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Interactions of cullin3/KCTD5 complexes with both cytoplasmic and nuclear proteins: Evidence for a role in protein stabilization

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ABSTRACT

Based on its specific interaction with cullin3 mediated by an N-terminal BTB/POZ homologous domain, KCTD5 has been proposed to function as substrate adapter for cullin3 based ubiquitin E3 ligases. In the present study we tried to validate this hypothesis through identification and characterization of additional KCTD5 interaction partners. For the replication protein MCM7, the zinc finger protein ZNF711 and FAM193B, a yet poorly characterized cytoplasmic protein, we could demonstrate specific interaction with KCTD5 both in yeast two-hybrid and co-precipitation studies in mammalian cells. Whereas trimeric complexes of cullin3 and KCTD5 with the respective KCTD5 binding partner were formed, KCTD5/cullin3 induced polyubiquitylation and/or proteasome-dependent degradation of these binding partners could not be demonstrated. On the contrary, KCTD5 or Cullin3 overexpression increased ZNF711 protein stability.

1. Introduction

As one of the 26 members of the KCTD protein family, KCTD5 is characterized by the presence of an amino-terminal BTB domain with similarity to the tetramerization domain (T1) of voltage-gated potassium channels. The BTB domain serves as protein interaction domain mediating the formation of both homomeric [1] and heteromeric [2] protein complexes.

Like other BTB proteins, different KCTD protein family members such as KCTD6 [3] and KCTD11 [4] have been characterized as substrate-specific adapters for cullin3-based ubiquitin E3 ligases [5,6]. The BTB domains of these adapter proteins mediate their interactions with cullin3 [5,6], whereas additional protein motifs such as zinc fingers (ZF), meprin and traf homology (MATH) or Kelch repeats are required for the binding of the respective substrate [2].

E3 ubiquitin ligases mediate the specificity of an enzymatic cascade responsible for both mono-and polyubiquitylation by recruitment of specific target proteins for the final step of the reaction [7]. Targeting proteins for proteasomal degradation is the main function of polyubiquitylation and involves linkage through internal ubiquitin lysine residue 48 [8,9]. In contrast, linkage through lysine residue 63 results in non-proteolytic regulation of substrate function [10]. Monoubiquitylationplays a role in regulation of histone activity, gene expression, DNA repair, endocytosis and protein sorting [11,12].

KCTD5 has been shown to interact specifically with cullin3, but not with other members of the cullin family [13]. On part of KCTD5, this interaction mainly requires the BTB domain [13], whereas on part of the cullin3 protein the N-terminal 75 amino acids already found to be important for the binding of confirmed substrate adapters [14] are required. These findings raised the possibility that KCTD5 also function as a substrate-specific adapter for cullin3-based ubiquitin E3 ligases. This hypothesis was addressed in the present study by searching for candidate KCTD5 interacting proteins, characterization of these interactions in mammalian cells and examination of a possible cullin3/KCTD5 mediated polyubiquitylation of these proteins.

2. Materials and methods

2.1. Plasmid constructs

All plasmid constructs were generated by standard restriction enzyme and PCR-based cloning techniques. pGBT9-KCTD5 contains

Abbreviations: AAV-2, adeno-associated virus type 2; BTB, bric-a-brac, tramtrak and broad complex; FAM193B, family with sequence similarity 193, member B; FOXO1, forkhead box O1; KCTD, Potassium channel tetramerization domain; MCM7, minichromosome maintenance protein 7; PAX3, paired box 3; ZF, zinc finger; ZNF711, zinc finger protein 711.

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the KCTD5 ORF derived from pCATCH-KCTD5 [15] in pGTB9 vector (Clontech). The complete ZNF711 ORF was amplified from HEK-293 cDNA and inserted into pCATCH vector [16] for expression of an N-terminal FLAG-tagged or pMyc-CS3+MT vector (Anne Vojtek, University of Michigan) for expression of a ZNF711 protein harboring 6 sequential N-terminal Myc-tags, respectively, both under control of the CMV promoter. The complete FAM193B ORF was assembled from PCR amplification of HeLa cDNA and one of the clones obtained in the two-hybrid screening and likewise cloned into pCATCH and pMyc-CS3+MT. The complete MCM7 ORF for cloning into pCATCH, pMyc-CS3+MT and a pCATCH derivate containing a 6 × His-tag was derived from pQBT7-MCM7 (Open Biosystems, MH51011-75176, MCM7, genbank accession number BC013375.2).

The pCATCH-KCTD5-47/234 and pCATCH-KCTD5-1/203 constructs have been described [15]. The additional KCTD5 deletion variants were generated by PCR amplification of the corresponding KCTD5 sequences.

The ubiquitin ORF and the cullin3 ORF for cloning into pMyc-CS3+MT vector were derived from PCR amplification of HeLa cDNA and plasmid expression clone IOH26262-pdEYFP-C1amp (imaGenes, Berlin, Germany), respectively.

2.2. Yeast two-hybrid screening

The full length KCTD5 ORF fused to the Gal4 DNA binding domain in the pGBT9 vector was used as a bait to screen a human kidney matchmaker® cDNA library from HEK-293 cells contained in the pACT2 vector (Clontech) by sequential transformation into yeast strain HF7c. Identification of positive transformants, isolation of library plasmids and elimination of false positives by retransformation with appropriate controls were performed as described in the manual for the matchmaker two-hybrid system 2 (Clontech).

2.3. Cell culture, transfection and drug treatment

Propagation and transfection of HeLa and HEK-293 cells were performed essentially as described [17]. Proteasome inhibitor MG132 (Sigma) was used at a final concentration of 3 μM for 16 h before harvesting, while protein synthesis inhibitor cycloheximide (CHX, Amresco) was applied at a final concentration of 10 μg/ml for the indicated time periods.

2.4. Cell synchronization and flow cytometry

For synchronization by a double thymidine block, HeLa cells were incubated 1 h post transfection with 2 mM thymidine for 18 h, followed by release into fresh medium for 9 h and a second treatment with 2 mM thymidine for 17 h. HeLa cells were collected at different time points after second release and subjected to western blot or immunofluorescence analysis. The percentage of synchronized cells was assayed by FACS analysis of DNA content after staining with 40 μg/ml propidium iodide (Sigma–Aldrich).

2.5. Immunoprecipitation and western blot analysis

Immunoprecipitation analysis was performed in RAF buffer as described [15] with anti-FLAG-M2 agarose (Sigma). Immunoprecipitates or whole cell extracts were subjected to western analysis as described [15].

2.6. Immunofluorescence

HeLa cells grown on coverslips were fixed and permeabilized as described [15]. Cells were incubated with primary antibodies at RT for 1–2 h, stained with FITC and/or TRITC conjugated secondary antibodies for 1 h and fixed with DAPI Fluoromount (Southern Biotech). The images were acquired either with Zeiss Axiophot 2 microscope with a Zeiss 63 × /1.4 NA Oil DIC objective or confocal microscope Zeiss LSM 510 META with a 63 × oil aperture objective.

2.7. Ubiquitylation assay

His-tagged proteins co-transfected into HEK-293 with N-terminally Myc-tagged ubiquitin were extracted and purified in NiNTA agarose (Qiagen) under denaturing condition as described [18], eluted in protein sample buffer complemented with 200 mM imidazole and analyzed by western blots.

3. Results and discussion

3.1. Determinants governing subcellular KCTD5 localization

Whereas the full-length KCTD5 protein of 234 amino acids displays a largely cytoplasmic localization [13,15], it can be translocated into the nucleus by the viral AAV-2 Rep78 protein [15]. With respect to potential cellular KCTD5 interaction partners, it was therefore of major interest to analyze the factors determining subcellular KCTD5 localization. With a comprehensive series of FLAG-tagged KCTD5 deletion variants (Fig. 1A) we could demonstrate that KCTD5 subcellular localization is influenced by its C-terminus in a complex manner (Fig. 1B). Deletion of 31 C-terminal amino acids (KCTD5-1/203) led to a nuclear localization of the corresponding protein variant. Deletion of additional 12 C-terminal amino acids (KCTD5-1/191) did not change this nuclear phenotype. Slightly larger deletions (KCTD5-1/181 and KCTD5-1/171), however, completely restored the cytoplasmic localization of the full-length protein. In contrast, a KCTD5 variant containing only amino acids 1 to 160 (KCTD5-1/160) showed a nuclear localization and deletion of the remaining C-terminal amino acids located outside the BTB domain (KCTD5-1/145) again switched the phenotype to a cytoplasmic localization. In addition to monomeric forms of the proteins, all the nuclear variants strikingly displayed a highly abundant band with reduced migration on western blots (Fig. 1C). The sizes of these additional bands were consistent with dimeric forms of the proteins stable under the conditions of SDS PAGE. By x-ray analysis, bacterially expressed KCTD5 has been shown to form pentamers [19] with the C-terminal 23 amino acids, which promoted cytoplasmic localization in our experiments, not visible on electron density maps and therefore predicted to be rather exposed to the outside. These and additional C-terminal sequences may thus regulate oligomere formation mediated by the BTB domain. The role of oligomerization in nuclear KCTD5 translocation was directly assessed by coexpression of the full-length protein together with the nuclear localized KCTD5-1/191 variant using two different tags for detection. In the presence of KCTD5-1/191, the full-length protein preferentially also was located in the nucleus in a high percentage of cells (Supplementary Fig. 1A). Coimmunoprecipitation analysis confirmed the formation of protein complexes between the full-length and the truncated protein (Supplementary Fig. 1B). Thus oligomerization most likely represents a prerequisite for nuclear translocation and not just a consequence of the later. A possible cell-cycle dependence of subcellular localization of the full-length KCTD5 protein was analyzed after HeLa cell synchronization through a double thymidine block (Fig. 1D) at several time points after cell release from G1/S into S phase of the cell cycle. During S phase, KCTD5 was predominantly located in the cytoplasm (Fig. 1E, t = 0 h and t = 3 h). An accumulation of KCTD5 in the perinuclear area was observed in the G2 phase at 6–7 h after release and at late stage M phase, KCTD5 was found

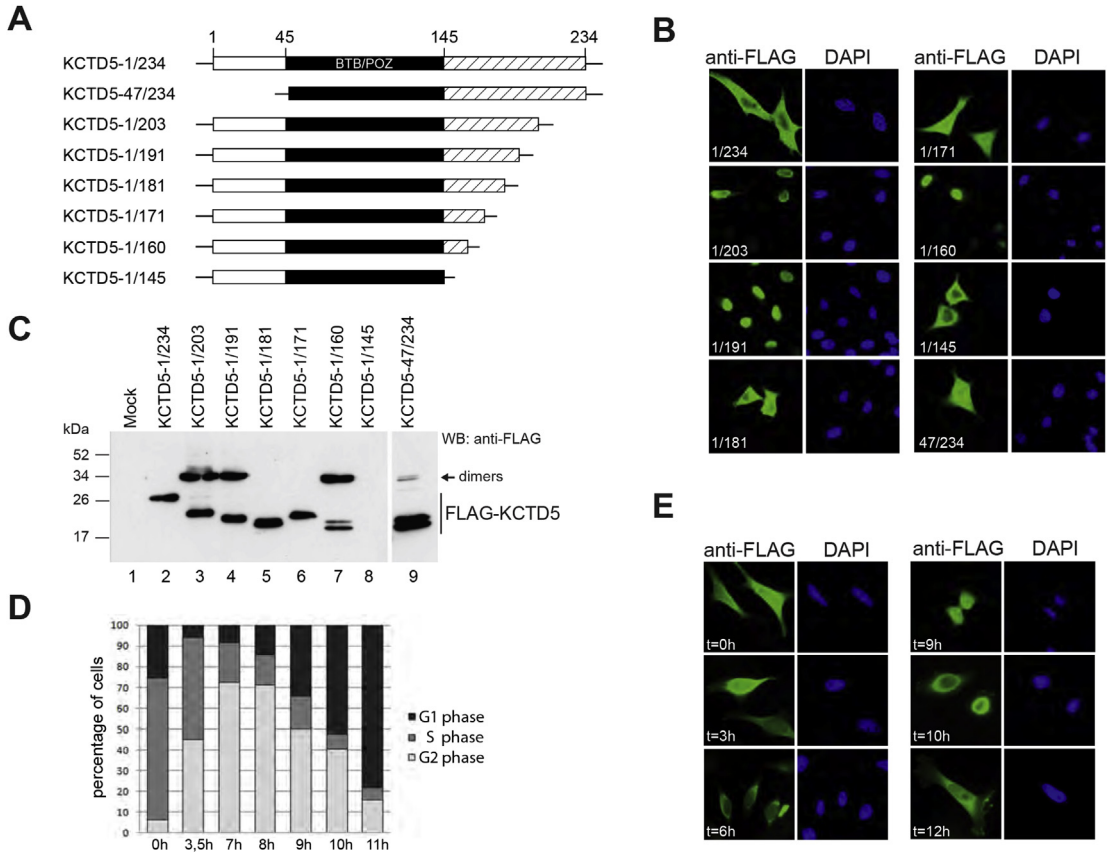

Fig. 1. Influence of individual protein domains and cell cycle phases on subcellular KCTD5 localization. (A) Schematic presentation of KCTD5 expression constructs with the BTB/POZ domain indicated by a filled box. (B) Subcellular expression pattern of N-terminally FLAG-tagged KCTD5 protein constructs determined by IF analysis in HeLa cells 40 h post transfection. (C) Western blot analysis of whole cell extracts harvested 40 h post transfection of HEK-293 cells with the indicated FLAG-tagged KCTD5 expression constructs. The KCTD5-1/145 variant could only be detected upon longer exposure in this particular experiment (not shown), but a band of the expected size was clearly observed in other experiments (also compare Fig. 2B to D). (D) Synchronization of HeLa cells by a double thymidine block. The percentage of cells in G1, S or G2 phase is shown at different time points after release from the block. (E) IF analysis of FLAG-tagged full-length KCTD5-1/234 at different time points after release from the double thymidine block.

homogeneously distributed in the nucleus (Fig. 1E, t = 9 h), followed by the formation of a perinuclear ring with the spreading of the cells (t = 10 h) and a predominantly cytoplasmic localization at later time points (t = 12 h).

3.2. Identification of cellular KCTD5 interaction partners

To identify potential substrates for the proposed role of KCTD5 as adapter for cullin3-based ubiquitin E3 ligases [13], we used the full-length KCDTD5 protein as bait in yeast two-hybrid interaction screening of a cDNA library from HEK-293 cells. Three of the cDNA clones isolated were shown to interact specifically with KCTD5 in retransformation assays. These comprised parts of the coding region of the minichromosome maintenance protein 7 (MCM7, GenBank accession no. BC013375), the zinc finger protein ZNF711 (GenBank accession no. NM_021998) and the FAM193B protein (predicted isoforms X2 to X6, GenBank accession no. XP_006714939 to XP_006714943). MCM7 encodes a subunit of the replication licensing factor RLF-M required for the initiation of cellular DNA replication [20,21]. The MCM7 region identified as KCTD5 interacting domain in the two-hybrid screen corresponds to the C-terminal amino acids 591 to 719 (Fig. 2A) spanning the so-called LG2 box shown to be required for MCM7 poly-ubiquitylation by E6-AP E3 ubiquitin ligase [22]. Zinc finger protein ZNF711 contains 12 zinc fingers of the C_2H_2 type (Fig. 2A) and appears to function as a transcription factor with more than 900 common binding sequences identified for ZNF711 and its interaction partner PHF8 [23]. FAM193B, also known as LOC54540 or IRIZIO, has been described to cooperate with PAX3-FOXO1 in alveolar rhabdomyosarcoma [24]. These KCTD5 interactions were confirmed by cotransfection of FLAG-tagged KCTD5 expression constructs with either MCM7 (Fig. 2B), ZNF711 (Fig. 2C) or FAM193B (Fig. 2D), all provided as Myc-tagged full-length expression constructs, into HEK-293 cells, followed by immunoprecipitation using FLAG-M2 agarose. Similar amounts of KCTD5 proteins could be precipitated for full-length KCTD5 protein and the prior characterized series of KCTD5 variants (Fig. 2B to D, upper panels). Both MCM7 and ZNF711 preferentially coprecipitated with the KCTD5 variants localized in the nucleus and showed only a weak, but clearly detectable interaction with the full-length protein and some of the cytoplasmic variants (Fig. 2B and C). Interactions with full-length KCTD5 may thus take place primarily during M phase as also suggested by additional immunofluorescence data (not shown) and may be involved in the regulation of cytokinesis. Of note, for ZNF711 a participation in cell division has already been suggested by RNA interference mediated knock-down [25]. KCTD5 interactions with both proteins were largely abolished by deletion of KCTD5 amino acids 146 to 160, may be partially due to the cytoplasmic localization of this KCTD5 variant.

In contrast to MCM7 and ZNF711, FAM193B most strongly bound to KCTD5 proteins located in the cytoplasm such as the full-length protein and truncated KCTD5-1/181 and KCTD5-1/171 variants

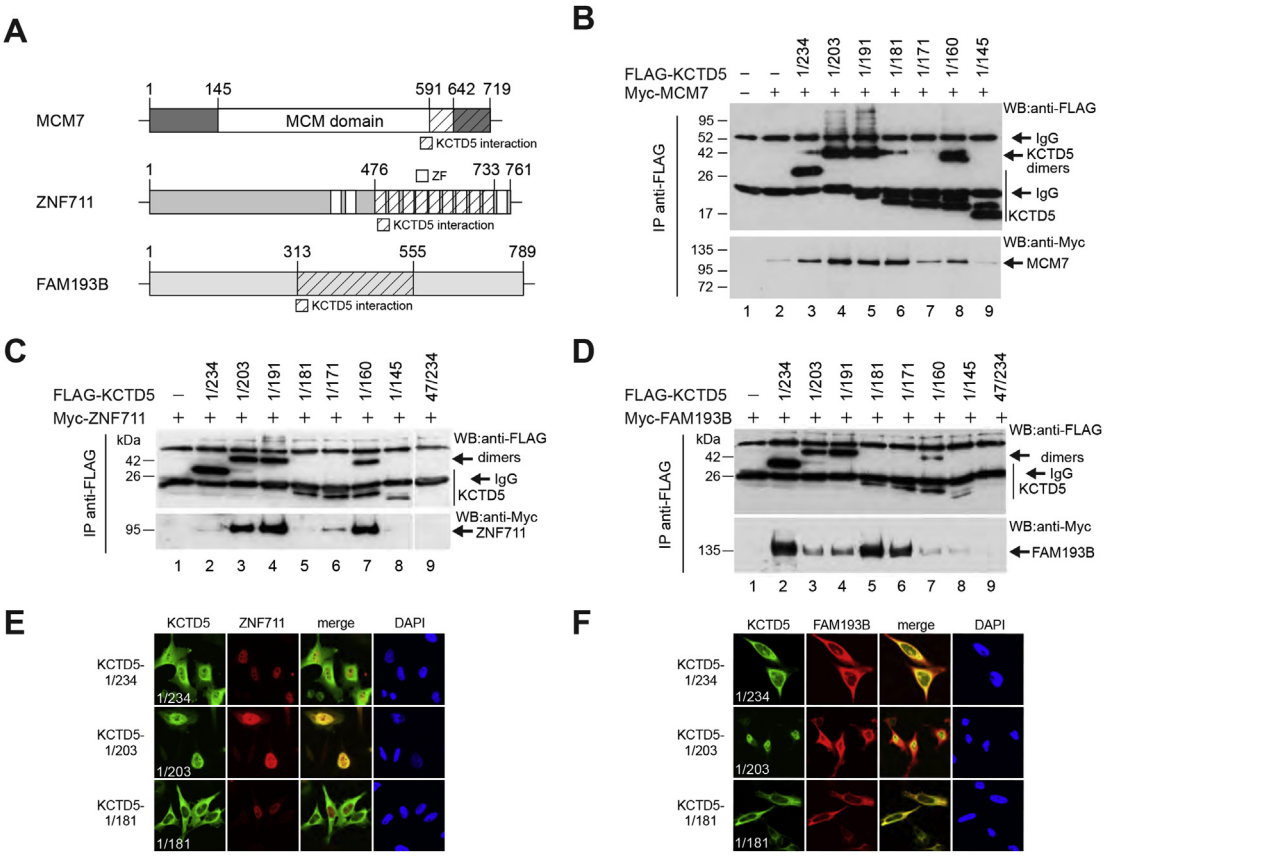

Fig. 2. Identification of cellular KCTD5 interaction partners. (A) Schematic presentation of the cellular KCTD5 interaction partners MCM7, ZNF711 and FAM193B identified by yeast two-hybrid screening. Characteristic domains like the so-called MCM domain of MCM7 or the 12 zinc fingers (ZF) of ZNF711 are indicated by open boxes. The domains identified as KCTD5 binding sequences in the two-hybrid screen are shown as hatched areas. Characteristic amino acid positions are provided above the boxes. (B to D) Coprecipitation of Myc-tagged full-length (B) MCM7, (C) ZNF711 and (D) FAM193B proteins with FLAG-tagged full-length KCTD5 and truncated protein variants after cotransfection into HEK-293 cells and purification on anti-FLAG M2 agarose. Precipitates were analyzed for the presence of FLAG-tagged KCTD5 proteins (upper panels) and co-precipitated MCM7/ZNF711/FAM193B (lower panels) by western blot analysis. (E and F) Cotransfection of (E) Myc-tagged ZNF711 or (F) Myc-tagged FAM193B and selected FLAG-tagged KCTD5 protein variants into HeLa cells, followed by double IF analysis through confocal laser scanning microscopy with antibodies detecting the two tags.

(Fig. 2D). However, some of the nuclear KCTD5 variants were still able to bind FAM193B, though less efficiently. Despite their cytoplasmic localization (compare Fig. 1B), KCTD5-1/145 and KCTD5-47/234 showed a strongly reduced FAM193B binding.

Double immunofluorescence analysis shown exemplarily for the full-length KCTD5-1/234, the nuclear KCTD5-1/203 and the cytosolic KCTD5-1/181 proteins with ZNF711 (Fig. 2E) and FAM193B (Fig. 2F) demonstrated colocalization of the nuclear KCTD5 variants with ZNF711 and MCM7 (not shown), whereas the full-length KCTD5 and all cytosolic variants colocalized with FAM193B. Thus the differential co-precipitation of the different KCTD5 variants with the identified cellular interaction partners may be mostly explained by their subcellular localization. No major translocations of KCTD5 or its interaction partners were observed after coexpression.

3.3. Formation of trimeric complexes of KCTD5 with cullin3 and the newly identified interaction partners

A function of KCTD5 as substrate-specific cullin3 adapter protein would require simultaneous binding to cullin3 and the potential substrate. To assay for the presence of such trimeric complexes, the KCTD5 interaction partners were cotransfected as FLAG-tagged expression constructs into HEK-293 cells together with Myc-tagged KCTD5 and Myc-tagged cullin3. In MCM7 immunoprecipitates, both KCTD5 and cullin3 could be detected (Fig. 3A). However, overexpression of KCTD5 was neither required for co-precipitation of cullin3 nor did it increase the amount of co-precipitated cullin3 (Fig. 3A, compare lanes 4 and 5). In ZNF711 immunoprecipitates, Cullin3 could be detected in the presence of KCTD5 (Fig. 3B) and overexpression of Cullin3 enhanced the amount of KCTD5 complexed with ZNF711 (Fig. 3B, compare lanes 3 and 4) without altering exogenous KCTD5 expression levels (Fig. 3B, lower part, whole cell extracts). Administration of proteasome inhibitor MG132 markedly increased ZNF711 expression levels and the amount of co-precipitated KCTD5 and cullin3 (Fig. 3B, lanes 6 to 8). With FAM193B, cullin3 could only be co-precipitated in the presence of exogenous KCTD5 (Fig. 3C, compare lanes 5 and 6), which demonstrates that KCTD5 serves as an adapter for the formation of the corresponding protein complex. Cullin3 in turn increased the binding of KCTD5 (Fig. 3C, compare lanes 4 and 6) pointing to a cullin3-mediated stabilization of a primary FAM193B/KCTD5 complex.

3.4. Stabilization of ZNF711 after KCTD5 and cullin3 overexpression

We then examined whether the identified cellular interaction partners might represent substrates for cullin3/KCTD5-mediated polyubiquitylation. In line with the findings of Kuhne et al. [22], we could demonstrate polyubiquitylation of a His-tagged MCM7 by

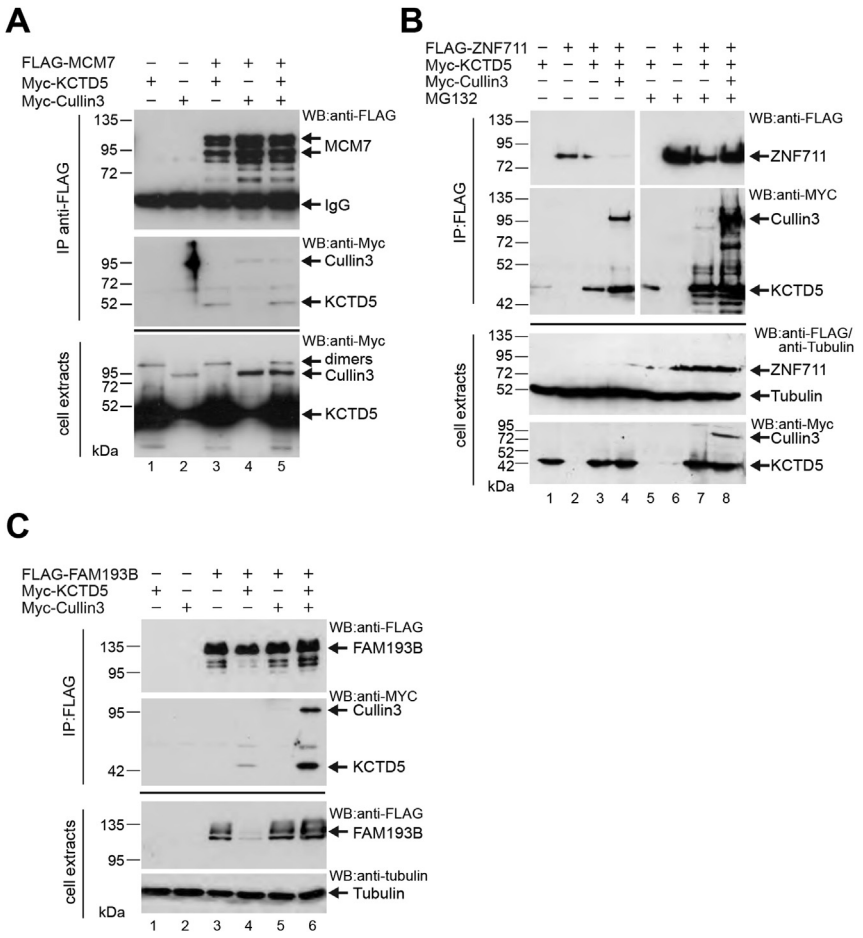

Fig. 3. Analysis of KCTD5 and cullin3 complex formation with MCM7, ZNF711 and FAM193B. (A) Coprecipitation of KCTD5 and cullin3, both provided as Myc-tagged expression constructs, with FLAG-tagged MCM7 after cotransfection into HEK-293 cells. Western analysis of anti-FLAG M2 agarose purified protein complexes with anti-FLAG (MCM7, upper panel) and anti-Myc (for cullin3 and KCTD5, middle panel) antibodies. KCTD5 and cullin3 expression levels were controlled by anti-Myc western analysis of whole cell extracts (lower panel). (B) Copurification of KCTD5 and cullin3 with FLAG-tagged ZNF711 as described in (A) for FLAG-tagged MCM7. In addition to KCTD5 and cullin3 expression levels (last panel), those of ZNF711 and tubulin (internal control) were also controlled by western analysis of whole cell extracts (third panel). In lanes 5 to 8, proteasome inhibitor MG132 was added at a final concentration of 3 μM for 16 h before harvesting. (C) Copurification of KCTD5 and cullin3 with FLAG-tagged FAM193B as described in (A) for FLAG-tagged MCM7. FAM193 and tubulin expression levels were controlled by western analysis of whole cell extracts (lower panels).

NiNTA purification under denaturing conditions (Fig. 4A). The polyubiquitylated MCM7 forms were subject to proteosomal degradation, since the levels of both the modified and the non-modified MCM7 forms were enhanced in the presence of the proteasome inhibitor MG132 (Fig. 4A, lane 5). MCM7 polyubiquitylation drives the disassembly of the replisome at terminating replication forks and involvement of functional cullin-dependent ubiquitin E3 ligases in this process has been suggested by preventing cullin neddylation with the inhibitor MLN4924 [26]. However, after overexpression of either KCTD5 alone or in combination with cullin3, neither a decrease in MCM7 protein levels nor significant changes in the ratios of polyubiquitylated to non-ubiquitylated forms of MCM7 were observed, regardless of the presence of the proteasome inhibitor MG132 (Fig. 4B). These data were corroborated by additional experiments with RNA interference vectors (data not shown) and strongly argue against a role of KCTD5 or cullin3 in MCM7 polyubiquitylation. For ZNF711 and FAM193B we were not able to unambiguously identify polyubiquitylated protein forms by an approach analogous to that used for MCM7 (data not shown). However, we noticed a strong increase in ZNF711 levels after KCTD5 or cullin3 overexpression (Fig. 4C). By inhibition of *de novo* protein synthesis with cycloheximide we could demonstrate that ZNF711 exhibited an increased half-life in the presence of KCTD5 and/or cullin3 (Fig. 4D, compare the lower three panels with the upper one). Thus at least for ZNF711, complex formation with the cullin3-KCTD5 scaffold is rather involved in protein stabilization than in degradation. Since co-expression of cullin3 enhanced the binding of full-length KCTD5 to ZNF711, we favor the hypothesis that a functional cullin3/KCTD5 complex mediates this ZNF711 protein stabilization. The interactions of KCTD proteins with cullin3 have been shown to induce changes in KCTD conformation [27], which may contribute to this enhanced binding of the substrate. With regard to the lack of cullin3/KCTD5 dependent polyubiquitylation of MCM7 it has to be noted that KCTD5 contains none of the substrate recognition domains such as zinc fingers, meprin and traf homology or Kelch shown to be required for substrate recognition in BTB proteins successfully characterized as cullin3 ubiquitin ligase adapters. The C-terminal region of KCTD5 hypothesized to function as a substrate recognition motif [13] was largely dispensable for the binding of MCM7, ZNF711 and FAM193B. Furthermore, in contrast to other BTB proteins, KCTD5 itself was not found to be ubiquitylated [13]. One has to keep in mind, however, that for some substrates ubiquitylation can only be demonstrated by very sensitive methods such as mass spectrometry [28,29].

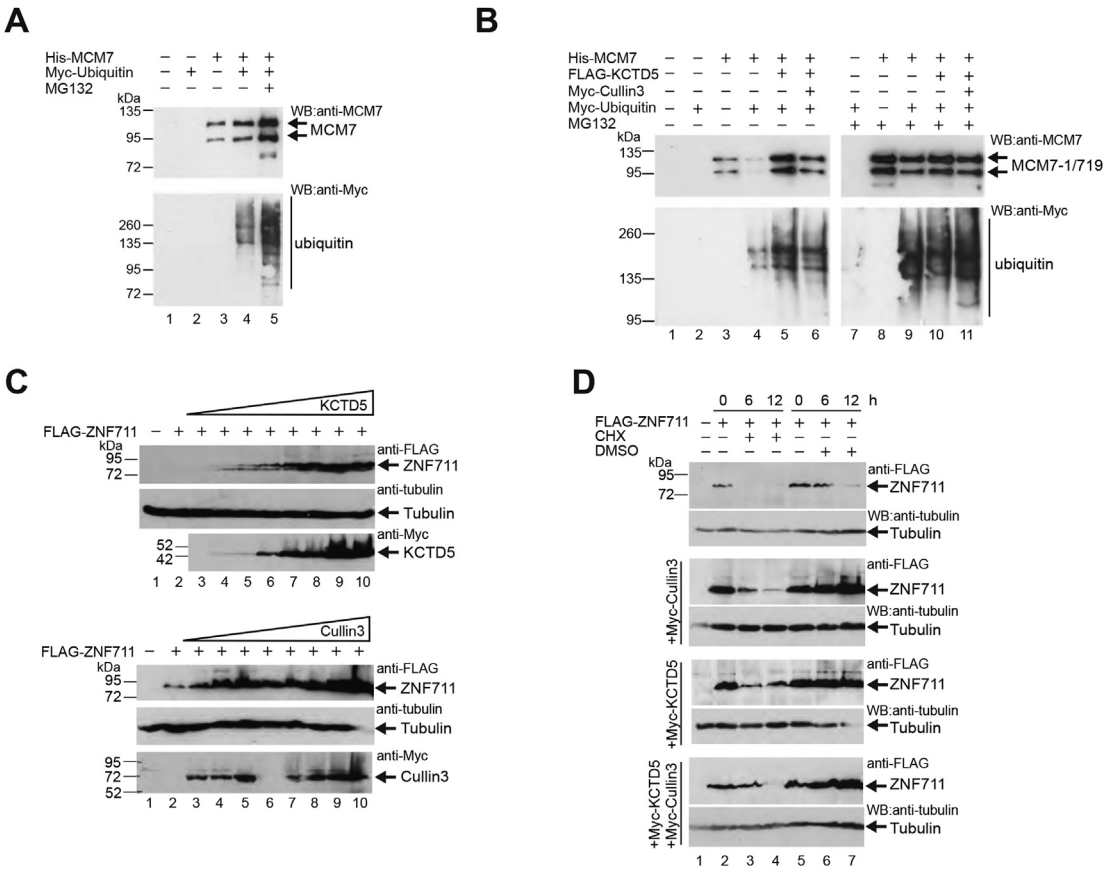

Fig. 4. Effects of KCTD5 and cullin3 overexpression on polyubiquitylation and protein levels of KCTD5 binding partners. (A) Analysis of polyubiquitylation of full-length MCM7 after cotransfection of His-tagged MCM7 constructs with Myc-tagged ubiquitin into HEK-293 cells, followed by Ni-NTA purification under denaturing conditions and western blot analysis for MCM7 (upper panel) and ubiquitin (anti-Myc, lower panel). Where indicated, proteasome inhibitor MG132 was added as described in Fig. 3(B) Analysis of wildtype MCM7 polyubiquitylation as described in (A) after additional cotransfection of FLAG-tagged full-length KCTD5 and Myc-tagged cullin3 expression constructs as indicated. (C) Western blot analysis of FLAG-tagged ZNF711 (1 μg) cotransfected into HEK-293 cells together with increasing amounts (0.1–3.0 μg) of KCTD5 (upper part) or cullin3 (lower part) expression constructs (total DNA amount was adjusted to 4 μg with empty vector DNA). The expression levels of the cotransfected proteins and tubulin as an internal control were monitored by western analysis as indicated. (D) Protein levels of exogenously expressed FLAG-tagged ZNF711 in HEK-293 cells after cycloheximide-mediated inhibition of *de novo* protein synthesis for the indicated time periods, starting 24 h post transfection. In the lower three panels, different combinations of KCTD5 and cullin3 expression constructs were cotransfected as indicated. Treatment of cells with DMSO used for solubilization of cycloheximide served as control (lanes 5 to 7).

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.069.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.069.

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