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FXR inhibits gankyrin in mouse livers and prevents development of liver cancer

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Abstract

One of the early events in development of liver cancer is a neutralization of tumor suppressor proteins Rb, p53, HNF4α and C/EBPα. The elimination of these proteins is mediated by a small subunit of proteasome, gankyrin, which is activated by cancer. The aim of this study was to determine mechanisms which repress gankyrin in quiescent livers and mechanisms of activation of gankyrin in liver cancer. We found that farnesoid X receptor, FXR, inhibits expression of gankyrin in quiescent livers by silencing the gankyrin promoter through HDAC1-C/EBPβ complexes. C/EBPβ is a key transcription factor which delivers HDAC1 to gankyrin promoter and causes epigenetic silencing of the promoter. We show that down-regulation of C/EBPβ in mouse hepatoma cells and in mouse livers reduces C/EBPβ-HDAC1 complexes and activates the gankyrin promoter. Deletion of FXR signaling in mice leads to de-repression of the gankyrin promoter and to spontaneous development of liver cancer at 12 months of age. DEN-mediated liver cancer in WT mice also involves the reduction of FXR and activation of gankyrin. Examination of liver cancer in old mice and liver cancer in human patients revealed that FXR is reduced; while gankyrin is elevated during spontaneous development of liver cancer. Searching for animal models with altered levels of FXR, we found that long-lived Little mice have high levels of FXR and do not develop liver cancer with age and after DEN injections due to failure to activate gankyrin and eliminate Rb, p53, HNF4α and C/EBPα proteins.

CONCLUSION—FXR prevents liver cancer by inhibiting the gankyrin promoter via C/EBPβ-HDAC1 complexes leading to subsequent protection of tumor suppressor proteins from degradation.

Keywords

Cancer; liver; gankyrin; C/EBP; HDAC1

The development of hepatocellular carcinoma (HCC) is a multistep process which includes the progressive alterations of gene expression leading to liver proliferation and to liver cancer.[1] The studies of liver regeneration after partial hepatectomy (PH) identified several critical steps of the initiation of the liver proliferation.[2] However, molecular mechanisms which trigger liver proliferation during development of liver cancer are not known. The quiescent stage of the liver is supported by a member of C/EBP family, C/EBPα.[3] Because three other tumor suppressor proteins p53, Rb and p16 protect liver from development of cancer[1], one would assume that the liver is well protected from the development of cancer.

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Moreover, the growth inhibitory activities of some of these proteins are increased with age.[3,4] Despite these activations, the frequency of liver cancer is increased with age[5,6] suggesting that the tumor suppressor proteins are eliminated by a specific mechanism. We recently found that the age-associated development of liver cancer is mediated by activation of gankyrin[5] which is a component of 26S proteasome.[7] Gankyrin also eliminates growth inhibitory activities of Rb, p53, and p16. Elimination of C/EBPα and Rb is mediated by a direct interaction of gankyrin with and subsequent degradation of these proteins.[5,8] Gankyrin-mediated elimination of p53 involves activation of MDM2 ligase which triggers degradation of p53 through UPS system.[9] Gankyrin also neutralizes p16 by the replacement of p16 from cdk4.[10]

Gankyrin has been first discovered as a small non-ATP subunit of 26S proteasome and as a protein which is increased in human hepatocellular carcinoma.[7,11] It has been shown that the development of liver cancer in animal models of carcinogenesis involves activation of gankyrin.[1,11] Moreover, the shRNA-mediated inhibition of gankyrin reduces the development of liver cancer in the nude mice.[12] Recent publications show that gankyrin expression is increased in colorectal carcinoma (CRC) samples, in pancreatic cancer and in human lung cancers.[13,14,15] In this paper, we show that of FXR represses gankyrin in quiescent livers and that liver cancer activates gankyrin via a release of this repression.

Experimental Procedures

Work with animals and human samples

Animal work is approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol AN-1439). Generation and characterization of FXR/SHP KO mice are described in previous paper.[15] Little mice are characterized in our previous reports.[16,17] Protein extracts from human liver tumors and from healthy patients were obtained from ORIGene company.

DEN-mediated liver carcinogenesis

Liver tumors were induced in WT and in Little mice by Diethylnitrosoamine tumor liver induction protocol (DEN) as described.[5] For FXR agonist treatment experiment, 8-week old mice were intraperitoneally injected with FXR agonist GW4064 (30mg/kg body weight). Control mice were injected with vehicle (corn oil).

Isolation of nuclear and cytoplasmic extracts and Western blotting

were performed as described our previous publications.[18, 19] A typical picture of the quality of the separation of cytoplasmic and nuclear proteins is shown in Supplemental Figure 2.

Reverse-Transcriptase PCR

Total RNA from liver tissues or Hep3B2 cells was extracted with RNeasy mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. cDNA was synthesized using SuperScript III First-strand (Invitrogen) and random primer hexamers. Sequences of primers used in these studies are presented in supplemental materials.

Chromatin Immunoprecipitation Assay

The chromatin immunoprecipitation assay (ChIP) assay was performed, as described in our previous articles[5,18] using the Chip-It kit.

EMSA Assay

EMSA assay was performed as described in our previous paper.[19]

Antibodies and Reagents

Antibodies to FXR (C20 and H130), gankyrin, C/EBPβ (C19), C/EBPα (A144), cdk4, cdc2, cyclin D3, Rb, p53 and HDAC1 (H-51) were from Santa Cruz Biotechnology. Antibodies to acetyl-histone H3 (Lys9) and histone H3 trimethyl Lys9 were from Abcam. Monoclonal anti-β-actin antibody was from Sigma. BrdU uptake assay kit was from Invitrogen. Co-immunoprecipitation studies were performed using TrueBlot reagents as described.[5,19]

Knockdown of C/EBPβin Hepa 1-6 cells and in liver

Hepa 1-6 cells were transduced with the shRNA-expressing lentivirus (Sigma-Aldrich, St. Louis, MO), and stable cell lines were generated by selection with puromycin for two weeks. For in vivo silencing experiments, 3-month old mice were injected through tail vein with C/EBPβ siRNA or non-target siRNA (50 μg of siRNA from Dharmcon complexed with in vivo-jet PEI, N/P ratio of 6, per mouse).

Results

The development of liver cancer in FXR/SHP KO mice involves activation of gankyrin and elimination of C/EBPα

FXR/SHP KO mice have hepatobilliary dysfunctions including increased liver proliferation[16] and development of liver cancer at age of 12 months (Anakk et al. MS submitted). Because gankyrin-mediated elimination of C/EBPα is one of the key events in development of liver cancer[5], we examined if this pathway is activated in livers of FXR/SHP KO mice. Figure 1A shows typical picture of liver of 17-month-old FXR/SHP KO mice with severe cancer. BrdU up-take confirmed that liver proliferation is increased in these animals (Figure 1B). Since C/EBPα needs to be phosphorylated at S193 by cdc2 and cdk4 to be degraded by gankyrin[5], we examined expression of C/EBPα, gankyrin, cdc2 and cdk4 in livers of FXR/SHP KO mice. Figure 1C shows that gankyrin is elevated in livers of FXR/SHP KO mice. The elevation of gankyrin correlates with elimination of C/EBPα protein, but not C/EBPα mRNA (Figure 1D). Protein levels of cdc2, cdk4 and cyclin D3 are increased in livers of FXR/SHP KO mice (Figure 1C). We next asked if gankyrin is activated in FXR/SHP KO mice at early stages of development of liver cancer. Examination of 6-month-old mice showed that gankyrin is significantly increased in livers of FXR/SHP KO mice; however, C/EBPα levels are only slightly reduced (Figures 1E). Because ph-S193 isoform of C/EBPα is a target of gankyrin, we suggested that the remaining 40-50% of C/EBPα might be not phosphorylated at S193. We previously showed that a phosphatase, PP2A, eliminates the phosphate from S193.[20] Our studies of FXR/SHP mice showed that PP2A is increased and ph-S193 isoform of C/EBPα is not detectable in nuclear extracts of livers of 6-month-old FXR/SHP KO mice (Figure 1E). We also found that the enzymes, which phosphorylate C/EBPα at S193, are weakly activated at this age of FXR/SHP KO mice (Supplemental Figure 1 A-B).

FXR is reduced and gankyrin is elevated in spontaneously developed mouse and human liver tumors

We next examined if spontaneous liver tumors might have reduced FXR. Western blotting with proteins from liver tumors of 24 months old mice showed a reduction of FXR and elevation of gankyrin (Figure 2A and B). Consistent with data in FXR/SHP KO mice, protein levels of C/EBPα are reduced in these tumor samples, while levels of C/EBPα mRNA are not changed (data not shown). We further examined expression of FXR, gankyrin and C/EBPα in livers of 4 patients with severe liver cancer and in 4 normal patients. Figure 2C shows that FXR is reduced to 15-20% in all examined tumor samples and that gankyrin is elevated in these samples. Western blotting showed that C/EBPα is

dramatically reduced in all human tumor samples. Thus, these studies revealed that spontaneous development of liver cancer in mice and in humans involves reduction of FXR, elevation of gankyrin and reduction of C/EBPα.

FXR inhibits expression of gankyrin

The search for the FXR binding sites showed no consensuses within 1.4 kb region of the mouse gankyrin promoter suggesting indirect mechanisms of the FXR-mediated repression of the promoter. Previous studies found that FXR directly binds to the C/EBPβ promoter[21] and that C/EBPβ-HDAC1 complexes are abundant in livers and repress C/EBP-dependent promoters.[19] Therefore, we hypothesized that FXR might repress the gankyrin promoter through C/EBPβ-HDAC1 complexes. We found that the gankyrin promoter contains two consensuses for C/EBPβ and that C/EBPα and C/EBPβ bind to the gankyrin promoter in vitro (Figure 3A-B). ChIP assay revealed that C/EBPα, C/EBPβ and HDAC1 occupy the gankyrin promoter in livers of WT animals. However, C/EBPβ and HDAC1 are not observed on the gankyrin promoter in livers of FXR/SHP KO mice (Figure 3C). In agreement with these data, the activation of FXR in cultured mouse Hepa 1-6 cells by ligands CDCA and GW4064 reduces levels of gankyrin protein (Figure 3D) and gankyrin mRNA (Figure 3E). We observed that activation of FXR also increases levels of C/EBPβ and surprisingly levels of HDAC1 (Figure 3D).

FXR-mediated inhibition of gankyrin requires C/EBPβ

We next examined if the inhibition of gankyrin involves C/EBPβ-HDAC1 complexes and found that activation of FXR in Hepa 1-6 cells increases amounts of the C/EBPβ-HDAC1 complexes (Figure 4A) and that C/EBPβ-HDAC1 complexes occupy the gankyrin promoter (Figure 4B). To examine if the FXR-dependent inhibition of gankyrin requires C/EBPβ, we generated two cell lines (C3a and C4a) expressing shRNA to C/EBPβ which dramatically inhibits C/EBPβ (Figure 4C). The activation of FXR by CDCA in the control clone inhibits expression of gankyrin; however, FXR fails to inhibit gankyrin in clones C3a and C4a (Figure 4D).

To determine if C/EBPβ is required for the repression of gankyrin in quiescent livers, we inhibited C/EBPβ by siRNA as it is shown in Figure 4E. The down-regulation of C/EBPβ leads to a significant reduction of C/EBPβ-HDAC1 complexes. The reduction of C/EBPβ-HDAC1 complexes correlated with the elevation of the gankyrin mRNA and protein (Fig 4E and F). These studies show that FXR represses the gankyrin promoter and that this repression requires C/EBPβ.

Gankyrin promoter is activated in liver cancer via a release of FXR-C/EBPβ-HDAC1 mediated repression

We next examined mechanisms which activate gankyrin during development of liver cancer after DEN injections. Since gankyrin is elevated at early steps of DEN-mediated cancer[5], we examined FXR-C/EBPβ-gankyrin pathway at days 2, 4 and 7 after DEN injection. FXR and C/EBPβ are reduced; while expression of gankyrin is elevated at days 2 and 4 (Figure 5A, upper). The decline of FXR and C/EBPβ leads to reduction of the C/EBPβ-HDAC1 complexes (Figure 5A, bottom). Examination of C/EBPβ and HDAC1 in FXR/SHP KO mice showed that, at the age of 12 mos, C/EBPβ expression is elevated in livers of these mice and amounts of C/EBPβ-HDAC1 complexes are also increased (Figure 5B). However, these complexes are not bound to the gankyrin promoter (Fig 5C). We next examined the status of the gankyrin promoter and found that C/EBPα/β-HDAC1 complexes occupy and repress the gankyrin promoter in quiescent liver since histone H3 is trimethylated at K9 on the promoter (Figure 5C). However, C/EBPβ and HDAC1 are removed from the gankyrin

promoter in livers of DEN-injected mice which leads to acetylation of histone H3 at K9. Consistent with these data, the gankyrin promoter is also activated in FXR/SHP KO mice.

Activation of FXR in DEN-treated mice inhibits elevation of gankyrin

To determine if the reduction of FXR is responsible for the elevation of gankyrin after DEN injections, we activated FXR by GW4064 and then treated mice with DEN. In control animals treated with corn oil, the expression FXR, C/EBPβ, HDAC1 and gankyrin is similar to that observed in mice without GW4064 treatments (Figure 5D). However, the activation of FXR by GW4064 supports high levels of C/EBPβ and C/EBPβ-HDAC1 complexes which correlate with the lack of activation of gankyrin (Figure 5E). ChIP assay revealed that the C/EBPβ-HDAC1 complexes occupy and repress the gankyrin promoter in GW4064-treated mice (Figure 5F).

Little mice express high levels of FXR and do not develop liver cancer after DEN-injection

Previous studies showed that long-lived Little mice have increased levels of genes involved in the xenobiotic detoxification and that crossing these mice with FXR knockout mice corrected their expression.[17] We performed Western blotting analysis and found a 4-5 fold elevation of the FXR in 24-36 month-old Little mice (Figure 6A and B). It has been shown that the frequency of liver tumor is increased with age and reaches around 30% at age of 24 months.[5] However, Little mice do not develop liver cancer with age. Therefore, we tested the hypothesis that high levels of FXR in old Little mice protect liver from development of cancer. WT and Little mice were treated with DEN and liver tumors were examined at 35-36 weeks after DEN injection. We have examined 5 WT mice and 5 Little mice and found that all WT animals have developed a severe liver cancer; while only two Little mice have few tumor nodules with a very small size (Figure 6C). Three other Little mice did not have liver cancer. Examination of liver sections by H&E staining revealed that the livers of wild type mice contain multiple diverse nodules of proliferating hepatocytes, including enlarged cells with moderate anisonucleosis on the left and a cluster of small uniform deeply basophilic cells to the right (Figure 6D). In contrast, livers of Little mice treated with DEN show unremarkable architecture and cytology with uniform hepatocytes containing minimal cytoplasmic lipid and glycogen. We found that number of replicating hepatocytes is significantly increased in WT mice (up to 25-30%); while around 5% of hepatocytes are BrdU positive in livers of Little mice (Figure 6E-F). These data show that Little mice are resistant to the development of liver cancer after DEN treatment.

Little mice do not activate gankyrin

We next determined molecular mechanisms by which Little mice are protected from the liver cancer. Recent report showed that gankyrin causes degradation of a liver specific transcription factor HNF4α[22]. Therefore, we included this protein in our studies. We found that gankyrin is elevated and it causes reduction of C/EBPα, Rb, HNF4α and p53 in control wild type mice (Figures 7A and B). FXR is slightly reduced in WT mice; however, in Little mice, FXR levels remain at high levels leading to the lack of activation of the gankyrin and to no reduction of C/EBPα, Rb, HNF4α and p53. The reduction of the tumor repressor proteins in WT mice takes place on the levels of protein degradation since levels of mRNA are not changed significantly (Figure 7C). To determine if gankyrin is responsible for the degradation of tumor suppressor proteins, we examined interactions of these proteins with gankyrin. In these experiments, we have used up to 1 mg of nuclear extracts for the Co-IP studies. These studies showed that the remaining C/EBPα, Rb, p53 and HNF4α are bound to gankyrin in WT mice; while these proteins are not detected in gankyrin IPs from Little mice (Fig 7A, bottom). Examination of the C/EBPβ and HDAC1 showed that C/EBPβ and HDAC1 are increased in livers of Little mice (Figure 7D). We found that the amounts of C/

EBPβ-HDAC1 complexes are higher in Little mice and that these complexes occupy and repress the gankyrin promoter in Little mice treated with DEN (Figure 7E).

Discussion

Gankyrin is a protein which is activated in liver cancer and which causes degradation or elimination of activities of five tumor suppressor proteins; Rb, p53, C/EBPα HNF4α, and p16.[1,5-7,22] This places gankyrin in a unique position to be a target for therapeutic approaches for prevention of liver cancer. In this paper, we elucidated mechanisms of activation of gankyrin during development of liver cancer. Four lines of evidence show that development of liver cancer involves the reduction of FXR and subsequent activation of gankyrin. First, DEN-mediated carcinogenesis in WT mice reduces FXR leading to the reduction of HDAC1-C/EBPβ complexes and activation of the gankyrin promoter. Second, the deletion of FXR signaling in FXR/SHP KO mice activates gankyrin in the liver leading to development of liver cancer. Third, high levels of FXR in Little mice prevent development of age-associated liver cancer and development of cancer under DEN protocol. Forth, levels of FXR are reduced in spontaneously developed mouse and human liver tumors; while gankyrin is elevated. Figure 7F summarizes our studies and presents our hypothesis, according to which the elevation of gankyrin triggers degradation of four tumor suppressor proteins and leads to the liver cancer. Based on literature data and on our observations, we suggest that the gankyrin-mediated elimination of C/EBPα is associated with phosphorylation at S193, while other proteins might be degraded by additional mechanisms such as activation of MDM2 (for p53) and direct interactions of gankyrin with Rb. These findings provide a basis for the generation of gankyrin-based therapeutic approaches to prevent liver cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FXR	farnesoid X receptor
C/EBP	CCAAT/Enhancer Binding Proteins
ChIP	chromatin immunoprecipitation assay
DEN	diethylnitrosoamine
EMSA	electrophoretic mobility shift assay

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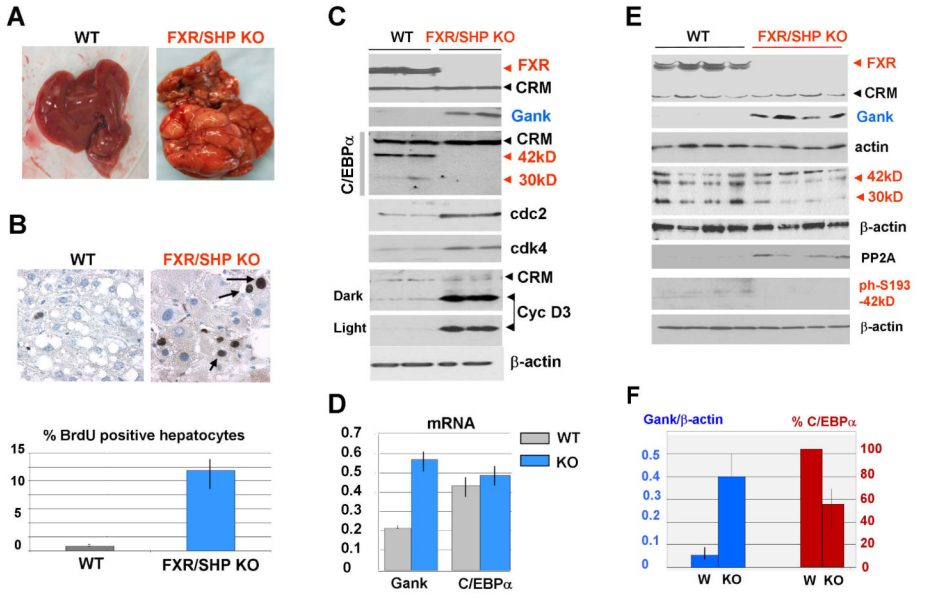

Figure 1. Development of liver cancer in FXR/SHP KO mice involves activation of gankyrin and elimination of C/EBPα

A. Liver tumors in 17-month-old FXR/SHP KO mice. B. A representative picture of BrdU staining of livers of WT and FXR/SHP KO mice. Arrows show BrdU positive hepatocytes. Bar graphs show a summary of BrdU staining for two animals. C. Expression of gankyrin is elevated in liver cancer samples from FXR/SHP KO mice. Western blotting was performed with antibodies to proteins shown on the right. Dark and light exposures are shown for cyclin D3. CRM; cross reactive protein and β-actin show protein loading. D. Levels of gankyrin and C/EBPα mRNA in livers of 17-month-old mice. E. Expression of gankyrin and C/EBPα in 6-month-old FXR/SHP KO mice. Liver proteins of WT and FXR/SHP KO mice were examined by Western blotting with Abs to FXR, gankyrin, cdk4, C/EBPα and with Abs to ph-S193 isoform of C/EBPα. Each membrane was reprobed with β-actin. F. Levels of gankyrin and C/EBPα are shown as a summary of three independent experiments.

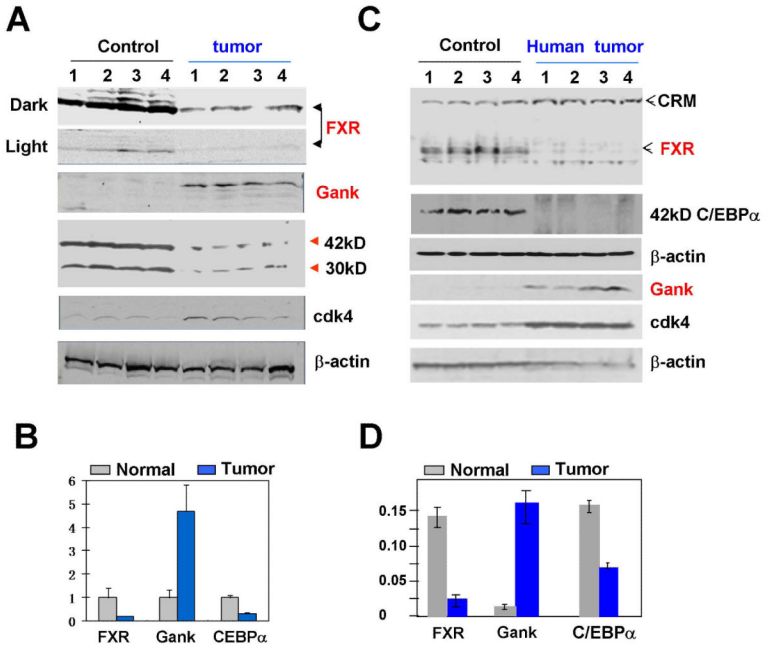

Figure 2. FXR is reduced in liver tumors of 24 mo old mice and in human liver tumors

A and C. Western blotting was performed with control livers and with liver tumor samples of mice (A) and humans (C) using antibodies to FXR, gankyrin, cdk4 and C/EBPα. **B and D.** Bar graphs show levels of the proteins as ratios to β-actin.

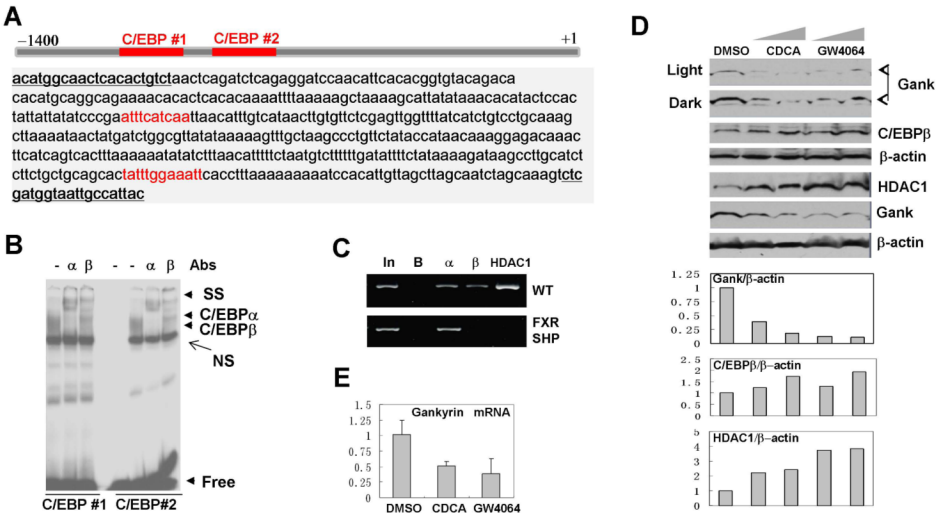

Figure 3. FXR inhibits expression of gankyrin

A. Gankyrin promoter contains two consensuses for C/EBP proteins. Upper. Two C/EBP consensuses within 1.4 kb gankyrin promoter are shown by red. **Bottom image**: Nucleotide sequence of the region containing C/EBP sites. The positions and sequences of primers used for ChIP assay are underlined. B. EMSA with C/EBP probes covering sites 1 and 2 using nuclear extracts from quiescent livers. Antibodies to C/EBPα and C/EBPβ were incorporated in binding reactions. Positions of C/EBPα, C/EBPβ, supershift (SS), non specific band (NS) and free probe are shown by arrows. **C. C/EBPβ**-HDAC1 complexes occupy gankyrin promoter in livers of WT mice, but are removed from the promoter in livers of FXR/SHP KO mice. ChIP assay was performed with chromatin solutions from WT quiescent livers and from livers of FXR/SHP KO mice. In; 1/100 of input, B; beads. D. Activation of FXR in mouse Hepa 1-6 cells increases levels of C/EBPβ and HDAC1 and inhibits expression of gankyrin. Western blotting was performed with protein extracts isolated from cells treated with DMSO (control) and with increasing concentrations of CDCA and GW4064. Bar graphs show levels of gankyrin, C/EBPβ and HDAC1 calculated as ratios to β-actin. E. FXR inhibits expression of gankyrin on the revel of mRNA. Expression of gankyrin mRNA was examined in mouse Hepa 1-6 cells treated with DMSO, CDCA and GW4064 by Q-RT-PCR.

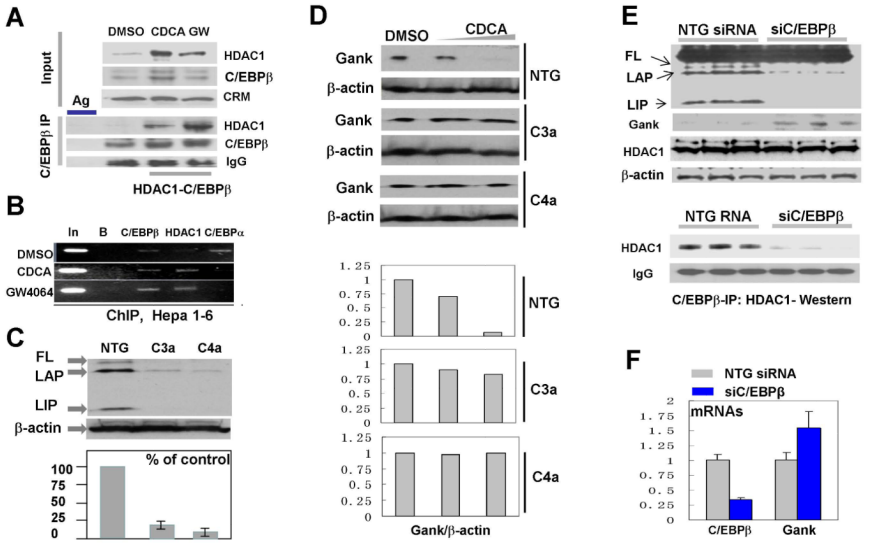

Figure 4. FXR-mediated inhibition of gankyrin requires C/EBPβ and C/EBPβ-HDAC1 complexes

A. Amounts of C/EBPβ-HDAC1 complexes are increased in cells with activated FXR. Input: Western blotting with nuclear extracts used for Co-IP studies. **C/EBPβ-IP**: C/EBPβ was immunoprecipitated from nuclear extracts and the IPs were probed with Abs to HDAC1 and C/EBPβ. Ag; agarose beads were incubated with nuclear extracts. CRM; cross-reactive molecule, serves as a loading control. B. Activation of FXR in Hepa 1-6 cells leads to the accumulation of the C/EBPβ-**HDAC1 complexes on the gankyrin promoter.** ChIP assay was performed with chromatin solutions from Hepa 1-6 cells treated with DMSO, CDCA and GW4064. In; 1/100 of input, B; beads. C. Expression of shRNA to C/EBPβ in stable clones inhibits C/EBPβ. Western blotting was performed with antibodies to C/EBPβ. Positions of full length (FL), LAP and LIP isoforms of C/EBPβ are shown by arrows. Bar graphs show the level of inhibition of C/EBPβ. **D. C/EBP**β is required for the FXR-dependent inhibition of gankyrin. Gankyrin was examined in control Hepa 1-6 cells (NTG) and in stable clones of Hepa 1-6 cells with inhibited C/EBPβ. Bar graphs show ratios of gankyrin to β-actin. **E. Knock-down of C/EBP**β in livers of WT mice activates expression of gankyrin. Expression of C/EBPβ and HDAC1 was examined in livers of mice were treated with control RNA (NTG) and with siRNA to C/EBPβ. **Bottom image:** C/EBPβ was immunoprecipitated from nuclear extracts and HDAC1 was examined in C/EBPβ IPs. **F. Expression of gankyrin and C/EBP**β **mRNAs in livers of siRNA-treated mice.**

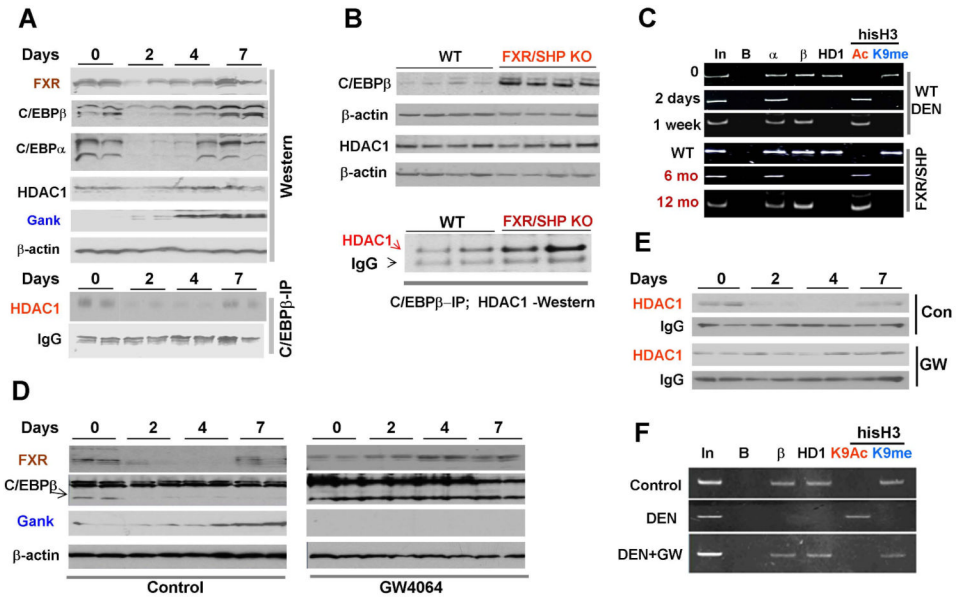

Figure 5. Gankyrin is activated in livers of DEN-treated WT mice via removing of C/EBPβ-HDAC1 complexes from the gankyrin promoter

A. Expression of FXR, C/EBPβ and gankyrin at early time points after injection of DEN. Western blotting was performed with nuclear extracts isolated at 2, 4 and 7 days after DEN injection. **Bottom image.** C/EBPβ was IP from nuclear extracts; and HDAC1 was determined in these IPs by Western blotting. IgG; heavy chains of IgGs are shown. **B. Expression of C/EBPβ and HDAC1 in FXR/SHP KO mice.** Western blotting was performed with nuclear extracts from livers of 4 mice of each genotype. **Bottom image:** C/EBPβ was immunoprecipitated from liver nuclear extracts of WT and FXR/SHP KO mice and IPs were probed with monoclonal antibodies to HDAC1. C. The gankyrin promoter is activated in livers of DEN-treated WT mice and in livers of FXR/SHP KO mice. ChIP assay was performed with chromatin solutions of WT, DEN-treated WT mice and FXR/SHP KO mice of different ages. D. Activation of FXR in DEN-treated mice prevents elevation of gankyrin. Expression of FXR, C/EBPβ and gankyrin was examined by Western blotting. E. Activation of FXR in DEN treated mice supports C/EBPβ-**HDAC1 complexes.** C/EBPβ was immunoprecipitated and HDAC1 was examined in these IPs. F. The gankyrin promoter is repressed in DEN-treated mice with activated FXR. ChIP assay was performed as described in legend to figure 5C.

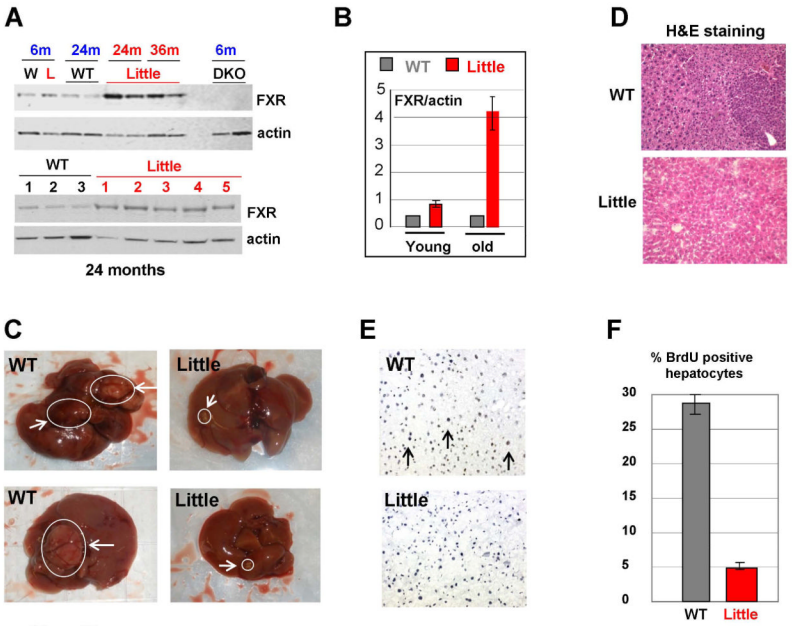

Figure 6. Long-lived Little mice contain high levels of FXR and are resistant to the DEN-mediated cancer

A. The elevation of FXR in livers of 24-36 month-old mice. Upper image shows Western blotting with proteins from livers of WT and Little mice of different ages. Nuclear extracts from FXR/SHP KO (DKO) livers serve as negative control. Bottom image shows Western blotting with five additional 24-month-old Little mice. **B. Levels of FXR were calculated as ratios to β-actin.** Bar graphs show a summary of multiple experiments with 9 mice of each genotype. C. Typical pictures of livers of WT and Little mice 35 weeks after DEN injection. Circles show the size of the tumor nodules. D. H&E staining of livers of WT and Little mice after DEN treatments. A typical picture of livers of WT and Little mice treated with DEN is shown. E. BrdU uptake in livers of WT and Little mice treated with DEN. Arrows show BrdU positive hepatocytes. F. Bar graphs show a summary of experiments with two animals of each genotype.

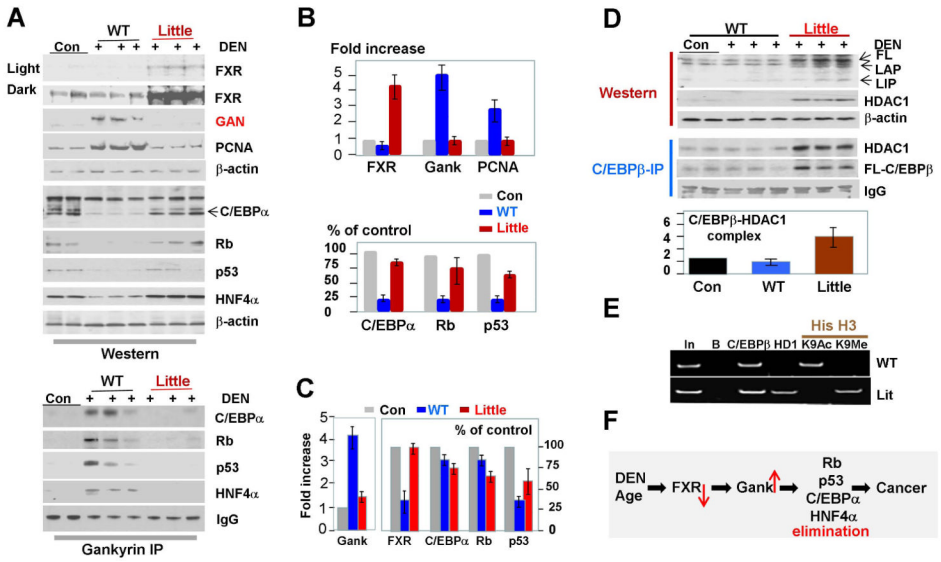

Figure 7. Inhibition of gankyrin by FXR prevents liver cancer in Little mice

A. Expression of proteins of FXR-gankyrin-pathway. Western blotting was performed with nuclear extracts using antibodies shown on the right. β-actin control is shown for the C/EBPα/Rb/p53 membrane. **Bottom image.** Gankyrin was immunoprecipitated and the IPs were probed with Abs to C/EBPα, p53, Rb and HNF4α. **B. Upper image.** Levels of FRX, gankyrin and PCNA were normalized to β-actin and calculated as a fold elevation compared to control WT animals. **Bottom image** shows levels of C/EBPα, p53 and Rb calculated as percentage of the levels observed in WT untreated mice. **C. Levels** of mRNA determined by qRT-PCR. D. Expression of C/EBPβ **and HDAC1 proteins and amounts of C/EBPβ-HDAC1 complex are increased in Little mice.** Western blotting shows levels of C/EBPβ and HDAC1. **C/EBPβ-IP:** C/EBPβ was immunoprecipitated from nuclear extracts and these IPs were probed with Abs to HDAC1 and to C/EBPβ. Bar graphs show amounts of C/EBPβ-HDAC1 complexes in WT and Little DEN treated mice as ratios to amounts in WT untreated livers. E. ChIP assay with gankyrin promoter in livers of WT DEN-treated mice and in Little DEN-treated mice. F. Hypothetical pathway by which liver cancer activates gankyrin and causes gankyrin-mediated degradation of tumor suppressor proteins.