The SAM domain of the RhoGAP DLC1 binds EF1A1 to regulate cell migration

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Summary
Deleted in liver cancer 1 (DLC1) is a multi-modal Rho-GTPase-activating protein (RhoGAP) and a tumor suppressor. Besides its RhoGAP domain, functions of other domains in DLC1 remain largely unknown. By protein precipitation and mass spectrometry, we identified eukaryotic elongation factor 1A1 (EF1A1) as a novel partner for the sterile alpha motif (SAM) domain of DLC1 but not the SAM domain of DLC2. The solution structure of DLC1 SAM revealed a new monomeric fold with four parallel helices, similar to that of DLC2 SAM but distinct from other SAM domains. Mutating F38, L39 and F40 within a hydrophobic patch retained its overall structure but abolished its interaction with EF1A1 with F38 and L39 forming an indispensable interacting motif. DLC1 SAM did not localize to and was not required for DLC1 to suppress the turnover of focal adhesions. Instead, DLC1 SAM facilitated EF1A1 distribution to the membrane periphery and ruffles upon growth factor stimulation. Compared with wild-type DLC1, the non-interactive DLC1 mutant is less potent in suppressing cell migration, whereas overexpression of the DLC1 SAM domain alone, but not the non-interactive mutant SAM or DLC2 SAM, greatly enhanced cell migration. This finding reveals a novel contribution of the SAM-EF1A1 interaction as a potentially important GAP-independent modulation of cell migration by DLC1.

Key words: DLC1, EF1A1, migration, RhoGAP, SAM

Introduction
Small guanine nucleotide triphosphatases (GTPases) constitute a large family of molecular switches that regulate cell motility, growth, differentiation, apoptosis and intracellular trafficking (Wemenberger and Der, 2004; Hall, 2005; Vega and Ridley, 2007). They are activated by guanine nucleotide exchange factors, which catalyze the exchange of their GDP for GTP, thus keeping them in active conformation, whereas GTPase-activating proteins (GAPs) promote their ability to hydrolyse GTP to GDP. The complexity of their regulation is further enhanced by the fact that all GAPs carry multiple protein modules, the functions of which remain largely unknown. Various combinations of these modules might spatiotemporally regulate the dynamic subcellular localization, activity and anchorage of molecular switches.

Deleted in liver cancer 1 (DLC1) is encoded by a locus, which upon its deletion, is linked to the formation of various cancers, especially liver and breast cancers (Yuan et al., 1998; Goodison et al., 2005; Liao and Lo, 2007). It is a human homolog of the rat p122RhoGAP, which is a GAP for RhoA GTPases (Homma and Emori, 1995). Recent studies show that DLC1 is a candidate tumor suppressor that acts primarily by inhibiting cell migration and tumor metastasis (Goodison et al., 2005) or as a potent apoptosis inducer (Zhou et al., 2004; Yuan et al., 2007). Two other homologs, DLC2 and DLC3, have also been identified and they too can inhibit tumor growth in culture (Leung et al., 2005; Durkin et al., 2007a; Liao and Lo, 2007). DLC1 has three distinct domains: the sterile alpha motif (SAM) at its N-terminus, the steroidogenic acute regulatory-related lipid transfer (START) domain at the C-terminus and a conserved RhoGAP (GAP) domain close to the middle of the protein (Fig. 1A). Like most GAPs, the molecular and cellular mechanisms of DLC1/p122RhoGAP remain elusive (Durkin et al., 2007b; Lahoz and Hall, 2008). The p122RhoGAP was first identified as a binding partner for phospholipase Cδ1 (Homma and Emori, 1995), which occurs at the distal region of the GAP and START domains. More recently, DLC1/p122RhoGAP was shown to be a substrate of protein kinase B (Hers et al., 2006) and tensin proteins at focal adhesions (Liao et al., 2007; Qian et al., 2007), both of which target different sites on the linker region between the SAM and GAP domains. The GAP activity, which was long thought to be essential for the anti-tumor function, was later shown to be specific toward RhoA, RhoB and RhoC. DLC1 dramatically reduced RhoA activity at the leading edge of cell protrusions, whereas the anti-tumor activity of DLC1 is mediated in both RhoGAP-dependent and RhoGAP-independent manners (Healy et al., 2008). However, the function of the DLC1 SAM domain remains uncharacterized. The SAM superfamily comprises members that have diverse cellular targets, such as SAM domains, proteins without SAM domains, and even RNAs (Qiao...
DLC1 binds EF1A1 to regulate cell migration (Bowie, 2005). Despite diverse interaction modes, most SAM domains with known 3D structures adopt a similar structure, consisting of five α-helices arranged in a globular manner. Unlike common SAM domain structures, the structure of the SAM domain of DLC2 features a unique four-helix bundle with a nearly parallel orientation of helices 1 and 4, and helices 2 and 5 (Li et al., 2007; Kwan and Donaldson, 2007).

Here, by using protein precipitation and MALDI-TOF mass spectrometry analyses, we have identified the eukaryotic elongation factor-1A1 (EF1A1, previously known as EF-1α) as a novel target of the SAM domain of DLC1. In addition to its role in protein synthesis (Thornton et al., 2003), EF1A1 is also involved in transporting β-actin mRNA (Liu et al., 2002), binding of G-actin (Murray et al., 1996), bundling of F-actin (Gross and Kinzy, 2005), binding of microtubules (Moore et al., 1998) and also conferring susceptibility to cell death induced by palmitate overload (Borradaile et al., 2006). Furthermore, its expression is also elevated in melanomas and tumors of the pancreas, breast, lung, prostate and colon (Thornton et al., 2003). All these data suggest that EF1A1 is a regulator of cell growth and the cytoskeletal network. Using a combination of structural, molecular and cellular approaches, we have examined the structure of the SAM domain of human DLC1 (DLC1 SAM) further and revealed a novel functional coupling between EF1A1 and DLC1. The DLC1 SAM adopted a four-helix fold similarly to DLC2 SAM but it utilized a unique motif on a hydrophobic surface to bind directly to EF1A1. Importantly, the SAM domain was necessary for DLC1 to translocate EF1A1 to the membrane periphery and ruffles upon fibroblast growth factor stimulation, acting as an auxiliary switch to the anti-metastatic RhoGAP domain. This novel mechanism could help modulate DLC1 function during cell migration and normal development.

Results

EF1A1 as a novel DLC1 interacting partner

Except for the catalytic GAP domain and the tensin-binding site, the biochemical and molecular bases for the functions of all other regions of DLC1 remain largely unknown. To explore the possible function of the SAM domain and its underlying mechanism of action, we set out to identify its putative partners by subjecting purified recombinant glutathione S-transferase (GST)-SAM to the rat liver homogenates. One unique band of 48 kDa was consistently observed (Fig. 1B, arrow). Further tryptic digests and analyses by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry revealed various peptides that formed different parts of EF1A1 (with 23% coverage of total sequence) (Fig. 1C).

To confirm that EF1A1 was indeed a true partner of DLC1, coimmunoprecipitation and GST pull-downs were conducted with HEK293T human embryonic kidney epithelial cells. Fig. 2A shows that endogenous EF1A1 could be co-immunoprecipitated with Flag-tagged DLC1, confirming their interaction inside the cells whereas pull-down studies using DLC1 GST-SAM also detected the endogenous EF1A1 (Fig. 2B). However, no binding was detected with either the GST control or the DLC2 GST-SAM, indicating the specificity of the interaction. Furthermore, His-tagged SAM was overexpressed and purified from HEK293T cells and shown to be
greatly enriched by GST-EF1A1 (Fig. 2C) and not by the GST control. To further substantiate their direct interaction, Flag-tagged DLC1 was transcribed and translated in vitro and was similarly shown to interact with GST-EF1A1 but not the GST control (Fig. 2D). Taken together, these results confirm the specific interaction between the SAM domain of DLC1 and EF1A1. The molecular basis and the cellular and physiological significance of such interaction are further addressed below.

Structure of DLC1 SAM
Given the pleiotropic roles of various members of the SAM superfamily (Qiao and Bowie, 2005), it is important to examine the structure-function relationship of DLC1 SAM and how it interacts with EF1A1. DLC1 SAM exists in a monomeric form even at a protein concentration of ~1 mM, as demonstrated by dynamic light scattering, gel filtration, NMR relaxation experiments and binding studies in HEK293T cells (data not shown). The three-dimensional structure of DLC1 SAM was determined using NMR to a high resolution (Table 1). It consists of four α-helices connected by well-defined loops (Fig. 3A). The polypeptide chain begins with an unstructured N-terminal region (residues 1-7) followed by α-helix 1 (residues 8-26). The chain then takes a turn into helix 2 (residues 29-38). After a long loop region, the chain enters helix 4 (residues 44-50), and then reverses direction into the long C-terminal helix (residues 56-74). Helices 1, 4 and helices 2, 5 are oriented in a nearly parallel manner. The N- and C-terminal helices are located on one side of the fold in the structure. The spatial arrangement of the helices is nearly the same as that of the DLC2 SAM domain (Li et al., 2007), but different from other SAM domains. Packing of these helices is mediated by several hydrophobic and aromatic residues including A16, A19, W22 and L23 (helix 1), Y31, L34, and Y35 (helix 2), I44 and V47 (helix 4) and I59, L62, L66 and L69 (helix 5). No large hydrophobic patches exist, and most charged residues are distributed evenly on the protein surface (Fig. 3B), which is consistent with its high solubility. Although the overall structures of DLC1 and DLC2 SAM domains are very similar (pairwise RMSD of 2.1 Å) because of their high sequence identity (76%), there are a number of significant differences, including their hydrophobic surfaces, length of helix 2, and structure of the loops (Fig. 3C,D). Loop 2 and loop 3 of DLC1 and DLC2 SAM domains are significantly different, because the average RMS deviations between the loop 2 and loop 3 of DLC1 and DLC2 SAM (1.19 Å and 1.83 Å), respectively, are much larger than the RMS deviations for loop 2 and loop 3 in ten DLC1 SAM structures (0.04 Å and 0.37 Å) and ten DLC2 SAM structures (0.09 Å and 1.00 Å). In addition, helix 2 of DLC1 SAM is longer than that of DLC2 SAM. Such differences could result in the selectivity in binding of the two homologous domains to EF1A1.

Identifying a key EF1A1-binding motif on the DLC1 SAM domain
Protein-protein association can result from both hydrophobic and electrostatic and/or hydrogen bonding interactions between interfaces that comprise complementary nonpolar and charged or
DLC1 binds EF1A1 to regulate cell migration

EF1A1 is a multi-functional protein that regulates general protein synthesis as well as the actin and microtubule network (Thornton et al., 2003; Liu et al., 2002; Gross and Kinzy, 2007; Moore et al., 1998). EF1A1 regulates the actin network through its G-actin-binding and F-actin-bundling activity (Liu et al., 2002; Gross and Kinzy, 2005; Murray et al., 1996). Since DLC1 is a human homolog of the rat p122RhoGAP, which acts on RhoA and is a key regulatory switch for actin network, we hypothesized that the DLC1 interaction with EF1A1 could be linked to actin-based dynamics. Actin is assembled into major cellular structures such as focal adhesions, stress fibers, microspikes, cortical actin network and membrane ruffles (Rodriguez et al., 2003). To better understand the functional relationship between DLC1 SAM and EF1A1 in actin-based cell dynamics, we used NIH3T3 cells treated with fibroblast growth factor (FGF) and confocal immunofluorescence microscopy to evaluate how DLC1 (wild type and mutants) and EF1A1 would regulate subcellular localization in some of these actin-rich structures. We first observed that expression of the full-length DLC1 in NIH3T3 fibroblasts led to drastic shrinkage of the cell body with resultant multiple protrusions. These cells also displayed major disruption at the focal adhesions. However, the expressed DLC1 SAM or EF1A1 was never localized to the focal adhesions, as shown by paxillin staining in those cells, nor did it lead to drastic change in actin cytoskeleton (supplementary material Fig. S3). These results indicate that the SAM domain is not required for the DLC1 role of

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<th>Table 1. Experimental restraints and structural statistics for ten lowest-energy NMR structures of DLC1 SAM</th>
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| Violations (mean ± s.d.)                        |
| Distance constraints (Å)                       |
| 0.16±0.02                                      |
| Dihedral angle constraints (°)                 |
| 2.31±0.62                                      |
| Max. dihedral angle violation (°)              |
| 3.43                                           |
| Max. distance constraint violation (Å)         |
| 0.19                                           |

| Deviations from ideal geometry                  |
| Bond lengths (Å)                                |
| 0.0112±0.0013                                   |
| Bond angles (°)                                 |
| 2.13±0.08                                      |
| Mean RMS deviation from the average coordinates (Å) |
| 0.41±0.13                                      |
| Backbone atoms (Cα, C, N, O)                    |
| 0.98±0.12                                      |
| All heavy atoms                                 |

*Generated by TALOS based on backbone-atom chemical shifts. †Protein residues 8-76. A Ramachandran plot of the final ten structures showed 88.1%, 9.9%, 1.9% and 0.1% of residues in the most favored, allowed, additional allowed and disallowed regions, respectively, for DLC1 SAM.

polar residues. A common type of interactive surface contains a hydrophobic patch surrounded by polar groups (Larsen et al., 1998). There are three such regions on the DLC1 SAM surface. The first region consists of six hydrophobic residues, Y31, L34, F40, I42, L46 and V47, and four charged residues, D37, D43, R49 and E50. The second region, which is adjacent to the first hydrophobic region, comprises four hydrophobic residues, A16, Y35, F38 and L39, and two charged residues, E36 and D37. The third one contains several hydrophobic residues centered on L54 and charged residues D55 and D57 (Fig. 3B).

To map the binding site(s) of DLC1 SAM to EF1A1, we designed several SAM mutants with mutated residues in or close to the potential binding interfaces mentioned above and also some charged residues that could mediate binding by electrostatic interaction. They were: one double mutant, K48A/R49A, one triple mutant F38G/L39G/F40G and two quadruple mutants, K48A/R49A/R64A/R65A and F28G/F53G/L54G/A58G. The mutations were chosen on the basis of the structure and van der Waals surface so that they can retain the 3D structure of the wild-type protein. Except for A58, all mutated hydrophobic residues were located in loop regions or the end of the secondary structure elements. Flag-tagged wild-type SAM or its mutants were coexpressed with HA-tagged EF1A1 and coimmunoprecipitations were carried out. The double mutant K48A/R49A and the quadruple mutant K48A/R49A/R64A/R65A retained the binding to EF1A1 (Fig. 4A), suggesting that the positively charged residues alone are not responsible for the binding. By comparison, F28G/F53G/L54G/A58G also retained the binding, yet the triple mutant F38G/L39G/F40G (abbreviated as FLF mutant hereafter) had significantly reduced binding. CD spectra showed that this triple mutant retained the structure of the wild type (supplementary material Fig. S1). 15N-1H heteronuclear single quantum coherence (HSQC) spectra demonstrated that the mutation did not change the global structure of the protein, because residues distant in sequence and space from the mutated region displayed negligible chemical shift changes upon mutation (supplementary material Fig. S2). Therefore, DLC1 SAM interacts with EF1A1 via either one or both of the two hydrophobic regions, which are centered on, or close to, F40. To define their interaction mechanism, we further designed mutants with single-point mutations at the three FLF residues, F38G, L39G and F40G, and carried out immunoprecipitation using HEK293T cells that coexpressed HA-tagged EF1A1 with Flag-tagged wild-type SAM, FLF triple mutant or the three single-point mutants. Fig. 4B shows that single-point mutations F38G and L39G reduced the binding to EF1A1 as significantly as triple mutant FLF, yet the F40G mutant still retained the full binding ability to EF1A1. These results suggest that the hydrophobic region containing F38 and L39 constitutes a key binding motif that mediates the interaction between DLC1 SAM and EF1A1. Interestingly, DLC2 SAM that lacks such a hydrophobic surface after these residues are substituted to S82 and Q83, respectively (Fig. 3C,D; Fig. 4C), did not bind EF1A1 (Fig. 2C; Fig. 4B). This finding is crucial, because comparing the cellular effects of EF1A1, together with the wild-type DLC1 SAM and its non-interactive FLF mutant, will allow immediate assessment of the functional consequence of their interactions.
dissolving focal adhesions, and are consistent with the findings of others (Liao et al., 2007; Qian et al., 2007).

Next, the subcellular localization of DLC1 and EF1A1 at cortical actin network and membrane ruffles in quiescent and FGF-treated NIH3T3 cells was examined (Fig. 5B). During the quiescent state, DLC1 (wild type or FLF) induced cell shrinkage indicative of an active RhoGAP. EF1A1 was mostly concentrated at the cell periphery, whereas DLC1 appeared to be partially colocalised with EF1A1 on cell periphery or distributed in some punctate structures (labeled P in Fig. 5B). By comparison, overexpression of the SAM domain and EF1A1 did not affect the cell morphology whereby SAM was seen colocalised strongly with EF1A1 in the cytosol and at cortical actin (box in Fig. 5B). However, SAM-FLF that did not interact with EF1A1 failed to colocalise with EF1A1 (dotted box in Fig. 5B). To gain a better insight into the nature of the subcellular localization of DLC1, the catalytic arginine finger R677 within the GAP domain of DLC1 was mutated to glutamic acid. This mutant (DLC1 R677E) enabled cell spreading, thus allowing easier study of the intracellular distribution of these two proteins. The majority of DLC1 R677E was concentrated in large punctate structures within the cytosol, whereas EF1A1 now appeared more diffuse in the cytosol than it was in the presence of a GAP-active DLC1. However, no colocalisation of DLC-R677E with EF1A1 was observed at the cell periphery. Compared with the result obtained with the SAM domain alone, this suggests that despite the presence of SAM domain, inactivation of the GAP activity caused the retention of DLC1 in specific subcellular compartments, thus preventing its interaction with EF1A1 during the quiescent state. Consistently, DLC1 R677E with further mutations on the FLF motif (DLC1 R677E-FLF) presented the same results as the DLC1 R677E mutant.

Interestingly, after FGF treatment, DLC1 full-length, SAM and GAP-inactive DLC1 R677E exhibited strong colocalization with EF1A1 in the cytosol and cortical region and also on the membrane ruffles (highlighted in boxes in Fig. 5B). However, introducing the FLF mutant to these constructs resulted in weaker if not complete loss of colocalisation of EF1A1 with DLC1 on these cellular structures. Indeed, EF1A1 appeared to be ‘retarded’ or delocalised to the perinuclear and cytosolic region (Fig. 5B, dotted boxes). Consistently, DLC2 SAM that lacks the hydrophobic surface formed by FLF residues in DLC1 SAM and which does not bind EF1A1, did not colocalise with EF1A1 on these structures either. Taken together, these results indicate the ability of the SAM domain of DLC1 to facilitate the targeting of EF1A1 to the cortical and ruffling membranes via its specific interaction with EF1A1.

Since formation of membrane ruffles is associated with cell migration, and little is known about the contribution of other protein modules on the effect of DLC1 on anti-metastasis other than the contribution from the linker region between the SAM and GAP or its GAP domain and/or activity, we sought to determine how DLC1 SAM could impact this important biological process. Motile NIH3T3 cells were transiently transfected with GFP plasmid together with greater excess of the Flag-tagged vector (Ctrl) or Flag-tagged wild-type DLC1 (DLC1 WT), or with full-length DLC1 with the specific triple FLF mutations (DLC1 FLF). After 4 hours, cells were allowed to migrate and the proportions of all transfected cells were then scored for their ability to exert migration as described in the Materials and Methods. Fig. 6A shows that wild-type DLC1 greatly inhibited the rate of migration by more than 70%. However, the DLC1 FLF mutant displayed only a slightly lower potency of inhibition compared with the wild type (P=0.06). DLC1 is known to exert its antimetastatic effect that requires its catalytic GTPase-

Fig. 3. Solution structure and van der Waals surface of DLC1 SAM. (A) Ribbon drawing of the ten lowest-energy conformers. The four helices are indicated in red and yellow. (B) Views of the van der Waals surface, illustrating potential protein-protein association interfaces. The side chains are colored: green, hydrophobic; blue, basic; red, acidic; and grey, polar (and main chain). (C) Superimposition of SAM domains of DLC1 (green) and DLC2 (violet). The helices (H) are represented as ribbons. These helices are labeled according to the conserved folds among all known structures of SAM, where helix 3 is absent only in DLC1 and DLC2 SAM. (D) Four hydrophobic residues A16, Y35, F38 and L39 (green) on the EF1A1-binding motif of DLC1 SAM and four corresponding residues of DLC2 SAM (i.e. A60, Y79, S82 and Q83) are shown. The figures were created with MOLMOL (Koradi et al., 1996).
activating protein domain. Interestingly, cells expressing DLC1 R677E did not completely abolish the ability of DLC1 to suppress cell migration (Fig. 6B) despite their complete loss of activity towards endogenous Rho, as evidenced by the Rho activity assay (Fig. 6C). This result indicates that GAP-independent modulation of cell migration exists. Since DLC-FLF exhibited weakener inhibition on cell migration, we questioned whether this mutant could confer weaker DLC1 GAP activity inside the cells. In contrast to this, the result in Fig. 6C shows that DLC1 FLF did not affect DLC1 GAP activity, thus it could constitute one of the GAP-independent modes of modulation on cell migration. It is likely that the weaker effect of DLC1 FLF on inhibition of cell migration is due to failure of DLC1 FLF to facilitate EF1A1 distribution in cells, including its recruitment to actin-rich regions as seen above.

To further address the possible role of EF1A1 on DLC1-mediated morphogenesis and cell migration, we used shRNA to knock down the endogenous EF1A1. Interestingly, such knockdown (with sh-42) resulted in major arborizations of cells that coexpressed the exogenous DLC1 SAM and DLC1 FLF to facilitate EF1A1 distribution in cells, including its recruitment to actin-rich regions as seen above.

For further details, please refer to the original article.
Fig. 5. DLC1 SAM domain facilitates recruitment of EF1A1 to the membrane periphery and membrane ruffles. NIH3T3 cells were transfected with various constructs and made quiescent followed by treatment for 20 minutes with 10 ng/ml fibroblast growth factor (FGF). Cells were then fixed, permeabilized, stained and visualized under confocal immunofluorescent microscopy. (A) SAM domain is not required for DLC1 function in dissolving focal adhesions. Cells were transfected with Flag-DLC1, Flag-DLC1 SAM, Flag-DLC1 ΔSAM or HA-EF1A1 and treated with FGF before fixation. Ectopic expression of the proteins was detected by anti-Flag or anti-HA (red), and the focal adhesions are shown by anti-paxillin (green), followed by appropriate fluorophore-conjugated secondary antibodies. Merged signals are presented as overlaid staining (yellow). Arrow indicates untransfected control cell. (B) Cells were cotransfected with HA-EF1A1 and Flag constructs of either DLC1 wild type (DLC1), DLC1 full-length mutant F38G/L39G/F40G (DLC1 FLF), DLC1 full-length mutant R677E (DLC1 R677E), DLC1 full-length with a mutant consisting of a combination of R677E and F38G/L39G/F40G (DLC1 R677E-FLF), DLC1 SAM domain wild type (SAM), SAM domain mutant F38G/L39G/F40G (SAM-FLF), or DLC2 SAM domain. Coexpressed HA-EF1A1 (a; blue), and different Flag-DLC1 constructs (b; green) were detected by appropriate anti-Flag and anti-HA antibodies followed by fluorophore-conjugated secondary antibodies. Cells were labeled with TRITC-phalloidin (c; red) to mark the cell border, the cortical actin on the cell periphery or membrane ruffles. Merged signals are presented in panels labelled d (cyan for panel a overlaid with panel b; purple for a overlaid with c; yellow for b overlaid with c; bright white for a overlaid with both b and c). The boxes highlight regions of tight colocalization whereas dotted boxes indicate regions of low or no colocalization. The P labels punctate structures. The intensity of images was enhanced to capture changes in the cell periphery and cell protrusions. Scale bars: 10 μm.

conceivable that the exogenous SAM domain might act as a dominant-active mutant that interrupts the interaction between endogenous DLC1 and EF1A, thus reversing the suppressive effect of the endogenous DLC1. Taken together, our results strongly support the notion that DLC1 could mobilize EF1A1 to the membrane periphery and membrane ruffles via its SAM domain, which could help establish actin-based dynamics potentially linked to the moderate suppression of cell migration. This finding therefore reveals a novel contribution of the SAM-EF1A1 interaction as part of the potentially important ‘GAP-independent’ modulation of cell migration by DLC1.

Discussion
A novel structure and function for the SAM domain of DLC1
SAM domains represent an emerging and important protein domain superfamily with diverse cellular functions (Qiao and Bowie, 2005). The number of encoded SAM domains (in brackets) roughly correlates with the complexity of genomes in S. cerevisiae (4), C. elegans (26), D. melanogaster (42), mice (178) and human (206).
They could serve as a docking site for kinases (e.g. ETS-1 transcriptional activator for ERK2 MAP kinase), as a polymeric device (e.g. for transcriptional repression function of TEL), as a RNA-binding domain (e.g. Smaug), or as a signaling scaffold through homophilic interactions (e.g. for Byr2 serine/threonine kinase, Eph receptor tyrosine kinase and diacylglycerol kinase). Some SAM domains also engage non-SAM proteins as their binding partners (e.g. interaction of BAR with Bcl-2 and Bcl-XL). Given the uniformity in structural folds but diversity in their functions (Kim and Bowie, 2003), our understanding on the precise role(s) of SAM domains awaits systematic and thorough examinations on their structures, interacting partners and cellular functions.

Combining proteomics, biochemical, structural and cellular studies, our current work has added yet another important aspect of SAM function for DLC1, a RhoGAP protein and a candidate tumor suppressor where it interacts specifically with an emerging multi-functional protein, the translational elongation factor EF1A1. Such interaction is specific and could provide an important step in suppressing cell migration. In connection with this, our finding provides an important link between cell metabolism and cell dynamics where it offers several new insights and possible functions.

**Functional coupling between DLC1 and EF1A1**

EF1A1 is involved in diverse biological processes. They range from protein synthesis (Thornton et al., 2003), oncogenic transformation (Tatsuka et al., 1992), cell proliferation (Gangwani et al., 1998), transporting mRNA (Liu et al., 2002), bundling of F-actin (Gross et al., 1998), conferring susceptibility to cell death induced by palmitate overload (Borradaile et al., 2006) or by hydrogen peroxide (Chen et al., 2000), and also conferring cytoprotection against ER-stress-induced apoptosis (Talapatra et al., 2002). EF1A1 expression is elevated in melanomas and tumors (Thornton et al., 2003) and correlates with metastasis (Taniuchi et al., 1992) and oncogenic transformation (Tatsuka et al., 1992). EF1A1 is therefore an emerging regulator in cell growth, cell death and cytoskeletal network control; however, the molecular mechanisms remain largely unknown.

Our finding provides an important link between cell metabolism and cell dynamics where it offers several new insights and possible underlying mechanism associated with the role of DLC1 in regulating tumor formation and metastasis. Here, we show that DLC1 facilitates the recruitment of EF1A1 to the membrane periphery and ruffles via its SAM domain. By contrast, SAM mutants devoid of EF1A1 binding fail to recruit EF1A1 to these structures and also fail to affect cell migration. Consistently, the corresponding DLC1 full-length mutant also becomes less potent in suppressing cell migration. In connection with this, overexpression of DLC1 SAM but not DLC2 SAM unexpectedly promoted cell migration. Such a paradox could best be explained

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**Fig. 6.** Effects of DLC1 SAM on cell migration. (A,B,D) The effects of different DLC1 constructs on cell migration were assayed in a Boyden chamber. Data are presented as the means ± s.d. for 3–4 independent experiments. Statistical comparison was made using ANOVA and Newman-Keuls multiple comparisons. NIH3T3 cells were migrated on fibronectin. (A) NIH3T3 cells were transfected with GFP-tagged plasmid as a cell marker together with twice the quantity of Flag-tagged vector (Ctrl), Flag-tagged DLC1 full-length wild-type (DLC1) or FLF full-length mutant (DLC1 FLF). Data denoted by different letters indicate significant difference at P<0.05. (B) NIH3T3 cells were transfected with GFP-tagged plasmid as cell marker together with twice the quantity of Flag-tagged vector (Ctrl), Flag-tagged DLC1 full-length wild-type (DLC1) or R677E full-length mutant (DLC1 R677E). (C) HEK293T cells were transfected with vector control or with Flag-tagged DLC1 or its various deletion or point mutants as indicated. Lysates were then subjected to GST-RBD pull-down assays as described in the Materials and Methods to assay the amount of active Rho being precipitated, hence a measurement for the GAP activity of DLC1. (D) MCF7 cells were transfected with GFP-tagged vector (Ctrl), GFP-tagged DLC1 SAM domain wild type (SAM), FLF mutant (SAM-FLF) or GFP-tagged DLC1 SAM domain. Data denoted by different letters indicate significant difference at P<0.02.
with the idea that overexpressed SAM acts as a dominant active form to displace endogenous DLC1-EF1A1 interaction, thus reversing the inhibition. However, this hypothesis has yet to be tested. Consistent with this is our observation that overexpression of DLC2 SAM, which does not bind EF1A1, failed to exert any stimulatory effect on cell migration. Based on our binding, localization and cell migration studies, the effect mediated by SAM and SAM-FLF are likely to be specific to EF1A1. However, we cannot completely rule out the likelihood that this same region might still be important in directly or indirectly mediating the binding of other protein(s) inside the cells, which themselves could also be important in regulating cell migration. Furthermore, how would targeting of EF1A1 affect the seemingly pro-metastatic and pro-migratory potentials of EF1A1? To partly resolve this issue, a specific mutant of EF1A1 that could not bind DLC1 SAM (but hopefully still retains its many other biological functions) needs to be identified and re-introduced to the EF1A1-knockdown background. How this mutant redistributes to the cell periphery and ruffles in the presence and absence of DLC1, and the impact on cell migration could then be ascertained. Given that EF1A1 is also involved in bundling of F-actin (Gross and Kinzy, 2007), and microtubule dynamics (Moore et al., 1998), it remains to be seen how DLC1 and EF1A1 function in these crucial biochemical processes. In this regard, disposition of EF1A1 with cortactin or Arp2/3 should be better defined to establish any direct link to actin dynamics. Together, we propose a model whereby the primary role of DLC1 SAM is to facilitate EF1A1 translocation to the sites of active actin assembly/disassembly during cell motility control. Interestingly, the wild type or the FLF mutant SAM domain could still be localized to those structures independently of other functional modules of DLC1. The basis for such autonomous translocation remains unknown.

DLC1 was previously reported to localize at focal adhesions through the linker region between SAM and RhoGAP domain, and it was recently shown to dramatically reduce RhoA activity at the leading edge of cellular protrusions (Liao et al., 2007; Qian et al., 2007; Healy et al., 2007). It is worth noting that the cellular relocalization of several other proteins found at the membrane ruffles and at focal adhesions were indicated as a key regulatory event in polarized cell migration (Wonzniak et al., 2004). As the effect of DLC1 SAM serves to recruit EF1A1 to the membrane periphery and ruffles, and helps to suppress cell motility, such an auxiliary effect should ensure better control of DLC1 activity in cell migration under physiological conditions.

Although our current work focuses on delineating the binding sites on DLC1 SAM, we have yet to find out precisely on which part of EF1A1 such interaction occurs. EF1A1 comprises multiple functional modules, and our preliminary data revealed that both the GTP-binding domain and the actin-binding domain of EF1A1 are probably involved in their interaction with DLC1 SAM (data not shown). Consequently, the involvement of these two distinct domains of EF1A1 suggest that although F38-L39 is the essential motif (possibly by initiating the interaction), it is plausible that other secondary binding sites on the SAM domain could still exist to aid further stabilization. All these results imply a complex regulation of EF1A1 function and interaction, the mechanism of which awaits further detailed structural and mutational studies. Given that EF1A1 is involved in actin-mediated cell morphogenesis in yeast (Gross and Kinzy, 2005; Gross and Kinzy, 2007), our results nonetheless provide the first evidence that EF1A1 is indeed important for cell migration by linkage to a RhoGAP protein in the mammalian system.

However, EF1A1 can be phosphorylated in vitro by Rho kinase, an effector for RhoA and that modification results in reduced actin bundling (Izawa et al., 2000). Interestingly, it also binds to Bni1p, the mDia homolog in yeast (Umikawa et al., 1998), which is another effector of Rho or the split pleckstrin-homology domain of phospholipase Cγ1 to regulate IP3 production (Chang et al., 2000). In plants, EF1A1 acts as an activator of phosphatidylinositol 4-kinase (PI4K) and regulates phosphatidylinositol (4,5)-biphosphate via its interaction with phospholipase Cγ1 (Yang et al., 1993; Yang and Boss, 1994). Recently, EF1A1 was identified as a binding partner of Akt2 (Lau et al., 2006), whereas the other isoform EF1A2 was shown to bind and activate PI4K and Akt, depending on the intracellular abundance of phosphatidylinositol (3,4,5)-trisphosphate (Amiri et al., 2006; Jeganathan and Lee, 2007). In this regard, DLC1 is also a target for Akt kinase signaling in response to insulin (Hers et al., 2006). Therefore, it is likely that DLC1 and EF1A1 could act in concert to regulate the PtdIns(3,4,5)P3-Akt pathway for cell growth, apoptosis and motility control.

Taken together, our results provide the first evidence that the RhoGAP DLC1 can functionally interact with EF1A1 through its SAM domain, which could facilitate the localization of EF1A1 towards actin-rich regions and modulate cell migration. As DLC1 has already been shown to exert its inhibition on cell migration, at least via its GAP activity, this finding reveals a novel contribution of the SAM-EF1A1 interaction that could represent an important GAP-independent modulation of cell migration by DLC1. The complexity of the function and regulation of DLC family proteins can now be further examined by simple modularity and then validated in the context of the whole protein. It remains to be explored just which functions each module confers towards the overall function and regulation of DLC family proteins.

Materials and Methods
Plasmid construction

Full-length cDNAs for DLC1 (Accession no. NM_006094) and EF1A1 (Accession no. NM_001402) were generated by reverse-transcription-based PCR from total RNA isolated from human HEK293T cells and cloned into Flag-tagged, HA-tagged, glutathione S-transferase (GST), or GFP-tagged pX410 vector, GST-tagged pGEX-4T1 vector or myc-His-tagged pcDNA3.1. Deletion mutants were generated by PCR using specific primers with appropriate restriction sites whereas point mutants were derived by site-directed mutagenesis, as described previously (Luo and Low, 2004). Clones were verified after sequencing entirely in both directions and propagated in Escherichia coli strain DH5α and XL1-blue.

Identification of DLC1-interacting partners

DLC1-interacting partners were identified as described previously (Luo and Low, 2004) with minor adjustment. Briefly, livers from adult female rats were homogenized in buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 5 mM sodium orthovanadate, 5 mM glycerol 2-phosphate and protease inhibitors). Glutathione-Sepharose beads (Amersham Biosciences) coated with 100 μg GST or GST-SAM were mixed with the lysate or just the lysis buffer (as control) and incubated overnight at 4°C. After extensive washing, bound proteins were eluted with lysis buffer containing 4 M urea, resolved by SDS-PAGE and visualized by silver staining (Bio-Rad). The unique bands were analyzed with MALDI-TOF as previously described.

Cell culture and transfection

HEK293T cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μM penicillin, and 100 μg/mL streptomycin and maintained at 37°C in a 5% CO2 atmosphere. MCF7 and NIH3T3 cells were grown in DMEM (high glucose) and maintained at 37°C in a 5% CO2 atmosphere. HEK293T cells were grown in DMEM (high glucose) with 10% fetal bovine, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Precipitation, direct binding studies and western blot analysis

Cells were lysed in lysis buffer 1 containing 150 mM sodium chloride, 50 mM Tris-HCl pH 7.5, 0.25 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and protease inhibitors.
Expression and purification of DLC1 SAM for NMR spectroscopy

The wild-type DLC1 SAM and FLP mutant were cloned to a modified pET-M vector and overexpressed in BL21 (DE3) growing in M9 minimal medium. Isotope-enriched protein was prepared using 13C-labeled glucose and 15N-labeled NH4Cl as the sole carbon and nitrogen sources. Purification was facilitated by an N-terminal His tag. Further purification was achieved by using size-exclusion chromatography.

NMR spectroscopy

All NMR experiments were performed at 25°C on a Bruker 500 MHz or 800 MHz NMR spectrometer. The protein sample concentration was typically 0.5–1 mM. For all measurements, the sample solution contained 50 mM sodium phosphate buffer (pH 7.0) in 90% H2O, 10% D2O. For assignment of the 1H, 13C, and 15N resonances, a standard set of heteronuclear NMR spectra were recorded with 15N-labeled or 13C, 15N-labeled DLC1 SAM. Aromatic resonances were assigned using a 3D 1D, 15N-edited NOE experiment (Lin et al., 2006). Proton-proton distance restraints were measured from the same NOE experiment. All data were processed with NMRPipe and analyzed with NMRView.

Structure calculation

NOE cross peaks were assigned using the automated NOE assignment using the new software CANDID and the torsion angle information. Structures were calculated with CYANA (Herrmann et al., 2002) using the standard protocol. Ten conformers with the lowest final target function values were selected from 100 calculated conformers for further energy minimization in AMBER (Case et al., 2002). The final ten structures were validated using PROCHECK-NMR (Laskowski et al., 1996) and deposited in the Protein Data Bank (PDB ID: 2GYT).

Immunofluorescence

Transfected NIH3T3 cells were immunostained as described previously (Luo and Low, 2004). Briefly, NIH3T3 cells seeded on sterilized glass coverslips were transfected with various epitope-tagged expression plasmids for DLC1 and/or EF1A1, starved for 18-24 hours in medium with 0.5% serum before treatment with 10 ng/ml fibroblast growth factor (FGF) (Promega) for 20 minutes. After fixing and permeabilization, they were incubated with monoclonal anti-paxillin (BD Transduction Laboratories), polyclonal rabbit anti-HA (Zymed Laboratories) and/or monoclonal M2-anti-FLAG (Sigma) followed by incubation with Alexa Fluor 594-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated donkey anti-mouse IgG, Pacific Blue-conjugated goat anti-rabbit IgG (Molecular Probes). Filamentous actin was identified by staining with TRITC-phalloidin (Sigma). Coverslips were examined by confocal fluorescence microscopy (Olympus FV500). All images were captured with a x100 objective lens.

Rho activation/inactivation assays

HEK293T cells were lysed (50 mM Tris-HCl pH 7.4, 2 mM magnesium chloride, 1% Triton-X-100, 10% glycerol, 100 mM sodium chloride, supplemented with 1 mM DTT and a mixture of protease inhibitors) and lysate diluted five times with lysis buffer and incubated with GST-RhoETK-RBD for 30 minutes at 4°C (Besson et al., 2004). The beads were collected by centrifugation and washed three times with lysis buffer. Bound proteins were eluted, separated by SDS-PAGE and analyzed by western blot analyses with polyclonal anti-Rho antibodies (Santa Cruz Biotechnology).

Cell migration assay

Migration assays were conducted in modified Boyden chambers (24-well Transwell, Corning Costar; 8 μm pore size) as previously described (Shang et al., 2003). The lower surface of the filter was coated with 5 μg/ml fibronectin (Sigma) as a chemoattractant. Transfected NIH3T3 or MCF7 cells were seeded at a density of 1.5×10^6 in 100 μl DMEM (high glucose) with 0.2% bovine serum albumin. 600 μl DMEM (high glucose) containing 10% serum was added to the lower compartment. Equal amounts of cells were seeded in six-well plates in DMEM with 10% serum. After 4 hours, the cells that did not penetrate the filters were removed with cotton swabs. The transfected cells in the six-well plates and the transfected cells that had migrated to the lower surface were fixed with 4% paraformaldehyde in 1× PBS and counted.

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Figure S1

The figure shows a graph with the x-axis labeled as Wavelength (nm) ranging from 180 to 270 and the y-axis labeled as Mean Residue Ellipticity ranging from -20,000 to 40,000. The graph compares the mean residue ellipticity of wild type DLC1-SAM (black line) and FLF mutant (red line) proteins. The wild type DLC1-SAM shows a significant decrease in mean residue ellipticity, while the FLF mutant maintains a relatively higher value.
Figure S2
Figure S3 (continued)
Figure S4 (continued)

**GFP + sh-22**

**GFP + sh-32**

**GFP + sh-42**

**DLC1**

**DLC1-R677E**