Structure and functions of the HSR domain of the nuclear body associated Sp100 protein

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Zusammenfassung

Das dominante Sp100-Protein lokalisiert in Säugerzellen in spezifischen Kernarealen, den sogenannten PML-Kerndomänen. Es scheint die Transkription spezifischer Gene, Chromatinstruktur, Funktionen von Tumorsuppressorproteinen sowie Metastasenbildung beeinflussen zu können. Weniger häufig vorkommende Spleißvarianten, welche sich von Sp100 nur durch zusätzliche C-terminale Sequenzen unterscheiden, lokalisieren entweder teilweise oder ausschließlich in Kerndomänen ohne nachweisbares PML-Protein. Die sogenannte HSR-Domäne nahe am 5` Ende des kodierenden Bereiches von Sp100 vermittelt im Sequenzkontext des dominanten Sp100-Proteins sowohl dessen Homo-Oligomerisierung als auch die Lokalisation in PML-Kerndomänen. Die gleiche Domäne bildet jedoch Filamente, wenn sie in isolierter Form exprimiert wird. In den Spleißvarianten ist die PML-Kerndomänenlokalisierungsfunktion dieser Domäne teilweise oder gänzlich inaktiviert.

Um Antworten auf einige der zahlreichen Fragen zu bekommen, welche sich hieraus ergeben, war vorrangiges Ziel dieser Arbeit, die strukturellen und funktionellen Eigenschaften der HSR-Domäne von Sp100 und seinen Varianten in verschiedenen Zelllinien und Spezies zu klären. Weitere Aufgaben waren die Eingrenzung der für die Bildung von Filamenten notwendigen minimalen HSR-Sequenz sowie die Analyse der Wirkung der HSR-Filamente auf Struktur und Zusammensetzung der PML-Kerndomänen. Zudem sollte die potentielle Rolle der HSR-Domäne bei der Lokalisation der Sp100-Spleißvarianten in Kerndomänen ohne nachweisbares PML erforscht werden. Schließlich sollte untersucht werden, ob und wenn ja, welche bekannten zellulären oder Virusprotein-haltigen Filamente mit HSR-Filamenten kolokaliseren und wenn ja, ob diese funktionell modifiziert werden.

Computeranalysen mit Hilfe von Datenbanken offenbarten Proteine mit starken Sequenzhomologien zum humanen Sp100-Protein sowohl in verschiedenen Mausspezies als auch in der Ratte. Der Proteinsequenzvergleich zeigte, dass die HSR-Domäne zwischen Ratte, Maus und Mensch hoch konserviert ist, während die übrigen Sequenzbereiche wesentlich höhere Abweichungen aufwiesen. Dies deutet auf eine wichtige, evolutionär konservierte Struktur und Funktion der HSR-Domäne. Das mit Hilfe eukaryotischer Vektoren exogen exprimierte Protein der HSR-Domäne offenbarte sich in der Mehrzahl der Zellen im Immunfluoreszenzmikroskop in Form von filamentösen Strukturen. Dies war unabhängig von

der verwendeten Zelllinie und nur wenig abhängig von der Höhe der Expression. HSR-Filamente blieben nach dem enzymatischem Abbau von DNA und RNA morphologisch unverändert. Dies spricht gegen eine Assoziation und stabilisierenden Beitrag der Nukleinsäuren für diese filamentösen Strukturen. Durch konfokale Mikroskopie wurden die HSR-Filamente zwar hauptsächlich im Zellkern lokalisiert, einige reichten aber auch bis in das Zytoplasma. Keine Kolokalisierung wurde mit bekannten zytoplasmatischen oder nukleären Filamenten des Zytoskeletts oder mit den Adenovirus-spezifischen E4ORF3-Filamenten beobachtet, wobei letztere dafür bekannt sind, die PML-Kerndomänen zu zerstören. Dies weist daraufhin, daß HSR-Filamente einen neuen Typ von Filamenten darstellen.

Bei Versuchen zur Kartierung der Sp100-Sequenzen, die zur Bildung von Filamenten führen und damit interferieren, wurde die HSR-Domäne als kleinste Filament-bildende Region bestätigt und gezeigt, dass bereits eine relativ kleine C-terminale Verlängerung mit authentischen Sp100-Sequenzen, welche eine durch Computeranalysen vorhergesagte „Coil-coil-Struktur“ enthielt, zur Bildung morphologisch veränderter Filamente führt. Diese Filamente schienen Veränderungen im zellulären Chromatin zu bewirken. C-terminale Verlängerungen der minimalen HSR-Domäne mit zusätzlichen authentischen Sp100-Sequenzen verhinderten die Bildung der Filamente vollständig. Zusammengefaßt deuten diese Daten auf eine durch C-terminale Sequenzen induzierbare Konfomationsänderung der HSR-Domäne. Diese Interpretation steht auch im Einklang mit der unterschiedlichen nukleären Lokalisation des dominanten Sp100-Proteins und der Sp100-Spleißvarianten.

Die Expression der HSR-Domäne führte zur spezifischen Rekrutierung aller getesteter PML-Kerndomänenproteine (inklusive endogenem Sp100 und PML) in Filamente, ließ die Lokalisation einer Vielzahl anderer, nicht mit PML-Kerndomänen assoziierter, Proteine aber unverändert. Die experimentell nachgewiesenen Interaktionen der HSR-Domäne mit dem endogen exprimierten dominanten Sp100-Protein und mit SUMO1 ist eine mögliche Erklärungen hierfür; die Bindung an die PML-IV-Isoform als mögliche Erklärung wurde ausgeschlossen. Diese Befunde implizieren eine dominant-negative Wirkung der überexprimierten HSR-Domäne auf die Struktur und möglicherweise auch die Funktion der PML-Kerndomänen. Außerdem deuten diese Ergebnisse auf eine mögliche strukturelle Funktion des Sp100-Proteins in der Organisation der PML-Kerndomänen.

Durch die Verwendung muriner PML-k.o.-Fibroblasten wurde herausgefunden, dass die Bildung der HSR-Filamente von PML unabhängig ist. Dieser Befund und fehlende Hinweise für eine direkte Sp100-PML Interaktion deuten daraufhin, dass das PML-Protein indirekt, wahrscheinlich durch ein bisher unbekanntes zelluläres Protein, in diese Filamente rekrutiert wird. Die teilweise Blockade der Bildung der Filamente durch Koexpression der enzymatisch aktiven SUMO-spezifischen Protease SuPr1, nicht aber durch die entsprechende inaktive Mutante, sind ein Hinweis für die Beteiligung eines sumolierten Proteins. Da die Überexpression von SUMO1 und Ubc9 die PML-Kerndomänenstruktur nicht wieder herstellte, scheint durch die Anreicherung von SUMO1 in Filamenten kein genereller Mangel an SUMO1 zu entstehen, der potentiell für die Auflösung der PML-Kerndomänen verantworltich hätte sein können. Es konnte auch gezeigt werden, dass SuPR1 mit dem dominanten Sp100-Protein interagiert, dieses desumoliert, ohne dessen Expressionsniveau zu beeinflussen.

Untersuchungen zu möglichen Funktionen der Sp100-Proteine in der Zellzyklus-regulation zeigten, dass das komplette Sp100-Protein und N-terminale, nicht aber C-terminale Fragmente das Überleben der Kolonien in „Colony Forming Assays“ hemmen und die Anzahl der apoptotischen Zellen in der subG1 Phase erhöhen. Das HSR-Protein induzierte auch einen leichten G2 Zellzyklusarrest und lokalisierte in sich teilenden Zellen an Strukturen, die der mitotischen Spindel ähnelten. Zusammengefaßt weisen diese Daten daraufhin, dass die HSR-Domäne für diese Zellzyklus- und Zelltod-modulierenden Funktionen von Sp100 verantwortlich ist.

Die experimentelle Suche nach Domänen in den Sp100-Spleißvarianten, welche für deren teilweise oder komplette Lokalisation in nukleären Kerndomänen ohne nachweisbares PML verantwortlich sein könnten, schloss die Möglichkeit der Existenz eines nukleären Kerndomänenlokalisierungssignals innerhalb Varianten-spezifischer Sequenzen aus. Dies ergab sich aus der Beobachtung, dass keines der korrespondierenden Spleißvarianten-spezifischen rekombinanten Proteine in nukleären Kerndomänen lokalisierte. Basierend auf diesen Ergebnissen scheint es wahrscheinlicher zu sein, dass die HSR-Domäne, welche sowohl im dominanten Sp100-Protein als auch in den Spleißvarianten enthalten sind, durch C-terminale, Spleißvarianten-spezifische Sequenzen in seiner Struktur modifiziert wird. Dadurch wird die Spezifität des Lokalisierungssignal in den Spleißvarianten wahrscheinlich geändert und führt zur Rekrutierung in Kerndomänen ohne nachweisbares PML.

Summary

The dominant Sp100 protein localizes in mammalian cells to distinct nuclear domains, designated PML bodies and appears to be able to modulate transcription of specific genes, chromatin structure, tumour suppressor protein functions and metastasis. Minor splice variants, which differ from Sp100 only by additional c-terminal sequences, localize in different cell lines either in part or exclusively to other nuclear domains devoid of detectable PML. The so-called HSR domain located close to the 5´-end of the Sp100 coding region is known to mediate both homo-oligomerization and PML body targeting in the context of the dominant Sp100 protein. However, it was shown to form filaments when expressed in an isolated form and its PML-body-localization function is obviously partially or fully inactivated in the Sp100 splice variants.

In order to shed light on the many questions resulting from these known facts, the major aim of this study was to elucidate structural and functional properties of the HSR domain of Sp100 and its variants in different cell lines and species. A further task was to define the minimal functional sequence for filament formation and the effects of these filaments on PML body structure and composition. Moreover, the potential role of the HSR domain in localization of Sp100 splice variants to nuclear dots without detectable PML protein should be investigated. Finally it should be examined which, if any, known cellular or viral protein containing filaments colocalize with the HSR filaments and may thus be functionally modified.

A computer based search in data bases revealed proteins with strong sequence similarities to human Sp100 proteins in various mice species and in rat. Protein alignments showed that the HSR domain is highly conserved from rat and mouse species to human, whereas the remaining sequences diverged substantially more, arguing for an important evolutionary conserved structure and function of this domain in Sp100 proteins. Visualization of the human Sp100 HSR protein domain expressed with the help of eukaryotic vectors revealed filamentous structures in the majority of cells. This was independent of the cell lines used and almost independent of the expression levels achieved by variation of the amounts of the corresponding expression plasmid. HSR filaments remained morphologically unaltered after enzymatic removal of DNA or RNA, arguing against an association or stabilizing contribution of nucleic acids for these filamentous structures. By confocal microscopy the HSR filaments were located mainly in the cell nucleus, but in some cases they extended also

into the cytoplasm. No colocalization was observed with any known nuclear or cytoplasmic cytoskeletal filaments or with the adenovirus-specific E4Orf3 filaments, the latter being known to disrupt PML bodies. This argues that HSR filaments represent a novel type of filaments.

The Sp100 region necessary for filament formation was mapped. The HSR domain was the minimal region tested necessary for filament formation. A relatively short c-terminal extension of this minimal HSR domain by authentic Sp100 sequences, which contained a computer-predicted coiled coil structure, led to formation of morphologically different filaments. These filaments appeared to induce changes in the cellular chromatin. C-terminal extensions of the minimal HSR domain by longer authentic Sp100 sequences prevented filament formation completely. Taken together, these data indicate a sensitivity of the HSR domain for conformational changes inducible by c-terminal sequences. This interpretation is also consistent with the alternative nuclear localization of the Sp100 splice variants.

Expression of the Sp100 HSR domain was found to specifically recruit all PML body proteins tested (including endogenous Sp100 and PML) into filaments, but did not change the localization of a variety of non-PML-body-associated nuclear proteins. Interaction of the Sp100 HSR domain with endogenously expressed dominant Sp100 protein and with SUMO1, as shown experimentally, are possible reasons for this observation. Binding to the isoform PML IV was excluded as a potential explanation. These findings imply a dominant negative function of the overexpressed HSR domain on the structure and possibly also on the function of PML bodies. In addition, the results point to a possible essential structural function of the Sp100 protein in PML body organization.

By using murine PML k.o. fibroblasts, HSR filament formation was found to be independent of PML. This finding and the lack of evidence for a direct PML-Sp100 interaction suggests that the PML protein is recruited to these filaments indirectly via an unknown cellular protein. Partial inhibition of filament formation by coexpression of enzymatically active SUMO-specific protease SuPr1, but not by that of an inactive mutant thereof, provided circumstantial evidence for involvement of a SUMOlated protein. Despite of SUMO1 accumulation in HSR filaments, a SUMO1-deficit eventually responsible for PML body disruption was not apparent, as evident by no rescue of the PML body structure by overexpression of SUMO1 and Ubc9. The dominant Sp100 protein was demonstrated to

interact with SuPr1 and was shown to result in its deSUMOlation without changing its expression level.

Investigations into possible functions of Sp100 proteins in cell regulation, full-length Sp100 and N-terminal but not C-terminal fragments thereof were found to inhibit colony survival in colony formation assays and to increase the number of apoptotic cells in the sub-G1 phase. The Sp100 HSR protein induced also a small G2 cell cycle arrest and localized in dividing cells at structures resembling the mitotic spindle. Taken together, these data suggest that the HSR domain is responsible for these cell cycle and cell death modulating functions of Sp100.

The experimental search for domains in Sp100 splice variants responsible for their partial or complete localization to nuclear bodies devoid of detectable PML, excluded the possibility for the existence of a nuclear dot localization signal in the variant-specific sequences. This was deduced from the observation that none of the corresponding splice variant-specific recombinant proteins localized to nuclear dots. Based on the results obtained it appears more likely that the HSR domain present both in the dominant Sp100 protein and in the splice variants is modulated by the c-terminal splice variant-specific sequences structurally and thus adopts a novel function as a localization signal recruiting the splice variants to nuclear dots devoid of detectable PML protein.

1. Introduction

1.1 - PML Bodies

PML bodies are multi-protein complexes located in the nucleus of higher eukaryotic cells. PML bodies are not the only structures observed in the nucleus. The nucleus is compartmentalized into different subnuclear domains. Several other structures can be found in the highly differentiated cell nucleus like the nucleoli, Cajal bodies or among others splicing speckles.

These studies focused on PML bodies and on the proteins contained in these bodies. PML bodies (also known as Kremer bodies, ND10 or POD – PML oncogenic domains) received their name because of the localization in these structures of the PML (promyelocytic leukemia) protein (Bouteille et al., 1983; Ascoli and Maul, 1991; Dyck et al., 1994; Koken et al., 1994; Weiss et al., 1994 and Hodges et al., 1998).

PML bodies are located in the interchromatin space of the nucleus. They are attached to the highly insoluble nuclear matrix but do not depend on DNA or RNA interaction for their structural integrity since DNAse and RNAse digestion does not change their morphology or localization (Stuurman et al., 1992). However, a recent report shows that PML bodies can become more dynamic and instable when the chromatin surrounding them is disrupted, possibly due to a loss of chromatin contacts (Eskiw et al., 2002).

PML bodies can be identified by immunofluorescence in interphase cells with the use of specific antibodies for PML body proteins. They are dynamic structures which vary in their number, size, composition and mobility. Although their size and number and composition is strong cell type dependent their numbers range from 5 to 30 per cell and their size from 0,2 to 1µm (Ascoli and Maul, 1991). The number and size of PML bodies is also cell cycle dependent as they are disrupted during mitosis (Koken et al., 1995; Chan et al., 1997; Everett et al., 1999; Kießlich et al., 2002). They are also altered by stress factors or when subjected to viral infection or transfection of viral proteins (Carvalho et al., 1995; Maul et al., 1993; Puvion-Dutilleul et al., 1995). PML bodies have a “doughnut-like” shape when observed by electron microscopy with a dense fibrillar ring surrounding the less dense core (Dyck et al., 1994; Weis et al., 1994).

Highly mobile Sp100 bodies that do not contain PML and less mobile bodies containing Sp100 and PML have been described (Wiesmejer et al., 2002). PML bodies also show metabolic-energy-dependent movement within the nucleus (Muratani et al., 2002). It was also observed that in response to stress microstructures form as a result of fission from the surface of 'parental' PML bodies which fuse again with parental PML bodies after the stress response. Thereby, PML bodies do not reform at new random locations and their relative size observed before stress is preserved after recovery, suggesting PML bodies are not random accumulations of protein (Eskiw et al., 2003).

The proteins that have been found in PML bodies so far have many different functions (Figure 1.1, adapted from Hofmann and Will, 2003). These functions range from transcriptional regulation, post-translational protein modification, DNA maintenance and repair, apoptosis, protein degradation to antiviral defense. Some of the proteins that are present in these bodies appear to transiently localize to PML bodies, others like Sp100 and PML, are tightly associated with PML bodies.

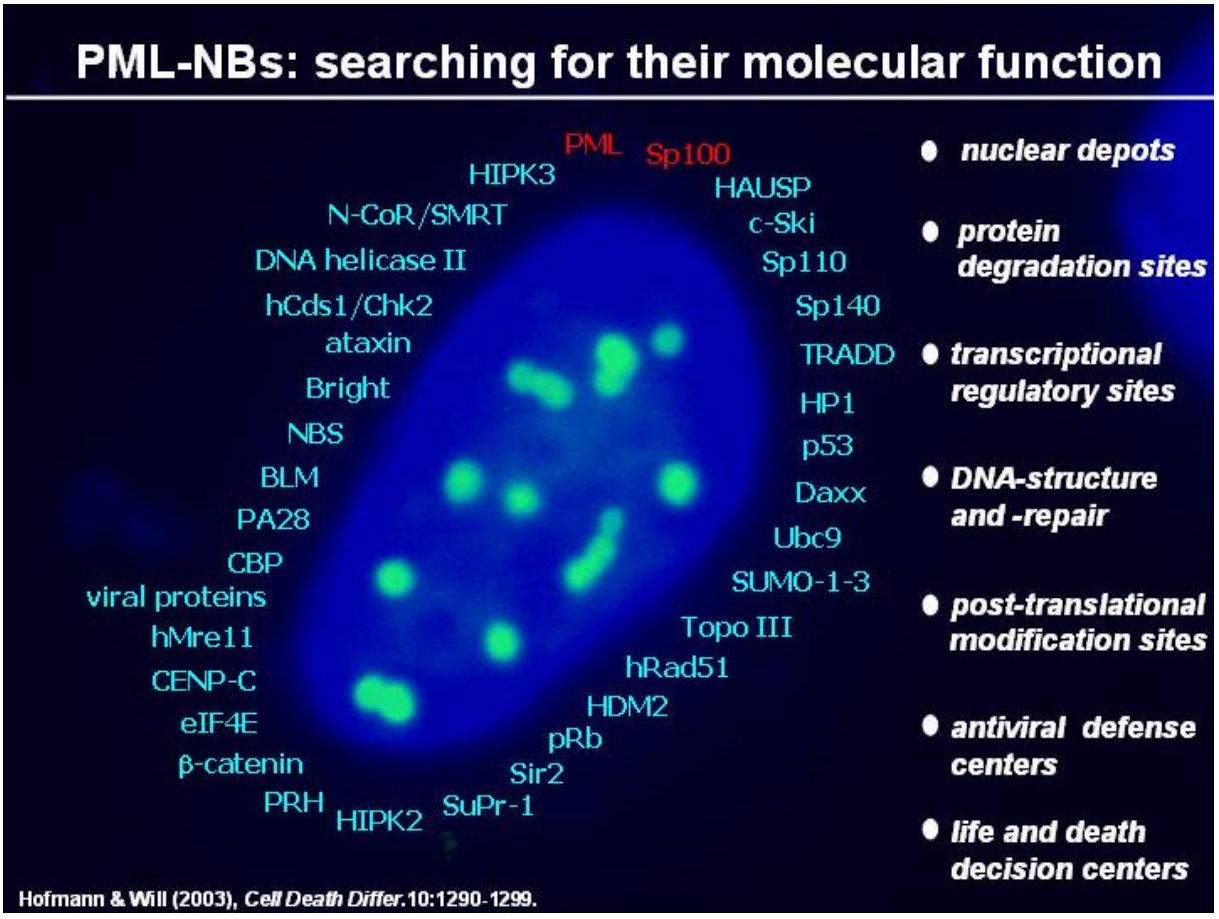

Figure 1.1 – Proteins that have been identified in PML bodies and the cellular processes they are involved in. Adapted from Hoffman and Will, 2003)

What is known from the functions of the proteins that form this multi-protein complex led to the conclusion that PML bodies play a role in gene regulation, cell growth, differentiation and apoptosis (Seeler and Dejean, 1999).

Although the knowledge of the individual functions of the proteins that are present in these bodies is often known, there is still no function for the PML body as an individual cellular organelle.

Based on the wide array of functions of the proteins that localize to PML bodies they have been suggested to serve as nuclear depots for proteins. They may be places where proteins can be stored and post-translationally modified so that in cases of cellular stress the cellular response could be faster than the *de novo* protein synthesis (Maul et al., 2000; Negorev and Maul, 2001). They may also regulate the bioavailability of nuclear factors and control the function of its resident proteins by regulating their post-translational modifications (Hoffman and Will, 2003).

Others have suggested that PML bodies could serve as sensors for foreign or inappropriately expressed proteins and then act as a potential nuclear immune system (Tsukamoto et al. 2000; Maul et al., 2000). This was supported by the fact that PML-bodies are adjacent to proteosomal components (Baumann et al., 2001; Lallemand-Breitenbach et al., 2001). Also the fact that both PML and Sp100 genes contains an interferon-inducible promoter and that all interferons (a, ß and ?) strongly induce PML (and Sp100) expression leading to an increase in the number and size of PML bodies (Maul et al., 1995; Regad et al., 2001a; Regad et al., 2001b) supports this option. All these data also suggest a role of PML bodies in antiviral defense.

Also worth noticing is that PML bodies are targeted by a number of different RNA and DNA viruses including herpesviruses, adenoviruses, papovaviruses, papillomaviruses and arenaviruses (reviewed by Everett, 2001; Moller et al., 2003). The interaction of viral proteins with PML bodies can lead to the disruption or a change in the composition of PML bodies. Also known is that the genomes of some nuclear-replicating DNA viruses associate preferentially with PML bodies that presumably assist in viral gene expression or replication.

A highly non-random association of PML bodies with a gene-rich major histocompatibilty complex (MHC) region on chromosome 6 was reported, which remained even when a subsection of this region was integrated into another chromosomal location, suggesting that PML bodies have specific chromosomal associations which occurred in this

case independent of transcription (Shiels et al., 2001). In contrast, it was also shown that in the G1 phase of the cell cycle more than 70% of PML bodies contain active transcription foci (Kießlich et al., 2002). Also supporting a localization of PML bodies at specific transcription sites is the fact that nascent RNA polymerase II transcripts can be found within this nuclear body (LaMorte et al., 1998).

Finally, concerning the structure of the PML bodies, some suggest that PML itself is the key protein for the formation of PML bodies (Ishov et al., 1999; Zhong et al., 2000) because in the absence of PML some other proteins that normally colocalize with PML show a different cellular localization whereas the lack of other PML body components, such as Sp100 (in NT2 cells) does not change the localization of PML or PML body components (Negorev et al., 2001).

There are also some reports which suggest that, since PML is not evolutionary conserved (no gene homologous to PML has been found in *Drosophilia melanogaster* nor in *Xenopus laevis*) it cannot be the underlying structure of PML bodies and propose the translation initiation factor eIF4E as the basilar stone of PML bodies because they could show that eIF4E still localizes to nuclear bodies in the absence of PML (reviewed in Borden, 2002; Strudwick and Borden, 2002).

1.2 - The PML protein

When studying the molecular events that lead to acute promyelocytic leukemia (APL) it was found that the gene that encodes for the retinoic acid receptor alpha (RAR) is fused to the *pml* gene by a chromosomal translocation t(15;17). The expression of this fusion protein PML-RAR is sufficient for the transformation of cells and induction of leukemias (Melnick and Licht, 1999; Grisolano et al., 1997) and for the disruption of PML bodies into hundreds of small dots in the nucleus and cytoplasm. The treatment with retinoic acid is not only a successful therapy for APL patients (Degos, 1994) but also restores the normal PML body distribution and leads to the differentiation of the cells (Daniel et al., 1993).

PML has been proposed to have a central role in the organization of the PML body as shown in cells from PML -/- mice that lack PML bodies where proteins like DAXX, Sp100, CBP or ISG20 (proteins that normally localize in PML bodies) localize in an aberrant distribution in aggregates (Ishov et al., 1999; Zhong et al., 2000). It was further shown that SUMOlation of PML is required for the localization of PML in bodies and the recruitment of other PML body components to these structures (Ishov et al., 1999).

The PML protein is expressed in several isoforms by alternative splicing of the nine exons encoded by the *pml* gene (Fagioli et al., 1992). All seven isoforms identified so far contain the TRIM motif but differ in their central and C-terminal regions (Fagioli et al., 1992). The PML-IV isoform was found to interact with the tumor suppressor p53 activating p53 dependent pathways (Pearson et al., 2000; Fogal et al., 2000; Guo et al., 2000). No functions specific for other PML isoforms are known.

The N-terminus of PML is formed by three cysteine-rich zinc binding motifs (Goddard et al., 1991): a RING domain, two B-boxes (Borden et al., 1996) followed by a coiled-coil homo-oligomerization domain (Kastner et al., 1992; Perez et al., 1993). These three motifs are called RBCC or tripartite motif (TRIM). A complete RBCC motif is necessary and sufficient for localization of PML to PML bodies. The coiled coil domain is followed by a serine rich domain that was speculated to have a regulatory function (de Thé et al., 1991; Kakizuka et al., 1991; Fagioli et al., 1992).

PML has been linked to several important functions like apoptosis (Wang et al., 1998; Quignon et al., 1998; Torii et al., 1999), cellular senescence (Ferbeyre et al., 2002; Pearson et al., 2000), regulation of transcription (Zhong et al., 2000; Khan et al., 2001; Zhong et al.,

2000), growth control (Mu et al., 1994), control of genomic stability (Zhong et al., 1999), DNA repair (Bischof et al., 2001), response to viral pathogens (Chelbi-Alix, 1998), antigen presentation by MHC class I antigens (Zheng et al., 1998) and protein degradation (Lallemand-Breitenbach et al., 2001). It is worth noticing that concerning transcriptional regulation, PML can both repress and activate transcription depending on the cellular context. PML interacts with a variety of transcription factors (CBP, DAXX, p53, HDACs, etc) and may thereby control transcription of a number of genes. The mechanism by which PML functions is however not yet known. Cells from PML -/- mice show a reduction in apoptosis induction by Fas, TNF, interferons and ceramides which implies that PML may have a role as a modulator in apoptotic pathways. PML -/- mice develop normally but are more prone to develop tumors in response to cancer promoting drugs and are more resistant to irradiation due to the defects in apoptotic pathways (Wang et al., 1998).

1.3 - The Sp100 protein

The main subject of these studies was the Sp100 protein, a 54 kD protein (named due to its “speckled” nuclear distribution and aberrant electrophoretic mobility at 100 kD in SDS-Page) which was first identified using human autoimmune sera from patients suffering from the autoimmune liver disease primary biliary cirrhoris (PBC) (Szostecki et al., 1990; Szostecki et al., 1992). Also isolated using the same approach was another Sp100-related protein, Sp140/LYSP100 (Dent et al., 1996; Bloch et al., 1996). EST database searches led to the discovery of yet another related protein called Sp110 (Bloch et al., 2000). Both Sp140 and Sp110 can only be found in blood cells. These proteins together with the autoimmune regulator protein (AIRE) form the Sp100 family of protein.

All these proteins share at their N-terminus a novel protein motif called HSR domain (Sternsdorf et al., 1999). The name derives from the mouse HSR domain that is in certain outbread mouse strains amplified several thousands of times leading to the formation of a homogeneously staining region (HSR) on chromosome 1 (Eckert et al., 1991; Grötzinger et al., 1996). All Sp100 splice variants contain at the N-terminus a sequence with similarity to parts of the peptide-binding groove of the MHC class I molecules and an HP1 (Heterochromatin Protein 1) binding site and a SUMO-conjugating site at the centre followed by a putative transactivation domain (Xie et al., 1993). Finally Sp100 also possesses a NLS (Nuclear Localization Signal) at its C-terminus.

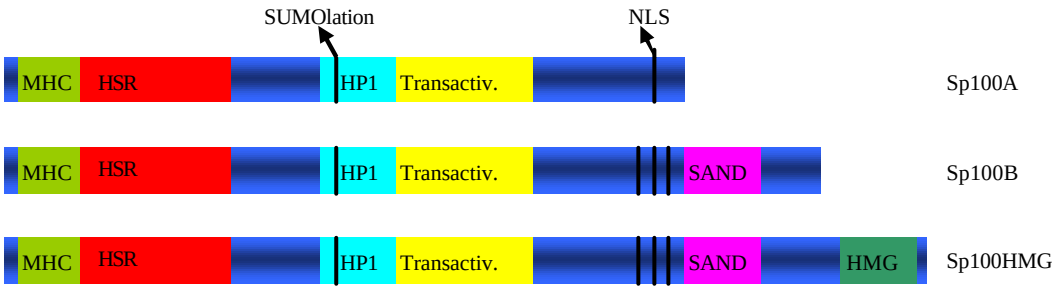

Figure 1.2 – Domains of Sp100 and its splice variants

Sp100 and PML share several common characteristics: both proteins are modified by the small ubiquitin-related modifier (SUMO-1) (Sternsdorf et al., 1997) but SUMOlation of Sp100 is not required for the localization of Sp100 in PML bodies (in contrast to PML). Both are up-regulated by interferons (Guldner et al., 1992; Grötzinger et al., 1996; Grötzinger et al., 1996) and Sp100 perfectly colocalizes with PML in PML bodies. The localization of Sp100 in

PML bodies is mediated by its HSR domain which is also responsible for its oligomerization (Sternsdorf et al., 1999; Negorev et al., 2001).

Sp100 exists in a number of splice variants (Guldner et al., 1999). The predominant form, termed Sp100A, is 480 amino acids long and exhibits an aberrant electrophoretic migration at 90-100 kD in SDS-PAGE. The larger splice variants SP100B (Dent et al., 1996) and Sp100 HMG (Seeler et al., 1998) encode additional functional domains at the C-terminus. Sp100 B possesses a SAND domain (also known as HNPP domain) which is normally found in proteins with transcription regulatory functions (reviewed in Wojciak and Clubb, 2001). The SAND domain is also known to bind DNA (Bottomley et al., 2001). Sp100 HMG has a SAND domain and in addition an HMG-box. This HMG-box is known to be a DNA binding domain that is supposed to recognize DNA predominantly at stem-loop structures and cruciform DNA (Grosschedl et al., 1994). It was observed that Sp100 splice variants localize to nuclear bodies that do not contain PML depending on the cell type examined. In HeLa cells 70-90% of the splice variants localize with PML whereas in U2OS cells it is only 0-30% (Guldner et al., 1999). The AIRE protein contains an HSR domain at its N-terminus and a SAND domain like SP100B but does not localize in PML bodies (Rinderle et al., 1999).

Concerning their function, all of Sp100 family members have been shown to be involved in transcription regulation (activation and repression). One of the interaction partners of Sp100 is HP1 (Seeler et al., 1998) a protein involved in transcriptional repression/gene silencing. Although Sp100 cannot bind directly to DNA, it can inhibit transcription when recruited by DNA-bound transactivation factors. Two other potential recruiters of Sp100 to the DNA are hHMG2/DSP1 (Lehming et al., 1998) and Bright (the B-cell regulator of IgG - Zong et al., 2000). More recently Sp100 has been found as a cofactor of ETS-1 enhancing or repressing ETS-1 dependent-transcription (Wasylyk et al., 2002; Yordy et al., 2004) and to work as a coactivator for HIPK2-mediated p53 transcriptional activation (Moller et al., 2003).

Also known is the colocalization of Sp100 and PML with centromeres in the G2 phase of the cell cycle (Everett et al., 1999). Although no direct interaction is known between Sp100 and the centromere component CENP-2, there is a colocalization of both proteins in the G2 phase. This colocalization of Sp100 and centromeres is probably mediated by the interaction of HP1 and centromeres during mitosis (Kourmouli et al., 2000; Furuta et al., 1997).

Sp100 also interacts with the Nijmegen breakage syndrome protein (NBS1) (Naka et al., 2002) and was shown to recruit NBS1 into PML bodies. This protein plays a central role in DNA repair, cell cycle check point and telomere maintenance (Tauchi et al., 2001; Ranganathan et al., 2001).

In these studies, except when stated otherwise, Sp100A was used and is refered to as Sp100 or wild type Sp100.

1.4 - The SUMO1 protein and (de)SUMOlation mechanism

SUMO1 (Small Ubiquitin-like Modifier) belongs to the growing group of ubiquitin-like proteins that covalently modify post-translationally their target proteins. SUMO-1 is also known has smt3, sentrin, GMP1 or PIC1. In humans, three SUMO forms, named SUMO1, SUMO2 and SUMO3, are expressed from three different genes. SUMO1, the best studied form of SUMO, shows about 50% sequence identity to SUMO2/3, which are almost identical. SUMO is a protein of 101 amino acids. SUMO1 and ubiquitin share 18% of sequence homology but share a common three-dimensional structure. One of the main differences between SUMO1 and SUMO2/3 is the fact that SUMO1 cannot form multimeric chains like SUMO2/3.

SUMOlation (the term that describes the modification of proteins by SUMO) is the formation of an isopeptide bond between the C-terminus of SUMO and a e-amino group of a lysine residue in the target protein. A consensus sequence for SUMOlation is ?KxE, where ? is a large hydrophobic residue. SUMOlation of the target protein starts with the ATP dependent activation of SUMO by the SUMO activating E1 enzyme (AOS1/UBA2) and the E2 conjugating enzyme Ubc9. Another enzyme, an E3 ligase recruits SUMO-conjugating enzymes to the target proteins. This enzyme is thought to determine target specificity. In the case of Sp100, this E3 ligase is RanBP2 (Pichler et al., 2002).

All types of SUMO E3 ligases localize to speci?c subcellular compartments. RanBP2, the E3 ligase for Sp100, is part of the nuclear transport machinery in the nuclear pore complex, where it functions as a docking factor of transport complexes. This was supported by the fact that the import factor RanGAP1 is targeted to the nuclear pore upon SUMOlation (Matunis et al., 1996; Mahajan et al., 1997).

Several proteins in the PML bodies are SUMOlated. In the case of PML it was found that SUMOlation is even required for the formation of the PML bodies (Müller et al., 1998). Other PML body proteins like Sp100, p53, DAXX and HipK2 are also SUMO modified. Moreover, the fact that the E2 conjugation enzyme Ubc9 and a known SUMO protease (SuPr1) also localize to PML bodies show the close interaction between the (de)SUMOlation mechanisms and PML bodies.

In contrast to (poly)-ubiquitination which usually leads to degradation, SUMOlation has been described to either stabilize proteins (by antagonizing ubiquitination as shown in the

case of MDM2 – Buschmann et al., 2000), influence their subcellular localization (as shown for the nuclear import factor RanGAP1 – Mahajan et al., 1997) or to promote the interaction with other proteins (RanGAP1).

As already mentioned, cells also possess the ability to cleave SUMO from SUMOlated proteins. This function is performed by so called SUMO proteases. Several proteases that hydrolyze SUMO from the target protein have been identified in yeast and in mammals (Kim et al., 2002). These proteins are weakly related to some viral cysteine proteases and all possess a conserved catalytic sequence His/Asp/Cys. One of these SUMO proteases is called SuPr1 and it localizes to PML nuclear bodies. SuPr-1 has the ability like some viral proteins to disrupt PML bodies (Best et al., 2002). This effect, as it was mentioned before, was also observed for some viral proteins that not only disperse PML bodies but can also cause proteasomal-dependent degradation of PML (Everett, 2001). In the case of SuPr1 it is worth noticing that this disruption of PML-bodies is time-dependent and only observed 36 hours after transfection by indirect immunofluorescence and that a catalytical defect mutant of SuPr1 does not dissolve the PML bodies to the same extent as the wild type SuPr1 (Best et al., 2002).

Possible functions of SUMO proteases are still unclear. As PML bodies are disrupted during the cell cycle, one of the possible functions of SUMO proteases may be related to this disruption. Studies in yeast support a function of SUMO proteases in cell-cycle regulation although these do not contain PML bodies (Best et al., 2002), implying that SUMO-1 protease function may be evolutionarily conserved though in different substrates. SUMO proteases are also involved indirectly in the regulation of transcription through (de)SUMOlation of transcription factors as described for example for Sp3 (Ross et al., 2002).

1.5 - Other PML body proteins

As mentioned before more than 50 proteins have been found in PML bodies. Most of these proteins localize to these structure only transiently while others like PML or Sp100 are constitutive members of PML nuclear bodies. In these studies, other PML body proteins were investigated.

DAXX

DAXX is a multi-functional adaptor protein with functions in several apoptosis pathways and transcription. It is recruited by SUMOlated PML to PML bodies. DAXX is also localized in the cytoplasm, at the chromatin and at centromeres.

It has been suggested that DAXX is a pro-apoptotic protein. Several studies indicate an important role of DAXX in regulating apoptotic signaling in various pathways (Yang et al., 1997; Chang et al., 1998; Perlman et al., 2001; Charette et al., 2001; Wu et al., 2002; Ohiro et al., 2003). There are also some contradictory reports because overexpression of DAXX does not always enhance apoptosis (Torii et al., 1999) and data also suggests that DAXX is not sufficient for Fas-mediated apoptosis in the absence of FADD or caspase 8 (Juo et al., 1998; Yeh et al., 1998; Zhang et al., 1998). What is known is that DAXX silencing just by itself has little effect on cell survival and proliferation but it enhances both Fas- and stress induced apoptosis which argues for an anti-apoptotic function (Chen and Chen, 2003).

Therefore, it seems that DAXX has pro-apoptotic and anti-apoptotic activity depending on cellular context (Torii et al., 1999; Chen and Chen, 2003). The same is true when it concerns transcription where DAXX can act both as a repressor and as an activator (Hollenbach et al., 1999; Li et al., 2000; Emelyanov et al., 2002; Hollenbach et al., 2002).

Concerning the function of DAXX in apoptosis it is known that localization of DAXX in PML bodies appears to correlate with its pro-apoptotic activity because DAXX mutants that do not localize to PML bodies are defective in promoting apoptosis (Torii et al., 1999). This suggests that DAXX may act together with PML through the PML bodies to influence apoptosis. It has also been reported that PML overexpression may inhibit the repressive function of DAXX in transcription by recruiting it to PML bodies (Li et al., 2000). DAXX can also bind and cooperate with another PML body protein, the CREB binding protein (CBP),

for transcriptional activation (Emelyanov et al., 2002). Altogether this suggests that recruitment of DAXX into PML bodies regulates its function in apoptosis and transcription.

HIPK2

Homeodomain-interacting protein kinase 2 (HIPK2) belongs to a family of enzymes together with HIPK1 and HIPK3. HIPK2 is a serine/threonine kinase involved in transcriptional regulation (Kim et al., 1998; Choi et al., 1999; Di Stefano et al., 2004), apoptosis (Hofmann et al., 2002; D'Orazi et al., 2002; Di Stefano et al., 2004) and growth control (Pierantoni et al., 2001; Hofmann et al., 2003). It localizes mainly in the so called HIPK2 bodies which do not contain PML but it can be recruited by PML-IV, UV and other stimuli to PML bodies.

HIPK2 was identified to have a function as an activator of the p53 tumor suppressor (Wang et al., 2001; D'Orazi et al., 2002; Hofmann et al., 2002; Kim et al., 2002). HIPK2 phosphorylates p53 (D'Orazi et al., 2002; Hofmann et al., 2002) promoting p53 acetylation and activation. HIPK2 was in addition shown to stabilize p53 by preventing the MDM2 mediated degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997; Di Stefano et al., 2004).

Overexpression of HIPK2 was described to disrupt PML bodies rendering the release of DAXX from PML nuclear bodies possible (Hofmann et al., 2003). It is also known that PKM (the hamster homologue of HIPK2) induces structural changes in PML bodies by releasing PML, Sp100, and DAXX from PML bodies. These changes can be attributed both to its kinase activity, posttranslationally modifying PML, and to the presence of a functional SUMO-1 interaction motif in its C-terminus modifying SUMO pathways (Engelhardt et al., 2003).

p53

PML bodies play an important role in the regulation of the tumor suppressor p53 by affecting the stability, activity and function of this protein. The tumor suppressor p53 is probably the most studied protein that can be recruited to PML bodies, by either PML-IV, coexpression of SUMO1/Ubc9, Ras activation, UV or ? irradiation (Fogal et al., 2000).

p53 has been considered as the guardian of genome by many authors, the protein that maintains genomic stability. Consistent with this is the fact that more than 50% of tumors have a p53 gene mutation (Hollstein et al., 1991). p53 is extensively post-translationally modified (phosphorylation, acetylation, ubiquitination, SUMOlation, ribosylation and glycosylation - Melchior and Hengst, 2002). Many of the proteins that posttranslationally modify p53 localize in PML bodies like HIPK2, CHK2, CBP, HAUSP and SIRT1. The importance of PML bodies in the posttranslational modification of p53 is illustrated by the example that phosphorylation of p53 by HIPK2 requires PML.

p53 is a short-lived protein that is stabilized and activated upon a wide range of cellular stresses (Prives and Hall, 1999; Appella and Anderson, 2000). When p53 is activated by external and internal stress signals its active form accumulates in the nucleus and induces either cell growth arrest, to allow DNA repair and preventing thus the proliferation of cells with damaged DNA, or apoptosis to eliminate irreparably damaged cells. p53 also contributes to cellular processes such as differentiation, DNA repair and angiogenesis. p53 exerts its function by acting as a transcription factor which regulates the expression of target genes involved in these processes but also has transcription-independent functions (Slee et al., 2004).

1.6 - The adenovirus 5 early protein E4Orf3

After infection with adenovirus the appearance of PML bodies is dramatically changed. The dot pattern characteristic for these structures is changed into a filamentous structure where PML and CBP colocalize with E4Orf3. The E4Orf3 protein is one of the first transcribed proteins of the adenovirus genome and is responsible for these changes (Carvalho et al., 1995; Doucas et al., 1996). There are some indications that PML body reorganization is linked to modulation of the immune response (Terris et al., 1995; Zheng et al., 1998) and cell-cycle independent virus growth, whereby the latter also involves the E4Orf6 protein as well as the E1B protein (Goodrum and Ornelles, 1999).

The E4orf3 protein has been shown to facilitate the malignant transformation of rodent cells (Nevels et al., 1999) and PML body disruption and cotransformation are activities shared with another viral protein, the IE1 protein of human cytomegalovirus (CMV) (Ahn et al., 1997; Shen et a, 1997; Ahn et al., 1998). The E4orf3 (and E4orf6) gene product have been shown to play an important role in adenovirus DNA replication (Bridge and Ketner, 1989; Huang et al., 1989).

Overexpression of PML enhances glucocorticoid-induced transcription. After transfection of E4Orf3, and despite the fact that PML body morphology is changed, activation of a glucocorticoid-responsive promoter by PML is not inhibited but on the contrary even increased (Wienzek and Dobbelstein, 2001). The authors suggested that PML body integrity may not be needed for PML and CBP functions in transcriptional activation or that E4Orf3 may affect posttranscriptional events such as RNA stability or translational efficiency.

1.7 - Cellular filaments and truncated proteins

In normal cells several proteins exist which oligomerize and form filaments. These filaments are not static structures in a cell. They are also assembled and disrupted to respond to several events that occur through the life of the cell.

The most common example are the proteins of the cytoskeleton. The cytoskeleton is important for the cell shape and intracellular transport. Proteins like actin, tubulin, vimentin and keratins are some of the proteins that form the cytoskeleton. Other cellular filaments are the lamins, some of which form the nuclear membrane (reviewed in Moir and Spann, 2001). The mitotic spindle that is formed during mitosis is also a filamentous structure of proteins that are thought to be part of the nuclear matrix like NuMA (Nuclear Mitotic Apparatus protein). For NuMA, it was also found that it not only forms filaments in the nucleus but also in the cytoplasm when expressed as a truncated version without its NLS (Saredi et al., 1996).

These examples are however examples that occur in "normal" cells. Some proteins involved in apoptosis possess a DED (Death Effector Domain) (reviewed in Tibbetts et al., 2003). These proteins can form so called death effector filaments that initiate the death signal (Siegel et al., 1998). Two of the examples referred to above (NuMA and Lamins) are also cleaved during apoptosis (Taimen and Kallajoki, 2001).

Most of these proteins have an intrinsic property to form filaments; even when they are expressed in different cells or cellular compartments these proteins keep their ability to form filaments. The secondary structure of these proteins often contains coiled coils that allow their oligomerization and the formation of filaments (reviewed in Burkhard et al., 2001). In summary, nowadays it is known that filamentous structures are very important for the normal cell life and play an important role in apoptosis.

The second part of this section concerns truncated proteins because in these studies truncated forms of Sp100 were used. There are numerous examples of studies performed with truncated proteins to identify a domain or to study the function of a determined domain.

Have truncated proteins a biological relevance? To answers this question one must remember that during apoptosis proteins are cleaved and that there are several studied cases where DNA mutations give rise to truncated proteins that are involved in several pathologies (Rinderle et al., 1999; Harris et al., 2003; Bardella et al., 2004). Sometimes like in the case of

PML, this truncation is followed by the fusion with another protein (PML-RAR) that causes the acute promyelocytic leukemia.

It is known that AIRE normally localizes in the nucleus and is involved in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (Rinderle et al., 1999). Mutations in this gene lead to the formation of truncated proteins that cause APECED. These C-terminally truncated versions of AIRE as well as the wild-type form filaments that colocalize with microtubules. The interaction of a nuclear factor with components of the cytoskeleton is not a singular case, the components of the nuclear matrix NMP125 is known to interact with vimentin (Marugg, 1992) and hnRNP S1 proteins can also interact with vimentin (Tsugawa et al., 1997). As mentioned before, AIRE shares with Sp100 its HSR domain and this domain is present in the AIRE truncated forms that can associate with microtubules, structures that play a very important role the cell life cycle or apoptosis.

1.8 - Programmed cell death (Apoptosis)

Apoptosis is a physiological process for killing cells and it is critical for the normal development and function of multicellular organisms. Disorders in apoptotic pathways can contribute to a variety of diseases, including cancer, autoimmunity, and degenerative disorders. Apoptosis leads to several morphological changes like chromatin condensation or cytoplasmic shrinkage (reviewed in Strasser et al., 2000).

Cells undergo apoptosis after internal or external signals, for instance, by ligation of death receptors. There are several apoptotic pathways but all share a common machinery of programmed cell death that is activated by a family of cysteine proteases (caspases) that cleave proteins at aspartate residues. Cell death is accomplished by proteolysis of vital cellular constituents, DNA degradation and phagocytosis of apoptotic cells by neighboring cells.

One of the apoptosis pathways is regulated by the mitochondria and is activated when proapoptotic members of the Bcl-2 protein family are translocated into the mitochondrial membrane which will then trigger the release of cytochrome c into the cytosol. Cytochrome c binds the apoptosis-activating factor 1 (APAF-1) which will then assemble into a multimeric caspase activation platform that recruits and activates caspase-9 and thus allows the activation of the downstream effector caspase-3 (Wang, 2001).

Another apoptosis pathway is initiated by a number of cell surface receptors upon binding their respective ligands (Krammer, 2000).The best understood death receptor systems are the tumour necrosis factor receptor (TNF-R) and CD95 (Fas/APO-1) pathways which when activated transmit an apoptotic signal via intracellular recruitment and assembly of a death-inducing signaling complex (DISC) (Kischkel et al, 1995). This DISC complex can then recruit initiator procaspases that then activate the caspase cascade.

Besides receptor induced apoptotic pathways, nuclear apoptotic pathways also exist. Several transcription factors like p53 can induce the expression of proapoptotic target genes that can directly trigger apoptosis via the mitochondrial pathway or by transcriptional upregulation of death receptors such as CD95.

PML bodies contain several proteins that are involved in apoptosis. Proteins like PML, DAXX, TRADD, HIPK2, p53, CBP, hSir2, Hdm2, FLASH and HIPK3 have been involved in apoptotic pathways (reviewed in Hoffman and Will, 2003).

1.9 – Aim of these studies

The aim of this thesis was the study of the properties of the PML body component Sp100 and in particular of its characteristic HSR domain which is known to be important for homo-oligomerization and the targeting of Sp100 to PML bodies.

One of the objectives of this work was the creation of a dominant negative-mutant of Sp100 since until the present date no knock-out Sp100 cell are available. The Sp100 HSR domain was one option for the creation of this dominant-negative mutant. Expression of the isolated HSR domain could potentially act as a dominant-negative mutant as it should bind to the wild-type Sp100 and may thereby inhibit Sp100 functions.

Early on in these studies it was observed that the HSR domain localizes predominantly in filamentous structures when expressed, suggesting a possible structural function for the Sp100 protein. To investigate this property of the Sp100 HSR domain to form filaments in more detail and potential effects this may have on PML body structure were subsequently a major topic of this work.

Finally, it was also one of the goals of this work to investigate if there exists in the C-terminal extensions of Sp100 splice variants any signal responsible for the their localization in PML independent bodies.

2 – Materials and Methods

2.1- Material

2.1.1 – Bacteria, Cells and Medium

2.1.1.1 - Bacteria

E.coli strain	Source
DH5a	Invitrogen
TOP10	Invitrogen

2.1.1.2 - Eukaryotic cells

Cell Line	Source
HeLa	Human cervical carcinoma cells
U2OS	Human osteosarcoma cells
293T	Human embryonic kidney cells transformed with SV40 T antigen
PML-/- MEF	Mouse embryonic fibroblasts from PML knock-out mice spontaneously immortalized (Dr. Hüseyin Sirma)
PML +/+ MEF	Mouse embryonic fibroblasts from 129VS mice spontaneously immortalized (Dr. Hüseyin Sirma)

2.1.1.3 - Medium

Medium	Source
DMEM	Invitrogen
Supplemented with:	
10% (V/V)	Heat Inactivated Fetal Bovine Serum (Biochrom AG)
0,01 mg/ml	Penicillin/Streptomycin (Invitrogen)
2 mM	L-Glutamin (Invitrogen)

2.1.2 - DNA

2.1.2.1 – Expression Vectors

Name	Use	Source
pSG5	Eukaryotic Expression Vector	Stratagene
pSG5-LINK	Eukaryotic Expression Vector	Stratagene
Modified to contain SV40 NLS and Flag-tag (T.Sternsdorf and K. Jensen)		
pSG5-Flag	Eukaryotic Expression Vector	Stratagene
pcDNA3	Eukaryotic Expression Vector	Invitrogen
TOPO	Subcloning	Invitrogen

2.1.2.2 - Plasmids

cDNA	Vector	Epitope-Tag/ External NLS	Source
Sp100	pSG5	--	Dr. Thomas Sternsdorf/Kerstin Jensen
Sp100	pSG5-Flag	Flag	Dr. Thomas Sternsdorf/Kerstin Jensen
Sp100Wing	pSG5-LINK	Flag + NLS	Dr. Thomas Sternsdorf/Kerstin Jensen
Sp100B	pSG5-Flag	Flag	Dr. Thomas Sternsdorf/Kerstin Jensen
Sp100HMG	pSG5-Flag	Flag	Dr. Thomas Sternsdorf/Kerstin Jensen
Sp100HMG 887-2670	pSG5-LINK	Flag + NLS	Dr. Cora Lüders
Sp100HMG 1016-2670	pSG5-LINK	Flag + NLS	Dr. Cora Lüders
Sp100HMG 1481-2670	pSG5-LINK	Flag + NLS	Dr. Cora Lüders
Sp100HMG 1855-2670	pSG5-LINK	Flag + NLS	Dr. Cora Lüders
Sp100HMG 2110-2670	pSG5-LINK	Flag + NLS	Dr. Cora Lüders
PML-III	pSG5	--	Dr. Thomas Sternsdorf/Kerstin Jensen
PML-IV	pcDNA3	--	Prof. Dr. Gianni Del Sal
SUMO1	pcDNA3	GFP	Prof. Dr. Gianni Del Sal
Ubc9	pcDNA3	HA	Prof. Dr. Gianni Del Sal
Ad5 E4Orf3	pcDNA3	HA	Dr. Thomas Dobner
SuPr1	pEGFP	GFP	Prof. Dr. Leonard Zon
SuPr1 C466S	pEGFP	GFP	Prof. Dr. Leonard Zon
p53	pcDNA3	--	Prof. Dr. Gianni Del Sal
Puromycin	pBabe	--	Dr. Thomas Hofmann
GFP	pCGFP	--	Dr. Hüseyin Sirma
OTT	pEGFP	GFP	Dr. Tilman Heise
PSF 297-707	pEGFP	GFP	Dr. Gritta Tettweiler
PSF 337-707	pEGFP	GFP	Dr. Gritta Tettweiler

2.1.3 - Synthetic Oligonucleotides

Name	Sequence (5'-3')
Sp100 1 Sense EcoRI	GAA TTC ATG GCA GGT GGG GGC GGC GAC CTG AGC
Sp100 9 Sense EcoRI	GAA TTC ATG AGC ACC AGG AGG CTG AAT GAA TGT ATT TC
Sp100 33 Sense EcoRI	GAA TTC ATG GAT TTG CAA AGG ATG TTC ACG GAA G
Sp100 69 Sense EcoRI	GAA TTC ATG AAA AAG ACA TTT CCA TTC CTC GAG GGC CTC
Sp100 149 Anti-Sense BglII	AGA TCT TTA ATG GAT TAC ATT TTC AAA GCC TTT ATA AAT GAG
Sp100 182 Anti-Sense HindIII	AAG CTT TTA TCG AAA AGA GTT TTC ACC AGT TCC TTG TTC
Sp100 253 Anti-Sense SalI	GTC GAC TTA GAC AGC AAT TTG TTC GCA GGA CTC TGT TGG
Sp100 334 Anti-Sense SalI	GTC GAC TTA GGA TCC TTC AGA GTC CTC ACT GCT GAT GAC

2.1.4 – Enzymes

Name	Source
EcoRI	Fermentas
BglII	Pharmacia Biotech
HincII	New England Biolabs
T4 DNA Ligase	Roche
Pfu DNA Polymerase	Stratagene
RNAse A	Roche
DNAse 1	Roche
Benzonase	Merck
Shrimp Alkaline Phosphatase	Boehringer Mannheim

2.1.5 - Antibodies

Primary antibodies

Antibody	Species	Source	Antigen	IFL	WB
Anti-FLAGM2	Mouse	Sigma	DYKDDDDK	1:1000	1:1000
Anti-Sp100 (AB)	Rabbit	Szostecki, C.	N-Terminus	1:250	
Anti-Sp100 (DF)	Rabbit	Szostecki, C.	C-Terminus	1:250	
Anti-Sp100 (GH)	Rabbit	Szostecki, C.	C-Terminus		1:5000
Anti-PML	Rabbit	Santa Cruz	N-Terminus	1:50	1:1000
Anti-DAXX	Rabbit	Santa Cruz	C-Terminus	1:50	1:1000
Anti-SUMO1	Mouse	Zymed Laboratories	SUMO-1	1:200	1:1000
Anti-Tubulin	Mouse	Sigma	C-terminus	1:5000	1:10000
Anti-PARP	Mouse	BD Biosciences	PARP		1:1000
Anti-HA	Rat	Roche	YPYDVPDYA	1:50	
Anti-Lamin A/C	Mouse	Santa Cruz	N-Terminus	1:50	
Anti-Lamin B	Mouse	BD Biosciences	aa 506-691	1:50	

Secondary antibodies

Antibody	Species	Source	Conjugate
Anti-Mouse	Goat	Dianova	Peroxidase
Anti-Rat	Goat	Dianova	Peroxidase
Anti-Rabbit	Donkey	Dianova	Peroxidase
Anti-Mouse	Goat	Molecular Probes	Alexa Fluor-488
Anti-Mouse	Goat	Molecular Probes	Alexa Fluor-546
Anti-Mouse	Goat	Molecular Probes	Alexa Fluor-594
Anti-Mouse	Goat	Molecular Probes	Alexa Fluor-633
Anti-Rabbit	Goat	Molecular Probes	Alexa Fluor-488
Anti-Rabbit	Goat	Molecular Probes	Alexa Fluor-546
Anti-Rabbit	Goat	Molecular Probes	Alexa Fluor-594
Anti-Rat	Goat	Molecular Probes	Alexa Fluor-594

2.1.6 – Fluorescence attached markers for cell organelles

Name	Target	Source
Hoechst	DNA	Sigma
DRAQ5	DNA	Apotech

2.1.7 – Molecular weight markers

2.1.7.1 – DNA marker

DNA Marker	Source
DNA Smart Ladder	Eurogentec

2.1.7.2 – Protein marker

Protein Marker	Source
Dual Color Precision	BioRad

2.1.8 - Antibiotics

Antibiotic	Concentration
Ampicillin	100 µg/µl
Kanamycin	50µg/ul

2.1.8 - Diverse materials and kits

Name	Source
Maxi Prep Kit	Qiagen
Gel Extraction Kit	Qiagen

2.1.9 – Chemical reagents

If not stated otherwise, all chemical reagents used in these studies were from Sigma (München), Roche Diagnostics (Mannheim), Roth (Karlsruhe), Merck (Darmstadt), Life Technologies (Eggenstein) and Biozym (Oldendorf).

2.2 – Methods

2.2.1 – Molecular Biology Methods

2.2.1.1 – Cloning of Sp100 fragments

During the course of these studies several Sp100 constructs were cloned into an expression vector. For this purpose, primers were first designed to PCR amplify fragments of Sp100. The fragments were inserted into the TOPO vector and then subcloned into eukaryotic expression vectors.

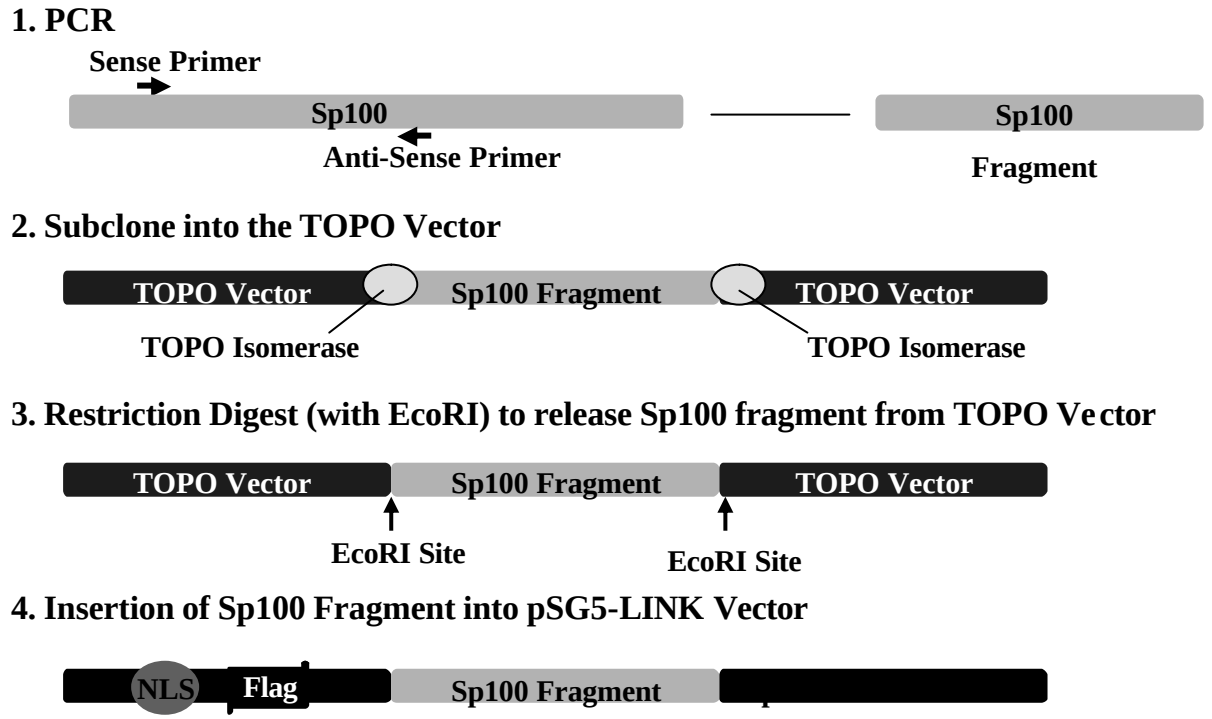

Figure 2.1 – Schematic representation of the cloning strategy for the several truncated forms of Sp100 prepared in these studies. A PCR reaction (1) was performed using designed primers containing a Start (sense) and Stop (anti-sense) codon to amplify the region of interest. The PCR fragments obtained were run on a TAE-agarose gel and gel extracted. The purified fragments were then inserted into the Blunt End TOPO vector (2), transformed into bacteria and cultures were grown to prepare mini and then maxi preparations of DNA. After this the TOPO vector containing the Sp100 fragment of interest was digested using EcoRI and the Sp100 fragments obtained were gel extracted and purified. The pSG5-LINK vector was also digested using EcoRI, followed by dephosphorylation using shrimp alkaline phosphatase. The digested Sp100 fragment from the TOPO vector and the digested and dephosphorylated pSG5-LINK vector were then ligated using a T4 DNA Ligase. Mini and maxi

preparations were prepared. The plasmid obtained was sequenced. For a more detailed description consult the following sections.

2.2.1.2 – DNA Amplification by the polymerase chain reaction (PCR)

The PCR allows the amplification of a specific DNA sequence defined by primers hybridizing to specific regions within a plasmid. For each PCR reaction the following mixture was prepared:

Reaction Mixture

1 µl	DNA (100 ng DNA)
2 µl	Primer 1 (10 pmol/µl)
2 µl	Primer 2 (10 pmol/µl)
5 µl	dNTP Mix (each 2 µM)
5 µl	Pfu Buffer
1 µl	Pfu DNA Polymerase
34 µl	dH_2O

The reaction was performed on a Robocycler Gradient 40 from Stratagene with the following program:

Temperature (°C)	Time (min)	Cycles
95	2:00	1
95	0:30	
58	0:45	26
70	1:20	
70	10:00	1

2.2.1.3 – Restriction of plasmid DNA

The size of a DNA fragment from mini/maxi preparation or the preparation of a DNA fragment for a ligation was performed by digesting the DNA with the use of restriction enzymes. The prepared mixture contained:

1-2µg	Plasmid DNA
0,5-1µl	Restriction enzyme(s)
2µl	10x reaction buffer for the restriction enzyme(s)
X µl	dH_2O
20µl	Total Volume

The restriction digest was incubated at 37°C for 1-2 hours in a heating block.

2.2.1.4 – Agarose Gel Electrophoresis for the Separation of DNA

The products of a DNA digestion reaction by restriction enzymes or the PCR products can be analyzed by TAE-agarose gel electrophoresis. In an electrophoresis the DNA fragments are separated by their sizes. The DNA was first mixed with a DNA loading buffer and then loaded in a TAE-agarose gel. The electrophoresis was performed using a constant current of 60-120V. The DNA stained by the ethidium bromide contained in the gel was visualized under UV light. The DNA Smart Ladder from Eurogentec was loaded to determine the size of the bands and to quantify the amount of DNA.

TAE (50x)

2 M	Tris-acetate
50 mM	EDTA

Agarose Gel

1-2% agarose in TAE buffer (w/V)
0.1 µg/ml ethidium bromide

DNA Loading Buffer

30% (V/V)	Glycerol
0,25% (w/V)	Xylene cyanol FF
0,25% (w/V)	Bromophenol blue

2.2.1.5 - Isolation and agarose gel extraction of DNA fragments

For ligation reactions the digested DNA was loaded in a 1-2% agarose-TAE gel and the desired bands were extracted from the gel. To extract the DNA from the agarose gel the Qiagen Gel Extraction Kit was used.

The DNA bands were cut from the agarose-TAE gel piece, and the gel containing the restricted DNA was dissolved using 600µl of the supplied buffer and 200µl of isopropanol at 50°C. After this the mixture was loaded on an anion exchange column. The DNA retained in the column was washed using 750µl of PE buffer supplied. The DNA was then eluted using 50µl EB buffer supplied with this kit.

To determine the purity and concentration of gel-extracted DNA fragments, an aliquot of the gel-extracted DNA was run on another agarose-TAE gel (2.2.1.4)

2.2.1.6 - Dephosphorylation of linear plasmids

For a more efficient ligation, after the digestion of the DNA with restriction enzymes, the vector DNA was on the 5'-terminus dephosphorylated using the shrimp alkaline phosphatase to reduce re-ligation of the vector.

10µl	digested vector (0.5-1µg)
1µl	Shrimp alkaline phosphatase
2µl	Shrimp alkaline phosphatase buffer
X µl	dH_20
20µl	Total

This mixture was incubated for 1 hour at 37°C and afterwards loaded on an agarose-TAE gel for gel purification (2.2.1.5)

2.2.1.7 - Ligation of DNA fragments with vector DNA

The ligation of an insert and the dephosphorylated vector DNA fragments was performed with the help of T4 DNA ligase. This enzyme catalyses the formation of a phosphodiester bond between a 3'-hydroxyl and a 5'-phosphate on double-stranded DNA.

For this reaction a molecular ratio of 3:1 from Insert:Vector was used. The amount of DNA used was estimated as described in section 2.2.1.4. The mixture was incubated overnight at room temperature.

~6µg	Insert DNA
~2µg	Vector DNA
2µl	T4 ligase buffer
1µl	T4 ligase
X µl	dH_20
20µl	Total

2.2.1.8 – Preparation of competent bacteria

For the preparation of competent bacteria, E.coli TOP10 were plated on an agar plate free of antibiotics and grown overnight at 37°C in an incubator. From this plate a colony was picked and grown in 500 ml of LB medium at 37°C until the OD at 600nm was between 0.6 and 0.8. The bacteria were then cooled 10 minutes on ice and then centrifuged 5 minutes at 6000g. The pellet was then resuspended in 10 ml of cold TFB-I buffer. After this 140 ml of TFB-I buffer was added and the bacteria cooled 20 minutes on ice. The bacteria were then centrifuged at 4°C with 3500g for 5 minutes. The pellet was then resuspended in cold TFB-II buffer and frozen in 500µl aliquots in an ethanol ice bath. The tubes with the aliquots were then stored at -80°C.

	TFB-I Buffer	**TFB-II Buffer (20 ml)**
RbCl	100mM	10mM
$MnCl_2$	50mM	
$CaCl_2$.2H2O	10mM	75mM
MOPS pH7		10mM
KCH_3COO	30mM	
Glycerol	15%	15%
	pH 5.8 with CH3COOH	pH 6.8 with NaOH
dH_2O		
	Filter sterilize	

2.2.1.9 – Transformation of bacteria

The product of a ligation (20µl) was careful mixed with 100µl of competent bacteria that were thawn on ice. This mixture was incubated on ice for 20 minutes. The bacteria were then heat shocked for 90 seconds at 42°C and immediately afterwards cooled on ice. To this mixture was added 1ml of LB medium and incubated for 1 hour at 37°C with 800 rpm in a heating block. This mixture was then centrifuged 2 minutes at 8000 rpm in a benchtop centrifuge and 1 ml of supernatant was removed. The bacterial pellet was redissolved in the remaining supernatant and afterwards plated on an agar plate supplemented with the appropriate antibiotic to select bacteria carrying the plasmid of interest conferring resistance.

LB medium	
Trypton	10 g/L
Yeast Extract	5 g/L
NaCl	10 g/L

2.2.1.10 - Growing transformed bacteria

For mini preparations, a single colony (2.2.1.9) was picked from an agar plate and added to 2ml of LB medium supplemented with the respective antibiotic. This mixture was incubated overnight with shaking at 200 rpm at 37°C.

For DNA maxi preparations 200µl of a culture used for DNA mini preparations was diluted in 200ml of LB medium containing the respective antibiotic. The mixture was then grown overnight with shaking at 200 rpm at 37°C.

2.2.1.11 - Plasmid isolation

Maxi preparation of DNA

For the preparation of large amounts of DNA the Qiagen Maxi-Plasmid-Kit was used. 200 ml bacterial cultures transformed with a plasmid of interest grown overnight in LB medium (2.2.1.10) were centrifuged and afterwards lysed with an alkaline buffer followed by neutralization. The lysates were then centrifuged and the supernatant containing the plasmid DNA was loaded on an anionic exchange column where the plasmid DNA is retained. After elution, the DNA was precipitated using isopropanol and washed with 80% ethanol. The pellet was then redissolved in EB buffer supplied with this kit. DNA concentration was then measured using a spectrophotometer.

Mini preparation of DNA

For the preparation of small amounts of DNA 2ml of bacterial culture grown overnight (2.2.1.10) were centrifuged and afterwards lysed with an alkaline buffer followed by neutralization. The lysates were then centrifuged and the supernatant transfered to another tube where the plasmid DNA was then precipitated using 2 times (V/V) 100% ethanol and 1/10 (V/V) of 3M sodium acetate. The pellet was washed with 80% ethanol. The pellet was then redissolved in EB buffer supplied with the Qiagen Maxi-Plasmid Kit.

2.2.1.12 - Preparing glycerol stocks

To store transformed bacteria for longer times, glycerol stocks were produced. For this purpose 500µl of an overnight culture were mixed with 500µl of 85% glycerol and then stored at -80°C.

2.2.1.13- Sequencing

All cloned plasmid DNAs were sequenced by Dr. Andreas Moeller, DKFZ, Heidelberg. Primer T7 and cloning primers were used for the PCR reaction. Sequences were aligned using the MacVector program (Accelrys) and plasmid maps generated.

2.2.2 - Cell culture techniques

2.2.2.1 - Culture of eukaryotic cells

All cell lines used in these studies were grown in 75 cm^2 flasks in 10 ml of DMEM-Medium supplemented with 10% FBS, Penicillin/Streptomycin and L-Glutamin at 37°C with 5% CO2 in an incubator.

For passaging of cells, the medium was removed from 80-90% confluent cells, cells were washed one time with 10 ml of PBS and afterwards incubated for 2-5 minutes in a trypsin solution to detach the cells. The cells were resuspended in 5 ml of DMEM medium and pipetted up and down about 10 times so that no cell aggregates were present. The cells were diluted 1:5-1:20 in a new flask in fresh medium.

For experiments, HeLa S3, U2OS and H1299 were plated on coverslips or petri dishes at a concentration of $5x10^4$ cells/ml. 293T, PML -/- and PML +/+ mouse embryonic fibroblast were plated at a concentration of $2,5x10^4$ cells/ml. Cells were counted using a cell counter (Z2 from Beckman Coulter).

Volume of cells plated (diluted to the concentration cited above)

0,5 ml	24-well plate
1 ml	12-well plate
5 ml	6 cm dish
10 ml	9 cm dish

PBS Buffer pH 7,4

137 mM	NaCl
2.7 mM	KCl
8.1 mM	Na_2HPO_4
1.5 mM	KH_2PO_4

2.2.2.2 - Transient transfection of eukaryotic cells by calcium phosphate precipitation

The transfection with calcium phosphate is one method for the introduction of a plasmid DNA into eukaryotic cells. The cells were plated 8-12 hours before transfection either on glass coverslips (Assistent™ 12mm∅) in 12 or 24 well plates or in 6 or 9 cm Petri dishes. For transfection the following mixture was prepared for 9 cm dishes (The mixture was scaled down for 6 cm dishes, 12 and 24 well plates):

Transfection mix (9 cm dish)

220µl	dH_20
10µg	DNA
250 µl	2x HBSS Buffer
31,6 µl	2M $CaCl_2$

2xHBSS Buffer

275 mM	NaCl
10 mM	KCl
1.5 mM	Na2HPO4
12 mM	Dextrose
50 mM	HEPES
pH 7.05 with NaOH	
Filter sterilize	

After diluting the DNA in water and adding the HBSS buffer, $CaCl_2$ was slowly added to the mixture. After this, the mixture was incubated 30 minutes at room temperature and

afterwards added to the cells for 24 hours at 37°C and 5% CO_2. Twelve hours after transfection the medium was replaced with fresh medium.

This method was used only for 293T cells.

2.2.2.3 - Transient transfection of eukaryotic cells with Fugene-6

Transient transfection of all other cell lines used in these studies was performed using Fugene6 (Roche) as transfection reagent. The cells were plated 8-12 hours before transfection either on coverslips in 12 or 24 well plates or in 6 or 9 cm petri dishes. The following mixture was prepared:

100µl	DMEM Medium
2X volume of desired amount of DNA (µl)	Fugene 6
X µg	DNA

The order by which the components were added to the mixture is the same as given in the table above. After adding each component the mixture was gently mixed. After this, the mixture was incubated at room temperature for 15 minutes and afterwards added to the cells for 24 hours at 37°C and 5% CO2.

2.2.2.4 – Harvesting of cells

The cells were prepared in several ways depending on the experiment performed:

Fixation on cover slips for indirect immunofluorescence:

The medium of cells grown on coverslips in 12 or 24 well plates was aspirated. The cells were washed once with PBS buffer. The PBS buffer was removed and the cells were fixed with a fresh solution of a cold mixture of methanol and acetone (1:1) for 90 seconds on ice. After this, immunofluorescence experiments were performed (Section 2.2.4).

Total cell extracts in 2xSDS loading buffer for immunoblotting:

Cells were grown in 6 well plates. The medium was removed and the cells were washed with PBS buffer. The cells were trypsinized, collected in PBS and transferred to a 15

ml tube and centrifuged. Cells were redissolved in PBS and transferred to a 1.5ml Eppendorf tube and centrifuged again in a benchtop centrifuge for 5 minutes at 8000 rpm. The supernatant was removed and 200µl of 2xSDS loading buffer were added. Cell lysates were then sonicated 4 times for 10 seconds. The samples were then boiled at 99°C for 10 minutes. Cells were centrifuged for 2 minutes at 132000 rpm in a benchtop centrifuge. After this, cells were either loaded on a SDS-polyacrylamide gel (2.2.3.1) or stored at -20°C.

2xSDS Loading Buffer

312.5 mM	Tris-HCl pH 6,8
50% (V/V)	Glycerol
10% (w/V)	SDS
0,01% (w/V)	Bromophenol blue
25% (V/V)	?-Mercaptoethanol

Cell extraction with lysis buffer for immunoblotting and immunoprecipitations:

Due to the insolubility of Sp100 and its truncated forms relatively strong buffers were used to prepare cells extracts for immunoblotting and immunoprecipitations. After several tests the RIPA buffer was chosen, as it solubilized most of the Sp100 protein.

Cells were grown in 6 cm (or 9 cm) plates. The medium was removed and cells were washed with PBS. Cells were then trypsinized and collected in PBS and transferred to a 15 ml Falcon tube and centrifuged for 5 minutes at 500g at 4°C. The supernatant was removed and the pellet was resuspended in 1ml of PBS and transfered to a 1.5 ml Eppendorf tube. Another centrifugation was performed in a benchtop centrifuge for 5 minutes at 8000 rpm and the supernatant was then discarded. The cells were lysed in 600 µl of RIPA buffer together with 1µl of Benzonase (250 U/µl) to digest RNA and DNA and incubated at room temperature for 30 minutes. The samples were then centrifuged for 2 minutes at maximum speed (13200 rpm) in a benchtop centrifuge and stored at -80°C.

RIPA Buffer

250 mM	NaCl
50 mM	HEPES pH 7.5
5 mM	EDTA
1% (V/V)	Igepal
0,5% (w/V)	SDOC
0,1% (w/V)	SDS
1 tablet for 50µl	Protease inhibitors, Complete™ Roche)

Cellular fractionation:

HeLa cells were plated on a 9 cm dish 12 hours before transfection with Fugene6 of the Sp100 33-149 construct. Twenty four hours after transfection cells were washed with 10 ml PBS and then incubated for 3 minutes with a Trypsin solution to detach the cells from the dish. The cells were then collected in a 15 ml Falcon tube with 10 ml of PBS and then centrifuged for 5 minutes at 4°C with 500g. The supernatant was removed and then cells were incubated on ice with 400µl of Buffer A for 15 minutes. With the help of a homogenizer the cells were crushed 20 times. To confirm complete lysis of the cells, a 10µl aliquot of the cell lysate was mixed with 10µl of Trypan Blue (which is excluded from intact cells and stains disrupted cells) and the cells were examined under a light microscope. After confirming that at least 95% of the cells were lysed, the lysate was transferred to a 1.5 ml Eppendorf tube and centrifuged for 10 minutes at 4°C with 700 rpm in a benchtop centrifuge. After centrifugation the supernatant containing the cytoplasmic fraction was transferred to a new tube. The pellet obtained by this centrifugation was the nuclear fraction.

Buffer A	
20 mM	HEPES-KOH, pH=7.5
10 mM	KCl
1.5 mM	$MgCl_2$
1 mM	Na-EDTA
1 mM	Na-EGTA
1 mM	DTT
250 mM	Sucrose
1 tablet of Protease Inhibitors, Complete™ Roche, per 50µl	

2.2.3 - Identification of Proteins by Antibodies

2.2.3.1 - SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Denatured proteins migrate due to their different molecular weights with different speed through a polyacrylamide gel. Due to this, they can be separated by electrophoresis according to their size.

The percentage of polyacrylamide in the gels was chosen according to the size of the proteins analyzed. Percentages ranging from 7.5 to 12.5 were used. The Mini-PROTEAN 3 gel apparatus from BioRad was used to run the SDS-polyacrylamide gels.

Mini-Gels	**Separating Gel (10 ml)**			**Stacking Gel (2ml)**
	7,5%	10,0%	12,5%	5,0%
Acrylamide (40%)	1,9 ml	2,5 ml	3,1 ml	250 µl
dH_2O	5,5 ml	4,9 ml	4,3 ml	1,5 ml
1,5 M Tris pH 8,8	2,5ml	2,5ml	2,5ml	
1 M Tris pH 6,8				250µl
SDS 10%			100µl	20µl
TEMED			14µl	12µl
APS (10%)			160µl	12µl

Samples were mixed with 2x-SDS loading buffer, boiled 5-10 minutes at 99°C and loaded on the gels. The gels were run in SDS-Page Running Buffer at 60-120V.

SDS-Page Running Buffer

960 mM	Glycine
125 mM	Tris
0.5% (w/V)	SDS

2.2.3.2 – Immunoblot

In an immunoblot, proteins that have been electrophoretically separated are transferred to a nitrocellulose membrane. Proteins on the membrane can be stained using antibodies. The protein transfer was performed using a semi-dry transfer chamber (BioRad) (45 minutes, 15 V). The proteins migrate towards the anode from the gel to the membrane. The membrane and gel were placed in-between two Whatman filter papers soaked with a transfer buffer.

The membranes were stained with Ponceau S (2.2.3.3) to check the transfer efficiency and then blocked with 5% milk in TBS for 20 minutes and then washed with TBS-0.1%Tween for another 20 minutes. The primary antibody against the target protein was diluted in 5% milk in TBS solution and then incubated for 1 hour at room temperature (or overnight at 4°C) followed by three washings with TBS-0.1% Tween. The second antibody, directed against the primary antibody and conjugated to horseradish peroxidase, was added for 1 hour and the blots again washed three times with TBS-0.1% Tween. The secondary antibody was always diluted 1:25000 in 5% milk in TBS solution. The blot was then incubated with the Super Signal West DURA reagent for 1 minute with mixing. The peroxidase conjugated to the secondary antibody degrades this reagent and the product of this reaction emits light which is captured on an X-Ray film.

Transfer Buffer

80% (V/V)	SDS-PAGE Running Buffer (2.2.3.1)
20% (V/V)	Methanol

TBS pH 7,4

10 mM	Tris-HCl pH 7,6
150 mM	NaCl

2.2.3.3 - Staining of nitrocellulose membranes with Ponceau S

After SDS-PAGE the transfer efficiency onto nitrocellulose membranes was checked by reversible staining of the membrane with a Ponceau S solution for 2-3 minutes followed by washing the membrane with TBS-0.1% Tween.

Ponceau S staining solution

0.5% (w/V)	Ponceau S
1% (V/V)	Acetic Acid

2.2.3.4 - Staining of nitrocellulose membranes with Amido Black

After the immunoblots were performed, proteins on the membranes were stained irreversibly with an Amido Black solution to estimate the amount of proteins loaded. Membranes were incubated with Amido Black staining solution for 10 minutes followed by destaining for 30 minutes in destaining solution.

Amido Black

0.1% (m/V)	Amido Black
45% (V/V)	Methanol
10% (V/V)	Acetic Acid

Destaining Solution

45% (V/V)	Methanol
10% (V/V)	Acetic Acid

2.2.4 - Indirect Immunofluorescence

Indirect immunofluorescence is used to examine the localization of proteins within a cell. Cells fixed on coverslips as described in 2.2.2.4 were incubated with the primary antibodies diluted in PBS for 1 hour at room temperature. The cells were then washed 3 times with PBS. The fluorescently labeled secondary antibodies, also diluted in PBS, were added to

the cells and the cells were incubated for 45-50 minutes at room temperature in the dark and subsequently washed 3 times with PBS. The coverslips were then glued on glass plates with Mowiol and left overnight at 4°C. The next day, the coverslips were sealed with nail varnish to prevent them from drying out.

Coverslips were then examined using the Axio Vert 200M confocal microscope from Zeiss.

Pictures were assembled with Adobe Photoshop.

Mowiol

6 g	Glycerol
2.4 g	Mowiol
6 ml	dH_2O
2 hours at room temperature	
12 ml	0.2M Tris pH=8,5
Incubate 10 minutes at 50°C	
Centrifuge 15 minutes	
Store at -20°C	

2.2.4.1 - Treatment of cells with nucleases

In some immunofluorescence experiments DNA and RNA were digested prior to the incubation with the antibodies. First, the cells grown on coverslips were washed with 1mM $MgCl_2$ in PBS and then incubated for 2 minutes at -20°C with methanol. Afterwards the cells were again washed shortly with 1mM $MgCl_2$ in PBS. Thereafter cells were treated with RNase (100µg/ml) and DNase (100U/ml) in 50µl of 1mM $MgCl_2$ in PBS for one hour at room temperature.

2.2.5 – Immunoprecipitations

Immunoprecipitations were used to investigate the interaction between proteins. The cells were lysed in 600µl RIPA buffer as described in 2.2.2.4. The supernatants were precleared by incubating them with 50 µl of washed Protein G Sepharose beads (Pharmacia) for 1 hour at 4°C while rotating them. To wash Protein G Sepharose beads, 3 times 1 ml of

RIPA buffer was used. In the end a 50% (V/V) solution Protein G Sepharose beads:RIPA buffer was prepared. After preclearing, the lysates where centrifuged in a benchtop centrifuge at 13200 rpm for 1 minute and 250µl of supernatant was transferred to each of 2 Eppendorf tubes.

To one of these tubes an isotype-matched control antibody was added and to the other tube the same amount of the specific antibody was added. This specific antibody will bind the target protein. The lysates were incubated with these antibodies for 3 hour at 4°C while rotating them. After the incubation, 50 µl of washed Protein G Sepharose beads were added to capture immunoprecipitated proteins and incubated with the lysates for 1 hour at 4°C while rotating them. Protein G binds to IgGs and is coupled to a sepharose matrix, which allows target proteins bound by the specific antibody and interacting proteins to be precipitated. Finally the beads were washed 5 times with 1 ml of RIPA buffer.

Bound proteins were eluted by boiling the beads in 50µl 2xSDS loading buffer for 10 minutes at 99°C. Samples were centrifuged at 13200 rpm before SDS Page (2.2.3.1).

2.2.6 - Colony forming assays

Colony forming assays were performed to investigate the effect of overexpressed proteins (Sp100 and fragments) on cell survival. HeLa cells were plated in 6 cm Petri dishes at a concentration of 5x104 cells/ml. Twelve hours later, cells were transfected with the indicated plasmids. A plasmid encoding resistance to puromycin was co-transfected (1/9th of total transfected DNA) to select for transfected cells in all but one dish (negative control). The same amount of DNA was transfected per dish by filling up the DNA with an empty vector. 24 hours after transfection the cells were split in 3 fractions. To one of these fractions (20%) 200 µl of 2x-SDS loading buffer was added for immunoblotting experiments. The other two fractions (40% each of the initial cells) were plated in 9 cm dishes and incubated with puromycin (1µg/ml) for the selection of transfected cells. Every 3 days the medium was removed, cells were washed with 10 ml PBS to remove floating dead cells and new medium supplemented with puromycin was added. After 14 days the medium was aspirated and cells were washed with 10 ml PBS prior to fixation with methanol for 10 minutes at room temperature. Colonies were then stained by using Giemsa. Colonies were counted using the FluorS Multimager from BioRad and the software “Quantity One”.

2.2.7 - FACS (Fluorescence Activated Cell Sorting) analysis

FACS analysis was used to determine the number of cells in different phases of the cell cycle. The cells were cultured in 9 cm dishes. One of the plates was left untransfected to serve as a negative control for the transfection effect. The cells were transfected using Fugene6 and 36 hours afterwards were trypsinized and collected in 10 ml PBS. In all transfected samples GFP was cotransfected ($1/9^{th}$ of total DNA). The cells were then centrifuged for 5 minutes at 300g and again washed with PBS and centrifuged again. The cell pellet was then dissolved in 0,5 ml of PBS and 5 ml of cold 80% ethanol solution was added while vortexing the samples slowly. The cells were then incubated for 20 minutes on ice and stored at -20°C.

On the next day cells were warmed 5 minutes to room temperature and centrifuged for 5 minutes at 300g and the supernatant was discarded. 5 ml of PBS were added to the pellet and again incubated 5 minutes at room temperature and centrifuged down for 5 minutes at 300g and the supernatant was removed.

Then, the RNA was digested and the DNA was stained using propidium iodide (PI). The substance binds to nucleic acids and can afterwards be detected by its fluorescence. To do this, cell pellets were incubated in 1,5 ml PBS containing PI (0,5 mg/ml) and RNAse A (10mg/ml DNA-free) for 30 minutes at 37°C in the dark and then left to cool to room temperature in the dark.

The samples were then measured using the FACS Calibur machine (Becton Dickinson). The measurements give the cell number versus DNA content. Doublets were first excluded by gating the measurements. The cells were also separated between GFP positive (transfected) and negative cells (untransfected). Stained cells with one copy of their genetic material (G1 phase – haploid cell) will be half as bright as cells with two copies (G2/M phase - diploid cell). The results were at the end exported with the Expo32 software.

3. Results

The main objectives of these studies were to study the sub-cellular localization of the Sp100 HSR domain and its potential to form filamentous structures and to investigate why Sp100 splice variants localize in part to alternative nuclear bodies not containing PML.

3.1 - Filament Formation by the HSR Domain of Sp100

Homologous Sp100 proteins are known both in rodents (mouse and rat) and in humans. The HSR domain of Sp100 is the most conserved region of this protein as evident in the protein alignment shown in Figure 3.1. The protein sequence similarity between human Sp100 and the Sp100 from other species is rather low (22-29%) when the whole protein is taken in account (Table 3.1). The amino acid sequence homology for the HSR domain (amino acids 35 to 145) however, shows a sequence similarity of Sp100 between human and the other species that is higher /43-54%) than when the whole protein is taken in account (Table 3.1).

Amino acid sequence homology of human Sp100 with Sp100 from other species	Sp100 protein	HSR domain (aa 35-145)
Rattus norvegicus	22,2%	43,2%
Mus musculus	28,7%	55,9%
Mus caroli	28,9%	54,1%

Table 3.1 – Sequence homologies between the human Sp100 and its homologue from other mammal species.

The Sp100 protein is known to localize in PML bodies, and this is mediated by the HSR domain (Sternsdorf et al., 1999). In this study the subcellular distribution of the isolated HSR domain was investigated in more detail, because it was initially noted that it not only localizes in PML nuclear bodies but also forms filaments. The overexpression of a truncated form of Sp100 from amino acid 33 to amino acid 149 containing the HSR domain of Sp100 (amino acids 35 to 145) forms filaments that can be seen by indirect immunofluorescence

microscopy (Figure 3.2A). These filaments are not the only structures observed, in some cells also rods/tracks and dots are formed (Figure 3.2-B and C).

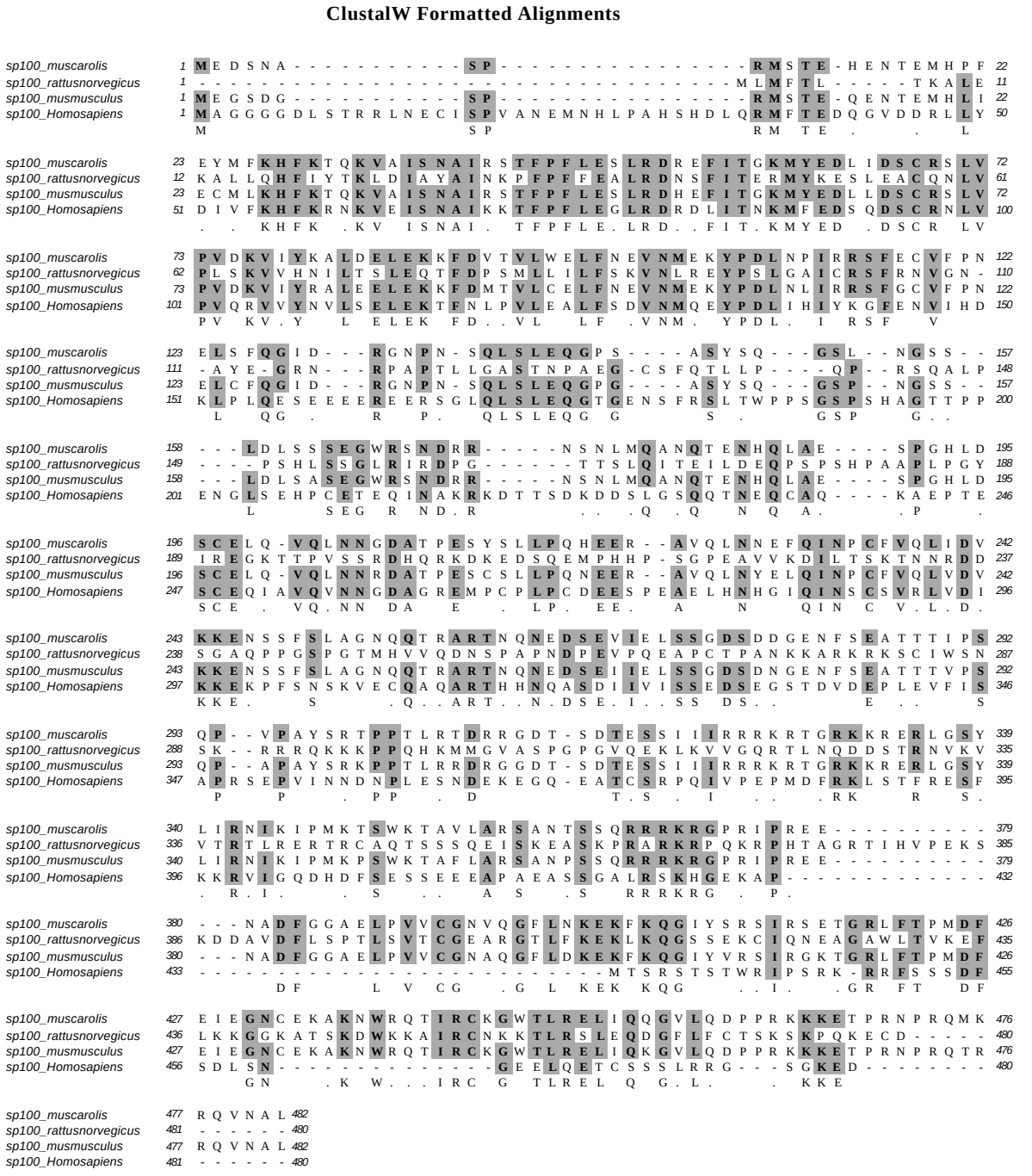

Figure 3.1 – Protein sequence alignment of Sp100 from human, mouse and rat (*Homo sapiens, Mus caroli, Mus musculus, Rattus norvegicus* – Top to bottom). The conserved amino acids are shaded in grey.

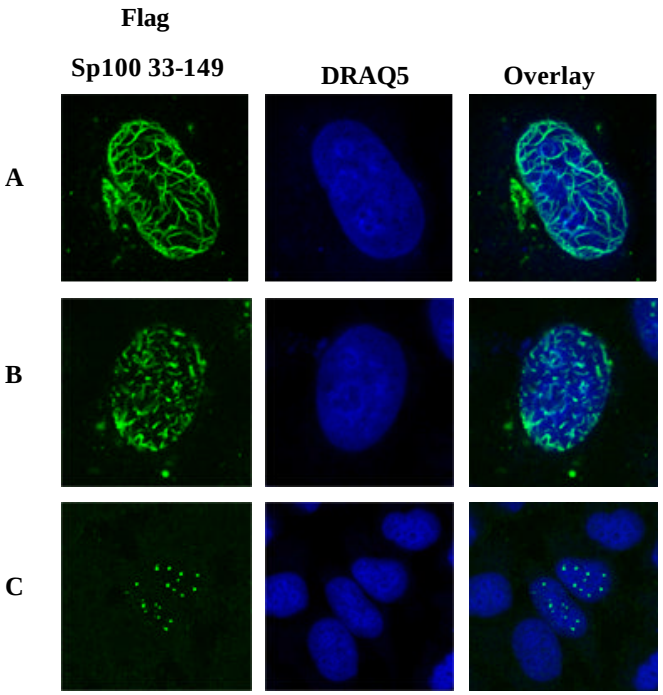

Figure 3.2 – Characterization of the structures created by Sp100 33-149. Different structures: filaments (A), tracks (B) and dots (C) are observed when this truncated form of Sp100 is expressed by transfection in HeLa cells and its expression examined by indirect immunofluorescence microscopy 24 hours after transfection. Sp100 33-149 was detected using the anti-Flag antibody and the DNA stained with DRAQ5.

To verify whether the formation of these structures by the isolated HSR domain (Sp100 33-149) is cell type dependent, several human cell lines (HeLa, U2OS and H1299) were transfected with the plasmid coding for this truncated form of Sp100 and examined by indirect immunofluorescence microscopy. No major differences in the formation of filaments (Figure 3.3), tracks or dots (data not shown) were observed in different cell types, indicating that the formation of these structures is independent of the cell type in which this protein is expressed.

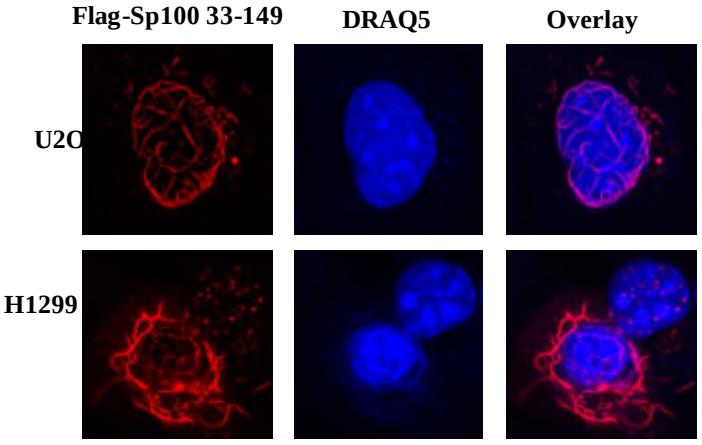

Figure 3.3 – Characterization of the filaments formed after transfection of Flag Sp100 33-149 in different cell lines (small tracks and dots are also observed - data not shown). Cells were fixed 24 hours after transfection and checked by indirect immunofluorescence microscopy. In these experiments Sp100 33-149 was detected by using the anti-Flag antibody and the DNA stained with DRAQ5.

The observation of filaments, small tracks and dots formed by Sp100 33-149 may lead to the conclusion that, because not all cells take up the same amount of plasmid DNA and express different amounts of this truncated form of Sp100, this may determine the different structures formed.

To clarify this point the same number of HeLa cells was transfected with increasing amounts of the plasmid that codes for Sp100 33-149. The total amount of DNA transfected was kept the same by adding empty vector DNA. After 24 hours the cells were fixed and examined by indirect immunofluorescence. Afterwards cells were counted for the structures observed. The results shown in Figure 3.4 indicate that the amount of transfected DNA does influence to some extent the relative percentage of the different structures observed (filaments: 73% 1000ng, 62% 100ng). However, even with ten times less transfected plasmid used the filaments were still the most abundant structure created by Sp100 33-149. Transfected amounts below the 100 ng plasmid shown (1, 10 and 50 ng) were not detectable by indirect immunofluorescence experiments.

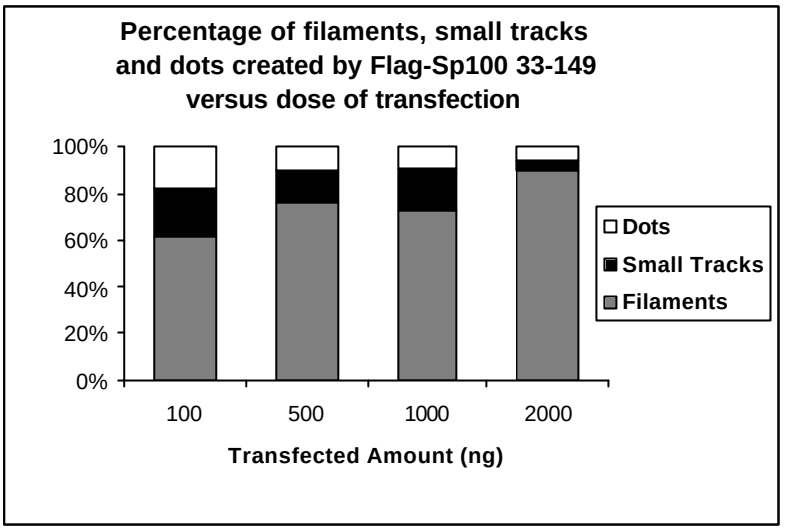

Figure 3.4 – Quantification of the percentage of the structures observed upon expression of Flag-Sp100 33-149 with several concentrations of plasmid transfected. At least 150 cells were counted in each experiment.

3.2 - Mapping of the Sp100 domain that forms filaments

When overexpressed, the wild type Sp100 localizes in nuclear dots, whereas approximately 70% of Sp100 33-149 localizes in filaments. To determine the regions of Sp100 that are responsible for the localization in filaments or dots several constructs encoding for fragments of Sp100 were cloned and checked by sequencing (see Figure 3.7 for a schematic representation). After their transfection into HeLa cells their expression was checked by Western blot. The sizes of the bands obtained are according to the predicted sizes (Figure 3.5).

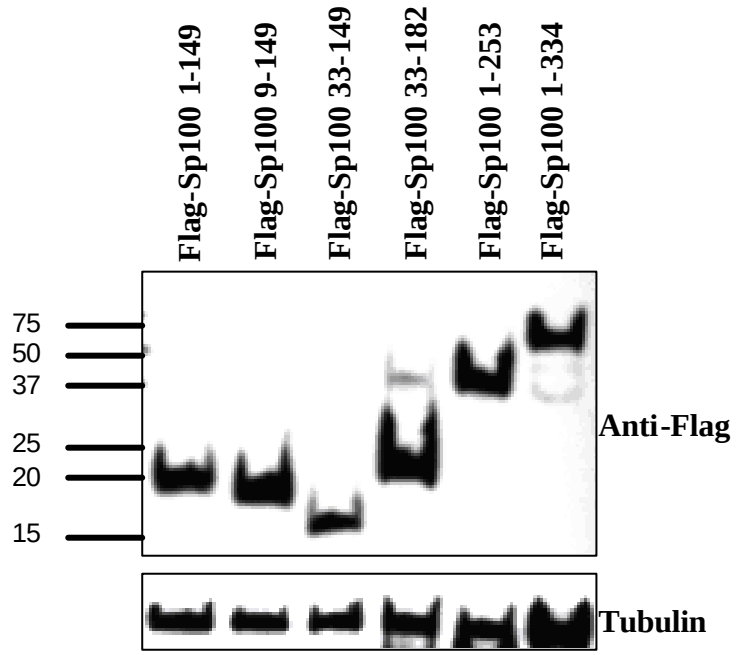

Figure 3.5 – Expression of the truncated forms of Sp100 in HeLa cells as detected by immunoblotting. The anti-Flag antibody was used to detect all truncated forms of Sp100. The tubulin was detected using an antibody against its full-length form.

The HSR domain of Sp100 is predicted to consist of four α-helices. A smaller of the HSR region (amino acids 69-149) in which the first of these a-helices (Figure 3.6) was not present was cloned but no expression of this protein could be detected neither by indirect immunofluorescence nor by immunoblotting. The sequence of this construct was correct as determined by sequencing. It was thought that the peptide expressed by this construct may be unstable and therefore degraded by proteasomes. However, addition of the proteasome

inhibitor MG132 after transfection of the plasmid containing this cDNA did not allow detection of the correspondent protein. Computer predictions using the Lupas algorithm revealed a coiled coil in the region 149-182 and this may explain the different morphology of filaments and the chromatin rearrangement by Sp100 33-182 (Figure 3.7).

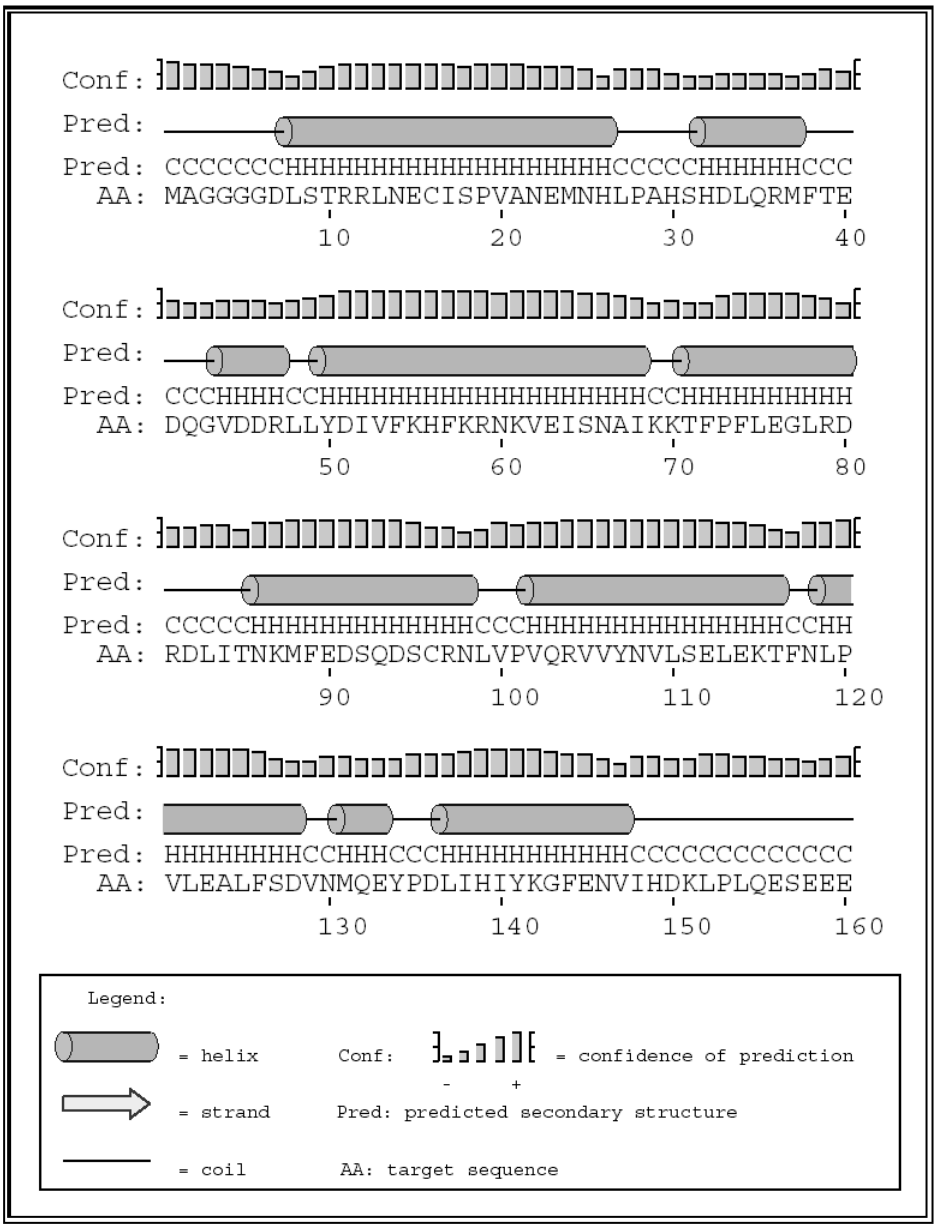

Figure 3.6 – Secondary structure of the HSR domain of Sp100 (aa 35-145)

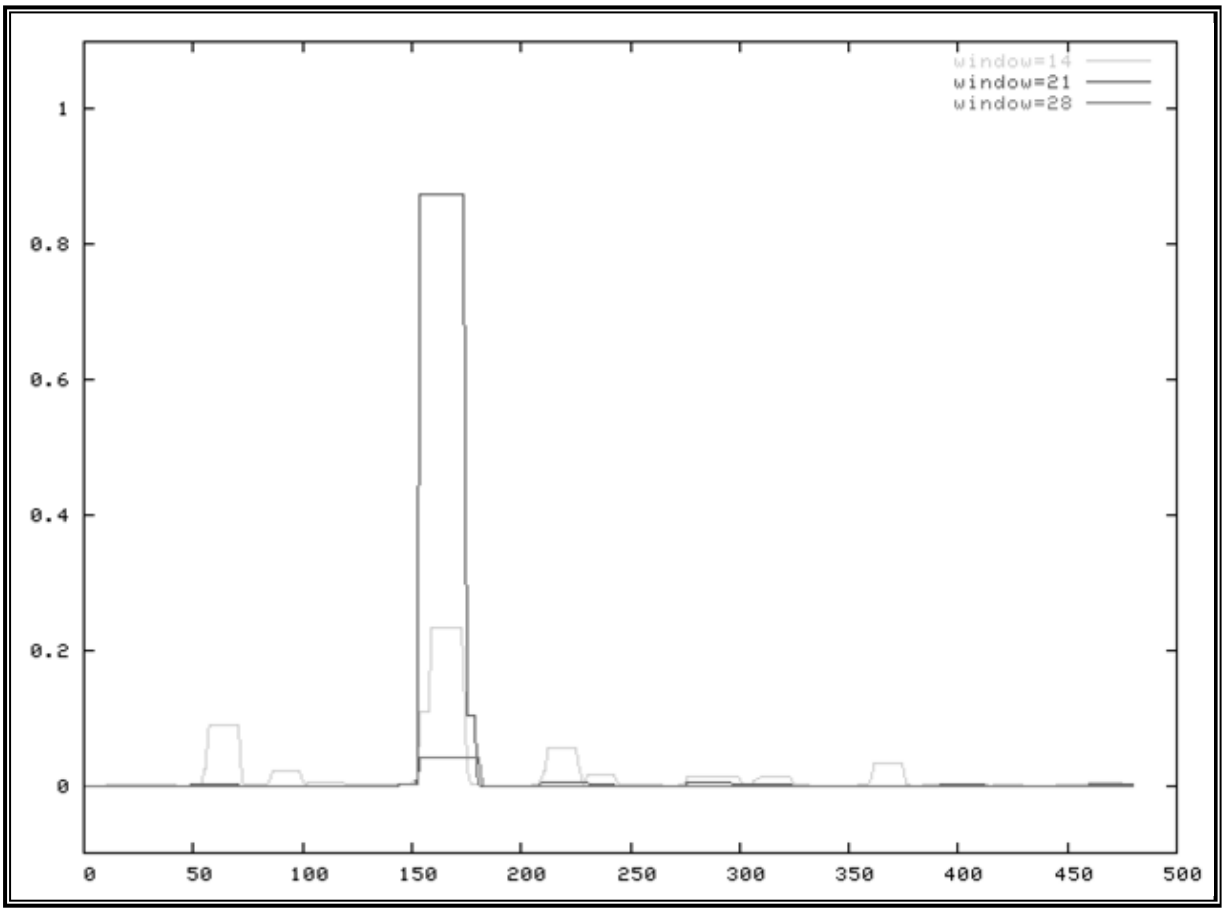

Figure 3.7 – Coiled coil computer prediction for Sp100 full length using the Lupas algorithm.

The intracellular localization of Sp100 fragments was analyzed by indirect immunofluorescence microscopy after transfection of the corresponding plasmids into HeLa cells. Sp100 1-149 was the longest truncated form of Sp100 containing the HSR domain that still formed filaments in the cells (Figure 3.8-C). The Sp100 1-253 (Figure 3.8-D) was still capable of making small tracks but no longer formed a large filament network like the smaller truncated versions of Sp100. It is of note that the truncated forms up to amino acid 149 (Figure 3.8 - B and C and Figure 3.2-A) showed a filament structure very similar while the Sp100 33-182 (Figure 3.8-A) showed filaments which were thicker and seemed to condense chromatin. Computer analysis using the Lupas algorithm (Lupas et al., 1991) also predicted a coiled coil structure in the region 149-182 and this may explain the different morphology of filaments and the chromatin rearrangement by Sp100 33-182 (Figure 3.7). The Sp100 1-334 (Figure 3.8-E) was no longer able to produce any filaments or small tracks but accumulated in the nucleus of the cells in nuclear dots, which were however larger than dots seen with wild

type Sp100, but colocalized with PML (data not shown) or in the cytoplasm unable, probably due to the overexpression, to be transported into the nucleus.

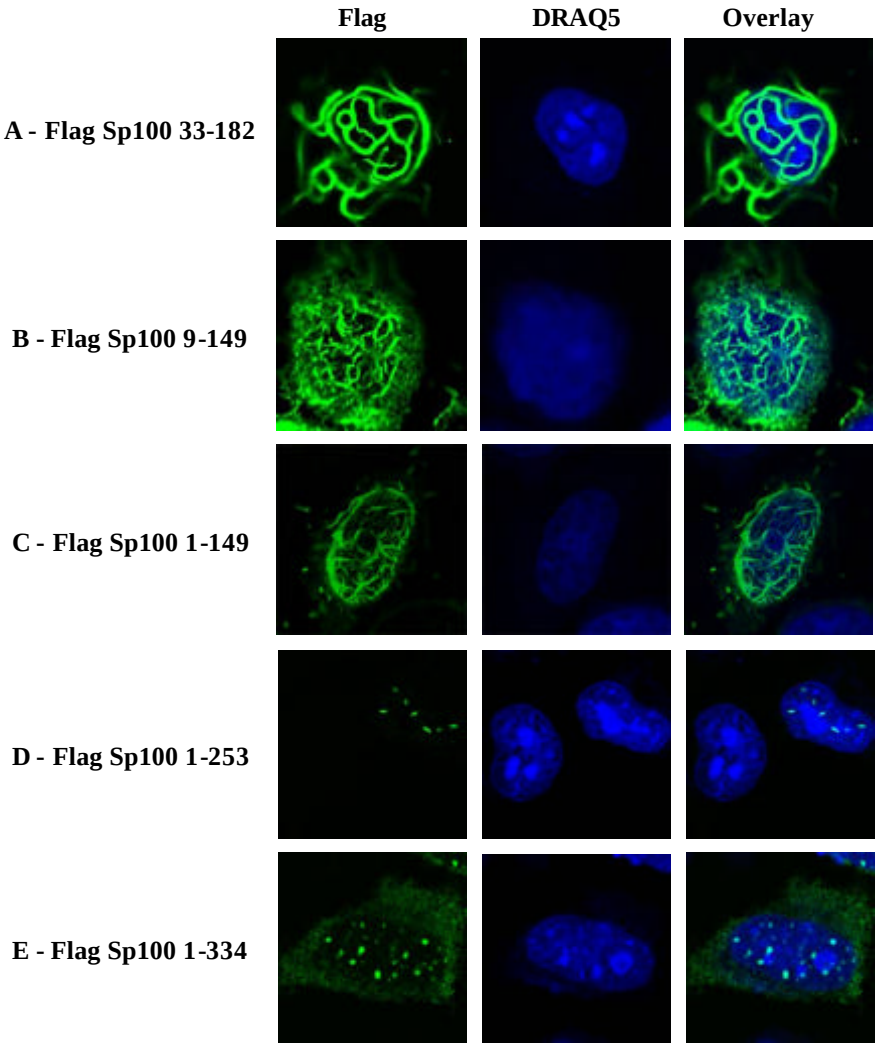

Figure 3.8 – Mapping of the domains of Sp100 that are able to form filamentous structures in the nucleus of the cell. Several truncated forms of Sp100 containing the HSR domain were transiently expressed in HeLa cells and checked by indirect immunofluorescence microscopy. Sp100 truncated forms were detected by using the anti-Flag antibody. The DNA was stained with DRAQ5.

The smallest part of Sp100 capable of creating a filamentous network was the Sp100 33-149 polypeptide which corresponds to the HSR domain of Sp100. The largest fragments of Sp100 tested which formed filaments were Sp100 1-149 and 33-182, but Sp100 1-253 no longer localized in filaments. It is of note that Sp100 1-253 still lacks the SUMOlation site of

Sp100. The constructs used and the localization of the corresponding polypeptides are summarized below (Figure 3.9).

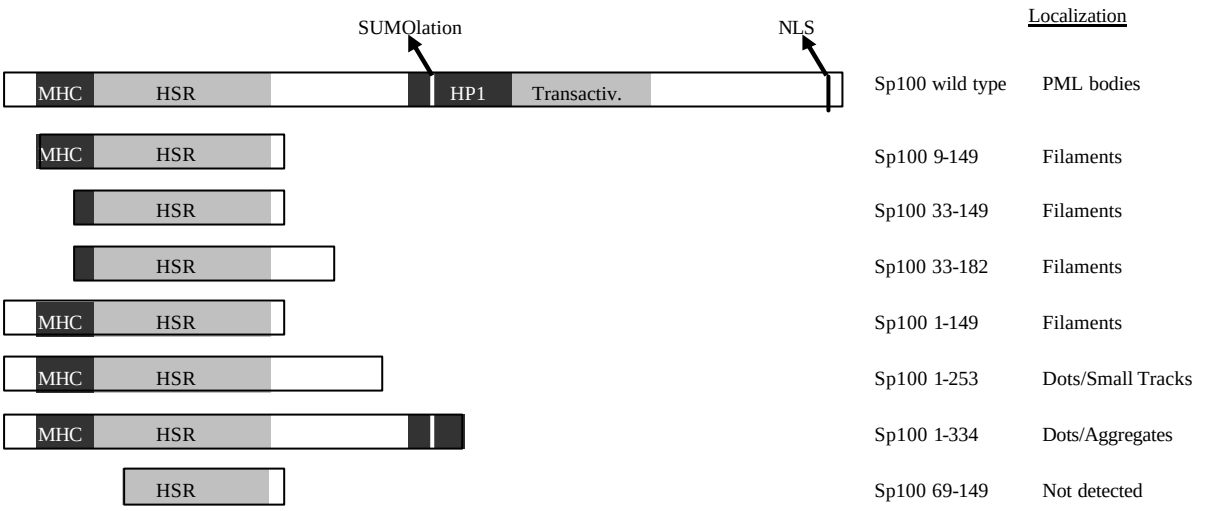

Figure 3.9 –Domains of Sp100 and schematic representation of the several truncated forms used in these studies as well as the major structures they localized to.

3.3 - Subcellular localization of the filaments of truncated forms of Sp100

The exact subcellular localization of filaments formed by truncated Sp100 forms was investigated. The several filament forming forms of truncated Sp100 were expressed and afterwards co-stained with lamin A/C antibody, as a marker for the nuclear membrane, to probe their sub-cellular localization. As it can be seen by some of the representative pictures shown in Figure 3.10, filamentous structures were present inside the nucleus, in a ring surrounding the nucleus and sometimes seemed to extend into the cytoplasm of the cells.

It was investigated whether the ring surrounding the nucleus formed by Sp100 truncated forms colocalizes with the nuclear membrane. As seen in Figure 3.10 the ring formed by truncated Sp100 forms only partially colocalized with lamin A/C. Moreover, all truncated Sp100 forms forming filaments surrounded the nucleus inside of the nuclear membrane (Figure 3.10 A, C and D), except for the Sp100 33-182, which surrounded the nuclear membrane on the cytoplasmic side (Figure 3.10 - B).

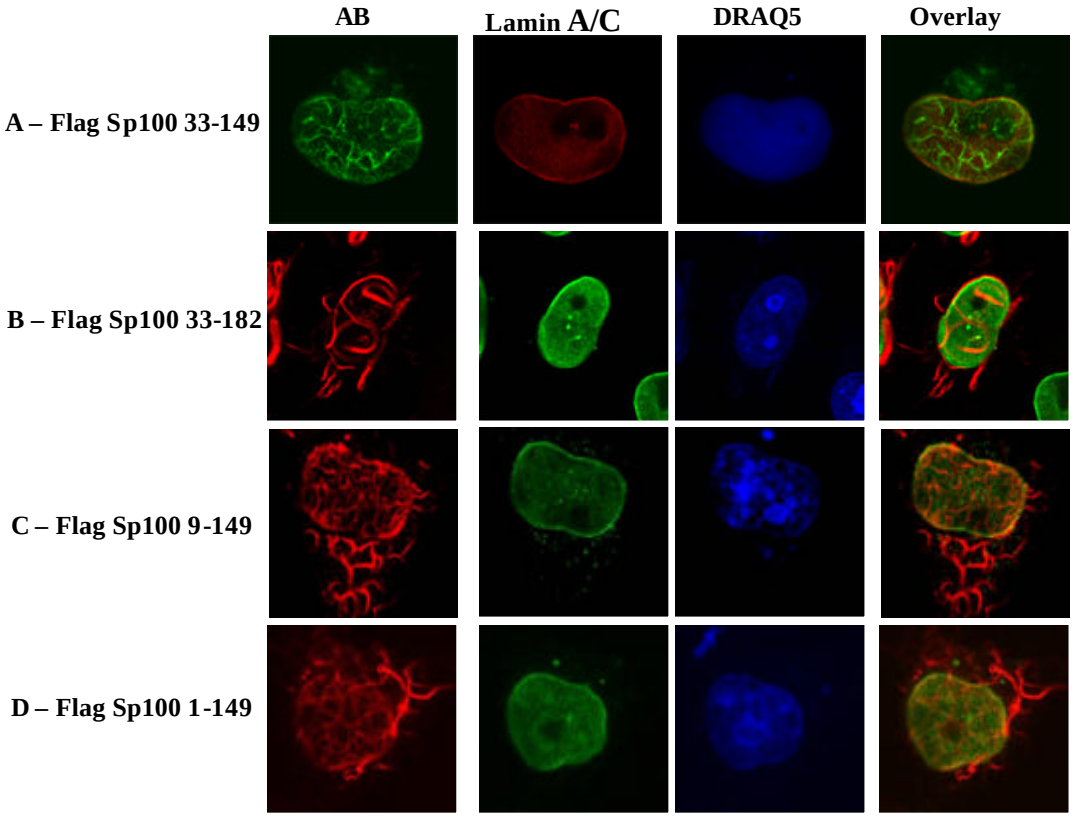

Figure 3.10 – Subcellular localization of the filaments. Co-staining of Lamin A/C with the truncated forms of Sp100 showed that the filaments are present mainly in the nucleus of the cells but also surrounding the nucleus and in cytoplasmatic extensions. All Sp100 truncated forms were detected using a rabbit polyclonal antibody directed against the N-terminus of Sp100 (AB). Lamin A/C was detected using a mouse monoclonal antibody from Santa Cruz.

Cell fractionation experiments were in addition used to analyze the subcellular localization of Sp100 filaments. Lysates from cells expressing Flag-Sp100 33-149 were separated into nuclear and cytoplasmatic fractions and analysed by immunoblotting. The results confirmed that the filaments are mainly located in the nucleus of the cells. With the method used, filaments that are strongly bound to the nuclear membrane will be contained in the nuclear fraction, because the nuclear membrane is not disrupted during the fractionation. It is likely that cytoplasmatic extensions of the filaments observed by indirect immunofluorescence are attached to the nuclear membrane and were therefore contained in the nuclear fraction.

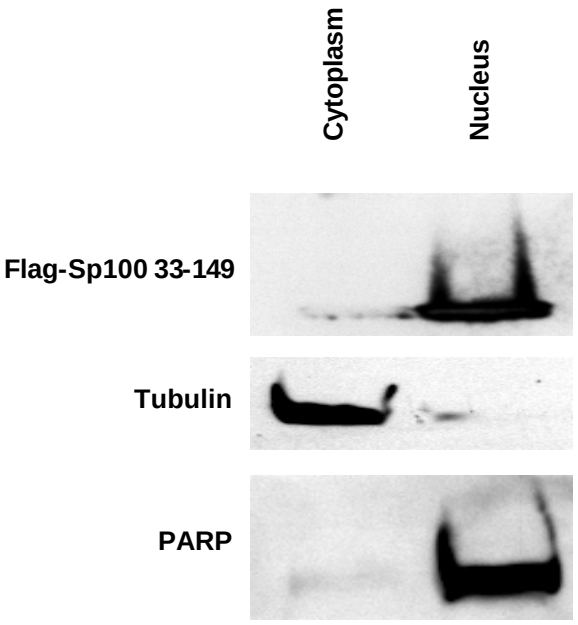

Figure 3.11 – Subcellular localization of the filaments. HeLa cells were transfected and subjected to cell fractionation and immunoblotting revealing that the filaments of Sp100 33-149 are present mainly in the nucleus of cells and/or attached to the nuclear membrane. Flag-Sp100 33-149 was detected using the anti-Flag M2 antibody. The purity of the fractions was determined by blotting for tubulin (cytoplasm) or PARP (nucleus). Tubulin was detected using the antibody against its C-terminus and PARP by using an antibody against the full length protein.

The phenomenon of cytoplasmatic extensions of filaments was examined in more detail. The amount of cells which showed filaments extending into the cytoplasm was quantified by indirect immunofluorescence microscopy in the case of Sp100 33-149. The majority of cells (71%) that contained filaments also showed cytoplasmic extensions (Figure 3.12).

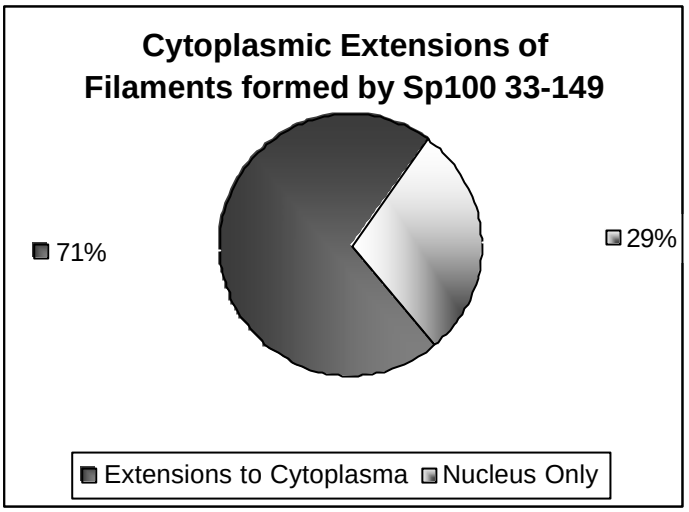

Figure 3.12 – Subcellular localization of the filaments. Quantification of the amount of cells with cytoplasmic extensions of filaments. HeLa cells were transfected with Sp100 33-149. After fixing, the cells were costained for Sp100 33-149 (with a rabbit polyclonal antibody directed against the N-terminus of Sp100) and for lamins A/C. Using a confocal microscope, 104 cells showing filaments were scored for extensions of filaments into the cytoplasm.

3.4 - No colocalization of Sp100 33-149 filaments with cellular filaments

Cells have filamentous structures such as the cytoskeleton. The cytoplasmic extensions of the filaments created by the truncated forms of Sp100 were investigated to examine whether they localize eventually with cytoskeleton proteins like tubulin. In Figure 3.13 it is demonstrated by indirect immunofluorescence staining that none of the cytoplasmatic extensions of the several truncated forms of Sp100 colocalizes with tubulin.

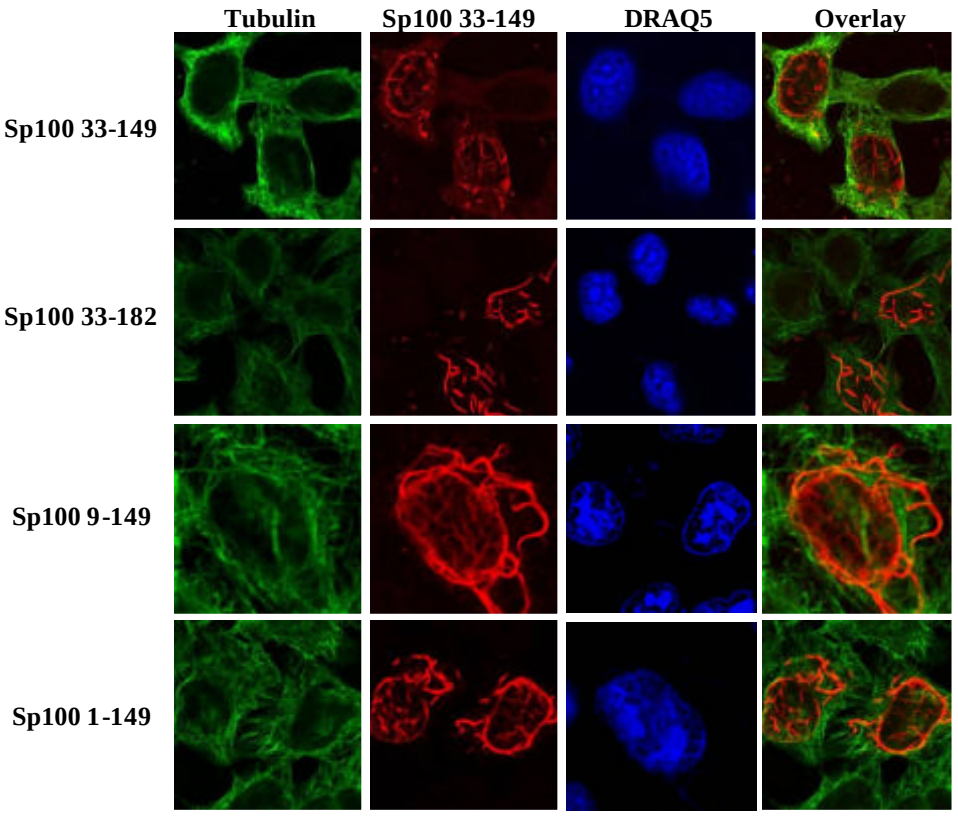

Figure 3.13 – Truncated forms of Sp100 that form filaments do not colocalize with tubulin in HeLa cells. HeLa cells were transfected with plasmids encoding several Flag tagged truncated forms of Sp100 and examined by indirect immunofluorescence microscopy. The truncated forms of Sp100 were detected with AB - a rabbit polyclonal antibody against the N-terminus of Sp100, tubulin with an antibody against its C-terminus, and DNA was stained with DRAQ5.

In the case of Sp100 33-149 a eventual colocalization with actin and vimentin was also investigated. However, no colocalization with these known cellular filaments was observed (Figure 3.14).

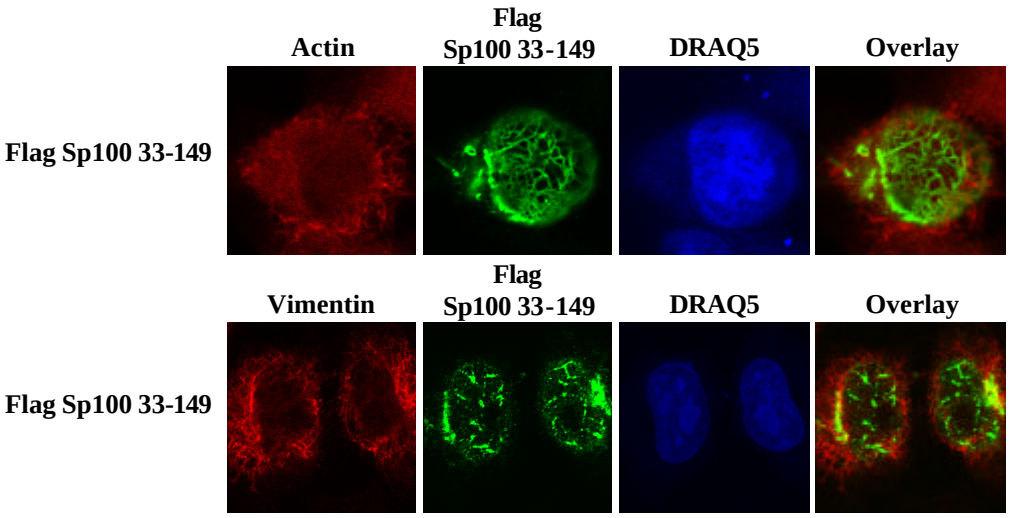

Figure 3.14 – Sp100 33-149 filaments do not colocalize with actin or vimentin in HeLa cells. HeLa cells were transfected with the Flag-Sp100 33-149 plasmid and expression examined by indirect immunofluorescence microscopy. The truncated form of Sp100 was detected with AB - a rabbit polyclonal antibody against the N-terminus of Sp100, actin by using phallloidin, a chemical compound that stains actin and vimentin using a mouse monoclonal. DNA was stained with DRAQ5.

3.5 – The filaments formed by Sp100 33-149 are not associated with DNA

PML bodies are known to be associated with the nuclear matrix but not bound to DNA (Stuurman et al., 1992). To investigate whether Sp100 33-149 localizes to filaments which are attached to RNA or DNA, HeLa cells were transfected with Flag-Sp100 33-149 and DNA and RNA were digested using DNAse and RNAse for 2 hours at 37°C prior to fixation. The cells were stained with an anti-Flag and an anti-PML antibody by indirect immunofluorescence microscopy. DNA was stained with DRAQ5. The results show that even when almost all DNA was digested as seen by the disappearance of the DRAQ5 staining (the DNA staining in the nucleolus remains – Figure 3.15), Sp100 33-149 (and PML) still remained in filaments (Figure 3.15) indicating that Sp100 33-149 filaments integrity does not depend on RNA or DNA.

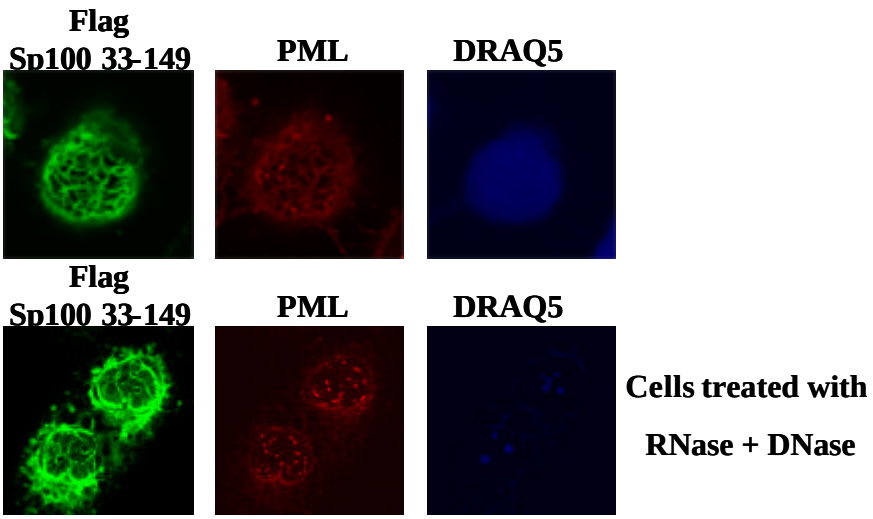

Figure 3.15 – Sp100 33-149 filament integrity does not depend on DNA. Indirect immunofluorescence microscopy was used to investigate the localization of Sp100 33-149 and PML without (A) and after DNA (DNAse) and RNA (RNAse) digestion (B). HeLa cells were transfected with Flag-Sp100 33-149. Cells were subjected to DNA and RNA digestion for 2 hours at 37°C prior to fixation. Sp100 33-149 was detected with an anti-Flag antibody and PML with a rabbit polyclonal antibody directed against its N-terminus. DNA was stained with DRAQ5.

3.6 - No colocalization of Sp100 33-149 filaments with Ad5 E4Orf3 viral filaments

After expression of the adenovirus protein Ad5 E4Orf3 in human cells this viral protein localizes in small tracks/filaments in the nucleus of the cells (Carvalho et al., 1995; Doucas et al., 1996) (Figure 3.16 –A). Expression of E4Orf3 has also been reported to disrupt PML bodies by recruiting PML into these filaments/small tracks (Carvalho et al., 1995; Doucas et al., 1996) (Figure 3.16 –C).

It was important to know whether these filaments created by Ad5 E4Orf3 were similar or not to those formed by Sp100 33-149. HeLa cells were cotransfected with Flag-Sp100 33-149 and with HA-E4Orf3 and examined by indirect immunofluorescence microscopy. The results shown in Figure 3.16-B demonstrate that the type of filaments created by these two proteins have different morphology and localization within the nucleus of the cells.

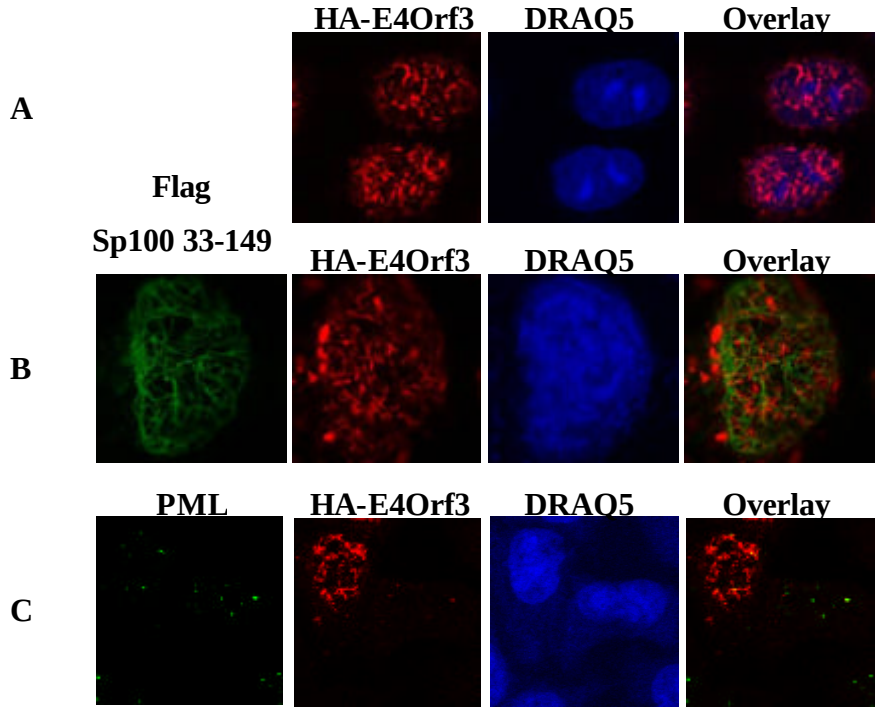

Figure 3.16 – No colocalization of the filaments created by Sp100 33-149 and Ad5 E4Orf3. The expression of Ad5 E4Orf3 in HeLa cells leads to the formation of filaments or small tracks in the nucleus of these cells. These filaments have a nuclear localization different to those formed by Sp100 33-149. HeLa cells were transfected with HA tagged E4Orf3 alone or together with Flag-tagged Sp100 33-149 and examined by indirect immunofluorescence microscopy. Sp100 33-149 was detected with an anti-Flag antibody, E4Orf3 with an anti-HA antibody and DNA was stained with DRAQ5.

3.7 - Recruitment of endogenous PML-Body proteins into the filaments created by Sp100 33-149

Sp100 is normally localized in PML bodies where it colocalizes with several other proteins. Because some filamentous structures like the E4Orf3 of adenovirus 5 can either recruit or disrupt PML body proteins, the localization of PML and other PML body proteins upon expression of truncated forms of Sp100 was examined.

HeLa cells were transfected with Flag Sp100 33-149 and co-stained for Flag and endogenous PML or other PML body components. PML was found in nuclear dots in untransfected cells (Figure 3.17-A). Sp100 33-149 localized to filaments and delocalized PML from PML nuclear bodies into these filaments (Figure 3.17-A). This was quite surprising because no direct interaction between Sp100 and PML has ever been shown, which could explain the delocalization.

Less surprising was the recruitment of endogenous Sp100 into filaments formed by Sp100 33-149 (Figure 3.17-B) because this truncated form of Sp100 is essentially the HSR domain of the Sp100 protein which is known to be the dimerization/oligomerization domain of Sp100. Also expected was the recruitment of SUMO1 (Figure 3.17-C) because it can be covalently attached to Sp100 and PML which were both recruited into these filaments although the SUMOlation site of Sp100 is not present in the Sp100 33-149. Also recruited by Sp100 33-149 into these filaments was DAXX (Figure 3.17-D) which is known to interact and to be recruited into PML bodies by PML (Zhong et al., 2000).

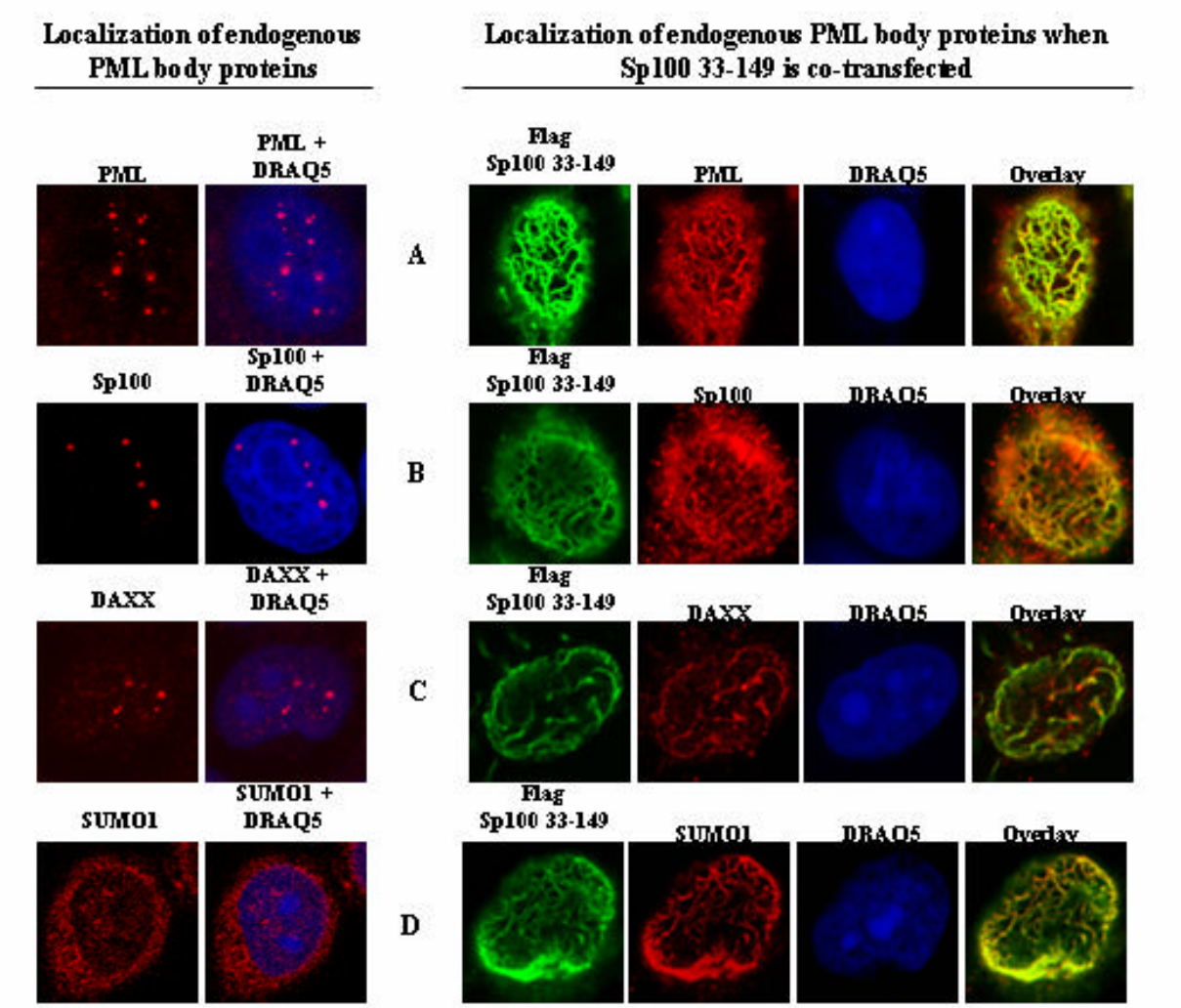

Figure 3.17 – The expression of Sp100 33-149 leads to the formation of filaments in HeLa cells and recruits/delocalizes other PML body proteins into these filaments. In this figure this effect is shown for endogenous PML, Sp100, DAXX and SUMO1. HeLa cells were transfected with Flag-tagged Sp100 33-149 and fixed 24 hours later. Indirect immunofluorescence experiments were performed and Flag-Sp100 33-149 was stained with an anti-Flag antibody, Sp100 with an antibody against its C-terminus, PML with an antibody against its N-terminus, DAXX with an antibody against its C-terminus and SUMO1 with an antibody against the full-length protein. DNA was stained using DRAQ5.

3.8 - Recruitment of endogenous PML and Sp100 by other Sp100 truncated forms that localize in filaments

The other three truncated forms of Sp100 that also localize in filaments (Sp100 33-182, Sp100 1-149 and Sp100 9-149) were also investigated for the recruitment of the two major PML body constituents (Sp100 and PML). Not surprisingly all of them were able to recruit endogenous Sp100 in filaments (Figure 3.18). All these truncated forms share with the previously shown Sp100 33-149 the entire HSR domain of Sp100 making the formation of dimers between the endogenous Sp100 and the truncated form possible.

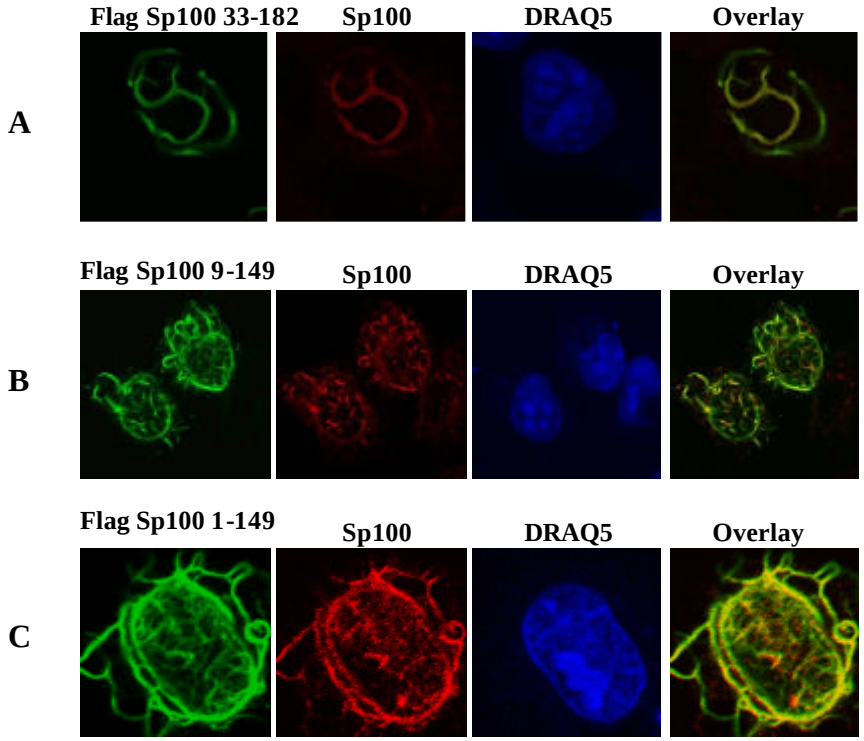

Figure 3.18 – Recruitment of endogenous Sp100 in by the truncated forms of Sp100 33-182, Sp100 9-149 and Sp100 1-149. HeLa cells were transfected with plasmids expressing the truncated forms of Sp100: Flag-Sp100 1-149, Flag-Sp100 9-149 and Flag-Sp100 33-182 - and fixed 24 hours later. Indirect immunofluorescence microscopy experiments were performed and the truncated forms were stained with anti-Flag antibody. Endogenous Sp100 was stained with DF - a rabbit polyclonal antibody against its C-terminus which does not recognize the Sp100 truncated forms. DNA was stained using DRAQ5.

Concerning endogenous PML, the type of recruitment was not the same for all truncated forms of Sp100. The two truncated forms extending from the Sp100 33-149 towards the N-terminus of Sp100 (Sp100 1-149 and Sp100 9-149) were also able to recruit PML into filaments, in a manner already seen for Sp100 33-149, where PML was evenly distributed along the filaments and PML bodies were disrupted (Figure 3.19 – B and C). The truncated form of Sp100 33-182 did not disrupt PML bodies, but recruited the PML bodies into filaments in a way that the PML was seen in dots which are changed in their distribution and are aligned along the filaments like "beads on a string" (Figure 3.19-A).

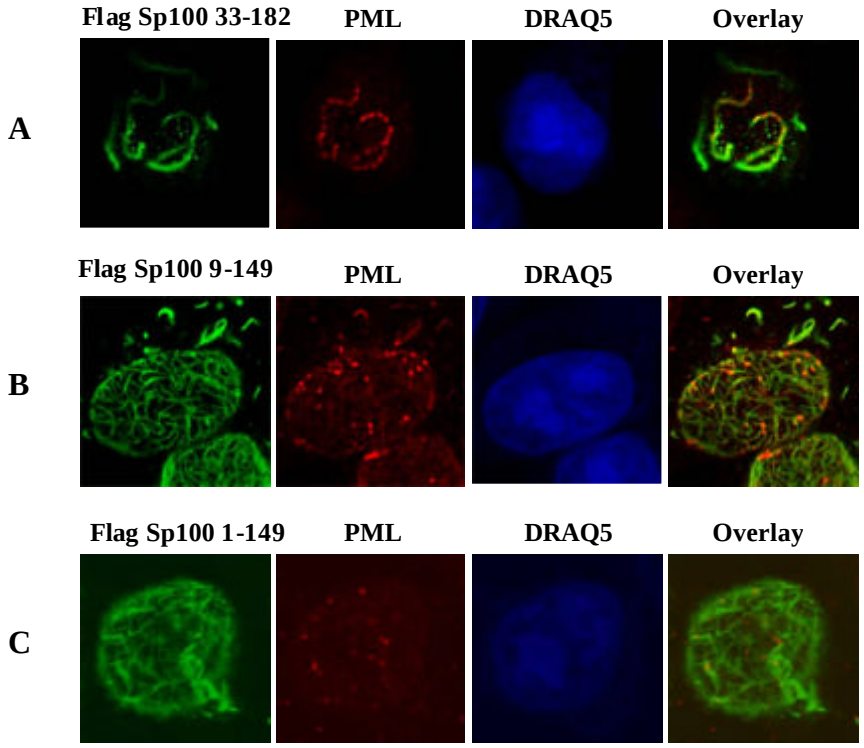

Figure 3.19 – Recruitment of endogenous PML in HeLa cells by the truncated forms of Sp100 33-182, Sp100 9-149 and Sp100 1-149. HeLa cells were transfected with plasmids expressing the truncated forms of Sp100: Flag-Sp100 1-149 (C), Flag-Sp100 9-149 (B) and Flag-Sp100 33-182 (A) and fixed 24 hours later. Indirect immunofluorescence microscopy experiments were performed and the truncated forms of Sp100 were detected using an anti-Flag antibody and endogenous PML with an antibody against its N-terminus. DNA was stained using DRAQ5.

3.9 - No recruitment of non-PML body proteins into filaments by Sp100 33-149

As all the proteins examined so far were recruited by the Sp100 33-149 into filaments and are normally localized in PML bodies it was relevant to investigate if this recruitment was specific for PML body components. To study this, several proteins or truncated forms of proteins that are normally localized in nuclear dots which are distinct from PML nuclear bodies were chosen to be examined for their localization in the presence of Sp100 33-149.

PSF (PTB associated splicing factor) is a 100 kD polypeptide that forms complexes with PTB although most of this protein is found attached to the nuclear matrix and not complexed with PTB. PSF is described to participate in the constitutive pre-mRNA splicing or to bind and retain defective RNAs in the nucleus (reviewed in Shav-Tal et al., 2002). Plasmids expressing GFP-PSF truncated forms were transfected into HeLa cells. These truncated forms localized in splicing speckles (Figure 3.20-A and C). When co-expressed with Sp100 33-149 they were not recruited into the filaments of Sp100 33-149 but remain in nuclear dots (Figure 3.20-B and D). This implies that the recruitment of PML nuclear body components by Sp100 33-149 is not an experimental artifact caused by overexpression of proteins but a process that remodels specifically the PML nuclear body.

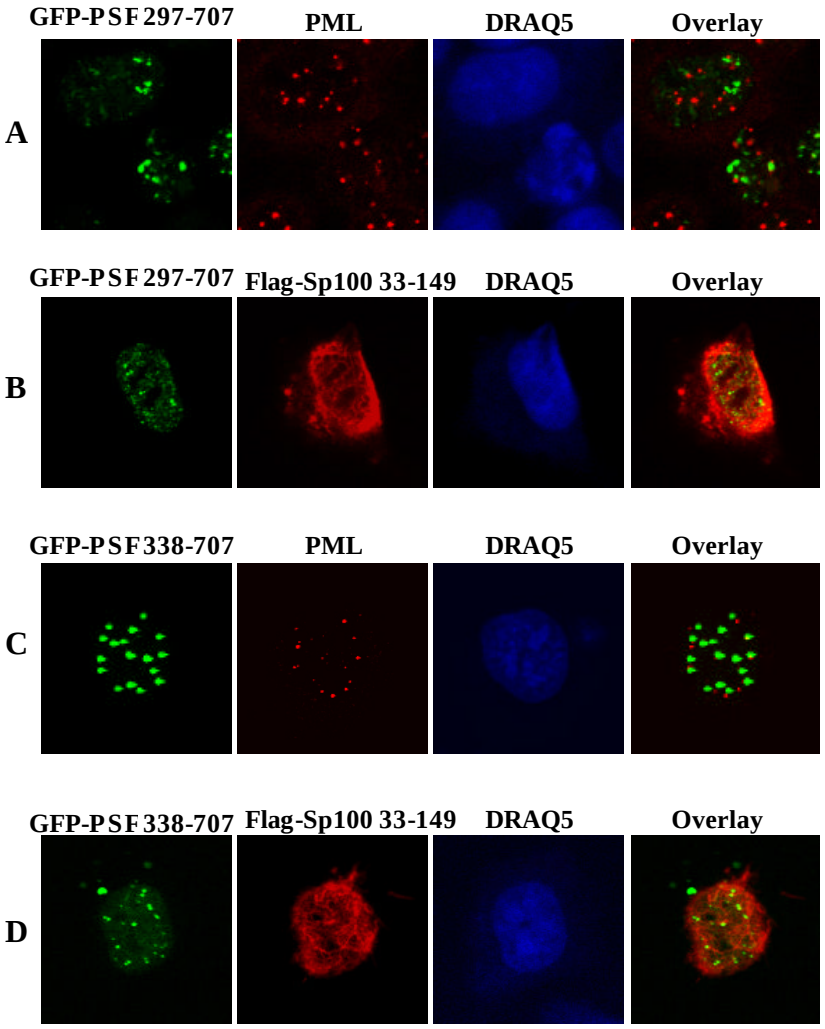

Figure 3.20 – No recruitment into filaments of GFP-PSF truncated forms by Sp100 33-149. HeLa cells were transfected with PSF truncated forms alone (A and C) or co-transfected with PSF truncated forms together with Flag-Sp100 33-149. 24 hours after transfection cells were fixed and the target proteins observed by indirect immunofluorescence (anti-Flag antibody against the Flag-Sp100 33-149 and a rabbit polyclonal against the N-terminus of PML) or by direct immunofluorescence in the case of GFP tagged PSF truncated forms. DNA was stained with DRAQ5.

The localization of GFP-OTT, another protein that localizes in nuclear dots that do not overlap with PML (Figure 3.21-A) (T. Heise, unpublished results) was also investigated by co-expressing it together with Sp100 33-149 in HeLa cells. OTT (One-Twenty-Two) is a protein related to the Drosophila split-end (spen) family of proteins (Mercher et al., 2001). OTT was found as a fusion protein with MAL (megakaryocytic acute leukemia protein) and

this fusion protein is exclusively associated with infant acute megakaryoblastic leukemia (Mercher et al., 2002).

Indirect immunofluorescence experiments performed in cells fixed 24 hours after transfection showed that the localization of GFP-OTT is not influenced after co-expression of Sp100 33-149 and that it remained in non-PML bodies (Figure 3.21-B).

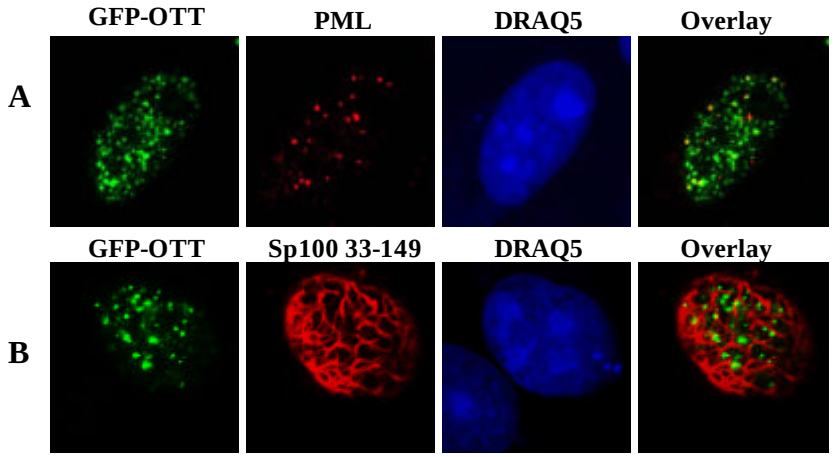

Figure 3.21 – No recruitment into filaments of GFP-OTT by Sp100 33-149. HeLa cells were transfected with GFP-OTT alone (A) or cotransfected with GFP-OTT and Flag-Sp100 33-149 (B). 24 hours after transfection cells were fixed and the target proteins analyzed by indirect immunofluorescence microscopy (Flag antibody against the Flag-Sp100 33-149 and a rabbit polyclonal against the N-terminus of PML) or by direct immunofluorescence microscopy in the case of GFP-OTT. DNA was stained with DRAQ5.

Other proteins that normally do not to a great extent localize to PML bodies (but which can be recruited into these bodies under certain circumstances, such as overexpression of PML-IV) were investigated. One of these proteins is p53 which was not recruited by Sp100 33-149 into filaments and its subcellular distribution seemed to be unaffected by the expression of this truncated form of Sp100 (Figure 3.22 A).

HIPK2 mainly localizes to HIPK2 bodies which do not contain PML but it can be recruited to PML bodies for example by PML isoform IV. When a GFP tagged form of HIPK2 was expressed in HeLa cells it showed partial colocalization with PML (Figure 3.22-B). When GFP-HIPK2 was coexpressed with Flag-Sp100 33-149 in HeLa cells, HIPK2 was still distributed in a nuclear dot fashion and was not recruited by Sp100 33-149 into filaments (Figure 3.22-C).

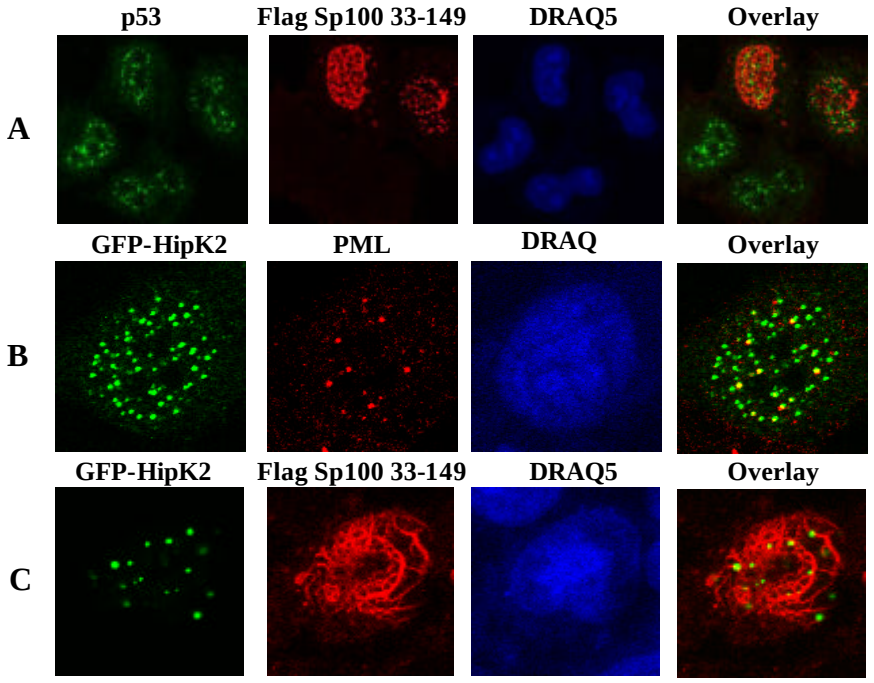

Figure 3.22 – No recruitment into filaments of endogenous p53 and GFP-HipK2 by Sp100 33-149. In HeLa cells Flag-Sp100 33-149 (A), GFP-HipK2 (B) and GFP-HipK2 and Flag-Sp100 33-149 (C) were expressed by transfecting the corresponding plasmids. Cells were fixed and the target proteins analyzed by indirect immunofluorescence microscopy (Flag antibody against the Flag-Sp100 33-149, a rabbit polyclonal against the full length p53 and a rabbit polyclonal against the N-terminus of PML). DNA was stained with DRAQ5.

Finally the behavior of a nuclear diffuse protein in the presence of Sp100 33-149 filaments was investigated. The La protein is a highly abundant nuclear phosphoprotein known to associate with newly synthesized RNA polymerase III transcripts and to play a major role in the stabilization of newly synthesized small RNAs (reviewed in Wolin and Cedervall, 2002). The La protein (and the GFP tagged version of this protein) showed a nuclear diffuse pattern when expressed in HeLa cells (Figure 3.23-A). When GFP-La was coexpressed with Flag-Sp100 33-149 it was not detected in filaments but displayed its normal nuclear diffuse staining (Figure 3.23-B).

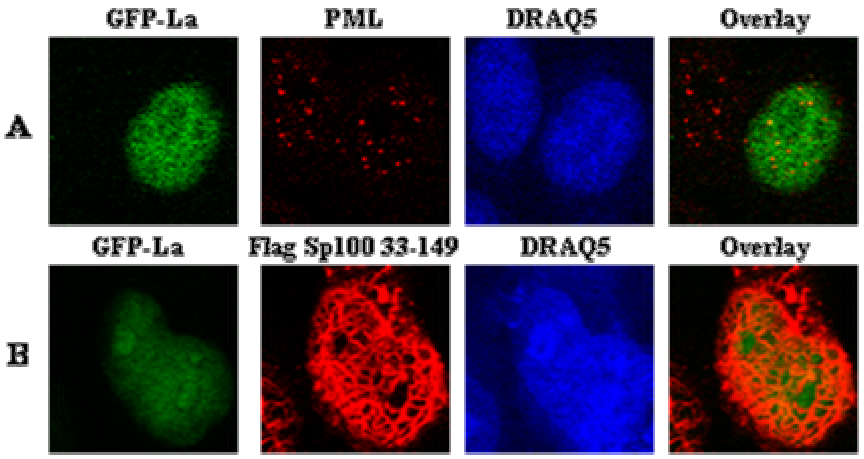

Figure 3.23 – No recruitment into filaments of GFP-La by Sp100 33-149. In HeLa cells GFP-La was expressed (A) and coexpressed with GFP-La and Flag-Sp100 33-149 (B). Cells were fixed and the target proteins analyzed by indirect immunofluorescence microscopy (Flag antibody against the Flag-Sp100 33-149 and a rabbit polyclonal against the N-terminus of PML). GFP-LA was detected by direct fluorescence microscopy. DNA was stained with DRAQ5.

Translation initiation factor eIF4E has been proposed to be the underlying structure of PML bodies (Borden, 2002). Therefore, it was important to examine the localization of this protein after cotransfection with a Flag-Sp100 33-149 expression vector. In contrast to a previous report (Cohen et al., 2001), in my studies eIF4E was never detected in PML bodies, although four different antibodies against this protein were tested, which all resulted in a diffuse eIF4E staining throughout the cell (data not shown).

In summary, Sp100 33-149 seems to recruit specifically PML body components, but not proteins which localize in other nuclear bodies (not containing PML/Sp100) or diffuse nuclear proteins.

To investigate the mechanism of recruitment of Sp100, PML, DAXX and SUMO1 into the filaments formed by Sp100 33-149 interaction studies were performed. For this purpose, coimmunoprecipitations were performed. Flag-Sp100 33-149 was coexpressed either with wild type Sp100, PML-IV or GFP-SUMO1. Flag tagged Sp100 33-149 was precipitated with an anti-Flag antibody and then the pelleted materials were probed for the presence of either wild type Sp100, PML or SUMO1. The results in Figure 3.24 show that Sp100 33-149 was coimmunoprecipitated with wild type Sp100 and also to some extent with SUMO1 but not PML-IV.

It has been described before that the HSR domain of Sp100 included in Sp100 33-149 is responsible for the oligomerization of Sp100. Oligomerization with itself or with wild type Sp100 is likely to be important for the formation of the filaments. For this reason, the coimmunoprecipitation with wild type Sp100 was expected, but the coimmunoprecipitation of GFP-SUMO1 was quite unexpected because this truncated form of Sp100 does not possess the SUMOlation site of the full-length Sp100.

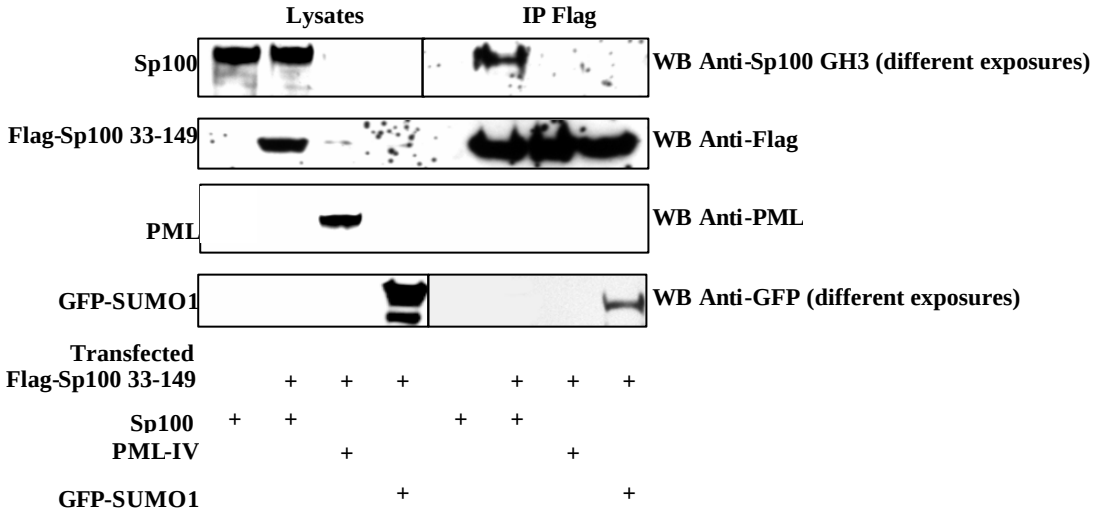

Figure 3.24 – Flag-Sp100 33-149 interacts with wild type Sp100 and GFP-SUMO1 but not with PML IV. In 293T cells Sp100 alone was expressed (as a negative control) and Flag-Sp100 33-149 was coexpressed with either Sp100, PML-IV or GFP-SUMO1. Cells were harvested after 24 hours and coimmunoprecipitations were performed using an anti-Flag antibody coupled to protein G beads. Lysates (Input – 10%) and immunopelleted material were analyzed by immunoblotting. Sp100 was detected by using an antibody against its C-terminus (GH3), Flag-Sp100 33-149 by using an anti-Flag antibody, PML with an antibody against its N-terminus and GFP-SUMO1 by using an antibody against GFP. Where indicated, different exposure times for lanes loaded with lysates and immunopellets are shown for the same blot.

3.10 - The role of PML for formation of Sp100 33-149 filaments

It is believed that PML is essential for the formation of PML bodies. In the absence of PML in PML -/- mouse embryonic fibroblasts, PML body components show an aberrant localization.

PML is an essential protein for the formation of PML bodies and for the localization of other proteins to PML bodies. PML-IV was shown to recruit DAXX, CBP and HIPK2 into PML bodies whereas PML-III does not recruit HIPK2 into PML bodies. After overexpression of PML isoforms III and IV increased amounts of PML in PML bodies and an increase in the number of PML bodies were observed as expected (Figure 3.25- A and C and data not shown).

The question was whether overexpression of PML would restore the PML body in cells expressing Sp100 33-149 filaments. The results show that the coexpression of PML isoforms III and IV with Sp100 33-149 did not disrupt the filament formation and that the overexpressed PML isoforms were also recruited into the Sp100 33-149 filaments (Figure 3.25 – B and D) implying that PML does not influence filament formation by Sp100 33-149.

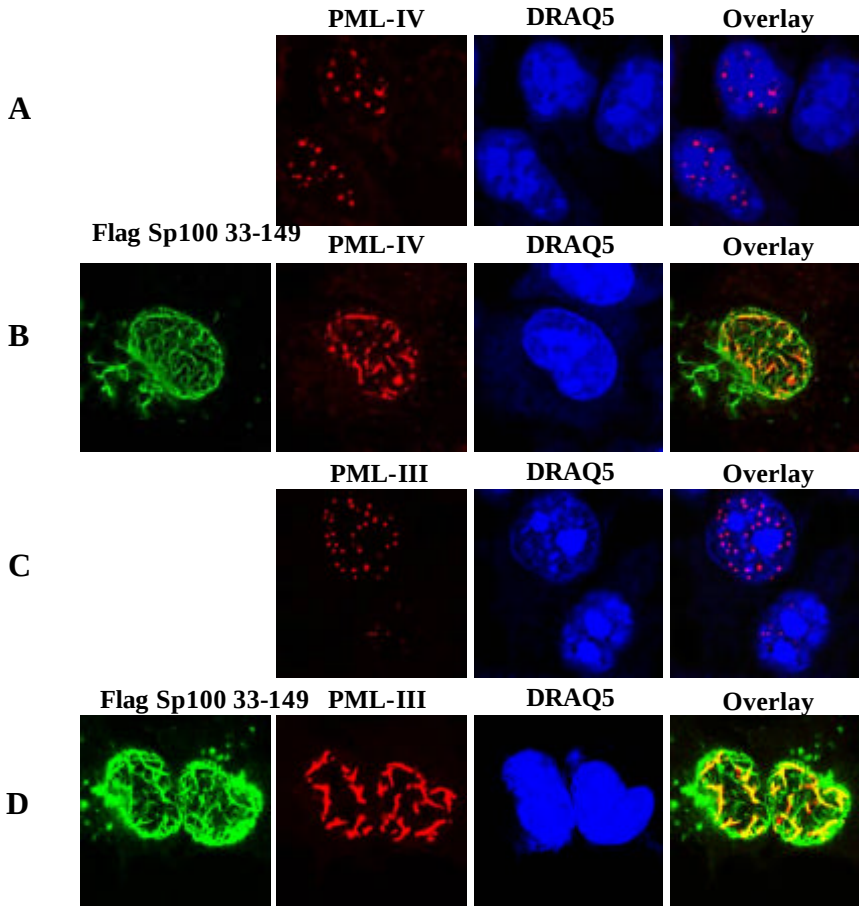

Figure 3.25 – Overexpression of PML isoforms does not inhibit the filament formation by Sp100 33-149. HeLa cells were transfected with PML isoforms IV and III (A and C, respectively) and their localization was investigated by indirect immunofluorescence microscopy in fixed cells, showing an enrichment of PML in PML bodies and in the number of PML bodies. Coexpression of Flag-Sp100 33-149 with PML isoforms IV and III (B and D, respectively) does not disrupt filament formation by Sp100 33-149 but shows a larger amount of PML in the filaments where Sp100 33-149 localizes.

To examine whether filaments formed by Sp100 33-149 depend on PML because delocalization of PML into filaments may be the mechanism how other PML body components (DAXX, CBP, SUMO1) are localized to filaments, PML -/- mouse embryonic fibroblast (Figure 3.26 - A) and mouse embryonic control fibroblasts expressing wild type PML (Figure 3.26 - B) were transfected with an expression vector for Sp100 33-149. Indirect immunofluorescence microscopy was performed and the results show that Sp100 33-149 localized to nuclear filaments in PML -/- and wild type cells, clearly indicating the

independence of filament formation for PML (Figure 3.26 - A). A semi-quantitative evaluation of the number of transfected cells indicated that the amount of cells that showed Sp100 33-149 filaments increased from the 70% observed in HeLa cells to about 90% in PML -/- and control mouse embryonic fibroblasts.

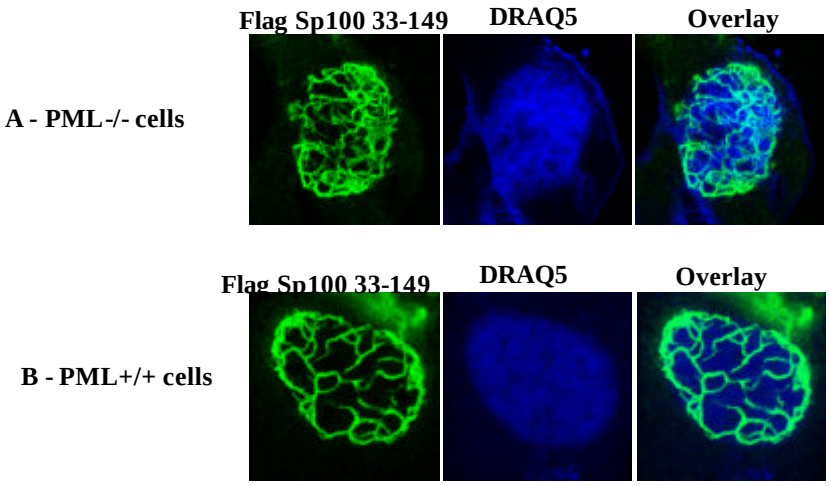

Figure 3.26 – PML is not needed for the formation of filaments by Sp100 33-149. In PML-/- cells as well as in PML +/+ control cells no difference was observed in the filament formation of transfected Flag-Sp100 33-149 by indirect immunofluorescence microscopy. Cells were fixed and then stained with anti-Flag antibody against Flag-Sp100 33-149. DNA was stained with DRAQ5.

3.11 - The role of SUMOlation in the formation of Sp100 33-149 filaments

Although Sp100 33-149 does not have a consensus SUMOlation site, it is still possible that it interacts with SUMO1 similarly as shown for HIPK2 eventually, through a SUMO1 interaction motif (SIM) (Engelhardt et al., 2003). This may cause a shortage of available SUMO1 in the cells and eventually even prevent PML from being SUMOlated. It is known that SUMOlation of PML is essential for targeting of PML to bodies and for recruitment of other PML body components into nuclear bodies (Ishov et al., 1999; Zhong et al., 2000).

The results presented in Figure 3.27 show Sp100 33-149, when co-expressed with GFP-SUMO1 (or GFP-SUMO1 and HA-Ubc9 which is an E2-conjugation enzyme in the SUMOlation machinery), is still capable of forming filaments. The results imply that the filament formation is not due to PML body disassembly which may have occurred because of low SUMO1 levels in the cells.

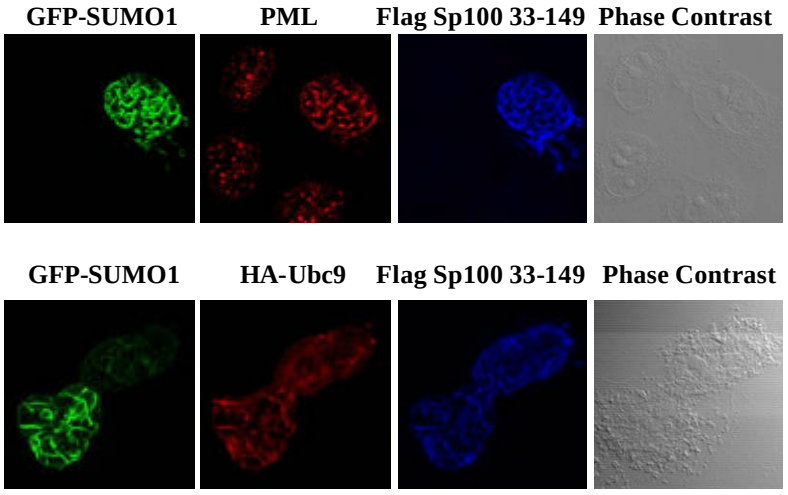

Figure 3.27 – Effect of SUMOlation on filament formation by Sp100 33-149. Indirect immunofluorescence microscopy was used to investigate the effect of overexpressed SUMO1 or SUMO1+Ubc9 on filament formation. HeLa cells were transfected with GFP-SUMO1 and Flag-Sp100 33-149 or with GFP-SUMO1, Flag-Sp100 33-149 and HA-Ubc9. Cells were fixed 24 hours after transfection and analyzed by indirect immunofluorescence microscopy. Flag-Sp100 33-149 was detected with an anti-Flag antibody, HA-Ubc9 with an anti-HA antibody, GFP-SUMO1 by direct fluorescence microscopy and PML with an antibody raised against its N-terminus and indirect immunofluorescence microscopy. DNA was stained with DRAQ5.

Sumo Protease 1 (SuPr1) removes SUMO1 from PML and this leads to the disassembly of PML bodies or to a reduced number of PML bodies when the protease is expressed for shorter periods (Best et al., 2002) (Figure 3.28-A). Therefore, the effect of the SUMO protease SuPr1 on the formation of Sp100 filaments was also investigated. When this protease was coexpressed with Sp100 33-149 the number of cells with filaments was diminished and an increase of cells with Sp100 33-149 in nuclear dot structures was observed (Figure 3.28-B).

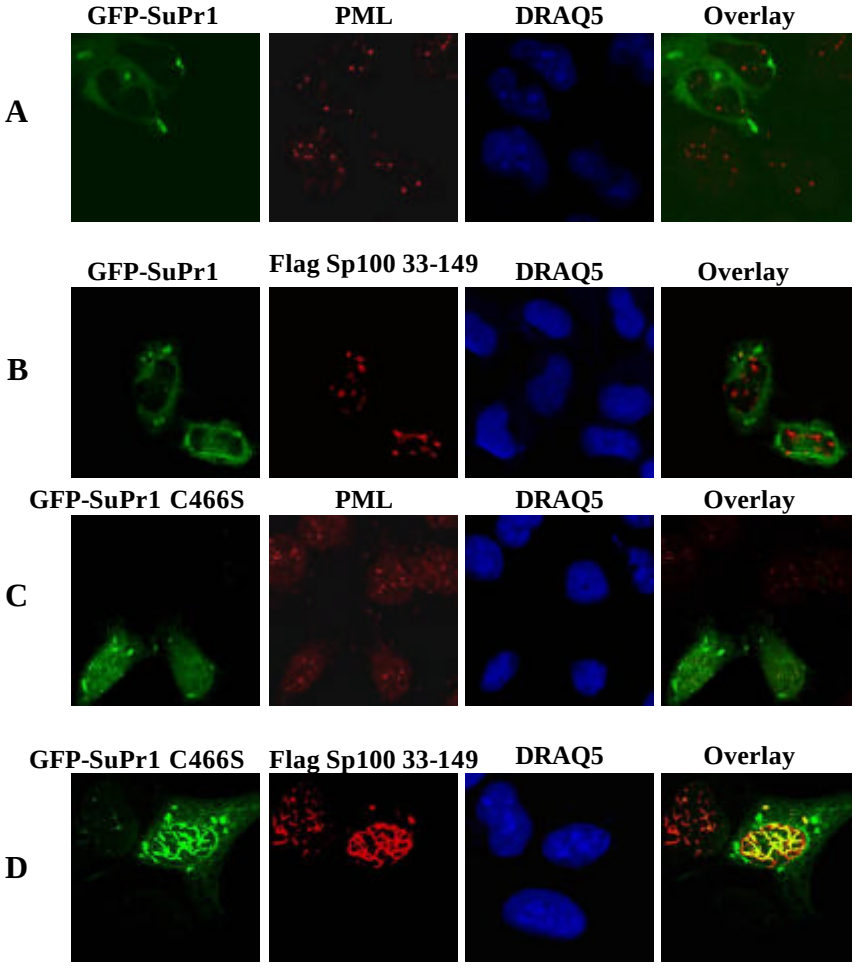

Figure 3.28 – Effect of SUMOlation on Sp100 33-149 filament formation. Indirect immunofluorescence microscopy was used to investigate the effect of overexpressed SUMO protease SuPr1 and its inactive form on the filament formation by Flag-Sp100 33-149. In HeLa cells GFP-SuPr1 was expressed (A), GFP-SuPr1 and Flag-Sp100 33-149 were coexpressed (B), GFP-SuPr1 C466S was expressed (C) and GFP-SuPr1 C466S and Flag-Sp100 33-149 were coexpressed (D). GFP-SuPr1 and GFP-SuPr1 C466S were detected by direct fluorescence microscopy, Flag-Sp100 33-149 with an anti-Flag antibody and PML with an antibody raised

against its N-terminus, then both visualized by indirect immunofluorescence microscopy. DNA was stained using DRAQ5.

When the catalytically inactive form of this protease was co-expressed with Sp100 33-149 the filaments were again the major structure observed (Figures 3.28-D and 3.29). This indicates that delocalization of Sp100 33-149 from filaments needs a catalytically active SuPr1.

It is s also worth mentioning that the cells were fixed 24 hours after transfection. This time point is shorter than the time point needed to disassemble PML bodies which was described to take about 36 hours (Best et al., 2002).

The number of cells in which Sp100 33-149 localized in dots, rods or filaments after transfection with the corresponding expression vector alone or when coexpressing GFP-SuPr1 or GFP-SuPr1-C466S was quantified (Figure 3.29). The results showed a strong reduction in Sp100 33-149 filament formation from 75% to 15% with SuPr1 expression but no significant change when catalytically inactive SuPr1-C466S was coexpressed.

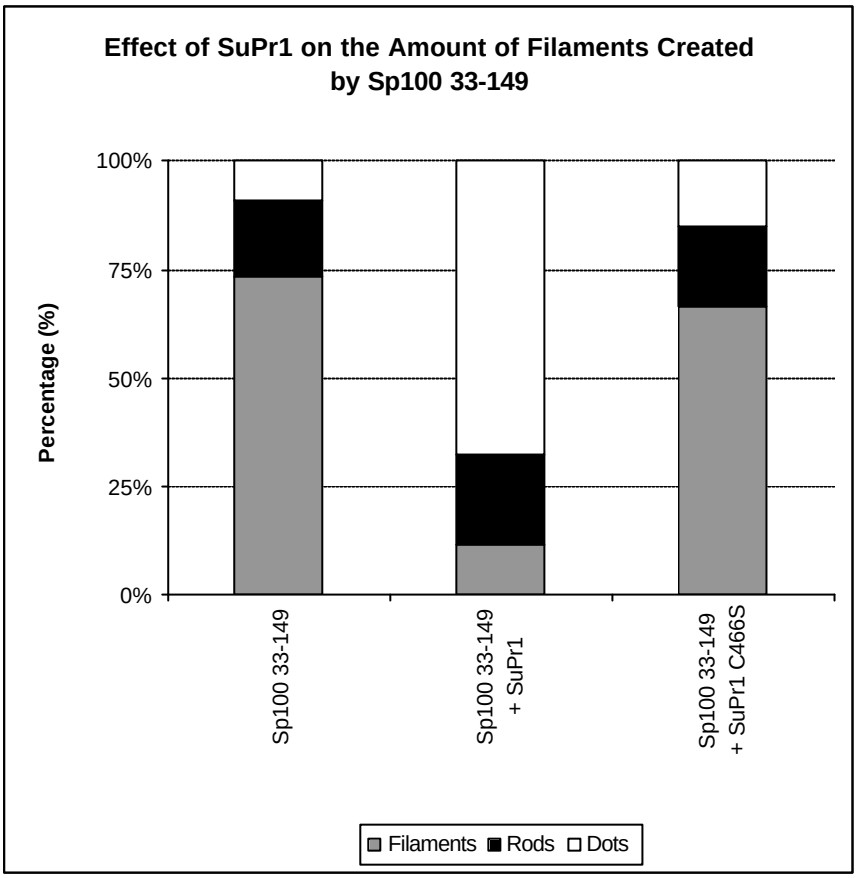

Figure 3.29 – Effect of SUMOlation on filament formation by Flag-Sp100 33-149. Quantification of cellular structures formed by Sp100 33-149 when expressed in HeLa cells with or without coexpression of GFP-SuPr1 and GFP-SuPr1 C466S. HeLa cells were transfected with Flag-Sp100 33-149 or cotransfected with GFP-SuPr1 or GFP-SuPr1 C466S and Flag-Sp100 33-149 and examined by indirect immunofluorescence microscopy. After staining this truncated form of Sp100 with an anti-Flag antibody, cells were examined by confocal microscopy and at least 100 cells were scored for the formation of filaments, rods and dots.

It was investigated whether this reduction in filament formation by SuPr1 was due to a down regulation of Flag-Sp100 33-149 protein levels. 293T cells were transfected with an Flag-Sp100 33-149 expression vector and cotransfected either with GFP-SuPr1 or its mutant GFP-SuPr1 C466S expression vectors. The immunoblots analyses showed (Figure 3.30) that when GFP-SuPr1 was present the protein level of Flag-Sp100 33-149 was slightly decreased but that when the catalytically inactive SuPr1 was expressed the Sp100 33-149 levels were increased. In all samples presented, GFP was coexpressed by cotransfection of a corresponding expression plasmid. The level of GFP was not changed by coexpression of

SuPr1 or its catalytically inactive mutant, demonstrating that the effect on Sp100 33-149 levels was specific.

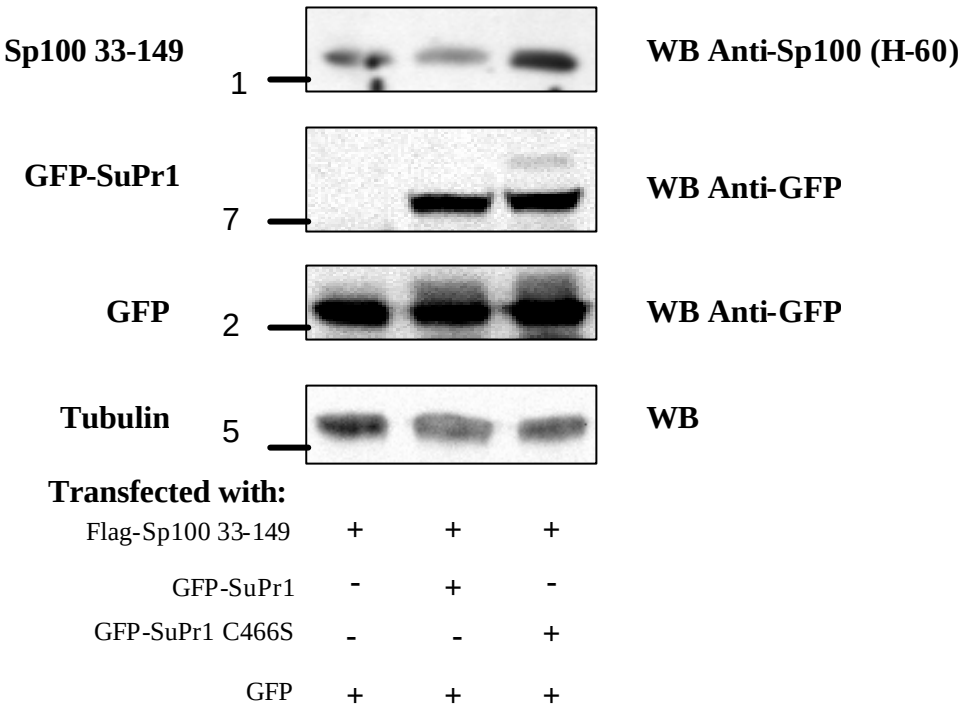

Figure 3.30 – Effect of SuPr1 on the protein levels of Flag-Sp100 33-149. 293T cells were transfected with a Flag-Sp100 33-149 expression vector alone or cotransfected with Flag-Sp100 33-149 and either with GFP-SuPr1 or GFP-SuPr1 C466S expression vectors. Flag-Sp100 33-149 was detected using an anti-Flag antibody. GFP, GFP-SuPr1 and GFP-SuPr1 C466S were detected using an antibody against GFP and tubulin with an anti-tubulin antibody.

The protein levels of Sp100 33-149 presented in the previous figure were quantified and corrected by the total amount of protein loaded by using the tubulin signals (Figure 3.31). The quantification showed a 25% decrease of Sp100 33-149 levels when SuPr1 was expressed and an increase of 80% when the catalytically inactive form of SuPr1 was expressed. The levels of both SuPr1 and SuPr1-C466S were also measured and showed no significant difference.

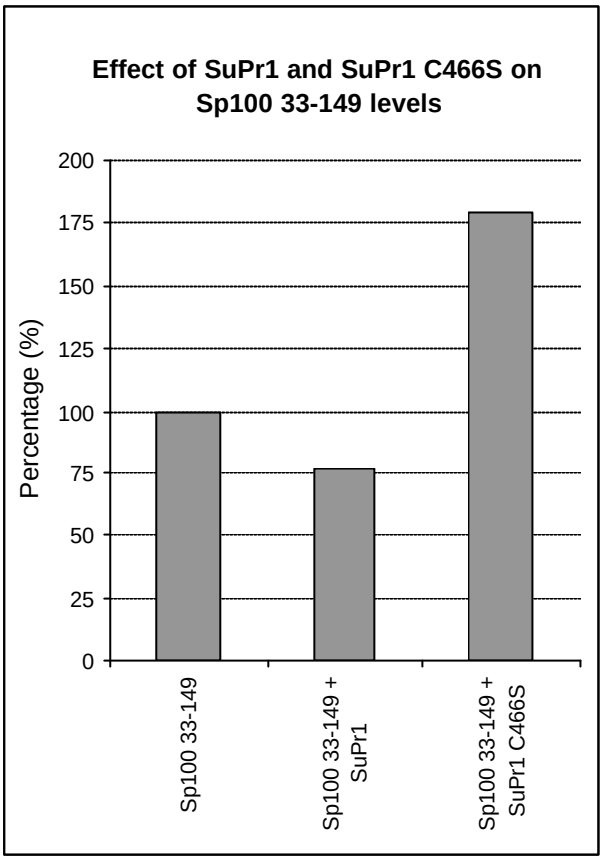

Figure 3.31 – Quantification of the effect of SuPr1 and SuPr1-C466S on the levels of Flag-Sp100 33-149. Tubulin levels were used to correct the levels of total amount of protein loaded. The levels of SuPr1 and SuPr1-C466S were also measured and showed no significant difference.

To investigate further how deSUMOlation may block Sp100 33-149 filament formation, it was tested whether Sp100 is deSUMOlated by SuPr1. Sp100 33-149 interacts with wild type Sp100 (Figure 3.24), but it is not known whether Sp100 is also deSUMOlated by SuPr1.

Analysis of immunoprecipitates and lysates revealed that Sp100 interacts with SuPr1 and its catalytically inactive form and is indeed deSUMOlated by cotransfection with SuPr1, but not by the mutant (Figure 3.32).

In contrast to the effect of SuPr1 on Sp100 33-149 protein levels, no changes in Sp100 protein levels were observed.

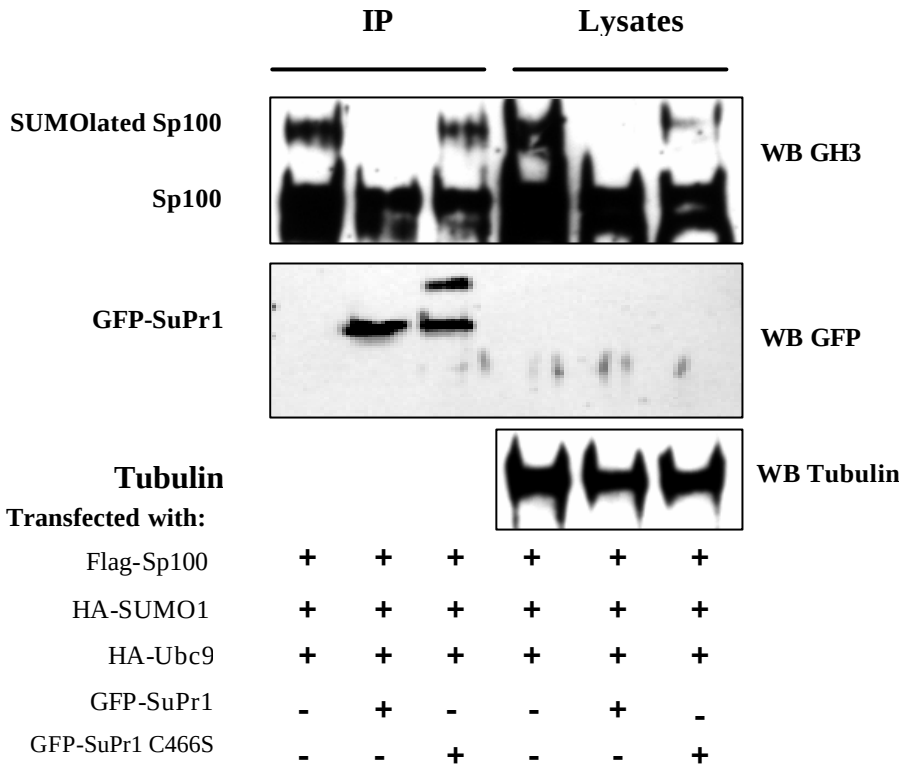

Figure 3.32 – DeSUMOlation of Sp100 by SuPr1. 293T cells were cotransfected with Flag-Sp100 33-149, HA-Ubc9, HA-SUMO1 either without SUMO protease or with GFP-SuPr1 or GFP-SuPr1 C466S expression plasmids. The cell lysates were immunoprecipitated using an anti-Flag antibody. Immunoprecipitates and cell lysates were analyzed by immunoblotting. Flag-Sp100 33-149 was detected by using an anti- Flag antibody, GFP-SuPr1 and GFP-SuPr1 C466S were detected by using an antibody against GFP and Tubulin with an antibody against full length tubulin.

3.12 – Inhibition of cell survival by Sp100 is conferred by its HSR domain

The effect of Sp100 on cell survival is not known to date. Using colony forming assays it was found that overexpression of Sp100 has a growth inhibitory effect on cells (data not shown). To investigate if a specific domain of Sp100 is responsible for this effect, colony forming assays were performed by using Sp100 deletion mutants. In these experiments HeLa cells were transfected with Sp100 or Sp100 deletion mutants and with a plasmid coding for puromycin resistance (all but the negative control cells) to allow the selection of transfected cells. Tranfected cells were selected with puromycin for 14 days and the number of surviving colonies determined.

As it can be seen in Figure 3.33-A, Sp100 33-149 inhibited colony formation (85% reduction) even more efficiently than wild type Sp100 (75% reduction). The construct expressing Sp100 208-480 did only show a modest reduction in colony growth (35% reduction) (Figure 3.33-A and B). Sp100 33-182 was as effective as wild type Sp100 in the reduction of colonies. Although the expression level of Sp100 33-182 was lower than that achieved with the other Sp100 constructs the total amount of protein loaded was also less in this case (Figure 3.33-C). In case of Sp100 208-408 the total amount of protein loaded was also less. However, because the protein levels were comparable with those of wild type Sp100 and Sp100 33-149, the results indicate that the C-terminus of Sp100 has no strong effect on colony growth (Figure 3.33-C).

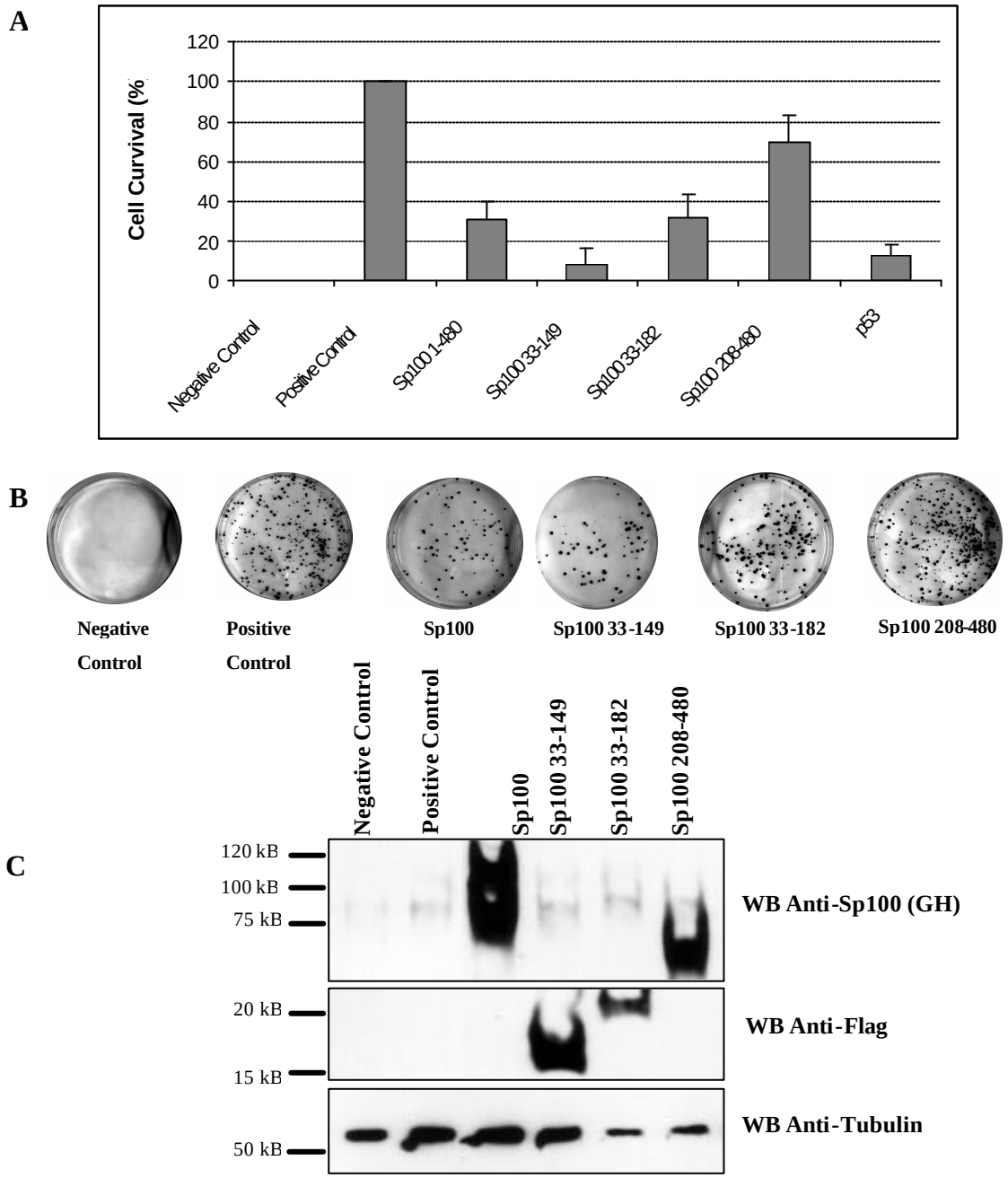

Figure 3.33 – The HSR domain of Sp100 is responsible for inhibition of colony growth by Sp100. Colony forming assays were performed. The cells were transfected with the indicated Sp100 constructs expression plasmids and a plasmid coding for puromycin resistance was cotransfected in all cases except for the negative control. The total amount of transfected DNA (3.6µg) was the same in all experiments. This was achieved by filling up the DNA with an empty vector when needed. The cells were selected with puromycin for 14 days and the number of surviving colonies was determined. The values shown in A are the average from 3 independent transfections. In A is shown the quantification and in B the plates of one representative experiment and in C the immunoblots of one representative experiment. Immunoblots were performed with anti-Flag antibody, a rabbit

polyclonal antibody against the C-terminus of Sp100 (GH) and a mouse monoclonal antibody against tubulin for normalization of total protein content.

To analyze in more detail why the several constructs of Sp100 induced different effects on colony survival, FACS analysis was performed. This should uncover potential changes in cell-cycle distribution of cells after overexpression of the corresponding Sp100 proteins. HeLa cells were transfected for 36 hours with several Sp100 constructs and p53 (as a positive control) and cotransfected with a GFP expression vectors to select transfected cells. For each sample, the DNA content/cell cycle distribution was determined for untransfected and transfected (GFP positive) cells. Transfection per se resulted in a small increase in apoptotic (subG1) cells and of cells in the G2/M phase (see vector transfected cells Figure 3.34-A)

The results obtained indicate a strong apoptotic effect (subG1) when cells were transfected with expression vectors to wild type Sp100, Sp100 33-149 and Sp100 33-182 (Figure 3.34 B-D). Especially in the case of Sp100 33-149 and maybe Sp100 33-182 there is an increase of cells in the G2/M phase above the level observed after transfection alone with empty vector. The expression of Sp100 C-terminal construct (Sp100 208-480) had no apoptotic effect (Figure 3.34-E). Cells were also transfected with p53 as a positive control for apoptosis/cell cycle arrest, however the effect was similar to vector transfected cells at the time point examined (Figure 3.34-F)

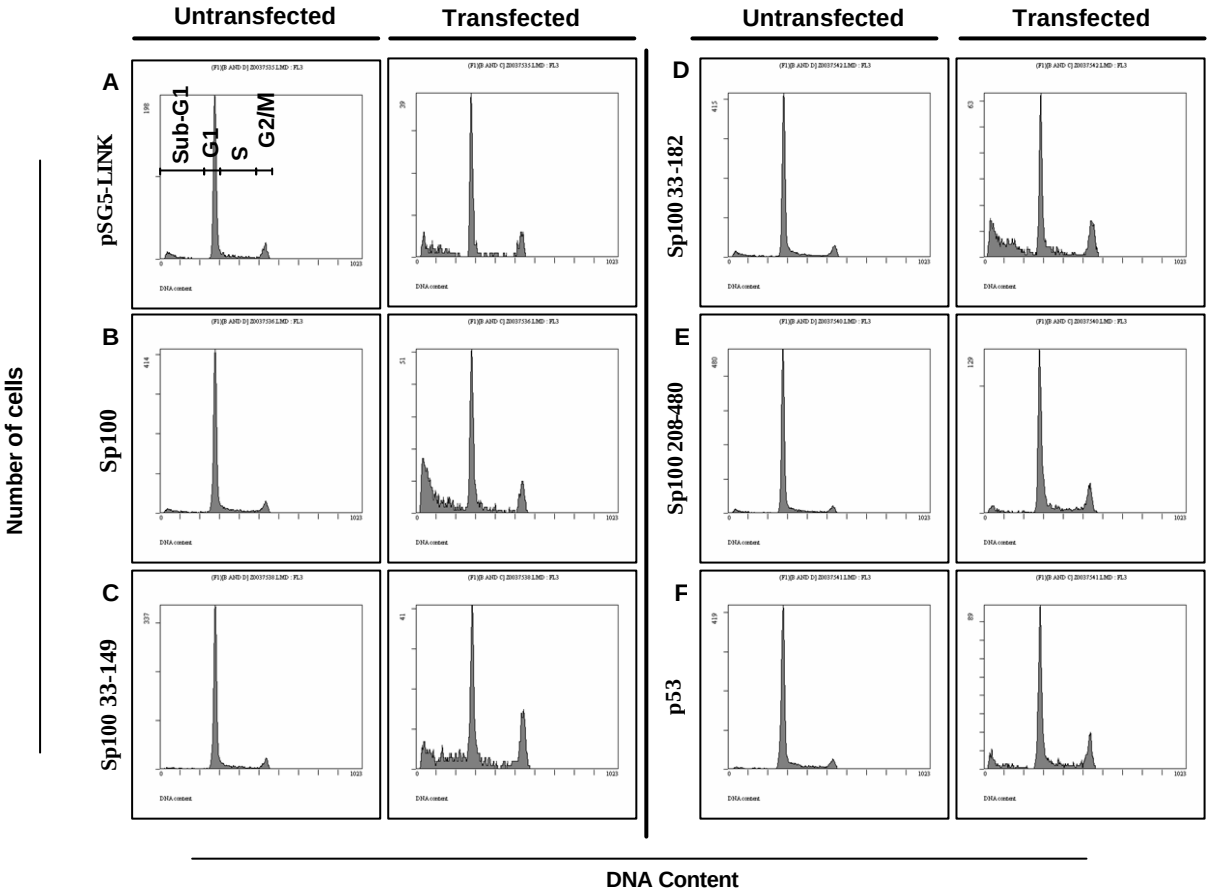

Figure 3.34 – FACS analysis testing the effect of wild type Sp100 and N-terminal polypeptides thereof on the cell cycle distribution. HeLa cells were transfected as described with the indicated constructs. After 36 hours the cells were fixed and DNA stained with PI. For each sample both the transfected (GFP positive) and untransfected cells were measured by FACS. The number of cells versus DNA content is shown.

Regarding a possible G2 cell cycle arrest, it was noticed that the filaments of Sp100 33-149 colocalized with what resembles the mitotic spindle in dividing cells (Figure 3.35). One other important information, although not quantified, was a large number of dividing cells observed by indirect immunofluorescence microscopy when Sp100 33-149 was expressed.

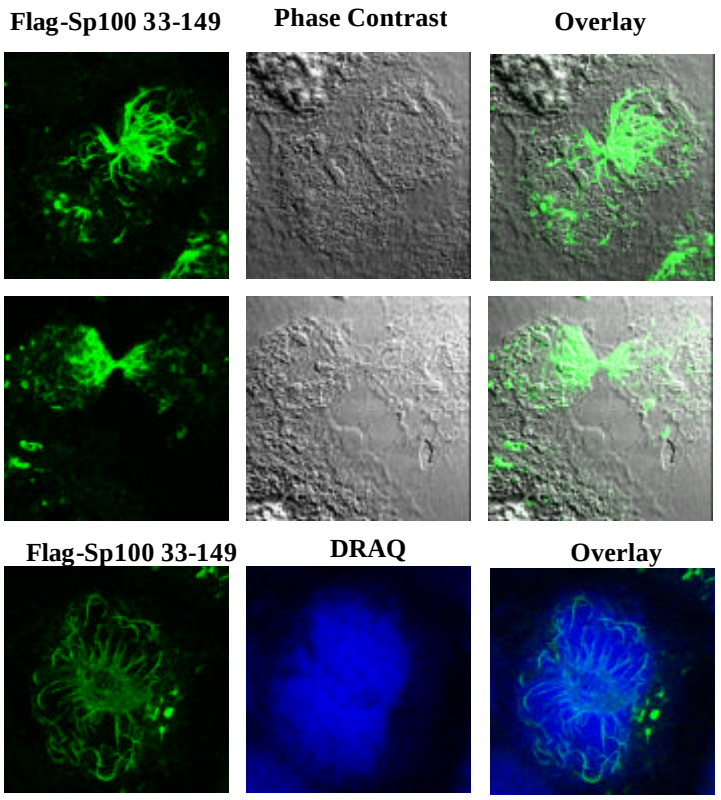

Figure 3.35 – Sp100 33-149 associate in mitotic cells with filaments that resemble the mitotic spindle. Sp100 33-149 was stained by using an anti-Flag antibody and analyzed by indirect immunofluorescence microscopy. DNA was stained with DRAQ5 and documented by direct immunofluorescence microscopy.

3.13 - Tracking a non-PML-body localization signal in the C-terminus of Sp100 splice variants

Sp100 is alternatively spliced in human cells. At least 4 alternative splice variants are known: Sp100A or wild type Sp100 (the major form of Sp100 – which was used so far), Sp100B, Sp100C and Sp100HMG. While Sp100C has only a few more amino acids at the C-terminus when compared to Sp100A, Sp100B and Sp100HMG have C-terminal extensions that contain additional domains. Sp100B and Sp100HMG have an HNPP (or SAND) domain which is known to bind DNA. Additionally, Sp100 HMG has an HMG domain which was suggested to bind DNA (Figure 3.36)

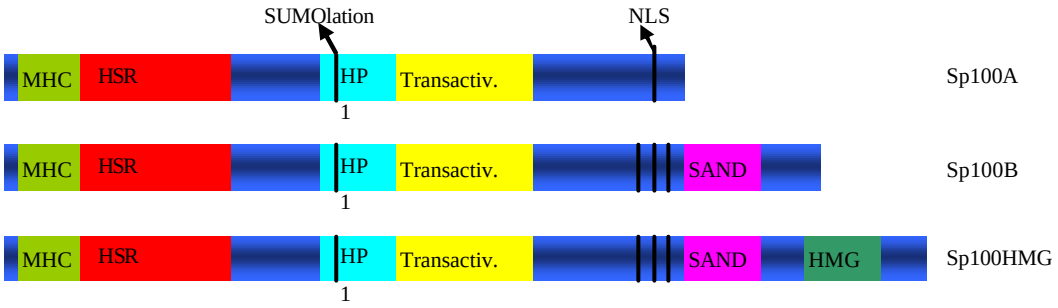

Figure 3.36 – Scheme of Sp100 splice variants domains

Curiously, Sp100 splice variants Sp100B and Sp100HMG colocalize only partially with PML in PML nuclear bodies, but localize also to nuclear dots which lack PML (so called alternative nuclear dots). This localization to alternative dots is dependent on the cell type examined (Guldner et al, 1999 and Figure 3.37). In HeLa cells 70-100% of the splice variants Sp100B and Sp100HMG colocalize with PML while in U2OS cells the colocalization is between 0-30% (Guldner et al, 1999).

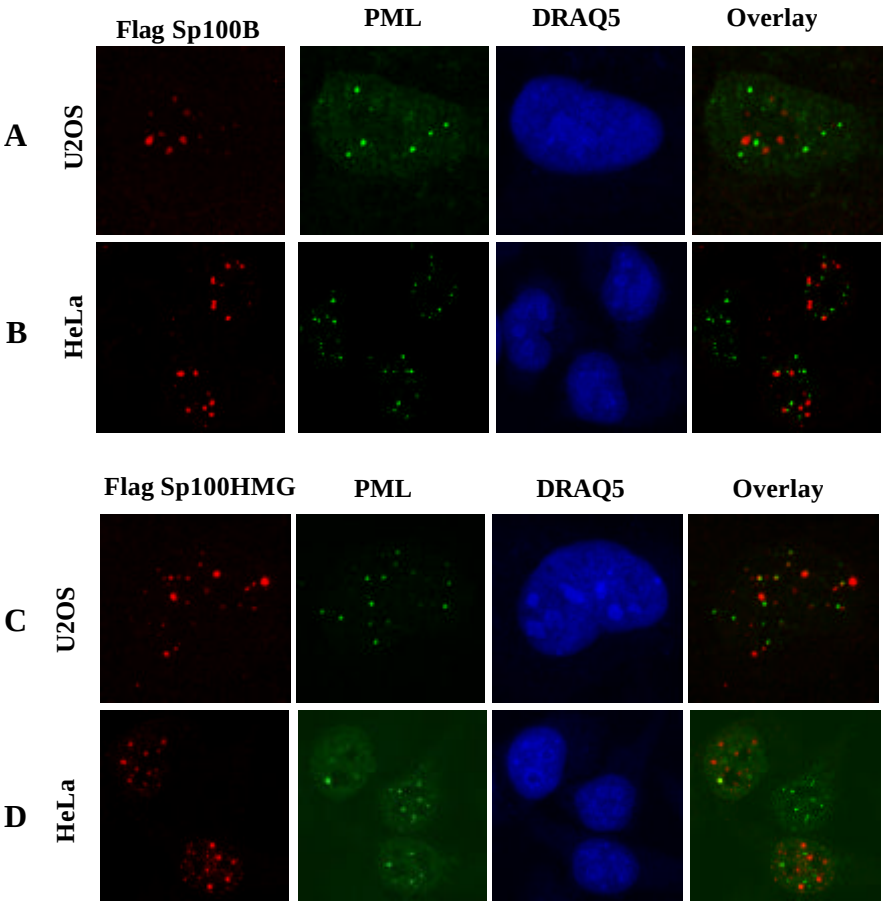

Figure 3.37 – Localization of Flag-Sp100B and Flag-Sp100HMG in U2OS and HeLa cells. Cells were transfected with Flag-Sp100B (A+B) or Flag-Sp100HMG (C+D) expression vectors. Cells were fixed and Flag-Sp100B and Flag-Sp100HMG were detected by with an anti-Flag antibody and PML by using a rabbit monoclonal against its N-terminus, and then detected by indirect immunofluorescence microscopy. DNA was stained with DRAQ5 and analyzed by direct fluorescence microscopy.

Several Flag-tagged constructs of Sp100HMG were cloned (Figure 3.38) to determine whether there exists a signal in the C-terminal part of the splice variants which directs these to alternative nuclear dots. These constructs were cloned by adding in to each new construct a new domain of the Sp100HMG splice variant starting from the C-terminus (Figure 3.38)

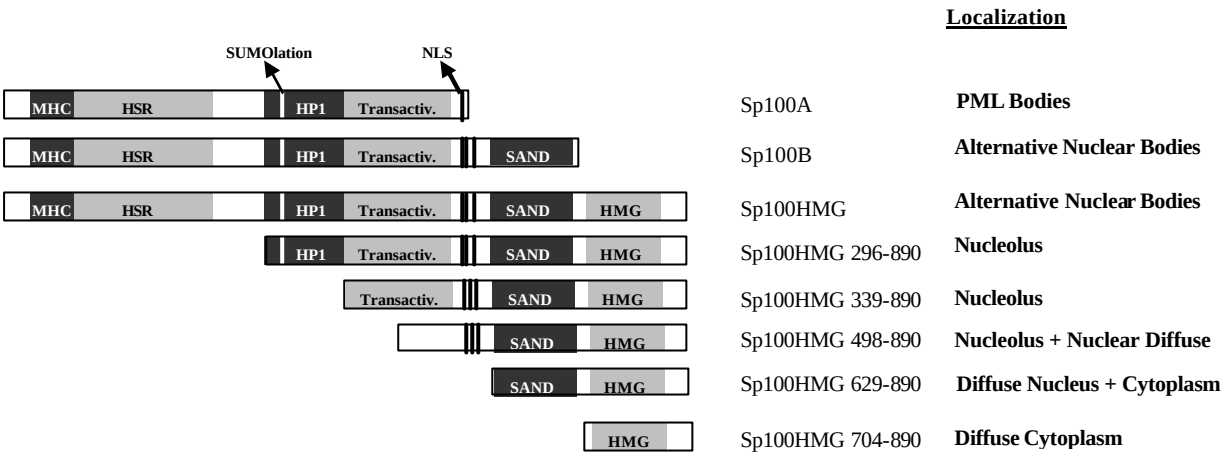

Figure 3.38 - Domains of Sp100A, Sp100HMG and schematic representation of the several truncated forms of Sp100HMG used in these studies as well as their subcellular localization as determined by indirect immunofluorescence in HeLa cells.

The constructs were expressed in HeLa cells and their localization was examined by indirect immunofluorescence. Sp100HMG 704-890, a construct that expressed mainly the HMG domain specific for the Sp100HMG splice variant and this protein showed a diffuse cytoplasmatic staining although it contained an external NLS. The Sp100HMG 629-890 possessed in addition to the HMG domain also the SAND domain, a domain common to both Sp100B and Sp100HMG splice variants but not found in Sp100A. This protein when expressed showed a diffuse staining throughout the cell. Neither of both proteins localized in PML bodies.

Sp100HMG 498-890 contain already a part in common with the Sp100A protein including the NLS in addition to the splice variants specific SAND and HMG domains. This protein localized in a nuclear diffuse pattern and unexpectedly also in the nucleolus. A nucleolar staining was also observed for the proteins Sp100HMG 339-890 and Sp100HMG 296-890 which contained either the transactivation domain of Sp100 (Sp100HMG 339-890) or in addition the HP1 binding site and SUMOlation site (Sp100HMG 296-890). Again, neither of these proteins showed a nuclear body localization.

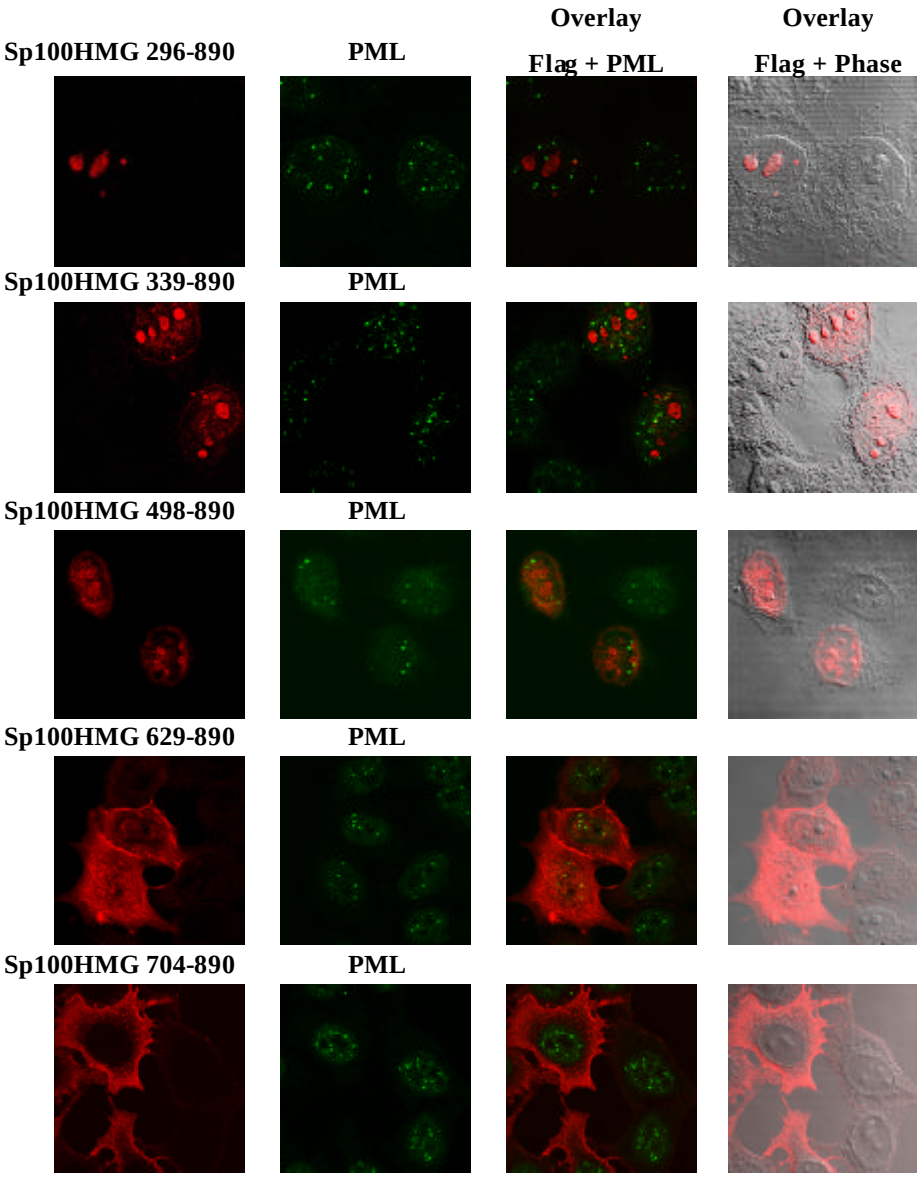

Figure 3.39 – Localization of Flag-Sp100HMG truncated forms in HeLa cells. Cells were transfected with expression vectors for Flag-Sp100HMG truncated forms as indicated. Cells were fixed and stained for Flag-Sp100HMG truncated forms with an anti-Flag antibody and detected by indirect immunofluorescence microscopy. DNA was stained using DRAQ5 and visualized directly by fluorescence microscopy.

4. Discussion

4.1 – Characterization of the HSR Domain of Sp100

One of the original objectives of this thesis was the creation of a dominant negative mutant of Sp100 which could potentially help to decipher better its little known functions. The HSR domain which is common to Sp100, its splice variants and related proteins, was the logical choice for the creation of this dominant negative mutant because the HSR domain is known to mediate homo-oligomerization of Sp100. When the isolated HSR domain of Sp100 is expressed it should bind to the wild type Sp100 and may thereby inhibit its normal function. It was also of general interest to study the Sp100 HSR domain in more detail as it is essential not only for the Sp100 homo-oligomerization but also for targeting of Sp100 to PML nuclear bodies (Sternsdorf et al, 1999; Negorev and Maul, 2001). Moreover, the HSR domain was determined to be the most conserved region of Sp100 when Sp100 protein sequences from different species were aligned at their amino acid level. Homologues of human Sp100 protein sequences are known for mouse (Mus caroli and Mus musculus) (Strausberg et al., 2002; Weichenhan et al., 1997) and rat (Rattus norvegicus) (Gene Bank Accession Number: XP_217470). When the full-length sequences from these species were aligned with the full length human Sp100 protein sequence, a similarity of 22% with the rat and of 28% to the mouse sequence was evident. However, when only the HSR domain was aligned this similarity increased to 44% for the rat and to around 55% for the mouse species Sp100 HSR domain.

When a construct with a Flag-tagged HSR domain of Sp100 (33-149) and containing an external NLS was expressed in HeLa cells the protein in the majority of the cells was predominantly in filamentous structures and located mainly in the nucleus of the cells. In some transfected cells small rods or dots were also observed but the percentage of cells that showed these structures was very low when compared to the percentage of cells presenting filaments.

This localization in filaments by the HSR domain of Sp100 has not been described before although it has been published before that a N-terminal GFP-tagged Sp100 protein (29-159) forms elongated rods and leads to the redistribution of PML body proteins. The authors explained this by the overexpression and self-aggregation of this peptide (Negorev and Maul,

2001). The construct used in this study (29-159) is almost identical to the construct used here (33-149). The discrepancy that only elongated rods but no extensive filaments were observed may be due to the use of GFP fusion proteins in the previous study in contrast to Flag-tagged proteins used here.

To verify that the localization in filamentous structures was not only due to overexpression of the HSR domain, different amounts of the corresponding plasmid were transfected. The filaments were always the most common structure observed and the percentage of transfected cells showing filaments was never below 60%. Although some plasmid dose dependency on filament formation was observed, this was of questionable significantly, as for instance with 20 times less plasmid transfected the percentage of cells showing filaments decreased only by 26%.

The localization of the Sp100 HSR domain in filamentous structures did not depend on the cell type used. This localization in filaments was even observed when the human HSR domain was expressed in cells of a different species, such as mouse cells, implying that the mechanism for the filament formation is conserved also in these rodent cells where only mouse homologues of human PML body proteins are present.

The wild type AIRE protein, as well as truncated forms of the AIRE protein, all of which contain the HSR domain, were reported to form filaments in the cytoplasm (Rinderle et al., 1999). Truncated forms of AIRE are generated *in vivo* when the coding region of AIRE is mutated and were found in patients suffering from autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. It is possible that also in this case it is the HSR domain which is mediating the filament formation.

The AIRE filaments, as well as Sp100 filaments analyzed in this study, located both in the nucleus, but also at the nuclear membrane and showed extensions into the cytoplasm. They did not colocalize with any of the cytoskeleton proteins examined (actin, tubulin and vimentin). In notable contrast the cytoplasmatic filaments of the AIRE protein were colocalized with vimentin and microtubules (Rinderle et al., 1999) suggesting that additional sequences in AIRE that are not present in Sp100 HSR are required for the interaction with cytoskeleton structures.

It was also important to exclude the possibility that the Sp100 filaments are only accumulations of the expressed protein in the freely accessible interchromatin space, as similar described for filaments formed upon oligomerization of NLS-tagged vimentin (Reichenzeller et al., 2000). DNA and RNA digestions showed that the Sp100 filaments are stable in the absence of RNA and DNA arguing against the hypothesis that the filaments formed by the HSR domain of Sp100 were only protein accumulations in the interchromatin space. The results may suggest that these filaments, similar as the PML bodies themselves, are tightly attached to the nuclear matrix. This interpretation is further supported by the observation that the solubility of Sp100 HSR was comparable low as that of wild type Sp100 in protein extracts.

One cannot decide at this point whether these filaments are created by the Sp100 HSR domain alone, or whether these peptides are associated with an underlying filamentous structure that exists in cells. To clarify this, it should for example be tested whether a purified Sp100 HSR domain is still able to form filaments *in vitro* in the absence of other cellular proteins or whether expression of the HSR domain from a construct lacking an external NLS in the cytoplasm still results in filament formation or whether it need some underlying structure only found in the nucleus. Despite intense efforts, it could not be determined in this work whether eIF4E may be this underlying structure, as previously proposed (Borden 2000; reviewed in Borden, 2002; Strudwick and Borden, 2002) because (unlike published) neither our nor other laboratories could reproduce localization of eIF4E to PML bodies (data not shown).

Taken together the experiments of this work indicate that the localization of Sp100 HSR domain in filaments is neither due to overexpression, nor cell-type dependent, or caused by an accumulation of this truncated protein in the freely accessible interchromatin space, but it is an intrinsic property of this protein.

4.2 – Mapping of the Sp100 domain that forms filaments

Wild type Sp100 does not localize in filaments when overexpressed. In order to analyze which part of Sp100 may be responsible for filaments formation observed with the HSR domain, several Sp100 cDNA constructs were cloned and expressed in HeLa cells. The results indicate that the minimal part of Sp100 tested and found to be able to form filaments was the HSR domain. N-terminal extensions to the HSR domain did not prevent filament formation indicating that the MHC-like region of Sp100 or the N-terminus of Sp100 neither play a role in filament formation nor can prevent it. C-terminal longer extensions inhibited the formation of filaments.

However, when a short C-terminal extension to the HSR domain was expressed (Sp100 33-182) this still localized to filaments but these showed a different morphology and possibly possess the ability to remodel the chromatin based on the observation of DNA staining from immunofluorescence microscopy experiments. This C-terminal extension of the HSR domain did not possess any known Sp100 domain and ends before the known HP1 binding domain and SUMO-conjugating site. This data may suggest a possible interaction of Sp100 149-182 region with chromatin or with proteins that interact with it. Interestingly computer predictions showed a coiled coil structure in this region. Coiled coils are domains known for protein-protein interactions (Burkhard et al, 2001) which further supports this hypothesis.

A protein spanning from the N-terminus of Sp100 and extending further to amino acid 253 including the HP1 binding domain/SUMOlation site was also tested. This protein (1-253), as well as the slightly longer one reaching to amino acid 334 (containing in addition the transactivation domain), did not form filaments. The shorter one of the two proteins formed in some cells (<25%) small rods. This indicates that either the three-dimensional structure of Sp100 or some interaction that occurs between the amino acids 182 and 253 inhibits filament formation in the experimental conditions tested. The SUMOlation of Sp100 does not seem to be important for the inhibition of filament formation as the Sp100 1-253 polypeptide lacking the SUMOlation site did not form filaments.

When examining the secondary structure of the HSR domain (amino acids 35-145 – Figure 3.6) by computer analysis it was predicted to consist of several a-helices. A smaller

construct that lacks the first of these helices was cloned, sequenced and expressed, with or without addition of proteasomes inhibitors. Unfortunately, this protein could neither be detected by indirect immunofluorescence nor by immunoblotting for reasons unknown. It was therefore not possible to determine whether the complete HSR domain is required for filament formation. Further experiments are needed to demonstrate whether the same minimal HSR-domain is needed for filament formation and targeting of Sp100 to PML bodies, the latter shown previously (Negorev and Maul, 2001).

4.3 – Recruitment of other proteins by the HSR domain

Several viral proteins are able to modify PML bodies, either by destroying them or by changing their morphology (reviewed by Everett, 2001; Moller et al., 2003). One of these proteins is the E4Orf3 from adenovirus 5 (Carvalho et al., 1995; Doucas et al., 1996). This protein when expressed in human cells, leads to the formation of filaments/small tracks in the nucleus and recruits PML and CBP into these filaments/tracks (Wienzek and Dobbelstein, 2001). Localization studies showed that the filaments formed by E4Orf3 and the filaments formed by the HSR domain of Sp100 did not colocalize. Thus, HSR filaments are not identical to these viral protein filaments.

To test if the filaments formed by the HSR domain of Sp100 were also able to disrupt endogenous PML from PML bodies, indirect immunofluorescence experiments were performed and indeed PML was recruited into filaments formed by the HSR domain of Sp100. This was quite a surprise because until now no direct interaction between Sp100 and PML has been shown. This suggests that either a direct interaction between the HSR domain and PML exists or that both proteins interact with some unknown bridging protein.

Several other proteins that normally localize in PML bodies were also investigated for their localization after expression of the HSR domain of Sp100. Endogenous Sp100, SUMO1 and DAXX were all recruited into filaments by the HSR domain of Sp100. The presence of Sp100 in filaments is consistent with the fact that the HSR domain of Sp100 is also the domain responsible for the oligomerization of Sp100. The localization of SUMO1 in filaments could also be expected because wild type Sp100 and DAXX which were recruited into filaments can all be SUMO-modified although in this case it was not distinguished between free and bound forms of SUMO1. Concerning DAXX, unpublished yeast-two-hybrid data from our laboratory identified DAXX as a possible interactor with the N-terminal part of Sp100 although an interaction of both full-length proteins could not be shown in human cells under several conditions tested (E. Akgün, personal communications). DAXX is also known to to be recruited by PML into PML bodies and relocalization of DAXX with filaments may also be a secondary effect to the recruitment of PML.

The fact that PML was recruited by the HSR domain into filaments and that no interaction was found between both full-length proteins still left open the possibility that the

HSR domain of Sp100 can interact with PML. Coimmunoprecipitations experiments were performed but no interaction was found between the HSR domain of Sp100 and PML. The HSR domain of Sp100 did indeed show an interaction with the wild type Sp100 as expected and surprisingly a weak interaction also with SUMO1. Although Sp100 33-149 protein did not possess the known SUMOlation site of Sp100 or any other consensus site for SUMOlation, both proteins interacted. It is possible that the HSR domain of Sp100 contains a SUMO-interaction motif (SIM) as for HIPK2 a protein previously shown to be involved in PML body disruption (Engelhardt et al., 2003)

As PML is thought to be essential for PML body formation and for recruitment of other proteins into PML bodies, overexpression of the HSR domain was also tested in mouse fibroblast with a knock-out of PML. The results showed that the HSR domain of Sp100 was also able to localize in filaments in these cells which clearly demonstrates that the filament formation can occur independently of the PML protein. It was also investigated whether PML isoforms III and IV could rescue the PML body integrity or if they could inhibit the formation of HSR-filaments. The results obtained showed that coexpression of both PML isoforms could neither prevent the filament formation nor recruitment of PML into these filaments. Thus, PML does not seem to influence HSR filament formation. To establish whether recruitment of DAXX or other PML body components into HSR filaments is a secondary event to relocalization of PML, appropriate studies should be performed in PML -/- cells in future.

To further investigate whether the association of proteins with HSR-filaments was specific for PML body proteins the localization of other nuclear proteins was examined in the presence of the overexpressed HSR domain polypeptide.

First, it was examined whether proteins that are normally located in nuclear dot structures which are not PML bodies were recruited into HSR-filaments. Truncated forms of PSF (a protein involved in pre-mRNA splicing) were not delocalized into HSR filaments but remained located in splicing speckles. The same was true for OTT (a protein which when fused with MAL leads to leukemia), the HSR domain did not affect the distribution of OTT in non-PML nuclear bodies.

Most interestingly, proteins that can be recruited to PML bodies in response to certain cellular events, such as p53 or HIPK2, were also not recruited into HSR filaments. Similarly,

nuclear diffusely distributed proteins such as the autoantigen La were not detected in HSR filaments. All these results point to a specific relocalization of the HSR domain of Sp100 on the localization of proteins that are normally located in PML bodies (and the PML body itself), but not on other nuclear proteins or nuclear dot structures.

N-terminal extensions of the Sp100 HSR domain which formed filaments (Sp100 1-149 and Sp100 9-149) were also able to recruit endogenous PML and Sp100 into filaments. The C-terminal extension to the HSR domain (Sp100 33-182) was able to recruit endogenous Sp100 into filaments but did not have the same effect on PML. This truncated version of Sp100 did not change the localization of PML into filaments but the PML remained in bodies which were however changed in their normal distribution and that were aligned along the filaments.

The observation that overexpression of the Sp100 HSR domain led to a redistribution of PML body components into filaments raises the possibility that wild type Sp100 may have a function in PML body formation which is disturbed by the binding of the HSR domain.

To prove a structural function of Sp100 in PML body integrity, the formation of PML bodies should be investigated in the absence of Sp100. To date only one study described normal PML body morphology in NT2 cells which do not show detectable levels of Sp100 (Negorev and Maul, 2001). However, a single cell clone was selected for these studies and the results should be confirmed in other cell types. As there are no Sp100 knock-out cells available to date, another strategy may be the use of siRNA to downregulate Sp100 expression. In studies in our laboratory so far only a partial downregulation was achieved of Sp100 protein expression with this method. This did not result in any obvious changes in PML distribution (H. Staege, personal communications).

Regarding the potential function of Sp100 HSR as a dominant-negative mutant, it seems likely that the dramatic changes in PML body structure, observed is also due or exclusively to the redistribution of all other PML body components, not just of Sp100. On the other hand the HSR polypeptide may be useful as a tool to investigate whether the dot-structures of PML bodies is required for the normal functions of its components. In this regard it is of note that the effects observed by genetic knock-out PML expression are also not likely to only reflect the loss of PML protein functions but are also due to altered functions of other

PML body components which no longer localize in PML bodies in these PML knock-out cells.

4.4 – The effect of SUMOlation/deSUMOlation on the filament formation and on Sp100

SUMOlation of PML is essential for formation of PML bodies (Müller et al., 1998). Interestingly, the HSR domain of Sp100, which does not possess a SUMOlation site, interacted with SUMO1 and this potentially could have sequestered SUMO1 to the HSR filaments implying reduced amounts of SUMO1 available to SUMOlate PML. This in turn could have inhibited or interfered with PML body formation.

The hypothesis was addressed by coexpression of the HSR domain of Sp100 with SUMO1. No change in the filament formation was observed and even when SUMO1 and Ubc9 (the E2 activating enzyme for SUMOlation) were coexpressed the PML body structure was not rescued and PML remained in the HSR-filaments. What is more, both SUMO1 and Ubc9 also localized to Sp100 HSR filaments. Therefore, the rearrangement of PML bodies into filaments by the HSR domain is not the result of a shortage in SUMO1 and the conjugating machinery.

Unexpected was the effect of the SUMO protease SuPr1 on filament formation. SuPr1 is known to disrupt PML bodies by removing SUMO1 from PML (Best et al., 2002). When SuPr1 was coexpressed with the HSR domain of Sp100 the amount of cells that showed filaments dropped from 75% to 25% concomitant with an increase of cells that showed the HSR domain of Sp100 in dots. When catalytically inactive mutant of SuPr1 was expressed no changes were observed in the relative percentages of cells that showed filaments, tracks or dots when compared to expression of the HSR domain alone. This suggests that some unknown interaction partner required for the HSR-filament formation is SUMOlated.

SuPr1 is known to deSUMOlate PML and was shown to lead to the disassembly of PML nuclear bodies (Best et al., 2002). However it was not known whether Sp100 is also deSUMOlated by this enzyme. And indeed, SuPr1 was also found to be able to deSUMOlate Sp100. When SuPr1 was expressed together with Sp100 no SUMOlated band was observed in the immunoblot experiments, in contrasts to the situation when the inactive form of SuPr1 was coexpressed where one could clearly identify the SUMOlated band of Sp100.

To summarize, the filament formation by the Sp100 HSR domain seems not to result from a lack of available SUMO (or Ubc9) which could be sequestered by the binding to the

HSR domain, but seems strongly reduced by a SuPr1 mediated deSUMOlation of PML or Sp100 and/or other proteins. Thus, the SuPr1-mediated disassembly/block in filament formation may be due to the loss of an interaction of the HSR domain with one or several SUMOlated protein(s). At this point it is important to remember that in PML -/- cells HSR filaments were formed and it is therefore not likely that deSUMOlation of PML is responsible for the inhibition of filament formation by SuPr1.

4.5 – Model for filament formation

Based on the data obtained, one can create a integrative model to explain the changes that PML bodies undergo when the HSR domain of Sp100 is expressed.

It is known that SUMOlated PML is necessary for PML body formation and recruitment of other PML body components like DAXX into PML bodies. SUMOlated PML binds directly to DAXX but no direct interaction between Sp100 and PML has been shown so far, therefore an unknown protein (X) may be the connecting link. SUMOlation of Sp100 is not required for PML body localization but the ratio of SUMOlated/unSUMOlated Sp100 in PML bodies is not known.

In the model presented below, it is assumed that HSR domain spanning polypeptide binds to wild type Sp100 and links it into filaments. Other PML body components align along these filaments and are dragged by wild type Sp100.

Upon expression SuPr1 cleaves SUMO from PML, Sp100 and maybe other PML body proteins and this disrupts Sp100-HSR filaments. The mechanism for filament disruption is not clear but one possibility may be that the HSR domain can only link SUMOlated Sp100 into filaments. One would expect that PML and DAXX no longer localize in PML bodies because PML is no longer SUMOlated and in addition SuPr1 is known to dissolve PML bodies at least at later time points. After deSUMOlation, the HSR domain localizes again in nuclear bodies probably bound to Sp100.

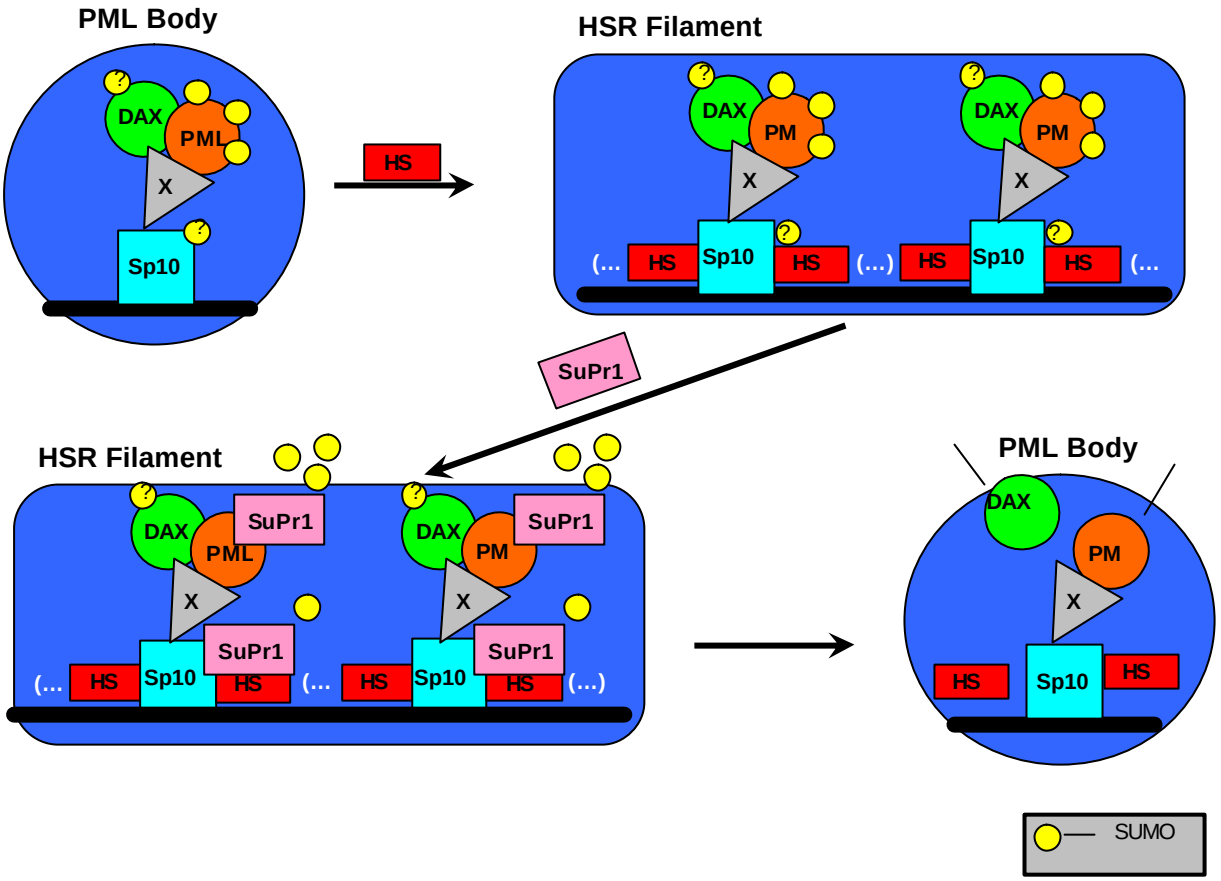

Figure 4.1 – Model to explain changes of PML bodies upon HSR-protein expression. For details see text.

4.6 – Growth inhibitory effect and apoptotic effect of the Sp100 HSR domain

In colony forming assays Sp100 showed a growth inhibitory effect. This effect was also observed and enhanced when only the HSR domain of Sp100 was expressed but not with the C-terminus of Sp100. Another N-terminal fragment that contained the HSR domain and also formed filaments had the same effect as the wild type Sp100. As a positive control for growth arrest p53 was used and the results showed that the effect of the HSR domain in inhibiting cell growth was as efficient as p53. This indicates that the HSR domain of Sp100 is responsible for the growth inhibitory effects of Sp100.

To further characterize the reason for fewer colonies in these experiments FACS analyses were performed and the results showed that Sp100 and its N-terminal constructs have an apoptotic effect on cells. Both wild type Sp100 and its N-terminal constructs were able to induce an apoptotic effect on cells and this was not observed when only the C-terminus of Sp100 was expressed. In addition to the proapoptotic effects, Sp100 HSR showed also a small G2 arrest transfected cells. In this regard it is of interest that in immunofluorescence experiments a higher than normal amount of dividing cells was observed when the Sp100 HSR domain was transfected. In these dividing cells Sp100 HSR could usually be localized with structures resembling the mitotic spindle. This suggests that Sp100 may also play a role in cell division through its HSR domain.

These results imply novel effects of Sp100 on cell survival which have not been noticed before. Until now, the only functions described for Sp100 concerned the regulation of transcription and eventually regulation of chromatin mediated by interaction with HP1. It is also of note that the HSR domain did not act as a dominant-negative mutant of Sp100 functions on cell-survival, but in contrast seemed to mediate these effects.

4.7 –C-terminal extensions of Sp100 splice variants do not contain an alternative nuclear dot localization

Sp100 splice variants Sp100B and Sp100HMG localize in part or completely (dependent on cell type) to alternative nuclear bodies devoid of detectable PML protein (Guldner et al, 1999). The N-terminus containing the HSR domain which targets Sp100 to PML nuclear bodies is also contained in the Sp100 splice variants. Therefore, other sequences located at the C-terminus of the splice variants override the HSR function as a dot localization signal of wild type Sp100. In order to investigate whether there is an alternative nuclear dot localization signal contained in the C-terminus of Sp100 splice variants several truncated Sp100HMG C-terminal constructs were expressed in HeLa cells.

The HGM and SAND domain, which are specific for the splice variants, showed a diffuse distribution when expressed, indicating that there is no separable signal domain contained in these domains which directs the splice variants to alternative nuclear dots. Interestingly, Sp100 splice variants constructs that did not possess the N-terminal region (including the HSR domain) localized to the nucleolus. It seems that also for the splice variants the HSR domain, which was absent from all these constructs, is needed for nuclear dot localization to PML as well as to non-PML bodies. Further, one can say that the non-PML body localization that is typical for Sp100B and Sp100HMG can only be obtained by the action of the HSR domain plus the C-terminal additions contained in these splice variants. These results confirmed and extended previous results of our laboratory from studies performed in a different cell line (C. Lüders, unpublished data).

Taken together these data suggests that C-terminal sequences of Sp100 splice variants induce an alternative conformation of at least the N-terminal half of Sp100 wild type protein region, which then may function as an alternative dot localization signal. Additional mutants of Sp100 splice variants need to be analyzed to test this hypothesis.

5 – Abbreviations

aa	Amino acid
AIRE	Autoimmune regulator
AOS1/UBA2	Ubiquitin activating enzyme 2
APAF-1	Apoptotic factor 1
APL	Acute promyelocytic leukemia
APS	Ammonium peroxide sulphate
ATP	Adenosine-5'-triphosphate
Bcl-2	B-cell lymphoma 2
Bright	B-cell regulator of IgH transcription
CBP	CREB binding protein
Caspase	Cysteine aspartase
cDNA	Complementary DNA
CENP-2	Centromeric protein 2
CMV	Human cytomegalovirus
DAXX	Fas death domain–associated protein
DED	Death effector domain
DEF	Death effector filament
DISC	Death inducing signaling complex
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic-acid
DNAse	Deoxyribonuclease
dNTP	Deoxynucleosidtriphosphate
E.coli	*Escherichia coli*
E4Orf3	Early protein 4 open reading frame 3
E4Orf6	Early protein 4 open reading frame 6
EDTA	Ethylenediaminetetraacetic acid
ETS-1	E twenty-six (Ets) domain transcription
eIF4E	Eukaryotic initiation factor 4E
FADD	Fas-associated death domain protein
FLASH	FLICE associated huge protein
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
HAUSP	Herpesvirus associated ubiquitin-specific protease
HDAC	Histone deacetylase
Hdm2	Human MDM2
HIPK2	Homeodomain interacting protein kinase-2
HMG	High mobility group
HP1	Heterochromatin protein 1
HRPO	Horse radish peroxidase
HSR	Homogenously staining region
JNK	cJun N-terminal kinase
kD	Kilo Dalton
MAL	Megakaryocytic acute leukemia protein
MDM2	Murine double minute clone 2
MHC	Major histocompability complex 1
NBS1	Nijmegen breakage syndrome protein 1
ND10	Nuclear domain 10
NLS	Nuclear localization signal
NMP125	Nuclear matrix protein 125
NuMA	Nuclear matrix protein
OD	Optical density
OTT	One-twenty-two protein
PBC	Primary biliary cirrhosis
PBS	Phosphate buffered solution

PCR	Polymerase chain reaction
PI	Propidium iodide
PML	Promyelocytic leukemia protein
PML-RAR	Promyelocytic leukemia protein-retinoic acid receptor fusion protein
POD	PML oncogenic domains
PSF	PTB splicing factor
RanBP2	Ran-binding protein 2
RanGAP1	Ran-GTPase-activating protein
RAR-a	Retinoic acid receptor alpha
RBCC	Ring finger-B-boxes-coiled coil
RING	C_3HC_4 zinc finger
RNA	Ribonucleic acid
RNAse	Ribonuclease
SAND	Sp100, AIRE-1, NucP41/75, DEAF-1
SDS	Sodium-dodecyl-sulphate
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis
SIRT1	Silent information regulator 1
snRNP	Small nuclear ribonuclear protein particles
Sp100	Speckled protein 100 kD
SUMO-1	Small ubiquitin-related modifier 1
SuPr1	SUMO protease 1
TAE	Tris-acetate-EDTA
TBS	Tris buffered solution
TEMED	N,N,N',N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
TRADD	TNF-alpha receptor-associated death domain protein
TRIM	Tripartite motif
Ubc9	Ubiquitin-like protein conjugating enzyme 9
wt	Wild-type

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7 - Bibliography

Ahn, J.H. and Hayward, G.S. (1997) The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. *J Virol*, **71**, 4599-4613.

Ahn, J.H., Brignole, E.J., 3rd and Hayward, G.S. (1998) Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. *Mol Cell Biol*, **18**, 4899-4913.

Appella, E. and Anderson, C.W. (2000) Signaling to p53: breaking the posttranslational modification code. *Pathol Biol (Paris)* , **48**, 227-245.

Ascoli, C.A. and Maul, G.G. (1991) Identification of a novel nuclear domain. *J Cell Biol*, **112**, 785-795.

Bardella, C., Costa, B., Maggiora, P., Patane, S., Olivero, M., Ranzani, G.N., De Bortoli, M., Comoglio, P.M. and Di Renzo, M.F. (2004) Truncated RON tyrosine kinase drives tumor cell progression and abrogates cell-cell adhesion through E-cadherin transcriptional repression. *Cancer Res*, **64**, 5154-5161.

Baumann, C.T., Ma, H., Wolford, R., Reyes, J.C., Maruvada, P., Lim, C., Yen, P.M., Stallcup, M.R. and Hager, G.L. (2001) The glucocorticoid receptor interacting protein 1 (GRIP1) localizes in discrete nuclear foci that associate with ND10 bodies and are enriched in components of the 26S proteasome. *Mol Endocrinol*, **15**, 485-500.

Best, J.L., Ganiatsas, S., Agarwal, S., Changou, A., Salomoni, P., Shirihai, O., Meluh, P.B., Pandolfi, P.P. and Zon, L.I. (2002) SUMO-1 protease-1 regulates gene transcription through PML. *Mol Cell*, **10**, 843-855.

Bischof, O., Kim, S.H., Irving, J., Beresten, S., Ellis, N.A. and Campisi, J. (2001) Regulation and localization of the Bloom syndrome protein in response to DNA damage. *J Cell Biol*, **153**, 367-380.

Bloch, D.B., de la Monte, S.M., Guigaouri, P., Filippov, A. and Bloch, K.D. (1996) Identification and characterization of a leukocyte-specific component of the nuclear body. *J Biol Chem*, **271**, 29198-29204.

Bloch, D.B., Nakajima, A., Gulick, T., Chiche, J.D., Orth, D., de La Monte, S.M. and Bloch, K.D. (2000) Sp110 localizes to the PML-Sp100 nuclear body and may function as a nuclear hormone receptor transcriptional coactivator. *Mol Cell Biol*, **20**, 6138-6146.

Borden, K.L. (2000) RING domains: master builders of molecular scaffolds? *J Mol Biol*, **295**, 1103-1112.

Borden, K.L. (2002) Pondering the promyelocytic leukemia protein (PML) puzzle: possible functions for PML nuclear bodies. *Mol Cell Biol*, **22**, 5259-5269.

Borden, K.L., Lally, J.M., Martin, S.R., O'Reilly, N.J., Solomon, E. and Freemont, P.S. (1996) In vivo and in vitro characterization of the B1 and B2 zinc-binding domains from the acute promyelocytic leukemia protooncoprotein PML. *Proc Natl Acad Sci U S A*, **93**, 1601-1606.

Bottomley, M.J., Collard, M.W., Huggenvik, J.I., Liu, Z., Gibson, T.J. and Sattler, M. (2001) The SAND domain structure defines a novel DNA-binding fold in transcriptional regulation. *Nat Struct Biol*, **8**, 626-633.

Bouteille, M., Bouvier, D. and Seve, A.P. (1983) Heterogeneity and territorial organization of the nuclear matrix and related structures. *Int Rev Cytol*, **83**, 135-182.

Bridge, E. and Ketner, G. (1989) Redundant control of adenovirus late gene expression by early region 4. *J Virol*, **63**, 631-638.

Burkhard, P., Stetefeld, J. and Strelkov, S.V. (2001) Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol*, **11**, 82-88.

Buschmann, T., Fuchs, S.Y., Lee, C.G., Pan, Z.Q. and Ronai, Z. (2000) SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell*, **101**, 753-762.

Carvalho, T., Seeler, J.S., Ohman, K., Jordan, P., Pettersson, U., Akusjarvi, G., Carmo-Fonseca, M. and Dejean, A. (1995) Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J Cell Biol*, **131**, 45-56.

Chan, J.Y., Li, L., Fan, Y.H., Mu, Z.M., Zhang, W.W. and Chang, K.S. (1997) Cell-cycle regulation of DNA damage-induced expression of the suppressor gene PML. *Biochem Biophys Res Commun*, **240**, 640-646.

Chang, H.Y., Nishitoh, H., Yang, X., Ichijo, H. and Baltimore, D. (1998) Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science*, **281**, 1860-1863.

Charette, S.J., Lambert, H. and Landry, J. (2001) A kinase-independent function of Ask1 in caspase-independent cell death. *J Biol Chem*, **276**, 36071-36074.

Chelbi-Alix, M.K., Quignon, F., Pelicano, L., Koken, M.H. and de The, H. (1998) Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. *J Virol*, **72**, 1043-1051.

Chen, L.Y. and Chen, J.D. (2003) Daxx silencing sensitizes cells to multiple apoptotic pathways. *Mol Cell Biol*, **23**, 7108-7121.

Choi, C.Y., Kim, Y.H., Kwon, H.J. and Kim, Y. (1999) The homeodomain protein NK-3 recruits Groucho and a histone deacetylase complex to repress transcription. *J Biol Chem*, **274**, 33194-33197.

Cohen, N., Sharma, M., Kentsis, A., Perez, J.M., Strudwick, S. and Borden, K.L. (2001) PML RING suppresses oncogenic transformation by reducing the affinity of eIF4E for mRNA. *Embo J*, **20**, 4547-4559.

Daniel, M.T., Koken, M., Romagne, O., Barbey, S., Bazarbachi, A., Stadler, M., Guillemin, M.C., Degos, L., Chomienne, C. and de The, H. (1993) PML protein expression in hematopoietic and acute promyelocytic leukemia cells. *Blood*, **82**, 1858-1867.

de The, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. (1991) The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell*, **66**, 675-684.

Degos, L. (1994) Is acute promyelocytic leukemia a curable disease? Treatment strategy for a long-term survival. *Leukemia*, **8**, 911-913.

Dent, A.L., Yewdell, J., Puvion-Dutilleul, F., Koken, M.H., de The, H. and Staudt, L.M. (1996) LYSP100-associated nuclear domains (LANDs): description of a new class of subnuclear structures and their relationship to PML nuclear bodies. *Blood*, **88**, 1423-1426.

Di Stefano, V., Blandino, G., Sacchi, A., Soddu, S. and D'Orazi, G. (2004a) HIPK2 neutralizes MDM2 inhibition rescuing p53 transcriptional activity and apoptotic function. *Oncogene*, **23**, 5185-5192.

Di Stefano, V., Rinaldo, C., Sacchi, A., Soddu, S. and D'Orazi, G. (2004b) Homeodomain-interacting protein kinase-2 activity and p53 phosphorylation are critical events for cisplatin-mediated apoptosis. *Exp Cell Res*, **293**, 311-320.

D'Orazi, G., Cecchinelli, B., Bruno, T., Manni, I., Higashimoto, Y., Saito, S., Gostissa, M., Coen, S., Marchetti, A., Del Sal, G., Piaggio, G., Fanciulli, M., Appella, E. and Soddu, S. (2002) Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser 46 and mediates apoptosis. *Nat Cell Biol*, **4**, 11-19.

Doucas, V., Ishov, A.M., Romo, A., Juguilon, H., Weitzman, M.D., Evans, R.M. and Maul, G.G. (1996) Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. *Genes Dev*, **10**, 196-207.

Dyck, J.A., Maul, G.G., Miller, W.H., Jr., Chen, J.D., Kakizuka, A. and Evans, R.M. (1994) A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell*, **76**, 333-343.

Eckert, W.A., Plass, C., Weith, A., Traut, W. and Winking, H. (1991) Transcripts from amplified sequences of an inherited homogeneously staining region in chromosome 1 of the house mouse (Mus musculus). *Mol Cell Biol*, **11**, 2229-2235.

Emelyanov, A.V., Kovac, C.R., Sepulveda, M.A. and Birshtein, B.K. (2002) The interaction of Pax5 (BSAP) with Daxx can result in transcriptional activation in B cells. *J Biol Chem*, **277**, 11156-11164.

Engelhardt, O.G., Boutell, C., Orr, A., Ullrich, E., Haller, O. and Everett, R.D. (2003) The homeodomain-interacting kinase PKM (HIPK-2) modifies ND10 through both its kinase domain and a SUMO-1 interaction motif and alters the posttranslational modification of PML. *Exp Cell Res*, **283**, 36-50.

Eskiw, C.H. and Bazett-Jones, D.P. (2002) The promyelocytic leukemia nuclear body: sites of activity? *Biochem Cell Biol*, **80**, 301-310.

Eskiw, C.H., Dellaire, G., Mymryk, J.S. and Bazett-Jones, D.P. (2003) Size, position and dynamic behavior of PML nuclear bodies following cell stress as a paradigm for supramolecular trafficking and assembly. *J Cell Sci*, **116**, 4455-4466.

Everett, R.D. (2001) DNA viruses and viral proteins that interact with PML nuclear bodies. *Oncogene*, **20**, 7266-7273.

Everett, R.D., Lomonte, P., Sternsdorf, T., van Driel, R. and Orr, A. (1999) Cell cycle regulation of PML modification and ND10 composition. *J Cell Sci*, **112 (Pt 24)**, 4581-4588.

Fagioli, M., Alcalay, M., Pandolfi, P.P., Venturini, L., Mencarelli, A., Simeone, A., Acampora, D., Grignani, F. and Pelicci, P.G. (1992) Alternative splicing of PML transcripts predicts coexpression of several carboxy-terminally different protein isoforms. *Oncogene*, **7**, 1083-1091.

Ferbeyre, G. (2002) PML a target of translocations in APL is a regulator of cellular senescence. *Leukemia*, **16**, 1918-1926.

Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Sternsdorf, T., Jensen, K., Pandolfi, P.P., Will, H., Schneider, C. and Del Sal, G. (2000) Regulation of p53 activity in nuclear bodies by a specific PML isoform. *Embo J*, **19**, 6185-6195.

Furuta, K., Chan, E.K., Kiyosawa, K., Reimer, G., Luderschmidt, C. and Tan, E.M. (1997) Heterochromatin protein HP1Hsbeta (p25beta) and its localization with centromeres in mitosis. *Chromosoma*, **106**, 11-19.

Goddard, A.D., Borrow, J., Freemont, P.S. and Solomon, E. (1991) Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. *Science*, **254**, 1371-1374.

Goodrum, F.D. and Ornelles, D.A. (1999) Roles for the E4 orf6, orf3, and E1B 55-kilodalton proteins in cell cycle-independent adenovirus replication. *J Virol*, **73**, 7474-7488.

Grisolano, J.L., Wesselschmidt, R.L., Pelicci, P.G. and Ley, T.J. (1997) Altered myeloid development and acute leukemia in transgenic mice expressing PML-RAR alpha under control of cathepsin G regulatory sequences. *Blood*, **89**, 376-387.

Grosschedl, R., Giese, K. and Pagel, J. (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends Genet*, **10**, 94-100.

Grotzinger, T., Jensen, K., Guldner, H.H., Sternsdorf, T., Szostecki, C., Schwab, M., Savelyeva, L., Reich, B. and Will, H. (1996a) A highly amplified mouse gene is homologous to the human interferon-responsive Sp100 gene encoding an autoantigen associated with nuclear dots. *Mol Cell Biol*, **16**, 1150-1156.

Grotzinger, T., Sternsdorf, T., Jensen, K. and Will, H. (1996b) Interferon-modulated expression of genes encoding the nuclear-dot-associated proteins Sp100 and promyelocytic leukemia protein (PML). *Eur J Biochem*, **238**, 554-560.

Guldner, H.H., Szostecki, C., Grotzinger, T. and Will, H. (1992) IFN enhance expression of Sp100, an autoantigen in primary biliary cirrhosis. *J Immunol*, **149**, 4067-4073.

Guldner, H.H., Szostecki, C., Schroder, P., Matschl, U., Jensen, K., Luders, C., Will, H. and Sternsdorf, T. (1999) Splice variants of the nuclear dot-associated Sp100 protein contain homologies to HMG-1 and a human nuclear phosphoprotein-box motif. *J Cell Sci*, **112 (Pt 5)**, 733-747.

Guo, A., Salomoni, P., Luo, J., Shih, A., Zhong, S., Gu, W. and Paolo Pandolfi, P. (2000) The function of PML in p53-dependent apoptosis. *Nat Cell Biol*, **2**, 730-736.

Harris, F.M., Brecht, W.J., Xu, Q., Tesseur, I., Kekonius, L., Wyss-Coray, T., Fish, J.D., Masliah, E., Hopkins, P.C., Scearce-Levie, K., Weisgraber, K.H., Mucke, L., Mahley, R.W. and Huang, Y. (2003) Carboxyl-terminal-truncated apolipoprotein E4 causes Alzheimer's disease-like neurodegeneration and behavioral deficits in transgenic mice. *Proc Natl Acad Sci U S A*, **100**, 10966-10971.

Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, **387**, 296-299.

Hodges, M., Tissot, C., Howe, K., Grimwade, D. and Freemont, P.S. (1998) Structure, organization, and dynamics of promyelocytic leukemia protein nuclear bodies. *Am J Hum Genet*, **63**, 297-304.

Hofmann, T.G. and Will, H. (2003) Body language: the function of PML nuclear bodies in apoptosis regulation. *Cell Death Differ*, **10**, 1290-1299.

Hofmann, T.G., Moller, A., Sirma, H., Zentgraf, H., Taya, Y., Droge, W., Will, H. and Schmitz, M.L. (2002) Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nat Cell Biol*, **4**, 1-10.

Hofmann, T.G., Stollberg, N., Schmitz, M.L. and Will, H. (2003) HIPK2 regulates transforming growth factor-beta-induced c-Jun NH(2)-terminal kinase activation and apoptosis in human hepatoma cells. *Cancer Res*, **63**, 8271-8277.

Hollenbach, A.D., McPherson, C.J., Mientjes, EJ., Iyengar, R. and Grosveld, G. (2002) Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. *J Cell Sci*, **115**, 3319-3330.

Hollenbach, A.D., Sublett, J.E., McPherson, C.J. and Grosveld, G. (1999) The Pax3-FKHR oncoprotein is unresponsive to the Pax3-associated repressor hDaxx. *Embo J*, **18**, 3702-3711.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C. (1991) p53 mutations in human cancers. *Science*, **253**, 49-53.

Huang, M.M. and Hearing, P. (1989) The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev*, **3**, 1699-1710.

Ishov, A.M., Sotnikov, A.G., Negorev, D., Vladimirova, O.V., Neff, N., Kamitani, T., Yeh, E.T., Strauss, J.F., 3rd and Maul, G.G. (1999) PML is critical for ND10 formation and recruits the PML-interacting protein daxx to this nuclear structure when modified by SUMO-1. *J Cell Biol*, **147**, 221-234.

Juo, P., Kuo, C.J., Yuan, J. and Blenis, J. (1998) Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr Biol*, **8**, 1001-1008.

Kakizuka, A., Miller, W.H., Jr., Umesono, K., Warrell, R.P., Jr., Frankel, S.R., Murty, V.V., Dmitrovsky, E. and Evans, R.M. (1991) Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML. *Cell*, **66**, 663-674.

Kastner, P., Perez, A., Lutz, Y., Rochette-Egly, C., Gaub, M.P., Durand, B., Lanotte, M., Berger, R. and Chambon, P. (1992) Structure, localization and transcriptional properties of two classes of retinoic acid receptor alpha fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. *Embo J*, **11**, 629-642.

Khan, M.M., Nomura, T., Kim, H., Kaul, S.C., Wadhwa, R., Zhong, S., Pandolfi, P.P. and Ishii, S. (2001) PML-RARalpha alleviates the transcriptional repression mediated by tumor suppressor Rb. *J Biol Chem*, **276**, 43491-43494.

Kiesslich, A., von Mikecz, A. and Hemmerich, P. (2002) Cell cycle-dependent association of PML bodies with sites of active transcription in nuclei of mammalian cells. *J Struct Biol*, **140**, 167-179.

Kim, K.I., Baek, S.H. and Chung, C.H. (2002) Versatile protein tag, SUMO: its enzymology and biological function. *J Cell Physiol*, **191**, 257-268.

Kim, Y.H., Choi, C.Y., Lee, S.J., Conti, M.A. and Kim, Y. (1998) Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. *J Biol Chem*, **273**, 25875-25879.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. and Peter, M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J*, **14**, 5579-5588.

Koken, M.H., Linares-Cruz, G., Quignon, F., Viron, A., Chelbi-Alix, M.K., Sobczak-Thepot, J., Juhlin, L., Degos, L., Calvo, F. and de The, H. (1995) The PML growth-suppressor has an altered expression in human oncogenesis. *Oncogene*, **10**, 1315-1324.

Koken, M.H., Puvion-Dutilleul, F., Guillemin, M.C., Viron, A., Linares-Cruz, G., Stuurman, N., de Jong, L., Szostecki, C., Calvo, F., Chomienne, C. and et al. (1994) The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *Embo J*, **13**, 1073-1083.

Kourmouli, N., Theodoropoulos, P.A., Dialynas, G., Bakou, A., Politou, A.S., Cowell, I.G., Singh, P.B. and Georgatos, S.D. (2000) Dynamic associations of heterochromatin protein 1 with the nuclear envelope. *Embo J*, **19**, 6558-6568.

Krammer, P.H. (2000) CD95's deadly mission in the immune system. *Nature*, **407**, 789-795.

Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) Regulation of p53 stability by Mdm2. **Nature**, *387*, 299-303.

Lallemand-Breitenbach, V., Zhu, J., Puvion, F., Koken, M., Honore, N., Doubeikovsky, A., Duprez, E., Pandolfi, P.P., Puvion, E., Freemont, P. and de The, H. (2001) Role of promyelocytic leukemia (PML) sumolation in nuclear body formation, 11S proteasome recruitment, and As2O3-induced PML or PML/retinoic acid receptor alpha degradation. *J Exp Med*, **193**, 1361-1371.

LaMorte, V.J., Dyck, J.A., Ochs, R.L. and Evans, R.M. (1998) Localization of nascent RNA and CREB binding protein with the PML-containing nuclear body. *Proc Natl Acad Sci U S A*, **95**, 4991-4996.

Lehming, N., Le Saux, A., Schuller, J. and Ptashne, M. (1998) Chromatin components as part of a putative transcriptional repressing complex. *Proc Natl Acad Sci U S A*, **95**, 7322-7326.

Li, H., Leo, C., Zhu, J., Wu, X., O'Neil, J., Park, E.J. and Chen, J.D. (2000) Sequestration and inhibition of Daxx-mediated transcriptional repression by PML. *Mol Cell Biol*, **20**, 1784-1796.

Mahajan, R., Delphin, C., Guan, T., Gerace, L. and Melchior, F. (1997) A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell*, **88**, 97-107.

Marugg, R.A. (1992) Transient storage of a nuclear matrix protein along intermediate-type filaments during mitosis: a novel function of cytoplasmic intermediate filaments. *J Struct Biol*, **108**, 129-139.

Matunis, M.J., Coutavas, E. and Blobel, G. (1996) A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol*, **135**, 1457-1470.

Maul, G.G., Guldner, H.H. and Spivack, J.G. (1993) Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). *J Gen Virol*, **74 (Pt 12)**, 2679-2690.

Maul, G.G., Negorev, D., Bell, P. and Ishov, A.M. (2000) Review: properties and assembly mechanisms of ND10, PML bodies, or PODs. *J Struct Biol*, **129**, 278-287.

Maul, G.G., Yu, E., Ishov, A.M. and Epstein, A.L. (1995) Nuclear domain 10 (ND10) associated proteins are also present in nuclear bodies and redistribute to hundreds of nuclear sites after stress. *J Cell Biochem*, **59**, 498-513.

Melchior, F. and Hengst, L. (2002) SUMO-1 and p53. *Cell Cycle*, **1**, 245-249.

Melnick, A. and Licht, J.D. (1999) Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood*, **93**, 3167-3215.

Mercher, T., Busson-Le Coniat, M., Khac, F.N., Ballerini, P., Mauchauffe, M., Bui, H., Pellegrino, B., Radford, I., Valensi, F., Mugneret, F., Dastugue, N., Bernard, O.A. and Berger, R. (2002) Recurrence of OTT-MAL fusion in t(1;22) of infant AML-M7. *Genes Chromosomes Cancer*, **33**, 22-28.

Mercher, T., Coniat, M.B., Monni, R., Mauchauffe, M., Khac, F.N., Gressin, L., Mugneret, F., Leblanc, T., Dastugue, N, Berger, R. and Bernard, O.A. (2001) Involvement of a human gene related to the Drosophila spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia. *Proc Natl Acad Sci U S A*, **98**, 5776-5779.

Moir, R.D. and Spann, T.P. (2001) The structure and function of nuclear lamins: implications for disease. *Cell Mol Life Sci*, **58**, 1748-1757.

Moller, A. and Schmitz, M.L. (2003) Viruses as hijackers of PML nuclear bodies. *Arch Immunol Ther Exp (Warsz)*, **51**, 295-300.

Moller, A., Sirma, H., Hofmann, T.G., Staege, H., Gresko, E., Ludi, K.S., Klimczak, E., Droge, W., Will, H. and Schmitz, M.L. (2003) Sp100 is important for the stimulatory effect of homeodomain-interacting protein kinase-2 on p53-dependent gene expression. *Oncogene*, **22**, 8731-8737.

Mu, Z.M., Chin, K.V., Liu, J.H., Lozano, G. and Chang, K.S. (1994) PML, a growth suppressor disrupted in acute promyelocytic leukemia. *Mol Cell Biol*, **14**, 6858-6867.

Muller, S., Matunis, M.J. and Dejean, A. (1998) Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus. *Embo J*, **17**, 61-70.

Muratani, M., Gerlich, D., Janicki, S.M., Gebhard, M., Eils, R. and Spector, D.L. (2002) Metabolic-energy-dependent movement of PML bodies within the mammalian cell nucleus. *Nat Cell Biol*, **4**, 106-110.

Naka, K., Ikeda, K. and Motoyama, N. (2002) Recruitment of NBS1 into PML oncogenic domains via interaction with SP100 protein. *Biochem Biophys Res Commun*, **299**, 863-871.

Negorev, D. and Maul, G.G. (2001) Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. *Oncogene*, **20**, 7234-7242.

Negorev, D., Ishov, A.M. and Maul, G.G. (2001) Evidence for separate ND10-binding and homo-oligomerization domains of Sp100. *J Cell Sci*, **114**, 59-68.

Nevels, M., Tauber, B., Kremmer, E., Spruss, T., Wolf, H. and Dobner, T. (1999) Transforming potential of the adenovirus type 5 E4orf3 protein. *J Virol*, **73**, 1591-1600.

Ohiro, Y., Usheva, A., Kobayashi, S., Duffy, S.L., Nantz, R., Gius, D. and Horikoshi, N. (2003) Inhibition of stress-inducible kinase pathways by tumorigenic mutant p53. *Mol Cell Biol*, **23**, 322-334.

Pearson, M., Carbone, R., Sebastiani, C., Cioce, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P.P. and Pelicci, P.G. (2000) PML regulates p53 acetylation and premature senescence induced by oncogenic Ras. *Nature*, **406**, 207-210.

Perez, A., Kastner, P., Sethi, S., Lutz, Y., Reibel, C. and Chambon, P. (1993) PMLRAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. *Embo J*, **12**, 3171-3182.

Perlman, R., Schiemann, W.P., Brooks, M.W., Lodish, H.F. and Weinberg, R.A. (2001) TGF-beta-induced apoptosis is mediated by the adapter protein Daxx that facilitates JNK activation. *Nat Cell Biol*, **3**, 708-714.

Pichler, A., Gast, A., Seeler, J.S., Dejean, A. and Melchior, F. (2002) The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell*, **108**, 109-120.

Pierantoni, G.M., Fedele, M., Pentimalli, F., Benvenuto, G., Pero, R., Viglietto, G., Santoro, M., Chiariotti, L. and Fusco, A. (2001) High mobility group I (Y) proteins bind HIPK2, a serine-threonine kinase protein which inhibits cell growth. *Oncogene*, **20**, 6132-6141.

Prives, C. and Hall, P.A. (1999) The p53 pathway. *J Pathol*, **187**, 112-126.

Puvion-Dutilleul, F., Chelbi-Alix, M.K., Koken, M., Quignon, F., Puvion, E. and de The, H. (1995) Adenovirus infection induces rearrangements in the intranuclear distribution of the nuclear body-associated PML protein. *Exp Cell Res*, **218**, 9-16.

Quignon, F., De Bels, F., Koken, M., Feunteun, J., Ameisen, J.C. and de The, H. (1998) PML induces a novel caspase-independent death process. *Nat Genet*, **20**, 259-265.

Ranganathan, V., Heine, W.F., Ciccone, D.N., Rudolph, K.L., Wu, X., Chang, S., Hai, H., Ahearn, I.M., Livingston, D.M., Resnick, I., Rosen, F., Seemanova, E., Jarolim, P., DePinho, R.A. and Weaver, D.T. (2001) Rescue of a telomere length defect of Nijmegen breakage syndrome cells requires NBS and telomerase catalytic subunit. *Curr Biol*, **11**, 962-966.

Regad, T. and Chelbi-Alix, M.K. (2001) Role and fate of PML nuclear bodies in response to interferon and viral infections. *Oncogene*, **20**, 7274-7286.

Regad, T., Saib, A., Lallemand-Breitenbach, V., Pandolfi, P.P., de The, H. and Chelbi-Alix, M.K. (2001) PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator. *Embo J*, **20**, 3495-3505.

Reichenzeller, M., Burzlaff, A., Lichter, P. and Herrmann, H. (2000) In vivo observation of a nuclear channel-like system: evidence for a distinct interchromosomal domain compartment in interphase cells. *J Struct Biol*, **129**, 175-185.

Rinderle, C., Christensen, H.M., Schweiger, S., Lehrach, H. and Yaspo, M.L. (1999) AIRE encodes a nuclear protein co-localizing with cytoskeletal filaments: altered sub-cellular distribution of mutants lacking the PHD zinc fingers. *Hum Mol Genet*, **8**, 277-290.

Ross, S., Best, J.L., Zon, L.I. and Gill, G. (2002) SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell*, **10**, 831-842.

Saredi, A., Howard, L. and Compton, D.A. (1996) NuMA assembles into an extensive filamentous structure when expressed in the cell cytoplasm. *J Cell Sci*, **109 (Pt 3)**, 619-630.

Seeler, J.S. and Dejean, A. (1999) The PML nuclear bodies: actors or extras? *Curr Opin Genet Dev*, **9**, 362-367.

Seeler, J.S., Marchio, A., Sitterlin, D., Transy, C. and Dejean, A. (1998) Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc Natl Acad Sci U S A*, **95**, 7316-7321.

Shav-Tal, Y., Cohen, M., Lapter, S., Dye, B., Patton, J.G., Vandekerckhove, J. and Zipori, D. (2001) Nuclear relocalization of the pre-mRNA splicing factor PSF during apoptosis involves hyperphosphorylation, masking of antigenic epitopes, and changes in protein interactions. *Mol Biol Cell*, **12**, 2328-2340.

Shen, Y., Zhu, H. and Shenk, T. (1997) Human cytomagalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation in cooperation with the adenovirus E1A proteins. *Proc Natl Acad Sci U S A*, **94**, 3341-3345.

Shiels, C., Islam, S.A., Vatcheva, R., Sasieni, P., Sternberg, M.J., Freemont, P.S. and Sheer, D. (2001) PML bodies associate specifically with the MHC gene cluster in interphase nuclei. *J Cell Sci*, **114**, 3705-3716.

Siegel, R.M., Martin, D.A., Zheng, L., Ng, S.Y., Bertin, J., Cohen, J. and Lenardo, M.J. (1998) Death-effector filaments: novel cytoplasmic structures that recruit caspases and trigger apoptosis. *J Cell Biol*, **141**, 1243-1253.

Slee, E.A., O'Connor, D.J. and Lu, X. (2004) To die or not to die: how does p53 decide? *Oncogene*, **23**, 2809-2818.

Sternsdorf, T., Jensen, K. and Will, H. (1997) Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J Cell Biol*, **139**, 1621-1634.

Sternsdorf, T., Jensen, K., Reich, B. and Will, H. (1999) The nuclear dot protein sp100, characterization of domains necessary for dimerization, subcellular localization, and modification by small ubiquitin-like modifiers. *J Biol Chem*, **274**, 12555-12566.

Strasser, A., O'Connor, L. and Dixit, V.M. (2000) Apoptosis signaling. *Annu Rev Biochem*, **69**, 217-245.

Strausberg,R.L., Feingold,E.A., Grouse,L.H., Derge,J.G., Klausner,R.D., Collins,F.S., Wagner,L., Shenmen,C.M., Schuler,G.D., Altschul,S.F., Zeeberg,B., Buetow,K.H., Schaefer,C.F., Bhat,N.K., Hopkins,R.F., Jordan,H., Moore,T., Max,S.I., Wang,J., Hsieh,F., Diatchenko,L., Marusina,K., Farmer,A.A., Rubin,G.M., Hong,L., Stapleton,M., Soares,M.B., Bonaldo,M.F., Casavant,T.L., Scheetz,T.E., Brownstein,M.J., Usdin,T.B., Toshiyuki,S., Carninci,P., Prange,C., Raha,S.S., Loquellano,N.A., Peters,G.J., Abramson,R.D., Mullahy,S.J., Bosak,S.A., McEwan,P.J., McKernan,K.J., Malek,J.A., Gunaratne,P.H., Richards,S., Worley,K.C., Hale,S., Garcia,A.M., Gay,L.J., Hulyk,S.W., Villalon,D.K., Muzny,D.M., Sodergren,E.J., Lu,X., Gibbs,R.A., Fahey,J., Helton,E., Ketteman,M., Madan,A., Rodrigues,S., Sanchez,A., Whiting,M., Madan,A., Young,A.C., Shevchenko,Y., Bouffard,G.G., Blakesley,R.W., Touchman,J.W., Green,E.D., Dickson,M.C., Rodriguez,A.C., Grimwood,J., Schmutz,J., Myers,R.M., Butterfield,Y.S., Krzywinski,M.I., Skalska,U., Smailus,D.E., Schnerch,A., Schein,J.E., Jones,S.J. and Marra,M.A. (2002) Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc. Natl. Acad. Sci. U.S.A, **99**, 16899-16903.

Strudwick, S. and Borden, K.L. (2002) Finding a role for PML in APL pathogenesis: a critical assessment of potential PML activities. *Leukemia*, **16**, 1906-1917.

Stuurman, N., de Graaf, A., Floore, A., Josso, A., Humbel, B., de Jong, L. and van Driel, R. (1992) A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. *J Cell Sci*, **101 (Pt 4)**, 773-784.

Suzuki, H., Seki, M., Kobayashi, T., Kawabe, Y., Kaneko, H., Kondo, N., Harata, M., Mizuno, S., Masuko, T. and Enomoto, T. (2001) The N-terminal internal region of BLM is required for the formation of dots/rod-like structures which are associated with SUMO-1. *Biochem Biophys Res Commun*, **286**, 322-327.

Szostecki, C., Guldner, H.H., Netter, H.J. and Will, H. (1990) Isolation and characterization of cDNA encoding a human nuclear antigen predominantly recognized by autoantibodies from patients with primary biliary cirrhosis. *J Immunol*, **145**, 4338-4347.

Szostecki, C., Will, H., Netter, H.J. and Guldner, H.H. (1992) Autoantibodies to the nuclear Sp100 protein in primary biliary cirrhosis and associated diseases: epitope specificity and immunoglobulin class distribution. *Scand J Immunol*, **36**, 555-564.

Taimen, P. and Kallajoki, M. (2003) NuMA and nuclear lamins behave differently in Fas-mediated apoptosis. *J Cell Sci*, **116**, 571-583.

Tauchi, H., Kobayashi, J., Morishima, K., Matsuura, S., Nakamura, A., Shiraishi, T., Ito, E., Masnada, D., Delia, D. and Komatsu, K. (2001) The forkhead-associated domain of NBS1 is essential for nuclear foci formation after irradiation but not essential for hRAD50-hMRE11-NBS1 complex DNA repair activity. *J Biol Chem*, **276**, 12-15.

Terris, B., Baldin, V., Dubois, S., Degott, C., Flejou, J.F., Henin, D. and Dejean, A. (1995) PML nuclear bodies are general targets for inflammation and cell proliferation. *Cancer Res*, **55**, 1590-1597.

Tibbetts, M.D., Zheng, L. and Lenardo, M.J. (2003) The death effector domain protein family: regulators of cellular homeostasis. *Nat Immunol*, **4**, 404-409.

Torii, S., Egan, D.A., Evans, R.A. and Reed, J.C. (1999) Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). *Embo J*, **18**, 6037-6049.

Tsugawa, K., Takahashi, K.P., Watanabe, T., Mai, A., Fujimoto, D. and Inoue, A. (1997) Association of hnRNP S1 proteins C2 and D2 with vimentin intermediate filaments. *Cell Struct Funct*, **22**, 239-246.

Tsukamoto, T., Hashiguchi, N., Janicki, S.M., Tumbar, T., Belmont, A.S. and Spector, D.L. (2000) Visualization of gene activity in living cells. *Nat Cell Biol*, **2**, 871-878.

Vlcek, S., Dechat, T. and Foisner, R. (2001) Nuclear envelope and nuclear matrix: interactions and dynamics. *Cell Mol Life Sci*, **58**, 1758-1765.

Wang, J., Chun, H.J., Wong, W., Spencer, D.M. and Lenardo, M.J. (2001a) Caspase-10 is an initiator caspase in death receptor signaling. *Proc Natl Acad Sci U S A*, **98**, 13884-13888.

Wang, Y., Hofmann, T.G., Runkel, L., Haaf, T., Schaller, H., Debatin, K. and Hug, H. (2001b) Isolation and characterization of cDNAs for the protein kinase HIPK2. *Biochim Biophys Acta*, **1518**, 168-172.

Wang, Z.G., Rivi, R., Delva, L., Konig, A., Scheinberg, D.A., Gambacorti-Passerini, C., Gabrilove, J.L., Warrell, R.P., Jr. and Pandolfi, P.P. (1998) Arsenic trioxide and melarsoprol induce programmed cell death in myeloid leukemia cell lines and function in a PML and PML-RARalpha independent manner. *Blood*, **92**, 1497-1504.

Wasylyk, C., Schlumberger, S.E., Criqui-Filipe, P. and Wasylyk, B. (2002) Sp100 interacts with ETS-1 and stimulates its transcriptional activity. *Mol Cell Biol*, **22**, 2687-2702.

Weichenhan,D., Kunze,B., Zacker,S., Traut,W. and Winking,H. (1997) Structure and expression of the murine Sp100 nuclear dot gene. Genomics, **43**, 298-306.

Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A. and Dejean, A. (1994) Retinoic acid regulates aberrant nuclear localization of PML-RAR alpha in acute promyelocytic leukemia cells. *Cell*, **76**, 345-356.

Weiss, M.A. and Warrell, R.P., Jr. (1994) Two cases of extramedullary acute promyelocytic leukemia. Cytogenetics, molecular biology, and phenotypic and clinical studies. *Cancer*, **74**, 1882-1886.

Wienzek, S. and Dobbelstein, M. (2001) Viral and cellular factors that target the promyelocytic leukemia oncogenic domains strongly activate a glucocorticoid-responsive promoter. *J Virol*, **75**, 5391-5397.

Wiesmeijer, K., Molenaar, C., Bekeer, I.M., Tanke, H.J. and Dirks, R.W. (2002) Mobile foci of Sp100 do not contain PML: PML bodies are immobile but PML and Sp100 proteins are not. *J Struct Biol*, **140**, 180-188.

Wojciak, J.M. and Clubb, R.T. (2001) Finding the function buried in SAND. *Nat Struct Biol*, **8**, 568-570.

Wolin, S.L. and Cedervall, T. (2002) The La protein. *Annu Rev Biochem*, **71**, 375-403.

Wu, S., Loke, H.N. and Rehemtulla, A. (2002) Ultraviolet radiation-induced apoptosis is mediated by Daxx. *Neoplasia*, **4**, 486-492.

Xie, K., Lambie, E.J. and Snyder, M. (1993) Nuclear dot antigens may specify transcriptional domains in the nucleus. *Mol Cell Biol*, **13**, 6170-6179.

Yang, X., Khosravi-Far, R., Chang, H.Y. and Baltimore, D. (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell*, **89**, 1067-1076.

Yeh, J.H., Hsu, S.C., Han, S.H. and Lai, M.Z. (1998) Mitogen-activated protein kinase kinase antagonized fas-associated death domain protein-mediated apoptosis by induced FLICE-inhibitory protein expression. *J Exp Med*, **188**, 1795-1802.

Yordy, J.S., Li, R., Sementchenko, V.I., Pei, H., Muise-Helmericks, R.C. and Watson, D.K. (2004) SP100 expression modulates ETS1 transcriptional activity and inhibits cell invasion. *Oncogene*.

Zhang, J., Cado, D., Chen, A., Kabra, N.H. and Winoto, A. (1998) Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature*, **392**, 296-300.

Zheng, P., Guo, Y., Niu, Q., Levy, D.E., Dyck, J.A., Lu, S., Sheiman, L.A. and Liu, Y. (1998) Proto-oncogene PML controls *Genes Dev*oted to MHC class I antigen presentation. *Nature*, **396**, 373-376.

Zhong, S., Hu, P., Ye, T.Z., Stan, R., Ellis, N.A. and Pandolfi, P.P. (1999) A role for PML and the nuclear body in genomic stability. *Oncogene*, ***18***, 7941-7947.

Zhong, S., Muller, S., Ronchetti, S., Freemont, P.S., Dejean, A. and Pandolfi, P.P. (2000a) Role of SUMO-1-modified PML in nuclear body formation. *Blood*, **95**, 2748-2752.

Zhong, S., Salomoni, P. and Pandolfi, P.P. (2000b) The transcriptional role of PML and the nuclear body. *Nat Cell Biol*, **2**, E85-90.

Zhong, S., Salomoni, P., Ronchetti, S., Guo, A., Ruggero, D. and Pandolfi, P.P. (2000c) Promyelocytic leukemia protein (PML) and Daxx participate in a novel nuclear pathway for apoptosis. *J Exp Med*, **191**, 631-640.

Zong, R.T., Das, C. and Tucker, P.W. (2000) Regulation of matrix attachment region-dependent, lymphocyte-restricted transcription through differential localization within promyelocytic leukemia nuclear bodies. *Embo J*, **19**, 4123-4133.