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Upstream binding factor-dependent and pre-rRNA transcription-independent association of pre-rRNA processing factors with rRNA gene

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ABSTRACT

The nucleolus is the ribosome biogenesis center. The nucleolar structure is disrupted upon entry into mitosis and is formed in early G1 phase. To understand the molecular mechanisms of nucleolar assembly and disassembly, we have studied the mechanism of association between factors involved in pre-ribosome RNA (rRNA) processing and rRNA gene chromatin (r-chromatin). We found that the pre-rRNA transcription-processing linking factor Nopp140 and pre-rRNA processing factors such as DKC1 and fibrillarin (FBL) associate with r-chromatin during interphase, while Nopp140, DKC1, and FBL were released from r-chromatin in mitosis. The association of these factors with r-chromatin was found to be restored independent of pre-rRNA transcription in early G1 phase, but a mature nucleolar structure was not formed, suggesting that nucleolar assembly can be divided into at least two steps with respect to pre-rRNA transcription. Moreover, we found that the r-chromatin association of Nopp140, DKC1, and FBL was dependent on the transcription factor upstream binding factor (UBF). However, we demonstrated that UBF alone was not sufficient to recruit these pre-rRNA processing factors to r-chromatin. Thus, UBF is necessary but not sufficient for the associations between pre-rRNA processing factors and r-chromatin.

1. Introduction

The primary function of the nucleolus is ribosome biogenesis, which includes ribosomal RNA precursor (pre-rRNA) transcription, modifications, and processing [1]. The 47S pre-rRNA is synthesized by RNA polymerase I (pol I). The transcribed 47S pre-rRNA is modified and processed successively by a variety of RNA–protein complexes (ribonucleoprotein, RNP).Modifications of pre-rRNA consisting of methylation and pseudouridylation are directed by two classes of small nucleolar RNAs (snoRNAs), box C/D and box H/ACA snoRNAs, respectively. Box C/D snoRNAs associate with four proteins, 15.5K, NOP56, NOP58, and fibrillarin (FBL), while box H/ACA snoRNAs associate with GAR1, NHP2, NOP10, and DKC1 (also known as dyskerin/NAP57) [2]. DKC1 was originally identified as a Nopp140-associated protein [3]. Nopp140 is a phosphoprotein localized in the nucleolus and Cajal body; it interacts with both box C/D snoRNPs and box H/ACA snoRNPs and may function as a snoRNP chaperone [4,5]. In addition, because Nopp140 was shown to associate with RNA polymerase I subunits [6], Nopp140 is suggested to link pre-rRNA transcription and processing.

The nucleolar structure is disrupted upon entry into mitosis and is formed in early G1 phase. During mitosis, pre-rRNA transcription and processing are silenced. Before nucleolar assembly, pre-rRNAs and factors involved in pre-rRNA modifications and processing form RNPs and are accumulated in small granules (prenucleolar body, PNB) outside the nucleolus. These RNPs are then recruited to chromosome regions termed nucleolar organizer regions (NORs) containing rRNA gene repeats to form the mature nucleolar structure [7]. However, the molecular mechanism by which these RNPs are assembled to NORs remains unknown. A previous study indicated that mature nucleolar structure formation is impaired but pre-rRNA processing factors are accumulated in segregated nucleoli when mitotic cells are released into G1 phase in the presence of a Pol I inhibitor [8,9]. However, it is currently unknown the assembly processes of pre-rRNA processing factors to NORs. It was also

Abbreviations: rRNA, ribosomal RNA; pre-rRNA, ribosomal RNA precursor; Pol I, RNA polymerase I; NOR, nucleolar organizer region; PNB, prenucleolar body; r-chromatin, rRNA gene chromatin; RNP, ribonucleoprotein; snoRNA, small nucleolar RNA; Act D, actinomycin D.

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reported that the transcription factor upstream binding factor (UBF) has the ability to recruit pre-rRNA transcription factors and factors linking pre-rRNA transcription and processing [10]. Thus, it is likely that UBF plays a crucial role in the formation of the nucleolus. Although immunofluorescence-based analyses have provided a clue to understand the nucleolar assembly and disassembly mechanism, physical association between r-chromatin and pre-rRNA processing factors have not been well understood. Here, we studied the association between pre-rRNA processing factors and r-chromatin to understand the molecular mechanism of nucleolar assembly and disassembly. We revealed that pre-rRNA processing factors are associated with r-chromatin in interphase and are released from r-chromatin in mitosis. We also found that the association of pre-rRNA processing factors with r-chromatin was restored in the presence of a pre-rRNA transcription inhibitor in early G1 phase. Furthermore, UBF was found to play an important role in the association of pre-rRNA processing factors with r-chromatin. Our results contribute to the understanding of the regulatory mechanism of nucleolar assembly and disassembly.

2. Materials and methods

2.1. Cell culture, transfection, cell cycle synchronization, and 5-fluorouracil incorporation assay

HeLa and U2OS cells were maintained in Dulbecco modified Eagle medium (Nacalai Tesque) containing 10% (v/v) heat-inactivated fetal bovine serum. U2OS 2–6–3 cells were maintained in the same medium supplemented with 50 μg/mL hygromycin B (Invivogen). Transient transfection of plasmid DNA was performed using GeneJuice (Novagen) according to the manufacturer's instructions. Small interfering RNA (siRNA) was transfected using Lipofectamine RNAi MAX (Life Technologies). Stealth RNAs for negative controls (Stealth RNAi negative-control duplex, catalog No. 12935-300, Life Technologies), UBF and RRN3siRNAs (UBTF-HSS111143 and RRN3-HSS123162 from Life Technologies) were used. To prepare mitotic cells, cells were incubated in medium containing 2 mM thymidine (Sigma) for 24 h, released into medium without thymidine for 3 h, and then incubated for additional 12 h in the presence of 0.5 μg/ml of nocodazole. Mitotic cells were collected by shaking dishes. For the experiment shown in Figs. 2 and 3, mitotic cells were released into culture medium in the absence or presence of 50 ng/mL of actinomycin D (Sigma). In 5-fluorouracil (5-FU) incorporation assay, cells were incubated for 60 min in the presence of 3 mM 5-FU (Sigma).

2.2. Antibodies

The following antibodies were used in this study: mouse monoclonal antibodies for β-actin (C4, Santa Cruz Biotechnology), BrdU (BU-33, Sigma), and RPA194 (C-1, Santa Cruz Biotechnology), rabbit polyclonal antibodies for dyskerin (DKC1) (H-300, Santa Cruz Biotechnology), fibrillarin (FBL) (H-140, Santa Cruz Biotechnology), histone H3 (ab1791, Abcam), Nopp140 (H-80, Santa Cruz Biotechnology), phospho-histone H3 (Ser10) (Millipore), RRN3 (ab112052, Abcam; Y-23, Santa Cruz Biotechnology), and UBF (H-300, Santa Cruz Biotechnology).

2.3. Chromatin immunoprecipitation

Chromatin extracts were prepared from cells fixed with 1% (v/v) formaldehyde by extensive sonication. In all ChIP assays, DNA

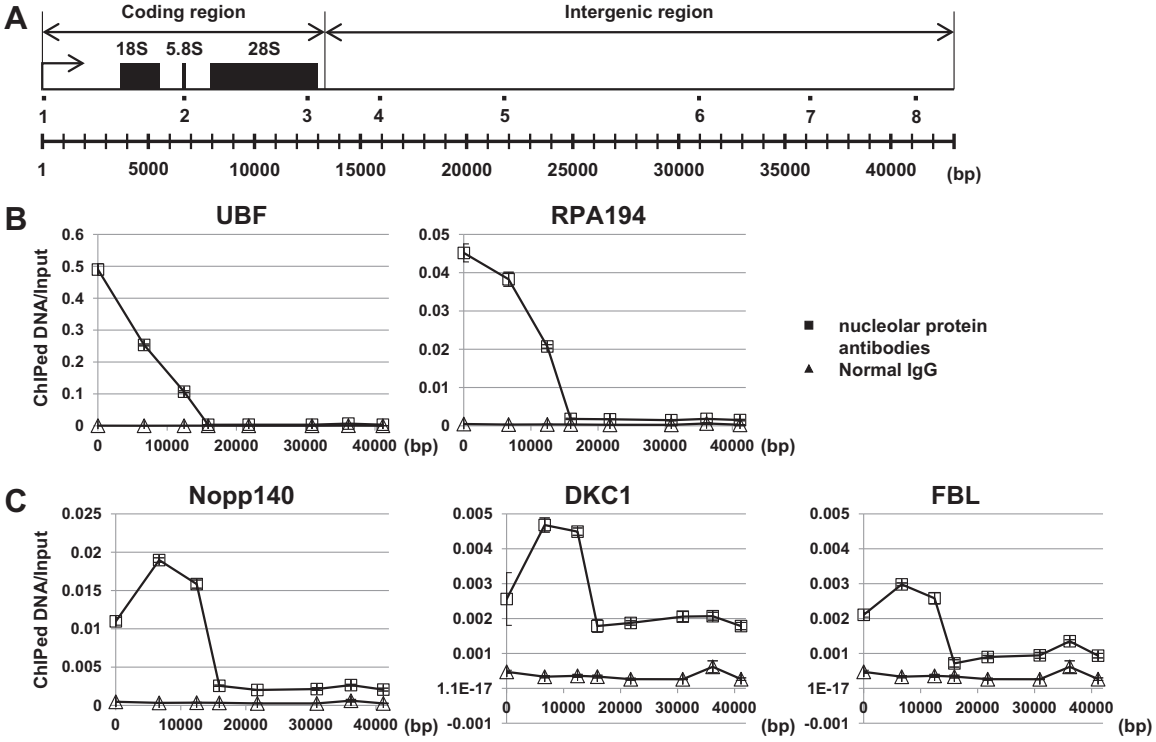

Fig. 1. Pre-rRNA processing factors were associated with r-chromatin in asynchronous cells. (A) Schematic representation of the human rRNA gene. The positions of 18S, 5.8S, and 28S rRNAs are shown schematically by black bars. Primer positions used for ChIP assay are shown by 1–8. Pre-rRNA transcription start site is set as 1. Primer sequences are listed in Table S1. (B and C) The r-chromatin association of nucleolar proteins in asynchronous. ChIP assays using antibodies against UBF and RPA194 (B), Nopp140, DKC1, and FBL (C) were performed with extracts derived from HeLa cells. Normal IgGs were used as controls. The amounts of precipitated DNAs relative to those of input DNAs were quantitatively analyzed with specific primer sets (1–8) for the rRNA gene shown in (A). Q-PCRs were performed in triplicate and error bars represent S.D. in one experiment. Two independent experiments showed similar results.

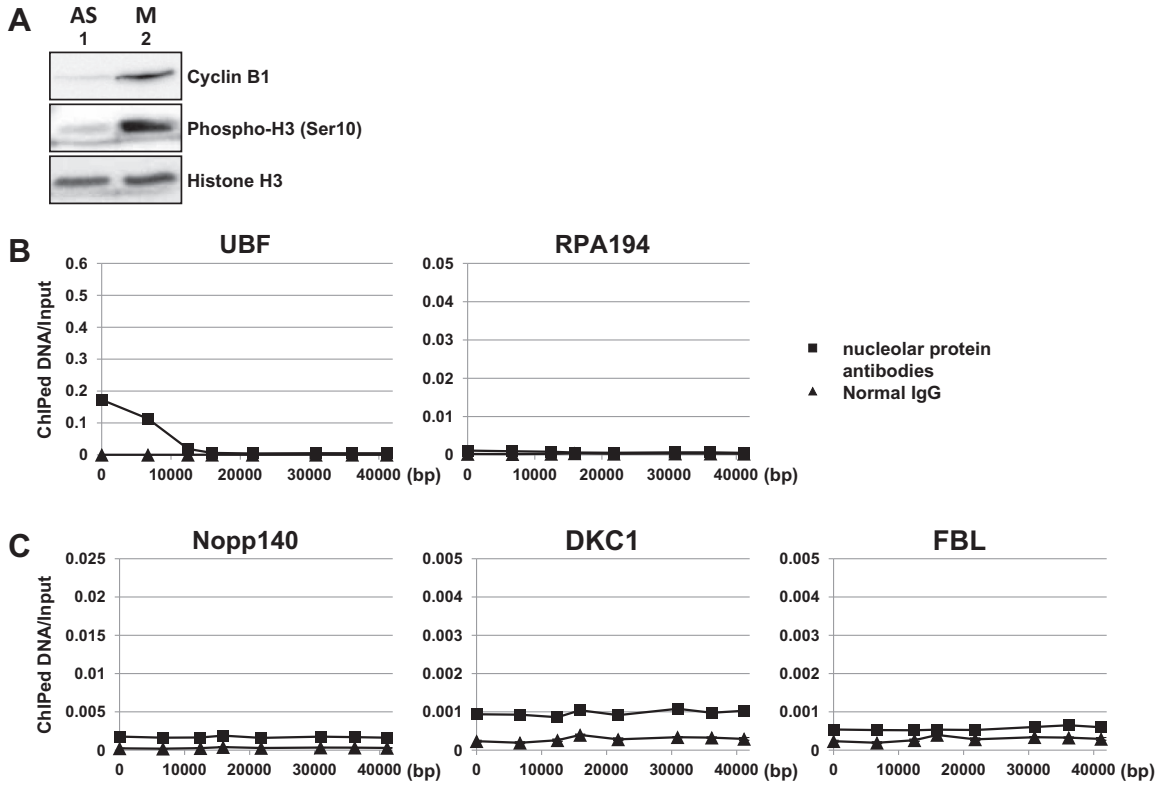

Fig. 2. Pre-rRNA processing factors were released from r-chromatin in mitosis. (A) Western blotting. Asynchronous (AS) and mitotic (M) cell extracts (lanes 1 and 2, respectively) were subjected to Western blotting with anti-Cyclin B1, phospho-histone H3 (Ser10), and histone H3 antibody. (B, C) ChIP assays using antibodies against UBF and RPA194 (B), Nopp140, DKC1, and FBL (C) were performed with extracts derived from mitotic HeLa cells. Normal IgGs were used as controls. The amounts of precipitated DNAs relative to those of input DNAs were estimated by Q-PCR with specific primer sets (regions 1–8 shown in Fig. 1A). Q-PCRs were performed in triplicate and error bars represent S.D. in one experiment. Two independent experiments showed similar results.

length extracted from cell lysates was between 200 and 500 base pairs. Cell lysates prepared from 4×10^6 cells were used for an immunoprecipitation assay. Precipitated DNA suspended in 10 mM Tris–HCl (pH 7.9) was used as templates for PCR. The primer sets used to amplify the specific regions in the human rRNA gene (GenBank accession U13369) are shown in Table S1. PCR reaction was quantitatively performed by FastStart Universal Master (ROX) (Roche).

2.4. Indirect immunofluorescence

HeLa or U2OS 2–6–3 cells grown on coverslips were fixed with PBS containing 1% or 3% (w/v) paraformaldehyde for 5–10 min, permeabilized for 10 min in cytoskeleton buffer (10 mM PIPES–KOH [pH 7.0], 100 mM NaCl, 3 mM $MgCl_2$, and 300 mM sucrose) containing 0.5% (v/v) Triton X-100, and incubated with PBS containing 0.1% (w/v) fat-free milk (Morinaga) and 0.1% (v/v) Tween-20. The fixed and permeabilized cells were incubated with primary antibody for 1 h, washed with PBS containing 0.1% (v/v) Tween-20, and incubated with secondary antibodies (Alexa Fluor 488 or 568 goat anti-mouse IgG antibody, Alexa Fluor 488 or 568 goat anti-rabbit IgG antibody, Life Technologies) for 30 min. DNA was stained with TO-PRO-3 iodide (Life Technologies) for 10 min. All images were captured by a confocal microscope (LSM 5 Exciter, Carl Zeiss) with a Plan Apochromat 63×1.4-numerical-aperture oil immersion objective lens.

2.5. Plasmid construction

To construct pEGFP-C1-Lac I-UBF1, the lac repressor (Lac I) cDNA cloned from pET-Duet vector (Novagen) by PCR and the human UBF cDNA were subcloned into Bgl II and Sal I sites and Kpn I and Xba I sites of pEGFP-C1 vector (Clonetech), respectively. The sequence of the plasmid was confirmed using ABI Prism BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) with appropriate primers.

3. Results

3.1. The association between pre-rRNA processing factors and r-chromatin in asynchronous and mitotic cells

To address how the nucleolar structure is maintained in interphase, we examined the association of nucleolar proteins with r-chromatin. We studied five nucleolar proteins involved in ribosome biogenesis: UBF and RPA194, a pol I subunit for pre-rRNA transcription and nucleolar chromatin formation, Nopp140 to link pre-rRNA transcription and processing, DKC1 and FBL for pre-rRNA modifications and processing. A chromatin immunoprecipitation (ChIP) assay was performed with antibodies against the nucleolar proteins described above (Fig. 1B and C) and PCR primer positions used for ChIP assay are shown by 1–8 (Fig. 1A). In asynchronous cells, UBF was enriched in the promoter proximal region and coding regions as previously reported [11,12]. RPA194 was also enriched in promoter and coding regions, suggesting that cells were actively transcribing pre-rRNAs (Fig. 1B). The pre-rRNA transcription and processing linking factor Nopp140 and snoRNP components DKC1 and FBL were similarly enriched in the coding region (Fig. 1C). These results suggested that the physical association between pre-rRNA transcription/processing factors and r-chromatin contributes at least in part to the maintenance of the nucleolar structure.

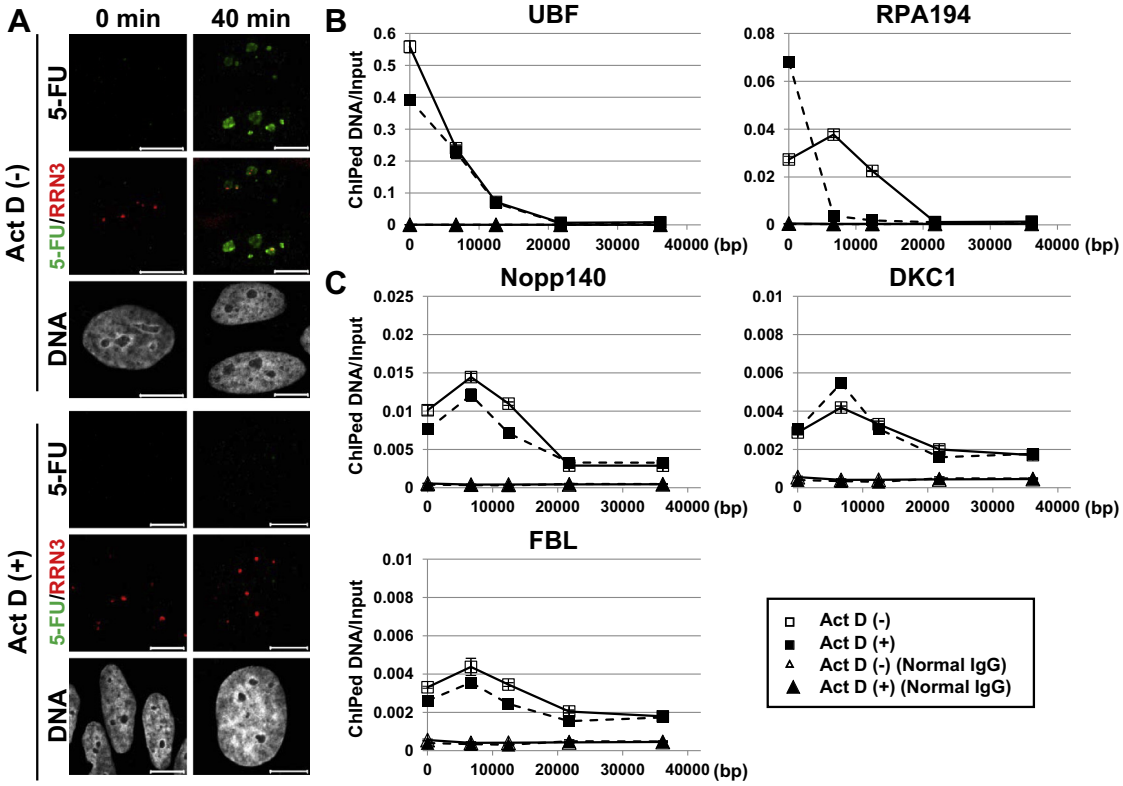

Fig. 3. The association between pre-rRNA processing factors and r-chromatin was restored in the presence of Act D in early G1 phase. (A) Effect of Act D on pol I-mediated transcription. 5-Fluorouracil (5-FU) incorporation assays were performed 3 h after release from mitotic block. Cells incubated with 5-FU for 0 or 40 min in the absence and presence of Act D (top and bottom panels, respectively) was subjected to immunofluorescence assays with anti-BrdU or -RRN3 antibodies as indicated at the left of the panels. Localization of proteins was observed under a confocal microscope. Bars at the bottom in each panel indicate 10 μm. (B, C) ChIP assays using antibodies against UBF and RPA194 (B), Nopp140, DKC1, and FBL (C) were performed 3 h after release. Normal IgGs were used as controls. The amounts of precipitated DNAs relative to those of input DNAs were estimated by Q-PCR with specific primer sets (regions 1–3, 5, and 7 shown in Fig. 1A). Open squares and triangles show the data from released cells in the absence of Act D and closed squares and triangles show the data from released cells in the presence of Act D. Q-PCRs were performed in triplicate and error bars represent S.D. Two independent experiments showed similar results.

Because nucleolar structure is disrupted during mitosis, we next investigated the association of pre-rRNA transcription/processing factors with r-chromatin using mitotic cell extracts. Mitotic synchronization was verified by the expression of Cyclin B1 and histone H3 phosphorylation status (Fig. 2A). In mitotic cells, the r-chromatin association of UBF was clearly detected, although the association level at the promoter and coding regions was decreased compared with that in interphase (Fig. 2B). Strikingly, the r-chromatin association of RPA194 was completely lost in mitotic cells (Fig. 2B). This result was consistent with the previous observation that the pol I subunits are transiently released from NORs during pro-metaphase [13,14].The enrichment of Nopp140, DKC1, and FBL at the rRNA coding region was also significantly decreased in mitosis (Fig. 2C). These results indicated that pol I and pre-rRNA processing factors were released from r-chromatin in mitosis. In addition, these results support a notion that the maintenance of nucleolar structure is mediated by the physical association between r-chromatin (NORs) and pre-rRNA transcription/processing factors.

3.2. The association between pre-rRNA processing factors and r-chromatin was restored independent of pre-rRNA transcription in early G1 phase

Pre-rRNA transcription was restored in telophase [15]. In addition, we found that the pre-rRNA processing factors associated mainly with the rRNA coding region (Fig. 1C). Therefore, it was hypothesized that transcription restoration after mitosis is required for re-assembly of pre-rRNA processing factors to r-chromatin. Although immunofluorescence analyses suggested that FBL and NCL were partially co-localized with NORs independent of pre-rRNA transcription in early telophase, it was unknown whether the association of pre-rRNA processing factors with r-chromatin was restored in the absence of pre-rRNA transcription. To address this issue, ChIP assays were performed using cells released from mitotic block in the presence of a pol I inhibitor, actinomycin D (Act D). Mitotic U2OS cells were released in G1 phase in the presence of Act D for 3 h followed by 5-fluorouracil (5-FU) labeling assays and ChIP assays. Newly transcripts were labeled with 5-FU to confirm the transcription inhibitory effect of Act D. The incorporated 5-FU signal was mainly detected around the foci labeled by a nucleolar transcription factor RRN3 antibody in cells incubated for 40 min without Act D, but the signal was significantly decreased when cells were incubated in the presence of Act D (Fig. 3A). This suggests that transcription by pol I was inhibited under the condition employed. ChIP assays indicated that UBF levels were similarly enriched in the promoter proximal region and rRNA coding regions in the presence and absence of Act D (Fig. 3B). The enrichment of RPA194 to the promoter proximal region and rRNA coding regions was restored 3 h after release from the mitotic block in the absence of Act D. However, the accumulation of RPA194 was detected only at the promoter proximal region when the cells were released in the presence of Act D (Fig. 3B). Interestingly, the recruitment of Nopp140, DKC1 and FBL to r-chromatin after mitosis was similarly observed even in the absence or presence of Act D in early G1 phase (Fig. 3C). These results suggested that pre-rRNA processing factors were recruited to r-chromatin before pre-rRNA transcription restoration, although the mature

nucleolar structure was not formed when pre-rRNA transcription restoration was impaired by Act D. These results also suggested that although Nopp140, DKC1, and FBL associated with the rRNA coding regions of r-chromatin, their r-chromatin association did not depend on the *de novo* synthesis of pre-rRNA.

3.3. UBF was necessary but not sufficient for the recruitment of pre-rRNA processing factors to r-chromatin

To understand the mechanism by which pre-rRNA processing factors are recruited to r-chromatin, we focused on the pol I transcription factor UBF because the recruitment of pol I and factors linking pre-rRNA transcription and processing to UBF binding sequence (pseudo-NORs) was shown to depend on UBF [10]. We also examined the effect of RRN3 as a control because RRN3 is an essential pol I transcription factor to recruit pol I to rRNA gene [16]. HeLa cells treated with UBF or RRN3 siRNA were subjected to ChIP assays with three primer sets for the promoter proximal and coding regions (regions 1–3 in Fig. 1A). The expression levels of UBF and RRN3 were efficiently decreased by siRNA treatment, whereas that of β-actin was not affected (Fig. 4A). The r-chromatin association of UBF was significantly decreased by UBF siRNA treatment, whereas it was not affected by RRN3 depletion (Fig. 4B). The association of RPA194 with r-chromatin was decreased by both UBF and RRN3 depletion (Fig. 4B). These results indicated that recruitment of pol I to the rRNA gene promoter region was mediated by transcription factors UBF and RRN3 in cells. Interestingly, the r-chromatin associations of Nopp140, DKC1, and FBL were significantly decreased by UBF depletion. Although RRN3 depletion slightly decreased the association level of these factors especially at the promoter proximal region (region 1), the effect of UBF depletion was more obvious than that of RRN3 depletion (Fig. 4C). These results suggested that UBF was required for the r-chromatin association of pre-rRNA processing factors. In addition, the function of UBF is not simply explained by pol I-mediated transcription alone because RRN3 depletion was not enough to efficiently disrupt the association between pre-rRNA processing factors and NORs.

To examine whether UBF was necessary and sufficient to recruit the pre-rRNA processing factors to r-chromatin, we used U2OS 2–6–3 cells, which contain about 200 copies of 256 × LacO sequences at a single chromosome locus [17]. This system allowed us to tether UBF1 at the ectopic Lac O locus. EGFP and LacI-fused UBF1 was expressed in U2OS 2–6–3 cells, and immunofluorescence assays were performed with antibodies against nucleolar proteins as described above (Fig. 4D). EGFP and Lac I-tagged UBF1 was detected in the nucleoli and Lac O sites. RPA194 and Nopp140 were efficiently recruited to the Lac O sites by EGFP-Lac I-UBF1 expression. On the other hand, pre-rRNA processing factors (DKC1 and FBL) were not detected on the ectopic UBF1 locus. These results indicated that UBF was necessary but not sufficient for the recruitment of pre-rRNA processing factors to NORs.

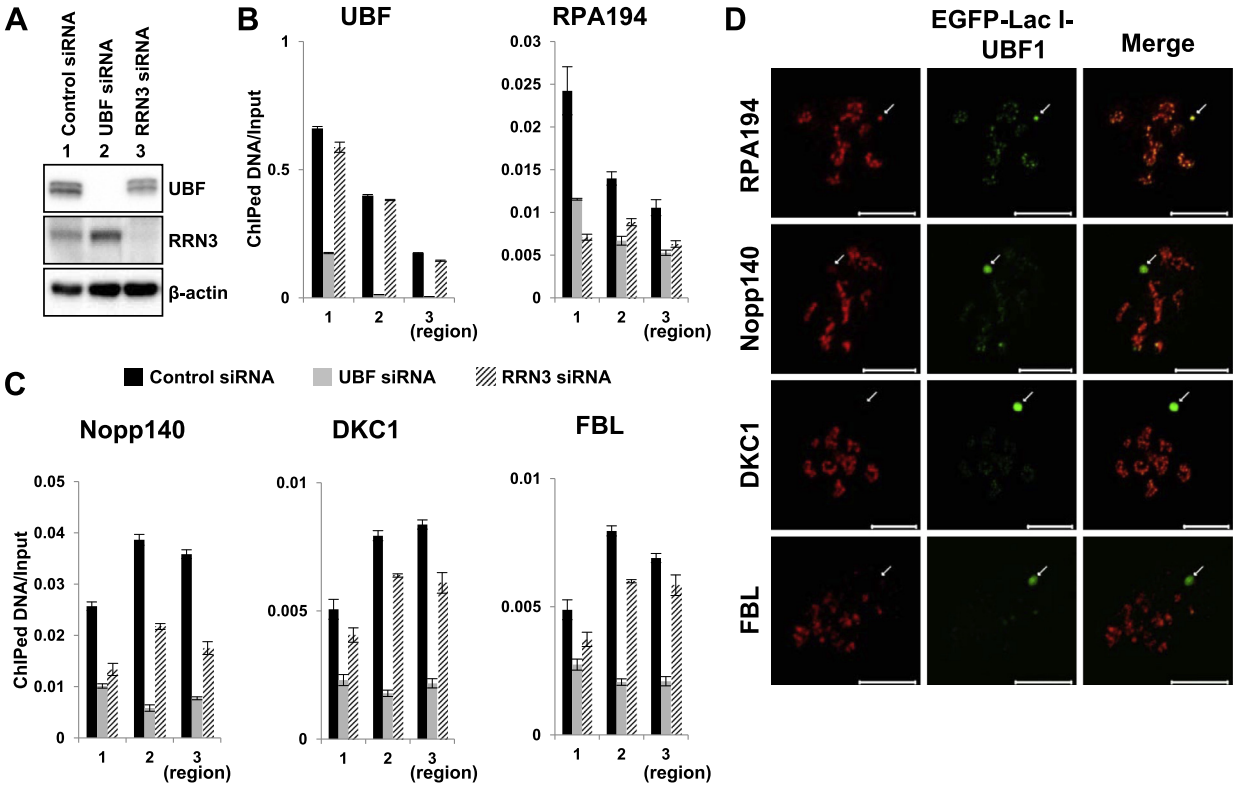

Fig. 4. UBF was necessary but not sufficient for the recruitment of pre-rRNA processing factors to r-chromatin. (A) Western blotting. The extracts prepared from HeLa cells transfected with control, UBF, and RRN3 siRNAs (lanes 1–3, respectively) were subjected to Western blotting with anti-UBF, RRN3, and β-actin antibodies 72 h after transfection. (B, C) Association between nucleolar proteins and r-chromatin. HeLa cells treated with siRNAs as in A were subjected to ChIP assays using (B) anti-UBF and-RPA194, and (C) anti-Nopp140, -DKC1, and -FBL antibodies. Normal IgGs were used as controls. The amounts of precipitated DNA relative to those of input DNAs were analyzed by Q-PCR with specific primer sets (regions 1–3 shown in Fig. 1A). Closed bar, gray bar, and cross-hatched bar show the results for cells transfected with control siRNA, UBF siRNA, and RRN3 siRNA, respectively. Q-PCR reactions were performed in triplicate and error bars represent S.D. Two independent experiments showed similar results. (D) Effect of UBF tethering at ectopic sites on the localization of nucleolar proteins. U2OS 2–6-3 cells were transfected with pEGFP-C1-Lac I-UBF1. Immunofluorescence assays were performed 42 h after transfection with anti-RPA194, -Nopp140, -DKC1, and-FBL antibodies as indicated at the left of the panels. Localization of proteins was observed under a confocal microscope. Arrows represent the ectopic locus containing Lac O repeats. Bars at the bottom in each panel indicate 10 μm.

4. Discussion

We demonstrated that not only pre-rRNA transcription factors but also the pre-rRNA processing factors such as DKC1 and FBL associated with r-chromatin (Fig. 1E). Although we examined the r-chromatin association of only FBL and DKC1, it would be reasonable to speculate that these factors associated with r-chromatin as snoRNP complexes.Nopp140 also associated with rRNA coding sequences (Fig. 1E). Given that Nopp140 was reported to interact with both snoRNPs [5] and pol I subunits [6], Nopp140 seems to serve as a scaffold to link pre-rRNA transcription and processing. Considering that the association was mainly detected on the rRNA coding sequences, the association between pre-rRNA processing factors (DKC1 and FBL) and r-chromatin is likely to contribute to coordinated pre-rRNA transcription and processing. Consistent with this idea, it was previously reported that yeast pre-rRNA processing factors associate with r-chromatin and regulate both optimal pre-rRNA transcription and processing [18]. Upon entry into mitosis, the association of pre-rRNA processing factors with r-chromatin was decreased. These data also suggest that the association of pre-rRNA processing factors with r-chromatin contributes to maintain the nucleolar structure in interphase.

Interestingly, we demonstrated that pre-rRNA processing factors (Nopp140, DKC1 and FBL) were re-assembled to r-chromatin after mitosis in the presence of a pol I inhibitor (Fig. 3C). These results suggest that ongoing pre-rRNA transcription alone is not the driving force to recruit the pre-rRNA processing factors to r-chromatin. Importantly, we found that UBF plays a crucial role in the assembly of pre-rRNA processing proteins to r-chromatin (Fig. 4C). Because RRN3 depletion did not significantly affect the association of pre-rRNA processing factors with r-chromatin, the function of UBF in the recruitment of pre-rRNA processing factors to r-chromatin is not simply explained by pre-rRNA transcription. Furthermore, we demonstrated that tethering UBF on ectopic sites failed to recruit the pre-rRNA processing factors using the Lac O-Lac I system. Thus, an additional factor(s) cooperating with UBF is required to mediate the assembly of pre-rRNA processing factors on r-chromatin. The cooperative function of UBF and transcribed pre-rRNAs may be required for the recruitment of pre-rRNA processing factors. Indeed, it was previously demonstrated that the U3 snoRNP components including FBL and U3 snoRNA are recruited to the plasmid vector containing the pol I promoter and 5′-ETS sequences when microinjected into the HeLa cell nuclei [19]. It should also be considered that the local concentration of pre-rRNA processing factors in the nucleolus is much higher than that in the nucleoplasm, which could be another reason why pre-rRNA processing factors were not recruited to the ectopic UBF locus in the nucleoplasm.

The initial recruitment of nucleolar RNPs such as FBL and NCL to NORs has been independent of pre-rRNA transcription [9]. We revealed that the r-chromatin association of these nucleolar proteins was restored even in the absence of pre-rRNA transcription restoration (Fig. 3). These results suggest that nucleolar formation consists of at least two steps; the first is recruitment of nucleolar RNPs including pre-rRNA processing factors and pre-rRNAs released during mitosis to NORs before pre-rRNA transcription restoration, and the second is the recruitment of PNB-associated pre-rRNA processing factors and ribosome particle assembly factors to NORs after pre-rRNA transcription restoration. Our results strongly suggest that the association between pre-rRNA processing factors (Nopp140, DKC1, and FBL) and r-chromatin is restored at the initial step of nucleolar assembly, which is mediated by UBF.

The function of UBF in the recruitment of pre-rRNA processing factors to NORs remains unknown. Since the pre-rRNA processing factors DKC1 and FBL were not recruited to the ectopically localized UBF sites, the function of UBF is not explained by simple protein–protein interactions with these pre-rRNA processing factors. To clarify the function of UBF will be crucial to understand the molecular basis of nucleolar assembly and disassembly.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.039.

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