Cajal-body formation correlates with differential coilin 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 coilin, the CB marker protein, might impact CB formation. We hypothesize that primary cells, which lack CBs, contain different phosphoisoforms of coilin compared with that found in transformed cells, which have CBs. Localization, self-association and fluorescence recovery after photobleaching (FRAP) studies on coilin phosphomutants all suggest this modification impacts the function of coilin and may thus contribute towards CB formation. Two-dimensional gel electrophoresis demonstrates that coilin 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 coilin 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 coilin is the target of an uncharacterized signal transduction cascade that responds to the increased transcription and snRNP demands found in transformed cells.

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Key words: Coilin, SMN, Small nuclear ribonucleoproteins, Nuclear organization

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; Ciocie and Lamond, 2005; Matera and Shpargel, 2006). CBs participate in spliceosomal small nuclear ribonucleoprotein (snRNP) biogenesis. Specifically, CBs contain small Cajal-body specific RNAs (scaRNAs) that guide modification of the snRNA moiety of the snRNP (Darzaq 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 spliceosomal 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; Tomlinson et al., 2006; Tomlinson 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 coilin (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 (Darzaq et al., 2002; Matera, 1999). For example, 70% of coilin 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 (Atcoolin) 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 Gemin, 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 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 phospho-residues that regulate the availability of the N-terminal self-interaction domain (Shapergel 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 RNA component of the newly made snRNP. 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 phospho-residues 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 to threonines (C6A) (Fig. 1), mimicking a dephosphorylated state. In the second mutant, the serines were changed to aspartates and the threonines were converted to...
 Constructs into cells in which endogenous coilin was knocked down using RNA interference. Previous work has shown that transiently transfected coilin duplex siRNAs reduce coilin levels and disrupt CBs (Lemm et al., 2006; Whittom et al., 2008). Additionally, the level of GFP-tagged coilin (or mutants thereof) in coilin-knockdown cells is approximately equal to the level of endogenous coilin in cells treated with control siRNA (supplementary material Fig. S1). By following this protocol, therefore, the impact of endogenous coilin on the CB formation potential of the various phosphomutants is greatly reduced. Compared with its localization in normal HeLa cells, WT GFP-coilin expression in the coilin-knockdown background increases the percentage of cells with only nucleoplasmic localization (Fig. 2C). An increase in the percentage of cells with nucleoplasmic localization was also observed for the C6D mutant (Fig. 2C). By contrast, the localization of C6A, ON and OFF mutants in the coilin-knockdown background did not vary dramatically from that observed in normal HeLa cells (compare Fig. 2B to 2C). We conclude from this analysis that endogenous coilin facilitates the incorporation of GFP-tagged WT and C6D mutant proteins into CBs.

**Colin phosphoresidues impact self-association and mobility**

Coilin is phosphorylated on several residues during interphase, with other residues becoming phosphorylated during mitosis (Carmo-Fonseca et al., 1993; Beausoleil et al., 2004; Olsen et al., 2006; 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 phosphosresidues, 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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**Fig. 1.** Known coilin phosphoresidues and mutations. Schematic of human coilin showing the locations of the self-association domain and RG box. Below is the C-terminal sequence from residue 562 to the end of the protein, residue 576. Residues shown to be phosphorylated by tandem MS/MS analysis are indicated in the schematic (T122, S271, S272, T303, S489) or by asterisks in the sequence (S566, S568, T570, S571, S572, T573). Mutations of these six C-terminal residues to alanine (C6A) or glutamate/aspartate (C6D/E, denoted in subsequent text as C6D) are shown. The OFF and ON coilin mutants contain mutations of all 11 phosphorylated residues.

**Fig. 2A to 2C.** We next investigated the localization of GFP-tagged coilin phosphomimics in which all 11 suspected phosphorylation sites were converted to a phosphorylated or a dephosphorylated state. These constructs (denoted as OFF for the dephosphorylated mimic and ON for the phosphorylated mimic) were transiently transfected into HeLa cells (Fig. 2A). For the OFF mutant, 40% of expressing cells displayed a normal coilin localization pattern (Fig. 2B). However, 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 coilin in subsequent text as C6D) are shown. The OFF and ON coilin mutants contain mutations of all 11 phosphorylated residues.
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
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 cell 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 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
Phosphorylation impacts CB formation

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.

Discussion

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.

![Graph A](image1)

![Graph B](image2)

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 the

This study, transient overexpression of SmB in a primary cell line that does not normally contain CBs is sufficient to induce the appearance of correspondingly transient CBs (Sleeman et al., 2001). Conversely, the overexpression of coilin in this cell line does not induce CBs. We hypothesize that the transient expression of Sm proteins in primary cell lines triggers a signal transduction cascade that changes the phosphorylation status of coilin from a CB-restrictive to a CB-permissive state. When Sm protein levels decrease, we suspect that coilin is restored to a CB-restrictive phosphorylation state and CBs disperse.

The data presented here provide evidence supporting the hypothesis that coilin phosphorylation impacts CB formation and may be part of an unknown signaling pathway initiated in response to the increased demand for snRNPs. Importantly, we demonstrate that coilin is hyperphosphorylated in the primary WI-38 line compared with that found in the transformed HeLa line (Fig. 5). We also show that PPM1G mRNA levels are reduced in WI-38 compared with HeLa cells (Fig. 6A). Since PPM1G is a known phosphatase of SMN and can dephosphorylate coilin in vitro (Fig. 6B), our results indicate that this phosphatase directly or indirectly, impacts coilin phosphorylation levels and thus explain in part why transformed cells have CBs whereas primary cells lack these structures. Other work shown here reveals that CBs can be induced to form in the WI-38 primary cell upon expression of 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 coils, 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.

The other study that suggests that coilin phosphorylation impacts CB formation is from the Lamond group (Sleeman et al., 2001). In this study, transient overexpression of SmB in a primary cell line that does not normally contain CBs is sufficient to induce the...
Phosphorylation impacts CB formation

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); 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.

Phosphorylation impacts CB formation

CDK2–cyclin-E and casein kinase 2 can phosphorylate coilin in vitro (Liu et al., 2000), 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 (Whittom et al., 2008) for 48 hours, followed by transfection with the various coilin constructs. The samples were centrifuged for 5 minutes at 17,000 × g of monoclonal antibodies to GFP (Roche), followed by a buffer change into 2-D solubilization buffer using an YM30 Microcon filter unit. The samples were then subjected to western blotting or immunoprecipitation. 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

Mitotic HeLa 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 2.5 μM recombinant 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 coin using appropriate antibodies.

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