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Review

Histone variants in archaea – An undiscovered country

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ABSTRACT

Exchanging core histones in the nucleosome for paralogous variants can have important functional ramifications. Many of these variants, and their physiological roles, have been characterized in exquisite detail in model eukaryotes, including humans. In comparison, our knowledge of histone biology in archaea remains rudimentary. This is true in particular for our knowledge of histone variants. Many archaea encode several histone genes that differ in sequence, but do these paralogs make distinct, adaptive contributions to genome organization and regulation in a manner comparable to eukaryotes? Below, we review what we know about histone variants in archaea at the level of structure, regulation, and evolution. In all areas, our knowledge pales when compared to the wealth of insight that has been gathered for eukaryotes. Recent findings, however, provide tantalizing glimpses into a rich and largely undiscovered country that is at times familiar and eukaryote-like and at times strange and uniquely archaeal. We sketch a preliminary roadmap for further exploration of this country; an undertaking that may ultimately shed light not only on chromatin biology in archaea but also on the origin of histone-based chromatin in eukaryotes.

1. Introduction

To regulate genome accessibility, different forms of life employ a wide range of tools. In eukaryotes, transcription factors, polymerases, repair enzymes and other DNA-binding proteins have to contend with the fact that most genomic DNA has been assembled into nucleosomes. One way to alter genome accessibility is to exchange bulk histones in these nucleosomes for paralogous variants that – either directly or indirectly – render chromatin more or less accessible. In eukaryotes, there is a large catalogue of these variants, which play specific roles in, for example, responding to DNA damage, establishing centromere identity and altering gene expression in response to temperature change [1–3]. These variants are encoded outside the main clusters of replicative histone genes. Their deposition is not coupled to the cell cycle and occurs at specific locations, regulated by dedicated chaperones and remodellers (reviewed in [4–7]).

Histone-fold proteins are also widespread in archaea [8–10] (Fig. 1f). In some species, they are relatively lowly expressed [10] and appear to behave more like transcription factors. HpyA, for example, the only histone found in the halophilic archaeon *Halobacterium salinarum,* binds to < 60 locations along the genome and regulates the expression of a small set of genes involved in the adaptation to low salt conditions [11]. In other species, histones mediate genome-wide packaging reminiscent of what is happening in eukaryotes. This includes members of the orders Thermococcales and Methanobacteriales where histones are amongst the most abundant proteins in the cell [10,12–14].

Many archaeal genomes encode multiple histone genes [8], which can differ significantly at the amino acid level (Fig. 1b). For example, the most dissimilar histone sequences in *Methanosphaera stadtmanae*, a methanogen found in the human gut, have only 41% identity. Can these homologs be considered histone variants in the sense we know them from eukaryotes? That is, do they play distinct (though perhaps overlapping) functional roles in the cell and make non-redundant contributions to genome function and fitness? Or are the amino acid differences that distinguish these variants largely immaterial - tolerated, rather than adaptive?

Below, we will review the evidence that histones encoded in the same archaeal genome do different things. We consider three lines of evidence: structural properties, regulation, and evolution. We will argue that histone variants with distinct, adaptive functions exist in archaea as they do in eukaryotes, but that we have barely scratched the surface when it comes to elucidating their physiological roles, an effort that will ultimately help us not only to understand chromatin biology in archaea but also the evolution of histone-based chromatin in eukaryotes.

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2. Structure

2.1. Similar building blocks, different assembly

Regarding both secondary and tertiary structure, the core histone fold in archaea and eukaryotes is remarkably similar. Monomers assemble into dimers in the familiar handshake configuration (Fig. 1c, d), mediated through hydrophobic contacts between the anti-parallel α2 helices of the two monomers and through hydrogen bonding between neighbouring loop regions [15,16]. Contacts with DNA are also made in a similar manner, principally via residues in the loop regions [17] (Fig. 1c,d).

In eukaryotes, side chain residues at the dimer interface form complementary shapes. As a consequence, eukaryotic histones form obligate heterodimers: H3-H4 and H2A-H2B [18]. H4 does not form dimers with another H4 molecule, H3 does not pair with H3, and so forth. Dimerization of (at least some) archaeal histones is comparatively more flexible as it allows formation of homo- as well as heterodimers. Direct experimental confirmation of this *in vivo* comes from *Methanothermus fervidus*, which has served as the model system for much of the early

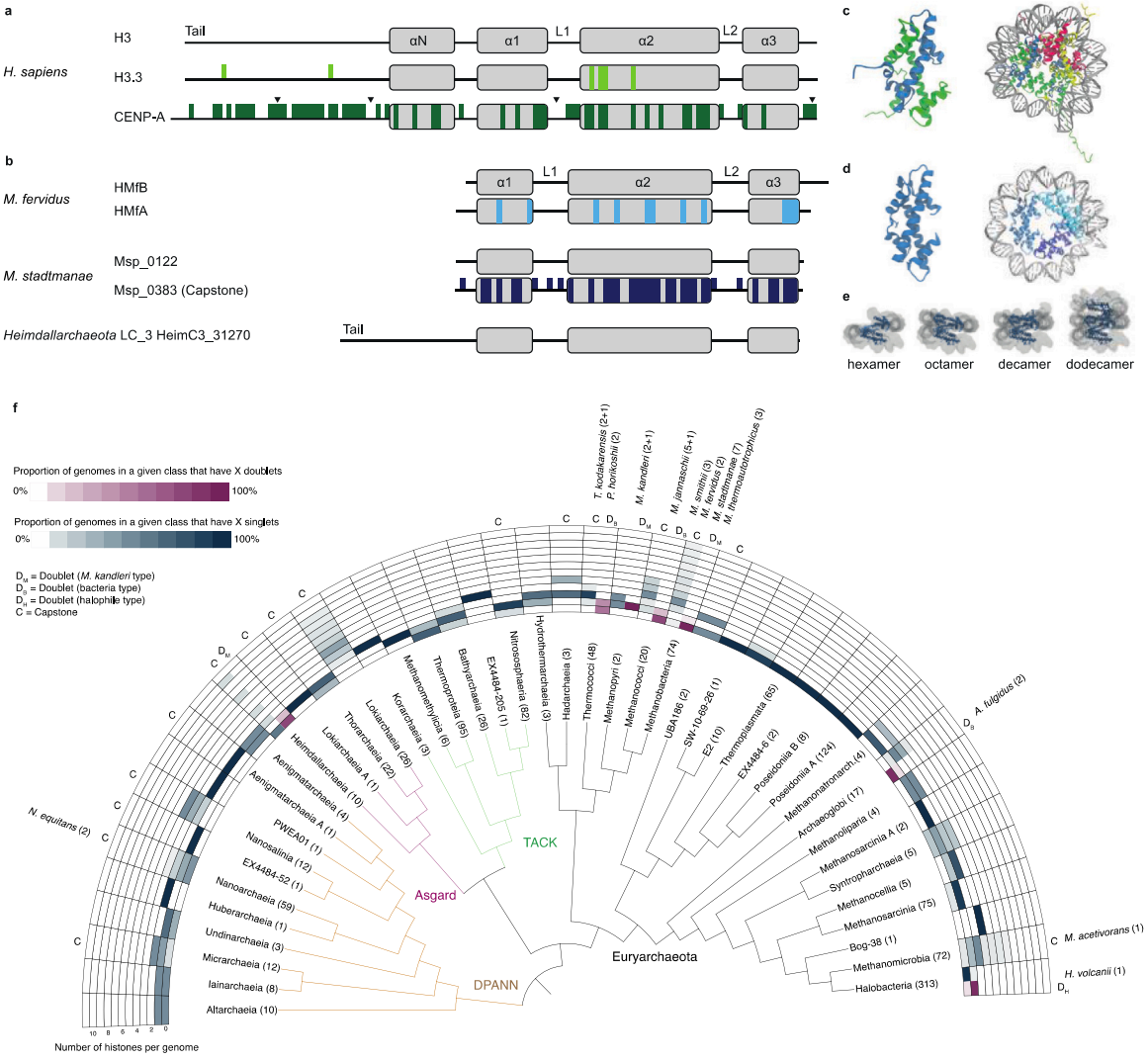

Fig. 1. Overview of archaeal and eukaryotic histone variants. a. Comparison of human histone variants H3.3 and CENP-A (cenH3) to replicative H3. Sequences were downloaded from HistoneDB 2.0 [68]. Sites which are not identical to replicative H3 are highlighted in coloured blocks on the secondary structure for each variant. Insertions are marked with an arrow. Alpha helices (αN-α3) and loop regions (L1/L2) are annotated. b. Comparison of histones from three archaea that each encode more than one histone. Top: for *Methanothermus fervidus*, residues which differ in HMfA relative to HMfB are shown on the secondary structure. Centre: in *Methanosphaera stadtmanae*, residues which differ in Msp_0383 relative to Msp_0122 are highlighted. Only two of the seven histones in this species are shown. Bottom: an example of a tailed histone from *Candidatus Heimdallarchaeota* archaeon LC_3. c. Structure of the H3-H4 dimer and the eukaryotic nucleosome (PDB: 1AOI [18]; red = H2B, yellow = H2A, green = H3, blue = H4). d. Structure of the HMfB dimer and hexamer (PDB: 5T5K [21]). e. Model of higher-order archaeal histone oligomers, showing sequential addition of HMfB histone dimers from hexamer to dodecamer. Structures in c. and d. were rendered using VMD [69]. f. Cladogram of archaea illustrating the distribution of histone proteins across different archaeal classes. The number of genomes available for each class is given in parentheses following the class name. The number of histones is shown separately for proteins containing either a single (singlet, blue) or end-to-end-duplicated (doublet, red) histone-fold domain. Clades containing putative capstone histones (see text and [66]) are annotated "C". Doublet histones are annotated by type (see [66]); bacteria-type doublet (D_B), *M. kandleri*-type doublet (D_M), Halobacteria-type doublet (D_H). Species mentioned in the main text are annotated with the number of singlet (+ doublet) histones that are present in their genome. The cladogram is based on GTDB (https://gtdb.ecogenomic.org), with the tree rooted at the split between DPANN and Euryarchaeota/Asgard/TACK. Information on histone presence/absence and number was obtained from [10]. Only information for species present in GTDB is shown, supplemented with additional Asgard species from Genbank (taxid 1935183) and [76]. Seven archaeal classes present in GTDB were not included in the histone survey dataset [10] and are therefore not included in the tree. For reference, Lokiarchaeia_A contains Odinarchaeota species. Some counts of species with no histones may be as a result of missing information/incomplete genome assemblies rather than indicating a definitive lack of histones.

biochemical work on archaeal histones by John Reeve and colleagues [19]. Homodimerization has also been extensively demonstrated *in vitro* using recombinant histones from *M. fervidus* [16,19–21], *Thermococcus kodakarensis* [22], *Pyrococcus horikoshii* OT3 [23] and *Methanobacterium formicicum* [24,25]. In addition, *in vitro* unfolding transitions indicate that homodimers are also formed in Pyrococcus GB-3a [25], *Archaeoglobus fulgidus* and *Methanocaldococcus jannaschii* [26]. Finally, there are several archaea that encode only a single histone gene, making homodimerization a necessary requirement for dimer formation [27]. Taken together, this suggests that the capacity for promiscuous homo- and heterodimerization is, if not a universal property of archaeal histones, then certainly widespread.

2.2. Variants that restrict promiscuous dimerization?

Some archaeal histone variants might break this promiscuity. Most members of the (confusingly named) Halobacteria as well as some distantly related species such as *Methanopyrus kandleri* [28,29] carry histone doublets, where two histone-fold domains are encoded back-to-back, connected through a flexible linker peptide. This physical tether should strongly favour dimerization between the fused histone-fold domains, which have begun to diverge in sequence and are thought to act – effectively – as obligate heterodimers [although structural modelling suggests that, if the tether were to be cut, both N- and C-terminal halves could still form homodimers (K. Stevens, unpublished results)]. Heterodimerization here is enforced by physical linkage, not structural incompatibility as observed for eukaryotic histones.

Outside of the forced marriage of histone doublets, we are not aware of any examples of obligate heterodimerisation in archaea. There are, however, at least two documented instances where heterodimerization appears to be required for DNA binding. One of the three histones of *Methanothermobacter thermoautotrophicus* OSU (HMtB) has lost its ability to bind DNA as a homodimer but can do so when assembled into a heterodimer with one of its homologs (HMtA2) [30]. Similarly, one of the two histones of *Nanoarchaeum equitans* can only bind DNA as a heterodimer [31]. These histones can still *form* homodimers but these dimers do not appear to interact with DNA, at least under the conditions tested *in vitro*. In both instances, the requirement for a non-identical histone to generate dimers capable of DNA binding has evolved through small changes to the protein sequence; via a single amino acid substitution in *M. thermoautotrophicus* [30] and a four-amino acid insertion in the L1 region in *N. equitans*, reminiscent of the extended L1 region in eukaryotic H3 compared to H4 [31]. The physiological role of these variants, how they are deployed and interact with other histones to affect chromatin properties *in vivo*, remains to be elucidated. Studying them further might help us understand the evolution of obligate heterodimerization, which – thus far – appears unique to eukaryotes and some viruses [32].

2.3. Archaeal histone variants modify the histone-DNA complexes that contain them

Model archaeal histone dimers assemble into tetramers very similar in structure to eukaryotic $(H3\text{-}H4)_2$ tetramers [21] (Fig. 1c,d). These tetramers can have different properties depending on their constituent histone variants, including altered stability, DNA binding affinity and propensity to assemble into larger complexes (see below). Most of our experimental knowledge in this regard again comes from *in vitro* work on the two histones of *M. fervidus*, HMfA and HMfB, which are 85% identical at the amino acid level. HMfA binds DNA at a lower concentration than HMfB but is associated with less extensive compaction [19,33]. The two histones of another hyperthermophile, *T. kodakarensis,* also differ in their binding properties: HTkB[1] has greater affinity for DNA and compacts DNA more extensively than HTkA [22]. Similarly, one of the histones in *M. jannaschii* (HMjA4) has lower affinity to DNA and is less effective at inhibiting transcription *in vitro* [34]. Interestingly, this homolog is encoded on a plasmid that is not found in closely related strains, suggesting that HMjA4 might be a recent acquisition, yet to be fully integrated into the workings of the new host genome.

Assembling combinations of histone paralogs with different properties into heterologous complexes can result in structures with intermediate properties. Tetramers built from fusion dimers of HMfA and HMfB, for example, have DNA binding affinities that are stronger than $(HMfA)_4$ and (slightly) weaker than $(HMfB)_4$ [35]. Is this true for archaeal histone variants in general? Making defined, homogeneous populations of heteromeric complexes is difficult (although feasible by way of making fusion proteins). As a result, this question has not been addressed experimentally at scale. However, recent systematic *in silico* modelling of all possible tetrameric complexes that can be built from the seven histones of *Methanosphaera stadtmanae* suggests that combinations of paralogs often yield tetramers with intermediate DNA binding affinities or tetramer stabilities and that the properties of the complex can change in a graded, dosage-dependent fashion depending on the number of copies of a given variant that are incorporated into the complex [36].

2.4. Archaeal histone variants modify the oligomerisation potential of higher-order complexes

Variant incorporation might also be important for larger, super-tetrameric complexes. In *M. fervidus* [13,14], *T. kodakarensis* [21,37,38], and likely other species [8], tetramers can extend into longer oligomers of variable length, where dimers are added in a stepwise fashion to wrap increasingly more DNA (Fig. 1e). Using a histone such as HMfA/B or HTkA/B to build these structures, previously referred to as hypernucleosomes [8] or archaeosomes [39], this process can – theoretically – go on forever. Footprints of these complexes as large as 450 bp have been found *in vivo* using micrococcal nuclease digestion, consistent with complexes composed of 30 histones [21,38,40], although complexes are typically smaller (4–10 histones). This variability in size represents a sharp departure from the fixed octameric architecture of eukaryotic nucleosomes.

Not all archaeal histones support oligomerization equally well. Tethered particle motion experiments have revealed weaker stacking and lower stability of oligomeric complexes that contain HMfA compared to HMfB, consistent with differences in DNA compaction between HMfA and HMfB [33]. In other species, variants might interfere with oligomer extension more radically: We recently identified histone variants in several Methanobacteriales archaea that we called 'capstone' variants. These capstones are characterized by unstable tetramer interfaces and are predicted to prevent further stable extension when incorporated at the end of a growing oligomer (Fig. 2) [36]. Structural modelling of histones from *M. stadtmanae* also suggests that this capstone property is easy to evolve: a single amino acid change can significantly destabilize the tetramer interface [36]. Using a single substitution (loss of histidine at the end of α2, see Fig. 2) as a guide for capstone identity, we discovered histones with predicted capstone function in several independent archaeal lineages (Fig. 1f), suggesting parallel evolution of capstone functionality. Previously, Henneman and colleagues had also highlighted residues likely to disrupt stacking interactions between dimers in the context of longer oligomeric complexes [8]. Histones with substitutions at those residues might act in a similar manner to that proposed for capstones and destabilize or outright prevent multimerization.

It remains to be established if, when, and why these histone variants are incorporated in archaeal chromatin *in vivo*. The same is true for heteromeric histone complexes in general. With the exception of HMfA/B heterodimers (see above), we have no experimental confirmation that specific heteromeric complexes exist *in vivo*. Yet we strongly suspect that they do given what we know about how these complexes assemble: in

[1] Note that the A/B labels here simply reflect naming conventions rather than ancient paralogy.

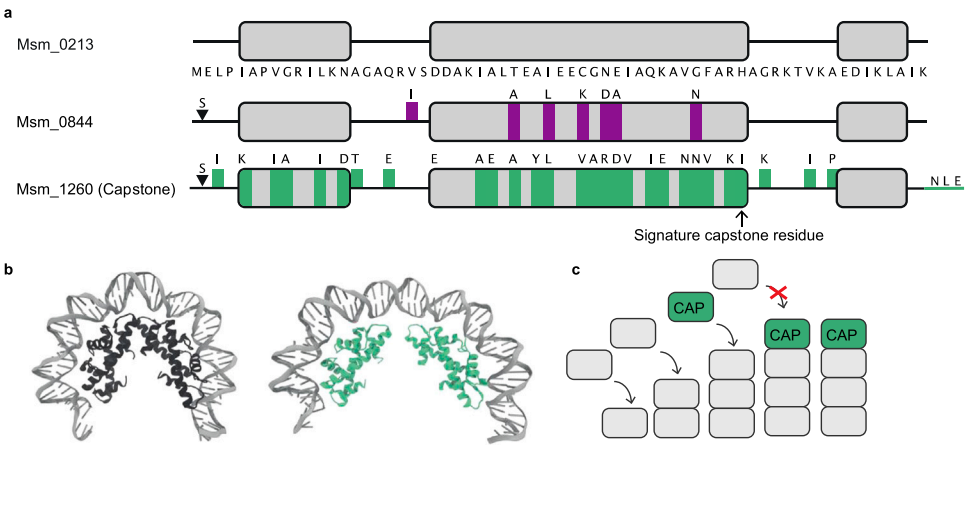

Fig. 2. Capstone histone variants. a. Histone secondary structure showing the positions of substitutions in the *Methanobrevibacter smithii* histones Msm_0844 and Msm_1260 (the putative capstone histone) relative to Msm_0123. Sites which are not conserved are highlighted in coloured blocks on the secondary structure for each histone. Insertions are marked with an arrow. The residue responsible for the capstone property (position 49 in HMfB coordinates) is highlighted. b. Representative frames from simulations of the most highly expressed histone (Msp_0122, left, black) and the capstone histone from *Methanosphaera stadtmanae* (Msp_0383, right, green). Images were rendered using VMD [69]. c. Model illustrating the potential capping function of capstone variants when integrated into oligomeric histone complexes.

contrast to eukaryotes, there are no known variant-specific (or even general) histone chaperones or remodellers in archaea. Instead, archaeal histones self-assemble onto DNA. This is not only true *in vitro* but also when histones are heterologously expressed in *E. coli* where – in the absence of any co-evolved deposition machinery – they associate with DNA and form complexes that protect DNA from micrococcal nuclease (MNase) digestion [14]. This self-assembly process is likely very dynamic. Recent work using cryo-EM and molecular dynamics simulations showed that HTkA oligomers can exhibit spontaneous breathing motions, where the oligomer opens up like a clam, providing both intermittent access to normally concealed DNA and perhaps a mechanism for exchange of histones paralogs without the need for dedicated remodelling [39]. We therefore suspect that variants will, perhaps somewhat haphazardly, be incorporated into and shape the properties of a heterogeneous population of complexes, which will affect DNA transactions throughout the nucleoid (Box 1).

2.5. Archaeal histone complexes and the nature of variants

The dynamic size of histone-DNA complexes and the capacity for homo- as well as heterodimerization have important implications for how we think about histone variants in archaea. Archaeal histone variants do not have positional identity in the same way that eukaryotic variants do. They are not swapped into a defined position in a fixed larger structure, like H2A.Z replacing replicative H2A in the octameric nucleosome. Archaeal histone complexes have rather more degrees of freedom in how different histones can be assembled relative to each other. In this regard, archaeal histones behave more like bacterial nucleoid-associated proteins, where homologous variants present in the same species (e.g. HupA and HupB in *E. coli*) can also often homo- and heterodimerise [41,42] and assemble into longer oligomeric complexes on DNA. When thinking about the nature and potential roles of histones variants in archaea, we must therefore avoid having our thoughts too narrowly guided by prior knowledge of (and pre-formed notions about) eukaryotic variants.

Box 1
Global versus local action

For lack of evidence to the contrary, we have assumed that assembly of tetramers and higher order structures is random, and that composition and assembly are consequently dictated primarily by mass action. Under such a model, chromatin architecture is altered on a global scale rather than locally. Changes in the expression of a particular histone variant tune chromatin properties such as genome accessibility genome-wide. This might be useful for a concerted response to environmental change (e.g. in response to heat stress) or in preparation for altered nutrient availability (e.g. during stationary phase). This is an obvious departure from the world of eukaryotes, where variants are often deposited in a precisely targeted fashion. Instead, the situation appears more like that found in bacteria where nucleoid composition and resultant architecture are constantly remodelled, as different nucleoid-associated proteins assume dominant roles in the chromatin landscape, depending on physiological need. For example, Dps is a major constituent of the *E. coli* nucleoid in stationary phase, where it mediates a compact, crystalline chromatin state, but barely detectable in exponential phase [70]. Archaeal histone variants might mediate similar global transitions in chromatin state, just in a more LEGO-like modular fashion, where one histone variant seamlessly slots in for another. Locally specific changes, on the other hand, require either variant-specific interactions with factors that confer specificity or that the variants themselves bind preferentially to different DNA sequences. To our knowledge, neither has been demonstrated for any archaeal histone *in vivo*. When expressed in *E. coli*, the binding footprints of HMfA and HMfB from *M. fervidus* are highly correlated, with only minor differences[14]. Similarly, in *T. kodakarensis*, Δ*htkA* and Δ*htkB* strains show similar MNase profiles [40]. If sequence preferences exist, they are likely to be subtle. SELEX experiments comparing the rank order and absolute differences in sequence preferences between paralogs would be informative, as would variant-specific pulldowns to establish topography of binding along the genome *in vivo*. Successful attempts at the latter have yet to be reported. In part, this might be because the lack of tails has made it harder (though likely not impossible) to introduce tags without disrupting native binding properties difficult (K. Sandman, personal communication). Similarly, obtaining antibodies with sufficient specificity to discern between paralogs for experiments such as ChIP-seq has been difficult, but would likely succeed eventually. As the capacity for locally specific incorporation is key to the compartmentalized nature of chromatin in eukaryotes, whether archaeal histones are deposited in a global or local manner remains one of the outstanding questions of archaeal histone biology.

3. Regulation

3.1. Archaeal histone variants exhibit dynamic differences in expression

Although we have a basic understanding of which variant complexes can form in principle, when and why those complexes are formed, how long they persist – and what their physiological relevance is – remains unknown. In the absence of known chaperones or remodellers, it seems reasonable to assume that complex assembly and deposition is more or less random and that specific variants are incorporated into dimers and higher-order complexes indiscriminately. As a result, chromatin properties would be altered globally rather than locally, a key difference to eukaryotes (Box 1). Under this model of chromatin assembly, controlled by mass action, the relative abundance of histone paralogs assumes a key role for chromatin structural change. How different variants are expressed, throughout the growth cycle and in the face of altered environmental conditions, might therefore give clues to their function.

What, then, do we know about differential expression of histone paralogs in archaea? To begin with, different histone paralogs, concurrently expressed in the same cell, often differ substantially in their relative expression level. Examples here include histone paralogs in *Methanococcus voltae* [43], *T. kodakarensis* [44,45], *M. fervidus* [19] and particularly in *M. stadtmanae,* where the abundance of different histone paralogs varies over a 27-fold range [36]. More importantly, these differences are often dynamic. Relative paralog dosage in *M. fervidus* [19], *M. voltae* [43] and *M. stadtmanae* [36], for example, changes at different stages of growth. In *M. fervidus,* HMfA is the most abundant histone in exponential phase, yet HMfB levels reach parity as cells enter stationary phase [19]. As recombinant HMfB compacts DNA more extensively than HMfA, this might suggest that stationary phase cells have more compact chromatin than cells at earlier growth stages [19]. Conversely, combining growth-phase specific expression data with structural modelling, we previously suggested that histone variants that increase in stationary phase in *M. stadtmanae* should generate *less* stable histone-DNA complexes on average than are found during exponential phase [36]. Both these predictions should be taken with a pinch of salt because they consider histones in isolation, rather than as part of a more complex chromatin ecosystem that, in archaea, often involves other highly expressed DNA-binding proteins [10,46]. How histones interact with these proteins, and whether histone variants display differential interactions, is very poorly understood and should be a key area of future research into archaeal chromatin [see [47] for a study breaking important ground in this regard].

3.2. Variants that change expression in response to environmental change

Regarding the role of histone variants in responding to environmental change, our knowledge remains similarly embryonic. The only information we have is that, at least on a few occasions, variants respond differentially to a change in external conditions. Expression of HTkA and HTkB in *T. kodakarensis,* for instance, varies with growth temperature. When shifted from their optimal growth temperature of 85 °C to 65 °C, the relative abundance of both paralogs drops, but more so for HTkA than HTkB (Table 1) [45]. Similarly, two of the six histones of *M. jannaschii* (MJ0168, MJ1258) are upregulated upon heat shock (98 °C vs 88 °C), one more strongly than the other (Table 1) [48]. Upon cold shock (65 °C), the same two histones, plus another (MJ0932), are downregulated [49]. MJ1258 is also more highly expressed following a pressure change from 7.8 atm to 500 atm alongside another, plasmid-encoded histone (MJECL17) [48]. Finally, only one of its six histone paralogs, MJ0932, is significantly upregulated in high H_2 environments and downregulated when *M. jannaschii* is grown alongside *Thermococcus paralvinellae* rather than in isolation [50]. Thus, differential responses of histone variants to a variety of stimuli are clearly evident. The physiological significance of these changes, however, remains unknown.

Meaningful progress here can be made at several levels. Even in species that remain genetically inaccessible, changes to chromatin composition and interaction partners under different conditions can be monitored using cross-linking mass-spectrometry, complemented by MNase-Seq and RNA-seq to monitor size and distribution of histone complexes and how they relate to gene expression. Ultimately, to assess changes in chromatin composition in different conditions, variant-specific labels or antibodies will need to be developed (see Box 1). Also desirable would be wider application of a classic approach to understanding gene function – deleting the gene of interest. In principle, deletion of histones with different structural properties may result in distinct changes to growth and gene expression. Where deletion studies in archaea with more than one histone gene have been carried out, variant-specific effects have been found: Deletion of *hstA* (but not *hstB*) in *M. voltae* [43] and *htkB* (but not *htkA*) in *T. kodakarensis* [51] reduces growth rate. Further, *T. kodakarensis* cells deficient in HTkA can no longer be transformed while deletion of *htkB* causes no such issue [51]. On a systems level, Δ*hstA* and Δ*hstB* strains of *M. voltae* exhibit distinct changes in gene expression [43], as do Δ*htkA* and Δ*htkB* strains in *T. kodakarensis* [51]. No obvious link was found between the positioning of tetrameric complexes and gene expression changes for each mutant, and MNase profiles from each strain were highly similar [40]. It is therefore tempting to speculate that structural properties of the variants are at fault for divergent effects on transcription. However, non-concordant deletion effects might also be caused by dosage effects, as deleting one histone might reduce overall histone titres more so than deleting another, an issue that has received insufficient attention in the current literature. Position effects might also play a role: deletions can disturb gene expression locally, which might have locus- and therefore variant-specific, system-wide knock-on effects. It is interesting to note in this context, that in *T. kodakarensis* expression of HTkA from a plasmid is toxic for wild-type but also Δ*htkA* cells, suggesting that ectopic expression cannot replace genomic expression [51]. Paralog swap experiments, where one histone variant is replaced in its native genomic location by a second variant (and vice versa), would be an interesting approach to try and disentangle these effects.

Table 1
Differential expression of archaeal histone variants in response to environmental change.

Organism	Conditions compared	Change in expression (log2-fold)	Reference
Methanocaldococcus jannaschii	Heat shock (98 °C/88 °C) at low pressure (7.8 atm)	MJ0168 = 1.1MJ1258 = 1.5	[48]
	Heat shock (98 °C/88 °C) at high pressure 500 atm	MJ0168 = 1.1MJ1258 = 1.8	[48]
	1.5 h after pressure shock (500 atm) at 88 °C	MJ1258 = 1.7MJECL17 = 1.3	[48]
	Gas substrate limited (600 ml/200 ml) at 7.8 atm	MJ0168 = 1.3MJ1258 = 1.3	[55]
	Gas substrate limited (600 ml at 500 atm/200 ml at 7.8 atm)	MJ0168 = 1.6MJ1258 = 1.2	[55]
	High H_2 v low H_2	MJ0932 = 1.4	[50]
	Co-culture with *Thermococcus paralvinellae* v monoculture	MJ0932 = −1.2	[50]
	Temperature change (65 °C/85 °C)	MJ0168 = −1.7MJ1258 = −1.5MJ0932 = −1.1	[56]
Thermococcus onnurineus	Nutrient change (modified minimal-CO/YPS)	TON_0185 = 1.6TON_1235 = 0.18	[57]
	Nutrient change (modified minimal-formate/YPS)	TON_0185 = 0.48TON_1235 = 0.56	[57]
Methanocella conradii	Co-culture v monoculture with *Pelotomaculum thermopropionicum*	Mtc_0089 = 0.17Mtc_0839 = −1.1	[58]
Thermococcus kodakarensis	Temperature change (65 °C/85 °C)	HTkA = −1.1HTkB = −0.51	[45]

Finally, a low-hanging fruit: Earlier investigations suggested that promoters of histone genes in several archaea harbour binding sites for heat shock regulators [52]. Pertinently, in species with two paralogs, one binding site was predicted to be stronger than the other [52]. Not all of these histones turned out to exhibit the predicted increase in expression following heat shock *in vivo* [53,54], suggesting that predictions here are not trivial. Nonetheless, with an increasingly diverse set of genomes and transcriptomes at our disposal, and an improved understanding of archaeal promoters and transcription factors, this area is ripe for renewed investigation and might take us closer to understanding the regulation of histone variants at the transcriptional level.

3.3. Post-transcriptional regulation

We know next to nothing about post-transcriptional regulation of histones in archaea, including how such regulation might differ between variants. This includes degradation of histones, about which we know nothing at all, but also an aspect of histone biology that takes up a prominent position in thinking about histone functional diversity in eukaryotes: post-translational modifications (PTMs).

In eukaryotes, histones are dynamically modified, particularly on their N-terminal tails. These PTMs, which include methylation, acetylation, and a myriad of other chemical modifications, can impact nucleosome dynamics and promote or restrict interactions with other proteins. Although some modifications are shared between replicative histones and their variants, others are variant-specific, often because the modified residue is unique to that variant [59]. For example, in response to DNA double strand breaks, H2A.X becomes phosphorylated on serine 139 in the C-terminal SQE/Dϕ motif (where ϕ is a hydrophobic amino acid), which is absent from replication-dependent H2A [60]. This phosphorylation event allows DNA repair through further interactions with a cascade of DNA repair proteins [2].

Almost all archaeal histones lack sizeable tails [8] and with them the major hotspots for modification in eukaryotes. The histone fold itself, however, can also be modified [61]. The finding that a number of archaeal genomes encode homologs of histone-modifying enzymes seemed to strengthen the case for histone PTMs in archaea, especially the discovery that, in some halophilic archaea, a putative histone deacetylase is located in an operon with the doublet histone [62]. Yet initial mass spectrometry analyses found no PTMs in histones from either *M. jannaschii* or *Methanosarcina acetivorans* [63] – what looked like a fire produced no smoke.

A more recent investigation, however, did find evidence for histone PTMs - specifically acetylation - in two members of the Thermococcales: *T. kodakarensis* and *Thermococcus gammatolerans* [64]. Both species encode two histone paralogs, each of which was found to be acetylated on several lysine residues. Interestingly, not all lysines are shared between the two paralogs, providing an opportunity for differential regulation of each variant via PTMs [64]. A few of the acetylated lysines, located towards the end of the core fold (L2; α3, Fig. 3), are found in near-orthologous positions to those in eukaryotes. PTMs of these residues might therefore have similar structural repercussions. Yet other lysines are found in parts of the protein, in particular the α2 helix, where eukaryotic histones rarely harbour lysines (Fig. 3), suggesting that any structural effects will have no eukaryotic equivalent. It is worth noting in this regard that four of the lysines in *T. kodakarensis* HtkB, including one in α2, have been predicted to play a role in hypernucleosome stability [8]. Of those, three showed evidence of acetylation (one is acetylated in both paralogs, the other two alternately acetylated in either HTkB or HTkA) suggesting a potential mechanism to control oligomer stability post-translationally in a variant-specific manner [64].

The discovery of histone PTMs is exciting, but comes with a major caveat: as of now, we lack direct information on modification stoichiometry, i.e. the fraction of histones that are modified. Establishing stoichiometry should be a priority for follow-up work; for those interested in physiological relevance, it matters whether 100% of histones are modified or 0.1%. The current data sketch out tantalizing possibilities for parallels with eukaryotes but fall short of demonstrating physiological significance. If modifications were indeed found to be common, future research should address how exactly those PTMs impact chromatin structure, how their deposition and removal is regulated, and how this process differs from variant to variant.

4. Evolution

The physiological role of histone variants is often hard to unravel experimentally. Many archaea remain genetically inaccessible or have eluded attempts to culture them. In the absence of experimental data, evolution can provide a powerful pointer to whether histone variants carry out distinct functions. From an evolutionary perspective, if sequence differences between paralogs have been maintained over long periods of time, this likely indicates that the paralogs play non-redundant physiological roles. In eukaryotes, H2A.Z from distantly related taxa cluster together on a phylogenetic tree, to the exclusion of other H2A variants, suggesting long-term maintenance of this particular variant [65]. Other variants, such as H2A.X, are polyphyletic, i.e. they have sequence or functional properties that have arisen multiple times independently in evolution [4,5].

We recently used this phylogenetic line of reasoning to search for evidence of long-term maintenance or parallel evolution of distinct histone types in archaea. We found evidence for both. In the Thermococcales, almost all species encode two paralogs, one with properties similar to *T. kodakarensis* HTkA and one more similar to HTkB (Fig. 4) [66]. Tracing the evolutionary history of these paralogs, we inferred that both were present in the last common ancestor of Thermococcales [66], approximately 750Mya [67]. Similarly, we uncovered several instances of long-term paralog maintenance in a distantly related order of archaea, the Methanobacteriales, which emerged around 1.6Gya [67]. Even if absolute divergence times are difficult to estimate and verify, a conservative interpretation of our results still indicates that a number of archaeal histone paralogs have been maintained as distinct entities for hundreds of millions of years [36,66], strongly suggesting that each plays a unique and important role in the cell. To note, these cases of long-term maintenance and initial diversification have happened in parallel to eukaryotes. So far, we have no evidence for shared ancestral paralogy (where histone variants in eukaryotes originated prior to eukaryogenesis and have also survived in extant representatives of some archaeal lineages).

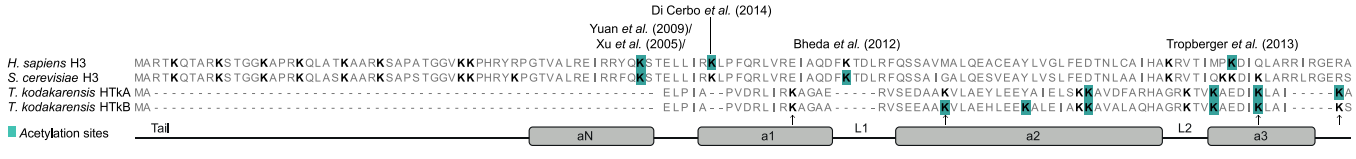

Fig. 3. Post-translational modifications of the core histone fold. Alignment of example eukaryotic histones with the two histones of *T. kodakarensis*. Lysines subject to post-translational modification in the globular domain of eukaryotic histone H3 (based on [61]) and those found acetylated in *T. kodakarensis* HTkA and HTkB [64] are highlighted with a blue shaded box. Lysines at position 56 [71], 64 [72] and 122 [73] have been found acetylated in human H3, as have lysines at positions 56 [74] and 79 [75] in H3 from *Saccharomyces cerevisiae*. The secondary structure for histone H3 is shown at the bottom. Arrows indicate lysines involved in potential stacking interactions for hypernucleosome formation in HTkB according to [8].

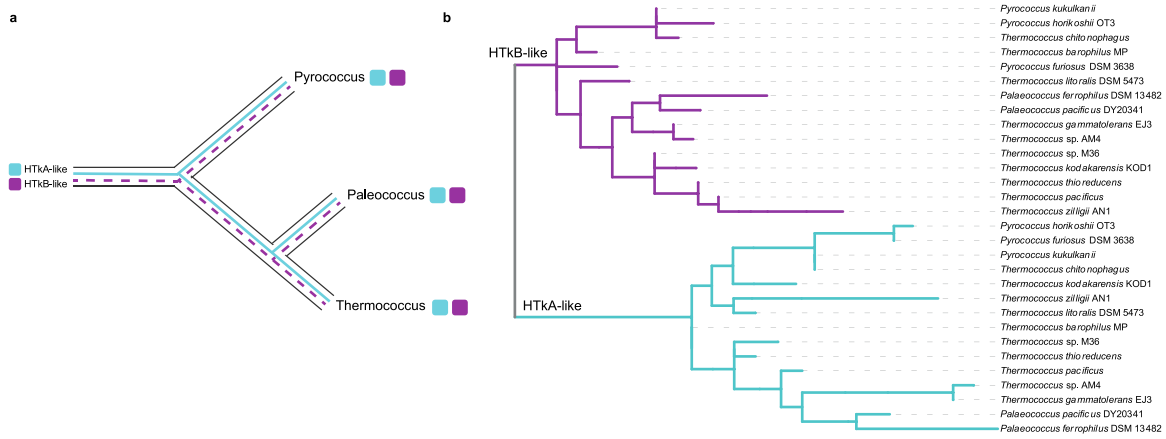

Fig. 4. Long-term maintenance of histone paralogs in the Thermococcales. a. Schematic Thermococcales tree illustrating long-term maintenance of histone paralogs with HTkA- and HTkB-like properties in Pyrococcus, Paleococcus and Thermococcus species, where both paralogs have been retained through multiple speciation events. b. Abridged protein-level tree for HTkA- and HTkB-like histones in the Thermococcales (see [66] for details), illustrating phylogenetic clustering of HTkA- and HTkB-like paralogs, which supports ancestral paralogy.

As highlighted above, we also find evidence for repeated independent emergence of particular histone properties, as histones to which we attribute capstone properties have evolved in several distantly related species. More cases like this will likely be revealed through systematic analysis of different clades of archaea, particularly as new genomes from diverse groups become available. Analysis here is complicated by the fact that archaeal histones are short and therefore typically yield poor phylogenetic signal. Combining evolutionary history with structural and functional data might therefore be critical to pin down conserved histone variants.

5. Conclusion

Our understanding of histone variants in archaea pales in comparison to what decades of diligent work have brought to light in eukaryotes. But this should not be taken as a lament. Instead, it should entice further, faster exploration of a country whose strange fruit are ripe for the picking. Prior work on eukaryotes has given us the tools and strategies to progress this exploration at speed, with a roadmap judiciously adjusted for the unique properties of archaeal histone complexes. Above, we have highlighted several areas where we are missing crucial information: Which other proteins do archaeal histones interact with, and are there interaction partners that are specific to certain variants? How is expression of archaeal histones controlled and how does control differ from one variant to another? Do different variants preferentially bind different sequences, and are differences in affinity large enough to matter *in vivo*. Is histone incorporation into larger oligomers really random? How does the composition of histone complexes change over time and are histones turned over quickly or slowly? What is the significance of post-translational modifications, how are they controlled, and are they widespread across archaea or limited to a few clades? Answers to all of these questions are within our grasp.

Conflict of interest

The authors declare that they are not subject to a conflict of interest.

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