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Brief Article

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Allosteric Targeting of the Fanconi Anemia Ubiquitin-Conjugating Enzyme Ube2T by Fragment Screening

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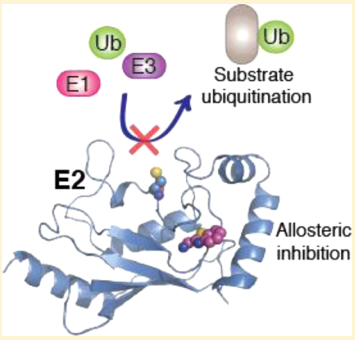
Supporting Information

ABSTRACT: Ube2T is the E2 ubiquitin-conjugating enzyme of the Fanconi anemia DNA repair pathway and it is overexpressed in several cancers, representing an attractive target for the development of inhibitors. Despite the extensive efforts in targeting the ubiquitin system, very few E2 binders have currently been discovered. Herein we report the identification of a new allosteric pocket on Ube2T through a fragment screening using biophysical methods. Several fragments binding to this site inhibit ubiquitin conjugation in vitro.



INTRODUCTION

Ubiquitination is a post-translational modification of proteins that regulates many cellular processes, from protein degradation to cell cycle progression and DNA repair.[1,2] Ubiquitin conjugation to substrate proteins is catalyzed by the sequential action of three enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase).[3] There are approximately 40 known E2s in humans that regulate diverse biological processes, making them attractive drug targets.[4,5] Structural information is available for many E2s, but the lack of deep active site clefts and the need to target the protein surface have led to E2s being considered challenging targets to small molecules.[5,6] Indeed, to date, the possibility of targeting E2s with small molecules has been largely unexplored and very few inhibitors are known.[7−10]

Ube2T is the E2 enzyme of the Fanconi anemia (FA) pathway,[11] which is essential for the repair of DNA interstrand cross-links. Together with FANCL (the E3 ligase), Ube2T catalyzes the monoubiquitination of the heterodimeric FANCI/FANCD2 complex, which is the key signaling event to activate the FA pathway for DNA repair.[11−14] Modulation of DNA repair pathways is an emerging strategy for the development of inhibitors of tumor cell growth, as it can either potentiate the effects of radiotherapy and conventional genotoxins or exploit synthetic lethal interactions.[15−17] The latter approach relies on genetic defects in DNA repair pathways in certain tumor cells, which lead to hypersensitivity toward inhibitors of compensatory pathways.

Ube2T has recently been found to be overexpressed in several tumors,[18−21] including breast[18] and prostate cancer,[20] and therefore it represents an attractive therapeutic target. Here we unveil an allosteric pocket on Ube2T that is ligandable to small molecules as identified via fragment screening using biophysical methods. Fragment-based drug discovery is now an established approach for the development of lead compounds.[22−25] "Fragments" are low molecular weight compounds (typically <250−300 Da), which bind to the target protein with weak affinities (high micromolar to low millimolar range). Fragments represent synthetically tractable starting points for medicinal chemistry to aid their elaboration into high affinity small molecules.[26,27] The weak affinities typically observed for fragments make it challenging to reliably detect and validate their binding. However, direct binding methods can aid detection of novel, secondary, and potentially allosteric sites on protein surfaces.[28] One way to enhance success rates is to deploy a combination or cascade of biophysical methods in order to complement the different detection and sensitivity capabilities of the single techniques.[29,30]

RESULTS AND DISCUSSION

Our biophysical cascade consisted of a first screen of a library of approximately 1200 fragments using two orthogonal techniques: differential scanning fluorimetry (DSF) and biolayer interferometry (BLI). This was followed by a secondary screen

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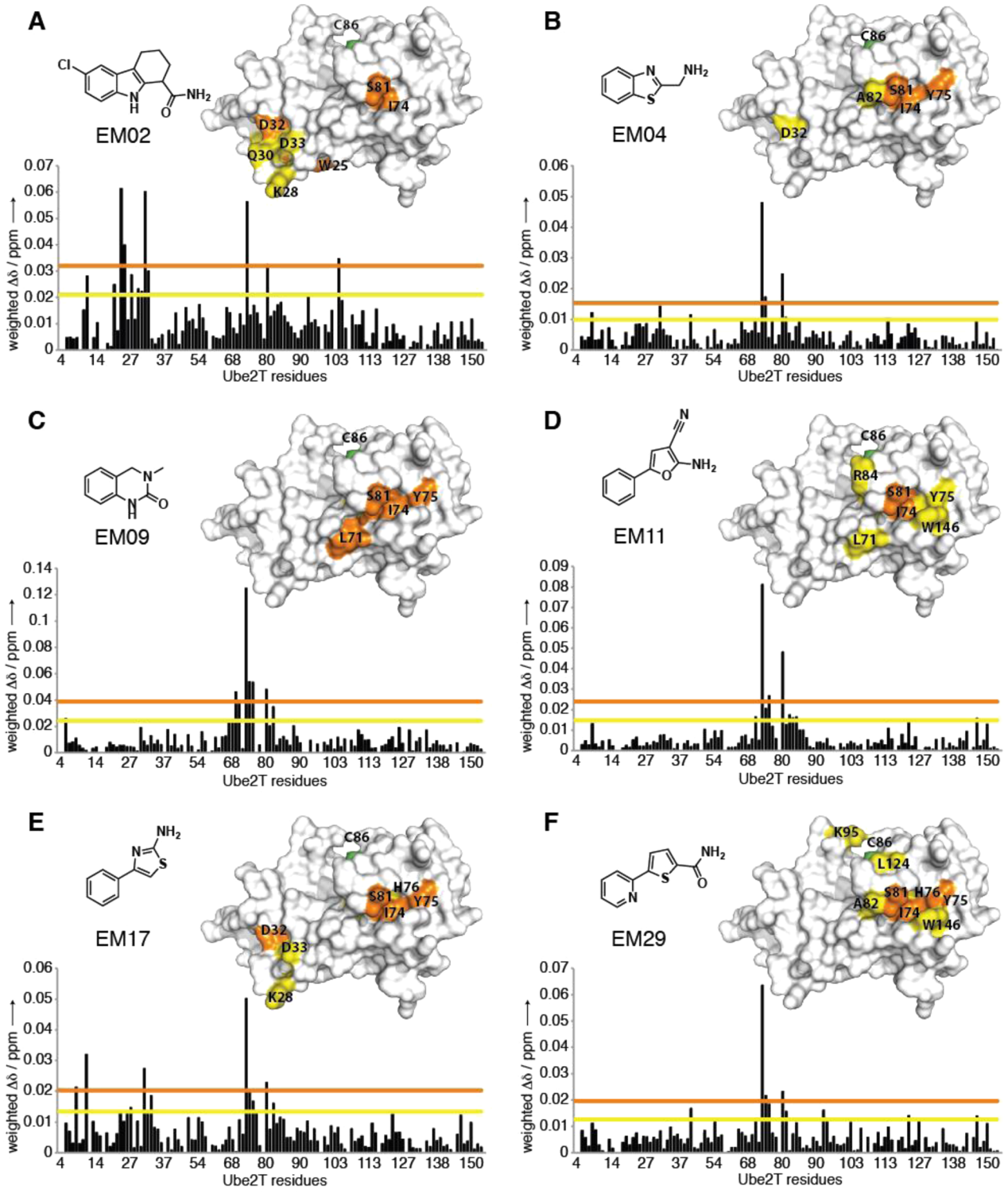

Figure 1. Chemical shift perturbations observed upon addition of 3 mM fragment* to 80 μM Ube2T$_{1-154}$. Weighted $\Delta\delta$s $\geq$ average + SD are indicated by a yellow line. Weighted $\Delta\delta$s $\geq$ average + 2 × SD are indicated by an orange line. Equivalent colors are used to highlight corresponding residues on the Ube2T surface. As a reference, the catalytic cysteine (C86) is colored in green. Chemical structures for individual fragments are shown. For HSQC spectra and weighted $\Delta\delta$s formula, see SI. *Concentration accuracy is limited by solubility issues for several fragments.

using one-dimensional ^{1}H NMR spectroscopy, binding site identification through protein-observed NMR spectroscopy, and X-ray crystallography.

DSF monitors the unfolding temperature of a protein using a fluorescent dye. We screened our fragment library measuring the effect of fragments as singletons on the melting temperature of Ube2T ($\Delta T_m = T_{m\ Ube2T+fragment} - T_{m\ Ube2T}$; Supporting Information (SI) Figure S1). We selected as primary hits nine fragments that induced stabilization of the protein ($\Delta T_m \geq +0.5$ °C) as well as 33 fragments that generated significant negative shifts ($\Delta T_m \leq -2.5$ °C). Previous work has indeed demonstrated that several destabilizers can be confirmed as real

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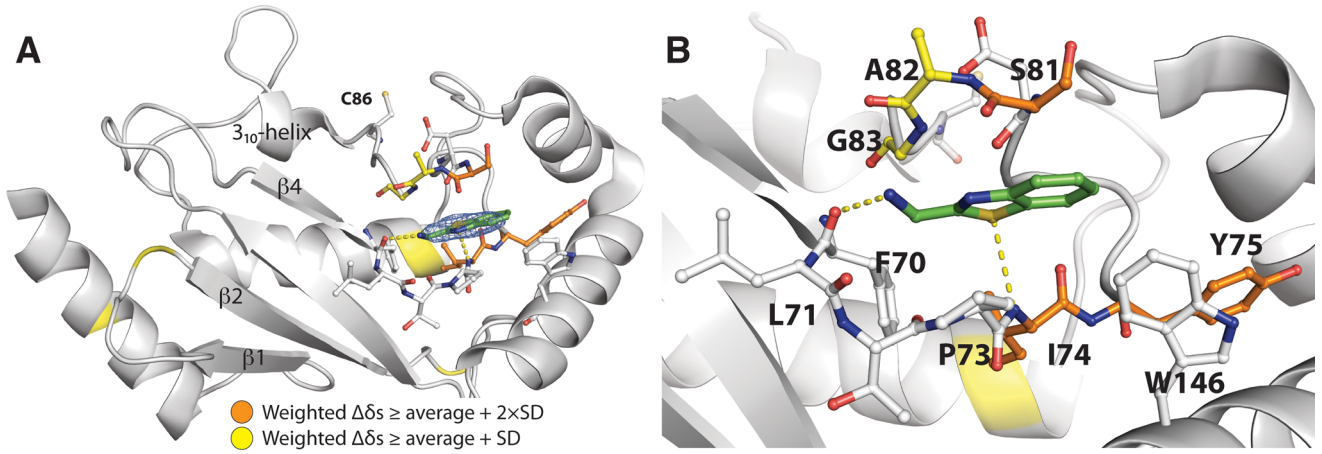

Figure 2. Crystal structure of EM04 in complex with Ube2T. (A) EM04-bound Ube2T structure (PDB 5NGZ) showing $2F_o - F_c$ electron density map contoured at 0.9σ level for fragment EM04. Amino acid side chains in the EM04 binding site are shown as ball and sticks and colored in orange and yellow consistent with the results of the CSP experiments, as described in Figure 1. (B) Zoom-in of the fragment's binding pocket. Hydrogen bonds are represented as dotted yellow lines.

binders.[31] In parallel, we screened the fragment library using BLI, an optical technique that requires immobilization of the target protein on a biosensor surface. Biotin-labeled Ube2T was attached to streptavidin-coated biosensors and screened against the fragment library at a concentration of 200 μM. A total of 90 fragments gave a statistically significant response in BLI (above a threshold value calculated as the sum of the robust standard deviation multiplied by three and the median). Interestingly, most of these 90 BLI hits caused negative ΔT_m in the DSF screening (SI, Figure S2). We then repeated the BLI experiment with the 90 fragments using a six-point concentration series, and 37 hits were selected after visual inspection of the individual binding sensograms. As a result of this first screen, we obtained 42 DSF hits (nine stabilizers and 33 destabilizers) and 37 BLI hits, out of which 10 were in common between the two screens (SI, Figure S3A), making a total of 69 unique hits (5.5% overall hit rate).

Of these initial hits, 34 fragments were chosen based on chemical diversity and commercial availability and submitted to a series of one-dimensional ^{1}H NMR spectroscopy binding experiments: water ligand observed gradient spectroscopy (WaterLOGSY),[32] saturation transfer difference (STD),[33] and Carr−Purcell−Meiboom−Gill (CPMG)[34,35] relaxation-edited sequences. Binding was assessed by comparing the proton signals of the fragments in the presence and in the absence of the protein. This secondary screen validated 13 of the 34 hits identified in the primary screen (61.8% attrition rate).

The next goal was the identification of fragments' binding sites on the Ube2T surface. X-ray crystallography is normally the method of choice for investigating the molecular details of protein−ligand interactions. Protein−fragment complexes are frequently obtained by soaking high-concentration solutions of fragments into apo protein crystals, however, this method has some limitations. Poor solubility and weak affinities of fragments can hamper the obtainment of cocrystal structures with high bound fragment occupancy. Furthermore, targeted pockets may be occluded by crystal packing contacts and limited structural rearrangement can occur without damaging the apo protein crystals, often preventing the identification of cryptic pockets. For the above reasons, we first used protein-observed NMR spectroscopy in solution to map the binding sites.

We expressed a truncated form of Ube2T ($Ube2T_{1-154}$, lacking the C-terminal flexible tail),[36] assigned the backbone amide protons of the HSQC spectrum (BMRB entry 27035), and performed chemical shift perturbation (CSP) analysis with each individual fragment. Out of the 13 fragments, six induced detectable shifts at 2 mM concentration after visual inspection of the spectra (the six validated binders were equally distributed between BLI and DSF hits; a summary of the confirmed hits is shown in SI, Figure S3, together with some representative data). CSPs shown by the six fragments were concentration-dependent, as demonstrated by a four-point titration of fragment into protein (SI, Figure S4).

Shifts mapped onto the X-ray structures of Ube2T (PDB 1YH2,[6] 4CCG[36]) were primarily clustered around a pocket adjacent to the catalytic cysteine (Cys86), formed by the loop between strand β4 and the 3_{10} helix of the E2 fold (Figures 1 and 2A). A slightly different behavior was observed for EM02 and EM17 (to a lesser extent), which also shifted several amino acids located between strands β1 and β2 (Figure 1A,E).

To elucidate the fragments binding modes, we next obtained well-diffracting protein crystals (1.7 Å for the apo form) that we found to tolerate relatively high concentrations of organic solvents that are needed for fragment soaking. The binding site identified by protein NMR was found to be solvent-accessible in our crystal form, suggesting that it would be suitable for soaking experiments. We could confirm this by solving the crystal structure of Ube2T in complex with EM04 at 2.4 Å resolution (PDB 5NGZ, Figure 2 and SI, Figure S5). The newly discovered pocket is distinct from the small molecules binding sites identified on the E2s Cdc34 and Ubc9 (SI, Figure S6).

EM04 binds Ube2T with a K_D = 1.3 mM (LE = 0.36 kcal mol^{-1}), as measured by isothermal titration calorimetry (ITC) (SI, Figure S7A). As shown in Figure 2B, the binding mode is driven by several hydrophobic contacts and by a hydrogen bond between the amino group of EM04 and the carbonyl group of Phe70 on Ube2T. An $-NH_2$ (hydrogen bond donor) appears to be a common structural feature between the identified fragments, as it is also present in EM02, EM11, EM17, and EM29, potentially suggesting a conserved interaction. A second hydrogen bond is formed between the backbone NH of Ile74 and the sulfur atom of the benzothiazole ring. These data are in agreement with the CSP experiments,

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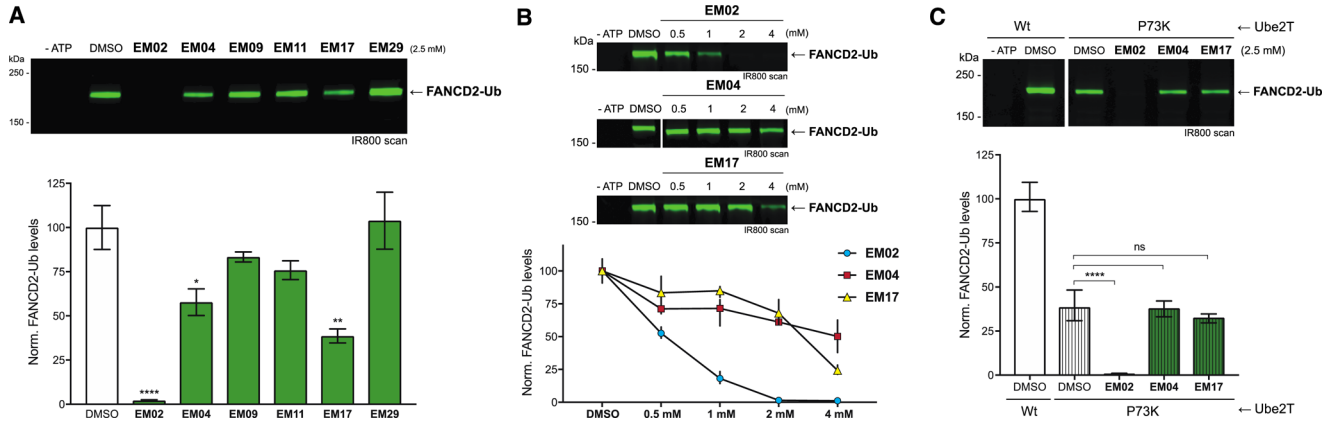

Figure 3. Fragments inhibit substrate ubiquitination by Ube2T. (A) Effect of the six fragments at 2.5 mM on Ube2T-mediated FANCD2 ubiquitination. A representative fluorescence scan shows the levels of monoubiquitinated FANCD2 using fluorescently labeled ubiquitin. The additional assays components (E1, Ube2T, FANCL) are not shown. The bar chart shows the integrated intensities of monoubiquitinated FANCD2 from three independent experiments normalized to DMSO reactions and plotted as mean ± SEM. (B) Concentration-dependent effect of fragments EM02, EM04, and EM17 in a four-point titration series. Representative fluorescence scans are shown and integrated intensities from three independent experiments are normalized to DMSO reactions and plotted as mean ± range. (C) Effect of 2.5 mM EM02, EM04, and EM17 on FANCD2 ubiquitination using Ube2T P73K mutant in the assay. A representative fluorescence scan is shown, and integrated intensities from four independent experiments are normalized to wild type Ube2T-DMSO reactions and plotted as mean ± SEM. Statistical significance was determined by one-way analysis of variance with Dunnett's multiple-comparison test. (n = 3 or 4, $^{****}P \leq 0.0001$, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, *ns* $P > 0.05$).

where major shifts were observed for Ile74 backbone NH upon fragment addition (Figure 1B and SI, Figure S4).

Fragments are typically thought as too weak binders to warrant testing in functional assays. However, fragments binding to secondary pockets have been found to exert allosteric effects on their targets.[37,38] To address this, we next investigated whether the validated fragments could impact Ube2T enzymatic activity using a biochemical assay, which measures the conjugation of fluorescently labeled ubiquitin to the substrate protein FANCD2.[39] A significant reduction of substrate ubiquitination was observed upon addition of fragments EM02, EM04, and EM17 (Figure 3A), suggesting that binding of small molecules to this pocket can lead to inhibition of Ube2T's biochemical activity. The assay was repeated in the presence of detergent as a control for aggregation (SI, Figure S8). None of the fragments affected the formation of the Ube2T–ubiquitin thioester (as demonstrated by an E2-charging assay, SI, Figure S9), suggesting that such inhibition involves the catalytic transfer of the thioester-linked ubiquitin molecule from Ube2T to the substrate protein FANCD2. The effect of EM02, EM04, and EM17 on FANCD2 ubiquitination was concentration-dependent (Figure 3B) as shown by a four-point titration series. Additionally, the three fragments did not show detectable inhibitory effect when tested in a similar biochemical assay set up using UbcH5c as ubiquitin-conjugating enzyme (SI, Figure S10), suggesting that selectivity toward Ube2T can be achieved.

To further confirm that the observed reduction in substrate ubiquitination is due to binding to the newly identified pocket, we introduced a point mutation on Ube2T (P73K), disrupting EM04 binding as measured by ITC (SI, Figure S7B). Such mutation abolished the effect of fragments EM04 and EM17, while EM02 still retained its inhibitory activity (Figure 3C). These results validate EM04 binding site as responsible for Ube2T inhibition. It is worth noting that Ube2T P73K was less active compared to the wild-type protein (Figure 3C), further suggesting the importance of this site for Ube2T function. Our data suggests a different mechanism of action for EM02, which exhibited a different pattern in CSPs (Figure 1A) and is the subject of current investigation in our laboratories.

CONCLUSIONS

In summary, using fragment screening, we discovered a new allosteric pocket on Ube2T, the E2 ubiquitin-conjugating enzyme of the FA pathway. Small molecules binding to this site can inhibit substrate ubiquitination in vitro, suggesting an allosteric modulation of Ube2T enzymatic activity, although we cannot rule out potential direct inhibition of specific protein–protein interactions. A cascade of biophysical methods was successfully implemented to screen and validate the binding of fragment-like small molecules and to elucidate their binding mode. Such fragments represent attractive starting points for further elaboration into high affinity inhibitors. A potent Ube2T inhibitor could find application in sensitizing cancer cells to cross-linking agents (such as cysplatin and mitomycin C) or be used to exploit specific synthetic lethal interactions. Very few E2 inhibitors are known to date, and targeting this newly discovered allosteric pocket could represent a successful strategy to develop high-affinity small molecules to effectively drug this class of enzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00147.

Experimental section: protein expression and purification, DSF, BLI, ligand-observed NMR spectroscopy, Ube2T backbone resonance assignment, CSP experiments, ITC, crystallization and structure determination, ubiquitination assays (PDF)

Molecular formula strings (CSV)

Accession Codes

Atomic coordinates can be accessed using PDB code 5NGZ. Authors will release the atomic coordinates and experimental

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data upon article publication. Backbone assignment of human Ube2T(1−154) can be accessed using BMRB accession number 27035.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

FA, Fanconi anemia; DSF, differential scanning fluorimetry; BLI, biolayer interferometry; SD, standard deviation; WaterLOGSY, water ligand observed gradient spectroscopy; STD, saturation transfer difference; CPMG, Carr−Purcell−Meiboom−Gill; CSP, chemical shift perturbation; ITC, isothermal titration calorimetry; SEM, standard errors of the mean

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