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Phosphorylation and the Cajal body: Modification in search of function

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Abstract

The Cajal body (CB) is a subnuclear domain that contains proteins and factors involved in a diverse range of activities including ribonucleoprotein maturation, histone gene transcription and telomerase assembly. Among these activities, the CBs' role in small nuclear ribonucleoprotein (snRNP) biogenesis is best characterized. Although CBs are found in plants, flies and mammals, not all cell types contain CBs. Rather, CBs are most prominent in transcriptionally active cells, such as cancer and neuronal cells. Many CB components, including the CB marker protein coilin, are phosphorylated in humans. The functional consequence of phosphorylation on CB assembly, activity and disassembly is largely unknown. Also unknown are the signaling pathways, kinases and phosphatases that act upon proteins which localize in the CB. The goal of this review is to demonstrate the need for a concerted effort towards elucidating the functional consequence of phosphorylation on CB formation and activity.

Keywords

Coilin; Kinase; Phosphatase; Signal transduction; SMN; Spinal muscular atrophy

Introduction

The eukaryotic nucleus contains an astonishing array of compartments, foci, domains and bodies. These subnuclear structures presumably allow for vital nuclear processes, such as RNA synthesis and processing, to occur in the most efficient manner for a given cell type. One of these structures is the Cajal body (CB). The initial discovery of CBs was made in 1903 by Ramon y Cajal, who called these subnuclear domains "nucleolar accessory bodies" due to their frequent association with nucleoli in neurons [1]. There are several excellent reviews that discuss major advances in the study of CBs, and the reader is encouraged to examine this literature for a comprehensive overview about CB function [2-7]. This review will focus on the role of phosphorylation on CB formation and function, as well as explore some of the signaling pathways that may impact phosphorylation of CB components. In the first section of the review, CB composition and activity will be described in brief. This will be followed by an examination of conditions that alter CBs, and signaling pathways triggered by these conditions. Phosphoproteins that accumulate in CBs will be discussed, as will the kinases and phosphatases known to modify these proteins. The review will end with an analysis of

important questions that need resolution in order to better understand the influence of phosphorylation on CB activity.

The Cajal body: What have we learned in the last 107 years?

A cursory PubMed search demonstrates that around 90% of publications on CBs have come since the identification in 1991 of antibodies that recognize coilin, the CB signature protein [8,9]. Interestingly, CBs are heterogeneous (described below) and some factors will co-localize with coilin in CBs during specific conditions, such as cell-cycle phase. Knockout and knockdown studies have shown that coilin is essential for proper CB formation and composition [10-14]. No obvious growth phenotype in mutants lacking coilin and CBs in *Arabidopsis* or *Drosophila* is detected [11,12]. However, HeLa cells depleted of coilin by RNAi proliferate slower than control treated cells [13,14], and knockout mice display reduced viability, fertility and fecundity [10,15]. Coilin and CBs, therefore, are not essential for survival, but their conservation from vertebrates to plants to flies strongly suggests that their presence must be of some benefit.

Many cell types do not have CBs [16,17]. Thus cells with CBs must have a physiological need for their existence, although the activities that take place within the CB likely can also occur in the nucleoplasm. Additionally, most (if not all) components found in the CB also localize to other cellular compartments such as the cytoplasm, nucleoplasm and nucleolus [18]. For some proteins that accumulate in the CB, it is not clear if their function in the CB is the same function found for these proteins in other cellular compartments, causing further complications in understanding the role of CBs in the cell. Moreover, some proteins initially thought to reside in CBs (e.g. NPAT and FLASH) have recently been shown to localize in histone locus bodies (HLBs) [19-23]. Replication-dependent histone gene clusters have been shown to associate with HLBs. This association allows for components in the HLB to orchestrate replication-dependent histone gene transcription and 3'-end pre-mRNA processing (reviewed in [22]). Interestingly, HLBs and CBs fuse during the S phase of the cell cycle [24]. Thus CB composition changes during the cell cycle. Collectively, the lack of CBs in some cell types, the localization of CB proteins to other cellular compartments, and the heterogeneity of CBs all conspire to make elucidation of CB function a challenge.

A key to understanding CB function stems from the finding that CBs contain the highest concentration of small nuclear ribonucleoproteins (snRNPs) in the nucleus [25]. Although snRNPs play a vital role in pre-mRNA splicing, active splicing does not take place within the CB [18]. Rather, the CB participates in snRNP modification and assembly. Evidence in support of this idea comes from detailed studies into snRNP biogenesis. Splicing snRNPs are comprised of a small nuclear RNA (snRNA), a septet of Sm proteins and additional snRNP-specific proteins [26]. U1, U2, U4 and U5 snRNA (but not U6 snRNA) are transported to the cytoplasm for processing and Sm protein assembly under the control of the survival of motor neuron (SMN) protein [27] [28]. Mutations in SMN cause most cases of spinal muscular atrophy (SMA), the leading genetic cause of infant mortality (reviewed in [27]). Upon nuclear re-entry, newly assembled snRNPs target first to the CB before they accumulate in speckles, another subnuclear domain [29]. In the CB, the snRNA moiety of the nascent snRNP is subject to modification reactions guided by small Cajal-body specific RNAs (scaRNAs) [30,31]. When certain scaRNAs are misdirected to the nucleolus, their cognate snRNAs are not properly modified [31]. snRNA modifications are necessary for proper snRNP function [32-34]. Interestingly, overexpression of one Sm protein (Smb) in a cell line that normally lacks CBs induces the formation of this subnuclear domain [35].

Taken together, the above findings strongly argue for a role of the CB in the efficient maturation of snRNPs. Again, however, cell types without CBs still have sufficient snRNP resources to

splice pre-mRNA, so CBs and the activities therein are not absolute requirements for cell function. ScaRNAs, for example, have been shown to properly modify snRNAs in flies lacking CBs [12]. Rather, it is thought that CBs facilitate snRNP biogenesis by providing a locally high concentration of the requisite materials. For example, elegant studies by Neugebauer and colleagues have shown that U4/U6 di-snRNP assembly is increased 11-fold in cells with CBs compared to the assembly rate in cells lacking CBs [36]. Since CBs are most prominent in cells that are transcriptionally active, such as neuronal and cancer cells [25,37-40], it is logical to assume that reactions facilitated by the CB contribute to the overall physiological robustness of these cell types.

It is notable that SMN, in most cell lines and adult tissues, is found in CBs in addition to the cytoplasm [41,42]. The exact role of SMN in the CB is not clear. Since SMN is critical for the nuclear import of nascent snRNPs to the CB [43], the accumulation of SMN in CBs may be reflective of this function. Support for this hypothesis comes from data showing that coilin can directly interact with SMN and Sm proteins [44-47]. Alternatively, or additionally, the SMN in CBs may facilitate the regeneration or recycling of snRNPs after a splicing reaction has taken place [46,48]. Curiously, in some cell lines and fetal tissues, nuclear SMN is found in Gems (for Gemini of CBs), which are nuclear bodies often found adjacent to CBs [49,50]. The function of Gems, which contain SMN and associated proteins known as Gemins but lack snRNPs, remains obscure. Interestingly, cells that display Gems still have CBs. These CBs are indistinguishable from CBs found in cells without Gems except that they are not enriched for the nuclear SMN complex proteins. In this review, we refer to 'canonical CBs'. Canonical CBs are those found in most cell lines and adult tissues and contain, among other factors, coilin, SMN, and snRNPs.

The composition of the CB and its nuclear dynamics suggest other functions besides those centered upon snRNP maturation and maintenance. CBs are mobile and associate with chromatin in an ATP- and transcription-dependent manner [51]. Furthermore, CBs have been shown to associate with snRNA genes (e.g. U2 genes) and histone gene clusters, as discussed above (reviewed in [7]), suggesting that CBs play a role in the transcription of these genes. Support for this hypothesis comes from the observation that basal transcription factors, such as RNA polymerase I and II, TFIIF, TFIIF, TFIIF, TBP and PTF- γ , are enriched within the CB, possibly indicating that these factors are assembled or processed in the CB before transport to their site of action [7,52,53]. The association of CBs with certain genes may therefore indicate that these genes require locally high concentrations of these factors. Alternatively, the fact that snRNPs are highly enriched in the CB supports the idea that CB association with U snRNA genes is the manifestation of some type of feedback regulatory control on the transcription of these genes [18].

Besides Gems and certain gene loci, CBs have been shown to occasionally associate with PML bodies with a high degree of statistical significance [54]. PML (promyelocytic leukemia) bodies contain numerous factors implicated in a variety of cellular functions, such as transcriptional regulation [18,55,56], and have been shown to associate with transcriptionally active gene loci [57,58]. We have demonstrated that a CB can simultaneously associate with an U2 gene locus and a PML body, possibly indicating that these two structures may work in concert to provide a hitherto unknown mechanism of regulatory control [59]. Additionally, CBs associate with telomeres [60,61]. This is an interesting result given that telomerase RNA has a structure similar to scaRNAs and is found within the CB. Telomerase RNA and scaRNAs require the WDR79/TCAB1 protein for localization to CBs [62,63]. In the CB, the telomerase RNA is most likely subject to modification reactions and holoenzyme assembly [64-66]. Thus CBs participate in the biogenesis and delivery of telomerase to telomeres.

CB dynamics: From cold carp to KO'ed flies

In order to better understand CB function, it is helpful to consider circumstances that either disrupt or promote canonical CBs assembly (Table 1). Strikingly, 13 of the 47 conditions listed increase coilin and/or CB component association with the nucleolus. Considering that Cajal initially called CBs “nucleolar accessory bodies” because he often observed them near nucleoli [1], the existence of biochemical dialog between CBs and nucleoli should not be surprising. Exactly why this happens, however, and what benefit it confers, is unclear. CBs and nucleoli share some components, Nopp140 and fibrillarin for example. Both of these proteins interact with snoRNAs (small nucleolar RNAs), which suggests that their presence in CBs may facilitate snoRNP (small nucleolar ribonucleoprotein) maturation [6]. Furthermore, Nopp140 and fibrillarin interact with CB proteins coilin and SMN, respectively [67-69], providing a direct link between the CB and nucleolus. Another link between CBs and nucleoli comes from studies in *Drosophila* that show that poly (ADP-ribose) polymerase 1 (PARP1) is important for CB integrity and may facilitate the localization of nucleolar proteins, like fibrillarin, to the CB [70]. An ancestral connection between CBs and nucleoli may be evident in budding yeast, which contains a structure known as the nucleolar body [71]. The nucleolar body contains components that are found within CBs, so it is possible that the CB assumed the nucleolar body capacity in higher eukaryotes. Situations listed in Table 1 that mislocalize CB or CB components to nucleoli, therefore, may be indications that the connection between CBs and nucleoli has been evolutionarily conserved, but not fully understood.

Viral infection by adenovirus or herpes simplex virus type 1 (HSV-1) has also been shown to alter typical CB localization. For instance, infection of HeLa cells with adenovirus induces the formation of between 50 and 100 coilin microfoci that do not contain snRNPs [72]. It was speculated that this phenotype was secondary to the inhibition of host protein translation as a consequence of the viral infection. In support of this hypothesis, CBs disassemble upon treatment with translation inhibitors [72]. HSV-1 infection also generates coilin microfoci that do not contain snRNPs [73]. This effect is mediated by the HSV-1 infected cell protein 0 (ICP0). Most interestingly, Lomonte and colleagues have shown that these microfoci correspond to centromeres, implying that coilin and some CB proteins are part of a novel response termed interphase centromere damage response (iCDR) [73]. It is not known at this point if the coilin microfoci induced by adenovirus also localize to centromeres.

With regard to the role of specific proteins on CB formation, many studies have employed RNA interference to knockdown proteins known to accumulate in CBs or be important for snRNP biogenesis. Other approaches have overexpressed a given protein or utilized dominant negative mutants. Together, these studies have shown that CB integrity is not mediated by just one component, but can be impacted by several different proteins that have dissimilar functions (Table 1). In excellent work by Luhrmann and colleagues, knockdown of hTGS1 (which is responsible for the 5'-cap hypermethylation step of snRNAs during snRNP biogenesis), PHAX (which is needed to export newly transcribed U1, U2, U4 and U5 snRNAs to the cytoplasm), and SMN (which is required for the assembly of Sm proteins onto the snRNA and subsequent re-import into the nucleus) were all shown to disrupt CBs [13]. Curiously, knockdown of hTGS1 and SMN, but not PHAX, also results in nucleolar coilin localization. Other studies have likewise shown that SMN knockdown disrupts CBs and mis-localizes coilin to the nucleolus [74,75]. A dominant negative mutant of SMN (SMN Δ N27), in contrast, forms large nuclear structures that accumulate CB proteins [48]. Knockdown of snurportin, which is needed for the import of newly generated snRNPs from the cytoplasm to the nucleus, results in the same phenotype as observed for SMN and hTGS1 knockdown [75] and overexpression of snurportin decreases CB number [76]. Inhibition of U snRNA export to the cytoplasm using leptomycin B also decreases the frequency of CBs [41]. Collectively, these findings demonstrate that snRNP biogenesis is required for CB formation. CB disruption, however, is

not limited to the inhibition of snRNP biogenesis, as evidenced by FLASH knockdown. FLASH is a component of histone locus bodies (HLBs) and during S phase, the proteins within HLBs and CBs are co-localized. Knockdown of FLASH causes CB disassembly and the accumulation of coilin in nucleolar caps [24,77], indicating that active replication-dependent histone gene transcription and 3'-end premRNA processing are necessary for CB integrity.

Coilin knockdown also triggers CB disappearance and reduces proliferation rates [13,14]. With no known enzymatic activity, coilin in the CB plays a fundamental role in the formation and composition of this structure. For example, immunodepletion of frog coilin results in CBs that lack snRNPs [78] and anti-coilin antibody injection in HeLa cells abolishes canonical CBs [79]. Coilin knockout models have been made for mouse [10], fly [12] and plant [11]. These models all show that coilin is required in order to form canonical CBs. Further verification of the importance of coilin for CB structure comes from several studies utilizing various dominant negative coilin mutants (see Table 1). Of particular note for this review are coilin phosphomutants that impact CB formation and localization [80-82]. Coilin was shown to be a phosphoprotein by Lamond and colleagues in 1993 [38], but the functional consequence of this modification on coilin and CB activity has yet to be fully understood. Before examining what is known about phosphorylation and CB activity, the role of another coilin modification, symmetrical dimethylarginine (sDMA), on CB formation will be discussed. Interactions between SMN and coilin are influenced by the methylation status of coilin [44,45,83] and hypomethylated coilin is correlated with Gem formation in HeLa cells [45,83]. Additionally, methylation inhibitors have been shown to decrease the frequency of CBs and increase Gem number [83,84]. Studies conducted by the Sleeman group nicely show that coilin methylation is not the sole determinant in Gem formation in SH-SY5Y neuroblastoma cells [84]. Rather, increasing levels of SMN observed during differentiation, and the resulting upsurge in snRNP biogenesis, appear to be instrumental in the fusion of CBs with Gems [84]. To further evaluate the role of methylation on coilin/SMN localization, PRMT5 levels in both HeLa and SH-SY5Y cells were reduced by RNAi. Knockdown of PRMT5, which is a methylase that adds sDMA modifications to coilin and Sm proteins, results in abnormal foci containing both coilin and SMN in HeLa cells, and no change in coilin/SMN localization in SH-SY5Y cells [84]. These findings suggest that different cell types regulate CB formation and composition by varied methods. Other work shows that the abnormal foci induced by PRMT5 knockdown are likely nucleolar caps, and reduction of PRMT7, which also modifies Sm proteins, produces a similar phenotype [85].

Further evidence demonstrating that CB structure and composition is influenced by a range of inputs comes from studies on proteins known to be part of signaling pathways or involved in transcription. One of these proteins is the nuclear isoform of fibroblast growth factor-2 (FGF-2²³). This protein indirectly regulates CB composition by binding SMN and influencing the number of Gems (overexpression of FGF-2²³ decreases Gem number) [86-88]. This is an important finding because it suggests that SMN may be a regulator of the integrative nuclear FGFR1 signaling (INFS) pathway. The INFS pathway, via activation of the CREB-binding protein, can upregulate gene activities associated with differentiation of neuronal cells [89]. Another signaling protein that impacts CBs is ZPR1. ZPR1 interacts with epidermal growth factor receptor (EGFR), the translation elongation factor-1 α (EF-1 α) and is part of a complex that includes SMN [90-92]. CBs and Gems are absent in the ZPR1 knockout mouse, clearly demonstrating a role for this protein in their maintenance [93] and hinting at a possible link between mitogenic signals and CB formation [94]. UV-C has been shown to disassemble CBs, with the proteasome activator subunit γ (PA28 γ) playing a role in this stress response [95]. In the absence of UV-C, overexpression of PA28 γ fragments CBs, showing that this protein also impacts CB integrity [95]. Interestingly, MEKK3, a kinase that responds to both mitogenic and stress signals, has been shown to interact with PA28 γ [96], indicating that CB formation and assembly might be a part of the MEKK3 signaling pathway. In regards to transcription

factors, the RNA polymerase II transcription elongation factor ELL and its associated protein EAF1 have been shown to accumulate in CBs [97]. MLL-ELL leukemia and expression of the MLL-ELL protein abolish canonical CBs [97], indicating that transcription elongation is important for CB formation. This finding thus confirms previous studies showing that transcription inhibition disrupts CBs [98,99].

CB formation: A response to increased snRNP demand

Upon further reflection of the information in Table 1, it is also important to appreciate that the dynamic nature of CBs is a manifestation of the interconnections between this subnuclear domain and overall cell metabolic state. Given what is known about the role of CBs, it makes sense, for example, that carp in the summer would have more CBs than those acclimatized to winter because fish in the summer will require more snRNPs [100]. It is also logical for cells to have more CBs during one stage of development compared to another in order to accommodate the level of RNA processing found during embryogenesis, as recently described in zebrafish [101]. The fact that CBs disassemble when transcription, translation, snRNA export or snRNP biogenesis is inhibited likewise clearly indicates that the formation of CBs is intimately tied to cellular activity. But how does this happen? What are the signals, messengers, modifiers and components that make the CB dynamic? Information that may assist us in the quest for these answers is found in conditions that induce CBs.

Compared to the number of investigations that report conditions that disrupt CBs, there are relatively few studies in which the formation of CBs was examined. The most seminal of these describes how CB frequency increases with transformation or immortalization [16]. In particular, one interesting finding from this study was that CBs were present in 24% of immortal Ref-52 cells, but this percent increased to 99% when the line was transformed by adenovirus. Transformation, therefore, directly correlates with CB formation. CB frequency has also been shown to increase upon osmotic shock of rat neurons [102], corresponding to increased gene expression. In another study, the WI-38 primary fibroblast line was examined and found to contain CBs in 2-3% of cells when incubated at 37°C, but 90% of cells contained CBs when cultured at 32°C [38]. It is not clear why CBs are induced in this line when grown at 32°C, but it is possible that modifications or expression levels of CB components underlie this phenomenon. In this same paper, the authors showed that coilin is phosphorylated during interphase and this modification increases during mitosis when CBs disassemble [38]. After mitosis, CBs reform by mid G₁ of the cell cycle [37,38]. Hyperphosphorylation of coilin during mitosis correlates with reduced self-association, providing one example of how phosphorylation may impact CB dynamics [103].

The remaining studies that induce CBs all rely upon exogenously added CB components. U7 snRNA, for example, when injected into frog oocytes induces CB formation [104]. Using a very sophisticated experimental model, Dundr and colleagues demonstrate that tethering a number of different CB components to a specific locus in the genome is sufficient to nucleate a CB at that location [105]. Knockdown studies using this system reveal that coilin and SMN work together in the generation of canonical CBs via a stochastic assembly model [2,105]. Further information regarding factors necessary for CB assembly comes from heterokaryon experiments using HeLa and DFSF1 cells [35]. Fusion of HeLa cells, which have CBs, with the primary DFSF1 cells, which lack CBs, leads to the induction of CBs in DFSF1 nuclei. These data demonstrate that factors in HeLa cells are capable of inducing CBs, and that DFSF1 cells are capable of receiving that signal. To parse out that factor, Sleeman and colleagues then showed that transient transfection of YFP-SmB is sufficient to induce correspondingly transient CBs in DFSF1 cells [35]. CB formation, therefore, may be exquisitely tuned to the flux of snRNPs in a given cell type.

Various studies on coilin have also showed that this protein can induce CBs, or at the very least, CB-like structures, depending on the species of the coilin used or expression construct. When GFP is fused to the N-terminus of coilin (either human or mouse), the GFP-coilin fusion protein localizes properly. In contrast, fusion of GFP to the C-terminus of coilin (coilin-GFP) results in the formation of numerous CB-like foci that contain Sm epitopes and fibrillarin [103]. Additionally, a mutant of human coilin (1-481), also produces numerous foci similar to that found for coilin-GFP [81]. Interestingly, while human GFP-coilin expressed in human cells (HeLa) generates normal coilin localization, human GFP-coilin expression in coilin knockout mouse embryonic fibroblast (MEF) cells very seldom produces CBs. In contrast, mouse GFP-coilin and frog GFP-coilin can readily form CBs in both HeLa and coilin KO MEF cells. Chimeric mapping experiments show that the very C-terminus of coilin plays a role in regulating CB number, and this region contains several phosphoresidues [47,81,82].

The pathways that may regulate CB formation

In summary with regard to conditions shown in Table 1 that lead to CB formation or disassembly, it is clear that this structure is responsive to the overall levels of transcription in general and cellular snRNP demand in particular. How this takes place is not very well understood, and may involve the posttranslational modification of important CB proteins by phosphorylation. Evidence in support of this hypothesis comes from work showing that the serine/threonine phosphatase inhibitor okadaic acid results in the accumulation of CBs in nucleoli, and a similar phenotype is observed in cells transfected with coilin containing a serine 202 to aspartate mutation [80,106]. In addition to coilin, phosphorylated proteins known to accumulate in or impact CBs are listed in Table 2. This table also lists proteins that are involved in signaling pathways or transcription, but not known to be phosphorylated. For most of the CB phosphoproteins, the kinases and phosphatases responsible for their modification are unknown, as are the signals and transducers that effect this change. Even more disheartening is the utter lack of knowledge about the functional consequence that these modifications have on the role of these proteins in the CB, if at all. Clearly the CB field has a long way to go before an archetypical signal transduction map can be generated that includes all the players, from primary messenger to cellular response and the role of the CB in meeting that desired response.

With what is known about other signaling pathways, can we find connections between these pathways and CB formation, composition, activity or disassembly? During serum starvation, CB numbers decrease [37]. Since serum contains growth factors and mitogens, it is germane to examine what pathways are triggered by these factors and if any CB associated proteins are in these pathways. The major pathway by which mitogens promote entry into the cell cycle involves stimulation of Cdk activity. In particular, mitogens, via Ras activation of a MAP kinase lead to increased expression of regulatory proteins such as Myc. The expression of many genes required for cell cycle progression (such as cyclin D) and cell growth is stimulated by Myc. Cdk4/cyclinD then phosphorylates Rb, resulting in active E2F that promotes S-phase gene transcription. Among these are genes that encode cyclin E and cyclin A, which are necessary for the G₁-S transition and S-phase, respectively. Could any of the proteins listed in Table 2 be substrates for the various phosphorylation events that take place in response to mitogens? Cdk2/cyclinE is enriched in the CB during the G₁-S transition and can phosphorylate coilin in vitro [107]. Additionally, NPAT was initially isolated as a substrate for cdk2/cyclinE and its phosphorylation is necessary for histone transcription and cell cycle progression [23, 58,108-111]. Along with FLASH, NPAT and other HLB proteins co-localize with CBs during the S phase [19], indicating that the crucial histone transcription and processing activities that occur in HLBs are present in CBs at this time. Along with cdk2/cyclinE, the cdk activating kinase cdk7/cyclinH/mat1 is enriched within CBs [99]. DNA damaged-induced down regulation of cdk2/cyclinE-mediated phosphorylation of NPAT has been proposed as a mechanism to reduce histone synthesis at the G₁ checkpoint [112]. In contrast, the exact roles

that cdk2/cyclinE and the cdk7/cyclinH/mat1 complex play in regulating CB activity during the cell cycle are unknown and clearly an interesting area for future investigations.

Other proteins that impact CBs and respond to mitogenic stimuli are FGF-2²³ and ZPR1. As discussed above, FGF-2²³, which interacts with SMN and influences Gem formation, is part of the integrative nuclear FGFR1 signaling (INFS) pathway and, as such, may be able to transmit mitogenic signals to SMN and the CB [86,113]. Although FGF-2²³ is not known to be phosphorylated, this protein can be methylated in vitro by PRMT5, and methylase inhibitors cause cytoplasmic accumulation of FGF-2²³ [88]. ZPR1 binds the cytoplasmic domain of the epidermal growth factor receptor (EGFR) and, upon binding of epidermal growth factor to EGFR, is released. Released ZPR1 can then interact with elongation factor 1 α and SMN. Interactions between ZPR1 and SMN facilitate SMN localization in CBs and ZPR1 knockout mice lack CBs [90,92,93]. Thus, ZPR1, like FGF-2²³, is able to transmit proliferation signals to the CB and influence its composition.

The mitotic signal transduction cascade also impacts CB protein phosphorylation. CBs disassemble during mitosis and reform by mid G₁ [37,38]. Coilin levels are relatively constant during the cell cycle [37], but the phosphorylation of coilin increases by at least two residues during mitosis [38]. Proteomic analysis of phosphorylated proteins during G₁ and M phase [114] corroborate that coilin contains residues that are specifically phosphorylated during mitosis [47]. Other phosphorylated CB proteins, Nopp140 for example, likewise show differential phosphorylation of certain residues during mitosis, whereas others, such as SMN, do not [114]. It is probable that these phosphorylation events are due to the activation of cdk1/cyclinB during the G₂/M transition, but, as with interphase phosphorylation, the exact kinases and phosphatases that act directly on CB proteins are largely undefined. Is there a functional consequence to the mitotic phosphorylation of CB proteins? For coilin at least, there is. Coilin is a self-associating protein, and this self-association is decreased during mitosis when coilin is hyperphosphorylated [103]. Moreover, coilin phosphomimics reduce self-association and increase mobility [82]. Together, these data indicate that coilin hyperphosphorylation during mitosis triggers CB disassembly. It is probable that coilin contains a certain contingent of phosphorylated residues that promote CB formation and another conformation that promotes CB disassembly. Interestingly, primary cells that lack CBs (WI-38), contain hyperphosphorylated interphase coilin compared to that found in transformed cells that have CBs (HeLa). In fact, the isoelectric point of coilin from interphase WI-38 cells is similar to that found for coilin from mitotic HeLa cells, suggesting that coilin phosphorylation status is one factor which regulates CB formation [82].

In addition to mitogenic and mitotic conditions, signals generated by stress also impact CBs and may alter protein phosphorylation in this structure. Adenovirus and HSV-1 infection induce coilin microfoci, as does inhibition of translation using anisomycin [72,115]. Such conditions may activate the double stranded RNA-activated kinase PKR, which is found in CBs [116]. Interestingly, the tumor suppressor protein p53 is found in a subset of CBs after inhibition of its degradation or activation of its expression [117]. SMN interacts directly with p53 and this interaction may influence p53-dependent apoptosis [117]. Viral infection may activate PKR and trigger the activation of other kinases that in turn modify p53 [118]. Stress resulting from UV-C exposure has also been shown to impact CBs in a mechanism dependent upon PA28 γ [95]. Since the levels of PA28 γ do not change substantially after UV-C treatment, Lamond and colleagues propose that the increased association between coilin and PA28 γ could be due to changes in the phosphorylation status of PA28 γ [95]. A candidate kinase for this modification is MEKK3.

Phosphorylation and the Cajal body: Where do we go from here?

To fully understand CB function, we need to appreciate the dynamic, heterogeneous, and enigmatically exasperating nature of this subnuclear domain. Deciphering the role of phosphorylation on CB formation, activity and disassembly is a difficult, but necessary step towards a functional comprehension of the CB. Most importantly, since CBs are connected to snRNP, histone and telomerase biogenesis, progress to this end may lead to the identification of novel cancer treatments designed to disrupt CB activity. As a first step in this direction, a thorough analysis of the kinases and phosphatases that modify CB proteins needs to be undertaken. The identification of these kinases and phosphatases will then facilitate the characterization of signaling pathways that impact the CB. Since coilin and SMN are key CB phosphoproteins [38,119,120], a modest effort has already been made to identify the kinases and phosphatases that modify these proteins. For example, coilin is an *in vitro* substrate for casein kinase II, cdk2/cyclinE and the nuclear phosphatase PPM1G [82,103,107]. PPM1G also modifies SMN [121]. Knockdown of PPM1G depletes SMN from CBs and may slightly fragment CBs [121], clearly indicating a role for PPM1G in the maintenance of CB integrity. Specifically, PPM1G may play a role in ensuring that nuclear SMN is hypophosphorylated relative to cytoplasmic SMN [120,121]. The Gruss lab is presently undertaking a large-scale effort to determine all the kinases and phosphatases that modify SMN (Oliver Gruss and Alma Husedzinovic, personal communication). When complete, this analysis will greatly contribute to our understanding of SMN regulation and indirectly provide information that will be relevant to the understanding of CB formation, activity, and disassembly.

One example of the functional insight that can be obtained from a thorough understanding of protein phosphorylation in the CB comes from a recent study showing that coilin phosphorylation differentially impacts SMN and SmB' interaction [47]. From these data, a model is proposed whereby SMN favors hypophosphorylated coilin while SmB' interaction with coilin is facilitated by phosphorylation (Figure 1). Such an arrangement may facilitate U snRNP cycling through the CB. Alternatively or additionally, phosphorylation-influenced interaction of coilin with SMN and Sm proteins may contribute to the targeting of SMN to CBs if coilin in the nucleoplasm is more phosphorylated compared to the coilin in CBs.

Upon consideration of the conditions that impact CBs (Table 1), the proteins in the CB that are phosphorylated (Table 2), the signaling proteins that affect CBs and the various signaling pathways that link to this structure, it is likely that activities and interactions of many CB proteins are modulated, as found for coilin, SMN and SmB', by phosphorylation. As if understanding the role of phosphorylation on CB function were not enough, work also needs to be conducted towards clarifying the relationship between phosphorylation and the PRMT5/7-mediated addition of sDMAs to CB proteins such as coilin. Furthermore, studies should also be conducted to determine if a phosphorylated protein in the CB contain a specific configuration of modified residues that differ compared to that found for this protein when it is localized to other cellular compartments. For example, does nucleoplasmic coilin have a different configuration of phosphorylated residues compared to coilin in the CB? Does fibrillarin in the nucleolus contain one set of phosphoresidues while fibrillarin in the CB has a different set? If so, then it is logical to assume that these proteins have a different or modified activity in the CB compared to other locales of the cells. Since there are at least 21 proteins that accumulate in the CB and are phosphorylated, much work remains. It is humbling, but in many ways the study of CBs has not advanced very much past a descriptive understanding of CBs akin to when Cajal first sketched a nucleolar accessory body. Hopefully it will not take 107 years to determine the role of phosphorylation on CB formation and activity.

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Fig. 1.

Phosphorylation of coilin's C-terminus may help disengage the SMN complex from the CB and compete Sm proteins away from SMN. Data from [47] demonstrate that a coilin C-terminal fragment (amino acids 362-576) recovers more SmB' in a pulldown assay when this fragment contains phosphomimic mutations compared to that bound to the wild-type fragment. In contrast, SMN binding to the phosphomimic coilin fragment is reduced compared to that recovered by the wild-type fragment. Based on this and other data reported in [47], the following model is proposed. In the CB, coilin may exist in two phosphoisoforms, one of which contains additional phosphorylated residues in the region of coilin that interacts with SMN (the RG box) and Sm proteins. Upon delivery of nascent snRNPs to the CB by the SMN complex, interactions between SMN and hypophosphorylated coilin (on left) displace snRNPs from the SMN complex. Released snRNPs can then bind hyperphosphorylated coilin (on right), allowing for snRNA modifications. Coilin phosphorylation by a kinase may promote SMN release from the CB, while phosphatase activity would release snRNPs. This mechanism may coordinate snRNP flux within the CB.

Table 1

Conditions that impact canonical Cajal bodies

Condition		Reference(s)	Notes
<i>Environmental</i>	Hibernation	[122]	In dormice, nucleolar CBs during hibernation; no CBs after hibernation
	Seasonal	[100]	More CBs in summer-acclimatized carp
	Mitosis	[38][37]	CBs disassemble during mitosis
<i>Cell cycle</i>	Fetal vs. adult	[17]	CBs present in fetal tissues that lack them as adult; the frequency of Gems also changes developmentally
<i>Developmental</i>	Zebrafish embryogenesis	[101]	CB number per nucleus varies during development
<i>Physiological changes</i>	Immortalization	[16]	Primary cells have the lowest frequency of CBs
	Transformation	[16]	Transformation increases frequency of CBs
	Breast cancer	[40]	CBs in nucleolus
<i>Treatments</i>	Temperature	[38]	CBs smaller at 39°C, bigger at 32°C; CB induction in primary line at 32°C
	Transcription inhibition	[98] [99]	Coilin mis-localizes to perinucleolar caps in cells treated with transcription inhibitors
	Translation inhibition	[72]	CBs disassemble upon treatment with anisomycin
	UV-C	[95]	Disassembly of CBs and partial mis-localization of coilin to speckles when treated with UV-C
	Adenovirus infection	[72][115]	Adenovirus infection induces coilin microfoci
	Herpes virus infection	[73]	HSV-1 and ICP0 cause some CB proteins to accumulate at centromeres (iCDR)
	Nuclear export inhibition	[41]	Leptomycin B depletes snRNPs from CB and reduces the number of CBs per cell
	Ser/Thr phosphatase inhibition	[80]	Okadaic acid causes coilin and snRNP accumulation in nucleolus
	Serum starvation	[37]	CB number decreases during serum starvation
<i>Alteration of specific factors</i>	Coilin mutants	[123]	Human coilin aa 1-291 can form large “pseudo-CBs”
		[80]	S202D mutant of human coilin mislocalizes to nucleolus
		[103]	Human coilin 1-248 and 1-315 localize to nucleoli and CBs, GFP fusion to C-terminus of coilin forms many CB-like foci
		[44]	Mutation of coilin RG box induces Gems
		[81]	Coilin C-terminal mutants impact CB number
		[82]	Coilin phosphomutants impact localization
		[13] [14]	Abolishes canonical CBs, but foci containing snoRNPs present
<i>Alteration of specific factors</i>	Coilin knockdown	[10]	Generates residual CBs that lack SMN and snRNPs
	Coilin knockout (mouse)	[12]	Abolishes CBs
	Coilin knockout (fly)	[11]	Abolishes CBs

Condition		Reference(s)	Notes
	Coilin knockout (plant)	[78]	In Frog, results in Cajal bodies that lack Sm proteins
	Coilin immunodepletion	[79]	Generate coilin microfoci; other Abs have no affect on CBs
	Coilin antibodies	[113]	Decreases the frequency of Gems
	FGF-2 ²³ overexpression	[24] [77]	Causes disappearance of CBs and perinucleolar localization of coilin
	FLASH knockdown	[105]	Promotes CB formation at specific genetic loci
	Immobilization of CB components	[83] [84]	Increase Gems and decrease CB numbers
	Methylation inhibitors	[97]	Delocalizes coilin from CBs
	MLL-ELL leukemia and MLL-ELL protein	[95]	Fragments CB
	PA28 γ overexpression	[70]	In <i>Drosophila</i> , fails to recruit fibrillarin to CBs
	PARP-1 mutant	[13]	Results in CB disassembly
	PHAX knockdown	[121]	Depletes SMN from CBs
	PPM1G knockdown	[85] [84]	Results in nucleolar caps or abnormal foci containing coilin and SMN
	PRMT5/7 knockdown	[35]	Induces CBs in primary line
	SmB overexpression	[75] [74] [13]	Disrupts CBs; accumulation of some coilin in nucleoli (same general phenotype as observed for knockdown of hTGS1 and snurportin)
	SMN knockdown	[48]	Generates large nuclear accumulations of CB components
	SMN deltaN27 mutant	[75]	Abolishes CBs, causes some coilin to localize in nucleoli
	Snurportin knockdown	[76]	Reduces CB number
<i>Alteration of specific factors</i>	Snurportin overexpression	[13]	Disrupts CBs and leads to the accumulation of some coilin in nucleoli
	TGS1 knockdown	[104]	Injection of U7 into <i>Xenopus</i> oocytes induces CBs
	U7 snRNA	[93]	CBs are abolished
	ZPR1 knockout mouse		

Table 2Phosphorylated CB components¹ and signaling proteins that impact CBs

Protein	Phosphorylated?	Notes
Coilin	Yes	Modified in vitro by CKII, cdk2/cyclinE, PPM1G [107] [103] [82]
Cdk2/cyclinE	Yes	Important regulator of cell cycle progression
Cdk7/cyclinH/mat1	Yes	Cdk activating kinase (CAK) in CBs [99]
EAF1	Yes	Transcription factor in CBs [97]
ELL	Yes	Transcription factor in CBs [97]
Fibrillarin	Yes	In CBs and nucleoli; modifies ribose by methylation
FLASH	Yes	Important for histone transcription and pre-mRNA processing [22]
Nopp140	Yes	Impacts RNA pol I transcription [124]
NPAT	Yes	In HLBs [19]; substrate for cdk2/cyclinE [108]
p53	Yes	Well known sensor of cell stress with important roles in vital cellular processes
PA28 γ	Yes	Mitogenic/stress kinase MEKK3 interacts with PA28 γ [96]
PARP1	Yes	Participates in recruitment of fibrillarin to CBs [70]
PHAX	Yes	Important for snRNA and snoRNA transport [125]
Pigpen (TLS)	Yes	Transcription factor and RNA-binding protein [126,127]
PKR	Yes	Double stranded RNA-activated kinase. In CBs and nucleoli [116]
PPM1G (not in CB)	Yes	Nuclear phosphatase that acts on SMN and coilin [121] [82]
Profilin 1	Yes	May play a role in CB movement [128]
PTF γ	Yes	snRNA gene-specific transcription factor [129]
SART3/p110	Yes	Localizes to CBs via interaction with U6 snRNA [130]
SMN	Yes	Modified by PPM1G phosphatase [121]
TCAB1	Yes	Controls telomerase localization to CB [62] [63]
TGS1	Yes	Catalyzes hypermethylation step on snRNA [13]
FGF-2 ²³	No	Responsive to mitogenic/differentiation stimuli [86] [113]
Peroxiredoxin V	No	Responsive to reactive oxygen species [131]
PRMT5/7 (not in CBs)	No	Methyltransferases that generate sDMAs [83] [85]
TFIIH	No	General transcription factor in CBs [99]
ZPR1	No	Responsive to mitogenic stimuli [90]

¹ Phosphorylation status from Uniprot.org