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N-TERMINAL ACETYLAATION STABILIZES PROTEINS BY BLOCKING N-TERMINAL UBIQUITINATION, AND BY DIRECTLY INHIBITING PROTEASOMAL PROCESSING

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ABSTRACT

More than 80% of all human proteins are modified by N-terminal (N-)acetylation, but the function of this modification is not well understood. N-acetylation has been suggested to affect protein stability, but in certain studies it inhibited protein degradation, while in others it acted as a degradation signal. To resolve this issue, we determined the contribution of N-acetylation to protein ubiquitination and turn-over. N-acetylation was modulated by substituting one or two N-terminal amino acids to generate good or poor substrates for N-acetyltransferases. Our data consistently indicate that N-acetylation stabilizes proteins by preventing their proteasomal degradation. It did so by two mechanisms: N-acetylation prevented N-terminal poly-ubiquitination on protein substrates and thereby prevented their degradation, and N-acetylation also inhibited proteasomal degradation regardless of its impact on substrate ubiquitination. In this way, it prevented proteasomal degradation of the naturally lysine-less cell cycle regulator p16 and it could even counteract degradation of ubiquitinated proteins. We conclude that in human cells, N-acetylation stabilizes protein substrates by inhibiting their ubiquitin-mediated targeting to the proteasome, and by inhibiting proteasomal processing of the substrate.
N-ACETYLATION INHIBITS PROTEIN DEGRADATION

INTRODUCTION

N-terminal acetylation (called N-acetylation from here on) is the covalent conjugation of an acetyl-group to the α-amino-group of the N-terminal residue of a protein. It is an irreversible modification that is highly conserved throughout eukaryotic evolution.\(^1\) The acetyl transfer is mediated by ribosome-associated N-terminal acetyltransferase (NAT) complexes, and occurs co-translationally, i.e. during synthesis of the target protein. NAT complexes are composed of a catalytic and an auxiliary subunit, and together target about 80-90% of all human proteins, making N-acetylation one of the most prevalent protein modifications in the cell.\(^1\)

Human cells express six different NAT complexes, termed NatA to NatF, that all have different substrate specificities (reviewed in ref. 2). NatA targets about 40% of all N-acetylated proteins and is selective for proteins from which the start-methione has been removed by methionine aminopeptidases (MetAPs). Removal of the start-methionine occurs when the adjacent residue has a side chain with a small gyration radius, as is the case for alanine, cysteine, glycine, proline, serine, threonine and valine.\(^3-4\) The start-methionine is often retained in proteins with other penultimate residues and such proteins are targeted by the other NAT-complexes. NatB targets proteins in which the start-methionine is followed by an acidic residue, while NatC, NatE and NatF mainly target proteins with a hydrophobic penultimate residue, with overlapping substrate specificities. So in general, targeting for N-acetylation by NAT complexes is specified by the nature of the first two amino acids of a protein. The atypical NatD complex forms an exception to this rule, since it only targets very rare substrates with a specific sequence in the N-terminal 30-50 residues.

These basic principles of NAT-specificity are mainly inferred from several proteome-wide studies of N-acetylation.\(^1, 4-5\) These studies have provided guidelines to predict whether a protein is a good or poor substrate for N-acetylation, but there are no general rules that predict N-acetylation status with 100% confidence. The proteomics datasets only predict reliably that a proline as ultimate or penultimate N-terminal residue prevents N-acetylation, which is known as the (X)PX-rule.\(^5\)

Despite its prevalence, the exact function of N-acetylation is poorly understood. It can contribute to protein-protein interaction, e.g. in the conjugation pathway of the ubiquitin-like molecule NEDD8, where N-acetylation of the yeast E2 enzyme Ubc12 enhances its affinity for the E3 ligase subunit Dcn1.\(^6\) N-acetylation might also be important for cytosolic retention of proteins, because it blocked ER-import of secretory proteins, which are generally N-acetyl-free.\(^7\) Lastly, it has been suggested that N-acetylation affects protein degradation, as outlined below.

The majority of cellular proteins is degraded by the proteasome, a multiprotease complex consisting of a 20S core particle that contains the proteolytic activity, and a 19S cap that regulates substrate binding and unfolding.\(^8\) Most proteins need to be ubiquitinated to permit their proteasomal degradation. In this process, the 76-amino acid protein ubiquitin is post-translationally conjugated to the substrate.\(^9\) Ubiquitin conjugation is performed by a three-step enzymatic cascade, involving an E1 ubiquitin-activating enzyme, an E2 conjugase, and an E3 ligase. The E1 activates and binds the ubiquitin and subsequently transfers it to the E2 conjugase. In most cases, the E3 ligase recognizes the substrate, while the associated E2 enzyme
conjugates ubiquitin to it. Some E3 ligases however possess enzymatic activity themselves and directly mediate the conjugation, after having received the ubiquitin from the E2.

Conventionally, the C-terminal carboxyl group of ubiquitin forms an isopeptide bond with the ε-amino-group of a lysine in the substrate. The seven lysines in ubiquitin can also be targeted, allowing the formation of poly-ubiquitin chains with different topologies. The functional consequence of ubiquitination largely depends on the type of chain: e.g. lysine (K)63-linked ubiquitin chains generally target membrane proteins for endocytosis, while K48-linked chains target proteins for proteasomal degradation.

In certain instances, N-terminal ubiquitination takes place, in which the α-amino-group of the substrate acts as ubiquitin acceptor site. This can only occur when the α-amino-group is free and for this reason N-acetylation is considered to block N-terminal ubiquitination. In agreement with this, initial biochemical experiments indicated that N-acetylation inhibits protein degradation. Recently however, it was shown in yeast that N-acetylation targeted a substrate for ubiquitination by the E3 ligase Doa10, followed by its degradation. Possibly therefore, N-acetylation may likewise serve as a degradation signal (degron) in mammalian cells. Given these contrasting data, we aimed to clarify the effect of N-acetylation on protein degradation in human cells. We show here, that in human cells, N-acetylation inhibits proteasomal degradation of target proteins. It does so by blocking N-terminal ubiquitination, but also by a ubiquitin-independent mechanism, suggesting that it directly inhibits proteasomal processing of the substrate.

RESULTS
N-terminal HA-tagging drives N-terminal ubiquitination of the anti-apoptotic protein Bcl-B

Our interest in the relation between N-acetylation, ubiquitination and protein stability was prompted by our study of the anti-apoptotic protein Bcl-B that we found to be ubiquitinated on internal lysine residues. To assess Bcl-B ubiquitination, we expressed hemagglutinin (HA)-tagged wild-type (WT) or lysineless (KR) Bcl-B in HEK 293T cells, together with FLAG-tagged ubiquitin. Cells were lysed under denaturing conditions to break all non-covalent protein-protein interactions, after which Bcl-B was immunoprecipitated with anti-HA mAb. This procedure ensured that the observed ubiquitination was direct and did not concern associated proteins. Bcl-B ubiquitination was examined by immunoblotting for Bcl-B and ubiquitin.

In agreement with our previous results, N-terminally tagged HA-Bcl-B (WT) was modified with one or more ubiquitin molecules (Fig. 1A; lane 2). C-terminally tagged Bcl-B-HA (WT) was likewise ubiquitinated (Fig. 1A; lane 4), indicating that the position of the HA tag did not obviously affect the ubiquitination status of wild-type Bcl-B. However, the position of the HA tag did affect the ubiquitination status of lysineless (KR) Bcl-B. Whereas C-terminally tagged Bcl-B-HA (KR) had lost all ubiquitination, N-terminally tagged HA-Bcl-B (KR) still carried ubiquitin (Fig 1A; lanes 3 and 5).

Since the α-amino-group of a protein may act as an alternative ubiquitin acceptor site (reviewed in 15), we hypothesized that ubiquitin was conjugated to the N-terminus of HA-Bcl-B
We performed mass spectrometry (MS) to test this. First, HA-Bcl-B (KR) was purified from denatured HEK 293T lysate using a tandem HA IP protocol. Next, the isolate was separated by SDS-PAGE, bands containing ubiquitin-Bcl-B conjugates were excised, proteins were digested with trypsin and the resulting peptides were analyzed by liquid chromatography tandem MS (LC/MS-MS). Trypsin cleavage leaves a 114 Da di-glycine remnant on a ubiquitin-modified peptide that can therefore be distinguished from a non-modified peptide by MS (reviewed in 16). This analysis revealed that N-terminal peptides of HA-Bcl-B (KR) were N-terminally modified with a di-glycine remnant (Fig. 1B). Together, these results demonstrated that N-terminal HA-tagging of lysineless Bcl-B drove poly-ubiquitin chain assembly at its N-terminus.

N-acetylation blocks N-terminal ubiquitination of HA-Bcl-B

We considered that HA-Bcl-B (KR), but not Bcl-B-HA (KR), was N-terminally ubiquitinated because of differential N-acetylation. The HA-tag we used contains a glycine residue between the start-methionine and the tyrosine of the HA-tag sequence (MG-YPYDVPDYA), which was inserted to generate a Kozak consensus sequence. The methionine will most likely be removed by methionine aminopeptidases (MetAPs),\(^3\) predicting that the mature N-terminus of HA-Bcl-B starts with glycine-tyrosine (GY) and therefore is a poor substrate for N-acetylation.\(^1\) However, N-acetylation status is difficult to predict on sequence alone and must be confirmed by MS.\(^2,17\)

For this purpose, we expressed Bcl-B-HA and HA-Bcl-B in HEK 293T cells, isolated them by IP and analyzed them by LC/MS-MS. Acetyl-free peptides have slightly different biochemical characteristics than their acetylated counterparts, which in theory could affect their detection by MS. Therefore, we chemically N-acetylated the free N-termini.

Figure 1. N-acetylation blocks N-terminal ubiquitination of Bcl-B. (A) HEK 293T cells were transfected to express FLAG-tagged ubiquitin, together with wild-type (WT) or lysineless (KR) Bcl-B variants that were fused to an N-terminal or C-terminal HA-tag (amino acid sequence YPYDVPDYA). Variants differed in their N-acetylation status (N-acetyl) as determined by MS. Bcl-B was isolated from denatured lysates by α-HA IP, followed by immunoblotting of the precipitates with α-HA mAb for Bcl-B and α-FLAG mAb for ubiquitin. (B) HEK 293T cells were transfected to express N-terminally tagged HA-Bcl-B without lysines (KR). Ubiquitin-Bcl-B conjugates were isolated and analyzed by LC/MS-MS as detailed in the materials and methods section. Shown is the tandem mass spectrum of the N-terminal tryptic peptide of HA-Bcl-B (KR), containing a di-glycine (GG) modified N-terminus. * Indicates m/z value of b\(_5\) ion minus H\(_2\)O.
post-isolation, but with deuterio-acetyl to allow the distinction between proteins that were N-acetylated post-isolation and those that were N-acetylated \textit{in vivo}.\textsuperscript{18} MS-analysis demonstrated that the methionine was removed from HA-Bcl-B with 100\% efficiency, generating a mature N-terminus starting with the sequence GY (Fig. S1). Moreover, both N-acetylated and acetyl-free N-termini were detected, indicating that HA-Bcl-B is a poor N-acetylation substrate that is only partially N-acetylated \textit{in vivo} (Fig. S1). For Bcl-B-HA, methionine removal was not complete, and peptides starting with the sequence MVD and VD were detected (Fig. S1). However, both peptides were completely N-acetylated, indicating that Bcl-B-HA is efficiently N-acetylated \textit{in vivo}. Thus, differential N-terminal acetylation explains why HA-Bcl-B, but not Bcl-B-HA can be ubiquitinated on its N-terminus.

To determine whether the poor N-acetylation of the (GY)HA-tag was the key feature that drove ubiquitination of lysineless Bcl-B, we created modified HA tags at the N-terminus of HA-Bcl-B that specified targeting by NAT enzymes. An alanine-proline sequence (AP) or a serine-aspartate (SD) sequence was inserted between the start-methionine and the GY sequence of HA-Bcl-B. The (AP)HA-Bcl-B variant is predicted to be N-acetyl-free based on the (X)PX rule, which states that a proline as ultimate or penultimate residue completely blocks N-acetylation.\textsuperscript{5} The (SD)HA-Bcl-B variant was predicted to be efficiently N-acetylated, and this was confirmed by MS-analysis (Fig. S1).\textsuperscript{1} Importantly, the lysine-less, N-acetylated (SD)HA-Bcl-B (KR) mutant had lost all ubiquitination, while the lysine-less (AP) and (GY) HA-Bcl-B (KR) mutants still carried ubiquitin (Fig. 2A). Together, these data indicate that N-acetylation blocks N-terminal ubiquitination of the lysine-less HA-Bcl-B mutant.

\textbf{N-acetylation stabilizes lysineless HA-Bcl-B}

We previously demonstrated that WT Bcl-B is modified on lysines by K48-linked polyubiquitination, targeting it for degradation by the proteasome.\textsuperscript{14} Because N-acetylation prevented N-terminal polyubiquitination of lysineless HA-Bcl-B, we hypothesized that it would also stabilize this protein. To test this, WT and lysineless HA-Bcl-B proteins were expressed in HEK 293T cells that were subsequently incubated with the translation inhibitor cycloheximide (CHX) for different time periods. The half-life of the HA-Bcl-B proteins was determined by immunoblotting of the total cell lysates and protein quantification. Green Fluorescent Protein (GFP) was used as a control, because it has a very long half-life.\textsuperscript{19-20}

In the analysis, we included the N-acetylated SD variant, and the (largely) N-acetyl-free AP and GY variants of HA-Bcl-B. N-terminal variants of wild-type HA-Bcl-B all were degraded with equal kinetics (Fig. 2B, C), which correlated with their equal ubiquitination status (Fig. 2A). In contrast, degradation kinetics differed among the lysine-less HA-Bcl-B variants (Fig. 2D, E). The lysine-less, N-terminally ubiquitinated (GY)HA-Bcl-B (KR) variant was degraded with kinetics comparable to WT HA-Bcl-B (Fig. 2D, E), indicating that N-terminal ubiquitination and lysine ubiquitination are equally capable to direct Bcl-B for degradation. The (AP)HA-Bcl-B (KR) variant was degraded to some extent, but with a slower rate than the (GY)HA-Bcl-B variant. In contrast, the N-acetylated (SD)HA-Bcl-B (KR) variant was completely stable (Fig. 2D, E). These degradation kinetics correlated well with the ubiquitination status of the HA-Bcl-B (KR)
Figure 2. *N*-acetylation stabilizes lysineless HA-Bcl-B by reducing its ubiquitination. (A) HEK 293T cells were transfected to express FLAG-tagged ubiquitin, together with wild-type (WT) or lysineless (KR) HA-Bcl-B variants. AP, GY and SD variants differed in the amino acid sequence of the mature N-terminus (N-residues) and their N-acetylation status (N-acetyl). HA-Bcl-B was isolated from denatured lysates by α-HA IP, followed by immunoblotting of the precipitates. Bcl-B and ubiquitin were detected by probing with α-HA or α-FLAG mAb respectively. 

(B) HEK 293T cells were transfected to express GFP as a stable protein control, together with HA-Bcl-B variants that differed in the amino acid sequence of the mature N-terminus and their N-acetylation status. The prefix Ac indicates an acetylated N-terminus, while ~ indicates a free N-terminus. Cells were treated with cycloheximide (CHX; 50 μg/ml) for indicated time periods, followed by immunoblotting of the total cell lysates. Bcl-B and GFP were detected by probing with α-HA or α-GFP antibody respectively. Depicted is a representative of three independent experiments. 

(C) Quantification of three independent experiments as the one shown in (A). Bcl-B signal intensity was normalized to GFP signal intensity. Normalized signal intensity at the 0 h time point was set to 100% and data points were connected by a one-phase decay curve fit. 

(D,E) Same experiments as depicted for WT HA-Bcl-B in panels B and C respectively, but now for lysineless (KR) HA-Bcl-B.
variants (Fig. 2A). Together, these data show that N-acetylation increases the stability of the lysine-less HA-Bcl-B mutant. The collective data argue, that an acetyl-free N-terminus may lead to protein degradation because it permits N-terminal poly-ubiquitination.

**N-acetylation inhibits degradation of HA-GFP regardless of its ubiquitination status**

Next, we addressed the possibility that an acetyl-free N-terminus may act as a protein-destabilizing region (degron), regardless of the fact that it permits N-terminal ubiquitination. To test this, we added the GY sequence to the stable protein GFP.\(^1\) N-acetylated WT and N-acetyl-free (GY)GFP were expressed in HEK 293T cells together with Blue Fluorescent Protein (BFP) as a stable protein control. Cells were treated with CHX for different time periods and analyzed for GFP content by flow cytometry. For both WT GFP and (GY)GFP, virtually no degradation was observed within the four hour chase period (Fig. 3A). These data indicated that the appended GY sequence did not affect GFP stability.

GFP lacks an unstructured region that can be used as a degradation initiation site, which is a requirement for efficient proteasomal degradation.\(^2\) Therefore, we added a loosely structured double HA-tag to GFP, which was either N-acetyl-free (GY) or N-acetylated (SD), and re-analyzed GFP stability. Interestingly, (GY)HA-GFP was now rapidly degraded within the four hour chase period while (SD)HA-GFP was as stable as untagged WT GFP (Fig. 3A). This result suggested that a loosely structured, acetyl-free N-terminus greatly destabilized GFP.

Next, we wanted to clarify whether it was the absence of N-acetylation that destabilized (GY)HA-GFP rather than its specific N-terminal sequence. Therefore, we studied the stability of three N-acetylated HA-GFP variants (AA, ME, SD), and three N-acetyl-free HA-GFP variants (AP, GY, VL). N-acetylation status of these specific N-terminal sequences was predicted with high confidence based on NAT-specificity and previously published analyses of whole proteome N-acetylation.\(^1,4\) In addition, for the sequences ME, SD and GY, our MS-analyses confirmed the predicted N-acetylation status (Fig. S1). Interestingly, all three N-acetylated HA-GFP variants were stable, but all three N-acetyl-free HA-GFP variants were rapidly degraded within the four hour CHX-chase period (Fig. 3B; Fig. S2). These data corroborated our results obtained with HA-Bcl-B (KR), and demonstrated that a protein can be destabilized by an acetyl-free N-terminus.

Next, we assessed whether the destabilization mediated by an acetyl-free N-terminus was dependent on ubiquitination of the substrate. To our surprise, all HA-GFP variants were ubiquitinated, including the N-acetylated ones, indicating that at least one of GFP’s 18 surface exposed lysines can accept ubiquitin (Fig. 3C). More importantly, the ubiquitination status of the N-acetyl-free HA-GFP variants was not quantitatively different from N-acetylated HA-GFP (Fig. 3D). Therefore, an acetyl-free N-terminus destabilized HA-GFP, without inducing quantitative changes in ubiquitination status.

**N-acetylation stabilizes lysineless p16 and prevents N-terminal ubiquitination**

The data obtained with GFP suggested that that N-acetylation can inhibit protein degradation regardless of the ubiquitination status of the protein. To further test this hypothesis, we
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Figure 3. N-acetylation stabilizes HA-GFP regardless of its ubiquitination status. (A) HEK 293T cells were transfected to express wild-type GFP, or GFP variants differing in the N-terminal amino acid sequence (GY, SD) that were either untagged, HA-tagged at the C-terminus (GFP-HA) or at the N-terminus (HA-GFP), together with BFP. Cells were treated with CHX (50 μg/ml) for indicated time periods and subsequently analyzed for GFP and BFP content by flow cytometry. GFP fluorescence intensity (FI) was normalized to BFP FI, and normalized GFP FI at the 0 h time point was set to 100%. Data points were connected by a one-phase decay curve fit. Depicted are mean and SD of three independent experiments. (B) As in (A), now including AA, ME, SD, AP, GY and VL HA-GFP variants that differed in the amino acid sequence of the mature N-terminus and their N-acetylation status. The prefix Ac indicates an acetylated N-terminus, while ~ indicates a free N-terminus. (C) HEK 293T cells were transfected to express FLAG-tagged ubiquitin, together with the indicated HA-GFP variants differing in the amino acid sequence of the mature N-terminus (N-residues) and their N-acetylation status (N-acetyl). HA-GFP was isolated from denatured lysates by α-HA IP, followed by immunoblotting of the precipitates. HA-GFP and ubiquitin were detected by probing with α-HA or α-FLAG mAb respectively. (D) Quantification of four independent experiments as the one shown in (C). For each HA-GFP variant, the signal intensity for total ubiquitinated HA-GFP was corrected for the signal intensity of non-modified HA-GFP, and normalized to the average signal of all six variants within the experiment. Normalized values of the N-acetylated AA, ME and SD variants and the N-acetyl-free AP, GY and VL variants were pooled and statistically analyzed (Student’s t-test; ns=non-significant).

studied the naturally lysineless p16, a tumor suppressor protein that inhibits cell-cycle progression by its interaction with cyclin-dependent kinase 4/6. The mature N-terminus of p16 starts with methionine-glutamate (ME), and proteomic studies have demonstrated that it is efficiently targeted for N-acetylation.

First, we examined the ubiquitination status of p16, and how this would be affected by its N-acetylation status. For this purpose, three N-terminal variants of p16-HA were studied:
wild-type p16 starting with ME, and two mutants starting either with SD or GY. MS-analysis demonstrated that (ME)p16-HA and (SD)p16-HA were completely N-acetylated, while (GY)p16-HA was N-acetyl-free (Fig. S1). Congruently, the N-acetylated variants (ME) and (SD) p16-HA were devoid of ubiquitination, but the N-acetyl-free (GY)p16-HA was ubiquitinated, most likely on the N-terminus because this was the only available acceptor site (Fig. 4A). Next, we tested whether the N-acetylation status of p16 determines its degradation rate, as found for lysineless HA-Bcl-B and for HA-GFP. Both N-acetylated variants were steadily degraded in a six hour CHX-chase period, but with much slower kinetics than N-acetyl-free (GY)p16-HA (Fig. 4B, C). Indeed, N-acetylated wild-type and SD variant p16 had a significantly longer half-life than the N-acetyl-free GY variant (Fig. 4D). Thus, we found, as for the lysine-less HA-Bcl-B mutant, that N-terminal acetylation protects the naturally lysineless p16 protein from N-terminal polyubiquitination and degradation.

![Figure 4](image)

**Figure 4. N-acetylation stabilizes p16, and blocks its ubiquitination.** (A) HEK 293T cells were transfected to express FLAG-tagged ubiquitin, together with C-terminally HA-tagged p16 variants. Variants differed in the amino acid sequence of the mature N-terminus (N-residues) and their N-acetylation status (N-acetyl). P16 was isolated from denatured lysates by α-HA IP, followed by immunoblotting of the precipitates. P16 and ubiquitin were detected by probing with α-HA or α-FLAG mAb respectively. (B) and (C) Primary data and quantification of p16 degradation. HEK 293T cells were transfected to express GFP as a stable protein control, together with wild-type ME-p16 and the SD and GY variants that differed in the amino acid sequence of the mature N-terminus and their N-acetylation status. Degradation was determined by CHX chase and quantified as outlined for figures 2B and 2C respectively. (D) The half-life of p16-HA was calculated from three independent experiments as shown in (A). Mean and SD are depicted, and asterisks indicate statistically significant differences (Student’s t-test; *P<0.05, ***P<0.001).
N-acetylation stabilizes lysineless p16 regardless of its ubiquitination status

The remaining question was whether an acetyl-free N-terminus destabilized (GY)p16-HA because it permitted N-terminal ubiquitination, or regardless of its effect on ubiquitination status. To examine this, we first made a p16 variant that was both ubiquitinated and N-acetylated. To accomplish this, a lysine residue was introduced in (SD)p16-HA close to the mature N-terminus (+K). This p16 variant was ubiquitinated to a similar extent as acetyl-free (GY)p16-HA, suggesting that the introduced lysine was as efficiently targeted as the free N-terminus (Fig. 5A). However, despite its ubiquitination, (SD)p16-HA (+K) had a similar half-life as lysineless (SD)p16-HA, and was more stable than (GY)p16-HA (Fig. 5B, C, D).

These data indicate that, as for modified GFP, p16 was stabilized by N-acetylation, even when it was ubiquitinated. However, we still needed to test whether a protein with an acetyl-free N-terminus requires ubiquitination to be destabilized. For this purpose, we required a p16 variant that was N-acetyl-free but not ubiquitinated. We hypothesized that introduction of a proline at the N-terminus might reduce ubiquitination of p16, since we observed this in the (AP)HA-Bcl-B (KR) variant (Fig. 2A). We therefore mutated p16-HA to make variants

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Figure 5. p16 stability is determined by its N-acetylation status, and not by its ubiquitination status. (A) HEK 293T cells were transfected to express FLAG-tagged ubiquitin, together with C-terminally HA-tagged wild-type ME-p16 and GY and SD variants, now with a with a lysine residue on P4 of the mature N-terminus (+K). Ubiquitination was determined as outlined for figure 4A. (B) and (C) Degradation of the p16-HA variants with a lysine residue on P4 of the mature N-terminus (+K), determined and quantified as outlined for figures 2B, C. (D) The half-life of p16-HA was calculated from three independent experiments as shown in (B). Mean and SD are depicted, and asterisk indicates statistically significant difference (Student’s t-test; *P<0.05).
with a mature N-terminus starting with either proline-glutamate (PE) or alanine-proline (AP). Both N-termini conform to the (X)PX rule and are therefore N-acetyl-free. Despite the absence of N-acetylation, we could not detect any ubiquitination of (PE)p16-HA or (AP) p16-HA (Fig. 6A), indicating that an N-terminus with an ultimate or penultimate proline is a poor ubiquitination site. Importantly, (PE)p16-HA and (AP)p16-HA were still rapidly degraded, with a half-life similar to (GY)p16-HA (Fig. 6B, C, D). These data demonstrate that ubiquitination is not required to target N-acetyl-free p16 for degradation.

The majority of proteins are degraded by the proteasome, but in some cases degradation can be performed by other proteolytic complexes, like the tripeptidyl peptidase II. To test whether p16-HA variants were degraded by the proteasome, we expressed them in HEK 293T cells, and treated with either CHX alone or in combination with the proteasome inhibitor MG132. For all variants, MG132 inhibited degradation, indicating that the proteasome degrades p16 and can do this in a ubiquitin-independent manner (Fig. 6E). Together, these data show that N-acetylation inhibits p16 proteasomal degradation by a mechanism that is independent of p16 ubiquitination.

P16 tumor suppressor capacity is determined by its N-acetylation status

P16 is a tumor suppressor protein, and its anti-tumor capacity will depend on its protein levels, and consequently on its stability. Therefore, we hypothesized that a difference in p16 N-acetylation status would affect p16 function. P16 function can be assessed in the osteosarcoma cell line U2OS, which does not express endogenous p16 and arrests in G1 cell-cycle phase upon ectopic p16 expression. Equal amounts of (ME)p16-HA and (GY) p16-HA cDNA were transfected in U2OS cells, together with spectrin-GFP cDNA to detect transfected cells. Cells were harvested and stained with propidium iodide (PI) followed by flow cytometry to analyze DNA content as a read-out for cell-cycle status. Compared to control cells, expression of (ME)p16-HA induced a significant accumulation of cells in G1 phase (Fig. 7A). When cells expressed (GY)p16-HA this accumulation in G1 phase was still present, but significantly reduced compared to the accumulation in (ME)p16-HA expressing cells (Fig. 7A). Although equal amounts of cDNA were transfected for both p16 variants, steady state protein levels of (ME)p16-HA were higher than of (GY)p16-HA (Fig. 7B), reflecting the difference in half-life (Fig. 4D). From these and above data we conclude that N-acetylation stabilizes p16 and therefore enhances the tumor suppressor capacity of p16.

DISCUSSION

The majority of human proteins are co-translationally modified by N-acetylation, but the function of this modification is incompletely understood. It has been suggested to affect protein stability, but whether it stabilizes a protein, or rather targets it for degradation is under debate. Here, we aimed to resolve this debate, and studied the role of N-acetylation in protein degradation in human cells. Our data clearly demonstrate that N-acetylation increases protein stability, by two different mechanisms (Fig. 8). First, N-acetylation blocks N-terminal ubiquitination, and thereby reduces ubiquitin-mediated targeting to
Figure 6. An acetyl-free N-terminus targets p16 for degradation, even in absence of ubiquitination. (A) HEK 293T cells were transfected to express FLAG-tagged ubiquitin, together with C-terminally HA-tagged wild-type ME-p16 and PE and AP variants. Ubiquitination was determined as outlined for figure 4A. (B) and (C) Degradation of the p16-HA variants, determined and quantified as outlined for figures 2 B, C. (D) The half-life of p16-HA was calculated from three independent experiments as shown in (B). Mean and SD are depicted, and asterisks indicate statistically significant differences (Student’s t-test; *P<0.05, **P<0.01). (E) HEK 293T cells were transfected to express GFP as a stable protein control, together with indicated p16-HA variants that differed in the amino acid sequence of the mature N-terminus and their N-acetylation status. The prefix Ac indicates an acetylated N-terminus, while ~ indicates a free N-terminus. Cells were left untreated, or treated with either CHX (50 μg/ml) alone or in combination with MG132 (50 μM) for six hours. Total cell lysates were analyzed by immunoblotting, p16 and GFP were detected by probing with α-HA or α-GFP respectively.

the proteasome. Second, N-acetylation can inhibit proteasomal degradation directly, independent from its effect on substrate ubiquitination.

Our results are in agreement with early biochemical experiments using isolated proteins, which indicated that blocking the N-terminal α-amino-group by N-acetylation inhibited protein degradation.11-12 However, they are in contrast with the recent observation that an acetylated N-terminus can function as a degradation signal in yeast.28-29 This N-acetyl-degron
is recognized by the E3 ligase Doa10, which will subsequently ubiquitinate the substrate and target it for proteasomal degradation. In theory, the stabilizing and degron functions of N-acetylation could co-exist in human cells, and it might depend on the specific protein or cellular state which function is more dominant. We hypothesize however, that the function of N-acetylation might have diverged during evolution to yeast or human, explaining the contrasting data. There are some notable differences in N-acetylation between the species: in yeast cells the NatA, NatB and NatC complexes are always ribosome-associated, while in human cells a significant fraction of these complexes is non-ribosomal. Furthermore, yeast cells do not have a NatF homologue and only 57-68% of all proteins are N-acetylated, compared to 84% in human cells. The increased incidence of N-acetylation in human cells might point to an acquired global function of N-acetylation, which is lacking or less dominant in yeast.

A global function of N-acetylation is also more likely because it is an irreversible modification. It is therefore difficult to imagine that protein stability is regulated by specific modulation of the N-acetylation status of individual proteins. We suggest that N-acetylation functions as a tag that licenses proteins to survive in the cell. Secretory proteins are mostly N-acetyl-free, which might facilitate their rapid degradation when they mistakenly end up in the cytosol. Proteolytic cleavage events generate C-terminal protein fragments that might be non-functional or only temporarily functional. Cleavage often occurs in unstructured loops, so the C-terminal fragment will in many instances contain a degradation initiation site, and an acetyl-free N-terminus, allowing for rapid degradation. The 16% of human proteins that are N-acetyl-free possibly escape this degradation by binding to stable interaction partners, or they might have functions which require rapid turnover.

Figure 7. N-acetylation determines p16 stability, and therefore its capacity to inhibit cell cycle progression. (A) U2OS cells were transfected to express spectrin-GFP, together with p16-HA variants that differed in the amino acid sequence of the mature N-terminus and their N-acetylation status. The prefix Ac indicates an acetylated N-terminus, while ~ indicates a free N-terminus. Cells were fixed and stained with propidium iodide (PI), followed by flow cytometry to analyze DNA content in the spectrin-GFP+ cells. Data represent mean and SD of three independent experiments. Asterisks indicate statistically significant differences (Student’s t-test; *P<0.05). (B) Expression control of (A). U2OS cells were transfected to express spectrin-GFP together with p16-HA variants that differed in the amino acid sequence of the mature N-terminus (N-residues) and their N-acetylation status (N-acetyl). Total lysates were immunoblotted for p16 (α-HA) and spectrin-GFP (α-GFP).
This does not necessarily mean that N-acetylation determines the stability of every protein in the cell. Indeed, our data indicate that degradation kinetics of wild-type Bcl-B do not depend on its N-acetylation status, most likely because Bcl-B is efficiently K48-polyubiquitinated on lysine residues. Also, preventing N-acetylation of GFP only destabilized the protein when an unstructured region was added to the N-terminus. This is most likely explained by the lack of a degradation initiation site in wild-type GFP. Hence, N-acetylation is expected to determine the stability of a subgroup of proteins, including proteins that are not (efficiently) ubiquitinated on lysine residues, and proteins that contain unstructured regions that can function as a degradation initiation site.

Perturbations of N-acetylation might not result in detectable changes in protein stability measured on a proteome-wide scale, unless other factors that determine protein stability are excluded as variables. These include ubiquitination, the presence of a degradation initiation site, protein structuredness and protein length. Indeed, two proteomic studies could not detect global changes in protein levels upon either knockout of NatB in yeast or knockdown of NatA in human cells. A possible extra complication with the knockdown study is that even a very small fraction of NatA is sufficient to N-acetylate all NatA substrates. Efficient knockdown of both the NatA auxiliary and catalytic subunit reduced N-acetylation of only 16 of the 242 identified NatA substrates. Therefore, insight into what substrates are stabilized by N-acetylation might mainly come from studying the mechanism of stabilization, rather than from proteomic studies.
Our data indicate that two mechanisms contribute to the increased stability of N-acetylated proteins. We demonstrate that N-acetylation blocks N-terminal ubiquitination, as was previously shown for the mouse protein p19Arf and the human cell cycle regulator p21. For lysineless Bcl-B, the N-terminal ubiquitination status clearly correlated with the degradation kinetics, suggesting that N-acetylation blocks targeting to the proteasome by preventing N-terminal ubiquitination (Fig. 8). However, we found that N-acetylation can also inhibit proteasomal degradation of a given protein independent from its effects on N-terminal ubiquitination (Fig. 8). N-acetylation stabilized the naturally lysineless protein p16, even when it was not ubiquitinated at all, and N-acetylation stabilized p16, also when it was deliberately ubiquitinated on an introduced lysine residue. HA-GFP was also stabilized by N-acetylation, and either of two mechanisms might have applied. N-acetylation of HA-GFP did not change its ubiquitination status quantitatively, but might have induced a qualitative switch from N-terminal ubiquitination to lysine ubiquitination.

Our findings suggest that N-acetylation can inhibit protein degradation by directly inhibiting proteasomal processing. It might impact on one or more of the following required steps: binding of the substrate to the proteasome, engaging the substrate via a degradation initiation site, unfolding the substrate, threading it into the proteolytic chamber, and degrading it. Substrates generally bind to the proteasome by means of ubiquitin, although they may also contain degrons that target them for ubiquitin-independent degradation, as demonstrated for the enzymes Thymidylate Synthase (TS) and Ornithine Decarboxylase. In agreement with our results, ubiquitin-independent degradation of TS required an acetyl-free N-terminus. Importantly, a free N-terminus was not sufficient to target TS for degradation, indicating that the N-terminal α-amino-group cannot function as an independent degron. The non-ubiquitinated p16 may also contain proteasomal docking elements, since we found that it was also targeted for degradation when it was N-acetylated, albeit more slowly than the acetyl-free variants. Hence, an acetyl-free N-terminus may assist proteasomal processing of substrates that are targeted to the proteasome by other elements, for example by degron sequences. A free N-terminus may enhance affinity of the substrate for the proteasome, facilitate entry into the proteasome, or serve as a ‘handle’ used to thread the substrate into the proteolytic chamber.

We changed the N-acetylation status of our substrates by mutating the N-terminus, but as an alternative method we also fused ubiquitin to their N-terminus. After synthesis of the fusion-protein, ubiquitin is efficiently removed by ubiquitin hydrolases, but during translation the ubiquitin moiety is expected to prevent N-acetylation. However, we found that p16 derived from a ubiquitin-p16 fusion protein is still completely N-acetylated (Fig. S1). Congruently, ubiquitin fusion did not alter the degradation rate of HA-GFP or p16 variants. (Fig. S3A-D) This indicates that ubiquitin may be removed during translation and prior to N-acetylation. Alternatively, N-acetylation may occur after translation, as is supported by proteomic studies that provided evidence for post-translational N-acetylation. Since ubiquitin fusion is often used to change the nature of the N-terminal amino acid, particularly in the N-end rule field, we caution that data interpretation may be confounded by effects on N-acetylation.
The naturally lysineless protein p16 is a cell cycle inhibitor and an important tumor suppressor that is deleted or mutated in many human cancers.\textsuperscript{41-42} Ordinarily, p16 is N-acetylated, but we showed that p16 is destabilized when this is prevented by N-terminal mutations. Such mutation resulted in decreased steady state p16 protein levels and a reduced capacity to inhibit cell cycle progression. Interestingly, this process may be relevant in cancer: a mutation was detected in the gene encoding for p16 in a melanoma cell line, and this mutant gene would encode for p16 with MK as N-terminal amino acids.\textsuperscript{43} This N-terminus is predicted to be a relatively poor target sequence for N-acetylation, so p16 levels are possibly low in this melanoma cell line.\textsuperscript{4} In this way, the MK mutation may have helped the melanoma cells to escape from cell cycle inhibition.

Interestingly, WT P16 has been suggested to be N-terminally ubiquitinated in 'sparsely' plated HeLa cells, suggesting that under these conditions N-acetylation of p16 was reduced.\textsuperscript{44} We consider the possibility that a cell can encounter conditions that affect the N-acetylation efficiency in general. Specifically, there might be a link between N-acetylation and metabolism. The carbon required to N-acetylate a protein is derived from glucose, which is processed to citrate in the TCA cycle and subsequently converted by ATP-citrate lyase into Acetyl-CoA, the donor-metabolite of the acetyl-group.\textsuperscript{45} A recent paper showed that overexpression of the anti-apoptotic protein Bcl-xL inhibited N-acetylation, because it reduced the levels of citrate and Acetyl-CoA.\textsuperscript{34} N-acetylation could be restored in Bcl-xL overexpressing cells by supplementing the medium with citrate. So the carbon flux through the cell might determine the levels of cellular N-acetylation, and therefore the stability of a potentially large amount of proteins.

**MATERIALS AND METHODS**

**Constructs**

All N-terminal protein variants were generated by PCR-mediated mutagenesis. For HA tagging, cDNA was subcloned into pHAN1 or pHAC2, that are variants of pEGFPN1 and pEGFPC2 (Clontech) in which the GFP tag is replaced by a double HA-tag (a kind gift of Lennert Janssen, Division of Cell Biology II, The Netherlands Cancer Institute, The Netherlands). Constructs encoding N-terminally (SD)HA-tagged Bcl-B WT or KR were previously described.\textsuperscript{14} P16 cDNA and spectrin-GFP were a kind gift of Rob Klompmaker and Indra Shaltiel respectively (Division of Cell Biology I, The Netherlands Cancer Institute, Amsterdam, The Netherlands). FLAG-ubiquitin construct was previously described,\textsuperscript{46} and was subcloned in a 3x FLAG-ubiquitin tandem repeat variant.

**Cell culture and transfection**

U2OS and HEK 293T cell lines were cultured in DMEM, supplemented with 8% FCS and antibiotics at 37°C, 5% CO\textsubscript{2}. Transfections were performed using polyethyleneimine (PEI) at a 1:3 (w/w) cDNA:PEI ratio. Cells were harvested 24 h after transfection, or 48 h after transfection when p16 ubiquitination was assessed.
**Immunoprecipitation**

To assess ubiquitination, cells were lysed by incubation for 5-10 min in pre-heated denaturing SDS buffer (50 mM Tris-HCl pH 8.0, 1% SDS, 0.5 mM EDTA, 10 mM DTT). SDS was quenched and diluted by adding nine volumes of NP-40 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM PMSF, Roche protease inhibitor cocktail, 1 mM EDTA). Lysates were centrifuged for 15 min at 13,000 g, 4°C to remove insoluble material. Next, protein concentration was determined by a Bradford assay, and IP was performed from equal amounts of protein with anti-HA mAb 12CA5 and Protein G Sepharose beads (GE Healthcare Life Sciences). Incubation was for 2 h at 4°C while rotating. Precipitate was washed 3-5 times with NP-40 buffer, and precipitated proteins were eluted by boiling for 10 min in LDS sample buffer with DTT. For MS analysis and analysis of p16 ubiquitination shown in figures 5A and 6A, N-ethylmaleimide was added to PBS used for washing the cell pellet, and to NP-40 buffer, to an end-concentration of 2 mM. After IP with the anti-HA mAb 12CA5, the precipitate was eluted by boiling in denaturing SDS buffer. Next, SDS was quenched by 1% NP-40, and a re-IP with 3F10 affinity matrix (Roche) was performed, followed by elution in sample buffer.

**Western blotting and antibodies**

Protein was separated by SDS-PAGE using 4-12% gradient NuPage gels (Invitrogen) according to manufacturer’s protocol. Next, protein was transferred to nitrocellulose membrane by semi-dry blotting using the Trans-Blot Turbo system (Bio-Rad). Membrane was blocked in 5% skim milk or Roche blocking buffer diluted in PBS, followed by antibody probing in 1% skim milk or Roche blocking buffer diluted in TBST. Fluorescence signal was analysed using the Odyssey Imaging System (LI-COR), chemiluminescence (Pierce Biotechnology) was analysed using the ChemiDoc Imaging System (Bio-Rad) or film (Kodak). Primary antibodies used were peroxidase-conjugated α-FLAG mAb M2 (Roche), DY-800 or DY-682 (Dyomics) fluorochrome-conjugated (conjugated in house) α-HA mAb 12CA5, and rabbit α-GFP pAb. Fluorochrome-conjugated goat-α-rabbit Ig (LI-COR) was used as secondary antibody.

**CHX-chase assay**

Transfected HEK 293T cells were treated with 50 μg/μl CHX (Sigma), in some instances in combination with 50 μM MG132 (Calbiochem) for the indicated time periods. For immunoblot read-out, cells were harvested and lysed in NP-40 buffer, followed by immunoblotting. Read-out was by the Odyssey Imaging System (LI-COR) to allow for quantification. Signal intensity of HA-tagged substrate was normalized to signal intensity of co-expressed GFP. For flow cytometric read-out of GFP, cells were harvested by trypsinization, and GFP and BFP signal intensity was determined by analyzing cells using a plate reader on the LSR Fortessa (BD Biosciences). For both read-outs signal at the 0 h time point was set to 100%. Mean and SD of three independent experiments was plotted, and points were connected by a one-phase decay curve fit. For curve fitting in case of GFP no plateau was set and in case of p16 and
HA-Bcl-B, a plateau was set at 0% and 52% respectively. Curve fitting and statistical analysis were performed using GraphPad Prism (Graph Pad software).

**Mass spectrometry**

Proteins were precipitated as described above, followed by SDS-PAGE and staining of proteins in the gel using SimplyBlue SafeStain (Life Technologies), after which bands of interest were excised. To assess Bcl-B ubiquitination, the N-methylated form of iodoacetamide was used as alkylation reagent instead of standard iodoacetamide, to avoid false-positive interpretation of ubiquitination. When N-acetylation was assessed, a chemical in-gel acetylation using deutero-acetyl was performed according to the protocol described in ref. Next, in-gel trypsin-digestion was performed using the Proteineer DP digestion robot (Bruker). Peptides were extracted from the gel, lyophilized, dissolved in 95/3/0.1 v/v/v water/acetonitril/formic acid and subsequently analyzed by on-line nanoHPLC MS/MS using an 1100 HPLC system (Agilent Technologies), as previously described. Peptides were trapped at 10 μL/min on a 15-mm column (100-μm ID; ReproSil-Pur C18-AQ, 3 μm, Dr. Maisch GmbH) and eluted to a 200 mm column (50-μm ID; ReproSil-Pur C18-AQ, 3 μm) at 150 nl/min. All columns were packed in house. The column was developed with a 30-min gradient from 0 to 50% acetonitrile in 0.1% formic acid. The end of the nanoLC column was drawn to a tip (5-μm ID), from which the eluent was sprayed into a 7-tesla LTQ-FT Ultra mass spectrometer (Thermo Electron). The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion-monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. Selected ions were fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post-analysis process, raw data were first converted to peak lists using Bioworks Browser software v3.2 (Thermo Electron), and then submitted to the Swissprot database, using Mascot v. 2.2.04 (www.matrixscience.com) for protein identification. Mascot searches were with 2 ppm and 0.5 Da deviation for precursor and fragment mass, respectively, and trypsin as enzyme. Collision-induced dissociation spectra were manually inspected. With the exception of a few very clear MS2 spectra, in most cases peptides of interest were synthesized to confirm spectrum assignment.

**ACKNOWLEDGEMENTS**

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### SUPPLEMENTARY FIGURES

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Figure S1. N-acetylation status of p16 and Bcl-B variants. (A) Indicated substrates were expressed in HEK293T cells and isolated by a tandem HA-IP protocol as detailed in the Materials and Methods section. Precipitate was separated by SDS-PAGE, and band of interest was excised. N-acetyl-free peptides were modified using an in-gel deutero-acetylation protocol, followed by trypsin-digestion and analysis by LC-MS/MS. Second column indicates whether the start methionine was retained, or removed in vivo by MetAPs. N-acetyl indicates whether: (+) the complete pool was N-acetylated (-) the complete pool was N-acetyl-free (-), or (+/-) part of the pool was N-acetylated.

Figure S2. N-acetylation inhibits degradation HA-GFP. Representative dot plots of the experiments depicted in figure 3B.
Figure S3. N-acetylation inhibits degradation of p16-HA and HA-GFP derived from ubiquitin-fusion proteins. (A) and (B) As in figure 2B and 2C respectively, but for ubi-p16-HA variants. Depicted in (A), and analyzed in (B) is p16-HA generated after ubiquitin removal. (C). The half-life of p16-HA derived from ubi-p16-HA was calculated from three independent experiments as shown in (A). Mean and SD are depicted, and asterisks indicate statistically significant differences (Student’s t-test; *P<0.05, ***P<0.001). (D) As in figure 3A, but for ubi-HA-GFP variants.