

# Residual Cajal bodies in coilin knockout mice fail to recruit Sm snRNPs and SMN, the spinal muscular atrophy gene product

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Cajal bodies (CBs) are nuclear suborganelles involved in the biogenesis of small nuclear ribonucleoproteins (snRNPs). In addition to snRNPs, they are highly enriched in basal transcription and cell cycle factors, the nucleolar proteins fibrillarin (Fb) and Nopp140 (Nopp), the survival motor neuron (SMN) protein complex, and the CB marker protein, p80 coilin. We report the generation of knockout mice lacking the COOH-terminal 487 amino acids of coilin. Northern and Western blot analyses demonstrate that we have successfully removed the full-length coilin protein from the knockout animals. Some homozygous mutant animals are viable, but their numbers are

reduced significantly when crossed to inbred backgrounds. Analysis of tissues and cell lines from mutant animals reveals the presence of extranucleolar foci that contain Fb and Nopp but not other typical nucleolar markers. These so-called “residual” CBs neither condense Sm proteins nor recruit members of the SMN protein complex. Transient expression of wild-type mouse coilin in knockout cells results in formation of CBs and restores these missing epitopes. Our data demonstrate that full-length coilin is essential for proper formation and/or maintenance of CBs and that recruitment of snRNP and SMN complex proteins to these nuclear subdomains requires sequences within the coilin COOH terminus.

## Introduction

The Cajal body (CB)\* was originally identified by Santiago Ramón y Cajal in vertebrate brain cells (Cajal, 1903). The structure was noteworthy for two reasons. The first was that, like the nucleolus, CBs are very argyrophilic. Upon silver staining, they become one of the most prominent structures in neuronal nuclei (Lafarga and Hervas, 1983; Lafarga et al., 1991). The second reason was a frequent physical proximity to nucleoli (Lamond and Carmo-Fonseca, 1993). In light of this morphological similarity and physical proximity, Cajal originally termed this structure the nucleolar “accessory

body.” In the near century that has transpired since their discovery, CBs have been identified in numerous species, including plants, insects, amphibians, and mammals (Gall, 1954; Hardin et al., 1969; Beven et al., 1995; Gall et al., 1995). However, understanding of CB function has lagged far behind the ability to identify this nuclear suborganelle.

Initial attempts to gain insight into CB function began with its molecular characterization, initiated over a decade ago (Andrade et al., 1991, 1993; Raska et al., 1991; Carmo-Fonseca et al., 1992; Matera and Ward, 1993). Although earlier studies gave important hints (Eliceiri and Rysere, 1984; Fakan et al., 1984), these later results demonstrated that high levels of small nuclear ribonucleoproteins (snRNPs) concentrate in CBs. Despite the fact that many of these snRNPs are involved in pre-mRNA splicing, the lack of poly A<sup>+</sup> RNA and serine-arginine proteins strongly suggests that splicing does not take place in CBs (Matera, 1999a). In fact, components of three major RNA processing pathways (rRNA, mRNA, and histone RNA) can be found in this nuclear compartment, suggesting a possible role for

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\*Abbreviations used in this paper: CB, Cajal body; ES, embryonic stem; Fb, fibrillarin; GFP, green fluorescent protein; Nopp, Nopp140; mAb, monoclonal antibody; MEF, mouse embryonic fibroblast; SMA, spinal muscular atrophy; SMN, survival motor neuron; snRNP, small nuclear ribonucleoprotein.

Key words: coilin; SMN; SMA; snRNPs; nuclear organization

CBs in preassembly and targeting of transcription complexes to their sites of action (for review see Gall, 2000). Recent data demonstrate that the CBs in adult animals and most cell lines are highly enriched in the survival motor neuron (SMN) protein (Liu and Dreyfuss, 1996; Matera and Frey, 1998; Carvalho et al., 1999; Young et al., 2000, 2001), although in fetal tissues SMN is typically localized independently in twin structures called “Gemini of coiled bodies” or gems (Liu and Dreyfuss, 1996; Young et al., 2001). Dreyfuss and colleagues have shown that SMN complexed with several other proteins is involved in the process of snRNP biogenesis (Fischer et al., 1997; Liu et al., 1997; Charroux et al., 1999, 2000; Pellizzoni et al., 1999). Interestingly, mutations in the *SMN1* gene in humans results in the autosomal recessive disease spinal muscular atrophy (SMA; Lefebvre et al., 1995), and deletion of the *Smn* gene from the mouse genome results in embryonic lethality (Schrank et al., 1997).

Other attempts to decipher CB function have involved perturbation of CB components, most notably p80 coilin. To date, the coilin protein is the only unambiguous marker for CBs. First identified from autoimmune patient sera in the early 1990s, the coilin protein is evident in both CBs and diffusely throughout the nucleoplasm (Andrade et al., 1991; Raska et al., 1991). Alignment of the protein from several species demonstrates a high degree of conservation at both the NH<sub>2</sub> and COOH termini, separated by a divergent central region (Tuma et al., 1993; Chan et al., 1994; Tucker et al., 2000). The only readily recognizable motifs are two nuclear localization signals (Tuma et al., 1993; Chan et al., 1994; Wu et al., 1994; Bohmann et al., 1995; Tucker et al., 2000). Mutational analysis of the coilin protein emphasizes the intimate relationship between CBs and nucleoli. Numerous truncations and point mutations of the protein result in redistribution of coilin to nucleoli or to the nucleolar periphery (Bohmann et al., 1995; Lyon et al., 1997; Hebert and Matera, 2000). Notably, treatment of cells with the phosphatase inhibitor Okadaic acid results in the relocation of not only coilin but also the splicing snRNPs from CBs to nucleoli (Lyon et al., 1997). Furthermore, our lab has reported recently the identification of a cryptic nucleolar localization signal within human coilin (Hebert and Matera, 2000). Coupled with data from Meier and colleagues demonstrating an interaction between coilin and the nucleolar protein Nopp140 (Nopp) both in vivo and in a yeast two-hybrid system (Isaac et al., 1998), it is not difficult to imagine a nucleolar phase in the life cycle of the coilin protein.

Although coilin is expressed in very early embryos (Ferreira and Carmo-Fonseca, 1995) and in all adult tissues (Chan et al., 1994; Tucker et al., 2000), CBs are not present in every tissue type (Young et al., 2000). Injection of coilin monoclonal antibodies (mAbs) into the nuclei or cytoplasm of HeLa cells resulted in the progressive disappearance of coilin foci (Almeida et al., 1998). Cells lacking CBs (as monitored by coilin mAbs; see Discussion) remained viable and competent to divide. Furthermore, the subnuclear localization of snRNPs in speckles and the overall nucleolar architecture appeared unaffected in antibody-injected cells (Almeida et al., 1998). Injected cells also retained the ability to splice viral RNA (Almeida et al., 1998).

To elucidate the role of coilin within CBs, Bauer and Gall (1997) took advantage of an experimental system, which al-

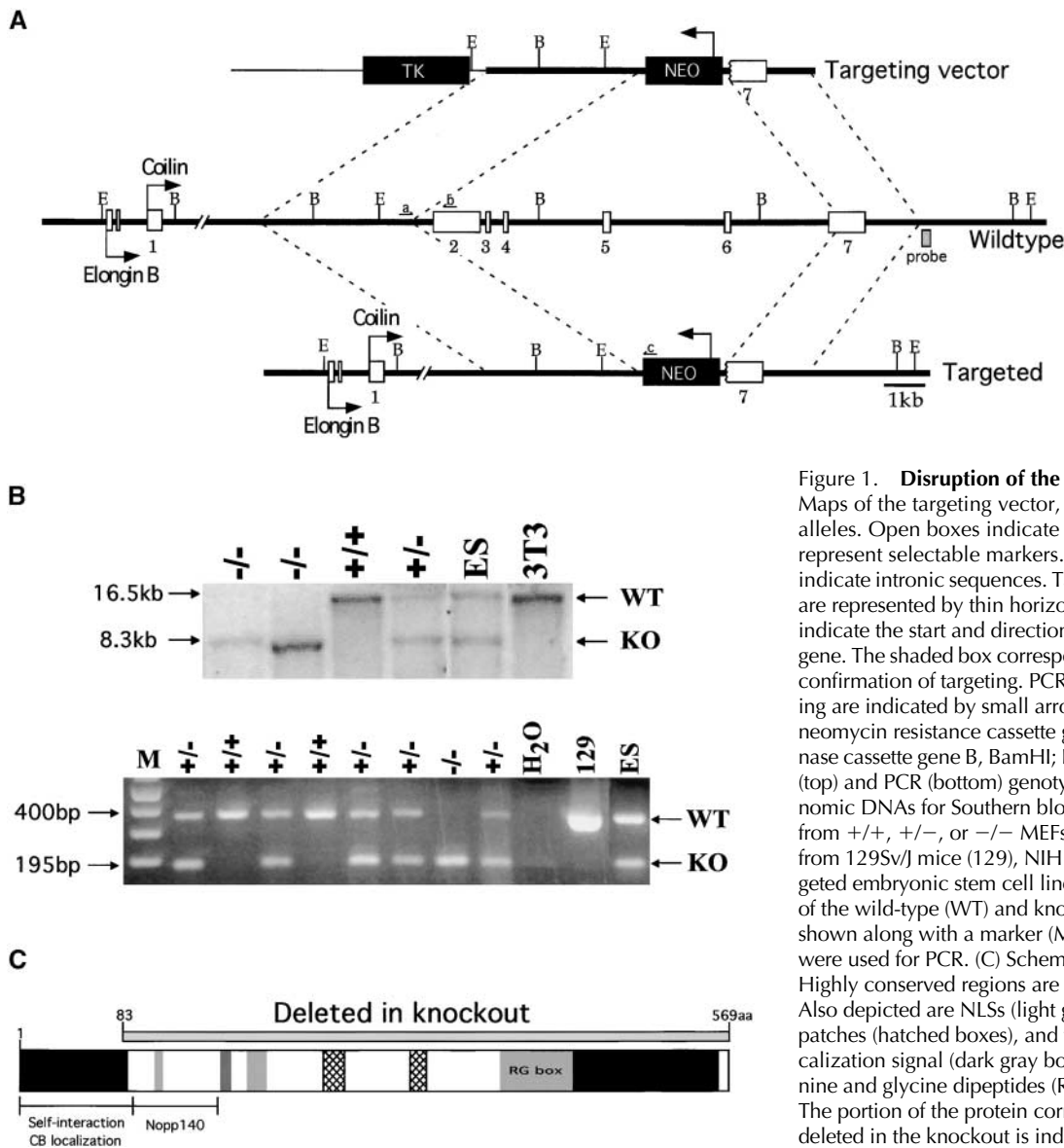
lows for the controlled formation of pronuclei in vitro. In this system, nuclei were assembled in vitro upon the addition of demembrated sperm heads to *Xenopus* egg extract. Nuclei assembled in this manner are devoid of nucleoli and “speckles” but contain nuclear bodies, which concentrate coilin, splicing snRNPs, fibrillarin (Fb), and several other CB and/or nucleolar epitopes (Bell et al., 1992; Bauer et al., 1994; Bauer and Gall, 1997). Coilin-immunodepleted extracts remained competent to form structures morphologically similar to the CBs formed with untreated egg extract. However, the structures thus formed not only lacked coilin but also failed to recruit splicing snRNPs. Similarly, when extracts were depleted of snRNPs by incubation with an antibody against Sm proteins CB-like structures formed but were devoid of coilin (Bauer and Gall, 1997). Unfortunately, addback experiments failed to reconstitute wild-type structures, revealing the need for a cleaner genetic system. These data suggest an interaction between coilin and Sm snRNPs and further indicate that these factors may not be essential for CB formation.

Here, we report the generation of a genetic model system to study coilin and CB function. Mice containing a deletion of 85% of the coilin protein, including exon 2 through the translational stop in exon 7, show reduced viability. Otherwise, homozygous mutant animals appear normal. We demonstrate that mutant tissues and cell lines derived from mutant embryos retain extranucleolar foci of similar size and shape to CBs. However, these “residual” CBs fail to concentrate splicing snRNPs or members of the SMN complex. Transient expression of wild-type mouse coilin in the knockout cell lines results in the formation of two different kinds of coilin-positive foci: (a) structures that appear to be fully intact CBs, capable of recruiting both Sm snRNPs and the SMN complex, and (b) a more diffuse accumulation of the coilin protein, which apparently lacks all other CB epitopes tested. Our data reveal that full-length coilin, although not required for nucleation of these extranucleolar foci (residual CBs), is a key player in the recruitment of SMN and the splicing snRNPs to the CB.

## Results

### Generation of the mutation

We have described previously the cloning and characterization of the mouse coilin cDNA and genomic locus (Tucker et al., 2000). Using these resources, we designed a targeting vector comprised of 129Sv/J genomic DNA to delete 85% of the coilin gene’s coding sequence. This 10-kb deletion encompasses exon 2 through the stop codon in exon 7 (Fig. 1 A) and removes coilin’s nuclear localization signals, several serine patches, and the highly conserved COOH terminus (Fig. 1 C; Hebert and Matera, 2000; Tucker et al., 2000). Chimeras were mated to CD-1, C57Bl/6J, and 129Sv/J females in order to establish lines on both outbred and inbred backgrounds. F1 heterozygotes were intercrossed to generate animals homozygous for the deletion. Genotypes of the resultant animals were determined by either Southern blot or PCR analysis (Fig. 1 B). Our data demonstrate that animals homozygous for the coilin mutation are both viable and fer-



**Figure 1. Disruption of the murine coilin locus.** (A) Maps of the targeting vector, wild-type, and targeted alleles. Open boxes indicate exons, and filled boxes represent selectable markers. Thick horizontal lines indicate intronic sequences. Targeting vector sequences are represented by thin horizontal lines. Large arrows indicate the start and direction of transcription for each gene. The shaded box corresponds to the probe used for confirmation of targeting. PCR primers for use in screening are indicated by small arrows (a, b, and c). NEO, neomycin resistance cassette gene; TK, thymidine kinase cassette gene; B, BamHI; E, EcoRI. (B) Southern blot (top) and PCR (bottom) genotyping of progeny. Genomic DNAs for Southern blot analysis were obtained from +/+, +/-, or -/- MEFs. Control DNAs were from 129SvJ mice (129), NIH 3T3 cells (3T3), or the targeted embryonic stem cell line 3G8 (ES). The positions of the wild-type (WT) and knockout (KO) alleles are shown along with a marker (M). Primers shown in A were used for PCR. (C) Schematic of the coilin protein. Highly conserved regions are indicated as black boxes. Also depicted are NLSs (light gray boxes), acidic patches (hatched boxes), and the putative nucleolar localization signal (dark gray box). A region rich in arginine and glycine dipeptides (RG box) is also indicated. The portion of the protein corresponding to the region deleted in the knockout is indicated by the bar.

tile (unpublished data) but show a reduction in viability on inbred backgrounds (Table I). Otherwise, the homozygous knockout animals cannot be overtly distinguished from their wild-type littermates. Whereas a more detailed examination of the organismal phenotypes will be presented elsewhere, this study is focused on cellular phenotypes.

### Expression analysis

To confirm that the wild-type coilin gene is not transcribed in our mutant animals, we conducted Northern blot analysis on polyadenylated RNAs extracted from the brains of wild-type, heterozygous, and homozygous mutant animals. Hybridization with probes from within the deletion interval (corresponding to exons 2 or 3–6) revealed an appropriately sized band of 2.6 kb (Tucker et al., 2000) in both the wild-type and heterozygote lanes and no band at all in the lane corresponding to the mutant animal (Fig. 2 A). However, when comparable blots were hybridized with probes corresponding to exon 1 (Fig. 2 A) or exon 7 (unpublished data) we observed apparently identical bands in all three lanes, regardless of genotype.

Because the first exon and most of the 3' untranslated region in exon 7 are retained in the knockout animals (Fig. 1 A), exon 1 may have spliced to somewhere within the neomycin resistance cassette, resulting in a hybrid RNA (Fig. 2 B). To test this hypothesis, wild-type and mutant coilin alleles were amplified from poly A<sup>+</sup> RNA by reverse transcriptase PCR. Digestion of the wild-type PCR products with HindIII resulted in the two predicted bands (1.3 and 0.9 kb), whereas the knockout product remained completely uncut (Fig. 2 B). Sequencing of PCR products from homozygous mutant animals confirmed that sequences within the neomycin resistance gene are present in the knockout transcript and identified the cryptic splice junctions (Fig. 2 B).

Western blotting of embryonic protein with the anticoinin antibody R288 confirmed the complete absence of wild-type coilin in the homozygous mutant animals (Fig. 2 C). However, because R288 was raised against the COOH-terminal portion of the human coilin protein (approximately amino acid residues 451–576; Andrade et al., 1993) this antibody is unable to detect the putative NH<sub>2</sub>-terminal coilin fragment.

Table I. Mice lacking coilin's COOH terminus show reduced viability

Strain	Results of F1 heterozygous intercrosses			Chi-square <sup>a</sup>
	+/+	+/-	-/-	
C57B1/6J	39	93	21	~0.003
129SvJ	92	155	35	~2.5 × 10 <sup>-6</sup>
CD-1	54	110	37	~0.1 <sup>b</sup>

<sup>a</sup>P value.

<sup>b</sup>Not significant.

To determine if the mutant coilin RNA was being translated, we generated polyclonal antibodies against a coilin peptide, corresponding to amino acids 67–81 of the coilin protein. Despite the low antigenicity profile of the coilin NH<sub>2</sub> terminus (this region is rather hydrophobic), these antibodies were able to detect the peptide in ELISA assays (unpublished data). However, we have been unable to detect any specific signals either by Western blotting or immunofluorescence. To simulate the expression pattern of this putative NH<sub>2</sub>-terminal fragment, we cloned the knockout allele transcript by reverse transcriptase PCR and expressed it as a green fluorescent protein (GFP) fusion in knockout cell lines (see below).

### Knockout tissues and cells display “residual” CBs

In an effort to understand the effect of the coilin deletion on CBs, we analyzed cryosections from adult animals and mouse embryonic fibroblast (MEF) cell lines derived from day 13.5–14.5 embryos (for details see Materials and methods and Table II). Labeling of tissues and MEFs from homozygous mutant animals with anticoin antibodies again confirmed the absence of the wild-type coilin protein in these animals (Fig. 3, A and B, and Fig. 4 A). In contrast, wild-type tissues and MEFs displayed prominent CBs when stained with this antibody (Fig. 3 A and Fig. 4 A). Importantly, sensory ganglion neurons from knockout animals lacked the robust extranucleolar silver staining common to the nuclei of wild-type neurons (Fig. 3 C).

We next wanted to investigate the distribution of other known CB components in knockout cells. Fb and Nopp are snRNP proteins. In wild-type tissues and cells, they localize to both nucleoli and CBs. Double labeling of wild-type tissues with antibodies against coilin and either Fb or Nopp revealed the expected localization patterns (Fig. 3 A; unpublished data). In agreement with published results (Alliegro and Alliegro, 1998; Platani et al., 2000), we noted heterogeneous levels of these epitopes among different CBs (Fig. 3 A; unpublished data).

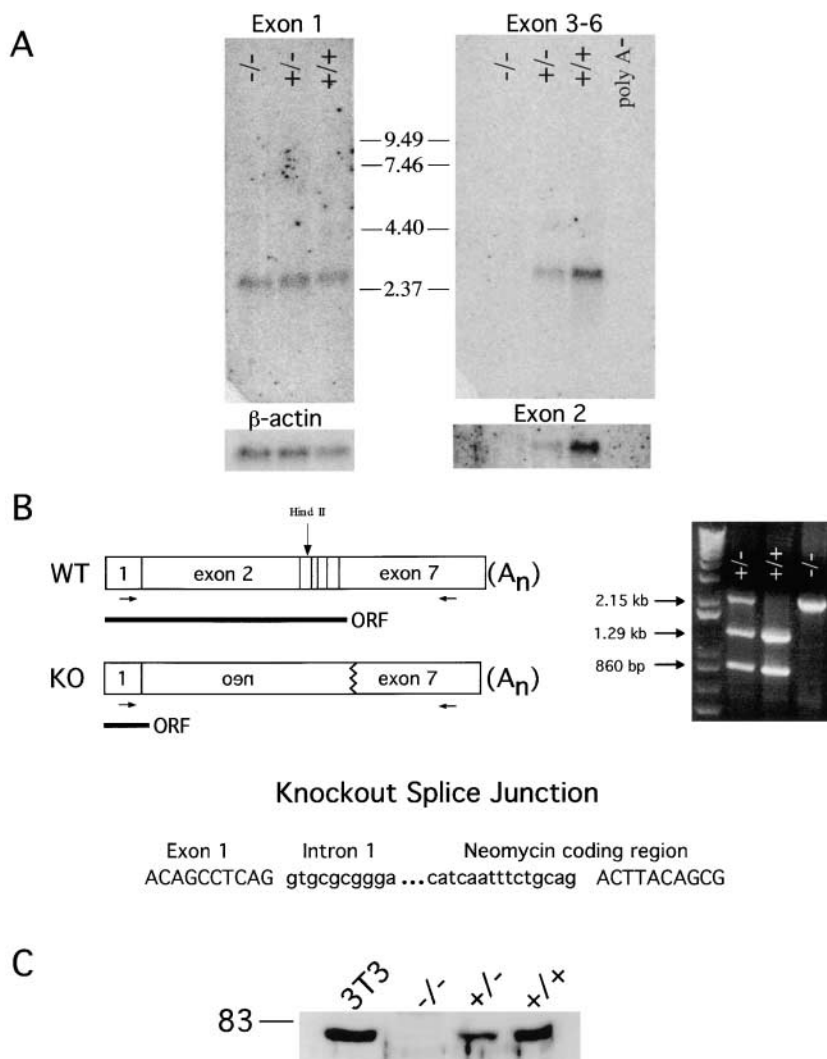


Figure 2. Analysis of coilin expression in mutant animals. (A) Northern blots of polyadenylated brain RNAs. The wild-type coilin transcript is ~2.6 kb. The indicated probes were PCR amplified from mp80-21 as described in Materials and methods. (B) Wild-type and mutant coilin alleles were amplified from polyadenylated RNA followed by digestion with HindIII. Open boxes represent mRNA from the indicated alleles; the length of each ORF is shown below. The small arrows represent the primers used for PCR. Sequence of the cryptic splice junction for the knockout allele transcript is shown below. (C) Western blot analysis of extracts from embryonic tissues with anticoin antibody R288, which recognizes epitopes in the coilin COOH terminus. The wild-type protein has a molecular weight of 80 kD. The knockout lane was overloaded intentionally to demonstrate the absence of wild-type coilin.



Table II. Analysis of SMN and extranucleolar foci in MEF cell lines

MEF	Genotype	Extranucleolar foci <sup>a,b</sup>	SMN foci <sup>a</sup>	SMN foci containing Nopp
			%	%
26	+/+	60–80 <sup>c</sup> (1.7/cell) <sup>d</sup>	20–30 (1.4/cell) <sup>d</sup>	90 (212 of 237)
42	-/-	10–30 (~1/cell) <sup>d</sup>	15–25 (1.5/cell) <sup>d</sup>	0 (0 of 368)
43	-/-	10–30 (~1/cell) <sup>d</sup>	<10 (1.5/cell) <sup>d</sup>	0 (0 of 268)

<sup>a</sup>Fraction of cells with one or more foci; ranges reflect variability between experiments.

<sup>b</sup>Foci scored with Fb or Nopp in the presence of NOH61 to exclude small nucleoli.

<sup>c</sup>Similar results were obtained when cells were scored with anticoilin antibodies.

<sup>d</sup>Scored for cells with one or more foci.

When applied to knockout tissues and MEFs, antibodies against Fb and Nopp revealed small extranucleolar foci in addition to nucleoli (Fig. 3, A and B, Fig. 4 B, and Figs. 5 and 6). Since Fb and Nopp label both CBs and nucleoli, it was important to use a nucleolus-only marker. Thus, to ensure that these foci were indeed extranucleolar we stained MEFs with antibodies against the 61 kD nucleolar helicase protein, NOH61. NOH61 is primarily localized to nucleoli and is not detectable in CBs (Zirwes et al., 2000). Staining of wild-type MEFs demonstrated that NOH61 is a good nucleolar marker, labeling even very small nucleoli (Fig. 5, arrowhead). When used on knockout MEFs, we found that the small foci identified by the Fb and Nopp antibodies did not stain with NOH61 (Fig. 5, large arrow, and Table II). Thus, these structures are not small nucleoli but are instead bona fide coilin-negative extranucleolar foci, reminiscent of the “coiled bodies without coilin” described by Bauer and Gall (1997) *in vitro*. Additional control experiments confirmed the nonnucleolar nature of the foci by hybridization of knockout MEFs with antisense oligos complementary to either 28S rRNA or MRP RNA followed by staining with anti-Nopp antibodies (see below). Since these extranucleolar foci lack coilin, we will henceforth refer to them as “residual” CBs.

### Residual CBs fail to recruit splicing snRNPs and the SMN complex

One of the most prominent features of the CB is the presence of high concentrations of splicing snRNAs (Carmo-Fonseca et al., 1992; Matera and Ward, 1993). The presence of trimethylguanosine cap and Sm protein epitopes within CBs suggests that the snRNPs accumulating there are at least partially mature (Matera, 1999a). To assess the status of these epitopes in our system, we stained our wild-type and knockout cell lines with antibodies against Nopp and the Sm proteins. To ensure detection of residual CBs rather than small nucleoli, we triple labeled cells with a probe against either 28S rRNA or MRP RNA (Fig. 6). In wild-type MEFs, accumulation of the Sm epitope in CBs was clearly evident (Fig. 6). In contrast, we were unable to detect accumulation of these proteins in residual CBs. The speckled nucleoplasmic distribution of snRNPs appears unaffected (Fig. 6). These results were recapitulated with antibodies against the trimethylguanosine cap (unpublished data) and the U2 snRNA specific protein, U2B'' (Fig. 3 B). Therefore, in the absence of the full-length coilin protein splicing snRNPs are not recruited to residual CBs.

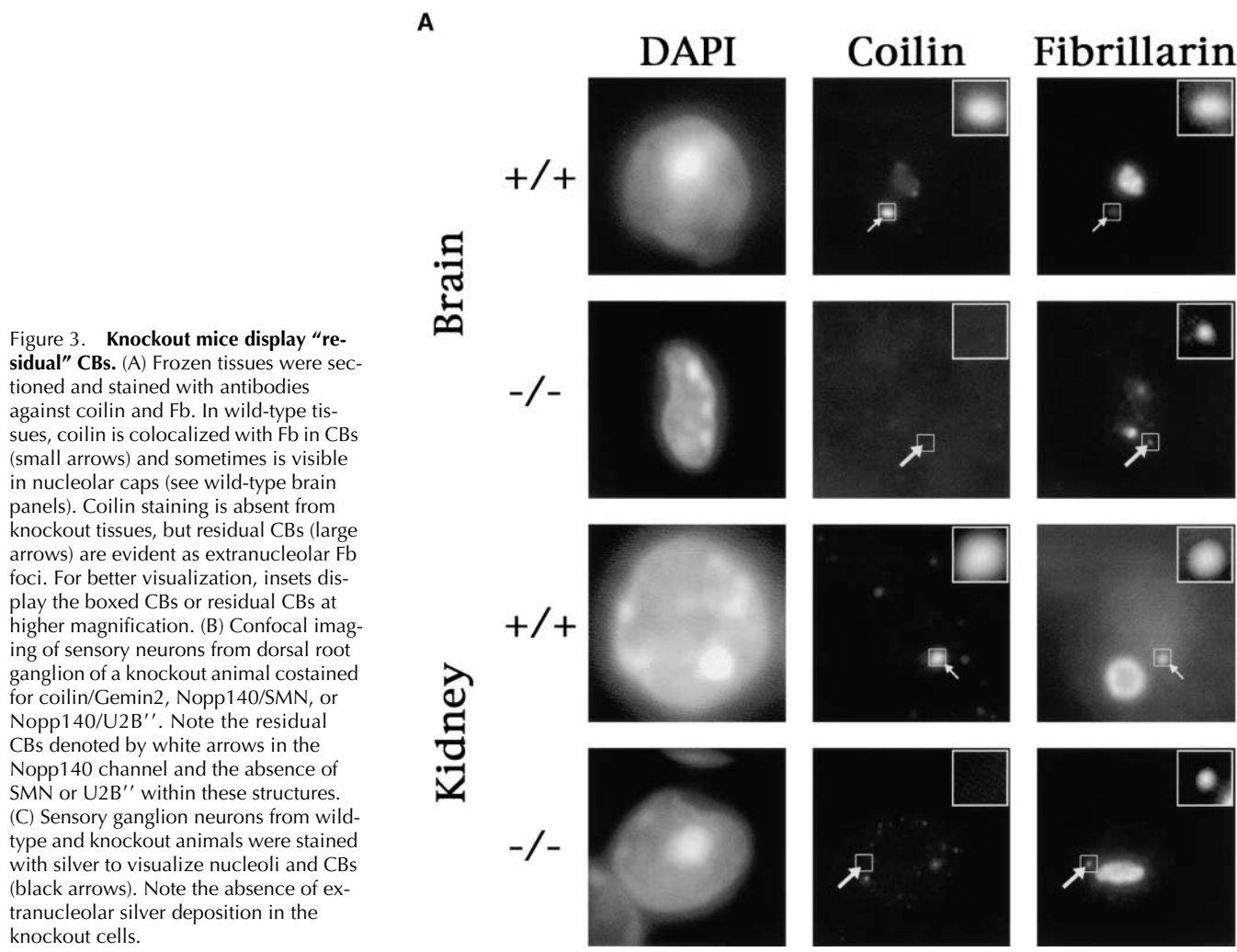
Before entry into the nucleus and subsequent localization in CBs, newly synthesized Sm snRNAs must undergo matu-

ration in the cytoplasm. This includes Sm core particle assembly (Mataj and Englmeier, 1998), which is mediated by the SMN–Gemin2 protein complex (Fischer et al., 1997). After nuclear import, snRNPs are thought to accumulate in CBs before proceeding on to interchromatin granules and perichromatin fibrils (Sleeman and Lamond, 1999a) where they participate in pre-mRNA splicing. It has been suggested recently that some form of “snRNP regeneration” is required between rounds of splicing and that nuclear SMN participates in this process (Pellizzoni et al., 1998; Matera, 1999b). Although SMN is distributed throughout the cytoplasm, within the nucleus it localizes to two types of foci, gems and CBs (Liu and Dreyfuss, 1996; Matera and Frey, 1998; Carvalho et al., 1999; Sleeman and Lamond, 1999b; Young et al., 2000). Furthermore, nuclear SMN complexes colocalize with snRNPs only in the CB; gems are devoid of snRNPs (Liu and Dreyfuss, 1996; Carvalho et al., 1999). These data along with the lack of snRNP accumulation in residual CBs prompted us to examine the relationship between SMN and residual CBs in our MEF cell lines.

Double labeling wild-type MEFs for coilin and SMN revealed that these epitopes colocalized in CBs 90–95% of the time (Table II and Fig. 4 A). Similar results were obtained when we used Nopp as the CB marker (90%; Table II and Fig. 4 B). However, double labeling of the knockout cell lines tells a dramatically different story. Although coilin-positive foci were absent in knockout MEFs (Fig. 4 A; unpublished data), gems were still detectable in a subset of cells (10–25%; Table II and Fig. 4). Costaining with Nopp (as an indicator of residual CBs) and either SMN or Gemin2 demonstrated that these epitopes were never coincident (Table II and Fig. 3 B and Fig. 4 B; unpublished data). In fact, both structures were sometimes observed in the same nuclei (Fig. 4 B). Thus, whereas gem formation is not precluded in coilin knockout cells, these data suggest that the SMN complex cannot properly target to CBs.

### Residual CBs do not contain the putative coilin NH<sub>2</sub>-terminal fragment

To determine if the knockout allele protein could be involved in the formation of residual CBs, we cloned its entire ORF (Fig. 2 B) behind that of GFP, generating pGFP–mcoilin<sup>KO</sup>. When transiently transfected into wild-type MEFs, GFP–coilin<sup>KO</sup> is primarily distributed throughout the cytoplasm and the nucleoplasm with additional accumulation in CBs (Fig. 7 A). Recruitment of the knockout construct to CBs in wild-type cells is presumably the result of binding to endogenous coilin and is consistent with the pres-



ence of a self-interaction domain within the coilin NH<sub>2</sub> terminus (Hebert and Matera, 2000). Upon expression in homozygous knockout MEFs, the GFP-coilin<sup>KO</sup> distribution was very similar to that of wild-type cells (Fig. 7 A), however no focal accumulations were detected. Thus, in the absence of wild-type coilin we conclude that the knockout allele protein is insufficient for formation of, or targeting to, CBs.

#### Ectopic expression of full-length coilin restores normal CBs to knockout cells

One caveat to previous *in vitro* studies of CB formation was that addback experiments failed to reconstitute normal CBs after coilin immunodepletion (Bauer and Gall, 1997). Therefore, we wanted to see if transfection of full-length coilin would reconstitute CBs in our knockout cell lines. To address this question, we cloned full-length mouse coilin into a GFP expression vector to generate an NH<sub>2</sub>-terminal fusion construct, pGFP-mcoilin<sup>FL</sup>. Control experiments in wild-type MEFs revealed proper targeting of the construct to nuclear foci (Fig. 7 B). However, in contrast to our observations using human coilin in human cells (Hebert and Matera, 2000), transfection of GFP-mcoilin into wild-type MEFs resulted in what appeared to be an increased number of CBs. Instead of the expected 1.5–2.0 foci per cell, we found that

transfected cells displayed on average 4.0 GFP-positive foci per cell (unpublished data). In HeLa cells, high levels of overexpression of full-length human coilin does not generate additional CBs. Instead, overexpression results in a general increase in intensity of the nucleoplasmic signal and ultimately the disassembly of CBs (Hebert and Matera, 2000). Thus, in contrast with human, mouse cells can seemingly tolerate a higher level of expression from the exogenous transcript before loss of coilin foci (unpublished data). Closer inspection of the transfected cells revealed two distinctly different types of foci in most cells expressing the GFP-mcoilin construct. Some foci have a very tight, bright, and compact appearance (Fig. 7 B, arrows), whereas others are more diffuse (Fig. 7 B, arrowheads). Additionally, cells with higher levels of nucleoplasmic GFP-mcoilin tended to have more of the diffuse variety, whereas cells with lower levels of nucleoplasmic expression had the more compact type (unpublished data).

To understand the differences between these two kinds of GFP-mcoilin dots, we stained transfected cells with antibodies against Nopp. This revealed that only the bright compact variety contained Nopp (Fig 7 B, arrows) and thus can be called CBs. To date, we have been unable to identify any other CB marker proteins that colocalize with GFP-mcoilin in the more diffuse foci. This demonstrates that, at

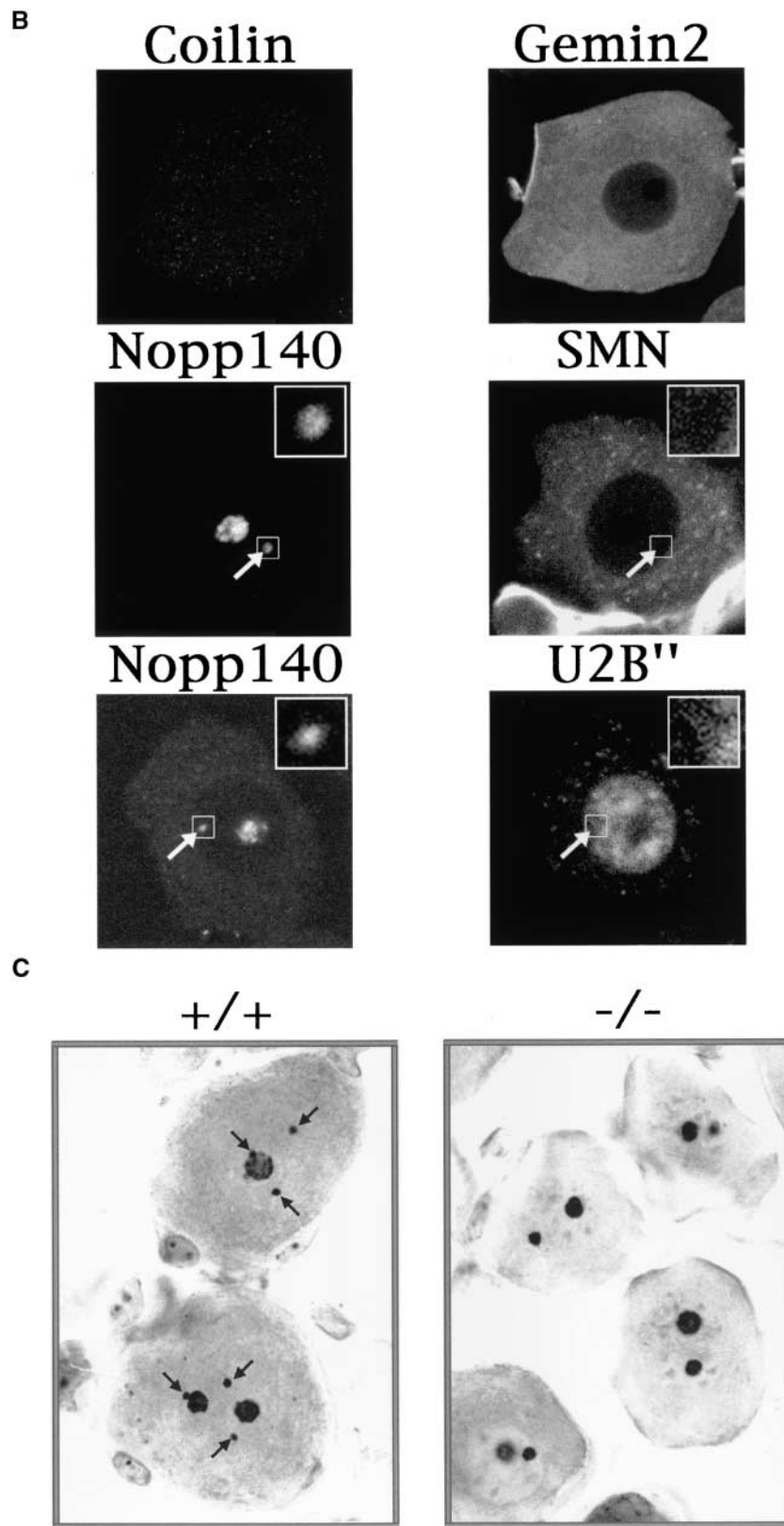


Figure 3 (continued)

least in mouse cells, coilin can form nuclear aggregates, which are not CBs (see Discussion).

In light of these two different types of foci observed after transfection of wild-type MEFs, we restricted our analysis of the knockout MEFs to those GFP foci that costained with

Nopp. Fig. 8 summarizes the results of our GFP–mcoilin “addbacks” to knockout cells. As shown, we found that the Nopp-positive GFP–mcoilin foci were able to recruit SMN (Fig. 8 A). It is important to note that in many of our knockout MEFs SMN does not form foci of any kind (Table II).

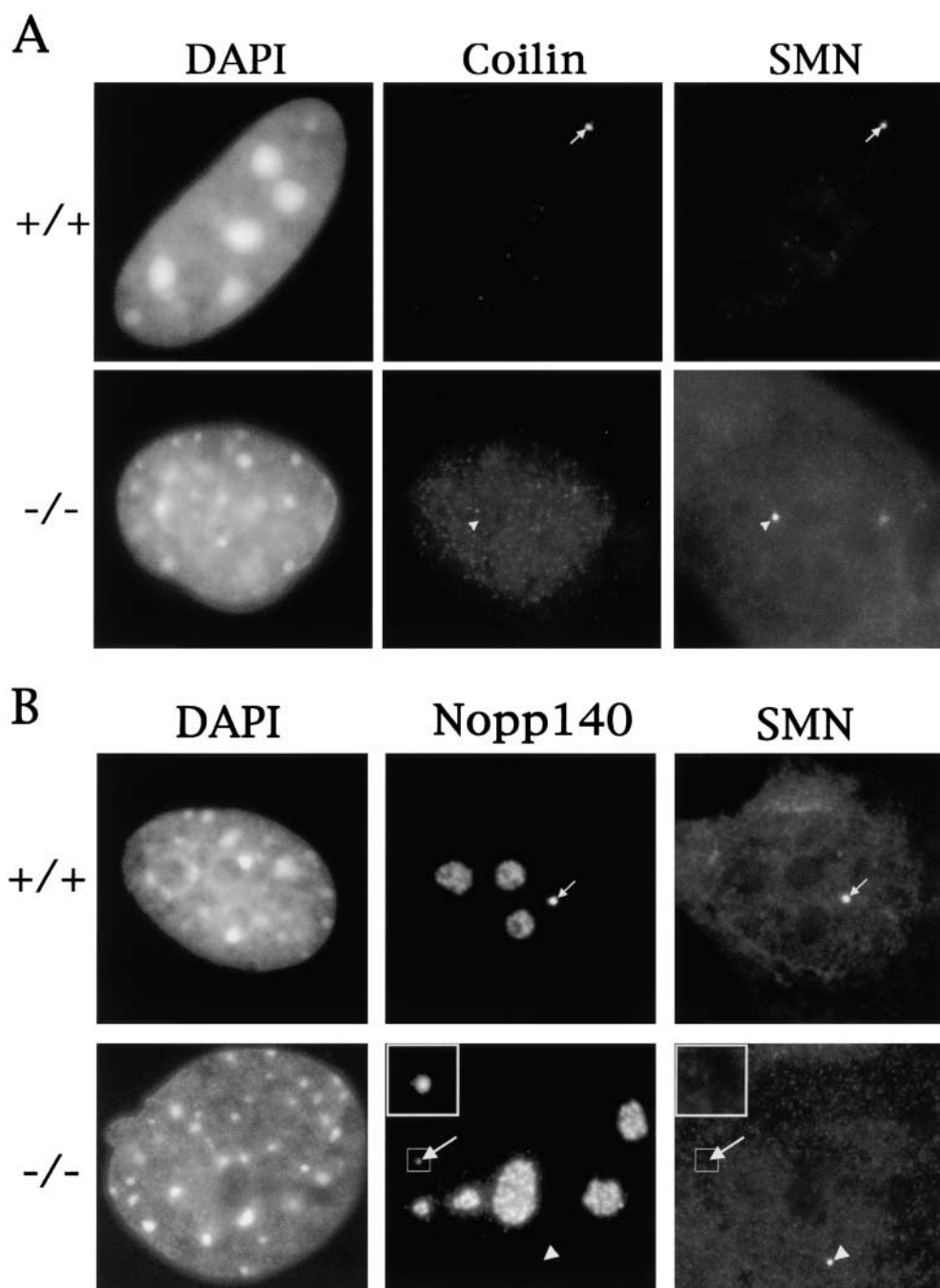


Figure 4. **Knockout MEFs display prominent gems.** Wild-type and knockout MEFs were costained for SMN and either coilin (A) or Nopp140 (B). SMN foci in wild-type MEFs were nearly always colocalized with coilin or Nopp140 in CBs (A and B, small arrows). In contrast, SMN foci present in knockout MEFs (arrowheads) were never found colocalized with Nopp140, a marker of residual CBs (large arrows). See Table II for additional details.

Thus, it was not surprising that some of the reconstituted CBs lacked detectable SMN. However, given that SMN and Nopp never colocalize in untransfected knockout cells (Table II), the fact that they do colocalize in cells transfected with coilin is significant. Additional experiments showed that the reconstituted CBs can also recruit splicing snRNPs (as assayed by the presence of the Sm epitope; Fig. 8 B). Together, these data demonstrate that sequences within the coilin COOH-terminal 487 amino acids are required for the recruitment of splicing snRNPs and the SMN complex to CBs.

## Discussion

We have generated a model system to assess the role of the p80 coilin protein in cellular function and CB formation. We show that at least some homozygous mutant animals are viable and fertile, but their numbers are significantly reduced when maintained on 129Sv/J or crossed to C57Bl/6J inbred backgrounds. Homozygous knockout animals are not reduced significantly in the outbred CD-1 mouse strain, suggesting the possibility of modifier loci. Since our knockout construct retains exon 1, it is formally possible that the



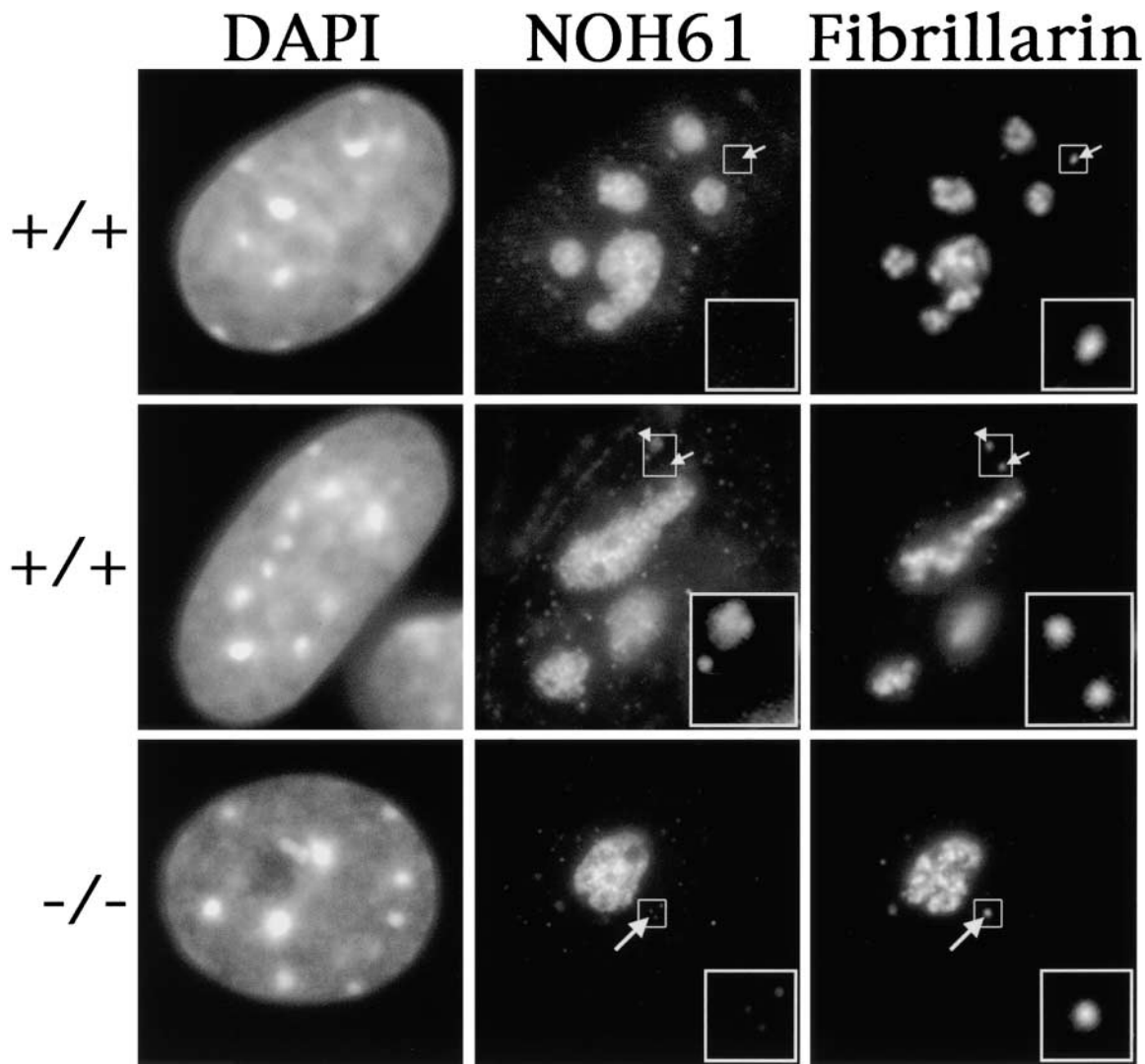


Figure 5. **Knockout MEFs display residual CBs.** Cells were costained with antibodies against Fb and NOH61 (a nucleolus-only marker) to allow discrimination of small nucleoli (arrowheads) from CBs (small arrows) and residual CBs (large arrow).

NH<sub>2</sub>-terminal 82 amino acids of coilin might perform an essential function. Expression of the GFP fusion of the knockout allele transcript showed that this construct is diffusely distributed throughout the cytoplasm and the nucleus in the knockout cell lines (Fig. 7 A). Thus, any putative essential function of the knockout allele protein would probably not be exerted within the residual CBs. Production of antibodies recognizing the NH<sub>2</sub> terminus of the coilin protein or generation of a demonstrably null coilin system will be required before this issue can be satisfactorily resolved.

As described above, coilin knockout animals and cells display extranucleolar foci that contain Nopp and Fb and have a similar size and shape to CBs. However, these residual CBs fail to recruit other factors common to wild-type CBs such as the SMN complex and the splicing snRNPs. Transient expression of GFP-tagged mouse coilin reconstituted CBs that also contained Fb, Nopp, SMN, and Sm snRNPs. Surprisingly, we also identified foci that appear to consist exclusively of GFP-coilin. Together, our data demonstrate a requirement for the COOH terminus of the coi-

lin protein in the recruitment of SMN and splicing snRNPs to CBs.

#### CBs without coilin?

Historically, CBs have been identified conclusively only on the basis of their structural morphology using various histological methods, including silver staining (Cajal, 1903, 1910; Hardin et al., 1969; Monneron and Bernhard, 1969; Lafarga and Hervas, 1983; Brasch and Ochs, 1992). The cloning of p80 coilin as a molecular marker for these structures (Andrade et al., 1991) has led to the nearly complete reliance upon this protein as a marker of CBs. However, in the absence of coilin there are presently no other known epitopes that uniquely tag CBs. Our finding that knockout cells also fail to display extranucleolar silver foci (Fig. 3 C) supports the idea that snRNP-rich CBs do not form in these animals. What then defines a CB? Bauer and Gall (1997) sought to explore the question in vitro using *Xenopus* egg extracts. They found that immunodepletion of coilin from the extracts resulted in formation of morphologically similar

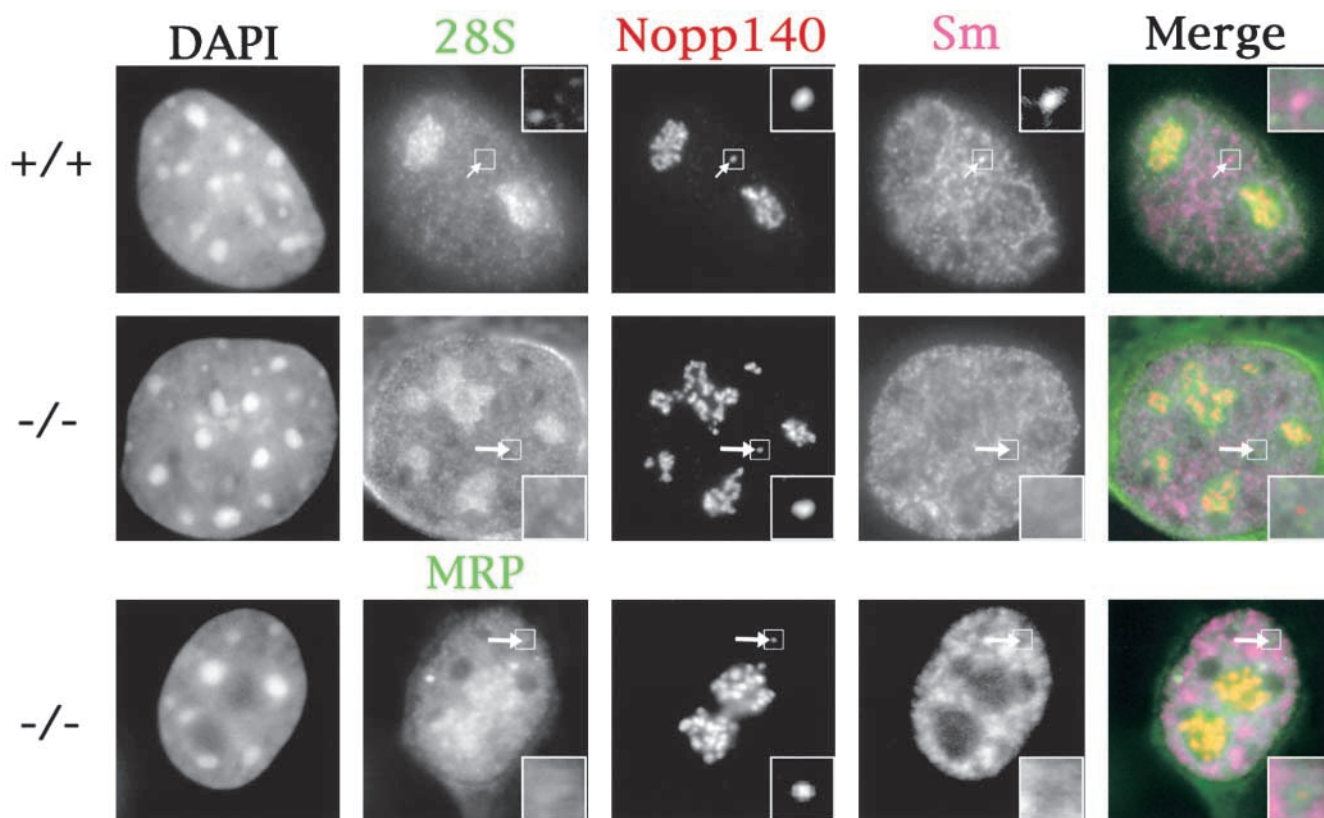


Figure 6. **Residual CBs do not recruit Sm proteins.** Triple labeling with antibodies against Nopp140 (red), the Sm proteins (magenta), and a nucleolus specific oligo (anti-28S or anti-MRP, green) demonstrates that Sm proteins are present in the CBs (small arrows) of wild-type MEFs but are absent from the residual CBs (large arrows) found in knockout MEFs.

structures that contained Fb but lacked Sm snRNPs. The presence or absence of SMN was not tested (Bauer and Gall, 1997). Unfortunately, *in vitro* coilin addback experiments failed to reconstitute CBs. These results suggest that left to their own devices nucleolar and CB factors can assemble into distinct supramolecular structures (Misteli, 2001).

Data from several labs demonstrate heterogeneity in the levels of certain epitopes within CBs (for review see Sleeman and Lamond, 1999b). We also detected variable levels of Fb among CBs of mouse cells (Fig. 3 A; unpublished data). SMN, which can localize to both gems and CBs, can thus be viewed as a heterogeneous CB component (Liu and Dreyfuss, 1996; Matera and Frey, 1998; Carvalho et al., 1999; this paper). Furthermore, Lamond and colleagues have suggested that these identifiable differences in epitope composition between CBs may indicate a family of related structures, each fulfilling different roles within the nucleus (Platani et al., 2000). Given such heterogeneity, it is important to note that the staining pattern of a single marker protein may not be indicative of the presence or absence of a CB. Although coilin is expressed in all adult tissues (Chan et al., 1994; Bohmann et al., 1995; Tucker et al., 2000), CBs are present in only a subset of cell types. In contrast, CBs are visible in all fetal tissues (Young et al., 2001). Young et al. (2000) proposed that in certain cells specific proteins (for example, phosphatases, kinases, and differentiation factors) may be important for CB assembly. Furthermore, we showed previously that coilin self-association correlates with its ability to localize in CBs (Hebert and

Matera, 2000). Cells that do not display CBs would presumably lack the proper coilin isoforms. Taken together, these results suggest a mechanism by which recruitment of specific components to CBs may be developmentally regulated.

#### Coilin participates in recruitment of specific components to the CB

Although much has been learned about the molecular composition of CBs, our understanding of the role that coilin plays in this structure was unclear. Bellini (2000) has summarized several possible roles for the coilin protein, all of which revolve around a central theme: the transport or recruitment of components to the CB. To serve as a recruitment factor, coilin must have the ability to interact with at least one other CB component. In fact, coilin has been shown to interact (directly or indirectly) with Nopp (Isaac et al., 1998), the U7 (Bellini and Gall, 1998) and U1 (Müller et al., 2000) snRNPs, and with itself (Hebert and Matera, 2000). In our genetic system, the loss of coilin's COOH terminus results in a failure of SMN and the Sm snRNPs to localize in residual CBs. Ectopic expression of wild-type coilin protein (Fig. 8) clearly demonstrates that coilin is required for reconstitution of CBs that contain SMN and splicing snRNPs. Thus, one novel function of coilin defined by our results is the recruitment of the SMN complex to CBs. Interestingly, we note that the coilin COOH terminus contains an arginine-glycine dipeptide motif (Fig. 1 C) similar to the COOH-terminal tails of the Sm proteins. Since the SMN

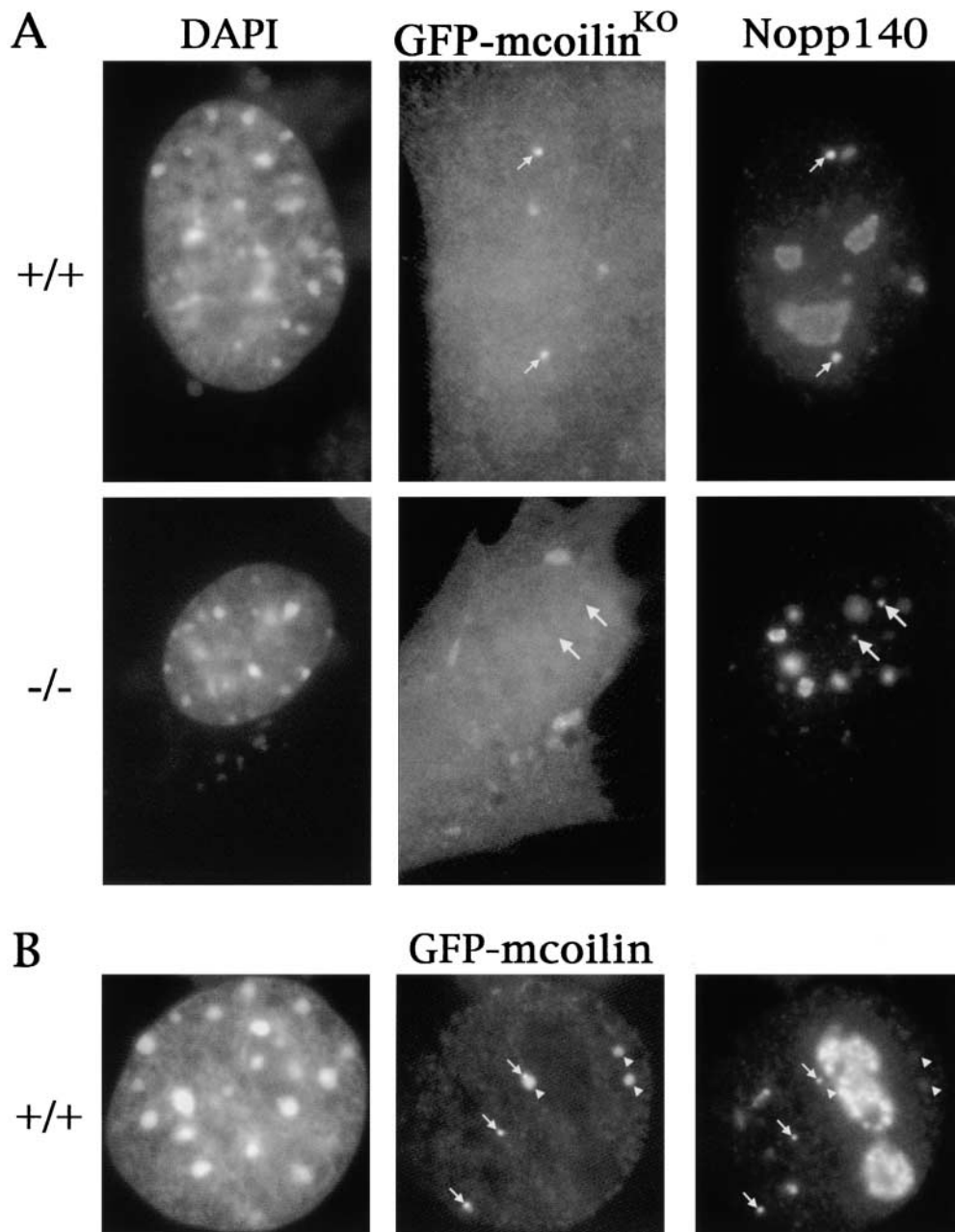


Figure 7. **Residual CBs do not accumulate mutant coilin constructs.** (A) The knockout allele protein (GFP-mcoilin<sup>KO</sup>) accumulates in CBs of wild-type MEFs but does not form foci in knockout cells. Cells were costained with anti-Nopp140 to identify nucleoli, CBs (small arrows), and residual CBs (large arrows). Costaining with anticoinin COOH-terminal antibodies in wild-type cells gave identical results (unpublished data). (B) Full-length mouse coilin localizes in two different types of foci when transiently transfected into wild-type or knockout (unpublished data) MEFs. Small arrows denote CBs that costain with Nopp140 (and other CB markers; see Fig. 8), whereas arrowheads mark positions of coilin-only foci.

protein is known to bind to arginine-glycine repeats (Friesen and Dreyfuss, 2000), we are currently investigating the function of this motif within coilin.

## Materials and methods

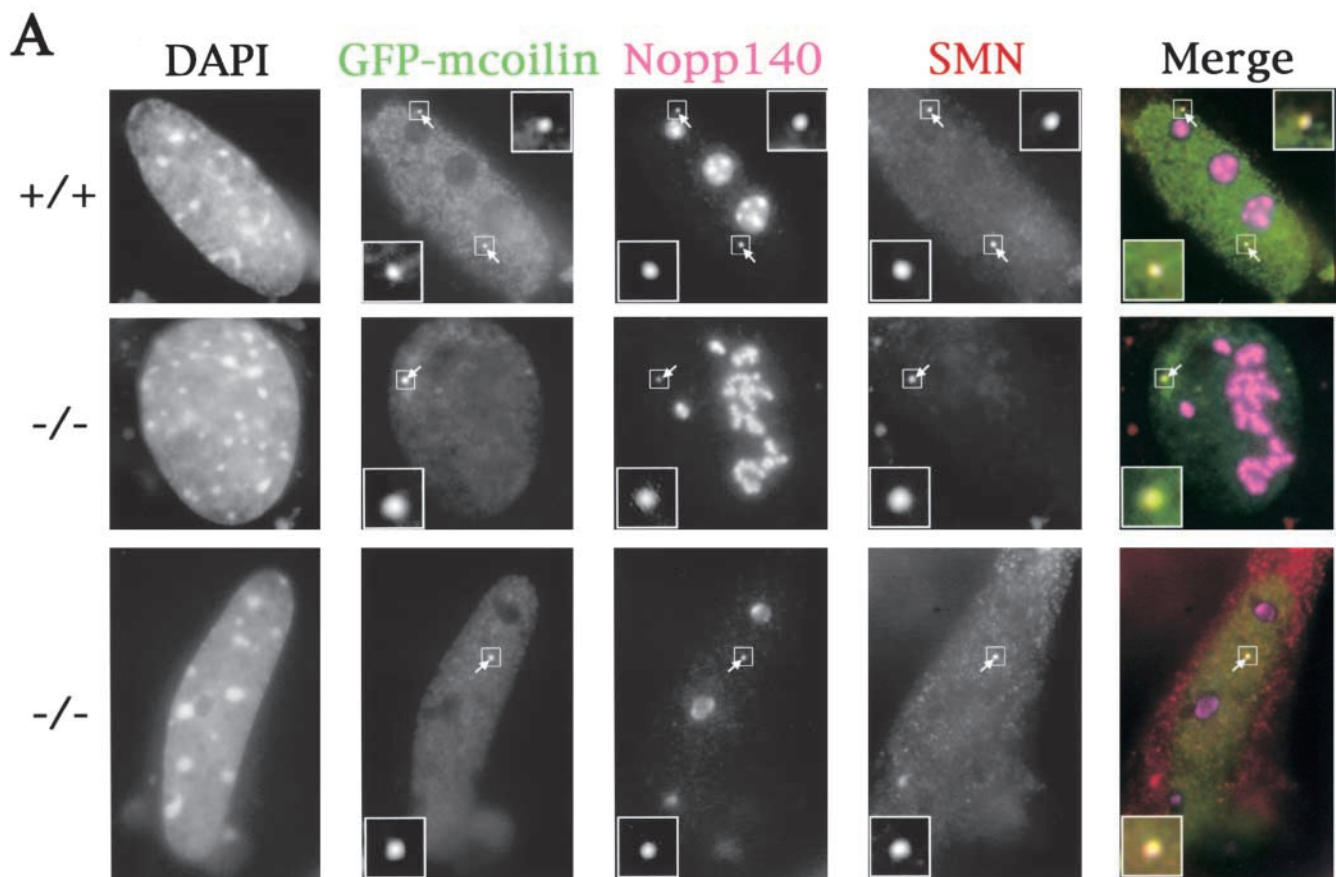
### Gene targeting

Mouse 129SvJ genomic clones containing regions of the coilin gene were described previously (Tucker et al., 2000). Targeting arms were generated by subcloning into the targeting vector, pPNT (Tybulewicz et al., 1991). A 4.2-kb KpnI/Bsp120I fragment from bacteriophage P1-11503 was inserted between the neomycin resistance and HSV thymidine kinase genes, both of which are driven by a PGK1 promoter. A 2.2-kb NotI/XhoI fragment

from  $\lambda$  clone 1A1 was inserted upstream of the neomycin resistance gene. The targeting vector (pMM6) was electroporated into 129Sv/R1 embryonic stem (ES) cells (Nagy et al., 1993) and selected with both G418 and gancyclovir. Successful recombination events were verified by Southern blot with a probe downstream of exon 7 (see Fig. 1). For amplification of the probe, the following primers were used: 200-bp forward, 5'-TCATGAGGCTGTGAAGAAGC-3' and 200-bp reverse, 5'-CCTCTCGAGAGTGCCACCAT-3'. Metaphase chromosomes were prepared from the targeted ES cells by standard techniques. Cells were then karyotyped to ensure 2N = 40 chromosomes were present in >95% of the spreads. The targeting efficiency of pMM6 was judged to be ~0.7% (3 out of 455 G418 resistant clones).

C57Bl/6J blastocysts were injected with the targeted ES line, resulting in several chimeras that displayed large contributions from the injected cells as assayed by coat color. Chimeras were mated to CD-1, C57Bl/6J, and 129SvJ females to generate F<sub>1</sub> heterozygotes. All offspring from CD-1





**Figure 8. Coilin recruits SMN and Sm proteins to CBs.** (A) Cells transiently transfected with GFP-mcoilin (green) were stained for Nopp140 (magenta) and SMN (red). In both wild-type and knockout MEFs, GFP/Nopp140 foci (arrows) also contained SMN. (B) Cells were transfected with GFP-mcoilin (green) and stained for Nopp140 (magenta) and the Sm proteins (red). Regardless of genotype, transfected cells displayed CBs (arrows) that also contained Sm.

mothers were either agouti or chinchilla (the other ES cell coat color allele), demonstrating transmission from the ES cell line. F<sub>1</sub> animals were intercrossed to generate coilin homozygous knockout mice. Resultant animals were genotyped by Southern blot and/or PCR analysis. Primers for PCR genotyping (Fig. 1) were as follows: forward primer, 5'-AAAGCAAGGTCAGACTATCGTTCC-3'; neo-reverse, 5'-TTTGCCAAGTTCTAATCCATCAG-3'; coilin reverse, 5'-TTCACGTGGCTGCTTTGTTTTATC-3'. Alternatively a four primer assay was also used: deletion allele, Neo forward, 5'-ACC-CAGCCGGCCACAGTCCG-3'; Neo reverse, 5'-GGCGCCCGGTTCTTTTGTGTC-3'; wild-type allele, intron 6 forward, 5'-AGCTTACGTGGAGTCAAGAATG-3'; exon 7 reverse, 5'-AACAAATGCGGTTACGGAGCG-3'.

#### Expression analysis

For Northern blotting, total brain RNA was extracted using the TRIZOL reagent (GIBCO BRL) according to the manufacturer's instructions. Polyadenylated RNAs were isolated with the Oligotex mRNA mini kit (QIAGEN) followed by fractionation on a 0.8% formaldehyde gel and transfer to Zeta-Probe Blotting Membrane (Bio-Rad Laboratories). Blots were hybridized with the indicated probes, which were PCR amplified from coilin cDNA clone mp80-21. Primers used: p80mEx1-1, 5'-GGTTAGGCTACGGCTTCAATTTGAC-3'; p80mEx2-3', 5'-GTCGCCACCATCGCTTACTATCAGG-3'; exon 2, mp80Ex2-15, 5'-CCTCCCCCAGAAGCCTCGGTA-3'; mp80Ex2-13, 5'-CGCCCCATCCTCTCCCAAAC-3'; exons 3-6, p80mEx3-5'; 5'-GCAGCTGCCCTCAAGTCCG-3'; p80mEx6-3', 5'-AGT-CACAGCGTATCCACCACCTC-3'.

Wild-type and mutant coilin alleles were amplified from cDNA synthesized from polyadenylated brain RNAs using coilin-specific primer mp80Ex7-4 (5'-CAGTTATGAGGAGCCCAAGTTC-3') for first strand synthesis. Resultant cDNA was used as template for nested PCR. The first round of amplification employed the primers m80-7 (5'-AAAGGAATTCAGATGGCAGCCTCCGAGAC-3') and mp80Ex7-4 followed by reamplifica-

tion with mp80Ex1-4 (5'-GCGCTTCGGCTTCAGTT-3') and mp80Ex7-2 (5'-GGCTGCACGGGCTCCTA-3').

For Western blotting, nuclear proteins were isolated from embryonic cells and separated by SDS-PAGE. Cell pellets were resuspended in 400  $\mu$ l of ice-cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 50 mM NaF, 1  $\mu$ M pepstatin, 10  $\mu$ M leupeptin) for every 0.5–1.0  $\times$  10<sup>6</sup> cells. After incubating on ice for 15 min, 25  $\mu$ l of 10% Nonidet P-40 was added and the tube vortexed for 10 s. After a brief centrifugation, the nuclear pellet was resuspended in 50  $\mu$ l of ice-cold buffer C (20 mM Hepes, 400 mM NaCl, 1 mM EDTA, 1 mM PMSF, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 50 mM NaF, 1  $\mu$ M pepstatin, 10  $\mu$ M leupeptin) by vigorous rocking at 4°C for 15 min. The extract was then centrifuged at high speed for 5 min at 4°C, and the supernatant was either immediately resolved by SDS-PAGE or stored at –80°C. After electrophoresis, the proteins were transferred to nitrocellulose, and the blots were probed with polyclonal anticoilin antibody R288 (1:500; Andrade et al., 1993) followed by detection with goat anti-rabbit HRP (1:10,000; Pierce Chemical Co.) and chemiluminescent detection.

#### Tissue fixation, sectioning, and staining

Fresh tissues were dissected from asphyxiated animals and sunk in 30% sucrose overnight at 4°C. The next day, tissues were embedded in OCT compound (Tissue-Tek) and frozen in dry ice. Blocks were stored at –80°C until sectioned. 4–8- $\mu$ m sections were cut using a CRYO-C knife (Hacker Instruments, Inc.) in a Leica CM3050 cryostat and melted onto gelatin-coated slides. Slides were stored at –25°C until use. Sensory ganglion neurons from dorsal root ganglia were processed and stained with silver using an adaptation of the original method of Cajal as described previously (Lafarga et al., 1991).

Staining for indirect immunofluorescence was conducted at room temperature. Sections were moistened in PBS followed by extraction with 0.5% Triton X-100/CSK (10 mM PIPES, 100 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.3 M



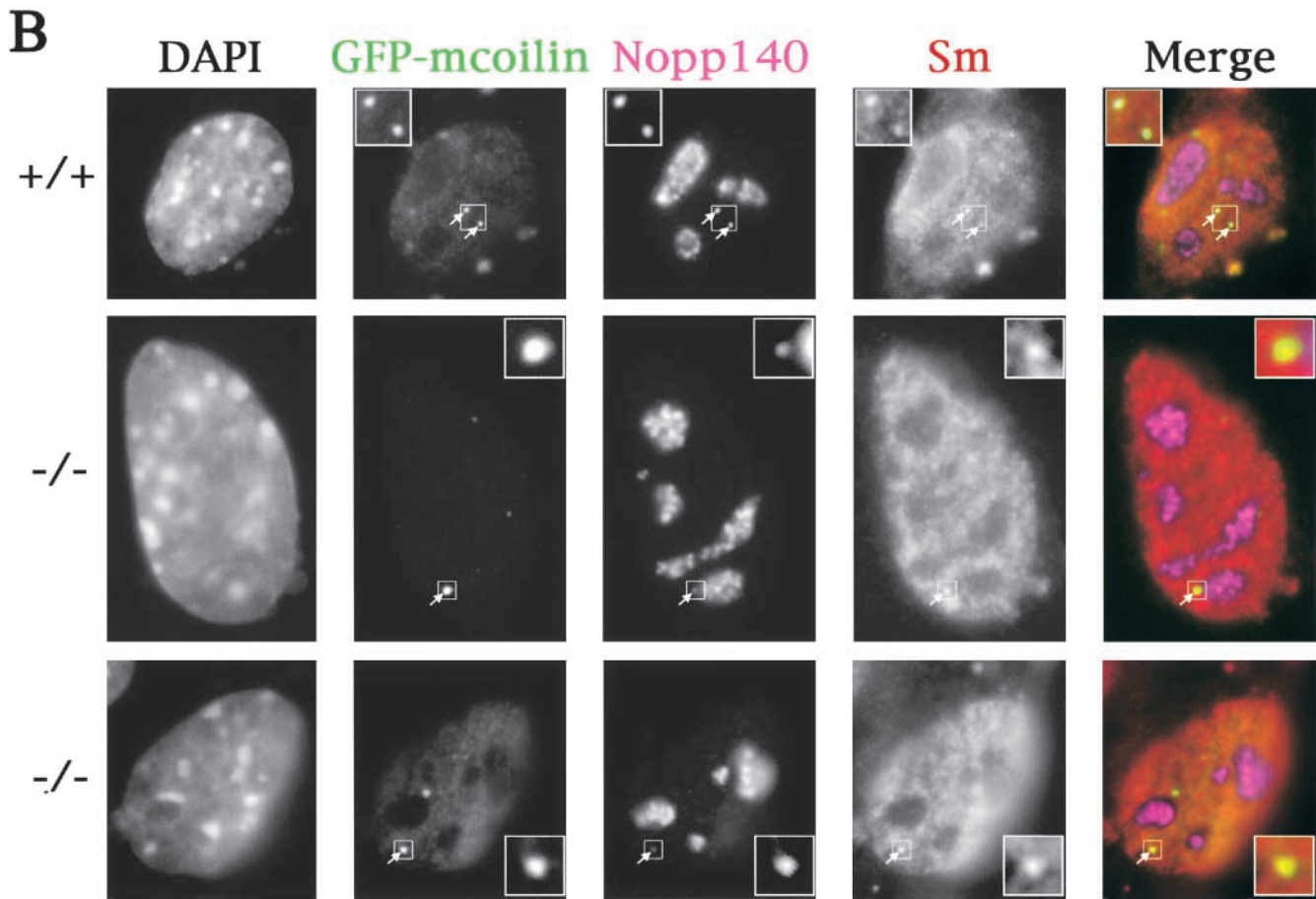


Figure 8 (continued)

sucrose, pH 7.2–7.4) for 3 min. Slides were rinsed in PBS and fixed in 4% PFA for 10 min followed by several PBS washes. Remaining reactive aldehydes were quenched by incubation in 0.1% sodium borohydride for 20 min followed by several PBS washes. Slides were then blocked for 1 h with 20% normal goat serum (Vector Laboratories)/0.1% Tween 20 followed by overnight incubation in primary antibody. The next day, slides were washed several times with PBS and blocked for 1 h followed by detection with secondary antibodies for 2 h. Primary antibodies used were: anticoinlin rabbit serum R288 (1:100), anti-Fb (mAb 72B9, 1:800), anti-Nopp rabbit serum (RF12, 1:200; a gift from T. Meier, Albert Einstein College of Medicine, Bronx, NY). Anti-rabbit Alexa 594 (1:200) and anti-mouse Alexa 594 (1:200) were obtained from Molecular Probes. Anti-mouse fluorescein (FITC, 1:200) was purchased from Vector Laboratories.

#### Establishment of MEF lines, cell culture, and transfection

MEF cell lines were derived by modification of the procedure of Todaro and Green (1963). Day 13.5–14.5 embryos were isolated from matings of +/- and +/-, +/- and -/-, or -/- and -/- mice. Hearts and livers were removed, and DNA for genotyping was isolated from these organs using the QIAmp Tissue Kit (QIAGEN). The remaining tissues were incubated in 0.05% trypsin-EDTA overnight at 4°C. The next day, embryos were incubated at 37°C for 30 min followed by addition of DME/10% FBS (GIBCO BRL) and disaggregation. Large tissue pieces were allowed to settle, and the supernatant was plated onto tissue culture-treated dishes (Falcon) and incubated at 37°C/5% CO<sub>2</sub> overnight. All cell lines were maintained at ~10–20% confluency throughout both the proliferative and crisis periods. During crisis, cells were passaged one to two times with at least one medium renewal between passages. Cell line establishment evidenced by a marked increase in cellular proliferation required ~3 m of continuous culture.

The mouse coilin cDNA (mp80-21, described above) was subcloned into pEGFP-C3 (CLONETECH) to generate pGFP-mcoilin<sup>FL</sup>. The coilin knockout allele (mcoilin<sup>KO</sup>) was amplified from brain RNA. Polyadenylated RNA was isolated as described followed by first strand synthesis (Superscript Preamplification System; GIBCO BRL) with coilin-specific primer

mp80Ex7-4. cDNAs were amplified with primers specific for exon 1 (m80-7 5'-AAAGGAATTCAAGATGGCAGCCTCCGAGAC-3', underlining denotes an engineered EcoRI site) and the neomycin resistance gene (KO-NEO3 5'-CCGGGATCCCGTGATATTGCTGAAGAGCTTG-3'; underlining denotes an engineered BamHI site). The resultant product was digested with EcoRI and BamHI and cloned into pEGFP-C2 (CLONETECH) to generate pGFP-mcoilin<sup>KO</sup>.

For transfections, subconfluent cells were split onto chambered slides (Nunc) and cultured overnight. The next day, GFP-mcoilin<sup>FL</sup> or GFP-mcoilin<sup>KO</sup> was transfected into MEFs using lipofectamine (GIBCO BRL). After a 20–30-h incubation, cells were fixed in 4% PFA, permeabilized with 0.5% Triton X-100 for 5 min, and processed for immunofluorescence.

#### Immunochemical methods and antibodies

For indirect immunofluorescence, cells were grown on chambered slides (Nunc). Untransfected cells were preextracted for 30 s to 1 min with cold 0.5% Triton X-100/CSK buffer followed by fixation in 4% PFA for 10 min at room temperature. Transfected cells were fixed in PFA followed by Triton X-100 extraction as described (Frey and Matera, 1995). Cells were then incubated with primary antibodies followed by detection with labeled secondary antibodies. The following primary antibodies were used: anticoinlin rabbit sera (R288, 1:100; 204.3, 1:150; a gift from A. Lamond, University of Dundee, Dundee, UK), anti-Nopp rabbit serum RF12 (1:200; Meier and Blobel, 1992), anti-Fb (mAb 72B9, 1:800), anti-Sm (mAb Y12, 1:800; Lerner et al., 1981), anti-SMN (mAb 2B1, 1:500; Liu and Dreyfuss, 1996; mAb MANSMA1, 1:4; Young et al., 2000), anti-Gemin2 (mAb 2E17, 1:500; Liu et al., 1997), anti-U2B'' (mAb 4G3, 1:15), and anti-NOH61 (mAb NOH61-4.2; Zirwes et al., 2000). The secondary antibodies used were: anti-rabbit Alexa 594 (1:200), anti-mouse Alexa (1:200), anti-mouse fluorescein (1:200), anti-mouse Texas red (1:100; Molecular Probes), anti-rabbit Texas red (1:100; Molecular Probes), anti-rabbit Cy5 (1:300; Jackson ImmunoResearch Laboratories), anti-mouse Cy5 (1:100; Amersham Pharmacia Biotech), and anti-guinea pig fluorescein (1:200; a gift from M.S. Schmidt-Zachmann, German Cancer Research Center, Heidelberg, Germany).

In situ hybridizations with the anti-MRP RNA and anti-28S rRNA oligonucleotides (Lee et al., 1996) were performed as described in Matera and Ward (1993). Slides were then processed for immunofluorescence as described above.

Polyclonal peptide antibodies GN-1971 and GN-1972 corresponding to amino acids 68–81 (PAESARLVDRNDL) of the coilin protein were prepared (Sigma-Aldrich). The synthesized peptide was conjugated to keyhole limpet hemocyanin. The peptide–keyhole limpet hemocyanin conjugate was mixed with either complete Freund's adjuvant (for the initial injection) or incomplete Freund's adjuvant (for all subsequent injections) and subcutaneously injected into two New Zealand white rabbits. A typical 2-wk schedule was followed and included six immunizations and four bleeds for each animal.

We are grateful to M. Hebert and E. Harkins for helpful advice and critical reading of the manuscript, and to J. Nicholson for blastocyst injections. We thank Lisa Massello for targeting vector construction and Liming Gao, Tabitha Barber, and Michael Mann for technical assistance. We also thank G. Dreyfuss, T. Meier, G. Morris, and M. Schmidt-Zachmann for gifts of antibodies.

This work was supported by grants to A.G. Matera from the National Institutes of Health (GM53034 and NS41617) and the Muscular Dystrophy Association, and by a grant to M. Lafarga from the "Fondo de Investigaciones Sanitarias" of Spain (FIS 00/0947).

Submitted: 20 April 2001

Revised: 11 June 2001

Accepted: 15 June 2001

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