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Ongoing U snRNP Biogenesis Is Required for the Integrity of Cajal Bodies

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Cajal bodies (CBs) have been implicated in the nuclear phase of the biogenesis of spliceosomal U small nuclear ribonucleoproteins (U snRNPs). Here, we have investigated the distribution of the CB marker protein coilin, U snRNPs, and proteins present in C/D box small nucleolar (sno)RNPs in cells depleted of hTGS1, SMN, or PHAX. Knockdown of any of these three proteins by RNAi interferes with U snRNP maturation before the reentry of U snRNA Sm cores into the nucleus. Strikingly, CBs are lost in the absence of hTGS1, SMN, or PHAX and coilin is dispersed in the nucleoplasm into numerous small foci. This indicates that the integrity of canonical CBs is dependent on ongoing U snRNP biogenesis. Spliceosomal U snRNPs show no detectable concentration in nuclear foci and do not colocalize with coilin in cells lacking hTGS1, SMN, or PHAX. In contrast, C/D box snoRNP components concentrate into nuclear foci that partially colocalize with coilin after inhibition of U snRNP maturation. We demonstrate by siRNA-mediated depletion that coilin is required for the condensation of U snRNPs, but not C/D box snoRNP components, into nucleoplasmic foci, and also for merging these factors into canonical CBs. Altogether, our data suggest that CBs have a modular structure with distinct domains for spliceosomal U snRNPs and snoRNPs.

INTRODUCTION

The interphase nucleus contains many morphologically distinct substructures, called nuclear bodies, which are non–membrane-bound, stable cellular compartments involved in the fidelity and efficiency of gene expression. One of the most extensively studied nuclear bodies, the Cajal body (CB), was originally identified more than 100 years ago (Cajal, 1903). CBs are present in the nucleus of vertebrates, invertebrates and plants in varying number and size depending on the cell type (Gall, 2000; Cioce and Lamond, 2005). They are molecularly defined by the presence of the protein coilin, which was discovered through the analysis of patient autoimmune sera (Andrade *et al.*, 1991; Raska *et al.*, 1991). The composition of CBs is very diverse, but a number of factors involved in RNA processing are found enriched within them: 1) spliceosomal U small nuclear ribonucleoproteins (snRNPs) and associated factors (Spector *et al.*, 1992); 2) small Cajal-body-specific (sca)RNAs which guide posttranscriptional modifications of spliceosomal U snRNAs (Darzacq *et al.*, 2002; Kiss *et al.*, 2002; Jády *et al.*, 2003); 3) small nucleolar (sno)RNPs involved in processing of pre-rRNA (Narayanan *et al.*, 1999; Verheggen *et al.*, 2002; Boulon *et al.*, 2004); 4) U7 snRNP, which is required for the 3′ trimming of histone mRNAs (Frey and Matera, 1995); and 5) basal transcription factors (Schul *et al.*, 1998; Gall *et al.*, 1999).

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The CB marker coilin is a multi-interacting protein that binds, among other things, directly to some of the spliceosomal Sm proteins (Hebert *et al.*, 2001; Xu *et al.*, 2005), and the snoRNP biogenesis factor Nopp140 (Isaac *et al.*, 1998). In addition, coilin contains an N-terminal self-oligomerization domain (Hebert *et al.*, 2000). It has been proposed that coilin provides a scaffold upon which the different types of CB components assemble, because upon immunodepletion of coilin from *Xenopus* egg extract, no visible foci of spliceosomal components can be observed in pronuclei formed in vitro (Bauer and Gall, 1997). Similarly, mouse embryonic fibroblast (MEF) cells lacking functional coilin fail to concentrate spliceosomal U snRNPs in the nucleus (Tucker *et al.*, 2001). Still, coilin is apparently not the only player in the formation of CBs. Overexpression of coilin leads to an increase of coilin in the nucleoplasm, but not in the formation of additional CBs, indicating that coilin is not limiting for CB formation. In contrast, the formation of CBs can be induced by increasing the relative abundance of spliceosomal U snRNPs (Sleeman *et al.*, 2001). Thus, the detailed requirements for CB formation and integrity are still somewhat elusive, also because—despite being stable substructures in the cell nucleus—all CB constituents including coilin are dynamically exchanged between CBs and the nucleoplasm (Snaar *et al.*, 2000; Handwerger *et al.*, 2003; Sleeman *et al.*, 2003; Dundr *et al.*, 2004).

There is considerable evidence that CBs are involved in the biogenesis of spliceosomal U snRNPs, which is an ordered multistep process that takes place in several subcellular compartments (Will and Lührmann, 2001; Bertrand and Bordonné, 2004). The snRNAs U1, U2, U4, and U5 are transcribed by RNA polymerase II in the nucleus as snRNA precursors containing a 5′ terminal 7-monomethylguanosine

(m^7G) cap structure and are then transported to the cytoplasm by the CRM1/RanGTP pathway using the U snRNA–specific adaptor PHAX (Ohno *et al.*, 2000). In the cytoplasm, the SMN complex, composed of the SMN protein and at least six other proteins (termed Gemin2–7; Yong *et al.*, 2004) facilitates the assembly of the seven Sm proteins B/B′, D1, D2, D3, E, F, and G on the conserved Sm binding site of the U snRNAs to form the Sm core structure. The formation of the Sm core is a prerequisite for hypermethylation of the m^7G cap generating the 2,2,7-trimethylated G (m_3G) cap (Mattaj, 1986; Plessel *et al.*, 1994). The methyltransferase responsible for the conversion of the m^7G to the m_3G cap of both U snRNAs and snoRNAs in yeast, Tgs1, has recently been identified (Mouaikel *et al.*, 2002). A human homologue of Tgs1 (called hTGS1) has been found on the basis of sequence homology (Mouaikel *et al.*, 2003). However, up to now it has not been verified that hTGS1 functions directly in the hypermethylation of m^7G caps in human.

The m_3G cap and the Sm core of the newly assembled U snRNPs form a bipartite nuclear localization signal (NLS) required for their subsequent reimport into the nucleus (Fischer *et al.*, 1993). In the nucleus, the newly imported U snRNPs first accumulate transiently in the CBs (Sleeman and Lamond, 1999), before moving to another nuclear body collectively termed speckles, from where they are believed to be recruited to active sites of splicing (Misteli *et al.*, 1997). The U snRNAs undergo site-specific pseudouridylation and 2′-*O*-methylation in the nucleus, processes that are thought to take place in the CBs, because the guide RNAs that direct these modifications—the scaRNAs—accumulate in CBs (Kiss *et al.*, 2002; Darzacq *et al.*, 2002; Jády *et al.*, 2003). In addition, a number of U snRNP-specific proteins are enriched in CBs; therefore, it has been proposed that they assemble on the U snRNA Sm cores within these nuclear bodies (Hetzer and Mattaj, 2000; Stanek *et al.*, 2003; Schaffert *et al.*, 2004; Tanackovic and Krämer, 2005). This is supported by the findings that proteins, which are required for the assembly of the U2, the U4/U6, and the U4/U6.U5 snRNPs, are enriched in CBs, and that upon depletion of these assembly factors the corresponding U snRNP components accumulate in CBs (Makarova *et al.*, 2002; Will *et al.*, 2002; Stanek *et al.*, 2003; Schaffert *et al.*, 2004; Stanek and Neugebauer, 2004; Tanackovic and Krämer, 2005).

CBs play also an important role in the biogenesis of snoRNPs, which are classified into two families, C/D box and H/ACA box, based on conserved RNA sequence elements, and which function in the nucleolytic processing and modification of pre-rRNA in the nucleolus (Bertrand and Bordonné, 2004). Indeed, in vertebrates most snoRNAs are encoded within introns of split genes and produced as a result of splicing. Only the U3, U8, and U13 snoRNAs are known to be transcribed by RNA polymerase II from independent genes. The primary transcripts from these genes obtain an m^7G cap structure, which becomes hypermethylated to an m_3G cap after assembly of core snoRNP proteins on the snoRNAs. These steps are believed to take place in CBs, because U3 and U8 snoRNAs, the snoRNP core proteins fibrillarin, Nop56, and Nop58, the snoRNP maturation factor Nopp140, and the putative cap methyltransferase hTGS1 are all partially enriched in CBs (Verheggen *et al.*, 2002; Boulon *et al.*, 2004).

In the present study we have investigated the role of ongoing U snRNP biogenesis on the structural integrity of CBs. We used RNAi to deplete HeLa cells of hTGS1 or SMN, two factors required for the cytoplasmic maturation of U snRNPs. Our results show that extracts from cells depleted of hTGS1 are impaired in m^7G cap methylation in an in vitro U snRNP hypermethylation assay. Methylation activity can be restored by the addition of recombinant wild-type, but not mutant, hTGS1. This demonstrated for the first time that hTGS1 is indeed actively involved in m_3G cap formation of U snRNPs. Using fluorescence microscopy, we also show that depletion of hTGS1 or SMN gives rise to coilin dispersion into numerous small foci in the nucleoplasm and also partially into the nucleolus. No colocalization of U snRNP components with coilin in the residual foci could be observed; however, the C/D box snoRNP components fibrillarin, Nop56, Nop58, and Nopp140 were found in the coilin-containing foci. Similar results were obtained upon depletion of PHAX, which blocks specifically the nuclear export of newly synthesized U snRNAs, although in this case coilin is excluded from nucleoli. Finally, we demonstrate by siRNA-induced depletion that snoRNP components condense into nucleoplasmic foci in the absence of coilin, whereas, again, no concentration of U snRNP components in such structures can be observed. Altogether, our data show that the formation of canonical CBs is dependent not only on coilin, but also on active U snRNP biogenesis. Furthermore, our results suggest that CBs have a modular structure with distinct domains containing either spliceosomal U snRNPs, snoRNPs, or one of the other CB components.

MATERIALS AND METHODS

Cell Culture and siRNA Transfection

HeLa SS6 cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen/BRL, Carlsbad, CA) and 10 U of penicillin/streptomycin per ml at 37°C and with 5% CO_2. For indirect immunofluorescence analysis and fluorescence in situ hybridization, cells were grown on glass coverslips.

siRNA duplexes directed against hTGS1, SMN, and PHAX were designed and synthesized in-house as 21mers with 3′-dTdT overhangs (Elbashir *et al.*, 2002). The siRNA duplex targeting coilin was designed and synthesized by Qiagen (Chatsworth, CA). For hTGS1 and PHAX, two siRNA duplexes were required in parallel to achieve effective reduction in gene expression. The sequences used to target each gene were as follows (GenBank accession numbers and the position of the targeted sequences relative to the start of the open reading frame are indicated in parentheses before and after each siRNA sequence, respectively): hTGS1 (NM_024831), 5′-GAUUGCCCUUGCUCGCAAUAA-3′ (2171–2192), and 5′-UCACCGUAUGAAAUGGAAACU-3′ (2670–2691); SMN (XM_041492), 5′-CCAAAUGCAAUGUGAAAUAUU-3′ (1224–1246); Phax (NM_032177), 5′-GUAUCAGCGAGGAACAAAUUA-3′ (939–961), and 5′-GAGUAUAUAGCACAGGAUUUA-3′ (1427–1449), coilin (NM_004645), 5′-GCAAUUAAAUGACGUGGUAAA-3′ (1342–1360). The GL2 siRNA, which targets the firefly (*Photinus pyralis*) luciferase gene, was used as a control (Elbashir *et al.*, 2002). siRNA transfections were performed as described previously (Elbashir *et al.*, 2002).

To monitor the effect of depletion of hTGS1, SMN, PHAX, or coilin on cell growth, cells were harvested 24, 48, and 72 h after siRNA transfection and the numbers of viable cells were determined by using the cell counter (CASY-counter TT, Schärfe Systems GmbH, Reutlinger, Germany). Different effects of the knockdowns on cell proliferation and cell survival were observed over time. Therefore, cells were assayed 48 h after transfection of siRNAs targeting SMN and PHAX and 72 h after transfection of siRNAs targeting hTGS1 and coilin. At these time points the targeted proteins were efficiently depleted, but no apoptosis was observed by the TUNEL assay performed according to the manufacturer's instruction (Roche, Indianapolis, IN).

To prepare S100 extract, cells were electroporated with siRNAs against hTGS1 or SMN according to Schaffert *et al.* (2004) and harvested after 72 h or 48 h, respectively.

Real-Time RT-PCR

hTGS1, SMN, and coilin knockdowns were analyzed by the relative quantification based on the relative amount of target mRNA in knockdown cells versus target mRNA in control cells with a one-step real-time RT-PCR using SYBR Green (QuantiTec, Beaverton, OR; SYBR Green RT-PCR Kit). For normalization of the target genes the intronless glutamate dehydrogenase 2 (GLUD2) was used as an endogenous standard. Total RNA was isolated from HeLa SS6 cells treated with GL2 control siRNA and with siRNAs targeting hTGS1, SMN, or coilin (RNeasy Mini Kit, Qiagen) and on-column was treated with DNase1. Total RNA, 12.5 ng from each sample, was used for analysis. The following PCR primers, which are exon/exon-spanning for all but GLUD2, were used: hTGS1-F 5′-GGCTATTACATCAGAGACAGTGG-3′;

hTGS1-R: 5′-GAATCAAGTTCACTTTCATCCAGGC-3′; SMN-F: 5′-TGCATTTACCCAGCTACCATTG-3′; SMN-R: 5′-GATCGGACAGATTTTGCTCCTC-3′; Coilin-F: 5′-CTTGAGAGAACCTGGGAAATTTG-3′; Coilin-R: 5′-GTCTTGGGTCAATCAACTCTTTCC-3′; GLUD2-F: 5′-TCGTGGAGGACAAGTTGGTG-3′; and GLUD2-R: 5′-TTGCAGGGCTTGATGATCCG-3′.

All real-time RT-PCRs were performed with the DNA Engine Opticon 1 System (Bio-Rad, Richmond, CA). The specificity of real-time RT-PCR products was verified by high-resolution gel electrophoresis, which showed a single product with the desired length in all cases. In addition, an Opticon melting-curve analysis was performed, which resulted in single-product-specific melting curves. The transcripts investigated showed real-time PCR efficiencies ranging from 1.8 to 2.1 (Pfaffl, 2001) in the range from 0.2 to 25 ng total RNA input with high linearity (Pearson $r > 0.98$). Relative quantification of knockdown versus control mRNA levels was accomplished according to the Pfaffl method (Pfaffl, 2001). Additionally, controls without RT mix were performed for each RNA to verify that there was no residual DNA contamination.

S100 Extract Preparation and In Vitro Hypermethylation Assay

Cells were harvested with a rubber policeman, washed in phosphate-buffered saline (PBS; pH 7.4), resuspended in one packed-cell volume of Roeder buffer A (Dignam *et al.*, 1983), and incubated on ice for 15 min. Then, the cells were lysed by rapidly pushing them six times through a narrow-gauge needle (25 g, 5/8, 15.9 mm). The nuclei were pelleted by centrifugation for 1 min at 13000 rpm and 4°C. The supernatant was transferred to a new tube, and 0.11 volumes of 10× Roeder B buffer were added (Dignam *et al.*, 1983) followed by centrifugation for 1 h at 100,000 × *g* and 4°C. Subsequently, the supernatant was dialyzed against reconstitution buffer (50 mM HEPES, pH 7.4; 50 mM KCl; 5 mM $MgCl_2$; 5% glycerol; 0.5 mM DTE; 0.5 mM phenylmethylsulfonyl fluoride). The concentration of the S100 extract was adjusted to 2 mg/ml, and the extract was then stored at −80°C until use.

The in vitro m_3G-cap hypermethylation assay was performed according to Plessel *et al.* (1994). In brief, 100 μl of S100 extract (2 mg/ml) was incubated, in the presence of 0.1 mM *S*-adenosylmethionine (SAM), with 70 pmol in vitro reconstituted U1 snRNP in reconstitution buffer (see above) for 1 h at 37°C. The reaction was stopped by the addition of 1 mg/ml proteinase K and further incubation for 30 min at 37°C. RNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 800 μl PBS at pH 7.4. The RNA suspension was split into two equal portions: one was incubated with anti-m_3G cap antibody R1131 bound to protein A-Sepharose and the other with anti-cap antibody H-20 bound to protein G-Sepharose. After head-over-tail incubation for 3 h at 4°C, the beads were washed with IPP300 (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.1% Nonidet P-40), and then treated with proteinase K for 30 min at 37°C and extracted with phenol/chloroform. Finally, the RNA was precipitated by ethanol and was analyzed on a 10% denaturing polyacrylamide gel. The signal intensity of precipitated RNA was determined with a phosphoimager plate.

Construction of Plasmids and Purification of Recombinant Proteins

To construct pGEX-hTGS1(aa 577–851) carrying the conserved methyltransferase domain, the hTGS1 coding sequence was amplified by using two oligonucleotides containing restriction sites for EcoRI and XhoI, respectively. The EcoRI-XhoI fragment was transferred into pGEX4T3 vector (Amersham, Indianapolis, IN) previously cut with EcoRI and XhoI. The D695A point mutation was generated by oligonucleotide-directed mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). pGEX constructs were transformed in BL21 *Escherichia coli* strain, and recombinant proteins were purified using glutathione-Sepharose beads (Amersham) essentially as described by Smith and Johnson (1988).

Immunoblotting, Indirect Immunofluorescence, Fluorescence In Situ Hybridization, and Fluorescence Microscopy

For Western blot analysis, 50 μg of total cell extract was separated on an 13% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Schleicher and Schüll, Keene, NH), and immunostained with antibodies by using the ECL detection kit as described by the manufacturer (Amersham).

Indirect immunofluorescence experiments with siRNA-transfected cells were performed as previously described (Ingelfinger *et al.*, 2002). Antibodies raised against the following proteins were used: hTGS1 (Verheggen *et al.*, 2002), SMN (7B10, kindly provided by Utz Fischer, University of Würzburg; Liu and Dreyfuss, 1996), coilin (5P10 π, kindly provided by M. Carmo-Fonseca, University of Lisbon; Almeida *et al.*, 1998), fibrillarin (ab5821 from AbCam, Cambridge, United Kingdom), Nop58 (Watkins *et al.*, 2002), and p110 (Schaffert *et al.*, 2004). For the detection of Sm proteins, an Sm-positive serum from a patient suffering from the autoimmune disease systemic lupus erythematosus was used. The secondary antibodies used for indirect immunofluorescence, labeled with either Alexa-488 or Alexa-647, were obtained from Molecular Probes (Eugene, OR).

Fluorescence in situ hybridization (FISH) was performed according to a protocol based on that of Taneja *et al.* (1992). The sequence of the U2 oligonucleotide used for FISH was as follows: 5′-GAACAGATACTACACTTGATCTTAGCCAAAAGGCCGAGAAGC-3′. The oligonucleotide was labeled at its 5′ and 3′ ends with Alexa-488 (Molecular Probes).

Fluorescence samples were visualized under identical conditions for control and knockdown cells by using a Zeiss LSM Meta 510 confocal microscope (Oberkochen, Germany). To obtain sharp images of the entire nucleus, a series of images was acquired in a 0.15-μm-thick optical slice from which a two-dimensional projection was subsequently obtained (Zeiss Software). Images were processed and merged by using Adobe Photoshop (version 7.0; San Jose, CA).

RESULTS

Depletion of hTGS1 and SMN by RNAi

To interfere with the cytoplasmic maturation of the spliceosomal U snRNPs before their reentry into the nucleus, we used RNAi to deplete HeLa cells of the putative cap hypermethylase hTGS1 or of the SMN protein. Real-time RT PCR analysis showed that 24 h after siRNA transfection the mRNA levels of hTGS1 and SMN were reduced to 8 and 13.5%, respectively, by the corresponding siRNAs compared with their levels in the presence of control siRNAs (set to 100%; Figure 1A). To demonstrate that the targeted proteins had been depleted from the cells, immunoblots with antibodies against hTGS1 or SMN were performed. As shown in Figure 1B, both the hTGS1 and the SMN proteins were reduced below the detection limit of the corresponding antibody after 72 (hTGS1) or 48 h (SMN) of siRNA treatment in comparison with control cells, in good agreement with the results from the real-time RT-PCR analysis. This shows that we are able to efficiently deplete HeLa cells of hTGS1 or SMN by RNAi. The siRNAs against hTGS1 and SMN were then tested for their effect on cell growth (Figure 1C). As a positive control, siRNAs against the essential splicing factor hPrp8 arrest cell growth after 24 h, with a decrease in cell number. In contrast, siRNAs against hTGS1 had only a marginal effect, if any at all, on the growth rate of the treated cells. Thus, hTGS1 seems not to be essential for cell viability, a finding consistent with results of studies in yeast demonstrating that deletion of the *TGS1* gene resulted only in a cold-sensitive phenotype (Mouaikel *et al.*, 2002). Depletion of SMN had an intermediate effect: cell growth is clearly reduced, but not as strongly as in the case of hPrp8. Still, at 72 h the cells start to undergo apoptosis (demonstrated by the TUNEL assay, unpublished data), indicating that SMN is essential for cell viability.

hTGS1 Is Required for Hypermethylating the m^7G Cap of U snRNAs

To find out whether hTGS1 is directly involved in the methylation of the m^7G cap of spliceosomal U snRNAs, we tested the function of hTGS1 in m_3G cap formation by using an in vitro hypermethylation assay (Plessel *et al.*, 1994). For this assay we used cytoplasmic S100 extract isolated from HeLa cells depleted of hTGS1 by RNAi. To obtain sufficient amounts of S100 extract for the biochemical experiments, the transfection procedure was scaled up, with transfection carried out by electroporation instead of the Oligofectamine method usually used. Note that with electroporation the knockdown efficiency for hTGS1 was slightly reduced (with 15% of the mRNA remaining after 24 h; unpublished data).

For the hypermethylation assay, S100 extracts from control cells and from cells depleted of hTGS1 were incubated, in the presence of the essential cofactor S-AdoMet, with in vitro reconstituted, radiolabeled U1 snRNPs bearing an m^7G cap. Hypermethylation of radiolabeled U1 snRNAs was detected by immunoprecipitation with an anti-m_3G-cap antibody (R1131), which does not recognize m^7G caps. As an internal control, a second antibody (H-20) was used, which

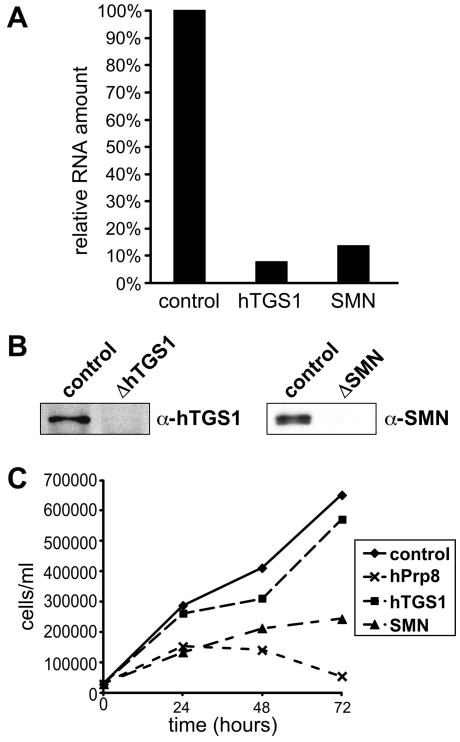

Figure 1. Validation of the targeting of hTGS1 and the SMN protein by siRNA duplexes. (A) Specific RNAi-mediated depletion of mRNAs encoding hTGS1 or SMN revealed by real-time RT-PCR analysis. HeLa cells were transfected either with luciferase siRNAs (control) or with siRNAs targeting hTGS1 or SMN. After 24 h cells were harvested, total RNA was isolated, and real-time RT-PCRs were performed. The diagram shows the relative levels of the target mRNAs compared with mRNA levels from cells transfected with control siRNAs directed against the nonhuman protein luciferase (set to 100%). (B) Knockdown of hTGS1 and SMN proteins by RNAi. Cells were treated as in A and harvested 72 h after transfection with siRNAs against hTGS1 (ΔhTGS1) or 48 h after transfection with siRNAs against SMN (ΔSMN), and the levels of the corresponding proteins were determined by immunoblotting (α-hTGS1 and α-SMN, respectively). (C) Cell survival after siRNA transfection. HeLa cells were harvested 24, 48, and 72 h after siRNA transfection and counted. The graphs show the growth curve of HeLa cells depleted of hTGS1 or SMN. For comparison, the growth rate of HeLa cells transfected with siRNAs targeting the nonhuman protein luciferase (control) and the essential splicing factor hPrp8 are also shown.

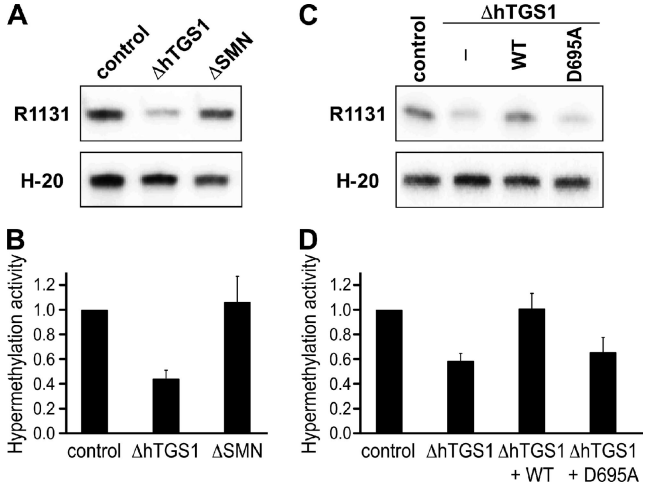

Figure 2. hTGS1 is required for efficient hypermethylation of U snRNAs in vitro. (A) Extract isolated from HeLa cells depleted of hTGS1 by RNAi shows a reduced hypermethylation efficiency. In vitro–reconstituted radiolabeled U1 snRNP containing an m^7G cap was incubated with S100 extract from HeLa cells depleted of hTGS1 (ΔhTGS1), SMN (ΔSMN), or the nonhuman protein luciferase (control) by RNAi. The reaction mixture was then split, and the U1 snRNP was immunoprecipitated with the m_3G-cap–specific antibody R1131, which immunoprecipitates only hypermethylated U1 snRNPs (product; top panel), and with the mAb H-20, which recognizes both m^7G and m_3G caps and thus binds to all U1 snRNPs in the reaction (total U1; bottom panel). (B) The results of three independent hypermethylation assays as described in A are summarized in the graph. The hypermethylation efficiency was calculated by the ratio of hypermethylation products immunoprecipitated with the R1131 antibody to the total U1 snRNA immunoprecipitated with the H-20 antibody. The hypermethylation efficiency was normalized to control extract (set to 1). Error bars, SD. (C) Recombinant hTGS1 restores the hypermethylation activity in hTGS1-depleted extract. Either recombinant hTGS1(aa 577–851) encompassing the methyltransferase domain (WT) or an inactive hTGS1(aa 577–851) mutant carrying a D695A mutation that affects S-AdoMet binding (D695A) was added to S100 extract depleted of hTGS1 (ΔhTGS1). The hypermethylation activity was then assayed as in A. (D) Summary of two independent hypermethylation assays as described in C and quantified as in B.

recognizes both m_3G and m^7G caps (Lührmann *et al.*, 1982; Bochnig *et al.*, 1987). Using both antibodies in parallel, we were able to distinguish between mono- and trimethyl-capped U1 snRNAs. The hypermethylation efficiency was calculated by determination of the ratio between the RNAs precipitated by the R1131 antibody (m_3G-capped U1 snRNA), and the RNAs were precipitated by the H-20 antibody (total U1 snRNA present in the reaction). Depletion of hTGS1 reduces the hypermethylation activity to ~40% (Figure 2, A and B). We believe that the remaining hypermethylation activity is due to residual hTGS1 after RNAi-mediated depletion (see above). Small amounts of the enzyme present in the extract are probably sufficient to hypermethylate m^7G caps in vitro. Interestingly, although the presence of an Sm core on the U snRNA is required for m_3G formation in vitro (Plessel *et al.*, 1994, and unpublished data), depletion of SMN has no influence on the hypermethylation efficiency of U1 snRNA in vitro (Figure 2, A and B), although it has been previously proposed that hTGS1 is recruited to U snRNAs by SMN. This proposal was based on the identification of a direct interaction of hTGS1 with the SMN protein (Mouaikel *et al.*, 2003). Altogether, these results show that in vitro hTGS1 can be efficiently recruited to preassembled U snRNA Sm cores in the absence of SMN.

To verify that the reduced hypermethylation activity observed in hTGS1-depleted extract is directly caused by the loss of hTGS1, recombinant hTGS1 encompassing the methyltransferase domain was added to the extract depleted of hTGS1 and investigated regarding its effect on the hypermethylation efficiency (Figure 2, C and D). An hTGS1 protein carrying a D695A mutation was used as a negative control. The corresponding mutation in yeast Tgs1 (D103A) prevents S-AdoMet binding and thus inactivates the enzyme (Mouaikel *et al.*, 2002). As shown in Figure 2C, only the wild-type protein, but not the mutant, restores hypermethylation activity. These results demonstrate clearly that 1) the inefficient hypermethylase activity is a direct result of the depletion of hTGS1 and 2) Asp695 of Tgs1 is essential for the methyltransferase reaction. Altogether, these results show directly, and for the first time, that hTGS1 is the human methyltransferase required for m_3G cap formation of U snRNAs.

Depletion of hTGS1 or SMN Leads to Loss of Canonical Cajal Bodies and Partial Localization of Coilin in Nucleoli

We next investigated possible effects of RNAi-mediated depletion of hTGS1 on the homeostasis of CBs by immunofluorescence microscopy employing an antibody specific for coilin, a marker protein for CBs. In control cells, we normally observe one to four larger CBs (Figure 3Aa). Strikingly, upon depletion of hTGS1 coilin is distributed throughout the entire nucleus in numerous smaller foci of different intensity and shape (Figure 3Ad). Counterstaining of the cells with an antibody against fibrillarin shows that coilin accumulates partially in nucleoli, where it forms more prominent structures (Figure 3A, d–f). Interestingly, the fibrillarin outside the nucleolus is also dispersed into numerous smaller foci in the absence of hTGS1 compared with control cells, where fibrillarin colocalizes with coilin in CBs (Figure 3A, b and e). Colocalization of coilin and fibrillarin can also be observed in the nucleoplasm of cells depleted of hTGS1 for some of the foci (shown in yellow in Figure 3Af), but in addition the two proteins reside in separate structures (colored green and red, respectively). This shows that depletion of hTGS1, which inhibits m^7G cap hypermethylation of U snRNAs in the cytoplasm and of snoRNAs in the nucleus, leads to loss of canonical CBs. Coilin redistributes into numerous small foci in the nucleoplasm and can be partially found in nucleoli.

To find out whether the effects on nuclear architecture observed upon depletion of hTGS1 are specific for this protein or are a general result of the interference with U snRNP biogenesis in the cytoplasm, we investigated the distribution of coilin in cells treated with siRNAs targeting SMN. Extracts from cells depleted of SMN are impaired in Sm core assembly in vitro, indicating that U snRNP maturation is indeed inhibited in cells lacking SMN (Feng *et al.*, 2005; Shpargel and Matera, 2005; Winkler *et al.*, 2005). As for hTGS1 depletion, and as shown in Figure 3A, g–i, depletion of SMN gives rise to the appearance of residual coilin-containing structures. The coilin-containing foci that are found in nucleoli are less prominent in the experiment shown, but we generally see some variation in the nucleolar pool of coilin upon depletion of hTGS1 or SMN (e.g., see Figure 4C). Similarly, we observe some heterogeneity in the number and intensity of coilin-containing nuclear bodies in individual cells (Figure 3A, d and g; see also below), but again foci containing both coilin and fibrillarin (shown in yellow in Figure 3Ai), and structures with only one of the two proteins can be found (colored green and red, respectively). However, in cells treated with siRNAs targeting SMN, there are fewer foci in which coilin and fibrillarin colocalize, compared with cells depleted of hTGS1. In summary, our experiments show that RNAi-mediated depletion of hTGS1 or SMN, which both inhibit the cytoplasmic maturation of spliceosomal U snRNPs and thus are expected to decrease reentry of U snRNA Sm cores into the nucleus, leads to loss of CBs and a partial concentration of coilin in nucleoli.

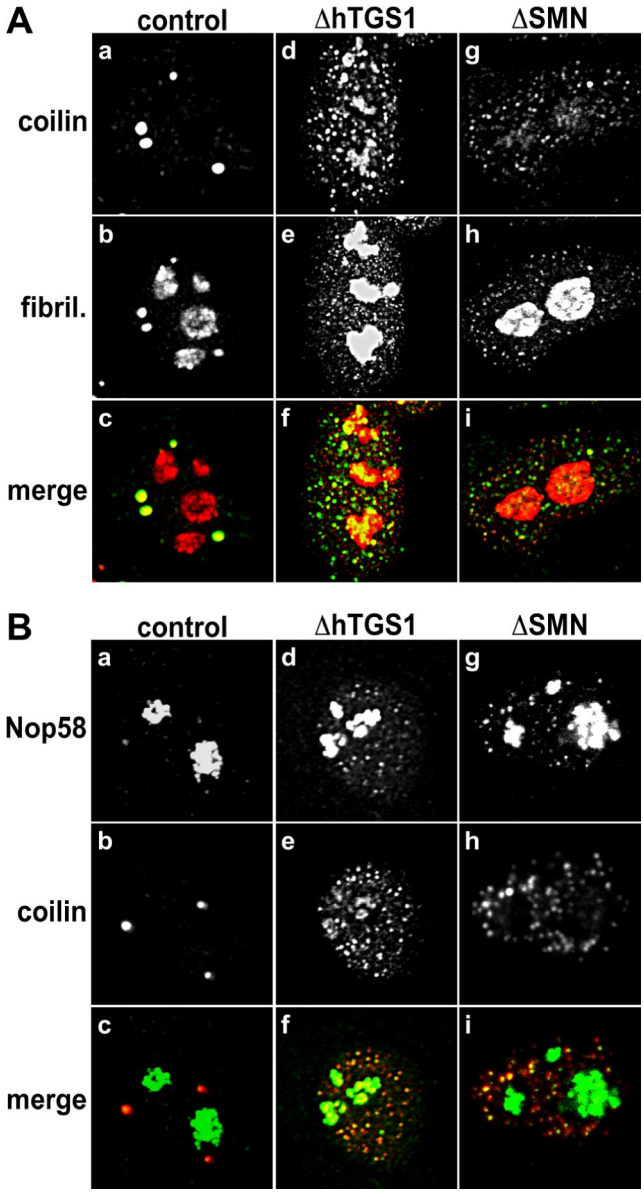

Figure 3. Depletion of hTGS1 or SMN leads to a relocalization of coilin into numerous small foci in the nucleoplasm and into nucleoli. (A) HeLa cells transfected with siRNAs targeting luciferase (control), hTGS1 (ΔhTGS1), or SMN (ΔSMN) were examined by indirect immunofluorescence. Cells were stained with antibodies against coilin (a, d, and g) and fibrillarin as a marker for nucleoli (b, e, and h). Panels c, f, and i show the merged picture of the two panels above, where coilin is shown in green and fibrillarin in red. Yellow indicates overlying signals. (B) HeLa cells as described in A were stained with antibodies against Nop58 (a, d, and g) and coilin (b, e, and h). Panels c, f, and i show the merged picture of the two panels above, where Nop58 is shown in green and coilin in red. Yellow indicates overlying signals.

Residual Nucleoplasmic Coilin-containing Foci in Cells Depleted of hTGS1 or SMN Contain snoRNP Components

We were interested in investigating the composition of the coilin-containing foci in the nucleoplasm of cells that have been depleted of hTGS1 or SMN by RNAi. As described above, we could show that fibrillarin, which we used as a marker for nucleoli, also colocalizes with coilin in a significant number of nucleoplasmic foci, but, in addition, structures that contained only coilin or fibrillarin could also be observed. Fibrillarin is a core component of mature C/D box snoRNPs. Therefore, we next investigated whether other snoRNP proteins that are usually found in CBs are distributed similarly to fibrillarin upon inhibition of U snRNP maturation. As shown in Figure 3Ba, in control cells, Nop58 is localized primarily in the nucleoli and is also found in CBs. On depletion of hTGS1 or SMN, Nop58 relocalizes in numerous small foci in the nucleoplasm (Figure 3B, d and g), as observed for fibrillarin. Counterstaining with an anti-coilin antibody reveals colocalization of Nop58 and coilin in

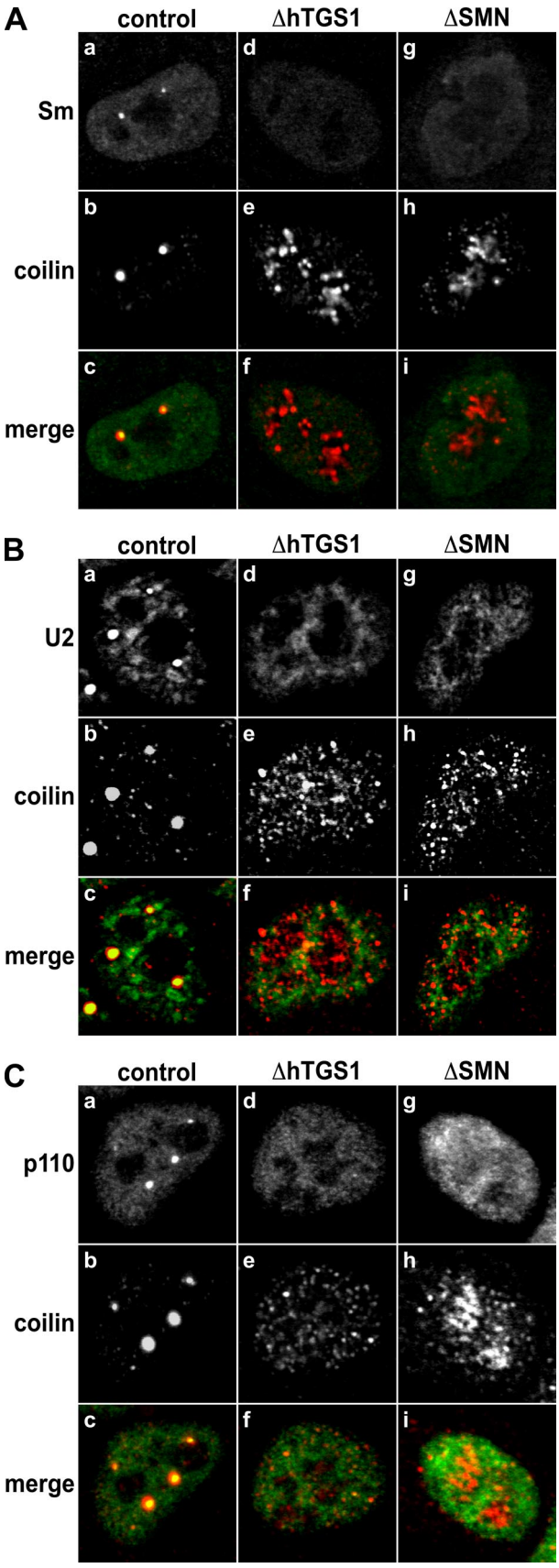

Figure 4. Sm class U snRNPs are predominantly localized in speckles and the U6 snRNP protein p110 is distributed diffusely throughout the nucleoplasm in cells depleted of hTGS1 or SMN. (A) HeLa cells transfected with siRNAs targeting luciferase (control), hTGS1 (ΔhTGS1), or SMN (ΔSMN) were examined by indirect immunofluorescence. Cells were stained with an autoimmune serum specific for Sm proteins (a, d, and g) and with an antibody against coilin (b, e, and h). Panels c, f, and i show the merged picture of the two panels above, where Sm proteins are shown in green and coilin in red. Yellow indicates overlying signals. (B) HeLa cells as described in A were examined using FISH for U2 snRNA (a, d, and g) and counterstained with an anti-coilin antibody (b, e, and h). Panels c, f, and i show the merged picture of the two panels above, where U2 snRNA is shown in green and coilin in red. Yellow indicates overlying signals. (C) HeLa cells as described in A were stained with antibodies against p110 (a, d, and g) and coilin (b, e, and h). Panels c, f, and i show the merged picture of the two panels above, where p110 is shown in green and coilin in red. Yellow indicates overlying signals.

some of the nucleoplasmic foci plus structures that contain only coilin or Nop58 (Figure 3B, f and i). Again, there are more foci with both coilin and Nop58 after knockdown of hTGS1 than of SMN, as seen for coilin and fibrillarin. Similar results were obtained upon hTGS1 or SMN depletion for the nuclear distribution of Nop56 and Nopp140 (unpublished data). Taken together, these experiments show that factors involved in snoRNP biogenesis are recruited to a subclass of residual coilin-containing bodies in knockdown cells.

Sm Class U snRNPs Do Not Colocalize with Coilin in Cells Lacking hTGS1 or SMN

Next, we investigated whether spliceosomal U snRNPs are enriched in some or all of the coilin foci in cells after knockdown of hTGS1 or SMN. Immunofluorescence studies were performed with human autoantibodies directed against the Sm proteins. To differentiate between speckles and any possible residual CB-like structures, cells were counterstained with an antibody against coilin, which is absent in speckles. In normal cells the Sm proteins are primarily distributed throughout the nucleus in the splicing speckles, and they accumulate in CBs (Figure 4Aa). In cells depleted of hTGS1 or SMN, the staining of Sm proteins characteristic for speckles can still be observed, but the concentration in discrete foci can no longer be detected (Figure 4A, d and g). Counterstaining with an antibody against coilin confirms that Sm proteins cannot be detected in the residual coilin-containing foci in knockdown cells (Figure 4A, f and i).

To ensure that the results obtained with autoantibodies against Sm proteins reflects the distribution of Sm cores assembled onto U snRNAs and not "free" Sm proteins, we also investigated the distribution of U2 snRNA after siRNA treatment. As shown in Figure 4B, U2 snRNA, normally predominantly localized in speckles and concentrated in CBs (panel a), was distributed throughout the nucleoplasm solely in speckles upon depletion of hTGS1 or SMN (panels d and g). There was no staining of U2 snRNA observed characteristic for canonical CBs or in the residual coilin foci (Figure 4B, f and i). Similar results were obtained for U4 snRNA (unpublished data). Furthermore, the U snRNP specific proteins U2-B" and U4/U6–60K, which in control cells are enriched in CBs, were also exclusively distributed in speckles throughout the nucleoplasm in cells depleted of hTGS1 or SMN (unpublished data). Altogether, these results show that there is no detectable recruitment of Sm class U snRNPs to residual coilin-containing bodies in cells lacking hTGS1 or SMN.

Immunofluorescence analysis failed to detect a significant cytoplasmic accumulation of U snRNPs in knockdown cells,

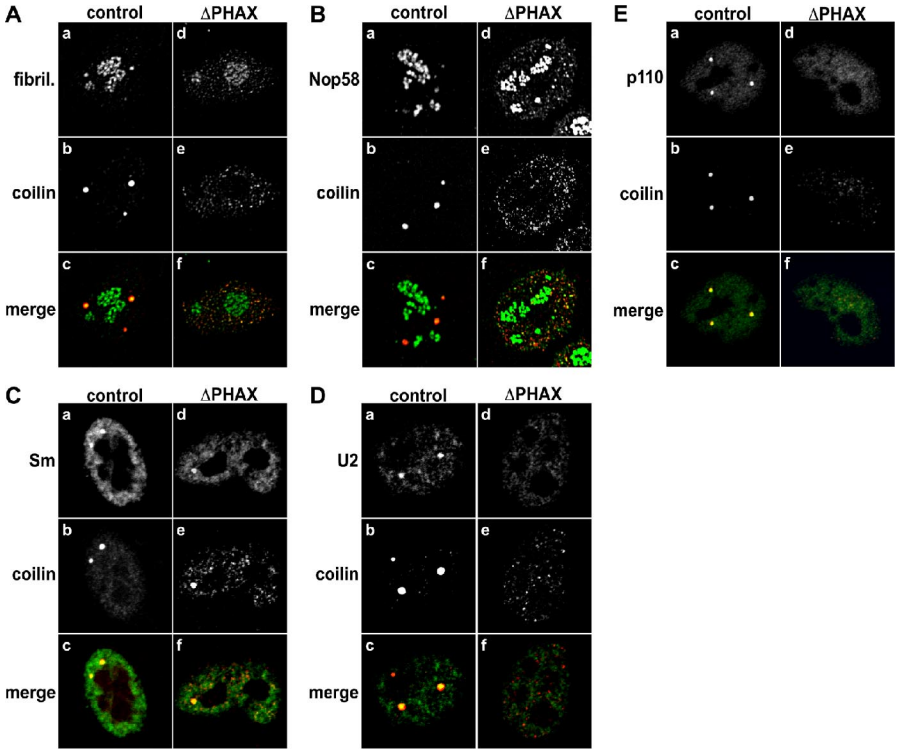

Figure 5. Depletion of PHAX leads to a distribution of U snRNPs and snoRNP components comparable to knockdown of hTGS1 or SMN. (A–E) HeLa cells transfected with siRNAs targeting luciferase (control) or PHAX (ΔPHAX) were examined by using indirect immunofluorescence or FISH. Cells were stained with an antibody against fibrillarin (A, a and d), an antibody against Nop58 (B, a and d), an autoimmune serum specific for Sm proteins (C, a and d), an oligonucleotide against U2 snRNA (D, a and d), an antibody against p110 (E, a and d), and an antibody against coilin (A–E, b and e). Panels c and f show the merged picture of the two panels above, where fibrillarin, Nop58, Sm proteins, U2 snRNA, or p110 are shown in green and coilin in red. Yellow indicates overlying signals.

as might be expected if unprocessed U snRNAs and Sm proteins are trapped in the cytoplasm after depletion of hTGS1 or SMN (Figure 4, A and B). Similarly, we could not observe a clear difference in the distribution of spliceosomal U snRNPs in nuclear and cytoplasmic extracts from cells lacking hTGS1 or SMN, when these were compared with control cells (unpublished data). The most likely explanation for this is that the high level and metabolic stability of mature U snRNPs in the cells before siRNA treatment obscure the detection of small amounts of newly assembled premature U snRNA Sm cores. In addition, the unprocessed cytoplasmic U snRNAs may be recognized by the cells as dysfunctional and then be degraded, in a process similar to other surveillance mechanisms that ensure that only functional RNAs are expressed and processed (Fasken and Corbett, 2005). Interestingly, Shpargel and Matera (2005) showed that newly synthesized Sm proteins still enter the nucleus in cells depleted of SMN, which the authors believe is due to a nuclear location property of the basic C-terminal domains of some of the Sm proteins (Girard *et al.*, 2004). However, the newly synthesized Sm proteins are not assembled onto U snRNAs, consistent with an inhibition of U snRNP maturation before the nuclear import of U snRNA Sm cores in cells depleted of SMN.

The U6 snRNP Is Absent from Coilin Foci in Cells Depleted of hTGS1 or SMN

The U6 snRNP differs from the other spliceosomal U snRNPs in that its RNA is transcribed by RNA polymerase III, thereby obtaining a different 5′ cap, and in that it lacks the Sm proteins, instead containing a set of seven Sm-like (LSm) proteins, numbered LSm2-8 (Will and Lührmann, 2001). Beside the LSm proteins, the U6 snRNP also contains the protein p110, which is involved in both the biogenesis and the recycling of U snRNPs by promoting the assembly of the U4/U6 snRNP and which is accumulated in CBs (Bell *et al.*, 2002; Stanek *et al.*, 2003; Schaffert *et al.*, 2004). Thus, we investigated the localization of p110 in HeLa cells treated with siRNAs targeting hTGS1 or SMN. Like the Sm class U snRNP components, p110 becomes distributed diffusely throughout the nucleoplasm upon depletion of hTGS1 or SMN and cannot be detected in the residual coilin-containing foci (Figure 4C). The same result was observed for U6 snRNA (unpublished data), indicating that all spliceosomal U snRNPs are absent from the residual coilin-containing foci in cells impaired in the biogenesis of Sm class U snRNPs.

RNAi-mediated Depletion of PHAX Also Leads to a Redistribution of Coilin, U snRNPs, and snoRNP Components in the Nucleus

Given that both hTGS1 and SMN are required for the maturation of U snRNPs in the cytoplasm, it seems likely that the interference with this process is responsible for the effects observed on CB integrity and coilin distribution when the cell is depleted of hTGS1 or SMN. To provide additional evidence for this, we blocked U snRNP biogenesis before the export of newly synthesized U snRNAs from the nucleus to the cytoplasm, by knockdown of PHAX, the export adaptor specific for U snRNAs (Ohno *et al.*, 2000). RNAi-mediated depletion of PHAX was carried out essentially as previously described, whereby PHAX protein is reduced by more than 95% (Watkins *et al.*, 2004). Immunofluorescence analysis of PHAX-depleted cells demonstrates that coilin is again dispersed in numerous small nucleoplasmic foci (Figure 5), but no relocalization of coilin to nucleoli can be observed. As is the case when cells are depleted of hTGS1 or SMN, fibrillarin and Nop58 can be found in some of the nuclear bodies defined by coilin, but coilin and snoRNP components can also be found in separate structures in the nucleoplasm (Figure 5, A and B). Furthermore, the coilin-containing nuclear bodies again lack Sm class U snRNP components, as well as the U6 snRNP protein p110 (Figure 5, C–E). Altogether, the reorganization of coilin, U snRNPs, and snoRNP components observed after knockdown of PHAX further

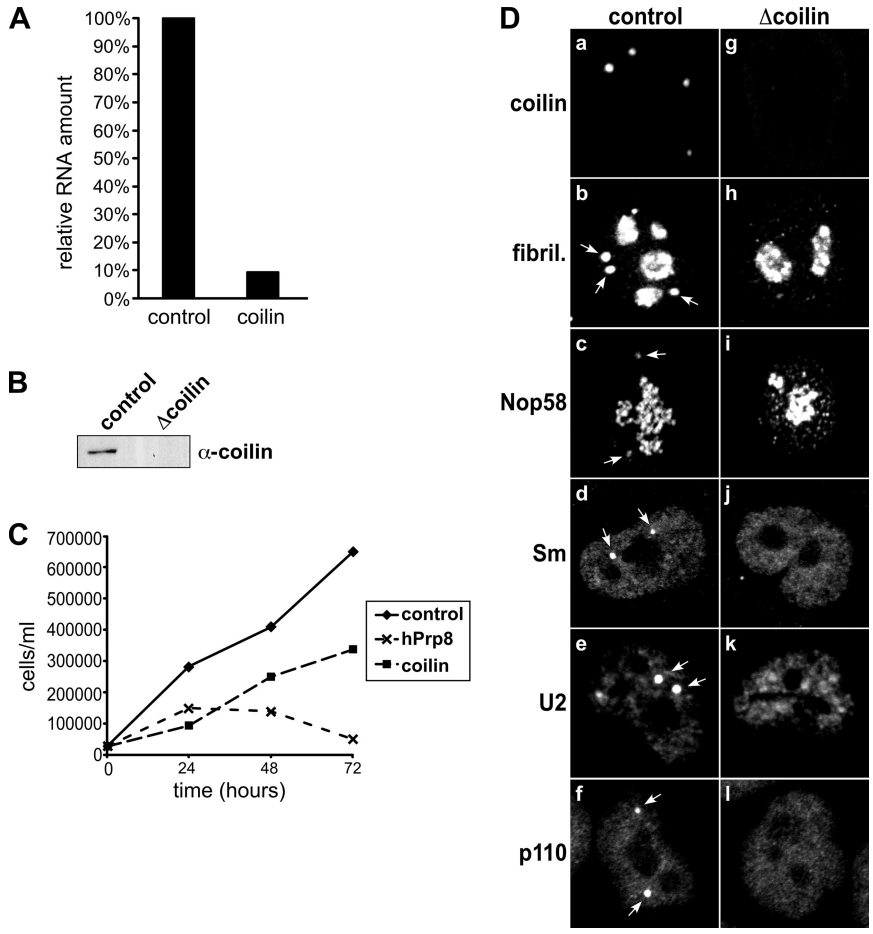

Figure 6. snoRNP components, but not spliceosomal U snRNPs, form detectable foci in coilin-depleted cells. (A) Specific RNAi-mediated depletion of coilin mRNA revealed by real-time RT-PCR analysis. HeLa cells were transfected with either the luciferase control siRNAs or with siRNAs targeting coilin. After 24 h cells were harvested, total RNA was isolated, and real-time RT-PCRs were performed. The diagram shows the relative level of the coilin mRNA compared with cells transfected with control siRNAs (set to 100%). (B) Knockdown of coilin protein by RNAi (Δcoilin). Cells were treated as in A and harvested 72 h after transfection, and the level of coilin protein analyzed by immunoblotting (α-coilin). (C) Cell survival after siRNA transfection. HeLa cells were harvested 24, 48, and 72 h after siRNA transfection and counted. The graphs show the growth curve of HeLa cells depleted of coilin. For comparison the growth rate of HeLa cells transfected with siRNAs targeting the nonhuman protein luciferase (control) and the essential splicing factor hPrp8 were determined in parallel. (D) HeLa cells transfected with siRNAs targeting luciferase (control) or coilin (Δcoilin) were examined using indirect immunofluorescence or FISH. Cells were stained with antibodies against coilin (a and g), fibrillarin (b and h), or Nop58 (c and i), with an autoimmune serum specific for Sm proteins (d and j), with an oligonucleotide against U2 snRNA (e and k), or with an antibody against p110 (f and l). In panels b–f, CBs are indicated with an arrow.

supports the idea that inhibition of U snRNP maturation leads to loss of CBs.

U snRNPs Fail to Form Prominent Foci after siRNA-induced Depletion of Coilin, whereas snoRNP Components Still Aggregate into Nucleoplasmic Dots

It has previously been proposed that coilin forms a platform upon which the other CB components assemble. Our results described above show that coilin fails to form structures of a size comparable to that of canonical CBs upon RNAi-induced depletion of hTGS1, SMN, or PHAX, indicating that the presence of U snRNA Sm cores is essential for CB formation. To investigate the requirement of coilin for the formation of nuclear bodies containing U snRNPs and snoRNP components, we examined the effect of RNAi-mediated depletion of coilin on the nuclear distribution of CB constituents in HeLa cells. As shown by real-time RT-PCR and immunoblot analysis (Figure 6, A and B), the coilin mRNA level decreased to <10% after 24 h of siRNA treatment, in comparison with control cells, and coilin protein levels decreased to below the detection limit after 72 h. HeLa cells depleted of coilin grow slightly more slowly than control cells, but are clearly viable (Figure 6C). Immunofluorescence with antibodies against fibrillarin and Nop58 demonstrates that these proteins still accumulate in small foci in the nucleoplasm in the absence of coilin (Figure 6D, a, b, f, and g), although the number of the snoRNP foci is reduced compared with cells depleted of hTGS1, SMN, or PHAX. The presence of coilin-independent snoRNP foci is consistent with the observation that fibrillarin and Nop58 form detectable nuclear bodies lacking coilin upon RNAi-mediated depletion of hTGS1, SMN, or PHAX (see above). For the spliceosomal U snRNPs, immunofluorescence studies of coilin-depleted cells show that the Sm proteins as well as U2 snRNA are distributed in the nucleoplasm in speckles without any clear enrichment in distinct foci (Figure 6D, c, d, h, and i). They also show that the U6 snRNP protein p110 is diffusely distributed throughout the nucleoplasm of cells lacking coilin (Figure 6D, e and j). Together, these data show that the condensation of U snRNPs into defined nuclear bodies is dependent on coilin, demonstrating that U snRNPs and snoRNP components differ in their requirement regarding coilin for the formation of detectable nuclear bodies.

DISCUSSION

Inhibition of U snRNP Maturation Leads to Disappearance of CBs and Loss of Association of Spliceosomal U snRNPs with Residual Coilin-containing Nuclear Bodies

CBs are prominent nuclear bodies found in vertebrates, invertebrates, and plants; however, the molecular requirements for the formation and stability of CBs are only poorly understood. In this work we have used fluorescence microscopy to determine the nuclear distribution of coilin, spliceosomal U snRNPs, and proteins found in C/D box snoRNPs—all usually concentrated in CBs—upon inhibition of the biogenesis of spliceosomal U snRNPs by RNAi-mediated

depletion of hTGS1, SMN, or PHAX. Knockdown of any one of these three proteins is expected to interfere with Sm class U snRNP maturation before reentry of U snRNA Sm cores into the nucleus.

Our most striking observation is that coilin, the marker protein for CBs, is dispersed in the nucleoplasm into numerous small foci upon knockdown of hTGS1, SMN, or PHAX. This indicates that CBs are lost when the biogenesis of Sm class U snRNPs is blocked, suggesting that premature U snRNA Sm cores and their ongoing processing in the nucleus are essential for the integrity of CBs. This view is supported by our findings that in the depleted cells, the spliceosomal U snRNPs found in the nucleus, which most probably represent the steady state population of mature U snRNPs, are distributed without concentration in any prominent foci in the nucleoplasm. It is also consistent with the fact that we could not observe a significant drop in the level of mature U snRNPs in the nuclei of depleted cells. In agreement with our results, Shpargel and Matera (2005) also reported the disappearance of CBs upon depletion of SMN.

Microinjection experiments in somatic cells have shown that the m_3G cap, although not being absolutely required for the nuclear import of U snRNAs, accelerates their transport, indicating that the m_3G cap has retained a signaling role for nuclear targeting of U snRNPs (Fischer *et al.,* 1994, Marshallsay and Lührmann, 1994). Our experiments show that the rate of U snRNP import in cells depleted of hTGS1 is sufficient for the cells to grow comparably to control cells, but, interestingly, the effect on CB integrity is indistinguishable from that of RNAi-mediated knockdown of SMN or PHAX, which is expected to efficiently block reentry of U snRNA Sm cores into the nucleus. One reason for this could be that the import of endogenous U snRNA Sm cores without m_3G caps is lower than implied by the microinjection experiments, because some of the U snRNA Sm cores with m^7G caps in the cytoplasm are identified as incorrect before their import into the nucleus and then degraded, as discussed earlier. Another possible reason may be that a certain level of U snRNA Sm cores is required for CB formation, and the amount of imported U snRNA Sm cores falls below this threshold in the absence of hTGS1. It is also possible that the m_3G cap, in addition to having a role in nuclear import, is also important to target U snRNA Sm cores to CBs. Finally, we cannot absolutely exclude the possibility that hTGS1 itself is important for the integrity of CBs, because hTGS1 is found in the cytoplasm and also localized in CBs (Mouaikel *et al.,* 2003). However, we regard this as highly unlikely, because the common principle in depleting hTGS1, SMN, or PHAX, which in all cases leads to loss of CBs, is the interference with U snRNP maturation before the transport of U snRNA Sm cores into the nucleus.

Our results showing an interrelationship between U snRNPs production and CB formation are further supported by the following observations. 1) The number and intensity of coilin-containing structures is decreased when Sm proteins are immunodepleted in in vitro–formed pronuclei from *Xenopus* egg extract (Bauer and Gall, 1997). 2) A loss of CBs is observed upon treatment of cells with leptomycin B (Carvalho *et al.,* 1999; Sleeman *et al.,* 2001), a general inhibitor of the CRM1 pathway exporting, among others, the newly transcribed U snRNAs to the cytoplasm (Cullen, 2003). 3) No CBs can be observed in cells lacking the nuclear import adaptor snurportin1, which recognizes the m_3G cap of U snRNA Sm cores (Huber *et al.,* 1998; Shpargel and Matera, 2005). 4) Transient overexpression of Sm proteins, which will increase the rate of U snRNPs in the nucleus, causes a corresponding transient appearance of CBs in primary cells usually devoid of these nuclear bodies (Sleeman *et al.,* 2001).

Residual Nuclear Bodies Containing Coilin and snoRNP Components

Although spliceosomal U snRNPs are clearly absent from coilin-containing nuclear bodies in cells depleted of hTGS1, SMN, or PHAX, our results show that common C/D box snoRNP components (fibrillarin, Nop56, and Nop58) and the snoRNP biogenesis factor Nopp140 still associate with coilin in the nucleoplasm and thereby form CB-like structures, albeit of smaller size. In addition, coilin-containing nucleoplasmic bodies of similar size but lacking snoRNP components can also be observed in depleted cells, demonstrating that at least two types of coilin-containing nuclear bodies arise when U snRNP maturation is inhibited. Given that depletion of hTGS1, SMN, or PHAX inhibits snoRNP maturation in the nucleus (Boulon *et al.,* 2004; Watkins *et al.,* 2004), our results further demonstrate that association of snoRNP components to detectable nuclear bodies is not dependent on active snoRNP biogenesis. Most snoRNP proteins are also components of scaRNPs, which guide post-transcriptional modifications of spliceosomal U snRNAs and which are structurally related to snoRNPs, but are found exclusively in CBs and absent from nucleoli (Darzacq *et al.,* 2002; Kiss *et al.,* 2002; Jády *et al.,* 2003). Therefore, the detection of fibrillarin, Nop56, and Nop58 in coilin-containing nuclear bodies could in part reflect the presence of scaRNPs in these structures. However, scaRNP biogenesis is also expected to be blocked in cells depleted of SMN or PHAX, and thus, the recruitment of fibrillarin, Nop56, and Nop58 as part of scaRNPs to the residual coilin-containing foci would similarly be independent of ongoing biogenesis. Altogether, our data suggest that the different behavior observed for spliceosomal U snRNPs and snoRNPs reflects a change in the stable association of these proteins with CBs after inhibition of U snRNP maturation. Spliceosomal U snRNPs depart from CBs in the absence of hTGS1, SMN, or PHAX and do not form detectable separate nuclear bodies. Without U snRNPs, CBs collapse, but snoRNP components can still associate with coilin, albeit in smaller nuclear structures.

Do Canonical CBs Have a Modular Structure with Distinct Domains for Different Activities?

Numerous small coilin-containing nuclear bodies are present in cells depleted of hTGS1, SMN, or PHAX, some containing snoRNP components and others being devoid of these proteins but probably containing distinct sets of factors. This suggests that canonical CBs have a modular structure with separate entities containing either spliceosomal U snRNPs, or snoRNPs, or other genuine CB components like the U7 snRNP or basal transcription factors. Under normal conditions, these multiple entities are held together and thereby form CBs. One factor required for the integrity of canonical CBs is coilin, as demonstrated in this study by the disappearance of CBs upon RNAi-mediated depletion of coilin. Consistently, MEF cells that have only the capability to express a short N-terminal fragment of coilin do not possess nuclear structures enriched in U snRNAs and associated proteins (Tucker *et al.,* 2001). Furthermore, it was shown that spliceosomal U snRNPs do not become concentrated in foci in pronuclei formed in vitro upon immunodepletion of coilin from *Xenopus* egg extract, and it was even suggested that CBs are aggregate structures composed of components of different RNA-processing systems (Bauer and Gall, 1997). Our results support this model and demonstrate moreover that U snRNPs and snoRNP components differ in their de-

pendence on coilin for the formation of detectable nuclear bodies. Indeed, we could show that snoRNP components still become concentrated in nuclear bodies after RNAi-induced depletion of coilin. A similar observation has been made in the above-mentioned coilin$^{-/-}$ MEF cells (Tucker *et al.*, 2001) and in *Xenopus* pronuclei formed in vitro (Bauer and Gall, 1997). However, in MEF cells the snoRNP-containing structures, called residual CBs, have a size and shape similar to those of canonical CBs, whereas the snoRNP foci in knockdown HeLa cells are smaller, but are present in larger number than CBs in control cells. These discrepancies may be due to the inhibition of snoRNP biogenesis upon RNAi-mediated depletion of hTGS1, SMN, or PHAX (Boulon *et al.*, 2004; Watkins *et al.*, 2004), or they could simply reflect differences between the two cell types (MEF vs. HeLa cells). It is also possible that the short N-terminal fragment of coilin expressed in the MEF cells, which is able to self-associate (Hebert and Matera, 2000), is responsible for the larger snoRNP foci.

CBs have a twin structure called gems for "gemini of CBs," which are defined by the presence of the SMN protein (Liu and Dreyfuss 1996). In many cell types these two substructures colocalize, but CBs and gems appear as distinct nuclear bodies in some cell lines and in many fetal tissues (Gall 2000; Cioce and Lamond 2005). Interestingly, although CBs disappear when the cells are depleted of hTGS1, PHAX, or coilin, we can still observe gems in these knockdown cells based on immunofluorescence staining for SMN (unpublished results). This indicates that formation and stability of gems are independent of CBs, further supporting the structural separability of these two substructures.

Our results demonstrate that active U snRNP biogenesis in the nucleus is essential for the formation of CBs. Interestingly, inhibition of snoRNP biogenesis by depleting fibrillarin, Nop56, Nop58, or Nopp140 does not change the appearance of canonical CBs in HeLa cells (unpublished results). This indicates either that the snoRNP domains in CBs might be minor compared with U snRNP modules or that snoRNP components still associate with and thereby stabilize CBs even in the absence of one of these proteins. In addition to having a role in U snRNP and snoRNP biogenesis, CBs have also been implicated in the recycling of U snRNPs during pre-mRNA splicing (Stanek *et al.*, 2003; Schaffert *et al.*, 2004). However, U snRNP recycling apparently does not contribute significantly to the formation and/or stability of CBs, because no U snRNP-containing nuclear bodies reminiscent of CBs can be observed in cells depleted of hTGS1 or PHAX, in which recycling should not be affected. Altogether, these observations argue that U snRNP maturation is the crucial activity required for the integrity of CBs in HeLa cells.

The results reported in this study support the following model for CB formation. First, the U snRNA Sm cores interact with coilin after their transport into the nucleus. Next, the recruitment of additional factors involved in the maturation of U snRNPs together with the self-oligomerization of coilin allow the formation of U snRNP subdomains of CBs. Finally, the association of other CB subdomains, e.g., containing snoRNP components and biogenesis factors, leads to the formation of canonical CBs. The whole process seems to be self-organized, as previously proposed for the formation of nucleoli (Misteli, 2001). The spatial organization of several RNA-processing activities, such as U snRNP and snoRNP maturation, into a single compartment may be helpful for the efficient processing of these RNPs, especially in light of the fact that U snRNP and snoRNP biogenesis is partially dependent on the same set of factors. However, this is not essential for cell growth, because cells without coilin, the absence of which leads to loss of CBs, are viable, indicating that U snRNP biogenesis and other processes usually occurring in CBs are not critically affected.

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