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Human UBL5 protein interacts with coilin and meets the Cajal bodies ☆

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ABSTRACT

UBL5 protein, a structural homologue of ubiquitin, was shown to be involved in pre-mRNA splicing and transcription regulation in yeast and *Caenorhabditis elegans,* respectively. However, role of the UBL5 human orthologue is still elusive. In our study, we observed that endogenous human UBL5 that was localized in the nucleus, partially associates with Cajal bodies (CBs), nuclear domains where spliceosomal components are assembled. Simultaneous expression of exogenous UBL5 and coilin resulted in their nuclear colocalization in HeLa cells. The ability of UBL5 to interact with coilin was proved by GST pull-down assay using coilin that was either *in vitro* translated or extracted from HEK293T cells. Further, our results showed that the UBL5–coilin interaction was not influenced by coilin phosphorylation. These results suggest that UBL5 could be targeted to CBs via its interaction with coilin. Relation between human UBL5 protein and CBs is in the agreement with current observations about yeast orthologue Hub1 playing important role in alternative splicing.

1. Introduction

Human UBL5 is a small protein consisting of 73 amino acid residues, which shares 22% sequence identity with ubiquitin. Despite this small sequence identity, both human UBL5 and its *Saccharomyces cerevisiae* ortholog Hub1p share similar fold with ubiquitin [1,2]. The main difference between ubiquitin-like proteins (UBLs) type I and UBL5 is, that mature UBLs bear invariant di-glycine motif at their C-termini required for their covalent attachment, while UBL5 possesses highly interspecies conserved di-tyrosine motif followed by one non-conserved residue [3,4]. It was shown that artificially exposed di-tyrosine motif at the very C-terminus was essential for covalent conjugation of Hub1p to some intracellular targets in *S. cerevisiae* [5]. However, this finding was not confirmed in other studies [6,7]. From the current view, Hub1p forms SDS-resistant non-covalent adducts with some cellular proteins in ATP-independent manner rather than covalent conjugates [8].

In yeast, Hub1, the sequential and structural homolog of UBL5 in higher eucaryotes, is part of splicing machinery that interacts with Snu66 [Ref. [7]], a component of spliceosomal complex U4/U6.U5-tri snRNP [9,10], and regulates Snu66 nuclear localization. Recent observations showed that Hub1 interacts with Snu66 via an HIND domain (Hub1-interaction domain) and that this interaction is necessary for an alternative splicing in *Schizosaccharomyces pombe* [11].

Studies on *Caenorhabditis elegans* showed that UBL5 is an indispensable nuclear-localized component of the signal pathway mediating the unfolded protein response in mitochondria (UPR^{mt}). It forms a non-covalent complex with the transcription factor DVE1, a previously reported member of the UPR^{mt} pathway, and activates transcription of mitochondrial heat shock proteins [12,13].

Cajal bodies (CBs) are subnuclear domains where assembly and/or modification of spliceosome components occur. They also contain factors for histone mRNA 3′-end processing, telomere maintenance and rRNA processing [14,15]. Main structural component of CBs is coilin [16–21], however, the CBs-like structures were observed also in yeast, in which any functional coilin homologue has not been identified so far [22,23]. It was shown that self-association of coilin via its N-terminal part is crucial for CBs formation [24,25]. Stability of CBs is influenced by phosphorylation and methylation of coilin [26–30]. Different activities of coilin modification factors are likely to be responsible for differences between CBs formation in primary and transformed cells [30,31]. CBs are highly mobile subnuclear domains [32,33] and they show fast exchange of deposited factors with the nucleoplasm [34,35]. Here we show that UBL5 colocalizes with CBs *in vivo* and interacts with coilin *in vitro* and that this interaction is not influenced by phosphorylation state of coilin.

2. Materials and methods

2.1. Cell lines and cultivation

HeLa and HEK293T cells (both from ATCC) were cultured in D-MEM medium (Sigma–Aldrich) supplemented with 10% fetal

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bovine serum (Gibco) and vitamins (Gibco) at 37 °C, 5% CO_2 atmosphere and 95% humidity.

2.2. DNA constructs

Vector pCMV-HA-UBL5 was prepared as follows: *ubl5* coding sequence was isolated by RT-PCR from the total RNA of HEK293T cells using primers U1+(cggaattctgatcgaggttgtttgcaacgaccg) and U2-(gcttctcgagctattgataataaagctccagg). The *ubl5* coding sequence was inserted into pCMV-HA (Clontech) through *Eco*RI and *Xho*I restriction sites. Vector pET41a-UBL5-GST was constructed as follows: the *ubl5* coding sequence was amplified by PCR using primers U3+(ggaattccatatgatcgaggttgtttgcaacgaccgtc) and U4- (ggaattccatatgttgataataaagctccaggt) and inserted into pET41a (Novagen) upstream of GST coding sequence through *Nde*I restriction site. Vector pCMV-cMyc-coilin was prepared as follows: *coilin* coding sequence was isolated by RT-PCR from total RNA of HEK293T cells using primers C1+(ccgctcgagttatggcagcttccgagacggttaggctacgg) and C2- (gatatttgcggccgctcaggcaggttctgtacttgatgtgttactt). The *coilin* coding sequence was inserted into vector pCMV-cMyc (Clontech) through *Xho*I and *Not*I restriction sites. Vector pGBKT7-coilin was constructed as follows: vector pCMV-cMyc-coilin was digested by endonucleases *Sfi*I and *Sma*I and the *coilin* coding sequence was inserted into pGBKT7 (Clontech) through the same restriction sites. Vector pGBKT7-SART1 was prepared as follows: *sart-1* coding sequence (previously obtained by RT-PCR) was amplified by PCR using primers S1+(ggaattccatatggggtcgtccaagaagcatcgcggagag) and S2-(ccgctcgagtcacttggtgatggtgttcgcgttcatg) and inserted into vector pGBKT7 through *Nde*I and *Sal*I restriction sites.

2.3. Transfection and immunofluorescence microscopy

Cells were plated on 35 mm Petri dish with sterile glass coverslips to 50% density. After 5 h, the cells were transfected with appropriate pCMV-derived mammalian expression constructs using FuGENE®HD (Roche) according to the manufacturer's instruction. After 24 h, the cells were washed once with preheated PBS (37 °C) and then fixed with either ice cold (−20 °C) acetone for 2 min or ice cold methanol for 5 min in a freezer at −20 °C. Fixed cells were washed twice by PBS containing 0.1% Triton X-100, then blocked using PBS with 1% bovine serum albumin and 0.1% Triton X-100 for 30 min and subsequently incubated with appropriate antibody for 1 h on ice. Then, the coverslips were washed twice in PBS and embedded in anti-fade reagent containing DAPI (Invitrogen). The cells were analyzed by fluorescence microscopy (Olympus IX81) or laser confocal microscopy (Leica TCS SP2). Rabbit polyclonal antibody α-coilin (sc-32860, Santa Cruz) was used in dilution 1:2000, mouse polyclonal antibody α-UBL5 (H00059286-A01, Abnova) in dilution 1:500, mouse monoclonal antibody α-UBL5 (H00059286-M01, Abnova) in dilution 1:500 and rabbit polyclonal antibody α-PML (sc-5621, Santa Cruz) in dilution 1:500. Secondary antibodies α-mouse IgG-TRITC and α-rabbit IgG-FITC (T6528 and F9887, respectively; Sigma–Aldrich) were used in dilution 1:150. The HA- or cMyc-fused proteins were visualized by α-HA-TRITC (H9037; Sigma–Aldrich) or α-cMyc-FITC (F2047, Sigma–Aldrich) antibody, respectively, both in dilution 1:100.

2.4. Expression and purification of recombinant proteins

Competent *Escherichia coli* BL21 (DE3) cells were transformed with plasmids pET41a and pET41aUBL5-GST for expression of GST and UBL5-GST, respectively. Transformed cells were inoculated to initial OD_{590} ~0.1 and incubated at 37 °C and 250 rpm. Protein expression was induced by 0.3 mM IPTG at OD_{590} ~0.5. After 3 h, the cells were pelleted at 4,000*g* and the pellet was resuspended in PBS (0.1 volume of initial culture volume) containing 5 μg/ml lysozyme, 1 μg/ml DNase, 1 μg/ml RNase and protease inhibitor Complete (Roche) and incubated at room temperature (RT). After 40 min, Nonidet P-40 (NP-40) was added to the final concentration 0.1% and the crude lysate was briefly sonicated on ice and centrifuged (25,000*g*, 20 min, 4 °C). The supernatant was collected, clarified by filtration through 0.45 μm PVDF filter and mixed with GST-bind resin (Novagen). After 1 h incubation at RT, the resin was washed by 100 resin volumes of PBS. Bound proteins were eluted by 10 mM reduced glutathione in PBS and then dialyzed against 10 mM Tris.HCl (pH 7.4) buffer containing 30 mM NaCl.

2.5. In vitro binding assay (pull-down)

[35-S]-radiolabeled proteins (SART1 or coilin) were translated *in vitro* using reticulocyte lysate (Promega) in the presence of TRAN35S-Label™ (MP Biomedicals) from pGBKT7-derived vectors as a template DNA. After 1 h, the lysate was diluted by interaction buffer (IB; 10 mM PIPES, 10% sucrose, 1 mM $MgCl_2$, 1 mM dithiothreitol, 150 mM NaCl, 0.5% NP-40, pH 6.8 and protease inhibitor Complete (Roche)) and GST-fused interaction partner was added. The mixture was incubated at room temperature for 1 h and then GST-bind resin was added. After 1 h incubation, the resin was washed five times with IB buffer and bound proteins were eluted by Laemmli buffer and analyzed by Tris-tricine SDS–PAGE followed by phosphorimaging

Coilin from HeLa cells was pulled-down as follows: HeLa cells were lysed by fresh IB buffer on ice and crude lysate was homogenized by passing through a 25G needle, incubated on ice for 20 min, briefly sonicated and then centrifuged at 20,000*g*, 4 °C for 10 min. The supernatant was mixed with GST-bind resin with immobilized GST or UBL5-GST and incubated 1 h at room temperature. The GST bind resin was then washed three times by IB and bound proteins were eluted by Laemmli buffer and analyzed by immunobloting. The hyperphosphorylated form of coilin was isolated from HeLa cells arrested in M-phase by 18 h nocodazole treatment and IB buffer additionally contained phosphatase inhibitor PhosSTOP (Roche).

2.6. Biochemical fractionation

HeLa cells were released from the surface of a cultivation dish to PBS using scraper. 10^6 cells was pelleted at 500*g* for 2 min and resuspended in 200 μl of TNE buffer (20 mM Tris. HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease inhibitor cocktail, pH 8.0) containing either 0.1% digitonin, Nonidet P-40 or sodium deoxycholate. The cell suspension was incubated 30 min on ice and, next, pelleted at 15,000*g* for 15 min to obtain supernatant and nuclear-rich pellet. The supernatant and pellet were separated on Tris-tricine SDS–PAGE, blotted to nitrocellulose membrane and immunochemically analyzed by α-UBL5, α-coilin and α-GAPDH antibody with chemiluminiscent detection.

3. Results

3.1. Intracellular localization of UBL5

As previous reports about intracellular localization of UBL5 vary in their conclusions [3,6,12,36], we first decided to investigate intracellular localization of endogenous UBL5 (e-UBL5). Immunostaining of e-UBL5 by a polyclonal or monoclonal antibody in HeLa cells resulted in observation of both cytoplasmic and nuclear localization of e-UBL5 (Fig. 1A). In the nucleus, interestingly, e-UBL5 formed foci in number of 1–5. The intracellular localization

of e-UBL5 was next examined using different approach. HeLa cells were lysed in different types of permeabilizing agents such as digitonin or nonionic and anionic detergents, Nonidet P-40 and sodium deoxycholate, respectively and biochemical fractionation was performed. Western blot analysis of cytoplasmic and nuclear rich fraction showed that they have comparable content of e-UBL5 (Fig. 1B, bottom panel). The Western blot analysis further showed that GAPDH is exclusively in the supernatant and coilin in the nuclear rich fraction. These observations are in agreement with localization of these proteins in living cell, namely, GAPDH is cytoplasmic and coilin nuclear protein. On the basis of these observations we conclude that e-UBL5 is localized both in the nucleus and in the cytoplasm.

3.2. Colocalization of UBL5 with Cajal bodies

The fact that in the nuclei e-UBL5 is present in a form of distinct foci led us to the question whether e-UBL5 colocalizes with some type of nuclear domain. We tested the possible colocalization of e-UBL5 with two types of nuclear domains, PML bodies and Cajal bodies (CBs), by fluorescent microscopy. The PML bodies were immunostained by α-PML protein antibody; however, colocalization of PML bodies with e-UBL5 foci was not observed (Fig. 2B). The CBs were visualized by α-coilin antibody, since coilin was reported to be the main structural component of CBs [16–18]. In this case, we observed discrete CBs and e-UBL5 foci, but on the other hand, we observed also a subset of CBs that was either in a close proximity to e-UBL5 foci or even entirely overlapped with e-UBL5 (Fig. 2A).

The link between UBL5 and CBs was also apparent from exogenous coexpression of HA-UBL5 and cMyc-coilin; both proteins colocalized in the nucleus of HeLa cells in an interchromatin region (Fig. 2C). Interestingly, when only HA-UBL5 was overexpressed in HeLa cells, we did not detect any nuclear bodies by α-coilin antibody, suggesting that the overexpression of HA-UBL5 could have negative effect on CBs formation or stability (Fig. 2D).

3.3. UBL5 interaction with coilin

The observed colocalization of e-UBL5 with coilin (CBs) and HA-UBL5 with cMyc-coilin in HeLa cells suggested possible mutual interaction of these proteins. To test this hypothesis, we performed a pull-down experiment (Fig. 3A) using immobilized UBL5 C-terminally extended with GST (UBL5-GST). The UBL5-GST fusion efficiently bound *in vitro* translated coilin, whereas the negative control, GST alone, did not (compare lanes 2 and 4 in upper panel Fig. 3A). Next, UBL5-GST but not GST alone was able to bind *in vitro* translated human SART-1 (Fig. 3A, lower panel), a homologue of yeast Snu66 positively interacting with yeast UBL5 [7,11], here used as a positive control.

It was previously shown that phosphorylation state of coilin affects stability/dynamics of CBs formation and retaining of nuclear components to them [30,37,38]. Therefore, we determined whether various extent of coilin phosphorylation affects its ability to interact with UBL5 *in vitro*. We used nuclear extract from HeLa cells synchronized in M-phase by nocodazole treatment, as a source of hyperphosphorylated coilin, because it was shown that hyperphosphorylation of coilin is responsible for CBs disassembly during mitosis [26]. To obtain dephosphorylated coilin, nuclear extract from interphase HeLa cells was treated by antarctic phosphatase (AP) for a complete protein dephosphorylation. The AP untreated extract from interphase cells served as a source of partially phosphorylated coilin. Different electrophoretic mobility of coilin from the mitotic, interphase and interphase AP-treated extracts confirmed their different phosphorylation state (e.g., compare lanes 1, 3 and 5 in Fig. 3B, upper panel). In a pull-down experiment, UBL5-GST, but not GST alone, bound coilin from all types of aforementioned nuclear extracts (compare differences

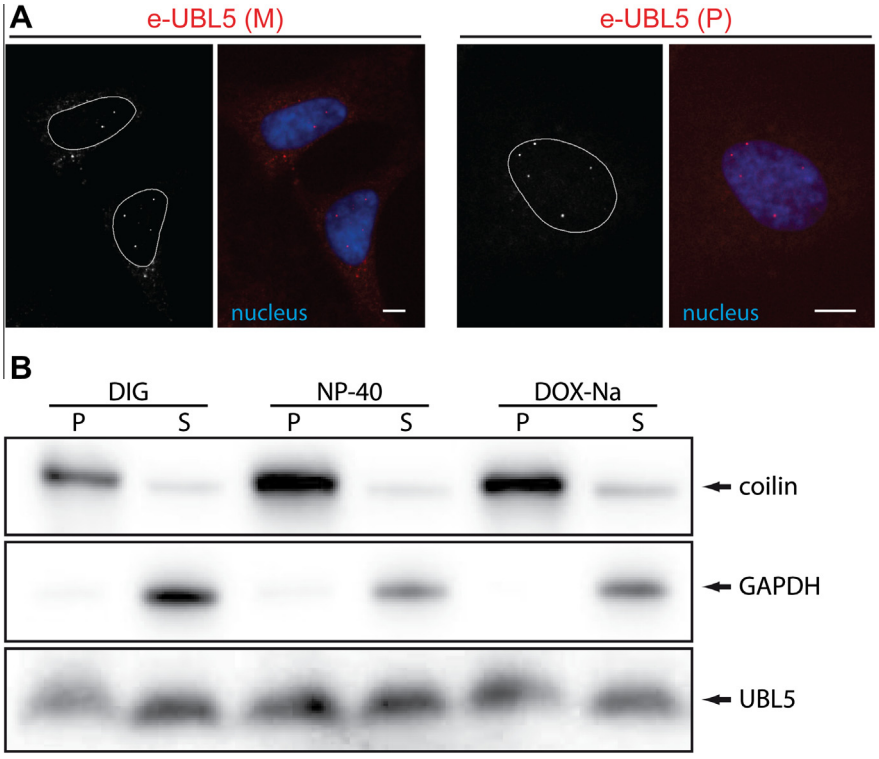

Fig. 1. Intracellular localization of endogenous UBL5. (A) HeLa cells were fixed and stained with α-UBL5 mouse monoclonal antibody (M; left panel) or α-UBL5 mouse polyclonal antibody (P; right panel) in combination with TRITC conjugated secondary antibody. e-UBL5 – endogenous UBL5. Nuclei were visualized by DAPI. Scale bar – 10 μm. (B) HeLa cells were lysed in presence of 0.1% digitionin (DIG), 0.1% Nonidet P-40 (NP-40) or 0.1% sodium deoxycholate (DOX-Na). Crude lysate was centrifuged and the supernatant (S) and pellet (P) were analyzed by Western blot with α-GAPDH (cytoplasmic marker), α-coilin (nuclear marker) and α-UBL5 antibodies.

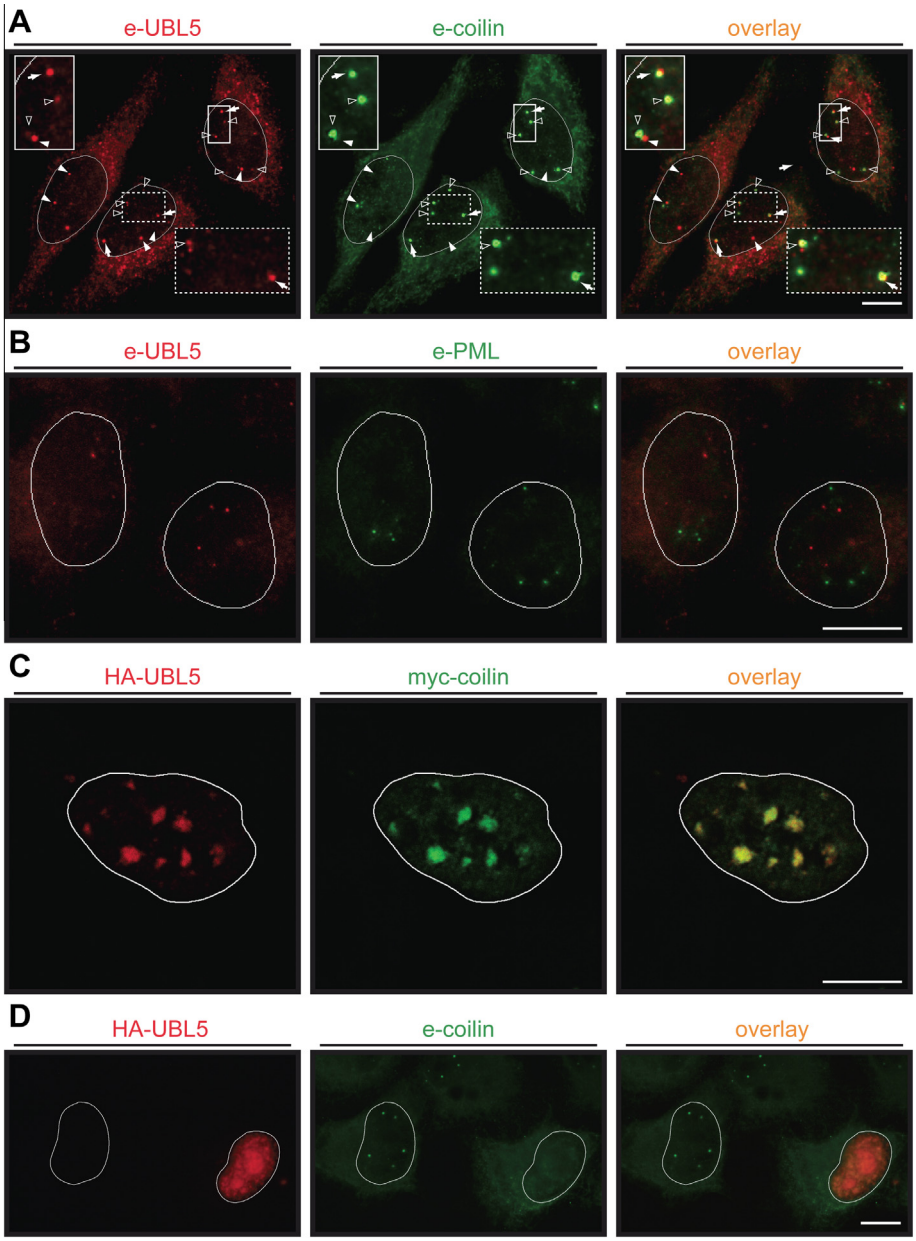

Fig. 2. Colocalization of UBL5 with coilin. (A) HeLa cells were fixed and stained by mouse polyclonal α-UBL5 antibody and rabbit polyclonal α-coilin antibody in combination with suitable secondary antibody. UBL5 foci which does not colocalize with CBs are pointed by *white filled arrowhead*, absolutely merged with CBs by *white delimited arrowhead* and partially overlapped with CBs by *white filled arrow*. Area bordered by *white curve* represents nuclei as stained by DAPI. (B) HeLa cells were fixed and stained by mouse polyclonal α-UBL5 antibody and rabbit polyclonal α-PML protein antibody. a. and b. were gained by epi-fluorescent microscope. (C) Hela cells were transfected by pCMV-HA-UBL5 and pCMV-cMyc-coilin, the cells were fixed 24 h post-transfection and stained with α-HA and α-myc monoclonal antibodies directly conjugated with FITC and TRITC, respectively. Pictures in panel c. were gained by laser scanning confocal microscope. (D) Overexpressed HA-UBL5 disrupts CBs. HeLa cells were transfected by pCMV-HA-UBL5, after 24 h the cells were fixed and coilin was visualized by rabbit polyclonal α-coilin antibody in combination with α-rabbit IgG-FITC conjugate secondary antibody. HA-UBL5 was stained by α-HA monoclonal antibody directly conjugated with TRITC. Scale bar – 10 μm.

between lines 1–2, 3–4 and 5–6 in Fig. 3B, lower panel - input). Based on these observations, we conclude that the mutual interaction between UBL5 and coilin is independent on coilin phosphorylation state.

4. Discussion

In NIH-3T3 cells, both cytoplasmic and nuclear localization of overexpressed Myc-UBL5 was reported and the nuclear/cytoplasmic level ratio decreased under hypoosmotic stress conditions [36]. Both cytoplasmic and nuclear localization was also reported for GFP-UBL5 in *Schizosaccharomyces pombe* [6]. In *C. elegans*, UBL5 was shown to translocate to the nucleus under specific mitochondrial stress induced by silencing of *spg-7*, *hsp-60* or *phb-2* [12]. Here we show that in HeLa cells endogenous UBL-5 (e-UBL5) is also localized both to the cytoplasm and nucleus. In the nucleus, e-UBL5 was observed in a form of irregular foci, in a number ranging from one to five foci per nucleus. A subset of these e-UBL5 foci either partially or entirely overlapped with Cajal bodies (CBs). The reason for such different superimposition of the e-UBL5 foci and CBs remains unknown. However, it is known that many components of CBs have highly dynamic behavior [34,35], so it is possible that the e-UBL5 foci represent some ordered complex colocalizing with CBs only transiently.

We propose that the interaction of UBL5 with coilin, the main structural component of CBs, could play important role in

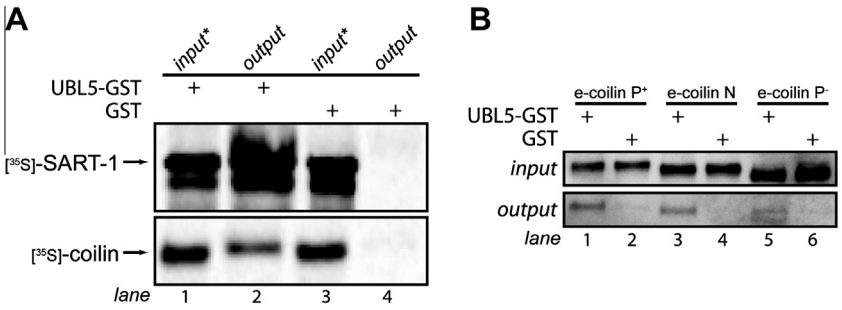

Fig. 3. Interaction of UBL5 with coilin *in vitro*. (A) ^{35}S-labeled coilin or SART-1 were prepared by *in vitro* translation and then incubated with UBL5-GST or GST for 1 h. Following that, GST-bind resin was added for next 1 h. The resin was then washed five times and bound proteins were analyzed by SDS–PAGE and visualized by phosphorimaging. Lanes 1 and 3 represent input of coilin or SART-1 (resin before wash); lanes 2 and 4 represent outputs of pulled coilin and SART-1 (washed resin). input* - 2%. (B) Coilin was extracted from the nuclei of nonsynchronized HeLa cells (*e-coilin N*) and cells arrested in prophase by nocodazole treatment (*e-coilin P*$^+$). Part of the lysate from nonsynchronized cells was treated by antarctic phosphatase to obtain dephosphorylated coilin (*e-coilin P*$^-$). All extracts were pulled-down as described in (A), samples were analyzed by SDS–PAGE and coilin was visualized by immunoblot. Input- 100%.

association of UBL5 with CBs. The UBL5-coilin interaction was proven by a pull-down experiment, where *in vitro* translated coilin efficiently bound to UBL5-GST. Interestingly, the overexpression of HA-UBL5 in HeLa cells had negative effect on the CBs formation or stability (Fig. 2D). Since the molecular principle of this effect is unknown, we cannot rule out the possibility that overexpressed UBL5 inhibits coilin oligomerization due to their mutual interaction.

The formation of CBs and their integrity was reported to be crucially influenced by coilin phosphorylation [38]. The phosphorylation state of coilin regulates its interaction with factors included in the spliceosome assembly, such as SmB′ and SMN proteins [37]. In contrast, our data show that the UBL5-coilin interaction is independent on coilin phosphorylation state, since UBL5 binds to both hyper- and dephosphorylated coilin *in vitro* (Fig. 3B). This finding is also supported by their almost exclusive colocalization when both are overexpressed (Fig. 2C), i.e. in the case when homogenous phosphorylation of coilin cannot be expected.

The physiological role of the UBL5-coilin interaction and the nature of the endogenous nuclear UBL5 foci remain to be determined. Proteomic studies showed an association of UBL5 with complexes controlling pre-mRNA metabolism in humans [39,40]. However, decreased UBL5 level in *C. elegans* using RNAi had no effect on pre-mRNA splicing [13], but the authors noted that residual amount of UBL5 in their experimental conditions can not be excluded. While there are several reports about UBL5 necessity for pre-mRNA splicing in *S. pombe* [6,7,11], the direct experimental proof of UBL5 role in this process in higher eukaryotes has not been reported yet. Since UBL5 protein is highly conserved among species and it was shown that disruption of *hub1* gene in *S. pombe* can be rescued by cDNA encoding human *ubl5* [7], it suggests that the role of human and yeast UBL5 may be conserved as well.

Here we show that UBL5 interacts *in vitro* with coilin, the main structural component of CBs, in phosphorylation independent manner and colocalizes with CBs *in vivo*. Since CBs are known to be involved in processes connected to pre-mRNA splicing, our data supports the hypothesis about the possible role of UBL5 in this process in mammalian cells as it was shown in yeast [6,7,11].

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