Nucleolus disassembly in mitosis and apoptosis: dynamic redistribution of phosphorylated-c-Myc, fibrillarin and Ki-67

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The nucleolus may undergo disassembly either reversibly during mitosis, or irreversibly in apoptosis, thus allowing the redistribution of the nucleolar proteins. We investigated here by immunocytochemistry the fate of three representative proteins, namely phosphorylated c-Myc, fibrillarin and Ki-67, and found that they behave independently in both processes: they relocate in distinct compartments during mitosis, whereas during apoptosis they may either be cleaved (Ki-67) or be extruded into the cytoplasm with a different kinetics and following an ordered, non chaotic program. The separation of these nucleolar proteins which occurs in early apoptotic nuclei continues also in the cytoplasm, and culminates in the final formation of apoptotic blebs containing different nucleolar proteins: this evidence confirms that the apoptotic bodies may be variable in size, content and surface reactivity, and include heterogeneous aggregates of nuclear proteins and/or nucleic acids.

Key words: apoptosis; fibrillarin; Ki-67; mitosis; nucleolus; phosphorylated-c-Myc.

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The nucleolus is a large nuclear domain directly involved in ribosome biosynthesis, that is a complicated process entailing the transcription of ribosomal genes (rDNA), the processing of rRNA and their assembly with ribosomal proteins to form the large and small ribosome subunits (see Gébrane-Younès *et al.,* 2005, for a recent review). A large number of proteins participate in these different steps, transiently interacting with either rDNA or rRNA, or becoming structural components of the mature subunits. In a recent review, Olson and Dundr (2005) suggest that the nucleolus is a highly dynamic organelle in which the protein movements should be necessary not only as a structural background for the ribosome factory, but also for the communication with other nuclear and cytoplasmic components. The structural integrity of the nucleolus is essential for the whole nuclear function; it is widely accepted that changes in nucleolar organization may represent diagnostic markers for different pathologies (Zimber *et al.,* 2004).

Nowadays, it is known that about 700 proteins (many with no apparent role in ribosome assembly) may either be stored or transiently localized within the nucleolus (Andersen *et al.,* 2005; see the database www.lamondlab.com/Nopdb). This is consistent with the present view that during interphase the nucleolus may serve a wide variety of functions besides ribosome assembly (Olson and Dundr, 2005; Hernandez-Verdun, 2006), including that of stress sensor. Rubbi and Milner (2003) suggested a nucleolar role in controlling p53 level (which suddenly increases whenever nucleolar function is impaired), while we recently proposed to consider this organelle as the *old guard* of nuclear homeostasis (Biggiogera *et al.,* 2004).

In eukaryotes, the nucleolus is disassembled when cells enter mitosis, while undergoing reassembling at the exit from cell division (Thiry and Goessens, 2004; Hernandez-Verdun, 2006). During prophase, nucleoli decrease in size and segregate, the granular

component (GC) undergoing disintegration; in metaphase, the silver-stained nucleolar material associates to the secondary constrictions of NORs-bearing chromosomes, while several RNPs and nucleolar proteins redistribute over the surface of all chromosomes (Thiry and Goessens, 2004); as a result, both rDNA transcription and rRNA processing are repressed. Conversely, at the transition from mitosis to the following interphase, the rDNA transcription machinery is reactivated and the reassembly of nucleoli allows rRNA processing to be resumed (reviewed by Hernandez-Verdun, 2006). Accordingly, nucleolar protein components may follow different redistribution pathways during the reversible mitotic disassembly of nucleoli.

The aim of the present investigation was to compare the dynamic mitotic redistribution of some nucleolar components with their rearrangement during apoptosis, when the nucleolus is irreversibly disassembled. In fact, during apoptosis, the dense fibrillar component (DFC) and the GC segregate and in late apoptosis, nucleolar remnants may also aggregate with other nucleoplasmic RNPs and even move into the cytoplasm in the form of Heterogeneous Ectopic RNP-Derived Structures (Biggiogera *et al.,* 2004). Remarkably, the extrusion from the nucleus of a wide and heterogeneous spectrum of proteins which survive in a partially degraded (or even in an undegraded) form during the late steps of apoptosis, legitimates the growing interest toward those novel and ectopic molecular complexes which may play a role in the ethiology of autoimmune diseases (Cline and Radic, 2004; Pellicciari *et al.,* 2005).

For the present study, three nucleolar proteins have been selected, which localize in nucleoli during interphase, namely fibrillarin that is considered as a marker of the nucleolar DFC, and Ki-67 and phosphorylated c-Myc, which are involved in cell proliferation control. In its unphosphorylated form, c-Myc predominantly locates in the cytoplasm of interphase resting cells, whereas, once phosphorylated (P-c-Myc), it exhibits a nuclear localization in proliferating cells. c-Myc is a leader factor in governing cell growth, proliferation and apoptosis (Pelengaris *et al.,* 2002; Amati, 2004; Secombe *et al.,* 2004; Vervoorts *et al.,* 2006), and plays a crucial role in regulating rRNA synthesis (Oskarsson and Trumpp, 2005). We have recently demonstrated that, possibly due to the inhibition of the ubiquitin-mediated degradation pathway (Secombe *et al.,* 2004), P-c-Myc accumulates in the nucleolus of tumor cells, where it colocalizes with fibrillarin (Soldani *et al.,* 2002): this is a small nucleolar ribonucleoprotein (RNP) involved in the early steps of rRNA processing and specifically located in the nucleolar DFC and in the Cajal bodies (Thiry and Goessens, 2004). As far as we are aware, mitotic redistribution of P-c-Myc and fibrillarin has never been described. Ki-67 protein too mostly associates to the DFC in interphase, but disperses from the nucleoli during prophase to redistribute (during metaphase-anaphase) at the chromosome periphery. Ki-67 is essential for the cell progress along the cycle (Brown and Gatter, 2002) and its immunocytochemical detection is widely considered as a suitable marker of the cell growth fraction (Endl *et al.,* 2001; Navarrete *et al.,* 2005); however, little is known on its specific function (Endl and Gerdes, 2000). To track the fate of P-c-Myc, fibrillarin and Ki-67 in HeLa cells, we used confocal fluorescence microscopy after dual-color immunolabeling.

Materials and Methods

Cells and treatments

HeLa cells were grown in D-MEM containing 10% fetal bovine serum, 2 mM glutamine and 100 units each of streptomycin and penicillin (Celbio, Italy) at 37°C in a humidified atmosphere containing 5% CO_2. For immunocytochemistry, the cells were seeded on glass coverslips whereas for Western blotting experiments they were grown in 25 cm^2 flasks. 24 h after seeding, when they were actively proliferating, cell cultures were treated for 20 h with either 1 µg/mL actinomycin D or 10 µM etoposide, to induce apoptosis. Control and treated cells on coverslips were fixed with 4% formaldehyde in phosphate buffered saline (PBS) and kept in 70% cold ethanol until use.

Immunocytochemistry

Samples were permeabilized for 15 min in PBS containing 0.1% bovine serum albumin and 0.05% Tween-20, and then immunolabeled for P-c-Myc and α-tubulin as follows: incubation with a polyclonal antibody recognizing P-c-Myc (Cell Signaling and Technology, Celbio, Italy, diluted 1:100) and with a goat anti-rabbit IgG conjugated with Alexa 594 (Molecular Probes, Invitrogen, Italy, diluted 1:200); incubation with a monoclonal antibody recognizing α-tubulin (Molecular Probes, diluted 1:100) then

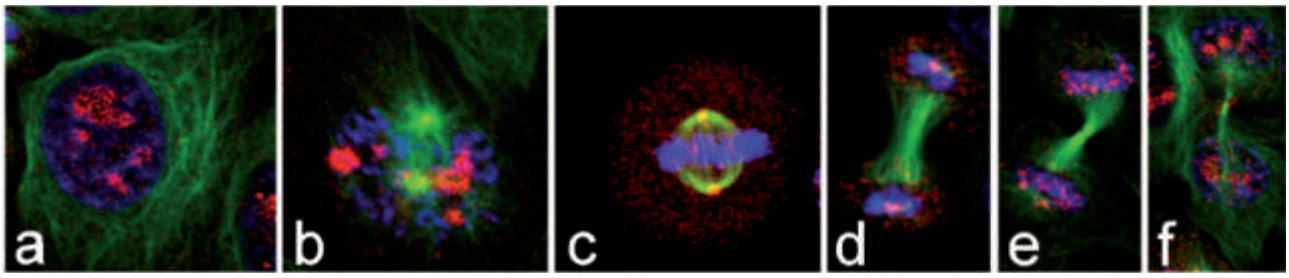

Figure 1. Redistribution of P-c-Myc during mitosis in HeLa cells. Confocal images of dual-immunolabeling for P-c-Myc (red fluorescence) and α-tubulin (green); DNA was counterstained with Hoechst 33258 (blue). a: interphase; b: late prophase; c: metaphase; d,e: ana-telophase; f: cytodieresis.

revealed with an anti-mouse secondary antibody conjugated with Alexa 350 or 488 (Molecular Probes, diluted 1:200) As a control, some slides were incubated in solutions without the primary antibodies or containing 0.1% rabbit or mouse serum, and finally exposed to the appropriate secondary antibody. All the incubations were performed at room temperature for 1 h and cells were finally counterstained for DNA with 0.1 µg/ml of Hoechst 33258 for 10 min, and mounted in a drop of Mowiol (Calbiochem, Inalco, Italy). Dual-immunolabeling experiments for the nucleolar proteins were also performed. After rehydration and permeabilization as described above, cells were incubated with the polyclonal antibody recognizing P-c-Myc and then with Alexa 488-conjugated goat anti-rabbit- IgG. After washings with PBS, the cells were incubated with either a human autoimmune serum recognizing fibrillarin (diluted 1:800) or with an anti-Ki-67 MoAb (diluted 1:50); fluorescent labeling was obtained using, respectively, an Alexa 594-conjugated goat anti-human-IgG or an Alexa 594-conjugated goat anti-mouse-IgG, (Molecular Probes, diluted 1:200). All the incubations were performed at room temperature for 1 h and cells were finally counterstained for DNA with 0.1 µg/mL of Hoechst 33258 for 10 min, and mounted in a drop of Mowiol.

Fluorescence confocal microscopy

For confocal laser scanning microscopy, we used a Leica TCS-SP system mounted on a Leica DMIRBE inverted microscope; for fluorescence excitation, an Ar/UV laser at 364 nm was used for Hoechst 33258, Ar/Vis laser at 488 nm for Alexa 488 and He/Ne laser at 543 for Alexa 594. Spaced (0.5 µm) optical sections were recorded using a 63x oil immersion objective. Images were collected in the 1024×1024 pixels format, stored on a magnetic mass memory and processed by the Leica Confocal Software.

Western Blot

After treatments, cells were washed twice with ice-cold PBS and resuspended at the concentration of 10×10^6/mL in the SDS loading buffer, according to a described procedure (Donzelli *et al.*, 1999). Cells were disrupted by sonication on ice, twice for 30s (60W) and heated for 5 min at 90°C. Samples were electrophoresed in a 7.5% or 12% SDS-PAGE minigel and transferred onto a nitrocellulose membrane (BioRad, Hercules, CA) by a semidry blotting for 1.45 h under a constant current of 70 mA. The membranes were saturated overnight with PBS containing 0.2% Tween-20 and 5% skim milk, and incubated for 1 h with the primary antibodies (anti-P-c-Myc, 1:1000; anti-fibrillarin, 1:8000; anti-Ki-67, 1:500). After several washings, the membranes were incubated for 30 min with the proper secondary antibodies conjugated with horseradish peroxidase (Dako, Italy). Visualization of immunoreactive bands was performed by an ECL System and Hyperfilm Photografic Film (Amersham Life Sciences, Little Chalfont, UK) using the manufacturer's instructions.

Results

To better elucidate the behaviour of the nucleolus during mitosis, we first addressed the distribution of P-c-Myc in HeLa cells during the different mitotic phases; then, we performed dual-color fluorescence immunolabelings, to describe the redistribution of P-c-Myc and fibrillarin or Ki-67 by colocalization experiments. Figure 1 shows the dynamic behaviour of P-c-Myc; in interphase P-c-Myc (red fluorescence) is confined to nucleoli (Figure 1a); during late prophase stage (b), when the chromosomes are visible, the P-c-Myc immunofluorescence overlaps the nucleolar shape. During metaphase (c), P-c-Myc is mainly located at the spindle peripolar regions, a few discrete immunopositive spots being also present in association with still undefined regions of the

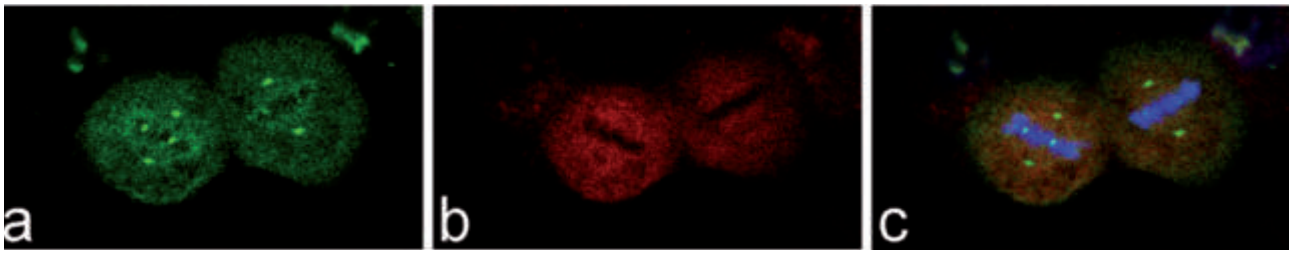

Figure 2. Dual immunolabeling of P-c-Myc and fibrillarin in metaphase cells. a: P-c-Myc, green fluorescence; b: fibrillarin, red fluorescence; c: merged image. DNA was counterstained with Hoechst 33258 (blue fluorescence).

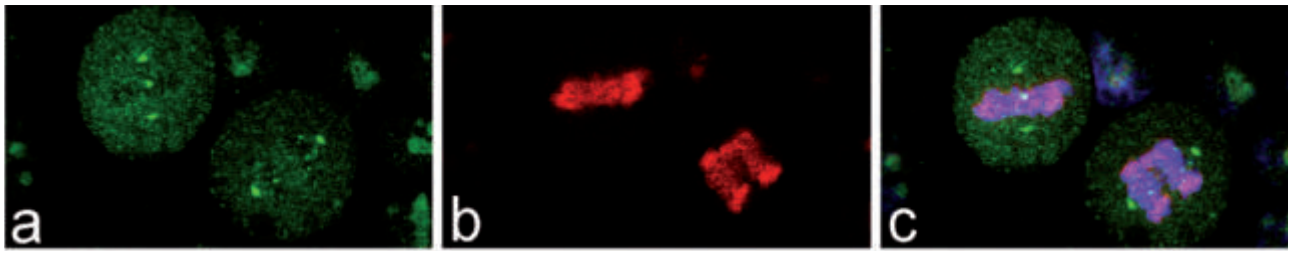

Figure 3. Dual immunolabeling of P-c-Myc and Ki-67 in metaphase cells. a: P-c-Myc, green fluorescence; b: Ki-67, red fluorescence; c: merged image. DNA was counterstained with Hoechst 33258 (blue fluorescence).

chromosomes; a weak diffuse fluorescence around the metaphase chromosomes was also observed. A similar immunolabeling for P-c-Myc was observed in anaphase (d), when the units of each metaphase chromosome move in opposite directions. In telophase (e), the fluorescent P-c-Myc spots increase in size and are exclusively located on the chromosomes. During cytodieresis (f), the immunolabeling of P-c-Myc finally reverts to the nucleolar location. In metaphase, the three proteins, P-c-Myc, fibrillarin and Ki-67 behave independently (Figures 2,3): P-c-Myc is mainly located at the spindle peripolar regions, a few discrete immunopositive spots being also present in association with the chromosomes (Figures 2a. 3a), whereas fibrillarin is diffusely distributed throughout the cytoplasm (Figure 2b), and Ki-67 relocates at the chromosome periphery (Figure 3b).

To induce apoptosis, HeLa cells were treated by either etoposide (a topoisomerase II inhibitor, which directly interferes with DNA molecules) or actinomycin D (an inhibitor of RNA-polymerase I, which mainly blocks rDNA transcription). Firstly we investigated immunocytochemically the distribution of P-c-Myc: it may be recognized within the cytoplasmic aggregates that are found in early apoptotic HeLa cells when chromatin starts to condense (Figure 4a,b). In fact, after treatment with either etoposide (a) or AMD (b), there was an increase of P-c-Myc-immunopositive spots in the nucleoplasm, which occurs in parallel with the chromatin damage after these different apoptotic stimuli (Fraschini *et al.,* 2005). In late apoptosis (Figure 4c,d), P-c-Myc is finally extruded in a still immunodetectable form within compact clusters in the apoptotic bodies blebbing at the surface, both after etoposide (c) or AMD (d). Consistent with the reports that during apoptosis proteolysis of nucleolar proteins is not an obligatory event (Horky *et al.,* 2002), western blot analysis (Figure 5) revealed that under conditions of PARP-1 cleavage (Soldani *et al.,* 2002; Biggiogera *et al.,* 2004; Fraschini *et al.,* 2005), Ki-67 is degraded in apoptotic cells whereas fibrillarin and P-c-Myc are not cleaved.

Discussion

The aim of the present study was to follow the dynamic behaviour of three nucleolar proteins, namely the phosphorylated c-Myc, fibrillarin and Ki-67, during reversible and irreversible disassembly of nucleoli. In fact, during mitosis the nucleolus breaks down with a temporaneous relocation of its components to different cell regions; conversely, apoptosis is accompanied by the final disruption of nucleolar structure and function. Figure 6 summarizes the results of our study. The micrographs in this figure refer to etoposide-treated HeLa cells, but similar data have been obtained after actinomycin D treatment (*not shown*). For sake of simplicity, we schematically defined three morphological stages of

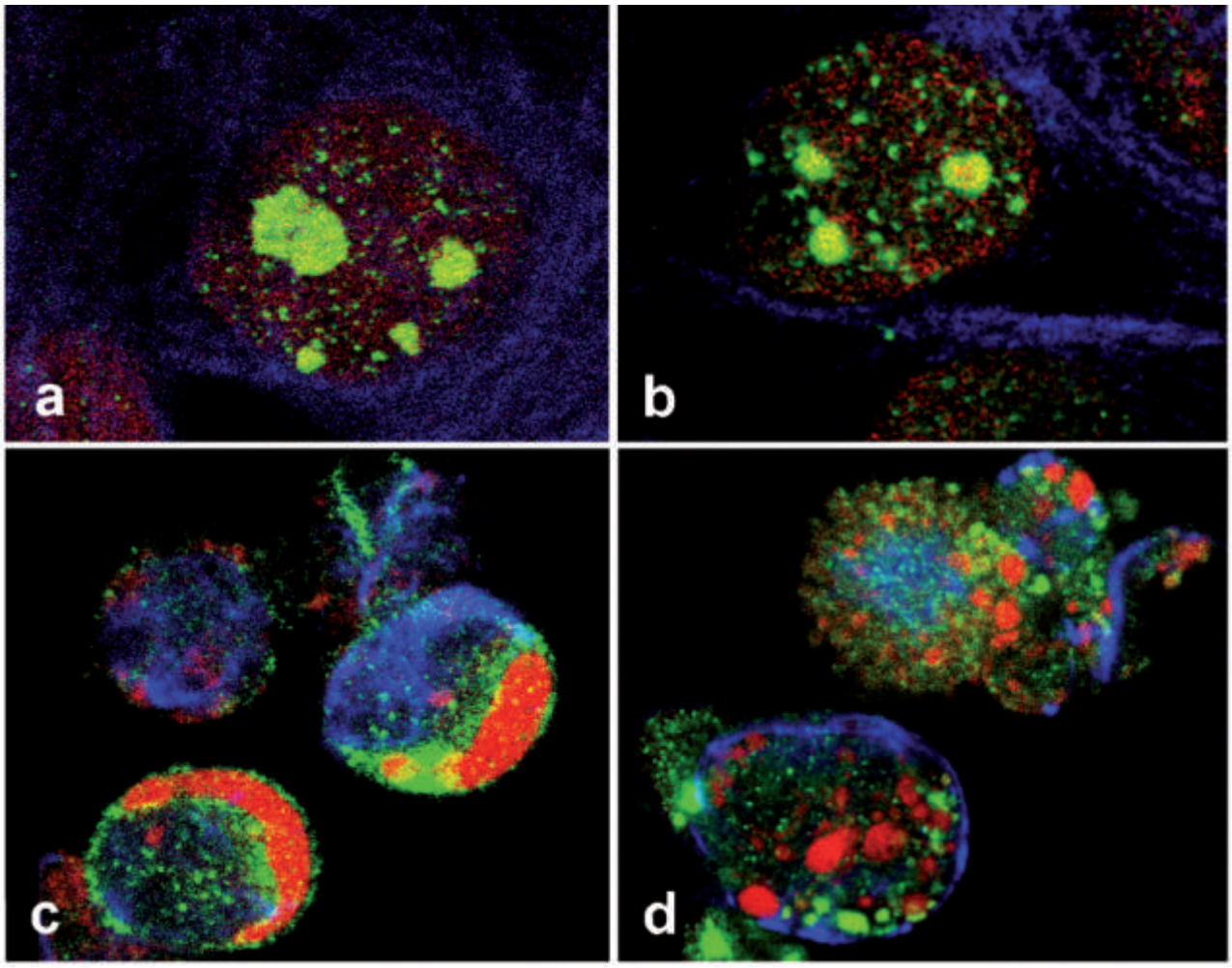

Figure 4. Distribution of P-c-Myc in apoptotic HeLa cells. Apoptosis was induced with etoposide (a,c) or actinomycin D (b,d) under the conditions described in Materials and Methods. Dual immunolabeling for P-c-Myc (green fluorescence) and α-tubulin (blue); DNA was stained with propidium iodide (red fluorescence). a,b: early apoptotic cells; c,d: late apoptosis with chromatin margination and nuclear fragmentation.

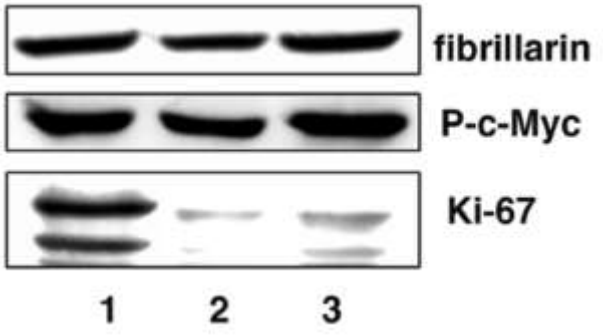

Figure 5. Western blot analysis of P-c-Myc, fibrillarin and Ki-67. 1: control HeLa cells; 2: etoposide-treated cells, 3: actinomycin D-treated cells.

apoptosis: early (with incipient chromatin condensation: *early*APO, in the Figure), intermediate (with marginated chromatin and cell surface blebbing: *middle*APO), and late (with karyorrhexic nuclei and massive blebbing: *late*APO). In early apoptosis, the immunolabelings for Ki-67 and fibrillarin were as during interphase, whereas P-c-Myc started moving to the nucleoplasm, as discrete immunopositive dots. In middle apoptosis, Ki-67 immunopositivity was lost; concomitantly, fibrillarin was extruded into the cytoplasm whereas P-c-Myc was still detectable in the interchromatin space as a diffuse signal. At late apoptosis, both fibrillarin and P-c-Myc were only found in the cytoplasm: P-c-Myc mainly formed dense aggregates close to the nuclear fragments, whereas fibrillarin was located in the peripheral cytoplasmic blebs.

Although nucleoli are stably organized and contain a structural framework, they are also highly dynamic. During mitosis, as the process of chromosome condensation proceeds, RNA synthesis either ceases completely or decreases sharply and resumes

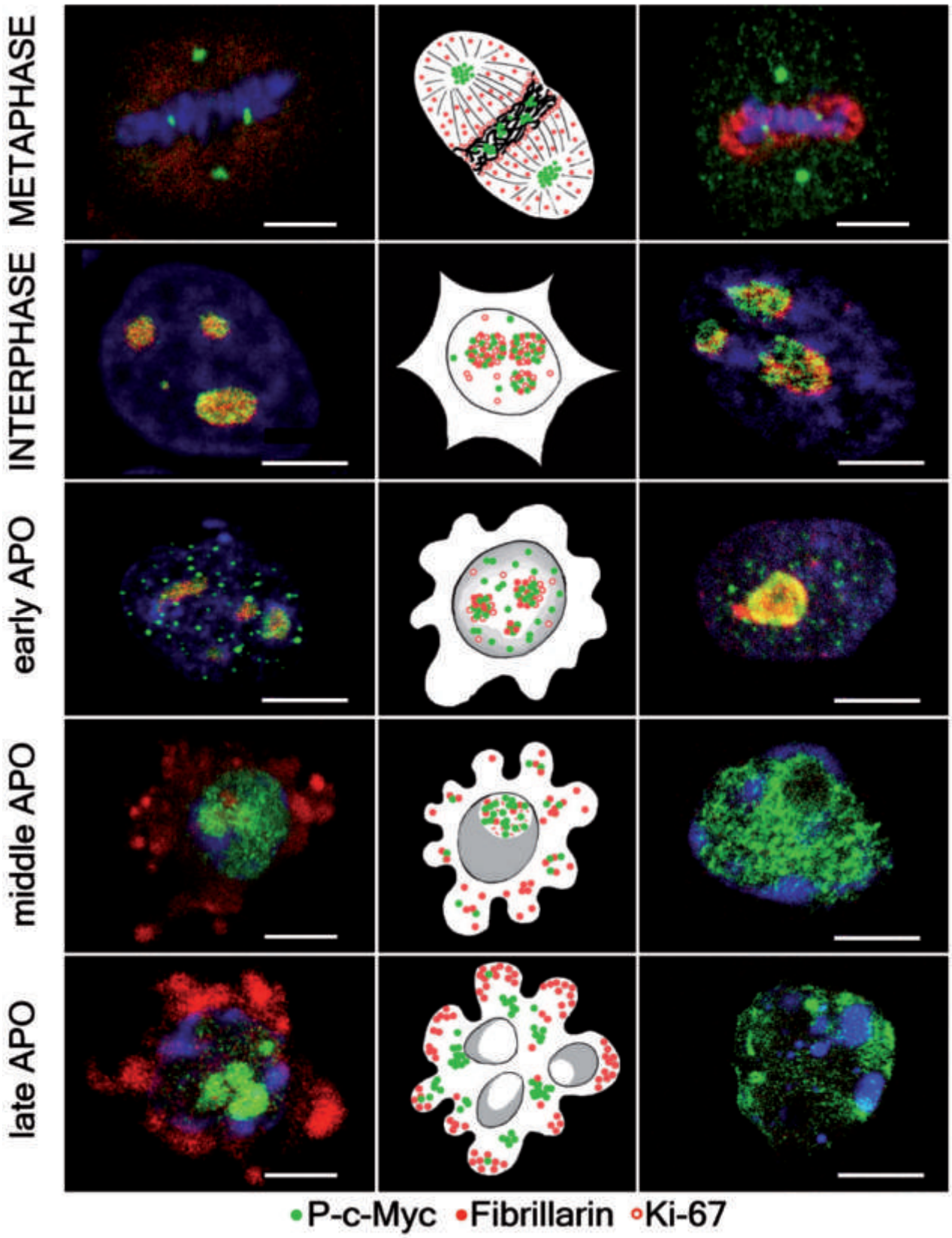

Figure 6. Localization of P-c-Myc, fibrillarin and Ki-67 in mitosis and apoptosis. Left column: double immunolabeling for P-c-Myc/fibrillarin (green/red fluorescence). Right column: double immunolabeling for P-c-Myc/Ki-67 (green/red fluorescence). DNA was counterstained with Hoechst 33258. Bars: 5 µm. Central column: schematic drawing of the distribution of the three nucleolar proteins in interphase and metaphase and at different apoptotic stages.

in late telophase. These changes in RNA synthesis explain why the *disappearance* of the nucleoli at the end of prophase and their reconstitution in daughter cells during telophase are common events of somatic cell division. In particular, during mitotic prophase nucleoli disintegrate and their various constituents are released and become differentially distributed within the dividing cells (Goessens, 1984; Scheer and Benavente, 1990). Immunocytological and *in situ* hybridization studies have clearly shown that the fate of several nucleolar components during mitosis depends on their functional roles in ribosome biogenesis. Many elements involved in rDNA transcription remain bound to the nucleolar organizer regions (NORs), while the nucleolar constituents not involved in rDNA transcription disperse in the cytoplasm during methapase. During telophase, the disparate nucleolar constituents rapidly reassemble in an apparently coordinate fashion and accumulate in the NORs, contributing to the reformation of functional nucleoli in the daughter cells (Scheer and Bonavente, 1990).

The definition of P-c-Myc distribution during mitotic progression represents an original set of data; in fact, much is known about the pattern of the unphosphorylated form of c-Myc (Smith *et al.,* 2004; Arabi *et al.,* 2005; Oskarsson and Trumpp, 2005) but the knowledge on its phosphorylated counterpart was far from being exhaustive, till now.

Immunocytochemistry at electron microscopy demonstrated that fibrillarin was localized exclusively in the fibrillar region of the nucleolus which includes both DFC and the fibrillar centers (Soldani *et al.,* 2002; Hernandez-Verdun, 2006). In metaphase and anaphase, immunolabeling for fibrillarin has been described on putative chromosomal NORs, while during telophase fibrillarin is considered as an early marker for the site of assembly of the newly forming nucleoli (Gébrane-Younès *et al.,* 2005; Hernandez-Verdun, 2006). Our experiments (Figure 2) showed that from prophase through metaphase, the immunolabeling for fibrillarin is mostly in the cytoplasm of HeLa cells (Figure 2), where it colocalizes with P-c-Myc. This observation suggests that the colocalization of P-c-Myc and fibrillarin is largely maintained also during mitosis. No apparent colocalization was found at the pericentriolar regions and at chromosome level: this suggests that the DFC reorganizes during mitosis with a partially independent redistribution of these two different protein components. It was previously reported that the intranuclear distribution of Ki-67 changes during the cell cycle, including mitosis (reviewed by Ross and Hall, 1995). We have shown here that during all the mitotic phases, Ki-67 relocates at the chromosome periphery as a typical 'chromosome passenger' protein (Hernandez-Verdun and Gautier, 1994), and here remains until the anaphase (Figure 3). No colocalization with P-c-Myc was ever visible during mitosis. To investigate the fate of P-c-Myc, fibrillarin and Ki-67 during apoptosis, we have treated asynchronous HeLa cell cultures with etoposide or AMD, under conditions that we have shown to activate a caspase-dependent apoptotic process (Biggiogera *et al.,* 2004). Previous studies about the destiny of nuclear RNP proteins during apoptosis demonstrated that already in early apoptotic cells, RNPs of both nucleolar and non-nucleolar origin segregate in the interchromatin space to form heterogeneous clusters called HERDS (for Heterogeneous Ectopic RNP-Derived Structures), which then may move into the cytoplasm (Biggiogera and Pellicciari, 2000).

It is known that several hundred proteins are specifically confined to the nucleolus, including histones and non-histone proteins, ribosomal and non-ribosomal proteins, enzymes (Andersen *et al.,* 2005). A few of these proteins have been well characterized (e.g. nucleolin or C23, B23, fibrillarin), although we have only hints about their precise biological functions. Consistent with the reports that during apoptosis proteolysis of nucleolar proteins is not an obligatory event (Horky *et al.,* 2002), western blot analysis revealed that Ki-67 is degraded in apoptotic cells whereas fibrillarin and P-c-Myc are not cleaved. Our data are in agreement with the report of Martelli *et al.* (2000), showing that in camptothecin-treated HL60 cells only UBF (Upstream Binding Factor) is proteolyzed, whereas fibrillarin, nucleolin and nucleophosmin are not.

The aim of the present study was to follow the dynamic behaviour of three nucleolar proteins, namely the phosphorylated c-Myc, fibrillarin and Ki-67, during reversible and irreversible disassembly of nucleoli. In fact, during mitosis the nucleolus breaks down with a temporaneous relocation of its components to different cell regions; conversely, apoptosis is accompanied by the final disruption of nucleolar structure and function. We have shown differences between the various nucleolar proteins in terms of their distribution in mitotic cells. The detailed definition of the localization of Ki-67 during mitosis and

apoptosis, and of its relationship with other nucleolar proteins provides relevant information about the complex behaviour of this protein. In general, our results demonstrate that the irreversible destruction of nucleoli during apoptosis is a non-chaotic process, leading to the extrusion of intact P-c-Myc and fibrillarin into the cytoplasm, as much as other nuclear proteins were found to do (Martelli *et al.,* 2000; Pellicciari *et al.,* 2005). Remarkably, we found that P-c-Myc and fibrillarin are released from the nucleus with a different kinetics, then following apparently distinct *disposal routes*. The careful analysis of early and late apoptotic phases allowed the demonstration that the separation of such nucleolar proteins occurring in early apoptotic nuclei continues also in the cytoplasm, and culminates in the final formation of apoptotic blebs with different contents. In fact, the sub-cellular particles which form the apoptotic bodies may be variable in size, content and surface reactivity, and may include heterogeneous aggregates of nuclear proteins and/or nucleic acids (Biggiogera and Pellicciari, 2000; Halicka *et al.,* 2000; Martelli *et al.,* 2000; Pellicciari *et al.,* 2005). At least some of these subcellular particles are highly enriched in autoantigens, and it has been proposed that they might be relevant in enhancing a risk of an autoimmune reaction in the presence of defective mechanisms of apoptotic clearance by phagocytes (Cline and Radic, 2004; Pellicciari *et al.,* 2005).

Acknowledgements

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