Title: Substrate binding allosterically relieves autoinhibition of the TRIB1 pseudokinase

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One Sentence Summary: Substrate binding to Tribbles-homolog 1 (TRIB1) pseudokinase induces allosteric changes that allow formation of a complex with the COP1 ubiquitin ligase.

Abstract: The Tribbles family of pseudokinases recruit substrates to the COP1 ubiquitin ligase for ubiquitination. CCAAT-enhancer binding protein (C/EBP) family transcription factors are crucial Tribbles substrates in adipocyte and myeloid development. Here we show that the TRIB1 pseudokinase can recruit various C/EBP family members, with binding of C/EBP β attenuated by phosphorylation. To explain the mechanism of substrate recruitment, we solved the crystal structure of TRIB1 in complex with C/EBP α . TRIB1 undergoes a significant conformational change relative to its substrate-free structure, to bind C/EBP α in a pseudo-substrate-like manner. Crucially, substrate binding triggers allosteric changes that link substrate recruitment to COP1 binding, which is consistent with molecular dynamics and biochemical studies. These findings offer a view of pseudokinase regulation with striking parallels to *bona fide* kinase regulation—via the activation loop and α C-helix—and raise the possibility of small molecules targeting either the activation loop-in, or loop-out, conformations of Tribbles pseudokinases.

Introduction

Protein kinases are a pervasive class of signalling protein that transduce all manner of biological signals. Kinase catalytic activity is controlled by a range of mechanisms, which converge on alignment of the catalytic and regulatory spines (1). Amongst the ~500 protein human protein kinase domains, approximately 10% are regarded as pseudokinases because they lack key catalytic features. Sequence variations that can turn a kinase into a pseudokinase include mutation of catalytic residues, substitutions that block ATP binding, or disruption of regulatory spine residues (2). Like their catalytically active counterparts, pseudokinases play roles in many different signalling pathways, but do so without phosphorylating substrates. These functions can be broadly categorised as allosteric regulation of active kinases, acting as scaffolds for protein–protein interactions, or acting as signalling switches (2, 3).

The Tribbles family of proteins occupy a dedicated branch of the kinome comprised of four members—TRIB1, TRIB2, TRIB3, and the more distantly related STK40 (also known as SgK495) (4). The family derives its name from the *Drosophila* Tribbles protein (5, 6), and share a common domain architecture: they have a variable N-terminal extension; a pseudokinase domain; and a C-terminal extension that binds to the COP1 ubiquitin E3 ligase (Fig. 1A). The pseudokinase and COP1-binding motif are key for the function of Tribbles proteins—by binding to substrates through their pseudokinase domain and to COP1 through their C-terminus they act as substrate adaptors for ubiquitination by COP1.

Human Tribbles proteins are implicated in wide-ranging signalling processes. TRIB3 is known to affect metabolism by binding to the metabolic regulatory protein kinase AKT, and recruiting acetyl-CoA carboxylase for degradation (7, 8). In contrast to inducing degradation, TRIB3 can stabilise the oncogenic PML-RAR α fusion protein preventing its sumoylation and

ubiquitination (9). In addition, TRIB1–3 have all been reported to control MAP kinase signalling (10–13).

Perhaps the most well-established role of Tribbles proteins is regulating CCAAT enhancer binding protein (C/EBP) family transcription factors (14–17). The C/EBP family is comprised of 6 members in humans (α , β , γ , δ , ϵ , and ζ). Further diversity arises because most family members can be translated as splicing isoforms, and family members can form either homo- or hetero-dimers (18). C/EBP transcription factors recognise a characteristic CCAAT DNA sequence to regulate proliferation, differentiation and metabolism, particularly of hepatocytes, adipocytes and hematopoietic cells (15, 18–20). Amongst the Tribbles, TRIB3 appears to not influence C/EBPs, whereas TRIB1, TRIB2 and STK40 are all capable of triggering degradation of selected C/EBPs by recruiting them to COP1 for ubiquitination (14, 21). Through their ability to control C/EBP and myeloid development, overexpression of TRIB1 and TRIB2 can cause development of acute myeloid leukaemia with high penetrance (14, 22), and TRIB1 deficiency results in a loss of tissue-resident M2-like macrophages (15). GWAS studies have also linked TRIB1 to levels of metabolic enzymes and plasma lipids (23, 24), which has subsequently been linked to post-translational control of C/EBP-regulated transcription (25).

Crystal structures of the TRIB1 pseudokinase domain have revealed a deprecated N-terminal lobe and ATP-binding site, which is consistent with the inability of TRIB1 to bind ATP (17). Importantly, the C-terminal COP1-binding motif of TRIB1 can bind to the pseudokinase domain in an autoinhibitory manner (17), which is mutually exclusive with the motif binding to the COP1 WD40 domain (26). Here we use a combination of crystallography, molecular dynamics and biochemistry to investigate the mechanism that allows release of TRIB1 autoinhibition. The structure of TRIB1 bound to its prototypical substrate, C/EBP α , shows that

TRIB1 undergoes a marked conformational change upon substrate binding. Such conformational changes have important implications for assembly of an active complex with COP1, and potentially for susceptibility of Tribbles pseudokinases to small molecule inhibitors.

Results

Structure of the TRIB1-C/EBPα complex

To understand the basis for recruitment of C/EBP family transcription factors we sought to solve the structure of the TRIB-C/EBPα complex. Crystals of the complex were successfully grown by fusing the TRIB1 recognition degnon of C/EBPα to the C-terminus of TRIB1 (fig. S1). The structure was solved to a resolution of 2.8 Å (Table 1). There are two complexes in the asymmetric unit, which have the C/EBPα peptide defined for residues 55–74 and 57–67, respectively. The former complex is displayed in subsequent figures. Both complexes and electron density maps are included in fig. S2. Remarkably, the activation loop of TRIB1 in both complexes is fully resolved (Fig. 1B), and adopts a position markedly different than the conformation seen in apo-TRIB1 (17). The activation loop folds towards the αC helix and generates a binding site for the C/EBPα degnon. This binding mode is effectively pseudo-substrate-like, as demonstrated by comparison with the prototypical substrate-like protein-kinase inhibitor peptide in complex Protein Kinase A (Fig. 1C). All of the C/EBPα degnon residues previously shown to be required for binding make direct contact with TRIB1 (17). Namely Ile55, Glu59, Ser61, Ile62 and Ile 64 are part of an extended peptide that spans TRIB1 and leads to a helical turn containing Tyr67 and Ile68, which lie in a cleft alongside the αG-helix of TRIB1.

To experimentally validate the TRIB1 residues involved in the interface, we generated a panel of mutants in a GST-TRIB1 fusion protein, and tested their ability to bind His₆MBP-C/EBPα (Fig. 1D,E). Mutants were selected that either directly contact C/EBPα or stabilise the activation loop in a conformation that is competent to form the binding site. In pulldown analysis, critical residues span the C/EBPα binding site—either directly contacting C/EBPα (His168, Phe293), or stabilising the substrate-competent conformation of the activation loop (Cys245, Pro246, and Ile253). The five TRIB1 mutants with abrogated C/EBPα binding are identical in TRIB2, consistent with the ability of TRIB1 and TRIB2 to bind C/EBPα with similar affinities (fig. S3), and to subsequently induce degradation of C/EBPα in cells and drive development of acute myeloid leukaemia in mice (14).

TRIB1 binds multiple C/EBP family members

Amongst their broad functions, C/EBP transcription factors coordinately regulate myeloid cell and adipocyte differentiation, with different family members predominating at specific phases of differentiation. However, there is some sequence variation in the Tribbles recognition degron of different C/EBP transcription factors (Fig. 2A)—C/EBPβ, δ and ε each contain a sequence similar to the C/EBPα degron, while C/EBPγ and ζ do not appear to have a comparable motif. We first sought to test whether C/EBPβ, δ, and ε could also be bound by TRIB1, and subsequently if sequence variations might alter the efficacy of TRIB1 binding. Employing isothermal titration calorimetry, we observed that TRIB1 bound to C/EBPα, β, δ and ε, peptides fused to maltose-binding protein with dissociation constants ranging from 7.4 to 17 μM (Fig. 2B). Such affinities suggest that C/EBPα, β, δ and ε all contain functional TRIB1 degrons, that bind TRIB1 with similar affinity.

To investigate which residues within the C/EBP degron contribute most strongly to TRIB1-binding, we performed a 1 μ s molecular dynamics simulation based on the TRIB1-C/EBP α structure. Plotting the root mean square fluctuation of C/EBP α peptide reiterates that the stretch from Glu59–Ala72 represents the most stable portion of the peptide and include the main binding determinants (fig. S4). This finding is consistent with several lines of evidence: previous binding studies of mutant C/EBP α peptides revealed a concentration of important binding residues in this region (17); the second complex within the asymmetric unit of the crystal structure was well defined only for residues 57–67; and this segment contains the highest levels of sequence identity between C/EBP family members.

C/EBP β phosphorylation antagonises TRIB1 binding

The observation that TRIB1 can bind various C/EBP family members with similar efficacy is interesting in light of previous observation that TRIB1 can affect C/EBP α but not C/EBP β in certain cases (15), and other scenarios where C/EBP α and C/EBP β are regulated equally by TRIB1 (27). These observations beg the question of whether further mechanisms control C/EBP degradation depending on cell type or developmental stage. To explore additional layers of regulation, we analysed data from phosphosite and observed that C/EBP β is phosphorylated at Ser77 and Tyr79 within its Tribbles degron. Tyr79 phosphorylation of C/EBP β occurs via c-Abl and Arg non-receptor tyrosine kinases, resulting in stabilisation of C/EBP β (28), while Ser77 is phosphorylated in a Ras-dependent manner (29). To test the effect of C/EBP β degron phosphorylation, we developed a competition assay based on fluorescence polarisation (Fig. 2D). In this assay, FITC-labelled C/EBP α peptide was competitively displaced by an unmodified

C/EBP β degron peptide. In contrast, Ser77-phosphorylated, or Tyr79-phosphorylated C/EBP β degron showed no ability to displace FITC-C/EBP α , even up to concentrations of 350 μ M.

Loss of TRIB1 binding after Tyr79 phosphorylation suggests a direct mechanism to explain post-translational stabilisation of C/EBP β by c-Abl (28), based on protection from degradation by TRIB1-COP1. Similarly, phosphorylation of Tyr79 by proline-directed Ser/Thr kinases such as CDK2 or MAPKs could represent a further mechanism by which C/EBP transcription levels can be protected from degradation by COP1-Tribbles under specific circumstances (29).

Conformational changes induced by substrate-binding

Previous structures of TRIB1 have shown that the pseudokinase domain contains an unusual α C-helix that forms a binding site for the C-terminal COP1-binding motif (17). TRIB1 and TRIB2 also contain a unique SLE sequence at the N-terminus of the activation loop—as opposed to DFG that is seen in most active kinases—which can block the putative ATP-binding pocket. The structure of substrate-bound TRIB1 now reveals distinct conformational changes upon substrate binding (Fig. 3). Analysis of these conformational changes delineate a clear allosteric mechanism to link substrate-binding on one side of TRIB1 to release of the COP1-binding motif from its binding site on the α C-helix—in effect release of TRIB1 autoinhibition (Fig. 3 and movie S1).

Upon binding to C/EBP α substrate, the activation loop becomes fully ordered, folding between the N- and C-terminal lobes to form the substrate-binding site (Fig. 3A; Fig. 1). With this rearrangement comes a conformational change in the SLE sequence at the N-terminus of the activation loop (Fig. 3B). Leu226 moves towards the α C helix, packing into a location homologous to the DFG phenylalanine of conventional kinases when they adopt their ‘DFG-in’

conformation (1). In TRIB1, rearrangement of Leu226 is facilitated by movement of Tyr134 within the α C-helix, which subsequently packs atop Leu226 to complete the regulatory spine (Fig. 3C). In this manner, the substrate-bound conformation of the TRIB1 pseudokinase can be thought to adopt a ‘SLE-in’ conformation that is comparable to the ‘DFG-in’ conformation of conventional kinases.

The existence of states that resemble inactive (SLE-out) and active (SLE-in) protein kinases structures is intriguing, but also has striking consequences for the function of TRIB1. In order for co-ordinated rearrangement of Tyr134 and Leu226 upon substrate-binding, the α C-helix of TRIB1 must rotate away from the active site of the kinase. For instance, the α -carbon positions of Tyr134, Ile135 and Gln136 move by 3.2, 4.8 and 4.4 Å, respectively, relative to their positions in the substrate-free structure. Further movement is propagated through the N-terminal portion of the α C-helix, and more subtle rearrangements occur within the β 4 and β 5 of the N-terminal lobe. Together these changes disrupt the docking site for the C-terminal tail of TRIB1 (Fig. 3D), making it incompatible with sequestration of the C-terminal COP1-binding site and TRIB1 autoinhibition.

Substrate-binding allosterically regulates TRIB1 autoinhibition

The C-terminal COP1-binding motif of TRIB1 binds to its own pseudokinase domain and the E3 ubiquitin ligase in a mutually exclusive manner (26); (Fig. 4A/B), which necessitates the allosteric changes outlined in the previous section. To test the functional effects of the allosteric changes we adopted an assay previously used to test COP1-binding by TRIB1, and STK40 (26, 30). Namely, TRIB1-COP1 association was inferred by displacement of a FITC-labelled TRIB1 C-terminal-peptide from the WD40 domain of COP1, which can be monitored by fluorescence

polarisation. Consistent with Uljon et al. and the autoinhibited crystal structure, we observed that the C-terminal COP1-binding motif is less effective at binding COP1 when it is attached to the pseudokinase (TRIB1(84–372)) than it is as an isolated peptide (residues 349–367; fig. S5). Strikingly, inclusion of the C/EBPα degron peptide with TRIB1(84–372) allows it to more effectively displace the FITC-labelled peptide (Fig. 4C). The displacement curve for TRIB1 with C/EBPα is shifted to approximately 10-fold lower TRIB1 concentrations relative to TRIB1 in the absence of substrate, supporting a model where substrate-binding releases autoinhibition and frees the tail to bind COP1.

To further investigate the structural basis of TRIB1 allostery we performed molecular dynamics simulations comparing the dynamics of TRIB1 in its autoinhibited form, in its active conformation but without substrate, and in its active conformation in the presence of substrate. These analyses could be used to: i) visualize the effect of substrate binding on stability of the C-terminal tail of TRIB1, and ii) infer key residues involved in the allosteric regulation of TRIB1.

Firstly, to understand the propensity of the TRIB1 C-terminal tail to occupy its autoinhibited conformation, we analysed the stability of the tail over the parallel simulations (movie S2). In the structure of autoinhibited TRIB1 (PDB: 5cem), the tail stably resides in its binding cleft atop the αC-helix, maintaining multiple hydrogen bonds with the β4 strand. In contrast, initiating a simulation of TRIB1 (SLE-in) bound to C/EBPα with the C-terminal tail in an equivalent position alongside the β4 strand shows the tail rapidly dissociating from its binding site. Beginning a simulation with TRIB1 in its active (SLE-in) conformation but without substrate present, causes the tail to remain bound for an intermediate length of time, but eventually dissociate. To quantify these observations, we plotted the number of hydrogen bonds between the TRIB1 C-terminal tail and the β4 strand over the course of the three simulations

(Fig. 4D). This analysis highlights that the C-terminal tail is less effectively sequestered when TRIB1 occupies an active state, and becomes fully destabilised when substrate binding completes the active complex.

Secondly, to understand the key residues inherent to the allosteric mechanism we compared the difference in torsion angle dynamics of each residue in TRIB1 using a Kullback-Leibler (KL) divergence based statistics (See methods; Fig. 5). Quantification of KL divergence of TRIB1 without the C-terminal tail (Fig. 5A) indicates that structural regions (such as the catalytic loop and activation loop) in direct contact with C/EBP α show significant difference between substrate-bound and unbound simulations. Interestingly, the KL divergence profile of TRIB1 with the C-terminal tail included reveals differences in torsion angle dynamics of residues not only in the substrate binding site, but distal regions of the pseudokinase domain, including the C-terminal tail, SLE-motif, G-loop, and α C-helix (Fig. 5A). In the presence of substrate, the SLE+1 aspartate (Asp228) displays large conformational flexibility in that it can make charge interactions with Lys220 from the catalytic loop, Arg102 from the glycine-rich loop and Lys130 from the α C-helix during the simulation (Fig. 5B). Therefore, the SLE-motif seems to be the global hub in propagating the allosteric signals of C/EBP α substrate binding. Together, these data reinforce the key functional role of sequence features that are unique to Tribbles proteins, which contributing to a mode of regulation reminiscent of active kinases.

Potential for nucleotide or small-molecule binding by TRIB1

A clear consequence of conformational changes induced by C/EBP α -binding is the opening of the TRIB1 active site, which in the SLE-out state is blocked by the activation loop (Fig. 6A). In contrast, the SLE-in state induces a large open binding pocket (Fig. 6B). Such an

open state would be consistent with reports that the closely-related pseudokinase TRIB2 can bind ATP, and to small molecule ligands (31, 32). We initially tested the effect of C/EBPα degron on the melting temperature of TRIB1(84–372) in a differential scanning fluorimetry (DSF) assay (33). The addition of C/EBPα degron peptide induced a significant stabilisation of TRIB1 (Fig. 6C; comparing orange to grey). However, further addition of 200 μM ATP did not shift the melting temperature of either TRIB1 alone (Fig. 6C; green) or the TRIB1-C/EBPα mixture (Fig. 6C; black) relative to their nucleotide-free equivalents. Thus, it appears that movement of the SLE-motif to its SLE-in conformation is insufficient to confer the ability to bind ATP.

Even though TRIB1 does not appear capable of binding ATP in either conformation, there is no obvious steric hindrance of the rear (adenine-binding) portion of the TRIB1 ATP-binding site, particularly in the SLE-in state. This is relevant because ATP-competitive inhibitors often occupy the adenine-binding portion of a kinase active site, rather than the phosphate-stabilising regions that are defunct in TRIB1. To explore this possibility we used DSF to screen TRIB1 against the publically available protein kinase inhibitor screen (PKIS; (34)). We found several compounds that induced thermal stabilization of TRIB1 by greater than 2 °C (Fig. 6D). The four hits were originally designed as inhibitors of either TIE2/VEGFR2 or EGFR/ErbB2 and were built upon either the benzimidazolyl diaryl urea, furopyrimidine (both TIE2/VEGFR2-targeting) or anilino thienopyrimidine (EGFR/ErbB2-targeting) scaffold (35–37). Moreover, three of the four also induced similar stabilisation of TRIB1 in the presence of C/EBPα degron peptide whereas GW607049C induced a different melting profile in the presence and absence of degron (Fig. 6E, fig. S7), which could indicate binding to different conformations of TRIB1. Although further characterisation is definitely required, these results offer the first suggestion that inhibitors could be developed to target TRIB1. Based on structural and mechanistic studies

presented here, development of specific ligands that bind the SLE-out or SLE-in conformations of TRIB1 could have exciting potential to either block substrate-binding, or release autoinhibition of TRIB1 to promote recruitment of the COP1 ubiquitin ligase.

Discussion

Tribbles pseudokinases function in concert with the COP1 ubiquitin ligase to degrade critical regulators of metabolism and transcription (4). In this study, we show that TRIB1 recognises conserved degrons in C/EBP family members with similar efficacy, and that binding of C/EBP β can be antagonised by C/EBP β phosphorylation. We also report the structure of TRIB1 in complex with the degron from its canonical substrate, C/EBP α . The mechanism of C/EBP α -binding by TRIB1—a pseudosubstrate-like binding mode—couples substrate-binding on one face of TRIB1 to release of the C-terminal tail on the opposing face of the molecule. Such a mechanism seems likely to have important consequences for protecting TRIB1 from unproductive degradation by COP1, but also offers exciting potential when considering ligands designed to bind the TRIB1 active site.

Pseudokinases are not constrained by the need to perform catalysis, and as such have evolved as diverse mediators of signal transduction via three major mechanisms; allosterically activating other enzymes, acting as signalling switches, or scaffolding protein–protein complexes (2, 3). There are few examples of pseudokinase structures captured in both inhibited and ‘active’ states, but the mechanism described here for TRIB1 bears remarkable resemblance to regulation of conventional kinases. However, it is facilitated by unique structural features of TRIB1. For instance, the α C-helix is commonly mobile in conventional kinases (1), but in TRIB1 it seems to be particularly malleable due several proline residues creating a non-ideal helix. Moreover, in

addition to possessing an SLE- rather than DFG-motif, TRIB1–3 all possess a glutamate preceding the SLE, which is unique, stabilises the autoinhibited state of TRIB1 and exhibits increased Kullback-Leibler divergence in molecular dynamics simulations of substrate-free and -bound TRIB1-C/EBPa (Fig. 5). Overall, the mechanism described in this work offers an intriguing example of sequence features that are divergent between pseudokinases and kinases maintaining orthologous function as an allosteric switch. In conventional kinases binding of regulators to the α C-helix and stabilisation of the regulatory spine generally facilitates substrate binding and activity (*1*); in the case of TRIB1 it appears that the reverse is true. Substrate binding promotes conformational changes in the α C-helix that disrupt sequestration of the TRIB1 C-terminal tail, and subsequent complex formation with COP1. Whether similar mechanisms control protein-protein interactions in other pseudokinases, or active kinases, is an interesting proposition.

It has recently been shown that TRIB2 also binds its own C-terminal tail (*32*), and thus potentially also adopts an autoinhibited conformation that facilitates allosteric regulation. Although no structures are available for TRIB2 or TRIB3, the structure of the STK40 pseudokinase domain was recently reported (*30*), which adopts a conformation similar to the substrate-bound state of TRIB1 (fig. S6). However, STK40 is the most divergent Tribbles family member and does not share the SLE-type motif or preceding glutamate residue. In this sense it appears unlikely to share the same allosteric switch mechanism as TRIB1, and may be constitutively-active with regards to substrate binding. The impact of such for STK40 stability, and biological functions independent of canonical Tribbles family-members will be an area of some interest.

C/EBP transcription factors play pleiotropic roles in development of adipocytes and myeloid cells. The importance of C/EBPs in myeloid development is illustrated by mutations in C/EBP α that occur in ~10% of acute myeloid leukaemia patients (38), and the critical transcriptional role of C/EBP β in multiple myeloma (39). Many of the biologically characterised roles of TRIB1 and TRIB2—for instance regulation of lipid metabolism or macrophage development—can be traced to their ability to control levels of C/EBP α in particular. The identification of the Tribbles degron present in the p42, but not the dominant negative p30 isoform of C/EBP α , suggested one mechanism by which Tribbles may selectively control a single transcription factor (17). We now show that the Tribbles degron is conserved in four of six C/EBP family members, of which all but C/EBP δ are similar to C/EBP α in that they also have short isoforms that lack a Tribbles degron. Because Leucine zipper transcription factors can form either homo- or hetero-dimers, which may contain no, one or two Tribbles degrons there appears to be ample opportunity for gradation of C/EBP ubiquitination by COP1-TRIB1/2, where complexes containing two degrons are preferentially degraded by Tribbles, over complexes that might be recruited less avidly. Furthermore, C/EBP β can apparently be protected from degradation by phosphorylation (Fig. 2D). Such protection may have clinical relevance when considering tumours with constitutively-activated kinases upstream of EBP β . Namely, given the role of EBP β in Ras-induced transformation and myeloid cancers it is tempting to speculate that pharmacologically preventing EBP β phosphorylation could make it susceptible to Tribbles-mediated degradation, and offer benefit in specific cancers (28, 29, 40). Phosphorylation of EBP β further adds to the complexity of COP1-based ubiquitination and phosphorylation, given that ETS-family transcription factor degradation is reduced upon phosphorylation by Src-family kinases (41, 42).

Although the capability of TRIB2 to bind ATP appears not to be realized in either the SLE-in or SLE-out forms of TRIB1 ((31);Fig. 6), this work opens the intriguing possibility that small molecule inhibitors targeted towards the active site of Tribbles could be used to block function. An exciting recent report suggests that small molecule inhibitors that covalently target a cysteine-rich segment in the α C-helix of TRIB2 can induce its degradation (32). The fact that these inhibitors were also originally designed against EGFR offers some overlap with the binding profile shown here for TRIB1. The chances of such overlap were likely increased by our having screened an identical library to Foulkes et al., which is enriched in EGFR targeted ligands. A more extensive search of chemical space is warranted to find more potent binders of TRIB1. The mechanism revealed in this work offers an exciting incentive for finding Tribbles-targeting small molecules—despite having poor ATP binding propensity the active-site pocket is intimately linked to regulation of TRIB1, and binding of inhibitors around the SLE-sequence and α C-helix may have drastic implications for the fate of TRIB1 or TRIB2 in cells.

Targeting pseudokinases with small molecules is an exciting area of development, for instance: small molecules that stabilize the KSR pseudokinase antagonize RAF heterodimerization and subsequently Ras signalling (43); and molecules targeting the HER3 pseudokinase modulate heterodimerization with kinase-active EGF-family receptors and induce degradation of HER3 (44). The distinct sequence features within the active site of TRIB1 may offer several possibilities for developing specific inhibitors. It remains a tantalising prospect that high affinity small molecules could manipulate the allosteric mechanism described here to protect substrates, or promote Tribbles degradation, in cancers promoted by Tribbles pseudokinases.

Materials and Methods

Protein expression and Purification

All bacterial constructs (TRIB1 and C/EBP) were expressed in *E. coli* BL21 (DE3) cells. COP1 was expressed in insect cells.

TRIB1/2 and C/EBP proteins: TRIB1(84–372), TRIB2(53–343) and TRIB1(84–343) linked by a -GSGSSGGPG- linker to C/EBPα(53–75) were cloned into modified pET-LIC vectors incorporating an N-terminal 6×His tag and a 3C protease cleavage site. C/EBPα(53–75), C/EBPβ(64–86), C/EBPδ(50–72) and C/EBPε(31–53) all fused to maltose-binding protein (MBP) were cloned into modified pET-LIC vectors incorporating an N-terminal 6×His tag followed by MBP and a 3C protease cleavage site.

Cell pellets were resuspended in purification buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% (v/v) glycerol and 10% (w/v) sucrose) supplemented with 0.2 mg/mL lysozyme and 80 U/mL benzonase. Cells were lysed by sonication (Sonifier, Heat Systems Ultrasonics). Protein was initially purified by Ni²⁺ affinity chromatography (His Select, Sigma). Proteins were eluted with purification buffer containing 300 mM imidazole. Protein containing fractions were pooled and digested overnight with 3C protease and 2 mM DTT. The protein was further purified by size-exclusion chromatography using a Superdex 200 Increase column (GE Life Science) (10 mM Hepes pH 7.6, 300 mM NaCl, 0.5mM TCEP) or anion exchange (Resource Q). The core peak fractions were pooled and snap frozen for storage at -80°C.

GST pulldowns: Mutations were generated in Trib1(84–372) in a modified pGEX-LIC vector incorporating an N-terminal GST tag using Quikchange mutagenesis. Cell pellets from expression of wild-type and mutant TRIB1 were resuspended in purification buffer and

supplemented with 0.2 mg/mL lysozyme and 80 U/mL benzonase and cells were lysed by sonication. The soluble fraction was bound to GST sepharose resin (Amersham Biosciences), and analysed for purity by SDS-PAGE. GST-TRIB1 was incubated with 500 μ M C/EBP α (53–75) fused to MBP at 4°C for 20 min using PBS pH 7.4. After incubation the resins were washed with PBS pH 7.4, 0.02% (v/v) Tween-20. Samples were then resuspended in SDS sample buffer and visualised on 12-18% SDS PAGE stained with coomassie R250.

COP1 WD40 domain: COP1 WD40 domain (376–731) was expressed in the *Trichoplusia ni* (*Tni*) and *Spodoptera frugiperda* (*Sf9*) cell lines using baculovirus produced in *Sf9* cells. All cells were purchased from Expression Systems, and cultured in ESF 921 medium at 27°C, shaken at 125 r.p.m. The Bac-to-Bac baculovirus expression system (Invitrogen) was used, as per the manufacturer's instructions, to produce COP1 WD40 baculovirus stocks, except FuGENE 6 (Promega) was used as the transfection reagent. Cells were plated at a density of 8.0×10^5 cells/mL and allowed to grow until logarithmic growth was achieved, as indicated by a density of 1.0×10^6 cells/mL. Subsequently, cells were inoculated with COP1 WD40 baculovirus with an MOI= 1, and incubated at 28°C for 72h (*Sf9*) or 48 h (*Tni*). Cells were harvested by centrifugation at $1000 \times g$, 5 min. Cell pellets were resuspended in 50 mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 150 mM NaCl and 10 mM imidazole supplemented with *DNaseI* (Applichem) and Protease Inhibitor Cocktail (Sigma; p8340). Cells were lysed using an EmulsiFlex-C3, and the soluble fraction purified by Ni²⁺ affinity chromatography (His-Select resin, Sigma). COP1 WD40 protein was eluted from resin in lysis buffer including 500 mM imidazole. The protein was further purified by size exclusion chromatography using a Superdex 200 Increase column (GE Life Science) (150 mM NaCl, 10 mM HEPES buffer pH 7.6).

Crystallisation and structure determination

TRIB1(84–343) with a GSGSSGGPG linker to C/EBP α (53–75) (~12.2mg/mL) was initially crystallised in 2.8 M Na Acetate (Salt) 0.1 M BIS-TRIS prop 7 pH. Initial hits were refined using additive screening and soaking experiments, and crystals for data collection were grown in 2.8 M Na Acetate 0.1 M BIS-TRIS prop 7 pH with 5% sodium citrate mother liquor diluted to 75 and 80% and drop ratios of 2:1 and 3:1. The crystals were cryoprotected in mother liquor supplemented with 25 % glycerol. In attempts to improve diffraction, the crystal that eventually yielded the best resolution included 1 mM ATP in the cryoprotectant, but there was no electron density attributable to nucleotide in the final structure. Data was collected at the MX2 beamline of the Australian Synchrotron (Table 1), and processed using XDS and AIMLESS (45, 46). The structure was solved by molecular replacement in Phaser using the pseudokinase portion of PDB 5CEM as a model (47). Rebuilding was performed in COOT (48), and the structure was refined against diffraction data to a final resolution of 2.8 Å using Refmac (49).

Molecular Dynamics

Structural Modelling: The SLE-out conformation of TRIB1(88–359) was modelled based on the PDB structure 5CEM (17). Missing residues of the α C- β 4 loop(140-141), activation loop (229–237) and part of the C-terminal tail(343–356) were fixed using the automodel class in Modeller (50). The C-terminal tail(341–364) was incorporated into the SLE-in-TRIB1:C/EPB α complex using PDB 5CEM as the template. The simulations were performed in the presence and absence of C/EPB α peptide.

MD System Preparation and Simulation: All MD simulations were performed using Gromacs version 5.1.2 (51). Specifically, the protein was parameterized using amber99sb-ildn force field and solvated with TIP3P water model (52). The protein was centered in a dodecahedron box with the distance between the solute and the box larger than 1nm in all directions. 0.1 mM ions (Na+

and Cl-) were added to the system by randomly replacing solvent molecules to neutralize the charge of the system. Steepest descent and conjugate gradient algorithms were employed in conjunction to minimize the potential energy of the system so that the maximum force (F_{max}) is less than 100 kJ/mol/nm. LINCS (Linear Constraint Solver) algorithm was used to constraint bonded interactions. Verlet cutoff scheme is used to maintain neighbor-list (53). Long range electrostatic interactions are calculated based on Particle-Mesh Ewald (PME) method (54). Temperature equilibration was done in canonical ensemble (NPT) for 200 ps using V-rescale thermostat (55). Isothermal-isobaric ensemble (NPT) was then maintained through Berendsen barostat at 1.0 bar for 200 ps. The productive simulations were carried out at NPT ensemble for 1 μ s. The torsion angle analysis of the trajectory was calculated using the gmx chi module of Gromacs.

Kullback-Leibler (KL) Divergence Analysis: Kullback-Leibler divergence is a statistical measure of how two probability distributions differ from each other. In this analysis, we focused on the distribution of the torsion angles (Φ , Ψ , χ) of each residue in Trib1 during the MD simulations. We used the C/EBP α unbound form as the reference state and C/EBP α bound form as the target state. MutInf package was used to perform the KL calculation (56). Specifically, we split the 100–1000 ns of our MD data into 4 equal length blocks as the bootstrapping set and discretized the torsion angle (Φ , Ψ , χ) distribution of each residue with a binwidth of 15 degrees. The sum of the KL divergence of all angles of a given residue was reported as the KL score for that residue. The KL score was then mapped to the Trib1 structure and visualized using the putty representation of PyMOL.

Biophysical measurements

Isothermal Titration Calorimetry (ITC): ITC experiments were performed at 30 °C using a VP-ITC calorimeter (GE Healthcare). TRIB1(84–372), and MBP fusion proteins of C/EBP α (53–75), C/EBP β (64–86), C/EBP δ (50–72) and C/EBP ϵ (31–53) were initially purified by Ni²⁺ affinity chromatography followed by size-exclusion chromatography using a matched buffer consisting of 10 mM HEPES (pH 7.6), 300 mM NaCl, 0.5 mM TCEP. C/EBP (200 mM) was injected into 20 mM TRIB1(84–372). Baseline corrections and integration were performed using NITPIC (57), isotherms were fit to a single site-binding model using SEDPHAT (58), and figures were generated using GUSSI ([http:// biophysics.swmed.edu/MBR/software.html](http://biophysics.swmed.edu/MBR/software.html)).

Fluorescence Polarisation: fluorescence polarisation of synthetic TRIB1 and C/EBP α peptides bearing an N-terminal fluorescein isothiocyanate (FITC) fluorophore (Mimotopes) was measured as indicated in combination with purified COP1 WD40 domain (376–731), TRIB1(84–372), TRIB2(53–343) or C/EBP β degron peptide. COP1-TRIB1 and TRIB1/2-C/EBP α K_D determination were performed in black 384-well microplates (Greiner Bio-One) format with a final reaction volume of 30 μ L, while TRIB1-C/EBP α displacement by C/EBP β was performed in 96-well format with a final reaction volume of 60 μ L. For COP1 displacement measurements, the COP1 WD40 domain and TRIB1-FITC peptide (349–367) were kept at a constant concentration of 1.5 μ M and 25 nM respectively, diluted in a fluorescence polarisation buffer (10 mM HEPES, 300 mM NaCl, 0.5 mM TCEP and 0.02% (v/v) Tween-20). The concentrations of competing ligand, either TRIB1 protein or TRIB1 peptide were varied from 0–20 μ M. Where necessary the reactions were supplemented with C/EBP α peptide (53–75) at a concentration of 10 μ M. Once all reactions were prepared they were allowed to incubate at room temperature for 20 minutes and measured using the PolarStar (BMG Tech) plate reader. Following this method, the data from three independent experiments were obtained and plotted as the mean $\pm$ S.E.M

using Prism 7. For TRIB1-C/EBP α displacement by C/EBP β , 1.5 μ M TRIB1, 25 nM C/EBP α -FITC peptide were incubated together and C/EBP β was titrated in at concentrations from 43 nM to 350 μ M and measurement was performed in an equivalent manner.

Differential Scanning fluorimetry: The PKIS screening library in 384-well format was received from SGC-UNC at 10 mM stock concentration. The stock was prediluted to 2 mM, before aliquoting into black 384 well plates (Greiner Bio-One) using the mosquito® LCP (TTP labtech) and screening against TRIB1 at a final compound concentration of 40 μ M. TRIB1(84–372) was diluted for use at 5 μ M (with or without 25 μ M C/EBP α peptide) with DSF buffer (10 mM Hepes pH 7.6, 300 mM NaCl, 0.5 mM TCEP). The protein was incubated in the plate at room temperature for 30 minutes. Sypro®Orange was then diluted with DSF buffer for use at 5 $\times$ and was pipetted into each well. The plate was then covered with PCR film and centrifuged for 5 minutes before being measured on a Roche lightcycler® 480 using the default Sypro®Orange protein programme. The data was initially condensed using R version 3.4.3. Data was then analysed in Microsoft Excel using the CS example DSF Analysis v3.0 template provided by the Structural Genomics Consortium (59, 60), and Boltzmann fitting in Graphpad Prism 7.

Supplementary Materials

Fig. S1. Construct Design for crystallization.

Fig. S2. C/EBP α degon electron density.

Fig. S3. Dissociation constant of TRIB1/2 binding C/EBP α .

Fig. S4. Stable regions of C/EBP α degon in complex with TRIB1.

Fig. S5. Displacement of the TRIB1 C-terminal tail from COP1.

Fig. S6. Comparison of TRIB1 to STK40.

Fig. S7. PKIS screening of TRIB1.

Movie S1. Morph between substrate-free (SLE-out) and C/EBP α -bound (SLE-in) structures of TRIB1.

Movie S2. Simulations of the TRIB1-C-terminal tail.

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Figures:

Fig. 1. The TRIB1-C/EBPα degron complex. **(A)** Schematic illustrating degradation of C/EBPα by TRIB1-COP1. **(B)** Crystal structure of the TRIB1-C/EBPα degron complex, with TRIB1 shown predominantly in orange with a green activation loop and C/EBPα in blue. **(C)** Comparison of the C/EBPα binding mode (blue) with that of the prototypic substrate-like protein kinase inhibitor (PKI; turquoise) in complex with protein kinase A (grey surface; PDB 1ATP) **(D)** GST-pulldown of His₆MBP-C/EBPα(53–75) by WT GST-TRIB1(84–372) and indicated mutants, separated by SDS-PAGE and visualised by Coomassie blue staining (upper) or Anti-His₆ immunoblotting (lower). A non-specific band that co-purifies with GST-TRIB1 is indicated with an asterisk. **(E)** Locations of TRIB1 mutations that disrupt C/EBPα binding, with TRIB1 shown as an orange surface and C/EBPα in blue.

Fig. 2. TRIB1 binds various C/EBPs and can be antagonized by phosphorylation. **(A)** Alignment of the TRIB1 degenon from various human C/EBP proteins, and the *Drosophila* C/EBP ortholog, Slbo. **(B)** Isothermal titration calorimetry of indicated MBP-fused C/EBP degenons injected into TRIB1(84–372). Data points represent mean of duplicate titrations, following buffer titration subtraction. **(C)** Detailed view of Ser65 and Tyr67 within the C/EBP α degenon (equivalent to Ser77 and Tyr69 in C/EBP β) in complex with TRIB1. **(D)** Fluorescence polarisation displacement assay of FITC-C/EBP α degenon from TRIB1 by: unmodified, Ser77-phosphorylated, or Tyr79-phosphorylated, C/EBP β peptide. Data points represent the mean of three technical replicates with error bars indicating SEM.

Fig. 3. TRIB1 conformational changes upon substrate binding. Comparison of structures of substrate-free (upper panels; PDB 5cem) and C/EBP α -bound TRIB1 (lower panels; this work). **(A)** Overview from the substrate binding side of the molecule. **(B)** The SLE-motif and surrounding residues within the active site, from the same orientation as panel A. **(C)** Simplified view showing the relative organisation of the α C-helix, Tyr134 and Leu226, from the opposite orientation to panels A/B. **(D)** Position of the α C-helix and residues that contact the C-terminal tail of TRIB1 in substrate-free TRIB1; orientation as for panel C. Movement of selected features upon EBP α binding are indicated with arrows in upper panels, and residues are selectively depicted as sticks for clarity.

Fig. 4. Allosteric release of the COP1-binding motif by substrate-binding. Structure of the C-terminal COP1 binding motif bound to (A) the pseudokinase domain of TRIB1, and (B) the WD40 domain of COP1. (C) Fluorescence polarisation displacement assay of FITC-TRIB1(349–367) from the WD40 domain of COP1 by either TRIB1(84–372) alone at varying concentrations, or TRIB1(84–372) in the presence of 10 μM C/EBP α degnon peptide. Points represent the mean of three technical replicates (generated using two separately prepared stocks of COP1), and error bars represent the SEM. (D) Summary of molecular dynamics simulations of SLE-out TRIB1, or SLE-in TRIB1 with and without substrate. The number of hydrogen bonds between the C-terminal tail and $\beta 4$ strand are plotted over the time course of the simulation above, and an indicative state during the latter part of the simulation is shown below. Animated trajectories are available in movie S2.

Fig. 5. Comparative analysis of molecular dynamics simulations of Trib1 pseudokinase domain with and without C/EBPα peptide. **(A)** The Kullback-Leibler (KL) divergence of the torsion angle of each residue is shown. Motifs and residues that display differential distribution between the C/EBPα unbound simulation and C/EBPα bound simulation are labeled. Left panel: KL divergence of Trib1 simulations without the C-terminal tail. Right panel: KL divergence of Trib1 simulations with the C-terminal tail. **(B)** Representative snapshots from TRIB1+C-tail:C/EBPα simulation. SLE+1 aspartate (Asp228) mediates charge interaction with Lys210 from catalytic loop, Arg102 from glycine-rich loop and Lys130 from αC-helix.

Fig. 6. TRIB1 can potentially bind small molecule ligands but not ATP. Representation of the available binding cavity of TRIB1 (grey) in **(A)** the SLE-out conformation from PDB 5cem, and **(B)** the SLE-in conformation when bound to C/EBPα. **(C)** DSF melting analysis of TRIB1 or TRIB1+C/EBPα degnon peptide in the presence or absence of ATP. **(D)** Summary of DSF analyses of TRIB1(84–372) against the PKIS library. T_m are expressed as difference between each compound and the mean of three DMSO-only controls. The four compounds shown in more detail in panel E are indicated. **(E)** Individual DSF melting curves of four selected compounds (left), with additional titrations for each that include C/EBPα degnon peptide (right).

Table 1. Crystallographic data summary

	TRIB1–cEBPα
Beamline	AS-MX2
Wavelength (Å)	0.9537
Resolution (outer shell) (Å)	48.7–2.8 (2.95–2.8)
Space group	P6 ₁ 22
Unit cell parameters	a = 98.8 Å
	b = 98.8 Å
	c = 332.7 Å
	α = 90°
	β = 90°
	γ = 120°
R _{merge} (outer shell)	0.077 (1.992)
R _{pim} (outer shell)	0.035 (0.879)
Mean I/σI (outer shell)	19.8 (1.5)
Completeness (outer shell)	99.8 (99.1)
Multiplicity (outer shell)	10.4 (10.7)
Total No. of reflections	258141 (37340)
No. of unique reflections	24777 (3491)
Mean (I) half-set correlation CC(1/2)	1.000 (0.609)
(outer shell)	
Wilson B factor (Å ²)	82.1
Refinement statistics	
R _{cryst}	0.220
R _{free}	0.276
Average B -factor overall (Å ²)	113
Ramachandran plot statistics (%)	
Favored regions	93.1%
Allowed regions	6.5%
Outliers	0.4%
PDB entry	

Supplementary Materials:

Supplementary Figure 1. Construct Design for crystallization. (A) Schematic illustration of the fusion construct that allowed successful crystallisation of the TRIB1-C/EBPα degron complex. (B) Illustration of the crystal packing within the unit cell. The linker between the TRIB1 pseudokinase and C/EBPα is not defined (dotted line), and could arise by C/EBPα being contributed by a TRIB1 molecule across a side-by-side packing or a packing between C-terminal lobes.

Supplementary Figure 2. C/EBPα degon electron density. (A) Electron density maps (2Fo-Fc blue, Fo-Fc (green)) contoured to 1.0 and 3.0 σ respectively, following a cycle of model refinement omitting the C/EBPα peptide. (B) 2Fo-Fc electron density map contoured to (1.0 σ) after refinement of the final structure.

Supplementary Figure 3. Dissociation constant of TRIB1/2 binding C/EBPα. Measurement of the dissociation constant of the TRIB1-C/EBPα degon monitored using fluorescence polarisation of FITC-C/EBPα degon peptide (25 nM) and varying concentrations of TRIB1(84–372) or TRIB2(53–343).

Supplementary Figure 4. Stable regions of C/EBPα degron in complex with TRIB1. RMSF plot of C/EBPα residues during 1 μ s simulation in complex with TRIB1. Simulations were performed both incorporating (red) and omitting (blue) the C-terminal tail of TRIB1 (see also Fig. 4D), indicating that the C-terminal tail of TRIB1 does not impart a significant stabilising or destabilising effect on substrate binding.

Supplementary Figure 5. Displacement of the TRIB1 C-terminal tail from COP1. Fluorescence polarisation displacement assay of FITC-TRIB1(349–367) from COP1 WD40 domain, by unlabelled TRIB1(349–367) (blue) compared to TRIB1(84 to 372) (black).

Supplementary Figure 6. Comparison of TRIB1 to STK40. Overlay of substrate-bound TRIB1 (Orange) with STK40 (light grey; PDB 5L2Q)

A

B

Compound	TRIB1(84-372)	Plus C/EBP	Compound	TRIB1(84-372)	Plus C/EBP	Compound	TRIB1(84-372)	Plus C/EBP	Compound	TRIB1(84-372)	Plus C/EBP
GW804482X	-1.593333	-0.936667	SB-759335-B	-0.613333	-0.286667	GSK938890A	-0.323333	-0.116667	GW809885X	-0.013333	0.303333
GSK237700A	-1.373333	-1.246667	GW301789X	-0.603333	0.103333	GW827105X	-0.323333	0.103333	GW627834A	-0.013333	0.153333
SB-437013	-1.323333	-1.316667	GSK319347A	-0.603333	-0.146667	GW416469X	-0.323333	-0.256667	SB-254169	-0.013333	-0.196667
SB-409513	-1.313333	-0.536667	GSK614526A	-0.603333	-0.336667	SB-210313	-0.313333	0.046667	SB-220025-A	-0.003333	0.393333
GW441756X	-1.213333	-0.926667	GW743024X	-0.593333	0.313333	SB-737198	-0.313333	-0.026667	GW631581B	-0.003333	-0.106667
SB-390523	-1.053333	-1.116667	GR105659X	-0.593333	0.413333	GW278681X	-0.313333	0.046667	GW572399X	-0.003333	0.343333
GSK200398A	-1.043333	-0.016667	SB-285234-W	-0.593333	-0.176667	GSK1030058A	-0.313333	-0.546667	GW300657X	0.006667	0.723333
GW654652X	-1.033333	-1.746667	SB-253226	-0.593333	-0.406667	GW785404X	-0.303333	-0.276667	GW781673X	0.006667	0.263333
GW770220A	-1.003333	0.393333	GW806776X	-0.583333	-0.336667	SB-242721	-0.303333	0.203333	GW817394X	0.026667	-0.006667
GW853606X	-0.973333	-0.126667	SB-686709-A	-0.583333	0.063333	GW434756X	-0.303333	-0.456667	GW574783B	0.026667	0.403333
GSK718429A	-0.953333	0.383333	SB-278538	-0.583333	-0.296667	GW441806A	-0.303333	-0.646667	DMSO_1	0.026667	0.083333
GSK35962X	-0.943333	-0.456667	GW779439X	-0.583333	0.123333	GW581744X	-0.303333	-0.456667	GW621970X	0.036667	-0.066667
SB-682330-A	-0.943333	-1.806667	GW549390X	-0.583333	-0.036667	GW619487A	-0.293333	0.113333	GW633459A	0.036667	0.133333
GSK1392956A	-0.933333	-0.136667	GW305178X	-0.583333	-0.116667	GW768505X	-0.293333	0.533333	G1261520X#4	0.036667	-0.266667
GW275944X	-0.933333	-0.116667	GW450241X	-0.573333	-0.086667	GW632580X	-0.283333	-0.156667	GW782612X	0.036667	-0.536667
GW622055X	-0.933333	-0.456667	GSK237700A_1	-0.563333	0.043333	SB-242719	-0.283333	-0.186667	GW769076X	0.046667	-0.106667
GW576924A	-0.933333	-0.226667	GW569293E	-0.563333	-0.016667	SB-739452	-0.283333	-0.396667	GW616030X	0.046667	-0.206667
SB-734117	-0.923333	-0.246667	SB-347804	-0.563333	-0.136667	GW435821X	-0.283333	-0.406667	DMSO_2	0.046667	-0.066667
SB-358518	-0.913333	0.373333	GW801372X	-0.563333	-0.276667	GW56804X	-0.283333	-0.856667	SB-264865	0.056667	-0.046667
GW806290X	-0.913333	-0.246667	SB-409514	-0.553333	-0.216667	GW549034X	-0.273333	-0.156667	SB-264866	0.076667	0.153333
SB-736302	-0.903333	-0.336667	GW642138X	-0.553333	0.093333	SB-725317	-0.273333	0.133333	GSK1220512A	0.086667	0.473333
GW575808A	-0.883333	-0.196667	SB-735467	-0.553333	-0.166667	GW680975X	-0.263333	0.193333	GW827106X	0.096667	0.333333
GW642125X	-0.883333	-0.276667	GW301888X	-0.553333	-0.096667	SB-223133	-0.263333	0.113333	GW778894X	0.096667	0.143333
GW290597X	-0.873333	0.053333	GW780056X	-0.543333	0.243333	GSK466314A	-0.253333	0.063333	GSK3206866A	0.096667	-0.166667
GW813360X	-0.853333	-0.316667	SB-742864	-0.543333	-0.196667	GW831090X	-0.253333	-0.346667	GW810372X	0.106667	0.143333
GSK466317A	-0.853333	-0.506667	GSK317315A	-0.543333	-0.476667	SB-610251-B	-0.253333	-0.386667	GW567808A	0.106667	0.933333
GW786460X	-0.843333	-0.036667	GW708336X	-0.543333	-0.496667	GW432441X	-0.243333	-0.016667	GW416981X	0.116667	0.033333
GW673715X	-0.843333	0.023333	GSK1023156A	-0.533333	-0.366667	GW641155A	-0.243333	0.003333	SB-400868-A	0.136667	0.233333
GW575533A	-0.833333	-0.396667	GW759710A	-0.533333	-0.026667	GW577921A	-0.243333	-0.266667	GW458787B	0.136667	0.153333
GW445015X	-0.833333	-0.316667	GW693481X	-0.523333	-0.036667	GW429374A	-0.243333	0.713333	GW869810X	0.146667	0.063333
SB-226879	-0.823333	-0.296667	SB-590885-AAD	-0.523333	-0.436667	GW29877X	-0.243333	0.113333	GW513184X	0.156667	0.303333
GW279320X	-0.823333	0.103333	GW771127A	-0.523333	-0.366667	GW711782X	-0.233333	-0.296667	GSK561866B	0.156667	0.123333
GW846105X	-0.823333	-0.156667	G185581X#3	-0.523333	-0.576667	GW811761X	-0.223333	0.123333	GW574782A	0.156667	0.603333
GW708893X	-0.803333	-1.166667	GSK1819799A	-0.513333	0.093333	GW280670X	-0.213333	0.433333	GSK1751853A	0.186667	0.103333
GSK711701A	-0.803333	-1.046667	GW679410X#4	-0.503333	0.003333	SB-678557-A	-0.213333	-0.146667	GW612286X	0.186667	0.343333
GW568377B	-0.793333	-0.086667	GSK949675A	-0.503333	-0.146667	SB-741905	-0.213333	-0.286667	GW615311X	0.206667	0.003333
GW627512B	-0.793333	-0.066667	SB-220455	-0.483333	-0.246667	SB-236687	-0.203333	-0.236667	GW589961A	0.226667	0.123333
GW445014X	-0.793333	-0.866667	SB-333612	-0.473333	0.233333	GW406731X	-0.203333	-0.416667	GW807982X	0.246667	0.743333
GSK182497A	-0.773333	0.433333	GSK2219385A	-0.473333	-0.066667	SB-250715	-0.203333	-0.286667	SB-216385	0.246667	0.043333
SB-743899	-0.753333	-0.076667	SKF-86055	-0.473333	-0.346667	GW775608X	-0.193333	-0.336667	GSK605714A	0.256667	0.143333
SB-245392	-0.753333	-0.596667	GSK625137A	-0.463333	-0.146667	GW784372X	-0.193333	-0.376667	GW807930X	0.266667	-0.456667
GW459057A	-0.743333	-0.076667	SB-278539	-0.453333	-0.406667	GR269666A	-0.183333	-0.296667	G1261520A	0.266667	-0.046667
GW809897X	-0.743333	0.063333	SB-739245-AC	-0.443333	-0.266667	GSK317354A	-0.173333	0.193333	GW811168X	0.316667	0.773333
GSK312948A	-0.733333	-0.496667	GW618013X	-0.443333	-0.166667	GW632046X	-0.173333	-0.276667	GW439255X	0.316667	-0.126667
GW784408X	-0.733333	0.103333	SB-360741	-0.443333	-0.126667	SB-242717	-0.173333	-0.206667	GW405841X	0.346667	-0.106667
GW458344X	-0.733333	-1.816667	GSK299115A	-0.443333	-0.546667	GW829874X	-0.163333	-0.006667	GW566221B	0.396667	0.193333
GSK30263A	-0.723333	0.023333	SB-738561	-0.433333	-0.226667	GW784752X	-0.163333	-0.066667	GW805758X	0.416667	-0.096667
SB-431542-A	-0.723333	-0.356667	SB-361058	-0.433333	0.073333	SB-735465	-0.163333	-0.066667	GW799251X	0.476667	0.293333
GW734508X	-0.713333	0.063333	GW820759X	-0.423333	-0.126667	SB-738482	-0.163333	-0.026667	GSK1326255A	0.516667	0.963333
GSK554170A	-0.713333	-0.396667	GW561436X	-0.423333	-0.246667	GW572401X	-0.153333	-0.296667	GSK1173862A	0.596667	0.793333
GW785804X	-0.713333	-0.136667	GW445012X	-0.423333	-0.736667	GW621431X	-0.153333	-0.076667	GSK1713088A	0.746667	2.473333
SB-633825	-0.703333	-0.826667	GW643971X	-0.403333	0.193333	GSK180736A	-0.153333	-0.306667	GW832467X	0.776667	0.463333
SB-390527	-0.703333	-0.596667	GW300660X	-0.403333	0.183333	GW607117X	-0.153333	-0.236667	GW700494A	0.826667	0.753333
GW282449A	-0.703333	-0.416667	GW682841X	-0.403333	-0.396667	GW445017X	-0.143333	-0.086667	GW694234A	0.906667	1.163333
GW580509X	-0.693333	-0.086667	GW276655X	-0.403333	0.043333	GW680191X	-0.143333	-0.286667	GW828525X	0.916667	0.503333
SB-614067-R	-0.693333	-0.196667	SB-242718	-0.393333	-0.016667	GW580496A	-0.143333	0.293333	GW819077X	0.916667	0.063333
GSK980961A	-0.693333	-0.446667	SB-675259-M	-0.393333	-0.146667	GW828536X	-0.133333	0.193333	GW680908A	0.956667	1.623333
GSK1030062A	-0.693333	-0.926667	SB-251527	-0.393333	-0.156667	GW352430A	-0.133333	0.073333	GW694590A	0.966667	1.303333
GW461104A	-0.693333	0.633333	SKF-86002-A2	-0.393333	-0.486667	GSK270822A	-0.133333	-0.516667	GSK192082A	0.996667	0.673333
GW576609B	-0.693333	-0.226667	GW831091X	-0.393333	-0.306667	GW678313X	-0.123333	0.433333	GW794607X	1.066667	-0.076667
SB-693162	-0.683333	-0.296667	GW282974X	-0.383333	0.093333	GW817396X_1	-0.113333	0.033333	GW784684X	1.256667	0.923333
SKF-62604	-0.683333	-0.376667	SB-376719	-0.383333	-0.336667	SB-476429-A	-0.113333	0.123333	GW709042A	1.356667	0.773333
GW284408X	-0.673333	-0.016667	SB-814597	-0.383333	-0.046667	GW651576X	-0.113333	0.093333	GW827396X	1.356667	-0.216667
GW275616X	-0.673333	-0.346667	GSK204925A	-0.373333	-0.276667	GW852849X	-0.103333	-0.306667	GW296115X	1.466667	0.763333
GW64007X	-0.673333	-0.886667	GW695874X	-0.373333	-0.286667	GW568326X	-0.103333	-0.166667	GW693917X	1.626667	1.883333
SB-239272	-0.663333	-0.096667	GW440139B	-0.373333	-0.246667	GW297361X	-0.093333	-0.016667	GW837331X	1.646667	0.123333
GW578748X	-0.663333	-0.156667	SB-736290	-0.363333	-0.363333	GSK2186269A	-0.083333	0.333333	GW576484X	1.676667	0.783333
GSK269962B	-0.663333	-0.806667	GW843682X	-0.363333	-0.116667	GW300653X	-0.083333	-0.116667	GW607049A	1.746667	4.223333

Supplementary Figure 7. PKIS screening of TRIB1. Differential scanning fluorimetry (DSF) screening of TRIB1(84 to 372) against the PKIS library. (A) T_m shift data, expressed relative to DMSO-only control, for TRIB1-C/EBPα degron peptide mixture. Samples are shown in the same order as Fig. 6D, and generally exhibit a similar binding profile with and without C/EBPα, with some exceptions. (B) DSF data for individual compounds, relating to Fig. 6D and fig. S7A.

Movie S1. Morph between substrate-free (SLE-out) and C/EBPα-bound (SLE-in) structures of TRIB1. Morph was created in PyMOL.

Movie S2. Simulations of the TRIB1-C-terminal tail. Pseudokinase-C-terminal tail interaction during molecular dynamics simulations of TRIB1 SLE-out (left), SLE-in without C/EBPα (centre), and SLE-in with C/EBPα (right).