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Investigations on Cellular Localization of Coiled-Coil Domain-Containing Protein 43 (CCDC43) Under Stress Conditions

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Abstract

Coiled-coil domain containing (CCDC) family proteins regulate multiple biological functions in the cell. These domain structures have physical properties favorable for liquid-liquid phase separation (LLPS) phenomena, which play important roles in cell biology dynamics. LLPS is a fundamental mechanism in forming membrane-less organelles, including stress granules (SG) and P-body, nucleolus, PML nuclear body, Cajal bodies, nuclear speckles, and centrosomes. One of the new members of the CCDC protein family is CCDC43. In this study, sequence-based analyses of CCDC43, performed as a first step to understanding the role of CCDC43 in the cellular network, revealed the LLPS potential of the protein. We report that CCDC43, which has an evolutionarily conserved protein sequence, structurally possesses RNA-binding sites and its amino acid composition analysis may show an aggregation propensity and LLPS behavior under cellular conditions. CCDC43 is known to show a diffuse localization in the cytoplasm. We investigated whether this cellular localization is present in SGs whose formation is based on LLPS. We found that CCDC43 does not aggregate at SGs under given stress conditions. These data provide the first insights into the intracellular behavior and roles of CCDC43 in a disease-independent manner.

Keywords: CCDC43, liquid-liquid phase separation, stress granules.

1. Introduction

The evolution of coiled-coil motifs may have been driven by the need to add dynamism to the structural conformation of proteins to enhance functional differentiation without creating new genes. This is evidenced by the high prevalence of coiled-coil motifs in the eukaryotic genome, up to 10% compared to the prokaryotic genome [1]. The CCDC protein family was first identified in the early 2000s. Family members are oligomers containing two or more helical domains. There are approximately 180 proteins containing coiled-coil motifs and members of this family are known to be associated with various metabolic pathways, embryonic development, and mitochondrial and epigenetic diseases [2, 3, 4]. Additionally, the CCDC structural motif is linked to numerous malignant cancers. CCDC proteins play a crucial role in various types of cancer, including nasopharyngeal, pancreatic, prostate, breast, and colorectal cancers, due to their altered expression and association with invasion and metastasis.

CCDC43 is a new member of this family, located on chromosome 17 and encoding a 224 amino acid protein. Based on the Genotype-Tissue Expression (GTEx) portal, the CCDC43 gene is overexpressed in musculoskeletal tissues. It is up-regulated in cancers such as head and neck cancer and urethral cancer. The IPTMnet database suggests 12 potential post-translational modification sites for CCDC43, including phosphorylations, ubiquitinations and sumoylation [5]. As another post-translational modification for CCDC43, Tanikawa *et al.* suggested that arginine at position 166 may be converted to citrulline. In extensive proteomic studies, they investigated the substrates of the PAD4 enzyme. They suggested that CCDC43 is citrullinated similarly to a group of proteins, particularly RNA-binding proteins that exhibit liquid-liquid phase separation [6].

The overall amino acid composition of the CCDC43 protein is hydrophilic and the charged residues and

putative RNA binding domain are consistent with the multivalent character of the protein [7, 8]. RNA-binding proteins can bind to specific RNAs that are their targets and alter their stability and function. Moreover, protein-RNA interactions play important roles in many cellular events and regulate various metabolic processes. RNA-binding proteins (RBPs) and translation factors form membrane-less organelles to respond to different stress conditions. Cellular stress can be triggered by oxidative stress, UV, heat shock, hypoxia, translational inhibition, starvation, viral infection, exposure to toxins, and chemotherapeutic agents that alter the expression of RBPs and translational factors [9].

The biological significance of CCDC43 has mainly been studied in cancer; limited research has focused on its biological role in cells [10, 11, 12]. Studies on the cancer-related properties of CCDC43 have revealed the following: CCDC43 expression is a critical factor in GC (gastric cancer) growth and evolution. It drives GC cell proliferation, invasion, and metastasis, leading to poor patient prognoses [11]. Similar results have been shown for colorectal cancer and induce FOXK1-mediated epithelial-mesenchymal transition (EMT) [13].

In this study, beyond cancer research, we investigated the cellular localization of CCDC43 in stress-induced cells to investigate its relationship with molecular mechanisms that we predicted based on the structural features of the CCDC43 protein, and the results showed that the intracellular localization of CCDC43 did not change under the stress conditions provided.

2. Materials and Methods

2.1. Cloning

The cloning procedure was based on the PCR cloning protocol of Sambrook *et al*. [13]. The cloning Ccdc43 were amplified by PCR using gene-specific primers designed against the complete coding sequence of the gene. XhoI/EcoRI sites were introduced on the primers which were used for cloning. The PCR products were digested with restriction enzymes as indicated and ligated into in-frame into the pEGFPN2 vector, which was similarly cut. The ligation was transformed into E. coli DH5α (Invitrogen) strains by heat shock using standard protocols. The insert lengths were checked by restriction digestions of the isolated plasmids (MN plasmid miniprep kit), and the sequence was verified by DNA sequencing with universal primers.

2.2. Cell culture

Human U2OS and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS-Sigma), 1% penicillin, and 1% streptomycin (Gibco). The cells were maintained at 5% CO2 at 37°C. The plasmids were transfected using Jetprime Transfection Reagent (Polyplus) according to the manufacturer's instructions.

2.3. Western blot analysis

HEK293T cells were harvested 48 h after transfection. Cells were lysed in NP-40 lysis buffer, loaded onto the 4-12% SDS-PAGE gel, and then transferred to the nitrocellulose membrane. The membrane was blocked with 5% non-fat milk for 1 hour at room temperature and then incubated with anti-GFP antibody overnight at 4°C. Membranes were washed with TBS three times and then incubated with HRP-conjugated secondary antibody [15]. Finally, the membranes were incubated with enhanced chemiluminescence (ECL) reagents, and the protein bands were visualized with Biorad Chemidoc Imaging System.

2.4. Immunofluorescence

U2OS cells were seeded on glass coverslips and transfected with the indicated plasmids. After 24 h transfection, cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Cells were washed three times with phosphate-buffered saline (PBS) and permeabilized with PBS containing 0.1 % Triton for 10 minutes. Then cells were incubated with primary antibodies at 4°C overnight. After washing steps, cells were then incubated with Alexa 594–conjugated secondary antibodies [16]. The nuclei were counterstained with Hoechst 33342 (Thermo). Images were acquired with a confocal laser scanning microscope (Zeiss, LSM880).

2.5. Stress treatments

U2OS cells were seeded on round glass coverslips in a 6-well plate at a density of 200,000 cells per well.Following a 24-hour incubation period, the cells were subjected to transfection with plasmids expressing CCDC43-GFP (1 µg per well) using Jetprime Transfection Reagent (Polyplus). The transfection reagent was added to the transfection buffer at a rate of 2 µl per µg of DNA. The mixture was then left to stand for 10 minutes. Following this, the cells were subjected to transfection in a dropwise manner.

For the induction of stress granules, 24 hours after transfection, cells were subjected to sodium arsenite (500 µM; Merck) for 30 minutes and hydrogen peroxide (200 µM H_2O_2; Sigma) for 2 hours at 37°C to induce oxidative stress [17]. In the context of heat stress, U2OS cells were exposed to heat shock at a temperature of 46°C for a duration of 30 minutes. Untreated cells maintained at 37°C were used as the negative control for all treatments. Subsequent to the stress regime, all cells were subjected to two washes with 1XPBS and fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. An anti-G3BP antibody (Abcam) was

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utilised to visualise stress granules. The nuclei were stained with Hoechst 33342 (Invitrogen).
Experiments were repeated 2 times on different days.

2.6. Imaging

Images were captured using a 63X oil objective mounted on a Zeiss LSM 880 Inverted Confocal Microscope. Immunostaining procedures and image exposure times were standardised between controls and stress condition samples. Image size was set to 512 X 512 pixels. Pinhole size was set to 0.92 AU. The gain per channel was the same in all experiments. The digital gain was 1.0. Finally, the image panels were evaluated with ZenBlue (Zeiss) software.

3. Results and Discussion

First, we analyzed the potential of CCDC43 as an RNA-binding protein using the catRapid program [18]. The database algorithm showed us two regions above the threshold (>0.5) between 86-193 aa. In particular, the mid-peak of CCDC43 at 0.67 suggests it may be an RNA-binding protein (Figure 1a). Besides this RNA-binding propensity of CCDC43, its structural features obtained from the AlphaFold2 program, and its sequence-based score predicted by FuzDrop analysis (https://fuzdrop.bio.unipd.it) showed similarity to the liquid-liquid phase separation characteristic of proteins forming membrane-less organelles (Fig 1b).
From this point of view, CCDC43 interaction with the stress granule, one of the most important membrane-less organelles in the cell, was investigated in this study.

To determine the cellular localization of CCDC43 in normal and stressed cells, we cloned CCDC43 protein into an overexpression vector by fusing it with green fluorescent protein. Western blot analysis using an anti-GFP antibody confirmed protein expression in plasmids obtained by transient transfection into HEK293T cells (Figure 2). Since the fused protein band of ~52 kDa was detected using antibodies against GFP, this result indicated that CCDC43 was successfully cloned at the N-terminus of GFP.

Given the role of RNA-protein interactions in SG formation and regulation of SG dynamics, and the disordered regions in the structure of CCDC43, a potential RNA-binding protein, we sought to determine whether this protein is a component of SGs. When cells are stressed by oxidative or thermal stress, cytoplasmic stress granules are formed by aggregation of G3BPs. We performed immunofluorescence experiments to determine the colocalization of CCDC43 with SGs under different stress conditions (Figure 3).

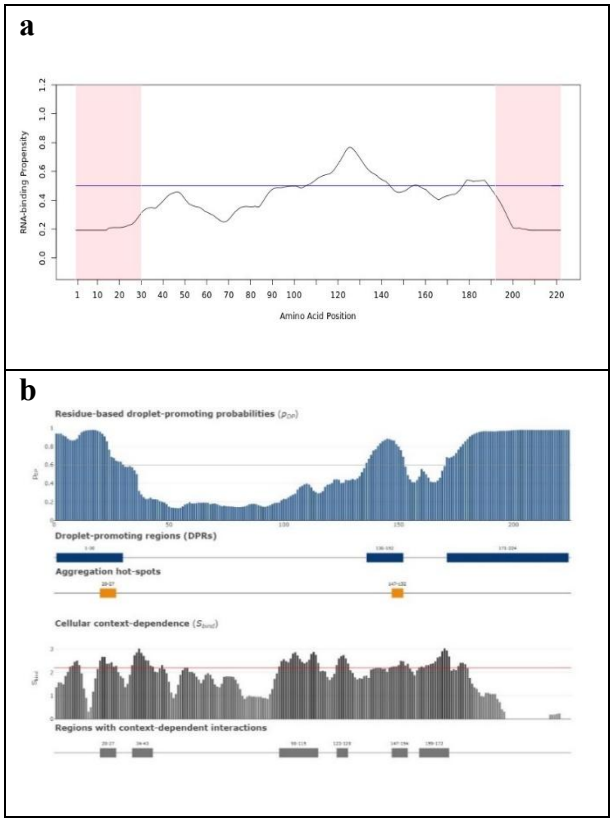

Figure 1. The RNA-binding propensity of CCDC43 protein by catRAPID signature (a) and residue-dependent droplet promoting profile of CCDC43 predicted by FuzDrop Server (b).

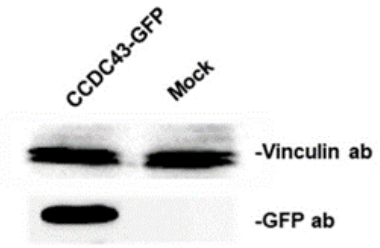

Figure 2. Western blot analysis of GFP fused CCDC43 protein. Hek293T cells were lysed and analyzed by Western blotting against anti-GFP antibodies to validate GFP tagged CCDC43 and anti-vinculin antibodies as a loading control.

The results indicated that the CCDC43 protein has a diffuse cytoplasmic distribution. Antibody staining in the literature shows that CCDC43 does not localize to a specific cellular region under normal conditions. A similar result was observed with GFP-labelled CCDC43. Some proteins are known to change their localization in response to changing conditions, especially stress, but this was not the case for CCDC43 under the stress conditions tested. Furthermore, SG induction was not affected by the overexpression of CCDC43 protein.

Although there is no essential cellular function predicted for the CCDC43 protein, the high evolutionary conservation of the protein sequence suggests that it may have a critically important role in eukaryotic cells. Consurf is a web tool that reveals evolutionary relationships between proteins by analyzing dynamic amino/nucleic acid substitutions between homologous sequences (available at consurf.tau.ac.il/). According to consurf results, the C-terminus of CCDC43 has a highly conservative motif [19, 20].

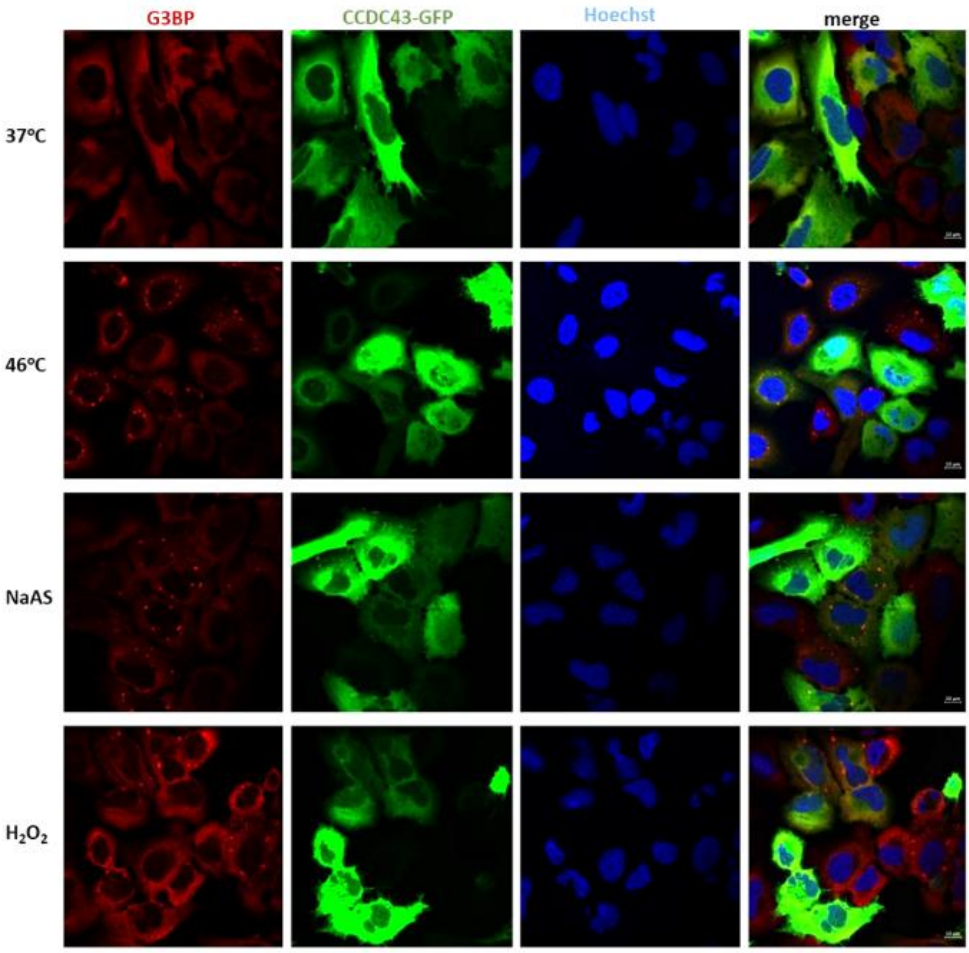

Fig 3. U2OS cells were transfected with the CCDC43-GFP construct and fixed at 24 h post-transfection. 1st column represents G3BP (red), 2nd column represents CCDC43-GFP (green), 3rd column represents hoechst (blue) and 4th column represents the merged state of all channels. The rows represent the normal (37 °C) and stress conditions (46 °C, NaAS, H_2O_2) of the cell. All scale bar is 10 µm .

CCDC43 belongs to the CCDC protein family, which is characterized by the presence of an alpha-helical helix domain and can fulfil various functional and functional biological roles within the cell through changes in their spatial conformation. Similar to these family members, the presence of regions that can provide multiple interactions is notable. In particular, the N-terminus of the protein shows low complexity (LC) domain features associated with its flexibility. As shown in Figure 4c, the Alfafold structure of CCDC43 has two major loops corresponding to the Consurf structural model as marked by variable color according to the conservation scale. This may indicate that the variable structure of the protein may have multiple functions in different cells and organisms and even at different stages of the cell.

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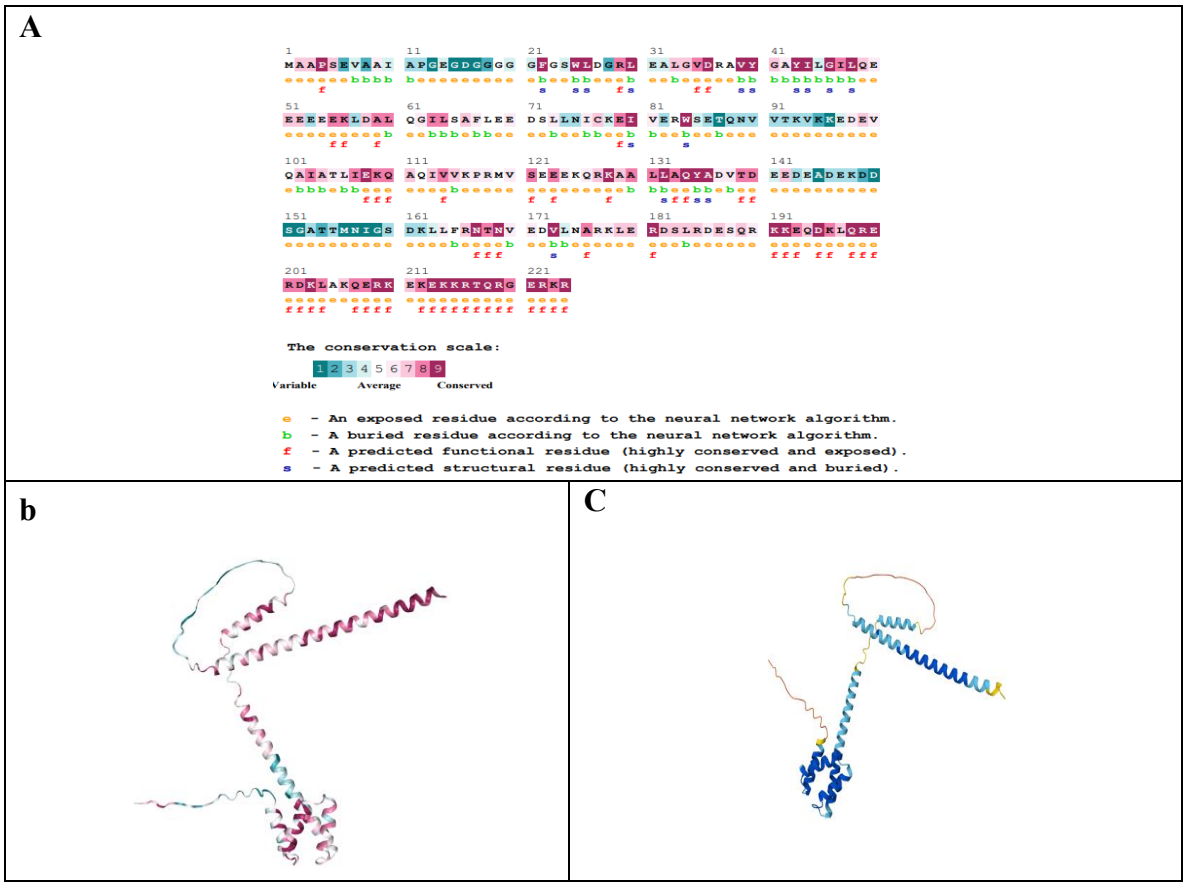

Fig 4. Consurf analysis of CCDC43 protein.
a. The evolutionary conservation colouring grades of the aminoacids are presented with the color scheme.
b. The corresponding evolutionary colored secondary structure of CCDC43.
c. Alphafold structural model of human CCDC43.

ConSurf shows the distribution of functional and structural residues of the modelled 3D structure of proteins and is a database that calculates evolutionary conservation levels for each amino acid, and accordingly the functional and structural residues of CCDC43 predicted by ConSurf are shown in Figure 4a. ConSurf results provide us with information for the identification of functionally important regions in proteins by surface mapping the level of evolutionary conservation of each amino acid region. The overlap of the evolutionary conserved residues of CCDC43 with the helix regions shown in Figure 4b and 4c, and the variable evolutionary nature of the flexible loop regions, indicate that this protein is evolutionarily conserved and phase separation compatible, similar to other CCDC family members.

Cells respond to stress conditions by activating defense mechanisms to prevent possible damage to gene expression and to activate apoptosis when necessary. The resulting SGs are involved in eukaryotic gene expression regulation and harbor a variety of RNA-binding proteins and mRNAs. A growing interest in recent years in understanding the dynamism, regulation, and functional mechanism of SGs and the mRNA decay cycle.

A stress granule is a type of RNP granule formed in eukaryotic cells in response to stress [21]. Ras-GTPase-activating protein (SH3 domain)-binding proteins, G3BP1 and G3BP2, are the undisputed core proteins of RNA-binding stress granules (SGs). Antibodies directed against these proteins and antibodies against TIA1 protein are used as markers for SG staining [22]. The number of SG-associated proteins is increasing in parallel with the researches conducted in this field. To date, CCDC43 has not been identified among the proteins interacting with SG-related proteins. Considering the highly dynamic nature of stress granules and the fact that cells form granules that differ in content other than certain basic proteins depending on changing stress conditions, it is clear that there are proteins involved in stress granules that have not yet been fully identified. Although overexpression of GFP-tagged CCDC43 in this study was not seen in stress granules, we suggest that this protein is associated with different phase-separating cellular structures. On the other hand, stress responses in cells are multifaceted and consist of a series of proteins and RNA molecules that activate stress granules in a dynamic process of association-dissociation [23, 24]. It is possible that CCDC43 proteins are involved in

different cellular pathways in a context-dependent manner.
Analysis of amino acid residue conservation in the CCDC43 protein structure points to its evolutionary significance. According to the Consurf neural network algorithm, high conservation degrees are numbered with degree 9 (dark purple). This server is a useful tool for calculating the probability of identifying key structural and functional residues.

Therefore, it can be assumed that the C terminal of CCDC43 is evolutionarily stable. In contrast to this part of CCDC43, the flexible loops corresponding to N and the middle part of the protein show a low rate of conservation. Therefore, due to the lack of a fixed structure and their conformational variability, it can be predicted that they may form multivalent interactions for phase separation. Proteins are probably conformationally heterogeneous in their native state and can change their biological activity under cellular conditions. They can trigger the formation of different phases in the cell due to ordered and disordered regions in their structure. The formation of biomolecular condensates by LLPS is essential in the cell, especially for the formation of membraneless organelles. The potential of proteins to form LLPS promotes their association with these dynamic structures. FuzDrop analysis of CCDC43 suggests that this protein may be involved in such dynamic phases.

Overall, this study has provided information for further studies to find the interaction partners of CCDC43 and for its detailed functional-structural characterization. Although the specific role of CCDC43 is not clearly understood, it is thought that it may modulate the expression of the genes as it is involved in the regulation of several types of cancer. Understanding the cellular pathways in which such a conserved and disease-associated protein plays an active role and how it reacts under different conditions will guide our future studies.

4. Conclusion

In conclusion, we have shown that CCDC43 can be a potential droplet-forming protein with its structural liquid-liquid phase separation propensity in cells. These results provide the first evidence for CCDC43 structural cellular properties and its behavior under stress conditions.

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Author's Contributions

Merve Tuzlakoğlu Öztürk: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ethics

There are no ethical issues after the publication of this manuscript.

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