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 protein is ubiquitously expressed in almost all mammalian organs, while UPF3A protein is hardly detectable in most of mammalian tissues, except in the testis. One hypothesis explaining this phenomena is the functional antognism between UPF3A and UPF3B in NMD. Thus, 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

38 **1 Induction**

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

49 NMD was first discovered in *Saccharomyces cerevisiae* (13). Its core factors include up-
50 frameshift protein (UPF)-1, UPF2 and UPF3 identified in the unicellular eukaryotic organism
51 (yeast) (13, 14), and suppressors with morphological effects on genitalia (SMG)-5, SMG6, and
52 SMG7 in multicellular organisms (*worms, frogs and mammals*), which provide additional layers
53 of regulations on NMD execution (5, 15-17). Among these NMD core factors, UPF3 is a
54 mysterious one. There is only a single UPF3 in invertebrate organisms, including yeasts, worms
55 and flies, while UPF3A and UPF3B, two paralogs of UPF3, are proposed to be generated by a
56 gene duplication event during the emergence of vertebrate animals (18, 19). In mouse and
57 human, UPF3B is considered as the dominant UPF3 paralog expressed in almost all tissues,
58 expect that UPF3A is highly expressed in male germlines (18, 20, 21). In human, Serin *et al.*
59 and Tarpey *et al.* used the Southern blotting method and found that *UPF3B* transcipts are highly
60 produced in all major ograns including bone marrow, brain, lung, testis and intenstine, etc.,
61 while *UPF3A* transcripts are highly enriched in human testis. Only little amounts of *UPF3A*
62 mRNAs are found in human bone marrow, brain, intestine samples (20, 21). In mouse, Upf3a
63 mRNAs in testis is around 40-folds higher than other organs. In consistent to the RNA data in
64 human and mouse, Shum *et al.* used antibody based-apprach and found UPF3A protein is almost
65 undetectable in the brain, heart, kidney, liver and spleen of adult mice, while UPF3B is

66 abundantly expressed in these tissues (18). The "under-represented" UPF3A protein in most
67 mammalian tissues was patially explained by the hypothesis that UPF3B competes over UPF3A
68 on binding to NMD factor UPF2, which destabilizes UPF3A protein (18, 22). In accordance
69 with this hypothesis, the depletion of UPF3B protein does result in a dramatic increase of
70 UPF3A protein (18, 22, 23). Thus, protein expressions of UPF3A and UPF3B could be mutually
71 exclusive in most mammalian organs, which supports the functional antognism between UPF3A
72 and UPF3B.

73 Another mystery on UPF3 paralogs is their biological functions in activating or suppressing
74 NMD. UPF3B is critical during the mammalian neurodevelopment (19, 21, 24, 25). UPF3B
75 mutations cause schizophrenia, intellectual disability and autism spectrum disorders in humans
76 (21, 24). Thus, the biology of UPF3B is better characterized in mammals (19). Recent findings
77 reconcile a conclusion that UPF3B is not only a weak NMD activator (16, 20, 26-28), but also
78 is involved in the regulation of translation termination (27, 29). UPF3A, when tethered to
79 mRNAs at a site downstream of a stop codon, elicits the NMD (26, 27). Furthermore, UPF3A
80 knockdown weakly stabilizes a subset of NMD targets (22). Thus, UPF3A, as UPF3B, could
81 positively regulate NMD. Intriguingly, Shum *et al.* reported that downregulation of UPF3A
82 leads to increased NMD activity in human and mouse cells, and overexpression of UPF3A
83 results in NMD inhibition (18). These results imply that UPF3A is an NMD repressor (18). Two
84 more recent studies revisited the interrelationship of UPF3 paralogs in several human cell lines.
85 They found that knockout of UPF3A or UPF3B alone weakly inhibits NMD, while
86 simultaneous knockout of UPF3A and UPF3B result in significant inhibition of NMD and the
87 strong accumulation of PTC containing mRNAs (16, 28). Thus, UPF3A remains as a weak
88 activator of NMD in human (16, 28).

89 To further investigate the role of UPF3A in murine NMD, we recently constructed a *Upf3a*
90 conditional knockout mouse line and generated a batch of *Upf3a* inducible knockout embryonic
91 stem cells and rib muscle derived fibroblasts. We found that UPF3A loss does not inhibit NMD
92 in mouse pluripotent and somatic cells (30), supporting the conclusion that UPF3A is not a
93 NMD repressor, but a weak activator (16, 28). Surprisingly, we found that UPF3A, as UPF3B,

is adequately expressed in all the mouse cell lines we established. This finding motivated us to re-investigate the physiological expression patterns of UPF3A and UPF3B in different tissues from wild type mice, which stands as the basics in UPF3A and UPF3B biology. We measured the expression of UPF3A and UPF3B at mRNAs and protein levels in nine major mouse tissues (liver, heart, spleen, lung, kidney, thymus, small intestine, cerebral cortex, and olfactory bulb) and reproductive tissues (testis and ovary) from males and females. We found that UPF3A and UPF3B are not mutual-exclusively but adequately and ubiquitously expressed in most of the tissue we investigated. Thus, our finding revises the previous long-standing assumption on UPF3A and UPF3B expression among tissues, and will serve as a reference for further in-depth study on the biology of UPF3 paralogs in mammals.

105 **2 Materials and Methods**

106 **2.1 Mice**

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

111

112 **2.2 Real-time quantitative PCR (RT-qPCR)**

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

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

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

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

121

122 **2.3 Western blotting**

123 Western blotting analysis followed a previously published method (31). Briefly, RIPA buffer
124 (Radioimmunoprecipitation assay buffer) supplemented with protease/phosphatase inhibitors
125 (APExBIO, Houston, TX, USA) was used for total protein extraction from mouse tissues. The
126 Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for
127 measuring the protein concentrations. Sodium dodecyl sulfate-polyacrylamide gel
128 electrophoresis was performed to separate the proteins, which were further transferred to PVDF
129 membranes (Merck Millipore Ltd., Darmstadt, Germany). After blocking with 5% milk-Tris-
130 buffered saline with Tween 20 (TBST) for 2 h, the membranes were incubated with rabbit anti-
131 UPF3A+3B antibody (1:1000, Abcam, Cambridge, UK) or mouse anti-GAPDH antibody
132 (1:5000, Proteintech, Tokyo, Japan) overnight at 4°C, then washed three times with TBST and

133 incubated with corresponding secondary antibodies for 1 h at room temperature. Protein bands
134 were visualized using BeyoECL Plus (Beyotime Biotechnology, Shanghai, China), and
135 quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).
136
137 **2.4 Statistical analysis**
138 Data were statistically analyzed by SPSS (v26.0) software, and all data are presented as
139 means ± standard deviation (SD). P value < 0.05 was considered significant. Kolmogorov-
140 Smirnov test was used to determine whether the data followed the normal distribution.
141 Comparisons between the two groups were executed by unpaired Student's *t*-test or Mann-
142 Whitney *U*-test.
143

144 **3 Results**

145 **3.1 mRNA expression of *Upf3a* and *Upf3b* among different tissues in adult mice**

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

156 Next, we compared the differences in the mRNA expression of *Upf3a* and *Upf3b* between
157 the sexes. In general, *Upf3a* and *Upf3b* mRNA expressions are comparable between sexes.
158 However, *Upf3a* mRNA expression in the olfactory bulbs from females, and *Upf3b* mRNA
159 expression in small intestines and thymuses of females are significantly higher than those of
160 male mice, while the mRNA expression of *Upf3b* in the lungs of males is higher than that in
161 females (Figure 1C).

162

163 **3.2 Protein expression of UPF3A and UPF3B among different tissues in adult mice**

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

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

200 **Discussion**

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

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

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

233 In our study, we found four types of tissue specific expression patterns related to protein
234 levels of UPF3A and UPF3B: 1) in spleens and lungs from both sexes and ovaries from adult
235 females, UPF3A is the major UPF3 paralog (***UPF3A***^{high}); in livers, kidneys, cerebral cortexes
236 and olfactory bulbs from both sexes, UPF3B is the dominant form of UPF3 paralog
237 (***UPF3B***^{high}); in the thymus of both sexes, UPF3A and UPF3B are comparable in protein
238 expression (***UPF3A/B***^{equal}); hearts and small intestines from both sexes express the least
239 amount of UPF3A and UPF3B (***UPF3A/B***^{null}). The biological mechanism of tissue specific
240 expressions of UPF3A and UPF3B are worthy of further detailed characterization. Previous
241 findings suggest that competition between UPF3B and UPF3A binding to UPF2, and thus
242 UPF3A is destabilized when UPF3B is expressed (18, 22). Our finding here suggested that,
243 under physiological conditions, UPF3A and UPF3B do not compete or destabilize each other.
244 Since the co-deletion of UPF3A and UPF3B in mammalian cells showed stronger NMD
245 inhibitory effects than UPF3A or UPF3B single deletion (16, 22, 28), our findings suggest that
246 UPF3A and UPF3B could synergistically regulate NMD. Furthermore, the ubiquitous
247 expression pattern of UPF3A indicates that, in addition to NMD, UPF3A may actively
248 participate in other NMD-independent mechanisms, such as epigenetic regulation in genetic
249 compensation, to maintain tissue homestasis (32).

250 In summary, in the current study, we characterized the mRNA and protein expression of
251 UPF3A and UPF3B in ten tissues from adult male and female mice. We found that UPF3A is
252 ubiquitously expressed, which may serve as a guidance of . Our study provides new evidence
253 on solving the mystery of UPF3A and UPF3B biology in mammals.

254

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.

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Disclosures

The authors have no conflicts of interest to declare.

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Figure legends:

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

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

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

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

Figures for Ma et al.

Figure 1

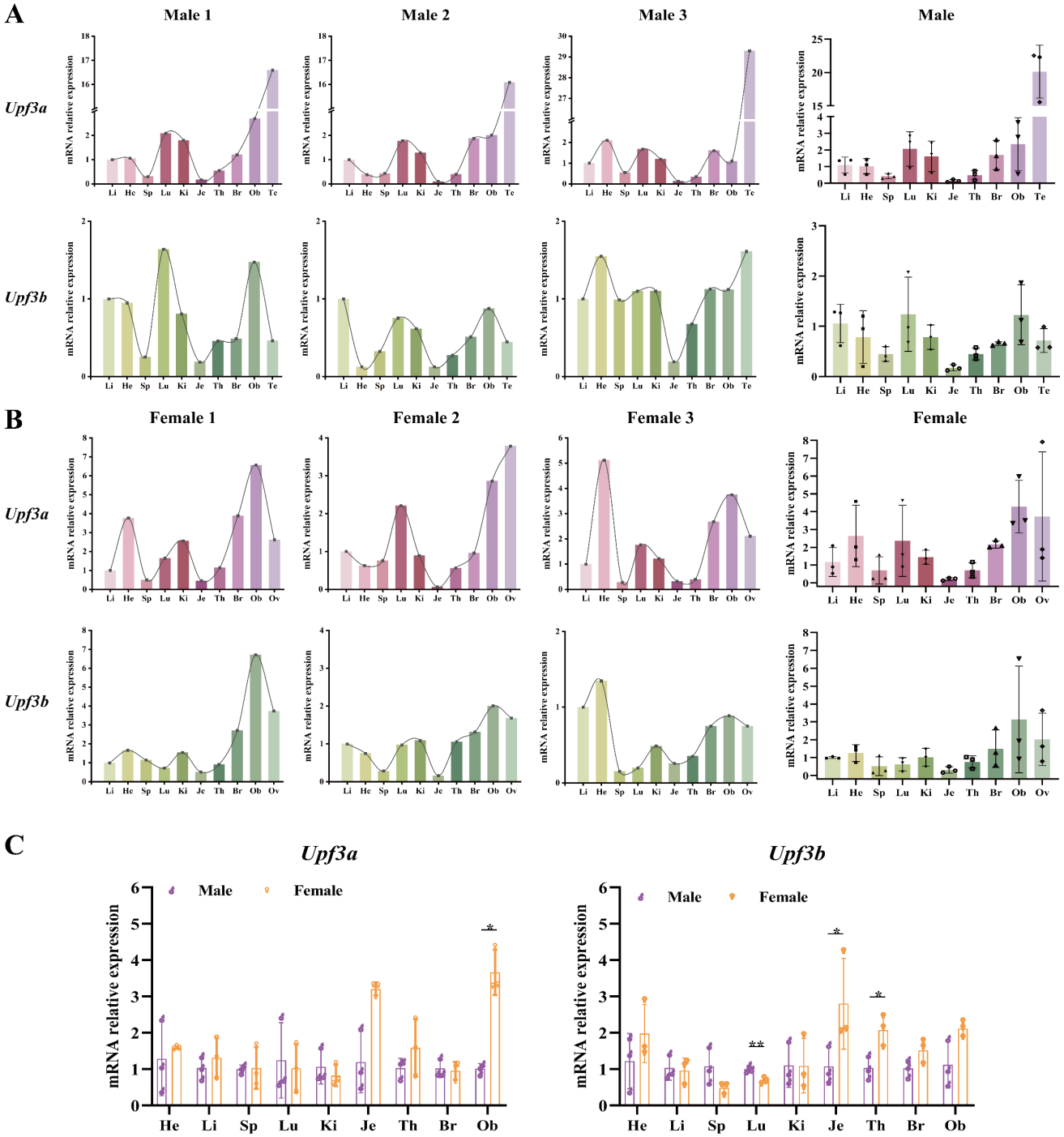

A
Male 1
Male 2
Male 3
Male
Upf3a
Upf3b
mRNA relative expression
Li He Sp Lu Ki Je Th Br Ob Te
B
Female 1
Female 2
Female 3
Female
Upf3a
Upf3b
mRNA relative expression
Li He Sp Lu Ki Je Th Br Ob Ov
C
Upf3a
Upf3b
Male
Female
mRNA relative expression
He Li Sp Lu Ki Je Th Br Ob
*
**

Figure 2

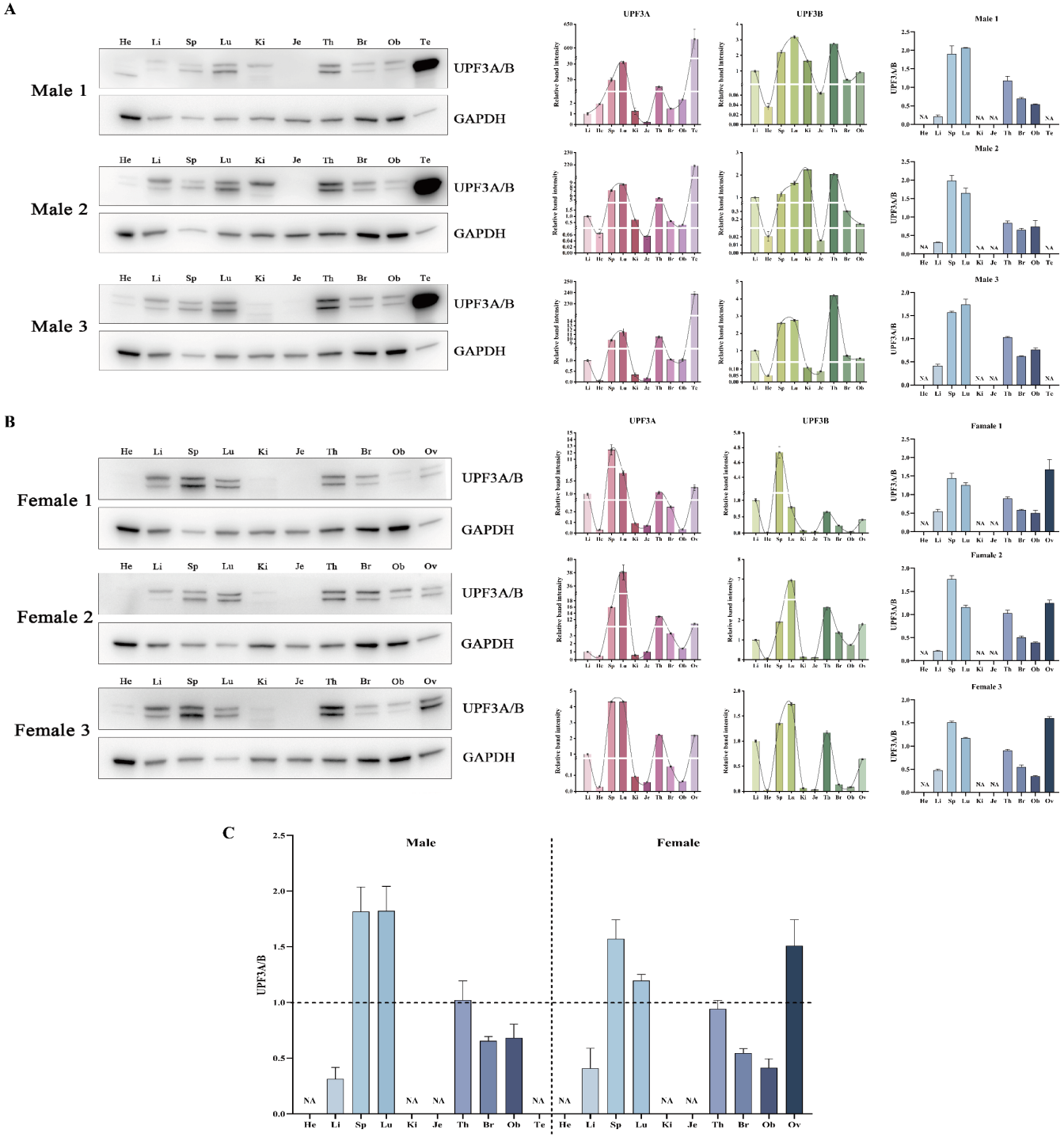

A
He Li Sp Lu Ki Je Th Br Ob Te
Male 1
Male 2
Male 3
UPF3A/B
GAPDH
UPF3A
UPF3B
Relative band intensity
B
He Li Sp Lu Ki Je Th Br Ob Ov
Female 1
Female 2
Female 3
Famale 1
Famale 2
C
Male
Female
UPF3A/B
NA

Figure 3

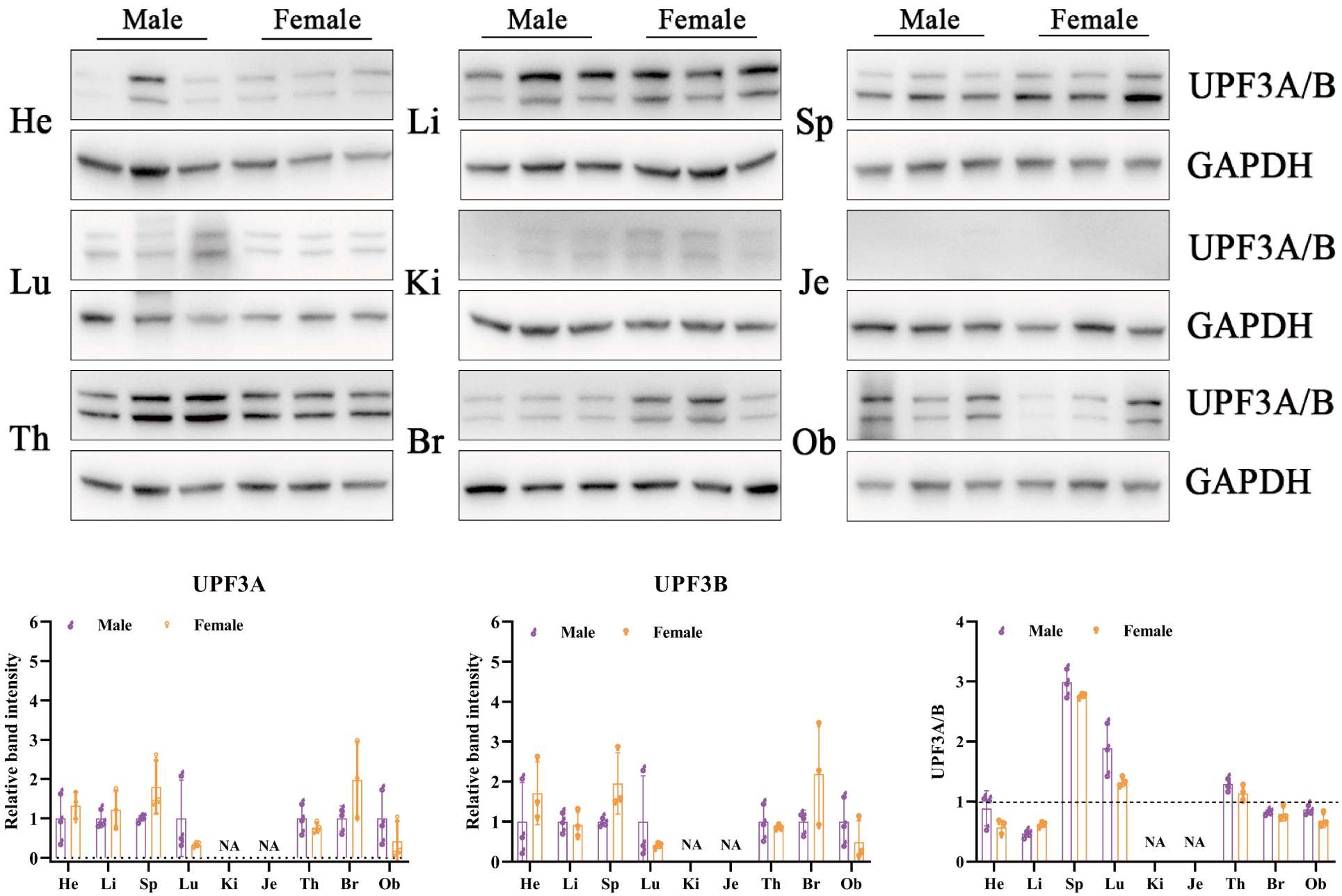
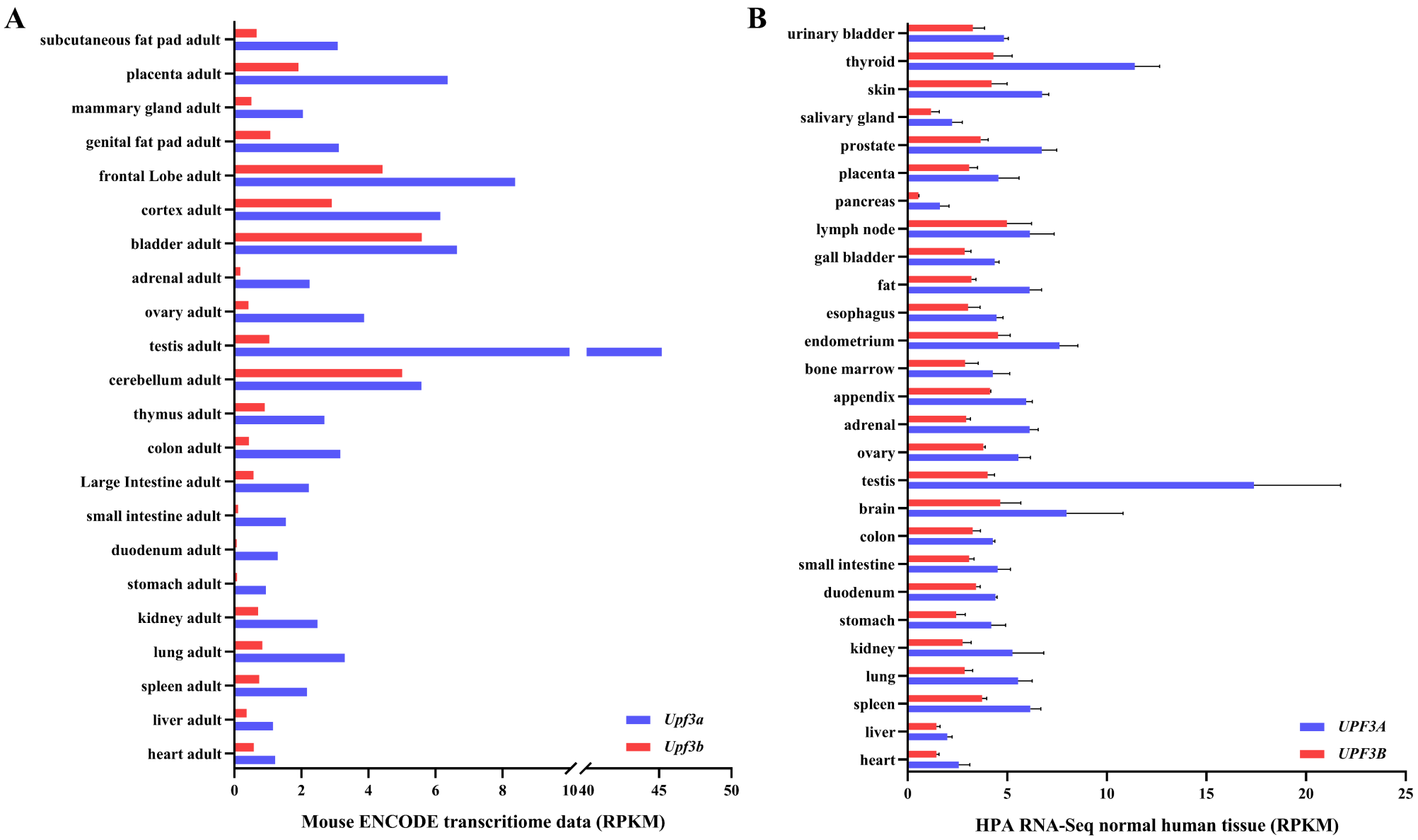

Male
Female
He
Li
Sp
UPF3A/B
GAPDH
Lu
Ki
Je
UPF3A/B
GAPDH
Th
Br
Ob
UPF3A/B
GAPDH
UPF3A
UPF3B
Male
Female
Relative band intensity
UPF3A/B
NA
He
Li
Sp
Lu
Ki
Je
Th
Br
Ob

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)