Upf3a is dispensable for nonsense-mediated mRNA decay in mouse pluripotent and somatic cells

Chengyan Chen[1,#], Yanmin Shen[1,#], Luqian Li[1], Zhao-Qi Wang[3], Tangliang Li[1,2,*]

1. State Key Laboratory of Microbial Technology, Shandong University, Qingdao, China
2. School of Basic Medical Sciences, Hangzhou Normal University, Hangzhou, China
3. Leibniz Institute on Aging - Fritz Lipmann Institute, Jena, Germany

[#], These authors contributed equally.

*, Correspondence:

Dr. Tangliang Li，State Key Laboratory of Microbial Technology, Shandong University, Qingdao, China; E-mail: li.tangliang@sdu.edu.cn

Running title: Upf3a does not repress NMD in mouse

Abstract

Nonsense-mediated mRNA decay (NMD) is a highly conserved post-transcriptional gene expression regulation mechanism in eukaryotic cells. NMD plays essential roles in mRNA quality and quantity control, and thus safeguards multiple biological processes including embryonic stem cell differentiation and organogenesis. UPF3A and UPF3B in vertebrate species, originated from a single UPF3 gene in yeast, are key factors in NMD machinery. While UPF3B is a well-recognized weak NMD promoting factor, whether UPF3A functions in promoting or suppressing NMD is under debate. In this study, we generated a *Upf3a* conditional knockout mouse strain, and established multiple lines of embryonic stem cells and somatic cells without Upf3a. We found Upf3a does not repress NMD in mouse embryonic stem cells, somatic cells and major organs including liver, spleen, and thymus. Our study reinforces that Upf3a is mainly dispensable for NMD and may weakly and selectively promote NMD in certain organs.

Keywords: Nonsense-mediated mRNA decay; Upf3a; NMD repressor; Embryonic stem cell

Introduction

In eukaryotic cells, transmission of genetic information from DNA to proteins are under stringent regulations at multiple layers. Nonsense-mediated mRNA decay (NMD) is a highly conserved gene expression regulation mechanism after DNAs are transcribed to form RNAs with RNA polymerases. Nonsense-mediated mRNA decay (NMD) surveillances transcriptome quality by eliminating mRNAs containing premature termination codons (PTCs), and thus prevents accumulation of N terminal truncated protein. Meanwhile, through recognizing other NMD features, such as long 3' UTRs and 5' uORFs, NMD regulates stability of around 3-10% of normal RNA transcripts, and participates in fine-tunning gene expression (Kurosaki et al 2019, Lykke-Andersen & Jensen 2015). Thus, NMD plays important roles in cell fitness, stress response, etc (Hug et al 2016). NMD is essential for embryonic development and tissue/organ maintenance (Han et al 2018).

UPF3A and UPF3B are unique NMD factors among all components of mammalian NMD machinery. UPF3A and UPF3B are two paralogues of yeast Upf3 (Lykke-Andersen et al 2000). Phylogenetic analysis indicates UPF3A and UPF3B could be generated by a gene duplication event during the emergence of vertebrate species (Shum et al 2016). UPF3B is a widely accepted mild NMD factor negatively regulating mRNA stability (Chan et al 2009, Chan et al 2007, Kunz et al 2006, Lykke-Andersen et al 2000). Furthermore, UPF3B participates in early and late translation termination, suggesting orchestrated roles of UPF3B in life regulation of RNA turnover and protein synthesis (Gao & Wilkinson 2017, Neu-Yilik et al 2017). Interestingly, although structural and biochemical analysis show that UPF3B is one of central factors in NMD, UPF3B loss causes very mild or even negligible NMD defects since UPF3B only regulates the stability of a small proportion of RNA targets (Huang et al 2018a, Tarpey et al 2007). Meanwhile, UPF3A has weak NMD promoting activity *in vitro* (Lykke-Andersen et al 2000); in UPF3B deficient mammalian cells, UPF3A may partially compensate UPF3B loss (Chan et al 2009, Chan et al 2007), since

UPF3A and UPF3B, through their middle domain, could bind to the MIF4GIII domain of NMD factor UPF2 (Bufton et al 2022).

Not like other NMD factors, knockdown of Upf3a, Upf3b or Upf3a/3b shows no obvious developmental defects in zebrafish (Wittkopp et al 2009). Upf3a or Upf3b knockout zebrafishes are all viable (Ma et al 2019). In mammals, knockout mice of Smg1, Upf1, Upf2 or Smg6 are embryonic lethal (Han et al 2018). Upf3b null mice generated by gene trapping strategy are viable and have very mild neurological symptoms (Huang et al 2018b). Interestingly, Upf3a knockout mice are early embryonic lethal (Shum et al 2016), suggesting Upf3a may have different roles in NMD. A detailed analysis on Upf3a functions in HEK293 cells，Hela cells, mouse pluripotent cells (P19: embryonic carcinoma cell), somatic cells, and major organs, such as olfactory bulbs and testis, identified a novel function of Upf3a as a general and strong NMD repressor in mammals (Shum et al 2016).

Inspired by the finding of Upf3a as a general NMD repressor (Shum et al 2016), we set to study whether constitutive NMD activation upon Upf3a knockout could affect mouse embryonic stem cell self-renewal, differentiation, and tissue homeostasis. To this end, we generated a Upf3a conditional knockout mouse by introducing 2 loxP sites floxing Upf3a exon 3, which is identical to the published Upf3a conditional gene targeting strategy (Shum et al 2016). To our surprise, with extensive analysis on NMD target transcripts expression in embryonic stem cells, somatic cells, as well as various tissues including liver, spleen, and thymus, we found that Upf3a does not play a role as a NMD repressor, but is a weak NMD promoting factor in certain mouse organs. Our results reinforce that UPF3A is generally dispensable for mammalian NMD (Wallmeroth et al 2022, Yi et al 2022).

Materials and methods

Mice and genotyping strategies

The Upf3a conditional knockout mouse (*Upf3a*$^{f/f}$) was generated by CRISPR–Cas9 gene editing in Cyagen. Cas9 protein, two gRNAs (gRNA1: AAATCTGTTGTTCGTACAGA, gRNA2: CTTGTTACAAGCTTTAGCCG) and a donor vector containing the two loxP sequences in intron 2 and intron 3 of mouse *Upf3a* gene were injected into mouse fertilized eggs. The embryos were transferred to recipient female mice to obtain F0 mice. For validating the two loxP site insertions, PCR products using two pairs of primers (F1: AAAGAACAGTGTGCAATTACTCGG, R1: TTCACAGGTAGGAACGATTCCATT; F2: TGTCCATTTTACCTATCCATTCG, R2: GAGCACTGCGCTACCACCTGACC) were sequenced. For routine genotyping of *Upf3a* alleles, three primers were used: F1, AAAGAACAGTGTGCAATTACTCGG; R1, TTCACAGGTAGGAACGATTCCATT; M1, AGCTCTTACTCTTGAGCCCAC (Wild-type allele: 133bps; Floxed allele: 200bps; knockout allele: 412bps) (Fig 1A). To generate the Upf3a inducible knockout mouse, *Upf3a*$^{f/f}$ mouse was crossed with Cre-ER^{T2+} transgenic mouse line. For genotyping of Cre-ER^{T2+} transgene, primers (Cre-ErF: ATACCGGAGATCATGCAAGC; Cre-ErR: GATCTCCACCATGCCCTCTA) were used (Cre-ER^{T2+} transgene: 552bps).

To delete Upf3a in adult Upf3a$^{f/f}$Cre-ER^{T2+} mice (age: 6 weeks), tamoxifen (Sigma-Aldrich, T5648) was intraperitoneally injected at a dose of 75mg/kg for 3 consecutive days. Three weeks after the last tamoxifen injection, mice tissues were collected and processed for WB and qPCR analysis.

All animals were maintained under specific pathogen-free conditions at the animal facility of the Shandong University, Qingdao, P. R. China. Animal care and experiments were performed in accordance with the ethic committee guideline.

Generation of Upf3a inducible deletion mESCs and somatic cells

mESC lines with Upf3a inducible deletion were generated and maintained by following a previously published protocol (Li et al 2015). The genotyping on sex of each ESC line was conducted with published primers (Tunster 2017).

Somatic cells from mouse ribs (RDSCs) were generated with finely chopped rib tissues from male Upf3a$^{f/f}$Cre-ER^{T2+} mice at 4 weeks of age. RDSCs were maintained with EF medium (High Glucose DMEM, supplemented with 10% FBS, 100 units/ml Penicillin, and 100ug/ml Streptomycin).

For the deletion of Upf3a in mESCs and RDSCs, 4-OHT (Sigma-Aldrich, H6278, 1uM) was used to treat cells for 5 successive days.

Expression of mUpf3a and mUpf3b in U2OS cells

cDNAs of mUpf3a and mUpf3b were amplified with PrimeSTAR® HS Premix (Takara) from an E14.1 ES cell cDNA library. The PCR products were further cloned in pEGFP-C1-EF1a vector with a seamless cloning kit (D7010S，Beyotime) (Li et al 2015). Sequences of pEGFP-C1-EF1a-mUpf3a and pEGFP-C1-EF1a-mUpf3b were validated through sequencing services provided by Tsingke. These plasmids were amplified with E.Z.N. A® Endo-free plasmid Midi kit (Omega BIO-TEK) and transiently transfected into U2OS cells with Lipofectamine™ 3000 (Invitrogen) following the company protocols. Protein samples were harvested 48 hours after transfection.

Protein extraction and analysis

Cells or mouse tissues were lysised with RIPA buffer (Sigma-Aldrich, 20-188) supplemented with combinations of Protease/Phosphatase inhibitors (APExBIO). Around 40-60ug protein was separated with gradient SDS-PAGE gel (4-20%, ACE Biotechnology). The following primary antibodies were used: rabbit anti-Upf3a/Upf3b (ab269998, Abcam, 1:1000); mouse anti-β-Actin (A5441, Sigma-Aldrich, 1:10,000); mouse anti-Lamin B1 (sc-374015, Santa

Cruz). The secondary antibodies used in these studies were HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:2,000; Proteintech).

qRT-PCR

Cells or mouse tissues were lysised with TRIzol Reagent (Sigma-Aldrich) and total RNAs were purified according to the company protocol. cDNAs were synthesized using HiScript® III 1st Strand cDNA Synthesis Kit (R312, Vazyme) according to the company manual. qRT-PCR in triplicate for each sample was performed using 2xTSINGKE® Master qPCR Mix (SYBR Green I) (TSINGKE) on the CFX96 Real-Time PCR system (Bio-Rad). The expression of β-Actin was used as the internal control. The primers used for the qPCR amplification of *Snhg12*, *Atf4*, *Gas5*, *1810032O08Rik*, *Ddit3*, and β-*actin* were synthesized according to a previous publication (Weischenfeldt et al 2008). qPCR primers for *Cdh11*, *Ire1*, *Smad5*, *Smad7* and *Snord22* were retrieved form Shum *et al.* (Shum et al 2016). qPCR primers for *Auf1* and *Hnrnpl* were from Li et al (Li et al 2015). qPCR primers of *Eif4a2* (PTC isoform) were from Huth *et al* (Huth et al 2022). Other gene specific primers designed and used in this study were listed as bellow:

Smg6: F, GAGAACCCAGAGCAGATTCG; R, CAAGCCCATCCATGTAGTCC;

Smg7: F, AACCCAAATCGAAGTGAAGTCC; R, ACACCGTACACAGTTCCTGTAA;

Eif4a1: F, GGGTCGGACGCTCTATAAGT; R, GTCGGGGCCATTGTCTCT

Upf3a: F, GCGCACGATTACTTCGAGGT; R, TCAAAACGGTCTCTGAACAGC;

Upf3b: F, AGGAGAAACGAGTGACCCTGT; R, CCTGTTGCGATCCTGCCTA;

Mettl23: F, ACCCAGCTCTTTCGGTTCC; R, AGGAGGGATTAAGGGCATGG.

For semi-quantitative RT-PCR analysis to identify the normal and the PTC$^+$ isoforms of *Pkm2*, *Rps9* and *Ptbp2*, primer sequences were retrieved from the previous study (Weischenfeldt et al 2012) . Primer sequences for *Eif4a2*, *Luc7l*, *Snrpb*, *Hnrnpa2b1*, *Srsf10*, *Cask*, *Alkbh3*, *Sf1*, *Nfyb*, *Ccar1*, *Slc38a2* and *Flot1* were described previously (McIlwain et al 2010).

Statistical analysis

The unpaired Student's t-test was used in this study. The statistical analysis in this study was performed with GraphPad Prism (Ver 6.00, GraphPad Software, San Diego, CA, USA).

Results

Upf3a is dispensable for NMD in mouse embryonic stem cells

To override embryonic lethality of Upf3a null (Shum et al 2016), we generated a Upf3a conditional knockout mouse (Upf3a$^{flox/flox}$: Upf3a$^{f/f}$) with Crispr-Cas9 technology (see "materials and methods" for details, Fig 1A) and crossed Upf3a$^{f/f}$ mouse with Cre-ER^{T2+} mouse, an inducible Cre transgenic line (Fig1B). Through intercrossing Upf3a$^{f/f}$ Cre-ER^{T2+} mouse with Upf3a$^{f/f}$ mouse, we isolated E3.5 blastocysts and established four Upf3a inducible deletion ESC lines. These ESC lines were genotyped as females. We treated these ESC lines with 4-OHT for 5 days, and confirmed Upf3a KO with normal genotyping and qPCR (Fig 1C, Fig 2A).

To further substantiate the successfully establishment of Upf3a KO ESCs, we screened a serial of commercial Upf3a antibodies and identified one newly released rabbit monoclonal antibody which could be used to detect exogenously and endogenously expressed mUpf3a and mUpf3b. Through WB, we found Upf3a protein was completely absent in 4-OHT treatment Upf3a$^{f/f}$ Cre-ER^{T2+} ESC lines, indicating Upf3a knockout ESC lines (designated as Upf3a$^{\triangle/\triangle}$ ESCs or Upf3a KO ESCs) are established (Fig 1D; Supp Fig 1A). Upf3a KO mESCs are all viable and show no phenotypic difference with their parent clones (Fig 1E, left panel).

To investigate Upf3a' role in NMD, we conducted qPCR analysis on mRNA transcripts from following sets of genes: Genes including *Cdh11*, *Ire1*, *Smad5*, *Smad7*, *Gas5* and *Snord22* were previously used to characterize Upf3a as a NMD repressor in mouse (Shum et al 2016); Genes including *Atf4*, *Auf1* (PTC specific), *1810032O08RIK*, *Smg5*, *Smg6*, *Hnrnpl* (PTC specific), and *Ddit3* were widely used or validated as mouse NMD targets (Li et al 2015, Weischenfeldt et al 2008, Weischenfeldt et al 2012); *Eif4a2* (PTC specific) was recently identified in Smg5, Smg6 and Smg7 knockout ESCs generated with Crispr-cas9 technology (Huth et al 2022); *Upf3a*, *Eif4a1* (PTC specific), *Smg6*, *Smg7* and *Mettl23* (PTC specific) are designed in current study. In our analysis,

previously generated Smg6 knockout ESC was used as positive control of NMD inhibition in ESC (Li et al 2015) (Supp Fig 1B). In contrast with strong increases of NMD target transcript in Smg6 KO ESC (Supp Fig 1B), Upf3a KO ESCs showed no obvious difference in transcript levels of all NMD target genes tested (Fig 2A). Furthermore, we conducted RT-PCR analysis on AS-NMD generated PTC$^+$ transcripts accumulation in Upf3a KO ESCs. We found a strong enrichment of PTC$^+$ isoforms in Smg6 KO ESCs, but no difference was detected between control and Upf3a KO ESCs (Fig 3A-B). Thus, Upf3a is dispensable for NMD in mouse embryonic stem cells.

Upf3a is dispensable for NMD in mouse somatic cells

To rule out a possible role of pluripotency on NMD activity, we generated somatic cell lines derived from rib tissues of 4 male Upf3a$^{f/f}$ Cre-ER^{T2+} mice. In this study, we named these cell lines as RDSCs (**R**ib muscle **d**erived **s**omatic **c**ells). PCR and WB analysis showed that five days of 4-OHT treatment successfully depleted Upf3a in RDSCs (Fig1C, lower panel; Fig 1D; Fig 2B; Supp Fig 1A). Loss of Upf3a does not cause visible cell viability changes (Fig 1E, right panel). We then used qPCR and analyzed expressions of NMD target gene transcripts in control and Upf3a KO RDSCs. Previously established Smg6 knockout fibroblast was used as a positive control of NMD inhibition in somatic cells (Supp Fig 1C) (Li et al 2015). We found Upf3a KO RDSCs, as compared with their parent cell lines, showed no obvious difference in transcript levels of all NMD target genes tested (Fig 2B). Furthermore, RT-PCR analysis on PTC$^+$ isoforms showed Smg6 KO fibroblasts had strong accumulation of PTC$^+$ isoforms. However, Upf3a KO did not result in any detectable change on the PTC isoforms tested (Fig 3C-D). Thus, in mouse somatic cells, such as fibroblasts generated from rib muscles, Upf3a is dispensable for NMD.

Upf3a deficiency weakly inhibits NMD in multiple tissues from adult mice

Since NMD may have tissue and cell type specificity, to expand our analysis to other cell types, we utilized Upf3a inducible knockout mice (Upf3a$^{f/f}$ Cre-ER^{T2+} mice) and treated them with tamoxifen to induce Upf3a deletion. After another 3 weeks, PCR analysis and WB show that Upf3a is efficiently deleted in liver, kidney and hematopoietic system, including spleen, thymus and peripheral blood from Upf3a$^{f/f}$ Cre-ER^{T2+} mice treated with tamoxifen (Supp Fig 2A-B). These mice are viable and have no visible behavior changes as compared with control animals (Upf3a$^{f/f}$ Cre-ER^{T2-} mice with TAM injection) (Data not shown). We isolated RNAs from livers, spleens, and thymus and used qPCR to determine NMD activity in samples from 4 controls (Upf3a$^{f/f}$ Cre-ER^{T2-} mice with TAM injection) and 4 Upf3a KO mice (Upf3a$^{f/f}$ Cre-ER^{T2+} mice treated with TAM). In Upf3a KO livers, RNA transcripts of well-conserved NMD targets, such as *Atf4*, *Snord22*, *Snhg12*, *1810032O08Rik* and *Hnrnpl*, are mildly but all significantly upregulated, while other gene transcripts, such as *Gas5*, *Auf1*, and *Eif4a2* have a trend of increasement (Fig 2C). In Upf3a KO thymus, only transcripts of *Auf1* are significantly increased, while transcripts of *Smad5*, *Gas5*, *Snord22*, *Hnrnpl*, *Ddit3*, and *Eif4a1* trend to accumulate (Fig 2D). It is interesting to note, in Upf3a KO liver and thymus, none of gene tested shows reduction at transcript level (Fig 2C-D).

In Upf3a KO spleens, expression of *Cdh11* is significantly upregulated, while UPR factors, including *Atf4 and Ddit3*, are significantly downregulated. Expressions of other 16 NMD targets have no difference between control and Upf3a KO samples. Overall, in spleens, Upf3a does not repress NMD (Fig 2E).

Next, we conducted RT-PCR analysis on AS-NMD generated PTC^{+} transcripts accumulation in Upf3a KO livers and spleens, we found no obvious change on PTC^{+} isoforms expression between control and Upf3a KO samples (Supp Fig 3).

Discussion

Upf3 is widely considered as one of core factors of NMD machinery, which is a highly conserved mRNA surveillance mechanism in eukaryotes cells (Hug et al 2016, Kunz et al 2006, Lykke-Andersen et al 2000, Lykke-Andersen & Jensen 2015, Yi et al 2021). In yeasts and worms, only one Upf3 locus is identified (Hug et al 2016). In vertebrate animals, two paralogs of UPF3, ie. UPF3A and UPF3B exist (Lykke-Andersen et al 2000, Serin et al 2001). It is even more intriguing that UPF3B is localized on X chromosome in mammals. Interestingly, although knockouts or knockdowns of NMD factors, such as Upf1, Upf2, Smg1, Smg5, Smg6 and Smg7, in various types of cells manifest strong NMD defects (Huth et al 2022, Li et al 2015), Upf3b knockdowns or knockouts have mild or even negligible effects on NMD (Chan et al 2007, Huang et al 2011, Lykke-Andersen et al 2000). Through generating a conditional knockout mouse model of Upf3a with traditional gene targeting strategy, Shum et al. found UPF3A represses NMD in mouse pluripotent cells (P19 embryonic carcinoma cell), tissue specific stem cells (neural stem cells and olfactory sensory neuronal precursors), somatic cells (mouse embryonic fibroblasts and neurons) as well as human 293T cells. NMD targets, such as *Cdh11*, *Smad5* and *Smad7*, are all downregulated in Upf3a knockdown settings (Shum et al 2016). Meanwhile, mRNAs transcripts of *Atf4*, *Gas5* and *Snord22* showed tissue specific NMD activation or repression upon Upf3a knockdown (Shum et al 2016). Due to the strong NMD repression upon Upf3a deficiency, Shum et al. found Upf3a KO mice are embryonic lethal.

Two recent studies revisited the function of UPF3A in humans (Wallmeroth et al 2022, Yi et al 2022). Yi *et al.* utilized HCT116 and Hela cells and found that UPF3A function as weak NMD activator (Yi et al 2022). Furthermore, Wallmroth et al. used HEK293 cells and found UPF3A overexpression or knockout does not affect NMD efficiency. UPF3A could compensate UPF3B loss and functions in NMD activation since UPF3A/B double knockout has profound NMD defects (Wallmeroth et al 2022). These

two new findings, together with most of previous results, strongly indicated that UPF3A and UPF3B are mainly NMD activators and function redundantly in human NMD (Chan et al 2009, Chan et al 2007, Kunz et al 2006, Lykke-Andersen et al 2000). These two studies raised several possibilities on UPF3A role in mammalian NMD: 1) Upf3a functions distinctly in mouse and human; 2) Upf3a plays different roles in immortal cells and primary cells (Yi et al 2022).

In this study, we used Crispr-Cas9 technology and adopted the identical gene targeting strategy to generate a new Upf3a conditional knockout mouse strain. Furthermore, we characterized a newly released rabbit monoclonal antibody which could detect expression of mouse Upf3a and Upf3b in a single immunoblotting. With these two key materials, we successfully produced Upf3a KO mESCs and somatic cells. we found that Upf3a and Upf3b protein expression are higher in ESCs than in somatic cells (mouse rib muscle derived somatic cells) (Fig 1D). Deletion of Upf3a is compatible with life of embryonic stem cells and somatic cells, which is similar to Upf3b null cells (Huang et al 2011). Through a large-scale qPCR and RT-PCR assays, we found Upf3a loss doesn't cause decrease of mRNA transcripts of well-recognized NMD target genes. On the contrary, in Upf3a KO ESCs, somatic cells, as well as in various animal tissues, including liver and thymus, transcripts levels of these well-documented NMD targets remain unchanged or slightly upregulated. Of note, in Upf3a KO livers, RNA transcripts of NMD target genes, including *Atf4*, *Snord22*, *Snhg12*, *1810032O08Rik and Hnrnpl* are mildly but significantly upregulated, indicating that Upf3a has tissue specificity in NMD. Thus, our study indicates that Upf3a is not a repressor of NMD, but rather functions as a weak NMD activator in mouse. Our study, together with majority of previous studies on UPF3A and UPF3B, indicates that UPF3A is a *bona fide* NMD activator (as weak as UPF3B) in mammalian cells (Chan et al 2009, Chan et al 2007, Kunz et al 2006, Lykke-Andersen et al 2000, Wallmeroth et al 2022, Yi et al 2022). In this study, we only use qPCR and RT-PCR assays and analyzed around 20 previously characterized NMD targets with featuring of PTCs,

uORFs and long 3' UTRs. However, we could not rule out the possibility that Upf3a may repress other un-characterized gene transcripts in certain unexplored tissues, such as testis with highest Upf3a expression (Shum et al 2016, Tarpey et al 2007). Future transcriptome-wide analysis in Upf3a KO cells and mice would precisely resolve Upf3a's role in NMD.

Acknowledgements

We thank all members of the Li laboratory for discussion and reading of the manuscript. We thank Dr. Haiyan Yu, Ms. Yuyu Guo and Ms. Xiaomin Zhao (Core Facilities for Life and Environmental Sciences, State Key laboratory of Microbial Technology, Shandong University) on helping with microscopy. Research projects in laboratory of Tangliang Li is 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.

Author contributions

C Chen: Investigation and data curation.

Y Shen: Investigation and data curation.

L Li: Investigation and data curation.

Z-Q Wang: Resources.

T Li: Conceptualization, supervision, funding acquisition, manuscript writing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References:

Bufton JC, Powers KT, Szeto JA, Toelzer C, Berger I, Schaffitzel C. 2022. Structures of nonsense-mediated mrna decay factors upf3b and upf3a in complex with upf2 reveal molecular basis for competitive binding and for neurodevelopmental disorder-causing mutation. Nucleic Acids Res. 50(10):5934-5947. doi:10.1093/nar/gkac421

Chan WK, Bhalla AD, Le Hir H, Nguyen LS, Huang L, Gecz J, Wilkinson MF. 2009. A upf3-mediated regulatory switch that maintains rna surveillance. Nat Struct Mol Biol. 16(7):747-753. doi:10.1038/nsmb.1612

Chan WK, Huang L, Gudikote JP, Chang YF, Imam JS, MacLean JA, 2nd, Wilkinson MF. 2007. An alternative branch of the nonsense-mediated decay pathway. EMBO J. 26(7):1820-1830. doi:10.1038/sj.emboj.7601628

Gao Z, Wilkinson M. 2017. An rna decay factor wears a new coat: Upf3b modulates translation termination. F1000Res. 6:2159. doi:10.12688/f1000research.12704.1

Han X, Wei Y, Wang H, Wang F, Ju Z, Li T. 2018. Nonsense-mediated mrna decay: A 'nonsense' pathway makes sense in stem cell biology. Nucleic Acids Res. 46(3):1038-1051. doi:10.1093/nar/gkx1272

Huang L, Lou CH, Chan W, Shum EY, Shao A, Stone E, Karam R, Song HW, Wilkinson MF. 2011. Rna homeostasis governed by cell type-specific and branched feedback loops acting on nmd. Mol Cell. 43(6):950-961. doi:10.1016/j.molcel.2011.06.031

Huang L, Low A, Damle SS, Keenan MM, Kuntz S, Murray SF, Monia BP, Guo S. 2018a. Antisense suppression of the nonsense mediated decay factor upf3b as a potential treatment for diseases caused by nonsense mutations. Genome Biol. 19(1):4. doi:10.1186/s13059-017-1386-9

Huang L, Shum EY, Jones SH, Lou CH, Dumdie J, Kim H, Roberts AJ, Jolly LA, Espinoza JL, Skarbrevik DM, et al. 2018b. A upf3b-mutant mouse model with behavioral and neurogenesis defects. Mol Psychiatr. 23(8):1773-1786. doi:10.1038/mp.2017.173

Hug N, Longman D, Caceres JF. 2016. Mechanism and regulation of the nonsense-mediated decay pathway. Nucleic Acids Res. 44(4):1483-1495. doi:10.1093/nar/gkw010

Huth M, Santini L, Galimberti E, Ramesmayer J, Titz-Teixeira F, Sehlke R, Oberhuemer M, Stummer S, Herzog V, Garmhausen M, et al. 2022. Nmd is required for timely cell fate transitions by fine-tuning gene expression and regulating translation. Genes Dev. 36(5-6):348-367. doi:10.1101/gad.347690.120

Kunz JB, Neu-Yilik G, Hentze MW, Kulozik AE, Gehring NH. 2006. Functions of hupf3a and hupf3b in nonsense-mediated mrna decay and translation. RNA. 12(6):1015-1022. doi:10.1261/rna.12506

Kurosaki T, Popp MW, Maquat LE. 2019. Quality and quantity control of gene expression by nonsense-mediated mrna decay. Nat Rev Mol Cell Bio. 20(7):406-420. doi:10.1038/s41580-019-0126-2

Li T, Shi Y, Wang P, Guachalla LM, Sun B, Joerss T, Chen YS, Groth M, Krueger A, Platzer M, et al. 2015. Smg6/est1 licenses embryonic stem cell differentiation via nonsense-mediated mrna decay. EMBO J. 34(12):1630-1647. doi:10.15252/embj.201489947

Lykke-Andersen J, Shu MD, Steitz JA. 2000. Human upf proteins target an mrna for nonsense-mediated decay when bound downstream of a termination codon. Cell. 103(7):1121-1131. doi:10.1016/s0092-8674(00)00214-2

Lykke-Andersen S, Jensen TH. 2015. Nonsense-mediated mrna decay: An intricate machinery that shapes transcriptomes. Nat Rev Mol Cell Biol. 16(11):665-677. doi:10.1038/nrm4063
Ma Z, Zhu P, Shi H, Guo L, Zhang Q, Chen Y, Chen S, Zhang Z, Peng J, Chen J. 2019. Ptc-bearing mrna elicits a genetic compensation response via upf3a and compass components. Nature. 568(7751):259-263. doi:10.1038/s41586-019-1057-y
McIlwain DR, Pan Q, Reilly PT, Elia AJ, McCracken S, Wakeham AC, Itie-Youten A, Blencowe BJ, Mak TW. 2010. Smg1 is required for embryogenesis and regulates diverse genes via alternative splicing coupled to nonsense-mediated mrna decay. Proc Natl Acad Sci U S A. 107(27):12186-12191. doi:10.1073/pnas.1007336107
Neu-Yilik G, Raimondeau E, Eliseev B, Yeramala L, Amthor B, Deniaud A, Huard K, Kerschgens K, Hentze MW, Schaffitzel C, et al. 2017. Dual function of upf3b in early and late translation termination. EMBO J. 36(20):2968-2986. doi:10.15252/embj.201797079
Serin G, Gersappe A, Black JD, Aronoff R, Maquat LE. 2001. Identification and characterization of human orthologues to saccharomyces cerevisiae upf2 protein and upf3 protein (caenorhabditis elegans smg-4). Mol Cell Biol. 21(1):209-223. doi:10.1128/MCB.21.1.209-223.2001
Shum EY, Jones SH, Shao A, Dumdie J, Krause MD, Chan WK, Lou CH, Espinoza JL, Song HW, Phan MH, et al. 2016. The antagonistic gene paralogs upf3a and upf3b govern nonsense-mediated rna decay. Cell. 165(2):382-395. doi:10.1016/j.cell.2016.02.046
Tarpey PS, Raymond FL, Nguyen LS, Rodriguez J, Hackett A, Vandeleur L, Smith R, Shoubridge C, Edkins S, Stevens C, et al. 2007. Mutations in upf3b, a member of the nonsense-mediated mrna decay complex, cause syndromic and nonsyndromic mental retardation. Nat Genet. 39(9):1127-1133. doi:10.1038/ng2100
Tunster SJ. 2017. Genetic sex determination of mice by simplex pcr. Biol Sex Differ. 8 doi:ARTN 31
10.1186/s13293-017-0154-6
Wallmeroth D, Lackmann JW, Kueckelmann S, Altmuller J, Dieterich C, Boehm V, Gehring NH. 2022. Human upf3a and upf3b enable fault-tolerant activation of nonsense-mediated mrna decay. EMBO J. 41(10):e109191. doi:10.15252/embj.2021109191
Weischenfeldt J, Damgaard I, Bryder D, Theilgaard-Monch K, Thoren LA, Nielsen FC, Jacobsen SEW, Nerlov C, Porse BT. 2008. Nmd is essential for hematopoietic stem and progenitor cells and for eliminating by-products of programmed DNA rearrangements. Gene Dev. 22(10):1381-1396. doi:10.1101/gad.468808
Weischenfeldt J, Waage J, Tian G, Zhao J, Damgaard I, Jakobsen JS, Kristiansen K, Krogh A, Wang J, Porse BT. 2012. Mammalian tissues defective in nonsense-mediated mrna decay display highly aberrant splicing patterns. Genome Biol. 13(5):R35. doi:10.1186/gb-2012-13-5-r35
Wittkopp N, Huntzinger E, Weiler C, Sauliere J, Schmidt S, Sonawane M, Izaurralde E. 2009. Nonsense-mediated mrna decay effectors are essential for zebrafish embryonic development and survival. Mol Cell Biol. 29(13):3517-3528. doi:10.1128/Mcb.00177-09
Yi Z, Arvola RM, Myers S, Dilsavor CN, Abu Alhasan R, Carter BN, Patton RD, Bundschuh R, Singh G. 2022. Mammalian upf3a and upf3b can activate nonsense-mediated mrna decay independently of their exon junction complex binding. EMBO J. 41(10):e109202. doi:10.15252/embj.2021109202

Yi Z, Sanjeev M, Singh G. 2021. The branched nature of the nonsense-mediated mrna decay pathway. Trends Genet. 37(2):143-159. doi:10.1016/j.tig.2020.08.010

Figure legends

Figure 1. Generation of Upf3a knockout ESCs and somatic cells. (A) Strategy to generate Upf3a conditional knockout mouse. Exon 3 of Upf3a is chosen to be conditionally deleted by Cre recombinase *in vitro* and *in vivo*. Wild-type (wt) allele, floxed allele (F) and knockout allele (△) are shown. **(B)** Mating strategy to generate Upf3a inducible knockout mouse line (Upf3a$^{f/f}$Cre-ER^{T2+}). Upf3a$^{f/f}$Cre-ER^{T2+} mice are further used to produce Upf3a inducible knockout embryonic stem cells (ESCs) and somatic cells from rib muscle (RDSCs). **(C)** PCR analysis on Upf3a locus (Exon 3) deletion in Upf3a$^{f/f}$Cre-ER^{T2+} ESCs and RDSCs after 4-OHT treatment. Wild-type allele (wt), floxed allele (F) and knockout allele (△) are marked. **(D)** WB analysis on Upf3a and Upf3b protein expression in Upf3a$^{f/f}$Cre-ER^{T2+} ESCs and RDSCs after 4-OHT treatment. Protein lysates from U2OS cells expressing GFP-mUpf3a and GFP-mUpf3b are used to validate antibody. Beta-actin is used as a loading control for WB. **(E)** Representative images of control ESCs/ RDSCs (Upf3a$^{f/f}$) and Upf3a knockout ESCs/RDSCs (Upf3a$^{△/△}$).

Figure 2. Upf3a does not repress major NMD targets expression.
(A-B) qPCR analysis on gene expression of NMD targets in mESCs. (**A**) and RDSCs **(B).** Due to considerable variances on expression levels of these NMD target genes between individual ESC and RDSC line, each data point (**A-B**) represents the relative expression of indicated gene in Upf3a KO related to its parent cell line. **(C-E)** qPCR analysis on gene expression of NMD targets in liver **(C)**, thymus **(D)** and spleen **(E)** from control (Co, Upf3a$^{f/f}$Cre-ER^{T2-} + TAM) and Upf3a KO (Upf3a$^{△/△}$: Upf3a$^{f/f}$Cre-ER^{T2+} + TAM) mice.
Note: *, P <0.05; ***, P <0.001; Unpaired Student's t-test is used.

Figure 3. RT-PCR analysis on AS-NMD in Upf3a KO ESCs (A, B) and RDSCs (C, D). Accumulations of PTC^{+} isoforms from exon inclusion (A, C) and exon skipping (B, D) are analyzed by RT-PCR. ESC lines (ES1, ES2, ES3) and

RDSC lines (M1, M3, M7) are used. Please note: AS-NMD defects in Smg6 knockout ESCs and fibroblasts (Smg6△) are used as a positive control of NMD inhibition for this analysis.

Supplementary Figure legends

Supp Figure 1. Generation of Upf3a KO ESCs and RDSCs. (A) WB analysis showed complete knockout of Upf3a in ESCs (Upper panel) and RDSCs (lower panel). Three independent ESC lines (ES1, ES2, ES3) and three independent RDSC lines (M1, M3, M7) are used for this analysis. Please note: Left panel summarized the predicted molecular weights of Upf3 proteins analyzed in our study. **(B, C)** NMD target expressions in Smg6 KO ESC **(B)** and Smg6 KO fibroblast **(C)**. please note: Error bars represent the variance of technical replicates in qPCR assay.

Supp Figure 2. Efficiencies of Upf3a deletion in TAM treated Upf3a$^{f/f}$ CreER^{T2+} mice. (A) PCR analysis of Upf3a gene locus in different organs from a Tamoxifen treated male Upf3a$^{f/f}$ CreER^{T2+} mouse. **(B)** WB analysis on Upf3a and Upf3b expression in thymus, spleen, liver and kidney from a pair of Tamoxifen treated male Upf3a$^{f/f}$ CreER^{T2-} and Upf3a$^{f/f}$ CreER^{T2+} mice.

Supp Figure 3. RT-PCR analysis on AS-NMD in Upf3a KO livers (A, B) and spleens (C, D). Accumulations of PTC^{+} isoforms from exon inclusion (**A, C**) and exon skipping (**B, D**) are analyzed by RT-PCR. Please note: AS-NMD defects in Smg6 knockout ESC (Smg6△) are used as a positive control of NMD inhibition for this analysis.

Figure 1

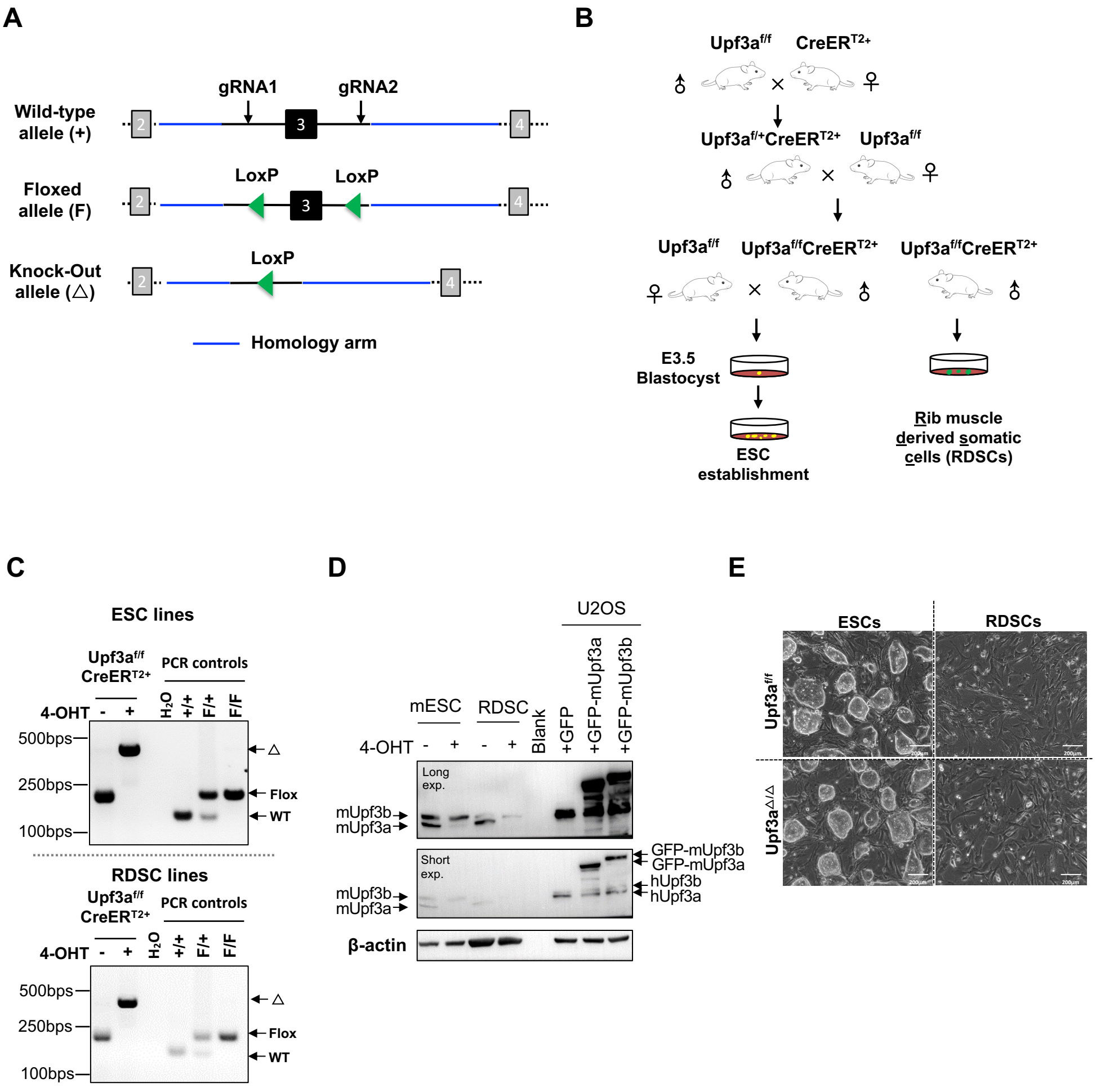

A
gRNA1
gRNA2
Wild-type allele (+)
Floxed allele (F)
Knock-Out allele (△)
LoxP
LoxP
LoxP
Homology arm
B
Upf3a f/f
CreER T2+
Upf3a f/+ CreER T2+
Upf3a f/f
Upf3a f/f
Upf3a f/f CreER T2+
Upf3a f/f CreER T2+
E3.5 Blastocyst
ESC establishment
Rib muscle derived somatic cells (RDSCs)
C
ESC lines
Upf3a f/f CreER T2+
PCR controls
4-OHT - +
H2O +/+ F/+ F/F
500bps
250bps
100bps
△
Flox
WT
RDSC lines
Upf3a f/f CreER T2+
PCR controls
4-OHT - +
H2O +/+ F/+ F/F
500bps
250bps
100bps
△
Flox
WT
D
U2OS
mESC
RDSC
Blank
+GFP
+GFP-mUpf3a
+GFP-mUpf3b
4-OHT - + - +
Long exp.
mUpf3b
mUpf3a
Short exp.
mUpf3b
mUpf3a
GFP-mUpf3b
GFP-mUpf3a
hUpf3b
hUpf3a
β-actin
E
ESCs
RDSCs
Upf3a f/f
Upf3a △/△

Figure 2

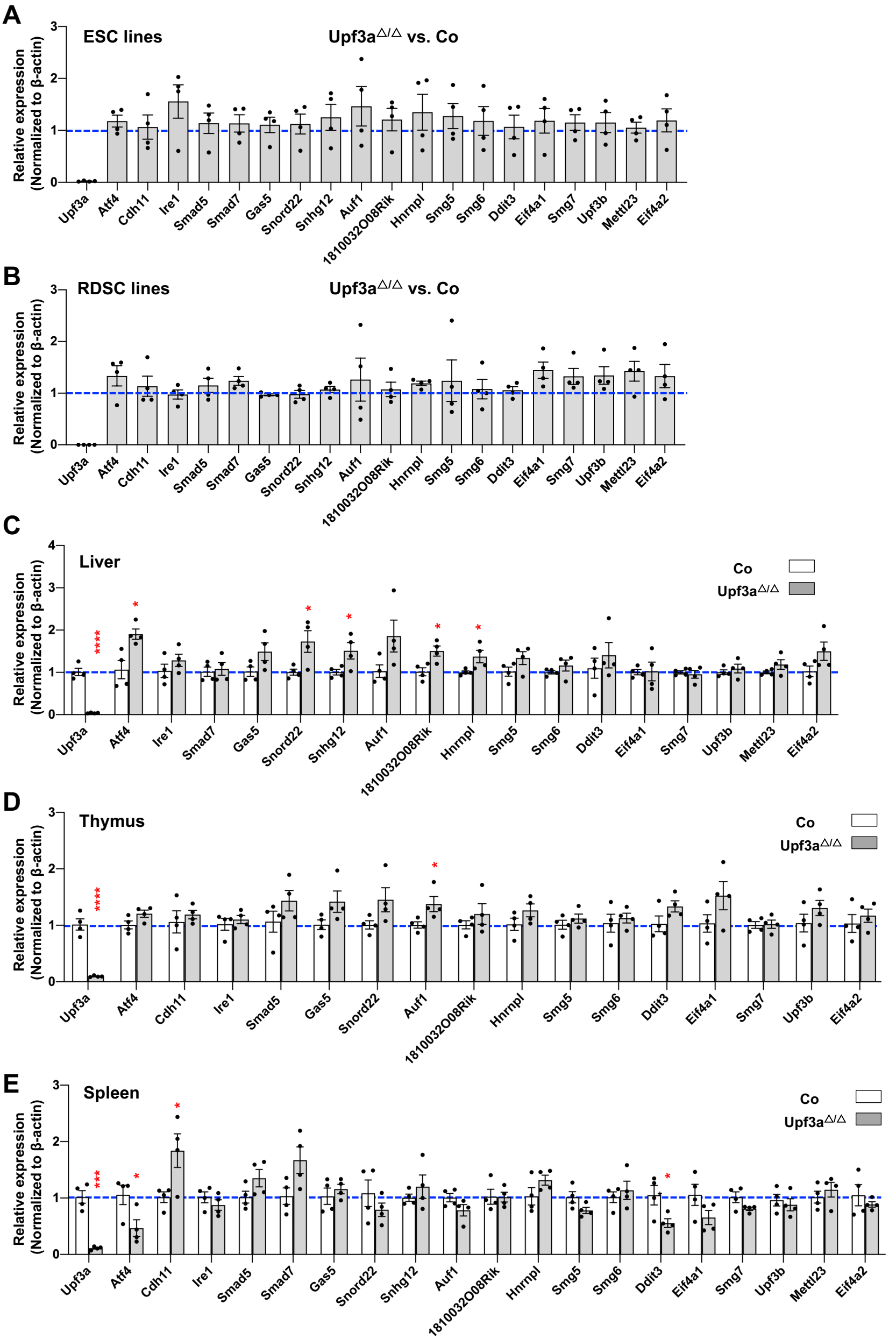
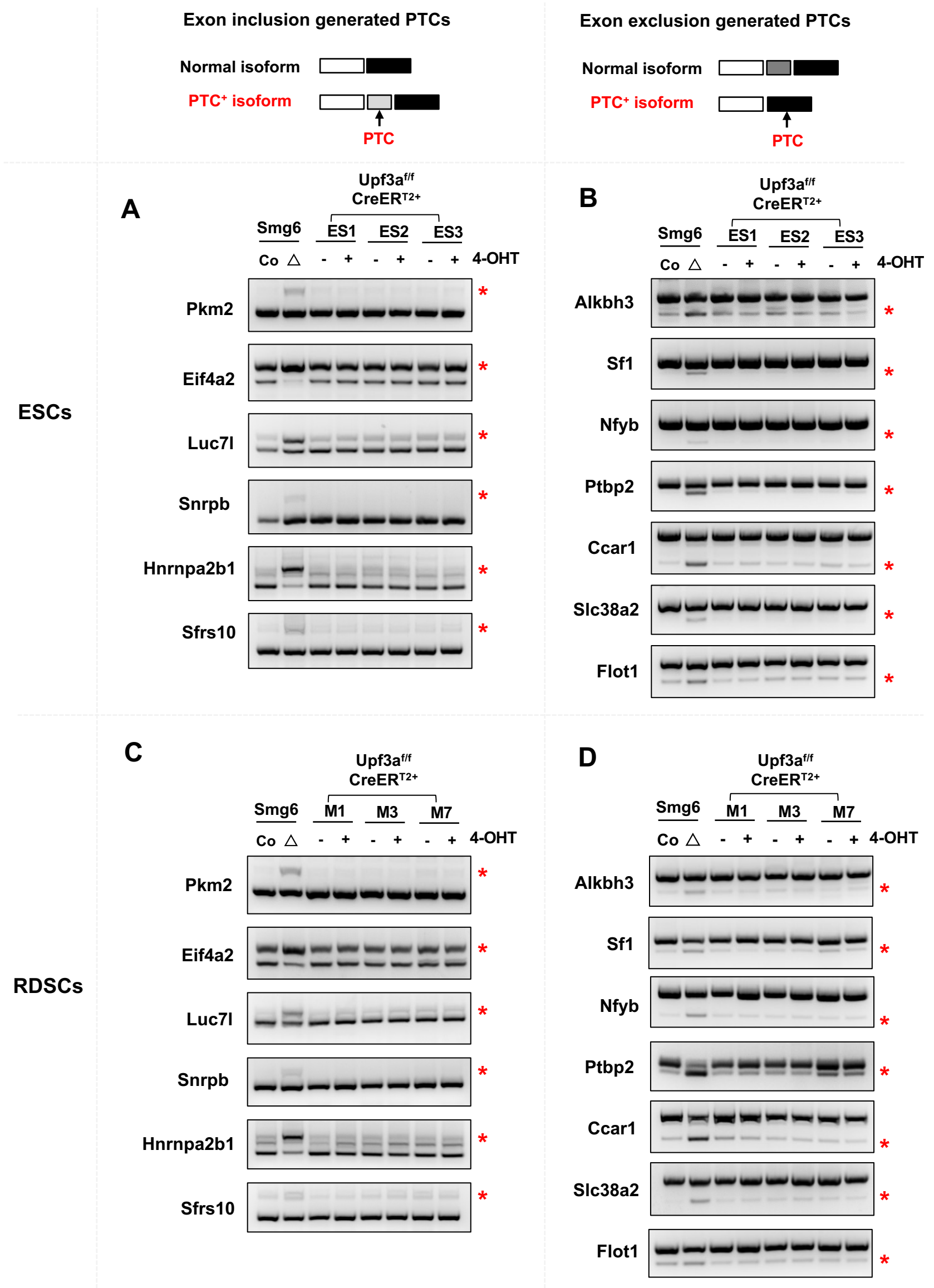

A
ESC lines
Upf3a△/△ vs. Co
Relative expression (Normalized to β-actin)
Upf3a
Atf4
Cdh11
Ire1
Smad5
Smad7
Gas5
Snord22
Snhg12
Auf1
1810032O08Rik
Hnrnpl
Smg5
Smg6
Ddit3
Eif4a1
Smg7
Upf3b
Mettl23
Eif4a2
B
RDSC lines
Upf3a△/△ vs. Co
C
Liver
Co
Upf3a△/△
D
Thymus
E
Spleen

Figure 3


Exon inclusion generated PTCs
Normal isoform
PTC+ isoform
PTC
Exon exclusion generated PTCs
Normal isoform
PTC+ isoform
PTC
ESCs
A
Upf3af/f
CreERT2+
Smg6
ES1
ES2
ES3
Co △ - + - + - + 4-OHT
Pkm2
Eif4a2
Luc7l
Snrpb
Hnrnpa2b1
Sfrs10
B
Upf3af/f
CreERT2+
Smg6
ES1
ES2
ES3
Co △ - + - + - + 4-OHT
Alkbh3
Sf1
Nfyb
Ptbp2
Ccar1
Slc38a2
Flot1
RDSCs
C
Upf3af/f
CreERT2+
Smg6
M1
M3
M7
Co △ - + - + - + 4-OHT
Pkm2
Eif4a2
Luc7l
Snrpb
Hnrnpa2b1
Sfrs10
D
Upf3af/f
CreERT2+
Smg6
M1
M3
M7
Co △ - + - + - + 4-OHT
Alkbh3
Sf1
Nfyb
Ptbp2
Ccar1
Slc38a2
Flot1