Differential expression of full length and N-terminally truncated FAM134B isoforms in normal physiology and cancer

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Abstract

Selective autophagy of the endoplasmic reticulum, namely ER-phagy, is mediated by ER-localized receptors, which are recognized and sequestered by GABARAP/LC3B-decorated phagophores, and transferred to lysosomes for degradation. Being one such receptor, *FAM134B* plays critical roles in cellular processes such as protein quality control and neuronal survival. *FAM134B* has also been associated with different cancers, although its exact role remains elusive. We report here that the *FAM134B* gene encodes not one but at least two different protein isoforms; the full length, and the N-terminally truncated forms. Their relative expression shows extreme variation, both within normal tissues

and among cancer types. Expression of full length FAM134B is restricted to the brain, testis, spleen and prostate. In contrast, N-terminally truncated FAM134B is dominant in the heart, skeletal muscle, kidney, pancreas and liver. We compared wild-type and knockout mice to study the role of the *Fam134b* gene in starvation. N-terminally truncated FAM134B-2 was induced in the liver, skeletal muscle and heart, but not in the pancreas and stomach following starvation. Upon starvation, *Fam134b*$^{-/-}$ mice differed from wild-type mice by less weight loss, less hyperaminoacidemic and hypocalcemic response, but increased levels of serum albumin, total serum proteins and α-amylase. Interestingly, either N-terminally truncated *FAM134B* or both isoforms were down-regulated in liver, lung and colon cancers. In contrast, upregulation was observed in stomach and chromophobe kidney cancers.

New & noteworthy

We reported tissues expressing FAM134B-2 such as the kidney, muscle, heart and pancreas, some of which exhibit stimulated expression upon nutrient starvation. We also demonstrated the effect of *Fam134b* deletion during *ad libitum* and starvation conditions. Resistance to weight loss and hypocalcemia, accompanied by an increase in serum albumin and α-amylase levels, indicate critical roles of *Fam134b* in physiology. Furthermore, the differential expression of FAM134B isoforms was shown to be significantly dysregulated in human cancers.

Keywords: Endoplasmic reticulum (ER), autophagy, reticulophagy, ER-phagy, gene expression, hepatocellular carcinoma, gene knockout

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Introduction

FAM134B (also called RETREG1) is a critical gene for neuronal survival. *FAM134B* loss-of-function mutations cause hereditary sensory and autonomic neuropathy type II, and its knockdown results in structural alterations of the *cis*-Golgi compartment, inducing apoptosis in some primary dorsal root ganglion neurons (25). This pathology is associated with a major role of FAM134B in selective ER degradation by autophagy, or ER-phagy. Mutant FAM134B proteins that cause sensory neuropathy in humans (25) are also unable to act as ER-phagy receptors (22). The autophagy receptor FAM134B is tethered to the ER membrane by its reticulon-homology domain (RHD), which is composed of two large transmembrane domains (TM12 and TM34), and binds to phagophore-associated LC3/GABARAB proteins via its C-terminally located LIR (LC3-interacting region) motif (6, 22).

FAM134B was shown to mediate lysosomal degradation of bulk ER fragments during starvation (22) or during ER stress response caused by misfolded luminal proteins (13, 14). On the other hand, FAM134B overexpression was found to induce ER stress, followed by cell death in HeLa cells (27). Early studies also reported that *FAM134B* expression is enhanced in fatty pig adipocytes, and that its experimental overexpression induced lipid deposition (43). Recently, the overexpression of *FAM134B* in mouse white adipose tissue was reported to increase obesity by promoting adipogenesis via enhanced mitophagy (8).

There are also several reports linking *FAM134B* to different cancer types as both a tumor suppressor and promoter. Initially, *FAM134B* was described as an oncogene that is able to transform the mouse NIH 3T3 cell line and found to be overexpressed in human esophageal cancer (38). Additional studies showed that its experimental downregulation induces significant reductions in cell proliferation, colony formation, wound healing, migration and invasion capacities of esophageal cancer cells (19). On the other hand, *FAM134B* displayed features of a tumor suppressor in colon cancer, since lower levels of the *FAM134B* protein expression are associated with larger tumor size, advanced cancer stages and higher recurrence rates (21). In breast cancer, the *FAM134B* expression is relatively higher in estrogen receptor-positive or non-triple negative subtypes and positively correlated with patient relapse-free survival, which

is suggestive of a tumor suppressor role (11). In further support of a tumor suppressive role, we also identified *FAM134B* as a hepatocellular senescence-associated gene, serving as a robust biomarker for the differentiation of hepatocellular carcinoma (HCC) from cirrhosis (42). In contrast, Zhang et al. recently reported a highly elevated expression of *FAM134B* in HCC compared to normal liver tissues (45). In addition, HCC patients with a higher expression of *FAM134B* have shorter overall survival and disease-free survival. Experimentally, the knockdown of *FAM134B* with shRNA inhibits cell growth and motility, as well as tumor formation and metastasis in nude mice, and its overexpression leads to increased cell proliferation and motility, as well as increased tumor formation and metastasis (45).

Given the discordant and controversial reports on the biological roles of FAM134B in literature, herein we performed a detailed exploration of this interesting gene by *in silico*, *in vitro* and *in vivo* approaches in terms of its structure, expression and implications in both normal physiology and cancer.

Methods

Genome and transcriptome data

The illustration of human and mouse FAM134B transcript variants was recreated using the NCBI reference sequence (RefSeq) database with the following accession codes: human FAM134B-1, NM_001034850.2; human FAM134B-2, NM_019000.4; mouse isoform-1, NM_001034851.2; mouse isoform-2, NM_025459.3; mouse isoform-3, NM_001277315.1; mouse isoform-4, NM_001277316.1; mouse isoform-5, NM_001277317.1; mouse isoform-6, NM_001277318.1. The exon annotation was based on the UCSC genome browser.

The expression data of FAM134B variants in human healthy tissues were retrieved from the Genotype Tissue Expression (GTEx) database, version 3 as RPKM (Reads Per Kilobase Million) values (3). Bar graphs were generated using Log2 (RPKM+1) excluding the standard deviation.

Cancer and normal tissue expression data of human FAM134B isoforms were downloaded directly from The Cancer Genome Atlas Splicing Variant Database (TSVdb) as RSEM values (RNA-Seq by

Expectation Maximization) (36). This database provides isoform level expression value of any human gene across 33 tumor types, and researchers can retrieve and interpret the isoform expression variations between or across clinical subgroups. Statistical analyses of tumor and non-tumor groups were performed using the nonparametric Mann-Whitney U test, and $p<0.01$ was accepted as significant.

Protein alignment and modeling

Human, mouse and bovine amino acid sequences of FAM134B isoforms were obtained from the University of California Santa Cruz (UCSC) Genome Browser. The sequences were aligned utilizing the alignment function of UniProt (5), and the reticulon domains of FAM134B were predicted through the PSIPRED workbench (7), using MEMSAT transmembrane topology prediction method (32). The LC3B binding LIR motif had previously been identified, and the homolog LIR amino acid sequence was used for illustration.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA from tissues were extracted using the NucleoSpin RNA isolation kit (Macherey-Nagel) and converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher) as per manufacturer's instructions. The PCR was set up in a 20µl reaction mixture with total 20ng of cDNA per reaction by using the MyTaq DNA polymerase kit (Bioline). The oligonucleotide sequences for human FAM134B isoforms are as follows: hFAM134B-1-F, 5'- GAGAAGCCTCAGTGAAAGCTG-3'; hFAM134B-2-F, 5'-TTTGGACCAGGCAAAAGCTGG-3', hFAM134B-Common-R, 5'-GCAACCGTGAGGCTAATCTTAGGA-3'. The oligonucleotides used for mouse *Fam134b* mRNA isoforms are as follows: isoform1-F, 5'- TTCTGGTTCCTTGCCTTGAC-3'; isoform2-F, 5'-TGCTGGAGTGAGAGAGCCTGT-3'; isoform3-F, 5'- CATAATAGTCCACTCCTCGGC-3'; isoform4-F, 5'- TCACGGTGCTGGAGTGAGAGC-3'; isoform5-F, 5'- CTCTGAGGTAATAGGCTCCTG-3'; isoform6-F, 5'- AGTGTTATGAAATGGGTCACAG-3'; common reverse, 5'-CAGAAGGTAGCTGAGTATGAC-3'; isoformX common-F, 5'-TCCTGTGCGTGCTTCTTGTGAG-3';

isoformX1-R, 5'- AAGCGCTCCTCCTCTCTC-3'; isoformX2-R, 5'-AAAGCGCTCCTCCTCTAC-3'; HPRT-F, 5'- CACAGGACTAGAACACCTGC-3'; HPRT-R, 5'- GCTGGTGAAAAGGACCTCT-3'.
The RT-PCR was performed under the following conditions: 3 minutes at 94°C (initial denaturation), 1 cycle; 30 seconds at 94°C (denaturation), 30 seconds at 58 °C (annealing), 30 seconds at 72°C (extension), 35 cycles; 3minutes at 72°C (final extension), 1 cycle. Samples were run at 1.5 % agarose gel for 45 minutes at 90V using Tris Acetic acid EDTA (TAE) in both agarose gel and running buffer. The DNA was stained with SafeView (ABM Inc.) by adding into both the agarose gel and the running buffer.

Ethical Statement

All animal experiments were approved by the IBG Animal Experiments Local Ethics Committee (IBG-AELEC). Human samples used in the RT-PCR were approved by the Ethics Committee of Dokuz Eylul University. Written consent was obtained from all patients prior to liver transplantation.

Animal studies

The *Fam134b*$^{+/-}$ mice (22) were a kind gift from Christian Hübner. Male and female mutant mice, along with their wild type counterparts, were housed and bred in individually ventilated cages (IVC) under a 12-hour light/dark cycle at 22±2 °C ambient temperature and 55±10 % humidity. All wild-type (*Fam134b*$^{+/+}$) and mutant (*Fam134b*$^{-/-}$) animals used in this study were derived by crossing founder *Fam134b*$^{+/-}$ mouse colony. Unless used for starvation experiments, all animals were fed *ad libitum* with standard sterile chow and water. For genotyping, tail DNA samples were extracted and subjected to PCR analysis using the following primers: Fam134b_wt_forward, 5'-ACCCCATAGTTCATACTAGGC-3'; Fam134b_mut_forward, 5'-CATGGCAATGACATTTCTCC -3'; and Fam134b_reverse, 5'-CGTAACAGAGGTTGGTGAGG-3. 280bp and 420bp product size represent the wild type and mutant alleles respectively.

Prior to starvation, the animals were synchronized by a pre-starvation for 24 hours, which is followed by free access to food for 2 hours (12). After 2 hours of feeding, the chow was removed from

cages of starvation groups (WT-ST and KO-ST), but *ad libitum* (WT-AL, KO-AL) groups were allowed to access chow. Access to drinking water was free at all times. All animals were weighed at the beginning and the end of the starvation procedure. Animal age and numbers are given in the related figure legends. For the termination of experiments, the animals were sacrificed using cardiac puncture-mediated exsanguination, followed by cervical dislocation under deep terminal anesthesia. Immediately after euthanasia, liver, spleen, kidney, heart and lung tissues were collected, weighed and snap frozen in liquid nitrogen. The whole heart tissue was used for further experimentation without compartmental dissection. The skeletal muscle tissues of mice were collected from gastrocnemius muscles. Animal and organ weight values were analyzed by the one-tailed nonparametric Mann-Whitney U test using the GraphPad Prism 8 software.

Bovine tissues were collected freshly from a local slaughterhouse, snap frozen in liquid nitrogen and transferred to the laboratory under the same conditions. Bovine kidney samples were taken from kidney cortex.

Serum Analyses

Whole blood was collected by cardiac puncture and immediately transferred to sterile microcentrifuge tubes with extra care to prevent hemolysis. Samples were left at room temperature for 20 minutes until the blood clot was formed and centrifuged for 10 minutes at 2000xg at 4°C. Mouse serum biochemistry analyses were performed using photometric methods by an automated analyzer, Architect c16000 (Abbott) as previously shown (24, 40). Total cholesterol, HDL cholesterol and triglyceride levels were directly measured, whereas the VLDL cholesterol levels were calculated by the Triglyceride/5 formula. LDL cholesterol levels were also calculated by the Friedewald equation: LDL=(Total Cholesterol-(VLDL+HDL))(15). Serum amino acids and catabolites were analyzed by the TSQ Endura Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, USA)(18). Due to insufficient serum materials, some parameters have not been tested in all experimental subjects, as indicated in related figures.

All parameters were analyzed by the one-tailed nonparametric Mann-Whitney U test, using the GraphPad Prism 8 software.

Plasmids

Full length FAM134B-1 and FAM134B-2 coding sequences were obtained using PCR and cloned into p3xFLAG-CMV-10 and p3xFLAG-CMV-14 vectors using HindIII and BamHI restriction enzyme digestion sites. Using these vectors, coding sequences were sub-cloned into Tet-inducible pcDNA4TO, pEGFP-C3 and pmCherry-C2 vectors. p3xFLAG-CMV-14 and pcDNA4TO vectors contain neomycin and zeocin antibiotic selection markers, respectively.

Cell culture and generation of stable cell lines

Cells were maintained in RPMI medium supplemented with 10% Fetal Bovine Serum, 100 IU/ml penicillin, 100µg/ml streptomycin and 1x non-essential amino acid (ThermoFisher). For immunofluorescence experiments, Huh7 cells were chemically transfected with mCherry-FAM134B-1- and EGFP-FAM134B-2. Cells were fixed after 24 hours without further antibiotic selection. Cell lines expressing inducible FAM134B variants were generated by transfecting pcDNA4TO-FAM134B-1 and pcDNA4TO-FAM134B-2 vectors into Hep3B-TRex, a Tet repressor (pcDNA6TR) expressing cell line. FAM134B-1 and FAM134B-2 containing p3xFLAG-CMV-14 vectors were transfected into Hep3B cells. The chemical transfection of plasmids was performed using the FuGENE HD (Promega) transfection reagent. After transfection, cells were maintained under Zeocin (pcDNA4TO, 10ug/ml) or Neomycin (p3xFLAG-CMV, 300ug/ml) selection for 3-4 weeks, and single cell derived clones were picked, expanded and screened by western blot.

Antibodies

The antibodies used in western blot are as follows: rabbit anti-FAM134B, (Sigma, HPA012077), mouse anti-FLAG (Sigma, F1804), rabbit anti-LC3B (Cell signaling, 2775S), rabbit anti-GOPC (Cell

Signaling, 8576), rabbit anti-Syntaxin 6 (Cell Signaling, #2869), rabbit anti-GM130 (Abcam, ab31561), mouse anti-58K (Abcam, ab27043) and mouse-anti Calnexin (Santa Cruz, sc23954).

Cell fractionation experiments

A fractionation experiment was performed using a slightly modified version of a previously established protocol (17). Briefly, equal amounts (5x10^6 from each) of FAM134B-1 and FAM134B-2 expressing Hep3B cell pellets were homogenized in 5 ml 0.35M sucrose containing homogenization buffer with 50mM Tris-HCL pH 7.6, 25mM KCL, 10mM MgCl2, 2mM DTT using a motor homogenizer (Stuart). After centrifugation at 14.000xg, 4 °C for 10 minutes, the supernatant was carefully loaded on top of the discontinuous sucrose gradient, which is made up of 10ml 1,5M sucrose (top) and 10ml 2M sucrose (bottom). Samples were centrifuged at 70.000xg, 4°C for 16h using SW32 Ti rotor (Beckman Coulter). By puncturing the bottom of the tubes, 500ul fractions were collected. Fraction samples were mixed with 4x Laemmli loading buffer (with 2-mercaptoethanol), making the total loading buffer concentration to 1x. Samples were directly loaded on polyacrylamide gel.

Western Blot

Protein extraction from animal tissues and cell pellets were performed using RIPA lysis buffer containing 150mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, %0.1 SDS, 25mM pH7.4 Tris, 1x protease inhibitor cocktail (cOmplete, ROCHE), 1mM NaOV3 and 1mM NaF. Cell pellets were directly lysed in RIPA buffer, while mouse liver, pancreas, muscle, heart, testis, fat, lung and stomach tissues were dipped in liquid nitrogen and immediately grounded into fine tissue powder with the help of a mortar and pestle. RIPA was directly added on the finely grounded tissue by taking extra care to prevent the tissue from thawing. Samples were kept on ice and vortexed every five minutes for 30 minutes, which was followed by centrifugation at 13.300xg, 4°C for 30 minutes. They were treated with Laemmli loading buffer containing 2-mercaptoethanol and boiled at 95°C for 3 minutes, and were then loaded on polyacrylamide gel and run at 90V for protein stacking and 110V for protein resolving. Proteins were wet

transferred to PVDF membrane at 400mA for 2 hours. Membranes were blocked with 5% Non-Fat Milk Powder dissolved in TBS-T (50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5% Tween-20) for 1 hour at room temperature. After blocking, the membranes were incubated with the primary antibody diluted in blocking solution overnight at 4 °C. Membranes were treated with HRP conjugated secondary antibodies diluted in blocking buffer for 1 hour at room temperature. Following the incubation step of each antibody, membranes were washed three times with TBS-T. Protein bands were viewed using SuperSignal West Dura (ThermoFisher), and membrane images were taken via western blot imaging system (Vilber Lourmat).

Immunofluorescence

Cells were seeded on coverslips before transfection. One day after transfection, they were fixed either with ice cold methanol (FAM134B-FLAG and 58K staining) for 20 minutes or 4% paraformaldehyde (EGFP and mCherry conjugated FAM134B) for ten minutes at room temperature. Fixed cells transfected with FLAG tagged FAM134B were blocked with 5% BSA in PBS-T (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4, pH 7.4, 0.1% Tween-20) for 1 hour at room temperature. Cells were then incubated with the primary antibody in 2% BSA overnight at 4°C. They were then incubated with anti-mouse and anti-rabbit secondary antibodies, which are conjugated with 488nm and 594nm fluorophores respectively, for 1 hour at room temperature. EGFP-FAM134B-2 and mCherry-FAM134B-1 transfected cells were not incubated with an antibody. Cells were stained with 100ng/ml DAPI solution for 1 minute. They were washed three times with PBS-T in between all staining procedures. Coverslips were mounted on glass slides using mounting medium (ProLong Gold Antifade Mountant, Invitrogen). Confocal images were taken using Zeiss LSM880 and processed using the ImageJ software.

Results

Genomic organization of FAM134B and its protein isoforms

The human *FAM134B* gene is located on chromosome 5p, and spans a 144 kb region. The structural organization of the human and mouse full-length orthologs are quite similar, with 9 exons and 8 introns. Two transcript isoforms, namely *FAM134B-1* and *FAM134B-2*, are generated from the human (Fig. 1A-top) and bovine (not shown) genes, while the mouse gene serves as a template to generate six isoforms (Fig. 1A-bottom). The *FAM134B-1* isoform is generated by transcriptional initiation from the DNA sequence 5' of exon 1, whereas other isoforms have alternative start sites all located in intron 3 in both human and mouse (Fig. 1A). The translation of these transcripts generates a full length FAM134B-1 protein isoform and an N-terminally truncated FAM134B-2 protein isoform in both human and bovine species. The mouse also generates a *FAM134B-1* isoform, as well as several N-terminally truncated isoforms (all short forms are referred to as *FAM134B-2* in this report for simplicity).

The domain organization of the full length FAM134B-1 is characterized by a hydrophilic N-terminal domain, followed by the hydrophobic RHD that spans the cytoplasmic leaflet of the ER membrane, and a hydrophilic C-terminal domain. The RHD of FAM134B is composed of two transmembrane domains, namely TM12 and TM34, which are separated by a hydrophilic loop facing the cytoplasm (6, 22). On the other hand, N-terminally truncated FAM134B-2 protein isoforms are composed of a short hydrophilic N-terminal domain, followed by the single hydrophobic TM34 domain of the RHD, and completed by an intact hydrophilic C-terminal domain (Fig. 1B). Both short and long isoforms harbor a LIR motif located near the end of the hydrophilic C-terminal domain (Fig 1B).

FAM134B isoforms are differentially expressed in normal adult tissues

The localization of transcription start sites of all short isoforms within intron 3 of the *FAM134B* gene strongly suggested that the expression of long and short *FAM134B* transcript isoforms are controlled by different factors and/or mechanisms. This led us to compare the tissue-specific expression of full-length and truncated *FAM134B* isoforms. First, we analyzed normal tissue expression data on human isoforms available at the GTEx database (3). We observed striking differences between tissues in terms of

FAM134B gene expression (Fig. 2A).

FAM134B-1 is strongly expressed in the brain, which also expresses *FAM134B-2* at lower levels. A stronger expression of *FAM134B-1* was also seen in the tibial nerve, adipose tissue and testis. Many other tissues including the skeletal muscle, heart, kidney cortex, colon, pancreas, liver and stomach displayed a strong expression of *FAM134B-2* compared to *FAM134B-1*. A few tissues such as the uterus and ovary were observed to have a weak expression of both *FAM134B* isoforms (Fig 2A). We strived to confirm the differential expression of *FAM134B* isoforms by an RT-PCR analysis of a panel of 14 human tissues. As shown in Fig. 2B, experimental data confirmed the differential expression of *FAM134B-1* and *FAM134B-2* transcripts in these tissues. For example, the brain, testis, spleen and prostate were rich in *FAM134B-1* expression. In contrast, the skeletal muscle, pancreas and liver displayed a stronger expression of *FAM134B-2*. As expected, no significant signal was observed in the ovarian tissue.

We also analyzed the expression patterns of *Fam134b* transcripts in several mouse tissues. As shown in Fig. 3A, mouse testis strongly expressed all isoforms except for isoform-5. The mouse brain showed a strong expression of isoform-1 with a weak expression of isoform-2, -4 and -6. The heart was weakly positive for isoform-1, -3, -4, -5 and -6. The muscle stood apart by a strong expression of isoform-3, together with a weak expression of isoform-1, -2, and-4.

Differential expression of FAM134B protein isoforms in normal tissues

A further verification of *FAM134B* isoform expression in different tissues was performed at protein level by western blotting. We initially applied western blotting experiments to bovine species, which express only two transcript isoforms, similar to human, as stated above. As shown in Fig. 3B, bovine FAM134B-1 and FAM134B-2 proteins migrated with apparent molecular weights of 70 kDa and 50 kDa, respectively, similar to human isoforms. The dominant expression of FAM134B-1 was observed in the brain, adipose, testis and kidney, while FAM134B-2 was dominant in the heart and muscle. The weak expression of both isoforms was detected in the lung tissue. The bovine liver stood apart by little to no expression of both FAM134B protein isoforms.

FAM134B isoforms differentially localize in sub-cellular compartments

FAM134B has previously been identified as an ER and Golgi-associated protein (22, 25). As the studied isoform(s) has not been specified in these studies, we went on to compare the two isoforms for their sub-cellular localization. Initial analysis of four well-differentiated (16, 44) liver cancer cell lines failed to detect FAM134B isoforms in these cell lines, except for the Hep40 displaying a weak FAM134B-1 expression (Fig. 4A). We constructed Hep3B-derived cells stably expressing FLAG-tagged FAM134B-1 or FAM134B-2.

In order to investigate the subcellular localization of both isoforms in the same experiment, we performed cell fractionation studies followed by western blot analysis. We first prepared cell extracts (Fig.4B, lines input-1 and input-2 respectively) prior to a sucrose gradient. Sucrose fractions were then collected from the bottom of the gradient making tube and subjected to western blotting using antibodies for FAM134B, as well as Golgi markers GOPC (9), GM130 (30) and STX6 (34) and CNX (ER, but leaky; ref. (33)).

FAM134B-2 co-localized with GOPC and cis-Golgi marker GM130, but not with trans-Golgi marker STX6 fractions, while calnexin was expanded to many fractions and co-localized with both isoforms. On the other hand, both FAM134B isoforms partially co-localized with Golgi marker 58K (4), demonstrated by immunofluorescent staining (Fig. 4C). Immunofluorescence analysis of FAM134B-1 and FAM134B-2 indicated that when ectopically expressed, they mostly co-localize with each other, and they also co-localize with Calnexin (Fig. 4D and E), suggesting ER-localization.

Starvation increases FAM134B-2 RNA and protein in the liver

As *FAM134B* is involved in ER-phagy during starvation (22), we studied its expressional changes in mice following starvation. Wild-type and *Fam134b*$^{-/-}$ mice were either fed *ad libitum* or starved up to 48h, followed by an expression analysis of *FAM134B* isoforms. Tissues obtained from *Fam134b*$^{-/-}$ mice

were used as negative controls. Our FAM134B antibody cross-reacted with several non-specific proteins in mouse tissues, thus limiting our analysis to only the FAM134B-2 isoform (Fig. 5A, C, D).

We first analyzed LC3B and FAM134B-2 expression in the liver after 48h of starvation. Autophagy induction in the liver as a response to starvation was shown by a decrease in p62 protein levels and induced levels of autophagosome-associated LC3B-II and LC3B-I (Fig. 5A). We also noticed that LC3B-I levels were more pronounced in *Fam134b*$^{-/-}$ mice compared to wild-type mice (Supplemental Fig. S1). FAM134B-2 was not detected in *Fam134b*$^{-/-}$ mice, as expected. *Fam134b*$^{+/+}$ and *Fam134b*$^{+/-}$ mice showed an induced expression after starvation, at 50 kDa (Fig. 5A and Supplemental Fig. 1). In addition to the 50kDa FAM134B-2, a specific smaller 35kDa protein was also induced in wild type animals (Fig. 5A, marked with a single asterisk). Additional starvation experiments performed for 24h have exhibited an even stronger stimulation than of 48h (Supplemental Fig. S2).

To test whether FAM134B protein accumulation in starved animal liver was associated with transcriptional upregulation, we performed an RT-PCR analysis of six mouse isoforms along with two additional putative protein coding transcripts, X1 and X2. The starvation induced the expression of isoform-3, -4 and -6 strongly and isoform-2 weakly, while the level of isoform-5 was not changed. There was little to no expression of isoform-1 in both fed and starved conditions (Fig. 5B). There was also no induced expression of putative transcripts X1 and X2.

Starvation increases FAM134B-2 in the muscle and heart

In order to observe possible starvation induced FAM134B-2 response in other organs, we expanded our analysis with mouse heart, muscle, pancreas and stomach. Similar to the liver, FAM134B-2 protein levels as well as shorter protein fragments near 35kDa in the muscle and heart were elevated following starvation (Fig. 5C). Stimulation of autophagy was confirmed by increased shift from LC3B-I to LC3B-II (Supplemental Fig. 3). Under the same conditions, there was no change in FAM134B-2 levels in the pancreas or stomach (Fig. 5D).

Upon starvation, Fam134b$^{-/-}$ mice display less weight loss and increased serum albumin levels along with disrupted amino acid release

In order to analyze the *in vivo* response of *Fam134b*-deficient tissues to starvation, we compared *Fam134b*$^{+/+}$ and *Fam134b*$^{-/-}$ mice for their response to nutrient starvation for 36h. The initial body weights of tested animals were similar with no significant differences (n=73) (Supplemental Fig. S4). Starvation induced significant whole body and tissue-specific weight loss in both wild-type and *Fam134b*$^{-/-}$ mice (Fig. 6). Among the tissues analyzed, there was significant weight loss in the liver, kidney, spleen and heart for both wild type and mutant animals, and significant loss of lung weight was observed only in mutant animals. Interestingly, starved mutant animals slightly resisted body weight loss compared to wild type mice, as shown by body weight change as percentage (Fig 6, Body weight change).

Autophagic proteolysis in the liver following starvation makes a significant contribution to the maintenance of blood glucose levels via conversion of amino acids to glucose, and accordingly, many amino acids are released into the blood by 24h of starvation (12). Therefore, we measured serum glucose and amino acid levels in both wild-type and *Fam134b*$^{-/-}$ mice after 36h of starvation. There was a significant decrease in serum glucose in both wild type and mutant mice, the fall in *Fam134b*$^{-/-}$ being more significant (Fig 7A). Wild-type animals displayed a significant increase in serum levels of amino acids glutamine, asparagine, alanine, serine, glycine, tryptophan, threonine and lysine. *Fam134b*$^{-/-}$ mice failed to increase the levels of these amino acids with the exception of threonine (Fig. 7B). There was no change in the levels of histidine, proline, valine, methionine and tyrosine in wild-type mice, but interestingly, all these five amino acids underwent a significant drop in *Fam134b*$^{-/-}$ mice (Fig. 7C).

Other changes in starved *Fam134b*$^{-/-}$ mice

We further investigated the *Fam134b* associated phenotype by analyzing serum levels of proteins, nutrients, electrolytes and enzymes. Interestingly, baseline High-density lipoprotein (HDL) cholesterol levels were significantly lower in mutant animals. Starvation induced a decrease in serum levels of

triglycerides, Very-low density lipoprotein (VLDL) and Low-density lipoprotein (LDL) cholesterol, but not in total cholesterol in either group of animals (Fig. 8).

Starvation also induced serum levels of albumin and total protein in *Fam134b*$^{-/-}$, but not in wild-type mice (Fig. 8). Wild type animals showed elevated levels of blood urea nitrogen (BUN), as reported previously (41), but no such significant change was observed in mutant mice.

Changes in serum ions such as sodium, potassium, chloride, calcium and iron were also observed (Fig. 9). Upon starvation, sodium, potassium and chloride displayed significant increases in both wild-type and *Fam134b*$^{-/-}$ mice. In contrast, calcium levels in wild-type animals and iron levels in both groups were decreased. The lack of starvation-induced hypocalcemia in *Fam134b*$^{-/-}$ mice was noteworthy.

Finally, starvation resulted in liver injury as evidenced by increased levels of serum AST and ALT in both groups of animals. Of particular interest, serum α-amylase activity was significantly upregulated in *Fam134b*$^{-/-}$, but not in wild-type mice (Fig. 10), suggesting starvation-induced pancreas injury.

Expression of *FAM134B* isoforms is suppressed or stimulated in different cancers

As stated earlier, the expression changes of *FAM134B* have been described in esophageal, colon, breast and liver cancers (9-14). Our observations described so far show that the human *FAM134B* gene encodes at least two different protein isoforms: one full length with an intact RHD domain (i.e. FAM134B-1) and an N-terminally truncated form with a disrupted RHD domain (i.e. FAM134B-2). Previously published data on FAM134B in cancers did not specify what type of FAM134B protein is implicated in different cancers. In order to clarify this issue, we collected following data sets from the TSVdb database: hepatocellular carcinoma (371 tumors and 50 non-tumor liver), lung adenocarcinoma (515 tumors and 59 normal), lung squamous carcinoma (501 tumors and 51 normal), colon adenocarcinoma (285 tumors and 41 normal) stomach adenocarcinoma (415 tumors and 35 normal) and kidney chromophobe cancer (66 tumors and 25 normal), and performed an isoform level differential expression analysis on these publicly available transcriptome data.

As shown in Fig. 11, both *FAM134B-1* and *FAM134B-2* isoforms were positive in non-tumor colon, lung, (Fig. 11A), stomach and kidney (Fig. 11B) tissues. In contrast, only *FAM134B-2* was positive with a marginal expression of *FAM134B-1* in non-tumor liver samples (Fig. 11A). HCC samples displayed a significant down-regulation of *FAM134B-2* (Fig. 11A). We confirmed this finding by performing an RT-PCR analysis on HCC tumors (n=11) and non-tumor liver tissues (n=16), which showed a loss of the *FAM134B-2* signal in most HCC tumors analyzed (Fig. 11C).

A significant down-regulation of both *FAM134B-1* and *FAM134B-2* was observed in colon and lung cancers (Fig. 11A). In contrast, FAM134B-2 was significantly upregulated in stomach adenocarcinoma. Finally, both FAM134B-1 and FAM134B-2 were significantly upregulated in chromophobe renal cell carcinomas (KICH). Other tumors including breast and prostate cancer did not display significant changes in the *FAM134B* isoform expression (not shown).

Discussion

We performed a comprehensive analysis of the *FAM134B* gene, its transcripts and encoded protein isoforms together with its implications in nutrient starvation and cancer. Our main findings indicate that human and bovine species express at least two different transcript isoforms encoding a full-length and an N-terminally truncated protein isoform. In contrast, mice encode at least six different transcript isoforms, giving rise to one full-length and five N-terminally truncated proteins. During the preparation of this report for publication, we became aware of a publication by Kohno et al. (23) on the identification of a novel *FAM134B-2* transcript isoform capable of encoding an N-terminally truncated protein in the livers of fasted mice. This transcript may represent either isoform-2 or isoform-3 described in our report (Fig. 1). In addition to isoform-2 and -3, mouse tissues exhibited the expression of isoform -4, -5 and -6, all capable of encoding four different N-terminally truncated proteins that differ from each other at the first 45 amino acids of the N-terminal region (Fig. 1B).

The FAM134B-2 protein lacks the N-terminal tail and the TM12 reticulon structure of the full-length protein, but retains the TM34 reticulon along with the C-terminal region, which harbors the LC3B binding LIR motif. It was previously shown that the remaining reticulon structure, namely TM34, is critical for membrane shaping (6), and that the LIR motif of FAM134B-2 still mediates ER-phagy (23). However, FAM134B-2 is unlikely to be a mere compensatory protein for FAM134B-1, since FAM134B-1 cleavage by the zika virus yields a truncated protein very similar to FAM134B-2 that is unable to promote reticulophagy (26).

Our subcellular localization studies with human FAM134B-1 and human FAM134B-2 indicated that loss of integrity of the RHD in the shorter isoform did not abolish the ability of FAM134B-2 to localize to the ER (Fig. 4B and D). However, the two isoforms of FAM134B did not completely co-localize, as shown in Fig. 4B. Co-localization of FAM134B-2 with the Golgi marker GOPC, GM130 (Fig. 4B) and 58K (Fig. 4C) but not with STX6 strongly suggested that this form may be preferentially localized at the ER membrane close to the Golgi apparatus. Indeed, mouse liver FAM134B-2 was shown to be localized at both the rough and smooth ER (23).

In order to detail the physiological relevance of *FAM134B* variants, we analyzed their distribution in human, bovine and mouse tissues. One of our most striking findings is the identification of the *FAM134B-2* transcript as the dominantly expressed form in the skeletal muscle, heart, kidney cortex, colon, pancreas, liver and stomach. This contrasts sharply with the dominant expression of *FAM134B-1* in the brain, tibial nerve, adipose tissue and testis. Our western blot analyses with bovine and mouse tissues have further shown that the tissue-specific expression of FAM134B is conserved during evolution (Figs. 3, 5). This may indicate that *FAM134B-1* and *FAM134B-2* are involved in some tissue-restricted functions. For example, *FAM134B-2* may be involved in the lysosomal degradation of a subgroup of ER components rather than bulk ER degradation (or turnover), which was previously elaborated for the liver (23). As the FAM134B-2 protein is distinctly co-localized to ER and Golgi, it may have a role during the transfer of protein and lipid molecules from ER to Golgi, or in autophagy-related changes during the trafficking, processing, and sorting of the newly synthesized membrane and secretory proteins and lipids.

Reticulon Homology Domain (RHD) containing proteins are well known as ER-shaping elements, and their deficiency results in a disturbed ER network (10). Furthermore, proteins harboring a single transmembrane structure (e.g. TM12 or TM34 alone) were previously shown to be involved in shaping ER tubules (46). Despite carrying a truncated RHD, a pronounced expression of FAM134B-2 in the muscle and heart might indicate a similar structural role in ER shaping and organization. However, besides the previously identified sensory neuropathy, no major phenotype has been observed even in aged animals, indicating that *Fam134b* deficiency is well-tolerated, possibly due to a compensatory mechanism which is yet to be understood.

From simple prokaryotes to humans, all living organisms are equipped with complex survival mechanisms to cope with nutrient scarcity. Starvation initiates a cascade of events including autophagy, thereby mobilizing energy sources to meet the energy demands of vital organs. In this complex mechanism, the liver, heart, skeletal muscle and kidney play critical roles in regulating the release and uptake of major energy sources such as amino acids, lipids, glucose and ketone bodies (37). Considering the critical role of starvation in autophagy, we performed comparative analyses of wild-type and *Fam134b*$^{-/-}$ mice (29). Starvation for 48h resulted in the accumulation of LC3B-I, as well as the autophagy-associated LC3B-II form in the livers of wild-type mice. A similar accumulation was observed in *Fam134b*$^{-/-}$ animals even in the absence of starvation (Supplemental Fig S1), which implies a possibly disturbed autophagic flux. The stimulated expression of FAM134B-2 in the muscle and heart but not in the pancreas and stomach strongly indicates a possible involvement of this protein in the physiological response against nutrient starvation.

The FAM134B-2 protein, which was weakly positive in the liver of fed animals, was induced strongly by starvation, as reported previously (23). However, besides the verification of the previously reported FAM134B-2 increase at 50kDa, we surprisingly observed another specific band at 35kDa, which was also increased upon nutrient starvation, not only in the liver but also in the muscle and heart. Indeed, this shorter isoform could be a post translational modification of FAM134B-2, as well as a protein product of short isoform transcripts.

The starvation-induced FAM134B accumulation in different tissues urged us to carry out a series of analyses to compare wild-type and *Fam134b*$^{-/-}$ mice under fed and starved conditions. *Fam134b*$^{-/-}$ mice under fed conditions did not differ from wild-type animals, with the exception of decreased serum HDL cholesterol levels. Since HDL production and clearance from blood are primarily performed by the liver (39), hepatic FAM134B-2 might be involved in the regulation and/or maturation of HDL particles.

Following 36h of starvation, both wild type and *Fam134b*$^{-/-}$ mice displayed significant weight loss in total body as well as in liver. Although we found some significant differences in other tissues, the weight loss was less pronounced. There were also significant changes in blood parameters, in line with the literature (20, 31, 41).

When we compared starvation responses of wild-type and mutant mice, we identified several important aberrations associated with *Fam134b* deficiency. Firstly, mutant mice were significantly more resistant to body weight loss, as opposed to wild-type mice. Secondly, mutant mice showed profound aberrations in serum amino acid dynamics. As reported previously, starvation-induced hypoglycemia and a decrease in insulin levels with maintained glucagon levels triggers an autophagic response in the liver, resulting in a surge of amino acids released into the blood at around 24h of fasting (12). Accordingly, we observed an increase in the serum levels of alanine, asparagine, glycine, lysine, glutamine, serine, tryptophan and threonine with wild-type mice. In contrast, mutant mice failed to elevate the levels of all these amino acids with only one exception. This observation strongly suggests that in the absence of FAM134B-2, liver tissue cannot undergo sufficient autophagic proteolysis to produce these amino acids. Another interesting observation with *Fam134b*$^{-/-}$ mice was a decrease in the serum levels of methionine, valine, tyrosine, histidine and proline. Thus, it appears that these normal constituents of serum are depleted during starvation, probably because they are converted to glucose. Such a compensation may be necessary when autophagic degradation of proteins is defective.

Glucagon in circulation is one of the key factors for energy mobilization upon nutrient starvation (12). Glucagon was previously shown to downregulate serum calcium levels by altering the gastrointestinal uptake (1). The steady state calcium levels in mutant mice during starvation strongly

suggest a function in calcium homeostasis, likely through intestinal absorption. Finally, *Fam134b*$^{-/-}$, but not wild-type mice, displayed abnormal increases in blood α-amylase levels upon starvation, which indicates pancreatic injury, salivary gland malfunction or other intraabdominal inflammations (28). As demonstrated in western blot experiments, FAM134B-2 is predominantly expressed in the pancreas, in which basal autophagy is necessary to maintain α-amylase secreting acinar cells (2). Furthermore, another ER-phagy receptor, CCPG-1, was shown to be critically involved in secretory acinar cells, and its absence results in the accumulation of insoluble α-amylase particles and unfolded protein response (UPR) (35). Therefore, the α-amylase increase in *Fam134b*$^{-/-}$ could be partially attributed to a defective ER-phagy, which may lead to a discharge or leak of excessive α-amylase into the bloodstream.

Our comprehensive analysis of *FAM134B-1* and *FAM134B-2* expression in the TCGA tumor collection has clearly established that *FAM134B-2*, and to a lesser degree *FAM134B-1*, are closely involved in cancer. The loss of *FAM134B-2* expression was observed in HCC, colon and lung cancers. In contrast, stomach and chromophobe renal cell carcinomas displayed a significant upregulation of the same transcript. *FAM134B-1* levels were generally low in non-tumor tissues, but a significant loss was observed in colon and lung cancers, as well as a significant increase in chromophobe renal cell carcinomas. Thus, cancers can be grouped into three classes depending on *FAM134B-2* gene expression; those with significant downregulation such as HCC, colon and lung cancers, those with significant upregulation such as stomach and a subtype of kidney cancers, and those with the *FAM134-2* expression likely not changing significantly in the third group of remaining cancers.

The past few years have been fruitful for the understanding of the molecular roles of *FAM134B,* but without defining its isoforms and their relevance in the whole organism. To our knowledge, this is the first paper to define the differential expression of *FAM134B* isoforms in various tissues under normal and starved conditions, as well as in cancer. These findings greatly help us further understand the functional roles of *FAM134B* isoforms in diseases such as cancer.

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Statement of contribution:

- UK: Designed and performed *in vivo* and *in vitro* experiments
- HEY, UE, SK, ZM: Carried out *in vivo* experiments and serum analyses
- AS, GK: performed statistical analyses
- EI, EB: performed and reported *in vitro* experiments
- ADC, NT: designed and cloned FAM134B plasmids, and carried out preliminary experiments
- NA: Contributed to the study by providing valuable HCC patient cDNA and by providing guidance on its usage
- MAS: Contributed to the study by providing results and interpretation of serum amino acid analyses.
- MO: Supervised, designing, planning, performing and reporting of all experimentation.

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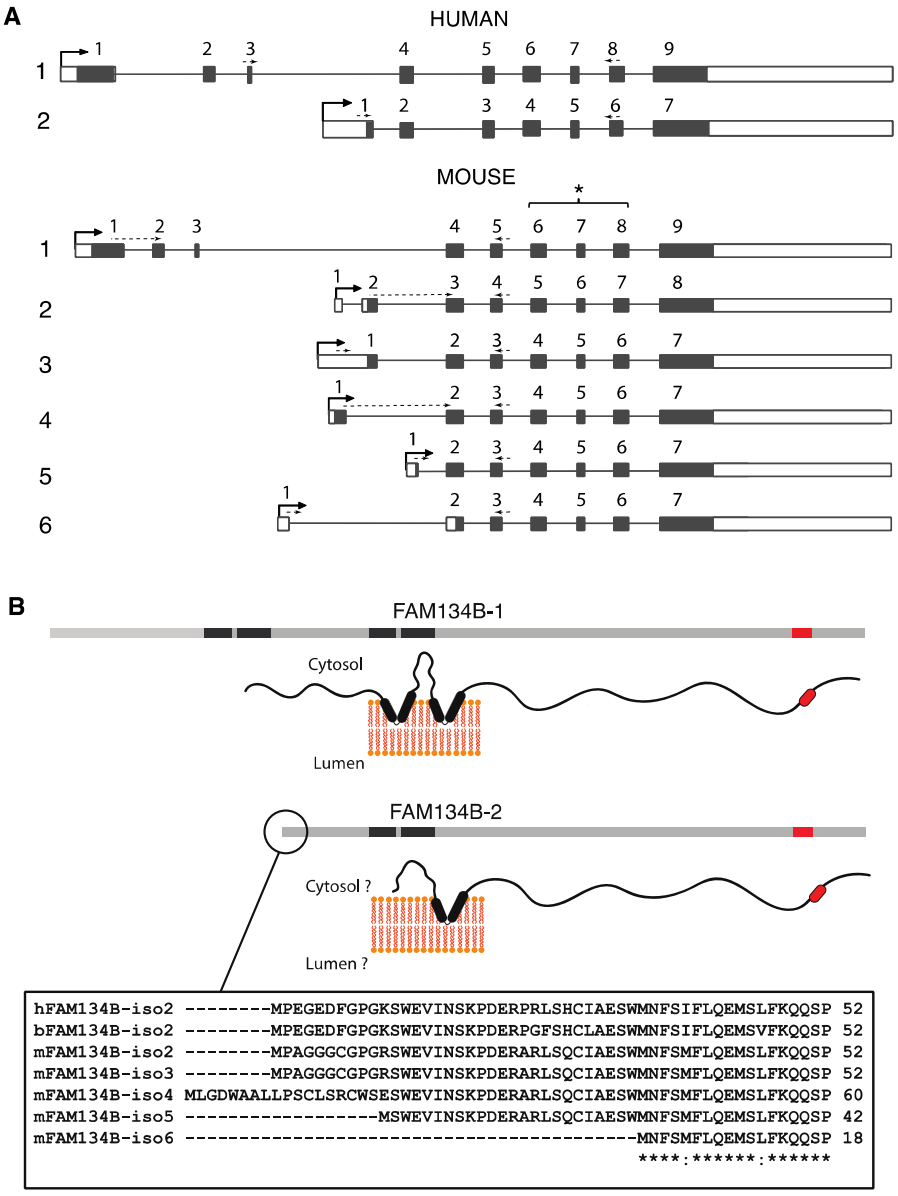


Figure 1. Gene and protein architecture of FAM134B variants.

(A) Human and Mouse *FAM134B* genome organization scheme based on the NCBI RefSeq, and the exon annotation based on the UCSC genome browser. Note that isoforms differ from each other by transcription start sites. Dashed arrows faced inwards indicate the location of selected oligonucleotides for RT-PCR. Asterisk shows the exons deleted for *Fam134b* knockout mouse model. (B) Schematics of human FAM134B-1 and FAM134B-2 proteins. The latter is further detailed as N-Terminal amino

acid sequence across species in the alignment box. V-shaped black rods represent hairpin structures of transmembrane regions. Red boxes/rods indicate the LIR motif, the target of GABARAP/LC3. Prefixes h-, b- and m- represent human, bovine and mouse *FAM134B* sequences, respectively.

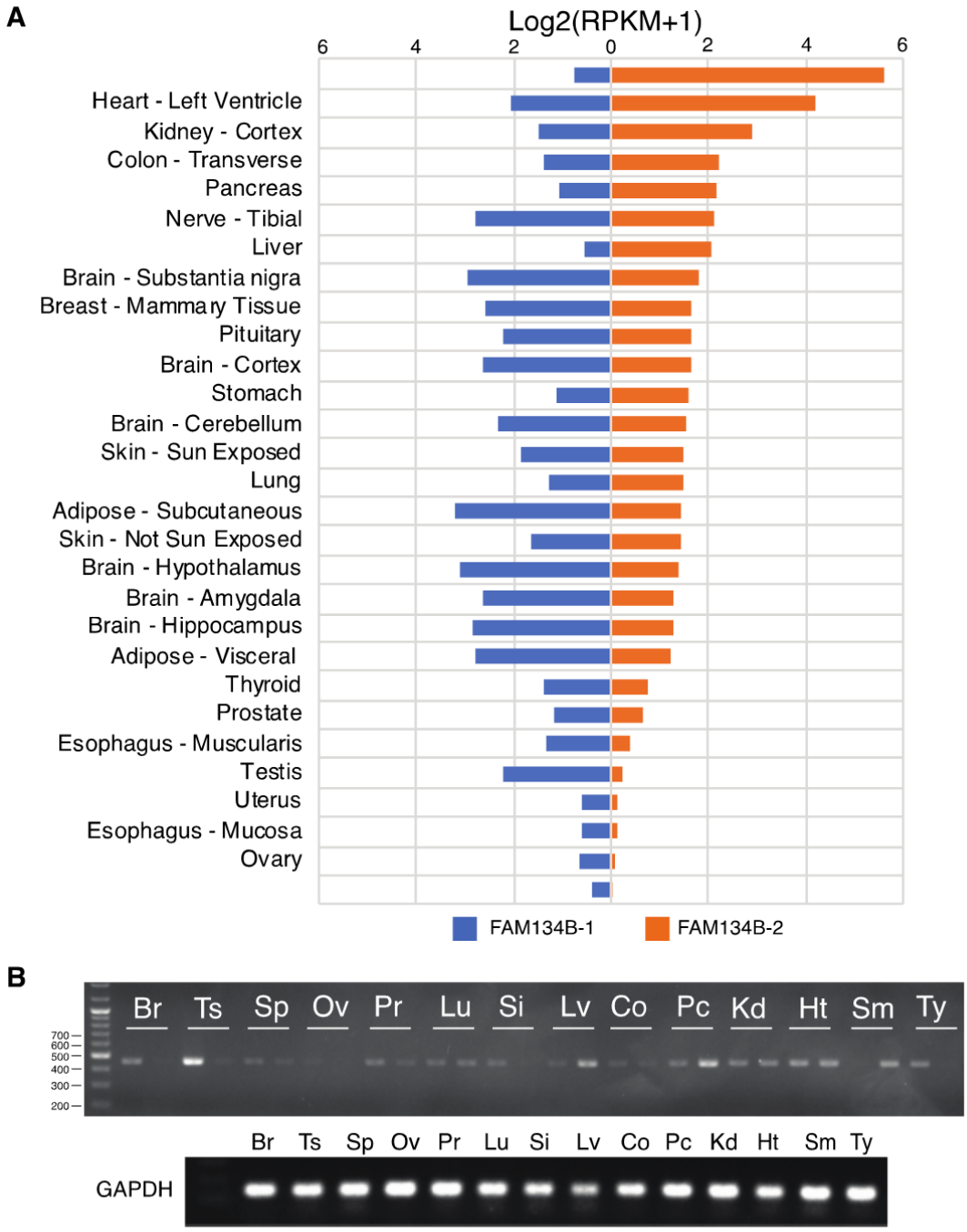


Figure 2. *FAM134B* isoforms are differentially expressed in human tissues.

(A) GTEx RNA-Sequencing data of human FAM134B isoforms represented as Log2 (Mean RPKM+1) (Standard Deviations are not shown). (B) FAM134B isoform expression levels in healthy human tissue panel demonstrated by the Reverse Transcriptase-PCR (RT-PCR). Equal loading control

PCR performed by using GAPDH primers. The tissue name coding is as follows: Br: Brain; Ts: Testis; Sp; Spleen; Ov: Ovaries; Pr: Prostate; Si: Small intestine; Lv: Liver; Co: Colon; Pc: Pancreas; Kd: Kidney; Ht: Heart; Sm: Skeletal Muscle; Ty: Thymus. For each tissue, FAM134B-1 and FAM134B-2 bands were loaded at left- and right-hand side respectively. Predicted band sizes according to in silico PCR is as follows: FAM134B-1, 450bp; FAM134B-2, 449bp.

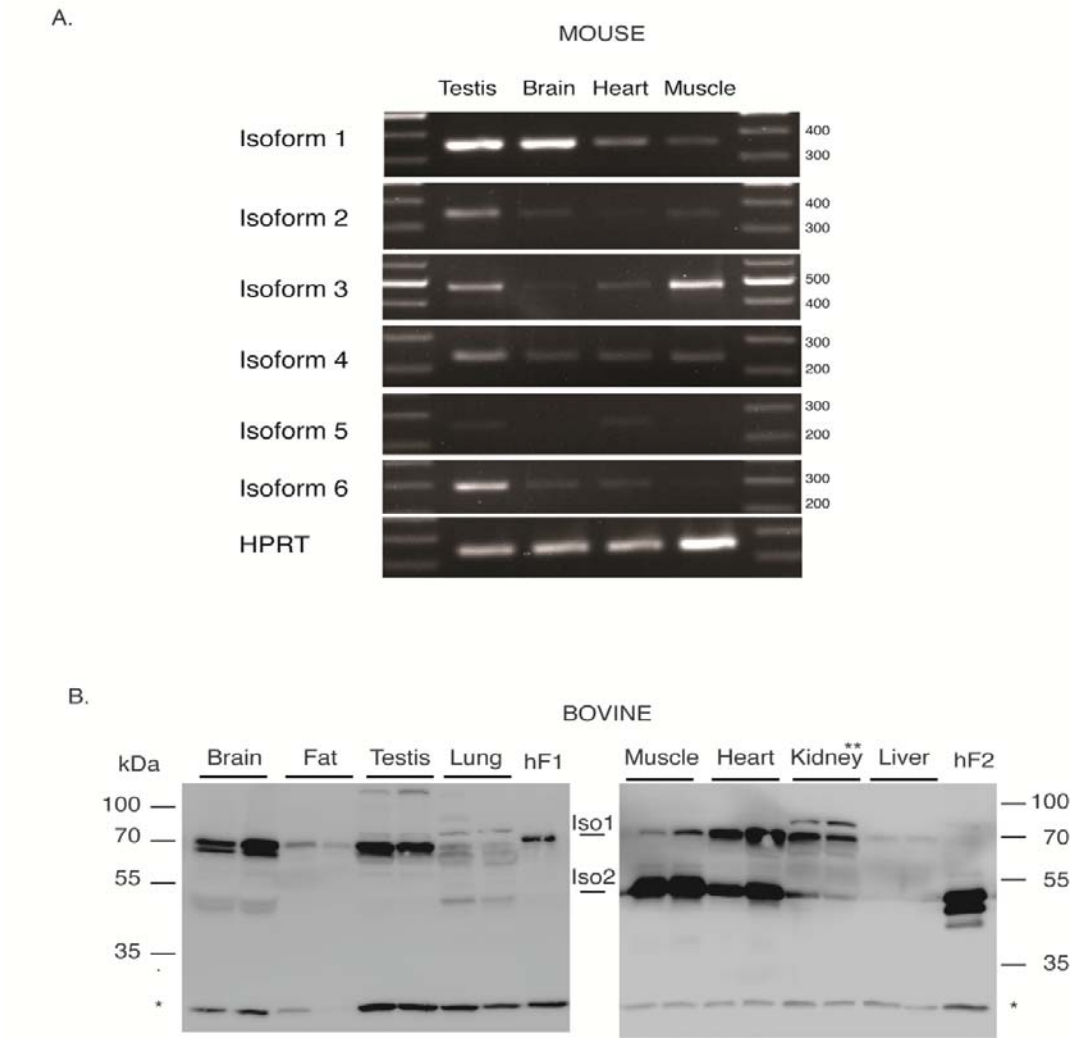


Figure 3. FAM134B isoforms are differentially expressed in mouse and bovine tissues

(A) Differentially expressed *Fam134b* variants in mouse testis, brain, heart and skeletal muscle, shown by RT-PCR. (B) Western blot results of bovine brain, fat, testis, lung, skeletal muscle, heart, kidney and liver probed by FAM134B antibody. hF1 and hF2 lanes represent positive control protein lysates of Hep3B cell line stably expressing human FAM134B-1 and FAM134B-2 respectively. Single asterisks show the nonspecific antibody binding. Double asterisk: kidney samples were taken from kidney cortex.

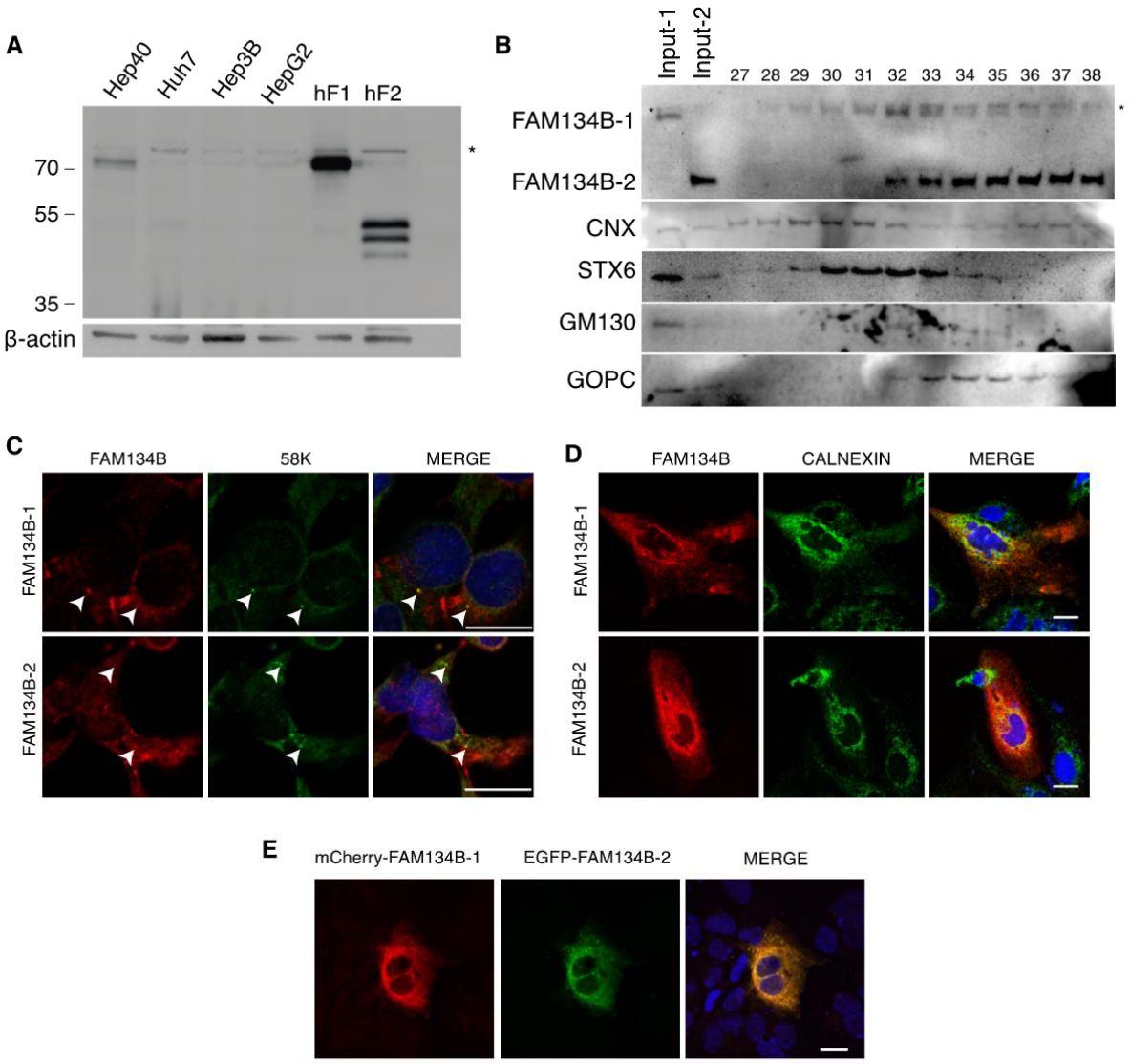


Figure 4. FAM134B isoforms are weakly expressed endogenously in cell lines and differentially localize in subcellular compartments

(A) Western blot result showing the basal levels of FAM134B in well-differentiated HCC cell lines. Asterisk indicates the non-specific antibody binding. hF1 and hF2 shows stable FAM134B-1 and FAM134B-2 expressions in Hep3B cell line respectively. (B) Sucrose gradient fractionation of Hep3B cells stably expressing FAM134B-1-FLAG and FAM134B-2-FLAG vectors. The numbers above the picture, from left to

right, represent the heavier to lighter fractions. FAM134B is probed by anti-FAM134B antibody. CNX (Calnexin) is used as ER marker; GOPC, GM130 and STX6 are used as Golgi markers. Note that specific FAM134B-1 bands reach a peak at fraction 32 and decrease thereafter. However, protein bands from non-specific antibody binding, marked with asterisk, start from fraction 31 and increase in following lighter fractions. (C) Partial co-localization of Golgi marker 58K with both FAM134B-1 and FAM134B-2. White arrowheads show FAM134B and 58K positive punctae. (D) Immunofluorescence imaging of FAM134B isoforms and ER resident protein Calnexin. (E) Immunofluorescence imaging of mCherry-FAM134B-1 and EGFP-FAM134B-2, co-expressed in Huh7 cell line. Scale bars in C, D and E represent 20μm.

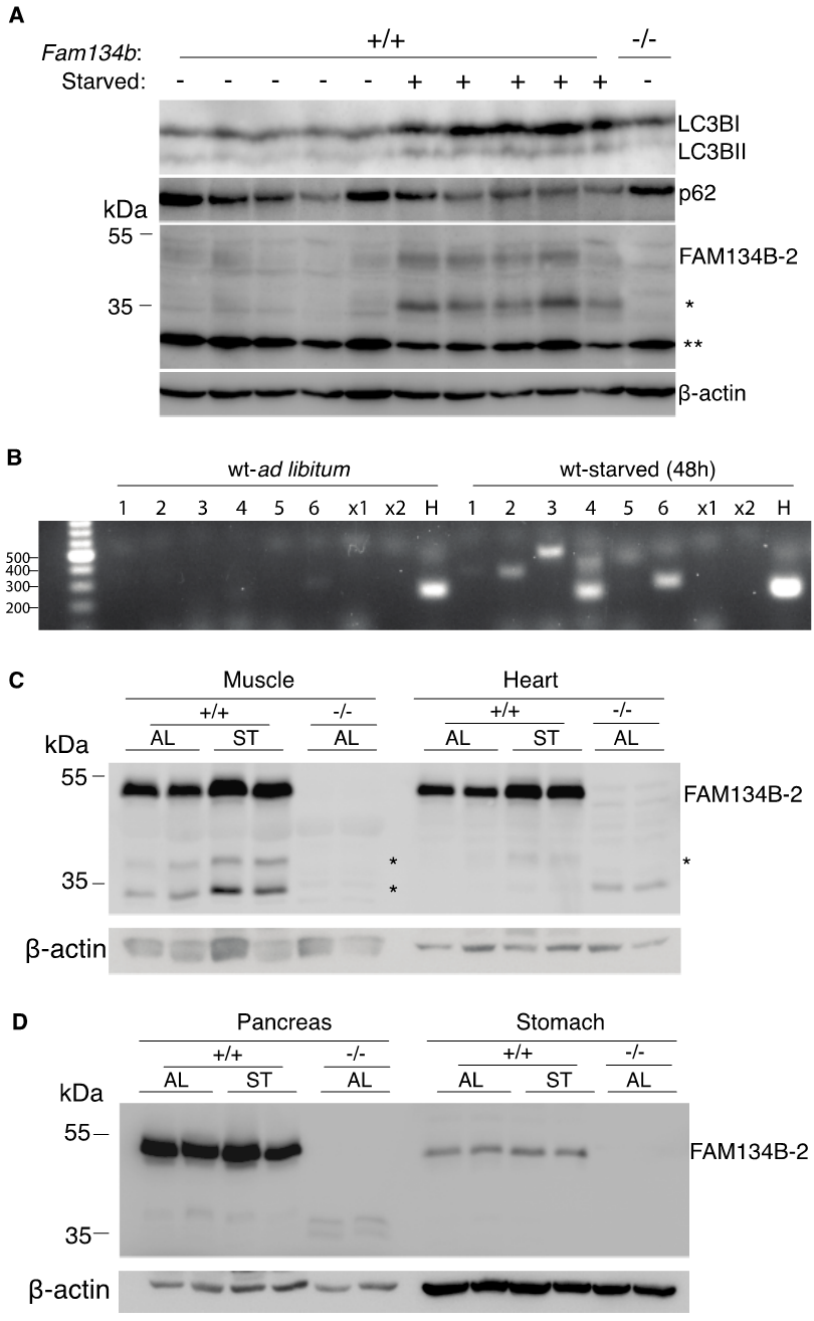
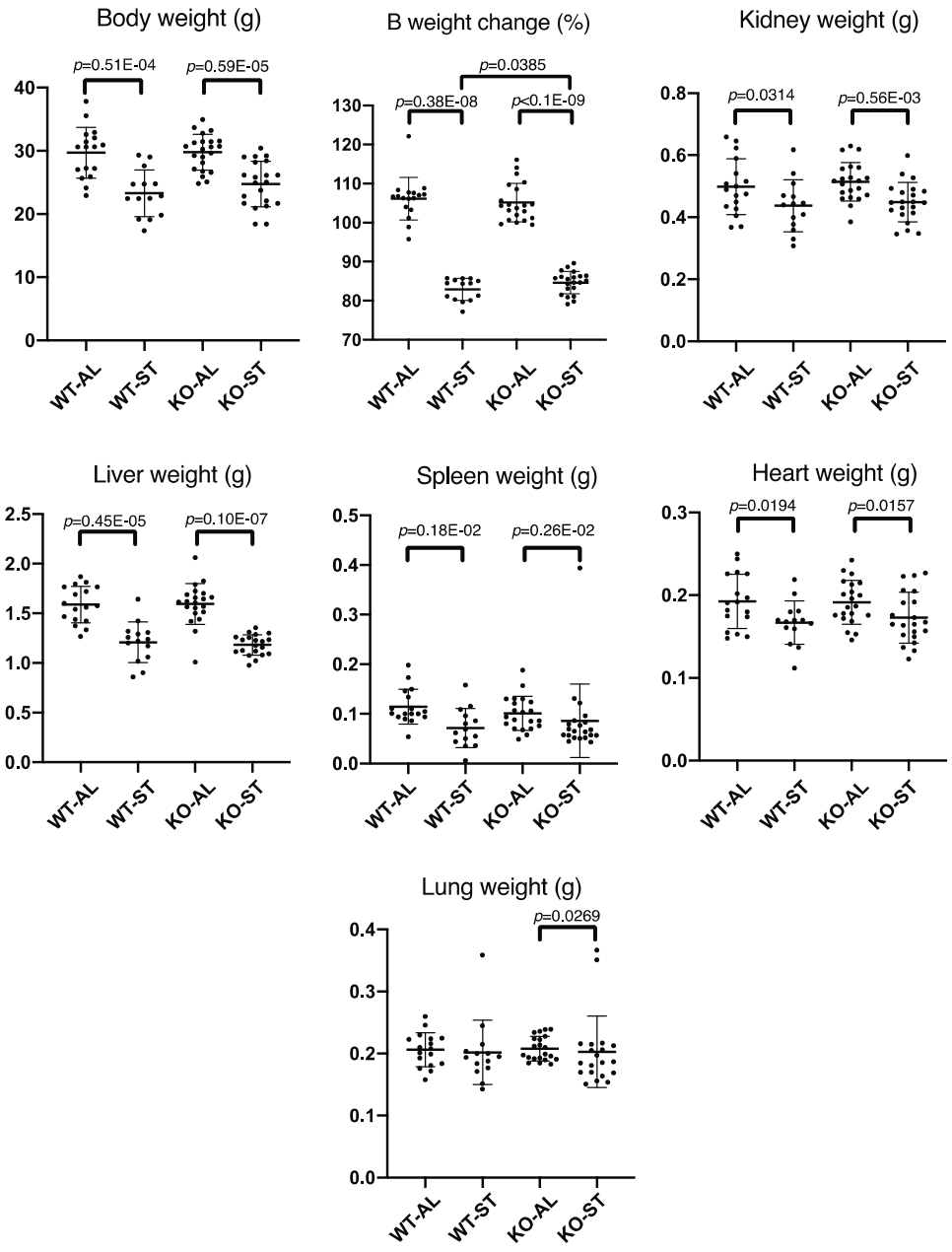


Figure 5. Starvation induces *FAM134B-2* in liver, muscle and heart in mice.

(A) Protein levels of FAM134B-2, p62 and LC3B in livers of mutant and wild type mice (12-week-old, male) upon 48h starvation. FAM134B-2 is observed near 55kDa, along with the additional band at 35kDa, which is marked with a single asterisk. Double asterisk indicates non-specific antibody binding. (B) mRNA levels of

Fam134b isoforms in 48h starved mouse liver. Numbers indicate the mouse *Fam134b* variants; H, mouse HPRT. Expected band sizes according to in silico PCR are as follows: isoform-1, 354bp; isoform-2, 343bp; isoform-3, 474bp; isoform-4, 231bp; isoform-5, 256bp; isoform-6, 286bp; Isoform X1, 469bp; Isoform X2, 473bp. (C) Tissue specific changes in FAM134B-2 levels upon 36h starvation (18-month-old mice, male) are demonstrated for skeletal muscle (gastrocnemius) and Heart; and (D) for pancreas and stomach.



783

784 **Figure 6**. Upon starvation for 36h, *Fam134b*$^{-/-}$ mice displayed resistance to total body weight loss.

785 18-month-old mice (n=73) were used for body and organ weight calculation with following groups:

786 *Fam134b*$^{+/+}$*-ad libitum* (WT-AL), n=17 (9 male, 8 female); *Fam134b*$^{+/+}$ Starvation (WT-ST), n=14 (8 male, 6

787 female); *Fam134b*$^{-/-}$*-Ad libitum* (KO-AL), n=21 (13 male, 8 female); *Fam134b*$^{-/-}$- Starvation (KO-ST), n=21

788 (13 male, 8 female). Mann-Whitney non-parametric test results are represented as mean and standard

789 deviation. $p<0.05$ was considered as significant. Non-significant results are not indicated.

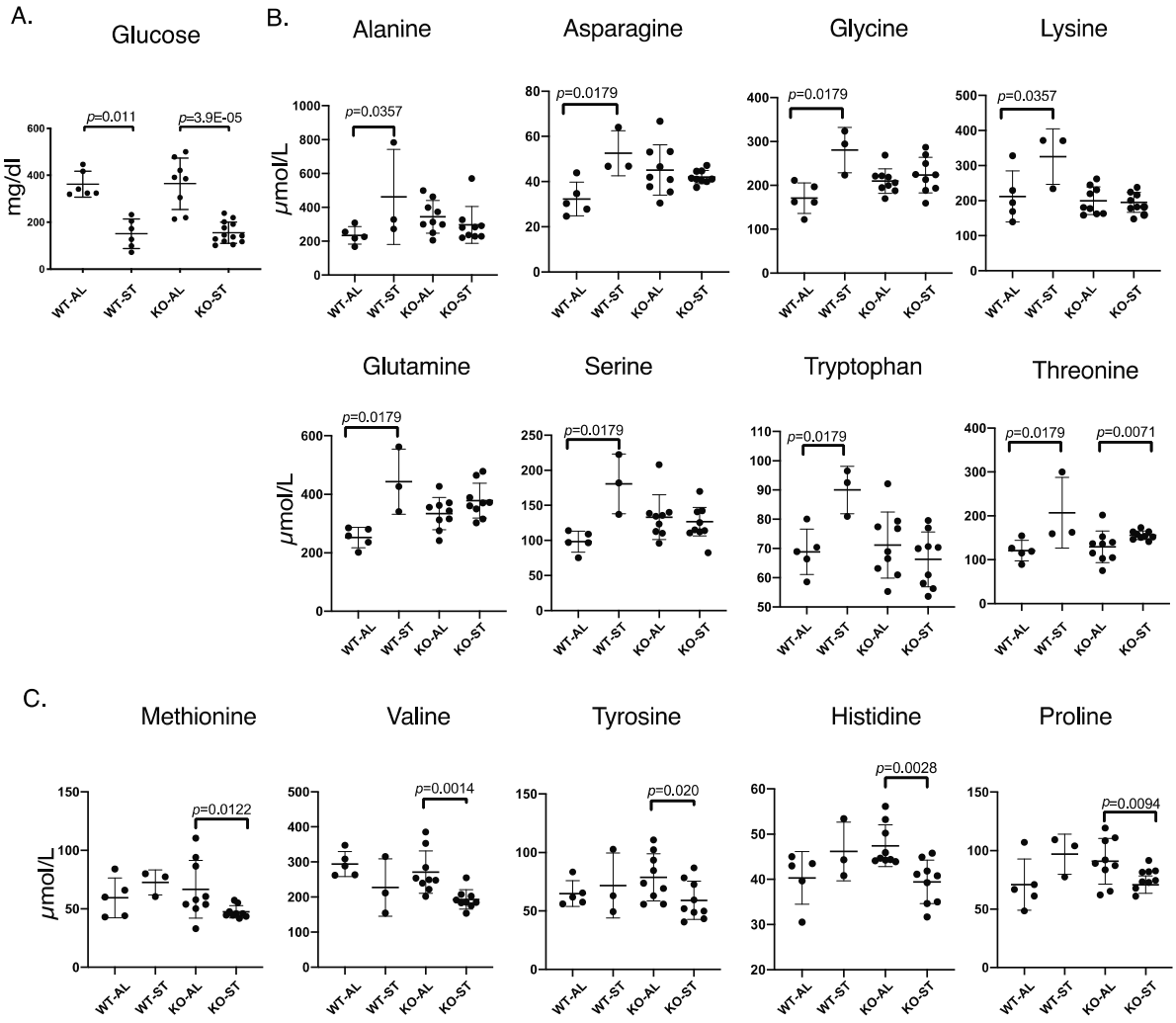


Figure 7. Amino acid release into blood following 36h of starvation is aberrant in *Fam134b*$^{-/-}$ mice.

(A)Serum glucose levels, (B) Serum amino acids with increased levels in wild type but not in *Fam134b*$^{-/-}$ mice. (C) Serum amino acids with decreased levels in *Fam134b*$^{-/-}$ but not in wild-type mice. Due to insufficient amount of serum some samples were unavailable for amino acid analyses. The animals included in amino acid analyses were average 18 months old and the animal groups are as follows: WT-AL, n=5 (4 male, 1 female); WT-ST, n=3 (2 male, 1 female); KO-AL, n=9 (6 male, 3 female); KO-ST, n=9 (8 male, 1 female) Mann-Whitney non-parametric test results are represented as mean and standard deviation. $p<0.05$ was considered as significant. Non-significant results are not indicated.

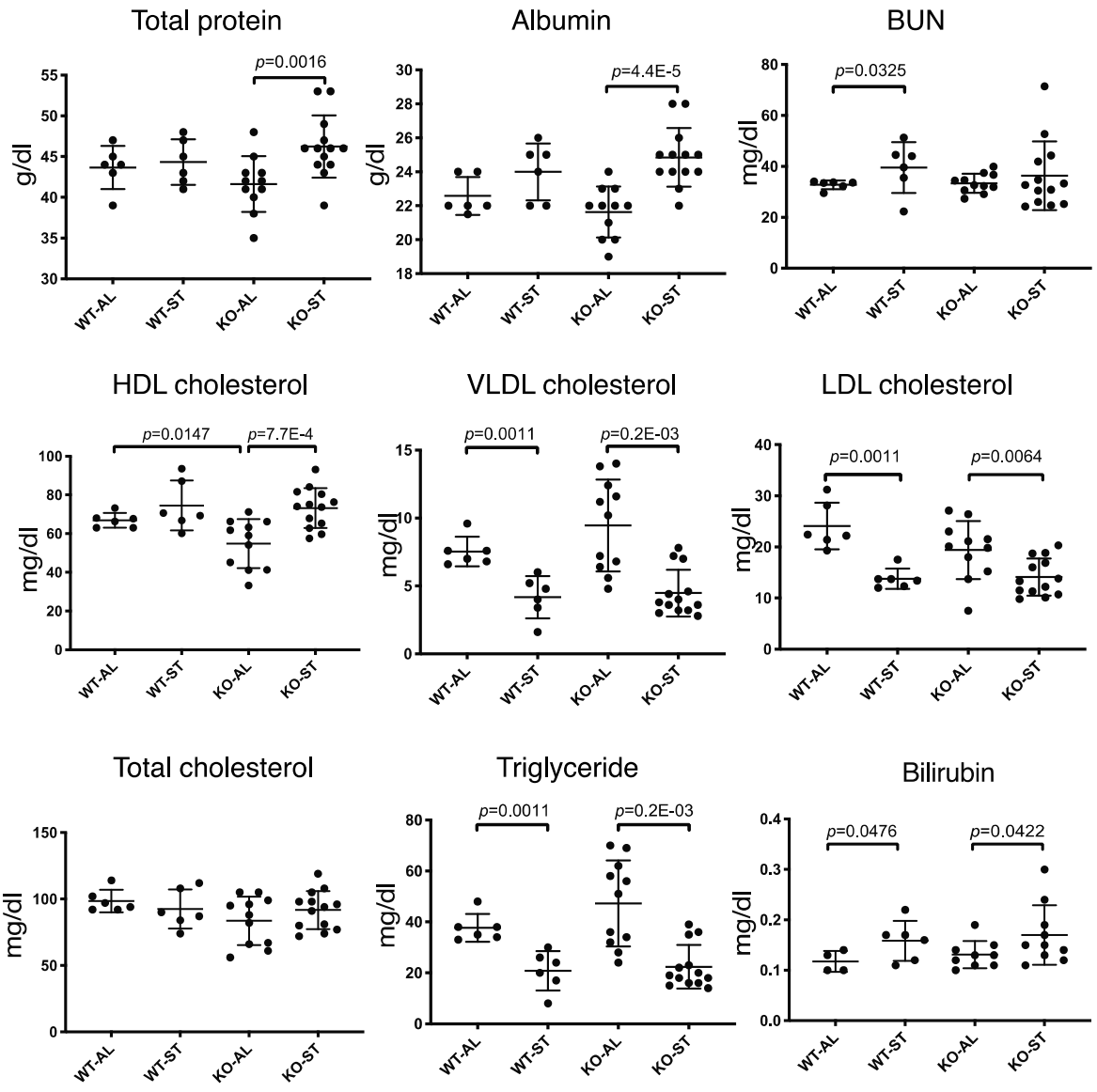


Figure 8. Upon 36h starvation, *Fam134b*$^{-/-}$ mice displayed a significant increase in serum albumin and total serum protein levels; but, there was no major difference from wild-type animals in urea, lipoproteins, lipids and bilirubin levels.

The animals tested for serum biochemistry were average 18 months old and the groups are as follows: *Fam134b*$^{+/+}$*-ad libitum* (WT-AL), n=6 (4 male, 2 female); *Fam134b*$^{+/+}$ Starvation (WT-ST), n=6 (4 male, 2 female); *Fam134b*$^{-/-}$*-Ad libitum* (KO-AL), n=11 (8 male, 3 female); *Fam134b*$^{-/-}$- Starvation (KO-ST), n=13, (9

male, 4 female). BUN, Blood Urea Nitrogen; VLDL, Very Low-Density Lipoprotein; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein. Mann-Whitney non-parametric test results are represented as mean and standard deviation. $p<0.05$ was considered as significant. Non-significant results are not indicated.

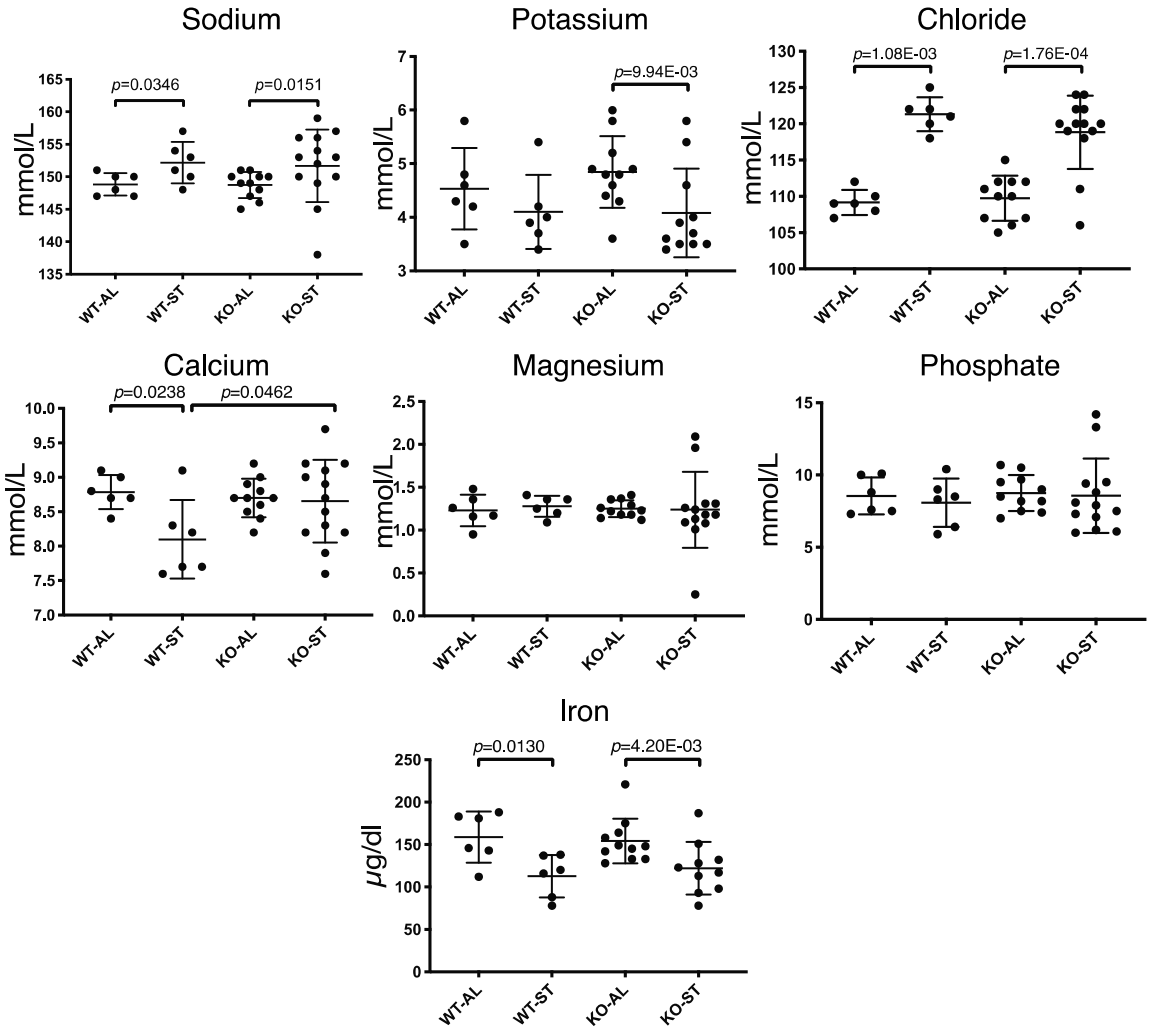


Figure 9. Upon 36h starvation, *Fam134b*$^{-/-}$ mice avoided starvation induced hypocalcemia as tested by serum calcium levels.

The animals tested for serum electrolyte levels were average 18 months old and the groups are as follows: *Fam134b*$^{+/+}$*-ad libitum* (WT-AL), n=6 (4 male, 2 female); *Fam134b*$^{+/+}$ Starvation (WT-ST), n=6 (4 male, 2 female); *Fam134b*$^{-/-}$*-Ad libitum* (KO-AL), n=11 (8 male, 3 female); *Fam134b*$^{-/-}$- Starvation (KO-ST), n=13, (9 male, 4 female). Mann-Whitney non-parametric test results are represented as mean and standard deviation. $p<0.05$ was considered as significant. Non-significant results are not indicated.

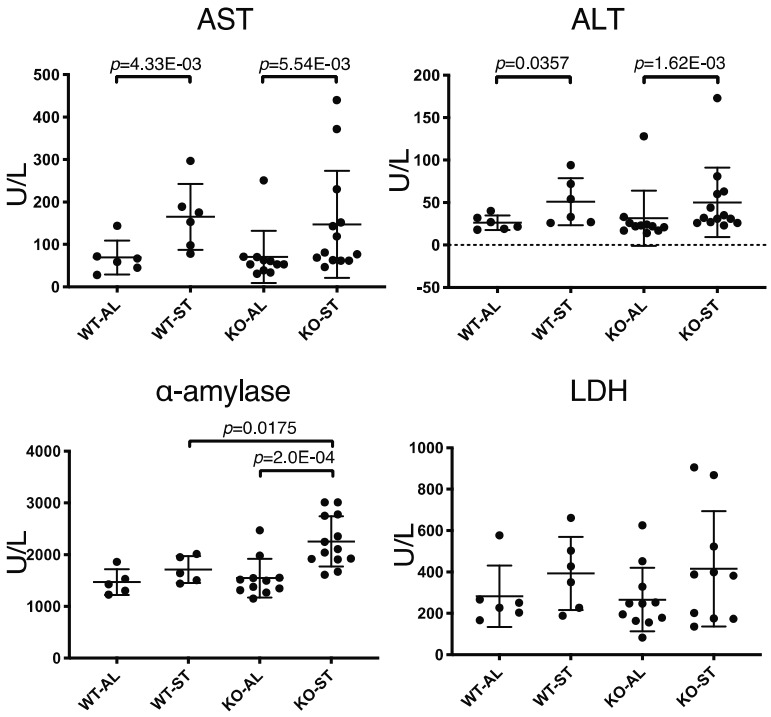


Figure 10. *Fam134b*$^{-/-}$ mice have an elevated serum α-amylase after 36h starvation.

The animals tested for serum enzyme levels were average 18 months old and the groups are as follows: *Fam134b*$^{+/+}$*-ad libitum* (WT-AL), n=6 (4 male, 2 female); *Fam134b*$^{+/+}$ Starvation (WT-ST), n=6 (4 male, 2 female); *Fam134b*$^{-/-}$*-Ad libitum* (KO-AL), n=11 (8 male, 3 female); *Fam134b*$^{-/-}$- Starvation (KO-ST), n=13, (9 male, 4 female). AST, aspartate aminotransferase; ALT, Alanine aminotransferase; LDH, lactate dehydrogenase. Mann-Whitney non-parametric test results are represented as mean and standard deviation. $p<0.05$ was considered as significant. Non-significant results are not indicated.

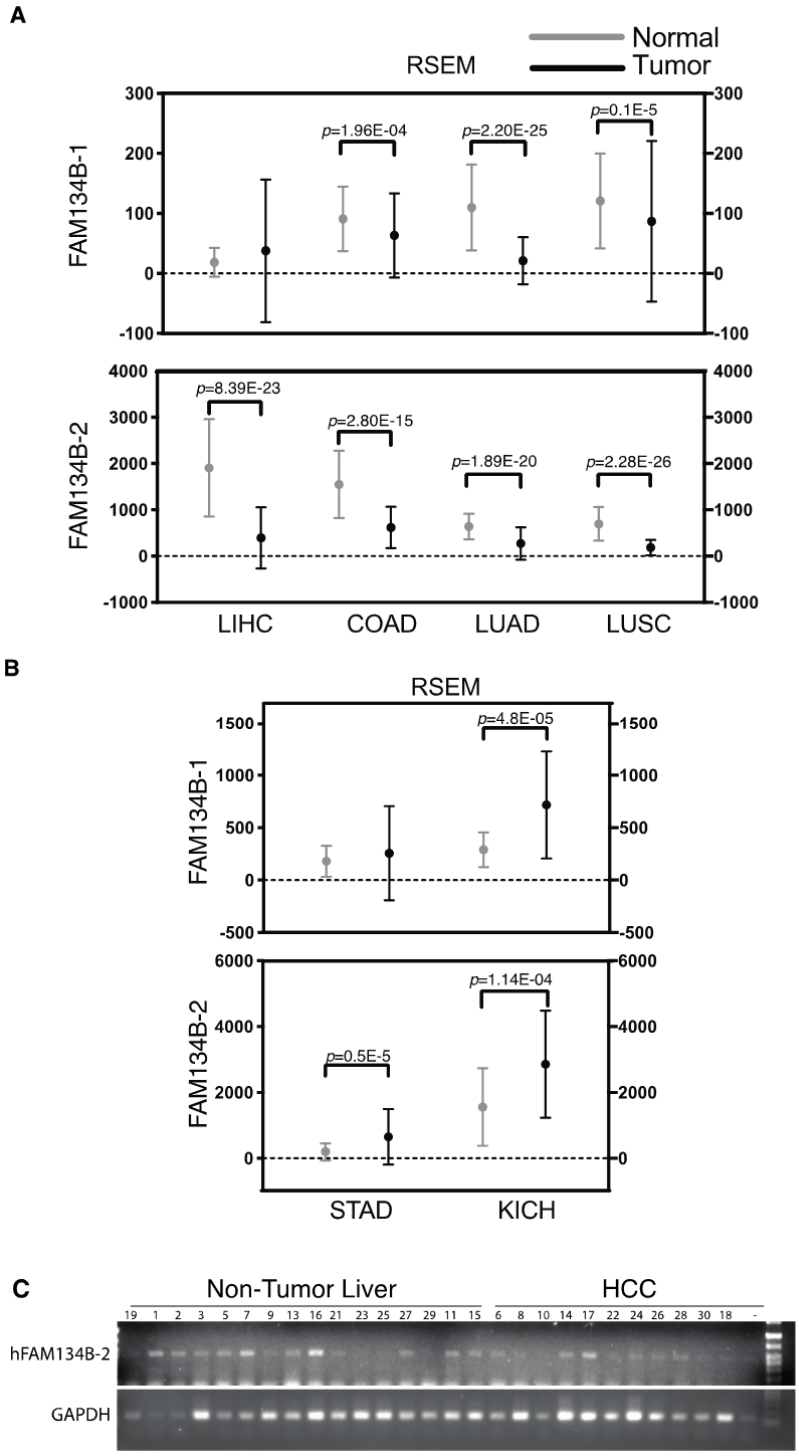
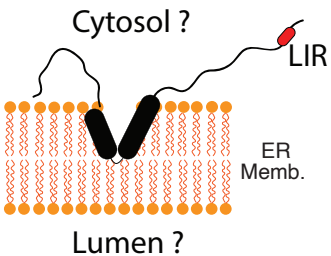


Figure 11. Significant changes in the expression of FAM134B-1 and FAM134B-2 respectively in different cancers.

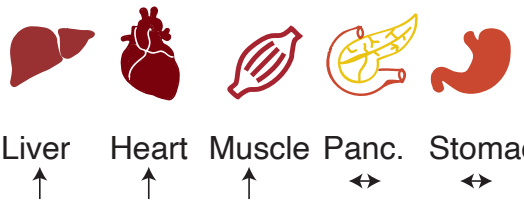
(A) Whisker plot of human *FAM134B* isoform levels in hepatocellular carcinoma (LIHC), colon adenocarcinoma (COAD), lung adenocarcinoma (LUAD) and lung squamous carcinoma (LUSC). (B) Whisker plot of human *FAM134B* isoform levels in stomach adenocarcinoma (STAD) and kidney chromophobe cancer (KICH). In Figure A and B, mean and standard deviation of RNA levels were represented as RSEM values that have been calculated using the data available at the TSVdb database. *p* values equal to 0.01 or more were considered as nonsignificant and not shown on the figure. (C) Confirmation of FAM134B-2 expression in hepatocellular carcinoma (HCC). Lanes 1-3 represent healthy liver samples, lanes 5, 7, 9, 13, 16, 21, 23, 25, 27 and 29 are cirrhotic liver, and lanes 6, 8, 10, 14, 17, 22, 24, 26, 28 and 30 are HCC samples paired with cirrhosis samples in the same order. Total RNA extracted from respective tissues was converted to cDNA and amplified by PCR, using selective primers for FAM134B-1 and FAM134B-2 respectively. GAPDH was used as control.

FAM134B-2 protein

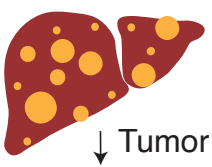


FAM134B-2 expression:

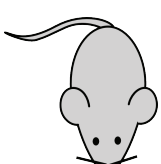
Nutrient Starvation:



HCC development:



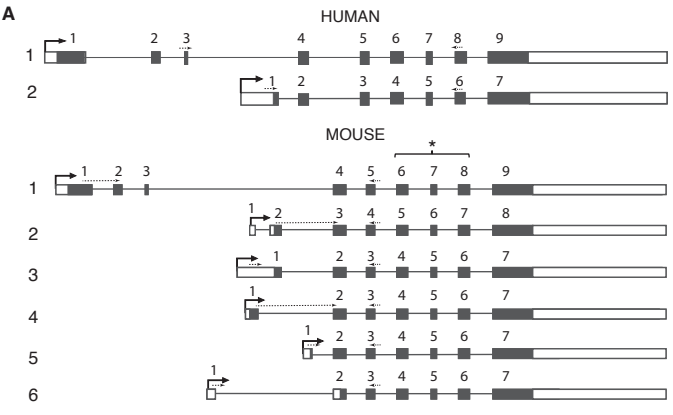
***Fam134b*$^{-/-}$ vs *Fam134b*$^{+/+}$**

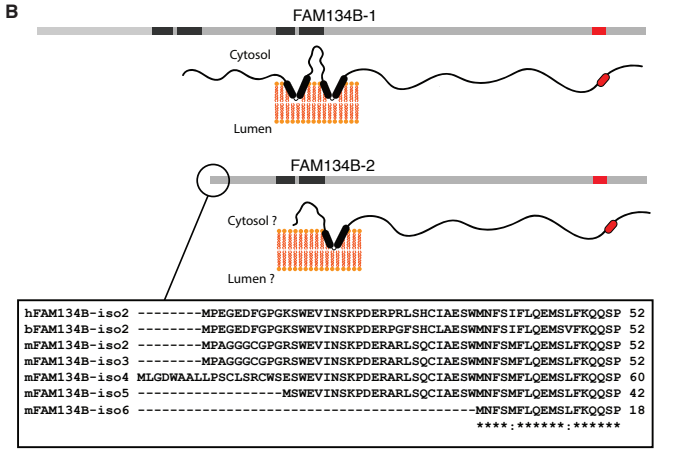


Fed: *Lower serum HDL

Starved:

*Decreased weight loss
*Lower hypocalcemic response
*Higher serum alpha-amylase
*Higher total serum albumin
*Disrupted amino acid release



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mFAM134B-iso2 --------MPAGGGCGPGRSWEVINSKPDERARLSQCIAESWMNFSMFLQEMSLFKQQSP 52
mFAM134B-iso3 --------MPAGGGCGPGRSWEVINSKPDERARLSQCIAESWMNFSMFLQEMSLFKQQSP 52
mFAM134B-iso4 MLGDWAALLPSCLSRCWSESWEVINSKPDERARLSQCIAESWMNFSMFLQEMSLFKQQSP 60
mFAM134B-iso5 ------------------MSWEVINSKPDERARLSQCIAESWMNFSMFLQEMSLFKQQSP 42
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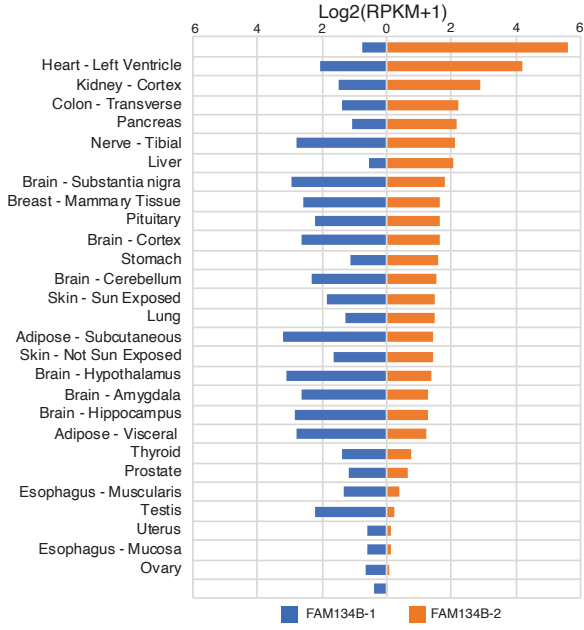
A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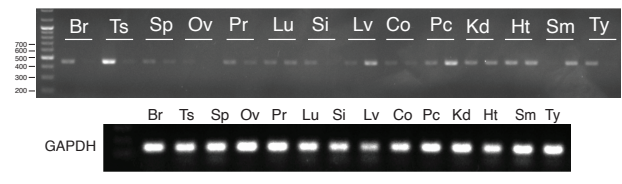
4
2
0
2
4
6
Heart - Left Ventricle
Kidney - Cortex
Colon - Transverse
Pancreas
Nerve - Tibial
Liver
Brain - Substantia nigra
Breast - Mammary Tissue
Pituitary
Brain - Cortex
Stomach
Brain - Cerebellum
Skin - Sun Exposed
Lung
Adipose - Subcutaneous
Skin - Not Sun Exposed
Brain - Hypothalamus
Brain - Amygdala
Brain - Hippocampus
Adipose - Visceral
Thyroid
Prostate
Esophagus - Muscularis
Testis
Uterus
Esophagus - Mucosa
Ovary
FAM134B-1
FAM134B-2

B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Sp
Ov
Pr
Lu
Si
Lv
Co
Pc
Kd
Ht
Sm
Ty
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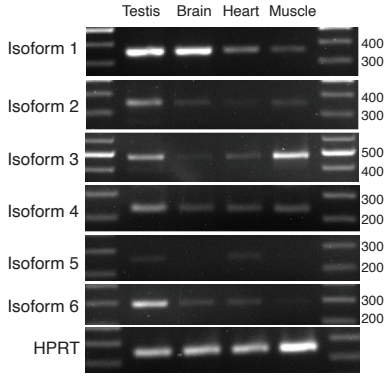
500
400
300
200

Br Ts Sp Ov Pr Lu Si Lv Co Pc Kd Ht Sm Ty
GAPDH

A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Brain
Heart
Muscle
Isoform 1
Isoform 2
Isoform 3
Isoform 4
Isoform 5
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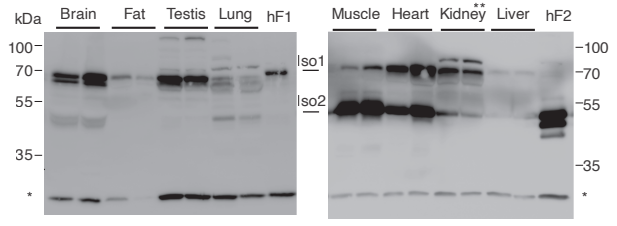
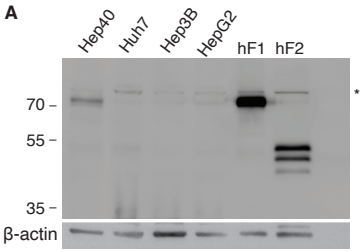
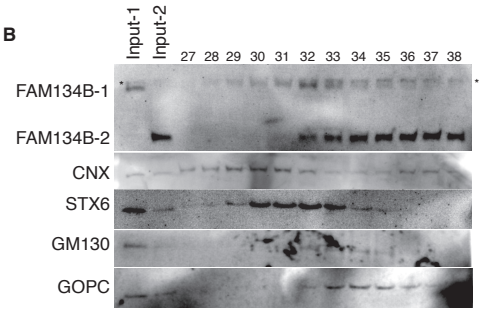
200
300
200

B


kDa
Brain
Fat
Testis
Lung
hF1
Muscle
Heart
Kidney
Liver
hF2
100
70
55
35
*
Iso1
Iso2
**


A
Hep40
Huh7
Hep3B
HepG2
hF1
hF2
70
55
35
β-actin
*


B
Input-1
Input-2
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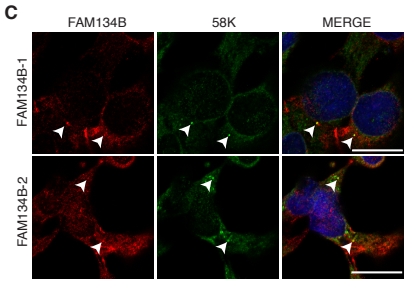
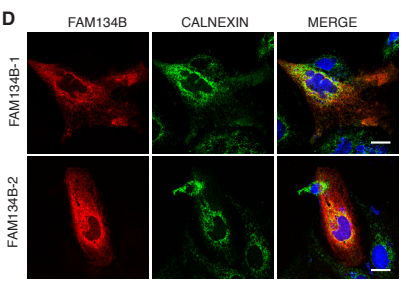
FAM134B-1
FAM134B-2
CNX
STX6
GM130
GOPC
*


C
FAM134B
58K
MERGE
FAM134B-1
FAM134B-2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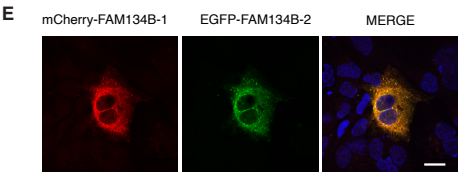
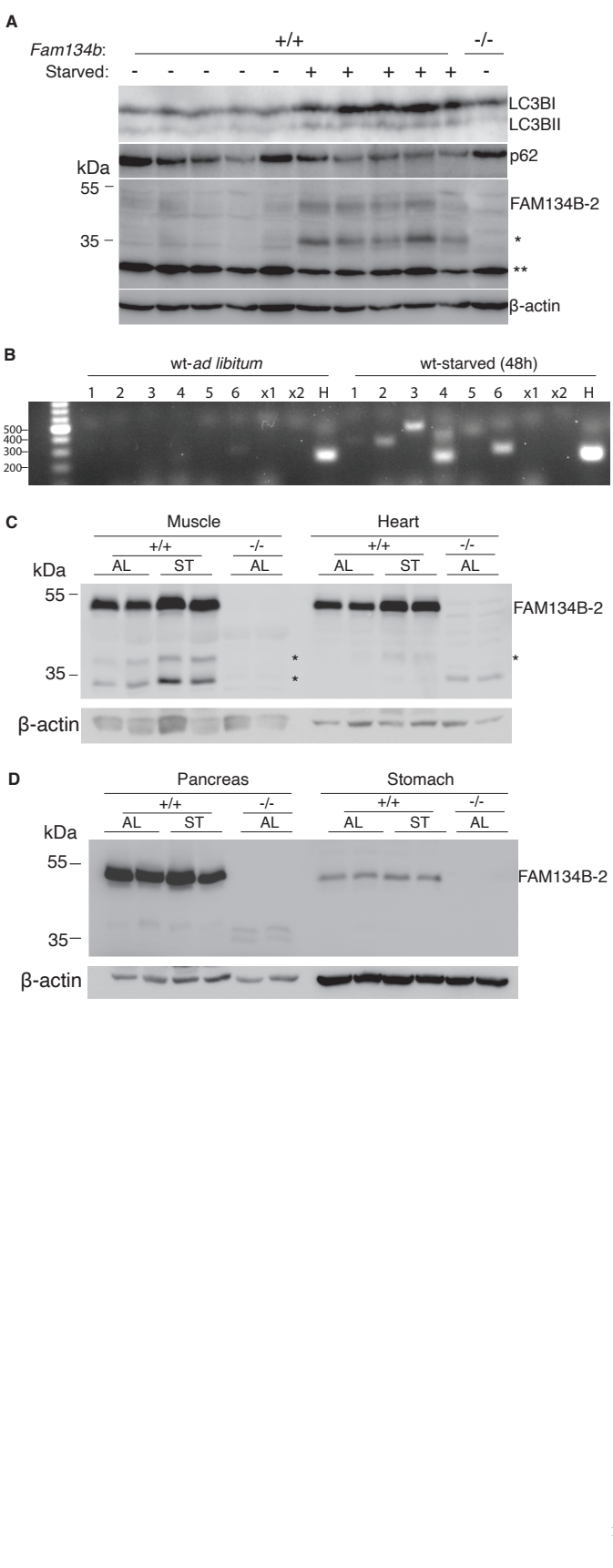
FAM134B
CALNEXIN
MERGE
FAM134B-1
FAM134B-2


E
mCherry-FAM134B-1
EGFP-FAM134B-2
MERGE


A
Fam134b:
+/+
-/-
Starved:
- - - - - + + + + + -
LC3BI
LC3BII
kDa
p62
55
FAM134B-2
35
*
**
β-actin
B
wt-ad libitum
wt-starved (48h)
1 2 3 4 5 6 x1 x2 H 1 2 3 4 5 6 x1 x2 H
500
400
300
200
C
Muscle
Heart
+/+
-/-
kDa
AL ST AL AL ST AL
55
FAM134B-2
35
*
β-actin
D
Pancreas
Stomach
+/+
-/-
kDa
AL ST AL AL ST AL
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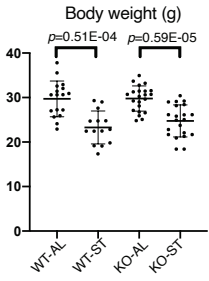
FAM134B-2
35
β-actin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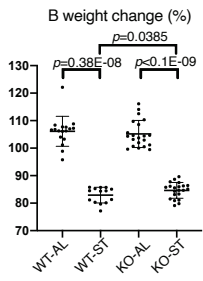
p=0.59E-05
40
30
20
10
0
WT-AL
WT-ST
KO-AL
KO-ST


B weight change (%)
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p=0.38E-08
p<0.1E-09
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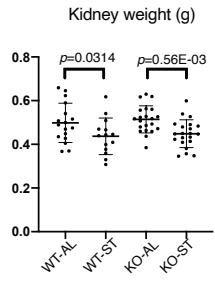
120
110
100
90
80
70
WT-AL
WT-ST
KO-AL
KO-ST


Kidney weight (g)
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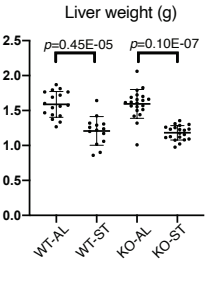
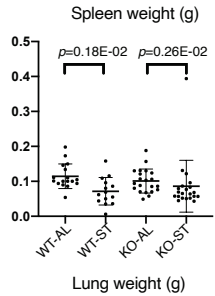
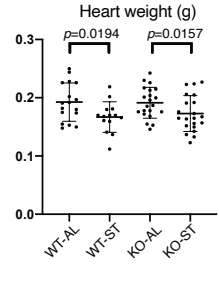
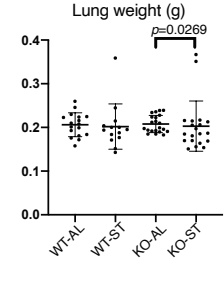
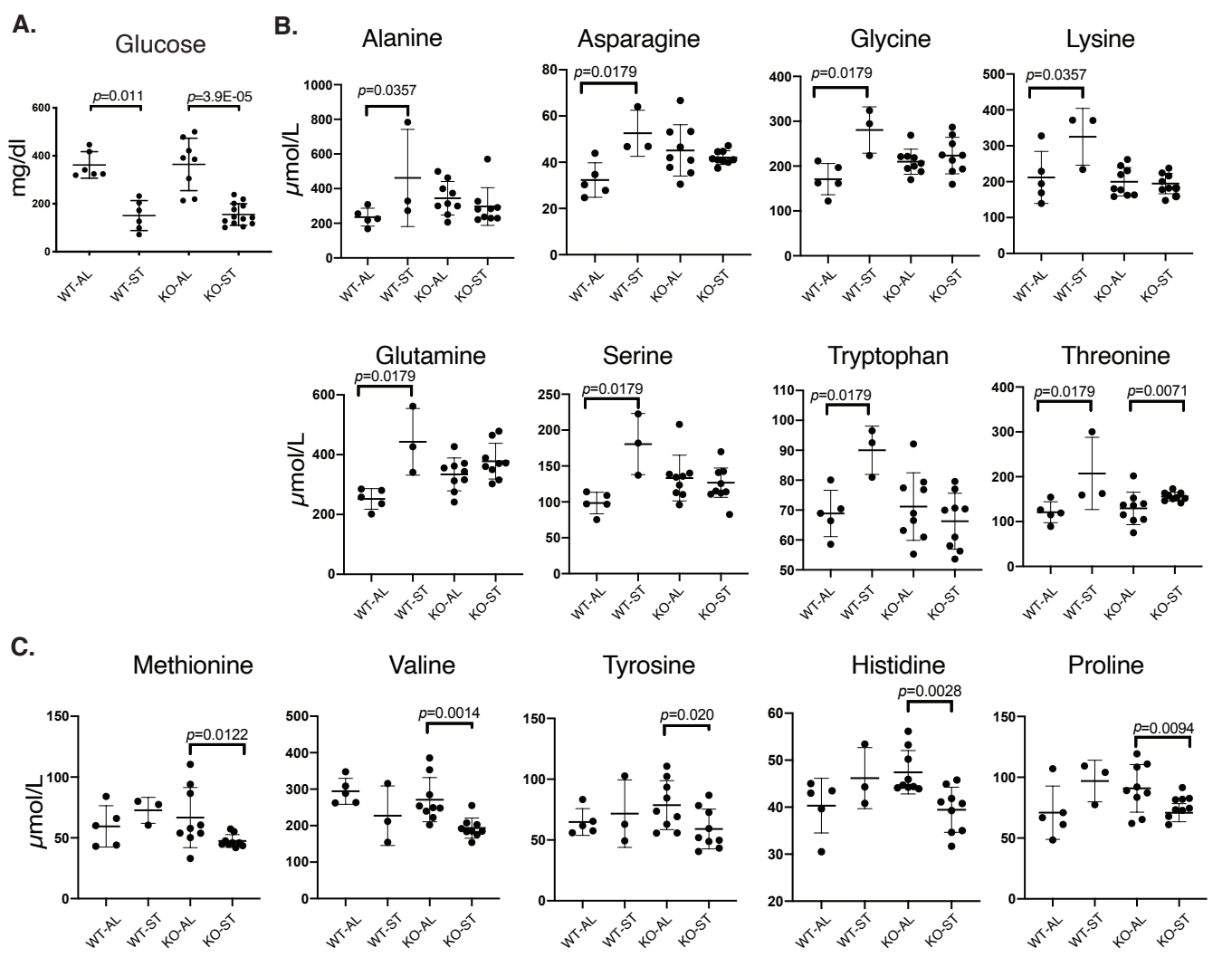
p=0.56E-03
0.8
0.6
0.4
0.2
0.0
WT-AL
WT-ST
KO-AL
KO-ST

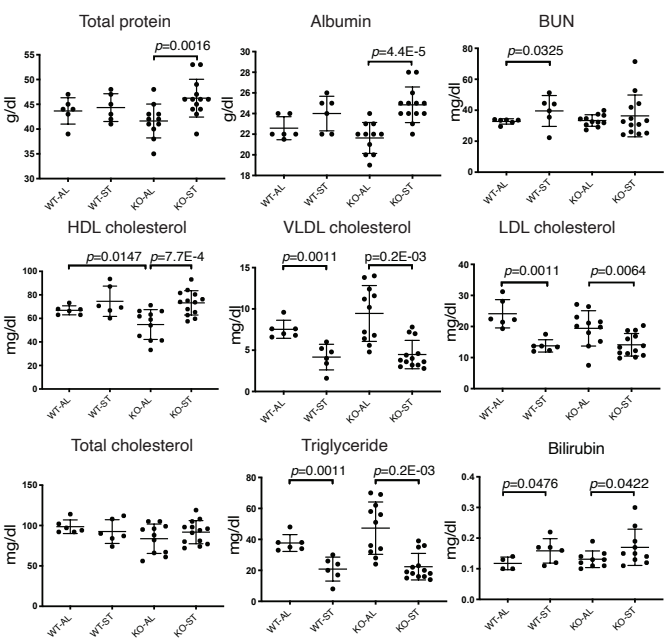

Liver weight (g)
p=0.45E-05
p=0.10E-07
2.5
2.0
1.5
1.0
0.5
0.0
WT-AL
WT-ST
KO-AL
KO-ST

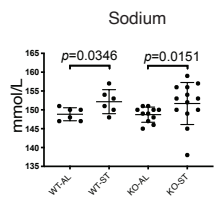

Spleen weight (g)
p=0.18E-02
p=0.26E-02
0.5
0.4
0.3
0.2
0.1
0.0
WT-AL
WT-ST
KO-AL
KO-ST

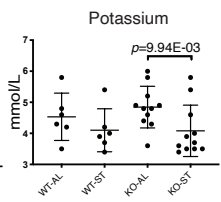

Heart weight (g)
p=0.0194
p=0.0157
0.3
0.2
0.1
0.0
WT-AL
WT-ST
KO-AL
KO-ST

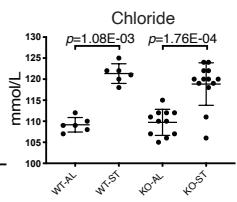

Lung weight (g)
p=0.0269
0.4
0.3
0.2
0.1
0.0
WT-AL
WT-ST
KO-AL
KO-ST

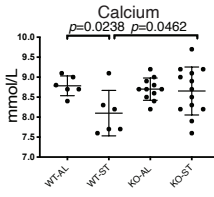

A.
Glucose
mg/dl
p=0.011
p=3.9E-05
B.
Alanine
Asparagine
Glycine
Lysine
Glutamine
Serine
Tryptophan
Threonine
µmol/L
p=0.0357
p=0.0179
p=0.0357
p=0.0179
p=0.0179
p=0.0179
p=0.0179
p=0.0071
C.
Methionine
Valine
Tyrosine
Histidine
Proline
µmol/L
p=0.0122
p=0.0014
p=0.020
p=0.0028
p=0.0094
WT-AL
WT-ST
KO-AL
KO-ST

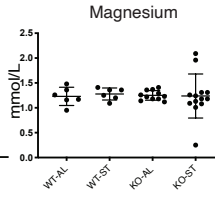

Total protein
p=0.0016
g/dl
55
50
45
40
35
30
WT-AL
WT-ST
KO-AL
KO-ST
Albumin
p=4.4E-5
g/dl
30
28
26
24
22
20
18
BUN
p=0.0325
mg/dl
80
60
40
20
0
HDL cholesterol
p=0.0147
p=7.7E-4
mg/dl
100
80
60
40
20
0
VLDL cholesterol
p=0.0011
p=0.2E-03
mg/dl
15
10
5
0
LDL cholesterol
p=0.0011
p=0.0064
mg/dl
40
30
20
10
0
Total cholesterol
mg/dl
150
100
50
0
Triglyceride
p=0.0011
p=0.2E-03
mg/dl
80
60
40
20
0
Bilirubin
p=0.0476
p=0.0422
mg/dl
0.4
0.3
0.2
0.1
0.0

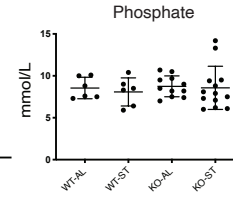

Sodium
p=0.0346
p=0.0151
mmol/L
165
160
155
150
145
140
135
WT-AL
WT-ST
KO-AL
KO-ST

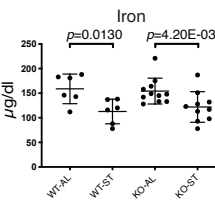

Potassium
p=9.94E-03
mmol/L
7
6
5
4
3
WT-AL
WT-ST
KO-AL
KO-ST

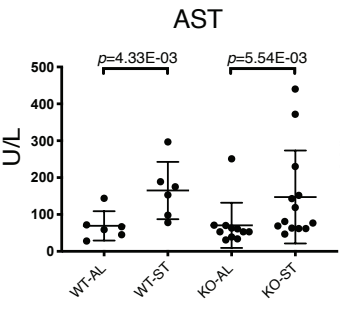

Chloride
p=1.08E-03
p=1.76E-04
mmol/L
130
125
120
115
110
105
100
WT-AL
WT-ST
KO-AL
KO-ST

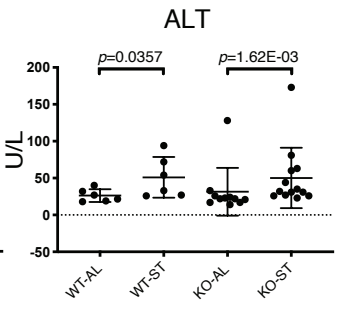

Calcium
p=0.0238
p=0.0462
mmol/L
10.0
9.5
9.0
8.5
8.0
7.5
7.0
WT-AL
WT-ST
KO-AL
KO-ST

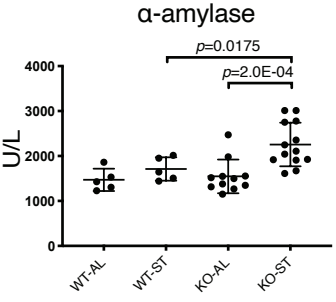

Magnesium
mmol/L
2.5
2.0
1.5
1.0
0.5
0.0
WT-AL
WT-ST
KO-AL
KO-ST

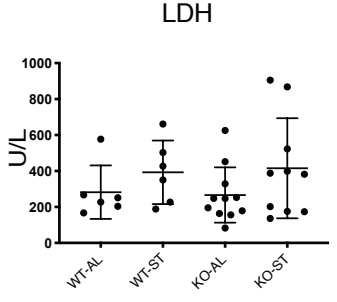

Phosphate
mmol/L
15
10
5
0
WT-AL
WT-ST
KO-AL
KO-ST

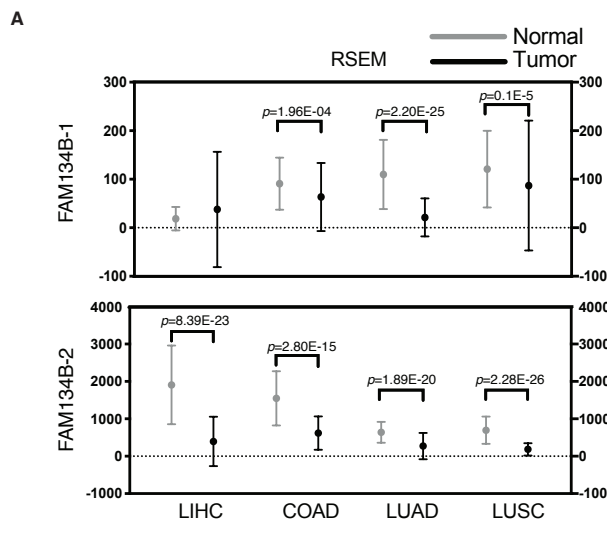

Iron
p=0.0130
p=4.20E-03
μg/dl
250
200
150
100
50
0
WT-AL
WT-ST
KO-AL
KO-ST

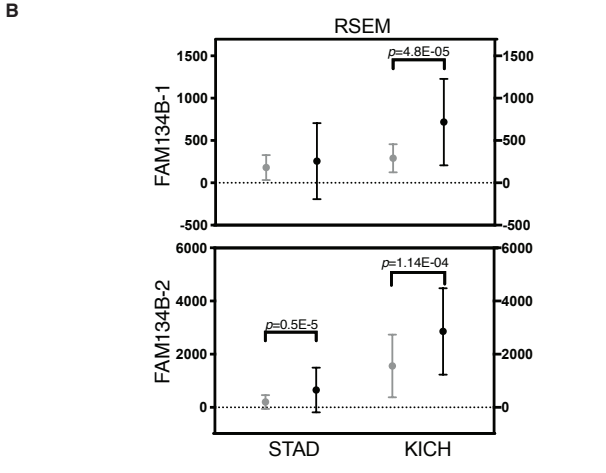

AST
p=4.33E-03
p=5.54E-03
U/L
500
400
300
200
100
0
WT-AL
WT-ST
KO-AL
KO-ST

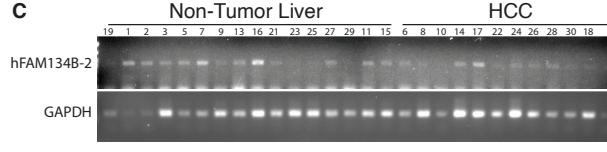

ALT
p=0.0357
p=1.62E-03
U/L
200
150
100
50
0
-50
WT-AL
WT-ST
KO-AL
KO-ST


α-amylase
p=0.0175
p=2.0E-04
U/L
4000
3000
2000
1000
0
WT-AL
WT-ST
KO-AL
KO-ST


LDH
U/L
1000
800
600
400
200
0
WT-AL
WT-ST
KO-AL
KO-ST


A
RSEM
Normal
Tumor
FAM134B-1
FAM134B-2
p=1.96E-04
p=2.20E-25
p=0.1E-5
p=8.39E-23
p=2.80E-15
p=1.89E-20
p=2.28E-26
LIHC
COAD
LUAD
LUSC


B
RSEM
FAM134B-1
FAM134B-2
p=4.8E-05
p=1.14E-04
p=0.5E-5
STAD
KICH


C
Non-Tumor Liver
HCC
hFAM134B-2
GAPDH