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PML nuclear bodies and their spatial relationships in the mammalian cell nucleus

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1. ABSTRACT

Promyelocytic leukaemia nuclear bodies (PML NBs) are found within the nucleus of mammalian cells, and are formed from the constituent proteins PML and Sp100. Numbering between 10 and 30 per cell, they are an obvious feature of the nuclear landscape, yet their functions have still to be unambiguously defined. In the mammalian nucleus, compartmentalization of functions is apparent, as reflected in the wide-range of other nuclear compartments that can be identified. These include nucleoli, transcription foci, splicing speckles, chromosomal topological markers such as centromeres and telomeres, the nuclear boundary, and the nucleoplasm itself. Quantification of the otherwise qualitative observations of relationships between mammalian nuclear compartments is essential for a complete understanding of nuclear processes. Here we describe some of the interesting known associations between PML NBs and other nuclear compartments, and comment upon their implications for PML NB function.

2. INTRODUCTION

2.1. The mammalian nucleus and functional compartmentalization

In the mammalian cell nucleus a number of substructures serve to compartmentalise the various functional activities. Nuclear architecture is complex, with many interactions described between functionally active compartments. Such substructures include the nucleoli, active sites of transcription (including nascent RNA and RNA polymerase II), Cajal bodies, splicing speckles, chromatin, chromosome topological markers such as centromeres and telomeres, the nuclear boundary, the nucleoplasm and promyelocytic leukaemia nuclear bodies (PML NBs). Unlike the nucleus itself, nuclear compartments are non-membrane bound and their formation is not fully understood. It is important to consider whether nuclear organization arises as a result of function, or vice-versa. At present little is known about the 3D spatial and temporal organization of nuclear processes, in comparison to their molecular basis.

2.2. PML nuclear bodies

PML NBs are a particularly interesting nuclear compartment due to their prominence in the nuclear landscape, which contrasts with their incomplete functional definition.

2.2.1. PML nuclear body composition

PML nuclear bodies (PML NBs), also known as ND10, PODs and Kremer bodies, number between 10 and 30 per cell, but this shows some variation across cell lines and according to cell cycle phase (1). Their principle protein component is PML (promyelocytic leukaemia) protein (as shown through the use of *Pml* knockout mice and cells (2)), which along with Sp100 makes up the two proteins that are constituently present at the bodies. In PML -/- cells typical PML NB proteins, such as Sp100, CBP, Daxx and SUMO-1, fail to localize within NBs (3). This is rectified by transfecting PML into PML -/- cells.

2.2.2. *PML* gene locus and PML isoforms

The PML genomic locus is about 35 kb long and consists of nine exons, from which a number of transcripts are produced via alternative splicing. This leads to the production of various PML isoforms and splice variants ranging from 48 to 97 kDa. There are seven isoforms of PML, PML I to VII, all of which share the N-terminal RBCC (RING-finger B-box, Coiled-coil)/TRIM (Tripartite motif) motif, but show C-terminal differences (4). Exons 2 and 3 contain the RBCC motif and exon 6 contains a nuclear localization signal (NLS). All isoforms except for PML VIIb contain the NLS and thus show a nuclear localization. A nuclear export sequence (NES) in exon 9 is retained in PML I, conferring upon it the ability to shuttle between the nucleus and cytoplasm (5). Therefore there is also a cytoplasmic population of PML protein, which may be implicated in the TGF-beta signalling pathway (6). At up to three lysines, (amino acid positions 65, 160 and 490), PML protein may be SUMO-1 (Small Ubiquitin-like Modifier-1) modified (7, 8). These sites are found within the RING-finger, first B-box and the NLS respectively. There is also a SUMO interaction motif (SIM) (9), found at amino acid positions 556-559 (10).

2.2.3. PML NB formation

PML protein found diffuse within the nucleoplasm lacks SUMO-1 modification (11). Homo-dimerization of PML protein via the coiled-coil moiety of the RBCC/TRIM leads to aggregation of primary PML bodies, which still lack sumolation (12) and differ in structure from the mature bodies. Upon covalent attachment of SUMO-1 to PML protein PML NBs mature into structures consisting of an outer PML protein shell that surrounds an inner core of other NB protein components (12, 13). There is a need for PML to be sumoylated for PML NB formation (3, 14). The other NB constituent protein Sp100 is also covalently modified by SUMO-1 (15). PML protein mutated so that SUMO-1 can no longer covalently bind to any of the three lysine residues at which SUMO-1 modification occurs forms aberrant nuclear aggregates and typical NB protein components such as Daxx, Sp100 and SUMO-1 are not recruited to these aggregates (3). PML forms a scaffold facilitating the accumulation of the other PML NB component proteins (3).

The SIM present in PML protein allows it to bind SUMO non-covalently. This is also a requisite for PML NB formation – following PML protein aggregation and sumoylation PML NB formation proceeds with the binding of PML to sumoylated PML through the SIM, along with the recruitment of other SIM-containing or sumoylated NB component proteins (9). These inherent biochemical properties of both PML and SUMO provide the molecular basis for PML aggregation and PML NB formation.

In the disease acute promyelocytic leukaemia (APL) the typical PML NB pattern is disrupted, and PML is found in a granular distribution throughout the nucleus (16-18). This is due to a reciprocal chromosomal

translocation at t (15;17) q (22;21), resulting in the formation of a PML-retinoic acid receptor alpha (RAR alpha) fusion protein, which is able to hetero-dimerize with wild-type PML and disrupt PML NB formation (19-22). Treatment with retinoic acid (RA) leads to reformation of PML NBs (17, 18). In non-APL cells where PML-RAR alpha has been transfected in, the disrupted PML NB organization can also be restored to the endogenous PML NB pattern by RA (16).

2.2.4. Functions of PML NBs

There are implications for PML NB function in wide-ranging nuclear activities (23) including tumour suppression (24), apoptosis (25), DNA replication and repair (26), gene regulation and transcription (27), and viral infection response (28). Further insight is provided by PML -/- mice, which although are viable, are more prone to tumours and viral infection (2). Since many of the proteins that co-localize with PML NBs do not interact in common pathways, it has therefore also been suggested that PML NBs act as protein storage depots (29).

3. SPATIAL RELATIONSHIPS IN THE MAMMALIAN NUCLEUS

Investigation of the spatial organization of the mammalian nucleus has thus far been dependent almost entirely upon observational studies using fluorescence microscopy methods to detect endogenous proteins and over-expressed exogenous proteins. Such studies are observer-subjective, and thus subject to observer biases. An alternative approach is to perform association studies, based upon the premise that when objects share a function they will show a closer association than is expected to occur stochastically. This is a more rigorous extension of observational studies.

3.1. Spatial organization of the nucleus – possible driving mechanisms

The mammalian nucleus is essentially a 3D volume and therefore understanding the mechanisms that drive its organization, and whether these are purely structural or functional, is essential for understanding nuclear function.

There are two main concepts that potentially could drive the organization of the nucleus; a scaffold-based nuclear architecture, or self-organization (30). In a scaffold based nuclear architecture structure must direct function – the nucleus has been "designed" to allow functions to be carried out efficiently, and organization is directed by a nuclear matrix structure. However, with self-organization the structure of the nucleus is determined by its functional status (31). Function is thus directing structure, incorporating the dynamism of a nucleus adapting to the conditions it finds itself presented with. Self-organization is paramount to the establishment and maintenance of nuclear organization (32). Apparently stable structures are actually the result of highly dynamic components – maintenance of the flux at steady-state equilibrium provides the illusion of stable complexes (31, 32). Stability and flexibility are combined, and both are essential for responsive nuclear functioning.

It is likely that macromolecular crowding, due to high concentrations of macromolecules in the nucleus, may also contribute to the assembly of nuclear compartments, which is in agreement with a self-organization model for nuclear organisation. Such crowding forces can increase association constants between molecules, and may separate different macromolecules into discrete phases (33).

3.2. Types of spatial relationships and association in the nucleus

The interplay between structure and function in the mammalian nucleus is manifested in its spatial organization. There are a number of types of spatial relationships between nuclear compartments that can be described. Examples of each can be found between PML NBs and other nuclear compartments.

Many different associations have been found to exist between PML NBs and various other nuclear substructures, mainly from qualitative observational studies using immunofluorescence detection or fluorescent protein fusions of marker proteins to define nuclear compartments. Complete co-localization is where a compartment is found to completely co-localize with all PML NBs in a cell nucleus. It may be the case that the compartment with which PML NBs are being compared is found at other locations within the nucleus in addition to PML NBs, such as diffuse or speckled throughout the nucleoplasm. An example of this is CREB binding protein (CBP), which is found at hundreds of discrete foci throughout the nucleus in addition to showing enrichment at the PML NBs (34). This indicates that there may be functional subsets of the other nuclear compartment, which adds complexity to the analysis of shared functional relationships with PML NBs.

Partial co-localization, where PML NBs and another nuclear compartment only partially overlap, may occur at all or only some PML NBs. This association also includes compartments that completely co-localize with only a subset of PML NBs, suggesting that PML NBs also may belong to subgroups that have different roles in the nucleus. This could explain the seemingly heterogeneous functions of PML NBs, or tie in with the different isoforms of PML (with their different C-termini) having different roles in the cell. Compartments may also be found adjacent to PML NBs, where there is no co-localization between the two. There may be a visible association between PML NBs and the compartment to varying degrees, but which again involves no co-localization. Such relationships can be quantified using distance-based approaches. Cell cycle phase specific associations, or induced associations between compartments and PML NBs caused by effectors of stress or viral infection may also occur, and thus are not seen all of the time.

Sometimes there are no obvious associations by eye, but they can be shown to occur more often than expected to stochastically – in cases where the compartment pattern is too complex to compute by visual inspection. Lastly, there may be no association above that which occurs by chance, likely showing that there is no relationship between the functions of PML NBs and the compartment with which they

are being compared. Such associations are not functionally relevant, yet may still occur because ultimately the nuclear volume can offer only limited locations for compartments to exist at. At the qualitative observational level these associations may easily give false positives with regard to potential PML NB functions, and as such should be reinforced with biochemical and, or, statistical data.

3.2.1. Complete co-localization

Classification of co-localization relationships tends to derive from observational studies. By far the most obvious spatial associations between PML NBs and other nuclear components are those that localize to the nuclear bodies themselves. Therefore, for complete co-localization full overlap between compartment and PML NB is a requirement at a site of co-localization.

Proteins may either transiently or covalently associate with the bodies. Those which localize to PML NBs include Sp100 (35), the other constituent protein of PML NBs, involved in transcriptional regulation, SUMO (36), CBP (37), BLM (14), Daxx (a transcriptional repressor) (14, 38), p53 (39) (a transcriptional activator) and pRB (40). Due to the large number of proteins that potentially interact with the PML NB it is difficult to tell whether the bodies play a functional role when they associate, or if they are simply acting as a store. Below we describe some of the proteins that share complete co-localization with PML NBs.

3.2.1.1. Sp100

Sp100 was first seen as a nuclear body component protein prior to PML (41). Together they make up the constituent proteins of PML NBs. Both PML and Sp100 are modified by SUMO-1 (15), and dissociate in mitosis, where they are no longer modified by SUMO-1 (15, 42). Sumoylation of Sp100 is not necessary for its targeting to PML NBs (43).

Sp100, and its splice variant Sp100-HMG (or Sp100b) have been shown to bind members of the heterochromatin 1 (HP1) family, and upon overexpression of Sp100, levels of endogenous HP1 have been shown to increase at PML NBs (44). This indicates a potential role for PML NBs within the control of heterochromatin architecture, and also in transcriptional repression as Sp100 may potentially act in a repression complex with HP1 and Sp100-HMG (44, 45).

3.2.1.2. CBP

PML protein and CREB binding protein (CBP) are found to co-localize within the nucleus at the PML NB (13). CBP is a transcriptional coactivator and a histone acetyl transferase (34), and is found throughout the nuclear body, whereas PML protein is only found in the outer shell (13). CBP is also seen in a finely speckled nucleoplasmic pattern in addition to being at the NBs (13). Accumulation of CBP at PML NBs is cell-type specific, and as demonstrated with Fluorescence Recovery After Photobleaching (FRAP), CBP moves rapidly between PML NBs and the nucleoplasm, whereas PML is comparatively stable (37). Compartmentalization of CBP at PML NBs indicates that PML NBs may play a role in transcriptional regulation at the level of histone modification, perhaps providing an environment for this to occur in.

3.2.1.3. SATB1 (MAR-binding protein)

The matrix attachment region (MAR)-binding protein special AT-rich sequence binding protein 1 (SATB1) directly interacts with PML protein, and is seen to show complete co-localization in HeLa and normal human WI38 fibroblasts (46). This interaction is required for the organization of the MHC class I locus into chromatin loop-structures, thus linking PML NB function to higher order chromatin organization, and possibly the nuclear matrix.

3.2.1.4. eIF4E

eIF4E (eukaryotic translation initiation factor 4E) is involved in nucleo-cytoplasmic messenger RNA (mRNA) transport, and its overexpression leads to increased cyclin D1 levels, cellular transformation and blocking of apoptosis in serum-starved cells (unlike PML which is a negative regulator of growth) (47). Endogenous eIF4E and PML co-localize at PML NBs. Dissimilar to PML protein, eIF4E has a discrete biochemical activity, and directly binds the 7-methyl guanosine 5' cap of mRNA (48). PML is able to repress eIF4E-dependent cyclin D1 mRNA transport (49), and mutation of the W73 amino acid residue in eIF4E abolishes its interaction with PML. Interaction of the PML RING domain with eIF4E causes a conformational change around the cap-binding site, inhibiting its ability to transport RNA. PML is also able to abrogate eIF4E-mediated transformation through its effects on eIF4E cap-binding (49). Thus, PML function can be directly linked with suppression of transformation.

Interestingly there is also a subset of eIF4E bodies in the nucleus that do not co-localize with PML NBs, and so although eIF4E is present at all PML NBs, not all eIF4E is associated with PML (50).

3.2.1.5. PLZF

Promyelocytic leukaemia zinc-finger (PLZF) protein localizes to around 14 0.3-0.5 μm nuclear domains in the KG1 myeloid cell line (51). These bodies appear similar to PML NBs, and about 30% of PLZF bodies co-localize with PML NBs. However, despite their apparent full co-localization at such sites, closer inspection of confocal microscopy data indicates that PLZF bodies and PML NBs may in fact be discrete structures, with variable degrees of overlap at their surfaces (51). Unlike PML NBs, PLZF bodies are not disrupted by E4 ORF3, and do not respond to treatment with IFN (51) (yet interestingly such treatment does result in the recruitment of PLZF to PML NBs without an increase in PLZF expression (52)). Therefore it is likely that PLZF bodies and PML NBs are functionally distinct, despite the PLZF/RARα fusion protein also being implicated in a rare form of APL (51).

3.2.2. Partial co-localization

Some nuclear proteins form compartments and bodies that only partially co-localize with PML NBs. This means that a proportion of the compartments overlap to

some degree with some or all PML NBs in a nucleus. However, as previously mentioned, partial co-localization also includes the complete co-localization of a compartment, but with only a subset of PML NBs. This indicates that not all PML NBs are equal, and some may behave differently to others.

3.2.2.1. Cajal bodies

Cajal, or coiled, bodies (CBs) are small spherical bodies composed of a large number of protein and RNA components including spliceosomal snRNPs (small nuclear ribonucleoproteins, various snoRNAs (small nucleolar RNAs), and the transcription factors TFIIF and TFIIH. Occasionally CBs can be found within the nucleolus, but they are generally located within the nucleoplasm. They are involved in the transport and maturation of snRNPs and snoRNPs, and associate with specific chromosomal loci, such as the tandemly repeated genes encoding U1 to U4, and U11 to U12 snoRNAs in mammalian interphase nuclei. PML NBs and CBs have been shown to partially co-localize (53-55), where typically at least one PML NB in a nucleus has an associated CB. A CB and its associated PML NB may localize to the same U2 small nucleolar RNA (snRNA) gene locus, where the CB appears positioned between the PML NB and the snRNA locus (55). Biochemical interactions between coilin (a CB marker protein) and PIASy (a PML NB component protein) could partially account for this co-localization. However, PML NBs do not contain U2-snRNP itself, which is found in Cajal bodies (53, 56). The function of this partial co-localization is so far unknown but may relate to PML NB functions in translational regulation.

3.2.2.2. Active transcriptional domains

Subpopulations of PML NBs show associations with sites of active transcription (57). By looking at fluorouridine (Fl-U) incorporation into nascent RNA, it has been shown that there are three types of interactions between PML NBs and active transcription sites as observed by indirect immunofluorescence; no overlap (but potentially adjacent localizations of the two foci), a partial overlap, and complete overlap, which was unrelated to the length of exposure to Fl-U. The majority of PML NBs (69%) show no overlap, with 28% showing partial overlap and 3% showing complete overlap; meaning that in unsynchronised cells over 30% of PML NBs show spatial association with transcription sites also demonstrated to contain (transcriptionally) active hyperphosphorylated RNA polymerase II. Cells in G1 phase, or treated with IFN show a spatial association of 70% and 80% respectively between PML NBs and sites of active transcription (57). Other studies have also provided evidence for the presence of nascent RNA or RNA polymerase II within PML NBs (13, 58). Collectively these strongly point to a role for PML NBs as an environment conducive to transcription.

However, there is some debate surrounding PML NBs' role as a site of active transcription. Using electron microscopy in HeLa cells PML NBs have instead been seen to be surrounded by RNA polymerase II or nascent RNA at distances greater than 25nm, with these not actually being found within the bodies themselves (59) confirming others' previous studies (53, 60, 61). To account for discrepancies in proximity one might consider that such observational differences may potentially be the result of alternative methodological approaches (discussed further in 4.2.1). Despite differences in opinion over the degree of association between PML NBs and nascent RNA transcripts, all of these studies are able to support a functional role for PML NBs at transcriptional compartments. On the other hand, in spite of their nearness to such compartments, it does not necessarily follow that PML NBs actively function in transcription. Indeed, PML NB immediacy to the transcriptional apparatus and nascent transcripts without co-localization may alternatively suggest a post-transcriptional function for the bodies.

3.2.2.3 Proteasomal protein degradation

Proteasomes are found in both the nucleus and cytoplasm, and proteasomal protein degradation foci have been demonstrated in the nucleus using immunofluorescence (62). Such foci partially overlap with a number of nuclear compartments, including PML NBs as shown by microinjection of DQ-ovalbumin, which becomes brightly fluorescent upon hydrolysis by proteases (62). The nuclear ubiquitin-proteasome system revolves around the 26S proteasome, which consists of the 20S "core" flanked by two 19S complexes that play a role in the regulation of substrate specificity (63). The immunoproteasome, which consists of the 11S complex (replacing the 19S complex at either or both ends of the proteasome) plus the 20S core, is induced by IFNγ, and aids in the proteolysis of peptides prior to their presentation to the MHC. The co-localisation of the 11S proteasome with PML NBs suggests that PML bodies may act as sites of active protein degradation, which may represent an underpinning biochemical function for PML bodies (12). The immunoproteasome's inducible association with PML NBs is discussed further in section 3.2.4.3.

3.2.2.4. Telomeres

A further example of partial co-localization between a nuclear substructure and PML NBs is that of telomeres. These subcellular structures consist of TTAGGG repeats bound by a protein complex that prevents chromosome ends from fusing or degrading (64, 65). The process of DNA replication results in gradual telomeric shortening, which in turn leads to cellular senescence. To overcome this problem, certain immortalized cell lines are able to maintain, or even extend, telomeres via a homologous recombination (66, 67) that does not require telomerase. Alternative lengthening of telomeres (ALT) associated PML NBs (APBs), a subset of PML NBs, co-localize with telomeres in such cells (65, 68, 69). APBs contain telomeric DNA, telomere-binding proteins TRF1 and 2, and various DNA recombination and replication proteins (68). The role of PML NBs in ALT mechanisms remains unclear but it has been suggested that they may promote the association or stability of recombination complexes, create a suitable chromatin environment for recombination (10), or even be a platform for the sumoylation of telomeric proteins in ALT cells (10, 70).

3.2.3. Adjacent localization

Adjacent localization includes relationships between nuclear substructures and PML NBs that include the two compartments being next to each other, or even touching, but not showing any co-localization. Here we discuss some of the nuclear compartments shown to share this relationship with PML NBs.

3.2.3.1. Middle-late S-phase replication domains

There are four different patterns of BrdU (bromodeoxyuridine, a thymidine analogue) incorporation that are found as DNA replication progresses – early (with many small domains), middle (with replication sites at the nuclear periphery and around nucleoli), middle-late (chain like structures of replication sites) and late (fewer large replication domains) (53). Whilst being excluded from such domains, PML NBs are often found next to a DNA replication domain in middle-late S-phase (53), with 50-80% of PML NBs in middle-late S-phase T24 bladder carcinoma cell nuclei directly adjacent to a replication domain, which may in part reflect the high proliferation rate of these cells. Since PML NB number is known to increase during S-phase it is possible that this is a response to chromatin organization changes, suggesting a role for PML NBs in DNA synthesis (1).

3.2.3.2. PLC-gamma 1

The gamma 1 phospholipase C (PLC-gamma 1) isoform, which is a phosphoinositide hydrolyzing enzyme, was found as an associated protein of PML NBs using mass spectrometry of PML co-precipitates derived from HCT116 human colon carcinoma cells (71). Phosphoinositides may play a role in nuclear signalling pathways and gene expression. Immunofluorescence reveals that PLC-gamma 1 puncta are often found adjacent to PML NBs in HCT116 cells. However in normal diploid fibroblasts the staining is cytoplasmic and vesicular suggesting that the observed association may be specific to transformed cells (71). PML NBs may thus also have a role in phosphoinositide nuclear signalling pathways.

3.2.3.3. Chromosome territories

PML NBs, (as well as Cajal bodies) co-localize with NLS-vimentin filaments indicating that they co-exist in an interconnecting nuclear compartment, from which chromosomes are excluded (72), also known as the inter-chromosomal domain compartment (ICD). Therefore PML NBs are likely to be found adjacent to chromatin compartments known as chromosome territories (CTs), which consist of both heterochromatin and euchromatin. However, by scoring individual gene loci (covering a range of locations on chromosomes 1, 6 and 9) for their position either peripheral, internal or external to their corresponding CT, and also whether or not they were touching a PML NB, Wang et al have shown that a locus' association with a PML NB is not dependent upon its position relative to its CT (supplementary data from (61)). Also, the PML NB – locus association is not dependent upon the CT to which the locus belongs. Therefore it is likely that PML NBs can also exist within CTs, as well as being peripheral and external to them.

3.2.3.4. Splicing speckles

Splicing speckles are dynamic nuclear compartments numbering between 25 and 50 per cell, and are rich in pre-messenger RNA (mRNA) splicing machinery such as snRNPs, spliceosomal subunits and other splicing factors (73). PML NBs often appear to be associated with the edges of splicing speckles, as defined by the SC35 spliceosome assembly factor ((74) and own observations). Their spatial location within interchromatin regions could account for their close association with PML NBs, and since they are storage sites for splicing factors, this could infer a similar nuclear function for PML NBs.

3.2.4 Induced relationships with PML NBs

Relationships between PML NBs and other nuclear compartments that are not normally seen can be induced under certain circumstances. Such events include DNA damage, viral infection, interferon (IFN) response and other cellular stresses. Proteins may either be recruited to PML NBs, or PML NBs are themselves recruited to other parts of the nucleus. The latter probably involves the fission products of PML NBs and not *de novo* formation of bodies (1). Such associations highlight the potential dynamic nature of the PML NB in response to cellular events.

3.2.4.1. DNA damage

3.2.4.1.1. Single strand DNA damage

In response to exogenous DNA damage, resulting from UV irradiation for example, PML NBs are able to co-localize with (75) and recruit single-stranded DNA (ssDNA) molecules (76). Imaging of live cells expressing YFP-PML shows that ssDNA foci form within existing PML NBs, and formation is inhibited in cells treated with siRNA directed against PML. Therefore ssDNA foci are either recruited into PML NBs (making it possible that PML NBs are DNA damage repair sites), or that PML NBs contain regions of chromosomal DNA that are processed into ssDNA upon DNA damage (76).

3.2.4.1.2. Double strand DNA damage

Gamma-H2AX, (histone H2AX phosphorylated on serine 139) localizes to large chromatin domains at sites of radiation-induced sites of double strand breaks (DSBs). Within hours of treatment with ionizing radiation PML NBs and DSBs co-localize (75, 77, 78). Eight to twelve hours subsequent to treatment, PML NBs partially or completely associate with hMre11 ionizing radiation-induced foci (IRIF), and later with p53 (75) in a stable association. Thus PML NBs are involved in DNA damage response and repair pathways, as both PML and DNA repair proteins are recruited to sites of DNA damage.

3.2.4.2. Viral infection and response to foreign DNA

3.2.4.2.1. Viral infection

Associations between PML NBs and the parental genomes of DNA viruses and early replication compartments have been shown to exist (79-81). Interestingly, PML NBs become disrupted upon infection with adenovirus, where the adenovirus type 5 (Ad 5) early region 4 open reading frame (E4 ORF3) product

reorganizes the bodies into thread-like structures within the nucleus (82, 83). The targeting of PML NBs by a variety of viruses suggests that disabling of PML NB function could be an important part of viral viability. In response to IFN, PML NBs may play a role in the antiviral activity of IFN, and thus viruses try to block IFN-meditated actions through the disruption of PML NBs as part of efforts to remove proteins that affect or interfere with efficient viral replication.

3.2.4.2.2. Foreign DNA

PML NBs may also help in preventing the expression of foreign DNA (84). Mouse polyomavirus-like particles (VLPs) are able to deliver transgenes into cell nuclei via pathways used by viruses but do not contain factors necessary for initiation and regulation of transcription. Such transgenes are positioned near centromeric heterochromatin to enable silencing. Upon transcriptional activation they are relocated to euchromatin and associate with PML NBs. Despite not playing a role in regulation of transgene expression, PML protein is required to enable interferon alpha (IFN alpha) inhibition of transgene expression, suggesting that PML is involved in type I IFN response in the prevention of foreign DNA expression (84).

PML NBs appear to associate with foreign DNA and its protein products, as seen above with viral infection, and also through integration of artificial constructs. In a system developed to directly visualize a gene and its protein product in living cells using the lac operator / repressor system, it was found that the integrated locus became surrounded by a PML NB in a transcription (of the locus) independent association (85). *In vivo* binding of enhanced yellow fluorescent protein (EYFP) / *lac* repressor and tetracycline receptor / VP16 transactivator to the locus was required, showing that the co-localization related to the high concentration of the expressed protein (EYFP / *lac* repressor or tetracycline receptor / VP16 transactivator) needed to associate at the gene locus for the assay, rather than the locus itself. The authors propose that in this case PML NBs may be acting as "sensors" of local accumulations of foreign proteins or DNA in the cell.

3.2.4.3. The Interferon Response

IFNs are secreted proteins that meditate a number of cellular responses including antiviral and antiproliferative activities (86). Treatment with IFN induces an increase in both PML mRNA and protein expression levels, as shown by a greater number and intensity of PML NBs in the nucleus (87). In response to IFN, a significant number of proteins are recruited to PML NBs.

3.2.4.3.1. NDH II

Nuclear DNA helicase II (NDH II) is a transient component of PML NBs (88), and links CBP to RNA polymerase II. It co-localizes with a small subset of PML NBs under normal conditions, but upon interferon alpha (IFN alpha) addition it co-localizes with almost all PML NBs in a transcriptionally dependent association, indicated by the presence of nascent RNA transcripts that are also found at PML NBs to which NDH II is recruited. Therefore PML NBs are implicated in the regulation of transcription of IFNα-inducible genes (88).

3.2.4.3.2. PA28 and the immunoproteasome

The interferon gamma (IFN gamma) inducible proteasome activator PA28 (also known as the 11S complex), which is involved in major histocompatibility (MHC) class I antigen presentation, can be found at PML NBs under basal conditions, but IFN gamma treatment increases the number and size of PA28-containing PML NBs (89). The 20S core proteasome, which is the target of PA28, is not typically found at PML NBs under basal conditions, but upon IFN gamma addition "immunoproteasomes" (consisting of the 20S core plus the 11S complex) are recruited to the bodies. Thus, PA28, a normal PML NB component, is able to help establish immunoproteasomes, which are then found at PML NBs under IFN gamma conditions. This co-localization suggests that PML NBs play some role in antigen presentation to the MHC, perhaps by providing an environment that is favourable to protein degradation. Alternatively, since PML NBs are targets for viral infection (see section 3.2.4.2), IFN produced in response to viral infection induces immunoproteasome formation and localization to PML NBs in an effort to degrade viral peptides for presentation to the MHC class I at the intranuclear site where they are most likely to be found. It is interesting to note that PML knock-out mice, despite being viable, are more prone to viral infection (90), suggesting that this process is less efficient in *pml* $^{-/-}$ mice.

3.2.4.4. Stress

3.2.4.4.1. Stress and nucleoli

UV-C or IR-gamma, and various types of stress such as chemical inhibition of transcription or DNA synthesis, and proteasome inhibition causes endogenous PML protein (specifically the PML I isoform with its C-terminal nucleolar targeting domain) to redistribute to nucleolar caps that eventually surround nucleolar components (91). Whilst there is little evidence to support an association between PML NBs and nucleoli in normal primary cells, in cells with DNA damage PML protein has been shown to sequester Mdm2 (a p53 ubiquitin-ligase) to the nucleolus, and therefore aids p53 stability (92). In human mesenchymal stem cells novel PML compartments have been observed in association with nucleoli (93).

3.2.4.4.2. Proteasomal inhibition and centromeres

An association between PML NBs and centromeres may exist as revealed in a significant number of G2 Hep2 cells that had been treated with the proteasome inhibitor MG132 (94). Initially Vmw100 (Herpes simplex virus type 1 regulatory protein) was observed to induce the proteolysis of both PML and Sp100 in a proteasome-dependent manner, in addition to that of CENP-C (a protein found at the centromere). This infers a dynamic link between PML NBs and centromeres in G2, which is only stabilized upon proteasomal inhibition. Thus a cell cycle-dependent link between PML NBs and centromeres can be suggested (94). However, as centromeres are

primarily heterochromatin structures this indicates that PML NBs may also be involved in the repression of gene expression (in a cell cycle specific manner), as well as in transcription.

3.2.5. Associations with PML NBs to a varying degree

Lastly, associations between PML NBs and other nuclear substructures may not involve direct physical associations, and instead must be measured using a distance-based approach, considering the degree of association and its relevance to shared functionality. This approach requires a strong statistical underpinning, and can also be used in the study of complex nuclear compartment interrelationships in which association cannot be decided by eye alone.

3.2.5.1. MHC

A highly non-random association between PML NBs and the major histocompatibility complex (MHC) gene cluster on chromosome 6 (specifically the centromeric end extending over 1.6 megabases) has been shown to exist (95). When this region was integrated into chromosome 18 the association with PML NBs remained suggesting that genomic determinants for PML NB association were specific. Association was compared between PML NBs, and two gene-rich regions (MHC on chromosome 6 and epidermal differentiation complex (EDC) on chromosome 1), and the gene-poor 6p24 region on chromosome 6. From observations alone it would seem that PML NBs could be seen to associate closely or overlap with either the MHC or EDC, both at the same time, or neither region. This highlights the need for more than just observations when showing that associations between nuclear compartments do or do not exist. To address this Shiels et al (95) developed a minimal distance nearest neighbour method to allow probabilities to be calculated for association or non-association showing for the first time that PML NBs associate specifically with particular gene rich regions of the genome.

These quantitative studies were extended by Wang et al (61) who found that the distance between a locus and its nearest PML NB correlates with both transcriptional activity and gene density around that specific locus. PML NBs were shown to associate with genomic regions of high transcription activity rather than highly transcribed individual genes. The association of PML NBs with transcriptionally active regions is further shown by the finding that genes on the active X chromosome are more significantly associated with PML NBs than their silenced counterparts, and that the histone-encoding gene cluster, transcribed only in S-phase is more strongly associated with PML NBs in S-phase than in G0/G1 (61).

4. WHAT AFFECTS PML NB SPATIAL RELATIONSHIPS AND ASSOCIATIONS?

Spatial relationships between PML NBs and other nuclear compartments and substructures are affected by both internal and external influences on the cell, including cell cycle related changes, and also by experimental methods. These may influence the characterisation of associations to some degree by affecting PML NB morphology or spatial locations, as well as that of the nuclear compartments being studied in conjunction with PML NBs.

4.1. Cell-related factors

4.1.1. Cell-line specific factors and disease states

PML NB number is known to show variation across cell lines (1). Variations in PML NB number, morphology and diameter across cell lines will result in differential PML NB – nuclear compartment relationships depending upon the choice of cell line of the researcher, important to consider when comparing studies between labs.

In certain cells it is possible to observe PML NB relationships with nuclear compartments that do not occur in others. For example, survival of motor neurons (SMN) protein, loss of which causes spinal muscular atrophy, co-localizes with most Cajal bodies. However interestingly in some transformed cell lines and primary foetal tissues SMN localizes to nuclear structures known as Gems, which are commonly found adjacent to Cajal bodies. In cells in which Gems are absent, it would thus be impossible to measure their interrelationships with PML NBs.

Primary cells and tumour cell lines often differ in expression levels of certain proteins, which may in turn alter nuclear organization or morphology, to the point of affecting the presence or absence of particular nuclear compartments. In tumour cells that are positive for telomerase, it is unlikely that they would need to employ ALT mechanisms to maintain telomere length, and thus PML NBs are not likely to show association with telomeres. Some proteins are not present at all in some cells, a good example of which are *pml* $^{-/-}$ cells. For obvious reasons it is not possible to measure relationships between endogenous PML NBs and other nuclear compartments in these cells.

A disease state may also influence the localization of proteins within a cell, as is evident in the PML-implicated disease acute promyelocytic leukaemia (APL). In APL cells the typical PML NB pattern is disrupted, with PML protein unable to form nuclear bodies, instead showing a microspeckled localization throughout the nucleus (16-18), due to wild-type PML and PML-RAR alpha fusion proteins hetero-dimerizing. In such cells normal spatial relationships are obviously disrupted as a consequence of PML protein delocalization from PML NBs. The pathology of APL is characterised by a block in promyelocyte differentiation, which becomes released with all-*trans* retinoic acid (ATRA) treatment (96), and a typical PML NB pattern returns to cell nuclei (97).

PML protein is found within giant bodies also containing heterochromatin HP1 proteins over the 1qh and 16qh juxta-centromeric heterochromatins in G2 cells of immunodeficiency, centromeric instability and facial dysmorphy (ICF) syndrome patients (98). Such bodies also contain satellite DNA unlike normal PML NBs, and

may be related to remodelling of heterochromatin in these specific disease cells. They are also likely to share different relationships with nuclear compartments than PML NBs, due to their differences in morphology.

4.1.2. PML cell cycle dynamics – protein expression, NB number and size

PML NB number is also known to vary according to cell cycle phase (1) and endogenous PML expression levels do differ within a cell population (99).

In late G1 cells, PML NBs are strongly labelled in around 15 bodies (99). In S phase cells there are also smaller bodies plus a diffuse nuclear fraction of PML protein, and the number of bodies increase two-fold from G1 (1). This increase is due in part to loss of PML NB structural stability, with fission and fusion events occurring. PML protein is redistributed as opposed to being synthesized *de novo* (1). Since the fission products are associated with chromatin, PML NBs probably respond to changes in chromatin organization and topology (1). Throughout G2 PML NB number decreases until a few large PML aggregates (known as mitotic accumulations of PML protein (MAPPs) are found in mitotic cells, (99, 100). Mitotic PML protein is de-conjugated from SUMO-1 (42). MAPPs also do not contain traditional PML NB component proteins such as Sp100 or Daxx, and contribute to reformation of PML NBs in G1 (100).

Senescence-associated nuclear bodies (SANB) are large PML NBs associated with spontaneous senescence, and include nucleolar proteins in addition to PML protein (91). Changes in PML NB number and morphology related to cell cycle progression or senescence are likely to result in differential PML NB – nuclear compartment relationships as a function of time or age of a cell. Therefore there is also a temporal aspect to studying nuclear compartment interrelationships and in particular when considering PML NB associations.

4.1.3. PML NB movement and dynamics

The spatial location of PML NBs within the mammalian nucleus is fairly restricted by the local chromatin environment that surrounds them. PML NBs exclude chromatin and thus it is likely that they reside in the channels and lacunae of the interchromatin space, whilst migrating though chromatin within a corral (101). Investigation of nuclear body movement using biologically inert Mx1-YFP expression bodies reveals that bodies diffuse within such a chromatin "corral", which is itself translocated within the nucleus as a result of chromatin diffusion (101). Therefore nuclear body mobility is a reflection of the accessibility and dynamics of the surrounding chromatin environment. Expression of NLS-Vimentin, a protein that forms filaments in the nucleus, can be used to map the interchromatin "free space" within the nucleus in which PML NBs are able to move (72), providing an idea of the limitations placed upon PML NBs in determining their nuclear locations.

PML NBs can be distinguished into three classes based upon their dynamic properties (102). Using EYFP fused to human Sp100, PML NB dynamics were studied *in vivo* over a 12 min period. PML NBs were divided into those which were positionally stable, (around a quarter of total PML NBs, and which was not size related as stationary bodies ranged in diameter from 0.2 to 1.4 μm), those with limited localized movement (which formed the majority of PML NBs), and finally those which showed rapid nuclear movements (starting and stopping several times during the 12 mins), roughly numbering 1-2 PML NBs per nucleus. Some nuclei contained over 10 of these fast moving PML NBs, and over half of nuclei studied contained at least one fast moving PML NB. Rapidly moving PML NBs were predominantly smaller bodies, and travelled at an average velocity of 4.0-7.2 μm min^{-1}, with a maximum of 18 μm min^{-1}. Note that whilst being seen in primary mouse embryonic fibroblasts and mouse embryonic stem cells, fast moving PML NBs were not seen in HeLa cells. Their movement is a metabolic-energy dependent mechanism as rapid longer movements ceased during ATP depletion (102).

Other studies have also shown that a population of PML NBs are stable in position and structure over extended periods of interphase (1, 103). Stresses including heat shock, heavy metal exposure and expression of adenovirus type 5 E1A protein, result in the fission of small PML-containing microstructures from the parental bodies, which lack in SUMO-1 and Sp100 (103). There are two subsets of microstructures, the first likely to be trapped within chromatin pockets and therefore sharing diffusion constants with that of mobile chromatin domains, and being able to move 50-70 nm per sec. The second group occupy large chromatin-free channels allowing diffusion constants much greater than the first subset. Hence, such microstructures show movement of up to 0.5 μm in 1 second and such movement is energy-independent. During recovery microstructures fuse with each other and also with the positionally stable PML NBs (103).

So whilst PML NB locations are fairly stable over time, PML protein itself is dynamic meaning that a subset of more mobile PML NBs may indeed possess much more variable associations with other nuclear compartments. Use of live cell imaging will be extremely useful in the investigation of such relationships.

4.1.4. PML isoforms

All PML isoforms have been found to co-localize at PML NBs. Although some PML NB – compartment relationships invoke specific PML isoforms, the whole PML NB would still be implicated. However it is interesting to note that when individually expressed in a *Pml*-null background, the different isoforms of PML show varying nuclear body morphologies (5), such as PML II's thread-like distribution, and PML V's large and dense bodies. Since specific isoform expression is not exclusive to certain cell lines or types (5), this should not bias PML NB relationships noted in particular cell lines or types. There are however differences between levels of particular isoform expression, with the major isoforms being PML I and II (5). Therefore PML NB relationships characterised

overall may possibly be more of a reflection of the roles of these isoforms in the nucleus.

4.2. Method-related factors

4.2.1. Fixation, permeabilization and immunofluorescence

Fixation and permeabilization are essential for the immunofluorescent staining and visualisation of mammalian cells at particular time points. Different methods may be used, usually those historical to the lab but this has an effect upon the distribution of proteins in the fixed cell, especially that of nuclear proteins. As an example, different cell fixation and permeabilization methods can be correlated with the relocation of active RNA polymerase II complexes to splicing speckles where conditions did not preserve cellular ultrastructure (104). Ominously the same methods also caused some redistribution of PML staining from PML NBs, but did not affect labelling of SC35 (splicing speckles) or coilin (Cajal bodies).

Some antibodies used in immunofluorescence recognise antigens better than others, and hence use of different antibodies to stain the same nuclear compartments in different publications may, in the extreme, result in noticeable differences in associations detected.

4.2.2. Imaging methods

Widefield and confocal microscopy are commonly used in the imaging of co-localization studies in the cell nucleus. The resolution limit of light microscopy poses a constant problem for the confirmation of true co-localization between two nuclear compartments. In 3D confocal imaging a further potential difficulty is the difference in resolution between the *z*-axis and those of the *x* and *y*. To some extent deconvolution can help resolve both drawbacks, but advances in imaging will be needed to improve the accuracy of quantification in association studies.

4.2.3. Choice of experimental time points

From the examples discussed it is clear that there are different subpopulations of PML NBs, and that when imaging PML NBs it is likely that all populations will be included in the analysis, especially in unsynchronised cells. Isoform-specific antibodies for PML will help to reveal any relationships that are related to particular isoforms in normal primary cells. Currently the vast majority of cell imaging is done in fixed cells, and not live ones. For PML microstructures that show movement, and are derived from larger PML NBs, their relationship with other nuclear compartments will be fairly dynamic, and imaging methods using fixed cells will only capture a snap shot of this PML NB population in time.

4.2.4. Effects of stress and handling of cells upon PML NBs

It is clear that PML NBs react to cellular stresses with changes in number, size and morphology (26, 103). When subjected to stress (including UV-C radiation, IR-gamma, and transcription or proteasome inhibition) endogenous PML (likely to be PML I) forms nucleolar caps (91). In response to type I interferon (IFN) PML expression is induced, resulting in a greater number of PML NBs, which are also larger in size than normal (87, 105). Although the stresses above will not occur in cells outside of experimental protocols, it may be that stresses on the cell resulting from culturing procedures and handling could also potentially have an effect on PML NB spatial relationships.

4.2.5. Over-expression of transiently and stably transfected PML

Although most PML live cell imaging is done with cells that stably express PML, the expression levels may not reflect exactly what is happening in normal cells. When using stably expressed PML, it is essential to choose the clones that best reflect *in vivo* levels of PML expression. However, usually only one isoform of PML is expressed, which as mentioned previously may result in different morphologies of PML NB (5). In cells where PML is transiently expressed the PML NBs that form do not always share normal morphology, and may simply be large aggregates of PML that do not necessarily behave in the same way as normal PML NBs.

5. HOW DO WE MEASURE PML NB – NUCLEAR COMPARTMENT RELATIONSHIPS?

When using an immunofluorescence approach to study nuclear compartment relationships, simple co-localization studies have disadvantages. They cannot discern with any significance if co-localization is due to functional homology, stochastic mechanisms, or a previously undetermined interaction with another nuclear compartment. For compartments showing complex localizations throughout the nucleus it is not always possible to identify trends through observations alone, and therefore a quantitative approach is required.

In order to measure the relationships between nuclear compartments quantitative distance-based studies become the obvious choice for the extrapolation of co-localization studies. Statistical methodologies should be employed to compare distance-based experimental data to simulated data generated using suitable hypotheses. Such studies are made more difficult by the fact that the nucleus is a three-dimensional space, as this poses problems for data modelling. It can then be determined if compartments associate more often than expected due to chance – in which case it is likely that an association exists. It is then prudent to reinforce such data using a biochemical approach, such as seeing if marker proteins from both compartments co-precipitate together. Proteins may directly associate with PML protein (either transiently or covalently), or with PML NB associated proteins, and then be dragged in to the bodies. However, if for example the relationship does not involve complete or partial co-localization, this may not be seen in the biochemical data. Also the wrong marker protein might be chosen, therefore revealing no relationship in the IP, when in fact one exists.

It is relatively simple to observe relationships between PML NBs and other nuclear compartments, but

much more difficult to translate such observations into meaningful conclusions, especially in relation to PML NB function. Through use of statistical methodologies to process association measurements it is possible to further investigate and confirm PML NB nuclear compartment interrelationships.

6. PERSPECTIVE

PML NBs show associations with many compartments in the nucleus. These include complete, partial, and adjacent associations, as well as associations to certain degrees, which can be quantified by distance-based methods. Occasionally such relationships are almost impossible to define by eye due to the complex nuclear localization of compartments, and thus statistical methodologies must be applied to look for associations in such data sets. More often than not observations are simply qualitative, and efforts in the future should be focussed upon moving away from this.

The inference that PML NB function relates to that of compartments with which it shows associations is useful when defining PML NB function, as exemplified by the many relationships described herein. Future application of such techniques to other nuclear compartments sharing undefined relationships with PML NBs may indeed help to highlight further functional relationships, or to discard less fruitful avenues of research. If there are indeed subtypes of PML NBs it will also be interesting to look at their spatial associations with each other, as this may provide more insight into the factors that underlie nuclear organization.

Since studies are often completed in unsynchronised cell populations or at only particular cell cycle phases it is difficult to describe the full spectrum of PML NB spatial interactions with other nuclear compartments as a function of time. Live cell imaging on a larger scale will no doubt aid our understanding of the role of PML NBs in relation to other nuclear substructures. There is a clear need for high throughput imaging methods to capture many cells, thus allowing a systems biology approach to be used in the investigation of nuclear organization. This subsequently results in large data storage requirements, and demands high throughput image processing and statistical methodologies. Such issues will need to be tackled in due course.

Ultimately our aim must be to have careful definitions of PML association in relation to as many nuclear compartments as possible, in a wide range of different cell lines, and under different cellular and cell cycle conditions. This will surely aid the quest to comprehensively describe PML NB function in the mammalian nucleus.

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