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Abstract

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ORIGINAL PAPER

Differential requirements for H/ACA ribonucleoprotein components in cell proliferation and response to DNA damage

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Abstract H/ACA ribonucleoproteins (RNPs) are comprised of four conserved proteins, dyskerin, NHP2, NOP10, and GAR1, and a function-specifying, noncoding H/ACA RNA. H/ACA RNPs contribute to telomerase assembly and stabilization, and posttranscriptional processing of nascent ribosomal RNA and spliceosomal RNA. However, very little is known about the coordinated action of the four proteins in other biologic processes. As described herein, we observed a differential requirement for the proteins in cell proliferation and identified a possible reliance for these factors in regulation of specific DNA damage biomarkers. In particular, GAR1 expression was upregulated following exposure to all forms of genotoxic stress tested. In contrast, levels of the other proteins were either reduced or unaffected. Only GAR1 showed an altered subcellular localization with a shift from the nucleolus to the nucleoplasm after ultraviolet-C irradiation and doxorubicin treatments. Transient siRNA-mediated depletion of GAR1 and dyskerin arrested cell proliferation, whereas loss of either NHP2 or NOP10 had no effect. Finally, loss of dyskerin, GAR1, NHP2, and NOP10, respectively, limited the accumulation of DNA damage biomarkers. However, the individual responses were dependent upon the specific type of damage incurred. In general, loss of GAR1 had the most suppressive effect on the biomarkers tested. Since the specific responses to genotoxic stress, the contribution of each protein to cell proliferation, and the activation of DNA damage biomarkers were not equivalent, this suggests the possibility that at least some of the proteins, most notably GAR1, may potentially function independently of their respective roles within H/ACA RNP complexes.

Keywords Dyskerin · GAR1 · Genotoxic stress · snoRNA · Cellular stress

Introduction

H/ACA ribonucleoproteins (RNPs) are composed of four evolutionarily conserved proteins, including DKC1 (dyskerin), NHP2, NOP10, and GAR1, and a function-specifying, noncoding H/ACA RNA (Meier 2006). Dyskerin, NHP2, and NOP10 form a core trimer that directly binds to H/ACA RNAs. The three proteins are interdependent upon each other for stability and also regulate stability of the bound RNAs (Grozdanov et al. 2009). GAR1 binds only to dyskerin and is needed for proper functioning of the H/ACA RNPs, but its absence does not reduce stability of the RNA (Darzacq et al. 2006).

After GAR1 binds, the fully matured H/ACA RNPs move from the nucleus to the nucleolus and Cajal bodies which are the sites where these complexes are thought to primarily function. There are over 350 unique H/ACA RNAs, including subsets of small nucleolar RNAs (snoRNA) and small Cajal body RNAs (Meier 2006; Jady et al. 2012). The majority of H/ACA RNAs have not yet been ascribed specific functions. The role of each RNP is dependent upon the RNA that it incorporates (Meier 2006). Thus, it is possible that H/ACA RNPs may regulate an array of important biologic processes.

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Among their known functions, H/ACA RNPs regulate posttranscriptional processing of nascent ribosomal RNA and spliceosomal RNA, respectively (Meier 2006). The four proteins are also components of the telomerase RNP. The trimer proteins bind to and stabilize telomerase RNA (TERC); TERC harbors an H/ACA RNA region at its 3′ end (Mitchell et al. 2003). Loss of function of any trimer protein reduces TERC stability and usually decreases telomerase activity; constitutive loss of function leads to excessive telomere attrition (Mitchell et al. 2003; Walne et al. 2007; Vulliamy et al. 2008). In contrast, GAR1 does not directly bind TERC, and its targeted depletion has no effect on TERC levels (Vulliamy et al. 2008). Thus, the role of GAR1 within the telomerase RNP remains unclear.

Of the four proteins, dyskerin has been the subject of the vast majority of studies reported in the literature. In general, rapidly proliferating cells express relatively high levels of dyskerin (Alawi and Lee 2007; Alawi and Lin 2011; Alawi et al. 2011). Dyskerin mRNA is upregulated in an array of cancer types, and high levels may be associated with poor prognosis (Sieron et al. 2009; Alawi et al. 2011; von Stedingk et al. 2013). Indolent tumors and slow-growing or arrested cells usually express lower levels of dyskerin, and loss of dyskerin function slows or arrests proliferation in most cell types (Alawi and Lee 2007; Gu et al. 2008; Alawi and Lin 2011; Lin et al. 2014). Through various loss-of-function approaches, emerging evidence suggests that dyskerin may regulate other cellular processes, including IRES-mediated translation, telomere maintenance independent of telomere length regulation, mitosis, transcription, and possibly microRNA processing (Yoon et al. 2006; Gu et al. 2008; Scott et al. 2009; Alawi and Lin 2010, 2013; Fong et al. 2014). It is unclear whether the observed effects were strictly related to decreased dyskerin function or whether they resulted from catastrophic disruption of H/ACA RNP function. Indeed, it is currently unknown whether any of the proteins can function independently of their presumed obligate binding partners. Moreover, apart from their roles in telomerase and H/ACA RNP biosynthesis and pseudouridination, very little is known about NHP2, NOP10, and GAR1.

As described herein, our findings suggest not only a differential expression of dyskerin, NHP2, NOP10, and GAR in normal and transformed cells, but also in response to specific types of cellular stress, notably DNA damage. We also observed a differential requirement for the proteins in cell proliferation and identified a possible reliance for these factors in regulation of specific DNA damage biomarkers. Together, our findings suggest the possibility that at least some of the H/ACA core proteins could potentially function independently of their respective roles within H/ACA RNP complexes.

Materials and methods

Cell culture

U2OS osteosarcoma cells (American Type Culture Collection, Manassas, VA) and HaCaT (immortalized skin keratinocytes) were grown in Dulbecco's minimal essential medium with GlutaMAX (Invitrogen, Carlsbad, CA) and 10 % fetal bovine serum. OKF6-TERT2 cells (TERT-immortalized primary oral keratinocytes) were cultured in keratinocyte serum-free media (Invitrogen) supplemented with growth factors as per the manufacturer's recommendations. The media were supplemented with 100 IU/mL penicillin and 100 IU/mL streptomycin (Invitrogen). Quality control was sporadically performed using the Mycoplasma Plus™ PCR Primer Set (Agilent Technologies, Santa Clara, CA).

siRNA and cDNA transfections

Custom-designed ON-TARGETplus SMARTPool siRNA duplexes targeting *DKC1*; pre-designed ON-TARGETplus SMARTPool siRNA targeting *GAR1*, *NHP2*, and *NOP10*; and a negative control, ON-TARGETplus nontargeting siRNA pool #2 were obtained from Dharmacon (Lafayette, CO). All transfections were performed using Lipofectamine 2000 (Invitrogen) as previously described (Lin et al. 2014).

Chemical reagents and antibodies

Aphidicolin, doxorubicin, rapamycin, chloroquine, trichostatin A, suberanilohydroxamic acid, etoposide, tunicamycin, MG132, nocodazole, and roscovitine were obtained from Sigma-Aldrich (St. Louis, MO). Thapsigargin (R&D Systems, Minneapolis, MN) was generously provided by Dr. Kelly Jordan-Sciutto. *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin B subunit was obtained from Dr. Bruce Shenker. Antibodies recognizing dyskerin and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GAR1 and NHP2 antibodies were obtained from Proteintech (Chicago, IL). NOP10, fibrillarin, and LC3B antibodies were from Abcam (Cambridge, MA). All other antibodies, including secondary antibodies, were obtained from Cell Signaling Technology (Danvers, MA).

Ultraviolet-C irradiation

A Stratalinker 2400 (Stratagene, La Jolla, CA) was used to irradiate the cells at 254 nm. The cells were first washed with PBS and then irradiated in PBS with various dosages.

The cells were then returned in media to the tissue culture incubator for the indicated times before analysis.

Protein extraction and immunoblotting

Protein extractions and immunoblots were performed as previously described (Alawi and Lin 2013). In most cases, the blots were stripped and re-probed with a different antibody.

Proliferation, cell cycle, and apoptosis assays

For the proliferation assay, U2OS cells were transfected with siRNAs in 4-well chamber slides. Fifty-four hours after transfection, 10 μM EdU was added to each well, and the cells were fixed with 4 % paraformaldehyde 16 h later. Analysis was performed using the Click-iT® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen) as per the manufacturer's protocol. As previously described (Lin et al. 2014), cell cycle and apoptosis analyses were performed 72 h after siRNA transfection. The Dead Cell Apoptosis Kit with Annexin V Alexa Fluor® 488 and Propidium Iodide (Invitrogen) was used as per the manufacturer's protocol. Data analysis was performed using FlowJo version 10 (Tree Star, Ashland, OR).

Indirect immunofluorescence and analysis

Cells were irradiated or treated with the indicated chemical agent in 4-well chamber slides. The cells were then permeabilized with 0.3 % Triton X-100 for 30 min, fixed with 4 % paraformaldehyde, permeabilized again with 0.3 % Triton X-100 for 1 h, and immunolabeled with the appropriate primary and secondary antibodies. For some experiments and where indicated, the cells were permeabilized for 1 h only after fixation. Coverslips were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen) and allowed to dry for at least 24 h before viewing. Cells were analyzed as previously described (Alawi and Lin 2013). All experiments were performed at least in triplicate.

RNA extraction and analysis

Total RNA was isolated using the miRNeasy kit (Qiagen, Valencia, CA). The miScript PCR system (Qiagen) was used for quantitative RT-PCR analysis on a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, California). All pre-designed Quantitect primers were obtained from Qiagen. For relative quantitation, the levels of the respective mRNAs were normalized to VIM. SnoRNA levels were normalized to the small nuclear RNA RNU5.

Relative quantitation and statistical analyses

NIH Image J was used to quantitate protein expression relative to the native protein or loading control. The "Cell Counting" function was used to quantitate EdU-positive and DAPI-positive nuclei, respectively, as described in Fig. 6. All statistical analyses were performed using Student's *t* test.

Results

Differential expression of dyskerin, GAR1, NHP2, and NOP10 mRNA and protein in cell lines

We began our studies by performing an in silico analysis of the relative mRNA expression levels of *DKC1*, *GAR1*, *NHP2*, and *NOP10* using six distinct human gene expression profile datasets available from BioGPS (Wu et al. 2009). Three datasets were comprised of primary human cells and the other three included transformed human cell lines. In general, there was mostly good correlation between DKC1, GAR1, and NHP2 mRNA expression in 5 of 6 datasets (Supp. Fig. S1). The strongest correlation was evident in the three primary cell datasets with more variability observed in the cancer cell line sets. In contrast, NOP10 levels exhibited no correlation to any of the other genes in the three cancer datasets and in two of the primary sample sets.

Differences in protein expression were also apparent in a limited number of cell lines and in response to specific growth conditions. In particular, all four proteins were markedly upregulated in the oral cancer cell line UM-SCC1 relative to OKF6-TERT2 cells which are TERT-immortalized primary oral keratinocytes (Fig. 1a). In contrast, the proteins were variably expressed in another oral cancer cell line FaDu and the MCF-7 breast carcinoma cell line. This reinforced the notion that these factors may be differentially expressed in cancer cells (Kim et al. 2012; von Stedingk et al. 2013). The proteins were expressed at low levels in normal B lymphoblasts (GM03798), with higher expression being noted in a B cell lymphoma cell line (P493-6). The c-MYC proto-oncogene is known to directly activate the expression of *DKC1*, *NHP2*, and *GAR1* (Schlosser et al. 2003; Alawi and Lee 2007; Wu et al. 2008). The levels of all four proteins were reduced in P493-6 cells lacking c-MYC relative to P493-6 cells expressing wild-type c-MYC levels. Thus, it is possible that c-MYC may regulate all four genes.

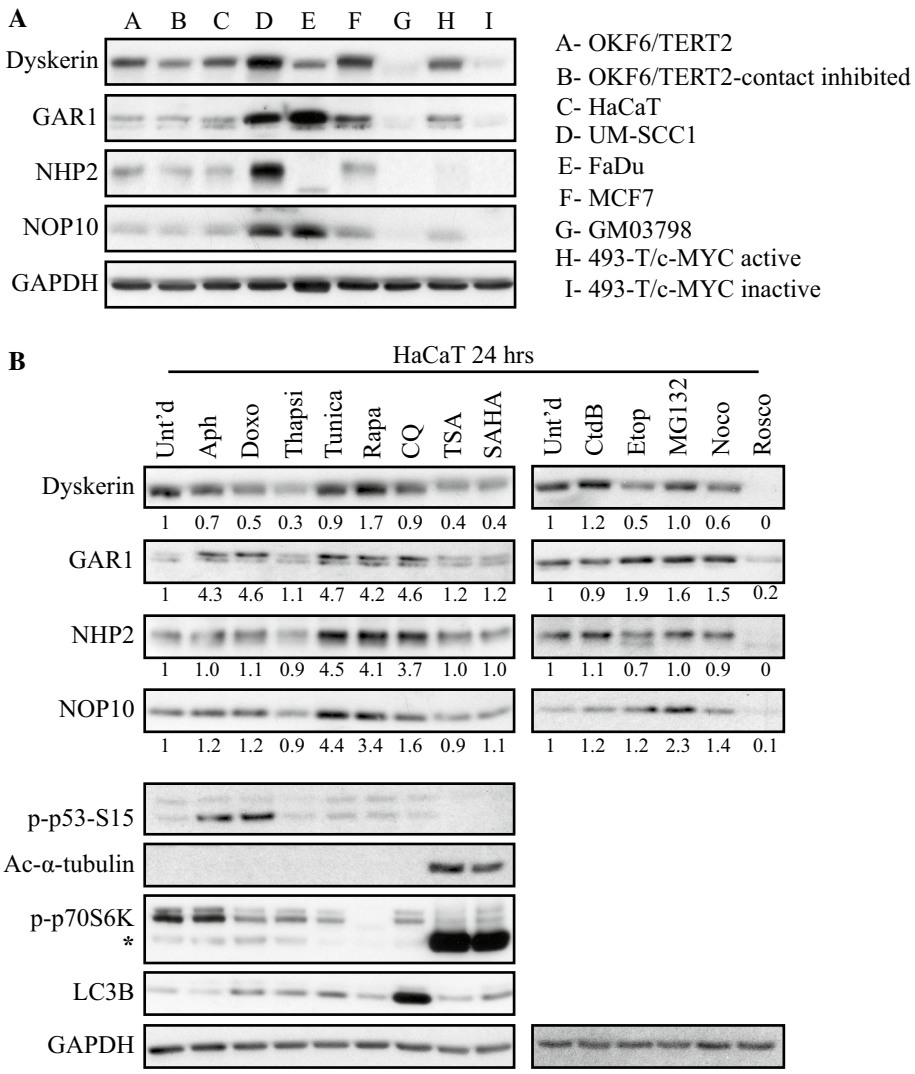

Fig. 1 H/ACA core proteins are variably expressed in human cell lines and after cellular stress. **a** Dyskerin, GAR1, NHP2, and NOP10 expressions were variably expressed in immortalized and transformed cell lines. The cell lines and their origins are listed on the right. **b** HaCaT cells were exposed to the chemicals listed and then harvested 16 h later. *APH* aphidicolin 10 μM, *Doxo* doxorubicin 0.5 μg/mL, *Thapsi* thapsigargin 0.5 μg/mL, *Tunica* tunicamycin 0.5 μg/mL, *Rapa* rapamycin 20 nM, *CQ* chloroquine 100 μM, *TSA* trichostatin A 1 μM, *SAHA* suberanilohydroxamic acid 2 μM, *Etop* etoposide 10 μM, *CtdB Aggregatibacter actinomyecetemcomitans* cytolethal distending toxin B subunit 0.225 μg/mL, MG132, 0.25 μM; *Noco* nocodazole 10 μM, *Rosco* roscovitine 50 μM. *p-p53-S15* phosphorylated p53-Serine 15, *Ac-α-tubulin* acetylated α-tubulin, *p-p70S6K* phosphorylated p70S6K. Expression of each of these markers was appropriate for the chemical treatment. p-p70S6K is suppressed following Rapa exposure. *denotes acetylated α-tubulin after re-probing the blot with anti-phospho-p70S6K

H/ACA RNP proteins exhibit a differential response to chemical stress

Dyskerin levels were reduced, whereas GAR1, NHP2, and NOP10 expressions remained unchanged after contact inhibition of OKF6-TERT2 cells (Fig. 1a, lane B vs. lane A). This led us to further examine the effects on expression of the proteins in response to various agents known to induce cell cycle arrest. Table 1 summarizes the data derived from HaCaT cells. Mostly similar findings were noted in other cell types including U2OS and OKF6-TERT2 cells (not shown). All agents tested triggered cell cycle arrest after 24 h of continuous exposure (not shown). There was no obvious correlation between changes in expression for any individual protein relative to the stage at which the arrest occurred. Actively proliferating cells have a high demand for protein synthesis and also typically exhibit relatively high levels of telomerase activity (Ruggero and Pandolfi 2003). However, there was also no overt relationship between the effects of these chemical agents on expression of the H/ACA proteins, and the reported effects of these agents on either telomerase or rRNA maturation. Of particular note, GAR1 was upregulated in response to all DNA-damaging agents tested, including aphidicolin (APH), doxorubicin (DOXO), and etoposide (Fig. 1b). In contrast, there was little or no effect on either NHP2 or NOP10, and dyskerin levels were consistently decreased relative to the untreated cells. In response to tunicamycin-induced endoplasmic reticulum stress, GAR1, NHP2, and NOP10 were markedly upregulated, whereas there was no effect on dyskerin. Conversely, dyskerin levels were reduced after treatment with thapsigargin with no appreciable effect on the other proteins. Thapsigargin induces endoplasmic reticulum stress through a mechanism distinct from tunicamycin (Ding et al. 2007).

Upregulation of GAR1, NHP2, and NOP10 also occurred after stimulatory (rapamycin) and inhibitory (chloroquine) modulation of autophagy. Dyskerin was only

Table 1 Effects of select agents on expression of the H/ACA core proteins

Agent	Well-recognized cellular effects	Reported effects on			Change in relative expression compared to untreated cells (*Down* regulated/*Up*regulated/*None*)				References
		Telomerase activity	rRNA synthesis	rRNA maturation	Dyskerin	GAR1	NHP2	NOP10	
UV-C	DNA DSBs and replication stress	Activate	Inhibit	Inhibit	Up	Up	Up	Up	Alfonso-De Matte et al. (2001), Calkins et al. (2013), Moore et al. (2013)
APH	DNA replication stress	None	Inhibit	None	Down	Up	None	None	Holt et al. (1997), Dimitrova (2011)
DOXO	DNA DSBs	Inhibit	Inhibit	None	Down	Up	None	None	Elmore et al. (2002), Burger et al. (2010)
ETOP	DNA DSBs	Activate	None	Inhibit	Down	Up	None	None	Jeyapalan et al. (2004), Burger et al. (2010)
Thapsi	ER stress	Inhibit	Inhibit	None	Down	None	None	Down	Rosenberger et al. (2007), Okamoto et al. (2010)
Tunica	ER stress	Inhibit	Inhibit	None	None	Up	Up	Up	Woo et al. (2009), Okamoto et al. (2010)
RAPA	Autophagy induction	Inhibit	Inhibit	Inhibit	Up	Up	Up	Up	Zhou et al. (2003), Mayer and Grummt (2006)
CQ	Autophagy inhibition	Unknown	Unknown	Unknown	None	Up	Up	None	
TSA	Chromatin relaxation	Activate (normal cells)	None	None	Down	None	None	None	Cong and Bacchetti (2000), Burger et al. (2010)
SAHA	Chromatin relaxation	Inhibit (transformed cells	None	None	Down	None	None	None	Burger et al. (2010), Li et al. (2011a, 2011b)

UV-C ultraviolet-C irradiation, *APH* aphidicolin, *DOXO* doxorubicin, *ETOP* etoposide, *Thapsi* thapsigargin, *Tunica* tunicamycin, *RAPA* rapamycin, *CQ* chloroquine, *TSA* trichostatin A, *SAHA* suberanilohydroxamic acid

modestly upregulated by rapamycin and showed no change after chloroquine treatment. Two different but functionally similar histone deacetylase inhibitors, trichostatin A and suberanilohydroxamic acid, reduced dyskerin expression by more than 50 % but did not affect the other proteins. NOP10 and GAR1 levels were increased following MG132-induced proteasome inhibition with no effect on the other proteins. Nocodazole, a microtubule depolymerizer, reduced only dyskerin expression. A bacterial cytolethal distending toxin had no effect on any of the proteins. Finally, the cyclin-dependent kinase inhibitor roscovitine completely attenuated the expression of all four proteins. This suggests that they may be phosphoproteins, and that phosphorylation by cyclin-dependent kinases contributes to their stability. Together, this suggests that the four proteins may be differentially regulated by cell stress-specific mechanisms, and that proliferative arrest alone does not sufficiently explain the effects on the proteins.

Upregulation of the four proteins in response to ultraviolet-C irradiation

Telomerase-negative U2OS osteosarcoma cells and two distinct telomerase-positive keratinocyte cell lines (HaCaT and OKF6-TERT2) were irradiated with increasing dosages of ultraviolet-C (UV-C). The cells were harvested 2 h after irradiation and in the absence of any measurable cell cycle arrest. U2OS cells lack TERC and telomerase reverse transcriptase (Alawi and Lin 2011). Thus, we postulated that any observed effects on the H/ACA core proteins would not be dependent on either their interactions with TERC or require the presence of functional telomerase activity.

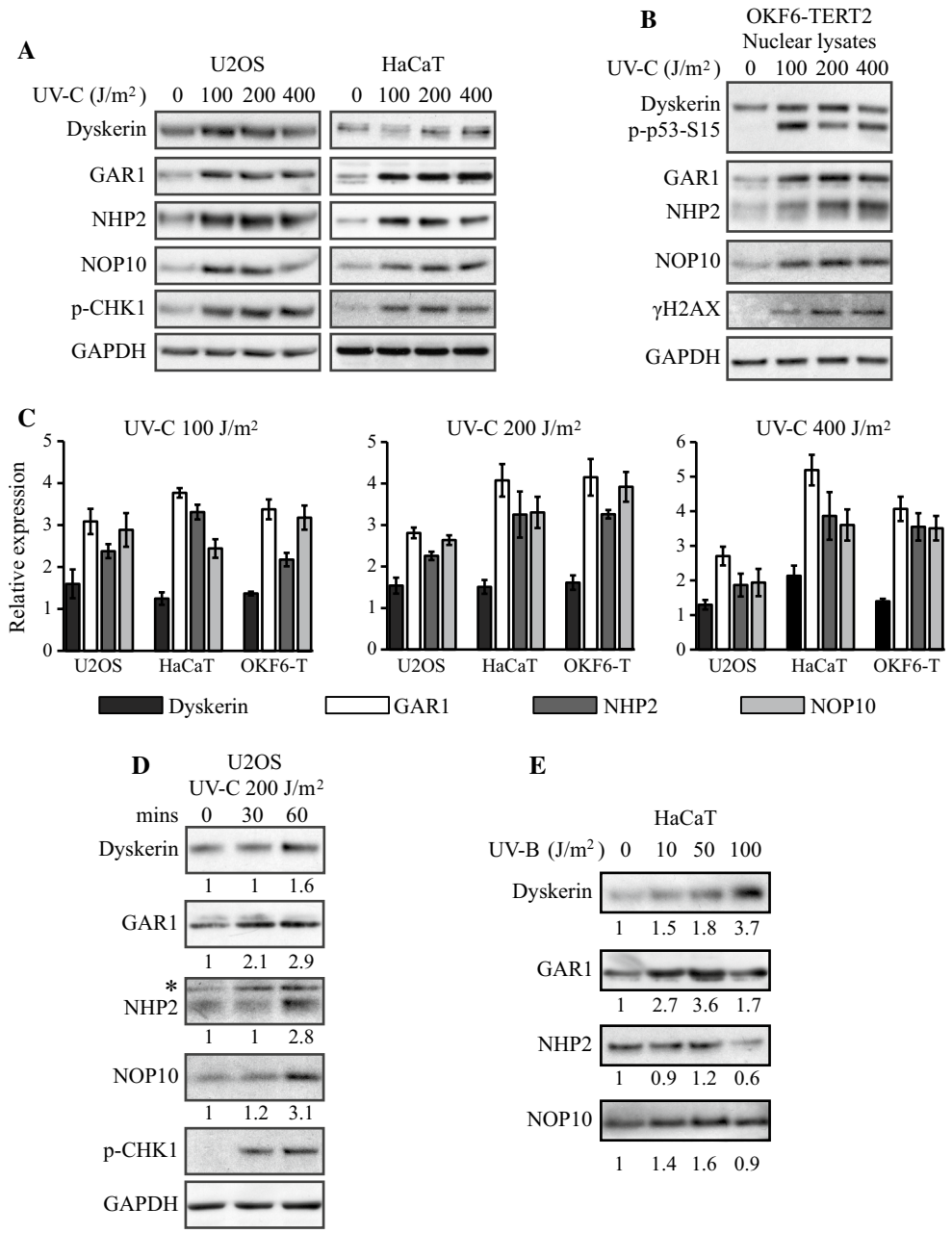

Fig. 2 Expression of H/ACA proteins after UV-C irradiation. **a** U2OS and HaCaT cells irradiated with UV-C at the indicated dosages and whole cell lysates were harvested 2 h later. **b** OKF6-TERT2 cells were similarly irradiated, and nuclear lysates were extracted 2 h later. **c** Relative expression of the four proteins (in relation to GAPDH) after irradiation in each cell line and at each dosage. Expression was normalized to the ratio of each protein to GAPDH in the untreated cells. *Error bars* denote standard deviations from three independent experiments. **d** HaCaT cells were irradiated with 200 J/m^2 and harvested 30 or 60 min later. **e** HaCaT cells were exposed to various dosages of UV-B and harvested 2 h later

Indeed, similar effects were seen irrespective of cellular telomerase status.

Markers indicative of activated DNA damage responses (DDR) to replication stress (phosphorylated CHK1^{Ser345}; p-CHK1) and DNA double-strand breaks (phosphorylated histone H2AX^{Ser139}; γH2AX) (Ciccia and Elledge 2010) were upregulated after UV-C exposure (Fig. 2a, b). Irrespective of the cell line and dosages, exposure to UV-C also led to a significant increase (>twofold) in expression of GAR1, NHP2, and NOP10 relative to their respective levels in untreated cells (Fig. 2a–c). In contrast, dyskerin expression was only increased between 1.3- and twofold over control cells. There was no effect on mRNA levels (not shown) suggesting upregulation

of the proteins occurred via unknown posttranslational mechanisms.

Under most conditions, GAR1 levels usually showed the greatest increase (≈three- to fivefold) relative to its expression in untreated cells. Moreover, GAR1 was upregulated twofold only 30 min after UV-C exposure, whereas the other proteins showed increases 60 min after irradiation (Fig. 2d). GAR1 also showed the greatest increase in expression after low (10 J/m^2) and moderately intense (50 J/m^2) exposures to UV-B. This indicated a differential response of the proteins, particularly GAR1, to UV irradiation.

Cellular localization of GAR1 is altered following exposure to specific genotoxic agents

All four proteins are primarily found within the nucleolus which is where rRNA processing occurs (Meier 2006). Unexpectedly, following exposure to DOXO (Fig. 3) and UV-C (Fig. 4 and Supp. Fig. S2), GAR1 translocated from the nucleolus to the nucleoplasm. None of the other proteins exhibited a similar change in localization. Fibrillarin, which is another protein implicated in posttranscriptional rRNA processing (Meier 2006), was retained within the nucleolus after both treatments (Fig. 3b). We note that when the cells were fixed before permeabilization with Triton X-100, GAR1 immunostaining was not observed within the nucleolus. However, when the cells were permeabilized both before and after fixation, GAR1 was observed within the nucleolus and nucleoplasm after DOXO. This latter technique was required to optimize the double-labeling of the cells with the GAR1 and fibrillarin antibodies.

To ensure the nucleoplasmic localization of GAR1 was not artifactual, we transiently expressed GFP-GAR1 into U2OS cells and then treated the cells with either DOXO or UV-C. GFP-GAR1 was localized exclusively to the nucleolus in untreated cells but was found in the nucleolus and nucleoplasm after treatment (Supp. Fig. S3). In contrast, GFP-dyskerin was retained within the nucleolus. APH had no effect on GFP-GAR1 localization (not shown).

While NHP2 and dyskerin remained nucleolar after DOXO treatment, both proteins were primarily confined to nucleolar cap structures around the periphery of the nucleolus (Fig. 3a and Supp. Fig. S4). GAR also co-localized with fibrillarin in nucleolar cap structures (Fig. 3b). This beaded staining pattern is similar to that reported for other nucleolar proteins after DOXO treatment (Burger et al. 2010). Ultrastructural studies will be needed to determine whether dyskerin, NHP2, and GAR1 interact together and/or with other nucleolar proteins within nucleolar caps following DOXO exposure. While NOP10 remained primarily within the nucleolus, we also observed a cytoplasmic membranous staining pattern after DOXO and UV-C treatments. This unusual pattern was consistently observed over the course of several experiments. We were unable to reliably observe nucleolar localization of either ectopic GFP-NOP10 or GFP-NHP2 after transfection. Thus, we could not confirm whether the membranous localization of NOP10 after DNA damage was either real or artifactual. APH and rapamycin did not appreciably alter localization of the proteins, including GAR1.

H/ACA RNP proteins regulate stability of other subunits within the complex

Several studies examining the cellular effects of dyskerin depletion have been reported in the literature (Sieron et al. 2009; Montanaro et al. 2010; Alawi and Lin 2011; von Stedingk et al. 2013). However, the effects of targeted depletion of GAR1, NHP2, and NOP10 are poorly characterized. After 72 h of siRNA-mediated knockdown in U2OS cells, the effects on protein, mRNA, and H/ACA snoRNA expression were assessed. As expected, targeted depletion of either dyskerin (siDKC1), NHP2 (siNHP2), or NOP10 (siNOP10), respectively, resulted in a 50–90 % reduction in levels of the other two components relative to their expression in control siRNA (siCTRL) transfected cells (Fig. 5a). There was no effect on GAR1 expression. Depletion of any trimer protein was also accompanied by a greater than 50 % decrease in H/ACA snoRNA levels (Fig. 5b). In contrast, GAR1 depletion (siGAR1) increased levels (>twofold) of dyskerin, NHP2, and NOP10 but had no effect on H/ACA snoRNAs. Transfection of any one siRNA reagent did not affect the mRNA levels of the other three genes (Fig. 5c). Thus, loss of GAR1 enhanced the stability of the trimer proteins. To our knowledge, this observation has not been previously reported. Levels of RUVBL1 and HSP90, both of which regulate H/ACA RNP biogenesis (Boulon et al. 2012), and NPM, a multi-functional nucleolar protein with no known links to H/ACA RNPs (Alawi and Lin 2013), were unaffected by depletion of the four H/ACA proteins.

Loss of dyskerin and GAR1 function arrests cell proliferation

As previously reported (Lin et al. 2014), dyskerin knockdown induced the proliferative arrest of U2OS cells as measured by a significant decrease in the incorporation of EdU ($p < 0.0001$) relative to siCTRL (Fig. 6a, b). GAR1 depletion also resulted in a significant decrease in EdU incorporation ($p < 0.0001$) comparable to that of dyskerin knockdown. Thus, loss of GAR1 induced cell cycle arrest through a mechanism that did not depend on reduced dyskerin expression, and vice versa. Surprisingly, NHP2 and NOP10 depletion did not trigger cell cycle arrest. Yet dyskerin expression was reduced under both conditions, albeit

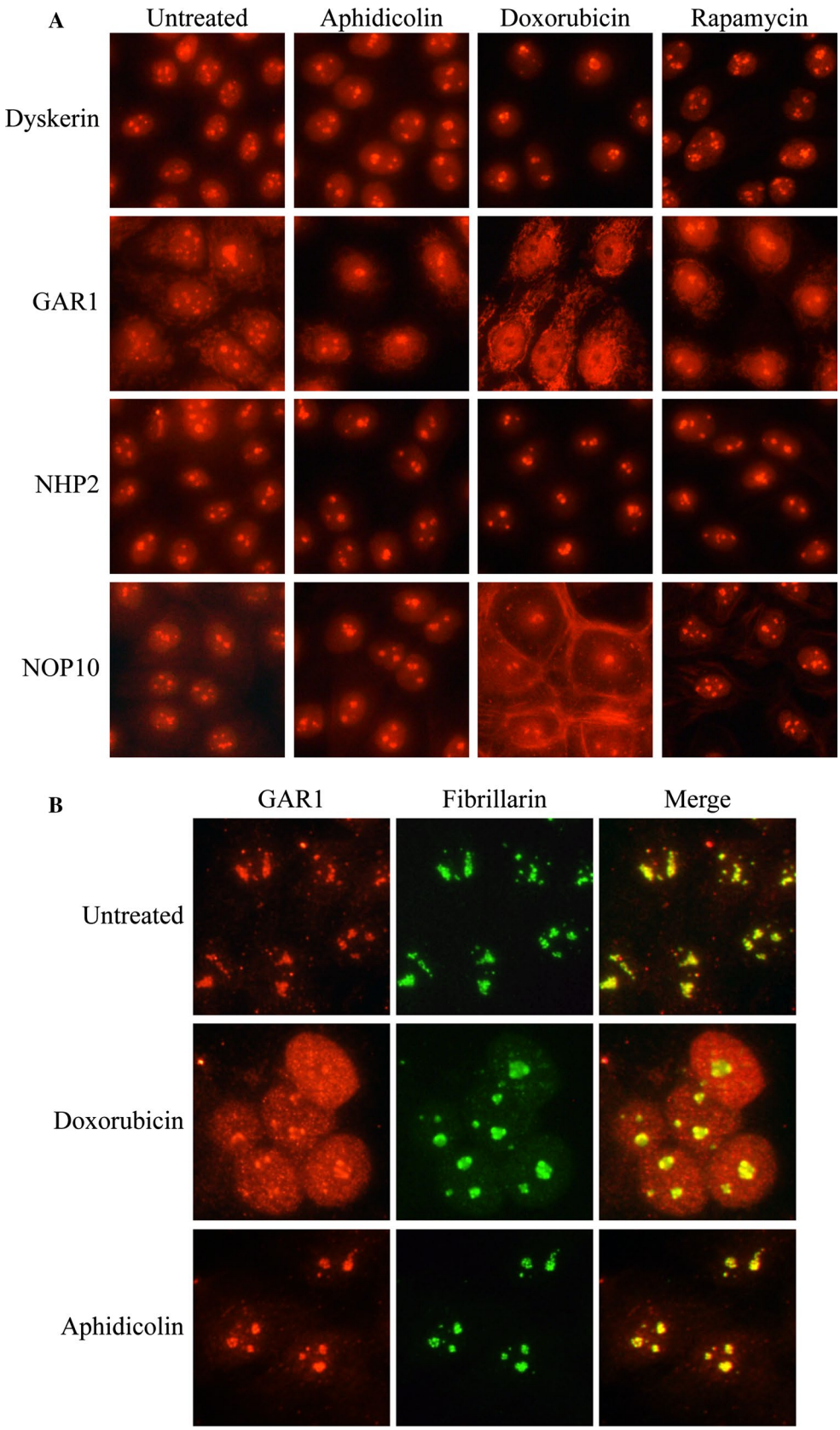

Fig. 3 GAR1 localizes to the nucleoplasm after exposure to DOXO. **a** OKF6-TERT2 cells were treated for 16 h with the chemicals shown. The cells were then fixed without pre-permeabilization. DOXO triggered GAR1 localization to the nucleoplasm, whereas dyskerin, NHP2, and NOP10 remained confined to nucleolar cap structures. NOP10 also showed a membranous pattern similar to that triggered by UV-C irradiation. There were no appreciable changes in nucleolar localization for any of the proteins after APH and RAPA treatments. In some experiments, there appeared to be GAR1 localization within the cytoplasm after damage. However, untreated cells also showed similar cytoplasmic staining. Thus, it is likely that this simply represented background staining associated with the particular antibody used. **b** HaCaT cells were treated with DOXO for 2 h. The cells were then permeabilized with 0.3 % Triton X-100 for 30 min prior to fixation and then permeabilized again for 1 h with 0.3 % Triton X-100 followed by immunolabeling. GAR1 was dispersed throughout the nucleoplasm but also co-localized with fibrillarin in nucleolar cap structures following treatment

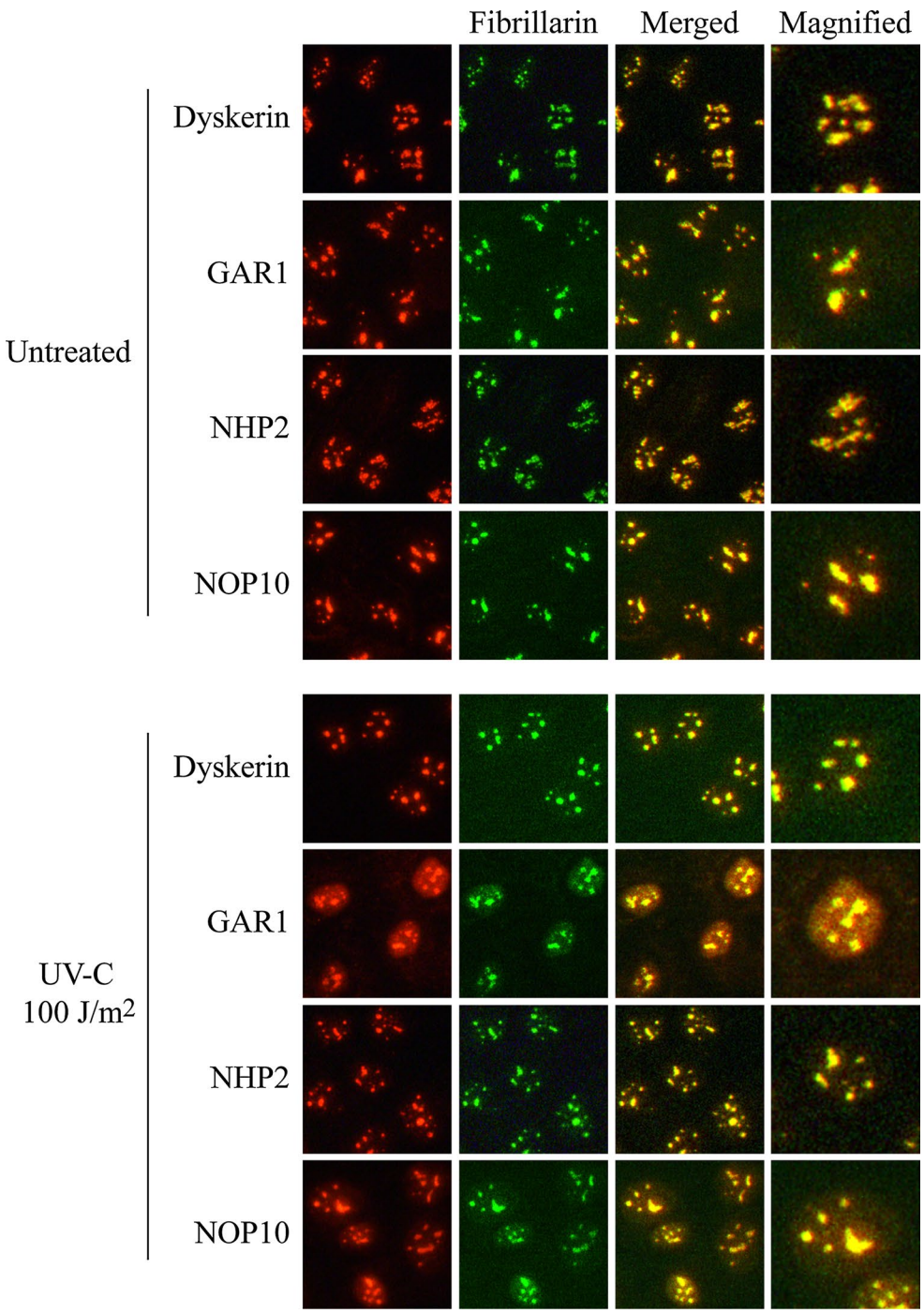

Fig. 4 GAR1 localizes to the nucleoplasm after exposure to UV-C. HaCaT cells were irradiated with 200 J/m^2. Two hours later, the cells were permeabilized with 0.3 % Triton X-100 prior to fixation and then immunolabeled as indicated. GAR1 was localized in the nucleolus within untreated cells and in the nucleolus and nucleoplasm after exposure. Fibrillarin remained confined to the nucleolus. Supp. Fig. S2 shows GAR1 nucleoplasmic localization following UV-C irradiation in cells that were fixed without pre-permeabilization

not as profoundly as when dyskerin was directly targeted for depletion. This suggests that either there may be a threshold below which loss of dyskerin function triggers cell cycle arrest or that dyskerin knockdown inhibits proliferation by dysregulating either GAR1 or other unknown factors. This will require further investigation. Flow cytometric analyses revealed mostly similar cell cycle profiles after depletion of the four proteins without evidence of a robust cellular accumulation within any specific stage (Fig. 6c). Depletion of the H/ACA proteins did not induce apoptosis (Fig. 6d).

Differential effects of H/ACA proteins on the DNA damage response

Although the four proteins exhibited different responses to chemical stress, their response to genotoxic stress was particularly interesting. Thus, we analyzed the effects of

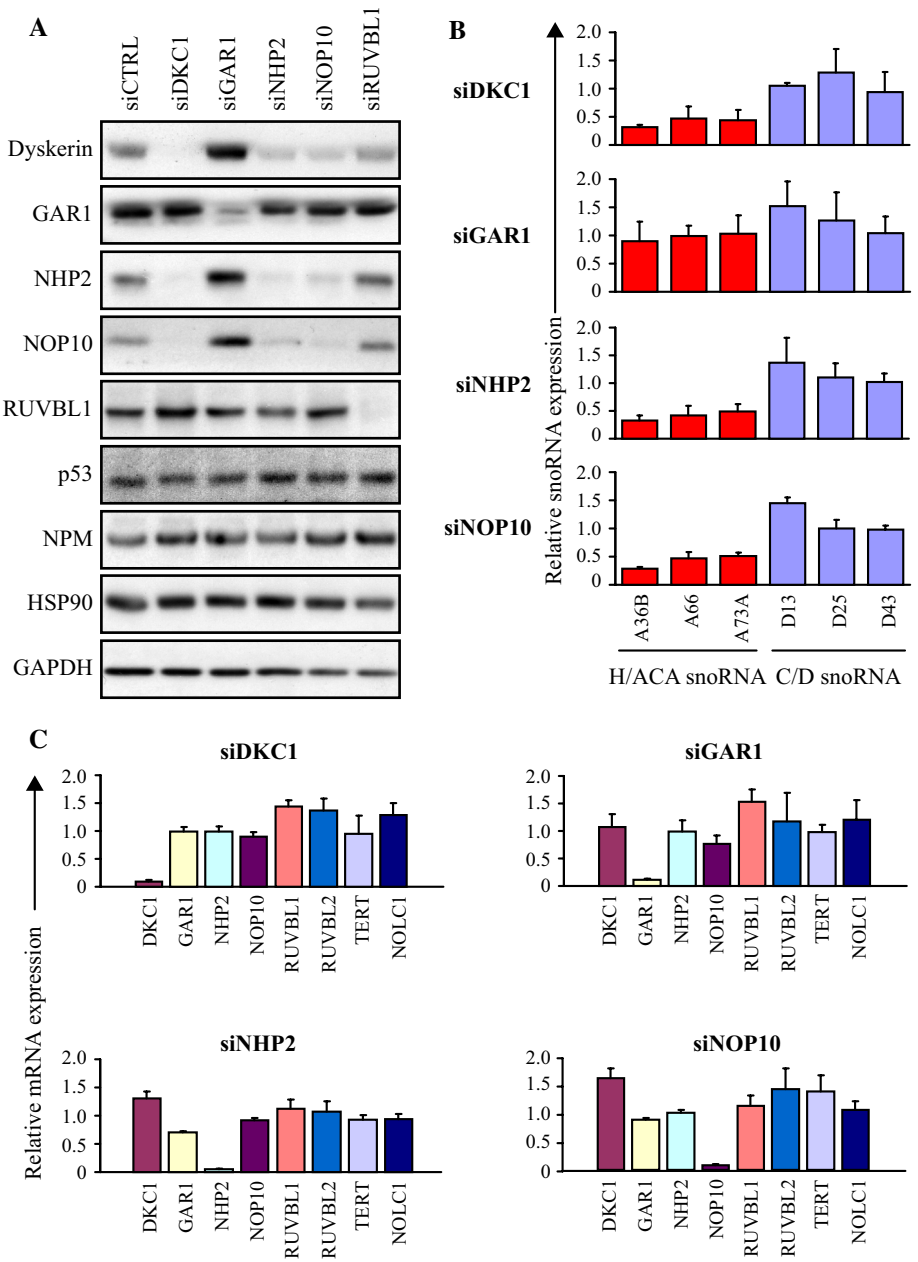

Fig. 5 Effects of siRNA-mediated knockdown on protein, snoRNA, and mRNA expression. **a** Immunoblot depicting protein expression 72 h after siRNA-mediated knockdown in U2OS cells. Loss of dyskerin, NHP2, and NOP10 expressions reduced the levels of its other two binding partners. GAR1 depletion stabilized the trimer proteins. RUVBL1 regulates H/ACA RNP biogenesis and is known to stabilize dyskerin. Loss of RUVBL1 expression (siRUVBL1) reduced dyskerin expression but had no apparent effect on the other proteins. **b** Loss of dyskerin, NHP2, and NOP10 expressions reduced the expression of three distinct H/ACA snoRNAs (ACA36B, ACA66, and ACA73A) by at least 50 % relative to the siCTRL cells. GAR1 depletion had no effect on H/ACA snoRNAs. C/D snoRNAs which are regulated by another macromolecular complex distinct from H/ACA RNPs were either unaffected or slightly increased after depletion of the H/ACA core proteins, including GAR1. Relative expression was first normalized to RNU5 expression and then tabulated as a measure of the snoRNA/RNU5 ratio in siCTRL cells. **c** Depletion of any one H/ACA mRNA did not reduce the expression of the other H/ACA genes or other factors that either regulate H/ACA RNPs (RUVBL1, RUVBL2), associate with H/ACA RNPs (TERT) or have no known association to H/ACA RNPs (NOLC1). *Error bars* denote standard deviations from at least five independent transfections

their depletion on the DDR, as measured by the accumulation of γH2AX, p-CHK1, and/or phosphorylated CHK-2^{Thr68} (p-CHK2). p-CHK2 is activated in response to double-strand breaks. For these experiments, we knocked down of each of the four proteins in U2OS cells for 72 h and then exposed the cells to APH, DOXO, or UV-C. APH

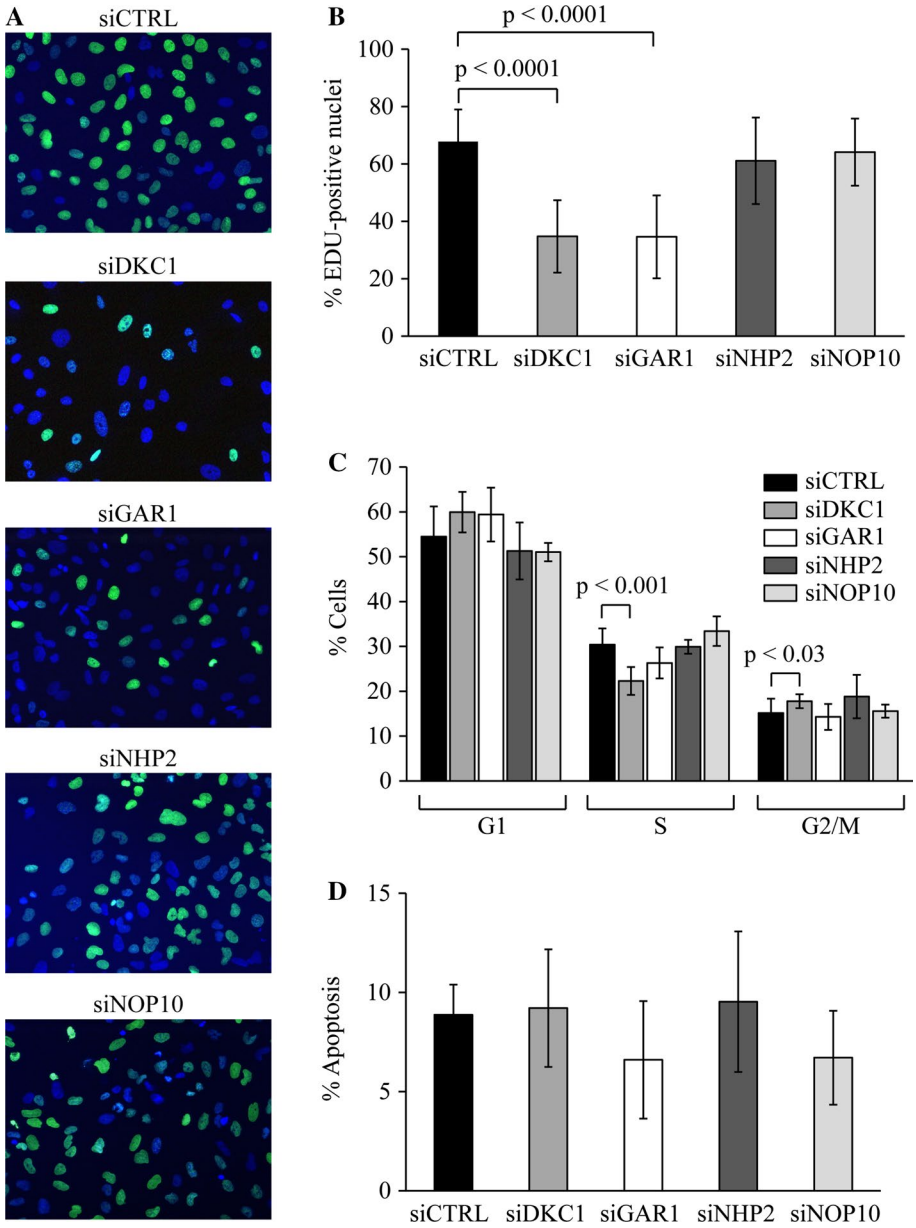

Fig. 6 Dyskerin and GAR1 are required for cell proliferation. **a** U2OS cells plated in 4-well chamber slides were transfected with siRNAs and analyzed by indirect immunofluorescence 72 h later. Sixteen hours before fixation and DAPI labeling, 10 μM EdU was added to the cells. Representative images are shown illustrating EdU incorporation following siRNA-mediated knockdown. EdU-positive nuclei are in *green*, and DAPI-positive nuclei are *blue*; the *green* and *blue* channels were overlayed. **b** For quantitation, at least 900 nuclei were counted per condition over five independent transfections. Compared to siCTRL cells, loss of dyskerin and GAR1, respectively, resulted in a significantly reduced percentage of EdU-positive nuclei/DAPI-positive nuclei counted (siCTRL = 67.8 ± 11.2 % vs. siDKC1 = 34.8 ± 12.6 %, $p < 0.0001$; vs. siGAR1 = 34.6 ± 14.5 %, $p < 0.0001$). In contrast, loss of NHP2 and NOP10 showed no significant decrease in EdU incorporation. **c** In parallel, cells were labeled with propridium iodide and analyzed by flow cytometry. As previously reported, there was a small but significant increase in the proportion of dyskerin-depleted cells in G_2/M ($p < 0.03$). However, the cell cycle profiles were essentially similar in all cases. **d** Cells were labeled with Annexin V Alexa Fluor 488 and propridium iodide and analyzed by flow cytometry. Depletion of the H/ACA core proteins did not induce spontaneous apoptosis. *Error bars* in **c** and **d** denote standard deviations from at least seven independent transfections

induces DNA replication stress, DOXO induces DNA double-strand breaks, and UV-C triggers replication stress and double-strand breaks (Zhang et al. 2007; Elmore et al. 2002).

After a 2-h DOXO treatment, depletion of each individual protein variably reduced the levels of p-CHK2 and γH2AX relative to siCTRL cells (Fig. 7a). Intriguingly, targeted dyskerin depletion reproducibly showed a

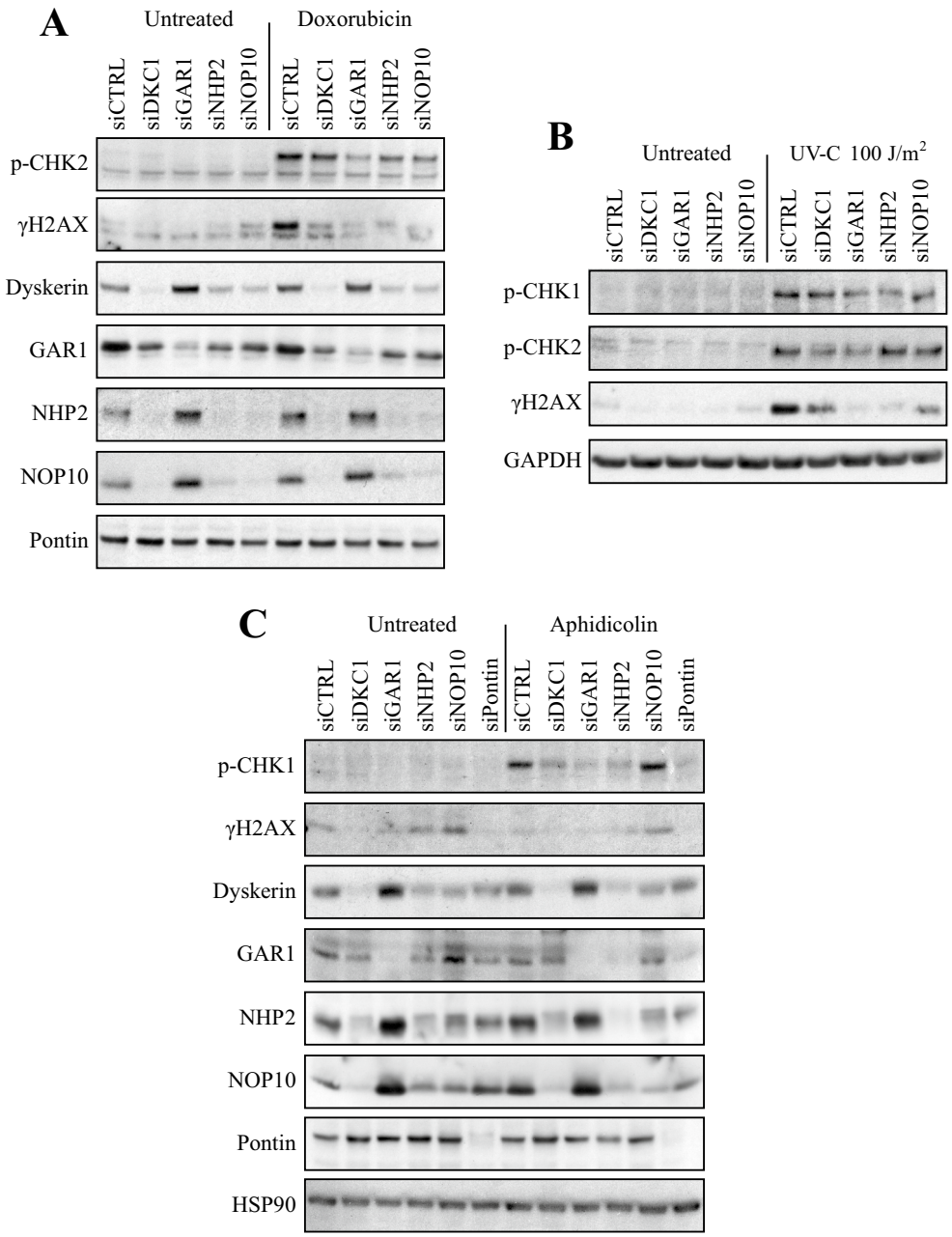

Fig. 7 Loss of H/ACA core proteins suppresses accumulation of activated DNA damage biomarkers. U2OS cells were transfected with siRNAs. 72 h later, the cells were treated with the indicated agent and harvested after 2 h. **a** Loss of the four proteins attenuated the accumulation of p-CHK2 and γH2AX after DOXO treatment. **b** The markers were broadly suppressed in siGAR1 cells following UV-C irradiation. p-CHK2 accumulation was only attenuated in siDKC1 and siGAR1 cells. **c** Loss of dyskerin, GAR1, and NHP2 markedly suppressed p-CHK1 after a 2-h APH treatment. NOP10 had no appreciable effect. Loss of RUVBL1 is known to suppress p-CHK1 after damage and was used as a positive control for this experiment. APH does not induce DNA DSBs. Accordingly, there was no effect on γH2AX

lesser inhibitory effect on p-CHK2 expression compared to its binding partners. However, a time course experiment using only siDKC1 cells showed significant inhibition of p-CHK2 activation with continued DOXO exposure (Fig. 8a).

Following UV-C exposure, siGAR1 and siNHP2 cells exhibited a more pronounced decrease in γH2AX and p-CHK1 levels compared to siDKC1 and siNOP10 cells (Fig. 7b). In contrast, p-CHK2 levels were similarly reduced in siDKC1 and siGAR1 cells relative to siCTRL cells but were unchanged after NHP2 and NOP10 depletion. A time course experiment using only UV-C treated siGAR1 cells confirmed the inhibitory effects of GAR1 depletion on the DDR biomarkers (Fig. 8b).

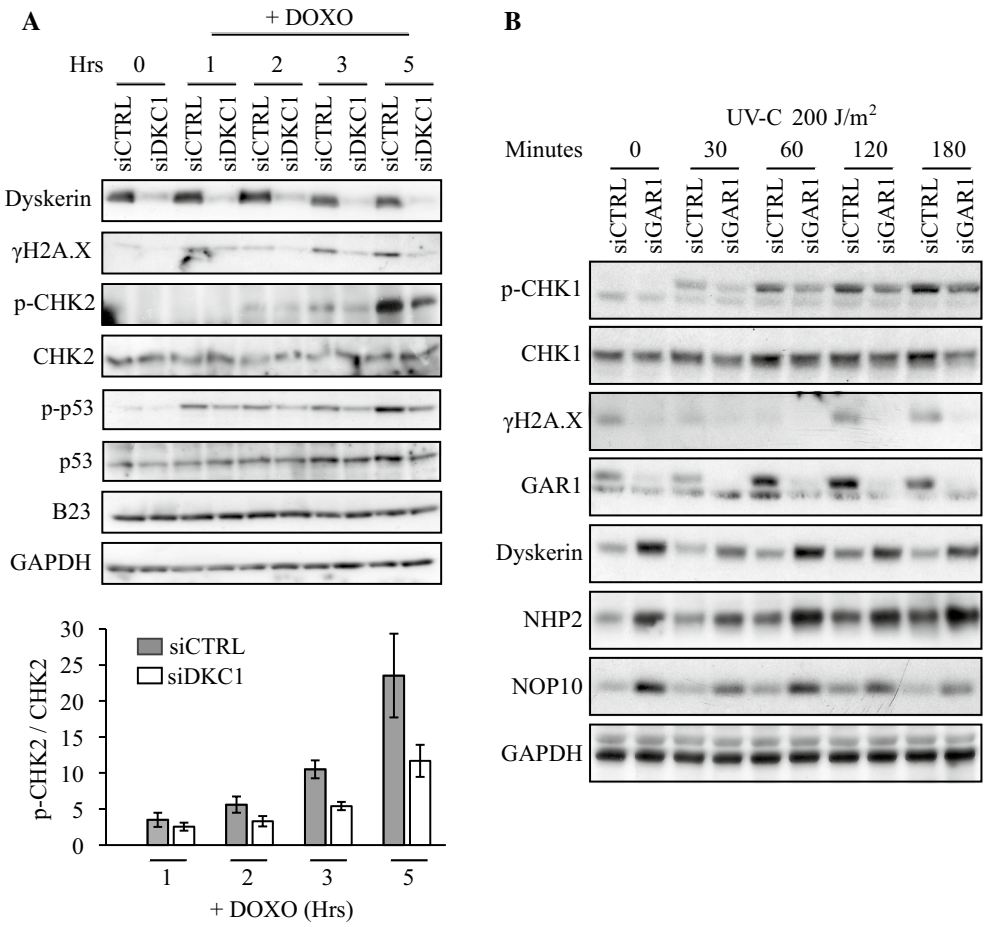

Fig. 8 Loss of GAR1 or dyskerin attenuates accumulation of DDR biomarkers after specific genotoxic stress. **a** siGAR1 cells were exposed to UV-C 200 J/m^2 and then harvested at the indicated times. p-CHK1 and γH2AX were suppressed in the absence of GAR1. **b** siDKC1 cells were continuously exposed to DOXO for the indicated times. γH2AX and DNA damage-specific phosphorylated form of p53 (p-p53^{Ser15}) were attenuated in the absence of dyskerin. p-CHK2 levels were significantly decreased in the siDKC1 cells relative to siCTRL cells especially after longer exposure. Native CHK2 expression remained constant. The relative p-CHK2/CHK2 ratios at each time point relative to untreated cells are illustrated in the graph

Accumulation of p-CHK1 in response to a 2-h APH exposure was markedly suppressed in the absence of dyskerin, GAR1, and NHP2 (Fig. 7c). Surprisingly, NOP10 depletion did not attenuate p-CHK1 expression. Together, these data suggest that irrespective of the physiologic responses of these proteins to genotoxic stress, they may each participate in regulation of DDR biomarkers but through mechanisms that may be dependent upon the specific type of damage incurred. The mechanisms by which this regulation occurs remain to be established. In general, loss of GAR1 elicited the most suppressive effect on these DDR biomarkers.

Discussion

One model of H/ACA RNP biogenesis suggests that GAR1 binds directly to dyskerin to form the mature RNP, but only after dyskerin is bound to NHP2 and NOP10 and after the H/ACA RNAs are incorporated and properly processed (Wang and Meier 2004). In other studies, dyskerin, GAR1, and NOP10 were found to form a complex independently of NHP2 and RNA (Rashid et al. 2006; Li et al. 2011a, b). In vitro, dyskerin and GAR1 can also directly interact independently of the other components (Wang and Meier 2004). If the H/ACA proteins or a subset of the proteins are always bound together with or without RNA, it is possible that other factors may influence their respective roles within specific biologic processes. To that end, during the course of H/ACA RNP maturation, dyskerin binds to several other proteins, including RUVBL1, RUVBL2, SHQ1, and NAF1; GAR1 replaces NAF1 in the final maturation step (Darzacq et al. 2006; Grozdanov et al. 2009). Dyskerin was also recently shown to bind to proteins that are not known to be implicated in H/ACA RNP synthesis or

maturation (Jobert et al. 2013). Similarly, GAR1 has other known binding partners, including factors implicated in the biogenesis of other macromolecular complexes (Pellizzoni et al. 2001). However, it remains unknown whether either dyskerin or GAR1 can independently bind to these other proteins or whether binding occurs in the context of H/ACA RNPs. Whether interactions with these or other unknown binding partners can help explain the observations described herein will require further investigation.

Of the four H/ACA proteins, analysis of GAR1 yielded the most unique results. Apart from its contribution to H/ACA RNP biogenesis and regulation of pseudouridination, very little is known about GAR1. GAR1 belongs to a class of proteins characterized by glycine–arginine-rich (RGG) domains (Thandapani et al. 2013). RGG motif proteins regulate an array of biologic processes including DNA damage signaling, pre-mRNA splicing, and transcription (Thandapani et al. 2013). Interestingly, germ line mutations in *DKC1*, *NHP2*, and *NOP10* have been implicated in the pathogenesis of dyskeratosis congenita, a multi-system disorder primarily associated with premature telomere attrition (Mitchell et al. 2003; Walne et al. 2007; Vulliamy et al. 2008; Kirwan et al. 2011). In contrast, GAR1 mutations have not been identified, and loss of GAR1 does not affect TERC stability (Vulliamy et al. 2008).

In our studies, GAR1 expression was upregulated following all types of genotoxic stress tested, and it showed the most robust response to UV irradiation. GAR1 was also the only protein to show an altered subcellular localization following DOXO and UV-C. We cannot exclude the possibility that one or more of the other proteins also translocated to the nucleoplasm after damage, and that we were not able to identify this using our antibodies. However, GFP-dyskerin remained in the nucleolus after damage. Similarly, Moore et al. (2011) reported that ectopic tagged dyskerin remained within the nucleolus after exposure to ultraviolet and ionizing radiation. To our knowledge, cellular localization of NHP2 and NOP10 after chemical stress has not been previously reported.

Loss of GAR1 also arrested cell proliferation and, irrespective of the genotoxin, suppressed the accumulation of DDR biomarkers via an unknown mechanism that did not depend on reduced expression of the other H/ACA proteins. Indeed, loss of GAR1 actually increased dyskerin, NHP2, and NOP10 levels in untreated cells. As these studies were performed in U2OS cells, it can be concluded that the role of GAR1 in proliferation and in regulation of the DDR biomarkers is telomerase-independent. Moreover, in light of the response of endogenous GAR1 to DNA damage, it is unlikely that the observed effects of GAR1 depletion on the DDR biomarkers were simply a consequence of cell cycle arrest or off-target effects of the siRNA. Although H/ACA snoRNA levels were unaffected by GAR1 depletion, GAR1 is thought to be necessary for proper functioning of H/ACA RNPs (Meier 2006). Nonetheless, our findings lead us to speculate that GAR1 may have functions that are independent of its role within H/ACA RNPs.

We previously reported that dyskerin depletion arrested cell proliferation independently of telomerase and with only a transient disruption of rRNA processing despite a significant decrease in H/ACA snoRNA expression (Alawi and Lin 2011). In the current study, while knockdown of dyskerin, NHP2, and NOP10 similarly reduced H/ACA snoRNA levels, only targeted dyskerin depletion triggered a proliferative arrest. This suggests the possibility that dyskerin may also regulate other factors or biologic processes that could potentially be uncoupled from its function with NHP2 and NOP10 in H/ACA RNPs. This is further supported by the observation that except following UV-C, dyskerin levels were reduced after exposure to genotoxic stress without appreciable downregulation of either NHP2 or NOP10. This suggests dyskerin levels may have to drop below a certain threshold to destabilize NHP2 and NOP10, or that the interdependency is not stringent.

Irrespective of the genotoxic stressor, dyskerin and NHP2 depletion decreased accumulation of the DDR biomarkers; this occurred independently of reduced GAR1 expression. Yet, the suppressive effect on the biomarkers was not typically as robust as after GAR1 depletion. While NOP10 depletion also suppressed the biomarkers after UV-C and DOXO treatments, its loss of function had no appreciable effect on p-CHK1 following APH. We typically achieved a 90–95 % decrease in dyskerin expression following its targeted depletion. Targeted NHP2 knockdown reduced NHP2 expression by at least 90 % in most experiments. In contrast, NOP10 depletion concomitantly reduced dyskerin and NHP2 levels to approximately 75 % of control cells. Thus, dyskerin and NHP2 levels may not have been sufficiently reduced following NOP10 depletion to impair APH-induced p-CHK1. Alternatively, it is possible that other factors associate with the H/ACA core proteins, either individually or collectively, following specific types of DNA damage, and that these interactions are needed for the H/ACA proteins to exert their respective influences on the DDR.

The DDR is a conserved signal transduction cascade whose activation is critical for the maintenance of genomic integrity (Ciccia and Elledge 2010). Inactivation of factors required for the DDR typically results in hypersensitivity to genotoxic stress; damaged cells undergo apoptosis at a greater rate than control cells (Ciccia and Elledge 2010). Yet, loss of dyskerin promotes cell survival in response to DNA damage (Bellodi et al. 2010; Montanaro et al. 2010; Lin et al. 2014). Loss of dyskerin function, including in primary human cells harboring germ line *DKC1* mutations, was shown to impair p53 activation in response to

genotoxic stress (Bellodi et al. 2010; Montanaro et al. 2010; Kirwan et al. 2011; Lin et al. 2014). p53 is a DNA damage-responsive factor. We previously reported that loss of dyskerin promotes survival after DOXO treatment independently of cellular p53 status (Lin et al. 2014). Our current findings suggest that loss of dyskerin may have a more broadly inhibitory effect on DNA damage signaling, albeit not as robustly as GAR1 depletion. The mechanisms by which loss of dyskerin attenuates the accumulation of DDR biomarkers yet promotes cell survival remain to be reconciled.

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