Phase transition of fibrillarin LC domain regulates localization and protein interaction of fibrillarin

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Abstract
A key nucleolar protein, fibrillarin, has emerged as an important pharmacological target as its aberrant expression and localization are related to tumorigenesis, chemoresistance and poor survival in breast cancer patients. Fibrillarin contains a N-terminal low complexity sequence (LC) domain with a skewed amino acid distribution, which is known to undergo a phase transition to liquid-like droplets. However, the underlying mechanism of the phase transition of the fibrillarin LC domain and its physiological function are still elusive. In this study, we show that the localization of fibrillarin and its association with RNA binding proteins is regulated by this phase transition. Phenylalanine-to-serine substitutions of the phenylalanine-glycine repeats in the fibrillarin LC domain impede its phase transition into liquid-like droplets, as well as the hydrogel-like state composed of polymers, and also its incorporation into hydrogel or liquid-like droplets composed of wild-type LC domains. When expressed in cultured cells, fibrillarin containing the mutant LC domain fails to localize to the dense fibrillar component of nucleoli in the same way as intact fibrillarin. Moreover, the phase transition of the fibrillarin LC domain is required for the interaction of fibrillarin with other RNA binding proteins, such as FUS, TAF15, DDX5 and DHX9. Taken together, the results suggest that the phenylalanine residues in the LC domain are critical for the phase transition of fibrillarin, which in turn regulates the sub-nucleolar localization of fibrillarin and its interaction with RNA binding proteins, providing a useful framework for regulating the function of fibrillarin.

Keywords; fibrillarin, LC domain, phase transition, nucleolus, RNA binding proteins

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Introduction

The nucleolus is the largest non-membrane-bounded organelle in the nucleus and is a factory for the biosynthesis of ribosomes. Ribosomal RNAs (rRNAs) are transcribed, processed and assembled with ribosomal proteins to comprise 60S and 40S ribosomal subunits in the nucleolus [1-3]. The complex processes that are involved in the formation of the ribosome subunits occur in distinct sub-compartments of the nucleolus, the fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC). Nucleolus also exerts various non-ribosomal functions including regulation of mitosis, cell-cycle progression and proliferation, post-translational modifications of the proteins involved in the cell cycle, and responses to cellular stress [4-6]. Many of the functions are associated with fibrillarin, an essential protein present in the nucleolus. Fibrillarin is known to be mainly located in the DFC region. As a core component of snoRNPs (small nucleolar ribonucleoproteins), the primary function of fibrillarin is methylation and processing of pre-rRNA during ribosomal biogenesis [7]. Fibrillarin also mediates the methylation of histone H2A, which is required for the transcription of ribosomal DNA [8]. In addition to its major functions in the biogenesis of different RNAs and ribosomal subunits, a growing body of evidence has revealed that fibrillarin is involved in the dynamics of nucleolar proteins, which are tightly linked with the stages of cell cycle, the metabolic condition, and stress stimuli [9-11]. Alterations in the expression and localization of fibrillarin are related to cancer, as well as other human diseases [11-13]. Aberrant fibrillarin expression contributes to the decreased p53 accumulation, tumorigenesis, chemoresistance, and is associated with poor survival in patients with breast cancer, indicating the potential application of fibrillarin as a therapeutic target [12, 14]. Thus, understanding the regulatory mechanisms for nucleolar localization and the activity of fibrillarin is important for both basic and translational research.

Human fibrillarin consists of 321 amino acids and two major structural domains: the N-terminal low complexity (LC) domain and the C-terminal methyltransferase domain [15]. The LC domain of fibrillarin has a highly skewed distribution of amino acids. Of the 76 residues within the LC domain, 88% are comprised of only three amino acids: glycine, arginine, and phenylalanine, corresponding to the LC or intrinsically disordered domains. The LC domains in RNA binding proteins can undergo phase transition into liquid-like droplets (LLDs) or amyloid-like reversible polymers [16-20]. The phase transition of the LC domain-containing proteins may be involved in facilitating protein-protein and protein-RNA interactions, contributing to a variety of cellular processes, including the formation of membraneless organelles, signaling complexes, the cytoskeleton, and numerous other supramolecular assemblies [21]. In recent studies, it has been reported that fibrillarin can phase separate into LLDs, which mediate the subcompartamentalized organization of nucleoli [15, 22]. The surface tension of fibrillarin LLDs is a key parameter that enables the multi-layered liquid characteristics of nucleoli [15]. Nascent pre-rRNA binding to the phase separated fibrillarin is shown to promote assembly of the DFC [22]. Despite these studies, whether phase separation contributes to the specific function of
fibrillarin is still unclear. Although phase separation has been observed and phase behavior characterized for many proteins both in vitro and in vivo, the molecular code that drives phase separation often remains enigmatic [23]. This has been a major barrier to understanding the role of phase separation in the physiological functions of fibrillarin in the context of living cells.

In the present study, we first uncovered the sequence determinants for the phase transition of the fibrillarin LC domain. *In vitro* assays showed that the fibrillarin LC domain can undergo phase transition into LLDs, as well as the hydrogel-like state composed of polymers. By interrogating the phenylalanine residues within the fibrillarin LC domain, we demonstrated that the F18/40-to-serine (S) substitution is the minimal variant required for interrupting not only the phase transition, but also partitioning into the existing LLDs or hydrogel droplets of the LC domain. Fibrillarin containing the F-to-S mutation in the LC domain also failed to localize in the DFC regions of the nucleoli in the same way as intact fibrillarin. Moreover, the phase transition of the fibrillarin LC domain is required for the interaction of fibrillarin with other RNA binding proteins such as FUS, TAF15, DDX5 and DHX9. Taken together, we can conclude that the phase transition of the fibrillarin LC domain is required for proper nucleolus localization of fibrillarin and its binding to different kinds of regulatory proteins.
Materials and Methods

Materials

The following primary antibodies were used for Western blotting, immunoprecipitation and cell imaging: anti-Flag M2 (F3165, Sigma-Aldrich, Germany), anti-fibrillarin (SC-25397, Santa Cruz, USA), anti-nucleophosmin (ab10530, Abcam, USA), anti-FUS (A300-294, Bethyl laboratories, USA), anti-TAF15 (NB100-566, Novus Biologicals, USA), anti-DDX5 (9877, Cell Signaling Technology, USA) and anti-DHX9 (PA5-19542, Thermo Fisher Scientific, USA).

Cell culture and transfection

HeLa and HEK-293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (PS, Thermo Fisher Scientific, USA). Cells were grown at 37°C and supplemented with 5% CO2. For transient transfection, Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA) was used according to the manufacturer’s protocol.

Cloning

To generate plasmids for the bacterial expression of the fibrillarin LC domain, the DNA fragment encoding residues 2-77 of fibrillarin was amplified by PCR from the cDNA library obtained from HEK-293T cells as a template and subcloned into pHis-mCherry or pHis-GFP parallel vectors to add N-terminal mCherry or GFP tags, respectively [24]. For cloning of the LC domain of nucleolin, the DNA fragment encoding residues 661-698 was amplified and subcloned into the pHis-GFP parallel vector. Bacterial expression plasmids for the LC domains of FUS (residues 2-214), TAF15 (residues 2-208), DDX5 (residues 2-167), DHX9 (residues 1151-1270) and hnRNPA2 (residues 181-341) were generated as described in previously published studies [16, 18]. For generation of the mammalian expression plasmids, DNA fragments encoding wild-type, F18/40S, FallS or ΔLC fibrillarin were amplified and then subcloned into pDsRed-N1 (C-terminal tag, confocal microscopy) or pCMV10-3XFlag (N-terminal tag, immunoprecipitation) vectors.

Protein purification

The recombinant proteins were purified using the method described in previously published studies [16, 18]. E. coli BL21 (DE3) cells were transformed with the bacterial expression plasmids and the transformed bacterial cells were spread onto Luria broth (LB) agar plates containing 100 μg ampicillin. A single colony was inoculated in 30 ml of LB^Amp media and stored in 37°C without shaking. After overnight incubation, 5 ml of the precultured bacterial cells were transferred into 1 L of LB^Amp media and grown until an OD600 of 0.7-1.0 was reached. To induce protein expression, 0.5 mM IPTG was added to the bacterial culture and the cells were incubated at 18°C overnight. After centrifugation, the cell pellets were resuspended in lysis buffer containing 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM β-mercaptoethanol (BME), 1%
Triton X-100, protease inhibitor cocktail (Sigma-Aldrich, Germany) and 0.4 mg/ml lysozyme. After incubating on ice for 30 min, the cells were sonicated at 60% power for 3 min (Model 705 Sonic Dismembrator, Fisher, USA) to lyse the bacterial cells. After centrifugation at 20,000 rpm for 1 h at 4°C, the supernatants were mixed with Ni-NTA resin (Qiagen, Germany) by gentle rocking at 4°C for 30 min. The Ni-NTA resin was packed in a glass column (Bio-Rad, USA) and then washed with 300 ml of wash buffer containing 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole, 20 mM BME and 0.1 mM PMSF. The bound proteins were eluted from the resin using elution buffer containing 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 200 mM imidazole, 20 mM BME and 0.1 mM PMSF. The purified recombinant proteins were stored at -80°C until further use.

**Liquid-like droplet assays**

To make the LLDs, the purified fibrillarin LC domain proteins linked to mCherry were dialyzed in gelation buffer containing 20 mM Tris-HCl pH 8.5, 200 mM NaCl, 20 mM BME and 0.1 mM PMSF overnight at room temperature. The dialyzed proteins were diluted to the indicated concentration in gelation buffer without NaCl to obtain the indicated salt concentration. Tev protease (Promega, USA) was added to the reaction mixtures to cleave off the N-terminal His-tags. The samples were transferred to a glass-bottomed 96 well plate pre-coated with 3% BSA (Perkin Elmer, USA). After 30 min of incubation at room temperature, the LLDs were visualized using light (DMi8, Leica, Germany) or confocal microscopy (LSM510, Zeiss, Germany). For the liquid-like droplet (LLD) incorporation assays, GFP-linked fibrillarin LC domain proteins (0.2 µM) were added to the LLD formation mixtures containing 5 or 2 µM of mCherry:FBL-LC. The intensity of the incorporated GFP proteins was analyzed using confocal microscopy (LSM510, Zeiss, Germany).

**Hydrogel formation**

Hydrogel droplets were generated as described in previously published studies [16, 18]. The mCherry-linked LC domains of fibrillarin, FUS, TAF15 or DHX9 were dialyzed in gelation buffer overnight at room temperature. Upon brief sonication (1 s, three times, Model 705 Sonic Dismembrator, Fisher, USA) and centrifugation (3 min at maximum speed) to remove the precipitates, the dialyzed proteins were concentrated to approximately 70-100 mg/ml using Amicon Ultra (Merck Millipore, USA). Small droplets (0.4 µl) of the protein solution were made onto the glass-bottomed 35 mm confocal dishes (SPL, Korea) and these were then incubated for 7-8 d at room temperature until gelation occurred. For the hydrogel binding assays, GFP-linked soluble proteins at a concentration of 3 µM were added to the hydrogel droplets and stored overnight at 4°C. The intensity of the trapped GFP-linked proteins was assessed using confocal microscopy.
siRNA-mediated knockdown of fibrillarin

Scrambled siRNA and siRNA targeting 3’ UTR of fibrillarin (Sense, 5’-CGAGAGATGTGTGTTGATATT-3’; antisense, 5’-UAUCAACACACAUCUCGCA-3’) were purchased from GenePharma. To validate the knock down of fibrillarin, HeLa cells were transfected with the indicated amounts of siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. Cells were lysed at 24, 48, 72, and 96 h after siRNA transfection and the lysates were analyzed using Western blotting. For confocal microscopy images of fibrillarin in Figure 4, HeLa cells were transfected with plasmid constructs encoding wild-type or mutant fibrillarin at 24 h after siRNA (10 μM) transfection.

Cell imaging

HeLa cells expressing dsRed-linked fibrillarin constructs were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. Upon permeabilization using 0.5% Triton X-100 in PBS (PBS-T), the cells were incubated overnight with anti-nucleophosmin antibodies at 4°C. The cells were then washed with 0.1% PBS-T and incubated with Alexa Fluor 488-conjugated secondary antibodies for 1 h at room temperature. The cells were analyzed using confocal microscopy.

Immunoprecipitation and Western blotting

HEK-293T cells were transfected with plasmid constructs of wild-type, F18/40S, FallS or ΔLC fibrillarin genes with N-terminal Flag tags. Following incubation for 48 h at 37°C, the transfected cells were collected and lysed by sonication in 500 μl of IP buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40 and 1 mM EDTA and a protease inhibitor cocktail (Thermo Fisher Scientific, USA). After centrifugation at 4°C for 10 min at maximum speed, the supernatants were collected and incubated with 2 μg of anti-Flag antibodies. Upon overnight rotation at 4°C, the immune complexes were supplemented with 100 μl of the protein G magnetic beads (Bio-Rad, USA) for 2 h and then washed three times with ice-cold PBS. The bound proteins were eluted with 2X SDS sample buffer by boiling at 95°C for 10 min. For Western blotting, the eluted samples were separated using 10% SDS polyacrylamide gels and the separated proteins were transferred onto the nitrocellulose membrane and detected using the indicated antibodies.
Results

Phase transition of the N-terminal LC domain of fibrillarin

To study the role of the N-terminal LC domain and its phase transition in the nucleolar distribution of fibrillarin, full-length or N-terminally deleted fibrillarin were first expressed in the HeLa cells. As shown in Figure 1A, the full-length fibrillarin with C-terminal dsRed tag (FBL-FL:dsRed) was evenly localized in the nucleolar DFC and FC regions that are surrounded by the GC region in which nucleophosmin is predominantly localized. However, the localization of fibrillarin that was deleted from the N-terminal LC domain (FBL-ΔLC:dsRed) was completely different from the full-length protein. Although still located inside the nucleoli, FBL-ΔLC:dsRed exhibited a dot-like expression pattern (Figure 1B). Knowing that the LC domain is required for proper nucleolar distribution of fibrillarin, we next validated the phase transition of the fibrillarin LC domain using the recombinant protein of the N-terminal LC domain (amino acids 2-77) of fibrillarin linked to the N-terminal mCherry fluorescent protein (mCherry:FBL). The solution of 5 μM of mCherry:FBL with 100 mM of NaCl was clear at room temperature and when observed under light microscopy, only a small amount of aggregated particles were observed on the surface of the glass slide (Figure 1C, left panel). Upon dilution of the NaCl concentration to 50 or 25 mM, the mCherry:FBL solution became turbid and the formation of LLDs with a spherical structure was observed by light microscopy (Figure 1C, middle and right panels). A reverse correlation was observed between the NaCl concentration and the size of the observed LLDs. These results are in agreement with the results of previous research on the phase transition of fibrillarin in vitro[15, 22].

Previously, it has been reported that the LC domains of a variety of RNA binding proteins, including FUS, TAF15, and hnRNPA2, can undergo phase transition to become hydrogel droplets that are composed of reversible amyloid-like polymers [16, 18, 20, 25]. Upon increasing the concentration up to 100 mg/ml and incubation at room temperature, the pure solution of the mCherry:FBL LC domain recombinant protein also became hydrogel droplets composed of a bunch of polymers that were visible using fluorescent microscopy (Supplementary Figure S2E). It was also reported in previous studies that the labile amyloid-like polymers and hydrogel droplets of different kinds of LC domains are specifically melted by the aliphatic alcohol, 1,6-hexanediol (HD), but not by the other, 2,5-HD[26]. The mCherry:FBL LC hydrogel droplets were also melted by 1,6-HD, but not by 2,5-HD, suggesting that the hydrogel droplets composed of the fibrillarin LC domain are composed of labile, amyloid-like polymers (Figure 1D).

Phenylalanine residues are critical to the phase transition of the fibrillarin LC domain

Many LC domains that undergo phase transition often contain repeats of aromatic amino acids, tyrosine and/or phenylalanine residues. In previous studies, it was revealed that repetitive tyrosine or phenylalanine residues are critical to the phase transition and function of LC
domains [16, 18, 20, 27]. Tyrosine-to-serine (Y-to-S) substitutions in the LC domain of FUS or TAF15 effectively disrupt the formation of the amyloid-like polymers, as well as the transcriptional activity of the LC domains [18]. In the case of hnRNPA2, a single phenylalanine-to-serine (F-to-S) substitution was sufficient to interrupt the incorporation of the LC domain to the pre-existing liquid-like droplets or hydrogel droplets [20]. The LC domain of fibrillarin contains six repeats of the FG (phenylalanine/glycine) motifs (Supplementary Figure S1A). To identify the phenylalanine residues responsible for the phase transition of the fibrillarin LC domain, six mutant LC domains with a single F-to-S (F5S, F12S, F18S, F30S, F40S, and F64S) substitution, two with a double F-to-S (F18/40S and F30/64S) substitution, two with a triple F-to-S (F5/18/40S and F12/30/64S) substitution and one in which all six phenylalanine residues were substituted to serine residues (FallS) were generated. Once recombinantly produced in the form of the fusion protein with the N-terminal GFP, wild-type or mutant LC domains were applied to the chamber slides containing hydrogel droplets composed of the mCherry-linked fibrillarin LC domain. After overnight incubation, the hydrogel trapping of the wild-type or mutant GFP-tagged LC domains of fibrillarin was analyzed using confocal microscopy. As shown in Figure 2A and B, the intensity of the hydrogel binding was decreased in all mutant LC domains when compared to the wild-type, and in particular, LC domains with F18/40S, F5/18/40S, or FallS substitutions exhibited the lowest trapping intensity, suggesting that residues F18 and F40 are critical to trapping by the hydrogel droplets. We then tested the incorporation of two mutant LC domains, F18/40S and FallS, into the LLDs composed of the wild-type fibrillarin LC domain. For this, LLD formation samples containing 5 or 2 μM of the mCherry-linked fibrillarin LC domain at 50 or 25 mM NaCl, respectively, were prepared in test tubes (Figures 2C and D). GFP-only or wild-type, F18/40S or FallS LC domains of fibrillarin linked to GFP (0.2 μM) were added to the LLD samples and the mixtures were then incubated in the chamber slides. Using confocal microscopy, as in the hydrogel binding assay, the robust incorporation of the GFP-linked wild-type LC domain into the mCherry LLDs was observed and the incorporation intensity was drastically decreased for the GFP-linked LC domains harboring F18/40S or FallS mutations (Figures 2C and D). No LLD incorporation was observed for the GFP-only (Figures 2C and D, top panels). We also confirmed that all the GFP-linked proteins tested possess the same ground-level fluorescent properties and purity (Supplementary Figures S1B and C).

Next, to test the effect of the F-to-S substitutions on the phase transition of the fibrillarin LC domain, the mCherry-linked wild-type LC domain of fibrillarin at varying concentrations ranging from 1 to 20 μM was incubated in a buffer containing 100, 50, 25, or 12.5 mM NaCl. LLD formation was observed using microscopy at all protein concentrations (1 to 20 μM) in the buffer containing 12.5 mM NaCl, 2 to 20 μM of proteins at 25 mM NaCl, 5 to 20 μM of proteins at 50 mM NaCl, and only 100 μM protein at 100 mM NaCl (Figures 3A, C and Supplementary Figure S2A). In all cases, the lower the NaCl concentration and the higher the
protein concentration, the larger the size of the LLDs. In samples with 10 μM protein in 12.5 mM NaCl and 20 μM protein in 12.5, 25, and 50 mM NaCl, LLD formation was very robust so that the bottom of the chamber slides was completely covered with droplets within 20-30 min of assembling the droplet samples (Figure 3A and Supplementary Figure S2A). Although still capable of making LLDs, the mCherry-linked LC domain with the F18/40S mutation failed to form droplets at proteins concentrations of 1, 2, 5 or 20 μM in buffers containing 12.5, 25, 50 or 100 mM NaCl, respectively (Figures 3B and C and Supplementary Figure S2B). In addition, under all conditions, the droplets formed by the F18/40S LC domain were smaller in size than the ones made by the wild-type LC domain. No droplets were assembled by the LC domain containing the FallS mutation. As shown in Supplementary Figure S2C and D, the wild-type LC domain formed LLDs at protein concentrations of 5 to 20 μM under a physiological salt concentration (150 mM NaCl) in the presence of 5% polyethylene glycol (PEG), while the F18/40S mutant LC domain formed smaller droplets at higher protein concentrations (10 to 20 μM). When subjected to hydrogel or polymer formation, mCherry-linked fibrillarin LC domains with F18/40S or the FallS mutation failed to make polymers, as well as hydrogel droplets, as did the wild-type LC domain (Supplementary Figures S2E and F). These results suggest that the two phenylalanine residues, F18 and F40, in the LC domain are critical for the phase transition and also for partitioning of the LLDs or hydrogel droplets.

**Role of the LC domain phase transition in nucleolar localization of fibrillarin**

We then investigated whether the F-to-S mutation that interrupted the phase transition of the LC domain also has an effect on the nucleolar localization of fibrillarin. To test this, full-length fibrillarin containing F18/40S or FallS LC domains with C-terminal dsRed tags were expressed in HeLa cells. Unexpectedly, fibrillarin containing mutant LC domains was well-localized in the nucleolar DFC regions as wild-type fibrillarin (Supplementary Figure S3). This might be because the mutant LC domains (F18/40S or FallS) are still trapped by the existing phase-separated structure in the nucleoli formed by the endogenous fibrillarin proteins. To test this possibility, HeLa cells were transfected with wild-type or mutant fibrillarin constructs with C-terminal dsRed tags in the presence or absence of the endogenously expressed fibrillarin. Regardless of the presence or absence of endogenous fibrillarin, fibrillarin without a LC domain (ΔLC) were localized to dot-like puncta in the nucleoli (Figures 4A and B, 2nd rows). As shown in Figure 4A, we again observed an almost normal nucleolar distribution of the fibrillarin containing F18/40S or FallS LC domains when they were expressed in cells that had been pre-transfected with scrambled siRNA. However, when the expression of endogenous fibrillarin was suppressed by the siRNA-mediated knock down (fibrillarin siRNA, Supplementary Figure S4), an aberrant, dot-like expression pattern was observed for the exogenously expressed fibrillarin with mutant LC domains (Figure 4B). Thus, these results suggest that the phase transition of the LC domain is critical for proper nucleolar distribution of fibrillarin.
Phase transition is required for the interaction of fibrillarin LC domains with RNA binding proteins

Having observed that the phase transition of the LC domain is required for nucleolar distribution of fibrillarin, we next tested whether the phase transition is also critical for fibrillarin to interact with other proteins. To test this, trapping of the GFP-linked LC domains from different kinds of RNA regulatory proteins was first analyzed using mCherry hydrogel droplets composed of the fibrillarin LC domain. As shown in Figure 5A, strong binding of the N-terminal LC domain of FUS was observed and the LC domains of TAF15, DHX9 or nucleolin were trapped at a moderate intensity by the hydrogel droplets. The LC domains of DDX5 or hnRNPA2 were only weakly bound to the hydrogel droplets (Figure 5A). Next, the trapping of the GFP-linked wild-type or mutant LC domains of fibrillarin by hydrogel droplets composed of FUS, TAF15, DDX5 or DHX9 LC domains fused to mCherry was analyzed (Figure 5B). Although the wild-type fibrillarin LC domain was readily trapped by all of the hydrogel droplets challenged, the hydrogel binding intensity was decreased for LC domains harboring F18/40S or FallS mutation (Figure 5B).

Although, the mutations that interrupted the phase transition of the LC domain also interfered with the interaction between the LC domains of fibrillarin and other RNA regulatory proteins in vitro, it was still unclear whether the phase transition capability of the LC domain is required for fibrillarin to interact with other proteins in living cells. To test this, Flag-tagged wild-type, F18/40S, FallS or ΔLC fibrillarin proteins were subjected to the immunoprecipitation assay in HEK-293T cells. As shown in Figure 5C, the interaction between the exogenously expressed wild-type fibrillarin and the RNA regulatory proteins including FUS, TAF15, DDX5 or DHX9 was observed. However, these interactions were disrupted by F18/40S, FallS or ΔLC mutations, indicating that the phase transition of fibrillarin is critical for the fibrillarin to interact with different kinds of RNA dependent regulatory proteins in living cells.
Discussion

Although phase transition has been observed for many proteins, the molecular code that drives the phase separation often remains enigmatic [23]. This has been a major barrier for understanding the role of phase transition in the physiological function of proteins. In the present study, we uncovered the sequence determinants for fibrillarin phase properties in order to study the roles of LC domain phase transition in fibrillarin function (Figure 2). Previous studies have shown that the LC domain of phase-separating proteins often contains repeats of aromatic amino acids, tyrosine or phenylalanine [16-18, 20]. The tyrosine residues are usually flanked by either glycine or serine residues (Y-motifs) while phenylalanine residues have a glycine residue at their amino or carboxyl sides (FG motifs). For the RNA binding proteins including FUS, TAF15, and heterogeneous nuclear ribonucleoprotein A2 (hnRNPA2), these repetitive tyrosine and phenylalanine residues are critical to phase separation or recruitment into the phase separated structures [16, 18, 20]. Fibrillarin possesses six repeats of the FG motifs in its LC domain. To determine if the phenylalanine residues in the fibrillarin LC domain are also critical for the phase transition, we generated mutant LC domains harboring one or more of the F-to-S substitutions. As shown in Figure 2, substitution of both F18 and F40 with serine residues was sufficient to interfere with the incorporation of the fibrillarin LC domain into both LLDs and hydrogel droplets. Likewise, the capability of phase separation was reduced by F18/40S and completely abolished by all F-to-S mutations (Figure 3 and supplementary Figure S2). Thus, our findings indicate that the phenylalanine residues in the LC domain are critical to the phase transition of fibrillarin.

One of the main concerns in the present study is whether the phase transition is important for proper nucleolar localization of fibrillarin. Without the N-terminal region, fibrillarin cannot localize to the DFC of the nucleoli, suggesting that the LC domain is required for the normal nucleolar distribution of fibrillarin (Figure 1). To verify the role of phase transition in fibrillarin localization, we introduced mutant fibrillarin with F-to-S substitutions into cells to replace the endogenous protein. When dsRed-linked wild-type, F18/40S or FallS fibrillarin constructs were expressed in HeLa cells pre-suppressed with the expression of the endogenous fibrillarin, F18/40S and FallS fibrillarin showed aberrant, dot-like expression patterns, while wild-type fibrillarin was evenly localized in the nucleolar DFC regions (Figure 4B). The correlating effects of the mutations of the FG repeats on the phase transition in vitro and the localization to nucleolar DFC in HeLa cells suggest that the localization of fibrillarin may be driven primarily by the phase transition of the LC domain. When F18/40S and FallS fibrillarin were expressed in HeLa cells without knockdown of the endogenous fibrillarin, however, they can associate with the nucleolar DFC regions as observed in Figure 4A and Supplementary Figure S3. The F18/40S and FallS mutants of the fibrillarin LC domain lack the capability to induce phase transition (Figure 3 and Supplementary Figure S2), but partially retain their ability to bind to either the LLDs or hydrogel droplets composed of wild-type LC domains (Figure 2).
Thus, it is plausible that exogenously expressed mutant fibrillarin proteins may be trapped by the pre-existing phase-separated structure formed by the endogenous fibrillarin within the nucleolar DFC region in cells. These observations are consistent with previous published research showing that the FUS mutants that lack the capability to form hydrogels partially retain their ability to bind to the wild-type FUS LC hydrogel in vitro and to stress granules in living cells [16].

In addition, phase transition appears to be critical to the interaction of fibrillarin with different kinds of RNA binding proteins (Figure 5). Using a hydrogel binding assay, we demonstrated that the LC domain of fibrillarin and RNA binding proteins including FUS, TAF15, DDX5 and DHX9 recruited each other, while the phase separation mutants (F18/40S or FallS) of fibrillarin showed a reduced binding affinity to the hydrogel droplets of the LC domain for those RNA binding proteins. Consistent with the results from in vitro experiments, pull-down studies performed in living cells showed that wild-type fibrillarin interacted with FUS, TAF15, DDX5 or DHX9, while the phase separation mutants (F18/40S or FallS) did not (Figure 5C). The results indicate that the ability of fibrillarin to interact with FUS, TAF15, DDX5 or DHX9 correlates with its phase separation capability. The nucleolus is a dynamic structure and its composition can vary dramatically under different cellular conditions [4]. Phase transition is one of the proposed mechanisms for the dynamic movement of RNA-binding proteins in and out of the nucleolus [21]. Liquid-like droplets show selective properties, admitting some proteins and RNAs and excluding others. However, there is a significant lack of information available about the role of phase separation in protein-protein interactions in the nucleolus. Our results suggest that the phase transition of the fibrillarin LC domain can control the organization of the nucleolar composition by recruiting specific RNA binding proteins. Whereas the phase-separated structures of the LC domain can trap specific molecules, it is not known how these proteins are released from fibrillarin in the nucleoli. In a previous study, we demonstrated that the binding of RNA Polymerase II to the FET (FUS/EWS/TAF15) protein LC domains is regulated in a phosphorylation-dependent manner, indicating posttranslational modification as a means of controlling the dynamic behavior of the LC derived phase-separated structures [18]. Moreover, the SR (serine-arginine rich) splicing factors first enter the nucleoli in a hypophosphorylated state, and then re-localize to nuclear speckles upon phosphorylation by CDC2-like kinases (CLKs)[28]. Thus, it is plausible that posttranslational modification of the LC domains in the RNA binding proteins might trigger their release from the phase-separated structures of fibrillarin.

Many of the proteins currently used as models for phase separation are linked to membraneless organelles such as nucleoli, Cajal bodies, and stress granules [29]. hnRNPA1 and FUS are the most extensively studied. The RNA binding proteins, hnRNPA1 and FUS, assemble into stress granules via a phase-separation mediated process [16, 30]. In hnRNPA1 and FUS, the aromatic residues phenylalanine and tyrosine in the LC domain contribute to the phase separation [16, 30]. Further characterization reveals that the phase separation of hnRNPA1 and
FUS can be tuned using the environmental conditions; in particular, a lower salt concentration, molecular crowding, and the interaction with RNA promote the phase separation [16, 31]. We observed that the phase separation of fibrillarin is dependent on the phenylalanine residues of the LC domain and environmental factors such as the salt concentration and molecular crowding. This indicates that the features of the sequence and the physical properties we have identified for fibrillarin phase separation may have more general implications for the phase separation of membraneless organelle proteins.

The results presented here provide the first direct evidence of the functional role of phase separation in the control of subnucleolar localization and protein interactions of fibrillarin. Fibrillarin plays a key role in the biogenesis of ribosomes, thus, any changes in the expression or localization of fibrillarin can lead to cell abnormalities often connected with cancer [12]. Hence, a mechanistic understanding of fibrillarin regulation may elucidate the role of fibrillarin in health and disease, contributing to novel treatments for diseases, such as cancer.
Data Availability Statement
All data required to evaluate the work are included in the manuscript and are available on request. The sequence of Fibrillarin gene is deposited in GenBank with accession number NM_001436.3.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations
LC domain, low complexity domain; FBL, fibrillarin; FC, fibrillar center; DFC, dense fibrillar component; GC, granular component; LLD, liquid-like droplet; FUS, Fused in sarcoma; TAF15, TATA-box binding protein associated factor 15; DDX5, DEAD-box helicase 5; DHX9, DExH-box helicase 9; NCL, nucleolin; hnRNP A2, heterogeneous nuclear ribonucleoprotein A2
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**Figure Legends**

**Figure 1. Phase transition of N-terminal LC domain of fibrillarin.** (A and B) HeLa cells were transfected with dsRed-linked full-length, wild-type fibrillarin (A, FBL:dsRed-WT) or mutant fibrillarin lacking the N-terminal LC domain (B, FBL:dsRed-ΔLC). Upon fixation and staining with anti-nucleophosmin antibodies, localization of dsRed-linked fibrillarin (red signal) or nucleophosmin (green signal) was visualized using confocal microscopy. Nuclei were detected by DAPI staining. (C) Liquid-like droplet (LLD) formation of N-terminal LC domain of fibrillarin. Assembled LLDs were observed using light microscopy. Scale bar, 20 μm. (D) Hydrogel droplets composed of mCherry-linked LC domain of fibrillarin (mCherry:FBL) were incubated with 20% levels of 1,6- or 2,5-hexanediols (HD) for indicated time periods. The fluorescence intensity of hydrogel droplets was analyzed by confocal microscopy at indicated time points upon addition of the HDs.

**Figure 2. Hydrogel or LLD incorporation of wild-type or mutant fibrillarin LC domains.** (A) Recombinant proteins of GFP-linked fibrillarin LC domains (GFP:FBL) harboring varying numbers of F-to-S substitution were applied to the chamber slides containing hydrogel droplets composed of wild-type fibrillarin LC domain (mCherry:FBL). After overnight incubation at 4°C, the intensity of the trapped GFP-linked proteins was assessed by confocal microscopy. (B) Confocal images shown in panel a were scanned to yield the signal intensity of bound GFP. The x axis indicates the scanned distance in μm, and the y axis indicates the GFP signal intensity in arbitrary units. (C and D) Recombinant proteins of GFP-only or GFP-linked wild-type, F18/40S or FallS LC domains of fibrillarin at 0.2 μM were added to the LLD assembly mixtures containing 5 μM (C) or 2 μM (D) wild-type fibrillarin LC domains fused to mCherry. Upon transfer to the chamber slides with glass bottom and incubation for 20 min at room temperature, incorporation of the GFP proteins to the mCherry LLDs were analyzed by confocal microscopy. Scale bar, 20 μm.

**Figure 3. Liquid-like droplet formation of wild-type or mutant LC domain of fibrillarin.** (A and B) Wild-type (A) or F18/40S (B) LC domains of fibrillarin linked to mCherry fluorescent protein were diluted to varying concentrations ranging from 1 to 20 μM in buffer containing 12.5, 25, 50 or 100 mM NaCl. Upon incubation at room temperature for 30 min, the red signals of assembled LLDs were observed by confocal microscopy. Scale bar, 50 μm. (C) Phase diagram of wild-type or F18/40S LC domains in (A) and (B). Black closed circle indicates wild-type LLD formation; black open circle indicates wild-type no LLD formation; red closed circle indicates F18/40S LLD formation; red open circle indicates F18/40S no LLD formation.
**Figure 4. Nucleolar localization of wild-type or mutant fibrillarin. (A and B)** Plasmid constructs expressing wild-type, ΔLC, F18/40S or FallS fibrillarin with C-terminal dsRed tags were transfected into the HeLa cells in the presence (A) or absence (B) of the endogenously expressed Fibrillarin. Cells were fixed and stained using anti-nucleophosmin and DAPI to visualize nucleoli and nuclei, respectively. Subcellular localization of exogenously expressed fibrillarin (red signals) and nucleophosmin (green signals) were visualized by confocal microscopy.

**Figure 5. Interaction between LC domains of fibrillarin and RNA regulatory proteins. (A)** Hydrogel droplets formed from a fusion protein linking mCherry to the LC domain of fibrillarin were incubated with 3 μM of GFP-linked LC domains of fibrillarin (FBL), FUS, TAF15, DDX5, DHX9, nucleolin (NCL), and hnRNPA2 (A2). The trapping of GFP-linked proteins to hydrogel droplets were analyzed using confocal microscopy. (B) GFP-only or GFP-linked wild-type, F18/40S, or FallS LC domains of fibrillarin were applied to the chamber slides containing hydrogel droplets formed from mCherry-linked LC domains of FUS, TAF15, DDX5 or DHX9. Upon overnight incubation at 4°C, hydrogel retention of the GFP proteins were analyzed by confocal microscopy. (C) HEK-293T cells were transfected with wild-type, F18/40S, FallS or ΔLC fibrillarin constructs with Flag tags. Upon pull-down using anti-Flag antibodies, co-precipitated proteins were analyzed by Western blotting using indicated antibodies.
Figure 1

A

FBL:dsRed  Nucleophosmin  Merge

WT

B

FBL:dsRed  Nucleophosmin  Merge

ΔLC

C

NaCl (mM)

100  50  25

D

mCherry:FBL LC domain hydrogel droplets

CTL  1,6-HD  2,5-HD

0  1  3  6  12  24  36  48 (hr)
Figure 2

A

mCherry : FBL

mCherry : FBL

mCherry : FBL

mCherry : FBL

WT

F30S

F30/64S

F5S

F40S

F5/18/40S

F12S

F64S

F12/30/64S

F18S

F18/40S

FallS

B

WT

F5S

F12S

F18S

F30S

F40S

F64S

F18/40S

F30/64S

F5/18/40S

F12/30/64S

FallS

C

mCherry : FBL

mCherry : FBL

mCherry : FBL

mCherry : FBL

GFP

GFP

WT

WT

5 μM

FallS

2 μM

FallS

D

mCherry : FBL

mCherry : FBL

mCherry : FBL

mCherry : FBL

GFP

GFP

WT

WT


Figure 3

A

mCherry:FBL-WT (μM)

B

mCherry:FBL-F18/40S (μM)

C

NaCl (mM)

- Wild-type, LLD
- Wild-type, No LLD
- F18/40S, LLD
- F18/40S, No LLD

mCherry:FBL (μM)
Figure 4

A  Scrambled siRNA

|       | FBL:dsRed | Nucleophosmin | Merge |
|-------|-----------|---------------|-------|
| WT    | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| ΔLC   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| F18/40S | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| FallS | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |

B  Fibrillarin siRNA

|       | FBL:dsRed | Nucleophosmin | Merge |
|-------|-----------|---------------|-------|
| WT    | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| ΔLC   | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| F18/40S | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) |
| FallS | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
Figure 5

A. mCherry:FBL LC domain hydrogel droplets

|          | GFP only | GFP:FBL | GFP:FUS | GFP:TAF15 | GFP:DDX5 | GFP:DHX9 | GFP:NCL | GFP:A2 |
|----------|----------|---------|---------|-----------|----------|----------|---------|--------|
|          |          |         |         |           |          |          |         |        |

B. mCherry:FUS GFP mCherry:TAF15 GFP mCherry:DDX5 GFP mCherry:DHX9 GFP

|          | GFP only | GFP:FBL WT | GFP:FBL F18/40S | GFP:FBL FallS |
|----------|----------|-------------|-----------------|---------------|
|          |          |             |                 |               |

C. Flag:FBL

| WT | F18/40S | FallS | ΔLC |
|----|---------|-------|-----|
|    | Flag:FBL | Input | IP  |

FUS  TAF15  DDX5  DHX9  Flag