The Epiallelic Nature of Mouse Ribosomal DNA

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Summary

Ribosomal DNA (rDNA) displays substantial inter-individual genetic variation in human and mouse. Here we report that 45S rDNA units in the C57BL/6J mouse strain are epiallelic, existing as distinct genetic haplotypes that influence the epigenetic state and transcriptional output of any given unit. Epigenetic dynamics at these haplotypes are dichotomous and lifestage specific: at one haplotype, the DNA methylation state is sensitive to the *in utero* environment, but refractory to post-weaning influences, whereas other haplotypes entropically gain DNA methylation during ageing only. rDNA epiallelism is influenced by total rDNA copy number, and also found in other inbred mouse strains and humans. In the future, it will be important to consider the impact of inter-individual rDNA (epi)genetic variation on mammalian phenotypes and diseases.

1 Introduction

2 The ribosome is one of the fundamental macromolecular complexes in all living cells, enabling
3 translation to occur in the cytoplasm. The mature mammalian 80S ribosome consists of small (40S)
4 and large (60S) subunits, both comprised of a different complement of proteins and RNA. Despite
5 the essential and highly conserved role played by the ribosome, it is now clear that it can be
6 compositionally diverse even within a given individual organism¹. It has been shown that the protein
7 composition of the ribosome can vary developmentally¹, and that the ribosomal RNA (rRNA)
8 components display inter- and intra-individual genetic variation^{2,3}. Such variation is thought to
9 ultimately influence preferential translation of some mRNAs i.e. the “ribosome filter hypothesis”⁴.

10
11 With respect to rRNA variation in mammals, several studies have reported genetic variation within
12 the 45S rDNA in human and mouse (**Fig. 1A**). The 45S rDNA codes for the 18S rRNA that is
13 incorporated into the 40S subunit, and the 5.8S and 28S rRNAs that are incorporated into the 60S
14 subunit (which additionally contains the 5S rRNA that is coded by the 5S rDNA present at an
15 unlinked genomic location). Because of the multi-copy and multi-allelic nature of 45S rDNA,
16 functional genomic analyses of this source of mammalian genetic variation has been difficult. Indeed,
17 rDNA clusters are typically excluded from genome assemblies, and none of the large-scale genomic
18 analyses of recent years have yielded any insights into rDNA genetic variation. Nonetheless, several
19 smaller studies have noted not only inter-individual genetic variation (in both sequence and copy
20 number) in mouse and human rDNA, but also epigenetic variation in the context of whole organism
21 stress responses^{2,5-8}. The latter observation raises the intriguing possibility that the impact of
22 mammalian rDNA sequence variation is also relevant at the DNA level, thereby impacting
23 transcriptional outputs in different biological contexts. Our aim in this study was to establish a deeper
24 understanding of the functional genomic impact of rDNA genetic variation in a mammalian model.

25 Results

26 **Mouse rDNA exists as distinct genetic haplotypes.** Using the C57BL/6J mouse strain, we focussed
27 on a ~15kb region of the rDNA termed the ‘coding unit’ (**Fig. 1A**, the intergenic sequence, IGS,
28 contains high repeat sequence density and was not analysed in detail). Short-read whole genome
29 sequencing (WGS) analysis of four different C57BL/6J mice identified 88 different coding unit intra-
30 and inter-individual single nucleotide variants (SNVs) (**Supplementary Materials, table S1**,
31 inclusion of indels did not affect SNV allele frequencies and were not explicitly considered further).
32 To achieve a deeper understanding of rDNA genomic architecture, we sequenced a C57BL/6J mouse
33 embryonic fibroblast (MEF) line using ultra-long read whole genome Nanopore technology⁹,
34 obtaining 932,683 reads (N50 ~72 kb), of which 1,760 contained one or more rDNA coding units

within a single read (**Supplementary Materials, table S2**). We confirmed 87/88 short-read rDNA coding unit SNVs in these reads (**table S1**). Previously, we reported a SNV at position -104 (relative to TSS, **Fig. 1A**) in the C57BL/6J rDNA that is associated with differential promoter methylation: 'A' variants at -104 are associated with 30-80% methylation, whereas 'C' variants display <25% methylation at the promoter^{6,7} (**fig. S1**). Using -104 as a starting point to explore the possibility of larger haplotypes within the rDNA, analysis of the SNVs throughout the coding unit revealed 4 different rDNA haplotypes, that we term 'ATA', 'ATG', 'CCA', 'CTA', in approximately equal proportions (**Fig. 1B**). ATA and ATG are more genetically similar to each other compared to either CCA or CTA (**Fig. 1B, Supplementary Materials**). Independent support for these haplotypes was obtained by pairwise correlation analysis of the relevant haplotype-associated SNVs in the four different short-read kidney WGS datasets (**fig. S2, table S3**). Analysis of reads containing two or more complete rDNA units revealed that rDNA haplotypes are found adjacent to each other (**Fig. 1C**). Although further refinement of C57BL/6J rDNA haplotypes may be possible, the analyses below demonstrate that the rDNA haplotypes underlie bona fide molecular differences.

The impact of rDNA haplotypes on functional genomic outcomes. Nanopore sequencing permits direct assessment of DNA methylation in unamplified DNA. Strikingly, we found that the ATA haplotype displays significant DNA methylation ($\geq 60\%$) across the length of the coding unit, CCA shows low methylation levels ($\leq 20\%$), and the other two haplotypes are largely unmethylated (**Fig. 2A**). Analysis of individual reads revealed that individual coding units are either almost completely methylated or unmethylated (**fig. S3**), and haplotype-specific methylation differences do not extend into the adjoining IGS regions, which are generally hypermethylated (**fig. S3**). To provide further support for rDNA haplotype-specific methylation using an orthologous method, we analysed 4 different kidney whole-genome bisulfite sequencing (WGBS) and 7 different sperm reduced representation bisulfite sequencing (RRBS) C57BL/6J datasets (11 different mice) (**table S3**). Short-read methods cannot directly define long-range (epi)genetic patterns, and in our case the major ATA-defining SNV is an 'A' at position 6832. However, if the proposed (epi)genomic architecture of the haplotypes is correct, then if we combine the ATA and ATG haplotypes into a single 'A' haplogroup, that can be distinguished from the CCA and CTA haplotypes at multiple positions, then in short-read data the allele frequency and DNA methylation state at these positions should be predictable based on the combined individual allele frequencies and DNA methylation levels of ATA and ATG at position 6832. This is indeed what we observe (**Fig. 2B, fig. S4**). The WGBS and RRBS datasets also demonstrate that whilst relative allele frequencies and DNA methylation show inter-individual variation, only the ATA rDNA haplotype shows substantial methylation *in vivo*.

70

71 DNA methylation at the mouse rDNA promoter has a strong effect on rRNA expression¹⁰. If
 72 methylation is restricted largely to ATA haplotypes, then methylation observed at the rDNA promoter
 73 of ‘A’ haplogroup should derive from ATA haplotypes only, and correlate with the relative frequency
 74 of ATA in the rRNA. We therefore performed ‘rRNA-seq’, which omits the rRNA depletion step in
 75 the standard mRNA-seq protocol (**Supplementary Materials**). Also, because of the sheer abundance
 76 of cellular rRNA, even internal transcribed spacer (ITS) RNA is readily detected (ITS2 contains the
 77 ATA-defining SNV at 6832). We observed a strong negative correlation between ‘A’ haplogroup
 78 promoter methylation levels and the relative frequency of A’s at position 6832 in muscle rRNA-seq
 79 (**Fig. 2C, table S4**). In fact, methylation even in the vicinity of position 6832 in the rDNA should be
 80 correlated with the variant ratios observed at position 6832 in rRNA-seq, which is what we observe
 81 using 4 different kidney WGBS datasets (**Fig. 2C**). Next, a CUT&Tag analysis of kidney samples
 82 showed that only ATA haplotypes display combined H3K9me3 enrichment/H3K27me3 depletion,
 83 consistent with previous reports of the relationship between DNA methylation and these histone
 84 modifications in other genomic regions¹¹ (**Fig. 2D, fig. S5, table S5**). Finally, it is known that the
 85 Upstream Binding Transcriptional factor (UBTF) binding to unmethylated rDNA is required for
 86 rRNA expression¹². Re-analysis of a previously published dataset of UBTF binding in B-cells of
 87 C57BL/6J mice¹³, showed that ATA is depleted for UBTF relative to the non-ATA haplotypes (**Fig.**
 88 **2E**). Collectively, these analyses demonstrate key functional genomic features of ATA vs non-ATA
 89 rDNA haplotypes in a variety of tissue types.

90

91 **DNA methylation dynamics at rDNA are dichotomous and life-stage specific.** Epigenetic
 92 silencing of rDNA is an integral component of the stress response in all eukaryotic cells. Recent
 93 studies using the C57BL/6J strain have shown that *in utero* exposure to nutritional stress, e.g.
 94 maternal protein restriction, obesogenic or high-fat diet, induces DNA hypermethylation of rDNA
 95 that persists into adulthood^{6,7,14}. We re-analysed our previous RRBS data from C57BL/6J individuals
 96 exposed to maternal protein restriction *in utero*⁶, and found that the hypermethylation occurs
 97 specifically at the ATA variants (**Fig. 3A, fig. S6**). In the GEO database, RRBS data is available for
 98 one other pure C57BL/6J mouse model of *in utero* nutritional stress in which the offspring were
 99 exposed to a high fat diet during gestation+lactation, or during gestation+lactation+post-weaning¹⁵.
 100 In both experimental groups, relative to controls, rDNA hypermethylation is observed at ATA
 101 variants only (**Fig. 3A**). The *in utero* nutritional stress models demonstrate that certain stimuli
 102 methylate and silence ATA rDNA units. But, can ATA units also lose methylation and re-activate?
 103 We re-analysed Dahlet et al.’s RRBS data of C57BL/6J CRISPR-based Dnmt1 knock out 8.5dpc
 104 embryos¹⁶, and found that Dnmt1 KO embryos show virtually no methylation at any of the rDNA

haplotypes (**Fig. 3B left panel, fig. S7**). In standard mRNA-seq protocols, rRNA is specifically removed. However, this depletion is never completely efficient and we find millions of rRNA reads in the Dahlet et al. mRNA-seq data (**Supplementary Materials**). Our own analysis of 22 matched C57BL/6J muscle mRNA-seq and rRNA-seq revealed an excellent correlation of rRNA variant frequencies ($R \geq 0.98$ in all cases, **fig. S8**). We then examined rRNA expression in Dahlet et al.'s mRNA-Seq data and found that ATA variants are expressed at considerably higher relative levels in the Dnmt1 KOs, proving that methylated ATA variants are not irreversibly silenced (**Fig. 3B right panel**).

Interestingly, previous studies show that a low protein diet in C57BL/6J mice post-weaning shows little, if any, rDNA hypermethylation^{7,17}. The study by Cannon et al. also includes a fourth group in which the mice were exposed to a high-fat diet post-weaning only, and in this group evidence for diet-induced epigenetic differences at rDNA is weaker¹⁵ (**fig. S6**). To further explore the idea that the epigenetic state of ATA rDNA shows only limited dynamics in adulthood, we leveraged the RRBS dataset of Petkovich et al., 2016, representing 193 different C57BL/6J mice spanning an age range from 3-35 months¹⁸. Ageing-associated DNA methylation dynamics are observed genome-wide in a large range of mammalian species¹⁹⁻²¹, including at rDNA in humans and rodents²²⁻²⁴. In the Petkovich et al. dataset, ATA showed no directional change with age (**Fig. 3C, fig. S9**). However, methylation at non-ATA haplotypes, and in particular CCA and CTA, display a positive correlation with ageing (**Fig. 3C**). By leveraging single-molecule level data, we were able to show that this was primarily driven by an increase in DNA methylation entropy within individual DNA molecules at non-ATA haplotypes (**Fig. 3D, fig. S10, S11**)²⁶⁻²⁸. Therefore, epigenetic dynamics at rDNA haplotypes are life-stage specific and dichotomous: the ATA haplotype displays environmentally-induced epigenetic dynamics during early development, but is less susceptible to further perturbations in later life. On the other hand, non-ATA haplotypes entropically accumulate methylation during ageing.

rDNA epialleles are present in other mouse strains and human. We then studied 5 additional inbred mouse strains (**Fig. 4A**). For each strain, we generated kidney WGS, WGBS, rRNA-seq, and droplet digital PCR (ddPCR) data from each of 6 different adult males (117 different datasets as 3 were discarded post-QC) (**table S3**). We confirmed that for any given sample, >82% of the rDNA coding unit SNVs called in the WGS data were also found in the WGBS data (**table S6-10, fig. S12**). We then asked which of these SNVs are associated with allelic methylation differences, i.e., are 'epivariants' (Wilcoxon rank sum test, FDR < 0.01), finding both strain-specific and multi-strain epivariants (**Fig. 4A, table S6-10**). Analysis of matched rRNA-seq data showed that epivariant-

associated methylation differences throughout the coding unit impact variant frequencies in the rRNA, with the exceptions being 129S1/SvImJ and C3H/HeJ (**Fig. 4B, fig. S13**). We noted that these two strains also showed the lowest levels of rDNA methylation (**Fig. 4A**). Given the known positive correlation between increasing total rDNA copy number and epigenetic silencing in lower organisms²⁹, we considered the possibility of copy number (CN) effects and calculated CN from three independent datasets - WGS, WGBS and ddPCR - for each individual mouse, as CN measurements in mammals are known to be technically challenging³⁰ (see Methods for CN calculations, **fig. S14**). Indeed, we found a significant positive correlation between total rDNA CN and DNA methylation across the 6 different inbred strains (**Fig. 4C, fig. S14**, note: intra-strain CN variation was considerably smaller and we did not have the sensitivity to examine CN vs methylation relationships within a strain). Finally, we generated WGBS and rRNA-seq data for 24 different human lymphoblastoid cell lines (LCLs) derived from ‘Gambian in Western Division – Mandinka’ individuals that were sequenced in the 1000 genomes project³¹. We first confirmed that for any given sample, >92% of the rDNA coding region SNVs called in the published WGS data were also called in our WGBS data (**table S11**). Even in this relatively small number of samples for an outbred population, we identified an epivariant at FDR <0.01. DNA methylation at this site (located within the 28S), was negatively associated with variant representation in rRNA and positively associated with total rDNA CN (**Fig. 4D**).

Discussion

Our data support the idea that mammalian rDNA is fundamentally epiallelic in nature. The genetic identity of a rDNA unit has a strong probabilistic influence on the homeostatic epigenetic state, and epigenetic dynamics at different life stages. The CN data suggests that the emergence of rDNA epialleles could be linked to the need of the genome to silence extra copies of rDNA. However, the silencing is not absolute, reminiscent of the epigenetic metastability associated with some mammalian retroelements^{32,33}. Could the loss/gain of specific rDNA variants underlie the CN differences? It has been shown that the number of different chromosomes harbouring rDNA clusters can vary from 3-5 amongst different inbred mouse strains³². The C57BL/6J genome contains rDNA clusters on 4 separate chromosomes³³, leading to the possibility that each chromosome harbours a different variant given that we find 4 different rDNA haplotypes in the C57BL/6J strain. Interestingly, the C3H strain has only 3 rDNA clusters³⁴, and shows very few DNA methylation-based rDNA epialleles. But could non-rDNA methylation based epialleles exist? A CUT&Tag analysis for H3K9me3 and H3K27me3 in the 129S1/SvImJ mice did not yield any variant-specific clusters (**fig. S15**). This will require further investigation, as will determining whether the human rDNA epialleles we report also display similar haplotypic structures. In this regard it is worth noting that Parks et al

175 reported that a significant number of rRNA variants are evolutionarily conserved between human and
176 mouse².

177

178 With regards to the molecular consequences of rDNA (epi)genetic variation in models of nutritional
179 stress and ageing, a tantalizing possibility is the production of variant ribosomes that have subtle but
180 measurable effects on translational output¹. It has been shown that endogenous rRNA sequence
181 variation regulates stress response gene expression in bacteria^{35,36}. Previous studies have shown that
182 human and mouse coding subunit rDNA genetic variation is located in rRNA regions with known
183 roles in translation¹. An initial Polysome-seq analysis shows that the ratio of 28S rRNA variants
184 incorporated into ribosomes is dependent on the ratio at which they are expressed (**fig. S16**). In
185 eukaryotes, there are examples of variation in either ribosomal proteins or rRNA modifications
186 influencing preferential translation of some mRNAs¹, yet the contribution of rRNA sequence
187 variation has remained largely unexplored and will require large-scale translome analyses in the
188 future. In summary, our work highlights a source of mammalian genetic variation that is largely
189 overlooked, and it will be interesting to explore the possibility that rDNA genetic variation underlies
190 some apparently ‘epigenetic’ phenomena³⁷ and/or contributes, in trans, to the ‘missing heritability’
191 in some human phenotypes and diseases.

References:

1. Genuth NR, Barna M. The Discovery of Ribosome Heterogeneity and Its Implications for Gene Regulation and Organismal Life. *Mol Cell*. **71**(3):364-374 (2018).
2. Parks MM, Kurylo CM, Dass RA, Bojmar L, Lyden D, Vincent CT, Blanchard SC. Variant ribosomal RNA alleles are conserved and exhibit tissue-specific expression. *Sci Adv*. **4**(2):eaao0665 (2018).
3. Tseng H, Chou W, Wang J, Zhang X, Zhang S, Schultz RM. Mouse ribosomal RNA genes contain multiple differentially regulated variants. *PLoS One*. **3**(3):e1843 (2008).
4. Mauro, VP, Matsuda D. Translation regulation by ribosomes: Increased complexity and expanded scope. *RNA Biol* **13**, 748-755 (2016).
5. Gibbons JG, Branco AT, Yu S, Lemos B. Ribosomal DNA Copy Number Is Coupled with Gene Expression Variation and Mitochondrial Abundance in Humans. *Nat Commun*. **11**(5):4850 (2011).
6. Holland ML, Lowe R, Caton PW, Gemma C, Carbajosa G, Danson AF, Carpenter AA, Loche E, Ozanne SE, Rakyan VK. Early-life nutrition modulates the epigenetic state of specific rDNA genetic variants in mice. *Science*. **353**(6298):495-8 (2016).
7. Danson AF, Marzi SJ, Lowe R, Holland ML, Rakyan VK. Early life diet conditions the molecular response to post-weaning protein restriction in the mouse. *BMC Biol*. **16**(1):51 (2018).
8. F. Shao, X. Liu, X. Zhang, Q. Wang, W. Wang Methylation of 45S Ribosomal DNA (rDNA) Is Associated with Cancer and Aging in Humans. *Int J Genomics* 2021, 8818007 (2021).
9. Jain M, Koren S, Miga KH, Quick J, Rand AC, Sasani TA, Tyson JR, Beggs AD, Dilthey AT, Fiddes IT, Malla S, Marriott H, Nieto T, O'Grady J, Olsen HE, Pedersen BS, Rhie A, Richardson H, Quinlan AR, Snutch TP, Tee L, Paten B, Phillippy AM, Simpson JT, Loman NJ, Loose M. Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nat Biotechnol*. **36**(4):338-45 (2018).
10. Santoro R, Grummt I. Molecular mechanisms mediating methylation-dependent silencing of ribosomal gene transcription. *Mol Cell*. **8**(3):719-25 (2001).
11. Rose NR, Klose RJ. Understanding the relationship between DNA methylation and histone lysine methylation. *Biochim Biophys Acta*. **1839**(12):1362-72 (2014).
12. O'Sullivan AC, Sullivan GJ, McStay B. UBF binding in vivo is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. *Mol Cell Biol*. **22**(2):657-68 (2002).
13. Diesch J, Bywater MJ, Sanij E, Cameron DP, Schierding W, Brajanovski N, Son J, Sornkom J, Hein N, Evers M, Pearson RB, McArthur GA, Ganley ARD, et al. Changes in long-range rDNA-genomic interactions associate with altered RNA polymerase II gene programs during malignant transformation. *Commun Biol*. **2**:39 (2019).

14. Denisenko O, Lucas ES, Sun C, Watkins AJ, Mar D, Bomsztyk K, Fleming TP. Regulation of ribosomal RNA expression across the lifespan is fine-tuned by maternal diet before implantation. *Biochim Biophys Acta*. **1859**(7):906-13 (2016).
15. Cannon MV, Buchner DA, Hester J, Miller H, Sehayek E, Nadeau JH, Serre D. Maternal nutrition induces pervasive gene expression changes but no detectable DNA methylation differences in the liver of adult offspring. *PLoS One*. **9**(3):e90335 (2014).
16. Dahlet T, Argüeso Lleida A, Al Adhami H, Dumas M, Bender A, Ngondo RP, Tanguy M, Vallet J, Auclair G, Bardet AF, Weber M. Genome-wide analysis in the mouse embryo reveals the importance of DNA methylation for transcription integrity. *Nat Commun*. **11**(1):3153. (2020).
17. Shea JM, Serra RW, Carone BR, Shulha HP, Kucukural A, Ziller MJ, Vallaster MP, Gu H, Tapper AR, Gardner PD, Meissner A, Garber M, Rando OJ. Genetic and Epigenetic Variation, but Not Diet, Shape the Sperm Methyome. *Dev Cell*. **35**(6):750-8 (2015).
18. Petkovich DA, Podolskiy DI, Lobanov AV, Lee SG, Miller RA, Gladyshev VN. Using DNA Methylation Profiling to Evaluate Biological Age and Longevity Interventions. *Cell Metab*. **25**(4):954-960.e6. (2017).
19. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol*. **14**(10):R115 (2013).
20. Hannum G, Guinney J, Zhao L, Zhang L, Hughes G, Sadda S, Klotzle B, Bibikova M, Fan JB, Gao Y, Deconde R, Chen M, Rajapakse I, Friend S, Ideker T, Zhang K. Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell*. **49**(2):359-67 (2013).
21. Mammalian Methylation Consortium. www.biorxiv.org/content/10.1101/2021.01.18.426733v1.
22. Swisshelm K, Distech CM, Thorvaldsen J, Nelson A, Salk D. Age-related increase in methylation of ribosomal genes and inactivation of chromosome-specific rRNA gene clusters in mouse. *Mutat Res*. **237**(3-4):131-46 (1990).
23. Oakes CC, Smiraglia DJ, Plass C, Trasler JM, Robaire B. Aging results in hypermethylation of ribosomal DNA in sperm and liver of male rats. *Proc Natl Acad Sci U S A*. **100**(4):1775-80 (2003).
24. D'Aquila P, Montesanto A, Mandalà M, Garasto S, Mari V, Corsonello A, Bellizzi D, Passarino G. Methylation of the ribosomal RNA gene promoter is associated with aging and age-related decline. *Aging Cell*. **16**(5):966-75 (2017).
25. Wang M, Lemos B. Ribosomal DNA harbors an evolutionarily conserved clock of biological aging. *Genome Res*. **29**(3):325-33 (2019).

26. Landan G, Mendelson Cohen N, Mukamel Z, Bar A, Molchadsky A, Brosh R, Horn-Saban S, Amann Zalcenstein D, Goldfinger N, Zundeleovich A et al. Epigenetic polymorphism and the stochastic formation of differentially methylated regions in normal and cancerous tissues. *Nat. Genet.* **44**:1207–1214 (2012).
27. Xie H, Wang M, de Andrade A, Bonaldo MDF, Galat V, Arndt K, Rajaram V, Goldman S, Tomita T, Soares MB. Genome-wide quantitative assessment of variation in DNA methylation patterns. *Nucleic Acids Res.* **39**:4099–4108 (2011).
28. Scherer M, Nebel A, Franke A, Walter J, Lengauer T, Bock C, Müller F, List M. Quantitative comparison of within-sample heterogeneity scores for DNA methylation data. *Nucleic Acids Res.* **48**(8):e46 (2020).
29. Jack CV, Cruz C, Hull RM, Keller MA, Ralser M, Houseley J. Regulation of ribosomal DNA amplification by the TOR pathway. *Proc Natl Acad Sci U S A.* **112**(31):9674-9 (2015).
30. Hall AN, Turner TN, Queitsch C. Thousands of high-quality sequencing samples fail to show meaningful correlation between 5S and 45S ribosomal DNA arrays in humans. *Sci Rep.* **11**(1):449 (2021).
31. 1000 Genomes Project Consortium, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. A global reference for human genetic variation. *Nature* **526**(7571):68-74 (2015).
32. Kazachenka A, Bertozzi TM, Sjöberg-Herrera MK, Walker N, Gardner J, Gunning R, Pahita E, Adams S, Adams D, Ferguson-Smith AC. Identification, Characterization, and Heritability of Murine Metastable Epialleles: Implications for Non-genetic Inheritance. *Cell.* **175**(6):1717 (2018).
33. Dominguez-Salas P, Moore SE, Baker MS, Bergen AW, Cox SE, Dyer RA, Fulford AJ, Guan Y, Laritsky E, Silver MJ, et al. Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. *Nat Commun.* **5**:3746 (2014).
34. Dev VG, Tantravahi R, Miller DA, Miller OJ. Nucleolus Organizers in *Mus Musculus* Subspecies and in the Rag Mouse Cell Line. *Genetics.* **86**(2): 389-98 (1977).
35. Kurylo CM, Parks MM, Juette MF, Zinshteyn B, Altman RB, Thibado JK, Vincent CT, Blanchard SC. Endogenous rRNA Sequence Variation Can Regulate Stress Response Gene Expression and Phenotype. *Cell Rep.* **25**(1):236-248.e6 (2018).
36. Song W, Joo M, Yeom JH, Shin E, Lee M, Choi HK, Hwang J, Kim YI, Seo R, Lee JE, Moore CJ, Kim YH, Eyun SI, Hahn Y, Bae J, Lee K. Divergent rRNAs as regulators of gene expression at the ribosome level. *Nat Microbiol.* 2019 Mar;4(3):515-526.
37. Bughio F, Maggert KA. The peculiar genetics of the ribosomal DNA blurs the boundaries of transgenerational epigenetic inheritance. *Chromosome Res.* **27**(1-2):19-30 (2019).

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Author contributions

Conceptualization: VKR; Methodology: AFD, RAES, HY, PPL, AH, SJM, MMP, MLH, ML, VKR, Software: FRA; Formal Analysis: FRA; Investigation: SY, AFD, RAES, HY, PPL, ZA, PD, NH, VAM, AB, MMP, MLH, ML, VKR; Resources: ML, MLH, VKR; Data Curation: FRA, SJM Writing – original draft: FRA, VKR; Writing – review & editing: FRA, SY, AFD, RAES, HY, PPL, PD, SJM, MMP, AH, MLH, ML, VKR; Visualization: FRA, VKR; Supervision: ML, MLH, VKR; Project administration: VKR; Funding acquisition: MMP, MLH, ML, VKR

Competing interests

Authors declare that they have no competing interests.

Data availability

All data generated for this study is available from BioProject ID: PRJNA733656.

Supplementary

Star Methods

Figs. S1 to S17, Tables S1 to S13

Figure 1. Long range haplotype characterization of rDNA in the C57BL/6J strain. (A) Schematic representation of rDNA in the C57BL/6J mouse strain. Adapted from *Refs 2 and 34*. **(B)** Delineation of rDNA coding unit haplotypes in a C57BL/6J MEF line using ultra-long read Nanopore sequencing. The top track shows representative coverage distribution from C57BL/6J kidney short-read whole genome sequencing data, and locations of the validated SNVs used to derive haplotypes in the Nanopore data. The bottom track shows only those SNVs that distinguish amongst haplotypes. Bars with bold contour denote alleles unique to that haplotype. Bars with non-muted colours and no contour indicate positions associated with the A/C haplogroups defined by their allele at position -104. Bold text in the x-axis indicates coding subunit SNVs (18S, 5.8S, or 28S). In the three letter names given to each haplotype, the first and third characters refer to the alleles at -104 and its top-linked coding subunit SNV at 12736 (Fisher exact test, FDR-adjusted p-value < 1e-46), whereas the middle character refers to the allele at the 8063 position that maximally splits the variation of -104 “C” reads into two largely consistent haplotypes (**fig. S2**, Supplementary Materials). It should be noted that, although these three positions combined distinguish the haplotypes, each individual position is not necessarily haplotype-specific. **(C)** Co-localization analysis of rDNA haplotypes in MEF. For Nanopore reads spanning multiple rDNA units, each cell shows the average proportion of units assigned to the corresponding column haplotype in a read, given that the read includes at least one unit of the haplotype indicated in its row.

Figure 2. The functional genomic outcomes of C57BL/6J rDNA haplotypes. (A) Direct CpG methylation profiles of rDNA haplotypes from ultra-long read Nanopore MEF data, from -1000 bp upstream of the TSS up to the end of the 3' ETS. **(B)** Example of rDNA haplotype-specific methylation analysis on C57BL/6J kidney whole genome bisulfite sequencing (WGBS) data (left panel) showing positional information for the CpG sites associated with each haplotype in a single mouse, and aggregate sperm Reduced Representation Bisulfite Sequencing (RRBS) data for seven mice (right panel). See also **fig. S4**. **(C)** Relationship between methylation levels and rRNA-Seq expression of the ATA rDNA haplotype in C57BL/6J muscle from 22 mice with methylation obtained from Bis-PCR in the rDNA promoter (squares, $p = 7.6 \times 10^{-6}$), and kidney from 4 mice (circles) with methylation levels obtained from ATA-associated WGBS reads. **(D)** The ATA haplotype is enriched for H3K9me3 but depleted for H3K27me3 on CUT&Tag C57BL/6J kidney data (biological replicate - **fig S5**). **(E)** The ATA haplotype is depleted for UBTF relative to other haplotypes in ChIP-Seq data from wild-type C57BL/6J B cells from Diesch et al., 2019¹³.

Figure 3. Epigenetic dynamics at C57BL/6J rDNA haplotypes are life stage specific. (A) Hypermethylation of the ATA haplotype (blue) is observed in different models of *in utero* stress

paradigms; the other three haplotypes (grey) are combined into a single category since they show similar effects. Methylation levels for ATA and non-ATA haplotypes for all panels are obtained on the CpG sites associated with the SNVs at 6007 and 6832. PR, LF, and HF stand for Protein-restricted, Low-fat and High-fat diet, respectively. **(B)** In Dnmt1 KO 8.5dpc embryos from Dahlet et al.¹⁴, the ATA haplotypes lose methylation in RRBS data (left panel) and display de-repression of rRNA (right panel). Both ATA and ATG expression are obtained from ITS2 variants to avoid differential rRNA depletion effects. **(C)** In RRBS data from Petkovich et al. (14), ageing-associated DNA hypermethylation is observed only at non-ATA haplotypes. The red line indicates the expected correlation coefficient for ATA haplotypes if they lost methylation at the same rate as the non-ATA haplotypes gain methylation. In this study, in the 3 month old mice the average methylation at ATA was ~75% and at non-ATA haplotypes was ~25%. **(D)** Only non-ATA haplotypes display increasingly disordered DNA methylation profiles with age (bottom track) in RRBS data from Petkovich et al. Disorder is estimated as the average of per-read Shannon entropy, exemplified here on two hypothetical mice (top track).

Figure 4. The epiallelic nature of rDNA is a feature of other mouse inbred strains and humans.

(A) Identification of epivariants in kidney of 5 inbred strains besides C57BL/6J ('BL6J'): 129S1/SvImJ ('129S'), C3H/HeJ ('C3H'), C57BL/6N ('BL6N'), CAST/EiJ ('CAST') and A/J ('AJ'). For each strain, displayed positions show statistically significant allele-specific methylation differences (FDR-adjusted p-value < 0.01) for over half of the mice. **(B)** Comparison of alternative allele frequencies (AAF) at the epivariant positions between rDNA (WGS) and rRNA for one representative mouse of each strain. Taking into account variant-specific methylation levels ('Adjusted' values, circle), assuming only unmethylated units are expressed, improves correlation coefficients in 4/6 strains. **(C)** Association between total copy number from ddPCR and methylation level from WGBS of the different strains. Correlation coefficients displayed use all mice as data points (black) or a single average point per strain (grey). The analysis was repeated using the highly conserved 18S subunit only to exclude any possibility that the results are due to strain-specific differences in mapping efficiency (**fig. S13**). **(D)** Analysis of male human Mandinka samples shows allele-specific methylation levels (left panel) and increased correlation between DNA (WGBS) and rRNA allele frequencies when accounting for methylation (middle panel) at position 7980 of the KY962518.1 rDNA reference. Average methylation levels around 7980 also appear correlated with rDNA copy number estimated from WGS (right panel).

Figure 1A

Figure 1B

Figure 1C

Figure 2A

Figure 2B

Figure 2C

Figure 2D

Figure 2E

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 4A

Figure 4B

Figure 4C

Figure 4D