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Effect of Roscovitine, a Selective Cyclin B-Dependent Kinase 1 Inhibitor, on Assembly of the Nucleolus in Mitosis

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Abstract—It is well known that at the beginning of mitosis the nucleolus disassembles but then reassembles at the end of mitosis. However, the mechanisms of these processes are still unclear. In the present work, we show for the first time that selective inhibition of cyclin B-dependent kinase 1 (CDK1) by roscovitine induces premature assembly of the nucleolus in mammalian cells in metaphase. Treatment of metaphase cells with roscovitine induces formation of structures in their cytoplasm that contain major proteins of the mature nucleolus participating in rRNA processing, such as B23/nucleophosmin, C23/nucleolin, fibrillarin, Nop52, as well as partially processed (immature) 46-45S pre-rRNA. This effect is reproducible in cells of various types; this indicates that general mechanisms regulate early stages of the nucleolus reassembly with CDK1 participation in mammalian cells. Based on our and literature data, we suggest that inactivation of the CDK1–cyclin B complex at the end of mitosis results in dephosphorylation of B23/nucleophosmin and C23/nucleolin; this facilitates their interaction with pre-rRNA and leads to formation of insoluble supramolecular complexes—nucleolus-derived foci.

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The nucleolus is an evolutionary conservative and the largest (1-5 μm) structural domain of the cell nucleus whose main function is ribosome synthesis [1-3]. In cells of higher eukaryotes, the nucleolus retains structural integrity and functional activity mainly in interphase. At the beginning of mitosis, the nucleolus disassembles simultaneously with condensation of chromatin into chromosomes and suppression of ribosomal gene (rDNA) transcription [4-8]. Phosphorylation of proteins participating in rDNA transcription [9-11] and rRNA processing [12-17] is first of all related to the starting mechanisms initiating nucleolus disassembly; among these proteins are upstream binding factor (UBF) (specific co-factor of RNA polymerase I), B23/nucleophosmin (main factor of pre-ribosome assembly), and C23/nucleolin (participates in various stages of rRNA maturation) [1, 2]. The main mitotic kinase CDK1 (cyclin B-dependent kinase 1) ($p34^{cdc2}$) active in the complex with cyclin B plays a key role in phosphorylation of these proteins [18-20]. Augmented phosphorylation level of B23 and C23 proteins results in retardation of their electrophoretic mobility [13], decreases binding to rRNA, and induces exit from the nucleolus [16]. In metaphase, the central stage of mitosis, the nucleolus completely disassembles, and its mass is dispersed in the cytoplasm. Exceptions are only components of the RNA polymerase I transcriptional complex, which are tightly bound to rDNA in the composition of nucleolar organizing regions (NORs) during all of the mitosis [5, 9, 11].

According to recent data, the nucleolus reassembly begins soon after chromosomes partition towards the poles of the mitotic spindle, that is, in anaphase or early

Abbreviations: BrUTP) 5-bromouridine-5′-triphosphate; CDK1) cyclin B-dependent kinase 1; DAPI) 4′,6-diamidino-2-phenylindole; 5′ETS) 5′-external transcribed spacer; FISH) fluorescent *in situ* hybridization; FITC) fluorescein isothiocyanate; iNDF) induced NDF; NDF) nucleolus-derived focus; NOR) nucleolar organizing region; PBS) phosphate buffered saline; pre-rRNA) 47-45S ribosomal RNA precursor; rDNA) ribosomal DNA; rRNA) ribosomal RNA; SSC) standard saline citrate; UBF) upstream binding factor.

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telophase. Initials stages of nucleolus assembly manifest themselves as two events: reactivation of rDNA transcription [4, 5, 21] and appearance of numerous discrete structures in the cell cytoplasm; these structures were named nucleolus-derived foci (NDF) [3, 4, 6-8]. This term was first suggested for describing bodies formed in monkey CMT3 telophase cells; the latter are stably transfected by plasmid expressing Rev protein of HIV [22]. Later NDF were described in other types of animal and plant cells; this indicates their universal character [23-26]. Using immunocytochemical approaches and specific antibodies, it was found that proteins participating in rRNA processing are main NDF components: fibrillarin (factor of pre-rRNA early processing), Nop52 (participates in the late stages of ribosome assembly), and also B23 and C23 phosphoproteins (their main functions are described above) [2, 27, 28]. Various rRNA types including mature 18S and 28S rRNA and partly processed 46-45S pre-rRNA were also found in NDF by fluorescence *in situ* hybridization [4, 8, 23, 29, 30]. The absence of rDNA and proteins bound to ribosomal genes including RNA polymerase I and UBF is typical of NDF [24, 26]. It is reasonable to suppose that the main function of NDF is participation in assembly of daughter nucleoli and in processing of immature rRNA of the mother nucleolus, which were synthesized in interphase preceding mitosis. The first suggestion is supported by observations of NDF material migration into daughter cell nuclei [29] and the second suggestion is supported by the presence in NDF of all major protein components participating in rRNA maturation in interphase nucleoli and also of immature pre-rRNA.

In spite of the fact that events accompanying nucleolus formation are described in detail, this process is not well studied on the molecular level. This is first of all related with the absence of reliable approaches to cell synchronization in the late stages of mitosis and also with the impossibility of modeling of assembly of functionally active nucleolus *in vitro*. Therefore, approaches that allow direct induction of nucleolus formation or reproduction of major stages of this process at the cellular level by the action of factors influencing the normal course of mitosis are of special interest. Roscovitine, an antitumor agent selectively suppressing CDK1 activity via interaction with its ATP-binding sites, is among these factors [31-35]. It is known that as normal mitosis comes to its end, CDK1 is inactivated due to disassembly of the CDK–cyclin B complex, this process being mainly promoted by cyclin B degradation [36]. It was shown that prolonged (3 h) treatment of cells in interphase by roscovitine results in drastic structural changes of the nucleolus, and that short-term (15-90 min) treatment in mitosis induces premature activation of chromosome NORs [37, 38]. In this study, we show for the first time that CDK1 inhibition also induces premature assembly of NDF, and this is accompanied by increased electrophoretic mobility of B23/nuleophosmin and C23/nucleolin indicating protein dephosphorylation. Based on literature data on B23 and C23 state in mitosis, we suggested that B23 and C23 dephosphorylation induces their binding to pre-rRNA and thus promotes formation of insoluble macromolecular complexes—nucleolus-derived foci. The data allow consideration of possible NDF formation in metaphase of mitosis on suppression of CDK1 activity as a novel experimental approach for evaluation of efficiency of inhibitors of this kinase, which may find application in tumor chemotherapy.

MATERIALS AND METHODS

Cells. Cultures of human HeLa, pig SPEV, mouse NIH/3T3, and green monkey CV1 cell lines were studied. HeLa, SPEV, and NIH/3T3 cell cultures were grown in DMEM medium from PanEco (Russia) with addition of 5-8% fetal calf serum from HyClone (USA), L-glutamine, penicillin, and streptomycin at the standard concentrations. CV1 cell culture was grown in 1 : 1 mixture of DMEM and F12 (PanEco) media with addition of 10% fetal calf serum, L-glutamine, and the above mentioned antibiotics at the standard concentrations. Cells were grown in an incubator from Sanyo (Japan) with 5% CO_2 and in a humid atmosphere at 37°C.

Antibodies. In this study we used the following antibodies: rabbit polyclonal antibodies to UBF protein from Santa-Cruz (USA), autoimmune sera to fibrillarin [39, 40] and Nop52 [26, 28], mouse monoclonal antibodies to C23/nucleolin [24, 26], and mouse monoclonal antibodies to B23/nucleophosmin from Sigma (USA). Antibodies to mouse, human, or rabbit immunoglobulins conjugated with fluorescein isothiocyanate (FITC) from Sigma, Alexa-688 from Molecular Probes (USA), or Texas Red from Jackson ImmunoResearch Laboratory (USA), respectively, were used as secondary antibodies.

Special cell treatments. Cells grown on cover glasses or in culture flasks were incubated with roscovitine (2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine) from Sigma at concentrations 75-150 μM for 10-60 min at 37°C. In parallel experiments, cells were treated with 1 μM okadaic acid (Sigma), an inhibitor of PP1 and PP2A phosphatases, for 20-60 min [37]. For accumulation of mitotic cells, 50 ng/ml nocodazole (Sigma) was added to the culture medium for 1.5-3 h.

Cell synchronization in mitosis. To synchronize HeLa cells, we used the double thymidine block method. Cells were incubated with 2 mM thymidine (Sigma) for 16 h, then were carefully washed free of thymidine, placed in thymidine-free medium for 9 h, and again incubated with 2 mM thymidine for 16 h. Cells were washed free of thymidine and incubated with 50 ng/ml nocodazole for 13-14 h. It is known that CDK1 activity is retained after such treatment [37]. The level of cell synchronization in K-metaphase was not less than 95%.

Immunocytochemistry. Cells were fixed with 2% paraformaldehyde (ICN, USA) in 0.1 M phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.2-7.4) for 20 min at room temperature, then treated with 0.5% Triton X-100 (ICN) for 7-10 min on ice and incubated with primary antibodies for 45-60 min at 37°C. Then cells were incubated with secondary antibodies for 40 min at room temperature and subsequently with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) for 10 min and afterwards mounted in Mowiol (Calbiochem, USA) containing 1,4-diazabicyclo[2,2,2]octane (DABCO) (Sigma). Preparations were studied using an Axiovert 200 fluorescence microscope (Carl Zeiss, Germany) with 40×/0.75 Plan-Neofluar, 100×/1.25 Fluar, and 100×/1.3 Achroplan objectives. Images were recorded using a 13-byte monochrome Cool $Snap_{cf}$ camera (Roper Scientific, USA) and processed using Adobe Photoshop CS software, version 9.2.

Preparation of a riboprobe for fluorescence *in situ* hybridization (FISH). The original plasmid containing human rDNA repeat fragment was kindly donated by Dr. M. O. J. Olson [30]. rDNA fragment 510 bp (+934/+1444) (pBss [41]) corresponding to a core region of the 5′-external transcribed spacer (5′ETS) was cloned in pBlueScriptSK(−) vector (Stratagene, USA) via restriction sites of *Sac*I and *Kpn*I endonucleases. A biotin-labeled riboprobe (biotin-16-uridine-5′-triphosphate, Biotin-16-UTP) (Roche, Germany) was obtained by transcription *in vitro* using a SP6/T7 transcription kit (Roche) according to the producer's instruction. Labeling efficiency was tested by a standard method for dot-blot hybridization, using streptavidin labeled by alkaline phosphatase (Roche).

RNA–RNA fluorescence *in situ* hybridization. Cells were fixed with 4% paraformaldehyde in PBS for 20 min, treated with 0.5% Triton X-100 in PBS for 10 min and then with 0.01% pepsin (Sigma) for 5 min at room temperature. Riboprobe (3 μl) obtained from 1 μg of DNA was mixed with 8.5 μl of deionized formamide (Sigma), and then the mixture was denatured for 10 min at 70°C and rapidly placed on ice. After cooling, 4 μl of 50% dextran sulfate (Sigma) and 2 μl of 10×SSC (standard saline citrate) (1×SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.3) were added to the mixture to final concentrations 2×SSC and 10% dextran sulfate. The hybridization mixture was incubated with cells in a chamber moistened with 50% formamide in 2×SSC for 16 h at 42°C. Preparations were sequentially washed with 50% formamide in 2×SSC (3 times for 10 min at 37°C) and with 2×SSC (10 min at 37°C and room temperature). For detection of the riboprobe avidin-conjugated rhodamine was used first (1 : 200 dilution in blocking solution; Roche), then cells were stained with antibodies to avidin (1 : 200) (Roche) and finally again with avidin conjugated with rhodamine. All reactions were performed for 30 min at room temperature.

If required, cells were also incubated with antibodies to B23 protein for 30 min at 37°C and then with antibodies to mouse immunoglobulins labeled with FITC for 40 min at room temperature. After staining with DAPI, preparations were mounted in Mowiol and studied using an Axiovert 200 microscope as described above.

Visualization of transcription of ribosomal genes. Activity of rDNA in nucleolar organizing regions (NORs) was evaluated by run-on transcription assay using 5-bromouridine-5′-triphosphate (BrUTP) (Sigma) as a labeled precursor of rRNA synthesis as described earlier [21]. CV1 cells were fixed in absolute acetone for 5 min at −20°C and washed with PBS for 1-3 min at 4°C. Then cells were incubated with initiating buffer solution containing 100 mM CH_3COOK, 30 mM KCl, 10 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), cocktail of protease inhibitors (1 : 100), 0.5 mM phenylmethylsulfonyl fluoride, pH 7.2, and also ATP, GTP, CTP, and BrUTP (Sigma) (each at concentration 150 μM), 100 U/ml RNase inhibitor (Boehringer, Germany) for 30 min at 33°C. Then cells were post-fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, treated with 1% Triton X-100 in PBS for 10 min on ice, and incubated with mouse monoclonal antibodies to 5-bromo-2′-deoxy-uridine (BrdU) (Roche) (1 : 50 dilution) for 1 h at room temperature. After washing with PBS, cells were incubated with antibodies to FITC-labeled mouse immunoglobulins for 30 min at room temperature, additionally stained with 1 μg/ml DAPI for 10 min, then mounted in Mowiol and studied using an Axiovert 200 microscope as described above.

Electrophoresis and immunoblotting. Proteins were separated electrophoretically according to Laemmli [42]. HeLa cells, asynchronized and synchronized in metaphase of mitosis, were lysed on ice in buffer containing 50 mM Tris-(hydroxymethyl)-aminomethane, pH 7.5, 150 mM NaCl, 10% glycerol (ICN), 0.5% Triton X-100, and cocktail of protease inhibitors (Sigma). Total protein concentration in lysates was determined according to Lowry (Peterson modification) using a Protein Assay Kit (Sigma) and following the producer's recommendations. Five-fold Laemmli buffer containing 250 mM Tris-(hydroxymethyl)-aminomethane, pH 6.8, 50% glycerol, 10% SDS, 500 mM β-mercaptoethanol, and 0.5% Bromophenol Blue was added to lysates before electrophoresis. Samples were heated for 5 min at 100°C. Proteins were separated by SDS-PAGE (10% polyacrylamide gel), not less than 30 μg of total protein was applied into gel pockets. After transfer of proteins onto a membrane (0.22 μm; Millipore, USA), the latter was incubated with antibodies to fibrillarin, B23, or C23 and then with corresponding secondary antibodies to human and mouse immunoglobulins conjugated with alkaline phosphate (Bio-Rad, USA). The membranes were visualized using Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) following the producer's recommendations.

To evaluate the quality of transfer, gels were stained with solution of Coomassie R-250 (Amresco, USA) in 10% acetic acid.

RESULTS

General regularities in formation of nucleolus-derived foci in normal mitosis and by the action of roscovitine. Earlier it was shown that fibrillarin, B23/nucleophosmin, C23/nucleolin, and Nop52 proteins are typical components of nucleolus-derived foci (NDF) in mammalian cells [4, 8, 22-26]. Using antibodies to any of these proteins, NDF were detected in cell cytoplasm as numerous (up to several dozens) discrete bodies up to 1.5 μm in diameter, beginning from anaphase (Fig. 1, b and g). NDF were not detected under any conditions in normal metaphase and also after cell incubation with nocodazole blocking cells in K-metaphase of mitosis. In metaphase cells, all NDF protein markers were uniformly distributed in the cytoplasm, and fibrillarin was also detected on the surface of chromosomes (Fig. 1a). Similar results were obtained after fluorescence *in situ* hybridization of metaphase and anaphase cells with 46-45S pre-rRNA probe containing a fragment of the 5′ETS core region of pre-rRNA (Fig. 1, c and h). As shown in Fig. 1 (g-i), local accumulation of premature rRNA (Fig. 1h) in anaphase cell coincides with NDF detected with antibodies to B23 protein (Fig. 1g). In pre-anaphase stages of mitosis, 46-45S pre-rRNAs were uniformly distributed in the cytoplasm similar to NDF protein markers (Fig. 1c) and were localized in nucleoli of interphase cells (Fig. 1k). This corresponds with data obtained for other cell cultures [29, 30].

Incubation of metaphase human HeLa (Figs. 1 and 2) and monkey CV1 (Fig. 3), pig SPEV, and mouse NIH/3T3 (picture not presented here) cells with 150 μM roscovitine resulted in drastic changes in fibrillarin, B23/nucleophosmin, C23/nucleolin, and Nop52 localization already after 15-30 min of action. After such treatment numerous round bodies 0.3-1.5 μm in diameter were detected in the cytoplasm. The data indicate that cells containing these bodies were in metaphase stage of mitosis. Namely, the position of chromosomes (Fig. 2, a and b) and maturation spindle morphology (picture not presented here) typical of this stage of mitosis were retained in cells with NDF. Roscovitine also induced formation of bodies in cells blocked with nocodazole in K-metaphase under the same treatment conditions (Figs. 2 (c-m) and 3). Similarly to NDF forming in anaphase of normal mitosis, the induced bodies contained all major protein markers: fibrillarin (Figs. 2 (a, c, e, h) and 3c), B23 (Fig. 2, i and k), nucleolin (Fig. 2f), and Nop52 (picture not presented here); this was proved by simultaneous cell staining with antibodies to two different proteins. Non-processed 46-45S pre-rRNA was also detected in bodies by FISH (Fig. 2l), but UBF protein was absent (picture not presented here). As shown in Fig. 2 (k-m), some of the B23-positive bodies contain a hybridized signal. It should be noted that action of roscovitine on interphase cells and on cells in other (except metaphase) stages of mitosis did not result in positional changes of the analyzed proteins and rRNA (Fig. 2, c and d). Similar morphological and immunocytochemical features of structures formed in anaphase (telophase) of normal mitosis and of those formed in metaphase by the action of roscovitine suggest the latter as analogs of natural NDF. These bodies were named induced NDF (iNDF).

Incubation of HeLa cells with 1 μM okadaic acid efficiently inhibiting the main phosphatases PP1 and PP2A [37] did not cause iNDF assembly in metaphase cells even when 60 min passed after treatment. iNDF assembly also was not detected if cells were incubated first with okadaic acid (1 μM, 60 min) and then with roscovitine (150 μM, 15-30 min) (picture not presented here).

As shown by us earlier, addition of 0.08 μg/ml actinomycin D to culture medium for 2-6 h results in partial (after 2-3 h) or complete (after 4-6 h) inhibition of NDF formation in mitotic monkey CV1 cells [26]. In this study, we showed that preincubation of CV1 and HeLa cells with actinomycin D for 4-6 h also completely suppressed iNDF formation by the action of roscovitine (picture not presented here). The data indicate that sufficient amount of pre-rRNA synthesized before mitosis is necessary for premature NDF assembly by the action of CDK1 inhibitor not only in normal mitosis, but also after cell treatment with roscovitine.

As shown in [37, 38], the action of roscovitine causes premature activation of rDNA *in vivo* transcription in metaphase [37, 38]. To determine whether this phenomenon is associated with iNDF assembly, we evaluated NOR activity in iNDF-containing cells by run-on transcription assay using BrUTP as a precursor of rRNA synthesis. It was shown that iNDF formation in metaphase CV1 cells correlates in time with resumption of rDNA transcription (Fig. 3, a-c). The data indicate that common mechanisms regulating reactivation of rDNA transcription in mitosis and NDF formation are possible.

Effect of roscovitine on electrophoretic mobility of major proteins composing NDF. It is well known that additional phosphorylation of B23 and C23 proteins in metaphase of mitosis manifests itself in retardation of their electrophoretic mobility on immunoblots of total lysates of chicken [13] and HeLa [16, 17, 25, 43] cells. To determine whether iNDF formation by the action of roscovitine is accompanied by change in mobility of these proteins, we prepared total lysates of HeLa cells synchronized in metaphase of mitosis in the absence of roscovitine and after treatment with 150 μM roscovitine for 20 min. After separation in 10% polyacrylamide gel, proteins were transferred onto a membrane and visualized with antibodies to B23 and nucleolin. The membranes

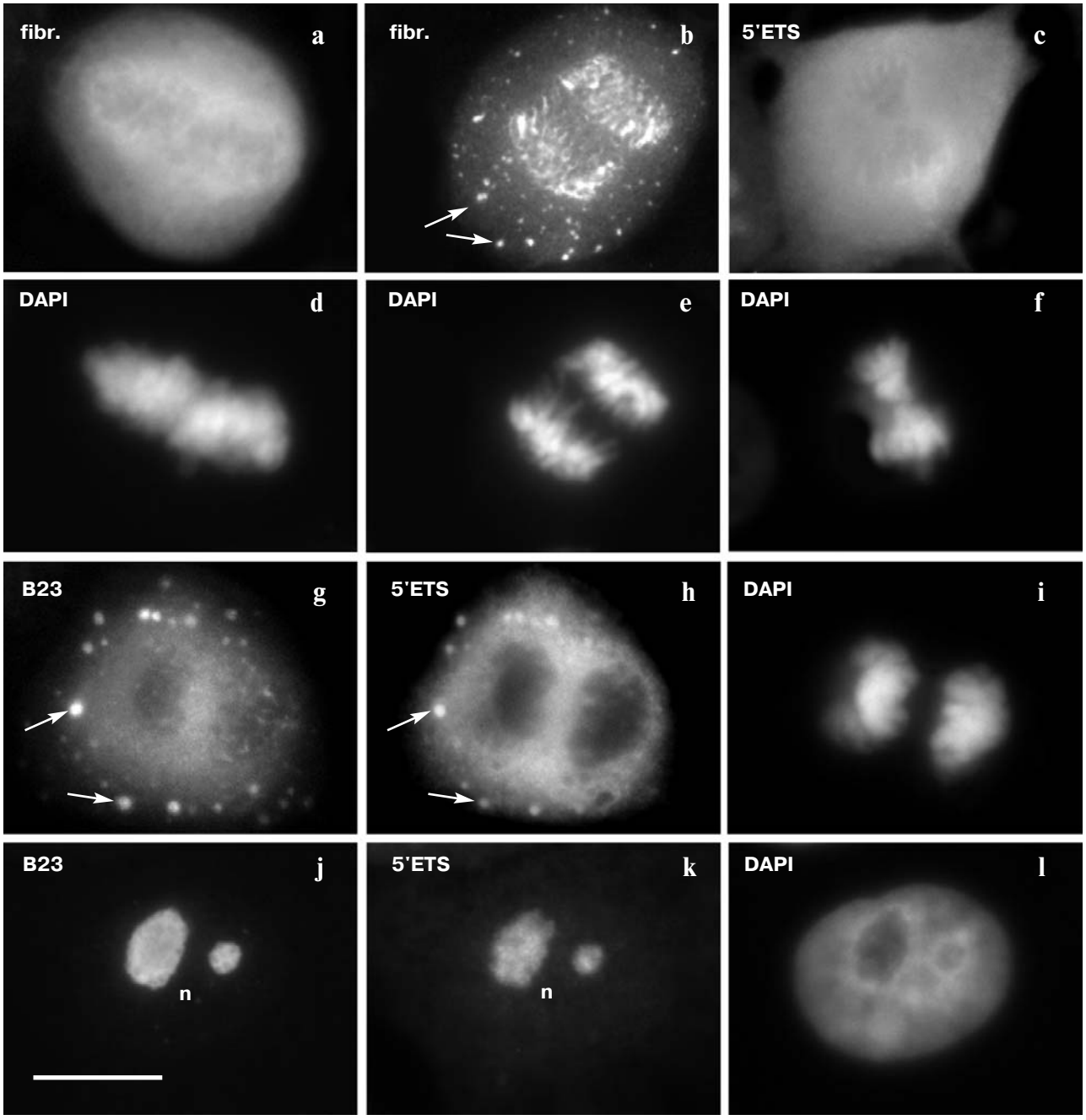

Fig. 1. Main molecular components of nucleolus-derived foci (NDF) in HeLa cells in normal mitosis. Immunochemical staining of HeLa cells with antibodies to fibrillarin (a, b) or B23/nucleophosmin (g, j). Detection of premature 46-45S pre-rRNA (c, h, k) containing a fragment of the 5′ETS core region of pre-rRNA by RNA–RNA fluorescence *in situ* hybridization (FISH). d-f, i, l) Chromosome staining with DAPI. NDF are absent from cell cytoplasm in metaphase of mitosis (a, c), but they are detected (arrows) in the final stages of mitosis (anaphase and telophase) (b, g). Stages of mitosis: a, c, d, f) metaphase; b, e, g-i) early anaphase; j-l) interphase. n) Nucleoli. Scale: 10 μm.

were also treated with antibodies to fibrillarin—a nuclear protein that composes natural and induced NDF, but is not subject to additional phosphorylation in mitosis [2]. The electrophoregram of pooled cell proteins in total cell lysates is presented in Fig. 4a.

As expected, on immunoblots of asynchronized HeLa cells specific antibodies to B23, C23, and fibrillarin revealed bands in 37-40, 105-110, and 34 kD regions, respectively. The positions of bands corresponding to B23 and C23 proteins were somewhat higher in cells blocked in metaphase than in asynchronized culture (Figs. 4b and 4c, lanes *1* and *2*). This is in accord with literature data on additional phosphorylation of proteins in mitosis and on retardation of their electrophoretic mobility on

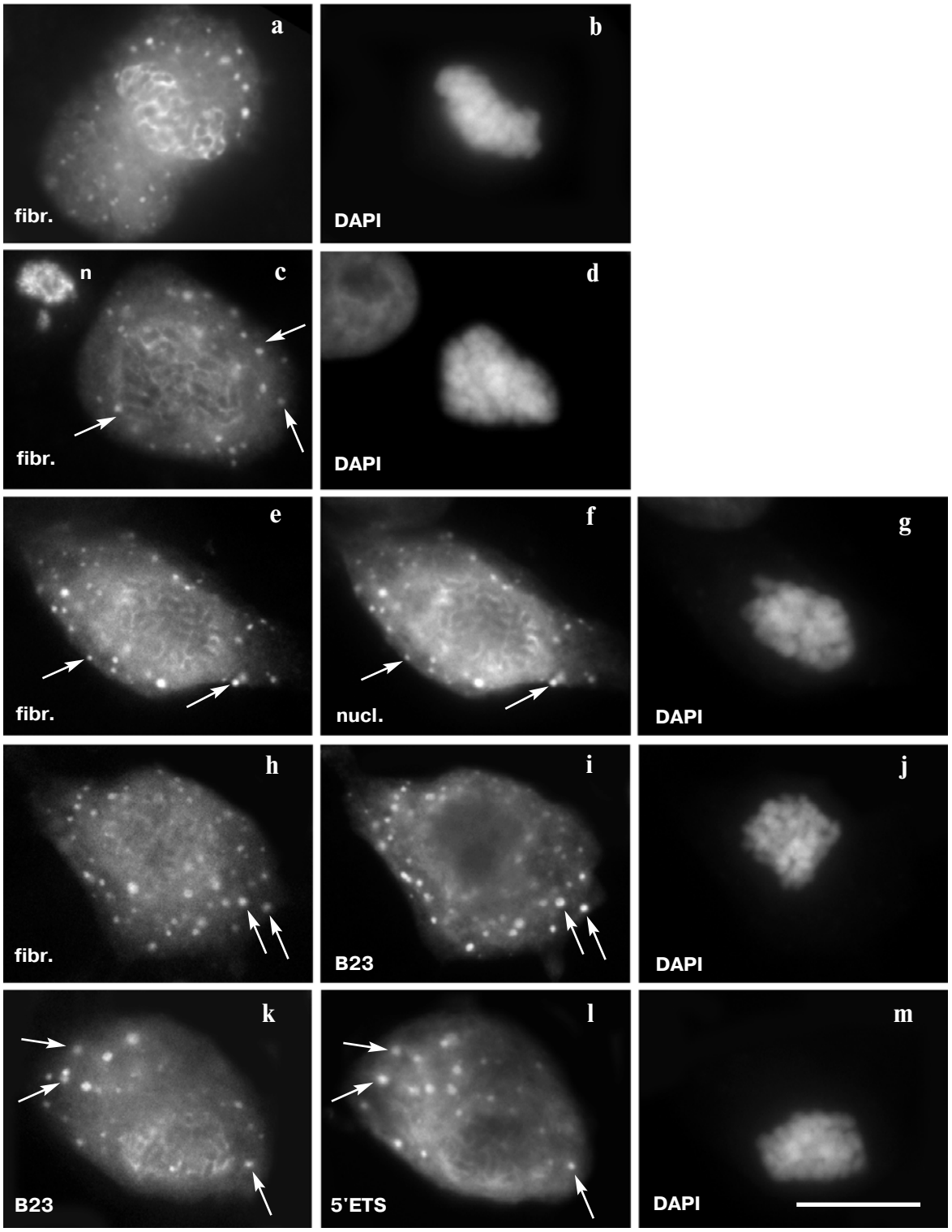

Fig. 2. Induction of iNDF formation in metaphase HeLa cells on their treatment with 150 μM roscovitine for 15 min. Immunocytochemical staining of cells with antibodies to fibrillarin (a, c, e, h), B23/nucleophosmin (i and k), or C23/nucleolin (f). Detection of premature 46-45S pre-rRNA (l), containing a fragment of the 5′ETS core region of pre-rRNA by RNA–RNA fluorescence *in situ* hybridization (FISH). b, d, g, j, m) Chromosome staining with DAPI. iNDF (arrows) are detected in cells treated by roscovitine in normal metaphase of mitosis (a, b) and also after cell synchronization in K-metaphase with 50 ng/ml nocodazole (c-m); these bodies contain major protein markers of natural NDF: fibrillarin (a, c, e, h), B23 (i, k), nucleolin (f), and partially processed pre-rRNA (l). n) Nucleoli. Scale: 10 μm.

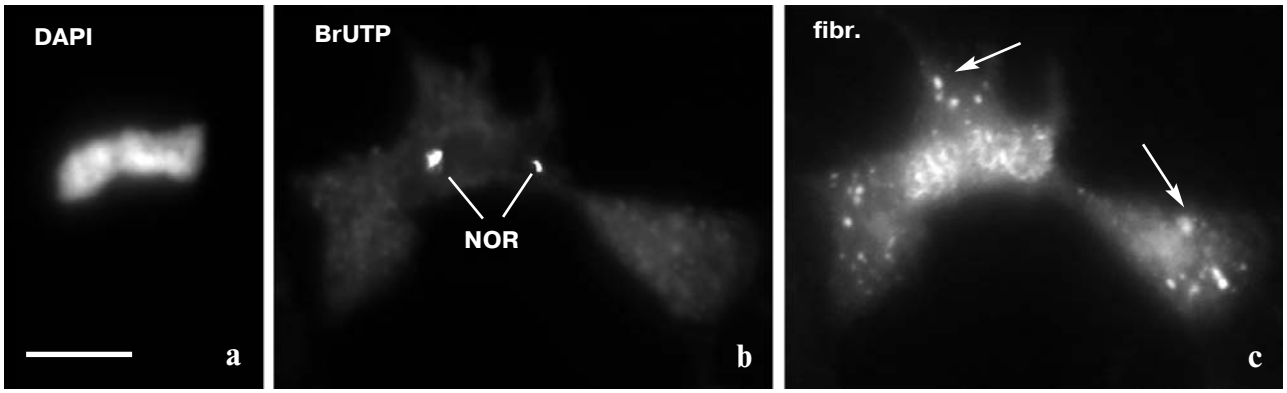

Fig. 3. Resumption of transcription of ribosomal genes and induction of iNDF formation in metaphase CV1 cells on cell treatment with 150 μM roscovitine for 15 min. Cells were synchronized in K-metaphase with 50 ng/ml nocodazole. Staining of chromosomes with DAPI (a), visualization of transcriptional activity of nucleolar organizing chromosome regions (NOR) by run-on transcription assay using BrUTP as labeled precursor of rRNA synthesis (b), immunocytochemical cell staining with antibodies to fibrillarin (c). Transcriptional activity of ribosomal genes is resumed in metaphase of mitosis in cells treated with roscovitine (b); iNDF (arrows) are formed in cytoplasm of these cells (c). Scale: 10 μm.

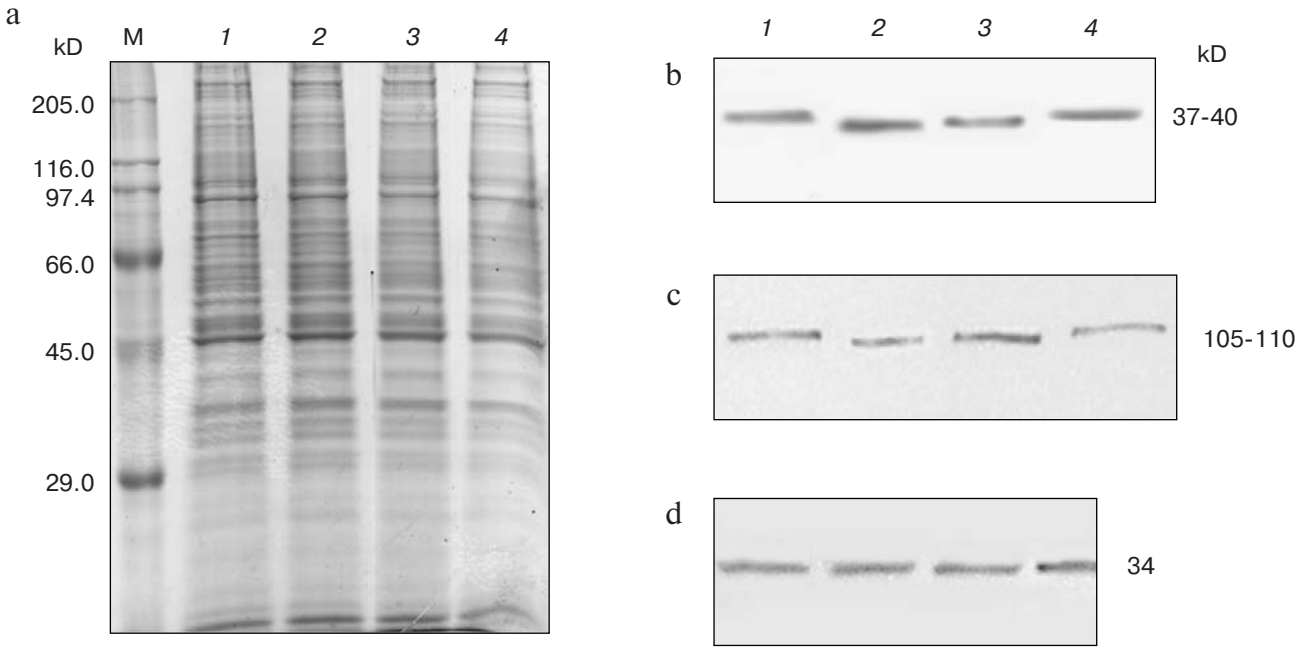

Fig. 4. Electrophoregram of pooled cell proteins in 10% polyacrylamide gel (a) and electrophoretic mobility of B23/nucleophosmin (b), C23/nucleolin (c), and fibrillarin (d) in total HeLa cell lysates. Lanes: M) protein markers; *1*) cells synchronized in K-metaphase; *2*) asynchronized culture; *3*) cells synchronized in K-metaphase and treated with 150 μM roscovitine for 20 min; *4*) cells synchronized in K-metaphase and treated with 1 μM okadaic acid for 30 min. Molecular masses of proteins are given in kD. Electrophoretic mobility of B23 and C23 proteins in lysates of mitotic cells increases to the values close to those for asynchronized cells by the action of roscovitine (b and c, lanes *2* and *3*). Treatment with okadaic acid does not influence the positions of bands on blots as compared with control metaphase cells (b and c, lanes *1* and *4*). The electrophoretic mobility of fibrillarin remains practically the same in all samples (d, lanes *1-4*).

immunoblots related with this phosphorylation. There were no visible changes in position of fibrillarin in mitotic cells and asynchronized culture (Fig. 4d, lanes *1* and *2*).

Electrophoretic mobility of B23 and C23 in lysates of mitotic cells increased to values close to those for asynchronized cells by the action of roscovitine (Figs. 4b and 4c, lanes *2* and *3*), whereas treatment with okadaic acid did not change electrophoretic mobility of proteins compared with normal metaphase cells (Figs. 4b and 4c, lanes *1* and *4*). Electrophoretic mobility of fibrillarin remained practically the same in all samples (Fig. 4d, lanes *1-4*).

So, the data indicate that cell treatment with roscovitine, a kinase CDK1 inhibitor, results in dephosphorylation of major nucleolar phosphoproteins B23 and C23.

DISCUSSION

According to modern concepts, the reassembly of the nucleolus in mitosis is a multi-stage process; its initial stages include activation of rDNA transcription and formation of mitotic derivatives (precursors) of the nucleolus called nucleolus-derived foci (NDF) [3-8]. By now, the main properties of NDF are described and their possible functions are discussed. The fact that proteins necessary for transcription of ribosomal genes and rDNA are absent from NDF [26] indicates that these formations are unable to achieve rRNA synthesis. It is probable that NDF material composes daughter nucleoli and/or participates in maturation of non-processed 46-45S pre-rRNA synthesized before mitosis [2, 6, 29]. However, rRNA composing NDF is an important structural component, because inhibition of rDNA transcription by actinomycin D before mitosis hinders normal NDF formation [26]. The fact that cell treatment with RNase A prevents NDF labeling with antibodies to B23 and fibrillarin—proteins bound to rRNA in interphase nucleoli—suggests that rRNA–protein interactions also have a place in NDF [26].

In spite of the interest for study of NDF participation in nucleolus formation and other processes in mitosis, mechanisms regulating NDF formation remained unclear by the beginning of our study. Accounting for data [34, 38] on cyclin B-dependent kinase CDK1 playing an important role in regulation of mitosis and mitotic phosphorylation of major nuclear proteins, we used roscovitine, an efficient CDK1 inhibitor, for this study. Data indicate that the action of roscovitine on mammalian metaphase cells, which differed in species and tissue origin, induces formation of structures with composition and properties corresponding to NDF formed in normal mitosis. Roscovitine-induced iNDF contain the same proteins as natural NDF including B23/nucleophosmin, C23/nucleolin, fibrillarin, and Nop52 and also 46-45S pre-rRNA (Figs. 1-3). Analogously to natural NDF, UBF protein was absent from iNDF. As shown by electronic microscopy, induced and natural NDF have similar morphology and are formed of fibrogranular material (unpublished data). It is significant that rRNA is necessary for formation of roscovitine-induced NDF, because suppression of its synthesis with actinomycin D before addition of roscovitine prevented iNDF formation.

It is known that B23/nucleophosmin and C23/nucleolin, two major nucleolar proteins, and also the main protein components of NDF are additionally phosphorylated on cell entry into mitosis with participation of cyclin B-dependent kinase CDK1. According to [15-17], B23 is phosphorylated via the threonine residues T199, T219, T234, and T237, and C23 – via the threonine residues in the nine-times repeating TPXK motif (X is nonpolar residue) at the N-terminus of the molecule [12, 13]. Change in phosphorylation level of these proteins results in distinct retardation of their electrophoretic mobility on immunoblots [13, 16, 17, 43]. As shown in this study, CDK1 inhibition under NDF-forming conditions increases electrophoretic mobility of B23 and C23 proteins compared with that of control metaphase cells (Fig. 4). The data indicate that enhanced level of B23 and C23 phosphorylation in mitosis is constantly maintained by CDK1 activity. This conclusion corresponds with experimental data on partial B23 dephosphorylation by the action of less selective CDK1 inhibitor staurosporine in mitotic HeLa cells [43]. The absence of visible changes in protein mobility on treatment of metaphase cells with okadaic acid, an inhibitor of cell phosphatases PP1 and PP2A, or first with okadaic acid and then with roscovitine, indicates the same (Fig. 4). Moreover, according to the latest data, in the composition of NDF, B23 fused with GFP is not stained with antibody specifically reacting with phosphorylated threonine T199-P [17]. Since additional B23 phosphorylation in mitosis decreases and dephosphorylation increases RNA-binding activity of the protein [16], it can be suggested that B23 dephosphorylation caused by roscovitine will induce its interaction with pre-rRNA present in the cytoplasm. It can also be suggested that the state of C23/nucleolin, a nucleolar phosphoprotein, with properties similar to those of B23 protein, will change analogously.

Summarizing our data and that of the literature, we suggest the following sequence of events related with nucleolus formation in the final stages of mitosis. Inactivation of the CDK1–cyclin B complex results in dephosphorylation of nucleolar proteins, additionally phosphorylated on cell entry into mitosis. Decreased level of phosphorylation of proteins bound to nucleolar organizers UBF and TTF (transcription termination factor) results in activation of rDNA transcription ([9, 11, 37, 38]; and present study (Fig. 3, a-c)). Besides this, dephosphorylation of B23 and possibly of C23 restores their ability for binding with pre-rRNA and promotes formation of NDF—supramolecular deposits detected on the microscopic level.

Possible premature induction of at least two features of nucleolus genesis—activation of ribosomal genes and formation of nucleolus-derived foci—on experimental CDK1 inhibition indicates that this kinase plays an important role in nucleolus re-assembly in mitosis of higher eukaryotic cells. The participation of CDK1 in final stages of mitosis is also supported by recent data on induction of premature cytotomy in the presence of CDK1 selective inhibitor BMI-1026 [44].

Data on possible premature NDF formation in metaphase of mitosis could be used as the basis for meth-

ods for induction of their assembly in extracts of mitotic cells *in vitro* for subsequent analysis of the total protein content by mass-spectrometry. Results obtained in the present work could also be of interest for tests of new drugs—potential CDK1 inhibitors. Induction of NDF formation in metaphase of normal mitosis may indicate efficient action of these preparations on human tumor cells.

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