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NAT6 acetylates the N-terminus of different forms of actin

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All forms of mammalian actin comprise at their N-terminus a negatively charged region consisting of an N-acetylated aspartate or glutamate followed by two or three acidic residues. This structural feature is unique to actins and important for their interaction with other proteins. The enzyme catalyzing the acetylation of the N-terminal acidic residue is thought to be NAA10, an enzyme that acetylates multiple intracellular proteins. We report here that this acetylation is essentially carried out by NAT6 (Fus2), a protein of unknown function. Tests of the activity of human recombinant NAT6 on a series of purified proteins showed that the best substrate had several acidic residues near its N-terminus. Accordingly NAT6 was particularly active on highly acidic peptides with sequences corresponding to the N-terminus of different forms of mammalian actins. Knocking out of *NAT6* in two human cell lines led to absence of acetylation of the first residue of mature beta-actin (Asp2) and gamma-actin-1 (Glu2). Complete acetylation of these two actins was restored by re-expression of NAT6, or by incubation of extracts of *NAT6*-deficient cells with low concentrations of recombinant NAT6, while NAA10 showed much less or no activity in such assays. Alpha-actin-1 expressed in *NAT6*-knockout cells was not acetylated at its N-terminus, indicating that the requirement of NAT6 for acetylation of actin N-termini also applies to the skeletal muscle actin isoform. Taken together, our findings reveal that NAT6 plays a critical role in the maturation of actins by carrying out the acetylation of their N-terminal acidic residue.

Introduction

About 80% of mammalian proteins are acetylated at their N-terminus and six different *N*-acetyltransferase complexes (called NatA to NatF; for nomenclature see Ref. 1) are known to be involved in this process in mammals [2]. Their specificity is mainly dictated by the identity of the first few residues of the sequence of their protein substrates. For example, NatA mainly acetylates proteins where the initiator methionine has been removed and which start with serine, alanine, threonine, valine or glycine [3] while NatB preferentially acetylates an N-terminal methionine followed by glutamate, aspartate, glutamine or asparagine [4]. The catalytic subunits of all these *N*-acetyltransferases belong to the GCN5 (general control of non

Abbreviations
DTNB, 3,3′-dithio-bis(6-nitrobenzoic acid); Fmoc, fluorenylmethoxycarbonyl; GCN5, general control of non derepressible 5; HCD, high-energy collision induced dissociation; IPTG, isopropyl-β-D-thiogalactoside; NSI, nanospray ionization; PMSF, phenylmethylsulfonylfluoride; SB4, 4× concentrated reducing sample buffer; XIC, extracted ion chromatogram.

derepressible 5) family of *N*-acetyltransferases. In some cases, these catalytic subunits (NAA10 in the case of NatA and NAA20 in the case of NatB) are physiologically associated with other subunits, while in other cases the catalytic subunits seem to function alone.

The physiological importance of N-terminal acetylation is underlined by the observation that knocking out of NAA10 is incompatible with life in different organisms such as *Trypanosoma brucei*, *Drosophila melanogaster* and *Danio rerio* [5–7] though not in the yeast *Saccharomyces cerevisiae* [8]. In specific cases, N-terminal acetylation has been shown to affect protein half-life, subcellular targeting and association with other proteins [2]. Yet, for most proteins, the physiological role of N-terminal acetylation is still unknown.

In the present work, we reveal that the acetyltransferase NAT6 (also known as Fus-2) [9] is necessary and sufficient for the N-terminal acetylation of actin proteins, which constitute one of the most abundant protein families with unique contractile properties [10]. NAT6, a member of the GCN5 family, is present in vertebrates and invertebrates, but only limited functional data was available on this enzyme. Recombinant NAT6 indeed was shown to catalyse the acetylation of a peptide starting with a Met-Asp sequence, but found to be inactive on two other peptides that were tested [9]. No characterization of the acetylation site has been performed, but based on this limited information it was concluded that the substrate specificity of this enzyme might be similar to the one of NatB.

Actins are very abundant proteins that play an important role in cell architecture and motility [10]. Vertebrates have six different actins, which are encoded by six different genes. Actin proteins are highly conserved during evolution. Furthermore, the different actin forms present in vertebrates show extraordinary similarity with more than 90% amino acid identity, although they perform quite distinct functions. Actins are categorized in 2 classes based on the sequence of the N-terminus in the non-mature forms [11]. In class I actins (beta-actin and gamma-actin-1), the initiator methionine is directly followed by 3 acidic residues, while class II actins (alpha-actin-1 and -2, cardiac-alpha-actin and gamma-actin-2) are characterized by the presence of a cysteine between the initiator methionine and the stretch of 3–4 acidic residues (Fig. 1).

The mature actin forms all start with an *N*-acetylated acidic residue at the first position [11] and this seems to be important for their efficient interaction with other proteins, particularly myosin [12–14] (see Discussion). Maturation of actin N-termini requires at least two distinct *N*-acetyltransferases, and we will briefly summarize the commonly accepted model of this process (Fig. 1). In the case of class I actins, the first methionine is acetylated by NatB (beta-actin; most likely also gamma-actin-1) [4]. The resulting *N*-acetylmethionine is then removed by acylaminoacyl peptidase, an enzyme that specifically removes the first *N*-acetylamino acid from proteins [15]. Subsequently, the exposed N-terminal acidic residue is acetylated by NAA10 [3]. For class II actins, the first methionine is removed co-translationally by methionine aminopeptidase exposing an N-terminal cysteine residue, which is acetylated by an unknown acetyltransferase and removed by acylaminoacyl peptidase [15]. Finally, as for class I actins, the first acidic amino acid residue is assumed to be acetylated by NAA10 [3]. Thus, for all actin proteins the successive action of at least two *N*-acetyltransferases and one or two peptidases results in an *N*-acetylated acidic amino acid at the N-terminus.

Here, we fundamentally revise the current model of actin maturation and reveal that NAT6 is responsible for the acetylation of the first acidic amino acid of beta-actin and gamma-actin-1 and likely other actins.

Results

NAT6 can acetylate a protein N-terminus containing acidic residues

To glean insights into its substrate specificity, we first checked the *N*-acetyltransferase activity of NAT6 on a series of 14 purified recombinant proteins that were available in the laboratory and had been produced in *Escherichia coli*. At baseline, we did not observe any evidence for N-terminal acetylation of these proteins by mass spectrometry (with N-terminal peptides being detectable in 10 out of 14 proteins). Loss of the initiator methionine was observed only in two proteins, in good agreement with the known specificity of *E. coli* methionine aminopeptidase [16]. Based on this specificity and the amino acid sequence, we may assume that the initiator methionine is also retained in the four proteins for which we did not detect N-terminal peptides. Of note, analysis of NAT6 indicated that its N-terminus was partially acetylated, as indicated by MS/MS analysis of tryptic peptides: nine PSMs (peptide spectrum match) corresponded to a doubly charged peptide with $m/z = 657.2$ and sequence AcMQELTLSPGPAK, while two corresponded to the same, non-acetylated peptide with $m/z = 636.2$. This indicated that an autoacetylation reaction had occurred during the production of the recombinant protein [9] (see also below).

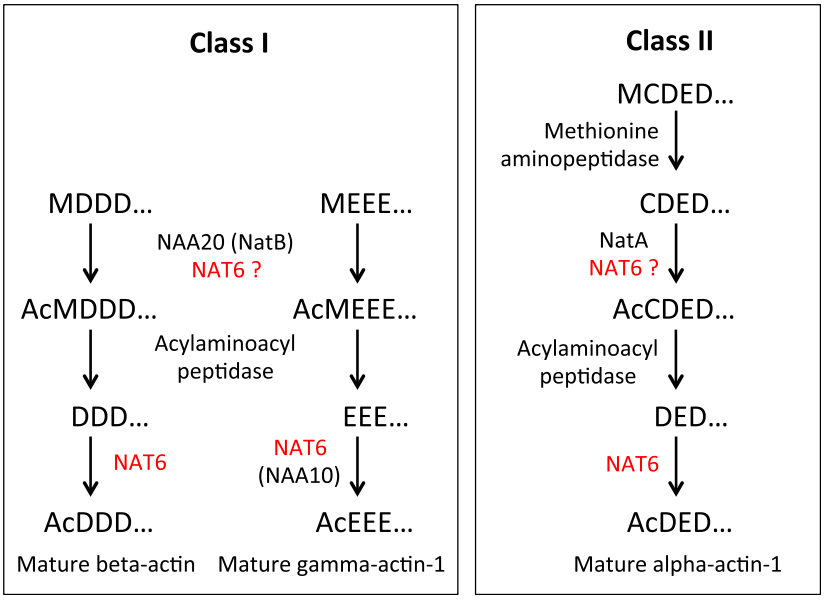


Fig. 1. Maturation of the N-terminus of different mammalian actins. Processing of class I actins (beta-actin and gamma-actin-1) involves the acetylation of the initiator methionine, removal of the *N*-acetylmethionine and acetylation of the amine of the second residue. Processing of class II actins involves removal of the initiator methionine, acetylation of Cys2, removal of *N*-acetylated cysteine and acetylation of the amine of the third residue. The role of NAT6 in actin acetylation is demonstrated in the present work.

Acetylation assays were performed by incubating NAT6 with these recombinant proteins in the presence of radiolabelled acetyl-CoA and stopping the reaction by quenching with acid. Incubation of NAT6 alone led to some time-dependent incorporation of radioactivity in this protein, confirming that NAT6 autoacetylates. Incorporation of radioactivity was considerably enhanced in the presence of FrlB, an *E. coli* protein that contains acidic residues in position +3 and +5 (Fig. 2A). Mass spectrometry analysis of the main radioactive fraction obtained by purification of the trypsin-digested protein by HPLC indicated that the N-terminal peptide (MLDIDK) was acetylated at its N-terminal methionine (Fig. 2B). These results suggested that NAT6 could acetylate the N-terminus of proteins that are rich in acidic residues.

NAT6 acetylates the N-termini of acidic peptides corresponding to actin N-termini

To further delineate the substrate spectrum of NAT6, we tested its activity on different synthetic peptides. Of particular interest was the possibility that NAT6 could acetylate the N-termini of actins, which in all cases are highly negatively charged. Mature actins start with a stretch of 3–4 acidic residues, where the N-terminal one is acetylated. In addition, acetylation of the initiator methionine (in beta or gamma-actin-1) or the cysteine in position 2 (in all other actins) is required during the maturation process of actin proteins (see Introduction). Hence, we synthesized peptides corresponding to the N-terminus of different forms of actin at various stages of maturation (Fig. 3) and also included some other peptides.

Activity of NAT6 on these peptides was assessed by measuring the release of free coenzyme A with DTNB. This revealed that the different peptides corresponding to the N-terminus of different forms of actin were excellent substrates for NAT6, much better than any of the other peptides that we tested (Fig. 3A). As expected, the presence of an acetyl group at the N-terminus of the peptides completely prevented the acetylation reaction (Fig. 3B). Remarkably, high activity was observed irrespective of the presence or absence of an N-terminal methionine (beta and gamma-actin-1) or an N-terminal Met-Cys dipeptide (alpha-actin-1) (Fig. 3B). These findings suggested that NAT6 might contribute to several steps in the course of actin protein maturation. On the one hand, it might participate in acetylation required for the removal of Met1 (class I actins) or Cys2 (class II actins) and on the other hand, it might be required for the final acetylation of the N-terminal Glu or Asp (Fig. 1).

Inactivation of NAT6 almost abolishes the immunoreactivity to antibodies targeting mature actin N-termini

To check the importance and determine the precise role of NAT6 in actin maturation, we studied cell models in which the *NAT6* gene had been inactivated. HAP1 cells in which a 17 bp deletion causes a frameshifting in exon 2 (the unique coding exon of the human *NAT6* gene) were obtained from a commercial source. U2OS cells in which the two copies of the *NAT6* gene were inactivated were produced with the CRISPR/Cas9 technique. Sequencing of the gene

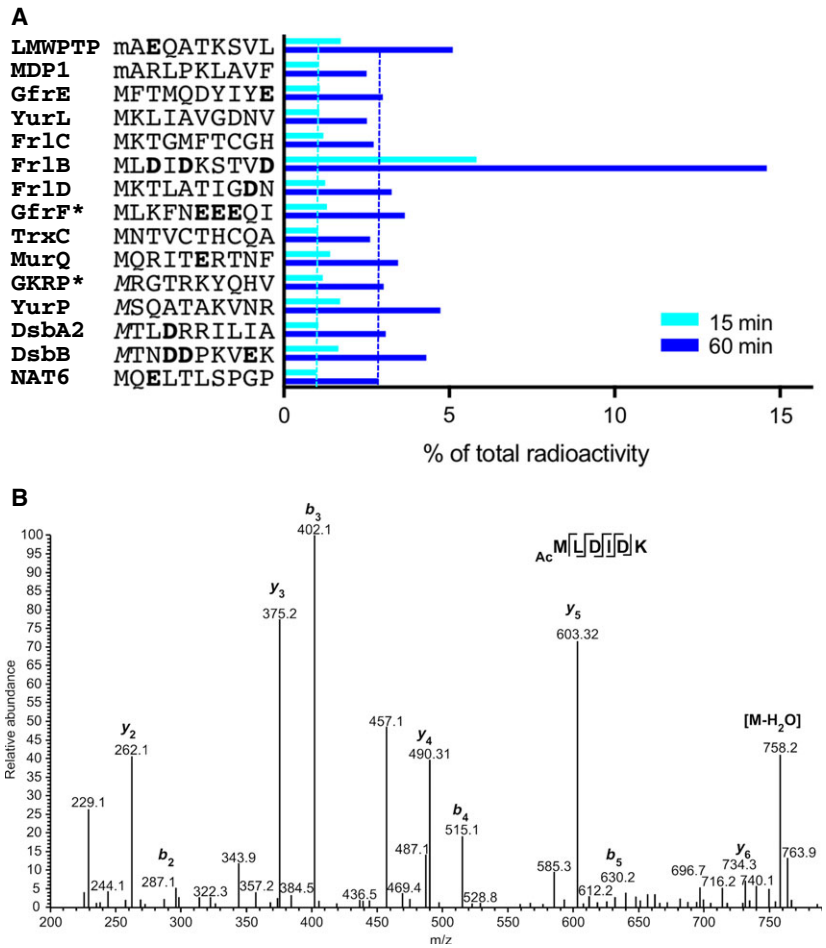


Fig. 2. Activity of NAT6 on a series of recombinant proteins and identification of the acetylated residue in FrlB. (A) Activity was measured with a radiochemical assay on the indicated proteins [31–39] by measuring the incorporation of [^{3}H] acetate into protein. The indicated protein substrates were incubated at 5 and 1 μM (*) in the presence of 1 μM NAT6 and 8 μM acetyl-CoA for 15 or 60 min. The N-terminal sequences of the different protein substrates are shown, with the acidic residues in bold. The presence (M) or absence (m) of the initiator methionine was determined by mass spectrometry analysis of tryptic peptides (see Materials and Methods). Italicized *M* indicates cases where in all likelihood the methionine is still present, though this could not be confirmed experimentally. The dotted lines represent the level of autoacetylation of NAT6. The figure shows one representative experiment out of two. (B) FrlB acetylated in the presence of radiolabelled acetyl-CoA for 60 min was digested with trypsin and the HPLC fraction containing the radioactive peptide was analysed by LC-MS/MS. The figure shows the MS/MS spectrum of the singly charged parent ion $[M + H]^{1+}$ = 776.2. The *y*5 fragment (*m/z* 603.32, i.e. loss of 173 amu) indicates that the N-terminal methionine is acetylated.

indicated the presence of two allelic 8 and 17 bp frameshifting deletions in exon 2 (data not shown).

Western blots performed on cell extracts with antibodies recognizing the mature forms (i.e. starting with an N-acetyl-Asp or N-acetyl-Glu) of beta-actin and of gamma-actin-1 showed a strong signal in extracts of wild-type cells, but no signal at all (beta-actin) or a very faint one (gamma-actin-1) in extracts of *NAT6*-deficient cells (Fig. 4A). Complementation of *NAT6*-deficient cells by infection with a lentiviral vector expressing mouse Nat6 restored a normal signal.

Furthermore, incubation of extracts of *NAT6*-KO cells with recombinant NAT6 and acetyl-CoA rapidly caused reappearance of the beta and the gamma actin bands with similar intensities as in extracts of wild-type cells (Fig. 4B). This demonstrated that the acetylation carried out by NAT6 is required for immunoreactivity. Similar incubations performed with wild-type cell extracts did not cause any modification in the apparent intensity of the beta-actin or gamma-actin-1 bands (Fig. 4B). It should be noted that the restoration of a normal signal by incubation of *NAT6*-deficient extracts with recombinant NAT6 only required very

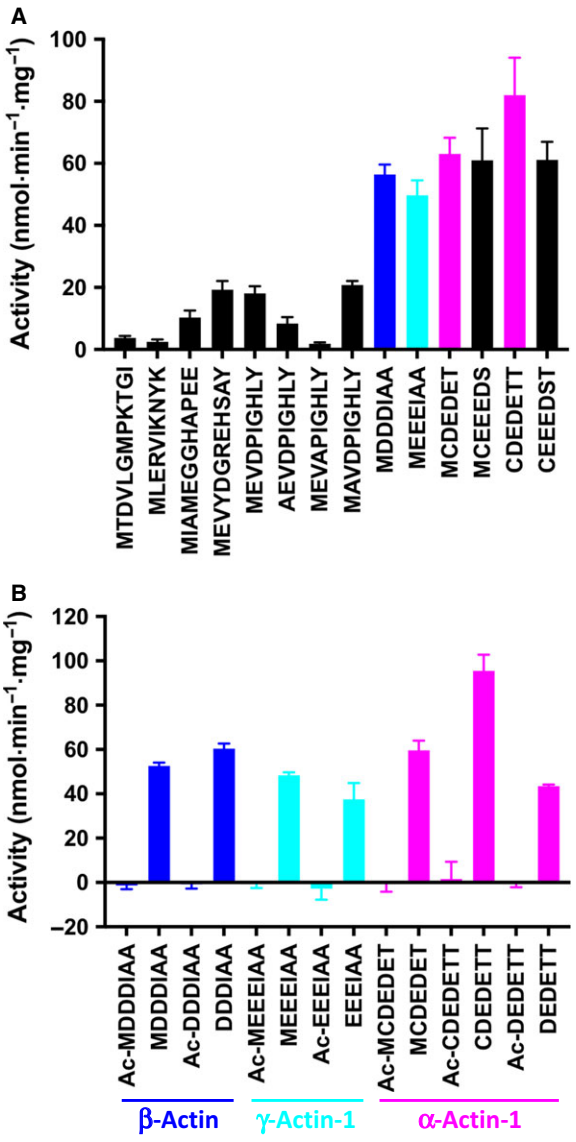


Fig. 3. Activity of NAT6 on different peptides. Activity was measured with 500 μM of the indicated peptides using a DTNB-based assay with 4.5 μg NAT6/100 μL. Mean of 4 experiments ± SEM. Bars corresponding to beta-actin peptides are colored in blue, to gamma-actin in cyan and alpha-actin in magenta. K_m and V_{MAX} were determined for peptide MDDDIAA and DDDIAA by measuring the activity on different concentrations of substrate (0.1, 0.2 and 0.5 mM, in triplicates). Care was taken to adjust the time and enzyme concentration so that no more than 15% of the substrates (peptide and acetylCoA) was used in the assays. Values of K_m of 0.5 and 0.4 mM and of VMax of 165 and 150 nmol·min^{-1}·mg^{-1} protein, respectively, were computed with the PRISM software (GraphPad Software, La Jolla, CA, USA). From these values, catalytic efficiencies of 192 and 219 s^{-1}·M^{-1} were calculated.

low amounts of NAT6 (0.25–1 μg·mL^{-1}) compared to what was used (50 μg·mL^{-1}) for the studies on recombinant proteins (Fig. 2). While the western blot signals corresponding to acetylated actins reaches a maximum 2.5 min after addition of 1 μg·mL^{-1} NAT6 in Fig. 4, the acetylation of recombinant proteins still progresses between 15 and 60 min after addition of 50 μg·mL^{-1} of NAT6 (Fig. 2). This suggests that NAT6 is very efficient at acetylating actins.

Taken together, these data indicated that the recognition of actins by the antibodies that we used critically depends on the acetylation of their N-terminus and that the acetylation of beta-actin and of gamma-actin-1 is strongly deficient in the absence of NAT6.

NAT6 is required for the acetylation of the N-terminal acidic amino acid of actin, but not the initiator methionine

Our observation that NAT6 can act on methionine-containing N-terminal actin peptides raised the question whether NAT6 might be required not only for the acetylation of the mature actin protein, but also for the removal of the methionine from beta and gamma-actin-1. To answer this question, we tested whether actin partially purified from *NAT6*-KO cells had retained the initiator methionine or whether it simply lacked the acetyl group on the first acidic amino acid. We therefore chromatographed cell extracts of control and *NAT6*-deficient HAP1 cells on Q-Sepharose. The fractions were analysed by SDS/PAGE with Coomassie blue staining and by western blotting with an anti-actin antibody (recognizing a C-terminal undecapeptide common to various forms of actins) to identify the fractions richest in actin (actually a mixture of beta and gamma-actin-1) (Fig. 5A,B). These fractions were digested with trypsin and analysed by mass spectrometry. We prepared also a third sample in which a portion of fraction 11 from *NAT6*-deficient cells was acetylated *in vitro* with acetyl-CoA and recombinant human NAT6.

Mass spectrometry analysis allowed the detection of N-terminal peptides of gamma-actin-1 and beta-actin in which the initiator methionine had been removed (Fig. 5C), but of none in which the N-terminal methionine was still present. Integration of the extracted ion chromatogram (XIC) allowed the quantification of the abundance of these peptides, leading to the conclusion that in control cells, gamma-actin-1 and beta-actin were almost totally (> 98%) acetylated while on the contrary acetylation was absent in actins extracted from *NAT6*-deficient cells. Incubation of actin from *NAT6*-deficient cells with recombinant NAT6 and acetyl-CoA restored the acetylation to about 76–84% of the normal value. These findings indicated that NAT6 is not required for the acetylation

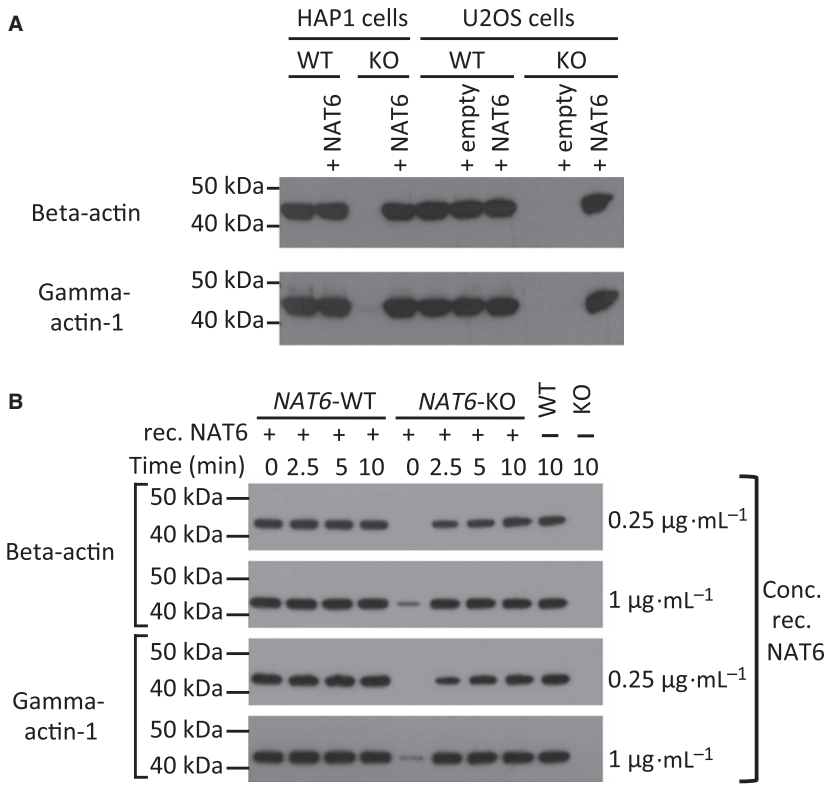
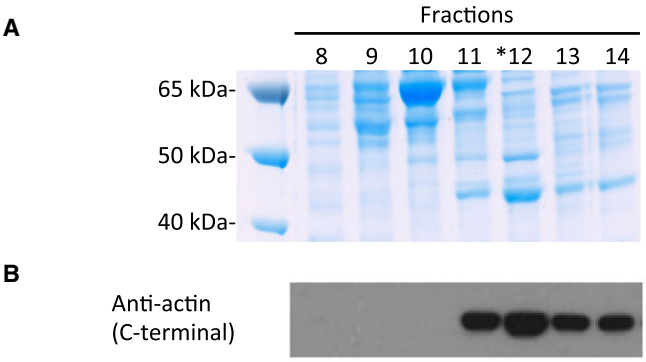


Fig. 4. Recognition of beta-actin and gamma-actin-1 by monoclonal antibodies targeting the mature N-terminus is abolished in *NAT6*-knockout cells and restored upon incubation with recombinant NAT6 and acetyl-CoA. (A) Western blotting of cell extracts. Extracts of control or *NAT6*-deficient HAP1 cells or U2OS cells were analysed by western blotting using antibodies recognizing the mature N-terminal part of beta-actin (upper lanes) or gamma-actin-1 (lower lanes). Samples corresponded to wild-type cells (WT), *NAT6*-deficient cells (KO), or cells transduced with lentiviral vectors containing (+NAT6) or not (+empty) an expression cassette for mouse Nat6. (B). Western blotting on cell extracts incubated with recombinant (rec.) NAT6 and acetyl-CoA. Extracts of wild-type or *NAT6*-KO HAP1 cells were incubated at 37 °C with 0.25 or 1 $\mu g \cdot mL^{-1}$ recombinant NAT6 and 200 µM acetyl-CoA. At the indicated times, aliquots (30 µL) were mixed with 10 µL of SB4 solution to stop the reaction, heated for 5 min at 90 °C and submitted to western blotting analysis as in (A). The last two lanes correspond to extracts of control (WT) or *NAT6*-KO (KO) cells that were incubated for 10 min with acetyl-CoA but no recombinant NAT6. Similar results were obtained using extracts of U2OS cells (not shown). Data shown correspond to one representative experiment out of at least 3.

of the initiator methionine of these two forms of actin, but that it is critical to acetylate the acidic residue that has been uncovered by the removal of the initiator methionine.

As NAT6 appeared to be involved in the acetylation of the first acidic residue of class I actin (after removal of the initiator methionine), we checked if this was also the case for a class II actin. Recombinant forms of skeletal muscle actin (alpha-actin-1) and of gamma-actin-1 fused to a C-terminal streptavidin binding peptide were expressed in wild-type and *NAT6*-KO HAP1 cells. After extraction from the cells, they were partially purified on streptavidin Sepharose beads (Fig. 6A) and analysed by trypsin digestion and mass spectrometry. Quantification based on the surface of the peaks in extracted ion chromatogram (Fig. 6B) indicated that alpha-actin-1 was essentially (84%) present as an acetylated, mature N-terminal sequence (Ac-DEDETT) in wild-type cell extracts, while extracts of the *NAT6*-deficient cells contained almost exclusively the non-acetylated sequence (DEDETT) from which the first two residues (Met and Cys) had been removed. In the case of gamma-actin-1, we similarly observed predominantly an acetylated (Ac-EEEIAA) N-terminus in the control cells and a non-acetylated (EEEIAA) N-terminus in the *NAT6*-deficient cells, in agreement with the data presented in Fig. 5C. A minority ($\leq 1\%$) of peptide starting with an *N*-acetylated methionine was also detected.

Taken together, these experiments demonstrate a key role of NAT6 in the acetylation of the N-terminal acidic amino acids of both class I and class II actin proteins. In contrast, NAT6 does not seem to be essential for the acetylation processes that are needed to remove N-terminal methionine and cysteine residues.



C

Protein	Peptide	WT cells area	WT cells %	KO cells area	KO cells %	Treated KO cells area	Treated KO cells %
γ-actin-1	EEE...CK	1.65×10^6	0.03	5.90×10^8	100	7.14×10^8	23.45
	AcEEE...CK	5.31×10^9	99.97	nd	0	2.33×10^9	76.55
	MEEE...CK	nd	0	nd	0	nd	0
	AcMEEE...CK	nd	0	nd	0	nd	0
β-actin	DDD...CK	6.30×10^7	1.24	1.76×10^9	100	2.66×10^8	15.58
	AcDDD...CK	5.03×10^9	98.76	nd	0	1.44×10^9	84.42
	MDDD...CK	nd	0	nd	0	nd	0
	AcMDDD...CK	nd	0	nd	0	nd	0

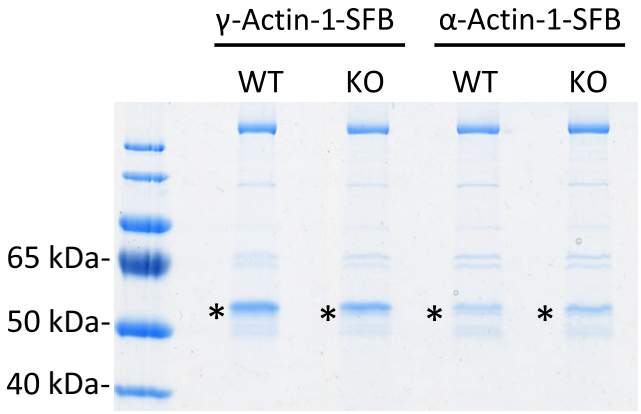
Fig. 5. Analysis of the N-terminus of beta and gamma actins partially purified from control and *NAT6*-deficient HAP1 cells. Cell extracts were prepared and chromatographed on Q-Sepharose, and the resulting fractions were submitted to SDS/PAGE/Coomassie blue and western blotting with anti-actin C-terminus antibodies, as illustrated in panel (A) and (B) for the wild-type cells extract. The fraction richest in actin (fraction 12 in wild-type samples; fraction 11 in *NAT6*-deficient samples; not shown) was digested with trypsin and analysed by mass spectrometry. A sample of fraction 11 (from the NAT6-deficient cells) was also incubated with recombinant NAT6 (10 $\mu g \cdot mL^{-1}$) and 200 μM acetyl-CoA for 15 min at 37 °C and analysed by mass spectrometry after trypsin digestion. (C) Results of the mass spectrometry analysis showing quantification based on peak integration of the extracted ion chromatogram for the different forms of N-terminal peptides in gamma-actin-1 and beta-actin isolated from control HAP1 cells (WT cells), *NAT6*-deficient HAP1 cells (KO cells) and extracts of the latter cells treated with recombinant NAT6 and acetyl-CoA (Treated KO cells). nd, not detected.

NAA10 only plays a minor role in the N-terminal acetylation of actin proteins

When present in the NatA complex, NAA10 is involved in the N-terminal acetylation of a multitude of proteins starting with serine, alanine, threonine, valine or glycine [3]. Previous work has indicated that the monomeric form of NAA10 acetylates the N-terminal acidic residues of actin peptides, but that this was much less the case with the NatA holoenzyme [3]. In our hands, recombinant NAT6 was much better at acetylating a gamma-actin-1 peptide and a beta-actin peptide than recombinant NAA10 (Fig. 7A).

To check if NAA10 could replace NAT6 in the acetylation of beta or gamma actin in *NAT6*-deficient cells, we overexpressed recombinant human NAA10 and mouse Nat6 using the same type of lentiviral vector (Fig. 7B,C). While NAT6 protein was strongly increased, NAA10 protein levels only increased by 20% (Fig. 7B) despite a five-fold increase in mRNA level (data not shown), presumably because NAA10 is much more stable as a complex with NAA15 than in free form [17]. Western blot analysis of beta-actin and gamma-actin with N-termini-specific antibodies indicated that NAA10 overexpression did not lead to the appearance of mature (i.e. acetylated) beta-actin and only to a faint band in the case of gamma-actin-1, while overexpression of NAT6 caused a complete recovery of the actin signal (Fig. 7C). These findings suggested that NAA10 plays at best a minor role in the acetylation of the mature forms of actin and that it cannot replace NAT6 in this function. Further characterization of the requirement of NAA10 for actin acetylation is difficult, since knockout of NAA10 strongly affects cellular viability and would be expected to lead to a plethora of non-specific effects.

A



B

Protein	Peptide	WT cells		KO cells	
		area	%	area	%
γ-Actin-1	EEE...CK	1.67×10^8	4.6	1.88×10^9	98.2
	AcEEE...CK	3.46×10^9	95.0	1.49×10^7	0.8
	AcMEEE...CK	1.32×10^7	0.4	1.82×10^7	1.0
α-Actin-1	DEDE...VK	3.31×10^8	16.5	1.01×10^9	99.9
	AcDEDE...VK	1.68×10^9	83.5	9.91×10^5	0.1

Fig. 6. Distribution of acetylated and non-acetylated N-terminal peptides in recombinant gamma-actin-1 and alpha-actin-1 isolated from HAP1 cells deficient or not in NAT6. Gamma-actin-1 and alpha-actin-1 were expressed as fusion proteins with a flag tag and a streptavidin binding peptide (SFB) and partially purified by binding to affinity beads. A portion of the beads was submitted to SDS/PAGE to visualize actins (A). Proteins were eluted from the rest of the affinity beads, digested with trypsin and analysed by mass spectrometry. Quantification is based on peak integration of the extracted ion chromatogram for the different forms of N-terminal peptides (B). In the case of gamma-actin 1, the peptide starting with acetylated methionine represented about 1% of the total (3rd line), while no peptide starting with a non acetylated methionine could be detected. In the case of alpha actin-1, no methionine/cysteine keeping forms of peptides could be detected.

Loss of NAT6 does not affect F-filament network

As beta-actin and gamma-actin-1 play a role in cell shape and dynamics, we examined cell morphology and actin organization in wild-type and *NAT6*-deficient cells stained with phalloidin (Fig. 8). We noted that there were some morphological differences between the wild-type and *NAT6*-deficient HAP1 cells: the wild-type clone that we got tended to grow as small cell clusters, while the NAT6-deficient clone grew with more cell spreading (Fig. 8, panels A,C). However, this morphological difference was not affected by complementation with Nat6 (Fig. 8, panels B,D), which indicates that it is unrelated to NAT6 deficiency. We did not observe such morphological differences in the U2OS cells (Fig. 8, panels E–H). Analysis of the actin fibers did not disclose any evident effect of NAT6 absence on the F-actin network either in HAP1 or in U2OS cells (Fig. 8).

Staining of the cells with gamma-actin-1 antibodies yielded, as expected, a much fainter signal in *NAT6*-deficient cells than in control cells. This loss of staining was corrected by complementation with Nat6 (Fig. 9).

Discussion

The main conclusion of the present work is that NAT6 is needed to acetylate the N-terminal acidic residue of beta-actin, gamma-actin-1 and most likely also other forms of actin. This conclusion is based on *in vitro* studies indicating that the recombinant enzyme acts very well on peptides corresponding to the N-terminus of mature actins, and also on studies in cells showing that deficiency in NAT6 leads to deficient acetylation of the N-terminal acidic residue of mature actins. This deficiency is documented both by mass spectrometry data and by a lack of reactivity towards anti-mature actin antibodies that can be

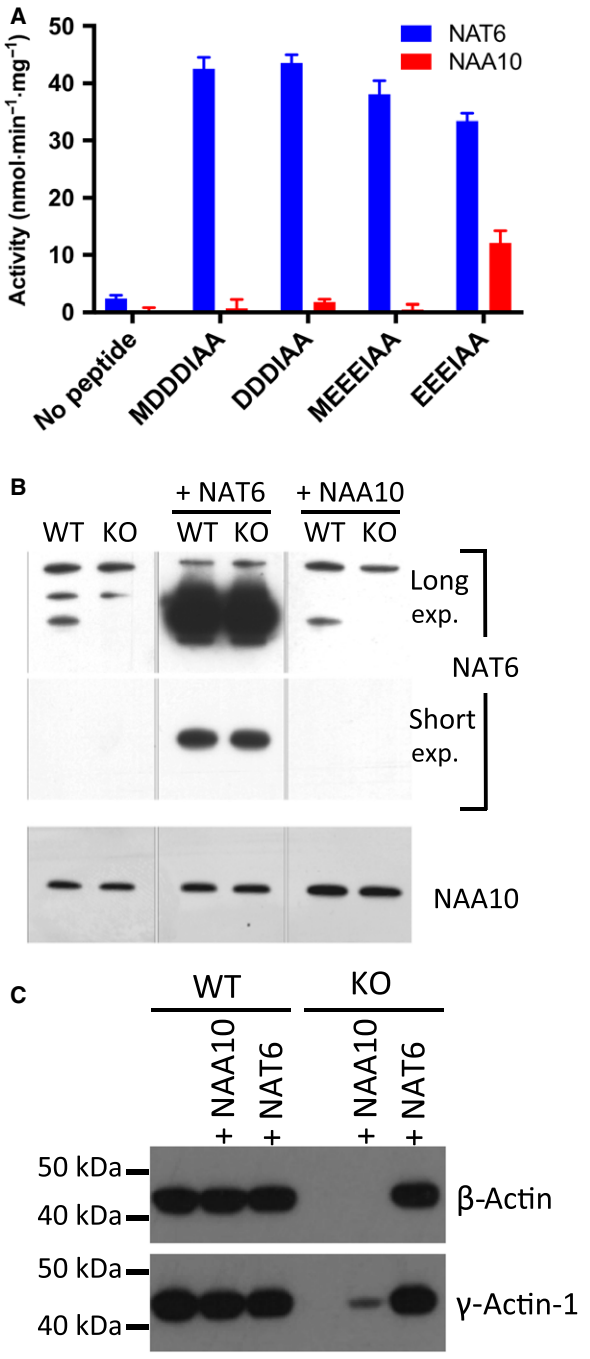


Fig. 7. Negligible activity of NAA10 to acetylate the N-terminus of beta-actin and gamma-actin-1. (A) *In vitro* acetylation activity was measured with 500 μM of the indicated peptides using a DTNB-based assay with 5 μg NAT6/100 μl or 5 μg NAA10/100 μl. (B) and (C) Wild-type or *NAT6*-deficient HAP1 cells were infected with lentiviral vectors allowing the expression of human NAA10 or mouse Nat6. The expression of NAA10 and NAT6 is shown in (B). Vertical bars indicate areas where markers were spliced out. Within each row, all the bands are derived from the same exposure (exp.) and the same gel. Western blotting analysis of mature beta-actin and gamma-actin-1 are shown in (C).

instantaneously corrected by *in vitro* acetylation with acetyl-CoA and recombinant NAT6.

Until now, NAA10 was thought to be the protein that acetylates the N-terminus of actin on the basis of its activity on a peptide corresponding to the N-terminal region of gamma-actin-1 [3]. However, this capacity of NAA10 is considerably reduced when NAA10 is associated with its physiological partners in the NatA complex. Accordingly, the crystal structure of the NatA complex of *Schizosaccharomyces pombe* shows that NAA15 surrounds NAA10 in a ring-like manner, thereby remodeling its catalytic site [18].

We confirmed that NAA10 has indeed a modest capacity of acetylating a gamma-actin-1 peptide, though much less than NAT6. The fact that no acetylation of beta-actin and very little acetylation of gamma-actin-1 are detected in *NAT6*-deficient cells indicate that NAT6 is the main enzyme that carries out this reaction in the cellular context. The virtual lack of activity of NAA10 under these conditions is presumably due to its association with NAA15 or NAA16 in the NatA complex.

NAT6 acts also on acidic peptides that have an initiator methionine. It might therefore be involved in the acetylation of the initiator methionine required for its removal by acylaminoacyl peptidase [15]. However, analysis of the extremity of the endogenous beta- and gamma-actin in *NAT6*-deficient cells shows that the initiator methionine has been removed, indicating that another *N*-acetyltransferase plays this role. The enzyme catalyzing this reaction is most likely NatB (i.e. NAA20), which is well known to acetylate N-terminal methionines preceding an acidic residue. The observation that decreased expression of NAA20 with siRNA causes a decreased acetylation of the small amount of methionine-retaining N-terminus of beta-actin [4] supports this conclusion in the case of beta-actin. The fact that no decreased acetylation was observed in the case of the methionine-containing form of gamma-actin-1 is difficult to interpret unambiguously, as NAT6 can also perform this reaction. An alternative (and maybe complementary) explanation would be that unprocessed gamma-actin-1 is a better substrate for NatB than beta-actin and that it is still efficiently acetylated by this enzyme even when it (NatB) is knocked down, while the acetylation of beta-actin is reduced under the same conditions.

Analysis of the supplementary table S3 of Van Damme *et al.* [4], which reports the sequence and acetylation level of the N-termini of ≈ 2100 human proteins, provides very useful information on the specificity of the acylaminoacyl peptidase removing *N*-acetylmethionine in beta and gamma-1 actins. In this

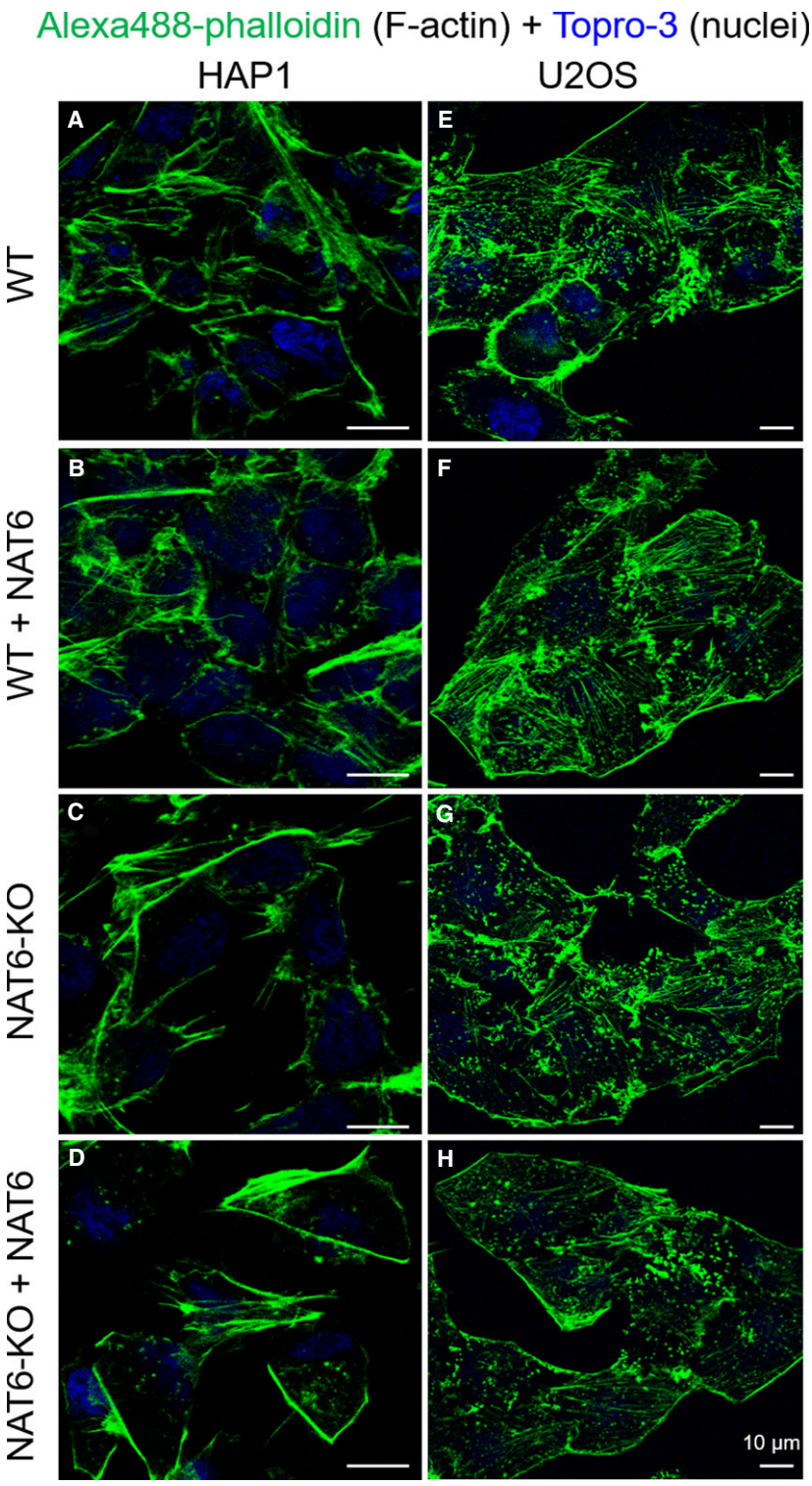


Fig. 8. Preservation of the F-actin network in *NAT6*-deficient HAP1 and U2OS cells. WT and *NAT6*-KO HAP1 or U2OS cells were used as such (panels A,C,E,G) or after infection with a lentivirus allowing the expression of Nat6 (WT+NAT6 and KO+NAT6; panels B,D,F,H). All cells were fixed/permeabilized and labelled for F-actin with Alexa 488-phalloidin (green) and TO-PRO-3-iodide (blue) to stain the nuclei. Images of basal sections were acquired to evidence membrane protrusions and stress fibers. Data shown are representative of 4 independent experiments in HAP1 (2 with rescue) and 2 independent experiments for the U2OS cells (1 with rescue). More than hundred cells were examined for the indicated conditions. Scale bars, 10 µm.

table, the only two proteins that start with a glutamate or an aspartate as a result of the removal of the initiator methionine are beta- actin and gamma-actin-1. The abundance of the forms of these two proteins where the initiator methionine is maintained is very low (< 1.5% in both cases). This contrasts with the presence in the same table of ≈ 500 other proteins starting with Met-Glu or Met-Asp: all of them are highly

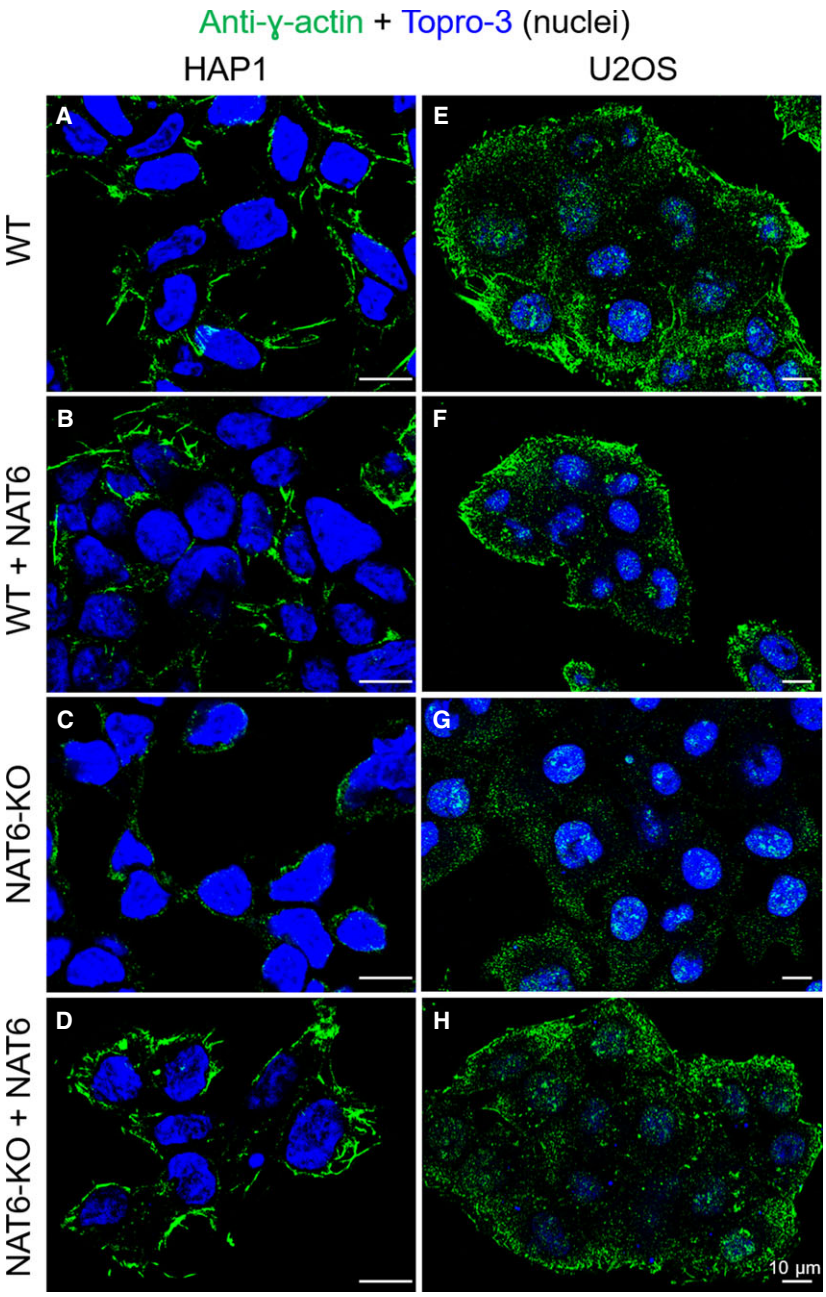


Fig. 9. Impairment of gamma-actin-1 recognition by immunofluorescence in *NAT6*-deficient HAP1 and U2OS cells. WT and *NAT6*-KO HAP1 or U2OS cells were used as such (panels A,C,E,G) or after infection with a lentivirus allowing the expression of Nat6 (WT+NAT6 and KO+NAT6; panels B,D,F,H). The cells were fixed/permeabilized, immunolabelled for gamma-actin-1 (green) and their nuclei stained with TO-PRO-3-iodide (blue). Data shown are representative of 5 independent experiments. Scale bars, 10 µm.

acetylated on their initiator methionine, which is apparently never removed. This last observation also applies to the proteins where the initiator methionine is followed by a stretch of at least 3 acidic amino acids: out of 13 proteins with this sequence motif reported in the data of Van Damme *et al.* [4], only two have their first methionine removed and both of them are actins (beta-actin and gamma-actin-1). All 11 other proteins have conserved their initiator methionine, which is acetylated to an extent of more than 98% in control cells.

These considerations indicate that the acylaminoacyl peptidase that removes *N*-acetylmethionine from actins is very specific for actins and that its selectivity is not simply due to the presence of a hot spot of negative charges near the N-terminus of the substrate. Because of this high selectivity, it is unlikely that this enzyme corresponds to APEH (acyl-peptide hydrolase), the only identified acylaminoacyl peptidase in vertebrates. The latter enzyme acts on small peptides [19–21] and poorly if at all on proteins [22].

Fig. 10. Alignment of NAT6 from various species. (A) Hsapiens, *Homo sapiens* NP_036323.2; Mmuscul, *Mus musculus* NP_062724.1; Drerio, *Danio rerio* XP_021334125.1; Onilotic, *Oreochromis niloticus*, XP_003444885.1; Ddiscoid, *Dictyostelium discoideum*, XP_643256; Celegans, *Caenorhabditis elegans* NP_498391.1; Dmelano, *Drosophila melanogaster*, NP_001014612.1; Amellif, *Apis mellifera* XP_001121320.1. Most of the sequences present a proline rich region, near the C-terminus (highlighted in yellow). The human and mouse sequences present ≈ 19 amino acid repeats at the N-terminus (highlighted in grey). Alignment generated with CLUSTAL OMEGA at the EMBL-EBI website. (https://www.ebi.ac.uk/Tools/msa/clustalo/)

These reflections lead us also to conclude that the critical role of NAT6 in acetylating the N-terminal acidic residue in actins most likely does not extend to other proteins. Thus, NAT6 deficiency has probably only one major consequence, which is to prevent the final maturation step of all forms of actin.

We summarize our views on the processing of the N-terminus of actins in Fig. 1. Compared to what was previously proposed, it is now clear that NAT6 plays a critical role in the acetylation of the N-terminal glutamate or aspartate once it has been uncovered. NAT6 may also play an ancillary role in other acetylation reactions, but we have no experimental proof for this.

The processing of actins to forms that have an acetylated N-terminus is conserved in many species. Many actins have an N-terminal acidic stretch of residues that interact with conserved, positively charged residues on myosin [12,13]. Acetylation masks the positive charge of the protonated N-terminal amine and this may indeed be important to facilitate an ionic interaction, as indicated by the finding that acetylation of the N-terminus of actin stimulates the myosin ATPase activity of heavy meromyosin [14]. Non-acetylated actin was indeed shown to be less good than acetylated actin to stimulate myosin ATPase.

Our studies on cultured cells did not reveal major changes in actin filaments, but these studies certainly do not allow us to conclude that lack of actin acetylation does not produce a functional impairment. It would be particularly interesting to investigate the lack of actin acetylation in context where movements are much more rapid, as is the case in skeletal muscle.

To the extent that we may judge, NAT6 orthologs (see alignment displayed in Fig. 10) are present in species in which the N-terminus of actins is acetylated on an acidic residue. This is the case for vertebrates, for *Dictyostelium discoideum* [23] and for *D. melanogaster* [10]. By contrast, there is no ortholog of NAT6 in *Acanthamoeba castellani*, in *S. cerevisiae* and in *S. pombe*, where there is no processing to an *N*-acetylated acidic residue [24,25]. Sequence comparisons of NAT6 and its orthologues with other proteins indicate that they belong to the GCN5 family, but that they are extremely distant from other enzymes known to catalyse N-terminal acetylation, such as NAA10, NAA20, NAA30, NAA50 and NAA60, whose sequences can be nicely aligned [26].

In conclusion, NAT6 is a new form of N-terminal *N*-acetyltransferase, which is characterized by a remarkable specificity for highly acidic peptides. It plays a crucial role in the final maturation step of the N-termini of actins, and this may be its sole critical role.

While our work was under review, two articles authored by Arnesen and colleagues [27,28] (commented in [29]), reported that NAT6, renamed NAA80, is responsible for actin acetylation in animal cells. In these two remarkable pieces of work, the authors provide information on functional consequences of lack of actin N-terminal acetylation on cell morphology and cell motility, as well as on the polymerization of actin [27]. Furthermore, they describe the 3D structure of *D. melanogaster* NAA80, which allows them to propose a mechanism for the recognition of the acidic N-terminus of actins by this enzyme [28]. While more restricted in its scope, our work is in general agreement with the data reported by Arnesen and colleagues, and provides in addition data on the ability of NAT6 to acetylate skeletal muscle actin, a type II actin. The main disagreement is that we did not succeed to find morphological effects caused by NAT6's absence in HAP1 cells or in another cell model. Of note, some morphological changes that we detected in NAT6-deficient HAP1 cells could not be corrected by complementation with NAT6.

Materials and methods

Cloning, expression and purification

NAT6 was amplified from human lymphoblast cDNA with primers 5′-C ATC CAT ATG CAA GAG CTG ACT CTG AG-3′ and 5′-GTA ATT CTC GAG GAT GTC TTT TTC CAT CCA GAA TAT G-3′. The resulting amplified fragment was digested with NdeI and XhoI and inserted between the corresponding restriction sites of plasmid pET22b (to fuse the protein with a C-terminal polyhistidine tag) using T4 DNA ligase. NAA10 (ARD1) was amplified from human HAP1 cells cDNA with primers 5′-AAT CAT ATG AAC ATC CGC AAT GCG AG-3′ and 5′-AA AGC GGC CGC GGA GGC TGA GTC GGA GGC CTC-3′. The resulting amplified fragment was digested with NdeI and NotI and inserted between the corresponding restriction sites of plasmid pET22b using T4 DNA ligase. The resulting constructs were checked by sequencing.

These plasmids were used to transform BL21 *E. coli* cells. These cells were grown in LB medium at 37 °C until the $OD_{600\ nm}$ reached 0.5-0.6. Protein expression was induced by 1 mM IPTG (isopropyl-β-D-thiogalactoside). After 24 h of expression at 20 °C, cells were harvested by centrifugation at 6000 ***g*** during 15 min at 4 °C. The pellet was resuspended in buffer containing 25 mM Hepes, pH 7.4, 300 mM NaCl, 2 $\mu g \cdot mL^{-1}$ leupeptin, 2 $\mu g \cdot mL^{-1}$ antipain, 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 1 $mg \cdot mL^{-1}$ lysozyme. Cells were lysed by 3 cycles of

freezing in liquid nitrogen and thawing at 37 °C. The extract was incubated with 0.1 mg·mL^{-1} DNaseI and 10 mM $MgSO_4$ during 30 min at 4 °C and then centrifuged at 20 000 ***g*** during 40 min at 4 °C.

The resulting supernatant was used for the purification on 1 mL HisTrap HP column (GE Healthcare, Little Chalfont, Bucks, UK). Twenty ml of the supernatant was diluted 4-fold in buffer A (50 mM phosphate buffer, pH 7.4, 300 mM NaCl, 25 mM imidazole, 5 μg·mL^{-1} leupeptin, 5 μg·mL^{-1} antipain and 10% glycerol) and loaded on the column, which had been previously equilibrated with buffer A. His-tagged protein was eluted with a linear gradient 0–100% of buffer B (same buffer as buffer A with 500 mM imidazole, pH 7.4). The fractions were analysed by SDS/PAGE and the ones containing the protein of interest were pooled and desalted on a G25 Sepharose matrix (PD10; GE Healthcare). The purified protein was conserved in 25 mM Hepes, pH 7.4, 300 mM NaCl, 5 μg·mL^{-1} leupeptin, 5 μg·mL^{-1} antipain and 10% glycerol at −80 °C.

Lentiviral CRISPR/Cas9 constructs were generated by ligating annealed oligos (CACCGgctggttcagcacccgtgac and AAACgtcacgggtgctgaaccagcC) into the BsmBI site of the plasmid lentiCRISPR v2 (Addgene plasmid 52961, a generous gift of Feng Zhang; Cambridge, MA, USA). To generate lentiviral expression constructs, Nat6 open reading frame (ORF) was amplified from mouse testis cDNA with primers 5′-ATA CAT ACT AGT CCA CGA TGG AGC TGA TCC TGA GTA-3′ and 5′-TTA TAT GGT ACC GAC AGA GCC ATC AGA TGT CT-3′ and NAA10 ORF from human HCT116 cells cDNA using primers 5′-ATA CAT GCT AGC ATG AAC ATC CGC AAT GCG AG-3′ and 5′-TAA TAT TGT ACA GGA TGG GGC AGG CTC TAG G-3′. The resulting PCR products were restriction-digested and inserted between the NheI and BsrGI sites of a bicistronic lentiviral vector driving expression of the gene of interest and a resistance marker for hygromycin [30], resulting in plasmids pJG140-2 and pJG204-1, respectively.

To generate lentiviral constructs for the overexpression of actin isoforms with a C-terminal affinity tag, we amplified gamma-actin-1 ORF from human embryonic kidney cells (HEK293T) cDNA with primers 5′-ATA CAT GCT AGC GGT CGC AAT GGA AGA AGA GAT C-3′ and 5′-TAA TAT TGT ACA GAA GCA TTT GCG GTG GAC GAT-3′ and alpha-actin-1 ORF from differentiated mouse C2C12 myoblasts cDNA using primers 5′-ATA CAT GCT AGC CAC CAT GTG CGA CGA AGA CGA-3′ and 5′-TAA TAT TGT ACA GAA GCA TTT GCG GTG CAC AAT G-3′. The resulting PCR products were inserted between the NheI and BsrGI sites of a lentiviral vector, which allows the expression of fusion proteins with a C-terminal SFB-tag (consisting of S-protein, FLAG tag and streptavidin-binding peptide) and selection with hygromycin [30]. This resulted in plasmids pJG141-1 (gamma-actin-1) and pJG143-1 (alpha-actin-1).

Assay of *N*-acetyltransferase activity on protein substrates

Activity on proteins was determined with a radiochemical assay. The reaction mixture (100 μL) comprised 25 mM Tris, pH 7.5, 1 mM $MgCl_2$, 25 mM KCl, 100 000 cpm of radiolabelled [^{3}H]acetyl-CoA (8 μM), 5 μM protein substrate and ≈ 1 μM purified recombinant NAT6. After 15 and 60 min of incubation at 30 °C, 40 μL of the reaction mixture was spotted on a P81 paper of 1.5 cm by 1.5 cm. These papers were immediately put in a solution of 70 mM phosphoric acid and washed for 10 min. This washing was repeated twice. Papers were dried and counted in the presence of 7 mL of scintillation liquid. The production of the proteins used as substrates has been previously described [31–39]. Assessment of their N-terminal sequence was determined by mass spectrometry analysis of trypsin peptides by LC-MS/MS using an LTQ XL IT mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as previously described [40].

Peptides synthesis and assay of *N*-acetyltransferase activity on peptide substrates

All peptides were synthesized in-house on solid phase using standard Fmoc (fluorenylmethoxycarbonyl) chemistry, and characterized by HPLC and mass spectrometry. Before usage, the lyophilized peptides were dissolved in water and pH was adjusted between 6.0 and 7.0 with NaOH.

Activity on synthetic peptides was determined with a DTNB-based assay. The reaction mixture of 100 μL comprised 25 mM Tris, pH 7.5, 0.25 mM acetyl-CoA, 1 mM $MgCl_2$, 25 mM KCl, 0.5 mM peptide and (unless otherwise indicated) 1 μM NAT6. After 30 min at 37 °C, 100 μL of 1.25 mM DTNB (prepared in KPi pH 8.0) was added to the reaction mixture and the absorbance at 412 nm was measured using a microplate reader.

Cell culture

HAP1 cells and *NAT6*-KO HAP1 cells (Horizon HZGHC003171c003, [41]) were cultured in IMDM supplemented with 10% FBS, 2 mM ultraglutamine and antibiotics (100 units·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin) at 37 °C in 5% CO_2. U2OS cells were cultured in DMEM supplemented with 10% FBS, 2 mM ultraglutamine and antibiotics at 37 °C in 5% CO_2.

Lentiviral transductions were performed essentially as previously described [30]. Briefly, we transiently transfected HEK293T cells with lentiviral vectors and second generation packaging plasmids psPAX2 and pMD2.G (kind gifts of Didier Trono, Addgene #12260 and #12259) using the calcium phosphate co-precipitation method [42]. About 24–48 h after transfection, target cells were infected in the presence of 8 μg·mL^{-1} polybrene (Sigma-Aldrich, St-Louis, MO, USA). Infected cells were selected for 4 days with

1.5 $\mu g \cdot mL^{-1}$ of puromycin (Thermo Fisher Scientific) or 300 $\mu g \cdot mL^{-1}$ of hygromycin (Invivogen) or 1 $mg \cdot mL^{-1}$ G418 (Invivogen, San-Diego, CA, USA). Empty lentiviral constructs were used to generate control cell lines. In the case of lentiviral CRISPR/Cas9 constructs, single cell clones were isolated. Loss of NAT6 was verified by western blotting and DNA sequencing.

Western blotting and incubation with recombinant NAT6

To prepare cell extracts, cells were grown to confluence in 10-cm diameter plates. After washing once with 5 mL cold PBS, cells were scraped into 0.5–0.8 mL of extraction buffer (25 mM Hepes, pH 7.4, 0.5 mM PMSF, 5 $\mu g \cdot mL^{-1}$ leupeptin and 5 $\mu g \cdot mL^{-1}$ antipain), submitted to 2 cycles of freezing in liquid nitrogen/thawing and lysed by vortex-mixing. Cell extracts were centrifuged for 15 min at 15 000 ***g*** and 4 °C. Protein concentration was determined with the Bradford assay using γ-globulin as a standard. The antibodies used were the following ones: monoclonal anti-beta-actin (A5441; Sigma-Aldrich), monoclonal anti-gamma-actin (A8481; Sigma-Aldrich), anti-actin (A2066; Sigma-Aldrich), anti-NAT6 (#15476-1; Proteintech, Rosemont, IL, USA) and anti-ARD1A (NAA10) (#9046, Cell Signaling Technology, Danvers, MA, USA).

To test the ability of recombinant NAT6 to restore the acetylation of actin in KO cells, supernatants (200 μg protein) of control and *NAT6*-KO cells extracts were incubated in a mixture (0.2 mL) containing 25 mM Tris pH 7.5, 25 mM KCl, 1 mM $MgCl_2$, 2 mM ATP and 0.2 mM acetyl-CoA. The reaction was initiated by the addition of 0.25 or 1 $\mu g \cdot mL^{-1}$ of recombinant NAT6. At the indicated times, 30 μL aliquots were taken off, mixed with 10 μL SB4 (4 × concentrated reducing sample buffer) loading solution and directly heated at 90 °C for 5 min.

F-actin labelling and immunolabelling of gamma-actin-1

Cells were seeded at 200 000 $cells \cdot cm^{-2}$ for HAP1 cells and 100 000 $cells \cdot cm^{-2}$ for U2OS cells in 24-well plates on glass coverslips in 1 mL complete medium and grown overnight. For immunofluorescence, cells were processed essentially as described in [43]. Briefly, they were rinsed with PBS, fixed with 4% formaldehyde, permeabilized with 0.05% saponin for HAP1 cells or with 0.5% Triton X100 for U2OS cells and quenched with Q-PBS (PBS with 0.01% saponin, 2% BSA and 0.1% lysine). Cells were then incubated for 1 h with the primary antibody (monoclonal anti-gamma-actin A8481, Sigma-Aldrich; 1 : 100 dilution) in Q-PBS, washed and further incubated with anti-IgG1 Alexa-488 secondary antibody (1 : 200 dilution) in Q-PBS with TO-PRO-3-iodide (Molecular Probes, Eugene, OR, USA; 1 : 500 dilution) for 1 h in the dark.

For F-actin staining, cells were incubated with Alexa-488 phalloidin (Molecular Probes; 1 : 150 dilution) in Q-PBS with TO-PRO-3-iodide for 1 h in the dark. After washing, coverslips were mounted in Faramount mounting medium (Dako-Agilent, Santa Clara, CA, USA) and examined with a LSM 510 META confocal microscope (Zeiss, Oberkochen, Germany) using a Plan-Apochromat 63×/14 oil DIC objective.

Mass spectrometry analysis of actin N-termini

For the mass spectrometry analysis of endogenous gamma-actin-1 and beta-actin in HAP1 cells (Fig. 5), 4 mL of the supernatant of a cell extract prepared as described above for HAP1 wild-type cells or *NAT6*-KO cells were diluted 5-fold in buffer C (25 mM Hepes, pH 7.4, 20 mM NaCl, 2 $\mu g \cdot mL^{-1}$ leupeptin, 2 $\mu g \cdot mL^{-1}$ antipain) and loaded onto a 1 mL-HiTrap Q HP (GE Healthcare) equilibrated with the same buffer. The column was washed with 5 volumes of buffer C and proteins were eluted with a linear NaCl gradient (20–750 mM in 20 mL buffer C). The elution fractions (1 mL) were analysed by SDS/PAGE followed by Coomassie blue staining and by western blotting with anti-actin antibody. Proteins of the samples to be analysed were precipitated with methanol/chloroform (4/1 vol/vol), resuspended in 50 μL of 50 mM ammonium bicarbonate and treated with 10 mM dithiothreitol (30 min at 56 °C) and with 60 mM chloroacetamide (30 min at room temperature and in the dark). After precipitation with methanol-chloroform, the proteins were resuspended and incubated with trypsin, before analysis by mass spectrometry.

For the analysis of the tagged alpha and gamma-actin-1 (Fig. 6), the supernatant (2.5 mL) of a cell extract corresponding to four 10-cm diameter plates was incubated with 25 μL Streptavidin Sepharose HP (GE Healthcare) during 90 min at 4 °C under rotation. The beads were then centrifuged at 1000 ***g*** during 5 min and washed three times with 1 mL of a buffer containing (20 mM Tris, pH 8.0, 1 mM EDTA and 150 mM NaCl). A portion of the beads (≈ 25%) was mixed with SB4 and 2 vol of water, and submitted to SDS/PAGE analysis. The rest of the beads was used to elute the retained proteins with 250 μL of 0.1% formic acid. Proteins were precipitated with methanol/chloroform (4 vol/1 vol) and further processed with dithiothreitol, chloroacetamide and trypsin as above.

Peptide analysis with the Orbitrap Lumos spectrometer (Thermo Fisher Scientific) was performed as follows. Peptides were dissolved in solvent A (0.1% trifluoroacetic acid in 2% acetonitrile), directly loaded onto reversed-phase pre-column (Acclaim PepMap 100; Thermo Fisher Scientific) and eluted in backflush mode. Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, 0.075 × 250 mm; Thermo Fisher Scientific) with a linear gradient of 4–27.5% solvent B (0.1% fluoroacetic acid in 98% acetonitrile) for 100 min, 27.5–40% solvent B for 10 min, 40–95% solvent B for 1 min and holding at 95% for the last 10 min at a constant flow rate of

300 nL·min^{-1} on an EASY-nLC 1000 UPLC system. The resulting peptides were analysed with an Orbitrap Fusion Lumos tribrid mass spectrometer (Thermo Fisher Scientific). The peptides were subjected to NSI (nanospray ionization) source followed by tandem mass spectrometry (MS/MS) in Fusion Lumos coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 120 000. They were selected for MS/MS using HCD (high energy collision induced dissociation) setting at 30; ion fragments were detected in the Orbitrap at a resolution of 30 000. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 5000 in the MS survey scan with 20.0 s dynamic exclusion. The electrospray voltage applied was 2.1 kV. MS1 spectra were obtained with an AGC target of 4×10^5 ions and a maximum injection time of 50 ms, and MS2 spectra were acquired with an AGC target of 5×10^4 ions and a maximum injection time of 100 ms. For MS scans, the m/z scan range was 350–1500. The resulting MS/MS data was processed using Sequest HT search engine within Proteome Discoverer 2.2 against a homemade protein database containing human actin protein sequences obtained from Uniprot. Trypsin was specified as cleavage enzyme allowing up to 2 missed cleavages, 5 modifications per peptide and up to 7 charges. Mass error was set to 10 p.p.m. for precursor ions and 0.2 Da for fragment ions. Oxidation of Met and Acetylation of N-termini (+42.010 Da) were considered as variable modifications. False discovery rate (FDR) was assessed using a fixed value PSM validator and thresholds for protein, peptide and modification site were specified at 1%. Sites of covalent modification were manually validated and peak integration of XIC was performed using FreeStyle 1.3 SP2 (Thermo Fisher Scientific).

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

EW, GT, DT, GTB and EVS planned experiments; EW, GT, DT and DV performed experiments; EW, GT, DT, DV, VS, GTB and EVS analysed data; VS contributed essential material; EW, GTB and EVS wrote the paper. All authors commented the manuscript and approved its final version.

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