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Integration of genomic and transcriptomic data of inbred mouse models for polygenic obesity and leanness revealed "obese" and "lean" candidate alleles in polyadenylation signals

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ABSTRACT

Most mammalian genes have multiple polyadenylation (PA) sites, and alternative polyadenylation (APA) has been linked to diseases such as obesity. Studies have shown that changes in the polyadenylation signal (PAS) can influence the efficiency of cleavage and affect disease susceptibility and phenotype. In our recent study we used inbred mouse models of polygenic obesity and leanness and identified single-nucleotide polymorphisms in PAS (PAS-SNPs) within several obesity candidate genes, including five with differential expression. Nevertheless, to date, there has been no systematic, whole-genome-level approach aiming to prioritise PAS-SNPs potentially affecting APA. Therefore, in this study, we build upon our previous work by integrating existing genomics data with transcriptomics. DEGs were identified in nine tissues by Affymetrix GeneChip. PA and PAS sites were from the PolyASite 2.0 portal. Prioritisation of candidate PAS-SNPs was performed based on whether they were located in DEG, the type of PAS changes they caused, locations of PA sites relative to PAS, and location(s) and expression(s) of Affymetrix probes within a given gene in various tissues. For the candidates, potential consequences due to the alteration in APA events were investigated using bioinformatics databases and tools. The analysis found 127 PAS-SNPs in 101 DEGs across different tissues and identified 12 high-priority and 7 moderate-priority PAS-SNP candidates in 10 and 7 DEGs, respectively. Candidate PAS-SNPs were in 3′ UTR of 12 protein-coding genes (Lean line: *Edil3*, *Eif2s1*, *Fbxl3*, *Hlf*, *Hsf2bp*, *Knop1*, *Lair1*, *Nmrk1*; Fat line: *Ehd1*, *Rpl14*, *Spon1*, *Txndc9*), introns of four protein-coding genes (Lean line: *Abi3bp*, *Prr16*; Fat line: *Agmo*, *Itga7*) and intron of one lncRNA (Lean line: *1700086O06Rik*). The integration of whole-genome sequencing and transcriptome analyses in this study has identified potential genome-wide candidate SNPs that could affect APA by altering/disrupting PAS motifs and be related to obesity in mice. The data provides a foundation for further research into these PAS-SNPs, their genes, and their contribution to the obesity/leanness phenotype, and contributes a part in explaining missing heritability commonly observed in complex traits.

1. Introduction

Polyadenylation (PA), i.e. the addition of a poly(A) tail to the 3′-end of the transcript, determines the mRNA stability, localisation, and translational potential of the mRNA. Approximately 70 % of mammalian genes contain more than one polyadenylation site (Derti et al., 2012). Alternative cleavage followed by polyadenylation (alternative polyadenylation, APA) increases the diversity of transcripts encoded by the same gene, which significantly affects gene expression and gene function (Yuan et al., 2021).

Abbreviations: 3′ UTR, three prime untranslated region; APA, alternative polyadenylation; BAT, brown adipose tissue; DEG, differentially expressed gene; eWAT, epididymal white adipose tissue; mWAT, mesenteric white adipose tissue; PA, polyadenylation; PAS, polyadenylation signal; PAS-SNP, single nucleotide polymorphism in polyadenylation signal; SNP, single nucleotide polymorphism; sWAT, subcutaneous white adipose tissue; WAT, white adipose tissue.

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Studies have shown the relationship between APA and cell developmental stage (Ji et al., 2009; Ulitsky et al., 2012), tissue-specificity (Lianoglou et al., 2013), and various diseases (Chang et al., 2017), including obesity (Brutman et al., 2018), one of the most significant public health challenges (Lancsar et al., 2022). For example, Brutman et al. (2018) identified 763 differentially expressed coding genes and one miRNA with APA in the hypothalamus of a high-fat-diet-induced obesity rat model. The alternative polyadenylation involved genes broadly involved in the development of neuron projection and synapse organisation (Brutman et al., 2018). More recently, 574 differentially expressed APA sites between the Fat and Lean selection mouse lines on high-fat diet were identified in hypothalamus by Mikec et al. (2023), including within seven genes previously associated with obesity or obesity-related traits (*Pdxdc1*, *Smyd3*, *Rpl14*, *Copg1*, *Pcna*, *Ric3*, *Stx3*) and ten potentially novel obesity candidate genes (*Ccdc25*, *Dtd2*, *Gm14403*, *Hlf*, *Lyrm7*, *Mrpl3*, *Pisd-ps3*, *Sbsn*, *Slx1b*, *Spon1*) (Mikec et al., 2023).

PA is regulated by both *cis*-elements and *trans*-factors (Xiao et al., 2016). For *cis*-elements, single nucleotide changes in the polyadenylation signal (PAS-SNP), typically located about 20-nt upstream of the PA site (Shulman and Elkon, 2020), have been shown to affect cleavage efficiency (Neve et al., 2017) and influence disease susceptibility (Fahiminiya et al., 2015; Wang et al., 2016b). For example, homozygosity for the G allele at rs10954213 in PAS (AAUAAA ➔ AAUGAA) favours the *IRF5* mRNA expression with longer 3′ UTR and is linked to human lupus (Graham et al., 2007). Meanwhile, genetic variants can decrease mRNA expression levels by increasing usage of intronic PAS (Mittleman et al., 2020).

Recently, Yang et al. (2020) developed a catalogue of whole-genome genetic variants associated with APA in human cancer, and Xiao et al. (2016) showed that SNPs near PA sites, where PAS is localised, significantly contribute to the differential whole-genome utilisation of PA sites between the mouse strains C57BL/6J and SPRET/EiJ. In our previous study (Šimon et al., 2023), we developed a catalogue of potential "obesity" or "leanness" PAS-SNP alleles present in our unique inbred mouse models for the polygenic obesity and leanness, which are the most common clinical manifestations in the human population (Huvenne et al., 2016), making our mouse models a valuable resource for investigating these conditions. By integrating genome (whole-genome sequencing (Mikec et al., 2022)) and bioinformatics data (PA and PAS sites from the PolyASite 2.0 portal (Herrmann et al., 2020)), we identified in those previous studies 682 PAS-SNPs within 583 genes involved in various biological processes, including transport, protein modification and degradation, cell adhesion and immune response. Orthologous genes in human have been linked to various diseases such as nervous system and physical disorders, immune system, endocrine system and metabolic diseases, and PAS-SNPs have been identified in genes associated with obesity (*Abcc6*, *Col4a1*, *Lhfpl3*, *Npc1*, *Lsamp*, and *Ppargc1a*) and broadly with PA (*Mnat1*, *Polr2c*, *Snd1*, *Ints11*, *Dhx15*, and *Eif3e*). Among these genes, *Car8*, *Itga7*, *Lat*, *Nmnat1*, and *Col4a1* were also differentially expressed genes (Šimon et al., 2023). These results suggest that some of the identified PAS-SNPs in the two lines could potentially contribute to their divergent body fat content. However, to our knowledge, no study has been conducted to prioritise genome-wide PAS candidates involved in obesity by potentially affecting alternative polyadenylation (Supplementary Fig. 1).

Therefore, in the present study, we have continued our previous work by integrating results obtained at a DNA level with transcriptome data (Affymetrix Mouse Genome 430-2.0 GeneChip) to 1) prioritise high-likelihood PAS-SNP candidates and 2) provide clues on the potential alterations in genes carrying candidate PAS-SNPs that may contribute to obesity and healthy leanness.

2. Material and methods

2.1. Genotyping of mouse selection lines

Whole-genome sequencing (WGS) of DNA samples isolated from the spleens of Fat and Lean mouse lines ($n = 2$ inbred animals), established by divergent selection on body fat percentage over more than sixty generations (Bünger and Hill, 1999; Sharp et al., 1984), and PAS-SNPs identification were previously performed (Mikec et al., 2022; Šimon et al., 2023) by overlapping the positions of SNPs identified by WGS with the positions of PAS motifs obtained from the PolyASite 2.0 portal (https://polyasite.unibas.ch/) (Herrmann et al., 2020). Although performing WGS of two animals in these lines may seem a low number, it's essential to consider the context of their high level of inbreeding. Lines first went through 60 generations of intense phenotypic selection on high and low body fatness (Sharp et al., 1984) which has already made lines genetically homogenous. A genome wide genetic mapping study using microsatellites (Horvat et al., 2000) revealed that by the end of the selection experiment, these lines exhibited minimal genetic diversity within themselves. Subsequently, a rigorous brother-sister inbreeding protocol was implemented, resulting in the animals chosen for WGS analysis being subjected to 68 and 70 generations of such inbreeding for the Lean and Fat lines, respectively. Regular genetic monitoring using microsatellites, performed across each generation, has consistently shown a lack of polymorphisms within these lines for the past two decades. While the possibility of new mutations and genetic drift introducing novel genetic variation remains, this risk is effectively mitigated by the stringent protocol of utilizing a single brother-sister mating procedure in each generation. Hence, it is reasonable to assert that the WGS of just two animals adequately represents the genome of all individuals within our Fat and Lean lines. Variant calling and hard filtering of WGS data were performed according to the Genome Analysis Toolkit (GATK) (McKenna et al., 2010) Best Practices recommendations (Depristo et al., 2011; Van der Auwera et al., 2013). Variants were annotated using the Ensembl Variant Effect Predictor (https://www.ensembl.org/Tools/VEP) (McLaren et al., 2016).

Sanger sequencing was used to validate selected PAS-SNPs. PCR products were purified using the QIAGEN Qiaquick PCR purification kit (Cat. No. 28104) and sequenced by Macrogen Europe (Macrogen Europe, Amsterdam, The Netherlands). The primer pairs used for PCR are listed in Supplementary Table 2.

2.2. Gene expression analysis

To investigate the expression of genes carrying PAS-SNPs, microarray transcriptome profiling was performed using the Affymetrix Mouse Genome 430-2.0 GeneChip on pooled RNA samples ($n = 1$) extracted using Qiagen RNeasy kits from various mouse tissues, including white adipose tissue (subcutaneous (sWAT), epididymal (eWAT), mesenteric (mWAT), and pooled (WAT)), brown adipose tissue (BAT), liver, muscle, adrenal gland, thymus and kidney. The data obtained were processed as previously described (Morton et al., 2011; Pedroni et al., 2014). A non-statistical approach was used to compare the Fat and Lean samples for each of the tissues due to a single replicate for each strain/tissue. The data was normalized using a variance-stabilizing transformation VSN. The fold-changes for each of the nine single tissue comparisons were the VSN moderated fold-changes with p-value artificially set to 1e-10 to facilitate analysis through the standard Fios Genomics analysis pipeline. Significance values were further controlled for false discovery, yielding adjusted p-value. For pooled WAT, the expression data from the three tissues depots (epididymal WAT, subcutaneous WAT, mesenteric WAT) were then joined and corrected for the batch effect using Empirical Bayes Analysis to obtain expression data in WAT. Expression of genes was considered differential if the expression of the single Affymetrix probe differed between Fat and Lean mouse lines by at least 1.5-fold at an adjusted $p < 0.05$. The

expression of Affymetrix probes within the differentially expressed genes (DEGs) carrying PAS-SNPs can be found in Supplementary Table 3.

2.3. PAS-SNP prioritisation

Affymetrix GeneChips often have probe sets that map to different locations within the gene and have been used to identify alternative splicing/polyadenylation events (Ji and Tian, 2009; Lembo et al., 2012). In the present study, the positions of the PAS-SNPs, PA sites and the positions and expressions of the Affymetrix probes were first visualized by the Golden Helix GenomeBrowse® v3.1.0 visualisation tool (http://www.goldenhelix.com) (Golden Helix, Inc., Bozeman, n.d.). Subsequently, all differentially expressed genes carrying PAS-SNPs were manually examined to prioritise PAS-SNPs that are most likely to influence the use of PA sites within a gene, according to the prioritisation criteria described below and shown in Fig. 1.

For the 3′ UTR APA, the effect of PAS-SNP on APA can be suggested by considering the type of changes in PAS motif caused by a PAS-SNP, the location of the PA sites relative to PAS, and the location of at least 2 Affymetrix probes with different expressions. For the intronic APA, the effect of PAS-SNP on APA can be suggested by considering the type of changes in PAS motif caused by a PAS-SNP, the location of the PA sites relative to PAS, and the location and expression of the Affymetrix probe (s).

In addition, genes, where the Affymetrix probe sets map to different transcripts, were discarded. For example, the gene *Abca5* (ATP-binding cassette, sub-family A (ABC1), member 5) has 5 transcripts. The

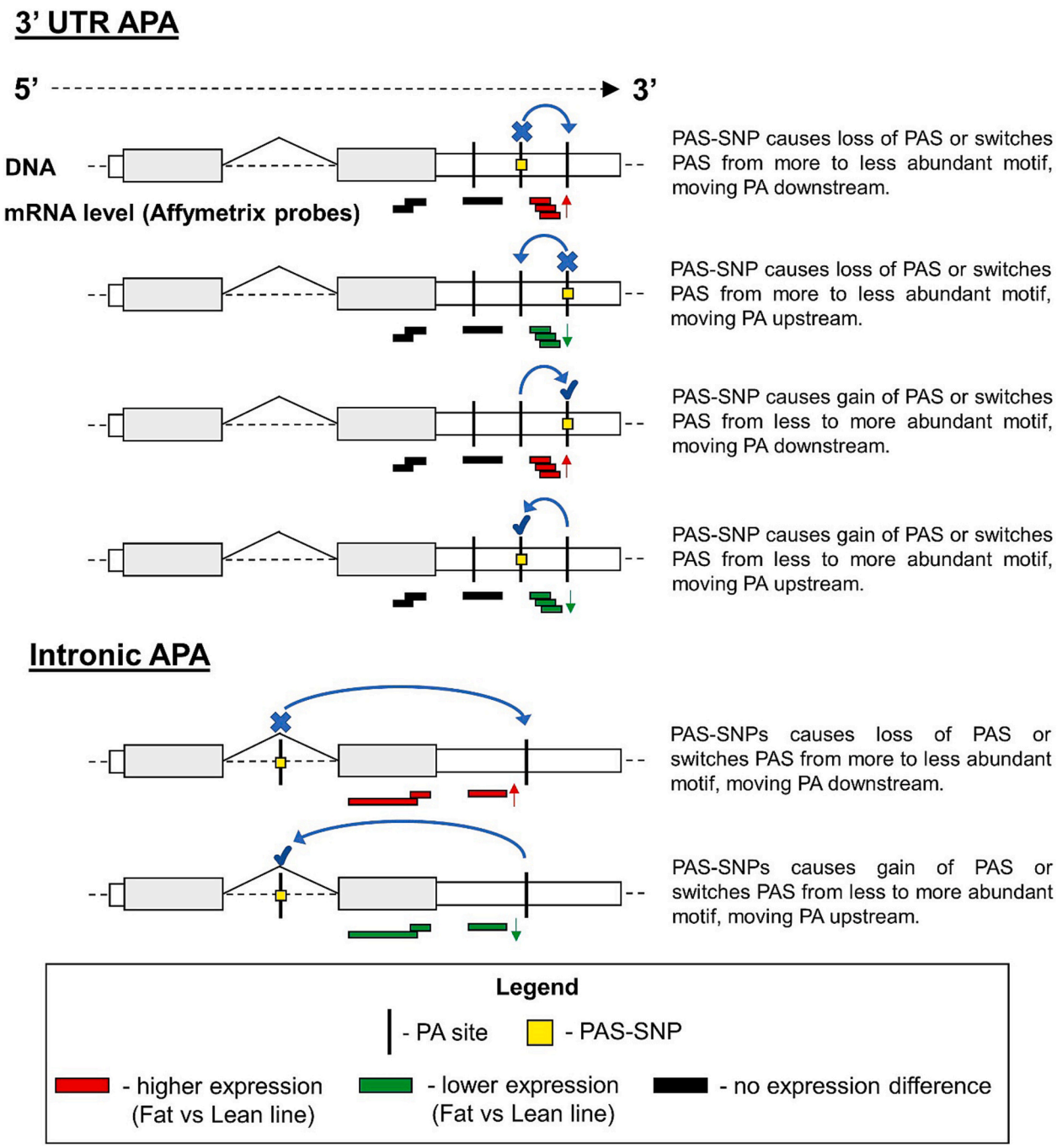

Fig. 1. Schematic representation of prioritisation criteria for PAS-SNPs.

Affymetrix Mouse Genome 430-2.0 GeneChip contains two probe sets for this gene (1434474_at and 1459391_at). While a probe set 1434474_at maps to the *Abca5* transcript ENSMUST00000043961 (higher expression in the Fat line), a probe set 1459391_at maps to the ENSMUST00000127318 transcript (no differential expression between the lines). Therefore, the conclusion about the potential effect of PAS-SNP rs52557469 in the Fat line on the 3′ UTR length cannot be drawn (Supplementary Fig. 2).

Finally, for the genes carrying candidate PAS-SNPs, we obtained SNPs and indels 60 bp upstream and within the corresponding PA-site cluster to check whether other genetic variants may generate PAS de-novo. In this way, we also obtained all PAS motifs for the corresponding PA site, which served to prioritise the PAS-SNPs.

2.4. Bioinformatics analyses

The number of DEGs carrying PAS-SNPs shared by different tissues was analysed and visualized using the UpSetR package for R (Conway et al., 2017; Lex et al., 2014). Functional annotation of DEGs carrying PAS-SNPs was performed using the g:Profiler web tool (https://biit.cs.ut.ee/gprofiler/gost) (Raudvere et al., 2019).

To investigate the potential effects of 3′ UTR PAS-SNPs on 3′ UTR length and the resulting abundance of sequence motifs for miRNAs and RNA-binding proteins (RBPs), RNA modifications and RNA-RNA interactions, we used the DIANA-TarBase v8 (Karagkouni et al., 2018) and RISE (RNA Interactome from Sequencing Experiments) (Gong et al., 2018) databases. The region between the PA site potentially affected by the PAS-SNPs and the PA site downstream or upstream, such that the differentially expressed Affymetrix probe lies within the two PA sites, was examined (Fig. 2).

For the intronic PAS-SNPs, the potentially lost protein domains and sites for RNA-RNA and protein-protein interactions were determined for the protein/lncRNA region downstream of the last potentially encoded exon using the Ensembl (Howe et al., 2021), RISE and UniProt (Bateman et al., 2021) databases (Fig. 2).

3. Results

In the present study, whole-genome sequencing, transcriptome and bioinformatics data were integrated to prioritise genetic variants that might affect APA of our mouse models of obesity and leanness. A total of 101 differentially expressed genes (DEGs) carrying 127 PAS-SNPs were identified. By manually examining the positions of the SNPs and the PA sites, as well as the positions and expressions of the Affymetrix probes within these genes, 19 PAS-SNPs within 17 genes were identified as priority “obese” or “lean” PAS-SNP candidates. The workflow of the study and the main results are shown in Fig. 3.

3.1. Analysis of differentially expressed genes carrying PAS-SNPs

A total of 309 and 373 PAS-SNPs specific to either the Fat or Lean line located in 583 genes (Fat: 257, Lean: 318, Both: 8) were identified by Šimon et al. (2023). Sanger sequencing confirmed the presence of selected PAS-SNPs (Supplementary Fig. 3).

Of the 583 genes carrying PAS-SNPs, differential expression of microarray probes was detected for 101 genes, most of which were differentially expressed in pooled WAT samples (29 genes), followed by the adrenal gland (27). The tissues with the lowest number of DEGs with PAS-SNPs were liver (15) and kidney (17). Four genes are shared by all tissues; *Gsn*, *H2-D1*, *Rpl14*, and *Snx6* (Supplementary Fig. 4).

The vast majority of the 101 genes are protein-coding (96 genes), while 5 genes encode lncRNAs. Affymetrix probe sets of 50 genes were

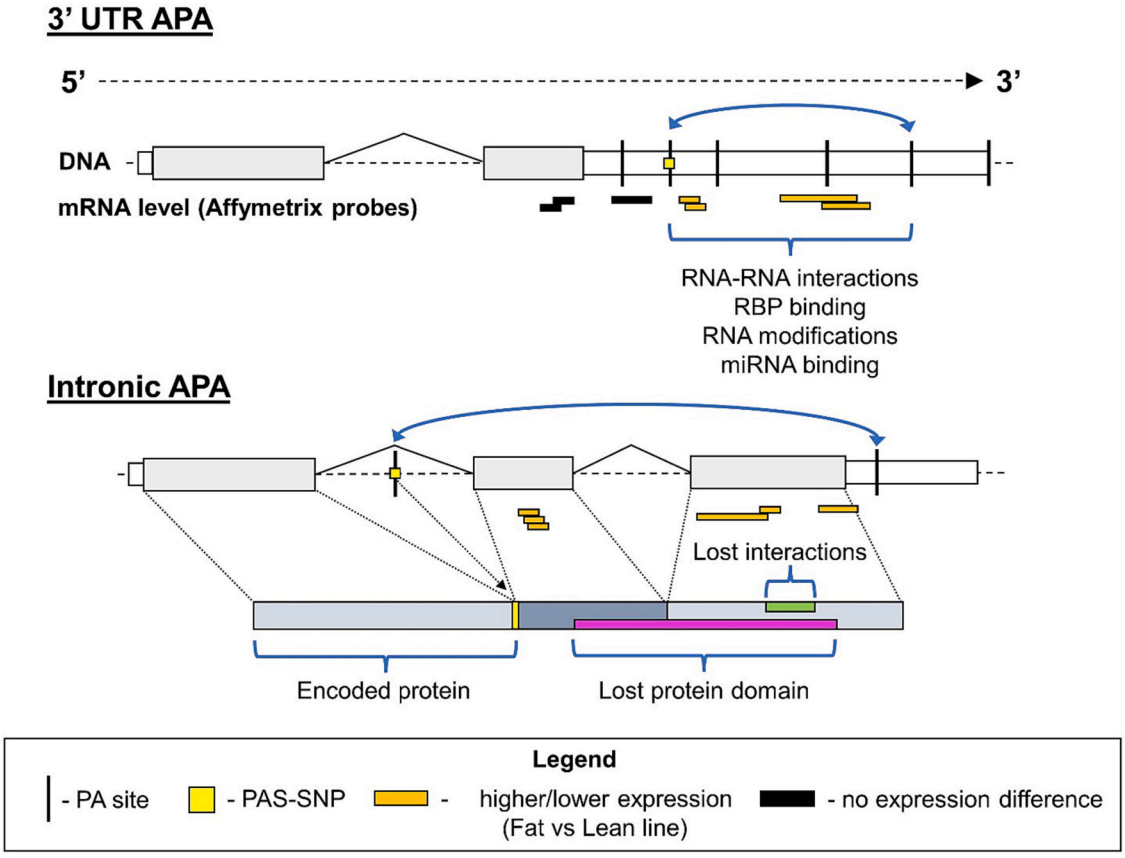

Fig. 2. Potential consequences of APA caused by PAS-SNPs on mRNA and protein. APA in 3′ UTR affects the number of mRNA interaction and modification sites, and protein truncation caused by intronic APA alters the protein function by reducing the number of interactions and disrupting protein domains.

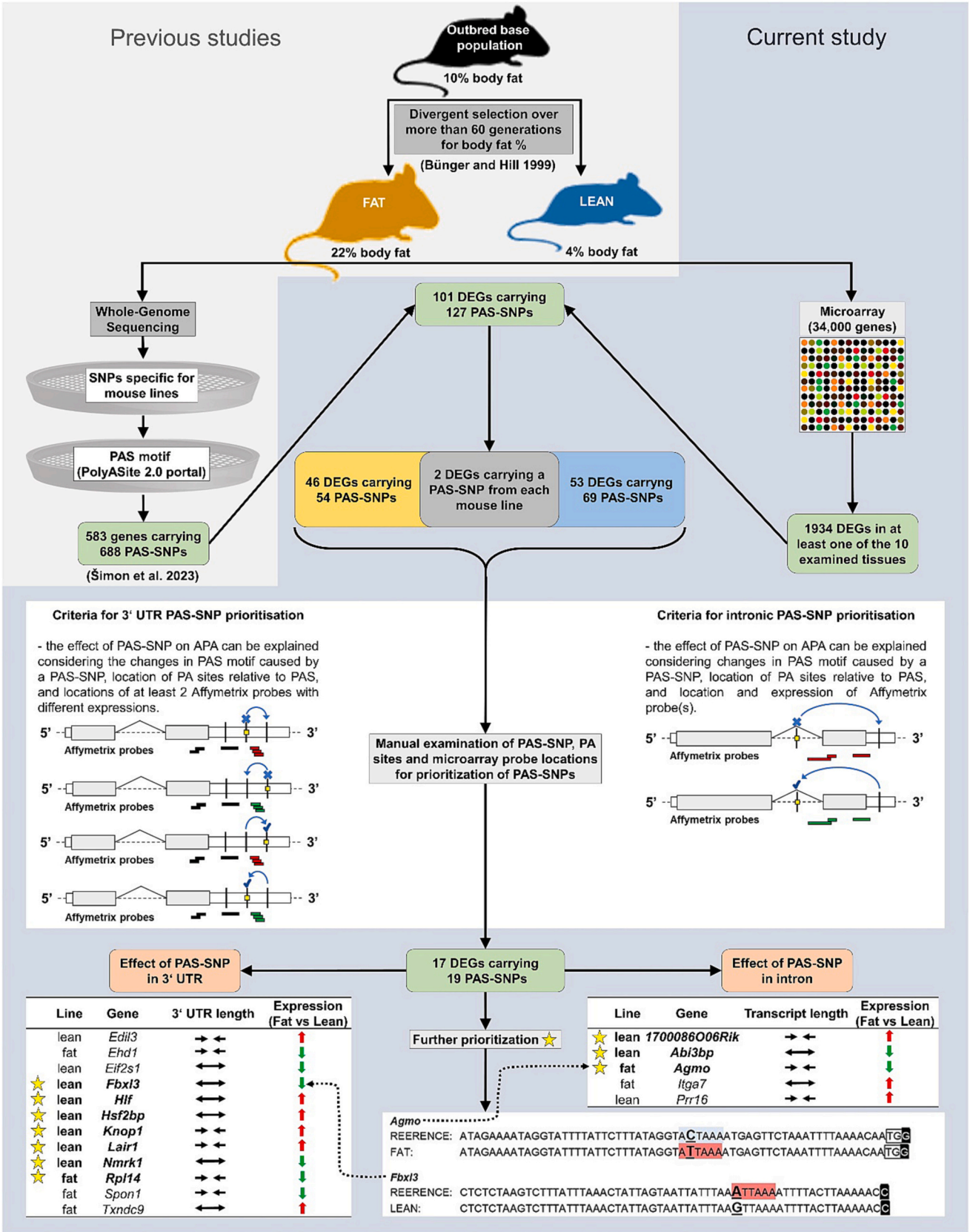

Fig. 3. Study workflow. The main steps include whole-genome sequencing, identification of PAS-SNPs, gene expression analysis, PAS-SNPs in DEGs, manual examination and PAS-SNPs prioritisation. Stars near the genes in the tables indicate genes carrying high-priority PAS-SNP candidates. Arrows in the 3′ UTR length and Transcript length columns indicate transcript truncation (→←) and lengthening (←→). Steps and results on the grey background, "Previous results", are from our previous studies (Bünger and Hill, 1999; Šimon et al., 2023).

expressed to a higher level and 43 to a lower level in the Fat line than in the Lean (not considering the tissue studies). In addition, probe sets of 8 genes were both up- and down-regulated in the Fat line: *Bltp1*, *Abi3bp*, *Fbln7*, *H2-Aa*, *H2-D1*, *Nrg4*, *Sptbn1*, and *Tenm4*. Functional enrichment analysis of DEGs with the g:Profiler showed that DEGs with higher abundance in the Fat line are involved in cell adhesion and are part of the organelle membrane. Meanwhile, DEGs with lower abundance are localised in the cytoplasm and plasma membrane, where they are part of neurons and the MHC immune complex. KEGG and Wikipathways enrichment analyses revealed that DEGs with higher expression may be involved in arrhythmogenic right ventricular cardiomyopathy and eicosanoid metabolism via cytochrome P450 monooxygenases, while the DEGs with lower abundance are involved in focal adhesion: PI3K-Akt-mTOR-signaling pathway. In addition, the more abundant DEGs were enriched as *mmu-miR-1932* and *mmu-miR-450a-5p* targets and those with lower abundance might be under the regulatory control of the miRNA *mmu-miR-5104* (Supplementary Fig. 5).

3.2. Candidate PAS-SNPs

To identify PAS-SNPs with potential impact on alternative usage of PA sites, we compared the positions of PAS-SNPs and PA sites with the positions and expressions of microarray probes within a single gene. A manual examination of 101 genes identified 19 PAS-SNPs with the likelihood to influence the APA of 17 genes. Among the 17 genes, PAS-SNPs could affect the 3′ UTR length of 12 genes, the length of 1 lncRNA (*1700086O06Rik*) and the length of proteins encoded by 4 genes (Table 1).

The examples of manual examination of PAS-SNPs in the 3′ UTR and intronic regions of two genes are given in Fig. 4. The rs245246928 and rs265523112 in *Nmrk1* of the Lean line cause the loss of the PAS motif (CATAAA→CATAGG) of the predominantly used 3′ UTR PA site of *Nmrk1*, possibly extending the 3′ UTR of *Nmrk1* in the kidney (Fig. 4a). Meanwhile, in *Agmo* of the Fat line, rs29207890 changes a less abundant motif ACTAAA to the second most abundant motif ATTAAA, possibly resulting in a very short protein in the adrenal gland and kidney encoded by only the first two exons (Fig. 4b).

3.3. Candidate 3′ UTR PAS-SNPs

The 14 identified PAS-SNPs (Fat: 4, Lean line: 10) could cause different 3′ UTR lengths of 12 protein-coding genes (Fat: 4, Lean line: 8) between the two lines. Among these, the 3′ UTR length of 8 transcripts would be shorter in the Fat line and 4 longer than in the Lean line. These differences would result in a different number of available sites for miRNA and RBP binding, RNA-RNA interactions and RNA modifications. For example, in the Lean line, the rs52133194 causes the loss of PAS (AACAAA→AACACA), which lengthens the 3′ UTR of *Eif2s1*, resulting in a higher number of miRNA-, RNA-RNA interaction- and RBP-binding sites in the 3′ UTR compared to the Fat line (Table 2).

3.4. Candidate intronic PAS-SNPs

5 PAS-SNPs (Fat line: 2, Lean line: 3) within 5 genes (Fat line: 2, Lean line: 3) may affect the length of 1 lncRNA (*1700086O06Rik*) and 4 protein-coding genes (*Abi3bp*, *Agmo*, *Itga7*, *Prr16*). Consequently, mRNAs transcribed from these genes may lose/maintain interactions with other RNAs (depending on the effect of PAS-SNP on the PAS motif), while proteins may lose/maintain functional domains (Table 3).

For example, the rs254851498 (ATTAAA→AATAAA) of the Lean line may truncate the lncRNA *1700086O06Rik*, however, the RNA-RNA interaction sites in this transcript would be conserved. The PAS-SNP rs29207890 (ACTAAA→ATTAAA) in the second intron of *Agmo* in the Fat line would result in a lost interaction with *Sf3a2* and a shorter protein with lost fatty acid hydroxylase and transmembrane domains (Supplementary Fig. 6a). Meanwhile, the rs32060094 PAS-SNP in *Prr16* of the Lean line changes AATAGA to a more abundant motif AATACA, possibly leading to the production of a shorter transcript with lost interaction sites for multiple RNAs and a shorter protein encoded by only a single exon with loss of the Largen/Inhibitory synaptic factor 1 domain (Supplementary Fig. 6b).

Table 1
Candidate PAS-SNPs identified in the fat and lean mouse selection lines.

Mouse line	PAS-SNP	Gene symbol	Region	Consequence (Fat vs Lean)	Tissue[b]
Lean	rs48329771	*Edil3*	3′ UTR	Longer 3′ UTR	Adrenal gland
Fat	rs37844368	*Ehd1*	3′ UTR	Shorter 3′ UTR	WAT
Lean	rs52133194	*Eif2s1* (*Fob2* QTL[a])	3′ UTR	Shorter 3′ UTR	Adrenal gland, liver, thymus, sWAT, mWAT
Lean	rs31062829	*Fbxl3*	3′ UTR	Shorter 3′ UTR	Muscle
Lean	rs229072835	*Hlf*	3′ UTR	Shorter 3′ UTR	Thymus
Lean	rs255472708, rs221503910	*Hsf2bp*	3′ UTR	Shorter 3′ UTR	Liver
Lean	rs32227744	*Knop1*	3′ UTR	Longer 3′ UTR	Adrenal gland
Lean	rs244789005	*Lair1*	3′ UTR	Longer 3′ UTR	WAT, sWAT, mWAT
Lean	rs245246928, rs265523112	*Nmrk1*	3′ UTR	Shorter 3′ UTR	Kidney
Fat	rs263963399	*Rpl14*	3′ UTR	Shorter 3′ UTR	Adrenal gland, kidney, liver, muscle, thymus, BAT, WAT, sWAT, mWAT, eWAT
Fat	rs31703795	*Spon1*	3′ UTR	Shorter 3′ UTR	adrenal gland, kidney, muscle, thymus, sWAT, mWAT, eWAT
Fat	rs32967435	*Txndc9*	3′ UTR	Longer 3′ UTR	muscle
Lean	rs254851498	*1700086O06Rik*	intron (1/1)	Longer lncRNA	Adrenal gland, BAT
Lean	rs50706522	*Abi3bp*	intron (14/34)	Shorter protein	Adrenal gland
Fat	rs29207890	*Agmo* (*Fob2* QTL[a])	intron (2/12)	Shorter protein	Adrenal gland, kidney
Fat	rs260246262	*Itga7*	intron (1/24)	Longer protein	WAT
Lean	rs32060094	*Prr16*	intron (1/1)	Longer protein	WAT, sWAT, mWAT

Genes: *Edil3*: EGF-like repeats and discoidin I-like domains 3; *Ehd1*: EH-domain containing 1; *Eif2s1*: eukaryotic translation initiation factor 2, subunit 1 alpha; *Fbxl3*: F-box and leucine-rich repeat protein 3; *Hlf*: hepatic leukemia factor; *Hsf2bp*: heat shock transcription factor 2 binding protein; *Knop1*: lysine rich nucleolar protein 1; *Lair1*: leukocyte-associated Ig-like receptor 1; *Nmrk1*: nicotinamide riboside kinase 1; *Rpl14*: ribosomal protein L14; *Spon1*: spondin 1, (f-spondin) extracellular matrix protein; *Txndc9*: thioredoxin domain containing 9; *Abi3bp*: ABI family member 3 binding protein; *Agmo*: alkylglycerol monooxygenase, transcript ENSMUST00000049874; *Itga7*: integrin alpha 7; *Prr16*: proline rich 16.

[a] The gene is located within the Fat-line obesity QTL 2 (*Fob2*) determined by (Horvat et al., 2000).

[b] sWAT - subcutaneous white adipose tissue (WAT), mWAT - mesenteric WAT, eWAT - epididymal WAT, WAT - pooled WAT, BAT - brown adipose tissue.

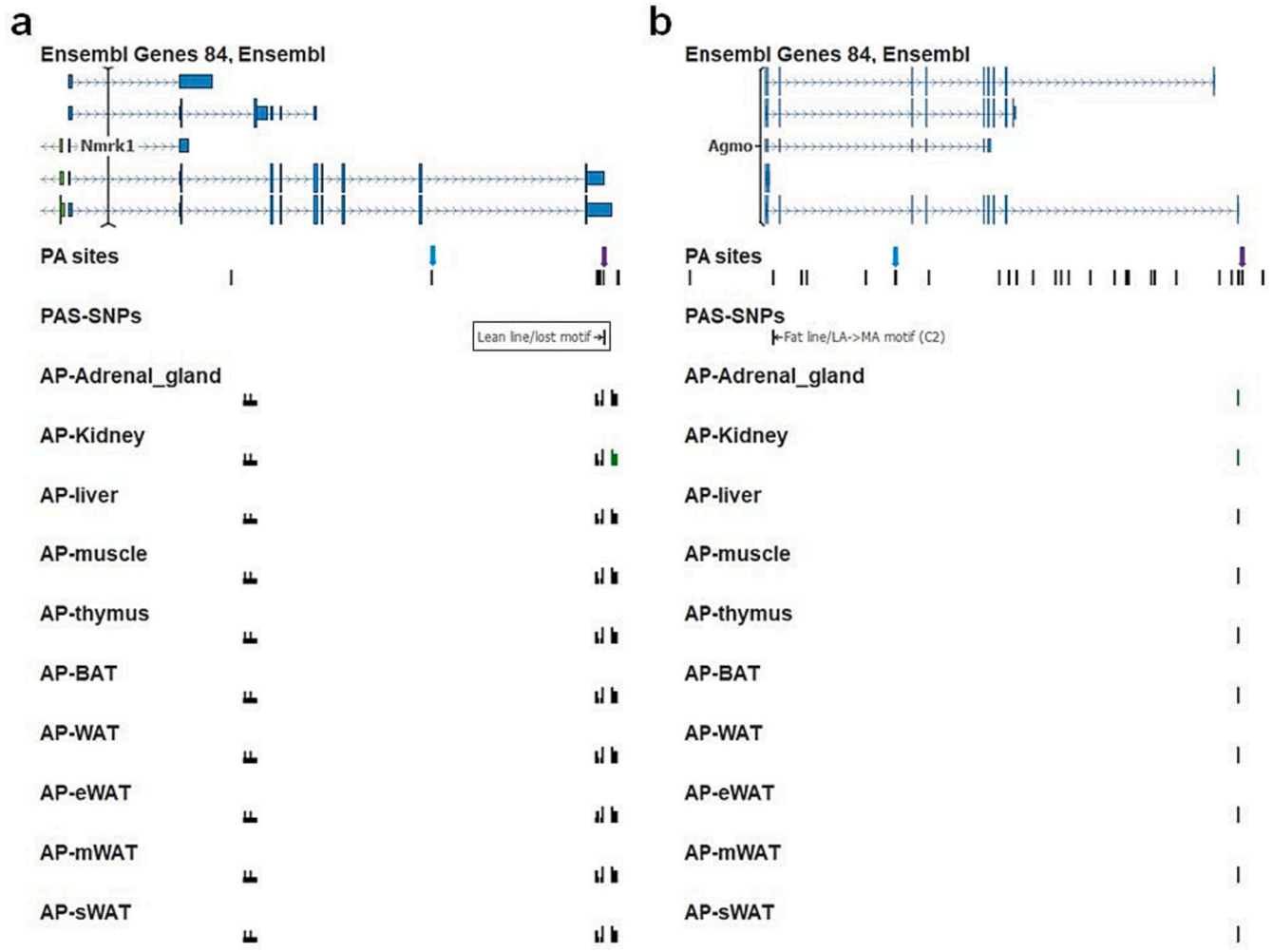

Fig. 4. Examples of manual examination for the prioritisation of PAS-SNP candidates involved in (a) 3′ UTR (gene *Nmrk1*) and (b) intronic (gene *Agmo*) APA. Legend: 2nd track, purple arrow – the most used 3′ UTR PA site, blue arrow – the most used intronic PA site, the purple box indicates the PAS-SNPs could affect the most used 3′ UTR PA site; 4th to 13th track (Affymetrix probes (AP) in different tissues), black rectangles – no expression difference between the lines, red and green rectangles – the expression being higher and lower in the Fat line compared to the Lean line, respectively, black box – tissue where PAS-SNP could affect APA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. DNA sequence analysis upstream of PA sites having candidate PAS-SNPs

Finally, we checked for the presence of other PAS sites in the region 60 bp upstream of PA sites with PAS-SNPs. In addition, we obtained other SNPs and indels 60 bp upstream for these PA sites to check whether other genetic variants could generate de-novo PAS. In prioritising the PAS-SNPs, we primarily considered their effects on the two major PAS motifs, AATAAA and its main variant ATTAAA, and in particular whether they affect a single PAS motif of the corresponding PA site. None of the other SNPs produce de-novo PAS. Fig. 5 shows the location and impact of the high-priority candidate PAS-SNPs. Of particular note here are PAS-SNPs in *Abi3bp*, *Fbxl3*, *Hsf2bp*, and *Nmrk1* of the Lean line, which cause a complete loss of the known PAS motifs of the corresponding PA sites, thus likely preventing PA from occurring here (Fig. 5).

4. Discussion

In the present study, WGS, transcriptome and bioinformatics data were combined to prioritise the PAS-SNP candidates that could affect the function and mRNA to protein translation of the corresponding genes. Out of 583 genes carrying 688 PAS-SNPs, 101 are differentially expressed between the Fat and Lean mouse lines. The manual inspection identified 12 high-priority and 7 moderate-priority PAS-SNP candidates within 10 and 7 genes, respectively, including within genes previously associated with obesity and obesity-related traits and comorbidities.

4.1. Pathway enrichment analysis

KEGG and WikiPatways enrichment analyses of DEGs carrying PAS-SNPs revealed that DEGs with higher expression may be involved in arrhythmogenic right ventricular cardiomyopathy and participate in eicosanoid metabolism via cytochrome P450 (CYP) monooxygenases, while those with lower abundance are involved in focal adhesion: PI3K-Akt-mTOR pathway. Obesity is a known risk factor for cardiovascular disease, and a study by Sokmen et al. (2013) found that obesity is associated with abnormalities in right ventricular structure and function. Regarding eicosanoid metabolism, it was suggested that the increase in CYPs-mediated eicosanoid metabolites contributes to the pathology of obesity and associated health problems (Wang et al., 2016c). Meanwhile, the dysregulated PI3K-Akt-mTOR pathway has been linked to obesity, diabetes, hyperglycaemia, and insulin resistance (Khan et al., 2016; Schultze et al., 2012; Yin et al., 2017), which were found in our Fat mice (Morton et al., 2005, 2016; Simončič et al., 2008b). Finally, DEGs with higher abundance in the Fat line were

Table 2
Candidate 3′ UTR PAS-SNPs and their potential effect on 3′ UTR lengths and, consequently, the abundance of regulatory motifs in their corresponding genes.

Mouse line	SNP	Gene symbol	Effect on PAS	Consequence (fat vs lean)	miRNA (Tarbase v8)	RNA-RNA interaction (RISE)	RBP binding (RISE)	RNA editing/ modification (RISE)
Lean	rs48329771	*Edil3*	lost motif	Longer 3′ UTR	*mmu-miR-19a-3p* *mmu-miR-19b-3p* *mmu-miR-7a-5p* *mmu-miR-1a-3p* *mmu-miR-206-3p* *mmu-miR-218-5p* *mmu-miR-706*	/	/	/
Fat	rs37844368	*Ehd1*	lost motif	Shorter 3′ UTR	*mmu-miR-26a-5p* *mmu-miR-26b-5p* *mmu-miR-155-5p* *mmu-miR-762* *mmu-miR-882*	*Optn*	CPSF6 PABPC1 UPF1	/
Lean	rs52133194	*Eif2s1*	lost motif	Shorter 3′ UTR	*mmu-miR-26a-5p* *mmu-miR-26b-5p* *mmu-miR-133a-3p* *mmu-miR-133b-3p* (also upstream) *mmu-miR-499-5p* *mmu-miR-677-5p*	*Gm42418*	U2AF2 (also upstream) SRRM4 (also upstream) EZH2 (also upstream) APC (also upstream) RBFOX2 (also upstream)	/
Lean	rs31062829	*Fbxl3*	MA → LA motif (C2)	Shorter 3′ UTR	*mmu-miR-20a-5p (also upstream)* *mmu-miR-669k-3p (also downstream)* *mmu-miR-106b-5p (also upstream)* *mmu-miR-142a-5p (also upstream)* *mmu-miR-26a-5p (also upstream)* *mmu-miR-23a-3p (also upstream)* *mmu-miR-669c-5p (also upstream)* *mmu-miR-499-5p (also upstream)* *mmu-miR-142a-3p* *mmu-miR-15a-5p* *mmu-miR-15b-5p* *mmu-miR-16-5p* *mmu-miR-21a-3p* *mmu-miR-23b-3p (also upstream)* *mmu-miR-26b-5p* *mmu-miR-31-3p* *mmu-miR-486a-5p* *mmu-miR-21a-5p* *mmu-miR-28a-3p* *mmu-miR-501-5p*	*U1*	CELF4 (also upstream) FUS (also upstream) RBFOX2 (also upstream) EZH2 (also upstream) LIN28A (also upstream) TARDBP (also upstream)	Y
Lean	rs229072835	*Hlf*	MA → LA motif (C1)	shorter 3′ UTR	/	*Ptbp1* *Gm22748* *Gm25855*	/	/
Lean	rs255472708, rs221503910	*Hsf2bp*	lost motif	shorter 3′ UTR	/	*U1* *Gm23105*	RBFOX2 (also upstream)	/
Lean	rs32227744	*Knop1*	LA → MA motif (C1)	longer 3′ UTR	*mmu-miR-1a-3p* (also upstream) *mmu-miR-206-3p* (also upstream) *mmu-miR-15a-5p* *mmu-miR-16-5p* *mmu-miR-15b-5p* *mmu-miR-532-5p* *mmu-miR-133a-5p* *mmu-miR-322-5p*	*U1* *Gm24119* *Snhg17* *Snora33* *Snora34* *Zc3h13* *Gm26224*	LIN28A (also upstream) EZH2 (also upstream) LIN28A (also upstream) RBFOX2 (also upstream) APC SRRM4 (also upstream) RBFOX2 (also upstream) U2AF2	m6A (also upstream - few)

(*continued on next page*)

Table 2 (*continued*)

Mouse line	SNP	Gene symbol	Effect on PAS	Consequence (fat vs lean)	miRNA (Tarbase v8)	RNA-RNA interaction (RISE)	RBP binding (RISE)	RNA editing/ modification (RISE)
					mmu-miR-125b-5p *mmu-miR-19a-3p* *mmu-miR-19b-3p* *mmu-miR-125a-3p* *mmu-miR-155-5p* *mmu-miR-18a-5p* *mmu-miR-195a-5p* *mmu-miR-26b-5p* *mmu-miR-29b-1-5p* *mmu-miR-378a-5p* *mmu-miR-381-3p* *mmu-miR-500-3p* *mmu-miR-501-5p* *mmu-miR-532-3p*		PABPC1 (also upstream)	
Lean	rs244789005	*Lair1*	MA → LA motif (C1)	longer 3′ UTR	/	/	/	/
Lean	rs245246928, rs265523112	*Nmrk1*	lost motif	shorter 3′ UTR	*mmu-miR-29b-3p*	/	/	/
Fat	rs263963399	*Rpl14*	MA → LA motif (C1)	shorter 3′ UTR	/	*Malat1* *U1* *Snord35a*	FUS (also upstream) LIN28A PABPC1	m6A
Fat	rs31703795	*Spon1*	LA → MA motif	shorter 3′ UTR	/	*Kif13a*	/	/
Fat	rs32967435	*Txndc9*	lost motif	longer 3′ UTR	/	/	PABPC1 (also upstream)	/

Column "Effect of PAS-SNP", MA – more abundant, LA – less abundant, C1 – canonical PAS AATAAA, C2 – most important canonical PAS analogue ATTAAA.

Table 3
Candidate intronic PAS-SNPs and their potential effect on lncRNA/protein lengths and, consequently, the abundance of interaction sites and functionality.

Mouse line	PAS-SNP	Gene symbol	Intron	Transcript	Domain (Ensembl)	RNA-RNA interaction (RISE)	Protein interactions (UniProt)	Effect (fat vs lean)
Lean	rs254851498	*1700086O06Rik*	–	ENSMUST00000181757	–	*Gm12896* *Sesn3* *Gm22620* *Snora44* *Mir5117* *Snord104* *Smg5* *Cntnap5b* *Plekha2*	/	Unchanged
Lean	rs50706522	*Abi3bp*	14/ 34	ENSMUST00000096012	C-terminal fibronectin type III	*Gm22457*	/	Less
Fat	rs29207890	*Agmo*	2/12	ENSMUST00000049874	Fatty acid hydroxylase; transmembrane domains	*Sf3a2*	/	Less
Fat	rs260246262	*Itga7*	1/24	ENSMUST00000099112	Integrin alpha chain; transmembrane domain	*Crtc2* *Plagl1* *Mer81* *Mettl7b*	/	More
Lean	rs32060094	*Prr16*	1/1	ENSMUST00000116639	Protein Largen/Inhibitory synaptic factor 1	*Snora34* *Tbc1d13* *Rab3b* *Gm26447* *Gm26448* *Mtd*	/	More

enriched as *mmu-miR-1932* and *mmu-miR-450a-5p* targets, while those with lower abundance might be under the regulatory control of the miRNA *mmu-miR-5104*. Interestingly, up-regulation of *miR-450a-5p* restored insulin sensitivity and reduced lipid accumulation (Wei et al., 2020). A more detailed exploration of how these pathways interact and contribute to the complex phenotype of obesity would be beneficial. Systems biology approaches based on larger datasets would provide more holistic view of the interplay between different genetic and metabolic pathways.

4.2. Potential effect of candidate PAS-SNPs in 3′ UTR

In the present study, PAS-SNPs within 12 genes could affect the 3′ UTR APA, resulting in transcripts with different 3′ UTR lengths and therefore abundance of binding motifs for RNAs in the Fat and Lean lines. Due to the PAS-SNPs, the 3′ UTR of 4 transcripts in the Fat line (*Edil3*, *Knop1*, *Lair1*, and *Txndc9*) would be longer and 8 (*Edh1*, *Eif2s1*, *Fbxl3*, *Hlf*, *Hsf2bp*, *Nmrk1*, *Rpl14,* and *Spon1*) shorter.

The PAS-SNP in the Lean line may shorten the 3′ UTR of *Edil3* in the adrenal gland, resulting in lost target sequences for 7 miRNAs. *Edil3* was recently identified as a novel candidate obesity-driven gene (Cobb et al.,

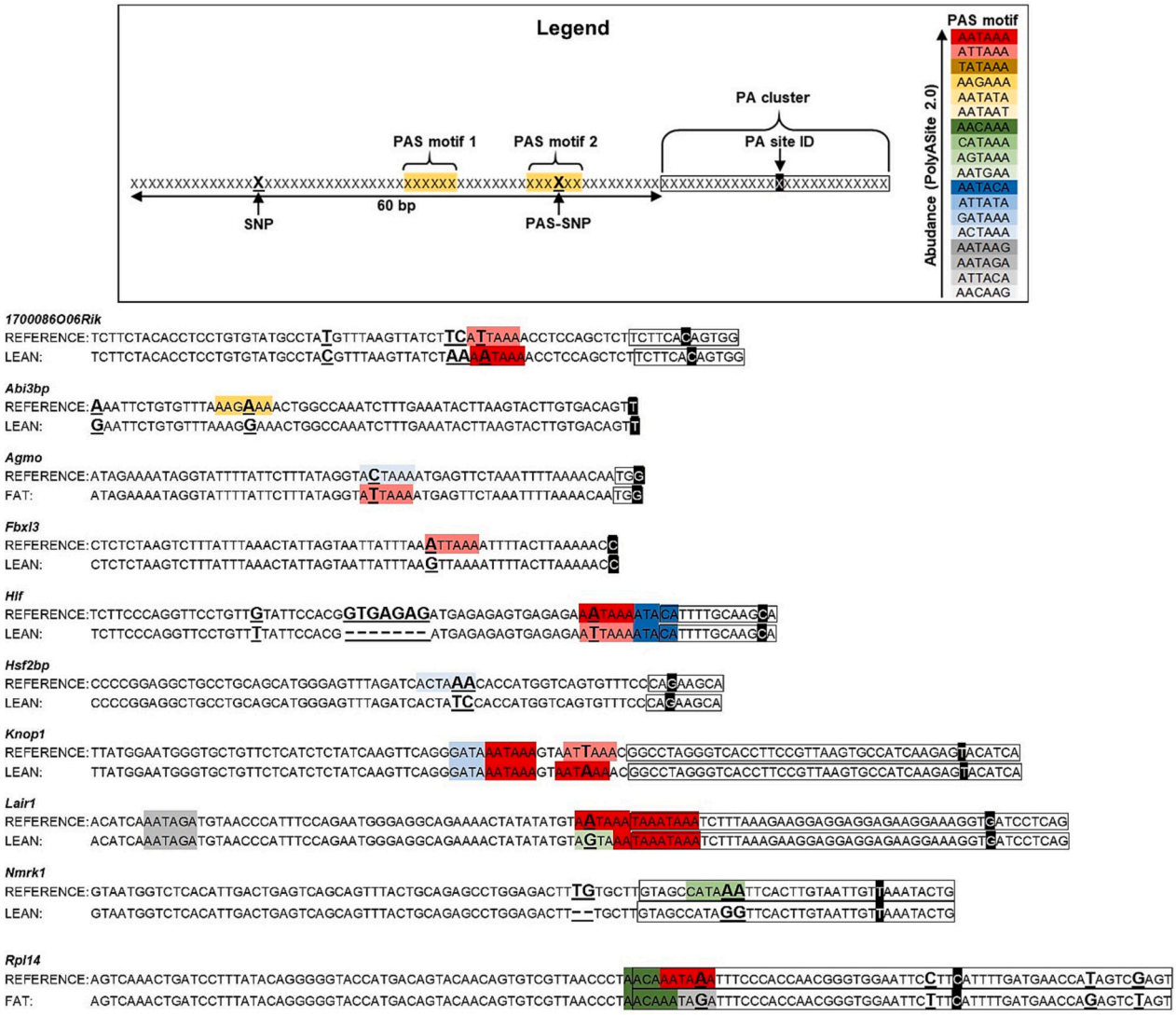

Fig. 5. DNA sequences of PA clusters and 60 bp upstream of genes carrying high-priority PAS-SNP candidates. PA site ID - most often cleavage site in this region according to PolyASite 2.0 portal, PA cluster - the longer the box the more variable the cleavage site. For each gene, the DNA sequence of the mouse line differing from the reference is provided.

2021), and may regulate the hypothalamic-pituitary-adrenal axis (Kanczkowski et al., 2013). Considering this, dysregulated *Edil3* expression observed in the present study could contribute to the distinct phenotypes of our mouse models. PAS-SNPs in the Lean line potentially also truncate 3′ UTR of *Knop1* and *Lair1* in the adrenal gland and WAT, respectively. *KNOP1* was recently considered a new candidate for body mass index in children (Yao et al., 2021). The 3′ UTR of *Knop1* in the Lean line would lose binding sites for several miRNAs, RNA and protein interactions, and most m^6A modification sites. Among the possible interactions with miRNAs, *miR-532-5p* (Ortega et al., 2013), *miR-19a-3p* (Huang et al., 2018), and *miR-125a-3p* (Chen et al., 2015) have been linked to obesity, while the long non-coding RNA *SNHG17* (small nucleolar RNA host gene 17) and the protein U2AF2 (U2 small nuclear RNA auxiliary factor 2) have been linked to diabetes (Li et al., 2021; Vastrad and Vastrad, 2021). Meanwhile, a higher expression of a longer *Lair1* isoform was noted in the present study. The interaction of human LAIR1 with adiponectin, a hormone modulating insulin resistance, glucose and lipid metabolism, has been demonstrated to suppress T cell activation (Zhang et al., 2021), suggesting *Lair1* and its PAS-SNPs rs244789005 as candidates that influence immune environment and metabolic health in WAT.

On the other hand, PAS-SNPs within *Eif2s1*, *Fbxl3*, *Hlf*, *Hsf2bp* and *Nmrk1* of the Lean line, could shift PA to more distal PA sites, resulting in transcripts with longer 3′ UTRs compared to the Fat line. Mice *Eif2s1* (eukaryotic translation initiation factor 2, subunit 1 alpha), located within the obesity quantitative trait locus, was genetically mapped in the F_2 cross between the Fat and Lean line, *Fob2* (Horvat et al., 2000). It has a function in minor initiation pathways and affects the translation of a small number of mRNAs (Anderson et al., 2021). EIF2S1 is essential for the integrity of the endoplasmic reticulum, and heterozygous *Eif2s1*$^{+/tm1Rjk}$ mice became obese and diabetic on a high-fat diet (Scheuner et al., 2005), linking endoplasmic reticulum stress and obesity. Expression of the longer transcript in the adrenal gland, thymus and WAT was lower in the Fat line, suggesting that some of regulators targeting 3′ UTR may promote *Eif2s1* translation in the Lean line. Given that *Eif2s1* maps to the *Fob2* QTL genomic interval in a Fat and Lean line cross (Horvat et al., 2000), that independent transgenic mice demonstrated obesity phenotype along with other physiological and functional relevance of EIF2S1 to metabolism and obesity development, makes *Eif2s1* and its PAS site polymorphism in the Lean line a high priority candidate gene for future experiments in examining the causal role of differential PA site usage in obesity or leanness.

Fbxl3 plays a crucial role in controlling the length of the circadian period (Siepka et al., 2007). Disruption of circadian clock in skeletal

muscle impairs glucose and energy metabolism and leads to insulin resistance (Gutierrez-Monreal et al., 2020), changes in muscle mass and strength and eventually to a decrease in daily physical activity (Aoyama and Shibata, 2017). More importantly, loss-of-*Fbxl3*-function mice exhibited longer free-running period (Srikanta and Cermakian, 2021), which was observed for our Lean compared to the Fat line (Simončič et al., 2008a), suggesting a higher *Fbxl3* expression in the Fat line may contribute to its lower physical activity and obese phenotype, making the rs31062829 a candidate "lean" allele.

The PAS-SNP in *Hlf* of the Lean line may result in a higher expression level of *Hlf* with a shorter 3′ UTR in the thymus of the Fat line. HLF is an oncogenic transcription factor that is a strong negative regulator of lymphoid development (Wahlestedt et al., 2017), also involved in T-cell differentiation in thymus (Smith et al., 1999). Its potential regulator *Ptbp1* (polypyrimidine tract binding protein 1) is also associated with thymocyte development (Bertonha et al., 2020). Interestingly, knock-down of *HLF* significantly reduced lipid content in 3T3-L1 cells (Dzitoyeva and Manev, 2013), suggesting that higher expression of a shorter transcript in the Fat line may contribute to its adiposity.

Our previous study found higher hepatic expression of cholesterol biosynthesis genes and protective HDL cholesterol levels in the Lean line (Simončič et al., 2011). In the present study, two PAS-SNPs were identified as high-priority candidates in the Lean line. They might cause higher expression of *Hsf2bp* with a shorter 3′ UTR in the Fat line liver. Oprea (2019) suggests that *HSF2BP* plays a role in lowering HDL cholesterol levels. The retained sequence in the 3′ UTR of *Hsf2bp* of the Lean line would attract the potential regulator *Rbfox2*, which has been shown to regulate genes involved in lipid and cholesterol biosynthesis (Wu et al., 2018).

Two PAS-SNPs in *Nmrk1* of the Lean line cause the region upstream of their PA site to be devoid of known PAS, likely rendering this PA site inactive, resulting in a longer 3′ UTR length. In kidneys, NMRK1 (also known as NRK1) plays a key role in NAD^+ biosynthesis, which is required for their proper function (Hershberger et al., 2017). Dysregulation of NAD^+ metabolism is associated with obesity and the development of diabetic kidney disease (Ralto et al., 2020). According to the Ensembl database, both SNPs are also present in obesity-resistant mice strain CAST/EiJ (Karunakaran and Clee, 2018), suggesting a positive effect of the longer transcript on leanness. Preclinical models show that NAD^+ pools decline also in obesity and replenishment prevents metabolic syndrome and reduces blood pressure (Abdellatif et al., 2021). Since our Lean line has higher expression of the longer variant of NMRK1 (Supplementary Table 3), this results further supports *Nmrk1* and genetic variation in PAS site as a high priority anti-obesity candidate.

In the Fat line, PAS-SNPs may cause shortening of the 3′ UTR of *Ehd1*, *Rpl14* and *Spon1*, but elongating the *Txndc9* transcript. Mice lacking EHD1 have less esterified cholesterol and triglycerides stored in lipid droplets (Naslavsky et al., 2007). A motif lost by PAS-SNP in a distal part of the *Ehd1* transcript would move the site of PA upstream. Considering that protein and transcript expression levels of EHD1 were among the highest in obesity-prone rats compared to obesity-resistant rats (Joo et al., 2011), miRNAs likely have a negative impact on the *Ehd1* transcript-to-protein conversion in the Lean line. For example, the expression level of *miR-26b* correlates negatively with increasing body mass index (Xu et al., 2015), suggesting that rs37844368 may be a candidate "obesity" allele.

The rs263963399 in the Fat line may shorten the 3′ UTR of the *Rpl14* in all tissues examined. *RPL14* expression is downregulated in obese individuals (Wang et al., 2016a), possibly increasing apoptosis (Germani et al., 2018; Wang et al., 2016a). Evidence suggests that pathways of programmed cell death are activated in hypertrophied adipocytes, contributing to metabolic abnormalities (Eguchi and Feldstein, 2014). Compared to the longer 3′ UTR of *Rpl14* in the Lean line, the 3′ UTR of the Fat line would lose target sites for PABPC1 (poly(A) binding protein, cytoplasmic 1), which is one of the key proteins in post-transcriptional regulation, acting as both a translational stimulator and repressor (Kini et al., 2016; Liu et al., 2016; Zhang et al., 2015). As in the case of *Rpl14*, a PAS-SNP in *Spon1* may shorten its 3′ UTR in several tissues of the Fat line, resulting in the loss of an interaction site for *Kif13a* (kinesin family member 13A). Lower SPON1 protein abundance was associated with body mass index (Lind et al., 2020). A potentially lost interaction with *Kif13a* could, according to previous studies (Delevoye et al., 2014; Gutiérrez et al., 2021; Nakagawa et al., 2000; Zhou et al., 2013), indicate changes in the localisation of SPON1 in the Fat line.

4.3. Potential effect of candidate intronic PAS-SNPs

Compared to the 3′ UTR APA (which accounts for about 80 % of APA events), intronic APA occurs less frequently because it can lead to mRNA decay or the production of proteins with altered functions (Nourse et al., 2020; Yuan et al., 2021). Here, we report 5 PAS-SNPs with a likelihood to influence the length and thus the functionality of 5 genes (*1700086O06Rik*, *Abi3bp*, *Agmo*, *Itga7* and *Prr16*).

LncRNAs play important regulatory roles in various human diseases (Chen et al., 2021). In the present study, a PAS-SNP in the *1700086O06Rik* of the Lean line potentially truncates the *1700086O06Rik* transcript. However, the position for several known interactions with other RNAs is retained in this short transcript, consistent with previous observations that only 15–45 % of conserved elements in lncRNAs localise after the first polyadenylation site (Chen et al., 2021). Nevertheless, alternative cleavage could direct the two transcripts of different lengths between the lines into different cellular compartments (Chen et al., 2021), where the potential biological significance of interactions with other RNAs could change. Among its RNA interaction partners, *Sesn3* (sestrin 3) has been linked to type 2 diabetes (Nascimento et al., 2013), dyslipidaemia (Sundararajan et al., 2021), insulin sensitivity/resistance (Tao et al., 2015), and adipogenesis (Lin et al., 2021).

The PAS-SNP within *Abi3bp* of the Lean line destroys PAS, possibly allowing the entire protein to be encoded. Should this site be active, transcription of this gene would be prematurely terminated in the adrenal gland of the Fat line, resulting in a protein with a partially lost C-terminal fibronectin type III domain. ABI3BP is a multifunctional autocrine/paracrine factor (Hodgkinson et al., 2013) linked to several diseases, including cardiovascular diseases (Delfín et al., 2019), and various cancers and tumours (Cai et al., 2020; Feng et al., 2023; Latini et al., 2008). Perhaps more noteworthy, its expression responded to refeeding (Qiao et al., 2019), and its interaction partner *Gm22457* (*Snora9*) (RISE) was identified as a candidate gene for intramuscular fat content (Cesar et al., 2018). The above results suggest that the Fat line may suffer from various comorbidities due to the alteration of *Abi3bp*, and its possible role in obesity (feeding behaviour, intramuscular fat) requires further investigation.

Meanwhile, a PAS-SNP in the second intron of *Agmo* in the Fat line may cause transcription termination at this site, which would result in a truncated protein with lost fatty acid hydroxylase and transmembrane domains in the adrenal gland and kidney. The result suggests changes in the lipidome in these two organs of the Fat line, which has been previously shown in obesity (Escasany et al., 2019; Witt et al., 2020).

In contrast, the PAS-SNPs within *Itga7* and *Prr16* in the Fat and Lean lines, respectively, may lead to longer proteins in the WAT of the Fat line. Extracellular vesicle populations expressing ITGA7 (integrin alpha 7) have been associated with human adiposity (Zhai et al., 2022). In the study by Chen et al. (2022), ITGA7 was demonstrated to transduce signals from extracellular matrix deposits such as collagens, activating phosphorylation cascades to promote adipogenesis. The *Itga7* RNA in the Fat line would retain interaction sites for three RNAs and the transposable element *Mer81*. As for the RNAs, *CRTC2*, *PLAGL1* and *METTL7B* have been linked to whole-body energy homeostasis (Han et al., 2020), diabetes (Kamiya, 2000) and cellular lipid accumulation (Yang et al., 2021), respectively. As for *Mer81*, human *MER81* serves as a precursor

for *hsa-mir-584* (Piriyapongsa et al., 2007), which has been linked to adipocyte growth and differentiation (Machal et al., 2015).

Consistent with this, the PAS-SNP in *Prr16* of the Lean line possibly causes the truncated protein with a loss of Largen/inhibitory synaptic factor 1 domain in WAT. PRR16/Largen protein has been demonstrated to regulate mammalian cell size independently of the mTOR and Hippo regulatory pathways (Yamamoto and Mak, 2017). Considering the findings of Petäjä et al. (2013), who showed that increased adipocyte size alone strongly promotes fat accumulation in the liver independent of other factors (Petäjä et al., 2013), the higher expression of active PRR16 in the Fat line compared to the Lean line could contribute to the increased adipocyte size and consequently increased fat accumulation. In other words, the lost functional domain in the Lean line protein could be a strategy to resist obesity and contribute to the lean phenotype, making the *Prr16* and its PAS polymorphism rs32060094 an interesting candidate for future functional studies.

In our study focused on alternative polyadenylation and its genetic determinants, we have identified 19 PAS-SNPs within 17 genes with a potential influence on APA. Prioritized candidate SNPs and genes provide a foundation for translation to human obesity, representing a step towards development of diagnostic markers and the identification of novel targets for therapeutic interventions.

However, it is imperative to address certain considerations for future research. The complexity, particularly related to Affymetrix probe signals amalgamating various splice forms, may be particularly relevant when considering intronic APA, where the distribution and number of probes further contribute to this challenge. Consequently, observed differences in probe set expressions may not solely arise from PAS-SNPs but could also be attributed to variations in transcript isoforms, as well as other expression regulations such as SNPs in promoters, methylation, and RNA-RNA interactions. In the future, eQTL analysis will be done to validate the proposed effect of PAS-SNPs on expression (transcript isoform abundance). Additionally, our reliance on PolyASite 2.0 portal data may not comprehensively represent all PA sites in our models. Looking ahead, advanced techniques for the detection of polyadenylation sites like whole transcriptome termini site sequencing (WTTS-seq) will facilitate the exploration of tissue-specific APA events and their functional consequences, with a particular focus on employing proteomics to confirm translation potential. Furthermore, extending our investigation to encompass genetic variants located in regulatory regions beyond PAS, such as upstream U-rich and downstream G-rich regions, will broaden our understanding of APA events; the whole genome SNP analysis of these mouse lines can be found in Šimon et al. (in press). Future research could employ RNA-seq for a more comprehensive and quantitative assessment of transcriptomic differences and APA events. These collective considerations will enrich our comprehension of APA's genetic mechanisms, particularly in obesity development.

To demonstrate functional effects for prioritized SNPs, it would be beneficial to examine protein expression levels or conduct RNA-binding protein immunoprecipitation assays. Techniques such as CRISPR-Cas9 mediated SNP editing and reporter assays could validate the effects on gene expression and phenotype. Since obesity is a complex trait influenced by numerous genetic and environmental factors, future studies should account for potential confounders such as diet, physical activity levels, and background genetic variations that could influence the observed associations.

5. Conclusion

The integration of whole-genome sequencing and transcriptome analyses in this study has identified genome-wide candidate SNPs that could affect APA by altering/disrupting PAS motifs and be related to obesity in mice. The analysis revealed 13 "lean" and 6 "obese" candidate PAS-SNPs in 11 and 6 genes, respectively, representing an important resource for future functional studies focusing on these PAS-SNPs, their effects on APA and their contribution to the obese/lean phenotype.

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Ethics approval and consent to participate

The FLI (Fat) and FHI (Lean) selection lines have been maintained in our laboratory for >70 generations. All mice used in this study were maintained according to local ethical and EU regulatory guidelines under the Veterinary Administration of Republic of Slovenia permit No. U34401-23/2020/6

CRediT authorship contribution statement

Martin Šimon: Formal analysis, Investigation, Visualization, Writing – original draft. **Špela Mikec:** Formal analysis. **Nicholas M. Morton:** Formal analysis, Writing – review & editing. **Santosh S. Atanur:** Formal analysis. **Simon Horvat:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Tanja Kunej:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

All authors declare that they have no competing interests and have read and approved the final version of the manuscript for submission.

Data availability

Data will be made available on request.

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