PML nuclear bodies are highly organised DNA-protein structures with a function in heterochromatin remodelling at the G2 phase

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Summary

We have recently demonstrated that heterochromatin HP1 proteins are aberrantly distributed in lymphocytes of patients with immunodeficiency, centromeric instability and facial dysmorphy (ICF) syndrome. The three HP1 proteins accumulate in one giant body over the 1qh and 16qh juxtacentromeric heterochromatins, which are hypomethylated in ICF. The presence of PML (promyelocytic leukaemia) protein within this body suggests it to be a giant PML nuclear body (PML-NB). The structural integrity of PML-NBs is of major importance for normal cell functioning. Nevertheless, the structural organisation and the functions of these nuclear bodies remain unclear. Here, we take advantage of the large size of the giant body to demonstrate that it contains a core of satellite DNA with proteins being organised in ordered concentric layers forming a sphere around it. We extend these results to normal PML-NBs and propose a model for the general organisation of these structures at the G2 phase. Moreover, based on the presence of satellite DNA and the proteins HP1, BRCA1, ATRX and DAXX within the PML-NBs, we propose that these structures have a specific function: the re-establishment of the condensed heterochromatic state on late-replicated satellite DNA. Our findings that chromatin-remodelling proteins fail to accumulate around satellite DNA in PML-deficient NB4 cells support a central role for PML protein in this cellular function.

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Key words: PML nuclear body, Heterochromatin, Chromatin-remodelling function, G2 phase, ICF syndrome, XY body

Introduction

The ICF syndrome (MIM 242860) is a rare autosomal recessive disorder characterised by immunodeficiency, centromeric instability and facial dysmorphy. It is caused by mutations in the *DNMT3B* gene encoding a DNA methyltransferase that specifically methylates the GC-rich satellite DNAs (Hansen et al., 1999; Xu et al., 1999). Hypomethylation of satellite DNA appears to promote a high decondensation that leads to cytogenetic abnormalities preferentially involving the heterochromatin of chromosomes 1 (1qh), 16 (16qh) and, to a lesser extent, 9 (9qh). Interestingly, in ICF syndrome, the cytogenetic abnormalities have been demonstrated to affect specifically the GC-rich juxtacentromeric satellite DNA, but not the adjacent AT-rich centromeric DNA (Jeanpierre et al., 1993).

We recently demonstrated that the in vivo distribution of the HP1 (heterochromatin protein 1) protein is altered in cells from ICF patients (Luciani et al., 2005). Indeed, in a large proportion of ICF cells at the G2 phase, HP1α, HP1β and HP1γ protein isoforms showed an aberrant signal concentrated into a large prominent nuclear body. Interestingly, and with high relevance to the cytogenetic anomalies that are part of the ICF pathology, this giant HP1 body colocalised with the undercondensed 1qh and 16qh heterochromatins. We also demonstrated that the PML (promyelocytic leukaemia) protein, the major component of normal PML nuclear bodies (PML-NBs), accumulates at the aberrant HP1 body, identifying this structure as a giant PML-NB.

PML-NBs have received much attention because they display an abnormal dispersed intranuclear pattern in blast cells from individuals with acute promyelocytic leukaemia (APL) (Seeler and Dejean, 1999). PML-NBs are present in most mammalian cell nuclei and a typical nucleus contains 5-30 bodies that appear as discrete foci varying in size from 0.2-1 μm in diameter (Melnick and Licht, 1999). Heat shock, heavy metal exposure, viral infection and rare pathological situations

can affect the integrity of PML bodies (Maul et al., 1995; Kamei, 1997; Ishov et al., 1999; Yeager et al., 1999; Nefkens et al., 2003; Eskiw et al., 2003). Such observations have suggested that the integrity of PML-NBs is of major importance for normal cell functioning. Nevertheless, the structural organisation and the precise functions of these nuclear bodies remain unclear.

Discordant studies have described the PML-NBs to be shaped either like a doughnut (Sternsdorf et al., 1997a) or like a sphere (Weis et al., 1994). Such contradictions have contributed to the uncertainty surrounding the real functions of these nuclear structures. Nevertheless, it is widely assumed that PML-NBs do not contain DNA material (Ascoli and Maul, 1991; Boisvert et al., 2000; Ching et al., 2005). The putative functions of PML-NBs are roles in transcriptional and cell growth regulation, tumour suppression, apoptosis and maintenance of genome integrity. In addition, the large number of PML-associated proteins that do not appear to act in a single pathway suggests that PML-NBs could be depots for storage and titration of nuclear proteins (Seeler and Dejean, 1999).

Here, we take advantage of the large size of the giant body to demonstrate that PML-NBs at G2 phase contain satellite DNA with proteins organised in ordered concentric spherical layers around it. In addition, we propose that PML-NBs have a specific function at the G2 phase: the re-establishment of the condensed heterochromatic state on late-replicated satellite DNA. Our analysis of APL-derived NB4 cells, which are considered to be deficient in PML protein function, indicates that PML protein and the PML-NBs play an important role in the specific organisation of chromatin remodelling proteins around satellite DNA.

Results

Protein composition of the giant HP1-PML body and the normal PML-NBs at G2 phase

To elucidate the basis for the large size of the giant HP1-PML body in ICF cells, we analysed its protein composition and compared it with that of normal PML-NBs. We have previously demonstrated that the giant body is specific to the G2 phase of the cell cycle (Luciani et al., 2005) (Fig. 1A,B). In this study, we therefore focused on proteins likely to be present in normal PML-NBs at G2 phase, which we tested by immunocytochemistry on ICF and control cells synchronised at this phase. Comparative results are summarised in Table 1.

We first examined the distribution of ATRX, a SWI-SNF chromatin-remodelling protein defective in ATRX syndrome (alpha thalassemia, mental retardation, X-linked), which colocalises with PML-NBs during S and G2 phases (Xue et al., 2003). Two different antibodies (H300 and 2H12) revealed the presence of ATRX in the giant body and the normal PML-NBs at G2 phase (Fig. 2A). We therefore also examined the distribution of DAXX, a transcription cofactor known to colocalise with ATRX in the PML-NBs (Ishov et al., 2004). We observed that DAXX accumulates in the giant body of ICF cells (Fig. 2B). Altogether, these findings demonstrate a specific association of DAXX and ATRX with the giant HP1-PML body, as well as with normal PML-NBs, at the G2 phase (Table 1).

We next investigated the distribution of BLM, a RecQ-like DNA helicase protein defective in Bloom syndrome, which has been reported to be in the PML-NBs during the S and G2 phases (Bischof et al., 2001). In addition, we investigated the distribution of topoisomerase III alpha (TOPOIIIα), which has been shown to be recruited to the PML-NBs by BLM (Hu et al., 2001). We observed that both proteins colocalise not only with the giant body of ICF cells (Fig. 2B) but also with PML-NBs of control G2 cells (Table 1). BLM has been suggested to associate with RAD51 for the repair of induced or spontaneous DNA damage (Bischof et al., 2001), but we did not find any RAD51 signal in the giant body.

We also examined the distribution of NBS1, a DNA-repair protein defective in Nijmegen breakage syndrome. In the absence of DNA damage, a portion of NBS1 has been shown to localise to PML-NBs (Lombard and Guarente, 2000). We found that NBS1 accumulates in a subset of giant HP1-PML bodies at the G2 phase, suggesting either that its presence is transient or that there is diversity in the composition of PML-NBs. Similarly, NBS1 colocalised with a proportion of PML-NBs in control G2 cells (Table 1).

Finally, we studied the distribution of CBP (CREB-binding protein), a transcriptional co-activator that has been shown to localise to the PML-NBs (LaMorte et al., 1998). We observed that it accumulates in both the giant body of ICF cells (Fig. 2B) and the PML-NBs of control G2 cells (Table 1). Nevertheless, the lack of RNA polymerase II in the giant body suggests that CBP protein is not involved in transcriptional activation (Kiesslich et al., 2002) in PML bodies of G2 phase.

Overall, our results demonstrate a very similar protein composition of both the ICF giant HP1-PML body and the normal PML-NBs at G2 phase.

Searching for DNA material within the giant body and the normal PML-NBs

We previously showed that the giant HP1-PML body in ICF cells consistently associates with the undercondensed hypomethylated satellite DNA. This association more frequently involves the satellite DNAs 1qh, 16qh and, to a lesser extent, 9qh (Luciani et al., 2005). To define the nature

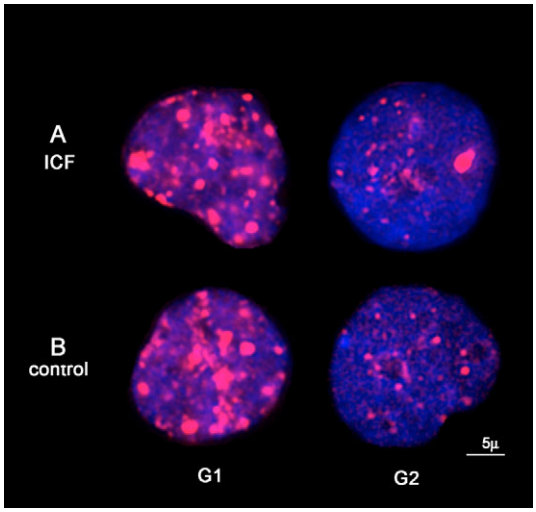

Fig. 1. Distribution of HP1α protein after immunostaining of ICF and control cells. (A) ICF nuclei and (B) control nuclei at G1 and G2 phases. At the G2 phase, there is an aberrant giant HP1 body in ICF nuclei (HP1 alpha, red; nuclei, blue with DAPI).

Table 1. Proteins of normal PML-NBs, the giant body, the XY body and the Xi*

Proteins	Normal PML-NBs			Giant body			XY body			Xi		
	Other studies		This study									
PML	+	(b)	+	+	(a)		+	(k)		0		
SP100	+	(c)	+	+	(a)		0			0		
SUMO-1	+	(d)	+	+	(a)		+	(k)		0		
HP1α	+	(e)	+	+	(a)		+	(l)		+	(s)	
HP1β	+	(e)	+	+	(a)		+	(l,m)		+	(s)	
HP1γ	+	(e)	+	+	(a)		+	(l)		+	(s)	
ATRX	+	(f)	+	+			0			0		
DAXX	+	(f)	+	+			+	(k)		0		
BLM	+	(g)	+	+			+	(n)		0		
TOPOIIIα	+	(h)	+	+			0			0		
RAD51	+/–	(g)	0	–			+	(o)		0		
NBS1	+/–	(i)	+/– 55%	+/–	53%		0			0		
CBP	+	(j)	+	+			0			0		
tri-K9 H3	0		0	–			0			+	(s)	
tri-K20 H4	0		0	–			–	(p)		+	(s)	
macroH2A	0		0	–			+	(q)		+	(t)	
BRCA1	0		+/– 20%	+			+	(r)		+	(u)	
γH2AX	0		+/– 10%	+/–	25%		+	(r)		0		

*Grey shading shows similarities between the giant body and other nuclear compartments. +, protein present; +/–, protein present in a subset of PML-NBs; –, protein absent; 0, untested.

Corresponding references: (a) Luciani et al., 2005; (b) Koken et al., 1994; (c) Szostecki et al., 1990; (d) Boddy et al.1996; (e) Seeler et al., 1998; (f) Tang et al., 2004; (g) Bischof et al., 2001; (h) Hu et al., 2001; (i) Lombard and Guarente, 2000; (j) Lamorte et al., 1998; (k) Rogers et al., 2004; (l) Metzler-Guillemain et al., 2003; (m) Motzkus et al., 1999; (n) Walpita et al., 1999; (o) Fernandez-Capetillo et al., 2003; (p) Kourmouli et al., 2004; (q) Hoyer-Fender et al., 2000; (r) Turner et al., 2004; (s) Chadwick and Willard, 2004; (t) Mermoud et al., 1999; (u) Ganesan et al., 2002.

of this association, we designed immuno-FISH experiments using the anti-PML antibody, the 1qh juxtacentromeric satellite DNA probe and the chromosome 1 centromere DNA probe. The analysis of 100 giant bodies revealed that the 1qh satellite DNA colocalises with the spherical PML signal but that the centromeric DNA was consistently located outside the PML sphere (Fig. 3A,B).

Second, we took advantage of the large size of the giant bodies to perform three-dimensional (3D) confocal analysis. This clearly demonstrated that, in most of the cases, the entire 1qh satellite DNA was contained within the spherical PML signal (Fig. 3C). Interestingly, in some cases we observed the 1qh satellite DNA extruding through a gap in the spherical PML signal. Altogether, these results demonstrate that the giant HP1-PML body contains undercondensed juxtacentromeric satellite DNA, but not centromeric DNA.

Given their similar protein composition, the presence of juxtacentromeric satellite DNA within the giant body indicated that normal PML-NBs might also contain satellite DNA, at least at the G2 phase. In support of this hypothesis, both the pan-centromeric DNA probe and the CREST antibodies, which specifically recognise centromeric regions, gave signals located outside, but close to, the PML-NBs (Fig. 4A). Such proximity might suggest that DNA sequences adjacent to the centromere are included within these nuclear bodies. We decided to examine the behaviour of the 1qh and the 9qh satellite DNAs which show, respectively, a high and low frequency of association with the giant body of ICF cells (Luciani et al., 2005). As the size variability of these satellite DNAs could influence their association with the PML-NBs, we performed experiments on three control subjects whose 1qh and 9qh heterochromatins were of equivalent size on metaphase chromosomes. We found that both 1qh and 9qh satellite DNAs colocalised with normal PML-NBs (Fig. 4B) with an equivalent frequency (Fig. 4C). We therefore studied the 16qh and the 15ph juxtacentromeric satellite DNAs, which are both smaller than the 1qh and the 9qh satellite DNAs but differ in size, the 16qh being larger than the 15ph. These results show that juxtacentromeric satellite DNAs colocalise with normal PML-NBs at the G2 phase with a frequency dependent on their length (Fig. 4C).

Moreover, since telomeric DNA has been shown to colocalise with large PML bodies in some ALT (alternative lengthening of telomeres)-tumour-derived cell lines (Yeager et al., 1999), we searched for the presence of telomeric DNA repeats in the giant body of ICF cells and in normal PML-NBs at the G2 phase. We did not detect any telomeric DNA or telomere-associated protein TRF2 in either of these structures (Fig. S1A, supplementary material). However, we did observe this colocalisation in the ALT cell line WI-38 VA-13 (Fig. S1B,

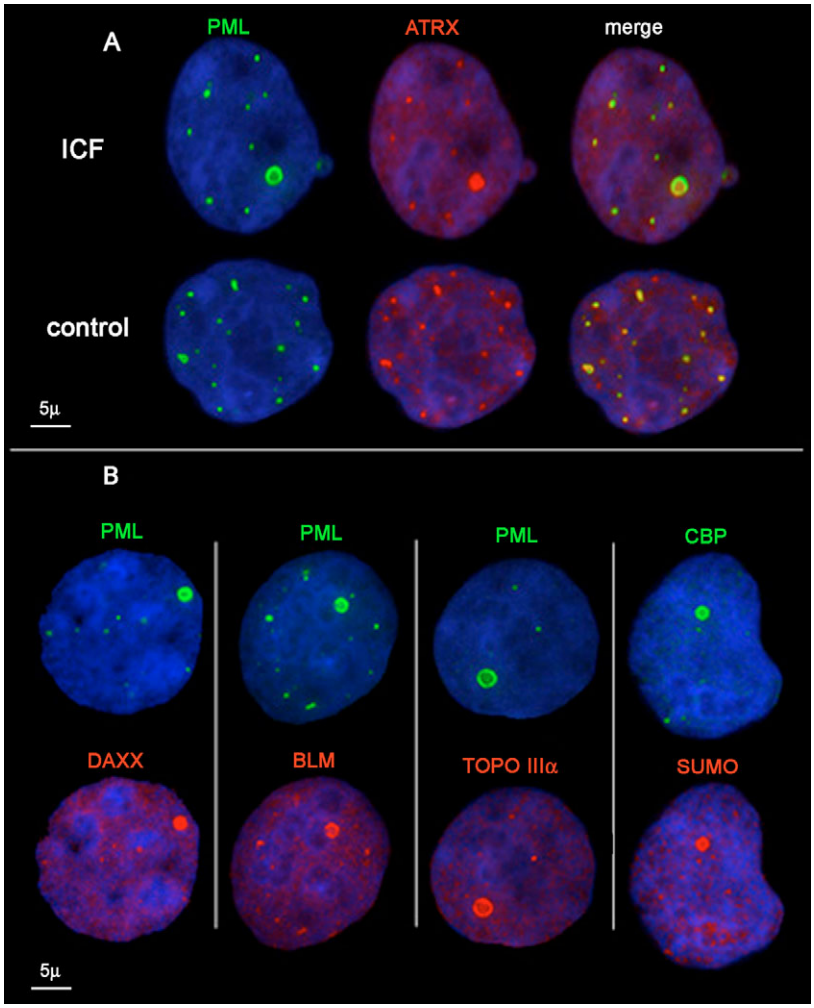

Fig. 2. Protein distribution in ICF and control G2 nuclei. (A) ATRX and PML proteins colocalise in the giant HP1-PML body and normal PML-NBs. A weak diffuse ATRX signal is also detectable in the nucleoplasm of ICF and control nuclei (PML, green; ATRX, red; nuclei, blue with DAPI). (B) Other proteins colocalise within the giant body as shown on ICF nuclei immunostained with antibodies against PML and DAXX, PML and BLM, PML and TOPOIIIα, CBP and SUMO.

supplementary material). Our results show that the ALT pathway is not activated in ICF, or control, Epstein-Barr virus (EBV)-transformed cell lines, demonstrating that ICF giant bodies and control PML-NBs that contain DNA are not ALT-PML bodies.

Taken together, our findings provide good evidence that the giant body of ICF cells and the normal PML-NBs contain DNA material, at least for a short time, during the G2 phase. They also demonstrate that only the juxtacentromeric satellite DNA is included within these structures, and not the core centromeric DNA, or the telomeric DNA.

Structural organisation of the giant body and normal PML-NBs

From previous studies, it is not clear whether normal PML-NBs are doughnut- or sphere-shaped structures. Moreover, nothing is known about the organisation of the different components within these structures, despite its probable relevance to PML-NB function. Thus, in order to gain insights into the spatial organisation of PML-NBs, we analysed in more detail the patterns of the different proteins that we have shown to colocalise with the giant body and the normal PML-NBs.

First, the fluorescent microscopic analysis of the giant body in ICF cells allowed us to distinguish clearly two types of protein pattern: one pattern consisted of a round-shaped signal surrounding a pale central core; the other appeared as a round-shaped homogeneously stained signal (Fig. S2, supplementary material). In addition, using triple immunostaining, we observed the DAXX signal to be larger than the ATRX signal, which in turn was larger than the HP1β signal (Fig. 5A). We also observed the BLM, SP100 and TOPOIIIα signals to be smaller than the PML signal, but larger than the DAXX signal (Fig. 5B-D).

To demonstrate that this spatial protein organisation is not EBV dependent, we analysed non-transformed peripheral lymphocytes of ICF patients, where the giant HP1-PML body is also present (Luciani et al., 2005). We confirmed the presence of the two types of protein signals in these cells (Fig. S3, supplementary material).

We next investigated whether the different patterns observed in the giant body were also distinguishable in the PML-NBs of control G2 cells. Microscope analysis showed that PML-NBs were extremely variable in size within the same nucleus, although they were clearly smaller than the giant PML body of ICF cells. Nevertheless, for some of the larger PML-NBs, co-immunostaining experiments produced distinct round-shaped protein signals that appeared similar to those observed in the giant PML body of ICF cells, suggesting that they share a similar organisation (Fig. S4, supplementary material).

Finally, taking advantage of the large size of the giant HP1-

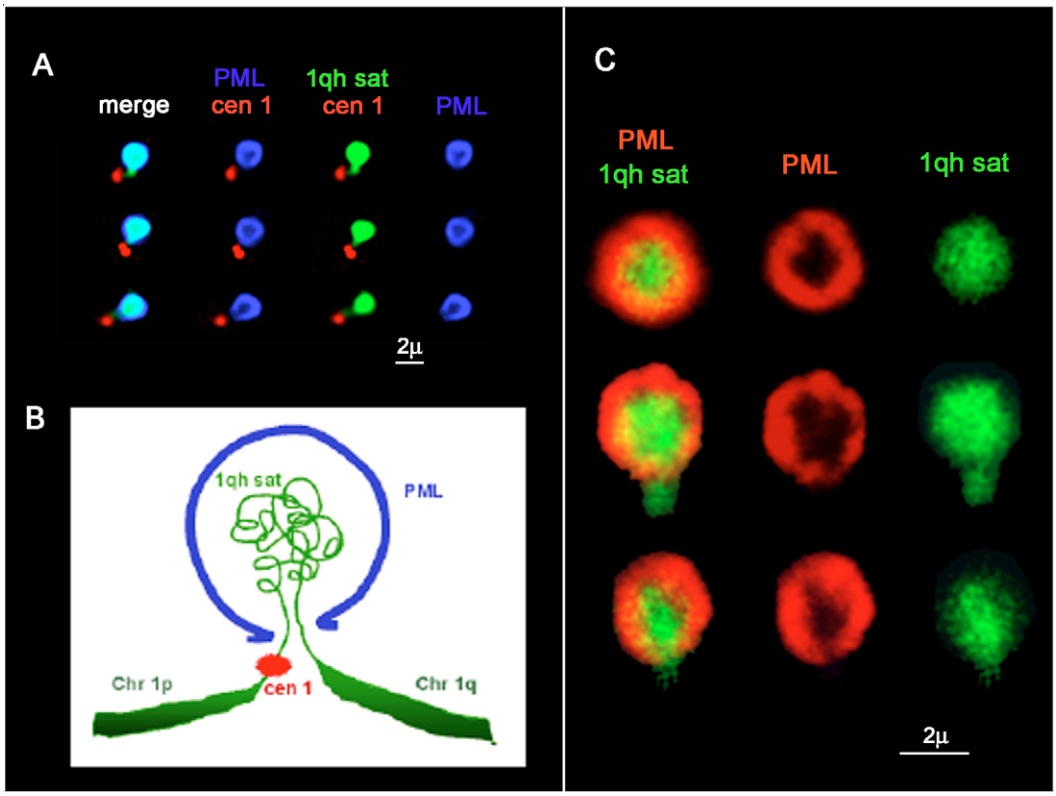

Fig. 3. The 1qh satellite DNA is included in the giant HP1-PML body in ICF cells. (A) Two-dimensional fluorescent microscopy: a giant body, identified with anti-PML antibody (blue), colocalises with 1qh satellite DNA (1qh sat, green), adjacent to the centromeric DNA (cen 1, red); (B) schematic representation. (C) Three-dimensional reconstruction: transverse section of three giant bodies after immuno-FISH with anti-PML antibody (red) and 1qh satellite DNA probe (green).

PML body, we analysed its spatial organisation using 3D confocal microscopy. Triple immunostaining and confocal analysis demonstrated that both PML and SP100 signals were organised in concentric spheres, with the PML sphere being larger than the SP100 sphere (Fig. 6A). By contrast, the HP1β signal consistently appeared as a full bowl located in the central area delineated by the PML and SP100 spheres (Fig. 6A). We also showed that DAXX and ATRX signals were organised in concentric spheres around the HP1β signal, with the DAXX sphere being larger than the ATRX sphere (Fig. 6B). Finally, we confirmed that the SP100 sphere was larger than the DAXX sphere, but we cannot differentiate its pattern from that of SUMO-1, CBP, TOPOIIIα and BLM.

Taken together, our results clearly demonstrate that the constituent proteins are organised in ordered concentric layers around the satellite DNA within the giant HP1-PML body. From the centre of the body to its periphery, they appear to be arranged in the following order: HP1α/HP1β/HP1γ at the centre, then ATRX, then DAXX, then CBP/BLM/TOPOIII/SUMO-1/SP100, and finally PML at the periphery (Fig. 7). Our results also indicate that, at G2 phase, the protein organisation is similar in both the giant body and the normal PML-NBs.

Finally, since neither DNAse nor RNAse treatment is able to disorganise normal PML-NBs, it has been suggested that these structures are attached to the nuclear matrix. To evaluate the degree to which nucleic acids contribute to the structural organisation of the giant body, we performed DNAse and RNAse treatments on unfixed ICF cells. We show that the integrity of the giant HP1-PML body does not appear to be affected by these treatments, indicating that it might be attached to the nuclear matrix, as suggested for normal PML-NBs (Ascoli and Maul, 1991). In particular, among the proteins tested, we show that the signal of PML, BRCA1 and HP1 proteins within the giant body is unaltered by DNAse or RNAse treatment (Fig. 8).

What kind of chromatin is within the giant HP1-PML body?

To understand the function of PML-NBs at the G2 phase better, we investigated the nature of the chromatin included in the giant body. The accumulation of HP1 proteins and the presence of satellite DNA within this structure indicates that it is a constitutive heterochromatin domain. Thus, we searched for post-translational histone modifications that are common hallmarks of the constitutive heterochromatin at the chromatin of the giant body. In particular, we tested the trimethylation of histone H3 on lysine 9, which creates a binding site for the HP1 proteins (Peters et al., 2003) and the trimethylation of histone H4 on lysine 20 (Schotta et al., 2004). Nevertheless, we detected no accumulation of these histone modifications in the giant body (Table 1), which suggests it contains an atypical constitutive heterochromatin.

These results led us to extend our study to components normally involved in the organisation of facultative heterochromatin, such as the inactive X (Xi) chromosome in female somatic cells and the XY body in male germ cells. The histone variant macroH2A is a consistent hallmark of the XY body (Hoyer-Fender et al., 2000) and is also enriched on the Xi chromosome (Mermoud et al., 1999). We thus investigated its presence in the giant body of ICF cells and found that the only detectable macroH2A signal was located at the centrosomes, a well-established location for this histone variant (Table 1).

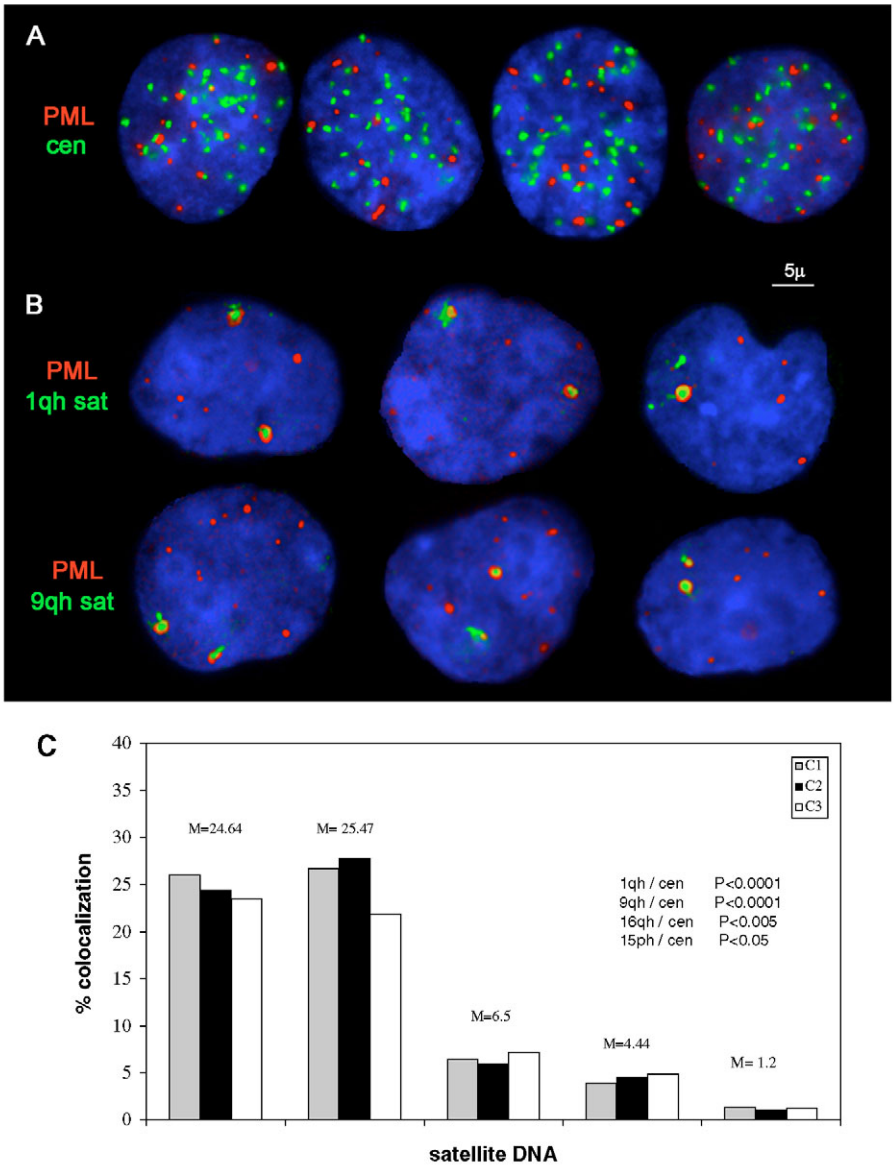

Fig. 4. In control G2 cells, the normal PML-NBs colocalise with juxtacentromeric satellite DNA, adjacent to the centromeric DNA. Immuno-FISH experiments demonstrate that PML protein signals (red) (A) are adjacent to the pan-centromeric DNA signals (cen, green) and (B) colocalise with the 1qh and 9qh satellite (1qh sat and 9qh sat) DNA signals (green). (C) Histogram showing the percentage of 1qh (M=24.64), 9qh (M=25.47), 16qh (M=6.5) and 15ph (M=4.44) satellite DNAs that colocalise with PML-NBs in three control subjects (C1, C2, C3) at G2 phase. The colocalisations have been scored from the analysis of 100 G2 cells and compared with those occurring between PML-NBs and chromosome 1 centromeric DNA, which is excluded from PML-NBs; *P* values were calculated by using Fisher's exact test, one sided (http://www.matforsk.no/ola/fisher.htm). M, mean.

We also investigated the distribution of the tumour suppressor BRCA1 protein as it has recently been demonstrated to be a component of both Xi and XY body heterochromatins (Ganesan et al., 2002; Turner et al., 2004). Co-immunostaining with anti-BRCA1 (clones SD118 and Upstate 07-434) and anti-SP100 or anti-PML antibodies gave a BRCA1 signal within nearly all 100 giant HP1-PML bodies analysed, with a pattern varying from a dot to a full bowl (Fig. 9A). We next investigated whether BRCA1 also colocalises with normal PML-NBs at the G2 phase. The analysis of 100 control G2 cells showed colocalisation in about 20% of the nuclei (Fig. 9B). Nevertheless, in a given nucleus, this colocalisation involved a small number of PML-NBs, which indicates either that the colocalisation is transient or that there is diversity in the composition of PML-NBs at G2.

We next searched for the presence of γH2AX, the phosphorylated form of the histone variant H2AX, in the giant body. γH2AX is a consistent element of the XY body (Turner et al., 2004). We found that a moderate proportion (25%) of the 100 giant bodies analysed contained a γH2AX signal that occupied the whole central area of the PML sphere. We therefore investigated the presence of γH2AX in 100 nuclei from control G2 cells and found it to colocalise with a subset of PML-NBs in 10% of nuclei (Table 1).

Furthermore, partial colocalisation of BRCA1 and γH2AX with PML-NBs has been shown in cells that maintain their telomeres by the ALT pathway (Wu et al., 2003), as well as in other cell lines in response to double-strand breaks of different origins (Carbone et al., 2002; Xu et al., 2003). In an attempt to detect possible DNA damage repair within the giant HP1-PML bodies, we carried out BrdU-incorporation experiments. BrdU pulses of 15 minutes were performed throughout the whole G2 phase and the cells co-immunostained with both anti-PML and anti-BrdU antibodies. No BrdU incorporation was detected in the 100 giant HP1-PML bodies analysed, indicating that few, if any, DNA synthesis and/or repair events

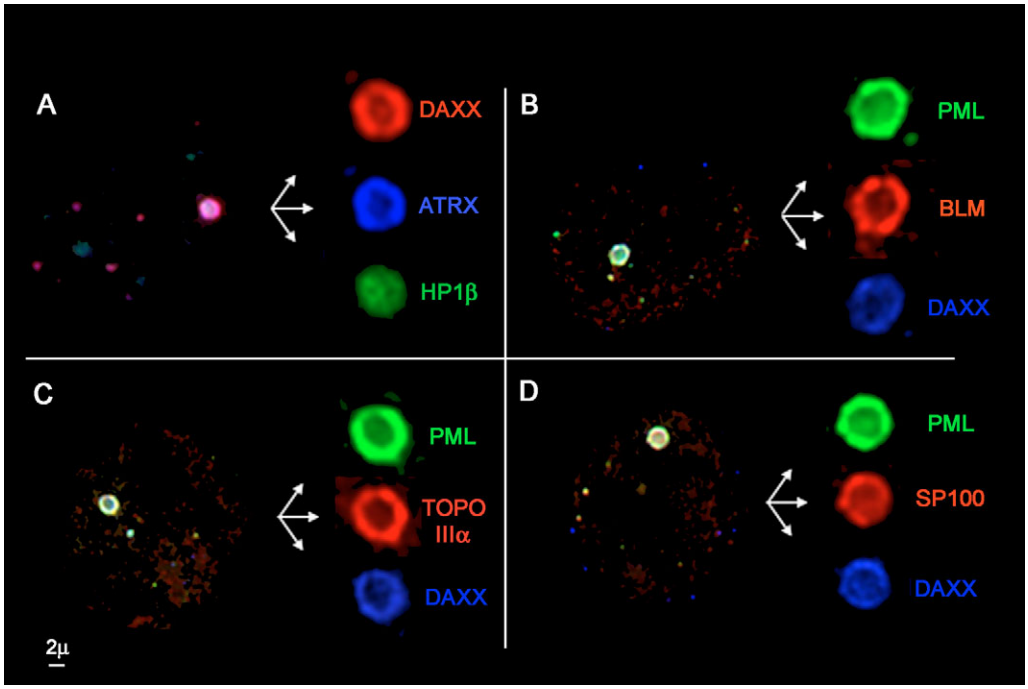

Fig. 5. In the giant body, protein signals are round shaped. Either they have a pale central core (PML, BLM, TOPOIIIα, SP100, DAXX, ATRX) or they are homogeneously stained (HP1β). These different patterns can be observed by decomposition into colours and enlargement (×2.8) of the protein signals colocalising in the giant body. (A) Triple immunostaining of an ICF nucleus showing that the DAXX layer (red) is larger than the ATRX layer (blue), which in turn is larger than the HP1β layer (green). (B-D) Triple immunostaining showing respectively that BLM, TOPOIIIα and SP100 layers (red) are located between PML (green) and DAXX (blue) layers.

occur within these structures during the G2 phase. In conclusion, the presence of BRCA1 and γH2AX suggests similarities between the giant body, the normal PML-NBs at the G2 phase, and the XY body heterochromatin.

Is PML protein required for the organisation of PML-NB-associated proteins around satellite DNA?

To address the question of whether PML protein is required for large body formation, we analysed the APL-derived NB4 cell line in which we investigated the localisation of protein components of the PML-NB relative to the satellite DNA. The NB4 cell line is derived from an APL patient carrying a t(15;17) translocation that generates a fusion protein between PML protein and a nuclear receptor for retinoic acid, RARα (Lanotte et al., 1991). In this cell line, PML-NBs are disrupted and PML protein shows a fine microspeckled nuclear distribution, as a result of forming a heterodimer with the PML-RARα fusion protein (De The et al., 1991; Melnick et al., 1997). The fusion protein therefore exerts a dominant-negative effect on wild-type PML, resulting in the loss of PML function in APL-derived NB4 cells (Zhu et al., 1997; Xu et al., 2003).

First, using unsynchronised NB4 cells, we performed co-immunostaining with antibodies directed against PML protein and any of the following proteins: SP100, DAXX, ATRX, TOPOIIIα, BLM and HP1α. With the exception of HP1α, all these proteins showed an altered nuclear pattern very similar to the microspeckled diffuse PML pattern. The HP1 protein pattern did not appear to be severely altered in unsynchronised NB4 nuclei as, in most of them, we observed typical irregularly shaped areas colocalising with DAPI-stained regions. This is in agreement with the observation that HP1α is primarily localised on the heterochromatic regions.

We therefore designed immuno-FISH experiments on NB4 cells synchronised at the G2 phase and searched for the formation of spheric structures around the 1qh satellite DNA by any of the proteins previously tested. In 100 nuclei analysed for each protein, none of these proteins was observed to accumulate on 1qh satellite DNA, or to form a 'body' around it (Fig. S5, supplementary material). In NB4 cells synchronised in G2, HP1 protein showed discrete small speckles, similar to those observed in normal control G2 cells; nevertheless, we did not detect any large, round-shaped body colocalising with 1qh satellite DNA. Together, these results strongly support an essential role for PML protein in the formation of large PML-NBs containing DNA.

Discussion

The PML-NBs contain DNA, at least transiently, during G2 phase

Electron spectroscopic imaging and immunofluorescence microscopy led to the conclusion that PML-NBs do not contain detectable DNA sequences (Ascoli and Maul, 1991; Boisvert et al., 2000; Ching et al., 2005). A colocalising DNA-PML body has been observed in some ALT-tumour-derived cell lines, but has been considered as a peculiarity arising from the abnormal functioning of these cells (Yeager et al., 1999).

The composition of the PML-NBs is known to vary greatly throughout the cell cycle and very few studies have taken this into account. Our findings, that the juxtacentromeric satellite DNA is included within a giant body in G2 cells of patients with ICF syndrome, has guided our study of the normal PML-NBs. Indeed, focusing on both specific genomic DNA sequences and the G2 phase, our study reveals a colocalisation of PML-NBs and juxtacentromeric satellite DNA that might be a normal event, previously undetected because of its short duration. Our results are supported by previous works that showed a short-lived juxtaposition of PML-NBs with all centromeres at the G2 phase (Everett et al., 1999) and with the chromosome 9 centromere (Wang et al., 2004).

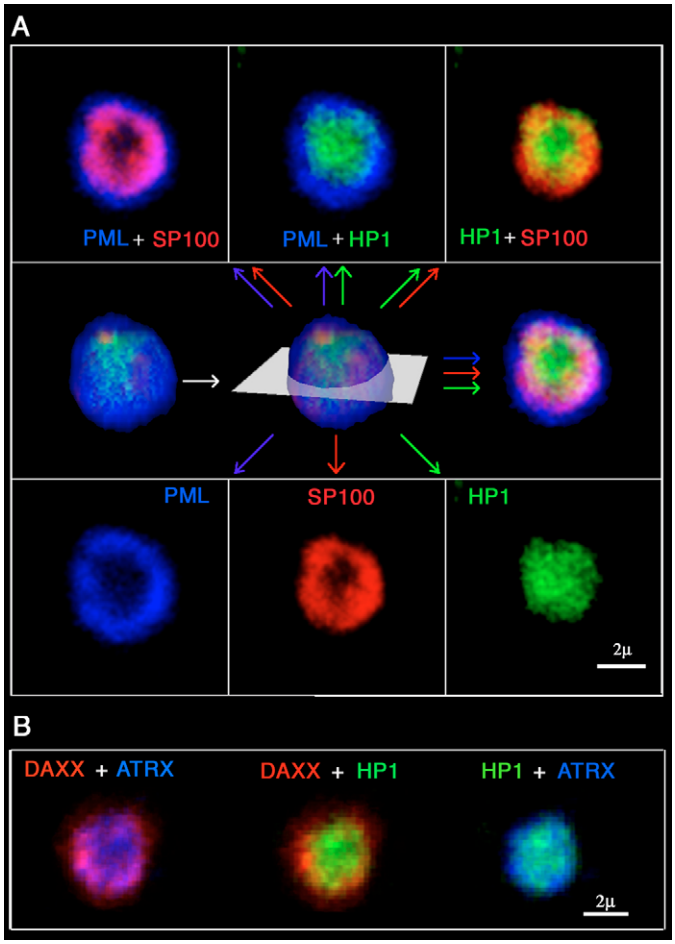

Fig. 6. Three-dimensional reconstruction of giant HP1-PML bodies. (A) Triple immunostaining showing PML (blue), HP1β (green) and SP100 (red). In the middle row, the left reconstruction shows the body viewed with a slight pitch, the centre reconstruction shows the equatorial plane corresponding to the transverse sections shown in the other rows, and the right reconstruction shows the transverse section with simultaneous rendering of the three fluorescent signals. The top row shows the transverse section with the various combinations of pairs of signals. The bottom row shows the transverse section with the signals alone: left, PML sphere; centre, SP100 smaller sphere; right, HP1 small bowl. (B) Triple immunostaining showing DAXX (red), ATRX (blue) and HP1β (green), in transverse section showing the various combinations of pairs of signals.

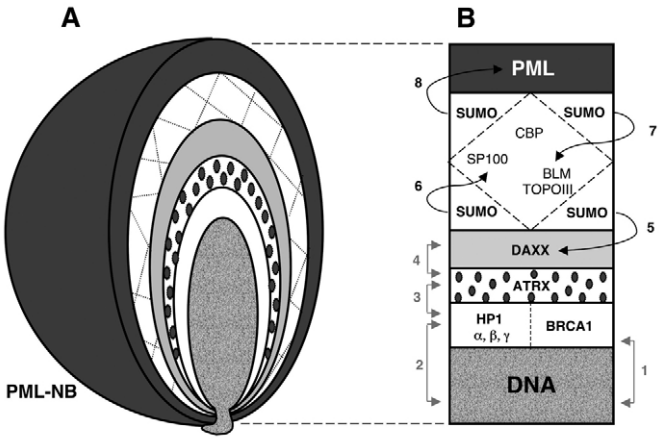

Fig. 7. Model of DNA-protein organisation in the PML-NBs at the G2 phase. (A) Transverse section of a PML-NB. (B) Detail of the different protein layers. The numbers indicate the following references: 1 (Paull et al., 2001; Cable et al., 2003); 2 (Zhao et al., 2000); 3 (Le Douarin et al., 1996; McDowell et al., 1999); 4 (Xue et al., 2003; Tang et al., 2004); 5 (Jang et al., 2002); 6 (Sternsdorf et al., 1997b); 7 (Eladad et al., 2005); 8 (Müller et al., 1998). The arrows for the references correspond to protein-protein or protein-DNA interactions (straight bi-headed arrows) and to protein-sumoylation (curved arrows).

We tested several juxtacentromeric satellite DNAs for colocalisation with normal PML-NBs and observed variable behaviours that could be related to the length of the satellite DNA sequences. This is considerably different from what we observed in ICF cells, where the giant body is seen to colocalise much more frequently with the 1qh satellite than the 9qh satellite (Luciani et al., 2005). This could be a result of the hypomethylation having a variable effect on different heterochromatins, dependent on their GC richness. Consistent with this, the 1qh heterochromatin contains more GC-rich satellite DNA than the 9qh one (Kokalj-Vokac et al., 1993).

Remarkably, the core centromeric DNA is not included in the giant body or in the normal PML-NBs, but instead lies consistently outside these structures. This particularity might be partially explained by the specific properties of the centromeric region. In humans, each pericentromeric heterochromatic region comprises two types of heterochromatin whose different composition is compatible with them having different functions (Blower and Karpen, 2001; Sullivan and Karpen, 2004; Henikoff and Dalal, 2005). One type of heterochromatin, corresponding to the core centromeric region, mainly comprises AT-rich α-satellite DNA repeats and is enriched in the histone H3 variant CENP-A. It is responsible for the attachment to spindle microtubules and must remain accessible to this machinery during mitosis. The other type of heterochromatin, corresponding to the juxtacentromeric heterochromatin region, mainly comprises a mix of satellite DNA repeats but lacks CENP-A enrichment. It is thought to condense rapidly before mitosis to ensure the correct cohesion and segregation of the sister chromatids. In conclusion, the presence of juxtacentromeric satellite DNA within PML-NBs does not arise by chance but reflects a physiological function that is specific to the G2 phase.

The PML-NBs are highly organised structures at the G2 phase

Previous studies on the spatial organisation of the PML-NBs are discordant, describing it as either a doughnut-shaped structure (Sternsdorf et al., 1997a) or a spherical one (Weis et al., 1994). In our study, the large size of the giant HP1-PML body has facilitated its analysis and its strong similarity with the normal PML-NBs suggests that they have the same spatial organisation. Our results indicate that, at least at the G2 phase, PML-NBs are spherical structures.

PML-NBs have been shown to display great heterogeneity in their protein composition throughout the cell cycle (Borden

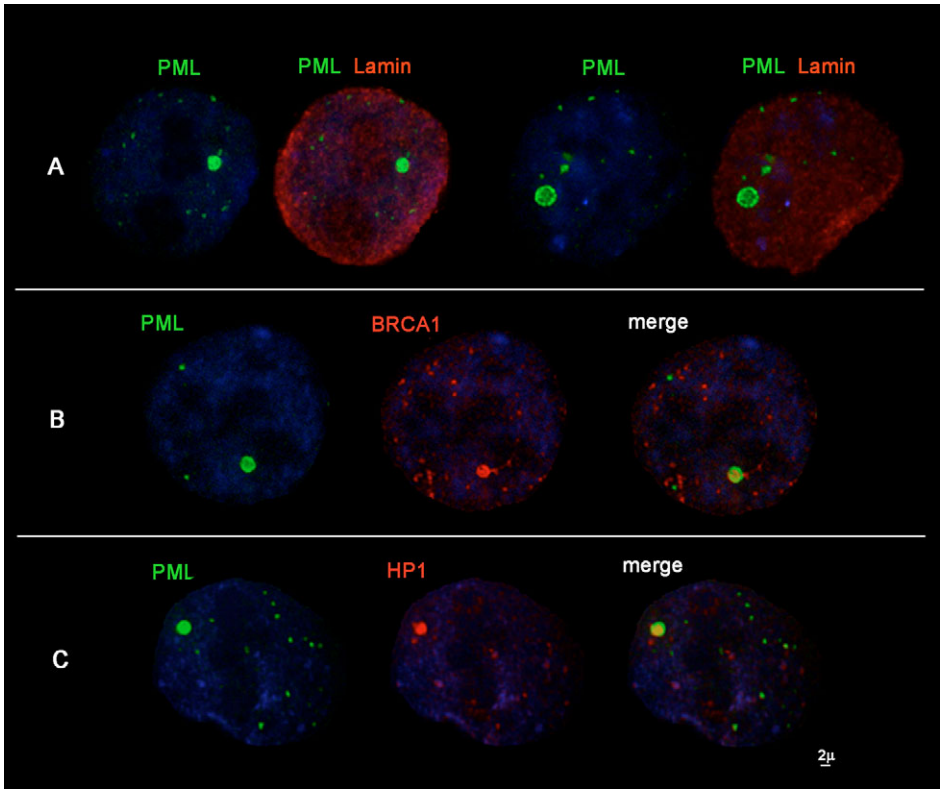

Fig. 8. In ICF cells, the integrity of the giant PML body is not affected by DNAse treatment. (A) Two pale DAPI-stained digested nuclei immunostained with anti-PML (green) and then with anti-lamin B (red) Abs to be easily detected. (B) One pale digested nucleus co-immunostained with anti-PML (green) and anti-BRCA1 (red) Abs. (C) One digested nucleus co-immunostained with anti-PML (green) and anti-HP1α (red) Abs.

et al., 2002). Here, we have focused on PML-NB organisation at the G2 phase, with the aim of gaining insights into their function during this brief interval of the cell cycle. We demonstrate for the first time that, at the G2 phase, PML-NBs display a high degree of protein organisation, with the different protein constituents distributed in ordered concentric layers around a core of juxtacentromeric satellite DNA. Such a reproducible organisation is almost certainly governed by specific protein-protein and protein-DNA interactions. We have therefore developed a model for the organisation of the PML-NBs based on both our results and these possible interactions (Fig. 7).

Theoretically, several PML-NB proteins could interact with the DNA, but our results indicate that only the distribution of BRCA1 and the HP1 isoforms is compatible with such an interaction. In support of our results, DNA interaction has been demonstrated for BRCA1 (Paull et al., 2001; Cable et al., 2003) involving the central domain of the protein, and for HP1 proteins through their hinge domain (Yamada et al., 1999; Zhao et al., 2000; Meehan et al., 2003). Taken together, these data raise the possibility that BRCA1 and HP1 interact, individually or simultaneously, with juxtacentromeric satellite DNA (Fig. 7). In our model, the ATRX layer is located between the BRCA1/HP1 layer and the DAXX layer (Fig. 7), which is supported by the fact that the ATRX protein can simultaneously interact with HP1 isoforms (Le Douarin et al., 1996; McDowell et al., 1999; Berube et al., 2000) and the DAXX protein (Tang et al., 2004). Confocal analyses indicated that BLM, TOPOIIIα, SP100, CBP and SUMO-1 are distributed between the DAXX and the PML layers, but are indistinguishable from each other. These findings could reflect the fact that SUMO-1 can be conjugated to DAXX (Jang et al., 2002), BLM (Eladad et al., 2005), SP100 (Sternsdorf et al., 1997b) or PML (Muller et al., 1998), and might therefore play a central role in PML-NB organisation. Finally, among the proteins tested, we found that PML was invariably located at the external border of the nuclear bodies. This peripheral location is of interest given that PML protein is absolutely required not only for the formation of PML-NBs, as demonstrated by knockout models (Zhong et al., 2000), but, based on our findings in PML-deficient NB4 cells, also for the specific protein organisation that takes place around satellite DNA at G2 phase. We therefore hypothesise that PML protein creates a spherical compartment (mould) into which the other proteins are packaged and interact in a coordinated manner. This hypothesis is also consistent with the capacity of PML protein to self-assemble in vitro into spherical macromolecular structures that resemble functional PML-NBs formed in vivo (Kentsis et al., 2002).

Moreover, in the ICF giant bodies, neither the BRCA1 signal nor that of HP1 was altered by DNAse treatment, suggesting that the PML protein mould might also have a protective structural role. If this were the case, then BRCA1 and HP1 would be expected to remain confined within the PML structure even after the disruption of their interaction with the DNA. In support of the high degree of protection conferred by the PML mould, mechanical stretching of ICF nuclei did not dissociate the satellite DNA from the giant body (unpublished results).

A chromatin-remodelling function for PML-NBs at G2 phase

Our demonstration that PML-NBs contain juxtacentromeric satellite DNA in addition to proteins that are specifically

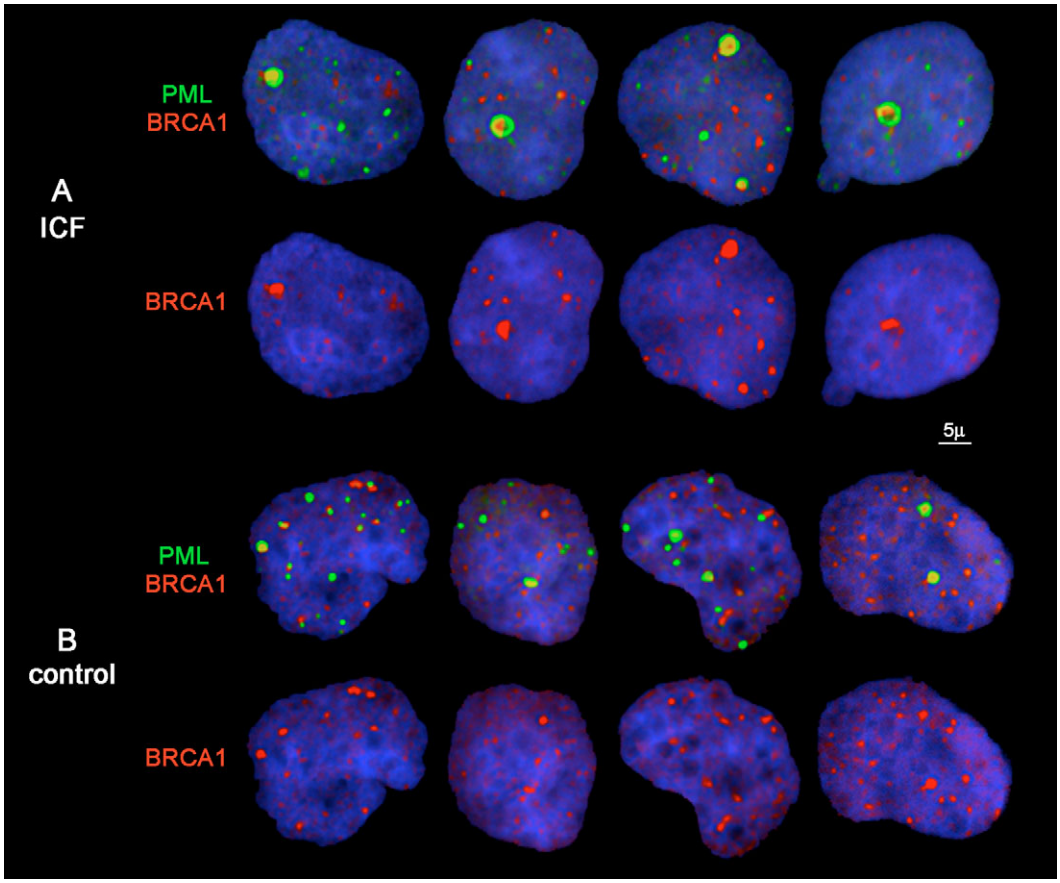

Fig. 9. Distribution of the BRCA1 protein in the giant body and normal PML-NBs. (A) BRCA1 colocalises with all giant bodies, varying from a dot to a full bowl. (B) BRCA1 colocalises with a small number of normal PML-NBs (BRCA1, red; PML, green; nuclei, blue with DAPI).

involved in chromatin remodelling suggests a specific function for these structures at the G2 phase. The juxtacentromeric satellite DNA, which is late replicating, participates in the somatic pairing of heterochromatic regions during the G2 phase (Schmid et al., 1983). Such properties imply that the satellite DNA needs to be disentangled and recover its condensed heterochromatic state rapidly before mitosis. The PML-NBs could be directly involved in this recovery, through several processes that might cooperate during this short interval.

One process might involve proteins acting at the nucleosomal and/or DNA level like RecQ helicases and DNA topoisomerases. These proteins could intervene before mitosis to disrupt inappropriate recombination events that frequently occur between DNA sequence repeats (Adachi et al., 1991; Wu and Hickson, 2001; Yankiwski et al., 2001). Our findings that BLM and TOPOIIIα proteins are present in PML-NBs during the G2 phase suggest that they exert a DNA-modifying function within these structures.

Other processes might participate in the re-establishment of the heterochromatic state, involving several proteins acting at the chromatin level. First, HP1 proteins are known to organise and maintain heterochromatin structure by interacting with histones in a methylation-dependent (Bannister et al., 2001; Lachner et al., 2001) or -independent manner (Nielsen et al., 2001), or again by direct DNA binding (Yamada et al., 1999; Zhao et al., 2000; Meehan et al., 2003).

Second, the ATRX-DAXX complex has been suggested to display ATP-dependent chromatin-remodelling activities (Xue et al., 2003), and to assemble with the juxtacentromeric heterochromatin specifically at the S- to G2-phase transition. An interesting model for this assembly has been proposed (Ishov et al., 2004). Nevertheless, in this model, the PML-NB never associates with the chromatin that is to be condensed, which excludes its direct participation in the chromatin-remodelling process. Our findings showing that DAXX, ATRX and the juxtacentromeric satellite DNA are present simultaneously within the giant body, as well as in the normal PML-NBs, strongly suggest that both structures have an active role in the remodelling process. In such a model, the PML-NBs would facilitate the recruitment and/or stabilisation of remodelling proteins to the juxtacentromeric satellite DNA specifically at the G2 phase (Fig. 10).

Third, both BRCA1 and γH2AX proteins have been shown to participate in the condensation and silencing of the XY body facultative heterochromatin (Starita and Parvin, 2003; Turner et al., 2004), together with HP1 (Metzler-Guillemain et al., 2003), BLM (Walpita et al., 1999), PML, DAXX and SUMO-1 (Rogers et al., 2004) (Table 1). The similarities between the giant body, normal PML-NBs and the XY body extend to the time at which they form during the cell cycle, as the XY body occurs in male pachytene spermatocytes, a phase that precedes the first meiotic division. Indeed, in the context of meiotic recombination, the close association of the X and Y chromosomes within the XY body might promote damaging non-homologous recombination events that need to be limited and/or corrected before the meiotic division (McKee and Handel, 1993). The XY body is therefore submitted to a

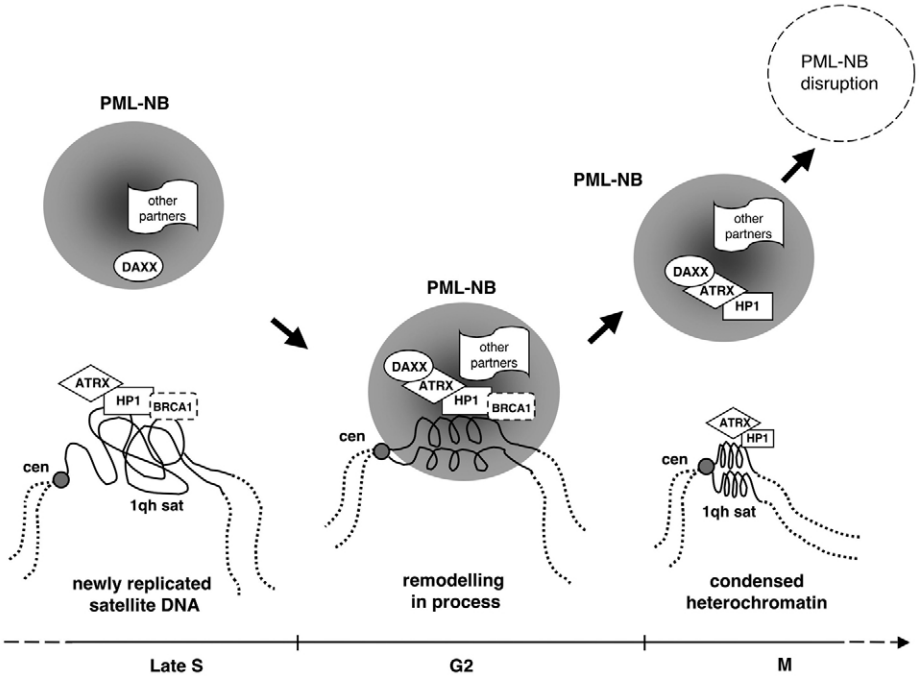

Fig. 10. Schematic representation of the chromatin-remodelling function exerted by PML-NBs on the juxtacentromeric satellite DNA. Chromatin-remodelling proteins and satellite DNA transiently colocalise within the PML-NBs during G2 phase. The mean percentages of colocalisation, obtained from the analysis of 100 cells of three control subjects (C1, C2, C3) at the S, G2 and M phases are respectively 8.5%, 24.6%, 6.1% for 1qh, and 8.3%, 25.4%, 5.8% for 9qh. These results support a chromatin-remodelling function of PML-NBs specific to the G2 phase (sat DNA, satellite DNA; cen, centromeric DNA).

remodelling process involving the resolution of entangled chromatids and chromatin condensation that could be compared with what occurs in PML-NBs at the G2 phase.

These parallels between the XY body, the giant body and normal PML-NBs at G2 phase suggest that they have functional similarities. Therefore, we propose that PML-NBs are sites of remodelling and/or condensing of the juxtacentromeric heterochromatin specific to the G2 phase prior to the mitotic division.

Why a giant HP1-PML body in ICF cells?

One striking feature that differentiates the giant HP1-PML body of ICF cells from the normal PML-NBs is its size. Indeed, the size of the giant body varies from 2-4 μm in diameter (Luciani et al., 2005), whereas that of the normal PML-NBs ranges from 0.2-1 μm (Melnick and Licht, 1999).

Despite the strong similarity between the giant body and the normal PML-NBs, we cannot exclude the possibility that the larger size of the giant body is a consequence of discrete differences in protein composition and organisation. Indeed, the methyl-binding proteins that participate in chromatin compaction through different mechanisms (Bird, 2002; Horike et al., 2005) are expected to bind inefficiently to the hypomethylated DNA that characterises the ICF syndrome.

Alternatively, the nature of the satellite DNA contained within the giant HP1-PML body could be a determining factor for the volume of this structure, through either its length or the degree to which the chromatin is condensed. Concerning the length, although juxtacentromeric satellite DNAs are highly polymorphic in normal individuals, no significant length increase has been observed in ICF patients that would explain the giant bodies. Concerning the satellite DNA condensation in ICF patients, GC-rich satellite DNA, and in particular 1qh, is highly undercondensed as a result of hypomethylation. It is therefore highly likely that the undercondensed satellite DNA in ICF occupies a larger volume than usual and this might directly lead to the giant size of the PML body.

Finally, we hypothesise that juxtacentromeric satellite DNA needs to be entirely included within PML-NBs for these structures to exert their remodelling and/or condensing function correctly during the G2 phase. This hypothesis is supported by our demonstration that the PML-NBs are spherical structures. Indeed, a sphere appears to be better adapted to the complete inclusion of DNA sequences than a doughnut. We further suggest that each PML-NB creates a domain containing the quantity of protein required for remodelling the juxtacentromeric DNA that it contains.

In conclusion, immuno-FISH analysis of lymphoblastic cells from patients with ICF syndrome has allowed us to demonstrate a giant HP1-PML body, occurring specifically at the G2 phase and containing juxtacentromeric satellite DNA. Altogether, the size of this body, its specificity to the G2 phase and the specific genomic sequence that it contains have oriented our investigation of normal PML-NBs at the G2 phase. We have been able to demonstrate that PML-NBs consist of a DNA core, with proteins highly ordered in concentric layers around it. We propose that these structures are involved in the re-establishment of the heterochromatic state on the late-replicated juxtacentromeric satellite DNA. Our study also highlights the importance of investigating the relationships between protein complexes and genomic DNA in situ, to elucidate processes in which they interact during a very short window of the cell cycle.

Materials and Methods

Cell lines

We used peripheral lymphocytes and freshly Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines from ICF patients and control subjects. Two ICF patients with the characteristic ICF syndrome phenotype, including chromosome 1 and 16 anomalies, were referred to as ICF1 and ICF2 in a previous study (Luciani et al., 2005). Two control subjects, a man and a woman who are in normal health, were used for analysing proteins in normal PML-NBs. For the colocalisation study of

juxtacentromeric satellite DNAs with normal PML-NBs, we selected three control subjects (C1, C2, C3) based on equal-sized 1qh and 9qh heterochromatins on metaphasic chromosomes.

We studied SV-40 transformed WI-38/VA-13 fibroblasts, which contain large ALT (alternative lengthening of telomeres)-associated PML bodies (Yeager et al., 1999) (ATCC, Cat: CCL-75.1). The PML-deficient NB4 cell line, derived from an APL patient carrying the t(15;17) translocation (Lanotte et al., 1991), was obtained from M. Lafage-Pochitaloff (Institut Paoli-Calmettes, Marseille, France). In this cell line, PML-NBs are disrupted and the PML protein shows a fine microspeckled nuclear pattern, as a result of forming a heterodimer with the PML-RARα fusion protein (De The et al., 1991; Melnick and Licht, 1999).

Cell preparations and immunocytochemistry

Cell cultures were synchronised at the G2 phase with our usual method: 5-fluorodeoxyuridine and uridine were added for 17 hours at final concentrations of 1.8 μM and 4 μM respectively, and the block was released by addition of thymidine (final concentration: 0.01 μM) for 5 hours (Luciani et al., 2005). Fibroblasts were similarly synchronised, tryspsinised, rinsed and resuspended in PBS (pH 7.2).

Cells collected by centrifugation were prepared as previously described (Mignon-Ravix et al., 2002). Briefly, cells were spread onto polylysine-coated slides by centrifugation (300 rpm for 5 minutes) with a cytospin 3 (Shandon). The cells were then fixed at room temperature for 10 minutes in 2% (w/v) paraformaldehyde in PBS pH 7.2. For indirect immunofluorescence experiments, each primary antibody was diluted and used under specific conditions, then detected following standard methods. After immunocytochemistry, preparations were fixed with 1% (w/v) paraformaldehyde in PBS for 5 minutes, rinsed again before staining with DAPI (50 ng/ml) diluted in Vectashield (Abcys). All antibodies were tested in individual staining reactions for their specificity and performance. Controls without primary antibody were all negative.

Antibodies

Primary antibodies (Abs) used for immunofluorescence staining were: three mouse Abs, 2HP-1H5, 1MOD-1A9 and 2MOD-1G6, directed against HP1α, HP1β and HP1γ, respectively, kindly provided by P. Chambon (IGBMC, Illkirch, France); a rat anti-HP1β Ab from Serotec (MCA1946); a rabbit anti-4x-dimethyl-H3-K9 Ab (gift from T. Jenuwein, IMP, Vienna, Austria); two rabbit anti-histone H3 and anti-histone H2B Abs (gift from S. Muller, IBMC, Strasbourg, France) used as control to evaluate the access to the histone cores within the giant body (all three Abs were used as recommended by A. Peters, IMP, Vienna, Austria). The rabbit anti-topoisomerase IIIα was a gift from J.-F. Riou (JE 2428; Onco-Pharmacologie, Université de Reims, Reims, France). We used a mouse anti-BRCA1 Ab (SD118) (J. Feunteun, IGR, Villejuif, France), a rabbit anti-BRCA1 Ab (07-434; Upstate), a rabbit anti-ATRX Ab (H-300, sc-15408; Santa Cruz) and a mouse anti-ATRX (2H12) Ab (Cardoso et al., 2000). We used a human CREST Ab (M. San-Marco, Laboratory of Immunology, Marseille, France), a rat anti-PML Ab (H. Will, Heinrich Pette Institute, Hamburg, Germany), a rabbit anti-SP100 Ab (AB1380; Chemicon International) and a mouse anti-TRF2 Ab (IMG-124A; Imgenex). The mouse anti-PML (sc-966), the rabbit anti-SUMO-1 (sc-9060), the mouse anti-SUMO-1 (D-11, sc-5308), the rabbit anti-BLM (H-300, sc-14018), the rabbit anti-RAD51 (H-92, sc-8349) and the goat anti-lamin B (sc-6217) Abs were purchased from Santa Cruz Biotechnology. The rabbit anti-DAXX (07-411), the mouse anti-CBP (06-297), the mouse anti-phospho-Histone H2AX (ser139, 05-636), the rabbit anti-histone macroH2A1 (07-219) and the rabbit anti-NBS1 (07-317) Abs were from Upstate Biotechnology. The anti-NBS1 Ab was used as previously described (Zhu et al., 2000). The mouse anti-DAXX-01 (ab9091) and the rabbit anti-trimethyl-H4-K20 (ab9053) Abs were purchased from Abcam. Multiple immunofluorescent experiments were performed using secondary antibodies conjugated either to Cyanin-3 or to FITC (tebu-bio) or again to AMCA (Abcys) fluorescent dyes.

Molecular cytogenetics

Fluorescent in situ hybridisation (FISH) was used to identify chromosomal regions specifically rearranged in the ICF syndrome and to localise them with respect to the protein signals. Nevertheless, to exclude potential artefacts in the immuno-FISH procedure, experiments were performed in a sequential manner, as previously described (Mignon-Ravix et al., 2002). Probes were chosen as follows: chromosome 1 was identified with a DNA paint probe (1066-1 B-02; Adgenix), a probe specific for the centromeric DNA sequences of chromosomes 1, 5 and 19 (LPE 05; Amplitech) and a probe specific for the 1qh juxtacentromeric satellite DNA (PSAT 0001; Q-Biogene). We also used a 16qh (clone pHuR195, 61081; ATCC), a 9qh (D9Z1; Q-Biogene), a 15ph (D15Z1; Q-Biogene) juxtacentromeric DNA probes. We used a pan-centromeric DNA probe (1141-F-01; Cambio) and a Cy3-labelled (CCCTAA)3 PNA probe (K5326; DAKO), which recognise all centromeres and all telomeres, respectively.

BrdU treatment

The possible involvement of the giant HP1-PML body in a DNA repair process was evaluated by incorporation of 5-bromodeoxyuridine (5-BrdU). After synchronisation and then releasing by thymidine, BrdU pulses for 15 minutes (final concentration 30 micrograms/ml) were successively performed during the time interval of 3-6 hours after release, which covers the whole G2 phase. After each pulse, cells were washed in PBS, cytospun onto slides, fixed in paraformaldehyde and immunostained with anti-PML Ab. The BrdU-incorporated DNA was then detected with a mouse anti-BrdU antibody coupled with fluorescein (1202693; Roche Diagnostics), as previously described (Stavropoulou et al., 1998).

DNAse and RNAse treatments

RNase and DNase treatments need to be performed prior to the fixation of the cells. When these treatments are performed on non-adherent cells spread onto slides by cytocentrifugation, they frequently result in cells lifting off the slide. Therefore, to overcome this inconvenience, the lymphoblastoid cells were treated in suspension, then fixed prior to being cytospun onto slides. DNAse treatment was performed with 0.1% triton/PBS buffer containing 0.25-0.5 μg/ml DNAse I (8047SA; Life Technologies) for 5-15 minutes at 4°C. Cells were then washed two times for 10 minutes each with PBS containing identical concentration of DNAse I at 4°C, fixed in suspension with 2% formaldehyde for 10 minutes, and cytospun onto slides. RNAse treatment was performed with 0.1% triton/PBS buffer containing 0.2-1.0 mg/ml RNAse A (R4642; Sigma-Aldrich), for 20-30 minutes at 23°C. Cells were then washed with PBS and fixed as for DNAse treatment. Immunocytochemistry was then performed with standard protocols.

Microscopic analysis

Nuclei preparations were observed using an Axioplan-2 Zeiss fluorescent microscope (Zeiss) and the images captured with a CCD camera (Photometrics 'SenSys'). Information was collected and merged using IPLab Spectrum software (Vysis). To analyse more precisely the colocalisation of the giant body with either satellite DNA or other proteins, several nuclei were photographed in 3D, using an axioplan-2 imaging Zeiss microscope equipped with a LUDL platform, motorised in *x*, *y* and *z* axes (Adgenix). This equipment allowed us to capture the images in different nuclear sections.

Confocal analysis

Confocal analysis was performed with a LSM510 NLO (Zeiss) confocal laser-scanning system equipped with an Argon Laser (488 nm), a HeNe laser (543 nm) and a femtosecond-pulsed infra-red laser (Tsunami-Millennium, Spectraphysics) for two-photon excitation (TPE) microscopy (wavelength 720 nm, pulse-width 120 femtoseconds, pulse repetition rate 80 MHz).

For triple labelling experiments, we used the following settings: AMCA fluorescence was obtained by TPE at 720 nm. The fluorescence signal was collected on channel 2 of the LSM510 after being reflected by a 545 nm beam splitter, then by a 490 nm beam splitter and was eventually selected by a 390-465 band-pass filter. FITC fluorescence was excited with the 488 nm line of the Argon laser. The fluorescence signal was collected on channel 3 of the LSM510 after being reflected by a 545 nm beam splitter, then passing through a 490 nm beam splitter and selected by a 500-550 band-pass filter with infra-red rejection. Cy3 fluorescence was excited with the 543 nm line of the Argon laser. The fluorescence signal was collected on channel 1 of the LSM510 after passing through a 545 nm beam splitter and selected by a 560 long-pass filter with infra-red rejection.

A series of ten optical sections were collected every 0.25 μ. Each optical section was stored as a 512×512 three-channel image with a 0.05 μ × 0.5 μ pixel size. Static three-dimensional reconstructions were generated with a homemade program Edit3D (Parazza et al., 1993; Metzler-Guillemain et al., 2000).

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