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The Nucleolus Takes Control of Protein Trafficking Under Cellular Stress

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Abstract

The nucleolus is a highly dynamic nuclear substructure that was originally described as the site of ribosome biogenesis. The advent of proteomic analysis has now allowed the identification of over 4500 nucleolus associated proteins with only about 30% of them associated with ribogenesis (1). The great number of nucleolar proteins not associated with traditionally accepted nucleolar functions indicates a role for the nucleolus in other cellular functions such as mitosis, cell-cycle progression, cell proliferation and many forms of stress response including DNA repair (2). A number of recent reviews have addressed the pivotal role of the nucleolus in the cellular stress response (1,3,4). Here, we will focus on the role of Nucleolin and Nucleophosmin, two major components of the nucleolus, in response to genotoxic stress. Due to space constraint only a limited number of studies are cited. We thus apologize to all our colleagues whose works are not referenced here.

Keywords

Nucleolus; Nucleolin; Nucleophosmin; Genotoxic stress

A role for the nucleolus in ribosome biogenesis was first proposed in the 1960's after the identification of RNA and ribosomal genes in these subnuclear compartments (5,6). These early studies pointed to the potential regulatory functions of the nucleolus for protein synthesis in response to a variety of cellular demands. At about the same time, modification of protein synthesis patterns was identified as one of the first phenomena occurring following cellular stress (7). Typically, an immediate arrest of protein synthesis followed by an increased rate of protein synthesis was observed after UV radiation (7). Down regulation of protein synthesis in response to stress is thought to be an adaptive response triggered to protect the cells and conserve the resources required to survive (8). On the other hand, induction of specific ribosomal proteins in response to stress may indicate the involvement of the translational machinery in sensing, responding and recovering from cellular stress (9). The association of several ribosomal proteins with the oxidative stress response (10) is additional evidence that translation regulation is a significant component of the cellular stress response. Several types of stress, including heat shock stress and several chemical

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Conflicts of Interest

No potential conflicts of interest to disclose.

compounds, can induce the synthesis of stress regulated proteins while inhibiting the rate of overall protein synthesis (11).

While the nucleolus can directly impact the rate of protein synthesis by regulating the levels of ribosome biogenesis it can also sense and respond to cellular stress by sequestering and releasing a variety of proteins affecting cell cycle and DNA repair. The nucleolus is thus in constant flux, assembling at the end of mitosis and disassembling at prophase while modifying its size and content in response to cellular demands throughout interphase. Thus, large nucleoli are a hallmark of active proliferating cells while terminal stages of differentiation such as lymphocytes are characterized by limited nucleolar size. In fact, silver-staining of the nucleolar organizer regions (AgNORs) is used as a marker for cancer progression in several tumors. This staining method selectively stains the major protein components of the nucleolus- Nucleolin (NCL), Nucleophosmin (NPM) and the upstream binding factor (UBF), the largest RNA polymerase 1 (RP1) subunit. Nucleolin is over-expressed in malignant tumors and its synthesis is associated with increased rates of cell division (12). NPM also known as B23, NO38 and numatrin (13), is 20 times higher in Novikoff hepatoma and 5 times higher in hypertrophic rat liver compared to normal rat liver (14). Both NCL and NPM are considered “hub” proteins in that they can interact with multiple proteins or nucleic acids and serve as scaffold proteins (15). This capacity to interact with multiple partners is mainly due to NCL and NPM disordered domains found in large part of the protein and corresponding to 55% and 47% respectively of the overall protein structure. In contrast to NPM, NCL does not have a canonical nucleolar localization sequence (NoLS) but its association with NPM warrants its nucleolar localization (16). There are several classes of NoLS but unlike the nuclear localization signal there is no consensus on a particular NoLS. Interaction with a nucleolar protein or RNA or rDNA seems to be the driving force behind nucleolar localization (17). Proteins can either associate transiently with nucleoli or accumulate only under specific metabolic conditions. The protein composition of the nucleolus is therefore not static and can be altered significantly in response to different metabolic status or stress. In fact, the nucleolus is depopulated of proteins due to a sharp migration of nucleolar proteins toward the nucleoplasm in response to cellular stress (Figure. 1). We have previously suggested that the nucleolus serves as a convenient depot for many proteins involved in DNA damage response (18). In response to stress, the nucleolus becomes a major traffic controller that allows key players to uncouple from their quiescent hub and quickly reach the scene of damaged DNA. Interactions with NPM and NCL are thus at the center stage of this critical response. Tables 1 and 2 list nuclear and nucleolar proteins interacting with NCL and NPM respectively.

Modulating repair enzymes activity

NCL and NPM can not only affect the nucleolar trafficking but can also influence the cellular stress response by modulating the activity of stress response proteins. We have recently shown that NCL interacts with PCNA and inhibits Nucleotide Excision Repair (NER) (19). There are two subpathways of NER: global genomic repair (GGR) and transcription coupled repair (TCR). Nucleolin helicase activity and its capacity to bind to a hairpin RNA structure (20) support its possible role in TCR. Nucleolin can also accelerate the annealing of oligonucleotides, including those containing mismatches (21) and thus might be involved in recombinational DNA repair. The multifunctional protein NCL can also inhibit replication by binding to the Replication Protein A (RPA) under stress conditions (22). Heat-shock for example triggers nucleolin translocation to the nucleoplasm, where it binds RPA (23), and consequently inhibits DNA replication initiation (24). Like many helicases, the Werner Syndrome protein (WRNp) is localized to the nucleoli under normal cellular growth but translocates to intranuclear repair foci in response to the Topoisomerase inhibitor camptothecin (CPT) (25) (Figure.1). In response to the genotoxic

agent 4-nitroquinoline-1-oxide or serum starvation, it translocates to the nucleoplasm (26,27). WRNp is one of the best characterized RecQ helicases and is known to have roles in DNA replication and repair, transcription, and telomere maintenance. WRNp owes its nucleolar location to its nucleolar targeting sequence (28) but may also interact with nucleolar proteins such as nucleolin. It has been proposed that tyrosine phosphorylation, either by direct modification of WRNp or of a putative “WRN-nucleolar carrier” may modulate the nucleolar trafficking of WRNp (26). In fact nucleolin is also translocated to the nucleoplasm in response to CPT (24). Thus, certain kinds of damage (CPT-induced DNA breaks, for example), prompt the release from the nucleolus of many proteins such as Topo1 (29), the AAA ATPase p97/VCP (18), WRNp and NCL that may be involved in DNA repair. The rapid dispersal of nucleolar DNA damage response proteins such as WRNp and nucleolin to the nucleoplasm enables an immediate and effective accumulation of these proteins at the damage sites where they can assemble into specific repair foci or be used as scaffold proteins for critical DNA repair enzymes.

In addition to its direct interaction with repair proteins, NCL could also modify repair efficiency indirectly by regulating transcripts affecting repair. In that respect we have identified the ribosomal protein S3a (RPS3a) mRNA as one of nucleolin’s putative targets (30). RPS3a has an Apurinic/Apyrimidinic (AP) endonuclease activity and can also cleave phosphodiester bonds within cyclobutane pyrimidine dimers (31). This activity is unusual since AP endonucleases are supposed to repair AP sites. It has thus been suggested that, at least in mammalian cells, the cleavage of AP sites by a β -lyase activity is not a DNA repair event in the classical sense but may rather represent a novel function such as forestalling of DNA repair (31). The cleavage of a phosphodiester bond is also a new function for a ribosomal protein. This activity could help relax DNA distortions brought about by the dimers and possibly help the replication process to pass over the dimers (32).

Our data indicate that NPM levels can set a threshold for p53 activation by ATR in response to UV radiation (33). NPM mediates the down regulation of the cyclin dependent kinase inhibitor p21 usually observed in response to low doses (10 Jm^{-2}) of UV radiation (33,34). This is probably due to the inhibitory effect of high levels of NPM on p53 phosphorylation at Ser15. Phosphorylation is usually not observed until 14 Jm^{-2} of UV radiation but when NPM levels are lowered, p53 phosphorylation occurs at 10 Jm^{-2} . NPM is also known to indirectly stabilize p53 by releasing ARF from the nucleolus in response to UV radiation. Once in the nucleoplasm, ARF binds to and inactivates mdm2, which leads to p53 stabilization (reviewed in (4)). These apparently contradictory results may actually be part of what are now becoming classical p53 regulatory loops, in which mechanisms involved in p53 up regulation also participate in its down regulation in a sequential manner. In this case it is very likely that the release of ARF from NPM also allow NPM to subsequently bind to p53 in order to prevent over activation (Figure.2). This is reminiscent of what has been described for mdm2 and S100B interaction with p53 where in both cases p53 can up regulate the proteins at the transcriptional levels and over expression of the proteins represses p53 expression (35,36).

Functional significance of NPM and NCL phosphorylation

A recent report indicates that dephosphorylation of NPM at Thr 199, 234 and 237 by the protein phosphatase 1 (PP1) facilitates Nucleotide Excision Repair (37). PP1 accumulates in nucleoli during interphase but diffuses into the cytoplasm at mitosis and associates with the kinetochore, while it relocates to chromosomes at the onset of anaphase and accumulates again in nucleoli (reviewed in (38)). PP1 does not have a NoLS but is sequestered to the nucleolus by its interaction with the nucleolar protein NOM1(39). PP1 is activated by Ionizing Radiation (IR) in an ATM-dependent manner (40). ATM regulates PP1 activity by

phosphorylating its inhibitor (I-2) on Serine 43 which leads to I-2 dissociation from PP1 (41). Activation of PP1 by ATM leads to a G2/M checkpoint through inhibition of Aurora-B kinase and down regulation of histone H3 phosphorylation at Serine 10. Our data (42) indicate that NPM is hyperphosphorylated at Ser125 in AT cells and that overexpression of a functional ATM in AT cells reduces NPM phosphorylation to the levels of normal cells. Moreover down regulating PP1 in ATM corrected AT cells results in NPM hyperphosphorylation, suggesting that the inability to activate PP1 in AT cells is responsible for NPM hyperphosphorylation, which could interfere with DNA repair (37). Nonetheless, phosphorylation of a small pool of NPM at Thr199 has been shown to be required for NPM recruitment to nuclear DNA damage foci induced by IR (43). Replacement of endogenous NPM with its nonphosphorylatable T199A mutant prolonged persistence of IR-induced RAD51 foci and correlates with unrepaired DNA damage (43). Therefore, phosphorylation of NPM could prevent or increase DNA repair depending on the source of DNA damage and the NPM phosphorylation sites involved. Nonetheless, it has also been suggested that NPM has a role in reducing the susceptibility of chromosomal DNA to damage rather than promoting DNA damage repair (44).

NPM phosphorylation at Ser125 could impact DNA repair indirectly by competing out with p53 for remaining active kinases in ATM deficient cells (42). In fact we have shown that NPM is a substrate for the ATM related kinase ATR and can compete, both in vitro and in vivo, with p53 for ATR phosphorylation (33).

Nucleolin phosphorylation for its part can either decrease (45) or increase (30) its RNA binding activity. We have already determined that phosphorylation of nucleolin by MAPK p38 increases nucleolin RNA binding activity (30). However, because SB203580, a specific inhibitor of MAPK p38, reduced the UV-induced RNA binding activity of nucleolin by only 50%, it is possible that other stress responsive kinases such as DNA-PK, ATM, ATR and human SMG-1 (hSMG-1) are involved in nucleolin activation. DNA-PK, ATM, ATR and hSMG-1 belong to a family of protein serine-threonine kinases whose catalytic domains share an evolutionary relationship with mammalian and yeast phosphoinositide-3 kinases (PI-3K) (46). DNA-PK activation requires free DNA ends and is thus considered a DNA damage sensor. ATM is activated by IR and is one of the primary sensors that can activate p53 and the cell cycle checkpoints in response to stress (46). ATR is an ATM and Rad 3-related kinase that was identified during a search of an EST database for gene products containing the catalytic domain of one of the phosphoinositide kinases. ATR is also activated by DNA damage including UV radiation and is important for the S-phase checkpoint where it is used as a damage sensor and scaffolding protein (46). hSMG-1 is involved in nonsense-mediated mRNA decay, and like ATM, plays a role in the recognition and repair of damaged DNA (47). The consensus sequence for phosphorylation by ATM/ATR overlaps extensively with the PI-3K site. Generally the sequence Ser/Thr-Gln-Glu is targeted. In the case of ATM, hydrophobic or acidic residues surrounding the Ser-Gln motif is favorable for phosphorylation, while positively charged amino acids are inhibitory (46). NPM and NCL do not contain perfectly matched ATM/ATR consensus sites but both are phosphorylated in vitro by ATM/ATR/hSMG-1 and are indirectly regulated by ATM through PP1 (42). Non consensus ATM/ATR sites have been identified in bona fide ATM substrates such as BRCA1 (48) and ATM itself (49), and proximity to the ATM/ATR kinases, rather than sequence context is believed to play a pivotal role in the selection of the substrates in vivo (46). Therefore, NCL and NPM abundance and their proximity to ATM in response to cellular stress could favor some direct phosphorylation in vivo.

The nucleolus is thus a large repository of stress responsive proteins poised to provide critical assistance to damaged DNA. Phosphorylation provides a rapid and effective way to uncouple and regulate the activity of several nucleolar proteins. By sequestering

phosphatases such as PP1 and Cdc14p to the nucleolus (38,50,51), the cells have evolved a mechanism to keep these proteins on call until their functions are urgently needed in the nucleoplasm or other cellular compartments. Given the diversity of possible post-translational modifications that can occur in response to genotoxic stress, it is likely that other modifications, either by themselves or in concert with phosphorylation, will prove to be important regulators of nucleolar proteins trafficking in response to cellular insults.

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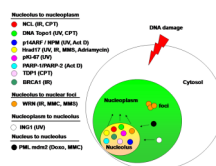


Figure 1. Nucleoli protein trafficking in response to DNA damage

The nucleolus is depopulated of its protein content in response to a variety of cellular stress. Proteins are grouped according to the direction they are moving. Arrows indicate the direction of the proteins movement. The Werner protein helicase accumulates in intranuclear repair foci in response to camptothecin (CPT). NCL: Nucleolin, IR: Ionizing Radiation, TDP1: Tyrosyl DNA phosphodiesterase 1, PML: Promyelocytic Leukemia protein, MMC: Mitomycin C, PARP: poly(ADPribose) polymerase (4,52) (53).

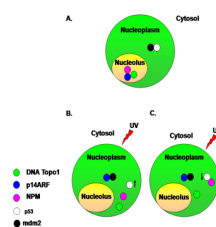


Figure 2. Schematic representation of potential NPM regulation of p53 in response to UV radiation

(A) Under normal conditions, ARF is retained in the nucleolus by its association with NPM and Topo1. (B) In response to UV radiation ARF dissociates from NPM and is released into the nucleoplasm where it associates with mdm2 and consequently stabilizes p53. (C) The dissociation of ARF from NPM frees NPM to interact with other proteins including p53 which represses its activation. ARF: Alternative Reading Frame, mdm2: murine double minute, NPM: nucleophosmin.

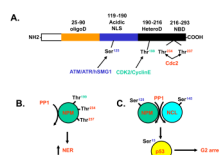


Figure 3.

(A) Schematic representation of NPM domains and phosphorylation sites. OligoD: Oligomerization domain, Acidic domain including nuclear localization signal (NLS), HeteroD: heterodimerization domain, NBD: nucleic acid binding domain. ATM/ATR, CDK2/CyclinE and Cdc2 phosphorylation sites are indicated. **(B) Dephosphorylation of NPM at Thr 199, 234 and 237 by PP1 facilitates NER.** **(C) Dephosphorylation of NPM at Ser 125 and possibly NCL at Ser145 by PP1 allows p53 phosphorylation at Ser15 in AT cells and facilitates G2 checkpoint in these cells.** NPM: Nucleophosmin, PP1: Protein Phosphatase 1, NCL: Nucleolin, NER: Nucleotide Excision Repair. See text for details.

Table 1

Nucleolin interacting proteins in the nucleus and nucleolus

Functions	Proteins	References
Ribosomal proteins	L3, L4, L5, L6, L7, L8, L9, L13a, L18, L18a, L28, L35a, L37a, S3a S8, S9, S11	(54)
DNA replication, recombination and repair proteins	hRPA, SWAP-70, topoisomerase I (Top1), hTERT, p53-inducible and death domain-containing PIDD/LRDD, PCNA, and NPM	(55) (56) (57) (58) (59) (16) (19)
Cell cycle and cell differentiation regulatory proteins	pRB, P53, CDC2 kinase, GDNF-inducible zinc finger protein 1(GZF1), casein kinase II (CKII), Histone H1, H2B and H3, interferon regulatory factor-2 (IRF-2), Hdm2, brefeldin A-inhibited guanine nucleotide-exchange protein (BIG1), A-Myb, C-Myb, glucocorticoid receptor (GR), RNA methyl transferase (NSUN2)	(60) (24) (61) (62) (63) (64) (65) (66) (67) (68) (69) (70)
Proteases	Granzyme A	(71)

Table 2

Nucleophosmin interacting proteins in the nucleus and nucleolus

Functions	Proteins	References
Ribosomal proteins	S9, L23	(72) (73)
DNA replication, recombination and repair proteins	ATR, BRCA1 and BRD1, APE1, Chk1, H2AX	(33) (74) (75) (76)
Cell cycle and cell differentiation regulatory proteins	NCL, NSUN2, ARF, p53, SENP3 and SENP5, p21WAF1/CIP1, Hdm2, YY1, PKR (eIF2 kinase), HEXIM1, Ebp1, Polo-like kinase 1 (Plk1), YB1, Nucleostemin, p120, USP36, CTCF, c-Jun, H2B, H3 and H4	(16) (70) (77) (78) (79) (80) (81) (82) (83) (84) (85) (86) (87) (88) (89) (90) (91) (92) (93)
Proteases	Granzyme M	(94)