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REVIEW

SUBJECT COLLECTION: EXPLORING THE NUCLEUS

Cellular effects of NAT-mediated histone N-terminal acetylation

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ABSTRACT

Histone acetylation involves the addition of acetyl groups to specific amino acid residues. This chemical histone modification is broadly divided into two types – acetylation of the amino group found on the side chain of internal lysine residues (lysine acetylation) or acetylation of the α-amino group at the N-terminal amino acid residue (N-terminal acetylation). Although the former modification is considered a classic epigenetic mark, the biological importance of N-terminal acetylation has been mostly overlooked in the past, despite its widespread occurrence and evolutionary conservation. However, recent studies have now conclusively demonstrated that histone N-terminal acetylation impacts important cellular processes, such as controlling gene expression and chromatin function, and thus ultimately affecting biological phenotypes, such as cellular ageing, metabolic rewiring and cancer. In this Review, we provide a summary of the literature, highlighting current knowledge on the function of this modification, as well as allude to open questions we expect to be the focus of future research on histone N-terminal acetylation.

KEY WORDS: N-terminal acetylation, Histones, Epigenetics, NAA40, NAT

Introduction

In eukaryotic cells, genomic DNA is packaged into a highly organised chromatin structure. The basic building units of chromatin are nucleosomes, each of them containing ∼146 base pairs of DNA wrapped around a protein octamer composed of the four core histones, H2A, H2B, H3 and H4 (Cutter and Hayes, 2015). Additionally, a linker histone H1 can bind at the entry and exit sites of DNA on the nucleosomes, thus promoting higher-order chromatin structure (Hergeth and Schneider, 2015; Zhao et al., 2021). Histone proteins are subjected to numerous post- or co-translational modifications (PTMs and CTMs, respectively), such as acetylation, methylation, phosphorylation and ubiquitylation, through the addition of chemical groups both on their globular domain and at the N-terminal tails (Tessarz and Kouzarides, 2014; Zhao and Shilatifard, 2019). Histone modifications constitute one of the main epigenetic mechanisms through which the cell regulates DNA-based processes, chromatin architecture and cellular phenotypes (Bannister and Kouzarides, 2011). Therefore, understanding the various pathways that drive misregulation of histone modifications and of their downstream activities in various pathologies, as well as identifying drugs to target these histone-related vulnerabilities have drawn considerable interest in recent years (Zhao et al., 2021).

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Acetylation is one of the most prevalent histone modifications and two distinct forms have been described so far: acetylation of the N-terminal amino group at the first amino acid (Nt-Ac; also called N-α-acetylation) or acetylation of the ε-amino group of internal lysine (K) side chains (N-ε-acetylation). These two types of histone acetylation not only affect distinct residues, but also utilise a different set of enzymes (Demetriadou et al., 2020). Although histone Nt-Ac and N-ε-acetylation were originally discovered around the same time, the latter has received considerably more attention through the decades, owing to it having been linked to transcriptional regulation early on (Box 1).

Nt-Ac is not restricted to histones but is in fact one of the most abundant protein modifications, estimated to occur in ∼80% of all soluble proteins (Ree et al., 2018). Protein Nt-Ac was for a long time considered a biochemically inert modification, with no major regulatory function. However, recently this notion has been overturned. At the protein level, Nt-Ac is now known to affect stability, localisation, aggregation and folding, as well as physical interactions of the substrate with other proteins. In a number of cases, the effects of protein Nt-Ac have been extended to cellular and organismal phenotypes (Aksnes et al., 2019). In the same vein, a number of recent studies have linked Nt-Ac of histones to physiological and pathological states. In this Review, we provide an overview of our current knowledge relating to the cellular and biological significance of histone Nt-Ac, highlighting the new findings as well as pointing out emerging concepts and gaps in our understanding of the process.

Histone Nt-Ac – occurrence and the enzymes involved

Following the initial discovery of Nt-Ac on histone fragments by Phillips in 1963, additional work validated the existence of this modification on two different calf thymus histones (Phillips, 1963; 1968). Since then, direct experimental evidence confirmed that Nt-Ac occurs on eukaryotic histones H1, H2A and H4, and additionally on histone H2B exclusively in yeast (herein yeast refers to *Saccharomyces cerevisiae* unless otherwise specified) (Demetriadou et al., 2020). Early support for the Nt-Ac of histones arose from failures in the efforts to sequence the histone H1 N-terminus through the use of an old protein sequencing methodology, known as Edman degradation. The effectiveness of Edman degradation for protein sequencing is based on the ability to sequentially label and cleave off amino acids on the N-termini of proteins, a process that can be hindered by the presence of modifications on the N-terminal amino group such as acetylation (Mann, 2016). The failure to apply Edman degradation to H1 proteins led to the assumption that the N-terminal amino acid of this histone was acetylated (Rall and Cole, 1971). Subsequently, mass spectrometry (MS)-based proteomic methods performed by independent groups, using samples from various mammalian and avian species have reported Nt-Acetylation of H1 isoforms, ranging in extent from almost 50% to fully acetylated N-termini (Garcia et al., 2004; Wiśniewski et al., 2007; Tweedie-Cullen et al., 2012). It has also been reported that the levels of Nt-Ac of H1.0 increase in

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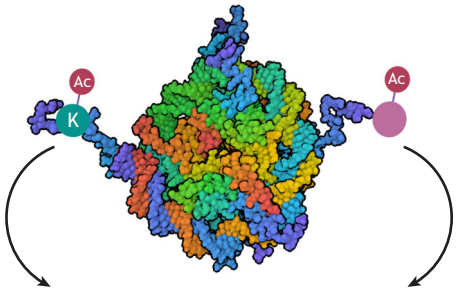
Box 1. The parallel lives of N-ε-acetylation and N-terminal acetylation

Histone N-terminal acetylation was first documented as an abundant modification in 1963 (Phillips, 1963), whereas N-ε-acetylation of histones was first detected in 1968 (Gershey et al., 1968). By 1964, it had already been proposed that histone acetylation can regulate RNA transcription (Allfrey et al., 1964). However, the potential biological importance of Nt-Ac was ignored for many decades, unlike histone lysine acetylation, whose cellular role was pursued upon its detection (see the figure for a timeline). Although writers, readers and erases were being identified for N-ε-acetylation, histone Nt-Ac was discussed mostly in relation to its capacity to hinder histone analysis by blocking their N-terminus from Edman degradation (Rall and Cole, 1971; Verreault et al., 1998). Only recently have studies started to address its physiological function, primarily stimulated by the discovery of the histone-specific NAT NAA40 in yeast in 2003 (Song et al., 2003) and in human cells in 2011 (Hole et al., 2011).



Histone N-ε-acetylation		Histone N-terminal-acetylation (Nt-Ac)
	1963	Discovery of histone Nt-Ac in calf thymus
Proposed role for histone acetylation on RNA synthesis	1964	
Discovery of histone N-ε-acetylation	1968	
Butyrate identified as a histone deacetylase (HDAC) inhibitor	1978	
Experimental link of active genes and acetylated histones	1988	
	1989	Yeast NAA10 (ARD1) shown to Nt-acetylate histones
Mutation of histone tail lysines affects gene regulation	1990	
Isolation of first HAT and HDAC enzymes	1995	
Bromodomain acetyl-lysine readers	1999	
Synthetic HAT inhibitor developed	2000	
	2003	Yeast histone-specific NAA40 (NAT4) identified
Vorinostat HDAC inhibitor FDA approved	2006	
Bromodomain inhibitors JQ1 and I-BET identified	2010	
	2011	Human NAA40 identified
	2013	Cross-talk of H4 Nt-Ac with internal modifications
Belinostat HDAC inhibitor FDA approved	2014	
Panobinostat HDAC inhibitor FDA approved	2015	
	2016	First biological phenotype affected by histone Nt-Ac
	2019	Oncohistone mutations affecting N-terminal residues
	2021	Bisubstrate inhibitors of NAA40 identified

ageing cells, although the underlying mechanism or significance remain unclear (Lindner et al., 1998, 1999). Abundant Nt-Ac of H2A and H4 has been detected following examination of biological samples from yeast, mouse and human cells (Song et al., 2003; Hole et al., 2011; Tweedie-Cullen et al., 2012; Wang et al., 2018a,b). In the case of H2B, this histone has been found to be Nt-acetylated in yeast, but this has not been shown to occur in other organisms so far (Mullen et al., 1989).

It is now established that deposition of histone Nt-Ac occurs through the action of a family of enzymes called N-terminal acetyltransferases (NATs) (Fig. 1A). Focused investigation over the past two decades has been highly successful in identifying and characterising the main classes of NAT enzymes. In eukaryotes, NATs are highly evolutionarily conserved complexes consisting of catalytic enzymes and, potentially, one or more auxiliary subunits (Aksnes et al., 2019). The specificity of a given NAT towards histone proteins firstly depends on whether or not the N-terminal initiator methionine (iMET) is removed from the nascent histone polypeptides and, secondly, on the identity of the first few N-terminal amino acids. Based on this information, it is possible to predict which of the NATs potentially target canonical histones and their variants (see Fig. 1B for details). Based on their N-terminal sequences, the majority of human canonical, variant and isoform histone proteins are potentially Nt-acetylated through the collective actions of NATs. Exceptions are the canonical H2B and H2A-Bbd variants, whose N-terminal amino acid is the non-acetylatable proline in humans. However, it should be noted the N-terminal sequence of H2B in yeast is methionine-serine, compared to methionine-proline in humans, possibly explaining why the former but not the latter has been shown to be N-terminally acetylated. In case of the H3 variant CENP-A, the second amino acid is glycine, a residue not acetylated efficiently, and this glycine amino group is instead methylated through the action of N-terminal methyltransferases (Bailey et al., 2013).

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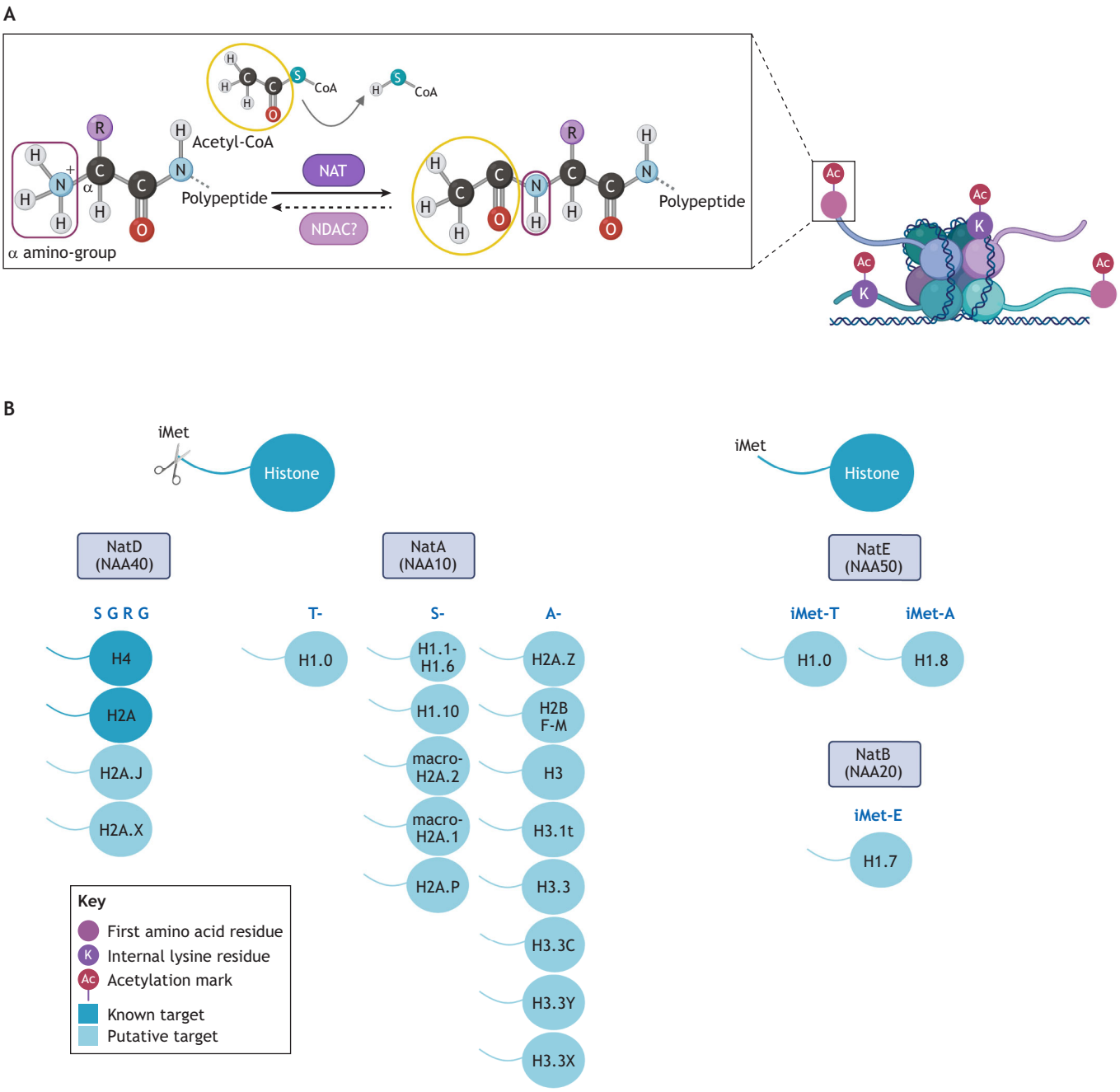

Fig. 1. NAT-mediated histone Nt-Ac. (A) Schematic diagram of histone Nt-Ac mediated by NAT. On the right, the location of Nt-Ac is indicated within the nucleosome compared to internal lysine acetylation; the former is located at the N-terminal tip of histone tails, whereas the latter is deposited on the side chain of lysine residues found within the N-terminal tail regions of the histones or on their globular domain. The chemical reaction of Nt-Ac is thought to occur co-translationally by NATs transferring an acetyl group from acetyl-CoA to the α-amino group of the first amino acid residue at the N-terminal end of histone proteins. N-terminal acetylation is considered irreversible to date, as no N-terminal deacetylases (NDACs) have been discovered. (B) Prediction of putative substrates for different NAT members based on the N-terminal sequence of human histones, which is also dependent on whether iMET will be cleaved or not. Histones in dark blue indicate known experimentally proven NAT targets, and those in light blue represent putative targets based on their N-terminal sequence. In cases where the iMET is maintained, histones can be Nt-acetylated through the action of NatB or NatE. After removal of iMET, histones can be Nt-acetylated through the action of NatA or NatD.

Beyond the activity of writers (i.e. the proteins that add the modification), the abundance of epigenetic modifications is also determined by the relative activity of erasers that can remove these modifications (Zhao et al., 2021). Dedicated deacetylases for N-ε-acetylation are crucial for achieving reversibility and dynamic responses to environmental stimuli. However, for Nt-Ac, no N-terminal deacetylase(s) (NDAC) has been identified for histones, or any other proteins for that matter. In the absence of dedicated NDACs, histone Nt-Ac could hypothetically be removed through other indirect mechanisms, for example, histone clipping or exchange (Santos-Roza et al., 2009; Venkatesh and Workman, 2015).

Taken together, currently, the Nt-Ac of canonical H1, H2A, and H4 isoforms has been reproducibly established experimentally. Canonical H2B is Nt-acetylated in yeast but unlikely to be modified in higher organisms, whereas canonical H3 is predicted to be Nt-acetylated based on its sequence, although this has not been experimentally demonstrated yet. Notably, there are also variants of H2A, H2B and H3 that could potentially be Nt-acetylated. Given that investigations on many of these histone modifications are still at their infancy, below we will focus our discussion on the cellular and biological roles of H2A and H4 Nt-Ac, which have been explored the most and for which interesting insights have emerged from recent studies.

Cellular effects of histone Nt-Ac

Key to elucidating the connection between histone modifications, such as N-ε-acetylation and biological phenotypes, has been the ability to detect and quantify such modifications in experimental and biological samples, using proteomic methods and modification-specific antibodies. Unfortunately, the standard proteomic methods that have been used to examine lysine acetylation are not suitable for detection of Nt-Ac. Moreover, so far antibodies able to detect Nt-Ac have only been raised against H2A and H4 (Schiza et al., 2013; Ju et al., 2017). Only more recently, has the development and application of newer methods, such as top-down proteomics (mass-spectrometry-based methods for analysing intact proteins) of histone proteoforms (Ueberheide and Mollah, 2007), uncovered instances where the levels of histone Nt-Ac are altered, such as during the mammalian cell cycle (Jiang et al., 2018), or in influenza-activated CD8+ T cells (Rezinciuc et al., 2020). Consequently, there is only limited information regarding the potential correlation between histone Nt-Ac levels and cellular conditions.

The major link between histone Nt-Ac and biological phenotypes has been elucidated so far for H2A and H4, partly due to the high substrate selectivity of NAA40, which targets these two histones, compared to that of other NATs. The first indication that Nt-Ac of histones can affect cellular phenotypes came from a study that reported growth phenotypes associated with H4 Nt-Ac in yeast (Polevoda et al., 2009). Specifically, yeast cells either lacking NAA40 or modified to possess a non Nt-acetylatable H4 (H4S1V mutant) could grow without any apparent problems in normal conditions but displayed increased sensitivity under specific stress conditions and upon treatment with certain chemicals. In particular, NAA40-deleted cells displayed reduced growth upon exposure to the general transcription inhibitor 3-amino-1,2,4-triazole (3-AT), to the antimitotic microtubule-destabilising drugs benomyl and thiabendazole (TBZ), as well as to the nonspecific membrane inhibitor dinitrobenzene (DBZ) (Polevoda et al., 2009).

A subsequent study published by our group demonstrated that histone Nt-Ac in yeast is a modification that can affect transcriptional activation, and we also uncovered that crosstalk with other internal modifications, as one mechanism through which this can occur (Schiza et al., 2013). Specifically, we identified that H4 Nt-Ac antagonises the asymmetric dimethylation of the neighbouring arginine 3 residue (H4R3me2a) and, in that manner, promotes the expression of the rRNA-encoding genes. Additionally, we demonstrated that this H4 Nt-Ac–rRNA axis is responsive to conditions of calorie restriction (CR) (Schiza et al., 2013).

This work was followed by a number of interesting studies, which together have permanently disproven the initial notion of a Nt-Ac being a functionally inert histone modification. As discussed below, investigations of NAA40-mediated Nt-Ac of histones H2A and H4 have uncovered links between this histone modification and key cellular processes and phenotypes, such as cellular ageing, metabolism and oncogenesis.

Cellular ageing

As stated above, histone Nt-Ac has been linked to the response of yeast to CR, which is achieved by limiting glucose levels in the medium (Schiza et al., 2013). CR is an established lifespan-extending strategy from yeast to primates (Balasubramanian et al., 2017). Although the underlying cellular signalling responses that initiate the lifespan extension in response to CR are complex and incompletely understood, they are known to involve adaptations at the epigenetic and transcriptomic levels (Molina-Serrano et al., 2019).

Investigations into the function of yeast NAA40 found prolonged replicative lifespan following deletion of the enzyme and the associated loss of H4 Nt-Ac (Molina-Serrano et al., 2016). Furthermore, deletion of yeast NAA40 mimicked the alterations in the transcriptome that are seen upon CR or glucose limitation, given that RNA-seq analysis found comparable transcriptome profiles in NAA40-deleted and CR-treated cells. Importantly, it has been shown that CR reduces both the levels of NAA40 and its associated H4 Nt-Ac modification (Molina-Serrano et al., 2016; Varland et al., 2018). The link between NAA40 and CR was further supported by the observation that constitutive expression of NAA40 reduced the beneficial effects of the glucose deprivation (Molina-Serrano et al., 2016).

Additionally, NAA40-deleted yeast cells displayed increased expression levels of stress-induced genes, including nicotinamidase (*PNC1*), glycogen phosphorylase (*GPH1*), glycogen (*GLC3*), hexokinase (*HXK1*), trehalose-6-phosphate synthase (*TPS2*), glycogen synthase (*GLY1*) and neutral trehalase (*NTH1*). Subsequent mechanistic analysis in the same study focused on *PNC1* (Molina-Serrano et al., 2016). This is a stress-response gene whose expression is CR-induced and has a well-described CR-mediated longevity effect by regulating cellular nicotinamide and NAD levels, resulting in increased activity of silent information regulator (Sir2) (Anderson et al., 2003). Investigations of this cellular lifespan effect revealed an antagonistic relationship between Nt-Ac of H4 and its adjacent mark, H4R3me2a (Schiza et al., 2013; Molina-Serrano et al., 2016). Specifically, increased expression of *PNC1* coincided with reduced H4 Nt-Ac across the *PNC1* locus, whereas this was not apparent in a non-methylatable H4R3K mutant (Molina-Serrano et al., 2016). Together, these data suggest the involvement of H4 Nt-Ac in a CR-controlled lifespan pathway in yeast, where it acts as gatekeeper of H4R3me2a and of the *PNC1* stress-induced gene (Fig. 2).

CR is a remarkably efficient intervention in slowing ageing in a number of species across the animal kingdom (Flanagan et al., 2020). However, histone marks have also been implicated in the physiological ageing processes independently of CR (Yi and Kim, 2020). Whether histone Nt-Ac is involved only in CR-induced pathways or also has a role in other ageing processes, either in yeast or other organisms, has not been examined to date.

Metabolism

The reciprocal interaction between metabolism and epigenetic modifications is currently a particularly active field of investigation, given their interrelated connection. On one hand, epigenetic enzymes use intermediate metabolites, such as acetyl-coenzyme A (CoA) and S-adenosylmethionine (SAM) among others, as substrates or co-factors when modifying DNA and chromatin, suggesting that epigenetic marks might be influenced by

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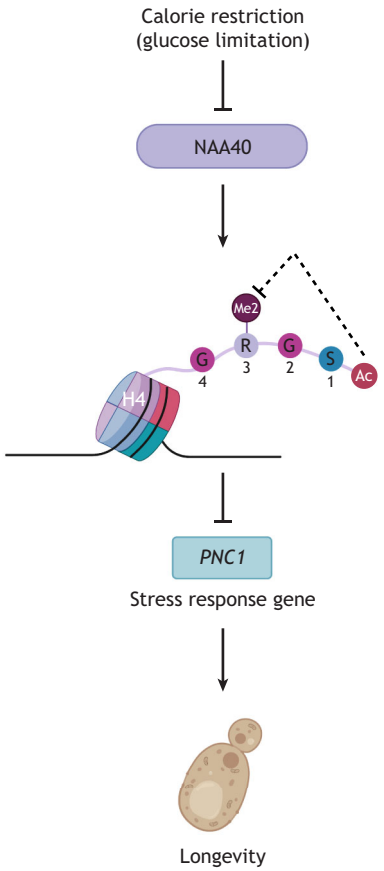

Fig. 2. Role of histone Nt-Ac in calorie restriction-induced longevity in yeast. In yeast, CR downregulates NAA40 expression, thereby decreasing the levels of H4 Nt-Ac in chromatin. H4 Nt-Ac normally antagonises the deposition of the H4R3me2a mark, so upon CR, the subsequent decrease in NAA40 and increase in the levels of H4R3me2a trigger the transcription of the stress-response gene nicotinamidase (*PNC1*), which promotes longevity in this organism.

the availability of such metabolites within cells. On the other hand, epigenetic mechanisms of gene regulation are involved in the control of metabolic enzymes and pathways, and consequently of the metabolic state of cells and tissues (Reid et al., 2017; Thakur and Chen, 2019). Accordingly, recent studies have reported that repression of NAA40 or loss of its acetyltransferase activity affects the expression of metabolic enzymes and pathways (Charidemou et al., 2022; Demetriadou et al., 2022). As discussed above, NAA40 itself and its downstream histone Nt-Ac are responsive to glucose deprivation in yeast (Molina-Serrano et al., 2016; Varland et al., 2018). However, it is not clear at the moment whether NAA40 or other NATs are responsive to other nutrient conditions, or whether this regulation also occurs in species other than yeast.

In a recent work addressing the role of NAA40 in human colorectal cells, we uncovered a novel link between the activity of NAA40 and one-carbon metabolism, a known metabolic network affecting both methylation reactions and nucleotide biosynthesis (Demetriadou et al., 2022). First, transcriptomic investigation revealed that NAA40 controls the expression levels of multiple enzymes within the one-carbon metabolism pathway. Specifically, high levels of NAA40 and of its N-terminal acetyltransferase activity are associated with expression of thymidylate synthetase (*TYMS*) and repression of methylenetetrahydrofolate reductase (*MTHFR*) and methionine adenosyltransferase 1A (*MAT1A*). Elevated expression of *TYMS* results in increased conversion of deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP). This could potentially facilitate DNA synthesis owing to an enhanced supply of the required nucleotides. Conversely, the reduced levels of MTHFR and MAT1A metabolic enzymes result in low SAM concentrations. In turn, global histone methylation is decreased because SAM is the universal methyl donor. Thus, the regulation of one-carbon metabolism by NAA40 is dependent on its acetyltransferase activity. In parallel with the reduced expression of one-carbon metabolism enzymes, we also observed concomitant changes in metabolites involved in this pathway and of various histone methylation marks. Collectively, our study provides evidence for NAA40 being a regulator of one-carbon metabolism (Demetriadou et al., 2022) (Fig. 3).

There are also data suggesting that NAA40 is involved in the regulation of lipid metabolism within liver cells (Liu et al., 2012; Charidemou et al., 2022). However, a caveat of these studies is the fact that histone Nt-Ac was not directly investigated, but instead its effects have been indirectly implied to be a consequence of loss of NAA40 activity. Specifically, Liu et al. developed liver-specific *Naa40*-knockout (KO) mice, but this was prior to the recognition that this protein acted as a mammalian NAT enzyme (Liu et al., 2012). In these mice, a decreased hepatic fat mass was observed specifically in male mice and not in female mice. Consistent with that, in the absence of NAA40, male mice exhibited less lipid accumulation and were protected against age-associated liver steatosis as their fatty acid uptake and lipid synthesis was reduced (Liu et al., 2012). Conversely, our recent study examining NAA40 in murine AML12 hepatic cells found that its depletion resulted in increased lipid synthesis and activation of *de novo* lipogenesis genes, as well as elevated diglyceride and triglyceride levels. This phenotype of accelerated lipid production was also observed when the enzyme was repressed *in vivo* in the larval fat body of *Drosophila melanogaster* (Charidemou et al., 2022). Nevertheless, the discrepancy between the two studies is currently unclear, and further investigations are required to address the opposing effects. Future work is needed to unravel the precise metabolic role of NAA40 in the liver and whether its effect is dependent on its histone acetyltransferase activity.

In the case of histone N-ε-acetylation, it has also been demonstrated that global and gene-specific histone acetylation levels can be affected by the state of intracellular metabolism (Friis et al., 2009; Wellen et al., 2009), given that acetyltransferases use the intermediate metabolite acetyl-CoA as an acetyl donor (Galdieri et al., 2014). Therefore, a reasonable hypothesis is that the levels of histone Nt-Ac are also sensitive to intracellular acetyl-CoA availability. This hypothesis was specifically examined for the first time in a study investigating the effects of glucose starvation on protein N-terminal acetylation (Varland et al., 2018). The authors assessed the outcome of nutrient starvation on the global levels of protein N-terminal acetylation as yeast cells progress through different growth phases of their lifecycle. Strikingly, upon nutrient limitation, genome-wide N-terminal acetylation levels remained generally unchanged, regardless of the significant reduction in the intracellular levels of acetyl-CoA following the entry of cells into the stationary phase (Varland et al., 2018). It is clearly intriguing that the Nt-acetylome is in general unresponsive to acetyl-CoA levels in yeast. Whether the same outcome is true in higher organisms has not been examined so far. One potential explanation for the minimal responsiveness of Nt-Ac to changing acetyl-CoA levels could be whether NATs have low K_m values for this substrate.

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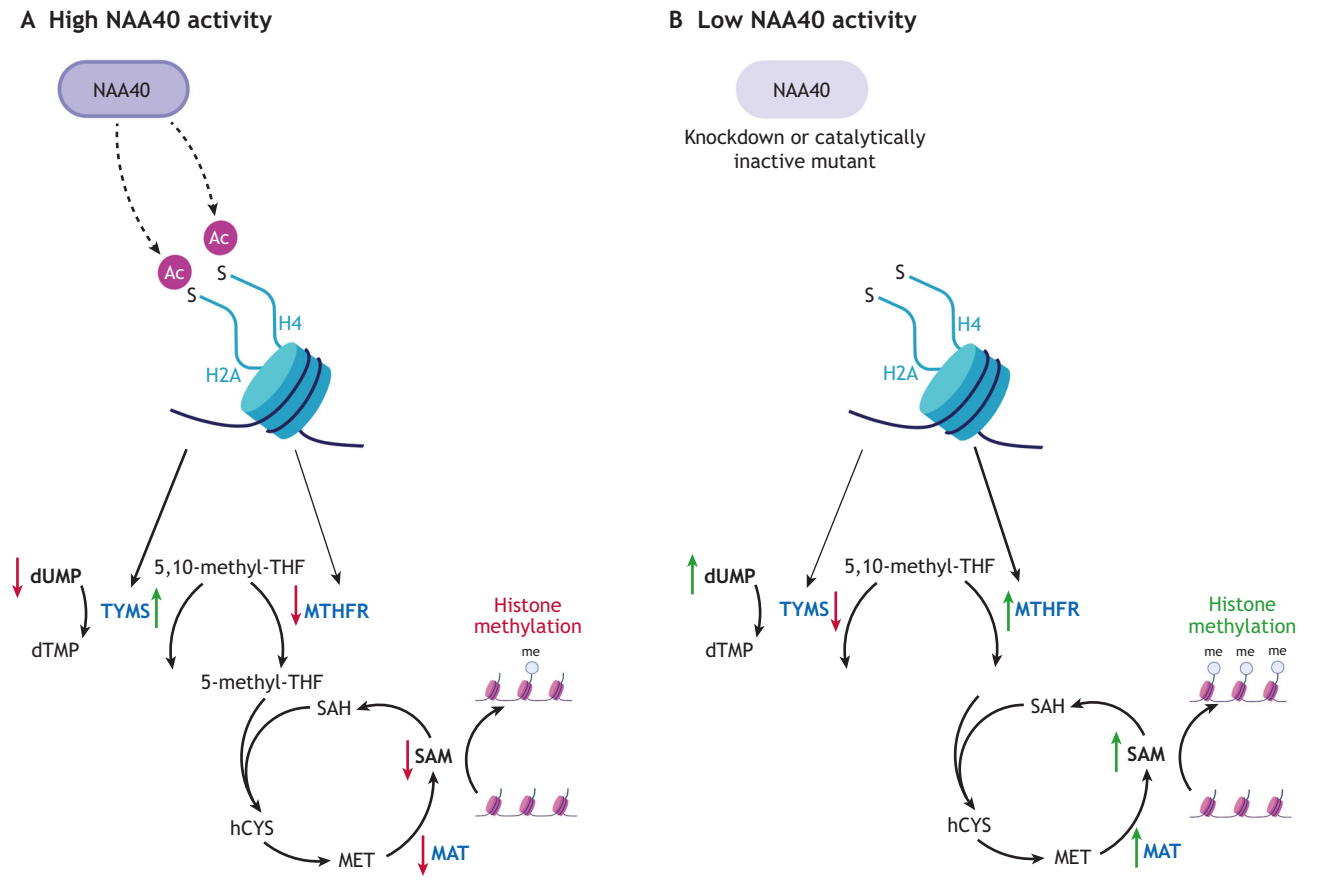

Fig. 3. Involvement of NAA40 in one-carbon metabolism. (A) High levels of NAA40 in colorectal cancer cells promote Nt-Ac of histones H2A and H4, inducing thymidilate synthase (TYMS) expression, while repressing methylenetetrahydrofolate reductase (MTHFR) and methionine adenosyltransferase 1A (MAT1A), all key enzymes of the one-carbon metabolism pathway. Induction of TYMS results in increased conversion of dUMP into dTMP. At the same time, the reduced levels of MTHFR and MAT1A result in low SAM concentrations, thereby reducing the global histone methylation levels. (B) Loss of NAA40 activity through knockdown or catalytic inactivation in colorectal cancer cells decreases the expression of *TYMS*, and increases the expression of *MTHFR* and *MAT1A*. As a result, levels of dUMP are reduced, whereas those of SAM are elevated, leading to increased global histone methylation. Catalytic mutant NAA40 can be generated by mutating glutamic acid 139 to glutamate, which abolishes NAA40 N-terminal acetyltransferase activity. Red arrows indicate reduced expression levels/metabolites, whereas green arrows indicate increased levels. THF, tetrahydrofolate; SAH, S-adenosylhomocysteine; hCYS: homocysteine; MET, methionine.

Evaluation of this hypothesis is currently hindered by the absence of reliable acetyl-CoA K_m values for the complete set of NATs. In the few cases where the information is available, the K_m values are comparable to those reported for N-ε-lysine acetyltransferases, which have a wide range of acetyl-CoA K_m from 0.2 µM to ∼50 µM (Reid et al., 2017). For instance, human NatD was reported to have acetyl-CoA K_m of ∼1 µM, for human NatA and *Saccharomyces cerevisiae* NatC this is ∼30 µM, for *Candida albicans* NatB, human NatB and *Schizosaccharomyces pombe* NatA it is ∼50 µM (Ho et al., 2021; Gottlieb and Marmorstein, 2018; Grunwald et al., 2020; Hong et al., 2017; Deng et al., 2020; Liszczak et al., 2013). More thorough measurements and comparison of acetyl-coA K_m values of NATs across species will allow a proper evaluation of this hypothesis.

Another more speculative possibility is that histone Nt-Ac could influence metabolism due to the high abundance of histone proteins and of the prevalence of their Nt-Ac. Considering that the extent of histone H1, H2A and H4 Nt-Ac could range from 50 to 99%, it has been estimated that the consumption of acetyl-CoA by histones would exceed the free intracellular pool of acetyl-CoA available in mammalian cells (Demetriadou et al., 2020). Consequently, Nt-Ac of histones could 'use up' the pools of this metabolite that otherwise could fuel alternative metabolic processes. Indeed, this might be the reason underlying our observation that loss of NAA40 results in a large rise in intracellular acetyl-CoA levels and induced lipid synthesis (Charidemou et al., 2022). However, there is presently no direct experimental support for a potential association between histone Nt-Ac and metabolic fluctuations.

Oncogenesis

It has been established for some time now that, beyond genetic mutations, epigenetic alterations are key to cancer development (Lu et al., 2020). Over the past few years, work by independent groups as discussed below has highlighted that NAA40 is altered in cancers, promoting oncogenesis and thus has been proposed as a putative therapeutic target.

One study pointing to such a role implicated the dysregulation of NAA40 and of its associated H4 Nt-Ac in lung cancer progression (Ju et al., 2017). In particular, NAA40 levels were found to be elevated in lung cancer tissue samples compared to normal tissue samples, and intriguingly this NAA40 overexpression was found to be associated with enhanced invasion and worse prognosis. Knockdown (KD) of NAA40 and subsequent reduction of H4 Nt-Ac did not have an effect on the proliferation or survival of lung

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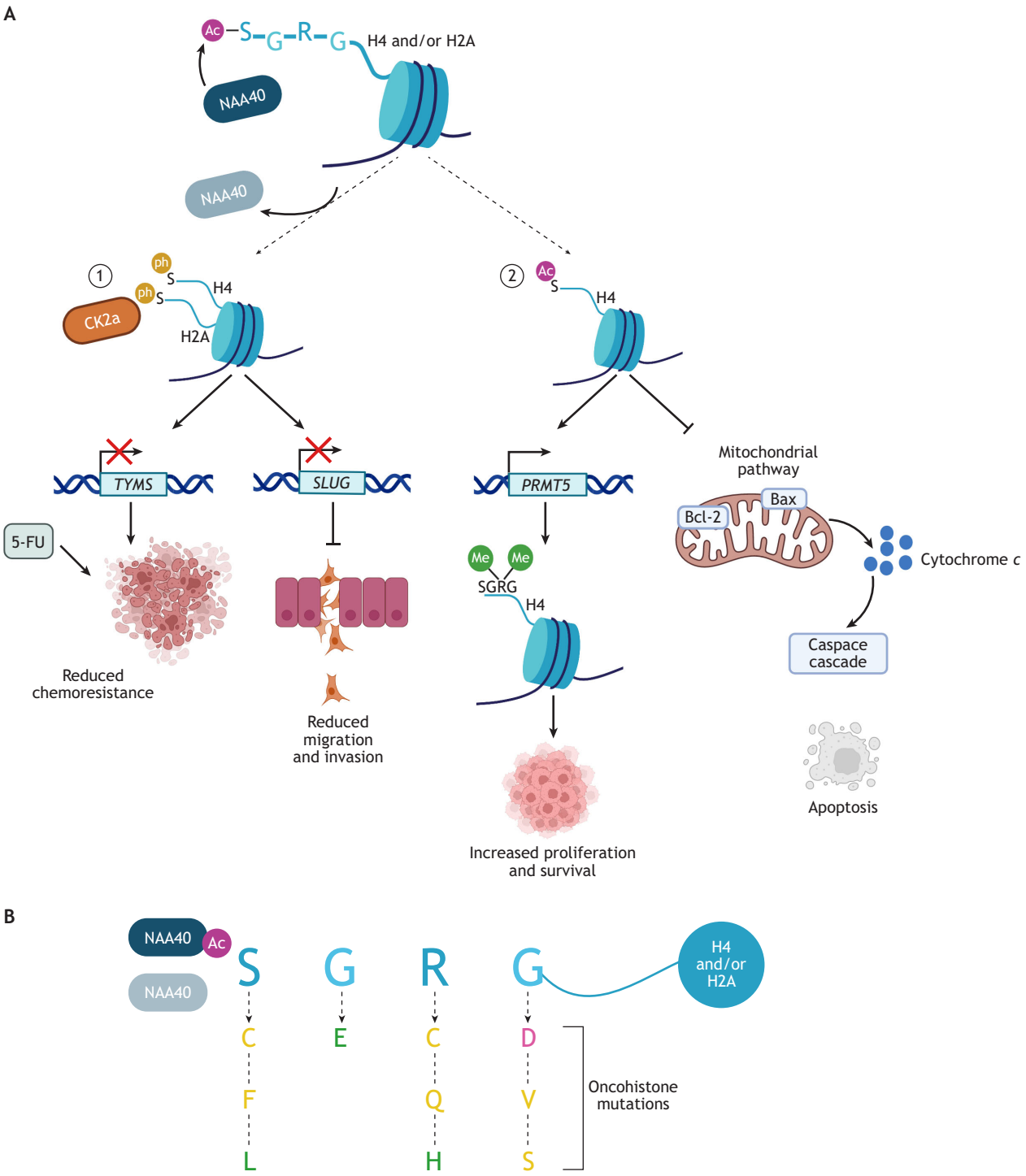

Fig. 4. Links between NAA40 and its associated histone Nt-Ac in cancer. (A) Representation of key cancer phenotypes that have been linked to NAA40 acetyltransferase activity. (1) Lack of NAA40 and of its corresponding Nt-Ac allows CK2α kinase to catalyse the phosphorylation (ph) of serine 1 (S1) on histones H2A (H2AS1ph) and H4 (H4S1ph). In colorectal cancer cells, this leads to silencing of *TYMS*, which encodes the molecular target of the chemotherapeutic agent 5-FU, therefore resulting in reduced chemoresistance. In lung cancer cells, the absence of NAA40 and the subsequent accumulation of H4S1ph leads to the silencing of the epithelial-to-mesenchymal transition (EMT) regulator *SLUG*, resulting in inhibition of invasion and migration. (2) NAA40 mediates the acetylation of serine 1 on histone H4. H4 Nt-Ac promotes the expression of PRMT5, which catalyses the symmetric dimethylation of histone H4 arginine 3 (H4R3me2s), leading to increased proliferation and survival through the deregulation of key oncogenes and tumour suppressor genes. NAA40 and its corresponding H4 Nt-Ac also inhibit the mitochondrial caspase-9-mediated apoptotic pathway. It remains unknown whether these effects are mediated through the antagonistic relationship between H4 Nt-Ac and H4S1ph. (B) Oncohistone mutations that affect the N-terminus of H2A and H4 occur with high frequency in cancer. Mutations detected both in H2A and H4 are shown in yellow, mutations detected only in H2A are shown in pink, mutations detected only in H4 are shown in green. All indicated oncohistone mutations negatively affect the ability of NAA40 to N-terminally acetylate peptides carrying these mutations.

cancer cells. However, NAA40 KD reduced migration and invasion of lung cancer cells *in vitro* and in mice models. Mechanistically, H4 Nt-Ac was shown to antagonise phosphorylation on the same serine residue, H4S1ph (a repressive mark), as it prevented the recruitment of CK2α, the kinase responsible for this modification, onto chromatin. Notably in the absence of NAA40, deposition of the repressive H4S1ph mark was increased within the promoter region of the transcription factor and epithelial-to-mesenchymal transition (EMT) regulator *SLUG* (also known as *SNAI2*). Thus, in lung cancer, there is evidence suggesting that NAA40 promotes cancer progression through its histone acetyltransferase activity, which antagonises the deposition of the repressive H4S1ph, thereby inducing a key EMT regulator and subsequently promoting metastasis (Ju et al., 2017) (Fig. 4A).

Our group has uncovered another oncogenic role for NAA40 and histone Nt-Ac in colorectal cancer, where it affects cell survival and growth rather than invasion and metastasis. In our initial study, we showed that NAA40 KD in colorectal cancer cells reduced cell survival and induced apoptosis through a p53 (also known as TP53)-independent, mitochondrial caspase-9-mediated apoptotic pathway (Pavlou and Kirmizis, 2016). In follow-up work, we found that NAA40 is frequently upregulated in colorectal cancer patients and, accordingly, depletion of NAA40 repressed colorectal tumour growth in mouse xenografts (Demetriadou et al., 2019). Moreover, NAA40 mediated colon cancer cell proliferation through an interplay between symmetric dimethylation of histone H4 arginine 3 (H4R3me2s) and H4 Nt-Ac. In particular, NAA40 stimulates the expression of protein arginine methyltransferase 5 (PRMT5), which in turn increases the levels of H4R3me2s, resulting in the activation of crucial oncogenes and repression of tumour suppressor genes that together promote colorectal cancer cell growth (Demetriadou et al., 2019). As mentioned above, in another study, we found that NAA40 deficiency leads to alterations in the levels of different one-carbon metabolism genes, and, interestingly, one of the most significantly downregulated genes upon knockdown of NAA40 was a key enzyme of one-carbon metabolism, TYMS. Importantly, TYMS is also the molecular target of the chemotherapeutic agent fluorouracil (5-FU) (Demetriadou et al., 2022). 5-FU is routinely used in colorectal cancer treatments, and *TYMS* expression is a predictor for 5-FU resistance in colorectal cancer patients (Abdallah et al., 2015). Importantly, we showed that NAA40 confers 5-FU resistance to colorectal cancer cells *in vitro* and in mouse xenografts, as well as in human samples, based on analysis of patient data. Loss of enzymatic activity of NAA40 results in an increased deposition of the antagonistic serine 1 phosphorylation repressive mark on histones H2A and H4 (H2A/H4S1ph) on the *TYMS* promoter. Additionally, upon NAA40 depletion, we observed increased localisation of H2A/H4S1ph in the inner nuclear membrane, where it colocalised with the nuclear lamina. This is further supported by the increased occupancy of the lamina major component, protein lamin A/C at the *TYMS* promoter. Collectively, this work provides evidence that NAA40 regulates *TYMS* expression by controlling the presence of the antagonistic H2A/H4S1ph modification at the nuclear periphery. Importantly, this in turn affects the response of cancer cells to chemotherapy (Demetriadou et al., 2022) (Fig. 4A).

Evidence thus far supports the notion that transcriptional upregulation of NAA40 contributes to oncogenesis in colorectal and lung cancers. In both cases, the underlying molecular mechanisms involve crosstalk with other histone modifications (H2AS1ph, H4S1ph and H4R3me2), although the affected gene networks and cancer pathways are distinct. This raises the possibility that the carcinogenic properties of NAA40 might be highly dependent on the cancer type. Given that the levels of *NAA40* transcripts are upregulated in several other cancer types compared to the respective non-cancer tissues, it is possible that NAA40 and histone Nt-Ac have oncogenic roles in other malignancies as well (Koufaris and Kirmizis, 2020). Interestingly, in liver cancer, increased expression of *NAA40* has been associated with decreased activity of the p53 network, pointing to p53 being a potential mediator of the transcriptional deregulation of NAA40 in malignancies (Koufaris and Kirmizis, 2021).

Interestingly, the link between histone Nt-Ac and cancer is further supported by a recent examination of oncogenic histone mutations – which are frequently referred to as 'oncohistone mutations' – based on a comprehensive screen for the frequency of histone mutations across tumour samples (Nacev et al., 2019). Intriguingly, S1C mutation in H2A and S1C/R3C in H4 were among the most common alterations of histone residues found in cancers. Notably, this was not the case for the N-terminal residues of other histones (Nacev et al., 2019). Our subsequent pan-cancer analysis of the Cancer Genome Atlas (TCGA) found that mutations that affect the N-terminal regions of proteins in cancers are rare, but amongst those mutations those affecting the NAA40-recognition motif (SGRG) of H2A and H4 were by far the most frequent (Koufaris and Kirmizis, 2020). Importantly, a recent report found that oncohistone mutations affecting the N-terminus of H2A and H4 resulted in a reduced ability of NAA40 to Nt-acetylate these mutants when tested *in vitro* (Ho and Huang, 2022) (Fig. 4B).

Based on the findings to date, there seems to be an apparent paradox, in that, although current evidence supports an oncogenic role for NAA40, somatic mutations prevalent in cancer are those that presumably disrupt histone Nt-Ac. Although the underlying molecular details need to be elucidated, a possible explanation for this paradox is that overactive NAA40 in cancer would affect the global Nt-Ac of all its target histones, whereas somatic histone mutations would impact only one of the many of H2A or H4 alleles – given that human H2A and H4 are encoded by multiple genes – possibly resulting in local or nucleosome-specific effects. Similarly, residues that are mutated in cancer have also been identified in other histone proteins; however, it remains to be elucidated how certain oncohistone mutations drive carcinogenesis (Mitchener and Muir, 2022).

Conclusions and future perspectives

As we have discussed here, there is now ample evidence linking Nt-Ac of histones H2A and/or H4 to important cellular processes. Given that these modifications have attracted attention only recently, it is likely that additional biological phenotypes will be discovered that are linked to them and potentially to Nt-Ac of other histones as well. The broad cellular functions of histone Nt-Ac are possibly associated with diverse molecular mechanisms acting at the chromatin level that have so far only been partly elucidated. One main mechanism revealed over the past decade is the crosstalk with other adjacent modifications, through the regulation of writers mediating H2A/H4S1ph and H4R3me2a (Schiza et al., 2013; Ju et al., 2017). Beyond the identified crosstalk with other histone modifications, further potential mechanistic pathways associated with histone Nt-Ac are currently speculative as supportive experimental evidence is currently unavailable. For instance, it is well established that Nt-Ac of proteins can affect protein stability, turnover and localisation (Ree et al., 2018). Hence, it would be interesting to examine whether Nt-Ac of histone isoforms and variants can affect their stability and/or incorporation into chromatin. On a separate note, specialised reader complexes

(i.e. complexes that identify the modifications) have been established to be key for linking N-ε-acetylation of lysine residues with downstream responses. Readers of N-ε-acetylation contain acetyl-lysine recognition domains, which commonly include bromodomains (BRD). BRD are specialised domains of ∼110 amino acids that can recognise the presence of acetylated lysine on proteins, including histones, and initiate downstream molecular responses (Marmorstein and Zhou, 2023). However, whether a similar mechanism is employed downstream of histone Nt-Ac remains elusive, and presently no such readers have been identified. Determining whether or not Nt-Ac readers exist will be highly important for further elucidating the biological function of this modification. Possible readers of Nt-Ac could potentially be identified using unbiased screening approaches, such as those that have been applied to N-ε-acetylation in the past, for instance using immobilised Nt-Ac histone peptides or nucleosomes as bait (Bartke et al., 2010). One notable distinction of Nt-Ac, however, is the high abundance of this modification compared to individual N-ε-acetylation marks, which might mean that it is the absence rather than the presence of Nt-Ac that is the defining state.

Taken together it is clear that, so far, we have only scratched the surface regarding the mechanisms by which Nt-Ac of histones within chromatin can ultimately affect cellular processes and phenotypes.

Nevertheless, the emerging insights into the cellular functions of histone Nt-Ac, especially in cancers, have already motivated the development of the first specific inhibitors against NAA40 (Deng et al., 2021). This inhibition approach relied on the use of bisubstrate molecules, which covalently connect the CoA cofactor and the short peptide SGRGK, potently inhibiting NAA40 at the nanomolar range, with an impressive 1000-fold specificity compared to other NATs (Deng et al., 2021). Although these relatively large molecules are limited in their ability to penetrate cells and inhibit NAA40 intracellularly, they provide proof-in-principle for the use of highly effective and selective inhibitors with therapeutic potential in cancers and/or metabolic diseases.

As has been outlined throughout this Review, compared to our understanding of N-ε-acetylation, there are many more open questions regarding the occurrence, frequency and biological significance of Nt-Ac. First, information on Nt-Ac of histones beyond H2A and H4 is currently limited. For instance, in the case of H3, it remains unknown if its N-terminus is modified, and for H1 isoforms that have been shown to be Nt-acetylated the responsible enzymes have not been examined. Second, although NATs have traditionally been thought to act mainly co-translationally, a recent study has reported that NAA40 generates distinct isoforms with specific nuclear or cytoplasmic localisation (Jonckheere and Van Damme, 2021). It will therefore be important to determine whether N-terminal acetylation of histones occurs co-translationally at the ribosomes, post-translationally in the nucleus or in both compartments. The potential N-terminal acetylation of histones within the nucleus could point to a dynamic regulation of this histone modification and of its associated gene network. Third, Nt-Ac of histone variants has not been examined so far and could potentially result in interesting biology. For example, beyond the canonical H2A and H4, other histone H2A variants contain the conserved motif targeted by NAA40 (Magin et al., 2015), but whether their N-termini are indeed acetylated remains to be examined. Fourth, the writers, readers and erasers form a trinity of regulators that mechanistically establish and propagate the effects of histone modifications within chromatin. For histone Nt-Ac, only the writers are currently understood; as mentioned above, it is not clear whether Nt-Ac readers or erasers exist. The discovery of any NDACs would demonstrate that histone Nt-Ac is a more dynamic modification than currently believed. Fifth, acetylation is one among a large number of acylations that are known to occur on histone proteins. Notably histone acylations beyond acetylation (e.g. propionylation, butyrylation and hydroxybutyrylation) can also affect chromatin dynamics and gene transcription (Nitsch et al., 2021). Nevertheless, the presence of acylations at the N-termini of histone proteins beyond acetylation has not been thoroughly examined so far, with one exception being the detection of propionylation on H2A terminus (Foyn et al., 2013). Further investigations are needed to determine the types and abundance of acylations on the N-termini of histones and their potential crosstalk with Nt-Ac.

In the field of epigenetics, long periods of stasis have been followed by intense activity and a rapid expansion of knowledge. For example, histone lysine acetylation was discovered in the late 1960s, yet the first histone acetyltransferases and deacetylases were not identified until three decades later (Peixoto et al., 2020). A decade ago, there was already ample evidence for the existence of histone N-terminal acetylation, but beyond that a limited understanding of the underlying mechanisms and biological significance remains. Within the past decade, considerable insights into NAA40-mediated histone interactions and their associated biology have been gained through the dedicated work of a number of research groups. During this period, we have learned about crosstalk between histone N-terminal acetylation and other modifications, recognised the presence of oncohistone mutations affecting residues related to H2A and/or H4 N-terminal acetylation and linked this modification to cellular and organismal phenotypes such as metabolism, ageing, and cancer. Nevertheless, much remains to be uncovered in the next decades regarding the N-terminal modifications of H2A and H4, as well as of other histones. Arguably, the histone tail tip has more tales to tell.

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