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## Epigenetic changes play critical role in age-associated dysfunctions of the liver

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### Summary

C/EBP family proteins are important regulators of liver functions. Here we show the critical role of C/EBP $\alpha$ -mediated chromatin remodeling in the age-associated dysfunctions of the liver and in the maintenance of physiological homeostasis. Because ph-S193 isoform of C/EBP $\alpha$  is increased in livers of old mice, we have generated C/EBP $\alpha$ -S193D knockin mice which mimic the ph-S193 isoform of C/EBP $\alpha$ . Analyses of these mice showed that the S193D mutation causes chromatin remodeling leading to histological appearance of “foci-like” nodules, which are also observed in livers of old mice. These “foci-like” structures contain K9 trimethylated histone H3, a marker of heterochromatin. The increase of heterochromatin regions in S193D mice correlates with the elevation of S193D-C/EBP $\alpha$ -HDAC1 complexes and with dys-regulation of gene expression including epigenetic silencing of cyclin D1 and D2 promoters and the inhibition of liver proliferation. The elimination of C/EBP $\alpha$ -HDAC1 complexes in S193D mice by inhibition of HDAC1 corrects chromatin structure and normalizes expression of cyclin D1 and D2. We found that epigenetic dys-regulation is also associated with the elevation of C/EBP $\beta$  and with the increase of C/EBP $\alpha/\beta$  hetero-dimers in S193D mice. The C/EBP $\alpha/\beta$  hetero-dimers activate transcription of Glut4 and increase levels of Glut4. As the result, S193D livers have accelerated uptake of glucose and accumulation of glycogen in the liver. Thus, this study demonstrates that phosphorylation of C/EBP $\alpha$  at S193 leads to the appearance of heterochromatin regions which correlates with the development of age-related dysfunctions of the liver.

### Keywords

epigenetic control; liver; body homeostasis; C/EBP $\alpha$ ; proliferation; aging

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### Author contributions

JJ has performed experiments with the examination of heterochromatin regions in S193D and old mice, CHIP assay, treatments of S193D and old mice with growth hormone, examination of glucose metabolism in S193D mice and genotyping. GW has performed Q-RT-PCR, immunostaining and a portion of Western blotting studies. PI has performed immunostaining, CHIP and Western blotting experiments. XS was responsible for genotyping and maintenance of mice. SH and MF have provided their expertise for the analyses of blood parameters and for examination of liver tissues. NT performed Co-IP and HPLC-based examination of protein-protein complexes, was overall supervisor of the research and contributed to the writing and editing the manuscript.

Supporting information.

Additional supporting information is included in on-line version of the manuscript.

## Introduction

Senescent liver is characterized by many alterations in biological processes leading to the decline of liver functions and to the alterations in body homeostasis (Schmucker, 2005; Timchenko, 2009). The most dramatic alteration in liver functions is the impaired liver regeneration after partial hepatectomy (PH) and after surgical resections (Bucher *et al.*, 1964; Fry *et al.*, 1984, Iakova *et al.*, 2003, Conboy *et al.*, 2005; Geilchinsky *et al.*, 2010). The decline of the regenerative capacity of old livers is mediated by the appearance of the C/EBP $\alpha$ -Brm-HDAC1 complex (Iakova *et al.*, 2003, Conboy *et al.*, 2005; Wang *et al.*, 2006) and following epigenetic silencing of E2F-dependent promoters (Wang *et al.*, 2007; 2008a; Timchenko 2009). Recent reports have shown that the reduction of GSK3 $\beta$  in aged livers causes the age-associated elevation of the C/EBP $\alpha$ -HDAC1 complexes (Jin *et al.*, 2009a; 2009b; 2009c). In agreement with these observations, Seo et al have found that the inhibition of GSK3 $\beta$  leads to development of senescence phenotype in human liver-derived Chang cells (Seo *et al.*, 2008). The aging liver is also characterized by changes in the size of hepatocytes. Chipchase et al have found that livers of two years old mice have increased number of hepatocytes containing enlarged nuclei with increased ploidy; while livers of 3 months old mice do not contain enlarged hepatocytes (Chipchase *et al.*, 2003). The premature liver polyploidy has been also described in DNA repair (Ercc1) deficient mice (Chipchase *et al.*, 2003). One of the characteristics of the old livers is a lipid accumulation which leads to the development of steatosis (Kuk *et al.*, 2009). It has been also shown that the activities of alanin transaminase (ALT) and aspartate transaminase (AST) and concentration of triglycerides are increased in the blood of old animals as the result of liver dysfunctions (Schmucker, 2005). Molecular basis for these age-associated dysfunctions of the liver has been not elucidated.

The liver is able to regenerate itself after partial hepatectomy (Fausto *et al.*, 2006). The regeneration of the liver after PH is a very complex process which includes reorganizations of the networks of several signal transduction pathways (Michalopoulos, 2007). Despite the global re-organization of these pathways, the remaining portion of the liver proliferates and it is able completely support its differentiation functions and body homeostasis. The molecular basis for these enormous capabilities of the small remaining portion of the liver is not known. Two members of C/EBP family, C/EBP $\alpha$  and C/EBP $\beta$ , are expressed in the liver and play critical role in the regulation of liver biology (Timchenko, 2009; Johnson, 2005). Transcription factor C/EBP $\alpha$  is an important component of network since C/EBP $\alpha$  knockout mice die shortly after birth due to impaired energy homeostasis (Wang *et al.*, 1995). C/EBP $\alpha$  displays many of its functions through direct interactions with proteins. C/EBP $\alpha$  interacts with the SWI/SNF chromatin remodeling complex and activates expression of genes involved in adipogenesis (Pedersen *et al.*, 2001). The interaction of C/EBP $\alpha$  with Brm is involved in the inhibition of cell proliferation (Iakova *et al.*, 2003, Conboy *et al.*, 2005; Muller *et al.*, 2004). In aging liver, C/EBP $\alpha$  is hyperphosphorylated at S193 by cyclin D3-cdk4 and is associated with histone deacetylase 1, HDAC1, and with heterochromatin protein 1 $\alpha$ , HP1 $\alpha$  (Wang *et al.*, 2008a; 2008b). In agreement with these observations, Demarco et al have identified a direct interaction of C/EBP $\alpha$  with HP1 $\alpha$  in the regions of centromeric heterochromatin (Demacro *et al.*, 2006).

We have recently generated S193D-C/EBP $\alpha$  knockin mice and have shown that liver proliferation after PH is completely inhibited in these mice (Wang *et al.*, 2010). Here we examined the role of phosphorylation of C/EBP $\alpha$  at S193 in the age-associated decline of liver functions. We found that the age-related mutation of C/EBP $\alpha$  leads to the significant increase of heterochromatin regions and that these alterations are similar to those observed in old mice which have abundant ph-S193 isoform of C/EBP $\alpha$ . The elevation of heterochromatin regions correlates with the development of age-like dys-functions such as

inhibition of liver proliferation, accumulation of glycogen in the liver and the elevation of ALT/AST and triglycerides in the blood.

## RESULTS

### The C/EBP $\alpha$ -S193D increases size of hepatocytes and alters chromatin structure

In these studies, we have examined liver functions in C/EBP $\alpha$ -S193D knockin mice, which have been recently generated (Wang *et al.*, 2010). The Fig 1A shows an example of genotyping of mice used for these studies. We have mutated serine to aspartate by TC-GA substitution which also created new BamHI site and destroyed MluI site (Fig 1A). In the course of examination of S193D mice, we found that S193D isoform significantly changes liver morphology. The H&E staining showed that the size of hepatocytes and size of nuclei are significantly increased in livers of C/EBP $\alpha$ -S193D mice compared to WT mice (Fig 1B). The size of nuclei in S193D mice is around 2-fold bigger than that in WT mice (see bottom part of the Fig 1B). Since the liver weight/body weight ratio is not changed in S193D mice, the increase of the size of hepatocytes is consistent with a reduction of the number of hepatocytes in the liver down to 50% (Fig 1C). Since further studies showed that liver proliferation is inhibited in S193D mice during postnatal liver development (Fig 6), these observations suggested that normal size of the S193D livers is supported by increased cell growth leading to the increased size of hepatocytes. To determine if the alterations of the size of nuclei in S193D mice change chromatin structure, we have performed DAPI staining of livers of 2 mo old mice. Consistent with H&E staining, the DAPI staining showed that hepatocytes in C/EBP $\alpha$ -S193D livers have a larger size of nucleus. In addition to that, we found that hepatocytes in S193D livers have a significant increase of “foci-like” nodules (Fig 1D). Since livers of old mice are characterized by increased size of nuclei (Chipchase *et al.*, 2003), we have next performed H&E and DAPI staining of livers of WT young (2 mo) and old (24 mo) mice (Fig. 1E–G). We found that up to 35–40% of hepatocytes are enlarged in livers of old mice (Fig 1F). Examination of chromatin structure by DAPI staining revealed the abundance of “foci-like” nodules in nuclei of livers from old mice, especially in cells with enlarged nuclei (Fig 1G and see additional images in Supplemental Figure 1).

### “Foci-like” structures in young S193D mice and in old wild type mice are heterochromatin regions

We have next examined if the “foci-like” structures in S193D and old WT hepatocytes might be heterochromatic regions. Livers of young WT, young S193D and old WT mice were stained with antibodies to the marker of heterochromatin, histone H3 trimethylated at K9 (H3K9Me). Figure 2 shows typical pictures of the staining. Nuclei of hepatocytes of young WT mice show mainly diffused staining of H3K9Me with a rare punctuated location of H3K9Me. On the contrary, nuclei in hepatocytes of young S193D mice and nuclei in large hepatocytes of old WT mice contain a much bigger number of H3K9Me located in “foci-like” structures. In S193D livers, around 80–90% of hepatocytes contain H3K9Me in “foci-like” structures. It is interesting to note that, in livers of old mice, “foci-like” structures determined by DAPI staining and H3K9me are mainly co-localized in enlarged hepatocytes. Thus, these studies show that the S193D mutation of C/EBP $\alpha$  leads to the elevation of heterochromatin regions. Similar alterations are observed in large hepatocytes of aged livers.

### Livers of S193D mice contain abundant C/EBP $\alpha$ -HDAC1 complexes and elevated levels of C/EBP $\beta$

We have next examined pathways by which C/EBP $\alpha$ -S193D mutant causes alteration of chromatin structure. Our previous studies have shown that the S193-ph isoform of C/EBP $\alpha$  form complexes with a chromatin remodeling protein histone deacetylase 1, HDAC1, in livers of old mice (Wang *et al.*, 2008a). Therefore, we determined if the C/EBP $\alpha$ -S193D

might affect chromatin structure via interactions with HDAC1. Staining of livers of 2 mo old mice with Abs to HDAC1 and C/EBP $\alpha$  showed that livers of WT mice have a diffuse nuclear staining for both HDAC1 and C/EBP $\alpha$ ; while HDAC1 and C/EBP $\alpha$  are observed in punctuate “foci-like” structures of the C/EBP $\alpha$ -S193D livers (Fig 3A). We have next examined interactions of C/EBP $\alpha$  with HDAC1 and with heterochromatin protein  $\alpha$ , HP1 $\alpha$ , using Co-IP approach and HPLC-based size exclusion chromatography. HP1 $\alpha$  has been included in the studies since this protein cooperates with HDAC1 in the repression of promoters and because this protein interacts with C/EBP $\alpha$  (Wang *et al.*, 2008a; Demacro *et al.*, 2006). Co-IP approach showed that larger amounts of HDAC1 and HP1 $\alpha$  are observed in the complexes with the mutant C/EBP $\alpha$ ; while C/EBP $\alpha$ -HDAC1 complex is detected in WT mice only after longer exposure (Fig 3B). Previous studies have shown that the livers of old mice contain the C/EBP $\alpha$ -Brm complex (Jakova *et al.*, 2003; Conboy *et al.*, 2005) and that this complex is up-regulated by phosphorylation of C/EBP $\alpha$  at S193 (Wang *et al.*, 2007). Therefore, we have examined if Brm might be in the complexes with C/EBP $\alpha$  in young S193D mice. Under sensitivity of our assay, we could not detect Brm in C/EBP $\alpha$  IPs (Fig 3B). The lack of Brm in C/EBP $\alpha$  complexes is perhaps associated with the very low levels of Brm in livers of 2 mo old mice. To confirm results of Co-IP and to determine the size of the C/EBP $\alpha$ -HDAC1 complex, we have utilized HPLC-based techniques. These studies showed that the complex is significantly larger (around 680kD) than the sum of masses of C/EBP $\alpha$ , HDAC1 and HP1 $\alpha$  suggesting that it might contain additional components (Fig 3C).

Given the elevation of heterochromatin regions in hepatocytes of S193D mice, we suggested that S193D mice might have changes in the transcription of many genes. Therefore, we have performed micro array studies with livers of 2 months old WT and S193D mice. These studies identified a number of genes with reduced or increased expression on livers of S193D mice (data not shown). In this paper, we have investigated in details a portion of these genes including C/EBP family proteins, genes which are involved in control of liver growth and in the control of glucose metabolism. The alteration in expression of these genes was confirmed by Q-RT-PCR and by Western blotting. These data are presented throughout the manuscript (see Figs 3, 5 and 8). C/EBP family is one of the groups of genes with altered mRNA levels in livers of S193D mice. Figure 3D shows differences in the levels of C/EBP family mRNAs which were further confirmed by Q-RT-PCR. We found that the levels of C/EBP $\beta$  and C/EBP $\epsilon$  mRNAs are increased in livers of S193D mice, while levels of C/EBP $\alpha$  and C/EBP $\gamma$  are slightly reduced. To examine if protein levels of C/EBP proteins are changed in S193D mice, we have performed Western blotting analyses. These studies have shown that, although C/EBP $\epsilon$  and C/EBP $\gamma$  mRNAs are detected by RT-PCR, these proteins are not detectable in livers of both WT and S193D mice by Western blotting (data not shown). Examination of C/EBP $\alpha$  showed that it is expressed as three isoform with MW 42kD, 40kD and 30kD. In agreement with a slight reduction of C/EBP $\alpha$  mRNA, the protein levels of these three isoforms of C/EBP $\alpha$  are slightly reduced in livers S193D mice (Fig 3E). However, this reduction is minor and represents less than 20% (Fig 3E, middle bar graphs). Quite different result was observed for C/EBP $\beta$  expression. C/EBP $\beta$  is also expressed in the liver as three isoforms: full-lengths protein (FL), C/EBP $\beta$ -LAP and C/EBP $\beta$ -LIP. We have found that protein levels of C/EBP $\beta$ -LAP are elevated in livers of S193D mice; while levels of a truncated isoform C/EBP $\beta$ -LIP are not changed. Calculation of C/EBP $\beta$ -LAP as ratios to  $\beta$ -actin showed around 2.5-fold increase in livers of S193D mice (Fig 3E, bottom). Taken together, these studies showed that the alterations of chromatin structure in S193D mice are associated with elevation of C/EBP $\alpha$ -HDAC1 complexes and with elevation of C/EBP $\beta$ .

## The inhibition of HDAC1 in S193D mice corrects size and chromatin structure of nuclei in hepatocytes

Given the abundance of the C/EBP $\alpha$ -HDAC1 complexes in livers of S193D mice, we suggested that these complexes might be involved in the alterations of chromatin structure. To test this hypothesis, we have inhibited expression of HDAC1 by siRNA in S193D mice and examined chromatin structure of the nuclei. We have previously established conditions for the efficient delivery of siRNA HDAC1 and for a strong inhibition of HDAC1 in the liver (Wang *et al.*, 2008b). The animals were injected with siRNA to HDAC1 for three days and livers were harvested and analyzed. Using these conditions, we have inhibited expression of HDAC1 to less than 15% in livers of C/EBP $\alpha$ -S193D mice (Fig 4A). Examination of C/EBP $\alpha$ -HDAC1 complexes by Co-IP approach showed that siRNA-mediated inhibition of HDAC1 eliminates the C/EBP $\alpha$ -HDAC1 complexes in livers of S193D mice (Fig 4B). H&E staining and DAPI staining showed that the elimination of C/EBP $\alpha$ -HDAC1 complexes correlates with the correction of the size and chromatin structure of the nuclei in hepatocytes (Fig 4C). Staining of the livers with antibodies to C/EBP $\alpha$  and to HDAC1 showed that the reduction of HDAC1 by siRNA re-distributes C/EBP $\alpha$  from foci-like structures to the diffuse location within nucleus. In addition to the correction of chromatin structure, the inhibition of HDAC1 reduces the size of hepatocytes. We suggest that the enlarged size of hepatocytes in S193D mice might be the result of epigenetic alterations and that the elimination of foci-like structures also corrects the size of existing hepatocytes.

Previous studies have shown that C/EBP $\alpha$ -HDAC1 complexes are also abundant in livers of old mice (Wang *et al.*, 2008a) suggesting that these complexes might be involved in the age-associated alterations of chromatin structure in old animals. To determine the role of the C/EBP $\alpha$ -HDAC1 complexes in these age-associated alterations, we have initially tried to reduce the complexes by inhibiting HDAC1; however, the siRNA-mediated inhibition of HDAC1 in old mice caused a massive cell death shortly after injection of siRNA. Therefore, we have used an alternative approach to eliminate the complex, which is the treatment of mice with growth hormone (GH). It has been shown that GH activates pathways which de-phosphorylate C/EBP $\alpha$  at S193 and reduce amounts of C/EBP $\alpha$ -HDAC1-Brm complex (Wang *et al.*, 2008a). In agreement with previous reports, the treatments of old mice with GH eliminated the C/EBP $\alpha$ -HDAC1-Brm complex (Fig 4E). H&E staining and examination of nuclei by DAPI staining revealed that the elimination of the C/EBP $\alpha$ -HDAC1-Brm complex also corrects the size and chromatin structure of the nuclei. The figure 4F shows typical pictures of H&E and DAPI staining. We found that the treatment of old mice with GH reduced the amounts of enlarged hepatocytes down to 10–15% (Fig 4G).

Previous studies showed that GH reduces the C/EBP $\alpha$ -HDAC1 complex by activation of pathways which de-phosphorylate C/EBP $\alpha$  at S193. Since S193D mutation prevents elimination of the negative charge on S193, we examined if S193D mutation will prevent GH-mediated correction of the chromatin structure in S193D mice. We found that the treatment of S193D mice with GH is not sufficient to alter chromatin structure and size of hepatocytes in S193D mice (Fig 4H). This result suggested that the S193D mutation took C/EBP $\alpha$  away from the GH-dependent control. Taken together, the studies of S193D mice and examinations of old mice showed that the elevation of the C/EBP $\alpha$ -HDAC1 complex correlates with alterations in chromatin structure and that elimination of these complexes in S193D and in old mice contributes to the correction of chromatin structure.

### **C/EBP $\alpha$ -HDAC1 complexes reduce levels of cyclin D2 in C/EBP $\alpha$ -S193D livers via repression of the cyclin D2 promoter**

We have next examined biological consequences of the change of chromatin structure in S193D mice. The micro array analysis has determined a group of mRNAs which have reduced levels in livers of S193D mice. This group represents genes which are involved in regulation of liver growth including cyclins D1 and D2 (Fig 5A). Since D-type cyclins play critical role in the liver proliferation, we focused our further studies on the promoters of cyclin D1 and D2. Western blotting confirmed that the expression of cyclin D2 is reduced in C/EBP $\alpha$ -S193D livers (Fig 5B); while cyclin D1 protein was not detected in quiescent livers. Therefore, we first examined if C/EBP $\alpha$ -HDAC1 complexes down-regulate cyclin D2 promoter in quiescent livers. Examination of the cyclin D2 promoter identified four consensus sites for C/EBP $\alpha$  (Fig 5C). To determine if C/EBP $\alpha$  binds to these sites, we have examined the interaction of C/EBP $\alpha$  with DNA probes covering C/EBP $\alpha$  sites within the cyclin D2 promoter using EMSA. These studies showed that C/EBP $\alpha$  binds to the cyclin D2 promoter in vitro (Figure 5D).

To test if C/EBP $\alpha$ -HDAC1 complexes down-regulate the cyclin D2 in S193D mice, we have examined expression of the cyclin D2 in livers of S193D mice treated with siRNA to HDAC1. Western blotting has shown that the levels of cyclin D2 are normalized in S193D mice by siRNA to HDAC1 and that this normalization correlates with the elimination of the C/EBP $\alpha$ -HDAC1 complexes (Fig 5E). To determine the causal role C/EBP $\alpha$ -HDAC1 complexes in the repression of the cyclin D2 promoter in the livers of S193D mice, we have performed chromatin immunoprecipitation (ChIP) analyses. ChIP studies showed that C/EBP $\alpha$ -HDAC1 complex is abundant on the cyclin D2 promoter in the C/EBP $\alpha$ -S193D livers, while only C/EBP $\alpha$  is observed on this promoter in WT mice (Fig 5F). The elimination of HDAC1-C/EBP $\alpha$  complexes leads to the removal of HDAC1 from the cyclin D2 promoter (Fig 5F). To determine if the cyclin D2 promoter is repressed in S193D livers, we examined modifications of histone H3 associated with the cyclin D2 promoter. K9 acetylated histone H3 occupies the cyclin D2 promoter in WT mice; while tri-methylated H3K9 is not detected. In contrast, the Ac-K9-histone H3 is dramatically reduced on the D2 promoter in livers of C/EBP $\alpha$ -S193D mice, but amounts of trimethylated K9H3 are increased (Fig 5F). The inhibition of C/EBP $\alpha$ -HDAC1 complexes reversed the patterns of modifications of histone H3 on the cyclin D2 promoter. These patterns of histone H3 modifications show that C/EBP $\alpha$ -HDAC1 complex inhibits the cyclin D2 promoter.

### **C/EBP $\alpha$ -HDAC1 complexes repress cyclin D1 promoter in the liver during post-natal development**

Since expression of cyclin D1 protein is not detectable in quiescent livers of 2 months old mice, we have examined the role of C/EBP $\alpha$ -HDAC1 complexes in the regulation of cyclin D1 using mice during early post-natal development when liver proliferates. We have first asked if the C/EBP $\alpha$ -S193D inhibits liver proliferation after birth. Proliferation of the livers during postnatal development was examined by measuring BrdU uptake in 1, 3, 7, 15 and 60 days old mice. Figure 6A shows that the rate of liver proliferation in WT mice is relatively high at day 1 and then declines with age. We have found that the phosphomimetic C/EBP $\alpha$ -S193D reduces liver proliferation at all stages of postnatal liver development. We have next examined mechanisms by which C/EBP $\alpha$ -S193D mutant and ph-S193 isoform inhibit liver proliferation during postnatal liver development. Figure 6B shows that total protein levels of both WT and S193D-C/EBP $\alpha$  are increased with age and the phosphorylation of WT C/EBP $\alpha$  at S193 is detectable at days 7, 15 and 60 in WT mice. Since antibodies to S193-ph isoform of C/EBP $\alpha$  do not recognize S193D mutant (Wang *et al.*, 2006; 2007), no specific signals were observed with protein extracts from the livers of C/EBP $\alpha$ -S193D mice. We have next examined expression of cyclin D1 during postnatal development. In addition to

cyclin D1, expression of FoxM1 was examined since this protein is expressed only in proliferating livers and serves as an additional marker of liver proliferation. Figure 6C shows that the levels of cyclin D1 and FoxM1B are significantly lower in C/EBP $\alpha$ -S193D mutant livers during whole course of the studies. Calculations of cyclin D1 levels as ratios to  $\beta$ -actin revealed 2–3-fold higher levels of cyclin D1 protein at days 1–7 in WT mice compared to the levels in S193D mice. The expression of cyclin D1 is inhibited and is not detectable at days 15 and 60 in both WT and S193D mice.

We next asked if the inhibition of cyclin D1 in S193D livers is mediated by C/EBP $\alpha$ -HDAC1 complexes. Examination of the cyclin D1 promoter identified a consensus for C/EBP $\alpha$  to which C/EBP $\alpha$  binds (Supplemental Figure 2). To determine if the C/EBP $\alpha$ -HDAC1 complexes inhibit the cyclin D1 promoter through interactions with the identified site, we have performed ChIP assay with primers covering this site. For the ChIP studies, 7 day old animals were used since the differences in the expression of cyclin D1 between WT and S193D mice are significant at that time point. ChIP studies showed that the cyclin D1 promoter is occupied by C/EBP $\alpha$  in WT mice; while HDAC1 is not detectable on the promoter and histone H3 is acetylated at K9 (Fig 6D). This pattern of histone H3 modifications shows that the promoter is active. On the contrary, the cyclin D1 promoter is occupied by C/EBP $\alpha$ -HDAC1 complex in S193D mice leading to partial de-acetylation of histone H3 and following trimethylation at K9. These alterations in the histone H3 modifications revealed that the cyclin D1 promoter is partially repressed in livers of S193D mice by C/EBP $\alpha$ -HDAC1 complex.

C/EBP $\alpha$  has several pathways of inhibition of cell proliferation. Previous studies showed that C/EBP $\alpha$  arrests liver proliferation in young mice through binding to and inhibition of cdk2 (Wang *et al.*, 2001; 2004; Tan *et al.*, 2005); therefore we have examined if this pathway might be also involved in the inhibition of liver proliferation during post-natal liver development. Figure 6E shows that cdk2 is associated with the mutant S193D-C/EBP $\alpha$  at all tested stages of postnatal liver development and that this association is proportional to amounts of C/EBP $\alpha$ . On the contrary, the association of cdk2 with C/EBP $\alpha$  in WT mice is observed only in livers of 15 and 60 days old mice. Examination of cdk2 IPs in *in vitro* kinase assay with histone H1 substrate shows that, in WT livers, cdk2 activity is high at days 1–7 and is reduced at later stages of liver development. However, cdk2 activity is inhibited in C/EBP $\alpha$ -S193D livers at all stages of postnatal development (Fig 6F). Taken together, these data demonstrate that the mutant S193D-C/EBP $\alpha$  and ph-S193 isoform of C/EBP $\alpha$  arrest liver proliferation during postnatal development through repression of the cyclin D1 promoter and via inhibition of cdk2.

### **Livers of C/EBP $\alpha$ -S193D mice have increased uptake of the glucose and accumulation of glycogen**

Examination of blood parameters showed that levels of glucose are reduced in S193D mice (Wang *et al.*, 2010 and see Fig 7A). Therefore, we have investigated pathways by which S193D mutation affects glucose levels. The glucose tolerance test revealed that the C/EBP $\alpha$ -S193D animals remove glucose from the blood much faster than WT mice (Fig 7B). To determine if the liver is involved in the increased removing glucose from the blood, we have injected radioactive glucose and have examined the uptake of H3-glucose by livers in WT and in S193D mice. These studies revealed that livers of S193D mice utilize glucose much more efficiently than livers of WT mice (Fig 7C). These data suggest that the reduction of the glucose in the blood of C/EBP $\alpha$ -S193D mice is due to an increased uptake of glucose by the liver. Since one of the pathways of the utilization of the glucose in the liver is the conversion of glucose into glycogen, we have isolated glycogen from WT and S193D livers after injection with H3-glucose. Measurements of total and H3-glycogen showed that the utilized H3-glucose is converted into glycogen (Fig 7D). In agreement with these data,

staining of WT and S193D livers for glycogen detected a significant increase of glycogen in livers of C/EBP $\alpha$ -S193D mice (Fig 7E).

### The C/EBP $\alpha$ -S193D increases expression of genes involved in utilization of glucose

We have next examined molecular mechanisms by which the S193D mutant increases liver-mediated uptake of glucose in C/EBP $\alpha$ -S193D mice. Examination of expression of genes involved in glucose metabolism by Q-RT-PCR showed that levels of mRNAs coding for the glucose transporters Glut2 and Glut4, GcK and GyS2 are increased in livers of C/EBP $\alpha$ -S193D mice (Fig 8A and B). Since Glut4 is also expressed in adipose tissues, we have examined the levels of Glut4 mRNA in adipocytes and found around 15-fold elevation of Glut4 mRNA in adipose tissue of S193D mice. Because levels of Glut4 mRNA are dramatically activated in both liver and adipocytes, we determined mechanisms of regulation of Glut4 by C/EBP $\alpha$ -S193D. We have focused the studies on the livers of S193D mice, since the S193D liver has increased uptake of glucose. Western blotting confirmed that livers of C/EBP $\alpha$ -S193D mice contain higher levels of Glut4 protein (Fig 8C). We have next asked if there is a difference in the ability of WT C/EBP $\alpha$  and the mutant S193D-C/EBP $\alpha$  to activate the Glut4 promoter. The Glut4 promoter was cloned into pGL3 vector and co-transfected with WT C/EBP $\alpha$  and with S193D-C/EBP $\alpha$  into Hep3B2 cells. In addition to these constructs, C/EBP $\alpha$ -R290A mutant was used since it does not bind to DNA (Miller *et al.*, 2003; Wang *et al.*, 2004). Co-transfection studies showed that the S193D mutation significantly increases the ability of C/EBP $\alpha$  to activate the promoter (Fig 8D). Our further studies in tissue culture systems showed that the cyclin D3-cdk4-mediated phosphorylation of endogenous C/EBP $\alpha$  at S193 enhances its ability to activate the Glut4 promoter (Supplemental Figure 3). Taking together these data, we conclude that the S193D mutation and phosphorylation of C/EBP $\alpha$  at S193 enhance the ability of C/EBP $\alpha$  to activate the Glut4 promoter.

### C/EBP $\beta$ forms hetero-dimers with C/EBP $\alpha$ -S193D and co-operates with C/EBP $\alpha$ -S193D in the activation of the Glut4 promoter

Because protein levels of C/EBP $\beta$  are increased in livers of S193D mice (Fig 3), we have examined if this elevation might be involved in the activation of the Glut4 promoter. We first performed EMSA with the probe covering C/EBP site within the Glut4 promoter. Specific antibodies were incorporated to distinguish C/EBP $\alpha$  and C/EBP $\beta$  isoforms. Consistent with elevation of protein levels of C/EBP $\beta$ , significantly higher amounts of C/EBP $\beta$  are bound to the Glut4 promoter in livers of S193D mice (Fig 8E). This is especially clear after supershift of the C/EBP $\alpha$  (Fig 8E, lane 8). Most important, the incorporation of antibodies to C/EBP proteins showed that the majority of S193D-C/EBP $\alpha$  binds to the Glut4 promoter as a heterodimer with C/EBP $\beta$  and that the binding of C/EBP $\alpha$  homodimers is not detectable after supershift with antibodies to C/EBP $\beta$  (Fig 8E, lane 9). It is important to note that antibodies to C/EBP $\beta$  specifically interact with C/EBP $\beta$  and do not cross-react and do not supershift C/EBP $\alpha$  in livers of WT mice (Fig 8E, lane 4). To determine if the elevation of C/EBP $\beta$  and C/EBP $\alpha$ / $\beta$  heterodimers up-regulate Glut4 in livers of S193D mice, we have examined interactions of C/EBP proteins with the Glut4 promoter and modifications of histone H3 on the Glut4 promoter using ChIP analysis. HDAC1 protein was also included in these studies since it forms complexes with C/EBP proteins in the liver (Wang *et al.*, 2008a; 2008b). These studies showed that the Glut4 promoter is occupied and partially repressed in WT mice by C/EBP $\beta$ -HDAC1 and C/EBP $\alpha$ -HDAC1 complexes because histone H3 is trimethylated at K9 on the Glut4 promoter (Fig 8F). On the contrary, HDAC1 is not detectable on the Glut4 promoter in S193D livers. Our data show that the Glut4 promoter is activated by S193D-C/EBP $\alpha$  and C/EBP $\beta$  because histone H3 is acetylated at K9 on this promoter.



## **C/EBP $\beta$ is required for the increased ability of C/EBP $\alpha$ -S193D to activate the Glut4 promoter**

EMSA and ChIP assays suggested that C/EBP $\alpha$ -S193D activates the Glut4 promoter via formation of heterodimers with C/EBP $\beta$ . To directly examine the role of C/EBP $\alpha$ :C/EBP $\beta$  hetero-dimers, we have performed experiments in cultured HEK293 cells. These cells were chosen for these studies since they do not express endogenous C/EBP $\alpha$ ; therefore, effects of the transfected S193D mutant will not be influenced by endogenous C/EBP $\alpha$ . The expression of C/EBP $\beta$  was inhibited in these cells by siRNA; and the WT and S193D mutant C/EBP $\alpha$  were co-transfected with the Glut4 promoter. As the control, the activity of Glut4 promoter was examined in cells transfected with C/EBP $\beta$  siRNA alone. Figure 8G shows that the inhibition of C/EBP $\beta$  in cells transfected with WT C/EBP $\alpha$  does not change significantly the activation of the Glut4 promoter. However, the inhibition of C/EBP $\beta$  in cells transfected with S193D mutant reduces the activity of the Glut4 promoter to the levels observed for activation by WT C/EBP $\alpha$ . These studies clearly demonstrated that the increased ability of C/EBP $\alpha$ -S193D to activate the Glut4 promoter is mediated via interactions with C/EBP $\beta$ , perhaps through the formation of hetero-dimers with C/EBP $\beta$ . Thus, these studies showed that the formation of C/EBP $\beta$ :C/EBP $\alpha$ -S193D heterodimers is involved in the activation of the Glut4 promoter and following increase of Glut4 protein in S193D mice.

## **DISCUSSION**

### **Age-related dysfunctions of the liver are mediated by the ph-S193 isoform of C/EBP $\alpha$**

Senescent liver is characterized by decline of several functions and alterations in liver morphology (Schmucker, 2005; Timchenko, 2009). In this paper, we have examined the role of age-specific S193-ph isoform of C/EBP $\alpha$  in the age-related alterations using recently generated C/EBP $\alpha$ -S193D mice. In our previous paper, we have shown that C/EBP $\alpha$ -S193D mutant inhibits liver proliferation after partial hepatectomy (Wang *et al.*, 2010). The impaired liver proliferation in S193D mice is similar to that observed in livers of old WT mice (Bucher *et al.*, 1964; Timchenko, 2009). Examination of liver morphology and liver functions in young C/EBP $\alpha$ -S193D mice showed that hepatocytes of S193D mice have enlarged size and have abundant heterochromatin regions which are similar to those observed in livers of old WT mice. These data are in agreement with previous reports showing that mammalian aging is associated with remodeling of chromatin structure (Sedivy *et al.*, 2008; Bandyopadhyay *et al.*, 2007; Narita *et al.*, 2003). Our data suggest that alterations of chromatin structure might be the cause of the increase of size of hepatocytes of old mice; however, it is also possible that the increased ploidy in old hepatocytes (Chipchase *et al.*, 2003) explains the appearance of DAPI-bright heterochromatic foci, because the increased number of foci could reflect the heterochromatic centromeres of additional chromosomes.

We have performed a detailed investigation of pathways by which S193D-C/EBP $\alpha$  and ph-S193 isoform of C/EBP $\alpha$  might change chromatin structure. Our data show that alterations in chromatin structure of hepatocytes correlate with the elevation of C/EBP $\alpha$ -HDAC1-HP1 $\alpha$  complexes. These complexes are abundant in livers of old mice, but contain some additional proteins such as Brm and E2F4 (Iakova *et al.*, 2003, Conboy *et al.*, 2005; Wang *et al.*, 2008a). The elevation of C/EBP $\alpha$ -HDAC1 complex seems to be responsible for the alterations of chromatin structure because the inhibition of the complex by siRNA to HDAC1 or by treatment of old mice with GH corrects chromatin structure in livers of S193D mice and in livers of old mice respectively. In this paper, we have shown that alterations of chromatin structure are involved in the inhibition of liver proliferation and in dys-regulation of gluconeogenesis. It is interesting to note that our previous paper has

identified an additional age-associated and S193-ph- dependent alteration which is a high frequency of development of liver cancer in old mice. We have shown that the development of liver cancer in old mice is associated with the specific elimination of ph-S193 isoform of C/EBP $\alpha$  by gankyrin-mediated degradation through ubiquitin proteasome system (Wang *et al.*, 2010). Taken together, the investigations of S193D mice show that the expression of the age-specific C/EBP $\alpha$ -S193D isoform in the liver of young mice leads to alterations which mimic liver dysfunctions observed in old animals (Schmucker, 2005, Timchenko, 2009). Alterations in S193D mice include enlarged hepatocytes, inhibition of liver proliferation, steatosis, accumulation of AST/ALT and TG in the blood and accumulation of glycogen in the liver (Fig 9).

It is interesting to note that Seo *et al.* have shown that the accumulation of glycogen itself led to senescence (Seo *et al.*, 2008). Therefore, the accumulation of glycogen in livers of S193D mice might contribute to the age-associated changes in S193D mice. Our findings also suggest that the correction of the chromatin structure might be sufficient to improve liver functions in elderly. One of the possible tools for the correction of chromatin structure is a treatment of mice with growth hormone. Consistent with this hypothesis, Krupczak-Hollis *et al.* have shown that the treatment of old mice with GH corrects liver regeneration via stimulation of proliferation of hepatocytes (Krupczak-Hollis *et al.*, 2003). A recent paper has shown another pathway which might correct liver regeneration in aged mice. Gielchinsky *et al.* found that activation of Akt/mTORC1 pathway in aged livers restores liver regeneration by activation of the process of cell growth (Gielchinsky *et al.*, 2010).

Examination of the S193D mice has provided an answer to the critical question of liver biology. Liver supports body homeostasis and, during liver regeneration, the small portion of remaining liver completely maintains its functions of the differentiated tissue. The majority of studies of liver regeneration were performed with 70% model of partial hepatectomy; however, there are many reports of successful liver regeneration after 90% partial hepatectomy (Moser *et al.*, 2001; Gorbin *et al.*, 2002; Benko *et al.*, 2010; Eipel *et al.*, 2010). How does the remaining, very small portion of the liver control the multiple functions? Data in this paper and our published observations (Wang *et al.*, 2010) present an example of pathways which liver uses to control proliferation and differentiation functions at the same time. Our data suggest that the enormous capabilities of the liver to control multiple functions are associated with a proper regulation of C/EBP $\alpha$  activities by timely phosphorylation/de-phosphorylation at a single amino acid residue and that disruption of this regulation causes pathological alterations in the liver and in blood.

### Multiple pathways of C/EBP $\alpha$ -mediated arrest of cell proliferation

C/EBP $\alpha$  inhibits cell proliferation mainly through the interactions with cell cycle proteins (Wang *et al.*, 2001, Tan *et al.*, 2005; Timchenko, 2009). The analysis of S193D mice allowed us to identify additional pathways by which C/EBP $\alpha$  inhibits liver proliferation and additional pathways which regulate the biological activities of C/EBP $\alpha$ . The additional pathway of growth arrest involves silencing the cyclin D1 and D2 promoters by C/EBP $\alpha$ -HDAC1 complexes through direct interactions with these promoters. Although this pathway requires protein-protein interactions with HDAC1, it differs from previously described pathways and also requires DNA binding activity of C/EBP $\alpha$ . This is a first observation showing that direct interaction of C/EBP $\alpha$  with the promoters of D-type cyclin genes is involved in the arrest of proliferation. Examination of the S193D mice has also identified additional levels of complexity of the regulation of C/EBP $\alpha$  activities. Data in our paper show that S193D-C/EBP $\alpha$  activates the Glut4 promoter, but it inhibits cyclin D2 and cyclin D1 promoters. How are these opposite activities of C/EBP $\alpha$  regulated? One possible pathway is supported by the findings that S193D mice have the increased amounts of S193D-C/EBP $\alpha/\beta$  heterodimers. Our data show that the C/EBP $\alpha/\beta$  heterodimers bind to the

Glut4 promoter and activate the promoter stronger than homo-dimers of each of these proteins. We have also shown that the interactions of C/EBP $\alpha$  with chromatin remodeling proteins might be one of the mechanisms which control these opposite transcriptional functions of C/EBP $\alpha$ . We have found that C/EBP $\alpha$  is observed on the cyclin D1 and cyclin D2 promoters in the complexes with HDAC1; while C/EBP $\alpha$  binds to the Glut4 promoter independently on HDAC1, mainly in the complexes with C/EBP $\beta$ . Thus, our data show that the transcriptional activities of C/EBP $\alpha$  are also controlled, at least in part, by phosphorylation at S193.

## Experimental Procedures

### Antibodies and Reagents

Antibodies to cyclin D3 (C-16), C/EBP $\alpha$  (14AA and N19), Glut4, C/EBP $\beta$  (C19), cyclin D1, cyclin D2 HDAC1, HP1 $\alpha$ , cdk4 and cdc2 are from Santa Cruz Biotechnology. Monoclonal anti- $\beta$ -actin antibodies were from Sigma.

### C/EBP $\alpha$ -S193D knockin mice

The generation of C/EBP $\alpha$ -S193D knockin mice was described in our previous paper (Wang *et al.*, 2010). Briefly, the C/EBP $\alpha$ -S193D mice were generated by replacement of the endogenous C/EBP $\alpha$  gene with knockin construct which contained a substitution of TC to GA in the position of Ser193 (See Fig 1A). This substitution leads to the mutation of Ser to Asp and to alterations in restriction sites for MluI and BamHI. Therefore, the genotyping of these mice included the examination of 858 bp PCR product by restriction enzymes MluI and BamHI. Our previous paper contains more details for the generation and genotyping of these mice (Wang *et al.*, 2010). For all experiments presented in this paper, we have bred heterozygous mice and have examined WT, hetero and homozygous mice from the same littermates. Animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (protocol AN-1439).

### Experiments with growth hormone (GH) and with siRNA to HDAC1

For GH treatment of mice, recombinant mouse growth hormone (rmGH) provided by the National Hormone and Peptide Program was injected into mice (2 mg/kg) subcutaneously for 3 days. The physiological saline (PS, 0.9% NaCl) was used as vehicle for rmGH. The control animals were injected with PS. Liver tissues were collected and used for the staining with H&E and DAPI and for the examination of protein expression as described below. The inhibition of HDAC1 by siRNA was performed as described in our previous paper (Wang *et al.*, 2008b). The control animals were injected with a control siRNA containing random composition of nucleotides (Wang *et al.*, 2008b).

### Blood test, H&E and DAPI staining

Examination of the blood parameters, H&E staining and DAPI staining were performed in Baylor College of Medicine Facilities.

### Immunostaining of livers with antibodies to C/EBP $\alpha$ , HDAC1 and K9 trimethylated histone H3

The immunostaining of the WT, S193D and WT old livers was performed as described in our previous papers (Wang *et al.*, 2008a; 2008b). The antibodies to C/EBP $\alpha$  (14AA) and HDAC1 were from Santa Cruz Biotechnology. Antibodies to H3K9-trimethyl were from Abcam.

### Examination of the Glut4 promoter

The proximal region of the mouse Glut4 promoter (containing C/EBP $\alpha$  site) was cloned into pGl3 vector. The reporter construct was co-transfected with WT C/EBP $\alpha$  and with C/EBP $\alpha$  mutants (S193D and R290A) into HEK293 cells. The activity of the promoter was calculated as a ratio to Renilla luc control.

### Size exclusion chromatography

Nuclear extracts were isolated from livers as described earlier (Timchenko *et al.*, 1999) and fractionated by size-exclusion column SEC400 (HPLC, BioLogic HR, BioRad). The detailed procedure for the analysis of C/EBP $\alpha$  complexes is described in our previous papers (Wang *et al.*, 2001; 2004). Briefly, Gel filtration fractions were loaded on denaturing gradient (4–20%) PAAG, blotted onto membrane and probed with antibodies to C/EBP $\alpha$  (14AA), HDAC1, HP1 $\alpha$  and Brm (Santa Cruz Biotechnology). To detect C/EBP $\alpha$  complexes, C/EBP $\alpha$  was immunoprecipitated from each fraction, and IPs were probed with antibodies to HDAC1.

### Protein isolation and Western blotting

Nuclear extracts were isolated from cultured cells and from livers as described in previous papers (Wang *et al.*, 2001; 2004). Briefly, tissues were homogenized with buffer A (20mM Tris-HCl pH 7.5, 30mM KCl, 10% glycerol and inhibitors of phosphatases) and spun down at 12,000rpm for 10 min at 4°C. Supernatant (cytoplasm) was frozen and the pellet was treated with high salt buffer (20mM Tris-HCl pH7.5, 0.42M NaCl, 25% sucrose, 5mM DTT and inhibitors of phosphatases). After centrifugation, the supernatant (NE) was used for the Western blotting and for Co-IP or frozen in –80°C freezer. Nuclear extracts were isolated from mouse livers of WT and S193D mice and examined by Western blotting as described below.

### Co-Immunoprecipitation

C/EBP $\alpha$  was immunoprecipitated from nuclear extracts or from gel filtration fractions with polyclonal antibodies (14AA, Santa Cruz Biotechnology), and the presence of HDAC1 and HP1 $\alpha$  in C/EBP $\alpha$  IPs was examined by Western blotting with monoclonal antibodies to the mentioned proteins.

### Electrophoretic mobility shift assay (EMSA)

EMSA was performed with the Glut4 promoter and with promoters of cyclin D1 and cyclin D2. The sequence of the Glut4 probe is as follows. 5'-CTGCACTCAATTCTTT**CAGAAATTT**CGCAGT-3' (C/EBP site is shown in bold). The sequences of the probes with C/EBP sites within cyclin D1 and cyclin D2 promoters are shown in Fig 5C and Supplemental Fig 2. The double stranded probes were gel-purified and labeled by Klenow “fill in” reaction with radioactive P<sup>32</sup>-dCTP. The DNA probes were incubated with nuclear extracts isolated from WT and from S193D livers. The binding buffer contained 20mM Tris-HCl pH 7.5, 100mM KCl, 5mM MgCl<sub>2</sub>, 5mM DDT, 1 $\mu$ g/10 $\mu$ l poly (dI-dC) and 10% glycerol. Antibodies were added to the binding reactions before probe addition. The reaction mixtures were separated by 5% native gel electrophoresis; the gel was dried and exposed with X-ray film.

### Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed using Chip-It kit according to the instruction. Briefly, chromatin solutions were prepared from livers of WT and S193D mice. C/EBP $\alpha$ , C/EBP $\beta$ , HDAC1, histone H3K9 and histone H3-trimethyl K9 were immunoprecipitated from the solutions. DNA was isolated and used for the PCR reactions with primers covering C/EBP $\alpha$  sites

within the Glut4, cyclin D1 and cyclin D2 promoters (see Figure 5 and Supplemental Figure 2). The sequences of these primers for the Glut4 promoter are as follows, 5'-ACACACACACACACACACAC-3' (forward) and 5'-TAAGGTTCCCGCTGCTTCTGAGTT-3' (reverse). PCR mixtures were amplified for one cycle of 95°C (5 min), 60°C (5 min) and 72°C (2 min). Then PCR mixtures were amplified for 31 cycles of 95°C (1 min), 60°C (2 min) 72°C (1.5 min). PCR products were separated by 8 % PAGE.

### Micro array studies

Total RNA was isolated from three animals of each genotype and used for micro array assay. The micro array was performed as described in our previous paper (Wang *et al.*, 2008c). Briefly, two hundred nanograms of total RNA were amplified using Illumina TotalPrep RNA Amplification Kit (Ambion, Cat# IL1791) following kit instructions. In vitro transcription was performed and biotinylated cRNA was synthesized by 14-hour amplification with dNTP mix containing biotin-dUTP and T7 RNA polymerase. Amplified cRNA was subsequently purified and concentration was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, DE). The amplified products were used for analysis of Illumina Sentrix Beadchip Array Mouse-6 arrays. The arrays were scanned with BeadArray Reader (Illumina, CA). Data were analyzed using BeadStudio software (Illumina, CA). Clustering and pathway analysis were performed with BeadStudio and Ingenuity Pathway Analysis (Ingenuity Systems, Inc.) softwares respectively.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

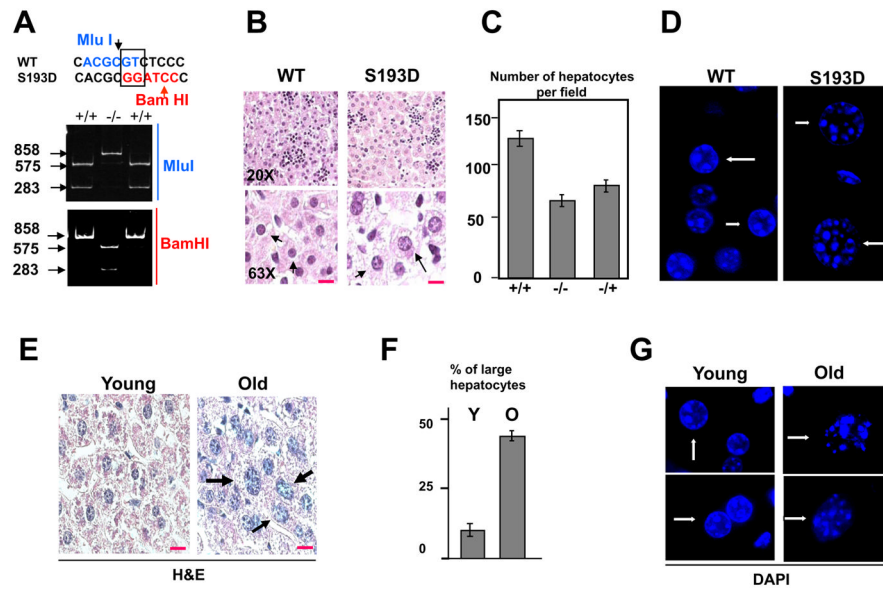
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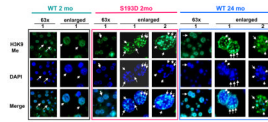
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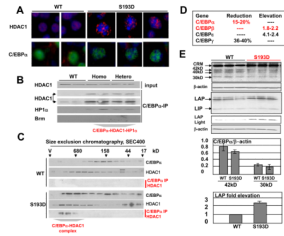


**Figure 1. The S193D isoform of C/EBP $\alpha$  causes alterations in liver morphology. A. An example of genotyping of the C/EBP $\alpha$ -S193D mice**  
 Upper image shows the sequence surrounding S193. TC nucleotides were mutated to GA leading to the disruption of the restriction site for MluI and creation of restriction site for BamHI. PCR products of 858 bp were generated using DNA from littermates and examined by restriction with MluI and BamHI (Bottom image). **B. Livers of C/EBP $\alpha$ -S193D mice contain larger hepatocytes with enlarged nuclei.** Livers of WT and S193D mice were stained with H&E. Images taken under 63X magnification are shown on the bottom. The hepatocytes are shown by arrows. Red lines show scale bars: 10  $\mu$ m. **C. Bar graphs show number of hepatocytes per field under 20X magnification.** **D. Chromatin structure is altered in hepatocytes of C/EBP $\alpha$ -S193D mice.** WT and C/EBP $\alpha$ -S193D livers were stained with DAPI. A typical picture of DAPI staining is shown. Arrows show nuclei of hepatocytes. Original magnification, x63. **E. A significant portion of hepatocytes in old mice has an increased size.** A typical picture of H&E staining of livers of young and old mice is shown. Arrows show enlarged hepatocytes. Red lines show scale bars: 10  $\mu$ m. **F. Bar graphs show percentage of enlarged hepatocytes in livers of young and old mice.** **G. DAPI staining of livers from young and old mice.** Two images of each age group are shown.



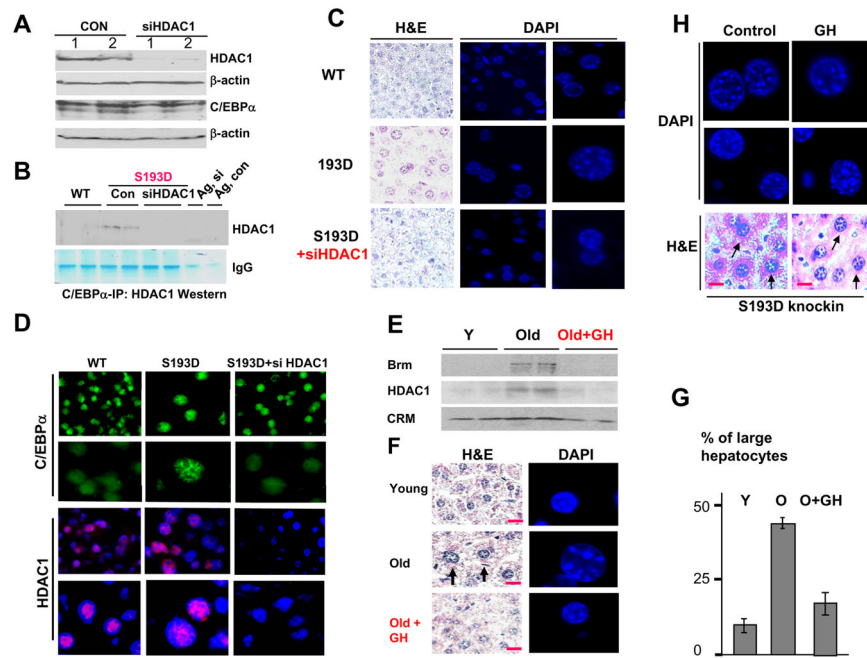


**Figure 2. “Foci-like” structures in hepatocytes of young S193D and old WT mice contain a marker of heterochromatin; histone H3 trimethylated at K9 (H3K9me)**  
 Livers of WT young (2mo), S193D young (2mo) and WT old (24 mo) mice were stained with antibodies to H3K9-trimethyl and with DAPI. Bottom panel shows the merge. 63x; shows bigger fields of the livers with multiple cells per field. These pictures were taken under original magnification 63X. Arrows mark nuclei of hepatocytes which are shown on the right side as enlarged images. Two-three typical pictures of enlarged nuclei are shown for each genotype on the right (enlarged). Arrows on enlarged images show big H3K9me foci which co-localize with DAPI foci.

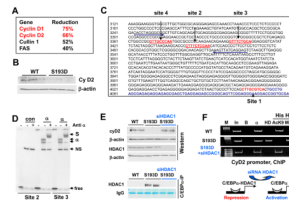


**Figure 3. Alterations of the chromatin structure in S193D mice are associated with an increase of C/EBP $\alpha$ -HDAC1 complexes. A. C/EBP $\alpha$  and HDAC1 are observed in foci-like structures in hepatocytes of S193D mice**

Livers were immunostained with antibodies to HDAC1 and C/EBP $\alpha$ . Two images for WT and three images for S193D mice are shown. Original magnification, x63. **B. HDAC1 and HP1 $\alpha$  form a complex with C/EBP $\alpha$  in C/EBP $\alpha$ -S193D mice.** Proteins were immunoprecipitated with Abs to C/EBP $\alpha$  and examined by Western blotting with Abs to HDAC1, Brm1 and to HP1 $\alpha$ . Light and dark exposures are shown for HDAC1. **C. Size exclusion chromatography.** Nuclear extracts of WT and S193D livers were fractionated by gel filtration and the fractions were examined by Western blotting with Abs to C/EBP $\alpha$  and HDAC1. **C/EBP $\alpha$ -IP: HDAC1 Western:** C/EBP $\alpha$  was immunoprecipitated from each fraction and examined by Western blotting with antibodies to HDAC1. **D. Alterations of mRNA levels of C/EBP family proteins in livers of S193D mice.** The table shows results of micro array studies which were further confirmed by Q-RT-PCR. **E. Protein levels of C/EBP $\alpha$  and C/EBP $\beta$  in WT and in S193D livers.** Three animals of each genotype were examined by Western blotting with Abs to C/EBP $\alpha$  and to C/EBP $\beta$ . Short exposure for the section with C/EBP $\beta$ -LAP isoform is shown. The filter was re-probed with  $\beta$ -actin. 42kD, 40kD and 30kD isoforms of C/EBP $\alpha$  and LAP and LIP isoforms of C/EBP $\beta$  are shown. CRM, cross reactive molecule. **Middle image.** Bar graphs show levels of C/EBP $\alpha$  42kD and 30kD isoforms as ratios to  $\beta$ -actin. **Bottom image.** Bar graphs show levels of C/EBP $\beta$ -LAP as ratios to  $\beta$ -actin.

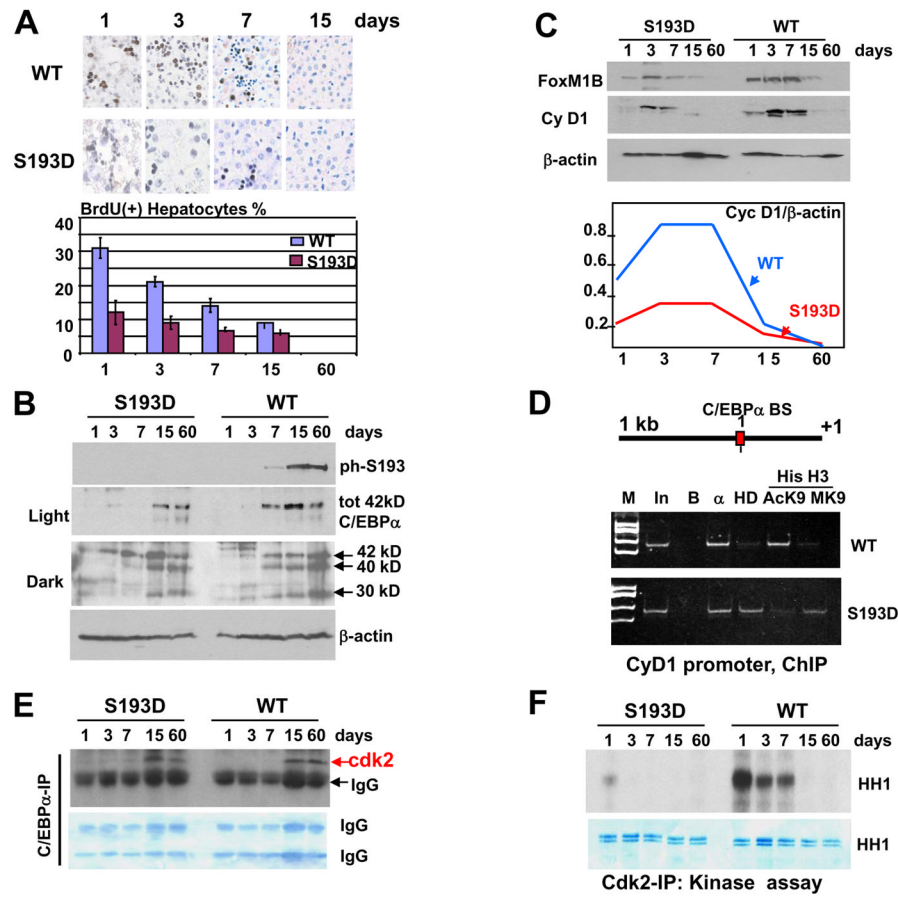


**Figure 4. Inhibition of HDAC1 and the elimination of the C/EBP $\alpha$ -HDAC1 complexes correct size of hepatocytes and chromatin structure.** **A. Inhibition of HDAC1 by siRNA to HDAC1** Western blotting was performed with nuclear extracts isolated from control S193D livers and from S193D livers of mice injected with siRNA to HDAC1. HDAC1 and C/EBP $\alpha$  levels were examined. Each membrane was re-probed with  $\beta$ -actin. **B. The inhibition of HDAC1 eliminates C/EBP $\alpha$ -HDAC1 complexes in S193D livers.** C/EBP $\alpha$  was immunoprecipitated from livers of WT, S193D and S193D siRNA injected mice and probed with Abs to HDAC1. Signals of heavy chains of IgGs are shown. **C. H&E and DAPI staining of the livers of WT, S193D and S193D mice treated with siRNA to HDAC1.** Right panel shows DAPI staining. Original magnification, x63. Enlarged images are shown on the right panels. **D. Immunostaining of siHDAC1 treated livers with C/EBP $\alpha$  and HDAC1.** Bottom images show pictures taken under 63X magnification. **E–F. Elimination of C/EBP $\alpha$ -HDAC1 complexes in livers of old mice corrects size of the nuclei and chromatin structure.** Old animals were treated with growth hormone (GH) for 3 days. **E.** C/EBP $\alpha$  was immunoprecipitated from the livers and probed with Abs to HDAC1 and to Brm. **F. H&E and DAPI staining of young, old and old mice treated with GH.** Representative pictures are shown. Red lines show scale bars: 10  $\mu$ m. Single cells for DAPI staining are shown in this picture. Supplemental figure 1 contains more images of the DAPI staining. **G. Percentage of enlarged hepatocytes in livers of young, old and old GH-treated mice.** Bar graphs show a summary of experiments with three animals of each group. **H. Treatment of young S193D mice with GH does not change size and chromatin structure of hepatocytes.** S193D mice were treated with GH and with PBS (control) and stained with DAPI (upper) and with H&E (bottom) at 3 days of GH injections. Two images of DAPI staining are shown. Arrows show hepatocytes on the H&E staining. Red lines show scale bars: 10  $\mu$ m.

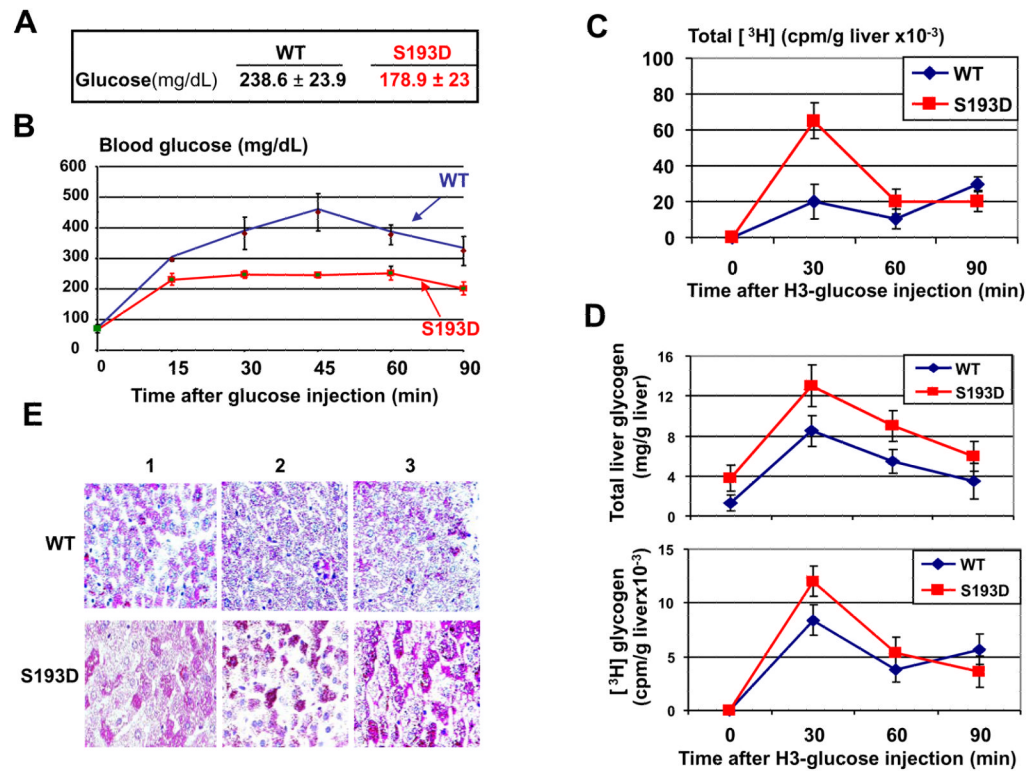


**Figure 5. C/EBP $\alpha$ -HDAC1 represses the cyclin D2 promoter. A. List of cell cycle related mRNAs which are reduced in livers of C/EBP $\alpha$ -S193D mice**

The alterations of mRNA were found in micro-array studies and then confirmed by Q-RT-PCR. **B. Protein levels of cyclin D2 are reduced C/EBP $\alpha$ -S193D mice.** Western blotting was performed with antibodies to cyclin D2. The filter was re-probed with  $\beta$ -actin. **C. Location of C/EBP $\alpha$  binding sites within the cyclin D2 promoter.** Four C/EBP $\alpha$  sites are shown by red. Arrows show positions of primers used for ChIP experiments. **D. C/EBP $\alpha$  binds to the cyclin D2 promoter.** C/EBP $\alpha$  was overexpressed in cultured cells and incubated with DNA probes covering sites 2 and 3 of cyclin D2 promoter. Antibodies to C/EBP $\alpha$  were added to the binding reactions as shown on the top. Positions of C/EBP $\alpha$  shift, supershift (S) and free probe are shown on the right. NS, non-specific band. **E. Elimination of C/EBP $\alpha$ -HDAC1 complexes in S193D mice increases expression of cyclin D2.** Western blotting of cyclin D2 and HDAC1 was performed with nuclear extracts isolated from WT, S193D and S193D mice injected with siRNA to HDAC1.  $\beta$ -actin control is shown for the cyclin D2 filter. Bottom image shows C/EBP $\alpha$ -HDAC1 complexes determined by Co-IP using the same nuclear extracts. **F. C/EBP $\alpha$ -HDAC1 complex represses the cyclin D2 promoter.** ChIP assay was performed with chromatin solutions from livers of WT, S193D and S193D +siHDAC1 mice using primers covering C/EBP $\alpha$  sites on the promoter of cyclin D2. M; molecular weight markers; In; input, B; agarose beads. HD; IP with antibodies to HDAC1, AcK9 and MK9 are IPs with antibodies to AcK9 and trimethyl-K9 histone H3.

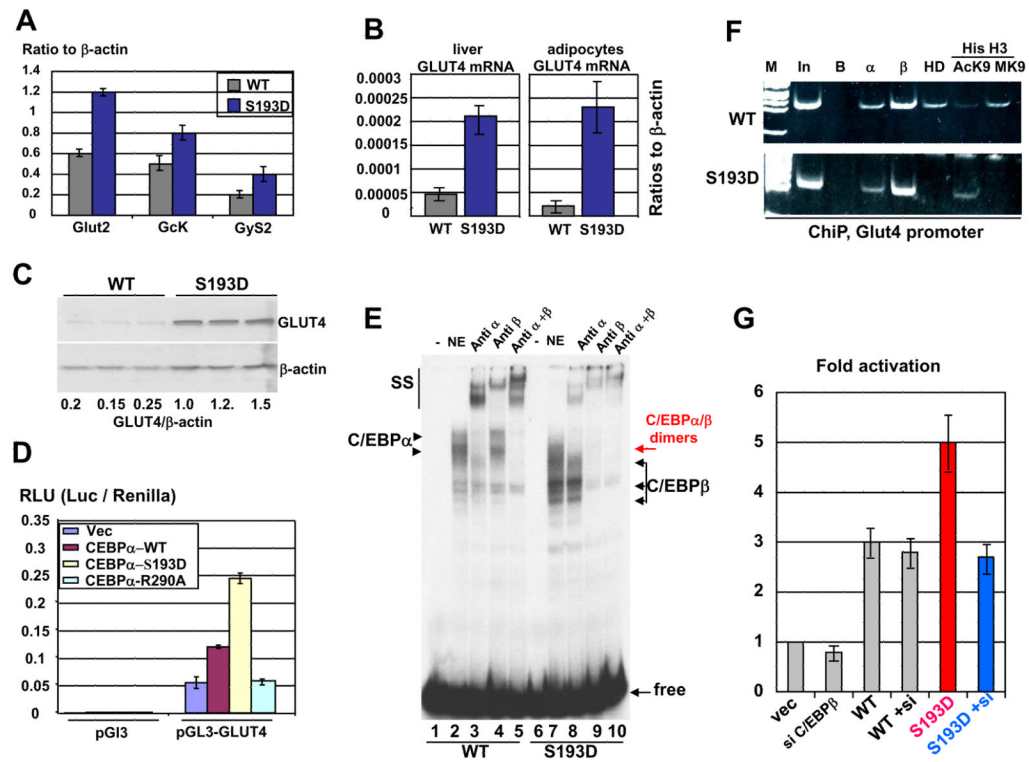


**Figure 6. C/EBP $\alpha$ -S193D inhibits liver proliferation during post-natal development via repression the cyclin D1 promoter and inhibition of cdk2.** **A. C/EBP $\alpha$ -S193D mutant inhibits hepatocyte DNA synthesis during postnatal liver development** BrdU uptake was examined in the livers of 1, 3, 7, 15 and 60 days old mice. Bar graphs show a summary of three independent experiments. **B. Expression of C/EBP $\alpha$  during postnatal development.** Western blotting was performed with nuclear extracts isolated from WT and S193D livers at different age after birth (shown on the top) using antibodies to total C/EBP $\alpha$  and to ph-S193 isoform of C/EBP $\alpha$ . Middle image shows a dark exposure of C/EBP $\alpha$  filter. The membranes were reprobred with  $\beta$ -actin. **C. Expression of FoxM1B and cyclin D1 was determined by Western blotting.** The levels of cyclin D1 were calculated as ratios to  $\beta$ -actin (bottom image). **D. C/EBP $\alpha$ -HDAC1 complex represses D1 promoter.** Upper diagram shows locations of C/EBP $\alpha$  sites on the cyclin D1 promoter. ChIP assay was performed with 7 day old animals. M; molecular weight markers; In; input, B; agarose beads. HD; IP with antibodies to HDAC1, AcK9 and MK9 are IPs with antibodies to AcK9 and Trimethyl-K9 histone H3. **E. C/EBP $\alpha$ -S193D is associated with cdk2 at all stages of postnatal development.** C/EBP $\alpha$  was immunoprecipitated from nuclear extracts; and cdk2 was examined in these IPs. Commassie stain of the membrane is shown below. **F. C/EBP $\alpha$ -S193D inhibits kinase activity of cdk2 in postnatal livers.** Cdk2 was immunoprecipitated from nuclear extracts and examined in kinase assay with histone H1 (HH1) substrate. The bottom image shows a coomassie stain of the membrane to verify loading of HH1.

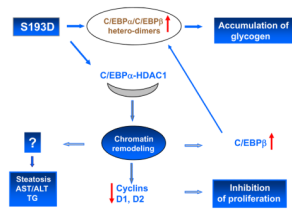


**Figure 7. C/EBP $\alpha$ -S193D mutant reduces the levels of glucose through activation of uptake of glucose by liver. A. Results of the examination of glucose in the blood of 2 months old WT and C/EBP $\alpha$ -S193D knockin mice**

The summary of analyses of eight animals of each genotype is shown. **B. Glucose tolerance test.** Mice were fasted for overnight and the glucose was injected. The concentration of glucose in the blood was examined at 15, 30, 45, 60 and 90 min after injection. **C. Glucose up-take by the liver in WT and C/EBP $\alpha$ -S193D mice.** H3-glucose was injected into mice and the level of H3 radioactivity was examined at different time points after injection. The picture shows amounts of H3 radioactivity per 1g of liver. **D. Accumulation of glycogen is increased in livers of C/EBP $\alpha$ -S193D mice.** Glycogen was isolated from the livers of mice injected by H3-glucose (see Fig 7C). Upper image shows amounts of total glycogen in livers of injected mice. Bottom image shows amounts of H3-glycogen in the livers of injected mice. **E. Livers of S193D mice contain larger amounts of glycogen than livers of WT mice.** Livers were stained with PAS. Livers of three animals of each genotype are shown.



**Figure 8. C/EBP $\alpha$ -S193D activates the Glut4 promoter in livers of S193D mice as a hetero-dimer with C/EBP $\beta$ .** **A. Expression of gluconeogenic genes in livers of C/EBP $\alpha$ -S193D mice** Q-RT-PCR was performed with primers specific to Glut2, Gck and Gys2 mRNAs. The levels of mRNAs were calculated as ratios to  $\beta$ -actin. **B. Levels of Glut4 mRNA are increased in livers and in adipose tissues.** Levels of Glut4 mRNA were determined in the liver (left) and in adipocytes (right) by Q-RT-PCR. **C. Levels of Glut4 protein are increased in livers C/EBP $\alpha$ -193D mice.** Western blotting was performed with nuclear extracts from livers of WT and S193D mice using antibodies to Glut4. The membrane was re-probed with Abs to  $\beta$ -actin. **D. S193D mutation enhances the ability of C/EBP $\alpha$  to activate the Glut4 promoter.** The Glut4 promoter was transfected with C/EBP $\alpha$  proteins into HEK293 cells and the activity of the promoter was calculated and normalized to activity of Renilla. **E. Binding of C/EBP $\beta$  and C/EBP $\alpha$ :C/EBP $\beta$  hetero-dimers to the Glut4 promoter is increased in livers of S193D mice.** EMSA was performed with DNA probe covering C/EBP site within the Glut4 promoter and with nuclear extracts isolated from WT and S193D livers. Antibodies to C/EBP $\alpha$  and C/EBP $\beta$  were added to the reactions before the probe addition. Positions of C/EBP $\alpha$  and C/EBP $\beta$  are shown by arrows. SS; supershift. **F. The Glut4 promoter is activated in S193D livers.** ChIP assay was performed as described in the text. In; 1/100 input. B; beads.  $\alpha$  and  $\beta$ ; C/EBP $\alpha$  and C/EBP $\beta$  correspondingly. HD; HDAC1. AcK9 and MK9; acetylated and trimethylated histone H3. **G. C/EBP $\beta$  is required for the increased ability of S193D-C/EBP $\alpha$  to activate the Glut4 promoter.** The expression of C/EBP $\beta$  was inhibited by siRNA. WT C/EBP $\alpha$  and S193D-C/EBP $\alpha$  were co-transfected with the Glut4 promoter into cells with inhibited C/EBP $\beta$ . The activity of the Glut4 promoter was calculated as described above.



**Figure 9.** A hypothetical model for the pathways by which S193D isoform of C/EBPα causes the age-associated dysfunctions of the liver.