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Cooperation of C/EBP family proteins and chromatin remodeling proteins is essential for termination of liver regeneration

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Abstract

Liver cancer is the fifth most common cancer. A highly invasive surgical resection of the liver tumor is the main approach utilized to eliminate the tumor. Mechanisms that terminate liver regeneration when the liver reaches the original size are not known. The aims of this work were to generate the animal model which fails to stop liver regeneration after surgical resections and elucidate mechanisms which are involved in termination of liver regeneration. Because epigenetic control of liver functions has been previously implicated in the regulation of liver proliferation, we have generated C/EBP α -S193A knockin mice, which have alterations in formation of complexes of C/EBP family proteins with chromatin remodeling proteins. The C/EBP α -S193A mice have altered liver morphology and altered liver functions leading to changes of glucose metabolism and blood parameters. Examination of proliferative capacity of C/EBP α -S193A livers showed that livers of S193A mice have a higher rate of proliferation after birth, but stop proliferation at the age of 2 months. These animals have increased liver proliferation in response to liver surgery as well as CCl₄-mediated injury. Importantly, livers of C/EBP α -S193A mice fail to stop liver regeneration after surgery when livers reach the original, pre-resection, size. The failure of S193A livers to stop regeneration correlates with the epigenetic repression of key regulators of liver proliferation C/EBP α , p53, FXR, SIRT1, PGC1 $\pm$ and TERT by C/EBP β -HDAC1 complexes. The C/EBP β -HDAC1 complexes also repress promoters of enzymes of glucose synthesis PEPCK and G6Pase.

Conclusions—Our data demonstrate that a proper co-operation of C/EBP and chromatin remodeling proteins is essential for the termination of liver regeneration after surgery and for maintenance of liver functions.

Keywords

liver regeneration; chromatin; C/EBP; p300; HDAC1

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A surgical resection of liver tumor sections is one of the most common ways employed today to eliminate liver cancer. This approach is based on the unique ability of liver to regenerate after a significant portion of the organ is removed. The studies of liver regeneration showed that the adult hepatocytes are the main source for the liver regeneration (1). Numerous studies of liver regeneration identified many regulatory intermediates including activation of signaling networks between Kupffer cells and hepatocytes in which cytokines NF- κ B and IL-6 play critical role (2, 3). One of the important events in the liver regeneration is activation or repression of transcription factors which are required for the initiation/inhibition of liver proliferation. This transcriptional shift includes activation of FOXO3, FOXI2, E2F1, c-jun, C/EBP β , Myb, USF (1, 3) and neutralization of inhibitors of liver proliferation such as Rb family and C/EBP family proteins (4). While mechanisms of initiation of liver regeneration have been investigated in detail, very little is known about the mechanisms that terminate liver regeneration. Global gene profiling of the liver 3 weeks after PH revealed alterations in cell cycle, apoptosis, TGF β and angiogenesis signaling (5). In other studies, PPAR signaling, lipid metabolism and coagulation have been implicated in the termination of liver regeneration (6). Two recent reports suggest that certain microRNAs may be involved in the termination of liver regeneration (7, 8). It has been also shown that the ablation of integrin-linked kinase leads to enhanced liver proliferation (9).

C/EBP α and C/EBP β belong to C/EBP family proteins that control multiple functions in different tissues (4, 10). In this paper, we found that the deregulation of complexes formation between C/EBP proteins and chromatin remodeling proteins alters liver biology and liver regeneration after PH and liver injury. This deregulation leads to early entry of hepatocytes into the cell cycle and to a loss of the termination of liver proliferation and regeneration.

Materials and Methods

Antibodies and reagents

Antibodies to C/EBP α (14AA), C/EBP β (C-19), cdc2 (L-19), cyclin D1 and PCNA (FL-261) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA).

Mice, partial hepatectomy and CCl₄-induced liver injury

Wild-type, S193A and S193D mice were maintained as previously described (11, 12).

Partial hepatectomy: PH was performed as described in our previous publications (11, 12).

Acute CCl₄ treatments: Mice (males) were injected intraperitoneally with a single dose of 10% CCl₄ in olive oil (5.0 ml/kg) or vehicle control. Data in this manuscript represent summaries of work with 4-5 mice per each time point after PH or after CCl₄ injection. Experiments with animals have been approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol AN-1439).

Histology

Mice were sacrificed and liver pieces were quickly removed and fixed in 10% neutral buffered formalin. BrdU was injected intraperitoneally 2 hours before mice were sacrificed. BrdU staining was performed using BrdU uptake assay kit from Invitrogen (Carlsbad, CA).

Apoptosis was detected by using “In situ Cell Death Detection” (TUNEL) kit from Roche Applied Science (Mannheim, Germany).

Protein isolation and Western blotting

Procedure for isolation of nuclear extracts is described in our previous publications (12-14).

Chromatin Immunoprecipitation Assay was performed using the ChIP-IT Kit (Active Motif). The procedure and sequences of primers are described in our previous papers (13-15) and in supplemental materials.

Examination of regulation of C/EBP α promoter using luciferase-reporter construct

The promoter region of mouse C/EBP α gene, containing C/EBP site, was cloned into pGl3-luc reporter vector. This construct was co-transfected with WT, S193D and S193A C/EBP α mutants into HEK293 cells. 24 hours after transfection, protein lysates were isolated and luciferase activity was determined.

Statistical analysis

All values are presented as means $\pm$ SD. Statistical analyses were performed using the Student's *t*-test. Statistical significance was assumed when $*p < 0.05$.

Results

C/EBP α -S193A mice have altered liver morphology and blood parameters

C/EBP α -HDAC1 and C/EBP α -p300 complexes are elevated during liver differentiation and aging (4, 11, 14). Since phosphorylation of C/EBP α at Ser193 is required for the formation of these complexes (11), we generated C/EBP α -S193A knockin mice in which serine 193 is mutated to alanine (Fig 1A-B). H&E staining showed that livers of S193A mice contain larger hepatocytes and have reduced levels of glycogen (Fig 1C and D). In agreement with this, the number of hepatocytes per visual field is reduced in S193A versus wild type livers (Fig 1C); however, liver/body weight ratio does not differ in WT and S193A mice. We also observed significant differences in the blood parameters between WT mice, S193A mice and the previously investigated C/EBP α -S193D mice. Levels of ALT and AST are reduced in S193A mice, while they are elevated in S193D mice (12). The levels of triglycerides (TG), glucose and VLDL are reduced; while albumin levels are increased in S193A mice. These data show that phosphorylation of C/EBP α at S193 is involved in control of liver functions.

Livers of S193A mice have a higher rate of proliferation during post-natal development than livers of WT mice

We next sought to determine if differentiation and proliferation of the S193A livers differs from that of WT mice during postnatal liver development. Measurement of DNA replication via BrdU uptake and examination of cyclin D1 showed that S193A livers have a higher rate of proliferation than WT livers (Fig 2A-B-C). Surprisingly, we found that the levels of the mutant C/EBP α -S193A in S193A mice are lower than levels of C/EBP α in WT mice at all stages of post-natal liver development (Fig 2D). qRT-PCR analysis revealed that levels of C/EBP α mRNA are also lower in livers of S193A mice (Fig 2E). Thus, both proliferation and

differentiation of S193A livers are impaired after birth and levels of mutant C/EBP α are reduced by around 40-50% compared to levels in livers of WT mice. Since heterozygous C/EBP α with total ablation of C/EBP α express 50% of C/EBP α , but did not show any alterations (16), we conclude that changes of liver functions and proliferation in S193A mice are caused mainly by the S193A mutation.

C/EBP β -HDAC1 complexes are increased in livers of S193A mice during postnatal development

We next examined mechanisms by which the S193A mutation within C/EBP α protein reduces levels of C/EBP α mRNA. Since another member of C/EBP family, C/EBP β , represses C/EBP-dependent promoters in the complexes with HDAC1 (17, 18), we examined if S193A livers might utilize this mechanism for the repression of the C/EBP α promoter. The full-length C/EBP β is gradually increased in livers of WT and S193A mice with an identical degree of increase at each time point; however, the elevation of HDAC1 takes place early in S193A mice and to a higher degree than in WT mice (Fig 3A and B). As the result, the amounts of C/EBP β -HDAC1 complexes are increased in S193A mice at early stages of post-natal development and stay at high levels at days 15 and 60 after birth (Fig 3C).

C/EBP β -HDAC1 complex inhibits an auto-regulation loop of activation of the C/EBP α promoter in livers of S193A mice

We next examined the hypothesis that the C/EBP β -HDAC1 complexes might interrupt the positive auto-regulation of C/EBP α promoter mediated by C/EBP α -p300 complexes. Since the phosphor-mimicking S193D mutation increases interactions of C/EBP α with p300 (14), we included C/EBP α -S193D mice in further studies. We found that C/EBP α -HDAC1 and C/EBP α -p300 complexes are elevated in S193D livers, but these complexes are reduced in S193A mice (Fig 3D). We next examined the ability of WT C/EBP α , C/EBP α -S193D and C/EBP α -S193A mutants to co-operate with p300 in the regulation of the C/EBP α promoter. The C/EBP α promoter-pGL3-luc reporter was co-transfected with WT and mutant C/EBP α proteins into HEK293 cells. Figure 3E shows that WT C/EBP α activates its own promoter and that S193D mutation significantly increases this activation; while S193A mutation reduces the ability of C/EBP α to activate the promoter. This auto-activation depends on p300 because the inhibition of p300 by siRNA significantly inhibits the auto-activation (Fig 3E). ChIP approach showed that the C/EBP α promoter is occupied by C/EBP α /p300 complexes in WT mice and in S193D mice; however, only C/EBP β and HDAC1 are observed on the C/EBP α promoter in S193A mice (Fig 3F). Histone H3 is acetylated at K9 on the C/EBP α promoter in livers of WT and S193D mice; but it is de-acetylated and trimethylated at K9 in livers of S193A mice. These data show that the activity of C/EBP α promoter is reduced in S193A mice due to removal of C/EBP α -p300 complexes from the promoter and subsequent repression by C/EBP β -HDAC1 complexes (Fig 3F, bottom). Further studies indicated that the switch of C/EBP α -p300 complexes to C/EBP β -HDAC1 complexes occurs on many other promoters.

Promoters of G6Pase and PEPCK are repressed in livers of S193A mice by the C/EBP β -HDAC1 complexes

Given the reduction of glucose in S193A mice, we examined the expression of enzymes that are involved in the glucose metabolism. These studies showed that levels of G6Pase, PEPCK and Glut2 are significantly reduced in S193A mice (Fig 4A), while protein levels of Glut4 and GyS2 are not changed significantly. qRT-PCR confirmed that the levels of G6Pase, PEPCK and Glut2 mRNAs are reduced in livers of S193A mice (Fig 4B). We found that the G6Pase and PEPCK promoters are repressed in S193A mice by C/EBP β -HDAC1 complexes. In WT mice, these promoters are activated by C/EBP α /p300 complexes (Fig 4C). These results suggest that the reduction of glucose levels in S193A mice is associated with the C/EBP β -HDAC1-mediated repression of the promoters of enzymes of glucose synthesis (Fig 4E).

CEBP β -HDAC1 complexes repress SIRT1, PGC1 α , FXR, p53 and TERT in livers of S193A mice

C/EBP binding sites have been found in promoters of key regulators of liver biology including C/EBP α itself (4, 17), p53 (15), FXR (19), SIRT1, PGC1 α (15) and TERT (13). Therefore, we examined if the expression of these proteins might be altered in livers of S193A mice. We found that protein and mRNA levels of SIRT1, PGC1 α , FXR, p53 and TERT are reduced in livers of S193A mice (Fig 4D-E, for PGC1 α and FXR mRNAs data are not shown). ChIP assay showed that C/EBP β -HDAC1 complexes are abundant at the promoters of SIRT1, p53 and TERT genes in S193A livers and that these promoters are partially repressed because histone H3 is trimethylated at K9 (Fig 4F). Thus, these results show that the key regulators of liver functions are repressed in S193A mice by C/EBP β -HDAC1 complexes (Fig 4F).

Hepatocytes of S193A mice enter cell cycle early and fail to stop proliferation after PH

We next examined the regeneration of the S193A livers after 70% PH. We found two critical differences. First, a significant portion of S193A hepatocytes enter DNA replication phase at 24 hours, while DNA replication is observed in WT mice only at 36 hours after PH. The second difference is that DNA replication continues at high levels at 48, 72 and 96 hours in livers of S193A mice (Fig 5A). Around 20% of hepatocytes continue proliferation at each time point within 48-96 hours in S193A mice. The final number of hepatocytes that proliferated in S193A mice within 96 hours after PH is more than 125% suggesting that some hepatocytes proliferated twice or more within this time period. In WT mice, around 60% of hepatocytes proliferated within 96 hours. We also found that mitosis takes place earlier in S193A mice and to higher degree (Fig 5B). The activation of cyclin D1 and PCNA occurs early in S193A mice and to higher degree than in WT mice (Fig 5 C-D). We found that the restoration of liver mass in S193A mice occurs much faster and that the S193A livers reach original size at days 7-10; while livers of WT mice restore original size at day 15 (Fig 5E). Importantly, livers of S193A mice do not stop growth when they reach the pre-surgery size. As the result, liver/body weight ratio is increased in S193A mice at day 15 after PH. To determine the proliferative status of the S193A livers at 7-15 days after PH, we examined cell cycle proteins and mitosis. Figure 6A and B shows that levels of PCNA and

cdc2 remain high at days 10 and 15 in livers of S193A mice; while expression of these proteins is reduced in WT mice to the levels observed in quiescent livers. Mitotic figures are also abundant in S193A livers at 15 day after PH; while no mitotic figures were found in livers of WT mice (Fig 6C-D). We have also examined liver regeneration in heterozygous S193A mice after PH, but did not find significant differences between WT mice and heterozygous S193A mice (data not shown).

Homozygous C/EBP α -S193A mice have increased rate of liver proliferation after CCl₄-mediated liver injury

To further examine proliferative capacities of S193A livers, we subjected S193A mice to CCl₄ treatment, which is known to cause liver damage and subsequent liver proliferation. H&E staining and examination of levels of ALT/AST showed that livers of S193A mice have much less damage (Fig 7A and B). TUNEL assay showed that S193A mice have reduced apoptosis (Fig 7C). We found that the peak of DNA replication occurs at the same time in both WT and in S193A mice; however, the number of BrdU positive hepatocytes is higher in S193A livers and S193A livers do not stop proliferation and show much higher rate of proliferation at 72 and 96 hours than livers of WT mice (Fig 7D). Livers of S193A mice also have a higher number of mitotic figures after CCl₄ treatments and higher levels of *cdc2* and cyclin D1 (Fig 7D, E and F). We observed that C/EBP α is reduced at early time points in both WT and S193A mice; however, it is returned to normal levels in WT mice; but not in S193A mice. We suggest that the failure of normalization of C/EBP α at 96 hours in S193A mice is due to disruption of the auto-regulatory loop.

Heterozygous C/EBP α -S193A mice have increased rate of liver proliferation after CCl₄ treatments, but stop proliferation within a normal timeframe

Given the reduction of levels of C/EBP α in homozygous S193A mice (Fig 2), we asked if the failure to stop liver proliferation is mediated by the S193A mutation or by the reduction of the protein levels of C/EBP α . To address this issue, we performed CCl₄ treatments on heterozygous C/EBP α -S193A mice which contain levels of C/EBP α close to those observed in WT mice in quiescent livers and in livers after CCl₄ treatments (Fig 8A-B). We found that liver proliferation is increased in heterozygous S193A mice; however, the hepatocytes of S193A heterozygous mice enter the cell cycle and stop proliferation at the same time points as hepatocytes of WT mice (Fig 8D). The expression of *cdc2* and PCNA is also higher in heterozygous S193A mice at 48 hours, but it is identical to that in WT mice at 96 hours after CCl₄ treatments (Fig 8A and C). These observations show that the reduction of total levels of C/EBP α contributes to the increased proliferation; however, the lack of phosphorylation of C/EBP α at Ser193 causes a loss of termination of liver proliferation.

Discussion

The liver possesses a unique capability to proliferate after surgical resections and after drug-mediated liver injury (3). Although it has been shown that integrin-linked kinase and glypican 3 are involved in the regulation of liver size after PH (9, 20), precise mechanisms of termination of liver growth are not known. In this paper, we present evidence that the cooperation of C/EBP family proteins with chromatin remodeling proteins is responsible for

the proper liver proliferation after surgical resections and after CCl₄-mediated injury. Figure 8E summarizes the main results of our studies. First, we found that S193A mice have significantly lower amounts of C/EBP α -HDAC1 and C/EBP α -p300 complexes than WT mice. In livers of WT mice, these complexes are formed at appropriate times of liver development and they are involved in the proper regulation of liver differentiation/proliferation after birth, in regulation of liver proliferation after PH and after liver injury. In S193A mice, these biological processes are impaired. One of the critical findings of this work is the elucidation of a key event in the termination of liver regeneration after surgical resections. We found that livers of S193A mice do not stop regeneration when the liver reaches the original size at days 7-10 and instead continue growth leading to an enlarged liver mass. Consistent with continuing liver proliferation after PH, livers of S193A mice also do not stop proliferation after CCl₄-mediated liver injury. Together, these findings show that phosphorylation of C/EBP α at Ser193 is required for proper entry of hepatocytes into cell cycle and for timely termination of liver proliferation.

Systemic investigations of liver functions in S193A mice revealed several serious dysfunctions, including metabolic changes such as an elevation of albumin, reduction of glucose levels and reduction of ALT/AST in the blood. The work presented in this paper was focused on the mechanisms of reduction of key regulators of liver functions PEPCK, G6Pase, Glut2, p53, C/EBP α , SIRT1, PGC1 α and telomerase. The promoters of these genes are repressed in S193A mice by the C/EBP β -HDAC1 complexes leading to reduction of the corresponding mRNAs and proteins. This global repression of the key regulators of liver proliferation is likely to be the main cause of the increased liver proliferation and failure of the liver to stop regeneration after partial hepatectomy in the S193A mouse model.

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
HDAC1	histone deacetylase 1

TERT	telomerase reverse transcriptase
ChIP	chromatin immunoprecipitation assay
CCl₄	carbon tetrachloride

Figure 1. Characterization of S193A mice

(A) Upper: WT C/EBP α gene contains a site for restriction enzyme MluI (M) and is resistant to KasI; while the S193A mutant is resistant to MluI but contains site for restriction enzyme KasI. Bottom image shows genotyping. MI; restriction of the PCR product with MluI. Kas; restriction of the PCR product with KasI. (B) Strategy for genotyping of S193A mice. (C) H&E staining of 2-month-old WT and S193A mice. Arrows show enlarged hepatocytes. Bar graphs show number of hepatocytes per field under 20X magnification. n = 5; *P < 0.05. (D) PAS staining of livers of WT and S193A mice. Bar graphs show % of hepatocytes containing glycogen. n = 5; *P < 0.05. (E) Examination of blood parameters in S193A mice. The table shows data for 8 animals of each genotype. Right column shows a comparison of the parameters with those in S193D mice (12).

Figure 2. Livers of S193A mice have higher rate of liver proliferation during post-natal development

(A) A typical picture of BrdU staining of livers of WT and S193A mice at days 1, 3, 7 and 15 after birth. (B) Bar graphs show percent of BrdU-positive hepatocytes as a summary of three independent experiments. $n = 3$ to 5; $*P < 0.05$. (C) Expression of cyclin D1 and PCNA. $n = 3$; $*P < 0.05$. (D) Protein levels of C/EBP α were determined by Western blotting assay. Bottom image shows levels of C/EBP α as ratios of both isoforms to β -actin. $n = 3$; $*P < 0.05$. (E) Levels of C/EBP α mRNA were determined by qRT-PCR. $n = 3$ -5; $*P < 0.05$.

Figure 3. C/EBP β -HDAC1 complexes are elevated in livers of S193A mice and repress the C/EBP α promoter

(A) Western blotting was performed using Abs to C/EBP β and HDAC1 with nuclear extracts isolated at different time points after birth. (B) Levels of C/EBP β and HDAC1 were calculated as ratios to β -actin. Data represent mean $\pm$ SD; $n = 3-5$; $*P < 0.05$. (C) Co-IP studies were performed. Bottom image shows amounts of HDAC1 in C/EBP β IPs as ratios to IgG signals. (D) C/EBP α was immunoprecipitated from nuclear extracts of WT, S193D and S193A mice and these IPs were probed with antibodies to p300 and to HDAC1. Dark and light exposures are shown. IgG; signals of immunoglobulins. Bar graph shows amounts of HDAC1 and p300 proteins in C/EBP α IPs as ratios to IgGs. (E) C/EBP α -luc promoter was co-transfected with WT, S193D and S193A C/EBP α mutants into HEK293 cells. In parallel experiments, these proteins were co-transfected in cells with inhibited p300. Internal figure shows the inhibition of p300 by siRNA. Data represent mean $\pm$ SD; $n = 3-5$; $*P < 0.05$. (F) ChIP assay was performed using chromatin solutions from livers of WT, S193D

and S193A mice with primers covering C/EBP site within the C/EBP α promoter. Bottom image shows a hypothesis for the regulation of C/EBP α promoter.

Figure 4. Key regulators of liver biology are repressed in S193A mice by C/EBPβ-HDAC1 complexes

(A) Western blotting was performed with antibodies shown on the right. Bar graphs: Protein levels of G6Pase, PEPCK and Glut2 were calculated as ratios to β-actin. Data represent mean ± SD; n = 3-5; *P < 0.05. (B) Levels of G6Pase, PEPCK and Glut2 mRNAs were determined by qRT-PCR. n = 3-5; *P < 0.05. (C) ChIP assay with G6Pase and PEPCK promoters. α and β; IPs with antibodies to C/EBPα and C/EBPβ; HD1; IPs with HDAC1; K9Ac and K9-me; IPs with antibodies to histone H3 acetylated or trimethylated at K9. Bottom part shows a hypothetical model for the mechanisms of reduction of glucose in S193A mice. (D) Protein levels of key regulators of liver functions were determined by Western blotting. Bar graphs: Levels of SIRT1, p53 and TERT proteins were calculated as ratios to β-actin. Data represent mean ± SD; n = 3-5; *P < 0.05. (E) Levels of SIRT1, p53 and TERT mRNAs were determined by qRT-PCR. n = 3-5; *P < 0.05. (F) Examination of SIRT1, p53 and TERT promoters by ChIP assay. Bottom image shows a hypothesis for the mechanisms which repress SIRT1, PGC1α, p53 and TERT in livers of S193A mice.

Figure 5. S193A hepatocytes start DNA replication at early time points after PH and do not terminate proliferation

(A) **Upper:** Typical pictures of BrdU staining of the WT and S193A livers. Bar graphs on the left show % of BrdU positive hepatocytes at each time point. Bar graph on the right shows total percent of hepatocytes which incorporated BrdU within 96 hours after PH. (B) Typical picture of H&E staining is shown. Bottom image shows number of mitotic figures per 1000 hepatocytes. (C) Expression of cell cycle proteins in WT and S193A mice after PH. (D) Levels of cyclin D1 and PCNA were calculated as ratios to β -actin. (E) Typical pictures of livers at day 15 are shown on the left. Right image shows the restoration of liver mass.

Figure 6. Livers of S193A mice have a high rate of proliferation at day 15 after PH
 (A) Levels of PCNA and cdc2 were determined by Western blotting. (B) Levels of PCNA and cdc2 were calculated as ratios to β -actin. (C) Typical pictures of mitotic figures in livers of WT and S193A mice at days 8 and 15 after PH. (D) Number of mitotic figures was calculated per 1000 hepatocytes.

Figure 7. Homozygous C/EBPα-S193A mice have reduced liver injury and apoptosis, but have increased liver proliferation after CCl₄ treatments
 (A) H&E and BrdU staining of livers after CCl₄ treatments. (B) ALT/AST levels were examined in WT and S193A mice at different time points after CCl₄ treatments. (C) TUNEL assay was performed with S193A and WT mice. Right image shows number of TUNEL positive hepatocytes. (D) Percent of BrdU positive hepatocytes (left) and number of mitotic figures (right) were calculated after CCl₄ treatments. (E) Expression of C/EBPα and cell cycle proteins was examined using Western blotting assay. (F) Levels of cdc2 and PCNA were calculated as ratios to β-actin.

Figure 8. Heterozygous S193A mice have increased liver proliferation, but enter the cell cycle and stop proliferation at the same time points as hepatocytes of WT mice
 (A) Expression of C/EBPα and cell cycle proteins was determined in WT and heterozygous S193A mice after CCl₄ treatments. (B) Levels of C/EBPα isoforms 42 and 30kD were calculated as ratios to β-actin at each time point after CCl₄ treatments. (C) Levels of cdc2 and PCNA were calculated as ratios to β-actin. (D) Typical pictures of BrdU staining of livers and number of BrdU positive hepatocytes at each time point after CCl₄ treatments. (E) A hypothetical role of C/EBP family proteins and chromatin remodeling proteins in liver proliferation, differentiation, liver injury and liver functions.