Conditional (intestinal-specific) knockout of the riboflavin transporter-3 (RFVT-3) impairs riboflavin absorption

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Running Title: Role of RFVT-3 in intestinal riboflavin transport.

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Abstract

Riboflavin (RF) is indispensable for normal cell metabolism, proliferation, and growth. The RFVT-3 protein (product of the \textit{Slc52a3} gene) is expressed in the gut with the expression being restricted to the apical membrane domain of the polarized intestinal epithelial cells. The relative contribution of RFVT-3 to total carrier-mediated RF uptake in the native intestine, however, is not clear. We addressed this issue in the current investigation using a conditional (intestinal-specific) RFVT-3 knockout (cKO) mouse model developed by Cre/Lox approach. All RFVT-3 cKO mice were found to be RF deficient and showed a significant growth and development retardation; also, nearly two-thirds of them died prematurely between the age of 6 and 12 weeks. \textit{In vivo} (intestinal and colonic loops) and \textit{in vitro} (native isolated intestinal epithelial cells) uptake studies showed a severe inhibition in carrier-mediated RF uptake in the cKO mice compared to control litter-mates. We also observed a significant increase in level of expression of oxidative-stress responsive genes in the intestine of the cKO mice compared to control litter-mates. Supplementation of the RFVT-3 cKO mice with pharmacological doses of RF led to a complete correction of the growth retardation and to normalization in the level of expression of the oxidative-stress responsive genes in the gut. These results show, for the first time, that the RFVT-3 system is the main transporter involved in carrier-mediated RF uptake in the native mouse small and large intestine, and that its dysfunction impairs normal RF body homeostasis.

Key words: Riboflavin, transporter, knockout, intestine
Introduction

The water-soluble vitamin riboflavin (RF) is required for normal cellular metabolism, proliferation, and growth. In the biologically active forms (flavin mononucleotide and flavin adenine dinucleotide), RF plays a key metabolic role in the transfer of electrons in biological oxidation-reduction reactions involving carbohydrate, lipid, amino acid, and certain water-soluble vitamins (pyridoxine and folate) (3, 32). Studies have also shown a role for RF in protein folding within the endoplasmic reticulum (38). More recent investigations have demonstrated anti-oxidant and anti-inflammatory properties for RF (13, 20, 33, 35), and further showed a role for the vitamin in normal immune function (23, 34). RF deficiency leads to a variety of abnormalities including degenerative changes of the nervous system, anemia, skin lesions, cataract, and growth retardation (28, 32); RF deficiency also leads to an increase in the susceptibility to cancer (24). Deficiency/suboptimal levels of this micronutrient occur in chronic alcoholism, diabetes mellitus, inflammatory bowel disease, Brown-Vialetto Van Laere (BVVL) and Fazio Londe syndromes (the latter are neurological disorders caused by mutations in RFVT-2 and -3; refs. 1, 2, 4, 5, 9, 10, 14, 16, 17, 19, 29).

Mammals cannot synthesize RF endogenously. Therefore, they must obtain the vitamin from exogenous sources via intestinal absorption. Two sources of RF are available to the intestine: a dietary source and a bacterial source [the latter is in regard to the vitamin produced by the microbiota of the large intestine; refs. 12, 15, 40]. Previous studies from our laboratory and others have shown that small and large intestinal RF uptake is specific and carrier-mediated in nature (30-32). The recently cloned RF transporters -2 & -3 (RFVT-2 and -3; products of the \textit{SLC52A2} & \textit{SLC52A3} genes, respectively; refs. 6, 41, 42) are both expressed in the gut with expression of RFVT-3 being significantly higher than that of RFVT-2 (36, 42); the former is also
more efficient in transporting RF than the latter (36). Also, live cell confocal imaging studies of polarized intestinal epithelial cells have shown that RFVT-3 is predominantly expressed at the apical membrane domain, while RFVT-2 is mostly expressed at the basolateral membrane domain (BLM) of these cells (36).

To date, however, the relative contribution of the RFVT-3 system to total carrier-mediated RF uptake in the native intestine \textit{in vivo} is not well defined. Our recent \textit{in vitro} study utilizing the intestinal Caco-2 cell line and \textit{SLC52A3} gene-specific siRNA approach showed an important role for RFVT-3 in RF uptake (36). The aim of this study was to define the role of RFVT-3 in RF uptake in native intestine \textit{in vivo}, and for that we have generated a conditional (intestinal-specific) RFVT-3 cKO mouse model. Our results showed that the RFVT-3 system is indeed the main transporter involved in RF uptake in the native small and large intestine, and that dysfunction of this system negatively impacts RF body homeostasis.

\textbf{Material and Methods}

\textit{Materials-} \textsuperscript{[\textit{3}H]}-RF and \textsuperscript{[\textit{3}H]}-biotin (specific activity 21.2 Ci/mmol and 60 Ci/mmol, respectively; radiochemical purity > 97% for both) were purchased from Moravek Biochemicals (Brea, CA) and American Radiolabeled Chemicals Inc. (St Louis, MO), respectively. Anti-RFVT-3 polyclonal and anti-\(\beta\)-actin monoclonal antibodies were obtained from Gene Tex, Inc., (Irvine, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-rabbit IRDye-800 and anti-mouse IRDye-680 antibodies were purchased from LI-COR Bioscience (Lincoln, NE). All chemicals and reagents were purchased from commercial vendors and were of analytical/molecular biology grade.

\textit{Generation of conditional (intestinal-specific) RFVT-3 cKO mice -} A 11.88 kb region of the \textit{Slc52a3} gene was subcloned from a C57BL/6 BAC clone (RP23:424F10) using a homologous
recombination-based technique (*inGenious* Targeting Laboratory Inc, Ronkonkoma, NY). The region was designed such that the 5' homology arm extends 6.55 kb to a single LoxP site. The 3' homology arm extends 2.61 kb 3' to a LoxP/FRT-flanked Neo cassette. A single LoxP site was inserted upstream of exon 2 in intron 1-2, and the LoxP/FRT-flanked Neo cassette was inserted downstream of exon 3 in intron 3-4. The size of the target region is 2.72 kb containing exons 2 and 3 (Fig. 1A).

*Generation of the homozygous LoxP+/+ mice:*

i) **Identification of somatic Neo-deleted mice:** Targeted iTL BA1 (129/SvEv x C57BL/6N) hybrid embryonic stem cells were microinjected into C57BL/6 blastocytes. Resulting chimeras with a high percentage agouti coat color were mated to C57BL/6 FLP mice to remove the Neo cassette. The Neo-deletion was confirmed by PCR using forward primer 5'-GTTGGTGATATGGTCTTCCAGATG-3' and reverse primer 5'-CCAGGTGGGTCTGTGTTTGTAG-3'. Subsequently, a primer set (forward 5'-CACTGATATTGTAAGTAGTTTGC-3' and reverse 5'-CTAGTGCGAAGTAGTGATCAGG-3') was used to screen mice for presence of the FLP transgene. Additionally, a PCR was performed to detect the presence of the distal LoxP site using a forward primer 5'-TACTCTGTACAAGGAGGAGCCCTC-3' and a reverse primer 5'-CAGATGATAACCTGGGAAAGATAGGG-3'. Finally the 3' homology arm integration was confirmed by PCR using forward primer 5'-TCACCAGGCTTCTTGTGTGTACAC-3' and reverse primer 5'-GCTGATCGGATCCGGTGTTCTTGAGGAC-3'.

ii) **Identification of germline Neo-deleted mice:** The confirmed somatic Neo-deleted male mice were mated with C57BL/6N wild-type female mice to generate germline Neo-deleted mice.
Resulting pups were genotyped for Neo-deletion and confirmed heterozygote mice (Slc52a3 LoxP+/−) for germline Neo-deletion and FLP absence.

**iii) Identification of homozygous germline Neo-deleted mice:** The confirmed germline Neo-deleted heterozygote mice (Slc52a3 Lox+/−) were mated to generate homozygous (Slc52a3) germline Neo-deleted mice. Resulting pups were genotyped for Neo-deletion as described above and confirmed homozygote (Slc52a3 LoxP+/+).

The generated homozygous mice (Slc53a3 LoxP+/+) were mated with mice expressing intestinal specific Cre-recombinase (The Jackson Laboratory) under the control of the villin-promoter. The villin gene is predominantly expressed in intestinal epithelial cells of the small bowel and the proximal portion of the large bowel (22). DNA isolated from ear samples of the offspring were genotyped for LoxP+/+ sites and the presence of the Cre-recombinase using specific primers (For Slc52a3 LoxP+/+, the forward 5'-TACTCTGTACAAGGAGGAGCCCTC-3', the reverse 5'-GTTCACAGATAACCTTTAACT-3'; For Cre, the forward 5'-GTGTGGGACAGAGAACAAACC-3', the reverse 5'-ACATCTTCAGGTTCTGCGGG-3'). Deletion of exons 2 and 3 in the small bowel by villin promoter driven Cre-recombinase expression was confirmed by PCR using intestinal mRNA. All breeding and animal studies were approved by the Long Beach VA Medical Center Institutional Animal Care and Use Committee (IACUC).

**3H-RF uptake in vivo by jejunal/colonic loops, and in vitro by isolated native mouse intestinal epithelial cells** - Jejunal/colonic loops (~1cm) were used for RF uptake experiments in vivo as described previously (7, 27). Briefly, the jejunal/colonic loops were filled with 100 µl of Krebs-Ringer (K-R) buffer (pH 7.4) containing 3H-RF (or 3H-biotin) with or without unlabeled 1 mM...
RF (or 1mM biotin). Uptake measurement was performed after 5 min (39, 43) and expressed in fmol/mg protein/5 min.

Jejunal epithelial cells from adult (6-12 weeks) cKO mice and their wild-type litter-mates were prepared for transport studies as described before (7, 27). Briefly, mice were sacrificed by cervical dislocation, their jejunum removed, stool contents washed out, and then incubated in Hank’s balanced salt solution supplemented with 0.5 mM DTT and 1.5 mM EDTA at 37°C water bath for 15 min. A suspension of enterocytes was transferred to 15 ml falcon tubes, centrifuged (1,500 g for 3 min), and the cells were re-suspended in K-R buffer (pH 7.4). Enterocyte viability was checked by the Trypan blue exclusion method and found to be greater than 87%. ³H-RF uptake was determined by rapid filtration method (11). Protein concentrations were determined using Bio-Rad kit (Hercules, CA).

Quantitative RT-PCR - One microgram of total RNA was treated with DNaseI and cDNA was synthesized using i-Script kit (Bio-Rad). RT-qPCR was performed using the CFX96 real-time i-Cycler (Bio-Rad) and SYBR green PCR kit (Bio-Rad). Primers used were specific for mouse RFVT-3 (forward 5'-GGATCAGTGGAAGCCAGTG-3’ and reverse 5’-GACCTGTTAGGCAGGAAGCAGTG-3’), mouse RFVT-2 (forward 5’-TGCTGGCCATCACCAA-3’ and reverse 5’-GCCAGAGACCTGCACA-3’). Additionally, RT-qPCR for stress response genes GPX1 (forward 5’-CTCTTTACCTTCTCTGCAGGAA-3’ and reverse 5’-GGACAGCAGGGTTTCTATGT-3’), SOD1 (forward 5’-GATGACTTGGCAAAGGTGG-3’ and reverse 5’-CTGCGCAATCCCATCACTC-3’), and FMO2 (forward 5’-CAGTTTCAGACCACCTGTCA-3’ and reverse 5’-TGTATTTCGCGGTATGGA-3’) was performed. The housekeeping gene used in all amplifications was mouse β-actin, (forward 5’-ATCCTCTTCCCTCCTGGA-3’ and reverse 5’-GACCTGTTAGGCAGGAAGCAGTG-3’).
TTCATGGATGCCACAGGA-3'). The relative expression was quantified by normalizing Ct values with corresponding β-actin as described before (21). The negative control reactions with respective gene specific primers, PCR mix and without RT product were used in every experiment.

**Western blot analysis:** Villus epithelial cells were isolated from the jejunum of RFVT-3 cKO mice and control litter-mates as described before (25). Cells were lysed in RIPA buffer (Sigma) containing complete protease inhibitor cocktail (Roche, NJ) and the soluble protein fraction was isolated by centrifugation at 12,000 rpm for 10 min. SDS-PAGE of 60 µg of protein was performed on a 4-12% mini gel (Invitrogen). The protein was transferred onto immobilon polyvinylidene difluoride membrane and the blot was blocked in LI-COR blocking buffer. The blot was simultaneously probed overnight with a mouse RFVT-3 antibody raised in rabbits along with a β-actin monoclonal antibody. The blot was washed three times in PBS-Tween 20 and then incubated with anti-rabbit IRDye 800 and anti-mouse IRDye 680 (LI-COR Bioscience) secondary antibodies (1:30,000) for 45 min at room temperature. The specific immunoreactive bands were captured using the Odyssey infrared imaging system (LI-COR Bioscience) and their densities were quantified using the LI-COR software (LI-COR Bioscience).

**Measurement of blood RF levels** - Blood levels of RF in the RFVT-3 cKO mice and sex-matched control litter-mates were determined using glutathione reductase assay kit (Cayman Chemical Company, Ann Arbor, MI) (8). Briefly, blood samples were collected from euthanized mice and the glutathione reductase activity was determined in the red blood cells as described in the manufacturer’s protocol.

**Phenotype assessment** - Body weight, bone density, and other changes were monitored in the RFVT-3 cKO mice and sex-matched wild-type litter-mates. Animals were anesthetized and
placed in a Kodak Image station (Kodak Molecular imaging Systems, New Haven, CT) for performing X-ray imaging analysis. Body weight was determined weekly using Mettler Toledo scale. Estimation of bone density and determination of bone length in X-ray images were performed using Scion Image 4.0.3 (Scion Corp., Frederick, MD).

**Histology** - The RFVT-3 cKO mice and sex-matched wild-type litter-mates were euthanized and the intestine, heart, liver, and kidneys were immediately removed and fixed in 10% formalin for overnight. The tissue samples were sectioned and paraffin embedded. H&E-stained slides were prepared using standard histological techniques (Long Beach Veterans Medical Center Clinical Histology Laboratory). In another study the oriented histology section of the small bowel wall were semi-quantitatively analyzed to measure the average villus height for each animal. Briefly, H&E stained sections were viewed in a conventional Nikon Exclipse 50i microscope equipped with a 10x ocular with a micrometer and a 10x objective. The surface of the bowel mucosa was identified as the junction between crypts and villi, and the height of 5 representative villi was measured in 5 separate 10x fields. The mean and standard deviation was calculated. All groups of data were compared using the Student $t$-test and the difference of the mean with the standard error of the mean calculated. The microscopic analysis was performed by a board-certified anatomic pathologist.

**Statistical Analysis** - All data (uptake, RT-qPCR, and Western blot analysis) presented are means ± SE of at least 3 independent experiments with at least three different sets of animals. Uptake of $^3$H-RF (and $^3$H-biotin) by the carrier-mediated process was determined by subtracting uptake in the presence of 1 mM of unlabeled RF (or biotin) from that in its absence. In all studies, simultaneously performed controls (sex-matched litter-mates) were used. The statistical significance was set at $P < 0.05$, and was calculated using the Student $t$-test.
Results

**Generation and establishment of the conditional (intestinal-specific) RFVT-3 cKO mouse colony**- Recent studies from our laboratory and others have shown that the RFVT-3 is expressed in the gut, and that the expression is restricted to the apical membrane domain of intestinal epithelial cells (36). The contribution of RFVT-3 to total carrier-mediated RF uptake by the native intestine, however, is not known. The mouse is a good animal model to study intestinal RF transport physiology because murine intestine shares similar intestinal RF uptake process with humans. Moreover, murine RFVT-3 shares high degree of sequence homology with the human RFVT-3 (www.Genome.jp/tools/clustlw). Accordingly, we chose this animal to develop a RFVT-3 cKO model and used the cKO mouse to study intestinal RF uptake in vivo. A homozygous mouse (Slc52a3 LoxP<sup>+/+</sup>) was generated (inGenious Targeting Laboratory) (Fig. 1A) and bred with a mouse that expresses the intestinal-specific Cre-recombinase under the control of the villin promoter to obtain an intestinal-specific RFVT-3 cKO mouse. Figure 1B shows the presence of Cre-recombinase and LoxP<sup>+/+</sup> sites in the offspring. We confirmed the intestinal-specific deletion of the Slc52a3 gene at both the mRNA (PCR) in the gut [and its normal expression in other tissues (liver and kidney)] (Fig. 1C & D) and the protein (Western blotting) levels (Fig. 1E). Finally, we did not observe any change in the level of expression of the other RF transporter that is also expressed in the gut, i.e., the RFVT-2, in the intestine of the RFVT-3 cKO mice (Fig. 1F). These findings confirm the intestinal-specific nature of the RFVT-3 cKO and shows that such a knockout does not lead to an induction in the level of expression of RFVT-2 in the gut.

**Phenotype assessment of the RFVT-3 cKO mice**- The phenotype of the RFVT-3 cKO mice showed a remarkable difference compared to sex-matched wild-type litter-mates (Fig. 2). All
RFVT-3 cKO mice showed significant growth retardation, and nearly two-thirds of them died prematurely between the age of 6 and 12 weeks. Also, the cKO animals exhibited lethargic behavior, hunched back posture, and ocular surface abnormalities compared to wild-type litter-mates (Fig. 2A and B). Total body weight of the RFVT-3 cKO animals was significantly (P < 0.01) lower than that of age- and sex-matched wild-type litter-mates (Fig. 2C). In addition, X-ray image analysis of RFVT-3 cKO mice compared to wild-type litter-mates showed a significant (P < 0.001) decrease in bone density (femur and tibia; Fig. 2D) and a significantly (P < 0.001) shorter femur, tibial and pelvic bones (Fig. 2E). However, no histological abnormalities were found in the heart, liver, kidney, jejunum, ileum, cecum, proximal, and distal colon of the RFVT-3 cKO mice (data not shown). In addition, no significant changes in the length of the jejunal crypts and villi of the cKO mice compared to control litter-mates were seen (data not shown). Finally, a significantly (P < 0.01) lower RF blood level (erythrocytes glutathione reductase activity was measured to reflect the level of RF in blood samples; see “Methods”) was found in the RFVT-3 cKO mice compared to their sex-matched litter-mate controls (Fig. 2F).

Effect of loss of RFVT-3 on intestinal RF uptake - We used both an in vivo (gut loops) and an in vitro (isolated intestinal epithelial cells) preparation to examine the effect of knocking out the intestinal RFVT-3 on gut RF uptake. Results were compared to RF uptake by the same preparation from sex-matched litter-mate controls. The results showed RF (0.24 µM) uptake to be severely (P < 0.01) inhibited in jejunal loops of RFVT-3 cKO mice compared to control litter-mates (Fig. 3A). No such inhibition was observed in the uptake of the unrelated vitamin biotin (83 nM) by jejunal loop of RFVT-3 cKO mice when compared to uptake by their control litter-mates (Fig. 3B). The latter finding confirms specificity of the RFVT-3 cKO model. Similarly, RF uptake by epithelial cells freshly isolated from the jejunum of RFVT-3 cKO mice was found
to be significantly (P < 0.01) suppressed compared to uptake by cells from control litter-mates (Fig. 3C) [the residual RF uptake observed in this preparation from the RFVT-3 cKO mice is most likely due to uptake across the exposed basolateral membrane where RFVT-2 is expressed (36)].

Previous studies have shown that the normal microflora of the large intestine synthesizes considerable amount of RF (12, 15, 40) and that colonocytes have an efficient carrier-mediated mechanism for RF uptake (37). Therefore, we also examined carrier-mediated RF (0.24 µM) uptake in vivo in the colon of the RFVT-3 cKO mice and compared to uptake with control litter-mates. The results again showed a significant (P < 0.01) inhibition in carrier-mediated RF uptake in the colon of RFVT-3 cKO mice compared to sex-matched control litter-mates (Fig. 4A). The residual expression of RFVT-3 in the colon (Fig. 4B) is likely due to the fact that the villin expression decrease in the distal portion of the colon (22), which leads to a decrease in Cre expression, and thus, less efficient RFVT-3 gene knockout in that part of the colon (22). No change in level of expression of the RFVT-2 mRNA was found in the colon of RFVT-3 cKO as compared to control litter-mates (2.6 ± 1 and 3.1 ± 0.6 arbitrary units, in wild-type litter-mates and cKO, respectively).

Level of expression of oxidative-stress responsive genes in the intestine of the RFVT-3 cKO mice - Recent studies have shown that RF deficiency leads to oxidative-stress in intestinal epithelial cells (18). Therefore, we examined the effect of loss of RFVT-3 expression in the intestine on level of expression of oxidative-stress responsive genes [flavin containing monooxygenase 2 (FMO2), glutathione peroxidase (GPX1), and superoxide dismutase (SOD1)] in the jejunum; results were compared to those obtained with control litter-mates. The results showed a significant (P < 0.01 for all) increase in level of mRNA expression of FMO2, GPX1,
and SOD1 in jejunal mucosa of RFVT-3 cKO compared to wild-type litter-mates (Fig. 5A-C, respectively). These findings are in line with a state of depletion of RF in intestinal epithelial cells and points to a role for RF in the maintenance of normal intestinal health (18, 26).

Effect of RF supplementation of the RFVT-3 cKO mice on animal phenotype: In this study, we examined the effect of supplementing the RFVT-3 KO mice with pharmacological amounts of RF on the observed growth retardation, changes in bone density and length, and on the ocular surface abnormalities as well as on the early death phenotypes. This was done by providing RF (500 µM) in the drinking water to dams during pregnancy and lactation, and to the RFVT-3 cKO mice throughout their life. The results showed that RF supplementation of the RFVT-3 cKO mice lead to correction of all the above described abnormal phenotypes and that the KO animals grew at a similar rate as their control litter-mates (Fig. 6A and B). In addition, the level of expression of the oxidative-stress responsive genes (FMO2, GPX1, and SOD1) was found to be similar in the jejunum of the RFVT-3 cKO mice and wild-type litter-mates (Fig. 6C-E, respectively).

Discussion

The aim of this study was to develop a better understanding of the role of the recently cloned RFVT-3 in intestinal carrier-mediated absorption process of RF in the native gut in vivo. RF, a member of the B-family of water-soluble vitamins, plays critical roles in normal cellular metabolism and function of humans and other mammals. However, it cannot be synthesized endogenously, and thus, must be obtained from exogenous sources via intestinal absorption. To achieve our aim, we developed a RFVT-3 cKO mouse model utilizing the Cre/Lox approach and verified the near absence of the RFVT-3 protein/mRNA in the gut of the cKO animals compared to their sex-matched wild-type litter-mates. We also showed that expression of RFVT-3 in other
tissues (liver and kidney) is not affected, i.e., similar in the cKO animals and their control litter-mates. Furthermore, we found that knocking out the RFVT-3 system in the intestine does not lead to induction in the expression of RFVT-2, a RF transporter that is also expressed in the gut.

Our results showed that all RFVT-3 cKO mice develop distinct phenotypes that include growth retardation (total body weight was markedly decreased compared to control litter-mates), decreased bone density and length, lethargic behavior, hunched back posture, and ocular surface abnormalities compared to wild-type litter-mates. In addition, their blood RF level was found to be significantly lower than that of control litter-mates, pointing to an important role for the RFVT-3 system in the maintenance of normal RF body homeostasis. Finally, almost two-thirds of the RFVT-3 cKO mice died prematurely between the age of 6 and 12 weeks. The cause of death is not clear but could not be attributed to histological abnormalities in critical organs of the RFVT-3 cKO mice as no such abnormalities were observed in the heart, liver, kidney, and the small and large intestine.

Our transport investigations in the native small intestine (jejunum) of the RFVT-3 cKO mice in vivo showed severe inhibition in carrier-mediated RF uptake compared to uptake by the jejunum of the wild-type litter-mates. In contrast, uptake of the unrelated vitamin biotin was found to be similar in the RFVT-3 cKO mice and to control litter-mates demonstrating substrate specificity of the knockout. Likewise, RF uptake in freshly isolated native jejunal epithelial cells in vitro was severely inhibited in the RFVT-3 cKO mice compared to control litter-mates. Similarly, carrier-mediated RF uptake in the native colon in vivo was found to be severely reduced in the RFVT-3 cKO mice compared to uptake in the colon of wild-type litter-mates. These findings clearly show that RFVT-3 plays an important role in the absorption of both
dietary RF (which takes places in the small intestine) and the microbiota-generated RF (which takes place in the large intestine).

Recent studies have shown that RF deficiency leads to oxidative-stress in intestinal epithelial cells (18, 26). Thus, we investigated the effect of loss of RFVT-3 expression in the intestine on level of expression of the oxidative-stress responsive genes FMO2, GPX1, and SOD1. Our results showed that expression of all these genes to be markedly increased in the jejunal mucosa of the RFVT-3 cKO mice compared to wild-type litter-mates. These data support an important role of RF in maintenance of normal intestinal health (18, 26).

Finally, the results of the effect of RF supplementation [RF (500 µM) was added to the drinking - water of dams during pregnancy and lactation, and to the cKO mice during their entire life span] of the RFVT-3 cKO mice showing reversal of all observed abnormal phenotypes (growth retardation, bone density and length, ocular surface abnormalities and early death) as well as the restoration in the level of expression of oxidative-stress responsive genes clearly demonstrate the importance of RF in overall health, growth and development.

In summary, results of these investigations provide clear evidence for the essential role played by RFVT-3 in carrier-mediated RF uptake in native intestine in vivo, and in the maintenance of normal RF body homeostasis. It is hoped that the development of this mouse model, which allows the generation of tissue- specific deletion of RFVT-3, would serve as a catalyst for further investigations into the role of this recently identified and important RF transporter in the physiology/pathophysiology of other critical tissues.


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Figure legends

Figure 1. Generation of the RFVT-3 cKO mice, genotyping and validation of RFVT-3 loss at the protein and mRNA expression level.

A) Schematic depiction of the homologous recombination to introduce the LoxP site upstream of exon 2 and downstream of exon 3 of the *Slc52a3* gene. A 5′ LoxP recombination site was inserted into intron 1 of the *Slc52a3* gene together with a Neo cassette and a second 3′ LoxP recombination site was introduced into intron 3. Exon 2 and 3 of the *Slc52a3* gene were crossed with transgenic mice expressing the Cre-recombinase in intestinal epithelial cells under the control of villin promoter. B) Genomic PCR analysis of mouse ear DNA samples using two primer pairs to show the presence of LoxP<sup>+/+</sup> sites and Cre-transgenes. C) Loss of RFVT-3 mRNA expression due to conditional deletion of Exon 2 and 3 of *Slc52a3* in the small intestine was confirmed by RT-qPCR. D) Quantitative PCR from reverse-transcribed total RNA of jejunum mucosal scraping, liver and kidney of cKO mice and their sex-matched litter-mates to determine the expression level of RFVT-3 as described in “Methods”. Data are means ± SE of 6-
9 sets of mice. * P < 0.01. E) Expression of RFVT-3 protein in the intestine of the RFVT-3 cKO and control litter-mates. Western blot analysis was performed using specific anti-RFVT-3 polyclonal antibodies as described in “Methods”. Data are means ± SE of at least 3 separate sets of mice. * P < 0.01. F) Quantitative PCR from reverse-transcribed total RNA of jejunum mucosal scraping, liver and kidney of cKO mice and their sex-matched litter-mates to determine the expression level of RFVT-2 as described in “Methods”. Data are means ± SE of 6-9 sets of mice.

**Figure 2. Effect of the RFVT-3 cKO on mouse phenotype (body weight, and bone density and length) and RF blood level.** A) A representative image of a cKO mouse (right) and sex-matched wild-type litter-mate (left) showing ~50% reduction in their body size. B) Representative X-ray image of a cKO mouse (right) and sex-matched wild-type litter-mate (left) showing difference in size and bone length. C) Bar graph showing marked difference in body weight of cKO mice compared to control litter-mates (*P < 0.01). D) Bar graph showing significant decrease in bone density in cKO mice (expressed as % relative to wild-type litter-mates) (** P < 0.001). E) Bar graph showing significant decrease in bone length in cKO mice (expressed as % relative to wild-type litter-mates) (** P < 0.001). F) RF levels in blood of cKO mice and wild-type litter-mates. *P < 0.01. Data are means ± SE of 6 sets of mice.

**Figure 3. Determination of effect and specificity of the RFVT-3 cKO on RF uptake in vivo and in vitro.** A) Carrier-mediated RF uptake by in vivo jejunal loop of cKO mice and sex-matched litter-mates. B) Carrier-mediated biotin uptake by in vivo jejunal loop of RFVT-3 cKO mice and control litter-mates. C) Carrier-mediated RF uptake by in vitro freshly isolated intestinal epithelial cells of cKO mice and wild-type litter-mates. Data are means ± SE of at least 3 separate uptake determinations performed on at least 3 different sets of mice. *P < 0.01.
Figure 4. Effect of the RFVT-3 cKO on carrier-mediated RF uptake in the colon, and on level of mRNA expression of RFVT-3. A) Carrier-mediated RF uptake by in vivo intact colonic loop of cKO mice and their sex-matched litter-mates. B) Quantitative PCR from reverse-transcribed total RNA of colon of cKO mice and their sex-matched litter-mates was performed to determine the expression level of RFVT-3. Data are means ± SE of 4 different determinations from 4 different sets of mice. * P < 0.01.

Figure 5. Effect of the RFVT-3 cKO on level of mRNA expression of oxidative-stress response genes. Quantitative PCR from reverse-transcribed total RNA of jejunum mucosal scraping was used to determine the mRNA expression level of FMO2 (A), GPX1 (B), and SOD1 (C). Data are means ± SE of 4 different sets of mice. * P < 0.01.

Figure 6. Effect of RF supplementation of the RFVT-3 cKO mice on their observed phenotype. A) A representative image of a RFVT-3 cKO mouse (right) and sex-matched control litter-mate (left) showing similar body size. B) Graph showing similar body weight of cKO mice compared to wild-type litter-mates. C-E) Quantitative PCR from reverse-transcribed total RNA of jejunum mucosal scraping was used to determine the level of mRNA expression of FMO2 (C), GPX1 (D), and SOD1 (E). Data are means ± SE of 4 different sets of mice.
Figure 1

A. Diagram illustrating the genetic constructs with LoxP and FRT sites and the Neo cassette.

B. Gel electrophoresis images showing Cre transgene and loxP (+/+) sites.

C. RT-PCR analysis of RFVT-3 mRNA expression in WT and KO litter-mate samples.

D. Bar graph showing relative expression of RFVT-3 mRNA in WT and KO litter-mate samples from jejunal mucosa, liver, and kidney.

Wild-type

Cre transgene

loxP (+/+ ) sites

RFVT-3

β-actin

Jejunal mucosa

Liver

Kidney

WT (litter-mate)

KO (litter-mate)

WT (litter-mate)

KO (litter-mate)

WT (litter-mate)

KO

*
**E. RFVT-3 Protein**

Relative expression of RFVT-3 protein (Arbitrary units)

- **Wild-type**
- **KO**

*RFVT-3* and *β*-actin

**F. RFVT-2 mRNA**

Relative expression of RFVT-2 mRNA

- **WT (litter-mate)**
- **KO (litter-mate)**

Jejunal mucosa

Figure 1
Figure 2

A) Images of WT and KO mice.

B) X-ray images showing bone structure differences.

C) Bar graph showing body weight comparison between WT (litter-mate) and KO.

D) Graph showing percentage decrease of bone density in Femur and Tibia.

E) Graph showing percentage decrease of bone length in Femur, Tibia, and Pelvis.

F) Graph showing RF levels in mouse blood (µmol/min/ml) comparison between WT (litter-mate) and KO.
**A. RF uptake-Jejunal loop *in vivo***

Carrier-mediated RF uptake (fmol/mg protein/5 min)

- WT (litter-mate)
- KO

**B. Biotin uptake-jejunal loop *in vivo***

Carrier-mediated biotin uptake (fmol/mg protein/5 min)

- WT (litter-mate)
- KO

**C. RF uptake-isolated enterocytes *in vitro***

Carrier-mediated RF uptake (fmol/mg protein/3 min)

- WT (litter-mate)
- KO

*Figure 3*
A. RF uptake-colon loop *in vivo*

Relative expression of RFVT-3 mRNA

Figure 4
A. Flavin containing Monooxygenase 2 (FMO2)-mRNA

B. Glutathione peroxidase 1 (GPX1)-mRNA

C. Superoxide dismutase 1 (SOD1)-mRNA
Figure 6

**A**

![Image of two mice](image)

**B**

![Graph showing body weight over months](graph)

**C**

![Bar chart showing relative expression of FMO2 mRNA](bar_chart)

**D**

![Bar chart showing relative expression of GPX1 mRNA](bar_chart)

**E**

![Bar chart showing relative expression of SOD1 mRNA](bar_chart)