Crystal Structure of HECT Domain of UBE3C E3 Ligase and its Ubiquitination Activity

Sunil Singh and J. Sivaraman*

Department of Biological Science
14 Science Drive 4
National University of Singapore
Singapore 117543

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Structure and function of HECT domain of UBE3C E3 ligase

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*Corresponding author: JS; dbsjayar@nus.edu.sg

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Abstract

The HECT family of E3 ubiquitin ligase is divided into three subfamilies: the NEDD4, the HERC, and the “other”. Previous studies have mostly targeted members of the NEDD4 subfamily for structural and functional analysis. The UBE3C E3 ligase is a member of the “other” subfamily HECT and influences several crucial cellular processes, including innate immunity, proteasome processivity, and cancer metastasis. Here, we report the crystal structure of the HECT domain of UBE3C (amino acids (aa) 744-1083) with an additional fifty N-terminal amino acids (aa 693-743) at 2.7 Å, along with multiple in vitro ubiquitination assays to understand its enzymatic activity. The UBE3C HECT domain forms an open, L-shaped, bilobed conformation, having a large N-lobe and a small C-lobe. We show that the N-terminal region (aa 693-743) preceding the UBE3C HECT domain as well as a loop region (aa 758-762) in the N-lobe of the HECT domain affect the stability and activity of UBE3C HECT domain. Moreover, we identified Lys903 in the UBE3C HECT domain as a major site of autoubiquitination. The deletion of the last three amino acids at the C-terminal completely abrogated UBE3C activity while mutations of Gln961 and Ser1049 residues in the HECT domain substantially decreased its autoubiquitination activity. We demonstrate that these region/residues are involved in the E2-E3 transthiolation process and affect the UBE3C mediated autoubiquitination. Collectively, our study identified key residues crucial for UBE3C enzymatic activity, and it may assist in the development of suitable inhibitors to regulate its activity in multiple cancers.
Introduction

Ubiquitination is a vital post-translational modification that affects several key signaling events, including protein homeostasis, DNA damage, innate immunity, and neurodegenerative diseases [1] [2]. Ubiquitination is brought about by the sequential enzymatic activities of three enzymes, namely ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) [3]. Mutations of E3 ubiquitin ligases have been implicated in numerous human diseases [4] and thus are a common focus of study. Based on the mechanism of ubiquitin transfer to the substrate proteins, E3 ubiquitin ligases are classified into 3 classes: RING (really interesting new gene) type; HECT (homologous to the E6-AP carboxy terminus) type, and RBR (RING between RING) type [5].

The HECT family of E3 ligases regulate several biological pathways by assembling linkage specific polyubiquitin chains on various substrates [6]. There are 28 members of the HECT-type E3 ligase family, which are further classified into three different subfamilies based on the diversity of their N-terminal substrate-binding domains: the “NEDD4” subfamily (9 members; each contain an N-terminal C2 domain and two to four WW domains), the “HERC” subfamily (6 members; each bearing the regulator of chromatin condensation 1 (RCC-1)-like domain), and the “other” subfamily (13 members; no uniformity) [7]. The 13 members of the “other” subfamily display significant sequence diversity (only 20% to 30% identity) and regulate many crucial cellular signaling pathways [8]. Previous studies on the NEDD4 subfamily has formed the basis of our understanding of the ubiquitination mechanism of HECT E3 Ligases [9], [10]. However, we have a limited understanding of the ubiquitination mechanism of the “other” subfamily of HECT E3 ligases.

UBE3C (aa 1-1083), also known as RTA-associated ubiquitin ligase (RAUL), is a member of the “other” subfamily of HECT E3 ligases. It contains an “IQ” motif (aa 45-74) at its N-
terminus and a HECT domain (aa 744-1083) at its C-terminus, which regulates several crucial cellular processes [11], [12]. The HECT domain of UBE3C displays ~30% sequence identity with the HECT domains of NEDD4 subfamily members, and about 32% sequence identity with the HECT domains of the “other” subfamily members. This highlights that the HECT domain alone (besides the full length HECT E3s) varies considerably among different members and thus more structural and biochemical studies are needed to fully decipher HECT E3 mediated ubiquitination.

Previous studies have shown that UBE3C assembles K29- and K48- linked ubiquitin chains [13], [14], [15], [16]. The K48-linked ubiquitin chains serve as a marker for proteasomal degradation while the biological significance of K29-linked chains is still emerging. Moreover, in the presence of an excess of ubiquitinated proteins in the cell, UBE3C localizes to the proteasome and enhances proteasome processivity [12]. Previous work has also implicated UBE3C to be compromised in several disease processes. One study showed that the activity of the UBE3C is affected in Kaposi’s sarcoma-associated herpesvirus (KSHV), whereby the viral RTA E3 ligase hijacks the activity of UBE3C to accelerate the degradation of interferon regulatory factor (IRF)-3 and IRF7 to inhibit interferon signaling [17]. This allows for rapid viral growth and leaves the host immunocompromised. Other studies have shown that abrupt activation of UBE3C is associated with epithelial-mesenchymal transition, which is associated with the progression of melanoma and glioma [18]. Despite the versatile nature of UBE3C-mediated ubiquitination and its involvement in disease processes, the structure and ubiquitination mechanism of this E3 ligase is not available.

As a continuation of our previous work on various E3 ubiquitin ligases [19], [20], [21], [22], [23], [24], here we report the structure and function of the UBE3C HECT domain (aa 744-1083) having N-terminal adjacent region (aa 693-743). The amino acids from the N-terminal region (aa 693-743) form hydrogen bonding contacts with the N-lobe of UBE3C HECT
domain which enhance the stability and activity of the UBE3C HECT domain. Furthermore, we show that the deletion/mutation of a loop region (aa 758-762) in the N-lobe affects the catalytic activity of UBE3C. Notably, we identified that Lys903 in the UBE3C HECT domain is a major site of autoubiquitination and UBE3C assembled K48-linked polyubiquitin chains. Moreover, mutations of Gln961 and Ser1049 residues significantly reduce UBE3C mediated autoubiquitination activity and deletion of the last three C-terminal amino acids rendered the UBE3C HECT domain inactive. This study may lead to the development of inhibitors to target the ubiquitin ligase activity of UBE3C across a broad range of cellular processes.

**Material and Methods**

**Protein expression and purification**

The details of protein constructs used in this study are listed in Table S1. The construct from amino acid (aa) 693-1083 of UBE3C consists of HECT domain (aa 744-1083) and the N-terminal extended region (aa 693-743) and hereafter referred to as WT UBE3C HECT domain. The clones were confirmed through DNA sequencing and all clones were expressed in *E. coli* BL21 (DE3). His6-SUMO-tagged WT UBE3C HECT domain and its mutants were purified by nickel-nitritotriacetic acid affinity chromatography and treated with PreScission Protease (GE Healthcare) for overnight cleavage at 4 °C, followed with gel filtration chromatography in 20 mM Tris pH 8.0, 150 mM NaCl, 5% Glycerol, 1 mM DTT. The WT UBE3C HECT domain eluted as a monomer (~ 43 kDa) in gel filtration chromatography (Superdex S75 column). UBA1 (E1) was purified by nickel-nitritotriacetic acid purification while GST-tagged ubiquitin and UbcH5B and UbcH7 (E2s) were purified by glutathione-affinity chromatography followed by gel filtration chromatography (Superdex S75 column).
The GST tag was cleaved from the proteins using PreScission Protease (GE Healthcare) before gel filtration chromatography.

Thermal shift assay to measure the melting temperature (Tm)

Thermal shift assay also known as differential scanning fluorometry was performed in a Real Time PCR machine (Bio-Rad). The measurements were recorded in 96-well plates. The reaction mixture of 20 μL consisted of 1.0 mg/mL protein sample (WT UBE3C HECT domain or its various mutants), 2 μL Sypro orange (10X, Life Technologies) in 20 mM Tris pH 8.0, 150 mM NaCl and 2 mM DTT. The temperature was increased from 10 °C to 95 °C at a ramp rate of 1% (1 °C / min). Raw temperature-dependent fluorescence signals were recorded based on thermal denaturation of UBE3C constructs. To process the data, the first derivative algorithm was used in a Microsoft Excel macro to calculate the temperature at which the upward slope of the fluorescence versus temperature curve is the steepest (Tm). Each measurements were carried out in triplicates (± SD).

Crystallization and structure determination of WT UBE3C HECT domain

For crystallization experiments, WT UBE3C HECT domain (aa 693-1083) was purified in 20 mM sodium citrate pH 7.0, 150 mM NaCl, 5% glycerol and 1 mM DTT. The crystal screening of WT UBE3C HECT domain was carried out using commercially available crystallization screens by incubating 1 μL protein solution (15 mg/mL) with 1 μL crystallization solution in a hanging drop vapor diffusion method. The initial condition obtained from the Molecular Dimensions structure screen was further optimized. The best diffraction quality crystals were obtained after three days at room temperature (23˚C) from a condition consisting of 100 mM HEPES 7.5, 20% PEG 10,000. The suitable crystals were immersed in mother liquor solution with 25% ethylene glycol as cryoprotectant for synchrotron data collection. A complete native diffraction data was collected up to 2.7 Å at
the beamline 24-ID-E, Advanced photon source at Argonne national laboratory (APS, US). Data was processed with HKL2000 program [25]. CCP4 online program Morda [26] generated initial model using NEDD4L HECT (PDB : 2ONI; 30% sequence identity) as the search model which was used in Phenix-Phaser for molecular replacement [27]. One molecule was present in the asymmetric unit. The model was verified and built manually in COOT [28]. The final model was refined using Phenix.Refine to a 2.7 Å resolution with an R factor of 0.23 ($R_{\text{free}}= 0.27$). The stereochemistry of the model was evaluated with PROCHECK [29]. PyMOL was used to prepare all structure related figures [30].

**Circular dichroism spectrometry**

Far UV spectra (260–200 nm) measurements of WT UBE3C HECT domain and its mutants were performed using a Jasco J-100 spectropolarimeter (Jasco Europe, MI, Italy) in 20 mM Tris pH 8.0, 150 mM NaCl, and 2 mM DTT at room temperature (23 °C) using a 0.1-cm path length and stoppered cuvettes. Ten scans were recorded, averaged, and the baseline was subtracted.

**In vitro ubiquitination assays**

Multiple in vitro ubiquitination assays were conducted to characterize the functional activity of WT UBE3C HECT domain. The reaction mixtures contained 0.05 μM UBA1, 1 μM UbcH7 or UbcH5B, 3 μM WT UBE3C HECT or its mutants, 10 μM WT ubiquitin or its mutants. The ubiquitination mixtures were incubated at 37 °C for 60 minutes in 50 mM HEPES pH 7.5, 100 mM NaCl, 5 mM ATP, 10 mM MgCl₂, and 1 mM DTT. The ubiquitination reaction was terminated with the addition of SDS loading buffer prior to 12.5% SDS-PAGE analysis. For time point analysis, the samples were taken at different intervals and the reactions were quenched using SDS loading buffer followed by SDS-PAGE analysis. The gels were scanned with GS-800 calibrated densitometer (Bio-Rad) and the intensity of
polyubiquitin chain formation corresponding to autoubiquitination (WT or mutant UBE3C HECT constructs) was quantified with ImageJ software. For thioester formation analysis, WT UBE3C HECT domain and its various mutants were incubated with E1, E2, Ub and ATP for 2 min at 30 °C. The reactions were stopped in the absence and presence of a reducing agent (DTT) followed by SDS-PAGE analysis. The bands corresponding to E3-Ub show the thioester linked ubiquitin due to their susceptibility to reduction by DTT.

**Western Blot**

SDS-polyacrylamide gels were transferred to polyvinylidene difluoride membranes (Sigma-Aldrich) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories; Hercules, CA) followed by incubation with mouse monoclonal anti-ubiquitin antibody (Santa Cruz Biotechnology (SantaCruz, CA)) at 1:2000 dilutions. Subsequently after washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) in a 1:10,000 dilution. The bands were visualized using ECL western blotting substrate (Pierce) as instructed by the manufacturer. PageRuler Prestained protein ladder (catalogue no. 26616; lot 00355719; Thermo Scientific) was used in all the gels to compare the protein bands.

**Mass spectrometry**

From the 12.5% SDS-PAGE gel, bands corresponding to ubiquitinated species were excised and subjected to exhaustive trypsin digestion. The TripleTOF 5600 system (AB SCIEX, Foster City, CA, USA) was used to perform MS analysis in an information dependent mode. Peptide identification and quantification was performed on the ProteinPilot™ 5.0 software Revision 4679 (AB SCIEX) using the Paragon™ database search algorithm (5.0.0.0, 4767) and the integrated false discovery rate (FDR) analysis function. The data was searched against a database consisting 2018_juneuniprot_sprot (total 40698 entries). The processing
was specified as follows: Digestion-trypsin; ID Focus-Biological Modifications; special factors- ubiquitin/SUMO enrichment; Search Effort-Thorough; Detected Protein Threshold-0.05 (10.0%). For FDR determination, data was searched against a concatenated database with in silico on-the-fly reversal for decoy sequences automatically by the software. Peptides identified with confidence interval $\geq 95\%$ were considered.

**Results**

**Structure of the WT UBE3C HECT domain (aa 693-1083)**

Previous studies of different HECT E3 ligases suggest an important role of the $\sim$50 residues preceding the HECT domain in conferring stability and activity (Fig. S1) [19], [31]. We therefore generated two constructs: the UBE3C HECT domain alone (aa 744-1083) and the extended version of the UBE3C HECT domain comprising an additional N terminal 50 residues (aa 693-1083) and hereafter referred to as WT UBE3C HECT domain (Fig. 1A). The UBE3C HECT domain alone was soluble but displayed a strong tendency to aggregate and could not be crystallized. The WT UBE3C HECT domain, on the other hand, was soluble and behaved well in solution, which suggested that the additional residues (aa 693-743) were needed to provide stability to the HECT domain. This construct (aa 693-1083) was crystallized, and the structure was solved and refined to 2.7 Å resolution (Table 1).

HECT domain is known to exist in two different conformations- an inverted T-shape and an open L-shape based on the movement of the C-lobe around the flexible hinge region [6]. The HECT domain of UBE3C adopts an open L-shape bilobed conformation with an N-lobe (aa 744-965) and a C-lobe (aa 973-1083) positioned away from each other but connected through a short, flexible hinge region (aa 966-972) (Fig. 1B). The N-lobe consists of a large subdomain (aa 744-848 and aa 929-965; green) and a small subdomain (aa 856-928). The large subdomain of the N-lobe is located below the C-lobe (dark pink), while the small
subdomain is located away from the C-lobe, and mostly adopts a loop conformation, due to which; this region (aa 856-928) is not well defined in the electron density map (shown as dotted line; Fig. 1 B). The N-terminal extended region (aa 693-743; shown in sky blue) covers the N-lobe large subdomain. The large subdomain of the N-lobe comprises eight α-helices (α1-α8) and three β-strands (β1-β3). The C-lobe contains four α-helices (α9-α12) and four β-strands (β4-β7). The catalytic cysteine (Cys1051) involved in the transthiolation reaction is located on the C-lobe (Fig. 1 B, C). Sequence alignment of UBE3C E3 ligase with other HECT E3 ligases showed that it contains a loop region (aa 758-762) in the large subdomain of N-lobe for which no electron density was observed in the crystal structure and is shown as dotted line in Fig. 1 B. A close inspection of the N-lobe and C-lobe allowed us to identify the residues involved in the interactions of the UBE3C HECT domain. Hydrogen bonding contacts were observed between an N-lobe Arg774 and the catalytic cysteine on the C-lobe (Cys1051), as well as between an N-lobe Glu836 and C-lobe Ser1019 (Fig. 1D).

Comparison of the WT UBE3C HECT domain with other HECT E3 ligases

Next, we performed a DALI [32] search to identify the structural homologs of the UBE3C HECT domain. The highest structural similarity was observed with Rsp5 (rmsd: 2.8 Å for 279 Ca) (Fig. S2 A). Notably, Rsp5 is a yeast homolog of the human NEDD4 HECT E3 ligase. Rsp5 (PDB: 3OLM) adopts a similar open L-shape to that of UBE3C HECT domain structure. Furthermore, the DALI search showed that it has structural similarities with the E6AP E3 ligase, a member of the “other” subfamily HECT (PDB: 1C4Z) which also exists in an open L-conformation (rmsd: 2.9 Å for 265 Ca) (Fig. S2 B).

HECT E3 ligases display diversity in the small subdomain of the N-lobe (~70 aa) to function with several E2 enzymes (Fig. S3A) [9]. Previous studies with the NEDD4 HECT subfamily have identified that ~10 residues in the small subdomain of the N-lobe are required for its interaction with the E2 enzymes [31]. Notably, the N-lobe small subdomain is highly
conserved (50% to 80% sequence identity) across the members of the NEDD4 subfamily. However, sequence comparison shows substantial differences between the small subdomains of the UBE3C HECT domain (aa 856-928) and the NEDD4 subfamily, with conservation of only four of the 10 residues in the UBE3C HECT E3 ligase (Fig. S3A). Moreover, there is only 20% to 35% sequence identity for the E2-binding region among members of the “other” subfamily, which lends further evidence to support that diversity in the small subdomain of the N-lobe is required to bring about specificity in ubiquitination across multiple HECT E3 ligases.

Many of the conserved residues known to be essential for the stability and activity of various NEDD4 subfamily HECT E3 ligases are replaced or missing in the UBE3C HECT domain. For instance, all members of the NEDD4 subfamily contain a conserved tyrosine in the N-lobe that is naturally deleted in UBE3C HECT domain (Fig. S3B). This tyrosine residue provides stability to the HECT domain through hydrogen bond formation with the C-lobe threonine [33]. Moreover, many HECT E3 ligases contain conserved histidine and threonine residues immediately before the catalytic cysteine in the C-lobe (His-Thr-Cys; HTC). Close inspection of crystal structures of members of the NEDD4 subfamily showed that this His residue is involved in extensive hydrogen bond formation with several conserved residues present after the catalytic cysteine. Notably, UBE3C has a serine residue in place of the conserved histidine (Ser-Thr-Cys) (Fig. S3C), with fewer hydrogen bonds formed, and the residues following the catalytic cysteine are not well conserved (Fig. S3C).

**The WT UBE3C HECT domain is catalytically active**

HECT E3 ubiquitin ligases perform autoubiquitination to regulate their activities and interaction with other proteins [31]. To explore this for UBE3C E3 ligase, we performed an *in vitro* ubiquitination assay, using UBA1 (E1), UbcH5B (E2), WT UBE3C HECT domain
(E3) and ubiquitin. Both the SDS-PAGE and western blot analysis using anti-ubiquitin antibodies showed that the WT UBE3C HECT domain was active, with the formation of higher molecular weight bands corresponding to the WT UBE3C HECT-Ub polymers (Fig. 2 A, B). Moreover, we observed that WT UBE3C HECT domain formed free ubiquitin chains in solution. Notably, we also found that the WT UBE3C HECT domain was active with the members of two different families of E2 enzymes (UbcH5B and UbcH7) and WT UBE3C HECT domain was ~ 4-fold more active with UbcH5B than with UbcH7, as noted by the higher band intensity and longer polyubiquitin chains (Fig. 2 C, D, E). Besides, we performed a control experiment of E1 with UbcH5B and UbcH7 to show that E2-ubiquitin remained charged so that E1-E2 was not a rate limiting step for WT UBE3C HECT domain mediated ubiquitination (Fig. 2 F).

**K903 is a major site of autoubiquitination in the WT UBE3C HECT domain**

Mass spectrometric analysis on the higher molecular weight bands corresponding to WT UBE3C HECT-Ubₙ identified the lysine residues (K903 and K907) as the possible sites of autoubiquitination in the WT UBE3C HECT domain (Fig. S4). We have mutated these lysine residues to arginine and generated single mutant (K903R and K907R) WT UBE3C HECT domain constructs. Subsequently, we performed an *in vitro* autoubiquitination assay with these mutants and compared it with WT UBE3C HECT domain. SDS-PAGE and western blot analysis using anti-ubiquitin antibodies showed that K903R mutant resulted in a significant reduction in the autoubiquitination (Fig. 3 A, B). However, the K907R mutation did not affect UBE3C autoubiquitination. We speculate that K907 residue could be involved in weak autoubiquitination and therefore its mutation did not result in a loss of autoubiquitination. To further validate the SDS-PAGE and western blot results, we performed time point autoubiquitination assay with the WT UBE3C HECT domain and its K903R and K907R mutants. Briefly, samples were collected at different time points and the
reactions were quenched using SDS-laemmli buffer followed by SDS-PAGE analysis. This experiment further corroborated our observation that autoubiquitination was substantially decreased in the K903R mutant as compared to the WT or K907R mutant UBE3C HECT domain constructs (Fig. 3 C-G). Collectively, our results show that K903 is a major autoubiquitination site in the WT UBE3C HECT domain.

The WT UBE3C HECT domain assembles K48-linked polyubiquitin chains

Next, we analyzed the specificity of the polyubiquitin chain linkages assembled by the WT UBE3C HECT domain. This in vitro ubiquitination assay was performed with the UBA1 (E1), UbcH5B (E2), WT UBE3C HECT (E3) and a panel of eight different Ub mutants bearing K-to-R mutations, such that each mutant retained only one lysine residue (K6, K11, K27, K29, K33, K48 and K63); K0 has all lysine residues mutated to arginine. SDS-PAGE and western blot analysis showed that monoubiquitination of WT UBE3C HECT domain was achieved with each of the eight different Ub mutants. However, compared with the other single lysine Ub mutants, the K48-linked chains were significantly longer and of higher intensity (Fig. 4 A, B). K48-linked polyubiquitin chains mark proteins for proteasomal degradation [34] and such chains might allow UBE3C to self-regulate its expression in the cell through autoubiquitination.

Role of N-terminal extended region (aa 693-743) in UBE3C HECT stability and activity

The N-terminal extended region has been shown to confer stability to HECT domains [19], [31] and thus we sought to study the effects of the extended region (aa 693-743) in UBE3C HECT domain stability and activity. A close inspection of the crystal structure of the WT UBE3C HECT domain identified the residues from the extended region involved in the interactions with the residues from N-lobe. Notably, Phe695 and Phe702 are placed into the hydrophobic pockets in the N-lobe (Fig. 4 C). Besides, Arg698, Asp709 and Lys710 from the
extended region form hydrogen bonding contacts with different residues from the N-lobe (Fig. 4 C; Table S2). Subsequently, we purified the UBE3C HECT domain (aa 744-1083) alone without the N-terminal extended region. Circular dichroism experiment showed that secondary structures were maintained in UBE3C HECT domain (aa 744-1083) (Fig. 4 D). To study the role of the extended region in UBE3C HECT stability, a thermal shift assay was performed to measure the melting temperatures ($T_m$), where higher $T_m$ is an indication of enhanced stability of the proteins [35]. Here, we observed that the UBE3C HECT domain alone showed temperature dependent thermal denaturation at 37.8 ± 0.35 °C as compared to WT UBE3C HECT domain (43.7 ± 0.30 °C) with additional N-terminal 50 aa (Fig. 4 E). This suggested that the extended region (aa 693-743) is required to confer stability to the UBE3C HECT domain. Moreover, to evaluate the role of the extended region in UBE3C activity, we conducted an in vitro autoubiquitination assay of the UBE3C HECT domain alone (aa 744-1083) and compared its activity with WT UBE3C HECT domain (aa 693-1083). Notably, deletion of the extended region resulted in catalytically inactive UBE3C HECT domain as no bands were observed both in SDS-PAGE and western blot analysis blotted with anti-ubiquitin antibodies (Figure 4 F, G). This shows that the extended region is indispensable for the stability and the activity of UBE3C HECT domain.

**Impact of mutations in the loop region (aa 758-762) on WT UBE3C HECT activity**

The sequence alignment of UBE3C with other HECT E3 ligases showed a loop region (aa 758-762) in the N-lobe of the UBE3C HECT domain (Fig. S1). DALI structural analysis of the UBE3C HECT domain showed that the equivalent regions to this loop region (aa 758-762 of UBE3C) are present in E6AP (aa 539-544) and Rsp5 (aa 491-495) HECT E3 ligases. Notably, the ternary complex structure of Rsp5 (PDB: 4LCD), in which its active site cysteine (C777) is crosslinked to both Ub’s C-terminus and Sna3C (substrate) showed that the Rsp5 loop region (aa 491-495) might adopt different orientations to facilitate Rsp5 mediated
ubiquitination [36]. To gain further insights we superimposed the structure of the UBE3C HECT domain onto Rsp5 HECT-Ub-Sna3C complex. This superimposed complex model of UBE3C showed that its loop region (aa 758-762) is positioned near to the Rsp5 loop region (aa 491-495) (Fig. 5 A). Thus, to understand the role of this loop region in UBE3C stability and autoubiquitination activity, we generated two constructs, one in which the loop was deleted (termed as Δ Loop mutant), and the other in which all five loop residues were mutated into alanine (termed as Loop to Ala mutant). The secondary structures of these two mutants were similar to WT UBE3C HECT domain (Fig. 5 B). Subsequently, the thermal stability analysis was performed which showed that the loop region provided the additional stability to the UBE3C HECT domain. This was evident as both Δ Loop (34.5 ± 0.6 °C) and Loop to Ala (35.4 ± 0.4 °C) mutants showed early temperature dependent thermal denaturation as compared to WT UBE3C HECT (41.0± 0.6 °C) (Fig. 5 C).

Next, we performed thioester formation analysis to evaluate the involvement of the loop region in the E2-E3 transthiolation step of WT UBE3C HECT domain mediated ubiquitination. Here, we found that deletion of this loop region did not affect E2-E3 transthiolation process whereas loop to alanine mutation significantly reduced it (Fig. 5 D).

Subsequently, to study the effects of this loop region on the ligation step of the WT UBE3C HECT domain, we conducted an in vitro autoubiquitination assay with these mutants and compared it with the activity of WT UBE3C HECT domain. Both SDS-PAGE and western blot analysis showed that the Δ Loop mutant was defective in autoubiquitination whereas the loop to alanine mutant was completely inactive (Fig. 5 E, F). Based on our observation, we propose that the loop region plays essential roles in WT UBE3C HECT domain activity and its mutation/deletion results in a substantial loss of UBE3C ubiquitination activity.

**Involvement of Gln961 and Lys1013 residues in the WT UBE3C HECT activity**
Next we observed that most HECT E3 ligases have a conserved glutamic acid (Glu) in the N-lobe and a conserved glutamine (Gln) in the C-lobe, which form hydrogen bonds with each other when HECT domain adopts inverted T conformation. Studies have suggested that glutamic acid and glutamine provide the majority of the stabilizing energy for the inverted T conformation observed in some of the HECT E3 ligases such as WWP1, WWP2 and HUWE1 [10]. Besides, studies have shown that mutation of conserved glutamic acid affects ubiquitination activity of the HECT E3 ligases such as WWP1 and AREL1 [10], [19]. However, the roles of conserved Glu and Gln residues remains unclear during the E2-E3 transsthiation and ubiquitin ligation steps. In the structures of NEDD4L bound to UbeH5B-ubiquitin (PDB: 3JVZ) and Rsp5-Ub-Sna3C complex (PDB: 4LCD), the conserved Glutamic acid and Glutamine were not found to be involved in the E2-E3 transsthiation step [37], [36]. Notably, in UBE3C the conserved Glu in the N-lobe is replaced by Gln (Gln961), and the conserved Gln in the C-lobe is replaced by Lys (K1013) (Fig. S3D, S3E). Interestingly, a close inspection of our crystal structure showed that these two residues are not involved in any hydrogen bonding contacts in UBE3C (Fig. 6 A).

To study the involvement of these two residues in WT UBE3C HECT enzymatic activity, we have mutated its Gln961 and Lys1013 residues to generate three different single mutants such as Gln961Ala (Q961A), Gln961Glu (Q961E), Lys1013Gln (K1013Q) and one double mutant Gln961Glu:Lys1013Gln (Q961E:K1013Q) to mimic the other HECT E3 members. CD profile of all these mutants showed that the secondary structures were maintained (Fig. 6 B). Furthermore, to study the effects of these mutations on the E2-E3 transsthiation step, we performed a thioester formation experiment with WT UBE3C HECT domain and its various mutants. The bands corresponding to E3-Ub show the thioester linked ubiquitin due to their susceptibility to reduction by DTT (Fig. 6 C). We found that the Gln961 mutations (Q961A and Q961E) substantially reduced the formation of E3-Ub thioester intermediate, indicating
defects in E2–E3 transthiolation reaction. In contrary, mutation of Lys1013 (K1013Q) increased the formation of E3-Ub thioester intermediate, suggesting enhanced E2–E3 transthiolation process. The subsequent SDS-PAGE and western blot analysis of in vitro autoubiquitination assay of these mutants showed that the mutation of Gln961 residue substantially decreased the UBE3C ubiquitination activity as compared to the WT UBE3C HECT domain (Fig. 6 D, E). Notably, the Lys1013Gln (K1013Q) mutant showed enhanced ubiquitination activity (~2-fold) compared to the WT UBE3C HECT (Fig. 6 D, E, F), whereas the double mutant Gln961Glu:Lys1013Gln (Q961E:K1013Q) showed reduced level of autoubiquitination activity (Fig. 6 D, E). Collectively, we show that Gln961 and Lys1013 residues are involved in the E2–E3 transthiolation and likely in the ubiquitin ligation step of UBE3C HECT domain mediated autoubiquitination process.

Role of C-terminal residues in the ubiquitination activity of WT UBE3C HECT domain

Studies have shown that C-terminal conserved amino acids (~60 aa) in the HECT domains are involved in the regulation of enzymatic activity and ubiquitin chain specificity [38], [39]. For instance, deletion of the last three C-terminal amino acids of E6AP E3 ligase did not affect E6AP substrate ubiquitination whereas deletion of last three C-terminal amino acids of Rsp5 (yeast homologue of NEDD4) HECT E3 ligase resulted in complete loss of autoubiquitination activity [40]. Moreover, as mentioned in the previous section (see “comparison of the WT UBE3C HECT domain with other HECT E3 ligases”) many HECT E3 ligases have conserved histidine and threonine residues immediately before the catalytic cysteine in the C-terminal of HECT domains (His-Thr-Cys; HTC). In the NEDD4L-UbcH5B-ubiquitin complex structure (PDB: 3JVZ), UbcH5B Leu119 makes van der Waals interactions with HECT NEDD4L His920 and the catalytic Cys922 and the authors showed that mutations of His920 resulted in reduced E2–E3 transthiolation reaction [37]. However,
UBE3C E3 ligase has a serine residue in the place of conserved histidine (Ser-Thr-Cys; STC).

To study the role of C-terminal residues in UBE3C autoubiquitination, we have deleted the last three amino acids at the C-terminal and generated a WT UBE3C Δ3 CT HECT mutant (aa 693-1080). Besides, we mutated Ser1049 into His to create a WT UBE3C S1049H HECT mutant (aa 693-1083) with the His-Thr-Cys (HTC) sequence order as found in other HECT E3 ligases (Fig S3 C). SDS-PAGE and western blot analysis using anti-ubiquitin antibodies of an in vitro ubiquitination assay with these mutants showed that autoubiquitination was completely inhibited with Δ3 CT mutant (aa 693-1080) (Fig. 7 A, B) while mutation of Ser1049 into His (S1049H) substantially reduced WT UBE3C HECT ubiquitination activity (Fig. 7 C, D). Furthermore, based on the thioester assay, we found that Δ3 CT and S1049H mutants of WT UBE3C HECT domain showed reduced transthiolation reaction with E2 enzyme which likely resulted in a significant loss of activity (Fig. 7 E). Here, we show that the presence of Ser residue (S1049) in UBE3C HECT domain in place of a conserved His just before the catalytic cysteine is important for its enzymatic activity.

Discussion

Members of the “other” subfamily of HECT E3 ligases play important roles in assembling linkage-specific ubiquitin chains on various substrates [41]. However, there has been limited structural characterization of the “other” subfamily HECT ligases, particularly compared with the NEDD4 subfamily. Previous work has indicated that the extended region preceding the HECT domain affects the stability and catalytic activity of many HECT E3 ligases such as AREL1 and HUWE1 [19], [31]. Here, we also demonstrate the importance of the 50 amino acids (aa 693-743) upstream of the UBE3C HECT domain (aa 744-1083) for its stability and catalytic activity. In our crystal structure we observed that the extended region interacts with
several N-lobe residues to maintain the UBE3C HECT domain (Fig. 4 C). Moreover, the UBE3C HECT domain alone was functionally inactive (Fig. 4 F, G).

A previous study with UBE3C found that it could form thioester bonds with these two families of E2 enzymes (UbcH5B and UbcH7) [42]. Here, we show that the WT UBE3C HECT domain performs autoubiquitination with these two families of E2 enzymes, with four-fold higher activity with UbcH5B than UbcH7 (Fig. 2). Furthermore, WT UBE3C HECT domain assembled K48-linked polyubiquitin chains (Fig. 4 A, B). We found that K903 is a major site of autoubiquitination in UBE3C HECT domain and a K903R mutant showed significantly less autoubiquitination as compared to WT UBE3C HECT (Fig. 3). A previous study with Rsp5 E3 ligase (Yeast NEDD4 homologue) reported that the autoubiquitination at Lys432 induces its oligomerization and subsequent inactivation [43]. We suspect that through autoubiquitination at K903, UBE3C could regulate its activation or interaction with other proteins in the cells. However, the biological implication of this K903 autoubiquitination in UBE3C HECT domain is yet to be established in vivo. We observed that the HECT domain of UBE3C contains a loop region (aa 758-762) in the large subdomain N-lobe and deletion/mutation of this loop region substantially decreased its autoubiquitination activity (Fig. 5 E, F). Our results suggest that the loop region plays important role in E2-E3 transthioylation and the transfer of ubiquitin to the substrate protein lysine residue. We speculate that mutating the loop residues into alanine might affect the relative positioning of critical residues which could be involved in the E2-E3 transthioylation step thus altering the UBE3C E3 ligase enzymatic activity.

Functional analysis of WWP1 E3 ligase (NEDD4 HECT subfamily) showed that mutation of its conserved glutamic acid to valine (E798V) substantially increased its autoubiquitination activity in human prostate cancer [44]. The UBE3C HECT E3 ligase has an N-lobe glutamine residue (Q961) in the equivalent position, and we show that mutations of Gln961 (Q961A
and Q961E) significantly reduce autoubiquitination activity (Fig. 6 D, E). Mutation of Gln961 residue affected the transthiolation activity of UBE3C E3 ligase which could be a reason for its decreased enzymatic activity (Fig. 6 C). Furthermore, we found that mutation of Ser residue (S1049H) and deletion of last three C-terminal amino acids greatly reduced the E2-E3 transthiolation step (Fig. 7 E) and resulted in a significant loss of UBE3C enzymatic activity (Fig. 7 A-D). Notably, some of the WT UBE3C HECT domain mutants such as Δ Loop, Q961A, Q961E, Q961E:K1013Q and S1049H could form di-ubiquitin however they are defective in autoubiquitination which indicates that UBE3C likely adopts a slightly different mechanism for di-ubiquitin formation.

The UBE3C ubiquitin ligase is involved in myriad cellular processes, such as proteasome processivity, interferon signalling, and cancer metastasis [12], [18]. In summary (Fig. 8), we solved the crystal structure of WT UBE3C HECT domain (aa 693-1083) at 2.7 Å and showed that the N-terminal extended region (aa 693-743) and a loop region (aa 758-762) are imperative for the UBE3C ubiquitin ligase stability and activity. Moreover, the conserved amino acids (N-lobe Glu, C-lobe Gln and His) in the HECT family are naturally mutated in UBE3C E3 ligase and we show that the equivalent residues (N-lobe Gln961, C-lobe Lys1013 and Ser1049) play critical roles in the E2-E3 transthiolation and affect its autoubiquitination activity. The UBE3C HECT domain utilizes K903 as a major ubiquitination site during in vitro ubiquitination assay and its last three C-terminus amino acids are needed to perform the autoubiquitination. Our study may lead towards the development of therapeutic inhibitor molecules that can alter the ubiquitination activity of UBE3C E3 ligase in various biological processes.

**Data Availability**: Three dimensional atomic coordinates, and structure factors of UBE3C have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 6K2C).

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Author contribution statement: S.S. and J.S. designed research; S.S. performed research; S.S. and J.S analysed the data; S.S. and J.S wrote the paper.

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Table and Figure Legends.

**Table. 1** Data processing and refinement statistics of WT UBE3C HECT domain (aa 693-1083).

**Figure 1. Domain architecture and the structure of WT UBE3C HECT domain.** (A) Domain architecture of full length UBE3C (top) and the construct taken for crystallization (bottom) i.e. WT UBE3C HECT domain (aa 693-1083). Full length UBE3C contains N-terminal IQ motif and C-terminal HECT Domain. (B) Crystal structure of UBE3C HECT domain showing the N-lobe and C-lobe connected through a flexible hinge region (colored orange) in an open L-shape conformation. The N-terminal extended region (aa 693-743) is colored sky-blue. The N-lobe large subdomain is shown as green and the C-lobe (colored dark pink) contains the catalytic cysteine (C1051). The region (aa 850-926) having N-lobe small subdomain is not observed in the electron density and indicated with dotted black line. The N-lobe loop region (aa 758-762) is not well defined in the electron density and it is indicated by dotted green line. (C) The representative $2F_o-F_c$ electron density map of UBE3C HECT region (aa 1049-1051) contoured at 1.6 $\sigma$ is shown. The catalytic cysteine (C1051) on UBE3C HECT is shown. (D) Close view representation of N-lobe and C-lobe in the UBE3C HECT domain showing the essential hydrogen bonding contacts. The N-lobe is colored green and the C-lobe is colored dark pink. All the interacting residues are shown in stick representation. The hydrogen bonds are highlighted as yellow dashed lines.

**Figure 2. Autoubiquitination activity of WT UBE3C HECT domain.** (A) The various *in vitro* ubiquitination reactions were conducted in the presence (+) or absence (-) of E1 (UBA1), E2 (UbcH5B), E3 (WT UBE3C HECT), Ub and ATP respectively. SDS-PAGE analysis shows the formation of polyubiquitin chains on the WT UBE3C HECT domain and formation of free ubiquitin chains in solution. (B) Western blot analysis of (A) to highlight the catalytic activity of WT UBE3C HECT domain. The products are immunoblotted with anti-ubiquitin antibody. * represents ubiquitin; ** represents diubiquitin formed in the reaction. (C) SDS-PAGE and (D) western blot analysis of similar *in vitro* autoubiquitination reactions to highlight the activity of WT UBE3C HECT with two E2 enzymes (UbcH5B and Ub)
UbcH7). The arrowheads in (A) and (C) represent the E1 enzyme used in the reaction. (E) The ubiquitination assays with two E2 enzymes were quantified by plotting the intensity of polyubiquitin chains formed by WT UBE3C HECT domain. The ubiquitination assays were carried out in triplicates (± SD). (F) SDS-PAGE analysis of E1 interactions with UbcH5B and UbcH7 to show that E2-ubiquitin remained charged so that E1-E2 step is not rate limiting when comparing the rate difference between UbcH5B and UbcH7.

Figure 3. K903 is a major site of autoubiquitination in the UBE3C HECT domain. (A) SDS-PAGE analysis of various in vitro ubiquitination reactions conducted with E1, E2 (UbcH5B), E3 (WT UBE3C HECT and its K903R and K907R mutants), Ub and ATP. (B) Western blot analysis of (A) using anti-ubiquitin antibodies show significant decrease in the autoubiquitination with K903R mutant. * represents ubiquitin; ** represents diubiquitin formed in the reaction. (C-F) Time dependent ubiquitination assay with WT UBE3C HECT domain and its K903R and K907R mutants. The samples were taken at the indicated time points followed by SDS-PAGE analysis. The arrowheads in (A), (D), (E) and (F) represent the E1 enzyme used in the reaction. (G) The quantification of above ubiquitination assays was performed by plotting the intensity of polyubiquitin chains formed corresponding to autoubiquitination as shown in the images. All the assays were carried out in triplicates (± SD).

Figure 4. Ubiquitin chain linkage specificity and the role of N-terminal extended region (aa 693-743) in UBE3C HECT stability and activity. (A) SDS-PAGE analysis of in vitro autoubiquitination assay of WT UBE3C HECT domain in the presence of WT ubiquitin and different single lysine Ub mutants. The reactions were incubated for 60 mins at 37°C. (B) Western blot analysis of (A) to highlight the ubiquitin chain linkages assembled by WT UBE3C HECT domain. The products were immunoblotted with anti-ubiquitin antibodies. * represents ubiquitin; ** represents diubiquitin formed in the reaction. (C) Close up representation of the extended region and large subdomain N-lobe to show the residues from the extended region which are involved in hydrogen bonding contacts with residues from the large subdomain N-lobe. Phe695 and Phe702 pack into the hydrophobic pocket in the large subdomain N-lobe. Extended region is colored sky blue and large subdomain N-lobe is colored green. All the interacting residues are shown in stick representation. The hydrogen bonds are highlighted as yellow dashed lines. (D) Circular dichroism analysis showed the structural similarities between HECT domain alone (744-1083 aa) and WT HECT domain (aa 693-1083) of UBE3C E3 ligase. (E) Thermal shift assay to measure the melting temperatures (Tm) of WT HECT and HECT domain alone of UBE3C E3 ligase. The temperature dependent fluorescence signals were recorded based on the thermal denaturation of UBE3C constructs. RFU, Relative fluorescence units. Each measurements were carried out in triplicates (± SD). (F) SDS-PAGE and (G) western blot analysis of in vitro ubiquitination assay to evaluate the role of extended region (aa 693-743) on UBE3C HECT domain autoubiquitination activity. The products were immunoblotted with anti-ubiquitin antibodies. The HECT domain alone (aa 744-1083) is catalytically inactive. * represents ubiquitin; ** represents diubiquitin formed in the reaction. The arrowheads in (A) and (F) represent the E1 enzyme used in the reaction.
Figure 5. Comprehensive analysis of the loop region (aa 758-762) on WT UBE3C HECT enzymatic activity. (A) Superimposition of UBE3C HECT domain with ternary complex of Rsp5:Ub:Sna3C (PDB: 4LCD) to show the positioning of UBE3C loop region (aa 758-762) relative to Rsp5 loop region (aa 491-495). UBE3C HECT domain is colored green and Rsp5 HECT domain is colored yellow. The loop regions are shown as dotted lines and highlighted with a black circle. The catalytic cysteines of UBE3C (C1051) and Rsp5 (C777) are shown in sphere representation. (B) Circular dichroism experiment showed the structural similarities among WT UBE3C HECT and its Δ Loop (aa 758-762) and Loop to Ala mutants. (C) Thermal shift assay to measure the melting temperatures (Tm) of WT UBE3C HECT domain and its Δ Loop (aa 758-762) and Loop to Ala mutants. The temperature dependent fluorescence signals were recorded based on the thermal denaturation of UBE3C constructs. RFU, Relative fluorescence units. Each measurements were carried out in triplicates (± SD). (D) Thioester formation analysis was performed by incubating WT UBE3C HECT domain and its Δ Loop and Loop to Ala mutants with E1, E2, Ub and ATP for 2 min at 30 °C. The reactions were stopped in the absence and presence of a reducing agent (DTT) followed by SDS-PAGE analysis. The bands corresponding to E3-Ub show the thioester linked ubiquitin due to their susceptibility to reduction by DTT. (E) SDS-PAGE analysis of in vitro ubiquitination reactions to compare the catalytic activities of WT UBE3C HECT and its Δ Loop mutant and Loop to Ala mutants. The arrowhead represents the E1 enzyme used in the reaction. (F) Western blot analysis of in vitro ubiquitination reactions in (E) to compare the catalytic activities of WT UBE3C HECT and its Δ Loop mutant and Loop to Ala mutants. The products were immunoblotted with anti-ubiquitin antibody. * represents ubiquitin; ** represents diubiquitin formed in the reaction.

Figure 6. Functional analysis of Gln961 and Lys1013 residues in the WT UBE3C HECT domain autoubiquitination activity (A) Close view representation of UBE3C HECT domain showing Gln961 and Lys1013 residues. The N-lobe is colored green and C-lobe is colored dark pink. The catalytic cysteine (C1051) is also shown. All the residues are shown in stick representation. (B) Circular dichroism experiment showed the structural similarities among WT UBE3C HECT and its various mutants. (C) Thioester formation analysis was performed by incubating WT UBE3C HECT domain and its various mutants (Q961A, Q961E, K1013Q and Q961E:K1013Q) with E1, E2, Ub and ATP for 2 min at 30 °C. The reactions were stopped in the absence and presence of a reducing agent (DTT) followed by SDS-PAGE analysis. The bands corresponding to E3-Ub show the thioester linked ubiquitin due to their susceptibility to reduction by DTT. (D) SDS-PAGE analysis of in vitro ubiquitination reactions to compare the catalytic activities of WT UBE3C HECT and its various mutants. The arrowhead represents the E1 enzyme used in the reaction. (E) Western blot analysis of in vitro ubiquitination reactions in (D) to compare the catalytic activities of WT UBE3C HECT and its various mutants. The products were immunoblotted with anti-ubiquitin antibody. * represents ubiquitin; ** represents diubiquitin formed in the reaction. (F) The quantifications of WT UBE3C HECT and its K1013Q mutant mediated ubiquitination were performed by plotting the intensity of polyubiquitin chains formed corresponding to autoubiquitination as shown in the images. All the assays were carried out in triplicates (± SD).
Figure 7. The role of C-terminal residues in the WT UBE3C HECT domain mediated ubiquitination. (A) SDS-PAGE analysis of in vitro ubiquitination reactions to compare the catalytic activities of WT UBE3C HECT and its Δ3 CT mutant. The reactions were incubated for 60 mins at 37°C. (B) Western blot analysis of (A) using anti-ubiquitin antibodies shows complete loss of the autoubiquitination activity in UBE3C Δ3 CT HECT mutant. * represents ubiquitin; ** represents diubiquitin formed in the reaction. (C) SDS-PAGE analysis of in vitro ubiquitination reactions to compare the catalytic activities of WT UBE3C HECT and its S1049H mutant. The arrowheads in (A) and (C) represent the E1 enzyme used in the reaction. (D) Western blot analysis of (C) using anti-ubiquitin antibodies shows significant decrease in the autoubiquitination activity of UBE3C S1049H HECT mutant. * represents ubiquitin; ** represents diubiquitin formed in the reaction. (E) Thioester formation analysis was performed by incubating WT UBE3C HECT domain and its Δ3 CT and S1049H mutants with E1, E2, Ub and ATP for 2 min at 30°C. The reactions were stopped in the absence and presence of a reducing agent (DTT) followed by SDS-PAGE analysis. The bands corresponding to E3-Ub show the thioester linked ubiquitin due to their susceptibility to reduction by DTT.

Figure 8. Schematic diagram to summarize the effects of various mutations on UBE3C E3 ligase autoubiquitination activity (A) The WT UBE3C HECT domain (aa 693-1083) shows high in vitro autoubiquitination activity. (B) The UBE3C HECT domain (aa 744-1083) alone was functionally inactive. (C-F) The deletion/mutation of loop region (aa 758-762) and mutation of Gln961 and S1049 residues, and deletion of last three amino acids at C-terminal significantly reduced the autoubiquitination activity of WT UBE3C HECT domain. Our study provides the structure and function of WT UBE3C HECT domain which may assist in the development of therapeutic inhibitor molecules.
Table 1 Data processing and refinement statistics of WT UBE3C HECT (aa 693-1083).

| Data | WT UBE3C HECT domain |
|------|----------------------|
| **Data collection** | |
| Wavelength (Å) | 0.9791 |
| Resolution range (Å) | 44.5-2.7 (2.79-2.7) |
| Space group | P43212 |
| a, b, c (Å) | 75.61, 75.61, 220.19 |
| α, β, γ (°) | 90, 90, 90 |
| Total number of reflections | 386840 |
| Unique reflections | 18318 (1791) |
| R-meas | 0.169 (0.444) |
| R-pim | 0.036 (0.106) |
| CC1/2 | 0.9923 (0.981) |
| Completeness (%) | 99.69 (99.16) |
| Redundancy | 20.9 (13.7) |
| I/σ | 32.45 (4.75) |
| Wilson B factor (Å²) | 54.14 |
| **Refinement** | |
| Resolution range (Å) | 44.5-2.7 (2.796-2.7) |
| Reflections used in refinement | 18278 (1777) |
| Reflections used for R-free | 687 (95) |
| R-workᵃ | 0.23 (0.25) |
| R-freeᵇ | 0.27 (0.35) |
| Number of protein atoms | 2339 |
| Number of water molecules | 23 |
| RMS (bonds) (Å) | 0.008 |
|                       |       |
|-----------------------|-------|
| RMS (angles) (Å)       | 1.21  |
| Ramachandran favored (%) | 96.55 |
| Ramachandran allowed (%) | 3.45  |
| Ramachandran outliers (%) | 0.00  |
| Rotamer outliers (%)   | 0.00  |
| Clashscore             | 6.64  |
| Average B-factor (Å²)  | 56.44 |
| Protein atoms (Å²)     | 56.43 |
| Water molecules (Å²)   | 57.81 |

Statistics for the highest-resolution shell are shown in parentheses.

\( aR_{\text{work}} = \frac{\Sigma |F_{\text{obs}}| - |F_{\text{calc}}|}{\Sigma |F_{\text{obs}}|} \), where \( F_{\text{calc}} \) and \( F_{\text{obs}} \) are the calculated and observed structure factor amplitudes, respectively.

\( bR_{\text{free}} = \) as for \( R_{\text{work}} \) but was calculated using ~5% of data excluded from refinement.
Figure 1

A

N-45 74 693 744 928 973 1083
IQ HECT Large subdomain 848 856 Small subdomain 973 C lobe

B

C-lobe

Hinge region

Loop region (758-762 aa)

N-lobe

926 aa 850 aa

C

D

C1051 S1049 T1050

C1051

S1019

2.9 Å

E836

2.6 Å

R774

3.1 Å
**Figure 2**

A

|   | E1 | E2 | E3 | Ub | ATP |
|---|----|----|----|----|-----|
| 1 |   | +  |    |    |     |
| 2 |   |    |    |    |     |
| 3 |   |    |    |    |     |
| 4 |   |    |    |    |     |
| 5 |   |    |    |    |     |
| 6 |   |    |    |    |     |

B

C

D

E

F

---

WB: anti-Ub

WB: anti-Ub

WB: anti-Ub

Relative rate of Ubiquitination

UbcH5B

UbcH7

UbcH5B-Ub

UbcH7-Ub

**UBE3C-Ub**

**UBE3C-Ub**

**UBE3C-Ub**

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Figure 3

A

WT UBE3C HECT (693-1063 aa)  
No ATP

UBE3C-Ubn

UBE3C

B

WT UBE3C HECT (693-1063 aa)  
No ATP

UBE3C-Ubn

WB: anti-Ub

C

WT UBE3C K907R HECT

E1

Ub

UbH58

D

WT UBE3C K903R HECT

E

WT UBE3C K907R HECT

F

WT UBE3C K903R HECT

G

Relative Amount of Ub Chains Formed

WT UBE3C HECT  
WT UBE3C K907R HECT  
WT UBE3C K903R HECT
Figure 4

A

B

C

D

E

F

G

WB: anti-Ub

WT UBE3C HECT (693-1083 aa)
UBE3C HECT alone (744-1083 aa)

WB: anti-Ub

WT UBE3C HECT (693-1083 aa)
UBE3C HECT alone (744-1083 aa)
Figure 8

A
Extended region + UBE3C HECT (693-1083)

Autoubiquitination → Activity; High

B
UBE3C HECT (744-1083)

Autoubiquitination → Inactive

C
Extended region + UBE3C Δloop/ Loop to Ala HECT

Autoubiquitination → Activity; Less

D
Extended region + UBE3C Q961 mutant HECT

Autoubiquitination → Activity; Less

E
Extended region + UBE3C Δ3 CT HECT

Autoubiquitination → Inactive

F
Extended region + UBE3C S1049H HECT

Autoubiquitination → Activity; Less