

The chromatin landscape of the euryarchaeon *Haloferax volcanii*

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Abstract

Archaea, together with Bacteria, represent the two main divisions of life on Earth, with many of the defining characteristics of the more complex eukaryotes tracing their origin to evolutionary innovations first made in their archaeal ancestors. One of the most notable such features is nucleosomal chromatin, although archaeal histones and chromatin differ significantly from those of eukaryotes. Despite increased interest in archaeal histones in recent years, the properties of archaeal chromatin have been little studied using genomic tools. Here, we adapt the ATAC-seq assay to archaea and use it to map the accessible landscape of the genome of the euryarchaeote *Haloferax volcanii*. We integrate the resulting datasets with genome-wide maps of active transcription and single-stranded DNA (ssDNA) and find that while *H. volcanii* promoters exist in a preferentially accessible state, modulation of transcriptional activity is not associated with changes in promoter accessibility, unlike the typical situation in eukaryotes. Applying orthogonal single-molecule footprinting methods, we quantify the absolute levels of physical protection of *H. volcanii*, and find that **Haloferax** chromatin is similarly or only slightly more accessible, in aggregate, than that of eukaryotes. We also evaluate the degree of coordination of transcription within archaeal operons and make the unexpected observation that some CRISPR arrays are associated with highly prevalent ssDNA structures. These results provide a foundation for the future functional studies of archaeal chromatin.

Introduction

Life on earth is now understood to be divided into two deep fundamental clades – Archaea and Bacteria. Archaea were only discovered as a separate branch of the tree of life in the 1970s¹, yet it was noticed very early on that they share a number of common features with the more organizationally complex eukaryotes, especially in the organization of their information processing cellular machinery. Based on these similarities it was suggested that eukaryotes evolved from archaea^{2,3}, a view strengthened in the phylogenomic era⁴, and eventually solidified with the discovery of archaeal lineages such as the Lokiarchaeota⁵. Thus, we now know that many of the complex cellular features that characterize

eukaryotes trace their origins to their archaeal ancestry^{6,7}.

One of the most notable such features is nucleosomal chromatin. Nearly all eukaryotic genomes are packaged by nucleosomes, consisting of two tetramers of the four core histones H2A, H2B, H3 and H4, wrapping around ~147 bp of DNA. These proteins are, with very rare exceptions^{8,9}, the most evolutionarily conserved among eukaryotes¹⁰, in large part because aside from their packaging function they are also subject to a large number of precisely regulated posttranslational modifications (PTMs) at key residues¹¹, through which they play a pivotal role in all aspects of chromatin biology (transcription and its regulation, DNA replication, DNA repairs, mitosis, and others).

As early as the 1980s it was noticed that some archaea

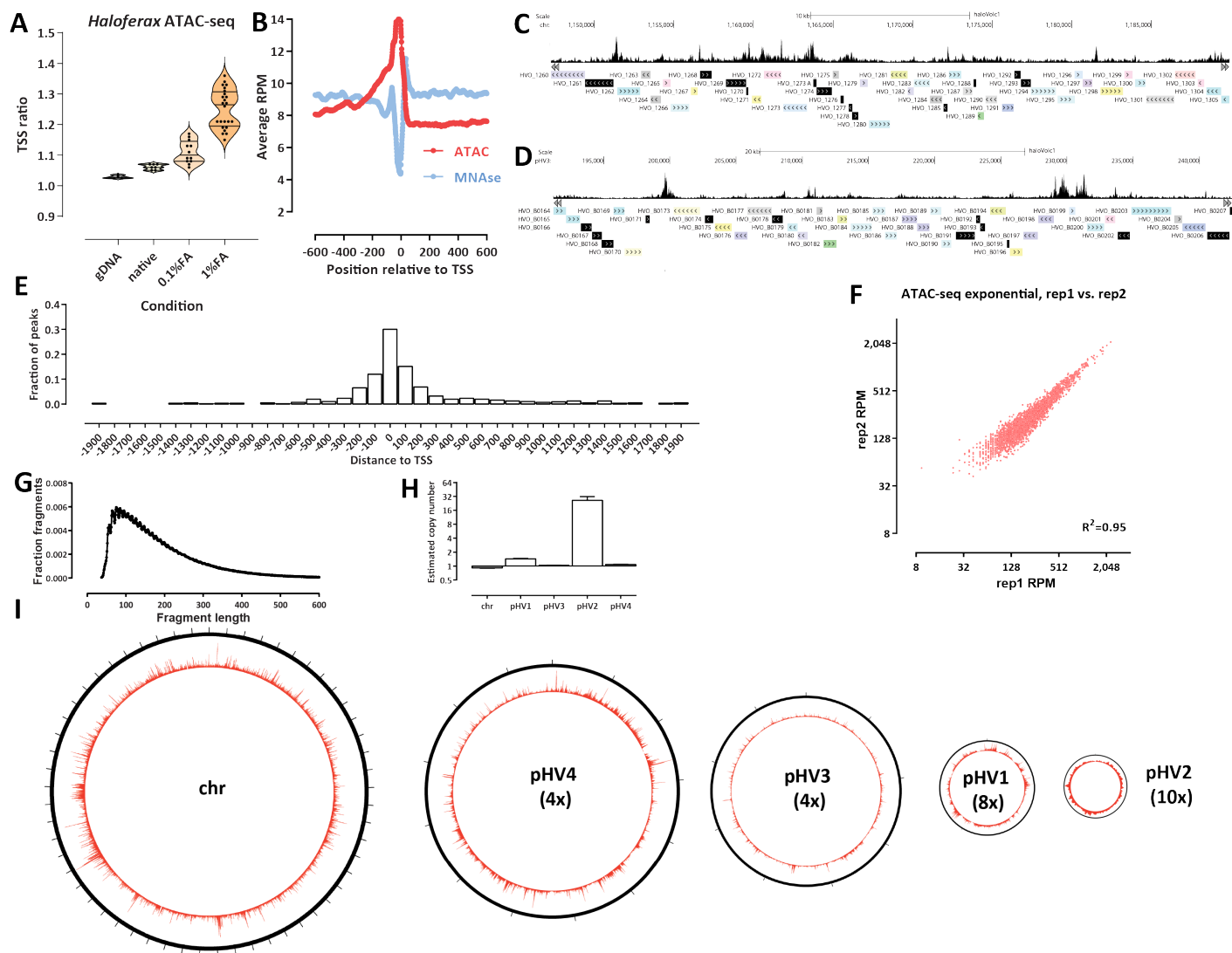


Figure 1: Archaeal ATAC-seq and the open chromatin landscape of *H. volcanii*. (A and B) Adaptation and optimization of the ATAC-seq assay to the archaeal context. (A) Distribution of TSS ratio scores (see Methods for details) for native, 0.1%- and 1%-formaldehyde ATAC-seq libraries. (C and D) Representative browser snapshots of ATAC-seq profiles along the *H. volcanii* genome. (E) Distribution of MACS2 ATAC-seq peaks relative to TSSs. (F) High reproducibility of *H. volcanii* chromatin accessibility measurements using ATAC-seq. Shown is the between-replicate correlation over TSSs in RPM (Read Per Million) units. (G) Fragment length distribution in *H. volcanii* ATAC-seq datasets. (H) Estimated relative copy number of *H. volcanii* chromosomes. Genomic DNA was tagged and amplified ($n = 4$) and normalized read coverage was estimated for each chromosome/plasmid. The average ratios are shown. (I) Global ATAC-seq profile over each of the five *H. volcanii* chromosomes. The number in brackets corresponds to the magnification of the true proportional size of plasmids relative to the main chromosome.

possess proteins and structures similar to eukaryotic histones and nucleosomes^{12,13}. We now know that most archaea have nucleosomal chromatin and histones^{14–16}, and that these histones are ancestral to the eukaryotic histones. This is not true for all archaea as some lineages use other proteins such as Alba/Sac10b, Sul7d, Cren7, and CC1^{17,18} to package their genomes, but the most often observed state is to have histones.

However, archaeal histones differ substantially from those in eukaryotes – while they share the core histone fold domain, they usually do not have the unstructured tails of H2A/H2B/H3/H4 that are the main sites of key PTMs. Archaeal histones also do not form octameric nucleosomes; instead, only one or a very small number of histone genes are found in archaeal genomes, and the structures they form are very different from those of eukaryotes. The diversity

of histone sequences across the whole archaeal phylogeny is very large and still largely unexplored experimentally, but the available structural¹⁹, biochemical and modeling work suggests that in at least some species histones can form so called “hypernucleosomes” or “archaeosomes”, consisting of a protein core of individual histones stacked next to each other, around which DNA is wrapped^{15,20}, ranging from 60 to 500 bp²¹. It has also been proposed that archaeal histones exhibit an inherently dynamic association with DNA, resulting in so called chromatin ‘slinkies’ that can easily slide along DNA²¹, in contrast to the much more stable association of nucleosomes with DNA in eukaryotes.

Despite the relevance of archaeal chromatin to understanding the deep evolution of chromatin organization, up until now the structure of archaeal chromatin has received direct experimental investigation using modern genomic tools, with the exception of early MNase-seq studies nearly a decade ago that mapped nucleosomal positioning in the euryarchaeotes *Haloferax volcanii*²² and *Methanothermobacter thermautotrophicus* and *Thermococcus kodakarensis*²³. Furthermore, very little is known about the relationship between chromatin structure and the regulation of gene expression in these organisms.

The picture has become even more complicated in recent years by the suggestion that in some archaea, in particular haloarchaea, the presence of histone proteins does not necessarily mean that chromatin is packaged by them^{24–29}. This tentative conclusion is based on the low abundance of the HpyA protein in *Haloferax* proteomics datasets^{28,29} and on data suggesting that it is not necessarily involved in genome packaging but has functions analogous to a transcription factor²⁶. Thus the precise nature of chromatin structure in these archaea remains an open question.

To fill these gaps in our understanding of the organization of archaeal chromatin, we mapped chromatin accessibility and active transcription in *Haloferax volcanii* using a combination of bulk and single-molecule techniques such as ATAC-seq³⁰ (Assay for Transposase-Accessible Chromatin using sequencing), NOMe-seq/dSMF³¹ (Nucleosome Occupancy and Methylome sequencing/dual Single-Molecule footprinting) and KAS-seq³² (Kethoxal-assisted single-stranded DNA sequencing). We find that chromatin in *H. volcanii* exhibits similar features to that of eukaryotes on a broad level, with preferentially accessible promoters regions. However, unlike in eukaryotes, chromatin accessibility at promoters does not relate to transcriptional activity. Using single-molecule footprinting we estimate absolute protein occupancy levels over the *H. volcanii* to be comparable to, or possibly slightly lower than those in eukaryotes. However, unlike what is seen in most eukaryotes, we do not observe stably positioned nucleosome protection footprints, but rather only statistically elevated accessibility around promoters. We also revisit the question about the degree of coordination of transcriptional activity and chromatin accessibility within *Haloferax* operons, and make the unexpected discovery that some CRISPR arrays are associated

with very strong ssDNA signatures.

Results

ATAC-seq reveals the open chromatin landscape of *H. volcanii*

To study chromatin accessibility in archaea we adapted the ATAC-seq assay³⁰ to the *Haloferax volcanii* archaeon. *H. volcanii* is a halophile with a strong preference for very high salt concentrations in the growth medium (see the Methods section), which grows optimally at 42 °C^{33,34}, and is a widely used archaeal model system.

The principle behind ATAC-seq is the very strong preference of the Tn5 transposase³⁰ for insertion into accessible DNA as opposed to tagmentation of protected (by nucleosomes, transcription factors, or other proteins) DNA. Tn5 insertion then tags accessible sites with landing sites for PCR primers, allowing for highly efficient amplification of open chromatin regions in the genome.

After extensive testing of a variety of different experimental protocols (fixation conditions and input cell numbers), we arrived at the following modifications of the standard ATAC protocol. First, because archaea are not eukaryotes and do not have a nucleus, we omitted the cell lysis and nuclei isolation step that is a standard feature of eukaryotic ATAC-seq protocols, such as the now standard omniATAC³⁵. Second, and most important, we reasoned that if the previously reported dynamic repositioning of archaeal nucleosomes along DNA occurs in *H. volcanii*, optimal results might be obtained by introducing a crosslinking step into the standard ATAC protocol, which would “freeze” any nucleosomes and other structural proteins in place and not allow transposition into DNA that might change from protected to accessible during the duration of the transposition reaction. Indeed, comparing the TSS (transcription start site) enrichment generated without fixation and with light (0.1% formaldehyde) and strong (1% formaldehyde) fixation showed that strong fixation produces optimal (by TSS enrichment) results (Figure 1A). The optimal input cell number was determined to be $\sim 1 \times 10^6$. We then compared the *H. volcanii* ATAC-seq TSS metaprofile with that from the previously published MNase-seq dataset and observed the expected inverse relationship (Figure 1B). *H. volcanii* TSSs exhibit elevated accessibility in the 0 to -400 bp upstream region, decreasing away from the TSS, and displaying a weak periodicity of ~ 60 -70 bp.

Genome browser visualization of ATAC-seq profiles (Figure 1C-D) revealed an accessibility landscape largely reminiscent of that in eukaryotes with compact genomes such as the budding yeast *Saccharomyces cerevisiae*³⁶, with accessibility concentrated at TSSs. Peak calling using MACS2³⁷ generalized that observation (Figure 1E) – nearly all ATAC-seq peaks are located within 200 bp of an annotated TSS.

ATAC-seq measurements in *H. volcanii* are also highly reproducible between experimental replicates (Figure 1F).

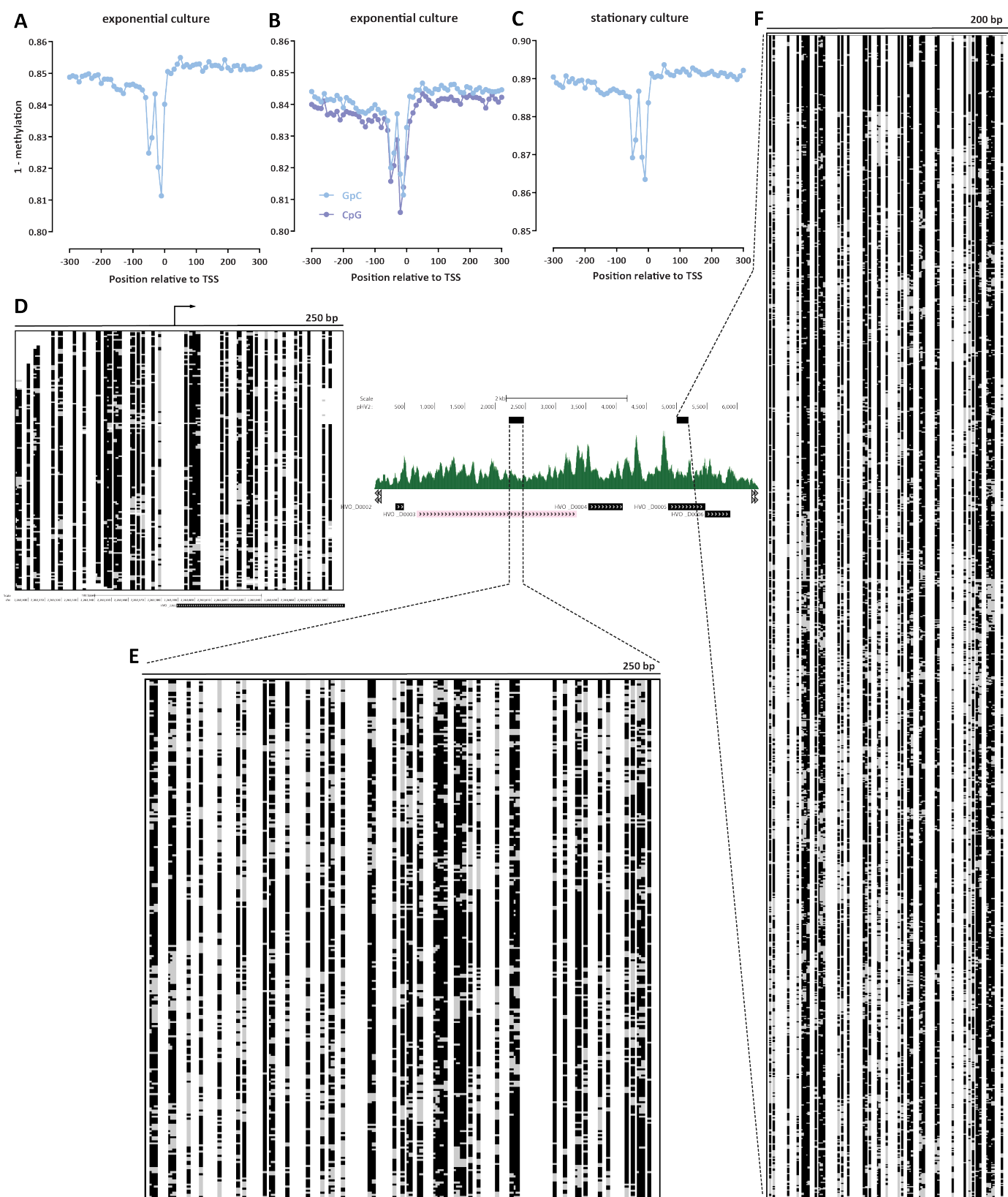


Figure 2: Absolute DNA occupancy/protection levels in *H. volcanii*. (A-C) TSS metaprofiles in different conditions (two replicates of an exponentially dividing culture, and a stationary culture). (D) Single-molecule map (250-bp) around a main-chromosome TSS. Black indicates unmethylated and therefore protected sites, gray indicates methylated and thus accessible sites. (E and F) Single-molecule maps over the pHV2 plasmid: 250 bp-window map (E) and very high coverage ($\geq 1,200$ single molecules) 200 bp-window map (F).

The *H. volcanii* ATAC-seq fragment length distribution is unimodal, peaking at 90-100 bp, and does not show the eukaryotic mono-, di- and tri-nucleosomal signature (Figure 1G).

The *H. volcanii* genome consists of multiple replicons³⁸, with a main chromosome (“chr”) and four plasmids of very different size – pHV4, pHV3, pHV1 and pHV2 (in order of decreasing size), which together comprise ~30% of the total genome. To properly interpret sequencing data (which is typically normalized to total read coverage), we determined the relative copy number distribution of these replicons using tagged naked genomic DNA control samples generated during the optimization of the ATAC-seq protocol (Figure 1H). The smallest plasmid – pHV2 – appears to exist in ~26 copies for each main chromosome, pHV1 is found at ~1.4 copies for each main chromosome, while the two large plasmids – pHV4 and pHV3 – exist in a 1:1 ratio to the main chromosome.

As previous studies of chromatin openness in bacteria have reported the existence of very large domains of lower and higher accessibility³⁹, we wondered whether the same is observed in *Haloferax*. We do not observe such domains in our datasets (Figure 1I). Recently, an ATAC-seq dataset was reported from the crenarchaeote *Sulfolobus islandicus*⁴⁰, which lacks histones, and instead packages its genome mainly through Alba/Sac10b proteins^{41,42}. In that species, large domains similar to those in bacteria were reported, suggesting that such large-scale domains of elevated chromatin accessibility might be a feature associated with the lack of nucleosomal chromatin in prokaryotes, while archaea that contain histone genes, such as *H. volcanii*, exhibit eukaryote-like organization. We also reexamined the *Sulfolobus islandicus* dataset and found that it displays a much more modest TSS enrichment than that seen in *H. volcanii*, which is also more narrowly concentrated around the TSS position (Supplementary Figure 1).

Finally, we observed an anti-correlation between ATAC-seq signal and genomic GC content (Supplementary Figure 2). *Haloferax volcanii* exhibits a rather high average GC content of 65%³⁸, decreasing to 58% in intergenic regions, and areas with even higher GC content (>65%) show markedly lower ATAC-seq signal. This observation is corroborated by the available external MNase-seq dataset, which shows positive correlation with GC content (i.e. the inverse of ATAC-seq, as expected) and naked DNA controls (which show no correlation with GC content, i.e. indicating that PCR biases during sequencing library preparation are not the reason for the observed patterns).

Absolute DNA occupancy/protection levels in *H. volcanii*

While ATAC-seq is immensely helpful for identifying the location of accessible regions in the genomes and measuring their relative accessibility, it is a bulk method that does not provide information about the absolute levels of protection/accessibility in the genome. Instead, absolute ac-

cessibility must be measured either restriction digestion-based or enzymatic labeling single-molecule methods. To quantify absolute occupancy/protection levels in the *H. volcanii* genome, we applied NOMe-seq⁴³ and dSMF³¹ to *Haloferax* chromatin. These methods rely on the preferential methylation of accessible cytosine nucleotides (5mC) by a recombinant methyltransferase that modifies specifically in GpC contexts (NOMe-seq) or a combination of methyltransferases that label both GpC and CpG (dSMF).

However, these methods are potentially confounded by the presence of endogenous methylation in either context. Fortunately, in the case of *H. volcanii* endogenous DNA modifications have been previously studied using PacBio single molecule sequencing, and no CpG and GpC modifications were found. Instead, only two restriction modification system-associated modifications in different contexts, specifically, 4-methylcytosine in a C(m4)TAG context and N6-methyladenine in a GCA(m6)BN6VTGC context were identified⁴⁴.

Figures 2A-C show the metaprofiles of average methylation around *H. volcanii* TSSs for NOMe-seq and dSMF datasets generated from exponentially growing and stationary cultures (post log-phase in the growth curve). We observe baseline absolute protection levels around 84-85% in the exponentially growing cells and ~89% in stationary cells. For comparison, analogous studies in eukaryotes, such as the budding yeast *S. cerevisiae*^{36,45,46}, have shown absolute protection levels around 90% ($\pm 5\%$). Thus, *Haloferax* chromatin exhibits broadly similar, though perhaps somewhat lower levels of protection than what is observed in conventional eukaryotes.

The base-pair resolved nature of these single-molecule methods enabled an observation of a feature not readily apparent in ATAC-seq and MNase-seq datasets – a protection footprint immediately upstream of the TSS. We also observe this feature as a protection footprint in a few percent of single molecules at individual promoters (Figure 2D). At present we are not able to confidently identify its functional association – its width is likely too small for it to be a positioned -1 nucleosome, and we hypothesize it may correspond to one of the complexes involved in the archaeal transcriptional cycle, analogous to how similar protection footprints associated with the RNA polymerase and the preinitiation complex (PIC) in eukaryotes have been observed in dSMF datasets³¹.

On the other hand, unlike this unique protection footprint, we do not observe strongly positioned individual nucleosome-like features along the *Haloferax* genome, such as those seen in conventional eukaryotes (Figure 2D). However, our NOMe-seq and dSMF datasets were sequenced at an effective depth of ~100 \times for fragments of width 200 bp. To determine if higher sequencing depth would clearly reveal such putative positioned nucleosomes (or other proteins), we turned to the pHV2 plasmid, which, as previously discussed, exists in high copy numbers in *H. volcanii* cells. Over the pHV2 plasmid we obtained ~200 \times coverage for

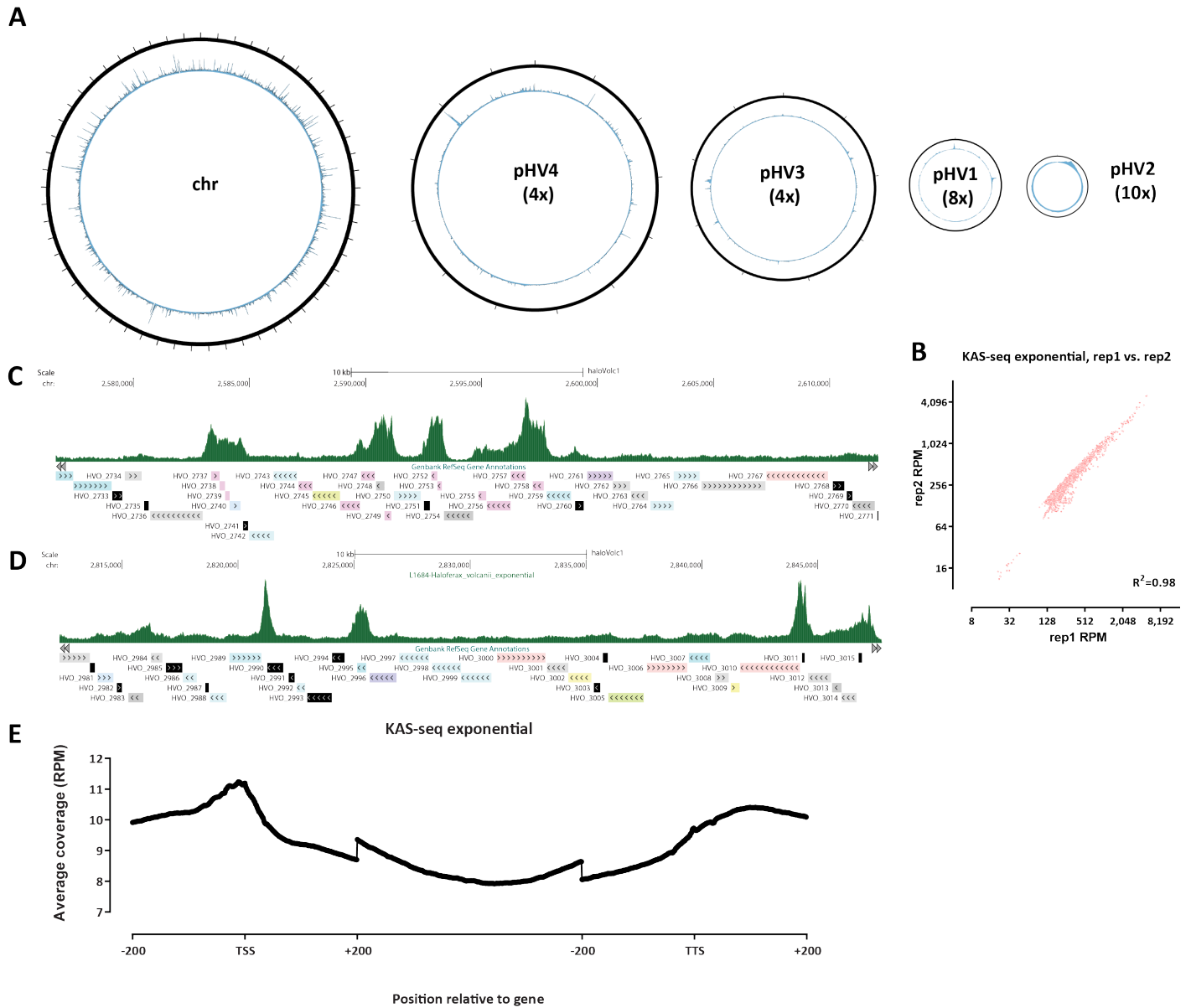


Figure 3: The ssDNA and active transcription landscape in the *H. volcanii* genome as measured by KAS-seq. (A) Global KAS-seq profiles over each of the five *H. volcanii* chromosomes in an exponential culture. (B) High reproducibility of active transcription measurements using KAS-seq. (C-D) Representative browser snapshots of KAS-seq profiles along the *H. volcanii* genome. (E) KAS-seq metaprofile along *H. volcanii* gene bodies.

fragments of length 250 bp (Figure 2E) and $\sim 1,200\times$ coverage for fragments of length 200 bp (Figure 2F). These high-depth maps also reveal considerable heterogeneity of footprints and accessible sites. **These observations are consistent with previous proposals for highly dynamic association of archaeal nucleosomes with DNA, but also with packaging by other, non-histone proteins that is similarly positionally unstable.**

The ssDNA and active transcription landscape in the *H. volcanii* genome

We then turned our attention to the landscape of active transcription in *H. volcanii*. To this end, we utilized the KAS-seq³² assay, which measures with high specificity the presence of single-stranded DNA in the genome, **by utilizing the labeling of unpaired guanine nucleotides with N_3 -kethoxal (which is then tagged with biotin in a click-chemistry reaction followed by very efficient enrichment for labeled DNA fragments via a streptavidin pulldown.** Most ssDNA is usually found within the transcriptional bubbles

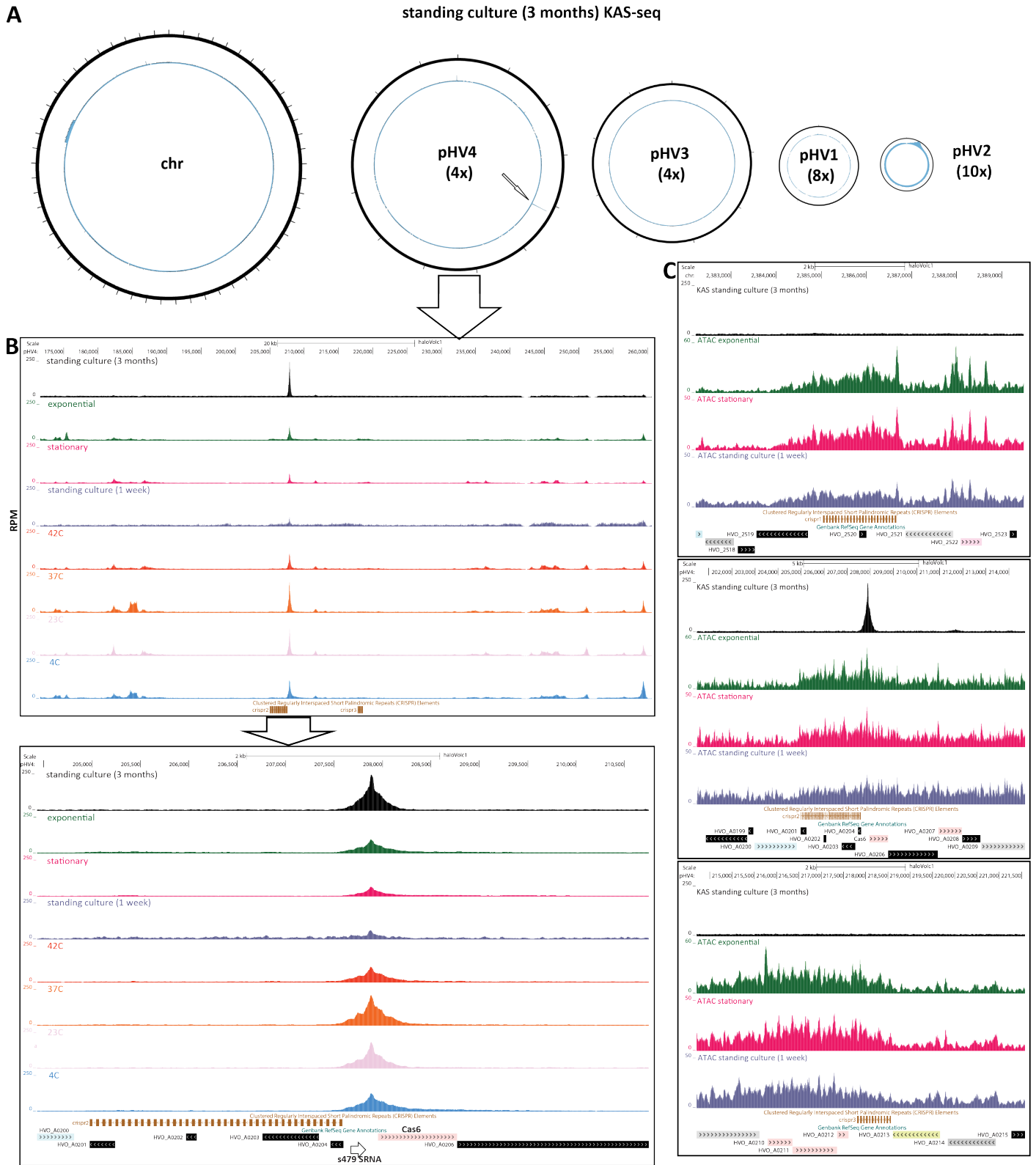


Figure 4: Abundant ssDNA structures associated with some *H. volcanii* CRISPR arrays in specific conditions. (A) Global KAS-seq profiles over each of the five *H. volcanii* chromosomes in a long-standing culture (~3 months) reveals an extremely strong ssDNA peak associated with one of the CRISPR arrays on the pHV4 plasmid. (B) KAS-seq signal levels around pHV4 plasmid CRISPR arrays in different condition. (C) KAS-seq and ATAC-seq levels around all three *H. volcanii* CRISPR arrays in different conditions.

associated with RNA polymerase molecules engaged with DNA. KAS-seq provides several advantages in the *H. volcanii* context. First, due to the absence of straightforward to execute methods for depleting *H. volcanii* ribosomal RNA (rRNA) from RNA sequencing libraries analogous to polyA-selection in eukaryotes^{47,48}, it enables measurement of transcriptional activity at much lower cost than deep RNA-seq experiments. Second, and most important, it measures the act of active transcription, unlike the steady-state transcript levels that conventional RNA-seq quantifies, and is thus the best measurement available of actual transcriptional activity, which is the property most relevant to understanding the relationship between chromatin accessibility and transcription. Third, it also identifies other ssDNA structures, such as those resulting from paused polymerase molecules, G-quadruplexes, and others.

We carried out a time course analysis of *Haloflexax* growth and applied both KAS-seq and ATAC-seq during the “exponential” log-phase of growth, and on the “stationary” post-log phase stage, as well as on “standing” cultures, which had been left at room temperature for ~1 week. We also carried out KAS-seq on exponentially growing cells that were then incubated at different temperatures – the typical growing temperature of 42 °C, 37 °C, 23 °C and a cold shock at 4 °C for 4 hours.

At the broadest global level, we observe uniform levels of KAS signal along the length of *H. volcanii* chromosomes (Figure 3A), with sharp localized peaks. Also, KAS-seq measurements in *H. volcanii* are highly reproducible between experimental replicates (Figure 3B). Locally, at the level of individual genes, we observe a combination of high peaks at the promoters of some genes and elevated KAS-seq signal along gene bodies (Figure 3C-D), which observation is generalized by KAS-seq metaprofiles over all genes (Figure 3E). This indicates that in *H. volcanii* RNA polymerases spend substantial amount of time associated with the TSS, perhaps to the point of full pausing analogous to that observed in metazoans⁴⁹, and also similar to recent observations in the archaeon *Sulfolobus solfataricus*⁵⁰.

Abundant ssDNA structures associated with some *H. volcanii* CRISPR arrays in specific conditions

We also carried out a KAS-seq on a *H. volcanii* culture that had been left standing at room temperature for 3~months, which resulted in a surprising observation about the chromatin structure of CRISPR arrays in this organism. CRISPR (clustered regularly interspaced short palindromic repeats) arrays are a key element in the defense systems against foreign genetic material of many prokaryotes and consist of multiple identical repeats interspersed with non-repetitive sequences that target foreign plasmids and phages, together with a set of Cas genes. *H. volcanii* is one of the prokaryotic systems where these elements were first originally observed^{51–53}.

In standing *Haloflexax* cultures, transcriptional activity is largely suppressed, as the cells enter a dormant state.

This is reflected in the largely flat global map for the long-term standing culture KAS-seq dataset (Figure 4A). However, we also observed a single extremely sharp peak of KAS-seq signal on the pHV4 plasmid. Close examination revealed that this peak resides between the second (as numbered in the available genome annotation) CRISPR array in the *H. volcanii* genome⁵⁴ and its associated Cas6 gene (Figure 4B); a previously reported small RNA (sRNA) – s479⁵⁵ is also located in between Cas6 and the CRISPR array, but the KAS-seq peak is not associated with its putative promoter; it is situated downstream of the array. This KAS-seq signal peak is also found in all other conditions we assessed, but it stands out particularly in the long-term standing culture due to the absence of all other peaks that result from the active transcription of regular genes.

Curiously, only the second CRISPR array in *H. volcanii* displays this strong ssDNA structure, while the other two do not (this is true across all assayed conditions), but all three arrays show elevated chromatin accessibility in ATAC-seq datasets, which is not focused on the beginning of the array but covers its whole length (Figure 4C). The possible interpretations of these observations is treated in detail in the Discussion section.

Coordination between chromatin accessibility and transcriptional activity within *H. volcanii* operons

Being prokaryotes, archaea often have genes organized into operons⁵⁶, with multiple genes transcribed as a single unit, which are therefore expected to share a common promoter and to exhibit similar levels of active transcription. However, transcription of these operons is still little studied using modern genomic tools. To address this gap, we used our KAS-seq and ATAC-seq data, which provides information about the chromatin accessibility and active transcription in different conditions, to investigate the extent of coordination between the transcriptional activity of different units in operons in the *H. volcanii* genome.

Perhaps the most conspicuous *H. volcanii* operon is the one that includes its rRNA genes⁵⁷. There are two copies of this operon in the genome. Figure 5A shows KAS-seq and ATAC-seq profiles along rRNA genes in the different conditions we assayed. We observe a largely uniform KAS-seq profile in exponentially growing cells, and generally elevated chromatin accessibility (which might be associated with very active transcription); a more non-uniform pattern is seen in cold-shocked cells kept at 4 °C, where lower KAS-seq levels are seen over the large subunit (LSU) rRNA relative to the small subunit (SSU), with various intermediate states in other conditions.

More interesting patterns are seen in operons comprised of regular protein coding genes. Figure 5B shows a gene array consisting of A-type ATP synthase subunits, for which distinct KAS-seq peaks are seen at the beginning of the operon as well as in between genes in the middle of the operon. Furthermore, KAS-seq levels are not uniform over the gene bodies of all genes.

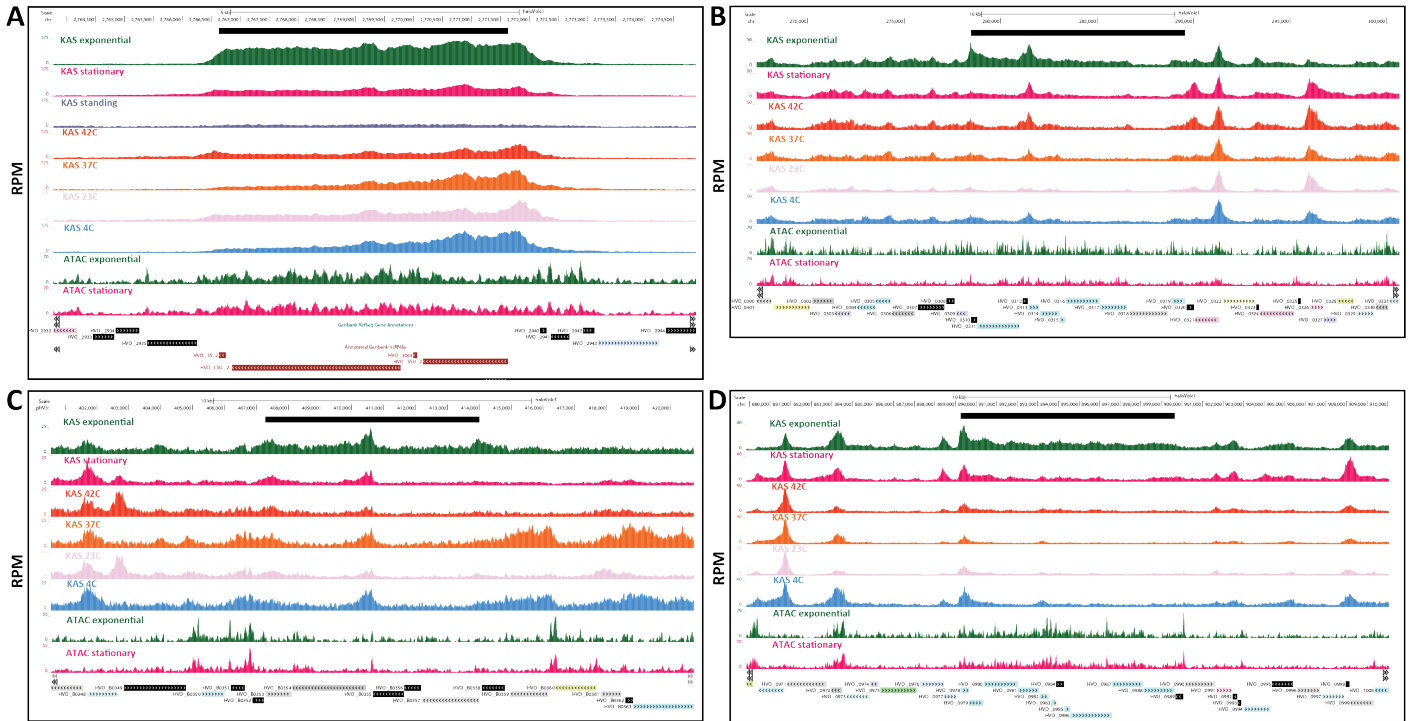


Figure 5: Coordination between chromatin accessibility and transcriptional activity within *H. volcanii* operons. Black bar shows the operon boundaries. (A) Ribosomal RNA operon. Note that the tracks shown here were generated by including multimapping reads (see Methods for details). (B) A-type ATP synthase subunits A, A, B, C, D, E, F, I, K, and H. (C) RNA polymerase II subunits. (D) NADH dehydrogenase-like complex subunits A, B, CD, H, I, J1, J2, K, L, M, N.

We examined multiple other operons (Figure 5C-D) and Supplementary Figure 3, which reveal a very diverse picture of the extent of coordination between the transcriptional activity over individual genes within an operon – internal operon peaks are observed for multiple operons, while there are also other operons where KAS-seq signal is more uniform.

In some cases (e.g. Figure 5C), these internal KAS-seq peaks are also associated with matched ATAC-seq peaks. Thus, one interpretation of these observations is that not all these operons are true operons (even though they consist of functionally related genes), but instead independent initiation and regulation of transcription from internal TSSs may be occurring. This interpretation is particularly supported in the cases where ATAC-seq peaks are seen at the beginning of genes, and where the baseline gene-body KAS-seq signal differs greatly between different sections of the operon, and it also fits with previous observations of internal promoters inside archaeal operons based on transcript 5'-end mapping^{47,58,59}. On the other hand, internal KAS-seq peaks might also arise from very strong and immediate coupling between transcription and translation, i.e. if the process of initiation of translation at internal positions in the operon somehow leads to the polymerase pausing at certain sites.

Chromatin accessibility does not correlate with transcriptional activity in *H. volcanii*

In eukaryotes, the regulation of chromatin accessibility at regulatory elements (promoters and enhancers) is key to gene regulation, as nucleosomal chromatin is generally refractive to occupancy by regulatory proteins and to active transcription⁶⁰, and while perfect correlation between accessibility levels at promoters and gene expression is rare, open chromatin states are generally associated with increased transcriptional activity.

In contrast, the relationship between chromatin accessibility and transcriptional activity in archaea has not been systematically studied as chromatin accessibility has not been mapped globally, across conditions, and in conjunction with global measurements of active transcription.

We first identified differentially accessible promoter regions between the different conditions we studied (Figure 6A). In contrast to an a priori expectation that changes in gene expression would be associated with shifts in chromatin accessibility levels around promoter regions, we did not find strong changes between exponentially growing and stationary cells (Figure 6A). We observed large apparent differences in ATAC-seq signal each of those two conditions and standing cultures (Supplementary Figure 4), but in those comparisons the profiles are highly skewed towards

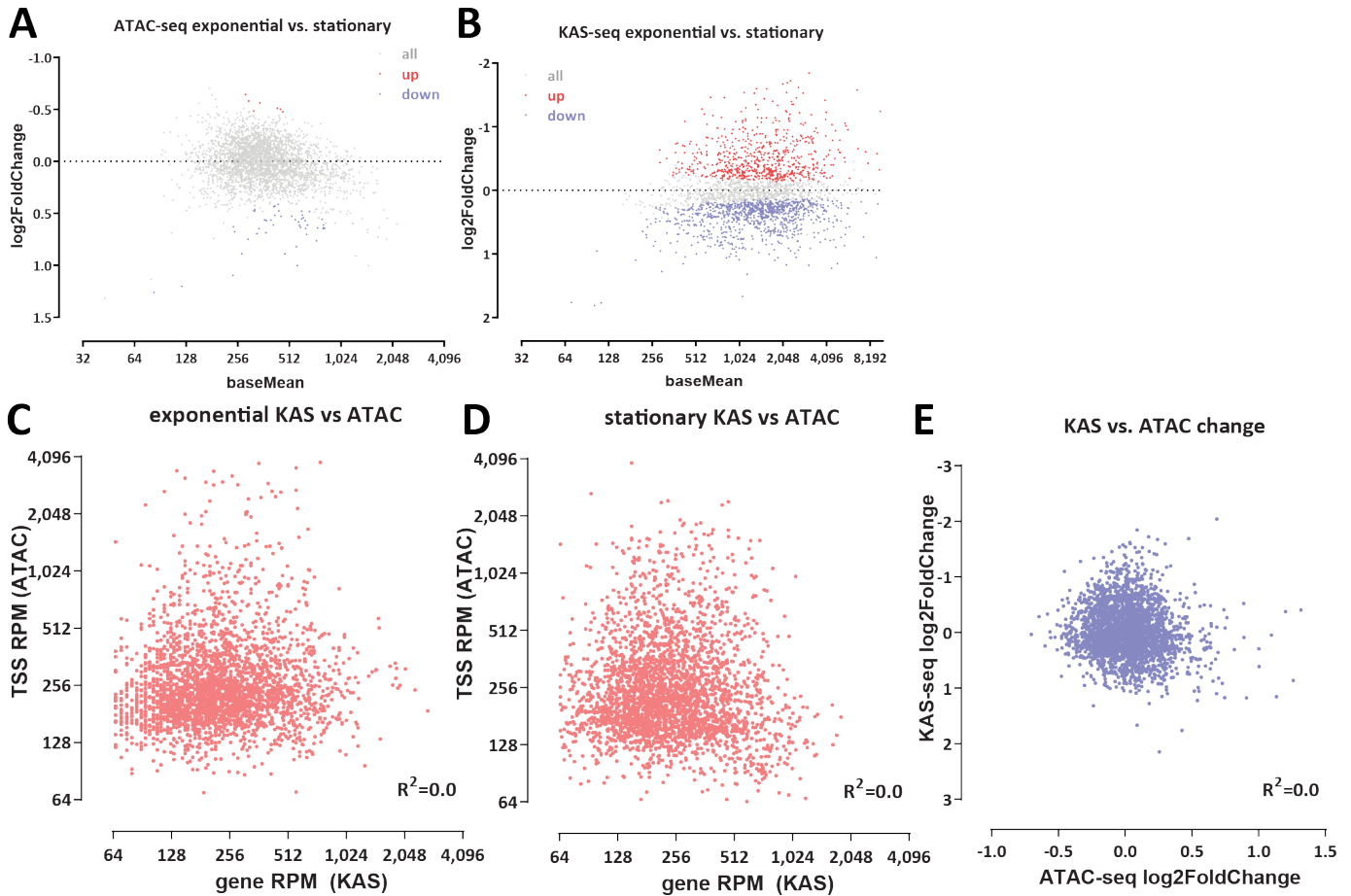


Figure 6: Chromatin accessibility does not correlate with transcriptional activity in *H. volcanii*. (A) Differential chromatin accessibility between exponential and stationary conditions; (B) Differential KAS-seq levels between exponential and stationary conditions; (C-D) Lack of correlation between KAS and ATAC signals in exponential and stationary conditions; (E) Lack of correlation between changes in chromatin accessibility and changes in transcriptional activity.

increased higher accessibility in the actively growing and stationary cells instead of showing the typical more symmetric changes (i.e. in both directions) between two conditions; the explanation for this pattern is that it is due to the dormant state in which standing cultures are in, and in which we do not observe as strong ATAC-seq peaks as in actively growing cells.

In stark contrast to the lack accessibility changes between non-dormant conditions, we find a large number of genes that display strong differential KAS-seq signal over their gene bodies (Figure 6B). The lack of major changes in chromatin accessibility is therefore not due to little changing in the overall cellular state between these conditions, as large-scale changes in RNA polymerase occupancy over the genome are in fact observed.

We then quantified the degree of correlation between KAS and ATAC signals (Figure 6C-D) and found no correlation between the two. We also found no correlation between the level of changes in chromatin accessibility and

changes in RNA polymerase association with DNA between exponential and stationary cells (Figure 6E).

These global observations are supported by the study of individual loci. Figure 7A shows ATAC-seq levels and KAS-seq levels over all genes across different conditions; the lack of correlation between them is readily apparent. Figure 7B and C show individual examples of genes for which transcriptional activity shifts between conditions yet ATAC-seq profiles are largely identical.

We thus conclude that based on the currently available data, the modulation of chromatin accessibility does not appear to be a major determinant/correlate of transcriptional activity in *Haloferox* archaea.

Discussion

In this study, we adapted and applied methods for global profiling of chromatin accessibility and ssDNA in the euryarchaeote *Haloferox volcanii*, revealing the chromatin ar-

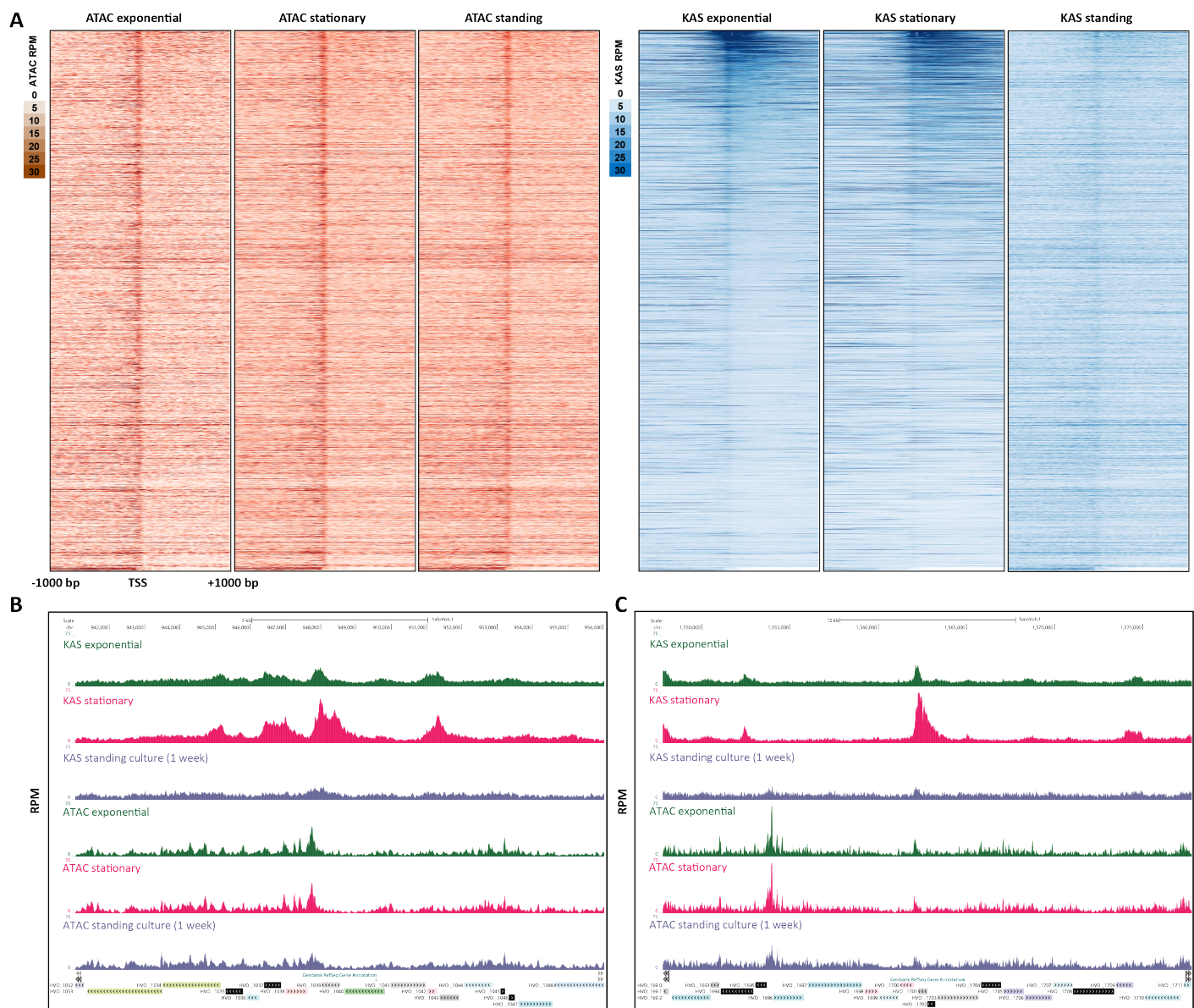


Figure 7: Chromatin accessibility does not correlate with transcriptional activity in *H. volcanii*. (A) Genome-wide heatmaps of ATAC-seq and KAS-seq signals around *H. volcanii* TSSs, sorted by KAS-seq levels in the exponential condition. (B-C) Representative snapshots of genes with significantly altered transcriptional activity between the exponential and standing condition, but no corresponding changes in chromatin accessibility.

chitecture of this representative of the haloarchaea. We identified several convergent and divergent with respect to those of conventional eukaryotes properties.

The *H. volcanii* genome displays a chromatin organization very similar to that of eukaryotes with compact genomes such as budding yeast – accessibility peaks are almost exclusively found very close to promoters. Absolute accessibility/protection levels are similar, perhaps slightly lower than those in budding yeast, with a baseline protection level of 85-90%. On the other hand, we do not observe the strongly positioned nucleosome-like features such as those typical in eukaryotes in *Haloferax*, but a more het-

erogeneous picture, consistent with the previously reported dynamic association of archaeosomes with DNA.

These are quite puzzling observations in the light of recent reports casting doubt on the nucleosomal packaging of haloarchaeal genomes^{24–26}. Once again, the picture of chromatin accessibility revealed by ATAC-seq in *Haloferax* is pretty much identical to that of conventional eukaryotes with nucleosomal chromatin and densely packed genomes. There is very little difference between a *Haloferax* ATAC-seq snapshot and a yeast ATAC-seq snapshot – strong localized peaks at promoters are observed in both – and absolute protection/occupancy levels are also very similar. No pro-

tein other than histones is known to confer such properties, so it is very tempting to conclude that *Haloferax* histones are the ones responsible for the observed accessibility patterns, but that view is discordant with the most recent experimental results on the subject. Experiments involving *in vitro* reconstitution on DNA combined with heterologous expression in yeast of the *Haloferax* histone protein and its other putative chromatin proteins might shed light onto this question in the future.

Unlike what has been reported about bacteria and archaea without histones the *H. volcanii* genome does not exhibit large-scale domains of diminished and elevated accessibility. This property could now tentatively be associated with the absence of such proteins in many prokaryotes.

In contrast to the norm in eukaryotes, accessibility at promoters does not correlate with transcriptional activity. This is a puzzling observation that will need to be functionally dissected in depth by future studies. The idea that chromatin accessibility is not necessary for transcription in archaea is supported by previous observations that transcription by the archaeal RNA Polymerase is slowed, but not blocked by archaeal nucleosomes⁶¹. However, we are still left with the question of what the determinants of the observed accessibility are. Nearly all promoters in *H. volcanii* show some level of accessibility (Figure 7A), but its levels differ greatly between individual genes. How these differential states are specified, and whether they might in fact change in conditions that we have not assayed remains to be determined. MNase-seq studies in *Methanothermobacter thermautotrophicus* and *Thermococcus kodakarensis*²³ resulted in the proposal that nucleosome positioning in those organisms is significantly influenced by DNA sequence, but no such strong association was reported for *Haloferax volcanii*²². In the currently available datasets we observe anti-correlation between chromatinization and high genomic GC content, but whether this is the primary determinant of nucleosomal or other protein occupancy, and whether this correlation can account for the large differences in promoter accessibility combined with a general absence of such strong peaks elsewhere in the genome observed in *Haloferax* remains uncertain. Generating chromatin accessibility and nucleosome positioning data across other archaeal clades and also in multiple closely related species will allow us to generalize these observations and to train fully powered models that relate sequence to chromatin accessibility, potentially identifying such determinants.

We also made the observation that operons in *Haloferax* display non-uniform levels of transcriptional activity, and may in fact consist of multiple distinct transcriptional units. The phenomenon of independent transcription of operon genes has been suggested by some previous studies in *Haloferax* and *Sulfolobus*⁶²⁻⁶⁴, but this is again an observation and a hypothesis that needs to be generalized to and tested in not only more archaeal species, but also in bacteria, where the application of KAS-seq to the study of transcriptional activity may also result in unanticipated

findings. In *Haloferax* we were only able to examine several dozen of unambiguous operons (e.g. unidirectional arrays of functionally related genes).

Bacteria are also highly relevant to the other surprising observation we made – the strong ssDNA structure present at the second *Haloferax* CRISPR array, especially in dormant cells that are otherwise mostly transcriptionally silent, but not at the other two CRISPR arrays. The second CRISPR array is uniquely associated with the Cas6 gene. Cas6 is the endoribonuclease that generates guide RNAs^{54,65}, thus one possible explanation for the strong ssDNA peak between the CRISPR array and Cas6 is that it represents paused RNA polymerase at the Cas6 promoter. If this explanation is correct, the fact that Cas6 is the only gene in the genome with this property in dormant cells is remarkable and perhaps points to the importance of retaining the ability to process CRISPR transcripts even in a dormant cellular state. Alternatively, the ssDNA structure might be related to the transcription of the CRISPR array itself; however, such an explanation does not explain the uniqueness of the KAS-seq signal at the second CRISPR array and the absence of the same strong KAS peaks at the other two CRISPR arrays. The second CRISPR array is also separated from both the Cas6 gene and the KAS-seq peak by the s479 sRNA, the reported function of which is involved in the regulation of zinc transport⁵⁵; how it might fit into the overall picture is not clear. The functional significance of elevated chromatin accessibility over CRISPR arrays is also currently unknown. As prokaryotes exhibit an immense variety of CRISPR systems and number and organization of CRISPR arrays, mapping these properties in multiple other additional prokaryotes should be highly informative.

The foundation provided by our work for the future application of functional genomic methods across different archaeal species and conditions should answer these and many other questions.

Methods

Except where explicitly indicated otherwise, data was processed using custom-written Python scripts (<https://github.com/georgimarinov/GeorgiScripts>)

Haloferax volcanii cell culture

H. volcanii cells were obtained from the DSMZ German Collection of Microorganisms and Cell Cultures GmbH (Cat # 3757), and cultured in *Halobacterium* media^{66,67}, prepared as follows: 7.50 g casamino acids, 10.00 g yeast extract, 3.00 g sodium citrate, 2.00 g KCl, 20.00 g MgSO₄ × 7 H₂O, 0.05 g FeSO₄ × 7 H₂O, 0.20 mg MnSO₄ × H₂O, and 250.00 g NaCl were mixed with distilled water in a total volume of 1 L. Media was then autoclaved and allowed to cool.

H. volcanii was typically grown at 42 °C, except for where otherwise indicated.

Cultures were stored at room temperature when not actively growing.

***Haloferax volcanii* genome assembly and annotations**

For all analyses, the genome assembly and annotation for the *Haloferax volcanii* DS2 strain, downloaded from the NCBI database, and also matching the haloVolc1 version on the UCSC Microbial Genome Browser⁶⁸ (<http://microbes.ucsc.edu/>), was used. The UCSC Microbial Genome Browser was used for visualization of genome browser tracks.

ATAC-seq experiments

Several variations of the ATAC-seq assays were tested. As *H. volcanii* is an archaeon, i.e. a prokaryote without a nucleus, and as it does not have a cell wall (as many other prokaryotes do), the nuclei isolation step typical for ATAC-seq protocols used in eukaryotes was omitted.

For native ATAC-seq, cells (~ 0.1 , ~ 1 or $\sim 10 \times 10^6$ cells as measured by OD₆₀₀) were pelleted at 10,000 *g* for 2 minutes, then resuspended in 50 μ L transposition mix (25 μ L 2 \times TD buffer, 2.5 μ L Tn5, 22.5 μ L ultrapure H₂O), and incubated at 37 °C for 15 minutes. The reaction was stopped by adding 250 μ L PB Buffer (Qiagen, Cat # 28006) and purified using the MinElute PCR Purification Kit (Qiagen, Cat # 28006), eluting in 10 μ L EB buffer. PCR was carried out by mixing the 10 μ L eluate, 10 μ L H₂O, 2.5 μ L i5 primer, 2.5 μ L i7 primer, and 25 μ L NEBNext High-Fidelity 2 \times PCR Master Mix, using the following thermocycler program: 3 minutes at 72 °C, 30 seconds at 98 °C, 10 cycles of: 98 °C for 10 seconds, 63 °C for 30 seconds, 72 °C for 30 seconds. Final libraries were purified using the MinElute PCR Purification Kit.

For crosslinked ATAC-seq, cells were fixed by adding 37% formaldehyde (Sigma) at a final concentration of either 0.1% or 1% and incubating for 15 minutes at room temperature. Formaldehyde was then quenched using 2.5 M glycine at a final concentration of 0.25 M. Cells were subsequently centrifuged at 10,000 *g* for 2 minutes, washed once in 1 \times PBS, and centrifuged again at 10,000 *g* for 2 minutes. Transposition was carried out as above for 15 minutes. The reaction was stopped with the addition of 150 μ L IP Elution Buffer (1% SDS, 0.1 M NaHCO₃) and 2 μ L Proteinase K (Promega, Cat # MC5005), then incubated at 65 °C overnight to reverse crosslinks. DNA was isolated by adding an equal volume of 25:24:1 phenol:chloroform:isoamyl solution, vortexing and centrifuging for 3 minutes at 14,000 rpm, then purifying the top aqueous phase using the MinElute PCR Purification Kit, eluting in 10 μ L EB buffer. Libraries were generated as described above.

ATAC-seq data processing

Demultiplexed FASTQ files were mapped to the *H. volcanii* genome as 2 \times 36mers using Bowtie⁶⁹ (version 1.0.1) with

the following settings: `-v 2 -k 2 -m 1 --best --strata`. Duplicate reads were removed using picard-tools (version 1.99).

TSS scores were calculated as the ratio of ATAC signal in the region ± 100 bp around TSSs versus the ATAC signal of the 100-bp regions centered at the two points ± 2 kbp of the TSS as previously described⁷⁰.

Peak calling was carried out using MACS2³⁷ with the following settings: `-g 4000000 -f BAM --to-large --keep-dup all --nomodel`.

DNA isolation and naked DNA sequencing

Genomic DNA was isolated by centrifuging cells at 10,000 *g* and resuspending the pellet in 200 μ L 1 \times PBS, then using the MagAttract HMW DNA Kit (Qiagen, Cat # 67563), following the manufacturer's instructions.

Genomic DNA libraries were prepared using 5 ng of DNA in a 50- μ L transposition reaction (x μ L DNA, 22.5 - x μ L H₂O, 25 μ L 2 \times TD buffer, 2.5 μ L Tn5). The reaction was carried out for 5 minutes at 55 °C, then stopped with 250 μ L PB buffer. DNA was isolated using the MinElute PCR Purification Kit and amplified as described above for ATAC-seq.

NOME-seq and dSMF experiments

NOME-seq/dSMF experiments were carried out as previously described³⁶, with some modifications. Cells were pelleted at 10,000 *g*, then crosslinked as described for ATAC-seq at 1% formaldehyde concentration.

Fixed cells were resuspended in 100 μ L M.CviPI Reaction Buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 10 mM DTT), then treated with M.CviPI by adding 200 U of M.CviPI (NEB), SAM at 0.6 mM and sucrose at 300 mM, and incubating at 30 °C for 20 minutes. After this incubation, 128 pmol SAM and another 100 U of enzyme were added, and a further incubation at 30 °C for 20 minutes was carried out. For dSMF experiments, M.SssI treatment followed immediately, by adding 60 U of M.SssI (NEB), 128 pmol SAM, MgCl₂ at 10 mM and incubation at 30 °C for 20 minutes. The reaction was stopped by adding an equal volume of Stop Buffer (20 mM Tris-HCl pH 8.5, 600 mM NaCl, 1% SDS, 10 mM EDTA).

Crosslinks were reversed overnight at 65 °C, and DNA was isolated using the MinElute PCR Purification Kit (Qiagen, Cat # 28006).

Enzymatically labeled DNA was then sheared on a Covaris E220, and converted into sequencing libraries following the EM-seq protocol, using the NEBNext Enzymatic Methyl-seq Kit (NEB, Cat # E7120L).

NOME-seq data processing

Adapters were trimmed from reads using Trimmomatic⁷¹ (version 0.36). Trimmed reads were aligned against the *H. volcanii* genome using `bwa-meth` with

default settings. Duplicate reads were removed using `picard-tools` (version 1.99). Methylation calls were extracted using `MethylDackel` (<https://github.com/dpryan79/MethylDackel>). Additional analyses were carried out using custom-written Python scripts (<https://github.com/georgimarinov/GeorgiScripts>).

KAS-seq experiments

KAS-seq experiments were carried out following the previously published protocol³² with some modifications in the sequencing library generation part.

Briefly, a 500-mM N₃-kethoxal solution was brought to 37°C, then added to 2 mL of culture at a final concentration of 5 μ M. Cells were then incubated for 5 minutes at 37°C in a ThermoMixer at 1000 rpm.

Cells were then pelleted by centrifugation at 10,000 *g* for 1 minute, resuspended in 200 μ L 1 \times PBS buffer, and DNA was immediately isolated using the Monarch Genomic DNA Purification Kit (NEB, Cat # T3010S), with the modification that elution was carried out with 50 μ L 25 mM K₃BO₃ solution (pH 7.0).

Biotin was clicked onto kethoxal-modified guanines by mixing 50 μ L DNA, 2.5 μ L 20 mM DBCO-PEG4-biotin (Sigma, Cat # 760749; DMSO solution), 10 μ L 10 \times PBS, and 22.5 μ L 25 mM K₃BO₃ and incubating at 37°C for 90 minutes.

DNA was isolated using AMPure XP beads and eluted in 130 μ L 25 mM K₃BO₃ (pH 7.0), then sheared on a Covaris E220 for 120 seconds down to \sim 150-200 bp.

Libraries were built on beads using the NEBNext Ultra II DNA Library Prep kit (NEB, Cat # E7645L). Biotin pull down was initiated by pipetting 20 μ L Dynabeads MyOne Streptavidin T1 beads (ThermoFisher Scientific, Cat # 65306) into DNA lo-bind tubes. Beads were separated on magnet, resuspended in 200 μ L of 1 \times TWB buffer (Tween Washing Buffer; 5 mM Tris-HCl pH 7.5; 0.5 mM EDTA; 1 M NaCl; 0.05% Tween 20), then separated on magnet again and resuspended in 300 μ L of 2 \times BB (Binding Buffer; 10 mM Tris-HCl pH 7.5, 1 mM EDTA; 2 M NaCl). The DNA (130 μ L) was added together with 170 μ L 0.1 \times TE buffer, and incubated at RT on rotator for \geq 15 minutes. Beads were separated on magnet, resuspended in 200 μ L of 1 \times TWB, and incubated at 55°C in a Thermomixer for 2 minutes with shaking at 1000 rpm. Beads were again separated on magnet and the 200- μ L 55°C TWB wash step was repeated. Beads were separated on magnet and resuspended in 50 μ L 0.1 \times TE.

End repair was carried out by adding 7 μ L NEB End Repair Buffer and 3 μ L NEB End Repair Enzyme, incubating at 20°C for 30 minutes, then at 65°C for 30 minutes.

End repair was followed by adaptor ligation by adding 2.5 μ L NEB Adaptor, 1 μ L NEB Ligation Enhancer and 30 μ L NEB Ligation Mix, incubating at 20°C for 20 minutes, then adding 3 μ L USER Enzyme and incubating at 37°C for 15 minutes. Beads were separated on magnet, resuspended in 200 μ L of 1 \times TWB, then incubated at 55°C in

a Thermomixer for 2 minutes with shaking at 1000 rpm. Subsequently beads were separated on magnet and resuspended in 100 μ L of 0.1 \times TE, separated on magnet again, resuspended in 15 μ L of 0.1 \times TE Buffer, and transferred to PCR tubes.

Beads were then incubated at 98°C for 10 minutes, and libraries were amplified by adding 5 μ L of i5 primer, 5 μ L of i7 primer and 25 μ L of 2 \times Q5 Hot Start Polymerase Mix, using the following PCR program: 30 seconds at 98°C; 15 cycles of 98°C for 10 seconds, 65°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes.

Beads were separated on magnet and the final libraries were purified from the supernatant using 50 μ L AMPure XP beads, eluting in 0.1 \times TE buffer.

KAS-seq data processing

Demultiplexed FASTQ files were mapped to the *H. volcanii* genome as 2 \times 36mers using Bowtie⁶⁹ with the following settings: `-v 2 -k 2 -m 1 --best --strata`. Duplicate reads were removed using `picard-tools` (version 1.99).

Multimapping reads analysis

For the purpose of examining repetitive regions in the genome, such as the rRNA operons, which exist in two identical copies in the genome, and are thus not uniquely mappable, reads were mapped with the `-a` option instead of `-k 2 -m 1`. Normalization was carried out as previously described and discussed⁷².

Differential accessibility/KAS-seq analysis

The analysis of differential chromatin accessibility as measured using ATAC-seq or enriched for KAS-seq signal was carried out using DESeq2⁷³. Read counts were calculated over promoters or gene bodies and used as input into DESeq2.

External sequencing datasets

MNase-seq datasets for *H. volcanii* were downloaded from NCBI accession PRJNA174818²², and processed as described above for ATAC-seq and KAS-seq.

ATAC-seq for *Suflolobus islandicus*⁴⁰ was downloaded through the Short Read Archive (SRA) from BioProject accession 814106.

Data availability

The sequencing datasets generated for and used in this study can be accessed from GEO accession GSE207470.

Author contributions

G.K.M. conceptualized the study, carried out cell culture, performed ATAC-seq, KAS-seq, and NOME-seq/dSMF, an-

alyzed the data and wrote the manuscript, with input from W.J.G. S.T.B. performed ATAC-seq, KAS-seq, and NOME-seq/dSMF experiments. T.W. and C.H. provided key reagents. A.K. and W.J.G. supervised the study.

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Competing interests

W.J.G. is a consultant and equity holder for 10x Genomics, Guardant Health, Quantapore, and Ultima Genomics, and cofounder of Protillion Biosciences, and is named on patents describing ATAC-seq.

A.K. is a consulting Fellow with Illumina, a member of the SAB of OpenTargets (GSK), PatchBio, SerImmune and a scientific co-founder of RavelBio.

References

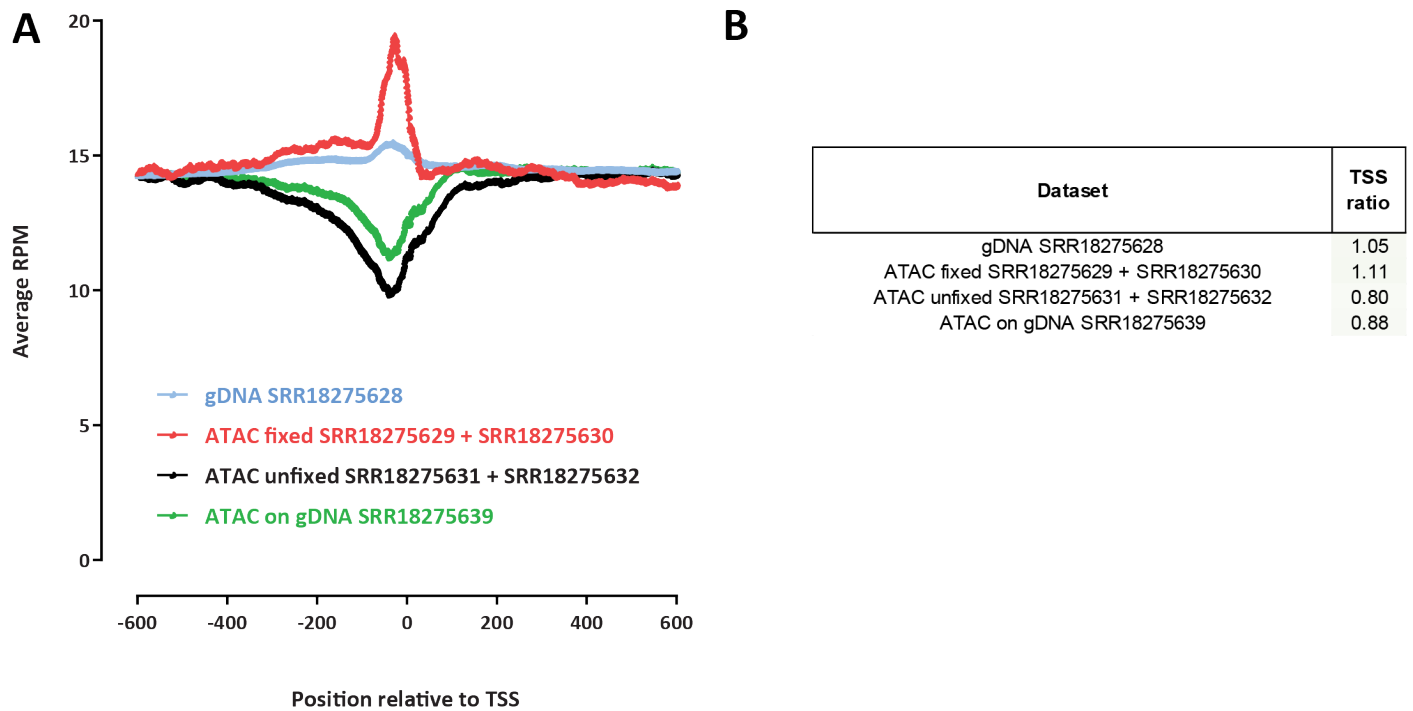
1. Woese CR, Fox GE. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc Natl Acad Sci U S A* **74**(11):5088–5090.
2. Lake JA, Henderson E, Oakes M, Clark MW. 1984. Eocytes: a new ribosome structure indicates a kingdom with a close relationship to eukaryotes. *Proc Natl Acad Sci U S A* **81**(12):3786–3790.
3. Lake JA. 1988. Origin of the eukaryotic nucleus determined by rate-invariant analysis of rRNA sequences. *Nature* **331**(6152):184–186.
4. Cox CJ, Foster PG, Hirt RP, Harris SR, Embley TM. 2008. The archaeobacterial origin of eukaryotes. *Proc Natl Acad Sci U S A* **105**(51):20356–20361.
5. Spang A, Saw JH, Jørgensen SL, Zaremba-Niedzwiedzka K, Martijn J, Lind AE, van Eijk R, Schleper C, Guy L, Ettema TJG. 2015. Complex archaea that bridge the gap between prokaryotes and eukaryotes. *Nature* **521**(7551):173–179.
6. Koonin EV. 2010. The origin and early evolution of eukaryotes in the light of phylogenomics. *Genome Biol* **11**(5):209
7. Koonin EV, Yutin N. 2014. The dispersed archaeal eukaryome and the complex archaeal ancestor of eukaryotes. *Cold Spring Harb Perspect Biol* **6**(4):a016188
8. Marinov GK, Lynch M. 2015. Diversity and Divergence of Dinoflagellate Histone Proteins. *G3 (Bethesda)* **6**(2):397–422.
9. Marinov GK, Lynch M. 2016. Conservation and divergence of the histone code in nucleomorphs. *Biol Direct* **11**(1):18.
10. Postberg J, Forcob S, Chang WJ, Lipps HJ. 2010. The evolutionary history of histone H3 suggests a deep eukaryotic root of chromatin modifying mechanisms. *BMC Evol Biol* **10**:259.
11. Jenuwein T, Allis CD. 2001. Translating the histone code. *Science* **293**(5532):1074–1080.
12. Searcy DG, Delange RJ. 1980. *Thermoplasma acidophilum* histone-like protein. Partial amino acid sequence suggestive of homology to eukaryotic histones. *Biochim Biophys Acta* **609**(1):197–200.
13. Searcy DG, Stein DB. 1980. Nucleoprotein subunit structure in an unusual prokaryotic organism: *Thermoplasma acidophilum*. *Biochim Biophys Acta* **609**(1):180–195.
14. Sandman K, Reeve JN. 2006. Archaeal histones and the origin of the histone fold. *Curr Opin Microbiol* **9**:520–525.
15. Henneman B, van Emmerik C, van Ingen H, Dame RT. 2018. Structure and function of archaeal histones. *PLoS Genet* **14**(9):e1007582.
16. Stevens KM, Swadling JB, Hoher A, Bang C, Gribaldo S, Schmitz RA, Warnecke T. 2020. Histone variants in archaea and the evolution of combinatorial chromatin complexity. *Proc Natl Acad Sci U S A* **117**(52):33384–33395.
17. Zhang Z, Guo L, Huang L. 2012. Archaeal chromatin proteins. *Sci China Life Sci* **55**(5):377–385.
18. Sandman K, Reeve JN. 2005. Archaeal chromatin proteins: different structures but common function? *Curr Opin Microbiol* **8**(6):656–661
19. Mattioli F, Bhattacharyya S, Dyer PN, White AE, Sandman K, Burkhart BW, Byrne KR, Lee T, Ahn NG, Santangelo TJ, Reeve JN, Luger K. 2017. Structure of histone-based chromatin in Archaea. *Science* **357**(6351):609–612.
20. Henneman B, Brouwer TB, Erkelens AM, Kuijntjes GJ, van Emmerik C, van der Valk RA, Timmer M, Kirolos NCS, van Ingen H, van Noort J, Dame RT. Mechanical and structural properties of archaeal hypernucleosomes. *Nucleic Acids Res* **49**(8):4338–4349.
21. Bowerman S, Wereszczynski J, Luger K. 2020. Archaeal chromatin 'slinkies' are inherently dynamic complexes with deflected DNA wrapping pathways. *bioRxiv* 2020.12.08.416859
22. Ammar R, Torti D, Tsui K, Gebbia M, Durbic T, Bader GD, Giaever G, Nislow C. 2012. Chromatin is

- an ancient innovation conserved between Archaea and Eukarya. *Elife* **1**:e00078.
23. Nalabothula N, Xi L, Bhattacharyya S, Widom J, Wang JP, Reeve JN, Santangelo TJ, Fondue-Mittendorf YN. 2013. Archaeal nucleosome positioning in vivo and in vitro is directed by primary sequence motifs. *BMC Genomics* **14**:391.
 24. Dulmage KA, Todor H, Schmid AK. 2015. Growth-Phase-Specific Modulation of Cell Morphology and Gene Expression by an Archaeal Histone Protein. *mBio* **6**(5):e00649–15
 25. Sakrikar S, Hackley RK, Pastor MM, Darnell CL, Vreugdenhil A, Schmid AK. 2022. Comparative analysis of genome-wide protein-DNA interactions across domains of life reveals unique binding patterns for hypersaline archaeal histones. *bioRxiv* 2022.03.22.485428
 26. Sakrikar S, Schmid AK. 2021. An archaeal histone-like protein regulates gene expression in response to salt stress. *Nucleic Acids Res* **49**(22):12732–12743.
 27. Hocher A, Borrel G, Fadhloui K, Brugère JF, Gribaldo S, Warnecke T. 2022. Growth temperature and chromatinization in archaea. *Nat Microbiol* **7**(11):1932–1942.
 28. Knüppel R, Trahan C, Kern M, Wagner A, Grünberger F, Hausner W, Quax TEF, Albers SV, Oeffinger M, Ferreira-Cerca S. 2021. Insights into synthesis and function of KsgA/Dim1-dependent rRNA modifications in archaea. *Nucleic Acids Res* **49**(3):1662–1687
 29. Jevtic Z, Stoll B, Pfeiffer F, Sharma K, Urlaub H, Marchfelder A, Lenz C. 2019. The Response of *Haloferox volcanii* to Salt and Temperature Stress: A Proteome Study by Label-Free Mass Spectrometry. *Proteomics* **19**(20):e1800491
 30. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ. 2013. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* **10**(12):1213–1218.
 31. Krebs AR, Imanci D, Hoerner L, Gaidatzis D, Burger L, Schübeler D. 2017. Genome-wide Single-Molecule Footprinting Reveals High RNA Polymerase II Turnover at Paused Promoters. *Mol Cell* **67**(3):411–422.e4.
 32. Wu T, Lyu R, You Q, He C. 2020. Kethoxal-assisted single-stranded DNA sequencing captures global transcription dynamics and enhancer activity *in situ*. *Nat Methods* **17**(5):515–523.
 33. Mullakhanbhai MF, Larsen H. 1975. *Halobacterium volcanii* spec. nov., a Dead Sea halobacterium with a moderate salt requirement. *Arch Microbiol* **104**(3):207–214.
 34. Pohlschroder M, Schulze S. 2019. *Haloferox volcanii*. *Trends Microbiol* **27**(1):86–87.
 35. Corces MR, Trevino AE, Hamilton EG, Greenside PG, Sinnott-Armstrong NA, Vesuna S, Satpathy AT, Rubin AJ, Montine KS, Wu B, Kathiria A, Cho SW, Mumbach MR, Carter AC, Kasowski M, Orloff LA, Risca VI, Kundaje A, Khavari PA, Montine TJ, Greenleaf WJ, Chang HY. 2017. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. *Nat Methods* **14**(10):959–962.
 36. Shipony Z, Marinov GK, Swaffer MP, Sinnott-Armstrong NA, Skotheim JM, Kundaje A, Greenleaf WJ. 2020. Long-range single-molecule mapping of chromatin accessibility in eukaryotes. *Nat Methods* **17**(3):319–327.
 37. Feng J, Liu T, Qin B, Zhang Y, Liu XS. 2012. Identifying ChIP-seq enrichment using MACS. *Nat Protoc* **7**(9):1728–1740.
 38. Hartman AL, Norais C, Badger JH, Delmas S, Haldenby S, Madupu R, Robinson J, Khouri H, Ren Q, Lowe TM, Maupin-Furlow J, Pohlschroder M, Daniels C, Pfeiffer F, Allers T, Eisen JA. 2010. The complete genome sequence of *Haloferox volcanii* DS2, a model archaeon. *PLoS One* **5**(3):e9605
 39. Melfi MD, Lasker K, Zhou X, Shapiro K. 2021. ATAC-seq reveals megabase-scale domains of a bacterial nucleoid. *bioRxiv* 2021.01.09.426053
 40. Badel C, Samson RY, Bell SD. 2022. Chromosome organization affects genome evolution in *Sulfolobus* archaea. *Nat Microbiol* doi: 10.1038/s41564-022-01127-7
 41. Cajili MKM, Prieto EI. 2022. Interplay between Alba and Cren7 Regulates Chromatin Compaction in *Sulfolobus solfataricus*. *Biomolecules* **12**(4):481.
 42. Bell SD, Botting CH, Wardleworth BN, Jackson SP, White MF. 2002. The interaction of Alba, a conserved archaeal chromatin protein, with Sir2 and its regulation by acetylation. *Science* **296**(5565):148–151.
 43. Kelly TK, Liu Y, Lay FD, Liang G, Berman BP, Jones PA. 2012. Genome-wide mapping of nucleosome positioning and DNA methylation within individual DNA molecules. *Genome Res* **22**(12):2497–2506.
 44. Ouellette M, Jackson L, Chimileski S, Papke RT. 2015. Genome-wide DNA methylation analysis of *Haloferox volcanii* H26 and identification of DNA methyltransferase related PD-(D/E)XK nuclease family protein HVO_A0006. *Front Microbiol* **6**:251
 45. Oberbeckmann E, Wolff M, Krietenstein N, Heron M, Ellins JL, Schmid A, Krebs S, Blum H, Gerland U, Korber P. 2019. Absolute nucleosome occupancy map for the *Saccharomyces cerevisiae* genome. *Genome Res* **29**(12):1996–2009.
 46. Chereji RV, Eriksson PR, Ocampo J, Prajapati HK, Clark DJ. 2019. Accessibility of promoter DNA is not the primary determinant of chromatin-mediated gene regulation. *Genome Res* **29**(12):1985–1995.
 47. Babski J, Haas KA, Näther-Schindler D, Pfeiffer F, Förstner KU, Hammelmann M, Hilker R, Becker A, Sharma CM, Marchfelder A, Soppa J. 2016. Genome-wide identification of transcriptional start sites in the

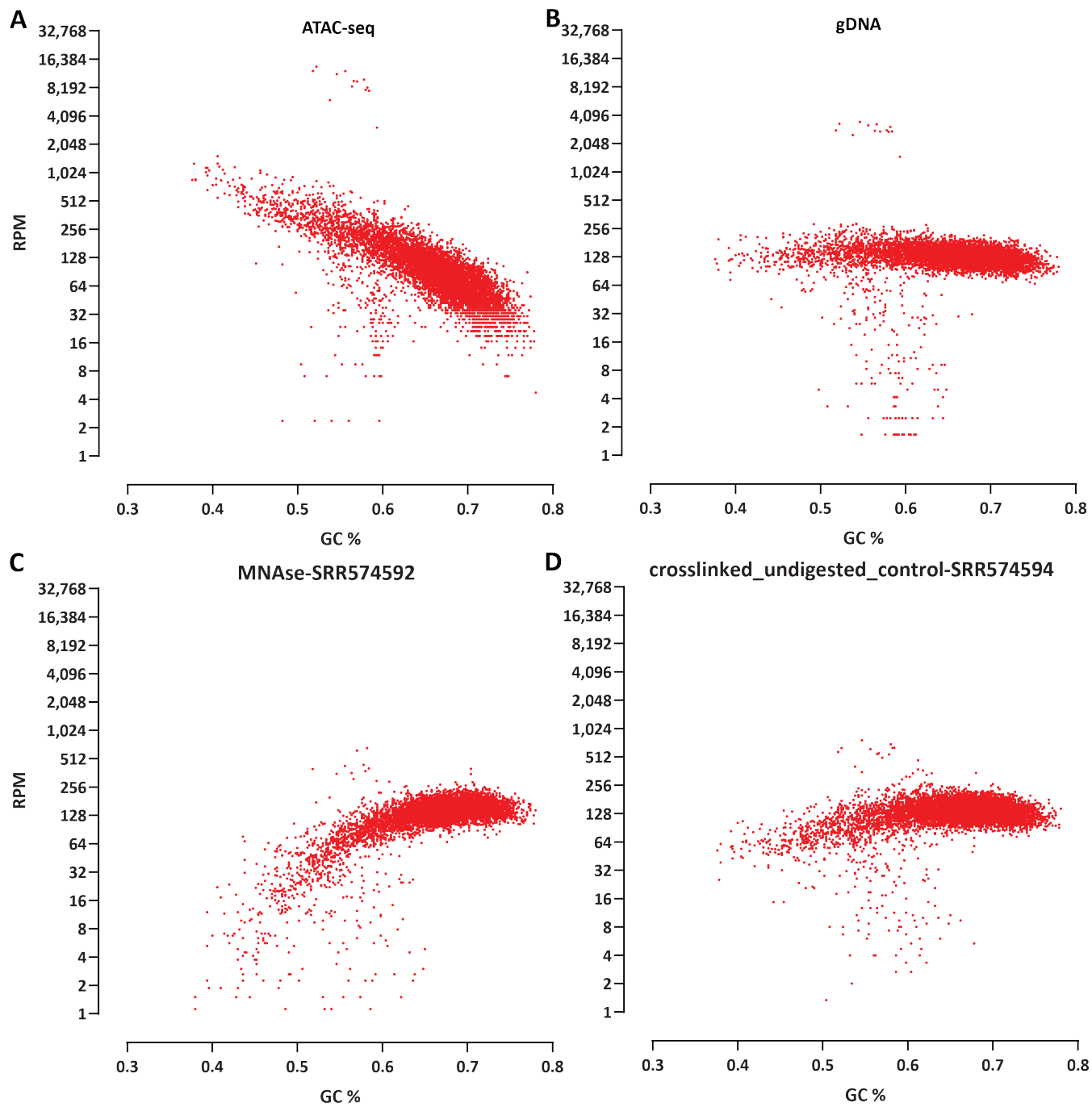
- haloarchaeon *Haloferax volcanii* based on differential RNA-Seq (dRNA-Seq). *BMC Genomics* **17**(1):629
48. Pastor MM, Sakrikar S, Rodriguez DN, Schmid AK. 2022. Comparative Analysis of rRNA Removal Methods for RNA-Seq Differential Expression in Halophilic Archaea. *Biomolecules* **12**(5):682.
 49. Core L, Adelman K. 2019. Promoter-proximal pausing of RNA polymerase II: a nexus of gene regulation. *Genes Dev* **33**(15–16):960–982.
 50. Blombach F, Fouqueau T, Matelska D, Smollett K, Werner F. 2021. Promoter-proximal elongation regulates transcription in archaea. *Nat Commun* **12**(1):5524
 51. Mojica FJ, Rodriguez-Valera F. 2016. The discovery of CRISPR in archaea and bacteria. *FEBS J* **283**(17):3162–3169.
 52. Mojica FJ, Díez-Villaseñor C, García-Martínez J, Soria E. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol* **60**(2):174–182
 53. Mojica FJ, Ferrer C, Juez G, Rodríguez-Valera F. 1995. Long stretches of short tandem repeats are present in the largest replicons of the Archaea *Haloferax mediterranei* and *Haloferax volcanii* and could be involved in replicon partitioning. *Mol Microbiol* **17**(1):85–93.
 54. Maier LK, Stachler AE, Brendel J, Stoll B, Fischer S, Haas KA, Schwarz TS, Alkhnabashi OS, Sharma K, Urlaub H, Backofen R, Gophna U, Marchfelder A. 2019. The nuts and bolts of the Haloferax CRISPR-Cas system I-B. *RNA Biol* **16**(4):469–480.
 55. Märkle P, Maier LK, Maaß S, Hirschfeld C, Bartel J, Becher D, Voß B, Marchfelder A. 2021. A Small RNA Is Linking CRISPR-Cas and Zinc Transport. *Front Mol Biosci* **8**:640440.
 56. Price MN, Arkin AP, Alm EJ. 2006. The life-cycle of operons. *PLoS Genet* **2**(6):e96.
 57. Garrett RA, Dalgaard J, Larsen N, Kjems J, Mankin AS. 1991. Archaeal rRNA operons. *Trends Biochem Sci* **16**(1):22–26.
 58. Koide T, Reiss DJ, Bare JC, Pang WL, Facciotti MT, Schmid AK, Pan M, Marzolf B, Van PT, Lo FY, Pratap A, Deutsch EW, Peterson A, Martin D, Baliga NS. 2009. Prevalence of transcription promoters within archaeal operons and coding sequences. *Mol Syst Biol* **5**:285.
 59. Laass S, Monzon VA, Kliemt J, Hammelmann M, Pfeiffer F, Förstner KU, Soppa J. 2019. Characterization of the transcriptome of *Haloferax volcanii*, grown under four different conditions, with mixed RNA-Seq. *PLoS ONE* **14**(4):e0215986
 60. Klemm SL, Shipony Z, Greenleaf WJ. 2019. Chromatin accessibility and the regulatory epigenome. *Nat Rev Genet* **20**(4):207–222.
 61. Xie Y, Reeve JN. 2004. Transcription by an Archaeal RNA Polymerase Is Slowed but Not Blocked by an Archaeal Nucleosome. *J Bacteriol* **186**:3492–3498.
 62. León-Sobrino C, Kot WP, Garrett RA. 2016. Transcriptome changes in STSV2-infected *Sulfolobus islandicus* REY15A undergoing continuous CRISPR spacer acquisition. *Mol Microbiol* **99**(4):719–728
 63. Maier LK, Benz J, Fischer S, Alstetter M, Jaschinski K, Hilker R, Becker A, Allers T, Soppa J, Marchfelder A. 2015. Deletion of the Sm1 encoding motif in the lsm gene results in distinct changes in the transcriptome and enhanced swarming activity of *Haloferax* cells. *Biochimie*. 2015 Oct;117:129–137
 64. Berkemer SJ, Maier LK, Amman F, Bernhart SH, Wörtz J, Märkle P, Pfeiffer F, Stadler PF, Marchfelder A. 2020. Identification of RNA 3' ends and termination sites in *Haloferax volcanii*. *RNA Biol* **17**(5):663–676.
 65. Carte J, Wang R, Li H, Terns RM, Terns MP. 2008. Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. *Genes Dev* **22**(24):3489–96
 66. Torreblanca M, Rodríguez-Valera F, Juez G, Ventosa A, Kamekura M, Kates M. 1986. Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov. and *Haloferax* gen. nov. *Syst Appl Microbiol* **8**:89–99.
 67. Rodríguez-Valera F, Juez G, Kushner DJ. 1983. *Halobacterium mediterranei* spec. nov., a new carbohydrate-utilizing extreme halophile. *System Appl Microbiol* **4**:369–381.
 68. Chan PP, Holmes AD, Smith AM, Tran D, Lowe TM. 2012. The UCSC Archaeal Genome Browser: 2012 update. *Nucleic Acids Res* **40**(Database issue):D646–52.
 69. Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* **10**(3):R25.
 70. Marinov GK, Shipony Z. 2021. Interrogating the Accessible Chromatin Landscape of Eukaryote Genomes Using ATAC-seq. *Methods Mol Biol* **2243**:183–226.
 71. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**(15):2114–2120.
 72. Marinov GK, Wang J, Handler D, Wold BJ, Weng Z, Hannon GJ, Aravin AA, Zamore PD, Brennecke J, Toth KF. 2015. Pitfalls of mapping high-throughput sequencing data to repetitive sequences: Piwi's genomic targets still not identified. *Dev Cell* **32**(6):765–771.
 73. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**(12):550.

Supplementary Materials

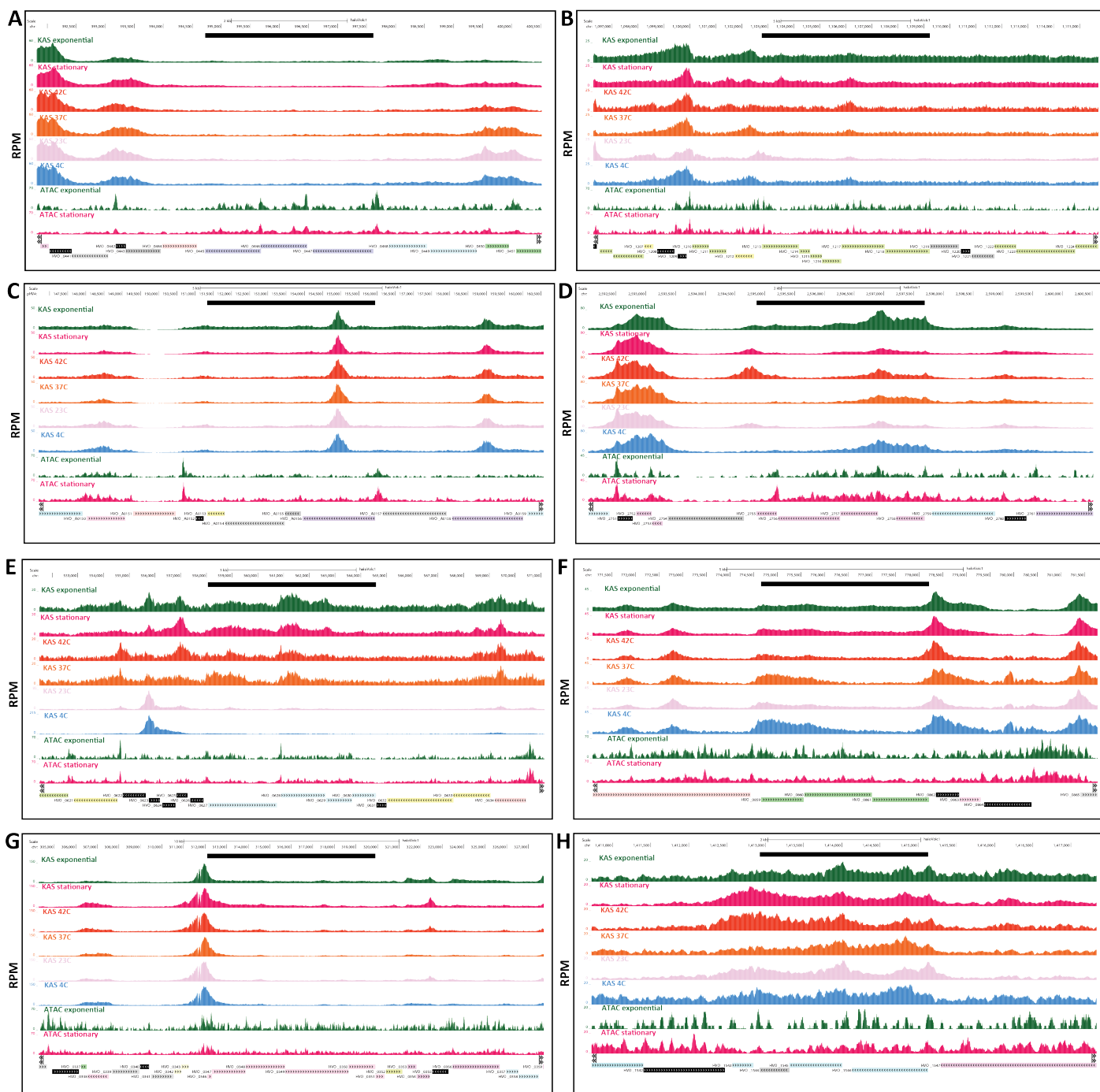
Supplementary Figures



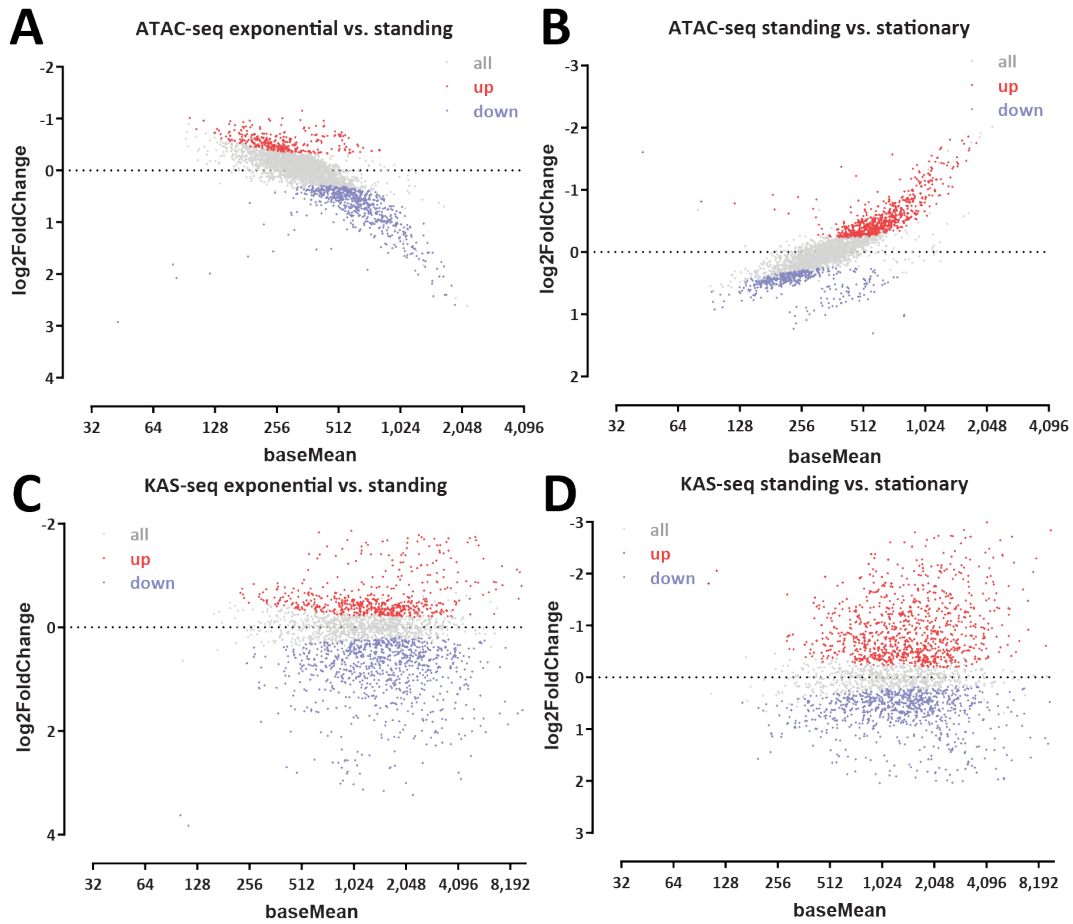
Supplementary Figure 1: TSS enrichment levels in ATAC-seq data for *Suflolobus islandicus*. (A) TSS metaprofiles for ATAC-seq in fixed and unfixed cells and for genomic DNA libraries. (B) TSS scores for each dataset.



Supplementary Figure 2: Correlation between the extent of chromatinization and GC content in *Haloferax*. The genome was split in 500-bp bins and RPM values and GC% were calculated for each bin. (A) ATAC-seq; (B) Tagmented naked genomic DNA control; (C) MNase-seq (external dataset); (D) Crosslinked undigested control (external dataset).



Supplementary Figure 3: Coordination between chromatin accessibility and transcriptional activity within *H. volcanii* operons. Black bar shows the operon boundaries. (A) Putative phosphate-phosphonate ABC transporter. (B) Flagellar cluster (FlaCE, FlaF, FlaG, FlaH, FlaI, FlaJ). (C) Urease accessory protein operon (UreG, UreD, UreE, UreF). (D) 50S ribosomal proteins L12, L10, L1, L11. (E) Putative ABC transporter. (F) FeS assembly genes SufC, SufB, SufD. (G) RNA Polymerase subunits. (H) Dihydroxyacetone kinase subunit L, subunit DhaK, and phosphotransfer subunit.



Supplementary Figure 4: Differential ATAC-seq in KAS-seq analysis for standing *H. volcanii* cells. (A-B) Differential chromatin accessibility; (C-D) Differential KAS-seq levels.