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Assembly and Maturation of the U3 snoRNP in the Nucleoplasm in a Large Dynamic Multiprotein Complex

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Summary

The assembly and maturation of box C/D snoRNPs, factors essential for ribosome biogenesis, occur in the nucleoplasm. To investigate this process, we have analyzed non-snoRNP factors associated with the nucleoplasmic human U3 snoRNA. We show that both the precursor and mature length nucleoplasmic U3 snoRNAs are present in larger multiprotein complexes that contain the core box C/D proteins as well as many non-snoRNP factors linked to snoRNP assembly (TIP48, TIP49, Nopp140), RNA processing (TGS1, La, LSm4, hRrp46), and subcellular localization (CRM1, PHAX). Using RNAi, we show that most of these factors are essential for box C/D snoRNA accumulation. Furthermore, we demonstrate that the core proteins undergo a restructuring event that stabilizes their binding to the snoRNA. Importantly, restructuring, which may be mediated by the putative remodeling factor TIP49, appears to be linked to nucleolar localization. We believe that the assembly complex coordinates snoRNA processing, snoRNP assembly, restructuring, and localization.

Introduction

Small nucleolar RNAs (snoRNAs) are involved in the cleavage and modification of the ribosomal RNA (rRNA). Several box C/D snoRNAs, including U3, U8, and U14, are essential for rRNA processing (Terns and Terns, 2002). These snoRNAs contain rRNA complementary regions that likely function as RNA chaperones in rRNA processing. Typically, the two major classes of snoRNA function as "guide RNAs" by base pairing with specific sites of modification in the ribosomal RNA in the nucleolus (Kiss, 2002). Box C/D snoRNAs guide 2′-O-methylation, whereas H/ACA snoRNAs direct pseudouridine formation. Mature box C/D snoRNAs are associated with four common core proteins, namely 15.5K, NOP56, NOP58, and fibrillarin (methyltransferase) (reviewed in Tran et al. [2004]). The U3 snoRNA is unique in that it is associated with many additional U3-specific proteins (Granneman and Baserga, 2004), however, the U3 snoRNP monomer has been shown to contain the four core box C/D proteins as well as hU3-55K (Rrp9p in yeast) (Watkins et al., 2000). The remaining U3-specific proteins are proposed to associate with the U3 snoRNP as part of the pre-rRNA processing complex (Granneman et al., 2003).

In vertebrates, many snoRNAs are encoded within the introns of protein-coding genes and are released from the pre-mRNA via a splicing-dependent pathway (Terns and Terns, 2002). In contrast, the essential U3 and U8 snoRNAs are encoded in snRNA-type RNA polymerase II genes. The transcripts produced from this type of gene (pre-snoRNAs) contain an m^7G cap structure and a short 3′ extension. During snoRNP biogenesis, the m^7G is hypermethylated to an m^3G cap structure and the short 3′ terminal extension removed (Terns and Terns, 2002). SnoRNP biogenesis appears to occur in two phases; first, the core snoRNP is assembled and the snoRNA processed in the nucleoplasm, most likely in the Cajal body, and second, the assembled snoRNP complex is transported to the nucleolus (Narayanan et al., 1999; Verheggen et al., 2002). Evidence suggests that Cajal bodies are nuclear bodies/compartments involved in RNP biogenesis and modification (Lamond and Sleeman, 2003).

The core box C/D proteins bind a conserved sequence and structure-based element termed the box C/D motif that is essential for the biogenesis and function of the box C/D snoRNPs (Tran et al., 2004). It has been demonstrated that the evolutionarily conserved box C/D motif folds into a stem-internal loop-stem structure that is also known as a k-turn. 15.5K is an RNA binding protein that binds directly to this k-turn motif (Kuhn et al., 2002; Szewczak et al., 2002; Watkins et al., 2000, 2002). The binding of 15.5K, in conjunction with the conserved sequence of stem II in the box C/D motif, is essential for the recruitment of the remaining box C/D snoRNP proteins (Watkins et al., 2002). The formation of the complete core box C/D complex is required for nucleolar localization (Verheggen et al., 2001; Watkins et al., 2002). Furthermore, NOP58 and 15.5K (Snu13p in yeast) are required for the accumulation/stability of all box C/D snoRNAs in yeast (Lafontaine and Tollervey, 1999; Watkins et al., 2000). Thus, the core proteins play essential roles in the biogenesis and accumulation of box C/D snoRNPs. However, it is unclear whether this assembly process can occur spontaneously or, as with the spliceosomal snRNPs (Yong et al., 2004), requires the assistance of assembly factors in vivo.

The complexity of RNP assembly, even of relatively small complexes such as the formation of the Sm core structure on the spliceosomal snRNAs, has recently become apparent. The nascent snRNAs, which are transcribed in the nucleus, are exported to the cytoplasm via an export complex containing PHAX and CRM1 (Ohno et al., 2000). In the cytoplasm, the large SMN-containing assembly complex drives the specific binding of the core Sm proteins (Meister et al., 2002; Yong et al., 2004). The mature snRNP is then reimported into the nucleus where it first localizes to the Cajal body before moving to the splicing speckles (Sleeman and Lamond, 1999). In comparison, relatively little is known about the factors

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involved in the assembly of the snoRNPs. In yeast, a number of proteins have been linked to the processing of the snoRNA precursor, these include TGS1 (Verheggen et al., 2002), the cap methyltransferase, as well as La, LSm proteins, and the exosome, factors linked to the removal of the 3′ extension (Kufel et al., 2003). However, yeast snRNA-type RNA polymerase II genes differ significantly from those found in higher eukaryotes, and it is quite likely that transcription termination and 3′ end formation of transcripts in vertebrates differs from that observed in yeast (Uguen and Murphy, 2003). Furthermore, SMN, Nopp140, TIP48, and TIP49 have been suggested to be involved in box C/D snoRNP assembly and/or localization (Jones et al., 2001; King et al., 2001; Newman et al., 2000; Pellizzoni et al., 2001; Verheggen et al., 2001; Watkins et al., 2002; Yang et al., 2000). However, it is not clear when or, in many cases, if these additional proteins bind the snoRNP in vivo and what role they perform.

Although a number of candidate proteins for snoRNP assembly and biogenesis have been identified, direct evidence for their involvement is in most cases limited. In addition, it is not clear whether snoRNP assembly and snoRNA processing are mediated by a large multiprotein complex similar to that seen with the spliceosomal snRNPs or whether snoRNP biogenesis occurs as a series of independent steps. We therefore set out to characterize the early stages of nucleoplasmic U3 box C/D snoRNP biogenesis. Here, we show that in the nucleoplasm, both the precursor and mature U3 snoRNA are present in large multiprotein complexes that contain the core box C/D proteins as well as factors linked to RNA processing, snoRNP assembly, and RNA export/localization. Furthermore, we provide evidence that a restructuring event, linked to nucleolar localization, results in the stabilization of the binding of the core box C/D proteins. Our data suggest that snoRNP biogenesis is mediated by a large multiprotein complex that coordinates snoRNP assembly, localization, and snoRNA processing events.

Results

Nuclear Extract Contains Both Mature and Precursor U3 snoRNA

In order to learn more about the nucleoplasmic process of snoRNP biogenesis, we characterized the snoRNP and pre-snoRNP complexes present in HeLa nuclear extract. Importantly, during the preparation of nuclear extract, the nucleoli, containing the majority of the mature snoRNPs, are removed by centrifugation. Therefore, we believe that the nuclear extract produced with this method represents the soluble nucleoplasmic fraction. Northern hybridization revealed that the mature form (U3-m) as well as a longer form of the U3 (U3-p) snoRNA were present in the nuclear extract (Figure 1A). In contrast, a single species of U3 was observed in total nuclear RNA.

We next characterized the pre-snoRNAs present in nuclear extract. RNA was isolated from nuclear extract and immunoprecipitated with either an antibody specific for the m^3G cap (R1131) or an antibody that recognizes both m^7G and m^3G cap structures (H20). The immunoprecipitated RNAs were then analyzed by Northern hybridization with a probe specific for U3 snoRNA. As shown in Figure 1B, the R1131 antibodies only immunoprecipitated the shorter, mature length U3-m. However, H20 antibodies precipitated both forms of U3 (U3-p and U3-m). Therefore, only the mature length U3 contains an m^3G cap, whereas the longer, precursor transcript contains an m^7G cap structure in accordance with earlier publications (Terns and Dahlberg, 1994; Verheggen et al., 2002). The longer U3 species contains an approximately 8-nucleotide 3′ extension (Figure 1A). Analysis of known mammalian U3 genes revealed that the sequences downstream of the coding sequence, i.e., the 3′ extension(s), are either uridine- or pyrimidine-rich (Figure 1C). Interestingly, yeast pre-U3 snoRNA also contains a U-rich 3′ extension that has been proposed to be the binding site of La and LSm proteins (Kufel et al., 2003), suggesting that this may also be the case in higher eukaryotes.

U3 snoRNP Present in Nuclear Extract Is Found in Large Multiprotein Intermediates

We next characterized and compared the complexes associated with the two forms of the U3 snoRNA, namely U3-m and U3-p. Nuclear extract was separated on a 10%–30% glycerol gradient. RNA was isolated from the gradient fractions, separated by PAGE, and the major RNAs revealed by silver staining (Figure 2A). The S value markers at the bottom of the figure are derived from the migration behavior of the characterized spliceosomal snRNP complexes. The distribution of the U3 snoRNA transcripts was revealed by Northern hybridization (Figure 2B). The longer form of U3 (U3-p), which peaks at 15S–16S, migrated significantly faster on the glycerol gradient than the mature length RNA (U3-m). This implies that the precursor transcript U3-p is associated with significantly more proteins than the mature length RNA U3-m, and at least some of these additional factors would be expected to be required for RNA processing.

In order to determine which proteins are associated with the U3 snoRNA and pre-snoRNA in nuclear extract, we first separated the distinct complexes present in HeLa nuclear extract by glycerol gradient centrifugation. Protein association in each gradient fraction of the U3 peak was then determined by immunoprecipitation (Figure 2, fractions 8–14) using a battery of antibodies raised against both mature snoRNP proteins and putative snoRNP biogenesis factors. The coprecipitated RNAs were subsequently analyzed by Northern blot hybridization.

Antibodies that recognize the mature snoRNP proteins NOP58, NOP56, and fibrillarin as well as the U3-specific protein hU3-55K coprecipitated both the precursor and mature U3 snoRNA in nuclear extract (Figure 3; see below). This confirms that these proteins associate with the snoRNA in the nucleoplasm and that core snoRNP assembly takes place on the precursor transcript. Fibrillarin antibodies preferentially coprecipitate mature length U3, suggesting either a later association and/or weaker association with the precursor relative to NOP58.

Immunoprecipitation also revealed that La, LSm4, and the exosome subunit hRrp46 were associated specifi-

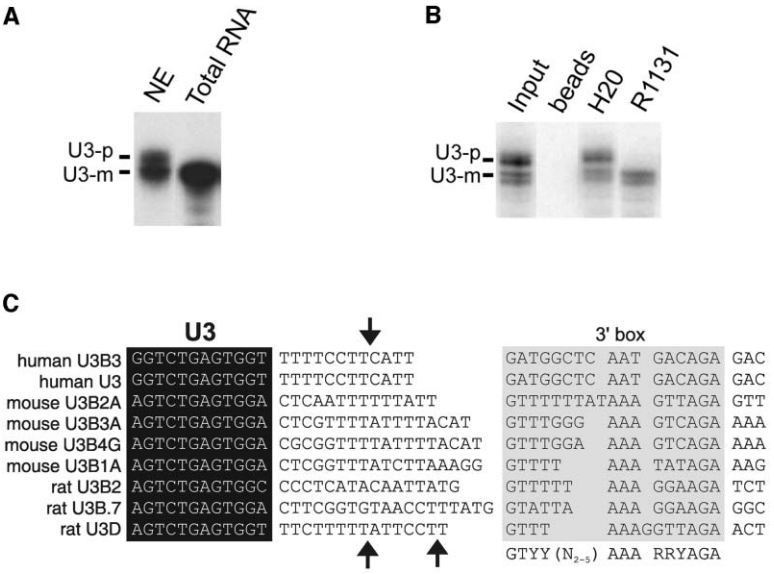

Figure 1. Nuclear Extract Contains U3 Pre-snoRNAs

(A) Northern analysis of U3 snoRNAs present in either nuclear extract (NE) or total nuclei (total RNA). Because of the relatively low abundance of the U3 snoRNA in nuclear extract, 100-fold more nuclear extract RNA was loaded compared to the total nuclear RNA. U3-p and U3-m denote the longer precursor transcript and mature length U3 snoRNAs, respectively.

(B) Analysis of cap structure of the nuclear extract U3 snoRNAs. RNA was isolated from nuclear extract and then immunoprecipitated with antibodies that recognize either an m^3G cap (R1131) or both an m^7G and m^3G cap structure (H20) or protein-A Sepharose alone (beads). Precipitated RNAs were then analyzed by Northern hybridization. Note that in some experiments, the two forms of U3 snoRNA appear slightly heterogeneous. This is likely due to loss of one or two nucleotides at the 3′ end of the transcripts during sample preparation.

(C) Phylogenetic comparison of mammalian U3 snoRNA 3′ extension sequences. DNA sequences, which begin three nucleotides upstream of box D, are aligned with respect to the coding region (white text on black background) and the 3′ box (gray box). The 3′ box was identified based on the work of Neuman de Vegvar et al. (1986), Hernandez and Weiner (1986), and Neuman de Vegvar and Dahlberg (1990). The predicted 3′ ends of the human U3 and rat U3D precursor transcripts (Stroke and Weiner, 1985) are indicated by arrows at the top and bottom of the alignment, respectively.

cally with the precursor U3 snoRNA (Figure 3). This is consistent with their predicted role in 3′ processing (Kufel et al., 2003). The LSm proteins and hRrp46 showed the same association profile and appeared to be present in the same 13–16S complex. In contrast, La was associated with a much slower (10–12S) migrating complex, distinct to that containing the LSm proteins and hRrp46. Importantly, the U3 complexes could not be immunoprecipitated by anti-LSm1 antibodies implying that the nuclear LSm2–8 complex was associated with the pre-snoRNPs (Figure 4).

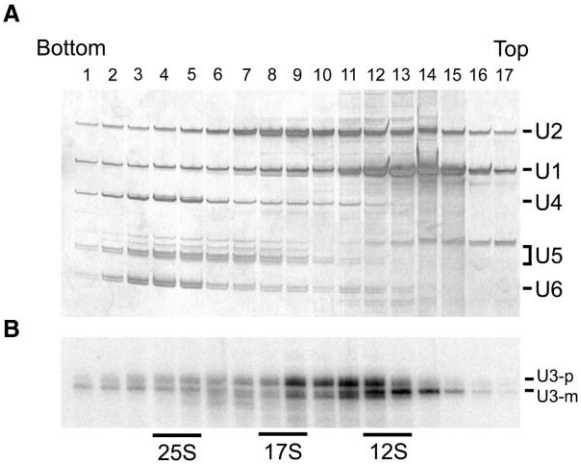

Figure 2. U3 Pre-snoRNA Is Found in a Separate, Larger Complex Than the Mature Length snoRNA

Sedimentation behavior of RNPs present in HeLa nuclear extract separated on a 10% to 30% glycerol gradient is shown. RNAs present in each fraction were isolated and separated on an 8% polyacrylamide-7 M urea gel. The sedimentation coefficients of the major snRNP peaks are indicated at the bottom, and the identities of the RNAs are marked on the right. Fraction numbers are indicated at the top. (A) Major snRNAs were visualized by silver staining. (B) U3 snoRNAs were detected by Northern blotting. U3-p and U3-m denote the longer precursor transcript and mature length U3 snoRNAs, respectively.

We also demonstrated that the putative biogenesis/assembly factors TIP48, TIP49, and Nopp140 were associated with the U3 snoRNP in nuclear extract. Both TIP48 and TIP49 were associated with U3-p; however, TIP48 was specifically associated with the precursor transcript, and TIP49 was also associated with U3-m. From this, we infer that TIP48 associates with the pre-snoRNP prior to TIP49. Interestingly, Nopp140 was associated with both U3-p and U3-m, however, it was found predominantly in the larger, faster migrating complexes. To our surprise, we could also demonstrate that CRM1 and PHAX, two components of the spliceosomal snRNP export complex (Ohno et al., 2000), were stably associated with the nucleoplasmic U3 snoRNP. PHAX was specifically associated with the precursor RNA, whereas CRM1 was associated with both precursor and mature U3 snoRNAs. Intriguingly, anti-SMN antibodies clearly did not coprecipitate the U3 snoRNA (Figure 4A), suggesting that this protein is not stably associated with snoRNPs during biogenesis.

Although we have shown that a multitude of proteins are associated with the U3 snoRNP in nuclear extract, the association of these proteins with the mature, nucleolar snoRNPs has not been analyzed in the majority of cases. This point is especially important for proteins such as Nopp140, which are predominantly found in the nucleolus. We therefore next analyzed the association of these proteins with mature snoRNPs in nucleolar extract to determine whether they are only associated with the pre-snoRNP complexes. As seen in Figure 4B, antibodies recognizing the mature snoRNP proteins, NOP56,

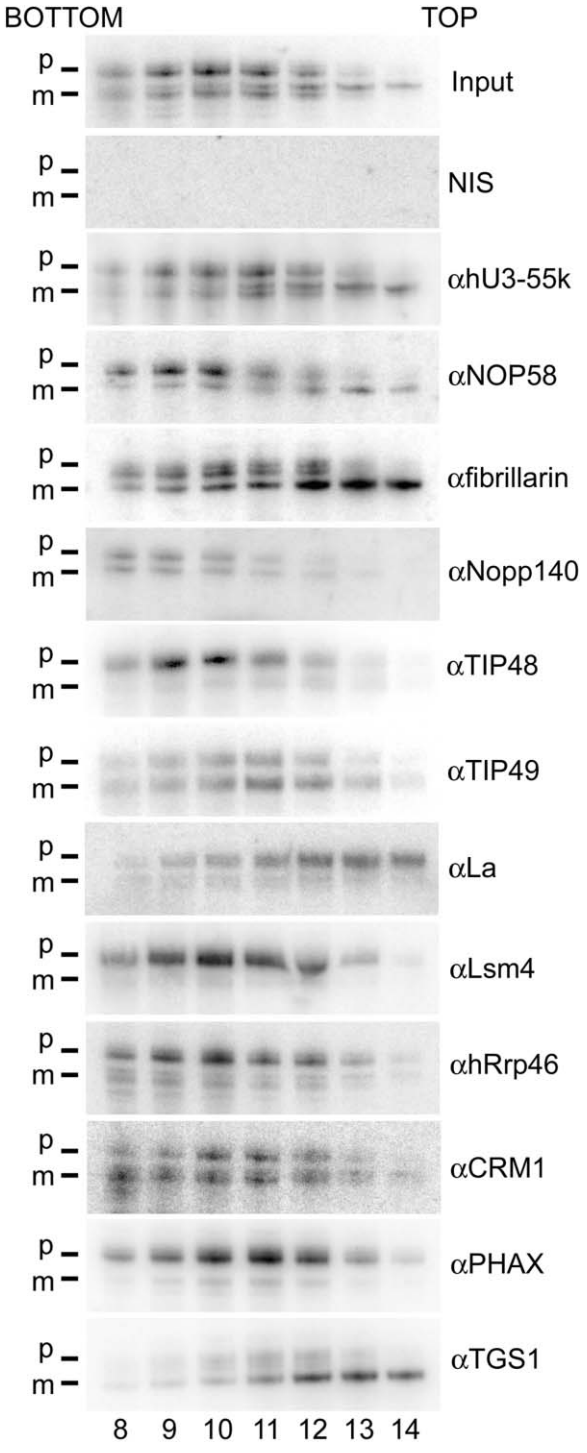

Figure 3. Differential Association of Proteins with the Precursor and Mature U3 snoRNAs

Nuclear extract was separated on a 10% to 30% glycerol gradient and the gradient fractions 8 to 14 (Figure 2) used for immunoprecipitation with either protein-specific antibodies or control nonimmune serum (NIS). The RNAs precipitated from each fraction were isolated, separated on an 8% polyacrylamide-7 M urea gel, and the U3 snoRNA revealed by Northern blotting. The antibody used is indicated on the right of each panel. The fraction numbers are indicated at the bottom. The U3 precursor and mature length transcripts are indicated by p and m, respectively. Input represents RNA derived from 10% of the fraction used for immunoprecipitation.

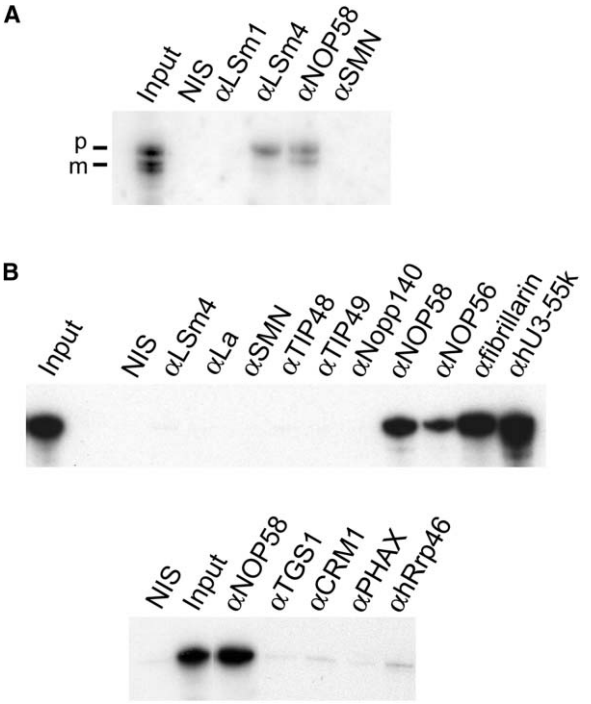

Figure 4. Comparison of Proteins Bound to the Nuclear and Nucleolar U3 snoRNAs

Immunoprecipitations were performed with either (A) nuclear or (B) nucleolar extract, and precipitated RNAs were analyzed as described for Figure 3. Antibodies used are indicated at the top of the panel. The U3 precursor and mature length transcripts in (A) are indicated by p and m, respectively. NIS, nonimmune serum. Input, RNA derived from either (A) nuclear or (B) nucleolar extract equivalent to 10% of the material used for immunoprecipitation.

NOP58, fibrillarin, and hU3-55K, all specifically coprecipitated the nucleolar U3 snoRNP. In contrast, none of the other antibodies coprecipitated the nucleolar U3 snoRNP. This clearly shows that the putative biogenesis factors are only associated during the nucleoplasmic phase of snoRNP biogenesis.

RNAi-Directed Depletion of snoRNA-Associated Proteins

We have demonstrated that many *trans*-acting factors are associated with the U3 snoRNP in nuclear extract and are, therefore, likely involved in the nucleoplasmic phase of its biogenesis. However, it is unclear what role these proteins play in snoRNP biogenesis. We, therefore, designed and synthesized synthetic siRNA duplexes to specifically deplete the factors associated with the nucleoplasmic snoRNP complexes according to Elbashir et al. (2002). HeLa cells were transiently transfected with specific siRNA duplexes. After 60 hr incubation, the cells were harvested and the viable cell number determined. Importantly, at this time point the cells had not undergone apoptosis (data not shown). As a control, the functional siRNA duplex, GL2, which targets the firefly luciferase mRNA, was used. This duplex has no natural target in HeLa cells and has little or no effect on cell growth and RNA levels (Elbashir et al., 2002; data not shown). Each of the siRNA duplexes specifically tar-

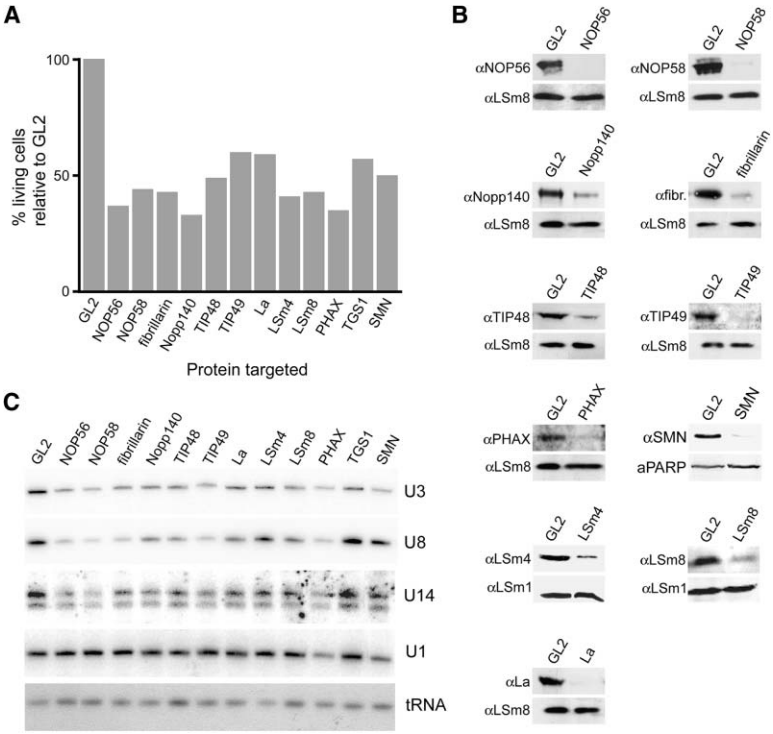

Figure 5. RNAi Knockdowns Reveal Essential Functions for Nucleoplasmic U3 snoRNA-Associated Proteins in Box C/D snoRNP Accumulation in HeLa Cells

(A) The number of living cells present 60 hr after transfection with each siRNA duplex was determined and expressed as a percentage of the control knockdown (siRNA GL2 against luciferase). The mRNA targeted is indicated on the horizontal axis.

(B) Western blot analysis of protein depletion. The protein targeted is indicated above each panel. The antibodies used are indicated on the left. Proteins derived from equal numbers of cells were loaded. GL2 is the control siRNA targeting luciferase.

(C) Protein requirement for box C/D snoRNA accumulation. Total RNA was extracted from HeLa cells 60 hr after transfection with the siRNA duplex. Northern blot analysis was used to determine the relative levels of the U3, U8, and U14 box C/D snoRNAs, the U1 snRNAs, and $tRNA^{Arg}$. The specific probe used is indicated to the left of each panel. The protein targeted is indicated at the top.

geting snoRNP biogenesis factors as well as the core snoRNP proteins resulted in a significant reduction in cell growth relative to the cells transfected with the control duplex (Figure 5A). Therefore, each of these proteins is essential for the maintenance of the cell growth rate. Western blot analysis of the transfected cells revealed that in each case, the siRNA duplexes significantly reduced the expression levels of the target protein (Figure 5B). In contrast, the level of the control protein, LSm8 (except LSm1 for LSm4 and LSm8 and PARP for SMN), remained constant, demonstrating the specificity of the siRNA-mediated depletion.

We next analyzed the effect of the siRNA-mediated depletion of individual proteins on snoRNA levels in HeLa cells. Total RNA was extracted from an equal number of cells 60 hr after siRNA duplex transfection and analyzed by Northern hybridization with probes specific for box C/D snoRNAs, snRNAs, and tRNA. We analyzed the levels of the m^3G-capped, independently transcribed U3 and U8 snoRNAs as well as the intron-encoded U14 snoRNA to determine whether the factors are specific for U3 biogenesis or essential for the accumulation of both independently transcribed and intronic box C/D snoRNAs. Consistent with their involvement in the biogenesis of both types of box C/D snoRNP, the loss of the majority of proteins resulted in the reduction of the levels of all tested box C/D snoRNAs when compared to cells treated with the control siRNA duplex (Figure 5C). Interestingly, in the majority of cases a significantly greater reduction in U8 and U14 levels, relative to U3 snoRNA, was observed. This may reflect the different turnover rates of the individual snoRNPs.

The loss of each of the core proteins resulted in a reduction in box C/D snoRNA levels (Figure 5C). The efficiency of protein depletion was approximately equivalent for NOP56, NOP58, and fibrillarin, therefore enabling a direct comparison on their relative importance in snoRNA accumulation. The strongest effect was observed with NOP58, a protein previously shown to be essential for snoRNA stability/accumulation in yeast (Lafontaine and Tollervey, 1999). Interestingly, NOP56 and fibrillarin appeared to be more important for U8 accumulation than for U3 and U14. Depletion of TIP49 and PHAX also resulted in a comparable reduction in box C/D snoRNA levels. Targeted loss of other proteins, such as the LSm proteins, La, TIP48, and Nopp140, had a lesser effect on box C/D snoRNA levels. Loss of SMN and, to a lesser extent, TGS1 resulted in reduced U3 levels but had no noticeable effect on U8 and U14 snoRNAs. Therefore, our data suggest that these proteins are not essential for the biogenesis of all snoRNAs. With the exception of PHAX and SMN, which are also involved in snRNP biogenesis, none of the other proteins were essential for the accumulation of the U1 snRNA (Figure 5C). Importantly, loss of either PHAX or SMN had no effect on the levels of tRNA. We therefore believe that the effects seen here are specific and that the majority, if not all, of these proteins are essential for box C/D snoRNA accumulation and, therefore, snoRNP biogenesis.

The Core Box C/D snoRNP Complex Undergoes a Stabilization Event before Entering the Nucleolus

After completion of the RNA processing events and the recruitment of fibrillarin, the nucleoplasmic U3 snoRNP was still associated with many proteins not present in the mature nucleolar snoRNPs (Figure 3). These proteins could function in the subnuclear trafficking of the snoRNP. Alternatively, it is possible that a further maturation step is necessary to generate the mature snoRNP complex.

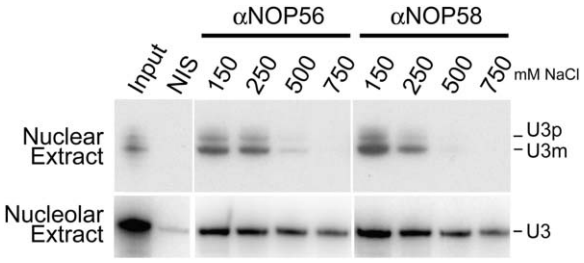

Figure 6. Nuclear and Nucleolar Core U3 snoRNPs Exhibit Different Salt Stabilities

SnoRNP particles present in either nuclear (top) or nucleolar (bottom) extract were immunoprecipitated with nonimmune serum (NIS) or antibodies against either NOP56 or NOP58. Bound particles were incubated with buffer containing 150 to 750 mM NaCl as indicated. The remaining coprecipitated U3 RNAs were isolated and analyzed by Northern blotting. Input represents 10% of the starting material.

This could include structural changes to the core box C/D complex. Indeed, the AAA+ protein TIP49, which is associated with U3-m, is a potential protein remodeling/restructuring factor. To test this latter possibility, we compared the salt sensitivity of the association of the core snoRNP proteins NOP56 and NOP58 with the U3 snoRNPs found in either nuclear extract or the nucleolus. U3 snoRNPs were immunoprecipitated from either nucleolar or nuclear extracts with either anti-NOP56 or anti-NOP58 antibodies. The bound material was then subjected to increasing concentrations of NaCl (Figure 6). The complexes remaining bound to the beads were then eluted and analyzed by Northern hybridization. Both forms of U3 (U3-m and U3-p) present in nuclear extract were stably associated with both NOP56 and NOP58 at 150 mM NaCl. However, upon increasing salt concentrations both U3-m and U3-p were dissociated. Indeed, the amount of associated U3 snoRNA was dramatically reduced at 500 mM and almost not detectable at 750 mM NaCl. In both cases, there was a >100-fold decrease in U3 snoRNA association upon increasing the NaCl concentration from 150 to 750 mM. Therefore, the association of the core proteins NOP56 and NOP58 with both forms of the U3 snoRNA in nuclear extract is salt sensitive. In contrast, the association of the mature U3 snoRNP, present in nucleolar extracts, with NOP56 and NOP58 was hardly affected by the increased salt concentrations. Indeed, only a 2- to 3-fold difference was observed between the bound material washed at 150 and 750 mM NaCl. This demonstrates a major difference in the stability of the core snoRNP complex between complexes found in the nucleoplasm and the nucleolus. Importantly, even though fibrillarin antibodies could not efficiently coprecipitate the precursor transcript, fibrillarin was associated with U3-m, ruling out the possibility that the difference in stability is due to the lack of a complete core box C/D complex. In summary, we propose that one or more of the biogenesis factors associated with U3-m complex may mediate this restructuring event, leading to the stabilization of the core box C/D complex.

Discussion

A Large, Structurally Dynamic Multiprotein Complex Essential for snoRNP Biogenesis

We have investigated the early phases of snoRNP biogenesis by characterizing pre-snoRNP complexes present in nuclear extract. On the basis of our analysis, we believe that nuclear extract represents the soluble nucleoplasmic material. Using this approach, we demonstrated that the U3 snoRNPs present in nuclear extract are larger than the mature nucleolar complexes and associated with a significant number of nucleoplasmic proteins that are not bound to the mature nucleolar complexes. In addition to the core box C/D proteins and U3-specific hU3-55K, these include factors linked to RNA processing (TGS1, La, LSm4, and the exosome subunit hRrp46), putative assembly factors (Nopp140, TIP48, and TIP49), and RNA export factors (CRM1 and PHAX). This suggests that as with the spliceosomal snRNPs, snoRNP assembly is mediated by a large multiprotein complex. Furthermore, using RNAi, we could demonstrate that these proteins are not only present in the pre-snoRNP complexes but that most are essential for maintaining box C/D snoRNA levels in HeLa cells. Importantly, this is the first direct functional study of the role of these proteins in snoRNP biogenesis in higher eukaryotes. On the basis of the fact that most of these proteins are essential for the accumulation of the U3, U8, and U14 snoRNAs, we conclude that many of these proteins are common box C/D snoRNP biogenesis factors.

In addition to the complexity and size of these pre-snoRNP complexes, our data also provide compelling evidence that these multiprotein complexes are structurally dynamic. Indeed, a combination of glycerol gradient and immunoprecipitation analysis enabled us to characterize several putative intermediate complexes, which we could characterize with respect to their temporal stage in snoRNA biogenesis by the processing status of the 5′ and 3′ ends of the U3 snoRNA. We have clearly demonstrated that the core box C/D proteins as well as hU3-55K are stably associated with the precursor U3 snoRNA (Figures 3 and 5), suggesting that the mature snoRNP proteins are associated very early in the biogenesis pathway. The reduced association of fibrillarin with the precursor suggests either that this protein binds later than NOP56 and NOP58 or that fibrillarin binds weakly at first, and the association is later stabilized, perhaps upon completion of 3′ processing. It was previously reported that 3′ extended rat pre-U3 snoRNAs, expressed from a transiently transfected plasmid, are bound by 15.5K (again transiently transfected), but not associated with GFP-tagged NOP58 and fibrillarin (Verheggen et al., 2002). We cannot clearly explain the discrepancy between our observation and this earlier work; however, it is possible that the overexpression of transiently transfected rat U3 constructs leads to saturation of the biogenesis pathway and, therefore, incomplete assembly of the snoRNP complex.

PHAX, TIP48, La, LSm4, and the exosome subunit hRrp46 are exclusively associated with the precursor U3 snoRNA. The association of La, LSm4, and hRrp46 with the 3′ extended form of U3 snoRNA correlates with

their involvement in the 3′ maturation of small RNAs (Perumal and Reddy, 2002). Indeed, these proteins likely dissociate from the pre-snoRNP once processing at the 3′ end is complete. In addition, PHAX and TIP48 also dissociate from the pre-snoRNP at this point, suggesting that extensive structural changes occur upon completion of 3′ processing and cap hypermethylation. The association of LSm4, but not LSm1, with the nucleoplasmic U3 snoRNP complex suggests that the LSm2 to LSm8 complex is associated with the pre-snoRNPs. Because of a lack of antibodies, we could not address which other LSm proteins are associated with the pre-snoRNP. However, the fact that LSm8 is essential for U3 accumulation (as shown by RNAi) supports the notion that multiple LSm proteins are associated with the U3 snoRNP. La and the LSm proteins both recognize U-rich sequences at the 3′ end of transcripts and could both bind the U-rich 3′ extension in the U3 pre-snoRNA (Figure 1C) (Achsel et al., 1999; Wolin and Cedervall, 2002). Because these proteins are in separate complexes, we postulate that the initial precursor transcript is likely bound by La and later replaced by the LSm proteins at the 3′ end of the snoRNA. The pre-snoRNP is associated with the exosome subunit hRrp46, and from this, we infer that the complete nuclear exosome complex is bound to the pre-snoRNP complex (Perumal and Reddy, 2002); however, further work is necessary to define which exosome components interact with the pre-snoRNP. Therefore, the LSm proteins, which have been shown in yeast to interact with exosome subunits, may function in the recruitment of the exosome and the regulation of 3′ processing (Fromont-Racine et al., 2000). In contrast, in *S. cerevisiae* the LSm proteins first bind pre-U3 snoRNA and are later replaced by La (Kufel et al., 2003). However, in yeast, the nascent transcript is significantly longer than observed in metazoans and, it would appear, undergoes a different maturation process.

The data presented in this manuscript strongly suggest that the multiprotein complex associated with the U3 precursor RNA mediates the assembly of the snoRNP complex as well as the processing of the pre-snoRNA. This implies that assembly and processing are coordinated events and that the use of the multiprotein assembly complex likely permits regulation of the various aspects of snoRNP biogenesis.

A Role for Nuclear Export Factors in U3 Biogenesis

In this manuscript, we have shown that the nuclear export factors CRM1 and PHAX are stably associated with the U3 snoRNP present in nuclear extract. Interestingly, PHAX was associated specifically with the precursor transcript, whereas CRM1 was found bound to both the precursor and mature U3 snoRNAs. Furthermore, using RNAi, we showed that PHAX is essential for snoRNA accumulation, demonstrating the functional significance of its interaction with the pre-snoRNP. The precursor U3 snoRNA contains an m^7G cap structure, a substrate for the cap binding complex (CBC) (Lewis and Izaurralde, 1997). Unfortunately, antibodies to CBC were not available, and we could not check association of CBC with the nuclear extract complexes. Ran GTP also associates with CRM1 bound to export cargoes and could also be associated with the nuclear extract U3 snoRNP. Indeed, the recent work of Boulon et al. (2004) (this issue of *Molecular Cell*) confirmed the association of PHAX and CRM1 with the snoRNA and also demonstrated that Ran and the CBC also bind the U3 snoRNP.

SnoRNP biogenesis was originally proposed to include a cytoplasmic phase (Baserga et al., 1992). Later work suggested that snoRNP biogenesis takes place in the nucleus and does not include nuclear export (Terns and Dahlberg, 1994). We were surprised to find the nuclear export factors PHAX and CRM1 stably associated with the U3 pre-snoRNPs. If snoRNPs remain in the nucleus, this would suggest an important nuclear function for these proteins. Interestingly, Cheng et al. (1995) previously suggested that RanGTP was essential for the correct localization of newly synthesized U3 snoRNA. Furthermore, the recent data of Boulon et al. (2004) provide evidence that PHAX is essential for Cajal body localization, whereas CRM1 is necessary for nucleolar localization. It is therefore possible that nuclear extract contains two populations of U3 snoRNP. The PHAX-associated complex could either be on the way to or extracted from the Cajal body. In contrast, the mature U3 snoRNA-containing complex, containing CRM1, could represent an intermediate that has left the Cajal body and is in the process of localising to the nucleolus. Because of the nature of the extract preparation, we cannot be absolutely certain of the in vivo localization of the pre-snoRNP complexes. However, the available data support the notion that snoRNP assembly and maturation are nucleoplasmic processes (Verheggen et al., 2002; Boulon et al., 2004). Furthermore, most of the biogenesis factors associated with the nuclear extract U3 snoRNP are found in the nucleoplasm, but not in the nucleolus (King et al., 2001; Ingelfinger et al., 2002; Verheggen et al., 2002; Boulon et al., 2004). In addition, the forms of the U3 precursor transcripts shown to concentrate in the Cajal bodies (Verheggen et al., 2002) are present in nuclear extract. Therefore, it is highly likely that the complexes found in nuclear extract represent the nucleoplasmic phase of snoRNP biogenesis. However, further work is required to define which complexes are found in the Cajal body and determine whether the CRM1 bound pre-snoRNP represents a complex in the process of localizing to the nucleolus. It is, however, important to note that none of the potential nuclear functions of PHAX and CRM1 rigorously exclude the possibility that the proteins may also function in the nuclear export of snoRNPs.

Restructuring of the Core Box C/D snoRNP Complex

The nucleoplasmic m^3G-capped mature length U3 snoRNA is still associated with a number of proteins, including Nopp140, TIP49, and CRM1, that are not bound to the mature nucleolar complex. This raises the possibility that snoRNP biogenesis is, at this point, not yet complete. CRM1 likely facilitates nucleolar localization (see above). TIP48 and TIP49 are related AAA+ ATPases that are involved in a number of functions including histone acetylation and DNA repair (Ikura et al., 2000). These proteins likely function as molecular motors in the remodeling of multiprotein complexes. We

therefore reasoned that after the binding of the core box C/D proteins and the processing of the snoRNA, the core snoRNP complex may undergo a structural change. Indeed, we could show that the core box C/D snoRNP complex undergoes a restructuring event that converts NOP56 and NOP58 association with the snoRNA from a salt-sensitive to a salt-stable form.

Our data suggest that the core box C/D proteins NOP56 and NOP58 are loaded onto the U3 snoRNA in an open or loose conformation. These proteins are likely recruited to the snoRNA as part of a large preformed multiprotein assembly complex that contains core box C/D proteins, TIP48, and several other snoRNP biogenesis factors (N.J.W. and R.L., unpublished data). However, it is unclear how many other proteins are involved in the binding of the core box C/D proteins. The difference in salt sensitivity likely indicates that in the intermediate snoRNP complexes, the protein-RNA, and/or protein-protein contacts differ to those found in the mature snoRNP. Interestingly, the SMN complex functions in both the docking of the core Sm proteins onto the Sm binding site of the snRNAs as well as providing an additional specificity factor in the assembly of these complexes (Yong et al., 2004). Although only the box C/D motif is essential for snoRNA accumulation, it is possible that the large assembly complex provides additional specificity through protein-protein and/or protein-RNA contacts.

All U3 snoRNP present in nuclear extract is in the salt-sensitive, open conformation. The mature nucleoplasmic U3 snoRNA is associated with CRM1 and TIP49. Boulon et al., (2004) have shown that CRM1 is involved in the nucleolar localization of the U3 snoRNP (see above). This implies that the CRM1-associated complexes are in the process of moving from the Cajal body to the nucleolus. The closed, salt-stable form of the U3 snoRNP is only present in the nucleolus. It is, therefore, interesting to speculate that this event may coincide with or occur after nucleolar localization. Indeed, the stabilization of the core complex could occur upon the release of the remaining nucleoplasmic factors such as the AAA+ protein TIP49 and CRM1. TIP49 is a likely candidate for the mediator of the restructuring event. TIP49 has been proposed to function as a DNA helicase though there is conflicting evidence on the ability of this protein to hydrolyse ATP and to unwind DNA (Ikura et al., 2000). It is conceivable that this protein could regulate either protein-protein or protein-RNA interactions within the box C/D snoRNP. Because we have yet to identify all the components involved in snoRNP biogenesis, it is also possible that an as-yet-unidentified protein mediates this structural change in the core box C/D complex.

It is possible that the open conformation may be important for other aspects of snoRNP biogenesis. Indeed, it may be that many biogenesis factors can only bind the open conformation of the core box C/D complex. This would enable these factors to differentiate between the mature and precursor complexes. This point is especially relevant for Nopp140, an abundant protein that is predominantly found in the nucleolus (Meier and Blobel, 1992), but which only interacts with snoRNPs in the nucleoplasm. In addition, the change in structure may reveal or create functional elements within the core complex necessary for nucleolar localization and/or function. Further work is necessary to define the functional significance of this structural change.

Experimental Procedures

Extract Preparation and Glycerol Gradient Analysis

HeLa nuclear extracts were fractionated on a 10% to 30% glycerol gradient containing 150 mM KCl (Schneider et al., 2002). Purified HeLa nuclei were resuspended in buffer DM (20 mM HEPES-NaOH [pH 7.9], 150 mM NaCl, 3 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 10% glycerol), sonicated three times for 30 s with a Branson microtip at a setting of 1.5, and then centrifuged for 30 min at 16,000 × g to prepare nucleolar extract. The pellet, containing the nucleoli, was then resuspended in buffer D (20 mM HEPES-NaOH [pH 7.9], 150 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 10% glycerol), sonicated, and then centrifuged for 30 min at 16,000 × g to release the snoRNPs from the nucleoli.

Immunoblots and immunoprecipitations were performed as described previously (Watkins et al., 2002). Antibodies recognizing NOP56, NOP58, TIP48, LSm4, LSm8, and LSm1 as well as the anti-cap antibodies R1131 and H20 were described previously (Achsel et al., 1999; Bochnig et al., 1987; Ingelfinger et al., 2002; Lührmann et al., 1982; Watkins et al., 2000, 2002). TIP49 antibodies were provided by Stuart Maxwell (Newman et al., 2000). Fibrillarin antibodies were provided by Michael Pollard. La and hRrp46 antibodies were provided by Ger Pruijn (Brouwer et al., 2001; Pruijn et al., 1995). SMN antibodies were provided by Utz Fischer (Liu and Dreyfuss, 1996) and Nopp140 antibodies by Tom Meier (Meier and Blobel, 1992). PHAX antibodies were provided by Iain Mattaj (Ohno et al., 2000) and TGS1 antibodies by Remy Bordonne (Verheggen et al., 2002). Anti-CRM1 antibodies were provided by Achim Dickmanns and Ralf Kehlenbach (Kehlenbach et al., 1998).

siRNA Transfection and Cell Culture

All siRNA duplexes were designed as 21-mers with 3′-dTdT overhangs (Elbashir et al., 2002). The sequences used to target each gene were as follows. Accession numbers are given. Position of the targeting sequences relative to the start of the open reading frame is indicated after the sequence.

NOP56 (NM_006392), 5′-CAAUAUGAUCAUCCAGUCCAUUA-3′ (495–517); NOP58 (NM_015934), 5′-CAAGCAUGCAGCUUCUACCG UUC-3′ (927–949); Fibrillarin (NM_001436), 5′-CAGUCGAGUUCU CCCACCGCUCU-3′ (625–647); TIP48 (NM_006666), 5′-GAGACCA UCUACGACCUGGGCAC-3′ (518–540) and 5′-GAGAGUGACAUGGC GCCUGUCCU-3′ (962–984); TIP49 (NM_003707), 5′-AAGGAACCA AACAGUUGAAACUG-3′ (570–592) and 5′-GAGUCUUCUAUCGCU CCCAUCGU-3′ (1034–1056); Nopp140 (NM_004741), 5′-AAAUUGA GGUGGAUUCACGAGUU-3′ (1894–1916); PHAX (NM_032177), 5′-UAG UAUCAGCGAGGAACAAAUUA-3′ (939–961) and 5′-AAGAGUAUAU AGCACAGGAUUUA-3′ (1427–1449); TGS1 (NM_024831), 5′-AAGAUU GCCCUUGCUCGCAAUAA-3′ (2338–2360) and 5′-UAUCACCGUAU GAAAUGGAAACU-3′ (2837–2859); SMN (XM_041492), 5′-AAGUGG AAUGGGUAACUCUUCUU-3′ (1020–1042); LSm4 (NM_012321), 5′-AAC GGCCGUCCCAAAGCUGGCUG-3′ (635–657); LSm8 (NM_016200), 5′-AAGAAACAGAUUCUGCGCUUGAU-3′ (224–225); and La (NM_003142): 5′-GAAUUAGGUCCACUUCAAUGUCC-3′ (1377–1399) and 5′-AAGAUUCUUCCAUUAAAUUGCCU-3′ (1518–1540).

In some cases, two siRNA duplexes were required to achieve an effective reduction in gene expression. The GL2 siRNA, which targets the luciferase gene, was used as a control (Elbashir et al., 2002). siRNA transfections were performed as described previously (Elbashir et al., 2002). Cells were analyzed 60 hr after transfection. The effect of the siRNA duplexes on cell growth was determined as described by Schaffert et al. (2004). For Northern and Western blot analysis of transfected cells, gels were loaded such that each lane contained material derived from the same number of cells.

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