

A human pan-genomic analysis reconfigures the genetic and epigenetic make up of facioscapulohumeral muscular dystrophy

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ABSTRACT

Facioscapulohumeral muscular dystrophy (FSHD) is the only human disease associated with epigenetic changes at a macrosatellite array. Almost 95% of FSHD cases carry a reduced number (≤ 10) of tandem 3.3 kilobase repeats, termed D4Z4, on chromosome 4q35; remaining cases bear variants in chromatin remodeling factors, such as SMCHD1, DNMT3B, LRIF1. Reduced CpG methylation is used for the molecular diagnosis of FSHD, but D4Z4-like sequences dispersed in the genome can generate ambiguous results. By analyzing complete haplotype level assemblies from the T2T-CHM13 human genome and 86 haploid genomes from the human pangenome project, we uncovered the extensive number of D4Z4-like elements and their widespread inter- and intra-individual variability. An original analytical approach was developed to elucidate this previously unaccounted wealth of D4Z4-like elements and to analyze CpG methylation at D4Z4 in bisulfite-treated DNA from 29 FSHD index cases and 15 relatives. Integrated analysis of clinical phenotype, D4Z4 CpG methylation level and gene variants showed that low D4Z4 methylation was associated with variants in the *SMCHD1* gene, but not with the patients' clinical phenotypes. This is the first study showing the relevance of the pangenome and T2T-CHM13 assemblies for investigating the genotype-phenotype correlation in genetic diseases. The extension and the variability of D4Z4-like elements scattered throughout the human genome and the inconsistent association of phenotypes with methylation profiles advocate for a critical revision of FSHD diagnostic tests based on D4Z4 CpG methylation assays and indicate that molecular investigations must be complemented by family studies for the proper interpretation of results.

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common form of hereditary myopathy with an estimated prevalence from 1/8,000 to 1/20,000^{1,2}. FSHD is the only hereditary human disease associated with reductions in copy number of a 3.3 kb macrosatellite³, termed D4Z4. In the general population, the size of the D4Z4 repeat varies between 11 and 150 units, whereas FSHD patients carry fewer than 11 repeats⁴. Each copy of the 3.3 kb D4Z4 repeat contains an open reading frame (ORF) with two homeobox domains, named *DUX4*-Like (*DUXL*)⁵, and two different classes of GC-rich repetitive DNA^{6,7}: hhspm3, a member of a low copy human repeat family⁸, and LSau, a middle repetitive DNA family^{8,9}. A tandemly arrayed D4Z4 repeat with 98% identity to the 4q35 array is located at 10q26¹⁰, but only alleles with reduced D4Z4 copy number on chromosome 4q35 have been associated with FSHD (Figure 1).

Two genetically distinct FSHD subtypes, FSHD1 and FSHD2, are currently described. In FSHD1 [OMIM #158900], the reduction of 4q35-D4Z4 repeats below a critical threshold (10 or fewer repeat units, RU)¹¹ is thought to determine epigenetic alterations and the inappropriate expression of nearby genes leading to disease^{12–14}. In FSHD2 [OMIM #158901] affected individuals carry two D4Z4 arrays in the healthy range (>10 RU), but bear damaging heterozygous variants in chromatin remodeling factor genes, such as *SMCHD1* (for structural maintenance of chromosomes flexible hinge domain containing 1)¹⁵, *DNMT3B* (for methyltransferase 3B)¹⁶ and *LRIF1* (for ligand-dependent nuclear receptor interacting factor 1)¹⁷. It has thus been proposed that similar to the reduction of D4Z4 RU, the inactivation of these genes alters the epigenetic configuration of the D4Z4 array at 4q35 (Figure 1). Recent studies linked the molecular etiology of FSHD with the inappropriate expression of the DUX4 protein from the terminal D4Z4 repeat. Only a specific haplotype labelled 4qA-PAS is considered to be permissive for DUX4 protein expression, due to the

100 presence of a 260-bp sequence, termed pLAM, which provides a 3'-terminal exon (Exon 3)
101 and a polyadenylation signal (PAS) (AUUAAA) in juxtaposition to the terminal copy of *DUX4*
102 ORF (hereafter *ter-DUX4*) on 4q^{18,19} (Figure S1). Although a similar arrangement is
103 observed also on chr 10q26, at this locus the PAS signal is disrupted by a single nucleotide
104 variant (AUCAAA), preventing the polyadenylation of the *ter-DUX4* mRNA. By analogy, 4qB,
105 an alternative (to 4qA-PAS) haplotype at 4q (Figure S1), is considered not permissive for
106 the expression of the *DUX4* protein due to the lack of pLam. Based on these observations
107 (D4Z4 reduced copy number or heterozygous variants in chromatin remodelers, associated
108 with 4qA-PAS haplotype) a reduction of CpG methylation of D4Z4 has been proposed as a
109 faithful indicator of anomalous *DUX4* gene expression and it is used as a proxy of disease
110 status in the clinical setting²⁰.

111 This model is in contrast with clinical and epidemiological data showing reduced
112 penetrance and clinical variability in FSHD families^{21–27}, as well as with observations
113 obtained by adopting an articulate neurological assessment for the study of genotype-
114 phenotype correlation in FSHD. This latter approach includes the phenotypic classification
115 of probands and relatives by applying the Comprehensive Clinical Evaluation Form
116 (CCEF)²⁸, which beside quantifying the degree of motor disability²⁹, classifies individuals on
117 the basis of detailed phenotypic features which include parameters such as age at onset or
118 site of disease onset beside the detailed description of muscle impairment. Four main
119 descriptive categories have been created. They identify: (1) subjects presenting facial and
120 scapular girdle muscle weakness typical of FSHD (category A, subcategories A1-A3), (2)
121 subjects presenting an incomplete phenotype with muscle weakness limited to scapular
122 girdle or facial muscles (category B, subcategories B1 and B2), (3) asymptomatic or healthy
123 subjects (category C, subcategories C1 and C2), and (4) subjects with myopathic
124 phenotypes presenting clinical features not consistent with FSHD canonical phenotype
125 (category D, subcategories D1 and D2)²⁸. The application of this methodology for the

stratification of clinical phenotypes has permitted the quantification of the large phenotypic variability observed in individuals carrying a D4Z4 Reduced Allele (DRA) which is in contrast to the indication that a positive molecular test is the only determining aspect for FSHD diagnosis^{30–37}. Studies also showed that the different categories are associated with different disease trajectories; in particular subjects assessed with a classical FSHD phenotype (Category A) display a steeper functional decline, than subjects with limited muscle impairment (Category B)^{31,35}. Large genotype-phenotype studies also highlighted differences between probands and relatives, including 30-50% of relatives remaining non-penetrant carriers. Consistent with these findings it has been assessed that DRA with 4-8 RU have the frequency of a common polymorphism (3% in the general population^{25,38–40}), which is not compatible with the incidence of FSHD; moreover the DRA 4qA-PAS haplotype has a 2% frequency in the human population³⁹. Finally, studies reported a wide variability in D4Z4 CpG methylation levels among FSHD index cases and FSHD families with no clear association between D4Z4 methylation status and disease manifestations or severity^{32,41}.

Here, we report the results of comparative analyses of 86 haplotype-level assemblies from the Human Pangenome Project dataset (hereafter PGR)⁴² and the outcome of high-throughput CpG methylation assay of the D4Z4 repeat based on the T2T-CHM13 human genome assembly (hereafter T2T). Our study displays all the potential of complete, high-quality haplotype level genome assemblies for the analysis of D4Z4 arrays in human diseases, confirms the high frequency of the permissive 4qA-PAS haplotype, which is comparable to a common polymorphism and shows that across the 3.3 kb D4Z4 elements only two sequences are 4q/10q-specific and amenable for the study of methylation patterns in FSHD. This refined CpG methylation assay reveals that 4q/10q-specific reduced methylation correlates with the presence of damaging *SMCHD1* heterozygous variants, but not with the clinical status of FSHD2 cases and advocate revisions of previous findings based on the GRCh38 reference (hereafter hg38).

152

153 **Subjects and methods**

154

155 **Participants**

156 The study cohort included 26 subjects (8F, 18M) reported to the Miogen Laboratory of the
157 University of Modena and Reggio Emilia for the diagnosis of FSHD⁴³. These cases carried
158 D4Z4 alleles with 10 or more repeats. In 8 cases the study was extended to one or both
159 parents, for a total of 14 subjects (7F, 7M). Overall, 5 trios and 3 parent-child couples were
160 analyzed. Four subjects carrying a 4U DRA were also included (2F, 2M). In three of these
161 subjects (Family C individuals III-1 and III-3; Family 1252 subject 297/08³²) D4Z4
162 methylation was previously assessed through the BSS assay using primer sets specific for
163 the distal 4qA and 4qA-Long (4qA-L) D4Z4 repeat regions³².

164 The clinical phenotype was determined (Table S1) according to the Comprehensive
165 Clinical Evaluation Form (CCEF), which evaluates the distribution and degree of motor
166 impairment, age at onset of motor impairment and site of muscle weakness at onset, the
167 presence of typical and atypical symptoms²⁴. The CCEF has been developed by the Italian
168 Clinical Network for FSHD to classify subjects on the basis of these clinical features. The
169 CCEF classifies: 1) subjects presenting facial and scapular girdle muscle weakness typical
170 of FSHD (category A, subcategories A1-A3), 2) subjects with muscle weakness limited to
171 scapular girdle or facial muscles (category B subcategories B1, B2), 3)
172 asymptomatic/healthy subjects (category C, subcategories C1, C2), 4) subjects with
173 myopathic phenotype presenting clinical features not consistent with FSHD canonical
174 phenotype (category D, subcategories D1, D2). Degree of motor impairment was also
175 established by applying a standardized evaluation that generates the FSHD score ²⁹.

176 Signed informed consent was obtained from all the subjects prior to the inclusion in
177 the study.

178

179 **Bisulfite sequencing**

180 Bisulfite sequence analysis was performed on high molecular weight genomic DNA
181 obtained from peripheral frozen blood through phenol-chloroform extraction. To assess the
182 methylation level at D4Z4 locus, specific primer sets were designed on hg38 genome
183 assembly using MethPrimer tool⁴⁴. No CpG sites were allowed in the primer sequence. The
184 amplicon selection was based on following criteria: i) the sequences contain SNPs allowing
185 to discriminate between the 4q and 10q D4Z4 repeats; and ii) include the maximum number
186 of CpG sites possible. Amplicons were representative of the whole D4Z4 array and included
187 functional domains such as the D4Z4 binding element (DBE)¹². Table S2 and Table S3,
188 Figure S2 summarize the characteristics of the four selected primer sets. Specific Illumina
189 adapters were added to each set of primers.

190 Bisulfite conversion was performed on 1 µg of genomic DNA by using the EpiTec
191 Bisulfite Kit (Cat N°59104 QIAGEN) following the manufacturer's protocol.

192 PCR amplification of both converted and non-converted DNA was performed using
193 the four selected primer pairs (Table S2). For every subject we generated two pools of
194 amplicons: one from bisulfite converted (BSC), one from non-converted DNA. Illumina
195 paired-end sequencing was then performed by Eurofins genomics on the amplicon pools
196 for a total of 36 Mb (60K read pairs per amplicon with 2 x 300 bp read mode).

197

198 **Bioinformatics analyses**

199 ***Identification and characterization of D4Z4-like sequences and D4Z4 associated*** 200 ***elements in human genome assemblies***

201 The complete sequence of a reference D4Z4 element was obtained from the hg38 assembly
202 in UCSC genome browser (<https://genome.ucsc.edu/>). Sequence similarity searches based
203 on the BLAST program⁴⁵ were performed to annotate D4Z4-like elements and functional

elements of the D4Z4 array (see below), in the hg38 reference assembly of the human genome [[GRCh38.p14 - hg38 - Genome - Assembly - NCBI](#)], the T2T assembly [[T2T-CHM13v1.1 - Genome - Assembly - NCBI](#)], and 86 distinct haplotype-level assemblies from the PGR, as available from https://github.com/human-pangenomics/HPP_Year1_Assemblies.

Results of BLAST sequences similarity searches were stored in simple tabular format and processed by custom Perl scripts. A similarity threshold of 85% and an alignment length threshold of 300 aligned or more aligned residues were used to define D4Z4-like elements. Matches were binned in 5 bins according to their size (<500bp; <1000bp; <1500bp; <2500bp; and <3300bp). Matches of size in-between 3200 and 3300 bp were considered to provide a complete representation of a D4Z4 macrosatellite (full match).

D4Z4 arrays were annotated based on the terminal repeat for the presence of qA or qB sequence variants; presence/absence of the pLam sequence and integrity of the AUUAAA polyadenylation signal (PAS). qA/B has been annotated based on the sequence of the respective probes used for the FSHD diagnostic protocol; pLam has been annotated based on the sequence reported in UCSC genome browser (<https://genome.ucsc.edu/>) (Supplemental Material and Methods).

Similarity-based clustering of D4Z4 like elements and terminal DUX4 coding sequences

D4Z4-like sequences of 3200 or more bp in size (full matches) were extracted from their respective genome assemblies and aligned with Kalign⁴⁶. Conserved alignment blocks were extracted with Gblocks⁴⁷, using the following parameter: Minimum Length Of A Block: 3, Allowed Gap Positions: none. A sequence similarity matrix was computed by means of the EMBOSS distmat program⁴⁸, using the Tajima-Nei correction for multiple substitutions⁴⁹. Phenetic clustering was performed by using the NJ algorithm, as implemented by hclust()

230 function from the cluster package in R⁵⁰. Six distinct groups with high sequence identity
231 were defined for the D4Z4 complete elements. D4Z4 from T2T were used to anchor/assign
232 each group to a chromosome.

233 The same method was applied to cluster *ter-DUX4* coding sequences by sequence
234 similarity. Terminal repeats were identified based on the presence of a qA or qB element *in*
235 *cis* and within 15 Kb from a complete *DUX4* ORF and tentatively assigned to chr 4 or 10
236 based on sequence similarity.

237

238 ***In-silico identification of target regions of primer sets in hg38 and T2T genome*** 239 ***assemblies***

240 Potential target regions of primer sets in the hg38 and T2T genome assemblies were
241 determined by a custom Perl script (Table S4). Sequence similarity searches of primer
242 sequences on the hg38 and T2T reference assemblies were performed by blastn, with
243 default parameters⁴⁵. All matches of 17 bp or longer and with at most 1 mismatch and all
244 perfect matches of 16 bp or longer were recorded. Genomic coordinates were cross-
245 referenced, and potential target genomic regions were identified as those spanned by a 5'
246 and 3' primer pairs, in the correct orientation, and within a distance >50 bp and <600 bp.
247 The same procedure was applied for the identification of potential primer target regions in
248 the BSC space, in this case both the genome and the primer sequences were converted *in-*
249 *silico*.

250

251 **Statistical analyses**

252 Distribution of values were compared by applying the non-parametric, two tailed
253 Kolmogorov Smirnov test, as implemented by the `ks.test()` function from the stats package
254 of the R programming language.

255

Analysis of bisulfite converted reads

Quality control and determination of target regions

Reads' quality was assessed by Fastqc^{51,52}. Individual reports were merged by MultiQC⁵³ and quality metrics were visually inspected.

Non-converted amplicons' reads were aligned to T2T and hg38 human genome assemblies using Bowtie2⁵⁴. Sample-level coverage profiles were computed by bedtools genomecov⁵⁵. Genomic regions covered by 100 or more reads in at least 50% of samples were considered for subsequent analyses. By this approach a total of 172 and 57 candidate target regions were identified in the T2T and hg38 assemblies, respectively. 50/57 and 169/172 had at least a high similarity match (17 bp with at most 1 mismatch) to one or more primer sequences.

Highly similar candidate regions were collapsed to provide a non-redundant representation of repetitive sequences and allow non-ambiguous mapping of BSC reads. Different sequence identity thresholds were tested through a titration analysis to identify the ideal sequence similarity threshold. The total number of non-ambiguously mapped BSC reads and the total number of CpGs covered by at least 10 BSC reads in at least 50% of samples (testable CpGs) were recorded (Table 1). Considerations based on the number and cumulative size of genomic regions that were merged, and on the number of testable CpGs, prompted us to select 95% identity as the most appropriate threshold. In conclusion, by comparing hg38 and T2T-based analyses of non-converted reads, we defined a total of 60 and 9 distinct consensus groups (groups of sequences GS) respectively (Table S5 and Table S6). For every target region the consensus sequence was determined by majority rule consensus, by applying the cons EMBOSS program⁴⁸. These consensus sequences were used for the further analysis of methyl-seq data.

Assessment of methylation levels

BSC reads were aligned to consensus target region sequences and methylation levels were determined by Bismark⁵⁶ +Bowtie2. The bismark_methylation_extractor tool was applied to compute CpG methylation levels. Only CpGs covered by more than 10 reads in at least 25 patients were considered for the delineation of methylation profiles.

SMCHD1 variants analysis

FSDH2 probands are routinely tested for the presence of *SMCHD1*, *DNMT3B*, *LRIF1* variants. We collected the identified *SMCHD1* variants in the cohort and performed a reannotation on GRCh37 genome assembly using wAnnovar⁵⁷ (<https://wannovar.wglab.org/>; the analysis was performed on the 27.9.22) and filtered. We excluded intron variants and considered only exonic (missense, stop or frameshift variants) and splicing variants with a GnomAD exome all MAF < 10⁻⁴ (GnomAD frequency for PM2 pathogenic moderate rule in Varsome)⁵⁸. Among the filtered variants we then excluded the ones which were benign and likely benign according to Varsome ACMG (American College of Medical Genetics and Genomics) prediction^{59,60} (<https://varsome.com/>; the analysis was performed on 27.9.22). As *SMCHD1* gene function has not been thoroughly investigated yet, we decided not to filter out the variants predicted as VUS (Variants of Uncertain Significance). Finally, variant validation through Sanger sequencing was performed on probands and on relatives (Table S7).

Results

T2T and PGR reveal the extended variability of D4Z4 repeats throughout the human genome

The extent of D4Z4-like repeat elements in the human genome was investigated by analyzing T2T and 86 haplotype-level genome assemblies from the PGR⁴².

Our analyses uncovered hundreds of “complete” (>3200 bp) as well as partial (300 – 3200 bp) D4Z4-like sequences (hereafter D4Z4-I), irrespective of the reported geographic origin of the subjects (Table S8). The cumulative size of D4Z4-I spanned in-between 700 Kb to 1.5 Mb of sequence in the haplotype-level assemblies of the 43 subjects from the PGR and accounted for approximately 1.2 Mb of sequence in T2T. D4Z4-I were scattered through several chromosomes in T2T and accounted for hundreds of kilobases of sequences on chr 1, chr 4, chr 10, chr 15, chr 21 and chr 22 (Figure 2A). Notably, in hg38 only 86.5 Kb of D4Z4-I were observed, representing a more than 10-fold reduction compared with T2T and PGR (Figure 2B). In hg38, D4Z4-I were prevalently associated with the long arms of chr 4 and chr 10 (Figure 2A) where they are arranged as two large arrays of 8 and 10 complete D4Z4 repeats, respectively. PGR and T2T instead harbored a large number (minimum 285, maximum 626, Table S8) of “incomplete” D4Z4-I repeats of < 2.5 Kb in size, which are lacking in hg38. Conversely (Figure 2B), the number of complete D4Z4 elements included in hg38 was within the range of variability observed in T2T and PGR (26 to 121). Interestingly, subjects from AFR (African) ancestry were associated with a higher variability in D4Z4-I copy numbers (Kolmogorov Smirnov test p-value = 0.0006403), while a narrower range was observed in individuals of EAS (Eastern Asia) and AMR (South America) ancestry (Figure S3).

Figure 2C shows the variability and the distribution of complete D4Z4-I elements. Six different groups were defined on the basis of sequence identity levels. D4Z4 from T2T were used to anchor/assign each group to a chromosome. The largest clusters (Table 2) and the majority of the sequences (87%) were assigned to either chr 4 (cluster-2) or chr 10 (cluster-1); this notwithstanding, complete D4Z4 elements were also observed on chr 14 (cluster-3), chr 22 (cluster-5 and cluster-6), chr 15 and chr 21 (cluster-4). The number of complete D4Z4 elements assigned to distinct clusters in the 86 PGR haplotypes is summarized in Figure 2D and Table 2. Chr 4 and chr 10 were associated with a median number of 22 and 19

334 repeats, respectively. The difference was statistically significant according to a Kolmogorov
335 Smirnov test (p-value: 0.02518). Complete D4Z4 repeats on chr 10 displayed lower
336 variability compared with those on chr 4. Substitution rates estimates at haplotype levels
337 suggested a high inter-individual variability, with rates ranging from 0.164 to 0.4312
338 (average of 0.245) substitutions for 100 bp for chr 4 and from 0.015 to 0.21 (average of
339 0.13) for chr 10 (Figure S4 and Figure S5). Interestingly, as shown in Table 2, more than
340 75% of the assemblies carried D4Z4-I assigned to cluster-6 (chr 22); whereas less than 50%
341 of the haplotype assemblies in the PGR had one sequence assigned to cluster-3 (chr 14),
342 cluster-4 (chr 15-21) and cluster-5 (chr 22).

343

344 **The pangenome assemblies show the high frequency of the 4qA-PAS haplotype** 345 **associated with FSHD in the world population**

346 The permissive DRA 4qA-PAS haplotype has been proposed to cause FSHD. However, we
347 previously reported a frequency of 2% for this haplotype³⁸, which is not compatible with the
348 prevalence of the disease⁶¹. We thus analyzed the T2T and PGR to investigate the
349 prevalence of this molecular signature in other populations and to study the conservation
350 and the integrity of *ter-DUX4*.

351 The pLam and telomeric qA and qB sequences (Supplemental Material and
352 Methods) were used to identify and classify terminal D4Z4 repeat in PGR, T2T and hg38. A
353 total of 172 D4Z4 terminal repeats were identified (Table S9) and tentatively assigned to chr
354 4 or chr 10 based on sequence similarity profiles (as described by the clustering of the
355 D4Z4-I sequences in Figure 2C). Haplotypes with inconsistent annotations (2 or more qA/qB
356 probes assigned to the same chromosome and none to the other chromosome) were not
357 included in subsequent analysis^{62,63}. By this approach the complete set of alleles at D4Z4
358 repeat loci at chr 4 and chr 10 was determined for 72 haplotypes from PGR (Table S9).
359 Patterns of conservation of *ter-DUX4* sequences were investigated. Figure 3A shows that

360 *ter-DUX4* assigned to the 10q D4Z4 terminal repeat are found to be highly similar and
361 formed a single cluster; while 4q *ter-DUX4* are more heterogeneous and were partitioned in
362 3 groups (Figure 3A-B). Interestingly, as shown in Figure 3C, in chr 10 and chr4_group 2
363 *ter-DUX4* displayed lower levels of variability (average 0.061 subs per 100 bp) compared to
364 both chr4_group 3 (average 0.12 subs per 100 bp) and chr4_group 1 (average 0.142 subs
365 per 100 bp); Figure 3A also shows that the most variable sequences (chr4_group 1 and
366 chr4_group 3) were preferentially associated with qB alleles. All *ter-DUX4* sequences were
367 found to be intact, and none was associated with frameshifts or premature stop codons.
368 Consistent with previous observations^{18,19}, all the alleles classified as qB did not carry a
369 pLam and were more proximal to the *ter-DUX4* compared to qA (Figure 3D-G). In the same
370 way, (Figure 3E-F) the vast majority of alleles classified as qA (112/119) were associated
371 with a pLam both on chr 4 (41/47) and chr 10 (71/72). Only terminal 4q pLam had valid
372 PAS⁶⁴; while in pLam assigned to 10q the PAS sequences were disrupted by a single
373 nucleotide substitution (AUUAAA→AUCAAA). Perfect conservation of PAS/PAS-like
374 elements was observed both on 4q and 10q terminal D4Z4 repeats. One qB haplotype was
375 assigned to 10q (Table 3).

376 Remarkably, in line with our previous observations^{38–40} (Table 3), 4/86 haplotypes
377 (4.6%) carried D4Z4 alleles with 6-8 RU and 4qA-PAS (Figure 3F). None of these
378 haplotypes carried disruptive variants in the *ter-DUX4* or in the pLam sequence element.

379 No statistically significant difference in the total number of chr 4 D4Z4 repeats was
380 observed when qA/qB alleles pLam+/pLam- alleles were compared (Figure 3H-I),
381 suggesting that alleles that are not permissive for the expression of the *ter-DUX4* are not
382 associated with significant differences in D4Z4 copy numbers compared with the permissive
383 qA-PAS haplotype.

384

D4Z4 hypomethylation correlates with *SMCHD1* damaging variants but not with FSHD clinical phenotype

Results shown above uncover all the heterogeneity of D4Z4 repeats in the human genome, including the presence of qA D4Z4 alleles with 8 repeats or fewer, and raise potential concerns about the reliability of methylation/diagnostic tests based on the hg38. As bisulfite treated DNA sequencing at targeted regions is commonly used to diagnose FSHD⁶⁵, the uncovered variability of D4Z4-I sequences prompted us to investigate D4Z4 CpG methylation comparing the hg38 and the T2T.

A set of primers spanning the entire length of the D4Z4 repeat and a total of 126 CpGs (Figure S2 and Table S3) were designed based on hg38 assembly to recapitulate previous findings in the field⁴¹. This experimental design was used to assess a carefully selected cohort of 26 index cases referred for FSHD2 diagnosis, and 14 relatives all carrying 10 or more RU. All the participants assayed were searched for damaging variants in the chromatin remodelers *SMCHD1*, *DNMT3B* and *LRIF1*. In addition, 3 FSHD1 index cases and 1 relative carrying 4 RU were studied.

Amplicon targeted regions were sequenced both in non-converted and in BSC DNA samples. This approach was undertaken: i. to test the 4q/10q-specificity of the primer sets commonly used and designed based on hg38 assembly⁴¹, ii. to facilitate the alignment of short BSC derived reads. Both non-converted and BSC NGS data were used for sequence alignment based on hg38 and T2T reference genomes (Table S4).

A total of 77% and 99.43% non-converted short reads mapped to hg38 and T2T respectively. Moreover, the majority of BSC reads (62% for T2T and 54% for hg38) did not align at 4q primer target regions, potentially suggesting “off target” sequences, and/or high levels of heterogeneity in methylation levels. To identify genomic regions targeted by our assay in an unbiased manner, genomic intervals covered by 100 or more non-converted reads in at least 50% of the samples were recorded. A total of 57 and 172 genomic regions

in hg38 and T2T displayed these remarkable levels of coverage. Notably, 50/57 and 169/172 of the target regions delineated by coverage analyses had at least a high similarity match (17 bp with at most 1 mismatch) to one or more primer sequences. Based on these observations sequences above 95% sequence similarity were collapsed to prevent inconsistent mapping of BSC short reads across highly similar D4Z4-I elements. By this approach, we defined a total of 60 and 9 distinct consensus groups (groups of sequences, hereafter GS) in T2T and hg38 respectively (Table S5 and Table S6). Consensus sequences were computed for each GS and used for the further analysis of methyl-seq data.

This *ad-hoc* bioinformatics workflow revealed remarkable differences in the completeness, number, and breadth of testable CpGs when the GSs derived from hg38 and T2T were compared: hg38 125 CpG; T2T 938 CpG. Global methylation patterns were summarized in the form of a heatmap (Figure S6 and Figure S7). In the T2T-based analysis a total of 4 distinct GS including 81 distinct CpGs had no missing data and complete methylation profiles across all samples, as shown in Figure 4 and Table 4: GS1 (primer D6): 28 CpGs; GS2 (primer D1): 32 CpGs; GS5 (chr 13-chr 15): 16 CpGs and GS6 (chr 13-chr 15): 5 CpGs). Notably, only GS1 and GS2 included D4Z4 5' 4q/10q-specific regions exclusively, for a total of 60 CpGs, while GS5 and GS6 mapped in D4Z4-I repeats on chr 13 and 15. The wide-range distributions of CpG methylation levels, as displayed in Figure S8, highlight a high variability at regions GS1 and GS2, while off-target regions GS5 and GS6 have high methylation levels, with a narrower distribution. Figure S9 shows a general overview of the CpG methylation patterns observed in all the 60 distinct GSs defined in our analysis. Taken together these findings indicate that a significant proportion of the BSC reads recovered by our targeted high throughput assay derive from off-target, hyper-methylated D4Z4-I regions. We also observed that when hg38 is considered as reference, the criteria we established for the T2T-based analysis (namely no missing data and

complete methylation profiles across all samples) identified only a single group, GS3 (primer D1) including 26 CpGs (Figure 4, Figure 5 and Table 4). In the light of these observations, methylation profiles inferred from T2T were considered more informative and were selected for subsequent analyses. Only primer D1 had complete data both in T2T and hg38 (Table 4); however, CpG methylation levels estimated on hg38 were slightly more elevated compared to those observed on T2T at the equivalent target region (Figure S10, median values: 49.13 hg38, 43.75 T2T). An observation that might be consistent with discrepancies in the mapping of BSC reads on different genome assemblies.

Further, our analysis revealed that 4q/10q-specific methylation patterns at GS1 and GS2 stratified samples into 3 groups: low methylation (average CpG methylation 24%), intermediate methylation (average CpG methylation 48%), and high methylation (average CpG methylation 71%). The methylation pattern of GS5 and GS6 was highly uniform across all samples, with an average of 89%.

Interestingly, the observed methylation profiles did not correlate with the disease status. As shown in Figure 6A, of 16 samples presenting low methylation 10 (62.5%) derived from cases presenting the classical FSHD features (CCEF category A), 6 (37,5%) derived from cases presenting incomplete (2, CCEF clinical Category B) or complex (4, CCEF clinical category D) phenotypes. Of 16 samples, presenting intermediate methylation 9 (56.3%) derived from cases assessed as clinical category A. Of 12 samples presenting high methylation 7 were from Category A cases (58.3%). Consistently, the methylation level distributions assessed in DNA from subjects presenting the CCEF clinical categories A, B and D was not statistically different (Figure S11). Instead, the majority (11/16) of participants in the low methylation group carried Pathogenic, Likely Pathogenic variants or Variants of Uncertain Significance according to ACMG classification⁶⁰ (P/LP/VUS variants) in *SMCHD1* (Fisher exact test p-value 0.0003). This association was confirmed by the comparison of the methylation profile distributions of *SMCHD1* P/LP/VUS variants carriers and non-carriers

(Figure 4, Kolmogorov-Smirnov test p -value $\leq 1e-16$). Identical patterns were recovered also when methylation levels on the hg38 assembly were assessed with our approach (Figure 5).

Discussion

The pangenome assemblies provide novel hints to evaluate the significance of the D4Z4-like sequences in the clinical setting

Thirty years ago, reduction below a critical threshold of the number of repeats belonging to the D4Z4 macrosatellite at 4q35 was causally associated with FSHD. D4Z4 is part a family of repetitive elements characteristic of heterochromatin and it was then known that many D4Z4-I were present in the human genome⁶⁶. At that time, the lack of a complete genome assembly hampered the possibility of fully deciphering the significance of molecular and epigenetic findings in FSHD. Very recently, these limitations have been overcome by the availability of complete, high quality haplotype level genome assemblies. By analyzing the haplotypes from the PGR we collected relevant information on the size, the distribution and the composition of the D4Z4 locus, including distal sequence elements that are considered a hallmark of the FSHD molecular signature. We established that the number of 3.3 kb D4Z4 repeats included in each array ranges from 6 RU to 89 RU⁴⁰. We observed that the distance between the qA sequences and the last D4Z4 repeat varies. We also confirmed that qA sequences are more distal compared to qB sequences; the pLam sequence is in association with qA haplotypes¹⁸, and valid PAS is exclusively associated with 4q⁶⁴; chr 10 D4Z4 repeats were all marked by qA, with a single exception: a 10qB allele carrying 1 RU. Notably, PGR haplotype analysis confirmed the high frequency of the permissive 4qA-PAS haplotype, which is comparable to a common polymorphism⁶¹. In fact, in 4.6% of cases we identified permissive haplotypes consisting of reduced 4q D4Z4 arrays with ≤ 8 RU and the

489 4qA-PAS. Since all *ter-DUX4* and pLam sequence elements associated with 4qA alleles
490 were found to be conserved and functionally intact, we speculate that disruptive genomic
491 variants at the *ter-DUX4* are unlikely to account for the staggering discrepancy between the
492 frequency of the presumed causative 4qA-PAS allele and the prevalence of FSHD.
493 Furthermore, no skewed association has been detected between 4q D4Z4 arrays copy
494 number and qA/B alleles¹⁹, indicating that non-permissive 4qB alleles and potentially
495 permissive 4qA alleles do not have a different selective pressure for the maintenance of
496 the physiological number of D4Z4 RU.

497 The consistency between data obtained from the analysis of PGR and previous
498 observations^{6,61,66} shows the validity of the PGR to advance knowledge of the molecular
499 signature used to diagnose FSHD on a world-wide scale.

500 The PGR analysis also identified 6 haplotypes with peculiar annotations where 2 or
501 more qA/qB probes were assigned to the same chromosome and none to the other
502 chromosome (Table S9). This condition has been reported before⁶² and is ascribable to the
503 spreading of repetitive and polymorphic D4Z4-I at different loci of the genome^{63,67}.

504 Our comparative analyses of complete D4Z4-I (Figure 2C) highlighted that D4Z4
505 repeats, as well as the *ter-DUX4*, located at chr 4 present a higher variability compared to
506 chr 10. In addition, PGR haplotypes from different ancestries showed different levels of
507 variability in the number of complete D4Z4-I, with a significant difference in D4Z4 copy
508 number between individuals of African ancestry, native Americans and far east Asians.

509

510 **D4Z4 methylation assay must consider the T2T sequence for the identification of** 511 **4q/10q-specific CpGs**

512 Reduced CpG methylation at D4Z4 has been widely studied and proposed as a biomarker
513 for the presence of FSHD. Previous studies of D4Z4 methylation presented some
514 drawbacks: technical limitations and lack of reproducibility led to discordant results

515 regarding which regions are the most representative for D4Z4 methylation status^{20,32,68–74}.
516 In addition, the correlation between D4Z4 methylation level and FSHD clinical status has
517 often been postulated, but different technical settings in the various studies, the lack of clear
518 clinical evaluation of patients and the prominent absence of family studies had hindered the
519 interpretation of results^{20,41,73,74}. In the present work, we comprehensively evaluated the
520 D4Z4 methylation status by considering a high number of reads and excluding technical
521 biases introduced by the previous studies based on hg38 and on standard bioinformatics
522 pipelines. We established (Figure S2) that the primer sets BSS-D1 and BSS-D6 identify
523 CpG methylation signals deriving from 4q/10q-specific regions only, whereas the primer
524 sets BSS-D3 and BSS-D5 amplify D4Z4-I distributed not only at 4q and 10q but also on
525 chromosome 1 and on the short arms of acrocentric chromosomes. Thus, the study of D4Z4
526 CpG methylation can be hazardous if the distribution of D4Z4-I elements and their variability
527 are not correctly taken into account.

528 Despite all this, the application of short read-based assays for the analysis of
529 repetitive elements of relatively large size has limitations. When non-converted reads were
530 aligned to both hg38 and T2T, more than 50% of mapped reads did not align at expected
531 target regions. Nevertheless, the total fraction of mapped reads and the number of testable
532 CpGs were higher when aligned to T2T compared to hg38, confirming that T2T provides a
533 more accurate representation of the human genome. We can anticipate that long read
534 sequencing technologies, such as Oxford Nanopore, will provide relevant technical
535 advances and open new possibilities for the study of repetitive elements' organization and
536 function in the genome. In this respect, Butterfield and colleagues (2023)⁷⁵ applied targeted
537 nanopore sequencing to FSHD patients and healthy subjects and showed that an
538 asymmetric methylation gradient forms in a length-dependent manner at the proximal end
539 of the D4Z4 repeat array reaching saturation approximately at the 10th repeat. They also
540 observed a highly similar methylation pattern at 4q and 10q which was irrespective of the

clinical phenotype (FSHD or healthy)⁷⁵. This notwithstanding, at present, long-read sequencing technologies are not mature for systematic large-scale applications in the clinical settings. Instead, the targeted CpG methylation analysis we set up is manageable on a large scale as it follows a simple protocol, is less expensive and highly reproducible. Remarkably, our data are consistent with the results obtained by Butterfield and colleagues.

D4Z4 CpG methylation level is not a predictor of FSHD disease

Beside the formal demonstration that primer design is a crucial point in the analysis of D4Z4 methylation, our study also revealed that contrary to common knowledge²⁰, not all DNAs from myopathic patients referred as FSHD2 (26 index cases) presented a low methylation level. Our analysis identified three clusters with low, intermediate and high D4Z4 CpG methylation (mean at 24%, 48% and 71% respectively). Figure 6 depicts the results of several comparisons regarding the following parameters: clinical phenotype, D4Z4 methylation level and *SMCHD1* mutational status. Figure 6A shows that some genotype-phenotype intersections are not in agreement with the current indications for FSHD diagnosis^{76,77}. Remarkably, we observed the full range of D4Z4 methylation levels in DNA from the 26 participants presenting a classical FSHD phenotype (CCEF Clinical Category A); of those 16 carriers of a damaging *SMCHD1* variant, 11 presented low 4q/10q-specific D4Z4 CpG methylation, 5 displayed intermediate CpG methylation. We also observed 5 samples with low CpG methylation with no damaging *SMCHD1* or *DNMT3B* or *LRIF1* variants and presenting classical (2), incomplete (1) or complex (2) phenotypes. underlying the genetic heterogeneity of this molecular phenotype.

The variants we identified were distributed all along *SMCHD1* gene body and did not provide information on specific *SMCHD1* region/domain that might account for the reduced methylation at 4q/10q-specific D4Z4 sequences (Figure 6B).

566 To further explore this aspect, we investigated genotype-phenotype correlation in
567 eight trios/parent-child pairs participating to this study (Figure 6C, Table S7). We observed
568 participants presenting the classical FSHD phenotype (CCEF Category A), healthy subjects
569 (CCEF Category C) or complex phenotypes presenting atypical features (CCEF Category
570 D). According to our data, only in families 1 and 26 subjects presenting a classic FSHD
571 phenotype displayed a reduced CpG methylation profile and carried a damaging
572 heterozygous *SMCHD1* variant. In family 22 the classic clinical phenotype, in presence of
573 D4Z4 reduced methylation, was not associated with variants in known chromatin modifiers.
574 In family 8 the probands presented the classic clinical phenotype associated with
575 intermediate D4Z4 methylation level in presence of a damaging *SMCHD1* variant, as the
576 healthy mother. In family 27 the proband presented the Category A phenotype associated
577 with reduced CpG methylation and a damaging *SMCHD1* variant, whereas intermediate
578 D4Z4 methylation level was detected in the healthy father carrying the same *SMCHD1*
579 allele. In family 30 father and daughter presented a complex phenotype with atypical clinical
580 symptoms (Category D1); both reported clinical onset at pelvic girdle and distal lower limbs.
581 They were heterozygous for *SMCHD1* damaging variant and presented reduced D4Z4 CpG
582 methylation. In families 9 both the probands and her father presented Category A phenotype
583 without displaying reduced D4Z4 CpG methylation and carrying WT *SMCHD1* alleles, as
584 the proband in family 11.

585 Altogether, these results suggest that *SMCHD1* modulates, directly or indirectly,
586 D4Z4 methylation at 4q and 10q, at the same time it seems that deleterious variants at
587 *SMCHD1* are not sufficient for the pathogenesis of FSHD and cannot be considered faithful
588 indicators of FSHD clinical status.

589 In conclusion, our work uncovered the complexity of the genomic setting of D4Z4-l
590 and showed that a molecular diagnostic test for FSHD2 based on *SMCHD1* inactivation and
591 D4Z4 hypomethylation must follow rigorous protocols to avoid biased interpretation.

Molecular analysis should be considered together with precise clinical assessment and complemented by family studies for the proper interpretation of results. Moreover, the variability unveiled by our analysis should warn about the risk of relying on a single ideal reference genome sequence in FSHD. In fact, this simplification could strongly narrow our capacity of observing the variability ascribable to each single human genome producing biased interpretations of sequencing data. Finally, our study indicates that a linear approach to diagnose FSHD is obsolete and novel genomic approaches integrated with the precise phenotypic description of patients and their families are needed for the comprehension of the molecular network leading to muscle wasting in FSHD.

Declarations

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Authors' contributions

RGT, VS, MC conceived the study concept and supervised the project; VS, SP conducted the molecular analysis; MC conducted bioinformatics analysis; PK and MK contributed to bioinformatic analysis; LR, SCP, MGD, CR, SB, LM, DL contributed to the patients clinical analysis and sample collection; FMS, GP contributed to results discussion; RGT, VS, MC, SP wrote the paper (original draft); all authors read and approved the final manuscript.

Declaration of interests

The authors declare no competing interests.

618

619 **Web sources**

620 The PGR dataset analyzed during the current study is available at

621 https://github.com/human-pangenomics/HPP_Year1_Assemblies.

622

623 **Availability of data and materials**

624 Data generated and/or analyzed during the current study and not included in this
625 published article are available from the corresponding author on reasonable request.

626

627 **Ethics approval and consent to participate**

628 Signed informed consent was obtained from all the subjects prior to the inclusion in the
629 study. The study was approved by the ethics committee of Emilia Romagna Area Vasta
630 Nord 743/2022/OSS/UNIMO SIRER ID 5111.

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910

911 **Figure titles and legends**

912

913 **Figure 1. Scheme of the proposed models for FSHD1 and FSHD2 pathogenesis**

914 Tandemly arrayed D4Z4 repeats (triangles) and the 4q35 haplotype elements are
915 represented along with the molecular signatures proposed for FSHD1 and FSHD2
916 pathogenesis.

917

918 **Figure 2. Stratification and variability of D4Z4-I elements**

919 (A) D4Z4-I elements in hg38 and T2T. Cumulative size per chromosome of D4Z4-I elements.
920 Only chromosomes for which at least 1 Kb of D4Z4-I sequence was identified, in any of the
921 2 assemblies, are reported. Size (in Kb) is shown on the Y axis. Different colors are used
922 for T2T and hg38 and to mark complete D4Z4-I (see legend). (B) Cumulative size (in Kb) of
923 D4Z4-I elements in the hg38, T2T and PGR haplotype level assemblies. Left: cumulative
924 size of D4Z4-I elements. Right: cumulative size of full D4Z4-I elements. Data is displayed in

the form of a barplot. Values are on the Y axis, labels on the X axis. For the human pangenome haplotype level assemblies, 3 distinct bars are used to indicate the minimum, maximum and average values. Color codes according to the legend. (C) Dendrogram of complete D4Z4-l elements. Red rectangles and color codes are used to delineate the 5 main groups of D4Z4-l elements, as identified by sequence similarity-based clustering. (D) Total estimated number of complete D4Z4 elements per sequence similarity group. Distributions of values are displayed in the form of a boxplot, with groups indicated on the X axis and copy numbers on the Y axis. The top-right panel provides zoom on groups chr 14, chr 15-21, chr 22-1 and chr 22-2.

Figure 3. Characterization of D4Z4 alleles

(A) Heatmap of *ter-DUX4*. Scaled identity levels are displayed by the green color gradient on the right. The dendrogram on the left shows clustering of *ter-DUX4* based on sequence identity. Colored vertical bars are used to indicate: presence/absence of pLam; classification as qA or qB; sequence similarity cluster (see top). Color codes are explained directly under each bar. (B) Numerosity of sequence similarity clusters. The barplot shows (y-axis) the total number of *ter-DUX4* in each cluster (x-axis). (C) Boxplot of substitution rates. Substitution rates (substitutions by 100 bp) by cluster. Distribution of values are shown as a boxplot. Clusters are on the y-axis, values on the x-axis. (D) Distance of qA and qB elements from *ter-DUX4*. Data are represented in the form of a histogram. Distances (in Kb) are indicated on the x-axis. qA and qB elements are marked with different colors (see legend). (E-G) Annotation of terminal D4Z4 repeats. Two barplots are displayed for the 3 (out of 4) clusters to which 5 or more sequences have been assigned. Each barplot reports, for qA and qB alleles respectively: the total count (tot); the number pLam-like elements (pLam); the number pLam-like elements with a valid PAS (plamPolyA); the total number of haplotypes with 8 or less D4Z4 repeats. Values are indicated on the y-axis, labels on the x-

axis. Distinct sequence similarity clusters are indicated by a corresponding label directly under each plot. (G-H) Total number of complete D4Z4-I elements in H) qA and qB haplotypes; I) haplotypes with (pLam+) and without (pLam-). Distribution of values are displayed in the form of a boxplot, with values on the X-axis and groups on the Y axis. Color codes are consistent throughout Figure 2.

Figure 4. CpG Methylation profiles of cohort subjects inferred on T2T

A total of 82 CpGs for which complete data are available for all the study subjects are shown. (A) Heatmap of % CpG methylation: each row represents the CpG methylation pattern of a single individual. Columns represent the methylation pattern of each analyzed CpG. The dendrogram shows the clustering of the subjects based on the observed methylation profiles. Colored vertical bars are used to display CCEF clinical category status, and the presence/absence of deleterious genomic variants in *SMCHD1*. Color codes are illustrated directly under each bar. The colored bar at the top demarcates each of the 4 distinct 95% sequence identity genomic region clusters with complete data for all the subjects. (B) Boxplots of methylation levels distributions by subject. The color-scale used for the heatmap and the boxplot is shown at the bottom. (C) Violin-plot of average methylation levels, restricted to CpGs in regions GS1 and GS6, in subjects with/without deleterious genetic variants in *SMCHD1*.

Figure 5. Methylation profiles of cohort subjects inferred on hg38

A total of 32 distinct CpGs, for which complete data are available for all the study subjects, are displayed. (A) Heatmap of % CpG methylation: individuals are reported on the rows and CpGs on the columns. The dendrogram shows a clustering of the subjects based on the observed methylation profiles. Colored vertical bars are used to display CCEF grades, and the presence/absence of deleterious genomic variants in *SMCHD1*. Color codes are

illustrated directly under each bar. The colored bar at the top demarcates each of the 4 distinct 95% sequence identity genomic regions clusters with complete data for all the subjects. (B) Boxplots of methylation levels distributions by subject. The color-scale used for the heatmap and the boxplot is shown at the bottom. (C) Violin-plot of average methylation levels in subjects with/without deleterious genetic variants in *SMCHD1*. Only CpGs in primer-targeted regions are considered.

Figure 6. Genotype-phenotype correlation

(A) Venn diagrams reporting the number of subjects presenting different combinations of 4q/10q-specific D4Z4 methylation level, *SMCHD1* mutational status and FSHD clinical category. (B) ACMG classification, median 4q/10q-specific D4Z4 methylation of variant carriers, variant type and location are reported for each *SMCHD1* variant. When the variant is carried by more than one subject, median D4Z4 methylation status of all the subjects is reported. (C) Distribution of 4q/10q-specific D4Z4 methylation, *SMCHD1* mutational status and FSHD clinical category in the 8 trios/parent-child couples included in the study. Circles/squares are colored according to the median 4q/10q-specific D4Z4 methylation level; the color is fading as it represents the variability of CpG methylation pattern in each individual. WT= subject carrying no *SMCHD1* variants, VAR= subject carrying a damaging *SMCHD1* variants.

Table titles and legends

Table 1. Titration analysis for the identification of the optimal sequence similarity threshold

Identity-level (%)	# regions	size (Kb)	uniquely mapped BSC reads (%)	# testable CpGs
99	145	67,41	5,43%	237
98	103	59,13	12,13%	411
97	87	54,01	26,74%	873
96	73	46,14	32,47%	997
95	60	40,03	49,17%	1176
94	56	38,71	51,01%	1083
93	53	37,55	53,43%	891
92	46	36,49	58,47%	766
91	45	33,02	72,71%	637
90	42	32,75	75,41%	451

Identity-level (%): sequence identity threshold. #regions: number of distinct regions defined by the threshold. size (Kb): total size of the regions in Kb. uniquely mapped BSC reads (%): percentage of uniquely mapping BSC reads. # testable CpGs: total number of distinct CpGs for which more than 10 reads were available for more than 25 subjects. Italic characters indicate the selected sequence similarity threshold.

Table 2. Characterization of complete D4Z4 elements clusters

Cluster	Chromosome	% sequences	Median number of complete repeats (per haplotype)	Average substitution rate per 100 bp	Substitution rates estimates at haplotype level
1	Chr 10	87%	19	0.13	0.015-0.21
2	Chr 4		22	0.245	0.164-0.4312
3	Chr 14	2.3%	0.5	1.73	NA
4	Chr 15, Chr 21	3.1%	1	1.81	NA
5	Chr 22-1	1.5%+6.1%	0	0.798	NA
6	Chr 22-2		2	1.24	NA

Table 3- Co

Haplotype	PGR analysis	Previous knowledge
4qA 4qA 4qA	- 6 to 89 RU alleles; - The majority of alleles classified as qA are associated with a pLam both on 4q and 10q; - AUAUAAA PAS at 4q; - 4.6% individuals carry a 4qA-PAS alleles with <8RU; - qA sequence is not always at the same distance from the last D4Z4 repeat (from 4.7 Kb to 8.3 Kb).	- 6 to ~90 RU alleles⁴⁰; - qA alleles are associated with a pLam both on 4q and 10q¹⁸; - AUAUAAA PAS at 4q⁶⁴; - 3% healthy population carry a DRA and 1.3% a DRA with 4qA-PAS haplotype³⁹.
4qB	- qB alleles do not carry a pLam sequence; - qB sequence is more proximal than qA (2.5 Kb and 7.3 Kb respectively); - No differences in D4Z4 repeat numbers between 4qA and 4qB alleles.	- qB alleles do not carry a pLam sequence¹⁸; - qB sequence is more proximal than qA¹⁹; - Observed equal frequency of 4qA and 4qB alleles¹⁹.
10qA 10qB	- AUAUAAA PAS at 10q; - One 10qB allele was identified.	- AUAUAAA PAS at 10q⁶⁴; - 10q is associated predominantly to qA haplotype¹⁹; - sporadic cases of 10qB alleles⁶².

1032 **Table 4: GS containing CpGs covered by at least 10 BSC reads in at least 50% of**
1033 **samples and no missing data across all samples**

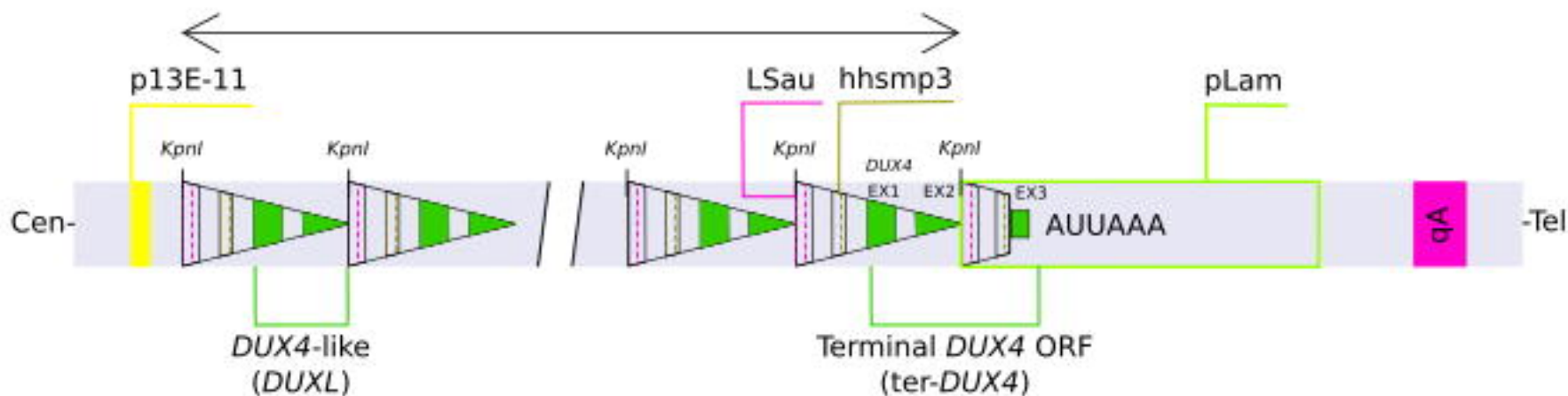
Assembly	Group	Chromosome	N° CpGs
T2T	GS1	Chr 4, Chr 10	28
	GS2		32
	GS5	Chr 13, Chr 15	16
	GS6		5
hg38	GS3	Chr 4, Chr 10	26

1034

FSHD

Prevalence 1:8.000 - 1:20.000

D4Z4 repeated units (RU)



FSHD 1

95% cases

FSHD 2

5% cases

D4Z4 reduced allele (DRA)
 ≤ 10 RU

+

4qA-PAS haplotype

>10 RU

+

4qA-PAS haplotype

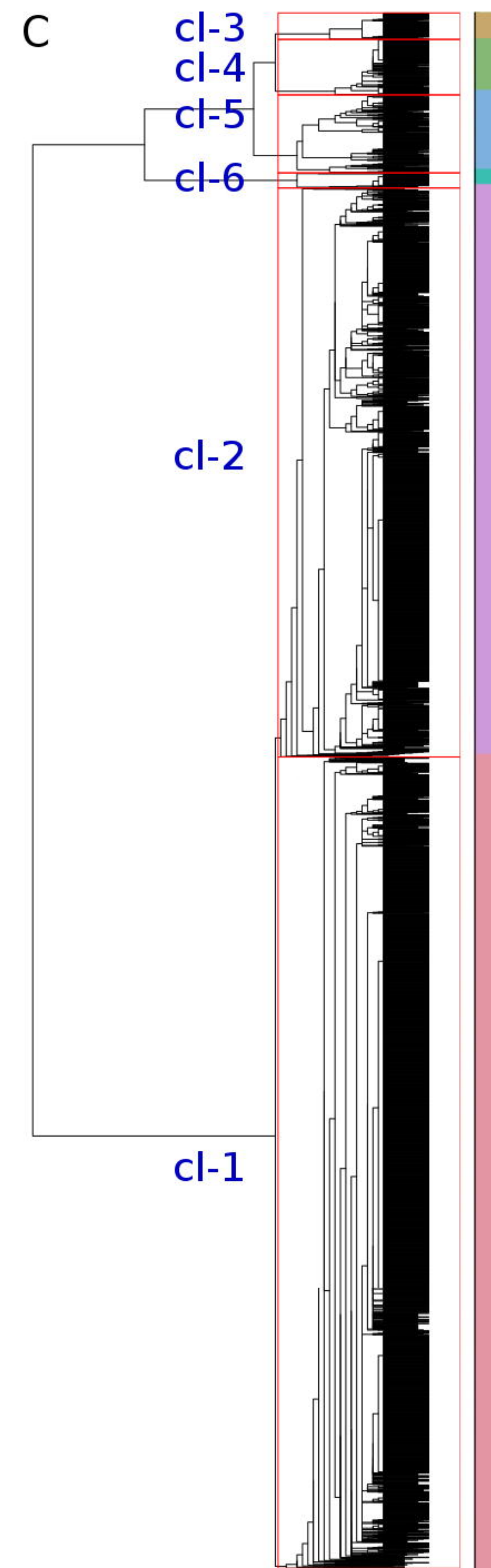
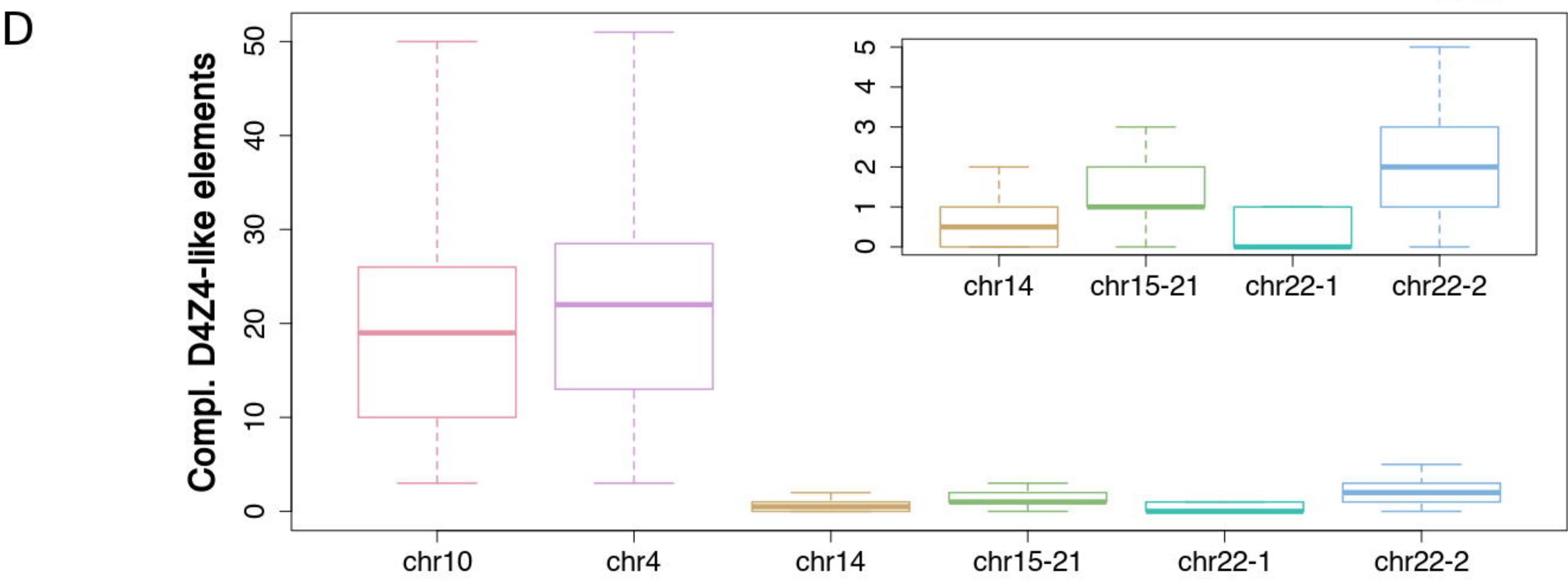
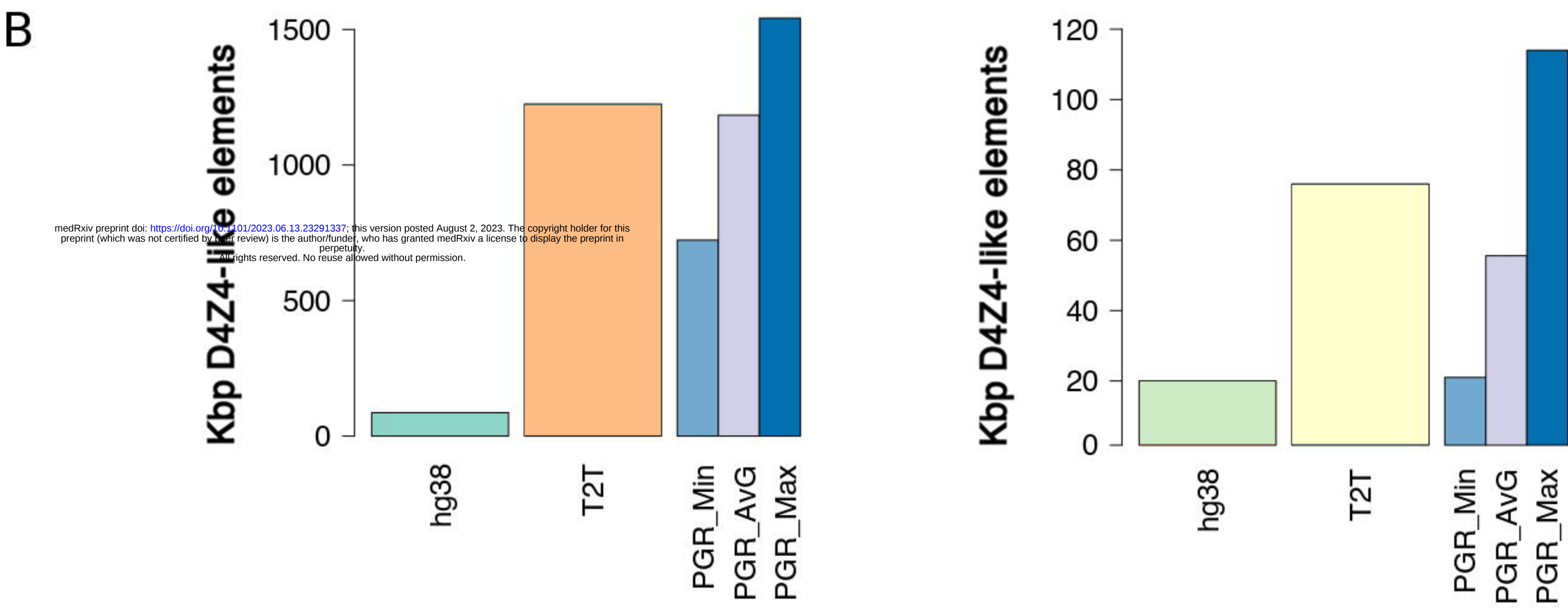
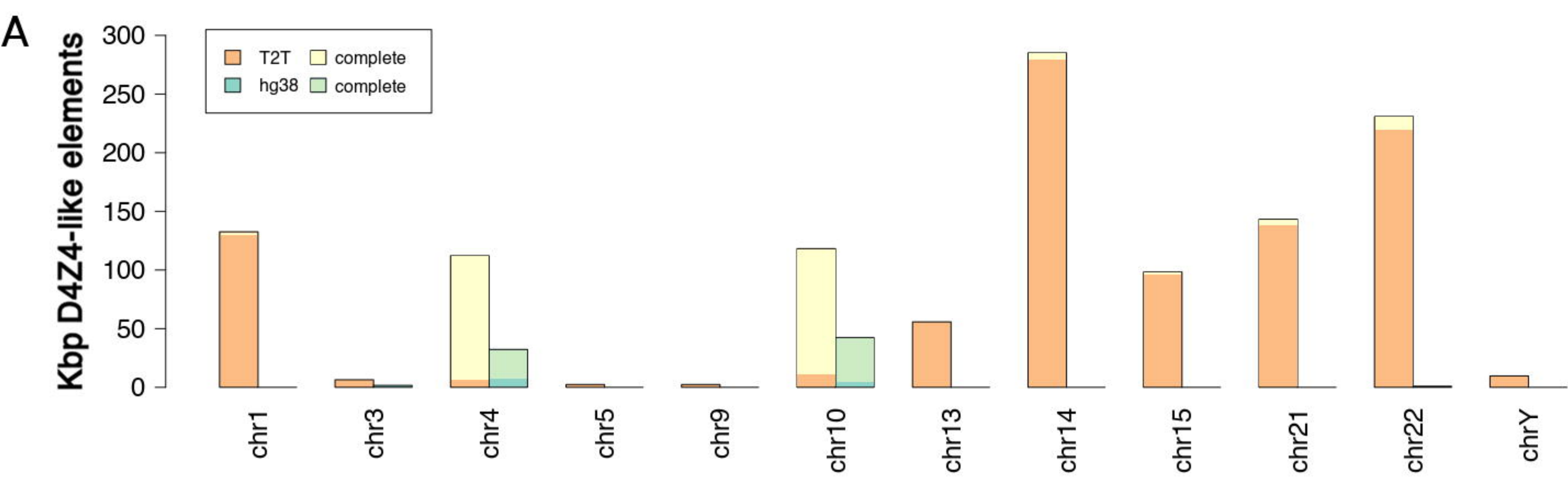
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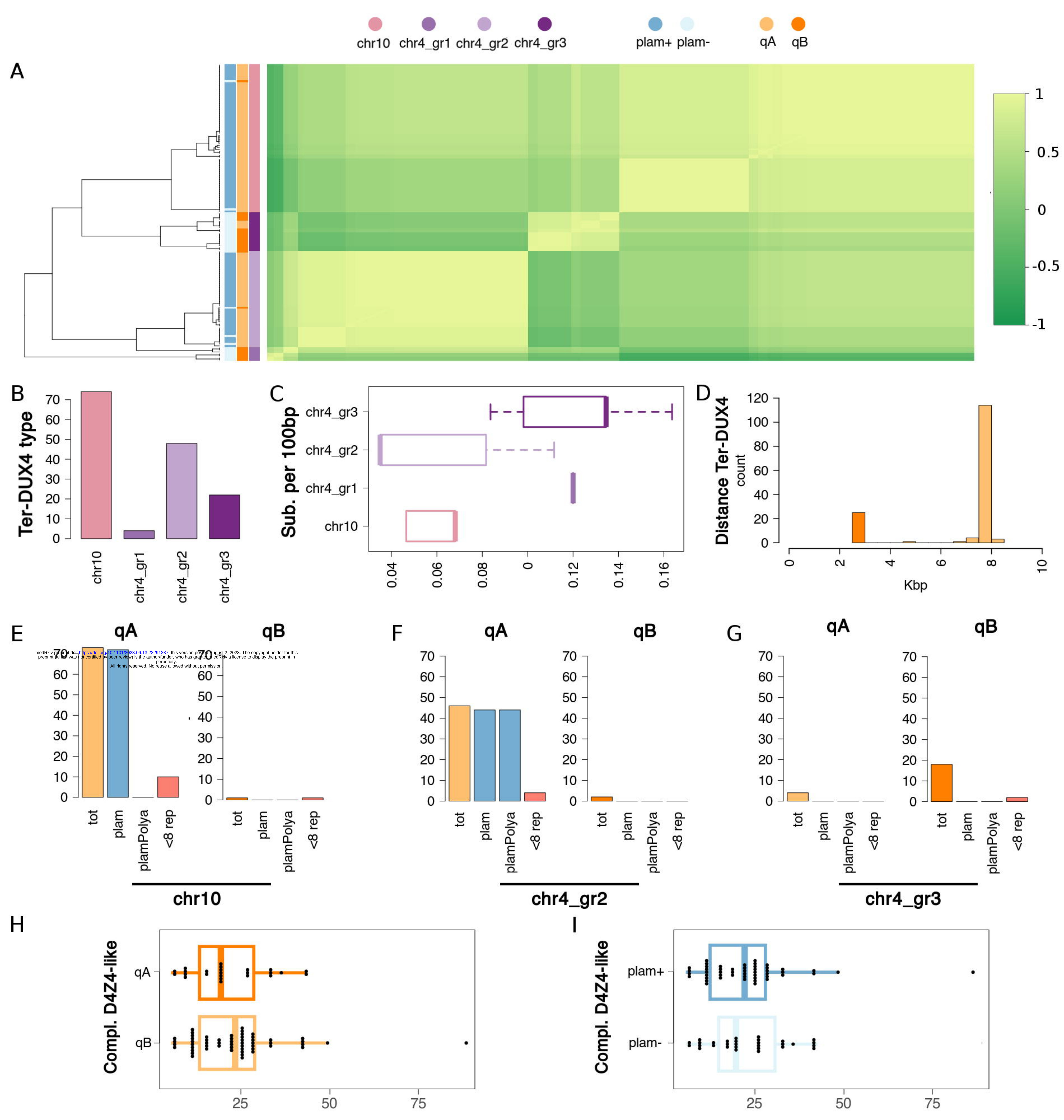
Variants in chromatin remodeling genes
(SMCHD1/DNMT3B/LRIF-1)

Terminal D4Z4
reduced methylation

D4Z4 repeats
reduced methylation

Terminal DUX4 ORF (ter-DUX4) toxic expression





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GS1

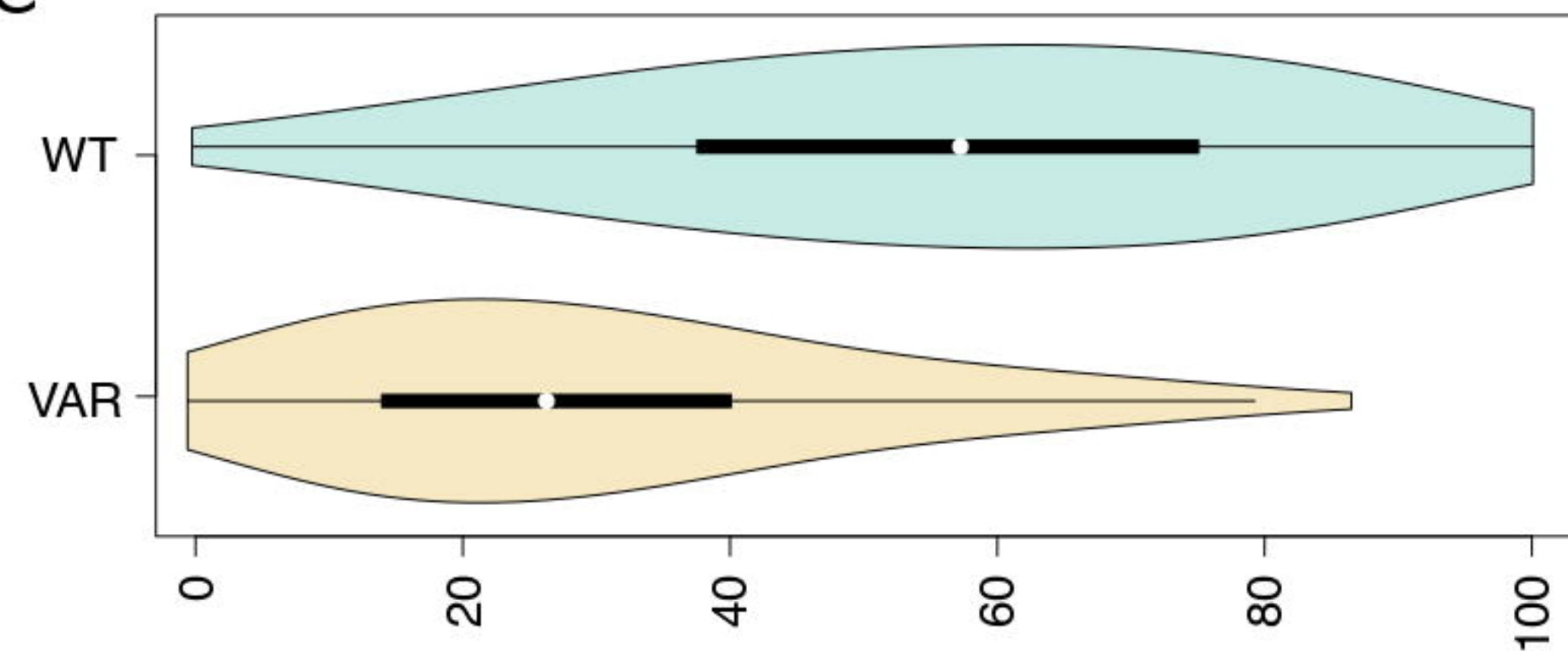
GS2

GS5

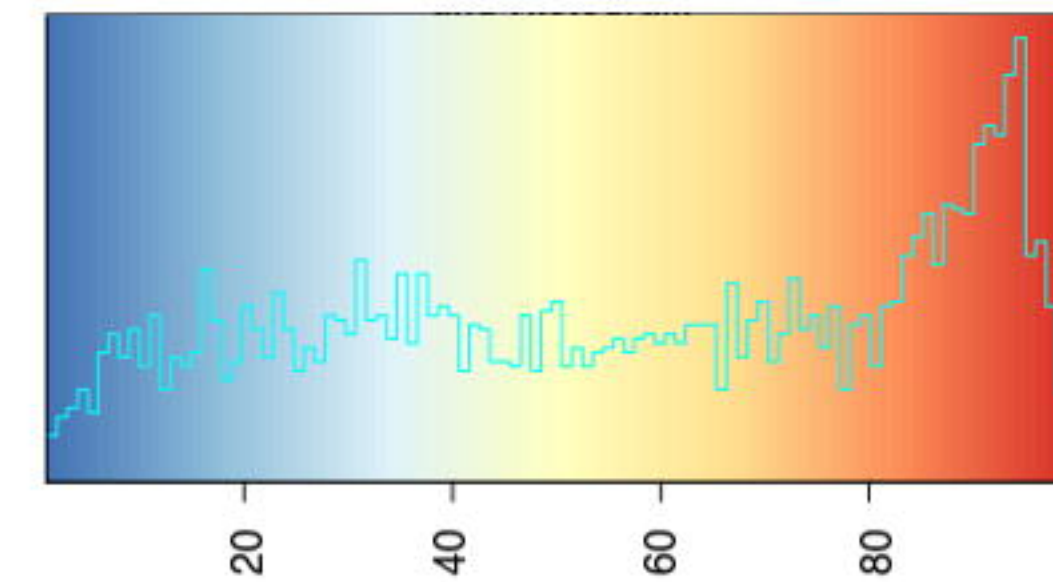
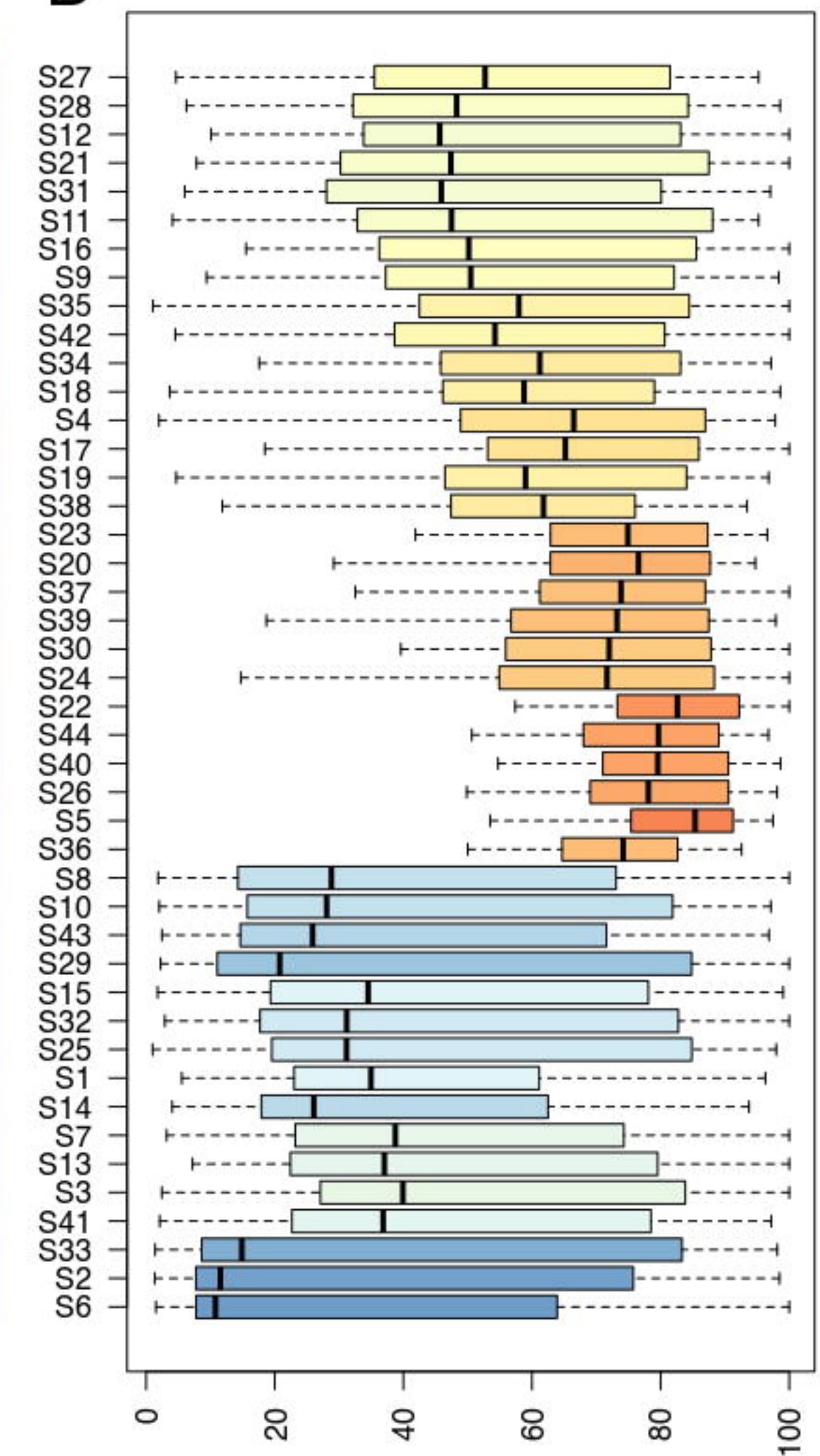
GS6

A1 VAR
 A2 WT
 A3
 B1
 C2
 D1

C



B



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