CICY

Centro de Investigación Científica de Yucatán, A.C.

Posgrado en Ciencias Biológicas

Estudio funcional de la fibrilarina como ribonucleasa

Tesis que presenta

Ulises Rodríguez Corona

En opción al título de

Doctor en Ciencias

(Ciencias Biológicas: Opción Bioquímica y Biología Molecular)

Mérida, Yucatán, México

2018

RECONOCIMIENTO

Por medio de la presente, hago constar que el trabajo de tesis titulado **"Estudio funcional de la fibrilarina** fue realizado en los laboratorios de la Unidad de Bioquímica y Biología Molecular de Plantas 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 de Plantas, perteneciente al Programa de Posgrado en Ciencias Biológicas de este Centro.

Atentamente,

_____

Dr. Manuel Martínez Estévez

Director Académico

Mérida, Yucatán, México, 08 de enero de 2018.

DECLARACIÓN DE PROPIEDAD

Declaro que la información contenida en la sección de Materiales y Métodos Experimentales, los Resultados y Discusión de este documento proviene de las actividades de experimentación realizadas durante el período que se me asignó para desarrollar mi trabajo de tesis, en las Unidades y Laboratorios del Centro de Investigación Científica de Yucatán, A.C., y que a razón de lo anterior y en contraprestación de los servicios educativos o de apoyo que me fueron brindados, dicha información, en términos de la Ley Federal del Derecho de Autor y la Ley de la Propiedad Industrial, le pertenece patrimonialmente a dicho Centro de Investigación. Por otra parte, en virtud de lo ya manifestado, reconozco que de igual manera los productos intelectuales o desarrollos tecnológicos que deriven o pudieran derivar de lo correspondiente a dicha información, le pertenecen patrimonialmente al Centro de Investigación Científica, A.C., y en el mismo tenor, reconozco que si derivaren de este trabajo productos intelectuales o desarrollos tecnológicos, en lo especial, estos se regirán en todo caso por lo dispuesto por la Ley Federal del Derecho de Autor y la Ley de la Propiedad Industrial, en el tenor de lo expuesto en la presente Declaración.

Firma: _____

M. en C. Ulises Rodríguez Corona

AGRADECIMIENTOS

Agradezco a mis Padres por la confianza depositada, el apoyo incondicional y la motivación necesaria en los momentos adecuados.

A Nayeli Romero quien estoicamente camina conmigo en busca de sueños mutuos, por el inicio de nuestra familia.

Amigos, compañeros, otros y demás, afortunado fui de compartir con ustedes, salud y felicidad donde sea que la encuentren.

Al CICY por la oportunidad de cursar mis estudios de doctorado en sus instalaciones, particularmente a mi asesor, el Dr. Enrique Castaño de Serna, por sus enseñanzas, consejos y hospitalidad durante estos años.

ÍNDICE

ÍNDICE DE FIGURAS

RESUMEN

La fibrilarina es una proteína con actividad metiltransferasa responsable de la metilación del grupo 2`-hidroxilo de la ribosa blanco del RNAr a partir de la S-adenosilmetionina (SAM) y de la glutamina 105 y 104, de humanos y levaduras respectivamente, en la histona H2A. Es responsable también de la progresión de diferentes virus interactuando con proteínas y RNA viral para llevar a cabo funciones aún desconocidas. Su localización es principalmente nucleolar sin embargo también se encuentra en los cuerpos cajales y distintos tipos de estrés celular provocan que salga de estos compartimentos. Dentro del nucléolo está involucrada en el procesamiento del pre-RNAr y de la biogénesis de los ribosomas, en los cuerpos cajales aún es desconocida su función. Durante la maduración del pre-RNAr la fibrilarina interactúa con el RNA nucleolar U3 ubicándose en los sitios iniciales de corte para la obtención de los RNAs ribosomales maduros, lo cual sugiere que tiene actividad como ribonucleasa. Esta tesis se enfocó en definir la actividad como ribonucleasa de la fibrilarina de *H. sapiens* y las de *A. thaliana*. Se determinó que el dominio GAR (rico en argininas y glicinas) es el responsable de esta actividad, así como de la interacción con distintos fosfolípidos, particularmente con los fosfoinosítidos. Su actividad como ribonucleasa es selectiva a ciertos RNAs y puede ser inhibida en la presencia de los fosfoinosítidos. Otros fosfolípidos como el ácido fosfatídico evitan la interacción entre la fibrilarina y el RNA nucleolar U3. Sugerimos que la actividad como ribonucleasa de fibrilarina puede ser dirigida a un punto específico mediante la interacción con un RNA guía y que este corte sea regulado en algún punto por los fosfoinosítidos o el ácido fosfatídico.

ABSTRACT

Fibrillarin is a protein with methyltransferase activity responsible for the methylation of the 2'-hydroxyl group of the target ribose in RNAr from S-adenosylmethionine (SAM), and the methylation of glutamine 105 and 104, in humans and yeasts respectively, of histone H2A. It is also responsible for the progression of different viruses, interacting with proteins and viral RNA to carry out yet unknown functions. Fibrillarin location is mainly nucleolar however it is also found in the cajal bodies and different types of cellular stress cause it to leave those compartment. Within the nucleolus is involved in the processing of pre-RNAr and the ribosome biogenesis, in cajal bodies its function is still unknown. During the maturation of the pre-RNAr, fibrillarin interacts with nucleolar RNA U3, locating in the initial processing sites for the obtation of mature ribosomal RNAs, which suggests that it has activity as ribonuclease. This thesis focused on defining the activity of *H. sapiens* and *A. thaliana fibrillarins* as a ribonuclease. It was determined that GAR domain (rich in arginines and glycine) is responsible for this activity, as well as the interaction with different phospholipids, particularly with phosphoinositides. Its activity as ribonuclease is selective to certain RNAs and can be inhibited in the presence of phosphoinositides. Other phospholipids such as phosphatidic acid prevent the interaction between fibrillarin and nucleolar RNA U3. We suggest that fibrillarin ribonuclease activity can be directed to cut at a specific site by interacting with a guide RNA and that this cleavage could be regulated at some point by phosphoinositides or phosphatidic acid.

INTRODUCCIÓN

La principal característica que define a la célula eucariota es su núcleo. Es el sitio que almacena gran parte de la identidad celular. Profundizando en él, se puede observar el nucléolo. El nucléolo es una estructura dinámica involucrada en una diversa gama de funciones que van desde la transcripción de DNAr, la maduración y el ensamblaje de los ribosomas hasta la respuesta a estrés e infecciones virales (Olson et al., 2000). Dentro del nucléolo se pueden encontrar diversas proteínas que participan en las funciones descritas anteriormente en las que resaltan la fibrilarina y la nucleolina. La fibrilarina es una proteína con secuencia y función altamente conservada en los distintos reinos biológicos, su principal actividad, hasta ahora descrita es como metiltransferasa, catalizando la transferencia del grupo metilo de la S-adenosilmetionina (SAM) al grupo 2´-hidroxilo de la ribosa blanco del RNAr. Así mismo la fibrilarina es capaz de metilar la glutamina 105 (en levadura) y 104 (en humano) de la histona H2A. Dicha metilación se restringe al nucléolo celular (Lafontaine and Tollervey, 2000; Tessarz et al., 2014). Para llevar a cabo la metilación del RNAr, la fibrilarina se asocia a las proteínas nucleolares Nop56/58 y L7a, así como a un RNA guía. La metilación del RNAr únicamente se ha podido reproducir utilizando la fibrilarina de arqueas (Peng et al., 2014), la cual carece de un importante dominio presente en la fibrilarina de eucariotas. Dicho dominio dirige a la fibrilarina hacia el nucléolo e interactúa con proteínas virales (Kim et al., 2007). Por el contrario para la metilación de la histona la fibrilarina no forma complejos proteicos.

Adicional a su función como metiltransferasa, la fibrilarina ha sido asociada a otros procesos celulares tales como el desarrollo celular, progresión viral, oncogénesis, estrés celular, biogénesis de los ribosomas (Tollervey et al., 1993; Newton et al., 2003; Kim et al., 2007; Marcel et al., 2013; Rodriguez-Corona et al., 2015). Un ejemplo éstas funciones se pudo observar al generar ratones knockout en la región que tiene la función como metiltransferasa de fibrilarina. Los embriones homocigotos de esa mutación no lograban desarrollarse más allá de la fase mórula (Newton et al., 2003). Con esto se concluyó que la fibrilarina es una proteína esencial para la vida. Dentro del nucléolo celular, donde se lleva a cabo el procesamiento y maduración del RNAr, la fibrilarina está asociada a los

sitios de corte del pre-RNAr, de los cuales uno de los más importantes es el sitio de transcripción externa (5´-ÉTS) ya que es el primer corte en la ruta de maduración del pre-RNAr. Este trabajo se enfocó a caracterizar funcionalmente a la fibrilarina de eucariotas como ribonucleasa. Para ello, se clono, expreso y purifico la fibrilarina de *Homo sapiens* y las fibrilarinas 1 y 2 de *Arabidopsis thaliana*. Así mismo, se generaron mutantes de fibrilarina para identificar el domino responsable de la degradación de RNAr. Esta tesis se presenta como una colección de cuatro artículos. El Capítulo I, correspondiente a los Antecedentes los cuales son una revisión publicada en *Biology of the Cell* y lleva título "Fibrillarin from Archaea to human" (Rodriguez-Corona et al., 2015). Los tres siguientes corresponden a los resultados obtenidos. El Capítulo II se centra en definir la actividad como ribonucleasa de la fibrilarina de *H. sapiens* y el dominio responsable de ello. Dicha actividad se vislumbraba desde 1990, cuando se demostró que una sonda de RNAr era degrada en presencia de fibrilarina (Kass et al., 1990). Este capítulo lleva por título "Fibrillarin shows a novel ribonuclease activity in its GAR domain" y actualmente se encuentra en revisión en la revista PLoS ONE.

En el Capítulo III utilizando como modelo de estudio dos de las tres fibrilarinas de *A. thaliana* se confirmó la actividad como ribonucleasa aunque esta es diferencial entre ambas proteínas, posiblemente a la estructural en el dominio que contiene la actividad. Este capítulo lleva por título "Novel ribonuclease activity differs between fibrillarins from *Arabidopsis thaliana*" y fue publicado en la revista Frontiers in Plant Science (Rodriguez-Corona et al., 2017). Así mismo, en el capítulo IV, utilizando la fibrilarina 2 de *A. thaliana* se logró reproducir la metilación de la histona H2A de *Brassica oleracea.* Dicha metilación fue reportada previamente en células humanas y levaduras (Tessarz et al., 2014) y a diferencia de lo que sucede en humanos, no se restringe al nucléolo sino que también se localiza en la periferia del núcleo. Este capítulo lleva por título "Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in *Brassica oleracea*" y fue publicado en la revista Frontiers of Plant Science (Loza-Muller et al., 2015).

Finalmente en el capítulo V se hace una discusión general de los resultados obtenidos y la comparación entre la fibrilarina de *H. sapiens* y las de *A. thaliana*.

CAPÍTULO I. Antecedentes

Fibrillarin from Archaea to human

Ulises Rodriguez-Corona*, Margarita Sobol†, Luis Carlos Rodriguez Zapata‡, Pavel Hozak† and Enrique Castano*[1]

*Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Colonia Chuburna de Hidalgo, Mérida, Yucatán, México, †Department of Biology of the Cell Nucleus, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, Prague 14220, Czech Republic, and ‡Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Colonia Chuburna de Hidalgo, Mérida, Yucatán, México

Abstract

Fibrillarin is an essential protein that is well known as a molecular marker of transcriptionally active RNA polymerase I. Fibrillarin methyltransferase activity is the primary known source of methylation for more than 100 methylated sites involved in the first steps of pre-ribosomal processing and required for structural ribosome stability. High expression levels of fibrillarin have been observed in several types of cancer cells, particularly when p53 levels are reduced, because p53 is a direct negative regulator of fibrillarin transcription. Here, we show fibrillarin domain conservation, structure and interacting molecules in different cellular processes as well as with several viral proteins during virus infection.

Key words: Cancer, Methylation, p53, Ribosomal biogenesis, RNA processing.

Abbreviations: aFIB, Archaea fibrillarin; CBs, Cajal bodies; DFC, dense fibrillar component; FACT, facilitates chromatin transcription complex; FCs, fibrillar centers; GAR, glycine- and arginine-rich domain; MTase, methyltransferase; NOP1, nucleolar protein 1; NORs, nucleolar organiser regions; NS1, Non-structural pro-tein; PIP2, phosphatidylinositol 4,5-bisphosphate; PNBs, pre-nucleolar bodies; pol I, RNA polymerase I; PRMT1, protein arginine N-methyltransferase 1; RAP, [RuLCl$_2$]H 4H$_2$ O; SAM, S-adenosylmethionine; ASF/SF2, alternative splicing factor/splicing factor 2; SMN, survival of

motor neuron; snoRNA, small nucleo-lar RNA; snoRNP, small nucleolar ribonucleoprotein; snRNA, small nuclear RNA; Tat, Trans-activator of transcription.

Introduction

The nucleolus is the largest visible structure inside the cell nucleus. It exists both as a dynamic and stable region depending of the nature and amount of the molecules that it is made of. The main function of this structure is ribosome biogenesis. This process involves transcription of rDNA, processing of RNAr and assembly of ribosomal proteins (Kressler et al., 1999). The nucleolus consists of three components: fibrillar centers (FCs), dense fibrillar component (DFC) and granular component. Over 4500 proteins were identified by multiple mass spectrometry and are involved in several cellular processes (Ahmad et al., 2009). Besides ribosome biogenesis in recent years, several other functions have been attributed to the nucleolus, such as genetic silencing, cell cycle progression, senescence and biogenesis of small nuclear RNA and tRNAs proliferation and many forms of stress response (Andersen et al., 2005; Boisvert et al., 2007; Shaw and Brown, 2012). Among nucleolar proteins (NOP), fibrillarin is an essential protein that has been conserved in its sequence and function throughout evolution (Ochs et al., 1985; Jansen et al., 1991). Normally during interphase, fibrillarin can be detected in the transition zone between FC and DFC, where rDNA transcription occurs, and in the DFC, where the pre-RNAr processing takes place in eukaryotic cells (Ochs et al., 1985; Sobol et al., 2013). Therefore, it is commonly used as a marker of active nucleoli. Depending on the organism, fibrillarin mass ranges between 34 and 38 KDa and was originally described in the nucleolus of *Physarum polycephalum* (Christensen et al., 1977). It is included in the superfamily of the Rossmann-fold S-adenosylmethionine (SAM) methyltransferases (MTases) (Wang et al., 2000). The characteristics of this superfamily include a con-served SAM-binding motif, the catalytic triad/tetrad [K-D-K-(H)] and seven-stranded β-sheet flanked by α-helices to form an α-β-α structure (Rakitina et al., 2011). Their primary and secondary structures are conserved and one of their principal characteristics is a site rich in arginine and glycine residues and a specific motif to bind RNA.

Fibrillarin transfers the methyl group of SAM to 2-hydroxyl group of ribose target (Omer et al., 2002; Ye et al., 2009). The MTase activity was confirmed by reconstruction of the small

ribonucleoprotein from Archaea *Sulfobus solfataricus*. Testing the recon-structed complex with mutations within the MTase domain of the *Fbl* gene helped confirm the methylation activity by Archaea fibrillarin (aFIB) (Omer et al., 2002). Recently, a new methylation activity has been attributed to fibrillarin. It mediates methylation of Gln-105 in histone H2A, which is a modification that impairs binding of the facilitates chromatin transcription complex (FACT) complex and is specifically present at 35S ribosomal DNA locus, thus having an epigenetic effect specific in active RNA polymerase I (RNA pol I) promoters. Abnormal levels of fibrillarin have been found in several types of cancers such as breast cancer and prostate cancer (Koh et al., 2011b; Miller et al., 2012) as well as interacting with viral proteins from the Influenza A virus and the trans-activator of transcription (Tat) protein from HIV (Yoo et al., 2003; Melen et al., 2012). Here, we would like to present a first view on what is known about this protein and what still remains to be cleared.

Fibrillarin phylogenetics

The term 'fibrillarin' has been used indistinctly for several proteins from many organisms; in particular, aFIBs that have significant differences and could confuse newcomers. Moreover, several synonyms exist in the literature such as 34 kDa nucleolar scleroderma antigen, Dmel CG9888, CG9888, Dmel CG9888ri, GCR-6, GCR6, Pen59C5, fib, pen59C5, Fib, FIB, FBL, Fbl, FIB1, FLRN, RNU3IP1, RNAr 2 -*O*-methyltransferase fibrillarin, NOP1, nop1, fibM and aFIB depending on the organism and the time when the reference was published. Here, to avoid confusion and to distinguish between different organisms, we will use the term 'fibrillarin' for all eukaryotic fibrillarins with the exception of yeast fibrillarin (NOP1) and define the data from the individual organisms by adding genus and species before the term when the observations could be unique. We will use the term aFIB for all archaeal organisms as used by Omer et al. (2002). We think it is particularly important to make this distinction considering the large amount of biochemical and structural data that has been obtained from archaeal organisms whose aFIB could not completely complement specific roles of eukaryotic fibrillarins. Furthermore, as in eukaryotic cells fibrillarin is localised primarily in the nucleoli and Archaea do not have nucleus, it would be unlikely that all functions are conserved. Archaeal fibrillarins have been used in recombinant protein purification due to their high yield expression and ease to remove other bacterial proteins with high temperatures. Higher plant or vertebrate fibrillarins show

a very poor expression in bacteria in a native form. They have to be purified from inclusion bodies and in most cases refolded before biochemical experiments can be performed (Pearson et al., 1999). On the other hand, most of the genetic experiments were carried out with NOP1 and detail localisation studies were done with vertebrate fibrillarins. It remains to be defined if the nonstandard interacting partners are the same throughout the different kingdoms as well as under the different stages of development and cell growth conditions. One clear difference is that aFIB shows poor RNA binding (Omer et al., 2002) while fibrillarin from different eukaryotic organisms shows well-defined RNA binding activity and, in some cases, up to two binding sites for RNA have been described (Rakitina et al., 2011).

NOP1 function is essential for the modification and processing of pre-RNAr. NOP1 can be replaced by the fibrillarin of *Arabidopsis thaliana* (Barneche et al., 2000) as well as by human and *Xenopus* fibrillarins (Schimmang et al., 1989; Jansen et al., 1991). However, the protozoan fibrillarin from *Tetrahymena termophila* does not complement yeast counterpart, possibly due to the differences in the amino terminal domain. A domain that is rich in glycine and arginine residues (termed the GAR domain) and has a low sequence similarity when compared between different organisms (David et al., 1997). This can suggest lower general conservation that is commonly believed. Moreover, replacement of NOP1 by human, *Xenopus* or *A. thaliana* fibrillarin changes the growth and nuclear morphology in yeast, thus show-ing that not all fibrillarin functions are conserved (Jansen et al., 1993).

The sequence alignments and comparison of 10 model eukaryotic fibrillarins and all aFIBs was carried (Figure 1.1A). Archaea bacteria present a large spectrum of cladograms that separate from other groups. The sequence comparison for all complete eukaryotic fibrillarins is included (Figure 1.1B).
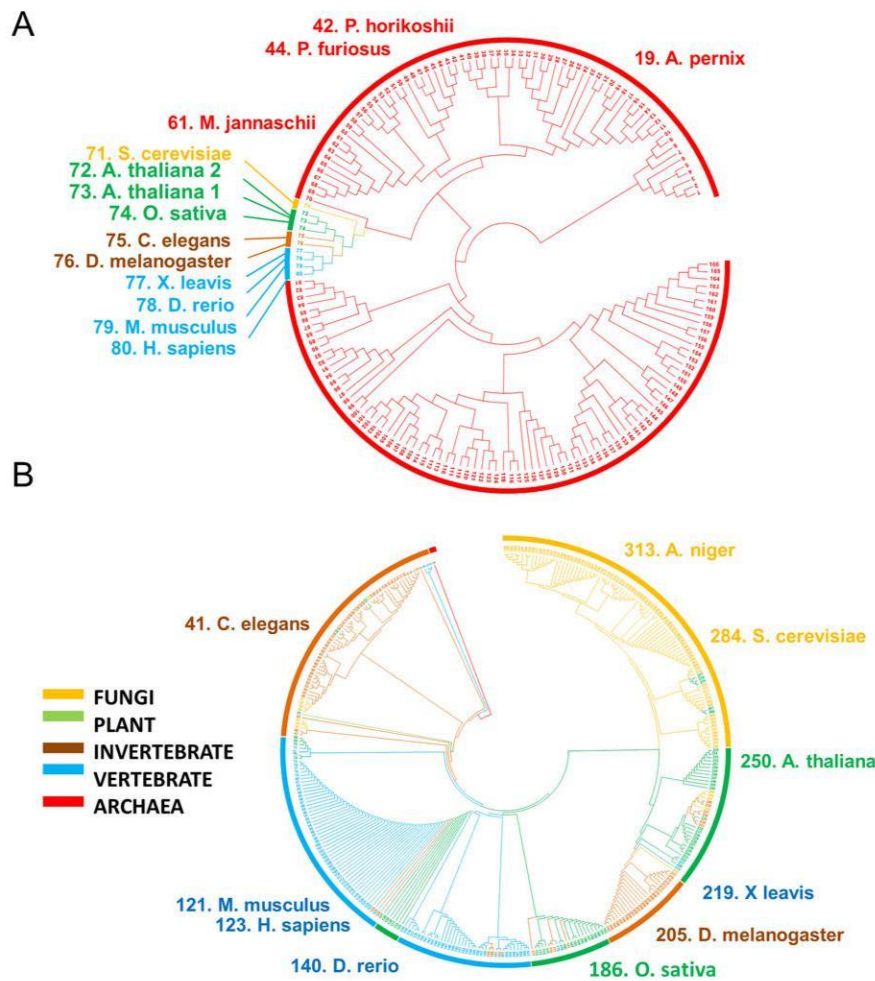
Figure 1.1 Evolutionary relationships of taxa. (**A**) The analysis involved 371 amino acid sequences of complete Archaea fibrillarins and eukaryotic fibrillarins lacking the GAR sequence to be comparable in size and domain composition. There were a total of 21 positions in the final dataset. The tree with the highest log likelihood is shown. (**B**) The analysis involved 212 amino acid sequences of complete eukaryotic fibrillarins. For both analyses, all positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6. CK1 protein was used to root the tree as a non-related protein. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model

The cladogram reveals nine primary branches that separate groups of fungi, invertebrate, plant and vertebrae. The over-all sequences vary significantly within each group. There are the greatest sequence similarities within plants (63%) and within vertebrates (61%), while invertebrates, fungi and Archaea show more diverse sequences (33, 27 and 20%, respectively). The obtained percentage is the sequence similarity between the most distantly located members of each class. Invertebrate fibrillarins are very diverse and several groups are located within branches of vertebrate and plant clads. It remains to define if the differences account for some specific functions. For example, *Xenopus* and human fibrillarins are separated in the two different clads and they have a different complementation level in NOP1 mutants (Jansen et al., 1993). From the sequence analysis, we obtain a particular signature that is unique to fibrillarins, located in the central region of the protein. as shown in Table 1.

Tabla 1 Secuencias firma de fibrilarina. Las secuencias muestran aminoácidos específicos comunes en todas las fibrilarinas. Estas firmas se localizan en el dominio de unión al RNA. Las firmas se obtuvieron mediante el software Pratt-Pattern Matching (http://www.ebi.ac.uk/Tools/pfa7pratt/).

Table 1 | Fibrillarin signatures

Taxa	Amino acid sequence
Archaea	L-Y-L-G-(A/I)-(A/S)-(S/A)-G-T-T-S-H-(V/L/I)-(S/A)-D
Fungi	V-L-Y-(L/I)-G-(A/S/G)- (A/S)-S-G-T-(S/T)-V-S-H-V-(A/S)-D-(L/I/V)-V-G
Plants	P-G-(A/T/S)-(K/R)-V-L-Y-L-G-A-(A/S)-S-G-(T/Y)-T-(V/I)-S-H-V-S-D-(L/I)-V-G
Invertebrate	K-(V/L)-L-Y-L-G-(A/G)-(A/S)-(S/N/T)-G-T-(T/S)-V-S-H-(V/C)-(S/A)-D
Vertebrate	V-(L/M)-Y-L-G-A-A-S-G-T-T-V-S-H-V-S-D-(I/V)-(V/I)-G

Several different families of fibrillarins can be proposed from the phylogenetic study but until more discriminatory biochemical and genetic data are available it would be too premature to do so. Taking into account that x-ray crystallographic data exist on very distinct fibrillarins clads as shown in Figure 1.2, we can see that apparent sequence difference between them only slightly alters the overall structure of the protein. Now it remains to be tested if different conformations and partners are adopted on fibrillarins from different clads.

Fibrillarin structure and domain functions

The fibrillarin protein sequence can be divided into two big domains: the N-terminal domain and the domain with the MTase. In *A. thaliana*, the N-terminal domain is divided into two regions: (1) the GAR domain with around 77 amino acids and (2) a spacer region with 61 amino acids (Figure 1.2A). The GAR domain is responsible for the interaction with different cellular and viral proteins and has a nucleolar retention signal. Snaar concluded that the GAR domain directs the protein to the nucleus and is involved in nucleoli retention. However, for nucleolar localisation it requires the RNA-binding motif (Snaar et al., 2000). Furthermore, this region is not required for localisation of the fibrillarin to the Cajal bodies (CBs). The GAR domain of the human fib-rillarin and the *Arabidopsis* fibrillarin is completely necessary for nuclear localisation (Pih et al., 2000; Levitskii et al., 2004) and is methylated on several arginine residues. Fibrillarin has been shown to be a substrate for arginine methylation by protein arginine *N*-methyltransferase 1 (PRMT1) and the methylated residues correspond to 45% of the total fibrillarin arginines (Lischwe et al., 1985). The methylations may promote specific binding with some proteins such as survival of motor neuron 1 (SMN1).

The MTase domain is divided into two regions: (1) the R or central region with 87 amino acids and (2) a region of 95 amino acids rich in *α*-helix structures. Inside the R region, there is the characteristic RNA-binding motif GCVYAVCF specific of proteins that bind RNA (Aris and Blobel, 1991). Rakitina et al. (2011) showed that the sequence GCVYAVCF is not completely necessary for the interaction with RNA. Using different constructs of a mutant *A. thaliana* fibrillarin 2, two additional regions for RNA binding were found. One is inside the R region between the amino acids 138 and 179, and the other one is in the region rich in *α*-helix structure between the amino acids 225 and 281. Both RNA-binding sites work independently and can interact with various RNAs; moreover, the deletion of either of the two regions has no negative effect on the RNA binding, but there is a synergistic effect when both are present as shown by the high Hill coefficient. The C-terminal end is characterised by the conserved structure composed of seven *β*-sheets and seven *α*-helix, and three conserved amino acids that surround the AdoMet-binding region (Deng et al., 2004). This region of the fibril-larin also interacts with Nop56 protein (Lechertier et al., 2009). Furthermore, amino acid residues of the MTase catalytic triad of

Arabidopsis fibrillarin 2, K138/D231/K260, reside within the R-(K138) and *α*-rich-(D231 and K260) RNA-binding sites. Yanagida et al. (2004) demonstrated that the GAR and spacer region of human fibrillarin interact with the splicing factor 2 associated p32 and the MTase domain interacts with PRMT5. Fibrillarin interacts with both PRMT1 and PRMT5 on different sites, which reflects the complexity of the methylation of its GAR domain or the possibility that it could also involve the process of protein methylation by one of these other enzymes while bound to fibrillarin (Yanagida et al., 2004).

In yeast, NOP1 was characterised by the Tollervey group, which obtained temperature-sensitive mutants and showed that different functions of NOP1 are controlled by different sites of the protein. NOP1 mutations in several positions (D186G, D223N, D263G, K138E, S257P and T284A) were found to be defective in the processing of 35S pre-RNAr. Mutations in positions V87G, E103G, A175V and P219S inhibit nucleolar methylation of the 35SRNAr. Mutations in positions E198G and A245V limited conformational changes of pre-ribosomal subunits. We would expect that point mutations in NOP1 protein that affect methylation and ribosome assembly would have conserved amino acids. However, the mutations in NOP1 protein affect pre-RNAr cleavage, which include S257, D263, T284 differ in other fibrillarins such as *Arabidopsis* fibrillarin 1 corresponding to A234, T240, A261, respectively, and in At-fibrillarin 2 to A245, S251, A272. Hence, the difference in key amino acids in plant fibrillarin can affect complete functional complementation in yeast (Barneche et al., 2000). Detailed structure was determined for a handful of fibrillarins, which provided information about the mechanism of RNA methylation and complex formation. We compare the known structures of fibrillarins from different organisms. The composite (Figure 1.2) reveals that fibrillarin structure is well conserved from Archaea to humans. The information taken from Molecular Modelling Database of the National Center of Biotechnology Information contains the crystal structures of the human fibrillarin in complex with S-adenosyl-L-homocysteine (unpublished data by Plotnikov's group, http://www.thesgc.org/structures/2ipx), the *Aeropyrum pernix* fibrillarin in complex with the SAM (de Silva et al., 2012), the fibrillarin in complex with the Archaea protein Nop56/58 (Oruganti et al., 2007), the hyperthermofilic Archaea *Pyrococcus furiosus* fibrillarin (Deng et al., 2004), the hyper-thermofilic Archaea *Pyrococcus horikoshii* fibrillarin (unpublished data by Boisvert, D.C. and Kim, S.H.; http://pdbj.org/emnavi//quick.php?id=1g8a), the

Methanococcus jannaschii fibrillarin (Wang et al., 2000), the fibrillarin in complex with the Nop5 protein of *S. solfataricus* (Ye et al., 2009) and with the small nucleolar ribonucleoprotein (snoRNP) of *S. solfataricus* (Ye et al., 2009). Surprisingly, the structures share an impressive level of structural conservation. The lessons learned from the X-raya data postulate an interesting mechanism of action of the methylation of RNA by forming a dual complex with four fibrillarins interacting at distinct times with the guide RNA in order to methylate different regions in RNAr (Lapinaite et al., 2013). The detailed information about the catalytic site of the enzyme has been also obtained. However, several questions can be raised with regard to the actual conservation of the fibrillarin structure. While most of the tested fibrillarins retain a similar shape, the human counterpart and the only tested eukaryotic fibrillarin starts at position 93 and therefore the GAR domain is missing, so it resembles the archaeal fibrillarins (Figure 1.2A). The lack of this part in the protein is significant for interpretation of other protein–protein interactions with eukaryotic fibrillarins. Since the information on how the human fibrillarin was crystallized has yet to be published, it remains to be seen if the structure is so well conserved.
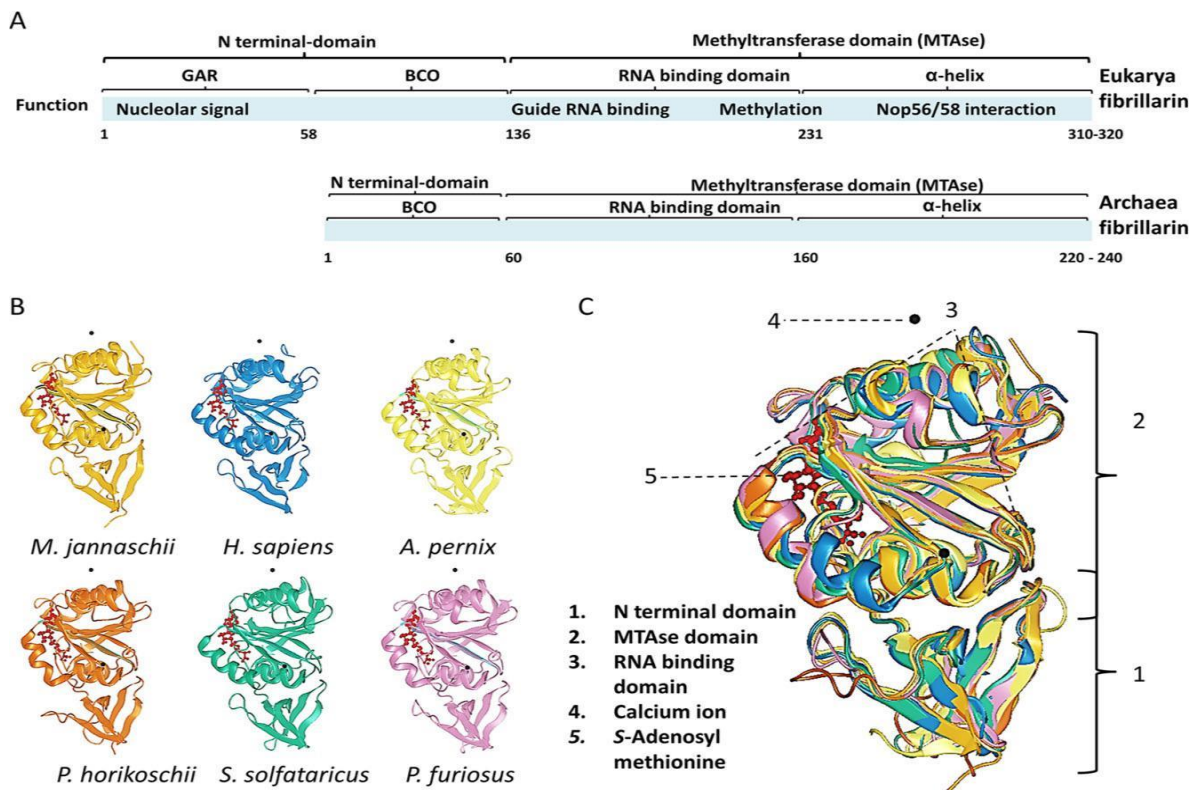
Figure 1.2 Structural alignment of different fibrillarin. (A) Representation of the primary structure of the Archaea and Eukarya fibrillarin. The fibrillarin sequence is divided in four regions: The GAR domain is a sequence rich in glycine and arginine. BCO: a sequence with undefined activity. The methyltransferase domain contains the enzymatic activity as well as a conserved RNA binding sequence. This domain can be subdivided into RNA binding domain and the α-helix region that interacts with Nop56/58. The average amino acid position of each domain is given below the bar. **(B)** Six crystal structures of the fibrillarin from different organism were compared: In orange is from Pyrococcus horikoshii (protein data base ID: 1G8A); in dark yellow from Methanococcus jannaschii (protein data base ID: 1FBN); in blue from Homo sapiens (protein data base ID: 2IPX); in light yellow from Aeropyrum pernix (protein data base ID: 4DF3); in purple from Pyrococcus furiosus (Protein Data Base ID: 1PRY); in green from Sulfolobus solfataricus (Protein Data Base ID: 3ID6). **(C)** The six crystal structures of fibrillarin were aligned to visualise the overlap of structures. Protean 3D was used to perform the structural alignment of fibrillarin with rigid-body alignment (TM-aling). The localisation of the calcium ion and the S-Adenosyl methionine are shown as well as the domain regions.

Post-transcriptional modifications by fibrillarin

Fibrillarin-specific complex is directly involved in different post-transcriptional processes such as pre-RNAr cleavage, RNAr methylation and ribosome assembly (Tollervey et al., 1993). Methylation of RNAr is carried out in more than 100 sites, with some variation depending on the organism. However, for all these sites fibrillarin is the main candi-date for methylation. The low RNA affinity of aFIB requires L7Ae protein to form a complex for facilitating RNA guide binding followed by interaction with Nop5. However, in other organisms, the complex of Nop56, Nop58 and 15.5K can direct methylation. This complex belongs to a family of similar complexes called snoRNP (and small ribonucleoprotein in Archaea) (Dunbar et al., 2000; Reichow et al., 2007; Lechertier et al., 2009). The snoRNP uses a small nucleolar RNA (snoRNA) guide to base pair with the target RNA molecule that will be modified. Each snoRNP has between 70 and 600 nucleotides and its own associated proteins. There are two main types of snoRNP, one with the C/D box having the methylation function on RNAr and a second with the H/ACA box having the

pseudouridylation function. The methylation guide snoRNA has the C box (RUGAUGA, R is purine) near to the 5 end and the box D (CUGA) near to the 3 end. The boxes C and D form a kink-turn. The guide snoRNA contains also the sequence of 10 to 21 bp complementary to the methylation site of the target RNA; the methylation takes place on the fifth nucleotide upstream to the D box (Cavaille et al., 1996; Kiss-Laszlo et al., 1996). Generally, the snoRNAs are encoded either by their own genes or by intronic sequences of the genes coding NOPs related to the biogenesis of ribosomes such as nucleolin and fibrillarin. For a review on the subject, we recommend the study of Bratkovic and Rogelj (2014).

The methylation complex is composed of aFIB, Nop5 and L7Ae protein in Archaea. In eukaryotic organisms, the paralogous proteins Nop56 and Nop58 replace the Nop5 and the protein 15.5K replaces L7Ae. Recombinant proteins have been used to show fibrillarin interaction with Nop5 as a first step followed by interaction with L7Ae protein that binds the RNA guide at an earlier step (Motorin and Helm, 2011). The N-terminal domain of Nop5 interacts with aFIB and the C-terminal domain of Nop5 binds to L7Ae protein to create an active complex with the aid of the guide RNA. The small RNAs known to interact with fibrillarin are U3, U8, U13, U14, U60, x, y, snR3, snR4, snR8, snR9, snR10, snR11, snR30, snR189 and snR190 (Schimmang et al., 1989; Fournier and Maxwell, 1993). The activity of the complex has been tested on recombinant proteins using an aFIB (Omer et al., 2002) and NOP1 on genetic yeast assays (Tollervey et al., 1993). From the multiple methylations that are carried on RNAr, no particular methylation seems to correlate with a specific function. Apparently several methylations are necessary to affect the ribosome architecture and function (Basu et al., 2011). Up to date, the role of individual methylation is still unknown; however, knockout studies indicate that incorrect methylation of RNAr is associated with a modified phenotype of the cell (Newton et al., 2003; Amsterdam et al., 2004; Marcel et al., 2013).

Recently, histone H2A glutamine methylation has been shown to be carried out by fibrillarin. This modification is specific for the nucleolus where the highest concentration of fibrillarin is observed. H2A, methylated in Q105 in yeast and Q104 in human, is the first epigenetic histone modification found only in the nucleolus and playing a possible role in

nucleolar architecture involving FACT as a chromatin remodeler (Tessarz et al., 2014).

In multicellular organisms, the role of fibrillarin-mediated methylation has been studied using fibrillarin knockdown in the mouse model. Native fibrillarin was substituted by one that lacked the MTase domain and the N-terminal domain. The result was a protein only with the GAR region. The homozygous knockdown embryos showed massive apoptosis and did not develop, unlike heterozygous knockdown animals that did not show any apparent defect. In a second generation of animals descendent from the heterozygous first generation, the proportion of homozygous animals without the mutations was higher than the heterozygous animal population. Therefore, some heterozygous embryos with likely reduced fibrillarin levels did not develop (Newton et al., 2003). Fibrillarin has also been shown as an essential gene for zebrafish during embryonic development as identified by insertional mutagenesis (Amsterdam et al., 2004). Furthermore, in plants the reduction in fibrillarin levels using RNAi showed a dwarf apoptotic phenotype when the levels of fibrillarin reduced more than 90% and no effect on phenotype in plants with a lower fibrillarin reduction (Kim et al., 2007).

Fibrillarin localisation and cell cycle

Fibrillarin such as other nuclear proteins (alternative splicing factor/splicing factor 2 (ASF/SF2), high mobility group protein 17) is highly dynamic, likely due to the flux of molecules required to fuel the ribosome biogenesis process. The dynamic studies of fibrillarin have been carried out by tagging the protein with green fluorescent protein followed by fluorescence recovery after photobleaching experiments. The observations with green fluorescent protein–fibrillarin showed a rapid exchanged between the fibrillarin in nucleoli and nucleoplasm, also showing slightly different kinetics depending on the location of the fibrillarin (Phair and Misteli, 2000; Snaar et al., 2000). Under these conditions, fibrillarin molecules are present in CB and nucleoli only for a short time. This argues against a simple localised methylation ac-tivity of RNAr processing and as suggested by Misteli group, it could indicate that fibrillarin may roam the nucleus in search of specific binding partners (Phair and Misteli, 2000).

The abundance and localization of fibrillarin during mitosis has also been studied in detail

in several models (Amin et al., 2007; Hernandez-Verdun et al., 2013). During the interphase, fibrillarin is localized in the DFC of the nucleolus and its concentration can double from G1 to G2 (Cerdido and Medina, 1995). Upon entering prophase, concomitantly to the chromatin condensation, rDNA transcription and RNAr processing are shut down and the nucleolus starts disintegrating. Fibrillarin together with components of the processing complex such as pre-RNAr, nucleolin, U3 and U14 snoRNAs are relocated to the chromosomal periphery, where it forms part of the perichromosomal sheath (Medina et al., 1995) or perichromosomal compartment (Angelier et al., 2005). Fibrillarin has been also found to be dispersed in cytoplasm of mitotic cells, which means that part of processing complexes are disassembled and can be targeted for degradation at this time. In telophase, before entering the reassembling nucleoli, fibrillarin is localized in the processing complex components from pre-nucleolar bodies (PNBs) (Medina et al., 1995). Interactions detected between proteins of the same RNAr processing machinery in both PNBs and nucleoli suggest that PNBs are pre-assembly platforms for RNAr-processing complexes (Angelier et al., 2005). Then, PNBs become associated with nucleolar organizer regions (NORs), which represent rDNA bound to components of the transcriptional complex such as upstream binding factor (in vertebrates) and RNA polymerase I. This association is temporally regulated and fibrillarin is the first one of the early processing factors that leaves PNBs to NORs (Leung et al., 2004). It has been demonstrated that the already restored active rDNA transcription is necessary for the recruitment of RNAr processing factors (Benavente et al., 1987; Azum-Gelade et al., 1994; Sobol et al., 2013); however, during *Xenopus laevis* embryogenesis, the presence of 'maternal' pre-RNAr is essential and sufficient for this recruitment (Verheggen et al., 2000). On the other hand, it has been proposed that kinases and/or phosphatases en-gaged in the transition from mitosis to interphase can regulate the initial recruitment of fibrillarin to NORs prior to rDNA transcription initiation (Dousset et al., 2000). In this case, the nucleolar assembly after mitosis is temporally and spatially orchestrated by the active mechanism of protein phosphorylation rather than an indirect effect of activation of pol I transcription (Dousset et al., 2000; Leung et al., 2004). Nevertheless, it is clear that PNBs are indispensable for the initial accumulation and/or pre-assembling of the components of the RNAr processing machinery before their sequential association with NORs.

Involvement of fibrillarin in viral infection

Several viruses (umbraviruses, Influenza A, HIV, etc.) with a nuclear phase interact with proteins localized in the CB and nucleoli for their replication and transport inside the cell. Fibrillarin dynamics between the CB and the nucleoli can be one of the reasons why this protein is targeted by several viruses. Among them, the nut rosette virus belongs to the family of umbraviruses that encodes ORF3 protein. Fibrillarin interacts directly with ORF3 through the lysine-rich domain of ORF3 and the arginine-rich domain of the fibrillarin, followed by shuttling this viral protein between CB and nucleolus (Kim et al., 2007). Two stages of the umbravirus life cycle suggest in-volvement of fibrillarin, which is redistributed to the cytoplasm and participates in the formation of viral ribonucleoproteins able to move through the plant phloem resulting in complete infection of the plant (Kim et al., 2007). Therefore, the interaction bet-ween plant viral nucleolar antigens and fibrillarin in the nucleolus is the key of systemic spread of this type of plant virus (Hiscox, 2002; Zheng et al., 2015).

Other known viruses that have the animal cells as a host and interact with fibrillarin have been studied. One example is Influenza A virus subtype H3N2 that causes flu. In this virus, a multi-functional protein (non-structural protein, NS1) inhibits the pre-mRNA processing in the host cell and counteracts cell antiviral responses. The NS1 protein of the human H3N2 virus interacts with fibrillarin and nucleolin via its C-terminal nuclear localization signal 2/nucleolar localization signal. Confocal microscopy has shown that NS1 protein colocalises with nucleolin in nucleoplasm and nucleolus and with B23 and fibrillarin in the nucleolus of influenza A/Udorn/72 virus-infected A549 cells. Since some viral proteins contain nucleolar localization signals, it is likely that viruses have evolved specific nucleolar functions (Melen et al., 2012). Another virus that uses fibrillarin is HIV. The HIV Tat protein has been reported to interact with fibrillarin and U3 complex. Tat protein affects the ribosome RNAr maturation and overall amount of 80S ribosome. The impairment of nucleolar pre-RNAr maturation through the interaction of Tat with fibrillarin-U3 snoRNA complex can be involved in the modulation of the host response, therefore contributing to the apoptosis and protein shut-off in HIV-uninfected cells.

Other viruses, like the porcine arterivirus, during their infection cycle have the

nucleocapsid protein colocalised and interacting with fibrillarin (Yoo et al., 2003). Still remains to analyse several more viruses with nuclear phases for possible fibrillarin interaction (Hiscox, 2002). Furthermore, it remains unknown the functional role between fibrillarin and viral proteins.

Fibrillarin as an oncogene

The nucleolus is involved in biogenesis of the machinery necessary for the overall protein translation and eventually cell growth and cell cycle progression (Tsai and Pederson, 2014). The specific alteration in many of the NOPs can result in growth behavior changes or altered cell viability. Fibrillarin is no exception, and it has been shown that fibrillarin is overexpressed in mouse and human prostatic intraepithelial neoplasia that can progress to prostate cancer (Koh et al., 2011a). In human adenocarcinoma, the amount of fibrillarin correlates *in vivo* with the amount of MYC protein, a well characterized oncogene that has been also shown to interact with fibrillarin (Koh et al., 2011b; Miller et al., 2012). The GeneAtlas U133A data show the tissue-specific pattern of fibrillarin mRNA expression in several tissues, and its more than twofold expression in different types of leukemia and lymphoma cells compared to normal cells. There is also high fibrillarin expression in cells like lymphoblasts, and in cells expressing proteins such as cd34, bdca and cd19, which require a high yield of proteins for continuous replication. Furthermore, p53 decreases the expression and protein level of fibrillarin by interacting with fibrillarin intron 1 sequence that contains a p53 regulatory site (Marcel et al., 2013). In breast cancer cells, lack of regulation of fibrillarin caused by p53 level reduction results in an increased level of fibrillarin and higher level of aberrant methylations in RNAr that leads to altered ribosome activity including impairment of translational fidelity, and increased internal ribosome entry site of key cancer genes (Marcel et al., 2013).

The key genes controlling growth and division of cancer cells are early growth response 1 (*EGR1*), *p53* and phosphatase and tensin homolog (*PTEN*), which form a network of regulation (Zwang et al., 2011). PTEN controls the levels of phosphatidylinositol 3,4,5-trisphosphate in cells by dephosphorylation of phosphoinositide into phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2 interaction with fibrillarin results in conformational changes of fibril-larin and affects its RNA binding (Sobol et al., 2013; Yildirim et al., 2013).

EGR1 is often downregulated in human cell lines and cancer tissues that lose cell cycle progression control. Typically, ribosomal protein levels increase in tumour cells and this process is required for tumour progression (Liu et al., 1996; Ponti et al., 2014). Therefore, many aggressive cancers show changes in nucleolar morphology, which could be caused by the increased amount of fibrillarin in these cells.

Fibrillarin is also overexpressed in murine and human breast cancer cells as well as in prostate cancer cells. The treatment with anti-cancer drugs such as ascorbate (vitamin C) and menadione (vitamin K3) known as Apatone has been shown to kill tumour cells by autoschizis in a ratio of 100:1. Because autoschizis entails sequential reactivation of DNase I and DNase II, and because the fibrillarin redistribution following DNase I and Apatone treatment is identical, it seems that the nucleolar and fibrillarin changes are the markers of autoschizis (Jamison et al., 2010). Fibrillarin was also found in low quantities in the FCs and in the nucleoplasm after the treatment with a newly synthesized antitumor complex [RuLCl$_2$]H 4H$_2$O (RAP). RAP has the same antitumor effects as cisplatin that cross-links to DNA and triggers apoptosis (Alderden et al., 2006). The low level of fibrillarin under these treatments reflects the reduced protein expression and cell cycle progression in the treated cells. Combined treatment with ascorbate and menadione exhibits synergistic antitumor activity and preferentially kill tumour cells by autoschizis. Fibrillarin staining shifted from FCs and adjacent regions to a more homogeneously stained of entire nucleolus. This finding was consistent with the percentage of autoschizic cells detected by flow cytometry (Jamison et al., 2010). We can conclude that a specific reduction in the amount of fibrillarin is required to control some types of cancer and its redistribution can mark certain types of cell death.

Fibrillarin and interacting partners

Tollervey in 1993 generated temperature-sensitive yeast mutants for NOP1 and showed that several key points of the ribosome assembly are dependent on NOP1. Mutations in this protein either cause synthesis inhibition of the subunits 18S and 25S or inhibit the nucleolar methylation of the 35S subunit; finally, affecting 60S subunit by an unknown mechanism (Tollervey et al., 1993). Most important, these experiments showed that NOP1

or fibrillarin is essential for cells to survive. Interestingly, the investigated mutations were located in different sites of the protein domains and had different effects on ribosome biogenesis. That suggests that different subsets of interacting partners can be involved. Therefore, this study shows that fibrillarin is a multifunctional protein involved in several processes of yeast ribosome biogenesis. Over the last 20 years, many fibrillarin interacting candidates have been identified. While the majority of fibrillarin is located in the nucleoli and CB during interphase, upon chromosomal condensation and nucleolar breakdown fibrillarin is relocated in the perichromosomal compartment, together with other molecules that can interact with fibrillarin in a cell cycle dependent manner. Moreover, fibrillarin in a low amount can be also located in other parts of the nucleus, and interactions may also depend on the cell growing conditions or the developmental cellular stage. Results from sucrose and glycerol gradients show different sedimentation peaks of fibrillarin, suggesting that fibrillarin is found in more than one complex in the cells (Dragon et al., 2002; Sasano et al., 2008).

The action of fibrillarin in ribosomal production is shown in Figure 1.3 where all the known ribosomal proteins interacting with fibrillarin are shown at each stage of action. The process starts from the methylation of histone H2A that leaves a mark that is recognised by FACT. Then, FACT remodels functional chromatin so that RNA pol I can start transcription. This modification is only found on active rDNA sequences. In yeast, RNA pol I sub-unit RPA49, a non-essential subunit of RNA pol I, interacts in a two-hybrid system with fibrillarin (Krogan et al., 2006). It remains to be tested if the human homolog PAF53 also interacts with fibrillarin and what is the functional significance of this interaction. However, this would indicate a direct link of interaction between RNAr transcription and its processing machineries. The typical fibrillarin interacting partners are Nop56/Nop58, 15.5K and snoRNA. They form snoRNP, which is involved in the methylation of RNAr (Lechertier et al., 2009).

The complex has a mass of 400 kDa that could involve fibrillarin tetramer that changes conformation in order to methylate different regions of the RNAr. Nop56/58 plays an important role in positioning the catalytic subunit on the target RNA by simultaneous contacting the guide RNA and fibrillarin. The snoRNA works as a guide and directs the

precise location of methylation. This mechanism has been described in detail based on the X-ray crystal data obtained from aFIB (Lapinaite et al., 2013). Obviously, it needs to be analysed if eukaryotic fibrillarins behave in the same way and how they are involved in the more than 100 modifications of RNAr that take place in the normal ribosome (Maden et al., 1995).

Among other interacting proteins, p32 and Nop52 interact with fibrillarin at different times but probably at the same binding region. The splicing factor ASF/SF2 binds p32 to regulate the mRNA splicing via inhibition of both phosphorylation and binding of ASF/SF2 to pre-mRNA (Petersen-Mahrt et al., 1999). Nop52 is a human homolog of yeast Rrp1p. It is associated with several distinct 66S pre-ribosomal particles in yeast cells and is involved in late events related to the production of 60S ribosomal subunit. However, Nop52 interaction with p32 competes with fibrillarin binding. Therefore, it is possible that at different stages of the ribosome biogenesis, the interaction of fibrillarin with each one of these proteins is necessary. We can suggest that p32 associates with the pre-ribosomal 90S particles through fibrillarin modifying the ribosome for maturation. In its turn, Nop52 replaces fibrillarin and interacts with p32 initiating the formation of the 60S and 28S ribosomal particles in the granular component (Yoshikawa et al., 2011). In CBs, fibrillarin can interact with the SMN protein. SMN is the protein coded by the spinal muscular atrophy disease gene. The SMN protein is found both in the cytoplasm and in the nucleus where it is concentrated in gems often associated with CB. The interaction between SMN and fibrillarin has been demonstrated using yeast two-hybrid system (Pellizzoni et al., 2001). Pull downs of SMN and its several mutants showed that fibrillarin and GAR1 ribonucleoprotein require a conserved Y/G box, which is found in some spinal muscular atrophy patients (Pellizzoni et al., 2001).
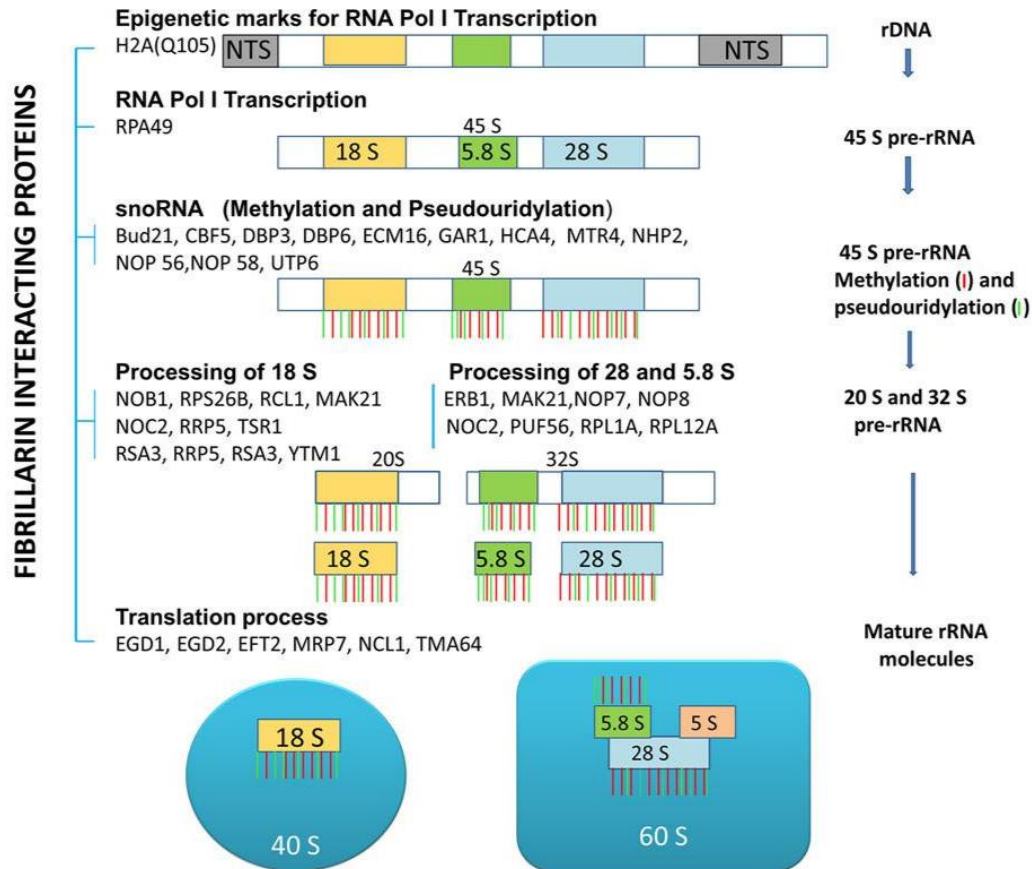
Figure 1.3 Fibrillarin interacting proteins involved at different stages of ribosomal processing. Epigenetic marks of RNA pol I promoter by directly methylating H2A histone at position 105 directs the start of action of fibrillarin. Transcription initiation with the interaction of the subunit of RNA pol I (RPA49) and its action in RNAr processing of methylation and further processing of the 45S pre-RNAr into 20S and 32S pre-RNAr. Finally, it can interact with proteins involved in the translation process.

Gene duplication of fibrillarin in plants can lead to specialised functions. Fibrillarin 2 from *A. thaliana* has been shown to be part of the mediator complex for RNA pol II transcription, something that may be unique for the plant group. Also fibrillarin may interact with RNA pol II basal transcription factors such as transcription factor IIB (Backstrom et al., 2007).

Many other proteins have been detected to interact with fibrillarin in low- and high-

throughput methods (Krogan et al., 2006; Chatr-Aryamontri et al., 2013). Figure 1.4 shows some of the possible processes in which the partners of fibrillarin are involved. The functional roles were segregated for a preliminary selection but proteins can have also additional functional roles inside a cell. Not surprisingly, most of the proteins are involved in the RNAr biogenesis and RNAr maturation processes. The early known temperature-sensitive fibrillarin mutants could have an effect on the interaction with some of these proteins and therefore this would explain the earlier phenotypes of ribosome instability when NOP1 is mutated on the amino acids E198G and A245V. Furthermore, other functional interaction can be specific only under a particular cell environment, for example toxic metals such as mercury and aluminium lead to fibrillarin relocation in different cells and can be involved in the autoimmune disease scleroderma probably affecting the degradation by 26S proteosome (Pollard et al., 1997; Chen et al., 2002; Jiang et al., 2014).

Besides protein interactions, the role of other molecules should be studied to understand fibrillarin function. We have shown that fibrillarin can bind to PIP2 for joint interaction inside the nucle-olus and for association with the nascent RNAr for further methylation and processing (Yildirim et al., 2013). Furthermore, the colocalisation of fibrillarin and PIP2 in actively transcribing cells was detected in the DFC region (Sobol et al., 2013).
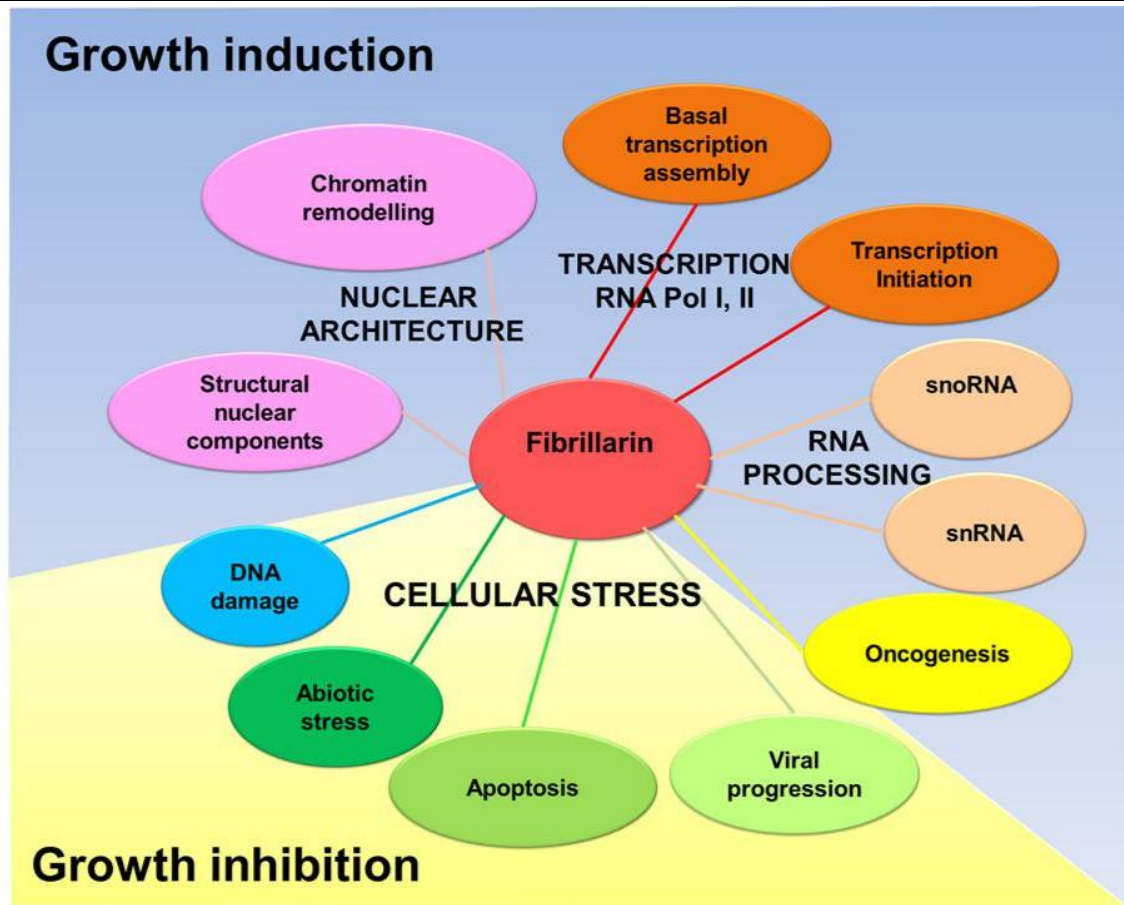
Figure 1.4 Schematic drawing of fibrillarin involved in cellular processes. We show the cellular processes in which fibrillarin interacting proteins could be involved. Depending on the interacting protein and status of the cell some interactions may favour cell growth or growth inhibition. Fibrillarin interactions with proteins involved in these processes can be varied in nature, from stable structural complex formation to substrates for its enzymatic methylation activity in specific cells or environments.

Conclusion

There is very limited evidence that fibrillarin is functionally involved outside the realms of RNAr pro-cessing. However, experiments carried out with the antibodies against fibrillarin at the different steps of mitosis resulted in RNA polymerase I transcription reduction. This study showed the nuclear morphological changes in 40% of the cells with alterations in chromatin condensation. Also, these cells do not progress to G1 phase (Fomproix et al., 1998). One possibility is the genomic instability caused by the absence of fibrillarin due to the formation of R loops, in which the nascent pre-RNAr anneals to the rDNA template

strand. This mechanism of genomic instability has been described with knockouts of the SR protein splicing factor ASF/SF2. The factor promotes recruitment of U1 snRNP to 5 splicing sites but its absence can cause R loops due to the hybridization of nascent RNA with the single-stranded DNA strand bubble after RNA pol II passing (Li and Manley, 2005). Interestingly, fibrillarin also interacts with ASF/SF2 and it is unknown if the absence of fibrillarin can affect ASF/SF2 leading to genomic instability by this factor and the nuclear aberrations that have been reported (Fomproix et al., 1998). This is also in agreement with the data obtained in HeLa cells, where fibrillarin was silenced by RNAi up to 70%. This led to an aberrant formation of the nuclei in 30–45% of the cells before 72 h. The fibrillarin silencing reduced also the cellular growth. The authors proposed that fibrillarin is an important factor in the maintenance of the nuclear envelope and in the cellular growth in HeLa cells (Amin et al., 2007). Although recent experiments were not successful in obtaining the same effect (Tessarz et al., 2014), it is not clear if the observed differences result from the depletion time or the level of depletion. In plants, RNAi experiments on fibrillarin showed that plants with lower than 90% protein level in fibrillarin had extensive necrosis and dwarf phenotype, while 50% reduction had no significant effect on the tobacco plants (Kim et al., 2007).

Fibrillarin is a very dynamic protein that can be involved in different processes inside the nucleus. The methylation activity of RNAr and H2A cannot explain all the processes in which the fibrillarin could be involved. Particularly, interesting is the observation that a group of interacting partners is related to the cell stress mechanism, in particular DNA damage, where little has been investigated. A more dotted pattern of fibrillarin staining was seen in irradiated U2OS human cell line as compared to the control (Foltankova et al., 2013). Upon DNA damage, other molecules such as check point kinase 1 kinase also migrate to the nucleolus and colocalise with fibrillarin (Peddibhotla et al., 2011). Moreover, therapeutic drugs such as RAP can relocate fibrillarin due to the DNA damage, which can indicate some additional roles of fibrillarin during DNA repair and apoptosis. Furthermore, fibrillarin, coilin and SMN can also be located at centromeres of human cells when infected by HSV-1 and in cells in which centromeres are damaged (Morency et al., 2007; Sabra et al., 2013).

Fibrillarin has been shown to interact with proteins in several processes as shown in Figure 1.4. However, we should also consider the possibility that many of the interactions do not translate into a stable functional complex formation. It can rather imply that fibrillarin uses these proteins as a substrate for methylation, for example the H2A, or it is itself a substrate for others such as PRMT1. This case scenario could explain the abundance of methylated ribosomal proteins that interact with fibrillarin and at the same time the lack of significant signal of fibrillarin in any particular ribosomal complex outside the nucleoli.

Conflict of interest statement

The authors have declared no conflict of interest.

Antecedentes directos: La fibrilarina como ribonucleasa

En 1990 Kass y colaboradores en un extracto nuclear de células de ratón, llamado S-100, identificaron al snoRNA U3 en complejo con distintas proteínas incluyendo la fibrilarina. Utilizando la región 312 – 1290 del RNAr de ratón transcrito *in vitro*, el cual comprende la región de transcripción externa del RNAr 5´ (5´-ETS), demostraron que el complejo S-100 es capaz de procesarlo. Al incubar al complejo S-100 con anticuerpos específicos contra fibrilarina el procesamiento del RNAr disminuyo significativamente. Ya que es posible inmunoprecipitar el complejo proteico de U3 mediante anti-fibrilarina concluyeron que U3 es esencial para el procesamiento de 5´ETS omitiendo de esta forma a la fibrilarina (Kass et al., 1990).

En 1991 Tollervey y colaboradores mediante Nop1, la cual es funcional y estructuralmente la fibrilarina homologa en levaduras demostraron que es una proteína vital y requerida para el procesamiento del pre-RNAr. Demostraron que la inhibición de fibrilarina deteriora la producción de ribosomas así como disminuye la producción del RNAr. Mediante northen blot evidenciaron que con Nop1 afectada, la ruta para la maduración del pre-RNAr es afectada, particularmente la ruta para la obtención del RNAr 18S (Tollervey et al., 1991). En 1993 nuevamente Tollervey y colaboradores utilizando Nop1 caracterizo mutantes termosensitivas. Mutaciones en los aminoácidos D186G, D223N, D263G y K138E, S257P, T284A (ubicados en el dominio metiltransferasa) son capaces de inhibir la síntesis de los RNAr 18S y 25S, particularmente el 18S. Mutaciones en los aminoácidos V87G, E103G, A175V, P219S inhiben la metilación del ARNr 35S y mutaciones en los aminoácidos A245V y E198G limitan los cambios conformacionales de las subunidades ribosomales. La inhibición de alguna de estas funciones no afecta otra. Concluyeron que la fibrilarina es responsable de la síntesis de ribosomas, el procesamiento del RNAr así como su modificación (metilación) (Tollervey et al., 1993). Algunas de estas mutaciones puntuales en Nop1 implican aminoácidos que se encuentran conservados en las fibrilarinas de *A. thaliana*, por lo tanto, es posible que organismos como *A. thaliana* que expresen más de una fibrilarina tengan funciones complementarias.

Para 2004 Saez-Vasquez aisló el complejo ribonucleoproteico NF D (nuclear factor D) de

coliflor compuesto por 30 proteínas, entre ellas a la fibrilarina y nucleolina, y a los snoRNAs U3 y U14. Determino que el complejo es capaz de interactuar con el DNAr y posteriormente traslocarse al mismo sitio en el pre-RNAr y cortar en el sitio P rio debajo de las secuencias A1, A2, A3 y B localizadas en el espacio de transcripción externa 5´ del pre-RNAr, sin embargo no fue asociada en particular alguna proteína (Saez-Vasquez et al., 2004).

En 2017 Sharma y colaboradores silenciaron a la fibrilarina por separado mediante dos RNAi en células HCT116. Este silenciamiento fue progresivo durante tres días durante los cuales se monitorio la presencia de fibrilarina, mediante western blot fue detectada a las 24 horas, de 48 horas en adelante no fue detectada. El gradual silenciamiento de la fibrilarina inicialmente inhibió el corte en los sitios A1 y A0 del 5´-ETS, el sitio 5´ final del RNAr 18S y el sitio 2 de la región de transcripción interna uno (ITS1). Cada uno de estos cortes se demostró por la acumulación del pre-RNAr 47S y 34S. La presencia del RNAr 34S únicamente es detectable cuando hay un deterioro en la correcta maduración del RNAr (Sharma et al., 2017).

En 2014 Tessarz y colaboradores describieron la metilación de la glutamina 105 en humanos y 104 en levadura de la histona H2A mediante la fibrilarina, Nop1. La glutamina modificada se encuentra presente sobre la unidad transcripcional del DNAr 35S, es especifica al nucléolo y forma parte del sitio de unión al facilitador de transcripción de la cromatina, FACT por sus siglas en inglés (Tessarz et al., 2014).

Los antecedentes mencionados involucran directamente a la fibrilarina en los sitios de procesamiento del pre-RNAr, sin embargo, ninguno de ellos ha comprobado que la fibrilarina sea la responsable de llevar a cabo dicha actividad. Es evidente que la fibrilarina es una proteína de vital importancia para la maduración del pre-RNAr. En el presente estudio la fibrilarina fue clonada, expresada y purificada logrando así determinar su actividad como ribonucleasa. Así mismo se verifico si en plantas la histona H2A se encuentra metilada en la posición antes descrita y si la fibrilarina de *A. thaliana* se encuentra involucrada.

JUSTIFICACIÓN

Como se puede observar la fibrilarina se encuentra involucrada en diversos procesos dentro del nucléolo celular, sin embargo, no es del todo comprendido cuál es su papel dentro de las funciones del nucléolo. No se conoce a fondo el mecanismo por el cual la fibrilarina lleva acabo el procesamiento del RNAr o como es que está involucrada en el desarrollo celular o la morfología del núcleo. La actividad única como metiltransferasa de la fibrilarina no explica todos los procesos en los cuales se ha involucrado a la proteína y es posible que tenga otro tipo de actividad. Parte de esta incógnita se debe a que aún no se conocen todas las moléculas que pudieran estar involucradas en la formación y función del núcleo y nucléolo, por lo tanto, gran parte de las interacciones y la señalización en este compartimento celular no es percibida aun. Así mismo la idea que distintos fosfolípidos ejerzan cierta función cuando no se encuentran anclados a una membrana no está del todo comprobada. Se requiere generar nuevas herramientas que nos permitan una visualización más amplia y profunda de todas las interacciones que se llevan a cabo. Es de importancia continuar con el estudio del núcleo y nucléolo así como de las proteínas, conocidas y desconocidas, que lo comprenden ya que gran parte de todos los procesos celulares tienen su origen aquí. Este trabajo se centró en definir a la fibrilarina como una ribonucleasa nucleolar.

HIPÓTESIS

La fibrilarina forma parte del proceso de biogénesis de los ribosomas y ha sido identificada en los sitios de procesamiento del pre-RNAr, por lo tanto, adicional a su actividad como metiltransferasa tiene actividad como ribonucleasa.

OBJETIVO GENERAL

- Evaluar la actividad de la fibrilarina como ribonucleasa.

OBJETIVOS ESPECÍFICOS

- Identificar si la fibrilarina tiene actividad como ribonucleasa.

- Identificar el dominio con actividad ribonucleasa de la fibrilarina.

- Definir el efecto de los fosfolípidos de inositol en la actividad como ribonucleasa de la fibrilarina.

- Definir la actividad como ribonucleasa de la fibrilarina interactuando con el snoRNA U3.

- Comparar la actividad de la fibrilarina de *H. sapiens* con las de *A. thaliana*.

- Determinar si la fibrilarina de *A. thalina* metila a la histona H2A.

ESTRATEGIA EXPERIMENTAL

La estrategia experimental (Figura 1.5) consistió en la clonación, expresión y purificación de la fibrilarina de *H. sapiens* y las fibrilarinas de *A. thaliana* para su posterior evaluación como ribonucleasa. El análisis incluyo generar mutantes de la proteína y la transcripción de distintos tipos de RNA. Mediante el ensayo fat blot se determinó la interacción de fibrilarina con distintos fosfolípidos.
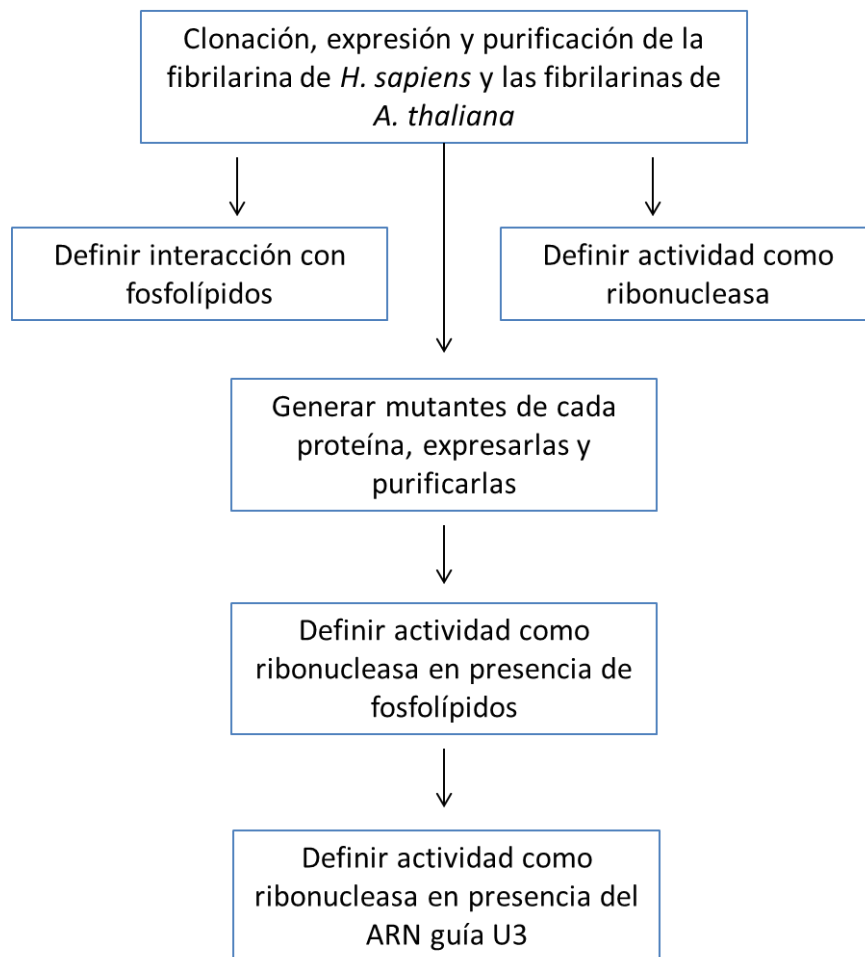
Figura 1.5 Diagrama de la estrategia experimental general empleada para el cumplimie objetivos.

CAPÍTULO II.

En este capítulo, el cual lleva por título "Fibrillarin shows a novel ribonuclease activity in its GAR domain", se demuestra que la fibrilarina de *H. sapiens* tiene actividad como ribonucleasa y que el dominio GAR es el responsable de esta. Para ello se clono, expreso y purifico la fibrilarina y se observó que la degradación del RNAr es directamente proporcional a la concentración de proteína presente. Para definir el dominio responsable de la actividad como ribonucleasa inicialmente se expresaron los dominios N-terminal (HsGB) y metiltransferasa (HsRα) fusionados a GST por separado. Al observar que la actividad como ribonucleasa se encuentra en Hs<u>GB</u> se expresó el dominio <u>GA</u>R y el dominio <u>B</u>CO fusionados a GST por separado definiendo así al dominio GAR (aminoácidos 1 a 59) como responsable de la degradación del RNAr.

Por otro lado, mediante el ensayo fat blot se definió la interacción de la fibrilarina con distintos fosfolípidos entre los que destacan los fosfoinosítidos monofosfato, bisfosfato y el ácido fosfatídico. Fue posible definir que la presencia de dichos fosfolípidos tiene un efecto inhibitorio de la fibrilarina como ribonucleasa, particularmente el fosfatidilinositol 3,4-bisfosfato, el fosfatidilinositol 3,5-bisfosfato y el fosfatidilinositol 3,4,5-trisfosfato, sin embargo permiten la interacción entre la fibrilarina y su RNA guía U3. Por el contrario la interacción fibrilarina-U3 es bloqueada en presencia del ácido fosfatídico, esto se demostró mediante ensayos EMSA. Así mismo la interacción fibrilarina-U3 impide la degradación del RNAr, lo cual sugiere que este RNA dirija el corte a un punto específico en el RNAr el cual no esté presente en el RNAr completamente procesado como el que se usó para estos ensayos.

Con los resultados obtenidos se cumple el objetivo principal el cual es definir si la fibrilarina tiene actividad como ribonucleasa y se sometió un artículo el cual actualmente se encuentra en revisión en la revista PLoS One.

Fibrillarin shows a novel ribonuclease activity in its GAR domain

Rodriguez-Corona U[1]., Pereira A[3]., Sobol M[2]., Rodriguez-Zapata L.C[3]., Aquino C[1]., Yildirim S[2]., Hozak P[2]., Castano E[1]

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Mexico[1], Department of Biology of the Cell Nucleus, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic [2].Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida, Mexico [3].

Abstract

Fibrillarin is a highly conserved nucleolar methyltransferase known to be in charge of ribosomal RNA methylation across evolution from Archaea to humans. Recently, it has been shown that this essential protein is functionally involved in the methylation of histone H2A in nucleoli as well as in other processes including viral progression, cellular stress, nuclear alteration and cell cycle progression. Here we show that fibrillarin has an additional activity as a ribonuclease, which may be regulated by certain phosphoinositides and phosphatidic acid when it is not bound to U3 snoRNA. The formation of a guide complex can help to target fibrillarin to RNA sequence-specific sites for methylation or processing. Furthermore, fibrillarin-U3 snoRNA complex is released in presence of phosphatidic acid, which may allow the formation of new complexes during cell cycle. The ribonuclease activity is associated with GAR domain, which is also involved in the interaction with phosphoinositides.

Introduction

Ribonucleases are involved in several important processes in cells including cytoplasmic and nuclear RNA degradation, RNAi, antiviral defense, DNA synthesis and RNA processing (Houseley and Tollervey, 2009; Bubeck et al., 2011; Arraiano et al., 2013; Deutscher, 2015; Moelling and Broecker, 2015).

Particularly, the maturation of pre-RNAr involves specific RNases and RNA- modifying enzymes (Fatica and Tollervey, 2002; Henras et al., 2015). It is known that pre-RNAr processing is mediated mostly by co-transcriptional association of nascent transcripts with ribosomal proteins (RPs) and small nucleolar ribonucleoprotein particles (snoRNPs) containing fibrillarin (Tschochner and Hurt, 2003).

Fibrillarin is a 2'-O-methyltransferase, which is essential for all eukaryotes. It is and involved in the methylation of RNAr and H2A, ribosome biogenesis, and viral progression among others (Rodriguez-Corona et al., 2015). In particular, fibrillarin has been shown to be involved in the methylation of RNAr in yeasts (Tollervey et al., 1993; Rodriguez-Corona et al., 2015) and histone H2A in plants and humans (Tessarz et al., 2014; Loza-Muller et al., 2015). A smaller version of fibrillarin in archaea has been shown to methylate RNA, but biochemical experiments with eukaryotic fibrillarins are lacking (Peng et al., 2014). Fibrillarin is known to associate with different nuclear and nucleolar RNAs, such as U3, U8, U13, U14, U60, x, y, snR3, snR4, snR8, snR9, snR10, snR11, snR30, snR189, and snR190 (Schimmang et al., 1989; Fournier and Maxwell, 1993), and other proteins to form a variety of snoRNPs. The most studied snoRNP is composed of the small nucleolar RNA (snoRNA) U3, which is involved as a guide RNA in the first steps of the pre-RNAr processing (Kass et al., 1990) and also is essential for the cleavage leading to the maturation of 18S RNAr (Hughes and Ares, 1991). For this early processing, several factors such as fibrillarin, nucleolin and U14 snoRNA are required (Dragon et al., 2002; Granneman et al., 2004; Saez-Vasquez et al., 2004). Furthermore, fibrillarin is also required for dissemination of several viruses in plants and animal cells, since fibrillarin blocking impairs infection by a wide range of paramyxoviruses (Kim et al., 2007).

Fibrillarin is able to interact with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) (Yildirim et al., 2013). Our earlier results showed that fibrillarin is capable of binding to PtdIns(4,5)P$_2$ and that phosphoinositides (PIs) together with DNA and RNA play crucial roles in function and dynamic architecture of the nucleus (Sobol et al., 2013; Yildirim et al., 2013; Hamann and Blind, 2017). The involvement of PIs in the crucial nuclear processes was shown for phosphatidylinositol (PtdIns), which inhibits epsilon DNA polymerase

(Shoji-Kawaguchi et al., 1995), PtdIns(4,5)P$_2$, which regulates activity of Pol I and Star-PAP (Mellman et al., 2008; Yildirim et al., 2013), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$) which mediates the mRNA export (Okada et al., 2008).

Here we show that fibrillarin has a ribonuclease activity for RNAr and this general activity is blocked when fibrillarin is bound to the U3 snoRNA. The ribonuclease activity is associated with G/R rich domain (GAR) of the protein, same domain responsible for the interaction with different PIs. We show that phosphatidylinositol 5-phosphate (PtdIns(5)P) increases the ribonuclease activity of fibrillarin, while phosphatidic acid (PA) has an opposite effect. Furthermore, the binding of fibrillarin to the U3 snoRNA appears to be dependent on the absence of PA.

Materials and Methods

Cell Lines, Cell Culture and Transfection Assays. HeLa cells used for the assays were standard Type Culture Collection cell lines. Cells were grown on sterile bottom-glass petri dishes with Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (all from Life Technologies, Carlsbad, California, USA). Cells were cultured at 37ºC in a 5% CO$_2$ atmosphere in standard petri dishes with glass slides or glass bottomed. Transient transfection of HeLa cells was performed at 80% confluence using PEi at 0.06% plus 10 μg of plasmid DNA (pStr-GFP-C2:fibrillarin) in 1 ml of DMSM without fetal calf serum. Transfection cocktails were vortexed for 60 seconds and incubated for 5 minutes at RT. Cocktails were added dropwise to the cell culture. Cells were analyzed by fluorescence microscopy 24-48 hr after transfection. Distribution patterns of wild-type and fibrillarin mutants coupled with GFP reporter were analyzed in 200 cells for at least three separated experiments. Plasmid DNA used for transfections was purified from bacterial cultures using maxiprep columns (QIAGEN, Hilden, Germany).

Cloning. 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 pET-15b vector (Fib_Fwd 5´-CCATGGATGAAGCCAGGATTCAGTCCCCGTG–3´; Fib_Rev 5´-GGATCCTCAGTTCTTCACCTTGGGGGGTGGC–3´). For protein expression in HeLa cells, fibrillarin sequence was cloned into XhoI and BamHI sites of pEGFP-N1 vector. The

N-terminal extreme (aminoacids 1 to 134) containing the GAR domain of fibrillarin was amplified from pET15b:HsFib vector (GB_forward 5´ - 3´ CCATGGATGAAGCCAGGATTCAGTCCC; GB_reverse 5´ - 3´ CTCGAGGTACTCAATTTTGTCATCTCCTTCC) and cloned into NcoI and XhoI sites of pET42b vector. Once cloned into pET42b, small fragments from fibrillarin were obtained by complete plasmid PCR with the following primers: BCO (Forward 5´ - 3´ AGAATGTGATGGTGGAGCCGCA, reverse pET42b 5´- 3´ CCATGGACCCGCGTCCCTCAA); HsGAR domain (Forward pET42b 5´- 3´ CTCGAGCACCACCACCACCA; reverse 5´- 3´ TCTTCCTCCTCCTCCACCGCC); miniGAR (Forward pET42b and reverse 5´- 3´ TCACCAAAGCCCCCTCGGCC).

Recombinant protein expression and purification. Fibrillarin was expressed individually in Escherichia coli BL21 gold competent cells induced with 1 mM isopropyl -D-1-thiogalactopyranoside at 25 °C for 5 hours. Harvested cells were suspended 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 by sonication. After clarification by centrifugation (17400 g x 15 minutes), the supernatant was loaded onto a Ni-NTA agarose column (Thermofisher, Waltham, Massachusetts, USA) and washed 3 times with the extraction buffer and eluted with a linear gradient from 70 to 200 mM imidazone in BC buffer and revised by SDS-PAGE 15%. Elution with most of the protein was passed through MonoQ sepharose (Amersham Pharmacia). Fibrillarin fractions were pulled dialyzed in BC-100 and 0.1mM AEBSF. After MonoQ sepharose, fraction with most protein was loaded on Mono S (Amersham Pharmacia) and eluted with a linear gradient from 0.1 to 0.5 M KCl in BC buffer with 0.1mM AEBSF. Purity of proteins was checked by SDS-PAGE 15% followed by silver stained. For peptides cloned into pET42b vector the supernatant was first load into Ni-NTA agarose column followed by loading into glutathione-sepharose column and eluted with BC buffer with 10 mM of reduced glutathione with 0.1 mM of AEBSF.

Fibrillarin complex purification from HeLa cells. HeLa cells were transfected with pStr-GFP-C2:fibrillarin and lipofectamin 2000 as stated by the protocol of Thermo Fisher Scientific (Waltham, Massachusetts, USA). Nuclear extract was according to Schreiber

method (Schreiber et al., 1990). HeLa cells were pre-incubated for two days after transfection. The cells are then washed with PBS, dislodged and pelleted by centrifugation. Resuspension in lysis buffer (10 mM HEPES; pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Nonidet-40 and 0.5 mM PMSF along with the protease inhibitor cocktail (Sigma)) The cells were allowed to swell on ice for 20 min. Tubes were vortexed and centrifuged at 12,000 g at 4°C for 10 min. The pelleted nuclei were washed with lysis buffer and suspended in nuclear buffer containing 20 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF with protease inhibitor cocktail for 30 min on ice. Nuclear extract was collected by centrifugation at 12,000 g for 15 min at 4°C. Nuclear extracts were then allowed to interact with Strep-Tactin resin and allowed to bind for 30 minutes at 4°C. After incubation the resin was washed 5 times with nuclear buffer and finally elution was carried out nuclear buffer containing 2.5 mM desthiobiotin. The elution's containing fibrillarin were frozen and stored at -80°C until required for the activity assays.

Western blot assay. Fibrillarin was loaded in 12% acrylamide gel to perform a SDS-PAGE. Subsequently protein was transferred to a nitrocellulose membrane and blocked with 3 % of BSA in PBS at room temperature for 1 hr. After blocked, membrane was incubated with anti-Fib antibody (1/3000) (Santa Cruz Biotechnology. H-140, rabbit polyclonal) for one hr at room temperature and secondary antibody (1/5000) one hour at room temperature, with three washes of 10 minutes each with PBS-T between incubations. Immunoblotting signals were analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences, Lincoln, Nebraska, USA). For small peptides primary antibody was anti-6xHis (1/5000) (abcam, ab18184, mouse monoclonal)

Fibrillarin mutagenesis. Mutagenesis of fibrillarin sequence was made with Thermo Scientific Phusion Site-Directed Mutagenesis Kit (Waltham, Massachusetts, USA) using specific primers for each mutation. For bacteria expression, the mutation of the sequence were made from vector pET15b:fibrillarin. For transfection the mutations were made from the vector pSTR - EGFP –C2: fibrillarin. Same primers were used for mutation of both vectors. R34A (fwd 5´ TTTGGCGGGGGCGCGGGTCGAGGCGGA 3´, rev 5´ GCCCCCTCGGCCTCCACGAC 3´), R45A (5´ AGGTCGTGGAGCGGGAGGAGGTG, 3´,

rev 5´ CTAAAGCCTCCGCCTCGACC 3´).

RNA preparation. Total RNAr was extracted from HeLa cells culture using the commercial kit GenElute™ Mammalian Total RNA Miniprep (Sigma-Aldrich, San Luis, Misuri, USA). snoRNA U3 was cloned into the pGEMT-easy vector and in vitro transcribed on sense by T7 RNA polymerase and on antisense by SP6 RNA polymerase (New England BioLabs, Ipswich, Massachusetts, USA).

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, gel was washed for 10 minutes with buffer I (10 mM Tris-HCl, 20% isopropanol, pH 7.5) and consequent incubation for 30 min in buffer II (10 mM Tris-HCl, pH 7.5) and buffer III (100 mM Tris-HCl, pH 7.5). Gel was resolved with 0.2% of toluidine blue and washed with water until activity band was visible (Dudkina et al., 2016).

Ribonuclease assay. Total RNAr extracted from HeLa cells was mixed with fibrillarin on BC200 buffer (20 mM Tris-HCl buffer, pH 8, 200 mM KCl, 0.2 mM EDTA, 10% glycerol), incubated for 1 hour at 37 ºC and then loaded in 1% agarose gel. The same procedure was performed for the snoRNA U3.

Phospholipid strip assay. PIP strips were used for the fat blot assay (P-6001, Echelon-Inc, Salt Lake City, Utah, USA). The assay was made according to the manufacture instructions with 0.4 µg of protein.

Ribonuclease assay in fixed HeLa cells. Cells were fixed in 4% formaldehyde solution in PBS for 15 mins and washed once in PBS. Then cells were permeabilized in PBS, 1% BSA, 0.1% Triton X-100 for 5 minutes and washed once with PBS. RNA was stained with pyronin Y for 5 minutes at room temperature and washed 3 times with PBS. All proteins tested were added in BC200 buffer, incubated for 1 hour at 37 ºC and then washed 3 times with PBS. Coverslips were picked and mounted on a slide; MOWIOL/DAPI was added and incubated for 10 minutes at room temperature. All measurements were acquired using

Leica DM6000B (LEICA TCS SP5 AOBS tandem, Germany) confocal fluorescence microscope. The excitation wavelength was 555 nm.

Fluorescence Recovery After Photobleaching. FRAP experiments were performed on a Delta Vision OMX Super resolution microscope. Cells expressing fibrillarin-GFP were grown for live cell analysis, exchanging DMSM for warm DPBS medium right before the experiment. Dishes were placed inside a temperature-controlled chamber on the microscope stage under the objectives. Images were obtained using a sCMOs camera, using the 60X oil 1.42 objective with fluorescence free immersion oil 518F. One CB or one nucleolus was selected per nucleus for each photobleaching experiment. The experiment consisted in 9 s before photobleaching the ROI, then monitoring fluorescence recovery trough 110 s, making each experiment last for 120 s, acquiring 120 images per stack. Photobleaching removed ~99% of total fluorescence in both nucleolus and CBs. Image processing of recovered fluorescence in bleached regions was performed with the software ImageJ® in order to acquire half-life ($\tau 1/2$) values plus mobile and immobile fractions; software R was used to carry out quantitative and statistical analysis. Estimation of the Diffusion coefficients (D) for wild-type and mutants was carried out as described by Chen & Huang (2001) (Chen and Huang, 2001).

Bioinformatic analysis. Sequences selection for the analyses and Hidden Markov Models (HMM) used to detect GAR domain. Complete proteomes of 35 chordate species and Saccharomyces cerevisiae were used to retrieve GAR containing proteins. To identity GAR containing proteins, HMMs were constructed and calibrated from the alignment of 250 sequences retrieved by PSI-Blast (Altschul and Koonin, 1998) against the Refseq-protein database. The fibrillarin (FIB; AAP36189.1) and hGAR1 (Q9NY12.1) proteins sequences from *H. sapiens* were used as queries against Chordata organisms database (taxid:7711). Fibrillarin and GAR1 sequences retrieved by PSI-Blast, with three iterations, were aligned independently by MUSCLE (Edgar, 2004) and manually curated. The constructed HMM model for fibrillarin protein retrieved by PSI-Blast, excluding the GAR domain, and using only the BCO region, the RNA-binding domain, and the a-helix region (Rodriguez-Corona et al., 2015). This model was used to detect fibrillarin proteins from the 36 complete genomes analyzed in this work and subsequently, fibrillarin proteins were

retrieved by EMBOSS 3.0 suite (Rice et al., 2000). All the fibrillarin sequences were subjected to a motif scan in the Pfam database v30.0 (Finn et al., 2014) to confirm the presence of the fibrillarin domain (PF01269) and each sequence was subjected to BLASTp search against nr database of the National Center for Biotechnology Information (NCBI). A 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. Two independently HMM models were constructed for the RGG-box regions of GAR1 protein. The RGG-box1 and RGG-box2 models were constructed from the alignment of the 250 GAR1 proteins retrieved by PSI-Blast (Altschul and Koonin, 1998). These RGG-box HMM models were used to detect RGG-box containing proteins in the 36 complete genomes analyzed in this work. All the HMM models were constructed using the HMMER software package v3.1 (Eddy, 2011).

Phylogenetic reconstructions from the alignment of the GAR region were done by RAxML v8.0.26 (Stamatakis, 2006) with the MtREV+ Γ substitution model and were visualized in FigTree v1.4 (http://www.molecularevolution.org). The substitution amino acid models were calculated with the statistical package ProtTest 2.4 (Abascal et al., 2005). HMM logos of the GAR domain, RGG-box1, and RGG-box2 domains were plotted with the Skylign tool (Wheeler et al., 2014). The amino acid alignments of the selected proteins were visualized by BOXSHADE v3.3.1C (http://boxshade.sourceforge.net/).

Results

Fibrillarin as a ribonuclease

During fibrillarin purification, we found its activity as a ribonuclease by serendipity as we noted that RNA was particularly degraded by fibrillarin unlike other recombinant proteins. Therefore, we decided to investigate if a ribonuclease interacted with fibrillarin. We purified recombinant fibrillarin to homogeneity (Figure 2.1A). Purification of recombinant fibrillarin to homogeneity was carried out after several chromatographic steps as stated in "Materials and Methods". Fibrillarin was identified with the specific antibody (Figure 2.1B). To ensure that ribonuclease activity was attributed to the purified fibrillarin, an in gel activity assay was performed. We found only two bands, where RNA, incorporated into the gel, was

degraded (Figure 2.1C, lane 2). According to the western blot, these two bands corresponded to the whole fibrillarin and the partially degraded fibrillarin, suggesting that only the part of the protein, which is certainly fibrillarin, had ribonuclease activity. RNAse A was used as a positive control. Using the purified fibrillarin, we studied if its ribonuclease activity is dose-dependent. For that, we used the protein of gradually increased concentration from 2 to 8 ng with a constant concentration of RNAr (2 µg). Degradation of RNAr was directly proportional to the amount of fibrillarin added (Figure 2.1D).
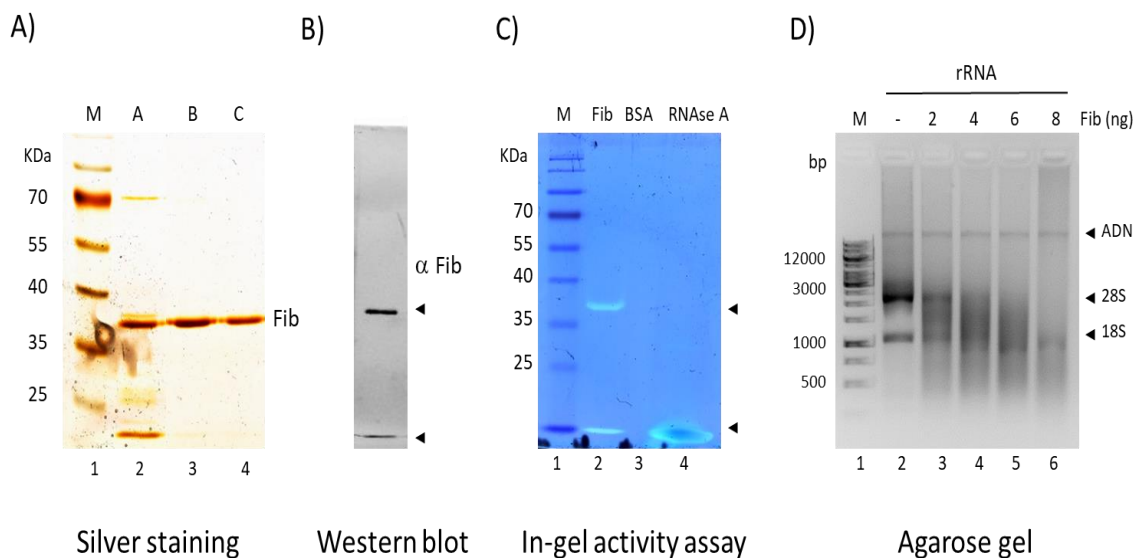
Figure 2.1 Fibrillarin as a ribonuclease. A) Silver stain of purify fibrillarin. M: molecular weight marker. A, B, and C: different fibrillarin elutions. Only the protein purification with the "C"-quality was used for further analysis. **B)** Fibrillarin western blot. Two bands marked by arrows were recognized by the specific fibrillarin antibody. **C)** *In gel* fibrillarin ribonuclease assay. Two signals marked by arrows corresponding to fibrillarin (line 2) appeared after washing. BSA and RNAse A were used as negative and positive controls, respectively (lines 3 and 4). **D)** Ribonuclease fibrillarin assay with the increasing concentration of protein (from 2 to 8 ng) added to a constant concentration of RNAr (2 µg). The level of RNA degradation was directly proportional to the amount of fibrillarin.

Fibrillarin-PIs interaction

We recently reported the interaction between fibrillarin and PtdIns(4,5)P_2 (Yildirim et al., 2013). Moreover, according to the molecular characteristics for PIs binding sites previously described (Rosenhouse-Dantsker and Logothetis, 2007; Morales et al., 2017), fibrillarin has negatively charged and aromatic rings-containing amino acids for binding to more than one phospholipid (Figure 2.2A). Using a phospholipid strip assay, we found that fibrillarin interacts with other negatively charged phospholipids, in particular, with PA (Figure 2.2B). Electrophoretic mobility shift assay with in vitro transcript U3 snoRNA showed that the interaction fibrillarin-RNA in presence of PIs changed the mobility of the transcript (Figure 2.2C): the presence of PA caused that the binding of fibrillarin to RNA was inhibit (Figure 2.2C line 8). The mobility of RNA in presence of PtdIns(3)P, PtdIns(5)P and PtdIns(3,4)P_2 (Figure 2.2C, lanes 5, 6, and 7, respectively) was reduced when compared with the mobility of fibrillarin-RNA without PIs, either due to a charge or a conformational change of fibrillarin bound to RNA, which altered RNA mobility (Figure 2.2C, lane 3). As a control, phenol was added after the incubation to verify that the RNA mobility was affected by fibrillarin. As it was expected, there was no mobility of U3 snoRNA (Figure 2.2C, lane 4).

Besides the studies of complex formation between fibrillarin-U3 snoRNA and fibrillarin-phospholipids, we decided to checked the ribonuclease activity of fibrillarin in the presence of phosphoinositides. As it is shown in Figure 2.2D, the presence of any phosphoinositide, particularly bis and trisphosphate phosphoinositides, significantly decrease the ribonuclease activity of fibrillarin. Calcium ions can be found in the fibrillarin crystal structure and it is well known that calcium activates a group of ribonucleases (Schwarz and Blower, 2014), so we decided to try the ribonuclease activity of fibrillarin by the addition of calcium and PA, as PA is the phospholipid with the major interaction as it is showed in the pipstrip assay. With a low concentration of fibrillarin (1ng) calcium increase its activity and PA inhibit it (Figure 2.2E lanes 3 and 4). With the addition of calcium and PA at the same assay the degradation of RNAr is less than the degradation with calcium without PA (Figure 2.2E lane 5).
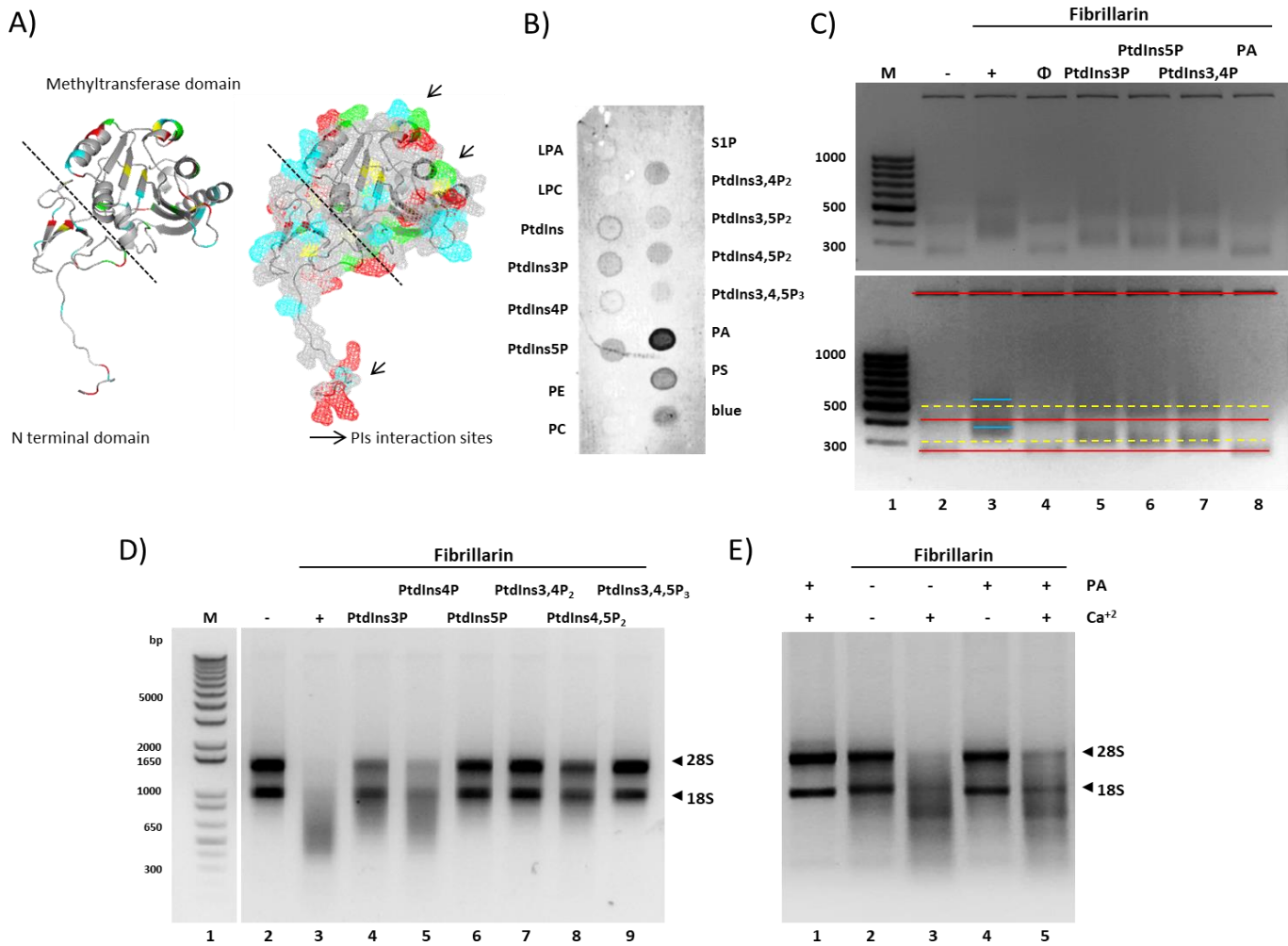
Figure 2.2 Fibrillarin-phosphoinositides interaction. A) Fibrillarin structure with characteristic aminoacids for PIs binding. According to the literature (Rosenhouse-Dantsker and Logothetis, 2007; Morales et al., 2017) from 3 to 5 amino acids are necessary for PIs binding. These amino acids should be positively charged and at least one with an aromatic ring. Red: arginine, blue: lysine, green: histidine, yellow: tyrosine. **B)** Fibrillarin phospholipid strip assay. Fibrillarin is able to interact with most of PIs, however, the highest interaction is revealed with PA. **C)** Electrophoretic mobility shift assay carried between fibrillarin and U3 snoRNA with different phospholipids tested. Three PIs used (Ptdins(3)P, PtdIns(5)P and PtdIns(3,4)P$_2$) altered the mobility of U3 snoRNA (lanes 5, 6 and 7) compared with the mobility without any phospholipid (lane 3). PA, interaction with fibrillarin causes the release of snoRNA (lane 8).

Lane 4 corresponds to a sample treated with phenol after incubation time, to eliminate the mobility due to fibrillarin. Blue lanes indicate the position of snoRNA and fibrillarin. Yellow lines indicate the position of snoRNA with fibrillarin with PI. Red lines show the mobility of snoRNA U3 alone. **D)** Fibrillarin ribonuclease assay in phosphoinositides presence. The addition of phosphoinositides, particularly bis and trisphosphate phosphoinositides, significantly decrease the ribonuclease activity of fibrillarin. All the assays were made with a final concentration of phosphoinitides of 50 ng/µl. **E)** Fibrillarin ribonuclease assay with PA and calcium. In presence of calcium, fibrillarin ribonuclease activity increases significantly (lane 3) and reduced with the addition of PA in a 20% (lane 5).

GAR domain, modular in fibrillarin

According to the structure of fibrillarin (Rodriguez-Crorona et al., 2015). we analyzed the N-terminal region (Hs<u>GB</u>: 1-134 a.a.), which comprises the <u>G</u>AR domain and <u>B</u>CO space region, in terms of holding the ribonuclease activity. In gel ribonuclease activity assay showed that HsGB domain fused with GST is able to degrade RNA, but its activity increased when GST was cleaved (Figure 2.3A, lines 3 and 5). Purified HsGB domain caused a significant degradation of RNAr and this effect was directly proportional to the domain concentration. 28S RNAr was the first target for degradation by HsGB domain (Figure 2.3B). To find the region responsible for the ribonuclease activity of fibrillarin, we split the HsGB domain into several regions (Figure 2.3C). As well as HsGB domain, all small peptides were expressed fused with GST and then recognized by anti-6xHis antibody (Figure 2.3D). Ribonuclease activity assay showed that even being fused with GST, HsGAR domain (amino acids 1 to 59) was able to degrade RNA (Figure 2.3E, lane 3), while the HsBCO region comprising amino acids 84 to 134 did not show any activity (Figure 2.3E, lane 5). Further deletions of GAR domain (miniGAR - amino acids 1 to 20) and a deletion from amino acids 13 to 68 (delGAR) lacked the activity (Figure 2.3E, lines 4 and 6, respectively). Considering that GAR domain is absent in fibrillarins of Archeabacteria and the possibility that it acts as modular domain that could be found in other proteins, we revised the published genomes for similar sequences. The phylogenetic tree constructed from the alignment of GAR domain of the retrieved fibrillarin proteins was grouped into three major clades (Figure 2.3F). The basal branch contains the GAR domain

of NOP1 protein from S. cerevisiae, the most ancestral species analyzed in this study, and sequences of non-mammalian species. The group A was divided in two subclades (A1 and A2) and contains almost all vertebrate species containing single fibrillarin sequences. Group A contains only mammalian sequences in comparison with the basal group. The subgroup A1 contains GAR sequences from none primates with exception of Gorilla GAR sequence. The GAR domain of human fibrillarin protein was clustered in A2 group; this branch contains all hominoid analyzed species. In the case of species containing a second fibrillarin sequence, they were clustered in group B, and contain a distinctive GAR pattern.

Figure 2.3 Fibrillarin domain with ribonuclease activity. A) *In gel* RNAse activity assay of fibrillarin HsGB domain (amino acids 1 to 134). Degradation of RNA is higher when the domain is not fused to GST (lane 5). GST and RNAse A were used as negative and positive controls, respectively. **B)** Ribonuclease activity assay. Degradation of RNAr is directly proportional to the amount of HsGB domain. **C)** Schematic representation of small peptides derived from HsGB expressed. **D)** Ponceau staining and western blot of the HsGB small peptides expressed. All peptides were GST-tagged. The band corresponding to each peptide is shown by a dot. Anti-6xHis antibody was used to identify each peptide, red dot. **E)** Ribonuclease activity assay for all small peptides of HsGB expressed. Under the same conditions, GST-GAR domain has higher activity than GST-HsGB domain (lines 2 and 3). The other peptides (miniGAR, BCO, and delGAR) have no acivity. GST was used as a negative control. **F)** Bioinformatic analysis of the R/G rich region (or GAR domain) of retrieved proteins from 36 complete genomes. The phylogenetic tree of GAR domain of fibrillarin proteins retrieved from chordate analyzed genomes. GAR domains were clustered in three major clades: clade A, clade B, and the basal group. The clade A was divided into A1 and A2 subgroups.

Mutation in GAR domain.

From the sequence analysis of GAR domain, the arginines in position 34 and 45 were chosen for mutagenesis as they are conserved in all studied Hominidae species (Figure 2.4A). Here we substituted them for alanine to define, if the positive charge had an effect on the ribonuclease activity and PIs/PA binding. Purified recombinant proteins were normalized prior to the experiments on ribonuclease activity or phospholipid binding (Figure 2.4B). Intriguingly, upon R34A and R45A fibrillarin mutants lost the ability to interact with the bisphosphate PIs, PA, and phosphatidylserine (PS), while interaction with PtdIns(3)P and PtdIns(5)P remained. Besides, R34A fibrillarin mutant interacted weakly with phosphatidylcholine (PC) and sphingosine-1-phosphate (S1P) (Figure 2.4C). Both mutations affected interaction with phospholipids as shown by phospholipid strip analysis, indicating that arginine positive charges are important for phospholipid binding. Wild type fibrillarin and the R34A fibrillarin mutant degraded RNAr 28S to the same degree, while the R45A fibrillarin mutant was found to be more active and degraded RNAr 28S up to 80% in a calcium independent manner as seen in Figure 2.4D.

The localization and dynamic behavior of fibrillarin in living cells was studied by transient transfection of HeLa cells with plasmids for wild type fibrillarin (Wt) and its mutant variants, all fused to GFP. We showed that mutated fibrillarins exhibited different patterns in live cells as compared to wild type (Figure 2.4E). As compared to Wt fibrillarin, R45A fibrillarin mutant had a 50% reduction in the nucleoli size (Table 2). FRAP analysis showed that fibrillarin mutants had a 40% lower diffusion coefficient in Cajal bodies (CBs), but only a minor reduction in mobility in the nucleous (Nco) (Figure 2.4E). Nucleolar R34A and R45A fibrillarin mutants showed a significant decrease in their dynamic speed (Diffusion Coefficient) compared to Wt fibrillarin, which was reflected in the increased value of their half-lives. In CBs, there was also an increase in R45A and R34A half-lives in comparison to Wt fibrillarin, which was consequently reflected in smaller diffusion coefficient values. This was observed as a longer residence time of the mutants inside the nucleolus and CBs in comparison with their wild type counterpart (Figure 2.4F).
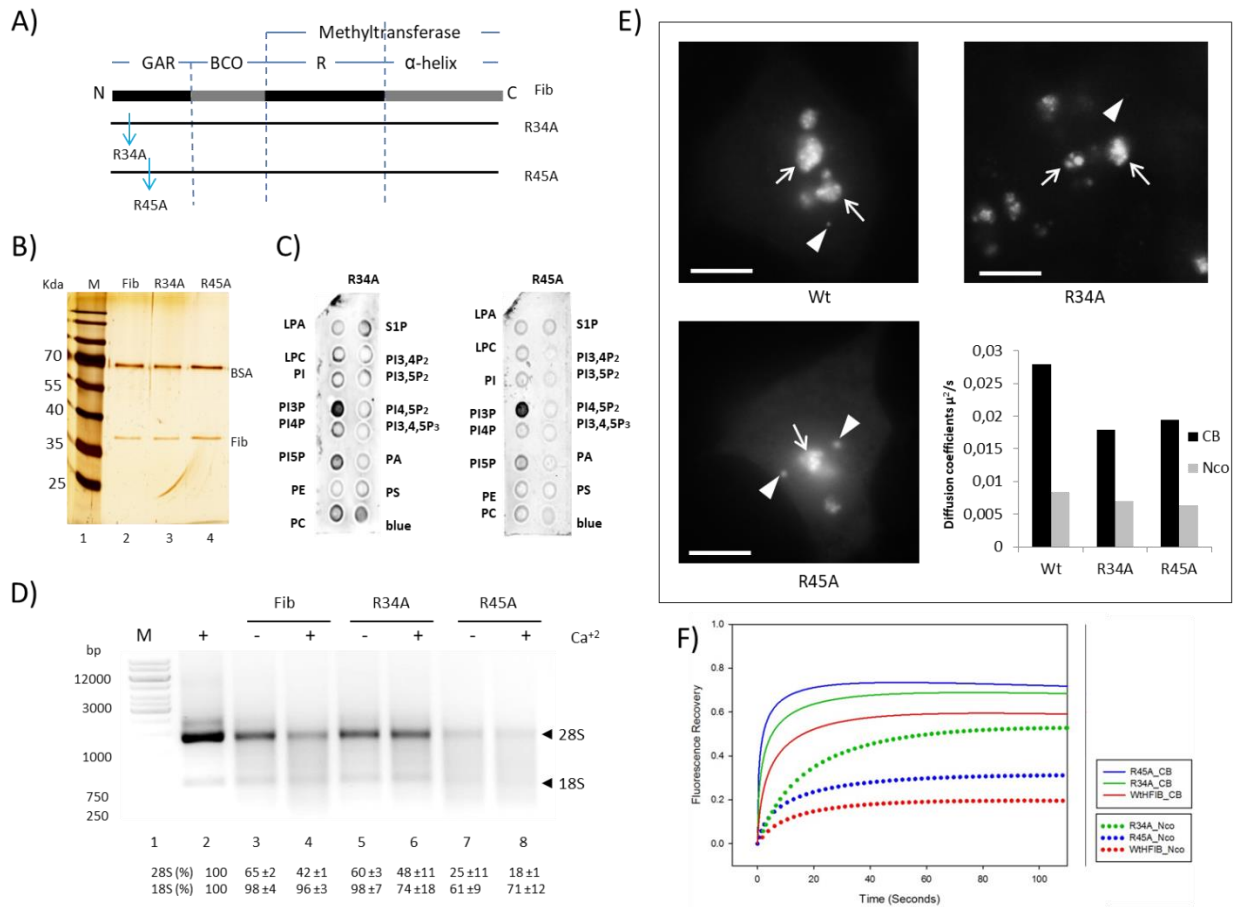
Figure 2.4 Activity of HsGAR domain mutants. A) Schematic representation of fibrillarin domains. GAR: glycine-arginine rich domain; BCO: space sequence; R: RNA binding domain; α-helix - alpha-helix domain. Blue arrows represent the mutations R34A and R45A in the fibrillarin GAR domain. **B)** Silver stain normalization of wild type fibrillarin (Fib) and mutated R34A and R45A fibrillarins. **C)** Phospholipid strip assay for R34A and R45A fibrillarin mutants. **D)** Ribonuclease activity assay of R34A and R45A fibrillarin mutants. Quantitative measurements of the density of 28S and 18S RNAr bands from 3 independent experiments are shown. **E)** HeLa cells transfected with either wild type fibrillarin-GFP or its mutated forms, R34A or R45A, both tagged with GFP. Typical localization patterns for Wt and the mutants observed in live cells are shown. FRAP analysis of all normalized dynamics for Wt and mutant fibrillarins in Cajal bodies (CB) and nucleoli (Nco) is shown as a graph data of diffusion coefficients. **F)** FRAP dynamics normalized from 200 independent measurements of Wt and mutant fibrillarins in Cajal bodies (CB) and nucleoli (Nco).

Tabla 1 Half-life and diffusion coefficients corresponding to each group of FRAP experiments and Scheffé analysis. Scheffé analysis (α=0.05) was carried out as a *pos hoc* statistic test taking both half-life and diffusion coefficients in account.

	Localization	Average half life ($\tau_{1/2}$)	Diffusion coefficient ($\mu m^2/s$)	Diameter of nucleoli (μm)	Scheffé test
FIB	Nucleolar	6.65 ± 1.51	0.008	5.41 ± 0.62	b
R34AMut	Nucleolar	7.99 ± 1.31	0.007	3.35 ± 0.69	ab
R45AMut	Nucleolar	8.78 ± 0.92	0.006	2.65 ± 0.87	a
FIB	Cajal bodies	2.00 ± 0.57	0.027		de
R45AMut	Cajal bodies	2.88 ± 1.10	0.019		d
R34AMut	Cajal bodies	3.13 ± 1.37	0.017		cd

Ribonuclease activity in fixed cells.

In fixed cells without any treatment, the cytosolic RNA and RNAr can be clearly observed with pyronin Y. Upon treatment with RNAse A, the entire signal belonging to RNA disappeared as well as by HsGAR domain, while the treatment with Wt fibrillarin or R45A mutant reduced the signal from the cytoplasm but they had a lower effect to the nucleolar RNA signal. Considering the small size of fibrillarin, it is unlikely that the difference is due to problems with protein accessibility and more likely to do with the overall amount of RNAr concentrated in the nucleoli (Figure 2.5).
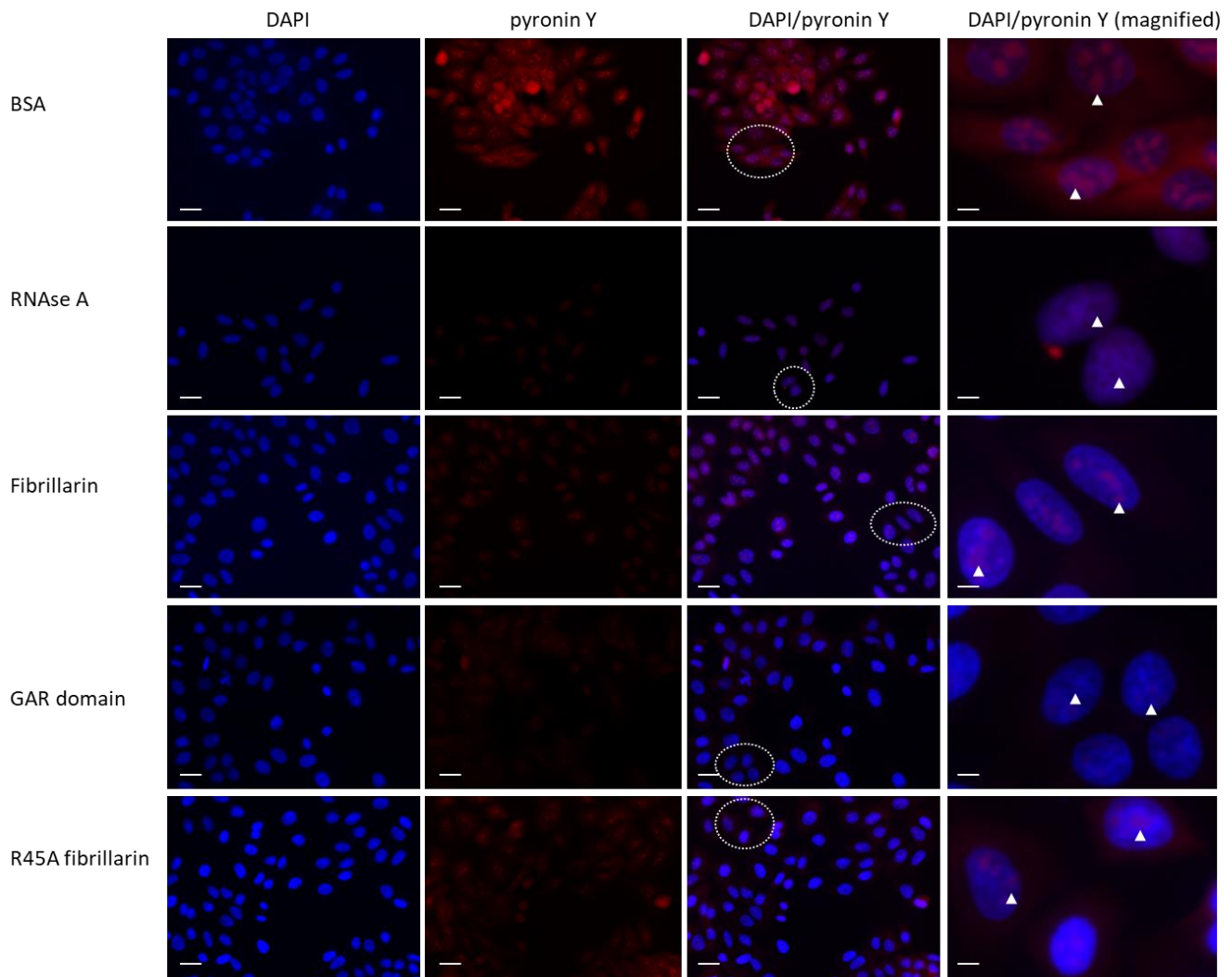
Figure 2.5 Ribonuclease activity assay in fixed HeLa cells. The ribonuclease activity for Wt fibrillarin, HsGAR domain, and R45A fibrillarin mutant was tested. RNA was stained with pyronin Y and DNA was stained with DAPI. BSA was used as a negative control for the ribonuclease activity, RNAse A was used as a positive control. As compared to the negative control, pyronin Y signal was significantly lower for all the proteins tested, particularly for HsGAR domain. DAPI signal was the same in each case. Dot white circles indicate the magnified area. Arrow heads indicate nucleolus.

Specificity of fibrillarin ribonuclease activity in complex with guide RNA.

The specificity of fibrillarin as a ribonuclease was tested against the U3 snoRNA. U3 snoRNA has been described as one of several RNA processing guidelines for RNAr which interacts with fibrillarin. Figure 2.6A shows that fibrillarin does not degrade U3 snoRNA but interacts with it resulting in retardation by electrophoretic mobility shift assay. Such interaction was inhibited in PA presence, while under the same conditions PtdIns(4,5)P_2 caused a minor alteration in the complex migration in a gel (Figure 2.6B, lines 4 and 5, respectively. We showed that fibrillarin ribonuclease activity was blocked by U3 snoRNA. For that, we pre-incubated fibrillarin with U3 snoRNA for 15 minutes followed by the addition of RNAr. Under these conditions fibrillarin was not able to degrade RNAr (Figure 2.6C, lane 7). The addition of calcium had no activation effect on fibrillarin when it was bound to U3 snoRNA (Figure 2.6C, lane 8). To test the activity of fibrillarin after pre-incubation, we incubated fibrillarin for 15 minutes without RNA, then we added RNAr and this was degraded as we expected. Native fibrillarin extracted from HeLa cells was also tested for the ribonuclease activity. The in gel activity assay showed five bands from the ribonucleoprotein complex, which contains fibrillarin. According to the western blot, four of those bands (marked by arrow heads) correspond to fibrillarin most likely due to the protein degradation, since fibrillarin is very prone to degradation even in the presence of a cocktail of protease inhibitors. The fifth band, marked by an asterisk, also has a ribonuclease activity, but we were not able to define its identity. Fibrillarin complex from HeLa cells was able to degrade RNAr, but, as well as the recombinant fibrillarin, it was unable to digest U3 snoRNA (Figure 2.6D).
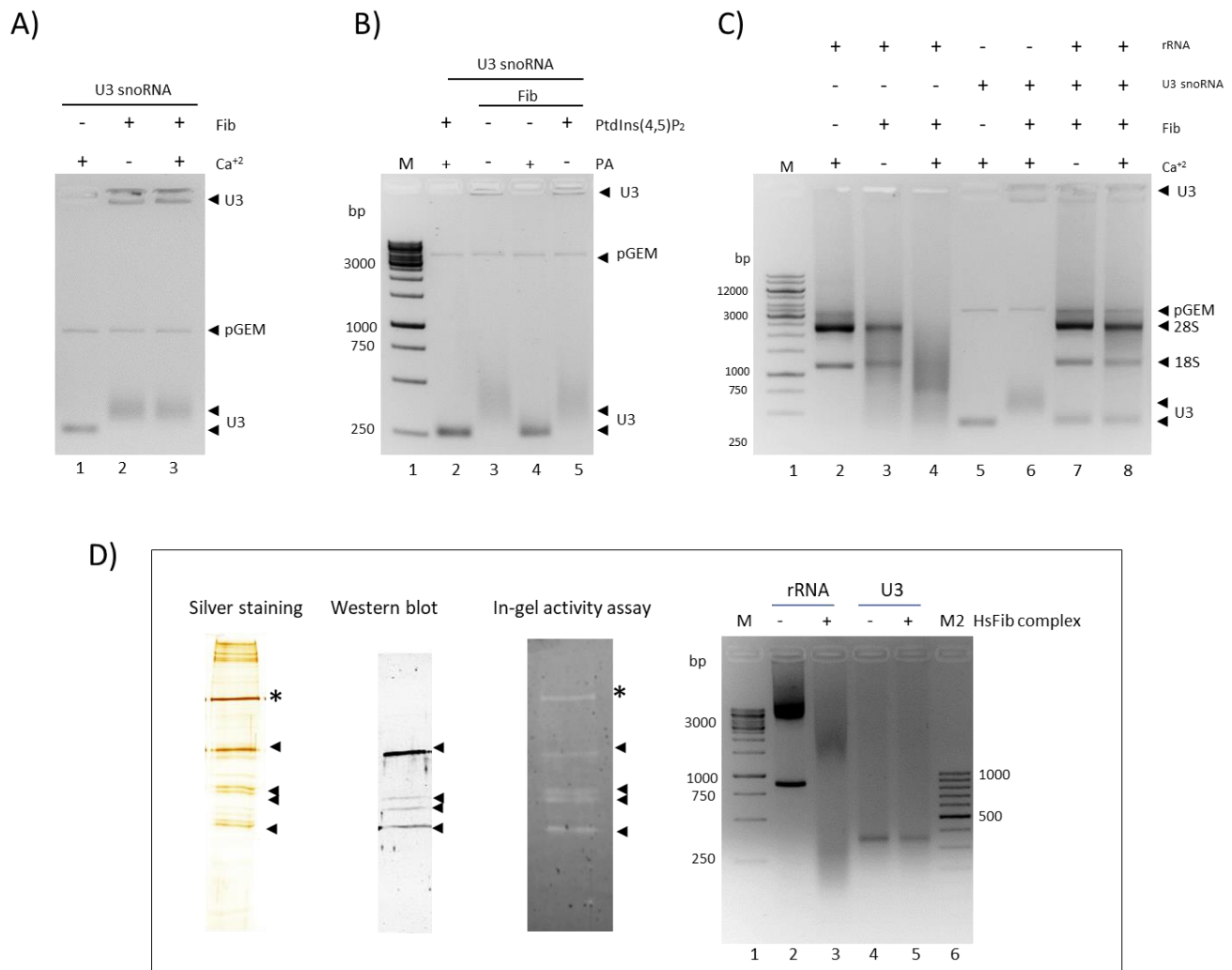
Figure 2.6 RNA-specific ribonuclease activity exhibited by fibrillarin in complex with U3 snoRNA. A) and **B)** Gel shift of U3 snoRNA with Wt fibrillarin, all incubations and procedures were carried out at 37 ^{o}C for A) and 4°C for B). **B)** PtdIns(4,5)P$_2$ and PA were added as stated in the Figure. Plasmid control pGEM was used to normalize each lane. **C)** Ribonuclease activity of fibrillarin with or without guide U3 snoRNA added. The reactions were carried out at 37 ^{o}C. **D)** Ribonuclease activity of the native fibrillarin extracted from HeLa cells with strep tag. The purified complex was visualized by PAGE with silver stain and western blot that showed 4 of the bands correspond to fibrillarin and parts of it due to degradation of the protein. Followed by in gel activity assay that show activity that correspond to bands in the same four positions as in western blot analysis. Ribonuclease assay showed high activity for the complex under native conditions.

Discussion

Fibrillarin is an essential protein for life, whose function and structure are highly conserved in all eukaryotic organisms (Jansen et al., 1991), reviewed by Rodriguez-Corona (Rodriguez-Corona et al., 2015). Its function has been described as a methyltransferase involved in RNAr processing (Tollervey et al., 1993) and methylation of histone H2A in Pol I promoters (Tessarz et al., 2014; Loza-Muller et al., 2015). Here we described its activity as a ribonuclease when is not forming a complex with Nop56, Nop58, and 15.5K proteins. Fibrillarin in an eukaryotic organism is structured by GAR domain, a spacer region that we called BCO, the central domain with RNA binding motif (R), and the α-helix domain (Aris and Blobel, 1991; Rodriguez-Corona et al., 2015). Fibrillarin is located mainly in the cell nucleolus (Andersen et al., 2005), where it is involved in RNAr processing. Also fibrillarin is located in Cajal bodies with unknown function up to date (Nizami et al., 2010).

The ribonuclease activity of fibrillarin was slightly foreshadowed previously. In 1990 Kass et al., using nuclear extracts from mouse cells, determined that a ribonucleoprotein complex, which contains fibrillarin and U3 snoRNA, was involved in RNAr processing. Using the antibody against fibrillarin, they showed that RNAr processing decreased significantly (Kass et al., 1990). Kass et al., concluded that some component of the extract was responsible for the ribonuclease activity; however, it was not attributed to fibrillarin. One year later Tollervey and collaborators, using yeast fibrillarin (NOP1), showed that its depletion reduced the level of mature RNAr, but increased the amount of pre-mature RNAr, resulting particularly affected the 18S RNAr and the ribosomes production (Tollervey et al., 1991). In cruciferous plants, Vasquez-Saez et al., isolated the ribonucleoprotein complex nuclear factor D (NF D) consisting of 30 proteins including fibrillarin, nucleolin, and the snoRNAs U3 and U14. They determined that the complex is able to 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-RNAr (Saez-Vasquez et al., 2004). However, this ribonuclease activity could not be associated with any particular protein at that time.

In the present study, fibrillarin was expressed, purified to homogeneity, and its specific ribonuclease activity was detected. Like other ribonucleases, the activity of fibrillarin increased by the addition of 1 mM calcium (Schwarz and Blower, 2014). The ribonuclease

activity of fibrillarin was also demonstrated within the fixed cells showing particular selectivity for mRNA as was shown for RNAse A or the RNAse catalytic 3D8 antibody (Seo et al., 2015).

In addition, we found that fibrillarin GAR domain is responsible for the activity as a ribonuclease. As it is shown here, GAR domain is present in several nucleolar proteins i.e. nucleolin (Lapeyre et al., 1987), NSR1 (Lee et al., 1991), SSB1 (Jong et al., 1987), and GAR1 protein (Girard et al., 1992). All of these proteins have RNA recognition motifs as well as fibrillarin does, besides nucleolin and GAR1 protein directly interact with RNAr and are involved in its processing (Bugler et al., 1987; Girard et al., 1992). In fibrillarin, GAR domain is needed to target it to nucleoli (Snaar et al., 2000) and for the interaction with nuclear phase viruses (Kim et al., 2007). Also, the ribonuclease activity could be used by viruses for their own processing mechanism as the essential role of fibrillarin in viral progression yet to be defined (Kim et al., 2007; Chang et al., 2016; Deffrasnes et al., 2016). Furthermore, interaction of phospholipids, including PIs and PA, with fibrillarin was established here. Within the cell nucleus, PIs are considered as the essential cofactors for various processes ranging from transcription regulation and differentiation to cell cycle control (Fiume et al., 2012). Previously, we described that PtdIns(4,5)P$_2$ interacts with fibrillarin affecting its RNA-binding mobility in native PAGE (Yildirim et al., 2013). PA is involved in various cellular processes, particularly within the nucleus, and regulates the location and activity of interacting proteins like transcriptional repressor Opi1p. Opi1p interaction with PA keeps the complex outside the nucleus and in the inactive form (Loewen et al., 2004; Yao et al., 2014). When fibrillarin interacts with PA, the general ribonuclease activity decreases and also releases fibrillarin from the interaction with U3 snoRNA. Similar results were obtained by Hatton and collaborators on the inhibition of RNAse A activity by PA (Hatton et al., 2015).

Negatively charged phospholipid binding domains usually require positive charges. A clear example is the γ-core in which arginines were mutated in a signature similar to that of fibrillarin GAR domain. Similar to the fibrillarin mutants described in this study, such mutations resulted in an alteration of PIs binding (Sagaram et al., 2013). The GAR domain R45A mutant has a greater effect on the activity of fibrillarin as a ribonuclease as

compared to Wt fibrillarin. Moreover, R45A mutated fibrillarin degrades RNAr in a calcium independent manner. Since the binding site for calcium is located within the alpha helix domain, the likely scenario is that arginine 45 keeps GAR domain in a non-active form by calcium binding to the rest of the protein. Mutation of this arginine prevents the binding and leaves GAR domain active in a calcium independent manner. Both mutants (R34A and R45A) modify the interaction with negatively charged phospholipids compared to Wt fibrillarin. It is known that phospholipids affect binding to proteins and can promote protein-protein interaction. Examples of this can be found in the literature as PDZ domain (PSD-95 / Discs large / ZO-1) of syntenin-2. This domain interacts with PtdIns(4,5)P$_2$ regulating the nuclear organization of the protein. Mutations of this domain, Lys113, Lys167, Lys197, and Lys244, result in a loss of binding with PtdIns(4,5)P$_2$ (Wawrzyniak et al., 2013b). Here we show that the nucleolar size is changed when the mutated fibrillarins are expressed in a cell, perhaps due to the changes in protein interactions as indicated by FRAP. Since fibrillarin interacts with at least 280 proteins, (https://thebiogrid.org/108399/summary/homo-sapiens/fbl.html), the mutations result in a decrease of a nucleolar size probably due to an improper complex formation partially compromising the nucleolar architecture.

Ribosome biogenesis is an essential cellular process, which consumes an important part of the cell energy (Warner, 1999). Here we show that, besides the methyltransferase activity of fibrillarin, this protein can also act as a ribonuclease regulated by PIs and PA. Fibrillarin ribonuclease activity may be directed by some guide RNA like U3 snoRNA. For the experiments shown here, we used a completely processed RNAr without the external and internal spacers, where U3 snoRNA guides the cleavages in at least 3 different positions (Kass et al., 1990). That is the reason, why the complex fibrillarin-U3 snoRNA was not able to degrade RNAr. In addition, recently NORD27 guide RNA has been shown to modulate pre-mRNA splicing. This guide RNA is known to bind fibrillarin although undetected by a western blot due to minor amounts. Localization experiments for fibrillarin show that it is located mainly in nucleoli and CB, however a faint signal appears throughout the nucleoplasm. This signal may result from nucleoplasmic fibrillarin involved in the splicing of mRNA in the complex with NORD27 guide RNA (Falaleeva et al., 2016). Fibrillarin has been shown to be an essential protein for life. In yeasts, fibrillarin mutants

result in different phenotypes affecting maturation of RNAr and ribosome biogenesis at different stages (Tollervey et al., 1993). This study for the first time shows that fibrillarin, in addition to methylation, has another extremely important activity, which is essential for its involvement in RNAr processing and finally ribosomes production and protein synthesis.

CAPÍTULO III.

En este capítulo, el cual lleva por título "Novel Ribonuclease Activity Differs between Fibrillarins from *Arabidopsis thaliana*", se logró reproducir la actividad como ribonucleasa de *H. sapiens* utilizando las fibrilarinas de *A thaliana*. Reproducir el resultado obtenido previamente era de importancia para corroborarlo con otro modelo de estudio, así mismo, para verificar si en un organismo con al menos dos fibrilarinas ambas tienen la misma actividad. Ambas fibrilarinas se expresan en un nivel similar en diferentes tejidos así como en las diferentes etapas del desarrollo de la planta. La identidad entre la fibrilarina 1 y 2 de *A. thaliana* (AtFib1 y AtFib2) es del 91%, el principal cambio dentro de su estructura se observa en el dominio AtGAR. Ambas poseen actividad como ribonucleasa la cual no es dependiente de calcio, sin embargo la actividad de AtFib2 es significativamente mayor. Así mismo, utilizando el dominio AtGAR y Atα de AtFib2 se confirmó que la actividad como ribonucleasa se encuentra en AtGAR.

Mediante el ensayo fat blot se determinó la interacción entre cada una de las fibrilarinas con los fosfoinosítidos. AtFib1 interactúa principalmente con los fosfoinosítidos monofostato y AtFib2 interactúa con cada uno de los fosfoinosítidos así como con el ácido fosfatídico, mismo con el cual, la actividad como ribonucleasa se inhibe. Por otra parte, se inmunolocalizo a la fibrilarina en el nucléolo de callos de *A. thaliana* y su colocalización con el fosfatidilinositol 4,5-bisfosfato. No fue posible discernir entre cada una de las fibrilarinas por la falta de un anticuerpo específico para cada una de ellas, por lo tanto, la señal corresponde al total de las fibrilarinas en el núcleo y nucléolos.

Con los resultados obtenidos se reproduce la actividad como ribonucleasa de HsFib en las fibrilarinas de *A. thaliana*, que a pesar de contar con una actividad a diferente nivel ambas son capaces de degradar el RNAr. Los resultados fueron publicados en 2017 en la revista Frontiers in Plant Science, doi:10.3389/fpls.2017.01878 (Rodriguez-Corona et al., 2017).

Novel Ribonuclease Activity Differs between Fibrillarins from *Arabidopsis thaliana*

Ulises Rodríguez-Corona[1], Alejandro Pereira-Santana[2], Margarita Sobol[3], Luis C. Rodríguez-Zapata[4], Pavel Hozak[3] and Enrique Castano[1]*

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Mexico[1], Biosystematics Group, Department of Plant Sciences, Wageningen University and Research, Wageningen, Netherlands[2], Department of Biology of the Cell Nucleus, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic [3].Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida, Mexico [4].

Abstract

Fibrillarin is one of the most important nucleolar proteins that have been shown as essential for life. Fibrillarin localizes primarily at the periphery between fibrillar center and dense fibrillar component as well as in Cajal bodies. In most plants there are at least two different genes for fibrillarin. In *Arabidopsis thaliana* both genes show high level of expression in transcriptionally active cells. Here we focus on two important differences between *A. thaliana* fibrillarins. First and most relevant is the enzymatic activity by AtFib2. The AtFib2 shows a novel ribonuclease activity that is not seen with AtFib1. Second is a difference in the ability to interact with phosphoinositides and phosphatidic acid between both proteins. We also show that the novel ribonuclease activity as well as the phospholipid binding region of fibrillarin is confine to the GAR domain. The ribonuclease activity of fibrillarin reveals in this study represents a new role for this protein in RNAr processing.

Keywords: nucleoli, fibrillarin, ribonuclease, phosphoinositides, phosphatidic acid, glycine-arginine rich domain.

Abbreviations: AtFib, A. thaliana fibrillarin; DFC, dense fibrillarin component; FAA,

formalin-acetic acid-alcohol;FC, fibrillar center; GAR, glycine arginine rich domain; GC, granular component; NV, nucleolar vacuole; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; RRM, RNA recognition motif.

Introduction

The nuclear architecture and gene regulation are some of the most relevant subjects in science today. During the last few decades, the study of the molecules involved in gene regulation has revealed several proteins, DNA and RNA as the main players. Recently, other smaller molecules like phospholipids also play a crucial process in the dynamic architecture and function of the nucleus (Sobol et al., 2013; Yildirim et al., 2013). Here we focus on the nucleoli as one of the most studied nuclear structures in eukaryotic cells. Besides ribosomal RNA (RNAr) production and ribosome pre-assembly the nucleolus is also involved in many relevant aspects of the cell life including biogenesis of small nuclear and nucleolar RNA (snRNA and snoRNA respectively), sensing cellular stress, nucleolar dysfunctions as cancer, genetic silencing, cell cycle and viral infection progression, senescence among others. (Jacobson and Pederson, 1998a; Cockell and Gasser, 1999; Garcia and Pillus, 1999; Olson and Dundr, 2001; Hernandez-Verdun et al., 2010). In plants the nucleolus consists of four components: fibrillar center (FC), dense fibrillar component (DFC), granular component (GC) and nucleolar vacuole (NV). Fibrillarin was first identified in dense fibrillar and granular regions of the nucleolus with autoimmune sera from a patient with scleroderma (Ochs et al., 1985). Also fibrillarin in plants was detected for first time in onion cells in the transition zone between the FC and the DFC (Cerdido and Medina, 1995). Ultrathin sections of rat neurons have shown fibrillarin localization at the periphery of FC and in the DFC (Desterro et al., 2003). Fibrillarin is a conserved S-adenosyl-L-methionine-dependent methyltransferase which is found in all eukaryotic cells and a shorter version exists in the Archaea kingdom as well (Rodriguez-Corona et al., 2015; Shubina et al., 2016). Therefore the only activity assigned to fibrillarin has been methylation of RNAr and Histone H2A (Tollervey et al., 1993; Tessarz et al., 2014; Loza-Muller et al., 2015). However this activity is not essential for life, while fibrillarin is an essential protein in eukaryotic organism so its precise role may still need to be defined. Reduced levels of fibrillarin in Drosophila melangonaster exposed to mTOR resulted in

lifespan prolongation and a decrease of the nucleolar size in the fat body and intestine cells (Tiku et al., 2016). Since mTOR also regulates p53 and higher levels of p53 directly reduce the amount of fibrillarin. It correlates well with several types of cancers that show the reduction of p53 and therefore an increase of fibrillarin and higher level of methylation in ribosomes causing errors during translation (Marcel et al., 2013). Human fibrillarin also forms a sub-complex with splicing factor 2-associated p32 with unknown function but independent from ribosomal processing (Yanagida et al., 2004). Furthermore, it was surprising that silencing of fibrillarin in human cells shows nuclear structure alterations in a cell cycle dependent manner before the cells death (Amin et al., 2007).

Fibrillarin in plants has been found in pulldowns of the RNA pol II transcription mediator complex as subunit 36a. *Arabidopsis thaliana* has three different genes of fibrillarin (Barneche et al., 2000). It is also involved in the viral progression and long distance trafficking of viruses in plants like the Bamboo mosaic potexvirus satRNA forms a ribonucleoprotein complex with fibrillarin and this complex allows the virus phloem based movement and infection in other tissues (Chang et al., 2016). Due to the several unknowns of this protein, we therefore decided to study both fibrillarin proteins: fibrillarin 1 (AtFib1) and fibrillarin 2 (FLP fibrillarin-like protein; AtFib2) from *A. thaliana* as a model plant. In most eukaryotic cells, fibrillarin localizes primarily in the FC and DFC regions of the nucleolus, where active ribosomal DNA (rDNA) transcription and RNAr processing takes place. Both proteins contain three domains; glycine-arginine rich domain (GAR domain), methyltransferase domain and alpha region. The domains are very well conserved with the exception of the GAR domain that does not exist in the Archaea. The GAR domain has been shown to be required for nucleolar localization of fibrillarin (Snaar et al., 2000), but no further studies have been carried out on the function of this domain. In human cells, recent work demonstrated how two nucleolar proteins, fibrillarin and nucleophosmin, can phase-separate into droplets similar to the subnucleolar compartments in vitro and in vivo (Feric et al., 2016). This is attributed to the physical properties of the GAR domain resulting in a disordered structure in fibrillarin. However, the combination sequence of GAR domain and at least one RNA Recognition Motif (RRM) of fibrillarin is required for proper subnucleolar compartment formation and maintenance (Feric et al., 2016).

In the last few years, questions as to the endonuclease activity required for the proper processing for RNAr has shown to involve a complex were several proteins are, including fibrillarin (Henras et al., 2015). In yeast depleted U3 snoRNA causes affect knob formation on nascent pre-RNAr and alter as seen on the promoters by electron microscopy (Dragon et al., 2002). During our studies throughout purifications we discover that fibrillarin has a ribonuclease activity, here we show a distinction on this activity between the two fibrillarins of *A. thaliana*. Furthermore, in this study we show the interaction of both fibrillarins with phosphoinositides, which is involved in several nuclear functions (Sobol et al., 2013; Yildirim et al., 2013), and therefore may provide clues for uncovering the fibrillarin nuclear dynamics.

Materials and Methods

Bioinformatic analysis. Amino acid alignment in Figure 1A was visualized by BOXSHADE v3.3.1C (http://boxshade.sourceforge.net/). Gene expression data for AtFib proteins in Figure 2.1B was taken from (Schmid et al., 2005). Treatment descriptions and gene expression information can be inspected in TAºIR (accession: ExpressionSet: 1006710873), and also can be inspected in www.PLEXdb.org (accession: AT40). The heatmap in Figure 2.1B was generated by using the ComplexHeatmap package (Gu et al., 2016) from Bioconductor project (Huber et al., 2015). Structural studies of Arabidopsis fibrillarins were modeled on the free software 3d-jigsaw (https://bmm.crick.ac.uk/~3djigsaw/) and edited with PyMOL v1.8.4.0.

Cloning. *Arabidopsis thaliana* plants were cultivated on soil in a controlled environment and photoperiod of 10-13 hours light at 23º C and 11-14 hours dark at 20º C (Yoo et al., 2007). RNA extraction was made with RNeasy® Plant Mini Kit (QIAGEN Sciences, Maryland 20874). Sequences of AtFib1 and AtFib2 were obtained with SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermofischer Scientific). The specific primers to amplify AtFib1 were: forward 5´ - 3´ CATATGATGAGACCCCCAGTTACAGGA and reverse 5´ - 3´ GGATCCCTATGA GGCTGGGGTCTTTTG. To amplify AtFib2 the specific primers were: forward 5´ - 3´ CATATGATGAGACCTCCTCTAACTGGAAG and reverse 5´ - 3´ GGATCCTCTAAG

CAGCAGTAGCAGCCTTTG. Forward primers have NdeI restriction enzyme sequence, reverse primers have BamHI restriction enzyme sequence for pET15b expression plasmid cloning. Same strategy was used for GAR (AtGAR2) and alpha helix (Atα2) domain cloning of AtFib2. AtGAR2 primers: forward: 5´ - 3´ CATATGATGAGACCTCCTCTA ACTGGAAG, reverse 5´ - 3´: GGATCCCACAATCACTTTGCTTCCTCC. Atα2 primers: forward 5´ - 3´: CATATGCTTGTAGGCATGGTTGATGT, reverse 5´ - 3´: GGATCC CAAAGGCTGCTACTGCTGCTTAG.

Protein Expression and Purification. *Arabidopsis thaliana* fibrillarins were expressed in *E. coli* Artic competent cells induced with 1 mM isopropyl -D-1-thiogalactopyranoside at 11 °C for 24 hours. Harvested cells were suspended in protein extraction buffer (500 mM NaCl, 25 mM tris pH 8, 10% glycerol, 20 mM imidazole, 0.1% triton X-100, 0.1 mM AEBSF and 0.1 mM DTT) and sonicated. After clarification by centrifugation (17400 g x 15 minutes), the supernatant was subjected to further purification steps. The clarified supernatant was loaded onto a Ni-NTA agarose column (Thermofisher Scientific) and washed 3 times with the extraction buffer. Fibrillarins were eluted (200 mM NaCl, 25 mM tris pH 8, 20% glycerol, 0.1 mM AEBSF and 0.1 DTT) in a linear gradient from 20 to 200 mM of imidazole. Fibrillarins containing fractions were further purified by Q sepharose chromatography leading to single band detection of fibrillarins. Same strategy was used for AtGAR2 and Atα2 domains.

In gel RNase activity. Proteins were separated in 15% SDS-PAGE gel. Prior to polymerization, running gel was supplied with 5 mg/mL of total RNA extracted from *A. thaliana*. After electrophoresis, gel was washed for 10 minutes with buffer I (10 mM Tris-HCl, 20% isopropanol, pH 7.5) and consequent incubation for 30 min in buffer II (10 mM Tris-HCl, pH 7.5) and buffer III (100 mM Tris-HCl, pH 7.5). Gel was stained with 0.2% of toluidine blue and washed with water (Dudkina et al., 2016).

In vitro transcription. *Arabidopsis thaliana* snoRNA U3 sequence was amplified and cloned into pGEM®-T Easy Vector (PROMEGA). Once cloned, vector was linearized with NdeI enzyme for 1h at 37 ºC. Transcription was made with T7 RNA polymerase (New England Biolabs Inc) for 2h at 37 ºC. Specific primers for AtsnoU3 used are: forward 5´- 3´

ACGACCTTACTTGAACAGGA, reverse 5´- 3´ CCTGTCAGACCGCCGTGC GAC.

Ribonuclease assay. Total RNA extracted from *A. thaliana* was mixed with each fibrillarin on BC200 buffer (20 mM Tris-HCl buffer, pH 8, 200 mM KCl, 0.2 mM EDTA, 10% glycerol), incubated for 1 hour at 37 ºC and then loaded in a 1% agarose gel.

Fat blot assay. PIP strip with spotted phosphoinositides (Echelon Biosciences, P-6001) was probed with anti-Fib antibody. For this, the membrane was blocked with 3% BSA in PBS for 1h followed by 3h at room temperature of 1% BSA in PBS and 0.4 µg of each protein. After that, PIP strip was washed 3 times, 10 minute each, with PBS-T and incubated with primary antibody for 1 h. Again washed with PBS-T and incubated with the appropriate IRDye secondary antibody for 1h. The immunoblotting signals were analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences, Lincoln, Nebraska, USA).

Western blot assay. Fifty ng of each fibrillarin was loaded in a 12% acrylamide gel to perform a SDS-PAGE. Subsequently we transfer the protein to a nitrocellulose membrane and blocked with 3 % of BSA in PBS at room temperature. Later was made incubation with anti-Fib rabbit antibody (1/5000) for two hours at room temperature and a third with secondary antibody (1/4000) one hour at room temperature, with three washes between incubations and revealed. The immunoblotting signals were analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences, Lincoln, Nebraska, USA).

Immunofluorescence. *Arabidopsis thaliana* callus were made according to (Sugimoto and Meyerowitz, 2013). Sample preparations for microscopy analysis was made as previously publish by our group (Loza-Muller et al., 2015) with callus from *A. thaliana* instead of leaves. Images were acquired in confocal microscope (Leica TCS SP5 AOBS TANDEM) and a laser-scanning microscope FV100 Olympus with 60X (NA 1.4) oil immersion objective lens.

Results

The comparison between the three fibrillarin genes in *A. thaliana* shows the greater amino acid difference in the GAR domain represented by a dotted blue contour (Figure 3.1A). Considering that this part of the protein resides the main difference we check if their expression would be tissue or developmental stage specific. Transcriptional patterns of AtFib genes (Figure 3.1B) demonstrate high expression levels in the different tissues and on different developmental stages in wild-type Arabidopsis Col-0 (Data can be inspected in www.PLEXdb.org, accession: AT40). Sequence differences between AtFib proteins (GAR domain + fibrillarin domain) could have major implications on protein activity. At transcriptional level, we found differences between AtFib genes. AtFib1 shows the highest expression levels but also little variations between treatments, while AtFib2 shows the most variation between treatments and seems to be more affected by development stages but remains expressed in all stages. AtFib3 shows the lowest level of expression values and almost no variation between tissues and development stages.

A)

B)

Figure 3.1 Alignment and ribonuclease activity of *A. thaliana* fibrillarins. (A) sequence comparison of the three AtFib proteins by aligning in MAFFT program.**(B)** Heat-map of the transcriptional expression patterns of AtFib1-3 genes in different tissues and different developmental stages in wild-type Arabidopsis Col-0. Data were taken from (Schmid et al., 2005).

We focused on AtFib1 and AtFib2 as they are expressed in almost all conditions. The in silico structure prediction between them (Figure 3.2A) shows that the main structural difference is due to an angle changed for the exposure of the GAR domain as can be seen in the overlay of the structures in figure 3.2B. As in other crystal structure of fibrillarin (Rodriguez-Corona et al., 2015), the regions of methyl transferase to alpha region maintain a similar structure. Our initial studies were to test gel mobility alterations by fibrillarin with RNA resulted in degradation of the RNA when a short incubation was carried out at room temperature. We therefore tested the purified fibrillarin with an *in gel* ribonuclease activity assay to make sure that no other protein was responsible for this activity. The *in gel* toluidine blue staining of RNA show a white band from the lack of RNA due to its degradation at the correct molecular weight for the purified fibrillarin (Figure 3.2C). Different eluates were loaded in the ribonuclease activity gel assay and show that both fibrillarins (AtFib1 and AtFib2) have ribonuclease activity. Western blot of the bands confirmed their correspondence to fibrillarin (Figure 3.2C). AtFib2 is more susceptible to degradation as showed by western blot and as *in gel* activity assay.
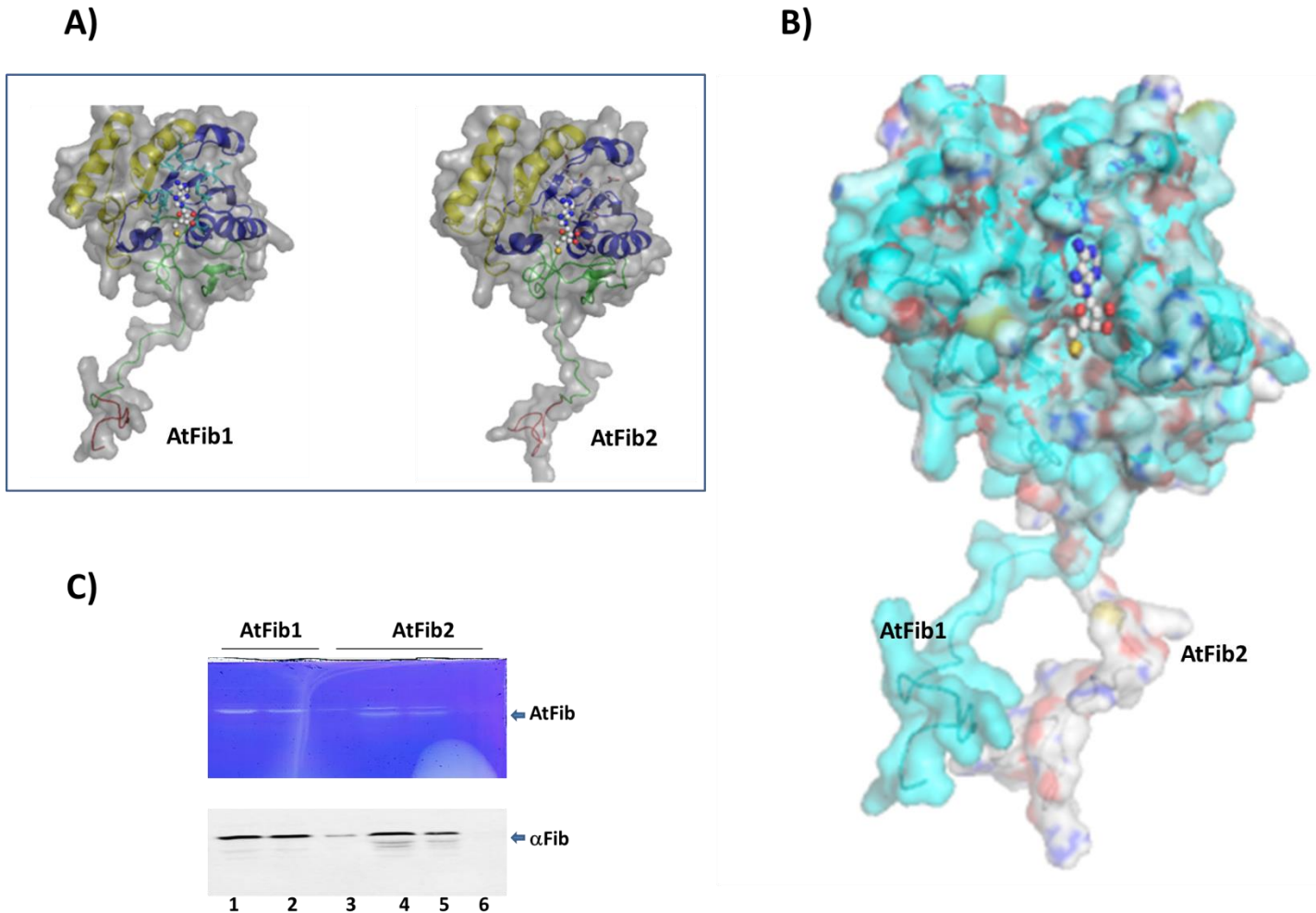
A)

B)

C)

Figure 3.2 Structural difference between fibrillarins from *A. thaliana*. (A) The domains are shown as follows. In Red the GAR domain, Green: BCO space region, Yellow: RNA binding domain, Blue: Alpha helix domain. SAM is showed as spheres. The molecular surface of the proteins is showed in gray color. **(B)** Structural alignment of AtFib1 and AtFib2. The alignment shows that GAR domain and BCO space region are oriented in opposite directions in these two proteins. **(C)** In gel ribonuclease activity assay. The white bands correspond to the spaces in the gel, in which RNA was degraded by fibrillarin, confirmed by Western blot. 1, 2, and 3 are three different elutions from the purification process of AtFib1. 4, 5, and 6 are three different elutions from the purification process of AtFib2.

We decided to characterize this novel ribonuclease activity and purified both proteins to

homogeneity (Figure 3.3A) in the exact same procedure and tested their activity under native conditions. Both fibrillarins were incubated with RNAr to test their ability to cleave RNAr. The reactions were carried out using the same amounts of fibrillarins as what is shown in the silver stained gel (Figure 3.3A). The results shown in figure 3.3B demonstrate that AtFib2 has a potent ribonuclease activity in a dose dependent manner while AtFib1 can only show activity under the greatest amount. This correlates well with the in gel activity assay which shows both proteins to have activity but AtFib2 show significant RNAr cleavage. We tested if *A. thaliana* fibrillarins are activated by calcium, as other ribonucleases (Schwarz and Blower, 2014), we found that AtFib1 is not activated by calcium, while AtFib2 shows minor activation (Figure 3.3C). Interestingly, the activation of the ribonuclease activity by calcium shown for AtFib differs from that of human fibrillarin that we tested.
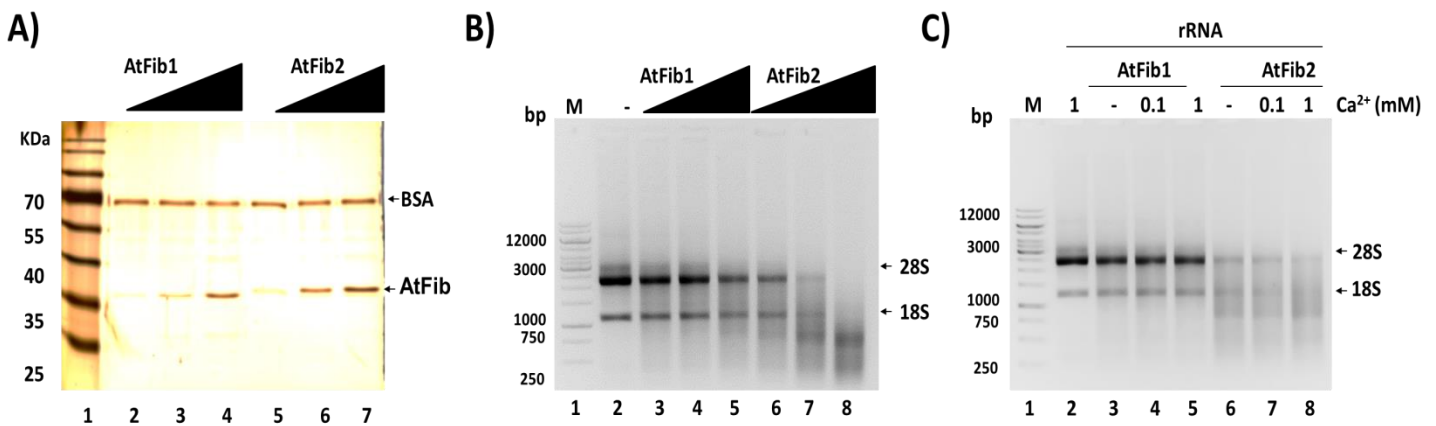

Figure 3.3 Ribonuclease activity of *A. thaliana* fibrillarins in calcium presence. (A) Concentration from 2 to 8 ng of both *A. thaliana* fibrillarins. BSA was added to normalize. **(B)** Fibrillarin ribonuclease activity. Increased amounts of fibrillarin were added to a constant concentration of RNAr. The assay clearly shows that AtFib1 is less active as compared to AtFib2 at the same concentration. **(C)** Using the concentration of fibrillarins as shown in lane 3 and 5 of Figure 2.3A, we tested further the activation of ribonuclease activity by calcium for AtFib1 and AtFib2 (lane 4 – 5 and 7 – 8, respectively). Calcium was added at the concentrations of 0.1–1 mM.

Our previous studies with human fibrillarin had shown its interaction with phosphoinositides (Yildirim et al., 2013). In figure 3.4A, AtFib1 primarily interacts with phosphatidylinositol 4-phosphate (PtdIns(4)P), while AtFib2 interacts with all phosphoinositides, as well as with phosphatidic acid (PA). This is similar to what we have detected with the unique human fibrillarin (Yildirim et al., 2013) and data not shown). PA is implicated in many stress events in plants and it is also involved in phosphoinositides metabolism. Here we detected a decrease of the ribonuclease activity by the addition of PA as seen in figure 2.4B, lane 9. PA inactivation is reversed by the addition of calcium (Figure 3.4B, lane 10).

Nuclear phosphoinositides have been extensively studied in plant membranes but studies are lacking on the nuclear forms. To provide more information on nuclear phosphoinositides, we took advantage of the phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) antibody. We carried out Confocal microscopy of Arabidopsis callus which had membrane bound PtdIns(4,5)P_2 removed by Triton X-100 as it was done in other publications (Laboure et al., 1999) (Figure. 3.4C). We show that nuclear PtdIns(4,5)P_2 has a partial colocalization with fibrillarin. Since the antibodies against fibrillarin detect both forms of fibrillarin it is impossible to discern between the two forms at this stage. We have unsuccessfully tried to raise antibodies, which would distinguish between these two fibrillarins that may lead to a better colocalization of one of them with phosphoinositides. The PtdIns(4,5)P_2 exhibits a dotted pattern in nucleoli regions and a diffuse pattern in other nuclear regions. Fibrillarin colocalizes with PtdIns(4,5)P_2 in the nucleolus but not in other regions like Cajal bodies.
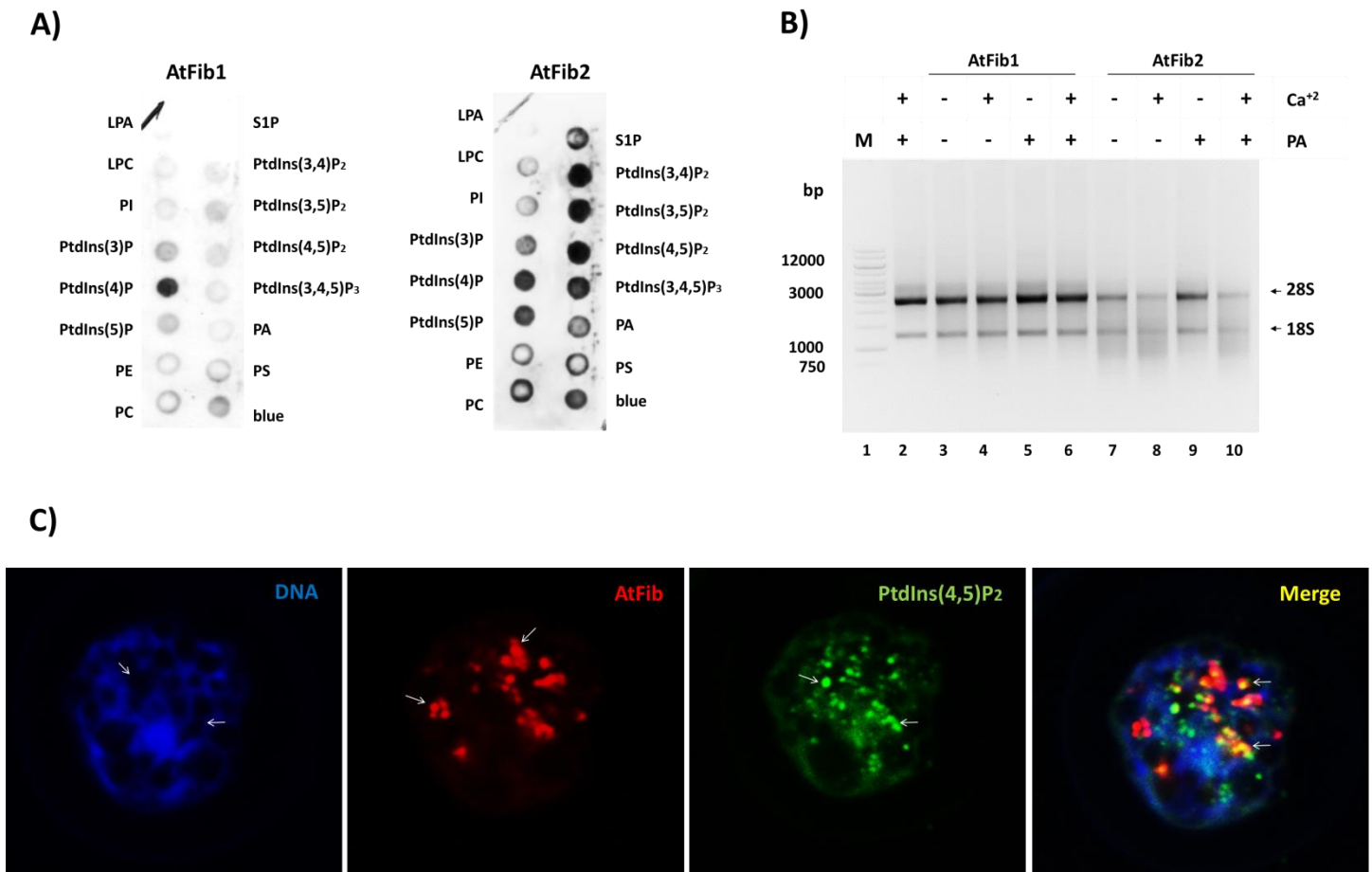
Figure 3.4 *Arabidopsis thaliana* fibrillarins and phosphoinositides. (A) Fat blot assay for AtFib1 and AtFib2. AtFib1 interacts mainly with the monophosphate phosphoinositides, in contrast AtFib2 interacts with all phosphoinositides and phosphatidic acid. **(B)** Ribonuclease activity in phosphatidic acid presence. With the same concentration for both fibrillarins as Figure 2.3A, lanes 3 and 5, its clear how in phosphatidic acid presence (30 ng) the ribonuclease activity of AtFib2 is inhibit (lane 9). **(C)** Colocalization between AtFib's and PtdIns(4,5)P_2 in *A. thaliana* callus.

In order to define the domain that has ribonuclease activity, we overexpressed two domains of the protein, which were shown to have an enzymatic activity assigned (Figure 3.5A). The N terminus contains the GAR domain and the C terminus the α domain. We

purified both domains (Figure 3.5B) and tested them for activity. Only AtGAR2 domain showed high ribonuclease activity both in an in gel activity assay with RNA as substrate, as well as under native conditions (Figure 3.5C and 3.5D). The ribonuclease activity of AtGAR2 domain is less selective than the full fibrillarin protein as it degrades both 28S and 18S simultaneously and gives a less selective pattern of bands as well; figure 3.5D lanes 4 and 5. The AtGAR2 domain is also the interacting domain for phospholipid binding, including all phosphoinositides species as well as PA and phosphatidylserine (PS) and resemble the full protein binding, while the alpha region of AtFib2 had no ribonuclease activity and only binds to phosphatidylinositol 5-phosphate (PtdIns(5)P) (Figure 3.5E).
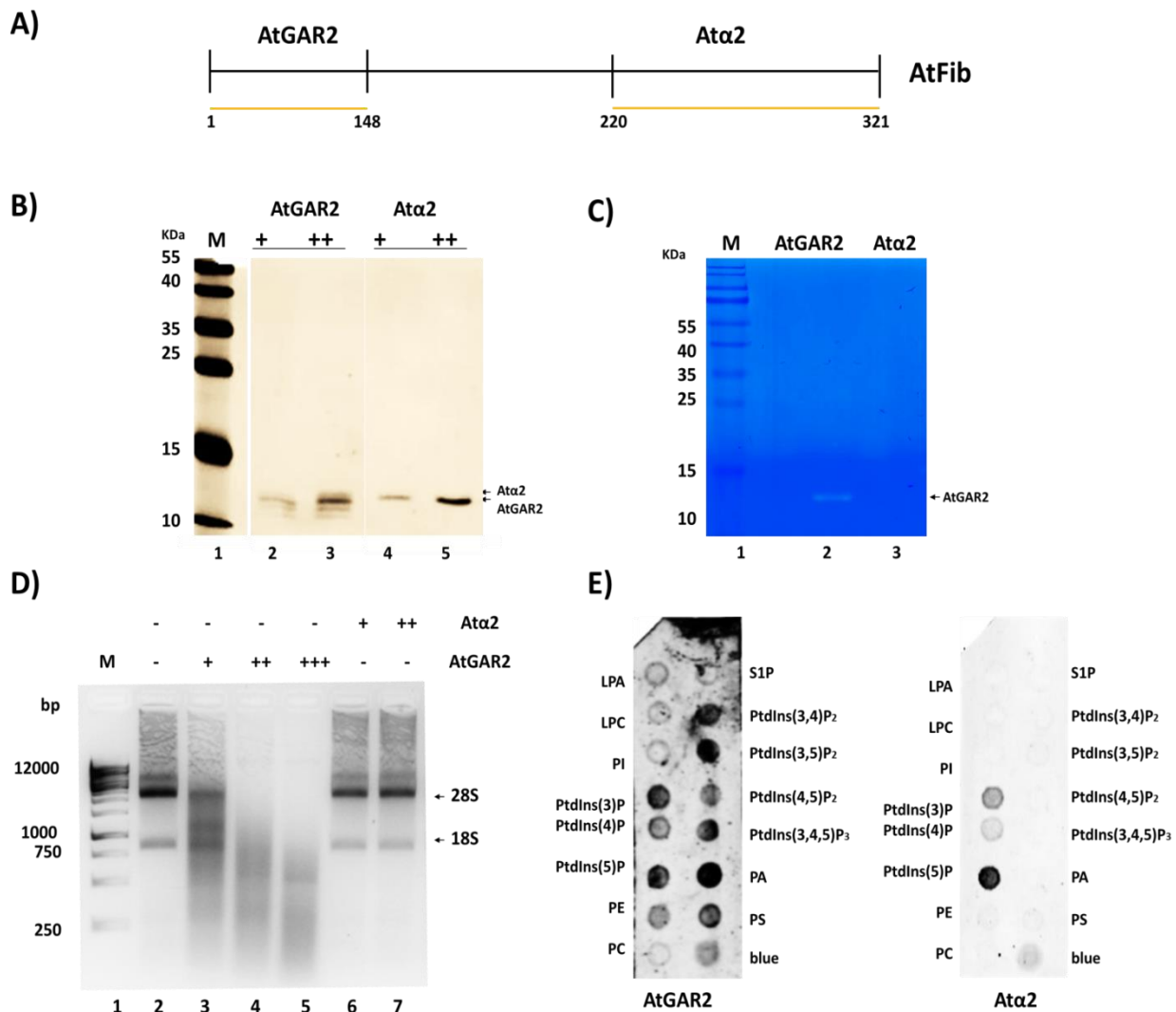
Figure 3.5 Ribonuclease activity of AtFib2 domains. (A) Schematic representation of AtFib. Yellow lines represent the expressed domains [AtGAR2 (1–48 aminoacids) and Atα 2 (220–321 amino acids)]. **(B)** Western blot for AtFib2 domains. Two different concentrations of AtGAR2 and At α 2 were recognized with anti-HIS primary antibody. **(C)** In gel ribonuclease activity assay. The white bands correspond to the spaces in the gel in which RNA was degraded by AtGAR2. **(D)** Ribonuclease activity of AtGAR2 and At α 2 domains. Degradation of RNAr was directly related to the amount of AtGAR2 domain added. **(E)** Fat blot assay for AtGAR2 and Atα2. AtGAR2 domain interacts with all phosphoinositides in the same way as the whole AtFib2 protein. By the other hand, Atα 2 interacts mainly with PtdIns(5)P. Note. + signal represent the addition of protein, ++ represents the same protein but twice the concentration.

Finally, we compared AtFib1 and AtFib2 for their ribonuclease activity on U3 guide RNA and overall RNAr. We found that AtFib2 was able to cut RNA as compared between figure 3.6A and 3.6B. AtFib1 showed only a minor reduction in the amount of ribosomal RNA but maintained the exact same pattern, while AtFib2 showed a different pattern of RNAr and U3 after interacting with this fibrillarin as seen in figure 3.6B lane 6 compared to lanes 7 and 8.
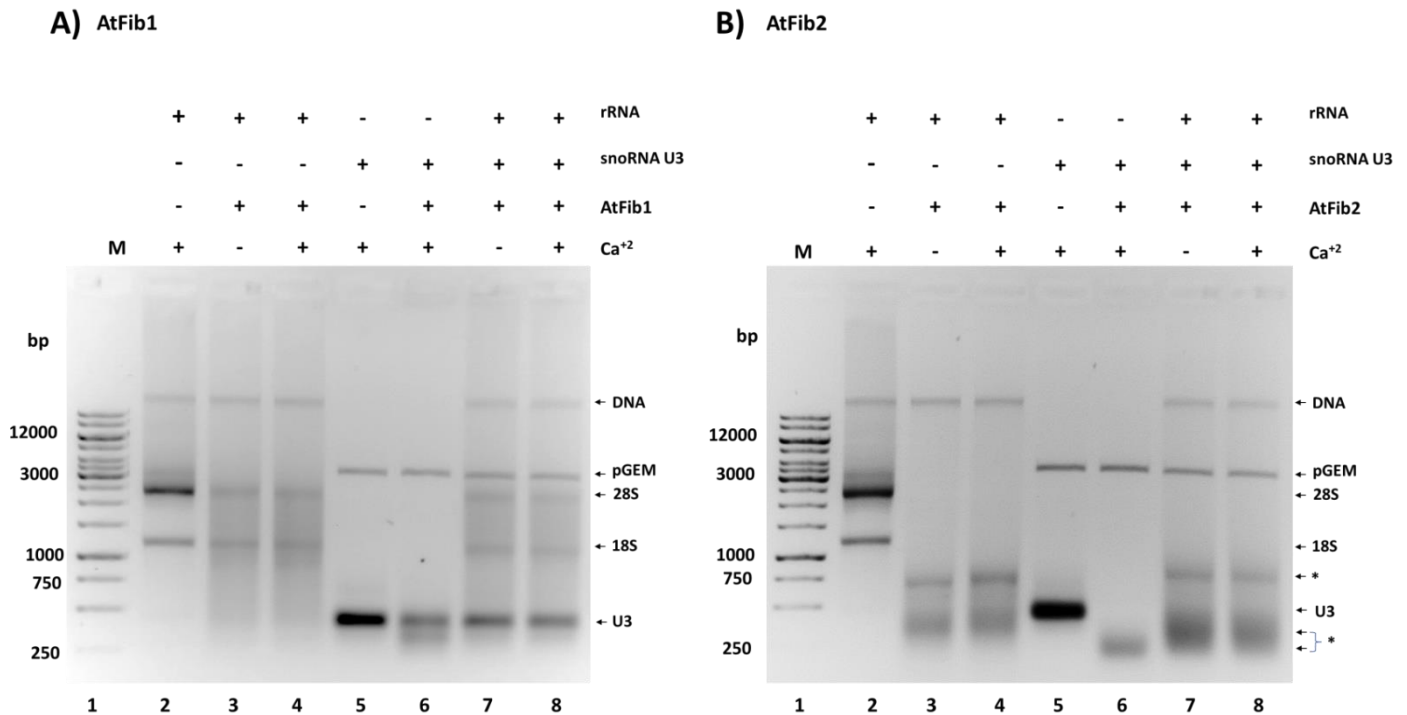
Figura 3.6 Ribonuclease activity against RNAr and snoRNA U3. (A) AtFib1 ribonuclease activity against RNAr and snoRNA U3. As expected, compared to AtFib2, AtFib1 has significantly lower ribonuclease activity either to RNAr (lanes 3 and 4) or to snoRNA U3 (lane 6–8). **(B)** AtFib2 ribonuclease activity against RNAr and snoRNA U3. As expected, compared to AtFib1, AtFib2 has ribonuclease activity against RNAr (lanes 3 and 4) and snoRNA U3 (lane 6–8). The asterisks show ∗ the positions of specific RNA bands originated from RNAs cleaved by AtFib2.

Discussion

The plants genomes are a mix of duplicated and triplicated regions, which results from the series of whole-genome duplication events (WGD) known as paleopolyploidy, events that occurred throughout plant evolution. These events have played a major role in Brassicaceae evolution. *A. thaliana* has undergone three paleopolyploidy events (At-α, At-β and At-γ; (Bowers et al., 2003; Schranz et al., 2012). These variations in gene copy number, retention of duplicated copies, and posterior sub- or neo-functionalization, increase the genetic variation (van den Bergh et al., 2016) which play an essential role in the environment adaptation (Dassanayake et al., 2011). The major transcriptional differences between AtFib genes indicate the great importance of the functional fate of duplicated copies, which could have implications on protein activity. AtFib1 and AtFib2 are expressed in large amounts and in all tissues as seen in figure 1B. Therefore, changes in the known functions can be expected for these proteins as they acquire different mutations. However the differences are localizing to the GAR domain. Fibrillarin is also well known to be involved in pre-RNAr processing in nucleolus in several organisms. However the mechanism of its action is still largely unknown and a variation of function may occur during gene duplication and subsequent differential mutagenesis. Since the early experiments of Tollervey and collaborators with temperature sensitive mutants of Nop1 (yeast fibrillarin), the main attributed activity of fibrillarin was a methyltransferase for RNAr (Tollervey et al., 1993) and more recent for histone H2A (Tessarz et al., 2014; Loza-Muller et al., 2015). However, even during the early experiments with mutant Nop1, the yeasts showed different phenotypes before dying at the non-permissive temperature, in particular, the nop1.2 and nop1.5 alleles showed a reduced level of synthesis for both 18S and 25S RNAr, moreover the production of all pre-RNAr species decreased except the

main 35S primary transcript (Tollervey et al., 1993). This indicates that some mutants are not able to cut the pre-RNA to produce the mature forms.

One of the main features of fibrillarin is the N-terminal GAR domain. It is the least evolutionary conserved domain of the protein; however, this sequence was added in the transition between Archaea to Eukariotic cells as it is absent in all Archeobacteria. This domain is also responsible for the phosphoinositide binding, which well correlates with the lack of it in Archaea kingdom (Amiri, 1994; Hickey et al., 2000; Wang et al., 2000). Furthermore, nucleolar localization requires the GAR domain (Snaar et al., 2000). Fibrillarin forms a complex with Nop56, Nop58, a guide RNA and 15.5k, we postulate that the fibrillarin ribonuclease activity is directed by the complex to selective sites. Currently, we and others have been unsuccessful to form an active eukaryotic ribonucleoprotein complex with fibrillarin (Peng et al., 2014). These complexes have been successfully carried out in Archaea that lack the GAR domain, but not with any of the eukaryotic counterparts (Peng et al., 2014).

One elusive question in regard to ribosomal processing is the nature of the endonuclease activity involved in catalysis of the primary pre-RNA cleavage in eukaryotic cells. Fractions carried out by Saez-Vasquez and collaborators showed a highly purified high-molecular-weight complex, which reproduce this cleavage in vitro. The authors could not discern which protein had the ribonuclease activity, but they identified nucleolin and fibrillarin as important proteins in this fraction (Saez-Vasquez et al., 2004). Other previous experiments suggested that fibrillarin is the ribonuclease protein involved in the cleavage of RNAr (Kass et al., 1990). They used specific antibodies against human fibrillarin native complex in an in vitro ribonuclease assay and showed a decrease in activity when the fibrillarin was blocked (Kass et al., 1990). Surprisingly the authors did not suggest that fibrillarin was involved in the cleavage of RNAr but assumed that it affected the complex. Also fibrillarin was identified in the "Christmas trees" as part of the pre-RNAr early processing complexes (Scheer and Benavente, 1990). From our work, we can speculate that AtFib2 ribonuclease activity is involved in the processing of RNAr and that when complex with Nop 56, 58 and 15.5K together with the guide RNA may direct fibrillarin for sequence specific breaks as was shown with the complex by Kass and colaborators, 1990.

Previously we showed that human fibrillarin was able to interact with phosphatidylinositol 4,5-bisphophate (PtdIns(4,5P)$_2$), one of seven phosphoinositides (Yildirim et al., 2013). Amino acids 9 to 25 of the GAR domain of both AtFib2 and human fibrillarin are absent in AtFib1 and may explain their similarities between both of these proteins. The nuclear phospholipids, in particular phosphoinositides, can be located in nuclear speckles, intra nuclear chromatin domains as well as nucleoli. They interact with a wide range of proteins like: Star-PAP poly(A) polymerase, Histone 1, TAF3, UBF etc. (Osborne et al., 2001; Yildirim et al., 2013; Divecha, 2016). The interaction of phospholipids with such proteins can result in the activation of the protein (like Start-PAP) or affect the stability with other proteins to form particular complexes like TAF3 with H3K4me3 (Stijf-Bultsma et al., 2015). The complex nuclear environment contains large amounts of these phospholipids in a non-membrane fashion for complex formation.

Here we show a differential binding of phospholipids to *A. thaliana* fibrillarins. Taken into account that phosphoinositides-protein interaction affects the protein ability to form new complexes it is therefore likely that both fibrillarins in *A. thaliana* bind to different partners. This may also explain why confocal microscopy of both fibrillarins does not colocalize 100% with the PtdIns(4,5)P$_2$ signal as it does in human cells (Sobol et al., 2013). Phosphatidic acid has been shown to inhibit RNase A (Hatton et al., 2015), here we show that it is also able to decrease the ribonuclease activity of fibrillarin.

It has been proposed that GAR domain can destabilize the RNA secondary structure during their interaction (Pih et al., 2000). However, it is unclear which structure can be generated when GAR domain is bound to phospholipids or during its interaction with RNA. The interaction of GAR domain with phospholipids may also explain the fibrillarin phase separation behavior for proper subnucleolar compartment formation and maintenance (Feric et al., 2016). However, the structural phase separation may be more complex involving phospholipids and their metabolism, as well as other ribonucleoproteins and guides RNA. The structure alterations of the nucleoli can be observed with different transcription inhibitors like Actinomycin D. Upon transcription inhibition, the separation of nucleolar compartments forms a two phase separated system similar to what is observed

whit a mix of hydrophobic molecules in water (Sobol et al., 2013; Feric et al., 2016).

Several questions arise from this work including the role of fibrillarin in Cajal bodies: does it have a role in mRNA processing? Is there a ribonuclease role of fibrillarin as mediator 36a? During cell cycle, does the alteration in nuclear structure in fibrillarin depleted cells is due to degradation of structural RNA? Do viral particles require fibrillarin due to its role in RNA processing? Does GAR domain methylation by any or all of the methyltransferases (PRMT1, PRMT3, PRMT5 etc.) affect ribonuclease activity?

CAPITULO IV.

En este capítulo titulado "Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in Brassica oleracea" se logró reproducir el resultado obtenido por Tessarz y colaboradores los cuales describieron la metilación de la glutamina 105 en humanos y 104 en levadura de la histona H2A mediante la fibrilarina, Nop1, sin la presencia de proteínas auxiliares o algún tipo de RNA guía. La glutamina modificada se encuentra presente sobre la unidad transcripcional del DNAr 35S, es especifica al nucléolo y forma parte del sitio de unión al facilitador de transcripción de la cromatina, FACT por sus siglas en inglés (Tessarz et al., 2014). Utilizando la fibrilarina 2 de *A. thaliana* (AtFib2) se comprobó la interacción entre AtFib2 - histona H2A y que la metilación en la glutamina es una modificación presente en plantas, que a diferencia de lo reportado por Tessarz y colaboradores esta también se encuentran en la periferia del núcleo.

Los resultados de este trabajo fueron publicados en 2015 en la revista Frontiers in Plant Science, doi: 10.3389/fpls.2015.00976 (Loza-Muller et al., 2015) y cumplen con el último objetivo planteado para esta tesis.

Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in Brassica oleracea

Lloyd Loza-Muller[1], Ulises Rodríguez-Corona[1], Margarita Sobol[2],Luis C. Rodríguez-Zapata[3], Pavel Hozak[2] and Enrique Castano[1]*

Unidad de Bioquímica y Biología Molecular de Plantas, Centro de Investigación Científica de Yucatán, Mérida, Mexico[1],Department of Biology of the Cell Nucleus, Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic [2].Unidad de Biotecnología, Centro de Investigación Científica de Yucatán, Mérida, Mexico [3].

Abstract

Fibrillarin is a well conserved methyltransferase involved in several if not all of the more than 100 methylations sites in RNAr which are essential for proper ribosome function. It is mainly localized in the nucleoli and Cajal bodies inside the cell nucleus where it exerts most of its functions. In plants, fibrillarin binds directly the guide RNA together with Nop56, Nop58, and 15.5ka proteins to form a snoRNP complex that selects the sites to be methylated in pre-processing of ribosomal RNA. Recently, the yeast counterpart NOP1 was found to methylate histone H2A in the nucleolar regions. Here we show that plant fibrillarin can also methylate histone H2A. In Brassica floral meristem cells the methylated histone H2A is mainly localized in the nucleolus but unlike yeast or human cells it also localize in the periphery of the nucleus. In specialized transport cells the pattern is altered and it exhibits a more diffuse staining in the nucleus for methylated histone H2A as well as for fibrillarin. Here we also show that plant fibrillarin is capable of interacting with H2A and carry out its methylation in the rDNA promoter.

Keywords: histones, methylation, RNA polymerase I, *Brassica*, phosphoinositide
Abbreviations: aFib, archaea fibrillarin; AtFib1, *Arabidopsis thaliana* fibrillarin 1; AtFib2, *Arabidopsis thaliana* fibrillarin 2; BoFib, *Brassica oleracea* fibrillarin; DABCO, 1,4-Diazabicyclo(2.2.2)octane; DAG, diacylglycerol; DAPI, 4 ,6-diamidino-2-phenylindole; DFC, dense fibrillar component; DTT, dithiothreitol; FAA, formalin – acetic acid – alcohol; FACT, facilitator of chromatin transcription; FC, fibrillar center; GAR, arginine glycine rich

domain; GC, granular component; GMSA, gel mobility shift assays; HRP: horseradish peroxidase; HsFib, *Homo sapiens* fibrillarin; IPTG, Isopropylthiogalactoside; IRES, internal ribosome entry site; NCBI, national center for biotechnology information; NE, nuclear extract; Nop1, nucleolar protein 1; Nop56: nucleolar protein 56; Nop58, nucleolar protein 58; PBS, phosphate-buffered saline; PBST, Phosphate-buffered saline tween; PI4,5P2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5 trisphosphate; PLC, phospholipase C; PVDF, polyvinylidene difluoride membrane; rDNA, ribosomal DNA; RNAr, ribosomal RNA; SAM, *S*-adenosyl methionine; SMN, survival of motor neuron; snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein; TBS, Tris-buffered saline; TBST, Tris-buffered saline tween; U2OS, human osteosarcoma cell line; UBF, upstream binding factor.

INTRODUCTION

The nucleolus is the largest structure inside the cell nucleus. The main function of this structure is ribosome biogenesis. This process involves transcription of rDNA, processing of RNAr and assembly of ribosomal proteins (Kressler et al., 1999). Ribosomal genes (rDNA) in eukaryotes are in a tandem arrayed of 100–1000s (depends on the species) copies at chromosomal loci, known as nucleolus organizer regions. Each RNAr gene is transcribed within the nucleolus by RNA polymerase I to produce a primary transcript that is processed to form the 18S, 5.8S, and 25–28S RNArs (Nemeth and Langst, 2011). However, the nucleolus is also involved in several other processes like genetic silencing, cell cycle progression, senescence and biogenesis of snRNA and tRNAs (Jacobson and Pederson, 1998b; Cockell and Gasser, 1999; Garcia and Pillus, 1999). In plants the nucleolus consists of four components: FCs, DFC, GC and the nucleolar vacuole (NV). Fibrillarin is a methyltransferase involved in the processing of the primary ribosomal transcript and is mainly located in the FC and DFC region of the nucleoli where it is directly involved in several steps of ribosome biogenesis (Rodriguez-Corona et al., 2015). Fibrillarin is known to be part of the snoRNP that methylate RNAr (Tollervey et al., 1993). Biochemical evidence for the process with eukaryotic fibrillarin is lacking but it has been demonstrated using aFib in order to recapitulate the methylation process on RNAr (Tran et al., 2003). High resolution crystal structure data from this complex has been obtained by several laboratories (Aittaleb et al., 2003; Oruganti et al., 2007; Ye et al., 2009) and have

shown a well conserved overall structure (Rodriguez-Corona et al., 2015). The snoRNA acts like a guide to help direct aFib together with Nop56/58 and L7Ae that interact with the RNAr in order to methylate at specific sites. In eukariotes fibrillarin has been shown to form a complex with Nop56, Nop58, protein 15.5Ka and different guide RNAs like U3, U6, etc. The guide RNA recognizes specific regions to be methylated on RNAr. Fibrillarin is also involve in the earliest steps of ribosomal transcription initiation and this steps require the interaction with PI4,5P2 (Sobol et al., 2013; Yildirim et al., 2013) linking the RNAr processing with RNAr transcription initiation where PLC can inhibit transcription initiation (Yildirim et al., 2013). Overproduction of fibrillarin in mammalian cells can lead to alteration on ribosomal methylation and as a result there is an alteration in the process of translation. Highly methylated ribosomes surpass IRES leading to misread translation that results in some types of cancers (Marcel et al., 2013). In plants, fibrillarin has been shown to be part of the mediator of RNA polymerase II transcription (subunit 36a) (Backstrom et al., 2007). Two different RNA binding sites have been determined in fibrillarin from *Arabidopsis thaliana* (Rakitina et al., 2011). Plant fibrillarin has also been a link between both RNAr gene binding and pre-RNAr processing by analyzing the fractions containing the snoRNP complex in both promoter complex and RNAr cleavage sites (Saez-Vasquez et al., 2004). Moreover, plant umbravirus life cycle suggest the requirement of fibrillarin. Fibrillarin is redistributed upon infection to the cytoplasm and participates in the formation of viral ribonucleoproteins able to move through the plant phloem resulting in complete infection of the plant (Kim et al., 2007). Recently, fibrillarin has been shown to be involved in epigenetic nucleolar mechanism. Fibrillarin methylate histone H2A in yeast and human cells at position Q105 and this methylation is unique to the nucleolus (Tessarz et al., 2014). The FACT (facilitates chromatin transcription) is a protein complex known to facilitate transcription elongation of RNA pol II derived transcription where it has a preferential interactionto histone H2A/H2B dimers. In RNA pol I transcription FACT interacts preferentially with the methylated H2A to reorganized nucleosomes in the active promoters for RNAr (Tessarz et al., 2014). Nevertheless, the ribosomal promoter has been shown to differ significantly between mammalian and plants (Perry, 2005; Knight et al., 2014). We show that plant fibrillarin is also capable to methylate histone H2A while bound to the rDNA. Our results also showed that *in vivo* methylated histone H2A in *B. oleracea* can also be found at other locations besides the nucleolar regions, this modification in

plants may have additional epigenetic roles than what is found in animal cells.

MATERIALS AND METHODS

Maintenance and Propagation of Cell Culture. U2OS osteosarcoma cells were kept in DMEM with 10% fetal calf serum in 5% CO_2/air, 37°C, humidified atmosphere.

Antibodies. Rabbit polyclonal anti-H2A (Q105Met) was a kind gift from Tessarz et al. (2014). Rabbit Fibrillarin Antibody (H-140): Santa cruz sc-25397); Anti-Histone H2A antibody ChIP Grade (ab15653) Abcam. Anti-Histone H3 (mono methyl K4) antibody – ChIP Grade (ab8895). Goat Anti-Rabbit IgG H&L (Alexa Fluor 647) (ab150079) Abcam. (Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate (Invitrogen) (A-11008).

Nucleotide Sequence Data base. Fibrillarin nucleotide sequence from *B. oleracea* (BoFib) was obtained from the database for *B. oleracea* (http:// www.ocri-genomics.org/bolbase/) with the accession number: Bol39546. All other nucleotide sequence were obtained from NCBI: Saccharomyces cerevisiae fibrillarin (Nop1: CAA98572.1), *Homo sapiens* fibrillarin (HsFib: CAA39935.1) and *Arabidopsis thaliana* fibrillarins 1 and 2 (AtFib1: NP_568772.3, AtFib2: NP_567724.1; respectively).

Plasmids. pET15b::Fibrillarin contain the sequence from *A. thaliana fibrillarin 2* (NP_567724.1). The *pHis::PLC* that expresses recombinant PLC were received from Dr. Hitoshi Yagisawa. All expression vectors were in frame with the histidine tag from the plasmid. pLLMP1 plasmid was constructed by cloning rDNA promoter (−265 to +163) from a PCR of the genomic DNA of *B. oleracea* into pGEM. The oligos used for the PCR of rDNA (fwd 5 -TCGGTAC CGAGTTTAGGATGTCAAGT-3 rev TAGGATCCGGAAAAGTCGCC GGAAAAG-3) (Chen and Pikaard, 1997). pUC18 was from Thermo Fisher Scientific.

Recombinant Protein Expression and Purification. Expression vectors were transformed in *Escherichia coli* BL21 (DE3) pLysE from Invitrogen and allowed to grow to an OD of 0.5 at 600 nm. 1 mM IPTG was added after and incubated at 25° C for 3 h.

Followed by 10 min centrifugation at 4000 × *g*, suspension was carried out in a denaturing buffer (20 mM Tris HCl, pH 7.9, 8M Urea, 0.1 M NaH$_2$PO$_4$, 0.5 M KCl, 20 mM imidazol) and sonicated three times. The re-suspended lysate was centrifuged at 4000 × *g* for 10 min to remove cell debris and the supernatant was allowed to binding 0.1 ml of Ni^{2+}-nitrilotric acetic acid resin for 1 h. The column was wash with 5 ml of the denaturing buffer. Finally 0.3 ml of elution where recovered in a denaturing buffer containing 250 mM Imidazole.

Nuclear Extract and Histone Purification. *Brassica oleracea* nuclear extraction was carried out as described by (Gustavsson et al., 1991). Briefly we used 60 g of fresh weight for the maceration in liquid nitrogen and suspended at 4°C with an extraction buffer 50 mM Tris-Cl pH 8.0, 3 mM EDTA, 2 mM EGTA and 0.2% NP 40. Debris was removed and the extract collected. Centrifugation of the extract was carried out and the nuclei were responded in a hypotonic buffer for 30 min at 4°C followed by addition of an extraction buffer 10 m M Tris-Cl pH 8.0, 1.5 M NaCl, 0.05% NP40 to obtain the NE after centrifugation at 6500 *g* for 10 min. The extraction of histones from *B. oleracea* was carried out from the left over nuclear pellet and high salt extraction buffer 10 m M Tris-Cl pH 8.0, 2.5 M NaCl, 0.05% NP40 was added for 30 min under rotation at 4°C. Centrifugation at 16000 *g* for 10 min. was carried out and the remaining extract contain a large amount of histones.

Western Blot Analysis. Proteins were separated on a 15% SDS-PAGE and transferred to nitrocellulose membrane (Pall Corporation, USA). After 1h of blocking with 5% non-fat milk in TBST (TBS, 0.1%Tween-20), the membrane was incubated with either anti-H2AQ105 or anti fibrillarin as mention in the legends in TBST with 5% milk over night at 4°C then washed with TBST. Immunoreactive bands were detected with anti-rabbit antibodies conjugated with HRP followed by AlkPhos direct labeling reagents (Amersham).

Immunofluorescence. The plant tissue was fixed in tubes containing FAA with aspiration for 24 h. They were dehydrated through an ethyl alcohol series and embedded in paraffin (melting point 54–56°C) with a graded series of tertiary butyl alcohol. The paraffin blocks were sectioned serially at 5 µm thickness using a microtome. The deparaffinization was

carried out with four washes with Histology grade Xylene for 2 min and by removal of xylene with absolute ethanol. Seventy percent ethanol followed by water for 1 min each. *B. oleracea* inflorescence and surrounding tissue were permeabilized with 0.1% Triton X-100 in PBS for 15 min, respectively. After washes with PBST they were either incubated with anti-H2AQ105me or anti-fibrillarin. Secondary antibodies donkey anti-rabbit IgG conjugated with Alexa 488 (Invitrogen), goat anti-rabbit IgG conjugated with Alexa 647 (Invitrogen). After being washed for 30 min with PBST cells were mounted with moviol (DAPI-DABCO). Images were taken in confocal microscope (Leica TCS SP5 AOBS TANDEM) and a laser-scanning microscope FV100 Olympus with 60X (NA 1.4) oil immersion objective lens. U2OS were treted as published previously (Sobol et al., 2013).

Gel Mobility Shift Assays. Gel mobility shift assays were carried as previously publish (Castano et al., 1997), with minor modifications. End-labeled rDNA promoter was incubated for 30 min with 10 ng of purified protein at room temperature using the binding reaction contained 5 ng of probe (5000 c.p.m./ng), 25 mM HEPES (pH 7.4), 80 mM NaCl, 10% glycerol, 0.5 mM PMSF and 1 mM leupeptin in a final volume of 20 µl. The misture was separated in a native 6% PAGE at 4° C followed by autoradiography.

Methylation. For assays on purified histones, 0.2 µg of AtFib2 was assayed on 1 µg of purified histones in the presence of 100 µM SAM (H^3) in 1∕2 TBS and 1 mM DTT for 30 min at 30°C. Half of the reaction was loaded on SDS–polyacrylamide gel electrophoresis for Coomassie staining and 20% of the reaction for western blotting or for scintillation counting.

Farwestern. Purified histones were separated on an 15% SDS PAGE and transfer to a PVDF membrane, Membrane was blocked with PBST with 5% of non-fat milk (PBS. 0.1% tween-20) for 1 h at room temperature, then washed three times with PBST. After blocking the membrane was incubated with AtFib2 (0.5 µg) as bait in protein binding buffer (20 mM Tris pH 7.6, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol, 0.1% tween-20, 2% non-fat milk, 1 mM DTT) at 25[a]C for 4 h, then washed three times with PBST and incubated with anti-Fibrillarin (for 12 h at 4[a]C). Immunoreactive bands were detected with anti-rabbit antibodies conjugated with HRP followed by AlkPhos direct labeling reagents (Amersham).

Striping of the membrane was incubated at 50°C for 45 min under agitation in a buffer (50 mM Tris HCL pH 6.8, 2% SDS and ß-mercaptoethanol(8ml/l)) followed by rinsing the membrane with water.

Transcription Pull-down *In Vitro.* Methodology published in (Castano et al., 2000). Brief explained a reaction mixture containing either NEs or purified transcription factors were mix with 100 ng of RNAr promoter in the presence of 0.5 mM NTP, 5 mM MgCl, 5 mM DTT, in 20 mM HEPES KOH pH 8.4, in 20 ul reaction volume. In order to assay if H2A methylation was bound during the transcription, rDNA promoter region was bound to magnetic beads (Dynabeads MyOne Streptavidin C1, 650.01, Invitrogen). The promoter was obtained by PCR from the plasmid containing the RNA pol I promoter sequence from *Brassica oleracea*. The oligos used were a 5 -biotin labeled oligo TCGGTACCGAGTTT AGGATGTCAAGT-3 (promoter region from −265 to −248) and a reverser oligo 5 - TAGGATCCGGAAAAGTCGCCGGAAAAG-3 from +142 to +163 (published by Chen and Pikaard,1997), Control oligos 5 -biotin labeled pUC18 CCC AGTCACGACGTTGTAA and a reverse CGCAACGCAATTAATGTGAG were purchase from Sigma–Aldrich. Before adding the NEs the bound sequences were blocked with 5% BSA for 1 h at 4°C. The beads were then incubated with NE in a transcription buffer without nucleotides for 1 h, after incubation the beads were washed six times with a buffer containing 20 mM Tris pH 7.9, 100 mM KCl, 0.1 mg/ml BSA, 10% Glycerol, 0.2 mM EDTA pH 8.0. The full amounts of beads were loaded into a PAGE for western blot analysis. PVDF membranes were soaked in Ponceau S stain [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] to verify protein transfer.

RESULTS

Fibrillarin sequence can be divided into four regions: The GAR domain, Space region, the Central domain with the RNA binding region and the Alpha helix rich domain. The GAR domain istypically the least conserved and contains a non-structural motif that is methylated in human cells. Figure 4.1 shows the sequence alignment and domain position for fibrillarins. The comparison between human, yeast, *A. thaliana* and *B. oleracea* reveal that the GAR domain contains the lowest degree of conservation with 31.82% of similarity between AtFib1 and Nop1 as the lowest and with 49.32% of similarity between AtFib2 and

BoFib as the highest. The RNA binding domain is well conserved in all species with 72.04% of similarity between AtFib1 and Nop1 as the lowest and with 98.92% of similarity between AtFib1 and AtFib2 as the highest. The alpha helix rich domain differs by 63.44% of similarity between HsFib and Nop1 as the lowest and with 90.72% of similarity between AtFib1 and AtFib2 as the highest, and is known to interact with other proteins in mammalian cells like SMN (Pellizzoni et al., 2001). The red marked amino acids indicate the sites for mutations that allowed Nop1 to be a temperature sensitive mutant. These are key amino acids in Nop1 and are essential for yeast viability at 37°C. We find that for the most part are well conserved, with two alterations between *B. oleracea* and Nop1 located at the N terminus. The two green slash boxes highlight the sequences defined by Rakitina et al. (2011) to be responsible for RNA binding in *Arabidopsis*, while the green letters define the human RNA binding domain. The bold blue label arginine amino acids in the sequence are known to be methylated in human cells. The yellow boxed serine is known to be phosphorylated and the black boxed lysine to be acetylated in human fibrillarin. Although the exact function of all the modifications has still to be defined in any species, and may reflect the high versatility of this protein in different complexes that may occur in the cells (Rodriguez-Corona et al., 2015).

Figura 4.1 Fibrillarin sequence comparison relationships of taxa. The analysis included the sequences from both *Arabidopsis thaliana* fibrillarin (AtFib1 NP_568772.3 and AtFib2 NP_567724.1), fibrillarin sequence from *Brassica oleracea* (BoFib Bol039546), fibrillarin sequence from *Homo sapiens* (HsFib CAA39935.1) and the yeast fibrillarin Nop1 CAA98572.1. All the domains are label in different colors GAR domain in blue, space region in gray, central domain in purple and the alpha rich domain in orange. Arginines known to be methylated are marked in blue. Key amino acids that were mutated in Nop1 are marked in red. The phosphorylated serine is marked in a yellow square and the acetylated lysine in a black square. The doted underline sequence marks the methyl transferase domain. The slash boxes in green indicate the RNA binding domains in *Arabidopsis thaliana* fibrillarins. Green label amino acids indicate the define RNA binding region.

In order to assay the effect of phospholipids in specific histone binding to the rDNA *in vitro* we tested the NE and purified histones from *B. oleracea* on rDNA promoter binding.

We used magnetic beads with the rDNA promoter region as bait. Figure 4.2A shows a typical pull-down experiment, the asterisks indicate the proteins that were specifically bound to the promoter. The amount of these bound proteins increased with the pre-incubation of PLC to the NE. PLC acts on PI4,5P2 which is a known phosphoinositide that binds several nuclear proteins including fibrillarin and histones (Yu et al., 1998; McLaughlin et al., 2002; Yildirim et al., 2013). Since several of the proteins bound to the promoter had a similar profile to that of histones, we further tested that PLC treatment would affect the interaction of histones to the promoter. We carried out a GMSA (Figure 4.2B) with purified histones from *B. Oleracea* and used them to bind the rDNA promoter. Histones bound readily to the promoter and PI4,5P2 degradation by PLC showed an increased histone binding to the promoter. However, the NE reduced significantly the binding of histones to the promoter. This indicates a competition for the interaction between the histones and the ribosomal promoter with NE components that prevent this interaction. PLC treatment under these conditions did not show a significant increase in binding.

Figure 4.2 *In vitro* interaction of the RNAr promoter and Histones in *B. oleracea*. (A) RNAr promoter pulldowns of *B. oleracea* nuclear extract. Specific bands label with an * represent selective binding proteins to the rDNA promoter. Larger amount was obtained upon PLC treatment to the extract prior to promoter pulldown. **(B)** GMSA with the rDNA promoter. NE was used in combination with purified histones with PLC pretreatment or without the arrows indicates protein-DNA complexes

Histone H2A found in active rDNA has been recently shown to be methylated in the nucleolus by fibrillarin in yeast and human cells (Tessarz et al., 2014), therefore we decided to test if plant fibrillarins can also methylate H2A at the rDNA promoter. Purified histones from *B. olearace* were used (Figure 4.3A) and tested for both protein–protein interactions and methylation using AtFib2 (Figure 4.3B) which is 88% identical to BoFib. To test for protein-protein interactions we used a far-western approach where the transferred histones were used as bait for AtFib2. Western blot of AtFib2 shows the amount used in the assay (Figure 4.3B). The binding of AtFib2 to histone H2A is shown by farwestern (Figure 4.3C). Histone H2A was verified by western blot after stripping (Figure 4.3D). We expected fibrillarin to tightly bind their substrates until the enzymatic reaction could be accomplished plus previously this possible interaction was obtained from a two hybrid system in the interactome data published (Krogan et al., 2006). Krogan et al. (2006) showed H2A among several other proteins that can bind human fibrillarin. After farwestern blot analysis we proceeded to carry out a methylation assay to verify if fibrillarin methylate histones *in vitro* (Figure 4.3E). This was done by mixing tritium radiolabeledvSAM, AtFib2 and histones from *B. oleracea*. After the reaction, the histones were separated in a 15% SDS PAGE and stain histones were measured on a scintillation counter showing specific addition of the radiolabeled SAM by the addition of AtFib2.

H2A methylation was further checked by western blot (Figure 4.3F). The aid of anti-H2AQ105me previously used to check H2A methylation by yeast fibrillarin Tessarz et al. (2014) showed successfully that AtFib2 methylate histones H2A from *B. oleracea*. Moreover, AtFib2 methylated H2A while bound to the rDNA promoter. We tested this by allowing the histones from a methylation reaction bind to the rDNA promoter attached to magnetic beads for 1 h. The rDNA promoter bound proteins were resolved on a 15% SDS PAGE and western blot was carried out with anti-H2AQ105me detecting large amounts of

methylated H2A as compared with a pUC18 sequence bound to magnetic beads used as control (Figure 4.3F). Since control and rDNA promoter beads were incubated in a buffer containing BSA, the loaded amount was verified by staining the membrane with ponceau and checking that BSA amounts should be equal.
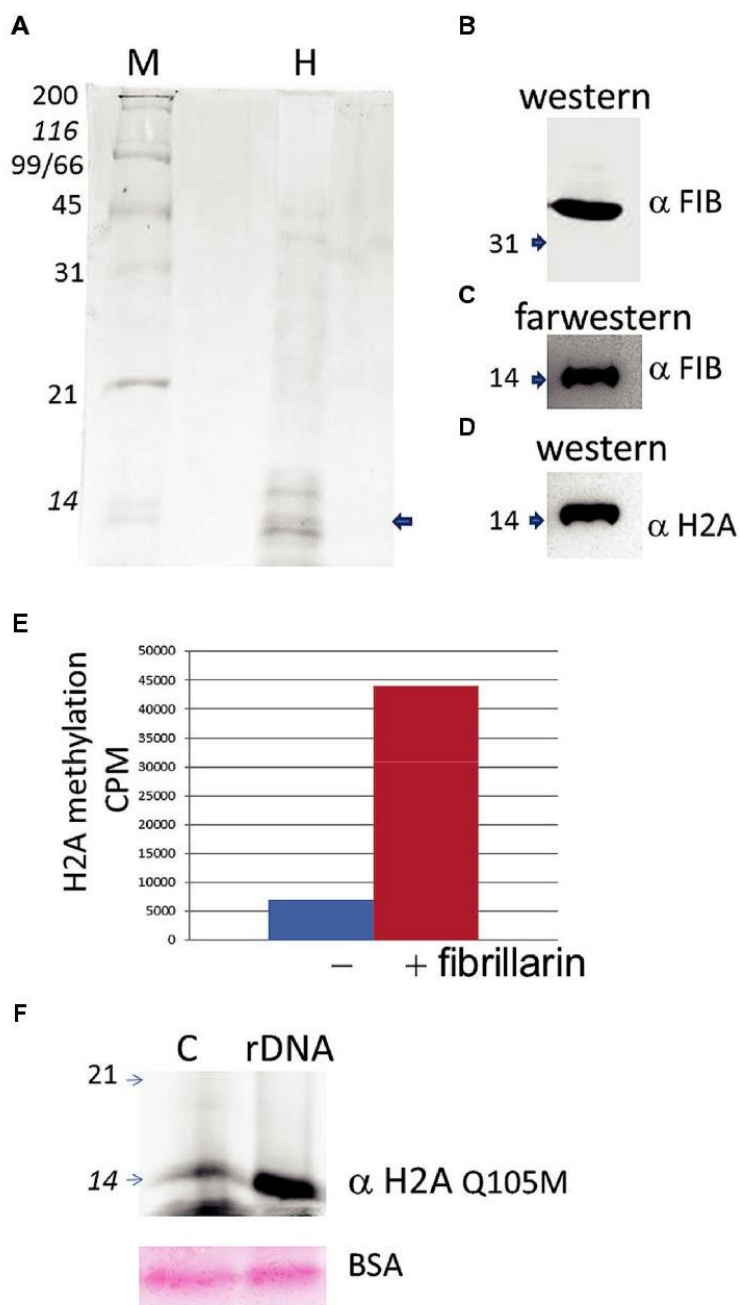
Figura 4.3 Histone H2A methylation in *B. oleracea*. (A) Purified histones from *Brassica* as seen in a coomasie stain. M indicate protein weight marker and H the purified histones. The arrow shows the band that is labeled by farwestern in the position of H2A. **(B)**. Western blot of the purified AtFib2. **(C)** Farwestern of the purified histone fraction. Purified AtFib2 was used to screen the histones and find specific interacting partners. **(D)** Western blot of H2A to mark the position of this histone after striping the membrane. **(E)** *In vitro* methylation assay with or without AtFib2 with the *B. oleracea* purified histones. **(F)** Western blot with anti H2A Q105me on histone pulldowns with a control beads (C) or with beads with the rDNA promoter (rDNA). Below is shown the ponceau stain of BSA from the transfer membrane. Numbers indicate the KDA by the marker.

We proceeded with the *in vivo* localization of methylated H2A by immunolocalization in cells of *B. oleracea*. The immunolocalization pattern of anti-H2AQ105me and fibrillarin was compared between human U2OS cells and *B. oleracea* cells. Both plant and human cell lines showed a primary stain of fibrillarin and methylated histone H2A in the nucleoli. Human U2OS cells were used as a control since the immunolocalized pattern for H2A Q105me had already been published (Tessarz et al., 2014). Here we show a higher magnification the staining of anti-H2AQ105me in human cells. As can be seen the stain at the nucleolus is not homogenous and there is a weak diffuse nuclear stain (Figure 4.4A). Floral meristem *B. oleracea* cells showed fibrillarin stain located in the nucleoulus (Figure 4.4B). The secondary antibody did not stain the cells and was used to set the intensities of the signals (Figure 4.4C). The staining with anti-H2AQ105me shows an additional stain on the periphery of the nucleus and additional stain outside the nucleus (Figure 4.4D). This pattern of stain was reproducible in three independent experiments and in all the fresh cauliflower floral meristem buds that have a round nucleus. The staining was specific to anti-H2AQ105me as addition of just secondary antibody did not stain the cells (Figure 4.4E). The additional stain of the anti H2AQ105me outside the nucleus also shows exactly in the same position a weak DAPI stain at the extra nuclear regions, we were surprise by this extra nuclear DNA, but it is been consistent in tree independent experiments with different reagents. This extranuclear DNA could be either an aggregation of organelles like mitochondria from the meristematic cells as previously shown by Kuroiwa et al. (1992).
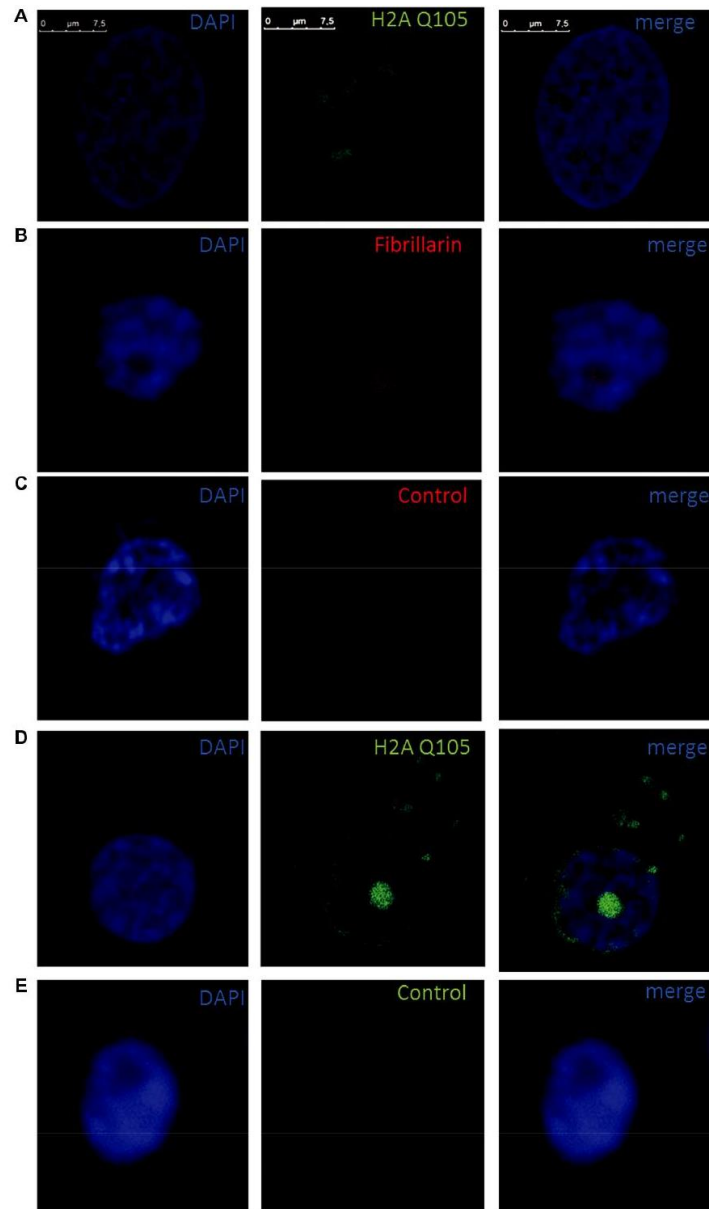
Figura 4.4 Immunolocalization of methylated H2A in *B. oleracea*. All cells where stained with DAPI. **(A)** U2OS cells immunostained with anti H2A Q105me show a specific nucleoli stain. **(B–E)** Nucleus from *B. oleracea* fresh cauliflower floral meristem cells with round nucleus. **(B)** Immunostained with antibodies against fibrillarin. **(C)** Control secondary antibody only couple to alexa 555. **(D)** Imunolocalization of methylated H2A. **(E)** Control secondary antibody only couple to alexa 488.

We also checked the pattern in specialized cells. The vascular inflorescence cells showed a different stain as seen in Figure 4.5. Due to the type of tissue, these cells are elongated in order for them to carry out their function. The nucleus is also elongated and thinner than in meristem cells. Here the fibrillarin stainwas not only localized to the nucleolus but showed a diffuse stain in most of the nucleus (Figure 4.5A). This is a typical localization of fibrillarin in cells that are under stress (Mironova et al., 2014). As well as in cells that overexpress fibrillarin. Specialized transport cells in plants may reflect this pattern for unknown functional roles at this time. None of these specialized cells showed additional extrachromosomal staining as compared with all of the meristem cells that had a weak extranuclear DAPI stain. The Anti-H2A (Q105M) staining showed a similar pattern to that of fibrillarin. However, these cells had no perinuclear staining or additional extra nuclear stain (Figure 4.5B). These are the first results that show nucleolar methylated histone H2A in plants and may involve a conserved epigenetic rDNA transcriptional mechanism for all eukaryotic cells nucleoli. The immunolocalization of Dimethylated lysine 4 in histone 3 in these cells shows an overall nuclear pattern, with no selectivity for the nucleolus as compared with methylated H2A (Figure 4.5C). Furthermore, the methylation of histone H2A in specialized cells can be involved in other epigenetic mechanism that can be specific to plants outside the nucleoli as shown by the immunolocalization pattern of H2A.
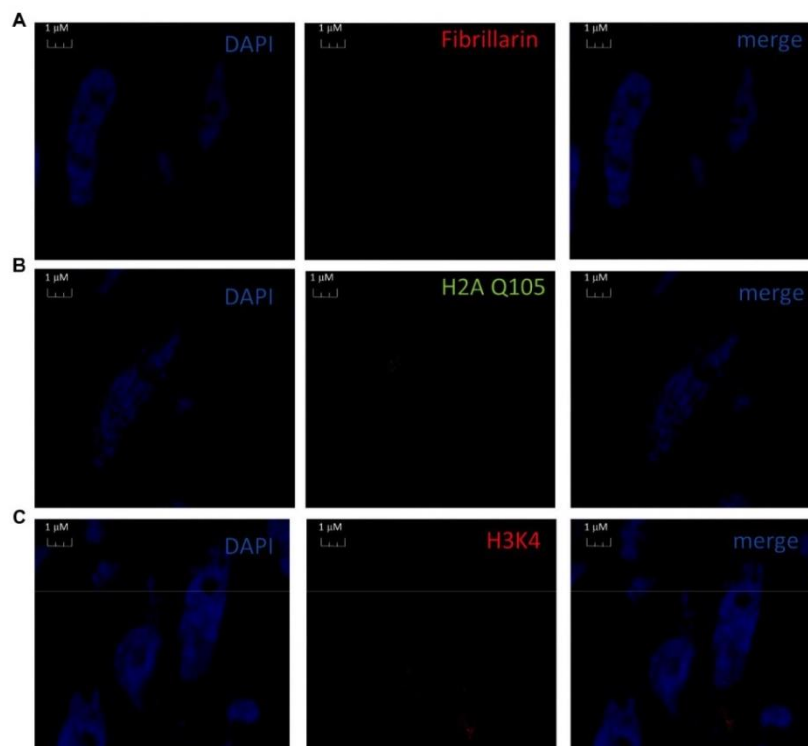
Figura 4.5 Nuclear immunolocalization of *B. oleracea* vascular cells. All vascular cells were stained with DAPI. **(A)** Immunolocalization of Fibrillarin. Immunolocalization of methylated H2A showing a similar pattern as fibrillarin immunostain. **(C)** Histone H3 dimethylated in lysine 4 was used as control for nuclear staining a different nuclear pattern from H2A Q105me pattern is observed.

DISCUSSION

Fibrillarin sequence in all eukaryotic cells differs from Archaea organisms by addition of the GAR sequence (Rodriguez-Corona et al., 2015); this highly methylated region is responsible for nucleolar localization and protein–protein interaction and is the less conserved sequence in all fibrillarins (Snaar et al., 2000). The lack of conservation in the GAR domain can indicate that only methylated arginine charges are involve for these activities. Although up to date, there is no biochemical data that provides clear function besides the nucleolar localization (Rodriguez-Corona et al., 2015). The central domain, the RNA binding region and the Alpha helix rich domain form the methyl transferase region that allow fibrillarin to methylate RNAr and histones. Tessarz et al. (2014) showed recently that yeast and human fibrillarin can methylate histone H2A and the previously thermo-sensitive yeast fibrillarin (Nop1) mutant (Tollervey et al., 1993) showed a reduction of methyl transferase activity of H2A from Nop1 at the non-permissive temperature after 3 h (Tessarz et al., 2014). Thus showing that Nop1 is responsible for this methylation and the methylation is under constant evaluation by the cell. Probably this is part of the mechanism that helps the cell define the number of ribosomal promoter regions that to be active. Interestingly the key mutated amino acids in the alpha helix in yeast are not well conserved in plants as seen in Figure 4.1. This may reflect the difficulty of some fibrillarins to recapitulate fully all the functions of fibrillarin in a yeast complementary assay (Jansen et al., 1991; Pih et al., 2000).

The promoter of the rDNA from *B. oleracea* was reported by (Chen and Pikaard, 1997) and has been used previously *in vitro* transcription assays. We used the same assay as bait for nuclear proteins in particular histones and fibrillarin. Since PtdIns4,5P$_2$ is known to interact with fibrillarin and histones we tested if the degradation of PtdIns4,5P$_2$ by the

recombinant PLC added into the assay would alter the amount of nuclear proteins that bind the promoter. The results correlates with the studies on histone H1 and H3 interaction with PtdIns4,5P$_2$ where it was suggested that this lipid may promote the formation of less accessible interaction of RNA pol II to the promoter due to higher binding of the histones (Yu et al., 1998). PtdIns4,5P$_2$ is well known phosphoinositide in the signal transduction mechanism in the cell membrane (McLaughlin et al., 2002; Lemmon, 2008; Boss and Im, 2012), where it is digested by PLC into PA and DAG. However, the nuclear form of this lipid has only come into play during the last decade (Osborne et al., 2001; Yildirim et al., 2013). PtdIns4,5P$_2$ is known to bind histone H1, H3 as well as fibrillarin, StarPAP, UBF etc. (Yu et al., 1998; Jiang et al., 2006; Mellman et al., 2008; Yildirim et al., 2013) and localized in transcriptionally active ribosomal promoters in human cells. Up to date it is not clear what is the mechanism by which PtdIns4,5P$_2$ is affecting transcription and it's interesting that its removal increases binding of several proteins to the rDNA promoter as seen in Figure 4.2. H1 was reported to increase its binding activityas a result of PtdIns4,5P$_2$ loss (Yu et al., 1998). However, there is no studies yet done on chromatin structure alteration by phosphoinositides.

The *in vitro* methylation of *B. oleracea* histones by AtFib2 is similar to the results obtained recently by Tessarz et al. (2014) with purified Nop1. This epigenetic mechanism involves fibrillarin marking histone H2A on active ribosomal promoters. Our pulldown experiments with the rDNA promoter show a preferential binding of methylated H2A as compared to a control sequence. As previously publish that fibrillarin and histone bind well to the human rDNA promoter (Yildirim et al., 2013). On a recent model (Leonhardt and Hake, 2014). Fibrillarin interacts with RNA pol I and such interaction represses FACT complex action on chromatin remodeling. This model is interesting considering that fibrillarin in plants has been shown to be part of the mediator for RNA pol II transcription, as up to date there is a missing functional data to explain the function of fibrillarin on the mediator. It may help in the process of chromatin remodeling in other parts outside the nucleolus. It was observed on the immunolocalization of both fibrillarin and methylated histone H2A in the *B. oleracea* nucleus. There is a clear label outside the nucleolus in plant cells that is not seen in human U2OS cells. This may indicate plant fibrillarin role with RNA pol II, however more experiments are required to test this hypothesis. Fibrillarin is primarily located in the

nucleoli, in particular in the DFC and FC regions. However, in these regions, several processes take place, the transcription initiation, elongation and first stages of RNAr processing take place in this region and may involve different functions of fibrillarin, which is well known to methylate RNAr for further processing. Methylation of H2A may help discriminate between active and inactive rDNA and its nucleoli organization. There is evidence that core histone H3 is also located in mitochondria in *B. oleracea*, however, this is not recognize by highly specific antibodies for the N terminal tail region of H3. One possibility is that the N terminal region of H3 is modified and is not detected with these antibodies (Iwasaki et al., 2013). A similar scenario could explain the methylated H2A signal in the extra nuclear stain in the fresh cauliflower floral meristem buds. Since *B. oleracea* meristem cells are the most exposed cells it would follow that it may also have this additional function. Furthermore other explanation may involve ribonucleoproteins known to interact with fibrillarin that can form U bodies structures found in the cytoplasm (Liu and Gall, 2007) although it is unclear why methylation of H2A would be required for this outside the nucleus. Although it is known those histones H2A/H2B have antimicrobial action in particular cells that are closer to the surface as publish (Stekhoven et al., 2004). The absence of this signal in vascular inflorescence cells can be due to a reduction in the number of mitochondria for this cell type or lack of U bodies. Plant viruses that interact with fibrillarin may take advantage of the broad distribution of this protein in this transport cell. The spread of the virus through the plant aided by fibrillarin has been published (Kim et al., 2007) and the diffusion of fibrillarin in vascular cells may help viruses tag alone for distribution through the phloem. The diffusion of the methylated H2A in transport cells correlates well with the diffusion pattern of fibrillarin. However, it is early to define the role of this epigenetic marker and its functional significance in this type of cells. Tessarz et al. (2014) had shown a particular interaction with FACT and it is known that in many cell types FACT facilitates the remodeling of RNA pol II promoter more than RNA pol I promoters. Recently it was shown that FACT– Histone interactions identifies a role of Pob3 C-terminus in H2A– H2B binding (Hoffmann and Neumann, 2015). So it is possible that methylation of H2A in specialized cells may reflect this interaction as suggested by hoffmann and Neumann that FACT interactions are altered by histone posttranslational modification.

CAPITULO V. DISCUSIÓN GENERAL

El nucléolo es una estructura dinámica dentro del núcleo involucrada en una diversa gama de funciones que van desde la transcripción de ADNr, el procesamiento del pre-RNAr, la maduración y el ensamblaje de los ribosomas hasta la respuesta a estrés, respuesta a infecciones virales y la morfología en el desarrollo celular (Olson and Dundr, 2001). Particularmente hablando acerca del procesamiento del pre-RNAr, todos los tipos de proteínas involucradas en el metabolismo del RNA se encuentran presentes en este evento, desde su transcripción mediada por la RNA polimerasa I, hasta la maduración del mismo mediada por distintas endo- y exo- ribonucleasas, así como proteínas que lo modifican (metilación o pseudouridilacion) (Fatica and Tollervey, 2002; Henras et al., 2015). En cuanto a la metilación del RNAr esta tiene lugar en 106 diferentes posiciones de las cuales 39 se encuentran en el RNAr 18S, 65 en el 28S y 2 en el 5.8S. Quince de estas 106 metilaciones, una en el RNAr 18S y 14 en el RNAr son particularmente dependientes a la presencia de la fibrilarina (Sharma et al., 2017). La fibrilarina, es una proteína esencial para la vida cuya función y estructura se encuentra altamente conservada (Jansen et al., 1991). Se estructura por un dominio rico en argininas y glicinas (GAR), una región espaciadora que hemos denominado BCO, un dominio central con un motivo de unión a ARN, y el dominio α-hélice (Aris and Blobel, 1991; Rakitina et al., 2011). Ha sido descrita como una metiltransferasa del grupo 2´- hidroxilo de la ribosa blanco del ARNr (Tollervey et al., 1991), de la glutamina 104 o 105 de la histona H2A (Tessarz et al., 2014) así como el auto antígeno de esclerosis (Aris and Blobel, 1991). Se ubica principalmente en el nucléolo celular (Andersen et al., 2005) donde lleva a cabo la metilación del ARNr, en el núcleo asociada a la proteína BIG 1 (Padilla et al., 2008) y en los cuerpos cajales (Nizami et al., 2010).

Para la obtención de un ribosoma maduro es necesaria la interacción de alrededor de 80 proteínas ribosomales y cuatro RNAr. Tres de éstos provienen de un solo transcrito que al asociarse en el nucléolo con proteínas ribosomales, factores de biogénesis de ribosomas y distintos snoRNA, se denomina pre-ribosoma 90S. Recientemente, utilizando crio microscopía electrónica se reportó la estructura del pre-ribosoma 90S de *Chaetomium*

thermophilum (Kornprobst et al., 2016). Dentro de la variedad de proteínas y complejos identificados en el pre-ribosoma 90S resalta la presencia del snoRNP U3, el cual se encuentra involucrado en el procesamiento del pre-RNAr. Como se ha mencionado anteriormente (Kass et al., 1990), se identificó que U3 se asocia a lo largo del transcrito del pre-ribosoma 90 en la región de transcripción externa 5´ (5´ ETS) y en la región correspondiente al pre-RNAr 18S. En ambos casos el snoRNA U3 fue identificado con las proteínas con la cuales interactúa tales como Nop56, Nop58, Snu13, Rrp9 y Nop1 (fibrilarina) (Kornprobst et al., 2016). El procesamiento del pre-RNAr en ambos sitios es crucial para la obtención de RNAr maduro. En 1990, utilizando un extracto nuclear de células de ratón, se identificó al snoRNA U3 en complejo con distintas proteínas, incluyendo fibrilarina y se demostró que este complejo es el responsable del primer corte en la región 5´ ETS del pre-RNAr (Kass et al., 1990). En 1993, a través de la generación de mutantes de fibrilarina, se demostró que esta proteína es esencial para el procesamiento del pre-RNAr en levaduras. Distintas mutantes a lo largo de la proteína inhibían el procesamiento del pre-RNA 35S resultando principalmente afectado la formación del RNAr 18S (Tollervey et al., 1993). Así mismo, en 2004, se aisló el complejo nuclear factor D (NFD) de células de *Brassica*, el cual está compuesto por 30 proteínas dentro de las que se encontraron los snoRNA U3 y U14 y a la fibrilarina. Utilizando la región 5´ ETS del pre-RNAr se demostró que el complejo NF D tiene actividad como ribonucleasa. Debido a la gran cantidad de proteínas presentes en este complejo, no fue posible identificar cuál de ellas es la responsable de la degradación del RNA (Saez-Vasquez et al., 2004). En este trabajo, utilizando las fibrilarinas recombinantes de *H. sapiens* y *A. thaliana* se logró demostrar su actividad como ribonucleasa. La actividad como ribonucleasa de la fibrilarina también fue demostrada mediante la hidrolisis de RNA dentro de la célula (Seo et al., 2015). Utilizando la pironina Y para teñir el RNA y a la fibrilarina para degradarlo es claro que la señal correspondiente al nucléolo, donde se encuentra el RNAr, disminuye significativamente.

Haciendo una comparación de las tres fibrilarina evaluadas en este proyecto, se puede observar que todas tienen actividad como ribonucleasa. En general, la fibrilarina de HsFib tiene identidades del 72 y 70% con AtFib1 y AtFib2 respectivamente, y las fibrilarinas de *A. thaliana* tienen una identidad del 91 % entre ellas. Derivado de este trabajo se identificó

que en el dominio GAR radica la actividad ribonucleasa y que éste tiene una identidad del 45 y 46% entre HsFib y AtFib1 y AtFib2 respectivamente. Entre las fibrilarinas de *A. thaliana* la identidad de este dominio es del 74%. Analizando las deleciones en el dominio GAR de HsFib realizadas en este trabajo se observa que la actividad como ribonucleasa se centra entre los aminoácidos 20 al 59, dentro de los cuales hay una región de entre 20 y 25 aminoácidos con 47% de identidad entre HsFib y ambas fibrilarinas de *A. thaliana* y de 70% entre AtFib´s. La actividad como ribonucleasa del dominio GAR pierde la selectividad a ciertos RNAs presente en la proteína. Al no ser el dominio de interacción con RNA, incluso el RNA guía U3 es degradado. Cabe destacar que el dominio GAR en HsFib se encuentra dimetilado en distintas posiciones (Lischwe et al., 1985), sin embargo, para este estudio se utilizó una fibrilarina recombinante, expresada en bacterias, por lo que carecía de estas modificaciones. Por lo tanto, es necesario contemplar que estas modificaciones podrían regular su actividad como ribonucleasa.

Dentro del dominio GAR de fibrilarina también se encuentra la señal que la dirige al nucléolo celular (Snaar et al., 2000). Así mismo, este dominio es el responsable de la interacción con proteínas virales. Silenciando a la fibrilarina el movimiento de las partículas virales a través del hospedero es significativamente menor o incluso inhibido. Esto ocurre tanto para virus de plantas (Zheng et al., 2015; Chang et al., 2016) como de animales (Deffrasnes et al., 2016). El mecanismo por el cual los virus utilizan a la fibrilarina aún no es del todo claro. Ejemplificando, se sabe que la proteína ORF3 del virus de la roseta del cacahuate interactúa con fibrilarina. Fibrilarina introduce a ORF3 al nucléolo y a los cuerpos cajales para posteriormente salir de estos compartimentos y ensamblar ribonucleopreoteinas virales para su difusión (Kim et al., 2007; Canetta et al., 2008). Tomando en cuenta la actividad como ribonucleasa de fibrilarina se puede hipotetizar que inicialmente la fibrilarina metila a la partícula viral introducida al núcleo y posteriormente ésta dirige a la fibrilarina a la formación de ribonucleoproteinas virales. Estas ribonucleoproteinas virales compuestas al menos por un RNA guía, una partícula viral y fibrilarina se transportan hacia otras células utilizando a la fibrilarina como una RNAsa en contra de los RNAi, permitiendo así su infección. Por ello, es necesario definir si el corte de la fibrilarina puede ser dirigido por un RNA guía, y cuáles son las características y los posibles blancos de éste.

Dentro de este proyecto también se determinó la interacción de la fibrilarina, tanto de *H. sapiens* como las de *A. thaliana*, con diversos fosfolípidos entre ellos los fosfoinosítidos y el PA. Dentro del núcleo celular los fosfoinosítidos son considerados co-factores esenciales para diversos procesos, que van desde la regulación de la transcripción, diferenciación y control del ciclo celular (Fiume et al., 2012). Anteriormente, se describió que el PtdIns(4,5)P_2 interactúa con HsFib regulando su unión al RNA (Yildirim et al., 2013) por lo tanto, era de importancia determinar el papel de los fosfoinosítidos y las fibrilarinas actuando como ribonucleasa. Se determinó que las fibrilarinas interactúan en mayor medida con los fosfoinosítidos PtdIns(3)P, PtdIns(5)P, y PtdIns(3,4)P_2 y con el PA, Además del PtdIns(4,5)P_2. El PA se encuentra involucrado en distintos procesos celulares, particularmente dentro del núcleo, regula la locación intracelular y actividad de proteínas con las que interactúa. Un ejemplo de esto es la interacción del PA con el represor transcripcional Opi1p. Al interactuar el PA y Opi1p este se mantiene inactivo fuera del núcleo (Loewen et al., 2004; Yao et al., 2014). Cuando la fibrilarina, tanto HsFib como AtFib2, interactúa con el PA la actividad como ribonucleasa disminuye hasta en un 10% indicando que el sitio de interacción con el PA y el dominio con actividad de RNAsa es el mismo. En trabajos anteriores también se ha encontrado que el PA es capaz de incrementar la expresión del receptor del factor de crecimiento epidermal al inhibir la actividad de RNAsas en el citoplasma ligadas a la degradación del RNAm (Hatton et al., 2015). Será de importancia determinar bajo qué condiciones los fosfoinosítidos y el PA son capaces de regular la actividad como ribonucleasa de la fibrilarina.

Mutantes de HsFib fueron generadas en las posiciones R34A y R45A del dominio GAR, estos cambios modificaron la unión con los fosfoinosítidos al interactuar únicamente con el PtdIns(3)P y el PtdIns(5)P. Esto indica que el dominio de unión a los fosfoinosítidos y el de actividad de ribonucleasa es el mismo, por lo que la actividad como ribonucleasa podría estar regulada por estas interacciones lipídicas. Ejemplos de esto pueden ser encontrados en la literatura como el del dominio PDZ (PSD-95/Discs large/ZO-1) de la syntenin-2. Este dominio interactúa con el PtdIns(4,5)P_2 regulando la organización nuclear de este fosfoinosítido. Mutando este dominio en 4 posiciones diferentes (Lys113, Lys167, Lys197 y Lys244) la interacción con el PtdIns(4,5)P_2 se pierde por completo, sin embrago

la unión al antígeno L6, perteneciente a la superfamilia de las tetraspaninas, se mantiene intacta (Wawrzyniak et al., 2013a).

Deacuerdo con la base de datos BioGrid [http://thebiogrid.org/108399/summary/ homo-sapiens/fbl.html] la fibrilarina interactúa con almenos 233 proteínas, cada uno de dichos complejos proteicos es de importancia para conservar los procesos biológicos. Es claro que las mutantes de HsFib en el dominio GAR (R34A y R45A) interactúan con diferentes proteínas dando así una idea de la importancia biológica de la fibrilarina, en la cual se debe profundizar. Lek et al., (Lek et al., 2016), llevo a cabo un análisis de variación genética codificante para proteínas en 60,706 humanos dentro de las cuales se encontraba la fibrilarina. Se determinó que la fibrilarina cuenta con 99 variantes con cambio de sentido de las cuales únicamente se encontró una variante homocigota (p.Arg74Trp). No se cuenta con los datos clínicos funcionales de ninguna de estas 99 variantes pero el hecho de que la presencia de homocigotos para cada una sea nula habla de lo esencial que es la correcta función de la proteína.

En cuanto a la fibrilarina como metiltransferasa del RNAr la evidencia indica la formación de un complejo formado por las proteínas Nop56, Nop58, L7a, fibrilarina y un RNA guía para dirigir la metilación (Omer et al., 2002; Peng et al., 2014). Recientemente se demostró *in vitro* utilizando como modelo de estudio la arquea *Pyrococcus abyssi* que la interacción fibrilarina-Nop5 es suficiente para llevar a cabo la metilación del RNA 16S y 23S in vitro, esto sin la unión a la proteína L7a o algún RNA guía (Tomkuviene et al., 2017). Sera necesario demostrar si el complejo fibrilarina – Nop5 es suficiente también en eucariotas para metilar el RNAr y analizar cuáles son las posiciones en la que se requiere un RNA guía. Como metiltransferasa de proteínas la metilación en la glutamina 104 o 105 de la histona H2A afecta la interacción entre la histona y el complejo FACT (facilitador de transcripción de la cromatina). El complejo FACT interactúa con la polimerasa I modificando los nucleosomas permitiendo la transcripción. La metilación en la glutamina de la histona H2A previene la interacción entre la esta y el complejo FACT, sin embargo permite la remodelación de la cromatina mejorado la transcripción de la polimerasa I. En humanos esta modificación es exclusiva al nucléolo celular (Tessarz et al., 2014). También se ha demostrado que el complejo FACT aumenta la transcripción mediada por

la polimerasa II al remover el dímero histona H2A/H2B sin la hidrolisis de ATP (Belotserkovskaya et al., 2004). En plantas el complejo FACT se compone por las proteínas Spt16 y SSRP1 y se localiza en el núcleo celular en las regiones de 5´-UTR y 3´-UTR de los genes transcripcionalmente activos (Duroux et al., 2004). En este trabajo se logró reproducir la metilación de la histona H2A mediante la fibrilarina y esta modificación se localizó en el núcleo de células de *Brassica*. Su localización en el núcleo celular es similar a la reportada para *A. thaliana* y también se lleva a cabo en el promotor del DNAr. Ya que el complejo FACT en plantas es necesario en diferentes aspectos del desarrollo como el crecimiento de hojas y floración (Van Lijsebettens and Grasser, 2010) la metilación de la histona H2A es un proceso importante en parte regulado por la fibrilarina.

Adicionalmente a su actividad como metiltransferasa, la fibrilarina tiene actividad como ribonucleasa, lo cual la convierte en una proteína multifunción o "moonlight". La principal característica de una proteína moonlight es llevar a cabo más de una función con el mismo polipéptido (Jeffery, 2014). A pesar que la actividad como metiltransferasa en células eucariotas no se ha logrado reproducir in vitro, únicamente en arqueas, (Peng et al., 2014) es claro, basado en mutantes termosensitivas de levadura que la fibrilarina tiene esta función (Tollervey et al., 1993). Por lo tanto, la actividad como metiltransferasa así como ribonucleasa se encuentra en el mismo polipéptido. Para la maduración del pre-RNAr además de su procesamiento y unión a diferentes proteínas ribosomales es necesaria su metilación, de la cual fibrilarina es responsable. En las posiciones en las cuales el RNAr es metilado, se ha evidenciado el corte del pre-RNAr, por lo que resulta posible hipotetizar que la fibrilarina lleve a cabo ambas funciones. Sumado a esto, la fibrilarina además de su localización en el nucléolo, se encuentra en los cuerpos cajales interactuando con ciertos scaRNA (RNA específicos de cuerpos cajales). Hoy en día no se conoce por completo la función de los cuerpos cajales, sin embargo, se sugiere que son cuerpos nucleares en donde se lleva a cabo el procesamiento de los RNA nucleares y del RNAm (Nizami et al., 2010; Morimoto and Boerkoel, 2013).

La fibrilarina es una proteína esencial para la vida cuyas funciones se encuentran involucradas en la biogénesis de los ribosomas, proceso en el cual la demanda de energía es importante para la célula (Warner, 1999). Esto implica que los procesos dentro

del nucléolo celular sean altamente regulados. Es por ello que la multifunción de fibrilarina en el procesamiento y modificación del pre-RNAr parece lógica. Las modificaciones postraduccionales en la proteína así como su interacción con distintos fosfolípidos encajan en un proceso de regulación sin el cual el RNA seria afectado. Serán necesarios estudios *in vivo* que corroboren los publicados aqui para mejorar el entendimiento de un proceso vital para la célula como lo es la biogénesis de los ribosomas.

Conclusiones

- La fibrilarina tiene actividad como ribonucleasa.

- La actividad como ribonucleasa de fibrilarina es selectiva a ciertos RNAs. Ej. el RNA guía U3 no es degradado sino que forma complejo con fibrilarina.

- La actividad como ribonucleasa de la fibrilarina se localiza en el dominio GAR. Dicha actividad es independiente al dominio metiltransferasa de fibrilarina y al motivo de unión al RNA.

- El PA reduce la formación del complejo fibrilarina-RNA guía.

- El sitio de unión a fosfolípidos de la fibrilarina es el dominio N terminal. Mutaciones en las argininas 34 y 45 inhiben la interacción con el ácido fosfatídico y los fosfoinosítidos bisfosfato.

Perspectivas

- Comprobar que la fibrilarina se encuentra involucrada en el corte del pre-RNAr.

- Definir el mecanismo para guiar la función como ribonucleasa de fibrilarina a un blanco específico. Caracterizar los RNA guía y blanco.

- Generar una herramienta que permita la discriminación entre fibrilarinas.

- Comprobar el efecto del silenciamiento de fibrilarina sobre el procesamiento del RNAr.

- Definir funciones de las ribonucleopreoteinas virales en complejo con fibrilarina.

Referencias

Abascal, F., Zardoya, R., and Posada, D. (2005). ProtTest: selection of best-fit models of protein evolution. *Bioinformatics* 21**,** 2104-2105. doi: 10.1093/bioinformatics/bti263

Ahmad, Y., Boisvert, F.M., Gregor, P., Cobley, A., and Lamond, A.I. (2009). NOPdb: Nucleolar Proteome Database--2008 update. *Nucleic Acids Res* 37**,** D181-184. doi: 10.1093/nar/gkn804

Aittaleb, M., Rashid, R., Chen, Q., Palmer, J.R., Daniels, C.J., and Li, H. (2003). Structure and function of archaeal box C/D sRNP core proteins. *Nat Struct Biol* 10**,** 256-263. doi: 10.1038/nsb905

Alderden, R.A., Mellor, H.R., Modok, S., Hambley, T.W., and Callaghan, R. (2006). Cytotoxic efficacy of an anthraquinone linked platinum anticancer drug. *Biochem Pharmacol* 71**,** 1136-1145. doi: 10.1016/j.bcp.2005.12.039

Altschul, S.F., and Koonin, E.V. (1998). Iterated profile searches with PSI-BLAST--a tool for discovery in protein databases. *Trends Biochem Sci* 23**,** 444-447. doi:

Amin, M.A., Matsunaga, S., Ma, N., Takata, H., Yokoyama, M., Uchiyama, S., et al. (2007). Fibrillarin, a nucleolar protein, is required for normal nuclear morphology and cellular growth in HeLa cells. *Biochem Biophys Res Commun* 360**,** 320-326. doi: 10.1016/j.bbrc.2007.06.092

Amiri, K.A. (1994). Fibrillarin-like proteins occur in the domain Archaea. *J Bacteriol* 176**,** 2124-2127. doi:

Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S., and Hopkins, N. (2004). Identification of 315 genes essential for early zebrafish development. *Proc Natl Acad Sci U S A* 101**,** 12792-12797. doi: 10.1073/pnas.0403929101

Andersen, J.S., Lam, Y.W., Leung, A.K., Ong, S.E., Lyon, C.E., Lamond, A.I., et al. (2005). Nucleolar proteome dynamics. *Nature* 433**,** 77-83. doi: 10.1038/nature03207

Angelier, N., Tramier, M., Louvet, E., Coppey-Moisan, M., Savino, T.M., De Mey, J.R., et al. (2005). Tracking the interactions of rRNA processing proteins during nucleolar assembly in living cells. *Mol Biol Cell* 16**,** 2862-2871. doi: 10.1091/mbc.E05-01-0041

Aris, J.P., and Blobel, G. (1991). cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proc Natl Acad Sci U S A* 88**,** 931-935. doi:

Arraiano, C.M., Mauxion, F., Viegas, S.C., Matos, R.G., and Seraphin, B. (2013). Intracellular ribonucleases involved in transcript processing and decay: precision

tools for RNA. *Biochim Biophys Acta* 1829**,** 491-513. doi: 10.1016/j.bbagrm.2013.03.009

Azum-Gelade, M.C., Noaillac-Depeyre, J., Caizergues-Ferrer, M., and Gas, N. (1994). Cell cycle redistribution of U3 snRNA and fibrillarin. Presence in the cytoplasmic nucleolus remnant and in the prenucleolar bodies at telophase. *J Cell Sci* 107 (Pt 2)**,** 463-475. doi:

Backstrom, S., Elfving, N., Nilsson, R., Wingsle, G., and Bjorklund, S. (2007). Purification of a plant mediator from Arabidopsis thaliana identifies PFT1 as the Med25 subunit. *Mol Cell* 26**,** 717-729. doi: 10.1016/j.molcel.2007.05.007

Barneche, F., Steinmetz, F., and Echeverria, M. (2000). Fibrillarin genes encode both a conserved nucleolar protein and a novel small nucleolar RNA involved in ribosomal RNA methylation in Arabidopsis thaliana. *J Biol Chem* 275**,** 27212-27220. doi: 10.1074/jbc.M002996200

Basu, A., Das, P., Chaudhuri, S., Bevilacqua, E., Andrews, J., Barik, S., et al. (2011). Requirement of rRNA methylation for 80S ribosome assembly on a cohort of cellular internal ribosome entry sites. *Mol Cell Biol* 31**,** 4482-4499. doi: 10.1128/mcb.05804-11

Belotserkovskaya, R., Saunders, A., Lis, J.T., and Reinberg, D. (2004). Transcription through chromatin: understanding a complex FACT. *Biochim Biophys Acta* 1677**,** 87-99. doi: 10.1016/j.bbaexp.2003.09.017

Benavente, R., Rose, K.M., Reimer, G., Hugle-Dorr, B., and Scheer, U. (1987). Inhibition of nucleolar reformation after microinjection of antibodies to RNA polymerase I into mitotic cells. *J Cell Biol* 105**,** 1483-1491. doi:

Boisvert, F.M., Van Koningsbruggen, S., Navascues, J., and Lamond, A.I. (2007). The multifunctional nucleolus. *Nat Rev Mol Cell Biol* 8**,** 574-585. doi: 10.1038/nrm2184

Boss, W.F., and Im, Y.J. (2012). Phosphoinositide signaling. *Annu Rev Plant Biol* 63**,** 409-429. doi: 10.1146/annurev-arplant-042110-103840

Bowers, J.E., Chapman, B.A., Rong, J., and Paterson, A.H. (2003). Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature* 422**,** 433-438. doi: 10.1038/nature01521

Bubeck, D., Reijns, M.A., Graham, S.C., Astell, K.R., Jones, E.Y., and Jackson, A.P. (2011). PCNA directs type 2 RNase H activity on DNA replication and repair substrates. *Nucleic Acids Res* 39**,** 3652-3666. doi: 10.1093/nar/gkq980

Bugler, B., Bourbon, H., Lapeyre, B., Wallace, M.O., Chang, J.H., Amalric, F., et al. (1987). RNA binding fragments from nucleolin contain the ribonucleoprotein consensus sequence. *J Biol Chem* 262**,** 10922-10925. doi:

Canetta, E., Kim, S.H., Kalinina, N.O., Shaw, J., Adya, A.K., Gillespie, T., et al. (2008). A plant virus movement protein forms ringlike complexes with the major nucleolar protein, fibrillarin, in vitro. *J Mol Biol* 376**,** 932-937. doi: 10.1016/j.jmb.2007.12.039

Castano, E., Vorojeikina, D.P., and Notides, A.C. (1997). Phosphorylation of serine-167 on the human oestrogen receptor is important for oestrogen response element binding and transcriptional activation. *Biochem J* 326 (Pt 1)**,** 149-157. doi:

Castano, E., Gross, P., Wang, Z., Roeder, R.G., and Oelgeschlager, T. (2000). The C-terminal domain-phosphorylated IIO form of RNA polymerase II is associated with the transcription repressor NC2 (Dr1/DRAP1) and is required for transcription activation in human nuclear extracts. *Proc Natl Acad Sci U S A* 97**,** 7184-7189. doi: 10.1073/pnas.140202297

Cavaille, J., Nicoloso, M., and Bachellerie, J.P. (1996). Targeted ribose methylation of RNA in vivo directed by tailored antisense RNA guides. *Nature* 383**,** 732-735. doi: 10.1038/383732a0

Cerdido, A., and Medina, F.J. (1995). Subnucleolar location of fibrillarin and variation in its levels during the cell cycle and during differentiation of plant cells. *Chromosoma* 103**,** 625-634. doi:

Cockell, M.M., and Gasser, S.M. (1999). The nucleolus: nucleolar space for RENT. *Curr Biol* 9**,** R575-576. doi:

Chang, C.H., Hsu, F.C., Lee, S.C., Lo, Y.S., Wang, J.D., Shaw, J., et al. (2016). The Nucleolar Fibrillarin Protein is Required for Helper Virus-Independent Long-Distance Trafficking of a Subviral Satellite RNA in Plants. *Plant Cell*. doi: 10.1105/tpc.16.00071

Chatr-Aryamontri, A., Breitkreutz, B.J., Heinicke, S., Boucher, L., Winter, A., Stark, C., et al. (2013). The BioGRID interaction database: 2013 update. *Nucleic Acids Res* 41**,** D816-823. doi: 10.1093/nar/gks1158

Chen, D., and Huang, S. (2001). Nucleolar components involved in ribosome biogenesis cycle between the nucleolus and nucleoplasm in interphase cells. *J Cell Biol* 153**,** 169-176. doi:

Chen, M., Rockel, T., Steinweger, G., Hemmerich, P., Risch, J., and Von Mikecz, A. (2002). Subcellular recruitment of fibrillarin to nucleoplasmic proteasomes: implications for processing of a nucleolar autoantigen. *Mol Biol Cell* 13**,** 3576-3587. doi: 10.1091/mbc.02-05-0083

Chen, Z.J., and Pikaard, C.S. (1997). Transcriptional analysis of nucleolar dominance in polyploid plants: biased expression/silencing of progenitor rRNA genes is developmentally regulated in Brassica. *Proc Natl Acad Sci U S A* 94**,** 3442-3447. doi:

Christensen, M.E., Beyer, A.L., Walker, B., and Lestourgeon, W.M. (1977). Identification of NG, NG-dimethylarginine in a nuclear protein from the lower eukaryote physarum polycephalum homologous to the major proteins of mammalian 40S ribonucleoprotein particles. *Biochem Biophys Res Commun* 74**,** 621-629. doi:

Dassanayake, M., Oh, D.H., Haas, J.S., Hernandez, A., Hong, H., Ali, S., et al. (2011). The genome of the extremophile crucifer Thellungiella parvula. *Nat Genet* 43**,** 913-918. doi: 10.1038/ng.889

David, E., Mcneil, J.B., Basile, V., and Pearlman, R.E. (1997). An unusual fibrillarin gene and protein: structure and functional implications. *Mol Biol Cell* 8**,** 1051-1061. doi:

De Silva, U., Zhou, Z., and Brown, B.A., 2nd (2012). Structure of Aeropyrum pernix fibrillarin in complex with natively bound S-adenosyl-L-methionine at 1.7 A resolution. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 68**,** 854-859. doi: 10.1107/s1744309112026528

Deffrasnes, C., Marsh, G.A., Foo, C.H., Rootes, C.L., Gould, C.M., Grusovin, J., et al. (2016). Genome-wide siRNA Screening at Biosafety Level 4 Reveals a Crucial Role for Fibrillarin in Henipavirus Infection. *PLoS Pathog* 12**,** e1005478. doi: 10.1371/journal.ppat.1005478

Deng, L., Starostina, N.G., Liu, Z.J., Rose, J.P., Terns, R.M., Terns, M.P., et al. (2004). Structure determination of fibrillarin from the hyperthermophilic archaeon Pyrococcus furiosus. *Biochem Biophys Res Commun* 315**,** 726-732. doi: 10.1016/j.bbrc.2004.01.114

Desterro, J.M., Keegan, L.P., Lafarga, M., Berciano, M.T., O'connell, M., and Carmo-Fonseca, M. (2003). Dynamic association of RNA-editing enzymes with the nucleolus. *J Cell Sci* 116**,** 1805-1818. doi:

Deutscher, M.P. (2015). Twenty years of bacterial RNases and RNA processing: how we've matured. *RNA* 21**,** 597-600. doi: 10.1261/rna.049692.115

Divecha, N. (2016). Phosphoinositides in the nucleus and myogenic differentiation: how a nuclear turtle with a PHD builds muscle. *Biochem Soc Trans* 44**,** 299-306. doi: 10.1042/BST20150238

Dousset, T., Wang, C., Verheggen, C., Chen, D., Hernandez-Verdun, D., and Huang, S. (2000). Initiation of nucleolar assembly is independent of RNA polymerase I transcription. *Mol Biol Cell* 11**,** 2705-2717. doi:

Dragon, F., Gallagher, J.E., Compagnone-Post, P.A., Mitchell, B.M., Porwancher, K.A., Wehner, K.A., et al. (2002). A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature* 417**,** 967-970. doi: 10.1038/nature00769

Dudkina, E., Ulyanova, V., Shah Mahmud, R., Khodzhaeva, V., Dao, L., Vershinina, V., et al. (2016). Three-step procedure for preparation of pure Bacillus altitudinis

ribonuclease. *FEBS Open Bio* 6, 24-32. doi: 10.1002/2211-5463.12023

Dunbar, D.A., Wormsley, S., Lowe, T.M., and Baserga, S.J. (2000). Fibrillarin-associated box C/D small nucleolar RNAs in Trypanosoma brucei. Sequence conservation and implications for 2'-O-ribose methylation of rRNA. *J Biol Chem* 275, 14767-14776. doi:

Duroux, M., Houben, A., Ruzicka, K., Friml, J., and Grasser, K.D. (2004). The chromatin remodelling complex FACT associates with actively transcribed regions of the Arabidopsis genome. *Plant J* 40, 660-671. doi: 10.1111/j.1365-313X.2004.02242.x

Eddy, S.R. (2011). Accelerated Profile HMM Searches. *PLoS Comput Biol* 7, e1002195. doi: 10.1371/journal.pcbi.1002195

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32, 1792-1797. doi: 10.1093/nar/gkh340

Falaleeva, M., Pages, A., Matuszek, Z., Hidmi, S., Agranat-Tamir, L., Korotkov, K., et al. (2016). Dual function of C/D box small nucleolar RNAs in rRNA modification and alternative pre-mRNA splicing. *Proc Natl Acad Sci U S A* 113, E1625-1634. doi: 10.1073/pnas.1519292113

Fatica, A., and Tollervey, D. (2002). Making ribosomes. *Curr Opin Cell Biol* 14, 313-318. doi:

Feric, M., Vaidya, N., Harmon, T.S., Mitrea, D.M., Zhu, L., Richardson, T.M., et al. (2016). Coexisting Liquid Phases Underlie Nucleolar Subcompartments. *Cell* 165, 1686-1697. doi: 10.1016/j.cell.2016.04.047

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y., Eddy, S.R., et al. (2014). Pfam: the protein families database. *Nucleic Acids Res* 42, D222-230. doi: 10.1093/nar/gkt1223

Fiume, R., Keune, W.J., Faenza, I., Bultsma, Y., Ramazzotti, G., Jones, D.R., et al. (2012). Nuclear phosphoinositides: location, regulation and function. *Subcell Biochem* 59, 335-361. doi: 10.1007/978-94-007-3015-1_11

Foltankova, V., Legartova, S., Kozubek, S., Hofer, M., and Bartova, E. (2013). DNA-damage response in chromatin of ribosomal genes and the surrounding genome. *Gene* 522, 156-167. doi: 10.1016/j.gene.2013.03.108

Fomproix, N., Gebrane-Younes, J., and Hernandez-Verdun, D. (1998). Effects of anti-fibrillarin antibodies on building of functional nucleoli at the end of mitosis. *J Cell Sci* 111 (Pt 3), 359-372. doi:

Fournier, M.J., and Maxwell, E.S. (1993). The nucleolar snRNAs: catching up with the spliceosomal snRNAs. *Trends Biochem Sci* 18, 131-135. doi:

Garcia, S.N., and Pillus, L. (1999). Net results of nucleolar dynamics. *Cell* 97**,** 825-828. doi:

Girard, J.P., Lehtonen, H., Caizergues-Ferrer, M., Amalric, F., Tollervey, D., and Lapeyre, B. (1992). GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. *EMBO J* 11**,** 673-682. doi:

Granneman, S., Vogelzangs, J., Luhrmann, R., Van Venrooij, W.J., Pruijn, G.J., and Watkins, N.J. (2004). Role of pre-rRNA base pairing and 80S complex formation in subnucleolar localization of the U3 snoRNP. *Mol Cell Biol* 24**,** 8600-8610. doi: 10.1128/MCB.24.19.8600-8610.2004

Gu, Z., Eils, R., and Schlesner, M. (2016). Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* 32**,** 2847-2849. doi: 10.1093/bioinformatics/btw313

Gustavsson, H.O., Rask, L., and Josefsson, L.G. (1991). Transcription in vitro of a napin gene, napA, from Brassica napus with a HeLa cell nuclear extract. *Hereditas* 115**,** 191-193. doi:

Hamann, B.L., and Blind, R.D. (2017). Nuclear phosphoinositide regulation of chromatin. *J Cell Physiol*. doi: 10.1002/jcp.25886

Hatton, N., Lintz, E., Mahankali, M., Henkels, K.M., and Gomez-Cambronero, J. (2015). Phosphatidic Acid Increases Epidermal Growth Factor Receptor Expression by Stabilizing mRNA Decay and by Inhibiting Lysosomal and Proteasomal Degradation of the Internalized Receptor. *Mol Cell Biol* 35**,** 3131-3144. doi: 10.1128/MCB.00286-15

Henras, A.K., Plisson-Chastang, C., O'donohue, M.F., Chakraborty, A., and Gleizes, P.E. (2015). An overview of pre-ribosomal RNA processing in eukaryotes. *Wiley Interdiscip Rev RNA* 6**,** 225-242. doi: 10.1002/wrna.1269

Hernandez-Verdun, D., Louvet, E., and Muro, E. (2013). Time-lapse, photoactivation, and photobleaching imaging of nucleolar assembly after mitosis. *Methods Mol Biol* 1042**,** 337-350. doi: 10.1007/978-1-62703-526-2_22

Hernandez-Verdun, D., Roussel, P., Thiry, M., Sirri, V., and Lafontaine, D.L. (2010). The nucleolus: structure/function relationship in RNA metabolism. *Wiley Interdiscip Rev RNA* 1**,** 415-431. doi: 10.1002/wrna.39

Hickey, A.J., Macario, A.J., and Conway De Macario, E. (2000). Identification of genes in the genome of the archaeon Methanosarcina mazeii that code for homologs of nuclear eukaryotic molecules involved in RNA processing. *Gene* 253**,** 77-85. doi:

Hiscox, J.A. (2002). The nucleolus--a gateway to viral infection? *Arch Virol* 147**,** 1077-1089. doi: 10.1007/s00705-001-0792-0

Hoffmann, C., and Neumann, H. (2015). In Vivo Mapping of FACT-Histone Interactions Identifies a Role of Pob3 C-terminus in H2A-H2B Binding. *ACS Chem Biol*. doi: 10.1021/acschembio.5b00493

Houseley, J., and Tollervey, D. (2009). The many pathways of RNA degradation. *Cell* 136**,** 763-776. doi: 10.1016/j.cell.2009.01.019

Huber, W., Carey, V.J., Gentleman, R., Anders, S., Carlson, M., Carvalho, B.S., et al. (2015). Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods* 12**,** 115-121. doi: 10.1038/nmeth.3252

Hughes, J.M., and Ares, M., Jr. (1991). 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* 10**,** 4231-4239. doi:

Iwasaki, W., Miya, Y., Horikoshi, N., Osakabe, A., Taguchi, H., Tachiwana, H., et al. (2013). Contribution of histone N-terminal tails to the structure and stability of nucleosomes. *FEBS Open Bio* 3**,** 363-369. doi: 10.1016/j.fob.2013.08.007

Jacobson, M.R., and Pederson, T. (1998a). A 7-methylguanosine cap commits U3 and U8 small nuclear RNAs to the nucleolar localization pathway. *Nucleic Acids Res* 26**,** 756-760. doi:

Jacobson, M.R., and Pederson, T. (1998b). Localization of signal recognition particle RNA in the nucleolus of mammalian cells. *Proc Natl Acad Sci U S A* 95**,** 7981-7986. doi:

Jamison, J.M., Gilloteaux, J., Perlaky, L., Thiry, M., Smetana, K., Neal, D., et al. (2010). Nucleolar changes and fibrillarin redistribution following apatone treatment of human bladder carcinoma cells. *J Histochem Cytochem* 58**,** 635-651. doi: 10.1369/jhc.2010.956284

Jansen, R., Tollervey, D., and Hurt, E.C. (1993). A U3 snoRNP protein with homology to splicing factor PRP4 and G beta domains is required for ribosomal RNA processing. *Embo j* 12**,** 2549-2558. doi:

Jansen, R.P., Hurt, E.C., Kern, H., Lehtonen, H., Carmo-Fonseca, M., Lapeyre, B., et al. (1991). Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in yeast. *J Cell Biol* 113**,** 715-729. doi:

Jeffery, C.J. (2014). An introduction to protein moonlighting. *Biochem Soc Trans* 42**,** 1679-1683. doi: 10.1042/BST20140226

Jiang, H., Sha, S.H., and Schacht, J. (2006). Kanamycin alters cytoplasmic and nuclear phosphoinositide signaling in the organ of Corti in vivo. *J Neurochem* 99**,** 269-276. doi: 10.1111/j.1471-4159.2006.04117.x

Jiang, Z., Zhang, H., Qin, R., Zou, J., Wang, J., Shi, Q., et al. (2014). Effects of lead on the morphology and structure of the nucleolus in the root tip meristematic cells of

Allium cepa L. *Int J Mol Sci* 15, 13406-13423. doi: 10.3390/ijms150813406

Jong, A.Y., Clark, M.W., Gilbert, M., Oehm, A., and Campbell, J.L. (1987). Saccharomyces cerevisiae SSB1 protein and its relationship to nucleolar RNA-binding proteins. *Mol Cell Biol* 7, 2947-2955. doi:

Kass, S., Tyc, K., Steitz, J.A., and Sollner-Webb, B. (1990). The U3 small nucleolar ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell* 60, 897-908. doi:

Kim, S.H., Macfarlane, S., Kalinina, N.O., Rakitina, D.V., Ryabov, E.V., Gillespie, T., et al. (2007). Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. *Proc Natl Acad Sci U S A* 104, 11115-11120. doi: 10.1073/pnas.0704632104

Kiss-Laszlo, Z., Henry, Y., Bachellerie, J.P., Caizergues-Ferrer, M., and Kiss, T. (1996). Site-specific ribose methylation of preribosomal RNA: a novel function for small nucleolar RNAs. *Cell* 85, 1077-1088. doi:

Knight, B., Kubik, S., Ghosh, B., Bruzzone, M.J., Geertz, M., Martin, V., et al. (2014). Two distinct promoter architectures centered on dynamic nucleosomes control ribosomal protein gene transcription. *Genes Dev* 28, 1695-1709. doi: 10.1101/gad.244434.114

Koh, C.M., Iwata, T., Zheng, Q., Bethel, C., Yegnasubramanian, S., and De Marzo, A.M. (2011a). Myc enforces overexpression of EZH2 in early prostatic neoplasia via transcriptional and post-transcriptional mechanisms. *Oncotarget* 2, 669-683. doi:

Koh, C.M., Gurel, B., Sutcliffe, S., Aryee, M.J., Schultz, D., Iwata, T., et al. (2011b). Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. *Am J Pathol* 178, 1824-1834. doi: 10.1016/j.ajpath.2010.12.040

Kornprobst, M., Turk, M., Kellner, N., Cheng, J., Flemming, D., Kos-Braun, I., et al. (2016). Architecture of the 90S Pre-ribosome: A Structural View on the Birth of the Eukaryotic Ribosome. *Cell* 166, 380-393. doi: 10.1016/j.cell.2016.06.014

Kressler, D., Linder, P., and De La Cruz, J. (1999). Protein trans-acting factors involved in ribosome biogenesis in Saccharomyces cerevisiae. *Mol Cell Biol* 19, 7897-7912. doi:

Krogan, N.J., Cagney, G., Yu, H., Zhong, G., Guo, X., Ignatchenko, A., et al. (2006). Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. *Nature* 440, 637-643. doi: 10.1038/nature04670

Laboure, A.M., Faik, A., Mandaron, P., and Falconet, D. (1999). RGD-dependent growth of maize calluses and immunodetection of an integrin-like protein. *FEBS Lett* 442, 123-128. doi:

Lafontaine, D.L., and Tollervey, D. (2000). Synthesis and assembly of the box C+D small nucleolar RNPs. *Mol Cell Biol* 20**,** 2650-2659. doi:

Lapeyre, B., Bourbon, H., and Amalric, F. (1987). Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. *Proc Natl Acad Sci U S A* 84**,** 1472-1476. doi:

Lapinaite, A., Simon, B., Skjaerven, L., Rakwalska-Bange, M., Gabel, F., and Carlomagno, T. (2013). The structure of the box C/D enzyme reveals regulation of RNA methylation. *Nature* 502**,** 519-523. doi: 10.1038/nature12581

Lechertier, T., Grob, A., Hernandez-Verdun, D., and Roussel, P. (2009). Fibrillarin and Nop56 interact before being co-assembled in box C/D snoRNPs. *Exp Cell Res* 315**,** 928-942. doi: 10.1016/j.yexcr.2009.01.016

Lee, W.C., Xue, Z.X., and Melese, T. (1991). The NSR1 gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *J Cell Biol* 113**,** 1-12. doi:

Lek, M., Karczewski, K.J., Minikel, E.V., Samocha, K.E., Banks, E., Fennell, T., et al. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 536**,** 285-291. doi: 10.1038/nature19057

Lemmon, M.A. (2008). Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* 9**,** 99-111. doi: 10.1038/nrm2328

Leonhardt, H., and Hake, S.B. (2014). Histone glutamine methylation afFACTing rDNA transcription. *Cell Res* 24**,** 261-262. doi: 10.1038/cr.2014.22

Leung, A.K., Gerlich, D., Miller, G., Lyon, C., Lam, Y.W., Lleres, D., et al. (2004). Quantitative kinetic analysis of nucleolar breakdown and reassembly during mitosis in live human cells. *J Cell Biol* 166**,** 787-800. doi: 10.1083/jcb.200405013

Levitskii, S.A., Mukhar'iamova, K., Veiko, V.P., and Zatsepina, O.V. (2004). [Identification of signal sequences determining the specific nucleolar localization of fibrillarin in HeLa cells]. *Mol Biol (Mosk)* 38**,** 483-492. doi:

Li, X., and Manley, J.L. (2005). Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. *Cell* 122**,** 365-378. doi: 10.1016/j.cell.2005.06.008

Lischwe, M.A., Ochs, R.L., Reddy, R., Cook, R.G., Yeoman, L.C., Tan, E.M., et al. (1985). Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in NG,NG-dimethylarginine. *J Biol Chem* 260**,** 14304-14310. doi:

Liu, C., Adamson, E., and Mercola, D. (1996). Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor beta 1. *Proc Natl Acad Sci U S A* 93**,** 11831-11836. doi:

Liu, J.L., and Gall, J.G. (2007). U bodies are cytoplasmic structures that contain uridine-rich small nuclear ribonucleoproteins and associate with P bodies. *Proc Natl Acad Sci U S A* 104**,** 11655-11659. doi: 10.1073/pnas.0704977104

Loewen, C.J., Gaspar, M.L., Jesch, S.A., Delon, C., Ktistakis, N.T., Henry, S.A., et al. (2004). Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. *Science* 304**,** 1644-1647. doi: 10.1126/science.1096083

Loza-Muller, L., Rodriguez-Corona, U., Sobol, M., Rodriguez-Zapata, L.C., Hozak, P., and Castano, E. (2015). Fibrillarin methylates H2A in RNA polymerase I trans-active promoters in Brassica oleracea. *Front Plant Sci* 6**,** 976. doi: 10.3389/fpls.2015.00976

Maden, B.E., Corbett, M.E., Heeney, P.A., Pugh, K., and Ajuh, P.M. (1995). Classical and novel approaches to the detection and localization of the numerous modified nucleotides in eukaryotic ribosomal RNA. *Biochimie* 77**,** 22-29. doi:

Marcel, V., Ghayad, S.E., Belin, S., Therizols, G., Morel, A.P., Solano-Gonzalez, E., et al. (2013). p53 acts as a safeguard of translational control by regulating fibrillarin and rRNA methylation in cancer. *Cancer Cell* 24**,** 318-330. doi: 10.1016/j.ccr.2013.08.013

Mclaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002). PIP(2) and proteins: interactions, organization, and information flow. *Annu Rev Biophys Biomol Struct* 31**,** 151-175. doi: 10.1146/annurev.biophys.31.082901.134259

Medina, F.J., Cerdido, A., and Fernandez-Gomez, M.E. (1995). Components of the nucleolar processing complex (Pre-rRNA, fibrillarin, and nucleolin) colocalize during mitosis and are incorporated to daughter cell nucleoli. *Exp Cell Res* 221**,** 111-125. doi: 10.1006/excr.1995.1358

Melen, K., Tynell, J., Fagerlund, R., Roussel, P., Hernandez-Verdun, D., and Julkunen, I. (2012). 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* 9**,** 167. doi: 10.1186/1743-422X-9-167

Mellman, D.L., Gonzales, M.L., Song, C., Barlow, C.A., Wang, P., Kendziorski, C., et al. (2008). A PtdIns4,5P2-regulated nuclear poly(A) polymerase controls expression of select mRNAs. *Nature* 451**,** 1013-1017. doi: 10.1038/nature06666

Miller, D.M., Thomas, S.D., Islam, A., Muench, D., and Sedoris, K. (2012). c-Myc and cancer metabolism. *Clin Cancer Res* 18**,** 5546-5553. doi: 10.1158/1078-0432.ccr-12-0977

Mironova, A.A., Barykina, N.V., and Zatsepina, O.V. (2014). [Cytological analysis of the reaction of the nucleolar RNA and RNA-binding proteins to oxidative stress in HeLa cells]. *Tsitologiia* 56**,** 489-499. doi:

Moelling, K., and Broecker, F. (2015). The reverse transcriptase-RNase H: from viruses to antiviral defense. *Ann N Y Acad Sci* 1341**,** 126-135. doi: 10.1111/nyas.12668

Morales, J., Sobol, M., Rodriguez-Zapata, L.C., Hozak, P., and Castano, E. (2017). Aromatic amino acids and their relevance in the specificity of the PH domain. *J Mol Recognit.* doi: 10.1002/jmr.2649

Morency, E., Sabra, M., Catez, F., Texier, P., and Lomonte, P. (2007). A novel cell response triggered by interphase centromere structural instability. *J Cell Biol* 177**,** 757-768. doi: 10.1083/jcb.200612107

Morimoto, M., and Boerkoel, C.F. (2013). The role of nuclear bodies in gene expression and disease. *Biology (Basel)* 2**,** 976-1033. doi: 10.3390/biology2030976

Motorin, Y., and Helm, M. (2011). RNA nucleotide methylation. *Wiley Interdiscip Rev RNA* 2**,** 611-631. doi: 10.1002/wrna.79

Nemeth, A., and Langst, G. (2011). Genome organization in and around the nucleolus. *Trends Genet* 27**,** 149-156. doi: 10.1016/j.tig.2011.01.002

Newton, K., Petfalski, E., Tollervey, D., and Caceres, J.F. (2003). Fibrillarin is essential for early development and required for accumulation of an intron-encoded small nucleolar RNA in the mouse. *Mol Cell Biol* 23**,** 8519-8527. doi:

Nizami, Z., Deryusheva, S., and Gall, J.G. (2010). The Cajal body and histone locus body. *Cold Spring Harb Perspect Biol* 2**,** a000653. doi: 10.1101/cshperspect.a000653

Ochs, R.L., Lischwe, M.A., Spohn, W.H., and Busch, H. (1985). Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. *Biol Cell* 54**,** 123-133. doi:

Okada, M., Jang, S.W., and Ye, K. (2008). Akt phosphorylation and nuclear phosphoinositide association mediate mRNA export and cell proliferation activities by ALY. *Proc Natl Acad Sci U S A* 105**,** 8649-8654. doi: 10.1073/pnas.0802533105

Olson, M.O., Dundr, M., and Szebeni, A. (2000). The nucleolus: an old factory with unexpected capabilities. *Trends Cell Biol* 10**,** 189-196. doi:

Olson, M.O.J., and Dundr, M. (2001). "Nucleolus: Structure and Function," in *eLS*. John Wiley & Sons, Ltd).

Omer, A.D., Ziesche, S., Ebhardt, H., and Dennis, P.P. (2002). In vitro reconstitution and activity of a C/D box methylation guide ribonucleoprotein complex. *Proc Natl Acad Sci U S A* 99**,** 5289-5294. doi: 10.1073/pnas.082101999

Oruganti, S., Zhang, Y., Li, H., Robinson, H., Terns, M.P., Terns, R.M., et al. (2007). Alternative conformations of the archaeal Nop56/58-fibrillarin complex imply flexibility in box C/D RNPs. *J Mol Biol* 371**,** 1141-1150. doi: 10.1016/j.jmb.2007.06.029

Osborne, S.L., Thomas, C.L., Gschmeissner, S., and Schiavo, G. (2001). Nuclear PtdIns(4,5)P2 assembles in a mitotically regulated particle involved in pre-mRNA splicing. *J Cell Sci* 114**,** 2501-2511. doi:

Padilla, P.I., Uhart, M., Pacheco-Rodriguez, G., Peculis, B.A., Moss, J., and Vaughan, M. (2008). Association of guanine nucleotide-exchange protein BIG1 in HepG2 cell nuclei with nucleolin, U3 snoRNA, and fibrillarin. *Proc Natl Acad Sci U S A* 105**,** 3357-3361. doi: 10.1073/pnas.0712387105

Pearson, D.L., Reimonenq, R.D., and Pollard, K.M. (1999). Expression and purification of recombinant mouse fibrillarin. *Protein Expr Purif* 17**,** 49-56. doi: 10.1006/prep.1999.1099

Peddibhotla, S., Wei, Z., Papineni, R., Lam, M.H., Rosen, J.M., and Zhang, P. (2011). The DNA damage effector Chk1 kinase regulates Cdc14B nucleolar shuttling during cell cycle progression. *Cell Cycle* 10**,** 671-679. doi:

Pellizzoni, L., Baccon, J., Charroux, B., and Dreyfuss, G. (2001). The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1. *Curr Biol* 11**,** 1079-1088. doi:

Peng, Y., Yu, G., Tian, S., and Li, H. (2014). Co-expression and co-purification of archaeal and eukaryal box C/D RNPs. *PLoS One* 9**,** e103096. doi: 10.1371/journal.pone.0103096

Perry, R.P. (2005). The architecture of mammalian ribosomal protein promoters. *BMC Evol Biol* 5**,** 15. doi: 10.1186/1471-2148-5-15

Petersen-Mahrt, S.K., Estmer, C., Ohrmalm, C., Matthews, D.A., Russell, W.C., and Akusjarvi, G. (1999). The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation. *Embo j* 18**,** 1014-1024. doi: 10.1093/emboj/18.4.1014

Phair, R.D., and Misteli, T. (2000). High mobility of proteins in the mammalian cell nucleus. *Nature* 404**,** 604-609. doi: 10.1038/35007077

Pih, K.T., Yi, M.J., Liang, Y.S., Shin, B.J., Cho, M.J., Hwang, I., et al. (2000). Molecular cloning and targeting of a fibrillarin homolog from Arabidopsis. *Plant Physiol* 123**,** 51-58. doi:

Pollard, K.M., Lee, D.K., Casiano, C.A., Bluthner, M., Johnston, M.M., and Tan, E.M. (1997). The autoimmunity-inducing xenobiotic mercury interacts with the autoantigen fibrillarin and modifies its molecular and antigenic properties. *J Immunol* 158**,** 3521-3528. doi:

Ponti, D., Bellenchi, G.C., Puca, R., Bastianelli, D., Maroder, M., Ragona, G., et al. (2014). The transcription factor EGR1 localizes to the nucleolus and is linked to suppression of ribosomal precursor synthesis. *PLoS One* 9**,** e96037. doi:

10.1371/journal.pone.0096037

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

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

Rice, P., Longden, I., and Bleasby, A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 16**,** 276-277. doi:

Rodriguez-Corona, U., Sobol, M., Rodriguez-Zapata, L.C., Hozak, P., and Castano, E. (2015). Fibrillarin from Archaea to human. *Biol Cell* 107**,** 159-174. doi: 10.1111/boc.201400077

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

Rosenhouse-Dantsker, A., and Logothetis, D.E. (2007). Molecular characteristics of phosphoinositide binding. *Pflugers Arch* 455**,** 45-53. doi: 10.1007/s00424-007-0291-6

Sabra, M., Texier, P., El Maalouf, J., and Lomonte, P. (2013). The Tudor protein survival motor neuron (SMN) is a chromatin-binding protein that interacts with methylated lysine 79 of histone H3. *J Cell Sci* 126**,** 3664-3677. doi: 10.1242/jcs.126003

Saez-Vasquez, J., Caparros-Ruiz, D., Barneche, F., and 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. *Mol Cell Biol* 24**,** 7284-7297. doi: 10.1128/MCB.24.16.7284-7297.2004

Sagaram, U.S., El-Mounadi, K., Buchko, G.W., Berg, H.R., Kaur, J., Pandurangi, R.S., et al. (2013). Structural and functional studies of a phosphatidic acid-binding antifungal plant defensin MtDef4: identification of an RGFRRR motif governing fungal cell entry. *PLoS One* 8**,** e82485. doi: 10.1371/journal.pone.0082485

Sasano, Y., Hokii, Y., Inoue, K., Sakamoto, H., Ushida, C., and Fujiwara, T. (2008). Distribution of U3 small nucleolar RNA and fibrillarin during early embryogenesis in Caenorhabditis elegans. *Biochimie* 90**,** 898-907. doi: 10.1016/j.biochi.2008.02.001

Scheer, U., and Benavente, R. (1990). Functional and dynamic aspects of the mammalian nucleolus. *Bioessays* 12**,** 14-21. doi: 10.1002/bies.950120104

Schimmang, T., Tollervey, D., Kern, H., Frank, R., and Hurt, E.C. (1989). A yeast nucleolar

protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. *EMBO J* 8**,** 4015-4024. doi:

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., et al. (2005). A gene expression map of Arabidopsis thaliana development. *Nat Genet* 37**,** 501-506. doi: 10.1038/ng1543

Schranz, M.E., Mohammadin, S., and Edger, P.P. (2012). Ancient whole genome duplications, novelty and diversification: the WGD Radiation Lag-Time Model. *Curr Opin Plant Biol* 15**,** 147-153. doi: 10.1016/j.pbi.2012.03.011

Schreiber, E., Harshman, K., Kemler, I., Malipiero, U., Schaffner, W., and Fontana, A. (1990). Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. *Nucleic Acids Res* 18**,** 5495-5503. doi:

Schwarz, D.S., and Blower, M.D. (2014). The calcium-dependent ribonuclease XendoU promotes ER network formation through local RNA degradation. *J Cell Biol* 207**,** 41-57. doi: 10.1083/jcb.201406037

Seo, Y., Jun, H.R., Lee, J., Park, H., Kim, M., Lee, Y., et al. (2015). In-Cell RNA Hydrolysis Assay: A Method for the Determination of the RNase Activity of Potential RNases. *Mol Biotechnol* 57**,** 506-512. doi: 10.1007/s12033-015-9844-7

Sharma, S., Marchand, V., Motorin, Y., and Lafontaine, D.L.J. (2017). Identification of sites of 2'-O-methylation vulnerability in human ribosomal RNAs by systematic mapping. *Sci Rep* 7**,** 11490. doi: 10.1038/s41598-017-09734-9

Shaw, P., and Brown, J. (2012). Nucleoli: composition, function, and dynamics. *Plant Physiol* 158**,** 44-51. doi: 10.1104/pp.111.188052

Shoji-Kawaguchi, M., Izuta, S., Tamiya-Koizumi, K., Suzuki, M., and Yoshida, S. (1995). Selective inhibition of DNA polymerase epsilon by phosphatidylinositol. *J Biochem* 117**,** 1095-1099. doi:

Shubina, M.Y., Musinova, Y.R., and Sheval, E.V. (2016). Nucleolar Methyltransferase Fibrillarin: Evolution of Structure and Functions. *Biochemistry (Mosc)* 81**,** 941-950. doi: 10.1134/S0006297916090030

Snaar, S., Wiesmeijer, K., Jochemsen, A.G., Tanke, H.J., and Dirks, R.W. (2000). Mutational analysis of fibrillarin and its mobility in living human cells. *J Cell Biol* 151**,** 653-662. doi:

Sobol, M., Yildirim, S., Philimonenko, V.V., Marasek, P., Castano, E., and Hozak, P. (2013). UBF complexes with phosphatidylinositol 4,5-bisphosphate in nucleolar organizer regions regardless of ongoing RNA polymerase I activity. *Nucleus* 4**,** 478-486. doi: 10.4161/nucl.27154

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22**,** 2688-2690. doi: 10.1093/bioinformatics/btl446

Stekhoven, F.M.a.H.S., Bonga, S.E.W., and Flik, G. (2004). Extranuclear Histones in Teleost Gills: An Evolutionary Study. *Fish Physiology and Biochemistry* 30**,** 201-211. doi: 10.1007/s10695-005-7442-5

Stijf-Bultsma, Y., Sommer, L., Tauber, M., Baalbaki, M., Giardoglou, P., Jones, D.R., et al. (2015). The basal transcription complex component TAF3 transduces changes in nuclear phosphoinositides into transcriptional output. *Mol Cell* 58**,** 453-467. doi: 10.1016/j.molcel.2015.03.009

Sugimoto, K., and Meyerowitz, E.M. (2013). Regeneration in Arabidopsis tissue culture. *Methods Mol Biol* 959**,** 265-275. doi: 10.1007/978-1-62703-221-6_18

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

Tiku, V., Jain, C., Raz, Y., Nakamura, S., Heestand, B., Liu, W., et al. (2016). Small nucleoli are a cellular hallmark of longevity. *Nat Commun* 8**,** 16083. doi: 10.1038/ncomms16083

Tollervey, D., Lehtonen, H., Carmo-Fonseca, M., and Hurt, E.C. (1991). The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *EMBO J* 10**,** 573-583. doi:

Tollervey, D., Lehtonen, H., Jansen, R., Kern, H., and Hurt, E.C. (1993). Temperature-sensitive mutations demonstrate roles for yeast fibrillarin in pre-rRNA processing, pre-rRNA methylation, and ribosome assembly. *Cell* 72**,** 443-457. doi:

Tomkuviene, M., Licyte, J., Olendraite, I., Liutkeviciute, Z., Clouet-D'orval, B., and Klimasauskas, S. (2017). Archaeal fibrillarin-Nop5 heterodimer 2'-O-methylates RNA independently of the C/D guide RNP particle. *RNA* 23**,** 1329-1337. doi: 10.1261/rna.059832.116

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

Tsai, R.Y., and Pederson, T. (2014). Connecting the nucleolus to the cell cycle and human disease. *Faseb j* 28**,** 3290-3296. doi: 10.1096/fj.14-254680

Tschochner, H., and Hurt, E. (2003). Pre-ribosomes on the road from the nucleolus to the cytoplasm. *Trends Cell Biol* 13**,** 255-263. doi:

Van Den Bergh, E., Hofberger, J.A., and Schranz, M.E. (2016). Flower power and the

mustard bomb: Comparative analysis of gene and genome duplications in glucosinolate biosynthetic pathway evolution in Cleomaceae and Brassicaceae. *Am J Bot* 103**,** 1212-1222. doi: 10.3732/ajb.1500445

Van Lijsebettens, M., and Grasser, K.D. (2010). The role of the transcript elongation factors FACT and HUB1 in leaf growth and the induction of flowering. *Plant Signal Behav* 5**,** 715-717. doi:

Verheggen, C., Almouzni, G., and Hernandez-Verdun, D. (2000). The ribosomal RNA processing machinery is recruited to the nucleolar domain before RNA polymerase I during Xenopus laevis development. *J Cell Biol* 149**,** 293-306. doi:

Wang, H., Boisvert, D., Kim, K.K., Kim, R., and Kim, S.H. (2000). Crystal structure of a fibrillarin homologue from Methanococcus jannaschii, a hyperthermophile, at 1.6 A resolution. *EMBO J* 19**,** 317-323. doi: 10.1093/emboj/19.3.317

Warner, J.R. (1999). The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci* 24**,** 437-440. doi:

Wawrzyniak, A.M., Kashyap, R., and Zimmermann, P. (2013a). Phosphoinositides and PDZ domain scaffolds. *Adv Exp Med Biol* 991**,** 41-57. doi: 10.1007/978-94-007-6331-9_4

Wawrzyniak, A.M., Kashyap, R., and Zimmermann, P. (2013b). "Phosphoinositides and PDZ Domain Scaffolds," in *Lipid-mediated Protein Signaling,* ed. D.G.S. Capelluto. (Dordrecht: Springer Netherlands), 41-57.

Wheeler, T.J., Clements, J., and Finn, R.D. (2014). Skylign: a tool for creating informative, interactive logos representing sequence alignments and profile hidden Markov models. *BMC Bioinformatics* 15**,** 7. doi: 10.1186/1471-2105-15-7

Yanagida, M., Hayano, T., Yamauchi, Y., Shinkawa, T., Natsume, T., Isobe, T., et al. (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. *J Biol Chem* 279**,** 1607-1614. doi: 10.1074/jbc.M305604200

Yao, H., Wang, G., and Wang, X. (2014). Nuclear translocation of proteins and the effect of phosphatidic acid. *Plant Signal Behav* 9**,** e977711. doi: 10.4161/15592324.2014.977711

Ye, K., Jia, R., Lin, J., Ju, M., Peng, J., Xu, A., et al. (2009). Structural organization of box C/D RNA-guided RNA methyltransferase. *Proc Natl Acad Sci U S A* 106**,** 13808-13813. doi: 10.1073/pnas.0905128106

Yildirim, S., Castano, E., Sobol, M., Philimonenko, V.V., Dzijak, R., Venit, T., et al. (2013). Involvement of phosphatidylinositol 4,5-bisphosphate in RNA polymerase I transcription. *J Cell Sci* 126**,** 2730-2739. doi: 10.1242/jcs.123661

Yoo, D., Wootton, S.K., Li, G., Song, C., and Rowland, R.R. (2003). Colocalization and interaction of the porcine arterivirus nucleocapsid protein with the small nucleolar RNA-associated protein fibrillarin. *J Virol* 77**,** 12173-12183. doi:

Yoshikawa, H., Komatsu, W., Hayano, T., Miura, Y., Homma, K., Izumikawa, K., et al. (2011). Splicing factor 2-associated protein p32 participates in ribosome biogenesis by regulating the binding of Nop52 and fibrillarin to preribosome particles. *Mol Cell Proteomics* 10**,** M110.006148. doi: 10.1074/mcp.M110.006148

Yu, H., Fukami, K., Watanabe, Y., Ozaki, C., and Takenawa, T. (1998). Phosphatidylinositol 4,5-bisphosphate reverses the inhibition of RNA transcription caused by histone H1. *Eur J Biochem* 251**,** 281-287. doi:

Zheng, L., Du, Z., Lin, C., Mao, Q., Wu, K., Wu, J., et al. (2015). Rice stripe tenuivirus p2 may recruit or manipulate nucleolar functions through an interaction with fibrillarin to promote virus systemic movement. *Mol Plant Pathol* 16**,** 921-930. doi: 10.1111/mpp.12220

Zwang, Y., Sas-Chen, A., Drier, Y., Shay, T., Avraham, R., Lauriola, M., et al. (2011). Two phases of mitogenic signaling unveil roles for p53 and EGR1 in elimination of inconsistent growth signals. *Mol Cell* 42**,** 524-535. doi: 10.1016/j.molcel.2011.04.017