Law and Order in the Nucleus

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Although the nucleus is the hallmark of eukaryotic cells, we still know remarkably little about its structure and function. As a matter of fact, already in 1836 Gabriel G. Valentin described not only nuclei in neurons but also their most conspicuous structure, the nucleolus. Nuclei come in different sizes, shapes and numbers. Some eukaryotic cells have multilobed nuclei (e.g., polymorphonuclear leukocytes), others may have two or more nuclei (e.g., cardiac and skeletal muscle cells) while some have none (e.g., platelets and mature red blood cells). Cytologists have, for some time, recognized a few subnuclear structures and even related them with the metabolic state of a cell. The best example is the nucleolus, which reflects whether or not cells are actively producing protein. The distribution of chromatin within the nucleus and its overall morphology are now routinely used to distinguish cell types, sex and diseases. The initial discovery of a prominent mass of chromatin seen only in female cells by Murray L. Barr (1948), led to the identification of the inactive X chromosome in females and represented the beginning of a new science called human cytogenetics.

For a long time, the nucleus has been underestimated as a mere repository of the genetic information packed into chromatin, freely floating like noodles in a soup of amorphous nucleoplasm. Since there are no dividing membranes within the nucleus that would allow for further compartmentalization, as is the case in the better-studied cytoplasm, subnuclear compartments were not expected. However, in the last decades the development of antibodies to nuclear components combined with the ability to fluorescently tag proteins uncovered a different picture of the nucleus with many discrete and distinguishable subnuclear compartments involved in DNA or RNA metabolism (reviewed in Leonhardt and Cardoso, 1995). Studies on chromatin architecture using initially mitotic chromosomes and later, with the development of in situ hybridization techniques, also interphase chromatin indicated a high degree of organization into discrete compartments called chromosome territories (reviewed in (Cremer and Cremer, 2001). A graphic summary of different subnuclear compartments is shown in Figure 1. This surprising discovery of nuclear structures raised the question how this organization of chromatin and nuclear proteins is actually achieved.

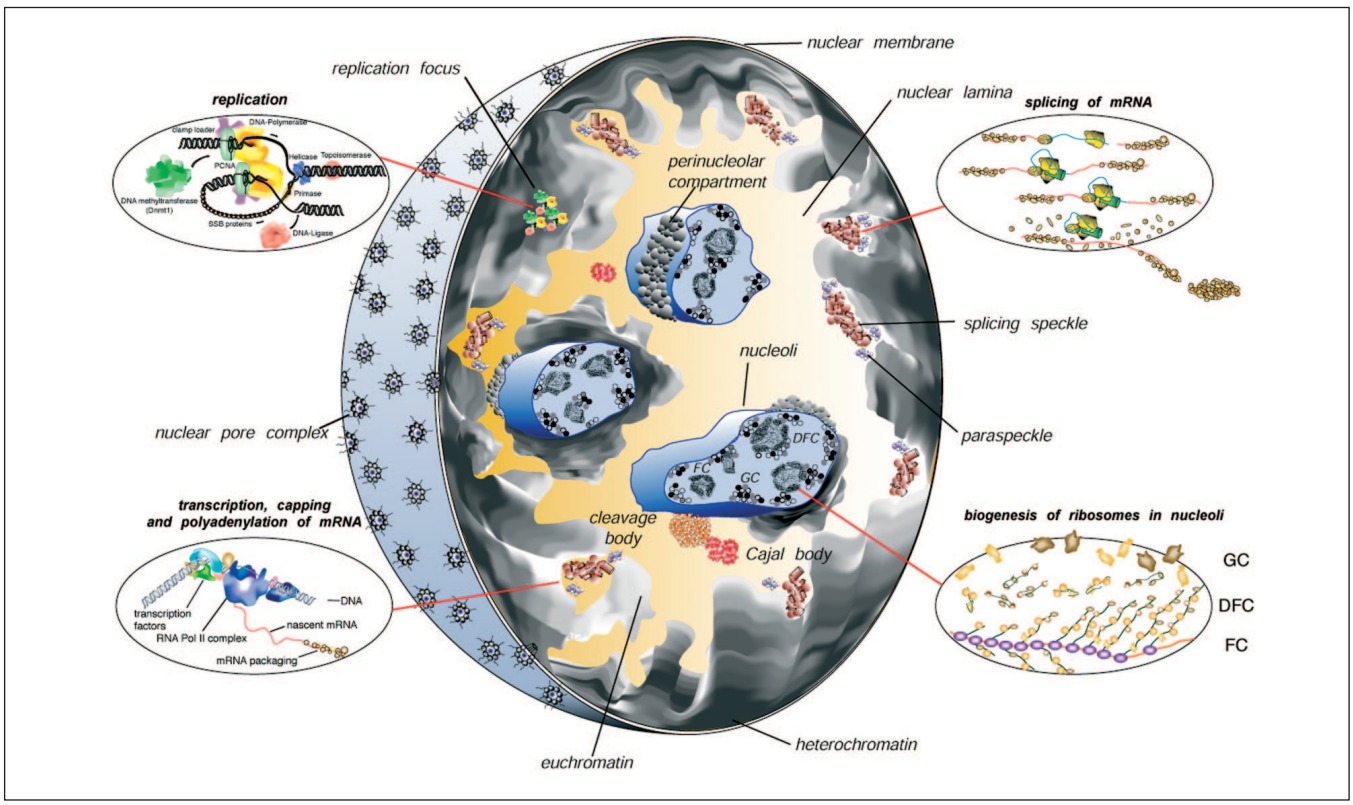

Fig. 1: *Law and order in the nucleus.*
The graphic illustrates several of the different subnuclear compartments identified in the nucleus with some of their functions highlighted and their relation to interphase chromatin organization. (Graphic courtesy of Robert Martin)

Nuclear organization and genome replication

Our interest in this field stems from ear-

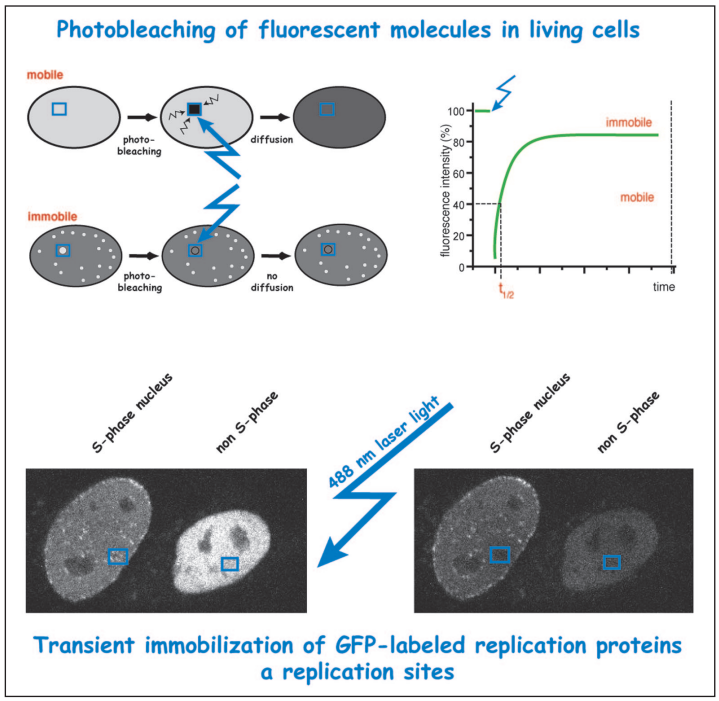

Fig. 2: *Dynamics of replication factors in living cells.*
In spot photobleaching experiments, a short intense pulse of laser light is used to render a fluorophore irreversibly non-fluorescent within a small micrometer-size region. Images are acquired before and after photobleaching and the fluorescence intensity is monitored as a function of time (see fluorescence intensity plot). Recovery of fluorescence occurs as a result of the exchange between bleached and unbleached population of the fluorescent protein and the fraction of fluorescent protein participating in this exchange is called mobile (top example). Immobile fluorescent proteins not moving during the duration of the experiment in the cell nucleus, are either attached to the nuclear envelope or to insoluble subnuclear structures (bottom example). Quantitative determination of the final fluorescence intensity relative to the initial fluorescence intensity gives information about the amount of fluorescent protein that is mobile. The time point after photobleaching where half of the final fluorescence intensity (t1/2) is regained can be used to calculate effective diffusion coefficients of the fluorescent proteins and thus reveal the dynamics underlying the steady-state distribution of this protein inside living cells.
The confocal images below depict two cells expressing GFP-tagged PCNA before and after photobleaching (blue square in both cell nuclei). While the cell in non S-phase lost during the bleaching period most of its fluorescence, the S-phase nucleus lost fluorescence locally at the photobleached area. This side-by-side example clearly shows that while the replication factor PCNA diffuses rapidly in the nucleoplasm of non-replicating cells it gets transiently immobilized at replication sites during S-phase.

lier work on terminally differentiated cells, which were induced to reenter the cell cycle. Using antibodies to proteins involved in cell cycle regulation and DNA synthesis, we found a cell cycle specific localization of some of them at nuclear sites of active DNA replication (Cardoso et al., 1993). In particular, we could show that the cell cycle factors cyclin A and cdk2 are concentrated together with replication factors (RPA and PCNA) at subnuclear replication sites. These results provided a temporal and spatial link from cell cycle regulation to DNA replication.
We extended this work by analyzing the cell cycle distribution of other replication factors and mapping sequences responsible for association with the replication foci. This lead to the identification of the replication foci targeting sequence in the DNA ligase I enzyme, which ligates the Okazaki fragments generated during lagging strand DNA synthesis (Cardoso et al., 1997; Montecucco et al., 1998). Likewise, targeting sequences have been mapped in nucleolar proteins, splicing factors, coilin, and proteins involved in the replication of the genetic and epigenetic information reviewed in (Cardoso and Leonhardt, 1998). These targeting sequences are often separated from the catalytic domains of the enzymes indicating that the localization of these factors at replication foci is not simply due to high substrate concentration but rather represents an active organization into large protein complexes also called factories. These subnuclear structures thus integrate and couple the numerous enzymatic activites requir-ed for genome replication.

Dynamics of replication proteins and other nuclear factors

To study the dynamic regulation of these nuclear structures during the cell cycle in vivo and in real time, we have established an approach for the visualization of DNA replication in living cells using translational fusions of different replications factors to green (GFP) or red fluorescent proteins (DsRed). Using cell lines expressing a GFP fusion to PCNA (proliferating cell nuclear antigen, a central component of the replication machinery) we could show by time lapse microscopy that replication foci patterns change throughout S-phase in a characteristic manner and that the changing patterns of replication foci are not due to movements of foci through the nucleus. Instead, changes occur by assembly and disassembly of foci throughout S phase (Leonhardt et al., 2000). In contrast, other subnuclear structures, like e.g., Cajal bodies involved in RNA metabolism, are very dynamic exhibiting large-scale movements within the nucleus (Boudonck et al., 1999).
To investigate whether the replication factors remain stably bound at replication foci or whether they are in constant exchange, we have used biochemical in situ extractions as well as fluorescence photobleaching techniques. The principle of photobleaching techniques and its application to determine the mobility of fluorescent macromolecules in living cells is explained in Figure 2. Both experimental approaches showed that the PCNA clamp was tightly bound at replication sites, showing only little exchange if any. In stark contrast, PCNA was highly mobile in the nucleoplasm of non-replicating cells (Figure 2). A comparison with another replication factor RPA (single-stranded DNA-binding protein) involved in the initiation of DNA replication, showed that while RPA exchanged in a time frame of seconds, PCNA showed virtually no turnover within several minutes (Sporbert et al., 2002). This has lead us to propose an alternative model for DNA replication, whereby the PCNA clamp stays bound throughout the synthesis of several Okazaki fragments. That could be achieved, as it was suggested for the DNA polymerase, by coupling the leading and lagging strand PCNA-polymerase complex together. We are currently testing this model by simultane-

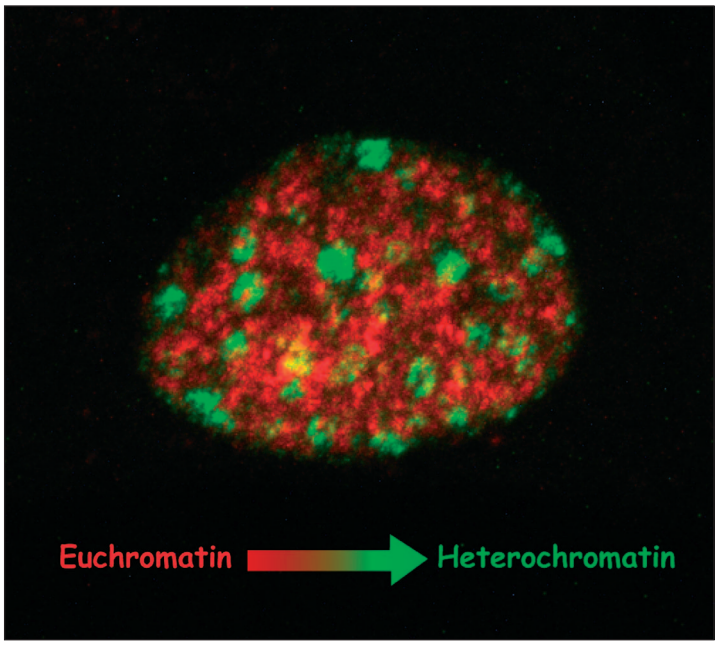

Fig. 3: *Genome replication program.*
Mammalian cells expressing a GFP tagged DNA ligase I were pulse labeled for 10 minutes with the thymidine analog bromodeoxyuridine, followed by chase with 10-fold excess of thymidine. Three hours later, the cells were fixed and the incorporated nucleotide detected with specific antibodies (red) together with simultaneous detection of the GFP-DNA ligase I (green) localization. The image shows a projection of a z-stack of confocal images.
The early replicating chromatin (labeled with the nucleotide) consisting mostly of euchromatic regions is shown together with the late replicating chromatin (labeled three hours later with the tagged replication protein) comprising mostly constitutive heterochromatic centers. This spatial and temporal program of replication is followed at every cell cycle. (Image courtesy of Anje Sporbert)

ously measuring the on/off rate of PCNA and PCNA-binding replication factors in living cells. Since these fluorescence imaging techniques are based on averages of thousands of molecules we are now, in collaboration with the groups of U. Kubitscheck and H. Leonhardt, tracing single molecules within living cell nuclei (Kues et al., 2001).

Genome replication program

Replication of the mammalian genome starts at tens of thousands of origins that are activated at specific times during S phase raising the question how this replication program is coordinated. Fluorescence photobleaching analyses showed that the transition from earlier to later replicons occurs by disassembly into a nucleoplasmic pool of rapidly diffusing subcomponents and reassembly at newly activated sites. A careful examination of the temporal and spatial assembly of new PCNA molecules by overlaying the images collected at consecutive times indicated that PCNA assembled in non-overlapping sites. These replication sites were in close proximity to earlier ones suggesting that activation of neighboring origins may occur by a domino effect possibly involving local changes in chromatin structure and accessibility (Sporbert et al., 2002). We are now trying to dissect the mechanism underlying the ordered activation of later replication origins and setting the replication program (Figure 3).

Last not least, at every cell division cycle, not only the genetic but also the epigenetic information has to be accurately replicated. This involves the disruption and reestablishment of chromatin structures as well as the maintenance of DNA methylation patterns. Both processes have to be coordinated with DNA replication opening windows of opportunities to introduce changes. We are, therefore, analyzing different proteins involved in the maintenance and change of epigenetic information and their dynamic interaction with replication machinery. Both, the replication of genetic and epigenetic information are required for stable gene expression patterns and genome stability. Our longterm goal is to study the architecture, assembly and regulation of these replication factories throughout the cell cycle including their interaction with other nuclear components and processes. Genome replication with its links to chromatin structure, gene expression, DNA repair and cell cycle regulation is more than ever an exciting field of research. We hope this work will ultimately contribute to the understanding of the principles connecting structure and function in this fascinating organelle we have seen for so long and still know so little about.

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