Identification of the human $N^\alpha$-acyetyltransferase complex B (hNatB): a complex important for cell-cycle progression

Kristian K. STARHEIM*, Thomas ARNESEN*†‡†, Darina GROMYKO*†, Anita RYNINGEN§, Jan Erik VARHAUG†‡ and Johan R. LILLEHAUG*†‡‡

*Department of Molecular Biology, University of Bergen, N-5020 Bergen, Norway, †Department of Surgical Sciences, University of Bergen, N-5020 Bergen, Norway, ‡Department of Surgery, Haukeland University Hospital, N-5021 Bergen, Norway, and §Department of Medicine, Haukeland University Hospital, N-5021 Bergen, Norway

INTRODUCTION

Protein $N^\alpha$-terminal acetylation is a conserved and widespread protein modification in eukaryotes. Several studies have linked it to normal cell function and cancer development, but nevertheless, little is known about its biological function. In yeast, protein $N^\alpha$-terminal acetylation is performed by the $N^\alpha$-acyetyltransferase complexes NatA, NatB and NatC. In humans, only the NatA complex has been identified and characterized. In the present study we present the components of hNatB (human NatB complex). It consists of the Nat3p homologue hNAT3 (human $N^\alpha$-acyetyltransferase 3) and the Mdm20p homologue hMDM20 (human mitochondrial distribution and morphology 20). They form a stable complex and in vitro display sequence-specific $N^\alpha$-acyetyltransferase activity on a peptide with the $N^\alpha$-terminus Met-Asp-. hNAT3 and hMDM20 co-sediment with ribosomal pellets, thus supporting a model where hNatB acts co-translationally on nascent polypeptides. Specific knockdown of hNAT3 and hMDM20 disrupts normal cell-cycle progression, and induces growth inhibition in HeLa cells and the thyroid cancer cell line CAL-62. hNAT3 knockdown results in an increase in G0/G1-phase cells, whereas hMDM20 knockdown decreased the fraction of cells in G0/G1-phase and increased the fraction of cells in the sub-G0/G1-phase. In summary, we show for the first time a vertebrate NatB protein $N^\alpha$-acyetyltransferase complex essential for normal cell proliferation.

Key words: cell cycle, human MDM20, human $N^\alpha$-acyetyltransferase 3 (hNAT3), human $N^\alpha$-acyetyltransferase B (hNatB), immunoprecipitation, protein $N^\alpha$-terminal acetylation.

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INTRODUCTION

$N^\alpha$-terminal protein acetylation is a widespread modification in eukaryotes. Despite this, its biological function and importance are largely unknown [1]. $N^\alpha$-terminal acetylation is believed to be a co-translational process, where nascent polypeptides are acetylated when protruding 20–100 residues from the ribosome [2,3].

In yeast, the NAT (N-acyetyltransferase) complexes NatA, NatB and NatC perform most co-translational $N^\alpha$-terminal acetylations. The three major NATs are heteromeric complexes, where the catalytic subunits Ard1p (arrest defective 1 protein), Nat3p and Mka3p respectively, form a subfamily within the GNAT (GCN5-related NAT) superfamily [1]. The amino acid sequence at the $N$-terminal end of the nascent polypeptides defines the substrate specificity of the three enzyme complexes. NatA acetylates serine, alanine, threonine, valine and glycine $N$-termini after initial methionine processing. NatB acetylates methionine residues when followed by an acidic residue or asparagine residues, whereas NatC acetylates methionine residues when followed by a hydrophobic residue. As the same pattern of $N^\alpha$-terminal acetylation is found in all eukaryotes studied, it has been suggested that the system of $N^\alpha$-acetylation is evolutionarily conserved [4].

hNatB (yeast NatB) contains the catalytic subunit Nat3p and the auxiliary subunit Mdm20p (mitochondrial distribution and morphology 20 protein). It acetylates the $N$-terminal methionine residue of substrates with the $N$-terminal sequence Met-Glu-, Met-Ala-, Met-Thr- and Met-Val-. hNAT3 knockdown results in an increase in G0/G1-phase cells, whereas hMDM20 knockdown decreased the fraction of cells in G0/G1-phase and increased the fraction of cells in the sub-G0/G1-phase. In summary, we show for the first time a vertebrate NatB protein $N^\alpha$-acyetyltransferase complex essential for normal cell proliferation.

Key words: cell cycle, human MDM20, human $N^\alpha$-acyetyltransferase 3 (hNAT3), human $N^\alpha$-acyetyltransferase B (hNatB), immunoprecipitation, protein $N^\alpha$-terminal acetylation.

Abbreviations used: Act, actin; ARD1, arrest defective 1; ACTH, adrenocorticotropic; CHX, cycloheximide; Cytc, cytochrome c; DAPI, 4′,6-diamidino-2-phenylindole; hARD1, human ARD1; HEK-293 cells, human embryonic kidney cells; hMDM20, human mitochondrial distribution and morphology 20; hNAT3, human $N^\alpha$-acyetyltransferase; hNatB, human NatB; hNAT3, human Nat3; hNAT, human NAT; NF-κB, nuclear factor κB; NLS, nuclear localization signal; siRNA, small interfering RNA; Tpm, tropomyosin; Tpr, tetra-tricopeptide repeat; zE GAP, zebrafish embryonic growth-associated protein.

1 To whom correspondence should be addressed (email thomas.arnesen@mbi.uib.no).
co-translationally and sequence-specifically on peptides with the N-terminal sequence Met-Asp-. Knockdown of hNAT3 and hMDM20 in the CAL-62 cell line demonstrated an abnormal cell-cycle distribution of the knockdown cells, pointing at a possible role of hNATB in cell-cycle progression.

**EXPERIMENTAL**

**hNAT3 and hMDM20 cloning and expression**

Using NCBI BLAST database searches with the yeast Nat3p and Mdm20p amino acid sequences as a query we identified homologues of these, and termed them hNAT3 (GenBank® accession number NP_057184) and hMDM20 (GenBank® accession number NP_079229) respectively. Plasmids encoding V5- and Xpress-tagged hNAT3 were constructed from a gene-specific PCR of cDNA made from total RNA isolated from HeLa cells (human cervix carcinoma, DSMZ number ACC 57). cDNA was made as described previously [20]. A plasmid encoding V5-tagged hMDM20 was sub-cloned from a plasmid purchased from Open Biosystems (accession number BC113585). PCR products were inserted into vectors pcDNA3.1/V5-His and pcDNA4/HisMax (Invitrogen). The primers for amplifying the hNAT3 gene were as follows: Pr. 1 (xp-hNAT3 for), 5′-ATGAC-CACGCCTACGG-3′; Pr. 2 (xp-hNAT3-stop rev) 5′-TTATTTAAG-TGCTTCCAGGCCTC-3′; Pr. 3 (hNAT3-V5 for), 5′-CGTAT-TACGGCCTACGG-3′; Pr. 4 (hNAT3-V5 rev), 5′-TTTACATG-TCTTCAAGGCTCAC-3′; hMDM20-primer: pr.1 (hMDM20-V5 for), 5′-ATGGCGAGCGGG-3′ and pr. 2 (hMDM20 rev), 5′-AATTTTATAGTCTTTCTTGTC-3′.

**Antibodies against endogenous hMDM20 and hNAT3**

An anti-hMDM20 antibody was made by Biogenes. Rabbits were immunized with the MDM20-derived peptide LNHPVEPTKNEKTD (amino acid position 703–716), and affinity-purified antibody was used for Western blotting. Anti-hNAT3 is a mouse monoclonal antibody raised against a full-length hNAT3 with a GST (glutathione transferase)-tag (Abnova, catalogue number H00051126-M01). These two antibodies were not applicable to staining of endogenous hNAT3 and hMDM20 using immunofluorescence.

**Cell culture and transfection**

HEK-293 cells (human embryonic kidney 293 cells; A.T.C.C. number CRL-1573) and CAL-62 cells (human thyroid anaplastic carcinoma; DSMZ number ACC 507) were cultured in DMEM (Dulbecco’s modified Eagle’s medium). HeLa cells (human cervix carcinoma; DSMZ number ACC 57) were cultured in EMEM (Eagle’s minimum essential medium). ONCO-DG-1 cells (human thyroid carcinoma; DSMZ number ACC 507) were cultured in RPMI 1640 medium. HCT116 cells (a gift from Dr Fred Bunz, Dr Bert Vogelstein and Dr Kenneth W. Kinzler, John Hopkins University School of Medicine and Howard Hughes Medical Institute, Baltimore, MD, U.S.A.) were cultured in McCoy’s 5A medium. All media were supplemented with 10% (v/v) FBS (fetal bovine serum), 3% L-glutamine and 0.1 mg/ml gentamicin. Cell cultures were grown in a cell culture incubator (Queue) with 5% CO₂ at 37°C. Plasmid transfection was performed using FuGENE® (Roche) according to the manufacturer’s protocol. siRNA (small interfering RNA) transfection was performed using Dharmafect (Dharmacon) according to the manufacturer’s protocol. Gene-specific smart pool siRNAs were purchased from Dharmacon and used at a final concentration of 20–50 nM to silence the hNAT3 and hMDM20 genes: sihNAT3, siGENOME SMARTPool, catalogue number M-oo8944-01; sihMDM20, ON-TARGET plus SMARTPool, catalogue number L-014530-01; negative control (siCtr), ON-TARGET plus non-targeting pool, catalogue number D-001810.

**Immunofluorescence**

HeLa cells grown on coverslips were washed in PBS, fixed in methanol, permeabilized in 0.1% Triton X-100 and blocked in 10% BSA. Primary antibodies were anti-V5 or anti-Xpress (both from Invitrogen). Secondary antibodies were Alexa Fluor® 488- or 594-conjugated IgGs (Invitrogen). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories). Images were acquired using an inverted Leica DMI 6000B microscope.

**Western blotting**

SDS/PAGE and Western blotting were performed as described previously [21]. Polyclonal rabbit antibodies against hMDM20, described above, were used at a 1:500 dilution, anti-actin (Santa Cruz Biotechnology) at 1:500, anti-CytC (anti-cytochrome c; BD Biosciences Pharmingen) at 1:500, anti-hNAT3 (Abnova) at 1:500, anti-L26 (Novus Biologicals) at 1:5000, anti-p21 (Santa Cruz Biotechnology) at 1:2000, anti-V5 (Invitrogen) at 1:2000 and anti-Xpress (Invitrogen) at 1:2000. HRP (horseradish peroxidase)-linked anti-mouse and anti-rabbit antibodies were from Amersham Biosciences.

**Immunoprecipitation**

Approx. 2 x 10⁶ cells were harvested for each sample. Cells were lysed in 300 μl of IPH lysis buffer [50 mM Tris/HCl (pH 8), 50 mM NaCl, 0.5% Nonidet P40, 5 mM EDTA, 1 mM NaVO₃ and 1 mM Pefabloc (Roche)] and incubated for 5 min on ice. Cell membranes and organelles were removed by centrifugation at 15 700 × g for 30 s, and the cell lysate was transferred to a new tube. To remove proteins that bind unspecifically to the agarose beads, Protein A/G–agarose (Santa Cruz Biotechnology) was added, and the lysate was incubated on a roller for 30 min at 4°C. The beads were removed by centrifugation at 1500 g for 4 min. The cell lysate was then incubated with 2 μg of specific antibody on a roller for 1–4 h at 4°C, before adding 50 μl of Protein A/G–agarose beads. The lysate was then incubated on a roller at 4°C for 4–10 h. The beads were collected by centrifugation as above, and washed with 1 × PBS. The supernatant was removed, and the samples were analysed by SDS/PAGE and Western blotting.

**In vitro N°-acetyltransferase assay**

HEK-293 cells were harvested and lysed in 300 μl of IPH lysis buffer. Typically, 5 x 10⁶ cells were used. Protein A/G–agarose (40 μl) was added to the lysates and incubated for 1 h at 4°C. After centrifugation at 1500 g for 2 min, the supernatants were collected and incubated for a further 2 h at 4°C with anti-hNAT3 or non-specific antibody (2 μg). Protein A/G–agarose (50 μl) was added to the samples and they were incubated for a further 16 h. After centrifugation (1500 g for 4 min at 4°C) and three washes in 2 × PBS and once in acetylation buffer [50 mM Tris/HCl (pH 8.5), 1 mM DTT (dithiothreitol), 800 μM EDTA, 10 mM sodium butyrate and 10% (v/v) glycerol], the samples were subjected to an *in vitro* acetylation assay. To pellets of Protein A/G–agarose-bound hNAT3–hMDM20 complexes was added 10 μl of peptide (0.5 mM, custom-made peptides from Biogenes or Sigma Genosys), 4 μl of [³¹⁵]Acetyl-CoA (50 μCi, 2.07 GBq/mmol; GE Healthcare) and 250 μl of acetylation buffer. 

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buffer. The mixture was incubated for 2 h at 37°C with rotation. After centrifugation (1500 g for 4 min at 4°C) the supernatant was added to 250 μl of SP Sepharose (50% slurry in 0.5 M acetic acid; Sigma) and incubated on a rotor for 5 min. The mixture was centrifuged (1500 g for 4 min at 4°C) and the pellet was washed three times with 0.5 M acetic acid and finally with methanol. Radioactivity in the peptide-containing pellet was determined by scintillation counting. Samples without peptide (containing distilled water instead) were subtracted from the obtained values in order to present the specific activity. All custom-made peptides contained seven unique amino acids at the N-terminus, since these are the major determinants for N-terminal acetylation. The next 17 amino acids were identical with the ACTH (adrenocorticotropic) peptide (amino acids 1–24) sequence to maintain a positive charge facilitating peptide solubility and effective isolation by cation-exchange Sepharose beads. The ACTH-derived lysine residues were replaced by arginine residues to minimize any potential interference by N-terminal acetylation. The peptide sequences were: TF2AA (P52655), [H]ANSANTNRWGPVGRRRRPVRVYP[OH]; NF-κB (nuclear factor κB) p65 (Q04206), [H]MDELFPLRWG-RPVGRRRPRVRVYP[OH]; hARL8b (Q9NVJ2), [H]MLA-\hbox{LISRWRGPVGRRRRPVRVYP[OH];} and kinesin KIF4A (O95239), [H]MKEEVKGRWPVGRRRRPRVRVYP[OH].

**Isolation of polysomes**

Total ribosome isolation was performed using a modification of previously described methods [22]. Approx. 2 × 10^7 cells were used per experiment. Prior to harvesting, cells were treated with 10 μg/ml CHX (cycloheximide) for 5 min at 37°C. Cells were harvested, and treated with the cross-linker diithiothreitol/succimidylpropionate (Pierce) according to the manufacturer’s protocol. Cells were then lysed with KCl ribosome lysis buffer [1.1% (w/v) KCl, 0.15% (w/v) triethanolamine, 0.1% (w/v) magnesium acetate, 8.6% (w/v) sucrose, 0.05% (w/v) sodium deoxycholate, 0.5% (v/v) Triton X-100 and 0.25% (v/v) Pefabloc], and incubated on ice for 15 min. After removing the nucleus and membranes by centrifugation at 400 g for 10 min, 700 μl of cell lysate was ultracentrifuged at 96 000 rev./min for 25 min on a 0.4 ml pillow of 25% sucrose in KCl ribosome lysis buffer using a MLA-130 rotor (Beckman). The pellet was dissolved in 50 μl of KCl total ribosome lysis buffer. Lysate control, top supernatant and ribosomal pellet were analysed by SDS/PAGE and Western blotting.

**Cell-cycle analysis by FACS**

After harvesting of approx. 1 × 10^7 cells by trypsin treatment, the cells were rapidly fixed in ice-cold 70% ethanol and incubated for at least 30 min at 4°C. They were then washed once, resuspended in 800 μl of PBS. RNase A (Sigma) (0.1 mg/ml) and 40 μg/ml PI (propidium iodide; Sigma), both final concentrations, was added before they were incubated without light exposure at 37°C for 30 min. Samples were immediately analysed by flow cytometry (FACS, Calibur, Becton Dickinson) as described previously [18].

**RESULTS**

The hNatB subunits: hNAT3 and hMDM20 proteins

Using NCBI BLAST database searches with the yNat3p and Mdm20p amino acid sequences as a query, we identified the human homologues and termed them hNAT3 and hMDM20 respectively. See Figure 1 for a schematic overview of hNAT3 and hMDM20.

**Figure 1** Schematic models of hNAT3 and hMDM20

hNAT3 is a protein of 178 amino acids, and contains a predicted acetyltransferase motif between amino acids 45–129. hMDM20 is a protein of 972 amino acids, with a predicted TPR motif between amino acids 21–146 and a putative NLS between amino acids 871–877.

**Figure 2** Expression of hNAT3 and hMDM20 in human cell lines

Human cell lines CAL-62 (human thyroid anaplastic carcinoma), HeLa (human cervix carcinoma), HEK-293 and HCT116 (human colon cancer) were lysed, and the cell lysates were analysed using SDS/PAGE and Western blotting for the expression of hNAT3 and hMDM20. Actin was used as a loading control. Molecular-mass markers (in kDa) are indicated on the left-hand side.

hMDM20. We then cloned the full-length hNAT3 from cDNA from total HeLa cell RNA, as described in the Experimental section. hMDM20 plasmid was purchased from Open Biosystems, and hMDM20 was subcloned into an eukaryotic expression vector. hNAT3 has a theoretical molecular mass of 20.4 kDa, as calculated by the ExPaSy compute Mw tool (http://au.expasy.org/tools). In silico analysis predicts that hNAT3 contains a conserved acetyltransferase motif between amino acids 45–129 (SMART; http://smart.embl-heidelberg.de) and a globular domain between amino acids 45–129 (ELM; http://elm.eu.org). This human homologue was previously included in a multiple alignment of Nat3p homologues performed by Polevoda et al. [5], and it shares 41.33% sequence identity with its yeast homologue (as calculated with JalView; http://www.jalview.org/).

In silico studies suggest that the hMDM20 protein has a molecular mass of 112.3 kDa (ExPaSy; http://ca.expasy.org) and contains two globular domains (ELM). Based on the protein sequence a TPR (tetra-tricopeptide repeat) region was predicted between amino acids 21–146 (ScanProsite tool at ExPaSy) and a NLS (nuclear localization signal) between amino acids 871–877 (PredictNLS Online; http://cubic.bioc.columbia.edu). Multiple alignment of hMDM20, Mdm20p and NP766310, the putative mouse homologue of Mdm20p, were performed using Mafft sequence aligner (see Supplementary Figure 1 at http://www.BiochemJ.org/bj/415/bj4150325add.htm). hMDM20 protein shares 20.36 and 92.90% sequence identity with its yeast and mouse homologues respectively. This also indicates that MDM20 is conserved in higher eukaryotes. To investigate whether endogenous hNAT3 and hMDM20 proteins are expressed in human cell lines, we analysed several different human cell lines by SDS/PAGE and Western blotting using anti-hNAT3 and anti-hMDM20 (Figure 2). Indeed, the two antibodies detected proteins and...
of the expected size in all tested cell lines, thus supporting that the two proteins are generally expressed.

hMDM20 interacts with hNAT3

To investigate whether or not hNAT3 and hMDM20 may form a complex in human cells, HEK-293 cells were lysed and subjected to immunoprecipitation using a hNAT3-specific antibody and then SDS/PAGE and Western blotting. The presence of a strong hMDM20 signal detected by the hMDM20 antibody in the anti-hNAT3 immunoprecipitate was taken to indicate that the hNAT3–hMDM20 complex is formed in cells (Figure 3A). Immunoprecipitation of hMDM20 using anti-hMDM20 was not successful. We therefore constructed a plasmid encoding a V5-tagged hMDM20, expressed hMDM20-V5 in HEK-293 cells and used an anti-V5 antibody for immunoprecipitation. When analysing the complexes by SDS/PAGE and Western blotting with anti-hNAT3 antibodies, we detected a strong hNAT3-specific signal as compared with the control (Figure 3B). Taken together, these experiments clearly demonstrate that hNAT3 and hMDM20 may form a complex as expected for hNatB in vivo.

Subcellular localization of hMDM20 and hNAT3

In silico analysis of the hMDM20 sequence indicated a putative NLS between amino acids 871 and 877 (KKKKKKK) (Figure 1). However, immunofluorescence analysis of hMDM20-V5 in HeLa cells demonstrated a cytoplasmic localization (Figure 4A). This indicated that the predicted NLS of the hMDM20 sequence does not function as a NLS in vivo under the conditions tested in the present study. On the other hand, Xpress-tagged hNAT3 was located in both the nucleus and the cytoplasm (Figure 4C). hNAT3-V5 displayed a similar localization pattern (results not shown). The observed subcellular localization pattern is consistent with a model where hNAT3 and hMDM20 may form a cytoplasmic complex in vivo.

The hMDM20–hNAT3 complex acetylates oligopeptides with Met-Asp- N-termini in vitro

To investigate whether the hNAT3–hMDM20 complex has protein NAT activity, the hNAT3–hMDM20 complex was immunoprecipitated from HEK-293 cells using an anti-hNAT3 antibody and used for in vitro NAT assays (see the Experimental section for details). A significant activity measured as [14C]acetyl incorporation was found towards the MDEL-peptide, whereas no significant activity was measured for the other peptides tested (Figure 5). Thus the hNAT3–hMDM20 complex acetylates peptides in a sequence-specific manner in vitro. As the yNatB is known to acetylate methionine residues that are followed by an acidic residue, the acetylation of the MDEL-peptide supports a conservation of substrate specificity of NatB from yeast to man.

The hMDM20–hNAT3 complex is associated with ribosomes

The hNAT3–hMDM20 is predicted to be associated with ribosomes to allow co-translational N-terminal acetylation. To investigate this hypothesis, we isolated polysomes from HEK-293 cells and analysed for the presence of hNAT3 and hMDM20 by SDS/PAGE and Western blotting. hMDM20 and hNAT3 were both detected in the polysomal fraction using anti-hMDM20 and anti-hNAT3 antibodies (Figure 6). Both proteins were also
The human NatB acetyltransferase complex

**Figure 5** *In vitro* N-acetylation activity of the hNatB complex

HEK-293 cells (approx. $5 \times 10^6$ cells per sample) were harvested, lysed and the lysate subjected to immunoprecipitation (IP) using anti-hNAT3 or negative control antibodies. The beads containing hNatB complexes were analysed for $N^\alpha$-acetyltransferase activity (see the Experimental section for details) using different peptides and [14C]acetylCoA. The acetyl incorporation was determined by isolation of the peptides followed by scintillation counting. Experiments were performed three times and values are means $\pm$ S.D. The presence of hNAT3 and hMDM20 in the various protein preparations was verified by Western blotting (results not shown).

**Figure 6** hMDM20 and hNAT3 co-sediments with polysomal fractions

HEK-293 cells were pre-treated with CHX and dithiobis(succinimidylpropionate) cross-linking before lysis. Cell lysates were ultracentrifuged through a 25% sucrose cushion (see the Experimental section for details). Cell lysate (L), supernatant post-ultracentrifugation (S) and pellet post-ultracentrifugation (P) were analysed by SDS/PAGE and Western blotting. The membrane was incubated with anti-hMDM20, anti-hNAT3, anti-L26 (ribosomal protein) and anti-CytC antibodies. Molecular-mass markers (in kDa) are indicated on the left-hand side.

Knockdown of hNAT3 and hMDM20 knockdown affects cell-cycle progression

yNatB has been linked to a potential role in cell-cycle-related processes [9]. To determine whether hNatB-mediated acetylation plays a role in cell-cycle regulation and cell proliferation, we performed siRNA-mediated knockdown of hNAT3 and hMDM20 in CAL-62 cells, and analysed the cells by microscopy, FACS analysis and SDS/PAGE and Western blotting.

After 72 h, a significant decrease in cell number could be observed in the sihNAT3-treated cells as compared with control cells treated with non-targeting siRNAs (Figure 7A). Similar observations were made using ONCO-DG1 cells (results not shown). Cell-cycle status in CAL-62 cells was analysed by FACs 72 h after siRNA transfection (Figure 7B). This revealed that knockdown of hNAT3 leads to $G_1/G_0$ arrest. Similar
results were obtained using HeLa cells (results not shown). We also specifically knocked down the other hNatB subunit, hMDM20, and these cells also displayed a significantly reduced cellular proliferation. However, cell-cycle analysis demonstrated an increase in sub-G0/G1 particles, most probably representing dead cells, and a significant decrease in the percentage of G0/G1 cells (Figure 7B). Western blotting of CAL-62 cells treated with sihNat3 and sihMDM20 using anti-hNat3 and anti-hMDM20 antibodies demonstrated a significant knockdown of both proteins (Figure 7C). This also suggested that knockdown of hMDM20 negatively affected hNat3 levels, whereas knockdown of hNat3 had a weak negative effect on hMDM20 levels (Figure 7C). Furthermore, this verified the specificity of the anti-hNat3 and anti-hMDM20 antibodies. Seeing that knockdown of hNat3 and hMDM20 affects the cell cycle, we investigated the level of p21 in the cells treated with sihNat3 and sihMDM20 (Figure 7C). In cells treated with sihNat3 we observed an increase in p21 levels, whereas a decrease in p21 levels is observed when hMDM20 is knocked down.

DISCUSSION

hNat3 and hMDM20 are the human homologues of the components of the yNatB complex. In yeast, NatB plays a role in Act filament stability through the acetylation of Act and Tpm [5], and regulates the protein kinase A pathway through the N-terminal acetylation of stress-induced carboxypeptidase inhibitor Ts1p [8]. Also, NatB has been predicted to play a role in cell-cycle regulation [9]. We are the first to identify, clone and describe hNat3 and hMDM20 as the components of the hNatB complex. hNat3 and hMDM20 are simultaneously expressed in several different human cell lines, and hMDM20-V5 and Xpress-hNat3 display overlapping cytoplasmic localization in HeLa cells. Co-immunoprecipitation shows that the two proteins form a stable complex in vivo. This, taken together with the finding that both hNat3 and hMDM20 co-sediment with ribosomal pellets, suggest a model where the hNatB complex is associated with the ribosome where it may act co-translationally. Even though hNat3 does not contain an identifiable NLS, we observed hNat3 in the nuclei of transfected cells. Taking into account its low molecular mass, we expect that hNat3 may be able to pass through the nuclear pore without being dependent on shuttle proteins. The yeast NatA, B and C complexes have previously been found to associate with the ribosome [3,23], thus N-terminal acetylation has been suggested to be a co-translational process. The findings of the present study are in agreement with this. The substantial amount of hNat3 and hMDM20 present in the non-polyribosomal fraction indicates that hNat3 and hMDM20 dynamically interacts with the ribosome and/or have other functions independent of ribosome binding. It is worth noting that the levels of sedimented and soluble hNat3 and hMDM20 are similar to those found for the hNatA subunits hARD1 and NATH, pointing to possible similarities in ribosome-binding dynamics between the two human NAT complexes [10].

Acetyltransferase assays show that the hNat3–hMDM20 complex displays sequence-specific NAT activity on the methionine residue of a peptide with the N-terminus Met-Asp-in vitro. yNatB is known to acetylate the N-terminal methionine residue of proteins with an acidic amino acid residue in the second position, thus NatB substrate specificity is at least partially conserved from yeast to human. ANSA-, MDEL-, MLAL- and MKEE-peptides represent the N-termini of human proteins that are predicted to be acetylated by different NATs, of which only MDEL was significantly acetylated by hNat3–hMDM20 (Figure 5). The ANSA-peptide represents the N-terminus of the general transcription factor TFIIA, that is a predicted NatA substrate due to its alanine residue at the first position. The MLAL-peptide contains the seven first amino acids of the human Arf-like GTPase hARL8b [24] and is a putative NatC substrate. The MKEE-peptide represents the N-terminus of kinesin KIF4A and does not match any known NAT substrate specificity. MDEL represents the N-terminus of the transcription factor NF-κβ subunit p65 and is a potential NatB substrate. NF-κβ is a regulator of genes that drive immune and inflammatory responses [25]. Several of the NF-κβ subunits, including p65, have been coupled to oncogenesis. Induction of NF-κβ by tumour necrosis factor or other stimuli leads to the transcriptional activation of genes that suppress apoptosis through the caspase cascade [26]. Based on our in vitro acetylation results, we suggest p65 to be a hNatB substrate in vivo. The possible functional role of N-terminal acetylation of p65 remains to be investigated.

Interestingly, knockdown of hNat3 and hMDM20 displayed differences in phenotypes. After hNat3 knockdown, CAL-62 and HeLa cells displayed arrest in the G0/G1-phase. Knockdown of hMDM20 also affected the cell cycle and decreased proliferation, but the cell-cycle analysis revealed a loss of cells from the G0/G1-phase, and also some induction of cell death. The finding that knockdown of hNat3 increased the level of p21, and knockdown of hMDM20 decreased p21 levels, are consistent with the effects on the cell cycle. p21 is a known inhibitor of traverse through the G1-phase [27]. Furthermore, it is also known that elevated levels of p21 may inhibit the induction of apoptosis, whereas in some cases it is known that preventing induction of p21 is necessary to induce apoptosis [28]. Thus the p21 level may explain why we detect cell death when analysing the hMDM20-knockdown cells, whereas no significant levels of apoptosis are detected for hNat3-knockdown cells. Combined, these results indicate that the human NatB complex is important for human cell growth and proliferation. Some of the effects induced by hNat3–hMDM20 knockdown may be related to loss/reduction of hNatB (or hNat3)-mediated N-terminal acetylation of specific substrates. Worth noting is that loss of hMDM20 also causes a reduction of hNat3, thus the hMDM20-knockdown phenotype may include the hNat3-knockdown phenotype. The differences in p21 levels and in cell-cycle distribution between hNat3- and hMDM20-knockdown cells, indicate that hNat3 and hMDM20 may have differential mechanisms of action with respect to cell-cycle progression. Thus the differences in phenotypes could indicate that one or both hNatB subunits may have individual functions in addition to those of the hNat3–hMDM20 complex. Then, individual effects on cell-cycle arrest or cell death may mask other effects. It should be noted that although significant similarities were found for mdm20 and nat3 yeast deletion strains in terms of Act and Tpm acetylation [5], yeast deletion strains nat3-Δ and mdm20-Δ have been shown to display some differences in phenotypes. This points in the direction of Nat3p and Mdm20p having distinct functions other than those of a Nat3p–Mdm20p complex [9], supporting that this may also be the case for hNat3 and hMDM20. The fact that hNat3 is present both in the cytoplasm and in the nucleus might indicate involvement of hNat3 in post-translational acetylation activities in the nucleus. Such an activity, if present, could explain the different cellular responses to hNat3 and hMDM20 knockdown since hMDM20 was localized exclusively to the cytoplasm.

With respect to function, the phenotypes of nat3-Δ and mdm20-Δ in yeast are to a large extent due to lack of acetylation of Act1p, Tpm1p and Tpm2p. Since the mechanism of N-terminal acetylation is believed to be conserved, it would be interesting to investigate whether hNatB plays a similar role in human
microfilament organization. Human TPM2 (GI: 42476296) has a Met-Glu-N-terminus, thus being an hNatB substrate candidate. In the case of actins, the human β-actin is additionally N-terminally processed [6], and the resulting acidic Act N-termini are acetylated by an unknown NAT. Thus the role of hNatB in human Act function is unclear. Clearly, the identification of hNatB substrates whose function depends on N-terminal acetylation is one of the most important future tasks. Of particular interest is the elucidation of the mechanism underlying the hNat3–hMDM20 knockdown-induced inhibition of cell proliferation.

In summary, in the present study we identify hNat3 and hMDM20 as components of the hNatB complex. We suggest that hNatB is a cytoplasmic complex that associates with the ribosome where it may act co-translationally. The hNatB complex displays sequence-specific Nα-acetyltransferase activity on peptides with the N-terminal Met-Asp-, thus hNatB is conserved from yeast both in respect to subunit composition and substrate specificity. Based on these studies, we suggest the NF-κB subunit p65 as a potential hNatB substrate. Knockdown of hNat3 leads to reduced cell proliferation and perturbation of cell-cycle progression linked to the G1/S–G2/M interphase, whereas knockdown of hMDM20 leads to a decrease in the G1/S phase, and some cell death. Thus one cannot out rule that hNat3 and hMDM20 have functions other than those in an hNat3–hMDM20 complex. These studies indicate the importance and complexity of Nα-terminal acetylation in human cells.

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Identification of the human $N^\alpha$-acetyltransferase complex B (hNatB): a complex important for cell-cycle progression

Kristian K. STARHEIM*, Thomas ARNESEN*†‡, Darina GROMYKO*†, Anita RYNINGEN§, Jan Erik VARHAUG†‡ and Johan R. LILLEHAUG*

*Department of Molecular Biology, University of Bergen, N-5020 Bergen, Norway, †Department of Surgical Sciences, University of Bergen, N-5020 Bergen, Norway, ‡Department of Surgery, Haukeland University Hospital, N-5021 Bergen, Norway, and §Department of Medicine, Haukeland University Hospital, N-5021 Bergen, Norway

Figure S1  Alignment of hMDM20, mouse NP766310 and Mdm20p

Matching residues are indicated with a dark grey background and conservative substitutions are indicated with a light grey background. Using yeast Mdm20p as the query sequence, mouse NP766310 was identified as a potential homologue.

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1 To whom correspondence should be addressed (email thomas.arnesen@mbi.uib.no).