Article

Nucleolin Interacts and Co-Localizes with Components of Pre-Catalytic Spliceosome Complexes

Iva Ugrinova 1,†, Mounira Chalabi-Dchar 2,†, Karine Monier 2 and Philippe Bouvet 2,3,*

1 “Roumen Tsanev” Institute of Molecular Biology, Bulgarian Academy of Sciences, BG1113 Sofia, Bulgaria
2 Cancer Cell Plasticity Department, Centre de Recherche en Cancérologie de Lyon, Université de Lyon, UMR INSERM 1052 CNRS 5286, Centre Léon Bérard, Lyon 69008, France
3 Ecole Normale Supérieure de Lyon, Université de Lyon, Lyon 69342, France
* Correspondence: pbouvet@ens-lyon.fr; Tel.: +33-6-2744-6212
† Authors contributed equally to this work.

Received: 4 June 2019; Accepted: 25 June 2019; Published: 1 July 2019
First Version Published: 14 June 2019 (doi:10.3390/sci1020033)

Abstract: Nucleolin is an RNA binding protein that is involved in many post-transcriptional regulation steps of messenger RNAs in addition to its nucleolar role in ribosomal RNA transcription and assembly in pre-ribosomes. Acetylated nucleolin was found to be associated with nuclear speckles and to co-localize with the splicing factor SC35. Previous nuclear pull down of nucleolin identified several splicing components and factors involved in RNA polymerase II transcription associated with nucleolin. In this report, we show that these splicing components are specifics of the pre-catalytic A and B spliceosomes, while proteins recruited in the Bact, C and P complexes are absent from the nucleolin interacting proteins. Furthermore, we show that acetylated nucleolin co-localized with P-SF3B1, a marker of co-transcriptional active spliceosomes. P-SF3B1 complexes can be pulled down with nucleolin specific antibodies. Interestingly, the alternative splicing of Fibronectin at the IIICS and EDB sites was affected by nucleolin depletion. These data are consistent with a model where nucleolin could be a factor bridging RNA polymerase II transcription and assembly of pre-catalytic spliceosome similarly to its function in the co-transcriptional maturation of pre-rRNA.

Keywords: nucleolin; spliceosome assembly; splicing factors; immunofluorescence; proteomic

1. Introduction

Nucleolin (Ncl) is one of the most abundant protein of the nucleolus, and it has been implicated in many molecular and cellular processes, although its functions remain poorly understood [1,2]. Ncl could be characterized as a moonlighting protein [3,4] as it is able to perform multiple and unrelated functions without partitioning these functions into different protein domains. Indeed, the central domain of Ncl composed of 4 classical RNA-Binding domains (RBD) allow the interaction of Ncl with numerous nucleic acids sequences (RNA and DNA) involving Ncl in diverse functions, such as pre-ribosomal RNA maturation, mRNA stability, mRNA translation regulation, transcriptional regulation of specific genes [5,6].

Ncl is also remarkable due to the fact that it is present in many different cellular localization [1]. Although historically, it was found as an abundant nucleolar protein (which gave it its name, Nucleolin) [7], it has now been shown to be present also in the nucleoplasm, cytoplasm and on the cell surface of many cell types. It is likely that for each of these cellular localizations, Ncl is associated with a specific set of proteins that give to Ncl a localization-dependent specific function.

These multiple cellular localizations and associated functions could be also determined by specific post-translational modifications (PTM) of Ncl. Indeed, several PTM have been described for Ncl, such as
phosphorylation [8–10], methylation [11], glycosylation [12], SUMOylation [13], ubiquitination [14,15], and acetylation [16].

The N-terminus of Ncl is highly phosphorylated by two major kinases casein kinase 2 (CK2) and mitotic cyclin-dependent kinase 1 (CDK1) [8,10]. Ncl phosphorylation by CK2 is required through the S-phase progression in cell cycle and hence proliferation [17]. At the other end of the protein, N-arginine dimethylation modulates the interaction between the c-terminal domain of Ncl (GAR or RGG domain) with nucleic acids [18]. Regulation of the interaction of Ncl with nucleic acids can be also achieved through SUMOylational modification at Lys-294 of the human protein, which facilitated the mRNA binding property of Ncl by maintaining its nuclear localization [13]. Cell surface Ncl is exclusively glycosylated and N-glycosylation is required for its expression on the cell surface [19]. We have previously shown that Ncl could also be acetylated (Ac-Ncl) on several lysines, which are present exclusively within the first 150 N-terminal residues of nucleolin [16]. Using a specific antibody against acetylated K88 (Ac-K88), it was shown that ac-K88 Ncl was not present in the nucleolus nor associated with rDNA, but instead it was found associated with specific nuclear domains that co-localized with SC35 (SRSF2; serine/arginine-rich splicing factor 2) [16]. This co-localization of Ac-Ncl with nuclear speckles that contains SC35 suggest that Ncl could be associated in spliceosome assembly and/or in pre-mRNA splicing. So far, there has been no report demonstrating a direct role for Ncl in splicing; however, Ncl has been found in an RNP complex formed on a specific HIV pre-mRNA splicing site (SLS2-A7 RNA) [20]. Ncl binds to specific RNA sequences in SLS2-A7 RNA, perhaps affecting its alternative splicing. Further links between Ncl and RNA processing are demonstrated by the interaction of Ncl with DGC8 and Drosha, the core components of the microprocessor complex and its involvement in the biogenesis of miR-15a/16, by a direct action during precursor processing [21]. Ncl was also found associated with Oct4-related lncRNAs, and perhaps regulates the production of Oct4 transcript [22].

A nucleolin interactome study using a Tap–tag purification of nuclear Ncl complexes allowed the identification of about 140 potential Ncl nuclear interacting partners [23]. About 50% of these proteins are involved in ribosome biogenesis which reflect the major nucleolar role of Ncl. Gene ontology of the purified proteins identify also 45 proteins implicated in RNA metabolism (ribosomal RNA excluded) including RNA stability, RNA nuclear export, pre-mRNA splicing. Interestingly, most of these proteins were still associated with Ncl after RNase treatment, suggesting a direct protein-protein association [23].

To gain more insight into the potential implication of Ncl in spliceosome assembly/function, in this work, we studied the subcellular co-localization and interaction of Ac-Ncl with a component of catalytically activated/active spliceosome, P-SF3b155 (phosphorylated SF3B1). Together with the re-analysis of the splicing components found in our previous proteomic data [23], our data suggest that Ncl interact mostly with the pre-catalytic B complex and could probably participate in the assembly of the pre-catalytic spliceosomes.

2. Results

2.1. Subnuclear Distribution of Acetylated Nucleolin in Nuclear Speckles

Acetylated Ncl co-localizes with SC35 in HeLa and PBMC stimulated cells [16]. Nuclear speckles containing SC35 most likely represent storage sites for splicing factors [24] as they contain high concentration of the spliceosome machinery components [25]. The exact functions of speckles in RNA splicing is still not completely understood as some reports describe the nuclear speckles as RNA processing sites while others support a view where RNA processing takes place in close proximity to the speckles [26,27]. Thanks to the biochemical characterization of the different complexes involved in splicing, specific proteins involved in the different steps of the spliceosome assembly have been described [28]. SF3b1 (SF3b155), a U2 associated protein is a critical component of the spliceosome active site and is phosphorylated during the splicing catalysis [29]. Antibodies that detect the phosphorylated
form of SF3B1 have been developed [30]. We used these antibodies to precisely compare the localization of Ac-Ncl with SC35 and P-SF3B1 (Figure 1).

**Figure 1.** Colocalization of acetylated nucleolin (Ac-Ncl) with SC35 and P-SF3B1. (A) Triple immunofluorescence (IF) microscopy of U2OS cells (Human Bone Osteosarcoma Epithelial Cells) displayed as a 3-color merged image of a single nucleus. Nuclear speckles are detected with the anti-SC35 antibody (blue) and active spliceosome with the anti-P-SF3B1 (green). Ac-Ncl (red) is visualized using the antibody detecting anti-ac-K88 Ncl. (B) Presentation of individual sections of one z-stack image. The white inset in panel A is enlarged and presented as individual sections. Z-stack sections were acquired (optical section of 200 nm) and deconvolved. Scale bars, 5 µm for the main images and 1 µm for the insets.

Anti-SC35 antibody (blue signals) defines punctuated structures corresponding to the nuclear speckles that are most of the time associated with P-SF3B1 (green signals). In contrast, P-SF3B1 (green signals) labeling detects higher number of punctuate structures that are not all associated with SC35. In the same nucleus, Ac-Ncl (red signals) are more often associated with P-SF3B1 (green signals) than with SC35 (blue signals). In addition, only sparse ac-Ncl red signals exhibit no association with blue SC-35 nor green p-SF3B1 signals. A close examination of a close-up z-section view of a nuclear speckle (white square in Figure 1A) shows that in several sections ac-Ncl, SC35 and P-SF3B1 colocalize but did not completely overlap (Figure 1B). Ac-Ncl seems to be on the periphery of the SC35 structure as does P-SF3B1.

To better analyze the colocalisation of Ac-Ncl signals compared to SC35 and P-SF3B1 signals, a quantification of the colocalization was performed using the JACoP plugin in the ImageJ software (Figure 2).

In our analyses, Pearson’s coefficients (PC) r were used to establish a first estimate of co-localization by correlating pixel intensities between images based on a coefficient. This approach gives the linear equation describing the relationship between intensities in the two images. The value of PC can range from 1 to −1, with 1 standing for complete positive correlation and −1 for a negative correlation, with zero standing for no correlation. PC is very sensitive to thresholding and evaluation of co-localization events using PC alone may be ambiguous, as values are highly dependent on noise, variations in fluorescence intensities or incomplete co-localization relationships throughout the sample [31]. Mid-range coefficients $r = 0.459$ and $r = 0.441$ for SC35 with Ac–Ncl or with P-SF3B1 respectively do not allow drawing clear conclusions (Figure 2A,B). However, the PC value obtained for Ac–Ncl and P-SF3B1 ($r = 0.738$) showed good co-localization (Figure 2C).
Mid-range coefficients $r = 0.459$ and $r = 0.441$ for SC35 with Ac–Ncl or with P–SF3B1 respectively do not allow drawing clear conclusions (Figure 2A, B). However, the PC value obtained for Ac–Ncl and P–SF3B1 ($r = 0.738$) showed good co-localization (Figure 2C).

**Figure 2.** 3D colocalization quantification of acetylated Ac–Ncl with SC35 and P–SF3B1. (A) Co-visualization of acetylated nucleolin (Ac–NCL) and P–SF3B1 together with SC35 in U2OS cells (displayed as a 3-color merged image of a nucleus). The white inlet is enlarged and presented in panel D. (B) Co-visualization of Ac–Ncl, P–SF3B1 and SC35 in U2OS cells. For better visualization of colocalization single channels and 2 color merged images are presented in the upper and lower rows respectively. Ac–Ncl was detected with a rabbit polyclonal antibody directed against Ac-K88 of human Ncl [16]; (Covalab, Lyon, France, ref: 00106706) visualized with an anti-rabbit secondary antibody coupled to Alexa555 [red]; P–SF3B1 was detected with an anti-pT313-SF3b155 [30] (gift from R. Lührmann and C. Girard) visualized with an anti-rabbit secondary antibody coupled to Alexa488 [green], while SC35 was detected with an monoclonal antibody (ab11826, Abcam, UK) and visualized with an anti-mouse secondary antibody coupled to Alexa647. The calculated Pearson’s coefficients are presented below the corresponding 2 color images. (C) Graphical presentation of calculated Mander’s coefficients for 54 individual cells analyzed in 3D for Ac–Ncl, P–SF3B1 and SC35. (D) Enlargements represented single channels and merged image inset (white square in panel A). Colocalization analyses were performed with the JACOP plugin in ImageJ software. All images were deconvolved and channel shifts were corrected before colocalization analysis. Scale bars, 5 µm for the main images and 1 µm for the insets.
To evaluate the co-localization events more rigorously, the Mander’s overlap coefficient was also calculated [32]. This coefficient varies from 0 to 1, the former corresponding to non-overlapping images and the latter reflecting 100% co-localization between both images. The advantage of using Mander’s overlap coefficient is that M1 (or M2) is a good indicator of the proportion of one signal coincident with a signal in the other channel over its total intensity. This estimation could even apply if the intensities in both channels are different from one another. The Mander’s coefficient is very sensitive to noise, so M1 and M2 were calculated, setting the threshold to the estimated value of background instead of zero. In our experiments, 54 nuclei were scored, and the statistical analyses are presented in Figure 2C.

It appears that an Ac–Ncl signal (red) co-localizes much more fully with the active splicing marker P-SF3B1 (70% in the green SF3B1 channel regarding Ac–Ncl, Figure 2C) and vice versa than with the general spliceosomal factor SC-35 (35% in the blue SC-35 channel regarding Ac-Ncl distribution, Figure 2C). Therefore, a higher percentage of Ac–Ncl signals colocalize with P-SF3B1 than with SC35.

It seems that SC35 is smaller and more centrally positioned than Ac–Ncl and P-SF3B1, which have higher signal volume and are located at the periphery of the SC35 structure (Figure 2D). This model matches very well to the calculated Mander’s coefficients presented on the bar graph (80% in the red and in the green channels regarding SC-35 distribution (Figure 2C).

Acetylation of Ncl is important for the co-localization with P-SF3B1. Indeed, colocalisation experiments with an Ncl Ab that detect acetylated and non-acetylated forms of Ncl [16] show very little co-localization with P-SF3B1 (Supplementary Figure S1). As the large majority of Ncl is not acetylated [16] and is localized in the nucleoli, this polyclonal Ab labeled mostly the nucleoli structures and only very weakly the speckles (Supplementary Figure S1).

2.2. Interaction of Ncl with Spliceosome Components

To identify the nuclear proteins that interact with Ncl, a proteomic analysis of the protein that can be pull down using a tap-tag approach was previously developed [23]. Tap-tagged Ncl was expressed in HeLa cells, and protein complexes were purified from nuclear extracts and analyzed by mass spectrometry. Out of the 140 proteins 33 were found in the spliceosome database [33] (Supplementary Table S1). As Ncl is an RNA-binding protein, the recovery of spliceosome components with Ncl could be indirect and mediated by an RNA molecule that bridges Ncl with the spliceosome. However, after RNAse treatment, only 5 (SNRPA, PUF60, U2SURP, USP39 and CDC5L) out of the 33 proteins were lost [23] (Supplementary Table S1, underlined proteins), showing that the pull down of the spliceosomal protein was probably not RNA mediated. SF3B1 was found in the Ncl pull down proteins (Supplementary Table S1). We validated this finding by immunoprecipitation using an anti-Ncl antibody (Figure 3). Although P-SF3B1 is not very abundant in these cells (Inp lane, Figure 3), we were able to clearly detect P-SF3B1 in the pull down with anti-Ncl antibody (IP lane-Ncl, Figure 3).

The spliceosome is a highly dynamic structure that bring together more than 200 components. The composition of the spliceosome changes at each step of the splicing reaction forming different complexes: A, B, Bcat, C and P [28,34]. Specific components are added or removed during this process in order to assemble, catalyze the splicing reaction, disassemble and recycle the spliceosome components. In order to define if Ncl was present all along the assembly of active spliceosome or if it is present only during a specific step of the spliceosome assembly, we determine in which complex(es) belong the 33 spliceosomes proteins pull down with Tap-tagged Ncl (Supplementary Table S1). Many proteins are presents in snRNPs that are common for the different spliceosome complexes. However, several proteins (DX15, U2AF1, U2AF2, PUF60 and U2SURP) of the 17S U2 snRNP which is specific of the A complex are present in the Ncl pull down. The pre-catalytic B complex is characterized by the presence of the U4/U6 snRNP which contain PRPF4, PRPF3, PPIH, PRPF31 that are also associated with Ncl complexes. SART1 and USP39 associated with the U4/U6.U5 tri-snRNP and MFAP1 and IK that are recruited at the B complex are also associated with NCL. In contrast, none of the specific protein present in the Bcat, C and P complex (except for the helicase DDX41, Supplementary Table S1) are present in the Ncl pull down. Altogether this analysis show that the spliceosomal proteins that
interact with Ncl belong to the early stage of spliceosome assembly (A and B complex, Supplementary Table S1).

![Table 3](image)

**Figure 3.** Nucleolin co-immunoprecipitate with P-SF3B1—A protein member of spliceosome complex and marker for active splicing. Immunoprecipitation (IP) assay was performed in U2OS cells with anti nucleolin polyclonal antibody (polyclonal antibody 5567). Following immunoprecipitation, proteins were separated on an SDS-PAGE gel and probed with an anti nucleolin polyclonal antibody and with anti P-SF3B1 polyclonal antibody and an anti-rabbit secondary antibody coupled to IRdye800 was used. IP with pre-immune serum (IgG) was used as control. Inp: input pre-immunoprecipitation fractions; IP: immunoprecipitated proteins; Sup: supernatant after immunoprecipitation.

**2.3. Depletion of Ncl Affects the Alternative Splicing of Fibronectin**

To determine if this co-localization of Ac–Ncl with P-SF3B1 and the interaction of Ncl with many spliceosome components had a functional role in splicing, we studied (Figure 4) the consequences of Ncl depletion on the alternative splicing of Fibronectin (FN) mRNA which is a well know model to study the co-transcriptional splicing [35,36]. Ncl depletion by siRNA in HeLa cells leads to a strong reduction in Ncl mRNA and protein accumulation (Figure 4A), however the levels of FN mRNA accumulation, measured by RT-QPCR did not significantly change (Figure 4C). However, when the products of these amplification were resolved on an agarose gel we could clearly observed a significant different accumulation of alternative spliced FN mRNA for the mRNA regions coding for the EDB and IIICS domains (Figure 4D,E). Therefore, although the global expression of FN is not changed after Ncl depletion, the co-transcriptional alternative splicing at IIICS and EDB sites is affected upon Ncl depletion.
Figure 4. NCL depletion impacts Fibronectin (FN) alternative splicing. (A) HeLa cells were treated with control siRNA (siCTL) or NCL siRNA (siNCL) respectively. After 5 days, protein extracts and total RNA were prepared. NCL mRNA level was measured by RT-qPCR (left panel) and NCL protein expression was assessed by western blot and normalized by comparison with β-actin (right panel). **: p-value < 0.005. (B) Partial structure of Fibronectin gene. The type III repeat domains are shown as boxes and the number of the repeat among 15 is shown in the box. Approximate positions of the primers (previously used in Kumakazi et al. 1999) [37] are shown by arrows under the boxes. (C) Different splice variants relative expressions were measured by RT-qPCR in HeLa cells upon the depletion of NCL. F.C; for fold change compared to siCTL. (D) Agarose gel analysis of PCR products: IIICS, EDB. v1 for variant1 and v2 for variant 2. (E) Quantification of FN splice variants from two independent experiments. The intensity of each band (from agarose gel in D) was measured by ImageLab® software. *: p-value < 0.05.
3. Discussion

Understanding how Ncl can have multiple functions in the cell is crucial to determine the role of Ncl. The multiple cellular localizations coupled with post-translational modifications and specific interacting proteins are probably key to elucidate the molecular functions of Ncl [1]. The discovery that Ncl could be acetylated on Lysine residues in the N-terminal domain and that this Ac–Ncl is specifically co-localized with nuclear speckles suggested that Ac–Ncl could be involved in some aspect of pre-mRNA processing [16]. Ncl has been implicated in different aspects of mRNA metabolism/regulation, such as mRNA adenylation [38,39], mRNA stability [13,40], translation regulation [41–46], nuclear export of RNP particles [47], but there are no report demonstrating a direct Ncl implication in pre-mRNA splicing. Ncl binds to SLS2-A7 stem-loop structure present in HIV RNA sequences [20] that are involved in HIV-RNA splicing and export, and seems to affects the processing of several miRNA [21], but there are no experimental demonstration of a direct function of Ncl in these processes.

The recent proteomic study on the Ncl–nuclear interacting proteins raised again the potential implication of Ncl in pre-mRNA splicing, as many splicesomal components were identified [23]. Out of the 140 identified proteins, 33 were found to be part or associated with splicesomes (Supplementary Table S1). Interestingly, only 5 of these proteins were lost upon RNase treatment, suggesting that these interactions are not RNA mediated. The pull down of P-SF3B1 by the Tap-tagged Ncl was mostly not affected by the RNase treatment [23]. The full splicesome machinery was not recovered, and only specific proteins of the different snRNPs were present in Ncl pull down. For instance, for the 17S U2 snRNP, only 3 out of the 13 constituents of this snRNP were found. In contrast, 5 out of the 9 U5 snRNP proteins and 4 out of the 7 U4/U6 snRNP were found in Ncl interactome data.

Splicesomes are highly dynamic complexes with many components that are present during all the splicing reaction and also many additional proteins that are recruited only in specific steps of the splicing event [48]. Thanks to the extensive biochemical and structural characterization of the splicesomal intermediates components [33,49–51] we could determine to which splicesomal complexes the Ncl associated proteins belong (Supplementary Table S1). Interestingly, proteins specific of the A and B pre-catalytic complex where found in the Ncl pull down (17S U2 snRNP associated proteins and U4/U6 snRNP respectively), while proteins recruited at the Bact, C and P complexes were completely absent in the pull down with the exception of DDX41 (Supplementary Table S1). As DDX1 has other functions that in pre-mRNA processing [52], the presence of DDX1 in Ncl pull down may not be related to splicesomal component. Interestingly, in a previous study, it was found that Ncl was associated with the A complex after treatment with anacardic acid an inhibitor of acetyltransferase [53]. Anacardic acid has inhibitory activity toward P300/CBP and PCAF acetyltransferases that efficiently acetylate Ncl [16]. This could suggest that this post-translational modification of Ncl could be involved in the regulation of association or release of Ncl with the splicesome complexes. These data suggest that Ncl may participate in the early assembly of the splicesome onto pre-mRNA.

It is believed that about 80% of pre-mRNA splicing is taking place during transcription. SF3B1, a critical component of the splicesome active site, is phosphorylated during the splicing catalysis and P-SF3B1 can be used as a marker for immunolocalization and biochemical characterization of the co-transcriptional splicesome [30]. P-SF3B1 can be pulled down with Ncl (Figure 3), and immuno-fluorescence experiments (Figure 2) show that Ac–Ncl is strongly co-localized with P-SF3B1 (Mander’s coefficient of 0.706), while the co-localization of Ac–Ncl with SC35 was only partial ($r = 0.298$). The partial co-localization of P-SF3B1 with SC35 (Figure 2; $r = 0.232$) is consistent with data presented in a previous report [30]. In addition, in this report, the authors suggested that the 20% of P-SF3B1 that was associated with the SC35 labeled nuclear speckles could correspond to the function of P-SF3B1 in the post-transcriptional splicing [30]. Although about 70% of Ac–Ncl co-localized with P-SF3B1, a marker of activated splicesome, in the proteomic data of the Ncl nuclear complex, we did not find the specific proteins that are recruited at the Bact complex. As P-SF3B1 detect active splicesome present in region of decompacted chromatin, and is present in chromatin fraction containing marks of transcriptionally active chromatin [30], it is possible that this Ac-Ncl colocalisation with P-SF3B1...
indicates rather the sites where are located the co-transcriptional P-SF3B1 spliceosomes rather than the colocalisation of active spliceosome per se. Indeed, basic components of the RNA polymerase II (RNAPII) complex (POLR2B, POLR2H, TFIIF), several transcription factors and subunits of chromatin remodeling complexes (SMARCA4, CHD4, SSRP1) were also found in Ncl nuclear pull down. In that case, Ac-Ncl could be a key component to bridge the assembly of pre-catalytic spliceosomes during the transcription process. Interestingly, we show that the alternative splicing of Fibronectin at the IIICS and EDB site is affected upon Ncl depletion (Figure 4). This function is reminiscent to the nucleolar function of Ncl [2,54,55]. Ncl is both required for the transcription of ribosomal RNA by RNA polymerase I [56–58] and for the co-transcriptional maturation of the pre-ribosomal RNA through direct interaction with rRNA and snoRNPs [59–61].

Future experiments coupling ChIP-seq and the characterization of Ncl bound pre-mRNA should allow to determine if Ncl is also a basic factor required for the co-transcriptional assembly of the spliceosome machinery or if it is only specific only to a subset of genes.

4. Materials and Methods

4.1. Antibodies

The following antibodies were used: rabbit polyclonal antibodies against human nucleolin no5567 (Covalab, Lyon, France, ref 00106860) developed in our laboratory and used previously [57,62], a rabbit polyclonal antibody against human K88-acetylated nucleolin (Ac-Ncl) [16]: Covalab, Lyon, ref: 00106706), anti-SC35 (ab11826, Abcam, UK), anti-P-SF3B1 (anti-pT313-SF3b155; gift from R. Lührmann and C. Girard). Secondary antibodies: DAMA647 (A31571, Molecular probes (Thermo Scientific, Rockford, IL, USA), DARA488 (A21206, Molecular probes), DARA555 (A31572, Molecular probes) and GAR-IRDye800 (926-32211, LI-COR, NE, USA).

4.2. Cell Cultures

HeLa cells were grown in αMEM medium containing Glutamax (PAA), complemented with 10% Fetal Calf Serum (FCS), 1% non-essential amino acids, and 1% penicillin/streptomycin. Osteosarcoma cells U2OS (ATCC number HTB-96 were cultivated in DMEM (PAA) + 10% FBS + 2 mM l-Glutamine + 100 units/mL penicillin + 100 micro-g/mL streptomycin (PAA). Cells were maintained at 37 °C in a 5% CO₂-humidified incubator.

4.3. Cell Transfection

A mixture of functional siRNAs (Eurogentec, France) specific for human nucleolin was used as previously described [62]. siRNAs (siRNA #4 (UUUCUUUGACAGGCUCUUCCU) and siRNA #2 (UCCAGGUAAACUUAAUUUCU) were reconstituted at a concentration of 100 nM and stored at −20 °C. As a siRNA control, we used stealth high GC siRNA (Invitrogen: Thermo Scientific, Rockford, IL, USA). HeLa cells were transfected in a 6-well dish using siRNA at 2 nM final concentration. siRNAs were diluted in 200 µL of Opti-MEM and plated in a well. 80 µL of INTERFERin (Polyplus, France) diluted 1:10 in RNase-free water were added. After 10 min incubation, 2 mL of medium containing 3 × 10⁵ cells were added. RNA extraction and protein analysis were performed 5 days after the initial transfection.

4.4. Western Blot Analysis

For Western blot analysis, total cell extracts were prepared and loaded onto a 10% SDS-polyacrylamide gel. Nucleolin antibody (Covalab, France) and beta-actin (Sigma (USA) A-5441) were used. Cells were detached, lysed in 2% SDS, 10% glycerol and 20% β-mercapto-ethanol for a final concentration of 1 × 10⁶ cells/mL, and boiled at 95 °C for 5 min. 1 × 10⁵ cells were loaded onto a 10% SDS poly acrylamide gel electrophoresis. The proteins were then transferred to Nitrocellulose membranes. Membranes were blocked in 5% milk and incubated with the primary antibodies over night at 4°C.
in PBS containing 1% milk. Membranes were washed in PBS and secondary anti-body incubations were performed for 45 min in PBS containing 1% milk at room temperature. After PBS washes, protein bands were visualized with the Pierce ECL Western blotting substrate (Thermo Scientific, Rockford, IL, USA), according to the provided protocol.

4.5. RNA Extraction & RT-PCR

Total RNA was prepared by TRIzol (Invitrogen, Thermo Scientific, Rockford, IL, USA) extraction. One hundred nanograms of total RNA was reverse-transcribed using hexamer random primers and first-strand cDNA synthesis kit (Fermentas, Thermo Scientific, Rockford, IL, USA) and the synthesized cDNA was used for RT-QPCR using FastStart Universal SYBR Green Master (ROX) (Roche, Germany). The primer sequences, previously used in Kumazaki et al. 1999 [37], are given in Table 1. A 40-cycle PCR amplification was conducted with parameters of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. 10 µL of the PCR products was analyzed on 1.5% agarose–ethidium bromide gels. The data analysis was performed with the ImageLab® (BioRad, France) program. The unpaired Student t test was used to determine significance. A P value of 0.05 was considered significant.

Table 1. Primer sequences used for RT-QPCR.

| Gene Name | Primer (S)       | Primer (AS)       |
|-----------|------------------|------------------|
| NCL       | ACCCAGGGGATCACCTAATG | CCTTTGGAGGACCCAGTTTC |
| EDB       | GGTCCTATGCTGATCAGAGTC | CAGGTGACACGCATGGTGTCTG |
| IIICS     | GGCTACTATTACTGGCCTGG | CTGAGAGAGAGCTTCTT |
| GAPDH     | TCTTCTTTTTCGTCGCCAGC | GAGGCAGGGATGATGTTCTG |

4.6. Co-Immunoprecipitation

Co-immunoprecipitation was performed as described previously [63]. Briefly, 5 × 10^6 cells were resuspended in 400 mL of lysis buffer (150 mM NaCl, 10 mM Tris-Cl pH 8, 1% NP-40 and a cocktail of protease inhibitor) and incubated on ice for 10 min. Lysates were then clarified (12,000 g for 10 min at 4 °C). To avoid unspecific binding to the beads, the supernatants were incubated with protein A sepharose beads (Sigma-Aldrich, USA #P3391) at 4 °C for 3 h. Beads were then eliminated by centrifugation. Meanwhile, 5 µL of the antibody (rabbit polyclonal anti-nucleolin 5567 antibody or pre-immune serum for controls) was incubated with beads at 4 °C for 3 h. Equal amounts of lysates were then added on the beads and incubated over night at 4 °C. Supernatants (supernatant unbound fraction) were mixed with 5× protein loading buffer and beads (IP fraction) were extensively washed and eluted at 95 °C for 5 min in protein loading buffer for western blot analysis. Finally, the membranes were probed with anti-nucleolin 5567 and anti P-SF3B1 antibodies, detected with an anti-rabbit secondary antibody coupled to IRdye800. Imaging was performed on an Odyssey Infrared Imaging System (LI-COR Biosciences).

4.7. Immunofluorescence

Cells were plated 5 × 10^4 cells/well in 24-well dishes onto glass coverslips. 2 days after plating, cells were fixed in cold methanol for 3 min at −20 °C and permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 2 × 10 min. All subsequent incubations were performed in a humidified chamber maintained at 37 °C. Nonspecific binding of antibodies was blocked by 10% FCS, 3% BSA, and 0.1% Triton X-100 in PBS (blocking buffer) for 30 min. Coverslips were next incubated with primary antibodies (purified or serum) diluted in the blocking buffer for 30 min as described [64]. After 3 washes at room temperature in PBS-T, they were incubated with secondary antibodies also diluted in the blocking buffer, for 30 min. After 3 more washes in PBS-T, coverslips were washed in PBS, rinsed
in ddH₂O and briefly dipped in absolute ethanol. After a quick dry, coverslips were mounted on a slide with Fluoromount G (FMG Southern biotech, Birmingham USA) containing 400 ng/mL DAPI.

4.8. Microscopic Image Acquisition

All images were acquired using a Cool Snap HQ charge-coupled-device (CCD) camera mounted on a Zeiss Axio-Imager Z1 equipped with a 63× oil-immersion objective lens (numerical aperture [NA] D 1.4/working distance 0.19 mm) and fluorescence filters suited for the visualization of DAPI, Alexa488, Alexa555 and Alexa647. For each field of view, z-stacks of about 10 images with a pixel size of 102 nm were obtained by setting the z-step at 200 nm. Image stacks were processed using a 3D constrained iterative deconvolution module running under Metamorph (Meinel Algorithm on Metamorph [iteration: 7x/s: 0.7/frequency: 5/without auto background]), using the Point Spread Functions (PSF) measured for the different channels under similar acquisition conditions for PS-speck beads (Molecular probes) mounted in the same mounting medium. The x, y, and z shifts between individual channels were corrected on the 3D stack by imaging 100 nm multi fluorescent microspheres under similar acquisition conditions (translation of red channel x–1 and z–1).

4.9. Colocalization Analysis

For colocalization analysis, the JACoP plugin of ImageJ software was used. Colocalization analysis was performed on deconvolved and shifts corrected 3D stacks n = 10–15 or on individual sections.

Supplementary Materials: The following are available online at http://www.mdpi.com/2413-4155/1/2/33/s1, Table S1: Protein composition of each spliceosome complexes was retrieved from the Spliceosome database [33]. In red are highlighted the proteins that are present in the pull-down of nuclear protein that interact with tap-tagged nucleolin [23]. Proteins that are underlined are lost in the pull-down assay when the complexes are treated with an RNase suggested that the association of these proteins with nucleolin is RNA mediated [23]. Figure S1: Colocalization of nucleolin (Ncl) and acetylated nucleolin (Ac-Ncl) with P-SF3B1. Double immunofluorescence (IF) microscopy of U2OS cells (Human Bone Osteosarcoma Epithelial Cells) displayed as a 2-color merged image and single channels black and white images. The white insets represent enlarged single nucleus for better visualization of co-localization. Scale bars, 20 µm for the main images and 5 µm for the insets. (A) Nuclear speckles corresponding to active spliceosome are detected with an anti-P-SF3b (gift from R. Lührmann and C. Girard) visualized with a secondary antibody coupled to AlexaFluor488) [green]. Ac-Ncl was detected with a rabbit polyclonal antibody detected against Ac-K88 of human Ncl [16]; Covalab, Lyon, ref: 00106706) visualized with a secondary antibody coupled to AlexaFluor555) [red]. (B) Double immunofluorescence (IF) microscopy of U2OS cells displayed as a 2-color merged image and single channels black and white images. P-SF3B1 (green) was visualized as in panel A. Nucleolin was detected with anti nucleolin polyclonal antibody (polyclonal antibody 134, Ghisolfi-Nieto L J.Mol. Biol. 260(1):34–53. 1996) has been made in our laboratory) and visualized with a secondary antibody coupled to Alexa555) [red].

Author Contributions: Conceptualization, P.B.; Methodology, I.U., K.M.; Investigation, I.U., K.M., M.C.-D.; Writing—Original Draft Preparation, P.B.; Writing—Review & Editing, I.U., K.M., M.C.-D., P.B.; Funding Acquisition, P.B., I.U.

Funding: This research was funded by the Ligue contre le Cancer (Allier and Saone et Loire, France), the Foundation pour la recherche sur le Cancer (ARC) the CNRS, the Ecole Normale Supérieure de Lyon, the Agence Nationale de la Recherche, project ANR Theranuc, ANR-16-CE17-0023 and Hubert Curien program RILA 2016, project N° 38647PM and project NO. DNTS 01/13.

Acknowledgments: We thank Lührmann, R. and Girard, C. for the kind gift of the anti-pT313-SF3b155antibody and member of the Cancer Cell Plasticity Department, Centre de Recherche en Cancérologie de Lyon for fruitful discussions.

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

References

1. Berger, C.M.; Gaume, X.; Bouvet, P. The roles of nucleolin subcellular localization in cancer. Biochimie 2015, 113, 78–85. [CrossRef] [PubMed]
2. Ginisty, H.; Sicard, H.; Roger, B.; Bouvet, P. Structure and functions of nucleolin. J. Cell Sci. 1999, 112 Pt 6, 761–772.
3. Huberts, D.H.; van der Klei, I.J. Moonlighting proteins: An intriguing mode of multitasking. Biochim. Biophys. Acta 2010, 1803, 520–525. [CrossRef] [PubMed]

4. Jeffery, C.J. An introduction to protein moonlighting. Biochem. Soc. Trans. 2014, 42, 1679–1683. [CrossRef]

5. Cong, C.; Das, S.; Bouvet, P. The multiple properties and functions of nucleolin. In The Nucleolus; Protein Reviews Series; Atassi, M.Z., Ed.; Springer: New York, NY, USA, 2011; p. 185.

6. Ugrinova, I.; Petrova, M.; Chalabi-Dchar, M.; Bouvet, P. Multifaceted nucleolin protein and its molecular partners in onogenesis. Adv. Protein Chem. Struct. Biol. 2018, 111, 133–164. [PubMed]

7. Olson, M.O.; Guetzow, K.; Busch, H. Localization of phosphoprotein C23 in nuclei by immunological methods. Exp. Cell Res. 1981, 135, 259–265. [CrossRef]

8. Belenguer, P.; Caizergues-Ferrer, M.; Labbe, J.C.; Doree, M.; Amalric, F. Mitosis-specific phosphorylation of nucleolin by p34cdc2 protein kinase. Mol. Cell. Biol. 1990, 10, 3607–3618. [CrossRef] [PubMed]

9. Caizergues-Ferrer, M.; Belenguer, P.; Lapeyre, B.; Amalric, F.; Wallace, M.O.; Olson, M.O. Phosphorylation of nucleolin by a nucleolar type II protein kinase. Biochemistry 1987, 26, 7876–7883. [CrossRef] [PubMed]

10. Peter, M.; Nakagawa, J.; Doree, M.; Labbe, J.C.; Nigg, E.A. Identification of major nucleolar proteins as candidate mitotic substrates of cdc2 kinase. Cell 1990, 60, 791–801. [CrossRef]

11. Lischwe, M.A.; Roberts, K.D.; Yeoman, L.C.; Busch, H. Nucleolar specific acidic phosphoprotein C23 is highly methylated. J. Biol. Chem. 1982, 257, 14600–14602.

12. Carpentier, M.; Morelle, W.; Coddeville, B.; Pons, A.; Masson, M.; Mazurier, J.; Legrand, D. Nucleolin undergoes partial N- and O-glycosylations in the extranuclear cell compartment. Biochemistry 2005, 44, 5804–5815. [CrossRef] [PubMed]

13. Zhang, D.; Liang, Y.; Xie, Q.; Gao, G.; Wei, J.; Huang, H.; Li, J.; Gao, J.; Huang, C. A novel post-translational modification of nucleolin, SUMOylation at Lys-294, mediates arsenite-induced cell death by regulating gadd45alpha mRNA stability. J. Biol. Chem. 2015, 290, 4784–4800. [CrossRef] [PubMed]

14. Ko, C.Y.; Lin, C.H.; Chuang, J.Y.; Chang, W.C.; Hsu, T.I. MDM2 degrades deacetylated nucleolin through ubiquitination to promote glioma stem-cell-like cell enrichment for chemotherapeutic resistance. Mol. Neurobiol. 2018, 55, 3211–3223. [CrossRef] [PubMed]

15. Wang, S.; Chen, X.; Wang, M.; Yao, D.; Chen, T.; Yan, Q.; Lu, W. Long non-coding RNA CYP4B1-PS1-001 inhibits proliferation and fibrosis in diabetic nephropathy by interacting with nucleolin. Cell. Physiol. Biochem. 2018, 49, 2174–2187. [CrossRef] [PubMed]

16. Das, S.; Cong, R.; Sandilya, J.; Senapati, P.; Moindrot, B.; Monier, K.; Delage, H.; Mongelard, F.; Kumar, S.; Kundu, T.K.; et al. Characterization of nucleolin K88 acetylation defines a new pool of nucleolin colocalizing with pre-mRNA splicing factors. FEBS Lett. 2013, 587, 417–424. [CrossRef] [PubMed]

17. Xiao, S.; Caglar, E.; Maldonado, P.; Das, D.; Nadeem, Z.; Chi, A.; Trinite, B.; Li, X.; Saxena, A. Induced expression of nucleolin phosphorylation-deficient mutant confers dominant-negative effect on cell proliferation. PLoS ONE 2014, 9, e109858. [CrossRef] [PubMed]

18. Raman, B.; Guarnaccia, C.; Nadassy, K.; Zakhariev, S.; Pintar, A.; Zanuttin, F.; Frigyes, D.; Acatrinei, C.; Vindigni, A.; Pongor, G.; et al. N(o mega)-arginine dimethylation modulates the interaction between a Gly/Arg-rich peptide from human nucleolin and nucleic acids. Nucleic Acids Res. 2001, 29, 3377–3384. [CrossRef] [PubMed]

19. Losfeld, M.E.; Leroy, A.; Coddeville, B.; Carpentier, M.; Mazurier, J.; Legrand, D. N-Glycosylation influences the structure and self-association abilities of recombinant nucleolin. FEBS J. 2011, 278, 2552–2564. [CrossRef]

20. Marchand, V.; Santerre, M.; Aigueperse, C.; Fouillem, L.; Salieu, J.M.; Van Dorsselaer, A.; Sanglier-Cianferani, S.; Branlant, C.; Motorin, Y. Identification of protein partners of the human immunodeficiency virus 1 tat regulatory exon 3 leads to the discovery of a new HIV-1 splicing regulator, protein hnRNP K. RNA Biol. 2011, 8, 325–342. [CrossRef] [PubMed]

21. Pickering, B.F.; Yu, D.; Van Dyke, M.W. Nucleolin protein interacts with microprocessor complex to affect biogenesis of microRNAs 15a and 16. J. Biol. Chem. 2011, 286, 44095–44103. [CrossRef]

22. Hawkins, P.G.; Morris, K.V. Transcriptional regulation of Oct4 by a long non-coding RNA antisense to Oct4-pseudogene 5. Transcription 2010, 1, 165–175. [CrossRef] [PubMed]

23. Salvetti, A.; Coute, Y.; Epstein, A.; Arata, L.; Kraut, A.; Navratil, V.; Bouvet, P.; Greco, A. Nuclear functions of nucleolin through global proteomics and interactomic approaches. J. Proteome Res. 2016, 15, 1659–1669. [CrossRef] [PubMed]
24. Spector, D.L.; Lamond, A.I. Nuclear speckles. *Cold Spring Harb. Perspect. Biol.* 2011, 3, a000646. [CrossRef] [PubMed]
25. Spector, D.L. The dynamics of chromosome organization and gene regulation. *Annu. Rev. Biochem.* 2003, 72, 573–608. [CrossRef] [PubMed]
26. Smith, K.P.; Moen, P.T.; Wydner, K.L.; Coleman, J.R.; Lawrence, J.B. Processing of endogenous pre-mRNAs in association with SC-35 domains is gene specific. *J. Cell Biol.* 1999, 144, 617–629. [CrossRef] [PubMed]
27. Xing, Y.; Johnson, C.V.; Dobner, P.R.; Lawrence, J.B. Higher level organization of individual gene transcription and RNA splicing. *Science* 1993, 259, 1326–1330. [CrossRef] [PubMed]
28. Bessonov, S.; Anokhina, M.; Krasauskas, A.; Golas, M.M.; Sander, B.; Will, C.L.; Urlaub, H.; Stark, H.; Luhrmann, R. Characterization of purified human Bact spliceosomal complexes reveals compositional and morphological changes during spliceosome activation and first step catalysis. *RNA* 2010, 16, 2384–2403. [CrossRef]
29. Wang, C.; Chua, K.; Seghezzi, W.; Lees, E.; Gozani, O.; Reed, R. Phosphorylation of spliceosomal protein SAP 155 coupled with splicing catalysis. *Genes Dev.* 1998, 12, 1409–1414. [CrossRef]
30. Girard, C.; Will, C.L.; Peng, J.; Makarov, E.M.; Kastner, B.; Lemm, I.; Urlaub, H.; Hartmuth, K.; Luhrmann, R. Post-transcriptional spliceosomes are retained in nuclear speckles until splicing completion. *Nat. Commun.* 2012, 3, 994. [CrossRef]
31. Bolte, S.; Cordelieres, F.P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc.* 2006, 224, 213–232. [CrossRef]
32. Manders, E.M.; Stap, J.; Brakenhoff, G.J.; van Driel, R.; Aten, J.A. Dynamics of three-dimensional replication patterns during the S-phase, analysed by double labelling of DNA and confocal microscopy. *J. Cell Sci.* 1992, 103 Pt 3, 857–862.
33. Cvitkovic, I.; Jurica, M.S. Spliceosome database: A tool for tracking components of the spliceosome. *Nucleic Acids Res.* 2013, 41, D132–D141. [CrossRef] [PubMed]
34. Galej, W.P. Structural studies of the spliceosome: Past, present and future perspectives. *Biochem. Soc. Trans.* 2018, 46, 1407–1422. [CrossRef] [PubMed]
35. Kornblith, A.R.; Pesce, C.G.; Alonso, C.R.; Cramer, P.; Srebro, A.; Werbajh, S.; Muro, A.F. The fibronectin gene as a model for splicing and transcription studies. *FASEB J.* 1996, 10, 248–257. [CrossRef] [PubMed]
36. White, E.S.; Baralle, F.E.; Muro, A.F. New insights into form and function of fibronectin splice variants. *J. Pathol.* 2008, 216, 1–14. [CrossRef]
37. Kumazaki, T.; Mitsui, Y.; Hamada, K.; Sumida, H.; Nishiyama, M. Detection of alternative splicing of fibronectin mRNA in a single cell. *J. Cell Sci.* 1999, 112 Pt 10, 1449–1453.
38. Zhang, X.; Xiao, S.; Rameau, R.D.; Devany, E.; Nadeem, Z.; Caglar, E.; Ng, K.; Kleiman, F.E.; Saxena, A. Nucleolin phosphorylation regulates PARN deadenylase activity during cellular stress response. *RNA Biol.* 2018, 15, 251–260. [CrossRef] [PubMed]
39. Woo, H.H.; Baker, T.; Laszlo, C.; Chambers, S.K. Nucleolin mediates microRNA-directed CSF-1 mRNA deadenylation but increases translation of CSF-1 mRNA. *Mol. Cell. Proton.* 2013, 12, 1661–1677. [CrossRef] [PubMed]
40. Ishimaru, D.; Zuraw, L.; Ramalingam, S.; Sengupta, T.K.; Bandyopadhyay, S.; Reuben, A.; Fernandes, D.J.; Spicer, E.K. Mechanism of regulation of bel-2 mRNA by nucleolin and A+U-rich element-binding factor 1 (AUF1). *J. Biol. Chem.* 2010, 285, 27182–27191. [CrossRef]
41. Izumi, R.E.; Valdez, B.; Banerjee, R.; Srivastava, M.; Dasgupta, A. Nucleolin stimulates viral internal ribosome entry site–mediated translation. *Virus Res.* 2001, 76, 17–29. [CrossRef]
42. Takagi, M.; Absalon, M.J.; McClure, K.G.; Kastan, M.B. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* 2005, 123, 49–63. [CrossRef] [PubMed]
43. Abdelmohsen, K.; Tominaga, K.; Lee, E.K.; Srikantan, S.; Kang, M.J.; Kim, M.M.; Selimyan, R.; Martindale, J.L.; Yang, X.; Carrier, F.; et al. Enhanced translation by Nucleolin via G-rich elements in coding and non-coding regions of target mRNAs. *Nucleic Acids Res.* 2011, 39, 8513–8530. [CrossRef] [PubMed]
44. Chen, J.; Guo, K.; Kastan, M.B. Interactions of nucleolin and ribosomal protein L26 (RPL26) in translational control of human p53 mRNA. *J. Biol. Chem.* 2012, 287, 16467–16476. [CrossRef] [PubMed]
45. Hung, C.Y.; Yang, W.B.; Wang, S.A.; Hsu, T.I.; Chang, W.C.; Hung, J.J. Nucleolin enhances internal ribosomal entry site (IRES)-mediated translation of Sp1 in tumorigenesis. *Biochim. Biophys. Acta* 2014, 1843, 2843–2854. [CrossRef] [PubMed]
46. Terenzio, M.; Koley, S.; Samra, N.; Rishal, I.; Zhao, Q.; Sahoo, P.K.; Urisman, A.; Marvaldi, L.; Oses-Prieto, J.A.; Forester, C.; et al. Locally translated mTOR controls axonal local translation in nerve injury. *Science* 2018, 359, 1416–1421. [CrossRef] [PubMed]

47. Terrier, O.; Carron, C.; De Chassey, B.; Dubois, J.; Traversier, A.; Julien, T.; Cartet, G.; Proust, A.; Hacot, S.; Ressnikoff, D.; et al. Nucleolin interacts with influenza A nucleoprotein and contributes to viral ribonucleoprotein complexes nuclear trafficking and efficient influenza viral replication. *Sci. Rep.* 2016, 6, 29006. [CrossRef] [PubMed]

48. Wahl, M.C.; Will, C.L.; Luhrmann, R. The spliceosome: Design principles of a dynamic RNP machine. *Cell* 2009, 136, 701–718. [CrossRef]

49. Herold, N.; Will, C.L.; Wolf, E.; Kastner, B.; Urlaub, H.; Luhrmann, R. Conservation of the protein composition and electron microscopy structure of Drosophila melanogaster and human spliceosomal complexes. *Mol. Cell. Biol.* 2009, 29, 281–301. [CrossRef] [PubMed]

50. Plaschka, C.; Lin, P.C.; Nagai, K. Structure of a pre-catalytic spliceosome. *Nature* 2017, 546, 617–621. [CrossRef] [PubMed]

51. Zhang, X.; Yan, C.; Zhan, X.; Li, L.; Lei, J.; Shi, Y. Structure of the human activated spliceosome in three conformational states. *Cell Res.* 2018, 28, 307–322. [CrossRef] [PubMed]

52. Jiang, Y.; Zhu, Y.; Liu, Z.J.; Ouyang, S. The emerging roles of the DDX41 protein in immunity and diseases. *Protein Cell* 2017, 8, 83–89. [CrossRef] [PubMed]

53. Kuhn, A.N.; van Santen, M.A.; Schwienhorst, A.; Urlaub, H.; Luhrmann, R. Stalling of spliceosome assembly at distinct stages by small-molecule inhibitors of protein acetylation and deacetylation. *RNA* 2009, 15, 153–175. [CrossRef] [PubMed]

54. Durut, N.; Saez-Vasquez, J. Nucleolin: Dual roles in rDNA chromatin transcription. *Gene* 2014, 556, 7–12. [CrossRef] [PubMed]

55. Tuteja, R.; Tuteja, N. Nucleolin: A multifunctional major nucleolar phosphoprotein. *Crit. Rev. Biochem. Mol. Biol.* 1998, 33, 407–436. [CrossRef] [PubMed]

56. Angelov, D.; Bondarenko, V.A.; Almagro, S.; Menoni, H.; Mongelard, F.; Hans, F.; Mietton, F.; Studitsky, V.M.; Hamiche, A.; Dimitrov, S.; et al. Nucleolin is a histone chaperone with FACT-like activity and assists remodeling of nucleosomes. *EMBO J.* 2006, 25, 1669–1679. [CrossRef] [PubMed]

57. Cong, R.; Das, S.; Ugrinova, I.; Kumar, S.; Mongelard, F.; Wong, J.; Bouvet, P. Interaction of nucleolin with ribosomal RNA genes and its role in RNA polymerase I transcription. *Nucleic Acids Res.* 2012, 40, 9441–9454. [CrossRef] [PubMed]

58. Rickards, B.; Flint, S.J.; Cole, M.D.; LeRoy, G. Nucleolin is required for RNA polymerase I transcription in vivo. *Mol. Cell. Biol.* 2007, 27, 937–948. [CrossRef] [PubMed]

59. Ginisty, H.; Amalric, F.; Bouvet, P. Nucleolin functions in the first step of ribosomal RNA processing. *EMBO J.* 1998, 17, 1476–1486. [CrossRef] [PubMed]

60. Ginisty, H.; Serin, G.; Ghisolfi-Nieto, L.; Roger, B.; Libante, V.; Amalric, F.; Bouvet, P. Interaction of nucleolin with an evolutionarily conserved pre-ribosomal RNA sequence is required for the assembly of the primary processing complex. *J. Biol. Chem.* 2000, 275, 18845–18850. [CrossRef] [PubMed]

61. Roger, B.; Moisand, A.; Amalric, F.; Bouvet, P. Nucleolin provides a link between RNA polymerase I transcription and pre-ribosome assembly. *Chromosoma* 2003, 111, 399–407. [CrossRef] [PubMed]

62. Ugrinova, I.; Monier, K.; Ivaldi, C.; Thiry, M.; Storch, S.; Mongelard, F.; Bouvet, P. Inactivation of nucleolin leads to nucleolar disruption, cell cycle arrest and defects in centrosome duplication. *BMC Mol. Biol.* 2007, 8, 66. [CrossRef] [PubMed]

63. Gaume, X.; Tassin, A.M.; Ugrinova, I.; Mongelard, F.; Monier, K.; Bouvet, P. Centrosomal nucleolin is required for microtubule network organization. *Cell Cycle* 2015, 14, 902–919. [CrossRef] [PubMed]

64. Zeitlin, S.G.; Barber, C.M.; Allis, C.D.; Sullivan, K.F. Differential regulation of CENP-A and histone H3 phosphorylation in G2/M. *J. Cell Sci.* 2001, 114, 653–661. [PubMed]