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GAP-independent functions of DLC1 in metastasis

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Running title: DLC1, a GAP-dependent and GAP-independent metastasis suppressor

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ABSTRACT

Metastases are responsible for most cancer-related deaths. One of the hallmarks of metastatic cells is increased motility and migration through extracellular matrixes. These processes rely on specific small GTPases, in particular those of the Rho family. Deleted in Liver Cancer-1 (DLC1) is a tumor suppressor that bears a RhoGAP activity. This protein is lost in most cancers, allowing malignant cells to proliferate and disseminate in a Rho-dependent manner. However, DLC1 is also a scaffold protein involved in alternative pathways leading to tumor and metastasis suppressor activities. Recently, substantial information has been gathered on these mechanisms and this review is aiming at describing the potential and known alternative GAP-independent mechanisms allowing DLC1 to impair migration, invasion and metastasis formation.

Keywords: DLC1; RhoGAP; GAP-independent; migration; invasion; metastasis

INTRODUCTION

Cancer remains the second leading cause of death worldwide [1]. An almost irretrievable outcome of cancer is metastatic progression [2]. Initiation of the metastatic cascade starts with a cellular reprogramming called epithelial-to-mesenchymal transition (EMT) that prompts malignant cells to escape the primary tumor site by simultaneous loss of cell-cell contact and gain of motility and invasiveness [3]. Control of motility is therefore a crucial step for malignancy prevention. Migration is finely tuned by pathways that modulate actin dynamics and focal adhesion (FA) formation [4]. FAs are complex multiprotein structures that allow attachment of the cell with the extracellular matrix (ECM) and that transduce various signaling pathways [5]. Small GTPases of the Rho family are major regulators of actin dynamics and FA formation [6]; their deregulation may therefore lead to tumorigenesis [7]. There are more than 60 members in the Rho family. Among these, Rho itself, Rac and Cdc42 are the best characterized proteins that regulate the cytoskeleton during migration [8]. Rho controls the formation of actin stress fibers through actomyosin bundling and contraction [9]. Rac and Cdc42 are responsible for actin polymerization leading to formation of two kinds of protrusions: filopodia and lamellipodia. Filopodia, which require the formation of long actin filaments, are controlled by Cdc42. These structures participate in environment sensing and directional migration. Lamellipodia, which are large and branched actin network protrusions, are controlled by Rac [9].

Small GTPases, including those from the Rho family, cycle between an inactive GDP-bound state and an active GTP-bound conformation. In their active form, they modulate cell signaling by interacting with effector proteins [10]. Activation of small GTPases is performed by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP. Small GTPase inactivation, on the other hand, is performed by GTPase-activating proteins (GAPs) that increase the low intrinsic GTP-hydrolysis activity of the small GTPases. There are 67 GAPs that modulate the Rho family GTPase members [11]. Intuitively, it could have been anticipated that loss of function mutations or epigenetic silencing of many of these GAPs would occur during carcinogenesis, yet only one of them, deleted in liver cancer-1 (DLC1), has been found to be widely down regulated in tumors [12].

DLC1 (also known as p122RhoGAP, ARHGAP7 and STARD12) is a RhoGAP protein with tumor and metastasis suppressor activities [13] that has been shown to be mutated almost as often as p53 in cancer [14]. DLC1 has been extensively investigated for its role as a tumor suppressor (reviewed in [13,15–17]) and during migration (reviewed in [18]). It is now known that DLC1 expression prevents cell migration [19–24], invasion [23,24] and metastatic progression [25–27] in various cancer types. There is accumulating evidence that DLC-1

exerts some of its functions in a GAP-independent manner by interacting with a series of newly identified partners. This review aims at describing the role of DLC1 during cell migration and metastatic progression with a particular emphasis on its GAP-independent functions.

DELETED IN LIVER CANCER-1

The deleted in liver cancer (DLC) protein family is composed of DLC1, DLC2, and DLC3. Members of this small family share high similarity and functional redundancy [13]. They all are GTPase activating proteins. The DLC family members contain a steroidogenic acute regulatory protein-related lipid-transfer (StART) domain and hence belong to a larger group of proteins called the StART family [17].

DLC2 and DLC3 are highly homologous with the conserved domains of DLC1 and are thought to act mainly as DLC1, they remain however much less studied than DLC1 [13]. Yet the observation that DLC2-null mice are viable in opposition to DLC1-null mice that die in utero indicate that the different DLC isoforms are not performing fully overlapping functions [28].

DLC1 was first identified in 1995 in the rat and was named p122RhoGAP [29]. The human orthologue was cloned and described three years later by the laboratory of Nicholas C. Popescu [30]. It has a wide expression in mouse and human (brain, heart, kidney, liver, lung and skin, etc.) [30,31]. DLC1 harbors potent GAP activities towards RhoA, B and C, a more limited GAP activity towards Cdc42, but it does not regulate the GTPase activity of Rac1 [26,32]. Rho and Cdc42 inactivation limits cell survival responses and also prevents cell migration by inducing actin stress fiber disruption [8]. DLC1 is also a platform protein that interacts with several partners, which modulates their activity in a GAP-independent manner.

First discovered as a tumor suppressor that was lost in hepatocarcinomas [30], DLC1 was later found to be down-regulated in several other malignancies including hematological malignancies, and lung, breast, prostate, kidney, colon, uterus, ovary and stomach cancers [13,33]. Importantly, DLC1 was found to be as frequently mutated as p53 in most aggressive cancers [12,14], which may not come as a surprise since Rho activation is required for tumor progression and for full transformation by oncogenes such as Ras [7,34]. Loss of DLC1 expression is associated with high metastatic potential [35]. In humans, deletion of the short arm of chromosome 8, which contains the *dlc1* gene, is a frequent event associated with cancer development [14,36]. More targeted mutations of the *dlc1* gene leading to reduced expression of the protein were also reported such as exonic missense mutations and intronic insertions/deletions in primary ovarian and colorectal [37]. DLC1 can also be epigenetically silenced [38,39]. In liver, breast, colon and prostate cancers, this results from hypermethylation of the CpG island in the DLC1 promoter [38]. In some tumors, like those of the prostate, loss of DLC1 is mostly carried out by epigenetic silencing and not a consequence of gene mutations [39]. Acetylation also modulates DLC1 activity since treatment of several cancer types with the histone deacetylase inhibitor trichostatin A reactivates DLC1 expression [39]. DLC1 is also the target of several kinases including protein

kinase A (PKA), Akt/PKB, PKC and PKD [40–43]. Phosphorylation of DLC1 on serine 567 by Akt and on serines 327 and 431 by PKC lead to inhibition of its GAP activity [40,42].

Initially, the tumor suppressor function of DLC1 was attributed to its GAP activity on Rho [12,14]. This notion was derived from observations such as that DLC1 silencing or expression of a constitutively active form of RhoA induced comparable accelerating effects on liver tumor growth [14]. However, GAP-independent mechanisms may also be involved in the tumor suppressive function of DLC1. Indeed, reintroduction of a GAP-dead DLC1 mutant in small cell lung cancer that did not express DLC1 cells impaired colony formation in soft agar, although to a lower extent than wild-type DLC1 [26]. Another observation supporting the GAP-independent tumor suppressive function of DLC1 is that ectopic expression of a GAP-dead DLC1 mutant in NIH3T3 fibroblasts partially abrogated migration [44]. Also in this case, expression of the wild-type DLC1 led to a stronger inhibition of migration. It is therefore likely that DLC1 uses both GAP-dependent and GAP-independent mechanisms to exert its tumor suppressive effects.

The question as to why DLC1 is the only RhoGAP, out of sixty-seven, that is lost in malignancies has been raised [12]. Possibly, DLC1, but not other RhoGAPs, prevents the activation of a non-canonical Rho pathway that would be specifically required for transformation. A more likely possibility to us is that it is the GAP-independent functions of DLC1 that strengthen its tumor suppressor activities. This does not mean that the other RhoGAPs do not have GAP-independent activities, some in fact do [45,46], but presumably their GAP-independent activities are not involved in counteracting tumorigenesis.

DLC1 structures

DLC1 interacts with its partners through conserved domains: a sterile alpha motif (SAM) domain, a GAP domain, a StART domain and a serine-rich (SR) region (**Fig. 1**). These domains are also found in the other members of the DLC family. SAM domains (amino acid 11 to 78) are almost as frequently found in proteins as SH2 domains [45]. SAM domains display great functional diversity. They can bind to one another, they can associate with non-SAM domains, they can interact with RNA, lipid molecules, etc. [45]. SAM domains may be involved in the dimerization capacity of DLC1 [43]. The middle part of DLC1 contains a serine-rich unstructured and relatively poorly conserved region with numerous potential sites for tyrosine phosphorylation (amino acids 86 to 638). Within this central part lie two highly conserved motifs: i) the LD motif (amino acids 469 to 476), that is known to be essential for DLC1 binding to FAK and talin, two proteins associated with focal adhesion [46], and ii) a binding site for tensin proteins (amino acids 440 to 445; more specifically tyrosine 442). The GAP

domain (amino acids 639 to 847) is highly conserved between the three DLC isoforms and displays selective GAP activity towards RhoA, B, and C, and Cdc42 [26]. Two arginine residues within the GAP domain, R677 and R718, are crucial for DLC1 GAP activity, most likely by acting as “arginine fingers” [46,47]. The C-terminal part of DLC1 is occupied by a StART domain (amino acids 878 to 1081). StART domains are conserved ~210 amino acid sequence forming a hydrophobic pocket that can bind lipids such as cholesterol [48]. However, no role in lipid transport has been reported for the DLC family. The physiological functions of the DLC1 StART domain remain relatively elusive at this point. However, interaction partners have been identified for each domain/region of DLC1, including the StART domain (Figure 1), suggesting that these sequences play important roles in DLC1’s functions.

Differential DLC1 expression levels

Varying DLC1 expression has an impact on focal adhesions, actin dynamics and the migratory capacity of the cells. DLC1 is reduced, or even lost, in many tumors and this, based on the capacity of DLC1 to regulate cell migration, may affect the metastatic potential of malignant cells [49]. It is indeed well accepted that diminished DLC1 levels during tumorigenesis results in more motile and invasive cell behavior [14]. Accordingly, RNA interference-mediated DLC1 reduction in cancer cells promoted increased migration [19]. On the other hand, restoring DLC1 expression in cancer cells that had down-regulated DLC1 led to inhibition of migration and invasion, particularly in breast cancer [25,50], hepatocarcinoma [23], multiple myeloma [24] and colon cancer [51]. Experimental overexpression of DLC1 resulted in disruption of focal adhesion, cell shrinkage and loss of stress fibers [22,23,47]. However, absence of DLC1 may also compromise cytoskeleton dynamics and migration. For example, silencing DLC1 in normal prostate epithelial cells inhibited their migration [52]. Additionally, DLC1 knock-out fibroblasts display disrupted stress fibers and fewer focal adhesions [53]. Therefore, overexpression and total absence of DLC1 may both compromise proper cytoskeleton dynamics and migration, while reduced expression of DLC1 could favor migration. Consequently, inhibition of DLC1 in malignant cells with reduced expression of the protein may represent a therapeutical approach to prevent metastasis development.

MOLECULAR BASIS OF MIGRATION AND METASTASIS

Cell migration is physiologically required during development, wound healing and immune responses, among other [54]. Its deregulation is involved in several pathological conditions such as metastatic progression and lissencephaly [54,55]. Cell migration involves spatiotemporally regulated events that can be divided into four main steps [9]: (i) polarization of the cell (ii) formation of protrusion and adhesion structures (iii) contraction of the cell body and (iv) retraction of the cell rear. In order to move, cells have to create new actin filaments that push the plasma membrane forward and depolymerize actin filaments at the cell rear [56]. Actin dynamics is a highly complex phenomenon involving dozens of proteins. How actin polymerization and depolymerization is achieved has been reviewed in details elsewhere [4,57]. The Rho GTPases, in particular Rho, Rac and Cdc42, play pivotal roles during the four processes mentioned above. Cdc42 establishes cell polarity by controlling the localization of the Golgi apparatus. Cdc42 also triggers filopodia formation [58]. Rac controls the generation of lamellipodia and is also responsible for early nascent focal contact formation [6]. Rho participates in the formation of FAs and stress fibers to generate the tension required for cell movement [59]. In addition, Rho triggers acto-myosin contraction that allows cell movement and tail retraction [8]. Inactivation of Rho, Rac and Cdc42 abrogates migration [58,60].

Metastasis occurs when malignant cells, often in response to stresses such as those resulting from gamma irradiation [61], undergo epithelial-mesenchymal transition (EMT). This process consists in changes in cell morphology that will be required for a cell to migrate in its extracellular environment [62]. EMT is characterized by the loss of E-cadherins [63], and hence loss of cell-cell adhesion structures, but also by increased expression of proteins involved in cell migration such as the Rho GTPases. Studies performed with dominant negative mutants and constitutively active forms of Rho, Rac and Cdc42 revealed that all three proteins are indispensable for migration into the extracellular matrix [64,65]. Overexpression of RhoA increases invasiveness and is often detected in metastatic tumors [66,67]. RhoB-null mouse embryonic fibroblasts display impaired motility and adhesion due to lower expression levels of the β 1-integrin adhesion receptor [68]. Studies on the RhoC knock-out mouse and analyses on highly metastatic melanoma cells revealed that RhoC is required for the metastatic process, but it is not involved in cancer cell proliferation [69,70]. RhoC favors tumor dissemination through sequential activation of Pyk2, a focal adhesion tyrosine kinase, FAK, MAP kinases, Akt and the upregulation of the MMP-2 and MMP-9 matrix metalloproteinases [71].

When Rho and Cdc42 are in the active GTP-bound form, they bind several effectors known to directly affect cytoskeleton dynamics [9,72]. Rho in particular modulates actin dynamics through ROCK activation [73].

ROCK facilitates acto-myosin contraction by inhibiting the myosin light chain (MLC) phosphatase and by directly phosphorylating MLC. Accumulation of phosphorylated MLC leads to actin bundling and contraction and the formation of stress fibers. ROCK also has the ability to phosphorylate the LIM kinase (LIMK) that in turn phosphorylates and inactivates ADF/cofilin, leading to actin filament stabilization [4]. Rho controls actin polymerization in at least two different ways. First, Rho-mediated activation of the mDia formins leads to actin nucleation and elongation [74]. Second, activated Rho binds and activates phosphatidylinositol-4-phosphate 5-kinase (PIP kinase), which leads to accumulation of phosphatidylinositol 4,5-bisphosphate (PIP₂) [9], which in turn promotes actin polymerization (discussed in detail below).

When activated, Cdc42 binds and stimulates several effectors that positively regulate actin polymerization and hence migration [75]. Cdc42 interacts with PAK1 and activates it leading to LIMK stimulation, cofilin inactivation and therefore prevention of actin depolymerization [9]. In addition, Cdc42 has the ability to stimulate WASP, which results in actin polymerization via activation of the Arp2/3 nucleator [75]. Cdc42 controls the orientation of the microtubule-organizing center (MTOC) that defines the positioning of the Golgi apparatus that is required for directed migration [76]. Cdc42 has recently been shown to increase trans-endothelial migration by modulating β 1-integrin expression level and activity [77].

DLC1 ROLE IN MIGRATION AND INVASION

What is regulated by the GAP activity of DLC1?

As discussed before, DLC1 has GAP activity towards RhoA, B, and C, and, albeit with lower potency, towards Cdc42 [26]. The negative effect of DLC1 on Cdc42, initially demonstrated *in vitro* [26], was recently corroborated *in vivo* [32]. The physiological consequences of this modulation still remain to be established though. Inactivation of RhoA by DLC1 results in loss of stress fibers and focal adhesions [22]. This can negatively impact on the ROCK pathway as shown in hepatocellular carcinoma where DLC1 prevents phosphorylation of MLC [78]. As indicated previously, DLC1 silencing increases Rho activity and favors migration. However, ROCK is not necessarily involved in this response. Indeed, DLC1 silencing in breast cancer increases migration in a mDia1-dependent but ROCK-independent manner [50]. Yet, the tumor suppressor activity of DLC1 still appears to depend on ROCK since colony formation induced by DLC1 silencing is abrogated upon treatment with the Y-27632 ROCK inhibitor [14]. Participation of other Rho effectors that are inhibited by Y-27632, like citron kinase and PKN [79], cannot be excluded however. Possibly, ROCK inhibition

by DLC1 contributes to its tumor suppressor function, while inhibition of mDia formins by DLC1 leads to a migration suppressor activity.

In addition to regulating the canonical Rho pathway, DLC1 is controlling adherens junction (AJ) stability [80]. AJs are structures that mediate cell-to-cell interactions and consist in complexes between the transmembrane E-cadherin protein and cytoplasmic p120 catenin and β -catenin [81]. By binding to β -catenin, α -catenin can be recruited to the AJ, which then leads to the attachment of the actin cytoskeleton to E-cadherins. This favors the maturation and the stability of AJs [82,83]. Loss of E-cadherin and α -catenin destabilizes AJs, a situation often documented during metastasis formation when tumor cells are no longer linked to one another, favoring their dissemination [63]. DLC1 binds to α -catenin and allows its interaction with AJs. This requires the GAP activity of DLC1 [80]. The presence of DLC1 in AJs contributes to its oncosuppressive activity and reinforces AJ stability. DLC1-mediated inhibition of RhoA and RhoC has been shown to promote expression of E-cadherin, increasing the number of AJs and strengthening cell-cell interactions [84]. It is now well accepted that decreased AJ stability is associated with increased motility and dissemination during EMT [63]. The consequence of the DLC1-mediated effects on AJ remains however to be directly evaluated during cell migration and invasion. Nevertheless, it remains quite clear that DLC1, through its negative regulation of Rho and Cdc42, via its GAP domain, contributes to inhibition of cell migration and invasion [4].

As mentioned in the introduction, DLC1 carries many potential phosphorylation sites in particular in its serine-rich region. Akt- and PKD-mediated phosphorylation of DLC1 can negatively affect its GAP activity [40,42]. PKD-mediated phosphorylation on serines 327 and 431 allows DLC1 to bind six out of the seven isoforms of the 14-3-3 scaffold protein family [40]. This binding sequesters DLC1 in the cytoplasm away from FAs, preventing it to fulfill its tumor and metastasis suppressor functions. Other phosphorylation events induce reverse effects. Very recently, the cyclic AMP-dependent kinase PKA was shown to phosphorylate DLC1 on serines 431 and 549. This induced DLC1 dimerization and enhancement of its GAP activity, leading to increased oncosuppressive effects and inhibition of migration, invasion and metastasis formation [43]. The GAP activity of DLC1 can therefore be differentially tuned by phosphorylation events.

The next section will discuss how DLC1 can also exert anti-metastatic activity independently of its GAP activity.

DLC1 GAP-independent signaling

Eleven DLC1 interacting proteins and four kinases that phosphorylate DLC1 have been described so far; these are listed in **Table 1**. Some of these interactions may modulate the GAP activity of DLC1 but may also result in DLC1 GAP-independent signaling and modulation of the DLC1-binding partner activity. The interaction map of DLC1 with other proteins is shown in **Figure 1**. The relevance of these interactions is discussed below.

PLC δ 1 and phosphoinositide signaling

Phosphoinositides are phosphorylated forms of phosphatidylinositol, a phospholipid found in cellular membranes. Phosphoinositides comprise phosphatidyl-inositol (PI) phosphate (PIP), PI bisphosphate (PIP₂) or PI trisphosphate (PIP₃). These molecules act as secondary messengers, and as scaffold and signaling molecules [85]. The phospholipase C proteins have the ability to hydrolyze the ester bond between the glycerol and the phosphate group and can therefore transform PIP₂ into inositol triphosphate (IP₃) and diacylglycerol (DAG) [85]. The first discovered binding partner of DLC1 was phospholipase lipase C delta 1 (PLC δ 1). By binding to PLC δ 1, DLC1 enhances its hydrolyzing activity resulting in increased IP₃ and DAG levels and a corresponding decrease of PIP₂. The consequence of this enzymatic reaction on the cytoskeleton is complex (reviewed in [85]). PIP₂ establishes a linkage between the actin cytoskeleton and the membrane and stimulates actin polymerization through several routes [85,86]. It primarily helps actin translocation to the plasma membrane where most actin polymerization proteins are localized [86]. It stimulates the WASP protein that in turn activates Arp2/3, an actin nucleator that allows the formation of protrusions pushing the membrane forward [4]. PIP₂ binds to several other actin regulators. By binding to the gelsolin capping protein, PIP₂ disrupts the actin:gelsolin complex. This leads to uncapping of the actin filament plus end, hence favoring actin polymerization [87]. Profilin is a protein that interacts with monomeric actin for delivery to the polymerizing barbed-ends of actin filaments [4]. PIP₂ then binds to profilin which dissociates from actin allowing the latter to be incorporated on the growing actin fiber. PIP₂ is also required for the conformational activation of vinculin and talin, two focal adhesion proteins the activation of which allows cell attachment, a step required for efficient migration.

Although PLC δ 1 was the first identified DLC1 binding partner, the nature of their interaction has still not been fully determined although the GAP-START domain region appears to be important (see **Figure 1**) [29]. The interaction of PLC δ 1 and DLC1 takes place at focal adhesions where DLC1 co-localizes with vinculin [88]. This localization is consistent with its potential role during migration. Interestingly, as DLC1, PLC δ 1 has been

defined as a tumor suppressor and inhibitor of migration [89]. Several approaches involving injection of PIP₂ or overexpression of negative regulators of PIP₂ have led to the notion that increasing PIP₂ levels results in actin polymerization while decreasing PIP₂ levels leads to actin depolymerization [85]. One can anticipate that the decrease of PIP₂ levels through DLC1-mediated activation of PLC δ 1 is a possible Rho-independent mechanism allowing DLC1 to modulate cytoskeleton dynamics and cell movement. The biological relevance of the DLC1:PLC δ 1 interaction on migration remain however to be evaluated.

DLC1 and focal adhesion regulation

DLC1 can be found in several subcellular structures [80,90,91] but location at focal adhesions is the one most often reported [21,22,44,46,50,90,92–100]. What is commonly called focal adhesions actually comprise all types of anchorage contacts. These are, from smallest to biggest/longest, focal contacts (FXs), focal adhesions (FAs) and fibrillar adhesions (FBs) [101]. When integrins are engaged by their ECM ligands, talin and paxillin are quickly recruited to form the FXs, which are transient adhesive structures. FXs that are not turned over transform into FAs by recruiting proteins such as FAK, vinculin, α -actinin and zyxin, and this provide an anchorage point to link actin bundles. FBs are much longer and stronger adhesive structures that are localized in central areas under the cell and that are characterized by the presence of tensins. The tensin family is composed of four members, tensin-1, tensin-2, tensin-3 and C-terminal tensin-like (cten) [102]. Tensins are often down-regulated in cancer, in particular in metastasis, suggesting that they play tumor and metastasis suppressor functions [102]. By linking the actin cytoskeleton and most of the integrin cytoplasmic tails (β 1, β 3, β 5 and β 7-integrins), tensins provide a crucial function required for cell migration [102–105].

DLC1 was reported to interact with each tensin family member [92–94,100]. The binding mode of DLC1 with the tensin proteins is however complex as it seems to vary between different tensin isoforms. The first identified binding mode involves the DLC1 SH2-binding motif containing serine 440 and tyrosine 442 (**Figure 1**) and the SH2 domain of tensin-1 and cten. This binding does not require phosphorylation of tyrosine residues though [94]. Other binding modes have been unraveled recently, including interaction of the 374 to 388 DLC1 sequence with the phosphotyrosine-binding (PTB) domain of tensin-2 [97] and the actin-binding motif of tensin-3 [100]. The latter contains a region, lacking in cten, that binds the DLC1 SAM domain [100]. DLC1-tensin binding occurs at focal adhesions where DLC1 most likely acts as a tumor [93,94] and migration [96] suppressor. Interestingly, mutational-driven disruption of this binding prevents DLC1 FA localization and abrogates its oncosuppressive activity, without negatively impinging on its GAP activity [46,94]. When fused to

a FA binding domain, the DLC1 mutant that is unable to bind tensins relocates to FAs but only partially recovers its tumor suppressor activity. Thus, the GAP activity at FAs does not appear to fully mediate some given DLC1 biological activities. DLC1 was reported to compete with β 3-integrin for binding with tensin-1 [93]. As a result, DLC1 engagement to tensin-1 prevents the connection between integrins and tensins, therefore impeding mechanical traction. Consistent with this notion is the fact that cells expressing truncated tensins lacking the SH2 domain, the very domain that interacts with DLC1, do not display enhanced migration [104]. Thus DLC1 negatively regulates migration by competing with tensins for integrin binding independently of its Rho GAP activity. How this competition is regulated is only partially understood.

FAK and talin are two other FA proteins that can interact with DLC1 [46]. Talin, like tensins, can directly interact with integrins, while FAK mediates the link between talin and the actin cytoskeleton [5]. An eight amino acid motif within DLC1, called LD-motif (amino acids 469 to 476), is responsible for this binding. DLC1 can interact simultaneously with talin and FAK [46]. Potentially, this increases the capacity of DLC1 to translocate to FAs, which, as mentioned earlier, is the place where DLC1 is supposed to exert its oncosuppressive functions. Paxillin is another focal adhesion protein that binds to FAK through its fourth LD motif [106]. DLC1 was shown to displace paxillin from FAK [46]. Interestingly, cells overexpressing a truncated version of paxillin that lacks the fourth LD motif display dramatically decreased migration [107]. While the effects of DLC1 binding on the structure of FAs have not yet been evaluated, one could predict that DLC1 binding to FAK negatively affects focal adhesion turnover and therefore migration by displacing paxillin from FAK. This represents yet another GAP-independent mechanism by which DLC1 inhibits migration and invasion.

The plasminogen connection

The urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) are activated upon binding to the uPAR membrane receptors. They then convert plasminogen into plasmin, a serine protease that degrades the ECM. They also stimulate matrix metalloproteinases (MMPs) that participate in ECM degradation [108]. The plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor that negatively regulates uPA and tPA. PAI-1 binds to uPA/tPA and leads to their internalization, preventing uPA/tPA to convert plasminogen into plasmin [109,110]. PAI-1 also directly inhibits the MMP activity, as well as the $\alpha_v\beta_3$ -integrin binding to vitronectin, two crucial processes during invasion [111]. The S100A10 protein is a cell surface receptor and a plasminogen activator. It competes with PAI-1 to bind to uPA/tPA. This results in augmented plasmin

production and promotion of invasiveness. S100A10 has also the ability to directly bind plasmin and activate its auto-proteolysis.

DLC1 modulates the plasminogen pathway to prevent dissemination. DLC1 binds to S100A10, increasing its susceptibility to ubiquitin-dependent degradation and this potently reduces, in a GAP-independent manner, S100A10-mediated plasminogen activation [112]. The binding to S100A10 occurs through the 348-354 DLC1 sequence. RNAi-mediated silencing of S100A10 in lung cancer cells leads to reduced colony formation and impaired invasiveness, as DLC1 does [112]. Another study investigating the role of DLC1 on the plasminogen pathway reported an unexpected effect of DLC1 on migration [52]. In this study, silencing DLC1 in human prostate epithelial cells led to increased PAI-1 expression that resulted in migration inhibition. Conversely DLC1 over-expression decreased PAI-1 expression levels. The reason of this discrepancy (i.e. DLC1/PAI-1 favors migration in lung cancer while it inhibits it in normal prostate cells) is unclear but may depend on the transformation status of a cell or possible differences in DLC1 residual expression after silencing. ECM integrity and composition are often affected in metastatic contexts [113,114]. While certain ECM components are up-regulated to favor motility, others are lost or degraded by MMPs allowing dissemination [114,115]. A study revealed that DLC1-mediated inhibition of liver metastasis formation was associated with the down-regulation of the ECM component osteopontin, and the MMP9 matrix metalloproteinase [27]. Osteopontin and MMP-9 have been associated with invasive phenotypes [115,116]. Therefore, this is potential alternative way for DLC1 to prevent metastasis formation. DLC1 has been shown to shuttle between the nucleus and the cytoplasm [40]. Although its function in the nucleus is not known, it is possible that DLC1 regulates the activity of transcription factors. This could be the basis of the DLC1-mediated repression of MMP-9 and osteopontin transcription.

DLC1 in caveolae

Caveolae are specialized plasma membrane subdomains that are involved in vesicular transport and compartmentalization of signaling proteins. Caveolin-1 is a major caveolae component, driving their formation and linking them to the cytoskeleton [117]. These organelles play an important role during adhesion and migration in several ways [117]. Caveolin-1 binds to filamin, an actin-binding protein linking stress fibers at integrin sites and controlling their stability. Caveolae promote adhesion, migration and invasion by interacting with and stabilizing uPAR and α_v -integrin [118] (see previous section). Stable knock-down of caveolin-1 induces decreased β_1 -integrin-dependent adhesion and disruption of the caveolin-1/ β_1 -integrin complex inhibits

migration [119]. Caveolae also play a role during invasion by favoring plasminogen activation and by hosting the matrix metalloproteinase-2 [120]. Another evidence for the involvement of caveolae during migration is their ability to control calcium release at the cell rear which is required for calcium-dependent protease-mediated cleavage of focal adhesion proteins [121].

In 2004, the rat DLC1 ortholog and caveolin-1 were reported to colocalize in caveolae and to interact through the DLC1 GAP domain [122]. DLC1 interaction with caveolin-1 leads to both internalization of DLC1 and caveolae. The physiological importance of caveolin-1-DLC1 interaction was recently evaluated and the mapping of this interaction was revisited. It was found that the human DLC1 StART domain was responsible for this interaction rather than the previously reported rat DLC1 GAP domain [91]. Stable expression of a DLC1 mutant that was unable to bind caveolin-1 in DLC1-negative lung cancer cells failed, unlike the wild-type DLC1, to inhibit colony formation, tumor migration into transwells, and *in vivo* tumor growth. Importantly, this DLC1 mutant retained a full GAP activity on RhoA, demonstrating that the migration inhibitory effect of DLC1 was GAP-independent. How the DLC1/caveolin-1 interaction inhibits migration is however not fully understood. One can hypothesize however that DLC1 and caveolae internalization prevents DLC1 from exerting a Rho GAP activity at the leading edge of the cell. Together with a decreased number of caveolae structures at the plasma membrane, this would be expected to markedly hamper cell migration

The SAM binders

In contrast to most DLC1 partners that interact with the SR and C-terminal DLC1 regions, EF1A1 and PTEN bind to the N-terminal SAM domain (**Figure 1**). EF1A1 (for eukaryotic elongation factor 1A1) is involved in protein translation. EF1A1 also binds to the actin cytoskeleton and regulates its dynamics through several mechanisms, independently from its role in protein synthesis, by (i) acting as a chaperone for β -actin mRNA at protrusion sites (i.e. plasma membrane sections that are pushed out by growing actin fibers) [123], (ii) through its actin-bundling activity [124], and (iii) by binding to G-actin and possibly sequestering it [125]. EF1A1 binds to the 38-40 sequence of DLC1 within the SAM domain [44]. This interaction seems to be an important GAP-independent event controlling cell migration since transfection of a DLC1 mutant unable to bind EF1A1 but with an unaffected GAP activity induces a weaker migration inhibitory effect than the wild-type construct in fibroblasts. The SAM domain was suggested to bind to the GAP domain and this is believed to generate an auto inhibitory signal. Indeed, the GAP domain of DLC1 is twenty times more active when isolated than when embedded in full-length DLC1 [22,26]. Consistent with these observations is that overexpression of

the SAM domain induces a higher motility rate than full-length DLC1. Instead, overexpression of the construct that does not bind EF1A1 did not stimulate migration. This indicates that modulation of migration by the DLC1 SAM domain depends on its ability to bind EF1A1 and is GAP-independent [44].

The phosphatase and tensin homolog (PTEN), a tumor suppressor, also binds to the DLC1 SAM domain [21]. This binding was proposed to mediate PTEN-induced dephosphorylation of FAK, leading to migration inhibition. However this hypothesis remains to be formally proven. Tensin-3 binds the DLC1 SAM domain through its actin-binding domain, the consequence of which is thought to release the SAM auto-inhibitory effect on GAP activity. The GAP-independent implications of tensin-DLC1 interaction are discussed above.

GAP-to-GAP

GAPs and GEFs rarely modulate a single GTPase. This is the case with DLC1 that regulate both Rho and Cdc42. Moreover, different GAPs and GEFs can modulate the same GTPase. This considerably increases the complexity of small GTPase regulation [126]. Additionally, binding between different GAPs has been reported. For instance p120 RasGAP and p190 RhoGAP interact and this is believed to control, on the one hand, the accessibility of the RasGAP SH3 domain and, on the other hand, the GAP activity of p190 [127,128]. Interestingly the RasGAP SH3 domain was shown to bind the DLC1 GAP domain resulting in inactivation of the latter's GAP activity [129] and consequently this should increase cell motility. We recently discovered that the 317-326 amino acid sequence within the SH3 of RasGAP mediates its binding to DLC1 (unpublished data). Treatment of cells with a cell-permeable version of the peptide corresponding to these 10 amino acids, the so-called TAT-RasGAP₃₁₇₋₃₂₆ peptide, resulted in increased adhesion associated with a potent inhibition of migration and invasion. This was an unexpected observation because, assuming that this peptide functions as the SH3 domain of RasGAP, it should also inhibit the GAP activity of DLC1 and hence favors migration. However, treatment with the C3 exoenzyme and Y-27632, which are inhibitors of Rho and ROCK, respectively, did not reverse this phenotype, suggesting that the RasGAP peptide exerts its activity in a DLC1 GAP-independent manner. Although the mechanisms induced by the RasGAP peptide downstream of DLC1 remain to be elucidated, this reflects once more the importance of GAP-independent events in the DLC1-mediated control of migration.

DLC1 phosphorylation

The serine/threonine kinase PKD phosphorylates DLC1 on serine 807 that lies within its GAP domain. This phosphorylation was reported to increase the inhibitory effect of DLC1 on colony formation and cell migration without modulating the GTP-bound state of RhoA [41]. Phosphorylation of DLC1 can therefore potentially inhibit dissemination and tumor growth in a GAP-dependent as well as in a GAP-independent manner. The *in vivo* relevance of PKD-mediated S807 phosphorylation remains however to be investigated.

DISCUSSION

DLC1 is convincingly more than just a GAP for small GTPases of the Rho family; it is also a scaffolding protein that triggers a wide range of signaling responses affecting cell survival and controlling cell motility. Specific down-regulation of DLC1 may be used by malignant cells to survive and become metastatic. Together with the two well-established cancer associated proteins BRCA1 and HIF1- α , loss of DLC1 has recently been recognized as a biomarker for early stage lung adenocarcinomas [130]. Therapeutic strategies aimed at restoring DLC1 expression represent an attractive anti-cancer strategy and this indeed has already been attempted. Using demethylation-inducing agents or dietary flavones, DLC1 expression could be restored in cell lines in which it was epigenetically silenced and this hampered proliferation and induced cell cycle arrest [131]. *In vivo* anticancer responses resulting from epigenetic restoration were however not reported [131]. Rho isoforms are often overexpressed in cancer. Reactivating DLC1 in cancers would be interesting in this context since two distinct anticancer signaling would be triggered; those that dependent on the GAP activity of DLC1 and those that do not. These two effects would hopefully synergize or at least be additive. We recently found that a tumor sensitizer cell-permeable peptide derived from RasGAP (called TAT-RasGAP₃₁₇₋₃₂₆) efficiently inhibits migration and invasion *in vitro* in a DLC1 GAP-independent manner (unpublished data). Whether TAT-RasGAP₃₁₇₋₃₂₆ inhibits metastasis formation *in vivo* is currently under investigation. The data obtained with TAT-RasGAP₃₁₇₋₃₂₆ indicate that DLC1-targeted druggable compounds with anti-metastatic potential can be generated.

Figure 2 summarizes schematically the different GAP-independent mechanisms presented in this review. These include actin depolymerization (via PLC δ 1 inhibition), FA remodeling (via interaction with tensins, FAK and talin), plasminogen production inhibition (via S100A10 degradation), caveolin internalization, and RasGAP and EF1A1-dependent anti-metastatic signaling. Such responses, in particular if happening simultaneously, could induce potent inhibition of migration and invasion.

Although not discussed in this review, DLC2 and DLC3 can add an additional layer of complexity to this picture. Overexpression of DLC2 was shown to induce loss of stress fibers and inhibit cancer cell growth [132,133]. Similarly, DLC2 loss is associated with enhanced migration [134]. In contrast, DLC2 associates with mitochondria [135] and interacts with α -tubulin [132], two features that are not shared with DLC1. Interestingly, DLC2 seems to bear some activity towards Rac and could therefore synergize with DLC1, which does not affect Rac, in preventing migration [136]. Akin to DLC1, DLC3 is a focal adhesion tumor suppressor broadly lost in various malignancies (reviewed in [17]) but its functions are not well understood at the moment. GAP-independent signaling has not been reported for DLC2 and 3 but it would not be surprising that the mechanisms mentioned in this review are also used by them.

There is a wealth of data supporting the notion that normal or increased DLC1 expression levels inhibit migration or invasion, while decreased DLC1 expression favors these processes. Intriguing data challenge however this simple view. For example DLC1-null fibroblasts display a complete loss of stress fibers [53]. As stress fibers are required for migration, cell movement should be inhibited, contradicting the idea that DLC1 suppresses invasion. In addition, DLC1 silencing in normal prostate cells leads to migration inhibition, the opposite of what is usually found in the literature [52]. Intriguingly, these two studies reporting a pro-migratory role of DLC1 were performed in non-malignant cells. Modulation of DLC1 expression levels in normal and cancer cells may therefore not have the same consequence. We recently generated data that also challenge the simple view that DLC1 loss favors migration. We found out that the TAT-RasGAP₃₁₇₋₃₂₆ peptide inhibited migration in a large panel of normal and malignant cells but failed to have this effect in DLC1-null mouse embryonic fibroblasts. DLC1 was expressed to very low, sometimes undetectable, levels in some of the cancer cells used in this study but TAT-RasGAP₃₁₇₋₃₂₆ was nevertheless still able to inhibit their migration. Here again, the consequence of DLC1 loss may differ between malignant cells and the normal mouse embryonic fibroblasts. Alternatively, complete (as in knock-out DLC1 cells) or partial (as in cancer cells) DLC1 loss may not necessarily lead to the same migratory phenotype. It will be very important in the future to solve this conundrum.

In conclusion, DLC1 contributes to growth and migratory suppressive effects through GAP-dependent and GAP-independent mechanisms. In contrast to what have been proposed previously, the GAP-independent mechanism may fulfill important tumor suppressor functions and represent therefore potential avenues of research to develop new anti-cancer therapies.

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FIGURE AND TABLE LEGENDS

Table 1 DLC1-interacting partners

This table lists the DLC1-interacting partners and the effects of the indicated interaction on DLC1 and the concerned binding partner. Positive and negative modulation of DLC1 are indicated by the + and – symbols, respectively

Fig. 1 DLC1 binding partners

The domains of DLC1 are represented by colored rectangles. Interactions with binding partners are indicated. DLC1 phosphorylation sites are indicated by P-containing circles and labeled as follows: green circle for GAP-inhibiting phosphorylations, blue circles for GAP-activating phosphorylations, and red circles for phosphorylations that trigger DLC1 GAP-independent effects

Fig. 2 DLC1 GAP-independent anti-metastatic mechanisms

This scheme highlights the anti-metastatic mechanisms used by DLC1 with a special emphasis on the GAP-independent signaling events. See main text for details

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Table 1

Protein	Binding site (aa)	Effect on DLC1	Effect on the target	Ref.
PLCδ1	StART domain	Not investigated	+ (increased activity)	[29]
Tensin	440-445	+ (GAP); FA localization	- (β -integrin binding inhibition)	[92–94,100]
Talin	469-476 (LD motif; SR)	+ (GAP); FA localization	Not investigated	[46]
FAK	469-476 (LD motif; SR)	+ (GAP); FA localization	Not investigated	[46]
S100A10	348-354	No effect (GAP)	- (degradation)	[112]
Caveolin-1	929-957 (StART)	No effect (GAP), Caveolae localization	- (Caveolin-1 dynamics)	[91]
EF1A1	38-39 (SAM)	No effect (GAP)	Membrane recruitment	[44]
p120-RasGAP	798-847 (GAP)	- (GAP)	Not investigated	[129]
PTEN	SAM	Not investigated	+ (increased activity)	[21]
14-3-3	S327 and S431 (SR)	- (GAP); sequestration	Not investigated	[40]
α-catenin	340-435 (SR)	+ (GAP); AJ localization	+ (AJ stability)	[80]
Kinase	Phosphorylation site (aa)	Effect on DLC1	Effect on the target	Ref.
PKA	S431 and S549 (SR)	+ (GAP)	Not investigated	[43]
Akt/PKB	S567 (SR)	- (GAP)	Not investigated	[42]
PKC/PKD	S327 and S431 (SR)	- (GAP)	Not investigated	[40]
PKD	S807 (GAP)	No effect (GAP); + (GAP-independent)	Not investigated	[41]

Figure 1

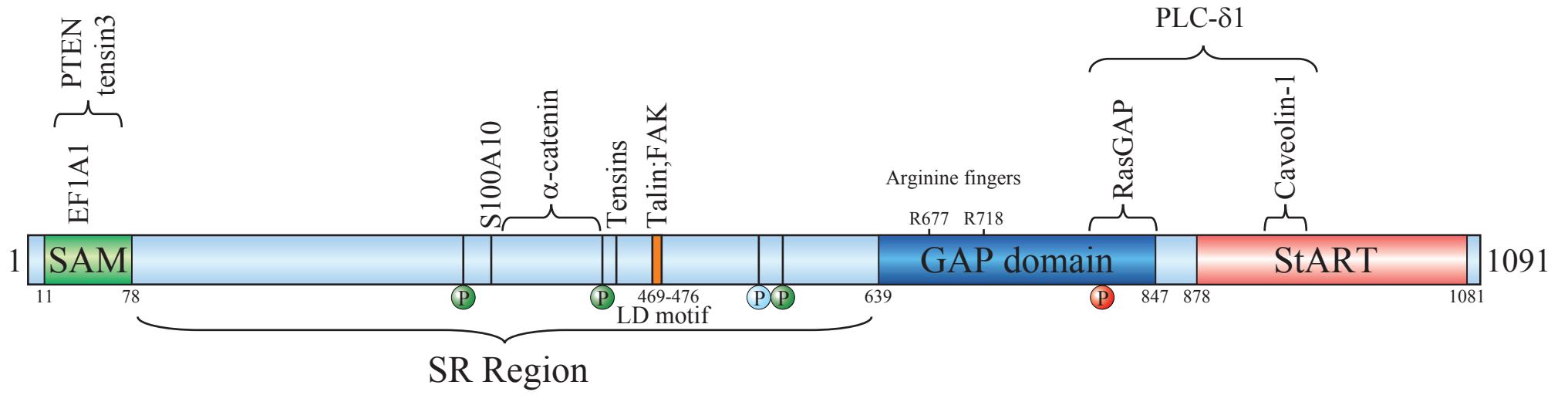


Figure 2

DLC1 GAP-independent signaling

