CHAPTER V

LOSS OF HISTONE DEACETYLASE 3 INCREASES SUSCEPTIBILITY FOR GENOMIC DAMAGE AND DISEASE DEVELOPMENT

Background and Significance

The metabolic syndrome (MetS) is defined in an individual who has three of the four following conditions: obesity, hypertension, dyslipidemia (increased serum triglycerides and decreased HDL), and hyperglycemia (393, 394). Obesity and insulin resistance, as well as environmental and genetic factors, can lead to the hepatic manifestation of MetS, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) (395-399). NAFLD is a form of hepatic steatosis, diagnosed when lipid levels in the liver exceed 5-10% by weight. The increase in hepatic triglyceride levels can originate from multiple sources, such as the break down of adipose tissue to form circulating free fatty acids for uptake by the liver, increased dietary consumption of carbohydrates, and de novo lipogenesis utilizing acetyl CoA (392). Persistent steatosis can eventually lead to NASH, which in addition to triglyceride accumulation, is characterized by degenerative/cytologic ballooning of hepatocytes, increased apoptosis, and varying degrees of inflammation (392, 400, 401). The degeneration from NAFLD to NASH most likely requires a “second hit”, in which the liver responds to an additional pathophysiological insult on top of triglyceride accumulation (402). The most well-studies of these insults include oxidative stress from either mitochondrial malfunction or enzymatic generation of ROS, and susceptibility to damage from inflammatory responses involving cytokines such as TNF-α (403-406).
Additionally, these insults activate resident hepatic stellate cells, which normally store vitamin A, and initiate deposition of extracellular matrix (ECM) upon activation. Deposits of ECM result in fibrosis, a form of scar tissue resulting from chronic liver damage (407-409). Progressive fibrosis eventually leads to liver cirrhosis, in which nodules of hepatocytes are surrounded by ECM, liver blood flow is impeded, and liver function deteriorates (410).

Cirrhosis is a major risk factor for developing hepatocellular carcinoma (HCC). Additional causes of cirrhosis other than NASH include chronic hepatitis B (HBV) or hepatitis C viral (HCV) infection, heavy alcohol consumption, and aflatoxin B exposure (411). HCC is one of the top five causes of cancer-related deaths worldwide, and rarely occurs in the background of a healthy liver. Thus, environmental and epidemiological factors play heavily on the development of HCC. Once diagnosed, HCC can be treated by radiofrequency ablation, liver resection, or chemoembolization (injection of cytotoxic drugs) (412), but unfortunately these therapies have not been proven to prolong survival of HCC patients.

HCC is a progressive disease, with many varying starting points, yet the molecular mechanisms that trigger the final malignant environment can be placed into distinct categories. There is a general frequency of gain or loss of chromosome regions occurring in defined dysplastic nodules and small-cell dysplasias, which also demonstrate increased proliferation and are characterized as pre-cancerous lesions (413, 414). Comparative genomic hybridization has identified at least 14 different chromosome arms that are amplified or deleted in HCC samples (415-418). It is likely that deleted regions contain tumor suppressors, such as p53, which is affected by loss of heterozygosity.
(LOH) in 40-50% of HCC cases, and amplified regions overexpress oncogenes, such as c-myc, which is amplified in 30% of HCC (419). Cirrhotic and HCC-affected livers also demonstrate shortening of telomeres (420-422), and in a mouse model of telomerase dysfunction, the development of pre-neoplastic hepatic lesions was accelerated in the background of liver carcinogen treatment (423). Shortening of telomeres may lead to decreased proliferation or senescence in hepatocytes, and limited liver regeneration capacity could eventually lead to activation of hepatic progenitors to repopulate the liver, but also predispose these proliferating progenitors to the mutagenic environment of the damaged liver (424).

In the mouse model of liver-specific deletion of Hdac3, hepatic steatosis occurred early on primarily as a benign phenotype. Data from continuing studies of the Alb:Hdac3fl/fl mice reveal that continuous loss of Hdac3, compounded with triglyceride accumulation, leads to NASH-like symptoms, such as fibrosis, apoptosis, and slight increases in inflammation. Endogenous DNA damage can be detected in P28 Hdac3-null hepatocytes, but not P17 cells, potentially from the accumulation of severe cellular damage and transcriptional changes (see Chapter IV). The increase in global acetylation, which occurs in the absence of Hdac3, lessens the amount of compact heterochromatin in hepatocyte nuclei, and renders the DNA more sensitive to inducible damage. As the Alb:Hdac3fl/fl animals age, small nodules are apparent on the liver surface within 5 months of age, with full blown HCC and benign adenoma development occurring between 7-12 months of age. This progressive phenotype of Alb:Hdac3fl/fl mice is representative of multiple insults to the liver, which likely cooperate to lead to the shortened lifespan of Alb:Hdac3fl/fl mice.
Results

Prolonged loss of *Hdac3* leads to endogenous DNA damage and increased sensitivity to irradiation

High levels of cellular damage, as measured by ALT (Figure 23), occurred in the absence of *Hdac3*, so the occurrence of other types of damage, such as DNA damage, were investigated as well. Although ALT levels were increased as early as P17, assessment of single- and double-strand DNA breaks by phosphorylated histone H2Ax (\(\gamma\)H2Ax) foci did not show any difference in DNA damage between *Hdac3*-null and control hepatocytes (Figure 31). However, by P28, the enlarged *Hdac3*-null livers showed an increased number of hepatocytes positive for \(\gamma\)H2Ax foci (Figure 31), demonstrating that continuous disruption of *Hdac3* expression leads to accumulation of DNA damage.

Microarray gene expression profiling using the PANTHER classification system (see Materials and Methods) identified a number of genes whose increased expression may correlate with the increases in DNA damage (Figure 32). Categories of identified genes included damaged DNA binding, DNA repair, oxidative stress/damage responses, and p53 feedback loops. The transcription of these genes may or may not be regulated by Hdac3, as the up-regulation mostly occurred as late as 4 weeks after initial deletion of *Hdac3* in the liver, suggesting secondary damage responses in the liver may be initiated in relation to the primary phenotypes caused by the loss of *Hdac3*.

Global increases in histone acetylation occurred in *Hdac3*-null hepatocytes, and this was hypothesized to affect chromatin structure by changing the amounts of compact heterochromatin compared to open euchromatin in the nucleus. To address this
Figure 31. Endogenous DNA damage occurs after chronic loss of Hdac3. Immunofluorescence of γH2Ax in P17 (upper panels) and P28 (lower panels) Alb:Hdac3 mice demonstrates occurrence of DNA damage is pronounced as nuclear foci (indicated by arrows) in Hdac3-null livers at P28, but not P17 (600X in all fields).
Figure 32. Up-regulated genes related to DNA damage in P28 Alb:Hdac3<sup>−/−</sup> mice. Using PANTHER software analysis, microarray data from P28 mice were sorted for up-regulated genes associated with presence of DNA damage in Hdac3-null livers. Ddb, damage specific DNA binding; Aptx, aprataxin; Blm, Bloom syndrome homolog; Xrcc, X-ray repair complementing defective repair in Chinese hamster cells; Ogg1, 8-oxoguanine DNA-glycosylase 1; Dusp, dual specificity phosphatase; Ywhah, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein; Amid, apoptosis-inducing factor (AIF)-like mitochondrion-associated inducer of death.

hypothesis, hepatocyte nuclei from P17 animals were examined at high magnification by electron microscopy (EM). Normal hepatocytes displayed areas of dark staining scattered throughout the nucleus (Figure 33, top panels), representing areas of heterochromatin. By contrast, EM revealed that Hdac3-null nuclei had much less
Figure 33. Nuclei of Hdac3-null cells have decreased amounts of heterochromatin. Control P17 hepatocyte nuclei (upper panels) contain dense staining regions of condensed chromatin as observed by electron microscopy. Loss of Hdac3 abolishes heterochromatin both in the center of the nucleus and at the periphery (11500X).

staining of dark, dense heterochromatic regions (Figure 33, bottom panels), suggesting the increased global acetylation was preventing regions of chromatin from forming a compact structure.

The more open chromatin structure may lead to the genomic DNA being more susceptible to endogenous damage, perhaps produced from increased oxidative stress and ROS production (Table 8). Additionally, this less-compact structure may be more sensitive to inducible DNA damage, such as whole-animal irradiation (IR). When 4-6 week old animals were given a 3 Gy non-lethal dose of IR, by 1 hour post-IR, control livers displayed little to no DNA damage as measured by γH2Ax foci (Figure 34, top
Figure 34. *Alb: Hdac3<sup>n/-</sup>* mice are more susceptible to DNA damage induced by irradiation. Mice were given an irradiation dose of 3 Gy, and livers were harvested at 1, 6, and 24 hours post-treatment. γH2Ax was detected using immunofluorescence in control and *Hdac3*-null livers. At the 1 hour time point, a small number of distinct foci representing DNA damage can be observed in control hepatocytes, and by 6 hours, no damage is observed (left panels in 1 Hr and 6 Hr panels, respectively). In *Hdac3*-null cells, an increased amount of γH2Ax foci are observed at 1 hour post-irradiation (right panel, 1Hr), which persist up to 6 and 24 hours (right panel, 6 Hr, and 24 Hr panel, respectively) (600X).
panels). *Hdac3*-null livers, though, displayed a visual increase in the amounts of DNA damage within the 1-hour timeframe post-IR. The damage persisted at the 6-hour time point, albeit to a lesser extent than the 1-hour time point (Figure 34), and by 24 hours, the amount of DNA damage in *Hdac3*-null cells had returned to basal levels (Figure 34). Therefore, loss of *Hdac3* affects chromatin condensation, which in turn may lessen the protective nature of the histone/DNA complex from both endogenous and inducible damage.

**Symptoms of NASH develop in *Hdac3*-null livers**

The increased hepatic steatosis seen in *Alb: Hdac3*<sup>fl/fl</sup> mice as early as P10 and P17 (Figure 24) is primarily a benign phenotype, but additional damaging hits to the liver can eventually lead to the development of NASH. Mouse models of NAFLD and NASH induced either by genetic mutation or dietary/pharmacological manipulation demonstrate phenotypes such as insulin resistance, obesity, and/or liver fibrosis, although these animal models do not entirely mimic the human disease (425). Similarly, genetic depletion of *Hdac3* specifically in the liver mimics some, but not all, of the phenotypes associated with NASH. The *Alb: Hdac3*<sup>fl/fl</sup> mice exhibited liver steatosis, but also had additional metabolic effects in peripheral tissue (which retain normal *Hdac3* expression), such as decreased adipose tissue leading to leaner mice, and decreased blood glucose and insulin levels (Figures 24 and 25). As the *Alb: Hdac3*<sup>fl/fl</sup> mice aged, the chronic hepatocellular damage resulted in increased apoptosis as measured by TUNEL (Figure 35A) and increased fibrosis throughout the liver tissue (Figure 35B). Activation of hepatic stellate cells is the likely cause of increased deposition of ECM, and expression of common
Figure 35. Chronic loss of *Hdac3* leads to symptoms of NASH. A. TUNEL immunohistochemistry of 14-week *Alb:Hdac3* liver tissue, with increased apoptosis evident in *Hdac3*-null tissue (100X). B. Masson Trichrome stain of liver tissue indicates increased fibrosis in *Alb:Hdac3* null mice by 14-weeks of age (200X). C. Increased expression levels genes associated with activated hepatic stellate cells, analyzed from biological replicates of P28 *Alb:Hdac3* microarray data.
markers of activated stellate cells are increased in *Alb:Hdac3<sup>fl/fl</sup>* mice (426) (Figure 35C).

The degree of disease is not only associated with the amount of fibrosis, but also the degree of hepatic progenitor activation, or ductular reactions (427, 428). These ductular reactions are a form of progenitor cell proliferation in chronically diseased states of the liver, in which the progenitor cell (or oval cell in rodents) can differentiate into either a mature hepatocyte or bile duct cell (cholangiocyte). A common immunohistochemical marker of oval cells are cytokeratins (429, 430), which are cytoplasmic filament proteins expressed in distinct combinations in specific cell types (431). For example, hepatocytes normally express the combination of type II keratin 8 (K8) and type I K18, while cholangiocytes express K7 and K19, in addition to K8 and K18. K14 can be found in embryonic hepatoblasts before committing to a specific hepatic lineage (429). Since multiple combinations of cytokeratins can be found in liver tissue, a broad range cytokeratin antibody was used for immunohistochemistry to identify increases in cytokeratin expression in *Alb:Hdac3<sup>fl/fl</sup>* mice. By H&E histology, a ductual reaction was present as early as 6 weeks of age in *Alb:Hdac3<sup>fl/fl</sup>* mice (Figure 36A), and in 14-week old mice, a significant increase of cytokeratin reactivity was observed spanning from portal vein areas, including cells of both bile duct and oval cell/hepatocyte morphology (Figure 36B). Thus, animals with liver-specific deletion of *Hdac3* exhibit some, but not all, characteristics of NASH, providing additional insights into mechanisms which may contribute to this disease in humans.

**Liver-specific loss of *Hdac3* leads to hepatocellular carcinoma**

Ductular reactions and increased number of oval cells are also highly associated
Figure 36. Activation of hepatic progenitors in Alb:Hdac3<sup>fl/fl</sup> mice. A. Representative H&E sections of P42 mice (400X). Arrows in Hdac3-null section represent the beginning of a ductual reaction. B. The broad spectrum cytokeratin antibody recognizes only individual bile ducts in control 14-week liver samples (left panel, indicated by solid arrows), but in Alb:Hdac3<sup>fl/fl</sup> mice, the ductular reaction is apparent as both ductular structures and smaller cells within the parenchymal cells (indicated by open arrow) (200X).

with development of HCC, with roughly half of human HCC cases expressing one or more progenitor cell markers (427). More specifically, increased expression of cytokeratins correlates with HCC (432-434). Significantly, increased transcript levels of K8 and K18 were evident as early as P17 in Alb:Hdac3<sup>fl/fl</sup> mice, with further up-regulation of K8 and K18 and additional cytokeratins K19, K14, and K6g (also known as K71) evident by P28 (Figure 37).

Furthermore, gross analysis of Alb:Hdac3<sup>fl/fl</sup> livers demonstrated the development of white nodules on liver lobes (Figure 38A, left panel) starting at approximately 5
Figure 37. Cytokeratin expression increases in liver with loss of Hdac3. Graphical representation of cytokeratin gene expression in P17 and P28 Alb:Hdac3fl/fl mice from biological replicates of microarray data.

months of age, which progressed rapidly into benign adenomas and liver tumors (Figure 38A, right panel) by 7-12 months of age. These observations were supported by histological H&E and Masson Trichrome analysis of the cancerous tissue (Figure 38B). Tumor regions, but not benign adenomas, were highly proliferative, as measured by Ki67 staining (Figure 39), and this was regardless of Hdac3 expression, because no regions of the liver had regained Hdac3 expression through mutation or loss of Cre expression (Figure 40).

Although HCC may take time to develop in the presence of liver insults (419), such as chronic viral infection or fatty liver, developed HCC becomes aggressive, fast-growing, metastatic, and a poor therapeutic target (435, 436). Thus, much focus has been placed on identifying the earliest biomarker possible for detection of HCC development. α-fetoprotein is a common serum marker that is diagnostic of HCC, but there can be large false-negative or -positive rates associated with α-fetoprotein measurements.
Figure 38. Gross histology and representative cellular morphology of HCC in aging Alb:Hetac3^{-/-} mice. A. Representative livers of 5-month (left panel) and 10-month (right panel) Alb:Hetac3^{-/-} mice. Note the small white nodules which develop by 5 months of age, indicated by arrows. A, adenoma; T, tumor. B. H&E (top panel) and Masson Trichrome (bottom panel) stained histological sections from 10-month Alb:Hetac3^{-/-} mice. Note areas of inflammation, small nodules of fatty cells, and lack of normal architecture in upper panel, and the large amount of fibrosis lining veinous areas and ringing hyperplastic cell nodules in lower panel.
Figure 39. Proliferation assessment in Alb:Hdac3^{fl/-} regions of tumor and benign adenoma. Upper panels depict representative H&E sections of adenoma, tumor, and internal tumor sections (left to right) from 10-12 month old Alb:Hdac3^{fl/-} mice. Bottom panels indicate proliferating cells in each region using Ki67 immunohistochemistry. Note that adenoma areas lack proliferation, while both cells at the tumor margin and internally are highly proliferative. Arrows indicate trabecular patterning, a common histological occurrence in HCC. A, adenoma; T, tumor.

Figure 40. Tumor-laden livers remain null for Hdac3. Immunohistochemistry using an Hdac3 antibody shows that normal hepatocytes stain positively for Hdac3 (left panel). Conversely, Alb:Hdac3^{fl/-} mice remain null both in tumor and liver regions for Hdac3 (right panel), demonstrating that re-expression of Hdac3 does not occur under periods of stress due to mutation or loss of Cre-recombinase expression, and the HCC phenotypes are the result of chronic loss of Hdac3. T, tumor; L, liver.
This has prompted the validation of additional molecular markers as diagnostic tools for HCC. Markers that are up-regulated and secreted from the liver into serum are useful to diagnosis and monitor HCC. One of these markers is gamma-glutamyl transpeptidase 1 (GGT1), expressed specifically in embryonic liver, but after birth expression is diminished. Reactivation of GGT1 occurs during HCC development, and GGT1 mRNA and protein can be detected both in liver tissue and serum samples, respectively, of HCC patients (435, 439). Insulin-like growth factor II (IGF2) is a peptide hormone with 50% similarity to insulin, with the highest expression during embryogenesis, and a likely role as a growth factor (440). Overexpression of Igf2 in a transgenic mouse model increased the incident of HCC (441), and a positive correlation exists between serum and liver tissue IGF2 expression levels and frequency of human HCC (435). Interestingly, hepatic levels of both Ggt1 and Igf2 were elevated as early as P28 in Alb:Hdac3fl/fl mice, as quantified by microarray analysis (2.21-fold and 2.71 fold increases, respectively). Levels of both Ggt1 and Igf2 were then measured by Q-RT-PCR in liver tissue of 5-6 week old Alb:Hdac3 mice, as well as from different liver sections from Alb:Hdac3fl/fl mice that developed HCC. The levels of Ggt1 in 5-6 week old Hdac3-null livers were highly elevated compared to the microarray detection at the P28 time point (Figure 41, left panel). Similarly, Igf2 levels were also increased, although not as highly as Ggt1, in the same tissue (Figure 41, right panel). Further elevation in both HCC markers was found in both non-tumor and tumor-laden tissue of 10-14 month old Alb:Hdac3fl/fl mice (Figure 41). These data suggest the development of HCC in a mouse model of liver-specific deletion of Hdac3 can mimic the human disease in regard to biomarker measurements.
Figure 41. Expression levels of HCC molecular markers are increased in *Hdac3*-null liver. Levels of *Ggt1* (left panel) and *Igf2* (right panel) were quantified by Q-RT-PCR in P42 *Alb:Hdac3* mice, and from non-tumor and tumor regions of 10-14-month *Alb:Hdac3* mice.

Metastasis of HCC commonly occurs to organs such as lung and bone (442). As peripheral tissues were affected by initial loss of *Hdac3* (e.g., adipose tissue and blood glucose levels), development of HCC in *Alb:Hdac3* mice was detrimental to peripheral organs such as lung, spleen, and kidney, which developed inflammation, disrupted architecture, and fatty lesions, respectively (Figure 42). Although the identification of liver-specific metastatic cells in each of these organs was inconclusive, these data suggest that damaged hepatocytes or their contents may leak into the bloodstream and affect peripheral organs, further complicating the *Alb:Hdac3* phenotype.

**Discussion**

Causing DNA damage is a major aspect of chemotherapeutic drug action, and is a result of HDI treatment on cancer cell lines. Interestingly, loss of the HDAC class I
Figure 42. Peripheral organs are affected in Alb:HDac3^{fl/fl} mice which develop HCC. Histological H&E analysis of lung, spleen and kidney from Alb:HDac3^{fl/fl} mice. Note the inflammation in the lungs (upper right panel), the disrupted architecture of the spleen (middle right panel), and lipid infiltration and disrupted architecture of the kidney (lower right panel) in Alb:HDac3^{fl/fl} mice. Littermate controls are depicted in left hand panels for comparison.
enzyme Hdac3 results in DNA damage in normal cells (Figure 31 and (136)), and in regard to hepatocytes, loss of Hdac3 also causes chromatin changes that increase the accessibility of endogenously produced damaging agents to harm DNA. Chronic loss of Hdac3 may lead to constant bombardment of the liver with damaging agents in addition to disrupted metabolic regulation. Accumulation of cellular damage, abnormal liver metabolism, DNA damage, and disrupted gene expression could all contribute to the switch from initial hepatic steatosis in Hdac3-null livers to NASH symptoms, correlating with the hypothesis that a second insult in addition to triglyceride deposits is required for the development of NASH (402).

The progression of NAFLD and NASH to HCC in the Alb:Hdac3<sup>fl/fl</sup> mice should not be completely surprising, as hepatocytes and progenitor cells that proliferate to replenish the damaged liver are also null for Hdac3, causing an endless cycle of cellular damage that ultimately leads to irreversible disease development. The progression to HCC in Alb:Hdac3<sup>fl/fl</sup> mice is also a quick-developing phenotype. When compared to other genetic HCC mouse models, which take more than a year to develop HCC (443-446), Hdac3-null livers develop tumors within 7-12 months. Markers of human HCC were also present in Hdac3-null murine liver tissue, as early as 4 weeks of age, which suggests the Alb:Hdac3<sup>fl/fl</sup> mouse model may be an excellent model for the human disease. In relation to NASH and HCC, obesity has more than doubled in adults and children over the last 20 years in the United States, which strongly correlates with increased risk for developing type II diabetes, cardiovascular problems, as well as fatty liver (447). And although HCC prevalence is not as high as in other countries, in the United States, the rate of HCC related-deaths has increased 2-fold within the last 20 years.
as well (411), and is likely to increase as the obesity epidemic persists.

Currently, there is no correlation with HDI treatment or HDAC3 loss and HCC development, but gene expression profiling of HCC, either HBV/HCV positive or negative, demonstrates genes that require HDAC3 for regulated function are commonly misregulated in patient samples compared to normal liver tissue (448-450). For example, the NR PPARγ is found to be up-regulated (450) in the majority of ~100 primary HCC samples compared to non-tumor liver tissue. This data correlates with loss of Hdac3 expression and up-regulation of Pparγ in the Alb:Hdac3^{−/−} mouse model, and may be significant in its contribution to HCC development. Conversely, N-CoR, a key component of the NR transcriptional repression complex, which requires HDAC3 for its repressor function, is down-regulated in HCC (449). Chromosome instability is well-defined in HCC, and N-CoR happens to be located on chromosome arm 17p, which is a common site of LOH in over 50% of HCC (451). Therefore, even though HDAC3 currently does not have a direct association with the development of human diseases such as NAFLD and HCC, multiple data sources demonstrate that disruption of key modulators such as PPARγ and N-CoR, which require HDAC3, are involved in liver diseases, further supporting the role of HDAC3 as an important and necessary enzyme required to regulate normal liver homeostasis.