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COMMENTARY

Turning a new page on nucleostemin and self-renewal

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ABSTRACT

A quintessential trait of stem cells is embedded in their ability to self-renew without incurring DNA damage as a result of genome replication. One key self-renewal factor is the nucleolar GTP-binding protein nucleostemin (also known as guanine-nucleotide-binding protein-like 3, GNL3, in invertebrate species). Several studies have recently pointed to an unexpected role of nucleostemin in safeguarding the genome integrity of stem and cancer cells. Since its discovery, the predominant presence of nucleostemin in the nucleolus has led to the notion that it might function in the card-carrying event of the nucleolus – the biogenesis of ribosomes. As tantalizing as this might be, a ribosomal role of nucleostemin is refuted by evidence from recent studies, which argues that nucleostemin depletion triggers a primary event of DNA damage in S phase cells that then leads to ribosomal perturbation. Furthermore, there have been conflicting reports regarding the p53 dependency of nucleostemin activity and the cell cycle arrest profile of nucleostemin-depleted cells. In this Commentary, I propose a model that explains how the many contradictory observations surrounding nucleostemin can be reconciled and suggest that this protein might not be as multi-tasking as has been previously perceived. The story of nucleostemin highlights the complexity of the underlying molecular events associated with the appearance of any cell biological phenotype and also signifies a new understanding of the genome maintenance program in stem cells.

KEY WORDS: Cell cycle arrest, DNA damage, p53, Nucleolus, Nucleostemin, GNL3, Ribosomal synthesis, Self-renewal, Stem cell

Introduction

The nucleolus is a well-recognized intranuclear organelle that functions in ribosome biogenesis. Increasingly, within recent years, nucleolar or ribosomal proteins have been reported to play dual roles in regulating the DNA damage response and repair as well as ribosomal synthesis (Antoniali et al., 2014). The emerging link between the nucleolus and genome maintenance (or other non-ribosomal events) makes it ever more challenging to determine the causative versus associative relationship between the non-ribosomal (e.g. DNA damage) and ribosomal phenotypes following a single event of gene perturbation. Does one lead to the other and, if so, which comes first? Or, do they occur independently? As the readout in any cell-centered study, be it based on a single cell or a population of cells, represents an integration of events that have happened up to the point of measurement, it should come as no surprise that the outcome of gene perturbation often varies in a cell-context-dependent manner that might seem paradoxical at first, but can be logically explained once all the pathways affected, either directly or indirectly, are considered. However, too often those variations are simply interpreted as yet 'one more function of a multi-tasking protein', a good example of which is the nucleolar protein nucleostemin. This Commentary attempts to resolve three existing debates on this molecule and uses our journey with nucleostemin (also known as guanine-nucleotide-binding protein-like 3, GNL3, in invertebrate species) to highlight how one perturbed function might be read differently by cells in more subtle ways than commonly perceived and reveal a new understanding of the self-renewal maintenance of stem cells and cancer cells.

History and mysteries

Nucleostemin was discovered because of its high expression in neuroepithelial stem or progenitor cells purified from the fetal forebrain (Tsai and McKay, 2002) and was later found to be highly expressed in other types of stem cells, tumors, and tumor-initiating cells or cancer stem cells (CSCs) (Baddoo et al., 2003; Lin et al., 2010; Ohmura et al., 2008; Tamase et al., 2009; Tsai and McKay, 2002; Yamashita et al., 2013). By contrast, in differentiated cells and tissues, this protein is expressed at a much lower level, except for those undergoing regeneration (Lin et al., 2013; Maki et al., 2007; Shugo et al., 2012; Siddiqi et al., 2008). It is a nucleolar protein that encodes four circularly permuted GTP-binding (CPG) motifs and undergoes rapid exchange between the nucleolar and nucleoplasmic compartments (Meng et al., 2006; Tsai and McKay, 2005) (see Box 1 for detail). Unlike its orthologs in yeast (Nug1) and fly (Ns1), the GTP hydrolysis (GTPase) activity of mammalian nucleostemin has always been assumed based on its GTP-binding property but never been experimentally shown. The lack of evidence of an intrinsic GTPase activity in mammalian nucleostemin might be due to the fact that this protein is highly unstable (R.T., personal observation) or the need of a yet unidentified GTPase-activating protein. Regardless, until such evidence is presented, it is scientifically inaccurate to refer to mammalian nucleostemin as a nucleolar GTPase or assume it functions as a GTPase enzyme. The biological importance of nucleostemin has never been in doubt, as it is well established that nucleostemin makes key contributions to blastocyst formation (Beekman et al., 2006; Zhu et al., 2006), embryogenesis (Meng et al., 2013), postnatal tissue regeneration (Lin et al., 2013; Shugo et al., 2012), cancer development (Lin et al., 2010; Sijin et al., 2004; Tamase et al., 2009) and reprograming to pluripotency (Qu and Bishop, 2012).

In contrast, the fundamental mechanism through which nucleostemin functions is less clear. Despite considerable interest, a consensus on this subject remains to be reached. However, within the past two years, several studies have been published that reveal the molecular mechanism of nucleostemin in reducing DNA damage on the telomeres and non-telomeric chromosomes. One study has shown that nucleostemin is able to reduce telomere damage through modification of telomeric repeat

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Box 1. Nucleolar localization and dynamic shuttling of nucleostemin

The translocation of nucleostemin between the nucleolar and nucleoplasmic compartments has been studied by chemical perturbation and mutation analysis (Meng et al., 2007; Tsai and McKay, 2005). Nucleostemin protein contains a stretch of basic residues at its N-terminus that functions as a nucleolar localization signal (NoLS). However, this basic NoLS can only confer a static nucleolar distribution; alone it cannot recapitulate the dynamic properties of the full-length nucleostemin, that is a long retention time in the nucleolus. These additional features require the GTP-binding domain of nucleostemin. The static accumulation and dynamic movement of nucleostemin is controlled by another domain that favors its nucleoplasmic localization. A key signal that controls the nucleolar trafficking of nucleostemin is GTP, which acts as a molecular switch to turn off the nucleoplasmic-anchoring activity. The nucleoplasmic distribution of non-GTP-bound nucleostemin mutants can be reversed back to the nucleolus by inhibiting the 26S-proteasome-mediated protein degradation pathway, suggesting that the degradation of nucleostemin protein is coupled to its nucleoplasmic distribution through GTP binding (Huang et al., 2009).

binding factor 1 (TRF1, also known as TERF1) and recruitment of promyelocytic leukemia protein isoform IV (PML-IV) in telomerase-negative human cancer cells (Hsu et al., 2012). Two reports have identified that it protects the genome integrity of stem or progenitor cells by promoting the recruitment of RAD51, the core component of the homologous recombination machinery, to stalled replication-induced DNA damage foci (Lin et al., 2013; Meng et al., 2013) (Fig. 1). Another study confirms its genome protective role in hematopoietic stem cells (HSCs) and its importance in maintaining the self-renewal of HSCs *in vitro* and *in vivo* (Yamashita et al., 2013). For the genome- or telomere-protective activity of nucleostemin, the nucleolus serves primarily a storage or sequestration role. What is interesting about these findings is that this genome- or telomere-protective role of nucleostemin offers a potential hypothesis that best unifies most of its *in vitro* and *in vivo* activities that have been reported to date, as discussed below. However, the road leading to this recent revelation is nothing but long and winding, as one has to plow through the various activities of nucleostemin that have been previously reported (e.g. p53 regulation, ribosomal synthesis and cell cycle regulation) and potentially more in the making.

Nucleostemin – a Jack of all trades or not?

Because of its nucleolar presence, there has always been an inclination to associate nucleostemin with ribosome biogenesis. Indeed, deletions of the nucleostemin orthologs in fission yeast, nematode and fruit fly have all been shown to reduce ribosomal production, either with or without interfering with the transcription or splicing of rRNAs (Du et al., 2006; Kudron and Reinke, 2008; Rosby et al., 2009). Two studies have reported a perturbed ribosomal effect following the knockdown of vertebrate nucleostemin in zebrafish and in human cancer cells (Essers et al., 2014; Romanova et al., 2009). Although these findings might appear to be consistent with a role of nucleostemin in ribosomal synthesis, there have been multiple lines of evidence that contradict a direct involvement of mammalian nucleostemin in ribosomal synthesis, including the failure of human nucleostemin to rescue the ribosomal phenotype of yeast null for GNL3 (the ortholog of nucleostemin), the non-congruent localization of nucleostemin and nascent 28S rRNA inside the human nucleolus, and the late occurrence of ribosomal defects following nucleostemin knockdown (Du et al., 2006; Kudron and Reinke, 2008; Lin et al., 2014; Politz et al., 2005). Whether nucleostemin has a ribosomal or non-ribosomal role is not the only issue under debate. Earlier studies have indicated that nucleostemin (mostly that of the mammal) is functionally linked to the p53 pathway and physically associated with MDM2, the negative regulator of p53 (Dai et al., 2008; Ma and Pederson, 2007; Meng et al., 2008; Tsai and McKay, 2002). However, the necessity of p53 in mediating the obligatory function of nucleostemin has been disputed by several independent studies that establish the indispensable role of nucleostemin in maintaining the continuous proliferation of cells that lack a functional p53 pathway (Beekman et al., 2006; Jafarnejad et al., 2008; Meng et al., 2013). Finally, even at the most basic and fundamental level, as is in all cell biological studies, consensus has yet to be reached on how loss of nucleostemin affects the cell cycle profile. Evidence linking nucleostemin depletion to any of the possible cell cycle arrest profiles (i.e. G1/S, intra-S, G2/M and mitotic arrest) can be found in the published literature (a point to be further illustrated later). If we believe that all the above studies are properly executed and reproducible, and that biology is not governed by random variables and probability, then how can we make sense of these contradictory findings? Is nucleostemin so ‘multi-talented’ or ‘permissive’ that opposite effects might be possible for a single event of nucleostemin perturbation? Or are those different outcomes of perturbation merely reflective of the complexity of

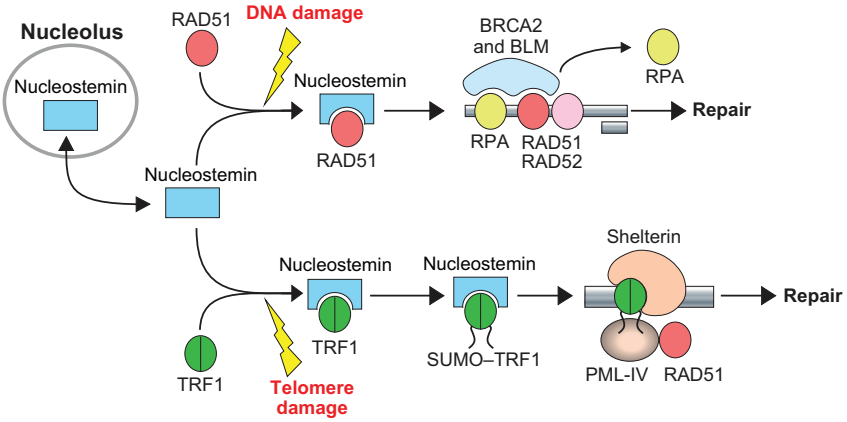

Fig. 1. Genome-protective role of nucleostemin. Shown here is a schematic illustration of the role of nucleostemin in the repair of replication-induced DNA damage on interstitial chromosomes and in the repair of telomere damage. The genome protective function of nucleostemin promotes the recruitment of the core homologous recombination protein, RAD51, which also requires breast cancer susceptibility gene 2 (BRCA2), Bloom syndrome protein (BLM), replication protein A (RPA) and RAD52. For damaged telomeres, nucleostemin regulates telomeric repeat binding factor 1 (TRF1) sumoylation and its subsequent association with promyelocytic leukemia protein isoform IV (PML-IV), which brings RAD51 to damaged telomeres for repair in telomerase-negative cancer cells.

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cells? Based on my understanding of nucleostemin, those seemly unrelated and at times contradictory observations might be the work of a multi-tasking cell rather than a multi-tasking gene.

The end does not always justify the means

A potential role of human nucleostemin in ribosomal synthesis was first claimed by a study that observed a delayed processing of 32S pre-rRNA to 28S rRNA and a reduction of 60S ribosomes in HeLa cells in which nucleostemin has been knocked down (Romanova et al., 2009). However, studies published by two independent groups argue that rRNA processing might not be the primary target of nucleostemin knockdown in human cells. One group showed that human nucleostemin is localized in subnucleolar regions that are deficient in nascent 28S rRNA (Politz et al., 2005). The other group found that knockdown of nucleostemin does not perturb the splicing of pre-rRNAs (supplementary data shown at http://www.ribogenesis.com; Tafforeau et al., 2013). It is worth noting that in the Romanova et al. study (Romanova et al., 2009), the ribosomal deficiency was measured after two rounds of small interfering (si)RNA treatment, which lasted for 5 (or more) days. This raises the concern that the initial direct effect of nucleostemin knockdown could have triggered secondary responses during this period of time, such as the observed ribosomal deficiency.

A recent discovery from my laboratory of a function of nucleostemin in genome maintenance (Lin et al., 2013; Meng et al., 2013) makes it possible to compare the time of onset of DNA damage to the time of onset of ribosomal deficiency in nucleostemin-knockdown cells. It is apparent that DNA damage arises at the earliest time point of measurement, that is 12 hours after the initiation of nucleostemin depletion, whereas no significant effect on rRNA synthesis or nucleolar structure can be noted up to 48 hours (Lin et al., 2014). This finding establishes that DNA damage occurs considerably before ribosomal perturbation in response to nucleostemin depletion. Of course, the time-of-onset evidence alone does not prove that ribosomal perturbation occurs secondarily as a result of DNA damage following nucleostemin depletion, as the manifestation of different phenotypes can take place in parallel and their times of onset might depend on the kinetics of the underlying mechanisms that lead to their activation. However, this finding does raise a reasonable doubt on the immediacy of ribosomal perturbation following nucleostemin depletion and acts as a reminder of what has been noted for over 15 years, that is that the transcription of rRNA is inhibited by the DNA-cross-linking agent cisplatin (Jordan and Carmo-Fonseca, 1998). There again, the question of whether rRNA transcription in cisplatin-treated cells is affected directly by rDNA lesions or indirectly by the DNA damage response signals has been long standing, as DNA cross-linking agents, such as cisplatin, can directly inhibit upstream binding factor (UBF) binding and RNA polymerase I (Pol I) transcription by forming rDNA adducts and blocking rDNA unwinding. A recent study by Calkins et al. addresses this issue by using UV and γ-irradiation, in addition to cisplatin, to introduce DNA damage (Calkins et al., 2013). They report that rRNA synthesis is also inhibited 24 hours after UV and γ-irradiation, and that the reverse signaling from cell cycle progression to ribosomal synthesis involves mechanism dependent on DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase 1 (PARP1). Another study in support of this notion uses DNA lesions induced by γ-irradiation and shows that Pol I inhibition is not a direct effect of DNA damage but is mediated by the complex between ataxia telangiectasia mutated (ATM), Nijmegen breakage syndrome 1 (NBS1) and mediator of DNA damage checkpoint 1 (MDC1) (Kruhlak et al., 2007).

As the end result of a gene depletion often does not reflect the cellular process that is affected first, in order to conclude a causal versus an associative relationship between a gene knockdown or knockout event and the observed phenotypes, multiple lines of evidence need to be considered, such as the sequence of onset of events, direct interactions between the protein of interest and pathway-specific components, functional rescue of phenotypic deficits, and protein localization to the site of action. Taking this into account, evidence for a direct role of nucleostemin in maintaining genome or telomere integrity is provided by the early onset of the observed DNA damage phenotypes, the direct binding of nucleostemin to RAD51 (genome repair) or TRF1 (telomere protection), its ability to rescue DNA or telomere damage that has been induced by stalling of replication or disruption of telomere-binding complex (known as shelterin dysfunction), respectively, as well as its physical recruitment to DNA damage foci. Last but not least, the complexity of the interconnecting signaling networks within cells and their versatile responses to any perturbation event should at least alert, if not prohibit, us biologists in drawing mechanistic conclusions based on a few end phenotypes – such is a valuable lesson we learned from working with nucleostemin.

Vertebrate and invertebrate nucleostemin tell two different stories

When attempting to correlate the activities of nucleostemin in different species, one must take into consideration that the invertebrate nucleostemin (named GNL3) is a shared ortholog of both nucleostemin and GNL3-like (GNL3L) in the vertebrates (Tsai, 2011; Tsai and Meng, 2009). Although there is no shortage of paralogous examples in biology, mammalian nucleostemin and GNL3L are unique in that they exhibit distinct functions. It has been shown that human nucleostemin lacks the ability to rescue ribosomal dysregulation phenotypes that are associated with GNL3 deletion in *Schizosaccharomyces pombe* (Du et al., 2006) and *Caernohabditis elegans* (Kudron and Reinke, 2008). Although the failure to rescue GNL3 deficiency with mammalian nucleostemin alone might simply reflect the inability of the latter to bind to the invertebrate rRNA or ribosome, the fact that human GNL3L is able to reverse the ribosomal phenotype of GNL3-depleted *S. pombe* poses the interesting possibility that mammalian nucleostemin might have functionally diverged from mammalian GNL3L and invertebrate GNL3 (Du et al., 2006). Indeed, recent work has shown that nucleostemin-knockdown triggers DNA damage without causing a significant disturbance of ribosomal synthesis, whereas depletion of GNL3L perturbs the processing of pre-rRNA without causing DNA damage in human breast cancer cells (Lin et al., 2014). On the basis of these studies, I propose the idea that during the evolution of vertebrate species, GNL3L might have retained the role of its ancestral gene in ribosome biosynthesis, whereas, nucleostemin, the paralog that arose, acquired a new function in genome protection. Another recent study has reported that nucleostemin mutation in zebrafish (*Danio rerio*) causes a reduction in the number of 60S ribosomes as well as of p53 stabilization, and that deletion of p53 can restore the decrease of 60S ribosomes in nucleostemin mutant fish and elevate 60S ribosomes in wild-type fish (Essers et al., 2014). This finding suggests that the ribosomal effect of nucleostemin-mutant fish can

be explained by the effect of nucleostemin mutation in stabilizing p53. Because nucleostemin and GNL3L proteins have 32.3% identity in human and only 28.7% in zebrafish, it is also conceivable that the functional divergence of nucleostemin and GNL3L is still ongoing as the vertebrate species continue to evolve. If so, zebrafish nucleostemin might have a greater functional resemblance to invertebrate GNL3 than human nucleostemin. Indeed, two major protein diversifications in the GNL3/nucleostemin family appear to occur in the evolution of vertebrate and mammalian species (Fig. 2).

Unlike nucleostemin and GNL3L, the story about their closest kindred, GNL2 (also known as Nug2/Nog2 in yeast and NGP1 in mammals), is quite different. GNL2 has always maintained the status of a single gene subfamily throughout evolution and has been known to function in the nuclear export of 60S ribosome, as detected by the Rpl25–GFP reporter (Saveanu et al., 2001). A recent study provides mechanistic insight into GNL2 function by showing that it works as a 'placeholder' that binds to the juncture of maturing pre-60S subunits and controls the proper timing in the transfer of pre-60S ribosomes to the nuclear export adaptor Nmd3 and the nucleolar export receptor Crm1 (Matsuo et al., 2014). In this event, the GTP-binding state of GNL2 is crucial for its binding to pre-60S ribosome, and its GTP hydrolysis is required for its release from pre-60S ribosome and the subsequent transfer of pre-60S ribosome to Nmd3. Notably, the GTPase activity of GNL2 is stimulated by KCl as well as a conformational change triggered by two pre-60S remodeling factors, the Rea1 ATPase and its co-substrate Rsa4.

The amazing diversity of cell-cycle-arrest profiles of nucleostemin-depleted cells

It is rather intriguing to see that loss of nucleostemin could have been associated with cell cycle arrest at the G1/S, intra-S, G2/M and M phases. Studies that reported a G1/S arrest phenotype following nucleostemin-knockdown include those performed in U2OS cells (Dai et al., 2008; Ma and Pederson, 2007), SW1710 cells (Nikpour et al., 2009), HeLa cells (Sijin et al., 2004), PC-3 cells (Liu et al., 2010) and bone marrow-derived stromal stem cells (Jafarnejad et al., 2008). In contrast, the finding that nucleostemin-depleted cells are arrested at the G2/M phase has also been reported in several studies performed in U2OS cells (Meng et al., 2008), HeLa cells (Romanova et al., 2009), 5637 cells (Nikpour et al., 2009) and mouse embryonic fibroblast cells (MEFs) (Zhu et al., 2006). It is noted that the studies describing G1/S arrest used cells with either wild-type or mutant forms of p53, retinoblastoma, p16 and alternate reading frame (p14ARF) protein (both encoded by *CDKN2A*), and studies describing G2/M arrest also used cells with wild-type or mutant forms of p53, retinoblastoma, p16 and p14ARF. This argues that none of these tumor suppressor genes is the cause of the observed cell cycle outcome of nucleostemin-depleted cells.

In a recent study from my laboratory, we observed a cell cycle arrest upon nucleostemin knockdown in human breast cancer cells (MDA-MB-231) after 12 hours, and found that, at this early time point compared with other related studies, nucleostemin depletion causes an increase in cells in S phase and a decrease of those in G2/M, indicative of an intra-S-phase arrest (Lin et al., 2014). Most importantly, our dose-dependent study revealed that the apparent discrepancy with regard to the cell cycle effects of nucleostemin depletion among different studies might be due to the level of protein knockdown. A more efficient loss of nucleostemin causes an early S-phase block with fewer cells in G2/M, whereas a less efficient loss of nucleostemin results in arrest in late S and G2/M with an increased number of G2/M cells (Lin et al., 2014). This idea resonates with the finding of an earlier study showing that HeLa cells with nucleostemin knockdown are unable to complete DNA synthesis to pass through S phase, resulting in an increase in the percentage of cells in G0/G1 and a concomitant decrease of S phase cells (Sijin et al., 2004). Another point to keep in mind is that a completely arrested S phase cell would not be able to incorporate exogenous mitotic markers (e.g. BrdU), which have been used to determine the percentage of cells in S phase as a measurement of nucleostemin knockdown in some studies. However, just when a more coherent picture on the cell cycle arrest profile of nucleostemin-depleted cells appears to emerge, one study published this year reports yet another new arrest phenotype in the mitotic phase in nucleostemin-depleted HeLa cells (Maida et al., 2014). At this stage, it is unclear whether this mitotic arrest phenotype occurs as a stand-alone event or in association with one of the many events caused by loss of nucleostemin, but it certainly contradicts what we have found in U2OS cells, where nucleostemin depletion causes cells to arrest at the G2/M phase before cells enter the mitotic prophase (Meng et al., 2008). In the same paper, the authors also showed that nucleostemin is a part of the telomerase reverse transcriptase (hTERT)–Brahma-related gene 1 (BRG1) complex.

p53 or no p53?

Our initial work on nucleostemin identified a physical connection between nucleostemin and the p53 protein complex (Tsai and

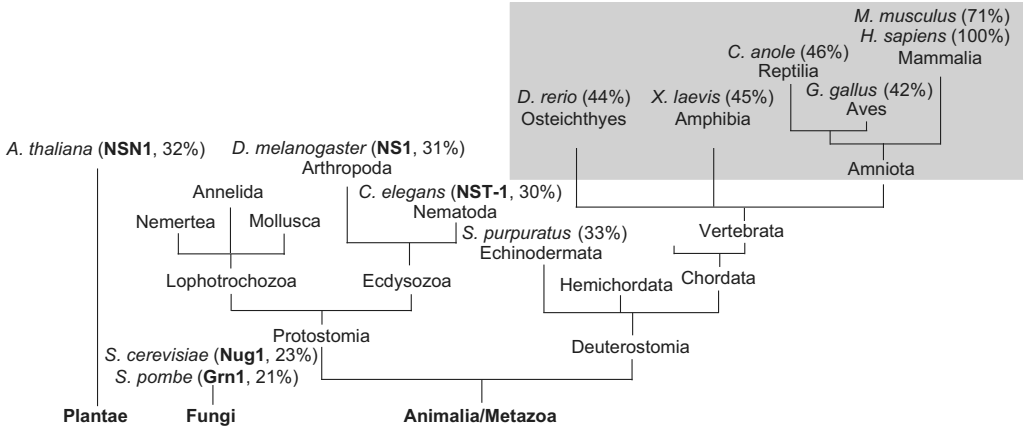

Fig. 2. A simplified phylogenetic tree of GNL3, nucleostemin and GNL3L. Percentages in parenthesis indicate protein identity between human nucleostemin and invertebrate GNL3, or nucleostemins (vertebrate) of different species. The gray box indicates species that have both nucleostemin and GNL3L genes. Alternative names for GNL3 in some invertebrate species are provided.

McKay, 2002). Although a number of subsequent studies have reported that loss of nucleostemin turns on p53 to induce cell cycle arrest in cells with wild-type p53, this finding did not address whether p53 is necessary for the obligatory function of nucleostemin in cell proliferation maintenance. There are some studies that addressed this question by investigating the effect of nucleostemin depletion in cells with wild-type and mutant p53, but they reached opposite conclusions. In support of the p53-dependency of nucleostemin activity, it has been shown that cell cycle arrest or apoptosis that is triggered by knockdown or knockout of nucleostemin can be completely or partially reversed by p53 knockdown (Ma and Pederson, 2007; Paridaen et al., 2011; Yamashita et al., 2013). Furthermore, nucleostemin has been found to directly interact with MDM2 to modulate the activity of p53 (Dai et al., 2008; Meng et al., 2008). In contrast, some studies have not observed any role pf 53 and have prompted the idea that an obligatory function of nucleostemin is required for cell proliferation even in the absence of p53 – a claim that is strongly supported by the observation that loss of p53 fails to rescue the growth-deficient phenotype of nucleostemin depletion in mouse blastocysts and MEFs (Beekman et al., 2006; Meng et al., 2013). In fact, we have found that loss of p53 might even accelerate the demise of nucleostemin-knockout MEF cells (Meng et al., 2013). As the gatekeeper for the genome integrity, most cytotoxic injuries converge on p53 to dispatch one or more of its downstream events, including cell cycle arrest, apoptosis, senescence and reduction in 60S ribosome numbers (as recently reported by Essers et al., 2014), among others. Therefore, it should not come as a surprise that p53 knockout might partially alter the outcome of nucleostemin depletion, in particular if events regulated by and downstream of p53 are used as the readout. However, this apparent involvement of p53 will not provide us with much insight into the specific mechanism of nucleostemin action. One can even argue that the primary target of nucleostemin, because it sits upstream of the p53 action, should not be affected by p53-knockout initially, as such is the case in DNA damage induced by nucleostemin knockout or knockdown (Meng et al., 2013).

To date, the only evidence linking nucleostemin directly to the p53 axis is its interaction with MDM2 (Dai et al., 2008; Ma and Pederson, 2007; Meng et al., 2008; Tsai and McKay, 2002). In our investigation of nucleostemin-mediated MDM2 regulation (not on p53 stabilization), we found that, compared to wild-type, mutants of nucleostemin that localize to the nucleoplasm more strongly bind to MDM2 and retain it in the nucleoplasm, thereby inhibiting its ubiquitylation and subsequent degradation, which results in p53 activation (Meng et al., 2008). However, the effect of nucleostemin-knockdown on MDM2 is much more evident in cells that are under nucleolar stress than in normal growing cells in interphase. Therefore, a more fitting model for the functional interplay between nucleostemin and p53 is that the nucleostemin-mediated regulation of MDM2 occurs mainly when the bulk of nucleostemin is released from the nucleolus into the nucleoplasm, which occurs during mitosis or nucleolar stress. Under conditions in which the nucleolus is intact, the amount of nucleostemin in the nucleoplasm is below the threshold of MDM2 and thus p53 regulation.

Nucleostemin brings a new perspective on self-renewal

The arguments presented so far indicate that most of the activities of nucleostemin reported to date, including genome and telomere protection, MDM2–p53 regulation, ribosomal synthesis and cell cycle regulation, can be explained without having to evoke the concept of multi-functionality (as compared to mono- or oligo-functionality) (Fig. 3). The obligatory function of nucleostemin in maintaining cell proliferation under normal growing conditions is most consistent with its role in protecting the genome from DNA damage, which is fulfilled by the nucleoplasmic pool of nucleostemin during genome replication in S phase. How DNA damage signals trigger nucleostemin localization to the site of damage is an important aspect in understanding the biology of nucleostemin in genome protection. The perceived signals that lead to the accrual of nucleostemin on damaged DNA in the nucleoplasm remain speculative but likely involve different sets of control that are distinct from those described for its nucleolar targeting and retention (see Box 1). In actively dividing cells with

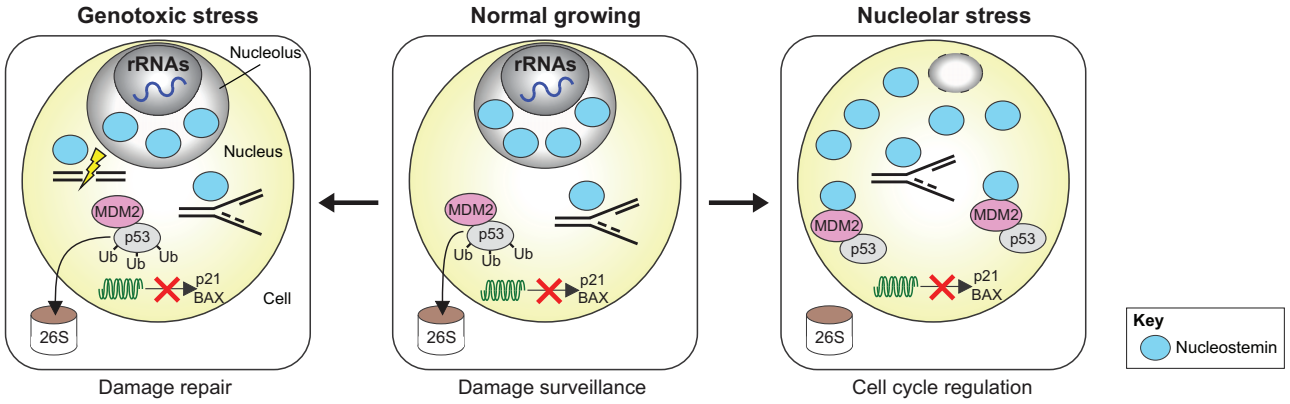

Fig. 3. Model of the mechanism underlying nucleostemin function. The central panel represents nucleostemin activity in normal growing interphase cells, whereas the left and right panels represent cells under increased genotoxic or nucleolar stress, respectively. The primary activity of nucleostemin is to protect against DNA damage caused by genome replication (indicated by the replication fork) in normal growing cells or by exogenous sources of radiation or chemicals (indicated by the lightning sign) in cells under genotoxic stress. In response to nucleolar stress, the release of nucleostemin from the nucleolus stabilizes MDM2 and prevents p53-mediated activation of its downstream targets involved in cell cycle regulation (represented by p21) and apoptosis (represented by BAX). Rounded rectangles, yellow circles and gray circles represent the cell, nucleus, and nucleolus, respectively. The nucleolus is divided into two domains that are either rich in or deficient in rRNAs (blue wave symbol). Ub, ubiquitin; 26S, 26S-proteasome complex; green double helix, p53-regulated genes.

intact nucleoli, loss of nucleostemin triggers replication-associated DNA damage, which then leads to S phase arrest, possibly through the ataxia telangiectasia and Rad3-related (ATR)–checkpoint kinase 1 (Chk1) and ATM–checkpoint kinase 2 (Chk2) pathways, which might activate p53, if available. When the nucleolus is disassembled under nucleolar stress or during mitosis, nucleostemin is released *en masse* from the nucleolus into the nucleoplasm. This sudden increase of nucleostemin in the nucleoplasm allows it to interact with and stabilize MDM2 independently of its function in DNA repair. Although invertebrate nucleostemin might indeed have a role in ribosomal synthesis, in mammalian cells, this activity has been inherited by GNL3L and not by nucleostemin. The apparent ribosomal defect that is seen upon nucleostemin depletion in mammalian cells might in fact be a result of increased DNA damage and subsequent cell cycle arrest.

This newly revealed activity of nucleostemin in genome protection, in conjunction with its established expression and importance in normal and cancerous stem cells, suggests that, compared with their differentiated progeny, mitotically active stem or progenitor cells might be differentially equipped to repair DNA damage caused by genome replication. Given that stem cells form the cornerstone in embryonic organogenesis and adult tissue homeostasis, it makes perfect sense that the integrity of their genome must be kept at the most pristine quality. As a testament to the importance of the DNA damage response and repair proteins in stem cell maintenance, defects in many of these proteins often result in phenotypes that can be traced to stem cell dysfunction, including premature aging, embryonic lethality, hematopoietic deficiency, increased UV and radiation sensitivity, and elevated cancer incidence. With regard to damage reduction, several mechanisms that might help achieve this goal have been proposed, including a specific microenvironment with low oxygen levels to minimize oxidative stress, a mitotically quiescent state that avoids replicative stress, and limited reactive oxygen species production from cell-intrinsic metabolism (Orford and Scadden, 2008). With regard to response and repair, damaged stem cells might choose to self-sacrifice for a greater good or to respond to and repair damaged DNA more efficiently (Blanpain et al., 2011). So which factor dictates the behavior of stem cells under DNA damage conditions? The answer might lie in the tissue origins of the cell.

Cord-blood-derived human embryonic HSCs (Milyavsky et al., 2010), interfollicular basal stem cells (Finlan et al., 2006) and intestinal stem cells are among those cell types that preferentially choose to commit suicide in response DNA damage. Their altruistic behavior might be driven mechanistically by an enhanced activity in the pro-apoptotic network or the lack of DNA repair activity. By comparison, more types of stem cells choose to live, as has been shown for adult hematopoietic stem cells (HSCs) (Mohrin et al., 2010), epidermal stem cells (Rachidi et al., 2007), hair follicle stem cells (Sotiropoulou et al., 2010) and mammary stem cells, as well as CSCs in breast cancer, leukemia and glioblastoma multiforme (Bao et al., 2006; Diehn et al., 2009; Jordan et al., 2006; Li et al., 2008). These cells might survive cell-intrinsic or extrinsic genotoxic insults through engaging specialized protective mechanisms that suppress the apoptotic pathway or promote the efficiency of DNA damage repair machinery – an area which nucleostemin might make its contribution in. Not surprisingly, stem cells also rely on different mechanisms for repair depending on their mitotic status, for example, non-homologous end-joining (NHEJ) is used by quiescent HSCs and homologous recombination is used by mitotically active HSCs (Mohrin et al., 2010). Some members in the core DNA damage response and repair pathway show particular enrichment in mitotically hyperactive cells. Breast cancer susceptibility gene 1 (BRCA1), a tumor suppressor with functions in DNA damage repair, the centrosome and mitotic spindle, is most highly expressed in the embryonic neuroepithelium, which contains highly proliferative progenitors (Lane et al., 1995). Deletion of this gene in the developing neural epithelium results in severe agenesis of the neocortex, hippocampus, cerebellum and olfactory bulb (Pao et al., 2014), which to some extent resembles the phenotype of nestin-driven nucleostemin-knockout mice (Meng et al., 2013). Indeed, when it comes to supporting the idea of specialized genome-protective mechanisms in self-renewing cells, nucleostemin does not stand alone.

Conclusion

Taking all that have been discussed in consideration, it is logical to think that nucleostemin exercises a primary and constitutive activity in safeguarding against replicative DNA damage and an induced activity in stabilizing MDM2 under conditions of nucleolar stress. Although invertebrate nucleostemin might function in the biogenesis of ribosome, the reported effect of nucleostemin depletion on rRNA synthesis in mammalian cells might rather represent a late event that occurs secondarily to cell cycle arrest. The discovery of its function in maintaining the integrity of replicating genome signifies a new chapter in the life of nucleostemin as well as in our understanding of self-renewal.

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Competing interests

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