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The extent of mRNA editing is limited in chicken liver and adipose, but impacted by tissular context, genotype, age and feeding as exemplified with a conserved edited site in COG3

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ABSTRACT

RNA editing is a post-transcriptional process leading to differences between genomic DNA and transcript sequences, potentially enhancing transcriptome diversity. With recent advances in high-throughput sequencing, many efforts have been made to describe mRNA editing at the transcriptome scale, especially in mammals, raising contradictory conclusions regarding the extent of this phenomenon. We show by a detailed description of the 25 studies focusing so far on mRNA editing at the whole-transcriptome scale that systematic sequencing artifacts are somehow considered in most studies, whereas biological replication is often neglected and multi-alignment not properly evaluated, which ultimately impairs the legitimacy of results. We recently developed a rigorous strategy to identify mRNA editing using mRNA and genomic DNA sequencings, taking into account sequencing and mapping artifacts, and biological replicates. We applied this method to screen for mRNA editing in liver and white adipose tissue from 8 chickens and confirm the small extent of mRNA recoding in this species. Among the 25 unique edited sites identified, 3 events were previously described in mammals, attesting this phenomenon is conserved through evolution. Deeper investigations on 5 sites revealed the impact of tissular context, genotype, age, feeding condition and sex on mRNA editing levels. More specifically, this analysis highlighted that the editing level at the site located on *COG3* was strongly regulated by 4 out of these factors. By comprehensively characterizing the mRNA editing landscape in chicken, our results highlight how this phenomenon is limited, and suggest a regulation of editing levels by various genetic and environmental factors.

INTRODUCTION

RNA editing has become a generic term for a wide array of post-transcriptional processes changing the mature RNA sequence relatively to the corresponding encoding genomic DNA matrix. This phenomenon, which is almost limited to eukaryotes with some exceptions, is characterized by nucleotide insertion, deletion or substitution in various types of RNAs, including mRNAs (Knoop 2011), tRNAs (Börner *et al.* 1996, Gott *et al.* 2010, Torres *et al.* 2014), miRNAs (Warnefors *et al.* 2014) and rRNAs (Eifler *et al.* 2013, Valach *et al.* 2014) and is likely to contribute to RNA diversity. Until recently this mechanism was considered relatively rare in vertebrates, mainly restricted to brain-specific substrates and repetitive regions of the genome (Bass 2002), and limited to extensively validated ADAR-mediated adenosine to inosine (A-to-I) substitutions and APOBEC-mediated cytosine to uracile (C-to-U) changes (Knoop 2011).

Since 2009, the advents of high-throughput sequencing technologies enabled to study this phenomenon at a transcriptome-wide scale and progressively challenged this view with estimates ranging from several hundred (Ju *et al.* 2011, Kleinman *et al.* 2012) to several thousand (Clop *et al.* 2006, Li *et al.* 2011, Bahn *et al.* 2011, Peng *et al.* 2012, Ramaswami *et al.* 2012, Park *et al.* 2012, Chen 2013, Kang *et al.* 2015) and even million (Bazak *et al.* 2013) mRNA edited sites throughout mammalian genomes. According to some of these mRNA editing screening studies, mRNA recoding appears as an extremely common process greatly contributing to transcript diversity. Furthermore, most of these studies report mRNA editing events leading to transversions that cannot be explained in the light of our current knowledge regarding the molecular bases of mRNA recoding (Li *et al.* 2011, Ju *et al.* 2011, Bahn *et al.* 2011, Peng *et al.* 2012, Chen 2013, Kang *et al.* 2015), suggesting the existence of so far uncharacterized mRNA editing mechanisms, and novel molecular components implied

in gene expression regulation. The conclusions raised by these studies regarding the extent and the nature of mRNA recoding, if further supported, would deeply impact our understandings of gene expression regulation and transcriptional modification.

Facing contradictory results regarding the extent of mRNA editing, a large number of studies and comments pointed the requirement of comprehensive and rigorous bioinformatics pipelines to limit technical artifacts in the editome characterizations (Schrider *et al.* 2011, Kleinman and Majewski 2012, Lin *et al.* 2012, Pickrell *et al.* 2012, Kleinman *et al.* 2012, Piskol *et al.* 2013, Lagarrigue *et al.* 2013). Working with short-read sequencing data for the detection of polymorphisms requires to carefully deal with technical artifacts related to mapping on paralogous or repetitive regions (Malhis and Jones 2010, Treangen and Salzberg 2012), mapping errors at splice sites (Park *et al.* 2012), or systematic and random sequencing errors (Nakamura *et al.* 2011, Meacham *et al.* 2011). This is especially the case when screening for mRNA editing events, since all of these artifacts are likely to generate artificial discrepancies between genomic DNA and mRNA further interpreted as edited sites. In this context, the huge variation regarding the extent of intra-tissue and intra-species mRNA editing revealed in literature could be in part due to the varying level of stringency of bioinformatics filters used to control these error prone artifacts, and whether biological replication is considered or not.

As shown in Table 1, most of the 25 RNA-seq based mRNA editing screening studies performed on vertebrates are not considering matched genomic DNA sequences to detect mRNA recoding, but rather consider either a consensus genomic sequence for the species studied, or EST databases to remove false positives arising from potential genomic polymorphisms, therefore occulting unreferenced individual variations (Bahn *et al.* 2011,

Danecek *et al.* 2012, Gu *et al.* 2012, Ramaswami *et al.* 2012, Cattenoz *et al.* 2013, Lagarrigue *et al.* 2013, Chen 2013, Bazak *et al.* 2013, Blanc *et al.* 2014, Toung *et al.* 2014, Sakurai *et al.* 2014, Chan *et al.* 2014, Zhang and Xiao 2015). More strikingly, while it is fully admitted that filtering on minor allele frequency is required to select high-quality genomic polymorphisms (Consortium 2010, 1000 Genomes Project Consortium *et al.* 2012), some mRNA editing screening studies still consider that reproducibility across biological replicates is not a mandatory criterion for considering a difference between DNA and RNA as a reliable editing event (Picardi *et al.* 2012, Peng *et al.* 2012, Ramaswami *et al.* 2012, Kleinman *et al.* 2012, Park *et al.* 2012, Cattenoz *et al.* 2013, Chen 2013, Bazak *et al.* 2013, Sakurai *et al.* 2014, Chen *et al.* 2014, Chan *et al.* 2014, Kang *et al.* 2015, Zhang and Xiao 2015). However, as depicted on Figure 1, considering biological replication clearly impacts the total number of editing events detected in high-throughput-based screening studies, since the number of differences between DNA and RNA reported appears to be directly negatively correlated with the number of biological replicates considered. From a methodological point of view, this study proposes a rigorous strategy to identify mRNA editing using both mRNA and genomic DNA high-throughput sequencings, taking into account sequencing and mapping artifacts, as well as biological replicates, to control the false positive rate. The efficiency of this approach has already been validated in our previous study on chicken embryo mRNA editing (Frésard *et al.* 2015). To strictly control multi-mapping, we looked for mRNA sequences spanning edited sites in unmapped genomic DNA sequences, allowing to consider potential errors and gaps in the reference assembly that still represent roughly 15% of the chicken genome (Wicker *et al.* 2005, Schmid *et al.* 2015).

From a biological perspective, in addition to our recent work screening mRNA editing in chicken whole-embryo (Frésard *et al.* 2015), this study is an answer to the evident lack of transcriptome-wide mRNA editing screening investigations focusing on non-mammalian vertebrates such as birds, that could contribute to our understanding of RNA editing evolutionary bases. Indeed, as depicted in Table 1, except our works, all mRNA editing screening studies in vertebrates are focused on primates or mouse transcriptomes. To date, the origins of RNA editing are still rather obscure, and even if it is currently proposed that it may have arisen several times in different phyla throughout evolution, it remains unclear whether selection was involved or not (Gommans *et al.* 2009, Gray *et al.* 2010, Gray 2012). While chicken is extensively used as a model organism in developmental biology (Davey and Tickle 2007), it also bridges the evolutionary gap between mammals and other vertebrates and therefore stands as an ideal species to explore conservation of mRNA editing events in vertebrates throughout evolution. In addition, our knowledge related to the regulation of mRNA editing level, and to factors enhancing or repressing mRNA recoding is still limited. Hitherto, few studies has been carried out to assess whether the genetic background, the sex, the feeding condition or the age influence mRNA recoding level. Most of these studies targeted the extensively studied APOBEC-mediated C-to-U editing event occurring in mammalian *APOBEC1* mRNA revealing the influence of ethanol intake (Lau *et al.* 1995, Van Mater *et al.* 1998), insulin (Wronski *et al.* 1998), obesity (Phung *et al.* 1996), and diet (Funahashi *et al.* 1995) on *APOBEC1* mRNA editing level. Others focused on previously described ADAR-mediated editing events in primate (Li *et al.* 2013), mouse (Gan *et al.* 2006) or rat (Holmes *et al.* 2013) transcriptomes, highlighting an insulin-dependent activity of ADAR in mouse pancreas (Gan *et al.* 2006), or suggesting influence of aging on ADAR-mediated mRNA editing in human, mouse and pig (Wahlstedt *et al.* 2009, Shtrichman *et al.*

2012, Venø *et al.* 2012). A better characterization of environmental and genetic factors influencing the level of mRNA recoding would definitely offer new insights on the role of mRNA editing in vertebrates.

In this study, we report the results from the first genome-wide characterization of the chicken liver and adipose mRNA editomes, based on both genomic DNA and mRNA high-throughput sequencings. Our results confirm the low extent of mRNA recoding in chicken and the absence of non A-to-I editing event in this species, in agreement with what has already been shown for chicken embryos (Frésard *et al.* 2015). We also highlight that mRNA editing level is impacted by genetic and environmental factors such as tissular context, genotype, age, and, to a minor extent, by feeding condition and sex. As exemplified with the recoding event located on *COG3* and confirmed at other positions, the mRNA editing level is tightly depending on several environmental and genetics factors.

MATERIALS AND METHODS

Ethics Statement

Chickens were bred at INRA, UE1295 Pôle d'Expérimentation Avicole de Tours, F-37380 Nouzilly in accordance with European Union Guidelines for animal care, following the Council Directives 98/58/EC and 86/609/EEC. Animals were maintained under standard breeding conditions, and subjected to minimal disturbance. The farm is registered with the French Ministry of Agriculture under license number C37–175–1 for animal experimentation. The experiment was performed under authorization 37–002 delivered to D. Gourichon.

Tissue collection, and library preparation

Two experimental meat-type chicken lines were divergently selected for seven generations using the ratio between abdominal fat weight and whole animal weight at 9 weeks as a fattening index, while maintaining live body weight constant (Leclercq *et al.* 1980). After selection, the two lines were maintained by carefully limiting inbreeding. Four 9 week-old males from the 35th generation in each line were slaughtered by electronarcosis and immediate bleeding. Liver and abdominal adipose tissue were then harvested and stored in nitrogen. Liver genomic DNA and total liver and adipose RNA were concurrently extracted according to the manufacturer's instructions using the AllPrep DNA/RNA Mini Kit (Agilent, Agilent Technologies, Santa Clara, CA). RNA quality was assessed on a BioAnalyzer 1000 (Agilent Technologies, Santa Clara, CA) and RIN (RNA Integrity Number) ≥ 9 were required.

Sequencing

RNA sequencing

Libraries with a mean insert size of 200 bp were prepared according to the manufacturer's instructions for RNA-seq library preparation, selecting polyadenylated mRNA using the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA) from each sample. Samples were tagged using a barcode sequence for subsequent identification, amplified by PCR and quantified by qPCR using the QPCR Library Quantification Kit (Agilent Technologies, Santa Clara, CA). A total of 16 libraries were sequenced in paired-ends 2 x 101 bp in triplicates on 3 different lanes on the Illumina HiSeq 2000 sequencer using the TruSeq PE Cluster Kit v3 (Illumina, San Diego, CA), the cBot SBS Kit v3 (Illumina, San Diego, CA) and the TruSeq SBS Kit v3 (Illumina, San Diego, CA). After quality checks and adapter trimming using CASAVA 1.8, matched libraries for a given sample were merged. Liver and adipose mRNA-seq raw data are available on Sequence Read Archive under accession SRP042257.

DNA sequencing

Liver DNA from the 8 animals was sequenced in paired-ends 2 x 101 bp on 4 lanes on an Illumina HiSeq 2000. Library preparation, DNA quantification and sequencing were performed according to manufacturers' instructions using TruSeq DNA Sample Prep Kit (Illumina, San Diego, CA), Agilent QPCR Library Quantification Kit (Agilent Technologies, Santa Clara, CA), TruSeq PE Cluster Kit v3 (Illumina, San Diego, CA) and cBot TruSeq SBS Kit v3 (Illumina, San Diego, CA). After quality checks, and adapter trimming using CASAVA 1.8, matched libraries for a given sample were merged. Liver genomic DNA-seq raw data are available on Sequence Read Archive under accession SRP042641.

Computational analyses

When not specified, analyses were performed with in-house Perl and R scripts.

Genomic sequences analyses

208 DNA sequences were aligned to the latest chicken genome assembly (Galgal4) using *BWA*
209 *v0.7.0* (Li and Durbin 2009) (*Command:* bwa aln). Sequences were then filtered based on
210 mapping quality (*Command:* samtools view -bS -q 30). *SAMtools v0.1.19* (Li *et al.* 2009a)
211 *rmdup* (*Command:* samtools rmdup) was used to remove possible PCR and optical
212 duplicates.

213 *mRNA sequences analyses*

214 mRNA sequences were aligned with *Tophat v2.0.5* (Kim *et al.* 2013) on the chicken reference
215 genome Galgal4 as described in (Frésard *et al.* 2014) (*Command:* tophat --min-intron-length
216 3 --max-intron-length 25000 --max-deletion-length 1 -mate-inner-dist 200 --read-realign-
217 edit-dist 0 --microexon-search). Uniquely mapped unduplicated sequences with a mapping
218 quality greater than 30 were selected, using *SAMtools v.0.1.19* (*Command:* samtools view -
219 bS -q 30) and in-house Python script.

220 **Identification of mRNA editing candidates**

221 Sequences were locally realigned and recalibrated before SNP detection, with *GATK v1.6.11*
222 for DNA (Van der Auwera *et al.* 2013) (*Commands:* GATK -T RealignerTargetCreator -R; GATK
223 -T BaseRecalibrator -R -knownSites; GATK -T PrintReads -R -BQSR), and *BamUtil* (*Command:*
224 bam recab) for RNA.

225 *SAMtools v0.1.19 mpileup* was used to detect SNPs between DNA and RNA samples from
226 each individual (*Command:* samtools mpileup -d 10000). We set a maximum coverage of
227 10,000 reads in pileup for each calling to take into account as many reads as possible in the
228 calling. SNPs were detected independently on each biological replicate. VCF files generated
229 by *SAMtools mpileup* were then used for subsequent analysis. For each biological replicate,
230 only variations where DNA was homozygous either for the reference allele or for the

alternative allele (MAF=1), and where RNA was heterozygous or homozygous for the alternative allele, were kept. Finally, we removed positions covered by less than 15 reads in both DNA and RNA alignments as well as tri-allelic sites.

Impact of biases on mRNA editing detection

To explore whether each editing event was likely related to sequencing errors or alignment artifacts, we developed custom R and Perl scripts. We computed information related to: (1) Extremity bias: an editing event was considered as biased if the edited allele was mostly supported by the 10 first or last bases of reads in the RNA-seq read pileup. In accordance with previous studies (Kleinman and Majewski 2012, Frésard *et al.* 2015), we chose to consider only the distribution of the edited nucleotide position, to increase the stringency of the method; (2) Strand bias: an editing event was considered as biased if the proportion of forward and reverse reads supporting it was markedly different ($\Delta > 0.5$); (3) Splice junction bias: an editing event was considered as biased if it was located within the region of a predicted splice site, *i.e.* within 1-3 bases of the exon or 3-8 bases of the intron. To perform this analysis, we determined the annotation and localization of editing events in transcripts using *Ensembl v71 Variant Effect Predictor* (McLaren *et al.* 2010); (4) Homopolymer and low complexity bias: an editing event was considered as biased if the 4 neighboring positions harbored the same nucleotide or if it was falling in a single sequence repeat (SSR). SSR were identified using *SciRoKo* (Kofler *et al.* 2007). The SSR patterns were investigated near candidate edited sites with an offset of ±3 bases; (5) Multimapping bias: for each editing events, we generated a consensus 40 bp sequence centered on the edited allele, based on the pileup of RNA-seq reads harboring the edited alleles in a given sample. We then used *fuzznuc* (Olson 2002) to search for this sequence throughout the whole genomic DNA-seq reads including unaligned reads from the same sample. An editing event was therefore

considered as biased if we found any match between its consensus surrounding sequence and genomic DNA-seq reads.

Impact of biological replication on mRNA editing detection

For each tissue independently, we explored how reproducible each unbiased editing event was across the 8 samples using custom R scripts. At each step, we computed the overall amount of events belonging to each class of substitution from DNA to RNA. Since our sequencing libraries were not strand-aware, the complement substitution of canonical editing events (*i.e.* A-to-G for ADAR-mediated editing and C-to-T for APOBEC-mediated editing) were also considered as canonical (*i.e.* C-to-T and G-to-A respectively).

Validation assays and editing yield quantification

DNA Sanger sequencing and RNA pyro-sequencing

To assess whether the DNA genotype of candidate editing events was homozygous or not, we performed Sanger sequencing on the liver genomic DNA from the 8 animals. We then assessed the mRNA genotype at these candidate sites on a PyroMark Q24 pyro-sequencer (Qiagen, Valencia, CA). Primers were designed with PyroMark Assay Design software (Table S2). PCR products were prepared using PyroMark PCR Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Data were analyzed with PyroMark Q24 v1.0.10 using default parameters.

Experimental designs used to test the effect of age, sex, genetic background and feeding on mRNA editing level

To measure the impact of age, sex, genotype and feeding on mRNA editing level, we used different independent experimental designs with animals contrasted for these factors: (1)

Genotype: broilers (N=8) and layers (N=8) in two unrelated experimental designs (*i.e.* N=2 x 16 in all); (2) Age: prepuberal (N=8) and postpuberal (N=8) layers; (3) Feeding: broilers slaughtered after a 24h fasting (N=8) or broilers fed *ad libitum* (N=8) in two independent studies (*i.e.* N=2 x 16 in all); (4) Sex: female (N=8) and male (N=8) layers. Liver DNA and liver RNA and/or adipose RNA were extracted and quality checked as described before (see *Tissue collection and library preparation*). We then performed both liver genomic DNA and liver and/or adipose mRNA pyro-sequencing following the aforementioned procedure for 5 edited sites (*i.e.* 2 liver specific, 1 adipose specific, 2 common to both tissues) previously validated in our main experimental design. The pyro-sequencing signal at the edited position in mRNA was standardized according to the signal obtained on DNA to avoid amplification and sequencing biases. The editing level was computed as the ratio between signals for the mRNA edited allele and allele on genomic DNA. We finally tested the effect of each factor on RNA editing level at the 5 selected sites using two-sided unpaired homoscedastic Student *t*-test. Statistical analyses were performed on R 3.2.0 using *t.test* (two sided unpaired homoscedastic Student *t*-test) functions from the *stats* package and graphical visualizations plotted using the *ggplot2* package.

In silico prediction of RNA editing impact on protein structure and function

To predict the putative effect of RNA editing on protein structure and function, we first identified genomic structures likely to be impacted using Ensembl v71 Variant Effect Predictor (McLaren *et al.* 2010). Focusing on missense coding editing events, we then recovered orthologous protein sequences from *Gallus gallus*, *Bos taurus*, *Rattus norvegicus*, *Mus musculus* and *Homo sapiens* to carry out multi-alignment. We finally used SIFT prediction tool (Kumar *et al.* 2009) that is based on both sequence homology and physical

300 properties of amino acids, to quantify the potential impact of coding editing events on

301 protein structure and function.

RESULTS

High-throughput sequence analyses

Liver DNA and liver and white adipose tissue RNA were extracted from 8 16-week-old male chickens, and paired-end sequenced on an Illumina HiSeq2000. After alignment on the current genome assembly Galgal4, and filtering on mapping quality and multi-mapping, we conserved an average of 157M DNA-seq reads, 30M liver mRNA-seq reads and 38M adipose RNA-seq reads per sample. On average, 93.5% of the genome was covered by at least 15 DNA-seq reads, and 14.3% and 18.4% by at least 15 mRNA-seq reads from liver and white adipose tissue, respectively.

mRNA editing detection and initial filtering

For each biological replicate, a base modification A (DNA base) → B (RNA base) was considered as a candidate mRNA editing event if: (1) the genotype inferred for the genomic DNA was homozygous AA with a minor allele frequency equal to 1 (*i.e.* all the reads are supporting a unique allele on the genomic DNA sequence); (2) the genotype inferred for the mRNA sequence was bi-allelic heterozygous AB or homozygous BB; (3) the position was covered by at least 15 reads of both genomic DNA and mRNA sequences; and (4) the mRNA editing event did not imply an insertion or deletion event. A total of 3,229 and 2,305 positions met these criteria in white adipose tissue (WAT) and liver, respectively (Figure 2).

Impact of sequencing and mapping artifacts on false editing discovery rate

In order to increase accuracy in the detection of editing events and to reduce the amount of false positives, a standard procedure consists to apply different *ad hoc* filters to remove suspicious candidates presenting error-prone splice junction bias, strand bias, extremity bias, splice bias, or repetitive region bias. We conducted a first analysis aimed at assessing

the amount of candidate mRNA editing events that were spurious with respect to each of these biases. This revealed that splice junction bias concerned about 15% of candidates in both WAT and liver (Figure 2). While the amount of repetitive region biased mRNA editing sites exceeded 30% of the candidate positions, this value increased to more than 45% considering strand or extremity biased positions (Figure 2) in both tissues. Considering altogether these sequencing and mapping artifacts, we showed that more than 85% candidates were subject to at least one source of bias. Thus, using these classical filters (*i.e.* usually applied in editing screening studies), the amount of candidate mRNA editing events dropped from 3,229 and 2,305 to 448 and 342 in WAT and liver respectively

Correction for multi-mapping based on DNA-seq raw sequences

Even if somehow taken into account during or after mRNA-seq read mapping procedure with splice-aware aligners such as TopHat or STAR, the reads multi-mapping may still have a great impact on mRNA editing false discovery rate because of gaps and miss-assemblies in the reference genome. To carefully control this artifact, we used the approach we first introduced in our previous study (Frésard *et al.* 2015), consisting in aligning back mRNA-seq reads harboring a candidate mRNA editing sites on corresponding individual DNA-seq reads, independently if they were aligned or not onto the genome. We revealed that among the 448 and 342 remaining candidates, 4.2% and 7.8% were multimapping-related false positives in WAT and liver respectively (Figure 2). Finally, considering both previously described filters and this last filter dealing with multi-mapping, we ended up with 429 and 315 unbiased mRNA editing sites in WAT and liver, respectively.

Impact of biological replication on canonical mRNA editing event identification

As previously mentioned in the introduction, the number of biological replicates (N) taken into account in mRNA editing screening studies based on RNA-seq data is highly variable, with N ranging from 1 to 6 for the study conducted by Hu and collaborators (Hu *et al.* 2015). As depicted on Figure 1, when the number of biological replicates is lower than 2, the total number of editing events is extremely variable, ranging from 15 (Picardi *et al.* 2012) to 1,586,270 (Bazak *et al.* 2013). This number decreases drastically between 40 (Frésard *et al.* 2015) and 253 (Gu *et al.* 2012) when N≥3, suggesting that considering biological replicates partly counteracts the lack of filters dealing with sequencing and mapping artifacts. Nevertheless, a substantial variability remains between studies, likely related to the differences in artifacts considered and stringency of bioinformatics filters and to the biological context (*e.g.* tissue, species) of each study.

With this observation in mind, we characterized the 429 and 315 mRNA editing events previously detected according to the number of biological replicates they were detected in, and the class of base substitutions they belong to. We differentiated canonical mRNA editing events (A-to-I and C-to-U interpreted as A-to-G and C-to-T by genome analyzers, and corresponding to editing events catalyzed by ADARs and APOBECs) from non-canonical events that are not explained by any of the two known editing mechanisms. As the RNA-seq libraries used in this study were not strand-specific, we considered complement bases of canonical changes as canonical editing events too (*i.e.* T-to-C and G-to-A). As can be seen in Figure 3, in the sets of 429 and 315 unbiased mRNA editing events detected in at least one individual described above, the amount of transversions (*i.e.* pyrimidine-to-purine, and purine-to-pyrimidine, 54.1% in WAT and 58.0% in liver) was greater than the amount of transitions (*i.e.* pyrimidine-to-pyrimidine, and purine-to-purine). Adding restriction based on

the number of biological replicates these events must be detected in, the amount of transversions progressively decreased from more than 50% considering no replication (N=1), to 25% (N=2) and finally to 10% (N=3) requiring editing events to be detected in at least 3 replicates. Considering that mRNA editing events were reproducible across at least 3 biological replicates, we finally conserved 19 and 11 positions in WAT and liver respectively, comprising 1 non-canonical transversion event in each tissue (Table 2), distributed across 13 chromosomes (Figure 4). Among these 27 unique events, 3 were common to both tissues. While most of these events were spatially isolated from each other, some of them were clustered in short genomic regions spanning a few bp (Figure 4), especially on chromosome 1 (3 mRNA editing events in a window of 1.391 bp downstream *NOX4* in WAT, and 2 in a window of 26 bp downstream *MPZL1* and *BRP44* in liver) and chromosome 12 (2 mRNA editing events in a window of 951 bp in *FLNB*).

Validations of candidate mRNA editing events

To assess the validity of some of the edited positions detected using the high-throughput screening approach, we performed Sanger sequencing on DNA to confirm their homozygous genotype and pyrosequencing on RNA to validate the mRNA base recoding at the corresponding position on the transcriptome. The validation revealed that non-canonical mRNA editing events were false positives either related to genomic SNPs undetected using genomic DNA-seq data for the one in WAT, or to unbalanced allelic expression not detected through mRNA-seq data for the one in liver (Table 2). Following the same approach, we selected 5 canonical events for validation: 2 out of the 3 candidates detected in both tissues, 2 specific to WAT, and 1 liver-specific. We first confirmed their homozygous status on genomic DNA and the mRNA recoding at these positions was then confirmed by mRNA pyrosequencing (Table 2) using samples from the tissue they were detected in.

Functional characterization of liver and white adipose editomes

Functional annotation of the 25 unique canonical mRNA editing events using Variant Effect Predictor revealed that most of them were located in non-coding regions, since 81% and 70% were situated in either 5kb gene flanking regions, intronic regions or intergenic regions in WAT and liver, respectively (Figure 5). Only 5 unique mRNA editing events were annotated as coding. Among these, 4 were likely to impact the mature protein: 1 common to both tissues on *COG3*, 2 WAT-specific on *CES1* and *FLNB*, and 1 liver-specific on *KCMA1* (Table 2). Focusing on these 4 missense mRNA editing events, we conducted a fine functional annotation analysis using the SIFT software to assess the impact of the amino acid substitution on these proteins. This revealed that none of these mRNA editing events was likely to be deleterious. Nevertheless, after carrying out multiple species protein alignments considering *Gallus gallus, Bos taurus*, *Rattus norvegicus*, *Mus musculus* and *Homos sapiens*, we showed that except for *CES1*, these missense mRNA editing events were impacting highly conserved amino acid residues (Figure 6).

Impact of genetic background, age, sex, feeding and tissular context on editing level

To test the impact of genetic background, age, feeding condition and sex on the mRNA editing level, we considered the aforementioned subset of 5 validated canonical mRNA editing events. We tested the effect of (1) the genotype, comparing mRNA editing level in liver and WAT between broilers and layers in two independent experimental designs (Figure 7A); (2) the age, comparing mRNA editing level in liver between prepuberal and postpuberal chickens (Figure 7B); (3) the feeding condition comparing mRNA editing level in both tissues between chickens slaughtered after a 24h fasting and feeding *ad libitum*, in two independent experimental designs (Figure 7C); (4) the sex, comparing mRNA editing level in liver between roosters and hens (Figure 7D). To test each effect, we performed both

genomic DNA Sanger sequencing and mRNA-derived cDNA pyro-sequencing on 8 independent biological replicates in each group. Our analysis on liver revealed a significant effect of genotype on mRNA editing level in the first design for 3 different positions among the 4 tested (*p*-values: 1.26×10^{-5}, 1.52×10^{-3} and 1.33×10^{-6}, Figure 7A and Table S1). In WAT, we highlighted 1 out of the 3 tested positions for which the editing level was significantly different between broilers and layers (*p*-value: 9.44×10^{-3}, Figure 7A and Table S1). Interestingly, while the general tendency is a greater editing level in broilers in comparison to layers in the liver (*COG3, PLA1A and MYO1B*), this trend is reversed for the highlighted site in WAT (*NDUFS6*). Regarding the age, we observed a significant effect on editing levels for two sites in liver (Figure 7B and Table S1). Concerning the effect of sex, one mRNA editing event showed a significant increase of mRNA editing level between males and females in liver (*p*-value: 1.90×10^{-3}, Figure 7D and Table S1). Finally, by analyzing the effect of the feeding condition, we highlighted a significant increase of the mRNA editing level after a 24h fasting for one site in liver in two independent experimental designs (Figure 7C and Table S1). Noticeably, for the edited position located on *COG3*, the mRNA recoding level was significantly impacted by genetic background, age and feeding.

DISCUSSION

To achieve whole-transcriptome screening for mRNA editing events in chicken liver and adipose tissue, we detected discrepancies between genomic DNA and mRNA sequences using matched genomic DNA-seq and mRNA-seq data in several biological replicates. Since 2009 (Li *et al.* 2009b), similar approaches based on mRNA-seq have been extensively used to characterize mouse, human and chimpanzee editomes in different tissues. According to the literature, the extent of mRNA editing is highly variable with estimates ranging from dozens to millions, even when comparing studies focusing on the same tissue in the same species. After an in-depth reading of mRNA editing screening studies, we highlighted that, despite recommendations for the use of rigorous bioinformatics pipelines to characterize editomes (Kleinman and Majewski 2012, Lin *et al.* 2012, Pickrell *et al.* 2012), many recent studies neglected the most reviewed sequencing and mapping artifacts related to mRNA-seq, such as strand bias, read extremity bias, splice junction bias and low complexity region bias (Table 1). In our study, these random or systematic biases were each impacting between 13% and 50% of the initial set of differences between DNA and RNA we detected. Overall, we showed that almost 90% of the candidate mRNA editing events initially detected were likely to be false positives arising from one of these artifacts, revealing how huge their impact is on false discovery rate as previously reported (Pickrell *et al.* 2012, Lagarrigue *et al.* 2013). Interestingly, the false positive rate we report is in agreement with the observations of Pickrell and collaborators (Pickrell *et al.* 2012), suggesting that among the 28,766 editing events detected in the study of Li and collaborators (Li *et al.* 2011), roughly 90% were likely false positives emerging from sequencing errors and mapping artifacts.

Even if taken into account during mRNA-seq read mapping procedures with splice-aware aligners, multi-mapping artifact could still be of a great impact on mRNA editing false

458 discovery rate. Indeed, current genome assemblies used to map short reads from high-
459 throughput sequencing experiments, even of high quality for human, mouse or chicken, are
460 still presenting missing sequences as well as misassembled regions (Groenen *et al.* 2011,
461 Genovese *et al.* 2013a, 2013b). Since these regions might harbor sequences that are
462 paralogous to properly assembled parts of genomes, this ultimately leads to shallow
463 identification of multimapped short reads when only considering the reference sequence.
464 To by-pass this error-prone issue for the identification of mRNA edited sites, we used in this
465 study the approach first introduced in our previous study (Frésard *et al.* 2015) consisting in
466 looking for mRNA sequences spanning edited sites in raw genomic DNA sequences, and
467 confirmed its efficiency. In our study, up to 8% of the initial candidate differences between
468 RNA and DNA were false positives related to errors and assemblies issues in the chicken
469 genome. This result suggests that thousands of edited sites reported in primates and mice
470 mRNA editing screening studies could be partly attributed to false positives resulting from a
471 spurious handling of multimapped mRNA-seq short reads. More strikingly, a huge
472 proportion of mRNA editing screening studies are based on mRNA-seq data solely, and do
473 not consider individual matched genomic DNA-seq data for the samples analyzed. In these
474 studies, candidate edited sites are filtered using the positions of known SNPs referenced in
475 databases such as dbSNP, rather than considering individual polymorphisms, thereby fully
476 occulting inevitable individual specific genomic variations. Even in the case of clonal mice
477 strains, considering a consensus strain-specific genomic sequence as exposed by Danecek
478 and collaborators (Danecek *et al.* 2012) indubitably leads to edited events false calls that
479 arise from somatic mutations, as we previously shown in our study on mice, invalidating
480 25% of mRNA editing candidates arising because of unreferenced genomic SNP (Lagarrigue
481 *et al.* 2013).

To further limit the amount of false positives among mRNA edited sites and to focus on biologically meaningful mRNA editing events, an obvious approach consists in considering biological replication. Surprisingly, a lot of mRNA editing screening studies report events without considering reproducibility across samples (Table 1, Figure 1). Although it is likely that mRNA editing is a partly individual-specific phenomenon (Gommans *et al.* 2009), short read sequencing technologies are error-prone when it comes to focus on slight variations and are not mature enough to allow investigation of private editing events. Therefore, biological reproducibility is uncontestably required in this scope. In our study, even after filtering to properly account for systematic and random sequencing artifacts as well as multimapping, we were still detecting more than 50% of non canonical mRNA editing events. Hitherto, the attempts by other group to validate such type of mRNA recoding using targeted Sanger sequencing were unsuccessful (Piskol *et al.* 2013), clearly ascertaining that they arise from unconsidered artifacts. When focusing only on editing events detected in at least 3 biological replicates, the proportion of canonical events raised 90% in the present work. If non-canonical recoding events were not related to artifacts, we would have not expected such an enrichment, which further ascertains they are false positives. With respect to this hypothesis, we invalided the 2 non-canonical events we were still detecting after considering biological replication. Overall, our results suggest that filtering to consider false positives arising from mapping artifacts and sequencing errors, even if mandatory, is not sufficient to remove all spurious editing events. While allowing focusing on the most biologically meaningful recoding events that are shared between individuals, considering biological replications is decisive to contain the amount of false positives in RNA editing screening studies based on current high-throughput sequencing technologies.

505 Altogether, considering filters dealing with systematic and random sequencing errors,
506 multimapping, mapping artifacts and biological replication, the number of edited sites
507 dramatically felt from 3.229 and 2.305 candidates to 19 and 11 robust events in WAT and
508 liver respectively. And even using highly drastic filters, two non-canonical false positives
509 were still detected, once again suggesting that hard filtering and biological replication are
510 still mandatory when working with current short-sequencing technologies. Even if a greater
511 sequencing depth would have allowed to detect a slightly higher number of edited sites, our
512 work reveals that the extent of mRNA editing is, at least in chicken, far below what has been
513 previously shown in most screening studies on human, mouse and chimpanzee.
514 Interestingly, most of the studies reporting an amount of mRNA editing events close to what
515 we record have been conducted on healthy tissues rather than immortalized cell lines or
516 tumors, and considered only sites edited in at least 2 biological replicates (Gu *et al.* 2012,
517 Lagarrigue *et al.* 2013, Frésard *et al.* 2015). The huge variation in mRNA editing extent
518 between our study and other screening studies in literature could be explained in different
519 ways. First of all, it is likely related to the differences in the stringency of filters applied and
520 in the false positive rate. Second, most of the mRNA editing screening studies were carried
521 out using transformed cell lines or cancer tissues (Table 1) and the extensive mRNA editing
522 reported may reflect real biological changes. Indeed, ADARs and APOBECs may become
523 more active during tumorigenesis, and may consequently increase mRNA editing, as it has
524 been highlighted in some cancer cell lines (Galeano *et al.* 2012). Third, it could also be
525 explained by the huge structural differences between mammalian and sauropsidian
526 genomes. Actually, ADAR-mediated A-to-I mRNA editing occurs in regions of double
527 stranded RNA (dsRNA). Yet, approximately half of typical mammalian genome contains
528 highly repetitive sequences (de Koning *et al.* 2011) such as retrotransposons, short

529 interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs).
530 While these sequences are often repeated in reverse tandems, they may generate dsRNA
531 structures that could be subsequently edited by ADARs (Nishikura 2010). In chicken, since
532 the amount of repetitive sequences across the genome falls below 15% (Wicker *et al.* 2005,
533 Schmid *et al.* 2015), it is expected that less A-to-I editing events occur.

534 At the end, among the 25 unique canonical mRNA editing events we report, only 3 (*i.e.* 13%)
535 located downstream *MPZL1* and *BRP44*, in *COG3* and upstream *NDUSF6*, are common to
536 both tissues. Comparisons with our previous study highlighted that only 4 mRNA edited sites
537 detected in chicken whole-embryo were also found in mature WAT or liver. Surprisingly,
538 only the edited sites located in *COG3* and upstream *NDUFS6* were common to WAT, liver
539 and whole embryo. These results are comparable to those reported by Danecek *et al.* and
540 Lagarrigue *et al.*, revealing a significant amount of tissue-specific edited sites (Danecek *et al.*
541 2012, Lagarrigue *et al.* 2013). Interestingly, while no homologue of *APOBEC1* has been
542 characterized in the chicken genome (Conticello *et al.* 2005), all the APOBEC-mediated C-to-
543 U mRNA editing candidate sites we initially detected were discarded along the filtering
544 pipeline, confirming that this specific mRNA editing mechanism is missing in chicken. We
545 also found that some of the mRNA editing events we detected were localized on mRNA
546 editing clusters, spanning regions of a few kilobases. This observation is supported by our
547 current knowledge regarding the mechanistic basis of ADAR-mediated A-to-I mRNA editing,
548 which occurs unspecifically in dsRNA, and doesn't involve specific mooring sequence as it is
549 the case for APOBEC-mediated C-to-U mRNA editing (Nishikura 2010).

550 Overall, most of the mRNA editing events we detected are falling in non-coding regions (*i.e.*
551 10 kb upstream or downstream genes, in introns, or in intergenic regions). Since these

552 regions are expressed, they are either corresponding to poorly annotated genomic regions,
553 non-mature mRNAs, or unannotated non-coding RNAs in which RNA editing is known to
554 occur (Picardi *et al.* 2014). Out of these 25 unique mRNA editing events, 5 are located in
555 coding sequences and only 4 are non-synonymous, impacting the sequence of *COG3*, *CES1*,
556 *FLNB* and *KCNMA1*. Interestingly, the edited sites falling in *COG3* and *FLNB* were already
557 described in mammalian species (Levanon *et al.* 2005, Shah *et al.* 2009, Danecek *et al.* 2012,
558 Holmes *et al.* 2013, Stulic and Jantsch 2013), revealing that some edited positions are
559 conserved throughout evolution between birds and mammals. Except for *CES1*, our analyses
560 show that each of these coding mRNA editing events are impacting highly conserved regions
561 in the protein sequence, as well as highly conserved amino-acid residues.

562 Our analysis finally shows that mRNA editing level is impacted by various genetic and
563 environmental factors such as the genetic background, the age, the feeding and the sex.
564 While the genetic background and the age influence the editing level at almost all the mRNA
565 editing sites we tested, the feeding and the gender tend to have effect on less positions. The
566 impact of aging on mRNA editing has already been reported in a few studies on mammals
567 (Wahlstedt *et al.* 2009, Shtrichman *et al.* 2012, Venø *et al.* 2012), as well as in our previous
568 study on chicken embryo (Frésard *et al.* 2015). In agreement with most of these studies, we
569 confirm that the level of edited transcripts increases with age, whatever the recoded site
570 considered. Nevertheless, an in-depth unbiased whole-transcriptome exploration of spatio-
571 temporal regulation bases of mRNA editing in vertebrates is still needed. We also observed
572 a significant effect of genotype on the liver mRNA editing level indicating another level of
573 regulation. Indeed, in one of the designs we used to assess the effect of genetic background,
574 3 out of 4 edited isoforms (in *COG3*, *PLA1A* and *MYO1B*) are 1.5- up to 2-fold more frequent

in broilers' liver compared to layers' liver. *PLA1A* encodes for the phosphatidylserine-specific phospholipase A1, mostly synthetized in liver, and implied in the release of free fatty acids and lysophosphatidic acid that acts as a lipid mediator in cell signaling. While it is established that this lipase does not catabolize triglycerides, its implication in global cellular process is still poorly understood (Aoki *et al.* 2002). *MYO1B* encodes for the widely expressed myosin 1B motors that functions in endocytosis, membrane trafficking, membrane retraction, and mechano-signal transduction. Even if the physiological landscape of myosin 1B is not fully depicted yet, some authors hypothesized its potential role on myogenesis (Wells *et al.* 1997, Redowicz 2007). While muscle mass stands as one of the most divergent phenotypic traits between layers and broilers, the mRNA editing in *MYO1B* could be part of the transcriptional bases leading to differences in muscle development between these strains, but it remains to test if this site is also differentially edited in muscular tissues between broilers and layers. Finally, *COG3* has a general cellular function related to the structure and function of the Golgi as further described bellow. Since the editing level at these sites located in *COG3*, *PLA1A* and *MYO1B* are differential between broilers' and layers' liver and because liver is a multi-function organ involved in many physiological processes, we hypothesized they might be implied in cellular and developmental processes leading to physiological differences between these two chicken strains. The genetic regulation of mRNA editing level at these sites could be linked to mechanisms acting in *trans*, involving ADARs, and further analyses comparing ADARs expression and activity between these two chicken genetic backgrounds are mandatory to investigate this hypothesis. But it could also be regulated by *cis*-acting mutations impacting surrounding mRNA sequence and secondary structure, as it has been recently suggested regarding mRNA recoding in drosophilia (Sapiro *et al.* 2015).

599 Whereas the extent of mRNA editing appears limited at the transcriptome scale in chicken
600 liver and white adipose, our results suggest that this phenomenon could be tightly
601 regulated. Indeed, the I/V non-synonymous recoding event impacting *COG3* is not only
602 conserved in mammals but is also under the influence of the genetic background, the age
603 and the feeding condition. For this last factor, this edited site was the only one impacted, in
604 two independent designs, which suggests that this observation is highly reliable. It is also
605 noticeable that *COG3* mRNA is almost exclusively edited in white adipose tissue with in
606 average 95% (in the "genotype" design) or 100% (in the "feeding" design) of the edited
607 isoform, in contrast with what we observed in the liver transcriptome. This further suggests
608 that these different isoforms are likely harboring different physiological functions and that
609 ADAR-mediated mRNA editing could act in a highly tissue-specific manner as previously
610 shown (Song *et al.* 2004), in a way that is similar to APOBEC1-mediated *APOB* mRNA editing,
611 which ultimately leads to the synthesis of two APOB isoforms – APOB100 in the liver, and
612 APOB48 in the small intestine – with distinct physiological functions. Since the edited site in
613 *COG3* is conserved through the evolution of vertebrates, and since it is tightly regulated by
614 multiple genetic and environmental factors, it is likely to have a functional role on the
615 encoded protein. *COG3* is one of the eight proteins of the oligomeric Golgi (*COG*) complex.
616 The *COG* complex is involved in intra-Golgi retrograde trafficking and in membrane
617 trafficking in eukaryotic cells (Loh and Hong 2004, Zolov and Lupashin 2005). Mutations
618 affecting *COG* subunits disturb both structure and function of the Golgi (Ungar *et al.* 2002)
619 and have been reported in congenital disorders of glycosylation (Kodera *et al.* 2015). These
620 different studies show an important role of the *COG* complex in eukaryotic cells. It is known
621 to be an evolutionarily conserved multi-subunit protein complex, but its exact cellular
622 function remains elusive. While this edited site in *COG3* is conserved across stages (embryos

and adult stages in chicken) and species (human, mouse, rat and chicken), it would require additional work to decipher its potential role on *COG3* functions and potentially on membrane trafficking pathways.

This study, which is complementary to our previous study conducted on chicken embryos, is the first describing the mRNA editing landscape in adult chicken. From a methodological point of view, we show how huge can be the impact of sequencing biases and mapping artifacts on the discovery of mRNA editing events if not properly considered. Moreover, we show the importance to consider biological replication with high-throughput sequencing data to filter spurious candidates, allowing focusing on the most biologically meaningful mRNA editing events. From a biological point of view, even if we cannot claim we are exhaustive, our results support the evidence that the extent of mRNA editing is limited in chicken, restricted to ADAR-mediated events. We also ascertain that some of editing sites are conserved throughout the evolution of vertebrates. Our study finally shows that mRNA editing level is strongly affected by the genetic background and age and - to a minor extent - by the feeding condition and the sex, which provides new insights into the comprehension of mRNA editing functions in vertebrates in relation to genetics and environmental components.

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TABLES

Table 1. Whole transcriptome mRNA editing screening studies in vertebrates.

Study	Species	Cells	Edited sites (N)	Matched DNA[1]	Replicates[2]	Potential biases
(Ju *et al.* 2011)	*H. sapiens*	Immortalized B cells	1,809	Yes	2/17	Splice, homopolymer, strand, extremity
(Li *et al.* 2011)	*H. sapiens*	Immortalized B cells	28,766	Yes	2/27	Splice, homopolymer, multimapping, strand, extremity
(Bahn *et al.* 2011)	*H. sapiens*	Glioblastoma cells	10,000	No	2/2	Splice, homopolymer, multimapping, strand, extremity
(Peng *et al.* 2012)	*H. sapiens*	Immortalized B cells	22,688	Yes	1/1	Splice, homopolymer, extremity
(Park *et al.* 2012)	*H. sapiens*	14 ENCODE cell lines	5,695	No	1/1	Homopolymer, extremity, multimapping
(Kleinman *et al.* 2012)	*H. sapiens*	Immortalized B cells	1,503	Yes	1/2	Homopolymer
(Ramaswami *et al.* 2012)	*H. sapiens*	ENCODE cell lines	150.865	No	1/2	Strand, multimapping
(Picardi *et al.* 2012)	*H. sapiens*	Spinal cord cells	15	Yes (Exome)	1/1	Homopolymer, multimapping, strand
(Bazak *et al.* 2013)	*H. sapiens*	16 tissues	1,586,270	No	1/1	Homopolymer, multimapping, strand
(Chen 2013)	*H. sapiens*	7 ENCODE cell lines	259,385	No	1/2	Multimapping
(Chan *et al.* 2014)	*H. sapiens*	Liver cells	20 007	No	1/3	Splice, homopolymer, multimapping, strand, extremity
(Mo *et al.* 2014)	*H. sapiens*	Prostate cancer cells	16,194	Yes	2/10	Homopolymer, multimapping, strand, extremity
(Sakurai *et al.* 2014)	*H. sapiens*	Brain cells	19,791	No	1/1	Splice, homopolymer, multimapping, strand, extremity
(Toung *et al.* 2014)	*H. sapiens*	Immortalized B cells	5,997	No	2/2	Multimapping, strand, extremity
(Zhang and Xiao 2015)	*H. sapiens*	Immortalized B cells	22,715	No	1/1	Multimapping, extremity
(Hu *et al.* 2015)	*H. sapiens*	Hepatocellular carcinoma cells	900	Yes	6/6	Splice, multimapping, strand
(Kang *et al.* 2015)	*H. sapiens*	Liver cells	485,684	Yes	1/9	Splice, multimapping, strand
(Danecek *et al.* 2012)	*M. musculus*	Brain cells	7,389	No	2/2	Homopolymer, multimapping
(Gu *et al.* 2012)	*M. musculus*	Liver, adipose and bone cells	253	No	3/3	Homopolymer, multimapping, extremity
(Lagarrigue *et al.* 2013)	*M. musculus*	Liver and adipose cells	63 and 188	No	4/6	Multimapping
(Cattenoz *et al.* 2013)	*M. musculus*	Brain cells	665	No	1/1	Splice, homopolymer, multimapping, strand, extremity
(Blanc *et al.* 2014)	*M. musculus*	Intestine and liver cells	500	No	1/1	Homopolymer, multimapping, extremity
(Chen *et al.* 2014)	*R. macaque*	Prefrontal cortex, cerebellum, muscle, kidney, heart, testis and lung cells	31,250	Yes	1/1	Homopolymer, multimapping, extremity
Frésard *et al.* 2015	*G. gallus*	Whole embryo	40	Yes	2/8	-
Roux *et al.* (The present study)	*G. gallus*	Liver and adipose cells	11 and 17	Yes	3/8	-

[1] If "yes": individual genomic DNA information is used to account for potential private individual genomic polymorphisms. If "no": potential private genomic polymorphisms are defined considering either genomic

variants databases such as dbSNPs, or strain-specific consensus genomic sequence in the case of studies based on clonal mouse strains.

[2] Ratio between the number of biological replicates considered for reporting a candidate difference between DNA and mRNA as a true mRNA editing event and the total number of biological replicates available in the study for a given cell type.

Table 2. mRNA editing screening in adult chicken liver and adipose tissue.

	Chromosome	Position	DNA allele	RNA allele	Canonical	Validation in Sanger	Validation in PyroMark	Replicates[1]	Gene names[2]	Localisation[3]
WAT	1	79605543	T	C	yes			3	PLA1A, POPDC2	Downstream gene
	1	*90873162*	T	C	yes			3	MPZL1, BRP44	Downstream gene
	1	103511385	T	C	yes			6	GRIK1	Intron
	1	***167109833***	A	G	yes	yes	yes	3	COG3	Exon (missense)
	1	169769193	A	G	yes			3	THSD1	Downstream gene
	1	187056174	A	G	yes			3	NOX4	Downstream gene
	1	187056183	A	G	yes			3	NOX4	Downstream gene
	1	187057565	A	G	yes			3	-	Intergenic
	2	***86000926***	T	C	yes	yes	yes	5	NDUFS6	Upstream
	4	17996546	T	C	yes			4	-	Intergenic
	5	22958596	T	C	yes			3	DGKZ	Intron
	11	10634278	A	G	yes			5	CES1	Exon (missense)
	11	19169664	A	C	no	no	no	3	DHODH, IST1	Upstream, Intron
	12	**8909946**	A	G	yes	yes	yes	6	FLNB	Exon (missense)
	12	8910897	A	G	yes			5	FLNB	Intron
	17	4705	T	C	yes			3	-	Intron
	17	94999	C	T	yes			6	-	Intergenic
	18	28378	T	C	yes			6	ZNF302	Upstream
	19	4812610	C	T	yes			4	CCLI8	Intron
Liver	**1**	**79608260**	T	C	yes	yes	yes	7	POPDC2, PLA1A	Downstream gene
	1	*90873162*	T	C	yes			3	MPZL1, BRP44	Downstream gene
	1	90873188	T	C	yes	yes	yes	4	MPZL1, BRP44	Downstream gene
	1	***167109833***	A	G	yes	yes	yes	5	COG3	Exon (missense)
	1	193343226	A	G	yes			6	MADPRT, ART7B	Upstream
	2	***86000926***	T	C	yes	yes	yes	5	NDUFS6	Upstream
	6	13236424	A	G	yes			3	KCNMA1	Exon (missense)
	7	**7564791**	A	G	yes	yes	yes	5	MYO1B	Intron
	8	25588113	T	C	yes			3	-	Exon (synonymous)
	28	518606	A	C	no	yes	no	3	HNRNPM	Missense
	LGE64	615592	T	C	yes			4	-	Intron

[1] Number of samples in which the mRNA editing event is detected.

[2] Name of the gene impacted by the mRNA editing event or name of the closest genes (< 10kb) if the mRNA editing event is falling in an intergenic region.

[3] Localization of the mRNA editing event inside genomic features, as predicted by Variant Effect Predictor (McLaren *et al.* 2010). If the mRNA editing event is falling inside a coding region, its impact on gene product is given in brackets.

In bold: mRNA editing candidate events subjected to Sanger genomic DNA sequencing and cDNA pyrosequencing; Underlined: mRNA editing events annotated as “coding - missense” and located in 4 different genes. In italics: mRNA editing events common to both tissues.

884 **FIGURE LEGENDS**

885 **Figure 1. mRNA editing screening studies based on high throughput sequencing in the**
886 **literature.**

887 This graph describes the numbers of mRNA editing events detected (log_{10}) across RNA-seq-
888 based screening studies as a function of the numbers of biological replicates considered to
889 declare an event as a true positive.

890 **Figure 2. Impact of sequencing and mapping biases on mRNA editing discovery.**

891 Contribution of random or systematic sequencing biases and mapping artifacts to the false
892 discovery of mRNA editing events using combined mRNA and DNA sequencings.

893 **Figure 3. Impact of biological replication on mRNA editing discovery.**

894 Distribution (in %) of unbiased mRNA editing events across the 12 classes of substitution
895 according to the number of replicates they are detected in, ranging from N=1 to N=3, in
896 white adipose tissue (WAT) and liver. The first two classes (AtoG and TtoC) are associated to
897 ADAR-mediated RNA editing, and the next two ones (CtoT and GtoA) to APOBEC-meditated
898 RNA editing. At the top-right of each graph, the total number of RNA editing events
899 detected for a given number of replicates is shown.

900 **Figure 4. Position of mRNA editing events across the chicken genome.**

901 **Figure 5. Distribution of mRNA editing events across genomic features.**

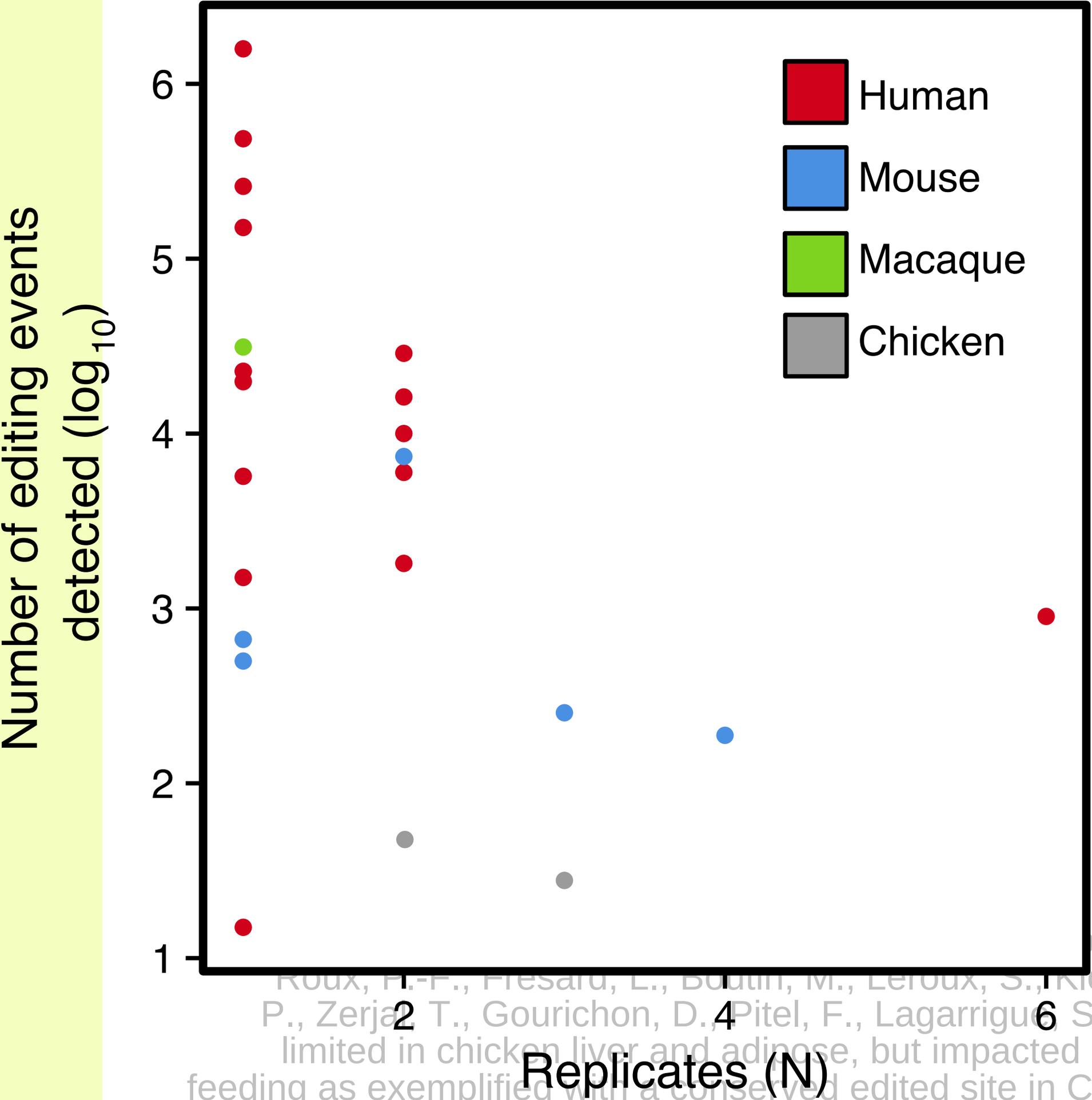
902 Annotations were assessed using Ensembl v71 Variant Effect Predictor (McLaren *et al.*
903 2010).

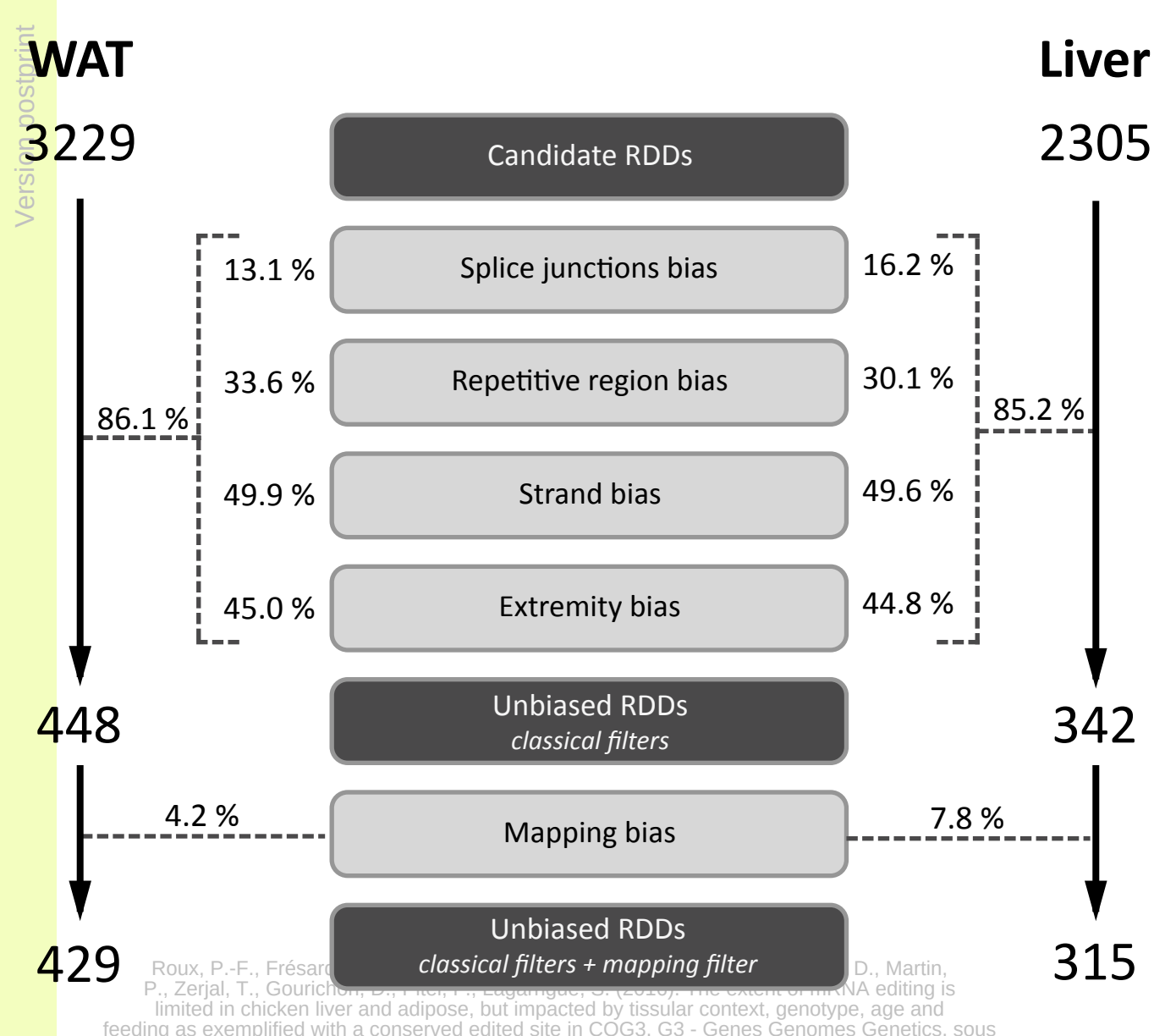
904 **Figure 6. Multispecies protein sequence alignments for coding mRNA editing events.**

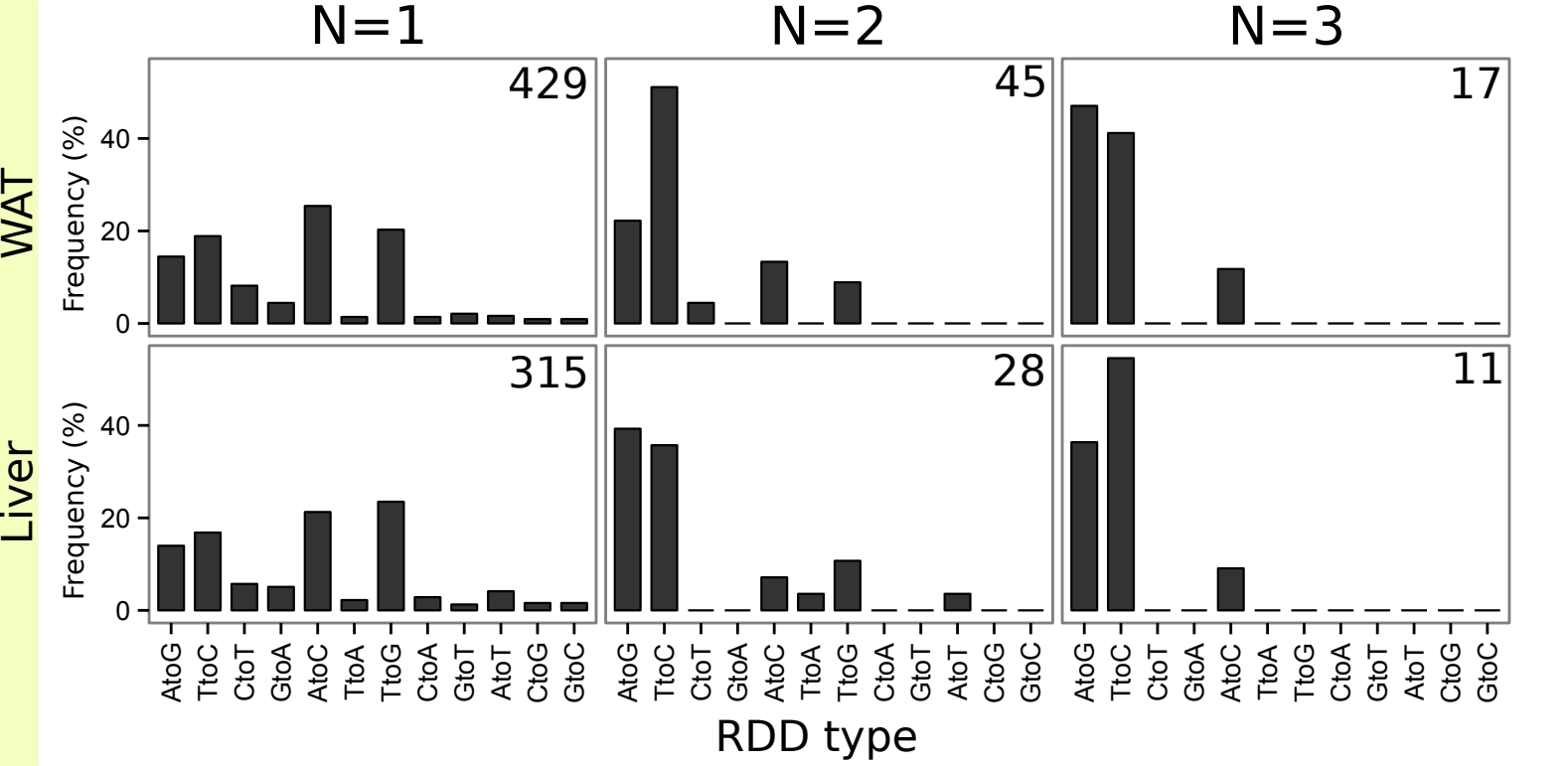
The red stars indicate the position of the amino acid impacted by coding mRNA events. The overall conservation across sequences is depicted below each alignment. The mRNA editing event impacting *COG3* was detected in both white adipose tissue and liver, while the ones impacting *CES1* and *FLNB* were WAT-specific, and the one impacting *KCMA1* was specific to liver

Figure 7. Impact of genetic background, age, feeding condition and sex on mRNA editing level.

Editing level (in %) at 5 genomic positions, in white adipose tissue (WAT) and liver according to (A) genetic background, (B) age, (C) feeding condition and (D) sex. Each boxplot shows the distribution of editing levels across N=8 biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, unpaired two-tailed Student *t*-test. Pre-pub.: prepuberal animals; Post-pub.: postpuberal animals; M: Males; F: Females.



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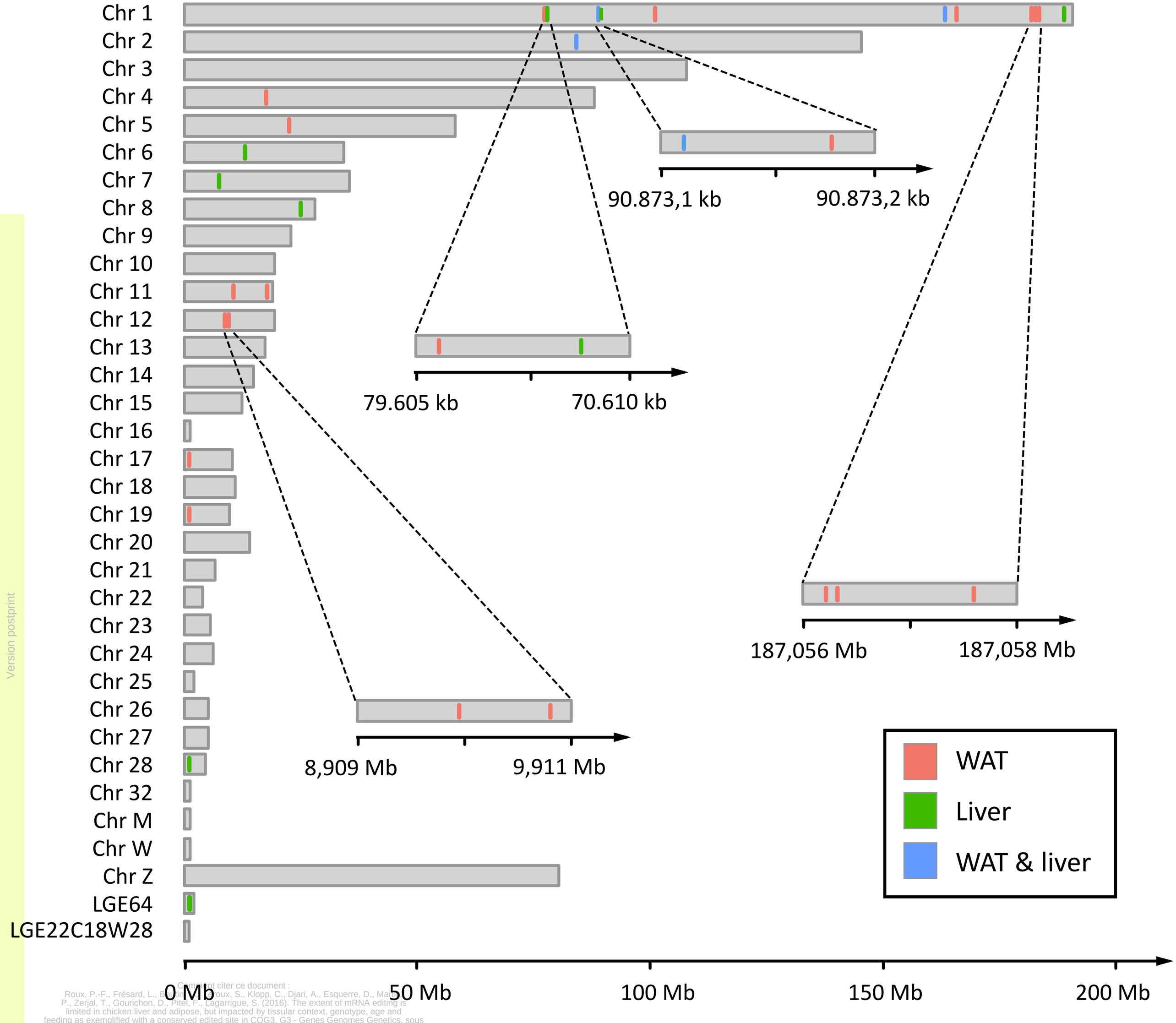

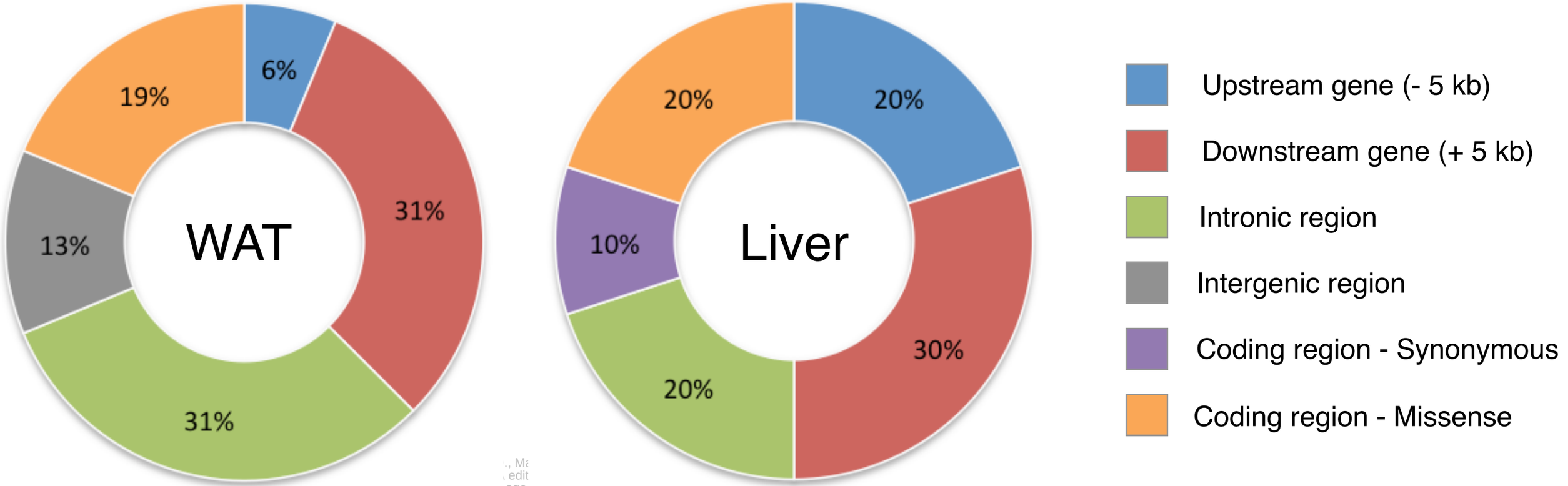
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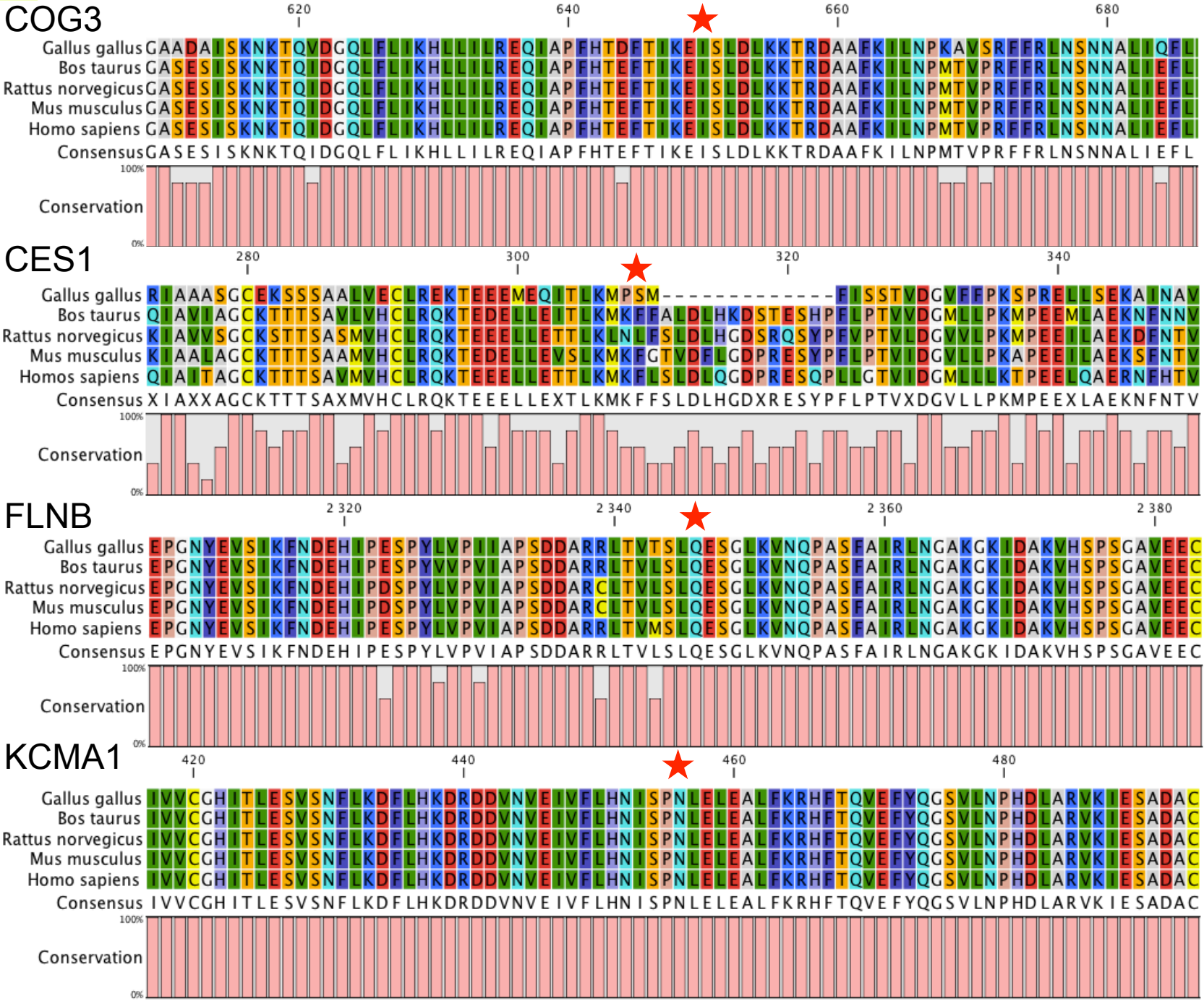
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CES1
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300
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100%
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FLNB
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