Disruption of FBXL5-mediated cellular iron homeostasis promotes liver carcinogenesis

Yoshiharu Muto1*, Toshiro Moroishi1*, Kazuya Ichihara1, Masaaki Nishiyama1, Hideyuki Shimizu1, Hitotoshi Eguchi2, Kyoji Moriya3, Kazuhiko Koike4, Koshi Mimori1, Masaki Mori2, Yuta Katayama3, and Keiichi I. Nakayama1

Hepatic iron overload is a risk factor for progression of hepatocellular carcinoma (HCC), although the molecular mechanisms underlying this association have remained unclear. We now show that the iron-sensing ubiquitin ligase FBXL5 is a previously unrecognized oncosuppressor in liver carcinogenesis in mice. Hepatocellular iron overload elicited by FBXL5 ablation gave rise to oxidative stress, tissue damage, inflammation, and compensatory proliferation of hepatocytes and to consequent promotion of liver carcinogenesis induced by exposure to a chemical carcinogen. The tumor-promoting outcome of FBXL5 deficiency in the liver was also found to be effective in a model of virus-induced HCC. FBXL5-deficient mice thus constitute the first genetically engineered mouse model of liver carcinogenesis promoted by iron overload. In addition, dysregulation of FBXL5-mediated cellular iron homeostasis was found to be associated with poor prognosis in human HCC, suggesting that FBXL5 plays a key role in defense against hepatocarcinogenesis.

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal and prevalent cancers worldwide. The molecular, cellular, and environmental underpinnings of HCC development and progression remain unclear, however, and few therapeutic options are available. Prominent factors associated with HCC include chronic hepatitis B or C virus infection, long-term alcohol intake, consumption of aflatoxin B1–contaminated food, and metabolic disorders such as nonalcoholic fatty liver disease and hereditary hemochromatosis (El-Serag, 2011). These factors are also associated with liver damage due to inflammation and oxidative stress (Farazi and DePinho, 2006). Oxidative stress in the liver promotes cell death and subsequent compensatory proliferation of hepatocytes (Kamata et al., 2005), with these effects being associated with modification of intracellular signaling pathways and the accumulation of genetic alterations that eventually lead to malignant progression (Maeda et al., 2005; Luedde et al., 2014; Marquardt et al., 2015). Homeostasis of cellular iron, a major elicitor of oxidative stress, is therefore likely integral to defense against hepatocarcinogenesis.

Iron is an essential factor for many metabolic processes in cells and organisms and also functions as cofactors such as heme and iron-sulfur clusters. On the other hand, iron catalyzes the Fenton reaction that results in generation of the hydroxyl radical, one of the most detrimental ROS in vivo that damages many biological macromolecules. It is therefore important that cellular iron levels undergo strict regulation (Hentze et al., 2010). Cellular iron level was shown to be regulated predominantly by the box and leucine-rich repeat protein 5 (FBXL5) and iron regulatory protein 2 (IRP2) in vivo (Moroishi et al., 2011). IRP2 is an RNA-binding protein that regulates the translation and stability of mRNAs encoding proteins that contribute to cellular iron homeostasis. Based on these actions, IRP2 regulates the size of the available iron pool in a manner dependent on cellular iron concentration. Under iron-replete conditions, the SCF<sub>FBXL5</sub> E3 ubiquitin ligase complex mediates the ubiquitylation and degradation of IRP2, with FBXL5 serving as the substrate recognition component for IRP2. Binding of iron to the hemerythrin domain of FBXL5 stabilizes this protein, whereas FBXL5 is unstable under iron-deficient conditions. This iron-sensing ability...
allows FBXL5 to control the abundance of IRP2 in an iron-dependent manner (Salahudeen et al., 2009; Vashishth et al., 2009). Mice deficient in FBXL5 manifest the failure of cells to sense increased cellular iron availability, resulting in constitutive accumulation of IRP2 and aberrant expression of its target genes. FBXL5-null mice show early embryonic mortality as a result of overwhelming oxidative stress, suggesting the essential role of FBXL5 in cellular iron homeostasis during early embryogenesis (Moroishi et al., 2011; Ruiz et al., 2013).

A substantial proportion of iron in the adult body is present in the hematopoietic system and liver. Conditional deletion of Fbxl5 in the hematopoietic system of mice results in iron overload in hematopoietic stem cells, with the consequent increase in oxidative stress impairing the ability of the cells to repopulate bone marrow (Muto et al., 2017). FBXL5 expression was also shown to be down-regulated in hematopoietic stem cells of some patients with myelodysplastic syndrome, suggesting that disruption of cellular iron homeostasis might contribute to this disease (Nilsson et al., 2007; Muto et al., 2017). In addition, conditional FBXL5 deficiency in mouse liver was found to result in iron accumulation and mitochondrial dysfunction in hepatocytes, leading to the development of steatohepatitis (Moroishi et al., 2011).

Iron homeostasis in the liver has been implicated in the pathogenesis of liver cancer. Individuals with hereditary hemochromatosis manifest iron overload in the liver and other organs and have a 200-fold greater risk of HCC development compared with the general population (Niederau et al., 1996; Bacon et al., 2011). Down-regulation of hepcidin expression is a characteristic finding in the liver of hemochromatosis patients and has been shown to be the cause of hepatic iron overload and subsequent hepatocarcinogenesis in such patients (Bacon et al., 2011). Hepatic iron excess is also a risk factor for HCC development in patients with nonalcoholic fatty liver disease (Sorrentino et al., 2009; Valenti et al., 2010) or those infected with hepatitis C virus (HCV; Chapoutot et al., 2000), with iron depletion therapy by phlebotomy and low-iron diet therapy having been shown to lower the risk for HCC development in individuals with chronic HCV infection (Kato et al., 2007). In addition, hepatic iron accumulation or elevated serum iron levels have also been associated with chronic hepatitis B virus (HBV) infection (Feltón et al., 1979; Senba et al., 1985) and to be correlated with the severity of HBV-related hepatitis (Martinelli et al., 2004; Mao et al., 2015). Furthermore, hepatic accumulation of iron was found to be predictive of HCC development (Nahon et al., 2008) or death (Ganne-Carrié et al., 2000) in patients with alcoholic cirrhosis. A clinical association between aflatoxin-induced HCC and iron overload has not been described to date, although exposure to aflatoxin B1 and dietary iron overload were shown to have a synergistic effect on the generation of oxidative stress or DNA damage in rat liver (Asare et al., 2007).

The abundance of hepcidin or FBXL5 mRNAs was found to be reduced in the liver of patients with chronic HCV infection (Fujita et al., 2007; Girelli et al., 2009; Nanba et al., 2016), further suggesting that altered iron homeostasis may be permissive for HCC in such patients. Moreover, hepcidin expression is down-regulated in the FBXL5-deficient mouse liver as a result of altered bone morphogenetic protein signaling (Moroishi et al., 2011), suggesting that the reduced amount of hepcidin mRNA in the liver of patients with chronic HCV infection might also result from dysregulation of FBXL5. In contrast, the abundance of hepcidin is increased in the liver of individuals with nonalcoholic steatohepatitis (Britton et al., 2016) or chronic HBV infection (Tseng et al., 2009), suggesting that down-regulation of hepcidin expression is not a universal mechanism for hepatic iron overload in chronic liver disease and that additional mechanisms remain to be elucidated.

Despite these various lines of evidence implicating iron dysregulation as a risk factor for liver cancer, the lack of a genetically engineered mouse model of hepatocarcinogenesis induced by hepatic iron overload has hampered research into pathogenic mechanisms. Here we provide evidence that altered FBXL5-mediated cellular iron homeostasis is associated with liver carcinogenesis, and that FBXL5-deficient mice constitute a genetically engineered mouse model of carcinogenesis promoted by dysregulation of iron homeostasis. We hypothesized that dysregulation of FBXL5 might promote the development of HCC on the basis of the previous epidemiologic and clinical findings.

To validate such a promoting effect on tumorigenesis, we adopted the diethylnitrosamine (DEN)-induced HCC model, given that a single DEN injection acts as an initiator of hepatocarcinogenesis (Sarma et al., 1986). Hepatocellular iron overload evoked by FBXL5 ablation in mice gave rise to oxidative stress, tissue damage, inflammation, and compensatory proliferation of hepatocytes as well as to consequent promotion of liver carcinogenesis induced by DEN exposure. This tumor-promoting effect of FBXL5 deficiency was confirmed in a model of carcinogenesis induced by expression of HCV core antigen. Furthermore, we found that a low level of FBXL5 expression in HCC patients is associated with poor prognosis, suggesting that the FBXL5–IRP2 axis is a potential therapeutic target for HCC associated with cellular iron dysregulation.

**Results**

**FBXL5 is essential for defense against HCC**

To explore the role of cellular iron homeostasis governed by FBXL5 in hepatocarcinogenesis, we generated mice in which Fbxl5 is specifically deleted in the liver by crossing Fbxl5<sup>F/F</sup> mice (which harbor floxed alleles of Fbxl5) with Alb-Cre transgenic mice (which express Cre recombinase under the control of the albumin gene promoter). The resulting Alb-Cre/Fbxl5<sup>F/F</sup> mice do not develop liver tumors spontaneously until ~750 d of age (data not shown), but they accumulate iron in the liver and develop steatohepatitis (Moroishi et al., 2011). We therefore investigated whether FBXL5 deficiency promotes liver carcinogenesis in association with exposure to a chemical carcinogen. A single administration of DEN to weaning male mice induces acute liver injury as well as the formation of multiple liver tumors within 10 mo (Sarma et al., 1986). Male Alb-Cre/Fbxl5<sup>F/F</sup> and Fbxl5<sup>F/F</sup> littermate control mice were thus injected i.p. with DEN at 15 d of age, and the resulting hepatic tumor nodules were examined at 36 wk of age (Fig. 1 A). Alb-Cre/Fbxl5<sup>F/F</sup> mice manifested a
higher tumor incidence and increased tumor size compared with Fbxl5<sup>F/F</sup> mice (Fig. 1 B). We also confirmed that FBXL5 deficiency promoted tumorigenesis in DEN-injected mice at 32 wk of age (Fig. S1). Histopathologic analysis of hepatic tissue sections also confirmed the increased number and size of tumorous lesions in the liver of Alb-Cre/Fbxl5<sup>F/F</sup> mice (Fig. 1 C). Of note, hepatic FBXL5 deficiency also promoted liver tumor development in female mice (Fig. 1 B), although females are less susceptible to DEN-induced liver carcinogenesis than are males (Naugler et al., 2007). Together, these data thus suggested that hepatic iron homeostasis governed by FBXL5 plays a prominent role in prevention of hepatocarcinogenesis.

To exclude the possibility that DNA damage potentially induced by Cre recombinase promotes liver carcinogenesis induced by exposure to DEN, we injected male Alb-Cre/Fbxl5<sup>F/F</sup> mice with DEN at 15 d of age and examined the incidence of hepatic tumor formation at 36 wk of age. Alb-Cre/Fbxl5<sup>F/F</sup> mice developed tumors with a number and size similar to those for Fbxl5<sup>F/F</sup> mice (Fig. S2 A). The survival rate of the DEN-injected Alb-Cre/Fbxl5<sup>F/F</sup> mice was also similar to that of control Fbxl5<sup>F/F</sup> mice (Fig. S2 B). We therefore concluded that Cre recombinase alone does not influence hepatic tumorigenesis in our experimental system.

Given that impaired degradation of IRP2 is primarily responsible for the embryonic mortality of Fbxl5<sup>−/−</sup> mice (Moroishi et al., 2011), we next investigated whether the promotion of carcinogenesis in the liver of Alb-Cre/Fbxl5<sup>F/F</sup> mice might also be attributable to IRP2 accumulation. Examination of DEN-induced carcinogenesis in the liver of male Fbxl5<sup>−/−</sup>Ir<sup>P2<sup>−/−</sup> mice revealed that the number and size of tumors at 36 wk of age were similar to those for Fbxl5<sup>F/F</sup> control mice (Fig. 1 B). Similar effects of IRP2 ablation on tumor promotion associated with FBXL5 deficiency were also observed in female mice (Fig. 1 B). This genetic evidence thus indicated that aberrant IRP2 activity in Alb-Cre/Fbxl5<sup>F/F</sup> mice is responsible for the tumor-promoting effect of FBXL5 ablation, with FBXL5 protecting the liver from carcinogenesis by suppressing IRP2 activity.

We next examined whether FBXL5 deficiency in the liver influences the survival of DEN-injected mice. The survival rate of male or female Alb-Cre/Fbxl5<sup>F/F</sup> mice was significantly
reduced compared with that for control Fbxl5+/f mice, whereas that of Fbxl5−/−Irp2−/− mice was similar to that for control mice (Fig. 2). These results thus showed that the poor prognosis of DEN-injected Alb-Cre/Fbxl5+/f mice is dependent on the accumulation of IRP2 that results from the lack of Fbxl5-mediated degradation of this protein.

Hepatic Fbxl5 deficiency elicits iron overload and oxidative stress
To determine whether cellular iron metabolism is dysregulated in both HCC and nontumor liver tissue of DEN-injected Alb-Cre/Fbxl5+/f mice, we examined the expression of IRP2 and its target proteins. Immunoblot analysis revealed that IRP2 accumulated in both nontumor liver tissue and HCC of Alb-Cre/Fbxl5+/f mice (Fig. 3 A). Consistent with this finding, expression of transferrin receptor 1 (TfR1), which is indicative of IRP2 activity, was also increased in nontumor liver tissue and HCC of the Fbxl5-deficient mice compared with control Fbxl5+/f mice (Fig. 3 A). Staining for both ferrous iron (Fe2+) and ferric iron (Fe3+) revealed marked accumulation of cellular iron in the liver of DEN-injected Alb-Cre/Fbxl5+/f mice, with the extent of iron accumulation in HCC being less pronounced than that in nontumor tissue (Fig. 3 B) despite the higher level of TfR1 expression in HCC (Fig. 3 A). The rapid proliferation of cancer cells might result in dilution of cellular iron. Indeed, these observations are consistent with previous results showing that iron depletion is a hallmark of HCC (Terada and Nakanuma, 1989; Deugnier et al., 1993). Collectively, our findings thus suggested that cellular iron metabolism is dysregulated in both HCC and nontumor liver tissue of Alb-Cre/Fbxl5+/f mice.

We next examined whether cellular iron overload in DEN-injected Alb-Cre/Fbxl5+/f mice induces oxidative stress in the liver. RT and real-time PCR analysis revealed significant up-regulation of mRNAs for proteins related to the NRF2-mediated oxidative stress response—such as NADPH quinone oxidoreductase 1 (Nqo1), heme oxygenase 1 (Hmox1), glutathione S-transferase A1 (Gstal), and metallothionein 2 (Mt2)—in nontumor liver tissue of the Fbxl5-deficient mice compared with that of control mice (Fig. 3 C). The abundance of Nqo1, Hmox1, and Gstal mRNAs was also increased in the tumors of Alb-Cre/Fbxl5+/f mice. The amounts of these mRNAs did not differ significantly between tumor and nontumor tissue of the Alb-Cre/Fbxl5+/f mouse liver, however, suggesting that a mechanism in addition to oxidative stress might increase the mutation rate of Fbxl5-deficient tumor cells. This notion is consistent with the fact that sequencing analysis of human HCC and experimentally induced rat or mouse HCC has revealed that the NRF2 gene is commonly mutated in the region encoding the domain of the transcription factor that interacts with its negative regulator Keap1 (Guichard et al., 2012; Zavattari et al., 2015; Ngo et al., 2017). We also tested whether expression of Cre recombinase alone activates NRF2 target genes in the liver by examining DEN-injected male Alb-Cre/Fbxl5+/f and Fbxl5+/f mice. Expression of the NRF2 target genes for Hmox1 and Nqo1 was similar for the two genotypes (Fig. S2 C), suggesting that Cre recombinase alone does not directly affect NRF2 activity in the liver. We further confirmed that Hmox1 expression was up-regulated at the protein level in nontumor liver tissue and HCC of Alb-Cre/Fbxl5+/f mice (Fig. 3 A). These results thus suggested that oxidative stress is induced in the liver of Alb-Cre/Fbxl5+/f mice.

ROS levels detected with the cell-permeable reagent 2′,7′-dichlorodihydrofluorescein diacetate or by deposition of 4-hydroxy-2-nonenal did not differ significantly between Fbxl5-deficient and control hepatocytes (data not shown), despite the significant activation of oxidative stress response genes apparent in the former (Fig. 3 C). We previously showed that acute dietary iron overload induces marked 4-hydroxy-2-nonenal deposition and massive cell death in the Fbxl5-deficient mouse liver (Moroiishi et al., 2011). The activation of ROS scavenger genes in the Fbxl5-deficient liver in the absence of acute dietary iron overload might limit oxidative stress to a level below that detectable by this method. Our findings thus suggested that chronic iron-induced oxidative stress at even a moderate level in the Fbxl5-deficient liver promotes carcinogenesis.

Fbxl5 deficiency induces the DNA damage response and inflammation in the liver
To explore in more detail the mechanism by which cellular iron overload promotes liver carcinogenesis, we performed RNA sequencing (RNA-seq) analysis for liver tumors and corresponding nontumor tissue in DEN-injected control and Alb-Cre/Fbxl5+/f mice. Consistent with our RT and real-time PCR data (Fig. 3 C), gene set enrichment analysis (GSEA) of the RNA-seq data indicated that the expression of NRF2 target genes is increased in both HCC and nontumor tissue of the Fbxl5-deficient liver.

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compared with the control liver (Fig. 3 D), suggesting that oxidative stress is indeed increased by the loss of FBXL5. The induction of DNA damage by oxidative stress contributes to the accumulation of somatic mutations and carcinogenesis in the liver (Farazi and DePinho, 2006; Cichoź-Lach and Michalak, 2014). Indeed, GSEA suggested that the G2-M checkpoint, the activation of which is a hallmark of the DNA damage response, is activated to a greater extent in FBXL5-deficient liver tumors than in control tumors (Fig. 4, A and B). Consistent with this finding, the number of somatic mutations found in tumor regions but not in nontumor liver tissue of the same animals was greater for Alb-Cre/Fbxl5F/F mice than for Fbxl5F/F mice (Fig. 4 C). These results confirmed that FBXL5 deficiency induces harmful oxidative stress and induction of the DNA damage response in the DEN-exposed liver.
Oxidative stress or tissue damage induces inflammation in the liver, which in turn further promotes tissue damage and can lead to carcinogenesis (Farazi and DePinho, 2006). Consistent with this notion, GSEA revealed an augmented inflammatory response in the FBXL5-deficient liver (Fig. 4, D and E). RT and real-time PCR analysis also confirmed that the expression of genes for several inflammatory cytokines—including TNFα, C-C motif chemokine ligand 2 (CCL) 2, and CCL5—was increased in the liver of Alb-Cre/Fbxl5F/F mice (Fig. 4 F). These cytokines were all previously shown to contribute to hepatocarcinogenesis in mice (Knight et al., 2000; Li et al., 2017; Mohs et al., 2017). Together, these data indicated that hepatic FBXL5 deficiency induces toxic oxidative stress that promotes DNA damage and chronic inflammation in the liver.

**Hepatic FBXL5 deficiency promotes liver damage and compensatory cell proliferation**

Oxidative stress also promotes hepatocyte cell death and chronic liver damage (Kamata et al., 2005; Cichoż-Lach and Michalak, 2014). We investigated whether oxidative stress in the liver of Alb-Cre/Fbxl5F/F mice gives rise to tissue damage. The activity of alanine aminotransferase (ALT) in serum at 48 h after DEN injection at 15 d of age did not differ between Alb-Cre/Fbxl5F/F and control mice (Fig. 5 A), suggesting that acute DEN-induced hepatotoxicity as the initiating event of carcinogenesis was not exacerbated by FBXL5 deficiency. In contrast, serum ALT activity at 20 or 36 wk of age was significantly higher in Alb-Cre/Fbxl5F/F mice that had been injected with DEN at 15 d of age than in corresponding controls (Fig. 5 B), indicative of chronic liver damage in the FBXL5-deficient animals. Consistent with these findings, GSEA revealed increased expression of genes related to the cell death pathway activated in response to DNA damage in the FBXL5-deficient liver (Fig. 5 C). Chronic liver damage in hemochromatosis model mice has been associated with ferroptosis, a form of iron-dependent cell death (Dixon et al., 2012; Wang et al., 2017). To examine whether the liver damage in our FBXL5-deficient mice was related to ferroptosis, we monitored...
the expression of genes associated with defense against (Slc7a11 and Gpx4) or promotion of (Acsl4) this form of cell death as well as that of ferroptosis marker genes (Ptgs2 and Chac1) in the liver at 20 wk of age (Yang et al., 2014; Chen et al., 2017; Doll et al., 2017). Whereas the abundance of Slc7a11 mRNAs was highly upregulated in the liver of Alb-Cre/Fbxl5F/F mice, the expression of ferroptosis marker genes was not significantly altered in the liver of these mice (Fig. 5 D). These results indicated that the oxidative damage apparent in the Fbxl5-deficient liver is not directly associated with ferroptosis, possibly as a result of the activation of antiferroptotic gene expression in response to chronic iron overload or oxidative stress.

The regenerative proliferation of hepatocytes that occurs in response to chronic liver damage has been shown to promote...
Liver carcinogenesis (Maeda et al., 2005; Luedde et al., 2014; Marquardt et al., 2015). Our observation that the weight of the FBXL5-deficient liver was greater than that of the control liver, despite the apparent absence of tumor formation, at 20 wk of age (Fig. 5 E) suggested that the regenerative proliferation of hepatocytes might be induced in the liver of FBXL5-deficient mice. Indeed, we found that the frequency of proliferative (Ki67-positive) hepatocytes in DEN-injected Alb-Cre/Fbxl5<sup>F/F</sup>/HCVcpTg mice at 20 wk of age was greater than that in corresponding controls (Fig. 5 F). These observations thus suggested that FBXL5 deficiency results in chronic hepatocellular damage and proliferation of hepatocytes.

**FBXL5 deficiency promotes carcinogenesis induced by HCV core protein**

To test the generality of the effect of FBXL5 deficiency on liver carcinogenesis, we next examined a mouse model of carcinogenesis induced by expression of a transgene for HCV core protein (HCVcpTg mice; Moriya et al., 1998). The number and size of liver tumors formed in Alb-Cre/Fbxl5<sup>F/F</sup>/HCVcpTg mice were significantly increased compared with those in Fbxl5<sup>F/F</sup>/HCVcpTg control mice (Fig. 6 A). Histopathologic analysis of hepatic tissue sections confirmed these findings (Fig. 6 B). Immunoblot analysis also showed that Hmox1 accumulated to a greater extent in the liver of Alb-Cre/Fbxl5<sup>F/F</sup>/HCVcpTg mice than in that of Fbxl5<sup>F/F</sup>/HCVcpTg mice (Fig. 6 C), suggesting that FBXL5 deficiency also exacerbated oxidative stress in the liver of HCVcpTg mice. Together, these data indicated that the tumor-promoting effect of FBXL5 deficiency in the liver is not limited to chemically induced HCC but is also operative in a model of virus-induced HCC.

**FBXL5 deficiency is associated with poor prognosis in human HCC patients**

Our mouse data indicated that cellular iron dysregulation induced by FBXL5 deficiency promotes liver carcinogenesis and thereby shortens survival time. Given that FBXL5 mRNA was previously shown to be down-regulated in the liver of patients with viral hepatitis (Nanba et al., 2016), we examined whether reduced expression of FBXL5 is associated with a poor prognosis in human HCC with The Cancer Genome Atlas (TCGA) Liver Hepatocellular Carcinoma (LIHC) dataset (Cerami et al., 2012; Gao et al., 2013) or data accessed at the Gene Expression Omnibus (GEO) database with the tag GSE14520 (Roessler et al., 2010). Patients in the TCGA-LIHC cohort were subdivided into quartile groups on the basis of FBXL5 expression level, and their survival was compared with Kaplan–Meier plots. The group with the lowest 25% of FBXL5 expression level showed the shortest survival and was thus considered the most clinically important among the quartile groups (Fig. 7 A and Fig. S3 A). Consistent with this finding, the group with the lowest 25% of FBXL5 expression level in the GSE14520 dataset also had a poorer prognosis compared with the group with the highest 75% (Fig. 7, B and C). Furthermore, the group with the lowest 25% of FBXL5 expression level in the TCGA-LIHC cohort harbored more somatic mutations than did the group with the highest 75% (Fig. S3 B). The abundance of TRi mRNAs, which is up-regulated by IRP2 and therefore represents an index of IRP2 activity, was also increased in HCC patients with a low FBXL5 expression level (Fig. 7 D). Furthermore, GSEA showed that genes whose expression was down-regulated in cultured cells by knockdown of TRi were significantly activated in HCC with a low level of FBXL5 expression (Fig. 7 E), suggesting that the up-regulation of TRi mRNAs in HCC with a low FBXL5 expression level had functional consequences. GSEA also showed that marker genes whose expression is up-regulated in proliferative HCC and genes highly expressed in HCC with a poor prognosis were significantly associated with a low level of FBXL5 expression in HCC (Fig. 7, F and G). FBXL5 expression was found to be significantly down-regulated in HCC associated with hemochromatosis.
Figure 7. Down-regulation of FBXL5 expression is associated with poor prognosis of human HCC. (A) Survival curves for HCC patients with high \( (n = 279) \) or low \( (n = 93) \) levels of FBXL5 expression were constructed from TCGA-LIHC dataset. ***, \( P < 0.005 \) (log-rank test). (B) Survival curves for HCC patients with high \( (n = 165) \) or low \( (n = 56) \) levels of FBXL5 expression were constructed from the GSE14520 dataset. *, \( P < 0.05 \) (log-rank test). (C) Recurrence-free survival for the HCC patients with high \( (n = 165) \) or low \( (n = 56) \) levels of FBXL5 expression in the GSE14520 dataset. *, \( P < 0.05 \) (log-rank test). (D) Relative TfR1 mRNA signal intensity for HCC with high \( (n = 165) \) or low \( (n = 56) \) levels of FBXL5 expression in the GSE14520 dataset. Box-and-whisker plots show median, interquartile range, and most extreme data point less than 1.5 interquartile range from box. ***, \( P < 0.005 \) (Student’s t test). (E–G) GSEA plots of differentially expressed genes in HCC with high \( (n = 165) \) or low \( (n = 56) \) levels of FBXL5 expression (GSE14520 dataset) for the list of genes down-regulated in P493-6 cells in response to knockdown of TfR1 mRNA (E), for the list of marker genes whose expression is up-regulated in proliferative HCC (F), or for the list of genes highly expressed in HCC with poor prognosis (G). (H) Meta-analysis and forest plot of the hazard ratio and 95% CI for the association between FBXL5 expression and overall survival in HCC patients \( (n = 914) \) of five different cohorts according to the random effects model.
compared with HCC associated with chronic viral hepatitis (Fig. S4), suggesting that loss of FBXL5 expression might play a greater role in liver carcinogenesis in patients with hemochromatosis. To reinforce the connection between our mouse data and human HCC, we retrieved an additional three human HCC transcriptomic datasets from the Array Express archive of the European Bioinformatics Institute (EBI). These datasets and the TCGA-LIHC dataset together include clinical data for a total of 914 patients. Meta-analysis of these five HCC cohorts revealed that the prognosis of the group with the lower 50% of FBXL5 expression level had a significantly poorer prognosis than did that with the higher 50% (hazard ratio, 0.665; P < 0.001; Fig. 7 H). Given that meta-analysis is one of the most reliable methods in medical statistics, we conclude that a low FBXL5 expression level is indeed strongly associated with poor prognosis in human HCC, although further investigations are still needed to validate our correlative findings based on published human data.

Given that FBXL5 deficiency resulted in an increase in the amounts of IRP2 and TR1 proteins in the liver of mice (Fig. 3 A), we postulated that IRP2 protein abundance might also be associated with a poor clinical outcome in human HCC. To examine this possibility, we analyzed surgical specimens of human HCC for the expression of IRP2 and TR1 (Fig. 8 A). The expression level of IRP2 was largely correlated with that of TR1 in the HCC specimens (Fig. 8, B and C), suggesting that an increase in IRP2 abundance was largely correlated with that of TfR1 in the HCC specimens (Fig. 8 D). Collectively, our findings thus implicate the cellular iron overload that results from down-regulation of FBXL5 in liver carcinogenesis and improved survival, suggesting that IRP2 might be a potential therapeutic target for the prevention or treatment of liver cancer associated with down-regulation of FBXL5 expression.

Our finding that tumor incidence in DEN-injected FBXL5-deficient mice is much greater than that in littermate controls (Fig. S1 and Fig. 6 A) suggested that hepatic tumors in the former mice are likely initiated earlier than those in the latter. The number of tumors in both DEN and HCV models was also greater for FBXL5-deficient mice than for control mice (Fig. 1 B, Fig. 6 A, and Fig. S1), suggesting that FBXL5 deficiency mainly affects tumor initiation rather than growth. Given that hepatic FBXL5 ablation alone essentially does not induce carcinogenesis, FBXL5 deficiency may promote the generation of tumor cells by accelerating the accumulation of mutations (Fig. 4, A–C). Dysregulation of FBXL5 may thus not be the cause (initiator) of carcinogenesis by itself but rather a risk factor for poor prognosis (promoter) in HCC. We therefore conclude that liver-specific FBXL5-deficient mice are a clinically relevant model for studies of HCC associated with hepatic iron overload.

FBXL5 is a master regulator of cellular iron metabolism by virtue of its role as the substrate recognition component of the SCF^{FBXL5} E3 ubiquitin ligase complex for IRP2 degradation (Salahudeen et al., 2009; Vashisht et al., 2009). Other proteins targeted by FBXL5 for proteasomal degradation include p150Glued and single-stranded DNA binding protein 1 (Zhang

**Discussion**

We have here uncovered a previously unrecognized role of cellular iron homeostasis governed by FBXL5 in defense against oxidative stress and hepatocarcinogenesis with the use of mouse models of conditional Fbxl5 deletion. Mechanistically, disruption of FBXL5-mediated cellular iron homeostasis results in iron overload and consequent oxidative stress in the liver of mice exposed to chemical or viral carcinogens. Iron-induced oxidative stress in such mice triggers liver damage, inflammation, and compensatory proliferation of hepatocytes, eventually leading to hepatocarcinogenesis. Analysis of public datasets revealed that down-regulation of FBXL5 expression was associated with poor prognosis of HCC patients. Additional ablation of IRP2 in conditional FBXL5-deficient mice prevented the promotion of hepatic carcinogenesis and improved survival, suggesting that IRP2 might be a potential therapeutic target for the prevention or treatment of liver cancer associated with down-regulation of FBXL5 expression.
FBXL5 is an iron-sensing oncosuppressor of the liver

FBXL5 has been shown to interact with and to trigger the degradation of Snail1 (Vinas-Castells et al., 2014; Wu et al., 2015) and CBP/p300-interacting transactivator 2 (CITED2; Machado-Oliveira et al., 2015). Snail1 was found to induce epithelial-to-mesenchymal transition and to confer characteristics of tumor-initiating stem cells in a human HCC cell line (Dang et al., 2011). This observation suggests that the accumulation of Snail1 might also contribute to the promotion of carcinogenesis in the FBXL5-deficient liver. In contrast, CITED2 was shown to down-regulate the expression of cyclin D1, telomerase reverse transcription, and matrix metalloproteinase 2 as well as to suppress the proliferation of a human HCC cell line (Cheung et al., 2013). However, our data provide genetic evidence that IRP2 accumulation is largely responsible for the promotion of HCC by FBXL5 ablation, given that the enhancement of tumorigenesis in the FBXL5-deficient liver was prevented by additional ablation of IRP2. This latter result suggests that any contribution of Snail1 or CITED2 to tumorigenesis in the FBXL5-deficient liver is limited. We therefore conclude that FBXL5 plays an essential role in the defense against hepatocarcinogenesis through suppression of IRP2 activity.

Our results show that FBXL5 ablation in the liver results in promotion of carcinogenesis through cellular iron overload. Disruption of systemic iron homeostasis leads to iron deposition in various organs including the liver and promotes the development of liver cancer in patients with primary or secondary hemochromatosis (Bacon et al., 2011), although disruption of cellular iron homeostasis has not previously been shown to promote liver carcinogenesis. We now demonstrate for the first time that cellular iron homeostasis is essential for suppression of liver carcinogenesis. Furthermore, the amount of Fe^{2+}, rather than that of Fe^{3+}, is primarily increased in FBXL5-deficient liver tissue (Moriishi et al., 2011), suggesting that Fe^{2+} specifically has a cancer-promoting effect. This notion is consistent with the fact that Fe^{2+} is unstable and readily participates in the Fenton reaction, resulting in the uncontrolled production of the hydroxyl radical. Selective chelation of intracellular Fe^{2+} in the liver or HCC tissue might thus be a more effective treatment than the systemic Fe^{3+} chelation therapy or phlebotomy currently administered in some HCC patients. Indeed, we propose that the FBXL5-IRP2 axis is a potentially valuable therapeutic target for human HCC.

We also show that FBXL5 is an essential regulator of ROS in the liver as a result of its function in cellular iron regulation. The expression of several genes that contribute to oxidative stress responses was up-regulated in FBXL5-deficient HCC or non-tumor liver tissue, indicative of iron-mediated cellular damage and disruption of redox homeostasis. These genes included Nqo1, Hmox1, Mt2, and Slc7a11, the last of which encodes a component of system xc(-). System xc(-) contributes to the maintenance of cellular redox homeostasis by mediating the import of cystine for synthesis of the major cellular antioxidant glutathione. Inhibition of system xc(-) by erastin in cancer cells triggers ferroptosis, a recently recognized form of iron-dependent cell death (Dixon et al., 2012). Up-regulation of Slc7a11 expression might therefore represent a mechanism to protect cells with cellular iron overload from ferroptosis. Indeed, SLC7A11 is highly expressed in human tumors including liver cancer, and its overexpression inhibits ROS-induced ferroptosis in xenograft models of human cancer (jiang et al., 2015). Marked up-regulation of Slc7a11 expression in the FBXL5-deficient liver might suppress ferroptosis, which has been detected in hemochromatosis model mice (Wang et al., 2017). This substantial activation of genes associated with protection against ferroptosis might contribute to the promotion of carcinogenesis in the FBXL5-deficient animals. Nqo1, Slc7a11, Hmox1, and Mt2 are also well-characterized oxidative stress response genes whose expression is regulated by the transcription factor NRF2, suggesting that oxidative stress induces NRF2 activation in the FBXL5-deficient liver and HCC. Expression of NRF2 was previously shown to be up-regulated in human HCC and to be correlated with tumor differentiation, metastasis, and size as well as associated with a poor prognosis (Zhang et al., 2015). Furthermore, NRF2 was recently shown to be essential for hepatocarcinogenesis in DEN-injected mice (Ngo et al., 2017), suggesting that constitutive activation of NRF2 target genes also contributes to the progression of liver cancer in Alb-Cre/Fbxl5F/F mice. These lines of evidence suggest that iron-dependent NRF2 activation might also contribute to the progression of HCC in the FBXL5-deficient liver.

Analysis of public datasets revealed that down-regulation of FBXL5 expression was associated with poor prognosis in HCC.
patients. However, no specific mutation in the FBXL5 gene has been found to be associated with HCC or mode of tumorigenesis. Although the regulation of FBXL5 expression remains largely unclear, mutations in the KDM5C gene for a histone H3 lysine-4 demethylase were found to induce loss of DNA methylation at the FBXL5 promoter in human samples (Grafodatskaya et al., 2013). The level of DNA methylation at the FBXL5 gene in human blood was also found to be correlated with the abundance of KDM5C, suggesting that the amount of FBXL5 mRNA might be regulated by KDM5C. Furthermore, KDM5C was shown to be abundantly expressed in invasive human HCC cells, and depletion of KDM5C by RNA interference inhibited HCC cell migration, invasion, and epithelial–mesenchymal transition in vitro (Ji et al., 2015). Collectively, these observations suggest that FBXL5 expression might be regulated at the epigenetic level, although further studies are required to verify this notion.

Our finding that suppression of aberrant IRP2 activity prevented the promotion of hepatocarcinogenesis induced by FBXL5 ablation suggests that targeting of IRP2 might be effective for mitigation of hepatocellular iron overload and therefore a candidate for therapeutic application. This notion is further supported by the finding that the abundance of IRP2 protein was correlated with that of TfR1 protein in human HCC, with increased IRP2 expression also being associated with reduced survival of HCC patients. Given that complete loss of IRP2 gives rise to microcytic anemia or neurodegeneration in mice (Cooperman et al., 2005; Galy et al., 2005), however, an appropriate level of IRP2 suppression would be needed for clinical benefit. Another potential limitation of such a therapeutic strategy is that no IRP2 inhibitor has been developed to date. Given that IRP2 is an mRNA binding protein, inhibition of such binding with antisense oligonucleotides might be a feasible approach to the treatment of HCC associated with FBXL5 down-regulation and cellular iron overload, although further studies will be necessary to validate the connection between FBXL5 down-regulation and human HCC.

Materials and methods

Mice

Generation of Fbxl5+/f and Fbxl5−/− mice was described previously (Moroshi et al., 2011). These mice were crossed with Alb-Cre transgenic mice (Postic and Magnuson, 2000), Irp2−/− mice (LaVaque et al., 2001), or HCVcpTg mice (Moriya et al., 1998) to generate Alb-Cre/Fbxl5+/f, Fbxl5−/−/Irp2−/−, or Alb-Cre/Fbxl5+/f/HCVcpTg mice. For chemically induced liver carcinogenesis, a single dose (25 mg/kg) of DEN (Sigma-Aldrich) was injected i.p. in mice at 15 d of age (Sarff et al., 1996). The injected mice were analyzed at 36 wk of age unless indicated otherwise. Alb-Cre/Fbxl5+/f/HCVcpTg or Fbxl5+/f/HCVcpTg mice were analyzed at 90 wk of age. Externally visible tumors (>1 mm in diameter) were counted and measured. All mice were maintained on a mixed genetic background (C57BL/6J;129Sv), and all mouse experiments were approved by the Animal Ethics Committee of Kyushu University.

Human specimens

Tissue specimens were obtained from 23 male patients with liver tumors who underwent hepatectomy at the Department of Gastroenterological Surgery, Osaka University Hospital (Table S1). The specimens were frozen immediately in liquid nitrogen and stored at −80°C for subsequent immunoblot analysis. The signal intensities of immunoblot bands were quantified with ImageJ software (National Institutes of Health). Specimen 10 was excluded from quantitative analysis because of the HCC-unrelated death of the patient within 3 mo after surgery. The cutoff value for classification of IRP2 expression level as high or low was determined with the minimal P value approach (Budczies et al., 2012; Hirata et al., 2016). The study protocol was approved by the Human Ethics Review Committees of Osaka University and Kyushu University, and a signed consent form was obtained from each patient for the use of tissue samples for medical research.

Analysis of published data

Overall survival was compared between HCC patients with high or low levels of FBXL5 expression in the TCGA-LIHC dataset accessed with cBioPortal (Cerami et al., 2012; Gao et al., 2013). Overall survival and recurrence-free survival were also compared between HCC patients with high or low FBXL5 expression levels with data accessed at GEO with the tag GSE14520 (Roessler et al., 2010). The data processed on the 96 HT HG-U133A 2.0 microarray platform were downloaded as a series matrix file and analyzed. The signal intensities for FBXL5 (209004_s_at) were then collated with those for TFR1 (208691_at) or the survival data included in the supplementary file “GSE14520_Extra_Supplement.txt.gz” downloaded from GEO. Survival curves were generated with the use of library ggplot2 in the R package (Fig. 7A and Fig. S3A) or JMP software (SAS Institute; Fig. 7, B and C). Normalized expression data accessed at GEO with the tag GSE14520 and used for the survival analysis in Fig. 7B were then analyzed with GSEA v2.0.13 software (Broad Institute) and the ODNNEll_TFRC_TARGETS_DN (Fig. 7E), CHIANG_LIVER_CANCER_SUBCLASS_PROLIFERATION_UP (Fig. 7F) and LEE_LIVER_CANCER_SURVIVAL_DOWN (Fig. 7G) gene sets. These gene sets were obtained from the Molecular Signatures Database v4.0 distributed at the GSEA web site. Patients were grouped on the basis of the abundance of FBXL5 mRNA, with the upper 75% and lower 25% representing the high and low expression groups, respectively (Fig. 7, A–G; and Fig. S3B). For the meta-analysis in Fig. 7H, we retrieved the TCGA-LIHC dataset from cBioPortal as well as four human HCC transcriptomic datasets from the Array Express archive of EBI, and patients were grouped on the basis of the median value of FBXL5 mRNA abundance. We retrieved the mutation data in Fig. S3B from cBioPortal. The signal intensity for FBXL5 (209004_s_at) in HCC for patients with HBV infection, HCV infection, or hemochromatosis was analyzed with the E-MTAB9500 dataset from the Array Express archive of EBI (Fig. S4).

RNA-seq analysis

Total RNA was extracted from nontumor and tumor tissue of a DEN-injected Alb-Cre/Fbxl5+/f male mouse (nontumor, n = 5; tumor, n = 5) and two DEN-injected littermate control Fbxl5+/f male mice (nontumor, n = 6; tumor, n = 6) at 45 wk of age with the use of a TRIzol Plus RNA Purification Kit (Life Technologies). Libraries were prepared with the use of a NEBNext Ultra
Directional RNA Library Prep Kit for Illumina. RNA-seq was performed as described previously (Katayama et al., 2016). Complementary DNA was sequenced with a HiSeq 2500 system (Illumina). The total amount of each transcript was calculated with the use of a series of programs including TopHat2 and Cufflinks. RNA-seq reads were mapped against the mouse (mm10) genome. Normalized expression data were then analyzed with GSEA v2.0.13 software (Broad Institute) and Hallmark (Fig. 4, A, B, D, and E), NFE2L2.V2 (Fig. 3 D), and GO_INTRINSIC_APOPTOTIC_SIGNALING_PATHWAY_IN_RESPONSE_TO_DNA_DAMAGE (Fig. 5 C) gene sets obtained from the Molecular Signatures Database v4.0 distributed at the GSEA Web site.

Variant calling for RNA-seq data

Somatic variant calling was performed largely based on the previously reported pipeline for processing of RNA-seq data from tumor samples in order to identify somatic mutations with the use of MuTect2 (McKenna et al., 2010; Coudray et al., 2018). The use of MuTect2 in somatic variant calling from RNA-seq data was also described by another group (Neums et al., 2018). After trimming the adapters (TrimGalore, v0.5.0) from fastq files, the data were aligned with STAR aligner (v2.4.2a) with the use of a two-pass procedure (Dobin and Gingeras, 2015). The aligned reads in BAM format were sorted, and duplicate reads were flagged (MarkDuplicates, Picard v2.5.0). RNA-seq reads were split into exons (SplitNCigarReads, minobenzidine in 1% H2O2. Sections from control and mutant room temperature then washed again with deionized water before incubation at 4°C with primary antibodies to TRA1 (13–6800; Invitrogen), to Hmox1 (sc-10789; Santa Cruz Biotechnology), to Hsp90 (610419; BD Biosciences), to human IRP2 (sc-33682; Santa Cruz Biotechnology), or to mouse IRP2 (generated by K. Iwai, Kyoto University, Kyoto, Japan). Immune complexes were detected with HRP-conjugated secondary antibodies (Promega) and enhanced chemiluminescence reagents (Thermo Fisher Scientific; Nagahama et al., 2001; Foster et al., 2003; Hirano et al., 2013). For analysis of the human HCC specimens, the membrane was also stained with Coomassie Brilliant Blue as a loading control.

Histopathology and immunostaining

Liver tissue was fixed with 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned at a thickness of 3 µm. Sections were stained with hematoxylin-eosin or immunostained with antibodies to Ki67 (550609; BD Biosciences). Immune complexes were detected with Alexa Fluor 488–conjugated goat secondary antibodies (Invitrogen; Li et al., 2004; Yokobori et al., 2009), and confocal images were acquired with an LSM 700 Laser Scanning Microscope (Carl Zeiss).

Iron histochemistry

The mouse liver was perfused with 50 mM hydrogen sulfide in deionized water and with 4% paraformaldehyde in PBS. Iron was detected in cryostat sections of the liver by enhanced Perls or Turnbull staining. Tissue sections were thus washed with deionized water and with 4% paraformaldehyde in PBS. Iron staining was performed as described previously (Katayama et al., 2016). Directional RNA Library Prep Kit for Illumina. RNA-seq was performed as described previously (Katayama et al., 2016). Complementary DNA was sequenced with a HiSeq 2500 system (Illumina). The total amount of each transcript was calculated with the use of a series of programs including TopHat2 and Cufflinks. RNA-seq reads were mapped against the mouse (mm10) genome. Normalized expression data were then analyzed with GSEA v2.0.13 software (Broad Institute) and Hallmark (Fig. 4, A, B, D, and E), NFE2L2.V2 (Fig. 3 D), and GO_INTRINSIC_APOPTOTIC_SIGNALING_PATHWAY_IN_RESPONSE_TO_DNA_DAMAGE (Fig. 5 C) gene sets obtained from the Molecular Signatures Database v4.0 distributed at the GSEA Web site.

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Iron histochemistry

The mouse liver was perfused with 50 mM hydrogen sulfide in deionized water and with 4% paraformaldehyde in PBS. Iron was detected in cryostat sections of the liver by enhanced Perls or Turnbull staining. Tissue sections were thus washed with deionized water, incubated for 30 min at room temperature with Perls reagent (5% potassium ferrocyanide and 5% HCl) or Turnbull reagent (5% potassium ferricyanide and 5% HCl), and then washed again with deionized water before incubation at room temperature first for 15 min with 0.05% diamino benzidine in deionized water and then for 10 min with 0.05% diaminobenzidine in 1% H2O2. Sections from control and mutant mice were processed at the same time to allow monitoring and detection of nonspecific staining.

Immunoblot analysis

Total protein extracts were prepared from mouse liver or human HCC specimens by lysis with a solution comprising 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, aprotinin (10 µg/ml), leupeptin (10 µg/ml), 1 mM PMSF, 400 µM Na2VO4, 400 µM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The extracts (40 µg of protein) were fractionated by SDS-PAGE on a 10% gel, and the separated proteins were then transferred to a polyvinylidene difluoride membrane (IPVH0010; Millipore). The membrane was exposed to 3% dried skim milk and then incubated overnight at 4°C with primary antibodies to TRA1 (13–6800; Invitrogen), to Hmox1 (sc-10789; Santa Cruz Biotechnology), to Hsp90 (610419; BD Biosciences), to human IRP2 (sc-33682; Santa Cruz Biotechnology), or to mouse IRP2 (generated by K. Iwai, Kyoto University, Kyoto, Japan). Immune complexes were detected with HRP-conjugated secondary antibodies (Promega) and enhanced chemiluminescence reagents (Thermo Fisher Scientific; Nagahama et al., 2001; Foster et al., 2003; Hirano et al., 2013). For analysis of the human HCC specimens, the membrane was also stained with Coomassie Brilliant Blue as a loading control.

RT and real-time PCR analysis

Total RNA was extracted from liver with the use of the Isogen reagent (Nippon Gene). Portions (1 µg) of the purified RNA were then subjected to RT with the use of ReverTra Ace (Toyobo), and the resulting cDNA was subjected to real-time PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems) and specific primers in a Step One Plus Real-Time PCR System (Applied Biosystems). Data were normalized by the abundance of attachment region–binding protein mRNA. The sequences of the various primers (sense and antisense, respectively) were as follows: 5′-GGACCGAGAGACCTCTCTT-3′ and 5′-GCACATCAC TCGAATTTCAAGG-3′ for attachment region–binding protein; 5′-AAGCCGAGATTGCTGATCCA-3′ and 5′-GGCTGTAG TATGATCAGGA-3′ for Hmox1; 5′-AGCGTTCGTTATTAC GATCC-3′ and 5′-AGTACAATCGAGGCTTCTTG-3′ for Nqo1; 5′-CATTAGAAGTTGGAAGCCAC-3′ and 5′-CAGTACTGCG TCAAGTG-3′ for Gsta1; 5′-GAAAGATCTTCTGTCG-3′ and 5′-AGGAGCAGCAGCTTTCCTTG-3′ for MT2; 5′-TTGGETGAAAA CTGCTGTAA-3′ and 5′-AGGATGTAGCGTCCAAATGC-3′ for Scl7a11; 5′-GATGAGGCCCTTCCGTGAAC-3′ and 5′-CGGCTGT ACTATACCGCAG-3′ for Gpx4; 5′-CCTGAGGGGTGTA ATCCAC-3′ and 5′-TTGGTTCTACTTGGGAAACG-3′ for ACSL4; 5′-GGACTGTCGAAATTTGGAAC-3′ and 5′-GTGCAACTGTA AGTACTGGACT-3′ for PTGS2; 5′-CTGGTGATTTCCGGTAC CC-3′ and 5′-CCCTATGCAAGGAGTCTCCC-3′ for Chac1; 5′-CCCT TACACTCATCATTCTT-3′ and 5′-GCTACGAGCTGGCTAC ACG-3′ for TNFa; 5′-TTTAAACCTGGATGGCAACAA-3′ and 5′-GCTGATGACGTTAACGATCT ATT-3′ for CCL2; and 5′-GCTGGTCCTTACCTTCC-3′ and 5′-TCAGTGCACAAACACAGAC TGC-3′ for CCL5.

Biochemical analysis

Serum ALT activity was measured with a standard clinical autoanalyzer.
Statistical analysis
No statistical methods were used to predetermine sample size. Experiments were not randomized. The investigator was blinded to clinical outcome for immunoblot analysis of human HCC specimens. Quantitative data are presented as means ± SD unless indicated otherwise. Comparisons between two groups were performed with the two-tailed Student’s t test or Mann-Whitney U test, and those among three groups were performed by one-way ANOVA followed by a post hoc Tukey test. Differences between survival curves were analyzed with the log-rank nonparametric test. Pearson’s correlation analysis was applied to evaluate the relation between protein expression levels (Fig. 8 B). For meta-analysis, a random-effects model (Riley et al., 2011) was adopted within Cochrane Review Manager (RevMan) V.5.3 software to synthesize the result as instructed by the Cochrane community. A P value of <0.05 was considered statistically significant.

Data availability
The RNA-seq data have been deposited in GEO under the accession no. GSE124370. All other relevant data are available from the corresponding authors on reasonable request.

Online supplemental material
Fig. S1 shows that FBXL5 deficiency increases DEN-induced hepatic tumor incidence. Fig. S2 shows that Alb-Cre alone does not influence DEN-induced liver carcinogenesis. Fig. S3 shows that a low level of FBXL5 expression is associated with an increased number of mutations in human HCC. Fig. S4 shows that FBXL5 expression level is lower in HCC associated with hemochromatosis than in that associated with HBV or HCV infection. Table S1 shows clinical information for the HCC patients and signal intensities of the immunoblot bands in Fig. 8.

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The authors declare no competing financial interests.

Author contributions: Y. Muto planned and performed experiments with human HCC samples, collected a large portion of mouse experimental data, and wrote the manuscript. T. Moroishi designed the study, performed and analyzed the mouse experiments, and wrote the manuscript. K. Ichihara performed the mouse experiments and RNA-seq. M. Nishiyama provided materials and intellectual support. H. Shimizu and Y. Katayama performed informatics analysis. H. Eguchi, M. Mori, and K. Mimori collected human HCC samples. K. Moriya and K. Koike generated HCVcpTg mice. K.I. Nakayama coordinated the study, oversaw collection and analysis of the results, and wrote the manuscript. All authors discussed the data and commented on the manuscript.

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