Research Article

Journal of PeptideScience

Received: 7 October 2010 Revised: 21 January 2011 Accepted: 26 January 2011 Published online in Wiley Online Library: 24 March 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1366

Design, synthesis and characterization of a peptide able to bind proteins of the KCTD family: implications for KCTD – cullin 3 recognition‡

Luciano Pirone,[a,b§] Stefania Correale,[a,b§] Ivan de Paola,[b] Laura Zaccaro,[a] Giuseppina De Simone,[a] Luigi Vitagliano,[a] Emilia Pedone[a]* and Sonia Di Gaetano[a]*

Pox virus Zinc/Bric-à-brac, Tramtrack and Broad (POZ/BTB) is a widespread domain detected in proteins involved in a variety of biological processes. Human genome analyses have unveiled the presence of POZ/BTB domain in a class of proteins (KCTD) whose role as important players in crucial biological processes is emerging. The development of new molecular entities able to interact with these proteins and to modulate their activity is a field of relevant interest. By using molecular modeling and literature mutagenesis analyses, we here designed and characterized a peptide that is able to interact with submicromolar affinities with two different members (KCTD11 and KCTD5) of this family. This finding suggests that the tetrameric KCTD11 and the pentameric KCTD5 are endowed with a similar cavity at the subunit–subunit interface deputed to the Cul3 binding, despite their different oligomeric states.

Keywords: protein–protein recognition; structure–function relationships; ubiquitination; POZ/BTB domains

Introduction

The Pox virus Zinc/Bric-à-brac, Tramtrack and Broad (POZ/BTB) domain is a module that has been found in a large number of functionally unrelated proteins [1–3]. The domain has been originally identified as a conserved motif present in the *Drosophila melanogaster* BTB complex transcription regulators, and in many POZ finger proteins [1–3]. Proteins containing the POZ/BTB domain are involved in diversified biological processes, which include transcription repression [4], cytoskeleton regulation [5], mediators of oligomerization and gating of ion channels [6] and protein ubiquitination/degradation [7–9]. It is commonly assumed that this domain plays a major role in protein–protein interactions. Structural characterizations of POZ/BTB domains isolated from different proteins have revealed the occurrence of variations on a common theme [1]. In all structures of POZ/BTB domains, so far reported it is possible to identify a common motif characterized by the presence of a three-stranded β-sheet and five α-helices [1]. Recent human genome investigations have highlighted the presence of the POZ/BTB domain in a new class of human proteins. The POZ/BTB domain of these proteins share a significant sequence identity (~30%) with the tetramerization domain of the K^+ voltage-gated channel T1 (T1-Kv) proteins (http://btb.uhnres.utoronto.ca/index.html). On this basis, this class of proteins has been denoted as KCTD (K^+ channel tetramerization domain containing proteins). Although KCTD proteins share a common POZ/BTB domain in their *N*-terminal regions, they present diversified *C*-terminal domains [10]. Although the biological role of these proteins is yet to be determined, recent investigations suggest that they are involved in important biological processes. It has been shown that a missense mutation of KCTD7 is linked to neurodegeneration and progressive myoclonic epilepsy [11]. Moreover, KCTD15 variants have been associated with adult obesity risk [12]. Very recently, it has been shown that KCTD12, previously identified as an important factor involved in the maturation of ear neurons, along with KCTD8 and KCTD16, plays a role in the pharmacology and kinetics of the gamma-aminobutyric acid (GABA(B)) receptor response [13,14]. The two members of the family that have been characterized in details are KCTD5 and KCTD11. KCTD5, the only member of the

* *Correspondence to: Emilia Pedone, Institute of Biostructures and Bioimaging, CNR, Via Mezzocannone 16, 80134, Napoli, Italy. E-mail: empedone@unina.it*

Sonia Di Gaetano, Institute of Biostructures and Bioimaging, CNR, Via Mezzocannone 16, 80134, Napoli, Italy. E-mail: digaetan@unina.it

a *Institute of Biostructures and Bioimaging, CNR, 80134 Napoli, Italy*

b *Department of Biological Sciences, "Federico II" University, 80134 Napoli, Italy*

‡ *Special issue devoted to contributions presented at the 12th Naples Worshop on Bioactive Peptides and 2nd Italy-Korea Symposium on Antimicrobial Peptides, 4-7 June 2010, Naples, Italy.*

§ *These authors equally contributed.*

Abbreviations used: *KCTD, potassium channel tetramerization domain containing proteins; PDB, Protein data bank; Cul3, cullin 3; KCTD11 BTB and KCTD5 BTB, POZ/BTB domains of KCTD11 and KCTD5, respectively; TrxA, Escherichia coli thioredoxin; Cul3wt_pep, peptide corresponding to the 49–68 region of Cul3; Cul3KK_pep, variant of Cul3wt_pep in which two Tyre residues were replaced with Lys residues.*

family whose three-dimensional structure has been determined experimentally, interacts with human Golgi reassembly stacking protein 55 (GRASP55) [10,15]. It has also been shown that KCTD5 also interacts with cullin 3 (Cul3) and is potentially involved in the E3 ubiquitin ligase complex [15].

KCTD11, a tumor suppressor, is an important antagonist of the Hedgehog pathway which is frequently deleted in human medulloblastoma [16–19]. Very recent studies have shown that it is widely downregulated in other human cancers [20]. KCTD11 is involved in the deacetylation and activation of Gli1 and Gli2, two key transcription factors of Hh signaling. Indeed, Gli1 is deacetylated by histone deacetylase 1 (HDAC1), which is ubiquitined for degradation by the E3 ubiquitin ligase, formed by Cul3 and KCTD11 [21].

Taking into account the role played by KCTD in important biological processes, the development of new molecular entities able to interact with these proteins and to modulate their activity is a field of relevant interest. We here report the design and the characterization of a Cul3-based peptide that is able to interact either with the pentameric KCTD5 or with the tetrameric KCTD11.

Experimental

Materials

All amino acids, Rink-amide 4-methylbenzhydrylamine (MBHA) resin, HBTU, HOBt and HATU were purchased from Novabiochem. DIPEA, TFA, piperidine, *N*-(+)-biotinyl-6-aminocaproic acid, acetic anhydride and scavengers were supplied from Fluka. The solvents used in the synthesis, purification and characterization of the peptides were from Romil.

Peptide Synthesis

Cul3wt_pep and Cul3KK_pep derived from Cul3 sequence (see below) were obtained by Fmoc solid-phase strategy (0.1 mmol). To mimic the charge status of the fragment within the parent protein, the *N*-terminus and the *C*-terminus of the peptides were acetylated and amidated, respectively. The syntheses were carried out with Rink-amide MBHA resin (substitution 0.51 mmol g^{-1}), using all standard amino acids. Coupling reactions were performed by using 10 equiv of Fmoc protected amino acids activated *in situ* with HBTU (9.8 equiv)/HOBt (9.8 equiv)/DIPEA (20 equiv) in DMF for 1 h.

Fmoc protecting group was removed by treatment with 30% piperidine in DMF two times for 10 min. Before the cleavage from the resin, both peptides were acetylated or biotinylated at *N*-terminus to obtain the corresponding derivatives. The acetylation reaction was carried out two times for 10 min using a solution of acetic anhydride (0.5 M)/DIPEA (0.015 M)/HOBt (0.125 M) in DMF (4.7 : 4 : 91.3 v/v/v). Biotinylated peptides were obtained using a solution of *N*-(+)-biotinyl-6-aminocaproic acid (2 equiv)/HATU (1.9 equiv)/DIPEA (4 equiv) in DMF overnight.

All peptides were cleaved off the resin by treatment with a mixture of TFA/H_2O/Ethanedithiol (EDT)/Triisopropylsilane (TIS) (94 : 2.5 : 2.5 : 1 v/v/v/v) for 2 h at room temperature. The resins were filtered and the crude peptides were precipitated with diethyl ether, dissolved in a H_2O/CH_3CN (1 : 1) solution and lyophilized.

The peptides were purified by preparative RP-HPLC on the Shimadzu system equipped with a UV-Vis detector SPD10A using a Phenomenex Jupiter Proteo C_{12} column (21.2 × 250 mm; 4 μm; 90 Å) and a linear gradient of H_2O (0.1% TFA)/CH_3CN (0.1% TFA) from 20 to 70% of CH_3CN (0.1% TFA) in 20 min at flow rate of 5 ml/min. The collected fractions containing the peptides were lyophilized. The identity and purity of the peptides were assessed by an ESI-LC-MS instrument (ThermoFinnigan, NY, USA) equipped with a diode array detector combined with an electrospray ion source and ion trap mass analyzer using a Phenomenex Jupiter Proteo C_{12} column (150 × 2 mm; 4 μm; 90 Å) and a linear gradient of H_2O (0.1% TFA)/CH_3CN (0.1% TFA) from 20 to 80% of CH_3CN (0.1% TFA) in 20 min at flow rate of 200 μl/min.

CD Spectroscopy Studies

Far-UV CD spectra were registered in the 190–260 nm range on a Jasco J-810 spectropolarimeter at 25 °C using a quartz cell with a 0.1-cm path length. The peptides were dissolved in 10 mM phosphate buffer (pH = 7.1). The final concentrations of Cul3wt_pep and Cul3KK_pep were 5.56 × 10^{-5} and 5.05 × 10^{-5} M, respectively.

Expression and Purification of POZ/BTB domains of KCTD11 and KCTD5

Expression and purification of the KCTD11 BTB were performed as described elsewhere [22]. Briefly, the His tag recombinant domain was expressed as a recombinant fusion protein with thioredoxin A (TrxA). A homogenous product was obtained upon purification.

The recombinant plasmid containing the POZ/BTB sequence of KCTD5 was a gift of Prof. Goldstein (Department of Pediatrics and Institute of Molecular Pediatric Sciences, University of Chicago). The His-tag recombinant protein was expressed and purified as already reported [10,22].

ELISA Assays

For ELISA assays, 5 μg/ml streptavidin in phosphate/citrate buffer pH 5.0 was incubated overnight at 37 °C for coating. First, binding was executed with 0.8 μM biotinylated Cul3 peptide in Phosphate Buffered Saline (PBS) 1X for 1 h at room temperature. Second, binding was performed with different concentrations of His-TrxA/KCTD11 BTB or His/KCTD5 BTB (0.8, 1.5, 3.8, 7.6 and 15.6 μM) in PBS 1X. His-TrxA was used as negative control in the same concentrations. As blocking solution 1% BSA, 0.05% Tween-20 in PBS 1X was used for 1 h at room temperature. To reveal the occurred interaction mouse anti-His monoclonal antibody was incubated in 1 : 1000 dilution at room temperature for 2 h; then, horseradish peroxidase-conjugated anti-mouse antibody (Pierce) was diluted 1 : 10 000 in PBS 1X and incubated at room temperature for 1 h. The colorimetric reaction has been carried out with SIGMA*FAST* OPD reagent (Sigma Aldrich), according to the manufacturer's instructions. Finally, a Model 680 Microplate Reader (Bio-Rad, Hercules, CA-USA) has been used for readings at 490 nm; data were processed by a Microplate Manager 5.2 program. The reported data are mean values of triplicate experiments.

Results and Discussion

Design of a Cul3-Based Peptide as a Potential KCTD11 Binder

Previous modeling and mutagenesis studies have provided some clues on the regions that mediate the recognition between Cul3 and KCTD11 BTB [21,22]. The complex is stabilized by a cluster

Journal of PeptideScience

Figure 1. Location of the 49–68 region (red), corresponding to the sequence of the Cul3wt_pep, in the three-dimensional structure of Cul3. Side chains of the residues Tyr58 and Tyr62 directly involved in KCTD11 are also shown.

Table 1. Analytical data of synthesized peptides obtained by ESI-LC-MS

Peptide	T_r (min)	Theoretical mass (Da)	Found mass (ESI-MS) (Da)
Ac-Cul3_wt	15.24	2412.2	2413.1
Biotin-Cul3_wt	15.40	2709.3	2711.8
Ac-Cul3_KK	11.51	2342.2	2343.6
Biotin-Cul3_KK	13.06	2639.4	2640.9

of aromatic residues which include Tyr58, Ty62 and Tyr125 of the cullin and Phe102 and Tyr103 of KCTD11 BTB. It has been shown that mutations of these residues inhibit the formation of the complex. An important role in Cul3-KCTD11 interaction is played by the helix 55–65 of Cul3 that is tightly bound at the interface of KCTD11 tetramer. Although other Cul3 residues are involved in KCTD11 recognition, we focused our attention on the role played by this region. A Cul3-based peptide as a potential KCTD11 binder was therefore designed by considering the peptide NSGLSFEELYRNAYTMVLHK, corresponding to the 49–68 region in Cul3 sequence (designed as Cul3wt_pep hereafter). The location of this fragment within Cul3 three-dimensional structure is shown in Figure 1.

Synthesis, Purification, and Characterization of the Cul3-Based Peptides

To verify the effectiveness of the designed peptide, a biotinylated form of Cul3wt_pep was synthesized on Fmoc solid-phase and then purified by RP-HPLC. To check the role of aromatic side chains in Cul3 – KCTD11 recognition, a Cul3wt_pep variant (Cul3KK_pep) in which Tyr58 and Tyr62 were substituted with Lys residues, was also prepared. Cul3KK_pep was synthesized and purified following the same procedures used for Cul3wt_pep. The identities of the obtained compounds were assessed by ESI-LC-MS (Table 1).

The CD spectra of both peptides, dissolved in a phosphate buffer (pH = 7.1), show a deep minimum at ~202–203 nm, a second minimum at ~222 nm, a cross over at ~192–193, and a maximum at a wavelength lower than 190 nm (Figure 2). The general features of the spectra are indicative for both peptides of the presence of a low, but significant, helical secondary structure content. The ratio between the CD signal at 222 nm and the signal of the other minimum at lower wavelength, which is often used as a mean to confirm the presence of helical content [23], is 0.48 and

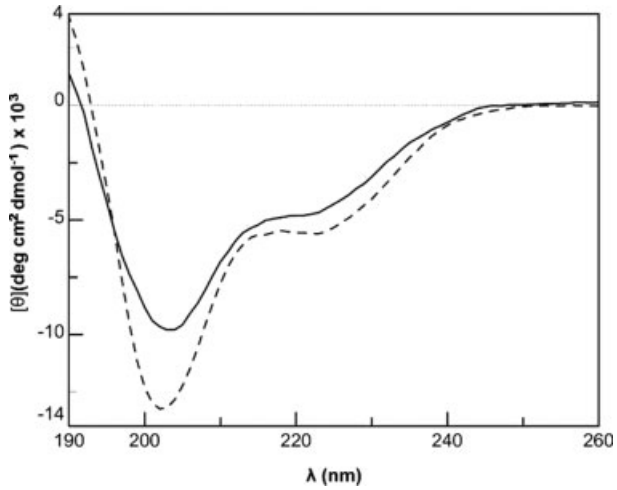

Figure 2. CD spectra of Cul3wt_pep (———) and Cul3KK_pep (- - - -) registered in phosphate buffer at pH = 7.1. θ is expressed as mean residue molar ellipticity [(θ)].

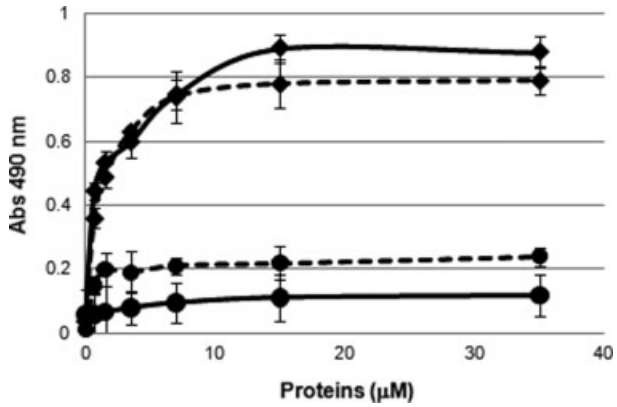

Figure 3. Binding curves obtained from ELISA assays on the proteins KCTD11 BTB (continue line) and KCTD5 BTB (dashed line) using the peptides Biotin Cul3wt_pep (diamond) and Biotin Cul3KK_pep (circle).

0.42 for Cul3wt_pep and Cul3KK_pep, respectively. These values, although much smaller than those observed for samples with elevated helical contents (close to the unity), are indicative of the presence of helical conformations. To quantify the helical content, we performed spectral deconvolution analysis using CDNN and CDPRO programs. The estimation of the helical content provided by these analyses was ~13% for both peptides.

Cul3 Peptide Interacts with POZ/BTB Domains with Different Oligomeric Organization

The ability of biotinylated Cul3wt_pep to bind the recombinant KCTD11 BTB domain, expressed as a fused protein with TrxA, was investigated by ELISA assay, following the procedure illustrated in the Methods section. The binding of the peptide to TrxA was monitored as negative control. As shown in Figure 3, the trend of the binding curve demonstrates that Cul3wt_pep specifically binds KCTD11 BTB with an apparent affinity constant of 0.8 μM. Similar results were obtained either coating KCTD11 BTB or the biotinylated peptide through streptavidin. The inability of the peptide variant Cul3KK_pep (Figure 3) to bind KCTD11 BTB

corroborates the indications provided by the modeling on the important role of the aromatic residues in the recognition process.

It has been reported that, in addition to KCTD11, another member of the KCTD, KCTD5, is able to bind Cul3. Intriguingly, the BTB domain of KCTD11 and KCTD5 exhibit different oligomeric states, being KCTD11 and KCTD5 tetrameric and pentameric, respectively. In this framework, we checked the binding of Cul3wt_pep to KCTD5 BTB. The binding curve (Figure 3) indicates that the peptide is also able to bind to KCTD5 BTB with an apparent affinity constant that is similar to that observed for the association with KCTD11 BTB.

Conclusion

We here show that a rather small peptide derived from a Cul3 fragment is able to interact with BTB domains of KCTD proteins despite their different oligomeric states. As KCTD are involved in crucial biological processes, the development of peptides able to interact with these proteins has important potential applications. Present findings also suggest strategies for future peptide optimization. Indeed, we here show that Cul3wt_pep only partially retains the helical state that the fragment assumes in the parent protein. It is likely that an increase in the helical propensity of Cul3wt_pep variants may be associated with an increased affinity for KCTD proteins. Several literature successful examples on other systems conducted using this strategy provide an indirect support to this approach [24,25].

Acknowledgements

The authors thank the Italian MIUR for financial support (PRIN 2007 and FIRB contract RBRN07BMCT).

References

1 Stogios PJ, Downs GS, Jauhal JJ, Nandra SK, Prive GG. Sequence and structural analysis of BTB domain proteins. *Genome Biol.* 2005; **6**: R82.
2 Zollman S, Godt D, Prive GG, Couderc JL, Laski FA. The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in drosophila. *Proc. Natl. Acad. Sci. U.S.A.* 1994; **91**: 10717–10721.
3 Bardwell VJ, Treisman R. The POZ domain: a conserved protein-protein interaction motif. *Genes Dev.* 1994; **8**: 1664–1677.
4 Melnick A, Ahmad KF, Arai S, Polinger A, Ball H, Borden KL, Carlile GW, Prive GG, Licht JD. In-depth mutational analysis of the promyelocytic leukemia zinc finger BTB/POZ domain reveals motifs and residues required for biological and transcriptional functions. *Mol. Cell Biol.* 2000; **20**: 6550–6567.
5 Kang MI, Kobayashi A, Wakabayashi N, Kim SG, Yamamoto M. Scaffolding of keap1 to the actin cytoskeleton controls the function of nrf2 as key regulator of cytoprotective phase 2 genes. *Proc. Natl. Acad. Sci. U.S.A.* 2004; **101**: 2046–2051.
6 Minor DL, Lin YF, Mobley BC, Avelar A, Jan YN, Jan LY, Berger JM. The polar t1 interface is linked to conformational changes that open the voltage-gated potassium channel. *Cell* 2000; **102**: 657–670.
7 Pintard L, Willis JH, Willems A, Johnson JL, Srayko M, Kurz T, Glaser S, Mains PE, Tyers M, Bowerman B, Peter M. The BTB protein mel-26 is a substrate-specific adaptor of the cul-3 ubiquitin-ligase. *Nature* 2003; **425**: 311–316.
8 Furukawa M, He YJ, Borchers C, Xiong Y. Targeting of protein ubiquitination by BTB-cullin 3-roc1 ubiquitin ligases. *Nat. Cell Biol.* 2003; **5**: 1001–1007.
9 Geyer R, Wee S, Anderson S, Yates J, Wolf DA. BTB/POZ domain proteins are putative substrate adaptors for cullin 3 ubiquitin ligases. *Mol. Cell* 2003; **12**: 783–790.
10 Dementieva IS, Tereshko V, McCrossan ZA, Solomaha E, Araki D, Xu C, Grigorieff N, Goldstein SA. Pentameric assembly of potassium channel tetramerization domain-containing protein 5. *J. Mol. Biol.* 2009; **387**: 175–191.
11 Van Bogaert P, Azizieh R, Desir J, Aeby A, De Meirleir L, Laes JF, Christiaens F, Abramowicz MJ. Mutation of a potassium channel-related gene in progressive myoclonic epilepsy. *Ann. Neurol.* 2007; **61**: 579–586.
12 Elks CE, Loos RJ, Sharp SJ, Langenberg C, Ring SM, Timpson NJ, Ness AR, Davey Smith G, Dunger DB, Wareham NJ, Ong KK. Genetic markers of adult obesity risk are associated with greater early infancy weight gain and growth. *PLoS Med.* **7**: e1000284.
13 Schwenk J, Metz M, Zolles G, Turecek R, Fritzius T, Bildl W, Tarusawa E, Kulik A, Unger A, Ivankova K, Seddik R, Tiao JY, Rajalu M, Trojanova J, Rohde V, Gassmann M, Schulte U, Fakler B, Bettler B. Native GABA(B) receptors are heteromultimers with a family of auxiliary subunits. *Nature* 2010; **465**: 231–235.
14 Bartoi T, Rigbolt KT, Du D, Kohr G, Blagoev B, Kornau HC. GABAB receptor constituents revealed by tandem affinity purification from transgenic mice. *J. Biol. Chem.* 2010; **285**: 20625–20633.
15 Bayon Y, Trinidad AG, de la Puerta ML, Del Carmen Rodriguez M, Bogetz J, Rojas A, De Pereda JM, Rahmouni S, Williams S, Matsuzawa S, Reed JC, Crespo MS, Mustelin T, Alonso A. Kctd5, a putative substrate adaptor for cullin3 ubiquitin ligases. *FEBS J.* 2008; **275**: 3900–3910.
16 Gallo R, Zazzeroni F, Alesse E, Mincione C, Borello U, Buanne P, D'Eugenio R, Mackay AR, Argenti B, Gradini R, Russo MA, Maroder M, Cossu G, Frati L, Screpanti I, Gulino A. Ren: a novel, developmentally regulated gene that promotes neural cell differentiation. *J. Cell Biol.* 2002; **158**: 731–740.
17 De Smaele E, Di Marcotullio L, Ferretti E, Screpanti I, Alesse E, Gulino A. Chromosome 17p deletion in human medulloblastoma: a missing checkpoint in the hedgehog pathway. *Cell Cycle* 2004; **3**: 1263–1266.
18 Di Marcotullio L, Ferretti E, De Smaele E, Argenti B, Mincione C, Zazzeroni F, Gallo R, Masuelli L, Napolitano M, Maroder M, Modesti A, Giangaspero F, Screpanti I, Alesse E, Gulino A. Ren(kctd11) is a suppressor of hedgehog signaling and is deleted in human medulloblastoma. *Proc. Natl. Acad. Sci. U.S.A.* 2004; **101**: 10833–10838.
19 Ferretti E, De Smaele E, Di Marcotullio L, Screpanti I, Gulino A. Hedgehog checkpoints in medulloblastoma: The chromosome 17p deletion paradigm. *Trends Mol. Med.* 2005; **11**: 537–545.
20 Mancarelli MM, Zazzeroni F, Ciccocioppo L, Capece D, Po A, Murgo S, Di Camillo R, Rinaldi C, Ferretti E, Gulino A, Alesse E. The tumor suppressor gene kctd11ren is regulated by sp1 and methylation and its expression is reduced in tumors. *Mol. Cancer* 2010; **9**: 172.
21 Canettieri G, Di Marcotullio L, Greco A, Coni S, Antonucci L, Infante P, Pietrosanti L, De Smaele E, Ferretti E, Miele E, Pelloni M, De Simone G, Pedone EM, Gallinari P, Giorgi A, Steinkuhler C, Vitagliano L, Pedone C, Schinin ME, Screpanti I, Gulino A. Histone deacetylase and cullin3-ren(kctd11) ubiquitin ligase interplay regulates hedgehog signalling through gli acetylation. *Nat. Cell Biol.* 2010; **12**: 132–142.
22 Correale S, Pirone L, Di Marcotullio L, De Smaele E, Greco A, Mazzà D, Moretti M, Alterio V, Vitagliano L, Di Gaetano S, Gulino A, Pedone EM. Molecular organization of the cullin e3 ligase adaptor kctd11. *Biochimie* 2011; **93**: 715–724.
23 Vuilleumier S, Mutter M. Synthetic peptide and template-assembled synthetic protein models of the hen egg white lysozyme 87–97 helix: importance of a protein-like framework for conformational stability in a short peptide sequence. *Biopolymers* 1993; **33**: 389–400.
24 D'Andrea LD, Iaccarino G, Fattorusso R, Sorriento D, Carannante C, Capasso D, Trimarco B, Pedone C. Targeting angiogenesis: structural characterization and biological properties of a de novo engineered VEGF mimicking peptide. *Proc. Natl. Acad. Sci. U.S.A.* 2005; **102**: 14215–14220.
25 Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, Korsmeyer SJ. Activation of apoptosis in vivo by a hydrocarbon-stapled bh3 helix. *Science* 2004; **305**: 1466–1470.

