Protein N-terminal Acetyltransferases Act as N-terminal Propionyltransferases In Vitro and In Vivo*§

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N-terminal acetylation (Nt-acetylation) is a highly abundant protein modification in eukaryotes catalyzed by N-terminal acetyltransferases (NATs), which transfer an acetyl group from acetyl coenzyme A to the alpha amino group of a nascent polypeptide. Nt-acetylation has emerged as an important protein modifier, steering protein degradation, protein complex formation and protein localization. Very recently, it was reported that some human proteins could carry a propionyl group at their N-terminus. Here, we investigated the generality of N-terminal propionylation by analyzing its proteome-wide occurrence in yeast and we identified 10 unique in vivo Nt-propionylated N-termini. Furthermore, by performing differential N-terminome analysis of a control yeast strain (yNatA), a yeast NatA deletion strain (yNatAΔ) or a yeast NatA deletion strain expressing human NatA (hNatA), we were able to demonstrate that in vivo Nt-propionylation of several proteins, displaying a NatA type substrate specificity profile, depended on the presence of either yeast or human NatA. Furthermore, in vitro Nt-propionylation assays using synthetic peptides, propionyl coenzyme A, and either purified human NATs or immunoprecipitated human NatA, clearly demonstrated that NATs are Nt-propionyltransferases (NPTs) per se. We here demonstrate for the first time that Nt-propionylation can occur in yeast and thus is an evolutionarily conserved process, and that the NATs are multifunctional enzymes acting as NPTs in vivo and in vitro, in addition to their main role as NATs, and their potential function as lysine acetyltransferases (KATs) and noncatalytic regulators. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.019299, 42–54, 2013.

Modifications greatly increases a cell’s proteome diversity confined by the natural amino acids. As more than 80% of human proteins, more than 70% of plant and fly proteins and more than 60% of yeast proteins are N-terminally acetylated (Nt-acetylated),¹ this modification represents one of the most common protein modifications in eukaryotes (1–5). Recent studies have pointed to distinct functional consequences of Nt-acetylation (6): creating degradation signals recognized by ubiquitin ligase of a new branch of the N-end rule pathway (7), preventing translocation across the endoplasmic reticulum membrane (8), and mediating protein complex formation (9). Nt-acetylation further appears to be essential for life in higher eukaryotes; for instance, a mutation in the major human N-terminal acetyltransferase (NAT), hNatA, was recently shown to be the cause of Ogden syndrome by which male infants are underdeveloped and die at infancy (10). Unlike lysine acetylation, Nt-acetylation is considered an irreversible process, and further, to mainly occur on the ribosome during protein synthesis (11–15). In yeast and humans, three NAT complexes are responsible for the majority of Nt-acetylation; NatA, NatB and NatC, each of which has a defined substrate specificity (16). NatA acetylates Ser-, Ala-, Gly-, Thr-, Val- and Cys- N-termini generated on removal of the initiator methionine (iMet) (1, 17–19). NatB and NatC acetylate N-termini in which the iMet is followed by an acidic (20–23) or a hydrophobic residue respectively (24–26). Naa40p/NatD was shown to acetylate the Ser-starting N-termini of histones H2A and H4 (27, 28). NatE, composed of the catalytic Naa50p (Nat5p) has substrate specificity toward iMet succeeded by a hydrophobic amino acid (29, 30). As largely the same Nt-acetylation patterns are found in yeast and humans, it was believed that the NAT-machineries were conserved in general (31). However, the recently discovered higher eukaryotic specific NAT, Naa60p/NatF, was found to display a partially distinct substrate specificity in part explaining the higher degree of Nt-acetylation in higher versus lower eukaryotes (4).

¹ The abbreviations used are: Nt-acetylation, N-terminal acetylation; Ac-CoA, Acetyl coenzyme A; But-CoA, Butyryl coenzyme A; HCD, Higher Energy Collision Dissociation; iMet, Initiator methionine; KAT, Lysine acetyltransferase; NAA#, N-alpha acetyltransferase # (gene/protein); NAT, N-terminal acetyltransferase; NBT, N-terminal butyryltransferase; NPT, N-terminal propionyltransferase; N-propionylation, N-terminal propionylation; Prop-CoA, Propionyl coenzyme A.
Human NatA is composed of two main subunits: the catalytic subunit hNaa10p and the auxiliary subunit, hNaa15p that is presumably responsible for anchoring the complex to the ribosome (14, 19). The chaperone-like HYPK protein is also stably associated with the NatA subunits and may be essential for efficient NatA activity (32). In addition, hNaa50p was shown to be physically associated with hNatA, however it is believed not to affect NatA activity (14, 33, 34). hNaa50p was also shown to exhibit N'-acyetyltransferase (KAT) activity (29), however, the structure of hNaa50p with its peptide substrate bound strongly indicates that the peptide binding pocket is specifically suited to accommodate N-terminal peptides, as opposed to lysine residues (35). The human NatA subunits are associated with ribosomes, but interestingly, significant fractions are also nonribosomal (19, 30, 32). Of further notice, the catalytic subunits, hNaa10p and hNaa50p, were also found to partially act independently of the hNatA complex (30, 36).

Recent studies have identified novel in vivo acetyl modifications of proteins. Mass spectrometry data of affinity-enriched acetylysine-containing peptides from HeLa cells showed the presence of propionylated and butyrylated lysines in histone H4 peptides (37). Similar analyses also showed the presence of propionylated lysines in p53, p300 and CREB-binding protein (38) besides the yeast histones H2B, H3 and H4 (39). Propionylated or butyrylated residues differ by only one or two extra methyl moieties as compared with their acetylated counterparts, thereby adding more hydrophobicity and bulkiness to the affected residue. To date, no distinct propionyl- or butyryltransferases responsible for these modifications have been identified. However, by using propionyl coenzyme A (Prop-CoA) or butyryl coenzyme A (But-CoA) as donors in the enzyme reaction, it was shown that some of the previously characterized lysine acetyltransferases (KATs) are able to respectively catalyze propionylation and butyrylation of lysine residues both in vitro (37, 40–42) and in vivo (38, 41). Similarly, it has been shown that lysine deacylases also are capable of catalyzing depropionylation (40, 41, 43, 44) and debutyrylation (44) (see review (45)).

Interestingly, mass spectrometry data also suggested that propionylated N-termini are present in human cell lines (46, 47). Until today, an N-terminal propionyl transferase (NPT) catalyzing N-terminal propionylation (Nt-propionylation) has to our knowledge not been identified.

In this study, we hypothesized that NATs might have the ability to act as NPTs. In vitro experiments using purified hNaa10p, hNaa50p or immunoprecipitated human NatA complex indeed confirmed their intrinsic capacity to catalyze Nt-propionylation toward synthetic peptides. NatA was also found capable of Nt-butyrylation in vitro. By means of Nterminomics, we further investigated the presence of yeast Nt-propionylated proteins in vivo. Indeed, we found evidence for Nt-propionylation being a naturally occurring modification in yeast. Interestingly, in a yeast strain lacking NatA, we observed a loss in Nt-propionylation and Nt-acetylation for several NatA substrates, as compared with a control yeast strain expressing endogenous NatA or a strain ectopically expressing hNatA. Thus, besides acting as NATs, yeast and human NatA can act as NPTs and we thus demonstrate for the first time that NATs have the capacity of both acetylating and propionylating protein N-termini in vivo and in vitro.

**Experimental Procedures**

*Yeasts and Sample Preparation—Saccharomyces cerevisiae* strains were made as follows. The haploid MATa strain BY4742 (Y10006, EUROSCARF) was transformed with an empty expression vector pBEVY-URA and used as a control strain termed yNatA. Y10976 (EUROSCARF) with NAA10::kanMX4 was transformed with an empty expression vector pBEVY-URA and used as a strain without yeast NatA termed yNatA. Y10976 was transformed with an expression vector pBEVY-URA-hNAA15-hNAA10 (1) and used as a strain expressing human NatA, but not yeast NatA termed y[NhNatA].

**Human Cell Culture—**Human A-431 cells (ATCC, CLR-1555) were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM l-glutamine, 50 mg/ml gentamicin, and 10% newborn calf serum (Lonza Group, Basel, Switzerland). Cells were cultured at 37 °C and in 5% CO2.

**Immunoprecipitation of NAT Complexes—**An aliquot of 5 × 10⁶ A-431 cells (per sample) were harvested and lysed in 500 μl of lysis buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 1 mM Pefabloc (Roche)). A total of 50 μl of protein A/G Agarose slurry (Santa Cruz) was added to the lysates and incubated rotating for 1 h at 4 °C. Following centrifugation at 2000 × g for 2 min, supernatants were collected and incubated for another 2 h at 4 °C with 1 μl anti-hNaa15p (anti-NATH in (19)) or custom-made unspecific rabbit IgG (Biogenes). Then, the samples were added 50 μl of Protein A/G Agarose. Following incubation for 16 h, three repetitive rounds of centrifugation and washing (twice in lysis buffer and once in acylation buffer), the samples were used for an in vitro peptide acylation assay.

**Plasmid Construction, Protein Expression, and Purification—**Plasmids used for expression of fusion proteins MBP-hNaa10p and GST-hNaa50p were pETM-41-hNaa10 and pETM-30-hNaa50, respectively. These plasmids as well as protein expression and purification were previously described (31).

**In Vitro Nt-Acetyltransferase Assay Using Synthetic Peptides—**Purified MBP-hNaa10p (5–300 nm) was mixed with selected oligopeptide substrates (300 μM) and Ac-CoA (100 μM) or Prop-CoA (100 μM) in a total volume of 60 μl in acylation buffer (50 mM Tris-HCl (pH 8.5), 1 mM dithiothreitol, 800 μM EDTA, 10% glycerol). The enzyme concentration was varied for the different substrates so that product formation would be detectable for weak substrates and would not cause product inhibition for strong substrates (48). The samples were incubated at 37 °C for 30 min, following which enzyme activities were quenched by the addition of 5 μl 10% trifluoroacetic acid. For the kinetic data, purified MBP-hNaa10p (5–50 nm) was mixed with a fixed amount of the EEEIA oligopeptide (300 μM) and varied amounts of Ac-CoA (5–150 μM) or Prop-CoA (5–150 μM) in 60 μl acylation buffer. Purified GST-hNaa50p (75–200 nm) was incubated with oligopeptide substrates (300 μM) and Ac-CoA (100 μM) and Prop-CoA (100 μM). The reaction mixture was incubated for 30 min at 37 °C before enzyme activity was quenched with 5 μl of 10% trifluoroacetic acid. For the kinetic data, purified GST-hNaa50p (75–250 nm) was mixed with a fixed amount of the MLGPE oligopeptide (300 μM) and varied amounts of Ac-CoA (5–75 μM) or Prop-CoA (5–75 μM) in 60 μl acylation buffer. The acylation reactions were quantified using RP-HPLC as described previously (49). When making use of immunoprecipitated material as enzyme input, the assay was carried out using 200
Identification of N-terminal Propionyltransferases

µM of oligopeptides and 200 µM of Ac-CoA, Prop-CoA, or But-CoA for 60 min. For kinetic data immunoprecipitated hNatA was incubated with a fixed amount of the SESSS oligopeptide (200 µM) and varied amounts of Ac-CoA, Prop-CoA, or But-CoA (25–150 µM) for 30 min. For time course experiments, the enzymatic reaction was stopped at 10, 30 or 60 min. For the immunoprecipitated material, the concentration of the hNaa10p used in the assays, was determined by SDS-PAGE and Western blotting analysis using anti-hNaa10p followed by quantification and purification recombinant hNaa10p using Image Gauge V 4.0.

Synthetic Peptide Sequences—Peptides (EEEIAAL, DDDIAAL, SESSSSK, MLGPEGG, MAPLDDL) were custom-made (Biogenes) to a purity of 80–95%. All oligopeptides contain seven unique acids at their N-termini, as these are the major determinants influencing Nt-acetylation. The next 17 amino acids are essentially identical to the adrenocorticotropic hormone peptide sequence (RWGRPV-GRRRRPVRV) however, lysines were replaced by arginines to minimize any potential interference because of N-acylation.

Proteome Preparation and (Differential) N-terminal COFRADIC Analysis—The yeast proteomes were prepared from 300 ml of culture at OD600 nm = 3.0 as described (1, 4). After harvesting, cells were washed twice in lysis buffer (50 mM Tris, 12 mM EDTA, 250 mM NaCl, 140 mM Na2HPO4, (pH 7.6)) supplemented with a complete protease inhibitor mixture tablet (1 tablet per 100 ml) (Roche Diagnostics) and glass beads were added before several rounds of vortexing and placing the samples on ice (10x). 1 ml of lysis buffer was used for a pellet resulting from 300 ml of yeast culture. The lysates were centrifuged at 5000 x g for 10 min and the retained supernatants were analyzed by N-terminal COFRADIC analysis (4, 50). Briefly, solid guanidinium hydrochloride was added to a final concentration of 4 M to inactivate proteases and denature all protein. Subsequently, proteins were reduced and alkylated simultaneously, using TCEP (1 mM final concentration (f.c.)) and iodoacetamide (2 mM f.c.) respectively, for 1 h at 30 °C in the dark. Subsequent steps of the N-terminal COFRADIC setups analyzed were performed as described previously (51) using 13C2D3-NHS-acetate and NHS-acetate at the protein level to block all primary protein amines for the comparative and differential analyses respectively. For the comparative analyses the subsequent steps of the N-terminal COFRADIC protocol were performed as described previously (50). For the differential analyses, the proteomes were digested at 37 °C overnight with sequencing-grade, modified trypsin (Promega, Madison, WI, USA) used at an enzyme/substrate ratio of 1/100 (w/w). After overnight tryptic digestion, the resulting peptide mixtures were vacuum-dried. Peptides starting with pyroglutamate were blocked prior to post-metabolic 15O-labeling and the SCX fractionation step. Here, 25 µl of pGAPase (25 U/ml, TAGZymeTM kit, Qiagen, Hilden, Germany) was activated for 10 min at 37 °C by adding 1 µl of 50 mM EDTA (pH 8.0), 1 µl of 800 mM NaCl, and 11 µl of freshly prepared 50 mM cystamine-HCl, 25 µl of Q-cyclase (50 U/ml, TAGZymeTM) was then added to the pGAPase solution. The dried peptides were re-dissolved in 212 µl of buffer containing 16 mM NaCl, 0.5 mM EDTA, 3 mM cystamine, and 50 µM aprotinin. The activated pGAPase and Q-cyclase mixture was added to the peptide sample and the mixture (275 µl total volume) was incubated for 60 min at 37 °C. The samples were further diluted separately to 1 ml in acetonitrile (CH3CN) and ammonium acetate (pH 5.5) to reach a f.c. of 2% CH3CN and 10 mM ammonium acetate. C18 solid-phase extraction (desalting step) of the pyroglutamate unblocked peptide mixtures was performed by loading these peptide mixtures onto an acucBONDII ODS-C18 SPE cartridge (1 ml tube, 100 mg, Agilent Technologies) as described previously (31). Peptides were eluted with 3 ml of 70% acetonitrile and vacuum-dried. For the differential N-terminal COFRADIC setups, post-metabolic 18O-labeling was used. Therefore, the dried peptide mixtures were re-suspended in a trypsin solution dissolved in H216O (yNatA sample) or in H218O (yNatAΔ and hNatA samples) respectively (equivalent of 1 µg of trypsin for each 100 µg of original protein input material), 5 µl of a 200 mM NH4HCO3 stock solution made in H216O or H218O respectively was added per 95 µl of each sample (f.c. of NH4HCO3 is 10 mM) and these samples were incubated overnight at 37 °C. Then, the 16O and the 18O-labeled samples were diluted separately in CH3CN and formic acid (HPLC-grade) to reach a f.c. of 50% CH3CN and 0.16% of formic acid (pH 2.6). Note that these conditions were optimized to avoid trypsin or acid-catalyzed oxygen back-exchange. Equal amounts of peptide material were mixed and N-terminal COFRADIC analysis was performed as described previously (4, 50).

LC-MS/MS analysis using an Ultimate 3000 RSLC nano-LTQ Orbitrap Velos system—Following COFRADIC enrichment of N-terminal peptides, each peptide fraction was re-dissolved in 40 µl of 2% acetonitrile prior to LC-MS/MS analysis. The peptide mixtures were introduced into an Ultimate 3000 RSLC nano LC-MS/MS system ( Dionex, Amsterdam, The Netherlands) in-line connected to an LTQ Orbitrap Velos (Thermo Fisher Scientific, Bremen, Germany). A 2.5 µl aliquot of the sample mixture was first loaded on a trapping column (made in-house, 100 µm I.D. × 20 mm length, 5 µl C18 Reprosil-HD beads). After back-flushing from the trapping column, the sample was loaded on a reverse-phase column (made in-house, 75 µm I.D. × 150 mm length, 3 µl C18 Reprosil-HD beads). Peptides were loaded with solvent A (0.1% trifluoroacetic acid in 2% acetonitrile) and were separated with a linear gradient from 2% of solvent A’ (0.1% formic acid in 2% acetonitrile) to 50% of solvent B’ (0.1% formic acid in 80% acetonitrile) at a flow rate of 300 nl/min followed by a steep increase to 100% of solvent B’.

The Orbitrap Velos mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS acquisition for the ten most abundant peaks in a MS spectrum. Full scan MS spectra were acquired in the Orbitrap at a target value of 16E6 with a resolution of 60,000. The ten most intense ions were then isolated for fragmentation in the linear ion trap, with a dynamic exclusion of 20 s. Peptides were fragmented after filling the ion trap at a target value of 1E4 ion counts. When operating the instrument in HCD, spectra were acquired in the Orbitrap with an effective FWHM resolution greater than 7500 around m/z 400. From the MS/MS data in each LC run, Mascot Generic Files were created using the Mascot Distiller software (version 2.3.2.0, Matrix Science, www.matrixscience.com/Distiller.html). While generating these peak lists, grouping of spectra was allowed with maximum intermediate retention time of 30 s and maximum intermediate scan count of 5. Grouping was done with a 0.005 Da precursor tolerance. A peak list was only generated when the MS/MS spectrum contained more than 10 peaks. There was no de-isotoping and the relative signal-to-noise limit was set at 2. The generated MS/MS peak lists were then searched with Mascot using the Mascot Daemon interface (version 2.3, Matrix Science). Searches were performed in the Swiss-Prot database with taxonomy set to yeast (S. cerevisiae) (7776 yeast sequences in UniProtKB/Swiss-Prot database version 2011_09 containing 532,146 sequence entries). For the differential setups; acetylation at lysines, carboxymethylation of cysteine and methionine oxidation to methionine-sulfoxide were set as fixed modifications and variable modifications were acetylation and propionylation of peptide N-termini and pyroglutamate formation of N-terminal glutamine. For the comparative analyses; 13C2D3-acetylation at lysines, carboxymethylation of cysteine and methionine oxidation to methionine-sulfoxide were set as fixed modifications and variable modifications were acetylation and 13C2D4-acetylation of peptide N-termini and pyroglutamate formation of N-terminal glutamine. Endoproteinase Arg-C/P (Arg-C specificity with arginine-proline cleavage allowed) was set as enzyme allowing no missed cleavages. The mass tolerance on the precursor ion was set to 10 ppm and on
fragment ions to 0.5 Da. The peptide charge was set to 1+, 2+ or 3+ and instrument setting was put on ESI-TRAP. Only peptides that were ranked one and scored above the threshold score, set at 99% confidence, were withheld. The estimated false discovery rate by searching decoy databases was typically found to lie between 2 and 4% on the spectrum level (51).

For the differential setups, all identified peptides were quantified using Mascot Distiller Toolbox version 2.3.2.0 (MatrixScience) in the precursor mode. The quantification configuration in Mascot was set to double $^{16}$O versus $^{18}$O labeling of C-termini (arginine). The software tries to fit an ideal isotopic distribution on the experimental data based on the peptide average amino acid composition. This is followed by extraction of the XIC signal of both peptide components (light (double $^{16}$O) and heavy (double $^{18}$O)) from the raw data. Ratios are calculated from the area below the light and heavy isotopic envelope of the corresponding peptide (integration method ‘trapezium’, integration source ‘survey’). To calculate this ratio value, a least squares fit to the component intensities from the different scans in the XIC peak was created. MS scans used for this ratio calculation are situated in the elution peak of the precursor determined by the Distiller software (XIC threshold 0.3, XIC smooth 1, Max XIC width 250). To validate the calculated ratio, the standard error on the least square fit has to be below 0.16 and correlation coefficient of the isotopic envelope should be above 0.97. Quantification of the degree of N-terminal acetylation was performed as described in (4). All data management was done by ms-lims (52).

Robust Statistical Analysis of the Measured Peptide Ratios—Following Mascot Distiller quantification and to identify up- or down-regulated proteins in a statistically significant way, the methods of robust statistics were applied to the base-2 logarithms of the calculated ratios of all the identified $^{16}$O/$^{18}$O peptide doublets being set as TRUE. Then, the R software package was used for statistical computing to calculate the probability distributions of log2 transformed peptide ratios. The ratios lying outside a 98% or higher level confidence interval, point to peptides with a statistical significant higher or lower concentration as compared with the control proteome digest and are thus indicated as such (setup 1; median $= 1.178$ and Huber scale 0.44; setup 2; median $= 1.22$ and Huber scale 0.46; the ratio means were also used to correct for small variations in initial protein concentrations). Ratios being set as FALSE (including all the singleton identifications) quantification software algorithms typically have difficulties to detect singletons; see also (53)) were all verified by visual inspection.

**Fig. 1.** NAT- and NPT-activity of recombinant hNaa10p toward synthetic N-terminal peptides. A, Product formation per minute per hNaa10p molecule. Purified MBP-hNaa10p (5–300 nM) was incubated with oligopeptide substrates and saturated levels of Ac-CoA or Prop-CoA in acylation buffer at 37 °C for 10 min. Product formation was determined using RP-HPLC. Experiments were performed in triplicates. B, Ac-CoA- and Prop-CoA saturation curves in the presence of EEEIA oligopeptide (300 μM) and purified MBP-hNaa10p (5 nm with Ac-CoA and 50 nm with Prop-CoA) in acetylation buffer at 37 °C for 30 min. Data were fitted to the Michaelis-Menten equation by Grafit 7 to determine kinetic parameters. Results shown are representative of three independent experiments.

**Fig. 2.** NAT- and NPT-activity of recombinant hNaa50p toward synthetic N-terminal peptides. A, Product formation per minute per hNaa50p molecule. Purified GST-hNaa50p (75 nM with Ac-CoA and 200 nm with Prop-CoA) was incubated with oligopeptide substrates and saturated levels of Ac-CoA or Prop-CoA in acylation buffer at 37 °C for 30 min. Product formation was determined using RP-HPLC. Experiments were performed in triplicates. B, Ac-CoA- and Prop-CoA saturation curves in the presence of MLGPE oligopeptide (300 μM) and purified GST-hNaa50p (75 nm with Ac-CoA and 250 nm with Prop-CoA) in acetylation buffer at 37 °C for 30 min. Data were fitted to the Michaelis-Menten equation by Grafit 7 to determine kinetic parameters. Results shown are representative of three independent experiments.
Identification of N-terminal Propionyltransferases

RESULTS

Human Naa10p and Naa50p are N-terminal Propionyltransferases In Vitro—The studies of Dormeyer et al. and Zhang et al. recently reported the existence of propionylated N-terminals in human cells (46, 47). We hypothesized that the existing human Nat-acetylation machinery would also be capable of Nat-propionylation as well as Nat-acetylation and therefore performed in vitro assays using purified hNaa10p and hNaa50p. hNaa10p was challenged with Ac-CoA or Prop-CoA and one of three representative NatA-type substrate peptides. SESSS represents a classical co-translational NatA substrate whereas DDDIA or EEEIA respectively represents the β- or γ-actin N-termini, likely representative of post-translational NatA/hNaa10p targets (30). Determination of product formation was carried out by a previously developed quantitative method based on RP-HPLC peptide separation (49). hNaa10p Nat-propionylated all peptides, but the level of Nat-propionylation was lower than for Nat-acetylation. For the EEEIA-peptide Nat-acetylation was ~10-fold higher, whereas for the DDDIA-peptide, Nat-acetylation was ~10-fold higher. hNaa10p was less active toward the SESSS-peptide as compared with the two other peptides, and the level of Nat-acetylation was ~threefold higher than for Nat-propionylation (Fig. 1A). Kinetic data was produced by varying the Ac-CoA and Prop-CoA concentrations while using a fixed amount of EEEIA-peptide. These results revealed that the $k_{cat}$ was much higher for Ac-CoA than for Prop-CoA, whereas the $K_m$ value was in the low $\mu$M range for both substrates (Fig. 1B, supplemental Table S1). The large difference in $k_{cat}$ rendered the specificity constant ($k_{cat}/K_m$) ~fivefold higher for Ac-CoA than for Prop-CoA (supplemental Table S1).

To investigate whether the NPT-activity was shared by other NATs, we also tested hNaa50p, hNaa50p Nat-propionylated both oligopeptides tested, but similar to hNaa10p, the extent was significantly lower compared with the degree of Nat-acetylation. For the MLGPE-substrate peptide, Nat-acetylation was ~sevenfold more effective, whereas for the MAPL-peptide, Nat-acetylation was ~25-fold more effective (Fig. 2A). To determine why Nat-acetylation is more efficient as compared with Nat-propionylation, kinetic studies with the MLGPE-peptide were performed. These studies revealed similar $K_m$ values in low $\mu$M range for both Prop-CoA and Ac-CoA (Fig. 2B, supplemental Table S1). The $k_{cat}$, however, is almost 10-fold higher for Ac-CoA than for Prop-CoA, rendering the specificity constant ~20-fold higher (supplemental Table S1).

These results demonstrate that both hNaa10p and hNaa50p are NPTs in vitro, and indicate that Nat-propionylation might be a general activity shared among different NATs.

The Human NatA Complex is an N-terminal Propionyltransferase In Vitro—To determine whether the endogenous hNatA complex possessed an intrinsic NPT-activity, we immunoprecipitated NatA using anti-hNaa15p. This ensured that all the catalytic hNaa10p molecules present in the sample were anchored to the complex. As observed for recombinant hNaa10p, NatA preferred catalyzing Nat-acetylation as compared with Nat-propionylation of the EEEIA- N-terminus. However, and in contrast to purified hNaa10p, for the DDDIA- and SESSS- N-termini, the human NatA complex was equally capable of carrying out Nat-propionylation as compared with Nat-acetylation (Fig. 3A), demonstrating that complexed hNaa10p or NatA is an NPT in vitro. The immunoprecipitated NatA was also challenged with But-CoA, but a decreased butyrylation activity as compared with acetylation and propionylation activity was observed for all N-termini tested. For the DDDIA- and SESSS- N-termini, the transferase activity was approximately halved, indicating that optimal enzymatic activity requires less than three methyl group on the CoA derivative (Fig. 3A). The kinetic data revealed that in contrast to the recombinant enzymes, the $K_m$ values of the three substrates were comparable. However, because of the decreasing $K_m$ values with each addition of a methyl group to the substrate,
the $K_{cat}/K_m$ values were over twofold and nearly fivefold higher for Prop-CoA and But-CoA respectively, compared with Ac-CoA (Fig. 3B and supplemental Table S1). Nt-propionylation was previously described to occur on 7 human proteins including three of which matched the (non-) canonical NatA acetylation substrate specificity, being $\beta$-actin (DD-), chromobox protein homolog 3 (AF-) and cytochrome b-c1 complex subunit 6 (DP-) (46, 47), the latter being a processed protein indicating post-translational rather than co-translational propionylation. All of these point to the $in vivo$ relevance of this observation also for human NatA.

N-terminal Propionylation in Yeast and NatA Acting as an $in Vivo$ N-terminal Propionyltransferase—To investigate the evolutionary conservation of Nt-propionylation and elaborate on our $in vitro$ data suggesting that NATs may act as NPTs, we decided to apply N-terminal COFRADIC positional proteomics to a yeast model (54). Briefly, two different proteome preparation strategies for the (comparative or differential) analyses of yeast N-terminomes were applied. First, differential proteome analyses were performed making use of trypsin-catalyzed C-terminal oxygen exchange. As such, the proteome digest of one sample was $^{16}$O-tagged (yNatA) and the other sample was $^{18}$O-tagged (yNatAΔ or hNatA). Equal amounts of these differentially labeled samples were mixed followed by SCX enrichment and N-terminal peptide sorting by means of N-terminal COFRADIC. Sorted fractions were subsequently analyzed by LC-MS/MS analysis. The left and right MS-spectra are representative of spectra belonging to an Nt-acetylated and Nt-propionylated N-terminus of a NatA substrate N-terminus. The Nt-acetylated N-terminus was recovered as an isotopic couple separated by 4 Da whereas the Nt-propionylated counterpart was uniquely identified in the yNatA setup (Single $^{16}$O). ‘Ac’ denotes $in vivo$ (black) or $in vitro$ (orange) Nt-acetylation whereas ‘Prop-’ indicates $in vivo$ Nt-propionylation.

**Fig. 4. Outline of the differential N-terminal COFRADIC strategy used to identify NatA mediated $in vivo$ Nt-propionylation.** Proteomes from yeast cells were prepared for differential N-terminal peptide analysis. After protein S-alkylation, reduction with tris(2-carboxyethyl)phosphine (TCEP), protein S-alkylation and $in vitro$ acetylation of primary amines, the proteomes are digested with trypsin. Pyroglutamate residues are enzymatically removed by the combined action of glutamyl cyclase and pyroglutamyl-carboxylase peptidase. Subsequently, a differential post-metabolic strategy was applied using trypsin-catalyzed C-terminal oxygen exchange. As such, the proteome digest of one sample was $^{16}$O-tagged (yNatA) and the other sample was $^{18}$O-tagged (yNatAΔ or hNatA). Equal amounts of these differentially labeled samples were mixed followed by SCX enrichment and N-terminal peptide sorting by means of N-terminal COFRADIC. Sorted fractions were subsequently analyzed by LC-MS/MS analysis. The left and right MS-spectra are representative of spectra belonging to an Nt-acetylated and Nt-propionylated N-terminus of a NatA substrate N-terminus. The Nt-acetylated N-terminus was recovered as an isotopic couple separated by 4 Da whereas the Nt-propionylated counterpart was uniquely identified in the yNatA setup (Single $^{16}$O). ‘Ac’ denotes $in vivo$ (black) or $in vitro$ (orange) Nt-acetylation whereas ‘Prop-’ indicates $in vivo$ Nt-propionylation.
Nt-propionylated N-termi and their Nt-acetylated counterparts identified in yeast. List of the 10 unique Nt-propionylated and corresponding yeast N-termi identified in the two differential proteome setups (i.e., the yNatA versus yNatA$^/$H9254 (setup 1) and the yNatA versus hNatA setup (setup 2)). N-termi 1 to 8 represents database annotated N-termi, whereas N-termi 9 and 10 represent N-termi with an internal protein position. Nt-propionylation for five out of the six NatA type N-termi identified in setup 1 was found to be yNatA dependent (single $^{16}$O/single yNatA). In each of the cases identified, ectopic expression of hNatA in yeast, (partially) restored Nt-propionylation. UniProt entry, start, N-terminal modification status, corresponding peptide sequence identified, difference in retention time between the Nt-propionylated and the Nt-acetylated N-termi, NAT type, number of identified spectra (per setup), corrected $^{16}$O/$^{18}$O ratios, statistical significance, highest Mascot ion score (max (score)) and percentage of Nt-acetylation in the control and yNatA$^/$H9254 yeast strains are indicated (N-termi found to be less than 2% or more than 98% Nt-acetylated, were considered as free or fully Nt-acetylated respectively). Whenever corresponding HCD spectra gave further evidence of the residence of an Nt-propionyl group, this is indicated with an asterisk. Whenever the N-termi was not identified this is indicated with N.I. (not identified), and when the NatA dependency of Nt-acetylation could not be calculated, this is indicated as N.D. (not determined).

| UniProt | Start | N-term | Modified_sequence | ΔRT (Rtprop-RTAc) | yNatA versus yNatA$^/$H9254 | yNatA versus hNatA | Max (score) | %Ac yNatA | %Ac yNatA | NatA substr. |
|---------|-------|--------|-------------------|-------------------|--------------------------|-------------------|-------------|-----------|-----------|--------------|
| 1       | 2     | Prop-  | STELVGSER         | 1.7 A             | 1 1.33                  | –                 | 45          | 100%      | 0%        |              |
|         |       | Ac-    |                   | 17 1.10            | –                       | 18 0.99           | –           |    76      |            |              |
| 2       | 1     | Prop-  | MNTEGASLSEQLLDAAR | 0.9 B             | –                       | 1 0.72             | –           | 46 100%   | 100% No   |              |
|         |       | Ac-    |                   | 17 0.75            | –                       | 24 1.41            | –           |    145     |            |              |
| 3       | 2     | Prop-  | AVSKVYAR          | 4.9 A             | –                       | 4 0.88             | –           | 47 0%     | 0%        | No           |
|         |       | Ac-    |                   | 26 1.35            | –                       | 579 0.88           | –           |    77      |            |              |
| 4       | 2     | Prop-  | SEVIEGNKIDR*      | 2.1 A             | –                       | 3 1.92             | –           | 68 100%   | 0%        | /            |
|         |       | Ac-    |                   | 26 1.24            | –                       | 117 1.03           | –           |    103     |            |              |
| 5       | 2     | Prop-  | GVEQILKR          | 3 A               | –                       | 1 1.32             | –           | 60 0%     | 0%        | No           |
|         |       | Ac-    |                   | 81 1.22            | –                       | 4 1.15             | –           |    66      |            |              |
| 6       | 2     | Prop-  | SGKGGKAGSAKASQR*  | 0.9 D             | –                       | 2 3.01             | –           | 99% yNatA | 89        | 100% 100% No |
|         |       | Ac-    |                   | 264 1.21           | –                       | 579 0.88           | –           |    77      |            |              |
| 7       | 2     | Prop-  | SQVYFDVAGQPQGR*   | 6.3 A             | –                       | 1 1.59             | –           | 102 100%  | 0%        | /            |
|         |       | Ac-    |                   | 17 1.47            | –                       | 35 0.91            | –           |    116     |            |              |
| 8       | 2     | Prop-  | SEITLGKYLFER*     | 1.5 A             | –                       | 1 1.59             | –           | 102 100%  | 0%        | /            |
|         |       | Ac-    |                   | 1 1.59             | –                       | 104 1.06           | –           |    144     |            |              |
| 9       | 37    | Prop-  | STKAQUTEVSSILEER  | 1 A               | –                       | 1 0.99             | –           | 85 100%   | 0%        | /            |
|         |       | Ac-    |                   | 4 2.20             | –                       | 85 0.99            | –           |    102     |            |              |
| 10      | 36    | Prop-  | SSSPEQKRY         | – A               | –                       | 2 2.31             | 98% yNatA  | 103       | 87% N.I.  | N.D.        |
In brief, all primary amines are chemically modified by acetylation at the protein level. On tryptic digestion, this creates a pool of two groups of peptides, one group that contains blocked N-termini (either \textit{in vitro} or \textit{in vivo} blocked protein N-terminal peptides) and one group that has a newly generated, free N-terminus (internal peptides), which is exposed to amino-reactive reagents in a diagonal chromatography setup following a strong cation exchange (SCX) based enrichment. It is of note in contrast to the second methodology (see below); the applied strategy does not allow to differentiate between \textit{in vitro} and \textit{in vivo} acetylated N-termini.

Second, and to assess the NatA-dependence of Nt-acetylation \textit{in vivo} (i.e. assign NatA substrates) (1), comparatively analyzed protein N-termini and their degrees of acetylation from the isolated N-terminomes of the yNatA and yNat\Delta strains. Therefore, all primary amines are chemically modified by $^{13}\text{C}_2\text{D}_3$-acetylation, before tryptic digestion. This creates a pool of two groups of peptides, one group that contains blocked N-termini (either \textit{chemically} or \textit{in vivo} blocked protein N-terminal peptides) and one group that has a newly generated, free N-terminus (internal peptides), which is exposed to amino-reactive reagents in a diagonal chromatography setup following a strong cation exchange (SCX) based enrichment. Further, differentiation between chemically acetylated N-termini and \textit{in vivo} acetylated N-termini is made possible by the 5 Da spacing between these two types which thereby allows the calculation of the extent of Nt-acetylation (1, 46).

Using the differential strategy, overall, 10 unique N-termini were identified in their \textit{in vivo} Nt-propionylated form (Table I and supplemental Fig. S2), clearly demonstrating the existence of Nt-propionylation in yeast. A representative example is shown in Fig. 5; both peptide forms show a similar fragmentation pattern, whereas the differential N-terminal modification state is evident from an unchanged y-ion series and mass-shifted b-ions. Because lower mass fragments ions are often missed when fragmenting peptides in ion traps and to confirm the exact localization of the propionyl group at the N-terminus, Higher Energy Collision Dissociation or HCD was applied (56) (Table I). A representative combined HCD/CID spectrum illustrates the additional presence of y$_1$ and the indicative b$_0^1$ and b$_0^2$ N-terminal propionyl reporter ions (Fig. 5). Furthermore, we observed a higher spectral count number for the Nt-acetylated peptides compared with only one or two identified spectra of their Nt-propionylated counterparts.
Although not very precise, spectral counts can be used as a rough quantitative measurement (57), and therefore suggest that Nt-propionylation occurs in yeast, albeit at a low frequency under the experimental conditions studied. Interestingly, the identified Nt-propionylated N-termini belong to several classes according to NAT-specificities: NatA (Ser-, Ala- and Gly- N-termini), NatB (Met-Asn- N-terminus) and NatD (Ser-Gly-Gly- of Histone H2A). This indicates that different yeast NATs may act as NPTs in vivo.

Further, two of the identified N-termini represent internal, nondatabase annotated N-termini of mitochondrial proteins of which the targeting sequence was lacking, thus pointing to either alternative translation initiation accompanied by co-translational Nt-propionylation or proteolytic cleavage followed by post-translational Nt-propionylation.

To address whether the in vivo Nt-propionylation observed in yeast is mediated by the NAT enzymes, we focused on the major NAT, NatA, of which our in vitro data suggested a role as NPT. We prepared and analyzed the N-terminomes of a control strain expressing yeast NatA (yNatA), a strain lacking NatA (yNatA/H9004) and a strain lacking yeast NatA while expressing human NatA (y[hNatA]) (supplemental Fig. S1). Eight out of the 10 identified Nt-propionylated N-termini displayed a NatA-type substrate sequence (Ser-, Ala-, Gly-). Importantly, in the yNatA and/or y[hNatA] strain, several of these NatA-type N-termini were identified in their in vivo Nt-propionylated form, whereas this variant remained undetected in the yNatA strain, implying that both yeast and human NatA are acting as both NATs and NPTs in vivo (Fig. 6 and supplemental Fig. S3).
In the analysis monitoring the extent of in vivo Nt-acetylation and to further strengthen the NatA-type substrate claim of the Nt-propionylated N-termini identified, the yNatA dependence of Nt-acetylation was determined (Table I). Of the 10 Nt-propionylated N-termini reported; four out of the six Ser-starting and thus NatA type N-termini were shown to be Nt-acetylated in a NatA dependent fashion, all except of one being >98% Nt-acetylated in vivo, while being Nt-free in the yNatA delta strain (Table I and Fig. 7). As expected, the prop-MNTEGASLSEQLLDAAR (NatB type) Fig. 7 and the prop-SGGKGGKAGSAAKASQSR (NatD type histone N-terminus) N-termini were both found to be Nt-acetylated in a NatA independent fashion (i.e. in yNatA and yNatAΔ identified as being >98% Nt-acetylated). The two remaining Ala- and Gly-starting N-termini however were never identified in their Nt-acetylated form.

**DISCUSSION**

As compared with the number of Nt-acetylated N-termini identified in vivo, there are only a few Nt-propionylated N-termini identified. Our data demonstrates that NATs act as NPTs and are responsible for Nt-propionylation in vivo. The NPT-activity of the purified enzymes however, was found to be lower than the NAT-activity toward most N-termini tested. Despite this, our data do not readily explain why there are not more Nt-propionylated N-termini in vivo. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide the in vitro NPT- and NAT-activities of the NatA complex were even comparable, raising the question of why we did not identify more Nt-propionylated N-termini in vitro. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide the in vitro NPT- and NAT-activities of the NatA complex were even comparable, raising the question of why we did not identify more Nt-propionylated N-termini in vitro. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide the in vitro NPT- and NAT-activities of the NatA complex were even comparable, raising the question of why we did not identify more Nt-propionylated N-termini in vitro. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide the in vitro NPT- and NAT-activities of the NatA complex were even comparable, raising the question of why we did not identify more Nt-propionylated N-termini in vitro. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide the in vitro NPT- and NAT-activities of the NatA complex were even comparable, raising the question of why we did not identify more Nt-propionylated N-termini in vitro. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide the in vitro NPT- and NAT-activities of the NatA complex were even comparable, raising the question of why we did not identify more Nt-propionylated N-termini in vitro. When solely relying on the in vitro data, the expected level of Nt-propionylation should be somewhere between 5 to 20% of the level of Nt-acetylation. For the DDDIA- and SESSS-peptide
Identification of N-terminal Propionyltransferases

approximately 20-fold (59). The latter observation was further supported in a recent study, which found that in murine hepatocytes the concentration of Ac-CoA was 20-fold higher as compared with Prop-CoA. Together, these studies clearly indicate that in a cellular context, Ac-CoA is more abundant than Prop-CoA and demonstrate the potential dynamicity of these modifications. Thus, even though the NAT-NPT-machinery in the cell is capable of catalyzing both Nt-acetylation and Nt-propionylation, the Ac-CoA/Prop-CoA ratio may greatly determine the modification status of the N-terminal proteome. Interestingly, the in vivo Nt-propionylated proteins identified in the current study and those identified previously (46) comprise a variety of N-terminal sequences. In fact, all the major NAT-clases, NatA, NatB and NatC/E/F are represented. This, and our results defining also hNaa50p/NatE as a potential NPT, clearly suggests that the NATs may at least to some extent act as NPTs in vivo. Interestingly, hNaa10p and hNaa50p were suggested to act as lysine acetyltransferases (KATs) besides acting as noncatalytic modulators. The major functional implications of protein Nt-acetylation are increasingly supported by experimental data. Future investigations will reveal whether Nt-propionylation mediates distinct functional effects as compared with Nt-acetylation, or whether these two modifications are interchangeable in terms of functional output.

In conclusion, Nt-propionylation is a naturally occurring modification in yeast and humans, and the eukaryotic NAT-enzymes have the intrinsic capability to act as combined NATs and NPTs in vivo.

Identification of N-terminal Propionyltransferases

This article contains supplemental Figs. S1 to S3 and Table S1.

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