The Role of Protein Nα-Terminal Acetylation in Protein Conformation

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Running title: Nα-Acetylation and Protein Conformations

Abbreviations:

Aβ: amyloid-beta

Ac-CoA: acetyl coenzyme A

ALS: amyotrophic lateral sclerosis

αS: alpha-synuclein

Chr: chromosome

C/EBP: CCAAT/enhancer binding protein

DSC: differential scanning calorimetry

FET : FUS/EWS/TAF15

FTLD: frontotemporal lobar degeneration

FUS: fused in sarcoma

GNAT: Gcn5-related N-acetyltransferase

HEAT: Htt/eEF3/PP2A/mTOR

Hsp: heat shock protein

Htt : Huntingtin

HYPK: Huntingtin-interacting protein K

iMet: initiator methionine

KDAC: Nε- or lysine deacetylase

KAT: Nε- or lysine acetyl transferase

LC domain: low-complexity domain

LiP-MS: limited proteolysis coupled to mass spectrometry

LLPS: liquid-liquid phase separation

NAC: non-amyloid-β component

NAT/Nat: Nα-terminal acetyltransferase

NES: nuclear export signal

NLS: nuclear localization signal

NMR: nuclear magnetic resonance

polyP: proline-rich domain

polyQ: polyglutamine repeat region

PTM: post-translational modification

RGG domain: arginine-glycine-glycine repeat domain

RRM: RNA-recognition motif

RNP: ribonucleoprotein

ssNMR: solid state nuclear magnetic resonance

TET: TLS/EWS/TAF15

ThT: Thioflavin T

TPP-TR: thermal proteome profiling over a temperature range

TLS: translocated in sarcoma

ZNF: zinc finger domain

Keywords: N-terminal acetylation, N-terminal acetyltransferases, Protein folding, Huntingtin, Alpha-synuclein, Fused in Sarcoma, Actin, Tropomyosin, Parvalbumin

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Abstract

Especially in higher eukaryotes, protein N-termini are subject to enzymatic modifications with the acetylation of the alpha-amino group of nascent polypeptides being a prominent one. In recent years, the specificities and substrates of the responsible enzymes, the Nα-terminal acetyltransferases, have been mapped in several proteome studies. Aberrant levels and mutant versions of these enzymes were found associated with several human diseases, explaining a growing interest in protein Nα-terminal acetylation. With some enzymes, such as the Nα-terminal acetyltransferase A complex, having thousands of possible substrates, researchers are now trying to decipher the functional outcome of Nα-terminal protein acetylation. In this review, we zoom in on one possible functional consequence of Nα-terminal protein acetylation being its effect on protein folding. Using selected examples of proteins associated with human diseases such as alpha-synuclein and huntingtin, we here discuss the, sometimes contradictory, findings on the effects of Nα-terminal protein acetylation of protein (mis)folding and aggregation.

The Diverse Nα-Terminal Acetyltransferase (NAT) Family

The acetylation of the nascent protein alpha-amino group is one of the most prevalent protein modifications as approximately 80% of all human proteins are Nα-terminally acetylated [1]. The enzymes responsible for this modification, the Nα-terminal acetyltransferases (NATs), catalyze the transfer of an acetyl moiety to the free α-amino group at the N-terminus of a newly synthesized protein, and consume acetyl coenzyme A (Ac-CoA) as a substrate. The NAT enzymes belong to the Gcn5-related N-acetyltransferase (GNAT) superfamily of enzymes which share the canonical GNAT-fold and have a wide repertoire of substrates [2]. Typically, Nα-terminal acetylation occurs when 20-50 residues of the nascent protein have emerged from the ribosome exit tunnel [3]. To date, the higher eukaryotic NAT family comprises eight members, NatA-H (Table 1).

Table 1. The Nα-Acetylation Transferase (NAT) Machinery.

NAT	Structure	Substrate specificity	Localization
NatA	**NAA10** HYPK NAA15	A-, S-, T-, V- and G-	Ribosome associated
NatB	**NAA20** NAA25	MD-, ME-, MN- and MQ-	Ribosome associated
NatC	**NAA30** NAA38 NAA35	ML- ,MI-, MF-, MY- and MK-	Ribosome associated
NatD	**NAA40**	Histone H2A and H4 (SGRGK…)	Ribosome associated
NatE	**NAA50** HYPK NAA15	MS-, MT-, MA-, MV, ML-, MI-, MF-, MY- and MK-	Ribosome associated
NatF	**NAA60**	ML-, MI-, MF-, MY- and MK-	Ribosome associated
NatG	**NAA70**	M-, A-, S- and T-	Chloroplast lumen
NatH	**NAA80**	Actin: DDD… and EEE…	Cytosol

All of its members except for NatG are present in humans [4]; NatG was exclusively identified in plants [5]. The NAT enzyme complexes consist of a catalytic subunit which can be accompanied by one or more auxiliary subunits that can establish ribosomal anchoring and may influence the substrate specificity of the catalytic subunit [6]. As the nascent polypeptide emerges from the ribosome, the acetyl group can be either transferred to the α-amino group of the initiator methionine (iMet) or to the α-amino group of the now-first amino acid after removal of iMet by ribosomal-bound methionine aminopeptidases [7, 8]. While each NAT targets distinct N-terminal residues there is some overlap between the different complexes (Table 1, Figure 1). To date, no enzymes capable of *in vivo* Nα-terminal deacetylation have been identified, suggesting that Nα-terminal acetylation is an irreversible process. This is in contrast to acetylation of the lysine ε-amino side-chain, which is a reversible process regulated by the opposing activities of lysine Nε-acetyl transferases (KATs) and lysine deacetylases (KDACs) [7].

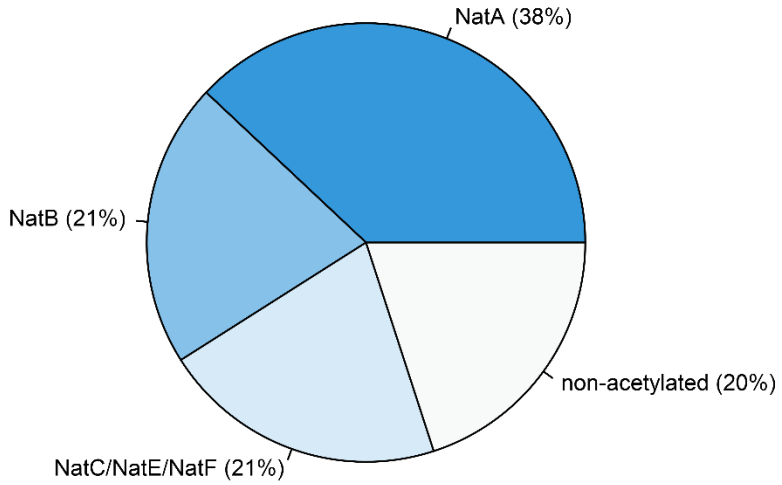


Figure 1. Theoretical distribution of the substrates of the human Nα-Acetylation Transferase (NAT) Family.

Approximately 80% of the human proteome undergoes Nα-terminal acetylation. NatA has affinity for small N-terminal residues following cleavage of the initiator methionine, covering approximately 40% of the human proteome. NatB targets methionine followed by an acidic residue. NatC, NatE and NatF exhibit overlapping substrate specificity, targeting predominantly hydrophobic residues and lysine following the initiator methionine. NatD and NatH are specialized NATs targeting respectively histones H2A and H4 and actin and are not depicted. Additionally, NatG, a plant-specific NAT, is excluded as well.

The involvement of Nα-terminal acetylation in the Pathogenesis of Several Diseases

Defects in the Nα-terminal acetylation machinery have been linked to several disorders in humans. The discovery of the X-linked Ogden Syndrome, caused by the S37P missense mutation in the catalytic subunit NAA10 of NatA, was the first of several rare, developmental disorders collectively known as NAA10-related syndromes [9]. A total of 8 deceased infant boys from two families were found to carry this mutation [10]. Some common symptoms associated with the Ogden syndrome include severe intellectual disabilities, facial dysmorphism and aged appearance, failure to thrive, hypotonia and cardiac abnormalities which are often fatal [9]. NAA10-related disorders range in severity depending on the functional implication of the mutant and the gender of the patient, as males carry only one copy of the X-chromosome [11]. Later, it became clear that defects in other subunits of the NAT complexes of which the genes are not located on the X-chromosome, such as NAA15 (Chr 4), NAA20 (Chr 20) and NAA30 (Chr 14), lead to similar phenotypes [12-16].

Various components of the NAT machinery are implicated in several tumors, such as hepatocellular carcinoma, breast cancer, lung cancer, urinary bladder cancer, colorectal cancer and prostate cancer, where their overexpression is most often linked to poor prognosis due to their stabilizing effect on oncogenes [17, 18]. For instance, in gliomas exhibiting upregulated NAA10 expression, the activity of various oncogenic pathways crucial to tumor progression is increased, such as cell proliferation, angiogenesis, DNA repair and epithelial-mesenchymal transition [19]. On the other hand, in several studies NAA10 has demonstrated tumor-suppressive activity, correlating with a more favorable prognosis [20-23]. Overall, the involvement of the NAT complexes in cancer appears to be dictated by context-depended effects, complicating our understanding of their precise contribution to tumorigenesis and progression.

Disturbance of the native protein conformation and protein aggregation are prominent features of several neurodegenerative disorders. These disorders are characterized by the aberrant accumulation of protein aggregates resulting in the progressive loss of nerve cells in the brain, leading to an increasing decline in cognitive and motor functions. Alzheimer's disease is the most common form of dementia which is characterized by deposits of amyloid-beta (Aβ) plaques and Tau tangles in various regions of the brain. These aggregates disrupt the normal cellular function leading to memory loss and eventually wide-spread cell death [24]. In Parkinson's disease, the aggregation of alpha-synuclein (αS) into inclusion bodies, so called Lewy bodies, cause the degeneration of dopaminergic neurons. This interferes with regular motor function giving rise to symptoms such as heavy tremors, rigidity and bradykinesia and can also cause cognitive impairment [25]. Lewy body dementia is also characterized by αS aggregates and shares features with both Alzheimer's and Parkinson's disease. Patients may

experience memory loss, hallucinations, fluctuations in alertness and attention, in addition to the loss of motor function similar to Parkinson's disease [26]. Nα-terminal acetylation has a direct impact on the function of αS and the accumulation of its aggregates into Lewy bodies. Notably, Lewy bodies have also been observed in 32-57% of sporadic Alzheimer's disease cases [27]. Additionally, there appears to be an intricate relationship between αS and Aβ aggregation [28]. Huntington's disease is a hereditary neurodegenerative disorder caused by a single mutation in the *HTT* gene encoding the Huntingtin (Htt) protein and splice variants. N-terminal fragments of mutant Htt form aggregates most commonly in neurons of the basal *ganglia,* particularly medium spiny neurons of the *striatum* [29]. Similarly, Huntington's disease patients display cognitive decline and neuropsychiatric symptoms as well as motor abnormalities as a result of the disturbed neuronal function. Interestingly, it has been demonstrated that Nα-terminal acetylation promotes rather than decreases the aggregation of N-terminal fragments of Htt in Huntington's disease [30, 31].

The Multifaceted Roles of Nα-Terminal Acetylation

Protein Nα-terminal acetylation has been linked to a diverse spectrum of protein functions linked to diverse physiological processes and disease pathogenesis. Despite its prevalence, the full extent of its functional consequences for the majority of the proteome remains largely elusive. This modification transforms the positively charged amino group into a neutral, more hydrophobic acetyl group which can influence protein biology in various ways. It may alter protein stability and half-life dynamics, as non-acetylated N-terminal residues are susceptible targets for E3 ligases, termed N-recognins. For instance, in *S. cerevisiae,* Ubr1 has been reported to target N-terminal methionine followed a large hydrophobic residue [32]. However, in the Ac/N-end rule pathway Nα-terminal acetylation itself may also serve as an Ac/N-degron, as evidenced by N-recognins like Doa10 and Not4 in *S. cerevisiae*, which recognize these N-degrons, thereby signaling protein degradation complementary to the Arg/N-end rule pathway [33-35]. It has been hypothesized that steric shielding of these N-degrons, e.g. by other members of a protein complex as has been reported for Hcn1 and Cog1 by respectively Cut9 and by Cog2 and Cog3, serves the cell as a strategy for dynamically regulating the half-life of these proteins [36].

Nα-terminal acetylation was also reported to alter protein-protein interactions and complex formation, exemplified by a family of NEDD8 E3 ligases, the DCN-like family, which was reported to selectively interact with Nα-terminally acetylated N-termini of the E2 ligases UBC12 and UBE2F [37-39]. Another function attributed to Nα-terminal acetylation is subcellular localization, as seen in *S. cerevisiae* with the Arf-like GTPase ArI3p, a member of the Raf superfamily, which is acetylated by the

NatC complex facilitating its localization to the Golgi apparatus via association with Sys1p, a Golgi membrane protein [40, 41]. In human physiology, the N-terminal tail of alpha-synuclein (αS), a critical factor contributing to pathogenesis in Parkinson's disease (see below), undergoes Nα-terminal acetylation and is involved in synaptic vesicle membrane binding under the influence of copper [42]. The removal of the N-terminal positive charge by the introduced acetyl group may also regulate protein-metal interactions involving the N-terminus, as is the case for αS where it alters the copper binding kinetics [43].

The impact of protein Nα-terminal acetylation also extends to protein folding and aggregation with notable ramifications for human pathology [44, 45]. Protein folding is a highly dynamic and complex process often involving chaperones that guide the (re)folding or unfolding processes. Important classes of chaperones amongst others include heat shock protein 60 (Hsp60) or chaperonins, heat shock protein 70 (Hsp70) and heat shock 90 (Hsp90). By Nα-terminally acetylating chaperones, NATs can indirectly influence protein folding. For instance, Nα-terminal acetylation of various Hsp10 orthologues appears to stabilize an N-terminal helix and protect these proteins against proteolytic degradation [46]. Furthermore, Hsp70 undergoes K77 acetylation by monomeric NAA10 in stress-induced conditions, stimulating refolding of denatured proteins. However, once a certain threshold of denatured proteins is reached, acetylation decreased and Hsp70 switches to inducing protein degradation [47]. Translational stalling and differential protein synthesis rates have been hypothesized to affect protein folding [48, 49]. Spontaneous folding of the nascent peptide chain can already start in the ribosome exit tunnel [50]. It is thus tempting to suggest that ribosomal binding proteins such as NATs can induce stalling and influence protein folding. Lastly, environmental factors such as temperature, pH, presence of ions and small molecules, protein concentration and molecular crowding may all impact native protein structures. Note that as NATs introduce a neutral acetyl group at the N-terminus, this may alter the electrodynamic interactions with the environment and with other secondary and tertiary elements of the protein itself. Many of our insights regarding protein folding and aggregation originate from proteins recombinantly produced in prokaryotic expression systems and hence lacking crucial modifications such as Nα-terminal acetylation. Therefore, such recombinant proteins may exhibit different stabilities, folding patterns and aggregation behaviors compared to their endogenous eukaryotic counterparts that exhibit *in vivo* acetylation.

In this review, we discuss the influence of Nα-terminal acetylation on protein aggregation and structure for several, well-studied proteins in this respect.

Nα-Terminal Acetylation and Other Factors Impacting Alpha-Synuclein Function and Conformation

Alpha-synuclein (αS) is an important regulator of multiple stages of the synaptic vesicle cycle, although its exact functions remain elusive [51]. This small protein, encoded by the *SNCA* gene, spans a length of 140 amino acids and has a molecular weight of 14.46 kDa [52]. The highest levels of αS are found in the cytosol of presynaptic terminals of neurons, with a fraction directly associated with synaptic vesicles. As mentioned above, αS is linked to several neurodegenerative disorders termed synucleinopathies. Under pathological conditions, αS monomers can aggregate, forming oligomeric intermediates that progress to amyloid fibril aggregates known as Lewy bodies [53]. These aggregates can further propagate via endocytosis to aggregate-free neurons in a prion-like manner [54]. Structurally, αS is composed of a positively charged N-terminal synaptic vesicle membrane binding region and a negatively charged C-terminal region (Figure 2.A). The N-terminal membrane binding region contains two zones of respectively four and three lysine-rich, 11-residue-long repeats containing the highly conserved KTKEGV motif, allowing the formation of two α-helical conformations [55]. Moreover, the non-amyloid-β component (NAC) domain, located within this region, acts as the nucleating factor for fibrillation as it enables cross-β sheet structures [56]. The first two residues of the N-terminus of αS are 'MD-', recognized as a high-affinity substrate for NatB [57]. Indeed, research has demonstrated consistent Nα-terminal acetylation of αS by NatB, effectively neutralizing the positive charge at the N-terminus [58].

As a monomer, native αS is intrinsically disordered, or more accurately, it remains natively unfolded while dynamically shifting between conformational states, both transiently and in response to various stimuli [42]. It was suggested that several of these states could potentially be more aggregation-prone [43]. Additionally, it is worth noting that non-membrane bound αS does not spontaneously aggregate. Instead, it is believed that its interaction with the lipid membranes of synaptic vesicles may initiate a seeding event that leads to its further aggregation [55]. *In vitro* studies using lipid vesicles, like unilamellar vesicles, show the formation of a partially α-helical structure at the N-terminal region of αS upon association with a membrane's surface. Specifically, the initial 14 residues at the N-terminus appear to be of high importance for the avidity of the protein-membrane interaction [59]. The impact of Nα-terminal acetylation on the formation of the N-terminal α-helical structure remains a subject of debate. This modification elevates the binding affinity toward lipid membranes by increasing the helicity of the N-terminal region, thereby decreasing the entropic cost associated with the transition from random coil to the partial α-helical structure at the N-terminal region. This stabilization arises from both short-range interactions with the N-terminus and long-range interactions involving several of the lysine-rich regions important for lipid binding [60]. However, Nα-terminal acetylation by itself is not sufficient to induce the formation of this structure, so it appears to be an intrinsic property of αS in the membrane-bound state [61]. Several other factors influence the interplay between αS and

vesicular membranes, such as the membrane composition and curvature. The lysine-rich repeats in the N-terminal membrane binding region dictate a preference for anionic membranes enriched in acidic lipids [59]. The helicity of the N-terminus appears to be an important factor in the early stages of the αS-lipid interaction [61]. Bell, et al. [62] have demonstrated that Nα-terminal acetylation decreases the lipid-induced aggregation using *in vitro* Thioflavin T (ThT) fluorescence experiments. Moreover, they showed that the morphology of the αS fibrils is altered and characterized by a higher quantity of secondary structure elements. Specifically, Nα-terminally acetylated αS forms shorter and more curved fibrils, whereas non-acetylated αS forms longer and straighter fibrils. However, solid state nuclear magnetic resonance (ssNMR) spectroscopy experiments reveal no substantial differences in the conformational states between membrane-bound, Nα-terminally acetylated αS and non-acetylated αS [63]. Furthermore, Bell, et al. [62] demonstrated that after the primary, lipid-induced seeding event, which initiates αS fibrillation, there is a higher amount of Nα-terminally acetylated αS oligomers formed during the secondary nucleation events compared to non-acetylated αS. Interestingly, several pathogenic mutations in the N-terminal domain of αS found in Parkinson's disease are also known to affect the folding propensity and lipid-binding capabilities [64, 65]. For instance, the V15A and A30P mutations may disrupt the formation of the first alpha-helical loop, which lowers the binding affinity for the membrane [66].

In the last decade, the idea that free cytosolic αS occurs as intrinsically disordered monomers has been criticized [67-71]. Bartels, et al. [68] provided the first evidence that endogenous, Nα-terminally acetylated αS, isolated from diverse human cell lines and clinical samples like human erythrocytes, partially exists as a folded tetramer of 58 kDa which exhibits greatly reduced aggregation kinetics [67]. An imbalance of the tetramer-monomer equilibrium could then result in an increase of aggregation-prone monomers and subsequently cause pathological αS aggregation [72]. These authors suggested that the tetrameric forms are lost during harsh cell lysis protocols or are simply not formed using recombinant, non-acetylated αS. Additionally, they noticed that Nα-terminally acetylated αS forms the α-helical structure at the N-terminal region even in the absence of lipid membranes. Similar findings indicating a tetrameric form using an Nα-terminally acetylated, recombinant αS construct or the full-length protein purified from *E. coli* have been reported [73-75]. However, several other studies contradicted these findings, as other groups were not able to replicate and prove the formation of αS tetramers in the same conditions, confirming the original hypothesis that cytosolic αS occurs primarily monomeric [60, 76-79].

Another factor impacting the aggregation of α-synuclein is copper homeostasis, or rather dyshomeostasis. Copper, a biologically active transition metal occurring in both monovalent Cu^{1+} and divalent Cu^{2+} oxidation states, serves as a vital cofactor for various crucial proteins involved in brain

development and metabolism [80]. Accumulation of not only copper but other bivalent metals like iron has been associated with increasing severity of Parkinson's disease progression in the brains of patients and aging mice [81, 82]. Copper toxicity triggers oxidative stress by disturbing reactive oxygen species generation through the Fenton and Haber-Weiss reactions, and through interfering with the functioning of mitochondria. There are indications that α-synuclein can exacerbate this effect of copper on oxidative stress [83]. *In vitro* ThT fluorescence measurements suggest that Cu^{2+}-α-synuclein interactions accelerate α-synuclein fibrillation rates by altering αS secondary and tertiary structures to an aggregation-prone state [84, 85]. Notably, α-synuclein-copper binding extends the partially α-helical structure induced at the α-synuclein N-terminal region, affecting the aforementioned lipid membrane interactions [86]. Three binding sites have been discerned for non-acetylated α-synuclein: the high-affinity site 1, coordinated by the sulfur atoms of Met-1 and Met-5; the lower-affinity site 2, centered around the imidazole ring of His-50; and the low-affinity site 3, involving the sulfur atoms of Met-116 and Met-127 [87-89]. Moriarty, et al. [90] showed that Nα-terminal acetylation of α-synuclein abolishes the high-affinity interaction between Cu^{2+} and site 1 in the N-terminal domain, reducing the aggregation kinetics compared to the non-acetylated holo-form. They demonstrated that copper now mainly bound to the His-50 and the Met-166/Met-127 sites. Furthermore, the acetylated α-synuclein His50Gln mutant has abolished copper binding and exhibits greatly reduced subsequent aggregation compared to the non-acetylated form under the same conditions [43]. This emphasizes the predominant role of the His-50 site over the N-terminal site in native, acetylated α-synuclein. Mason, et al. [43] hypothesized that Nα-terminal acetylation reduced the Cu^{2+}-induced structural shift of native αS toward an aggregation-prone intermediate. However, Miotto, et al. [88] reported that Nα-terminally acetylated αS-Cu^{1+} complexes exhibited similar binding tendencies to the non-acetylated complex. Nevertheless, they demonstrated a substantial increase in α-helical propensity within the first 10 residues of the acetylated complex.

The Involvement of Nα-terminal acetylation in Huntington's Disease Pathogenesis

Huntingtin (Htt), the central component in Huntington's disease, is a large protein with a molecular weight of 347 kDa and which forms a superhelical, solenoid-like structure [92]. Although expressed throughout the body, its highest levels are found in the neurons of the central nervous system as well as in non-neuronal tissue such as muscle fibers, liver and testes [29, 93]. Htt can be found in the nucleus and in the cytoplasm where it is associated with mitochondria, the Golgi apparatus and the endoplasmic reticulum [93, 94]. Htt is predominantly comprised of HEAT repeats, a structural motif named after four HEAT repeat-containing proteins: **H**untingtin (**H**tt), Eukaryotic Translation **E**longation

Factor 3 (e**E**F3), Protein Phosphatase 2 regulatory **A** Subunit (PP2**A**), Mechanistic **T**arget of Rapamycin (m**T**OR) (Figure 2.B) [95]. These repeats, approximately 38 amino acid in length, consist of helical hairpin repeat regions, which serve as scaffolds for various protein-proteins interactions [96]. These regions provide Htt structural fluidity to adapt its conformation to accommodate its partners. Given its extensive network of interaction partners, Htt is involved in many different biological processes, including vesicle trafficking, metabolism, transcription and protein turnover [29]. Of note, the number of reported interaction partners in Biogrid of Htt in *H. sapiens* is 331 (Biogrid database version 4.4, accessed on 2 January 2024, https://thebiogrid.org/109314/summary/homo-sapiens/htt.html) [97].

The N-terminal domain of Htt differs from the rest of the protein. It consists of a region containing the nuclear export signal (NES), also referred to as the N17 domain, the polyglutamine (polyQ) repeat region and the proline-rich domain (polyP), the latter appearing to exhibit a stabilizing effect on the polyQ domain and thus inhibiting aggregation [98]. Huntington's disease is caused by a single mutation leading to an expansion of poly CAG repeats in the *HTT* gene. This leads to increases of the polyQ tract length, which has been correlated with cell toxicity as it strongly impacts the biophysical and biochemical properties of Htt [99]. There appears to be an increase in proteolytic cleavage of the N-terminal domain of mutant Htt in Huntington's disease patients by caspases, calpains and other endoproteases [29]. It was hypothesized that specific polyQ-containing N-terminal Htt fragments may mediate neurodegeneration in Huntington's disease as these fragments appear more susceptible to misfolding and aggregation [100, 101]. Further, the increase in polyQ repeat length correlates with the pathology of Huntington's disease which only occurs in patients with a repeat length of over 35 [98].

Post-translational modifications add additional layers of complexity to the regulation of Htt activity. So far, various modifications like lysine acetylation, palmitoylation, phosphorylation, SUMOylation, ubiquitination, and proteolytic cleavages were found to influence Htt function in Huntington's disease [102]. Post-translational modifications of the N-terminal region notably impact the aggregation propensities of Htt. Despite the observation that Htt is Nα-terminally acetylated by NatA both *in vitro* and *in vivo*, the effect of Nα-terminal acetylation remains less understood, [30]. Using a bacterial system to produce Htt with an alpha-acetylated N-terminus or without, Gottlieb, et al. [30] were able to demonstrate that Nα-terminal acetylation promotes the aggregation of an N-terminal Htt fragment. Furthermore, circular dichroism (CD) spectroscopy data showed, despite a relatively small increase of α-helical content, no significant alterations of the random coil content.

Huntingtin-interacting protein K (HYPK) interacts with Htt by binding to the N-terminal N17 domain and is able to diminish mutant Htt aggregates upon overexpression, attributed to its inherent

chaperone activity [103]. Arnesen, et al. [31] found HYPK as a stable interactor of NatA, establishing an additional connection between Huntington's disease progression and Nα-terminal acetylation. Weyer, et al. [104] provided insights into the structural mechanisms by which HYPK regulates Nα-terminal acetylation through interaction with the NatA complex. They found that the HYPK C-terminus closely binds to the NAA15 subunit of NatA, while its N-terminus obstructs the entrance to the active site of NAA10. In this manner, HYPK may be able to exhibit an inhibitory effect on NatA and, consequently, decrease the aggregation of Htt. Notably, HYPK expression was reportedly reduced in both cellular and animal models of Huntington's disease [30, 105].

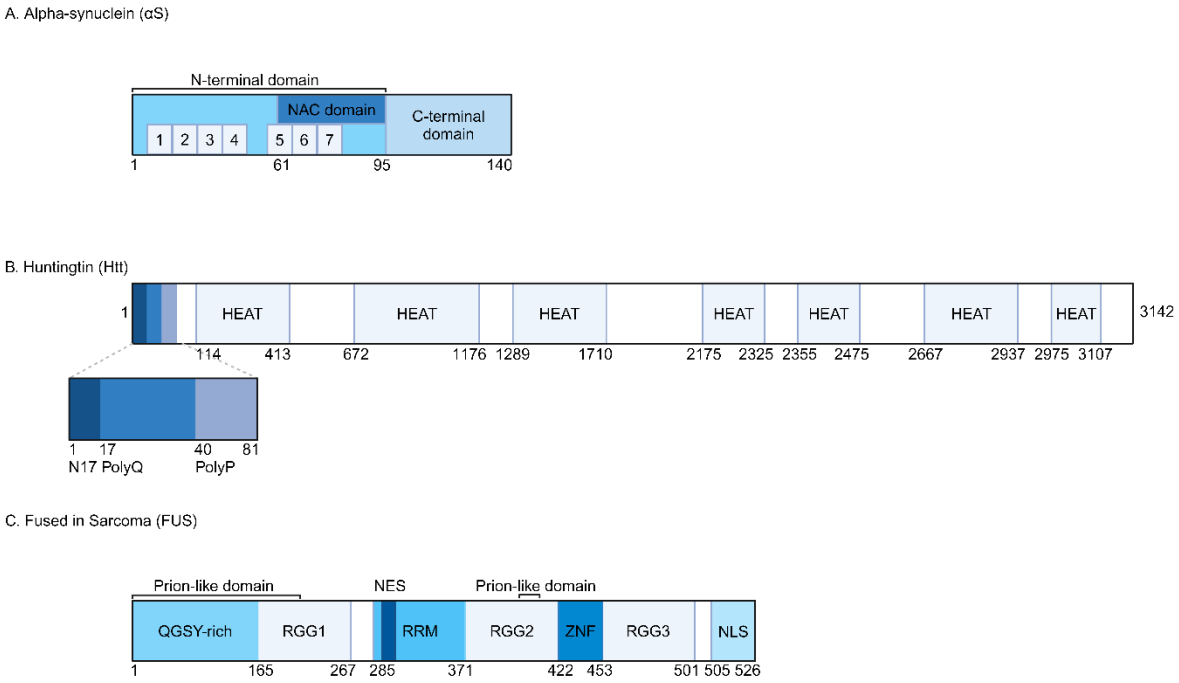
The RNA-binding Protein FUS

The RNA-binding protein fused in sarcoma (FUS)/translocated in sarcoma (TLS) shares several features with αS. It is an aggregation-prone protein, exhibiting prion-like properties. Canonical FUS has a length of 526 amino acids and a molecular weight of 53 kDa [52]. The residue following the iMet is Ala, making it a potential NatA substrate. FUS spans an intrinsically disordered transcriptional activation domain enriched in glutamine, glycine, serine and tyrosine (QGSY) residues termed the N-terminal low-complexity (LC) domain, three disordered arginine-glycine-glycine repeat (RGG) domains, a RNA-recognition motif (RRM), a zinc finger domain, a nuclear localization signal (NLS) and a nuclear export signal (NES) (Figure 2.C) [106]. Furthermore, two prion-like domains have been predicted ranging from residues 1-239 and 391-407 [107]. FUS is part of the FET (FUS/EWS/TAF15)/TET (TLS/EWS/TAF15) family sharing the characteristic RRM and the abolishment of the auto-repression of the transcriptional activation domain in oncogenic TET/FET-fusion proteins [108].

FUS was originally identified in myxoid liposarcoma where the FUS LC undergoes fusion with DDIT3, a member of the CCAAT/enhancer binding proteins (C/EBPs) family of transcription factors, as a result of the t(12;16)(q13;p11) translocation [109, 110]. The fusion protein likely drives sarcomagenesis similar to the EWS/FLI fusion protein in Ewing sarcoma. In addition, FUS has been associated with the formation of amyloid fibrils in various subtypes of neurodegenerative disorders, namely in amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD) and polyQ diseases such as Huntington's disease [111, 112]. FUS is a moonlighting protein involved in processes ranging from translation and transcription to degradation, as well as DNA damage repair and various messenger- and microRNA processing pathways [111]. Notably, it is a component of the ribonucleoprotein (RNP) complexes, essential for regulating RNA metabolism. Moreover, FUS is heavily involved in RNA and stress granule dynamics, undergoing self-assembly via liquid-liquid phase separation (LLPS) mediated by its N-terminal LC domain [113, 114]. LLPS is the phenomenon in which

macromolecules coalesce to form liquid droplets within the surrounding solvent, creating distinct micro-environments. FUS can translocate between the cytosol and nucleus, and FUS mutations linked to both familial and sporadic cases of ALS often affect the C-terminal NLS, resulting in cytosolic FUS accumulation [115]. In this aggregation-promoting environment, the N-terminal LC domain appears to be of high importance for the toxic gain-of-function and aggregation of retained FUS in nuclear inclusions [112]. These inclusions initially closely resemble the abovementioned stress granules and are likely the result of aberrant phase separation resulting in an irreversible liquid-to-solid phase transition [116].

The FUS LC domain contains several sites for post-translational modifications (PTMs) which appear to be important for mediating its function, especially phosphorylation [117, 118]. Bock, et al. [114] provide evidence for FUS Nα-terminal acetylation by NatA and explored the impact on the properties of the FUS LC domain. They reported no evident changes in the secondary structure comparing the acetylated and non-acetylated variants when applying NMR spectroscopy-based techniques and molecular simulation. However, it is worth noting that this analysis was performed with N-terminal fragments spanning the LC domain, which may not account for potential long-range interactions, such as observed in the stabilization of the helical structure at the N-terminus of αS. Several other regions beyond the LC domain have been reported to play an important role in the self-assembly of FUS so it is not unlikely that similar mechanisms as in αS affect the highly dynamic conformational states of the largely intrinsically disordered FUS [106]. Additionally, employing protocols that utilize gentle methods for the isolation of FUS, without potentially protein denaturing steps, might be required to better preserve any structural elements, as discussed above for αS. Although the secondary structure remains unaffected, Bock, et al. [114] demonstrated that Nα-terminally acetylated FUS LC exhibits an elevated tendency for phase separation. Furthermore, they observed slower and reduced aggregation for acetylated FUS LC. These authors speculated that similar findings are possible for the other members of the FET-family, which could provide more insights in the dynamics of the oncogenic N-terminal fusion proteins observed for these proteins.



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395 **Figure 2. The Structure of Three Nα-Terminally-Acetylated, Aggregation-Prone Proteins Involved in**
396 **the Pathogenesis of Various Neurodegenerative Disorders.**

397 (A) The structural domains of monomeric alpha-synuclein consisting of the positively charged N-
398 terminal domain involved in synaptic vesicle membrane docking; the aggregation-prone non-amyloid-
399 β component (NAC) region; the negatively charged C-terminal domain involved in metal ion and small
400 molecule binding and Ca^{2+}-mediated membrane binding. The N-terminal region comprises 7 lysine-
401 rich, 11-residue-long repeats containing the conserved KTKEGV motif which are involved in the
402 formation of two alpha-helical structures during membrane binding. (B) Huntingtin (Htt) mainly
403 comprises large HEAT-domains. The N-terminal domain of Htt, which is cleaved off at an increased
404 rate in Huntington's disease patients, comprises the N17 region containing a nuclear export signal, the
405 PolyQ domain being an aggregation-prone region and the PolyP domain. (C) Fused in Sarcoma (FUS)
406 contains the N-terminal low-complexity (LC) domain which is an intrinsically disordered transcriptional
407 activation domain enriched in glutamine, glycine, serine and tyrosine (QGSY) residues. FUS further
408 contains three disordered arginine-glycine-glycine repeat (RGG) domains, a RNA-recognition motif
409 (RRM), a zinc finger domain (ZNF), a C-terminal nuclear localization signal (NLS) and a nuclear export
410 signal (NES). Furthermore, two prion-like regions have been indicated ranging from residues 1-239
411 and 391-407.

412

413 **Nα-Terminal Acetylation Affects Fish Parvalbumin Allergenicity**

Parvalbumins are small, calcium-binding proteins (approximately 12 kDa) expressed in the cytosol of higher vertebrates muscles [52]. By maintaining Ca^{2+}-homeostasis, parvalbumins are involved in the regulation of muscle relaxation, apart from their role in various other biological processes [119]. The first residue following the non-retained iMet, mostly alanine or serine, has been observed to be acetylated [120]. The folding and function of fish parvalbumin, a major allergen, appears to be especially affected by this Nα-terminal acetylation. Similar to α-synuclein, differences have been observed between the non-acetylated variants produced in prokaryotic expression systems and the native variants carrying the Nα-terminal acetyl group found in fish [120, 121]. Permyakov, et al. [122] used a combination of differential scanning calorimetry (DSC), CD spectroscopy and limited proteolysis to assess the thermostability and secondary structure of rat and pike parvalbumin species. The N-terminal domain of pike parvalbumin displays a higher degree of disorder compared to rat parvalbumin which may contribute to its allergenicity. They demonstrated that Nα-terminal acetylation of fish parvalbumin shifts the thermal denaturation profile of the metal-bound protein state compared to acetylated rat parvalbumin where no such shift is observed. The authors attributed this effect to changes in protein folding, which were confirmed by the alterations in secondary structure observed in the CD data. Moreover, limited proteolysis of the parvalbumins showed that Nα-terminally acetylated fish parvalbumin had an increased resistance to proteolytic digestion by α-chymotrypsin. As such, this increased resistance of native fish parvalbumin to digestion upon consumption could contribute to its allergenicity in humans. Furthermore, Permyakov, et al. [122] demonstrated that the Nα-terminal acetylation status affects the association and dissociation of Ca^{2+} and Mg^{2+} to parvalbumin and, in turn, could affect its function in muscle relaxation. Lastly, the authors showed that Nα-terminal acetylation of fish parvalbumin increases the structural stability of the protein and, in turn, can prevent oligomerization and aggregation of the protein.

Strengthening the Scaffold: Nα-Terminal Acetylation's Influence on an Actin-Associated Protein Family and Cytoskeletal Stability

Tropomyosins, forming a diverse family of actin regulatory proteins with numerous isoforms as a result of alternative splicing, play crucial roles in various cellular functions [52]. Among these, striated muscle α-tropomyosins are pivotal not only for muscle contraction in striated muscles, but also for cytoskeletal dynamics in non-muscle cells. Their intricate structure, consisting of a highly dynamic and complex two-chained, parallel coiled coil, facilitates polymerization into filaments and enables them to warp along the major grove of actin filaments anchored by troponins. In a relaxed state tropomyosins cover the binding sites on the actin filament, preventing myosin from attaching and

initiating muscle contraction [123]. Due to their dynamic structure, tropomyosins are able to respond promptly to impulses such as myosin binding and fluctuations in Ca^{2+}-concentration [124]. It has been demonstrated that several tropomyosins are Nα-terminally acetylated and based on the N-terminal residues (often MD- and MA-), it is a likely occurrence for various other isoforms [125, 126]. The necessity of Nα-terminal acetylation of striated muscle α-tropomyosin for its structure and proper actin binding has been documented since the 1980s. It has been demonstrated that non-acetylated tropomyosins display an up to a 100-fold lower affinity for actin and have reduced capabilities to form filaments upon troponin binding [127-129]. This effect may stem from a local stabilization of the coiled-coil structure in the first 30 amino acids at the N-terminus, which potentially enhances interactions with the C-terminus of the adjacent tropomyosins in the filament [130, 131]. Additionally, Nα-terminal acetylation could influence the interactions between tropomyosins and the different myosin classes depending on their cellular location. More specifically, Nα-terminal acetylation of the sole tropomyosin Cdc8 of *S. pombe* by NatB has been demonstrated by Coulton, et al. [132] to promote the controlled binding of multiple myosin II heavy chains, thereby enhancing their coordinated function, along actin strands of the contractile ring formed during mitosis, towards the end of the telophase. Furthermore, it preserves the integrity of the actin filaments which appear to be defined by a wavy appearance and an increased fragility in the conditions with unacetylated Cdc8.

Emerging Techniques for Probing Protein Structural Differences

In the different sections above, several technologies have been described to evaluate protein structures and changes therein caused by Nα-terminal acetylation. With most of these, purified (recombinant) proteins have been analyzed, and by nature of the technologies used, such analyses took place outside of the endogenous cellular environment of the studied proteins.

To some extent, protein thermostability could be considered as a proxy for protein structures. In 2014, Savitski and colleagues introduced a proteome-wide technology for studying protein thermostability; thermal proteome profiling over a temperature range (TPP-TR) [133]. This technology both works on cell lysates and in living cells, the latter thus providing an opportunity to assess protein structural changes in a native environment. Whilst proven a powerful approach, when studying for instance the effect of reduced levels or even the absence of Nα-terminal acetylation in cellular models (e.g., [134]), careful interpretation of thermostability data is warranted as changes in thermostability might be caused by effects other than protein structural changes such as differences in protein interaction partners or subcellular localization caused by non-physiological Nα-terminal acetylation. One of the advantages of the limited proteolysis technology, LiP-MS, introduced by the Picotti lab over TPP-TR is

that it allows to define structural changes to regions of about 10 amino acids in length in a protein's structure, and this by mapping so-called conformotypic peptides to these regions [135]. However, thus far LiP-MS has been used on cell or tissue lysates in which proteins are taken out of their endogenous environment. Hence, it is clear that there are opportunities to develop novel technologies that evaluate protein structural changes directly in their native environment.

Author Contributions

S.C. and K.G. jointly wrote the review.

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