Deleted-in-liver cancer 1 (DLC1) exerts its tumor suppressive function mainly through the Rho-GTPase–activating protein (RhoGAP) domain. When activated, the domain promotes the hydrolysis of RhoA-GTP, leading to reduced cell migration. DLC1 is kept in an inactive state by an intramolecular interaction between its RhoGAP domain and the DLC1 sterile α motif (SAM) domain. We have shown previously that this autoinhibited state of DLC1 may be alleviated by tensin-3 (TNS3) or PTEN. We show here that the TNS3/PTEN-DLC1 interactions are mediated by the C2 domains of the former and the SAM domain of the latter. Intriguingly, the DLC1 SAM domain was capable of binding to specific peptide motifs within the C2 domains. Indeed, peptides containing the binding motifs were highly effective in blocking the C2-SAM domain-domain interaction. Importantly, when fused to the tat protein-transduction sequence and subsequently introduced into cells, the C2 peptides potently promoted the RhoGAP function in DLC1, leading to decreased RhoA activation and reduced tumor cell growth in soft agar and migration in response to growth factor stimulation. To facilitate the development of the C2 peptides as potential therapeutic agents, we created a cyclic version of the TNS3 C2 domain-derived peptide and showed that this peptide readily entered the MDA-MB-231 breast cancer cells and effectively inhibited their migration. Our work shows, for the first time, that the SAM domain is a peptide-binding module and establishes the framework on which to explore DLC1 SAM domain-binding peptides as potential therapeutic agents for cancer treatment.

Deleted-in-liver cancer 1 (DLC1) is a tumor suppressor that was initially implicated in hepatocellular cancers (1–4). It is now known to be deregulated, via deletion or down-regulation by epigenetic mechanisms, in malignancies of the lung, stomach, colon, kidney, uterus, ovary, pancreas, prostate, and breast (5). DLC1 has four known isoforms, α, β, γ, and 4i. The ubiquitous, 1091-residue DLC1α and the longer, 1528-residue DLC1β isoforms have been associated with focal adhesions (6, 7). Both isoforms harbor three structurally defined domains and an intervening, unstructured serine-rich (SR) region. An N-terminal sterile alpha motif (SAM) domain is separated from a Rho-GTPase activating protein (RhoGAP) domain by the SR region (6). The SR region is a hot spot for protein–protein interaction that plays important roles in the regulation of DLC1 function. Of note, the SR region has been shown to bind the 14-3-3 adaptor protein (8), tensins (9–11), the focal adhesion kinase and talin (12). The region has also been shown to assist in protein kinase A-induced dimerization of DLC1 (13). Moreover, the SR region undergoes phosphorylation mediated by the cyclin-dependent Ser/Thr kinase CDK5 (14). Recently, we identified a phosphorylation-mediated molecular interaction switch comprising DLC1, tensin-3 (TNS3), phosphatase and tensin homologue (PTEN), and phosphoinositide 3-kinase (PI3K) (10). We further showed that dynamic interactions of these proteins with each other in response to motility cues such as the epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) play an important role in the migration of mammary epithelial cells and breast cancer cells. Remarkably, phosphorylation of specific Thr residues within the C2 domains of TNS3 and PTEN, following the growth factor stimulation, triggers the switch of binding partners for DLC1 and phosphoinositide 3-kinase to promote cell migration (10).

The function of DLC1 as a tumor suppressor and regulator of cell migration is primarily dependent on its RhoGAP domain, which catalyzes the hydrolysis of GTP-bound RhoA (2, 15). Although the RhoGAP activity may be regulated by phosphorylation of the SR region in DLC1 (14, 16), we have shown that a direct intramolecular interaction between the SAM and RhoGAP domains keeps DLC1 in an autoinhibited state (11, 18, 19). This work was supported, in part, by grants from the Canadian Institute of Health Research (to S. S. C. L.), the Canadian Cancer Society (to S. S. C. L.), and the Natural Science Foundation of Hubei Province, China Grant 2016CFA053 (to X. C.). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S12.

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§The abbreviations used are: DLC1, deleted-in-liver cancer 1; SR, serine-rich; SAM, sterile α motif; Rho-GTP, Rho-GTPase–activating protein; TNS3, tensin-3; PTEN, phosphatase and tensin homologue; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; Fmoc, N-(9-fluorenyl)methoxycarbonyl; aa, amino acid; GST, glutathione S-transferase; HRP, horseradish peroxidase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; DAPI, 4',6-diamidino-2-phenylindole; HGF, hepatocyte growth factor; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt.
The SAM domain of DLC1 binds directly to the TNS3 and PTEN C2 domains

We have previously shown that the DLC1 SAM domain is capable of binding to PTEN and TNS3 through a homologous region (10, 11). Binding by TNS3 or PTEN may activate DLC1 by releasing the intramolecular interaction between the SAM and RhoGAP domains, thereby resulting in increased RhoGAP activity, decreased Rho-GTP level, and reduced cell migration (Fig. 1A). To exploit this mechanism to control cell migration, we first examined if the homologous C2 domains in TNS3 and PTEN would bind directly to the DLC1 SAM domain. To this end, we transfected HEK293 cells with expression constructs for the TNS3-C2 or PTEN-C2 domain fused to green fluorescence protein (GFP) together with the FLAG epitope-tagged DLC1, the SAM domain, or a DLC1 mutant in which the SAM domain was deleted (DLC1-H9004). Immunoprecipitation of the GFP-C2 domains followed by Western blotting showed that both the full-length DLC1 and the SAM domain co-immunoprecipitated with the TNS3 or PTEN C2 domain, whereas DLC1-H9004 did not (Fig. 1B). This indicates that the SAM domain is required for DLC1 binding to the C2 domain.

The DLC1 SAM domain recognizes specific peptides within the TNS3 or PTEN C2 domain

We report here that the DLC1 SAM domain binds directly to the TNS3 and PTEN C2 domains. Using peptide-walking arrays, we defined the regions in the two C2 domains that mediate SAM-binding and identified specific peptides that bound the SAM domain with micromolar affinities. Our work show, for the first time, the SAM domain is a peptide-interaction module capable of binding to a variety of different proteins. Intriguingly, binding of TNS3 to the DLC1 SAM domain blocks interaction of the latter with the RhoGAP domain, thereby promoting RhoGAP activity and resulting in decreased cellular RhoA-GTP levels (10). These studies suggest that targeting the SAM domain-mediated protein-protein interactions may be an attractive strategy to control cell migration by manipulating cellular RhoA-GTP levels via DLC1.

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the spot array is 12-aa in length with 2-aa overlap between neighboring peptides in the array (Fig. S1, A and B). The peptide arrays were subsequently probed for binding to the purified GST-SAM (Fig. 2A) or GST (for background binding, Fig. S2) and the bound protein visualized by anti-GST Western blots.

This led to the identification of three distinct regions in the PTEN-C2 domain, with the 230GPTR, 257FFHK, and 334NRYF motifs, respectively, that were recognized by the DLC1-SAM domain (Fig. 2, A, upper panel, and B). In contrast, only a single motif on the TNS3-C2 domain, 244CYHK, was recognized by the SAM domain (Fig. 2, A, lower panel and B). Intriguingly, this motif shares significant sequence identity to the 257FFHK motif in PTEN-C2 (Fig. 2B), suggesting similar mechanisms may be used by both C2 domains for SAM-binding.

Although the C2 peptide arrays included phosphorylated versions of the bound peptides, none was found to bind the DLC1-SAM domain (white boxes in Fig. 2A; Fig. S1, A and B). The SAM-binding peptides mapped to specific β-strands or surface loops at one side of the PTEN-C2 structure (Fig. 2C). Although the TNS3-C2 domain structure is not yet available, it should be noted that the 244CYHK peptide from TNS3-C2 aligns with the 257FFHK motif of PTEN-C2 domain (Fig. 2, B and C).

To measure the binding affinities of the identified C2 peptides for the SAM domain in solution, we synthesized peptides representing the different motifs with a fluorescein tag. Fluorescence polarization binding assays were conducted for the peptide-SAM complexes and the equilibrium binding curves were used to derive the corresponding dissociation constants (KD) (Fig. 2, D and E; Table 1; Fig. S3). The strongest interaction was observed for the 230GPTR motif-containing peptide from PTEN-C2 (called the PTEN-C2 peptide for short hereafter) with a KD of 0.87 μM (Fig. 2D; Table 1). In contrast, the 244CYHK motif-containing peptide (called the TNS3-C2 peptide hereafter) had a KD of 2.18 μM (Fig. 2E; Table 1), which is 7-fold stronger than the equivalent 257FFHK peptide from the PTEN-C2 domain (Fig. 2B; Table 1). A peptide containing the other PTEN-C2 motif, 334NRYF, was much weaker in binding, with a KD of 25.87 μM (Table 1; Fig. S3A). To rule out that the interaction was disulfide bond-dependent, a 244SYHK motif-containing variant of the TNS3-C2 peptide was also synthesized and tested for binding, which showed a minor change in affinity (KD = 3.58 μM) compared with the 244CYHK peptide (Table 1; Fig. S3A).

The importance of the 244CYHK and 230GPTR motifs for SAM-binding was investigated by Ala-scanning peptide spot arrays. The N-terminal half of each peptide was found to play an important role as substitution of any residue within this region led to a marked decrease or loss of SAM-binding (Fig. S4, A and B).
SAM-binding peptides activate DLC1 to inhibit cell migration

Table 1
Equilibrium dissociation constants for the SAM-C2 peptide interactions

| Peptide (Sequence)       | $K_d$ | μM |
|-------------------------|-------|----|
| TNS3-C2 peptide (CYHKKYSRATRD) | $2.18 \pm 0.45$ |
| PTEN-C2 peptide (PPTREDKFMYF) | $0.87 \pm 0.18$ |
| PTEN-C2-FFHK (FFHKQNKMLK| $14.05 \pm 1.51$ |
| PTEN-C2-NRYF (NRYSPNFKVLY) | $25.87 \pm 2.87$ |

a $K_d$ values were derived from fluorescence polarization measurements.

B). To confirm this finding, we synthesized analogues of the C2 peptides in which the 2nd and 3rd residues in the two motifs were substituted by Ala. Fluorescence polarization assay showed that the resulting peptides, containing the GAAR or CAAK motif, lost binding to SAM (Fig. S4, C and D).

The C2 peptides disrupt the SAM domain-C2 domain interaction

To find out if the two C2 peptides could inhibit the corresponding SAM-C2 interaction in cells, we resynthesized these peptides with an N-terminal, HIV-derived tat-motif (27) to facilitate cellular transduction and a fluorescein tag for easy detection. We also synthesized a scrambled version of the fluorescein-tat-TNS3-C2 peptide as control. The tat-peptides were able to transduce the MDA-MB-231 (breast cancer), HCC78 (liver cancer), and HEK293DSL.C1 (HEK293 overexpressing DLC1) cells with equal efficiency (Fig. S5) and the transduced peptides appeared as punctate structures that colocalized with actin (Fig. S6). With confirmation of cellular transduction, we then tested if the C2 peptides could block the interaction between GFP-TNS3-C2 or GFP-PTEN-C2 and GST-DLC1-SAM that were co-expressed in the HEK293DSL.C1 cells. To this end, we first established the conditions for binding between the SAM and two C2 domains. In agreement with previous findings (10), GST pulldown showed that the SAM domain bound more robustly to the TNS3-C2 domain in the absence, than in the presence, of EGF in serum-starved HEK293DSL.C1 cells (Fig. 3A). In contrast, the SAM-PTEN-C2 interaction was stronger in the presence of EGF stimulation (Fig. 3C). This discrepancy may be due to the distinct effect of phosphorylation of the two C2 domains on SAM-binding (10).

To assess the efficacy of the C2 peptides in disrupting the SAM-C2 interaction, an incremental amount of a fluorescein-tat-C2 peptide was added to the cells in the absence or presence of EGF followed by GST pulldown. As shown in Fig. 3, A and B, the TNS3-C2 peptide blocked TNS3-C2 binding to the SAM domain at a concentration as low as 5 μM, whereas the PTEN-C2 peptide exhibited a significant inhibitory effect only at 10 μM or above (Fig. 3, A and B). Intriguingly, in the presence of EGF, the PTEN-C2 peptide disrupted SAM-binding by the PTEN-C2 domain at 5 μM, whereas the TNS3-C2 peptide was unable to completely inhibit the SAM-C2 domain-domain interaction even at 15 μM (Fig. 3, C and D). The scrambled TNS3-C2 peptide control, in contrast, was unable to block SAM binding to either C2 domain even at 30 μM regardless of EGF. These results indicate that the C2 peptides have distinct abilities in disrupting SAM-binding to the corresponding C2 domains. Nevertheless, at high peptide concentrations (e.g. 30 μM), both peptides are effective in blocking the SAM-C2 interactions.

The C2 peptides inhibited RhoA activation and anchorage-independent cell growth

Because the TNS3-C2 domain can act as inhibitor of cell migration (10), apparently by binding to the SAM domain and mitigating its inhibition of DLC1-RhoGAP, we inquired whether the C2 peptides were sufficient to activate the RhoGAP and thereby, promoting RhoA-GTP hydrolysis (10, 11). To this end, the HEK293DSL.C1 cells were cultured in serum-free and EGF-containing medium with increasing concentrations of a C2 peptide (from 0 to 18 μM) or scrambled control (at 18 μM). Rhotekin-RBD beads were used to pulldown GTP-bound RhoA from the cell lysate followed by anti-RhoA Western blotting (10). Although both C2 peptides were capable of blocking RhoA activation at 18 μM, the PTEN-C2 peptide was more effective as it abolished cellular GTP-RhoA at 10 μM (Fig. 4A). This result is consistent with the finding above (Fig. 3, C and D) showing that the PTEN-C2 peptide inhibited the C2-SAM interaction more effectively than the TNS3-C2 peptide in the presence of EGF. To ascertain that the inhibitory effect of the C2 peptide on RhoA activation was not limited to the specific cell type or growth factor examined, we repeated the assay on MDA-MB-231 (a breast cancer line) under EGF stimulation and HCC78 (a liver cancer line) under the treatment of HGF (hepatocyte growth factor) and obtained essentially identical results (Fig. 4, B and C). It should be noted that the peptide treatment did not alter the cellular level of DLC1 in any of three cell lines tested (Fig. 4A; Fig. S7). Furthermore, the C2 peptides had no effect on Rac1 activation (Fig. S8), consistent with DLC1 being a Rho-specific GAP.

Because RhoA is known to play a role in tumorigenesis (4, 28, 29), we investigated if the corresponding tat-C2 peptides would inhibit cellular transformation using an anchorage-independent cell growth assay. To this end, the HEK293DSL.C1 cells were seeded on soft agar and allowed to grow in the absence or presence of a C2 peptide. Relative to the scrambled TNS3-C2 peptide control that showed no significant effect on colony formation, both tat-C2 peptides significantly ($p < 0.01$) reduced the number of colonies formed in soft agar. Moreover, the tat-PTEN-C2 peptide showed a more remarkable effect than the tat-TNS3-C2 peptide (Fig. 4, D and E), again reinforcing earlier results (Fig. 4A). It should be noted that the C2 peptides had no significant effect on cell proliferation or apoptosis (Figs. S9 and S10).

The C2 peptides reduced anchorage-independent growth and growth factor-induced migration of cancer cells

To corroborate the results obtained using HEK293DSL.C1 cells, we next tested the efficacy of the C2 peptides in inhibiting transformation and migration of MDA-MB-231, a triple negative breast cancer cell line with endogenous expression of DLC1 (10). To this end, we subjected the MDA-MB-231 cells to soft-agar growth assays in the presence of a C2 or control peptide. To ensure that the effect of C2 peptides was mediated by the
SAM domain, we overexpressed the SAM domain deletion mutant of DLC1, DLC1ΔSAM, in the MDA-MB-231 cells. The two C2 peptides and DLC1ΔSAM caused a significant reduction in colony formation in soft agar (Fig. 5, A and B). Specifically, the tat-TNS3-C2 peptide reduced colony formation by 38%, whereas the tat-PTEN-C2 peptide reduced the colony number by 55% compared with the scramble control (Fig. 5, A and B). This result is in excellent agreement with that obtained using the HEK293DLC1 cells (Fig. 4, D and E).

Because RhoA plays a critical role in cell migration (10, 29), we next determined the effect of the C2 peptides on cell migration using wound-healing assays. The C2 or control peptides were added, respectively, into the culture of serum-starved MDA-MB-231 under EGF stimulation (10). We found that both the C2 peptides, but not the scrambled control, effectively inhibited RhoA activation at the 16th hour. These results suggest that the C2 peptides inhibited cell transformation and migration through inactivating RhoA. The C2 peptides showed the same inhibitory effect on the migration of the HCC78 and HEK293DLC1 cells (Fig. S11).

**Cyclization of the TNS3-C2 peptide facilitated plasma membrane penetration without loss of anti-migration effect**

Because the target (DLC1) of the C2 peptides is intracellular, the inability of these peptides to cross the lipid bilayer of the plasma membrane greatly limits their therapeutic potential. Although we have shown that this limitation may be overcome by fusing the peptide with the tat sequence, we also wanted to test if cyclization of the C2 peptides themselves would provide an alternative for their intracellular delivery. Unlike linear peptides, cyclic peptides, exemplified by cyclosporine (30, 31), have been shown to penetrate the plasma membrane. Compared with the PTEN-C2 peptide that required all 12 aa for optimal binding, the TNS3-C2 peptide appeared to be less stringent in sequence requirement (Fig. S4, A and B). We therefore focused on the cyclization of the latter. To optimize efficiency of cyclization, we first subjected the TNS3-C2 peptide to serial truncations to identify the minimal sequence for SAM-binding. Probing the peptide truncation arrays with GST-SAM allowed the identification of a 7-residue motif, CYHKKYR, which retained full binding ability of the parent peptide (Fig. 6A, red asterisk).
To enable cyclization, we added the Gly-Cys dipeptide to the C terminus of this motif and used oxidation by air to promote the cyclization of the resulting peptide (now a 9-mer) through formation of an intramolecular Cys-Cys disulfide bond (32, 33). Ellman’s reagent assay, HPLC separation, and MALDI-MS verified the cyclic peptide with a final yield of 52% (Fig. S12).

Confocal microscopy suggested that the fluorescein-labeled TNS3-C2 cyclic peptide penetrated the MDA-MB-231 cells more efficiently than the corresponding linear peptide fused to the tat sequence as the former was detected intracellularly within 30 min, whereas the latter at 4 h (Fig. 6B). Moreover, the cyclic peptide, but not the linear counterpart, was detectable at 72 h, suggesting the cyclic peptide was more stable than the linear version (Fig. 6B). Importantly, the cyclic TNS3-C2 peptide showed a similar level of efficiency as the linear peptide in inhibiting the migration of the MDA-MB-231 cells triggered by EGF (Fig. 6C).

Figure 4. The C2 peptides decreased RhoA activity in multiple cell lines and reduced colony formation by DLC1-expressing HEK293 cells. A, both TNS3 and PTEN C2 peptides were able to inhibit RhoA activation in the DLC1-overexpressing HEK293DLC1 cells in a concentration-dependent manner. B and C, the same RhoA-GTP inhibitory effect was observed for the C2 peptides in EGF-treated MDA-MB-231 cells (B) or HGF-treated HCC78 cells (C). D, colony-formation in soft agar for the HEK293DLC1 cells in the absence (no treatment) or presence of a C2 peptide or the scrambled TNS3-C2 peptide. E, quantify of colony-formation data in D; *, denotes a p value < 0.01 (n = 3), Student’s t test; IB, immunoblotting.

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Discussion

DLC1 has been implicated in numerous cancers, including metastatic breast cancer (10, 11, 25, 34, 35). Recent studies have underscored the importance of DLC1 in actin skeleton reorganization and directional cell migration via its interactions with TNS3 and PTEN (10, 11, 36). TNS3 and PTEN act as alternate binding partners for DLC1 or P13K, depending on the phosphorylation states of the TNS3 and PTEN C2 domains (10). The binding partner-switch for DLC1 has been shown to regulate the spatiotemporal activation of Rac1 and RhoA under growth factor stimulation and govern directional cell migration fate (10). These previous studies suggest that the DLC1-TNS3/PTEN interactions are mediated by the C2 domains of TNS3 and PTEN and the SAM domain of DLC1 (10, 11, 25). Furthermore, these studies also indicate that the SAM domain plays a crucial role in the autoinhibition of the RhoGAP domain. Lowy and colleagues (12, 14, 16, 37) have shown that the autoinhibition of the DLC1-RhoGAP domain may also be mediated by Ser phosphorylation of the SR region by Akt1 and CDK5. It is therefore likely that either or both of the SAM domain and SR region may regulate the RhoGAP activity in DLC1, depending on cellular context.
The SAM domain is a small (60–90 residue) helical domain present in numerous proteins in the animal and plant kingdoms, including >200 in human proteins (17, 22). The modular domain primarily acts as scaffolding entity in larger, multidomain proteins involved in orchestrating cellular signaling. SAM domains display a remarkable promiscuity in binding macromolecules that include proteins, lipids, and RNA (18, 19, 22). There have been extensive investigations into the structure and function of the SAM domain of the EphA2 tyrosine kinase receptor (38–44). In comparison, the function of the DLC1 SAM domain is not fully understood despite a few early studies on the topic (17, 24, 45, 46). We have shown here that the DLC1 SAM domain binds directly to the TNS3 or PTEN C2 domain with micromolar affinities. Moreover, multiple peptides from the two C2 domains are capable of binding the DLC1 SAM domain. These findings have several important implications. First, our study shows, for the first time, that the DLC1 SAM domain is both a protein- and a peptide-interaction module. Although the SAM domain has been shown to form homo- or heterodimers, it has not been characterized for binding to other proteins or peptides. The versatile nature of the DLC1 SAM domain in protein/peptide binding greatly expands the repertoire of proteins that this modular domain may interact. It is likely that other SAM domains may have the same characteristics and therefore warrant further investigation. Second, our study suggests that the DLC1 SAM domain is capable of binding to peptides with distinct sequences. It would be interesting to find out how this versatility in ligand-binding is accomplished by such a small modular domain. Third, our study identifies the C2 domain as a protein-binding module. Like the SAM domain, the C2 domain has been found in numerous proteins (47). Studies to date have been focused on the calcium-dependent lipid-binding property of the C2 domain. In this regard, the PTEN/TNS3 C2 domains bind phospholipids, but in a calcium-independent manner (47, 48). Our work supports the notion that the C2 domain is also a protein-interaction module (49–51). It would be interesting to investigate if other C2 domains possess the same protein-binding capability and whether some C2 domains have the dual properties of lipid- and protein-binding.

Protein-protein interactions are frequently mediated by modular domains and targeting protein-protein interaction is an attractive approach for cancer therapeutics (50, 51). We explored this principle in the current study by identifying and developing peptides that block the SAM-C2 interaction. We have shown that peptides derived from the TNS3 or PTEN C2...
domains are potent inhibitors of the SAM-C2 interaction in cells. These peptides, when fused to the tat protein transduction sequence (27, 52) or cyclized, effectively activated DLC1 RhoGAP in both DLC1-expressing HEK293 cells or cancer cells, including MDA-MB-231 and HCC78. The ensuing inactivation of RhoA led to markedly reduced tumorigenic potential for these cells as assessed by anchorage-independent growth and decreased migratory potential as measured by wound-healing assay. Although DLC1 expression can be silenced by epigenetic mechanisms, many cancers express DLC1 (53). Therefore, peptide-based strategies to activate DLC1 RhoGAP, as illustrated by this study, may be a feasible approach in cancer therapy. Our finding that both the linear tat-C2 peptide and the cyclic version were capable of effectively inhibiting tumorigenic and migratory potential of cancer cells raises the possibility that these peptide inhibitors may be exploited for potential therapeutic applications.

**Experimental procedures**

**Antibodies**

Rabbit anti-DLC1 (H-260; sc-32931) and mouse anti-DLC1 (C-12; sc-271915) were obtained from Santa Cruz Biotech Inc. Anti-RhoA (catalog number ARH03) was supplied in the RhoA pulldown kit from Cytoskeleton Inc. Anti-GST-HRP (catalog number A7340), rabbit anti-GFP (catalog number G1544), and mouse anti-FLAG (M2; catalog number F1804) were obtained from Sigma-Aldrich. Rabbit anti-Rac1 was obtained from GenScript (A00660). Goat anti-mouse IgG (H+L)-HRP (catalog number 170–6516) and anti-rabbit IgG (H+L)-HRP (catalog number 170–6515) conjugates were obtained from Bio-Rad Laboratories.

**Expression constructs**

DNA sequence encoding the DLC1-SAM (residues 13–78) was cloned into the pGEX-2T vector (Addgene) for the expression of GST-SAM in *Escherichia coli*. FLAG-DLC1, FLAG-DLC1-SAM, FLAG-DLC1ΔSAM, GFP-TNS3-C2, and GFP-PTEN-C2 for mammalian cell expression were cloned as reported previously (10).

**Cell culture and transfection**

HEK293, MDA-MB-231, and HCC78 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured as monolayers as described (10). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing antibiotics and 10% fetal bovine serum (FBS; Sigma-Aldrich). MDA-MB-231 cells were grown in DMEM/F-12 containing antibiotics and 10% FBS. HCC78 cells were grown in RPMI1640 medium containing antibiotics and 10% FBS. Serum-free/starved growth media contained no FBS and growth factor. EGF treatment medium contained 20 ng/ml of EGF, and EGF treatment was for 30 min at 37 °C in 5% CO₂. Plasmids (2 μg) were transiently transfected using X-tremeGENE HP transfection reagent (Roche) at 70% confluence, using the manufacturer’s protocols. Cultures were allowed to grow for an additional 16 h before further treatments.

**Peptide arrays and soluble peptide synthesis**

Cellulose spot peptide arrays and soluble peptides were synthesized on a Multipep synthesizer from Intavis AG Bioanalytical Instruments. Peptide arrays were printed on cellulose membranes and soluble peptides were synthesized on TFA-cleavable resins (Rink resin; AnaSpec Inc.) using Fmoc chemistry. Design, probing, and blotting (Far Westerns) of peptide arrays followed the same procedures as reported previously (54). Peptide walking arrays were designed with 2-amino acid residue overlaps. Truncation peptide arrays were designed to interrogate single-amino differences. N-hydroxysuccinimide (NHS) fluorescein (Pierce) was used to label the N-terminal of soluble peptides when required. 6-Aminohexanoic acid (AnaSpec Inc.) linkers separated the Tensin3 (TNS3) and PTEN C2 peptide motifs from the cell-penetrating peptide (HIV-based tat motif, GRKKRRQRRRPQ) and the NHS-fluorescein label, to avoid possible steric interference during protein-peptide interactions. Peptides were purified by repeated cold-ether precipitation and desalted on Sephadex® G10 (Sigma-Aldrich) columns or purified on HPLC (C18 columns; Waters). Peptide masses were validated by MALDI MS.

**Determination of equilibrium dissociation constant (K_d) by fluorescence polarization**

Binding affinities of GST and the GST-DLC1-SAM domain for the fluorescein-labeled peptides from TNS3-C2 or PTEN-C2 were determined using a Multi-label Reader (PerkinElmer Life Sciences). Fluorescein-labeled peptides were diluted and incubated with increasing concentrations of DLC1-SAM in 20 mM Tris (pH 7.0), 150 mM NaCl, 3 mM DTT. The FP data (experiments performed in triplicates) was fitted to a non-linear regression model as described previously (55).

**Pulldown assays for RhoA-GTP**

Rhotekin-RBD beads pulldown and immunoblotting for Rho-GTP and total RhoA followed the manufacturer’s protocol (Cytoskeleton Inc., BK036). For peptide penetration, *tat*-peptides (at concentrations of 0–18 μM) were incubated with the cell culture at 37 °C for 0.5 h before lystate preparation, pulldown, and immunoblotting. For the GFP-C2 domain pulldown assay, 0–30 μM *tat*-C2 peptides were used to treat the HEK293DLC1 cells. Western blots were quantified using ImageJ.

**Pulldown assays for Rac1-GTP**

PAK-PBD beads pulldown and immunoblotting for Rac1-GTP and total Rac1 followed the manufacturer’s protocol (Cytoskeleton Inc., BK035). For peptide penetration, *tat*-peptides (at concentrations of 0–18 μM) were incubated with the cell culture at 37 °C for 0.5 h before lystate preparation, pulldown, and immunoblotting. Western blots were quantified using ImageJ.

**Fluorescence microscopy and confocal microscopy**

MDA-MB-231 cells were grown in glass-bottom dishes until 20–40% confluence. Cultures were incubated for 30 min to 72 h with linear (9-mer) and cyclic (9-mer) fluorescein-tagged *tat*-labeled or nonlabeled peptides (18 μM) in serum-free media containing EGF. Cells were fixed by incubation in 3.7% formal-
SAM-binding peptides activate DLC1 to inhibit cell migration

dehyde for 5 min. The fixed cells were washed with 1× phosphate-buffered saline (PBS) twice and treated with 0.2% Triton X-100 for 10 min. The fixed cells were rhodamine phalloidin stained and washed with 1× PBS (twice). Cells were imaged using a LSM-510 Zeiss META/ConfoCor2 microscope or an Olympus FV1000 microscope, after adding VectaShield® mounting medium containing DAPI to the fixed cells.

Anchorage-independent growth (soft-agar) assay

HEK293 and MDA-MB-231 cells (70% confluence) were transfected with plasmids encoding DLC1-SAM or treated with a tat-peptide (30 μM). After incubation with peptides for 4 h, the cells were trypsinized and plated at a density of 5 × 10^4 cells in 0.3% agarose in DMEM (10% FBS), on a layer of 0.6% agarose suspended in DMEM (10% FBS) in 6-well-plates (in triplicates). A small aliquot of cells was saved for ascertaining cell counts using a hemocytometer, post-peptide treatment (4 h) or transfection (16 h). The soft agar bilayers, with proliferating colonies, were replinished with DMEM (10% FBS) containing ± tat-peptides and EGF, twice a week. Colonies were allowed to grow in 37 °C in 5% CO2 for 22 days. Soft agar wells were stained for 1 h with crystal violet (Sigma-Aldrich) and washed in PBS. Colonies were counted and the numbers normalized to untreated cell cultures grown in soft-agar. Colonies >100 μm in diameter were counted. Representative micrographs were taken using an Infinity Capture Imaging system (Lumenera Corporation) mounted on a Motic AE31 inverted microscope (Matic Microscope).

Wound-healing assay

Cell monolayers at ~100% confluence in serum-free medium were scratched using a 200-μl pipette tip and cell debris were rinsed off with PBS. Wounded monolayers were incubated for 24 h in ± serum, ± EGF, and tat-peptides (18 μM) or cyclic peptides (30 μM). Images were captured at 0 and 24 h using the Infinity Capture Imaging System (Lumenera Corporation) mounted on a Motic AE31 inverted microscope (Matic Microscope). The cell migration front was established as one that had a continuous line of live migrating cells. As all wounds created were not the exact size between wells in which the cells were allowed to migrate, the wound areas at the end points were calculated by normalizing it to the wound area at 0 h for each well. Wound area dimensions were then determined using the analyze and measure functions of the ImageJ software and are expressed as a percentage.

Optimization of TNS3-C2 peptide

The 7-mer motif of TNS3-C2 was used as the core sequence for synthesizing cyclic peptides with two additional amino residues (Gly-Cys) at the C-terminal end to provide a linker and a disulfide-bond partner. The 9-mer linear peptide was synthesized as described before and all further cyclization steps were performed with the cysteine side chains de-protected first before cyclization. The 9-mer was sized as described before and all further cyclization steps were performed. After a week. Colonies were allowed to grow in 37 °C in 5% CO2 for 22 days. Soft agar wells were stained for 1 h with crystal violet (Sigma-Aldrich) and washed in PBS. Colonies were counted and the numbers normalized to untreated cell cultures grown in soft-agar. Colonies >100 μm in diameter were counted. Representative micrographs were taken using an Infinity Capture Imaging system (Lumenera Corporation) mounted on a Motic AE31 inverted microscope (Matic Microscope). The cell migration front was established as one that had a continuous line of live migrating cells. As all wounds created were not the exact size between wells in which the cells were allowed to migrate, the wound areas at the end points were calculated by normalizing it to the wound area at 0 h for each well. Wound area dimensions were then determined using the analyze and measure functions of the ImageJ software and are expressed as a percentage.

Flow cytometry

Cells were culture until 70% confluence. After 16 h serum starvation, cells were incubated with 18 μM peptides for 24 h. Subsequently, cells were harvested and resuspended in annexin binding buffer (10 mM Hepes (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2) and stained with annexin V-FITC (Biolegend) and SYTOX™ AADvanced (ThermoFisher Scientific). All flow cytometry samples were analyzed using LSRII flow cytometer (BD Biosciences) and FlowJo V10 (FlowJo LLC). A minimum of 20,000 events was recorded.

Cell proliferation assay

Cells were cultured in 100 μl of medium in 96-well-plates. After 16 h serum starvation, cells were incubated with 18 μM peptides for 24 h. Subsequently, cell number was evaluated using Sigma WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), 10 μl of WST-8 solution was directly added to the 100 μl of medium. Cells were then returned to the incubation chamber for 30 min. The 96-well-plates were then read for absorbance at 460 nm.

Peptide transfection efficiency detection by fluorescein absorbance measurement

Cells were cultured in 96-well-plates. After 16 h serum starvation, cells were incubated with or without 18 μM peptides for 4 h. After washing twice with PBS, 200 μl of RIPA buffer (25 mM Tris, 75 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.6) was used to lyse the cells. Intracellular fluorescence was detected by 480 and 535 nm for excitation and emission, respectively. For comparison, the absorbance of the scramble peptide was set as 100% (Fig. S5).

Statistical analysis

All statistical analyses were based on paired Student’s t test using Excel and GraphPad®. A p value of <0.01 was considered to be statistically significant.

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