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Farnesoid X receptor protects human and murine gastric epithelial cells against inflammation-induced damage

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Short title: Farnesoid X receptor in gastric cancer

Abbreviations: CDCA chenodeoxycholic acid, K13 human/mouse keratin 13, EV empty vector, FXR farnesoid X receptor, IBABP ileal bile acid binding protein, IM intestinal metaplasia, KO knock-out, PARP Poly ADP ribose polymerase, RARE retinoic acid receptor responsive element, SHP small heterodimer partner, WT wild-type.

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1 **SYNOPSIS**

2

3 Bile acids from duodenogastric reflux promote inflammation and increase the risk for gastro-
4 esophageal cancers. Farnesoid X receptor (FXR) is a transcription factor regulated by bile acids,
5 such as chenodeoxycholic acid (CDCA). FXR protects the liver and the intestinal tract against
6 bile acid overload, however, a functional role for FXR in the stomach has not been described.
7 We detected FXR expression in the normal human stomach and in gastric cancer (GC). FXR
8 mRNA and protein were also present in the human GC cell lines MKN45 and SNU5 but not in
9 AGS. Transfection of FXR into AGS cells protected against TNF α -induced cell damage. We
10 identified Keratin 13 (K13), an anti-apoptotic protein of desmosomes, as a novel CDCA-
11 regulated FXR-target gene. FXR bound to a conserved regulatory element in the proximal
12 human K13 promoter. Gastric expression of *K13* mRNA was increased in an FXR-dependent
13 manner by a chow diet enriched with 1% (w/w) CDCA and by indomethacin (35 mg/kg i.p.) in
14 C57BL/6 mice. FXR-deficient mice were more susceptible to indomethacin-induced gastric
15 ulceration than wild-type littermates. These data suggest that FXR increases the resistance of
16 human and murine gastric epithelial cells to inflammation-mediated damage, and may thus
17 participate in the development of GC.

18

19 **Key words:** bile acid; CDCA; FXR; keratin; gastric cancer; stomach

1 INTRODUCTION

2

3 Overexposure to bile acids leads to damage of gastrointestinal (GI) epithelia. For example,
4 patients with recurrent heartburn are at increased risk of esophageal cancer [1]. Chronic
5 inflammation upon duodenogastric reflux of bile acids, e.g. after gastric resection, is an
6 important risk factor for gastric cancer (GC) [2-5]. Efficient detoxification mechanisms, some
7 involving members of the nuclear receptor superfamily [6], counteract the detrimental actions of
8 excess bile acids on gastrointestinal (GI) organs.

9

10 Farnesoid X receptor (FXR) is nuclear receptor and transcription factor activated by
11 physiological bile acids [6, 7] including chenodeoxycholic (CDCA) and deoxycholic (DCA)
12 acid. FXR suppresses the *de novo* synthesis of bile acids in the liver and promotes excretion and
13 entero-hepatic circulation of conjugated bile acids [8]. Studies in FXR-deficient mice suggest a
14 beneficial role for FXR against cholestasis [7] and colitis [9]. FXR facilitates liver regeneration,
15 supports the differentiation of the intestinal epithelium and promotes antibacterial defense in the
16 GI tract [7]. In contrast, FXR-deficiency exacerbates hepatic [7] and intestinal [9] inflammation
17 and promotes carcinogenesis in mice [10]. These data corroborate the idea that FXR acts as a
18 protective factor in the liver and the GI tract.

19

20 Aberrant FXR expression or function has also been detected in human adenocarcinomas of the
21 colon [11], breast [12], prostate [13] and esophagus (Barrett) [14]. These findings pointed to
22 either causative or protective roles for bile acids and/or FXR in the pathogenesis of these tumor
23 types. FXR mRNA variants have been identified in normal stomach tissue of humans and mice
24 [15, 16], however, a functional role for gastric FXR has not yet been defined. We therefore have
25 evaluated the role of bile acid-activated FXR on cell survival of human and mouse gastric
26 epithelial cells. Our data indicate that FXR increases the resistance to inflammation-induced cell
27 damage accompanied by the up-regulation of protective target genes, such as keratin 13 (K13).

1 MATERIALS AND METHODS

2 Subjects

3 Tumor specimens were obtained by surgical resection from GC patients and processed
4 according to standard methods [17]. Tissue microarrays (TMAs) were generated by AW. GC
5 was classified histologically into diffuse (D-T), intestinal (I-T) and undifferentiated types
6 according to Lauren. Intestinal metaplasia (IM-T) was assessed within I-T tissue specimens.

8 Animal studies

9 Wildtype (WT) and FXR knockout (KO) mice (strainB6; 129XFVB-Nr1h4-tmlGonz/J, Jackson
10 Laboratory, Bar Harbor, Maine) were maintained on a pure C57BL/6 background. Male mice (4
11 weeks old) received a chow diet (Altromin, Lage, Germany) containing 1% (w/w) CDCA
12 (Chemos GmbH, Regenstauf, Germany) (n=5 per group and genotype). Experimental gastric
13 ulceration (n ≥ 6 per group and genotype) was performed with indomethacin as published [18].

15 Cell culture and Western blotting (WB)

16 Human embryonic kidney (HEK293), hepatoma HepG2 and the GC cell lines AGS, NCI-N87,
17 SNU1, SNU5, KATOIII (ATCC, Rockville, MD), MKN7 and MKN45 (Japan Cell Bank,
18 Tokyo, Japan) were cultivated as described [17]. Stable AGS clones were generated [17] upon
19 transfection with full-length FXR α 1 cDNA in pTarget (AGS/FXR) or pTarget empty vector
20 (AGS/EV), respectively. SDS-PAGE and Western blotting (WB) of cell and tissue lysates (25
21 μ g protein per gel lane) were performed as published [17].

23 Antibodies

24 The FXR (H-130, Santa Cruz Biotech., Santa Cruz, CA) and K13 (NCL-K13, Novocastra,
25 Newcastle, UK) antibodies were used for immunohistochemistry (IHC) and Western blotting
26 (WB). FXR (Clone A9033A, R&D Systems, Wiesbaden-Nordenstadt, Germany), cleaved
27 caspase-8 (Asp391, 18C8) and -3 (Asp175, Cell Signaling, Danvers, MA), cleaved PARP
28 (Asp214, 7C9, Cell Signaling) and β -actin (clone AC-74, Sigma, Taufkirchen, Germany) were
29 used for WB analysis.

1 **Immunohistochemistry (IHC)**

2 Sections from paraffin-embedded tissues (3 μm) were stained (1:100, FXR H-130) according to
3 a published procedure [19]. Cryosections (10 μm) were stained (1:50, K13) following antigen
4 retrieval using pepsin digestion and the Vectastain M.O.M. kit (Vectorlabs, Burlingame, CA).
5 Tissue microarrays (TMAs) were subjected to an automated staining protocol and quantitatively
6 evaluated by the expert pathologist AW.

8 **DNA-constructs**

9 The FXR-RE reporter plasmid consists of the human bile salt export pump (BSEP) promoter
10 driving the luciferase gene in pTK-luc (FXR-RE-luc) [20]. The human K13 promoter
11 (AF049259, 1 to 525 bp) containing a putative FXR site (RARE2) was subcloned into pGL3-
12 basic-luc (Promega GmbH, Mannheim, Germany) (RARE2-WT-K13p-luc) [21]. A second
13 plasmid (RARE2-MUT-K13p-luc) was constructed, in which the ACT GGG TGG GGC TCA
14 (RARE2) element in the human K13 promoter was deleted using the Quickchange Kit
15 (Stratagene, Amsterdam, NL). The 1428 bp full-length human FXR cDNA (476 aa, α 1 splice
16 variant with MYTG, NM_005123) [16] was inserted into pTarget (Promega). SiGENOME
17 SMARTpool siRNA against human FXR and K13 was from Dharmacon (Perbio Science, Bonn,
18 Germany). Knockdown efficiency was verified by RT-qPCR and WB and varied between 40 to
19 80 %. PCR products for human FXR α mRNA variants [22] (**Table S1**) were purified from
20 agarose gels (Pure Link Kit, Invitrogen, Darmstadt, Germany) and confirmed by sequencing
21 (GATC, Konstanz, Germany).

23 **Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR)**

24 Reactions were performed as published [19]. CT-values were normalized to CT-values of β 2-
25 microglobulin and compared to “no template” controls (CT > 36).

26
27
28

1 **DNA microarrays**

2 AGS/FXR and AGS/EV cells were treated for 16 h with 100 μ M CDCA. cRNAs were produced
3 as described by the manufacturer (One-Cycle cRNA labelling kit, Affymetrix, Wycombe, UK)
4 and hybridized to GeneChip® Human Genome Arrays (HG U133 Plus 2.0, Affymetrix) [20].
5 Gene sets (**Table S2**) were identified using the tools at <http://david.abcc.ncifcrf.gov>.

7 **Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assay (EMSA)**

8 Both methods were performed using oligonucleotides (**Table S1**) as previously described [20].

10 **Cell viability assays**

11 Colorimetric 1-(4,5-dimethylthiazol-2-yl)3,5-diphenylformazan (MTT) assays were performed
12 according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).
13 Flow cytometry was done on cells grown to confluency in 6-well plates and treated with TNF α
14 (100 ng/ml) (Roche) and cycloheximide (CHX) (20 μ g/ml) for 6 h. Floating and adherent cells
15 were harvested into PBS and collected by centrifugation (200 x g, 4 min, RT). Annexin and
16 propidium iodide (PI) double staining was performed according to the manufacturer's protocols
17 (Roche) and analysed on a FACScalibur device (BD Biosciences, San Jose, CA).

19 **Statistics**

20 Results are expressed as means \pm S.E. from at least 5 animals per group and genotype or 3
21 independent cell experiments. P-values were calculated using Graphpad Prism 4 software with
22 one-way ANOVA for mice, Student T test for cells, and Wilcoxon test for patient data.

1 RESULTS

2

3 Expression of FXR in human GC tissues and cell lines.

4 To assess the expression of FXR in human GC, IHC on tissue microarrays was performed using
5 a rabbit polyclonal FXR antiserum [13, 23, 24] (**Figure 1A**). In tissues from healthy individuals,
6 FXR was found in the nuclei of cells of the non-neoplastic foveolar epithelium from gastric
7 glands. In a series of GC specimens (n=70), consisting of both intestinal and diffuse types of
8 GC, FXR was undetectable (score 0) in 32 cases (46 %), showed moderate (score 1+)
9 expression in 31 cases (44 %) and strong expression (score 2+) in 7 cases (10 %) in the tumor
10 cells. FXR was most prominently expressed (score 2+) in 6 of 7 specimens of intestinal type GC
11 which contained regions of intestinal metaplasia (IM), as evident by translucent goblet cells, and
12 was localized both in the nucleus and the cytoplasm. FXR was detected by Western blot (WB)
13 in gastric tissue lysates as a ~56 kDa band similar in size to that detected in normal human liver
14 using a mouse monoclonal antibody [14, 25] (**Figure 1B**). Collectively, these data indicated that
15 FXR is expressed in the normal human stomach and in GC.

16

17 In humans [16] and rodents [15], two major FXR mRNA variants $\alpha 1/2$ and $\alpha 3/4$ have been
18 identified that result in divergence in the N-terminal region of the protein (**Figure 2A**) [22]. In
19 addition, the $\alpha 1$ and $\alpha 3$ variants contain a four amino acid (MYTG) insertion of unknown
20 function [22]. We amplified the full-length FXR $\alpha 1/2$ mRNA with a forward primer specific for
21 exon 1 and the truncated Δ FXR $\alpha 1/2$ transcript with a primer specific for exon 3 that contains
22 the start codon. FXR $\alpha 3/4$ mRNA was detected by an exon 3a specific primer [16]. A common
23 reverse primer against exon 4 was applied for all RT-PCRs, and all PCR products were
24 confirmed by DNA-sequencing. We detected full length FXR $\alpha 1/2$ mRNA in the human
25 hepatoma HepG2 cells. FXR $\alpha 3/4$ mRNA was detected in the human GC cell lines MKN45 and
26 SNU5. None of the other human GC cell lines (AGS, SNU1, N87, KATOIII, MKN7) expressed
27 either full-length FXR $\alpha 1/2$ mRNA or the $\alpha 3/4$ mRNA. However, the Δ FXR $\alpha 1/2$ mRNA was
28 detectable in all GC lines. Consistent with the results obtained from the PCR analyses, ~56 kDa

1 FXR protein was observed in SNU5 (*lane 3*) cells as compared to protein lysates from human
2 hepatoma HepG2 cells, hepatocellular carcinoma (HCC) and normal liver tissue (**Figure 2B**).
3 No FXR protein was detectable in AGS, SNU1, N87, MKN7 and KATOIII cell lysates (data not
4 shown), indicating that the Δ FXR α 1/2 transcript is not translated into a detectable protein. Real-
5 time quantitative RT-PCR using primers against the common DNA-binding domain of FXR
6 (**Figure 2C**) confirmed that SNU5 expressed the highest levels of total FXR α mRNA of all
7 human GC cell lines tested.

9 **FXR protects against cell damage.**

10 To explore the function of gastric FXR in cells, AGS cells were stably transfected with an
11 expression plasmid harbouring the full length human FXR α 1 cDNA (AGS/FXR) or with empty
12 vector (AGS/EV). Chronic inflammation is a risk factor associated with human GC [26]. We
13 tested, whether FXR modulates the sensitivity of AGS cells towards inflammation-induced
14 stress in the presence of TNF α and cycloheximide (CHX) which specifically induce apoptotic
15 cell death [27]. AGS/FXR and AGS/EV cells were treated for 24 h with a mixture of TNF α
16 (100 ng/ml) and CHX (20 μ g/ml). Results from MTT assays demonstrated that FXR-expressing
17 AGS/FXR cells retained higher viability (3-fold, * p <0.05) than AGS/EV cells (**Figure 3A**).
18 CDCA (at 50 μ M) further increased the protective effect of FXR (4-fold, * p <0.05) compared to
19 both vehicle and CDCA-treated AGS/EV cells. SNU5 cells, which expressed the highest level
20 of endogenous FXR, were the most resistant to the apoptotic effect of TNF α +CHX of all
21 parental GC lines and comparable to HepG2 cells (data not shown), suggesting endogenous
22 FXR is also protective.

24 Increased apoptosis was confirmed by WBs that showed cleaved caspase-8 appearing after 6 to
25 8 h of TNF α +CHX-treatment, followed by appearance of cleaved caspase-3 and PARP after 10
26 h (**Figure 3B**). The abundance of cleavage products was decreased and the time of their
27 appearance was delayed in AGS/FXR compared to AGS/EV cells. These data suggest that FXR
28 delays and limits the activation of TNF α -triggered apoptosis. To quantitatively assess apoptotic

1 cell numbers, annexin-propidium iodide (PI) dye exclusion flow cytometry was performed.
2 Statistical analysis of FL1 histograms revealed that approximately 5-fold more annexin-positive
3 apoptotic cells were present following 6 h treatment with $\text{TNF}\alpha$ +CHX in AGS/EV
4 ($25\pm 5\%$, $*p<0.05$) than in AGS/FXR ($5\pm 10\%$) cells (**Figure 3C**). These data corroborate the
5 protective effect of FXR against inflammation-mediated cell death.

7 **Human K13 is a novel FXR target gene.**

8 To identify potential mechanisms underlying cell protection by FXR, AGS/FXR and AGS/EV
9 clones were treated with 100 μM CDCA for 16 h, and total RNAs were subjected to DNA
10 microarray analysis (**Supplement 1**). Comparative analysis identified K13 mRNA as the most
11 highly upregulated transcript in AGS/FXR compared to AGS/EV cells. Other keratin types
12 including K6, keratin associated protein 2-1 and plakophilin 4, a structural component of
13 desmosomes, were also increased (**Table S2**). The mRNAs for $\text{TNF}\alpha$ -induced protein 8
14 (TNFAIP8), an inhibitor of caspases and anti-apoptotic factor, and the gastroprotective protein
15 plasminogen activator inhibitor 2 (SERPINB2) were also specifically increased in AGS/FXR
16 cells [28]. Induction of the human solute transporter $\text{OST}\alpha$, a well-characterized FXR target
17 gene, confirmed that FXR activity was increased by CDCA in AGS/FXR cells.

18
19 Quantitative RT-qPCR expression analysis was performed to confirm the microarray results.
20 Treatment for 24 h with 75 μM CDCA up-regulated K13 mRNA (4-fold) and the FXR-target
21 genes $\text{OST}\alpha$ (2-fold) and ileal bile acid binding protein (IBABP) (36-fold) in AGS/FXR
22 ($*p<0.05$ CDCA *versus* vehicle) but not in AGS/EV cells (**Figure 4A**). WB demonstrated that
23 the ~54 kDa K13 protein was also increased in parallel to its mRNA (**Figure 4B**). CDCA also
24 increased K13 and $\text{OST}\alpha$ mRNAs (~ 2-fold, $*p<0.05$ *versus* vehicle) in the parental SNU5 and
25 MKN45 cells (data not shown), indicating that K13 is also regulated by endogenous FXR. To
26 further test their role in apoptosis protection, both FXR and K13 expression were knocked down
27 by transient transfection of siRNA-oligonucleotides into AGS/FXR cells followed by a 24 h
28 challenge with $\text{TNF}\alpha$ +CHX (100 ng/ml+20 $\mu\text{g}/\text{ml}$) in absence and presence of 50 μM CDCA

1 (Figure 4C). The survival rate of RNAi-treated cells was lower (~ 25 to 60 %, * $p < 0.05$) than for
2 mock-transfected cells, both in vehicle and CDCA-treated cultures. Similar results were
3 obtained after FXR silencing in MKN45 and HepG2 cells (data not shown), suggesting an anti-
4 apoptotic role also for endogenous FXR.

5
6 **FXR directly binds to and transactivates the human K13 gene promoter.**

7 In the human K13 proximal promoter binding sites for retinoic acid (RAR) [21] and vitamin D₃
8 (VDR) receptors have been predicted (Ebeling, unpublished observation), and its regulation by
9 retinoids has been shown experimentally *in vitro* [29, 30] and *in vivo* [31, 32]. To determine if
10 FXR binds to these elements, we designed ChIP primers flanking the first 500 bp upstream to
11 the predicted transcriptional start site (Figure 5A). AGS/EV and AGS/FXR cells were
12 incubated for 16 h with either vehicle or 75 μ M CDCA, and cell lysates were subjected to ChIP.
13 Genomic qPCR reactions were visualized by gel electrophoresis and quantified (Figure 5B). No
14 pull-down of genomic DNA was observed with agarose beads coupled to control IgG (lane 3).
15 ChIP against FXR revealed an increased FXR binding (2-fold, * $p < 0.05$) to the K13 promoter
16 amplicon in presence of CDCA (lane 2) compared to vehicle (lane 1) in AGS/FXR but not in
17 AGS/EV cells. These data indicate that CDCA increases FXR binding within the proximal K13
18 promoter.

19
20 To determine if this genomic region mediates CDCA- and FXR-dependent transactivation of a
21 heterologous gene [20], we cloned the proximal human K13 (-500/ATG) promoter into pGL3-
22 basic luciferase vector (Figure 5C). HEK293 cells [20] were transiently transfected with either
23 EV or FXR expression plasmids, together with the reporter plasmid driven under the control of
24 the K13 promoter (RARE2-WT-K13p-luc) or a control reporter plasmid containing a cognate
25 FXR-RE from the BSEP enhancer (FXR-RE-luc). Cells were treated for 24 h with 30 and 100
26 μ M CDCA, and luciferase activity was determined in cell lysates. CDCA strongly stimulated
27 the activity of the K13 promoter (7 to 9-fold, * $p < 0.05$ FXR *versus* EV) only in cells cotrans-
28 fected with the FXR expression plasmid (Figure 5C).

29

1 Despite these findings, we did not identify a classical inverted repeat-1 (IR1) element in the
2 proximal 500 bp of the K13 promoter. Nevertheless, FXR also recognizes hexameric direct
3 repeat (DR) elements with a variable number of intervening nucleotides between the halvesites
4 [33]. Within the region amplified in our CHIP experiments, we localized three halvesite AGG
5 TCA-like elements, that had previously been described as retinoic acid receptor (RAR)
6 responsive elements (RAREs) [21]. One of these halvesites (RARE2) was well conserved
7 between human and rodents and overlapped with an experimentally proven RARE [34] and a
8 predicted SP 1[21] site (**Table S3**). We specifically deleted the RARE2 element in the K13
9 promoter-luciferase expression construct (RARE2-MUT-K13p-luc) and repeated the reporter
10 gene assay in HEK293 cells. Deletion of RARE2 abrogated the CDCA and FXR-dependent
11 transcriptional activation of the K13 promoter (**Figure 5C**) demonstrating the RARE2 is
12 necessary for the transcriptional activity of FXR.

13
14 To explore the direct interaction of FXR with the K13 promoter, EMSAs were performed [20]
15 using a biotin-labelled oligonucleotide, which contained a consensus IR1 element. Nuclear
16 extracts were prepared from HEK293 cells that had been transiently cotransfected with FXR or
17 EV plasmids. Increased protein binding to the IR1 element was visible in nuclear extracts from
18 CDCA-treated FXR transfected cells (lane 4) compared to untransfected cells (lane 2) (**Figure**
19 **5D**). Both the basal (lane 3) and CDCA-dependent (lane 4) complexes were effectively reduced
20 by competition with an unlabelled RARE2 oligonucleotide (lanes 5,6) but not by a mutated
21 RARE2 sequence (lanes 7,8). We then tested for direct binding of FXR to a biotin-labelled
22 RARE2 oligonucleotide (**Figure 5E**). Nuclear extracts of FXR-transfected and CDCA-treated
23 cells (lane 2) produced an enhanced mobility shift compared to vehicle-treated samples (lane 1),
24 which was efficiently competed by an excess of unlabelled RARE2 oligonucleotide (lane 3) but
25 not by the mutated RARE2 oligonucleotide (data not shown). Addition of polyclonal FXR-
26 antiserum (1µg), which recognizes the DNA-binding domain of FXR, interfered with the band
27 shift (lane 5), corroborating that the DNA-protein complex observed in lane 2, contains FXR
28 bound to the RARE2 element. These data indicate that FXR binds the RARE2, driving bile acid
29 responsiveness of the K13 promoter.

1 **K13 is also regulated by FXR *in vivo*.**

2 To examine K13 regulation by FXR *in vivo*, WT and FXR-KO mice were fed a chow diet
3 enriched in 1 % (w/w) CDCA for 7 days (n=5 per group and genotype). This diet was well
4 tolerated and elicited a general FXR response in the GI tract [20]. Nuclear FXR was visualized
5 by IHC in the corpus and antral glands of the mouse stomach using the rabbit polyclonal Ab
6 (**Supplement 2**). Murine K13 protein was mainly expressed in the non-epithelial compartment
7 facing the basolateral side of the gastric epithelial cells and in the squamous epithelium of the
8 forestomach. The gastric mRNAs of *K13* (5 to 6 -fold, *p<0.05, CDCA *versus* chow) and the
9 cognate FXR-target gene *Ibabp* (30-fold, *p<0.05, CDCA *versus* chow) were robustly increased
10 by CDCA in WT but not in FXR-KO mice. These data indicated that K13 is also regulated by
11 FXR in murine gastric epithelial cells.

12
13 To determine if K13 is co-expressed with FXR in the human stomach, IHC was performed. In
14 normal gastric tissue, K13 staining revealed a net-like pattern within the foveolar epithelium
15 which was absent in GC (**Supplement 2**). The ~54 kDa K13 protein was present in whole tissue
16 lysates of the normal gastric mucosa and in GC biopsies. Total FXR α mRNA levels were
17 determined in a larger series of GC specimens using RT-qPCR primers directed against its
18 common zinc-finger DNA-binding domain. FXR α mRNA was reduced in 25 of 30 (83 %, *
19 p<0.05 T *versus* N) biopsies of GC patients as compared to matched tumor-free tissue. K13
20 mRNA was also decreased in 24 out of 30 (80 %, *p<0.05 T *versus* N) GC biopsies. These data
21 indicate that K13 expression may be dependent on FXR also *in vivo*.

23 **FXR-deficiency promotes susceptibility to gastric injury *in vivo*.**

24 To examine the role of gastric FXR *in vivo*, stomach ulcers were induced in C57BL/6 wild-type
25 (WT) (n=9) and FXR-KO (n=8) mice by i.p. administration of 35 mg/kg indomethacin (Indo)
26 for 24 h [18]. The control WT (n=6) and FXR-KO (n=6) animals received a mock injection of
27 0.9 % (w/v) sodium chloride (NaCl). Gastric tissue sections were stained for H&E and injury
28 scores were determined for each mouse stomach (**Figure 6A**). Control mice did not show any
29 lesions in either genotype. WT mice on indomethacin displayed no (n=2, score 0) or superficial

1 erosive gastritis (n=4, score 1+) or had moderate discrete erosive gastritis (n=2, score 2+). In
2 contrast, almost all of the indomethacin-treated FXR-KO mice exhibited moderate discrete
3 erosive gastritis (n=3, score 2+) or suffered from severe gastritis with elongated erosions and
4 ulcerations of the gastric mucosa (n=3, score 3+) (**Table S4**). The mean injury score and the
5 area of the lesions (mm²) were significantly increased in FXR-KO compared to WT animals
6 (*p<0.05 KO *versus* WT) (**Figure 6B**). The percentage of mice having ulcers was 50 % (4 of 8)
7 in FXR-KO and 22 % (2 of 9) in WT (**Figure 6B**). All mice on indomethacin expressed
8 elevated gastric mRNAs for *Tnfα*, *IL1β* and *IL6* (data not shown) as compared to NaCl-treated
9 mice. Interestingly, only the indomethacin-treated WT mice showed an increase of gastric *Fxr*
10 and *K13* mRNAs (2-folds, *p<0.05) as compared to NaCl-treated littermates. These mRNAs did
11 not change in FXR-KO mice whether treated with indomethacin or NaCl (**Figure 6C**). Thus,
12 FXR-KO mice showed an enhanced susceptibility to gastric ulceration compared to WT mice.
13 Collectively, these findings *in vivo* confirmed our *in vitro* data that gastric FXR prevents
14 apoptosis and cell damage.

1 DISCUSSION

2

3 In the present study, we describe a novel role for bile acid-activated FXR in the protection of
4 human and murine gastric epithelial cells against inflammation-induced damage, and identified
5 K13 as a novel FXR target gene.

6

7 FXR mRNAs variants have been described in stomach tissue of human, rodents and other
8 species [15, 16]. We detected FXR α 1/2 and FXR α 3/4 mRNAs, as defined by Huber et al. [15],
9 and the ~56 kDa FXR protein in the non-neoplastic human stomach and in human GC cell lines.

10 In human GC specimens, FXR protein expression was most abundant in intestinal type GC
11 tissue with intestinal metaplasia (IM). IM is considered a potential preneoplastic lesion of
12 cancer of the upper GI tract that is known to be associated with bile acid reflux [2, 26, 35]. IM is
13 characterized by aberrant transcription factor regulation and altered gene expression [26, 36].
14 Recently, bile acids were shown to up-regulate intestinal differentiation markers (CDX2) and
15 the FXR-target gene SHP in human and rat gastric epithelial cell lines [37, 38]. Thus, our
16 findings are consistent with the previously described elevation of FXR expression in esophagitis
17 and in Barrett's intestinal metaplasia (IM) of the esophagus [14, 25]. Gastroesophageal reflux
18 disease (GERD) promotes IM in the gastric cardia [2] and contributes to Barrett's metaplasia
19 and esophageal carcinogenesis. Duodenogastric reflux, that occurs, for example as a
20 complication of surgical resection, increases the risk for GC [3]. Thus, bile acid-activated FXR
21 may be part of a protective defense reaction against the chronic irritation and inflammation of
22 the gastric mucosa, which is thought to facilitate the formation of intestinal type GC.

23

24 In support of this concept, we have shown that ectopic FXR confers resistance to inflammation-
25 induced apoptosis in the human GC cell line AGS, which is naturally devoid of FXR. We
26 identified K13, that together with other desmosomal components (K6, keratin associated protein
27 2-1, plakophilin 4) is up-regulated in response to FXR and its ligand CDCA, and showed that
28 K13 appears to mediate at least some of the anti-apoptotic effects of FXR. This particular
29 cluster of structural proteins suggests a possible role of FXR in maintaining the epithelial barrier

1 by preserving cell junctions. K13 is a differentiation marker of stratified mucosal epithelia and
2 is also expressed in human GC [39-42]. Consistent with the well-characterized regulation of
3 K13 by retinoids in the squamous epithelium [29-32], we discovered that K13 is up-regulated by
4 CDCA-activated FXR via a conserved retinoic acid-responsive element (RARE) *in vitro* and by
5 a CDCA-enriched diet *in vivo*. In addition to their structural functions, keratins, such as K8/18
6 in columnar epithelia, directly interfere with apoptotic signaling. Keratins interact with and
7 inhibit the death pathway components TNFR/Fas and TRADD/FADD [27, 43-47], suggesting a
8 direct mechanistic link between FXR and K13 to these proteins.

9

10 In further support of a protective role of gastric FXR, we found that tissue injury evoked by
11 indomethacin-mediated ulceration [18] of the gastric mucosa was more severe in FXR-deficient
12 mice compared to wild-type animals. This *in vivo* observation is consistent with previous
13 studies in mouse models of colitis and colon cancer, in which FXR-deficiency exacerbated both
14 inflammation [23] and tumorigenesis [10]. In those studies, it was suggested that one important
15 mechanism was FXR-mediated trans-repression of proinflammatory nuclear factor kappa B-
16 regulated genes in myeloid cells [10]. In our studies, both *Fxr* and *K13* mRNAs were
17 upregulated upon indomethacin-induced gastric ulceration, suggesting a similar protective role
18 in the non-neoplastic stomach.

19

20 In sum, our results suggest that FXR protects against inflammation-induced cell damage in the
21 normal stomach under stress conditions. As a consequence of chronic inflammation, FXR may
22 contribute to the development of intestinal type GC by promoting resistance to apoptosis in
23 transformed cells. Down-regulation of FXR in GC, similar to what has been described for
24 human colon [10] and Barrett esophageal [14] cancers, may be a secondary event that leads to a
25 loss of the protective functions exerted by FXR in non-malignant tissue.

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3

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6

7 **Competing interests:** None.

8

9 **Ethics approval:** Tissue sample collection of human GC specimens was approved by the Ethics
10 Committees of the Technische Universität München and the Charité Berlin. Animal housing and
11 experiments were performed in agreement with the ethical guidelines of the Technische
12 Universität München and the Ludwig Maximilians Universität München and were approved by
13 the appropriate government authorities.

14

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1 LEGENDS TO FIGURES

2

3 **Figure 1 Expression of FXR in human GC tissue**

4 (A) Detection of FXR on human GC tissue microarrays (n=70) by IHC using the rabbit
5 polyclonal Ab: N-N = normal non-neoplastic gastric epithelium with nuclear FXR (score 1+);
6 IM-T = Intestinal metaplasia in GC with strong nuclear and cytoplasmic FXR (score 2+); I-T =
7 Intestinal type GC positive for nuclear FXR (arrow, score 2+); D-T = Diffuse type GC with
8 nuclear FXR (arrow, score 1+). LP = lamina propria, GL = gland. Magnifications x100 and
9 x200. (B) Detection of ~56 kDa FXR protein in whole tissue lysates (25 µg protein per lane) of
10 GC specimens by Western Blot (WB) using the mouse monoclonal Ab; T = tumor, N = matched
11 non-tumor sample, P = patient, Con = normal liver.

12

13 **Figure 2 Expression of FXR in human GC cell lines**

14 (A) Detection of FXR α mRNA splice variants in human GC cell lines by RT-PCR and ethidium
15 bromide (EtBr) gel electrophoresis. Lanes numbers (upper panel): 1=AGS, 2=MKN45,
16 3=KATOIII, 4=SNU1, 5=SNU5, 6=N87; Lane numbers (lower left panel): 1=AGS, 2=SNU1,
17 3=N87, 4=KATOIII, 5=MKN7, 6=MKN45, 7=SNU5. Human hepatoma cells HepG2 were used
18 as a positive control (lower right panel). (B) Detection of ~56 kDa FXR protein in whole cell
19 and tissue lysates (25 µg protein per lane) by WB using a mouse monoclonal Ab. Lane
20 numbers: 1,2= hepatocellular carcinoma (HCC) from 2 individual patients, 3= SNU5, 4-
21 6=human HepG2 hepatoma cells from 3 independent culture passages; 7= normal human liver
22 tissue.(C) Quantification of total FXR α mRNA in SNU5 cells compared to other human GC
23 cell lines (AGS, SNU1, N87, KATOIII, MKN7, MKN45), HepG2 cells and human liver tissue.
24 Normalized CT-values from RT-qPCRs are -fold \pm S.E (n=3); *p<0.05 SNU5, HepG2, Liver
25 *versus* all remaining GC cell lines.

26

27

28

1 **Figure 3 FXR protects against apoptotic cell death**

2 (A) AGS clones were stably transfected with FXR (AGS/FXR) or empty vector (AGS/EV),
3 respectively, and treated for 24 h with TNF α +CHX (100 ng/ml+20 μ g/ml) in presence of
4 DMSO or CDCA (50 μ M). O.D. values from MTT assays are presented as % survival \pm S.E.
5 (n=4) of controls without TNF α +CHX; *p<0.05 CDCA *versus* vehicle, FXR *versus* EV.

6 (B) Detection of cleaved caspase 8, caspase 3 and PARP by WB. AGS clones were treated for 6
7 h (upper panel) or the times indicated (lower panel) with TNF α +CHX as in (A). (C) Flow
8 cytometric (FACS) analysis of apoptosis. AGS clones were treated for 6h as in (B). Annexin-
9 positive and PI-negative apoptotic cells were counted as % \pm S.E. (n=4) of total; *p<0.05 EV
10 *versus* FXR. Insert: WB validating FXR overexpression in AGS clones.

11

12 **Figure 4 Human K13 is a novel FXR-target gene *in vitro***

13 (A) CDCA induces mRNA of K13 and FXR-target genes. AGS clones were treated with 50 μ M
14 CDCA for 24 h, respectively. Representative EtBr agarose gels of RT-PCRs are shown above
15 quantitative analyses. Normalized CT-values of RT-qPCRs were calculated as -fold \pm S.E.
16 (n=3) of vehicle-treated EV cells; *p<0.05 CDCA *versus* DMSO. (B) CDCA upregulates K13
17 protein. Representative WBs are shown. (C) SiRNA knock-down of FXR or K13 reduces cell
18 survival. AGS/FXR cells were mock transfected or with siRNA for 24 h and then treated as in
19 (A). Data from MTT assays were calculated as in Figure 3; *p<0.05 mock *versus* RNAi, CDCA
20 *versus* DMSO (n=3). Insert: Knock-down efficiency of FXR/K13 mRNA and/or protein by
21 siRNAs compared to β -actin (WB) or β 2-microglobulin (RT-PCR).

22

23 **Figure 5 The human K13 promoter is bound and transactivated by FXR**

24 (A) Scheme of the human K13 gene locus with predicted binding sites for retinoic acid receptor
25 (RAREs). (B) ChIP. AGS/EV and AGS/FXR clones were treated for 24 h with vehicle or
26 CDCA (50 μ M). Cell lysates were subjected to IP with rabbit polyclonal FXR Ab or control IgG
27 as indicated (lanes 1 to 6). CT values of IP-ed DNA normalized to CT-values of input DNA are
28 expressed as -fold \pm S.E. (n=2) pull-down compared to control IgG. Agarose gels (left panel)

1 and quantitative summary of genomic PCRs (right panel) are presented together; * $p < 0.05$
2 CDCA (lane 2) *versus* DMSO (lane 1). (C) HEK293 cells were transiently cotransfected with
3 reporter plasmids containing the human proximal K13 promoter (RARE2-WT-pK13-luc), a
4 mutated K13 promoter (RARE2-MUT-pK13-luc) or a positive control plasmid containing a
5 cognate FXR-responsive element from the BSEP enhancer (FXR-RE-luc) together with FXR or
6 EV expression plasmids. Cells were then treated for 24 h with CDCA (0, 30, 100 μM).
7 Luciferase counts normalized to protein content are presented as -fold \pm S.E. (n=3) compared to
8 vehicle; * $p < 0.05$ CDCA *versus* DMSO. (D-E) EMSA. HEK293 cells were transfected with
9 FXR or EV expression plasmids and stimulated with CDCA (100 μM) for 16 h. Nuclear
10 extracts were incubated with biotin-labelled (D) inverted repeat-1 consensus FXR-binding
11 element (IR1-BIO) [20] or (E) K13 promoter oligonucleotide (RARE2-BIO). Reactions were
12 coincubated with rabbit polyclonal FXR Ab directed against the DNA-binding domain (lane 5)
13 or unlabelled competitor oligonucleotides (RARE2-WT, RARE2-MUT), respectively.

14

15 **Figure 6 FXR protects against indomethacin-induced gastric ulceration *in vivo***

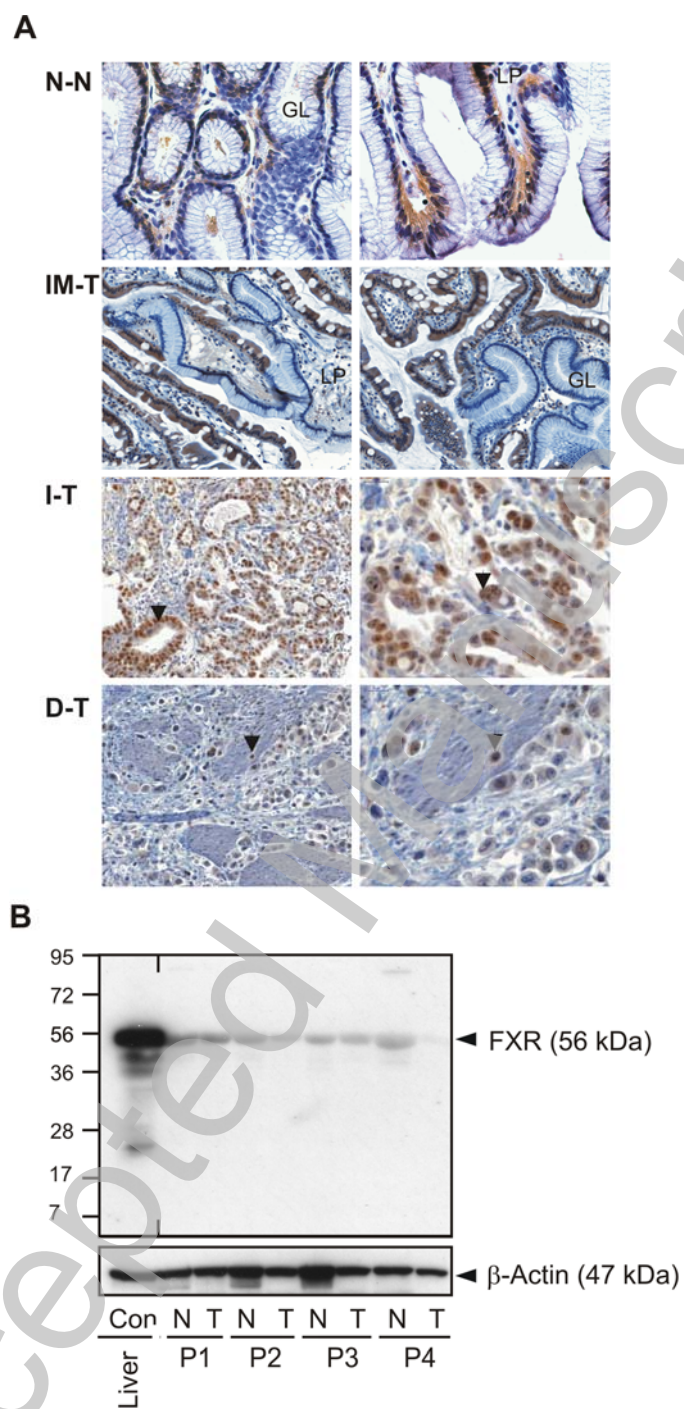
16 WT and FXR-KO C57BL/6 mice were injected i.p. with 35 mg/kg indomethacin (Indo) or
17 sodium chloride (NaCl) (n \geq 6 per group) for 24 h. (A) H&E staining with arrows mark the areas
18 of erosion and ulceration. (B) Left panel: Injury scores in H&E-stained sections of gastric
19 tissues, * $p < 0.05$ WT+Indo *versus* FXR-KO+Indo: score 0= no damage, score 1+ = superficial
20 gastritis, score 2+ = moderate discrete gastritis and erosions, score 3+ = severe gastritis with
21 elongated erosions and ulcerations (see also Table S4); Middle panel: Area of lesions in mm^2 ;
22 Right panel: Σ = total number of indomethacin-treated mice, U = number of mice with ulcers
23 per genotype. (C) Indomethacin up-regulates *K13* and *Fxr* mRNAs in stomachs of WT but not
24 FXR-KO mice. Normalized CT-values of RT-qPCRs are calculated as -fold \pm S.E.; * $p < 0.05$
25 NaCl *versus* Indo in the same genotype (n \geq 6 per group).

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**Figure 1**

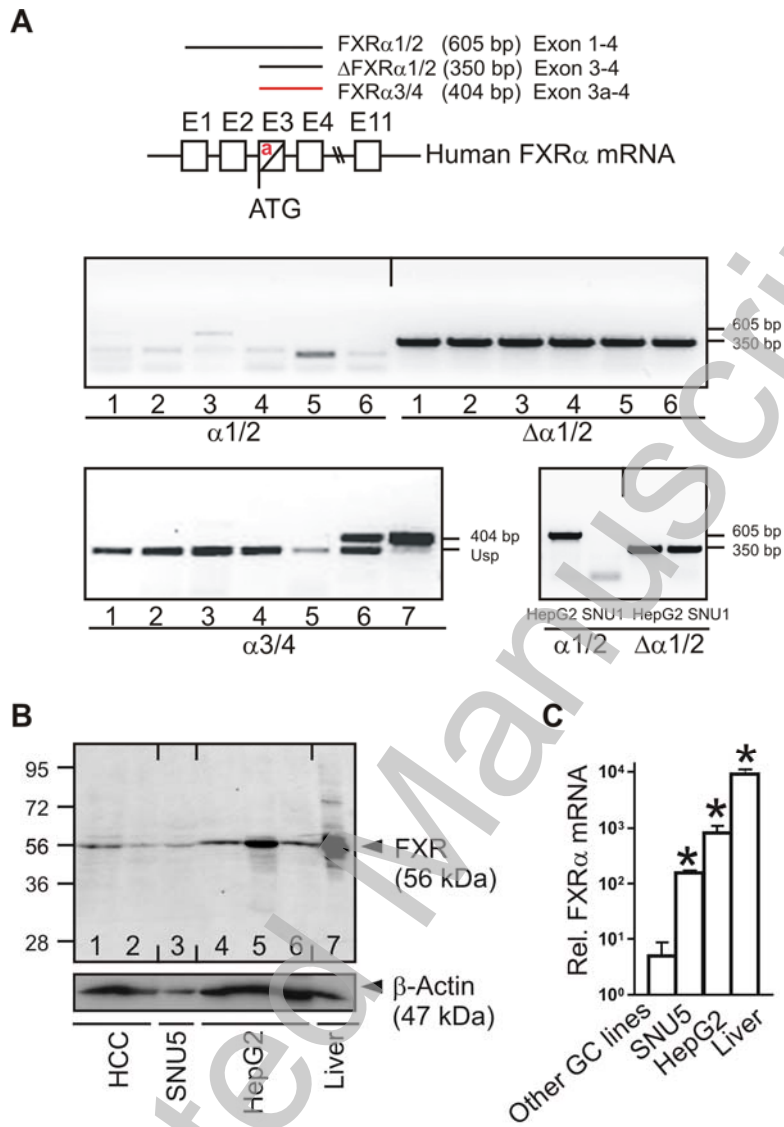


Figure 2

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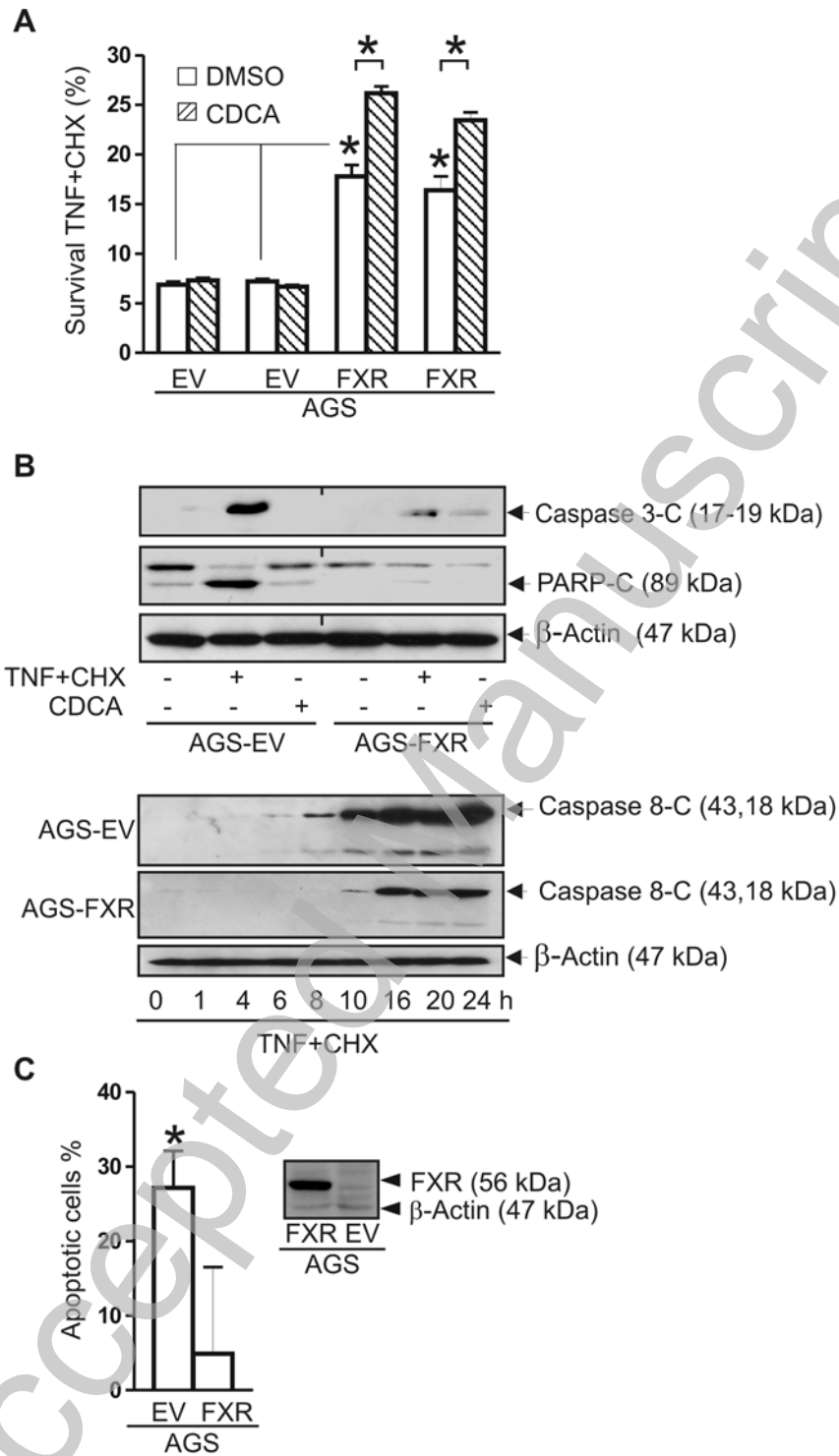


Figure 3

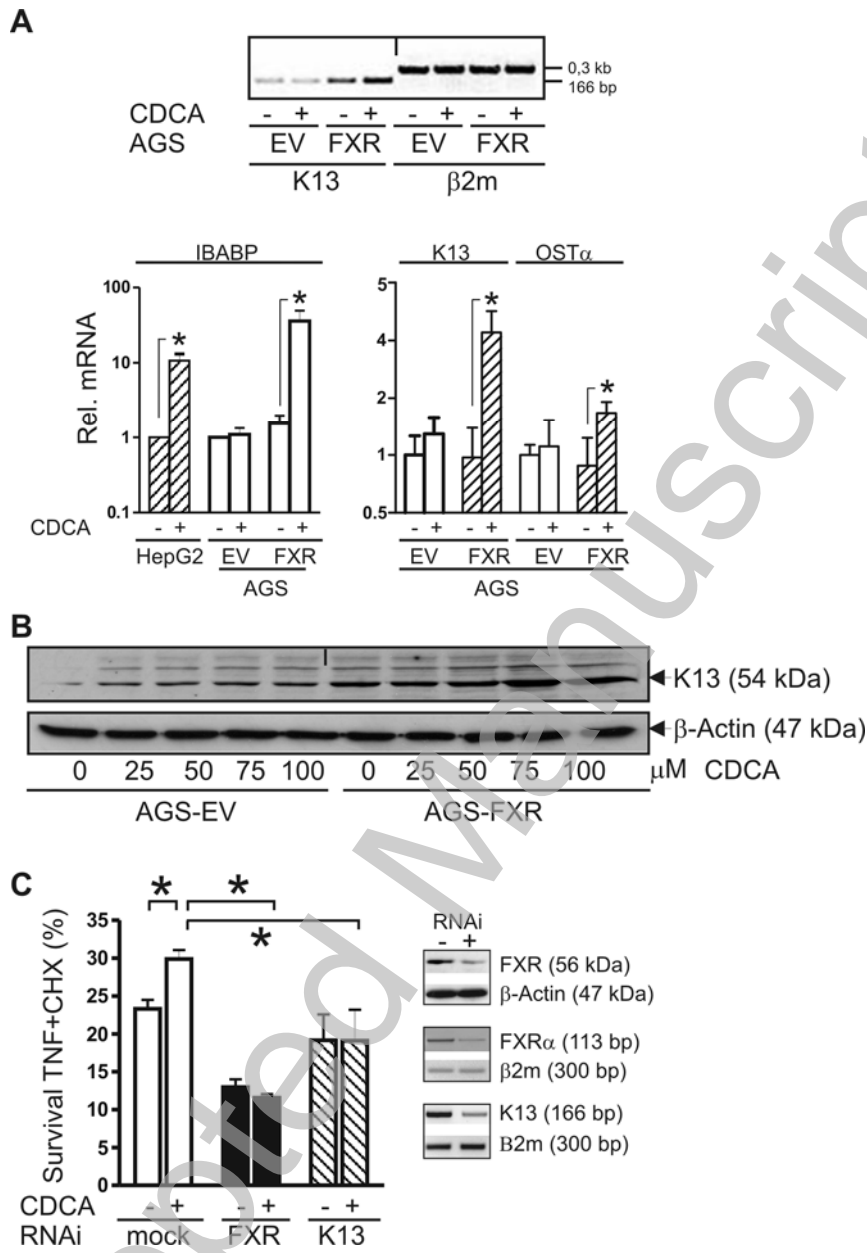


Figure 4

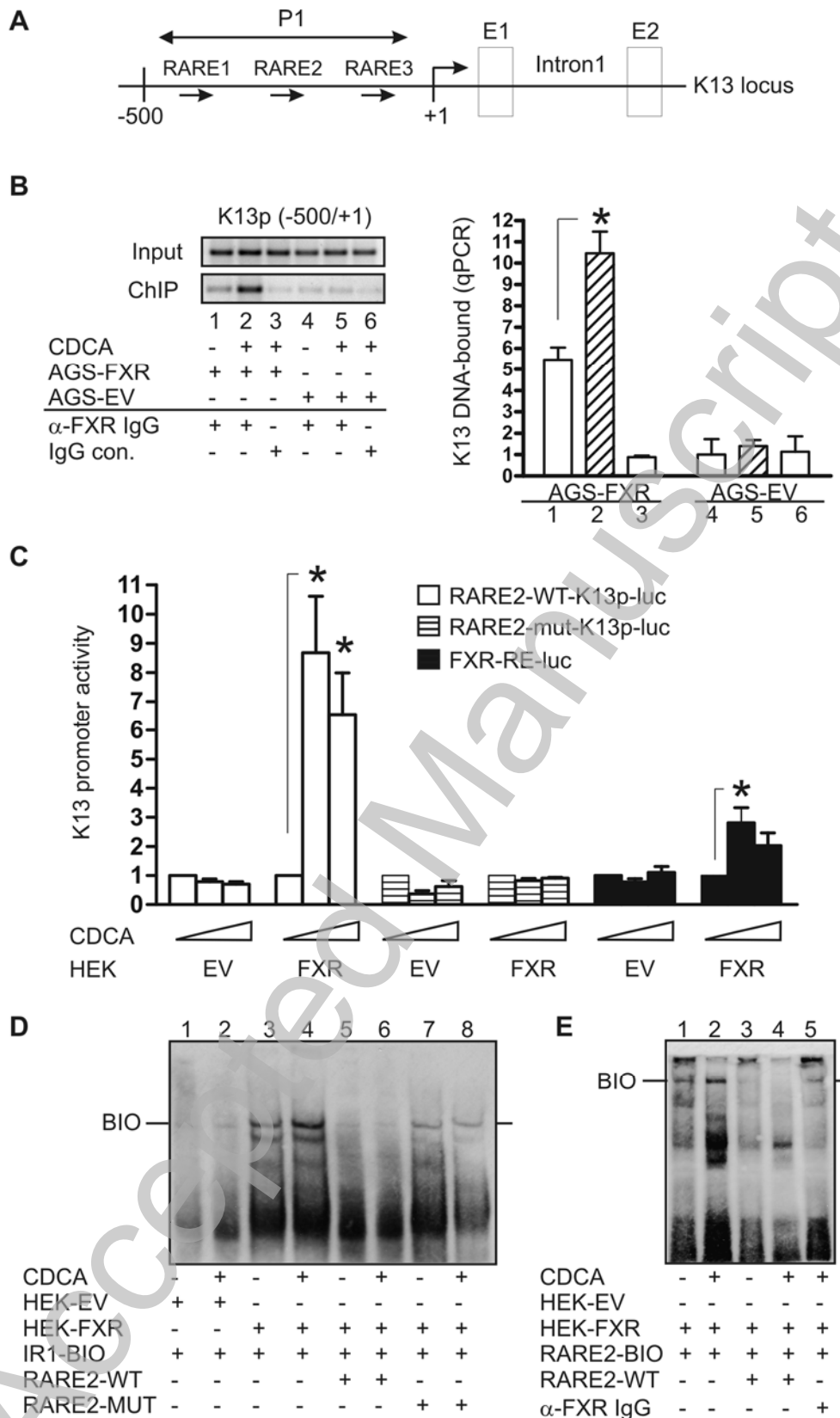


Figure 5

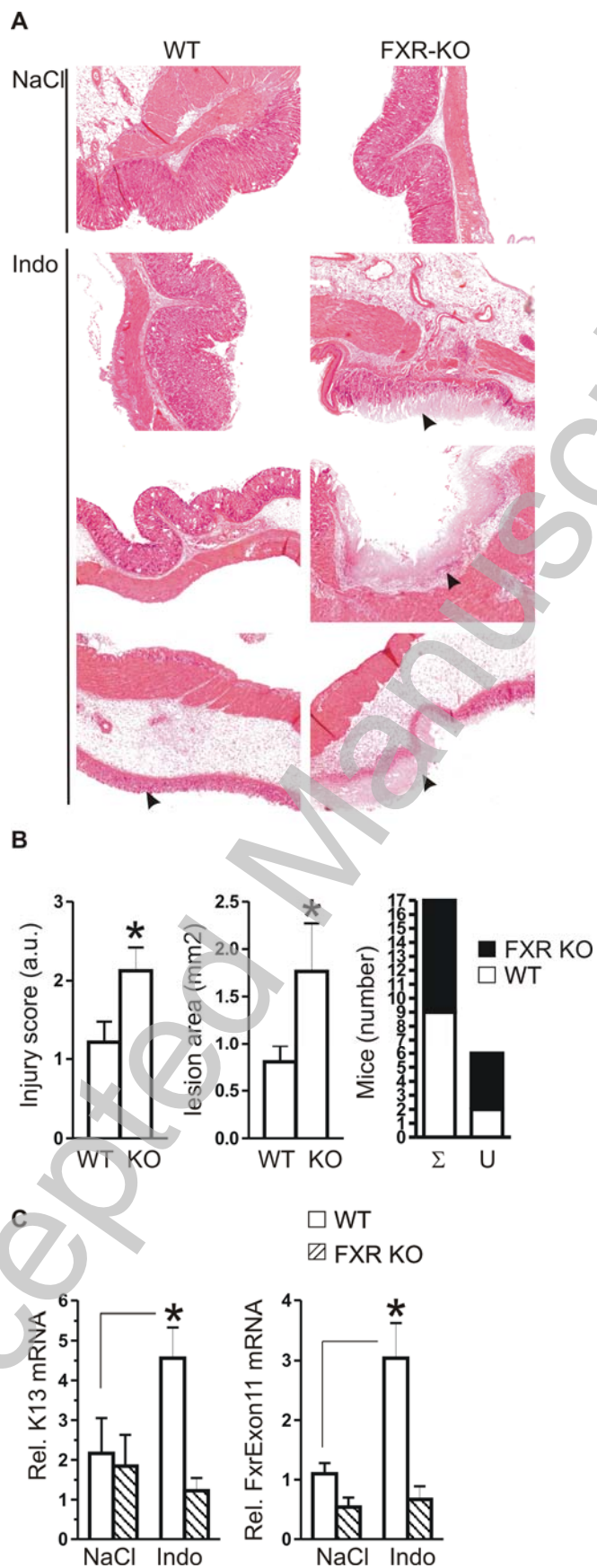


Figure 6