Trends in Biochemical Sciences

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Review

First Things First: Vital Protein Marks by N-Terminal Acetyltransferases

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N-terminal (Nt) acetylation is known to be a highly abundant co-translational protein modification, but the recent discovery of Golgi- and chloroplast-resident N-terminal acetyltransferases (NATs) revealed that it can also be added post-translationally. Nt-acetylation may act as a degradation signal in a novel branch of the N-end rule pathway, whose functions include the regulation of human blood pressure. Nt-acetylation also modulates protein interactions, targeting, and folding. In plants, Nt-acetylation plays a role in the control of resistance to drought and in regulation of immune responses. Mutations of specific human NATs that decrease their activity can cause either the lethal Ogden syndrome or severe intellectual disability and cardiovascular defects. In sum, recent advances highlight Nt-acetylation as a key factor in many biological pathways.

Your Favorite Protein Is Probably Acetylated at its N-Terminus

N-terminal acetylation (Nt-acetylation), also called Nα-acetylation (see Glossary and Figure 1), is a protein modification that is poorly represented in textbooks and, as such, many scientists are unaware that 80% of all human proteins receive an acetyl group at their N-terminus. Owing to several recent advances in the field, however, there is now a growing interest in this protein modification. Several recent contributions shed light on the molecular mechanisms through which the **N-terminal acetyltransferases** (NATs) exert their important biological functions. This review aims at advancing our current understanding of the protein, cellular, and physiological consequences of Nt-acetylation based on a multitude of recent reports. Consequently, it also highlights the fact that Nt-acetylation can no longer be viewed as an automatic negligible modification, but instead emerges as a crucial component in many biological pathways.

How Many and Which Proteins Are N-Terminally Acetylated?

Nt-acetylation has been established as a highly abundant protein modification in eukaryotic cells [1–4]. In fact, it is so common that it is reasonably safe to presume that your favorite protein is probably subject to this modification. The determinant for undergoing Nt-acetylation is mainly the identity of the first two amino acids at the N-terminus. Several NATs (NatA to NatF in humans) collectively catalyze Nt-acetylation of a majority (80%) of the different types of N-termini occurring in the proteome, resulting in the **Nt-acetylome** (Figure 2A). NatA has specificity towards A-, S-, T-, V-, C-, and sometimes G-starting N-termini, whose initiator methionine (iMet) has been removed by methionine amino peptidases (MetAPs) [2,5]. NatD also acts within the iMet-processed group; however, it is far more selective because it has only been shown to Nt-acetylate histones H2A and H4, making its contribution to the Nt-acetylome negligible [6,7]. Those N-termini that retain the iMet are modified by NatB, NatC, NatE, and NatF. In this group NatB acetylates Met-'Asx/Glx'-type N-termini (MD-, ME-, MN-, and MQ-starting) [8,9] whereas

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The identification of the first membrane-associated NAT, Naa60/NatF, and the first chloroplast NAT, Naa70/NatG, established new modes of the NAT machinery in their capacity to acetylate transmembrane and lumenal chloroplast proteins, respectively.

The structure of the NatA complex revealed molecular determinants for substrate-specific acetylation, including the significant impact of the auxiliary Naa15 on the specificity of the catalytic Naa10.

Nt-acetylation has been shown to regulate protein complex stoichiometry through the Ac/N-end rule pathway, which has also been connected to hypertension. Further, a link was established between Nt-acetylation and global protein folding.

Nt-acetylation has been found to play essential roles in *A. thaliana* drought-stress and immune responses, in *C. elegans* development and metabolism, as well as in human diseases.

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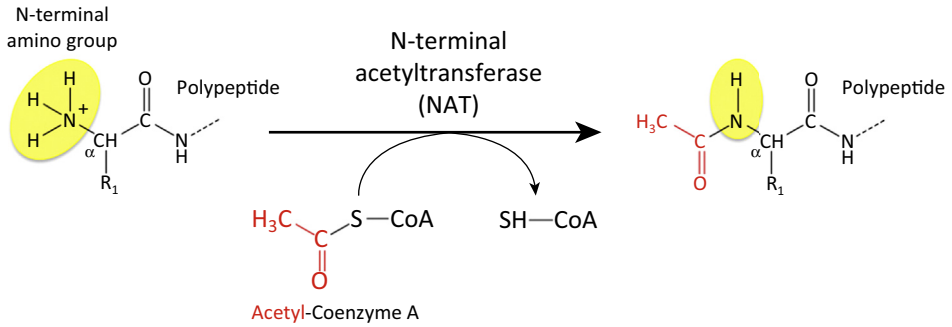

Figure 1. The Addition of an Acetyl Group to a Protein N-Terminus Is Catalyzed by an N-Terminal Acetyltransferase (NAT). N-terminal acetylation is a protein modification that changes the chemical properties of the N-terminus by neutralizing its positive charge through the addition of an acetyl group. The reaction is enzymatically catalyzed by N-terminal acetyltransferases (NATs) that transfer the acetyl group (-$COCH_3$, red) from acetyl-CoA (Ac-CoA) onto the Nα-group (NH_3^+, yellow) of the very first amino acid residue of the substrate protein. This modification is often referred to as Nα-acetylation to distinguish it from the acetylation of Nε-groups on lysine side chains.

NatC, NatE, and NatF act on Met-'hydrophobic/amphipathic'-type N-termini (ML-, MI-, MF-, MY-, and MK-starting) [10–15]. The recently identified plant NatG acetylates M-, A-, S-, T-starting N-termini, however it lacks a human ortholog [16].

Although proteomic technologies are incapable of directly defining the Nt-acetylation status of the entire proteome, an estimate of the total Nt-acetylation events is possible by extrapolation of proteomics data to all Swiss-Prot entries (Figure 2, based on [17]). Through this approach, it is calculated that the NAT family Nt-acetylates 80% of the human proteome (Figure 2A). This amount relates to both full and partial Nt-acetylation because many proteins exist as both Nt-acetylated and unacetylated variants. Hence it is very likely that a given protein, for example your favorite protein, is subject to Nt-acetylation. In determining the likelihood of a particular protein being Nt-acetylated based on its two first amino acids one can make use of the chart in Figure 2B where the size of subgroups within each NAT substrate class is shown as well as the frequency of Nt-acetylation events within the subgroups. If the N-terminus of the protein in question starts with alanine, which is in the NatA substrate class, there is a 95% chance that it is Nt-acetylated, but if it starts with valine there is a 80% chance that its N-terminus is unacetylated. NatB is distinguished in this sense because it has near 100% coverage of the Met-'Asx/Glx'-type N-termini. The combined action of NatC, E and F within the Met-'hydrophobic/amphipathic'-group will probably undergo further refinement in substrate specificity-profiling because it acts only on ~75% of the sequence-predicted substrates. A recent study on NatF revealed that it has selectivity for membrane proteins and that the NatF substrate category is enriched for transmembrane proteins [17]. This study was also the first to investigate the membrane-bound part of the Nt-acetylome, which was found to be equally large in the amount of proteins Nt-acetylated, although these were typically Nt-acetylated to a lesser degree, meaning that there were more partial Nt-acetylation events.

The Enzymes Catalyzing N-Terminal Acetylation

The NAT Compilation

The eukaryotic NAT machinery known to date is composed of seven NATs (NatA–NatG) (Figure 3) of which five (NatA–NatE) are present in *Saccharomyces cerevisiae* (reviewed in [18,19]), whereas multicellular eukaryotes have a sixth NAT, NatF [15,17], and in addition in the plant *Arabidopsis thaliana* a seventh NAT, NatG [16] has been identified. The eukaryotic NAT machinery is more complex than that of prokaryotes, which only have three known NATs. It is not well understood why eukaryotic cells have evolved different NATs. NAT diversification could

Glossary

GCN5-related N-acetyltransferase (GNAT) superfamily: protein superfamily related to general control nondepressed 5 (GCN5) comprising, among others, the catalytic Nα-acetyltransferases.

N-terminal acetyltransferase (NAT): enzyme that catalyzes Nt-acetylation.

N-terminal acetylation (Nt-acetylation): also known as Nα-acetylation, this is a protein modification involving the addition of an acetyl group to the free amino group (Nα-group) of a substrate protein (Figure 1).

***NAA*/Naa:** Nα-acetyltransferase gene/protein, respectively. Names given to human N-terminal acetyltransferases are numerically organized by acetyltransferase activity type: NatA activity is mediated by the catalytic subunit Naa10 and the auxiliary subunit Naa15, while NatB activity is mediated by the catalytic Naa20 and auxiliary Naa25 subunits, and so on.

Nt-acetylome: the part of the proteome that is subjected to Nt-acetylation.

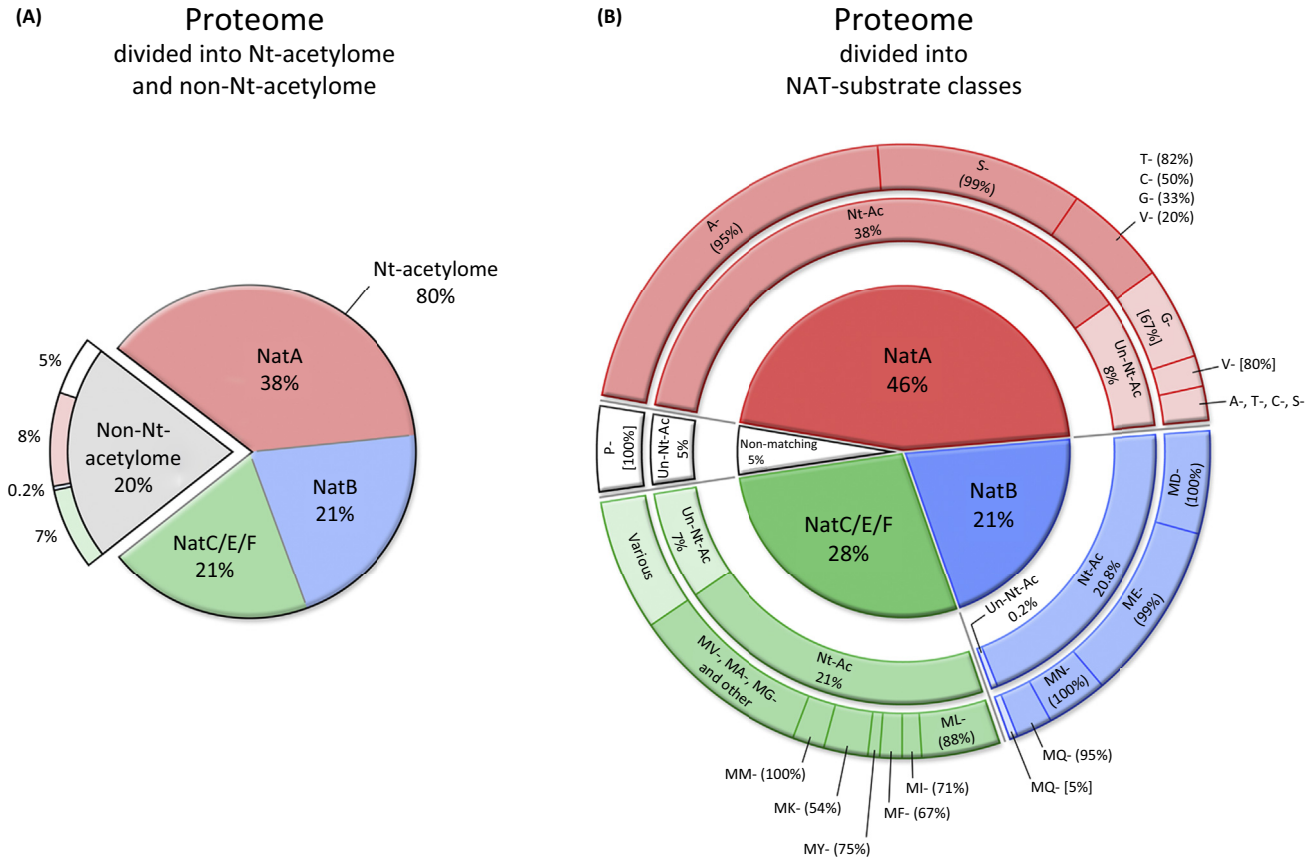

Figure 2. Occurrence of N-Terminal Acetylation in the Human Proteome. (A) To visualize the common occurrence of Nt-acetylation and N-terminal acetyltransferase (NAT) activity, the human proteome is divided into the Nt-acetylome (80%) and the non-Nt-acetylome (20%), and subdivided based on NAT-type substrate categories to illustrate the contribution by the different NATs. Note that the NAT substrate classes described to date also contain some part of the non-Nt-acetylome, as indicated by the color-coding. (B) To provide a tool for determining the likelihood that a particular protein N-terminal sequence undergoes Nt-acetylation, the human proteome was divided into NAT substrate classes with the contribution of different N-termini indicated. The inner pie shows the size of the NAT substrate class; the middle circle matches the pie in (A) and indicates the amount of Nt-acetylated versus unacetylated proteins within each substrate group; and the outer circle provides a more detailed overview of the different N-termini constituting a substrate class that can be used to predict the Nt-acetylation status of a given protein. For example, alanine A-starting proteins constitute the largest subgroup within the NatA-type substrate class, and 95% of A-starting proteins are Nt-acetylated. For NatB, the substrate category accounts for 21% of the proteome with only 0.2% being unacetylated, allowing the assumption that any MD-, ME-, MN-, MQ-starting protein is Nt-acetylated. NatC/E/F-type substrates are more difficult to predict based on current substrate profiles because this group accounts for 28% of the proteome, but with 7% unmodified. ML- is the most common substrate within the NatC/E/F class, and 88% of ML-N-termini are Nt-acetylated. Numbers are based on an estimate of total Nt-acetylation events made by extrapolation of the most recent proteomics data (also including organellar proteins) [17] to all Swiss-Prot entries based on the occurrence of the first two amino acids. Partial Nt-acetylation is considered as an Nt-acetylation event, meaning that a given protein can exist in both Nt-acetylated and non-Nt-acetylated forms, referred to as a partially Nt-acetylated protein. Proteome occurrences of partial iMet retention for MA-, MS-, MT-, MG-, and MV- were calculated based on their detection as iMet-processed/unprocessed. Data were lacking for MW- and MR-starting proteins, and these numbers were inferred based on structural similarity.

conceivably have co-evolved with the increasing complexity of the proteome. However, based on the currently identified enzymes, NATs did not diverge during the evolution of eukaryotes in which proteome complexity vastly increased [19]. The more complex eukaryotic NAT machinery entails a specialization towards different substrate groups that could provide a more flexible system in terms of regulation. In fact, some examples of upstream regulation of NATs have recently been uncovered (discussed in following sections). The recently discovered NATs, NatF targeting transmembrane proteins and NatG targeting chloroplast proteins, are clear examples of specialization, and likely exist owing to a need for particular subgroups of these substrate types to be Nt-acetylated.

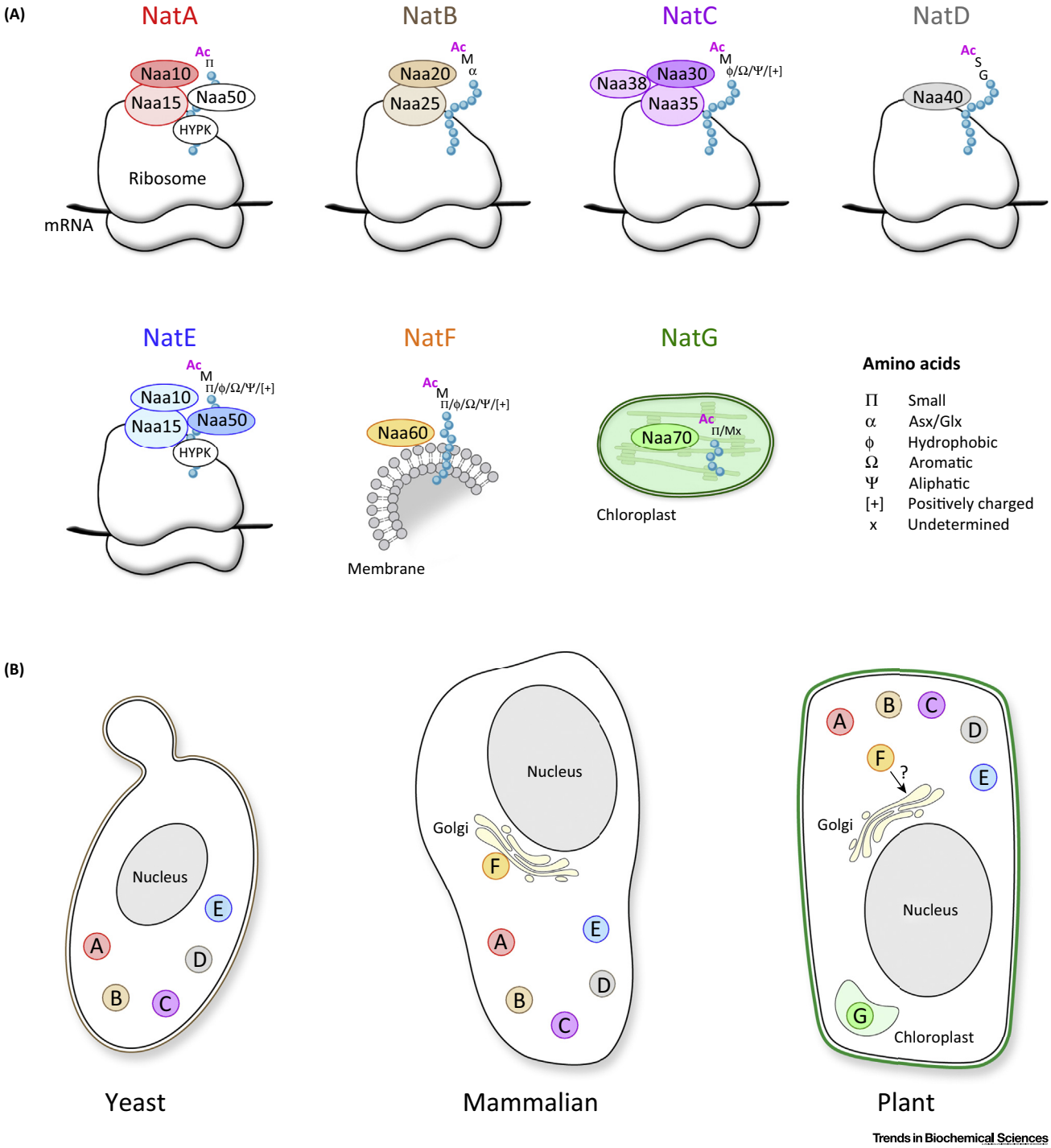

Figure 3. The N-Terminal Acetyltransferase (NAT) Machinery in Eukaryotic Cells. (A) Subunit composition of the seven currently known eukaryotic NATs, NatA–NatG. The catalytic subunits (Naa10, Naa20, Naa30, Naa40, Naa50, Naa60, and Naa70) typically associate with up to two auxiliary subunits (Naa15, Naa25, Naa35, and Naa38) that contribute to activity of the NAT complex through ribosome anchoring and/or substrate specificity-modulation. Amino acids are coded as follows: π, small; ∝, Asx/Glx; ϕ, hydrophobic; Ω, aromatic; Ψ, aliphatic; [+], positively charged; and x, undetermined. Specific N-termini of the different NAT classes are shown in Figure 2. (B) Intracellular distribution of the different NAT complexes from unicellular (yeast) to multicellular (mammals and plants) eukaryotes. While NatA–NatE reside in the cytosol, the two most recently identified NATs are organellar. NatF is found in both mammals and plants where it, at least in human cells, associates with the Golgi surface. NatG is found inside plant chloroplasts. In addition, some of the NAT catalytic subunits appear to localize to the nucleus (e.g., Naa10 and Naa50; not shown).

NAT activity typically requires a NAT complex in which the catalytic transferase subunit joins up to two auxiliary subunits that may mediate ribosome anchoring and in some cases contribute to substrate specificity (Figure 3A) [20–22]. The NatA complex is formed by the catalytic subunit **Naa10** and the auxiliary subunit Naa15 [5,23]. Naa50 is also attached to the NatA complex together with HYPK (Huntingtin-interacting protein K) [20,24,25]. Naa50 also forms part of the NatE complex (together with Naa15 and Naa10), which displays distinct substrate specificity and a distinct depletion phenotype compared to NatA [12,14,20,21,26]. NatB is composed of the catalytic subunit Naa20 and the auxiliary subunit Naa25 [27,28]. The catalytic subunit of NatC is Naa30, which associates with two noncatalytic subunits, Naa35 and Naa38, of which Naa35 mediates ribosomal association, whereas the role of Naa38 is less well described [11,13,22]. For NatD (Naa40), NatF (Naa60), and NatG (Naa70) no auxiliary subunit has been identified so far (Figure 3A). Interestingly, a recent study revealed that an N-terminal segment of Naa40, which is unique compared to the other catalytic Naas, might play a role that is analogous to the ribosomal-binding auxiliary subunits found in the other NATs [29].

The Workplace of NATs

Most NATs are localized in the cytosol (Figure 3B). NatA–NatE are associated with ribosomes, where they perform co-translational Nt-acetylation (Figure 3A) [20,22]. In addition, some catalytic subunits may localize to the nucleus [7,17,23] and Naa10 and Naa50 also display NAT activity in the absence of the ribosome-anchoring subunit, Naa15, but with altered substrate specificities [12,14,17,21]. Given that these NATs also exist in nonribosomal forms, this suggests that NATs might also act post-translationally [14]. In fact, several internal peptides, produced by post-translational cleavage *in vivo*, are Nt-acetylated [30,31] and a specific example of a protein undergoing such post-translational processing is actin [32].

Of particular interest are two newly identified NATs with organellar localization (Figure 3B). NatF is associated with the Golgi apparatus facing the cytosolic side where it Nt-acetylates transmembrane proteins [17]. NatG was identified as the first NAT localized inside an organelle, in this case in chloroplasts of the plant *A. thaliana* [16]. These recent studies underpin the post-translational nature of Nt-acetylation and finally connect this modification to cellular organelles.

Nt-Acetylation from a Structural Viewpoint: A Blueprint

Structural information on the NATs is currently emerging as the crystal structures of several Nα-acetyltransferases and NAT complexes have been solved. The recently developed bisubstrate analog-based Nα-acetyltransferase inhibitors [33] have been useful in some of these structural studies.

A crystal structure with both subunits of the NatA complex (Naa10 and Naa15) has been solved (Figure 4A) [21]. The Naa15 subunit consists of 37 α-helices arranged into 13 TPR (tetratricopeptide repeat) motifs [21]. These are conserved motifs composed of sequences of 34 amino acids that generally serve as protein–protein interaction motifs [34]. Some of the TPR motifs in Naa15 are involved in Naa10 binding [21], and it is likely that they are also crucial for the associations of Naa15 with Naa50, HYPK, and the ribosome (Figure 3A). The overall tertiary structure of Naa15 forms a ring-like structure with a cavity into which Naa10 binds (Figure 4A). The Naa15–Naa10 binding is mainly conducted by a large hydrophobic interface as well as by a few hydrogen bonds [21].

The catalytic subunits of NATs belong to the **GCN5-related N-acetyltransferase (GNAT) superfamily** together with some of the lysine acetyltransferases (KATs). All the NATs share the structural GNAT-domain (Figure 4B), which is an evolutionarily conserved characteristic of NATs, as shown by structural analyses of the bacterial NAT RimI [35] and the NAT from the archaea *Sulfolobus solfataricus* [36]. The GNAT domain consists of a central acetyl-CoA binding motif

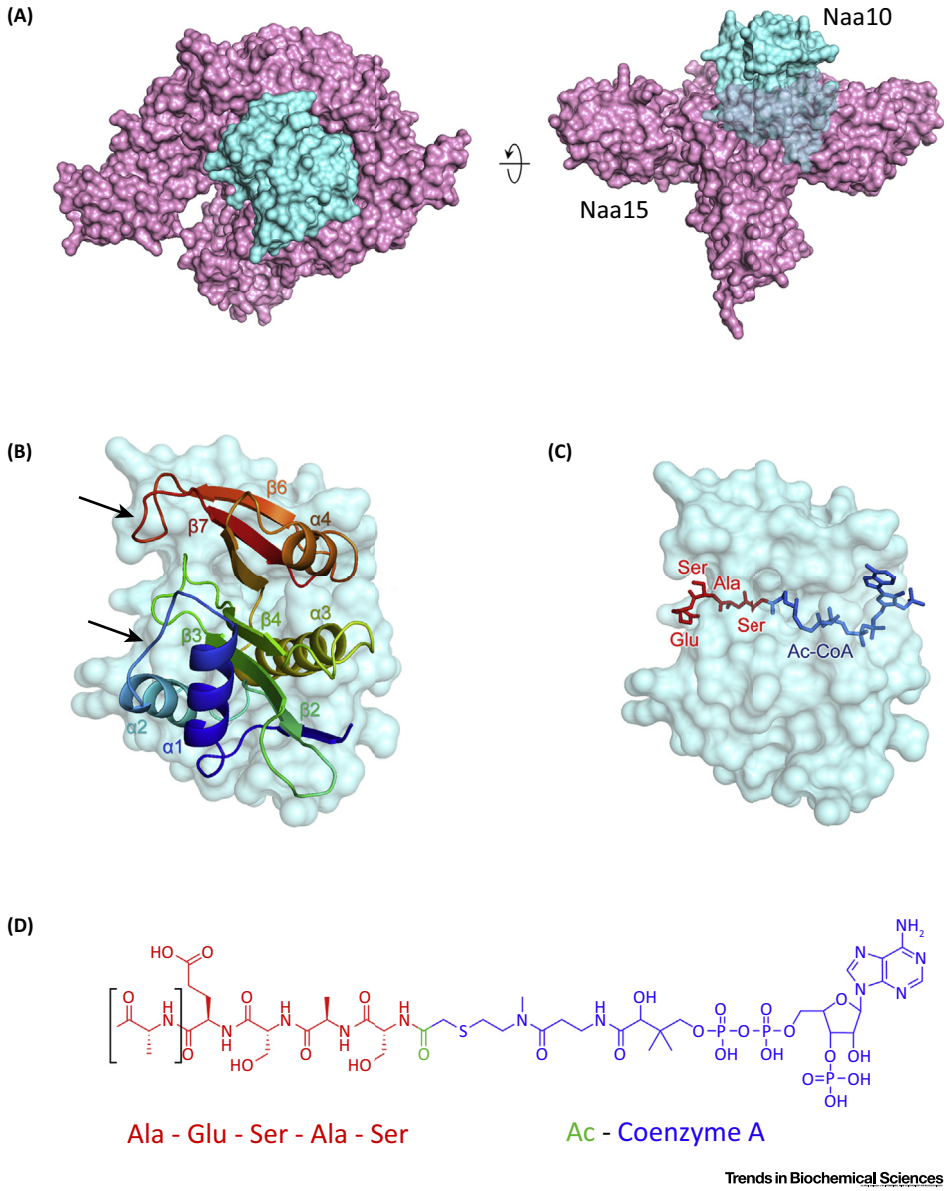

Figure 4. Structural Models of the NatA Complex. (A) Crystal structure of the *Schizosaccharomyces pombe* yeast NatA complex showing the interaction between the catalytic subunit Naa10 (cyan) and the auxiliary unit Naa15 (pink). The semi-transparency of Naa15 shows the insertion of Naa10 into the Naa15 binding cavity. (B) X-ray structure of the GNAT-fold (cartoon) depicted on Naa10 (surface) showing the positions of the acetyl-CoA binding domain-forming α-helix α3 (yellow) and β-sheets β2 (blue), β3, and β4 (green tones) as well as the peptide substrate-binding pocket-forming N-terminal α1-loop-α2 (blue/cyan, lower arrow) and the C-terminal β-hairpin of β6-loop-β7 (orange/red, upper arrow). (C) Association of a bisubstrate analog-based inhibitor with the peptide-acetyl-CoA substrate-binding pockets of the Naa10 monomer enzyme in its NatA complex configuration. (D) Chemical structure of the bisubstrate conjugate shown in (C), consisting of acetyl-CoA (blue) covalently linked to a NatA substrate peptide fragment Ser-Ala-Ser-Glu-Ala. Brackets represent the segment of the substrate peptide not crystallized and not shown in (C). The X-ray diffraction structures in (A–C) are modified from protein data bank (PDB) entry: 4KVM [21].

(Q/RxxGxG/A) flanked by four α-helices and seven β-sheet segments [16,21,29,37,38]. The acetyl-CoA binding domain is formed by several secondary elements (α3 and β2, β3, and β4) [37]. Substrate binding and the catalytic bi-bi reaction occur in a semi-open cavity that harbors both acetyl-CoA and the first 4–5 amino acids of the N-terminal peptide (Figure 4B,C).

Substrate specificity is assured by an N-terminal helix-to-helix loop and a C-terminal β-hairpin loop (Figure 4B) [21,37]. This C-terminal β-hairpin in NATs has an extended loop compared to KATs of the same superfamily, restricting the substrate specificity to α-amino groups [39,40]. Thus, these recent structural advances and enzymatic investigations reveal that it is improbable that NATs can acetylate ε-amino groups on lysine residues [39], as suggested for Naa10 and other NATs by previous *in vitro* studies [41,42]. However, NATs may act as N-terminal propionyltransferases, transferring slightly larger chemical groups onto protein N-termini [43].

The structure of the highly selective Naa40 differs from Naa10 and Naa50. In this case the substrate specificity-providing β-hairpin is flipped away from the substrate-binding site, and in compensation the α1-loop-α2 adopts a new conformation owing to an extended N-terminal segment [29]. This unique conformation of the extended loop is also the reason for the selectivity of Naa40. In contrast to Naa10, Naa50, and Naa60, which have the highest preference for peptide residue one and decreasing selectivity towards the succeeding residues, Naa40 requires the first four residues for substrate binding [21,29,37,38].

In the NatA complex, binding to Naa15 affects the conformation of the Naa10 α1-loop-α2, which alters the substrate specificity of Naa10 from acidic N-termini towards S- or A-starting (NatA-type) N-termini [14,21]. It is possible that future structural studies of NatB and NatC complexes will show similar modulating effects by the large auxiliary subunits (Naa25, Naa35) on their respective catalytic subunits.

To summarize, a majority of proteins receive an acetyl group at their N-terminus through the enzymatic activity of several NATs (NatA to NatF in humans) acting on different subtypes of N-termini. The NATs are often complexes in which a catalytic GNAT fold-containing enzyme (e.g., Naa10) is paired with a ribosome-binding subunit (e.g., Naa15), and the recently solved structures reveal how auxiliary subunits may also modulate substrate specificity. Nt-acetylation was recently connected to the organelles through the identification of Golgi- and chloroplast-resident NATs. The following section focuses on how the proteins subjected to Nt-acetylation are affected at the molecular level.

Substrate Responses to the Nt-Acetyl Group

With the wide array of proteins undergoing Nt-acetylation, it is not surprising that the substrate proteins are affected very differently (Figure 5). There are several examples of proteins that crucially depend on the Nt-acetyl group for some part of their functioning. However, the overall roles of Nt-acetylation are only recently beginning to emerge.

Nt-Acetylation of Cellular Proteins Affects Proteasomal Half-Life Regulation

A fundamental function of Nt-acetylation in promoting protein degradation was proposed in 2010 when it was shown that the acetyl group at the N-terminus of a protein can act as a specific degradation signal (termed Ac/N-degron) that is targeted by the Ac/N-end rule pathway, a novel branch of the previously characterized N-end rule pathway [44]. In this case, acetylated N-termini starting with M, S, A, T, or V were identified as Ac/N-degrons, and Doa10 [44] and Not4 [45] E3 ubiquitin ligases were described as specific recognition components (Ac/N-recognins) of the Ac/N-end rule pathway (Figure 5-1). Recently, Ac/N-degrons were described as conditional in that they may be shielded from recognition through participation in a protein complex (Figure 5-2) [45]. This work also identified two Ac/N-degron-containing substrates, Hcn1 and Cog1, which were each found to have their Ac/N-degron shielded upon complex formation with Cut9 and Cog2/3, respectively. The acetylated N-terminal Met residue of Hcn1 is enclosed within a TPR superhelix chamber in Cut9 [46]. This was the first crystal structure showing an Nt-acetylated Met at a protein–protein interaction site.

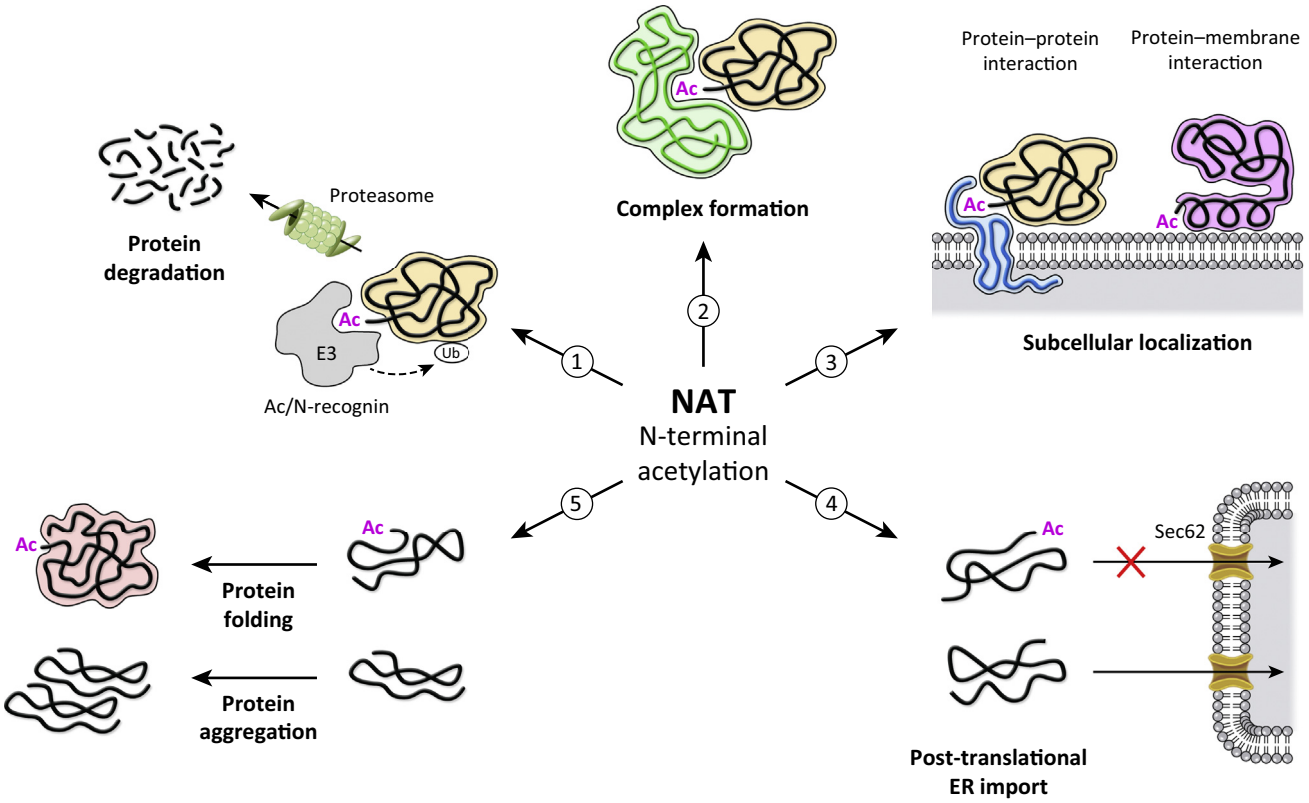

Figure 5. N-Terminal Acetylation May Affect Proteins in Several Different ways. Summary of described examples of protein functional effects of Nt-acetylation. NAT activity leads to an Nt-acetylated protein. This modified protein may be recognized by an Ac/N-recognin E3 ligase (e.g., Doa10) and targeted for proteasomal degradation through the Ac/N-end rule pathway, in which case the acetylated N-terminus acts as an Ac/N-degron (1). Another fate of an Nt-acetylated protein may be participation in a complex in which the Nt-acetyl group has a particular role in the protein–protein interaction site, acting as an interaction mediator (2). Complex formation may also represent a way for newly correctly folded proteins to shield their Ac/N-degron and prevent Ac/N-end rule targeting. Nt-acetylation-dependent targeting to the correct subcellular localization (3) may occur through protein–protein interactions or direct protein–membrane interactions; in the latter, the Nt-acetyl group possibly stabilizes a membrane-interacting secondary structure, such as an N-terminal α-helix like in the case of α-synuclein. Another example of Nt-acetylation-dependent targeting is the finding that acetylated N-termini prohibit post-translational import of proteins into the ER via the SRP-independent pathway using the Sec62 translocation channel (4). Finally, NAT-depletion is also associated with protein aggregation, suggesting that Nt-acetylation is involved in global protein folding (5). Abbreviations: Ac, acetyl group; ER, endoplasmic reticulum; NAT, N-terminal acetyltransferase; SRP, signal recognition particle; Ub, ubiquitin.

Continued work on the Ac/N-end rule pathway demonstrated that it is complemented by another branch of the N-end rule, the Arg/N-end rule pathway [47]. In this case Ubr1, the ubiquitin ligase of the Arg/N-end rule pathway, was shown to recognize unacetylated Met if followed by a bulky hydrophobic residue; that is, unacetylated NatC/E/F-type N-termini (Figures 2 and 3). This means that both Nt-acetylation and lack thereof can target proteins for degradation via the N-end rule pathway. A physiologically relevant example of such dual targeting was later provided by a study on Rgs2, a regulator of mammalian G-protein signaling [48]. The wild-type MQ-Rgs2 is subjected to degradation by the Ac/N-end rule pathway subsequent to full NatB-mediated Nt-acetylation (i.e., all Rgs2 molecules are Nt-acetylated). By contrast, a naturally occurring N-terminal mutant associated with hypertension, ML-Rgs2, is only partially Nt-acetylated by NatC (i.e., both Nt-acetylated and un-Nt-acetylated variants of Rgs2 are produced), and thus may be degraded via either of the two branches of the N-end rule. Overall, this leads to the mutant having a shorter half-life. The result is an imbalance in signaling governing blood pressure control in patients bearing that mutant, thus connecting the N-end rule pathway and Nt-acetylation to human physiology [48,49].

The generalizability of the Ac/N-end rule is challenged by the fact that fewer than 10 proteins have been demonstrated to follow this degradation pathway [44,45,47,48]. In addition, several previously known substrates of an Ac/N-recognin, such as Doa10, do not appear to require Nt-acetylation for their degradation [50], suggesting that Doa10, similarly to Ubr1 (the N-recognin of the Arg/N-end rule pathway), may, in addition to the Ac/N-degron recognition site, also contain other substrate-binding sites. Moreover, a more complex picture is suggested by studies describing increased protein half-lives due to Nt-acetylation, such as the *Drosophila* Hyx [3] and the human THOC7 [51].

Nt-Acetylation as a Mediator of Protein Interactions and Complex Formation

Complex formation may not only shield proteins from Nt/Ac-mediated degradation but may also be involved in enhancing protein complex formation *per se* (Figure 5-2). For example, NatC-mediated Nt-acetylation increases the affinity of Ubc12 for Dcn1, and yeast and human crystal structures revealed that Met-1 of the N-terminus of Ubc12 is buried in a hydrophobic pocket in Dcn1 [52]. Later, this concept was expanded because this distinctive Nt-acetylation-dependent interaction is structurally conserved across a family of mammalian NEDD8 ligation enzymes [53]. X-ray structures of Sir3 binding to the nucleosome revealed an Nt-acetylation-dependent interaction in which the Nt-acetyl group does not directly participate at the interaction site, but instead stabilizes a binding loop [54,55]. Another Nt-acetylation-dependent interaction is exemplified by phosducin-like 3 (PDCL3), a chaperone involved in the regulation of vascular endothelial growth factor receptor-2 (VEGFR-2). Interaction between these to proteins depends on NatB-mediated Nt-acetylation of PDCL3 and results in VEGFR-2 protection from misfolding and aggregation [56].

Nt-Acetylation as a Determinant for Protein Subcellular Localization

Several examples in which Nt-acetylation acts as a localization determinant in yeast have been known for some time [18,57,58]. However, despite more recent efforts [59,60], potential additional yeast substrates depending on the Nt-Ac group for localization remain unknown. Some of the examples of Nt-acetylation-dependent protein subcellular targeting (Figure 5-3) occur through protein–protein interaction. However, a direct interaction between an Nt-acetylated protein and the membrane can be rationalized by the increased hydrophobicity of an acetylated N-terminus (Figure 1) contributing to the overall hydrophobicity or stabilization of a membrane-interacting region. A recent clinically relevant example is the Parkinson's disease-implicated protein α-synuclein. Nt-acetylation stabilizes an N-terminal α-helix in α-synuclein, which increases its affinity for membranes [61]. Structural characterization further revealed an Nt-Ac-α-synuclein–Cu(I) complex, in which Nt-acetylation as well as copper binding to the N-terminal region contribute to stabilization of the α-helical structure [62]. The Nt-acetyl group likely stabilizes the helix macrodipole and cause more favorable H-bonds as a result of the absence of the α-amino positive charge. These recent reports on α-synuclein not only substantiate the link between Nt-acetylation and targeting to membranes but also suggest a possible role of Nt-acetylation in metallobiology.

Another mode of Nt-acetylation-dependent subcellular targeting was indicated by a study demonstrating that the absence of Nt-acetyl groups is part of an early determining step in the cellular sorting of nascent polypeptides following the signal recognition particle (SRP)-independent pathway for post-translational translocation across the endoplasmic reticulum (ER) membrane (Figure 5-4) [63]. In this case the N-termini of cytosolic proteins were reported to typically be among the highly Nt-acetylated NAT substrate classes, NatA or NatB, as compared to the N-termini of secretory proteins, which were typically enriched for NatC/E/F-type sequences. Moreover, mutating such secretory proteins into more Nt-acetylatable variants inhibited their targeting to the ER. However, note that many of the proteins harboring secretory signal sequences match the NatC/E/F-target class (Figure 2) meaning that they cannot *per se* be considered as non-Nt-acetylated.

Nt-Acetylation Affecting Protein Folding and Aggregation

The presence or absence of charge at the N-terminus may be a central factor in protein folding. Indeed, a potential role of Nt-acetylation in global protein folding (Figure 5-5) was recently uncovered [64]. It was shown here that Nt-acetylation deficiency in NatA-deleted yeast leads to the accumulation of misfolded proteins and increased levels of chaperones. This study also suggested that NatA might be implicated in the Sup35/PSI^+ prion cycle in yeast. Prions are heritable elements transmitted via protein, typically ordered into filamentous aggregates (amyloids). In [PSI^+] yeast cells the Sup35 protein forms amyloids. Sup35 is a NatA substrate, and deletion of NatA in [PSI^+] phenotypic cells decreases the stability of unacetylated Sup35 amyloid and relieves the phenotype of the [PSI^+] cells [64].

NatA depletion in human cells was also shown to cause aggregation of Huntingtin (Htt). HYPK (Htt-interacting protein K) stably interacts with NatA (Figure 3A), which is essential for its optimal Nt-acetylation activity [25]; HYPK also acts as a chaperone that prevents aggregation of Htt [65]. Knockdown of HYPK, but interestingly also Naa10 or Naa15, increases the aggregation tendency of mutant polyQ Htt, which is found in patients with Huntington's disease [25]. Thus, it appears that the NatA–HYPK complex is essential for proper Htt folding, possibly as a result of the direct Nt-acetylation of Htt. Another example of disease-related aggregation that is connected to Nt-acetylation is α-synuclein in Parkinson's disease, described above.

Interplay Between Nt-Acetylation and Other Protein Modifications

It was recently found that Ube2w, an E2 enzyme, can ubiquitylate the α-amino groups of protein N-termini [66,67], whereas ubiquitylation more commonly occurs on the ε-amino group of lysines. These reports indicate the possibility of an interplay between E2s and NATs that both act on protein N-termini, in which Nt-acetylation may potentially block E2s and ubiquityl-mediated proteasomal degradation, or inhibit signaling events mediated by N-terminal monoubiquitylation. Another example of such interplay with other modifications is the finding that NatD-mediated Nt-acetylation of histone H4 blocks the methylation of H4Arg3, which in turn regulates ribosomal DNA silencing [68]. A recent study also revealed kinetic competition between Naa50 and MetAPs, and proposed that Naa50-mediated Nt-acetylation may act to retain the iMet of otherwise MetAP-susceptible N-termini [69].

Several studies have now demonstrated the importance of Nt-acetylation for many different proteins. The molecular mechanisms in which Nt-acetylation take part are diverse and encompass half-life regulation, protein–protein and protein–membrane interactions, subcellular localization, folding, and aggregation. The following sections discuss the physiological consequences of Nt-acetylation as revealed by recent studies on model organisms and human diseases.

Physiological Functions of NATs in Model Organisms

The consequences of Nt-acetylation are also manifested in biological systems, and studies using different model organisms demonstrate NATs to be essential for a variety of physiological processes (Figure 6). Specific links between organism phenotypes and molecular regulation of or by NATs are now in the process of being uncovered.

In *Arabidopsis*, Nt-acetylation was recently shown to impact on drought-stress adaptation because NatA-depleted plants have increased drought-tolerance (Figure 6A-1) [70]. In that study, one of the very few known examples of upstream regulation of NAT activity was also revealed. Wild-type plants subjected to drought stress were shown to have decreased Nt-acetylation levels caused by a rapid transcriptional downregulation of NatA subunits by abscisic acid, a plant stress hormone. The finding that NatA function is indispensable in *Arabidopsis* supports its physiological importance, and overall this report suggests that decreases in NatA-mediated Nt-acetylation is an important switch in cellular stress responses [70].

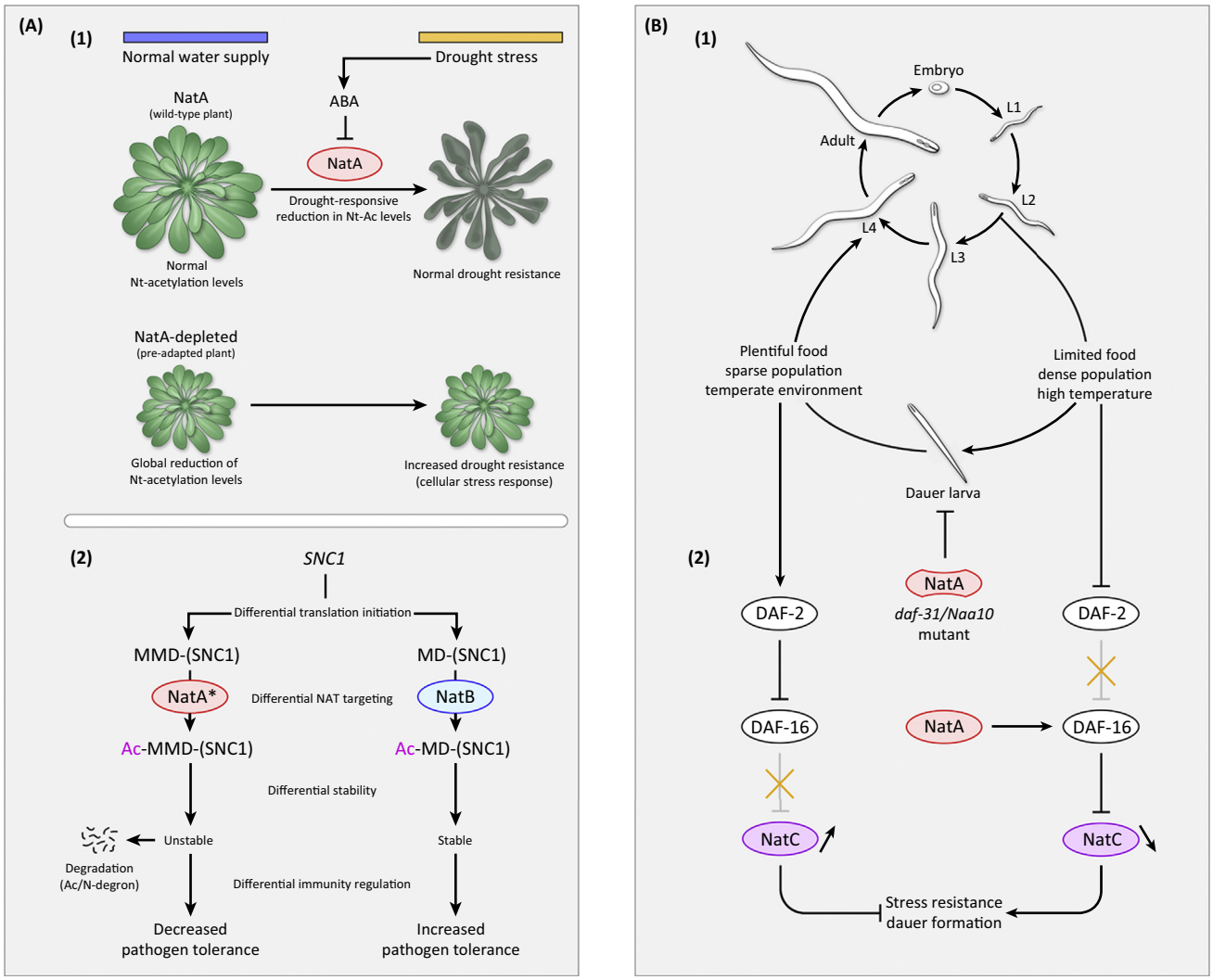

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Figure 6. Regulatory Consequences of NAT Activity at the Organism Level. (A) Impact of regulated Nt-acetylation in *Arabidopsis*. (A1) Under drought, plants produce the phytohormone abscisic acid (ABA) that induce part of the drought-stress response through transcriptional regulation, allowing the plant to adapt to the drought environment. As part of this process, ABA negatively affects the activity of NatA, causing a drought-responsive reduction in Nt-acetylation levels. Experimental induction of NatA-depletion with the concomitant reduction in global Nt-acetylation triggers constitutive induction of the drought-stress response, thus pre-adapting these plants to drought and increasing tolerance and survival, although NatA-depletion under normal water supply conditions causes vegetative growth retardation [70]. (A2) SNC1 is a Nod-like receptor serving as a immune receptor, and thus plays an important role in defense against pathogens in plants. Differential translation initiation generates two Nt-variants: MMD-(SNC1) and MD-(SNC1), which are each subject to Nt-acetylation, but by different NATs and with different consequences. For the NatA substrate, MMD-(SNC1), the Ac-Met likely acts as an Ac/N-degron, destabilizing SNC1 and thereby causing decreased pathogen tolerance. By contrast, the shorter MD-SNC1 form is Nt-acetylated by NatB and stable, conferring increased immune resistance [71] (* indicates some uncertainty about which NAT actually performs Nt-Ac of the MMD variant; see text). (B) Influence of NATs in *C. elegans* metabolism and development. (B1) The *C. elegans* lifespan and reproductive cycle consists of four larval stages (L1–L4), adulthood, and a dormant larvae dauer stage that is induced by an unfavorable environment. daf-31/NAA10-mutated larvae do not adopt a proper dauer stage under starved conditions. In addition, these daf-31/NAA10-mutated, starved larvae do not resume development upon exposure to nutrients [75]. (B2) Molecular model of how NATs affect the pathway balancing dauer stage formation and growth, starting with the transmembrane receptor DAF-2. Naa10/NatA stimulates the activity of DAF-16, a transcription factor regulating stress-response genes and whose inhibition by DAF-2 controls dauer formation. NatC acts further downstream in this pathway because it is repressed by DAF-16. Under rich environmental conditions NatC negatively impacts on stress resistance and dauer formation [76].

Another recent plant study identifies Nt-acetylation as a key switch in immune regulation and reports the antagonistic regulation of two different N-terminal variants of the same protein by two NATs (Figure 6A-2) [71]. The key plant immunity protein, SNC1, exists as two Nt-variants, MD-SNC1 (NatB substrate) and MMD-SNC1 (therein reported as a NatA substrate). Peculiarly, these

two variants respond very differently to Nt-acetylation: Ac-MMD-SNC1 is destabilized and Ac-MD-SNC1 is stabilized, leading to a decreased or enhanced immune response, respectively. Hence, this study connects protein half-life regulation by Nt-acetylation (discussed earlier) to biological regulation, and also demonstrates how this may interplay with alternative Nt-variants to provide homeostatic regulation. It can be speculated that such a two-faced regulation might apply to other MM-starting proteins as well [71,72]. Note that MMD-SNC1 does not match the canonical NatA specificity, and modification could conceivably involve Naa50/NatE (Figure 3A).

Previous studies in *Arabidopsis* have identified phenotypes associated with NAT loss-of-function. Pleiotropic developmental defects were associated with loss of NatB activity as observed in tcu2/NAA25-mutated plants [73]. In addition, NatC-mediated Nt-acetylation is necessary for normal growth and efficient photosynthesis, as revealed by the mak3-1/NAA30 mutant in which the synthesis of two key photosynthetic proteins, D1 and CP47, is affected [74].

In *Caenorhabditis elegans*, Nt-acetylation has been linked to development and metabolism as daf-31/NAA10 mutants failed to properly enter and exit the dauer stage, which is essential for *C. elegans* survival during starvation (Figure 6B-1) [75]. These findings indicate that NatA plays an important role in regulating adaptive metabolic behavior through an insulin-like IGF1 signaling pathway by inducing the activity of the transcription factor DAF-16. Interestingly, the same pathway was found to involve NatC, acting as a modulator of stress resistance downstream of DAF-16 [76]. In this case, loss-of-function mutations in natc-1/NAA35 increased resistance to several stressors, including oxidative stress. This study thus represents another example where a NAT is both regulated by and a key regulatory factor in important (in this case, metabolic) physiological processes. Such a metabolic involvement by NatC has also previously been suggested by yeast nutrient stress studies (reviewed in [18]). NatC could also possibly be linked to aging because a recent yeast study found this NAT to affect proteasome distribution (an age-dependent process) and showed that loss of NatC specifically affects growth of replicative old cells, in other words, those that have produced a high number of daughter cells [77].

The involvement of Naa10 in development was also supported by a *Drosophila melanogaster* study in which reduced Naa10 levels caused pleiotropic oogenesis defects [78]. Further, naa10-null mutations were shown to affect cell growth and division, and caused lethality. *Drosophila* flies mutated in Naa50 (NatE) [24] and fly cells depleted for Naa60 (NatF) [15] are viable, but have phenotypes related to cellular chromatid cohesion.

The zebrafish *Danio rerio* was recently adopted in NAT studies, and here the essentiality of Naa10 for development was again demonstrated. Developmental abnormalities such as a bent axis, abnormal eyes, and bent tails were observed after morpholino-mediated knockdown of naa10 [79]. These morphants were further described to display increased lethality and growth retardation. An earlier zebrafish study also connected NatC to development, and embryonic growth control and vessel development were affected, possibly through changes in mTOR signaling [80]. The Nt-acetylation-dependent protein interaction between Ac-MQ-PDCL3 and VEGFR-2 described above is associated with angiogenesis in zebrafish and mice [56]. The same report showed that a mutant PDCL3 that is unable to undergo Nt-acetylation was unresponsive to hypoxia, a known stimulator of angiogenesis which normally regulates the expression of PDCL3.

Overall, these organism-based studies undoubtedly testify that NATs play important physiological roles in development, stress signaling, and metabolic adaptation, and they will no doubt continue to provide important contributions to our understanding of the function of Nt-acetylation in humans.

Impact of NATs in Human Diseases

The importance of Nt-acetylation in human development and physiology has recently been established through the discovery of NAT mutations in several pathological conditions. Various mutations impairing the stability and/or catalytic activity of Naa10 are known to cause genetic disorders. The lethal X-linked Odgen syndrome is caused by a Ser37Pro mutation in Naa10 that impairs its catalytic activity as well as complex formation with Naa15 and Naa50 [51,81,82]. Other missense mutations in the *NAA10* gene result in severe human disorders in both males and females, including Tyr43Ser, Arg83Cys, Phe128Leu/Ile, Val107Phe, and Arg116Trp [83–86]. These patients have partially overlapping phenotypes including global developmental delay, cardiac anomalies, and intellectual disabilities.

A recent study connected Nt-acetylation with human blood pressure regulation by showing altered NAT targeting and half-life (described earlier) for a hypertension-associated variant of Rgs2, a blood pressure-regulating G-protein [48]. As described earlier, Nt-acetylation was also connected to Parkinson's disease through α-synuclein [87,88], and to Huntington's disease through NatA downregulation, leading to HYPK depletion and aggregation of the polyQ Huntingtin protein [25].

Several NATs, especially the NatA subunits, show an involvement in cancer cell survival and proliferation. In particular, Naa10 is able to act both as oncoprotein and tumor suppressor, depending on the type of tumor and tissue [89–92]. Other NATs are also involved in tumor development, as shown by *NAA30* knockdown experiments resulting in reduced tumorigenicity in glioblastoma cells. The *NAA30* knockdown cells had reduced levels of HIF1α and increased levels of the apoptosis factor p53 [93]. Similar effects were observed for Naa40, where *NAA40* knockdown in colorectal carcinoma cells led to increased apoptosis by activation of the mitochondrial caspase 9-mediated apoptotic cascade [94]. The manifold involvement of NATs in various signal cascades important for tumor development and metastasis makes it challenging to generally summarize the impact of NAT on cancer. Nevertheless, this also encourages the development of specific NAT inhibitors as potential new cancer drugs [33].

Concluding Remarks and Future Perspectives

The majority of proteins are acetylated at their N-terminus, and the identity of the first two amino acids can be used to predict whether your favorite protein is modified and by which N-terminal acetyltransferase. With as much as 80% of the proteome being substrates for Nt-acetylation, the work of understanding its functions is still ongoing. Our understanding of Nt-acetylation has recently advanced significantly. Protein features that may be affected by Nt-acetylation are half-life, interaction ability, subcellular targeting, folding, and aggregation. In particular, the biological consequences of NAT activities, which we knew nearly nothing about only a few years ago, are now in the process of being elucidated. Several important roles for NATs in physiological processes such as development, stress signaling, and metabolic adaptation have recently emerged. However, we are still unable to answer the fundamental question of why the cell spends vast amounts of energy on attaching acetyl moieties to the N-termini of so many proteins. Part of this enigma may be resolved by upcoming future studies (see Outstanding Questions).

Outstanding Questions

Are there more NAT enzymes to be discovered in eukaryotes? Most of the Nt-acetylome appears to be accounted for by the known NATs. However, proteomics and phylogenetic data indicate that there may exist further so-far unidentified NATs, for example in chloroplasts and mitochondria.

How do NATs interact with ribosomes? Are specific NATs targeted to distinct ribosome populations? The distinct ribosome-binding auxiliary subunits of the different NATs may potentially serve as adaptors for particular ribosome populations that specifically translate the substrates of the different NATs. Such a specific NAT–ribosome system would facilitate rapid and efficient Nt-acetylation, avoiding potentially time-consuming scanning events.

What are the biological functions of Nt-acetylation, and how do they connect to protein-level effects? Our understanding of the cellular as well as physiological functions of Nt-acetylation is still limited. Of particular interest is the linkage of phenotypes observed in model organisms and human diseases to particular NAT substrates.

Is Nt-acetylation regulated? Plant drought-stress-induced reduction of Nt-acetylation via NAT-downregulation was recently demonstrated. NAT regulation could also possibly occur through enzyme activation or inactivation, directly via acetyl-CoA levels (as for histone acetylation), or by reversibility of the modification. Although it might be unlikely that the majority of Nt-acetylated proteins are Nt-deacetylated, it is plausible that a few key proteins may be deacetylated for specific regulatory purposes.

Could specific inhibition or activation of NATs be utilized in disease treatment? Compounds specifically targeting NATs are being developed, and such compounds have potential in the treatment of NAT-related diseases. In the near future it is very likely that these compounds may be used to investigate NAT function *in vivo* and contribute to structural studies.

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