Citation for published version


DOI

https://doi.org/10.1016/j.str.2016.04.016

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LD Motif Recognition by Talin: Structure of the Talin-DLC1 Complex

Highlights
- The DLC1 LD motif forms a helix that binds the talin R8 rod domain
- Talin R8 also binds paxillin LD motifs and recruits paxillin to focal adhesions
- Charge complementarity is key to the interaction between LD motifs and talin R8

In Brief
DLC1 activity depends on binding of its LD motif to talin. Zacharchenko et al. report the structure of the DLC1/talin R8 rod domain complex. They define charge interactions critical for LD-motif recognition by the R8 helical bundle and identify paxillin as a novel talin binding protein.

Accession Numbers
5FZT

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**SUMMARY**

Cell migration requires coordination between integrin-mediated cell adhesion to the extracellular matrix and force applied to adhesion sites. Talin plays a key role in coupling integrin receptors to the actomyosin contractile machinery, while deleted in liver cancer 1 (DLC1) is a Rho GAP that binds talin and regulates Rho, and therefore actomyosin contractility. We show that the LD motif of DLC1 forms a helix that binds to the four-helix bundle of the talin R8 domain in a canonical triple-helix arrangement. We demonstrate that the same R8 surface interacts with the paxillin LD1 and LD2 motifs. We identify key charged residues that stabilize the R8 interactions with LD motifs and demonstrate their importance in vitro and in cells. Our results suggest a network of competitive interactions in adhesion complexes that involve LD motifs, and identify mutations that can be used to analyze the biological roles of specific protein-protein interactions in cell migration.

**INTRODUCTION**

Integrin-mediated cell adhesion to the extracellular matrix (ECM) involves the assembly of dynamic adhesion complexes and requires the spatial and temporal coordination of signaling and force-transmitting events (Gardel et al., 2010; Wehrle-Haller, 2012). Such complexes form on the cytoplasmic tails of integrin receptors and mature into larger structures called focal adhesions (FA) in response to force exerted by the actomyosin contractile apparatus (Roca-Cusachs et al., 2012). The dimeric adaptor proteins talin1 and talin2 (molecular weight ~270 kDa) play a key role in the assembly of adhesion complexes (Zhang et al., 2008), and talin-null cells cannot adhere or spread on ECM, a phenotype corrected by expression of talin cDNAs (Atherton et al., 2015).

Talin comprises an N-terminal FERM domain (~50 kDa) that binds to and activates integrins, connected to a large flexible rod (~200 kDa) that interacts with multiple ligands, including vinculin and F-actin (Calderwood et al., 2013). Integrin activation is implicated in cancer progression (reviewed in Seguin et al., 2015), and talin overexpression may therefore contribute to cancer metastasis (reviewed in Desiniotis and Kyprianou, 2011). The talin rod constitutes a force-sensing module that regulates the assembly and maturation of adhesion complexes, and is composed of 13 four- and five-helical bundles connected by short linkers, forming an extended flexible chain (Figure 1A) (Goult et al., 2013b). Several rod domains contain cryptic vinculin binding sites (VBSs) that become exposed as the talin domains unfold in response to force, enhancing vinculin binding (del Rio et al., 2009; Fillingham et al., 2005; Papagrigoriou et al., 2004; Yao et al., 2014). Disruption of the talin force-sensing mechanism has strong effects on adhesion assembly, cell polarization, and cell migration (Atherton et al., 2015).

Talin also binds a number of proteins that regulate adhesion dynamics, including the Rap1-GTP interacting protein RIAM (Goult et al., 2013a; Lee et al., 2009), the Rac GEF Tiam1 (Wang et al., 2012), and the Rho GAP DLC1 (Li et al., 2011). Recruitment of Tiam1 and DLC1 to adhesion complexes by talin is likely to have complementary effects, balancing Rac and Rho activity, thus creating a feedback mechanism between actin polymerization, membrane protrusion, assembly of nascent adhesions, actomyosin-driven FA maturation, and FA turnover (Devreotes and Horwitz, 2015; Lawson and Burridge, 2014). The DLC1 binding site in talin has been mapped by deletion analysis to the four-helix R8 domain (Li et al., 2011) that forms a unique protrusion in the C-terminal part of the rod that is otherwise composed of a linear chain of five-helix bundles (Figure 1A) (Gingras et al., 2010). Interestingly, R8 also contains binding sites for RIAM and vinculin, suggesting that the three ligands may compete for binding (Goult et al., 2013b). The talin binding site (TBS) in DLC1 contains an LD-like motif that features in a wide range of other proteins, including the FA protein paxillin (Alam et al., 2014). The TBS in DLC1 interacts with the FA-targeting (FAT) domain of FAK (Li et al., 2011), which also binds the LD motifs in paxillin (Alam et al., 2014). The DLC1 interactions with talin and FAK contribute to the biological activity of DLC1, including its tumor-suppressor activity, establishing the physiological importance of these interactions (Li et al., 2011).
and show that the talin R8 rod domain plays a significant role in recruiting paxillin to FAs. We propose that LD-motif recognition sites in adhesion proteins such as talin and FAK are to a large degree interchangeable, creating a network of competing protein-protein interactions that regulate the properties of adhesion complexes.

RESULTS

Structure of the DLC1/Talin Complex

The TBS in DLC1 has been shown to require an 8-residue peptide LDDILYHV located in the largely unstructured linker region (residues 78–639) between the SAM and GAP domains of DLC1 (Figure 1B) (Li et al., 2011). However, consensus secondary structure prediction using the NPSA server (https://npsa-prabi.ibcp.fr) indicates that the DLC1 peptide is located at the N terminus of a larger region with high helical propensity (residues 465–488, Figure 1C), suggesting that the TBS in DLC1 may extend beyond residues 469–476. To explore this possibility, we used two synthetic DLC1 peptides (residues 461–489 and 467–489) that span the putative helical region. The shorter fragment starts with a proline residue, which usually disrupts helical structure, and is often located at the beginning or end of a helix.

The minimal talin fragment required for interaction with DLC1 (Li et al., 2011) maps to the four-helix bundle R8 domain in the talin rod (Figure 1A) (Gingras et al., 2010; Goult et al., 2013b). Addition of the DLC1(467–489) peptide induced large chemical-shift changes in the heteronuclear single-quantum coherence (HSQC) spectra of 15N-labeled talin R8 (Figure 1D) as did the larger peptide (data not shown), demonstrating the formation of a stable complex. Although the majority of resonances showed significant chemical-shift changes, the overall pattern of cross-peaks was similar to that of free R8, suggesting that the R8 fold does not change upon DLC1 binding.

The shorter DLC1(467–489) peptide was less soluble than the longer fragment, and was therefore less suitable for the NMR titration experiments. However, its lower solubility favored crystallization of a DLC1 peptide/talin complex. For these reasons, we used the longer DLC1 fragment for solution binding studies and the shorter fragment for crystallization experiments. We crystallized a complex of DLC1(467–489) with the talin R7R8 fragment, the structure of which we previously determined in the free form (Gingras et al., 2010), and solved the structure of the complex by molecular replacement (Figure 2A; statistics in Table 1). As in the free form, the R7R8 talin rod fragment adopts a unique fold where the R8 four-helix bundle is inserted into the loop connecting helices α3 and α4 of the R7 five-helix bundle. Individually, the structures of R7 and R8 in the DLC1 complex are nearly identical to that of the free form (root-mean-square deviation [RMSD] 0.35 Å and 1.75 Å, respectively), the main difference being the relative orientation of the two domains (Figure 2B).

The linker region between R7 and R8 is well defined in the crystal structure and shows clear electron density at the 1σ level. It forms a twisted, two-stranded, anti-parallel β sheet stabilized by hydrogen bonds. Each end of the linker has a pair of residues that make close contacts with the helical bundles (Figures 2B and 2C). Despite the different angle between the R7 and R8

Figure 1. DLC1(467–489) Interacts with the Talin R8 Domain
(A) Model of the talin rod based on the structures of individual domains. Domain R8 interacts with DLC1.
(B) Domain composition of DLC1. The location of the talin binding site (TBS) in the largely unstructured serine-rich linker region is indicated.
(C) Secondary structure prediction for the TBS in DLC1, which includes an LD motif marked by the red box. “h” denotes a region of high helical propensity and “c” a random coil region. Fragments used in this study are indicated by the thick blue lines.
(D) Superposition of the 1H,15N-HSQC spectra (298 K, 800 MHz) of 100 μM talin R8 domain in the free form (blue) and in the presence of 4-fold excess of DLC1(467–489) (red).

See also Figure S3.

Here we report the crystal structure of the talin R7R8 domains in complex with the TBS of DLC1; the DLC1 LD motif forms a helix that binds to talin R8 in a consensus triple-helix arrangement between the contacting DLC1 and talin helices. We identify the main electrostatic interactions that stabilize the complex and use mutations to demonstrate the importance of the talin/DLC1 interaction in cells. Based on the talin/DLC1 structure, we predicted that talin R8 might also bind paxillin LD motifs; we demonstrate such an interaction by nuclear magnetic resonance (NMR) and glutathione S-transferase (GST) pull-downs,
domains in the complex and free forms, these contacts are maintained in both structures, suggesting that the freedom in domain orientation is mainly defined by the twist and bend of the β-sheet linker. The linker may increase the stability of both domains by bringing together the ends of the helices connected to the linker. In support of the latter possibility, we found a strong effect of surface mutations (R1523E, K1530E, and K1544E) on the solubility of the isolated R8 domain, likely caused by partial unfolding. The same mutations did not affect the fold of the R7R8 double domain (see later).

As expected from sequence analysis and NMR data, the DLC1 peptide forms an α-helix that interacts only with the talin R8 domain (Figures 2A and 2D). The peptide is well defined in the structure, with clear electron density at the 1σ level (Figure S1A) and average B-factor values similar to those of the protein (Table 1). Only limited crystal packing contacts were observed between the external surface of the DLC1 helix and the edge of the R7 domain of the neighboring molecule (Figure S1B). The minimal DLC1 binding region (469–476) identified by Li et al. (2011) corresponds only to the N-terminal half of the DLC1 helix, justifying the use of the extended fragment. The helix starts at E468, with the preceding Pro residue having an extended conformation. At the C terminus, the helix ends at W486 with the adjacent SEK sequence (Figure 1C), forming an extended structure.

The DLC1/Talin R8 Complex Resembles a Talin Five-Helix Bundle

The DLC1 helix docks into the hydrophobic groove formed by helices α2 and α3 of talin R8 (Figures 2 and 3), forming a canonical left-handed anti-parallel triple-helix coiled-coil arrangement (Figure S1C) (Lupas and Gruber, 2005). The topology and structure of the DLC1(467–489) complex with talin R8 have a striking resemblance to the five-helix bundles of the talin rod (Figures 2D and 2E). The DLC1 helix is equivalent to the N-terminal helix (designated as α0) of the five-helix bundle that is located at the distant interface between helices α2 and α3 of the four-helix core of the structure in a cross-over arrangement (Goutel et al., 2010, 2013b).

As part of the five-helix bundle, the α0 helix makes a set of hydrophobic contacts with the four-helix core. These contacts are mediated by aliphatic side chains located on the hydrophobic face of the amphipathic helix α0, which fits into the hydrophobic pockets at the interface between helices α2 and α3, following the general principle of “knobs-into-holes” packing found in helical bundles (Lupas and Gruber, 2005). The α0, α2, and α3 form a left-handed anti-parallel triple-helix coiled coil that is similar to the triple-helix coiled coil formed by DLC1 with the α2–α3 hairpin of R8 in the complex. The overall structure of the five-helix bundles of the talin rod can thus be classified as conjoined three-/four-stranded coiled coils (Moutetelis and Woolfson, 2009), adding a significant number of new members to this rare fold.

DLC1 Recognition by the Talin R8 Domain

The contacts between DLC1 and R8 are mediated by the hydrophobic side chains of L469, I472, V476, M479, V483, and W486 located on the hydrophobic face of the DLC1 helix (Figure 3D). These residues follow a typical heptad repeat of a coiled coil (Lupas and Gruber, 2005), starting with L468 in position “a” (marked by letters at the top of Figure 3E); the contacting residues occupy positions “a” and “d” of the three sequential repeats. Additional hydrophobic contacts are made by the side chain of L473 in position “e” of the first repeat. At the N-terminal end of the DLC1 helix, corresponding to the LD motif, residues L469, I472, L473, and V476 are embedded between the hydrophobic side chains of L1492 of the R8 α2 helix, and V1540, K1541, and I1543 of the α3 helix in a “knobs-into-holes” packing arrangement (Figure 3D) (Lupas and Gruber, 2005).
arrangement typical for the coiled-coil packing, creating a small hydrophobic core (Figure 3B). The negatively charged DLC1 residue D470 that is conserved within LD motifs (the “D” residue) makes direct contact with the positively charged side chain of K1544 in R8. The complementary hydrophobic surface of R8, together with the positively charged K1544, creates an LD-recognition box that matches the consensus features of LD binders. For example, it is extended by charge contacts between E488 of DLC1, which is wedged between the positively charged groups R1523 and K1530 of R8. These polar residues are not part of the LD motif, but generate DLC1-specific contacts that may contribute to recognition. The interaction between DLC1 E488 and R1523 and K1530 of talin R8 may explain why the DLC1 helix is disrupted at the C terminus: in a continuous helix, E488 would be pointing away from the talin surface.

We tested the role of positively charged residues in talin by selectively reversing the charge of R1523, K1530, and K1544 (Figures 3B and 4A). Surprisingly, when these mutations were introduced into the isolated R8 domain, a large fraction of the protein was found in inclusion bodies and the soluble fraction contained partially degraded protein. These observations suggest that although the mutations were at solvent-exposed positions, the R8 fold was destabilized. In contrast, the talin R7R8 fragment bearing the same mutations was soluble and stable. Similarity of the NMR spectra of the wild-type and mutated R7R8 demonstrate that the protein fold was not affected.

Single-residue mutations in talin R8 had variable effects on DLC1 binding to talin. The spectral changes for the R1523E talin R7R8 mutant were the closest to those of wild-type, with large shifts and broadening of the signals indicating minimal effects on DLC1(461–489) binding (Figure S2A). Somewhat reduced shift changes and significantly less broadening was observed for the K1544E mutation (Figure 4C), and very limited shift changes with no additional broadening were observed for the K1530E mutant (Figure 4D). From these results, we conclude that K1530 makes the largest contribution to the interaction with DLC1. The contribution of K1544 is significant, but smaller, while the contribution of R1523 is negligible. However, none of the single mutations completely abolished the interaction with DLC1. To enhance the effects of the mutations, we generated the K1530E/K1544E double mutant; this 2E R7R8 double mutant showed negligible chemical-shift changes on addition of DLC1 (Figure 4E), effectively disrupting the interaction between talin R8 and DLC1.

To validate the ion pairing between D470 and E488 of DLC1, and K1530 and K1544 of talin R7R8, we introduced charge-reversal mutations D470K/E488K in DLC1, complementary to K1530E/K1544E of talin. The addition of the double D470K/E488K DLC1 mutant to the K1530E/K1544E talin R7R8 induced significant chemical-shift changes (Figure 4F). These changes were not as large as those observed with the wild-type proteins, but were comparable with changes observed with the K1530E mutant. The D470K/E488K DLC1 mutant also showed some interaction with the wild-type R7R8, although not as strong as with the wild-type DLC1 (Figure S2B). The incomplete recovery of the interaction and residual binding of the mutated DLC1 may reflect the ability of the peptide to adopt a slightly different conformation in the complex due to its small size and flexibility. Although further optimization will be required to enhance the interaction between the DLC1/talin R8 charge-reversal mutants,
the results support the roles of the charged residues in DLC1 recognition by talin.

**Comparison of DLC1, RIAM, and Paxillin Complexes**

The talin binding LD motif of DLC1 interacts with the LD binding FAT domain of FAK and was initially identified through its homology with paxillin LD motifs (Li et al., 2011). From the sequence homology and structural similarity, we predicted that paxillin LD motifs should also interact with the talin R8 domain. Indeed, we observed large chemical-shift changes in the LD motifs should also interact with the talin R8 domain. Indeed, we observed large chemical-shift changes in the LD motifs relative to the α2 and α3 helices of talin R8 (green). DLC1 and talin residues that make contacts in the complex. Side chains of the residues involved in hydrophobic interactions are shown as balls; charged and hydrophilic interactions are shown with sticks. Blue rectangle identifies the “polar ridge” of the complex.

- (C) DLC1-interacting residues on the talin surface. LD-recognition box is marked by a red rectangle.
- (D) Talin-interacting residues on the surface of DLC1 helix. The helix is rotated by 180° around the vertical axis relative to the orientation in (B).
- (E) Sequence alignment of DLC1 with RIAM TBS and paxillin LD domains. Peptide fragments used to solve the structures of the complexes are underlined. Residues involved in the interactions with the corresponding proteins are highlighted in magenta (hydrophobic interactions) and orange (charged and hydrophilic interactions). Red box indicates the DLC1 LD-motif identified from sequence comparison. For paxillin LD1 the underlined region corresponds to the LD motif. Positions of the coiled-coil heptad repeat are shown above the sequences. The underlined positions “a” and “d” correspond to the interacting hydrophobic residues in coiled coils.

(F) Comparison of the positions of DLC1 and RIAM helices in the complexes with the talin R8 domain.

(G) Locations of the hydrophobic residues on the surfaces of the DLC1 and RIAM helices involved in the interaction with talin R8. The helices are rotated by 180° around the horizontal axis relative to the orientation in (F).

See also Figure S1.

Using an LD motif deletion mutant of DLC1, we previously demonstrated that the DLC1/talin interaction contributes to DLC1-adhesion targeting (Li et al., 2011). To assess whether the interaction with talin R8 has similar effect on paxillin localization, we compared talin/paxillin and talin/DLC1 ratios in talin1 and talin2 knockout (TKO) cells (Atherton et al., 2015) transfected either with wild-type talin or a talin mutant lacking the R8 domain (talΔR8). The relative abundance of both DLC1 and paxillin in adhesions was significantly and comparably reduced in cells expressing talΔR8 (Figure S5D). Reduced DLC1 localization was analogous to what we had seen earlier with the DLC1 mutant (Li et al., 2011), providing evidence that talin R8 is the interaction site for DLC1, thus validating our approach. The reduced localization of paxillin in FA provides the first evidence that talin directly contributes to paxillin recruitment to FA.

Besides DLC1 and paxillin, the R8 domain also binds RIAM (Goult et al., 2013b). The recently reported structure of the R8/RIAM complex (PDB: 4W8P) (Chang et al., 2014) shows that, similar to DLC1, RIAM forms a helix that fits into the hydrophobic groove of the α2 and α3 helices of talin R8 (Figure S5F; Chang et al., 2014). Although not identified as an LD motif, the sequence of RIAM has a characteristic distribution of negatively charged and hydrophobic residues (Figure S3E) that suggests the interaction with the LD-recognition surface of R8. In support for the similarity of DLC1 and RIAM recognition by R8, we observed a

### Figure 3. Recognition of the DLC1(467–489) Helix by the Talin R8 Domain

- (A) Position of the DLC1(467–489) helix (orange) relative to the α2 and α3 helices of talin R8 (green).
- (B) DLC1 and talin residues that make contacts in the complex. Side chains of the residues involved in hydrophobic interactions are shown as balls; charged and hydrophilic interactions are shown with sticks. Blue rectangle identifies the “polar ridge” of the complex.

(F) Comparison of the positions of DLC1 and RIAM helices in the complexes with the talin R8 domain.

(G) Locations of the hydrophobic residues on the surfaces of the DLC1 and RIAM helices involved in the interaction with talin R8. The helices are rotated by 180° around the horizontal axis relative to the orientation in (F).

See also Figure S1.
strong reduction in RIAM binding affinity for the R1530E/K1544E mutant (Figures S2E and S2F).

Interestingly, in the R8/RIAM complex (Chang et al., 2014) the RIAM helix has an unusual kink, which causes its displacement relative to DLC1 (Figure 3F). However, the critical hydrophobic side chains that make contacts with the surface of talin R8 are located in similar positions, and make contacts with similar residues on R8, particularly at the N- and C-terminal ends of the helices (Figure 3G). These residues occupy equivalent positions in the sequences of the two proteins, showing that the DLC1 and RIAM helices are generally in register relative to each other (Figure 3E).

The kink in the RIAM helix appears to be forced by the hydrophobic contacts of the aromatic ring of F12, which is inserted between helices α2 and α3 of talin R8. In DLC1, the equivalent L473 occupies a peripheral position and is partly exposed to solvent. The helical kink is energetically unfavorable, but may be partially compensated by the hydrogen bond involving RIAM S13, as suggested by Chang et al. (2014). Significantly, no kink is present in the RIAM helix in complex with vinculin determined by X-ray crystallography (Goult et al., 2013b), nor with the talin F3 domain determined by NMR (Yang et al., 2014). These arguments support an induced kink model, rather than a stable kinked helix model proposed by Chang et al. (2014). Additional contributions

Figure 4. Interactions of Charge-Reversal Mutations of Talin R7R8 and DLC1(461–489)
(A) Location of the mutated residues in the structure of talin R8/DLC1(467–489) complex.
(B–F) Superposition of the HSQC spectra of 0.2 mM talin R7R8 free (blue) and in the presence of 0.8 mM DLC1(461–489) (red). Mutations are marked on the spectra. wt, wild-type form of the protein.

See also Figure S2.
to the kink in the RIAM helix may be due to crystal packing (Figure S1D). Changes in the NMR spectra of R8 on ligand addition suggest different affinities for the interactions between talin and DLC1, RIAM, and paxillin. The strongest effects on the spectra were observed for RIAM, where many signals shifted and broadened significantly at R8/peptide ratio as low as 1:0.1. For DLC1 similar broadening and shifts were observed, but required a higher ratio of 1:0.5, while for paxillin only chemical-shift changes were detected. For each peptide, the chemical-shift changes of the signals that showed only limited broadening throughout the titration (corresponding to a fast exchange regime) could be successfully fitted to the theoretical binding curves, with similar dissociation constants (Figure S3). In agreement with the qualitative analysis, the $K_D$ values determined by fitting were 48, 3.5, and 168 $\mu$M for DLC1, RIAM, and paxillin, respectively. Overall, the measured $K_D$ values are within the range of the low to high-micromolar values reported for biologically relevant LD-motif interactions (Alam et al., 2014), and the value for RIAM is in excellent agreement with that reported earlier (Chang et al., 2014). The high affinity of talin R8 for RIAM likely reflects the larger contribution of hydrophobic side chains to binding, while the lower affinity for paxillin correlates with the smaller binding region.

Biological Implications for DLC1-Talin Interaction from Mutational Analysis

We reported previously that wild-type talin R8 is sufficient to form a complex with full-length DLC1 in cells (Li et al., 2011). To evaluate the effects of the single K1530E and K1544E and double K1530E/K1544E (2E) R8 mutants on the complex formation in vivo, we engineered GST-tagged R8 constructs into isogenic mammalian expression plasmids and co-transfected them with GFP-DLC1 into HEK 293T cells. Complex formation was determined by a GST pull-down assay. Consistent with the NMR results, the talin R8 K1530E mutation caused a greater reduction in DLC1 binding than K1544E, while the 2E double mutant reduced binding to a greater extent than either single mutant (Figure S2D).

We next compared the ability of the wild-type talin R8 and mutant constructs to compete with binding of endogenous talin to GFP-DLC1 in cells, to see whether the GFP-DLC1-dependent biological effects require the interaction with talin R8. For this experiment we used three pairs of GST-tagged talin constructs that each contained R8; (1) the wild-type talin R8 and 2E constructs described above (encoding amino acids 1,453–1,580), (2) talin R7R8 and equivalent 2E constructs (encoding amino acids 1,352–1,580), and (3) wild-type and 2E talin constructs spanning residues 1,288–1,646 that were used...
previously (Li et al., 2011). GST served as negative control in the assay. We first confirmed that complex formation with GFP-DLC1 as determined by GST pull-downs was greater for each wild-type talin fragment than for the respective 2E mutant (Figure 6A). The wild-type versions of each talin construct should therefore compete with endogenous talin for binding to
GFP-DLC1 more effectively than the 2E mutant. Talin was immu-
noprecipitated from the supernatants of the GST pull-downs and
blotted for GFP-DLC1 to evaluate this; co-expression of GST
with DLC1 or with vector served, respectively, as a positive
and negative control (Figure 6B). Substantially less GFP-DLC1
co-immunoprecipitated with talin in cells co-transfected with
constructs containing wild-type R8 versus the 2E mutants (Fig-
ure 6B). We conclude that each wild-type GST-talin polypeptide
inhibits binding of GFP-DLC1 to endogenous talin more effect-
vively than the respective 2E mutant.

To assess the biological effects of inhibiting the interaction be-
 tween endogenous talin and GFP-DLC1, we tested the ability of
each talin wild-type and 2E mutant pair to antagonize the activity
of co-transfected GFP-DLC1 in the A549 human non-small cell
lung cancer line. Equivalent expression levels of each talin
construct were confirmed by western blotting (Figure 6C). We
used three different bio-assays (for details see Supplemental
Experimental Procedures): monolayer colony growth (Figure 6D),
growth in soft agar (Figure 6E), and transwell cell migration (Fig-
ure 6F). In the absence of any co-transfected talin fragment,
GFP-DLC1 was inhibitory in all three assays, while the GST-R8
talin construct (wild-type or 2E mutant) by itself had no detect-
able biological activity, as its effects were similar to that of the
GST negative control (Figure 6D). However, each wild-type talin
polypeptide attenuated the inhibitory activity of GFP-DLC1 in all
three bio-assays, consistent with its efficient displacement of
endogenous talin from GFP-DLC1. By contrast, each 2E mutant
had only a marginal effect on the inhibitory activities of GFP-
DLC1. The results clearly demonstrate that the biological activity
of DLC1 is associated with its interaction with talin and confirm
the importance of the talin R8 residues K1530 and K1544 to
the interaction.

However, as talin R8 interacts with RIAM (Goult et al., 2013b)
and paxillin (shown here) in addition to DLC1, we used several
approaches to evaluate whether binding of talin R8 to endoge-
nous RIAM or paxillin might have contributed to the observed
results. For RIAM, the level of expression in the cell lines used here
varied from very low to undetectable. To detect RIAM protein in
any of the cell extracts, we had to use an anti-RIAM immunopre-
cipitation step followed by anti-RIAM immunoblotting. Using
these conditions, endogenous RIAM was detected in A549 and
H358 cells, but not in 293T cells (Figure S3A). In A549 cell ex-
tracts, which contain endogenous RIAM, anti-RIAM immuno-
blotting did not detect a GST-R8 complex (Figure S3B, left),
whereas the wild-type GST-R8, but not the 2E mutant, did bind
GFP-DLC1 under the same conditions (Figure S3B, right). The
failure to detect an R8/RIAM complex despite the higher affinity
of R8 for RIAM versus DLC1 suggests that the biological effects
induced by GST-R8 are unlikely to be mediated via RIAM.

To investigate whether the biological effects of GST-R8 might
be partly mediated via paxillin, we first confirmed that endoge-
nous paxillin is expressed in cell lines A549, H358, and 293T
(Figure S6A). However, the levels of endogenous paxillin in
A549 and H358 cells, in combination with its relatively low
affinity for DLC1, were insufficient to detect binding to GST-R8
using the pull-down assay (Figure S6B top and bottom, respec-
tively). As a positive control, HEK293 cells were co-transfected
with a paxillin-DDK construct (OriGene) and GST-R8 (wild-
type, R1544E and 2E mutants), followed by a GST pull-down
assay. Under these conditions, wild-type GST-R8 did bind pax-
illin-DDK, and did so more efficiently than 2E GST-R8 talin
mutant (Figure S6C).

Taking these data together, we conclude that the ability of
wild-type GST-talin R8 to inhibit growth and migration in A549
cells is largely attributable to its interaction with DLC1, as no ef-
fect was observed in the absence of DLC1, and binding to
endogenous RIAM and paxillin in cell extracts was undetectable
under conditions associated with a strong DLC1 interaction.

DISCUSSION

The interaction between talin and DLC1 plays a key role in re-
cruiting DLC1 to FAs and contributes to the tumor-suppressor
activity of DLC1 (Li et al., 2011). Although deletion analysis has
been successfully used to identify regions that are critical for talin
interaction with DLC1 (Li et al., 2011), the exact location of the
binding sites and the mechanism of the interaction have re-
mained unknown. Here, we refine the boundaries of the TBS in
DLC1 and report the crystal structure of this region in complex
with the talin R7R8 rod domains. Analysis of the structure iden-
tifies the general features of the DLC1 binding site in the talin
R8 four-helix bundle and the specific residues involved. Thus,
a talin R8 K1530E/K1544E double mutant markedly reduced
binding to DLC1 peptides in vitro, and to full-length DLC1 in cells,
compromising the ability of GST-talin R8 constructs to displace
DLC1 from endogenous talin and thereby to attenuate the tumor-
suppressor activity of DLC1. Sequence similarity between the
TBS in DLC1 and paxillin LD motifs suggested a possible interac-
tion between talin and paxillin, and we have confirmed this novel
interaction by NMR and have shown that it is an important factor
in determining paxillin levels in FAs. Taken together, our results
explain how talin R8 recognizes LD motifs in both DLC1 and pax-
illin, and suggest that talin forms part of an LD-motif-based
network of interacting proteins that contribute to the assembly
and regulation of adhesion complexes.

Our structure of the talin R7R8/DLC1 complex demonstrates
that the TBS in DLC1 forms a helix that packs against the two
adjacent α2 and α3 helices of the talin R8 four-helix bundle in a
consensus left-handed triple-helix coiled-coil arrangement. The
DLC1 binding site in talin is fully accessible to solvent, and
the conformation of the R8 domain does not change on binding.
The resulting five-helix coiled-coil complex can be classified as a
hybrid conjoined three-/four-stranded coiled coil (Moutevelis
and Woolfson, 2009). A similar structure is formed in the talin
R8/RIAM (Chang et al., 2014) and paxillin/FAK (Hoellrer et al.,
2003) complexes. Although classified as a rare fold (Moutevelis
and Woolfson, 2009), the three-/four-stranded coiled coil is likely
to be a relatively common topology for complexes between four-
helix bundles and isolated helices, as it minimizes the rearrange-
m ent of the four-helix core.

Recognition that the interaction between DLC1 and talin R8 in-
volves coiled-coil packing allowed us to analyze the interaction,
using well-established rules for coiled-coil structures. The TBS in
DLC1 contains a typical heptad repeat identified in left-handed
coiled coils (Lupas and Gruber, 2005) (Figure 3) that creates a
hydrophobic interaction surface. Flanking this region are polar
residues that contact complementary polar residues in talin
R8. We identified three regions on the talin R8 surface that aid
recognition of the DLC1 helix: (1) an LD-recognition box consisting of a hydrophobic cluster with an embedded positive charged 
amino acid that matches the consensus LD motif, (2) a polar 
ridge that generates a network of polar contacts and hydrogen 
bonds between DLC1 and R8, and (3) a small hydrophobic patch 
that contacts the C-terminal hydrophobic residues of the DLC1 
helix. In addition, the R8 binding surface lacks any charged or 
large polar residues along the whole interface between the α2 
and α3 helices, thus avoiding any unfavorable contacts with the 
hydrophobic residues in the middle of the DLC1 helix. 
Together, these features create a complementary surface that 
can accommodate the entire length of the DLC1 TBS helix (Figure 3).

Among the contacts identified between DLC1 and talin R8, 
charge complementarity within the polar ridge (Figure 3) is likely 
to define ligand selectivity. We confirmed this prediction by 
reversing the charges of K1530 and K1544 at opposite ends of 
the binding region in R8. While double charge reversal 
completely abolished DLC1 binding, single charge reversals 
had only a partial effect, demonstrating that both interactions 
contribute to ligand recognition. Paxillin LD motifs form signifi-
cantly shorter helices that correspond to the N-terminal half of 
the DLC1 helix, and interact only with the LD-recognition box. 
In this case charge reversal of K1544 in the LD-recognition box 
of R8 (Figure 3C) had a much stronger effect on the interaction 
with paxillin, practically abolishing binding. This observation 
highlights charge complementarity as a general feature of LD 
motif recognition, with additional contributions outside the LD 
box fine-tuning the interactions with specific ligands.

Our results further support the important contributions of weak 
interactions to the adhesion mechanisms. Despite the relatively 
low affinities of DLC1 and paxillin for talin R8 (Kd of 48 and 
168 μM, respectively), these interactions can be detected in 
cells, and their disruption strongly reduces the abundance of 
DLC1 and paxillin in FAs (Figure 5D). For DLC1 this affects adhe-
sion-dependent colony growth and migration, although the bio-
logical role of the talin-paxillin interaction is currently unclear 
and will need further investigation. Large differences in the dissocia-
tion constants of DLC1, RIAM, and paxillin interactions with 
talin R8 are in line with the low- to high-micromolar range of constants 
determined for other LD motif interactions (Alam et al., 2014). 
These interactions are likely to be enhanced through the high 
concentration of the binding sites within adhesion complexes.

Although not previously identified as an LD motif, the N-termi-
nal part of the TBS in RIAM shows a pattern similar to that of 
DLC1, with hydrophobic and charged residues that fit the LD-
recognition box in talin R8 (Figure 3). DLC1 also binds to the 
FAK FAT domain, a recognized partner for paxillin LD motifs, 
and R8 itself interacts with paxillin. Extending this set of interac-
tions, other LD motif binding proteins that have four-helix bundle 
structures, such as PYK2 (Alam et al., 2014), may also interact 
with DLC1 and RIAM. In turn, LD motifs of other proteins, 
including members of the paxillin family, such as leupaxin 
and Hic-5, may interact with talin. The combination of an LD-like helix 
and a four-helix bundle containing an LD-recognition box may be 
a common feature among interacting adhesion proteins serving 
alongside other interacting pairs such as SH3 domain/polypro-
line sequences. The critical contribution of charged residues 
to recognition of the LD motif and additional interactions outside 
the LD motif can be used to selectively modulate the binding 
of specific ligands, as we demonstrated for DLC1, paxillin, and 
RIAM using charge-reversal mutations.

Comprehensive analysis of talin has revealed multiple ligand 
binding sites in the 13 talin rod domains, often arranged in com-
plex overlapping patterns (Goult et al., 2013b). There are 11 
VBSs in the talin rod, and the talin/vinculin interaction plays a 
key role in stabilizing FAs (Carisey et al., 2013). There are five pu-
tative RIAM binding sites in talin (four in the rod) that have the po-
tential to regulate the initiation of adhesion complex assembly 
(Goult et al., 2013b; Yang et al., 2014). In addition, we now iden-
tify a paxillin binding site in the talin rod, and more talin interac-
tions may be discovered. In turn, RIAM itself has two TBSs that 
can also bind vinculin (Goult et al., 2013b), and paxillin has five 
LD motifs, several of which interact with vinculin and FAK (Hoell-
erer et al., 2003). A direct link between talin and FAK has also 
been reported (Lawson et al., 2012; Lawson and Schlaepfer, 
2012), although molecular details of this interaction are missing. 
DLC1 has at least one binding site that interacts with talin and 
FAK in a similar way. All these interactions create a complex 
network at the core of adhesion complexes, where mechano-
sensing molecules such as talin and vinculin link to each other 
and to signaling molecules such as FAK and DLC1, either directly 
or indirectly, through adaptor proteins such as RIAM and paxillin.

Strikingly, all talin rod five-helix bundles, except the C-terminal 
R13 actin-binding domain (Gingras et al., 2008; Goult et al., 
2013b), have the same conjoined three-/four-stranded coiled-
coil topologies. The significance of this is currently not under-
stood, although some speculation can be made based on 
comparison with the DLC1/talin R8 complex, which has the 
same helix arrangement as a talin rod five-helix bundle (Figure 2). 
The core of the fold is a typical four-helix bundle that is likely 
to remain stable when the N-terminal x0 helix is removed: the talin 
R8 four-helix bundle is perfectly stable in the absence of DLC1, 
and removal of the N-terminal x0 helix from the R10 domain gen-
erates a stable four-helix bundle (Gingras et al., 2006; Goult 
et al., 2010) that is similar to R8. This suggests that under 
some conditions, talin five-helix domains may exist as four-helix 
bundles, raising the exciting possibility that removal of the x0 
helix might expose cryptic binding sites that can interact with 
helical regions homologous to the x0 sequence. The VBSs in 
the talin rod are buried in the hydrophobic core of the helical bun-
dles in which they are contained (Calderwood et al., 2013), 
and force exerted on talin is required to expose these sites (del Rio 
et al., 2009; Yao et al., 2014). It is therefore tempting to speculate 
that force may also play a role in displacing the x0 helix in talin 
rod five-helix bundles, exposing cryptic binding sites for proteins 
such as those containing LD motifs.

Although talin is widely recognized as a key player in adhesion 
complex assembly, the extent of the talin interaction network is 
unclear, and no comprehensive proteomic study on talin binding 
partners has been reported. Rather, the majority of studies have 
concentrated on individual interactions that are often prominent 
under specific conditions. Experiments in live cells demonstrate 
that adhesion complex assembly has a high tolerance for dele-
tion of individual proteins, as well as deletions or mutations of in-
dividual binding sites. This implies a high level of redundancy in 
the system, some of which may be due to the multi-site interac-
tions between FA proteins.
**EXPERIMENTAL PROCEDURES**

**Peptides and Protein Preparation**

Recombinant wild-type mouse talin1 fragment R7/R8 (residues 1,357–1,653) was previously cloned into pET15/D-TOPO expression vector (Gingras et al., 2010). Site-directed R7/R8 mutants were produced by overlap extension PCR and subsequent ligation-independent cloning into pOPINB vector (OPPF-UK). Protein was produced in BL21 STAR (DE3) cultured in Luria-Bertani or 2 x M9 minimal medium containing 1 g/l [15N]-labeled NH₄Cl, and purified using nickel-affinity chromatography followed by ion exchange.

**X-Ray Crystallography**

Sitting-drop sparse matrix crystallization screens were set up using a 300-mM nickel-affinity chromatography followed by ion exchange.

**NMR Spectroscopy**

NMR spectra were collected on Bruker Avance III 600- and 800-MHz spectrometers equipped with CryoProbes. Experiments were performed at 298 K in 20 mM sodium phosphate (pH 6.5) and 50 mM NaCl with 5% (v/v) 2H₂O. Dissociation constants were evaluated from the 1H,15N-HSOCT chemical-shift changes in the titration experiments conducted using 0.1 mM [15N]talin R8 domain. Peptides were added from 5- to 10-mM stock solutions to generate titration points at peptide/protein ratios 0.1, 0.2, 0.5, 0.75, 1, 2, 4, and 8.

**Cell-Based Assays**

The plasmids expressing GFP-DLC1 and GST fusion proteins with talin rod fragments encoding talin amino acids 1,288–1,646 and 1,453–1,580 (R8) were described previously (J. et al., 2011). The plasmid encoding 1,352–1,580 (R7/R8) was engineered by PCR and subcloned into a eukaryotic expression vector, PEBG. HEK293T cells were transfected by Lipofectamine 2000, and DLC1-null lung adenocarcinoma cell lines A549 and H358 cells were transfected by Lipofectamine 3000 according to the manufacturer’s instructions (In VitroGen). Cells were co-transfected with plasmids expressing GFP-DLC1 or Paxillin-DDK and GST, GST-talin fragments, or vector at a ratio of 1:2.5. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere. In vivo pull-down assay, co-immunoprecipitation, immunoblotting, G418 colony growth, soft agar growth, and cell migration assays were described previously (Qian et al., 2009). All experiments were conducted in triplicate.

**Ratio Imaging**

Talin1 and talin2 knockout cells were generated and cultured as described in Atherton et al. (2015). Transient transfections were performed using Lipofectamine and Plus reagents (Life Technologies) as per the manufacturer’s instructions. Cells transfected with GFP-talin proteins were incubated overnight on glass-bottomed dishes (MatTek), fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 (Sigma). Samples were incubated with the primary antibody for 60 min and then washed thrice with PBS. Secondary antibody staining followed the same procedure. Fixed samples were imaged using a Delta Vision RT microscope (Applied Precision) equipped with a 60X/1.42 Plan Apo oil-immersion objective (Zeiss). Images were acquired with a CoolSnap HQ camera (Photometric). Images were background subtracted, a region of interest was selected around an individual peripheral adhesion (five per cell), and the integrated density measured for both channels. Dividing the values from paxillin or DLC1 by talin then produced a ratio. Further details can be found in Supplemental Experimental Procedures.
from the talin rod unfolds to form a complex with the vinculin head. Structure 13, 65–74.