Tissue Specific Induction of p62/Sqstm1 by Farnesoid X Receptor

Jessica A. Williams¹, Ann M. Thomas¹, Guodong Li¹, Bo Kong¹, Le Zhan¹, Yuka Inaba³, Wen Xie³, Wen-Xing Ding¹, Grace L. Guo¹*

¹Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, Kansas, United States of America, ²Department of Abdominal Surgery, Cancer Treatment Center, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, People’s Republic of China, ³Center for Pharmacogenetics, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America

Abstract

Background: Farnesoid X Receptor (FXR) is a member of the nuclear receptor superfamily and is a ligand-activated transcription factor essential for maintaining liver and intestinal homeostasis. FXR is protective against carcinogenesis and inflammation in liver and intestine as demonstrated by the development of inflammation and tumors in the liver and intestine of FXR knock-out mice. However, mechanisms for the protective effects of FXR are not completely understood. This study reports a novel role of FXR in regulating expression of Sqstm1, which encodes for p62 protein. p62 plays an important role in maintaining cellular homeostasis through selective autophagy and activating signal transduction pathways, such as NF-κB to support cell survival and caspase-8 to initiate apoptosis. FXR regulation of Sqstm1 may serve as a protective mechanism.

Methods and Results: This study showed that FXR bound to the Sqstm1 gene in both mouse livers and ileums as determined by chromatin immunoprecipitation. In addition, FXR activation enhanced transcriptional activation of Sqstm1 in vitro. However, wild-type mice treated with GW4064, a synthetic FXR ligand, showed that FXR activation induced mRNA and protein expression of Sqstm1/p62 in ileum, but not in liver. Interestingly, FXR-transgenic mice showed induced mRNA expression of Sqstm1 in both liver and ileum compared to wild-type mice.

Conclusions: Our current study has identified a novel role of FXR in regulating the expression of p62, a key factor in protein degradation and cell signaling. Regulation of p62 by FXR indicates tissue-specific and gene-dosage effects. Furthermore, FXR-mediated induction of p62 may implicate a protective mechanism of FXR.

Introduction

Autophagy was strictly thought of as a bulk protein degradation pathway until the discovery that it also performs selective degradation of polyubiquitinated proteins via sequestosome-1 (Sqstm1), which encodes for p62 protein. p62 is often found in cellular protein aggregates because it interacts with ubiquitinated proteins through its C-terminal ubiquitin associated (UBA) domain [1]. p62 also interacts with microtubule light chain 3 (LC3), an autophagy protein, via its LC3 interacting region (LIR). In addition to protein aggregates, recent studies indicate that p62 is also recruited to damaged mitochondria via binding to ubiquitinated outer mitochondrial membrane proteins, although this role of p62 in mitophagy is controversial [2,3]. Therefore, p62 may serve as an autophagy receptor for ubiquitinated proteins and damaged mitochondria.

In addition to its role in autophagy, p62 also has a role in signal transduction and aids in a cell’s decision to undergo apoptosis or survival through its organization of signaling complexes in the cytoplasm [1,4,5]. Upon cytokine stimulation, p62 is able to activate the nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) pathway [5–7]. Activated NF-κB induces the expression of pro-survival genes, such as anti-apoptosis and cell proliferation genes. Activated NF-κB also induces the expression of inflammatory genes such as cytokines, chemokines, and adhesion molecules [8]. In addition, p62 activates nuclear factor erythroid 2-related factor 2 (Nrf2) by binding to kelch-like ECH-associated protein 1 (Keap1), which is important for inducing expression of genes involved in the oxidative stress response [9–11]. Finally, p62 is able to fully activate caspase-8 in the extrinsic apoptosis pathway, which results in the initiation of apoptosis and cell death [4]. Ultimately, p62 helps maintain cellular homeostasis through its participation in autophagy and signal transduction. Therefore, a defect in autophagy can cause an accumulation of damaged organelles and p62-bound protein aggregates or defects in signal transduction, which can lead to tissue injury and disease.
Animals and Treatment

Animals for Chromatin Immunoprecipitation (ChIP) studies were treated as previously described [37]. Briefly, 10-week old FXR knockout (FXR−/−) and wild-type (WT) mice with a C57BL/6 background were fasted overnight and then given a one-time treatment of vehicle (PBS with 1% Tween-20 and 1% methylcellulose) or GW4064 (75 mg/kg) by oral gavage for four hours before harvesting of their livers or two hours before harvesting of their ileums for RNA and protein extraction. The VP-FXR transgenic mice were generated as previously described [38]. Briefly, constitutively active FXR was overexpressed in the liver and intestine using the tetracycline-inducible transgenic system. Briefly, constitutively active FXR was overexpressed in the liver and intestine using the tetracycline-inducible transgenic system. Transgenic mice were generated as previously described [38]. Briefly, constitutively active FXR was overexpressed in the liver and intestine using the tetracycline-inducible transgenic system. WT mice were fasted overnight and received a one-time treatment of GW4064 (150 mg/kg) or vehicle by oral gavage for either 4 or 16 hours before harvesting of their livers and ileums for RNA and protein extraction. The VP-FXR transgenic mice were generated as previously described [38]. Briefly, constitutively active FXR was overexpressed in the liver and intestine using the tetracycline-inducible transgenic system. VP-FXR was generated by fusing the VP-16 transactivation domain from the herpes simplex virus to the 3′ end of the FXR cDNA. FXR−/− mice were generated as previously described [34]. All animal protocols were approved by the University of Kansas Medical Center Animal Care and Use Committee (protocol number 2010-1947), and the mice were cared for according to standard guidance. All efforts were made to minimize suffering.

ChIP-Seq

Chromatin immunoprecipitation (ChIP) followed by massive parallel sequencing (ChIP-seq) analysis was performed as previously reported [37]. Briefly, cross-linked sonicated genomic DNA extracted from ten week-old fasted WT and FXR−/− male mouse livers or ileums gavaged with vehicle or GW4064 for 2 hours (ileum) or 4 hours (liver) were immunoprecipitated with antibody against FXR. Immunoprecipitated DNA fragments were then prepared for massive parallel sequencing analysis as previously described [37]. Enriched intervals, referred to as peak values, were identified when a given genomic region containing more than one enriched interval overlapping by at least one base pair appeared more than 20 times. Histograms of FXR binding to the Sqtstm1 gene in liver and ileum were generated by loading sequencing BAR files into Affymetrix Integrated Genome Browser (IGB) [39].

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ChiP-quantitative PCR (ChiP-qPCR)

ChiP was performed as previously described [37]. Briefly, ChiP assay was performed using anti-FXR antibody (H-130, Santa Cruz, CA), and immunoprecipitated DNA was analyzed by quantitative PCR (qPCR) using SYBR Green chemistry (Fermentas, Glen Burnie, Maryland). QPCR was performed to amplify FXR binding sites located in the Nrho2 and Ostb genes, which are positive control regions for FXR binding, as well as for the novel FXR binding site in the Sqtstm1 gene. A novel FXR binding site identified by ChiP-seq analysis was located 13.1 kb downstream of the Sqtstm1 transcription start site (TSS). This site was amplified by ChiP qPCR analysis using primers: Nrho2 3′ binding site F: 5′-CAGTTCAGCGCCTCAGGCC-3′ and R: 5′-GGCAGGGGAGTTGTGCTGCAAACGC-3′; Ostb F: 5′-CCGCAATGCGGACATCATAC-3′ and R: 5′-GTGAAACCCACGAGTG-3′, and Sqtstm1 F: 5′-CAGTTCAGCGCCTCAGGCC-3′ and R: 5′-GGCAGGGAGTTGTGCTGCAAACGC-3′. ChiP-qPCR results were normalized to input and expressed as fold over IgG negative controls.

RNA Isolation and Real-Time qPCR

RNA was isolated using TRI Reagent (Ambion, Applied Biosystems, Austin, TX) according to the manufacturer’s instructions, and RNA concentration was determined by spectrophotometry. cDNA was generated using standard RT-PCR protocols, and qPCR was performed using SYBR Green chemistry. The following primers were used for Real-Time qPCR: Shp F: 5′-CAGATCCCTTCTCAGCACAGAGT-3′ and R: 5′-AGGGGTCAAGGATTTGTGTG-3′; Sqstm1 F: 5′-CCGCAATGCGGACATCATAC-3′ and R: 5′-GTGAAACCCACGAGTG-3′. cDNA was generated using standard RT-PCR protocols, and qPCR was performed using SYBR Green chemistry. The following primers were used for Real-Time qPCR: Shp F: 5′-CAGATCCCTTCTCAGCACAGAGT-3′ and R: 5′-AGGGGTCAAGGATTTGTGTG-3′; Sqstm1 F: 5′-CCGCAATGCGGACATCATAC-3′ and R: 5′-GTGAAACCCACGAGTG-3′. qPCR results were normalized to 18 s and expressed as fold over WT vehicle control.

Construction of Plasmids

A 2 kb region of the Sqtstm1/p62 gene containing a FXR response element, which is an inverted repeat separated by one nucleotide (IR1), was cloned into a PGL4-TK luciferase vector. This IR1 was located 13.1 kb downstream of the Sqtstm1/p62 gene TSS. The cloned construct was confirmed by DNA sequencing, and the new plasmid was named PGL4-p62-TK luciferase vector. This IR1 was mutated in the PGL4-p62-TK vector using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions utilizing the following primers: F: 5′-GCAATCTACGTTGGCCCGCCCGCCGCC-3′ and R: 5′-TCGTCTCCTCCTGAGCAGTT-3′.
CAAGTTCACTGATGTGGTGTTCAAAGTTGTC-3 and R: 5’-GACAACTTTGAACACCACATCAGTGAACTTGGGGC-CAACGTAGGATTGC-3’. These primers generated an IR1 mutant by changing the IR1 sequence 5’CGTCACTGACCT-3’ to the mutant sequence 5’AGTTCACTGATGT-3’. The mutated base pairs are underlined in the original sequence. The mutation was confirmed by DNA sequencing, and the mutated plasmid was named p62-M.

Cell Culture, Transient Transfection, and Luciferase Reporter Gene Assay

HepG2 cells, purchased from the American Type Culture Collection (Manassas, VA), were cultured in DMEM supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA) and 1% penicillin/streptomycin in a 5% CO2 humidified atmosphere at 37°C. Cells were plated at a density of 5,000 cells per well in 200 µL medium in a 96-well plate and incubated overnight. Transient transfection was performed as previously described [40]. Briefly, cells were transfected with 0.2 µg plasmid per well containing either PGL4-p62-TK or p62-M along with human FXR, human RXRa and PCMV-renilla luciferase vector (Promega, Madison, WI) using TurboFect in vitro transfection reagent (Fermentas, Glen Burnie, Maryland) according to the manufacturer’s instructions. The previously described PGL4-Shp-TK plasmid [40] was used as a positive control for FXR activation. Six hours after transfection, medium was changed and cells were treated with 1 µM GW4064 or 0.1% DMSO as a control. Thirty six to forty eight hours later, firefly and renilla luciferase activities were measured using a Dual-Glo Luciferase Assay kit (Promega, Madison, WI). Firefly luciferase activity of each well was normalized as a ratio to that of renilla luciferase and expressed as fold over PGL4-TK empty vector control.

Western Blot

Cytoplasmic extracts from FXR −/− and WT mouse liver and ileum were isolated using a NE-PER kit (Thermo Scientific, Fremont, CA) according to the manufacturer’s instructions. Protein concentration was measured using BCA assay (Thermo Scientific, Fremont, CA). Western Blot was performed using 20 µg of protein separated on a 10% SDS/PAGE gel and transferred to a 0.45 µm PVDF membrane (Millipore, Billerica, MA). The membrane was blocked with 5% non-fat milk in TBS before adding p62 antibody (1:1000, Abnova, Walnut, CA). A chloroquine-treated HeLa cell lysate sample was used as a positive control for p62 labeling (molecular weight 62 kDa), and β-Actin (molecular weight 42 kDa) was used as a loading control. Band density was determined using ImageJ software.

Statistics

A student’s t-test was used to determine statistical significance for samples that demonstrated equal variance. A Mann-Whitney Rank Sum test was used to determine statistical significance for samples that did not demonstrate equal variance. A p-value of
0.05 was considered statistically significant. A p-value of 0.05 is indicated by * and a p-value of 0.01 is indicated by **.

**Results**

FXR Binding to the Sqstm1 Gene in Mouse Liver and Ileum

Binding of FXR to two regions at the 3' end of the Sqstm1 gene in the liver and ileum was discovered by our genome-wide ChIP-seq analysis [37]. These two FXR binding sites were located 13.1 and 15.8 kb downstream of the Sqstm1 TSS on chromosome 11. Abundance of FXR binding to novel and known target genes in the liver and ileum in ChIP-seq results were interpreted by a binding peak value. The peak value of FXR binding to the Sqstm1 gene at the 13.1 kb site was 755 in liver and 815 in ileum (Figure 1). The peak value of FXR binding to the Sqstm1 gene at the 15.8 kb site was 330 in liver and 500 in ileum (Figure 1). Binding of FXR to the 13.1 kb site of the Sqstm1 gene represented one of the highest peak values detected by ChIP-seq analysis, and it is relatively high compared to FXR binding to other known FXR target genes. For example, the peak value of FXR binding to the Nr0b2 gene encoding small heterodimer partner (Shp) was 498, and the peak value of FXR binding to the Ostβ gene encoding organic solute transporter β (Ostβ) was 572 [37]. Furthermore, sequence analysis of the 13.1 kb FXR binding site within the Sqstm1gene by NUBIScan [41] revealed the presence of a classical IR1. The chromosomal location of this IR1 and its sequence are shown in Figure 1. The 15.8 kb binding site did not have an IR1 present according to NUBIScan [41]. Therefore, the 13.1 kb site was further analyzed as a functional FXR binding site.

The binding results from ChIP-seq were confirmed by ChIP-qPCR as shown in Figure 2. Vehicle-treated WT mice showed binding of FXR to Sqstm1/p62 in both liver (126-fold) and ileum (18-fold) when compared to IgG controls. This binding was reduced to IgG control levels in liver and ileum for vehicle-treated FXR−/− mice. Ostβ was used as a positive control for FXR binding in both liver and ileum. In the liver, there was a 36-fold increase in binding to Ostβ compared to IgG controls, and there was a 32-fold increase for binding to Ostβ in the ileum compared to IgG controls. This binding was reduced to IgG control levels in liver and ileum of vehicle-control treated FXR−/− mice (Figure 2A). Ostβ is a known FXR target gene and was used as a positive control for liver and showed a 3.5-fold increase after GW4064 treatment. In ileum, there was a 10-fold increase in FXR binding to Sqstm1/p62 with GW4064 treatment. The FXR target gene Ostβ was used as a positive control for ileum and showed a 4-fold increase after GW4064 treatment (*indicates p<0.05, **indicates p<0.01, N = 3 WT and 4 FXR−/− mouse livers or ileums per group).

<0.05 was indicated by * and a p-value of <0.01 is indicated by **.
Activation of FXR Enhances Transcriptional Activation of \( \text{S}q\text{stm1} \) as Revealed by the Luciferase Reporter Gene Assay

A luciferase reporter assay was performed to determine if FXR binding to the \( \text{S}q\text{stm1/p62} \) gene was functional in enhancing transcription. Activation of FXR by GW4064 increased the luciferase activity of p62 3 to 7 fold \((p<0.01, \text{Figures 3A and 3B})\) when driven by an IR1 FXR response element \((5'-\text{CGGGTCACCT-GACCT-3}')\) found 13.1 kb downstream of the \( \text{S}q\text{stm1/p62} \) gene TSS compared to PGL4-TK vector control \((\text{Figure 3A})\). In addition, mutation of this FXR response element \((\text{p62-M, 5'-AGTTGACTGATGT-3}')\) reduced luciferase activity to levels similar to the PGL4-TK vector control \((\text{Figure 3B})\). As a positive control, activation of FXR by GW4064 significantly enhanced luciferase activity approximately 3 to 4 fold \((p<0.01)\) when driven by a FXR response element identified in the \( \text{Nr0b2/Shp} \) gene regulatory region \((\text{Figures 3A and 3B})\).

Activation of FXR Induces mRNA Expression of \( \text{S}q\text{stm1} \) in Ileum but not in Liver

Binding of FXR to the \( \text{S}q\text{stm1} \) gene does not guarantee activation of the gene’s transcription because many factors are involved in gene transcriptional activation. Therefore, \( \text{S}q\text{stm1/p62} \) mRNA expression levels were determined following FXR activation using \( \text{Nr0b2} \) or \( \text{Fabp6} \) as positive controls. \( \text{Fabp6} \) is the gene encoding for ileum bile acid binding protein \((\text{Ibapb})\). Shp is a classical target gene of FXR in the liver, and Ibapb is a direct target gene of FXR in the ileum. A significant GW4064-mediated induction of Shp mRNA was observed in both the 4- (2.3-fold) and 16-hour (2.4-fold) treatment groups for WT mouse livers \((p<0.01)\), as shown in Figure 4A. However, no induction of \( \text{S}q\text{stm1/p62} \) mRNA was seen for either time point in WT mouse livers \((\text{Figure 4A})\).

In contrast to results seen in the liver, a GW4064-mediated induction of both Ibapb and \( \text{S}q\text{stm1/p62} \) mRNA was observed in the 4- and 16-hour GW4064 treatment groups for WT mouse ileum when compared to vehicle controls \((\text{Figure 4B})\). Treatment with GW4064 resulted in a significant 2.5 and 6.9-fold induction of Ibapb \((p<0.05)\) and a 1.6 and 1.7-fold induction in \( \text{S}q\text{stm1/p62} \) mRNA in the 4 and 16-hour treatment groups in WT mouse ileum, respectively. Only the 4-hour GW4064 treatment group induction of \( \text{S}q\text{stm1/p62} \) mRNA was statistically significant \((p<0.05)\).

We then used FXR \(-/-\) mice to confirm that the GW4064-mediated induction of \( \text{S}q\text{stm1} \) gene expression was due to FXR activation. As shown in Figure 4A, a significant decrease in baseline Shp expression levels in liver was seen in both vehicle- and GW4064-treated FXR \(-/-\) mice for the 4- and 16-hour treatment groups \((p<0.01)\). However, FXR deficiency did not seem to affect \( \text{S}q\text{stm1/p62} \) baseline expression in mouse livers. For mouse ileums, a significant decrease in baseline Ibapb expression was seen in both vehicle- and GW4064-treated FXR \(-/-\) mice \((p<0.01, \text{Figure 4B})\). In addition, FXR \(-/-\) mice showed a baseline decrease in \( \text{S}q\text{stm1/p62} \) expression in ileum in both 4- and 16-hour GW4064 treatment groups, but this finding was only statistically significant for the 16-hour treatment group \((p<0.05, \text{Figure 4B})\).

Protein Expression of p62 in Mouse Liver and Ileum

It is known that p62 is expressed in intestinal epithelia \([42]\). After we observed induction of \( \text{S}q\text{stm1/p62} \) mRNA by FXR activation in ileum, we determined whether increased mRNA levels translated into protein induction. As shown in Figure 5, GW4064 treatment significantly increased p62 protein expression 2-fold \((p<0.05)\) over vehicle controls in mouse ileum. Furthermore, the GW4064-mediated induction of p62 protein expression was abolished in FXR \(-/-\) mouse ileums. However, there was no effect of GW4064 treatment on p62 protein expression in mouse liver.

mRNA Expression of \( \text{S}q\text{stm1} \) in FXR Transgenic Mouse Liver and Ileum

We used VP-FXR transgenic mice to determine whether genetically constitutive activation of FXR could also regulate the expression of \( \text{S}q\text{stm1/p62} \). As shown in Figure 6, \( \text{S}q\text{stm1/p62} \) mRNA expression was significantly increased in both liver and ileum from VP-FXR transgenic mice when compared to WT controls \((p<0.05)\). Shp and Ibapb were used as positive controls and were also significantly increased in VP-FXR transgenic mouse.
liver and ileum, respectively, when compared to WT mice (p < 0.05).

**Discussion**

p62 is the protein encoded by the Sqstm1 gene and has important cellular functions. In addition to the well-known role of p62 in facilitating selective autophagy, p62 also activates NF-κB [5–7], which is well known for its regulation of genes needed to promote cell survival and inflammation. In addition, p62 activates apoptosis to promote cell death [4] and activates the Nrf2 pathway to respond to oxidative stress [9–11].

FXR is a nuclear receptor suspected to have a role in the regulation of homeostasis in both liver and intestine. In the current study, we revealed that Sqstm1 is a bona fide FXR target gene by showing a novel FXR binding site located within the Sqstm1 gene in mouse liver and ileum using ChIP-seq analysis (Figure 1). In addition, treatment with the synthetic ligand of FXR, GW4064, increased binding of FXR to this novel target gene in both liver and ileum, and this binding was significantly reduced in FXR−/− mice (Figure 2). Furthermore, binding of FXR to the Sqstm1 gene regulatory region led to increased transcriptional activation as confirmed by luciferase reporter assay, and this transcriptional activation was abolished when the IR1 FXR response element was mutated (Figure 3). However, it appears that induction of Sqstm1 is tissue-specific because mice treated with GW4064 to activate FXR only had an induced expression of Sqstm1 in the ileum but not in the liver (Figure 4). The results from this study provide a potential mechanism by which FXR regulates the inflammatory response and/or promotes cellular homeostasis by inducing transcription of p62.

The tissue-specific induction of Sqstm1/p62 mRNA, despite the fact that FXR binds to a gene regulatory region of Sqstm1 in both liver and ileum, is an intriguing observation. This suggests that p62 is regulated by multiple transcription factors. For example, another known transcriptional regulator of p62 is Nrf2, which is important for regulating the oxidative stress response [9]. Therefore, the presence and/or balance of these various transcription factors, and possible inhibitory factors, that regulate p62 expression may determine whether FXR binding will be translated into transcriptional activation of the Sqstm1 gene. In addition, the fact that Sqstm1/p62 expression is not induced in the liver with FXR activation could be due to higher basal expression of Sqstm1/p62 in the liver than in the ileum.

Even though induction of Sqstm1 mRNA expression and p62 protein expression was only seen in mouse ileum and not in mouse liver after FXR activation (Figures 4 and 5), there was an increase in Sqstm1 mRNA expression in both the liver and ileum of the VP-FXR transgenic mice (Figure 6). This increase in Sqstm1 mRNA...
expression in the liver of VP-FXR transgenic mice could be due to the presence of constitutively active FXR, which is not present in WT mice treated with GW4064 to activate FXR. Assuming the inability of FXR activation to induce Sqstm1 expression in the liver is due to the presence of a co-repressor or inhibitory transcription factor, then constitutively active FXR in the transgenic mice might be strong enough to remove or override a competing transcription factor or co-repressor bound to the Sqstm1 promoter, and therefore, promotes transcription of the gene.

The specific IR1 sequence found in mouse is not conserved in human. However, there are several IR1 sites located within this downstream region on Chromosome 5 in human according to analysis by NUBIScan [41]. FXR may regulate Sqstm1 gene expression in human by binding to one of these IR1 response elements. Furthermore, the ileum-specific regulation of Sqstm1/p62 by FXR may have an implication in intestinal diseases. FXR deficiency has been shown to cause intestinal injury and disease such as inflammation [24,26,33] and tumorigenesis [30–32]. In addition, FXR has been shown to play a role in maintaining intestinal epithelial cell proliferation to protect against tumorigenesis [31,32]. Therefore, it is possible that FXR regulates the expression of the Sqstm1 gene in the ileum in order to mediate selective autophagy or signal transduction to maintain cellular homeostasis, regulate the inflammatory response, and/or conduct tissue repair. If FXR is indeed regulating Sqstm1/p62 expression for these processes, then tissue-specific drug development of a synthetic activator of FXR in the intestine could be beneficial for treating or preventing intestinal diseases. This tissue-specific role of FXR in Sqstm1 gene regulation is a novel finding and subsequent studies will further investigate the role of FXR in the regulation of Sqstm1/p62.

In conclusion, it is known that both p62 and FXR have beneficial effects in maintaining cellular homeostasis and preventing disease. We have shown that FXR transcriptionally regulates p62 expression in the intestine. If FXR is indeed regulating p62 expression for these processes, then tissue-specific drug development of a synthetic activator of FXR in the intestine could be beneficial for treating or preventing intestinal diseases. This tissue-specific role of FXR in p62 gene regulation is a novel finding and subsequent studies will further investigate the role of FXR in the regulation of Sqstm1/p62.

Author Contributions
Conceived and designed the experiments: GLG, JAW, WXD. Performed the experiments: JAW, AMT, GL, BK, LZ, YI, WX. Analyzed the data: JAW, AMT, GL, WX. Wrote the paper: JAW, AMT, GL, WX.
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