Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.9, No.6, 179-186, 2022

The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Role of cytoplasmic acetyltransferases, NAA60 and HAT1, in cellular protection against genotoxic agents

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(Received September 26, 2022; Accepted October 7, 2022)

ABSTRACT — Histone acetyltransferases (HATs) are separated into two types. Type A HATs act on nucleosomal histones and thus primarily function in transcriptional regulation, while cytoplasmic HATs (type B) are known as enzymes that modify free histones before their assembly into chromatin, and may also function outside the nucleus. N-alpha-acetyltransferase 60 (NAA60) is the most recently discovered type B HATs, which are also known as N-terminal acetyltransferases (NATs) and are found only in multicellular eukaryotes. NAA60 localizes to the Golgi complex and possesses a unique ability to catalyze the acetylation of membrane-anchored proteins at the N-terminus and free histones at the lysine side chains, the biological significance of which remains unclear. To investigate the cellular functions of NAA60 and its relation to other cytoplasmic HATs, Hat1, we generated *NAA60*- or *HAT1*-deficient cells and *NAA60/HAT1*-double deficient cells using a chicken B lymphocyte leukemia DT40 cell line. Although NAA60-deficient cells did not show any impairment in cell growth and showed a slight sensitivity to DNA damage agents, *NAA60/HAT1*-double deficient cells exhibited an additive increase in sensitivity to methyl methanesulfonate (MMS) and 4-nitroquinoline 1-oxide (4-NQO) when compared to *HAT1*-deficient cells, which were previously reported to be moderately sensitive to these agents. These results predict that each type B HATs might contribute differently in regulation of repair of chemical induced DNA lesions.

Key words: Histone acetylation, HAT1, NAA60, DNA repair

INTRODUCTION

Different histone modifications are known to modulate the chromatin accessibility for binding proteins and subsequently contribute to the regulation of all DNA-based processes, including gene expression, DNA replication and DNA repair (Fischle *et al*., 2003; Miller and Jackson, 2012). Among these modifications, histone acetylation is catalyzed by histone acetyltransferases (HATs), which are classified into two distinct groups (types A and B) based on their subcellular localization and substrate specificity (Poziello *et al*., 2021). Type A HATs (e.g., GCN5, PCAF, etc.) are nuclear enzymes which target the nucleosomal histones and function in the regulation of gene transcription (Poziello *et al*., 2021). On the other hand, the enzymes that catalyze the acetylation of free but not nucleosomal histones are called B-type HATs (Parthun *et al*., 1996). In all eukaryotes, the N-terminal 5 and 12 Lys of the newly synthesized cytoplasmic histone H4 are highly acetylated, and these acetylations might affect the

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formation of new nucleosomes and histone metabolism (Sobel *et al*., 1995). Among cytoplasmic histone acetyltransferases (Type B HATs), HAT1 is the founding member of this class of enzymes and has been shown to be responsible for the diacetylation (K5/K12) of newly synthesized histone H4 (Barman *et al*., 2006; Parthun, 2012). Despite the evolutionary conservation of acetylated pattern of newly synthesized histone by HAT1 and its implications for de novo chromatin assembly, deletion of HAT1 in yeast and chicken B cell line, DT40, did not show significant impact on cell proliferation, but exhibited mild sensitivity to DNA damaging agents, MMS and CPT, suggesting its implications for tolerance to DNA damages via histone deposition (Barman *et al*., 2008, 2006; Ma *et al*., 1998; Parthun *et al*., 1996). On the other hand, recently, N-alpha-acetyltransferase 60 (NAA60) has also been identified as a type B HAT as it localizes to the Golgi apparatus and acetylates on K91 of cytoplasmic histone H4, which is also known as N-terminal acetyltransferase (NAT) (Yang *et al*., 2011).

Protein N-terminal acetylation is one of the most abundant and conserved protein modifications, occurring in over 60% of eukaryotic proteins (Ree *et al*., 2018). Many NATs are highly conserved from lower to higher eukaryotes and the substrate appears to be partially overlapped (Deng and Marmorstein, 2021). However, NAA60 is unique among other NAT enzymes because it localizes to the Golgi apparatus, possesses the ability to catalyze the acetylation of membrane-anchored proteins at the N-terminus and is found only in multicellular eukaryotes (Aksnes *et al*., 2017, 2015). It was shown that NAA60 knockdown in MCF7-cell inhibits cell proliferation, sensitizes cells to DNA damage and induces cell apoptosis (Yang *et al*., 2011). In Drosophila cells, NAA60 knockdown induces chromosomal segregation defects during anaphase including lagging chromosomes and chromosomal bridges (Van Damme *et al*., 2011). Most recently, it has been found that NAA60 knockdown causes Golgi apparatus fragmentation in HeLa cells, suggesting its importance in regulation of organellar structure (Aksnes *et al*., 2015). Moreover, some NATs have been shown to associate with cancer biology and therapeutic potential, especially in liver cancer where a novel oncogenic role were reported in multiple studies and its association with patient survival (Koufaris and Kirmizis, 2020).

The chicken B-lymphocyte line DT40 is characterized by a high efficiency of gene targeting and phenotypic stability, providing us with a unique opportunity of studying the physiological role of histone acetylation by comparing the phenotypes of a variety of HATs mutants (Takami *et al*., 1999; Barman *et al*., 2006). In this study, to investigate the cellular functions of NAA60 and its relation to another cytoplasmic histone acetyltransferase, HAT1, we compared phenotypes among *NAA60*- or *HAT1*-deficient cells and *NAA60/HAT1*-double deficient cells using DT40 cells. Although *NAA60*-deficient cells did not show any impairment in cell growth or cell cycle and were slightly sensitive to DNA-damaging agents, *NAA60/HAT1* double deficient cells showed an additive increase in sensitivity to MMS and 4-NQO when compared to *HAT1*-deficient cells, which is previously shown to have moderate sensitivity to these agents. These results suggest that each type B HATs might contribute differently in regulation of repair of chemical induced DNA lesions.

MATERIALS AND METHODS

Plasmid constructs

The cDNA of NAA60 was cloned from DT40 cells. The NAA60 expression vector (pHA-NAA60) was constructed by inserting HA-tagged full-length HAT1 cDNA into pCMV plasmid. Deletion mutants of NAA60 including NAA60-ΔA, NAA60-ΔB and NAA60-ΔAB were generated by subcloning corresponding fragments from HA-NAA60 by PCR (Satrimafitrah *et al*., 2016).

To obtain NAA60 disruption constructs, 2 kb upstream arm, carrying exon 1-3, and 2 kb downstream arm, were amplified by genomic PCR using primers (primer up5' ATGACATAGACGCGGTGAAGCAGCTCTG and primer up3' AGGAAAATTGGAAGCTAGGATGTCTCCATC, and primer down5' TGACTCCATCAGCGGATGTACACAGCACAAAG and primer down3' CTCCTCCTAGGTCTAAACCTGACCCAGACT), then inserted into pBluescript II. Neomycin (Neo) and Histidinol (HisD) resistance cassettes, driven by β-actin promoter and flanked by loxP sites, were inserted independently between the upstream and downstream arms.

Gene targeting constructs for HAT1(pΔHAT1loxhyg and pΔHAT1loxpuro) was described previously.

Cell Culture, transfection, and chemical reagents

The DT40 *NAA60*$^{-/-}$ cells were created by replacing exons 3 of the chicken NAA60 gene by selection marker genes (Fig. 2). The targeting vectors used in this experiment were constructed so that drug resistance markers were replaced the the NAA60 gene within exon 3. DNA transfections have been described previously (Takami *et al*., 1999). After transfection with the neomycin-resistance vector, resistant colonies were grown in the presence of geneticin (1 mg/mL) (Gibco). One geneticin-resistant clone of 24 assayed was found to harbor a homologous recombinant, and this clone was expanded and transfect-

ed with the Histidinol–resistance vector. Colonies were selected in Histidinol (800 μg/mL). The desired gene disruption was verified by genomic PCR and RT-PCR (Fig. 2) using the primers NAA60-sense: 5′AAAATCGCCATCTCACTTGG3′ and NAA60-antisense: 5′CACAGCCTGAGTCACATTTTGG3′.

HAT1-deficient cells were described previously (Barman *et al*., 2006). *NAA60/HAT1*-double deficient cells were created by transfecting with two targeting constructs for HAT1 into *NAA60*-cells.

The cells were cultured in DMEM (Gibco) supplemented with 10% FCS, streptomycin and penicillin (each 100 U/mL), 1% chicken serum and β-mercaptoethanol as described previously (Takami *et al*., 1999).

For cell treatments, MMS and 4-NQO were diluted in DMEM and added to cells at the indicated final concentrations.

Immunofluorescence microscopy

Cells (2×10^5) were spotted onto glass slides, fixed with 2% paraformaldehyde in PBS for 15 min, and immersed in cold methanol for 30 min. After washing with 0.5% Triton X-100, cells were probed with rabbit anti-HA antibody (WAKO). Primary antibody was detected by Alexa 594-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene). DNA was counterstained with 4′-6-diamidino-2-phenylindole (DAPI) at 0.1 μg/mL. Stained samples on slides were examined under Axiovert M-200 fluorescence microscope (Zeiss, Germany), and the images were captured with cooled CCD camera (ORCA-ER, Hamamatsu, Japan).

Cell cycle analysis by flow-cytometry

2×10^6 cells were washed with cold PBS, resuspended in 80% cold EtOH and stored at 4°C. After centrifugation, the cells were incubated at 37°C with 500 μL of PI solution in PBS (50 μg/mL Propidium Iodide, 0.1 mg/mL RNaseA, 0.05% Triton-X-100, PBS) for 30 min in the dark. After addition of PBS, centrifugation and supernatant removal, the cells were resuspended in 500 μL PBS and transferred to round-bottom tubes for subsequent flow-cytometric analysis.

Clonogenic assays

Serially diluted cells were plated in duplicate onto six-well plates containing 5 mL/well of 1% methylcellulose (MC) (Sigma-Aldrich, USA) in DMEM supplemented with 15% FBS and 1.5% chicken serum (Barman *et al*., 2006). To test sensitivities to MMS (Nacalai Tesque, Japan), 4-NQO (Sigma-Aldrich, USA), serially diluted cells were plated in MC plates, containing various doses of each drug. Plates were incubated at 37°C for 8–10 days, and resulting visible colonies were counted. Percentage survival was estimated relative to numbers of colonies from untreated cells.

Protein extractions and western blots

Cells seeded in 10 cm dishes and treated as indicated were harvested. Whole cell (5×10^6) extracts were prepared by re-suspending the cell pellets in SDS lysis buffer prior to sonication and centrifugation at 18000 × g to clear the lysate and protein concentration was determined by the Lowry assay. Same amount of proteins were separated by 12.5% SDS-PAGE, followed by electro-blotting onto a PVDF membrane filter as described. The filter was probed with a 1/1000 dilution of anti-γH2AX antibodies (Millipore), anti-β-actin antibodies (Millipore), followed by incubation with a 1/1000 dilution of secondary antibodies (horseradish peroxidase conjugated anti-mouse IgG or anti-rabbit IgG antibodies (Dako)). The signal was developed using a Super SignalTM CL-HRP system (Pierce, Rockford, IL, USA) and visualized with a LAS-3000 (Fuji Film).

RESULTS

To ascertain the common nature of NAA60 anchoring to the Golgi apparatus across species, we first analyzed its subcellular localization in chicken DT40 cells. After transfection with HA-tagged chicken NAA60, DT40 cells were fixed and immunostained with HA antibodies. Immunofluorescent imaging revealed a highly reproducible perinuclear distribution pattern reminiscent of the intracellular distribution pattern of the Golgi apparatus (Fig. 1). Furthermore, deletion of potential transmembrane domains A and B, individually, in its N-terminal region led to a dispersion of NAA60 in the cytoplasm (Fig. 1), indicating a disappearance of NAA60 from the Golgi apparatus and suggesting that chicken NAA60, similarly to its human homolog, might be anchored on the Golgi apparatus in DT40 cells.

Defects of N-terminal acetylation in the Golgi apparatus and histone acetylation in the cytoplasm may consequently affect the growth and homeostasis of cells. In order to investigate whether NAA60 is required for the cellular response physiologically, we determined the effect of loss-of-function of NAA60 on the growth and proliferation in DT40 cells. We generated homozygous ($NAA60^{-/-}$) DT40 mutant cells by sequential transfection of the Neo and HisD constructs (Fig. 2A). The mutant clones were isolated and verified by genomic PCR (Fig. 2A and B), and the loss of NAA60 expression in these clones was further confirmed by RT-PCR (Fig. 2A and B). Next,

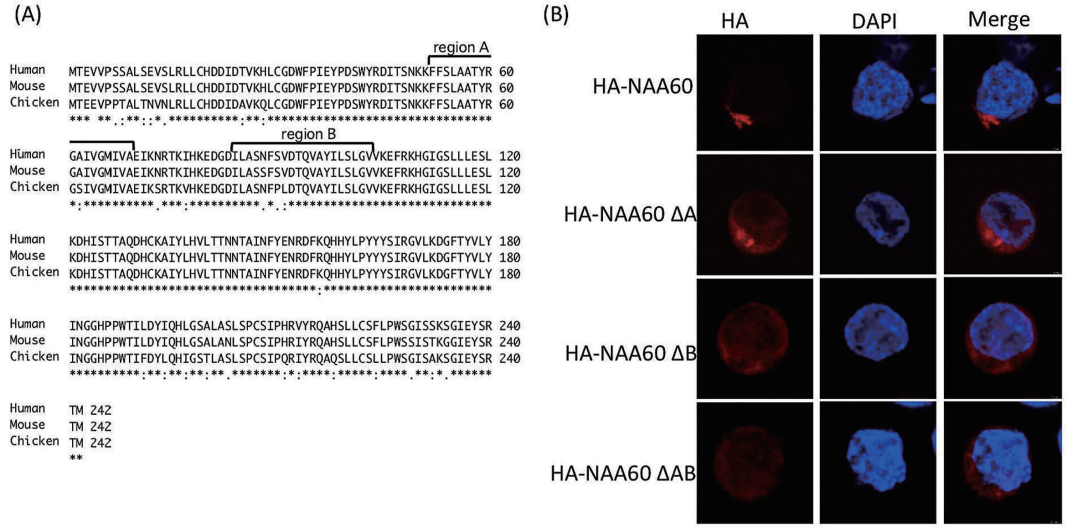

Fig. 1. Characterization of chicken NAA60. (A) Amino-acid sequence alignment of NAA60 from different species. Putative transmembrane regions A and B are marked with two bars. (B) Subcellular localization of NAA60. NAA60 is mainly localized in a portion of cytoplasmic apparatus mediated by its double transmembrane domain in DT40 cells. DT40 cells were transfected with expression plasmids encoding HA-NAA60 and corresponding mutants (ΔA: deletion of transmembrane region A [52-69aa], ΔB: deletion of transmembrane region B [84-103aa], Δ(AB):deletion of transmembrane A and B [52-103aa]). After 24 hr, fixed cells were stained with anti-HA antibody followed by Alexa 594 conjugated 2nd antibody, and then nuclei were counterstained with DAPI.

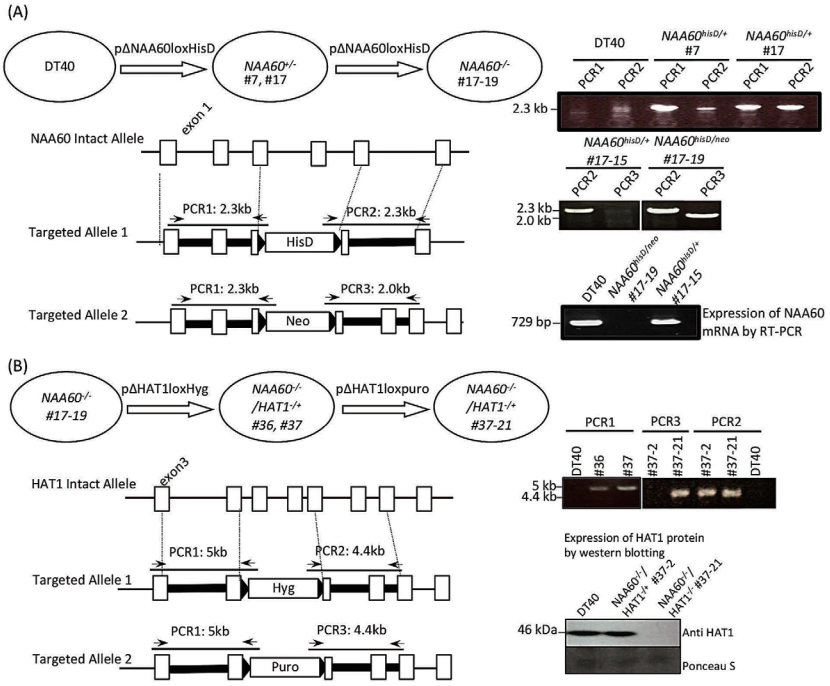

Fig. 2. Gene targeting of NAA60 and HAT1 locus. (A) Left, Schematic representation of intact (top) and targeted (bottom) NAA60 alleles. Replaced regions are indicated by bold line. The loxP sequences flanking Neo and HisD genes are shown as triangles. Exons are indicated by opened boxes. Right, PCR analysis for DT40 and *NAA60*-deficent cells (B) Left, Schematic representation of intact and targeted HAT1 alleles. Right, PCR and western blotting analysis for DT40 and *NAA60/HAT1*-double deficient cells.

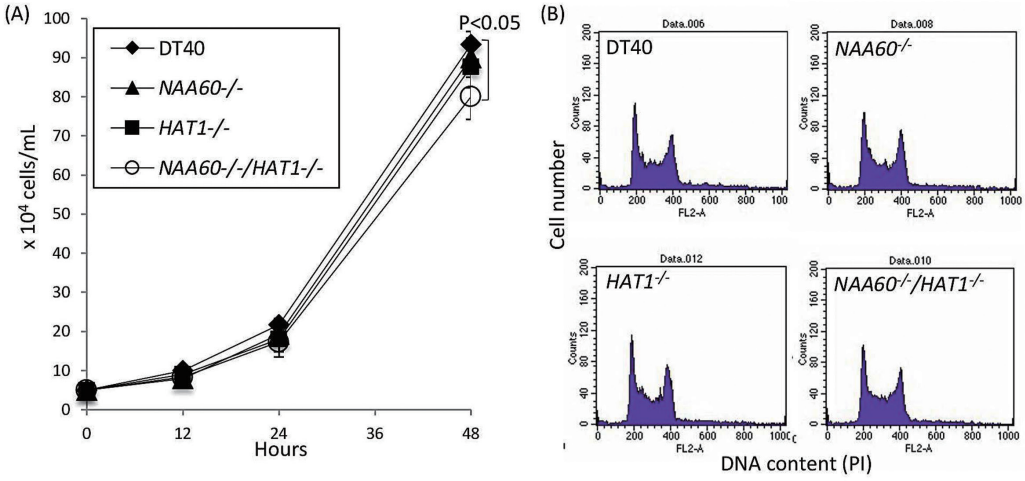

Fig. 3. Growth curve and Flow cytometric analysis of *NAA60*- and *HAT1*-deficient cells. (A) Growth curve of wild type DT40, *NAA60*$^{-/-}$, *HAT1*$^{-/-}$, *NAA60*$^{-/-}$/*HAT1*$^{-/-}$cells. Data represent means of three independent experiments. (B) Flow cytometric analysis of asynchronously grown DT40, *NAA60*$^{-/-}$, *HAT1*$^{-/-}$, *NAA60*$^{-/-}$/*HAT1*$^{-/-}$cells. Cells were fixed and stained with PI to detect total DNA (x-axis, linear scale).

we compared the proliferative rate and cell cycle distribution of *NAA60*$^{-/-}$ cells and *HAT1*$^{-/-}$ cells by flow cytometric analysis. We examined at least two independent clones of each genotype to assess the extent of variation of their phenotypes because of the genome instability associated with the cytoplasmic HAT defects. The growth rate of *NAA60*$^{-/-}$ cells and *HAT1*$^{-/-}$ cells was essentially same, compared with that of wild-type DT40 cells (Fig. 3A). Also, distribution of the cell cycle stage of mutant cells was not significantly different from that of wild type DT40 cells (Fig. 3B)

To further explore the *in vivo* function of NAA60 and/or the biological significance of N-terminal acetylation protein, we examined the effects of several DNA damaging agents on *NAA60*-deficient cells. Our previous studies revealed that *HAT1*-deficient cells are mildly sensitive to killing by a variety of genotoxic stresses (Barman *et al*., 2006). In the present study, we compared the sensitivities of the two cytoplasmic HAT deficient cells to genotoxic treatments using colony survival assays. Methylating agents such as MMS are powerful genotoxicants that induce chromosomal breaks and translocations. Figure 4A shows the MMS sensitivity of the representative wild-type, *NAA60*- and *HAT1*-deficent cells. Interestingly, the data show that the sensitivities of the two cytoplasmic HAT deficient cells to MMS were similar and exhibited slight sensitivity compared to wild type. 4NQO was initially considered a UV-mimetic carcinogen but it actually exhibits more complex effects, inducing the formation of various covalent adducts, oxidative damage, and DNA single-strand breaks. The sensitivity of each cytoplasmic HAT deficient cells to 4-NQO was also similar and exhibited slight sensitivity compared to wild type.

To examine the epistatic relationship of the two cytoplasmic HAT molecules, we generated two independent HAT1and NAA60 doble deficient clones (Fig. 2B). Briefly, two *NAA60*$^{-/-}$/*HAT1*$^{-/-}$ clones were made from a *NAA60*$^{-/-}$ clone and then investigated for growth rate, cell cycle and sensitivities to genotoxic treatments. Interestingly, the growth rate of *NAA60*$^{-/-}$/*HAT1*$^{-/-}$ clones were decreased than that of each cytoplasmic HAT single deficient cells while the cell cycle stage of double deficient cells was not significantly different from that of single deficient cells (Fig. 3). Furthermore, double deficiency of HAT1 and NAA60 exhibited remarkably more increased sensitivity to MMS (Fig. 4A) and 4-NQO (Fig. 4B) compared to each cytoplasmic HAT single deficient cells. These additive effects suggest that two cytoplasmic HAT molecules might cooperate in the normal cell growth and in the repair or tolerance of DNA damage.

The increased sensitivity to DNA damaging agents of the *NAA60*$^{-/-}$ cells described above could be the result of cell death due to the inability of the cells to properly organize their genome or a reflection of cell apoptosis due to NAA60 deficiency-induced stress. In order to further explore the cellular function of NAA60, we examined the effect of NAA60 depletion on cell apoptosis. For this purpose, after treatment of 4-NQO (0 hr, 12 hr, 24 hr, 48 hr), cells were stained with propidium iodide and then the number of dead cells and live cells were counted (Fig. 5).

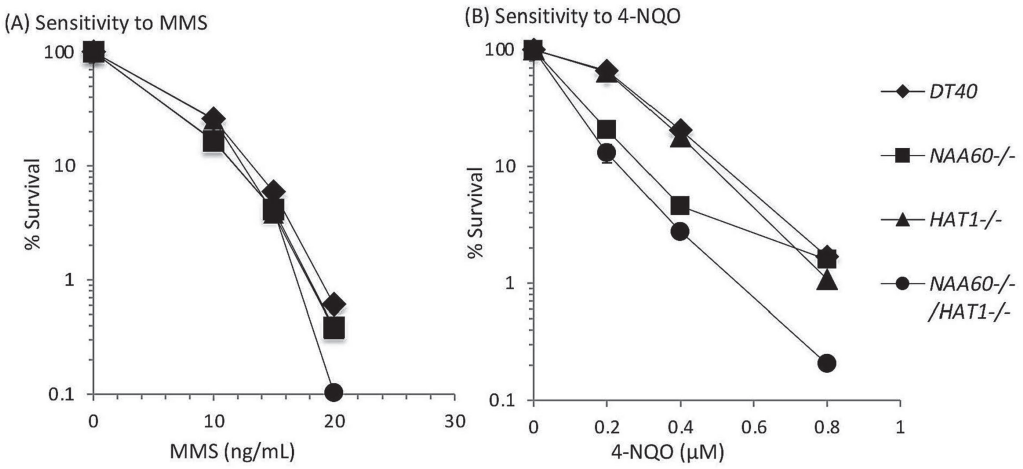

Fig. 4. Sensitivity of DT40, *HAT1*- and *NAA60*-deficient cells to DNA-damaging agents by colonogenic survival assay. Double deficiency of NAA60 and HAT1 sensitizes cells for specific DNA-damaging agents. Serially diluted cells (50, 500, and 5000 cells) were plated in duplicate in Methyl Cellulose media containing various doses of each (A) 4-NQO and (B) MMS. Plates were incubated at 37°C for 8–10 days, and resulting visible colonies were counted.

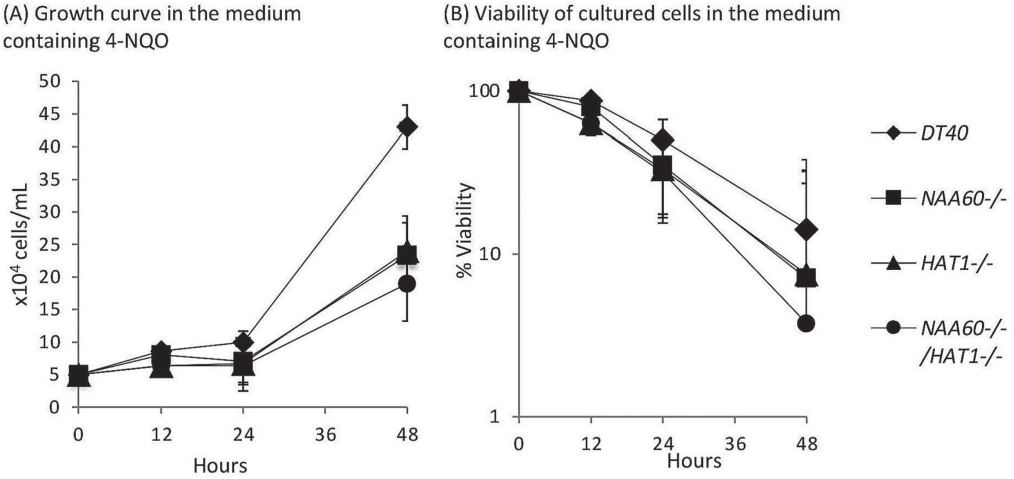

Fig. 5. Cells proliferation and viability assay of DT40, *HAT1*- and *NAA60*-deficient cells against DNA-damaging agents 4-NQO. Wild type DT40, *NAA60*$^{-/-}$, *HAT1*$^{-/-}$, *NAA60*$^{-/-}$/*HAT1*$^{-/-}$cells were separately cultured in 500 μL of culture medium containing 4NQO (0.2 μM) in 24-well plates at a density of 5 x 10^4 cells/mL, followed by incubation for 48 hr. Cells were stained with propidium iodide and then count the number of dead cells and living cells with reference to the viability of mock-treated cells. (A) represents living cells proliferation in the medium containing 4-NQO. (B) represents viability of cultured cells in the medium containing 4-NQO. Data represent means of three independent experiments.

Deficiency of either NAA60 or HAT1 resulted in a slight decrease in cell number. Notably, double deficiency of HAT1 and NAA60 had a more pronounced negative effect on cell proliferation after 4-NQO treatment (Fig. 5A). These analysis revealed that deficiency of HAT1 or NAA60 resulted in a reduced number of living cells and an increase in the number of cells containing fragmented nuclei, showing apoptosis. Double deficiency of HAT1 and NAA60 led to an even higher percentage of cells undergoing apoptosis (Fig. 5B).

We next assessed phosphorylation of histone H2AX (γH2AX), a marker of DNA damage, by Western blot.

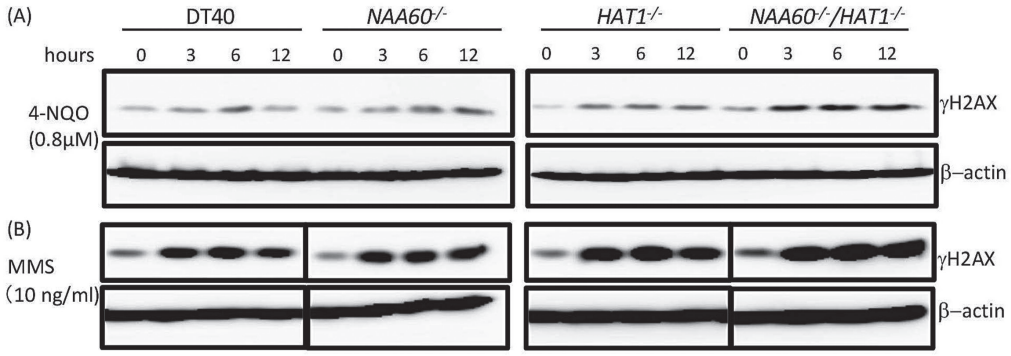

Fig. 6. Effects of deficiency of NAA60 and/or HAT1 on DNA damage response after treatment of 4NQO or MMS. Phosphorylate histone H2AX (γH2AX) levels were evaluated with immunoblotting analysis. The indicated cells were cultured in the presence of 4NQO (0.8 μM) or MMS (10 ng/mL) for indicated time periods, and whole cell extracts were prepared, followed by Western blotting, using antibodies against phosphorylated H2AX (γH2AX), and β-actin as a loading control.

The spreading of γH2A.X at both sides of a strand break is one of the earliest events involved in the DNA damage response (DDR) to different genotoxic stresses (Paull *et al*., 2000). The 4-NQO and MMS treatments drastically increased the level of γH2AX in all cell lines (Fig. 6A and B). Although DNA damages represented by γH2AX after exposures to these two agents and/or immediate follow-up response to the resultant lesions were essentially same between DT40, *HAT1*$^{-/-}$ and *NAA60*$^{-/-}$ cells, but more prominent DNA damages were retained in *NAA60*$^{-/-}$/*HAT1*$^{-/-}$cells (Fig. 6A and B). These results imply that the increased sensitivities of double deficient cells to 4-NQO or MMS could be due to the accumulation of an elevated amount of DNA damages that is hard to be repaired, leading to the apoptotic response in these cells.

DISCUSSION

The majority of HATs are A-HATs, which target nucleosomal histones and function in the regulation of gene transcription. Type B-HATs, on the other hand, are responsible for modifying newly synthesized histones that have not yet been incorporated into chromatin. They have received less attention and their role in acetylation of cytosolic histones is not well understood. One B-HAT, HAT1, is responsible for di-acetylation of K5 and K12 of newly synthesized histone H4, which is accompanied with *de novo* chromatin assembly. However, several studies have shown that, in yeast, the absence of di-acetylation on H4 has no effect on chromatin assembly or cell proliferation (Parthun *et al*., 1996). We have also previously reported that chicken DT40 cells lacking HAT1 show no apparent defects in nascent nucleosome formation and cell proliferation (Barman *et al*., 2006). These studies suggest that HAT1 may not be the only enzyme involved in the acetylation of newly synthesized histone H4 that associate with de novo chromatin assembly, which is a complex, multistep process, including the diverse patterns of acetylation of free histones (Parthun, 2012). Later, human Naa60, also known as N-terminal acetyltransferase, was identified as a type B HAT because it acetylates K20, K79, and K91 of cytosolic histone H4 and localizes to the Golgi apparatus (Yang *et al*., 2011).

In this study, we found that chicken NAA60 also localizes to the Golgi apparatus in the same fashion as human NAA60 (Fig. 1) and has a protective role against DNA damaging agents. Golgi is considered to be a distribution center because of its function of preparing proteins and lipids for export out of the cell or transport to other locations inside the cell, which is associated with a variety of enzymatic activities including protease, glycosylase, acetyltransferase and phospholipase (Wilson *et al*., 2011). Although acetylation of newly synthesized histones in the Golgi apparatus is consistent with the general function of this organelle, it is still puzzling that it links Golgi apparatus function to chromatin biology such as chromatin assembly and chromatin stability.

In the current report, we showed a cooperative and/or functional relationships of HAT1and NAA60 in the regulation of cell growth and sensitivity to DNA damaging agents. We found that each single deficiency of the two type B HATs displayed similar slight sensitivities to a variety of DNA damage (Fig. 4, 5). This phenotypic similarity can be interpreted in two different ways. First, HAT1 and NAA60 proteins may be equally required for efficient recovery as a single functional entity such as

newly nucleosome formation through histone acetylation past a variety of DNA damage. Alternatively, although NAA60 may have another contribution to cellular tolerance of DNA damage through the disordered acetylation of membrane anchored proteins which associate with signaling pathway for cell apoptosis, as suggested from its associations with Golgi apparatus, the contribution might be little in DT40 cells, resulting in *NAA60*$^{-/-}$ cells lacking a more prominent phenotype than *HAT1*$^{-/-}$ cells. We favor the later idea, because our observation showed that HAT1 and NAA60 double deficiency caused an additive increase in MMS and 4-NQO sensitivities (Fig. 4, 5, 6). Thus, it is likely that NAA60 can act independently of the HAT1 molecules in tolerance to exogenous DNA damages. Taken together, the available data suggest that N-terminal acetylation in membrane proteins and/or cytoplasmic histone acetylation play a role in repair of DNA lesion and/or DNA damage signaling pathways. Further investigations are needed to reveal the influence and significance of these modification on the molecular interplay in a variety of DNA damage pathways.

ACKNOWLEDGMENTS

We thank Harishkumar Madhyastha for useful comments and critical reading of the manuscript. This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of interest---- The authors declare that there is no conflict of interest.

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