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FORUM REVIEW ARTICLE

Emerging Roles of the Nucleolus in Regulating the DNA Damage Response: The Noncanonical DNA Repair Enzyme APE1/Ref-1 as a Paradigmatical Example

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Abstract

Significance: An emerging concept in DNA repair mechanisms is the evidence that some key enzymes, besides their role in the maintenance of genome stability, display also unexpected noncanonical functions associated with RNA metabolism in specific subcellular districts (*e.g.,* nucleoli). During the evolution of these key enzymes, the acquisition of unfolded domains significantly amplified the possibility to interact with different partners and substrates, possibly explaining their phylogenetic gain of functions. ***Recent Advances:*** After nucleolar stress or DNA damage, many DNA repair proteins can freely relocalize from nucleoli to the nucleoplasm. This process may represent a surveillance mechanism to monitor the synthesis and correct assembly of ribosomal units affecting cell cycle progression or inducing p53-mediated apoptosis or senescence. ***Critical Issues:*** A paradigm for this kind of regulation is represented by some enzymes of the DNA base excision repair (BER) pathway, such as apurinic/apyrimidinic endonuclease 1 (APE1). In this review, the role of the nucleolus and the noncanonical functions of the APE1 protein are discussed in light of their possible implications in human pathologies. ***Future Directions:*** A productive cross-talk between DNA repair enzymes and proteins involved in RNA metabolism seems reasonable as the nucleolus is emerging as a dynamic functional hub that coordinates cell growth arrest and DNA repair mechanisms. These findings will drive further analyses on other BER proteins and might imply that nucleic acid processing enzymes are more versatile than originally thought having evolved DNA-targeted functions after a previous life in the early RNA world. *Antioxid. Redox Signal.* 20, 621–639.

Overview on RNA Oxidative Damages, a Glimpse on Human Pathologies

An emerging body of evidence links DNA repair proteins to specific aspects of RNA metabolism associated with quality control processes toward damaged RNA molecules (*e.g.,* oxidized or abasic RNA) (Fig. 1). Due to its intrinsic nature (*i.e.,* mostly single-stranded and with bases not protected by hydrogen bonding or binding to specific proteins) and to its relatively higher amount, RNA may be more susceptible to oxidative insults than DNA (107). Not only 8-hydroxyguanosine (8-OHG) but also 5-hydroxycytidine, 5-hydroxyuridine, and 8-hydroxyadenosine have been identified in oxidized RNA (160). While oxidative damage to DNA is essentially repaired through the base excision repair (BER) pathway, no evidences of similar repair processes have been described for RNA molecules, even though some BER proteins recently entered the arena of the RNome world (138). If not repaired, damage to RNA molecules may lead to ribosomal dysfunctions and erroneous translation; thus, significantly affecting the overall protein synthesis mechanism (38, 135). Moreover, RNA damage has been shown to cause cell cycle arrest and cell death with or without the contribution of p53 and inhibition of protein synthesis (11). Oxidative RNA modifications can occur not only in protein-coding RNAs, but also in noncoding RNAs that recently have been revealed to

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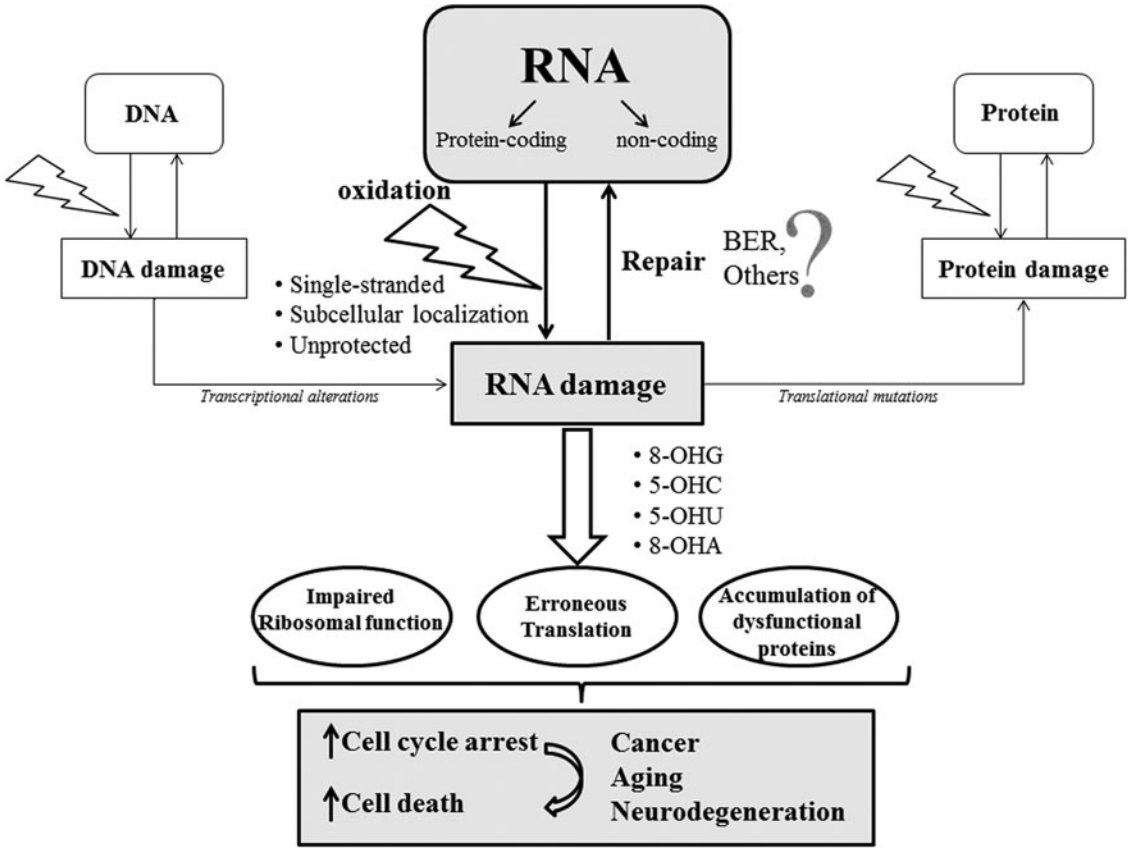

FIG. 1. Potential consequences of unrepaired RNA damage. RNA intrinsic nature renders it more susceptible to damage, such as oxidation. The molecular and surveillance mechanisms that cope with RNA damage are still poorly understood. If unrepaired, aberrant RNA may give rise to translation of defective and toxic protein aggregates that eventually leads to cell cycle arrest and consequently to cell death. These molecular processes have been associated with cancer onset, aging, and neurodegeneration.

contribute to the complexity of the mammalian brain (109). It has been hypothesized that RNA oxidation, causing aberrant expression of microRNAs and proteins, may initiate inappropriate cell fate pathways. Such sublethal damage to cells, while less toxic than genomic mutations and not inheritable, might be associated with underlying mechanisms of degeneration, such as age-associated neurodegeneration.

Currently, little is known about how cells may cope with damaged RNA, either modified or oxidized, but it is clear that such RNA can impair protein synthesis; thus, affecting cell function and viability. Therefore, specific surveillance mechanisms are needed to remove damaged molecules from the RNA pool to guarantee the biological integrity of cells. The idea that quality control mechanisms might exist to repair RNA was put forward after the identification of the biochemical activities of the mammalian AlkB homologs. In particular, it was discovered that AlkB (from *Escherichia coli*) and the human homolog hABH3, besides being able to directly reverse alkylation damage on DNA bases, were able to demethylate damaged bases on RNA; thus, playing a key role in the repair of specific RNA lesions (1, 111). While repair mechanisms have been demonstrated for alkylated RNA, the existence of such cleansing activities has not yet been identified for oxidatively damaged RNA. However, their existence appears unlikely, in the absence of direct reversal strategies, due to the lack of a template for accurate repair, as happens in the case of double-stranded DNA. The observation that under oxidative stress RNA modifications can occur up to a 10–20-fold higher extent than DNA (88), raises the question of how oxidized RNA may be specifically removed or repaired. The recent findings by Berquist *et al.* (12), Barnes *et al.* (7), and Vascotto *et al.* (149) highlight a novel "moonlighting" role for the repair apurinic/apyrimidinic (AP) endonuclease 1 (APE1) in RNA metabolism, both as a possible "cleansing" factor for damaged abasic RNA and as a regulator of *c-myc* gene expression through mRNA decay. In agreement with this hypothesis, a significant reduction in the protein synthesis rate occurs upon silencing of APE1 expression (149). Enzymatic (*e.g.*, by specific N-ribohydrolases, including the toxin ricin) (126), besides spontaneous (91), generation of abasic sites occurs upon ribosomal RNA (rRNA) oxidation. A role for the Y box binding protein 1 (YB-1) in recognizing 8-OHG sites has also been hypothesized (56), but no specific enzymatic mechanisms that can remove the oxidized base have been described yet. The accumulation of the 8-OHG substrates, which occurs upon silencing of APE1 expression (149), may thus, be explained under the assumption that enzymatic removal of oxidized RNA bases represents the limiting step in the process. According to this model, as already hypothesized for DNA substrates, APE1 could act through stimulation of a yet unknown glycosylase activity, by allowing a faster turnover (152). The APE1 interaction with RNA and with proteins

involved in ribosome assembly (*i.e.,* RSSA, RLA0) and RNA maturation (*i.e.,* PRP19) within the cytoplasm (149), could also represent a molecular proof of concept for the "extranuclear" functions of this noncanonical DNA repair enzyme. The RNA-mediated association with some of the APE1 protein partners would reinforce the view of APE1 as an essential factor in the RNA quality control process and may also explain the cytoplasmic accumulation of APE1 observed in a number of tumoral cell types (136, 137). An age-associated increase in oxidative nucleic acids damage, predominantly to RNA, has been recently highlighted in neurons from human and rodent brains; this phenomenon may play a fundamental role in the development of age-associated neurodegeneration (109). Oxidative damage to RNA molecules, both coding for proteins (mRNA) or performing translation (rRNA and tRNA), has been recently associated with the occurrence of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson disease (PD), dementia with Lewy bodies, and amyotrophic lateral sclerosis (107) and its impact in cancer development cannot be excluded, at present (11). Remarkably, studies performed on either human samples or experimental models, show that RNA oxidation is a characteristic of aging neurons. Its prominent occurrence in vulnerable neurons at early stage of age-associated neurodegenerative disorders indicates that RNA oxidation actively contributes to the development of the degeneration. Therefore, all the hypotheses concerning the involvement of APE1 in the development of such pathologies should be re-interpreted in light of these findings. The role played by APE1 in RNA-related processes needs further investigations, since its ability to recognize and cleave the RNA abasic sites (12, 42, 89, 138, 149) is compatible with a leading role in the early stages of the RNA quality control process. Additional studies aiming at the understanding the mechanisms related to oxidative RNA damage processing and their consequences may provide significant insights into the pathogenesis of neurodegenerative disorders, leading to improvements in the current therapeutic strategies.

The vast majority of cellular RNA is transcribed, assembled, and processed within nucleoli. These subcellular compartments appear to be perfect sensors for cellular stress, as they integrate RNA damage and growth control with signals to the DNA repair machineries. The following paragraphs will describe the emerging dynamics roles of this organelle in regulating the trafficking of DNA repair proteins during genotoxic damage.

Structure, Composition, and Classical Functions of the Nucleolus

The nucleolus is considered the ribosome factory of eukaryotic cells (18, 62) in which synthesis, maturation, and processing of rRNA, as well as assembly of rRNA with ribosomal proteins (RPs) take place (62) (Fig. 2). This membraneless organelle is considered a dynamic structure (4, 84), where protein complexes are continuously exchanged with the nucleoplasm. Its classical tripartite organization has been dissected using electron microscopy and reflects the different steps of ribosomal biogenesis (130): (i) the fibrillar center, where the RNA polymerase I (Pol I) transcription starts; (ii) the dense fibrillar component, where the initial stages of pre-rRNA processing occur and (iii) the granular component involved in the late processing steps (62). Transcription of the ribosomal DNA (rDNA) repeats generates a 47S pre-rRNA precursor that is further cleaved and processed into 28S, 18S, and 5.8S rRNAs and concomitantly assembled into large and small ribosomal subunits together with the 5S rRNA molecules (18, 32a). These complex series of events is controlled, in yeast, by roughly 150 small nucleolar RNAs (snoRNAs) and two large ribonucleoprotein complexes, named small subunit processosome (for the 40S ribosomal subunit) and large subunit processosome (for the 60S ribosomal subunit) (130). Two types of nucleotide modifications (2′-*O*-methylation and pseudouridylation) are introduced during the maturation process by snoRNAs belonging to the box C/D or box H/ACA families and mediate endonucleolytic cleavages of pre-RNAs (130, 32a). In addition, ribosomal gene transcription is regulated through the modulation of the transcriptional apparatus and epigenetic silencing (131). Large and small mature ribosome particles are independently exported to the cytoplasm through an exportin 1 (CRM1) and Ran-GTP-dependent mechanism: export of 60S subunit requires the exchange of complexes Noc1-Noc2 by Noc3-Noc2 (102) and the association with the adaptor shuttling protein NMD3 (142), whereas the 40S needs the heterodimer Noc4p/Nop14p (103). The work by Hinsby *et al.* exploited a machine learning-based predictor of nuclear export signals to analyze the late stage pre-40S complex, suggesting a role also for the human homolog of yeast DIM2p in the targeting and translocation of the late 40S to the cytoplasm (63).

The organization, the number, and the size of nucleoli in each cell is directly linked to the nucleolar activity (*i.e.,* Pol I transcription rate), which, in turn, depends on cell growth and metabolism (20). Generally, highly proliferating cells present many small nucleoli (62, 130). Ribosomal biosynthesis is a highly energy- and resources-consuming process (134); this explains why this process is tightly regulated by changes in cell proliferation, growth rates, and metabolic activities. Nucleoli constantly integrate different signaling events, maintaining the ribosomal subunit pool required to properly support protein synthesis during cell growth and division (18).

Biosynthesis of ribosomes is a very efficient process, since it has been estimated that 14,000 new ribosomal subunits can be synthesized every minute in an exponentially growing cell (125). The process has to be fine-tuned and several evidences indicate that ubiquitin and ubiquitin-like proteins-based regulatory circuits may control different stages of ribosome formation (129). Lam *et al.* (83) demonstrated that the levels of unassembled RPs within nucleoli exceed the required RP:rRNA ratio and identified an ubiquitin-proteasome-mediated mechanism, that monitors and degrades this excess (115). Ribosomal structural proteins are imported from the cytoplasm in supra-stoichiometric complexes and exported in the form of assembled ribosomal subunits; this ensures that RPs are never rate-limiting for the ribosomal assembly (83). The excess of protein undergoes ubiquitylation and degradation in the nucleoplasm through the proteasome system (114, 129), indicating that mammalian cells produce large amount of these proteins and degrade those that are not assembled with rRNA.

"Dynamic Trafficking:" Keywords of the Nucleolar Physiology

The nucleolus appears as a very dynamic organelle and different types of rearrangements involve this subnuclear

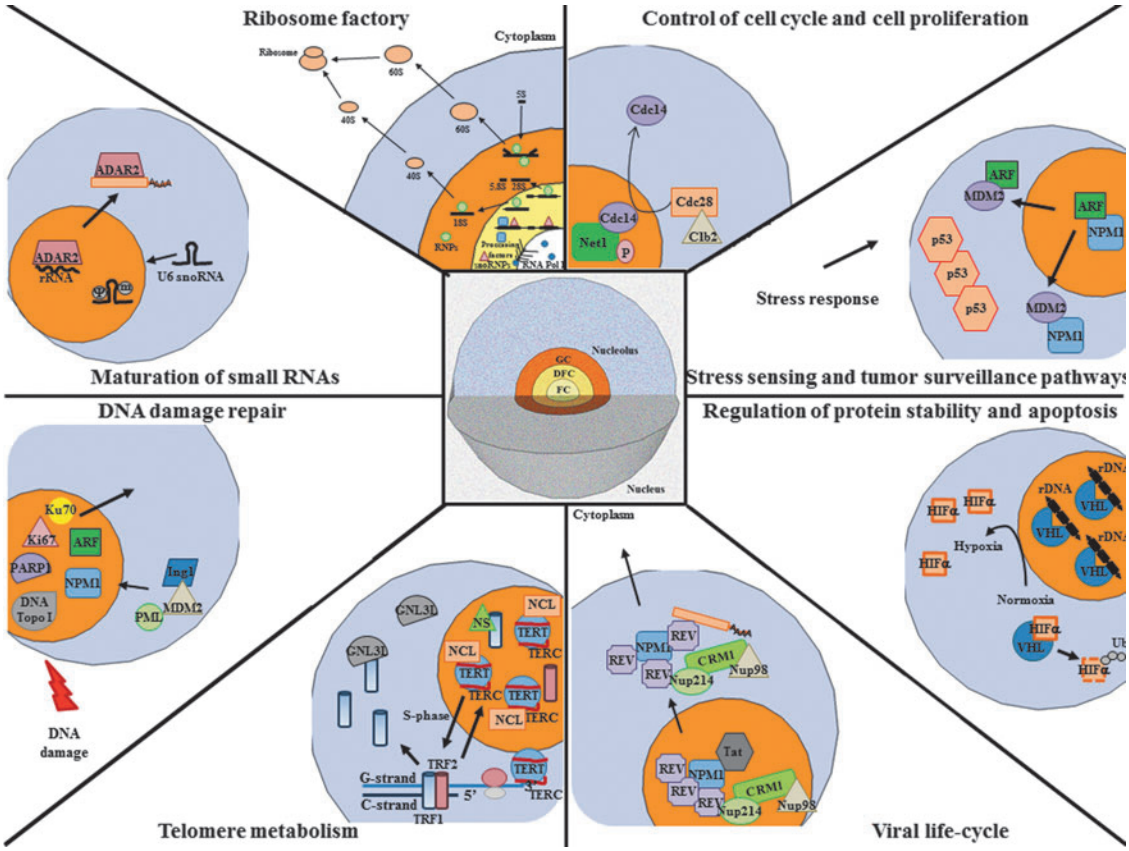

FIG. 2. The nucleolus, a multifunctional domain. The unveiling of the "nucleolome" allowed the understanding of the complexity of the nucleolus, firstly described only as a ribosome factory. The multifunctionality of the nucleolus includes different noncanonical functions as the control of cell proliferation and cell growth, regulation of protein stability, stress and DDR, telomere metabolism, maturation of small RNAs, and control of viral life-cycle. An illustrative mechanism for each function is summarized. MDM2, mouse double minute 2 homolog; ARF, p14 alternative reading frame; ADAR2, double-stranded RNA-specific adenosine deaminase 2; NS, nucleostemin; VHL, von Hippel-Lindau protein; HIF, hypoxia-inducible factor; ING1, inhibitor of growth family protein, member 1; PML, promyelocytic leukemia protein; DNA Topo I, DNA topoisomerase I; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase; GNL3L, guanine nucleotide binding protein-like 3 (nucleolar)-like; TRF, telomeric repeat-binding factor 2; REV, regulator of expression of virion protein; Tat, trans-activator of transcription; Ub, ubiquitin; DDR, DNA damage response. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

district. First, the ribosome assembly is a vectorial process, in which the ribosomal particles move away from their biogenesis sites. The second dynamic aspect is represented by the constant flux of proteins from the nucleoli to the nucleoplasm. Lastly, nucleolar components present scheduled reorganization every cell cycle, with assembly and disassembly steps required for the redistribution of nucleolar components between the two daughter cells (110).

The cyclic reorganization observed during cell cycle involves the nucleolus as a whole, as this organelle assembles at the end of each mitosis to remain functionally active until the beginning of the next one (4, 62). The nucleolar disassembly observed during mitosis is linked to rDNA transcriptional repression induced by CDK1-cyclin B-directed phosphorylation of components of the rDNA transcription machinery (130). Conversely, the formation of functional nucleoli at the exit of mitosis is not uniquely controlled by the resumption of rDNA transcription, but by a two-steps process regulated by cyclin-dependent kinases that connects the resumption of rDNA transcription and the restoration of rRNA processing (130). During late telophase, nucleoli form around the nucleolar organization regions, which are chromosomal domains where rDNAs, clustered in head-to-tail arrays (62, 84), are transcribed. This sub-nuclear compartmentalization allows the cells to locally concentrate all the factors required for the ribosomal biogenesis (18, 20).

The Way to Move: Visitors *Versus* Resident Nucleolar Proteins

Differently from nuclear localization signals (NLSs), nucleolar localization sequences (NoLSs) are not well characterized and no clear consensus sequences have been described yet (115, 130). NoLSs identified so far are rich in basic residues (*e.g.*, Arg and Lys) (40), but also Trp residues were described to play a functional role for the nucleolar localization of nucleophosmin 1 (NPM1) (108a). Since the mass per unit nucleolar volume is only twofold higher compared with the nucleoplasm, all diffusing macromolecules should theoretically be able to enter nucleoli: it is now clear that the residence time of nucleolar proteins depends on their relative affinity for preanchored complexes present within the nucleolus itself

(114, 115). The ability of different proteins to localize within the nucleolus has been linked to the stable interaction with anchored resident proteins, such as NPM1 or nucleolin (NCL), that may act as carriers, or as a retention scaffolds, supporting the idea that the residence time of proteins within the nucleolar compartment is strongly related to their specific interactions in a process known as "nucleolar sequestration" (40, 115, 130). Though all the molecules resident in the nucleoplasm may, in principle, enter into nucleoli, only those with an affinity for nucleolar resident proteins, are retained for longer times (110). For this reason some authors suggested the concept of "retention signal" in place of "targeting signal" for the nucleoli (121). Since the residence time is quite short and most of the nucleolar proteins shuttle from nucleoli to nucleoplasm and vice versa, the interaction of visitor proteins with anchored nucleolar residents has to be reversible (110, 115). Current models show that proteins and RNAs continuously flow and freely diffuse through the nuclear space (131): the average residence time for most nucleolar proteins within nucleoli is estimated to be only a few tens of seconds. Thus, nucleolus appears as a steady state structure with its component in a dynamic equilibrium with the surrounding nucleoplasm (121). However, in spite of this continual exchange of molecules between these two compartments, nucleolar domains are maintained because the number of retained proteins is higher than the amount of molecules that are released from the same domain (32a). Resident factors, able to connect and bind multiple protein partners, are considered as "hub proteins" and might be responsible for the nucleolar localization of the vast majority of visitor proteins in the absence of RNA–protein interactions. Each hub protein may have different recognition requirements; thus, possibly explaining the occurrence of different NoLSs. Two typical examples of hub proteins are NCL and NPM1, which contain disordered regions involved in protein–RNA interactions (40).

Recently, an additional protein retention mechanism based on GTP-driven cycles has been identified for nucleostemin (121). The GTP-GDP exchange mechanism is not the only intracellular signal able to move nucleolar proteins: stimuli as the hydrogen ion were already described by Mekhail *et al.* (98), who demonstrated how the nucleolar sequestration of the von Hippel-Lindau protein after a pH change promotes the stabilization of the hypoxia-inducible factor, triggering a general cell response to hypoxic conditions (115).

New Concepts in the Nucleolus Physiology: Beyond the Ribosome Factory

In the 60s, after the identification of the localization of ribosomal genes, the main function of the nucleolus was nicely summarized as "an organelle formed by the act of building a ribosome" (63). In the last decades, thanks to the development of technologies, such as protocols for the isolation of large amounts of nucleoli and the improvement of high-throughput mass-spectrometry-based proteomic approaches, several proteomic analyses were undertaken, unveiling the "nucleolome" (4, 5, 18, 32a, 125). Over 4500 proteins were described to localize within nucleoli and both bibliographic and bioinformatics analyses allowed to classify eight major functional groups (RPs, ribosome biogenesis, chromatin structure, mRNA metabolism, translation, chaperones, fibrous proteins, and others [see Fig. 3 for further details]) (3, 18, 32a, 108). Characterization of the nucleolar proteome under different stress conditions (*e.g.*, actinomycin D) further underlined the complexity of the nucleolome dynamics (5).

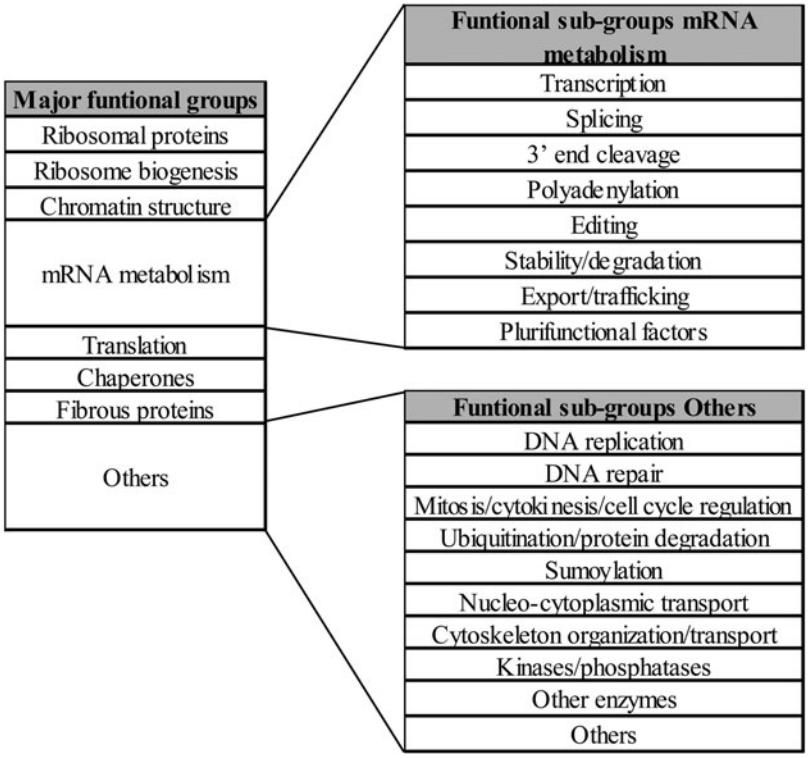

FIG. 3. Classification of functional groups and subgroups for the "nucleolome."

The nucleolome includes proteins related to cell cycle regulation, DNA damage and pre-mRNA processing (84), suggesting that this organelle may act as a multitasking district, being more than a simple ribosome factory (18, 62, 63, 84, 125). These particular multifunctional features are possibly related to the presence of many different nucleolar proteins endowed with multiple roles (*e.g.,* NCL and Nopp140) (90a). Nucleolar proteins unrelated to ribosome assembly mostly contain an RNA-binding motif or have a chaperone function and are able to shuttle between the nucleolus and the nucleoplasm (90a). It is currently known that nucleoli carry out many nonribosomal activities, such as: control of cell cycle and proliferation (62, 63, 130), stress sensing (18, 21, 64, 96, 108, 124, 139), tumor surveillance, DNA damage repair (18, 41, 84), regulation of protein stability and apoptosis, telomere metabolism (18), maturation of small RNAs, including tRNAs and small nuclear RNAs, maturation of the spliceosome and of the signal recognition particle (63, 115, 121), and also control of viral lifecycle (62, 71), acting in several ways as a sequestration core facility (Fig. 2) (21, 40, 63).

The assumption that a protein acts and executes its functions where it is more abundant is invalid for some nucleolar components: a high protein concentration in a given cellular compartment might not correspond to a quantitative signal for its activity (114). A clear example is represented by the many DNA repair proteins that reside within nucleoli (see Table 1 for more details), which in this instance, appear to act as storage sites (see below). After nucleolar stress or DNA damage, these proteins can relocalize to the nucleoplasm. The nucleolar stress response may therefore, act as a mechanism of surveillance monitoring the synthesis and the correct assembly of ribosomal units: it may halt the cell cycle progression until enough functional ribosomes are built or it may induce p53-mediated apoptosis or senescence (134). This represents an elegant and efficient way to coordinate arrest of cell growth and induction of DNA repair by controlling only the subcellular distribution of proteins.

Linking Nucleolar Physiology to Human Diseases

Several reports highlighted the presence of a layer of heterochromatin, called perinucleolar compartment (PNC), at the nucleolar periphery. The PNC is associated with specific DNA loci and it is enriched in RNA-binding proteins and RNA polymerase III transcripts (62, 114). These discoid-shaped caps are usually found in mammalian transformed cells, tumor-derived cell lines, and tumor biopsies, accounting for a possible connection between PNC and tumor initiation and progression (114). Despite the apparent link between cancer and PNC, the exact role of these nucleolar subdomains has not been identified yet.

Nucleoli are closely linked to cellular homeostasis and human health (62): rapidly proliferating cells, such as cancer cells, usually present a strong upregulation of genes involved in the ribosomal biogenesis. This phenomenon accounts for the presence of several prominent nucleoli that are a typical cytological feature of neoplastic cells (129). Morphological and functional changes associated with cancer usually correlate with quantitative and qualitative differences in the ribosome biosynthesis rate (62) and are a consequence of the increased metabolic needs of the cell (105). This could indicate that the nucleolus undergoes an adaptive response upon cellular transformation; on the other hand, the increased rate of ribosome biogenesis might be actively involved in, and contribute to, tumorigenesis (105). More recently, the accumulation of chemically modified ribosomes upon oxidative stress (*e.g.,* bearing 8-OHG modifications, or cross-linked rRNA-RPs complexes) is emerging as a contributing factor in the progression of neurodegenerative diseases, such as AD and PD (62). In addition, a novel link between nucleolar damage and neurodegeneration has recently been established through the association of PD with nucleolar integrity. These interesting findings also establish the existence of a direct signaling axis connecting the ribosomal synthesis rate and oxidative stress (122). Under this perspective, the deep understanding of changes related to tumor progression and the composition and dynamics of the nucleolome might be important to clarify the mechanisms of tumorigenesis and for designing new therapeutic strategies.

Dynamics of DNA Repair Proteins During Genotoxic Damage: Nucleolus and RNA Binding

Cellular life is continuously threatened by stressful conditions, such as viral infections, oncogenic activations, temperature shocks, and genotoxic damage, which must be immediately dealt with by the cell to maintain its homeostasis. DNA damage typically involves the storage sites of the genetic material, such as nucleus and mitochondria. Depending on the origin of the genotoxin (exogenous *vs.* endogenous), its nature (physical *vs.* chemical) or its uptake mechanisms, differential burden of damage can be accumulated both in nuclear and in mitochondrial DNA. Upon genotoxic insults a dynamic redistribution of DNA repair proteins to the site of damage is frequently observed; this phenomenon leads to the accumulation of DNA repair factors, often into spatially restricted foci. Relocalization events usually occur through cytoplasmic-mitochondrial, cytoplasmic-nucleoplasmic, and nucleolar-nucleoplasmic shuttling regulated by post-translational modifications (PTMs) and triggered by the damage itself (51).

Why nature selected such a time-consuming process involving reorganization of cell proteome upon DNA damage, instead of constitutively accumulating each DNA repair factor within the proper target compartment? Is there any functional relationship between RNA metabolism and the DNA damage response (DDR), which may involve an active site of RNA production, such as the nucleolus? These legitimate questions are still a matter of debate; however, based on existing data at least four possible explanations, not mutually exclusive, appear reliable: (i) the concentration, under physiological levels of DNA damage, of high amounts of DNA repair proteins in specific subcellular regions might be deleterious for nucleic acids integrity. Many repair proteins, in fact, are endowed with DNA trimming or modifying activities. Examples include APE1 or the DNA polymerase β (Polβ); overexpression of these proteins has already been shown to promote genomic instability, possibly due to the lack of coordination of their enzymatic activities (26); (ii) repair factors may exert more than one specific function that cannot be directly linked to their DNA repair activity. This situation is nicely exemplified by the APE1 protein, which nucleolar localization is necessary for cellular proliferation (see below), or by proteins, such as the Werner syndrome helicase (WRN), or the flap

Table 1. DNA Repair Protein Composition in the Nucleolus

Protein name	Putative role inside the nucleolus	Role(s) outside the nucleolus	Genotoxic stress leading to protein relocalization		References
			Imported	Exported	
APE1	RNA processing	AP endonuclease		Actinomycin D	(5, 149)
ATM		DNA damage signaling			(106)
ATR		DNA damage signaling			(106)
BLM	rDNA transcription	DNA helicase		Camptothecin	(5, 120, 163)
BRCA1/BARD1		E3 Ligase involved in HR		IR	(53)
CSB		Core NER factor			(99)
DNA-PK catalytic subunit		Kinase involved in NHEJ		UV, cisplatin	(36, 106)
FEN1	Resolution of stalled replication forks	Structure-specific nuclease		UV	(54)
ILF2 and ILF3		dsRNA binding proteins interacting with Ku70/80 and DNA-PK		UV	(106)
Ku70/80		DSB recognition in the NHEJ pathway		UV, cisplatin, IR, Actinomycin D, mitomycin C, hydroxyurea, doxorubicin	(2, 36, 106)
LigIII		DNA ligase involved in the BER pathway and in the SSB repair		UV, IR	(106)
MRE11		Nuclease involved in DSB repair			(5, 108)
MSH6		MMR factor		MNNG	(95)
Mus81	rDNA repair	Structure-specific endonuclease			(48)
OGG1		DNA glycosylase	Ro19-8022 + light (405 nm), $KBrO_3$		(25, 99)
p53		Multifunctional tumor suppressor, auxiliary roles in NER and BER		UV	(123, 139)
PARP1 and PARP2		DNA damage signaling and transcriptional modulation		Actinomycin D, H_2O_2, MMS, camptothecin, UV	(5, 97, 106)
PCNA		Sliding clamp involved in DNA repair and replication			(161)
PNK		DNA end trimming kinase/phosphatase			(5, 63)
Polβ		DNA polymerase involved in the BER pathway			(63)
Rad9B	rDNA repair	Poorly understood	UV, Actinomycin D		(117)
Rad17		DNA repair and replication modulator		UV, IR, MMS, adriamycin	(28)
Rad50		Part of the MRN complex			(5)
Rad52	rDNA repair	DSB responsive protein			(32)
RECQL4		DNA helicase	H_2O_2, streptonigrin		(156)
RNF8	Modulation of ribosome biogenesis	E3 ligase involved in HR		IR	(53)
RPA		Single-strand DNA binding protein involved in DNA repair and replication			(108)
Slx1-Slx4 complex	rDNA repair	Structure-specific endonuclease			(32)
SMUG1	rRNA quality control	DNA glycosylase			(73)
SSRP1		Structure-specific protein binding to cisplatin-DNA adducts		Cisplatin	(36)
TDP1		Tyrosyl DNA phosphodiesterase		Camptothecin	(8)
TOPBP1		DNA repair and replication modulator			(106)
Topo I		DNA topoisomerase		UV, camptothecin	(22, 93, 104)
Top2a and Top2b		DNA topoisomerases			(5)
WRN	rDNA transcription	DNA helicase implicated in BER and recombination		UV, IR, MMC, MMS, bleomycin, camptothecin, etoposide, cisplatin	(5, 16, 36, 75, 94)
XPC		Core NER factor	Ro19-8022 + light (405 nm)		(99)
XPG	rDNA transcription	NER endonuclease		UV	(112)
XRCC1		Scaffold protein in the BER pathway		Camptothecin	(5, 63, 120)

APE1, apurinic/apyrimidinic endonuclease 1; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; BER, base excision repair; DSB, double-strand break; FEN1, flap endonuclease 1; HR, homologous recombination; IR, ionizing radiation; NER, nucleotide excision repair; NHEJ, nonhomologous end joining; OGG1, 8-OHG DNA glycosylase; PARP, poly [ADP-ribose] polymerase; PCNA, proliferating cell nuclear antigen; Polβ, DNA polymerase β; rDNA, ribosomal DNA; rRNA, ribosomal RNA; RPA, replication protein A; SSB, single-strand break; WRN, Werner syndrome helicase; XRCC1, X-ray repair cross-complementing protein 1.

endonuclease 1 (FEN1) (54), that are required for the active transcription of ribosomal genes in the nucleolus. All these factors promptly relocalize to the nucleoplasm to exert their DNA repair function upon UV irradiation; (iii) while a lower steady-state amount of DNA repair enzymes is sufficient to grant a basal level of genome stability maintenance, it is likely that genotoxin-induced protein accumulation mechanisms have evolved, to cope with sustained DNA damage. Thus, increasing the local concentration of DNA repair factors may be a simple and fast way to amplify the local DNA repair capacity; (iv) disruption of the nucleolar structure, often observed occurring upon DNA damage, may halt rRNA production; thus, leading to a block of the protein synthesis machinery and allowing proper DNA repair. This may represent an efficient coupling mechanism involving nucleolus dynamics, RNA-binding, and DNA repair, which allows synchronization of DDR and arrest of cellular growth.

While many examples of proteome dynamics have been reported to occur during DDR, this review will focus on the relocalization of nucleolar proteins; for detailed information concerning general subcellular reorganization, please refer to Tembe and Henderson (139) and references therein.

The Nucleolus as DNA Damage Sensor and Storage Facility for DNA Repair Proteins

As already mentioned, recent proteomic analyses have pointed at the nucleolus as a mediator of the cell cycle regulation, tumor suppressing or protumorigenic activities, and DDR (5, 17, 63, 108). The presence of rRNA and hub proteins, such as NCL and NPM1 is likely the critical factor for the nucleolar accumulation of many proteins not uniquely related to ribosome biogenesis (27, 40). Among these, many DNA repair factors have been shown to localize within the nucleolus (Table 1), which acts as a stress response organelle and responds, in a unique damage-specific manner, to different cellular stresses (5, 17, 30, 106). During a stress response a broad reorganization of the nucleolar proteome occurs; interestingly, the vast majority of nucleolar proteins migrate toward the nucleoplasm and not vice versa, indicating that the nucleolus may act as a reservoir able to release critical factors upon DNA damage (108, 139). Very few proteins have been reported to migrate to the nucleolus during the stress response. Among these ING1 and PML, which are thought to participate in triggering of apoptosis and in cell cycle arrest, respectively (139). Intriguingly, chaperones, such as Hsc70 are targeted to the nucleolus after the stress response, possibly to restore the nucleolar function during the cellular recovery from stress. Typically, during the response to DNA damage or transcriptional inhibition, one of the first events is represented by the interruption of the ribosomal synthesis (108). This mechanism likely represents a cellular strategy to maintain homeostasis, indeed, as already pointed out, the ribosomal biogenesis is a rather expensive energy-consuming process (20). The impairment of rRNA transcription and processing is often, but not always, associated with nucleolar disintegration and condensation phenomena that lead to the formation of caps and necklace-like structures (20, 106). An extensive analysis of the effects of chemotherapeutic drugs on ribosome biogenesis and nucleolar integrity has recently been carried out by Burger *et al.* (23). For a broader list of responses to different stress stimuli, please refer to Boulon *et al.* (20). Along with the inhibition of ribosomal biogenesis, massive reorganization occurs, with a rapid outflow of "nucleolar effectors", such as $p14^{Arf}$, NCL, and NPM1, which slow down or arrest the cell-cycle in both p53-dependent and independent manners (20, 31, 34, 49, 86). Concurrently, also DNA repair factors stored within nucleoli and frequently bound to NCL and NPM1 are released into the nucleoplasm; the transient arrest of the cell-cycle progression possibly facilitates the DNA repair process. Only after resolution of DNA damage, rRNA synthesis is restored, as suggested by the inverse correlation existing between the rDNA transcription rate and γ-H2AX foci number (80). Notably, many nucleolar effectors also play a role within the nucleoplasm: beside the contribution to the modulation of the cell-cycle, several reports have pointed out that NCL or NPM1 may directly participate in the DDR. For instance, NCL has been shown to tune proliferating cell nuclear antigen (PCNA) activity in the nucleotide excision repair (NER) pathway (161), while NPM1 has been involved in the BER modulation (see below). Interestingly, both these proteins display strand annealing activity *in vitro* (19, 55) and have been identified as stress-responsive RNA-binding proteins, suggesting that upon genotoxic damage these factors may modulate DNA, as well as RNA repair or cleansing (162). More importantly, emerging evidences further highlight the importance of nucleolar hub proteins in human pathology. The link between NPM1 and oncogenesis, for example, is a well-established paradigm (31, 52, 151) and we recently demonstrated that in acute myeloid leukemia bearing NPM1 mutations, the cytoplasmic de-localization of the mutant NPM1c+ leads to extensive BER impairment due to APE1 nuclear deprivation (150). This evidence denotes how the deregulation of an important nucleolar factor might impact on the overall cellular dynamics and not only on the ribosome biogenesis, supporting the view of the nucleolus as a multifunctional and versatile organelle. Recent evidences from Lewinska *et al.* showed that, in yeast, the nucleolus acts as stress sensor also for oxidative stress. Their work linked the nucleolar exit of the Pol I-specific transcription factor Rrn3p, to the response to oxidation, suggesting that oxidative damage is indeed a cellular stress that is sensed from the nucleolus leading to arrest of the ribosome transcription (87). Interestingly, diethyl maleate-induced oxidative stress has been demonstrated to modulate also the whole nuclear export system through impairment of the CRM1-mediated nuclear export, coupled to relocalization of the nuclear pore component Nup98 to the nucleolus (33). Altogether, these observations underline the role of the nucleolus as stress sensor, further confirming the validity of the aforementioned model involving arrest of ribosome biogenesis, followed by nucleoplasmic and nucleolar proteome rewiring.

The damage-specificity of the nucleolar response is clearly depicted by the differential response to diverse DNA damaging stimuli: DNA repair factors undergo selective mobilization upon specific genotoxic conditions (Table 1). UV and ionizing radiation (IR), for instance, elicit markedly differential responses in terms of nucleolar proteome reorganization. Whereas UV stress is characterized by fast and persistent (hours), fluctuations of nucleolar nonhomologous end joining (NHEJ) proteins, IR lead to quicker (minutes), but less persistent responses, which are more limited, in terms of magnitude (106). The dynamic reorganization of nucleolar proteins upon DNA damage has been nicely described by

Adelmant *et al.*, who showed that microinjection of sheared DNA, to mimic the presence of strand breaks, leads to the rearrangement of Ku complexes. Intriguingly, in the absence of DNA damage, Ku associates with rRNA- and RP-containing complexes; a DDR onset elicits the exit of Ku from nucleoli and the modification of its interactome (2). This process likely represents a general response mechanism, where the nucleolus acts as a sensitive "antenna" for stress and DNA damage and as central hub for the coordination of the cellular response to stress conditions. The ability to undergo highly dynamic and selective reorganization allow for prompt release of DNA repair factors that are stored within this organelle. An essential question still remains to be answered: which is the triggering event that begins the signaling cascade linking DNA damage to the early nucleolar response? PTMs are usually a quick way to rewire the cellular proteome. APE1 and WRN translocation from the nucleolus is for instance triggered by acetylation (16, 89); on the contrary, FEN1 has been shown to migrate to the nucleoplasm upon UV irradiation in a phosphorylation-dependent manner. Remarkably, mutations that mimic or impair the UV-induced FEN1 phosphorylation, cause UV sensitivity (54), suggesting that DNA damage-induced protein translocation is essential for a correct DDR. Griffiths *et al.* pointed to SUMOylation as the major PTM targeting DNA glycosylases in yeast (51); this PTM has also been implicated in the modulation of rDNA repair through export of the rDNA double-strand breaks (DSBs) to the nucleoplasm by the homologous recombination (HR) machinery (39). In addition, the nucleolus has been reported to contain several DNA damage sensors (*e.g.*, the ataxia telangiectasia mutated [ATM], the ataxia telangiectasia and Rad3-related [ATR], and p53) (5, 123) and it has been demonstrated that, upon DNA damage induction, Pol I-mediated transcription is blocked in an ATM-dependent manner, and not by the DNA damage itself. Interestingly, through microirradiation studies, Kruhlak *et al.* showed that transcription of rDNA is transiently arrested only in damaged nucleoli, whereas the neighboring ones maintain normal transcriptional activity (80). Moreover, Rubbi and Milner, have elegantly shown that nucleolar disruption, rather than DNA damage, may lead to p53 stabilization, suggesting that the nucleolus may constitutively promote p53 degradation, unless DNA damage occurs (124). It would be interesting to understand if and how the extra-nucleolar DNA damage is ultimately signaled to the nucleolus and which is the event that triggers the nucleolar segregation.

The Paradigmatic Example of the APE1/NPM1 Interaction

APE1 is a typical example of DNA repair protein activated during the nucleolar stress response. APE1 is a multifunctional and essential factor in mammals that was identified about 20 years ago as the major AP-endonuclease in the BER pathway, as well as a redox coactivator of transcription factors (37, 157). The recent observation that APE1 is able to bind and cleave RNA highlighted previously unsuspected roles for the protein (77, 138). We found indeed that this protein associates with NPM1 in the nucleolus, where it may have a functional role as RNA cleansing factor. This hypothesis is corroborated by studies performed with inducible HeLa APE1 knock down cell lines, which showed how APE1 depletion leads to a widespread accumulation of unrepaired oxidized RNA species (*i.e.*, 8-OHG) upon oxidative stress. Notably, under unstressed conditions, APE1 knock down cells display impaired translation ability, lower protein content and overall cell growth impairment (149). These evidences point to a major contribution for APE1 as cellular scavenger of damaged RNA species. The nucleolar storage of APE1 is mediated by the interaction of the flexible and evolutionarily acquired N-terminal extension of the protein with both rRNA and NPM1 (42, 89, 116). However, the protein does not constitutively accumulate in the nucleoli; in fact, upon Pol I inhibition with actinomycin D, APE1 shuttles to the nucleoplasm (149). The evidence that treatment with the E3330 redox inhibitor (45) causes APE1 nucleolar exit and its accumulation to the nucleoplasm (147) suggests that the redox status of APE1 may play a significant role in controlling its subcellular trafficking. Interestingly, the APE1/NPM1 association is also impaired during oxidative stress (149), suggesting that the protein may be released from the nucleolus during stress conditions, possibly to operate within the BER pathway. In accordance with this observation, we delineated a complex regulatory pattern of NPM1 on APE1 endonuclease activities: NPM1 acts as an inhibitor of the APE1 ribonuclease function, but as an activator of the AP-endonuclease function on DNA (149, 150). This model suggests that when APE1 resides within the nucleolus, its activity is mainly focused on the rRNA quality control machinery, possibly modulated by NPM1. Whereas, during the DDR, the simultaneous outflow of APE1 and NPM1 to the nucleoplasm, leads to the activation of the APE1 AP-endonuclease function (Fig. 4). In agreement with this view, many reports point to the involvement of NPM1 in different aspects of the DDR, yet, the exact contribution(s) of this protein to the stress response is currently elusive (31, 79, 127). It is worth pointing out that the lack of NPM1 has been proved to sensitize cells to genotoxins that elicit a BER response and that APE1 catalytic activity is impaired in NPM1 knock out cells (150). These elements suggest that NPM1 plays a direct role in the BER modulation, which is still poorly understood. As discussed in the previous section of this review, the key event that triggers the APE1 release from nucleoli upon genotoxic stress is still a matter of debate. It is known that the APE1/NPM1 interaction is modulated by acetylation on the N-terminal domain of APE1 (42, 89). Acetylation of this protein region is induced upon genotoxic stress (89, 128); it is therefore, likely that, once again, stress-induced PTMs drive APE1 shuttling to the nucleoplasm in response to DNA damage. In an effort to characterize the response of APE1 to genotoxic stress, we generated a quadruple lysine to alanine substitution that mimics constitutive APE1 acetylation. As anticipated, the inability of this mutant to interact with NPM1 leads to nucleolar exclusion of the APE1 mutant. Interestingly, the lack of APE1 nucleolar accumulation causes a severe impairment of cellular proliferation, indicating that the presence of APE1 within nucleoli is required to ensure a proper cellular growth rate (89). These aspects of the APE1 biology still deserve thorough investigation and fascinatingly open novel perspectives for antitumor therapy, as targeting the APE1/NPM1 interaction may prove effective in counteracting cellular proliferation.

The APE1 protein is commonly renowned as DNA repair protein and only recently it has been identified as an enzyme active on abasic RNA molecules, unveiling its noncanonical function. Perhaps earlier examples of this versatility of function in dealing with genotoxic damage were described for the RP S3, both in *Drosophila* and humans. Specifically, S3 has

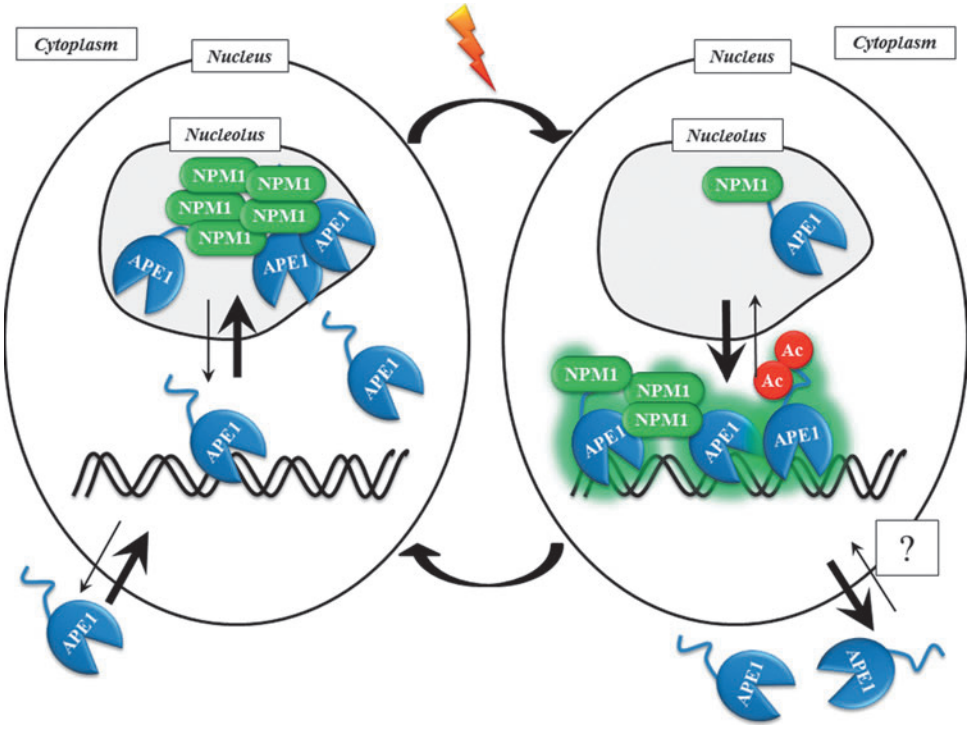

FIG. 4. Dynamic turnover of the APE1/NPM1 complex in response to cellular stress. Under basal conditions (left) the cellular APE1 pool is dynamically distributed throughout the cell, with prevalent accumulation in the nucleus and nucleoli. This accounts for the maintenance of a basal DNA repair capacity (both nuclear and mitochondrial), redox-mediated transcriptional modulation, cell proliferation, and RNA cleansing activity. Upon genotoxic stress and/or arrest of Pol I transcription (right) the dynamic equilibrium of APE1 localization is tipped towards a nucleoplasmic accumulation of the protein (149). The APE1 relocalization is likely mediated by simultaneous migration of NPM1 outside the nucleolus and hyperacetylation of the N-terminal region of APE1 itself (90). This situation ensures a potentiated DNA repair response, as both the nucleoplasmic APE1/NPM1 association and its acetylation have been linked to increased catalytic activity of the protein. The absence of APE1 from nucleoli, moreover, might favor a temporary arrest of cellular proliferation, useful to allow for more efficient DNA repair. If the DNA damage is sustained, it is likely a redistribution of a pool of APE1 to the cytoplasm. This phenomenon should boost the mitochondrial BER and possibly contribute to the cellular RNA cleansing capacity. APE1, apurinic/apyrimidinic endonuclease 1; BER, base excision repair; NPM1, nucleophosmin 1; Pol I, RNA polymerase I. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

been found to protect cells from genotoxic stress through: (i) its DNase activity on abasic sites in *Drosophila* (155); (ii) its ability to stimulate, the activity of the uracil-DNA glycosylase hUNG in human cells (78); (iii) its ability to bind with high affinity the oxidative lesion 8-OHG in humans (60) and (iv) its functional interaction, always in human cells, with other well-known DNA repair proteins, such as the 8-OHG DNA glycosylase (OGG1) and APE1 (61). Recent findings are increasingly pointing to the involvement of noncanonical proteins (57) and even of noncoding RNA molecules (46) in the DDR, adding more layers of complexity to this mechanism. An intriguing emerging evidence is that the association of DNA repair proteins to noncanonical binding partners (*i.e.*, RNA and RNA-binding proteins) is mainly driven by the presence of unfolded protein domains acquired during phylogenesis, which may be responsible for novel gain of function activities.

Relevance of the Unfolded Domains in BER Proteins: The Missing Link for a Phylogenetic Gain of Function?

Until recently, BER was considered the simplest among the DNA repair pathways since an *in vitro* reconstituted nuclear BER required only four or five core enzymes. However, recent studies have revealed that BER is much more complex, involving a network of distinct and integrated cell cycle- and genome-specific sub-pathways in which numerous noncanonical proteins take part (58, 59). Notably, many of these proteins are involved in RNA-metabolism processes. Actually, several noncanonical factors have been demonstrated to participate BER, even though their *in vivo* functions are yet to be fully unraveled. The list of non-BER proteins includes for instance: YB-1 [which has been shown to interact with the endonuclease VIII-like 2 (NEIL2) glycosylase (35), the endonuclease III-like glycosylase (NTH1) and APE1 (29)], NEIL2 [which was also found to interact with the RNA-binding protein hnRNP-U (6)], HMGB1 [which has been implicated in single-strand break (SSB) repair involving Polβ (90)] and the tumor suppressor p53, which was also shown to play a role in DNA damage repair through direct binding to APE1 and Polβ (168). The list of these non-BER proteins is still growing, supporting the notion that BER *in vivo* is far more complex than the simple model that we can reconstitute *in vitro*. In particular, the characterization of the BER interactome identified multiprotein complexes; thus, suggesting that complete

repair occurs through the action of BER complexes formed by core proteins and noncanonical factors (BERosomes) (59). The previous and simplistic view of the BER mechanism, based on the analysis of cocrystal structures of substrate-bound proteins, proposed a pathway consisting of sequential steps in which individual repair enzymes carry out reactions independently. In contrast with this original concept, recent discoveries showed that early BER enzymes stably interact with most of the downstream repair components. The initial view of BER as a "hand-off" or "passing the baton" process has been revisited by Hegde *et al.* who introduced, rather, a new paradigm in which the dynamic interplay of highly coordinated interactions between different BER proteins would increase the efficiency and the versatility of the process.

It has been observed that frequently those machines whose operations must be tuned rapidly in response to specific and diverse cellular needs, present components that are not fully structured. Such "malleable machines" (47), conversely to rigid entities, might presumably better respond to different conditions, for instance by promoting conformational rearrangements and facilitating multiple targets recognition. Structural analyses exploiting both experimental and modeling approaches have indeed evidenced the presence of disordered segments preferentially localized at the N- or C-terminus of different mammalian DNA repair proteins, a peculiar feature which is absent in their bacterial prototypes. These observations thus, suggest that, during evolution, higher organisms have acquired these binding domains to regulate multiprotein interactions and to improve pathways (*e.g.,* BER) efficiency, in an increasingly oxidizing environment. Long disordered segments are a common feature observed in a large percentage of proteins; being prevalent especially among proteins involved in vital processes, such as transcription, translation, signal transduction, and protein phosphorylation (47, 145). Such unstructured regions may provide versatility in recognizing multiple targets, promoting communication with many proteins in response to environmental changes; thus, expanding the capacities of ordered complexes and representing a powerful strategy selected by nature to quickly explore a vast interaction space with unique thermodynamic advantages (132). Disordered regions were shown to be prevalent in DNA binding proteins, particularly in those involved in targeted sequence binding (*e.g.,* repair proteins and transcription factors) (143, 153). Disordered prediction tools (PONDR and PrDOS) have been used to compare the secondary structure of human and bacterial early BER proteins (59). These analyses showed that mammalian DNA glycosylases are endowed with unique nonconserved extensions at their N- or C-termini, which are absent in their homologs in lower organisms (58). The human NEIL1 glycosylase, for instance, contains an extended disordered region spanning about 100 residues at the C-terminus, absent in the *E. coli* Nei-like protein. Similar comparisons between the human DNA glycosylases hNTH1, the MutY homolog (hMYH), and their *E. coli* prototypes (*i.e.,* endonuclease III and MutY, respectively), indicate that both hNTH1 and hMYH have extended disordered tails at the N-terminus that are absent in the *E. coli* enzymes (66, 140). Likewise, the N-terminal disordered region present in the human APE1 is absent in exonuclease III (Xth), its *E. coli* ortholog. The size range of the unstructured extensions is about 50–100 residues. In the case of human APE1 it consists of ~65 residues, being mostly disordered (59).

These disordered regions, due to their structural flexibility and plasticity have been shown to provide BER proteins with functional advantages and appear to be essential for their biological functions, including damage sensing (153), protein–protein interactions, repair regulation *via* PTMs and containing NLSs (118). Furthermore, the presence of disordered segments only in eukaryotic proteins, with the highest degree of disorder in mammals, suggests their evolutionary acquisition and well correlates with the increase in regulatory complexity observed in higher organisms.

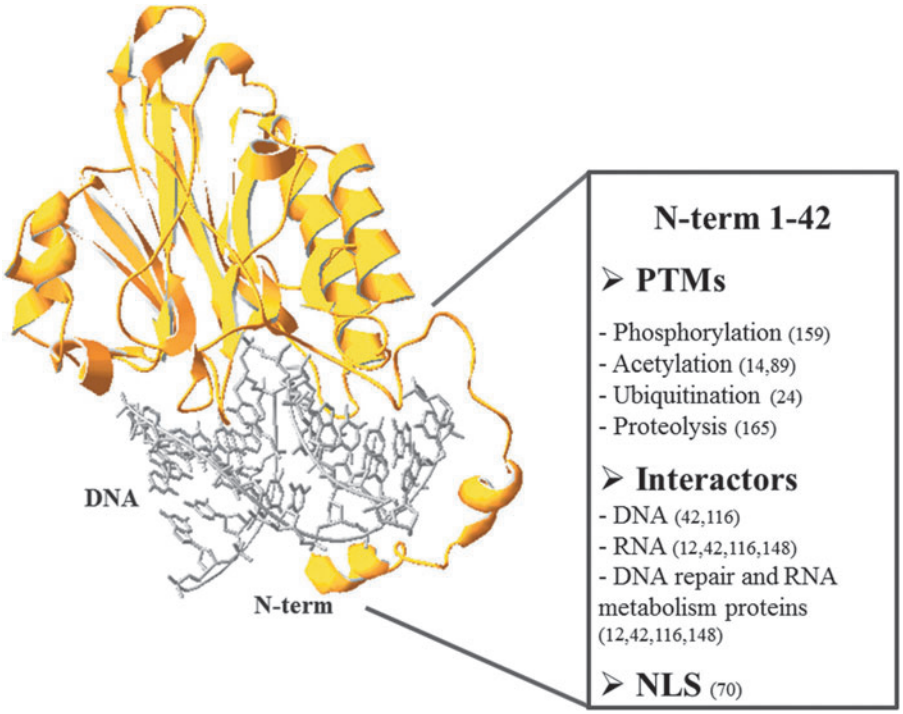

FIG. 5. Schematic representation of multiple regulatory functions of the APE1 disordered N-terminal region. APE1 crystal structure (yellow) bound to abasic DNA (grey) is from the pdb (1DEW) and displayed using the PDBV software. The deposited APE1 crystal structure was obtained using a truncated APE1 form (residues 40–318); missing residues have been manually added. The unstructured N-terminal portion of APE1 (residues 1–42) is essential for APE1 biological functions being site of PTMs, target of many interactions and, including the NLS. NLS, nuclear localization signal; PTMs, post-translational modifications. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Among the BER enzymes, APE1 offers a paradigmatic example of how a disordered tail can endow a protein with unique activities. The first crystallographic structure of the human APE1 in complex with DNA was obtained using a truncated protein lacking the first 35 amino acids and reveled that this protein consists of two symmetric alpha/beta fold with a significant structural similarity to both bovine DNase I and its *E. coli* homologue Xth (50). A crystal structure of the full-length protein has also been reported, but again, the N-terminal region was unresolved (10). Three functionally independent domains can be distinguished within the APE1 protein (Fig. 5): (i) the first 33–35 amino acid region consists of a structurally disordered segment (133) essential for the interaction with other proteins (148, 149) and harboring sites for PTMs (14, 24, 70, 89, 159, 165, 166) and RNA interaction (89); (ii) the redox domain is located in a region between amino acids 35 and 127; and (iii) the DNA repair domain, which spans the C-terminal domain of the protein from about residue 61 onwards (42, 68). Whereas the APE1 C-terminal domain involved in AP endonuclease activity is conserved from bacteria to humans, the N-terminal region is unique to mammals suggesting a recent acquisition during evolution.

Remarkably, this peculiar region also accounts for noncanonical activities that have been ascribed to APE1, including its role in RNA metabolism (42, 89, 116, 138, 148, 149). Over the past two decades, knowledge of the biological functions, mechanisms of action, interactions, and regulation of the APE1 protein has increased exponentially. In particular, it has become apparent that APE1 participates in multiple cellular processes not only confined to the maintenance of genome stability, in accordance with the current general view that many DNA repair enzymes may exert miscellaneous activities, being implicated, for example, in different steps of gene regulation (74, 113). As already mentioned, RNA decay and processing events require a wide spectrum of proteins, including RNA helicases, polymerases and, above all, exoribonucleases and endoribonucleases. For many years, it has been assumed that eukaryotes RNA cleavage relies mostly on the action of exoribonucleases, in contrast with prokaryotes, where RNA decay is mainly mediated by endoribonucleolytic processes (82, 101). In recent years, the unexpected implication of numerous endoribonucleases in the RNA turnover in eukaryotes significantly contributed to a change of perspective on the eukaryotic RNA metabolism (141). APE1 is among the several examples of recently identified enzymes whose endoribonucleolytic activity has been found to associate with the regulation of RNA stability. Evidence of its RNase H-like activity was first suggested by Barzilay *et al.* who demonstrated that APE1 is able to bind with relatively low affinity undamaged single- and double-stranded RNA molecules, albeit not exhibiting unspecific nuclease activity (9). Later on, it has been discovered that APE1 possesses the ability to cleave AP sites within single-stranded RNA molecules and that the nucleic acid secondary structure significantly influences the APE1 incision activity (12). Despite these first *in vitro* suggestions of APE1 biological relevance in the removal of AP-site-containing RNA, the unequivocal demonstration of APE1 involvement in RNA processing was brought only few years later. Very recently, in fact, Barnes *et al.* identified APE1 as the major endonuclease associated with polysomes and capable of cleaving the coding region determinant of the *c-myc* mRNA; thus, influencing *c-myc* half-life in cells (7). Surprisingly, recent biochemical studies performed using recombinant APE1 demonstrated that the APE1 endoribonuclease function is not limited to *c-myc* mRNA, but it may potentially influence the biogenesis and hence, the stability of other transcripts, including also miRNAs (77). These works demonstrated that APE1-mediated RNA cleavage occurred, *in vitro*, at single-stranded or weakly paired regions, preferentially 3′ of pyrimidines at UA, UG, and CA sites. This latter finding, in particular, led to hypothesize the possible involvement of APE1 in mRNA splicing events, since CA repeats are renowned as potent splicing modulators (15). Furthermore, the *in vivo* involvement of APE1 in RNA metabolic pathways was further corroborated by the observation of the APE1 association, through its N-terminal domain, with rRNA and the ribosome processing protein NPM1 within nucleoli (149). Furthermore, APE1 has been shown to interact with factors involved in the splicing process, such as the heterogeneous nuclear ribonucleoprotein L (81) [which is a key regulator of splicing that binds CA repeats with high affinity (65)], YB-1 (29, 119), as well as with proteins involved in the ribosome assembly and RNA maturation within cytoplasm (149).

So far, many studies have characterized the involvement of the APE1 C-terminal domain in RNA metabolism, demonstrating that the APE1 endoribonuclease activity and its nuclease function on DNA share the same active site (76). However, few groups have investigated the role of the unstructured N-terminal extension, possibly as a consequence of its disordered nature. A recent work published form our group systematically characterized the binding properties of the APE1 N-terminal disordered region towards nucleic acids and NPM1. We demonstrated that the N-terminus, in particular acquired Lys residues therein located, appear to be essential for the stabilization of both protein–protein and nucleic acids–protein interactions, as well as influencing the thermal stability of the protein. These evidences clearly support the notion that this unstructured domain might represent an evolutionary gain function necessary for mammals to cope with a progressively complex cellular environment (116).

In light of these recent findings and taking into account also previous reports on the pivotal role of APE1 disordered N-terminal region, we speculate that the targeting of this unfolded protein domain could be a valuable tool to interfere with the different APE1 functions *in vivo*.

Relevance of the Unstructured Domain of BER Proteins for Designing Novel Anticancer Strategies

BER proteins have been broadly explored as targets for cancer therapy (67); in particular, current approaches to cancer treatment report more effective results when specific DNA repair inhibitors are used in combination with DNA damaging drugs. The foremost rationale of the combined therapy is that impairment of BER enzymes is likely to sensitize cancer cells to chemotherapeutic agents. Druggable BER targets for cancer treatment include: FEN1, Polβ, APE1, and the poly [ADP-ribose] polymerase 1 (PARP1); while targeting of DNA glycosylases results inefficacious because of the functional redundancy of this class of enzymes (58).

In the last decade, remarkable attention has been posed on the development of PARP1 and APE1 inhibitors. The APE1 relevance for cell survival is demonstrated by the fact that

knocking out the APE1 gene induces either apoptosis in differentiated cells (69) or developmental failure during embryogenesis (158). Accumulating evidences have indicated that deregulation of APE1 in both expression and subcellular localization is indeed associated with different tumorigenic processes: APE1 upregulation or dysregulated expression has been described in a variety of cancers, including prostate, pancreatic, ovarian, cervical, germ cell, rhabdomyosarcoma, and colon (43, 92). Furthermore, it has been reported that elevated APE1 levels and anomalous intracellular localization are also typically correlated with aggressive proliferation and increased resistance to chemotherapeutic drugs and IR, implying that APE1 enhances repair and survival of tumor cells (92). Therefore, considering that APE1 expression appears to be linked to chemoresistance and taking into account that several studies have shown that decreasing APE1 levels may lead to cell growth arrest and to an increased cellular sensitivity to DNA damaging agents (44, 45, 85, 154), APE1 represents a promising target for pharmacological treatment. A description of all the APE1-targeting molecules currently under investigation is beyond the focus of this review, for an exhaustive review on APE1 inhibitors, the reader is redirected to (146). All the APE1 small-molecule inhibitors developed so far were designed to target specific APE1 functions, namely the DNA repair or the redox activity of the protein. It is however, still a matter of debate whether the enhanced sensitivity to cytotoxic agents observed upon APE1 inhibition is solely related to the loss of DNA repair activity, or is also linked to the loss of its transcriptional regulatory function, or both. Despite the efforts aimed at determining the relative importance of the attenuation of the APE1's repair or transcriptional functions, all currently available APE1 inhibitors display limited specificity for cancer cells. Therefore, exploration of novel opportunities for APE1 targeting is obviously a path that deserves further consideration. Based on several observations attesting the critical role of disordered segments in many BER enzyme functions, we propose that the N-terminal unstructured portion of the APE1 protein could be considered as a new potential target for cancer therapy. Classical pharmacological strategies usually target structured regions of proteins; however, considering the biological relevance of intrinsically disordered proteins, the ability to interfere with their interactions opens enormous potentials for drug discovery. Actually, there is a continuous progress in the development of small molecules directed against disordered protein regions; several low molecular weight compounds are effective in the specific inhibition of molecular interactions based on intrinsically disordered domains. For example, small molecules binding the disordered regions of *c-myc*, Aβ, EWS-Fli1 have recently been discovered (100, 144). Although the binding of a small molecule to a disordered region/protein may appear counterintuitive due to the intrinsic poor selectivity, this may also be considered a major advantage because it would facilitate the screening of initial compounds, which affinity and specificity could be successively improved through standard molecular optimization procedures.

In conclusion, we suggest that it would be interesting to investigate novel pharmacological approaches aimed at interfering with the APE1 N-terminal region in light of its important role in the coordination of many different functions of the protein, both in DNA repair and RNA metabolism.

Conclusions and Speculations

Different independent studies provided *in vitro* and *in vivo* evidence that many DNA repair proteins, particularly in the BER pathway (*e.g.,* APE1), are involved in RNA metabolism. Interestingly, many of these proteins are also part of the nucleolar proteome where they bind specific carrier proteins (*e.g.,* NPM1, NCL) and rRNA. Upon genotoxic stress, many DNA repair proteins exit from nucleoli and switch their interactome network from proteins involved in RNA metabolism to DNA repair complexes. Although still in the early phases, these findings have raised many questions and speculations concerning the role of these proteins, including APE1 as a paradigmatic example, in RNA metabolism. For instance:

1. Why should a protein involved in DNA repair play a role in RNA metabolism? One possible explanation is that this duality would preserve genetic stability not only through the DNA repair activity, but also through the ability to cleanse damaged RNA that may otherwise be inaccurately translated, or degrade unwanted foreign RNA (*e.g.,* viral RNA).
2. Do the redox function of APE1 and its role in RNA metabolism represent two sides of the same coin devoted to modulate gene expression through transcriptional and post-transcriptional mechanisms? Modulation of APE1 subcellular distribution through its redox status may represent an elegant, specific, and energetically economic mechanism to tune gene expression upon genotoxic stress conditions.
3. Is APE1 an ancient protein with a newly identified and yet unappreciated role? The current information regarding the primary amino acid sequence of APE1 across species seems to suggest a phylogenetic "gain-of-function" and hence, support this hypothesis. However, further experimental and bioinformatics studies of APE1 orthologs may reveal additional insights into this question.
4. Suppressing the amount of APE1 has proven effective in sensitizing cancer cells to chemotherapeutic agents. This finding has led to the proposal that selective inhibition of the APE1 DNA repair activity is an attractive avenue for the development of novel anticancer therapies. Similarly, one can envision targeting the non-DNA repair functions of APE1, namely its RNA-repair and/or RNA-cleavage activities, as novel approaches for the treatment of cancer or neurological disorders. Nonetheless, such therapeutic aims still need further studies to increase our understanding of the role of APE1 in its noncanonical functions.

In closing, a productive cross-talk between DNA repair enzymes and proteins involved in RNA metabolism seems reasonable and nucleolus is emerging as a dynamic functional hub that coordinates cell growth arrest and DNA repair mechanisms. These findings will drive further analysis of other BER proteins, such as FEN1, and might imply that nucleic acid processing enzymes are more versatile than originally thought and may have evolved DNA-targeted functions after a prior life in the early RNA world. The observation of cytoplasmic localization for canonical DNA repair proteins, such as APE1, simply beyond their mitochondrial targeting, may therefore, suggest much more than just an "abnormal" distribution pattern.

Acknowledgments

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Abbreviations Used

8-OHG = 8-hydroxyguanosine
AD = Alzheimer's disease
AP = apurinic/apyrimidinic
APE1 = apurinic/apyrimidinic endonuclease 1
ATM = ataxia telangiectasia mutated
ATR = ataxia telangiectasia and Rad3-related
BER = base excision repair
CRM1 = exportin 1
DDR = DNA damage response
DSB = double-strand break
FEN1 = flap endonuclease 1
hMYH = human MutY glycosylase homolog
HR = homologous recombination
IR = ionizing radiation
NCL = nucleolin
NEIL2 = endonuclease VIII-like 2
NER = nucleotide excision repair
NHEJ = nonhomologous end joining
NLS = nuclear localization signal
NPM1 = nucleophosmin 1
NoLS = nucleolar localization sequence
NTH1 = endonuclease III-like glycosylase
OGG1 = 8-OHG DNA glycosylase
PARP1 = poly [ADP-ribose] polymerase 1
PCNA = proliferating cell nuclear antigen
PD = Parkinson disease
PNC = perinucleolar compartment
Polβ = DNA polymerase β
Pol I = RNA polymerase I
PTMs = post-translational modifications
rDNA = ribosomal DNA
rRNA = ribosomal RNA
RP = ribosomal protein
RPA = replication protein A
snoRNA = small nucleolar RNA
SSB = single-strand break
WRN = Werner syndrome helicase
XRCC1 = X-ray repair cross-complementing protein 1
Xth = exonuclease III
YB-1 = Y box binding protein 1