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Review

Targeting the nucleolus for cancer intervention☆

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ABSTRACT

The contribution of the nucleolus to cancer is well established with respect to its traditional role in facilitating ribosome biogenesis and proliferative capacity. More contemporary studies however, infer that nucleoli contribute a much broader role in malignant transformation. Specifically, extra-ribosomal functions of the nucleolus position it as a central integrator of cellular proliferation and stress signaling, and are emerging as important mechanisms for modulating how oncogenes and tumor suppressors operate in normal and malignant cells. The dependence of certain tumor cells to co-opt nucleolar processes to maintain their cancer phenotypes has now clearly been demonstrated by the application of small molecule inhibitors of RNA Polymerase I to block ribosomal DNA transcription and disrupt nucleolar function (Bywater et al., 2012 [1]). These drugs, which selectively kill tumor cells in vivo while sparing normal cells, have now progressed to clinical trials. It is likely that we have only just begun to scratch the surface of the potential of the nucleolus as a new target for cancer therapy, with "suppression of nucleolar stress" representing an emerging "hallmark" of cancer. This article is part of a Special Issue entitled: Role of the Nucleolus in Human Disease.

1. Introduction

Over 200 years ago the development of light microscopy led to the revelation that cells are not fixed entities but dynamically responsive to environmental cues. This was exemplified with the observation that the most prominent structure within the nucleus, the nucleolus, disassembles and then reassembles during each cell cycle. Today it is well established that the nucleolus acts as a hub coordinating the synthesis and assembly of the core protein synthesizing machinery of the cell, the ribosome. In mammals this entails transcription by the dedicated RNA polymerase I (Pol I) enzyme of the ribosomal RNA genes (rDNA) that give rise to the 47S ribosomal RNA (rRNA) precursor, which is subsequently processed into the mature 28S, 18S and 5.8S rRNA. These rRNAs, together with the 5S rRNA synthesized by RNA polymerase III (Pol III) and the numerous ribosomal proteins (RPs) encoded by RNA polymerase II (Pol II) transcribed genes, are then assembled within the nucleolus into the 40S and 60S ribosomal subunits before export to the cytoplasm (reviewed in [2]). The nucleolus, while not membrane bound, represents a discrete yet dynamic structural domain within the nucleus into which various proteins can be sequestered and released. Its assembly and disassembly is a result of its dependence upon Pol I transcription, as nucleoli form around actively transcribing rDNA repeats (reviewed in [3,4]). Intriguingly, recent studies demonstrate that eukaryotic cells have evolved to use the nucleolar domain for an extensive and varied repertoire of cellular activities in addition to ribosome biogenesis. Additional roles now ascribed to the nucleolus include modulation of the cellular stress response, regulation of senescence and cell cycle progression, RNA and ribonucleoprotein (RNP) biogenesis, and even organization of the epigenome (see Section 4).

A strong correlation between nucleolar morphology and cancer was recognized by pathologists over 100 years ago, when it was first observed that large and abnormal nucleoli were common in cancer cells [5]. More contemporary studies have demonstrated that the dysregulated nucleolar morphology reflects hyperactivation of rDNA transcription (reviewed in [6]). For the larger part, it has been considered

Abbreviations: DDR, DNA Damage Response; DSB, Double Stranded Break; IR, Ionizing Radiation; NAD, Nucleolus Associated Domain; NCL, Nucleolin; NOR, Nucleolar Organizer Region; NPM, Nucleophosmin; PIC, Pre-Initiation Complex; Pol I, DNA-dependent RNA Polymerase I; Pol II, DNA-dependent RNA Polymerase II; Pol III, DNA-dependent RNA Polymerase I; rDNA, ribosomal RNA genes; RNP, Ribonucleoprotein; RP, Ribosomal Protein; rRNA, ribosomal RNA; S6K, ribosomal protein S6 Kinase; SL-1, Selectivity complex; snRNP, small nucleolar Ribonucleoprotein; TTF-1, Pol I Transcription Termination Factor; UBF, Upstream Binding Factor; Xi, inactive X chromosome

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that the primary function of accelerated ribosome biogenesis in cancer is to simply enable the increased proliferative growth frequently associated with malignancies. However, recent studies strongly suggest that the ability of the nucleolus to regulate non-ribosomal functions, in particular controlling the activity of various critical tumor suppressors and oncogenes, is likely to contribute significantly to malignant transformation. For example, it is now evident that impairing any one of a number of steps in ribosome biogenesis leads to activation of a nucleolar stress/surveillance mechanism that can result in the accumulation of the tumor suppressor protein p53 (reviewed in [7–9]). The direct coupling of ribosome biogenesis to proliferative and stress signaling pathways makes evolutionary sense: cells must on the one hand ensure sufficient protein synthetic capacity prior to committing to cell cycle progression; on the other, they need to reduce the enormous energy expended in making ribosomes if they are not undergoing division. Thus, for example, p53 is activated in response to impairment of ribosome biogenesis, or conversely suppressed by increased ribosome biogenesis driven by proto-oncogenic growth and survival signals [10].

These observations have led to the proposal that acute inhibition of ribosome biogenesis could form the basis of a tumor-specific mechanism to non-genotoxically activate p53, for cancer therapy. This hypothesis has been tested by Bywater et al., who reported that the small molecule inhibitor of Pol I transcription, CX-5461, killed B-cell lymphoma cells in vivo, while sparing the normal B-cell population [1]. These data provided the first direct evidence that targeting a nucleolar process (i.e. rDNA transcription) was a viable strategy for cancer therapy, and has led to a Phase 1 clinical trial of CX-5461 in patients with hematologic malignancies commenced in 2013 (Peter MacCallum Cancer Centre, Melbourne). The focus of this review is to evaluate the current understanding of the role of the nucleolus in cancer and to discuss how it can be targeted as a novel cancer treatment.

2. Regulation of the nucleoli

Eukaryotic cells typically contain multiple nucleoli per nucleus, which form around active clusters of the 47S rRNA genes (rDNA) known as the nucleolus organizer regions (NORs). There are ~200 copies of the rRNA gene located in tandem arrays at 5 locations in the genome, on the short arms of the acrocentric chromosomes; only a proportion of these (approximately half) are transcriptionally competent, with an 'open' structure associated with euchromatic histone modifications (H4Ac and H3K4Me), while the rest are transcriptionally silenced, with a heavily CpG methylated heterochromatic structure (H3K9Me, HK27Me and H3K20Me) (reviewed in [11–15]). Transcriptionally competent rDNA repeats are not necessarily active, but those that are characterized by association with the transcription factor UBF can achieve very high rates of transcription by Pol I [16], accounting for >30% of transcriptional activity of an exponentially growing cell [17] and [12].

Although the nucleoli are not membrane bound organelles, they segregate into three distinct regions: the pale-staining fibrillar center (FC), surrounded by the compact dense fibrillar component (DFC), which in turn is encased by an outer granular compartment (GC) (reviewed in [3] and [4]). These regions can also be defined by their affiliated proteins or RNA, and as such the processes functioning within these zones. Production of the rRNA requires formation of a competent pre-initiation complex (PIC) - comprising the regulatory factors UBF, RRN3 and the selectivity complex (SL-1) - at the promoter of the active rDNA genes, and subsequent transcription of the 47S precursor rRNA by Pol I (reviewed in [18–20]). These elements are concentrated at the boundary of the FC and DFC. The newly synthesized 47S precursor is extensively processed by splicing and post-translational modification, the early stages of which occur in the DFC, and the later stages in the peripheral GC. The GC contains the highest density of proteins due to it also being the location of assembly of the mature rRNAs along with the RPs into the 40 and 60S ribosome subunits. In addition, the epigenetically silent rDNA repeats associate with a shell of heterochromatin that surrounds the nucleolus (perinucleolar heterochromatin). Hence, the structure and biological functions of the nucleoli are mutually dependent (Fig. 1).

Importantly, nucleoli are dynamic in nature, and exquisitely regulated by multiple signaling pathways that primarily converge directly upon the Pol I transcription factors, such as PIC components RRN3, UBF and SL-1. This enables the nucleoli to respond rapidly to changing proliferative or environmental cues — a far cry from early studies that suggested rDNA transcription responded slowly and indirectly to nutrient status (reviewed in [21]). During the cell cycle, the mammalian nucleoli disassemble at the start of mitosis (prophase), coinciding with the inactivation of rDNA transcription; nucleoli then reassemble during telophase in a precisely controlled manner, enabling reactivation of rDNA transcription as cells enter G1. Rates of rDNA transcription are also increased during S and G2, as cells grow in preparation for cell division. This is achieved through the direct regulation of Pol I transcription factors by cell cycle regulatory proteins [22–25]. Further to cell cycle regulation, control of the rate of Pol I transcription is mediated by additional signaling pathways that control cell growth and division (reviewed in [20,26]). Broadly, these can be separated into those that upregulate ribosome biogenesis and promote cellular growth and proliferation, for example by cellular energy, nutrient and growth factor sensing signaling pathways; or those that downregulate ribosome biogenesis and prevent growth and proliferation in response to challenges such as metabolic or genotoxic stress, or senescence signaling pathways (Fig. 2). Significantly, these pathways inherently contain oncogenes and tumor suppressors, and their dysregulation enables cells to achieve the uncontrolled growth and proliferation that is a hallmark of cancer.

3. The nucleoli in cancer

Almost all cancer types display large and/or increased number of nucleoli [27] (reviewed in [28]). In fact, nucleolar size can in some cancers be used as a parameter for predicting clinical outcome, with increased size corresponding to worse prognosis [29,30]. Changes in nucleolar size have also been utilized as a measure of response to chemotherapeutic drugs [31]. Consistent with the above, as nucleolar size is related to its function in ribosome biogenesis, increased rates of Pol I transcription are similarly observed in cancer and correlated with adverse prognosis [32,33].

Perhaps somewhat surprisingly, the accelerated rates of rDNA transcription associated with cancer do not appear to be due to "gain of function" mutations in the Pol I apparatus, its associated factors or other components involved in maturation and biogenesis of mature ribosomes (reviewed in [34]). By contrast, loss of function mutations in components of ribosome biogenesis have been extensively reported, which collectively lead to the rare genetic diseases termed Ribosomopathies (reviewed in [26]). Instead of "gain of function" mutations, during malignant transformation rDNA transcription rates are upregulated as the result of activation by oncogenic signaling, or release from repression by tumor suppressor pathways, some of which are described in more detail below.

3.1. Cell cycle regulatory proteins implicated in cancer

Dysregulation of cell cycle control is a common feature of most cancer types (reviewed in [35]). Specifically, the CDK-cyclinD/INK4/pRB/E2F pathway regulating G1/S transition is commonly compromised in cancer. The tumor suppressor retinoblastoma protein (pRb), when active, prevents progression from G1 phase of the cell cycle (reviewed in [36,37]). Mutation of the retinoblastoma gene (*RB1*) was first identified in retinoblastoma, however loss of pRb activity, such as by cyclin D overexpression, CDK4 and CDK2 hyper-activation, or activation of caspase dependent proteolytic pathways, is observed in most cancers

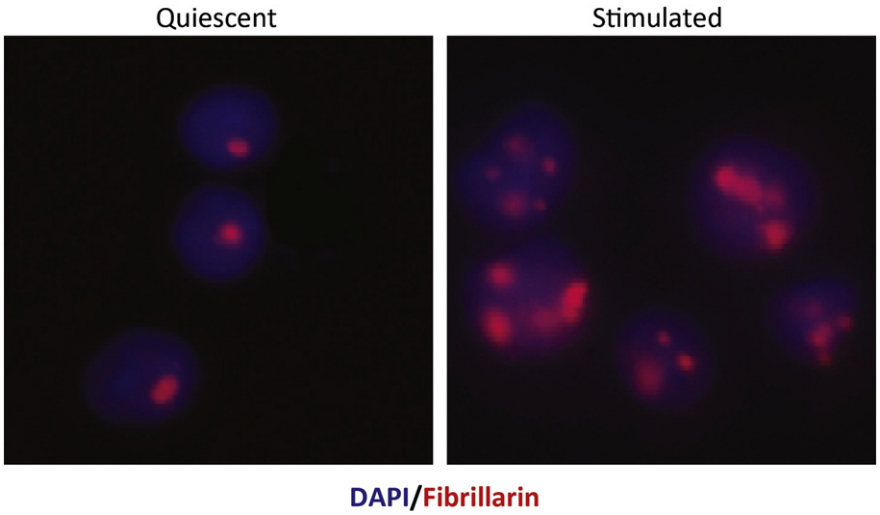

Fig. 1. Dynamic nucleolar structure. Non-malignant primary human T cells were grown under quiescent conditions (RP10 media) or under proliferative conditions (RP10 media with 5 ng/ml anti-CD28 in anti-CD3 coated wells). After PFA fixation the nucleolar structure was visualized by fibrillarin immunofluorescence (red).

(reviewed in [38]). pRb inhibits Pol I transcription by interacting with UBF, preventing it from recruiting SL-1 to the PIC [39–41], and/or binding to the rDNA promoter [42]. Conversely, its negative regulators CDK4-cyclin D and CKD2-cyclin E/A can enhance Pol I transcription rates through activating phosphorylation of UBF [24,25].

3.2. MYC

The transcription factor MYC (product of the *c-MYC* oncogene) is one of the most frequently activated oncoproteins, overexpressed in ~50% of all cancers (reviewed in [43,44]). MYC regulates transcription of a large

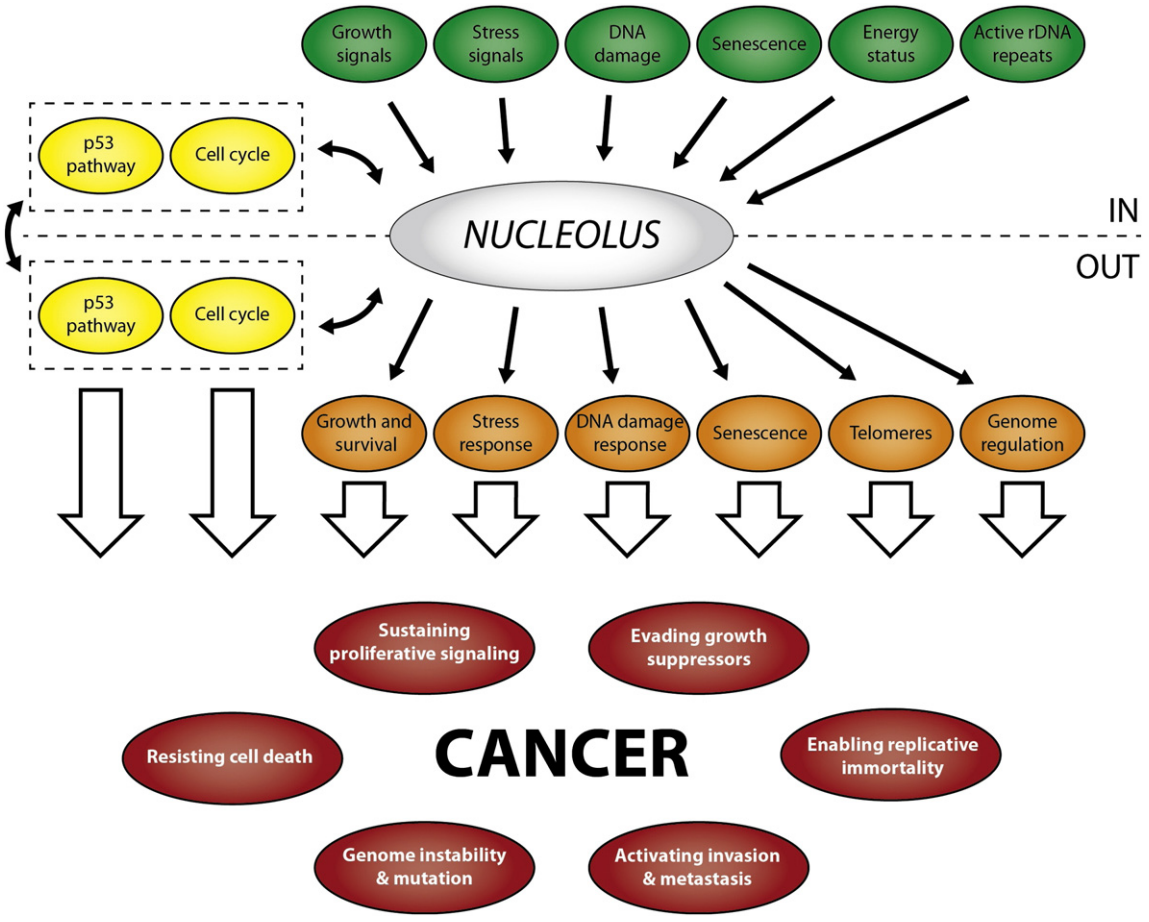

Fig. 2. The "inputs" and "outputs" of the nucleolus. The nucleolus is responsive to multiple proliferative or stress signaling pathways, which can directly regulate rates of Pol I transcription at the rDNA. Thus the nucleolus acts as a central integrator of signaling pathways, with its activity determined by the overall status of the cell. In addition to responding to signaling to drive ribosome biogenesis, the nucleolus controls cell cycle regulation, DNA damage response, stress response, senescence, telomere biogenesis, RNA and RNP biogenesis, and organization of the epigenome. Dysregulation of the nucleolus may corrupt these processes, and consequently drive tumorigenesis through the acquisition of key hallmarks of cancer (adapted from #115).

cohort of genes, particularly those which drive cell growth, including key factors involved in ribosome biogenesis and protein synthesis [45,46] (reviewed in [47–50]). MYC modulates Pol I transcription in multiple ways: it upregulates transcription of core Pol I subunits and transcription factors (such as UBF and RRN3) [51–53]; while directly, it associates with SL-1 to stabilize the UBF/SL1 complex, and binds to the rDNA to promote Pol I recruitment [54–56]. In addition, MYC promotes transcription of factors required for rRNA maturation and assembly [57,58], 5S rRNA by Pol III [59], and the RP genes by Pol II. Thus, MYC acts as a master regulator of ribosome biogenesis (reviewed in [60,61]).

3.3. RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways

The most prominent growth/nutrient regulatory pathways known to modulate Pol I transcription are the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signaling cascades. The RAS/RAF/MEK/ERK pathway can regulate Pol I transcription via multiple kinases, including ERK, MAPK, RSK2, and JNK [62–64] (reviewed in [20]). For example, ERK directly phosphorylates, and thus activates: i) UBF to increase Pol I transcription elongation [21,65] and ii) RRN3 to promote Pol I initiation [63]. PI3K/AKT/mTOR/S6K pathway components modulate the activity of Pol I through, for example: ribosomal protein S6 kinase (S6K), which indirectly modulates UBF and RRN3 phosphorylation, thus enhancing Pol I transcription [66,67]; PTEN, an upstream negative regulator of PI3K/AKT/mTOR, which represses Pol I transcription by promoting dissociation of the SL-1 complex [68]; or AKT, which is able to activate Pol I transcription at multiple levels [69]. Further, both pathways cooperate with MYC: ERK stabilizes MYC by phosphorylation [70], while AKT cooperates with MYC to activate Pol I transcription [69]. Numerous components of these signaling cascades act as oncoproteins, amplifying proliferative signaling down their respective pathway. For example, RAS and RAF are mutated in 30% and 6–7% of human cancers respectively (reviewed in [71–73]). PTEN expression is reduced in a range of cancers, the result of which is enhanced PI3K signaling (reviewed in [74,75]). In the PI3K pathway itself, the PIK3CA catalytic subunit of PI3K is most commonly reported to be amplified or mutated, while for a smaller subset of cancers other molecules including AKT, 4EBP1, eIF4E, Rheb and S6K1 are overexpressed and/or hyperactivated [76–78] (reviewed in [79,80]).

3.4. p53

The archetypal tumor suppressor p53 is assigned the title of "guardian of the genome", due to its role as a key mediator of stress signaling responses, including cell cycle arrest, senescence and apoptosis. The diverse roles by which p53 has been reported to perform tumor suppressive functions are too extensive to be discussed here, but have been covered in a number of in depth reviews [81–83]. Correspondingly, p53 is mutated in approximately half of all human tumors [84], and in the majority of remaining tumors expressing wild-type p53 its function is compromised. p53 directly inhibits Pol I transcription by binding to SL-1, thus preventing its interaction with UBF and formation of the PIC [85]. Further, downstream targets of p53-dependent stress signaling also negatively regulate Pol I transcription, including activation of pRb (reviewed in [86]), or repression of MYC [87].

3.5. ARF

In response to aberrant growth or oncogenic stress, the tumor supressor p14ARF engages anti-proliferative pathways. Its best characterized function is as a key regulator of p53, resulting in p53 stabilization and activation [88] (reviewed in [89]), but it can also act independently of p53 to induce cell cycle arrest or apoptosis [90,91] (reviewed in [89]). Loss of p14ARF is almost as common as loss of p53, occurring in as many as 40% of human cancers (reviewed in [92,93]). ARF can directly repress Pol I transcription via altering UBF phosphorylation and hence its ability to recruit the PIC, and also prevent Pol I transcription termination factor (TTF-1) nucleolar import [94] and [95].

In addition to the well-known pathways discussed here, numerous other oncoproteins and tumor suppressors have also been convincingly shown to modulate Pol I transcription. These include, for example, ATM, ATR and DNA-PK [96–98], CK2 [99–101], AML-ETO [102], RUNX2 [103–105], and NPM [106], (reviewed in [26]). Also, the recent use of 'omics' technologies has enabled the identification of many possible new components including, for example, deltaN isoform of netrin-1 [107], DDX31 [108], and ZNF545/ZFP82 [109].

Importantly, the above examples consistently demonstrate that ribosome biogenesis is directly targeted by pathways that drive the process of transformation. Such hijacking of the nucleoli is necessary to enable increased rates of protein synthesis and cell growth that are characteristic of cancer. However, the extent to which changes in ribosome number actually contribute to transformation, rather than being merely a reflection of the transformed phenotype, had not until recently been tested. Now though, there is strong evidence to suggest that accelerated ribosome biogenesis is both necessary for, and a driver of, the malignant phenotype [1]. Most intriguingly, it appears that it is the additional functions of the nucleolus that are corrupted by the accelerated ribosome biogenesis, independent of changes in cell capacity for protein synthesis, and are the critical determinant of malignant transformation.

4. Extra-ribosomal nucleolar functions and cancer?

There is now overwhelming evidence that the nucleolus has extra-ribosomal functions, which add an additional layer of complexity to the relationship between dysregulated ribosome biogenesis and cancer. In particular, recent advances in proteomic analysis of the nucleolus have demonstrated its plurifunctional nature [110–114]. Of the over 4500 proteins reported in the nucleolar protein database (NOPdb) (http://lamondlab.com/NOPdb3.0/), less than half have defined functions in ribosome biogenesis. Rather, proteins that localize to the nucleolus are involved in a diverse range of functions including regulating tumor suppressor and proto-oncogene activities, cell-cycle control, DNA replication and repair, and stress signaling. Importantly, dysregulation of many of these processes is known to drive malignant transformation [115].

Critically, the nucleolar proteome is not static, but dynamically altered in response to physiological and pathological signals such as nutrient and growth factor signaling or stress [116–120]. Rather than a consequence of passive diffusion, nucleolar residence of many proteins is regulated through controlled sequestration and release [121–128]. It follows that perturbations in nucleolar function and structure will lead to disruption of this regulation by nucleolar localization and as a consequence affect multiple cellular functions. Indeed it is apparent that many of the very same pathways containing oncogenes and tumor suppressors that modulate Pol I transcription during tumorigenesis (see Section 3), are themselves subject to regulation by the nucleolus. Thus the nucleolus is both the target of cancer signaling and also functions as an upstream regulator of pathways important for cancer. This homeostatic feedback loop clearly positions the nucleolus as central to the processes that are known to drive the hallmarks of cancer. Overcoming the ability of the nucleolus to correctly mediate these additional functions increasingly appears to be a key permissive step for malignant transformation and is described in more detail below (Fig. 2).

4.1. Nucleolar regulation of p53

A key function of the nucleolus that relates to cancer is its role in the regulation of the tumor suppressor p53. Typically p53 protein is maintained at basal levels in the cell by MDM2, which inhibits p53 activity by two mechanisms: i) ubiquitination, which targets it for

proteosomal degradation and ii) direct binding, which inhibits its transactivation activity. In order to stabilize and activate p53, it must be released from inhibitory association with MDM2. Further, post-translational modification and association with co-factors mediate p53 activity towards its transcriptional targets (reviewed in [129,130]). It is now clear that activation of p53 and its tumor suppressor function is mediated, in part, by the nucleolus. This is achieved through multiple mechanisms including the NPM1-MDM2-p14ARF axis, the RP-MDM2 nucleolar stress pathway, and via regulation of p53 trafficking.

4.1.1. NPM1-p14ARF-MDM2

The predominantly nucleolar protein, p14ARF binds MDM2 and inhibits its activity toward p53. Under normal conditions p14ARF is maintained at low levels by ubiquitin-mediated degradation, however it is transcriptionally upregulated and stabilized by the multifunctional nucleolar chaperone protein nucleophosmin (NPM1) in response to a variety of signals, particularly oncogenic or genotoxic stress. Nucleolar localization mediates the NPM1-p14ARF-MDM2-p53 pathway in two ways: i) translocation of NPM1 from the nucleolus to the nucleoplasm promotes the interaction of p14ARF with MDM2, thus disrupting MDM2's association with p53 and subsequent degradation of p53 [131-133], ii) increased nucleolar localization of p14ARF may result in its further stabilization, by preventing its degradation, as well as potentially sequester MDM2 from p53 [134] (reviewed in [129]).

4.1.2. RP–MDM2 nucleolar stress pathway

Following abrogation of ribosome biogenesis – for example, by inhibition of rDNA transcription or disruption of the 40S and 60S ribosomal subunit biogenesis – several RPs are released from the nucleolus, which bind MDM2 and inhibit its ubiquitin ligase activity towards p53, resulting in p53 accumulation (Fig. 3). These include RPs L5, L11, L23, L26, L37, S7, S15 and S20, although the best characterized and most robust data suggests RPL5 and RPL11 are the most important [135–146]. Upon nucleolar stress, RPL5 and RPL11 together with 5S rRNA are mutually stabilized, and as part of the RPL5/RPL11/5S rRNA complex bind MDM2 inhibiting both its E3 ligase function and its association with p53 [147–149]. This nucleolar stress pathway appears to be regulated at a number of levels, for example RPL11 can be sequestered in the nucleolus by factors such as PICT1 [123], or through post-translation modification by NEDD8, the inhibition of which promotes association with MDM2 [150,151,128]. Importantly, the requirement of RP binding to MDM2 in p53 activation by nucleolar stress has been substantiated in an in vivo mouse model [152].

Additional nucleolar factors also regulate p53 under conditions of nucleolar stress, either by stabilization as described above, or by alternative mechanisms (Fig. 3). For example, p53 mRNA is stabilized by the multifunctional nucleolar protein nucleolin (NCL) and RPL26, which bind to the 5′UTR of p53 mRNA and mediate increased p53 translation [153]. Interestingly under normal conditions, MDM2 targets RPL26 for degradation; thus decreased MDM2 activity following nucleolar stress would result in RPL26 stabilization and further increase p53 translation and abundance [154]. A number of proteins can dissociate the interaction between p53 and MDM2, thus resulting in p53 stabilization. For example RPS3 directly interacts with p53 [155], while Nucleostemin can bind directly to MDM2 [156,157]. Further, NCL associates with both p53 and MDM2, and depending on post-translational regulation can either antagonize their interaction or promote p53 degradation [121,158,159]. Finally, p53 transcriptional activity is mediated by acetylation by its coactivator p300/CBP; this is facilitated by neddylated RPL11 and MYBBP1A, which is sequestered in the nucleolus and released upon nucleolar stress [160,161,151,162].

4.1.3. p53 trafficking

In addition to sequestration of factors that regulate p53, the nucleolus may play a direct role in p53 transport and its degradation [163]. Both MDM2 and ubiquitinated p53 traffic through the nucleolus, and this may be required for the cytoplasmic export and subsequent degradation of p53. If p53 and MDM2 are co-transported with ribosomal subunits, then disruption of ribosome biogenesis could result in p53 accumulation, in part due to abrogation of this process sequestering p53 away from the proteasome [164–166,163].

The varied mechanisms by which the nucleolus and its components modulate p53 activity are indicative of its importance to the fundamental cellular p53 response. Further, p53 and its downstream targets can negatively regulate rDNA transcription (see Section 3) resulting in a feedback loop enhancing p53 regulation by the nucleolus. In fact, proteomic analysis of p53 wild-type compared to p53 null cell lines (HCT116) demonstrated that the population of proteins that translocate from the nucleolus upon nucleolar stress, including the RPs, is markedly different when p53 is absent [167]. Thus the nucleolus is potentially the focal point for the integration of multiple stress signals, enabling it to mediate an appropriate p53 response. While such surveillance and regulation is potentially critical for the prevention of cancer, until recently, data supporting a role for ribosome biogenesis in the promotion of tumor development through suppression of p53 function was largely circumstantial. Donati et al. demonstrated that upregulation of rRNA transcription in human cancer cell lines in vitro, and a regenerating rat liver model in vivo, decreased the p53 response to cytotoxic stress [10]. Macias et al. also reported that mice expressing an MDM2 mutant that no longer binds RPL5 or L11, exhibited earlier onset and more frequent tumors when crossed with Eμ-*MYC* transgenic mice compared to MDM2 wild-type mice [152]. These findings support a model where, under conditions of hyperactivated rDNA transcription, nucleolar components that mediate p53 signaling (such as RPs) are constantly consumed by the process of ribosome biogenesis, preventing p53 activation.

4.2. Nucleolar regulation of the cell cycle

While nucleolar control of cell cycle progression is exemplified by induction of a p53-mediated cell cycle arrest following inhibition of rDNA transcription, the nucleolus additionally can mediate the function of many other cell cycle regulatory proteins. For example, p14ARF regulates the activity of a number of genes involved in cell growth and proliferation, and can sequester cell cycle regulatory proteins in the nucleolus in response to cellular stress and inhibit cell cycle progression [91,168,124] (reviewed in [89]). On the other hand, NCL translocates from the nucleolus in response to stress, where it interacts with replication protein A (RPA) thus preventing activation of DNA replication [169,121]. Cyclin E, which is essential for DNA replication in S phase, is rapidly inactivated by nucleolar sequestration where it is ubiquitinated by nucleolar SCFFbw7gamma [127]. Interestingly, Fbw7 mediates the turnover of numerous proteins required for growth and proliferation (such as MYC), suggesting that a similar mechanism of nucleolar sequestration may be employed to rapidly inactivate these proteins also. Finally, Cdc14B is sequestered in the nucleolus during interphase, and then released at mitosis to regulate correct mitotic progression [170].

Correct duplication of the centrosome and chromosome segregation is an essential process during the cell cycle, especially critical for genomic stability. A number of nucleolar factors are reported to mediate this process including: i) NPM1 and NCL, which associate with the mitotic poles, and mediate correct centrosome duplication and formation of the mitotic spindle [171–173]; ii) HCA66, which is a component of the centrosome required for both centriole duplication and formation of the mitotic spindle, also localizes to the nucleolus in interphase and is required for maturation of the 40S ribosomal subunit [174]; and iii) RPL41, which associates with the microtubules during mitosis and is required for centrosome integrity [175].

Nucleolar control of the cell cycle requires its appropriate assembly and functional regulation throughout the processes of cell replication and division (see Section 2). Aberrant functioning of the nucleolus therefore has the potential to compromise both cell cycle processes, such as correct DNA replication and mitosis, and the checkpoints that

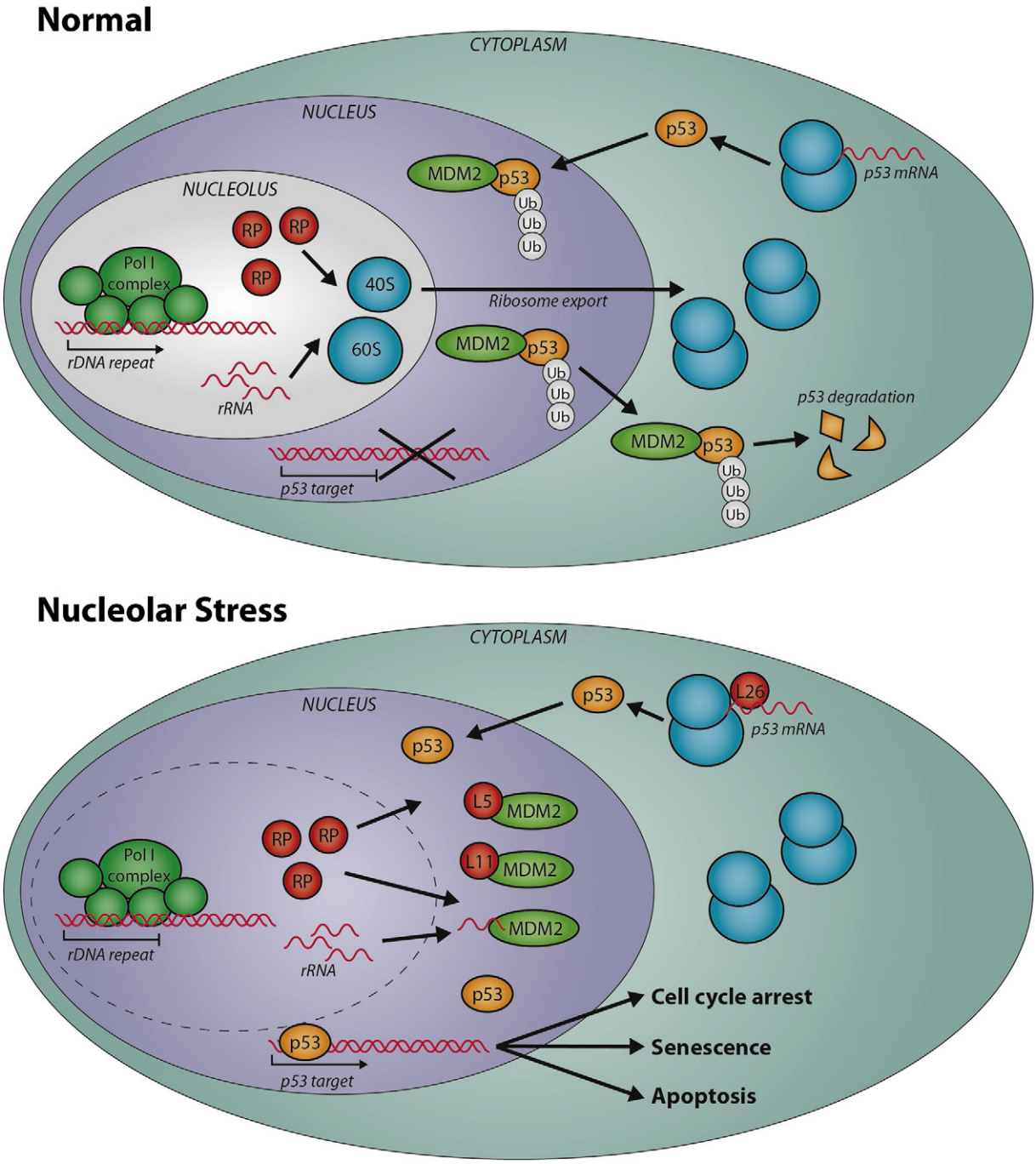

Fig. 3. The nucleolar stress pathway. Under normal growth conditions, levels of tumor suppressor p53 are suppressed by MDM2 binding leading to ubiquitination and degradation of p53. Upon nucleolar stress, nucleolar factors, including ribosomal proteins (RPs) and rRNAs are released from the nucleolus into the nucleoplasm and cytoplasm. Ribosomal proteins (predominantly L5 and L11) and 5S rRNA bind directly to MDM2, thereby releasing p53 allowing the tumor suppressor to accumulate. In addition, p53 mRNA expression has been shown to be upregulated by free ribosomal protein L26. The resultant activation of the p53 pathway can lead to cell cycle arrest, senescence or apoptosis.

prevent continuation of defective cell cycle progression, a hallmark of cancer [115].

4.3. Nucleolar regulation of the DNA damage response

Genome integrity is monitored by the DNA damage response (DDR) network, which activates cell cycle checkpoints and DNA repair pathways in response to specific types of DNA lesions. Compromised DDR results in genomic instability, a key underlying cause of cancer. At the nucleolus, DNA damage results in inhibition of rDNA transcription and reorganization of nucleolar structure [96,176,119,97]. At the same time, the nucleolus is increasingly reported to play an active role in the DDR. Proteomic and fluorescent imaging analyses have shown not only that a number of DDR proteins localize to the nucleolus, but that in response to different genotoxic insults the nucleolus undergoes reorganization with a distinct population of proteins translocating between the nucleolus and the nucleoplasm [177,117,167,119]. For example, double stranded break (DSB) repair factors RNF8 and BRCA1 translocate from the nucleolus to DNA-damage response foci in the nucleoplasm following ionizing radiation (IR), then revert to the nucleolus after several hours, presumably following DSB repair [178]. NCL interacts with a number of proteins involved in DNA repair (Topo I [179], Rad51 [180], WRNp [181]), and relocalizes from the nucleolus to the nucleoplasm where it associates with gamma-H2A.X and DDR foci [182]. Cdc14B is

phosphorylated by chk1 and released from the nucleolus following DNA damage [122], leading to Cdc14B-induced activation of the G2–DNA damage cell cycle checkpoint [170]. PARP-1 translocates from the nucleolus to the nucleoplasm following DNA damage [183,119], where it is involved in both DNA repair and induction of cell death; Notably, delocalization of PARP-1 from the nucleolus, such as occurs during stress response, sensitizes cells to DNA-damage induced apoptosis [183].

It has been proposed that as the rDNA repeats are particularly vulnerable to genomic instability, due to their repetitive nature and high rates of transcription [184], the nucleolus may activate DDR in a highly sensitive manner, performing a protective function overall for the genome [185]. To this end, Rubbi and Millner proposed that DNA damage at the nucleolus, but not at nucleoplasmic DNA, is necessary and sufficient to activate p53-dependent DDR [186]. Importantly, upregulated rRNA synthesis in different models was responsible for both increased DNA damage at the rDNA [187], and decreased p53-mediated response to cytotoxic stress [10]. This suggests that nucleolar dysregulation may result in both genomic instability at the rDNA repeats, and compromised DDR across the whole genome.

4.4. Nucleolar regulation of senescence

The activation of senescence, the process that drives a permanent cessation of cell proliferation, is emerging as an important mechanism for preventing or treating cancer (reviewed in [188]). Typically senescence is seen as preventing both hyperproliferation and genomic instability induced by telomere shortening, both of which are observed in cancer [115]. The involvement of the nucleolus in senescence has been clearly established, as inhibition of Pol I transcription induces senescence in both immortalized and transformed human cell lines [189] (reviewed in [190]).

The key signaling pathways mediating senescence include p53 and p16INK4a-pRb (reviewed in [191]). Thus, the nucleolus may induce senescence through its function in p53 pathway activation, discussed above. In addition, pRb transiently localizes with the nucleolus and nucleolar proteins, though the function of these associations in the regulation of the pRb pathway is less well-defined [41,192–194].

Nucleolar involvement in telomere stability is mediated by nucleolar localization of components of telomerase, and the telomere binding complex shelterin (reviewed in [190]). Telomerase is an RNP, composed of an RNA component (TERC) and reverse transcriptase (TERT), responsible for preventing telomere shortening during replication. In early S-phase, TERT moves to the nucleolus while TERC accumulates in Cajal bodies at the nucleolar periphery [195]. This nucleolar localization may be a pre-requisite for telomerase biogenesis [196–198,195,199]. Shelterin modulates telomerase activity and protects telomeres (reviewed in [200]). The shelterin component telomeric repeat binding factor 2 (TRF2) localizes to the nucleolus during G1 and S phase, and diffuses to the nucleoplasm in G2 [201]. Another shelterin component telomeric repeat binding factor 1 (TRF1) is stabilized or degraded by nucleolar proteins nucleostemin (NS or GNL3) and GNL3L respectively [202,203]. Telomere dysfunction, via loss of telomeric repeats or protective structures, triggers DDR at chromosome ends. This can lead to: i) end-to-end fusions, thus gross chromosomal aberrations and changes in ploidy; and eventually ii) the acquisition of new telomeres (reviewed in [200]).

Nucleolar dysregulation may therefore facilitate malignant transformation through: a) an escape from the senescence signaling pathways; b) driving genomic instability as a result of telomere dysfunction; and c) 'telomere healing', as a result of continual rounds of DDR at telomeres, enabling stable proliferation of malignant clones.

4.5. Nucleolar mediated epigenetic regulation of the genome

The organization of the genome within the nucleus contributes to the regulation of physiological processes such as transcription, replication, and establishing chromatin. Particularly, the spatial localization of chromosome regions can be required for the establishment of heterochromatin states, and as such can both regulate expression and maintain genomic integrity [204]. Recent genome wide analysis classified approximately 4% of the genome, in addition to regions containing NORs, as nucleolus-associated chromatin domains (NADs) [205]. Chromosomal regions – including telomeric and centromeric regions [206–208,205,209], satellite repeats [205], the Y chromosome [210], the inactive X chromosome (Xi) [211], imprinted chromatin regions [212,213], and repressed gene clusters specific to different cell types [205] and [209] – can be constrained at the nucleolar periphery, signifying a role for the nucleoli in organization of the genome (reviewed in [214]). Common characteristics of NADs are repressive histone marks and reduced gene expression; this includes repetitive regions, regions with low gene density and regions enriched in repressed genes [205,209]. In fact, the Xi is targeted to the perinucleolar heterochromatin during its inactivation, and it is proposed that its ongoing association with this region during S-phase is required to maintain its heterochromatic state [211]. Additionally, depletion of Tip5, a factor required for epigenetic silencing of the rDNA, resulted in loss of repressive histone marks and destabilization not only at the rDNA but also at associated satellite repeats [215]. Therefore, perinucleolar heterochromatin is proposed to function in the maintenance of repressive epigenetic state as a general strategy to prevent genomic instability.

Actively transcribed RNA pol III-dependent 5S and tRNA genes can also be found at the nucleoli [205], and enrichment of such genes has been documented in the perinucleolar regions surrounding the nucleolus [216–219]. Interestingly, Pol III transcribed 5S rDNA genes can induce association of the genomic region in which they are integrated with the nucleoli. As non-coding repetitive elements derived from Pol III transcripts make up a large proportion of the genome, it has been proposed that these can significantly contribute to nucleolar association [220]. Importantly, this association can result in the repression of linked genes, demonstrating the association between rRNA transcription, nucleolar localization and regulation of gene expression [220]. Thus, increased rDNA transcription could conceivably contribute to cellular transformation through altered organization and epigenetic regulation of the genome: Increased Pol I transcription as a result of loss of rDNA silencing could correspond to reduced epigenetic silencing of other NADs, resulting in genomic instability, particularly at repetitive regions; altered association of genomic regions with the perinucleolar heterochromatin may result in altered epigenetic regulation and expression from a number of loci.

In conclusion, the nucleolus controls many cellular processes whose dysregulation drive the acquisition of the hallmarks of cancer (Fig. 2). For the nucleolus to properly regulate these functions it needs to be exquisitely responsive to qualitative and quantitative changes in cellular stress signals. However in cancer, acquired oncogenic drivers (for example the overexpression of MYC), or loss of tumor suppressors (for example p53) result in the consistent hyperactivation of rDNA transcription. This effectively deadens nucleolar response to upstream signaling, preventing appropriate regulation of both ribosome biogenesis and extra-ribosomal nucleolar functions. Thus the nucleolus is a potential target in cancer therapy, with the inhibition of rDNA transcription predicted to not only reduce ribosome biogenesis and the protein translation capacity of growing cancer cells, but also restore appropriate regulation of many processes that are hurdles to acquisition of the cancer phenotype, such as activation of p53.

5. Targeting the nucleolus in cancer

The concept of targeting the nucleolus and ribosome biogenesis in cancer has proven to be controversial. This is due, in a large part, to its essential housekeeping role in sustaining the proliferation of normal cells. Thus it has been considered that drugs targeting ribosome biogenesis would not discriminate between highly proliferating normal cells

and tumor cells leading to a lack of a therapeutic window. This is despite the fact that it has been known for over two decades that a number of conventional chemotherapeutic agents impair ribosome biogenesis and/or nucleolar size, number and morphology [221–228]. A recent screen of common chemotherapeutic drugs demonstrated that out of 36 agents tested, 21 were found to affect ribosome biogenesis at the level of: (i) rDNA transcription, (ii) early rRNA processing (measured by the occurrence of the 32S rRNA intermediate) and (iii) late rRNA processing (measured by the occurrence of the mature 28S or 18S rRNAs) [225]. Moreover, a nucleolar disruption phenotype, characterized by the mislocation of NPM1, was consistently associated with the inhibition of rDNA transcription or early rRNA processing steps, but not late rRNA processing steps [225].

While the drug screen of Burger et al. [225] did not demonstrate whether the tested chemotherapeutic agents directly modulated the Pol I transcriptional machinery or the various rRNA processing factors, there is good evidence from a number of chemotherapeutics in current clinical use that their therapeutic efficacy is mediated in part by their ability to directly inhibit ribosome biogenesis (Fig. 4). For example, Dacitnomycin (also called Actinomycin D), a naturally occurring polypeptide antibiotic that intercalates GC-rich regions of DNA, is highly selective for the rDNA gene at concentrations as low as 5 nM and prevents the elongation stage of rDNA transcription by Pol I (Figs. 4 and 5) [229]. In addition, the platinum-containing compound cisplatin inhibits Pol I transcription with a high degree of specificity [221] through its ability to cross-link DNA at HMG-protein affinity sites thus

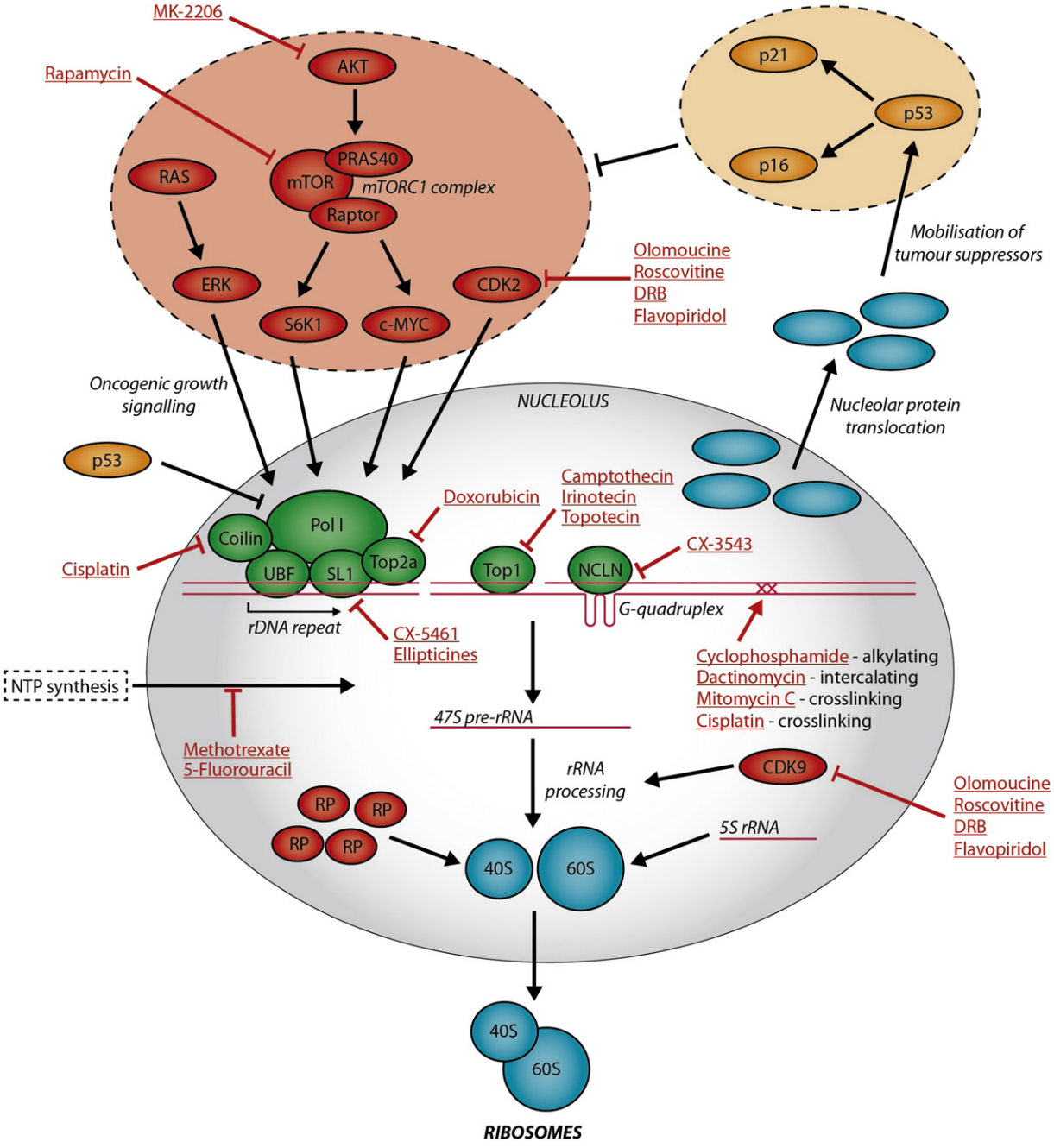

Fig. 4. Targeting the nucleolus in cancer therapy. A diverse range of anti-cancer drugs target ribosome biogenesis and nucleolar function. These agents can act directly at the level of rDNA transcription and pre-rRNA processing, or may impair the activity of members of upstream signaling pathways that regulate ribosome biogenesis and the nucleolus. Anti-cancer agents are highlighted in red and underlined.

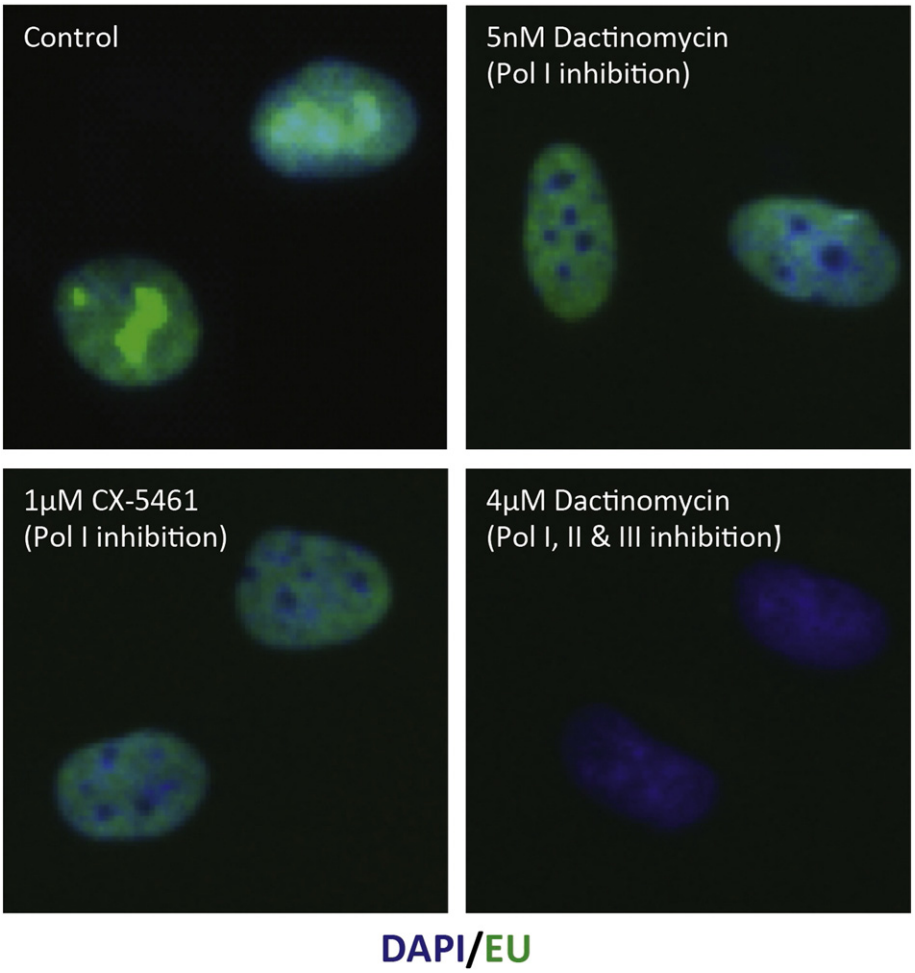

Fig. 5. Inhibition of RNA transcription by CX-5461 and Dactinomycin. Human fibroblast immortalized with hTERT were treated for 2 h with either 5 nM Dactinomycin or 1 μM CX-5461 to selectively inhibit Pol I, or with 4 μM Dactinomycin to inhibit all three RNA Polymerases (Pol I, II and III). After 1 h of treatment, media containing 1 mM of ethynyl uridine (EU) was added and the EU that had been incorporated into newly transcribed RNA was conjugated to fluorescently-tagged sodium azide by "click chemistry" {Jao, 2008 #305} (green). Following selective Pol I inhibition, no RNA is transcribed in the nucleolar regions as measured by EU staining.

preventing the transcription factor UBF from associating with the rDNA gene promoter [222]. Similarly, the anti-metabolite 5′fluorouracil (5′ FU), a well-characterized inhibitor of nucleotide synthesis [230], disrupts rRNA processing by preventing the cross-linking of rRNA binding proteins at key processing sites of the precursor rRNA transcript [223,224] (Fig. 4). In addition, inhibitors of Topoisomerase I activity, specifically Camptothecin, Irinotecan and Topotecian have also been demonstrated to potently disrupt Pol I transcription [231,232] (reviewed in [6]) (Fig. 4). In particular the ellipticine drug family of planar alkaloids [233] demonstrated anti-tumor activity in clinical trials [234–236] that was historically proposed to be the result of DNA breakage following the formation of an ellipticine–topoisomerase II–DNA ternary complex [237]. Recently the ellipticine derivative 9-hydroxyellipticine (9HE) was shown to specifically inhibit Pol I transcription by preventing the interaction between SL-1 and the rDNA gene promoter [238] (Fig. 4). Presumably 9HE via intercalating DNA at GC rich sites [239,240], which are common to the rDNA gene promoter, promoted DNA unwinding and the interruption of DNA-SL-1 interactions [238].

5.1. Nucleolar functions for targeted therapeutics

In addition to well-established conventional chemotherapeutic agents, many emerging anti-cancer drugs have been demonstrated to impair ribosome biogenesis in pre-clinical models. Various inhibitors of protein kinases have been demonstrated to inhibit ribosome biogenesis, in keeping with the regulation of this process by cellular growth and proliferation signaling pathways. Chemotherapeutic inhibitors of protein kinases that regulate the progression of cells through the cell cycle also disrupt ribosome biogenesis [225], consistent with the well-documented link between cell cycle control and the nucleolus (see Section 2). For example, the Cdk2 inhibitors roscovitine and olomoucine and the casein kinase 2 (CK2) inhibitor 5,6-dichloro-1-beta-ribofuranosylbenzimidazole (DRB) have been demonstrated to disrupt nucleolar integrity and drive the mislocalization of unprocessed rRNAs and rRNA processing factors, while rRNA processing has been demonstrated to be highly sensitive to the Cdk9 inhibitor Flavipiridol [241–244] (Fig. 4). Rapamycin, a naturally occurring specific inhibitor of the mammalian target of rapamycin complex 1 (mTORC1) is both an anti-cancer and immunosuppressive agent, reviewed in [245] (Fig. 4). Rapamycin has been well documented to suppress rDNA gene transcription by impairing signaling downstream of mTORC1 [66,246] and the new-generation 'rapalog' everolimus, which is FDA-approved for the treatment of renal cell carcinoma and advanced ER-positive/HER2-negative breast cancer [247,248], displays potent anti-tumor activity in a *MYC*-driven lymphoma model characterized by enhanced Pol I transcription [1,249]. Inhibitors of the protein kinase AKT, which acts upstream of mTORC1 and has important roles for the control of cell survival, proliferation, metabolism and angiogenesis, reviewed in [250], also impair ribosome biogenesis with the allosteric pan-AKT inhibitors AKTi-1/2 and MK-2206 demonstrated to suppress rDNA

gene transcription and induce apoptosis in a transgenic mouse model of *MYC*-driven lymphoma (Eμ-*MYC*) in culture and in vivo respectively [69,251] (Fig. 4).

5.2. Targeting the nucleolus by design: engineering specific inhibitors of ribosome biogenesis

The recent reevaluation of the nucleolus as a target for cancer therapy has been driven by our increased understanding of: (i) the importance of ribosome biogenesis in malignant transformation; (ii) the discovery of the nucleolar control of p53; and (iii) the inhibition of ribosome biogenesis being identified as key features of many chemotherapeutic and anti-cancer drugs. In particular, the development of a series of small molecules by Cylene Pharmaceuticals that specifically target ribosome biogenesis at the level of rDNA gene transcription by Pol I has met with some impressive success in identifying novel anti cancer agents.

5.2.1. CX-3543 (quarfloxin)

The first of the "selective Pol I transcription inhibitors" developed, CX-3543 (quarfloxin) specifically inhibits the elongation stage of Pol I transcription by preventing the stabilizing interactions between NCL and G-quadruplexes in the rDNA gene [252] (Fig. 4). NCL, which mediates the stabilization of G-quadruplex structures and prevents the renaturation of template DNA in the GC-rich rDNA gene facilitating rapid Pol I transcription [253], is selectively displaced from DNA by CX-3543 resulting in its redistribution from the nucleolus to the nucleoplasm and the inhibition of rDNA transcription [252]. This activity of CX-3543 is specific for NCL, with no observed impact on the association of Pol I transcription factors, such as UBF and SL-1, with the rDNA promoter, and was associated with the stabilization of p53 in keeping with the established nucleolar stress response discussed above (see Section 4.1) [252]. In pre-clinical studies utilizing a panel of cancer cell lines, CX-3543 exhibited a broad anti-proliferative effect and induced apoptosis of cancer cells independent of their p53 status. Furthermore CX-3543 demonstrated anti-tumor growth properties in xenograft models of breast (MDA-MB-231) and pancreatic (MIA PaCa-2) cancer [252] and progressed through a Phase I dose-escalation study in advanced solid tumors [254] (ClinicalTrials.gov NCT00955786) and a Phase II trial in low to intermediate grade neuroendocrine carcinoma (ClinicalTrials.gov NCT00780663) but was withdrawn from further trials due to issues with bioavailability [255].

5.2.2. CX-5461

The search for the next generation of direct Pol I inhibitors yielded the lead compound CX-5461 (now owned by Senhwa Biosciences), a small molecule that prevents PIC assembly by interfering with SL-1 binding to the rDNA promoter thereby preventing transcription initiation [189] (Fig. 4). CX-5461 was shown to be a highly selective inhibitor for Pol I activity, 300–400 folds more selective than for Pol II or Pol III transcription (Figs. 5 and 6). Indeed CX-5461 demonstrated limited direct effects on the transcription of Pol II target genes (*c-MYC*, *ACTB*) or on DNA synthesis even at very high drug concentrations [189].

CX-5461 in vitro exhibited a high anti-proliferative efficacy over a broad panel of human cancer cell lines (at low nano-molar concentrations) with a higher EC_{50} in non-cancer cell lines [189]. Robust

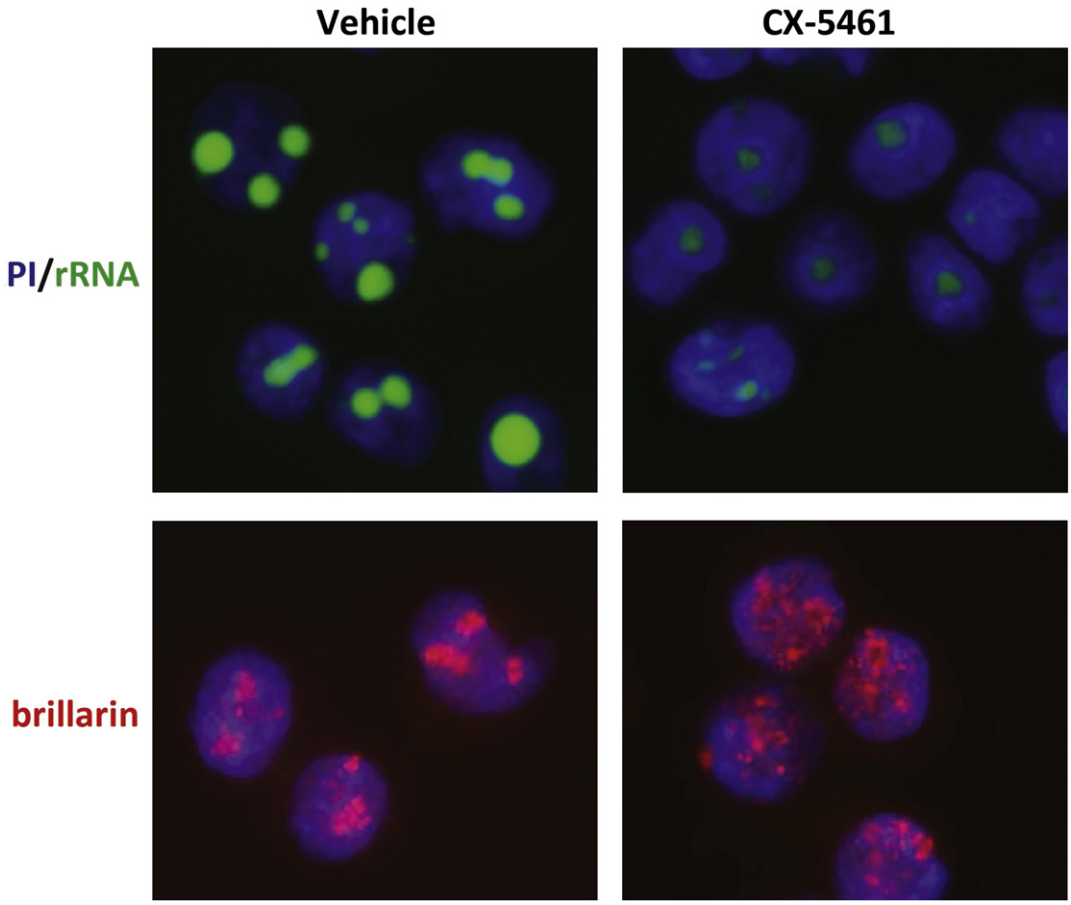

Fig. 6. Pol I activity and nucleolar structure in response to CX-5461 treatment. Acute myeloid leukemia cells (KG-1 cells) were treated ±500 nM CX-5461 for 4 h before PFA fixation. The cells were assayed by RNA-FISH with a probe designed against the 47S pre-rRNA external transcribed sequence (ETS) region which is rapidly processed following transcription, and is commonly used as a surrogate measure for Pol I activity (green, top panels). Also, the nucleolar structure was visualized by fibrillarin immunofluorescence (red, bottom panels).

anti-tumor growth properties were also demonstrated in melanoma (A375) and pancreatic (MIA PaCa-2) xenograft models, with CX-5461 inducing autophagy and senescence, in preference to apoptosis, in solid tumor cells.

Subsequent studies with CX-5461 in mouse transgenic models of hematologic malignancies have provided the best evidence to date that accelerated rDNA transcription and nucleolar integrity are critical for oncogenic activity tumor cells [1]. Specifically it was demonstrated that Pol I transcription could be targeted in vivo to selectively activate p53-dependent apoptosis in cancer cells, effectively treating tumors in both genetically engineered and xenograft models of lymphoma and leukemia [1]. Of particular interest, the induction of p53 mediated apoptotic death of the hematologic tumor cells was rapid, occurring within hours of treatment as a result of nucleolar stress and was independent of changes in total ribosome levels or protein translation. This observation is critical as it clearly demonstrates that Pol I transcription and nucleolar integrity are acutely required for the survival of certain tumor cells, independent of the level of functional ribosomes and thus protein synthesis rates and cell proliferation.

5.3. What confers selectivity and sensitivity of drugs targeting the nucleolus?

The above studies strongly suggest that cancers with altered genetic programs driving enhanced rDNA transcription and ribosome biogenesis will be vulnerable to the induction of a nucleolar stress response by agents that target the nucleolus. Cancers characterized by *c-MYC* gene amplification or overexpression, are a key example of malignancies that are likely to respond well to nucleolar-targeting agents. As mentioned above, MYC has a broad transcriptional program that is geared towards ongoing cell growth and proliferation, and through its ability to modulate the transcriptional activity of Pol I, Pol II and Pol III MYC acts as a global regulator of ribosome biogenesis (see Section 3). Enhanced ribosome biogenesis, characterized by increased rDNA transcription rates and Pol I machinery abundance [1], is a key feature of transgenic Eμ-*MYC* mice that constitutively overexpress MYC in B-lymphocytes and develop aggressive B-lymphomas [256]. Consistent with the above hypothesis that cancers characterized by oncogene driven up-regulation of ribosome biogenesis should be vulnerable to Pol I inhibition, MYC driven Eμ-*MYC* lymphomas are exquisitely sensitive to CX-5461. Indeed we believe MYC overexpression alone, independent of transformation, can be sufficient to sensitize cells to Pol I inhibition, as pre-malignant Eμ-*MYC* lymphoma cells demonstrated the same high sensitivity to Pol I inhibition and apoptotic response as the fully malignant Eμ-*MYC* lymphoma cells, despite exhibiting few genetic lesions in addition to elevated MYC expression [1]. Moreover we have found that MYC overexpression but not activation of other oncogenes such as RAS is sufficient to increase sensitivity to Pol I inhibition in human fibroblasts (R. Hannan, unpublished data).

Importantly, the apoptotic activity of CX-5461 in vivo was specific for MYC overexpressing lymphoma cells with no deleterious effect on the normal B-lymphocyte population observed in vivo [1]. This is because the targeted inhibition of rDNA transcription in non-tumorigenic B-lymphocytes in vivo by CX-5461 did not result in p53 pathway activation or the induction of apoptosis, in contrast to genotoxic insults such as γ-irradiation which activate DNA damage and p53 in both normal and tumor cells [1]. This ability of CX-5461 to induce p53-mediated responses in a cancer-cell specific manner will be a major benefit for moving this agent into the clinic due to the absence of genotoxic damage-associated side effects on the normal cell population. Finally, as with any anti-cancer agent, the development of resistant disease is likely to be an issue, and the potential combination of CX-5461 with conventional and novel chemotherapeutic drugs or other targeted therapies will be an important area to explore further.

6. Conclusions

While traditionally the contribution of the nucleolus to tumorigenesis has largely been seen to be centered on its role in facilitating ribosome biogenesis and proliferative capacity, more contemporary studies demonstrate the nucleolus is likely to play a much broader role in malignant transformation. In particular, the extra-ribosomal functions of the nucleolus as a central integrator of cellular stress are emerging as new mechanisms by which oncogenes and tumor suppressors can modulate functions in normal and malignant cells. The high dependency of certain tumor cells to co-opt nucleolar processes to maintain their cancer phenotype has clearly been demonstrated by the application of small molecule inhibitors of Pol I to selectively kill tumor cells in vivo [1]. Indeed it is likely we have only begun to scratch the surface of the potential of the nucleolus as a new target for cancer therapy. In 2011 Hanahan and Weinberg updated their "hallmarks of cancer" review [115]. It appears that "suppressing nucleolar stress" deserves a place in the next iteration of this classic text.

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