Cajal-body formation correlates with differential colinin phosphorylation in primary and transformed cell lines

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Summary

Cajal bodies (CBs) are nuclear structures that are thought to have diverse functions, including small nuclear ribonucleoprotein (snRNP) biogenesis. The phosphorylation status of colinin, the CB marker protein, might impact CB formation. We hypothesize that primary cells, which lack CBs, contain different phosphoisoforms of colinin compared with that found in transformed cells, which have CBs. Localization, self-association and fluorescence recovery after photobleaching (FRAP) studies on colinin phosphomutants all suggest this modification impacts the function of colinin and may thus contribute towards CB formation. Two-dimensional gel electrophoresis demonstrates that colinin is hyperphosphorylated in primary cells compared with transformed cells. mRNA levels of the nuclear phosphatase PPM1G are significantly reduced in primary cells and expression of PPM1G in primary cells induces CBs. Additionally, PPM1G can dephosphorylate colinin in vitro. Surprisingly, however, expression of green fluorescent protein alone is sufficient to form CBs in primary cells. Taken together, our data support a model whereby colinin is the target of an uncharacterized signal transduction cascade that responds to the increased transcription and snRNP demands found in transformed cells.

Introduction

Cajal bodies (CBs) are subnuclear domains conserved in insects, including Drosophila (Liu et al., 2006a; Liu et al., 2006b), yeast, plants and mammals (reviewed by Gall, 2000; Matera, 2003; Cioce and Lamond, 2005; Matera and Shpargel, 2006). CBs participate in splicingosomal small nuclear ribonucleoprotein (snRNP) biogenesis. Specifically, CBs contain small Cajal-body specific RNAs (scaRNAs) that guide modification of the snRNA moiety of the snRNP (Darzacq et al., 2002; Jady et al., 2003). The modifications on the snRNAs are necessary for proper snRNP function (Pan and Prives, 1989; Segault et al., 1995; Yu et al., 1998). The CB also takes part in the assembly of splicingosomal subcomplexes (Schaffert et al., 2004; Stanek and Neugebauer, 2004; Xu et al., 2005; Stanek et al., 2008) and the final steps of U2 snRNP biogenesis (Nesic et al., 2004). Other work has shown that CBs participate in the biogenesis and delivery of telomerase to telomeres (Jady et al., 2004; Lukowiak et al., 2001; Jady et al., 2006; Tomlison et al., 2006; Tomlison et al., 2008). Interestingly, CBs are mobile, contain basal transcription factors and can associate with RNA genes (e.g. genes encoding U2), histone gene clusters and PML (promyelocytic leukemia) bodies (Gall, 2000; Ogg and Lamond, 2002; Bongiorno-Borbone et al., 2008; Grande et al., 1996; Sun et al., 2005). Finally, studies in Arabidopsis thaliana show that certain steps in micro-RNA and small-interfering RNA biogenesis might occur in plant CBs (Li et al., 2006; Pontes et al., 2006).

The marker protein for CBs is considered to be colinin (also known as P80C) (Raska et al., 1990; Raska et al., 1991). It is also notable that, in addition to the cytoplasm, the survival motor neuron protein (SMN) localizes to CBs (Carvalho et al., 1999; Matera and Frey, 1998). SMN is a vital component in the cytoplasmic phase of snRNP biogenesis (Meister et al., 2002; Massenet et al., 2002), and might have a role analogous to its cytoplasmic functions in the CB by ensuring that nuclear snRNPs remain functional after a splicing reaction has taken place (Pelizzoni et al., 1998; Xu et al., 2005). Phosphorylation impacts SMN activity and localization (Grimmler et al., 2005; Petri et al., 2007). In particular, dephosphorylation of SMN by the nuclear phosphatase PPM1G is needed for SMN localization to CBs (Petri et al., 2007).

With the exception of scaRNAs, all of the factors enriched in the CB also localize to other cellular compartments such as the cytoplasm, nucleoplasm or nucleolus (Darzacq et al., 2002; Matera, 1999). For example, 70% of colinin is nucleoplasmic (Lam et al., 2002). The fact that almost all the components of the CB can be found in other locations in the cell makes the description of the exact roles of the CB difficult. Indeed, any possible function ascribed to the CB has to be reconciled with the reality that many cell types (e.g. adult lung tissue) do not have CBs (Spector et al., 1992; Young et al., 2001). Thus, the activities that take place within the CB can probably also occur in the nucleoplasm. A key to understanding the function(s) of the CB comes from observations showing that CBs are most prominent in cells that are transcriptionally active, such as neuronal and cancer cells (Matera, 2003). Moreover, inhibition of transcription with actinomycin D or α-amanitin disrupts CBs (Carmo-Fonseca et al., 1992). Active U snRNA transcription and snRNP biogenesis is required for CB integrity (Shpargel and Matera, 2005; Lemm et al., 2006; Girard et al., 2006). Clearly, therefore, CB formation and activity are dynamic and balanced by the transcriptional demands of the cell.
Colin-knockout mice have been generated to better understand the role of this protein and CBs (Tucker et al., 2001). Inbred strains of colin-knockout mice have significant viability defects (Tucker et al., 2001). Cell lines derived from colin-knockout mice do not have typical CBs but instead have two kinds of ‘residual’ CBs (Tucker et al., 2001; Jady et al., 2003). One kind of residual CB contains scaRNAs (Jady et al., 2003) and the other contains proteins such as fibrillarin and Nopp140 (Tucker et al., 2001). SMN does not accumulate in either kind of residual CB, underscoring the role of colin in the formation of canonical CBs (Tucker et al., 2001; Jady et al., 2003). Studies on Arabidopsis have identified a colin orthologue (Atcolin) that, along with other loci, impacts CB formation and size (Collier et al., 2006). Very recent work has shown that a colin orthologue is present in Drosophila melanogaster, and this protein is required for normal CB organization (Liu et al., 2009). Mutants lacking CBs in Arabidopsis and Drosophila did not display any obvious growth phenotypes, but it has been found that HeLa cells depleted of colin by RNAi proliferate more slowly than control treated cells (Lemm et al., 2006) and are impaired in their ability to splice an artificial reporter (Whittom et al., 2008). Consequently, it is evident that colin is not an essential protein and CBs are not required for survival, yet their presence must be advantageous, because genes encoding colin and CBs are conserved in vertebrates, flies, and plants.

It is possible that colin might serve as the scaffold of CBs and bring together various factors necessary for a range of functions into one nuclear subdomain, resulting in the most efficient platform to prepare these factors for their activities. Additionally, colin has a role in the association of CBs with PML bodies (Sun et al., 2005) and Gems (Hebert et al., 2001). Gems are subnuclear domains found in some cell lines and fetal tissue, and are often found adjacent to CBs (Liu and Dreyfuss, 1996; Young et al., 2000). Gems contain SMN and associated proteins known as Geminis, but lack snRNPs and colin. In cell lines that normally lack Gems, reduction of colin by RNA interference abolishes CBs and induces Gem formation (Lemm et al., 2006; Whittom et al., 2008). Post-translational modification of colin also has a role in whether or not a cell contains Gems. Specifically, colin contains symmetrically dimethylated arginines that are important for direct interaction with SMN (Hebert et al., 2001; Hebert et al., 2002) and the presence of Gems correlates with a decrease in colin methylation (Hebert et al., 2002; Boisvert et al., 2002). Colin also binds directly to several Sm proteins of snRNPs (Hebert et al., 2001; Xu et al., 2005), suggesting that direct colin interaction with both SMN and snRNPs mediates their localization to CBs. The interplay between colin, SMN and snRNPs at the CB might facilitate snRNP biogenesis and recycling. It is unknown whether colin in the nucleoplasm, where the majority of the protein resides, contributes to its putative role in the CB or possesses distinct nucleoplasmic-specific activities. For example, a recent study has shown that colin is recruited to centromeres in response to damage or depletion of CENP-B, indicating that colin has an undefined role in some type of centromere-repair pathway (Morency et al., 2007).

In addition to symmetrically dimethylated arginines, human colin is a known phosphoprotein (Carmo-Fonseca et al., 1993). During mitosis, the level of phosphorylation on colin increases (Carmo-Fonseca et al., 1993). Cell cycle analysis reveals that CBs disassemble during mitosis and reform in the cell cycle at early- to mid-G1. However, throughout the cell cycle, colin levels remain constant (Andrade et al., 1993), giving rise to the hypothesis that the phosphorylation status of colin has a role in CB formation. Support for this idea comes from studies showing that phosphatase inhibitors alter CB localization (Lyon et al., 1997), as does mutation of a critical serine in colin (S202) to aspartate (Lyon et al., 1997; Sleeman et al., 1998). Additionally, we have shown that colin is a self-interacting protein and this interaction is reduced in mitosis when colin is hyperphosphorylated (Hebert and Matera, 2000). Furthermore, we have also shown that the C-terminus of human colin contains potential phosphoresidues that regulate the availability of the N-terminal self-interaction domain (Shargel et al., 2003). Recent work using tandem mass spectrometry (MS/MS) for the large-scale analysis of phosphoproteins has revealed that colin has at least eleven phosphorylated residues, with six of these residing in the very C-terminus of colin (Beausoleil et al., 2004; Olsen et al., 2006; Beausoleil et al., 2006; Nousiainen et al., 2006) (www.phosida.com). Hence, both cell biological and MS/MS analyses support the hypothesis that colin activity is regulated by its phosphorylation status, which changes during the cell cycle.

Colin might need to contain the proper phosphorylation pattern in order for CBs to form during interphase and a different contingent of phosphorylated residues to trigger CB disassembly during mitosis. Transient expression of small nuclear ribonucleoprotein-associated protein B (SmB; official symbol RSMB), but not colin, induces correspondingly transient CB formation in cells that normally lack this structure (Sleeman et al., 2001). A possible explanation for this finding is that the expression of Sm proteins signals to the cell the need to upregulate the snRNP biogenesis machinery. Part of this upregulation might include the formation of CBs to efficiently modify the snRNA component of the newly made snRNPs. To achieve CB formation, the phosphorylation status of colin might need to be altered. Thus, we hypothesize that colin is a target of an unknown signaling cascade that responds to increases in the demand for splicing resources. The exact residues on colin that may be subjected to this putative phosphorylation pathway are not known. Nor is it known whether some of the same factors that modify SMN phosphorylation (e.g. PPM1G) can also modify colin.

In this work, we provide evidence for this hypothesis by demonstrating that colin phosphomutants, particularly in the very C-terminus, display altered localization, self-association and mobility characteristics. We also show that colin in a primary cell line is hyperphosphorylated relative to that found in a transformed cell line. This hyperphosphorylation correlates with decreased PPM1G mRNA levels. Interestingly, expression of additional PPM1G in primary cells induces CB formation. Hence, these data support a role for colin in the formation of CBs, and indicate that hyperphosphorylated colin in primary cell lines inhibits CB formation.

Results

Mutation of colin phosphoresidues disrupts colin localization

Tandem MS/MS analysis has shown that colin has at least 11 phosphorylated residues (Fig. 1) (Beausoleil et al., 2004; Olsen et al., 2006; Beausoleil et al., 2006; Nousiainen et al., 2006) (www.phosida.com). Interestingly, 6 of the last 11 amino acids of colin are phosphorylated. To address the functional consequence of these residues with regards to CB formation, two mutant colin cDNAs were generated in the GFP-colin background. In the first, the 6 residues subjected to phosphorylation were changed from serines or threonines to alanines (C6A) (Fig. 1), mimicking a dephosphorylated state. In the second mutant, the serines were changed to aspartates and the threonines were converted to

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Coilin phosphoresidues impact self-association and mobility

Coilin is phosphorylated on several residues during interphase, with other residues becoming phosphorylated during mitosis (Carmofonseca et al., 1993; Beausoleil et al., 2004; Olsen et al., 2006; Fonseca et al., 1993; Beausoleil et al., 2006). We have shown that coilin is a self-associating protein and that this association is reduced when coilin is hyperphosphorylated during mitosis (Hebert and Matera, 2000). To address how these phosphomutations affect self-association, if at all, extracts obtained from HeLa cells expressing WT, C6A, C6D, OFF or ON GFP-coilin proteins were subjected to immunoprecipitation with anti-GFP antibodies followed by western blotting with anti-coilin antibodies (Fig. 3). We found that less endogenous coilin is co-immunoprecipitated with the C6D mutant (lane 6) compared with that recovered by the WT (lane 4) or the C6A mutant (Fig. 3A, lane 5). We also observed a slight reduction in the amount of endogenous coilin recovered by the ON mutant compared with the WT (Fig. 3B, compare coilin signal in lane 1 with that in lane 2). No coilin was recovered when cells expressed GFP only (Fig. 3B, lane 4). These results demonstrate that phosphoresidues of coilin impact self-association.

To further characterize the role of coilin phosphoresidues, we performed fluorescence recovery after photobleaching (FRAP) on Cajal bodies in cells expressing GFP-tagged WT, C6A, C6D, OFF or ON coilin proteins. Recovery curves were generated by double normalization (supplementary material Fig. S2) and the time to half maximal recovery (T_50) was calculated for each protein (Fig. 4). Compared with the WT, both the C6D and ON proteins had a faster T_50, suggesting that, in this time frame, there is a greater exchange of the constitutively phosphorylated protein with an individual Cajal body. By contrast, both the C6A and OFF proteins had a slower T_50 than the WT, implying that these proteins are less mobile.

**WI-38 cells contain hyperphosphorylated coilin**

Human primary foreskin fibroblasts cells (DFSF1), which normally lack CBs, can be induced to form CBs by transient expression of SmB, but only for a limited time (Sleeman et al., 2001). Additionally, this same study demonstrated that fusion of DFSF1 cells to HeLa cells leads to the formation of CBs in DFSF1 nuclei. Consequently, DFSF1 cells are capable of forming CBs if the appropriate factors and/or signals are provided by HeLa cells.
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The fact that exogenous Sm protein, but not coilin or SMN, expression can transiently induce CB formation in DFSF1 cells (Sleeman et al., 2001) suggests that the cell is responding to a direct need to upregulate snRNP biogenesis. We hypothesize that part of this upregulation is the post-translational modification of coilin by phosphorylation, rendering it in a conformation conducive for CB formation. To test this idea, we used the WI-38 primary cell line that has been shown to have CBs in only 2-3% of cells (Spector et al., 1992). It is possible that CBs are rare in the WI-38 primary cell line because coilin phosphorylation levels and/or phospho-residues are different in this line compared with that found in HeLa cells, which have CBs. To further support this hypothesis, we conducted two-dimensional gel electrophoresis experiments using western blotting to detect coilin. The predicted pI of unphosphorylated coilin is 9.2 (www.phosphosite.org). As a control experiment, we compared the pI of coilin from untreated or phosphatase-treated HeLa lysate and observed that, as expected, coilin is shifted to a higher pI when dephosphorylated (Fig. 5A). It is also important to note that coilin in untreated HeLa lysate focused close to the position corresponding to pI 7 on a pH 7-10 strip, in agreement with previous results demonstrating that coilin is a phosphoprotein (Carmo-Fonseca et al., 1993). We then assessed the position of focused coilin, β-tubulin and SMN from both HeLa and WI-38 interphase cells on pH 5-8 strips. β-tubulin was used as an internal standard to help gauge whether the pI of coilin differed in the two lines. The pI of SMN was monitored to determine whether SMN phosphorylation was also correlated with CB formation, as we suspected was the case for coilin. The predicted pI of unphosphorylated SMN is 6.1, whereas β-tubulin is expected to be approximately 5.3. The focused position of β-tubulin was unchanged in HeLa versus WI-38 extracts. By contrast, the pI of coilin from HeLa differed compared with WI-38 coilin (Fig. 5B,C). Specifically, coilin from HeLa cell extracts was focused to several different pIs (arrows), implying that a range of phosphoisoforms exist, and the majority of the protein migrates to a more basic pI than that observed for WI-38 coilin. In fact, using β-tubulin as an internal standard, we conclude that coilin from WI-38 is more uniformly phosphorylated in this line compared to coilin from HeLa (Fig. 5B,C), and its pI is consistent with hyperphosphorylated coilin from HeLa mitotic lysate (Fig. 5D). SMN, a known phosphoprotein, was focused to several distinct foci in both HeLa and WI-38 extracts, but the overall migration of these foci did not appear to differ in the two lines relative to β-tubulin (Fig. 5E,F). Thus the lack of CBs in the

Fig. 2. Coilin phosphomutants impact localization. (A) HeLa cells expressing wild-type (WT) GFP-coilin or GFP-coilin mutants (C6A, C6D, OFF or ON) were fixed and GFP (green), SMN (red) and DAPI (blue) signals were detected. The right column shows the overlay of all three images (Merge). Some CBs are labeled with arrows. Arrowheads indicate nucleolar staining observed in cells expressing C6A and OFF. Scale bars: 10 μm. (B) Quantification of WT and coilin mutant localization in coilin-knockdown HeLa cells. At least 100 cells were counted for each construct. (C) Quantification of WT and coilin mutant localization in coilin-knockdown HeLa cells. At least 50 cells were counted for each construct.
primary cell line WI-38 correlates with an increase in the degree of phosphorylation of coilin.

**WI-38 cells contain reduced PPM1G phosphatase message levels**

The kinases and phosphatases responsible for coilin phosphorylation have not been clearly identified. Moreover, the kinases that phosphorylate SMN are likewise unclear. However, recent work has defined PPM1G as the phosphatase that governs SMN localization to CBs (Petri et al., 2007). Specifically, knockdown of PPM1G in HeLa cells results in the loss of SMN from CBs, but does not drastically alter coilin localization to CBs, or abolish CBs, although numerous small coilin foci were observed (Petri et al., 2007). It is possible that the small coilin foci induced upon knockdown of PPM1G indicates that this phosphatase also modifies coilin, and the small foci might contain relatively hyperphosphorylated coilin compared with that found in normal CBs. Since WI-38 cells only very rarely contain CBs, and coilin is hyperphosphorylated in this line compared with that found in HeLa cells, we suspected that PPM1G activity might be reduced in WI-38 compared with HeLa cell extracts. To explore this possibility, we conducted qRT-PCR on PPM1G mRNA levels from both lines and found that, relative to actin, PPM1G levels are significantly reduced (approximately 50%) in WI-38 compared with HeLa extracts (Fig. 6A). Consistent with previous results comparing the expression levels of SMN in transformed versus primary lines (Sleeman et al., 2001), we found that SMN message levels are significantly reduced in WI-38 cells (Fig. 6A). By contrast, coilin levels showed a slight but statistically insignificant increase in WI-38 (Fig. 6A). Incubation of recombinant PPM1G with mitotic HeLa cell lysate results in a shift of coilin on SDS-PAGE consistent with its dephosphorylation (Fig. 6B, compare the mobility of coilin in lane 4 to that in lane 5). Thus, at least in vitro, coilin is a substrate for PPM1G. Taken together, these results suggest that reduced PPM1G and SMN levels contribute to the lack of CBs in WI-38 cells. The most straightforward interpretation of this data is that reduced PPM1G levels in WI-38 cells leads to hyperphosphorylated coilin promoting CB disassembly. Reduced SMN levels in WI-38 cells might also contribute to an environment in which CB formation is not favored, although correspondingly reduced PPM1G levels might result in no change in the overall phosphorylation of SMN compared with that observed in HeLa cells.

**PPM1G phosphatase expression in WI-38 cells induces CBs**

To determine whether PPM1G expression could induce CBs in a primary cell line, YFP-PPM1G was transfected into WI-38 cells, followed by detection of CBs using anti-coilin antibodies. Most of the cells overexpressing YFP-PPM1G did not have CBs (Fig. 7A, row a). However, approximately 30% of these cells had clear CBs (Fig. 7A, rows b and c, arrows) and these CBs contained both SMN and snRNPs (our unpublished observations). Interestingly, cells clearly overexpressing YFP-PPM1G, as evidenced by cytoplasmic localization in addition to nuclear accumulation (Fig. 7A, row c), nearly always had at least one CB. Since CBs are normally found in only 2-3% of WI-38 cells (Spector et al., 1992), we conclude that PPM1G has an important role in the regulation of CB formation.

To further validate the ability of PPM1G to induce CBs in primary cells, we also scored WI-38 cells transiently transfected with a catalytically inactive form of PPM1G, D496A (Murray et al., 1999). To reduce possible overexpression artifacts, we only scored cells with nuclear YFP-PPM1G (or YFP-inactive PPM1G) localization and found that 17% of YFP-PPM1G-expressing cells had CBs compared with 5% of YFP-inactive PPM1G-expressing cells (Fig. 7C). WI-38 cells expressing GFP-coilin, GFP-SmB or GFP alone were also scored. Neither GFP-coilin nor GFP-SmB expression triggers significant CB formation (Fig. 7B). A previous study has shown that YFP-SmB expression for 2 hours can induce CBs in another primary cell line, but CBs are absent after 16 hours of expression (Sleeman et al., 2001). At neither time point did GFP-coilin expression induce CBs (Sleeman et al., 2001). Thus, our results for FP-SmB and GFP-coilin, obtained after 24 hours of expression, are consistent with previously published findings.

In stark contrast to the failure of GFP-SmB and GFP-coilin to form CBs, expression of GFP alone induces CB formation significantly above the 2-3% of WI-38 cells that normally have CBs (Fig. 7B). In fact, expression of GFP alone yields the highest percentage of cells with CBs amongst the constructs tested, with approximately 30% of transfected cells displaying CBs (Fig. 7C). In summary, expression of YFP-PPM1G induces CB formation in a primary cell line more than inactive YFP-PPM1G, GFP-coilin and GFP-SmB, suggesting that this phosphatase participates in the regulation of CB formation. The induction of CB formation in cells expressing GFP alone is unexpected, and might be a compensatory response by the cell to accommodate the transcription and processing of the GFP message. It should be pointed out that only
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low to moderately expressing GFP cells were scored (similarly to that shown in Fig. 7B), and the GFP message does not contain an intron, and thus is not spliced.

Discussion

Our previous findings, and data from other groups, support a model whereby the phosphorylation status of human coilin impacts CB formation. Central to this model are the observations that coilin hyperphosphorylation during mitosis correlates with reduced self-interaction and CB disassembly (Carmo-Fonseca et al., 1993; Hebert and Matera, 2000). Thus CBs might share a common feature with the nucleolus and the nuclear membrane in that increased phosphorylation of vital proteins in these compartments promotes their disassembly. There is also indirect evidence supporting the ideas that proper coilin phosphorylation is required to form CBs, and coilin hyperphosphorylation during mitosis triggers CB disassembly. First, the overexpression of human coilin in HeLa cells does not result in the formation of more CBs. One interpretation of this result is that the CB has a limited number of coilin-binding sites. We do not favor this interpretation, because our work shows that the overexpression of human coilin in HeLa cells in fact abolishes CBs, as assessed by SMN staining (Hebert and Matera, 2000). It is possible that newly overexpressed coilin does not contain the proper composition of phosphoresidues for CB localization, but is able to disrupt CBs by binding to and titrating out endogenous coilin in CBs. MS/MS analysis has confirmed our initial belief that the C-terminus of human coilin contains several phosphoresidues (Shpargel et al., 2003; Nousiainen et al., 2006; Olsen et al., 2006). Second, our studies into the CB formation potential of human, mouse and frog coilins in both human and mouse cell lines demonstrate that these coilins all contain an intrinsic nuclear body formation potential, but this potential is subject to increasing layers of regulation from frog, to mouse, to human (Shpargel et al., 2003). We suspect that phosphorylation of coilin is a major contributor to the regulation of CB formation and number in humans.

Additionally, two previous studies provide compelling indirect evidence that coilin phosphorylation changes upon transformation or in response to the RNP biogenesis needs of the cell. It should be pointed out, however, that neither of these papers contains direct data concerning changes in coilin phosphorylation. In the first study, the Spector group showed that cells of limited passage number have the fewest CBs, immortalized cells contain an intermediate number of CBs, and transformed cells have the greatest number of CBs (Spector et al., 1992). Most importantly, it was found that an immortalized cell line (Ref-52) had a dramatically higher frequency of CBs upon transformation (24% of Ref-52 cells have CBs compared with 99% of transformed Ref-52 cells). Therefore, transformation correlates with CB formation, and we believe that changes in the phosphorylation state of coilin underlie CB formation.

Fig. 5. IEF of endogenous coilin from HeLa and WI-38 cells. (A) Lysate from HeLa cells was untreated or treated with CIP, followed by IEF (pH 7-10 IPG strip), SDS-PAGE and western blotting. Coilin was detected using anti-coilin antibodies. (B) Interphase HeLa cell lysate was subjected to IEF (pH 5-8 IPG strip), SDS-PACE and western blotting. Coilin and β-tubulin were detected on the same blot using appropriate antibodies. (C) Lysate from interphase WI-38 cells was subjected to IEF (pH 5-8 IPG strip), SDS-PAGE and western blotting, followed by the detection of coilin and β-tubulin. (D) Mitotic HeLa cell lysate was subjected to IEF (pH 5-8 IPG strip), SDS-PAGE and western blotting, followed by the detection of coilin and β-tubulin. In panels E and F, HeLa and WI-38 extracts were treated as described for B and C, except that β-tubulin and SMN were detected. Representative gels are shown. Note that interphase HeLa coilin contains more phosphoisoforms (arrows) than found in WI-38, and coilin is hyperphosphorylated in WI-38 (arrow) relative to HeLa.

Fig. 6. Quantitative PCR analysis of coilin, SMN and PPM1G expression in HeLa and WI-38 cell lines and dephosphorylation of coilin by PPM1G. (A) Coilin, SMN and PPM1G expression levels relative to β-actin are shown. HeLa values for each message of interest are normalized to 100%. Error bars represent percentage error about the mean. The difference between relative coilin levels in HeLa compared with WI-38 is not significant (P=0.25). However, there is a significant decrease in the relative expression levels of SMN (P=0.0023) and PPM1G (P=0.000058) in WI-38 compared with HeLa cells. (B) Lysate from mitotic HeLa cells was untreated or treated with CIP or recombinant His-tagged PPM1G, followed by SDS-PAGE, western blotting and detection of coilin using appropriate antibodies.
that does not normally contain CBs is sufficient to induce this study, transient overexpression of SmB in a primary cell line from the Lamond group (Sleeman et al., 2001). In this particular cell, a nuclear aggregate of YFP-PPM1G was detected (arrowhead) next to a CB (arrow). Approximately half of the cells with CBs strongly overexpressed YFP-PPM1G, which accounted for 14% of the 36 cells scored (row c, note also cytoplasmic localization of YFP-PPM1G). In this particular cell, a nuclear aggregate of YFP-PPM1G was detected (arrowhead) next to a CB (arrow). Scale bars: 2 μm. (B) GFP-coilin, GFP-SmB and GFP alone expression in WI-38 cells. Merged images are shown and an arrow indicates the location of a CB (arrow). (C) Induction of CBs in WI-38 cells expressing YFP-PPM1G but inactive PPM1G, GFP-coilin and GFP-SmB do not form CBs (Fig. 7). The finding that GFP alone is the most efficient of the constructs tested at inducing CBs is extremely interesting (Fig. 7), although the mechanisms underlying this observation are not entirely obvious. We speculate that CBs are triggered to form in GFP expressing cells owing to the increased transcription demand imposed on the cell by the vector. Since CB formation is balanced by the level of transcription, as demonstrated by studies using transcription inhibitors such as actinomycin D (Carmo-Fonseca et al., 1992), it is possible that WI-38 cells expressing GFP alone are inducing CBs to accommodate the flux of GFP mRNA through the RNA-processing pathway, despite the fact the GFP message is not spliced. Clearly, more studies will be necessary to understand these findings and assess whether they are coupled to changes in coilin phosphorylation.

Tandem MS/MS analyses by several groups have found that at least 11 residues of coilin are phosphorylated (Fig. 1) (Beausoleil et al., 2004; Olsen et al., 2006; Beausoleil et al., 2006; Nousiainen et al., 2006) (www.phosida.com). Three of these residues, T122, S489 and S566, have been shown to be phosphorylated during mitosis. It is unclear, however, as to the exact contingent of phosphorylated amino acids of coilin during interphase and mitosis. To address this issue, we generated mutations in the C-terminal phosphoresidues of coilin (Fig. 1). We found that a mutant mimicking a constitutively dephosphorylated state (C6A) disrupts normal coilin localization in half of the transfected cells (Fig. 2) and co-immunoprecipitates endogenous coilin (Fig. 3A). By contrast, a constitutively phosphorylated-like mutant (C6D/E) localized normally, yet had greatly reduced amounts of co-immunoprecipitated coilin. These findings suggest that phosphorylation of coilin C-terminal residues impacts self-association, yet does not affect the ability of the mutant protein to incorporate into CBs. However, the ON mutant, which contains D/E changes in all 11 suspected phosphoresidues, shows a majority of transfected cells displaying only nucleoplasmic localization (Fig. 2A) and a faster recovery in CBs compared with WT or OFF mutant coilins, as assessed by FRAP analysis (Fig. 4). Thus, it appears that hyperphosphorylated coilin is more mobile and more nucleoplasmic than WT or OFF coilin. However, this interpretation is complicated.
Phosphorylation impacts CB formation

In summary, the work presented here strongly suggests that coilin phosphorylation impacts the organization of the nucleus with regard to CB formation in HeLa cells. Furthermore, in the WI-38 primary line that does not contain many CBs, coilin is hyperphosphorylated relative to that found in HeLa cells, which have CBs. This hyperphosphorylation correlates with reduced PPM1G mRNA levels. Transformation, therefore, might increase snRNP demand and signal to the cell the need to form CBs to efficiently generate snRNPs. To achieve CB formation in primary cell lines, we propose that SMN and PPM1G expression have to be elevated coupled with a dephosphorylation of coilin. Our finding that PPM1G expression in WI-38 cells induces CBs (Fig. 7) supports this belief. Recent findings elegantly demonstrate that a wide range of CB components, including coilin and SMN, can trigger CB formation at an artificial gene stably integrated into the HeLa genome (Kaiser et al., 2008). This study supports a self-assembly model wherein cooperation amongst CB components is necessary for CB formation. We suggest that an additional requirement for the CB self-assembly model is that coilin must be in the correct phosphorylation state. The next step towards deciphering the repertoire of phosphatases and kinases that control coilin phosphorylation. We cannot detect a direct interaction between the CB self-assembly model is that coilin must be in the correct phosphorylation state. The next step towards deciphering the repertoire of phosphatases and kinases that control coilin phosphorylation. We cannot detect a direct interaction between coilin and PPM1G (our unpublished observations), so it is possible that PPM1G does not directly act on coilin, or that the interaction is transient and weak. Previous work has shown that phosphatase inhibitors alter CB localization (Lyon et al., 1997), although the exact phosphatase(s) responsible is unknown. With regards to kinases that modify coilin, we have shown that CDK2–cyclin-E and casein kinase 2 can phosphorylate coilin in vitro (Liu et al., 2000; Hebert and Matera, 2000); therefore, these kinases will be obvious targets of our future investigations. It will also be important to determine whether the phosphorylation of coilin influences its symmetrical dimethylation and thus interaction with SMN.

Materials and Methods

Cell lines, cell culture, DNA constructs and transfection

HeLa and WI-38 cells were obtained from the American Type Culture Collection. All cells were cultured and imaged as previously described (Sun et al., 2005). Where indicated, HeLa cells were treated with 0.4 μg/ml nocodazole for 16 hours to arrest cells in mitosis. The GFP-coilin clone has been described previously (Hebert and Matera, 2000). The GFP-coilin construct was used as a template to generate C-terminal mutations of residues known to be phosphorylated. This was accomplished using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA) and appropriate mutagenesis primers (supplementary material Table S1). Mutations were verified by sequencing. YFP-PPM1G and inactive YFP-PPM1G has been described previously (Petri et al., 2007; Murray et al., 1999). DNA was transfected into HeLa and WI-38 cells using either SuperFect (Qiagen), Lipofectamine 2000 (Invitrogen), or FuGene 6 according to the manufacturer’s directions. HeLa stable cell lines expressing GFP-coilin, GFP-coilin(ON) or GFP-coilin(OFF) were generated by G418 selection. For studies in coilin-knockdown cells, HeLa cells were transfected with coinplex duplex siRNAs (Whittom et al., 2008) for 48 hours, followed by transfection with the various coilin constructs for 24 hours. GFP-coilin signal can still be detected, albeit faintly, in the coilin-knockdown cells given that the EGFP vector has a CMV promoter. Cells were verified for endogenous coilin knockdown by assessing if SMN was present in CBs. Coilin knockdown was measured at the protein level by western blotting and comparing GFP-coilin (or mutants thereof) and endogenous coilin to tubulin after treatment with control or coilin siRNA.

Antibodies, immunofluorescence, coimmunoprecipitation and western blotting

Immunofluorescence, western blotting and image acquisition were performed as previously described (Sun et al., 2005). Monoclonal antibodies against SMN were from BD Biosciences. Colun polyclonal antibody H-300 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tubulin monoclonal antibody TUB 2.1 was purchased from Sigma (St Louis, MO). Antibodies were used in a modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate and 1% NP-40), followed by brief sonication. Cell debris was removed by centrifugation for 10 minutes at full speed in a 4°C microcentrifuge. The samples were then subjected to western blotting or immunoprecipitation. For immunoprecipitation, samples were first pre-cleared with 20 μl 50% protein-G-Sepharose (GE Healthcare) for 1 hour at 4°C with gentle inversion. After the incubation, the beads were collected by centrifugation at 3000 r.p.m. in a microcentrifuge for 5 minutes and the supernatant was placed in a new tube. To the supernatant was added 2 μg of monoclonal antibodies to GFP (Roche), followed by incubation for 1 hour at 4°C with gentle inversion. After the incubation, 30 μl of 50% protein-G-Sepharose was added to the samples and they were incubated for an additional 1 hour at 4°C with gentle inversion. The samples were then centrifuged at 3000 r.p.m. for 5 minutes and the beads were washed with 1 ml RIPA. The procedure was repeated two more times, after which the beads were resuspended in 20 μl ×5 SDS loading buffer and subject to SDS-PAGE and western blotting as described (Sun et al., 2005). Coilin (endogenous or fused to GFP) was detected using a polyclonal antibody (H-300) from Santa Cruz Biotechnology.

Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed on a Zeiss 510 Meta confocal LSM. Images were collected with the 488 line of an argon laser (30 mW output, detection 500-575 nm) with a Plan-Apochromat ×63/1.4 Oil DIC objective lens. During imaging, cells were maintained in LabTek II chambered coverslips (Nunc) and maintained at 37°C with an open chamber or a heated chamber equipped with the microscope. For each scan, three pre-bleach images were taken, and a single 2 μm spot containing a Cajal body was bleached with the 488nm line at 100% transmission. Images were obtained by scanning at 3% transmission at 3 second intervals and fluorescence recovery in the bleached area was monitored until the plateau was reached. Z-stacks were aligned with the stackreg plugin for ImageJ and resulting normalized FRAP recovery curves were subjected to double normalisation as previously described (Phair et al., 2004a; Phair et al., 2004b). The resulting recovery curves were plotted with Origin 6.1 (Microcal). The recovery curves for each cell were fitted to a double exponential equation and the time necessary to reach half-maximal recovery (T50) was determined as previously described (Phair et al., 2004a; Kimura and Cook, 2001).

Two-dimensional gel electrophoresis

Lysates were prepared from mitotic HeLa, interphase HeLa or WI-38 by resuspending cell pellets in 2-D solubilizer buffer containing 9.5 M urea, 2% NP-40 and 2% β-mercaptoethanol. The cells were then disrupted using the Sonex Dismembranator, Model 100 (Fisher Scientific, Pittsburg, PA), with an output of 1 for three pulses of 5 seconds each, with cooling on ice between each pulse. After sonication, the samples were centrifuged for 5 minutes at 17,000 g. Immobilized pH gradient (IPG) strips (Bio-Rad, Hercules, CA) were used according to the manufacturer’s protocol. Briefly, 125 μl sample was used to rehydrate 7 cm pH 7-10 or pH 5-8 IPG ready strips for 12-15 hours before isoelectric focusing (IEF). Following IEF using a Protein IEF cell (Bio-Rad) for 10,000 volt-hours at 50 μA per strip with rapid ramping, the strips were rehydrated for 20 minutes with equilibration buffer containing 6 M urea, 2% SDS, 0.05 M Tris-HCl pH 8.8, 20% glycerol, and 2% β-mercaptoethanol, followed by SDS-PAGE (10%) and Western blot analysis. Images were obtained using the Bio-Rad Molecular Imager ChemiDoc XRS system and processed as previously described (Sun et al., 2005). For treatment with calf intestinal alkaline phosphatase (CIP), cell pellets were first lysed in RIPA as described above, followed by the addition of 10 μl of 10 U/μl CIP from New England Biolabs (Ipswich, MA) in 1× New England Biolabs buffer 2. The reactions were incubated for 1 hour at 37°C, followed by a buffer change into 2-D solubilizer buffer using an YM30 Microcon filter unit (Millipore Corporation, Bedford, PA) according to the manufacturer’s instructions.

cDNA synthesis and quantitative real-time PCR

cDNA was synthesized from RNA isolated from HeLa and WI-38 cells using the iScript cDNA Synthesis kit from Bio-Rad according to the manufacturer’s protocol. Samples were incubated at 25°C for 5 minutes followed by 42°C for 30 minutes and then 85°C for 5 minutes using a PCT-200 Peltier Thermal Cycler (MJ Research). For qRT-PCR, cDNA and primers outlined in supplementary material Table S2 were added to the 2X iScript SYBR green (Bio-Rad) and the reactions were subjected to a 10 minute incubation at 95°C, followed by 40 amplification cycles (95°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute) and a dissociation curve-analysis step. The reactions were conducted using a MX3000P or a MX3005P real-time PCR system (Stratagene). Amplification rates, Ct values and dissociation curve analyses of products were determined using MxPro (version 4.01) software. Relative expression was determined using the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001). Three independently isolated RNA samples were used for each cell line, and each sample was conducted in triplicate. Student’s t-test was used to determine statistical significance (a P-value of less than 0.05 is considered significant).
In vitro phosphatase assay
MitoHeLa cells were lysed and sonicated in RIPA buffer. The lysate was untreated, treated with 5 μl of 10 U μl CIP from New England Biolabs (Ipswich, MA), or treated with 2.6 μM recombiant His-tagged PPM1G in 1× New England Biolabs buffer 2 for 1 hour at 37°C, followed by SDS-PAGE, western blotting, and detection of coilin using appropriate antibodies.

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References
Phosphorylation impacts CB formation


**Fig. S1**

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<th>coilin KD</th>
<th>GC-WT</th>
<th>GC-ON</th>
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GFP-coilin or mutant

coilin

tubulin

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<th>Table S1.</th>
<th>Primers used for coilin mutagenesis</th>
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