forward cell movement, since expression of dominant-negative Rac1 blocks lamellipodia formation and cell movement in wound edge migrating cells [67, 68]. In a similar way, expression of dominant-negative Cdc42 impedes cell movement in the same cell model because of the disruption of the polarized phenotype of migrating cells [67]. Specifically, inhibition of Cdc42 activity causes the formation of lamellipodia all around the cell periphery and loss of Golgi/centrosome reorientation, showing that Cdc42 is required to restrict Rac1 activity to the front of the cell. More recently, the Cdc42 downstream effector FMNL2 was found to regulate actin polymerization at the edge of the lamellipodia thus causing the cell to move in a mesenchymal way [69].

Rho regulates cell-matrix adhesions which are regarded as necessary for cell movement [67]. What is more, RhoA, Rac1 and Cdc42 are tightly coordinated to modulate protrusions and adhesions at or near the leading edge of migrating cells thus controlling forward cell movement [70]. While Rac1 acts in the front of the cell where it causes the

formation of lamellipodia and Cdc42 the formation of filopodia, RhoA acts in the back part of the cell where it leads to the formation of actin stress fibers and focal adhesions, thus yielding a contractile force necessary for forward cell movement [2]. A recent study showed that a phosphorylation switch that transforms a protein complex under the EGF/PDGF signaling is responsible for this spatiotemporal activation of Rho GTPases in directional cell migration [71]. The degradation of ECM constituents is required for mesenchymal migration and is accomplished by MMPs whose activity can be controlled by Rho proteins. An example of this is the production of MMP-13 in an osteosarcoma cell line through Rac1 activity [72].

Cells with a rounded morphology usually adopt the amoeboid type of migration which is governed by Rho/ROCK signaling [73]. ROCK is localized at the plasma membrane where it competes with RhoE to induce actomyosin contractility necessary for squeezing between gaps in the ECM [74]. In addition, Cdc42 activation via DOCK-10 is important for this process [75].

Groups of cells also have the ability to migrate collectively as a unit, where cell junctions are maintained. Cancer cells are increasingly thought to perform collective migration in metastasis. In the leading layer of the cellular group a mesenchymal mode of migration is sustained, whereas in the internal cells the Rho/ROCK pathway is inhibited through the action of RhoE [76] ultimately halting membrane protrusions and Rac1 activity at cell contacts [77]. Another element that is controlled by Rho GTPases in this type of migration is stromal cells, as they seem to create tracks for the migrating cells. Firstly, RhoA activity in stromal cells remodels the ECM [78] and then the migrating cells move into the track with Cdc42 and ROCK to modulate their actomyosin contractility [79].

It has to be mentioned that in 3D ECMs additional ways of cell movement have been discovered that implicate not only lamellipodia and filopodia but also lobopodia, invadopodia and podosomes constituting different actin-rich structures complicating the signaling network of cell migration (reviewed in [80].

d) Cell-cell adhesions and polarity

Epithelial cells have an apical domain, which faces a central lumen or outside environment, and a basolateral domain that comes in contact with neighboring cells and the basement membrane [81]. The formation and maintenance of cell-cell adhesions is important for this polarization process. Cell-cell adhesions provide structural integrity and other critical functions to epithelial tissues. They additionally act as a fence preventing

diffusion of apical and basolateral membrane components that are required for the establishment and maintenance of apicobasal polarity. Adherens junctions physically maintain cell-cell contacts, but are also important sites for scaffolding signaling proteins, which in turn regulate cell division, differentiation and migration.

The actin cytoskeleton is connected with adherens junction complexes via interacting with afadin [82] and possibly α-catenin (this is controversial according to Benjamin et al. [83] and Drees et al. [84]. Since Rho GTPases can regulate the actin cytoskeleton, Rho, Rac1 and Cdc42 can also regulate epithelial cell-cell junctions [85] and based on their crosstalk a tightly regulated balance of their activities is important for proper organization and functioning of these epithelial barriers.

Cdc42-null embryonic stem cells lack apical polarity and have defective cell-cell junctions [86], while cell-cell contacts can activate Cdc42 [87], [88], which in turn cooperates with Par6 and aPKC to regulate the stability of AJ [89].

Rho is needed for the establishment and maintenance of AJ, as its inactivation results in the loss of E-cadherin-based cell-cell adhesions [90] while overexpression or deletion of Rho perturbs the gate function of TJ and general epithelial morphology [91, 92]. In fact, the spatial coordination of a RhoGEF and a RhoGAP at cell junctions ultimately activates RhoA thus keeping them intact and stable [93].

Rac1-mediated lamellipodia formation has been shown to expand initial cadherin-based cell-cell contacts in a zipper-like fashion [94, 95], but Rac1 is lost from mature cell-cell contacts indicating that it is not required at the later stages of cell-cell adhesion assembly [95]. Constitutively active Rac1 reverts the fibroblastoid phenotype of Ras-transformed MDCKF3 cells to an epithelial morphology, restoring E-cadherin-based cell-cell adhesion [96]. There is evidence that Rac1 can also negatively regulate AJ, which could be applied in conditions such as cell scattering or tumorigenesis [97]. Constitutively active Rac1 promotes Ras-induced transformation and the loss of AJ in primary epithelial cells [98, 99]. Moreover, elevated Rac1 activity has been found in primary mouse keratinocytes from squamous mouse epithelia with defective cell-cell junctions [100]. Regarding TJ, Rac1 has also been implicated in their regulation. It was reported that constitutively active Rac1 increases paracellular permeability in MDCK cells by disrupting TJs [101]. They also showed that both constitutively active and dominant-negative Rac1 disrupt the localization of several TJ proteins. Furthermore, expression of dominant-negative Rac1 inverts apicobasal polarity in cysts of MDCK II cells grown in Collagen I matrix via defective Laminin (LN) assembly [102]. Low Rac1 activity in TJ and higher Rac1 activity in AJ has been observed with two main

polarity proteins, Par3 and β2-syntrophin, an inactivator and an activator of Tiam1, respectively, regulating proper polarization [103]. Thus Rac1 seems to contribute to the assembly/disassembly of epithelial cell-cell adhesions and the establishment of epithelial apicobasal polarity. This is why the maintenance of tissue homeostasis requires a tight regulation of Rac1 activity in addition to its spatiotemporal control.

Similarly to regulating cell migration, Rho GTPases work either synergistically or antagonistically to modulate cell adhesions and polarization. For instance, when Rac1 is inhibited the RhoA-ROCK1-myosin II pathway is activated and leads to an inversion of polarity, a phenomenon that can be seen in malignant cells [104]. At cell-cell adhesions there are localized zones of Rac and Rho activity spatially separated the one from the other that cause the initiation and expansion of these contacts [94]. Furthermore, Rho acts opposite to Cdc42 at AJ to limit the tension on the apical side of epithelial cells that is necessary for sustaining the cell shape [105]. On the contrary, Rac1 and Cdc42 cooperate to regulate actin-based protrusions of epithelial cells [106] and to stabilize E-cadherin-mediated cell-cell adhesions [107].

e) Angiogenesis

Angiogenesis is essential for the establishment of solid tumors in order to be provided with the appropriate factors for their development. Rho GTPases can regulate various functions in endothelial cells which constitute the structural units of blood vessels (reviewed in [108]). They also control branching and lumen formation and contribute to neo-angiogenesis per se [109]. Rac1, being a master regulator of cell mobility, has become a favored candidate to attempt to halt tumor angiogenesis following metastasis [110]. Emerging is also the role of GEFs as they appear to fine-tune this multi-step process by regulating the different Rho GTPases especially in the vasculature [111].

1.1.4 Crosstalk between Rho GTPases

Rho GTPases exert multiple actions that are usually tightly integrated and coordinated. An example of this is the coordination of RhoA with Rac and Cdc42 at the cell edge to regulate the actin cytoskeleton [112]. Furthermore, as mentioned earlier, Cdc42 is

required to restrict Rac1 activity to the leading edge of migrating cells to drive directional cell migration [67]. Initial activation of Cdc42 is proposed to mediate the Nectin-based cell-cell contact induced activation of Rac1 [113]. Despite their many functional similarities, Rho GTPases can also act antagonistically. For instance, both Rac1 and Cdc42 can downregulate Rho activity and specifically Rac1-mediated inhibition of Rho is important for maintaining an epithelioid morphology [114]. Inactivation of Rho also enhances Rac1 signaling which is important for inducing neurite formation [115]. The same study showed that Rac1 and Rho have contradictory actions in processes of neuronal morphology [115]. The antagonism between Rac1 and RhoA can further be displayed by their spatial separation at the front of migrating cells [70]. In 3D migration models Rac1 and Cdc42 are responsible for the formation of different cell protrusions, while only inhibition of RhoA can lead to lamellipodia formation [116]. Following this, we previously described the same relationship of Rho GTPases in the regulation of apicobasal polarity and cell-cell contacts; Rho usually antagonizes with Rac and Cdc42, while the last two often cooperate. In many cases, the activity of one GTPase can either stimulate or counteract the activity of another. This can be done, respectively, by the activation of either a GEF or a GAP protein [2]. As will be discussed in detail later activating mutations have been discovered in the Rho GTPase genes themselves with their study leading to an interesting observation. Supporting the kind of crosstalk that has been described between Rho GTPases, mutations in RHOA gene until now are associated with reduced RhoA activity, while the opposite applies for RAC1 and CDC42, having in fact been characterized as oncogenes. In any case the balance between Rho GTPases changes leading to alterations in physiological cellular processes and causing tumorigenic events. These data along with others indicate the importance of crosstalk between members of the Rho family of GTPases and pinpoint their complicated signaling networks.

1.1.5 Rho GTPases in cancer

The fact that Rho GTPases regulate a great number of biological processes which are involved in cancer strongly suggests that they should play a role in oncogenesis, a hypothesis which is under vigorous investigation. Several lines of evidence currently implicate Rho GTPases in cancer development and progression.

Mutations within Rho GTPases, except for RhoH, were believed to be rare in cancer until recently [117]. Mutations had been identified within RhoH family member such as the

rearrangement of RhoH/TTF gene and the mutation of the 5'-UTR of RhoH gene in some hematopoietic malignancies [118, 119] reaching now the number of 57 mutations, but these were thought to be the exception. With the development of faster and cheaper deep sequencing technology [120] this idea has been strongly challenged, as Rho GTPases per se are found mutated in a wide variety of cancer types. Additional mutations have been found in the Rac1, Rac2, Cdc42, and RhoA genes [121], especially through the use of next-generation sequencing. Moreover, copy number alterations (CNA) including amplification, deletions or both have been found in Rho GTPase genes in various cancer types, which could justify the causal role of these proteins in tumorigenesis. Both mutations and CNA can be found using for example the cBio portal, a database that collects cancer genomics data sets from tumor samples across cancer studies [122, 123].

1.1.5.1 Direct mutations of Rho GTPases

a) Rac1

One early study trying to detect RAC1 mutations in human brain tumors identified deletions, frame shift and point mutations in the gene [124], suggestive of a role for Rac1 in brain tumor development. Among the 89 mutations identified in Rac1 gene so far a hot-spot one appears to be the P29S mutation. It was firstly identified by the groups of Hodis and Krauthammer in 2012 [125, 126]. In the first study, whole-exome sequencing was performed in melanoma samples and identified 5% of them to harbor missense mutations in RAC1 gene, making Rac1 the third most highly mutated gene in melanoma (after BRaf and NRaf mutations). The functional effect of the P29S mutation is that it induces a 'fast cycling' form of Rac1, as opposed to the more common gain-of-function mutations found at G12 and Q61 which tend to block GTPase activity. P29S is considered as a gain-of-function mutation that likely promotes oncogenic events during melanoma that implicate the disruption of cell proliferation, adhesion, migration and invasion [125]. A second study revealed P29S as a somatic recurrent mutation that leads to increased effector activation [126]. Expression of the mutant form of Rac1 in melanocytes led to enhanced cell proliferation and migration rendering P29S an activating mutation. Another study confirmed the transforming potential of this mutation *in vitro* among others that included C157Y as well as the discovery of RAC1 N29I as an oncoprotein [127]. These three mutants were revealed to be fast-cycling mutants which accounted for the constitutive activation of Rac1. P29S was further validated as a rapid-cycling mutant, similar to the spontaneously activated F28L mutant while maintaining

its GTPase ability [128]. A 2014 study revealed that RAC1 P29S expression in melanoma cell lines and *in vivo* conferred resistance to RAF and MEK inhibitors [129]. Along with that, a clinical study of the same year [130] suggested the potential of RAC1 P29S as a predictive biomarker for resistance to therapy in melanoma patients under treatment with these inhibitors. Further histological and clinical evidence showed that this hot spot mutation may be responsible for the early metastatic progression of BRAF mutant and wild-type melanoma [131]. A more recent biological insight of P29S mutation showed increased expression of PD-L1 in RAC1 P29S patients possibly indicative of reduced anti-tumor immune response [132].

b) Rac2 and Rac3

In the 2012 study conducted by Hodis' team, a homologue to RAC1 P29 residue was found to be mutated in RAC2 gene, substituting Proline (P) with Leucine (L) (P29L mutation) [125]. Later, in the 2013 study performed by Kawazu's team, two RAC2 mutants P29L and P29Q were confirmed for their transforming potential in fibroblastic cell lines [127]. Among 45 mutations the R102Q was found as a hot spot mutation according to The Cancer Genome Atlas (TCGA) with no further reports. Despite the reference of 29 mutations in RAC3 gene in cancer samples according to the cBio database, no RAC3 mutants have been identified so far in individual cancer studies. However, it is plausible that RAC3 mutants exist even in low frequency, based on the great sequence similarity between Rac1, Rac2 and Rac3 proteins as well as the conservation of amino acids that are mutated in Rac1 and Rac2.

c) RhoA

RHOA mutations have been identified by several groups in 2014 [133, 134] (with the total numbering 147 so far). Kakiuchi and colleagues firstly reported mutations of RHOA in diffuse-type gastric carcinoma [133]. These were: R5Q/W, G17E, L22R, V38G, Y42C, E54K, W58S, R68P, L69R and Y74D with R5Q and Y42C being recurrent. Functionally studying these mutants led to the conclusion that when the Y42 or the G17 residue is mutated, cancer cell lines have a similar growth advantage, even if these mutations are found in different regions of RhoA protein. Another study conducted with gastric adenocarcinoma samples added to the RHOA mutation list the following; Y34C, F39C, E40K, N41K, Y42S, L57V, D59Y, T60K, A61D and G62R/E [134]. These were accumulated in regions that participated in the interaction of RhoA protein with effector molecules. Depending on the target affected, this altered RhoA activity could account for the increased cell spread and the absence of cell

cohesion observed in this kind of tumors, given the vast number of cellular processes regulated by diverse Rho effectors, as described earlier. Inactivation of RhoA additionally promotes tumor formation in colorectal cancer, so it would be expected some of these mutations to disrupt RhoA activity [135].

d) RhoB, RhoC and RhoT1

RHOB was found to be mutated in bladder cancer [136] and among 74 mutations present, P75S/T/L is a hot spot mutation even though it has not yet been studied functionally. In a similar way, RHOC gene has come with a total number of 29 mutations and the hot spot S73A/Pfs*31 mutation without any functional analysis of the mutants. RHOT1 family member was identified with 91 mutations including the mutation in the homologous residue of RAC1 P29, P30L, suggesting that P29 residue may be a target for mutagenesis in Rho family GTPases.

e) Cdc42

Regarding CDC42 among 52 mutations, the classical G12D activating mutation of Ras has been identified in the gene, transformed into a G12V amino acid substitution rendering CDC42 another melanoma oncogene [125]. Despite the appearance of additional mutations on cBio portal for Cdc42, no function has yet been ascribed to them.

1.1.5.2 Overexpression of Rho GTPases in cancer

An indirect way of disrupting Rho GTPase signaling and function in cancer is the overexpression of either Rho GTPases themselves or their regulators or the presence of mutations in their regulators. Indeed, before the finding that Rho GTPases can themselves be mutated in cancer this was the only explanation for misregulated Rho GTPases, data that have been collected and reported by a number of reviews. In particular, Rac1, its splice variant Rac1b, Rac2, Rac3, RhoA, RhoB, RhoC, RhoG, Rnd3/RhoE and Cdc42 are overexpressed in various cancer types (as reviewed in [120, 121] and previously in [137]. This increased expression of Rho proteins is often associated with tumor progression, an indication of positive contribution of increased Rho GTPase activity to tumorigenesis [117]. In contrast to this, loss or reduced expression of RhoB was observed in lung cancer and head and neck squamous cell carcinoma [117]. In addition, Rnd3/RhoE, a ROCK inhibitor, is

downregulated in HCC (hepatocellular carcinoma) and correlated with poor prognosis and tumor progression [138, 139], while it is upregulated in gastric cancer cells under hypoxic conditions promoting EMT [140]. What is more, Rac3 activity was found to be increased in highly proliferative breast cancer cell lines, even if it was not increased at the protein level [141, 142]. The latter along with the fact that altered protein expression does not necessarily mean a causal role in cancer development, indicate the necessity to study both protein activity and expression [141, 142].

1.1.5.3 Modulation of GEFs in cancer

Several of their regulators are expressed differently or mutated in tumors, further supporting the contribution of Rho activity changes to tumorigenesis. The GEFs Tiam1, Ect2, MyoGEF, P-Rex1, LARG, Dock180, Vav1, Vav3 and β-PIX, the GAPs DLC-1, DLC-2, ARHGAP8 and P190RhoGAP and the GDIs Rho GDIα, D4-GDI and Rho GDIγ are overexpressed or genetically altered in various tumors [11, 120, 137]. A more recent study identifies mutations in PREX2 gene that encodes a Rac GEF, in melanoma increasing the list of indirect misregulation of Rho GTPases in cancer [143]. Ect2 has been recognized as an oncogene in human cancer since 2010, being aberrantly overexpressed and mislocalized in various types of tumor [144]. High expression of the GEFs Vav, Trio and Tiam1 is found in breast cancers with poor prognosis [145] and the GEF β-PIX in many breast cancers [146]. Furthermore, overexpression of Tiam1 in adenocarcinoma tissues as well as in squamous-cell carcinoma of the head and neck (SCCHN) is observed and is associated with advanced TNM stage and poor patient survival [147, 148]. Regarding Vav2 and Vav3, they seem to regulate a lung-metastasis specific transcriptome that leads to breast cancer progression [149]. Vav3 is also overexpressed in gastric cancer [150] as well as in prostate cancer where a novel nuclear function was found responsible for its malignant progression [151]. In addition, mutations in the PH domain of Tiam1, a Rac1-specific GEF, have been reported in renal carcinoma cells and correlated with transformation of NIH3T3 cells [152]. Furthermore, overexpression of the Rac activator STEF/Tiam2 was found to promote proliferation and invasion in liver cancer and thus be implicated in the pathogenesis of HCC [153]. Moreover, several Rho proteins' effectors present an altered expression pattern in various cancers. For instance, Pak isoforms which are Rho, Rac1 and Cdc42 effectors display increased expression in some tumors [141, 154].

1.1.5.4 Drug targeting

Small molecule inhibitors of Rho proteins are being developed as potential new cancer therapeutics. For instance, the ROCK inhibitor Y-27632 that targets the Rho signaling pathway retards migration of human prostate cancer cells in mice [155] and blocks the invasive activity of cultured rat hepatoma cells [141, 156]. Specific inhibitors are now being developed, instead of indirectly targeting Rho protein activity through downstream effectors or post-translation modifiers of Rho GTPases. For example, secramine has been identified as a small molecule inhibitor that perturbs Ddc42 activity in a RhoGDI1-dependent manner [157]. In addition, NSC23766 was found as a Rac GTPase-specific small molecule inhibitor that can halt the proliferation, anchorage-independent growth and invasion of prostate cancer cells [158]. Another small molecule, EHT 1864, was shown to specifically inhibit Rac downstream effector signaling by displacing its bound nucleotide [159]. Finally, a team in 2012 developed a high-throughput compound-screen assay hoping for a more rapid and effective identification of general and selective inhibitors for Rho proteins [160].

1.1.5.5 *In vivo* data for the role of Rho proteins in tumorigenesis

Mouse models that are deficient for either Rho GTPasesor their GEFs, have revealed some convincing evidence that link Rho GTPase signaling to cancer. In a model of Ras-driven skin cancer, Liu and colleagues showed that the RhoB-null mice had increased skin tumors compared to the heterozygote mice and that RhoB-deficient MEFs transformed with E1A and Ras showed greater resistance to DNA-damage induced apoptosis [161].C3 toxin-mediated inactivation of RhoA, B and C causes the development of aggressive malignant thymic lymphomas in mice [162]. Such findings indicate a tumor suppressor role for RhoB. In addition, deletion of RhoC from mice has been observed to reduce the frequency and growth of tumors [163]. Additionally, Malliri and colleagues reported that in a model of Ras-driven skin carcinogenesis Tiam1-KO mice had reduced tumor numbers and growth [164], [165] following TPA (12-O-tetradecanoylphorbol 13-acetate)-induced proliferation of the tumors. These data revealed an oncogenic function of Tiam1 and hence Rac1 activity during Ras-driven carcinogenesis. In agreement with this, Wang and colleagues reported that mice with Rac1 deletion specifically from keratinocytes are also resistant to developing tumors in the same mouse model of Ras-induced skin cancer [166]. Furthermore, Kissil and colleagues found that Rac1 is required for K-Ras-induced lung tumors in mice [167], a study that along

with the previous one shows the promotion of hyperproliferation in Ras-transformed, but not normal, cells by Rac1 activity. What is more, Rac2-KO and Rac3-KO mice showed slightly increased survival in a CML and ALL background, respectively [168, 169]. However it should be noted that the tumours which did develop in the Tiam1 knockout mice were more aggressive and invasive than in wildtype mice, showing a contrasting role for Tiam1 as a tumour suppressor, and highlighting the complexity of RhoGTPase signaling. Furthermore, deletion of Cdc42 form hepatocytes leaded to spontaneous tumor formation [170] indicating the involvement of the whole family of Rho GTPases in cancer.

1.2 Tiam1, a Rac1-specific GEF

Tiam1 is a Rac1-specific GEF which activates Rac1 by replacing GDP with GTP in the active center of the Rho enzyme. Despite its specificity, Tiam1 additionally activates Rac2, Rac3, Cdc42 and RhoA but to a lesser extent than Rac1 [171]. It is involved in Rac1-mediated cellular functions including cytoskeletal changes, cell migration and adhesion, cell proliferation and polarity. However, Tiam1 acts not only through its activity as a GEF but also in a Rac1-independent manner, making Tiam1's functions more difficult to identify. Characteristic examples are the induction of anoikis-resistance and the inhibition of c-Myc induced apoptotic cell death without involving Rac1 signaling [172, 173]. Furthermore, as previously mentioned, there are over 70 different GEFs regulating only 20 Rho family member proteins indicating the existence of a GEF redundancy. In addition, Rac1 is regulated not only by Tiam1 but also by other GEFs. These can explain the fact that Tiam1 depletion is not lethal in mice, since another GEF can compensate for its loss and lead to Rac1 activation and downstream signaling which was described above.

1.2.1 Tiam1 regulates

a) Cell proliferation

Mouse models of carcinogenesis that lacked Tiam1 developed fewer and smaller tumors in the skin [165] and intestine [174] than control mice. Additionally, knockdown of Tiam1 leads to decreased proliferation of epithelial cells such as the colon cancer cell line DLD-1 [174] and the MDCK II cell line [175]. Furthermore, MDCK II cells with depleted Tiam1

delay in mitosis because of accelerated centrosome separation and chromosome congression errors.

b) Apoptosis and survival

On one hand, when the cell receives apoptotic signals Tiam1 is inactivated thus decreasing Rac activation and its anti-apoptotic effects [176]. Tiam1 has also been described to promote apoptosis in human leukemia cells [177]. On the other hand, silencing of Tiam1 upregulates apoptotic genes and reduces viability in retinoblastoma cells [178]. Moreover, Tiam1 is necessary for preventing apoptosis in various tumors, as its knockdown is correlated with increased Ras-induced apoptosis [165, 174, 179].

c) Cell adhesions

Tiam1 degradation has been found to be required for Src-induced disassembly of adherens junctions (AJ) in MDCK II cells [180]. However, Tiam1 is mostly implicated in cell adhesion establishment and maintenance. Overexpression of Tiam1 has been found to assist in AJ and E-cadherin-mediated adhesion formation [181]. Furthermore, via restoring E-cadherin-mediated cell adhesion, Rac activation through Tiam1 did not allow MDCK II cells to obtain a fibroblastoid phenotype in response to Ras induction [96]. In accordance with this, it was later shown that Tiam1 expression is required for E1A-induced epithelioid morphology of primary MEFs and MDCKF3 cells [175]. The same study also showed that downregulation of Tiam1 in MDCK II cells causes a flattened phenotype with destroyed E-cadherin-based adhesions [175]. In endothelial cells an association of Tiam1 with cadherin-based adhesions is also present, as expression of VE-cadherin is correlated with an increase in Tiam1 levels and membrane localization [182]. Defective TJ assembly has also been reported in Tiam1-KO and Tiam1 knockdown keratinocytes [183]. Tiam1-KO cells had also reduced levels of occludin, claudin and ZO-1 that were rescued along with TJ assembly after expression of wild-type Tiam1. In fact, Tiam1 was found to interact with the Par polarity complex leading to Rac1 activation to control TJ biogenesis in keratinocytes [183]. In contrast to this, Chen et al. reported that increased Tiam1-mediated Rac1 activation impedes TJ assembly. When Par3 was downregulated in MDCK II cells Rac1 activity was increased and TJ assembly was disrupted showing the necessity of Par3-mediated inhibition of Tiam1 for efficient TJ assembly [184]. Going back and supporting the positive role of Tiam1 in TJ assembly, Guillemot et al. later in 2008 showed that paracingulin (CGNL1) recruits Tiam1 to epithelial

junctions where the latter promotes TJ assembly of MDCK II cells [185]. More recently and consistent with the data from Chen et al. regarding the disruption of TJ assembly due to elevated Tiam1/Rac1 activation, it was shown that a new Tiam1 interactor, β2-syntrophin, acts as a positive regulator of Tiam1 in contrast to the negative regulator Par3, with both of them resulting in the modulation of TJ assembly [103]. A more detailed reference to this study will be given later.

d) Cell migration

Tiam1 stands for T-cell lymphoma invasion and metastasis 1 indicating its ability to promote invasion of T-lymphoma cells [186]. Tiam1 has been shown to promote migration in numerous cancer cell lines including lung carcinoma, breast cancer, colorectal carcinoma, gastric cancer, osteosarcoma and ovarian cancer [172, 187-191]. Furthermore, α6β4 integrin is implicated in migration and invasion of pancreatic carcinoma cells via upregulating Tiam1 [192]. In addition to this, others showed that the 14-3-3 protein dimer localizes Tiam1 to integrin complexes thus activating Rac1 there and initiating the migration procedure [193]. In contrast to these data, other studies support the negative role of Tiam1 in cell migration and invasion which is consistent with its positive role in cell-cell adhesion, as previously described. Engers et al. reported that ectopic expression of Tiam1 impeded migration and invasion in renal cell carcinoma cells by increased cell-cell adhesions [181]. Moreover, overexpression of Tiam1 halts invasion of metastatic melanoma cells by inducing an epithelial phenotype [194]. Similarly, Tiam1 downregulation leads to increased migration of MDCK II cells in a wound healing assay [175]. Furthermore, Tiam1-dependent maintenance of AJ represses Src-induced focal adhesion turnover and collective cell migration [180]. What is more, skin and intestine tumor progression in mice takes place when Tiam1 is absent indicating a repressive role for Tiam1 in cell migration and invasion [165, 174].

1.2.2 Context-dependent Tiam1 functions

Tiam1's duality is evident in several cellular processes as displayed so far. This could be attributed to the fact that different cell types develop different cell-cell and cell-matrix adhesions. Even when the same cell type is used but the substrate changes, cell-matrix adhesions are again altered. For instance, Tiam1/Rac1 promotes cell-cell adhesion when MDCK cells are plated on fibronectin or laminin, while it promotes cell motility when they are plated on collagen [195]. In the first case, Tiam1 localizes to AJ, whilst in the second case

it is found to membrane ruffles and lamellipodia, highlighting the importance of Tiam1 localization in determining the substrate-dependent response. Another explanation could be the balance between Rac and Rho activation, since the one usually antagonizes the other as described in a previous session. For example, Rac signaling leads to ROS (reactive oxygen species) production which eventually causes the activation of p190Rho-GAP that halts Rho activity [196]. However, the same GAP can also decrease Rac activity [197], thus it could be responsible for establishing a Tiam1/Rac1 to RhoA balance that could determine the migratory behavior of cells [198].

1.2.3 Tiam1 in cancer

Deregulation of Tiam1 has been reported to contribute to tumor progression. Tiam1 overexpression is associated with poor prognosis in esophageal squamous cell carcinoma and high grade tumor in breast cancer [199, 200]. As mentioned above, Tiam1 seems to promote migration and invasion of various types of cancer. However, it is not required for the invasion of DMBA-induced skin tumors, β-catenin-induced intestinal tumors and Neu-induced mammary tumors [165, 174, 179]. In addition, Tiam1 protein expression is decreased during breast cancer progression [201]. Moreover, the inverse relationship between Tiam1's expression and invasiveness in RCC (renal cell carcinoma) and the transformation potential of a Tiam1 mutant propose a metastatic repressive role for Tiam1 [152]. In 2002 Malliri et al. showed that Tiam1-KO mice are less susceptible to developing Ras-induced skin tumors because of increased apoptosis of epidermal keratinocytes upon tumor initiation. In addition and as previously pointed, the mice developed fewer and smaller tumors, however these were more prone to progressing into malignancy [165]. This was attributed to the requirement of Tiam1 for the maintenance of cell junctions which prevent invasion [175]. Alternatively, Tiam1 deficiency in tumor cells could account for the decreased tumor growth and the lack of Tiam1 in the surrounding fibroblasts could account for the increased invasiveness of surviving tumors [202]. Likewise, Tiam1 is needed for the formation of APC-min mice, while it prevents invasion and progression of intestinal tumors [174].

Overall, the role of Tiam1 in cancer seems to be context-dependent. Disparate cell types, microenvironment, tissue and oncogenes determine Tiam1's participation in different signaling pathways. Whether these pathways are pro- or anti-tumorigenic and the balance between them yields the final effect of Tiam1 in tumor initiation and progression.

1.2.4 Regulation of Tiam1

The GEF Tiam1 is tightly regulated in space and time depending on the tissue, cell and subcellular site. The control mechanisms involve intramolecular inhibition, intracellular localization, post-translational modifications, protein-protein interactions and abundance of protein. The various structural domains of Tiam1 contribute to this tight control of its activity (Figure 2). Tam1 is a large protein of approximately 180 kDa consisting of a N-terminal mysistoylation site, two N-terminal PEST domains (P), two Pleckstrin homology (PH) domains; one N-terminal (PHn) and one carboxy-terminal (PHc) domain, a coiled-coil region with adjacent sequence (CC-Ex) following the PHn domain, a Ras-binding domain (RBD), a PSD-95/DlgA/ZO-1 (PDZ) domain, and a catalytic Dbl homology (DH) domain followed by the PHc domain. The PEST domains regulate Tiam1's stability and the upstream N-terminal region that harbors a myristoylation site is also involved in stabilization and localization of Tiam1 [6, 171]. Both PH domains are involved in membrane localization through interacting with phosphoinositides. The entire module PHn-CC-Ex is essential for plasma membrane association and the interaction with scaffold proteins such as spinophilin, JIP2/IB2 and Par3 transducing downstream signals [203]. Moreover, the RBD can mediate a direct interaction with Ras which is necessary for Rac1 activation downstream of Ras [204]. The catalytic DH-PH (PHc) combination is the minimal unit necessary for nucleotide exchange with the DH domain binding to Rac1 protein and causing nucleotide exchange thus leading to Rac1 activation [205]. Furthermore, an α-helix in the N-terminus of Tiam1 can interact with the DH domain and inhibit Rac1 association conferring an auto-inhibitory mechanism to the protein that may be released via phosphorylation or protein binding [206].

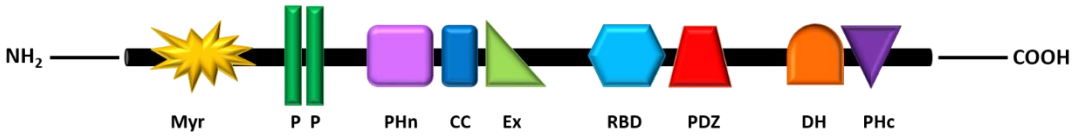


Figure 2: Structure of full-length Tiam1. It contains, from the amino terminus to the carboxy-terminus, Myr: myristylation sequence; P: PEST domain; PHn: N-terminal PH domain; CC: coiled-coil region; Ex: Extended domain; RBD: Ras binding domain; PDZ: PSD-95/DlgA/ZO-1 domain; DH: Dbl homology domain; PHc: C-terminal PH domain (adapted from Boissier et al., 2014 [203]).

It is noteworthy that the scaffolding functions of Tiam1 specify signaling downstream of Rac1. The interactions of Tiam1 with scaffold proteins mentioned above are determined by the stimuli received by cells, which in turn result in activation of distinct Rac-

dependent pathways by recruiting specific Rac effectors. Each interaction is further coordinated by Tiam1's subcellular localization which has to do with the binding partner.

1.3 β2-syntrophin is a Tiam1 interactor

1.3.1 Interaction of Tiam1 with the DGC complex

The dystrophin glycoprotein complex (DGC) is a multimeric protein complex associated with either dystrophin or its paralog utrophin. An utrophin-associated DGC complex has been found in MDCK II cells consisting of utrophin, β2-syntrophin and dystrobrevin-beta and localizing to cell-cell junctions (Figure 3; [207]). Tiam1 was shown to interact with all these three complex constituents [103]. The DGC complex may in general be comprised of dystroglycans, syntrophins, dystrobrevins, sarcoglycans, sarcospan, and dystrophin or utrophin [208] and exist in a variety of compositions due to the large number of isoforms of some of these protein families.

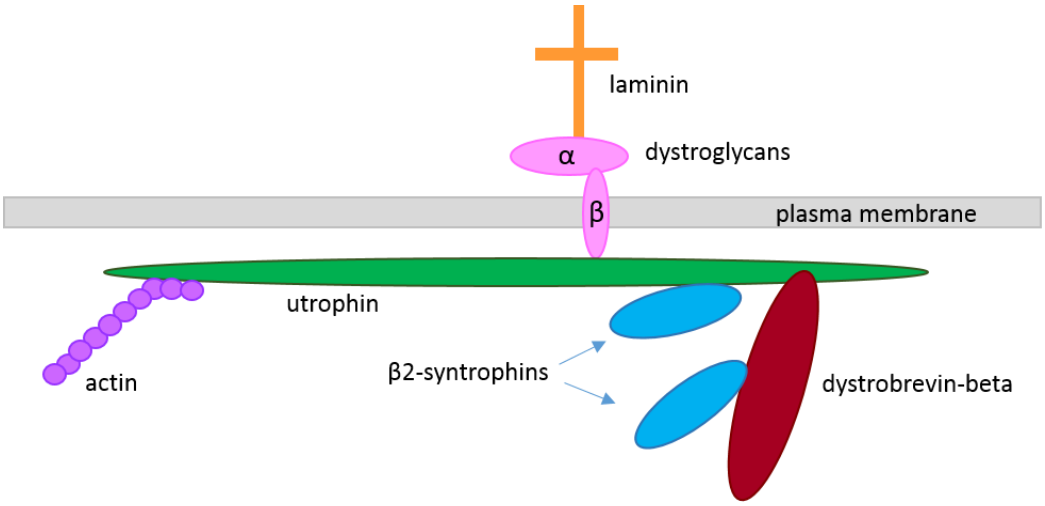


Figure 3: The β2-syntrophin/utrophin/dystrobrevin-beta complex. A schematic representation of the composition of the utrophin-associated DGC complex that exists at cell-cell junctions in MDCK II cells, which connects extracellular laminin to the intracellular actin cytoskeleton (adapted from Kachinsky et al., 1999 [207]).

Among these, utrophin is ubiquitously expressed, found at high levels in the lung, kidney, nervous system and vascular endothelial and smooth muscle cells [208], while dystrophin expression is confined to muscle and neuronal tissues. Both utrophin and dystrophin directly bind to actin and dystroglycan laminin (LN) receptors thereby linking the extracellular matrix (ECM) to the intracellular actin cytoskeleton [209]. Moreover, the dystrobrevin family has two members, alpha and beta, encoded by different genes with both of them binding to dystrophin and utrophin [210]. Furthermore, the syntrophin family has five members encoded by different genes: α1, β1, β2, γ1, and γ2. β2-syntrophin associates with utrophin at the neuromuscular junction (NMJ) in skeletal muscle, whereas α1, β1, and γ2 syntrophins associate with dystrophin at the sarcolemma [211]. γ1-syntrophin is only expressed in neurons [212]. Syntrophins possess multiple domains, including two Pleckstrin homology domains (PH1 and PH2) with one of them being split in two (PH1a and PH1b) by a PDZ domain, and one syntrophin unique (SU) domain [207] (Figure 4). The PDZ domain mediates interactions with a variety of proteins, including: inwardly rectifying potassium channels [213], voltage gated sodium channels [214], neuronal Nitric Oxide Synthase (nNOS) [215], PH domain-containing adaptor proteins TAPP1/2 [216], stress-activated protein kinase 3 (SAPK3) [217], heterotrimeric G proteins [218], islet cell autoantigen 512 (ICA512) [219], ATP-binding cassette transporter 1 (ABCA1) [220] and serine/threonine kinases MAST205 and SAST [221].

Figure 4: Structure of β2-syntrophin. PH1a-PH1b: split PH (Pleckstrin homology) domain; PDZ: PSD-95/Dlg/ZO-1 domain; PH2: second PH domain; SU: Syntrophin Unique domain.
(Adapted from Adams et al., 1995 [222]).

As already mentioned, the DGC is found in numerous forms in all tissue types, each one of them performing distinct functions. It is thus implicated in a variety of cellular functions, including the regulation of membrane integrity, apicobasal polarity, transmembrane signaling and ion homeostasis [208]. In particular, due to the large number of protein-protein interactions associated with the DGC, the complex acts as a scaffold to assemble multiple structural and signaling molecules to coordinate and mediate cytoskeletal changes.

Mouse models in which DGC components have been deleted have revealed some functional redundancy between DGC components. For example, redundancy was

demonstrated between dystrophin and utrophin, since the double knockout mouse displays more severe muscle weakness than the single knockouts [223]. Furthermore, dystrophin and utrophin double knockout mice have poor survival rates and display complete disruption of the DGC in the kidney, whereas the single knockout mice show compensatory mechanisms to maintain kidney DGC function [224]. Functional redundancy is also apparent between α1- and β2-syntrophin, since the double knockout mice have more severe NMJ defects than the α1 knockouts. Surprisingly, the β2-null mouse appears to develop normally and lives healthily for a normal lifespan, suggesting α1 may compensate for its loss [225]. However, α1 cannot bind to MAST205 [221], suggesting that this interaction is not important for survival. In a similar way, β2 cannot fully compensate for the loss of α1, since nNOS is absent from synapses in α1-null mice [226]. Moreover, mice lacking dystrobrevin-beta develop normally, but have reduced expression of syntrophins and Dp71 isoforms at the membranes of kidney tubules and liver sinusoids, however, no defects in kidney or liver membrane structure or kidney function are observed [227]. In contrast, dystroglycan deletion is embryonic lethal [228], demonstrating the importance of ECM-cell linkage through this LN receptor.

The DGC complex is known to regulate intracellular signaling through its association with multiple signaling proteins via the syntrophins. The recently discovered interaction of the complex with Tiam1 mediated by β2-syntrophin suggests its implication in Tiam1-Rac signaling [103]. Furthermore, the association of β2-Syntrophin with ICA512 controls intracellular signaling to regulate insulin secretion [219]. Furthermore, α1-syntrophin regulates stress-activated MAPK signaling through its interaction with SAPK3 [217]. Interestingly, the DGC has previously been implicated in the regulation of Rac1 signaling in skeletal muscle [229-231]. Oak et al. described a signaling cascade that by way of a β-dystroglycan/syntrophin/Grb2/Sos1 complex linked extracellular LN binding to intracellular Rac1 activation [229]. They also showed that activated Rac1 promoted the activation of PAK1 and JNKp46, ultimately resulting in c-Jun phosphorylation on Ser-65 which is thought to promote cell growth and survival. Later, it was shown that LN binding stimulates Src-mediated tyrosine phosphorylation of syntrophin which increases Grb2 binding and switches the Grb2 binding site on syntrophin, which is necessary for the recruitment of Sos1 to the complex and activation of Rac1 [230, 231]. This syntrophin-Rac1 signaling pathway is active *in vivo* since it is stimulated by muscle contraction and stretching [231]. Intriguingly, Tiam1 is known to associate with the Grb2-Sos1 complex downstream of Src signaling [180] and the

Tiam1-DGC interaction raises the possibility that Tiam1 is involved in LN-induced Rac1 activation.

The direct association of utrophin and dystrophin with actin means that signaling via the DGC can coordinate changes in the actin cytoskeleton. For example, the interaction of syntrophins with TAPP1 regulates PDGF-stimulated changes in the actin cytoskeleton [216]. It is likely that the Tiam1-DGC interaction provides another linker of the DGC to Rac1 signaling and subsequent cytoskeletal regulation.

Several reports have implicated DGC complexes in the regulation of epithelial apicobasal polarity through their regulation of endogenous LN assembly [232-235]. Loss of dystroglycan in mammary epithelial cells disrupts LN-induced polarity and the cytoplasmic region of dystroglycan is not required to rescue LN assembly defects [236]. These findings indicated that other members of the DGC are not required for LN assembly. However, inconsistent with this, it has since been shown that utrophin is also required for LN assembly and apicobasal polarity in MDCK II cells [235]. Whether syntrophins play any role in LN assembly is not yet known.

Importantly, various members of the DGC have been implicated in tumorigenesis. Numerous reports describe the frequent loss or reduced expression of dystroglycans in a variety of human cancers, indicating a tumor suppressor role for dystroglycans [237-245]. Moreover, mice deficient for dystrophin or sarcoglycan develop spontaneous embryonal rhabdomyosarcoma containing tumor-associated p53 mutations [246, 247]. Li et al. discuss various findings that imply utrophin functions as a tumor suppressor and consequently, they propose that down-regulation of utrophin is likely to be an early step in tumorigenesis [248]. They report that the utrophin gene is located at chromosomal region 6q24, a region which is frequently targeted in tumors by loss of heterozygosity (LOH) and inactivating deletion mutations. Moreover, they show that down-regulation of utrophin with expression of antisense utrophin results in cellular transformation. Consistent with this, they showed that overexpression of G-utrophin-GFP inhibited tumor cell growth and colony formation. Furthermore, DGC dysfunction has been implicated in cancer-induced Cachexia (muscle wasting), a debilitating condition that is thought to contribute to almost a third of all cancer-related deaths [249]. Inconsistent with tumor suppressor functions, the expressions of utrophin and syntrophin were found to be retained in B-chronic lymphocytic leukemia (B-CLL) cells, and higher levels of syntrophin and its interactor TAPP2 were observed in the more aggressive subset of these leukemias [250].

1.3.2 The role of β2-syntrophin in cell-cell adhesions

For normal epithelial tissue homeostasis cell-cell adhesions must be tightly regulated and continuously modulated. Defects in TJ have been attributed to a wide array of human diseases, including cancer, immune diseases, kidney dysfunction, congenital deafness, inflammatory bowel disease, and many others [251]. In particular, the loss of cell-cell adhesions and apicobasal polarity in tumorigenesis is well established and known to contribute to tumor progression and metastasis [252]. Cell-cell adhesions are also modulated during development, differentiation, cell proliferation and cell migration. For example, cells within the lining of the gut and the epidermis of the skin require rapid turnover, which involves rearrangements of their cell-cell contacts. Consistent with this, cell-cell adhesions are dynamic structures that undergo continuous remodeling [253, 254].

The evidence demonstrating the importance of Tiam1 and Rac1 to the regulation of cell-cell adhesions was described earlier. As discussed, the exact contribution of Tiam1 to the establishment and maintenance of epithelial cell-cell adhesions remains unclear with both positive and negative regulation of Tiam1 activity having been reported to be necessary for epithelial TJ assembly [183-185].

Prior to the 2012 study by Mack et al. the contribution of syntrophins and therefore the signaling functions of the DGC complex to the establishment of cell-cell adhesions and apicobasal polarity was not known, apart from the contribution of some DGC members to the latter. After studying the interaction of Tiam1 with the DGC complex it was found that this actually regulates these processes through the β2-syntrophin DGC member and that its disruption has implications for tumorigenesis [103]. Specifically, it was shown that Tiam1 interacts with the PDZ domain of β2-syntrophin through a PDZ-binding motif (PBM), while an interaction with associated utrophin-dystrobrevin-beta was indirect, mediated by β2-syntrophin. This suggested that the DGC could regulate Tiam1-mediated Rac1 signaling. Further studying its function they found that in particular β2-syntrophin regulates cell-cell adhesions, since depletion of β2-syntrophin from MDCK II cells led to a delay in TJ assembly. They also observed impaired AJ assembly in β2-syntrophin knockdown cells that was hypothesized to result in the TJ defects. β2-syntrophin downregulation was additionally found to impede cell-cell aggregation and to compromise cell-cell adhesion strength. They then observed that Tiam1 knockdown in MDCK II cells did not retard TJ assembly but instead enhanced it. In addition to this, Rac1 disruption led to accelerated TJ assembly showing that Tiam1-induced Rac activity perturbs TJ assembly, a finding that agreed with the data from Chen et al. referred above [184]. By trying to link β2-syntrophin with Tiam1/Rac1 activity,

they also showed that the interaction between β2-syntrophin and Tiam1 is necessary for Rac1 activation at cell-cell junctions. Furthermore, β2-syntrophin knockdown did not increase the inhibitory Par-3-Tiam1 interaction, meaning that disruption of Rac activation was independent from that. The question therefore became how a positive regulator, β2-syntrophin, could promote a negative regulator of TJ assembly, Tiam1-Rac. It was revealed that the activator β2-syntrophin and the inhibitor Par3 of Tiam1 are found in different places across cell-cell junctions leading to a spatial regulation of Tiam1-Rac activity and subsequently to an optimal TJ assembly. β2-syntrophin localizes more basally compared to Par3 and seems to maintain the correct Tiam1 localization across the apicobasal axis. It was also shown that β2-syntrophin and Tiam1 are found at AJ while Par3 is mainly present at TJ, thus forming two spatially separate complexes that regulate Tiam1-Rac activity at distinct cell-cell junction locations. In fact, these complexes create an apicobasal gradient of Rac1 activity (Figure 5). This gradient is present even in the absence of Tiam1 suggesting that maybe compensatory mechanisms result in the differential Rac activity across the apicobasal axis. After disrupting the apicobasal Rac activity gradient, it was shown that this spatial regulation at cell-cell junctions is essential for proper TJ formation and polarity. What is more, β2-syntrophin was found to be inversely correlated with prostate cancer progression, since β2-syntrophin is lost from cell-cell adhesions when the tumor becomes malignant. In accordance with the observed overexpression, described above, of Tiam1 and Rac1 in prostate cancer samples with advanced stage of malignancy, it seems that the whole β2-syntrophin-Tiam1-Rac1 signaling pathway is deregulated in human prostate cancer [103].

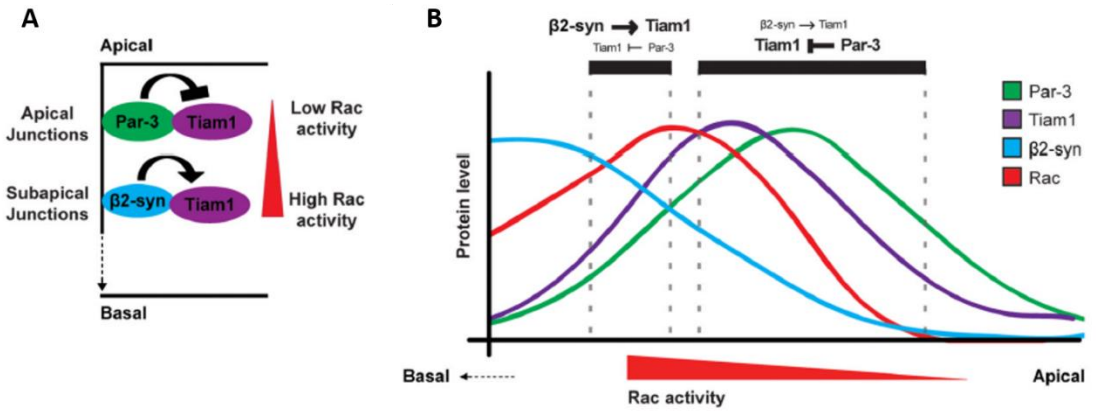


Figure 5: (A) A model depicting the differential localisations of Par-3 and β2-syntrophin and their differential effects on Tiam1-Rac activity at cell-cell junctions. (B) A model displaying the differential but overlapping localizations of β2-syntrophin, Par-3, Tiam1 and Rac at cell-cell junctions, which enable them to promote an apicobasal junctional Rac activity gradient. β2-syn, β2-syntrophin (adapted from Mack et al., 2012 [103]).

1.3.3 Polarity proteins regulate cell migration and proliferation

Many other polarity proteins except for β2-syntrophin have been implicated in the regulation of cell-cell adhesion. They are also involved in other cellular processes such as cell migration and proliferation. For instance, the polarity regulator Scribble has been found to inhibit cell migration via promoting cell-cell adhesion, because absence of Scribble increases cell migration and weakens cell-cell adhesions [255, 256]. This is similar to Tiam1 loss that disrupts AJ and promotes migration of MDCK II cells [175]. Nevertheless, there are cases where polarity proteins regulate cell-cell adhesion but also promote cell migration. In contrast, Scribble has also been reported to regulate the polarity of migrating cells and be necessary for directed cell movement [255, 257]. Additionally, members of the Par complex, including Par3, Par6 and aPKC have been observed to localize to the leading edge of migrating cells where they act to drive forward cell movement [255]. Furthermore and as shown before, the Tiam1-Par complex is believed to be important for promoting cell-cell adhesion in polarized epithelial cells [183, 184], however, in migrating keratinocytes the complex has been shown to stabilize the microtubule network, thereby promoting directed and persistent migration [258].

Regarding cell proliferation, a great deal of proteins regulating cell-cell adhesions and polarity are known to regulate this process as well. An example of this is members of the Par complex [259] and as shown above, Tiam1 [175, 260]. What is more, there is increasing evidence that cell-cell adhesion status is tightly linked to events in the nucleus [261]. Many proteins can localize to both the nucleus and the cell membrane with this localization being dependent on cell density and differentiation status. When found in the nucleus they modulate transcription, a function that is abrogated when upon cell confluency they translocate to the plasma membrane. For instance, Cask concentrates in the nuclei of undifferentiated cells where it positively regulates cell proliferation, but upon acquirement of mature cell-cell contacts it translocates to the membrane [262]. ZO-2 mainly localizes to the nuclei of sub-confluent cells, but accumulates at TJ in confluent cell cultures [263]. In addition, upon maturation of cell-cell contacts, the transcription regulator ZONAB accumulates at the cell membrane interacting with regulators of cell-cell adhesion, including ZO-1 and RalA [264-266]. This translocation abolishes its transcriptional regulation and results in the loss of its proliferation-promoting activities. Beta-catenin also positively regulates cell proliferation through its nuclear localisation in sub-confluent cells [267].

2 Materials and Methods

2.1 Buffers and solutions

Cell freezing medium	50% FBS (v/v), 10% DMSO (v/v), 40% culture medium (v/v)
IP Lysis buffer	50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton-X-100, 10% (v/v) glycerol, 2 mM EDTA, 25 mM NaF, and 2 mM NaH_2PO4, containing a protease inhibitor cocktail (Sigma, 1:100) and phosphatase inhibitor cocktails 1 and 2 (added fresh) (Sigma, both 1:100)
SDS-PAGE sample buffer	4x NuPAGE® LDS sample buffer (Invitrogen) and 10x NuPAGE® sample reducing agent (Invitrogen)
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4
PBST	0.1% Tween in PBS (v/v)
Western blocking buffer	5% non-fat milk powder in PBST (w/v)
Western stripping buffer	0.2 M Glycine, 1% SDS (v/v), pH 2.5 (titrated by addition of HCl)
IF blocking buffer	1% BSA in PBS (w/v)
Cell permeabilisation solution	0.5% Triton X-100 in PBS (v/v)
Crystal violet fix solution	3.7% of 100% w/v formaldehyde in H_2O (v/v)
Crystal violet stain solution	0.2% crystal violet in H_2O (w/v)
Crystal violet extract solution	0.2% Triton X-100 in PBS (v/v)
SRB fix solution	50% TCA in H_2O (v/v)
SRB stain solution	Sulforhodamine B (SRB) solution 0.4% in 1% acetic acid (w/v)
SRB extract solution	10 mM Tris base

2.2 Cell clones

In the experiments that were performed, MDCK II cell clones were used. Clones with reduced levels of β2-syntrophin and clones that overexpressing β2-syntrophin were both available in the lab. To deplete β2-syntrophin from MDCK II cells, we used a dox-inducible RNAi system (Figure 6; [260, 268]). In this system, the target sequence is under the control of an H1 promoter and a downstream Tet operator (TO). Additionally, the plasmid constitutively expresses a Tet repressor under the control of an actin promoter. Molecules of the repressor normally bind the TO sequence and do not allow the expression of the target sequence that is located downstream of the TO. However, when doxycycline is present, it binds the Tet repressor resulting in its sequestration and removal from TO, thus allowing the H1 promoter to drive the expression of the target sequence. Plasmids carrying two different short hairpin RNA (shRNA) sequences that specifically targeted β2-syntrophin mRNA were used, with both leading to reduction of the respective protein levels (Table 1). The canine genome is less well annotated than that of human or mouse, and therefore the β2-syntrophin gene in MDCK II cells was sequenced to verify available sequences on PubMed. Regions of the gene whose sequences matched that on PubMed were used to design the β2-syntrophin-specific short hairpin RNA (shRNA) sequences using the online siDESIGN® Center from Thermo Scientific. The sequences were also put through BLAST analysis to check they were specific to their targets. After the design of the shRNA oligonucleotides the procedure that had been followed was: cloning of these into the RNAi shuttle vector, screening for their knockdown ability by transient transfection in HEK293T cells, further sub-cloning into the pA'-TO dox-inducible RNAi vector [260, 268] and final transfection of MDCK II cells with the RNAi constructs. MDCK II cell clones expressing the two different shRNA sequences were named as shRNA #1 and shRNA #2. In addition to these another MDCK II clone, called Scrambled shRNA which does not target any sequences in the published dog genome, was generated that expressed a dox-inducible Scrambled shRNA sequence, having pRetro-Puro as the backbone vector. It acted as negative control, testing for potential effects from doxycycline treatment and activation of the RNAi machinery.

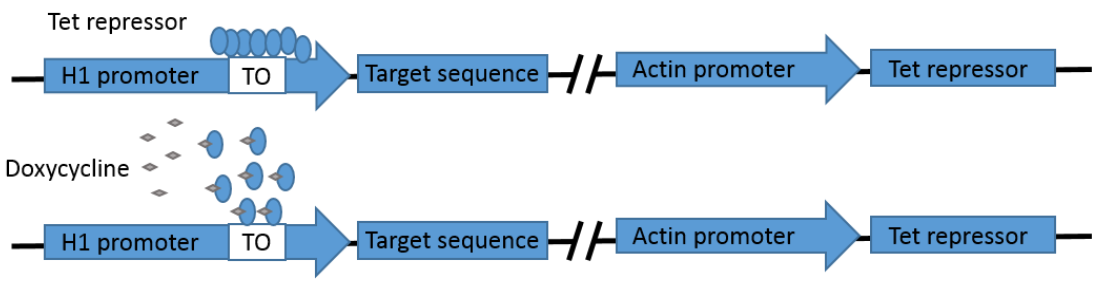


Figure 6: Schematic representation of the Dox-inducible RNAi system. Addition of dox relieves repressor binding and mediates shRNA expression. Further details are written in the text above.

A clone overexpressing β2-syntrophin was used in subsequent experiments to test the respective cell behavior with elevated protein levels. The procedure had been the following: mutation of GFP-Syn-FL to render it resistant to β2-syntrophin shRNA #1 and subsequent sub-cloning of Syn-FL into the pcDNA4/TO vector to generate β2-syntrophin-rescue, which was transfected to shRNA #1 MDCK II cells. These constructs and clones containing them had been used extensively in the lab [103].

Table 1: Details of β2-syntrophin shRNA oligos

Oligo name	**Oligo sequences**	**Starting base number on gene**	**Predicted species reactivity**
β2-syntrophin shRNA #1	Sense: GATCCCGCAGCATATTGTACCGCTATT CAAGAGATAGCGGTACAATATGCTGC TTTTTGGAAA Antisense: AGCTTTTCCAAAAAGCAGCATATTGTA CCGCTATCTCTTGAATAGCGGTACAAT ATGCTGCGG	1514	Human, dog, mouse
β2-syntrophin shRNA #2	Sense: GATCCCGACTGTATGCCGTGGACAATT CAAGAGATTGTCCACGGCATACAGTCT TTTTGGAAA Antisense: AGCTTTTCCAAAAAGACTGTATGCCGT GGACAATCTCTTGAATTGTCCACGGCA TACAGTCGG	1165	Human and dog Not mouse

2.3 Cell culture techniques

2.3.1 Cell maintenance

All cells were cultured at 37 °C with 5% CO_2. Dox-inducible MDCK II cell clones were used expressing two different shRNA sequences targeting β2-syntrohin, named as shRNA #1 and shRNA #2, and a Scrambled sequence as negative control. A rescue clone was also used, generated after the sub-cloning of the full-length β2-syntrophin into the shRNA #1 clone. The MDCK II cell clones were maintained in low glucose DMEM (Dulbecco's modified eagle medium, Gibco) supplemented with 10% (v/v) FBS (fetal bovine serum, Gibco), 1% (v/v) penicillin-streptavidin (Gibco) as antibiotics and 400 µg/ml G418 (Sigma) as selection marker. Serum-free medium was used for some experiments. The rescue clone was grown in the same medium with the addition of 0.25 mg/ml Zeocin (Invitrogen) as a second selection marker.

For passaging, the cells were washed twice with PBS and detached from the flask bottom after incubation with trypsin at 37 °C. Trypsin was then inactivated with the addition of medium and the cells were pelleted at 1200 rpm for 5 min. The cells were then plated at a lower density. To conduct the experiments, cell numbers were determined using a cell counter (Beckman Coulter).

2.3.2 Cell freezing

Cells from a confluent flask were pelleted by centrifugation at 1200 rpm for 5 min and then resuspended in 3 ml of cell freezing medium. They were separated into 3 cryovials each containing 1 ml and stored at -80 °C. For longer storage, the vials were transferred to tanks of liquid nitrogen.

2.4 Protein detection techniques

2.4.1 Cell lysis

Protein was harvested from cultured cells by washing once with ice-cold PBS, incubating the cells with lysis buffer for 10 min on ice while in agitation, scraping the cells and collecting the supernatant after centrifugation at 13,000 rpm for 10 min at 4 °C.

2.4.2 Determination of protein concentration

Concentrations of lysates were determined using the Advanced Protein Assay reagent (Cytoskeleton Inc.). The absorption was measured at a wavelength of 595 nm on a UV/Visible spectrophotometer.

2.4.3 SDS-PAGE and immunoblotting

An appropriate volume of protein lysate corresponding to 25 µg of protein was prepared adding ddH_2O and SDS-PAGE sample buffer to a concentration of 1x. The samples were heated at 70 °C for 10 min prior to gel loading. They were then run on NuPAGE® gels (Invitrogen) of the appropriate percentage and/or gradient of acrylamide, using the XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen). Along the samples, a Blue wide range molecular weight marker (Geneflow Ltd) was loaded. Proteins were transferred from the gels onto PVDF membranes (Immobilon-P, Millipore) using the XCell SureLock™ transfer system. After transfer, membranes were incubated with Western blocking buffer for at least 1 h at RT with agitation. They were then incubated with primary antibody, diluted in Western blocking buffer, for 1 h with agitation at RT or O/N at 4 °C depending on the antibody type. Membranes were washed three times for 10 min with PBST and then incubated with the relevant HRP-conjugated secondary antibody (GE Healthcare) for 1 h at RT with agitation. Immunoblots were visualized with ECL (Perkin-Elmer). To reprobe the membranes, these were stripped by incubation with Western stripping buffer for 25 min, then incubation with Western blocking buffer for at least 1 h and finally incubation with the appropriate antibodies as previously described.

Table 2: Antibodies used for immunoblotting

Antibody	Species	Manufacturer	Dilution
Syntrophin [1351]	mouse	abcam	1:4000
α-tubulin [DM1A]	mouse	abcam	1:2000
anti-mouse-HRP	sheep	Amersham	1:10000

2.5 Cell migration assay (Scratch Assay)

Cells were grown in T75 flasks either in the presence or absence of doxycycline (+/- Dox) for 3 days, with a change of medium on the 2nd day, and then plated in 12-well plates at a density of 4 x 10^5 cells per well. The next day a scratch was created using a P100 filter tip on a confluent cell monolayer. After washes with PBS to discard any cell leftovers from the cell-free area the respective type of medium was added. Images were taken at 0, 16 and 20 hours post-scratching to record the closure of the wound on a Zeiss Axiovert 25 inverted microscope with a 5x objective lens. Each cell clone treated with or without doxycycline (+/- Dox) was plated in 4 replicates in the 12-well plate and 4 pictures were taken for each replicate yielding in total 16 photos. 5 lines were drawn on the bottom of the plates to mark the area to be photographed, which was right above each line to ensure the same regions were imaged at each time point. The wound area from each picture was then calculated using the ImageJ software (NIH) by manually annotating the area of the scratches in each image.

For immunofluorescence: shRNA #1 and Scrambled shRNA MDCK II cells were plated on glass coverslips, placed inside 6-well plates, and grown for 3 days, changing the medium on Day 2, either in the presence or absence of doxycycline (+/- Dox) at a density of 4 x 10^5 cells per coverslip. On the second day the medium was switched to serum-free and on the third day a scratch was made on the confluent cell monolayer that was formed with a P2 filter tip. After washes with PBS to discard any semi-detached cells the respective type of medium with serum was added. The coverslips were fixed by incubation with 3.7% formaldehyde for 20 min at RT with agitation at 0 and 8 hours after the scratch. These were then stained via immunofluorescence accordingly.

2.6 Cell proliferation assays

2.6.1 SRB staining

Cells were grown with or without doxycycline (+/- Dox) for 3 days, changing the medium on the 2nd day, before plating at a density of 1000 cells/well in 96-well plates. At 0, 12, 24 and 48 hours after plating, the cells were fixed by incubation with ice-cold SRB fix solution for 1 h at 4 °C, then the plates were washed 5 times with H_2O and then stained by incubation with sulforhodamine B (SRB) stain solution for 10 min at RT. The dye was extracted with 10 mM Tris base and the absorption was measured at 490 and 560 nm on an

automated plate reader, subtracting the first values from the second for cell growth quantification.

2.6.2 Crystal violet staining

Cells were grown with or without doxycycline (+/- Dox) for 3 days, with a change of medium on Day 2, prior to plating at a density of 5000 cells/well in 12-well plates. The cells were fixed with 3.7% formaldehyde solution for 15 min, washed with PBS and stained with crystal violet solution for 30 min at 0, 12, 24, 48, 72 and 96 hours after plating. The dye was extracted with the addition of 400 µl of 0.2% Triton solution with agitation for 30 min. 100 µl from each well were then added into a 96-well plate to measure the absorption at 595 nm on an automated plate reader.

2.7 Cell imaging techniques

2.7.1 Microscope systems

Low light: The widefield Low light system is a high resolution imaging system that utilizes an Andor iXon 88 camera and CRI Muance 500-1000 nm spectrophotometer. Excitation can be set at 406, 436, 488, 512, 555 and 647 nm.

Spinning disc: The spinning disc inverted microscope performs real time confocal imaging (18-20 frames per second). Excitation via laser can be set at 406, 488, 555 and 647 nm.

Immunofluorescence images were taken either on the Low light or Spinning disc microscope system. In both systems, images were taken with a x100 oil immersion objective lens and were captured using Metamorph (Molecular Devices) software.

2.7.2 Immunofluorescence

After formaldehyde fixation, the coverslips were washed twice with PBS and the cells were permeabilized by incubation with 0.5% Triton solution for 4 min at RT and then washed three times with PBS. Blocking was performed with IF blocking buffer for 1 h at RT

and after two washes with PBS, 200 µl of primary antibody solution diluted in IF blocking buffer was used to cover the coverslips for 1 h at RT. After washing three times with PBS 200 µl of secondary antibody solution diluted in IF blocking buffer was added for 1 h at RT. After washing four times with PBS, the coverslips were mounted on slides with mounting solution containing DAPI and left covered O/N. The next day the slides were sealed with nail varnish. Note: when conjugated antibodies were used only one incubation of 1 h was performed after the blocking step. In the current study, conjugated antibodies were applied for immunofluorescence of focal adhesions and actin cytoskeleton.

Table 3: Antibodies used for immunofluorescence

Antibody	Species	Manufacturer	Dilution
Vinculin-FITC [hVIN1]	mouse	Sigma	1:500
Alexa Fluor® 568 Phalloidin	N/A	Life Technologies	1:100

2.7.3 Measuring the focal adhesion area

Images of cells immunostained with vinculin-FITCwere taken along a wound. Starting from the bottom of the wound on its right side and moving downwards 10 pictures each with 8 stacks were taken with the 100x objective lens with both the Low light and the Spinning disc microscope. Only photos from the Spinning disc microscope are shown here as the image quality was significantly better than for the low light microscope (see “Results” section). The images were then imported into ImageJ software: using the actin staining, the most in-focus focal adhesion plane was selected, background was subtracted with a rolling ball algorithm (size set to 50 pixels) and the threshold was adjusted to make focal adhesions black on a white background by selecting the order apply/auto/set (Figure 7). For measuring then the area of the focal adhesions in μm^2 the ImageJ command “Analyze particles” was used. To initially set the upper and lower limits for particles’ size, a number of representative focal adhesions’ areas was measured using the wand tool, yielding 0-3 μm^2 as the desired size for a particle in order to be recognized as a focal adhesion.

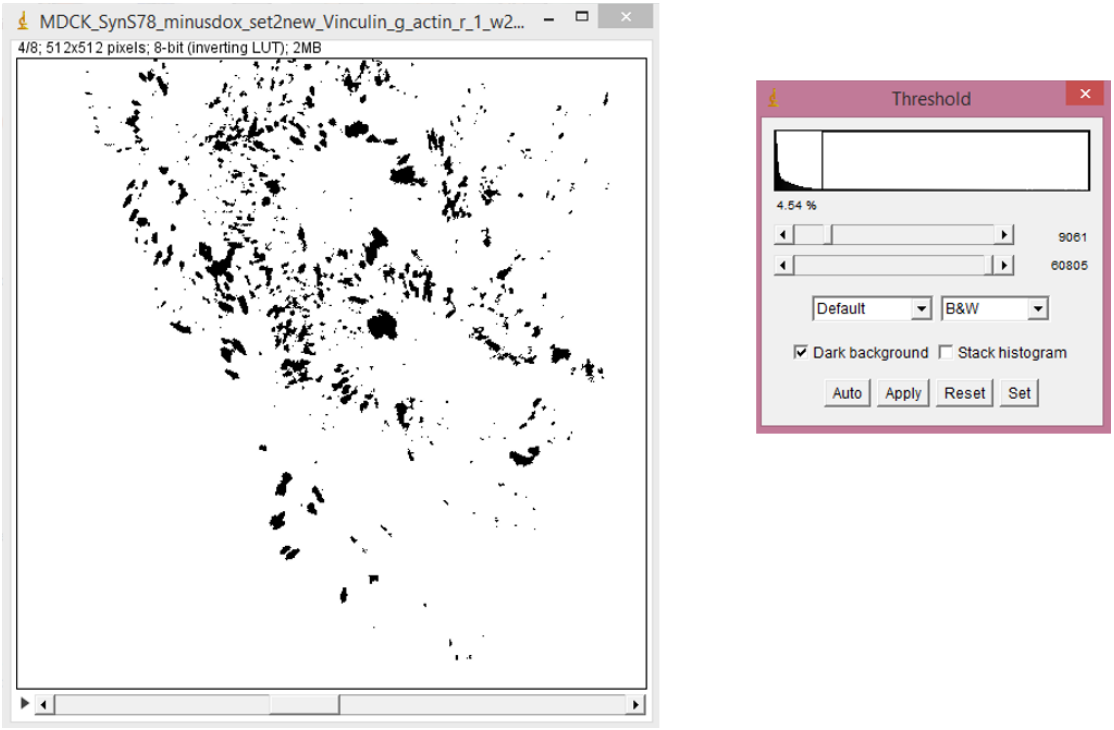


Figure 7: A thresholded image on ImageJ software (see text for details).

3 Results

3.1 shRNA targeting of β2-syntrophin decreases its protein levels

Clones expressing two different shRNA sequences against β2-syntrophin were screened for their level of protein knockdown following dox treatment (using the system described previously, see Figure 6). Following dox treatment, β2-syntrophin was successfully down-regulated using both β2-syntrophin shRNA #1 or #2 (Figure 8). There was less β2-syntrophin protein over time for both β2-syntrophin shRNA #1 and shRNA #2 compared to Scrambled shRNA, with the shRNA #1 displaying the greatest knockdown. The clones were tested in parallel for the time point that would give the maximum knockdown. For this purpose, MDCK II cells were harvested at 0, 24, 48, 72, 96 and 120 hours after doxycycline treatment and compared to the respective cell populations that remained untreated. The time point for the greatest knockdown was the 72 hours. At the same time, Scrambled shRNA was validated as a negative control, since β2-syntrophin levels did not change over time with the addition of dox (there was a small fluctuation in β2-syntrophin levels only in the minus dox controls throughout the 120 hours).

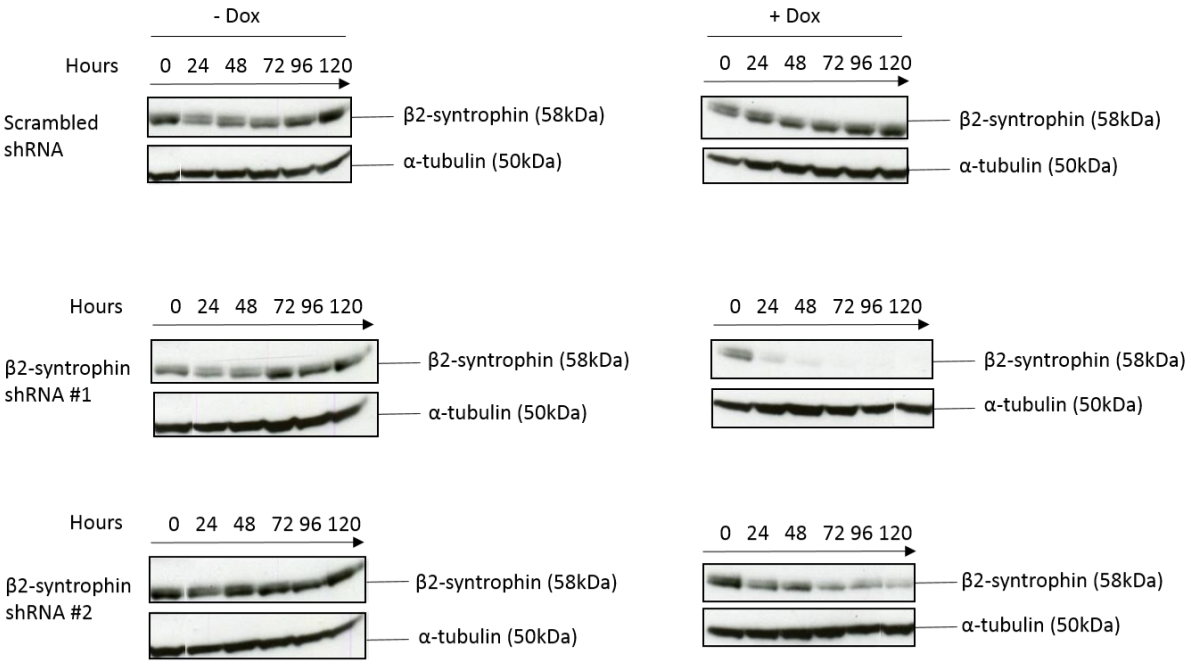
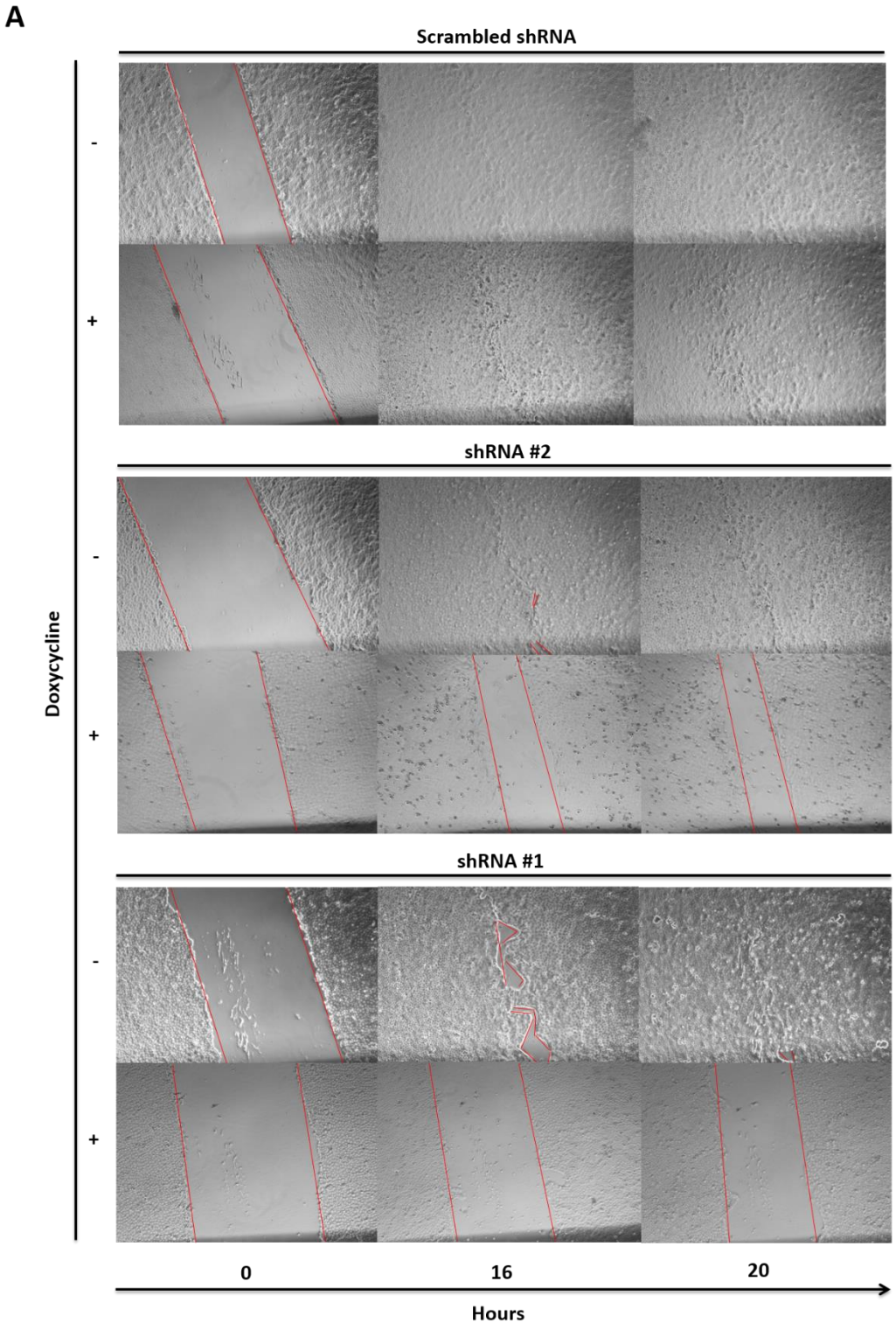


Figure 8: MDCK II clones with dox-inducible β2-syntrophin knockdown. β2-syntrophin shRNA #1, #2 and Scrambled shRNA clones were grown in the absence or presence of doxycycline (-/+ Dox) and were harvested after 0, 24, 48, 72, 96 and 120 hours. β2-syntrophin levels were detected by immunoblotting the corresponding lysates. α-tubulin is shown as a loading control. This time course was tested once.

3.2 β2-syntrophin promotes the migration of MDCK II cells

As described in the introduction, polarity proteins can regulate cell migration. In order to investigate whether β2-syntrophin also does this, we utilised a wound healing assay to look at migration in cells with reduced levels of this protein. Based on the dox treatment test (Figure 8), two MDCK II cells bearing two different shRNA sequences that target β2-syntrophin were grown in the presence or absence of dox for 3 days. On the 4th day a scratch was made on a confluent cell monolayer and images were taken at 0, 16 and 20 hours after scratching to capture the closure of the wound over time. The time points were selected, based on their more profound effect, from an initial wound healing assay that included 4, 8, 12, 16, 20 and 24 hours as time points. Scrambled shRNA cells were treated in the same way to act as a control for the experiment. The quantification of wound areas showed that cells with reduced β2-syntrophin did not reach a complete closure of the wound within 20 hours after scratch formation (Figure 9). This means that β2-syntrophin regulates cell motility and its presence is necessary for optimal migration of MDCK II cells.


A
Scrambled shRNA
shRNA #2
shRNA #1
Doxycycline
-
+
-
+
-
+
0
16
20
Hours

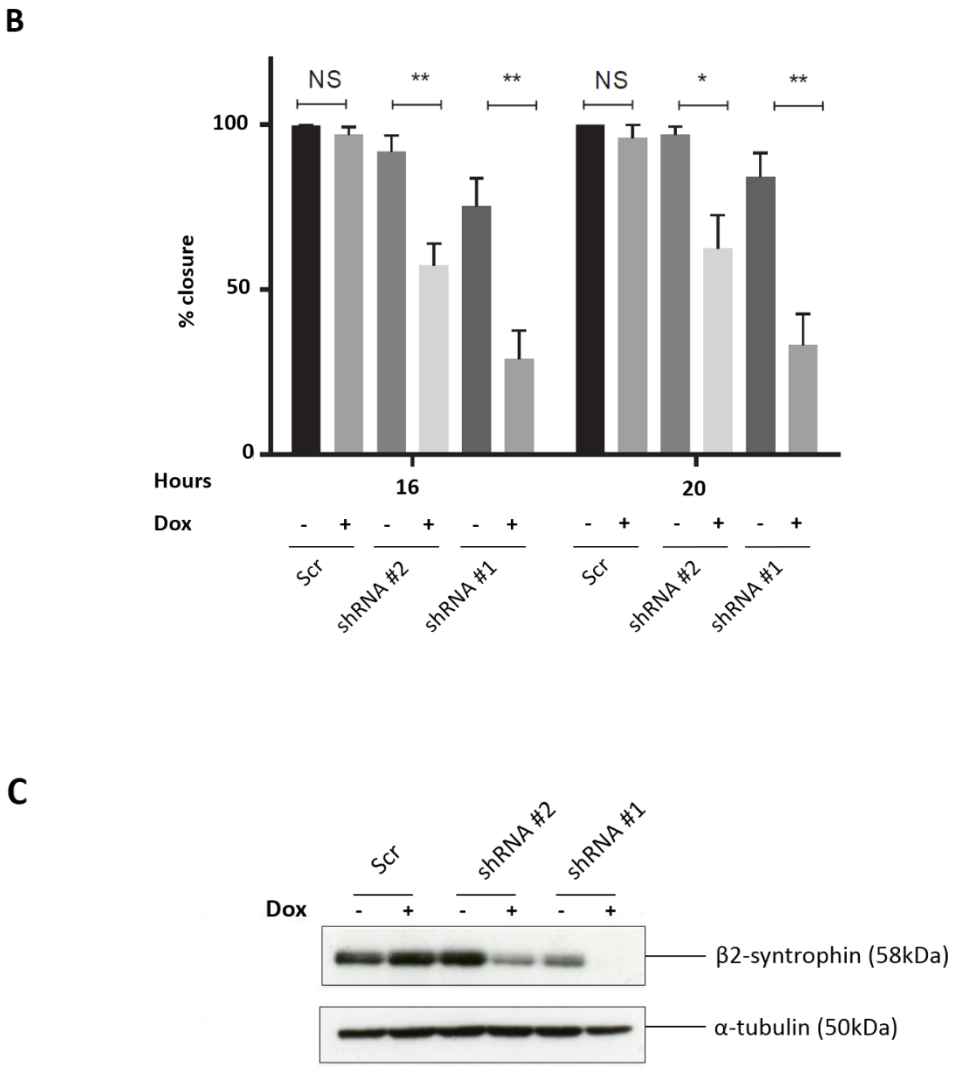
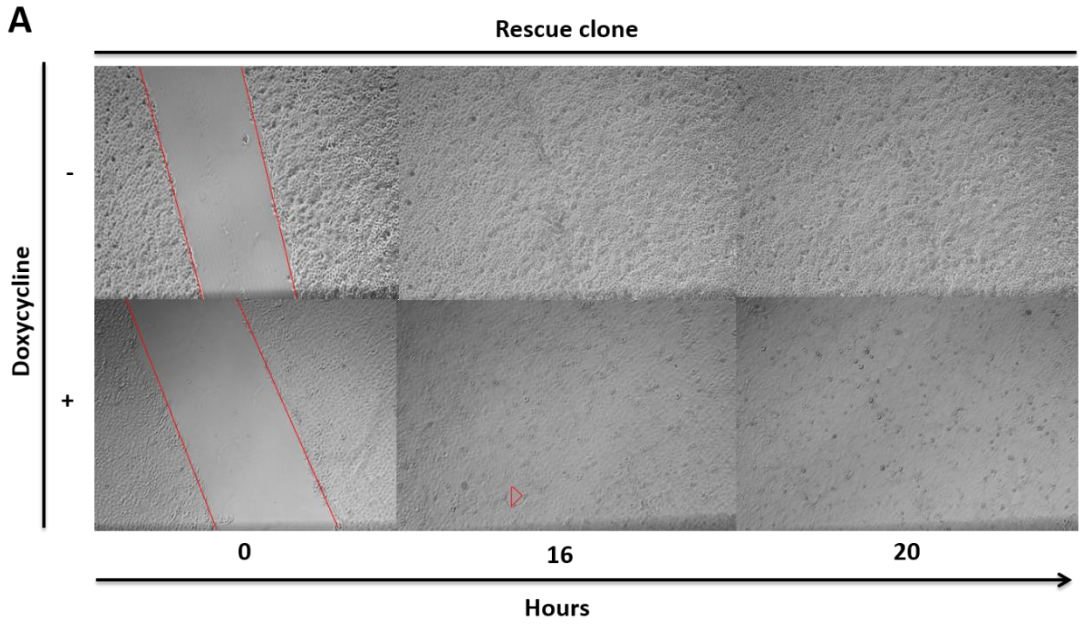
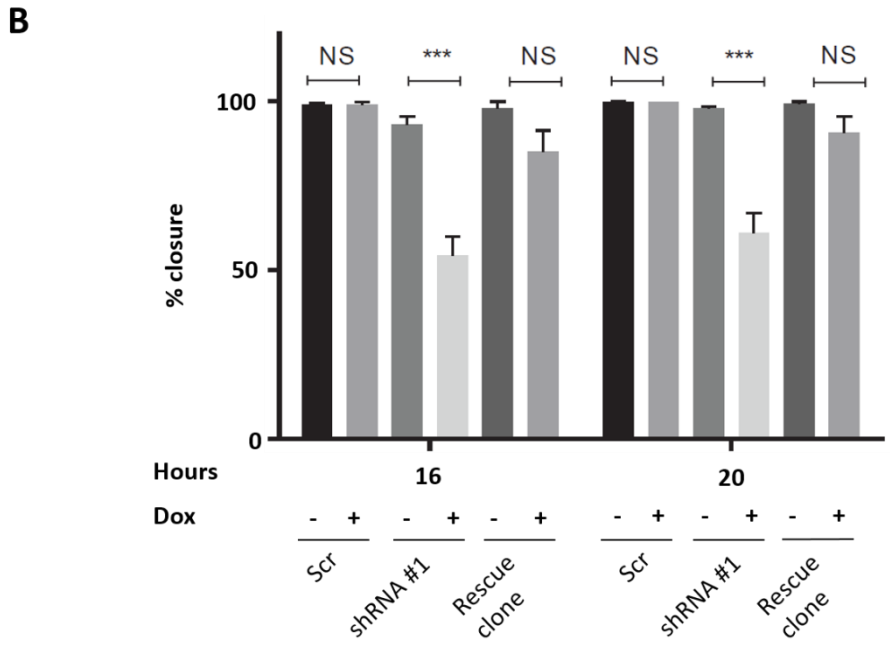


Figure 9: Knockdown of β2-syntrophin reduces cell migration. (A) MDCK II cells expressing either a dox-inducible Scrambled shRNA, or two different shRNA sequences targeting β2-syntrophin were scratched in the presence or absence of doxycycline (+/- Dox). Pictures were taken at the indicated time points post-scratching. (B) Quantification of wound areas. The mean was calculated from four independent experiments. Error bars represent standard error (SE). The statistical analysis was done via GraphPad Prism 6 software and the students-t test (NS = non-significant; * = p-value ≤ 0.05; ** = p-value ≤ 0.01; GraphPad Prism 6 software). (C) Western blot analysis of samples from (A-B). The blot is one from four independent experiments.

3.3 Overexpression of β2-syntrophin rescues the migration defect observed in β2-syntrophin depleted MDCK II cells

To test whether this is an on-target effect, we tried to rescue the migration defect by overexpression of β2-syntrophin. A wound healing assay was performed with shRNA #1 cells along with the Scrambled shRNA and the rescue clone cells that were expressing at the same time the shRNA #1 and the full-length β2-syntrophin (which is resistant to shRNA knockdown). After quantifying the wound areas, it was shown that the rescue clone cells reached complete closure of the wound the same time that Scrambled shRNA cells did (Figure 10). This meant that increased levels of β2-syntrophin restored the migration potential of MDCK II cells and indicated firstly that only reduced levels of the protein disrupt cell migration and secondly the absence of off-target shRNA effects on the observed phenotype.





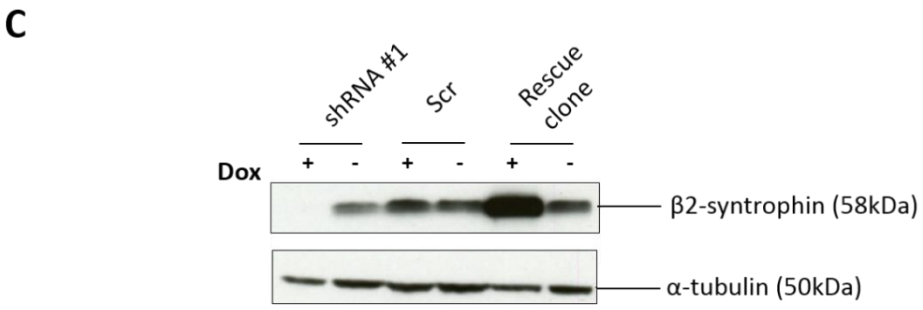


Figure 10: Rescue of β2-syntrophin knockdown restores the migration potential of MDCK II cells. (A) MDCK II cells expressing β2-syntrophin resistant to β2-syntrophin shRNA #1 clone cells were scratched in the presence or absence of dox (+/- Dox) and images were taken at the indicated time points. Pictures of shRNA #1 and Scrambled shRNA clones can be seen in Figure 9, but the quantification of wound areas was done using photos taken from experiments corresponding to this part of the study. (B) Quantification of wound areas. The mean was calculated from four independent experiments. Error bars represent standard error (SE). The statistical analysis was done via GraphPad Prism 6 software and the students-t test (NS = non-significant; *** = p-value ≤ 0.001). (C) Representative western blot analysis of lysates from (A-B). The blot is one from four independent experiments.

3.4 Downregulation of β2-syntrophin does not significantly alter the size of focal adhesions in leading edge cells

After observing that downregulation of β2-syntrophin reduces cell migration, we wanted to investigate the mechanism by which this occurs. The hypothesis that we worked on was that β2-syntrophin may regulate focal adhesions (FA) thus affecting the movement of cells towards the wounded area. Despite not knowing whether β2-syntrophin itself localizes in focal adhesions, dystroglycan that interacts with β2-syntrophin in the context of the DGC complex described in the introduction has been found to localize in FA [269-271]. Furthermore, manipulation of dystroglycan changes the levels of FA components [272] and affects the size and number of adhesions [271]. Additionally, syntrophins specifically concentrate at the trailing edge of migrating C2 myoblasts [273] where they could possibly have a role in modulating focal adhesions. The study in our lab searching for interactors of Tiam1 also checked for interactors of its paralog STEF, and β2-syntrophin was one of them. From previous studies in our lab, STEF has been shown to be necessary for proper migration of P1 cells via regulating the turnover of focal adhesions [268]. In this way, β2-Syntrophin could regulate FA through its interaction with STEF. In addition, Tiam1 has been shown to regulate FA dynamics downstream of Src [180]. So it could be possible that through

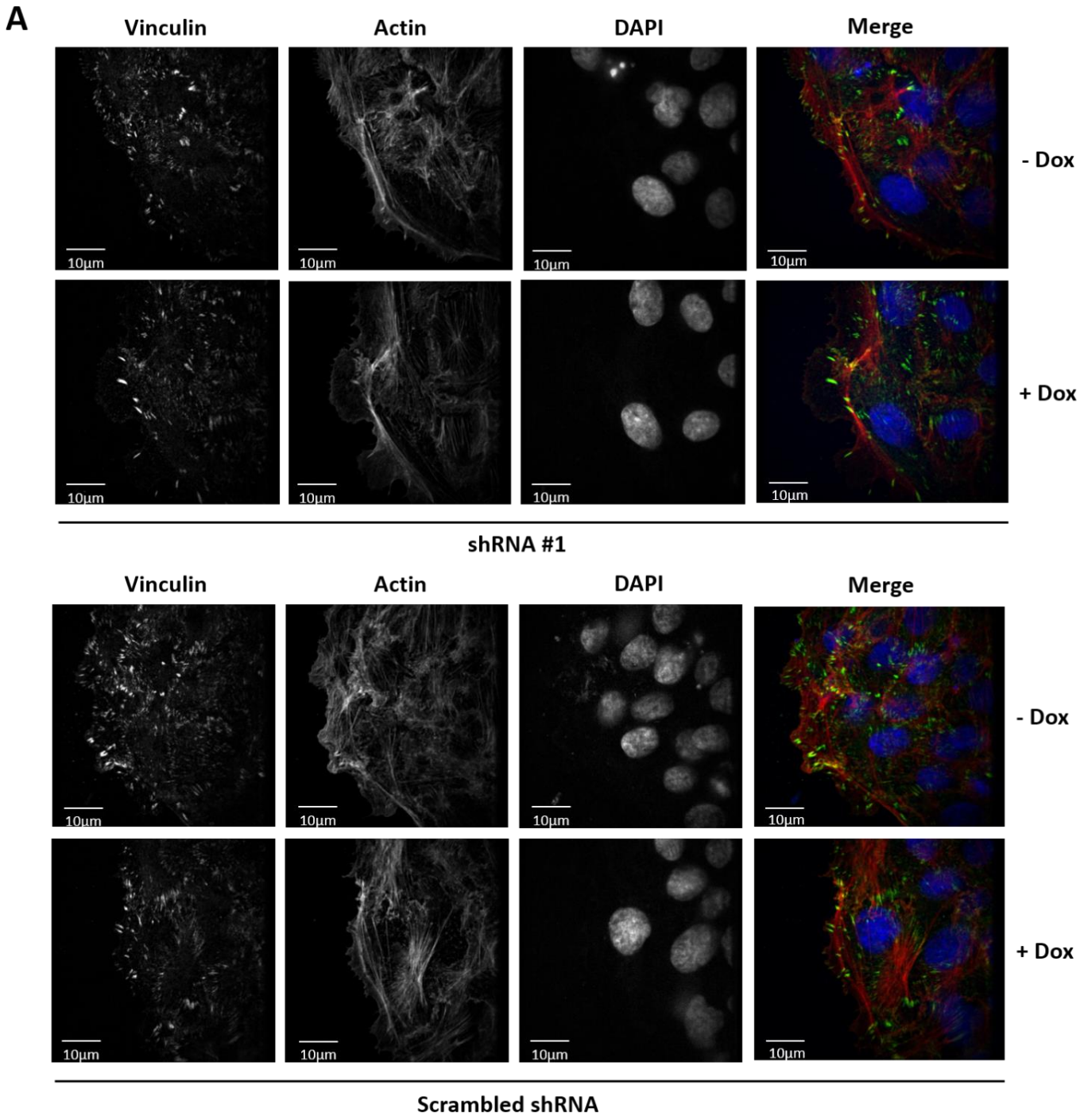
association with β2-syntrophin, Tiam1/STEF-mediated Rac1 activation controls FA dynamics which is vital for optimal cell migration.

Focal adhesions are integral to cell migration. They are continuously formed and disassembled as a cell moves along the ECM. Assembly of these adhesions is required to serve as traction points between the cell and the ECM, allowing the cell body to move forward. Equally important is the disassembly of these adhesions which allows net translocation of the cell in the direction of movement.

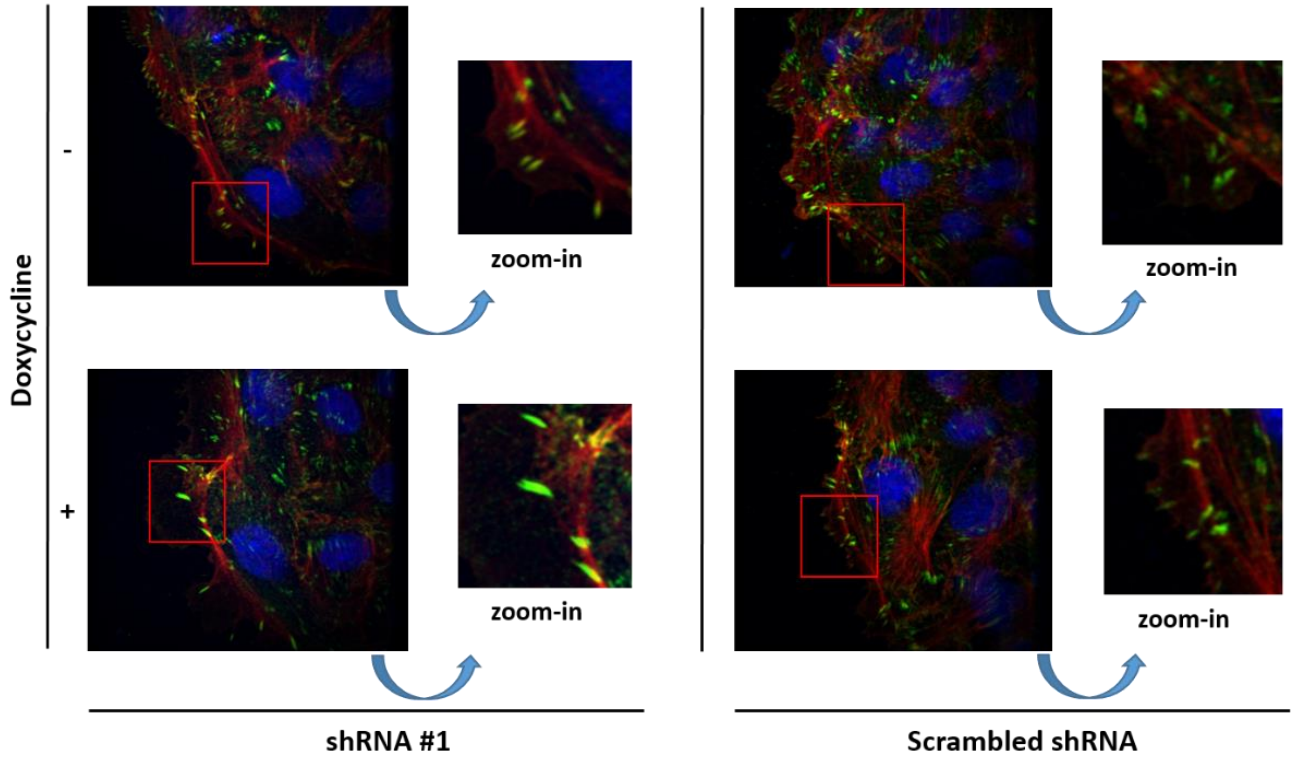
To study focal adhesions in migrating cells, immunofluorescence microscopy of wounded monolayers was utilized. The shRNA #1 cell clone was used to investigate the migratory defect further as this cell clone showed a more pronounced defect in cell migration than the shRNA #2 cell clone (consistent with a better knockdown of β2-syntrophin at the protein level). shRNA #1 and Scrambled shRNA cells were plated on coverslips in the presence or absence of doxycycline (+/- Dox) for 3 days. On the 3rd day when a confluent monolayer was reached, a scratch was created with a pipette tip to induce cell migration into the resulting wound. Cells were fixed with 3.7% formaldehyde at 8 hours post wounding, as this was sufficient time for leading edge cells to acquire a migratory phenotype, with a distinct lamellipodium visible at the leading edge. Cells were immunostained with anti-vinculin-FITC, since vinculin is a well-known focal adhesion marker, and rhodamine-phalloidin for staining of the actin cytoskeleton. The focal adhesions were examined in the leading edge cells, migrating into the wound. Intriguingly, leading edge β2-syntrophin downregulated cells (+ Dox) appeared to have focal adhesions with increased size compared to control shRNA #1 cells (- Dox). This observed focal adhesion phenotype was quantified. Images of cells immunostained with vinculin-FITC were taken along the wound, as described in detail in the “Materials and Methods” section. Background was subtracted from these images using the rolling ball algorithm in ImageJ software and focal adhesions were categorized into groups of different sizes to yield a frequency distribution. The groups of focal adhesion sizes were: 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75 and 3 μm^2. Focal adhesions having a size equal or less than 3 μm^2 were accordingly put in these categories with the result depicted in Figure 11 for both shRNA #1 and Scrambled shRNA cells, treated or untreated with Dox. Data was obtained from ten pictures for each cell type-condition from three independent experiments. The shRNA #1 clone treated with dox showed focal adhesions that were larger than those in the same clone when left untreated. However, after a student’s t-test this observation was not found to be statistically significant. The same analysis was done after changing the bin sizes to 0-1, 1-2, 2-3 and 3- >3

and taking into account only focal adhesions at the very edge of the wound. The difference between β2-syntrophin downregulated cells (shRNA #1 +Dox) and control cells (shRNA #1 – Dox) was again statistically insignificant (see “Discussion” section for suggestions for further study).

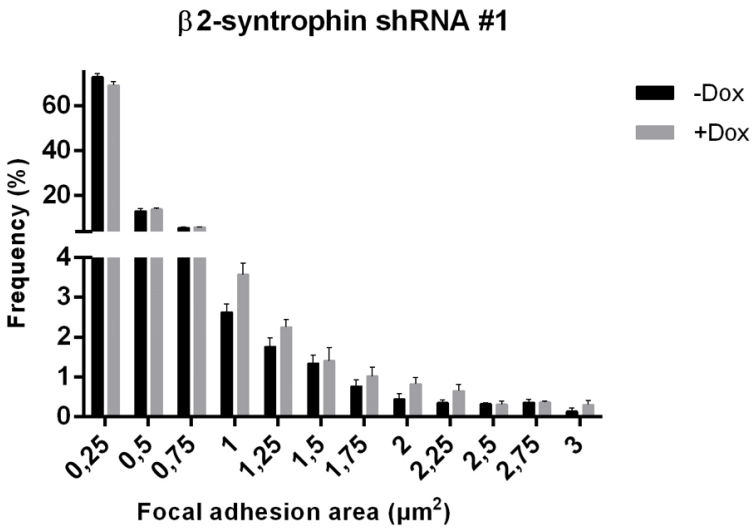
Focal adhesions connect the extracellular matrix to the actin cytoskeleton. At focal adhesions, filaments of the actin cytoskeleton bundle into stress fibres. Staining of the actin cytoskeleton and focal adhesions with rhodamine-phalloidin and anti-vinculin-FITC, respectively, allowed visualization of actin associated with focal adhesion. In control cells (-Dox), actin cables appeared thin and traversed the cell. In β2-syntrophin downregulated cells (+ Dox), however, thicker actin bundles were seen associated with the larger focal adhesions, and these did not traverse the cell, appearing instead to bundle at the cell cortex (Figure 11. A).

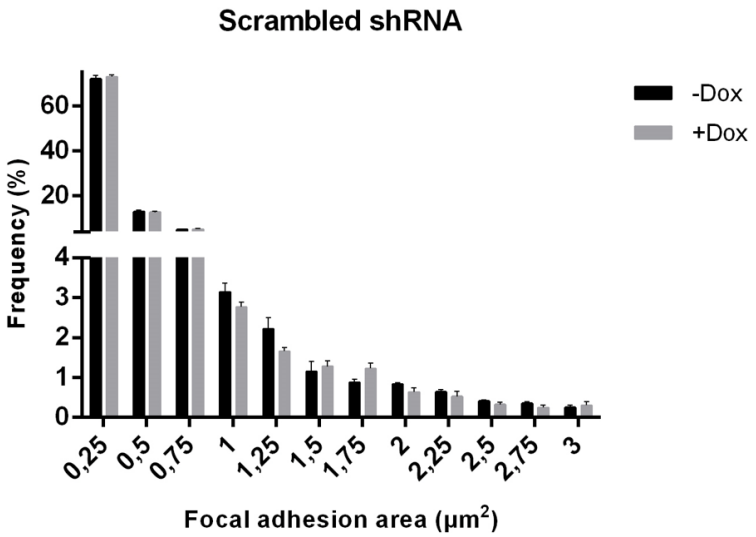

A
Vinculin
Actin
DAPI
Merge
- Dox
+ Dox
10µm
shRNA #1
Scrambled shRNA

B


Doxycycline
-
+
zoom-in
zoom-in
zoom-in
zoom-in
shRNA #1
Scrambled shRNA

C


β2-syntrophin shRNA #1
-Dox
+Dox
Frequency (%)
60
40
20
4
3
2
1
0
0,25
0,5
0,75
1
1,25
1,5
1,75
2
2,25
2,5
2,75
3
Focal adhesion area (μm²)


Scrambled shRNA
-Dox
+Dox
Frequency (%)
60
40
20
4
3
2
1
0
0,25
0,5
0,75
1
1,25
1,5
1,75
2
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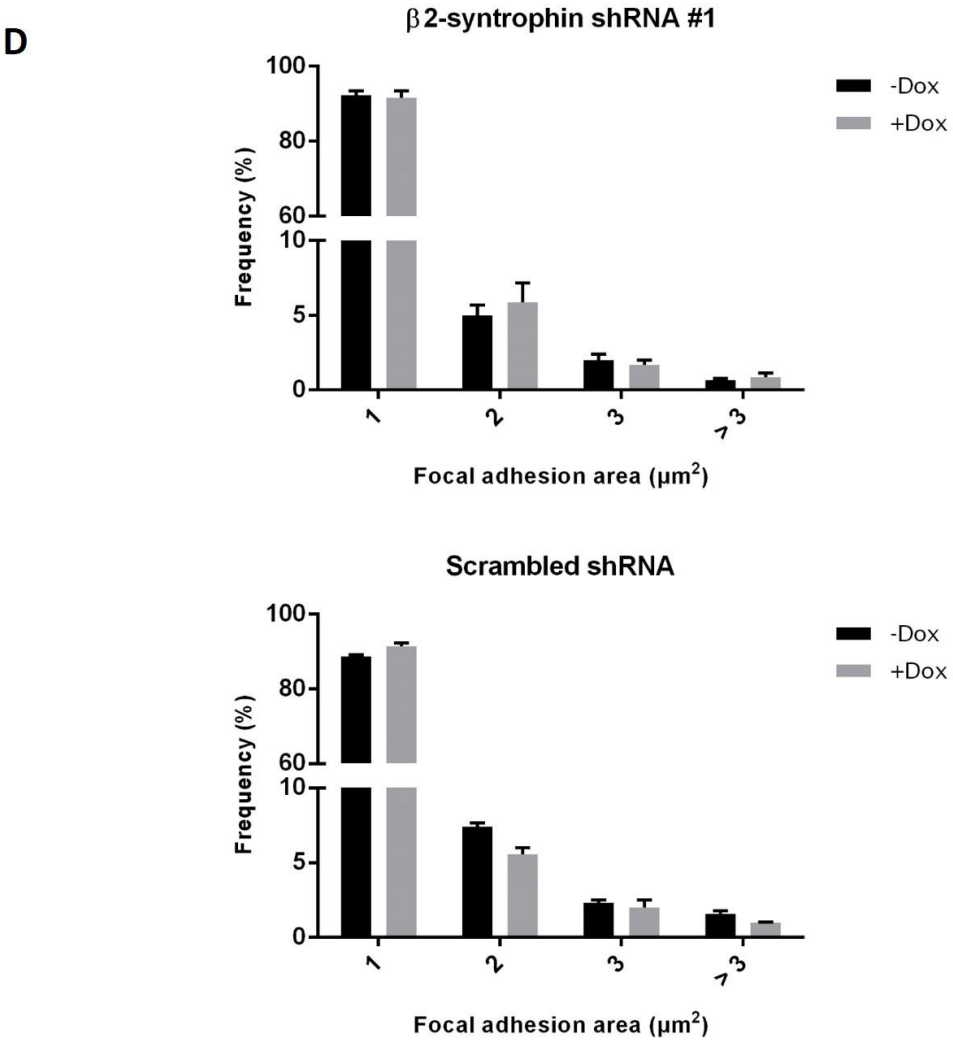
2,5
2,75
3
Focal adhesion area (μm²)



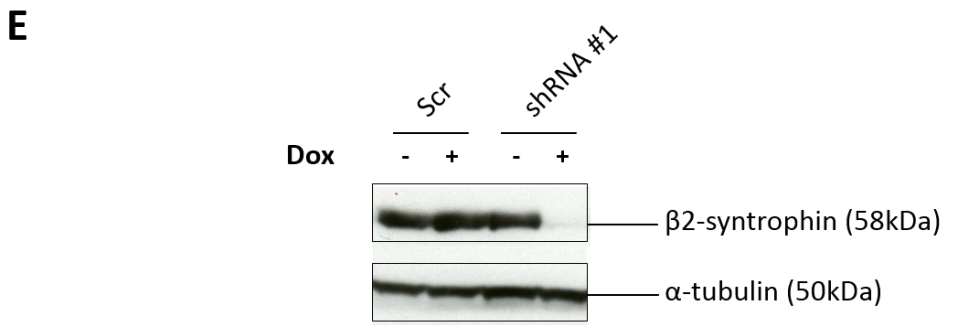


Figure 11: Knockdown of β2-syntrophin does not affect the FA size of leading edge cells. (A) Cells expressing either an inducible Scrambled shRNA, or an shRNA sequence targeting β2-syntrophin were scratched in the presence or absence of dox (+/- Dox) and immunostained with anti-vinculin-FITC (→ focal adhesions) and phalloidin (→ actin). (B) Pictures from (A) are shown after zooming-in. (C) Frequency distribution of focal adhesions in cells located at the edge of the wound. (D) Same analysis as (C), but after changing the bin sizes and taking into account only focal adhesions at the very edge of the wound. For both (C) and (D) the mean was calculated from three independent experiments. Error bars represent standard error (SE). The statistical analysis was done via GraphPad Prism 6 software and the students-t test (student's t test → NS). (E) Representative western blot analysis from (A-D) lysates. The blot is one from three independent experiments.

3.4.1 Selection of the microscope system

In order to decide which microscope system to use for analysing focal adhesions that underwent immunofluorescence, pictures were taken with both the Low Light and the Spinning disc microscope. Initially, Scrambled MDCK II cells (control cells) were grown in the absence of doxycycline (- Dox) for 3 days on glass coverslips and then scratched to induce cell migration. After 8 hours the cells were fixed with 3.7% formaldehyde and then focal adhesions and the actin cytoskeleton were stained with anti-vinculin-FITC and rhodamine-phalloidin, respectively. Then the pictures taken from the two microscope systems were compared (Figure 12). Focal adhesion complexes were more discrete with the Spinning disc rather than the Low Light microscope and the pictures had a lower background. Likewise the background with actin staining was also reduced. With the Low Light, actin seemed to be found in the cell membrane while the staining under the Spinning disc was indicative of actin filaments, stress fibers and lamellipodia as it would be expected. The analysis was thus conducted upon the photos taken with the Spinning disc microscope due to a better displayed picture.

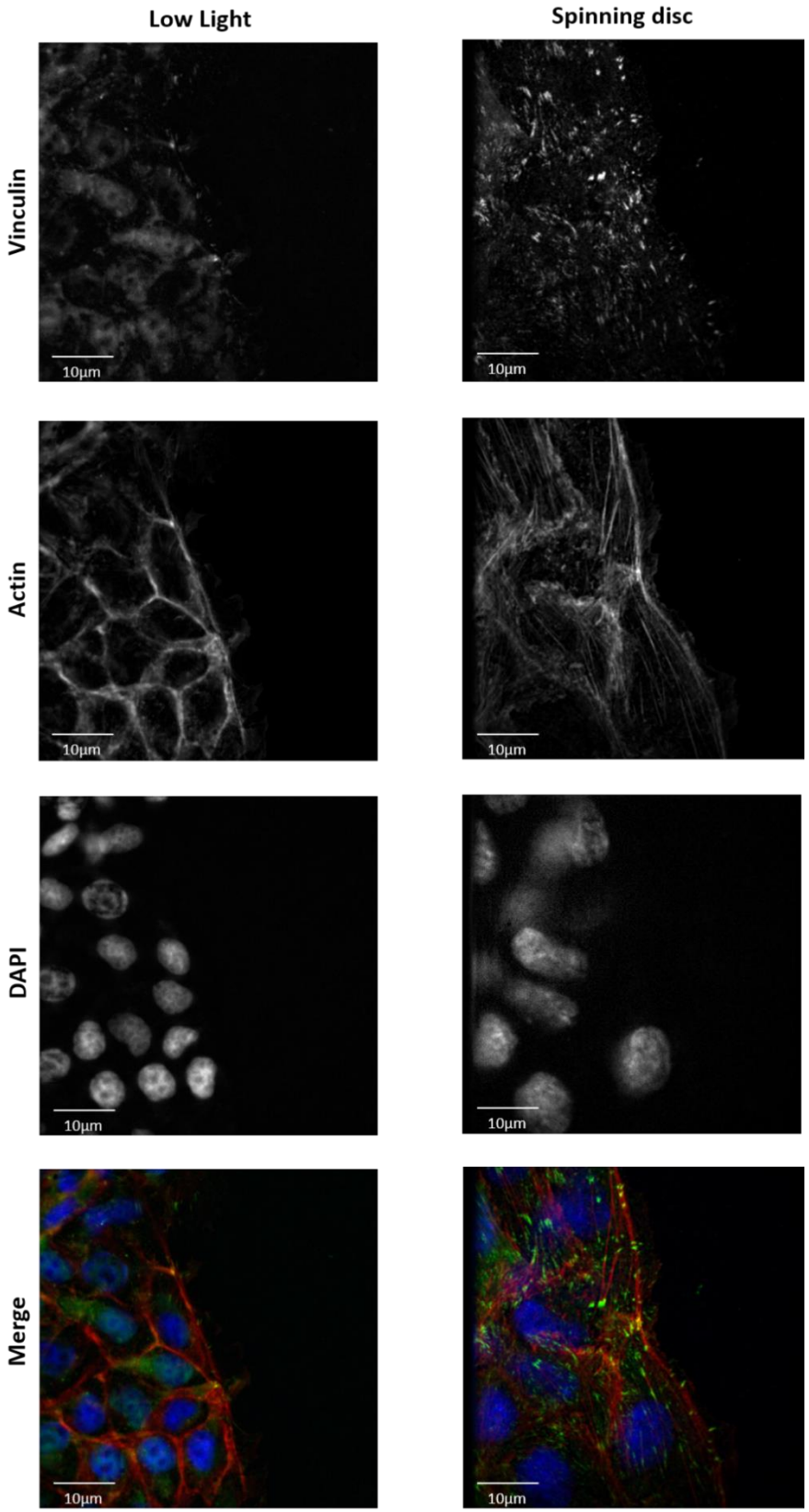
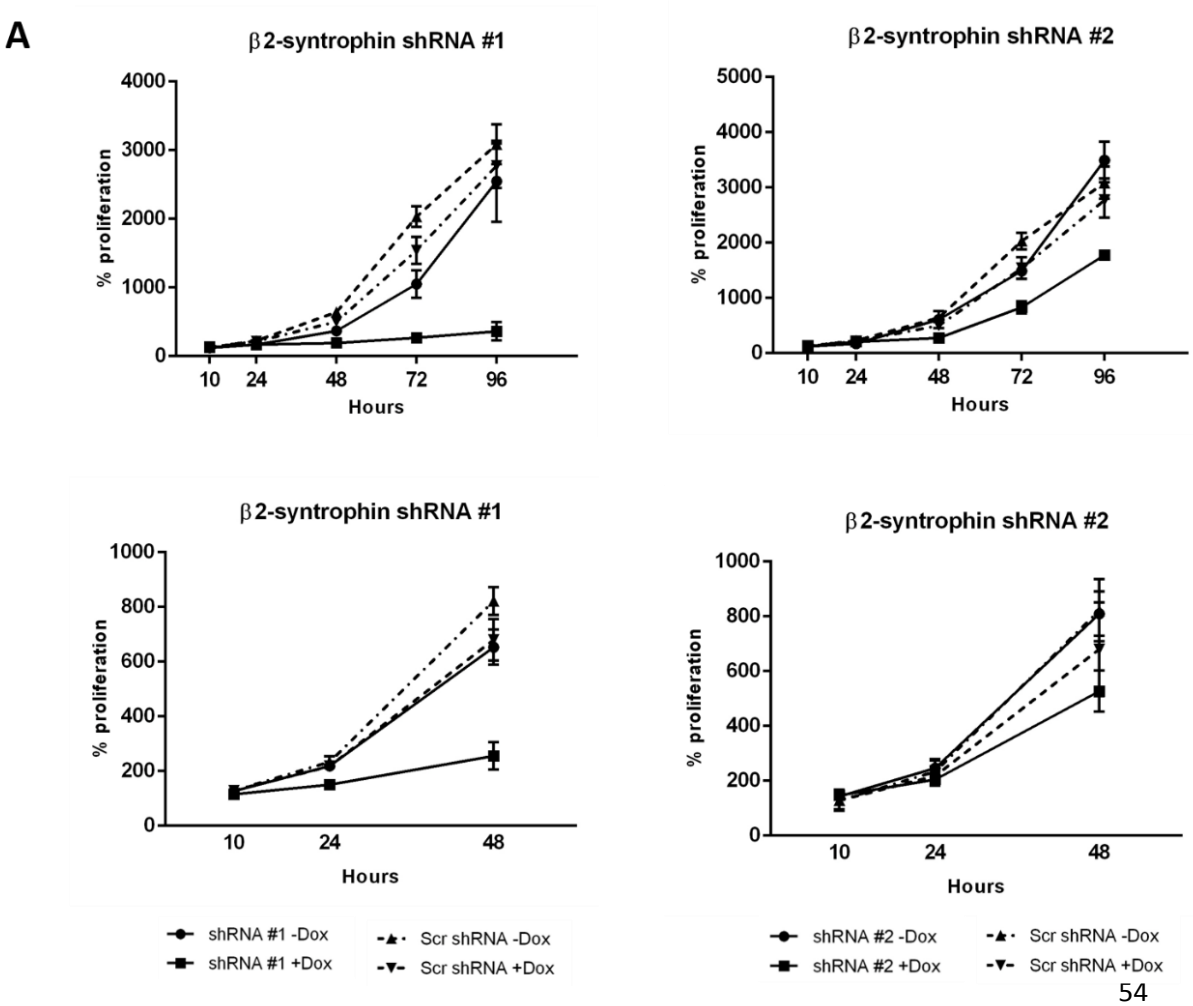


Figure 12: Images taken with the Spinning disc microscope display a better picture than those taken with the Low Light microscope. Scrambled MDCK II cells were scratched in the absence of dox (- Dox) and immunostained with anti-vinculin-FITC (→ focal adhesions) and phalloidin (→ actin) to compare the staining seen under the Spinning disc and the Low Light microscope.

3.5 β2-syntrophin promotes cell proliferation

Based on the fact that polarity proteins regulate cell proliferation, we asked whether β2-syntrophin contributes to the same cell process. Cells expressing a dox-inducible Scrambled shRNA and two shRNA sequences against β2-syntrophin (shRNA #1 and #2) were pre-treated with or without dox for 3 days to induce β2-syntrophin knockdown and then grown for several time intervals keeping the dox conditions stable for each clone. The time points for the crystal violet assay were 0, 12, 24, 48, 72 and 96 hours while for the sulforhodamine B (SRB) assay only 0, 12, 24 and 48 hours. At each time point cells were fixed with 3.7% formaldehyde and stained with either crystal violet or sulforhodamine B (SRB) dye. Then the dye was extracted and the absorption was measured on a spectrophotometer to quantify cell growth overtime. Both assays showed that downregulation of β2-syntrophin resulted in decreased cell proliferation (Figure 13.A). In fact statistically different results for crystal violet staining come from the 48hr (shRNA #1) and 72hr time-point (shRNA #2), while for SRB staining from the 24hr (shRNA #1) and 48hr (shRNA #2) time-point and the effect is maintained throughout the next time-points.



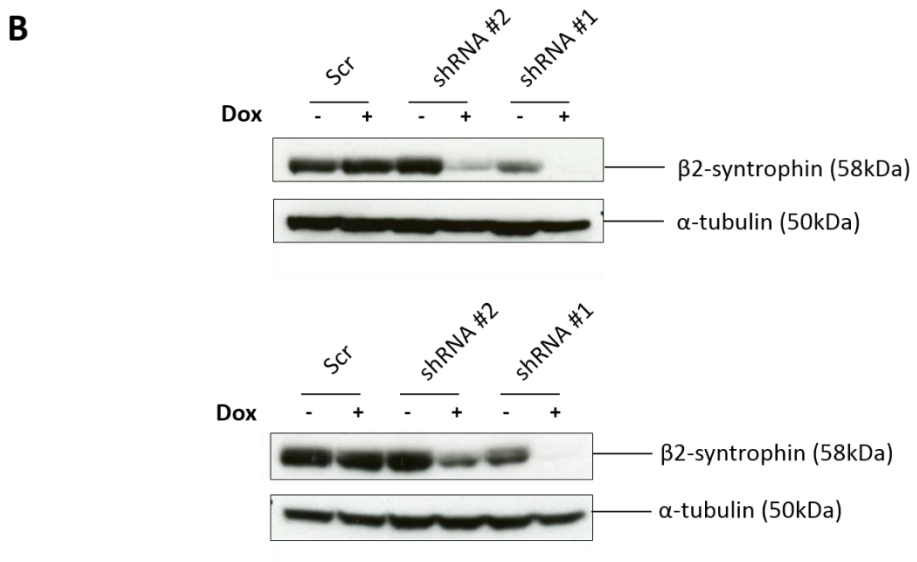


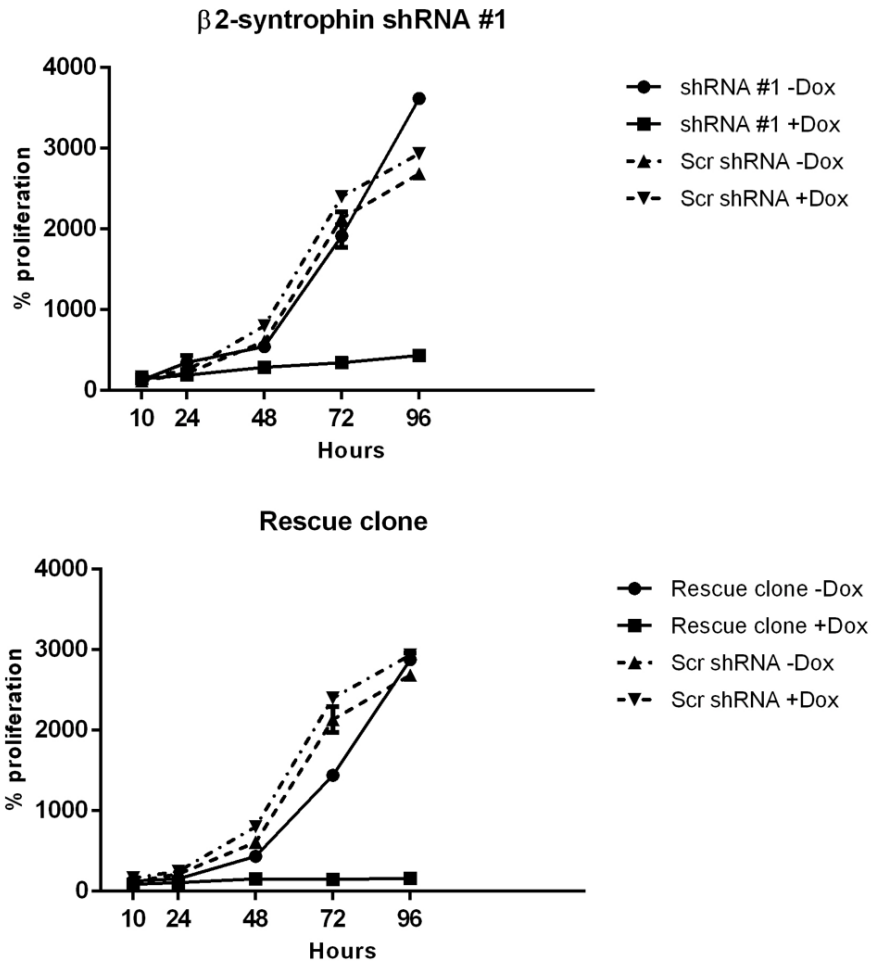
Figure 13: Knockdown of β2-syntrophin results in decreased cell proliferation. (A) Cells expressing either an inducible Scrambled shRNA, or two different shRNA sequences targeting β2-syntrophin were grown in the presence or absence of dox (+/- Dox) and stained with crystal violet (1st row) and sulforhodamine B (2nd row). The mean was calculated from four independent experiments. Error bars represent standard error (SE). The statistical analysis was done via GraphPad Prism 6 software and the students-t test. Statistical significance for: crystal violet staining at 48, 72, 96hr (shRNA #1) and 72, 96hr (shRNA #2), and SRB staining at 24, 48hr (shRNA #1) and 48hr (shRNA #2). (B) Representative western blot analysis from (A). The blot is one from four independent experiments.

3.6 Overexpression of β2-syntophin does not rescue the growth defect observed in the absence of β2-syntrophin

After observing that downregulation of β2-syntrophin leads to reduced cell proliferation, we wanted to check whether overexpression of the protein leads to an opposite, thus rescuing the defective phenotype, or same result. Crystal violet growth assay was utilized for Scrambled shRNA, shRNA #1 and rescue clone cells. They were seeded, after treatment with or without dox for 3 days, and at time intervals of 0, 12, 24, 48, 72 and 96 hours the cells were fixed and stained with crystal violet. Then in the same way quantification of cell growth was performed by extracting the dye and measuring the absorption in a spectrophotometer (Figure 14). The result suggests that overexpression of β2-syntrophin has the same effect as the downregulation of β2-syntrophin, that is an observed reduction of cell proliferation overtime. However, we cannot yet rule out that the

effects on cell proliferation are in fact an off-target effect of the shRNA, although we have seen this downregulation with two shRNA sequences targeted to different parts of the β2-syntrophin mRNA. Further experiments with cells expressing β2-syntrophin at levels closer to those of the endogenous protein would be required here to separate these two hypotheses.

A



B

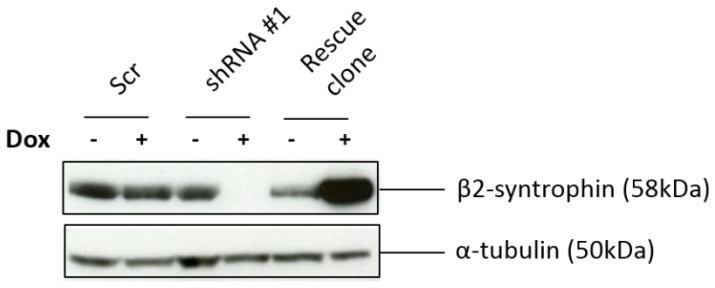


Figure 14: Overexpression of β2-syntrophin leads to reduced cell proliferation. (A) MDCK II cells expressing β2-syntrophin resistant to β2-syntrophin shRNA #1 and MDCK II Scrambled shRNA cells were grown in the presence or absence of dox (+/- Dox) and stained with crystal violet. The mean was calculated from three independent experiments. Error bars represent standard error (SE). The statistical analysis was done via GraphPad Prism 6 software and the students-t test. Statistical significance at 48, 72, 96hr (shRNA #1 and Rescue clone). (B) Western blot analysis of lysates from (A). The blot is one from three independent experiments.

4 Discussion

4.1 Outcome

The outcome from this study implies the involvement of β2-syntrophin in cell migration and proliferation. Collectively, for all the experiments the responses to β2-syntrophin downregulation through RNAi were dose-dependent, meaning that the level of downregulation was proportionally correlated with the magnitude of defect in both cell migration and proliferation. It has to be noted though that the two shRNA sequences give different levels of knockdown, but they are two different clones and may have additional differences that could account for this effect. We firstly showed that β2-syntrophin is required for optimal cell migration. In the absence of β2-syntrophin, which was achieved by targeting the protein with two different shRNA sequences, cells exhibit a reduced capacity for cell migration in comparison to control cells. Moreover, rescue of the migrative phenotype associated with β2-syntrophin knockdown was accomplished through re-expression of RNAi resistant β2-syntrophin, indicating that the phenotype is not due to off-target shRNA effects.

Then in seeking a mechanistic way of regulation of cell migration by β2-syntrophin we tried to assess its role in modulating focal adhesions. We observed that β2-syntrophin depletion does not affect the size of focal adhesions in migrating cells. Although there is a tendency of leading edge cells with depleted β2-syntrophin to form larger focal adhesions, it cannot be supported statistically. However, in order to re-test this hypothesis we could select different time points or do the experiment in a different way. For instance, we could use FRAP (Fluorescent analysis after photobleaching) analysis as used by Rooney et al., in 2010 [268] or perform live imaging so as to investigate focal adhesion turnover. Alternatively, the effect of β2-syntrophin absence on cell migration may as well be attributed to another mechanism.

Results from another study in the lab showed that Tiam1 protein levels were reduced following β2-syntrophin knockdown in sub-confluent but not in confluent MDCK II cell cultures (Malliri A., personal communication). These results indicate that Tiam1 protein levels are regulated by β2-syntrophin in proliferating and migrating cells, since these processes are mainly inactive post-confluency. Tiam1 has previously been shown to be required for optimal growth of MDCK II cells and implicated in the regulation of cell migration, although its role in this process remains unclear (discussed in the “Introduction”

section). It is therefore possible that the proliferation and migration defects observed in β2-syntrophin knockdown cells could be a result of decreased Tiam1 protein levels. In addition, it has been shown that the Tiam1-β2-syntrophin interaction is retained in the absence of cell-cell adhesions, suggesting that the interaction could contribute to the pro-migratory and/or pro-proliferative functions of β2-syntrophin.

An alternative way of cell migration control by β2-syntrophin could be thus accomplished via Tiam1. Tiam1 regulates LN5 deposition in keratinocytes and is required for efficient LN5-driven wound healing *in vivo* [274]. LN5 has been shown to promote cell migration [274-277], proliferation [275, 278] and wound healing [274, 277, 279]. Moreover, function-blocking antibodies of LN5 impairs migration and proliferation of sub-confluent MDCK II cells [275]. It is proposed that the reduced Tiam1 levels in β2-syntrophin knockdown cells leads to defective LN5 deposition, which could be responsible for the observed migration and proliferation defects. It is therefore hypothesized that β2-syntrophin is a key regulator of Tiam1-induced LN5 function that could have profound implications for the regulation of migration and proliferation in MDCK II cells.

Furthermore, we displayed that β2-syntrophin is necessary for efficient cell proliferation. Both growth assays, crystal violet and SRB cell staining, showed that depletion of β2-syntrophin reduces cell proliferation at the 24hr time-point and the effect is maintained at later time-points. However, overexpression of β2-syntrophin had the same effect on cell proliferation, suggesting that both reduced and elevated levels of β2-syntrophin lead to a defect in cell proliferation. This may further indicate that an intermediate dose of β2-syntrophin is essential for maintaining optimal cell proliferation. The further use of cells expressing β2-syntrophin at levels that are similar to those of the endogenous protein would be required to exclude any off-target effects of the shRNA.

The question of how β2-syntrophin regulates cell proliferation could be further explored through performing cell cycle analyses and examining the effects of β2-syntrophin depletion on the expression of various cell-cycle regulators. Moreover, it could be investigated whether β2-syntrophin promotes cell proliferation through a change in subcellular localization and if this depends on the cell density, as for many other polarity proteins [262, 263].

That is why, further work is required to establish the mechanisms by which β2-syntrophin regulates and particularly promotes cell migration and proliferation.

4.2 Potential roles of β2-syntrophin in cancer

The fact β2-syntrophin is involved in cell migration and proliferation (shown here) and cell-cell adhesion (already known), and that these are deregulated in cancer, could be indicative of a role of β2-syntrophin in tumorigenesis.

Starting from cell-cell adhesion, β2-syntrophin is a regulator of TJ assembly and may thus be implicated in the modulation of apicobasal polarity. This is often lost in cancer [280], therefore regulators of epithelial cell polarity are regarded as tumor suppressors. In accordance with this, the loss of utrophin is an early step in tumorigenesis [248] and is required to localize β2-syntrophin to cell-cell junctions [207]. It could be possible that loss of utrophin results in removal of β2-syntrophin from cell-cell junctions which would promote loss of apicobasal polarity and tumor development (Figure 15). Moreover, in cells that have lost β2-syntrophin and apicobasal polarity, Tiam1 levels may be decreased. In fact the loss of Tiam1 is linked to cancer progression *in vivo* [165, 174], and thus β2-syntrophin could stimulate tumor progression via downregulating Tiam1.

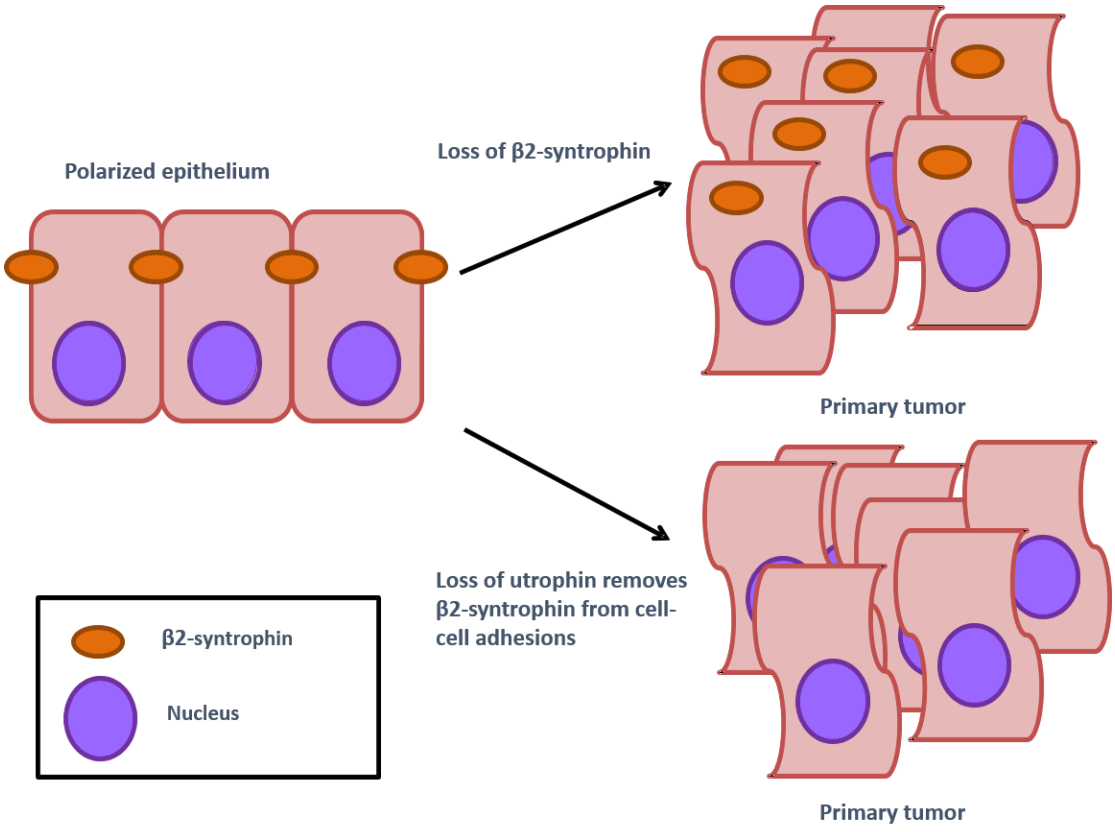


Figure 15: Loss of β2-syntrophin function may disrupt apicobasal polarity. Schematic representations are shown here depicting the potential mechanisms by which β2-syntrophin function could be lost at cell-cell adhesions and induce the loss of apicobasal polarity and in turn promote tumor development.

The finding of this study that β2-syntrophin is also necessary for optimal cell migration and proliferation comes in contrast with its function at cell-cell adhesions and its action as a tumor suppressor and it suggests that β2-syntrophin could additionally act as an oncogene. This dual role of β2-syntrophin is the same as with other polarity proteins and as described above it can be regulated by cell density and differentiation status [262, 263]. The loss of apicobasal polarity and the acquisition of an undifferentiated phenotype in cancer cells alters the function of polarity proteins. Their translocation from cell-cell adhesions to the nucleus may subsequently change their function from inducing cell-cell adhesions to promoting cell motility and proliferation therefore contributing to tumor progression. A similar change in β2-syntrophin localization could account for the effects observed upon its absence and its subsequent role in tumor progression (Figure 16).

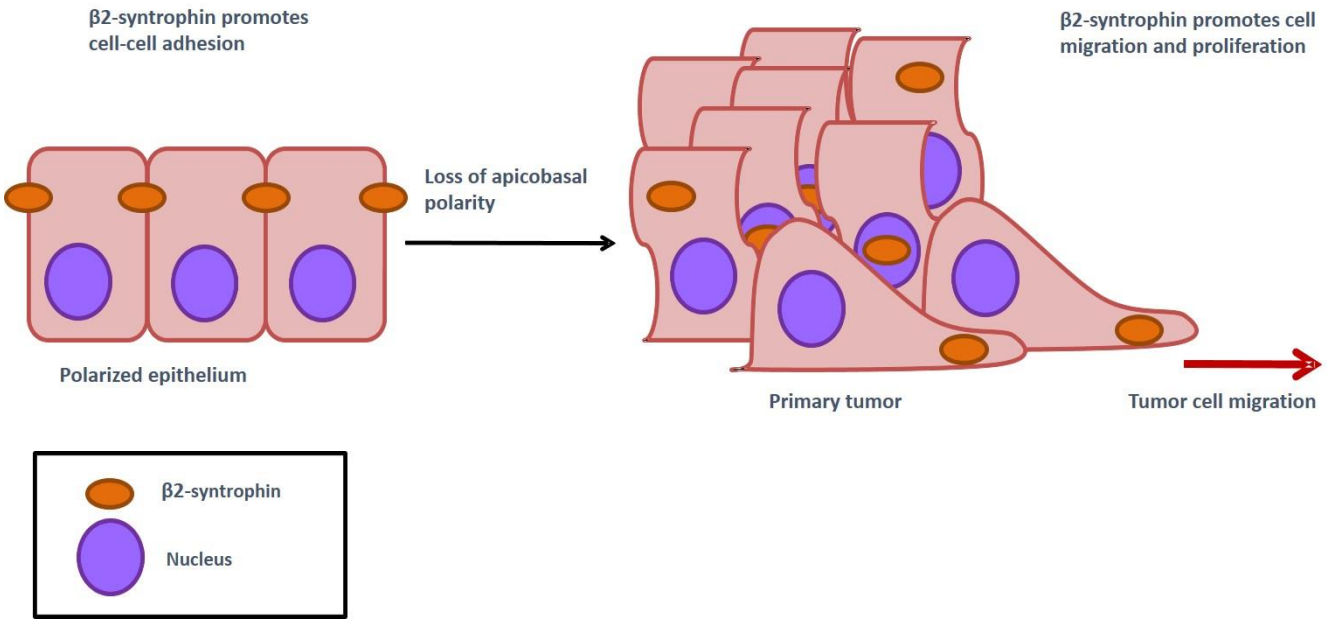


Figure 16: β2-syntrophin may promote tumor cell migration and proliferation. A hypothetical model involves the change in sub-cellular localization of β2-syntrophin after the acquirement of mutations that lead to apicobasal polarity loss. This in turn promotes the pro-proliferative and/or pro-migratory functions of β2-syntrophin and thus tumor progression.

As β2-syntrophin likely modulates several aspects of cancer, it could prove to be a novel therapeutic target. Specifically targeting its PDZ domain through which it seems to mediate protein-protein interactions that are essential for its multiple functions would be ideal. Such an interaction is the Tiam1-β2-syntrophin which is possibly important for the pro-proliferative and pro-migratory role of β2-syntrophin. This interaction is maintained in

cancer cells with lack of apicobasal polarity but presence of both Tiam1 and β2-syntrophin. So if Tiam1-β2-syntrophin indeed can promote tumor progression then inhibition of their interaction would be a new therapeutic strategy. In the study by Mack et al. in 2012 it was shown that the internal sequence KETDI of Tiam1 matches the consensus syntrophin PDZ-binding motif and its deletion abolishes the interaction with β2-syntrophin [103]. Thus targeting the KETDI sequence could be an alternative way of inhibiting Tiam1-β2-syntrophin interaction.

The discovery that β2-syntrophin regulates several biological processes that are associated with cancer has shown that the function of this molecule in epithelial cells is worthy of further study. Loss of apicobasal polarity could be either a cause or a consequence of β2-syntrophin absence. Therefore better understanding of the mechanisms regarding the role of β2-syntrophin in cell proliferation, cell migration as well as apicobasal polarity, will provide insight to normal tissue homeostasis and potential tumorigenic functions of β2-syntrophin and its related DGC complex. Polarity proteins act differentially under certain biological contexts and the study here provides further evidence that these regulators are implicated in multiple biological processes. This work along with others could lead to the identification of β2-syntrophin as a novel cancer biomarker providing diagnostic and/or prognostic value and possibly as a new drug target to help control tumor progression.

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