

Fibrillarin from Archaea to human

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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 preribosomal 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.



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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 rRNA 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; Hinsby et al., 2006; 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-rRNA 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 conserved 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.

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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 protein; PIP2, phosphatidylinositol 4,5-bisphosphate; PNBs, pre-nucleolar bodies; pol I, RNA polymerase I; PRMT1, protein arginine N-methyltransferase 1; RAP, [RuLCl₂]H·4H₂O; SAM, S-adenosylmethionine; ASF/SF2, alternative splicing factor/splicing factor 2; SMN, survival of motor neuron; snoRNA, small nucleolar RNA; snoRNP, small nucleolar ribonucleoprotein; snRNA, small nuclear RNA; Tat, Trans-activator of transcription.

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 *Sulfolobus solfataricus*. Testing the reconstructed 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., 2011; Miller et al., 2012) as well as interacting with viral proteins from the Influenza A virus and the transactivator 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, rRNA 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

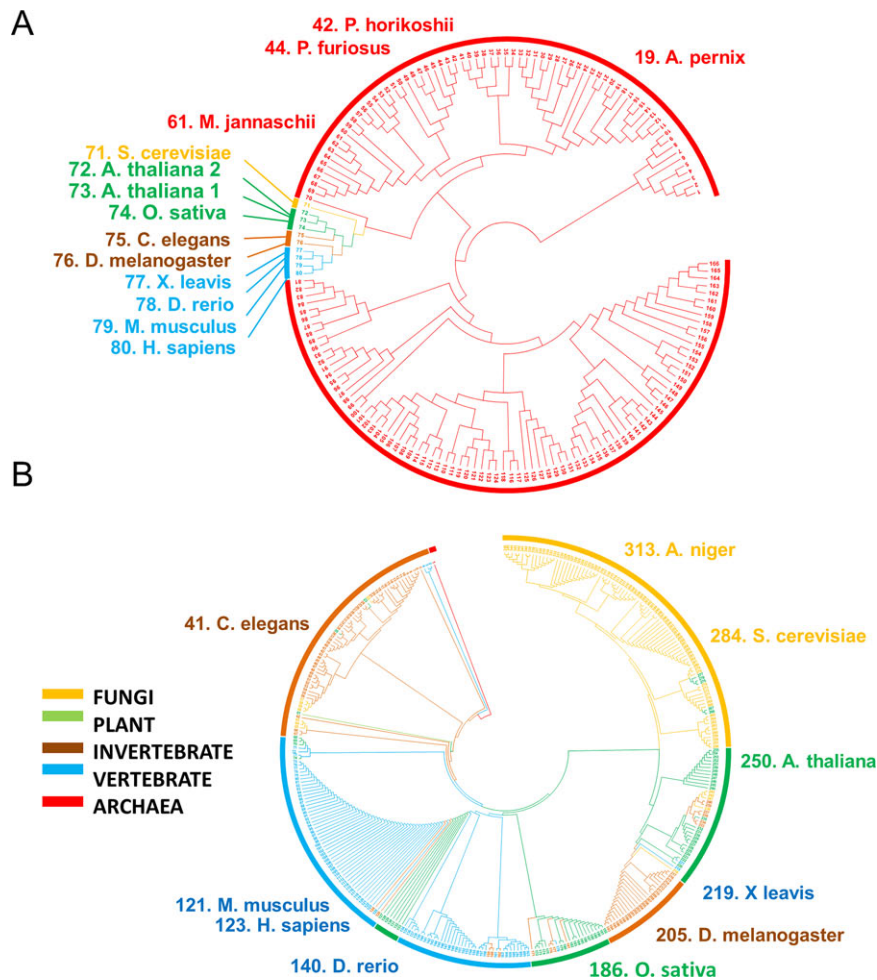
not completely complement specific roles of eukaryotic fibrillarins. Furthermore, as in eukaryotic cells fibrillarin is localised primarily in the nucleoli and Archaea do not have nucleus, it would be unlikely that all functions are conserved. Archaeal fibrillarins have been used in recombinant protein 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-rRNA. 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 thermophila* 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 showing 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 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 1B). The cladogram

Figure 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.



reveals nine primary branches that separate groups of fungi, invertebrate, plant and vertebrates. The overall 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.

Table 1 | Fibrillarins 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/M)-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/M)-(V/I)-G

The sequences show the specific sequence of amino acids that are common in all fibrillarins. This signature is localised in the RNA binding domain in all of the taxa and was obtained using Pratt-Pattern Matching (<http://www.ebi.ac.uk/Tools/pfa/pratt/>).

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 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.

Fibrillarins structure and domain functions

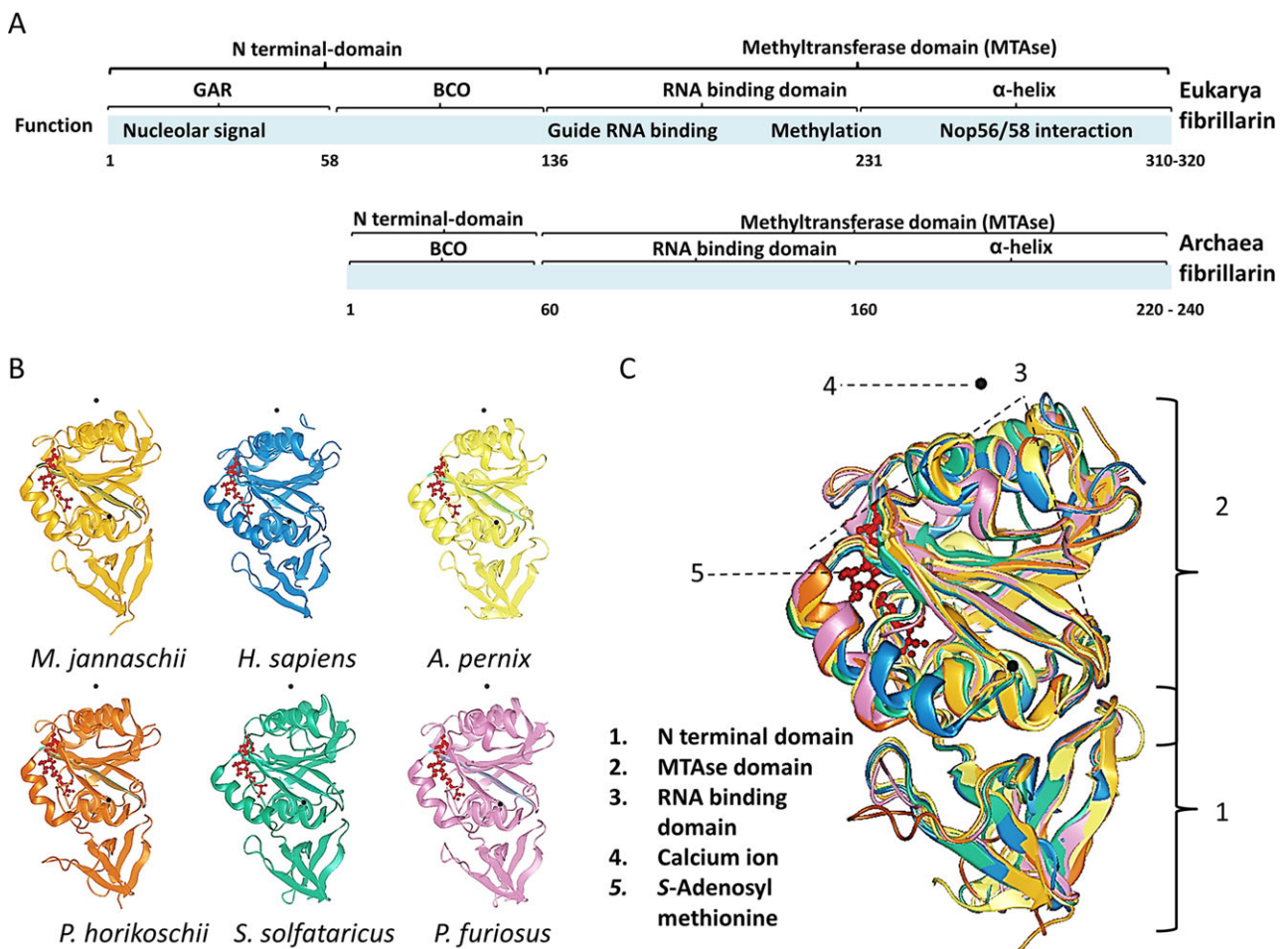
The fibrillarins protein sequence can be divided into two big domains: the N-terminal domain and the domain with the MTase. In *A. thaliana*, the N-terminal domain is divided into two regions: (1) the GAR domain with around 77 amino acids and (2) a spacer region with 61 amino acids (Figure 2A). The GAR 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 fibrillarins to the Cajal bodies (CBs). The GAR domain of the human fibrillarins and the *Arabidopsis* fibrillarins is completely necessary for nuclear localisation (Pih et al., 2000; Levitskii et al., 2004) and is methylated on several arginine residues. Fibrillarins 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 fibrillarins 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* fibrillarins 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 fibrillarins also interacts with Nop56 protein (Lechertier et al., 2009). Furthermore, amino acid residues of the MTase catalytic triad of *Arabidopsis* fibrillarins 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 fibrillarins interact with the splicing factor 2 associated p32 and the MTase domain interacts with PRMT5. Fibrillarins 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

Figure 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-align). The localisation of the calcium ion and the S-Adenosyl methionine are shown as well as the domain regions.



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-rRNA. Mutations in positions V87G, E103G, A175V and P219S inhibit nucleolar methylation of the 35S

rRNA. 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-rRNA cleavage, which include S257, D263, T284 differ in other fibrillarins such as *Arabidopsis* fibrillarins 1 corresponding to A234, T240, A261, respectively, and in *At*-fibrillarins 2 to A245, S251, A272. Hence, the difference in key amino acids in plant fibrillarins 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 2) reveals that fibrillarins 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 fibrillarins in complex with S-adenosyl-L-homocysteine (unpublished data by Plotnikov's group, <http://www.thesgc.org/structures/2ipx>), the *Aeropyrum pernix* fibrillarins in complex with the SAM (de Silva et al., 2012), the fibrillarins in complex with the Archaea protein Nop56/58 (Oruganti et al., 2007), the hyperthermophilic Archaea *Pyrococcus furiosus* fibrillarins (Deng et al., 2004), the hyperthermophilic Archaea *Pyrococcus horikoshii* fibrillarins (unpublished data by Boisvert, D.C. and Kim, S.H.; <http://pdj.org/emnavi//quick.php?id=1g8a>), the *Methanococcus jannaschii* fibrillarins (Wang et al., 2000), the fibrillarins 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-ray 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 rRNA (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 fibrillarins structure. While most of the tested fibrillarins retain a similar shape, the human counterpart and the only tested eukaryotic fibrillarins starts at position 93 and therefore the GAR domain is missing, so it resembles the archaeal fibrillarins (Figure 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 fibrillarins was crystallised has yet to be published, it remains to be seen if the structure is so well conserved. Furthermore, seven key amino acids of NOP1 at positions 87, 103, 138, 176, 257, 263 and 284 are not conserved in the aFIB, which raises the question if these key amino acids have other roles in eukaryotic cells.

Post-transcriptional modifications by fibrillarins

Fibrillarins-specific complex is directly involved in different post-transcriptional processes such as pre-rRNA cleavage, rRNA methylation and ribosome assembly (Tollervey et al., 1993). Methylation of rRNA is carried out in more than 100 sites, with some variation depending on the organism. However, for all these sites fibrillarins is the main candidate 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 rRNA 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 rRNA, 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 rRNA 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 activity of rRNA 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 localisation 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 localised 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 rRNA processing are shut down and the nucleolus starts disintegrating. Fibrillarin together with components of the processing complex such as pre-rRNA, 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 localised in the processing complex components from pre-nucleolar bodies (PNBs) (Medina et al., 1995). Interactions detected between proteins of the same rRNA processing machinery in both PNBs and nucleoli suggest that PNBs are pre-assembly platforms for rRNA-processing complexes (Angelier et al., 2005). Then, PNBs become associated with nucleolar organiser 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 rRNA 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-rRNA is essential and sufficient for this recruitment (Verheggen et al., 2000). On the other hand, it has been proposed that kinases and/or phosphatases engaged 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 rRNA 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 localised 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 involvement 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 between 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., 2014).

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 localisation signal 2/nucleolar localisation 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 localisation signals, it is likely that viruses have evolved specific nucleolar functions (Mellen 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 rRNA maturation and overall amount of 80S ribosome (Ponti et al., 2008). The impairment of nucleolar pre-rRNA 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.

Review on fibrillarin

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 behaviour 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., 2011). In human adenocarcinoma, the amount of fibrillarin correlates *in vivo* with the amount of MYC protein, a well-characterised oncogene that has been also shown to interact with fibrillarin (Koh et al., 2011; 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 leukaemia 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 rRNA 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 dephosphory-

lation of phosphoinositide into phosphatidylinositol 4,5-bisphosphate (PIP2). PIP2 interaction with fibrillarin results in conformational changes of fibrillarin 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 autschizis in a ratio of 100:1. Because autschizis 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 autschizis (Jamison et al., 2010). Fibrillarin was also found in low quantities in the FCs and in the nucleoplasm after the treatment with a newly synthesised antitumor complex [RuLCl₂]H·4H₂O (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 autschizis. Fibrillarin staining shifted from FCs and adjacent regions to a more homogeneously stained of entire nucleolus. This finding was consistent with the percentage of autschizic 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 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 subunit 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 rRNA 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 rRNA (Lechertier et al., 2009).

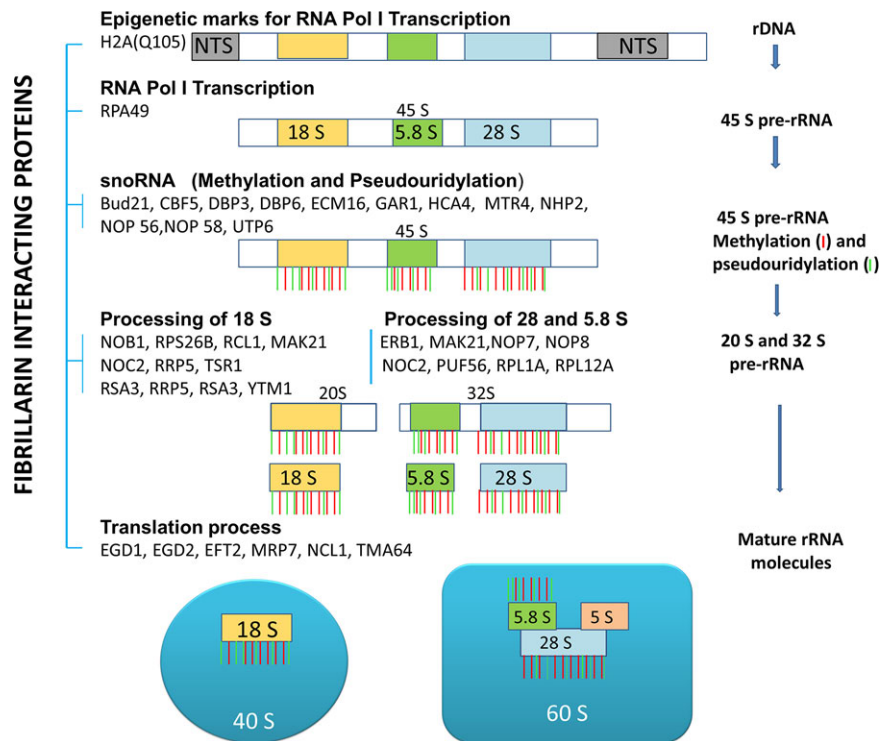
The complex has a mass of 400 kDa that could involve fibrillarin tetramer that changes conformation in order to methylate different regions of the rRNA. Nop56/58 plays an important role in positioning the catalytic subunit on the target RNA by simultaneously contacting the guide RNA and fibrillarin.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

Gene duplication of fibrillarin in plants can lead to specialised functions. Fibrillarin 2 from *A. thaliana*

Figure 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 rRNA processing of methylation and further processing of the 45 S pre-rRNA into 20 S and 32 S pre-rRNA. Finally, it can interact with proteins involved in the translation process.



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).

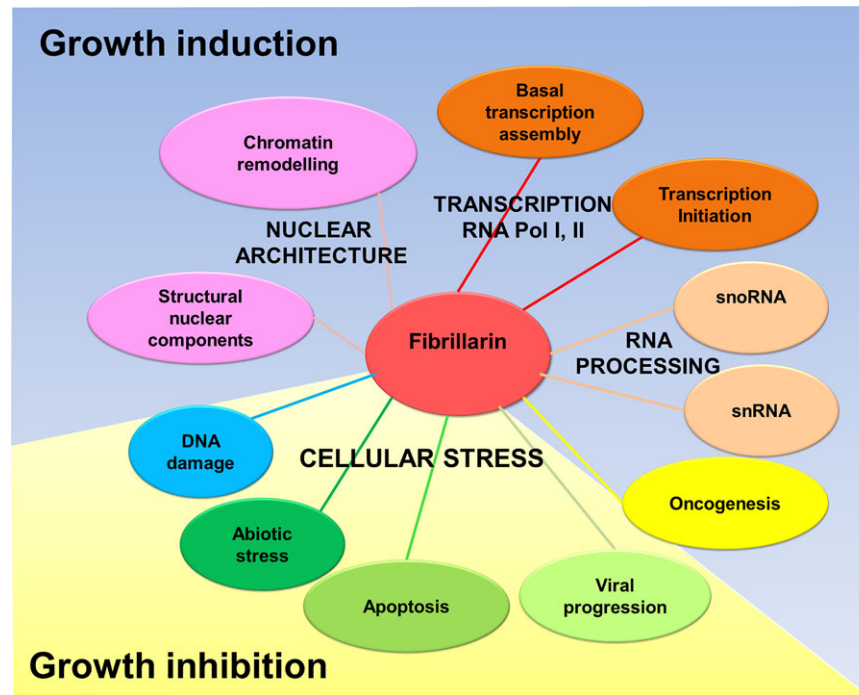
Many other proteins have been detected to interact with fibrillarin in low- and high-throughput methods (Krogan et al., 2006; Chatr-Aryamontri et al., 2013). Figure 4 shows some of the possible processes in which the partners of fibrillarin are involved. The functional roles were segregated for a preliminary selection but proteins can have also additional functional roles inside a cell. Supplementary Table 2 compiles the complete list of known interacting partners and processes they are involved. Not surprisingly, most of the proteins are involved in the rRNA biogenesis and rRNA maturation processes. The early known temperature-sensitive fibril-

larin 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 proteasome (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 nucleolus and for association with the nascent rRNA for further methylation and processing (Yildirim et al., 2013). Furthermore, the colocalisation of fibrillarin

Figure 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.



and PIP2 in actively transcribing cells was detected in the DFC region (Sobol et al., 2013).

Conclusion

There is very limited evidence that fibrillarin is functionally involved outside the realms of rRNA processing. 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-rRNA 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 hybridisation 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 rRNA 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 4 (for a complete list, see Supplementary Table 2). 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.

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Conflict of interest statement

The authors have declared no conflict of interest.

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