Centro de Investigación Científica de Yucatán, A.C.

Posgrado en Ciencias Biológicas

Estudio de la actividad ribonucleasa de Fibrilarina

Tesis que presenta

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En opción al título de

DOCTOR EN CIENCIAS

(Ciencias Biológicas: Opción Bioquímica y Biología Molecular)

Mérida, Yucatán, México
2021

CENTRO DE INVESTIGACIÓN CIENTÍFICA DE YUCATÁN, A. C.
POSGRADO EN CIENCIAS BIOLÓGICAS

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Por medio de la presente, hago constar que el trabajo de tesis de **Francisco Alejandro Guillén Chable** titulado **Estudio funcional de la actividad ribonucleasa de fibrilarina,** fue realizado en la unidad de **Bioquímica y Biología Molecular de Plantas**, laboratorio 23 del **Centro de Investigación Científica de Yucatán, A.C.** bajo la dirección del **Dr. Enrique Castaño de la Serna**, dentro de la opción de Bioquímica y Biología Molecular, perteneciente al Programa de Posgrado en Ciencias Biológicas de este Centro.

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Este trabajo se llevó a cabo en la Unidad de Bioquímica y Biología Molecular de Plantas del Centro de Investigación Científica de Yucatán, y forma parte del proyecto titulado Estudio funcional de la interacción entre fibrilarina y fosfolípidos en la progresión viral de plantas bajo la dirección del Dr. Enrique Castaño de la Serna con financiamiento del Consejo Nacional de Ciencia y Tecnología (CONACyT FC 1572, 576664.)

AGRADECIMIENTOS

Con especial agradecimiento se hace mención al Consejo Nacional de Ciencia y Tecnología por los recursos otorgados bajo el número de becario 576664, además de las Convocatorias 2017-2018 de la Beca Mixta de movilidad en las que fui receptor de las mismas.

Al Dr. Enrique Castaño de la Serna, por su confianza, paciencia y la forma en que me instruyó durante este proceso de formación. Por la amistad construida también se agradece en este espacio.

A mis asesores Dr. Felipe Vázquez Flota, Dra. Luisa López Ochoa, Dr. Dimitris Georgellis por las múltiples formas en las que fui receptor de sus consejos, ayuda y soporte, la finalización de esta tesis doctoral tiene mérito debido a su ayuda y soporte. Muchas gracias.

Al apoyo técnico de la Ingeniera Wilma Gonzales, y soporte en el mismo periodo.

A mi comité revisor en el que se incluye las colaboraciones de los Dra. Ana Ly Arroyo Herrera, Dra. Ileana Echevarría Machado y Dr. Luis Carlos Rodríguez Zapata.

A mis amigos del laboratorio #23, siguen siendo parte indispensable, los tengo siempre en la memoria y estima.

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ABREVIATURAS

FBL: Fibrilarina

HsFib: fibrilarina de *Homo sapiens*

AtFib1: fibrilarina 1 de *Arabidopsis thaliana*

Atfib2: fibrilarina2 de *Arabidopsis thaliana*

NORs: Nuclear organization regions

Nop: Nucleolar protein

ADN: Ácido desoxirribonucleico

RNA: ácido ribonucleico

CF: componente fibrilar

CG: componente granular

CFD: componente fibrilar denso

GAR: Región rica en glicinas y argininas

SAM: S-Adenosilmetionina

PCR: Reaccione en cadena de la polimerasa

PIPs: fosfoinositidos

PI(3)P: fosfatidilinositol 3 fosfato

PI(4)P: fosfatidilinositol 4 fosfato

PI(5)P: fosfatidilinositol 5 fosfato

PI(3,4)P2: fosfatidilinositol 3, 4 fosfato

PI(3,5)P2: fosfatidilinositol 3, 5 fosfato

PI(4,5)P2: fosfatidilinositol 4, 5 fosfato

PI(3,4,5)P3: fosfatidilinositol 3,4, 5 fosfato

PLC: phospholipase C

PLD: phospholipase D

PBS: Amortiguador de fosfatos salino

PBS-T: Amortiguador de fosfatos salino más Tween 20

SDS-PAGE: electroforesis en gel de poliacrilamida con dodecilsulfato de sodio

RESUMEN

La síntesis de los RNA ribosomales (rRNA) constituye uno de los eventos que más demanda energética requieren dentro de la célula, puesto que numerosos factores enzimáticos están inmersos de principio a fin de la biogénesis ribosomal. El mantenimiento de la estructura nucleolar es de importancia para el correcto funcionamiento de la actividad transcripcional de los rRNA. Recientemente se ha descrito que el nucleolo es una de los mayores cuerpos que utilizan parte de los principios de separación de fases para el mantenimiento de la estructura nucleolar y por ende del funcionamiento de la maquinara de síntesis de rRNA. Numerosas proteínas, entre ellas la proteína nucleolar fibrilarina se encuentra ampliamente conservada estructuralmente desde arqueas hasta humanos, por lo que es descrita como marcador de la actividad transcripcional de la Pol I. Además de residir en el nucléolo, la actividad metiltransferasa durante la biogénesis de los RNAs ribosomales y la modificación de alrededor de 100 o más residuos específicos es considerada de importancia debido a su relación con la fidelidad de la actividad traduccional de ribosomas funcionales. Además de las funciones relacionadas con la modificación y procesamiento de RNAs, se sabe que esta proteína metila de igual forma a la histona H2A en la glutamina 105 en humanos y plantas. Esta metilación es una marca epigenética única para el nucléolo. Además de estas funciones, en nuestro laboratorio se ha descrito que la fibrilarina 2 *Arabidopsis thaliana* posee actividad de ribonucleasa y que esta actividad reside en el dominio GAR, en la porción N terminal de la proteína. Se desconocen los mecanismos que permiten a esta proteína regular la degradación de RNA, posibles sustratos o la influencia de otras moléculas sobre la actividad ribonucleasa de fibrilarina. El siguiente trabajo de tesis doctoral presenta evidencia sobre la actividad ribonucleasa de la fibrilarina humana, que al igual que su contraparte en *A. thaliana*, la actividad reside en la región desordenada rica en Glicinas y Argininas. Importante para la regulación, formación y estabilidad de las estructuras nucleolares, estas regiones desordenadas presentan interacción con lípidos. Describimos en detalle que fibrilarina interactúa y co-localiza con ácido fosfatídico además de que estos fosfolípidos regulan la actividad ribonucleasa de fibrilarina de forma *in vitro*. Además de mutantes sitio-específicas de la misma región reducen el tiempo de vida media de residencia en el nucléolo de las fibrilarinas mutantes. Al formar parte de los complejos de metilación, nosotros nos preguntamos y evaluamos si en el contexto del complejo de metilación fibrilarina podría conservar dicha actividad ribonucleasa, hallamos que está actividad continua presente aún formando parte del complejo y además confirmamos que la región modular GAR posee dicha actividad, al igual que otras regiones similares que se encuentran en otras proteínas con asociación a RNA y lípidos.

ABSTRACT

The synthesis of ribosomal RNA (rRNA) constitutes one of the events that requires the most energy within the cell, since numerous enzymatic factors are involved from beginning to end in ribosomal biogenesis. The maintenance of the nucleolar structure is of importance for the correct functioning of the transcriptional activity of the rRNAs. Recently it has been described that the nucleolus is one of the largest bodies that uses part of the principles of phase separation for the maintenance of the nucleolar structure and therefore the operation of the rRNA synthesis machine. Numerous proteins, including the nucleolar fibrillarin protein, are structurally widely conserved from archaea to humans, which is why it is described as a marker of the transcriptional activity of Pol I. In addition to residing in the nucleolus, methyltransferase activity during biogenesis of the Ribosomal RNAs and the modification of about 100 or more specific residues is considered of importance due to its relationship with the fidelity of the translational activity of functional ribosomes. In addition to the functions related to the modification and processing of RNAs, this protein is known to methylate in the same way to histone H2A in glutamine 105 in humans and plants. This methylation is a unique epigenetic mark for the nucleolus. In addition to these functions, in our laboratory it has been described that the fibrillarin 2 Arabidopsis thaliana possesses ribonuclease activity and that this activity resides in the GAR domain, in the N-terminal portion of the protein. The mechanisms that allow this protein to regulate RNA degradation, possible substrates or the influence of other molecules on fibrillarin ribonuclease activity are unknown. The following doctoral thesis work presents evidence on the ribonuclease activity of human fibrillarin, that like its counterpart in *A. thaliana*, the activity resides in the disordered region rich in Glycines and Arginines. Important for the regulation, formation and stability of nucleolar structures, these disordered regions present interaction with lipids. We describe in detail that fibrillarin interacts and co-localizes with phosphatidic acid, in addition to the fact that these phospholipids regulate fibrillarin ribonuclease activity in vitro. In addition, site-specific mutants of the same region reduce the half-life of residence in the nucleolus of mutant fibrillarins. As part of the methylation complexes, we asked ourselves and evaluated whether in the context of the fibrillarin methylation complex it could preserve said ribonuclease activity, we found that this activity continues to be present even as part of the complex and we also confirmed that the GAR modular region possesses said activity, as well as other similar regions found in other RNA- and lipid-associated proteins.

1. INTRODUCCIÓN

El nucleolo es una de las mayores estructuras identificables en la célula, tanto que su descripción ha estado desde hace un centenar de años en la literatura científica. En las siguientes décadas a su descripción mucha información perteneciente a esta super estructura nuclear ha salido a la luz, con ella la principal de sus funciones, la biogénesis de los rRNA, indispensables para la fidelidad en la tracción proteica de los ribosomas. Se sabe que durante la biogénesis ribosomal participan más de cien factores, independientes de la maquinaria de transcripción de la polimerasa I, encargada de la elongación de los rRNA sintetizados a partir de las regiones organizadores del nucleolo (NORs) las cuales poseen los genes de rDNA. Fibrilarina es una proteína metiltransferasa que actúa sobre el RNA ribosomal, encargada de la metilación residuo-específico de la posición 2-OH de la ribosa. Esta proteína se encuentra conservada en diferentes organismos, desde arqueas hasta eucariotas, sin embargo, diferentes funciones que se detallan en los antecedentes permiten asumir los diferentes aspectos funcionales en los que la fibrilarina participa. Fibrilarina forma parte de los complejos de metilación, conocidos como ribo-nucleoproteínas, compuestas de una parte proteína y un RNA guía. Estos complejos de metilación modifican co-transcripcionalmente residuos sitio específicos del rRNA durante la biogénesis ribosomal. Éste último, es uno de los procesos que más demanda energética requiere la célula pues se requiere de un estricto control y regulación, en cada paso del proceso, participando un complejo de enzimas y proteínas accesorias, además de ácidos nucleicos que permitan el flujo de procesamiento del rRNA. Este flujo de procesamiento forma parte de un nuevo campo de la biología celular en el que se ha reconocido que el procesamiento del rRNA forma parte de un contexto termodinámico conocido como separación de fases, o separaciones líquido-líquido, en el que se concentran proteínas y ácidos nucleicos con el fin de crear un volumen definido cuya actividad principal consiste en retener y propiciar las actividades enzimáticas que poseen las proteínas que componen estos cuerpos o bio condensados. Regiones que se encuentran en proteínas como fibrilarina, conocidas como regiones intrínsicamente desordenadas cumplen la función de interacción con otros componentes nucleolares, que cuya actividad enzimática no ha sido descrita por completo. Se presenta evidencia de la existencia de una maquinaria regulatoria que permite el establecimiento y regulación de componentes enzimáticos y estructurales que permiten que la biogénesis tenga lugar en un tiempo y orden estrictamente definidos. La participación de moléculas en el interior del nucléolo, justamente como lípidos también es considerada en

la razón de su posible interacción con proteínas con actividad enzimática y la regulación de las mismas o de procesos en los que se requieren interacciones transitorias con lípidos.

En este trabajo doctoral se presenta información relevante de las funciones de fibrilarina en diferentes especies y contextos en los que ha sido descrita, principalmente como metiltransferasa. Se da también una breve introducción a la localización nucleolar de la proteína en las diferentes subestructuras sub-nucleolares durante interfase.

Se detallan las actividades regulatorias y de interacción con otras proteínas y con RNAs durante el proceso de biogénesis ribosomal y otros procesos relacionados con la inmunidad en plantas. De igual manera, como última instancia, se describe la información recientemente publicada acerca de la función de ribonucleasa de fibrilarina de *Arabidopsis thaliana* y su posible relevancia en otros aspectos transcripcionales, regulatorios y epigenéticos. Rodríguez-Corona describió que la región rica en glicinas y argininas de fibrilarina 2 de *Arabidopsis thaliana* posee actividad ribonucleasa, es decir la capacidad de degradar RNA. Además, fibrilarina ha sido reportada como metiltransferasa de la histona H2A fuera del contexto de las ribonucleopartículas.

Tomando como base el trabajo de nuestro laboratorio relativo a la fibrilarina 2 de *A. thaliana*, el objetivo general de la siguiente tesis radica en la evaluación de la capacidad degradativa de la fibrilarina humana, su interacción con lípidos y el efecto de las mutaciones en aminoácidos conservados en esta región desordenada conocida como dominio GAR, utilizando también el complejo de metilación, su expresión y purificación, diseñamos una estrategia para la evaluación de la actividad ribonucleasa en el contexto de un complejo snoRNP. Aunado a la información que proporciona nuestro trabajo, asumimos las siguientes preguntas que en el desarrollo de la tesis funcionaron de guía para la estrategia experimental: ¿Es conservada la actividad ribonucleasa de fibrilarina en otras especies? ¿Se requieren de los mismos cofactores para la regulación de la actividad ribonucleasa de fibrilarina? ¿si fibrilarina posee dicha actividad, es posible que existan otras proteínas con capacidad de corte sitio específica con regiones similares al de fibrilarina?

CAPÍTULO I

ANTECEDENTES

1. NUCLEÓLO

En células eucariotas el nucléolo es un organelo altamente estructurado y especializado encargado del proceso de biogénesis ribosomal, constituido por dominios nucleares formados alrededor de las regiones de organización nucleolar (NORs, nuclear organizar regions). Estas regiones NOR son sitios cromosómicos en los que existen múltiples copias de DNA ribosomal (rDNA). Basándose en la textura y contrastes observados por microscopia electrónica se definen tres componentes fundamentales del nucléolo: los centros fibrilares (FCs, fibrillar centers), el componente fibrilar denso (DFC, dense fibrillar component) y el componente granular (GC, granular component) (Figura 1).

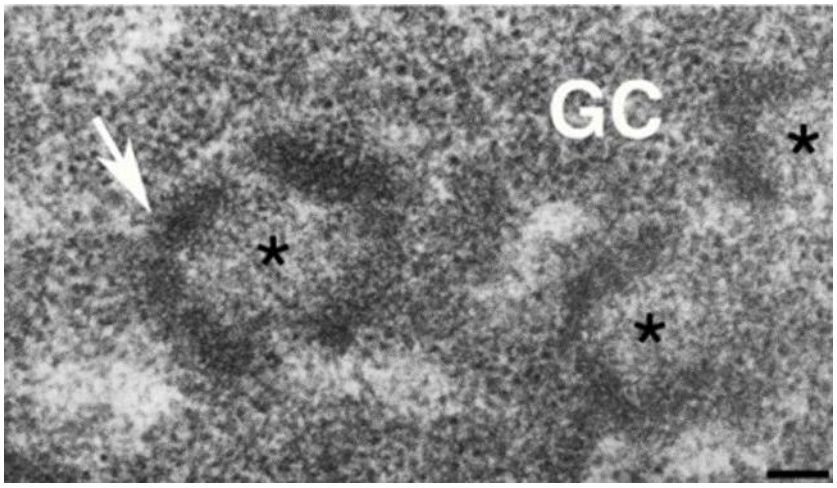


Figura 1. Organización nucleolar en eucariotas. El componente fibrilar se señala con asteriscos, el componente fibrilar denso con flecha en blanco y el componente granular (GC). Escala 0.1 µm. Tomado de Hernández-Vetun et al., 2010.

Considerado como un organelo sin membrana, el nucléolo es el sitio donde ocurren los diferentes pasos de la biogénesis ribosomal: la transcripción de los genes ribosomales (rDNAs), la maduración y el procesamiento de los RNA ribosomales (rRNAs) y el ensamble de los rRNAs y las proteínas ribosomales (RP). Siendo la unidad donde se da biogénesis ribosomal, cada componente del nucléolo contiene una actividad particular: en la interfase del FC y el DFC se encuentran los sitios activos de la transcripción mediada por la polimerasa I (Pol I), mientras que en el DFC residen rRNA en procesamiento temprano, y

el GC se encarga de la maduración de los pre-RNAs. Aun poco claro es el rol de los FCs, considerado como el sitio de localización de los rDNA no transcritos, los complejos de Pol I y los de la maquinaria de transcripción. Actualmente se considera que este organelo posee -además de la biogénesis ribosomal- funciones relaciones con el ciclo y proliferación celular, estrés rutas de sobrevivencia tumoral, apoptosis, formación de telómeros, modificaciones a RNAs de transferencia (tRNA), ciclo de vida viral, entre otras (Shaw & Doonan, 2005; Olson, 2010; Hernández-Verdun et al., 2010, Rodríguez-Corona et al., 2015).

1.1.1. El nucléolo y la biogénesis ribosomal

El ensamble ribosomal es un proceso caracterizado por una serie de eventos regulados espacial y temporalmente dentro del nucléolo. Estos eventos incluyen la transcripción y el procesamiento de los pre-rRNAs, su modificación, el ensamble con las proteínas ribosomales, la maduración de las pre partículas ribonucleoproteicas (pre-RNP) y finalmente el exporte de las unidades pequeñas y grandes hacia el citoplasma (Correll, Bartek, & Dundr, 2019; Langhendries, Nicolas, Doumont, Goldman, & Lafontaine, 2016; Nazar, 2004; Woolford & Baserga, 2013).

1.1.2. Biogénesis ribosomal en eucariotas

Los ribosomas constituyen la maquinaria de traducción y síntesis de proteínas, por tanto, esenciales para los organismos vivos. Están conformados por dos subunidades que consisten, ambas, en múltiples proteínas ribosomales y rRNA.

La subunidad pequeña, 40S, contiene al rRNA 18S y ~33 proteínas ribosomales, funcionando como la unidad decodificante del RNA mensajero (mRNA). La subunidad grande, 60S, contiene los rRNAs 25/28S, 5.8S y 5S junto con ~47 proteínas, que en conjunto se encarga de la formación de los enlaces peptídicos. Estas dos subunidades conforman la unidad ribosomal 80S (Weis et al., 2015).

El ensamble de los ribosomas es un proceso complejo altamente regulado en el que intervienen alrededor de 200 factores de ensamble: proteínas de unión a RNA, endo y exonucleasas, RNA helicasas, GTPasas y ATPasas (Peña et al., 2017). Todos estos factores en conjunto promueven el procesamiento del pre-rRNA, el re-modelamiento de interacciones proteína-proteína y RNA-proteína, además del tráfico nuclear y el aseguramiento del control del proceso en general.

1.2 TRANSCRIPCIÓN Y PROCESAMIENTO DEL rRNA

El ensamble de los ribosomas requiere, además de las proteínas ribosomales, cuatro rRNAs diferentes, tres de los cuales (18S, 5.8S y 25S/28S) son transcritos como un precursor primario de RNA (pre-rRNA), codificados por los genes ribosomales (rDNA) y transcritos por la Polimerasa I (Pol I) (Henras et al., 2015). La molécula primaria de rRNA trascrita, 47S pre-rRNA (~13000 nt) consiste en una secuencia tándem de los rRNA 18S, 5.8S y 28S (en eucariotas) flanqueadas en los extremos 5' y 3' por secuencias externas (ETS, external transcribed spacers) y por espaciadores internos (ITS, internal transcribed spacers), este precursor sufre una serie de intervenciones enzimáticas que en última instancia generan los productos maduros constituyentes de las subunidades ribosomales. Este proceso de remoción enzimática requiere de una serie de cortes mediadas por endo y exo nucleasas nucleolares que permiten la maduración del pre-rRNA. Esto implica que la remoción de los espaciadores no es resultado de un simple corte en las cercanías de los rRNAs 18S, 5.8S y 28S sino de una estricta y ordenada serie de pasos que derivan en la producción de los rRNAs. Aunando a la remoción de los espaciadores y el procesamiento, la modificación de residuos específicos es otro de los procesos que ocurren co y/o post transcripcionalmente durante la biogénesis de los rRNAs.

Estas modificaciones covalentes tienen la finalidad de extender la topología y asegurar la estabilidad del rRNA. Dichas modificaciones incluyen metilaciones en la mitad ribosa o en la base de nucleótidos específicos, acetilaciones y amino-carboxilaciones. Por otra parte, las modificaciones de isomerización incluyen la conversión de uridinas a pseudo-uridinas. En su conjunto, ambas contribuyen a la diversidad ribosomal, es decir a la posibilidad de tener diferentes poblaciones de ribosomas con capacidades de traducción diferentes en diversas situaciones celulares (Sharma et al., 2017).

Una observación general es que estas modificaciones confieren una función adecuada durante cada paso del proceso de traducción proteica, es decir influenciando la iniciación de la misma, mantenimiento del marco de lectura y el reconocimiento del codón de paro. En otro particular las mismas modificaciones influencian la estructura ribosomal de manera local, afectando propiedades intrínsecas como la capacidad de unión de los rRNA y RNA mensajeros (mRNA) (King et al., 2003, Baxter et al., 2007; Piekna-Przybylska et al., 2008; Baudin-Baillieu et al., 2009).

1.2.1. Complejos de metilación del rRNA

La metilación 2'-O de la ribosa es una de las modificaciones más abundantes y ampliamente distribuidas en los organismos; por ejemplo, se han elucidado 33 posiciones/residuos en bacterias, 112 en levaduras y alrededor de 210 en humanos (Decatur et al., 2002; Wilson et al., 2012; Sharma et al., 2015). Esta modificación es añadida post-transcripcionalmente, mediada por complejos conocidos como ribonucleopartículas (snoRNP) los cuales se constituyen por una familia de RNAs no codificantes, los snoRNA (small nucleolar RNA); proteínas estructurales que proveen la plataforma de ensamble del complejo y la enzima que añade el grupo metilo (-CH3) al carbono 2 de la ribosa en un residuo particular del rRNA (Monaco et al., 2018). La mayoría de estas modificaciones ocurren en regiones funcionalmente importantes del ribosoma, por ejemplo, el dominio peptidil-transferasa, el centro decodificante y el puente inter-subunidades (Decatur et al., 2002). Los complejos especializados que guían esta modificación sitio específica son conocidos como ribonucleopreoteínas C/D Box (RNP) debido a su porción nucleica, es decir los snoRNAs que los constituyen y que sirven de guía para la metilación: los snoRNAs con cajas C/D (Watkins et al., 2012).

1.2.2. Ribonucleopartículas C/D box

Tanto en como las RNP de arqueas como en las de eucariotas, los snoRNAs constituyentes posen un motivo conocido como cajas C/D en los extremos 5' y 3', además de un motivo C'/D' internamente (Reichow et al., 2007). Ambos motivos conservan una secuencia consenso RUGAUGA, donde R es una purina (en las cajas C y C') y CUGA en las cajas D y D'. Estas regiones consenso flanquean la región anti-sentido, la cual es complementaria a la región con el residuo específico del rRNA que será modificado. La región anti-sentido abarca alrededor de 10-21 nt de longitud y son necesarios específicamente 10 nt para la complementación (Kiss et al 1996; Dragon et al., 2006; Yang et a., 2016). La porción proteica que constituye los RNPs la integran las proteínas 15.5K, a las cuales se unen a RNA, un heterodímero de Nop58 y Nop56 (parálogos derivados de un evento de duplicación génica), y la parte catalítica es proporciona por la metiltransferasa fibrilarina (Schimmang et al., 1989; Gautier et al., 1997, Watkins et al., 2000). Esta última utiliza S-adenosil-metionina como el donador del grupo metilo. Algo interesante es la manera en que el complejo de metilación posiciona el dominio catalítico de fibrilarina directamente en contra posición al nucleótido que será metilado en el segmento de unión del rRNA y el snoRNA guía, y que en general este se encuentra a 5 nucleótidos rio arriba de una caja D (o D') (Bechellerie et al., 1997; Kiss et al., 1996).

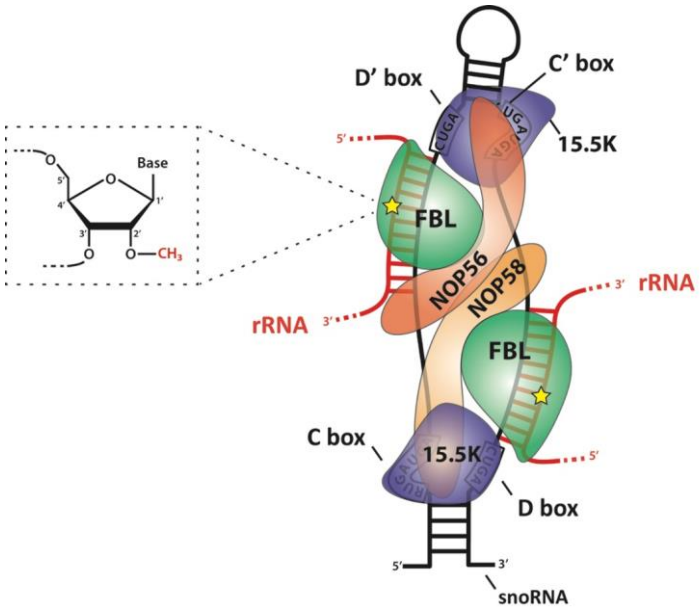


Figura 2. Representación esquemática de un complejo de metilación snoRNP. Aunque la mayor parte de la información estructural y bioquímica se basan en los estudios sobre los complejos de origen de arqueas, se sabe que se requieren dos moléculas de fibrilarina adyacentes a un soporte estructural formado por las proteínas Nop58/Nop56 y 15.5K, además de la necesidad de un snoRNA que sirva de guía para la metilación sitio específica de los residuos de rRNA durante la biogénesis ribosomal. Tomado de Monaco et al., 2018).

Aunque su localización es predominante en el nucléolo, se sabe que algunos snoRNAs se acumulan también en los cuerpos Cajales (Darzacq et al., 2002) -regiones subnucleares caracterizados por la presencia de proteínas y RNAs encargados del procesamiento de mRNA; así como por ser el sitio de ensamble de los snoRNPs (Shaw & Brown, 2004).

Además de las funciones relacionadas con las modificaciones sobre el rRNA, algunos snoRNPs se requieren para el corte de los precursores de rRNA tempranos, entre ellos las snoRNPs que contienen los snoRNAs U3 y U14, conservados en plantas y animales (Venema et al., 2000). Esto último sugiere que una población de las enzimas que realizan los cortes específicos durante el procesamiento de los pre-rRNAs requieren de la especificidad dictada por el apareamiento de bases de la misma manera en que las modificaciones como la metilación de la 2-OH ribosa se dan durante el proceso de biogénesis ribosomal (Olson, 2010; Rodríguez-Corona et al., 2017).

1.3 FIBRILARINA

Entre las proteínas nucleolares involucradas en el procesamiento de rRNA se encuentra la fibrilarina, una proteína esencial en el proceso de biogénesis ribosomal y, por tanto, para la vida. Posee, dependiendo del organismo, una masa de 34-38 kDa. Descrita generalmente por su actividad de metiltransferasa, fibrilarina es parte del complejo de ribonucleoproteínas asociadas al nucléolo (snoRNP) cuya función es la metilación del rRNA. En eucariotas, los snoRNP contienen snoRNAs que guían a la fibrilarina hacia sitios específicos del pre-rRNA para ser metilado, además de los snoRNA, los complejos de metilación contienen otras proteínas nucleolares: Nop56, Nop58 y la proteína 15.5Ka.

Fibrilarina es reconocida como un marcador de los sitios de actividad transcripcional de la Pol I, de manera que su localización se relaciona con los centros fibrilares y los componentes fibrilares densos, justo en la interface de estas subestructuras nucleares (Sobol et al., 2013). Wang et al., 2000 asignó a fibrilarina en la familia de metiltransferasas de Rossmann S-adenosilmethionina (SAM), familia caracterizada por la presencia de un domino de unión a SAM, el sitio catalítico [K-D-K-(H)] y siete hojas beta (β) flanqueadas por hélices alfa (α) en el dominio C-terminal, formando una estructura α-β-α. Así mismo se describió que la fibrilarina 2 de *Arabidopsis thaliana* posee dos sitios de interacción/unión con RNA, uno en la región de los aminoácidos 138 y 179 y un dominio en el C-terminal, entre los aminoácidos 225 y 281 (Rakitina et al., 2011).

Conservada estructuralmente en los diferentes reinos, desde arqueas a eucariotas (Rodríguez-Corona et al., 2015; 2017), fibrilarina se encuentra principalmente en el nucléolo, y considerando que en arqueas no existe tal estructura, las fibrilarinas de estos organismos poseen diferencias estructurales que los distinguen de sus contrapartes eucariotas, por ejemplo, la presencia del dominio GAR en el N terminal de la proteína.

Diferentes intentos se han orientado a la obtención del complejo de metilación eucariota sin éxito, sin embargo, la mayoría de los estudios utilizan los complejos de arqueas, como en los experimentos planteados por Omer et al., 2002; en el que se logra la reconstitución *in vitro* del complejo de metilación de la arquea termofílica *Sulfolobus solfataricus* capaz de llevar a cabo la metilación de un fragmento de rRNA complementaria la región guía del snoRNA utilizado en el ensayo. Así mismo se describe que existe un orden de ensamblaje: aL7a, aNOP5 y fibrilarina sobre el snoRNA guía. Por otro lado, mutaciones dentro del sitio de unión a SAM no impiden el ensamble del complejo, pero si la capacidad de metilación *in vitro* del mismo.

En la figura 3 se detalla la composición modular de las fibrilarinas de eucariotas con respecto a arqueas. Es clara la ausencia del dominio GAR en el N terminal, considerando que este dominio es particularmente importante en células eucariotas se le atribuyen funciones que van desde la localización nucleolar hasta su interacción con proteínas y RNAs virales, incluidos factores de splicing alternativo (Yanagida et al., 2004). Dentro del dominio metiltransferasa se encuentran las regiones central o R y la región rica en hélices alfa en el dominio C-terminal, con 87 y 95 aminoácidos (aa), respectivamente (Rodríguez-Corona et al., 2015). Además, un motivo de unión a RNA GCVYAVCF se encuentra dentro de esta región metiltransferasa (Aris and Blobel, 1991).

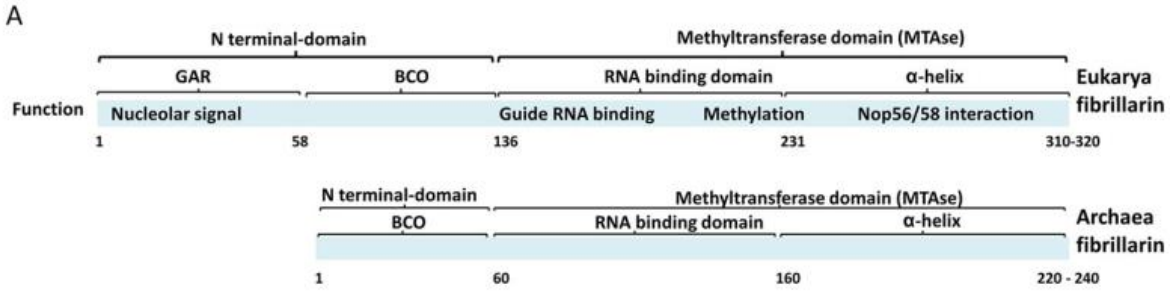


Figura 3. Representación de la estructura y funciones de los dominios cuya actividad ha sido descrita para fibrilarina. Se detallan las regiones en la que se divide la proteína en eucariotas y arqueas: La región N-terminal y el dominio metiltransferasa (MTAse). GAR: dominio rico en glicina y arginina. BCO: dominio blanco. Dominio de unión a RNA y el dominio de metilación. En el extremo C-terminal se muestra el dominio rico en hélices alfa. Destaca la ausencia de la región GAR en fibrilarina de arqueas, el cual posee actividad de dirigir a la proteína al nucléolo (tomado de Rodríguez-Corona et al., 2017).

En uno de los trabajos más importantes que señalan la actividad metiltransferasa de fibrilarina y por tanto las implicaciones de esta actividad en el procesamiento y modificación del rRNA fue el del laboratorio de D. Tollervey (Tollervey et al., 1993), en el que mutantes termosensibles en Nop1 (fibrilarina de *Saccharomyces cerevisiae*) reducían dramáticamente la metilación en los transcritos primarios de rRNA en las condiciones de temperatura permisible, más aún, estas mutaciones residen en el dominio de unión a SAM en las posiciones 157 y 219, alanina por valina y prolina por serina, respectivamente.

1.3.1 Otras funciones de fibrilarina en Eucariotas

Recientemente Tessarz et al., 2014 describió que la actividad de metiltransferasa de fibrilarina no se restringía a la metilación de residuos específicos en el rRNA, sino que esta misma función modificaba a la histona H2A en la posición 104 humanos y 105 en levaduras, modificando un residuo de glutamina (Q) (Figura 4). Esta posición en la histona H2A se encuentra altamente conservada en humanos, ratones y levaduras, mientras que en la variante H2A.Z, una variante relacionada con la activación o represión de genes regulados por la Polimerasa II (Pol II) (Rudnizki et al., 2016), han sido sustituidos por glicinas o serinas.

```
                 *******.*:**:.*: ..*** ***:*:*:  *:
hH2A      82 HLQLAIRNDEELNKLLGRVTIAQGGVLPNIQAVLLP 117
mH2A      82 HLQLAIRNDEELNKLLGKVTIAQGGVLPNIQAVLLP 117
yH2A      83 HLQLAIRNDDELNKLLGNVTIAQGGVLPNIHQNLLP 118
hH2A.Z    83 HLQLAIRGDEELDSLI-KATIAGGGVIPHIHKSLIG 120
mH2A.Z    83 HLQLAIRGDEELDSLI-KATIAGGGVIPHIHKSLIG 120
yH2A.Z    88 HLQLAIRGDDELDSLI-RATIASGGVLPHINKALLL 125
```

Figura 4. Alineamiento múltiple de secuencias de variantes de la histona H2A. La figura que muestra la región que abarca la glutamina en la posición 104 en humanos/ratón y 105 en levaduras. Esta misma posición es sustituida por glicinas o serinas en la variante H2A.Z (Tessarz et al., 2014).

Es importante reconocer aquí que diferentes modificaciones en las histonas pueden influenciar diferentes eventos, los cuales van desde la disrupción de la cromatina hasta el reclutamiento de proteínas de tipo no histona hacia la misma. Estas modificaciones poseen la habilidad de remodelar la estructura de la cromatina y, por tanto, el empaquetamiento del DNA, así mismo influenciar el reclutamiento de ordenado de complejos enzimáticos que modifiquen de la misma manera el DNA (Kouzarides, 2007). En este mismo orden de ideas Tessazrs describe mediante un ensayo de inmunoprecipitación de la cromatina y análisis de secuenciación (ChIP-seq) que la metilación en el la glutamina 104/5 en eucariotas se encuentra particularmente enriquecida en las unidades transcripcionales correspondientes al rDNA 35S (en levaduras) y que esta modificación sobre la histona H2A es específica para el nucléolo. Se sabe que la región que incluye la Q104/5 abarca un sitio de unión del complejo de facilitación de la transcripción en la cromatina (FACT, facilitator of chromatin trancription) (VanDemark et al., 2008; McCullough et al., 2011), por lo que la sustitución de Q por alanina o la metilación sobre este residuo impiden la unión de FACT *in vitro*, impidiendo así la transcripción de los genes 35S ribosomales. Se concluye de este estudio que la metilación en la Q104/5 es una marca epigenética que regula la interacción de FACT y los nucleosomas, y que fibrilarina cumple parte de la función epigenética metilando tal posición.

Otro de los estudios que demuestran la capacidad de fibrilarina de mediar la transcripción de los rRNA es el realizado por Yildirim et al., 2013. En este trabajo se demuestra la participación del fosfatidilinositol 4,5 bisfosfato (PIP2) en el proceso de transcripción de la Pol I interactuando con componentes de la maquinaria de esta polimerasa, localizándose en las regiones fibrilares del nucléolo, es decir, donde la transcripción de los rRNA tiene lugar. Poco se sabía de la participación de lípidos nucleares en los eventos que tienen lugar en el nucléolo, hasta hace dos décadas (Yu et al., 1998; Zhao et al., 1998). Yildirim (2013) reporta que la actividad transcripcional de Pol I es inhibida por la adición de anticuerpo anti-PIP2 o de igual forma de la adición de la enzima fosfolipasa C (PLC, phospholipase C), es decir eliminando o degradando a PIP5, respectivamente, de la transcripción de Pol I.

El reporte de Yildirim et al., determinó que PIP2 no se une a toda la maquinaria de transcripción de la Pol I, sino que de una manera selectiva con dos componentes de la misma: el componente de unión rio arriba (UBP, upstream binding factor) y fibrilarina, como lo demuestran los ensayos de pull down en los que se asume la formación de complejos de estas proteínas unidos a PIP2. UBP es una proteína estructural conocida por unirse al promotor de rDNA, además de plegar esta región asegurando así el establecimiento de una

estructura de DNA-proteína óptima para que el complejo de iniciación de la transcripción de Pol I tenga lugar en estos sitios (Stefanovsky et al., 2001). La observación de que PIP2, UBP y fibrilarina co-localizan es una evidencia que permite sugerir que tanto como el proceso de transcripción y modificación o procesamiento del rRNA, donde UBF y fibrilarina participan, respectivamente; están ligados a la asociación con PIP2 creando un entorno propicio para que ambos procesos continúen a través de los componentes estructurales del nucléolo.

Por último, además de la función de metilación, fibrilarina juega un papel importante en el proceso temprano de la transcripción de los genes de rDNA, y cuya participación en este proceso depende de la interacción con fosfolípidos, particularmente fosfatidilinositol 4,5 bisfosfato (PI4,5P2).

1.3.2 Fibrilarina en Plantas

En plantas se ha caracterizado la función y la importancia de esta proteína nucleolar en diferentes aspectos relacionados con la metilación de la histona H2A, la progresión viral y su asociación con RNA no codificantes (lncRNAs, long non coding RNAs) (Loza-Muller et al., 2015; Kim et al., 2007; Seo et al., 2018). Entre los primeros trabajos que describen la función de fibrilarina en plantas está el de Barnache et al., 2000; identificando los dos genes codificantes para fibrilarina en *A. thaliana*: AtFib1 y AtFib2, ambas cercanamente idénticas entre sí, y homólogas a otras fibrilarinas de eucariotas. Además, en un contexto en el que la expresión de Nop1 (levaduras) es nula; su expresión es recuperada si sus homólogos en *A. thaliana* son expresados en este fondo genético, señalando la conservación funcional de fibrilarina entre especies. Por otra parte, Sáez-Vázquez et al, 2004 describe en rábano la purificación de un complejo multiproteico de alrededor de 600 kDa, llamado NF D, que puede llevar a cabo *in vitro* el primer corte en la región 5' ETS del pre-rRNA. Mediante micro secuenciación identifican a nucleolina y fibrilarina como componentes de este complejo NF D, además de dos snoRNAs conservados en eucariotas, U3 y U14, los cuales se asocian a fibrilarina. Lo que supone que fibrilarina y otros componentes de estos complejos conservan la función primaria de procesamiento y modificación del pre-rRNA, sin embargo, no se le atribuye a ninguna de estas enzimas la actividad ribonucleasa que permite el primer corte en 5' ETS.

En Brassica, Loza-Muller et al., 2015 demuestra que fibrilarina puede metilar a la histona H2A *in vitro* y qué además la localización de esta modificación se encuentra principalmente en el nucléolo, sin embargo, a diferencia de células humanas y de levaduras, también se

encuentra en la periferia del núcleo. Además de que la metilación puede ser llevada a cabo por fibrilarina, esta modificación puede darse sobre los promotores del rDNA. Esta capacidad de metilación y la misma marca epigenética es un claro indicio de la conservación de mecanismos que regulan la transcripción y el procesamiento de los rRNA. Estos resultados concuerdan con los presentados en Tessarz et al., 2014, señalando la importancia de esta metilación en la Q para la iniciación de la transcripción mediada por Pol I.

Otras actividades que incluyen a fibrilarina son las planteadas en Kim et al., 2007, en las que la proteína viral ORF3 (llamada así por ser la codificada por el marco de lectura 3, en inglés open reading frame 3), una proteína viral, puede asociarse a fibrilarina, junto con el RNA viral (vRNA), y esta asociación es indispensable para la diseminación de las partículas virales de manera sistémica. Los umbravirus pertenecen a una familia de virus que posee como material génico una molécula de RNA viral, y se diferencian de otros virus por no codificar una recubierta proteica o cápside, por lo que no comparten procesos de infección similares en plantas (Taliansky et al., 2003-a; 2003-b). En ausencia de una cubierta proteica, ORF3 desempeña las funciones correspondientes, como el transporte a larga distancia del material vRNA a través del floema. El floema, por un lado, constituye el sistema vascular especializado en plantas, cuya función es el transporte de macromoléculas, compuestos de defensa, así como señales o rutas de información (Turgeon & Wolf, 2008). Se ha demostrado que el umbravirus de la roseta del cacahuate GRV (groundnut rosette virus) posee la habilidad de moverse a través del floema fuera del sitio de inoculación, gracias a su interacción con fibrilarina. Se ha señalado que esta interacción es importante para diferentes estados del ciclo de vida viral de GRV, los cuales van desde el importe nucleolar de ORF3 a través de los cuerpos Cajales, la re localización de fibrilarina hacia el citoplasma y la formación de ribonucleopartículas indispensables para el tráfico a larga distancia (Kim et al., 2007; Canetta et al., 2008).

ORF3 interactúa con fibrilarina de manera *in vitro* como lo demuestran los ensayos de far Western en Kim et al, 2007. Esta interacción es dependiente de la región N terminal de fibrilarina donde se localiza el dominio rico en glicinas y argininas (GAR) atribuyendo esta interacción de ORF3 a través de este dominio, necesariamente, ya que mutaciones en la región correspondiente al dominio GAR de fibrilarina, impedía la interacción con la proteína viral. Como se aprecia en la microscopia electrónica, la asociación de fibrilarina con la protein ORF3 y el RNA viral de manera *in vitro* forman estructuras filamentosas, de tipo

helicoidal, lo cual suma información a la importancia de estas asociaciones importantes para el ciclo de vida viral del umbravirus GRV.

La interacción de fibrilarina con diferentes especies de RNA ha sido descrita por Rakitina et al., 2011, sin embargo, ningún sustrato de RNA pertenece a la familia de RNAs no codificantes cuya relevancia en la regulación transcripcional, epigenética y silenciamiento de diferentes procesos ha crecido en los últimos años (Liu et al., 2015; Wang & Chekanova, 2017; Nejat & Mantri, 2017; Deng et al., 2018).

Seo et al., 2017 describe a ELENA1 (ElF18-Induced Long Non Coding RNA 1), un lncRNA, asociado a la respuesta inmune en *A. thaliana* tras el reconocimiento del patrón molecular asociado a patógenos, elf18, un fragmento peptídico derivado del factor de elongación Tu (EF-Tu) (Kunze et al., 2004). ELENA1 funciona como un regulador positivo de genes de la respuesta inmune, interactuando con la subunidad 19a del complejo de Mediador (MED19a), permitiendo el reclutamiento de la maquinaria de transcripción al promotor del gen PR1 (Pathogenesis-Related Gene 1). Mediante experimentos *in vitro* de interacción proteína-RNA se demuestra que fibrilarina es capaz de interactuar con ELENA1, y de manera *in vivo* se constata la interacción física de fibrilarina con MED19a en el nucleoplasma y en el nucléolo. Usando un fondo genético en el que fibrilarina se encuentra silenciada se detalla que el rol de fibrilarina es la de fungir como un regulador transcripcional negativo para genes de la respuesta inmune. La función de ELENA1 en el control de la transcripción de PR1 interesantemente, consiste en que puede disociar la interacción entre fibrilarina y MED19a, asegurando así el aumento de la expresión de PR1. En la figura 5 se detalla la posible participación de fibrilarina, MED19a y ELENA1 en la respuesta inmune tras el reconocimiento de elf18. Este estudio añade nueva información relevante acerca de la regulación epigenética de los lncRNAs en un contexto de expresión de genes relacionados con la respuesta inmune en plantas.

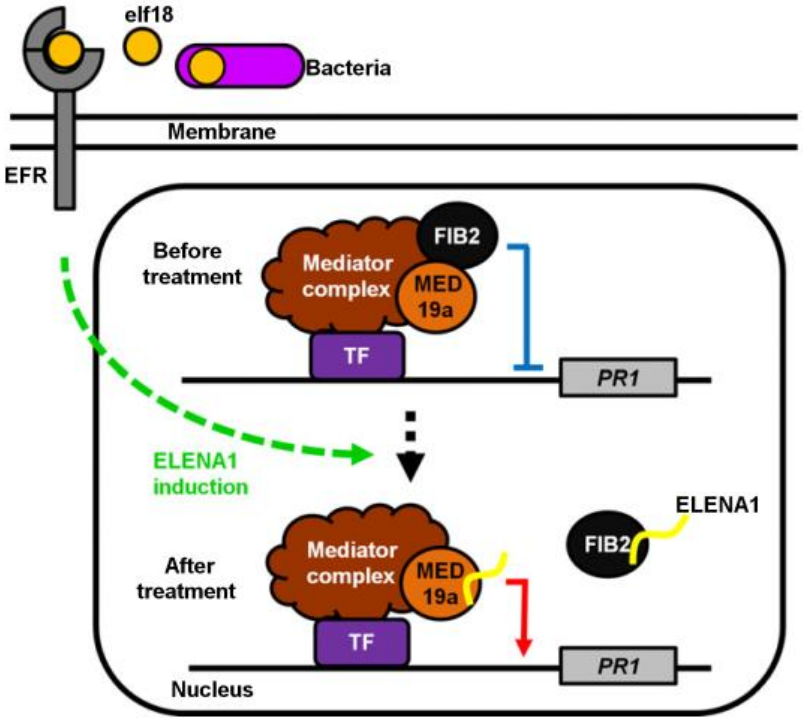


Figura 5. Modelo de la actividad transcripcional regulada por ELENA1. En ausencia de la señal propiciada por elf18, fibrilarina se mantiene unida a MED19a evitando así la transcripción de PR1. Tras la inducción de la señal, los niveles de ELENA1 aumentan, secuestrando a fibrilarina del complejo de Mediador, permitiendo la transcripción de genes relacionados con la respuesta inmune (tomado de Seo et al., 2018).

1.4 La actividad ribonucleasa de Fibrilarina

Recientemente, Rodríguez-Corona et al., 2017 describió dos aspectos relevantes de dos de los genes de fibrilarina en *Arabidopsis thaliana*: 1) la actividad ribonucleasa y 2) la capacidad de interacción con fosfoinosítidos y ácido fosfático (PA, phosphatidic acid); y que está actividad difiere entre los genes que codifican para las fibrilarinas en *Arabidopsis*: AtFib1 y AtFib2. En este estudio, se demuestra que la actividad de ribonucleasa es diferente entre las fibrilarinas, siendo AtFib2 más activa que AtFib1, además de que la actividad es proporcional a la cantidad de proteína presente en la reacción. Como otras ribonucleasas, AtFib2 es activada por calcio a una concentración óptima de 1 mM, mientras que la presencia de calcio no influye en la actividad de AtFib1. Además, se define que el dominio GAR de AtFib2 presenta la actividad ribonucleasa, con menor selectividad hacia los sustratos de RNA 28 y 18S.

Es importante hacer notar que las principales diferencias en estructura primaria y estructural entre Fib1 y Fib2 de *A. thaliana* residen en la región N terminal, es decir en el dominio GAR. Esto hace suponer que las diferencias corresponden a diferentes actividades que las fibrilarinas puedan llevar a cabo.

Algo realmente interesante es la interacción con fosfolípidos de inositol, ya que al igual que la actividad ribonucleasa, difiere entre ambas fibrilarinas, puesto que en un ensayo fat-blot de manera *in vitro* se demuestra que AtFib1 interactúa principalmente con fosfatidilinositol 4 fosfato, mientras que AtFib2 interactúa con todos los fosfolípidos incluido ácido fosfatídico, evaluados en el ensayo. Además, estas interacciones con fosfoinosítidos influencian la actividad ribonucleasa de AtFib2 inhibiéndola en presencia de ácido PA, mientras que ninguna alteración se percibe con AtFib1 en las mismas condiciones de reacción. La actividad ribonucleasa de Fib2 puede ser recuperada por la adición de calcio a la reacción.

De este información derivada de nuestro grupo de trabajo surgen otras preguntas interesantes relacionadas con la actividad ribonucleasa de fibrilarina: ¿tendrán la misma actividad fibrilarinas de otras especies?; si fibrilarina se encuentra en los cuerpos Cajales, ¿es posible que esta nueva actividad se encuentre relacionado con el procesamiento de mRNA o splicing alternativo?, ¿la actividad ribonucleasa se ve influenciada por las modificaciones postraduccionales que puedan residir en el dominio GAR? ¿Cómo se regula la actividad ribonucleasa durante el ciclo celular?

RECAPITULACIÓN DE LOS ANTECEDENTES

Como breve recapitulación de los antecedentes presentados con anterioridad haremos un resumen puntual de algunos aspectos relevantes que permitieron dirigir la actividad experimental de la presente tesis, estos los detallamos como oraciones puntuales:

- El nucleolo es la mayor de las estructuras sin membrana encargada de la regulación y biosíntesis de los rRNA.
- Numerosas proteínas accesorias y enzimáticas participan desde la transcripción hasta procesamiento de los rRNA
- Fibrilarina es una de las enzimas cuya abundancia y actividad enzimática reflejan la demanda energética de la biogénesis ribosomal
- Regiones ricas en repeticiones de aminoácidos como glicinas y argininas componen regiones modulares en ciertas enzimas como fibrilarina
- Fibrilarina de *A. thaliana* posee actividad ribonucleasa, y que dicha actividad radica en el dominio GAR de la proteína.
- Lípidos asociados al nucléolo podría tener influencia en la actividad enzimática de ciertas enzimas como fibrilarina
- Procesos como la biogénesis ribosomal están inmersos en una rama de la biología conocida como separación de fases en la que se describen eventos termodinámicos que regulan la formación y mantenimiento de las estructuras sub-nucleares.
- Colocalización previamente reportada entre lípidos y enzimas abren la posibilidad de un tipo de regulación estructural e incluso de la actividad enzimática *perse*
- Complejos de metilación entre los que se incluyen el perteneciente a fibrilarina han sido reportados con anterioridad y a los cuales se les adjudica la capacidad de corte de rRNA
- RNA guías podría modular y dirigir la actividad de fibrilarina como ribonucleasa en el mismo modo que guían las metilaciones sitio específicas de los residuos en el rRNA

JUSTIFICACIÓN

El proceso de biogénesis ribosomal es un evento que requiere de la participación de cientos de proteínas, RNAs y otras moléculas que definen la formación de las subestructuras nucleolares donde se dan lugar cada una de las etapas de la maduración del pre-rRNA. Es claro que durante todo este proceso se requieren de diferentes actividades enzimáticas: metiltransferasas, ribonucleasas, fosfatasas, así como entre un enorme número de actividades conocidas con anterioridad, sin embargo, se conoce parcialmente la relevancia de cada una de ellas durante el procesamiento del rRNA. Muchas moléculas como los snoRNA, requieren de complejos proteicos para llevar a cabo sus funciones, de manera que es posible suponer que otros procesos de modificación y procesamiento nuevos se requieran durante la formación de complejos, de la interacción proteína-proteína, así como de las interacciones ácido nucleico-ácido nucleico y ácido nucleico-proteína. Numerosas interacciones entre fibrilarina y otras moléculas permitirían suponer diferentes actividades reguladas por otro número importante de señales, las cuales siguen siendo un aspecto relevante que requiere de atención. Una de las grandes incógnitas es la localización y relocalización de fibrilarina entre diferentes estructuras nucleares sin tener una clara perspectiva de cuales sean sus funciones en ellas y por su puesto cuales sean los medios que permiten su movilización. La duración de las interacciones moleculares define la amplitud de la extensión de la señalización dentro de los complejos multi-organizados por lo cual la nueva actividad nueva actividad reportada de fibrilarina abre especio para la consideración de que se cumplan este tipo de funciones bajo un estricto control regulatorio, y que las interacciones que se dan durante el procesamiento de rRNA influyen en dicha actividad.

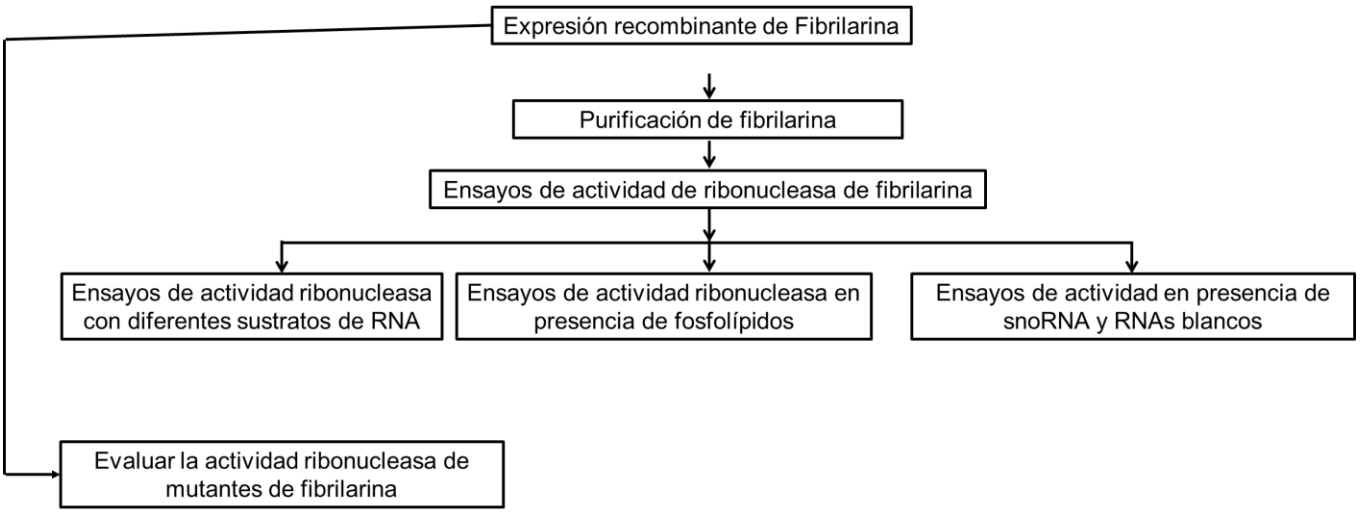
OBJETIVO GENERAL

Estudiar el mecanismo de la actividad ribonucleasa de la proteína fibrilarina de *Homo sapiens*

OBJETIVOS ESPECÍFICOS

1. Evaluar la actividad ribonucleasa de fibrilarina humana utilizando diferentes sustratos de RNA.
2. Evaluar la actividad ribonucleasa de fibrilarina humana en presencia de fosfoinosítidos.
3. Evaluar la actividad ribonucleasa del dominio GAR de las proteínas de unión a RNA GAR1, Nucleolina humana, TGB1 y Fibrilarina
4. Evaluar la actividad ribonucleasa del complejo de metilación snoRNP.
5. Determinar si la actividad de ribonucleasa es guiada en presencia de snoRNAs y RNAs blancos.

ESTRATEGIA EXPERIMENTAL



La siguiente estrategia experimental inicia con la expresión de fibrilarina de forma recombinante, con diferentes etiquetas para su purificación, y detección por western blot. Principalmente usaremos las etiquetas de 6Histidinas proporcionadas por el vector pET15b (Novagen TM) y GST del vector pET42b que contiene además una etiqueta de 8 histidinas, también de Novagen. Estas etiquetas permiten la purificación por afinidad a sustratos en particular: níquel para las etiquetas de histidinas y glutatión unidas a perlas de agarosa. Posteriormente, las fracciones puras de fibrilarina expresadas con estas etiquetas siguen un protocolo de purificación por exclusión molecular en diferentes resinas. Una vez conseguida las fracciones más puras de fibrilarina y revisadas por electroforesis SDS-PAGE, se llevan a cabo los ensayos de actividad ribonucleasa, descritos en Rodríguez-Corona et al., 201, consistentes en la mezcla e incubación de rRNA total y las fracciones de fibrilarina pura, y revelando en geles de agarosa al 1.5-3 %. Además, estas reacciones pueden suplementarse con diferentes sustratos de RNA, lípidos y otros factores proteicos con el fin de evaluar su influencia en la actividad de degradación de RNA que se presume fibrilarina desempeña. De manera que la capacidad degradativa del RNA total pueda ser evaluada con la ayuda de software especializo con el objeto de evaluar el cambio en el contenido de pixeles de las figuras digitalizadas resultantes de la degradación de RNA y su visualización por electroforesis en geles de agarosa.

BREVE RESUMEN DE LOS CAPÍTULOS II, III, IV, V.

El siguiente bloque de apartados corresponden a los capítulos en los que se engloban los objetivos específicos que detallan la presente tesis, organizados de la siguiente manera:

Asociados a los objetivos específicos 1) Evaluar la actividad ribonucleasa de fibrilarina utilizando diferentes sustratos de RNA y 2) Evaluar la actividad ribonucleasa de fibrilarina en presencia de fosfoinosítidos, los capítulos II y III detallan la actividad de fibrilarina humana como una ribonucleasa de RNA, la influencia de los fosfoinosítidos de inositol sobre dicha actividad, así como los protocolos de purificación establecidos para la obtención de una fracción de fibrilarina pura y sin contaminantes que pudieran intervenir en la degradación del rRNA. De igual manera el objetivo específico 5) Determinar si la actividad de ribonucleasa es guiada en presencia de snoRNAs y RNAs blancos, se contempla en el capítulo III, sin embargo, dichos resultados requieren más atención para poder establecer si la actividad degradativa de fibrilarina es guiada en la misma forma que la actividad de metiltransferasa.

El capítulo IV detalla el objetivo específico 3) Evaluar la actividad ribonucleasa del dominio GAR de las proteínas de unión a RNA GAR1, Nucleolina, TGB1 y Fibrilarina. En este capitulo se abordan las regiones ricas en glicina y arginina de las regiones intrínsicamente desordenadas de las proteínas nucleolares Nucleolina, GAR1 y la proteína viral TGB1 la cual contiene una región desordenada.

El capítulo V se asocia al objetivo específico 4) Evaluar la actividad ribonucleasa del complejo de metilación snoRNP, en el que la hipótesis de que la región GAR de fibrilarina contiene el módulo enzimático y se evalúa en el contexto del complejo de metilación, utilizando los constructos que poseen las 3 proteínas accesorias del complejo snoRNP (Nop59, Nop56 y 15.5K), fibrilarina completa y el complejo con una fibrilarina sin dominio GAR.

Capítulos VI y VII presentan los trabajos de investigación producidos durante la tesis experimental y sus correspondientes artículos, en el capítulo VI se presenta el artículo publicado en la revista Cells-MPDI y en el capítulo VII se presenta la revisión de literatura sobre las regiones intrínsicamente desordenadas y las propiedades termodinámicas

inherentes a la formación, establecimiento y mantenimiento de estructuras sin membranas como el nucleolo, sometido a International Journal of Molecular Sciences.

CAPÍTULO II. Fibrillarin as a ribonuclease

2.1 Introduction

The mayor nucleolar fibrillarin protein has been reported to be involved in several aspects more beyond the classical view of fibrillarin as a ribosomal rRNA production enzyme: nuclear structure dynamics, association with other molecules such as long non coding RNA, lipids and protein partners during different stage of development and cell cycle. Ribosomal biogenesis requires a strict control and tightly regulation in order to keep a high-quality order of protein production (Koš & Tollervey, 2010; Sirri, Urcuqui-Inchima, Roussel, & Hernandez-Verdun, 2008). Recently, it has been getting attention that liquid-liquid phase separations (LLPS) are mostly implicated in the synthesis and processing modifications during ribosomal production as some of the mayor proteins that participate contain intrinsically disordered regions (IDRs) in its sequences (Lin et al., 2015), i.e., fibrillarin, nucleolin, and GAR1 proteins, in charge of methylation, pseudo-uridylation and transcription of rRNA, respectively. LLPS, in that sense, ensures a membrane-less environment for the production and high ordered process of ribosome assembling. Fibrillar (FC) and dense fibrillar components (DFC) of the nucleolus are highly ordered structures that guide the correct transition of the pre-rRNA in order to keep a strict step by step processing.

2.2 Material and Methods

2.2.1 Plasmid constructs and E. coli strains used

The previously constructed pET42b:fibrillarin and pET15b:fibrillarin plasmids containing the human fibrillarin gene were transformed into *Escherichia coli* Bl21 by heat shock. The corresponding mases for the fusion proteins were ~ 66 kDa for the GST-fibrillarin and ~35 kDa for the 6 histidine-fibrillarin (6His-tag), both tags were in the N terminal region of fibrillarin. For plasmid amplification and cloning strategies DH5-α and TOP10 Competent cells were used.

2.2.2 Expresión y purificación de Fibrilarina de Homo sapiens (HsFib)

Recombinant 6xHis-fibrillarin was expressed in *E. coli* BL21 with 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) at 25 °C for 5 h. Harvested cells were resuspended in protein extraction buffer (500 mM NaCl, 25 mM tris pH 8, 10% glycerol, 20 mM imidazole, 0.1% Tween 20, 0.1 mM AEBSF, and 0.1 mM DTT) and broken down by sonication. After clarification by centrifugation (17,400× g for 15 min), the supernatant was loaded onto a Ni-NTA agarose column (Thermo Fisher) and washed three times with the extraction buffer and then eluted with a linear gradient from 70 to 200 mM imidazole in BC-100 buffer (20 mM Tris-HCl buffer, pH 8, 100 mM NaCl, 0.2 mM EDTA, 10% glycerol) and revised by 15% SDS-PAGE. The fraction containing fibrillarin was passed through MonoQ sepharose (Amersham Pharmacia, Buckinghamshire UK), utilizing a 0.1 to 0.5 KCl gradient to elute the recombinant protein 6xHis-fibrillarin. Fibrillarin containing fractions were pulled and dialyzed against BC-100 and 0.1 mM AEBSF. After the MonoQ sepharose purification step, the fraction containing fibrillarin was loaded on a MonoS (Amersham Pharmacia) column resin and eluted with a linear gradient from 0.1 to 0.5 M KCl in BC-100 buffer with 0.1 mM AEBSF. The purity of proteins was revised by 15% SDSPAGE, followed by silver staining. For -GST-fibrillarin, the supernatant was first loaded into a Ni-NTA agarose column followed by loading into a glutathione–sepharose column and eluted with BC-100 buffer with 10 mM of reduced glutathione and 0.1 mM of AEBSF.

2.2.3 Western blot

6xHis-Fibrillarin was loaded in 12% acrylamide gel to perform SDS-PAGE, then transferred to a nitrocellulose membrane and blocked with 3% of BSA in cold PBS at room temperature for 1 h. The membrane then was incubated with mouse monoclonal anti-histidine tag antibody (dilution 1/3000) (Abcam™, ab18184, mouse monoclonal) for 12 h at 4 °C, then following incubation with secondary antibody anti-mouse IgG (GE Healthcare, code NXA931) with three washes of 10 min each with PBS-T between incubations. Immunoblotting signals were analyzed by ChemiDoc MP Imagen System (Bio-Rad Laboratories Inc., cat.: 17001402).

2.2.3Total RNA Isolation

Total rRNA was extracted from either U2OS cell culture or *Brassica olerease* inflorescences with RiboZolTM (Solon, OH, USA), following the manufacturer's instructions. RNA pellets were resuspended in RNAse-free water treated with DEPC.

2.2.4 *Ribonuclease activity assay of recombinant fibrillarin*

Total RNA extracts from *Brassica oleracea* inflorescences were mixed with recombinant expressed GS or 6His tagged fibrillarin in RNAse activity buffer (25 mM Tris-HCl pH8, 100 mM NaCl, 0.1 mM EDTA, 0.1 DTT, and glycerol 10%) + 0.8 to 1 U/µL of RNAse inhibitor (Thermo Fisher) and incubated for 45 min at 37 °C, then loaded in a 3% agarose gel.

2.3 Results

2.3.1 Fibrillarin possess ribonuclease activity

As shown in the Figure 6, human recombinant 6xHis-fibrillarin was expressed in bacteria and detected in both fractions of the bacterial extract using monoclonal anti-histidine antibody (A) lanes2 and lane 3), a high-quality protein was obtained using a gradient of imidazole as the figure 6B shows. Also, the protein pure was passed through Amicon® protein concentrator (Figure 7). The GST tagged fibrillarin was also purified by two-step IMAC, and a silver staining was used to evaluated the purity of the obtained protein (Figure 8). Ribonuclease activity of fibrillarin was evaluated at different amounts of mass and with or without calcium, a known cofactor for other described ribonucleases. rRNA degradation was observed using 2-4 ng of total protein added to the reactions, figure 9. Temperatures was a key factor influencing the activity of fibrillarin as shown by the fact that null activity was detected at 4 °C, while a full degradation was observed at 37 °C after 1 hour of incubation. Also, time of incubation and zinc addition were evaluated in order to observe the effects of these two parameters in the reaction. As shown in the figure 10, a total degradation was evident at 45 min of incubation and the addition of zinc shown no influence in ribonuclease activity.

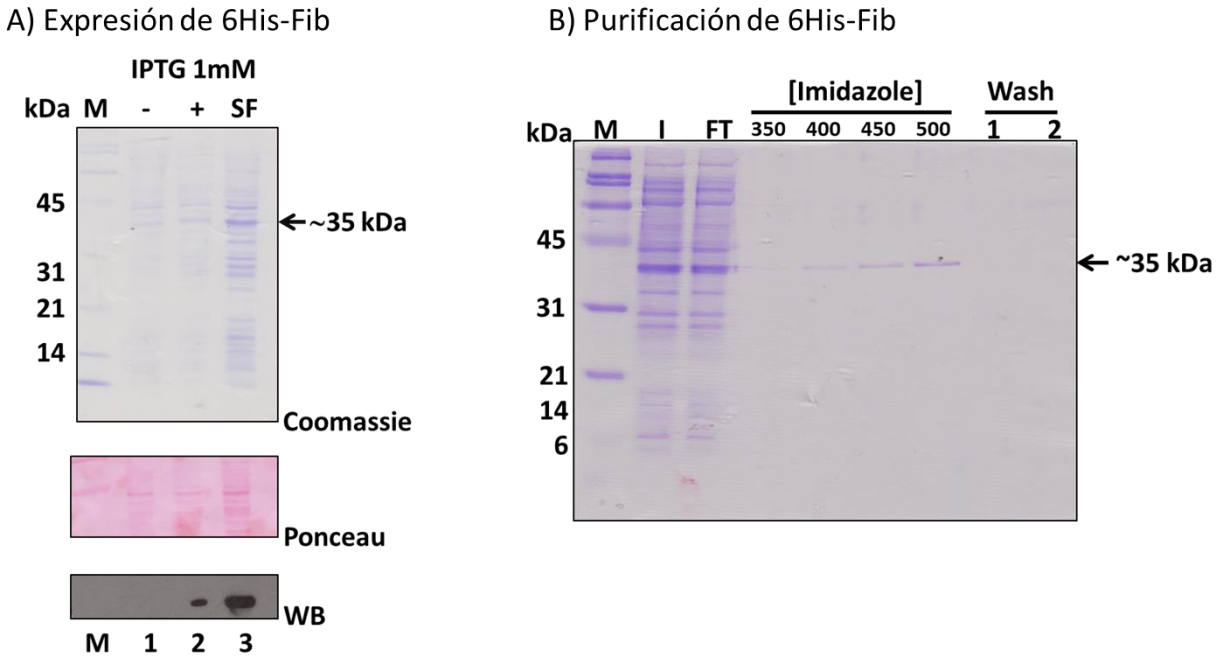


Figure 6. Expression and Inmuno detection of recombinant 6xHis-fibrillarin. A) Expression and Western blot immunodetection with monoclonal anti his antibody and B) Imidazole gradient purification of 6Histidine tagged fibrillarin. Total extract was loaded in A) as follow: lane 1 total E. coli extract without IPTG induction, lane 2 E. coli total extract after IPTG induction and lane 3 soluble fractions after E. coli lysate. In B) samples from purification by IMAC His-Ni affinity were loaded as indicated by the figure as follow, I: input, soluble fraction, FT: flowthrough, imidazole gradient from 350 to 500 mM and washes.

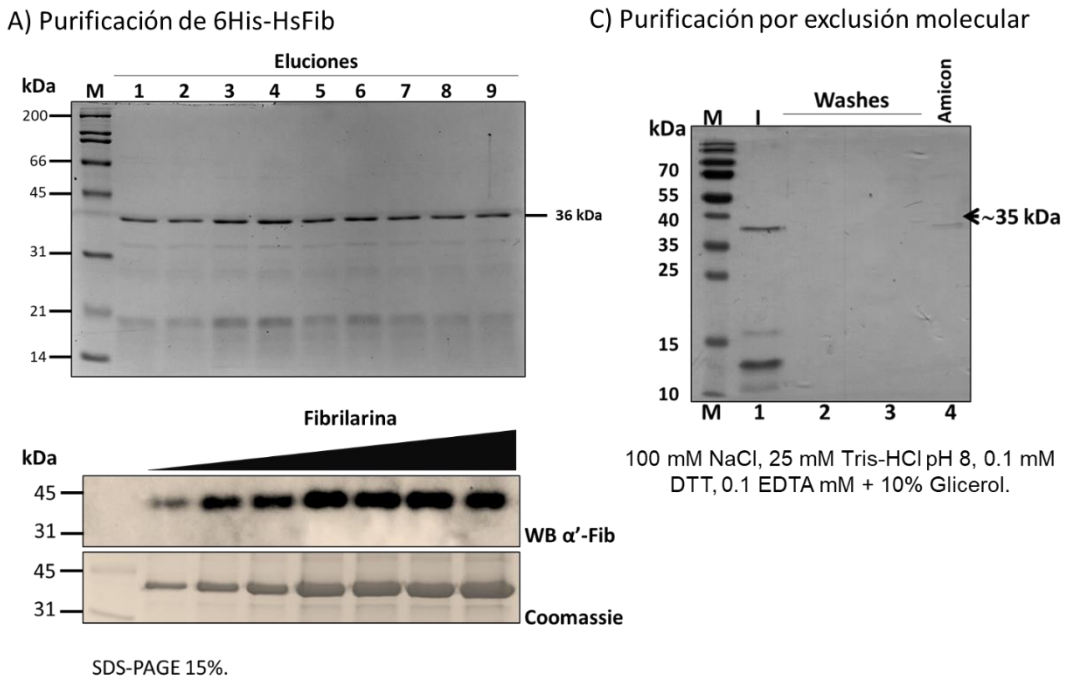


Figure 7. Molecular exclusion purification of 6xHis-fibrillarin. A) Elutions samples from IMAC affinity to Ni purification were loaded on SDS-PAGE and stained with Brilliant Coomassie blue to check the purity of the protein samples. B) Western blotting with increasing amounts of recombinant fibrillarin loaded. C) After a pooled mix from A) 6xHis-Fibrillarin samples were concentrated with AmiconTM devices and buffer exchange chromatography. Recombinant fibrillarin was detected with Coomassie blue and buffer considerations are described in the figure.

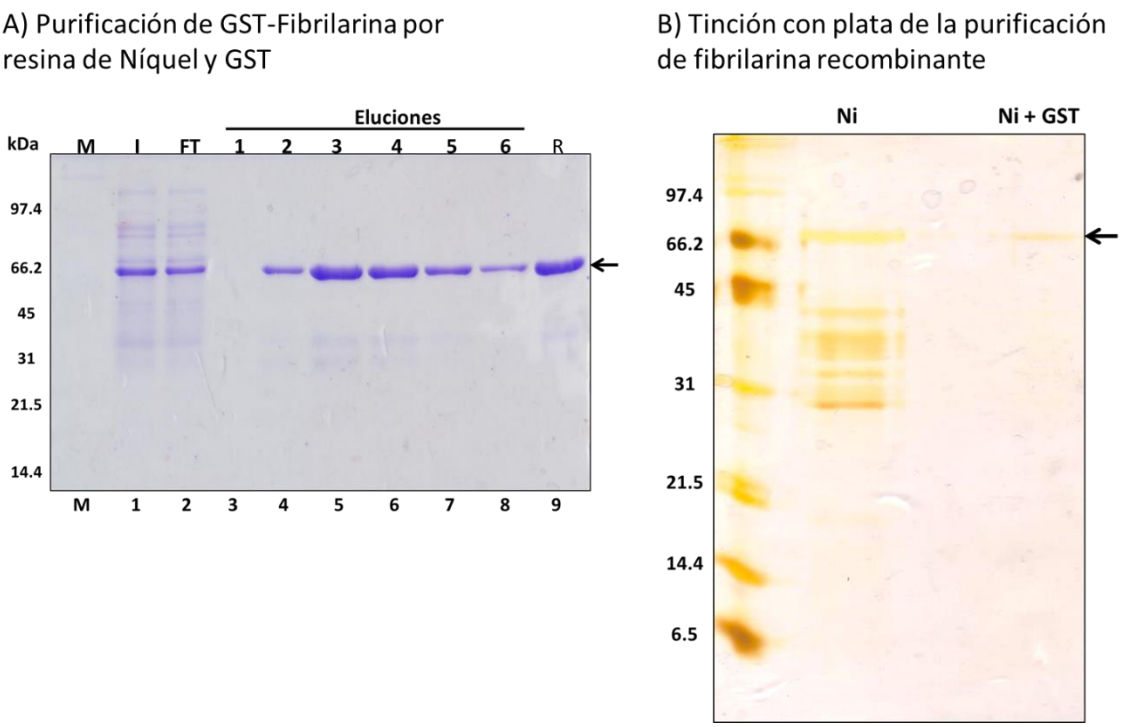


Figure 8. Double tag affinity purification of GST-fibrillarin. As detailed by the vector pET42b, recombinant proteins were fused to GST and a 8 histidine tag. We take ventage from this to consider a double step purification using IMAC affinity by histidine showed in A) ad using a GST tagged after. A double step purification strategy was employed in order to obtain a highly pure GST-fibrillarin. As shows in A) Nickel affinity purification was carried out with a 300 mM of imidazole. And then following an GST affinity purification. Showing in B) A silver stained SDS-PAGE after the double step purification was evaluated in order to see the purity degree of the protein preparations. Ni: shows samples loaded after the first IMAC purification and Ni-GST: shows samples from the double step purification. Amounts from proteins samples were adjusted to overcome the high sensitivity of silver staining.

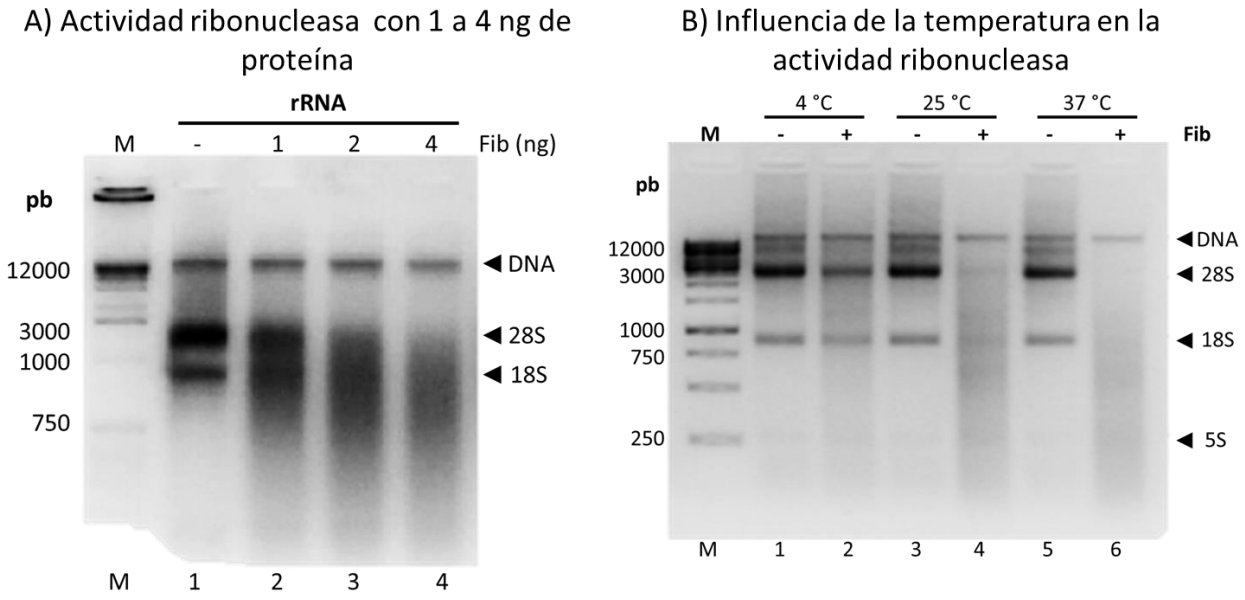


Figure 9. Fibrillarin as a ribonuclease. Human recombinant fibrillarin tagged with 6 Histidine were used for ribonuclease activity assays. A) shows activity of ribonuclease activity of fibrillarin under activity buffer using 1, 2 and 4 nanograms of pure protein. As appreciated in the figure there is a linear correlation with the degradative activity of fibrillarin and the amount of protein used in the assays. Following in vitro activities were carried out using 2 nanograms of protein. B) shows the dependency of ribonucleasa activity on temperature, as detailed by the figure at 4 °C and 25 °C no activity was observed, but at 37 °C a full degradation can be observed of the 2 micrograms of total RNA used in the assay.

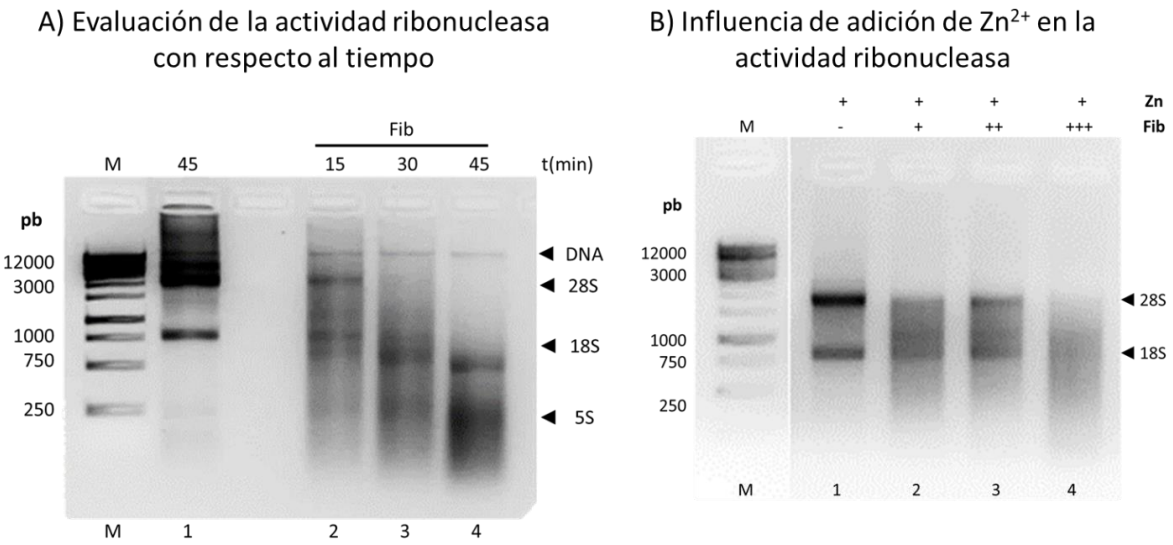


Figure 10. The influences of the incubation time and the presence of Zinc ions in the ribonuclease activity of fibrillarin. The figure shows in A) the influence of time incubation of fibrillarin and total RNA in ribonuclease buffer activity. 45 min with no protein added to the reaction preserves its integrity while in the incubations with 2 nanograms of recombinant fibrillarin at 15, 30 and 45 min a dependence on the degradation of RNA can be appreciated. Showed in B) some cofactors have been described as modulators of ribonuclease activity, here we tested the influence of zinc ion with increased amount of fibrillarin. 1 mM of zinc ion was used in the reactions and protein amount was increased in order to see the effects on the degradative activity of fibrillarin. No clearly effects were observed as the activity increased with amount of fibrillarin used, so we discard a possible influence of zinc ion in the activity of fibrillarin.

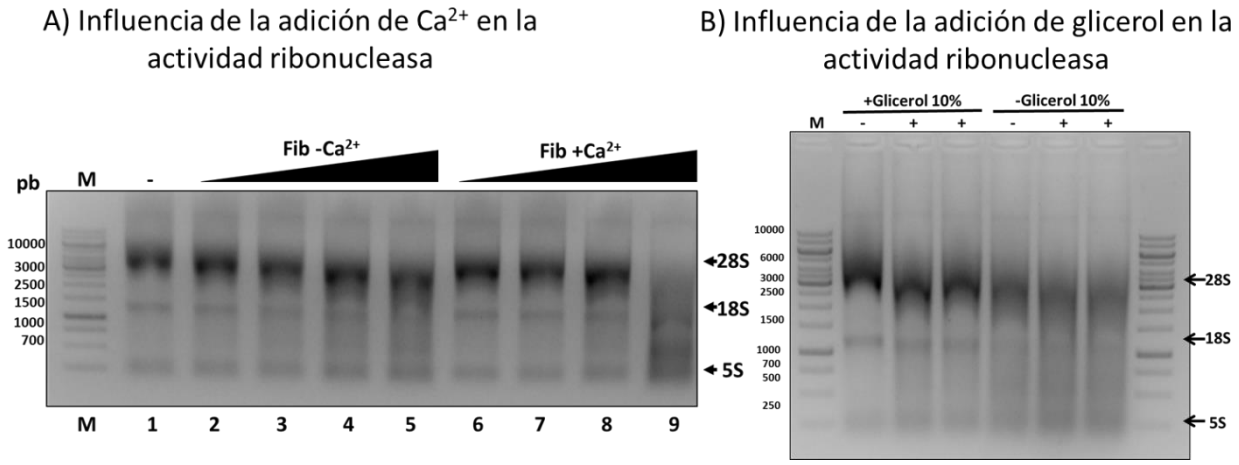


Figure 11. Calcium Ion and glycerol influence on the ribonuclease activity of fibrillarin as detailed in Material and methods section. Calcium ion also was described as a ribonuclease activity cofactor, following that idea we addressed the possibility that fibrillarin activity as a ribonuclease was related with the addition of calcium ion. Showing in A) we tested the ability of fibrillarin to degraded RNA in the absence/presence of calcium. No clearly induction or stimulation of the activity was observed in the following experiment. Also, we considered that our protein preparations were in a high glycerol concentration preventing the ribonuclease activity to be observed. Showed in B) we evaluated our protein preparation with or without the 10% of glycerol added to the activity buffer. As appreciated the addition of glycerol inhibit the degradative activity of fibrillarin against total RNA. Considering this we developed an activity buffer without ions or high glycerol content to evaluated the activity of fibrillarin as a ribonuclease.

2.4 Conclusions of the chapter

The human fibrillarin was purified to homogeneity according to the established protocols with an imidazole gradient for the 6xHis tagged fibrillarin and with a double step purification for the GST tagged protein. Also, a molecular exclusion strategy was employed for a highly pure fraction of 6xHis-fibrillarin. The *in vitro* ribonuclease activity was evaluated with this purification process and al the procedures were conducted in the same degree of protein purification. We concluded and demonstrate the ribonuclease activity of the human protein fibrillarin.

RNase contamination was discarded using DEPC treated solutions for all the purifications steps. Interesting to note was the fact that as the *A. thaliana* fibrillarin, our protein preparations was able to degraded total RNA with amounts of protein between 1 to 4 nanograms. With an optimal temperature of the activity at 37 °C. incubation time was evaluated from 15 to 45 min, with a clear correlation between time and degradation of rRNA increasing the time of incubation with equals amount of fibrillarin at 2 nanograms and 3 nanograms of total rRNA in the reactions. Reducing the amount of fibrillarin under 1 nanogram with test the ability of fibrillarin to degrade rRNA in presence of ions like zinc and calcium, both described as a cofactor for ribonucleases. To note, zinc and calcium ions in the activity buffer no clearly enhance of the degradative activity of fibrillarin was observed in the *in vitro* assays.

Capítulo III. Phospholipid influence on ribonuclease activity of fibrillarin

Phosphoinositide dependence of the ribonuclease activity of fibrillarin against total rRNA

3.1 Introduction

For several years the involvement of lipids and phosphoinositides inside the nuclear and nucleolar bodies has remained as an unclear field (Hoboth et al., 2021; Ulicna, Kalendova, Kalasova, Vacik, & Hozák, 2018). Important clues come from experimental data observing its localization using confocal microscopy and antibody fluorescence strategies to reveal how spatial and co-localizing partners plays important roles in transcription, association with chromatin remolders and regulation of enzyme activity inside the nuclear environment. Sobol et al., 2016 described the involvement of PI4,5P as a key regulator of Pol I RNA transcription and the association of important players as UBF1 and fibrillarin nucleolar protein to this phosphoinositide. In the nucleolus exist different populations of RNA, some involved in RNA processing and other forming complexes with proteins as the snoRNAs and lncRNAs. We asked if fibrillarin can degrade other RNA populations. We found that a different pattern of degradation using RNA extracted from the nuclear pellet, indicating that a subset of RNAs is subjected to site specific cleavage. Here we also evaluated if the activity of fibrillarin as a ribonuclease was affected in presence or absence of phosphoinositides.

3.2 Material and Methods

3.2.1 Cytoplasmatic and Nuclear RNA Extraction from U2OS Human Cells

The dishes containing U2OS Human cells were washed three times with cold PBS, then 500 µL of Lysis buffer (50 mM Tris-HCl pH 7, 150 mM NaCl, 2 mM MgCl2, 0.5% NP-40, 0.02U/µL RNAse Inhibitor) was added, scraped and re-suspended by pipetting up and down. The cells in lysis buffer were transferred on 1.5 mL tubes and centrifuged for 1 min at 1000 x g at 4 °C and the supernatant transferred to a fresh tube. The pellet and the supernatant were

used for nuclear and cytoplasmatic RNA extraction following the RibozolTM manufacturer' instructions.

3.2.2 Ribonuclease activity assay with lipids

Total RNA extracts frm U2OS cells or *Brassica oleracea* inflorescences were mixed with recombinant expressed 6His tagged fibrillarin in RNAse activity buffer (25 mM Tris-HCl pH8, 100 mM NaCl, 0.1 mM EDTA, 0.1 DTT, and glycerol 10%) + 0.8 to 1 U/µL of RNAse inhibitor (Thermo Fisher) and incubated for 45 min at 37 °C, then loaded in a 3% agarose gel. Phosphoinositides and PA were added to the final amount of 50 ng.

3.3 Results

Ribonuclease activity assay were carried out using 1 nanogram of recombinant human fibrillarin preincubated with phosphoinositides (50 nanograms) and mixed with total rRNA (2 µgrams), figure 12. Surprisingly, the preincubation with the phosphoinositides PI3,4P and PI3,5P inhibit the ribonuclease activity observed in absence of them. On the other hand, in presence of PI4,5P, the same pattern of degradation can be detectable in the agarose gel, in contrast to PI3,4P and PI3,5P (Figure 12). The tri-phosphorylated phosphoinositide PI3,4,5P inhibit the degradative activity of fibrillarin. Something to note is the inhibition of the activity by the presence of phosphatidic acid (PA) and the rescue when calcium is added to the reaction. The ability to enhance the activity of the mono phosphoinositide PI5P was previously observed in our group. We next evaluated its enhanced activity in presence of PI5P preincubated with recombinant fibrillarin, Figure 13. Following the idea that other populations of RNA can be found in the nucleolar context we developed a ribonuclease assay to test if fibrillarin can degraded this different RNA population. Nuclear peller associated RNA was isolated with previously reported protocol in our lab, then mixed with different amounts of fibrillarin from1 nanogram to 2 nanograms and lipids added to the reactions. Figure 14 details this experiment as follow:

Following the idea that a site-specific cleavage can be the mechanism by which fibrillarin is able to degrade RNA we designed a subset of synthetic RNA probes as depicted in the figure 15. However, no RNA degradation can be detected using the synthetic RNAs but a

changed in the electrophoretic mobility can be appreciated (Figure 16). Synthetic RNAs were designed to test the ability of fibrillarin to degrade RNA in a specific manner. The RNA probes containing C/D boxes, secondary structures and double stranded RNA molecules (dsDNA) are shown in as shown in figures 16 A and B. Interestingly, no degradation was observed in presence of these type of RNA molecules. An EMSA assay was conducted to determine if fibrillarin binds to these synthetic RNA probes. A slightly shift in the mobility of some species was observed in the figure 16.

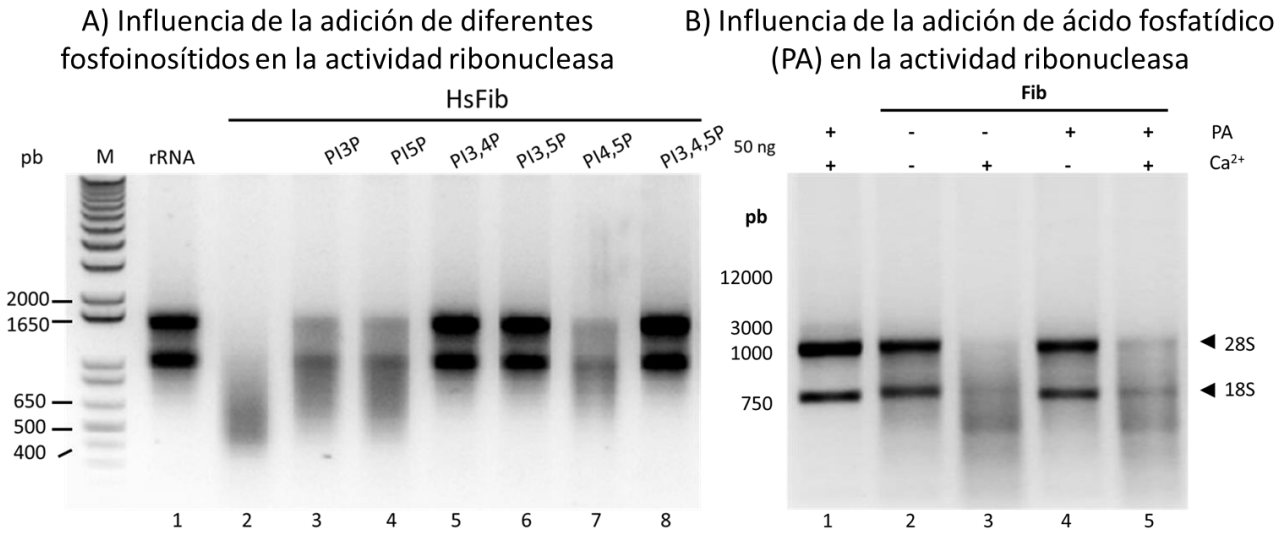


Figure 12. Ribonuclease activity of human fibrillarin and the influence of phosphoinositides on its activity. A) influences of the mono and bis phosphoinositides was evaluated, the lanes 3 and 4 correspond to PI3P y PI4P; lanes 5, 6 and 7 correspond to PI3,4P, PI3,5P and PI4,5P. interesting to note was the fact that the mono phosphoinositides inhibit the activity with a slower degree, while the presence of bis phosphoinositides inhibit fully the activity of the total rRNA. phosphoinositide PI3,4,5P (lane 8) inhibit the ability of fibrillarin to degraded rRNA. Also, the activity of fibrillarin was **inhibit by the presence of**

PI4,5P (A, lane 7 y B, lane 8). B) The addition of phosphatidic acid to the reaction inhibits the degradative effect of fibrillarin.

A) PI5P activa la actividad ribonucleasa de fibrilarina, dependiente de la concentración de proteína

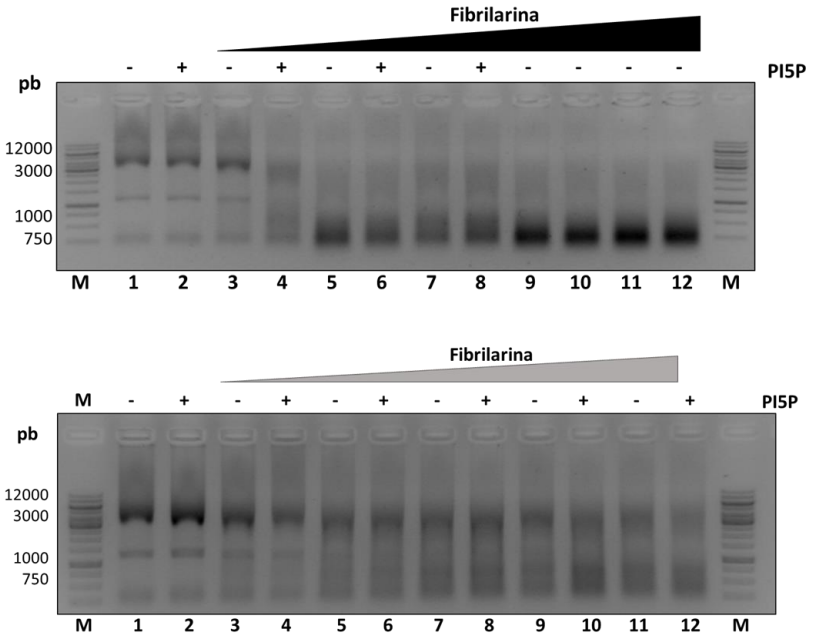


Figure 13. Phosphoinositol 5 phosphate influence on the ribonuclease activity of fibrillarin. In this experiment wit considered the activity of fibrillarin influenced by the addition of phosphoinositides in the last in vitro assay. Here we show in the upper panel the influence of PI5P on the activity of fibrillarin from 1 to 5 nanograms. Lanes 1 and 2 corresponding to the total RNA with PI5P as a control of no degradative effect. Lanes 3 and 4 shows 1 nanogram of fibrillarin with or without PI5P, lanes 5 and 6 shows 2 nanograms of fibrillarin with or without PIP5, lanes 7 and 8 shows 3 nanograms of fibrillarin with or without PI5P and the lanes 9 and 10 shows 4 nanograms of fibrillarin without IP5 and lanes 11 and 12 shows 5 nanograms of fibrillarin without PI5P. No clearly effect was observed so we reasoned that the possibility of influence could be observed at concentration bellow the 1 nanogram order. Lower panel shows and describes the activity of fibrillarin under the influence of PI5P using 0.5 to 2.5 nanograms of fibrillarin. More the interesting result comes from the lanes 2 and 3 were the addition of PI5P clear enhance the activity of 0.5 nanograms of fibrillarin. Lanes 4 to 12 shows increasing amounts of fibrillarin in a 0.5 nanogram order. Most of the evidence are related with a singular pattern observed after preincubation of fibrillarin with PI5P. A set of bands can be appreciated in the lower part of the agarose gel. Describing a possible mechanism of activity of recombinant fibrillarin.

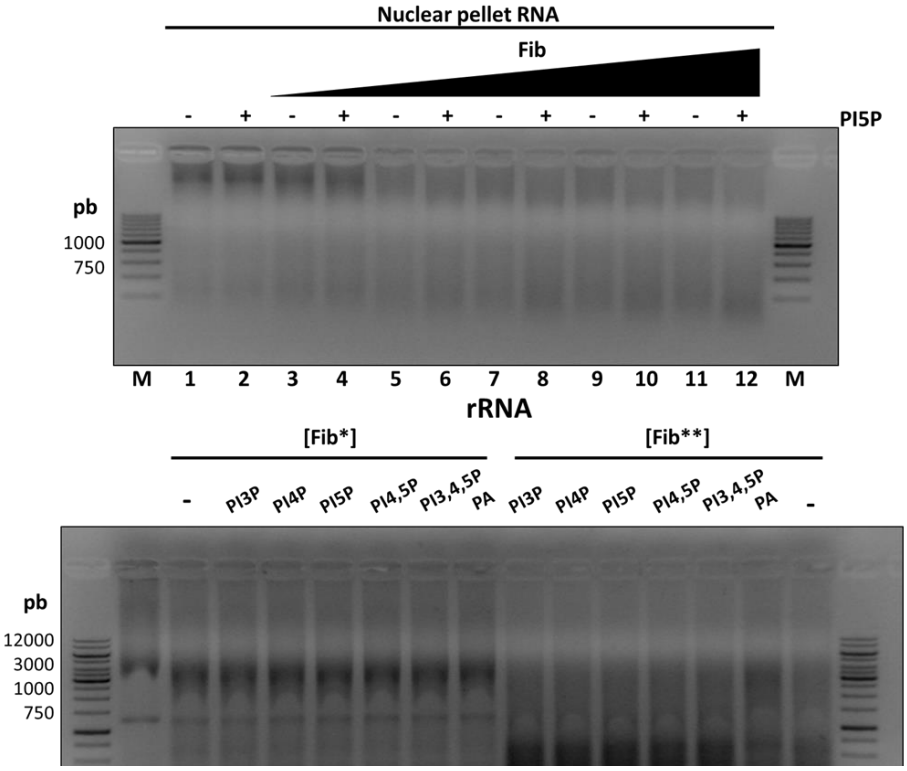


Figure 14. Fibrillarin ribonuclease activity against Nuclear associated RNA. Considering the other RNA populations present inside the nucleolus we isolate nuclear pellet RNA associates as previously described in methods. Also, we consider to test the ability of fibrillarin to degraded nuclear pellet associated RNA in presence of phosphoinositides. Figure 14, upper panel describes the influence of PI5P on the ribonuclease activity of fibrillarin against nuclear associated RNA. 0.5a and 2 to 2.5 nanograms of fibrillarin was used for the assay, as showed in the figure no clearly influence was observed. Lanes 1 and 2 shows control of RNA and lipid, and following lanes shoes increasing amounts of fibrillarin in a 0.2 nanogram order. Lower panel describes the activity of fibrillarin using only 0.5 and 1 nanograms, but adding PI3P, PI4P, PI5P, PI4,5P, PI3,4,5P and PA to the reactions. With the 0.5 nanograms of fibrillarin again a pattern was observed in the degradative activity of fibrillarin against pellet RNA. While using 1 nanograms of fibrillarin, no clearly effect was observed. A minor inhibition was observed in presence of PA in the lane 14 of the lower panel describing a possible role of PA under these conditions.

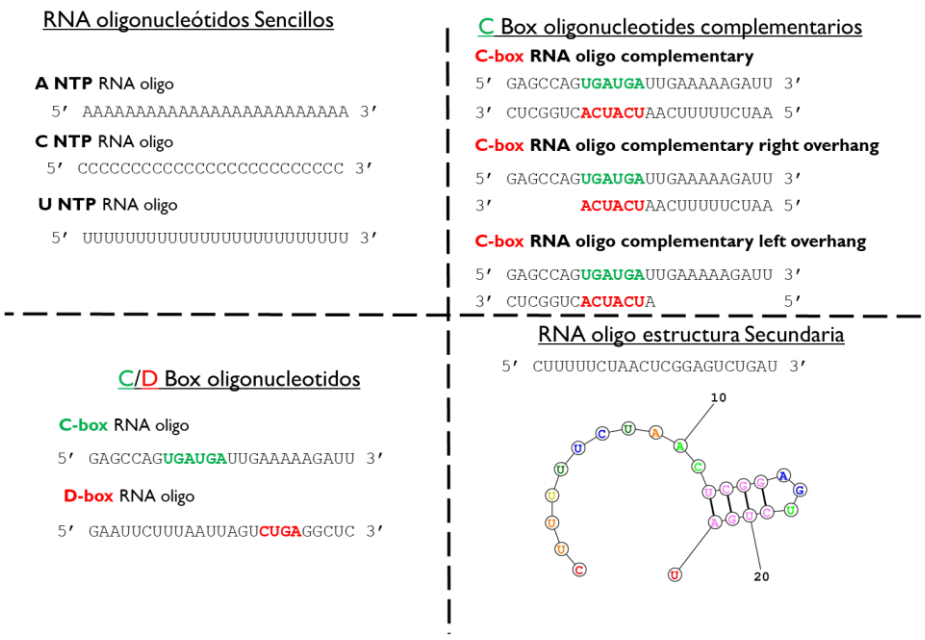


Figure 15. Synthetic RNA primers designed to test the specificity of the ribonuclease activity of fibrillarin. Following the idea related to a site-specific activity of fibrillarin as a possibility of the mechanism of degradation activity we developed a series of synthetic RNA sequences to test this specificity. The figure details the RNA sequences designed as follows: single nucleotide RNA sequences with only A, C and U. C/D box oligonucleotides depicted in green and red. Complementary sequences, in order to define if there is a possibility of double stranded degradation of RNA. And finally, an RNA sequence with a secondary structure.

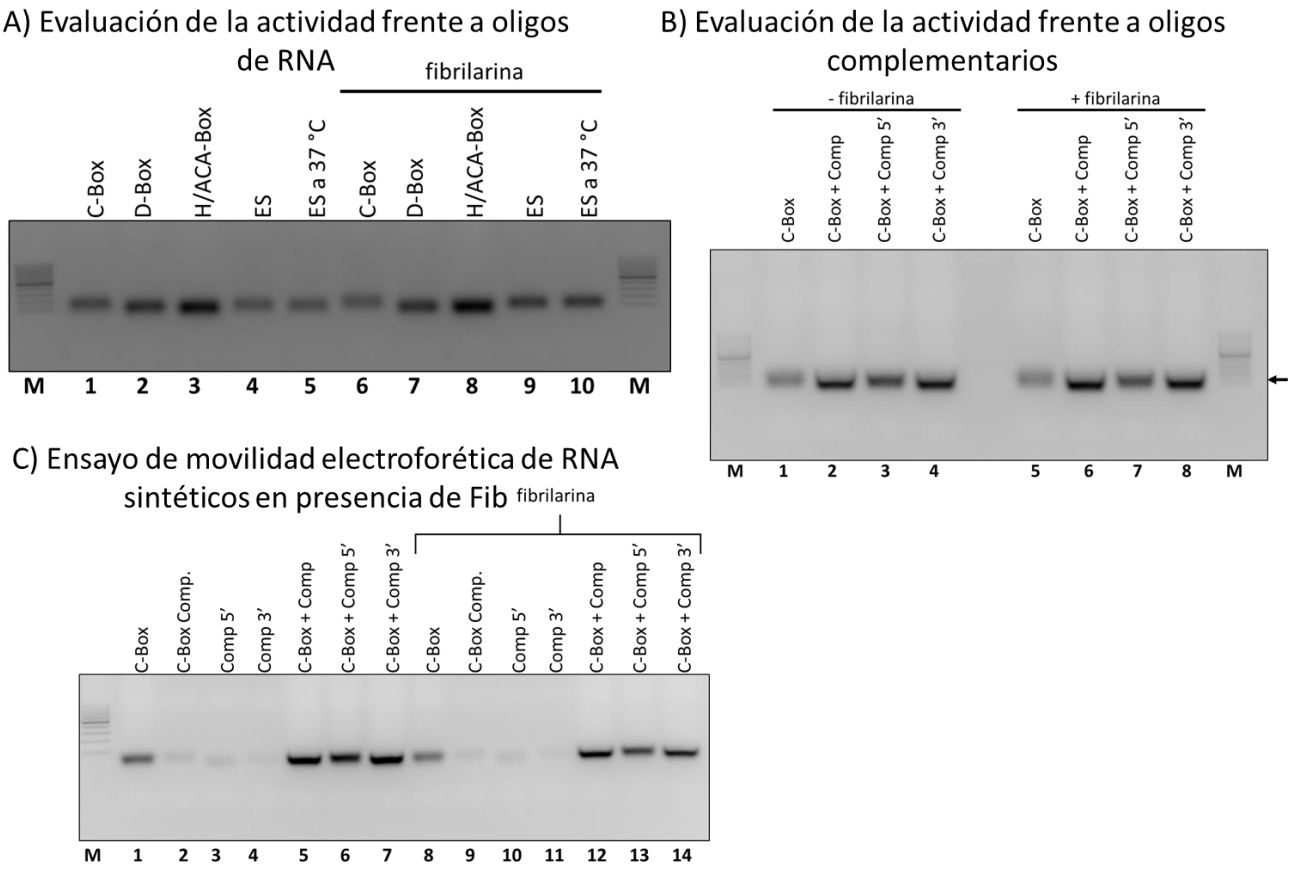


Figure 16. Ribonuclease activity assays against synthetic ssRNA and dsRNA. Showing in A) the ribonuclease activity of fibrillarin against the synthetic RNA sequences designed, as can be appreciated no degradation was visible even the amount of fibrillarin of 2 nanograms, in contrast of total RNA. B) shows the respectively assay using the complementary RNA sequences designed to create double stranded RNA molecules, also no degradations were appreciated. Controls were load on the gel with fibrillarin incubation and with 2 nanograms of fibrillarin. C) Electrophoretic mobility shift assay using synthetic RNA and 2 nanograms of fibrillarin. The addition of fibrillarin in lanes 8 to 12 shows a slight change of electrophoretic mobility of the double stranded RNA molecules.

3.4 Guided Ribonuclease activity of fibrillarin in presence of guides snoRNA

The methyltransferase activity of fibrillarin coupled to a ribonucleoparticle is guided by the participation of a set snoRNA, which contains specific stretches of complementary to the residues to be methylated (Falaleeva et al., 2016; A Fatica, Galardi, Altieri, & Bozzoni, 2000; Martin et al., 2014; Nazar, 2004). We reasoned that a ribonuclease activity residing in fibrillarin could be guided in the same way as the methyltransferase activity. Following these ideas, we cloned the snoRNA guides U3 and U8 in to pGEM T-Easy plasmid under the T7 promoter in order to transcribe them *in vitro*.

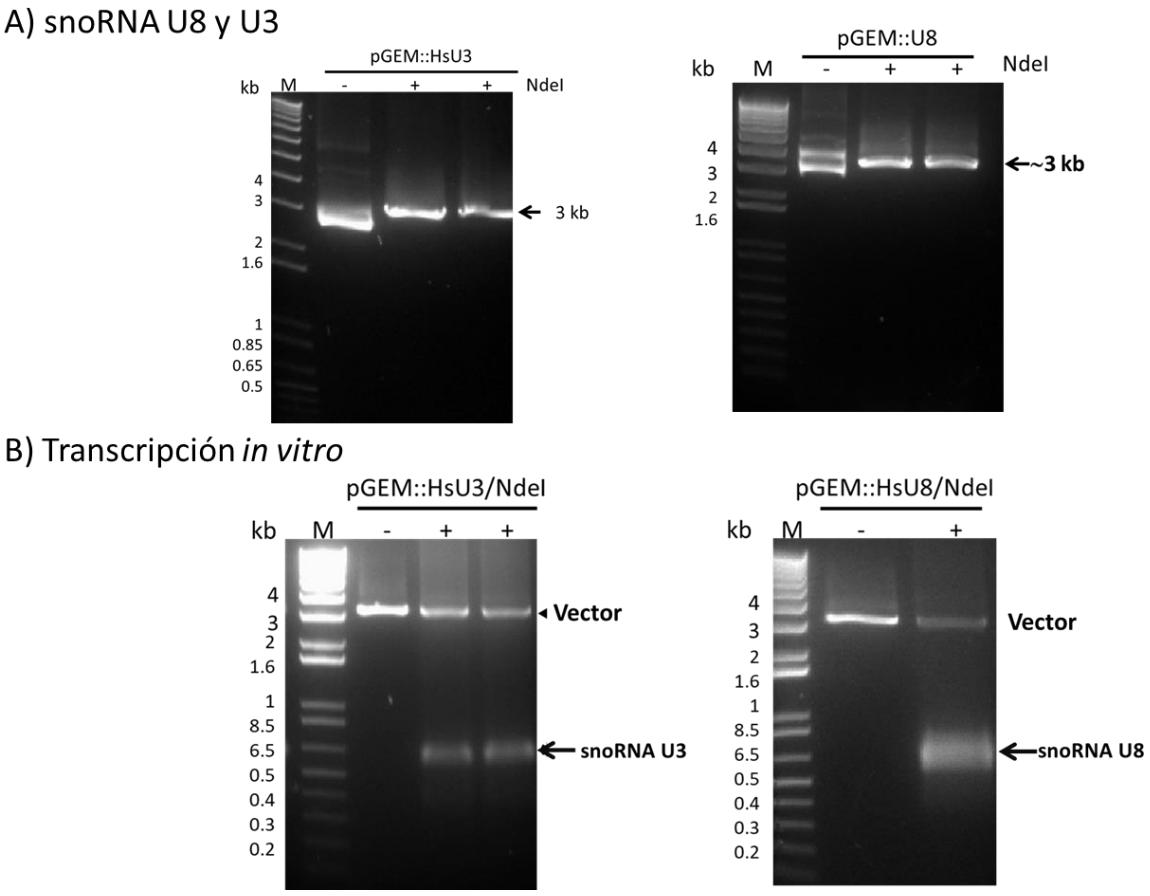
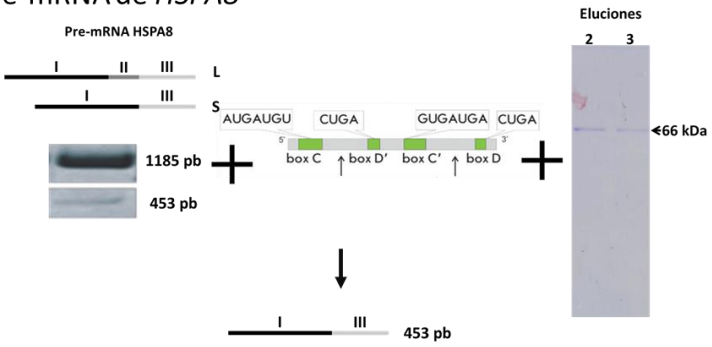


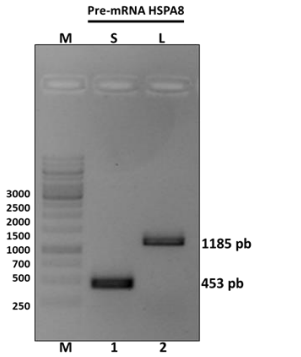
Figure 17. In vitro transcription of snoRNA guides U3 and U8. A) Plasmids containing the U3 and U8 genes previously cloned by Rodriguez Corona et al., 2017 were linearized using NdeI restriction enzyme. B) *In vitro* transcription using T7 RNA polymerase following NEB specifications with minor changes. Agarose gel 1% was stained with sybr safe (Thermo Scientific™).

In the other hand a specific gene known as the Hshp70 was cloned and used as an RNA target, cloned into the pJET1.2 vector under T7 promoter. This idea comes from a report were a pre mRNA was modified by the presence of snoRNP, including complexes containing fibrillarin (Frottin et al., 2019), indicating a possible role for a site specific activity of the ribonuclease containing snoRNPs.

A) Variantes en el pre-mRNA de *HSPA8*



B) RT-PCR del pre-mRNA *HPSA8*



Fw HSPA8 5'-ACTGAACGGTTGATCGGTGA-3'
Rv HSPA8 5'-AGATGAGCACGTTTCTTTCT-3'

C) Clonación de las variantes del pre-mRNA *HPSA8*

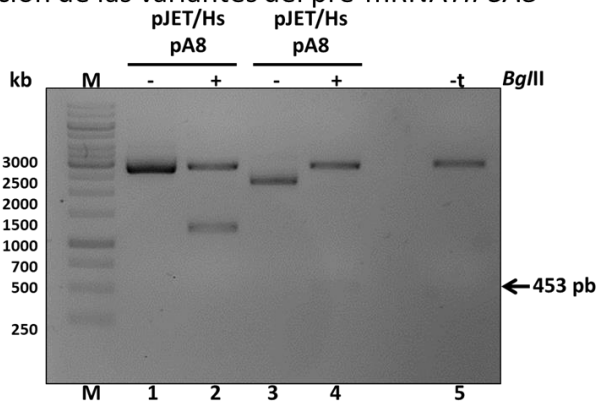


Figure 18. Cloning of the Hsp70 gene under T7 promoter. Two variants of the gene were cloned, a 1185 pb and 453 bp population was observed in the report from Frottin et al., 2019 as depicted in A). B) shows the amplification by RT-PCR of the variants using the primers designed in the figure and c) Shows the cloning of the variants long and short of Hspa8 into pJET1.2. Insert was observed after a BglII restriction enzyme flanking the multiple cloning site treatment.

3.5 Conclusion of the chapter

In this chapter we concluded with the findings related to the addition of phosphoinositides on the ribonuclease activity of fibrillarin and the relevance of the modifications of its activity under the presence of several types of RNA sequences designed to revel some insights on the mechanism of fibrillarin ribonuclease activity. We found that some type of phosphoinositides specially the PI5P can modulates the activity in order to make more visible the degradative effect of fibrillarin against total RNA and nuclear associated RNA. For the other hand PA can inhibit the activity of fibrillarin in the conditions used in the *in vitro* assays. PI4,5P also can inhibit the activity of fibrillarin during the pre-incubation with this phosphoinositide. Moreover, there is a possibility that a conformational change can be produced with the disordered region of fibrillarin called GAR domain. AIso, our data presented in this chapter clearly shows that no site-specific activity was observed, but this is not fully discarded as a possibility of the principles of activity and mechanism of fibrillarin as a ribonuclease. The evaluation of pre mRNA modification activity was not carried out yet, and this still is a topic to do some research and shed light into the mechanism of fibrillarin. Considering these results, we move on the hypothesis that fibrillarin can be dissected to define the region were the ribonuclease activity reside, and taking together this information and the published in our lab we evaluated the GAR domain as a possible region of activity.

CAPÍTULO IV. GAR Domains from Fibrillarin, SLM14 and Nucleolin possess Ribonuclease Activity

4.1 Introduction

Intrinsically disordered domains are characterized by the presence of stretches of particular amino acids: arginine, glycine and polar and aromatic residues. A great example of this is the repeated motif RGG founded in several proteins, also known as the GAR domain(Chong, Vernon, & Forman-Kay, 2018; Z. Li et al., 2018; McBride, Conboy, Brown, Ariyachet, & Rutledge, 2009; Smith et al., 2019; Yao et al., 2019). The GAR domain is present in several nucleolar proteins, i.e., nucleolin, NSR1, SSB1, and GAR1 protein, which have RNA recognition motifs like fibrillarin, directly interact with rRNA, and are involved in its processing. In fibrillarin, the GAR domain is needed to target nucleoli and for interaction with nuclear phase viruses. In this chapter we evaluated the ribonuclease activity of other proteins that possess intrinsically disordered domains with RGG repeats in its sequence.

4.2 Material and Methods

4.2.1 Sequence cloning of the GAR domain from fibrillarin, Lsm14, GAR1 and nucleolin.

Total RNA from U2OS cells was extracted with RiboZol™ (Solon, OH, USA), following the manufacturer's instructions. The sequences from fibrillarin, GAR1, and SLM14 were obtained using cDNA from the reverse transcriptase strategy of TaqMan™ and amplified by DreamTaq™ polymerase (Thermo Fisher Scientific, Waltham, MA, USA), following the provider's instructions. The N-terminal domain (amino acids 1 to 134) containing the GAR domain from human fibrillarin was amplified from the pET15b:HsFib vector with the following specific primers GB_forward 5'-CCATGGATGAAGCCAGGATTCAGTCCC-3'; GB_reverse

5’-CTCGAGGTACTCAATTTTGTCATCTCCTTCC-3’, and cloned into the *NcoI* and *XhoI* sites of the pET42b vector.

The specific primers used for cloning the GAR domains from GAR1 and Lsm14 were FwGAR1 5’-CCATGGATATGTCTTTTCGAGGCGGAGG-3’, and RvGAR1 5’-CTCGAGATGTCCTCTCCCTCTGAAACC-3’ between *NcoI* and *XhoI* restriction sites, respectively. For GAR-Lsm14, the primers were Fw 5’-AAGAATTCGATGACAATAGAGAA-3’ and Rev 5’-TAAGCTTAG GGTCCAAAAGCTGTGCTGT-3’ between *EcoRV* and *HindIII* restriction sites, respectively.

4.2.2 *Expression and purification of GAR domains from Fibrillarin, GAR1, Slm14 and TGB viral protein.*

Homo sapiens GAR1, Lsm14, HsGAR-Fib, and the viral protein TGB1 were expressed in *E. coli* Rosetta competent cells. The recombinant production of these proteins was induced with 1 mM IPTG at 25 ◦C for 4 h. Induced cells were harvested by centrifugation at 4000 × *g* for 20 min at 4 ◦C, resuspended in lysis buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 20 mM Imidazole, 10% glycerol, 0.1% of Triton X-100) and supplied with 0.1 mM AEBSF and 0.1 DTT as a protease inhibitor to reduce respective agents, then sonicated 10 times with 30 s ON/30 s OFF cycles. The fragmented cells were clarified by centrifugation at 15000 × *g* for 20 min at 4 ◦C, and the supernatant was clarified for the IMAC purification strategy, using 100 µL of nickel beads (Thermo Fisher^{TM}) and incubated for 30 min at 4 ◦C in a rotor. The column was washed using 10 volumes of beads lysis buffer, along with an increased concentration of NaCl from 100 to 500 mM. The proteins were eluted with 50 mM-Tris-HCl pH 8, 100 mM NaCl, and 250 mM imidazole and supplied with 10% glycerol, 0.1 mM AEBSF, and 0.1 mM DTT. All purification steps were done at 4 ◦C to reduce proteolysis. Proteins were stored at -80 ◦C until use in the next experimental procedures.

4.2.3 *In vitro Ribonuclease activity*

Total RNA extracts from HeLa cells were mixed with recombinant expressed GAR domains form fibrillarin, GAR1, Lsm14, nucleolin or TGB1 in RNAse activity buffer (25 mM Tris-HCl pH8, 100 mM NaCl, 0.1 mM EDTA, 0.1 DTT, and glycerol 10%) + 0.8 to 1 U/µL of RNAse

inhibitor (Thermo Fisher) and incubated for 45 min at 37 ◦C, then loaded in a 3% agarose gel. Phosphoinositides and PA were added to the final amount of 50 ng.

4.3 Results

As detailed in figure 19, GAR domain from fibrillarin was exhaustively purified by the combination of two tags containing in the cloned vector. IMAC purification was carried out first with Ni resin and followed by glutathione resin affinity purification. Ribonuclease activity of GAR domain from fibrillarin was evaluated against rRNA. Our data confirm, that the GAR domain of Hs-Fibrillarin contains the ribonuclease activity, as previously seen for the *A. thaliana* fibrillarin GAR domain (Rodriguez-Corona et al., 2017). Moreover, GAR domains subjected to denaturalization at 95 °C were able to degraded rRNA.

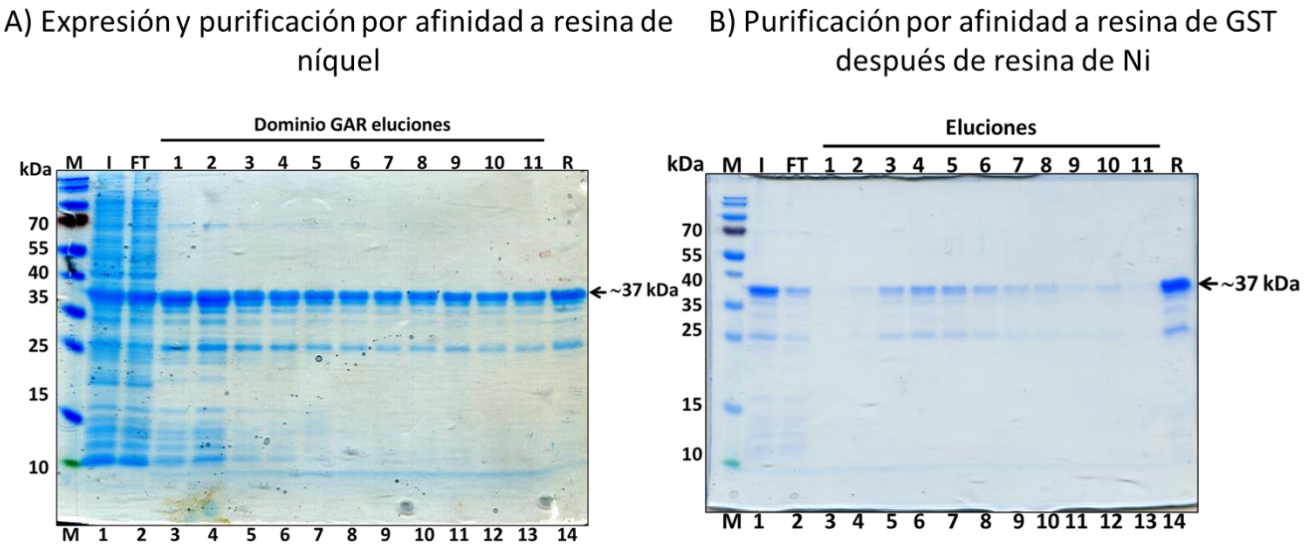


Figure 19. Results from the His and GST affinity purification of the GAR domain from fibrillarin. The GAR domain from fibrillarin was cloned into pET42b vector, fused to a GST and a 8 Histidine tag with the advantage to address a double step purification. Panel A) depicts the initial step of purification by a IMAC affinity to nickel, lanes 3 to 11 shows fractions eluted from the nickel resin as described in material and methods. B) shows the purification after GST affinity resin using as input the elutions from A). The corresponding molecular weight is marked by an arrow. I: input and FT: flowthrough.

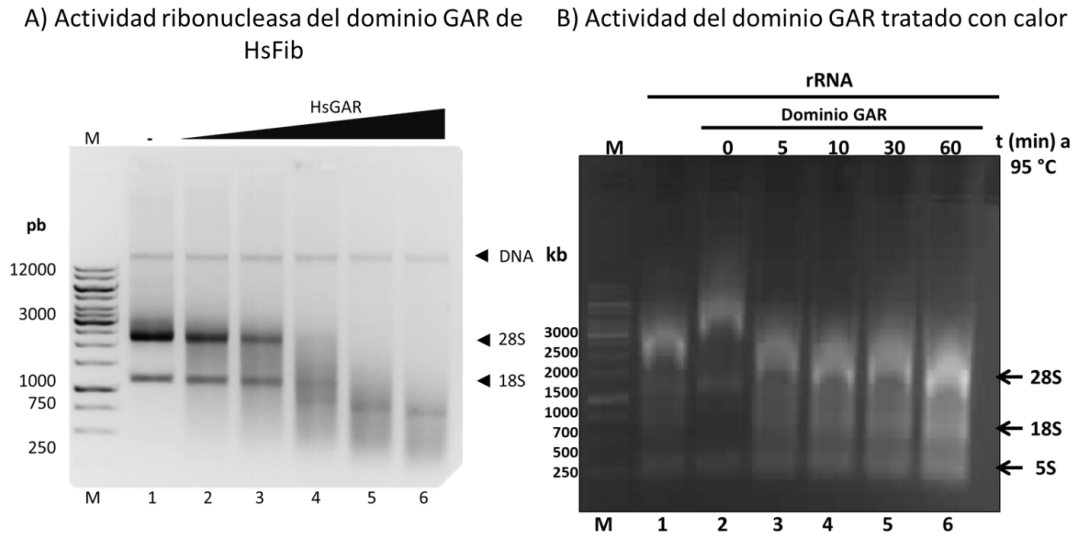


Figure 20. Ribonuclease activity evaluation of the fibrillarin GAR domain and the effect of denaturalization in the ribonuclease activity. The figure 20 panel A) describes an increasing amount of double step purified GAR domain. Showing in lanes 3 to 6 smaller amounts of GAR domains under 2 nanograms are more extensive activity against RNA. Panel B) shows a denatured GAR domains previously with time of heat incubation from 0 to 60 min, as can be observed the activity is similar to the non-denatured protein preparation indicating no relation between activity and structural stability. Lanes 2 to 6 shows time incubation time at 95 °C for 0, 5, 10, 30 and 60 min. After heat incubation proteins preparation was loaded on the ribonucleasa activity buffer against total RNA.

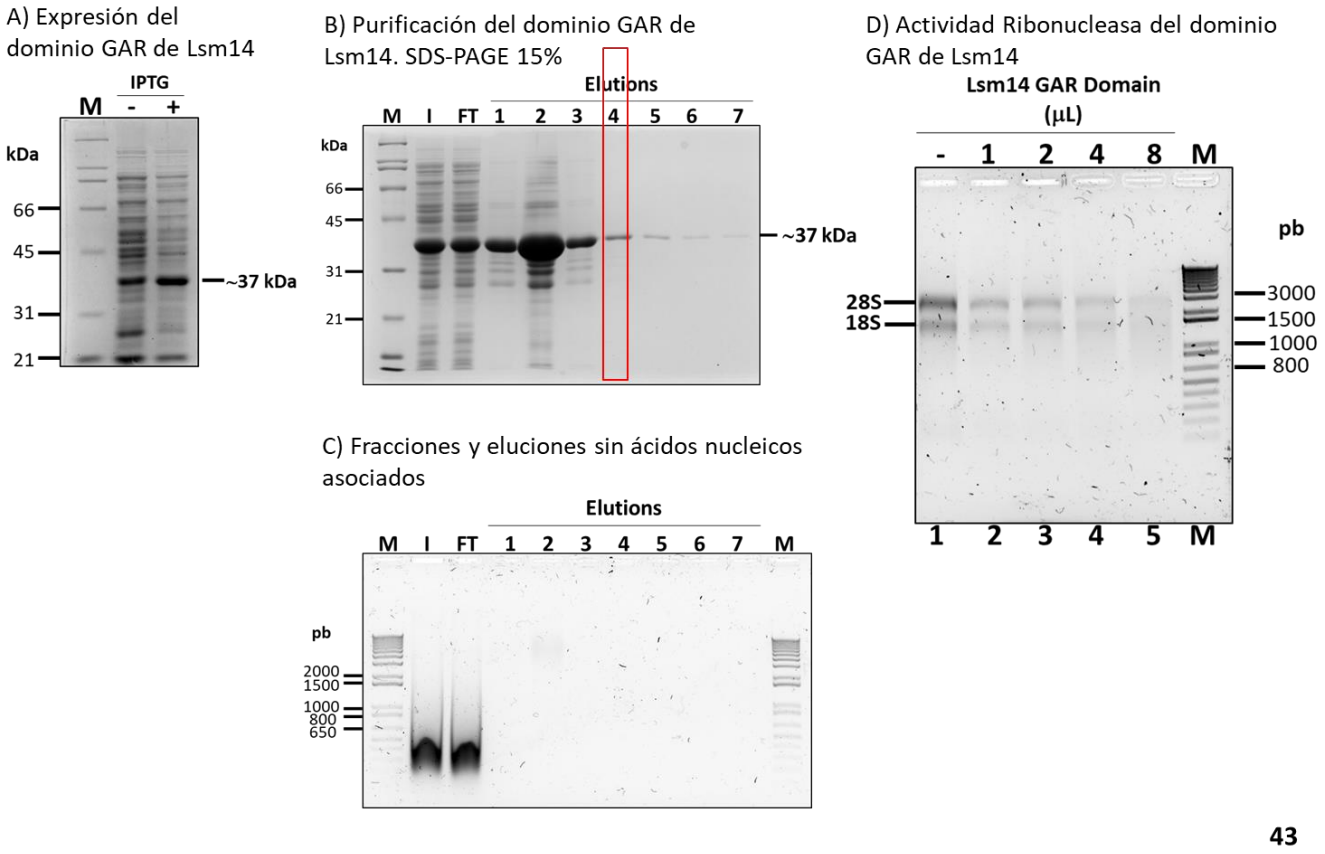


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Figure 21. Expression, purification and ribonuclease activity evaluation of the fusion GST-GAR-Slm14 protein. GAR domains from SLM14, an mRNA factor processing enzyme, was cloned to evaluating its degradative activity against total RNA. On A) induction of the expression was carried out with the addition of IPTG on the bacterial culture. A molecular weight is marked by an arrow around a 37 kDa band. B) depicts IMAC affinity purification of GAR-Slm14 to nickel resin, lanes 1to 7 shows fractions eluted from the resin. C) describes the no nucleic acids associated to the protein preparations, as can be observed in lanes 1 to 7. Agarose 1.5% stained with sybr safe (Thermo ScientificTM). Showing in D) the ribonuclease activity assay in vitro of the GAR-Lsm14 domain, here the amount was no quantified but the protein was loaded in the microliter scale to see an approximation of the ribonuclease activity. Lane 1 corresponding to the total

RNA and the lanes 2 to 5 shows the increasing volumes of GAR-Lsm14 added to the reactions.

4.4 Conclusion of the chapter

This chapter we concluded the finding regarding intrinsically disordered region contained in some RNA processing enzyme factors. Here we expressed and purified previously isolated genes codifying GAR domains from fibrillarin and from the mRNA processing factor Lsm14. Our finding points that these regions could support heat denaturing treatment and still activity can be observed for example the GAR from fibrillarin. Also, we cloned, expressed and purified other GAR domains, here we show only the corresponding GAR-Lsm14 domain but GAR domains from nucleolin, from GAR1 protein and the viral protein TGB1 were purified (data not showed here) in order to test the ability to degraded total RNA. Is interesting to note that a more activity can be detected against RNA when only the GAR doming from fibrillarin is present on the reaction.

CAPÍTULO V. The SnoRNP Complex containing fibrillarin possess ribonuclease activity

5.1 Introduction

Fibrillarin forms part of one of the main snoRNP complexes that modify rRNA during ribosomal RNA biogenesis; this complex guides the specific methylation site of about 100 residues in the rRNA (Samaha et al., 2010; Tollervey, Lehtonen, Carmo-Fonseca, & Hurt, 1991; Watkins & Bohnsack, 2012). The complex consists of three other well-known proteins: Nop58, Nop56, 15.5K, and one snoRNA guide. In this chapter we ask if the ribonuclease activity of fibrillarin is maintained in the snoRNP context. To resolve this question, we introduce the GAR domain sequence of fibrillarin in the mega construct expressing the complex with a truncated fibrillarin in N domain following the Exponential Mega Primer PCR technique.

5.2 Materials and Methods

5.2.1 *Exponential Megaprimer PCR (EMP) technique to introduce the GAR Domain coding region into Mega plasmid*

Following the EMP strategy (Ulrich et al., 2012) to introduce long DNA sequences into plasmids we cloned the GAR domain of fibrillarin into the mega plasmid containing the coding sequences for NOP56/NOP58, 15.5K and Fibrillarin, previously reported by Peng et al., 2014, because the coding region of fibrillarin is truncated in amino acid 83, without the coding sequence for the GAR domain.

For the synthesis of the megaprimer i.e. the GAR domain we used the following primers FW1-GARFib-EMP: 5’ ATGAAGCCAGGATTCAGTCC 3’ and RV1-EMP: 5’ GGACTGAATCCTCGCTTCATCACATTCTTCCCCGACTGGT 3’ in a reaction containing 1X HF Phusion buffer (Thermo Fisher, CAT F530S), 200 µM of each dNTP, 0.5 µM primer FW1, 0.5 µM primer RV1, 25 ng of pET15b:Fibrillarin (with full sequence) DNA plasmid as template and 0.02 U/µL Phusion DNA Polymerase (Thermo Fisher) in a final volume of 50

µL; following this PCR conditions: 98 °C, 30 seg as an initial denaturing step, followed by 25 cycles of denaturing (98 °C, 10 seg), annealing (63 °C, 30 seg) and extension (72 °C, 15 seg). And adding a 5 min at 72 °C final extension step to complete the PCR. The 266 bp product of this first PCR was analyzed by agarose gel 1% electrophoresis and purified with QIAquick Gel Extraction Kit (Qiagen ID: 28704).

In order to introduce the GAR domain DNA region amplified in the first EMP-PCR we conducted the second reaction using the primers FW1, RV2-GARFib-EMP 5' GGATCCCATAGTTAATTTCT 3' and the PCR product from the first PCR (as a mega primer) in a reaction containing 1X HF Phusion buffer, 200 µM of each dNTP, 0.5 µM primer FW1, 0.5 µM RV2, 25-50 ng megaprimer, 25 ng of Mega plasmid as template, and 0.02 U/µL Phusion DNA Polymerase (ThermoFisher) in a final volume of 50 µL. The PCR conditions were: an initial denaturing step a 98 °C for 30 seg, 25 cycles with a denaturing step (98 °C, 10 seg), annealing step (56 °C for 30 seg) and extension (72 °C for 30 seg) with no final extension step.

5.2.2 In vitro ligation and transformation of the EMP product

Using the KLD Enzyme Mix from NEB (Cat M0554S) the PCR product from the second reaction was phosphorylated, ligated and the template DNA removed with the Kinase, Ligase and DpnI enzymes contained in the enzyme mix. Using the following components (in a 10 µL reaction: 1X KLD reaction buffer, 1 µL KLD Enzyme mix, 1-4 µL EMP product and nuclease free H20 up to 10 µL). The reaction was incubated at 25 °C for 5 min, and 5 µL of the reaction were used to transformation into 50 µL chemically competent E. coli Top10 cells.

5.2.3 Recombinant protein expression and purification

The ribonucleoprotein complexes with or without the GAR domain sequence were expressed using *E. coli* BL21 DE3 competent cells. The recombinant production of these proteins was induced at an optical density (OD) of the culture between 0.6 - 0.8 absorbance units with 1 mM of isopropyl-D-1-thiogalactopyranoside (IPTG) at 25 °C for 4 h. The induced cells were harvested by centrifugation at 4000 x g for 20 min at 4° C, re suspended in lysis buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 20 mM Imidazole, 10% glycerol, 0.1% of Triton

X-100) and supplied with 0.1 mM AEBSF and 0.1 DTT as protease inhibitor and reducing agents, respectively. Then sonicated 10 times with 30 seg ON/ 30 seg OFF cycles. The fragmented cells were clarified by centrifugation at 15000 x g for 20 min at 4°C and the supernatant was used for IMAC purification strategy using Nickel beads from ThermoFisher. 100 µL of Ni beads were equilibrated with lysis buffer and then the soluble fraction was loaded into the beads and incubated for 30 min at 4° C in a roller, the column was washed using 10-volume of the beads for 5 times with lysis buffer with 50 mM imidazole a the RNP complexes were eluted with 50 mM-Tris-HCl pH 8, 100 mM NaCl, 250 mM imidazole, 10% glycerol and 0.1 mM AEBSF and 0.1 mM DTT. All the purification steps were conducted at 4° C in order to reduce proteolysis. The proteins were stored at -80 °C until used in the following experimental procedures.

5.2.4 Cytoplasmatic And Nuclear RNA Extraction from U2OS Human Cells

The dishes containing the cells were washed three times with cold PBS, then 500 µL of Lysis buffer (50 mM Tris-HCl pH 7, 150 mM NaCl, 2 mM MgCl2, 0.5% NP-40, 0.02U/µL RNAse Inhibitor) was added, scraped and resuspended by pipetting up and down. The cells in lysis buffer were transferred on 1.5 mL tubes and centrifugated for 1 min at 1000 x g at 4 °C and the supernatant transferred to a fresh tube. The pellet and the supernatant were used for nuclear and cytoplasmatic RNA extraction. We follow the manufacturer instructions for RibozolTM.

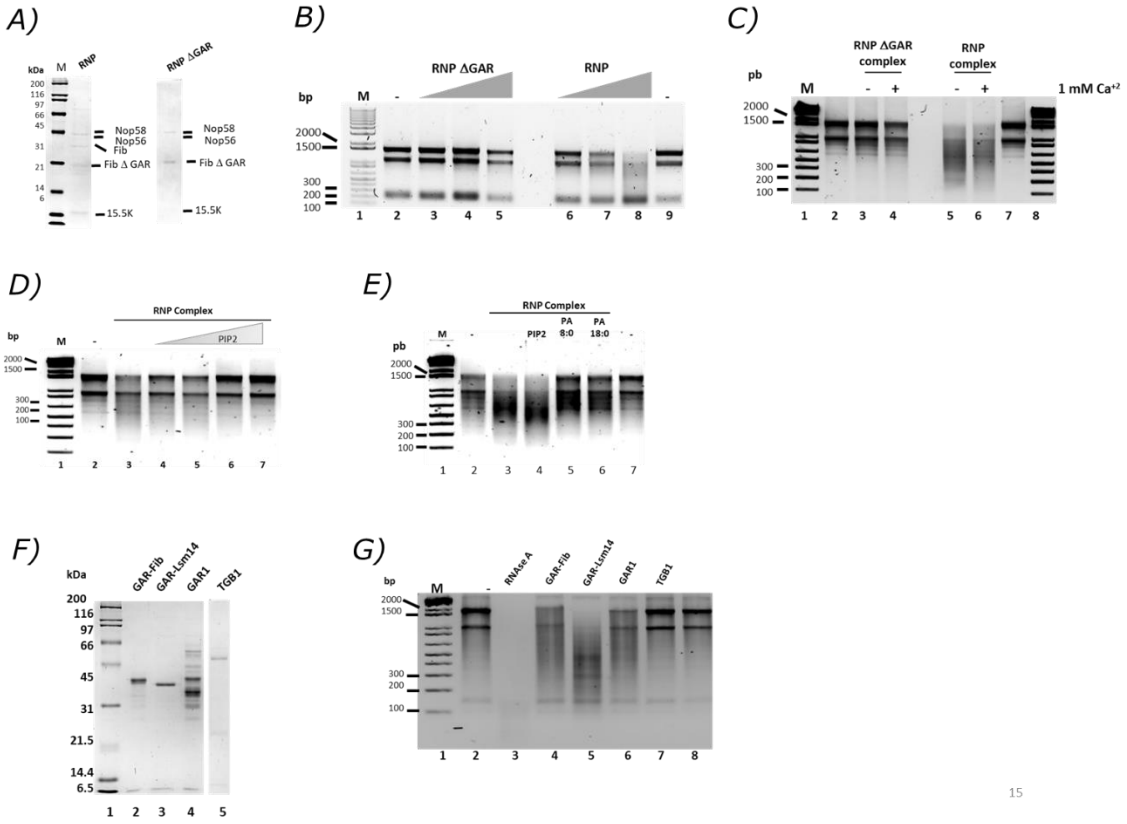
5.2.6 In vitro Ribonuclease Activity assay

Cytoplasmatic, nuclear or total RNA extracted from broccoli inflorescences or U20S cells was mixed with native fibrillarin-complexes with or without the GAR domain or recombinant fibrillarin alone in RNAse activity buffer (25 mM Tris-HCl pH8, 100 mM NaCl, 0.1 mM EDTA, 0.1 DTT 10%) + 0.2 U/µL of RNAse Inhibitor (Thermo Fisher) and incubated for 45 min at 37 °C, then loaded in a 1% agarose gel.

5.3 Results

Following the idea in which fibrillarin as part of the snoRNP methylation complex we hypostatized that even in this context we can still observe ribonuclease activity. We evaluated the in vitro ribonuclease activity of this complexes with or without the GAR domain from fibrillarin. To address this question, we previously have the construct for bacterial expression of the snoRNP methylation complex composed by the proteins Nop58, Nop56, 15.5k and a truncated fibrillarin, previously reported by (Peng, Yu, Tian, & Li, 2014). The truncated fibrillarin was deleted in its glycine-arginine rich domain because the previously work was oriented in the methyltransferase domain of fibrillarin located at the C-terminal domain. We decided to introduce the full length of fibrillarin on the snoRNP complex with a PCR technique called Exponential mega primer PCR (Ulrich, Andersen, & Schwartz, 2012) as detailed in material and methods. After the experimental amplification, introduction and cloning of the GAR domain into the snoRNP complex we expressed in *E. coli* Bl21 both of the complexes calling them as follow: RNP and RNP ΔGAR, with and without the GAR domain, respectively. As showing in the figure 22, A) we were able to introduce the region coding for the GAR domain and a shift can be observed for the electrophoretic mobility of the band corresponding to fibrillarin. The corresponding molecular masses and names are given in the figure 22 A). The purification conditions were carried out with aseptic conditions, majority of them at 4°C in order to obtain a good quality protein complex. We reasoned that ribonuclease activity could be still observable, so we carried out a ribonuclease activity *in vitro* assay as depicted by figure 22 B). Here we evaluated the increasing volume of complexes added to the reactions, and as can be appreciated the amount of RNP ΔGAR no degradation was detected against total RNA. But when the RNP complexes with full length fibrillarin was added to the reactions a highly degradative effect can be detected, suggesting that the preliminary observation that the GAR domains was the active module with the ribonuclease activity. Also taking the results from fibrillarin alone experiments, we evaluated the influence of calcium 1 mM on the activity of both complexes. Figure 22 C) shows the effect of the addition of calcium to the reactions, no clear induction or inhibition can be detected as RNP complex still was able to degraded total RNA in the same order with or without calcium. The idea that phosphoinositides can modulate the activity of fibrillarin during cell cycle was also tested in this RNP context, suggesting that a ribonuclease activity is dependent on lipid binding partners. With tested first the ability of PI4,5P to modulate the

ribonuclease activity of the RNP complex. As figure 22 D) shows an increasing amount of lipid from 5 to 50 nanograms was enough to inhibit the activity of the full complex, interesting as the first suggestion with fibrillarin alone experiments. For the other hand PA was also tested in the in vitro ribonuclease activity experiment, as figure 22 E) shows the PA with a 8 and 16 carbon lateral chain saturation was able to inhibit the ribonuclease activity of the complex, accorded to the fibrillarin experiments as described earlier. Finally, as a part for the final version for the experimental work for the publication we address the observation that multiple GAR domains are present in proteins with RNA processing relation, and the idea that a ribonuclease activity can be common to all these regions we evaluated them and the ability of total RNA degradation. Early we have cloned, expressed and purified GAR domains from the fibrillarin, Lsm4 and GA1 proteins, Figure 22 F). Also, the TGB1 viral protein was purified, TGB1 possess an intrinsically disordered region but different in amino acid composition. Interesting to note, was the fact that all the GAR domains presented in vitro ribonuclease activity, but TGB1 was not able to degraded RNA and suggesting that only glycine-arginine rich regions can share this degradative effect. In fact, this is only a hypothesis but our results points that there is a link between these regions and the in vitro activities observed.


A)
kDa
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RNP ΔGAR
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31
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Nop58
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Nop58
Nop56
Fib Δ GAR
15.5K
B)
RNP ΔGAR
RNP
bp
M
2000
1500
300
200
100
1 2 3 4 5 6 7 8 9
C)
RNP ΔGAR complex
RNP complex
pb
M
1 mM Ca+2
2000
1500
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200
100
1 2 3 4 5 6 7 8
D)
RNP Complex
bp
M
PIP2
2000
1500
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200
100
1 2 3 4 5 6 7
E)
RNP Complex
pb
M
PIP2
PA 8:0
PA 18:0
2000
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1 2 3 4 5 6 7
F)
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GAR-Fib
GAR-Lsm14
GAR1
TGB1
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116
97
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31
21.5
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RNAse A
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GAR-Lsm14
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Figure 22. Ribonuclease activity of SnoRNP-containing fibrillarin is dependent on GAR domain. A) Expression and purification of complexes with and without of GAR domain, the figure describe the induction expression and purification for the complexes containing a truncated and full length fibrillarin as depicted in the figure. An electrophoretic shift can be appreciates indicating the fibrillarin full length, marked by a line. All the protein factors conserved in the snoRNP are marked on the SDS PAGE. B) Activity of both complexes against total RNA, lanes 3 to 5 are increasing amount of RNPΔGAR against total RNA, intriguingly no degradations were observed. Lanes 6 to 8 increasing amounts of the complex RNP, as can be observed the degradative effect is enough to evaluated the activity only by the addition of the sequence of glycine and arginine to the expression vector. C) The activity of the complexes evaluated with 1 mM Calcium, lanes 3 and 4 shows the effect on the addition of calcium to the complex RNP ΔGAR, no clear effect of induction or inhibition can be detected, by the other hand, the addition of calcium to the RNP complex, lanes 5 and 6, also not shows an effect on total RNA degradation. D) Influence of PIP2 in the degradation pattern of complex with full fibrillarin sequence, the addition of small amounts of this lipid can modulate the activity, as example the induction occurs at amounts of 5 to 10 nanograms of PI4,5P (lanes 4 and 5) while amounts above theses the activity of the complex seems to be inhibited (lanes 6 and 7). E) Influence of PA with different length chains in the ribonuclease activity of complex with GAR domain, PA seems to inhibit the ribonuclease activity of RNP complex containing full length fibrillarin, and this inhibition is independent on the length of the lateral chains of PA, lanes 5 and 6. F) Expression and purification of different GAR domains from other proteins, GAR from fibrillarin, Slm14, GAR1 and the viral protein TGB1 were expressed in bacteria, purified to homogeneity and visualized on a SDS-PAGE stained with Coomassie blue. G) Ribonuclease activity of the 4 different domains with similar sequences as GAR domains compared with RNAse A, lane 3. Lanes 4, 5 and 6 shows the ribonuclease activity pattern observed for the respective GAR domains as detailed in the figure. Lane 7 was the TGB1 viral protein incubated with total RNA, no degradation was observed as the proteins contains a IDP region.

5.4 Conclusion of the chapter

For this chapter we addressed the hypothesis that the ribonuclease activity of fibrillarin can be still present in the snoRNP complex. In fact, our results point those complexes with the full length fibrillarin can still degraded total RNA, moreover we were able to observe an

influence on the RNP activity with the lipids PI4,5P and PA, suggesting a relation with the fibrillarin alone experiments. PI4,5P seem to inhibit the ribonuclease activity of full length fibrillarin containing snoRNP, while lower concentration between 5-10 nanograms of lipid induce an activation of the activity. For the other hand PA seem to inhibit the ribonuclease activity with no relation to the length of the lateral chain, as figure 22 shows. Also, we demonstrate that other GAR rich regions can hold ribonuclease activity and the relation with intrinsically disordered regions are dependent on the nature of the amino acid containing regions. Taking together our results shows a ribonuclease activity present not only in the fibrillarin-containing assays but in the RNP complexes can be observed the full activity of degradation of RNA.

CAPÍTULO VI. FIBRILLARIN RIBONUCLEASE ACTIVITY IS DEPENDENT ON THE GAR DOMAIN AND MODULATED BY PHOSPHOLIPIDS

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MDPI

Article

Fibrillarin Ribonuclease Activity is Dependent on the GAR Domain and Modulated by Phospholipids

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Received: 26 March 2020; Accepted: 29 April 2020; Published: 6 May 2020

Abstract: Fibrillarin is a highly conserved nucleolar methyltransferase responsible for ribosomal RNA methylation across evolution from Archaea to humans. It has been reported that fibrillarin is involved in the methylation of histone H2A in nucleoli and other processes, including viral progression, cellular stress, nuclear shape, and cell cycle progression. We show that fibrillarin has an additional activity as a ribonuclease. The activity is affected by phosphoinositides and phosphatidic acid and insensitive to ribonuclease inhibitors. Furthermore, the presence of phosphatidic acid releases the fibrillarin-U3 snoRNA complex. We show that the ribonuclease activity localizes to the GAR

(glycine/arginine-rich) domain conserved in a small group of RNA interacting proteins. The introduction of the GAR domain occurred in evolution in the transition from archaea to eukaryotic cells. The interaction of this domain with phospholipids may allow a phase separation of this protein in nucleoli.

Keywords: nucleolus; ribonucleolar particle; rRNA; fibrillarin; phosphoinositides; viral progression

1 . Introduction

The nucleolus is a well-studied nuclear structure which has been shown for a long time to separate into three components depending on the particular step of ribosome biogenesis: the fibrillar center (FC) where RNA pol I is found, the dense fibrillar component (DFC) where protein fibrillarin is concentrated, and the granular component (GC) that is enriched in nucleophosmin (NPM1) [1,2]. The nucleolar proteome consists of hundreds of different proteins, as well as a large set of different types of RNA that need to be in a particular compartment in order to carry their function [3]. All these components form a multi-layer core found in a liquid gel structure. The nucleolar assembly is controlled in a concentration-dependent phase transition, as shown earlier by [4], where the nucleoli behave as multi-component liquid-phase droplets ruled by thermodynamic forces [5]. An elegant set of experiments using a microfluidic device showed the phase dynamics of fibrillarin and other nucleolar components [6]. Furthermore, the composition, morphology, and fusion dynamics of nucleoli are altered by ATP depletion, as shown using fibrillarin-GFP in a lapse movie. Thus, fibrillarin condensates into dozens of small droplets throughout the nucleus [7]. Transcription of pre-ribosomal RNA at active nucleolar organizer regions (NORs) lowers the critical concentration for phase separation of nucleolar proteins, such as fibrillarin and NPM1, and nucleates the assembly of the fibrillarin-rich DFC [8]. The spatial separation of the nucleolar sub-compartments is dictated by differences in their viscoelastic properties, especially in the surface tensions of the fibrillarin- and NPM1-rich phases with respect to the surrounding nucleoplasm [9].

Fibrillarin in eukaryotic organisms contains a glycine/arginine-rich domain (GAR), a spacer region called BCO, a central domain with RNA binding motif, and an alpha helix domain [10,11]. Fibrillarin is an essential 2’-O-methyltransferase for all eukaryotes, mediating the methylation of rRNA and histone H2A at a glutamine in position 104 in humans and 105 in yeast required for the epigenetic regulation of ribosomal genes [12,13]. Fibrillarin is important in ribosome biogenesis and also plays a role in other processes, including viral progression. RNA viruses like coronavirus, infectious bronchitis virus (IBV), influenza, and human immunodeficiency virus (HIV) require fibrillarin for cell-to-cell infection, both in plants and humans [10,14–16]. Fibrillarin is known to associate with different nuclear and nucleolar RNAs (U3, U8, U13, U14, U60, x, y, snR3, snR4, snR8, snR9, snR10, snR11, snR30, snR189, and snR190) [1,17] and other proteins to form a variety of snoRNPs. The best-characterized and most studied snoRNP is composed of the small nucleolar RNA (snoRNA) U3. snoRNA U3 is involved as a guide RNA in the initial steps of pre-rRNA processing [18] and cleavage that leads to the maturation of 18S rRNA [19]. For this early processing, several factors, such as

fibrillarin, nucleolin, and the U14 snoRNA, are required [20–22]. It has been suggested that liquid–liquid phase separations (LLPSs) are implicated in synthesis and processing modifications during ribosomal production. Phase separation has been observed in proteins that contain intrinsically disordered regions (IDRs) in their sequences [23,24], i.e., fibrillarin, nucleolin, and Gar1 proteins, contain IDR. LLPS in this process ensures a membrane-less environment for the production and highly ordered process of ribosome assembly. Earlier results indicate that fibrillarin can bind to phosphatidylinositol 4,5-bisphosphate (PIP2). Lipids play crucial roles in nuclear function and dynamic architecture, and fibrillarin is involved in the lipid phase and RNA binding in the cell [25–27].

Phosphoinositide pathways are regulated by several enzymes in both cytosol and the nucleus. Each of the seven different phosphorylated phosphoinositides [28] is known to localize in different parts of the cell [29]. Therefore, a particular environment for these phosphoinositides, together with their partners, can be in a particular phase that can be differentiated by metabolic changes in the lipids. This hypothesis involves a specific localization for a protein–lipid complex together with a particular function that can be altered by lipid metabolism and thus results in a re-localization of the components or alteration of complex formation, leading to the movement of some components due to the biochemical properties of the new lipids. We are still far from showing that RNA, lipids, and proteins can organize in particular structures that can phase separate by means of lipid metabolism and posttranslational protein modifications. However, such processes would reduce the energy requirements needed for cells to organize particular structures and to relocate particular complexes upon phosphorylation changes in the lipids. Dynamic proteins like fibrillarin are intriguing subjects to test as they are known to exist in particular phase separation. However, their link to structural changes by RNA alteration suggests that this component is also essential for proper localization, as during RNA inhibition [30]. Therefore, fibrillarin localization is not a simple phase separation that requires a protein–lipid complex, but also RNA to have a functional role through ribosome biogenesis.

Here we show that fibrillarin has a ribonuclease activity for rRNA, and this general activity is blocked when it is bound to snoRNA U3. The ribonuclease activity of fibrillarin is carried by the GAR domain, which is also responsible for interaction with different phosphoinositides. Moreover, we also show that the ribonuclease activity of fibrillarin increases with phosphatidylinositol 5-phosphate (PI5P) and reduces in the presence of phosphatidic acid (PA) or during binding to Guide RNA U3. Ribonucleases are involved in vital cellular functions, including cytoplasmic and nuclear RNA degradation, RNAi, antiviral defense, DNA synthesis, and RNA processing [31–35]. Pre-rRNA maturation involves the activity of specific RNases and RNA modifying enzymes to achieve functional rRNA [36,37]. Most pre-rRNA processing is mediated by the co-transcriptional association of nascent transcripts with ribosomal proteins (RP) and several small nucleolar ribonucleoprotein particles (snoRNP) containing fibrillarin [38].

2. Materials and Methods

2.1. Cell Lines, Cell Culture, and Transfection Assays

HeLa cells were cultured as previously published [25]; transient transfection was performed at 80% confluence using polyethylenimine (PEI) with 10 µg of plasmid DNA in 1 mL of Dulbecco´s modified Eagle medium (DMEM) without fetal calf serum. Distribution patterns of the wild type and fibrillarin mutants, coupled with GFP reporter, were analyzed in 200 cells for at least three separate experiments. Plasmid DNA for transfections was purified from bacterial cultures using maxiprep columns (QIAGEN, Hilden, Germany).

A stable SNAP-tag-Fib cell line was generated as follows: transfection with PEI was performed in an 80% confluence 60-mm plate of U2OS osteosarcoma cell line. The cocktail for transient transfection (1.5 µg of pfSNAP-tag-fibrillarin plasmid DNA and 9 µL of PEI) was incubated for 15 min at RT and added dropwise. After 48 hours of transfection, 1000 µg/mL of geneticin G-418 of Sigma-Aldrich®was added and incubated for 9 days. The selection of positive transfected colonies was made after washing the dead cells with PBS and changing the culture media. Cells were stained with a fluorescent substrate that can be used to label SNAP-tag®fusion proteins, and positive colonies were selected prior to use in a bigger culture. The fluorescent substrate used for this work was SNAP-Cell®Oregon Green®. Specific primary anti-PIP2 from Echelon™ (Z-A045) was used for immunofluorescence detection of PIP2 and labeled by anti-mouse IgM secondary antibody conjugated with Alexa Flour®555 from Life Sciences. The PA sensor Spo20p-GFP was described previously [39].

2.2. Microscopy

Wide-field microscopy was performed on a Leica DM6000 (Leica Microsystems, Wetzlar, Germany; filter cubes: DAPI (Ex: 360/40, Em: 470/40), FITC (Ex: 480/40; Em: 527/30), TRITC (Ex: 546/12; Em: 600/40), Cy5 (Ex: 620/60; Em: 700/75)) using an HCX PL APO 100x/1.40-0.70 OIL objective, a Leica EL6000 with an HXP 120W/45C Vis Hg light source, Type F immersion liquid (Leica Microsystems), a Leica DFC350 FX camera, and Las X software.

2.3. Structured Illumination Microscopy

Images were acquired using the 3D-SIM system DeltaVision OMX (GE Healthcare Life Sciences, Marlborough, MA, USA) with PLAN APO N 60x/1.42 OIL objective, a 4x pco.edge 5.5 sCMOS camera and DeltaVision OMX (Buckinghamshire, UK) controlling software. Lasers with wavelengths 405, 488, 568, and 642 nm with filters for DAPI (Ex: 395.5/29; Em: 435.5/31), FITC (Ex: 477/32; Em: 528/48), and Alexa Fluor 568 (Ex: 571/19; Em: 609/37). Immersion oil laser liquid (Cargille Laboratories, Cedar Grove, NJ, USA; Code: 5610; n = 1.5160). Image reconstruction and registration were processed with DeltaVision softWoRx 6.5.2. software. Wiener filters were set to 0.001. Samples were mounted to 90% glycerol supplemented with 5% N-propyl gallate.

2.4. Cloning

Total RNA from U2OS cells was extracted with RiboZol™ (Solon, OH, USA), following the manufacturer's instructions. The sequences from fibrillarin, GAR1, and SLM14 were obtained using

cDNA from the reverse transcriptase strategy of TaqMan™ and amplified by DreamTaq™ polymerase (Thermo Fisher Scientific, Waltham, MA, USA), following the provider's instructions. The gene encoding fibrillarin (NM_001436.3) was amplified by RT–PCR from total RNA extraction of HeLa cells and cloned into the NcoI and BamHI sites of the pET-15b vector (Fib_Fwd 5'-CCATGGATGAAGCCAGGATTCAGTCCCCGTG-3'; Fib_Rev 5'-GGATCCTCAGTTCTTCACCTTGGGGGGTGGC-3'). For protein expression in HeLa cells, the fibrillarin sequence was cloned into the XhoI and BamHI sites of the pEGFP-N1 vector to generate pGFP-FIB. The N-terminal part (amino acids 1 to 134) containing the GAR domain of the fibrillarin was amplified from the pET15b:

HsFib vector (GB_forward 5'-CCATGGATGAAGCCAGGATTCAGTCCC-3'; GB_reverse 5'-CTCGAGGTACTCAATTTTGTCATCTCCTTCC-3', and cloned into the NcoI and XhoI sites of the pET42b vector). Once cloned into pET42b, small fragments from fibrillarin were obtained by complete plasmid PCR mutagenesis with the following primers: BCO (Forward 5'-AGAATGTGATGGTGGAGCCGCA-3', reverse pET42b 5'-CCATGGACCCGCGTCCCTCAA-3');

HsGAR domain (Forward pET42b 5'-CTCGAGCACCACCACCACCA-3'; reverse 5'-TCTTCCTCCTCCTCCACCGCC-3'; and miniGAR (Forward pET42b and reverse 5'-TCACCAAAGCCCCCTCGGCC-3').

The specific primers used for cloning the GAR1 and GAR domains from Lsm14 genes were FwGAR1 5'-CCATGGATATGTCTTTTCGAGGCGGAGG-3', and RvGAR1

5'-CTCGAGATGTCCTCTCCCTCTGAAACC-3' between NcoI and XhoI restriction sites, respectively.

For GAR-Lsm14, the domains were Fw 5'-AAGAATTCGATGACAATAGAGAA-3' and Rev 5'-TAAGCTTAG GGTCCAAAAGCTGTGCTGT-3' between EcoRV and HindIII restriction sites, respectively.

2.5. Recombinant Protein Expression and Purification

Recombinant fibrillarin was expressed in *Escherichia coli* BL21 gold with 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) at 25 °C for 5 h. Harvested cells were resuspended in protein extraction buffer (500 mM NaCl, 25 mM tris pH 8, 10% glycerol, 20 mM imidazole, 0.1% Tween 20, 0.1 mM AEBSF, and 0.1 mM DTT) and broken down by sonication. After clarification by centrifugation (17,400× *g* for 15 min), the supernatant was loaded onto a Ni-NTA agarose column (Thermo Fisher) and washed three times with the extraction buffer and then eluted with a linear gradient from 70 to 200 mM imidazole in BC-100 buffer (20 mM Tris-HCl buffer, pH 8, 100 mM NaCl, 0.2 mM EDTA, 10% glycerol) and revised by 15% SDS-PAGE. The fraction containing fibrillarin was passed through MonoQ sepharose (Amersham Pharmacia, Buckinghamshire UK), utilizing a 0.1 to 0.5 KCl gradient to elute the fibrillarin. Fibrillarin containing fractions were pulled and dialyzed against BC-100 and 0.1 mM AEBSF. After the MonoQ sepharose purification step, the fraction containing fibrillarin was loaded on a MonoS (Amersham Pharmacia) column resin and eluted with a linear gradient from 0.1 to 0.5 M KCl in BC-100 buffer with 0.1 mM AEBSF. The purity of proteins was revised by 15% SDSPAGE, followed by silver staining. For peptides cloned into pET42b vector, the supernatant was first loaded into a Ni-NTA agarose column followed by loading into a glutathione–sepharose column and eluted with BC-100 buffer with 10 mM of reduced glutathione and 0.1 mM of AEBSF.

Homo sapiens GAR1, Lsm14, HsGAR-Fib, and the viral protein TGB1 were expressed in *E. coli* Rosetta competent cells. The recombinant production of these proteins was induced with 1 mM IPTG at 25 ◦C for 4 h. Induced cells were harvested by centrifugation at 4000 × *g* for 20 min at 4 ◦C, resuspended in lysis buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 20 mM Imidazole, 10% glycerol, 0.1% of Triton X-100) and supplied with 0.1 mM AEBSF and 0.1 DTT as a protease inhibitor to reduce respective agents, then sonicated 10 times with 30 s ON/30 s OFF cycles. The fragmented cells were clarified by centrifugation at 15000 × *g* for 20 min at 4 ◦C, and the supernatant was clarified for the IMAC purification strategy, using 100 µL of nickel beads (Thermo Fisher™) and incubated for 30 min at 4 ◦C in a rotor. The column was washed using 10 volumes of beads lysis buffer, along with an increased concentration of NaCl from 100 to 500 mM. The proteins were eluted with 50 mM-Tris-HCl pH 8, 100 mM NaCl, and 250 mM imidazole and supplied with 10% glycerol, 0.1 mM AEBSF, and 0.1 mM DTT. All purification steps were done at 4 ◦C to reduce proteolysis. Proteins were stored at -80 ◦C until use in the next experimental procedures.

2.6. Exponential Megaprimer PCR (EMP) Strategy to Introduce the GAR Domain Coding Region into RNP Complex

Following the EMP strategy [40] to introduce long DNA sequences into plasmids, we cloned the N-domain of fibrillarin into the pLink plasmid containing the coding sequences for NOP56/NOP58, 15.5K, previously reported by [41], owed to the fact that the coding region of fibrillarin is truncated in amino acid 83, i.e., lacking the GAR domain coding sequence.

For the GARdomain primer synthesis, we used the followingprimers:

FW1-GARFib-EMP: 5’-ATGAAGCCAGGATTCAGTCC-3’ and RV1-EMP: 5’-

GGACTGAATCCTCGCTTCATCACATTCTTCCCCGACTGGT-3’ in a reaction containing 1X HF Phusion buffer (Thermo Fisher, CAT F530S), 200 µM of each dNTP, 0.5 µM primer FW1, 0.5 µM primer RV1, 25 ng of pET15b:fibrillarin (with full sequence) as plasmid DNA template, and 0.02 U/µL Phusion DNA polymerase (Thermo Fisher) in a final volume of 50 µL. Following this, PCR conditions were 98 ◦C, 30 s as an initial denaturing step, followed by 25 cycles of denaturing (98 ◦C, 10 s), annealing (63 ◦C, 30 s), and extension (72 ◦C, 15 s). The 266-bp product of the first PCR was analyzed by agarose gel 1% electrophoresis and purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany, ID: 28704).

The GAR domain DNA region was introduced with a second PCR using the primers FW1, RV2-GARFib-EMP: 5’-GGATCCCATAGTTAATTTCT-3’, and the PCR product from the first PCR (as a mega primer) in a reaction containing 1X HF Phusion buffer, 200 µM of each dNTP, 0.5 µM primer FW1, 0.5 µM RV2, 25–50 ng mega primer, 25 ng of megaplasmid as a template, and 0.02 U/µL Phusion DNA polymerase (Thermo Fisher™) in a final volume of 50 µL. The PCR conditions were an initial denaturing step at 98 ◦C for 30 s, 25 cycles, with a denaturing step (98 ◦C, 10 s), an annealing step (56 ◦C for 30 s), and an extension (72 ◦C for 30 s), with no final extension step.

2.7. In Vitro Ligation and Transformation of the EMP Product

Using KLD Enzyme Mix from NEB (Cat M0554S), the PCR product from the second reaction was phosphorylated, ligated, and the template DNA removed with kinase, ligase, and DpnI enzymes contained in enzyme mix. The following components were used in a 10-µL reaction: 1X KLD reaction buffer, 1 µL KLD enzyme mix, 1–4 µL EMP product, and nuclease-free H20 up to 10 µL. The reaction was incubated at 25 °C for 5 min, with 5 µL of the resulting product used for transformation into 50 µL chemically competent *E. coli* Top 10 cells.

2.8. Recombinant RNP Complex Expression and Purification

Ribonucleoprotein complexes (RNP), with or without the GAR domain sequence, were expressed using *E. coli* BL21 DE3 competent cells. The induced cells were harvested by centrifugation at 4000 × *g* for 20 min at 4 °C, resuspended in lysis buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 20 mM Imidazole, 10% glycerol, 0.1% of Triton X-100), and supplied with 0.1 mM AEBSF and 0.1 DTT as protease inhibitor and reducing agent, respectively. The fragmented cells from sonication were clarified by centrifugation at 15000x *g* for 20 min at 4 °C, and the supernatant used for the IMAC purification strategy, as stated above. Imidazole elution was carried out with 50 mM-Tris-HCl pH 8, 100 mM NaCl, 10% glycerol, and 0.1 mM AEBSF and 0.1 mM DTT. MonoQ purification was carried out to remove the remaining contaminants. All purification steps were done at 4 °C to reduce proteolysis. The proteins were stored at ¯80 °C until use in experimental procedures.

2.9. SNAP-Tag-Fibrillarin Purification from HeLa Cells

The media from the HeLa cell cultures was discarded, and the cell monolayer washed twice with cold PBS and the excess discarded. Then, 500 µL of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40, 0.5 mM AEBSF, and 0.1 DTT) was added to each plate, the cells were scraped and resuspended with up- and down-pipetting, transferred to a fresh 1.5-mL tube and incubated for 10 min at room temperature, then centrifugated at 15,000 × *g* at 4 °C for 5 min. The cytoplasmic upper layer (supernatant) was stored at −80 °C. The pellet was resuspended in 150 µL of buffer B (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 0.5 mM AEBSF, and 0.1 mM DTT) and placed on ice for 2 h, vortexing every 15 min, and then centrifugated at 15,000 × *g* at 4 °C for 5 min; the upper layer was stored at −80 °C as the nuclear protein fraction. Proteins were visualized in SDS-PAGE. In-vitro labeling with SNAP-Biotin strategy was done following the NEB manufacturer' instructions in a reaction tube: 1 mM DTT, 10 µM SNAP-tag substrate, 600 µL nuclear protein extract, and volume adjusted with PBS to 800 µL with 0.5 mM AEBSF. The tube was incubated at 37 °C for 30 min and stored for purification steps. Biotin–streptavidin magnetic beads were used for purification, following the manufacturer's instructions (Thermo Fisher Scientific).

2.10. Western Blot Assay

Fibrillarin was loaded in 12% acrylamide gel to perform SDS-PAGE, then transferred to a nitrocellulose membrane and blocked with 3% of BSA in PBS at room temperature for 1 h. The membrane then was incubated with rabbit polyclonal anti-Fib antibody (1/3000) (H-140, Santa Cruz Biotechnology, Dallas, TX, USA), for 1 h at room temperature and with the IRDye® 800CW goat anti-rabbit IgG secondary antibody from LICOR for 1 h at room temperature, with three washes of 10

min each with PBS-T between incubations. Immunoblotting signals were analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences, Lincoln, NE, USA). For small peptides, the primary antibody was anti-6xHis (1/5000) (Abcam, ab18184, mouse monoclonal) and IRDye® 800CW goat anti-mouse IgG secondary antibody.

2.11. Fibrillarin Mutagenesis

Mutagenesis of the fibrillarin sequence was performed with the Thermo Scientific Phusion Site-Directed Mutagenesis Kit, using specific primers for each mutation: fwd 5'-TTTGGCGGGGGCGCGGGTCGAGGCGGA-3', rev 5'-GCCCCCTCGGCCTCCACGAC-3' and 5'-AGGTCGTGGAGCGGGAGGAGGTG-3', rev 5'-CTAAAGCCTCCGCCTCGACC-3' for R34A and

R45A mutant, respectively.

2.12. RNA In Vitro Transcription

T7 RNA polymerase was used to in vitro transcribe on sense the U3 snoRNA cloned into the pGEM T-easy vector and SP6 RNA polymerase in antisense (New England BioLabs, Ipswich, MA, USA).

2.13. RNA Extraction from HeLa Human Cells

Total rRNA was extracted from the HeLa cell culture using a commercial kit, GenElute™ Mammalian

Total RNA Miniprep (Sigma-Aldrich, St. Louis, MO, USA).

2.14. In-Gel RNAse Activity Assay

Measurements were performed as zymography. Protein samples were separated in 15% SDS-PAGE gel. Resolving gel was supplied with 5 mg/mL of total extracted HeLa RNA prior to polymerization. After electrophoresis, the gel was washed for 10 min with buffer I (10 mM Tris-HCl, 20% isopropanol, pH 7.5) and incubated for 30 min in buffer II (10 mM Tris-HCl, pH 7.5) and buffer III (100 mM Tris-HCl, pH 7.5). The gel was resolved with 0.2% of toluidine blue and washed with water until the activity band was visible [42].

2.15. In Vitro RNA Activity Assay

Total RNA extracts from HeLa cells were mixed with HeLa expressed and purified SNAP-tag–fibrillarin complexes with or without the GAR domain; alternatively, recombinant fibrillarin was used alone in RNAse activity buffer (25 mM Tris-HCl pH8, 100 mM NaCl, 0.1 mM EDTA, 0.1 DTT, and glycerol 10%) + 0.8 to 1 U/µL of RNAse inhibitor (Thermo Fisher) and incubated for 45 min at 37 °C, then loaded in a 3% agarose gel. Phosphoinositides and PA were added to the final amount of 50 ng.

2.16. Fat Blot Assay

PIP strips were used for the fat blot assay (P-6001, Echelon™, Santa Clara, CA, USA). The assay was made according to the manufacturer's instructions with 0.4 µg of protein.

2.17. Fluorescence Recovery after Photobleaching

FRAP experiments were performed on a DeltaVision OMX Super-resolution microscope. HeLa cells were transiently transfected with pGFP-FIB and mutant constructs for the live cell analysis. DMEM media was replaced with warm phosphate-buffered saline medium (Dulbecco, Darmstadt, Germany) before the experiment. Dishes were placed in a temperature-controlled chamber supplemented with CO_2. Images were obtained with a CMOs camera, using the 60X oil 1.42 objective with fluorescence free immersion oil 518F. For each photobleaching assay, one Cajal Body (CB) or nucleolus (Nco) was selected per cell and stated as the region of interest (ROI). The ROI was selected manually for immediate lase photobleaching (single point FRAP), then the fluorescence recovery was monitored, each experiment lasting 120 s, with one image acquired per second. Photobleaching removed ~99% of total fluorescence in both the nucleolus and CB. Image processing was performed with ImageJ®software for half-life ($\tau_{1/2}$) value extraction plus mobile and immobile fractions. R software was used to carry out quantitative and statistical analysis. Estimation of the diffusion coefficients (D) of wild-type and mutants was carried out as described in [43]. Each mutant was compared to wild-type (WT) under independent measurements; therefore, the SD in the graph for WT is $n = 50$ for Nco and $n = 50$ for CB compared to each mutant $n = 25$ for Nco and CB, respectively.

2.18. Bioinformatic Analysis

Complete proteomes of 35 chordate species and *Saccharomyces cerevisiae* were used to retrieve GAR containing proteins. The complete list of species and their proteome files are listed in Table S1. To identity GAR containing proteins, hidden Markov models (HMM) were constructed and calibrated from the alignment of 250 sequences retrieved by PSI-Blast [44] against the Refseq-protein database. The fibrillarin (*FIB*; AAP36189.1) and *hGAR1* (Q9NY12.1) protein sequences from *H. sapiens* were used as queries against the Chordata organism database (taxid:7711). The complete list of retrieved fibrillarin proteins is shown in Table S2. An HMM model of the GAR domain was constructed from the alignment of the fibrillarin retrieved proteins and was subsequently used to detect other GAR domain-containing proteins in the 36 complete genomes. The list of retrieved proteins containing a GAR domain structure is given in Table S3.

3. Results

3.1. Fibrillarin as a Ribonuclease

Unlike other proteins studied in our laboratory, we unexpectedly detected RNA degradation activity during the process of recombinant fibrillarin purification. Therefore, this effect was studied more deeply to clarify whether recombinant fibrillarin was the cause of degradation or whether a ribonuclease interacted with the fibrillarin preparations. We purified recombinant fibrillarin to near homogeneity (Figure 1a) after several chromatographic steps, as stated above.

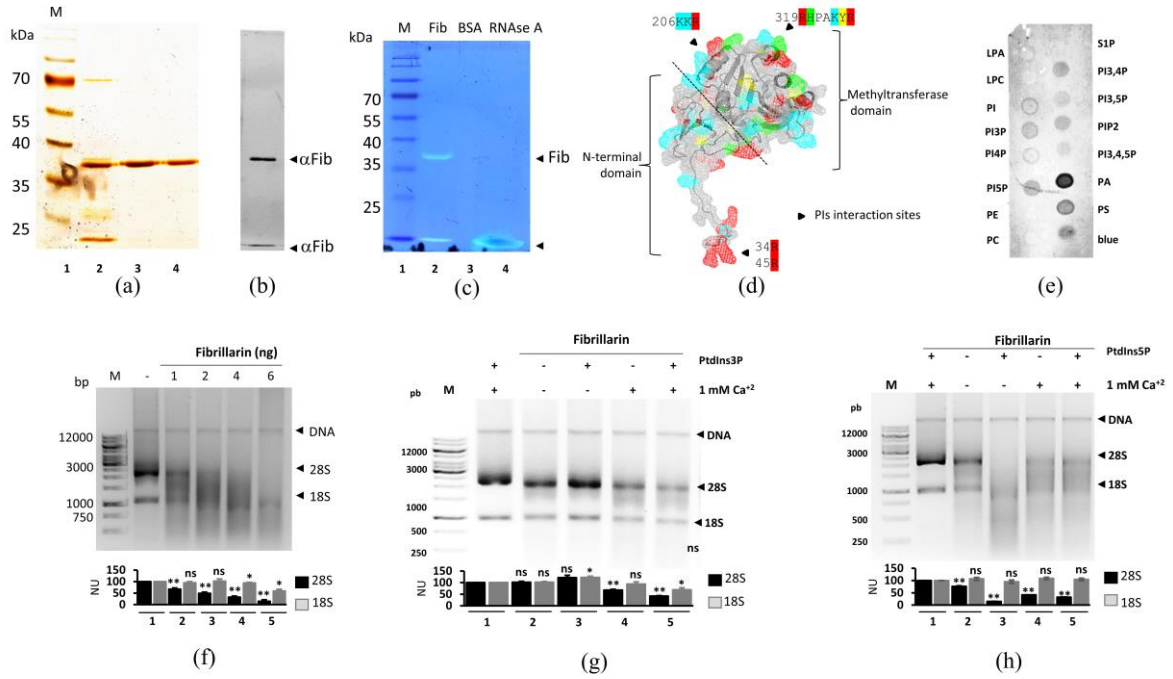

Figure 1. Fibrillarin acts as a ribonuclease. (**a**) Silver stain of purified fibrillarin. M: molecular weight marker. 2, 3, and 4: different fibrillarin elution profiles. Only elution 2 and 3 (major quality of protein purification) were used for further analysis. (**b**) Fibrillarin Western blot revealed two bands marked with arrow-heads that were recognized by specific fibrillarin antibody. (**c**) In-gel fibrillarin RNA assay shows two signals marked with arrowheads corresponding to fibrillarin (Lane 2). BSA and RNAse A were used as negative and positive controls, respectively (Lanes 3 and 4). (**d**) Computational inference of possible binding sites for phosphoinositides, one residing in the GAR domain and two in the globular C-terminal domain, are marked with arrows. The fibrillarin sequence with characteristic amino acids for phosphoinositide-binding is noted. Three to five amino acids are necessary for phophoinositide-binding [45,46]. These amino acids should be positively charged with at least one having an aromatic ring. Red: arginine, blue: lysine, green: histidine, yellow: tyrosine. (**e**) Fibrillarin fat blot assay shows that fibrillarin interacts modestly with several phosphoinositides and strongly with PA. Figure S1 depicts the quantification signals made by ImageJ software. (**f**) Total RNA in vitro assay was performed while increasing the concentration of protein (from 2 to 8 ng) added to a constant concentration of rRNA (2 µg). Degradation of RNA is directly proportional to the amount of fibrillarin. Copurified DNA from TriZol extraction is indicated by head arrows; the rRNA populations, i.e., 28S and 18S, are indicated. Influence of PI3P (**g**) and PIP5 (**h**) on human fibrillarin in vitro ribonuclease assay. (**f**–**h**) Quantification of 28S and 18S signals were made by ImageJ software and represented in normalized units (NU), statistical significance was determined by *t*-test (* p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001, ns= not significant p-value > 0.05) and plots are indicated below the gels from n = 3.

Fibrillarin identity was verified Western blotting using an anti-Fib primary antibody (Figure 1b), and two bands were detected. The upper band corresponds to the expected size of full-length fibrillarin, whereas a lower molecular weight band may correspond to the degradation product of the main protein. An *in-gel* activity assay was used to confirm that the ribonuclease activity was from the purified fibrillarin (Figure 1c). One band coincided with the full-length of fibrillarin confirmed by

Western blot (Figure 1b,c, Lane 2). In gel zymography, a small size band also exhibiting ribonuclease activity was noted (Figure 1c, Lane 2). This band corresponded with the potential fibrillarin degradation product detected by Western blot using an anti-Fib primary antibody (Figure 1b). Therefore, ribonuclease activity may be modular and present in a particular domain inside the fibrillarin sequence.

The interaction between fibrillarin and PIP2 has been reported [25]. Furthermore, according to the molecular characteristics for phosphoinositide binding [45,46], fibrillarin has the necessary amino acids (negatively charged and with aromatic rings) to bind phosphoinositides (Figure 1d). And indeed, in a phospholipid strip assay, fibrillarin interacted with phosphoinositides, but more strongly with the negatively charged PA (Figure 1e). From the homogeneous fibrillarin preparations, a dose-dependent ribonuclease activity in vitro assay was carried out by increasing the protein concentration from 1 to 6 ng with a constant amount of 2 µg of rRNA. The major 28/18S rRNA populations and their integrity used for the assay are depicted in Figure 1f Lane 2, and a DNA band co-purificated by TriZol®protocol is marked with arrowheads. rRNA degradation was directly proportional to the amount of fibrillarin added (Figure 1f, Lanes 2 to Lane 5); 28S rRNA was affected but not 5S rRNA (data not shown). The ribonuclease activity of fibrillarin was also found to be dependent on temperature, time, and display ion sensitivity (Figure S2).

Calcium ions have been detected in the crystal structure of human fibrillarin [47], and other enzymes with ribonuclease activity are either dependent on [48,49] or inhibited by calcium [50]. Our previous work showed that the AtFib2 from *A. thaliana* is activated with a small amount of calcium in ribonuclease activity assays, but no effect was observed in the same context with AtFib1 enzyme [51]. Ribonuclease activity was tested with PIP5 and PIP3 phospholipids with and without 1 mM Ca^{+2}. The experiments were carried out with a reduced amount of fibrillarin (<1 ng) to observe the likely activation of the enzyme (Figure 1g,h). The addition of PI3P had an inhibitory effect for rRNA digestion (Figure 1g, Lane 3), whereas the addition of PI5P resulted in a dramatic increase of ribonuclease activation (Figure 1h, Lane 3). Of note, these phospholipids did not affect rRNA in the absence of fibrillarin. Thus, strikingly, PIP3 and PIP5 differently affected the ribonuclease activity of fibrillarin.

3.2. Ribonuclease Activity of the Recombinant Ribonucleoparticle Complex Involving Fibrillarin

Fibrillarin forms part of one of the main snoRNP complexes that modify rRNA during ribosomal RNA biogenesis; this complex guides the specific methylation site of about 100 residues in the rRNA. The complex consists of three other well-known proteins: Nop58, Nop56, 15.5K, and one snoRNA guide [52–55].

To examine the ability of fibrillarin to degrade rRNA in the RNP context, a construct [41] containing a truncated fibrillarin (from amino acid 82 to 321) with the GAR domain absent in the coding sequence was compared to full-length fibrillarin. The first purified complex with full-length fibrillarin was termed RNP, and the second purified complex with truncated fibrillarin was termed RNP ΔGAR. The two versions of the RNP complex were visualized on SDS-PAGE (Figure 2a); a shift in the fibrillarin corresponding size was observed.

The ribonuclease activity of RNP and RNP ΔGAR complexes was tested in vitro using 2 µg of total RNA (Figure 2b); the complex with full-length fibrillarin degraded RNA, whereas RNP ΔGAR showed no significant activity. The addition of 1 mM Ca^{2+} did not affect the ribonuclease activity of either complex (Figure 2c). Similar to the experiments with purified fibrillarin, the influence of phosphoinositides were evaluated at a final concentration of 5 ng, but no effect of PIP3 and PIP5 was observed (Figure S3). Alternatively, PIP2 inhibited the ribonuclease activity of the complex in a concentration-dependent manner (Figure 2d). We also checked by structured illumination microscopy (SIM) the localization of PIP2 and fibrillarin using a SNAP-tag–fibrillarin construct in a stable cell line, indicating that in the nucleoli, the majority of fibrillarin surrounded PIP2 in the DFC (Figure 2e) and that the ring-like structure involved a change of phase which lacks the lipidic environment outside the DFC. Fibrillarin ring structures are likely formed by the interaction of rRNA, non-coding RNA, and phospholipids. The ring-like structure has a diameter with an average of 488 nm (SD ± 7 nm), with a PIP2 core of 188 nm (SD ± 3.6 nm). An analysis of the ring-like structure and its comparison to other proteins can be found in Figure S4 to Figure S8. In conclusion, the ability of fibrillarin to interact with several phospholipids may provide different functions in different phases from FC to DFC.

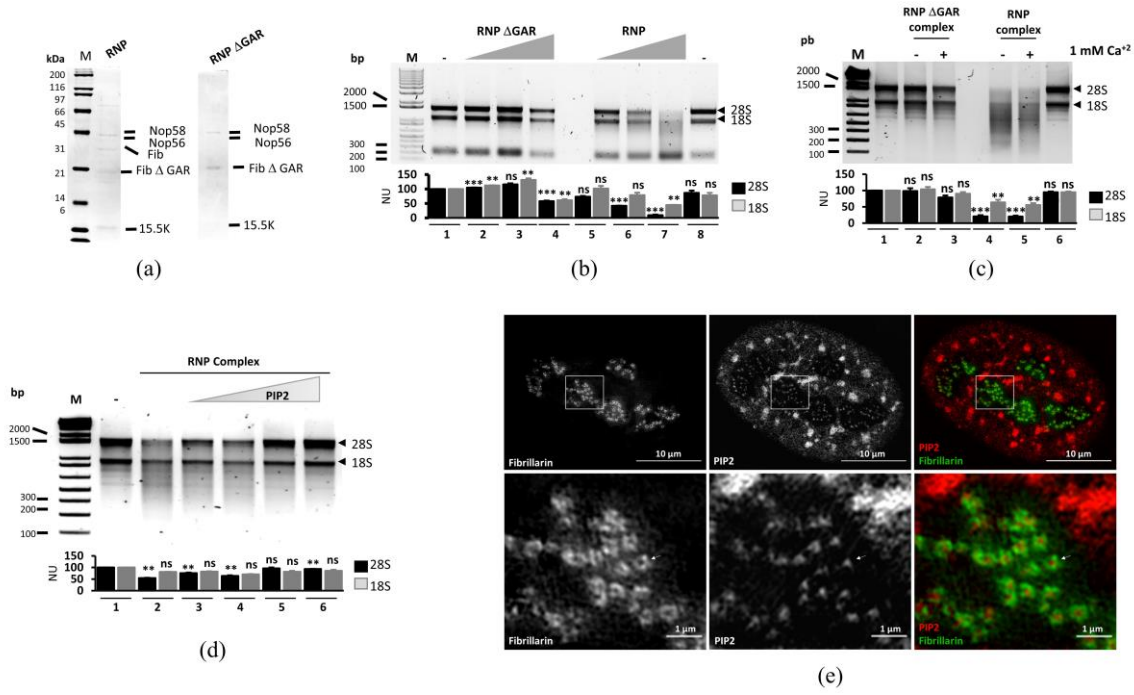

Figure 2. RNA activity of the recombinant ribonucleoprotein complex containing a truncated (RNP ΔGAR) and full-length complex (RNP). (**a**) IMAC affinity purification under native conditions of RNP ΔGAR and RNP complexes. The sequence coding GAR domain was inserted into the truncated fibrillarin–RNP complex, using the EMP–PCR strategy. A shift in the band size corresponding to fibrillarin is shown. The Nop58, Nop56, and 15.5K proteins are copurified as expected and labeled in the figures in both RNP purifications (**b**) Comparative activity of complexes with or without GAR domains. No activity was detected using increasing amounts of native RNP ΔGAR, while RNA degradation was observed with full-length fibrillarin in the complex. (**c**) Influence of calcium at 1 mM on the RNA activity. No significant modification of activity was observed

between the complexes. (**d**) *In vitro* RNA assay using increasing amounts of PIP2 lipid. Increasing amounts of phospholipid from 1 to 100 ng inhibited the of RNA activity. (**b**–**d**) Quantification of 28S and 18S signals were made by ImageJ software and statistical significance was determined by *t*-test (* *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001, ns = not significant *p*-value > 0.05) and plots are indicated below the gels from *n* = 3. (**e**) Colocalization of SNAP-tag–fibrillarin and immuno-labeled PIP2 (left panel) in HeLa cell nucleus. White inset is magnified in the right panel; the red signal corresponds to PIP2 and the green one to SNAP-tag–fibrillarin.

3.3. Fibrillarin Specificity Ribonuclease Activity in Complex with RNA Guide

The specificity of the SNAP-tag–fibrillarin complex as a ribonuclease was tested against U3 snoRNA, one of several RNA processing guidelines for rRNA that interacts with fibrillarin (Figure S9). Fibrillarin did not degrade U3 snoRNA, but interacted with it, resulting in retardation, as confirmed by GMSA (Figure 3a). The addition of PA dissociated the complex from U3 snoRNA (Figure 3a), whereas PIP2, under the same conditions, showed a minor alteration in the complex migration on gel (Figure 3a, Lanes 3 and 5).

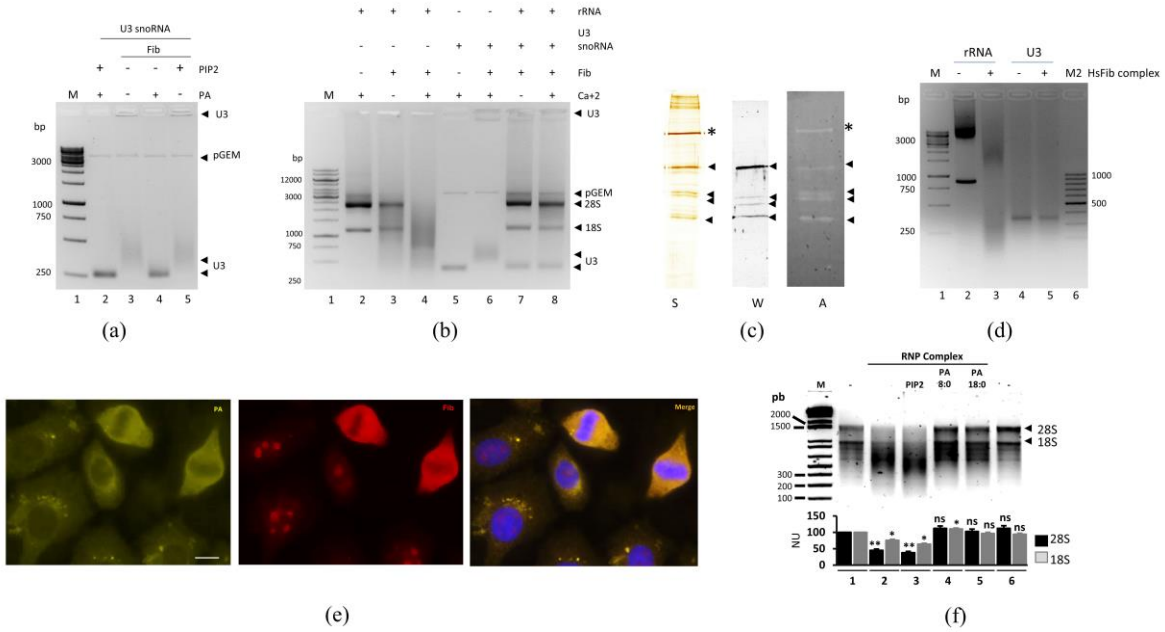

Figure 3. RNA specific RNA activity by fibrillarin with snoRNA U3. (**a**) Gel shift of snoRNA U3 with WT fibrillarin. All incubations and procedures carried out at 4 °C, PIP2 and PA were added as indicated. Plasmid control pGEM was used to normalize each lane. (**b**) RNA activity of fibrillarin with or without guide snoRNA U3 added. Reactions were carried out at 37 °C. (**c**) In-gel RNA activity of native fibrillarin RNP complex, labeled with silver staining (S), revealed by Western blot (W) and tested by activity assay (A). Arrowheads indicate fibrillarin and potential degradation products, and an asterisk indicates an undetermined ribonuclease activity (**d**) In vitro RNA activity of HeLa expressed and purified SNAP-tag–fibrillarin complex against total RNA and snoRNA U3. (**e**) Immunofluorescence showing the colocalization of PA and fibrillarin. The signal corresponds as follows: Blue–DAPI (nucleus signal), Yellow–PA sensor, and RED–fibrillarin. (**f**) In vitro RNA assay using PA. An inhibition effect was observed using PA containing 8 or 18 carbons and no saturation in the lateral chains (8:0 or 18:0), whereas little effect on the activity was observed with PIP2.

Quantification of 28S and 18S signals were made by ImageJ software and statistical significance was determined by *t*-test (* *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001, ns = not significant *p*-value > 0.05) and plots are indicated below the gels from *n* = 3.

Fibrillarin was not able to degrade rRNA when pre-incubated with snoRNA U3 for 15 minutes, prior to rRNA addition, suggesting that U3 snoRNA blocked the ribonuclease activity of fibrillarin (Figure 3b, Lane 7). When fibrillarin was incubated for 15 minutes without U3 snoRNA followed by the addition of rRNA, degradation was observed as expected. HeLa expressed and purified SNAP-tag–fibrillarin complex also showed ribonuclease activity (Figure 3c). An in-gel activity assay of the HeLa purified fibrillarin complex had 5 bands with ribonuclease activity corresponding to four bands revealed by Western blotting with anti-fibrillarin antibody (Figure 3c; marked with arrowheads). Fibrillarin degradation is likely to have occurred during the complex purification, as fibrillarin is prone to degradation even in the presence of protease inhibitors. The fifth band (asterisk) showed as-yet-undetermined ribonuclease activity. Fibrillarin complex from HeLa cells was unable to cut snoRNA U3 (Figure 3d). The subcellular distribution of PA was determined using a PA sensor described previously [39]. In HeLa cells during interphase, on one side, fibrillarin is concentrated in the nucleolus and, on the other side, the majority of a PA sensor was detected in perinuclear structures reminiscent of the Golgi apparatus with very little overlap of the signals (Figure 3a). Upon nuclear membrane rupture during mitosis, fibrillarin is abundantly relocated to the cytoplasm, where it colocalized with the PA sensor, as shown in Figure 3e. Note that during mitosis, the vesicular distribution of the PA sensor completely disappears. It is thus possible that fibrillarin interacts with PA in the cytoplasm during mitosis. The presence of PA inhibited the in vitro activity of the full-length fibrillarin containing the recombinant complex. This inhibition was not dependent on the size of the acyl chains of PA (Figure 3f, Lanes 4 and 5). A small change in the digestion pattern was, however, observed with low amounts of PIP2 (Figure 3f, Lane 3).

3.4. GAR Domain, Modular in Fibrillarin

Next, we evaluated the ability of the GAR domain from the recombinant complex to act as an active domain. The N-terminal region (GB: 1-134 aa) of fibrillarin includes the GAR domain and a sequence of amino acids 58 to 136 (the BCO) that has no defined function [10]. The GB (GAR and BCO regions) domain was cut into several sections, as depicted in Figure 4a. Constructs fused to GST and 6xHis were expressed and purified by a double-step purification and detected by a specific antibody (Figure 4b,c). The GAR domain exhibited ribonuclease activity, whereas the BCO did not show any activity (Figure 4d, Lane 5). Further deletions of amino acids 1 to 20 and 13 to 68 of the GAR domain abolished activity (Figure 4d, Lanes 4 and 6, respectively).

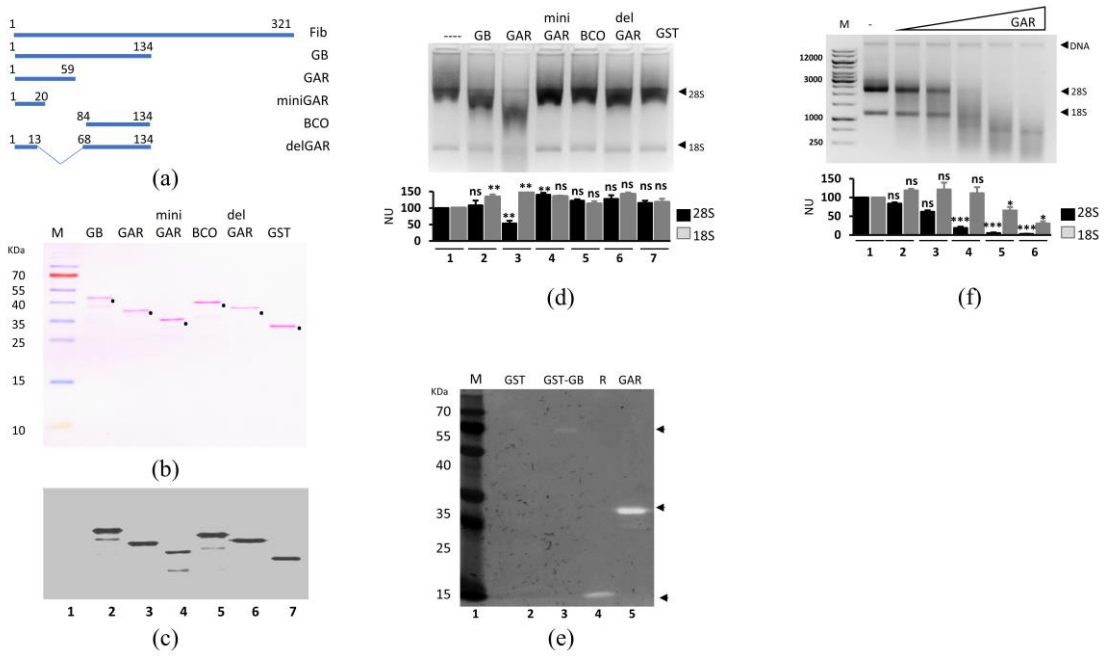

Figure 4. The GAR domain of fibrillarin is a modular domain with RNA activity. (**a**) Schematic representation of small peptides expressed from GB. (**b**) Ponceau staining of the HsGB small peptides expressed. All peptides were fused to the GST and 6 His tags. The band corresponding to the expected peptide is shown by a dot. **(c)** Anti-6xHis antibody was used to identify each peptide by Western blot. (**d**) RNA assay from the purified peptides. Under the same conditions, the GST-GAR domain has higher activity than the GST-GB domain (Lanes 2 and 3). The other peptides (miniGAR, BCO, and delGAR) have no activity. GST was used as a negative control. (**e**) In-gel RNAse assay of fibrillarin GB domain (amino acids 1 to 134). Degradation of RNA is higher when GST was cleaved from the GAR domain (Lanes 3 and 5). GST and RNAse A (R) were used as negative and positive controls, respectively. (**f**) RNA activity assay of the GAR domain. rRNA degradation is directly proportional to the amount of the GAR domain. (**d**,**f**) Quantification of 28S and 18S signals were made by ImageJ software and statistical significance was determined by *t*-test (* *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001, ns = not significant *p*-value > 0.05) and plots are indicated below the gels from *n* = 3.

An in-gel ribonuclease activity assay showed that the GAR domain fused with GST degraded RNA, and its activity increased when GST was cleaved (Figure 4e, Lanes 3 and 5). Increasing concentrations of purified GAR domain caused significant degradation of rRNA at the highest amount tested. Interestingly, 28S rRNA was the first target for the degradation using this domain (Figure 4f).

3.5. Mutation of the GAR Domain

From sequence analysis of the GAR domain, the conserved arginine in position 34 and 45 was chosen to be substituted with alanine (Figure 5a). Presumably, the alteration of amino acids causes a conformational change that better exposes the GAR domain in the R45A mutant, but not in R34A. The amount of all pure recombinant fibrillarins was normalized for ribonuclease activity or phospholipid-binding assays (Figure 5b,c). Surprisingly, R34A and R45A fibrillarin mutants lost the ability to interact only with the PIP2, PA, and phosphatidylserine (PS), while interaction with PI3P

and PI5P remained. R34A mutant also interacted weakly with phosphatidylcholine (PC) and sphingosine-1-phosphate (S1P) (Figure 5c). In conclusion, both mutations affected interaction with several anionic phospholipids, as shown by fat blot analysis, in agreement with the model where the positive arginine charges are essential for anionic phospholipid binding. WT fibrillarin and the R34A fibrillarin mutant degraded the rRNA 28S to the same degree, but the R45A fibrillarin mutant was found to be more active and degraded up to 80% of 28S rRNA (Figure 5d).

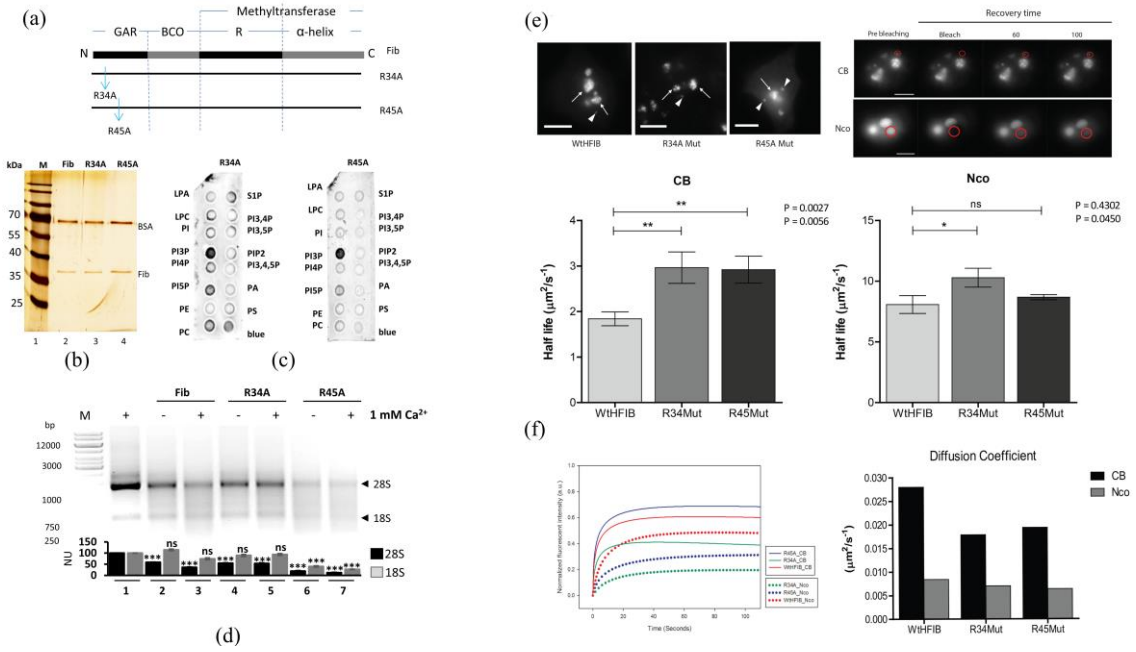

Figure 5. Activity of GAR domain mutants. (**a**) Schematic representation of fibrillarin domains GAR, BCO, and RNA binding alpha-helix domain. Blue arrows represent mutations R34A and R45A in the fibrillarin GAR domain. (**b**) Silver stain normalization of WT, R34A, and R45A fibrillarin. (**c**) Fat blot assay for fibrillarin mutants R34A and R45A. Figure S1 depicts the quantification signals made by ImageJ software. (**d**) RNA activity of fibrillarin mutants R34A and R45A. Quantification of 28S and 18S signals were made by ImageJ software and statistical significance was determined by *t*-test (* *p*-value < 0.05; ** *p*-value < 0.01; *** *p*-value < 0.001, ns = not significant *p*-value > 0.05) and plots are indicated below the gels from *n* = 3. (**e**) Transiently transfected HeLa cells expressing GFP-WT-fibrillarin, R34A, or R45A mutant coupled to GFP. In the upper left panel, representative images of the conventional localization of human fibrillarin in nucleoli (arrows) and Cajal bodies (arrowheads) are shown for WT and mutants. Intranuclear localization for WT fibrillarin and its mutants was observed in live cells. On the upper right panel, one representative photobleaching experiment is shown for a Cajal body and, for a nucleolus, red circles delimit the ROI, which was subsequently photobleached. The lower graphs plot the half-life coefficients obtained from 200 independent FRAP experiments comparing WT and mutants fibrillarins in CBs and NCOs; error bars correspond to SEM. (**f**) The left graph represents the normalized dynamics from 200 independent photobleaching events for WT and mutant fibrillarins in CBs and NCOs; the curves in the graphs represent the normalized values of the mean (*n* = 200) for each condition for each time point. The bar graph on the left shows a resume of the single diffusion coefficient values obtained from the previous analysis.

WT fibrillarin, mutant R34A, or R45A genes fused to GFP showed the previously reported intranuclear localization (enrichment in nucleoli and Cajal bodies) [1,2], but different intranuclear behavior in transiently transfected in HeLa cells. FRAP analysis showed that fibrillarin mutants had a 40% lower diffusion coefficient in Cajal bodies (CBs) showing statistical difference in *p*-values CB (WT R34) $p = 0.0056$ (**), and (WT - R45) $p = 0.0027$ (**). The mutants showed minor reduction in mobility in the nucleus (Nco) with *p*-values for Nco (WT - R34) $p = 0.0450$ (*) and for (WT - R45) $p = 0.4302$ (ns). Nucleolar R34A and R45A fibrillarin mutants showed a significant diminution in dynamic speed compared to WT, as reflected in increased half-lives. Compared to WT fibrillarin, R45A had a 50% reduction in nucleoli size, of which the intranuclear localization is shown in Figure 5e. In CBs, there was also an increase in the half-lives of R45A and R34A versus WT fibrillarin, reflected in smaller diffusion coefficient values observed as the longer residence time of the mutants within the nucleolus and CBs in comparison with WT (Figure 5f).

3.6. Ribonuclease Activity of GAR-Like Domains Containing Proteins

The phylogenetic tree constructed from the alignment of the GAR domain of the retrieved fibrillarin proteins was grouped into 3 major clades (Figure 6a; protocol and data in Figure S10). The basal branch contained the GAR domain of NOP1 protein from *S. cerevisiae*, the most ancestral species analyzed, and sequences of non-mammalian species. Group A was divided into 2 subclades (A1 and A2) and contained nearly all vertebrate species with single fibrillarin sequences. Group A contained mammalian sequences only, as compared to the basal group. Subgroup A1 contained GAR sequences from non-primates except for the Gorilla GAR sequence. Species with a second fibrillarin sequence were clustered in group B and exhibited a distinctive GAR pattern (Figure S11).

Using the HMM model constructed from the GAR domain of fibrillarin proteins, five different protein classes were retrieved: the fibrillarin proteins (fibrillarin domain), LSM14-A proteins (containing LSM14 (Scd6-like Sm domain and FDF domain), a predicted autoimmune regulator in armadillo (*Dasypus novemcinctus)*, containing HSR domain and PHD-finger), a predicted nucleolin isoform in rabbit (*Oryctolagus cuniculus*, containing 4 repeats of RRM1 (RNA recognition motif, RRM, RBD, or RNP domain), a nucleolar RNA helicase 2 in Zebrafish (*Danio rerio*, containing a DEAD (DEAD/DEAH box helicase), and a Helicase C (Helicase conserved C-terminal domain) and a GUCT (NUC152) domain). All 5 retrieved proteins contain domain regions with repeats of glycine-arginine amino acids known as RGG boxes, which are present in several proteins that participate in transcription, RNA binding and splicing and protein interactions [56–58]. A representation of the GAR domain location in each of the 5 retrieved proteins is depicted in Figure 6b. HMM for RGG-box 1 and RGG-box 2 repeats from GAR1 proteins were constructed, and other GAR1 proteins were detected in the analyzed genomes (Figure S12). LMS14-A was detected in 20 different species and contained 2 different R/G boxes, one before the FDF domain (a 20 aa R/G box I) and the other after the FDF domain (a 44 aa R/G box II). A multiple-sequence alignment of 23 LSM14-A proteins was performed and visualized with the Boxshade tool available at https://embnet.vital-it.ch/software/BOX_doc.html. The structure of the R/G rich region in such proteins followed the characteristic motif of the GAR domain (Figure S13). Considering that the GAR domain is absent in Archaebacteria fibrillarins and that it acts as a modular domain in other proteins, the published genomes for similar sequences were revised.

The Barley Stripe Mosaic Virus (BSMV) Triple Gene Block1 (TGB1) is a 58 kDa movement protein that possesses RNA-binding, RNA helicase, and ATPase activities [59–61]. The BSMV TGB1 protein contains a nuclear localization signal (NLS) and a nucleolar localization signal (NoLS) between 227–238 and 95–104 amino acids, respectively. BSMV TGB1 protein interacts with the GAR domain of the nucleolar fibrillarin (Fib2) from *Nicotiana benthamiana* [62]. Other examples correspond to the ORF3 viral protein that also interacts with the fibrillarin GAR domain [63]. Some NLS and NoLS contain intrinsically disordered regions [64,65] that help direct viral proteins to the nucleolus during viral infection. Therefore, the potential for TGB1 to degrade RNA was evaluated.

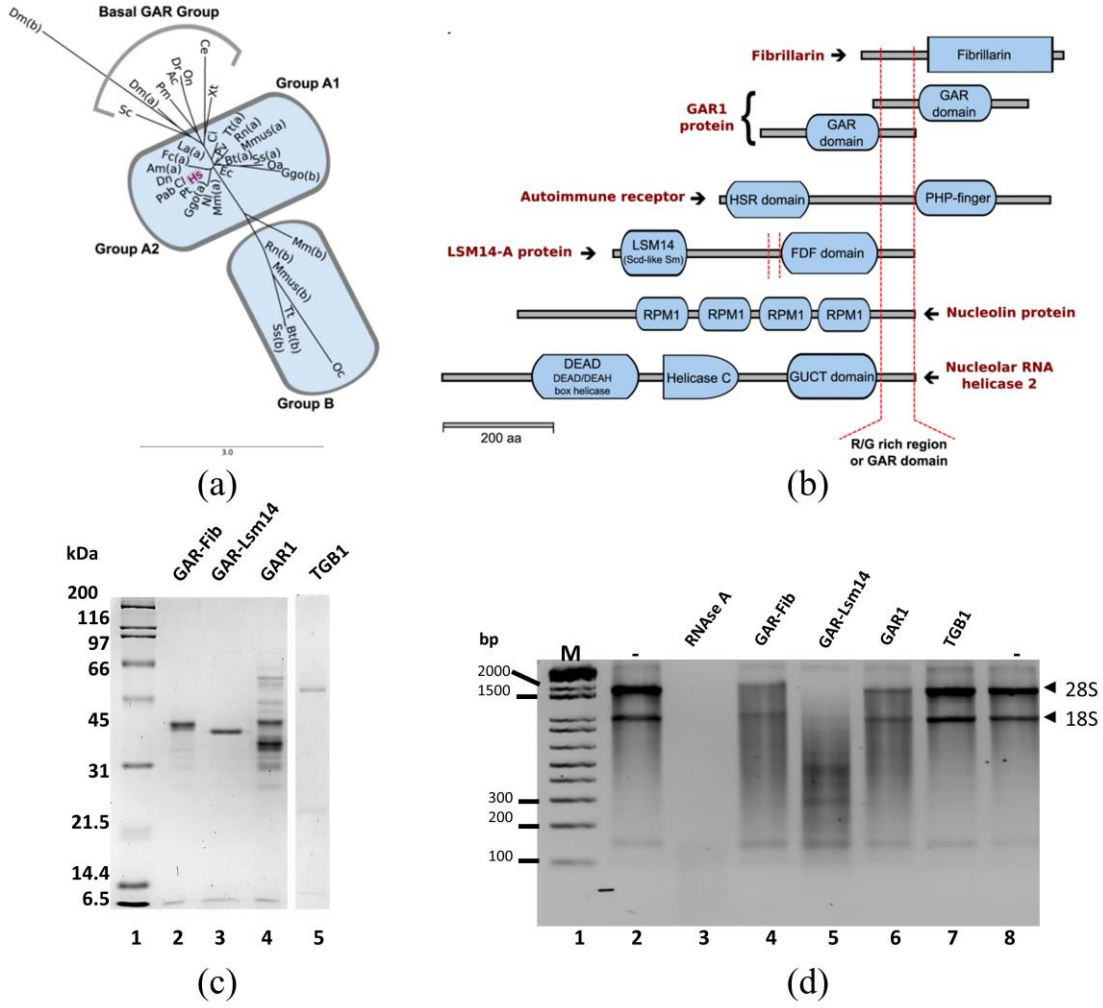

Figure6. RNA activity of GAR domains from different RNA-binding proteins. (**a**) Bioinformatic analysis of R/G rich region (or GAR domain) of retrieved proteins from 36 complete genomes. The phylogenetic tree of GAR domains of fibrillarin proteins was retrieved from chordates genome data. GAR domains were clustered in three major clades: A, B, and the basal group. Clade A was divided into A1 and A2 subgroups. (**b**) Schematic representation of different modular proteins shows the presence of GAR domains in N- or C-terminal regions. Five proteins contained GAR domains similar to fibrillarin: GAR1 protein, an autoimmune receptor, Lsm14, Nucleolin, and a nucleolar RNA helicase 2. (**c**) IMAC affinity purification of GAR domains from fibrillarin, Lsm14, GAR1, and viral protein TGB1 with a disordered domain similar to GAR. (**d**) In vitro RNA

assay of four GAR domains expressed and purified from *E. coli.* TGB1 and GAR1 (Lanes 6 and 7) showed no activity against total RNA. RNAse A diluted 1:20,000 was used as control.

Cloning and expression in *E. coli* were performed for the GAR domains from Lsm14, full-length GAR1, and the viral protein TGB1, before IMAC purification (Figure 6c), and their ribonuclease activity was compared in vitro with that of the GAR domain of fibrillarin. As shown in Figure 6d, the GAR domains from fibrillarin, LMS14, and GAR1 presented different degrees of activity as compared to the activity of RNAse A (Lane 3), which degraded total rRNA. In contrast, GARs from fibrillarin (Lane 4), Lsm14 (Lane 5), and GAR1 (Lane 6) presented a different pattern of degradation and null activity, as in the case of TGB1. Thus, not all the intrinsically disordered regions presented activities related to rRNA processing (Lane 7). We hypothesized that glycine–arginine repeat domains raised during evolution as a part of the lipid complexity and functional compartmentalization are required in the nuclear environment.

4. Discussion

Fibrillarin is an essential protein whose function and structure are highly conserved in all eukaryotic organisms [10,66]. It functions as a methyltransferase involved in the processing of rRNA [67] and the methylation of histone H2A in RNA Pol I promoters [12,13]. Here we describe its activity as a ribonuclease even in the absence of a complex with Nop56, Nop58, and 15.5K proteins. Ribosomal biogenesis requires strict control and tight regulation to maintain a high order of rRNA production [68,69]. The 47S pre-rRNA transcribed binds co-transcriptionally to fibrillarin in the periphery of the FC/DFC in its 5' end, and the self-assembly of fibrillarin in its GAR domain facilitates a directional sense of the pre-rRNA processing [70], forming specific clusters of RNA-protein complexes that mediate subsequent modifications of pre-rRNA after transcription. The ring-like structures are highly ordered and may involve a phase separation for fibrillarin activity. RNA pol I, together with UBF, are localized in the center with the majority of the nucleolar PIP2 pool. The majority of fibrillarin is found in this surrounding area, as seen in Figure 2e. The particular ring structures are similar in size and shape to those previously identified by labeling SLERT. The RNA SLERT is known to associate and regulate pre-rRNA transcription and rRNA production, leading to increased tumorigenesis [71].

The initial possibility for ribonuclease activity of fibrillarin was suggested by Kass et al. [18], who determined that a ribonucleoprotein complex containing fibrillarin and snoRNA U3 was involved in specific ribonuclease activity, using nuclear extracts from mouse cells. Furthermore, an antibody against fibrillarin decreased rRNA digestion significantly [18]. Tollervey and collaborators [67], using NOP1 (yeast fibrillarin), showed that its depletion reduced the level of mature rRNA but increased the amount of uncut premature rRNA [72]. In cruciferous plants, Vasquez-Saez et al. [22] isolated the ribonucleoprotein complex nuclear factor D consisting of 30 proteins, including fibrillarin, nucleolin, and the snoRNAs U3 and U14. The complex can interact with rDNA and cut into the P site, downstream from the A1, A2, A3, and B sequences located in the 5' ETS of pre-rRNA. However, the ribonuclease activity was not associated with any particular protein. In *C. elegans*, fibrillarin is a key regulator of pathogen resistance during bacterial pathogen infection [73]. In vitro and biomolecular fluorescence complementation assays [74] showed in *A. thaliana* that fibrillarin (FIB2 gene) can

interact with the ELF18-INDUCED LONG NONCODING RNA 1 (ELENA1) lncRNA and directly with MED19a in the nucleoplasm and the nucleolus, demonstrating that the FIB2 gene is a negative transcriptional regulator of immune responsive genes like PR1. We found here that the GAR domain of fibrillarin is responsible for its activity as a ribonuclease. The GAR domain is present in several nucleolar proteins, i.e., nucleolin [75], NSR1 [76], SSB1 [77], and GAR1 protein [78], which have RNA recognition motifs like fibrillarin, directly interact with rRNA, and are involved in its processing [78,79]. In fibrillarin, the GAR domain is needed to target nucleoli [80] and for interaction with nuclear phase viruses [63].

There is an increasing suggestion about how LLPSs are regulated in relation to the post-translational modifications (PTMs) on the disordered domains of different nucleolar proteins that promote phase separation behavior. Cross-talk between PTMs such as phosphorylation and methylation regulate and affects interactions between protein partners or other molecules. NOP1, the fibrillarin yeast counterpart, can be methylated at six of nine SRGG motifs, which belong to the GAR domain, and this arginine methylation increases the ability of NOP1 to interact with the major RNP proteins Nop56 and Nop58, also regulating the localization of NOP1 during ribosomal processing [24]. The mutations we carried on two arginines in fibrillarin led to an alteration in nucleolar size and dynamics associated with an alteration to phosphoinositide binding.

Furthermore, we established the interaction of various phospholipids with fibrillarin, including phosphoinositides and PA. Within the cell nucleus, phosphoinositides are essential cofactors for various processes ranging from transcription regulation and differentiation to cell cycle control [28,81]. Previously we described that PIP2 interacts with fibrillarin affecting its RNA binding mobility in native PAGE [25]. Here we also show that fibrillarin also interacts with PA. The general ribonuclease activity decreases upon interaction with PA and releases fibrillarin from the interaction with the guide snoRNA U3. We suggest that during mitosis, the interactions between PA and fibrillarin may prevent unspecific degradation of RNA and allow different complex formation.

Negatively charged phospholipid-binding domains usually require positive charges. A clear example is the γ-core, where the arginine has been mutated in a signature similar to that of the GAR domain of fibrillarin. Such mutations show similar alteration of phosphoinositide binding [82] as in our mutants. The mutation made in the GAR domain of R45A has a more significant effect on the activity of fibrillarin as a ribonuclease. Both mutants (R34A and R45A) modify the interaction with negatively charged phospholipids compared to WT fibrillarin. Phospholipids affect binding to proteins and can promote protein-protein interaction. An example is the PDZ domain (PSD-95/Discs large/ZO-1) of syntenin-2 [83]. This domain interacts with PIP2, regulating the nuclear organization of some proteins bearing this domain. The mutants for this PDZ domain (in positions Lys113, Lys167, Lys197, and Lys244) result in a loss of binding with PIP2 and functional alteration [83]. The nucleolar size changes in mutants may result from changes in protein interactions, as indicated by our FRAP experiments. Since fibrillarin interacts with at least 235 proteins [10], the mutations may lead to a reduction in size due to improper complex formation, resulting in a partial compromise in nucleoli architecture.

Ribosome biogenesis is an essential cellular process that consumes cell energy [84]. During interphase, where a high degree of ribosome synthesis is required, fibrillarin is mostly deacetylated by the NAD+ dependent deacetylase SIRT7, increasing H2AQ104 methylation levels and ensuring rRNA synthesis by Pol I complex activity. Here we show that fibrillarin also acts as a ribonuclease regulated by phosphoinositides and PA. It is favorable for the cell to inhibit a ribonuclease like fibrillarin during metaphase, and PA may provide such a mechanism. The PA found in membranes in the cytosol may interact with fibrillarin once the nuclear membrane is broken during metaphase. However, clearly more research in the subject is needed before this could be concluded. Fibrillarin ribonuclease activity may be directed by some guide RNA, like snoRNA U3, together with a phase change for functional localization. We used a complete processed rRNA without the external and internal transcription spaces, in which U3 was involved to guide the cleavages in at least three different positions [18]. This is the reason why the complex fibrillarin-U3 snoRNA was not able to degrade rRNA. Yeast mutants of NOP1 show different phenotypes that affect ribosome function and rRNA biogenesis at different stages, including a lack of cleavage of the pre-rRNA. Here we show for the first time that human fibrillarin has a second activity that is essential for such processes [10,51].

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4409/9/5/1143/s1, Figure S1: Quantification of Fat blot signals, Figure S2: Evaluation of the ribonuclease activity of fibrillarin, Figure S3: Ribonuclease activity assay of fibrillarin in the RNP complex or fibrillarin alone in the presence of different phosphoinositides, Figure S4: SIM image of SNAP FIB and Immunolocalize PIP2, Figure S5: SIM images taken along Z stack with 0.125 µM, Figure S6: Co-localization of GFP and SNAP tagged Fibrillarin, Figure S7: o-localization of immunolocalization fibrillarin and SNAP tagged Fibrillarin, Figure S8: SIM images of Snap-Fibrillarin, PA andPI4P during interphase, Figure S9: GMSA of fibrillarin in complex with snoRNA guides, Figure S10: Flow diagram of sequences analyzed by bioinformatics tools, Figure S11: GAR domain amino acid alignment, Figure S12: Phylogenetic analysis of the GAR domain branches founded in the proteomes of different species, Figure S13: Boxshade of the Lms14-A protein sequences. Table S1: List of chordate species and thier proteome files used in the analyses of GAR domain (Fibrillarin and GAR1 proteins), Table S2: Table. List of fibrillarin protein sequeces retrieved from 36 complete genomes of vertebrate species and a yaest (S. cerevisiae), Table S3: List of sequence proteins containing a GAR domain structure, retrieved from 36 complete genomes species by a HMM model from the GAR domain of FIB proteins.

Author Contributions: F.G.-C., U.R.C., and E.C. conceived the project and designed experimental strategies.

F.G.-C. and U.R.C. performed the experiments. A.P.-S. performed the bioinformatic analysis. A.B. and L.Š. performed the imaging and confocal microscopy. C.A. performed the FRAP analysis. F.G.-C. and U.R.C. wrote the manuscript. E.C., L.C.R.-Z., N.V., and P.H. critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the CONACYT project FC 1572, Grant Agency of the Czech Republic (Grant nos. 19-05608S and 18-19714S); by the Czech Academy of Sciences (Grant no. JSPS-20-06); by the Institutional Research Concept of the Institute of Molecular Genetics (Grant no. RVO: 68378050); by the MEYS CR (COST Inter-excellence internship LTC19048) and the project: BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University" (CZ.1.05/1.1.00/02.0109), from the European Regional Development Fund. The Microscopy Centre was supported by the MEYS CR (LM2018129 Czech-BioImaging) and by the European Regional Development Fund-Project "Modernization and support of research activities of the national infrastructure for biological and medical imaging CzechBioImaging" (no. CZ.02.1.01/0.0/0.0/16_013/0001775)., and the CONACYT fellowship to FGC with number 576664.

Acknowledgments: We would like to thank Michaela Blažíková and Ivan Novotný for their excellent help in protein dynamics as well as Pavel Krˇíž, Angela Ku, and Wilma Gonzalez for their technical help. Lisa DeTora provided editorial advice for manuscript revisions after peer review.

Conflicts of Interest: The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Schimmang, T.; Tollervey, D.; Kern, H.; Frank, R.; Hurt, E.C. A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *EMBO J.* **1989**, *8*, 4015–4024. [CrossRef] [PubMed]
2. Hernandez-Verdun, D.; Roussel, P.; Thiry, M.; Sirri, V.; Lafontaine, D.L.J. The nucleolus: Structure/function relationship in RNA metabolism. *Wiley Interdiscip. Rev. RNA* **2010**, *1*, 415–431. [CrossRef] [PubMed]
3. Narayanan, A.; Speckmann, W.; Terns, R.; Terns, M.P. Role of the Box C/D Motif in Localization of Small Nucleolar RNAs to Coiled Bodies and Nucleoli. *Mol. Biol. Cell* **1999**, *10*, 2131–2147. [CrossRef] [PubMed]
4. Weber, S.C.; Brangwynne, C.P. Inverse size scaling of the nucleolus by a concentration-dependent phase transition. *Curr. Biol.* **2015**, *25*, 641–646. [CrossRef] [PubMed]
5. Brangwynne, C.P.; Mitchison, T.J.; Hyman, A.A. Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 4334–4339. [CrossRef] [PubMed]
6. Falahati, H.; Wieschaus, E. Independent active and thermodynamic processes govern the nucleolus assembly in vivo. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 1335–1340. [CrossRef]
7. Berry, J.; Weber, S.C.; Vaidya, N.; Haataja, M.; Brangwynne, C.P. RNA transcription modulates phase transition-driven nuclear body assembly. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E5237–E5245. [CrossRef]
8. Mitrea, D.M.; Kriwacki, R.W. Phase separation in biology; functional organization of a higher order. *Cell Commun. Signal.* **2016**, *14*, 1. [CrossRef]
9. Mitrea, D.M.; Cika, J.A.; Stanley, C.B.; Nourse, A.; Onuchic, P.L.; Banerjee, P.R.; Phillips, A.H.; Park, C.G.; Deniz, A.A.; Kriwacki, R.W. Self-interaction of NPM1 modulates multiple mechanisms of liquid-liquid phase separation. *Nat. Commun.* **2018**, *9*, 1–13. [CrossRef]
10. Rodriguez-Corona, U.; Sobol, M.; Rodriguez-Zapata, L.C.; Hozak, P.; Castano, E. Fibrillarin from Archaea to human. *Biol. Cell* **2015**, *107*, 159–174. [CrossRef]
11. Aris, J.P.; Blobel, G. cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 931–935. [CrossRef] [PubMed]
12. Tessarz, P.; Santos-Rosa, H.; Robson, S.C.; Sylvestersen, K.B.; Nelson, C.J.; Nielsen, M.L.; Kouzarides, T. Glutamine methylation in histone H2A is an RNA-polymerase-I-dedicated modification. *Nature* **2014**, *505*, 564–568. [CrossRef] [PubMed]
13. Loza-Muller, L.; Rodríguez-Corona, U.; Sobol, M.; Rodríguez-Zapata, L.C.; Hozak, P.; Castano, E. Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in Brassica oleracea. *Front. Plant Sci.* **2015**, *6*, 976. [CrossRef]
14. Chen, H.; Wurm, T.; Britton, P.; Brooks, G.; Hiscox, J.A. Interaction of the coronavirus nucleoprotein with nucleolar antigens and the host cell. *J. Virol.* **2002**, *76*, 5233–5250. [CrossRef] [PubMed]
15. Ponti, D.; Troiano, M.; Bellenchi, G.C.; Battaglia, P.A.; Gigliani, F. The HIV Tat protein affects processing of ribosomal RNA precursor. *BMC Cell Biol.* **2008**, *9*, 32. [CrossRef] [PubMed]
16. Melén, K.; Tynell, J.; Fagerlund, R.; Roussel, P.; Hernandez-Verdun, D.; Julkunen, I. Influenza A H3N2 subtype virus NS1 protein targets into the nucleus and binds primarily via its C-terminal NLS2/NoLS to nucleolin and fibrillarin. *Virol. J.* **2012**, *9*, 167. [CrossRef]
17. Fournier, M.J.; Maxwell, E.S. The nucleolar snRNAs: Catching up with the spliceosomal snRNAs. *Trends Biochem. Sci.* **1993**, *18*, 131–135. [CrossRef]
18. Kass, S.; Tyc, K.; Steitz, J.A.; Sollner-Webb, B. The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell* **1990**, *60*, 897–908. [CrossRef]
19. Hughes, J.M.; Ares, M. Depletion of U3 small nucleolar RNA inhibits cleavage in the 5' external transcribed spacer of yeast pre-ribosomal RNA and impairs formation of 18S ribosomal RNA. *EMBO J.* **1991**, *10*, 4231–4239. [CrossRef]
20. Dragon, F.; Lemay, V.; Trahan, C. snoRNAs: Biogenesis, structure and function. *Encycl. Life Sci.* **2006**, 1–7. [CrossRef]

21. Granneman, S.; Vogelzangs, J.; Lührmann, R.; van Venrooij, W.J.; Pruijn, G.J.M.; Watkins, N.J. Role of pre-rRNA base pairing and 80S complex formation in subnucleolar localization of the U3 snoRNP. *Mol. Cell. Biol.* **2004**, *24*, 8600–8610. [CrossRef]
22. Sáez-Vasquez, J.; Caparros-Ruiz, D.; Barneche, F.; Echeverría, M. Characterization of a crucifer plant pre-rRNA processing complex. *Biochem. Soc. Trans.* **2004**, *32*, 578–580. [CrossRef] [PubMed]
23. Lin, Y.; Protter, D.S.W.; Rosen, M.K.; Parker, R. Formation and maturation of phase-separated liquid droplets by RNA-binding proteins. *Mol. Cell* **2015**, *60*, 208–219. [CrossRef] [PubMed]
24. Smith, D.-L.; Erce, M.A.; Lai, Y.-W.; Tomasetig, F.; Hart-Smith, G.; Hamey, J.J.; Wilkins, M.R. Crosstalk of phosphorylation and arginine methylation in disordered SRGG repeats of S. cerevisiae fibrillarin and its association with nucleolar localisation. *J. Mol. Biol.* **2019**, *432*, 448–466. [CrossRef] [PubMed]
25. Yildirim, S.; Castano, E.; Sobol, M.; Philimonenko, V.V.; Dzijak, R.; Venit, T.; Hozak, P.; Hozák, P. Involvement of phosphatidylinositol 4,5-bisphosphate in RNA polymerase I transcription. *J. Cell Sci.* **2013**, *126*, 2730–2739. [CrossRef] [PubMed]
26. Sobol, M.; Yildirim, S.; Philimonenko, V.V.; Marášek, P.; Castaño, E.; Hozák, P. UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity. *Nucleus* **2013**, *4*, 478–486. [CrossRef] [PubMed]
27. Hamann, B.L.; Blind, R.D. Nuclear phosphoinositide regulation of chromatin. *J. Cell. Physiol.* **2017**, *233*, 107–123. [CrossRef]
28. Castano, E.; Yildirim, S.; Fáberová, V.; Krausová, A.; Ulicˇná, L.; Paprcˇková, D.; Sztacho, M.; Hozák, P. Nuclear phosphoinositides—Versatile regulators of genome functions. *Cells* **2019**, *8*, 649. [CrossRef]
29. Kalasova, I.; Fáberová, V.; Kalendová, A.; Yildirim, S.; Ulicˇná, L.; Venit, T.; Hozák, P. Tools for visualization of phosphoinositides in the cell nucleus. *Histochem. Cell Biol.* **2016**, *145*, 485–496. [CrossRef]
30. Sobol, M.; Krausová, A.; Yildirim, S.; Kalasová, I.; Fáberová, V.; Vrkoslav, V.; Philimonenko, V.; Marášek, P.; Pastorek, L.; Capek, M.; et al. Nuclear phosphatidylinositol 4,5-bisphosphate islets contribute to efficient RNA polymerase II-dependent transcription. *J. Cell Sci.* **2018**, *131*, jcs211094. [CrossRef]
31. Arraiano, C.M.; Mauxion, F.; Viegas, S.C.; Matos, R.G.; Séraphin, B. Intracellular ribonucleases involved in transcript processing and decay: Precision tools for RNA. *Biochim. Biophys. Acta* **2013**, *1829*, 491–513. [CrossRef] [PubMed]
32. Deutscher, M.P. Twenty years of bacterial RNases and RNA processing: How we've matured. *RNA* **2015**, *21*, 597–600. [CrossRef] [PubMed]
33. Moelling, K.; Broecker, F. The reverse transcriptase-RNase H: From viruses to antiviral defense. *Ann. N. Y. Acad. Sci.* **2015**, *1341*, 126–135. [CrossRef] [PubMed]
34. Bubeck, D.; Reijns, M.A.M.; Graham, S.C.; Astell, K.R.; Jones, E.Y.; Jackson, A.P. PCNA directs type 2 RNase H activity on DNA replication and repair substrates. *Nucleic Acids Res.* **2011**, *39*, 3652–3666. [CrossRef] [PubMed]
35. Houseley, J.; Tollervey, D. The many pathways of RNA degradation. *Cell* **2009**, *136*, 763–776. [CrossRef] [PubMed]
36. Henras, A.K.; Plisson-Chastang, C.; O'Donohue, M.F.; Chakraborty, A.; Gleizes, P.E. An overview of pre-ribosomal RNA processing in eukaryotes. *Wiley Interdiscip. Rev. RNA* **2015**, *6*, 225–242. [CrossRef]
37. Fatica, A.; Galardi, S.; Altieri, F.; Bozzoni, I. Fibrillarin binds directly and specifically to U16 box C/D snoRNA. *RNA* **2000**, *6*, 88–95. [CrossRef]
38. Tschochner, H.; Hurt, E. Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends Cell Biol.* **2003**, *13*, 255–263. [CrossRef]
39. Kassas, N.; Tanguy, E.; Thahouly, T.; Fouillen, L.; Heintz, D.; Chasserot-Golaz, S.; Bader, M.-F.; Grant, N.J.; Vitale, N. Comparative characterization of phosphatidic acid sensors and their localization during frustrated phagocytosis. *J. Biol. Chem.* **2017**, *292*, 4266–4279. [CrossRef]
40. Ulrich, A.; Andersen, K.R.; Schwartz, T.U. Exponential Megapriming PCR (EMP) Cloning-Seamless DNA Insertion into Any Target Plasmid without Sequence Constraints. *PLoS ONE* **2012**, *7*, e0053360. [CrossRef]

41. Peng, Y.; Yu, G.; Tian, S.; Li, H. Co-expression and co-purification of archaeal and eukaryal box C/D RNPs. *PLoS ONE* **2014**, *9*, e0103096. [CrossRef] [PubMed]
42. Dudkina, E.; Ulyanova, V.; Shah Mahmud, R.; Khodzhaeva, V.; Dao, L.; Vershinina, V.; Kolpakov, A.; Ilinskaya, O. Three-step procedure for preparation of pure Bacillus altitudinis ribonuclease. *FEBS Open Bio* **2016**, *6*, 24–32. [CrossRef] [PubMed]
43. Chen, D.; Huang, S. Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. *J. Cell Biol.* **2001**, *153*, 169–176. [CrossRef] [PubMed]
44. Altschul, S.F.; Koonin, E.V. Iterated profile searches with PSI-BLAST–a tool for discovery in protein databases. *Trends Biochem. Sci.* **1998**, *23*, 444–447. [CrossRef]
45. Rosenhouse-Dantsker, A.; Logothetis, D.E.; Levitan, I. Cholesterol sensitivity of KIR2.1 is controlled by a belt of residues around the cytosolic pore. *Biophys. J.* **2011**, *100*, 381–389. [CrossRef]
46. Morales, J.; Sobol, M.; Rodriguez-Zapata, L.C.; Hozak, P.; Castano, E. Aromatic amino acids and their relevance in the specificity of the PH domain. *J. Mol. Recognit.* **2017**, *30*. [CrossRef]
47. Min, J.; Wu, H.; Zeng, H.; Loppnau, P.; Weigelt, J.; Sundstrom, M.; Arrowsmith, C.H.; Edwards, A.M.; Bochkarev, A.; Plotnikov, A.N.; et al. Resour. *Stud. Biol. Macromol. Protein Data Bank.* Available online: https://www.rcsb.org/structure/2IPX. (accessed on 20 April 2019).
48. Schwarz, D.S.; Blower, M.D. The calcium-dependent ribonuclease XendoU promotes ER network formation through local RNA degradation. *J. Cell Biol.* **2014**, *207*, 41–57. [CrossRef] [PubMed]
49. Seidel, C.W.; Peck, L.J. Purification of a calcium dependent ribonuclease from Xenopus laevis. *NucleicAcidsRes.* **1994**, *22*, 1456–1462. [CrossRef]
50. Rosta, E.; Yang, W.; Hummer, G. Calcium inhibition of ribonuclease H1 two-metal ion catalysis. *J. Am. Chem. Soc.* **2014**, *136*, 3137–3144. [CrossRef]
51. Rodriguez-Corona, U.; Pereira-Santana, A.; Sobol, M.; Rodriguez-Zapata, L.C.; Hozak, P.; Castano, E. Novel Ribonuclease Activity Differs between Fibrillarins from Arabidopsis thaliana. *Front. Plant Sci.* **2017**, *8*, 1878. [CrossRef]
52. Tran, E.J.; Zhang, X.; Maxwell, E.S. Efficient RNA 2'-O-methylation requires juxtaposed and symmetrically assembled archaeal box C/D and C'/D' RNPs. *EMBO J.* **2003**, *22*, 3930–3940. [CrossRef] [PubMed]
53. Watkins, N.J.; Bohnsack, M.T. The box C/D and H/ACA snoRNPs: Key players in the modification, processing and the dynamic folding of ribosomal RNA. *Wiley Interdiscip. Rev. RNA* **2012**, *3*, 397–414. [CrossRef] [PubMed]
54. Newman, D.R.; Kuhn, J.F.; Shanab, G.M.; Maxwell, E.S. Box C/D snoRNA-associated proteins: Two pairs of evolutionarily ancient proteins and possible links to replication and transcription. *RNA* **2000**, *6*, 861–879. [CrossRef] [PubMed]
55. Tomkuviene, M.; Licˇyte, J.; Olendraite, I.; Liutkevicˇiute, Z.; Clouet-D'Orval, B.; Klimašauskas, S. Archaeal fibrillarin-Nop5 heterodimer 2^0-O-methylates RNA independently of the C/D guide RNP particle. *RNA* **2017**, *23*, 1329–1337. [CrossRef]
56. Thandapani,P.; O'Connor,T.R.; Bailey,T.L.; Richard,S.DefiningtheRGG/RGMotif. *Mol. Cell***2013**,*50*,613–623. [CrossRef]
57. McBride, A.E.; Conboy, A.K.; Brown, S.P.; Ariyachet, C.; Rutledge, K.L. Specific sequences within arginine-glycine-rich domains affect mRNA-binding protein function. *Nucleic Acids Res.* **2009**, *37*, 4322–4330. [CrossRef]
58. Gendra, E.; Moreno, A.; Albà, M.M.; Pages, M. Interaction of the plant glycine-rich RNA-binding protein MA16 with a novel nucleolar DEAD box RNA helicase protein from Zea mays. *Plant J.* **2004**, *38*, 875–886. [CrossRef]
59. Donald, R.G.; Lawrence, D.M.; Jackson, A.O. The barley stripe mosaic virus 58-kilodalton beta (b) protein is a multifunctional RNA binding protein. *J. Virol.* **1997**, *71*, 1538–1546. [CrossRef]

60. Lawrence, D.M.; Jackson, A.O. Requirements for cell-to-cell movement of Barley stripe mosaic virus in monocot and dicot hosts. *Mol. Plant Pathol*. **2001**, *2*, 65–75. [CrossRef]
61. Lawrence, D.M.; Jackson, A.O. Interactions of the TGB1 protein during cell-to-cell movement of Barley stripe mosaic virus. *J. Virol.* **2001**, *75*, 8712–8723. [CrossRef]
62. Li, Z.; Zhang, Y.; Jiang, Z.; Jin, X.; Zhang, K.; Wang, X.; Han, C.; Yu, J.; Li, D. Hijacking of the nucleolar protein fibrillarin by TGB1 is required for cell-to-cell movement of Barley stripe mosaic virus. *Mol. Plant Pathol.* **2018**, *19*, 1222–1237. [CrossRef] [PubMed]
63. Kim, S.H.; Macfarlane, S.; Kalinina, N.O.; Rakitina, D.V.; Ryabov, E.V.; Gillespie, T.; Haupt, S.; Brown, J.W.S.; Taliansky, M. Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 11115–11120. [CrossRef] [PubMed]
64. Emmott, E.; Hiscox, J.A. Nucleolar targeting: The hub of the matter. *EMBO Rep.* **2009**, *10*, 231–238. [CrossRef] [PubMed]
65. Dixon, S.E.; Bhatti, M.M.; Uversky, V.N.; Dunker, A.K.; Sullivan, W.J., Jr. Regions of intrinsic disorder help identify a novel nuclear localization signal in Toxoplasma gondii histone acetyltransferase TgGCN5-B. *Mol. Biochem. Parasitol.* **2011**, *175*, 192–195. [CrossRef]
66. Jansen, R.P.; Hurt, E.C.; Kern, H.; Lehtonen, H.; Carmo-Fonseca, M.; Lapeyre, B.; Tollervey, D. Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in yeast. *J. Cell Biol.* **1991**, *113*, 715–729. [CrossRef] [PubMed]
67. Tollervey, D.; Lehtonen, H.; Jansen, R.; Kern, H.; Hurt, E.C. Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell* **1993**, *72*, 443–457. [CrossRef]
68. Peña, C.; Hurt, E.; Panse, V.G. Eukaryotic ribosome assembly, transport and quality control. *Nat. Struct. Mol. Biol.* **2017**, *24*, 689–699. [CrossRef]
69. Correll, C.C.; Bartek, J.; Dundr, M. The Nucleolus: A multiphase condensate balancing ribosome synthesis and translational capacity in health, aging and ribosomopathies. *Cells* **2019**, *8*, 869. [CrossRef]
70. Yao, R.-W.; Xu, G.; Wang, Y.; Shan, L.; Luan, P.-F.; Wang, Y.; Wu, M.; Yang, L.-Z.; Xing, Y.-H.; Yang, L.; et al. Nascent Pre-rRNA Sorting via Phase Separation Drives the Assembly of Dense Fibrillar Components in the Human Nucleolus. *Mol. Cell* **2019**, *76*, 767–783. [CrossRef]
71. Xing, Y.-H.; Yao, R.-W.; Zhang, Y.; Guo, C.-J.; Jiang, S.; Xu, G.; Dong, R.; Yang, L.; Chen, L.-L. SLERT Regulates DDX21 Rings Associated with Pol I Transcription. *Cell* **2017**, *169*, 664–678.e16. [CrossRef]
72. Tollervey, D.; Lehtonen, H.; Carmo-Fonseca, M.; Hurt, E.C. The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *EMBO J.* **1991**, *10*, 573–583. [CrossRef] [PubMed]
73. Tiku, V.; Kew, C.; Mehrotra, P.; Ganesan, R.; Robinson, N.; Antebi, A. Nucleolar fibrillarin is an evolutionarily conserved regulator of bacterial pathogen resistance. *Nat. Commun.* **2018**, *9*, 3607. [CrossRef] [PubMed]
74. Seo, J.S.; Diloknawarit, P.; Park, B.S.; Chua, N.H. ELF18-INDUCED LONG NONCODING RNA 1 evicts fibrillarin from mediator subunit to enhance PATHOGENESIS-RELATED GENE 1 (PR1) expression. *New Phytol*. **2019**, *221*, 2067–2079. [CrossRef] [PubMed]
75. Lapeyre, B.; Bourbon, H.; Amalric, F. Nucleolin, the major nucleolar protein of growing eukaryotic cells: An unusual protein structure revealed by the nucleotide sequence. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 1472–1476. [CrossRef]
76. Lee, W.C.; Xue, Z.X.; Mélèse, T. The NSR1 gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *J. Cell Biol.* **1991**, *113*, 1–12. [CrossRef]
77. Jong, A.Y.; Clark, M.W.; Gilbert, M.; Oehm, A.; Campbell, J.L. Saccharomyces cerevisiae SSB1 protein and its relationship to nucleolar RNA-binding proteins. *Mol. Cell. Biol.* **1987**, *7*, 2947–2955. [CrossRef]
78. Girard, J.P.; Lehtonen, H.; Caizergues-Ferrer, M.; Amalric, F.; Tollervey, D.; Lapeyre, B. GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J.* **1992**, *11*, 673–682. [CrossRef]

79. Bugler, B.; Bourbon, H.; Lapeyre, B.; Wallace, M.O.; Chang, J.H.; Amalric, F.; Olson, M.O. RNA binding fragments from nucleolin contain the ribonucleoprotein consensus sequence. *J. Biol. Chem.* **1987**, *262*, 10922–10925.
80. Snaar, S.; Wiesmeijer, K.; Jochemsen, A.G.; Tanke, H.J.; Dirks, R.W. Mutational analysis of fibrillarin and its mobility in living human cells. *J. Cell Biol.* **2000**, *151*, 653–662. [CrossRef]
81. Fiume, R.; Keune, W.J.; Faenza, I.; Bultsma, Y.; Ramazzotti, G.; Jones, D.R.; Martelli, A.M.; Somner, L.; Follo, M.Y.; Divecha, N.; et al. Nuclear phosphoinositides: Location, regulation and function. *Subcell. Biochem.* **2012**, *59*, 335–361.
82. Sagaram, U.S.; El-Mounadi, K.; Buchko, G.W.; Berg, H.R.; Kaur, J.; Pandurangi, R.S.; Smith, T.J.; Shah, D.M. Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: Identification of an RGFRRR motif governing fungal cell entry. *PLoS ONE* **2013**, *8*, e82485. [CrossRef] [PubMed]
83. Wawrzyniak, A.M.; Kashyap, R.; Zimmermann, P. Phosphoinositides and PDZ domain scaffolds. *Adv. Exp. Med. Biol.* **2013**, *991*, 41–57. [PubMed]
84. Warner, J.R. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* **1999**, *24*, 437–440. [CrossRef]

Capítulo VII. Revisión bibliográfica sobre proteínas con regiones desordenadas que participan en el proceso de separación líquido-líquido en biología. Sometido a International Journal of Molecular Sciences.

Review

Intrinsically disordered nucleolar proteins drive important process inside the cell

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Abstract: Multiple phase separation process can allow for multi structure and dynamic system in the cell to provide a level of organization that can be achieved with low energy requirements. Chemical modifications of proteins, RNA and Lipids can provide different environments for molecules to carry out enzymatic reactions at higher concentrations in particular locations of the cell. Here we show how membraneless process can maintain a dynamic situation and a high level of organization.

Keywords: Nucleolus, Intrinsically Disordered, Phase Separation, Fibrillarin, Phosphoinositides

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Int. J. Mol. Sci.* **2021**, *22*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

Compartmentalization around a lipid membrane organelle is one of the current paradigms that allows specific cellular process to take place inside the cell (Sonnino & Prinetti, 2010). However, a high complexity system requires more freedom to interchange components with low energy investment from the cell. Recently a growing body of evidence pointing that membraneless organelles show the same high order of structural and functional features (Banani et al., 2016; Boeynaems et al., 2018; Gomes & Shorter, 2019). Membraneless organelles inside the cells are getting more attention in biology not only for the cellular process that include them: ribosome biogenesis, transcription and RNA processing but also the dynamics of molecule exchange and organization (Zhao & Zhang, 2020). Membraneless organelles displays a drop-like behavior, characterized as Liquid-Liquid Phase Separation (LLPS) behavior (Falahati & Haji-Akbari, 2019; Strom & Brangwynne, 2019; Zhu et al., 2019). Based in their composition, membranelles organelles are enriched in proteins, RNA molecules, and lipids (Castano et al., 2019; Riback et al., 2020). These molecules, especially proteins, possess several post-translationally modifications in their sequences, leading to a complex mechanism of interactions between them and other membraneless body components. RNA constituents are also important in the regulation of dynamics, formation and deregulation of membraneless bodies (Alshareedah, Moosa, Raju, Potoyan, & Banerjee, 2020; Latonen, 2019). Maintenance of membraneless organelles (also bio-condensates) through cell cycle remains obscure and several questions rise about the nature of the process of LLPS into a non-equilibrium system like cells. Basic thermodynamics support partially the principles that governs bio-condensates formation and stability, but other important features still ambiguous, like the contribution of energy dependent process, chromatin influence on LLPS and epigenetics driving condensation.

2. Thermodynamics driving LLPS

Thermodynamic free energy state is currently the hypothesis driving liquid-liquid phase separation processes inside the cell (Alshareedah et al., 2020; Lafontaine, 2019). The simplest view of the hypothesis suggests that the drop formation process is a passive transition involving only weak interactions between different molecules, i.e., RNA and proteins with in a matrix defined as α. LLPS assembled bodies exhibits special features like the almost spherical configuration, fusion events when two droplets touch each other, and free diffusion of the molecules inside the condensate or b (Brangwynne,

Mitchison, & Hyman, 2011b; Weber & Brangwynne, 2012). But recently was demonstrated that also involves active processes related to rRNA transcription, post translational modifications and partner interactions that can be added as an additional term as c that may keep partial phase separation or amorphous structure of the form structure. Moreover, the coalescence assembly of the nucleolar structure for example, may involve two mayor driving forces: a passive, thermodynamically dependent process and an active, energy-consuming process (Falahati & Haji-Akbari, 2019; Falahati, Pelham-Webb, Blythe, & Wieschaus, 2016; Falahati & Wieschaus, 2017). However, this hypothesis was recently assayed considering that two specifically features of drop coalescence formation are temperature dependency and reversibility. LLPS are in that sense, a spontaneously driven mechanism that builds it up the nucleolar region, and possibly mediate nucleolus formation and function by spinodal decomposition primarily in the free Gibbs energy. Interesting to note is that not only LLPS forces mediate the nucleolus formation, but also energy consuming process, suggesting that enzymatically dependent process is a second force that contributes to the nucleolar formation and function that we added as the c term (Falahati & Wieschaus, 2017). In a molecular scale, LLPS is dependent on the multi-valency and multi-interaction networks inherent at the molecules like RNA and proteins that possess binding regions or repetitive motifs characterized by the non-stable conformational structure (P. Li et al., 2012; Yoshizawa, Nozawa, Jia, Saio, & Mori, 2020). This multi-valency and multi-interaction networks provide at the molecular level, the features that confer material properties to membraneless bio-condensates, like the viscoelasticity and the fusion dependent on temperature (Brangwynne, Mitchison, & Hyman, 2011a; Caragine, Haley, & Zidovska, 2018). Using Cryo2-blue light conformational change (Zhu et al., 2019) demonstrate that the material properties of the nucleolus in human cells are key features to modulate the proper function and processing of rRNA transcripts. Also supporting the idea that this function/regulation dependent on material properties are involved in some diseases but also in other membraneless organelles like speckles, Cajal Bodies and other functional bio-condensates. The use of optogenetics in these types of experiments lead to a fine tuning the mechanical and physical property of the nucleolar body, as demonstrated. Ribosomal RNA transcription requires a huge number of factors some of them with a size enough to prevents free diffusion when a gel like mesh is induced by blue light, given a buildup of the early species of rRNA during its processing (47S, 46S, 45S) and a decreased population of fully processed rRNA species (41S, 36S).

n the spinodal region of the phase diagram, the free-energy can be lowered by allowing the components to separate, thus increasing the relative concentration of a component material in a particular region of the material. The concentration will continue to increase until the material reaches the stable part of the phase diagram. Very large

regions of material will change their concentration slowly due to the amount of material which must be moved. Very small regions will shrink away due to the energy cost in maintaining an interface between two dissimilar component materials. [18] [19] [20]

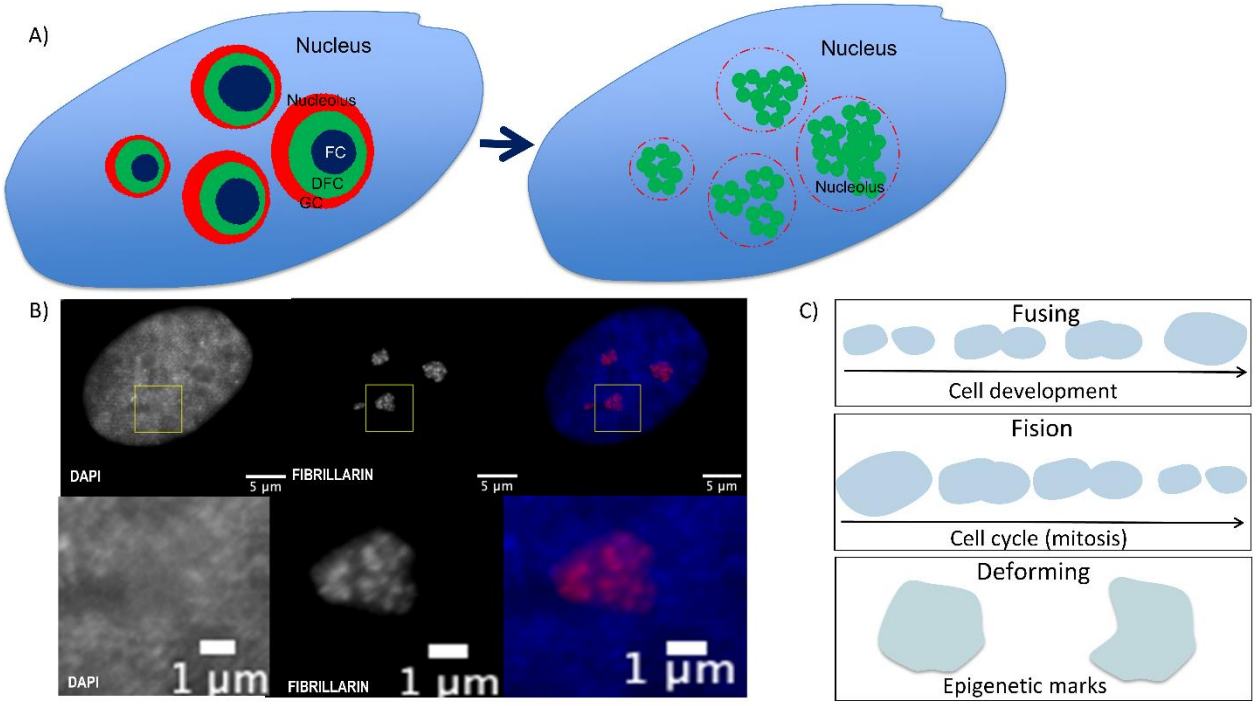


Figure 1. The multiphase structure of the nucleolus. Our understanding of the behavior and composition of the nucleolus has been changed in the past years, as showed in A), key insights come from SIM and dSTORM confocal analysis to yield a more precise composition of the molecular crowded nucleolus. B) Shows the classical localization of fibrillarin as a marker of the multicomponent dense fibrillar component (DFC) rim. C) Shows key concepts regarding the LLPS behavior of nucleolus. As a bio condensate nucleolus cand fuse to another one during cell development, can be break into small hubs during mitosis or changing its form during specific epigenetic marks to coupling rRNA metabolism.

3. The membraneless nucleolar compartment

The nucleus is the mayor membrane enclosed compartment inside eukaryotic cells, and the main of several important process during cell cycle. Nevertheless, other specific subnuclear structures are visible inside nucleus, i.e., nucleolus, lipid islets and Cajal bodies. These are, in contrast to nucleoli membraneless organelles that drives crucial tasks in order to keep a highly ordered and regulated biological process. Nucleolus, in fact, is the central membraneless organelle that drives the synthesis, processing and assembling of rRNA into ribosomal proteins to produce functional ribosomes(Correll et al., 2019; Emmott & Hiscox,

2009; Nazar, 2004; Woolford & Baserga, 2013). These important processes and their dysfunction and deregulation are related with several human medical conditions including cancer. The nucleolus is structured in a multilayer organization composed by three sub-compartments detailed as the fibrillar component (FC), the Dense fibrillar component (DFC) and the Granular component (GC). However, a recently study describes a fourth sub-compartment around the GC: *the nucleoli rim* (Stenström et al., 2020). Nucleolus assembly occurs at the ending of mitosis and proceeds until interphase, and then disassembly on the mitosis onset in each nuclear cycle. As an important key factor driving nucleolus formation in the nucleus during embryogenesis in *Drosophila* is the necessary of seeding the process, mainly by the presence of rDNA into the cells, but most importantly is the currently transcription of rRNA that condensates a highly saturated points to start the beginning of the nucleolar assemble, a process called nucleation. Suggested as a highly stochastic process, nucleation is the first step in order to build it up the nucleolus, moreover, nucleolar assembly is strictly dependent on rRNA transcription, as its inhibition blocks the coalescence membraneless body formation. In the other hand, the presence of rDNA is not a pre-requisite to assembly a highly saturated point as demonstrated in *D. melanogaster* in which the nucleolar protein fibrillarin and the Rpl135 RNA Pol I factor still congregate at inhomogeneous point into the nucleoplasm. Recently, it was demonstrated that transcriptional regulation of the activity of Pol I mediating rRNA transcription is negatively regulated by a LLPS process (Ide, Imai, Ochi, & Maeshima, 2020). Following single molecule tracking, the authors evaluated the dynamics of two main proteins of the Pol I machinery, the largest subunit of Pol I, RPA194; and the UBF, a chromatin upstream binding factor related to transcriptional activation. In the transcription inhibition treatment with the drug CX-5461 and, also in the RPA194 E593Q mutant related to a craniofacial disorder, the dynamics of Pol I bounded to the chromatin changed to a dissociated form, creating a newly sub compartment with LLPS behavior known as the nucleolar cap, in which more diffusive and free moving molecules of Pol I can be found.

Nucleolar structure has been considered intrinsically related to its highly rRNA transcription activity. Although nucleolar structure has been elucidated at different levels super resolution imaging are still sparse. Some clues rise that the nucleolar organization relies on the basis that interacting factors like UBF and RNA Pol I machinery factors form a structural platform to shape the classical three sub-compartment distribution of the nucleolus. Using 3D-Structured imaging microscopy (3D-SIM) ring-shaped structures were visualized with UBF coupled to GFP and immune-stained by nano antibodies. Moreover, the diameter size for the UBF nucleation foci were measured for mouse and human nucleoli in 240 nm and 170 nm, respectively. Also, the largest Pol I subunit RPA194 displayed a ring-shape structure as visualized by 3D-SIM. The data suggest that single ring structures of UBF correspond to

single transcription units looped in a way that exclude the intergenic spacers (IGS) between each rDNA unit, but the possibility with two rDNA repeats actively transcribed is not discarded (Maiser et al., 2020).

4. LLPS and disease

Although in the past decades the link between how membraneless organelles and bio-condensates are related to diseases was obscure, some light has been shed on the mechanisms in which the transition and more specifically, the aberrant transitions from a liquid-like to solid-like state promotes pathologies like cancer, viral infections, and other complexes diseases like the neurodegenerative pathologies as Alzheimer disease, Amyotrophic lateral sclerosis, Parkinson and Huntington diseases; literature summarizing these progress are given in the following (Aguzzi & Altmeyer, 2016; Alberti & Dormann, 2019; Alberti & Hyman, 2016). Also, with the advent of more research and compression of the principles governing phase to phase separation new avenues in the diagnosis, treatment and early detection of these pathologies could be improved. The mechanism by diseases and phase separation are linked still obscure but there are some clues that set the hypothesis that changes in the environmental micro conditions in the cell, dysregulation of some specific factors or enzymes or the molecule thermodynamics itself could lead to aberrant bio-condensate functionality. For example, *Fused in Sarcoma* (FUS) protein, a well-documented protein related to mRNA splicing and transcription have been demonstrated to phase separate *in vitro* and *in vivo*, indeed, possess prion-like low complexity domains in its sequence. Recently it was demonstrated that the transition of dynamically active liquid separation of FUS protein to a more solid and rigid state is determinant for Amyloid lateral sclerosis (ALS) and mutations in patients with ALS reveals that intrinsically disordered domains could explain this transition (Patel et al., 2015). Although this first evidence on how prion-like intrinsically disordered domains and LLPS are involved in disease, there are some other questions arising because FUS protein form functionally active bio-condensates as P granules and also co-localizing in nucleoli, and the tightly regulation to prevent to become a more rigid and solid state (known as hydrogels) are not fully understood (Murakami et al., 2015; Patel et al., 2015). Dysregulation of RNA-Binding proteins often are related to aging diseases, like Frontotemporal dementia and ALS, which possess fibrillar-like markers in affected cells. The clues come from key proteins as the heterogeneous nuclear ribonucleoprotein (hnRNP) A1. hnRNPA1 is able to phase separate by its intrinsically disordered domain, but not sufficient to drive LLPS in an RNA body context, indeed hnRNPA1 like other proteins related to RNA metabolism possess RNA recognition and binding domains. Multivalent low complexity domains are key in the maintaining and functionality of RNA-protein bio-condensates bodies (Molliex et al., 2015).

Other key aspects linking phase separation and disease is the propensity of proteins to phase separate changing its thermodynamic behavior. T cell-restricted intracellular antigen-1 (TIA1) proteins was described as a molecular marker of degenerative disease, and the mutations related in its low complexity domain are signatures of disease. Important to know is the fact that these mutations affects the solubility and dynamics of TIA1 promoting to phase separate in a liquid-like body (Mackenzie et al., 2017).

Wide spread cancer is another pathology recently described by a LLPS aberrant transition component in its molecular mechanism (Bouchard et al., 2018; Hanahan & Weinberg, 2011). The tumor suppressor SPOP (speckle-type POZ protein) was demonstrated to localize into liquid nucleolar bodies and mutations in cancer specific amino acids W131G and F33V disrupt this localization.

5. Intrinsically disordered proteins promote LLPS in the Nucleolus

The nucleolus protein content is highly heterogeneous, but intriguingly most of their tertiary composition involves stretches of intrinsically disordered regions along enzymatically active sequences. From rRNA transcription to RNA modifications, several proteins involved in these processes possess signatures corresponding to nucleolar targeting, IDRs, and an active motif uncharged to mediated the binding, modification or post-translational modifications during the nucleolus whole activity. Despite the fact that the different bio condensates bodies are very crowded some of the key structure features are promoted by a certain set of proteins known as a) scaffolds, and the increasing body of proteins that binds are known as b) clients. Interactions between scaffolds and clients promote the organization and maintaining of structures like P-bodies and PML nuclear bodies. The key features underlying the organization of these bodies reside in their stoichiometry of scaffolds and the valency of clients, as demonstrated by (Banani et al., 2016) using a small set of 3 combinatory proteins known to phase separate in vivo and in vitro.

The tunable physicochemical properties of proteins involved in several aspects of cell cycle and development had risen questions about how post translational modifications (PTMs) could explain the manner of how bio-condensates are regulated in an active, energy consuming process. Several proteins related to rRNA transcription, processing and assembling possess low complexity domains often called as intrinsically disordered domains, characterized by the presence of charged and aromatic amino acids. A well-documented signature is the RGG/RG repeat, characterized by the fact that this region is modified by methyl transferases adding a methyl group to the terminal guanidino nitrogen atom of the arginine. Fibrillarin and nucleolin proteins highly expressed in the nucleolus contain stretches of RGG/RG repeats in their sequences (Guillen-Chable et al., 2020; Masuzawa & Oyoshi, 2020; Thandapani, O'Connor, Bailey, & Richard, 2013) but how

they impact the fluidity and the maintaining of the sub nucleolar organization are still obscure. Arginine methylations has an inhibition property at least in vitro but in a in vivo context arginine methylation involve two key mechanisms to propitiate LLPS: 1) modifying protein-RNA interactions or 2) promoting partner interactions between proteins, promoting phase separation rather than inhibition. Phosphorylation of serine or tyrosine residues has been getting more attention about their relevance in phase separation. Rather than arginine methylation, phosphorylation and de phosphorylation can occur quickly and, in that sense, changing the properties of bio condensates in a very tunable manner. Further information about PMT as key regulators of LLPS can be found in the excellent review (Hofweber & Dormann, 2019).

Nucleolin is 710 aa highly conserved protein founded in GC of the nucleolus, composed of three domains: an N-terminal domain integrated by HMG-like domain, a central domain with 2 different stretches of RNA binding domains, and a C-terminal domain rich in arginine and glycine repeats similar to that found in fibrillarin (Lapeyre, Bourbon, & Amalric, 1987; Pontvianne et al., 2010; Serin et al., 1997).

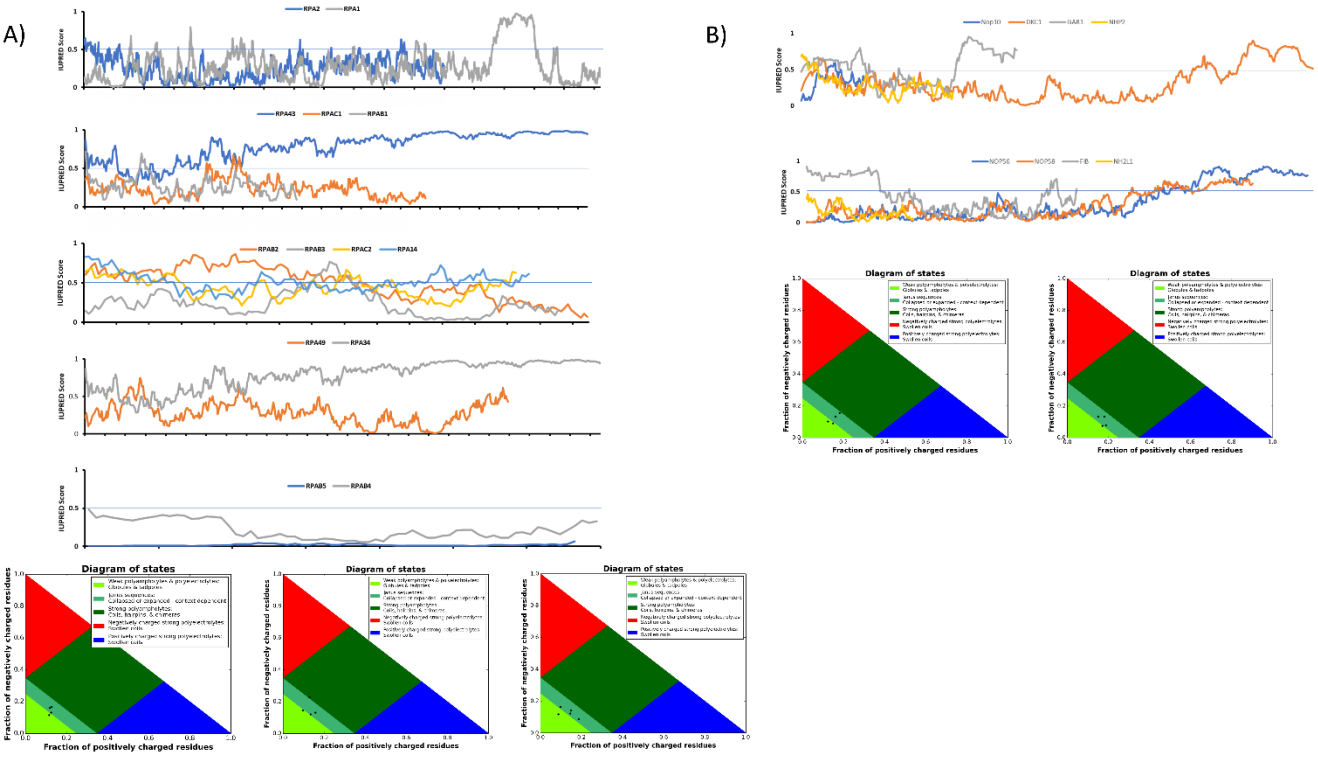
Two main proteins residing at the DFC, fibrillarin and GAR1, are the catalytic centers of two different complexes that guide the specific site methylation of ribosomal RNA (rRNA) nucleotide residues and the pseudo-uridylation of uridine residues in the rRNA, respectively (Girard et al., 1992). These two proteins contain intrinsically disordered regions (IDRs) at the N- and C- terminal regions, respectively (Guillen-Chable et al., 2020). It is well known that the N-terminal IDR of fibrillarin is a glycine-arginine rich region (GAR-domain) containing several repetitions of [RG] boxes in the whole domain (Chong et al., 2018). The GAR-domain of fibrillarin functions as a signal that direct the protein to the nucleolus in order to produce high quality ribosomes, also, it is documented that this region is involved with recognition of lipids, some specific RNA partners and other proteins. Recently the GAR-domain of plant *Arabidopsis thaliana* fibrillarin was described like a modular domain with ribonuclease activity, adding another layer of complexity of this domain and the whole protein itself (Rodriguez-Corona et al., 2017).

GAR1 protein is the central catalytic unit of the H/ACA ribonucleoparticle complexes, mainly involved in the modification of specific residues on the rRNA, but it is not clear if the C- terminal IDR is playing some important roles in the interaction with other partners and localization in the nucleolus (Girard et al., 1992).

Nucleophosmin (NPM1 also B23) is a highly conserved nucleolar phosphoprotein related to the last stages of ribosome biogenesis, and located at the granular component (GC) (Mitrea et al., 2016). As other important nucleolar proteins, NPM1 is a modular protein composed with several domains: an oligomerization domain (OD), two intrinsically disordered regions located on the inter sequences between

the OD and the C termina domain, which possess DNA and RNA binding activity, binding G-quadruplex DNA sequences (Bah et al., 2015; Mitrea et al., 2016). NPM1 function are often related to last ribosome processing and assembly with ribosomal proteins (r proteins), but also several other functions are described: regulation of centrosome duplication, genome stability, stress response, apoptosis and cancer and rRNA gene remodeling. Although NPM1 is important for rRNA assembly with r proteins, its function is dispensable for ribosome biogenesis. But its depletion causes the disruption of the nucleolar structure. Nucleolar localization of NMP1 dependents on the formation of a pentamer oligomer, and the disruption of this by a single amino acid phosphorylation on the serine 48 located in the OD changes its localization to the nucleoplasm. Oligomerization relies on OD, but also on the acidic IDR adjacent (Gibbs, Perrone, Hassan, Kümmerle, & Kriwacki, 2020).

Other important player in the LLPS system are lipids. Today it's no clear how lipid could be involved in the formation, nucleation or maintaining of bio condensates bodies. Recently it was described the nuclear nano scale localization of PI(4,5)P2, PI(3,4)P2 and PI(4)P by dSTORM. A subset of PI(4,5)P2 and PI(3,4)PI was detected on close proximity of the RNA Pol II machinery, specifically on nuclear speckles, a bio condensate known to concentrate mRNA and proteins related to RNA modifying routes. Also, another population of PI(4,5)P2 was detected in the nucleolar FDC, surrounded by fibrillarin, displaying a ring-shaped structure (Hoboth et al., 2021). Lipid islets has



been related to create nucleation points in the membrane surface concentrating key signaling proteins in response to several stress and developmental conditions (Snead & Gladfelter, 2019).

Figure 2. Intrinsically disordered regions in some key protein components during rRNA metabolism drive the LLPS behavior of the nucleolus. A) Intrinsically disorder tendency of the RNA Pol I machinery and their diagram phase showing and B) disorder tendency of the methylation and pseudo-uridylation complexes and their respective diagram phases. Although the disorder tendency shows a set of proteins containing IDR their corresponding diagram phases indicate a little tendency to phase separate. This is in correspondence with the idea that LPPS is coupled to other energy consuming processes that in an last step promote a multi component phase separated nucleolus.

6. Relevance of IDP and Cellular Cycle

Considered as one of the mayor processes in cell biology, the cell cycle involves a multiple and huge protein factors that regulate, signal and cooperate to procced at every step of development (Iyer-Bierhoff & Grummt, 2019; Iyer-Bierhoff et al., 2018; Tyson, Csikasz-Nagy, & Novak, 2002). Present in all the kingdoms of life, intrinsically disordered regions-containing proteins are getting attention with regard their regulatory activity during important stages of cell development (Galea, Wang, Sivakolundu, & Kriwacki, 2008; Lim & Kaldis, 2013). Even though their specific relationships between regulation and activity still are elusive, some light is shedding into it. In this way, two enzymatically active proteins, p21 and p27, regulate the mammalian cell division cycle by the inhibition of cyclin-dependent kinases. In their sequences only a stretch of the enzyme possesses an IDR, and a portion corresponding to the active site (Yoon, Mitrea, Ou, & Kriwacki, 2012). Upon binding to their target's theses IDR change their 3D configuration getting more folded but a region remains flexible. Other examples are present in the cyclosome, APC/C (anaphase-promoting complex) in which their IDR can bind tightly to their targets in a synergy action during cell division to promote ubiquitin mediated degradation of cyclins and other proteins uncharged with the regulation of the cell cycle (Olson, 2010).

7. Nuclear and nucleolar proteomes LLPS

The nucleus and the nucleolar structures are characterized by a highly protein content, most of the enzymes enrolled with specifics tasks during the cell cycle, ribosome biogenesis and stress responses. Several examples of nuclear and nucleolar proteome works are shedding light on the content and nature of the proteins residing both organelles. Until the 2000s, only a small subset of proteins have been characterized by protein content and function as detailed in (Andersen et al., 2005, 2002). Around 350 nucleolar proteins were identifying, and only a small subset of these are related to ribosomal biogenesis suggesting that a high protein content are engaged with other functions

inside the nucleolus. However, some of these proteins are continuously diffusing and their residence time inside the nucleolus change over time, describing a highly dynamic behavior (Andersen et al., 2005). These observations suggest that nuclear and nucleolar bodies possess liquid-like behavior, a well-documented feature describing the nature of nucleoli as a membraneless organelle (Brangwynne et al., 2011a; Deiana, Forcelloni, Porrello, & Giansanti, 2019; Feric et al., 2016).

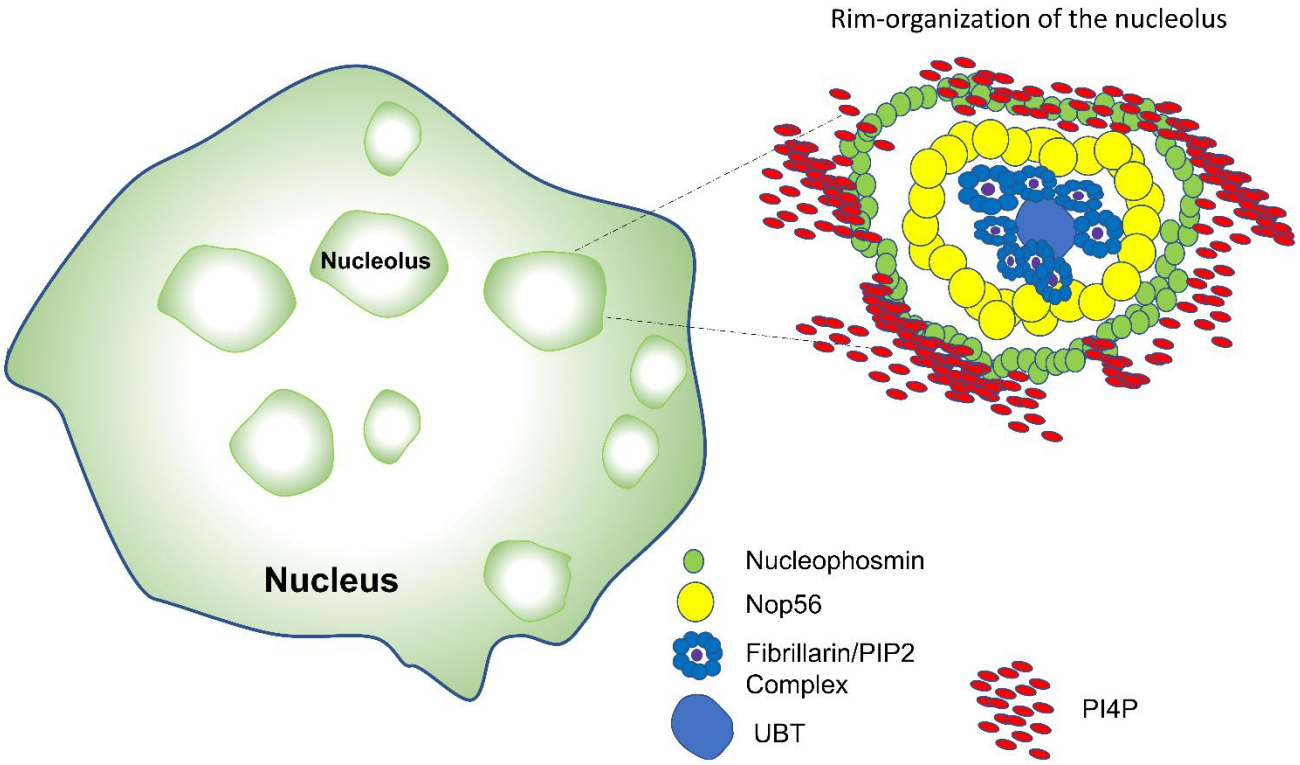


Figure 3. The rim organization of the nucleolus. A more current view of the nucleolus as a bio condensate body includes a small-scale organization of specific molecules, as described in other studies for fibrillarin and PIP2 forming small nucleation foci inside the DFC, and also with other phosphoinositides (not showed in the figure). Ring structures appears as a scaffold to confers the properties of highly dynamic multi-organized nucleolar body.

8. Conclusions

Cells relies on a multitude of compartments to highly conserve their fitness, development and cell cycle. A particular scenario of compartmentalization comes with the growing evidence of bio-condensates regulates several key aspects of the biochemical and physiological status of the cell. How the interplay between low complexity domains, proteins, lipids, and RNA populations can highly regulate their formation and maintaining are still explorable topics in

cell biology, biophysics and thermodynamics. Its intriguing capacity of assemble and disassemble come in fine evidence with the cell cycle when nucleolar disorganization comes and assemble each cycle. Numerous PTMs could explain the interaction network of some key proteins and their behavior, but more attention is needed to other energy consuming process that also phase separate without a multi valency protein network, in order to get a full landscape of the condensate relevance. New therapeutic strategies arise with the growing evidence supporting that LLPS could be a target to prevent or treat some neurodegenerative diseases and cancer.

Author Contributions: Conceptualization, F.G.C and E.C.; software, F.G.C, A.B..; formal analysis, E.C.; investigation, F.G.C, E.C.; writing—original draft preparation, F.G.C.; writing—review and editing, E.C., M.S., Pe.H., P.H.; project administration, E.C.; funding acquisition, E.C. and H.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the CONACYT project FC 1572, Grant Agency of the Czech Republic (Grant nos. 19-05608S and 18-19714S); by the Czech Academy of Sciences (Grant no. JSPS-20-06); by the Institutional Research Concept of the Institute of Molecular Genetics (Grant no. RVO: 68378050); by the MEYS CR (COST Inter-excellence internship LTC19048) and the project: BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University" (CZ.1.05/1.1.00/02.0109), from the European Regional Development Fund. The Microscopy Centre was supported by the MEYS CR (LM2018129 Czech-BioImaging) and by the European Regional Development Fund-Project "Modernization and support of research activities of the national infrastructure for biological and medical imaging Czech-BioImaging" (no. CZ.02.1.01/0.0/0.0/16_013/0001775)., and the CONACYT fellowship to FGC with number 576664.

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflict of interest.

References

Aguzzi, A., & Altmeyer, M. (2016). Phase Separation: Linking Cellular Compartmentalization to Disease. *Trends in Cell Biology*, *26*(7), 547–558. https://doi.org/10.1016/j.tcb.2016.03.004

Alberti, S., & Dormann, D. (2019). Liquid-Liquid Phase Separation in Disease. *Annual Review of Genetics*, *53*, 171–194. https://doi.org/10.1146/annurev-genet-112618-043527

Alberti, S., & Hyman, A. A. (2016). Are aberrant phase transitions a driver of cellular aging? *BioEssays*, *38*(10), 959–968. https://doi.org/10.1002/bies.201600042

Alshareedah, I., Moosa, M. M., Raju, M., Potoyan, D. A., & Banerjee, P. R. (2020). Phase transition of RNA−protein complexes into ordered hollow condensates. *Proceedings of the National Academy of Sciences*, 201922365. https://doi.org/10.1073/pnas.1922365117

Andersen, J. S., Lam, Y. W., Leung, A. K. L., Ong, S.-E., Lyon, C. E., Lamond, A. I., & Mann, M. (2005). Nucleolar proteome dynamics. *Nature*, *433*(7021), 77–83. https://doi.org/10.1038/nature03207

Andersen, J. S., Lyon, C. E., Fox, A. H., Leung, A. K. L., Lam, Y. W., Steen, H., … Lamond, A. I. (2002). Directed proteomic analysis of the human nucleolus. *Current Biology*, *12*(1), 1–11. https://doi.org/10.1016/S0960-9822(01)00650-9

Babinchak, W. M., & Surewicz, W. K. (2020). Liquid−Liquid Phase Separation and Its Mechanistic Role in Pathological Protein Aggregation. *Journal of Molecular Biology*, *432*(7), 1910–1925. https://doi.org/10.1016/j.jmb.2020.03.004

Bah, A., Vernon, R. M., Siddiqui, Z., Krzeminski, M., Muhandiram, R., Zhao, C., … Forman-Kay, J. D. (2015). Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch. *Nature*, *519*(7541), 106–109. https://doi.org/10.1038/nature13999

Banani, S. F., Rice, A. M., Peeples, W. B., Lin, Y., Jain, S., Parker, R., & Rosen, M. K. (2016). Compositional Control of Phase-Separated Cellular Bodies. *Cell*, *166*(3), 651–663. https://doi.org/10.1016/j.cell.2016.06.010

Boeynaems, S., Alberti, S., Fawzi, N. L., Mittag, T., Polymenidou, M., Rousseau, F., … Fuxreiter, M. (2018). Protein Phase Separation: A New Phase in Cell Biology. *Trends in Cell Biology*, *28*(6), 420–435. https://doi.org/10.1016/j.tcb.2018.02.004

Bouchard, J. J., Otero, J. H., Scott, D. C., Szulc, E., Martin, E. W., Sabri, N., … Mittag, T. (2018). Cancer Mutations of the Tumor Suppressor SPOP Disrupt the Formation of Active, Phase-Separated Compartments. *Molecular Cell*, *72*(1), 19-36.e8. https://doi.org/10.1016/j.molcel.2018.08.027

Bouffard, S., Dambroise, E., Brombin, A., Lempereur, S., Hatin, I., Simion, M., … Jamen, F. (2018). Fibrillarin is essential for S-phase progression and neuronal differentiation in zebrafish dorsal midbrain and retina. *Developmental Biology*, *437*(1), 1–16.

https://doi.org/10.1016/j.ydbio.2018.02.006

Brangwynne, C. P., Mitchison, T. J., & Hyman, A. A. (2011a). Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes. *Proceedings of the National Academy of Sciences*, *108*(11), 4334 LP – 4339. https://doi.org/10.1073/pnas.1017150108

Brangwynne, C. P., Mitchison, T. J., & Hyman, A. A. (2011b). Active liquid-like behavior of nucleoli determines their size and shape in Xenopus laevis oocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(11), 4334–4339. https://doi.org/10.1073/pnas.1017150108

Caragine, C. M., Haley, S. C., & Zidovska, A. (2018). Surface Fluctuations and Coalescence of Nucleolar Droplets in the Human Cell Nucleus. *Physical Review Letters*, *121*(14), 148101. https://doi.org/10.1103/PhysRevLett.121.148101

Castano, E., Yildirim, S., Fáberová, V., Krausová, A., Uličná, L., Paprčková, D., … Hozák, P. (2019). Nuclear Phosphoinositides—Versatile Regulators of Genome Functions. *Cells*, *8*(7), 649. https://doi.org/10.3390/cells8070649

Chang, C.-H., Hsu, F.-C., Lee, S.-C., Lo, Y.-S., Wang, J.-D., Shaw, J., … Lin, N.-S. (2016). The Nucleolar Fibrillarin Protein Is Required for Helper Virus-Independent Long-Distance Trafficking of a Subviral Satellite RNA in Plants. *The Plant Cell*, *28*(10), 2586–2602. https://doi.org/10.1105/tpc.16.00071

Chong, P. A., Vernon, R. M., & Forman-Kay, J. D. (2018). RGG/RG Motif Regions in RNA Binding and Phase Separation. *Journal of Molecular Biology*, *430*(23), 4650–4665. https://doi.org/10.1016/j.jmb.2018.06.014

Correll, C. C., Bartek, J., & Dundr, M. (2019). The Nucleolus: A Multiphase Condensate Balancing Ribosome Synthesis and Translational Capacity in Health, Aging and Ribosomopathies. *Cells*, *8*(8), 869. https://doi.org/10.3390/cells8080869

Deiana, A., Forcelloni, S., Porrello, A., & Giansanti, A. (2019). Intrinsically disordered proteins and structured proteins with intrinsically disordered regions have different functional roles in the cell. *PLoS ONE*, *14*(8), 1–16. https://doi.org/10.1371/journal.pone.0217889

Emmott, E., & Hiscox, J. A. (2009). Nucleolar targeting: the hub of the matter. *EMBO Reports*, *10*(3), 231–238. https://doi.org/10.1038/embor.2009.14

Falahati, H., & Haji-Akbari, A. (2019). Thermodynamically driven assemblies and liquid-liquid phase separations in biology. *Soft Matter*, *15*(6), 1135–1154. https://doi.org/10.1039/c8sm02285b

Falahati, H., Pelham-Webb, B., Blythe, S., & Wieschaus, E. (2016). Nucleation by rRNA dictates the precision of nucleolus assembly. *Current Biology*, *26*(3), 277–285. https://doi.org/10.1016/j.cub.2015.11.065

Falahati, H., & Wieschaus, E. (2017). Independent active and thermodynamic processes govern the nucleolus assembly in vivo. *Proceedings of the National Academy of Sciences*, *114*(6), 1335 LP – 1340. https://doi.org/10.1073/pnas.1615395114

Falaleeva, M., Pages, A., Matuszek, Z., Hidmi, S., Agranat-Tamir, L., Korotkov, K., … Stamm, S. (2016). Dual function of C/D box small nucleolar RNAs in rRNA modification and alternative pre-mRNA splicing. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(12), E1625–E1634. https://doi.org/10.1073/pnas.1519292113

Fatica, A, Galardi, S., Altieri, F., & Bozzoni, I. (2000). Fibrillarin binds directly and specifically to U16 box C/D snoRNA. *RNA (New York, N.Y.)*, *6*(1), 88–95. https://doi.org/10.1017/S1355838200991623

Fatica, Alessandro, & Tollervey, D. (2002). Making ribosomes. *Current Opinion in Cell Biology*, *14*(3), 313–318. https://doi.org/https://doi.org/10.1016/S0955-0674(02)00336-8

Feric, M., Vaidya, N., Harmon, T. S., Mitrea, D. M., Zhu, L., Richardson, T. M., … Brangwynne, C. P. (2016). Coexisting Liquid Phases Underlie Nucleolar Subcompartments. *Cell*, *165*(7), 1686–1697. https://doi.org/10.1016/j.cell.2016.04.047

Frottin, F., Schueder, F., Tiwary, S., Gupta, R., Körner, R., Schlichthaerle, T., … Hipp, M. S. (2019). The nucleolus functions as a phase-separated protein quality control compartment. *Science*, *365*(6451), 342–347. https://doi.org/10.1126/science.aaw9157

Galea, C. A., Wang, Y., Sivakolundu, S. G., & Kriwacki, R. W. (2008). Regulation of cell division by intrinsically unstructured proteins: Intrinsic flexibility, modularity, and signaling conduits. *Biochemistry*, *47*(29), 7598–7609. https://doi.org/10.1021/bi8006803

Gibbs, E., Perrone, B., Hassan, A., Kümmerle, R., & Kriwacki, R. (2020). NPM1 exhibits structural and dynamic heterogeneity upon phase separation with the p14ARF tumor suppressor. *Journal of Magnetic Resonance*, *310*, 106646. https://doi.org/10.1016/j.jmr.2019.106646

Girard, J. P., Lehtonen, H., Caizergues-Ferrer, M., Amalric, F., Tollervey, D., & Lapeyre, B. (1992). GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *The EMBO Journal*, *11*(2), 673–682. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1531632

Gomes, E., & Shorter, J. (2019). The molecular language of membraneless organelles. *Journal of Biological Chemistry*, *294*(18), 7115–7127. https://doi.org/10.1074/jbc.TM118.001192

Guillen-Chable, F., Corona, U. R., Pereira-Santana, A., Bayona, A., Rodríguez-Zapata, L. C., Aquino, C., … Castano, E. (2020). Fibrillarin Ribonuclease Activity is Dependent on the GAR Domain and Modulated by Phospholipids. *Cells*, *9*(5), 1–22. https://doi.org/10.3390/cells9051143

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hernandez-Verdun, D., Roussel, P., Thiry, M., Sirri, V., & Lafontaine, D. L. J. (2010). The nucleolus: Structure/function relationship in RNA metabolism. *Wiley Interdisciplinary Reviews: RNA*, *1*(3), 415–431. https://doi.org/10.1002/wrna.39

Hoboth, P., Sztacho, M., Šebesta, O., Schätz, M., Castano, E., & Hozák, P. (2021). Nanoscale mapping of nuclear phosphatidylinositol phosphate landscape by dual-color dSTORM. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, *1866*(5), 158890. https://doi.org/10.1016/j.bbalip.2021.158890

Hofweber, M., & Dormann, D. (2019). Friend or foe-Post-translational modifications as regulators of phase separation and RNP granule dynamics. *Journal of Biological Chemistry*, *294*(18), 7137–7150. https://doi.org/10.1074/jbc.TM118.001189

Ide, S., Imai, R., Ochi, H., & Maeshima, K. (2020). Transcriptional suppression of ribosomal DNA with phase separation. *Science Advances*, *6*(42), eabb5953. https://doi.org/10.1126/sciadv.abb5953

Iyer-Bierhoff, A., & Grummt, I. (2019). Stop-and-Go: Dynamics of Nucleolar Transcription During the Cell Cycle. *Epigenetics Insights*, *12*, 1–3. https://doi.org/10.1177/2516865719849090

Iyer-Bierhoff, A., Krogh, N., Tessarz, P., Ruppert, T., Nielsen, H., & Grummt, I. (2018). SIRT7-Dependent Deacetylation of Fibrillarin Controls Histone H2A Methylation and rRNA Synthesis during the Cell Cycle. *Cell Reports*, *25*(11), 2946-2954.e5. https://doi.org/10.1016/j.celrep.2018.11.051

Kass, S., Tyc, K., Steitz, J. A., & Sollner-Webb, B. (1990). The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell*, *60*(6), 897–908. https://doi.org/10.1016/0092-8674(90)90338-F

Kim, S. H., Macfarlane, S., Kalinina, N. O., Rakitina, D. V, Ryabov, E. V, Gillespie, T., … Taliansky, M. (2007). Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(26), 11115–11120. https://doi.org/10.1073/pnas.0704632104

Koš, M., & Tollervey, D. (2010). Yeast Pre-rRNA Processing and Modification Occur Cotranscriptionally. *Molecular Cell*, *37*(6), 809–820. https://doi.org/10.1016/j.molcel.2010.02.024

Lafontaine, D. L. J. (2019). Birth of Nucleolar Compartments: Phase Separation-Driven Ribosomal RNA Sorting and Processing. *Molecular Cell*, *76*(5), 694–696. https://doi.org/10.1016/j.molcel.2019.11.015

Langhendries, J.-L., Nicolas, E., Doumont, G., Goldman, S., & Lafontaine, D. L. J. (2016). The human box C/D snoRNAs U3 and U8 are required for pre-rRNA processing and tumorigenesis. *Oncotarget*, *7*(37). https://doi.org/10.18632/oncotarget.11148

Lapeyre, B., Bourbon, H., & Amalric, F. (1987). Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. *Proceedings*

of the National Academy of Sciences of the United States of America, *84*(6), 1472–1476. https://doi.org/10.1073/pnas.84.6.1472

Latonen, L. (2019). Phase-to-phase with nucleoli - Stress responses, protein aggregation and novel roles of RNA. *Frontiers in Cellular Neuroscience*, *13*(April), 1–10. https://doi.org/10.3389/fncel.2019.00151

Li, P., Banjade, S., Cheng, H. C., Kim, S., Chen, B., Guo, L., … Rosen, M. K. (2012). Phase transitions in the assembly of multivalent signalling proteins. *Nature*, *483*(7389), 336–340. https://doi.org/10.1038/nature10879

Li, Z., Zhang, Y., Jiang, Z., Jin, X., Zhang, K., Wang, X., … Li, D. (2018). Hijacking of the nucleolar protein fibrillarin by TGB1 is required for cell-to-cell movement of Barley stripe mosaic virus. *Molecular Plant Pathology*, *19*(5), 1222–1237. https://doi.org/10.1111/mpp.12612

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. *Development (Cambridge)*, *140*(15), 3079–3093. https://doi.org/10.1242/dev.091744

Loewen, C. J. R., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A., & Levine, T. P. (2004). Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. *Science (New York, N.Y.)*, *304*(5677), 1644–1647. https://doi.org/10.1126/science.1096083

Loza-Muller, L., Rodríguez-Corona, U., Sobol, M., Rodríguez-Zapata, L. C., Hozak, P., & Castano, E. (2015). Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in Brassica oleracea. *Frontiers in Plant Science*, *6*(November), 976. https://doi.org/10.3389/fpls.2015.00976

Ma, N., Matsunaga, S., Takata, H., Ono-Maniwa, R., Uchiyama, S., & Fukui, K. (2007). Nucleolin functions in nucleolus formation and chromosome congression. *Journal of Cell Science*, *120*(12), 2091–2105. https://doi.org/10.1242/jcs.008771

Mackenzie, I. R., Nicholson, A. M., Sarkar, M., Messing, J., Purice, M. D., Pottier, C., … Rademakers, R. (2017). TIA1 Mutations in Amyotrophic Lateral Sclerosis and Frontotemporal Dementia Promote Phase Separation and Alter Stress Granule Dynamics. *Neuron*, *95*(4), 808-816.e9. https://doi.org/10.1016/j.neuron.2017.07.025

Mahmoudabadi, G., Rajagopalan, K., Getzenberg, R. H., Hannenhalli, S., Rangarajan, G., & Kulkarni, P. (2013). Intrinsically disordered proteins and conformational noise Implications in cancer. *Cell Cycle*, *12*(1), 26–31. https://doi.org/10.4161/cc.23178

Maiser, A., Dillinger, S., Längst, G., Schermelleh, L., Leonhardt, H., & Németh, A. (2020). Super-resolution in situ analysis of active ribosomal DNA chromatin organization in the nucleolus. *Scientific Reports*, *10*(1), 1–11. https://doi.org/10.1038/s41598-020-64589-x

Martin, R., Hackert, P., Ruprecht, M., Simm, S., Brüning, L., Mirus, O., … Bohnsack, M. T. (2014). A pre-ribosomal RNA interaction network involving snoRNAs and the Rok1 helicase, 1173–1182. https://doi.org/10.1261/rna.044669.114.

Masuzawa, T., & Oyoshi, T. (2020). Roles of the RGG Domain and RNA Recognition Motif of Nucleolin in G-Quadruplex Stabilization. *ACS Applied Materials and Interfaces*. https://doi.org/10.1021/acsomega.9b04221

McBride, A. E., Conboy, A. K., Brown, S. P., Ariyachet, C., & Rutledge, K. L. (2009). Specific sequences within arginine-glycine-rich domains affect mRNA-binding protein function. *Nucleic Acids Research*, *37*(13), 4322–4330. https://doi.org/10.1093/nar/gkp349

Mitrea, D. M., Cika, J. A., Guy, C. S., Ban, D., Banerjee, P. R., Stanley, C. B., … Kriwacki, R. W. (2016). Nucleophosmin integrates within the nucleolus via multi-modal interactions with proteins displaying R-rich linear motifs and rRNA. *ELife*, *5*, 1–33. https://doi.org/10.7554/elife.13571

Molliex, A., Temirov, J., Lee, J., Coughlin, M., Kanagaraj, A. P., Kim, H. J., … Taylor, J. P. (2015). Phase Separation by Low Complexity Domains Promotes Stress Granule Assembly and Drives Pathological Fibrillization. *Cell*, *163*(1), 123–133. https://doi.org/10.1016/j.cell.2015.09.015

Morales, J., Sobol, M., Rodriguez-Zapata, L. C., Hozak, P., & Castano, E. (2017). Aromatic amino acids and their relevance in the specificity of the PH domain. *Journal of Molecular Recognition : JMR*, *30*(12), 10.1002/jmr.2649. https://doi.org/10.1002/jmr.2649

Murakami, T., Qamar, S., Lin, J. Q., Schierle, G. S. K., Rees, E., Miyashita, A., … St George-Hyslop, P. (2015). ALS/FTD Mutation-Induced Phase Transition of FUS Liquid Droplets and Reversible Hydrogels into Irreversible Hydrogels Impairs RNP Granule Function. *Neuron*, *88*(4), 678–690. https://doi.org/10.1016/j.neuron.2015.10.030

Nazar, R. N. (2004). Ribosomal RNA processing and ribosome biogenesis in eukaryotes. *IUBMB Life*, *56*(8), 457–465. https://doi.org/10.1080/15216540400010867

Newton, K., Petfalski, E., Tollervey, D., & Cáceres, J. F. (2003). Fibrillarin is essential for early development and required for accumulation of an intron-encoded small nucleolar RNA in the mouse. *Molecular and Cellular Biology*, *23*(23), 8519–8527. https://doi.org/10.1128/MCB.23.23.8519-8527.2003

Olson, M. O. (2010). Nucleolus: Structure and Function. *Encyclopedia of Life Sciences*, (December). https://doi.org/10.1002/9780470015902.a0005975.pub2

Patel, A., Lee, H. O., Jawerth, L., Maharana, S., Jahnel, M., Hein, M. Y., … Alberti, S. (2015). A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell*, *162*(5), 1066–1077. https://doi.org/10.1016/j.cell.2015.07.047

Peng, Y., Yu, G., Tian, S., & Li, H. (2014). Co-expression and co-purification of archaeal and eukaryal box C/D RNPs. *PLoS ONE*, *9*(7), 1–8. https://doi.org/10.1371/journal.pone.0103096

Pontvianne, F., Abou-Ellail, M., Douet, J., Comella, P., Matia, I., Chandrasekhara, C., … Sáez-Vásquez, J. (2010). Nucleolin is required for DNA methylation state and the expression of rRNA gene variants in Arabidopsis thaliana. *PLoS Genetics*, *6*(11), 1–13.

https://doi.org/10.1371/journal.pgen.1001225

Pritišanac, I., Vernon, R. M., Moses, A. M., & Forman Kay, J. D. (2019). Entropy and information within intrinsically disordered protein regions. *Entropy*, *21*(7), 1–24. https://doi.org/10.3390/e21070662

Rakitina, D. V., Taliansky, M., Brown, J. W. S., & Kalinina, N. O. (2011). Two RNA-binding sites in plant fibrillarin provide interactions with various RNA substrates. *Nucleic Acids Research*, *39*(20), 8869–8880. https://doi.org/10.1093/nar/gkr594

Riback, J. A., Zhu, L., Ferrolino, M. C., Tolbert, M., Mitrea, D. M., Sanders, D. W., … Brangwynne, C. P. (2020). Composition-dependent thermodynamics of intracellular phase separation. *Nature*, *581*(7807), 209–214. https://doi.org/10.1038/s41586-020-2256-2

Rodriguez-Corona, U., Pereira-Santana, A., Sobol, M., Rodriguez-Zapata, L. C., Hozak, P., & Castano, E. (2017). Novel Ribonuclease Activity Differs between Fibrillarins from Arabidopsis thaliana. *Frontiers in Plant Science*, *8*(October), 1–10. https://doi.org/10.3389/fpls.2017.01878

Rodriguez-Corona, U., Sobol, M., Rodriguez-Zapata, L. C., Hozak, P., & Castano, E. (2015). Fibrillarin from Archaea to human. *Biology of the Cell*, *107*(6), 159–174. https://doi.org/10.1111/boc.201400077

Sáez-Vasquez, J., Caparros-Ruiz, D., Barneche, F., & Echeverría, M. (2004). A plant snoRNP complex containing snoRNAs, fibrillarin, and nucleolin-like proteins is competent for both rRNA gene binding and pre-rRNA processing in vitro. *Molecular and Cellular Biology*, *24*(16), 7284–7297. https://doi.org/10.1128/MCB.24.16.7284-7297.2004

Samaha, H., Delorme, V., Pontvianne, F., Cooke, R., Delalande, F., Van Dorsselaer, A., … Sáez-Vásquez, J. (2010). Identification of protein factors and U3 snoRNAs from a Brassica oleracea RNP complex involved in the processing of pre-rRNA. *Plant Journal*, *61*(3), 383–398. https://doi.org/10.1111/j.1365-313X.2009.04061.x

Serin, G., Joseph, G., Ghisolfi, L., Bauzan, M., Erard, M., Amalric, F., & Bouvet, P. (1997). Two RNA-binding domains determine the RNA-binding specificity of nucleolin. *Journal of Biological Chemistry*, *272*(20), 13109–13116. https://doi.org/10.1074/jbc.272.20.13109

Shin, Y., Chang, Y. C., Lee, D. S. W., Berry, J., Sanders, D. W., Ronceray, P., … Brangwynne, C. P. (2018). Liquid Nuclear Condensates Mechanically Sense and Restructure the Genome. *Cell*, *175*(6), 1481-1491.e13. https://doi.org/10.1016/j.cell.2018.10.057

Sirri, V., Urcuqui-Inchima, S., Roussel, P., & Hernandez-Verdun, D. (2008). Nucleolus: The fascinating nuclear body. *Histochemistry and Cell Biology*, *129*(1), 13–31. https://doi.org/10.1007/s00418-007-0359-6

Smith, D.-L., Erce, M. A., Lai, Y.-W., Tomasetig, F., Hart-Smith, G., Hamey, J. J., & Wilkins, M. R. (2019). Crosstalk of phosphorylation and arginine methylation in disordered SRGG repeats of S. cerevisiae fibrillarin and its association with nucleolar localisation. *Journal of Molecular*

Biology. https://doi.org/10.1016/j.jmb.2019.11.006

Snead, W. T., & Gladfelter, A. S. (2019). The Control Centers of Biomolecular Phase Separation: How Membrane Surfaces, PTMs, and Active Processes Regulate Condensation. *Molecular Cell*, *76*(2), 295–305. https://doi.org/10.1016/j.molcel.2019.09.016

Sobol, M., Krausová, A., Yildirim, S., Kalasová, I., Fáberová, V., Vrkoslav, V., … Hozak, P. (2018). Nuclear phosphatidylinositol 4,5-bisphosphate islets contribute to efficient RNA polymerase II-dependent transcription. *Journal of Cell Science*, *131*(8), jcs211094. https://doi.org/10.1242/jcs.211094

Sobol, M., Yildirim, S., Philimonenko, V. V, Marášek, P., Castaño, E., & Hozák, P. (2013). UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity. *Nucleus (Austin, Tex.)*, *4*(6), 478–486. https://doi.org/10.4161/nucl.27154

Sonnino, S., & Prinetti, A. (2010). Lipids and membrane lateral organization, *1*(November), 1–9. https://doi.org/10.3389/fphys.2010.00153

Stenström, L., Mahdessian, D., Gnann, C., Leonetti, M., Uhlén, M., Cuylen-Häring, S., … Lundberg, E. (2020). Mapping of the nucleolar proteome reveals spatiotemporal organization related to intrinsic protein disorder, 1–16. https://doi.org/10.1101/2020.01.30.923003

Strom, A. R., & Brangwynne, C. P. (2019). The liquid nucleome – phase transitions in the nucleus at a glance. *Jcs*, *132*, 1–7. https://doi.org/10.1242/jcs.235093

Tessarz, P., Santos-Rosa, H., Robson, S. C., Sylvestersen, K. B., Nelson, C. J., Nielsen, M. L., & Kouzarides, T. (2014). Glutamine methylation in histone H2A is an RNA-polymerase-I-dedicated modification. *Nature*, *505*(7484), 564–568. https://doi.org/10.1038/nature12819

Thandapani, P., O'Connor, T. R., Bailey, T. L., & Richard, S. (2013). Defining the RGG/RG Motif. *Molecular Cell*, *50*(5), 613–623. https://doi.org/10.1016/j.molcel.2013.05.021

Tollervey, D., Lehtonen, H., Carmo-Fonseca, M., & Hurt, E. C. (1991). The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *The EMBO Journal*, *10*(3), 573–583. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1825809

Tyson, J. J., Csikasz-Nagy, A., & Novak, B. (2002). The dynamics of cell cycle regulation. *BioEssays*, *24*(12), 1095–1109. https://doi.org/10.1002/bies.10191

Ulicna, L., Kalendova, A., Kalasova, I., Vacik, T., & Hozák, P. (2018). PIP2 epigenetically represses rRNA genes transcription interacting with PHF8. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1863*(3), 266–275. https://doi.org/10.1016/j.bbalip.2017.12.008

Ulrich, A., Andersen, K. R., & Schwartz, T. U. (2012). Exponential Megapriming PCR (EMP) Cloning-Seamless DNA Insertion into Any Target Plasmid without Sequence Constraints. *PLoS ONE*, *7*(12), 1–9. https://doi.org/10.1371/journal.pone.0053360

Watkins, N. J., & Bohnsack, M. T. (2012). The box C/D and H/ACA snoRNPs: Key players in the modification, processing and the dynamic folding of ribosomal RNA. *Wiley Interdisciplinary Reviews: RNA*, *3*(3), 397–414. https://doi.org/10.1002/wrna.117

Weber, S. C., & Brangwynne, C. P. (2012). Getting RNA and protein in phase. *Cell*, *149*(6), 1188–1191. https://doi.org/10.1016/j.cell.2012.05.022

Woolford, J. L., & Baserga, S. J. (2013). Ribosome biogenesis in the yeast Saccharomyces cerevisiae. *Genetics*, *195*(3), 643–681. https://doi.org/10.1534/genetics.113.153197

Wright, P. E., & Dyson, H. J. (2015). Intrinsically disordered proteins in cellular signalling and regulation. *Nature Reviews Molecular Cell Biology*, *16*(1), 18–29. https://doi.org/10.1038/nrm3920

Xing, Y.-H., Yao, R.-W., Zhang, Y., Guo, C.-J., Jiang, S., Xu, G., … Chen, L.-L. (2017). SLERT Regulates DDX21 Rings Associated with Pol I Transcription. *Cell*, *169*(4), 664-678.e16. https://doi.org/10.1016/j.cell.2017.04.011

Yao, R. W., Xu, G., Wang, Y., Shan, L., Luan, P. F., Wang, Y., … Chen, L. L. (2019). Nascent Pre-rRNA Sorting via Phase Separation Drives the Assembly of Dense Fibrillar Components in the Human Nucleolus. *Molecular Cell*, *76*(5), 767-783.e11. https://doi.org/10.1016/j.molcel.2019.08.014

Yoon, M. K., Mitrea, D. M., Ou, L., & Kriwacki, R. W. (2012). Cell cycle regulation by the intrinsically disordered proteins p21 and p27. *Biochemical Society Transactions*, *40*(5), 981–988. https://doi.org/10.1042/BST20120092

Yoshizawa, T., Nozawa, R. S., Jia, T. Z., Saio, T., & Mori, E. (2020). Biological phase separation: cell biology meets biophysics. *Biophysical Reviews*, *12*(2), 519–539. https://doi.org/10.1007/s12551-020-00680-x

Zarin, T., Strome, B., Nguyen Ba, A. N., Alberti, S., Forman-Kay, J. D., & Moses, A. M. (2019). Proteome-wide signatures of function in highly diverged intrinsically disordered regions. *ELife*, *8*, 1–26. https://doi.org/10.7554/eLife.46883

Zhao, Y. G., & Zhang, H. (2020). Phase Separation in Membrane Biology: The Interplay between Membrane-Bound Organelles and Membraneless Condensates. *Developmental Cell*, 1–15. https://doi.org/10.1016/j.devcel.2020.06.033

Zhu, L., Richardson, T. M., Wacheul, L., Wei, M. T., Feric, M., Whitney, G., … Brangwynne, C. P. (2019). Controlling the material properties and rRNA processing function of the nucleolus using light. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(35), 17330–17335. https://doi.org/10.1073/pnas.1903870116

CAPITULO VIII. Discusión, conclusiones generales y perspectivas

El nucleolo constituye uno de las mayores estructuras multi-organizadas y cuya función es de vital importancia para la supervivencia, el desarrollo y mantenimiento de la célula. Aunque su volumen no se defina por la delimitación de una membrana lipídica esta estructura nuclear constituye uno de los ejemplos más estudiados que rompen el paradigma de la organización y definición de volumen delimitada por una barrera física como las membranas lipidias. Muchos de los constituyentes proteicos del nucléolo lo constituyen proteínas que poseen módulos o dominios enzimáticos específicos para cada una de las tareas que constituyen el proceso de la biogénesis ribosomal (Hernandez-Verdun, Roussel, Thiry, Sirri, & Lafontaine, 2010; Latonen, 2019). Estos procesos van desde la transcripción, la modificación y el acoplamiento del rRNA a las proteínas ribosomales (Alessandro Fatica & Tollervey, 2002; Nazar, 2004). Maquinarias como la de la polimerasa I encargadas de la transcripción de los rRNA y los complejos snoRNP encargados de la metilación y modificación de residuos específicos de los rRNA constituyen ejemplos de las partículas que se encuentran mediando la formación, establecimiento y mantenimiento del nucleolo. Estos complejos proteicos se encuentran constituidos por una variedad de proteínas con actividades enzimáticas caracterizadas por la presencia de regiones en su secuencia que no poseen regiones globulares o estructuras terciarias. Estas regiones conocidas por el inglés como *Intrinsically disordered regions*, o regiones intrínsicamente desordenadas (IDP), se encuentran ampliamente distribuidas en proteínas que constituyen el nucleolo (Chong et al., 2018; Zarin et al., 2019) (lo anterior se discute más adelante en el presente escrito). Acopladas a regiones enzimáticamente activas, permiten el manteamiento y aislamiento de una región de volumen termodinámicamente diferente al del volumen restante del nucleolo. Este tipo de aislamientos mediados por proteínas y ácidos nucleicos son descritas por la biología y termodinámica como separaciones líquido-liquido (Babinchak & Surewicz, 2020; Frottin et al., 2019; Pritišanac, Vernon, Moses, & Forman Kay, 2019). De hecho, el proceso de transcripción de los rRNA es conocido por ser uno de los procesos cuya regulación es dependiente de esta condición termodinámica. Los trascritos de rRNA 47S recién sintetizados sufren una serie de reacciones, modificaciones y empaquetamientos que ocurren simultáneamente y gracias a las condiciones facilitadas por las separaciones de fases las moléculas presenten poseen una mayor probabilidad de colisión, y por ende de cercanía física y modificación enzimática en última instancia (Iyer-

Bierhoff & Grummt, 2019; Yao et al., 2019). Por lo cual quedan varias incógnitas en lo que se refiere a la capacidad de acoplar funciones enzimáticas y la regulación y mantenimiento de separación de fases en la célula. Una de esas sería la capacidad de separación de fases de fibrilarina y su actividad enzimática durante el proceso de biogénesis ribosomal (véase a la figura 3 de la revisión de desorden y separación de fases).

Fibrilarina constituye una de las poblaciones proteicas con mayor abundancia dentro del nucleolo, descrita por su actividad metiltransferasa durante la modificación del rRNA (Bouffard et al., 2018; Falaleeva et al., 2016; Newton, Petfalski, Tollervey, & Cáceres, 2003). Esta proteína modular constituida por diferentes regiones en su secuencia las cuales van desde la unión a RNA, señales de localización nucleolar, además del sitio católico que promueve la metilación sitio específica de los residuos de rRNA que serán modificados (Rakitina, Taliansky, Brown, & Kalinina, 2011; Rodriguez-Corona, Sobol, Rodriguez-Zapata, Hozak, & Castano, 2015). Fibrilarina forma parte de los complejos con cajas C/D snoRNP cuya función es particularmente importante dado la errores en la metilación sitio específica de residuos de rRNA están directamente relacionados con condiciones como el cáncer (Hernandez-Verdun et al., 2010; Sirri et al., 2008). Además de la metilación sitio específica, fibrilarina ha sido descrita como metiltransferasa de la histona H2A, añadiendo una nueva capa de información con respecto a las funciones e importancia de fibrilarina durante el ciclo celular y el desarrollo (Loza-Muller et al., 2015; Tessarz et al., 2014).

En el presente trabajo de tesis doctoral se presenta evidencia de la actividad de fibrilarina previamente descrita por (Rodriguez-Corona et al., 2017) en el que se describe la capacidad de degradación de rRNA de la proteína perteneciente a *Arabidopsis thaliana*, esta actividad reportada en la región desordenada rica en glicinas y argininas conocida como dominio GAR es parte importante del procesamiento de rRNA en las células eucariotas. Con anterioridad se ha descrito la capacidad de corte de un complejo de metilación que contienen a fibrilarina pero sin asignarle la función a esta enzima en particular (Kass, Tyc, Steitz, & Sollner-Webb, 1990). En este trabajo los autores señalan que uno de los primeros eventos de procesamiento del rRNA es la incisión del rRNA 47S recién sintetizado, y con la ayuda de sondas marcadas radiactivamente se siguen el acumulamiento de las poblaciones de rRNA de acuerdo a su abundancia. La evidencia que sugiere que el complejo C/D snoRNP que contiene a fibrilarina se sustenta en que este complejo es capaz de incidir un RNA sintético similar al 47, aunque de manera *in vitro*, este es el primer reporte en el que

se hace evidente una actividad relacionada con una ribonucleasa de RNA. Los resultados de la presente tesis sugieren que HsFib posee la capacidad de degradar rRNA total, con mayor detalle aquel que se encuentra totalmente procesado, esto anterior es interesante, ya que es posible que existan regiones sitio específicas que promuevan la unión, corte y por ende la degradación de rRNA total. Al comparar la actividad degradativa de HsFib con la de una ribonucleasa A, encontramos que tiene una posible similitud entre dichas actividades. Por lo cual sería importante evaluar la actividad degradativa de ambas enzimas *in vitro* con el fin de determinar el mecanismo de actividad de fibrilarina. Algo interesante de notar también es el hecho de que cantidades tan pequeñas de masa usada para los experimentos con fibrilarina continúan cortando RNA, lo que sugiere también la dependencia de la cantidad de moléculas de fibrilarina disponible y de su capacidad enzimáticamente para llevar a cabo dicha función degradativa.

Gran parte de la atención relacionada con el mantenimiento de diferentes cuerpos nucleares está relacionada con la interacción de diferentes factores, entre ellos lípidos. Se sabe de la existencia de lípidos en el núcleo desde hace décadas, pero la relevancia de ellos con respecto al mantenimiento, organización y regulación de complejos proteicos, complejos de separación de fases y regulación genética apenas están siendo explorados (Loewen et al., 2004; Morales, Sobol, Rodriguez-Zapata, Hozak, & Castano, 2017; Sobol et al., 2013; Ulicna et al., 2018). Es interesante desde el punto de vista regulatorio la existencia de pequeños cuerpos lipídicos en el interior del núcleo, por ejemplo, aquellos conocidos como *lipid islets*, estas estructuras descritas por (Sobol et al., 2018) funcionan como agradados lipídicos cuya composición es mayoritariamente por $PI4,5P^2$ funcionan para el manteamiento regulatorio de ciertos cuerpos en los que la mayor de las actividades están relacionas con el procesamiento de diferentes poblaciones de RNA así como el mantenimiento de estructuras nucleares. Y generalmente asociadas a los *foci* de transcripción mediada por la Pol II, es decir con el procesamiento de mRNA. De la misma manera han sido descritas estructuras con forma de anillo que promueven interacciones entre proteínas, lípidos y RNA (Xing et al., 2017). Por ejemplo, el RNA largo no codificante SLERT, interactúa con la helicasa DEAD-box RNA DDX21 a través de una secuencia de 143-nt del tipo de un non-snoRNA formando estructuras de tipo de anillo que en lo sucesivo interactúan con la maquinaria de transcripción de la Pol I, inhibiendo la transcripción de los rRNA, el cual en condiciones como cáncer suponen un desbalance en la producción de

ribosomas funcionales. Estas estructuras de tipo anillo también se encuentran mediando la organización de los rDNA, en los que factores como UBF1 y mayor de las unidades de la Pol, la RPA194 interactúan con el rDNA para formar estas estructuras en las que la forma de anillo continúa siendo un factor característico (Iyer-Bierhoff & Grummt, 2019; Maiser et al., 2020; Yao et al., 2019). En nuestro trabajo de tesis encontramos que fibrilarina presente en el nucleolo posee esta configuración del tipo anillo, lo cual es evidente en la co-localización de fibrilarina y PI4,5P. Aunque la función de los lípidos asociados a la transcripción y organización de las unidades transcripcionales de la Pol I, no se describe por completo en la literatura, una hipótesis que resulta de nuestro trabajo es precisamente que lípidos como el fosfatidilinositol 4,5 bis fosfato y el ácido fosfatídico funcionan como plataformas para la interacción y secuestro de ciertos factores enzimáticos durante los procesos de inhibición y activación de la transcripción de los rRNA, lo cual es de importancia dado que nuestros resultados detalla que la actividad de ribonucleasa de fibrilarina también es modulada por la interacción con lípidos y las posibles cambios conformacionales a los que podría estar sujeta fibrilarina tras su interacción con PI4,5P o PA. Se desconocen otras moléculas que establezcan una relación estructural proteína-lipidio que permitan entender la consecución de la separación de fases en la célula (véase la figura 1 de la revisión de desorden y separación de fases).

Por otra parte, se sabe que las regiones desordenadas que se encuentran en numerosas proteínas nucleares y nucleolares, tales como ejemplo las que participan en la transcripción del rRNA que contienen repeticiones ricas en aminoácidos polares y cargados, cuya configuración electrostática promueve la interacción transitoria con moléculas de diferente naturaleza, en particular RNA y lípidos (Deiana et al., 2019; Mahmoudabadi et al., 2013; Shin et al., 2018; Wright & Dyson, 2015; Yoshizawa et al., 2020). Así mismo, proteínas virales que poseen regiones desordenadas han sido descritas por su capacidad de interactuar con RNA y secuestrar proteínas nucleolares, fibrilarina por ejemplo (Chang et al., 2016; Kim et al., 2007; Z. Li et al., 2018). Nuestros ensayos de actividad de ribonucleasa incorporaron la hipótesis de que regiones ricas en glicinas y argininas como las que se encuentran en fibrilarina, en su dominio N terminal, constituido por aproximadamente 120 aminoácidos, poseen también algún tipo de capacidad degradativa en este tipo de ensayos. En este punto se clonaron diferentes proteínas con regiones similares a las repeticiones GRR que se encuentran en la región desordenada de fibrilarina. Las regiones de GAR de

las proteínas GAR1, Lsm14, Nucleolina, así como la proteína de movimiento TGB1 fueron expresadas y purificadas bajo las mismas condiciones y su activad ribonucleasa fue evaluada en presencia de RNA total. Como es de suponerse diferentes actividades relacionadas con la modificación, procesamiento y ensamble de diferentes poblaciones de RNA están asociadas a estas proteínas (Ma et al., 2007; McBride et al., 2009; Nazar, 2004). En estos experimentos encontramos, a pesar de las similitudes en las regiones repetitivas en glicinas y argininas de las proteínas evaluadas, únicamente la que posee fibrilarina actúa como ribonucleasa, aunque no es posible descartar del todo esta hipótesis puesto que se observa algún grado de actividad ribonucleasa de las regiones GAR de Slm14 y GAR1.

Dado que determinamos que la interacción física correspondiente a la co-localización de fibrilarina con PI4,5P, determinamos mediante mutaciones puntuales el efecto sobre los cambios en las posiciones R34A y R45A, en cuanto a la residencia temporal en el nucléolo, además de su interacción con lípidos de inositol. Mientras que la residencia y recambio de estas dos mutantes con respecto la fibrilarina WT, cambian, haciendo que permanezcan menos de la vida media reportada previamente, se asumió la hipótesis de que estas mutaciones puntuales inhiben las posibles interacciones transitorias con los componentes de la matriz nucleolar. El perfil de lípidos de estas mutantes fue evaluado tomando en consideración que si las interacciones lípido-proteína provienen de las interacciones electrostáticas de los grupos fosfato presentes en los lípidos y los aminoácidos cargados eléctricamente, respectivamente, se esperaría un cambio con respecto a al perfil de unión con la fibrilarina WT. Como resultado de estos experimentos *in vitro* se observó que la mutante R45A cambia su patrón de unión a lípidos, orientándose la interacción hacia el fosfolípido PI3P. Por el otro lado, fibrilarina R34A cambia su perfil ligeramente con respecto a la fibrilarina WT, variando ligeramente las interacciones con los fosfolípidos bis fosfato y mono fosfato. Tomando en consideración que estas variaciones en cuanto a su unión con lípidos es posible que de forma *in vivo* (véase la figura 5 del artículo de investigación).

Por último, parte de la estrategia experimental de la presente tesis estuvo orientada sobre la hipótesis de si la actividad ribonucleasa se encuentra ligada al contexto de fibrilarina en un complejo de metilación como los que se encuentran de manera *in vivo*. Se ha descrito con anterioridad la posibilidad de purificación del complejo de metilación descrito en el que fibrilarina forma parte (Peng et al., 2014). La construcción del vector múltiple de expresión contiene una versión de fibrilarina truncada en el dominio GAR, por lo que con la

metodología de (Ulrich et al., 2012). En el contexto del complejo snoRNP generalmente se describe como la porción de la maquinaria que se encarga de la metilación sitio específica de los residuos de rRNA, nuestros resultados apuntan a que la actividad ribonucleasa también se encuentra presente en este complejo, debido que observamos degradación de RNA cuando el complejo con la fibrilarina ha sido integrado la región GAR. Interesante de notar es el hecho de que en ausencia del dominio GAR, no se observa degradación alguna de RNA, asumiendo de esta manera que la actividad ribonucleasa pertenece únicamente a fibrilarina y no a otro componente del complejo de metilación, confirmando lo que en (Kass et al., 1990; Sáez-Vasquez, Caparros-Ruiz, Barneche, & Echeverría, 2004) había descrito con anterioridad sin mencionar la posibilidad de que fibrilarina conservara un módulo con actividad ribonucleasa.

En el presente trabajo, además de describir que esta función se conserva en la fibrilarina humana nos permite la construcción de la hipótesis de que las regiones modulares que contienen regiones intrínsicamente desordenadas poseen actividad enzimática en un sentido y momento específico, determinados por la interacción con otras moléculas, como lípidos y RNA, además que unidos a la formación de estructuras de tipo anillo, estas actividades van organizando y digiriendo la síntesis, procesamiento y ensamble de moléculas de rRNA durante el ciclo celular (véase a la figura 1 de la revisión y la figura 2 del artículo de investigación). Posiblemente la razón de estas estructuras de tipo anillo permanece como una incógnita en el sentido de que se desconoce su participación en la regulación y mantenimiento de las estructuras nucleolares, pero dada la información recuperada de nuestras imágenes de microscopia de SIM es posible anticipar que el origen proviene de las interacciones termodinámicas que promueven la separación de fases, conocida como separaciones líquido-líquido, que requiere mínima cantidad de energía acopladas a actividades y procesos celulares cuya demanda energética es elevada como precisamente la síntesis de rRNA. La presente tesis es un inicio para poder entender las regulaciones termodinámicas inherentes a las moléculas presentes en el nucleolo, y en general aquellas en las que la regulación energéticamente demandante y la necesidad de membranas lipídicas no es la prioridad para organelos sin membrana.

Conclusiones

Como conclusiones del presente trabajo de tesis doctoral

1) Fibrilarina posee actividad ribonucleasa
2) La actividad ribonucleasa de fibrilarina es dependiente de fosfoinosítidos
3) La actividad ribonucleasa también esta presente en el contexto de los complejos de metilación.

Perspectivas

Con las siguientes aportaciones nosotros planteamos las siguientes perspectivas de trabajo para futuras investigaciones en el área de la actividad ribonucleasa de fibrilarina.

- Evaluar la actividad de ribonucleasa de las mutantes de fibrilarina de humanos que se poseen en el laboratorio.
- Evaluar las actividades de fibrilarina de plantas con respecto a diferentes sustratos de RNA.
- Evaluar la actividad ribonucleasa de fibrilarina en presencia de RNA guías y RNA blancos.

Bibliografía

Aris, J. P., & Blobel, G. (1991). Cdna cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proceedings of the National Academy of Sciences*, *88*(3), 931-935. https://doi.org/10.1073/pnas.88.3.931

Bachellerie, J.-P., Cavaillé, J., & Hüttenhofer, A. (2002). The expanding snoRNA world. *Biochimie*, *84*(8), 775-790. https://doi.org/10.1016/S0300-9084(02)01402-5

Baudin-Baillieu, A., Fabret, C., Liang, X., Piekna-Przybylska, D., Fournier, M. J., & Rousset, J.-P. (2009). Nucleotide modifications in three functionally important regions of the Saccharomyces cerevisiae ribosome affect translation accuracy. *Nucleic Acids Research*, *37*(22), 7665-7677. https://doi.org/10.1093/nar/gkp816

Baxter-Roshek, J. L., Petrov, A. N., & Dinman, J. D. (2007). Optimization of ribosome structure and function by rrna base modification. *PLoS ONE*, *2*(1), e174. https://doi.org/10.1371/journal.pone.0000174

Brown, J. W. S., & Shaw, P. J. (1998). Small Nucleolar RNAs and Pre-rRNA Processing in Plants. Plant Cell, 10(5), 649–658. http://doi.org/10.2307/3870654

Brown, J. W. S., Echeverria, M., Qu, L. H., Lowe, T. M., Bachellerie, J. P., Hüttenhofer, A., … Marshall, D. F. (2003). Plant snoRNA database. Nucleic Acids Research, 31(1), 432–435. http://doi.org/10.1093/nar/gkg009

Canetta, E., Kim, S. H., Kalinina, N. O., Shaw, J., Adya, A. K., Gillespie, T., … Taliansky, M. (2008). A plant virus movement protein forms ringlike complexes with the major nucleolar protein, fibrillarin, in vitro. *Journal of Molecular Biology*, *376*(4), 932-937. https://doi.org/10.1016/j.jmb.2007.12.039

Darzacq, X. (2002). Cajal body-specific small nuclear RNAs: a novel class of 2'-O-methylation and pseudouridylation guide RNAs. *The EMBO Journal*, *21*(11),

2746-2756. https://doi.org/10.1093/emboj/21.11.2746

Decatur, W. A., & Fournier, M. J. (2002). Rrna modifications and ribosome function. *Trends in Biochemical Sciences*, *27*(7), 344-351. https://doi.org/10.1016/S0968-0004(02)02109-6

Deng, P., Liu, S., Nie, X., Weining, S., & Wu, L. (2018). Conservation analysis of long non-coding RNAs in plants. *Science China Life Sciences*, *61*(2), 190-198. https://doi.org/10.1007/s11427-017-9174-9

Dragon, F., Lemay, V., & Trahan, C. (2006). SnoRNA: biogenesis, structure and function. John Wiley & Sons, Ltd (Ed.), *Encyclopedia of Life Sciences*. Chichester, UK: John Wiley & Sons, Ltd. https://doi.org/10.1038/npg.els.0003813

Echeverría, M., Steinmetz, F., & Barneche, F. (2000). Fibrillarin genes encode both a conserved nucleolar protein and a novel small nucleolar rna involved in ribosomal rna methylation inarabidopsis thaliana. *Journal of Biological Chemistry*, *275*(35), 27212-27220.

Fatica, A., Galardi, S., Altieri, F., & Bozzoni, I. (2000). Fibrillarin binds directly and specifically to U16 box C/D snoRNA. RNA (New York, N.Y.), 6(1), 88–95. http://doi.org/10.1017/S1355838200991623

Gautier, T., Bergès, T., Tollervey, D., & Hurt, E. (1997). Nucleolar KKE/D repeat proteins Nop56p and Nop58p interact with Nop1p and are required for ribosome biogenesis. *Molecular and Cellular Biology*, *17*(12), 7088-7098. https://doi.org/10.1128/MCB.17.12.7088

Henras, A. K., Plisson-Chastang, C., O'Donohue, M. F., Chakraborty, A., & Gleizes, P. E. (2015). An overview of pre-ribosomal RNA processing in eukaryotes. Wiley Interdisciplinary Reviews: RNA, 6(2), 225–242. http://doi.org/10.1002/wrna.1269

Hernandez-Verdun, D., Roussel, P., Thiry, M., Sirri, V., & Lafontaine, D. L. J. (2010). The nucleolus: Structure/function relationship in RNA metabolism. Wiley Interdisciplinary Reviews: RNA, 1(3), 415–431. http://doi.org/10.1002/wrna.39

Kass, S., Tyc, K., Steitz, J. A., & Sollner-Webb, B. (1990). The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. Cell, 60(6), 897–908. http://doi.org/10.1016/0092-8674(90)90338-F

King, T. H., Liu, B., McCully, R. R., & Fournier, M. J. (2003). Ribosome structure and activity are altered in cells lacking snornps that form pseudouridines in the peptidyl transferase center. *Molecular Cell*, *11*(2), 425-435. https://doi.org/10.1016/S1097-2765(03)00040-6

Kiss-László, Z., Henry, Y., Bachellerie, J.-P., Caizergues-Ferrer, M., & Kiss, T. (1996). Site-specific ribose methylation of preribosomal rna: a novel function for small nucleolar rnas. *Cell*, *85*(7), 1077-1088. https://doi.org/10.1016/S0092-8674(00)81308-2

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell*, *128*(4), 693-705. https://doi.org/10.1016/j.cell.2007.02.005

Leader, D. J., Clark, G. P., Watters, J., Beven, A. F., Shaw, P. J., & Brown, J. W. S. (1997). Clusters of multiple different small nucleolar RNA genes in plants are expressed as and processed from polycistronic pre-snoRNAs. EMBO Journal, 16(18), 5742–5751. http://doi.org/10.1093/emboj/16.18.5742

Liu, X., Hao, L., Li, D., Zhu, L., & Hu, S. (2015). Long non-coding rnas and their biological roles in plants. *Genomics, Proteomics & Bioinformatics*, *13*(3), 137-147. https://doi.org/10.1016/j.gpb.2015.02.003

Loza-Muller, L., Rodríguez-Corona, U., Sobol, M., Rodríguez-Zapata, L. C., Hozak, P., & Castano, E. (2015). Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in Brassica oleracea. Frontiers in Plant Science, 6(November), 976. http://doi.org/10.3389/fpls.2015.00976

McCullough, L., Rawlins, R., Olsen, A., Xin, H., Stillman, D. J., & Formosa, T. (2011). Insight into the mechanism of nucleosome reorganization from histone mutants that suppress defects in the fact histone chaperone. *Genetics*, *188*(4), 835-846. https://doi.org/10.1534/genetics.111.128769

Monaco, P., Marcel, V., Diaz, J.-J., & Catez, F. (2018). 2′-o-methylation of ribosomal

rna: towards an epitranscriptomic control of translation? *Biomolecules*, *8*(4), 106. https://doi.org/10.3390/biom8040106

Nazar, R. N. (2004). Ribosomal RNA processing and ribosome biogenesis in eukaryotes. IUBMB Life, 56(8), 457–465. http://doi.org/10.1080/15216540400010867

Nejat, N., & Mantri, N. (2018). Emerging roles of long non-coding RNAs in plant response to biotic and abiotic stresses. *Critical Reviews in Biotechnology*, *38*(1), 93-105. https://doi.org/10.1080/07388551.2017.1312270

Newman, D. R., Kuhn, J. F., Shanab, G. M., & Maxwell, E. S. (2000). Box C/D snoRNA-associated proteins: two pairs of evolutionarily ancient proteins and possible links to replication and transcription. Rna, 6(6), 861–879. http://doi.org/10.1017/S1355838200992446

Olson, M. O. (2010). Nucleolus: structure and function. En John Wiley & Sons, Ltd (Ed.), *Encyclopedia of Life Sciences*. Chichester, UK: John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470015902.a0005975.pub2

Omer, A. D., Ziesche, S., Ebhardt, H., & Dennis, P. P. (2002). In vitro reconstitution and activity of a C/D box methylation guide ribonucleoprotein complex. *Proceedings of the National Academy of Sciences*, *99*(8), 5289-5294. https://doi.org/10.1073/pnas.082101999

Peña, C., Hurt, E., & Panse, V. G. (2017). Eukaryotic ribosome assembly, transport and quality control. *Nature Structural & Molecular Biology*, *24*(9), 689-699. https://doi.org/10.1038/nsmb.3454

Piekna-Przybylska, D., Przybylski, P., Baudin-Baillieu, A., Rousset, J.-P., & Fournier, M. J. (2008). Ribosome performance is enhanced by a rich cluster of pseudouridines in the a-site finger region of the large subunit. *Journal of Biological Chemistry*, *283*(38), 26026-26036. https://doi.org/10.1074/jbc.M803049200

Pontvianne, F., Abou-Ellail, M., Douet, J., Comella, P., Matia, I., Chandrasekhara, C., … Sáez-Vásquez, J. (2010). Nucleolin is required for DNA methylation state and the expression of rRNA gene variants in Arabidopsis thaliana. PLoS Genetics, 6(11), 1–13. http://doi.org/10.1371/journal.pgen.1001225

Rakitina, D. V., Taliansky, M., Brown, J. W. S., & Kalinina, N. O. (2011). Two RNA-binding sites in plant fibrillarin provide interactions with various RNA substrates. Nucleic Acids Research, 39(20), 8869–8880. http://doi.org/10.1093/nar/gkr594

Reichow, S. L., Hamma, T., Ferre-D'Amare, A. R., & Varani, G. (2007). The structure and function of small nucleolar ribonucleoproteins. *Nucleic Acids Research*, *35*(5), 1452-1464. https://doi.org/10.1093/nar/gkl1172

Rodriguez-Corona, U., Pereira-Santana, A., Sobol, M., Rodriguez-Zapata, L. C., Hozak, P., & Castano, E. (2017). Novel Ribonuclease Activity Differs between Fibrillarins from Arabidopsis thaliana. Frontiers in Plant Science, 8(October), 1–10. http://doi.org/10.3389/fpls.2017.01878

Rodriguez-Corona, U., Sobol, M., Rodriguez-Zapata, L. C., Hozak, P., & Castano, E. (2015). Fibrillarin from Archaea to human. Biology of the Cell, 107(6), 159–174. http://doi.org/10.1111/boc.201400077

Rudnizky, S., Bavly, A., Malik, O., Pnueli, L., Melamed, P., & Kaplan, A. (2016). H2A.Z controls the stability and mobility of nucleosomes to regulate expression of the LH genes. *Nature Communications*, *7*(1). https://doi.org/10.1038/ncomms12958

Saez-Vasquez, J., Caparros-Ruiz, D., Barneche, F., & Echeverria, M. (2004). A plant snornp complex containing snornas, fibrillarin, and nucleolin-like proteins is competent for both rrna gene binding and pre-rrna processing in vitro. *Molecular and Cellular Biology*, *24*(16), 7284-7297. https://doi.org/10.1128/MCB.24.16.7284-7297.2004

Schimmang, T., Tollervey, D., Kern, H., Frank, R., & Hurt, E. C. (1989). A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *The EMBO Journal*, *8*(13), 4015-4024. https://doi.org/10.1002/j.1460-2075.1989.tb08584.x

Seo, J. S., Diloknawarit, P., Park, B. S., & Chua, N.-H. (2018). ELF18-INDUCED LONG NONCODING RNA 1 evicts fibrillarin from mediator subunit to enhance *PATHOGENESIS-RELATED GENE 1* (*pr1*) expression. *New*

Phytologist. https://doi.org/10.1111/nph.15530

Seo, J. S., Sun, H.-X., Park, B. S., Huang, C.-H., Yeh, S.-D., Jung, C., & Chua, N.-H. (2017). Elf18-induced long-noncoding rna associates with mediator to enhance expression of innate immune response genes in arabidopsis. *The Plant Cell*, *29*(5), 1024-1038. https://doi.org/10.1105/tpc.16.00886

Sharma, K., & Tollervey, D. (1999). Base pairing between u3 small nucleolar rna and the 5′ end of 18s rrna is required for pre-rrna processing. *Molecular and Cellular Biology*, *19*(9), 6012-6019. https://doi.org/10.1128/MCB.19.9.6012

Sharma, S., & Lafontaine, D. L. J. (2015). 'View from a bridge': a new perspective on eukaryotic rrna base modification. *Trends in Biochemical Sciences*, *40*(10), 560-575. https://doi.org/10.1016/j.tibs.2015.07.008

Sharma, S., Marchand, V., Motorin, Y., & Lafontaine, D. L. J. (2017). Identification of sites of 2′-O-methylation vulnerability in human ribosomal RNAs by systematic mapping. Scientific Reports, 7(1), 11490. http://doi.org/10.1038/s41598-017-09734-9

Shaw, P., & Doonan, J. (2005). Nd Sc What Makes a Nucleolus? Cell Cycle, (January), 102–105.

Sobol, M., Yildirim, S., Philimonenko, V. V, Marášek, P., Castaño, E., & Hozák, P. (2013). UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity. Nucleus (Austin, Tex.), 4(6), 478–86. http://doi.org/10.4161/nucl.27154

Stefanovsky, V. Y., Pelletier, G., Bazett-Jones, D. P., Crane-Robinson, C., & Moss, T. (2001). DNA looping in the RNA polymerase I enhancesome is the result of non-cooperative in-phase bending by two UBF molecules. *Nucleic Acids Research*, *29*(15), 3241-3247.

Stepanov, G. A., Semenov, D. V., Kuligina, E. V., Koval, O. A., Rabinov, I. V., Kit, Y. Y., & Richter, V. A. (2012). Analogues of Artificial Human Box C/D Small Nucleolar RNA As Regulators of Alternative Splicing of a pre-mRNA Target. Acta naturae, 4(1), 32-41.

Taliansky, M. E. (2003). Molecular biology of umbraviruses: phantom warriors.

Journal of General Virology, *84*(8), 1951-1960. https://doi.org/10.1099/vir.0.19219-0

Taliansky, M., Brown, J. W. S., Haupt, S., Gillespie, T., Ryabov, E. V., Rakitina, D. V., … Kim, S. H. (2007). Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. *Proceedings of the National Academy of Sciences*, *104*(26), 11115-11120. https://doi.org/10.1073/pnas.0704632104

Taliansky, M., Roberts, I. M., Kalinina, N., Ryabov, E. V., Raj, S. K., Robinson, D. J., & Oparka, K. J. (2003). An umbraviral protein, involved in long-distance rna movement, binds viral rna and forms unique, protective ribonucleoprotein complexes. *Journal of Virology*, *77*(5), 3031-3040. https://doi.org/10.1128/JVI.77.5.3031-3040.2003

Toiiervey, D., Lehtonen, H., Jansen, F., Kern, H., & Hurt, E. C. (1993). 72, 443–457.

Tomecki, R., Sikorski, P. J., & Zakrzewska-Placzek, M. (2017). Comparison of preribosomal RNA processing pathways in yeast, plant and human cells – focus on coordinated action of endo- and exoribonucleases. FEBS Letters, 591(13), 1801–1850. http://doi.org/10.1002/1873-3468.12682

Tran, E. J., Zhang, X., & Maxwell, E. S. (2003). Efficient RNA 2???-O-methylation requires juxtaposed and symmetrically assembled archaeal box C/D and C???/D??? RNPs. EMBO Journal, 22(15), 3930–3940. http://doi.org/10.1093/emboj/cdg368

Turgeon, R., & Wolf, S. (2009). Phloem transport: cellular pathways and molecular trafficking. *Annual Review of Plant Biology*, *60*(1), 207-221. https://doi.org/10.1146/annurev.arplant.043008.092045

VanDemark, A. P., Xin, H., McCullough, L., Rawlins, R., Bentley, S., Heroux, A., … Formosa, T. (2008). Structural and functional analysis of the spt16p n-terminal domain reveals overlapping roles of yfact subunits. *Journal of Biological Chemistry*, *283*(8), 5058-5068. https://doi.org/10.1074/jbc.M708682200

Venema, J., Vos, H. R., Faber, A. W., van Venrooij, W. J., & Raué, H. A. (2000).

Yeast Rrp9p is an evolutionarily conserved U3 snoRNP protein essential for early pre-rRNA processing cleavages and requires box C for its association. *RNA (New York, N.Y.)*, *6*(11), 1660-1671.

Wang, H.-L. V., & Chekanova, J. A. (2017). Long noncoding rnas in plants. En M. R. S. Rao (Ed.), *Long Non-Coding RNA Biology* (Vol. 1008, pp. 133-154). Singapore: Springer Singapore. https://doi.org/10.1007/978-981-10-5203-3_5

Watkins, N. J., & Bohnsack, M. T. (2012). The box C/D and H/ACA snoRNPs: key players in the modification, processing and the dynamic folding of ribosomal RNA: Box C/D and H/ACA snoRNPs. *Wiley Interdisciplinary Reviews: RNA*, *3*(3), 397-414. https://doi.org/10.1002/wrna.117

Watkins, N. J., Ségault, V., Charpentier, B., Nottrott, S., Fabrizio, P., Bachi, A., … Lührmann, R. (2000). A common core rnp structure shared between the small nucleoar box c/d rnps and the spliceosomal u4 snrnp. *Cell*, *103*(3), 457-466. https://doi.org/10.1016/S0092-8674(00)00137-9

Weis, B. L., Kovacevic, J., Missbach, S., & Schleiff, E. (2015). Plant-specific features of ribosome biogenesis. *Trends in Plant Science*, *20*(11), 729-740. https://doi.org/10.1016/j.tplants.2015.07.003

Wilson, D. N., & Doudna Cate, J. H. (2012). The structure and function of the eukaryotic ribosome. *Cold Spring Harbor Perspectives in Biology*, *4*(5), a011536-a011536. https://doi.org/10.1101/cshperspect.a011536

Yanagida, M., Hayano, T., Yamauchi, Y., Shinkawa, T., Natsume, T., Isobe, T., & Takahashi, N. (2004). Human fibrillarin forms a sub-complex with splicing factor 2-associated p32, protein arginine methyltransferases, and tubulins alpha 3 and beta 1 that is independent of its association with preribosomal ribonucleoprotein complexes. *The Journal of Biological Chemistry*, *279*(3), 1607-1614. https://doi.org/10.1074/jbc.M305604200

Yang, Z., Lin, J., & Ye, K. (2016). Box C/D guide RNAs recognize a maximum of 10 nt of substrates. *Proceedings of the National Academy of Sciences*, *113*(39), 10878-10883. https://doi.org/10.1073/pnas.1604872113

Yildirim, S., Castano, E., Sobol, M., Philimonenko, V. V, Dzijak, R., Venit, T., & Hozák, P. (2013). Involvement of phosphatidylinositol 4,5-bisphosphate in RNA polymerase I transcription. Journal of Cell Science, 126(Pt 12), 2730–9. http://doi.org/10.1242/jcs.123661

Yu, H., Fukami, K., Watanabe, Y., Ozaki, C., & Takenawa, T. (1998). Phosphatidylinositol 4,5-bisphosphate reverses the inhibition of RNA transcription caused by histone H1. *European Journal of Biochemistry*, *251*(1-2), 281-287.

Zhang, L., Lin, J., & Ye, K. (2013). Structural and functional analysis of the U3 snoRNA binding protein Rrp9. Rna, 19(5), 701–711. http://doi.org/10.1261/rna.037580.112

Zhao, K., Wang, W., Rando, O. J., Xue, Y., Swiderek, K., Kuo, A., & Crabtree, G. R. (1998). Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell*, *95*(5), 625-636.

Anexos

Avance del semestre 2020-I

Clonación del gen de fibrilarina en los vectores pET32a y pET22b

Con objeto de obtener nuevas construcciones de expresión del gen de fibrilarina se realizó la amplificación, clonación y subclonación a los vectores de expresión pET32a y pET22b, ambos con etiquetas de 6His-tag en el extremo C-terminal. Además, cuentan con una etiqueta de S-tag (pET32a) y un péptido señal pelB (pET22b), útiles para la detección por western blot, además de otras estrategias de purificación. Se diseñaron los siguientes oligonucleótidos para la amplificación del gen de fibrilarina y el ajuste al marco de lectura de los vectores de expresión: F22bFib: GCGGATCCATGAAGCCAGGATT R22bFib: GCCTCGAGTGAGTTCTTCACCT. F32aFib: GCGGATCCATGAAGCCAGGATT y R32aFib: GCCTCGAGTGAGTTCTTCACCT. Ambos pares de cebadores con sitios de restricción BamHi y XhoI. Como templado se utilizó la constracción pET15b:Fibrilarina usada para la expresión en E. coli.

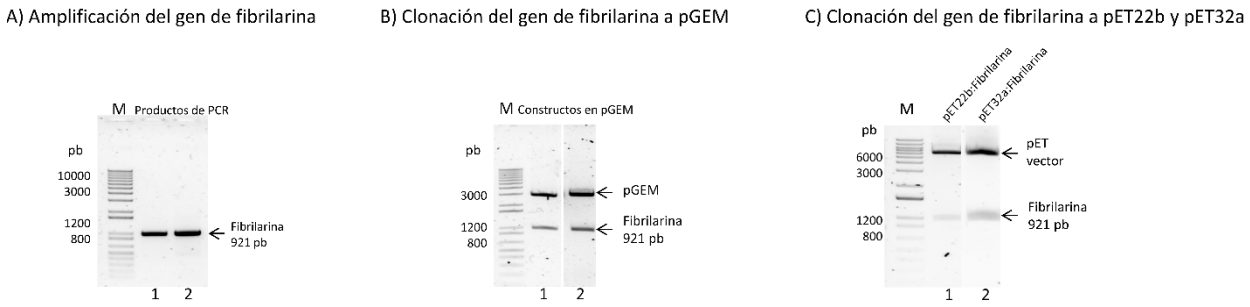


Figura 1. Clonación del gen de fibrilarina a los vectores pET con etiqueta 6His en el C-terminal. A) amplificación del gen de fibrilarina usando los cebadores diseñados para la subsecuente clonación en los vectores pET. B) Los productos de PCR fueron clonados al vector pGEM y digeridos con EcoRV para liberar el inserto correspondiente. C) Subclonación de los fragmentos clonados en pGEM a los vectores pET22b y pET32a, digeridos con las enzimas de restricción BamHI y XhoI.

Expresión y purificación recombinante de 6His-tag-fibrillarin

Las construcciones pET22b:Fibrilarina y pET32a:Fibrilarina fueron transformadas en E. coli BL21 DE3 para la inducción de la expresión mediante IPTG 1 mM (Figura 2A), como se detalla en la sección de Materiales y Métodos. Así mismo, se detectó la presencia de los productos de la inducción mediante WB anti 6His-tag (Figura 2B) y los ensayos de purificación de los mismos por cromatografía de afinidad a níquel (Figura 2C).

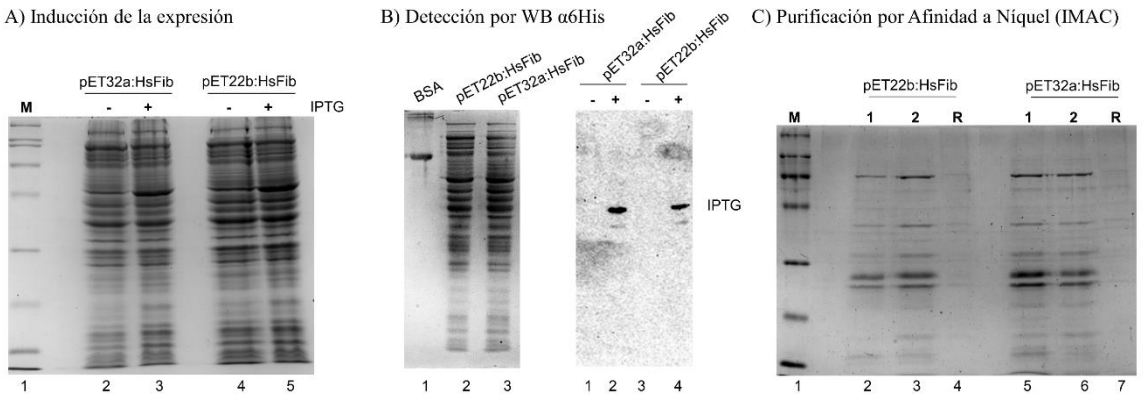


Figura 2. Análisis de la expresión de las construcciones del gen de fibrilarina en pET22b y pET32a. A) Análisis de la inducción mediante IPTG en SDS-PAGE 12%. B) Detección de la expresión de los constructos por WB anti histidinas. C) Análisis de los ensayos de purificación por IMAC de los constructos, en SDS-PAGE 12%