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Chapter 7

START-GAP/DLC Family Proteins: Molecular Mechanisms for Anti-Tumor Activities

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Additional information is available at the end of the chapter

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1. Introduction

The Rho family of GTPases belongs to the superfamily named "Ras-like" proteins, which consists of over 150 varieties in mammals [1]. Significant progress has recently been made in understanding the biological functions mediated by this family of small (~21 kDa) G proteins (guanine nucleotide-binding proteins). Rho GTPases affect crucial biological processes such as transcriptional regulation, cell cycle progression, apoptosis and membrane trafficking [2, 3]. Thus far, a total of 23 Rho proteins have been identified [4], among which RhoA, Rac1 and Cdc42 are characterized in detail. Rho GTPases are also involved in the cytoskeleton formation of the cell via the regulation of actin dynamics [5, 6]. RhoA induces stress fiber formation and focal adhesion assembly, thereby regulating cell shape, attachment and motility, whereas Rac1 promotes extension of lamellipodia and membrane ruffling [7]. Cdc42 has been shown to play a role in the formation of filopodia [8].

Like other small G proteins of the Ras-like protein family, Rho GTPases act by switching between an inactive GDP-bound and an active GTP-bound form, with the latter form capable of interacting with a myriad of downstream effectors (so far, more than 70 proteins have been identified [4]) to be activated by them. The activation of Rho GTPases is stimulated by guanine nucleotide exchange factors (GEFs) that exchange GDP for GTP and is inhibited by GTPase-activating proteins (GAPs) that hydrolyze the GTP to GDP [9]. Rho GTPases are also negatively regulated by guanine nucleotide dissociation inhibitors (GDIs), which bind to the GDP-bound form and not only prevent nucleotide exchange, but also remove Rho GTPases from the plasma membrane to the cytoplasm [4, 10, 11]. Taken together, cell morphology requires spatiotemporally restricted regulation of Rho GTPases through these regulatory proteins. Nonetheless, detailed insight into regulation of Rho GTPases has not been provided.

The effect on the wide spectrum of biological functions suggests the involvement of Rho GTPases and their regulators in cancer progression. Findings from extensive in vitro and in vivo studies shows that deregulated signaling of Rho GTPases may lead to tumorigenesis [12] and thus Rho GTPases are taken as potential targeting candidates for cancer therapy [13]. Since no constitutively active Rho mutants have been reported in human cancers, it is likely that aberrant Rho GTPase signaling in malignancy is caused by the alterations of their regulators [4, 12]. Extensive studies so far have revealed that regulators of Rho proteins are over- or underexpressed in various types of human cancer cells [14-17]. The most common alteration reported for Rho regulators in cancer is inactivation of RhoGAPs, especially of the START-GAP/DLC family RhoGAPs [18]. "START" stands for "steroidogenic acute regulatory protein (STAR)-related lipid transfer" and "DLC" stands for "Deleted in Liver Cancer (DLC)," a gene (or its product) found to be commonly deleted in liver tumors. The START-GAP/DLC family proteins have become the focus of attention on their roles in tumorigenesis. This type of genetic loss was also found in a number of other cancers [17, 19-21].

START-GAP1/DLC1 was originally cloned from rat cDNA library as a binding partner of phospholipase C-δ1 (PLCδ1) [22]. It has been shown that the C-terminal region of START-GAP1/DLC1 and the PH domain of PLCδ1 are responsible for the interaction [23, 24]. START-GAP1/DLC1 enhances the activity of PLCδ1, which generates two second-messengers, inositol 1,4,5-trisphospate (Ins(1,4,5)P_3) and diacylglycerol via hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2). Ins(1,4,5)P_3 is accepted by receptors on ER, resulting the elevation of intracellular calcium concentration, whereas diacylglycerol acts as the activator for PKC [25, 26]. Indeed, microinjection of START-GAP1/DLC1 into the cytosol elevated intracellular calcium concentration [23].

As mentioned in detail in the following sections, each member of the START-GAP/DLC multi-domain protein family contains one GAP domain for Rho GTPases. Overexpression of START-GAP1/DLC1 in cultured cells was first to demonstrate induction of drastic morphological changes accompanied by the disruption of actin stress fibers and elevation of intracellular Ca^{2+} concentrations [23]. START-GAP1/DLC1 was therefore originally designated ARP (adaptor for both Rho and PLC) [22], but later the name p122RhoGAP (or just p122) was used to avoid confusion with another ARP (actin related proteins) [23, 24, 27, 28]. We have then introduced the new name, START-GAP1, based on the characteristic domain structure of this Rho GTPase family [29-32]. Meanwhile the antioncogenic properties of the protein family have been revealed and the DLC nomenclature was introduced [21]. In this chapter, the combination of both the structure- and function-based nomenclatures is used throughout, since the use of either of them would not be appropriate to reflect the fundamental properties of the protein family accurately.

In the following sections we will focus on the structure, localization and expression-function relationship of the START-GAP/DLC family proteins in physiological conditions and in human diseases.

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2. The START-GAP/DLC gene family

In mammalian genome, there are three genes encoding structurally-related RhoGAPs containing the START domain (Figure 1). There are three groups of START domain-containing RhoGAP proteins in vertebrates, while a worm (*Caenorhabditis elegans)* or a fly (*Drosophila melanogaster)* possesses only one START-containing RhoGAP.

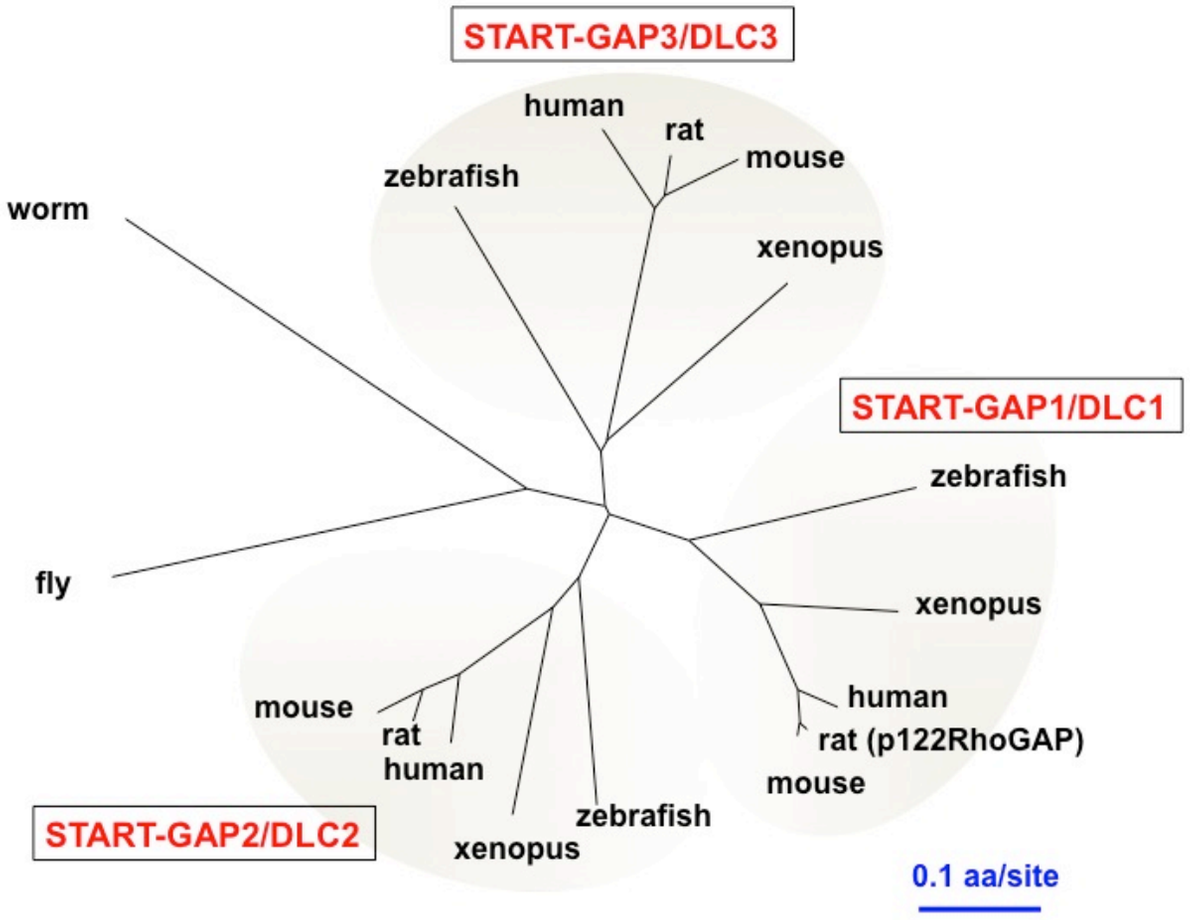

Figure 1. A phylogenetic analysis of the START domain-containing RhoGAPs generated with a "Treeview" after CLUSTAL W analysis (http://clustalw.ddbj.nig.ac.jp/). Modified from Kawai et al. [31].

2.1. Human START-GAP/DLC genes and their expression in various tumor cells

There are about 70 human genes encoding RhoGAPs that share a conserved GAP domain and are capable of switching off the Rho signal [33]. The *START-GAP1/DLC1* (also named as STARD12 or ARHGAP7) gene is localized on chromosome 8p21-22 and encodes a 1,091-amino acid protein with a molecular mass of 122 kDa. Using the quantitative RT-PCR method, Ko et al. have reported that START-GAP1/DLC1 is widely expressed in normal tissues, with high abundance in the lung and ovary, and moderately in the thyroid, spleen, intestine and kidney [34]. START-GAP1/DLC1 has also been known as a tumor suppressor gene product. It is

frequently underexpressed or not expressed in several tumor cells and inhibits cell growth, invasion and metastasis [35, 36]. Studies have indicated that downexpression of START-GAP1/DLC1 either by genomic deletion or DNA methylation [37] is associated with a variety of cancer types including lung [38], breast, prostate, kidney, colon, uterus, ovary, and stomach [38] [39] [40]. These phenotypes require the GAP activity of START-GAP1/DLC1 [35].

Negative regulation of the Rho/Rho-kinase (ROCK)/myosin light chain (MLC) pathway in hepatocellular carcinoma (HCC) cell lines by START-GAP1/DLC1 was shown to be RhoGAP-dependent [23, 41, 42]. The RhoGAP defective mutant failed to inhibit stress fiber formation in HCC lines [41], whereas the overexpression of START-GAP1/DLC1 resulted in morphological change with disruption of actin stress fibers [23, 42]. Using various cancer cell models, START-GAP1/DLC1 was shown to inhibit cell proliferation, suppress cell migration and invasion, and induce apoptosis [35, 36, 41, 43-45]. Restoration of START-GAP1/DLC1 expression in metastatic cell lines has been shown to cause the inhibition of cell migration and invasion as well as a significant reduction in metastases in nude mice [45].

Underexpression of START-GAP1/DLC1 was associated with either heterozygous deletions of the *START-GAP1/DLC1* gene or hypermethylation of the gene promoter region [17, 37, 46-49]. This protein is therefore thought to be under the epigenetic regulation for expression.

The *START-GAP1/DLC1* gene is transcribed from two different promoters, resulting in transcripts encoding three isoforms [47]. To date, most of studies on START-GAP1/DLC1 have focused on the so-called isoform 2. Low et al. have recently identified a new isoform of START-GAP1/DLC1, isoform 4 (DLC1-i4), using 5′-RACE method [50]. This novel isoform encodes a 1,125-amino acid protein with distinct N-terminus as compared with other three isoforms. Similar to them, DLC1-i4 is expressed ubiquitously in normal tissues and immortalized normal epithelial cells, suggesting a role as a major START-GAP1/DLC1 transcript. Differential expression of the four START-GAP1/DLC1 isoforms, however, is found in tumor cell lines: Isoform 1 (the longest isoform) and isoform 3 (short and probably nonfunctional) share a promoter and are silenced in almost all cancer and immortalized cell lines, whereas isoform 2 and isoform 4 utilize different promoters and are frequently downregulated. Isoform 4 is significantly downregulated in multiple carcinoma cell lines, including nasopharyngeal, esophageal, gastric, breast, colorectal, cervical and lung carcinomas. Ectopic expression of DLC1-i4 suppresses tumor cell colony formation. Differential expression of the isoforms suggests interplay in modulating the complex activities of START-GAP1/DLC1 during carcinogenesis.

There are two additional members of the START-GAP/DLC family. *START-GAP2/DLC2* (or *STARD13*) gene is located on chromosome 13q12 [51] and *START-GAP3/DLC3* (or *STARD8/KIAA0189*) gene is located on the X chromosome at q13 band [31, 52]. The *START-GAP2/DLC2* encodes a 1,113-amino acid protein with a molecular mass of 125 kDa, whereas the protein product of the START-GAP3/DLC3 transcript has 1,103 amino acids with a molecular mass of 121 kDa. START-GAP2/DLC2 has a broad tissue distribution, with the highest levels in the brain, heart and liver [15, 53]. Human START-GAP2/DLC2 protein shares 51% identity and 64% similarity to human START-GAP1/DLC1 at the level of the amino acid sequence [51]. Introduction of human START-GAP2/DLC2 into mouse fibroblasts suppress Ras signaling and

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cell transformation in a GAP dependent manner, suggesting the role of START-GAP2/DLC2 for growth suppression and carcinogenesis [51].

START-GAP3/DLC3 is also detected in a variety of human tissues with high abundance in the lung, kidney and placenta [53].

Following the findings of dysregulation of the START-GAP1/DLC1 gene function in a variety of solid tumors, downregulation of START-GAP2/DLC2 and START-GAP3/DLC3 genes was also shown to be involved in human cancer development.

START-GAP2/DLC2 was found to be downregulated in breast, lung, ovarian, renal, uterine, gastric, colon, rectal, and liver tumors [15, 52, 54]. The comparison of *START-GAP1/DLC1* and *START-GAP2/DLC2* gene expression in the same cell lines revealed that START-GAP2/DLC2 is more frequently downregulated than START-GAP1/DLC1 in HCC cell lines [15]. Moreover, the overexpression of START-GAP2/DLC2 suppresses cell proliferation, motility and anchorage-independent growth in the human hepatoma cell line, HepG2 [55]. START-GAP2/DLC2 was also reported to have an inhibitory effect on the growth of breast cancer cells in vitro [56].

Decreased START-GAP3/DLC3 expression in primary tumors from kidney, lung, uterine, ovary and breast has been reported [52]. Kawai et al. have demonstrated that START-GAP3/DLC3 serves as a GAP for both RhoA and Cdc42 in in vitro assays. Furthermore, the overexpression of START-GAP3/DLC3 in HeLa cells disrupts actin stress fibers and changes cell morphology in a GAP-dependent manner [31]. Ectopic expression of START-GAP3/DLC3 in human breast and prostate cancer cell lines inhibits cell proliferation, colony formation and growth in soft agar [52].

The structures of the three human START-GAP/DLC transcripts are depicted in Figure 2.

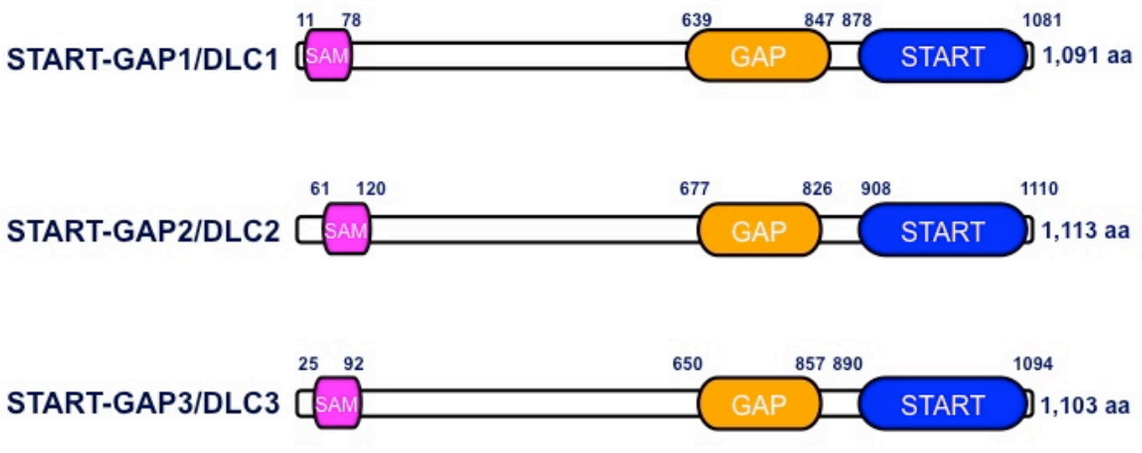

Figure 2. Schematic representation of the START-GAP/DLC family proteins. Each member of the family comprises of three distinct domains, namely the sterile α motif (SAM), RhoGAP (GAP) domain and START (steroidogenic acute regulatory protein (STAR)-related lipid transfer) domain.

2.2. Gene knockout studies of START-GAP/DLC proteins

Using a mouse model a gene knockout study of START-GAP1/DLC1 has been carried out [57]. The mouse *START-GAP1/DLC1* gene was inactivated by homologous recombination. Mice heterozygous for the targeted allele were phenotypically normal, but homozygous mutant embryos did not survive beyond 10.5 days postcoitum. Cultured fibroblasts from START-GAP1/DLC1-deficient embryos displayed alterations in the organization of actin filaments and focal adhesions [57]. In addition, a gene knockdown of START-GAP1/DLC1 in the background of c-*myc* overexpression promotes the formation of liver tumors [58].

Although the START-GAP1/DLC1 gene deficient mice were embryonic lethal, deletion of the *START-GAP2/DLC2* gene from mice resulted in survival to adulthood, indicating that the gene, unlike the *START-GAP1/DLC1* gene, was dispensable for embryonic development [59]. Neither did the authors observe a higher incidence of liver tumor formation in the *START-GAP2/DLC2* gene knockout mice. Nevertheless, they reported smaller phenotype with less formation of adipose tissue [59].

To the best of our knowledge, no reports describing the gene knockout study of the START-GAP3/DLC3 gene are currently available.

2.3. Homologs of the START-GAP/DLC family

As mentioned earlier, there are homologs of the mammalian START-GAP/DLC family proteins in invertebrates in the BLAST database. A *Drosophila* ortholog of START-GAP1/DLC1, *Crossveinless-c* (*Cv-c or RhoGAP88C*), was identified in search for genes that regulate *Drosophila* morphogenesis [60]. The function of *Cv-c* has been revealed to be a key regulator for unidirectional growth of dendritic branches of the fly via downregulating the activity of Rho1, the *Drosophila* Rho GTPase [61]. In the *Cv-c* mutant, two subclass of multidendritic sensory neurons formed dorsally directed branches; however, dendritic branches had a difficulty in growing along the anterior–posterior (A–P) body axis, suggesting that *Cv-c* contributes to sprouting and subsequent growth of the A–P-oriented branches through negative regulation of Rho1 [61]. Thus *Cv-c* plays a key role in directional dendritic growth presumably via its RhoGAP activity, localizing the GAP activity to sites undergoing cytoskeleton rearrangements during morphogenesis.

3. Structure and function of the domains

The START-GAP/DLC family proteins are composed of multi-domain structures. START-GAP/DLC proteins have three distinct domains: The sterile α motif (SAM) localized at its N-terminus, a conserved RhoGAP (GAP) domain in the middle and the steroidogenic acute regulatory (StAR)-related lipid transfer (START) domain at the C-terminus [51-53]. (Figure 2)

Although the START-GAP/DLC family proteins contain a potential lipid-binding START domain, the proteins are produced in soluble forms. The intracellular localization of these proteins, therefore, is determined by their specific interactions with target proteins resided at

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various cellular structures. Each domain of the START-GAP/DLC family proteins may contribute to different subcellular localization patterns.

3.1. The GAP domain

The biological activity of START-GAP1/DLC1 is mainly executed by the GAP domain (~150-200 amino acid), which promotes the hydrolysis of GTP bound to the Rho GTPases. This catalytic activity is mediated by the 'arginine-finger' present in the GAP domain [62]. START-GAP1/DLC1 and START-GAP3/DLC3 contain a conserved 'arginine-finger' at position 677 and 688, respectively [52, 53]. In in vitro assays both the full-length START-GAP1/DLC1 and the isolated GAP domain reveal activity on RhoA, RhoB and RhoC, to a lesser extent on Cdc42, and no activity on Rac1 [17, 32, 63]. By inactivating these small GTPases, START-GAP1/DLC1 affects cell morphology and control actin cytoskeletal remodeling [22, 23]. Similar to START-GAP1/DLC1, both START-GAP2/DLC2 and START-GAP3/DLC3 contain a RhoGAP domain and exhibit the GAP activity for RhoA and Cdc42 but not Rac1 in vitro [31, 32, 51].

3.2. The sterile α motif (SAM)

The SAM domain (~70 amino acids) has been found in signaling proteins (e.g., p53 related proteins p73 and p63, Eph-related tyrosine kinases and Ets transcription factors) [64, 65]. The SAM domain is thought to act as a protein interaction module via homo- or hetero-oligomerization with other SAM domains [64]. As an example, the EphA2 receptor that plays key roles in many physiological and pathological events including cancer has the SAM domain. Recently, a structural study of the EphA2 receptor SAM domain has validated structural elements relevant for the heterotypic SAM-SAM interactions: two SAM domains interact with a head-to-tail topology characteristic of several SAM-SAM complexes [66]. The SAM domain even interacts with RNA [67].

Structural studies of the SAM domain of START-GAP2/DLC2 have suggested that it binds to lipids such as phosphatidylglycerol [68]. Nevertheless, we know little about exact roles of the SAM domain in START-GAP/DLC function. Kim et al. have shown that the expression of the amino-terminal domain of START-GAP1/DLC1 acts as a dominant negative and profoundly inhibits cell migration by displacing endogenous START-GAP1/DLC1 from focal adhesions [69]. The SAM domain of START-GAP1/DLC1 may serve as an autoinhibitory domain for intrinsic RhoGAP catalytic activity. Eukaryotic elongation factor-1A1 (EF1A1) was found to be a target of the SAM domain of START-GAP1/DLC1 [70]. EF1A1 is involved in protein synthesis [71] and also in transporting β-actin mRNA [72]. EF1A1 is a regulator of cell growth and the cytoskeletal network controlling the actin network through its G-actin-binding activity [73], F-actin-bundling activity [74] and by stabilizing microtubules [75]. EF1A1 is overexpressed in various human cancers, including pancreas, lung, prostate, breast and colon cancers [71]. The SAM domain of START-GAP1/DLC1 adopts a four-helix fold similarly to the SAM domain of START-GAP2/DLC2, but it utilizes a unique motif on a hydrophobic surface to bind directly to EF1A1 [70]. Importantly, the SAM domain is necessary for START-GAP1/DLC1 to translocate EF1A1 to the membrane periphery and ruffles upon fibroblast growth factor stimulation, acting as an auxiliary oncogenic switch to the GAP domain [70].

3.3. The START domain

The START domain (~210 amino acids) is a well-conserved lipid binding domain, which is primarily found in proteins that transfer lipids between organelles and are involved in lipid metabolism as well as in modulation of signaling events involved in lipid processing [76, 77]. The mammalian START domain protein family is well characterized and is composed of 15 members that are classified into 6 subfamilies based on the sequence and ligand specificity: STARD1/3 and STARD4/5/6 subfamilies bind cholesterol and oxysterols, STARD2 (PCTP: phosphatidylcholine transfer protein)/7/10/11(CERT: ceramide transfer protein) subfamily binds phospholipids and ceramides/sphingolipids, STARD14 binds possibly fatty acids. They all may have roles in non-vesicular lipid transport. STARD14/15 subfamily consists of proteins with the thioesterase activity such as the Acyl-CoA thioesterase (ACOT) family proteins. The START-GAP/DLC family proteins fall into the STARD8/12/13 subfamily [76, 77]. Lipid binding properties of START-GAP1/DLC1 (STARD12), START-GAP2/DLC2 (STARD13) and START-GAP3/DLC3 (STARD8) have not well characterized yet.

Among these proteins, STARD1 (or steroidogenic acute regulatory protein, StAR) and STARD3 (or metastatic lymph node 64 kDa protein, MLN64) appear to be the most well characterized [78]. Both STARD1 and STARD3 bind cholesterol [79], and are also known to play a role in lipid transport into mitochondria [80, 81]. In particular, STARD1 localizes to the mitochondria and stimulates the translocation of cholesterol from the outer to the inner mitochondrial membranes [79]. It has been suggested that STARD1 is an essential component in steroid hormone production in steroidogenic cell [82, 83]. It is noteworthy, therefore, that START-GAP2/DLC2 has been found to localize in mitochondria and was found in proximity to the lipid droplets through the START domain [84]. Future research is required in order to establish the lipid ligand of the START domain of START-GAP2/DLC2 and clarify whether the START domain plays a role in mitochondrial lipid transport. START-GAP2/DLC2 also mediates ceramide activation of phosphatidylglycerolphosphate (PGP) synthase and drug response in Chinese hamster ovary cells [85].

The crystal structures for the START domains of human STARD3/MLN64 (PDB entry: IEM2) [86] and murine STARD4 (PDB entry: 1JSS) [87] were the first to be solved and showed an α-β helix-grip fold with a nine-stranded anti-parallel β-sheet forming a U-shaped hydrophobic cleft that binds the ligand and is flanked by N- and C-terminal α helices. The C-terminal α helix is proposed to serve as a 'cap' to the ligand-binding site, with lipid access to the binding pocket requiring a conformational change in the START domain and movement of the C-terminal helix. To date, the crystal structures for a limited number of the START domain-containing proteins were solved: human STARD1/StAR (PDB entry: 3P0L) [88], human STARD5 (PDB entry: 2R55) [88], human STARD2/PCTP (PDB entry: 1LN1) [87], STARD11/CERT (PDB entry: 2E3R) [89], human STARD13/START-GAP2/DLC2 (PDB entry: 2PSO) [88] and human STARD14 (PDB entry: 3FO5) [88]. The data confirm the basic helix-grip fold structure across the five mammalian subfamilies that defines this family of proteins [88].

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3.4. The FAT region

Between the SAM and GAP domains there is a long unstructured region (~190 amino acids) termed the FAT (focal adhesion targeting) region, due to the fact that its presence determines of focal adhesion localization of the START-GAP/DLC proteins [27]. START-GAP1/DLC1, START-GAP2/DLC2 and START-GAP3/DLC3 are recruited to focal adhesion sites via their FAT sequence, which binds to the *Src* homology 2 (SH2) domains of focal adhesion proteins and interacts with tensins [32, 43, 90]. The same region of START-GAP1/DLC1, however, has been found to possess the ability to interact with the PTB domain of tensin2 [30, 91, 92] and tensin1 [43]. The role of the interaction with the PTB domain in the localization of START-GAP1/DLC1 to focal adhesions remains controversial and requires further investigation. Using pull-down assay, Kawai et al. reported the interaction between the other members of the START-GAP/DLC family, START-GAP2/DLC2 and START-GAP3/DLC3, and tensin2 PTB [30].

Since the START-GAP/DLC family proteins are rich in serine residues, especially in their FAT regions, it is natural to propose that these proteins could be phosphorylated by the AGC (protein kinases A, G, and C) family protein kinases. Indeed, a numerous potential phosphorylation motifs by these kinases can be found in the START-GAP/DLC family proteins. Protein kinase B (PKB or Akt) is among the member of the kinase family. Since PKB/Akt plays an essential role in the actions of growth factors as well as the regulation of many other cellular processes, such as apoptosis and anoikis, neuronal development and degeneration, and the cell cycle [93], its involvement in function of the START-GAP/DLC family proteins is argued [94].

Hers et al. have demonstrated that Ser^{322} in the FAT region of rat START-GAP1/DLC1 is phosphorylated upon insulin stimulation of intact cells and that this site is directly phosphorylated in vitro by PKB/Akt and ribosomal S6 kinase (RSK1), another member of the AGC family of protein kinases [95], suggesting the phosphorylation via both the PKB/Akt and MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK)/RSK pathways by growth factors. In other words, this site has the potential to integrate the activities of two different signal transduction pathways in a manner dependent on the cellular context. As Ser^{322} falls within the FAT region, its phosphorylation may be involved in regulating the targeting of STASRT-GAP1/DLC1 to focal adhesions. However, despite the profound morphological changes, both $S^{322}A$ and $S^{322}D$ mutants showed similar localizations to focal adhesions as the wild-type STASRT-GAP1/DLC1. The function of the phosphorylation in signaling events downstream of PKB/Akt, such as GLUT4 translocation, the activation of RhoA effectors and cellular transformation, therefore awaits further studies.

A recent report by Ko et al. [96] has also postulated a central role of PKB/Akt phosphorylation of human START-GAP1/DLC1 in the regulation of its tumor suppressive activity, but it argues against the results obtained from the previously-mentioned study on rodent STASRT-GAP1/DLC1. Although human START-GAP1/DLC1 has three characteristic phospho-PKB/Akt substrate motifs, the authors showed that only Ser^{567} was phosphorylated by PKB/Akt. Only active PKB/Akt was able to interact with STASRT-GAP1/DLC1. Since phosphorylated START-GAP1/DLC1 forms a more stable interaction with PKB/Akt, there seems to be a cooperative

increase in binding. Furthermore, Ko et al. showed that unphosphorylated START-GAP1/DLC1 is sufficient to suppress proliferation and anchorage-independent cell growth. Using a *ras* transformed, *p53*-deficient murine hepatoma line, the authors demonstrated that only wild-type START-GAP1/DLC1 or a phosphorylation-dead mutant ($S^{567}A$) was able to inhibit tumor formation in nude mice, whereas the $S^{567}D$ mutant, simulating constitutive phosphorylation, did not inhibit tumor growth. PKB/Akt, suggesting that all START-GAP/DLC family members may share common mechanisms of post-translational regulation, also phosphorylated human START-GAP2/DLC2 in the corresponding motif.

The central region of START-GAP1/DLC1 containing the FAT region was reported to target caveolae by interacting with caveolin-1 [28, 92].

4. Regulation of intracellular localization

Generally, members of the START-GAP family, START-GAP1/DLC1, START-GAP2/DLC2 and START-GAP3/DLC3, START-GAP/DLCs do not localize evenly in the cytoplasm. Rather, they are localized in the specialized place in intracellular spaces. All three members are localized to focal adhesions of attached cells. A conserved region among them (the FAT region), responsible for targeting to focal adhesions, has now been identified. It is now established that the tensin family, which is the major component of the focal adhesion complex, is responsible for recruiting the START-GAP/DLC family proteins to focal adhesions. Nevertheless, many proteins are now revealed to interact with START-GAP/DLCs. Vinculin, another member in the focal adhesion complex, can also interacts with the START-GAP/DLC family proteins. In addition, PLCδ1 interacts with all three isoforms of START-GAP/DLCs and make a molecular complex in lipid rafts upon stimulation with extracellular stimuli. Moreover, 14-3-3 binds to START-GAP1/DLC1. Binding of 14-3-3 proteins often sequesters the target protein in a particular subcellular compartment and the release of 14-3-3 proteins then allows the target to be relocated. Since START-GAP1/DLC1 has a nuclear localization signal (NLS) sequence and found in the nucleus, it is suggested that it also interacts with importins.

Thus expected function of the START-GAP/DLC family proteins varies from one cell type to next, depending on the spatiotemporal regulation according to the binding proteins. Nevertheless, as a whole, they regulate cell shapes and motility via remodeling of actin cytoskeleton.

4.1. Focal adhesion targeting via interaction with tensin and vinculin

START-GAP1/DLC1 is localized in focal adhesions via the FAT region located in its N-terminal half and interacts with tensin family proteins, that constitutes focal adhesion components. Evidences that the interaction between START-GAP1/DLC1 and tensin2 occurs in a PTB domain-dependent manner have been provided. It was revealed that FAT3, the third subregion of the FAT region divided into five (39 amino acids), binds directly to the PTB domain of tensin2 [30]. This interaction does not require protein phosphorylation, since the interaction was detected with proteins expressed in bacterial expression system.

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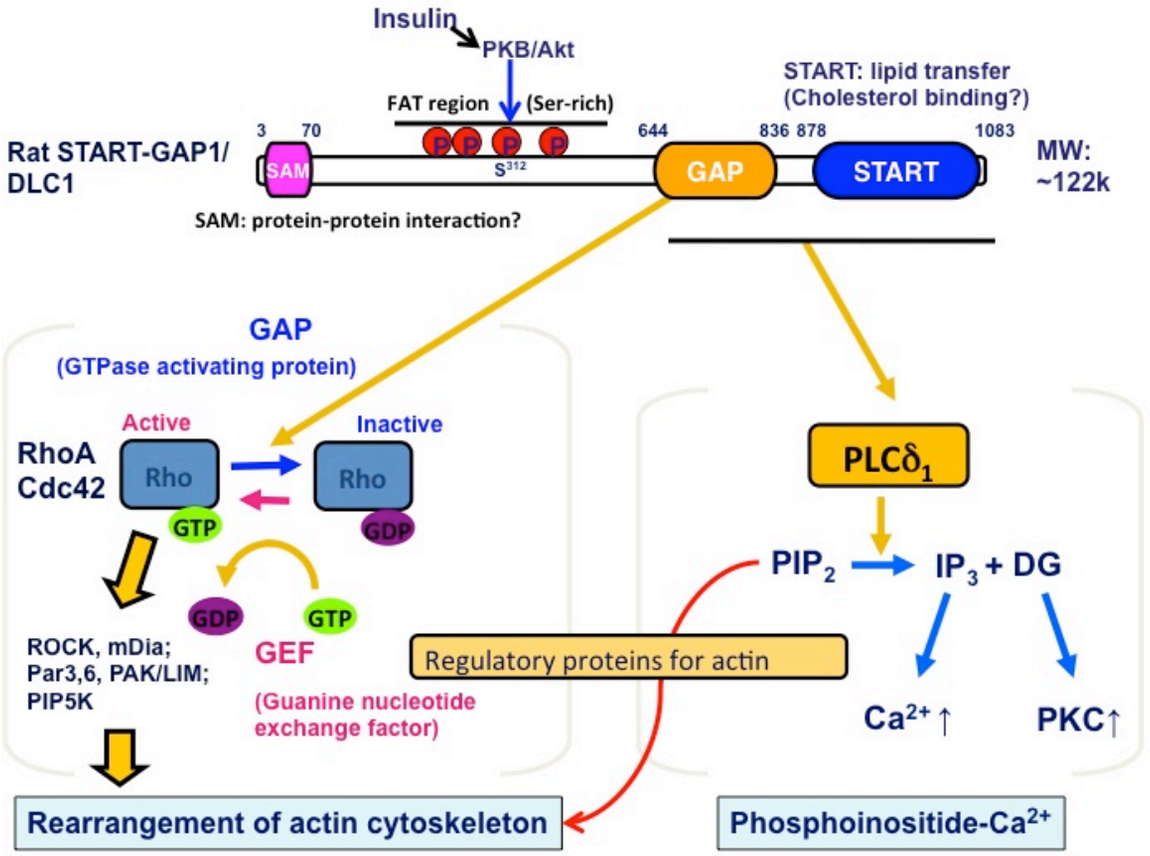

Figure 3. START-GAP1/DLC1 contributes to at least two signaling pathways. START-GAP1/DLC1 consists of about 11 hundred amino acids (for rat: 1,083 and for human: 1, 093) and contain the sterile α motif at the N-terminus, which is known to function in protein-protein interaction. In the C-terminal half there are "GAP domain" followed by the START domain, which is generally thought as a lipid binding or transfer domain. The GAP domain shows a GTPase-activating function, specific for Rho family GTPases, RhoA and Cdc42 but not Rac1 [31], converting "the active, GTP-bound form" to "the inactive, GDP-bound form." The GAP activity of START-GAP1/DLC1 therefore may be implicated in control of the cytoskeleton, cell polarity and cell migration. As for phosphoinositide signaling, one of the downstream effectors of RhoA is PIP5K that generates PtdIns(4,5)P_2. So by inhibition of activated Rho, PtdIns(4,5)P_2 generation is expected to be reduced. The C-terminal half of START-GAP1 is responsible for stimulation of PLCδ1 and breaks down PtdIns(4,5)P_2. This not only causes the generation of two second messengers, Ins(1,4,5)P_3 and diacylgrycerol, but also alters the activity of several PtdIns(4,5)P_2-dependent actin-regulating proteins. As a whole, in microenvironment surrounding active START-GAP1/DLC1, RhoA and Cdc42 are inacitvated and a loss of PtdIns(4,5)P_2 is expected. The region between the SAM and GAP domains is rich in serine residues and does not fall on any known protein domains and has been thought to form disordered conformation.

START-GAP2/DLC2 and START-GAP3/DLC3, as well as STRT-GAP1/DLC1, bind to the PTB domain of tensin2, presumably due to the presence of highly conserved residues in the center of FAT3. Deletion of this sub-region abrogates the interaction with the tensin PTB domain. The tensin2 PTB domain seems to determine the subcellular localization of FAT3. Nevertheless, our study with deletion mutants revealed that FAT3 is essential but not sufficient for the focal adhesion localization of START-GAP1/DLC1. These results suggest that the interaction between the tensin PTB domain and FAT3 contributes to START-GAP1/DLC1 localization but only partially. Other factors could affect the START-GAP1/DLC1 localization.

Amino acid sequence of START-GAP3/DLC3 contains a segment similar to the START-GAP1/DLC1 tensin binding site (^{353}STYDNL^{358}) and full-length START-GAP3/DLC3 was shown to bind the SH2 and PTB domains of tensin1 [43].

We noticed that tensin2 does not always colocalize with START-GAP1/DLC1 and that this interaction was insufficient for targeting START-GAP1/DLC1 to focal adhesions. We thereby explored if there is another molecule that interacts with START-GAP1/DLC1 by the GST pull-down assay using GST-START-GAP1/DLC1 transfected HeLa cells. As a result, we found that START-GAP1/DLC2 can bind with the C-terminus (730-1066) of vinculin. Moreover, START-GAP1/DLC1 and vinculin were found colocalized in foal adhesions. The domain in START-GAP1/DLC1 required for interaction with vinculin was narrowed down to residues 460-470 including an LD motif, a motif in paxillin required for interaction with vinculin. A deletion mutant and point mutants of this motif were fully localized to focal adhesions, although they lost binding ability to vinculin. It is likely that when START-GAP1, vinculin and tensin2 form a stable complex, they are localized to focal adhesions.

PtdIns(4,5)P_2 has an important role in regulating cytoskeleton assembly by inducing conformational changes in actin binding proteins such as vinculin and talin [97]. Thus, START-GAP1/DLC1 could influence cytoskeletal dynamics by altering local PtdIns(4,5)P_2 levels by activating PLCδ1 in the vicinity of focal adhesions as well as by regulating Rho GTPase activity there.

4.2. Raft localization via interaction with PLCδ1

The C-terminal region of START-GAP1/DLC1 covering the GAP and START domain is known to interact with PLCδ1, enhancing its activity [22]. Although START-GAP1/DLC1 binds with PLCδ1, it is not usually localized at the plasma membrane where PLCδ1 targets itself through its PH domain under unstimulated conditions.

Caveolae are plasma membrane domains that appear as flask-shaped invaginations at the cell surface and are enriched in cholesterol and sphingolipids. Yamaga et al. have shown that endogenous START-GAP1/DLC1 was found to sediment with caveolin-1 in low density cholesterol-rich membrane fractions [28], and both endogenous and exogenous START-GAP1/DLC1 were co-immunoprecipitated with caveolin-1 [28, 92]. Since multiple functions have been proposed for caveolae [98] and caveolin-1 has been found in membrane subdomains other than caveolae, including focal adhesions [98] the physiological significance of the interaction between START-GAP1/DLC1 and caveolin-1 requires further investigation.

In their previous study [28], Yamaga et al. provided supportive evidences for START-GAP1/DLC1 localization in caveola, which is cholesterol-enriched membrane microdomain, using an expression system of GFP-tagged proteins, an immunoprecipitation assay and a sucrose density gradient centrifugation analysis of cell lysates. GFP-tagged START-GAP1/DLC1 was observed as patch-like structures at the cell surface and in the cytoplasm of attached cells. The patches were dependent on the levels of the membrane cholesterol and co-localized with caveolin-1. START-GAP1/DLC1 interacts with caveolin-1 in vivo and is fractionated into low-density caveolin-enriched membrane fractions. These results support the idea that START-GAP1/DLC1 is targeted to caveolae via binding to caveolin-1. Yamaga et al. also found the C-

terminal half of START-GAP1/DLC1 was responsible for its patch-like distribution. The authors found amino acid sequences that resemble putative caveolin-binding motifs (ΦXΦXXXXΦ, ΦXXXXΦXXΦ or ΦXΦXXXXΦXXΦ), where Φ is an aromatic residue and X is any amino acid [99] in the N-terminal region (^{14}WLRVTGFPQY^{23} and ^{93}WTFQRDSKRWSRLEEFDVF^{111}) and in the GAP domain (^{690}YVNYEGQSAY^{699} and ^{725}FLQIYQY^{731}) of START-GAP1/DLC1. Since GFP-tagged START-GAP1/DLC1–534ΔC does not seem to reside in caveolin-1-containing membranes, the sequences in the N-terminal region may not function as caveolin-binding motifs. Either (or both) the sequence(s) in the GAP domain, however, could be important for caveolin-1 binding and therefore responsible for the distribution of START-GAP1/DLC1. It is therefore possible that the RhoGAP domain of START-GAP1/DLC1 contributes not only to the catalytic activity but also to the intracellular localization of START-GAP1/DLC1. The exact roles of the N-terminal region have to be clarified. GFP-tagged START domain was also observed as patches, but unlike GFP-tagged full-length protein or GFP-tagged GAP domain, it was just partially co-localized with caveolin-1. The patch-like localization of GFP-tagged START domain was similar to the distribution of cholesterol visualized by filipin, a fluorescent molecule that specifically binds to free cholesterol. The result suggests that the START domain can associate with cholesterol but it is not sufficient to recruit START-GAP1/DLC1 to caveolin-1-enriched membrane microdomains.

Recruitment and activation of PLCδ1 in lipid rafts by a muscarinic agonist was examined using PC12 cells [24]. Sucrose density gradient centrifugation analyses of cell lysates revealed that only a small amount of PLCδ1 was recovered in low-density membrane fractions. The amount, however, significantly increased when cells were treated with a muscarinic agonist carbachol. Immunoprecipitation studies demonstrated that carbachol also enhanced the interaction between PLCδ1 and START-GAP1/DLC1, which is constitutively localized in lipid rafts. The PH domain of PLCδ1 is likely responsible for the interaction. Since carbachol elevates intracellular Ca^{2+}levels in PC12 cells, Yamaga et al. next examined whether a rise of intracellular Ca^{2+} levels participates in the carbachol-induced raft recruitment of PLCδ1. After treatment with a Ca^{2+} ionophore ionomycin PLCδ1 was also translocated to lipid rafts in PC12 cells. Chelating extracellular Ca^{2+} by EGTA did not inhibit the carbachol-induced translocation of PLCδ1 to lipid rafts, whereas treatment of cells with thapsigargin to block the intracellular Ca^{2+} mobilization inhibited its translocation. These results suggest that PLCδ1 is recruited to lipid rafts and binds to preexisting START-GAP1/DLC1 in Ca^{2+} dependent manner, and this process can be triggered by external stimuli activating GPCRs (Figure 4).

Although the physiological function and significance of START-GAP1/DLC1 in caveolar localization remain unclear, it appears that caveolae are one of the compartments whereby START-GAP1/DLC1 may exhibit its tumor-suppressive role and possibly vasoregulatory role which will be mentioned in Section 7, and affect the cytoskeletal reorganization by its RhoGAP activity.

4.3. START-GAP2/DLC2 localization in mitochondria

Apart from focal adhesion localization, START-GAP/DLCs could be localized in mitochondria. Ng et al. has disclosed that START-GAP2/DLC2 is targeted to mitochondria through the

START domain using Huh-7 hepatoma cells [84]. They could observe the expression of ectopic START-GAP2/DLC2 in the cytoplasm especially in a punctate structure, suggesting that START-GAP2/DLC2 was concentrated in cytoplasmic speckles. It is noteworthy that the localization patterns of full-length START-GAP2/DLC2, START-GAP2/DLC2-ΔSAM lacking the N-terminal SAM domain and START-GAP2/DLC2-START, that consists of only from the START domain were very similar, indicating that the START domain is responsible for the intracellular localization. Analysis of the amino acid sequence has revealed that no noticeable localization signal in the START domain. START-GAP2/DLC2-START was in dot-like structure throughout the cytosol, although some cells showed aggregates in the perinuclear region, while START-GAP2/DLC2-ΔSTART, a mutant lacking the START domain, was found to distribute homogenously in the cytoplasm. Notably, almost all START-GAP2/DLC2 signals overlapped those of mitochondria, but not all mitochondria were targeted by START-GAP2/DLC2. They also biochemically fractionated mitochondrial fraction from myc-tagged START-GAP2/DLC2 expressed Huh-7 cells and found that it was found in both the cytoplasmic and mitochondrial fraction. Taken together, the START domain plays a pivotal role in intracellular localization and function of START-GAP2/DLC2. Whether these results could also apply for endogenous START-GAP1/DLC1 or START-GAP3/DLC3 in other cell types awaits future experiments.

4.4. START-GAP2/DLC2 localization around lipid droplets

In addition to demonstrate the mitochondrial localization of START-GAP2/DLC2, Ng et al. also examined whether START-GAP2/DLC2 and/or the START domain of START-GAP2/DLC2 can interact with intracellular lipids [84]. Since localization pattern of START-GAP2/DLC2 and its START domain are very similar, they examined whether lipophilic dyes such as Nile red and Sudan III overlap with signal of the START domain of START-GAP2/DLC2. The speckles containing the START domain were localized around the lipid droplets stained by Nile red. Nevertheless, the two signals did not overlap substantially with each other, despite that a major portion of the START domain was found to be in proximity to the lipid droplets. The authors suggested that the START domain of START-GAP2/DLC2 likely serves a lipid related function in the cell and it targets START-GAP2/DLC2 to areas proximal to the lipid droplets [84].

4.5. START-GAP1/DLC1 localization in the nucleus

Recently, using lung carcinoma cells Yuan et al. showed that START-GAP1/DLC1 harbors a functional bipartite nuclear localization signal (NLS) in serine-rich domain in the FAT region, which works together with the GAP domain to mediate START-GAP1/DLC1 nuclear import and subsequent apoptosis [38]. The potential NLS sequence was found as amino acids ^{415}RRENSSDSPKELKRRNS^{431} including a pat7 NLS spanning residues ^{423}PKELKRR^{429} [100]. A deletion mutant START-GAP1/DLC1-Δ372 lacking the N-terminus 372 amino acids accumulates in the nucleus, suggesting the presence of nuclear export signal (NES) in this region, whereas mutants with disruption of this NLS sequence could only localize in the cytosol.

The function of START-GAP1/DLC1 in the nucleus remains to be defined. Some Rho GTPases have C-terminal polybasic region that could be function as an NLS [101]. Therefore these GTPases may serve as substrates for START-GAP1/DLC1 in the nucleus. In the nucleus START-GAP1/DLC1 may also interact with non-GTPase substrates such as PLCδ1 [22], which is under nuclear import-export equilibrium and accumulate in the nucleus under specific phases of the cell cycle [102, 103] or extracellular stimuli that causes aberrant increase in intracellular Ca^{2+} [103-106]. Nuclear translocation of START-GAP1/DLC1 was proposed to be associated with apoptosis by a yet unknown mechanism [38].

START-GAP1/DLC1 interacts with 14-3-3 proteins, resulting inhibition of the GAP activity and block nucleo-cytoplasmic shuttling [100]. In GST pull-down assays, START-GAP1/DLC1 interacted with all 14-3-3 isoforms except 14-3-3σ. The other six 14-3-3 isoforms are ubiquitously expressed, readily form homo- and heterodimers, and could therefore potentially participate in the regulation of START-GAP1/DLC1 function. 14-3-3 proteins often regulate cellular processes by modulating target protein localization. The pat7 NLS spanning residues of START-GAP1/DLC1, ^{423}PKELKRR^{429}, was demonstrated to be masked by phorbolester-induced phosphorylation and the 14-3-3 interaction. Inactivation of this NLS by exchange of critical arginine residues impaired but did not prevent nuclear import of START-GAP1/DLC1. This suggests the presence of another NLS that contributes to START-GAP1/DLC1 nuclear shuttling.

Taken together, it was suggested that START-GAP1/DLC1 functions both as a cytoplasmic and nuclear tumor suppressor.

5. Negative regulation of carcinogenesis not dependent on the RhoGAP activity

5.1. RhoGAP activity-dependent pathway

As mentioned earlier, the START-GAP/DLC family proteins may exert their suppressive function by decreasing the levels of active, GTP-bound Rho proteins or inhibiting the GDP-GTP cycling process, affecting cytoskeletal remodeling, cell shape, motility, proliferation and apoptosis. Nevertheless, molecular mechanisms through which START-GAP/DLC family proteins are capable of suppressing cell motility and cell growth are still unclear.

Holeiter et al. have shown that enhanced migration of cells lacking START-GAP1/DLC1 is dependent on the Rho effector, Dia1, and does not require the activity of ROCK [107]. Leung et al. provide evidence for a key mechanism through which the START-GAP/DLC family proteins act as tumor suppressors [108]. In this study, the authors demonstrated that the expression of START-GAP2/DLC2 is involved in the inactivation of the Raf/MEK/ERK/RSK pathway, which is crucial for cell proliferation. This inhibitory activity of START-GAP2/DLC2 is attributed to RhoGAP function [108].

By binding to PLCδ1, START-GAP1/DLC1 enhances the hydrolysis of PtdIns(4,5)P_2, which interacts with a variety of actin regulatory proteins that affect the actin cytoskeleton [22, 23,

28]. In addition to the PLCδ1 activation and the modulation of RhoGAP activities, proper focal adhesion localization and interaction with tensins have been demonstrated to be essential for the growth-suppression function of START-GAP1/DLC1 [43, 90, 92, 109].

START-GAP1/DLC1 overexpression induces a significant reduction of stress fibers and filopodia, as well as membrane blabbing and cellular decomposition, nuclear condensation or fragmentation in NCI-H358 cells known as highly sensitive cells to tumor suppression functions of START-GAP1/DLC1 [110]. These effects were due to the collapse of actin cytoskeleton during apoptosis [111].

To overcome the embryonic lethality of homozygous START-GAP1/DLC1 knockdown [57], Zender et al. combined in vivo RNAi and a 'mosaic' mouse model of HCC to address the impact of the loss of START-GAP1/DLC1 on liver carcinogenesis [112]. Genetically modified liver progenitors ($p53^{-/-}$ cells infected with a retrovirus expressing *c-myc* and another expressing a START-GAP1/DLC1 shRNA) were transplanted into the liver of syngenic mice to assess their ability to generate tumors in situ. In contrast to control shRNA, START-GAP1/DLC1 shRNA accelerates the formation of liver tumors, which mimics aggressive HCC. Conversely, reintroduction of START-GAP1/DLC1 in hepatoma cells co-expressing oncogenic Ras results in a dramatic reduction of tumor growth in situ. This study demonstrates that START-GAP1/DLC1 loss, when combined with other oncogenic lesions, efficiently promotes the development of HCC.

Activation of RhoA, and thereby of its downstream effector ROCK, is both necessary and sufficient to promote HCC in vivo. Therefore, START-GAP1/DLC1, due to its RhoGAP activity, is capable to antagonize the activities of RhoA and its downstream effectors in HCC [58, 113, 114]. Moreover, RhoA is required for tumorigenesis induced by the loss of START-GAP1/DLC1 and constitutively active RhoA mimics loss of START-GAP1/DLC1 in promoting HCC in the 'mosaic' mouse model [58].

START-GAP1/DLC1 was also implicated in tumor metastasis. The expression levels of START-GAP1/DLC1 mRNA are significantly lower in highly invasive tumors than in less invasive ones [115]. Restoration of START-GAP1/DLC1 expression in vitro reduces the migration and the invasiveness of HCC cells [35, 41, 113]. This mechanism also seems to be dependent on RhoGAP activity and its downstream effectors ROCK and MLC [42].

Finally, reexpression of START-GAP1/DLC1 in HCC cells downregulated the expression of osteopontin and matrix metalloproteinase-9, which are found overexpressed in most primary metastatic liver tumors [114]. START-GAP1/DLC1 restoration also suppresses the distant dissemination of cells from subcutaneous tumors developing after inoculation of HCC cell lines in mice. This process is also dependent on the RhoA activity and reorganization of actin cytoskeleton in tumor cells [114].

5.2. RhoGAP activity-independent pathway

Angiogenesis is another form of cancer development. In addition to its direct effect via activation of Rho pathway, START-GAP1/DLC1 is responsible for regulation of angiogenesis in an indirect manner [116]. START-GAP1/DLC1 negatively regulates angiogenesis in a

paracrine fashion. START-GAP1/DLC1 silencing promoted pro-angiogenic responses through vascular endothelial growth factor upregulation, accompanied by the accumulation of hypoxia-inducible factor 1 and its nuclear localization.

6. Stability of START-GAP/DLCs

Stability of proteins may also contribute to the expression levels of the START-GAP/DLC family proteins. Generally, degradation of proteins is controlled by ubiquitination-dependent and independent proteolysis. START-GAP1/DLC1 seems to be ubiqutinylated and processed at least partly by ubiquitinylation-dependent proteasomal degradation [117]. Nevertheless, the sites and the mode of ubiquitinylation (mono- or poly-) on the molecule are unclear. In addition, a proinflammatory protein S100A10, a key cell surface receptor for plasminogen and a regulator of proteolysis, was found to be a novel binding partner of START-GAP1/DLC1 [118]. S100A10 colocalizes with START-GAP1/DLC1 in the cytoplasm via interaction between the C-terminus of S100A10 and the central domain of START-GAP1/DLC1, regulating tumor cell invasion [118]. These results strongly suggest that proteolysis dependent on post-transcriptional modification of START-GAP1/DLC1 plays an important role in the regulation of its tumor suppressive activity.

7. Possible roles of START-GAP1/DLC1 in the development of vascular diseases

Recently, another potential role of START-GAP1/DLC1 in the pathogenesis of a human disease has emerged. It has been known that the PLC activity with its effect on the regulation of intracellular Ca^{2+} levels has potential roles in cardiac regulation. PLCδ1 activity has shown to be enhanced in patients with coronary artery spasm, CSA (coronary spastic angina) [119, 120]. In this context, a new finding that START-GAP1/DLC1 protein levels and mRNA levels in fibroblasts from Japanese patients with CSA were enhanced compared with levels in control subjects is noteworthy [121]. The authors also found in the START-GAP1/DLC1 promoter analysis, the -228G/A and -1466C/T variants found associated with CSA patients using 5′-RACE method revealed the increase in luciferase activity [121]. The incidence of -228G-A was more frequent in male patients with CSA than in male control subjects, suggesting that this variant is a possible candidate responsible for upregulation of START-GAP1/DLC1 protein in CSA. Thus, it was postulated that START-GAP1/DLC1 is up-regulated in patients with coronary spasm, possibly by mutations in the promoter region, causing increased $[Ca^{2+}]_i$ to acetylcholine, and thereby seems to be related to enhanced coronary vasomotility.

As stated in the earlier section, START-GAP1/DLC1 plays two important roles in signaling pathways: One of the dual functions is the ability to enhance the PtdIns(4,5)P_2-hydrolyzing activity of PLCδ1 and the other is the GAP activity specific for RhoA and Cdc42. The former leads to both the protein kinase C-mediated pathway and Ca^{2+}-dependent pathway, resulting

in enhanced myosin light chain phosphorylation that plays an important role in the constrictor response of the coronary artery smooth muscle to transmitters such as serotonin and histamine. Homma and Emori showed that recombinant PLCδ1 catalyzes the hydrolysis of PtdIns(4,5)P_2 in a Ca^{2+}-dependent manner; in the presence of START-GAP1/DLC1, its activity is 5- to 10-fold increased in the range of physiological Ca^{2+} concentration [22]. Thus, it is conceivable that upregulation of START-GAP1/DLC1 protein observed in patients with CSA is responsible for the high activity of PLCδ1. These results are consistent with the previous finding of the $H^{257}R$ variant of PLCδ1, in which the conformational change is associated with upregulation of PLCδ1 activity [120]. These characteristics seem to explain the pathogenesis of CSA in which both the basal vascular tone and the vasoconstrictor response to the diverse stimuli were enhanced. Previously, PLC activity was shown to positively correlate not only with basal coronary artery tone but also with the maximal and averaged constrictor responses of the coronary artery to acetylcholine [119]. This finding also suggests a critical role of START-GAP1/DLC1 protein in the genesis of coronary spasm.

The START-GAP1/DLC1 protein has another function, a GAP activity for Rho GTPases. Alterations in RhoA/ROCK pathway have been implicated in the development of a variety of cardiovascular diseases, including hypertension, atherosclerosis, and cerebral and coronary vasospasm [122, 123].

It was reported that the ROCK inhibitor fasudil attenuated the constrictor response of the coronary artery to ACh and prevented the occurrence of chest pain in patients with CSA [124]. By its GAP activity, START-GAP1/DLC1 may antagonize the development of coronary spasm like the ROCK inhibitor. On the other hand, Ca^{2+} mobilization induced by PLCδ1 activation may upregulate Rho and ROCK in the vascular smooth muscle [125]. Thus, the role of START-GAP1/DLC1 in the regulation of Rho is complicated, and the relation of START-GAP1/DLC1 to the genesis of coronary spasm via Rho activity remains to be determined.

The mechanisms of enhancement of START-GAP1/DLC1 promoter activity by the -228G/A variant may be related to transcription factor SP because this variance causes loss of binding to SP1 in its region [121]. Upregulation of START-GAP1/DLC1 protein in the coronary arteries of CSA patients should be confirmed. It remains unclear whether upregulation of START-GAP2/DLC2 or START-GAP3/DLC3 is associated with CSA.

Regarding the caveola localization of START-GAP1/DLC1, the recent report by Nuno et al. that RhoA colocalization with caveolin-1 in caveolae regulates vascular contractions to serotonin is of interest [126]. Caveolins are not only structural components of caveola microdomains, but also regulate assembly and activation of a variety of receptors and signaling molecules such as PtdIns(4,5)P_2, glucose transporter 4 (GLUT4), epidermal growth factor receptors (EGFR) and endothelial nitric oxide synthase (eNOS). Localization of RhoA to caveolae versus noncaveolar lipid rafts differentially regulates its activation and contractions to RhoA-dependent agonists with greater activation associated with its localization to noncaveolar rafts. Presumably, translocalization of START-GAP1/DLC1 to caveolae by agonists reported by Yamaga et al. [24, 28] may also contribute to modulate the activity of RhoA/ROCK pathway in vascular cells.

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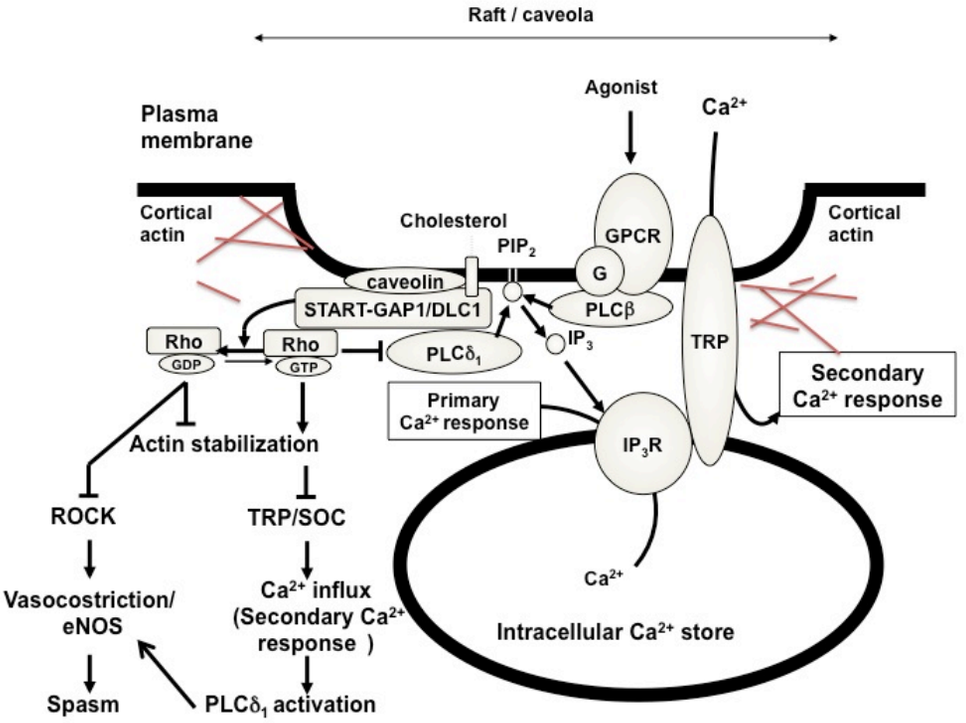

Figure 4. Roles of START-GAP1/DLC1 in a lipid raft/caveola and its possible involvement in the pathogenesis of coronary vasospasm. When G protein coupled receptors (GPCRs) such as muscarinic receptors are activated by agonists they initially stimulate β-type PLC resulting in the production of Ins(1,4,5)P_3 from PtdIns(4,5)P_2. Ins(1,4,5)P_3 binds to the receptors (IP3R or ER Ca^{2+} pump) at the intracellular Ca^{2+} stores such as ER and mobilizes Ca^{2+} from the stores. PLCδ1 is localized at the plasma membrane and in the cytosol in unstimulated cells, whereas START-GAP1/DLC1 is localized in lipid rafts via binding with cholesterol (or with caveolin if the lipid rafts are caveolae). The agonist-induced primary increase in Ca^{2+} recruits PLCδ1 into lipid rafts from other parts of the plasma membrane or from the cytosol by unknown mechanisms. PLCδ1 in the lipid rafts then binds START-GAP1/DLC1 to be activated in the presence of Ca^{2+}, resulting in a robust hydrolysis of PtdIns(4,5)P_2 that forms clusters in lipid rafts. Released Ins(1,4,5)P_3 then empties the internal Ca^{2+} stores via activation of IP3R followed by stimulation of TRP channels, a component of store-operated channels (SOCs) to increase Ca^{2+} influx (secondary Ca^{2+}). Thus the recruitment of PLCδ1 to rafts confers a general positive feedback mechanism for phosphoinositide/Ca^{2+} signaling in the cells. START-GAP1/DLC1 also modulates the RhoA/ROCK signaling pathway. Modified from [24].

8. Conclusion

The START-GAP/DLC family proteins are a group of Rho GTPases whose aberrant function suggests dysregulation of numerous cell processes that can develop diseases such as cancers and vascular spasm. Both in vitro and in vivo studies have provided strong evidence that START-GAP/DLCs have roles in regulating actin cytoskeleton organization. Binding to tensins and other molecules may target the RhoGAP activity of the START-GAP/DLCs to particular subcellular domains. Accumulating reports indicate that the START-GAP/DLC proteins are regulated in various ways at a genetic level by gene deletion, at an epigenetic level by the aberrant promoter methylation, or at a cellular level by the regulation of localization and stability. More information is required on the mechanisms responsible for these regulations,

including post-translational modifications such as phosphorylation and ubiquitination. In addition, dissection of the interacting partners of START-GAP/DLCs by proteomic analyses will reveal detailed signaling pathways that regulate cell shape, motility and proliferation. Future studies on the difference among START-GAP/DLCs may reveal which member of this family plays a key role in individual types of cancer or in vascular abnormality and whether restoration of the proper function of the START-GAP/DLCs is capable of restraining cell transformation or abnormal signaling. These efforts would result in identification of the member that becomes the subject of future studies as a potential drug target for therapy.

Nomenclature

CSA: cardiac spastic angina, DLC: deleted in liver cancer, GAP: GTPase activating protein, HCC: hepatocellular carcinoma, Ins(1,4,5)P_3: inositol 1,4,5-trisphospate (IP_3), NLS: nuclear localization signal, PLC: phosphoinositide-specific phospholipase C, PKB: protein kinase B, PtdIns(4,5)P_2: phosphatidylinositol 4,5-bisphosphate (PIP_2), ROCK: Rho-kinase, START: steroidogenic acute regulatory protein (STAR)-related lipid transfer

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