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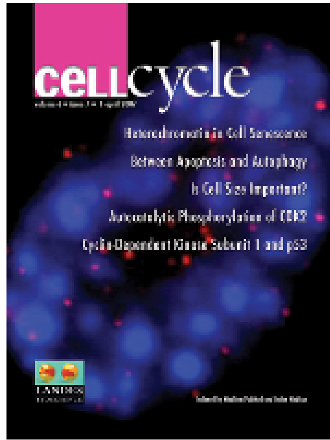

Cell Cycle
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/kccy20

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Published online: 04 Apr 2007.

To cite this article: Ryan Cawood, Sally M. Harrison, Brian K. Dove, Mark L. Reed & Julian A. Hiscox (2007) Cell Cycle Dependent Nucleolar Localization of the Coronavirus Nucleocapsid Protein, Cell Cycle, 6:7, 863-867, DOI: 10.4161/cc.6.7.4032

To link to this article: http://dx.doi.org/10.4161/cc.6.7.4032

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[Cell Cycle 6:7, 863-867, 1 April 2007]; ©2007 Landes Bioscience

Report

Cell Cycle Dependent Nucleolar Localization of the Coronavirus Nucleocapsid Protein

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Original manuscript submitted: 01/31/07
Revised manuscript submitted: 02/20/07
Manuscript accepted: 02/20/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=4032

KEY WORDS

nucleolus, cell cycle, coronavirus, trafficking, nucleocapsid protein

ACKNOWLEDGEMENTS

This work was funded by the award of BBSRC project grant (number BBS/B/03416) to J.A.H. and a BBSRC DTA/CASE studentship with Intervet UK, Ltd. to J.A.H.

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ABSTRACT

The nucleolus is a dynamic sub-nuclear structure which is involved in ribosome subunit biogenesis, modulation of cell growth and response to cell stress. The nucleolar proteome varies particularly with regard to the cell cycle. Viral proteins can localize to the nucleolus and using the coronavirus nucleocapsid (N) protein as a model, the cell cycle dependent trafficking of viral proteins to the nucleolus was investigated. Cell synchronization studies coupled to live cell confocal microscopy indicated that nucleolar localization of N protein was greater in the G_2/M phase of the cell cycle than at other stages. This result was reinforced when FRAP and FLIP analysis indicated that N protein was more mobile within the nucleoplasm and nucleolus in the G_2/M phase of the cell cycle. The data suggested that viral nucleolar proteins can also localize to the nucleolus in a cell cycle dependent manner and this may be related to dynamic trafficking.

INTRODUCTION

The nucleolus is a sub-nuclear compartment involved in many processes crucial to the efficient functioning of an eukaryotic cell.[1,2] These include, but are not limited to, ribosomal RNA synthesis,[3] modulation of cell growth[4,5] and response to cell stress.[6] The nucleolus is composed of over 700 proteins[7] which can be grouped into separate classes depending on their role in the nucleolus and wider cell function.[8,9] The nucleolar proteome and structure is dynamic and changes in response to the metabolic profile of the cell, including the cell cycle.[10-12] For example, the nuclear/nucleolar localization of B23 varies between interphase and mitosis.[13,14]

Viral proteins also target the nucleolus during infection and these are not limited to one particular type of virus and include examples from DNA, RNA and retroviruses,[15,16] however, whether viral proteins which traffic to the nucleolus can do so in a cell cycle dependent manner is unknown. Several coronavirus nucleocapsid (N) proteins have been shown to localise to the nucleolus,[17-21] although the severe acute respiratory syndrome coronavirus (SARS-CoV) N protein may do so at low efficiency.[22] The N protein has roles in both the replication of the virus, for example binding to viral RNA[23,24] and modulation of cell processes.[25-27] Coronavirus infection can also result in alteration of the cell cycle.[28-30] The avian coronavirus, infectious bronchitis virus (IBV) N protein, localizes to the nucleolus[17] and contains an eight amino acid motif (WRRQARFK) which is necessary and sufficient for directing nucleolar localization[31] and a nuclear export signal.[32] The sub-cellular localization of the IBV N protein has two phenotypes, localization to either the cytoplasm or both the cytoplasm and the nucleolus.[17-19] To investigate the hypothesis that trafficking of N protein to the nucleolus could be cell cycle related the differential sub-cellular localisation and dynamic trafficking of N protein was investigated in cell populations enriched in different stages of the cell cycle.

MATERIALS AND METHODS

Cells and cell cycle manipulation. Cell culture experiments were performed within sub-confluent cells to avoid artefact G_0/G_1 populations due to contact inhibition and in the absence of antibiotic or anti-fungal agents. Vero cells were cell cycle enrichment as described previously[29] by serum deprivation (G_0), double thymidine treatment (G_1/S) and nocodazole treatment (G_2/M).[29] Vero cells were G_0/G_1 phase enriched using serum deprivation by maintenance of cells in DMEM containing no FCS supplementation for

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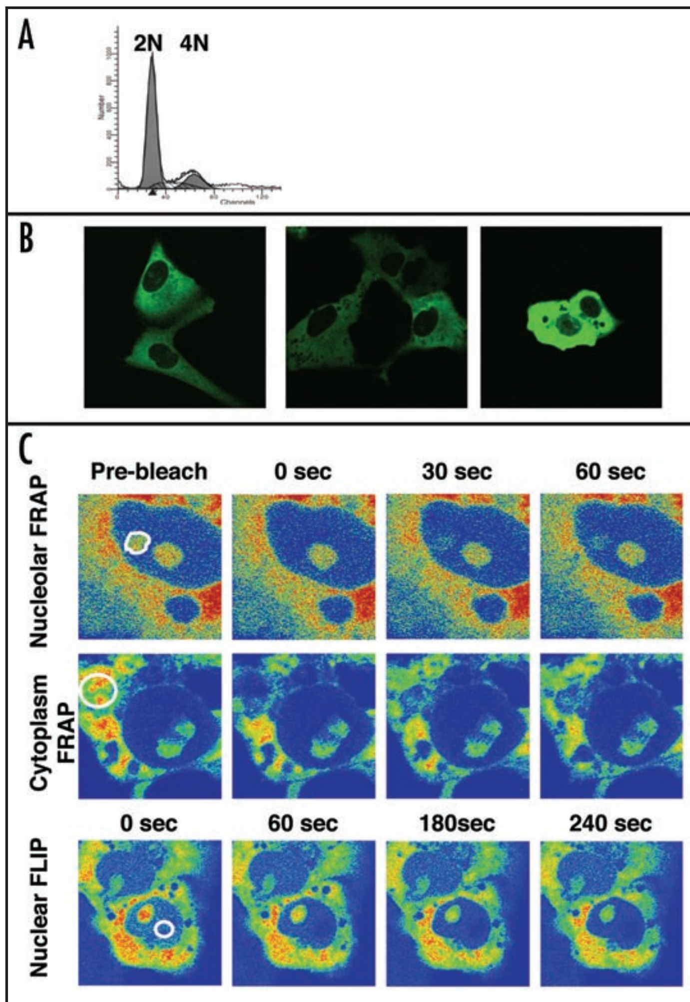

Figure 1. (A) Flow cytometry analysis of cell enriched in the G_0 phase of the cell cycle by serum starvation. Shown are the propidium iodide trace (shaded), principally showing the proportion of cells with a 2N or 4N DNA content. Fluorescent intensity is shown on the X-axis and number of cells on the Y-axis, and arrow heads indicate significant intensity. (B) Examples of the localisation of EGFP-N protein in serum staved cells taken with live cell confocal microscopy (note the linear range is exceeded for some cells). (C) Examples of cells in which the EGFP-N protein in one nucleolus has been photo-bleached (labelled nucleolar FRAP), a defined portion of the cytoplasm has been photo-bleached (labelled cytoplasm FRAP) and where a defined portion of the nucleoplasm has been continuously photo-bleached (labelled nuclear FLIP). In all three cases the photo-bleach area is denoted by white line. The images are false coloured using the Zeiss LSM 5 browser 'rainbow' feature where red represents the highest protein concentration and blue no protein present.

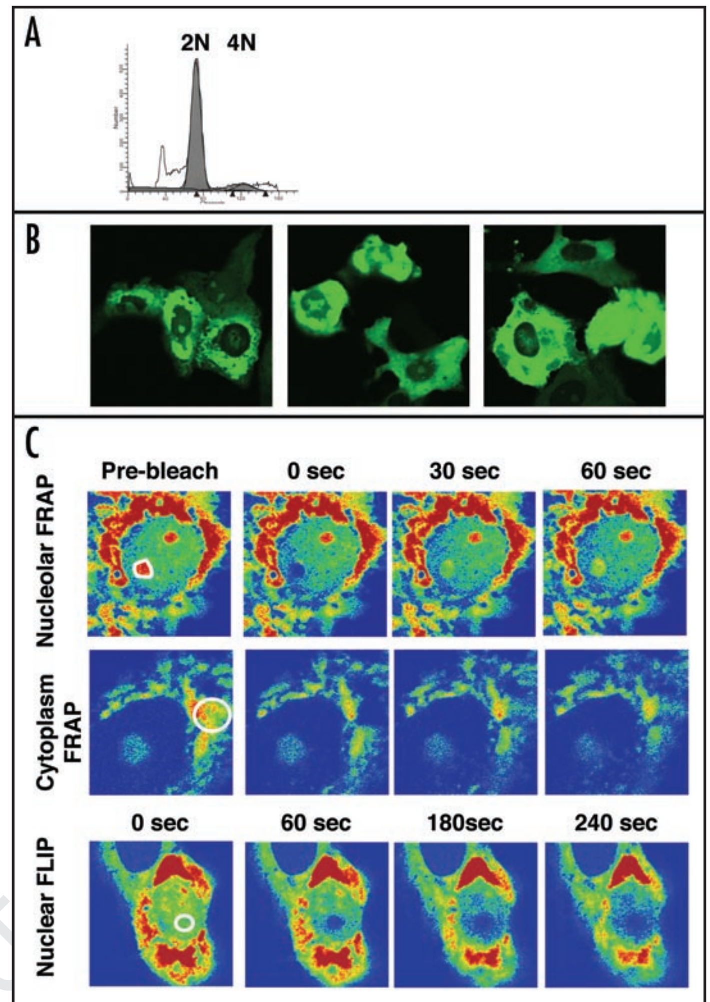

Figure 2. (A) Flow cytometry analysis of cell enriched in the G_1/S phase of the cell cycle by double thymidine block. Shown are the propidium iodide trace (shaded), principally showing the proportion of cells with a 2N or 4N DNA content. Fluorescent intensity is shown on the X-axis and number of cells on the Y-axis, and arrow heads indicate significant intensity. (B). Examples of the localisation of EGFP-N protein in cells treated with the double thymidine block taken with live cell confocal microscopy (note the linear range is exceeded for some cells). (C) Examples of cells in which the EGFP-N protein in one nucleolus has been photo-bleached (labelled nucleolar FRAP), a defined portion of the cytoplasm has been photo-bleached (labelled cytoplasm FRAP) and where a defined portion of the nucleoplasm has been continuously photo-bleached (labelled nuclear FLIP). In all three cases the photo-bleach area is denoted by white line. The images are false coloured using the Zeiss LSM 5 browser 'rainbow' feature where red represents the highest protein concentration and blue no protein present.

72 hr. Vero cells were enriched at the G_1/S phase border using double thymidine treatment (15) by incubation for 12 hr in maintenance media supplemented with 2 mM/ml thymidine (Sigma), cells were then washed three times with PBS and incubated for 12 hr in maintenance media followed by an additional 12 hr incubation in maintenance media supplemented with 2 mM thymidine. Vero cells were G_2/M phase enriched using nocodazole treatment by incubation of cells in maintenance media supplemented with 60 ng/ml nocodazole (Sigma) for 16 hr. Cell cycle enrichment was determined by flow cytometric analysis.[29]

Plasmids and transfection. Vero cells were cultured in six well plates until 70% confluent, then transfected with 1μg of plasmid DNA using Lipofectamine transfection reagent (Invitrogen).

Confocal imaging. Confocal sections of fixed samples were captured on an LSM510 META microscope (Carl Zeiss Ltd., Germany) equipped with a 40x and 63x, NA 1.4, oil immersion lens as described previously.[31] Note that some cells (data presented in Fig. 1B, 2B and 3C) are not within the linear range, as these can be above or below the focal plane of analysis and exhibit differential levels of transient protein expression.

FRAP microscopy. Vero cells were plated onto glass based 33mm culture dishes, transfected and imaged 24 h later on an inverted LSM 510 META confocal microscope (Carl Zeiss, Herts, UK). Cells were maintained at 37°C with a heated stage throughout the experiments. For imaging cell culture medium was exchanged for CO_2 independent medium (Gibco) to maintain cell homeostasis throughout the

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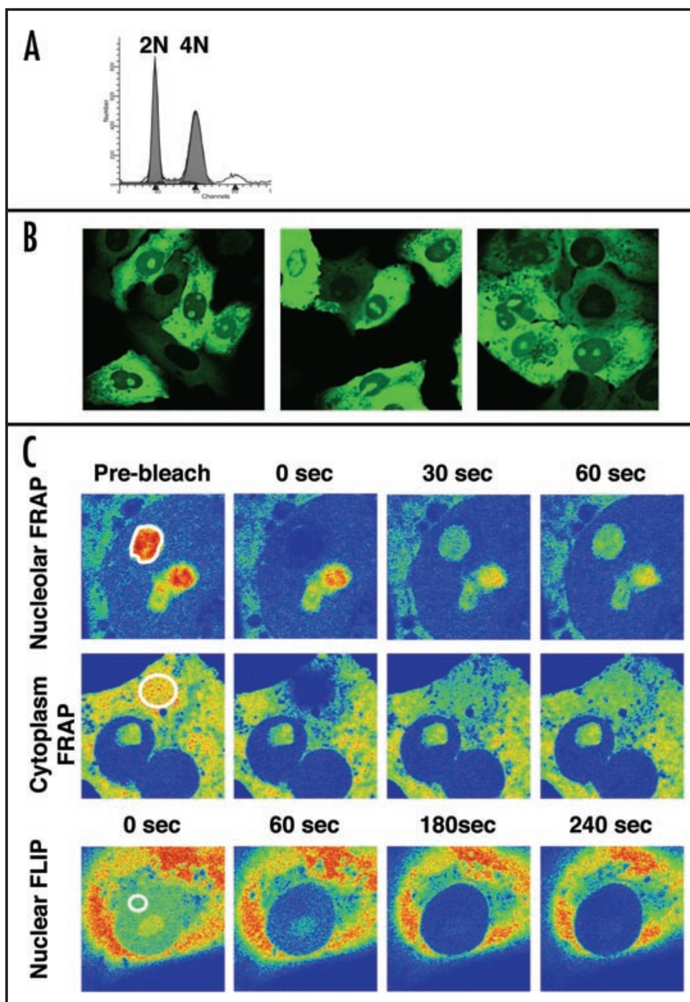

Figure 3. (A) Flow cytometry analysis of cell enriched in the G_2/M phase of the cell cycle by treatment with nocodazole. Shown are the propidium iodide trace (shaded), principally showing the proportion of cells with a 2N or 4N DNA content. Fluorescent intensity is shown on the X-axis and number of cells on the Y-axis, and arrow heads indicate significant intensity. (B) Examples of the localisation of EGFP-N protein in cells treated with the nocodazole, taken with live cell confocal microscopy (note the linear range is exceeded for some cells). (C) Examples of cells in which the EGFP-N protein in one nucleolus has been photo-bleached (labelled nucleolar FRAP), a defined portion of the cytoplasm has been photo-bleached (labelled cytoplasm FRAP) and where a defined portion of the nucleoplasm has been continuously photo-bleached (labelled nuclear FLIP). In all three cases the photo-bleach area is denoted by white the white line. The images are false coloured using the Zeiss LSM 5 browser 'rainbow' feature where red represents the highest protein concentration and blue no protein present.

experiments. All images were captured using a 63x objective and a digital zoom factor of 4 within the software. EGFP was excited with the 488 nm laser line delivered from a 30mW argon laser running at 6.1A and 1% power output; these settings were established as causing no residual background bleaching of the sample with the appropriate controls.

Photo-bleaching was performed on a defined area of 12 pixels squared, which equated to 20.16 μm^2 area, within the nucleolus with the 488 nm laser line at 100% power output for 100 iterations, bleaching took approximately 1.2 s. Five images were collected prior to the bleach and images were collected continually for 120 s

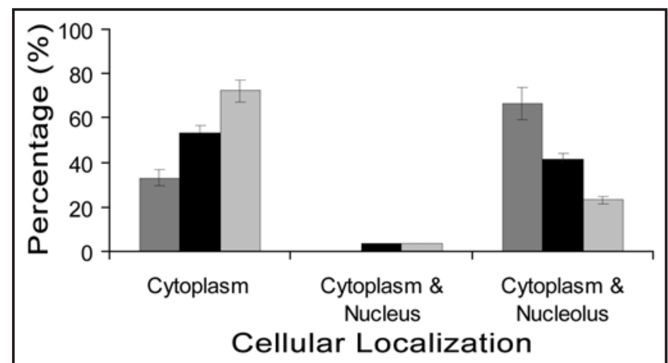

Figure 4. Histogram of the sub-cellular localization of EGFP-N protein in cells enriched in the G_0 (light grey), G_1/S (black) and G_2/M (dark grey) phases of the cell cycle.

subsequently. Recovery of fluorescence was detailed using the ROI Mean module of the LSM510 software.

FLIP microscopy. Transfected Vero cells were imaged in glass base dishes as outlined above. Imaging and photo-bleaching was performed with the same laser settings as detailed in the FRAP microscopy. In each FLIP experiment a single cell nucleus was imaged five times followed by a period of photo-bleaching for a total time of three minutes. Photo-bleaching was performed on one nucleolus within the cell for 50 iterations (mean bleach time 2.1 s). Pixel intensities of the unbleached nucleoli were detailed using the ROI Mean module of the LSM510 software.

RESULTS AND DISCUSSION

N protein is differentially located to the cytoplasm and nucleolus in asynchronously replicating cells. To investigate whether the sub-cellular localization of N protein is related to the proliferation state of the cell, Vero cells were transiently transfected at the same density to express EGFP-N protein and then grown to either a sub-confluent or confluent monolayer. Ten fields of view were counted (in three separate experiments) and the proportion of EGFP-N protein in either the cytoplasm or cytoplasm and nucleolus were determined in fluorescent cells. The data demonstrated that in sub-confluent cells there was a greater portion of EGFP-N protein in the cytoplasm and nucleolus (69 ± 6%) versus cytoplasm (31 ± 7%) only compared to the sub-cellular localization of EGFP-N protein in confluent cells, cytoplasm and nucleolus (49 ± 4%) versus cytoplasm (51 ± 6%). The data indicated that the nucleolar localisation of EGFP-N protein was linked to the proliferation state of the cell. Confluent cells undergo contact inhibition and become quiescent[33] and are thus in the G_0 phase of the cell cycle.

The sub-cellular localization of N protein is related to cell cycle stage. Given that EGFP-N protein exhibited differential localisation between sub-confluent and confluent cells, the hypothesis was tested that this may be related to the cell cycle stage of the cell. Cells were enriched in the G_0 phase (using serum starvation), the G_1 phase (using a double T block) or the G_2/M phase transition (using nocodazole). Flow cytometry was used to determine the cell cycle stage. The data indicated that in serum starved cells approximately 87% were in the G_0 phase, 10% were in S phase and 3% were in the G_2/M phase of the cell cycle (Fig. 1A). In double-T treated cells approximately 88% were in the G_1 phase, 3% were in S phase and 9% were in the G_2/M phase of the cell cycle (Fig. 2A). In nocodazole

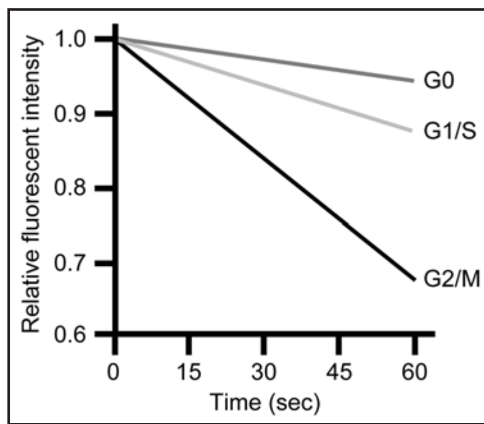

Figure 5. Relative loss of fluorescent signal in the unbleached nucleolus in cells containing two nucleoli, one of which has been photo-bleached, data is shown for cells enriched in the G_0, G_1/S or G_2/M phase of the cell cycle. Data fromfive independent experiments were normalized to allow comparison of the pre-bleach intensity and post-bleach intensity at a given time point (in seconds). The data is presented as a best-fit analysis.

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treated cells approximately 20% were in the G_1 phase, 23% in S phase and 57% in the G_2/M phase of the cell cycle (Fig. 3A). Asynchronously replicating sub-confluent cell populations had an average of 58% of cells in the G_0/G_1, 38% in S phase and 4% in the G_2/M phase (data not shown and ref. 29).

To determine the proportion of EGFP-N protein in the cytoplasm, cytoplasm and nucleolus and cytoplasm/nucleus/nucleolus in cells enriched in different stages of the cell cycle ten fields of view were examined (in three separate experiments) and the number of fluorescent cells matching each criterion was counted. Three representative fields of view are shown in (Fig. 1B, 2B and 3B) for cells enriched in the G_0, G_1/S and G_2/M phases of the cell cycle, respectively, and the proportion of cells showing each sub-cellular localisation pattern is compared in Figure 4. The data indicated that EGFP-N protein localised to the cytoplasm and nucleolus in approximately 23% of cells enriched in the G_0 phase of the cell cycle, 41% of cells enriched in the G_1 phase of the cell cycle and 65% of cells enriched in the G_2/M phase.

Dynamic trafficking of N protein in cells enriched in different phases of the cell cycle. The localization of N protein to the nucleolus may be related to the ability of N protein to traffic in the nucleus in cells enriched in different stages of the cell cycle. To test this hypothesis fluorescent recovery after photo-bleaching (FRAP) and fluorescent loss in photo-bleaching (FLIP) was used to investigate the dynamic trafficking of N protein to the nucleolus and within the nucleoplasm. In the FRAP experiments a cell in which two nucleoli were present was selected and one of these nucleoli was photo-bleached and the relative ability of EGFP-N protein to refill this area, and loss from the unbleached nucleolus was compared between cells enriched in different stages of the cell cycle. In the FLIP experiments, cells were imaged in which an defined area of the nucleus continuously photo-bleached in order to investigate protein trafficking within this structure.

To control for the general movement of EGFP-N protein FRAP was used to photo-bleach a defined area of the cytoplasm. This was particularly important in cells treated with nocodazole as this drug has been reported to disrupt the trafficking of several viral nucleoproteins,[34] although there is no evidence to suggest this occurs with the coronavirus N protein. Indeed the recovery of EGFP-N protein to the photo-bleached area in the cytoplasm of cells enriched either in the G_0, G_1 or G_2/M phase of the cell cycle was not significantly different (Fig. 1C, 2C and 3C, respectively). Both FRAP analysis of EGFP-N protein in the nucleolus and FLIP analysis of EGFP-N protein in the nucleoplasm indicated that EGFP-N protein was more mobile in cells enriched in the G_2/M phase of the cell than cells enriched in either the G_1/S or G_0 phases of the cell cycle (Fig. 1C, 2C and 3C, respectively). For example, comparison of the relative loss in fluorescence between the unbleached nucleolus in cells enriched in the G_0, G_1/S or G_2/M phase of the cell cycle (nucleolar FRAP) indicates that EGFP-N protein is more mobile in the latter phase of the cell cycle (Fig. 5). This data suggests that the cell cycle stage can effect the dynamic trafficking of N protein in the nucleus/nucleolus.

To our knowledge this is the first description that the trafficking of a viral protein to the nucleolus is related to the cell cycle stage of the cell. Recent data suggests that the trafficking and association of the nucleolar protein B23.1 to and with the nucleolus is different between interphase and mitosis and this is dependent on the phosphorylation status of the protein.[13] Taken together these data support the concept of the nucleolus as a dynamic structure whose protein content varies with different metabolic status of the cell.[9-11,35] Perhaps more importantly the data indicates that proteins can display differential trafficking within and to the nucleolus during interphase.

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