Spatial and dynamic organization of molecular structures in the cell nucleus

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CHAPTER 6

Summary and discussion

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The aim of this Thesis work was to gain insight into the spatio-temporal organization of the cell nucleus by using live cell imaging approaches. Chromatin domains and protein containing nuclear bodies were visualized in living cells by expressing fluorescently-tagged proteins that localize specifically to these compartments. Chromatin organization in the cell nucleus is nonrandom and its spatial organization is related to the transcriptional activity of genes. Besides chromatin, the cell nucleus contains variable numbers of different nuclear protein compartments, also referred to as nuclear bodies. These bodies are characterized by the presence of a distinct set of proteins although some proteins travel between two or more nuclear compartments. The specific protein content of nuclear bodies has linked them to various cellular activities, including transcription, RNA processing and to more general phenomena like apoptosis and senescence. Thus, the various nuclear bodies are believed to be essential in facilitating and coordinating specific nuclear activities. However, there is still a lot unknown about the underlying mechanisms that link bodies to specific nuclear activities, about the architecture of the nuclear bodies, and about the way nuclear bodies are formed. A key question is whether the spatial organization of nuclear compartments and the associated nuclear functions are supported by an underlying nuclear matrix structure. This question dominated the field of nuclear organization for many years and still remains elusive. Because the existence of a network structure resembling that of a cytoskeleton has not been demonstrated by immunocytochemistry many scientists doubt its existence in the cell nucleus. If such a structure exists it is very dynamic indeed and not comparable to the well described cytoskeleton architecture. Throughout this Thesis the term “nuclear matrix structure” has been used. By combining tools in live cell imaging, immunocytochemistry and molecular cell biology, this Thesis offers new insights in the structural organization of the cell nucleus and in the formation of one of the most intriguing nuclear bodies, the promyelocytic leukemia (PML) body.

Image analysis using STACKS

In order to understand the structural organization of the cell nucleus, it is essential to study the nuclear components in living cells as these components are dynamic in nature. To describe the dynamic properties of structures that reside in the cell nucleus we developed a software program called STACKS. Despite the fact that many software packages for quantitative image analysis were already commercially or freely available, we considered it important to develop a new software program in which the possibilities for image segmentation, object tracking, distance measurement, displacement quantification and cell movement correction are all integrated. Also, the program should offer fast image processing and enable user interaction. The features of this program are described in detail in chapter 2 and illustrated by describing the dynamic behavior of telomeres in normal as well as in tumor cells in different phases of the cell cycle. In general, when studying the dynamic behavior of structures inside the cell nucleus it is important to know whether this behavior is related to a specific cell cycle stage. For example, it has been shown in yeast, in which a centromere, a telomere, and an internal chromatin site were fluorescently tagged, that the internal chromatin loci moved more rapidly and over larger distances in G_1- and S-phase as compared to the other stages of the cell cycle (Heun et

al., 2001). Smaller, saltatory movements (<0.2 μm) were shown throughout interphase in both yeast and flies (Heun et al., 2001). Analysis of telomere movement by the STACKS program confirmed observations that telomeres move faster in G1 cells as compared to cells in other cell cycle stages. This was also expected because when cells exit from mitosis chromatin is unfolding and moves to its favored position in the cell nucleus.

As mentioned, the STACKS program contains various options for quantitative data processing and image transformations, which are not offered by the commercial software that comes with most wide-field and confocal microscopes. Because image analysis operations become complex and have to be performed on large data sets computation time is an issue. For this reason, the STACKS program allows GPU processing using the GPU processor of the video board. A direct comparison of image operations based on CPU and on GPU processing showed that GPU processing indeed greatly increases processing speed, which is necessary when large 4D image data sets have to be analyzed. The STACKS program is able to operate directly on data formats as produced by the commercial microscope systems, and has a convenient user interface. Furthermore, STACKS provides feedback on each operating step and offers great flexibility in order to cope with various cell types and images of varying quality or intensity. As research questions can be diverse, STACKS has been developed in a way that additional software tools can easily be added to the program, creating great flexibility for the user. In conclusion, STACKS is a major step ahead for scientists who wish to track and quantify the movements of objects in living cells or the movements of entire cells in cell or tissue cultures. In the coming years, the STACKS program will be developed further by adding specific applications to facilitate a broad spectrum of research questions.

Telomeres anchor at a protein complex containing lamin A/C, emerin and actin

In chapter 3 of this thesis the program STACKS is used to answer the question whether telomeres are associated with a nuclear matrix structure. To this end, cells have been incubated with shRNAs or drugs to disturb the nuclear matrix structure and movements of fluorescently labeled telomeres have been measured. Previous studies indicated that the movement of telomeres is constrained in the cell nucleus. In yeast, this constrained movement was explained by the attachment of telomeres to the inner nuclear membrane by the Ku and Sir proteins (Hediger et al., 2002). In human cells, however, telomeres are supposed to be associated to an inner nuclear matrix structure as they are not removed from cell nuclei after extensive extraction procedures that remove all soluble proteins and most DNA. Because this retention of telomeres could be an artifact of the extraction procedure, we wished to investigate whether a selective reduction of potential nuclear matrix proteins would increase the dynamics of telomeres by a lack of anchorage sites. RNA interference and specific drugs have been used to knock down or disturb proteins that are thought to be members of the nuclear matrix structure. We quantified the dynamics of telomeres using the program STACKS and found that latrunculin A-induced depolymerization of actin filaments resulted in an increase in telomere dynamics. Also a reduction of lamin A/C or emerin expression by RNA interference was shown to increase telomere dynamics. Although lamin B2 is also a constituent of the nuclear lamina structure, a reduction of lamin B2 did not result in an increase in telomere dynamics. These observa-

tions suggest that in mammalian cells telomeres are attached to a complex containing lamin A/C, emerin and actin proteins. Although lamin A/C and actin have been found present in the nucleoplasm, these findings raise many interesting questions. More experiments are needed to determine whether the association of telomeres with this complex is direct or indirect. Also, biochemical experiments need to be performed to address the full composition of the protein complex telomeres are attached to. Furthermore, it needs to be investigated whether telomeres are the only genomic regions that are anchored to lamin A/C containing protein complexes. These are important issues because dysfunctional telomeres and mutations in lamin proteins are associated with ageing and age-related diseases (Cooke & Smith, 1986; Merideth et al., 2008). Consistent with our observations, an increase in telomere mobility has been measured recently in fibroblasts derived from a patient carrying a mutation in lamin A (Vos et al., 2010).

A role for telomeres in *de novo* PML body formation

Besides chromatin, the cell nucleus contains nuclear bodies that vary in protein composition, number and shape. Knowledge about the function of these bodies is steadily increasing but still little is known about the mechanisms that lead to their formation. In chapter 4 the *de novo* formation of PML nuclear bodies is described. PML bodies disperse after treatment with the (DNA) alkylating agent methylmethane sulfonate (MMS) but reassemble when MMS is absent. Surprisingly, it was observed that PML bodies formed at telomeric DNA of U2OS cells and similar results were obtained in mouse embryonic fibroblasts. Also in cells that were derived from a patient suffering from acute promyelocytic leukemia and lack intact PML bodies we observed that expression of PML protein or treatment with arsenic trioxide resulted in the formation of PML bodies at telomeric sequences. The reason why the nucleation of PML bodies occurs at telomeres is currently not known. Initial experiments indicate that SUMO modification of telomere binding proteins might be involved. It was observed that SMC5, a component of the SUMO ligase MMS21-containing SMC5/6 complex, localizes temporarily at telomeric DNA during PML body formation. Obviously, this observation needs additional experimental support. A knockdown of SMC5/6 may confirm that the localization of this protein at telomeres is essential for PML body formation. Furthermore, it should be clarified which telomere binding proteins are sumoylated. Initial experiments were not conclusive. Knockdown of SMC5 by siRNAs prevented the formation of PML nuclear bodies after MMS treatment, but not in all cells. Furthermore, it needs to be investigated whether a reduction of SMC5 leads to a stress response preventing PML body formation. These and other experiments may unravel the mechanism by which PML bodies are formed *de novo*. This knowledge is important because PML bodies have been implicated in virus replication, monogenic transformation, cellular stress and senescence and could possibly play a role in age related disorders.

Chromatin is intimate with nuclear bodies

Nuclear bodies are positioned in the interchromatin space and an intermingling of chromatin with nuclear bodies, with exception of the nucleolus, has not been reported thus far. Nevertheless, nuclear bodies show in general little movement in the nucleus which might suggest that they interact with chromatin at their surface. Indeed, specific genomic regions have been shown to be associated with PML bodies, Cajal bodies or speckles. In

chapter 5 it is shown that during MMS treatment nuclear bodies become more dynamic in the cell nucleus while the dynamic behavior of chromatin is not altered. This suggests that due to MMS induced alkylating DNA damage, the interaction between nuclear bodies and chromatin is lost. To analyze the interaction of chromatin with nuclear bodies, cells have been cotransfected with constructs expressing different fluorescent marker proteins. One is a fluorescent fusion protein that localizes to a nuclear body and the other is a photoactivatable variant of GFP (PA-GFP) fused to a histone protein that is incorporated into chromatin. By selective activation of PA-GFP-histone proteins using 405 nm laser light the chromatin that surrounds nuclear bodies could be visualized selectively and tracked in time. The time-lapse images show clearly that nuclear bodies lose their contact with chromatin as a result of MMS treatment, suggesting that nuclear bodies are usually intimately connected with chromatin. What this means in functional terms has to be clarified yet. Recent work suggests that speckles function as nuclear hubs that connect various chromatin regions in order to facilitate and coordinate gene expression. Also, it is possible that some nuclear bodies are for their structural integrity dependent on chromatin.

A view of the cell nucleus at high resolution

Important insights in the spatial-temporal organization of the cell nucleus have mainly been obtained using wide-field fluorescence microscopy and fluorescence laser scanning confocal microscopy providing a spatial resolution in xy of about 200-300 nm and in z of about 500 nm. Using these imaging systems, detailed structural information about nuclear bodies and the way they interact with chromatin cannot be obtained. Electron microscopy offers the highest spatial resolution but its application in the study of nuclear bodies has been limited. The main reason is that the bodies show little or no contrast in standard EM preparations and as such cannot be identified. Immunogold labeling techniques have been used to identify nuclear bodies but the labeling is often poor and provides little architectural detail. One exception is the nucleolus whose architecture has mainly been resolved by EM studies.

Fascinating possibilities concerning high resolution imaging lay ahead with the development of high resolution optical imaging systems. Among others, important innovations have been developed by Stefan Hell by introducing Stimulated Emission Depletion (STED) microscopy, a technique that uses the non-linear de-excitation of fluorescent dyes to overcome the resolution limit imposed by diffraction with standard confocal laser scanning microscopes and conventional far-field optical microscopes (Hell et al., 1994). The resolution of a confocal scanning microscope is limited to the spot size to which the excitation spot can be focused. STED microscopy reduces the size of the excitation spot by using a short excitation pulse that is directly followed by a doughnut-shaped depletion pulse that acts only on excited dye molecules at the periphery of a spot and quenches them. As a result the fluorescence at the center of the doughnut remains unaffected and a 12-fold increase in spatial resolution can be obtained (Donnert et al., 2006). In addition to STED, Stefan Hell also pioneered 4Pi microscopy using two objective lenses opposing each other reaching an axial resolution of about 100 nm. The impact that high resolution microscopy can have on elucidating nuclear body architecture has recently been demonstrated by elucidating the ultrastructure of PML bodies using 4Pi fluorescence laser scanning microscopy (Lang et al., 2010). It was shown that the PML bodies consist

of a sphere having a 50 – 100 nm thick shell of PML and Sp100 proteins and that telomeric repeat DNA and HP1 was found positioned inside the PML bodies.

Forthcoming high resolution microscopy techniques are photo-activated localization microscopy (PALM) (Hess et al., 2006; Egner et al., 2007), a similar technique called stochastic optical reconstruction microscopy (STORM) (Betzig et al., 2006) and structured illumination microscopy (SIM) (Gustafsson, 2005). In PALM/STORM the imaging area is filled with many dark fluorophores that can be photoactivated into a fluorescing state by a flash of light. Because photoactivation is stochastic, only a few well separated molecules will "turn on." Then Gaussians are fit to their PSFs at high precision. After the few bright dots photobleach, another flash of the photoactivating light again activates a random collection fluorophores, and the PSFs that belong to these well spaced objects are determined. This process is repeated many times, building up an image molecule-by-molecule; and because the molecules were activated at different times, the precise localization of all fluorophores can be accurately determined by calculating their centers of mass. The result is that the resolution of the final image can be much higher than that limited by diffraction. With the PALM/STORM technique a resolution of 25 nm can be reached (Betzig et al., 2006; Rust et al., 2006). The image formation in SIM is based on the illumination of samples with patterned light resulting in interference patterns from which a multicolor high 3D (100 nm) resolution image can be reconstructed (Schermelleh et al., 2008). The main limitation of the PALM/STORM technique is that it is, like other high resolution imaging techniques, less suitable for analyzing dynamic structures in living cells. It is expected, however, that image acquisition and reconstruction will become much faster in the near future making PALM/STORM systems ideal tools for live cell imaging at high resolution. In this respect, faster image reconstruction software has been developed recently providing a 20-fold enhancement of the analysis routine (Hedde et al., 2009). The limitation that PALM/STORM imaging could be achieved in 2D only has been solved recently in a way that high resolution 3D images of cells can be obtained (Huang et al., 2008).

It should be mentioned that also correlative light-electron microscopy is a rapidly evolving field allowing the examination of fluorescently labeled objects in cells at nanometer-scale EM resolution. Currently, this approach is mainly used for analyzing complex structures in the cytoplasm of cells but it is in principle also applicable to structures that reside inside the cell nucleus (Lang et al., 2010). It is expected that with the rapid development of high, also mentioned super, resolution microscopy more detailed information about the structural organization of the cell nucleus will be obtained. This detailed knowledge about the structure of nuclear compartments is of crucial importance to understand the architecture of chromatin and nuclear bodies better, as well as the functional processes that are associated with these structures and dictate cellular function and behavior.

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