UPF3A is a ubiquitously expressed NMD factor among mouse tissues

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Running title: UPF3A is ubiquitously expressed in mouse and human tissues

Abstract

Nonsense-mediated mRNA decay (NMD), an important post-transcriptional regulatory mechanism in gene expression, is actively involved in a series of cellular and physiological processes, thus controlling cell fate and tissue homeostasis. Defects in NMD cause human diseases such as neurodevelopmental disorders, tumorigenesis and autoimmunity. UPF3 (Up-frameshift protein 3), first identified in the baker's yeast, is a core NMD factor. UPF3A and UPF3B, the two UPF3 paralogs emerging in vertebrates, have either activating or suppressing roles in NMD. Previous studies found that UPF3B is ubiquitously expressed in almost all mammalian organs, while UPF3A is exclusively expressed in the mammalian testis. UPF3B competitively binds to UPF2 with higher affinity than UPF3A, which finally destabilizes UPF3A protein. In the present study, we quantitatively evaluated the expression of UPF3A and UPF3B in nine major tissues and reproductive organs of wild type male and female mice. Our study confirmed that UPF3A has the highest expression in male germlines. To our surprise, we found in most tissues, including brain and thymus, the protein level of UPF3A is comparable with that of UPF3B. In spleen and lung, UPF3A is higher than UPF3B. These findings are further supported by publicly available gene expression data. Thus, our study demonstrated that UPF3A protein is ubiquitously expressed in mouse tissues, and may play important roles in the homeostasis of multiple mammalian tissues.

Key words: Nonsense-mediated mRNA decay; UPF3A; UPF3B

36 **1 Induction**

37 Nonsense-mediated mRNA decay (NMD) is a highly conserved post-transcriptional gene
38 regulatory mechanism in eukaryotes (1-6). NMD is considered as an RNA surveillance
39 machinery, which is an essential biological pathway to regulate mRNA degradation and
40 eventual protein expression. NMD can recognize and degrade aberrant mRNAs, which contain
41 premature termination codons (PTCs) located at least 50~55 nucleotides upstream of the last
42 exon-exon junction, to prevent possible toxic effects from truncated proteins translated (1-3, 5,
43 6). Furthermore, recent studies show that NMD can recognize the special structures of mRNAs
44 with physiological functions, such as long 3′ untranslated region (3' UTR) and upstream open
45 reading frames of mRNAs, so as to regulate cell fate (7). Thus, NMD is essential for embryonic
46 development and tissue homeostasis in mammals (2, 8-12).

47 NMD was first discovered in *Saccharomyces cerevisiae* (13). Its core factors include up-
48 frameshift protein (UPF)-1, UPF2 and UPF3 identified in the unicellular eukaryotic organism
49 (yeast) (13, 14), and suppressors with morphological effects on genitalia (SMG)-5, SMG6, and
50 SMG7 in multicellular organisms (*worms, frogs and mammals*), which provide additional layers
51 of regulations on NMD execution (5, 15-17). Among these NMD core factors, UPF3 is a
52 mysterious one. There is only a single UPF3 in invertebrate organisms, including yeasts, worms
53 and flies, while UPF3A and UPF3B, two paralogs of UPF3, are proposed to be generated by a
54 gene duplication event during the emergence of vertebrate animals (18, 19). In mouse and
55 human, UPF3B is considered as the dominant UPF3 paralog expressed in almost all tissues,
56 expect that UPF3A is highly expressed in male germlines (18, 20, 21). UPF3A protein is
57 undetectable in the brain, heart, kidney, liver and spleen of adult mice, while UPF3B is
58 abundantly expressed in these tissues (18). The "undetectable" expression of UPF3A protein
59 was explained by the hypothesis that UPF3B competes over UPF3A on binding to NMD factor
60 UPF2, which destabilizes UPF3A protein (18, 22). In accordance with this hypothesis, the
61 depletion of UPF3B protein does result in a dramatic increase of UPF3A protein (18, 22, 23).
62 Thus, protein expressions of UPF3A and UPF3B could be mutually exclusive in most
63 mammalian organs.

64 Another mystery on UPF3 paralogs is their biological functions in activating or suppressing
65 NMD. UPF3B is critical during the mammalian neurodevelopment (19, 21, 24, 25). UPF3B
66 mutations cause schizophrenia, intellectual disability and autism spectrum disorders in humans
67 (21, 24). Thus, the biology of UPF3B is better characterized in mammals (19). Recent findings
68 reconcile a conclusion that UPF3B is not only a weak NMD activator (16, 20, 26-28), but also
69 is involved in the regulation of translation termination (27, 29). UPF3A, when tethered to
70 mRNAs at a site downstream of a stop codon, elicits the NMD (26, 27). Furthermore, UPF3A
71 knockdown weakly stabilizes a subset of NMD targets (22). Thus, UPF3A, as UPF3B, could
72 positively regulate NMD. Intriguingly, Shum *et al.* reported that downregulation of UPF3A
73 leads to increased NMD in human and mouse cells, and overexpression of UPF3A results in
74 NMD inhibition (18). These results imply that UPF3A is an NMD repressor (18). Two more
75 recent studies revisited the interrelationship of UPF3 paralogs in several human cell lines. They
76 found that knockout of UPF3A or UPF3B alone weakly inhibits NMD, while simultaneous
77 knockout of UPF3A and UPF3B result in significant inhibition of NMD and the strong
78 accumulation of PTC containing mRNAs (16, 28). Thus, UPF3A remains as a weak activator
79 of NMD in human (16, 28).
80 To further investigate the role of UPF3A in murine NMD, we recently constructed a *Upf3a*
81 conditional knockout mouse line and generated a batch of *Upf3a* inducible knockout embryonic
82 stem cells and rib muscle derived fibroblasts. We found that UPF3A loss does not inhibit NMD
83 in mouse pluripotent and somatic cells (30), supporting the conclusion that UPF3A is not a
84 NMD repressor, but a weak activator (16, 28). Surprisingly, we found that UPF3A, as UPF3B,
85 is adequately expressed in all the mouse cell lines we established. This finding motivated us to
86 re-investigate the physiological expression patterns of UPF3A and UPF3B in different tissues
87 from wild type mice, which stands as the basics in UPF3A and UPF3B biology. We measured
88 the expression of UPF3A and UPF3B at mRNAs and protein levels in nine major mouse tissues
89 (liver, heart, spleen, lung, kidney, thymus, small intestine, cerebral cortex, and olfactory bulb)
90 and reproductive tissues (testis and ovary) from males and females. We found that UPF3A and
91 UPF3B are not mutual-exclusively but adequately and ubiquitously expressed in most of the

92 tissue we investigated. Thus, our finding revises the previous long-standing assumption on
93 UPF3A and UPF3B expression among tissues, and will serve as a reference for further in-depth
94 study on the biology of UPF3 paralogs in mammals.
95

96 **2 Materials and Methods**

97 **2.1 Mice**

98 Male and female C57BL/6 mice aged 8-10 weeks were maintained under specific pathogen-
99 free conditions at the animal facility of Shandong University (Qingdao, China). All mice had
100 free access to water and food. All animal care and experiments were performed according to
101 the guidelines of the ethics committee (License number: SYDWLL-2022-083).

102

103 **2.2 Real-time quantitative PCR (RT-qPCR)**

104 Total RNAs of mouse tissues were extracted by TRIzol reagent (Takara Bio, Tokyo, Japan),
105 then were used for cDNA synthesis with HiScript® II Q Select RT SuperMix for qPCR (Vazyme
106 Biotech Co., Ltd., Nanjing, China). RT-qPCR was performed on the CFX Real-time PCR
107 system (Bio-Rad, Hercules, CA, USA) using SYBR Green Master qPCR Mix (Tsingke
108 Biotechnology Co., Ltd., Beijing, China). The primers used are listed as bellow:

109 *mUpf3a*: F, GCGCACGATTACTTCGAGGT; R, TCAAAACGGTCTCTGAACAGC;

110 *mUpf3b*: F, AGGAGAAACGAGTGACCCTGT; R, CCTGTTGCGATCCTGCCTA;

111 *mβ-actin*: F, AGAGGGAAATCGTGCGTGAC; R, CAATAGTGATGACCTGGCCGT.

112

113 **2.3 Western blotting**

114 Western blotting analysis followed a previously published method (31). Briefly, RIPA buffer
115 (Radioimmunoprecipitation assay buffer) supplemented with protease/phosphatase inhibitors
116 (APExBIO, Houston, TX, USA) was used for total protein extraction from mouse tissues. The
117 Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for
118 measuring the protein concentrations. Sodium dodecyl sulfate-polyacrylamide gel
119 electrophoresis was performed to separate the proteins, which were further transferred to PVDF
120 membranes (Merck Millipore Ltd., Darmstadt, Germany). After blocking with 5% milk-Tris-
121 buffered saline with Tween 20 (TBST) for 2 h, the membranes were incubated with rabbit anti-
122 UPF3A+3B antibody (1:1000, Abcam, Cambridge, UK) or mouse anti-GAPDH antibody
123 (1:5000, Proteintech, Tokyo, Japan) overnight at 4°C, then washed three times with TBST and

incubated with corresponding secondary antibodies for 1 h at room temperature. Protein bands were visualized using BeyoECL Plus (Beyotime Biotechnology, Shanghai, China), and quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.4 Statistical analysis

Data were statistically analyzed by SPSS (v26.0) software, and all data are presented as means ± standard deviation (SD). P value < 0.05 was considered significant. Kolmogorov-Smirnov test was used to determine whether the data followed the normal distribution. Comparisons between the two groups were executed by unpaired Student's *t*-test or Mann-Whitney *U*-test.

135 **3 Results**

136 **3.1 mRNA expression of *Upf3a* and *Upf3b* among different tissues in adult mice**

137 We examined the mRNA expression of *Upf3a* and *Upf3b* in nine major organs (liver, heart,
138 spleen, lung, kidney, thymus, small intestine, cerebral cortex, and olfactory bulb), and
139 reproductive tissues (testis and ovary) of adult C57BL/6 mice. RT-qPCR analysis showed that
140 small intestine has the lowest expression of *Upf3a* and *Upf3b* in both sexes (Figure 1A and 1B).
141 In adult males, the mRNA level of *Upf3a* is the highest in the testis, 20 times more than that in
142 the liver (Figure 1A). In hematopoietic organs, including spleen and thymus, mRNAs of *Upf3a*
143 and *Upf3b* are generally lower than those in liver, heart, lung, kidney, cerebral cortex and
144 olfactory bulb from males. In females, no significant difference in the mRNA expression of
145 *Upf3a* and *Upf3b* is found between tissues (Figure 1B), although we could still notice that
146 hematopoietic organs have lower expression of *Upf3a* and *Upf3b*.

147 Next, we compared the differences in the mRNA expression of *Upf3a* and *Upf3b* between
148 the sexes. In general, *Upf3a* and *Upf3b* mRNA expressions are comparable between sexes.
149 However, *Upf3a* mRNA expression in the olfactory bulbs from females, and *Upf3b* mRNA
150 expression in small intestines and thymuses of females are significantly higher than those of
151 male mice, while the mRNA expression of *Upf3b* in the lungs of males is higher than that in
152 females (Figure 1C).

153

154 **3.2 Protein expression of UPF3A and UPF3B among different tissues in adult mice**

155 RT-qPCR analysis with a different set of primers does not allow for the direct comparison of
156 the expression of two genes. Furthermore, antibody-based approach normally could distinguish
157 the relative expression of protein isoforms of a single gene, and could not determine the relative
158 expression of two gene products in a single blot. We previously characterized a commercial
159 antibody which has cross-reactivities with mouse UPF3A and UPF3B proteins. This antibody
160 allows us to directly compare the relative expression of UPF3A and UPF3B in one
161 immunological reaction. We first explored the tissue specific expression of UPF3A and UPF3B
162 in male and female mice, respectively.

163 As expected, we found UPF3B is ubiquitously expressed in most of mouse tissues, including
164 the liver, spleen, lung, kidney, thymus, cerebral cortex, and olfactory bulb from both sexes and
165 ovaries in females (Figure 2). However, hearts and small intestines from both sexes have almost
166 negligible expression of UPF3B. For UPF3A, as expected, it is highly expressed in male testis.
167 Interestingly, we found UPF3A is evidently expressed in almost all the mouse tissues, including
168 the liver, spleen, lung, thymus, cerebral cortex, and olfactory bulb from both sexes and ovaries
169 in females.
170 We further investigated the relative expression of UPF3A and UPF3B in all tissues we
171 analyzed. We found that in spleens and lungs from both sexes, UPF3A protein levels are higher
172 than those of UPF3B. In livers, kidneys, cerebral cortexes, and olfactory bulbs from both sexes,
173 UPF3B is the dominant UPF3 paralog. Intriguing, we found a higher protein expression of
174 UPF3A over UPF3B in ovaries (Figure 2). Furthermore, we went on to ask whether the
175 expression of UPF3A and UPF3B expresses differently between sexes at given tissues. We
176 found there was no significant difference in the protein expression of UPF3A and UPF3B in all
177 tissues between male and female mice (Figure 3).
178
179 **3.3 UPF3A is ubiquitously expressed in mouse and human tissues**
180 To further substantiate our unexpected finding that UPF3A, as UPF3B, is ubiquitously
181 expressed in mouse tissues, we retrieved the *Upf3a* and *Upf3b* expression data from mouse
182 ENCODE transcriptome and used RPKM value (Reads Per Kilobase per Million mapped reads)
183 data to reveal relative expressions of *Upf3a* and *Upf3b* in different tissues. The mouse ENCODE
184 transcriptome dataset further confirmed that *Upf3a* is highly expressed in testis. Furthermore,
185 the RPKM value of *Upf3a* is higher than *Upf3b* in all tissues investigated (Figure 4A).
186 In our analysis, we further surveyed the HPA RNA-Seq data from normal human tissue, and
187 we found that the *UPF3A* is also highest expressed in human testis (Figure 4B), implying that
188 the expression of UPF3A is conservative. Furthermore, *UPF3A* expression is not lower than
189 *UPF3B* expression in most tissues of human and mouse, indicating that UPF3A is ubiquitously
190 expressed among human and mouse tissues (Figure 4A and 4B).

191 **Discussion**

192 Nonsense-mediated mRNA decay is an important post-transcriptional regulatory mechanism
193 of gene expression in eukaryotic cells (1, 3, 8-10). NMD controls the quality of transcriptome
194 and quantity of gene transcripts, thus playing vital roles in animal development and diseases (2,
195 9). Although UPF3 is among the 1st set of trans-regulatory NMD factors found in unicellular
196 eukaryotic organism - yeast (13, 14), the biology of UPF3 and its vertebrate paralogs (UPF3A
197 and UPF3B) are poorly understood. The functions of UPF3A and UPF3B are diverse and
198 controversial (18, 22, 26-28). One of the mysterious phenomena of UPF3A and UPF3B biology
199 is that while UPF3B is abundantly expressed in most mammalian tissues, UPF3A protein is
200 hardly detectable (18, 20, 21). Thus, proteins of UPF3A and UPF3B could be mutually
201 exclusively presented in most mammalian organs. We recently characterized an antibody which
202 could detect UPF3A and UPF3B proteins simultaneously ("one tube reaction"), and found that
203 in mouse embryonic stem cells and rib muscle derived fibroblasts, UPF3A and UPF3B are
204 adequately expressed. This finding motivated us to investigate whether the mutually exclusive
205 expression of UPF3A and UPF3B do exist under the physiological condition in mouse.

206 RT-qPCR analysis showed that mRNAs of *Upf3a* and *Upf3b* are ubiquitously presented in
207 all tested tissues from adult male and female mice. Furthermore, testis has the highest
208 expression of *Upf3a*. These findings are in good constancy with previous studies (18, 20). With
209 the antibody which could detect UPF3A and UPF3B proteins simultaneously ("one tube
210 reaction"), we investigated the proteins of UPF3A and UPF3B in nine tissues of adult male and
211 female mice, and compared the relative expression of UPF3A and UPF3B in these tissues. Our
212 study confirmed that UPF3B is ubiquitously expressed, which supported the general notion that
213 UPF3B is a major NMD related paralog of UPF3 in mammals (28). Furthermore, UPF3A is
214 highly expressed in testis, indicating an essential role of UPF3A in spermatogenesis (18, 20).
215 However, the most striking finding in the current study is that UPF3A protein is also
216 ubiquitously expressed. This finding is discrepant to most of previous studies conducted with
217 RNA-hybridization based or antibody-based approaches (18, 20, 21). We reasoned here that our
218 "one tube reaction" avoids several drawbacks when using immunoblotting: 1) Performance

219 variances of individual antibody against UPF3A and UPF3B; 2) Techniques including signal
220 detection and membrane processing. Furthermore, the ubiquitous expression nature of UPF3A
221 is supported by publicly available RNA-seq data in mouse and human tissues. Since UPF3A
222 knockout mice are embryonic lethal (18), the ubiquitous expression pattern of UPF3A indicates
223 that UPF3A is essential for organ development and tissue homeostasis.

224 In our study, we found four types of tissue specific expression patterns related to protein
225 levels of UPF3A and UPF3B: 1) in spleens and lungs from both sexes and ovaries from adult
226 females, UPF3A is the major UPF3 paralog (***UPF3A***^{high}); in livers, kidneys, cerebral cortexes
227 and olfactory bulbs from both sexes, UPF3B is the dominant form of UPF3 paralog
228 (***UPF3B***^{high})； in the thymus of both sexes, UPF3A and UPF3B are comparable in protein
229 expression (***UPF3A/B***^{equal}); hearts and small intestines from both sexes express the least
230 amount of UPF3A and UPF3B (***UPF3A/B***^{null}). The biological mechanism of tissue specific
231 expressions of UPF3A and UPF3B are worthy of further detailed characterization. Previous
232 findings suggest that competition between UPF3B and UPF3A binding to UPF2, and thus
233 UPF3A is destabilized when UPF3B is expressed (18, 22). Our finding here suggested that,
234 under physiological conditions, UPF3A and UPF3B do not compete or destabilize each other.
235 Since the co-deletion of UPF3A and UPF3B in mammalian cells showed stronger NMD
236 inhibitory effects than UPF3A or UPF3B single deletion (16, 22, 28), our findings suggest that
237 UPF3A and UPF3B could synergistically regulate NMD.

238 In summary, in the current study, we characterized the mRNA and protein expression of
239 UPF3A and UPF3B in ten tissues from adult male and female mice. We found that UPF3A is
240 ubiquitously expressed. Our study provides new evidence on solving the mystery of UPF3A
241 and UPF3B biology in mammals.

242

Authors' contributions

Tangliang Li designed the experiments and supervised the project; Xin Ma performed the experiments and analyzed the data; Xin Ma and Tangliang Li wrote the manuscript, which was edited by Yan Li and Chengyan Chen. All authors read and approved the final manuscript.

Acknowledgements:

We thank all members of Li laboratory for their helpful discussions and critical comments on the manuscript. The research projects conducted in Li laboratory are supported by: Grant No. LY22C050003, from the Natural Science Foundation of Zhejiang Province, China; Grants No. KF2020005 and KF2021008 from NHC Key Laboratory of Birth Defect for Research and Prevention (Hunan Provincial Maternal and Child Health Care Hospital, Changsha, China), Grant No. 31770871 from National Natural Science Foundation of China, and Qilu Youth Scholar Startup Funding of Shandong University.

Disclosures

The authors have no conflicts of interest to declare.

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366 **Figure legends:**

367 **Figure 1. The mRNA relative expression of *Upf3a* and *Upf3b* in tissues of C57BL/6 mice**
368 **measured by Real-time quantitative PCR. (A)** Male mice. **(B)** Female mice. **(C)** Comparison
369 of male and female mice. n=3. Results are represented as mean ± SD. *, $P < 0.05$; **, $P < 0.01$.
370 He: heart; Li: Liver; Sp: spleen; Lu: lung; Ki: kidney; Je: jejunum; Th: thymus; Br: brain; Ob:
371 olfactory bulb; Te: testis; Ov: ovary.

372 **Figure 2. The expression of UPF3A and UPF3B proteins in tissues from C57BL/6 mice**
373 **determined by Western blotting** (GAPDH was used as the loading control). **(A)** Male mice.
374 **(B)** Female mice. **(C)** the relative protein expression of UPF3A/B. n=3. Results are represented
375 as mean ± SD. He: heart; Li: Liver; Sp: spleen; Lu: lung; Ki: kidney; Je: jejunum; Th: thymus;
376 Br: brain; Ob: olfactory bulb; Te: testis; Ov: ovary; NA: not available.

377 **Figure 3. The comparison of UPF3A and UPF3B proteins in tissues from male and female**
378 **mice.** n=3. Results are represented as mean ± SD. He: heart; Li: Liver; Sp: spleen; Lu: lung; Ki:
379 kidney; Je: jejunum; Th: thymus; Br: brain; Ob: olfactory bulb; Te: testis; Ov: ovary; NA: not
380 available.

381 **Figure 4. *Upf3a* and *Upf3b* mRNA levels in different organs in mouse and human.** Mouse
382 ENCODE transcriptome data **(A)** and HPA RNA-seq normal tissues **(B)** of *UPF3A* and *UPF3B*
383 were retrieved from NCBI. RPKM, Reads Per Kilobase per Million mapped reads.

Figures for Ma et al.

Figure 1

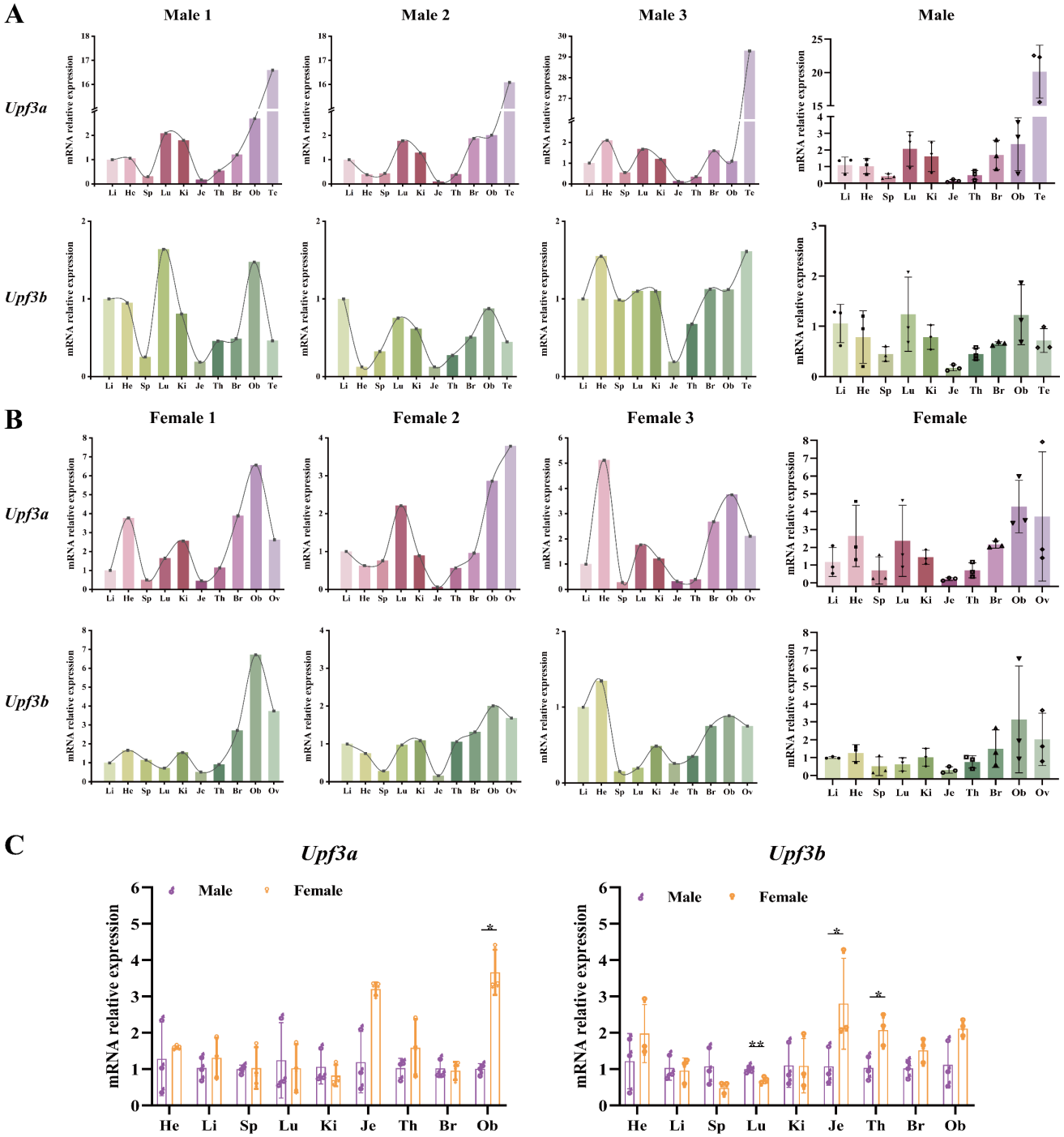

A
Male 1
Male 2
Male 3
Male
Upf3a
Upf3b
mRNA relative expression
Li He Sp Lu Ki Je Th Br Ob Te
B
Female 1
Female 2
Female 3
Female
Upf3a
Upf3b
mRNA relative expression
Li He Sp Lu Ki Je Th Br Ob Ov
C
Upf3a
Upf3b
Male
Female
mRNA relative expression
He Li Sp Lu Ki Je Th Br Ob
*
**

Figure 2

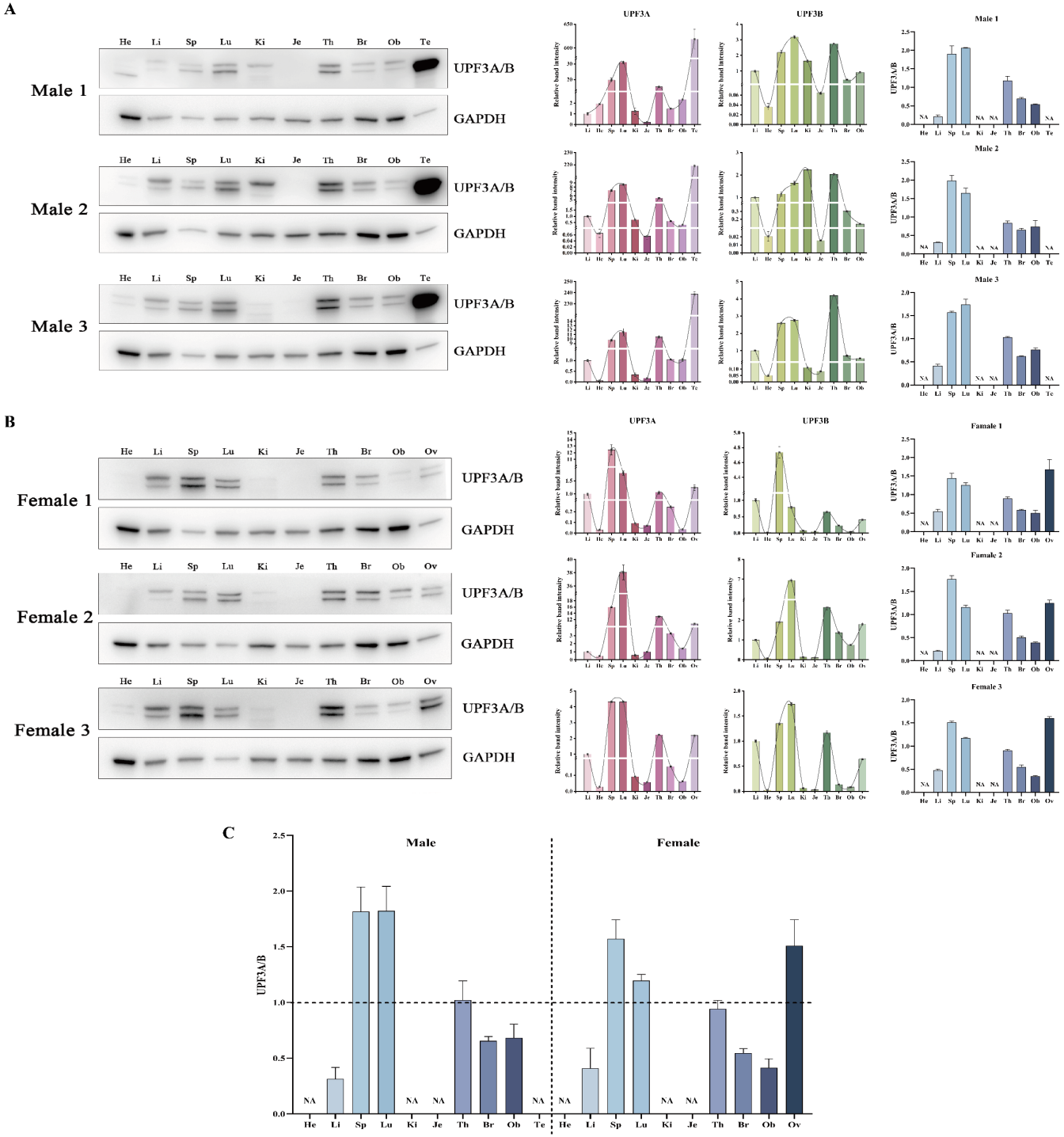

A
He Li Sp Lu Ki Je Th Br Ob Te
Male 1
Male 2
Male 3
UPF3A/B
GAPDH
UPF3A
UPF3B
Relative band intensity
B
He Li Sp Lu Ki Je Th Br Ob Ov
Female 1
Female 2
Female 3
Famale 1
Famale 2
C
Male
Female
UPF3A/B
NA

Figure 3

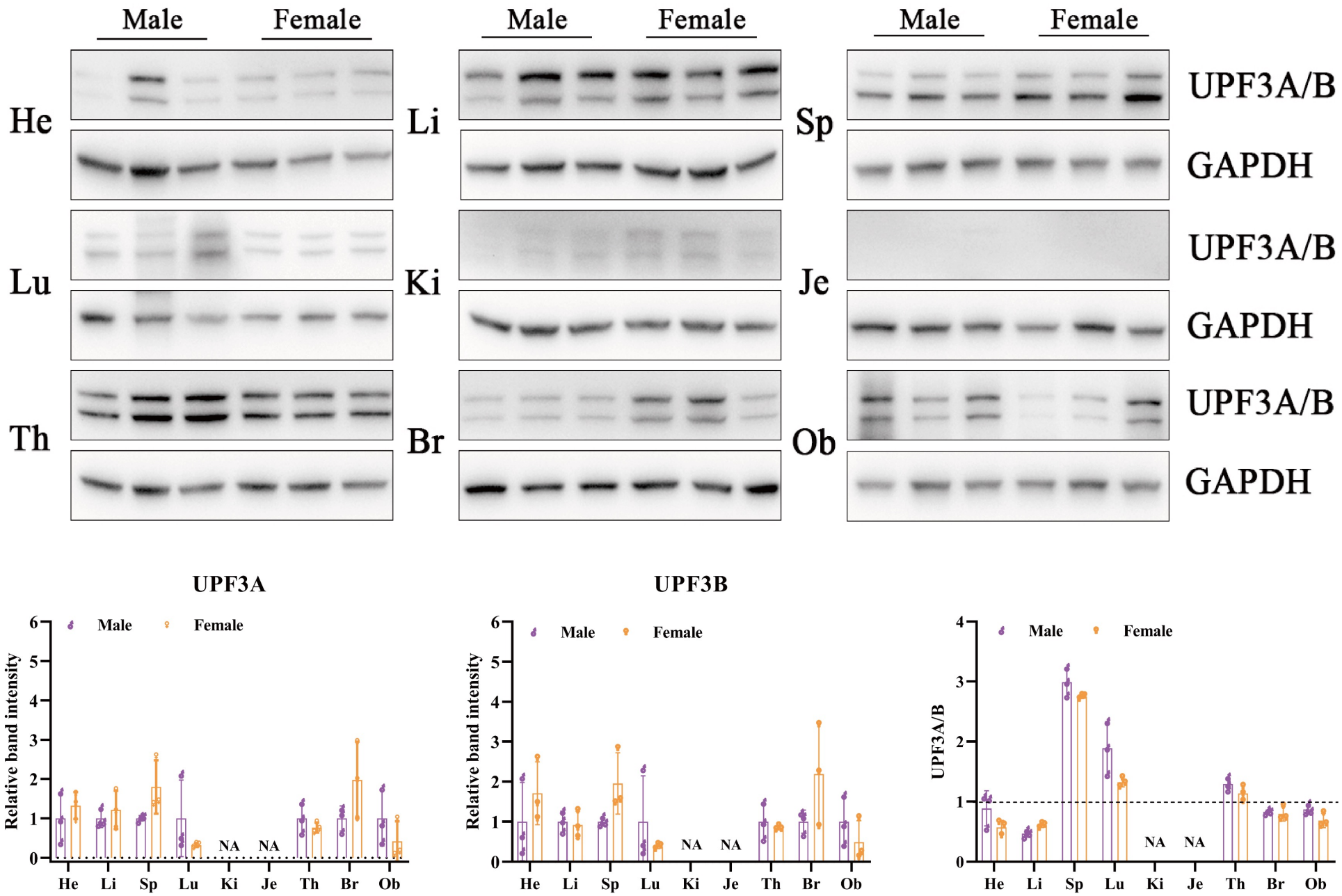
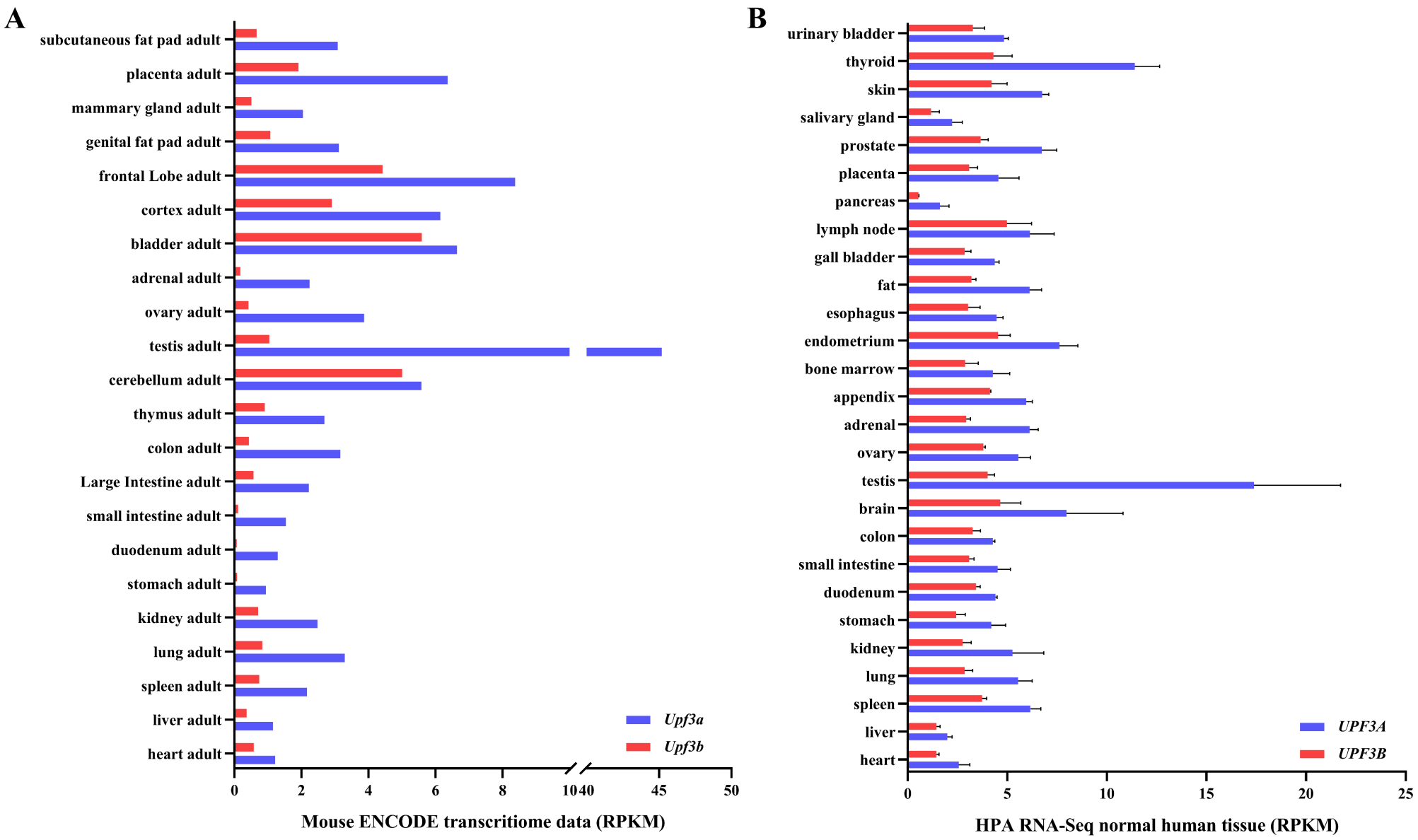

Male
Female
He
Li
Sp
UPF3A/B
GAPDH
Lu
Ki
Je
UPF3A/B
GAPDH
Th
Br
Ob
UPF3A/B
GAPDH
UPF3A
UPF3B
Male
Female
Relative band intensity
UPF3A/B
NA
He
Li
Sp
Lu
Ki
Je
Th
Br
Ob

Figure 4


A
subcutaneous fat pad adult
placenta adult
mammary gland adult
genital fat pad adult
frontal Lobe adult
cortex adult
bladder adult
adrenal adult
ovary adult
testis adult
cerebellum adult
thymus adult
colon adult
Large Intestine adult
small intestine adult
duodenum adult
stomach adult
kidney adult
lung adult
spleen adult
liver adult
heart adult
Upf3a
Upf3b
0
2
4
6
8
10
40
45
50
Mouse ENCODE transcritiome data (RPKM)
B
urinary bladder
thyroid
skin
salivary gland
prostate
placenta
pancreas
lymph node
gall bladder
fat
esophagus
endometrium
bone marrow
appendix
adrenal
ovary
testis
brain
colon
small intestine
duodenum
stomach
kidney
lung
spleen
liver
heart
UPF3A
UPF3B
0
5
10
15
20
25
HPA RNA-Seq normal human tissue (RPKM)