KCTD5 is endowed with large, functionally relevant, interdomain motions

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The KCTD family is an emerging class of proteins that are involved in important biological processes whose biochemical and structural properties are rather poorly characterized or even completely undefined. We here used KCTD5, the only member of the family with a known three-dimensional structure, to gain insights into the intrinsic structural stability of the C-terminal domain (CTD) and into the mutual dynamic interplay between the two domains of the protein. Molecular dynamics (MD) simulations indicate that in the simulation timescale (120 ns), the pentameric assembly of the CTD is endowed with a significant intrinsic stability. Moreover, MD analyses also led to the identification of exposed β-strand residues. Being these regions intrinsically sticky, they could be involved in the substrate recognition. More importantly, simulations conducted on the full-length protein provide interesting information of the relative motions between the BTB domain and the CTD of the protein. Indeed, the dissection of the overall motion of the protein is indicative of a large interdomain twisting associated with limited bending movements. Notably, MD data indicate that the entire interdomain motion is pivoted by a single residue (Ser150) of the hinge region that connects the domains. The functional relevance of these motions was evaluated in the context of the functional macromolecular machinery in which KCTD5 is involved. This analysis indicates that the interdomain twisting motion here characterized may be important for the correct positioning of the substrate to be ubiquitinated with respect to the other factors of the ubiquitination machinery.

**Keywords:** twisting; molecular dynamics; structure–function relationships; substrate recognition domain; ubiquitination

**Introduction**

Proteins frequently present multiple domains. Depending on the specific system, these domains contribute to the protein function either independently or through strict cooperation. In multi-domain proteins, a relevant role is played by the dynamics of the system. Indeed, a large variety of different motions has been documented (Lennon, Williams, & Ludwig, 2000; Taylor, Cawley, & Hayward, 2013).

An emerging class of multi-domain and modular proteins is represented by the KCTD (potassium channel tetramerization domain containing proteins) protein family (Liu, Xiang, & Sun, 2013; Skoblov et al., 2013). These proteins share a common POZ/BTB (Bric-à-brac, Tramtrack, Broad complex) domain in their N-terminal region. The POZ/BTB domain is a widespread module that is believed to mediate both homo- and hetero-oligomerization (Stogios, Downs, Jauhal, Nandra, & Privé, 2005; Zollman, Godt, Prive, Couderc, & Laski, 1994). In the different members of the KCTD family, the BTB domain is linked to a highly variable C-terminal region (Dementieva et al., 2009). The variability of this region is likely related to the involvement of KCTD proteins in a variety of fundamental biological processes. These proteins play crucial roles in the physiopathology of processes that can lead to severe human diseases including epilepsy, autism, cancer, and obesity (Azizieh et al., 2011; Bartoi et al., 2010; Boada et al., 2014; Canetitieri et al., 2010; Chen et al., 2009; De Smale et al., 2011; Golzio et al., 2012; Marneros et al., 2013; Mencacci et al., 2015; Schwenk et al., 2010). Natural mutations of these proteins also underlie genetic diseases. In particular, mutations affecting the gene of KCTD1 cause the scalp-ear-nipple syndrome (Mencacci et al., 2013), while mutations of KCTD7 lead to the insurgence of the progressive myoclonic epilepsy (Azizieh et al., 2011). Very recently it has been demonstrated that a missense mutation in KCTD17 causes autosomal dominant Myoclonus–Dystonia (Mencacci et al., 2015).

Despite the important roles played by these factors, the definition of their biochemical role is still fragmentary. In general terms, the members of the protein family may be divided in two subgroups: (a) those acting as...
substrate adaptors in CRL (Cullin RING Ligase) E3 ligase complex (Bayón et al., 2008; Chen et al., 2009; Smaldone et al., 2015; Zarelli & Dawid, 2013), and (b) those that, being unable to bind Cullin 3 (Cul3) (Smaldone et al., 2015), are involved or potentially involved in other processes such as participation in the assembly of the GABAB2 receptors (KCTD8, KCTD12, KCTD12b, and KCTD16) (Bartoi et al., 2010; Correale et al., 2013; Schwenk et al., 2010) or transcriptional repression of AP-2α (KCTD1 and KCTD15) (Ding et al., 2009; Zarelli & Dawid, 2013).

In the KCTDs of the first group, the N-terminal domain binds Cul3, whereas the variable C-terminal domain (CTD) recruits different substrates to be ubiquitinated. For the members of the second group, information on the functional interplay between the different regions of the protein is even more limited. In all cases, structural data are lacking since, with the only exception of the pentameric Cul3 binding protein KCTD5 (Dementieva et al., 2009), experimental three-dimensional models for these proteins are not available.

In this scenario, KCTD5 represents the only possible model that allows an analysis of the structural and dynamic mutual interplay between the BTB (residues 44–149) and the substrate binding domain (residues 155–210) of the protein. The BTB/POZ domain-containing protein KCTD5 consists of 234 amino acid residues and presents a pentameric organization. This protein is able to bind both Cul3 and Cul3-based peptides (Balasco et al., 2014; Bayón et al., 2008; de Paola et al., 2015; Pirone et al., 2011). It is important to note that KCTD5 is likely involved in the formation of a multimeric giant machinery that also includes the other factors (Cul3, Rbx1, E2 enzyme, and ubiquitin) needed for the ubiquitination process (Balasco et al., 2014). It has been suggested that the simple build up of these complexes on the basis of structural crystallographic data may not be functionally relevant as the dynamic properties of the components of these systems are crucial for the functionality of the machinery (Lydeard, Schulman, & Harper, 2013).

In order to shed light on the dynamics of the KCTD5 pentamer with a specific focus on the relative motions of the two domains, we here report the results of extensive molecular dynamics (MD) simulations. The analysis highlights large but confined movements of the two domains that may have implications for the protein function. Moreover, it has been recently pointed out that the role of the BTB domains in KCTDs may be more important for partner recognition rather than for their oligomerization (Correale et al., 2013). This implies that the C-terminal regions of some of these proteins are intrinsically endowed with the ability to form stable oligomers. The intrinsic insolubility of the CTD of KCTD5 has prevented an experimental check of this possibility (Dementieva et al., 2009). In this framework, to gain insights into the intrinsic stability of the pentameric assembly formed by the C-terminal region, we also performed a MD simulation on a truncated variant of the protein.

**Materials and methods**

**Notations and molecular modeling**

The structured portion of the protein (residues 44–210), denoted as full-length KCTD5 (KCTD5FL), was considered in this work. KCTD5FL presents two well-defined modules (N-terminal and CTDs) separated by a short linker (hinge residues 150–154). The N-terminal region corresponds to the folded BTB domain (KCTD5BTB, residues 44–149) with a motif of five α-helices and a three-stranded β-sheet. The C-terminal region (KCTD5CTD, residues 155–210) is composed by a three-stranded β-sheet and one α-helix. Helices and strands of KCTD5 have been indicated using Greek letters.

Two different crystallographic structures of KCTD5FL have been reported in the Protein Data Bank. They were determined at 3.1 and 3.3 Å resolution after crystallization in two different salt conditions (Dementieva et al., 2009). The structure refined at higher resolution in high-salt buffer (PDB entry code 3DRX) was considered as the starting model for MD simulations. Model building was used to rebuild the missing regions (residues 79–82 and 188–199 of chain A, residues 190–195 of chain B, residue 197 of chain D, and residues 189–198 of chain E) in four of the five chains of the crystallographic model, using the structure of these regions in chain C as template. The structure of the C-terminal region (residues 155–210) of this model was used in the simulation of KCTD5CTD system.

**Simulation procedures**

MD simulations were performed using GROMACS software package 4.5.5 (Van Der Spoel et al., 2005). Two MD simulations of the full-length protein were carried out using different force fields, Amber03 and OPLS-AA, and TIP4P as water model. The protein was immersed in triclinic boxes of 10.83 × 8.66 × 9.97 nm³ (52,493 water molecules) and 13.04 × 10.86 × 12.17 nm³ (52,783 water molecules) in Amber03 and OPLS-AA simulations, respectively. A single MD simulation was performed for the CTD using Amber03 as force field. The triclinic box sizes were 5.82 × 5.20 × 5.82 nm³ with 14,112 water molecules. To neutralize the systems, forty and twenty-five water molecules were replaced by sodium counterions in KCTD5FL and KCTD5CTD, respectively.
The simulations were run with periodic boundary conditions. The temperature and pressure of the systems were stabilized at 300 K and 1 atm, respectively. In order to allow relaxation of solvent molecules, energies were minimized by fixing the protein atoms. Energies of the systems were then minimized without restraints. Before starting constant temperature MD at 300 K, 100 ps MD runs were carried out at 50, 100, 150, 200, 250, and 300 K. The overall timescale of the simulations was 70 ns and 120 ns for KCTD5FL and KCTD5CTD, respectively. An integration time step of 0.002 ps was used. All bond lengths were constrained using the LINCS procedure. A no-bonded cutoff of 10 Å was applied for Lennard–Jones interactions. The particle mesh Ewald method with a grid spacing of 0.12 nm was used to account for the electrostatic interactions.

The collective motions of the protein in the MD simulations were examined by extracting the essential degrees of freedom according to the ED method (Amadei, Linssen, & Berendsen, 1993). The covariance matrix of the coordinate fluctuations was diagonalized to obtain eigenvectors and eigenvalues. Aimed at demonstrating that the simulation reached an adequate convergence in the essential space, we calculated the root-mean-square inner product (RMSIP) between two halves of the equilibrated trajectory (Amadei, Ceruso, & Di Nola, 1999; Amadei et al., 1993; Merlino, Vitagliano, Ceruso, & Mazzarella, 2003, 2004). In the simulations of KCTD5FL, the 60-ns interval corresponding to the equilibrated trajectory (10–70 ns) was divided in two halves of 30 ns. Similarly, the 100-ns interval (20–120 ns) was divided in two halves of 50 ns in the simulation of KCTD5CTD. The RMSIP between the first 10 eigenvectors is defined as:

\[ \sqrt{\frac{1}{10} \sum_{i=1}^{10} \sum_{j=1}^{10} (\eta^2_i \eta^2_j)^2} \]

where \( \eta^2_i \) and \( \eta^2_j \) are the ith and jth eigenvectors from the first and second half of the equilibrated trajectory, respectively.

The quality of the simulation was evaluated by checking trajectories using GROMACS routines and the program VMD (Humphrey, Dalke, & Schulten, 1996). The GROMACS tool g_rmsf was used in order to calculate the root-mean-square fluctuations (RMSF) of the Cα atoms. Firstly, each frame of the trajectory was superimposed with the first frame through a structural alignment of the protein Cα atoms of either KCTD5FL or the single domains (BTB or CTD). Firstly, trajectory structures were superimposed with the first frame through a structural alignment of the protein Cα atoms of either KCTD5FL or the single domains (BTB or CTD).

Results

Overall structural stability and flexibility of KCTD5

To gain insights into the structural stability and into the intrinsic flexibility of the structured region of KCTD5 (residues 44–210 hereafter defined as KCTD5FL), we performed MD simulations using different force fields (see Methods for details). The stability of system in these simulations was checked by evaluating the evolution of several structural indicators. The trends of root-mean-square deviation (RMSD) of the trajectory structures from the starting X-ray structure are reported in Figure 1(A) and (B) for both Amber03 and OPLS-AA force fields. In both cases, the system reaches a stable state in the early stages of the simulations (~10 ns). A deep inspection of the figure indicates that the overall RMSD is larger for the simulation performed using OPLS-AA (RMSD values of ~6.5 vs. ~4 Å). However, the RMSD values computed on the individual domains of the protein are similar for the two simulations. The RMSD values computed on the two domains are smaller than those observed for the entire protein. Altogether these observations indicate that KCTD5FL undergoes significant interdomain variations when compared to the starting structure, although the structure of each domain is well preserved. This concept is also confirmed by the analysis of the time evolution of the secondary structure elements (Figure 1(C) and (D)). As shown in figure, the protein preserves its proper folding during the simulations. However, when the OPLS-AA force field was applied, the a1 and a2 helices of the N-terminal BTB domain are less well preserved also showing a tendency to assume the 3 (10) helical conformation. The overall stability of the system is also corroborated by other indicators such as the number of hydrogen bonds (~630) and the radius of gyration (~36 Å) which were both rather constant along the simulations (Supplementary Figure S1).

In order to evaluate the convergence of the two simulations in the potentially equilibrated region (10–70 ns) of the trajectories, an RMSIP analysis was performed. Following literature protocols (Amadei et al., 1993, 1999; Merlino et al., 2003, 2004), the 60-ns interval corresponding to the equilibrated trajectory was divided in two halves of 30 ns each. On both halves, an essential dynamics analysis was performed. The RMSIP, computed considering the first ten eigenvectors derived from the diagonalization of the covariance matrices for each half, was 0.76 for both the simulations. The high RMSIP values indicate that the essential subspace spanned by the first 10 eigenvectors of the two different sets considered is largely overlapped. This assures that each simulation achieves an adequate level of convergence.

The same RMSIP-based approach was used to evaluate the consistency between simulations performed
using two different force fields. In particular, the RMSIP value was computed considering the overlap between the essential subspaces spanned by the first 10 eigenvectors of each equilibrated trajectory (10–70 ns).

The large resulting value of RMSIP (0.73) indicates that in the two simulations, a similar conformational space is sampled by the protein despite the use of different force fields.

Figure 1. Structure stability of KCTD5FL throughout the simulations.
Notes: The Cα-based RMSD values of trajectory structures against the starting model in the simulations performed using Amber03 and OPLS-AA force fields are reported in panels A and B, respectively. The RMSD values are calculated on the whole structure (black) and on the single domains (BTB in red and CTD in blue). The time evolution of the secondary structure content of KCTD5FL is shown in panel C for Amber03 and in panel D for OPLS-AA.
Moreover, the RMSD values, computed on the Cα atoms, between the average structures generated with the two force fields are rather low for the single domains, being 1.2 and 1.9 Å for the BTB and CTD domains, respectively. On the other hand, the RMSD value is higher when the entire protein is considered (4.1 Å). This is indicative of slight different orientations of the two domains in the two average structures.

In this framework, we report the analysis of the dynamic properties of KCTD5FL obtained using Amber03.

Using these MD data, we analyzed the preservation in the trajectory structures of the determinants of the KCTD5FL pentamer stability. Crystallographic studies have shown that some specific interactions in the two domains are fundamental for the formation and preservation of the pentameric assembly (Dementieva et al., 2009), although the resolution of the full-length protein is limited. In particular, important electrostatic interactions at the interface between adjacent subunits are established between the side chains of residues Asp93 and Glu124 with Arg107 and Lys110, respectively. The most important H-bonds involve (i) the backbone atoms of Leu56 and Gly51, (ii) the backbone nitrogen atom of Ala118 and the hydroxyl of Tyr 98 with the side chain of Asn114, (iii) the main chain carboxyl and N-amino groups of Asp116 and Lys115, (iv) the hydroxyl of Tyr158 with the backbone nitrogen atom of Phe181, and (v) the side chain of Gln183 with the main chain of Leu184. The analysis of these interactions along the simulations unveiled that they are well conserved (Supplementary Figure S2) thus confirming the stability of the entire pentameric assembly.

The availability of these MD simulations also offered us the possibility to evaluate the impact of a causaldisease missense mutation (Arg145His) found in a close KCTD5 paralog denoted as KCTD17 (74% sequence identity) (Mencacci et al., 2015). This mutation causes Myoclonus–Dystonia, a rare non-neurodegenerative movement disorder. KCTD17 Arg145, which is conserved in KCTD5 (Arg159) along with the local context, is located in the first strand of the β-sheet of the CTD. In KCTD5 crystallographic structures, Arg159 side chain is involved in intermolecular interactions with symmetry-related copies of the protein (Dementieva et al., 2009). Therefore, its location is somewhat influenced by crystal packing. To gain insights into the interactions that this side chain may form in solution, we analyzed its rotameric states in the trajectory structures. This analysis indicates that Arg159 side chain is essentially solvent exposed and only forms sporadic interactions with other residues. This suggests that the Arg to His mutation should not affect the overall structure and stability of KCTD5 and of its homolog KCTD17. It is likely that the mutation may have an impact on the interaction of KCTD17 with its biological partners.

**MD simulations highlight large interdomain motions in KCTD5FL**

The main motivation of this work was the analysis of the interdomain motions occurring in KCTD5. Taking into account the paucity of structural data on this class of protein, the analysis was conducted also to gain insights extensible to the other KCTDs.

Initial information on KCTD5FL flexibility has been obtained by the analysis of the RMSF values calculated on Cα atoms in the equilibrated region of the trajectory. Following the expected trend, residues which belong to structural elements of the protein display a lower flexibility. Indeed, RMSF values of helical and sheet regions are lower than 1.5 Å, whereas the RMSF values of loop residues could be as high as 3 Å. As shown in Figure 2, residues of the CTD show higher Cα RMSF values than the BTB ones. In particular, the most evident fluctuations are exhibited by residues of the interdomain waist (hinge region) that connects the two domains (residues 150–154) and of the loop regions of the CTD. The CTD exhibits higher RMSF values also when the domains are considered separately in this analysis (see Methods for details) (Supplementary Figure S3).

In order to gain insights into the dynamics between the two domains of KCTD5FL, the collective motions of the protein have been analyzed by essential dynamics. This analysis showed that most of the atomic displacements were contained in the first few eigenvectors. In particular, the essential subspace spanned by the first 10 eigenvectors covers about 80% of the total fluctuations. Of particular relevance are the motions described by the first eigenvector which represents 37% of the total

![Figure 2](image.jpg)

**Figure 2.** Root-mean-square fluctuations (Cα-based RMSF values) of residues of KCTD5FL calculated in the equilibrated region of the trajectory in the simulation performed using Amber03.

Note: The secondary structure elements of the protein are reported as bars (helices in blue and strands in red).
protein fluctuations. The visualization of the protein motion along the first eigenvector in a film-like fashion is indicative of large interdomain motions (Figure 3). In detail, the protein undergoes a relevant twisting motion of the CTD with respect to the BTB.

Interdomain rotations were quantified using the program DynDom (Poornam, Matsumoto, Ishida, & Hayward, 2009). In particular, taking as a reference the average structure of KCTD5FL computed in the equilibrated region of the trajectory (10–70 ns), relative interdomain rotations were evaluated for 120 trajectory structures sampled at every 0.5 ns of the plateau region (10–70 ns).

As shown in Figure 4(A), rotations as large as 20° were detected. Since deviations from the average structure could be in both clock-wise and anti-clockwise directions, pairs of trajectory structures may display relative rotations larger than 40°. For these extreme cases, the superimposition of the BTB domain leads to a significant displacement of the CTD units. As shown in Figure 4(B), the superimposition of the BTB domains of two extreme structures may lead to a striking observation, i.e., the CTD domain of a chain of the one structure (in red) becomes closer to the CTD of the adjacent chain than to its own one of the other structures (in green).

Further analyses on trajectory structures were performed to unravel other possible motions of KCTD5FL. The occurrence of interdomain bending motions was evaluated by considering the angle between the center of mass of the BTB domain, of the CTD, and of the hinge region (residues 150–154) connecting the two domains. As shown in Supplementary Figure S4, this angle adopts an average value of 175° with very limited fluctuations (±5°). These findings suggest that KCTD5FL retains its tube-like structure despite large twisting motions. A slightly wider bending angle is exhibited by the protein in the MD conducted under OPLS force field. The angle between the centers of mass assumes an average value of 169° with fluctuations of ±10°.

In this scenario, a major role is played by the hinge region linking the two domains that simultaneously assures large twisting movements while preserving the overall shape of the protein. We therefore analyzed the conformational states adopted by the residues of the hinge region. As expected, the C-terminal residues of the BTB domain, which belong to the helix α5, retain a helical conformation throughout the simulation (Figure 5). Most of the residues of the hinge (residues 151–154) populate the extended region of the Ramachandran plot (Figure 5). Interestingly, the only versatile residue of the hinge region is Ser150 which can adopt both the helical and the extended conformations (Figure 5). These observation indicate that Ser150, being able to adopt different conformations, functions as a pivot point in the global motions of KCTD5FL.

**Dynamics and stability of the pentameric KCTD5CTD**

MD analyses described in the previous paragraph indicate that the CTD domain of the protein retains its folded state although it is not stabilized by non-local interactions with residues of the BTB domain. This may indicate that the CTD pentamer is endowed with a significant stability. Since the role of the BTB domain as an essential oligomerization factor in KCTD proteins is being revisited in the recent literature (Correale et al., 2013), we performed a MD analysis on the truncated KCTD5CTD pentamer (residues 155–210) to gain insights into its intrinsic stability. In this context, it is worth mentioning that this topic could not be experimentally evaluated due to the CTD insolubility (Dementieva et al., 2009).

The overall stability of the system throughout the simulation was monitored by analyzing the RMSD values and the time evolution of both the radius of gyration and the secondary structure elements within the simulation. As shown by the trend of the RMSD and Rg, computed on the Cα atoms, the model reaches a stable equilibrated state after 20 ns with an average RMSD value in the plateau region of ~2.5–3 Å and a radius of gyration of ~19 Å (Figure 6(A) and (B)). The secondary structure of the CTD is well preserved although some residues belonging to the α-helix (residues 167–169) often assume a 3(10)-helix structure (Figure 6(C)). The flexibility of this model was checked by computing of
the RMSF values considering the equilibrated trajectory (20–120 ns). As shown in Supplementary Figure S5, loop regions presented higher values (up to ~1.8 Å), whereas lower values (~0.5 Å) were found in correspondence of the structural elements. The convergence of the trajectory was checked by computing the RMSIP value (see Methods for details). The resulting RMSIP value that was as high as 0.77 assures that an adequate convergence in the simulation was achieved.

Trajectory structures were then used to evaluate the impact of interdomain interactions in the stability of the pentameric assembly. The interface between adjacent chains in the CTD is characterized mainly by hydrophobic residues. The crystal structure of KCTD5 suggests that the CTD may be stabilized by both hydrophobic and H-bonding interactions (Dementieva et al., 2009). In particular, the hydrophobic interactions are formed by two clusters (Tyr158, Val160, Val185, Leu202, Val204 and Leu168, Val172, Phe181, Leu184) of apolar residues belonging to two adjacent chains within the pentamer. H-bonds are established between Tyr158 and Gln183 of one subunit and Phe181 and Leu184 of the adjacent chain. The inspection of trajectory structures shows that both hydrophobic and H-bond interactions are well preserved along the simulation (Supplementary Figure S6). Collectively these results indicate that the CTD pentamer is stable in the simulation timescale and that both hydrophobic and H-bonding interactions contribute to its stability.

**KCTD5**<sup>CTD</sup> exposes potentially reactive regions

The juxtaposition of the β<sub>4</sub>/α<sub>6</sub> and β<sub>5</sub>/β<sub>6</sub> motifs is a peculiar feature of KCTD5<sup>CTD</sup>. This arrangement leads to the solvent-exposure of the backbone of the β<sub>4</sub>-strand residues. It is well known that nature tends to avoid the exposure of regular β-strands since they are sticky and may lead to uncontrolled aggregation with potentially harmful consequences (De Simone, Esposito, Pedone, & Vitagliano, 2008; Hoskins, Lovell, & Blundell, 2006; Remaut & Waksman, 2006; Richardson & Richardson, 2002). The analysis of the MD simulation indicates that the exposure of this strand is preserved in the trajectory structures. The inspection of the β<sub>4</sub>-strand residues unravels that the carbonyl oxygen atoms of Val160 and Gln162 are particularly exposed to the solvent. The limited resolution of the crystallographic structure (3.1 Å) hampered an accurate analysis of the hydration state of these atoms. However, the analysis of the trajectory structures indicates that Val160 and Gln162 backbone are particularly exposed to the solvent. The limited resolution of the crystallographic structure (3.1 Å) hampered an accurate analysis of the hydration state of these atoms. However, the analysis of the trajectory structures indicates that Val160 and Gln162 are involved in the formation of several H-bond interactions with water molecules throughout the entire simulation. On average, each of these residues is involved in H-bonds with two water molecules.

**Discussion**

The KCTD family is an emerging class of proteins that are involved in important biological processes (Skoblov et al., 2013). Nevertheless, their biochemical and
structural properties are rather poorly characterized or even completely undefined. A large subclass of these proteins is involved in protein ubiquitination acting as simultaneous binders of both Cul3 and substrates. These functions are carried out by the different domains of these proteins. The conserved BTB domain is dedicated to the Cul3 recognition, whereas the variable CTD binds substrates for ubiquitination. We here used KCTD5, the only member of the family whose structure has been determined (Dementieva et al., 2009), to gain insights into the intrinsic structural features of the CTD and into the mutual dynamic interplay between the two domains of the protein. The MD simulation indicates that the pentameric assembly of the CTD is endowed with a significant stability in the simulation timescale (120 ns). Moreover, MD analyses lead to the identification of exposed β-strand residues that are known to be hot spot in molecular recognition. On this basis, we suggest that these regions may be involved in substrate binding. The promiscuity of these exposed regions may also be important for the binding of different types of substrates.

More importantly, our analysis provides interesting information of the relative motions of the two domains. The physical separation of the two domains, which do not form specific interactions, could have led to large uncorrelated motions. On the other hand, our analysis is suggestive of a significant overall flexibility of the protein that is, however, confined in a well-defined direction. Indeed, the most relevant element of KCTD5 dynamics is the twisting motion between the CTD and the BTB domain. This motion somewhat resembles the structural differences that can be detected from the comparison of the high and low salt crystallographic structures determined from non-isomorphous crystals (Dementieva et al., 2009). Interestingly, MD data indicate that the entire interdomain motion is pivoted by a single residue (Ser150) of the hinge region that connects the domains. It is likely that the orientation of the α5

Figure 5. Ramachandran plots of several residues of KCTD5<sub>FL</sub> throughout the simulation performed using Amber03 force field: (A) Thr149, (B) Ser150, (C) Gln151, and (D) Val152.
helix which protrudes outside the BTB domain may be important for this type of motion. It is worth mentioning that the same helix folds against the main body of the domain in other BTB-containing proteins as voltage-gated potassium channels (Supplementary Figure S7) (Bixby et al., 1999).

To evaluate the functional relevance of these motions, we analyzed the protein dynamics highlighted in the present investigation in the context of the functional macromolecular machinery in which KCTD5 is involved. This global assembly, generated using the strategy adopted in Balasco et al. (2014), includes KCTD5, Cul3, the RING finger-like domain-containing protein Rbx1, the E2 enzyme UbcH5b, and ubiquitin. As shown in Figure 7, the twisting motion between the BTB domain and the CTD may be important for the ideal positioning of the substrate to be ubiquitinated.

Along with recent reports (De Simone, Aprile, Dhulesia, Dobson, & Vendruscolo, 2015; De Simone, Montalvao, Dobson, & Vendruscolo, 2013; Lydeard et al., 2013), present findings indicate that in this large multiprotein assemblies, protein dynamics plays a relevant role. The interdomain twisting, which is a less frequently found interdomain motion in proteins (Lennon et al., 2000; Taylor et al., 2013) compared to other structural rearrangements of KCTD5, confines the relative motions of the two domains in a well-defined direction. It is likely that such movements are a general fingerprint of the KCTD members acting as ligases in ubiquitination. Although structural studies are eagerly needed to assess this point, a preliminary secondary structure prediction approach on these proteins (data not shown) indicates that the BTB and the substrate binding domain of these proteins are separated by rather
short unstructured regions. This may be an important requisite of the hinge region to perform the pivotal role in the interdomain twisting motion.

List of abbreviations

| Abbreviation | Description |
|--------------|-------------|
| MD           | molecular dynamics |
| RMSF         | root-mean-square fluctuation |
| RMSD         | root-mean-square deviation |
| RMSIP        | root-mean-square inner product |
| PDB          | Protein Data Bank |
| Cul3         | Cullin3 |
| CRL          | Cullin-RING ligase |
| GABA         | g-aminobutyric acid |
| BTB          | Bric-à-brac, Tramtrack, Broad complex |
| KCTD5FL      | structured portion of KCTD5 (residues 44–210) |
| KCTD5CTD     | C-terminal domain of KCTD5 (residues 155–210) |

Supplementary material

The supplementary material for this paper is available online at [dx.doi.org/10.1080/07391102.2015.1090343](http://dx.doi.org/10.1080/07391102.2015.1090343).

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Disclosure statement

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References

Amadei, A., Ceruso, M. A., & Di Nola, A. (1999). On the convergence of the conformational coordinates basis set obtained by the essential dynamics analysis of proteins’ molecular dynamics simulations. *Proteins: Structure, Function, and Genetics*, 36, 419–424.

Amadei, A., Linssen, A. B., & Berendsen, H. J. (1993). Essential dynamics of proteins. *Proteins: Structure, Function, and Genetics*, 17, 412–425. doi:10.1002/pro.340170408

Azizieh, R., Orduz, D., Van Bogaert, P., Bouschet, T., Rodriguez, W., Schiffmann, S. N., … Abramowicz, M. J. (2011). Progressive myoclonic epilepsy-associated gene KCTD7 is a regulator of potassium conductance in neurons. *Molecular Neurobiology*, 44, 111–121. doi:10.1007/s12035-011-8194-0

Balasco, N., Pirone, L., Smaldone, G., Di Gaetano, S., Esposito, L., Pedone, E. M., & Vitagliano, L. (2014). Molecular recognition of Cullin3 by KCTDs: Insights from experimental and computational investigations. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, 1844, 1289–1298. doi:10.1016/j.bbapap.2014.04.006

Bartoi, T., Rigbolt, K. T., Du, D., Köhr, G., Blagoev, B., & Kornau, H. C. (2010). GABA B receptor constituents revealed by tandem affinity purification from transgenic mice. *Journal of Biological Chemistry*, 285, 20625–20633. doi:10.1074/jbc.M109.049700

Bayón, Y., Trinidad, A. G., de la Puerta, M. L., del Carmen Rodríguez, M., Bogetz, J., Rojas, A., … Alonso, A. (2008). KCTD5, a putative substrate adaptor for cullin3 ubiquitin ligases. *FEBS Journal*, 275, 3900–3910. doi:10.1111/j.1742-4658.2008.06537.x

Bixby, K. A., Nanao, M. H., Shen, N. V., Kreusch, A., Bellamy, H., Pfaffinger, P. J., & Choe, S. (1999). Zn2+-binding and molecular determinants of tetramerization in voltage-gated K+ channels. *Nature Structural Biology*, 6, 38–43.

Boada, M., Antúnez, C., Ramírez-Lorca, R., DeStefano, A. L., González-Pérez, A., Gayán, J., … Ruiz, A. (2014). ATP5H/KCTD2 locus is associated with Alzheimer’s disease risk. *Molecular Psychiatry*, 19, 682–687. doi:10.1038/mp.2013.86

Canettieri, G., Di Marcotullio, L., Greco, A., Coni, S., Antonucci, L., Infante, P., … Gulino, A. (2010). Histone deacetylase and Cullin3–RENKCTD11 ubiquitin ligase interplay regulates Hedgehog signalling through Gli acetylation. *Nature Cell Biology*, 12, 132–142. doi:10.1038/nclb2013

Chen, Y., Yang, Z., Meng, M., Zhao, Y., Dong, N., Yan, H., … Shao, F. (2009). Cullin mediates degradation of Rhoa through evolutionarily conserved BTB adaptors to control actin cytoskeleton structure and cell movement. *Molecular Cell*, 35, 841–855. doi:10.1016/j.molcel.2009.09.004

Correale, S., Esposito, C., Pirone, L., Vitagliano, L., Gaetano, S., & Pedone, E. (2013). A biophysical characterization of the folded domains of KCTD12: Insights into interaction with the GABA B2 receptor. *Journal of Molecular Recognition*, 26, 488–495. doi:10.1002/jmr.2291

Figure 7. Global assembly which includes KCTD5 (BTB in red and CTD in orange), Cul3 (blue), Rbx1 (lime), UbcH5b (dark cyan), and the ubiquitin (magenta).

Note: The circular arrow depicts the interdomain twisting motion unraveled in this study.
KCTD5 is endowed with large, functionally relevant, interdomain motions.