

## Review

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# Elimination of Tumor Suppressor Proteins during Liver Carcinogenesis

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## ABSTRACT

Liver cancer is one of the most lethal cancers. Quiescent liver expresses up to 20 tumor suppressor proteins including Rb, p53, CCAAT-Enhancer-Binding Protein (C/EBP) $\alpha$ , Hepatocyte Nuclear Factor (HNF4) $\alpha$  and p16 and it is well protected from development of liver cancer. However, the negative control of liver proliferation by these factors and other tumor suppressor genes is eliminated in liver cancer. Studies of liver regeneration after surgery and injury have provided fundamental mechanisms on how liver neutralizes tumor suppressor proteins for the time of regeneration; however, studies of liver cancer in animal models and in human samples showed several additional pathways of this neutralization. One of these additional pathways includes activation of a small subunit of the proteasome, Gankyrin. Gankyrin is dramatically increased in human hepatocellular carcinoma (HCC) and in animal models of carcinogenesis. Once activated Gankyrin triggers degradation of main tumor suppressor proteins during development of liver cancer using slightly different mechanisms. Recent studies identified mechanisms which repress Gankyrin in quiescent livers and mechanisms of activation of Gankyrin in liver cancer. These mechanisms involve a communication between Farnesoid X Receptor (FXR) signaling and chromatin remodelling proteins mediated by members of C/EBP family. It has been recently shown that C/EBP $\alpha$  plays a critical role in this network and that the activation of C/EBP $\alpha$  in cirrhotic livers with HCC inhibits cancer progression. This C/EBP $\alpha$ -dependent inhibition of liver cancer involves activation of a majority of tumor suppressor genes and repression of tumor initiating pathways such as  $\beta$ -catenin and c-myc. These recent findings provide a background for FXR-based and C/EBP $\alpha$ -based approaches to treat liver cancer.

**KEYWORDS:** Liver cancer; Tumor suppressor genes; Gankyrin; C/EBP $\alpha$ ; Rb, p53; HNF4 $\alpha$ .

## INTRODUCTION

The development of hepatocellular carcinoma (HCC) has a long history of affecting mainly adults. In the majority of cases, HCC develops in patients which have chronic liver diseases and/or are under chemical treatments. These chronic diseases affect many signaling pathways leading to liver cancer. One of the critical events in the development of HCC is the loss of hepatocytes to properly control proliferation mainly associated with inability of hepatocytes to stop proliferation. This failure to terminate liver proliferation in HCC patients is associated with the reduction or neutralization of a negative control of liver proliferation. In this review, we summarize recent publications which provide new insight into mechanisms of termination of liver proliferation under normal conditions when liver proliferates but does not develop liver cancer and recent reports that show how these mechanisms of termination are eliminated during development of HCC leading to continued proliferation and tumor growth. Mechanisms of

normal liver proliferation/termination have been investigated in several models including liver proliferation/termination during postnatal development, liver proliferation/termination after surgical resections (partial hepatectomy) and liver proliferation after acute treatments with carbon tetrachloride (CCl<sub>4</sub>). These systems provided general principles of termination of liver proliferation under conditions when liver does not develop cancer. Investigations of liver cancer in animal models were mainly focused on the development of liver cancer after treatments with diethylnitrosamine (DEN), while fewer studies have been done with the chronic treatments by CCl<sub>4</sub>.

#### **PARTIAL HEPATECTOMY AS A MODEL FOR THE STUDY OF MECHANISMS WHICH TERMINATE LIVER PROLIFERATION**

One of the key characteristics of liver cancer is uncontrolled liver proliferation. It is well recognized that malignant cells lose the ability to stop proliferation. The understanding of mechanisms which stop liver proliferation is important for development of therapeutic approaches to treat liver cancer. One of the best systems for the studies of mechanisms that terminate liver proliferation is Partial Hepatectomy (PH). The most common model of PH involves resections of 2/3 of the liver which leads to initiation of liver proliferation and restoration of the original size. While mechanisms of initiation of liver proliferation after PH are well investigated and are described in several recent reviews,<sup>1-6</sup> very little is known about the mechanisms that terminate liver regeneration. Global gene profiling of the liver 3 weeks after PH has identified alterations in cell cycle, apoptosis, TGF $\beta$  and angiogenesis signaling.<sup>7</sup> PPAR signaling and lipid metabolism have also been implicated in the termination of liver regeneration.<sup>8</sup> It has been shown that certain micro RNAs may be involved in the termination of liver regeneration.<sup>9,10</sup> In addition, the ablation of integrin-linked kinase leads to enhanced liver proliferation.<sup>11</sup> A recent paper by Koral et al. have shown that leukocyte-specific protein (LPS) serves as a tumor suppressor and inhibits proliferation of hepatoma cell lines.<sup>12</sup> It has been shown that termination of liver regeneration after PH and after liver injury requires a tight cooperation of chromatin remodeling proteins and a family of C/EBP proteins and that disorganization of this cooperation leads to a failure of the liver to stop regeneration.<sup>13</sup> A number of key regulators of liver biology are under control of C/EBP family proteins and are properly regulated during liver development, differentiation and regeneration. These proteins include SIRT1, PGC1 $\alpha$ , p53, FXR, TERT, enzymes of glucose metabolism PEPCK, G6Phase, Glut2 and Glut4 as well as enzymes of triglyceride syntheses.<sup>14-18</sup> The ability of C/EBP proteins to activate or repress these genes depends on their association with p300 or with HDAC1. Using specific knock-in animal models, Jin et al. found that these known targets are mis-regulated in the liver if the C/EBP-chromatin remodeling complexes are not controlled in a proper way which leads to the lack of termination of liver regeneration.<sup>13,19</sup> Among additional candidates for the termination of liver proliferation, Yap (Yes-associated protein) has been implicated in the regulation

of tissue growth and size.<sup>20</sup> It has been shown that Yap protein is activated in the liver after surgical resections and in hepatocellular carcinoma.<sup>21,22</sup> The expression of Yap is under tight control of Hippo signaling which is also changed after PH and in hepatocellular carcinoma.<sup>22</sup> Most important, Yimlamai et al. have shown that Hippo-Yap pathway is critical for maintenance of differentiation state of hepatocytes.<sup>23</sup> In summary, studies of liver regeneration after PH have identified several candidates which might terminate liver proliferation, but are eliminated by liver cancer. Although these studies are important and useful for understanding of mechanisms of liver cancer, it has become clear that development of liver cancer includes several additional pathways to block termination of proliferation. In this review, we focus on the mechanisms by which liver cancer eliminates liver-specific tumor suppressor proteins.

#### **LIVER SPECIFIC TUMOR SUPPRESSOR GENES**

The quiescent status of the liver is supported by many Tumor Suppressor Genes (TSG). It has been shown that the activity of more than 20 different TSGs is lost in HCC due to mutations or due to hyper-methylation of their promoters.<sup>24</sup> The TSGs include micro-RNAs which behave as tumor suppressors.<sup>25-27</sup> Epigenetic control is also involved in support of TSGs as it has been shown by genome-wide methylation analysis.<sup>28,29</sup> Further studies provided convincing evidence that many of these TSGs are involved in the protection of liver from development of cancer. Detailed information for these tumor suppressor genes of the liver has been discussed in several recent reviews.<sup>24,30</sup> Therefore, we will here briefly discuss some of these TSGs which are related to the focus of our review. One of the important TSGs is Deleted in Liver Cancer (DLC1) tumor suppressor gene. This gene is located on chromosome 8p22 and plays a critical role in multiple liver functions. It has been shown that DLC1 is deleted in 40% human HCC<sup>31,32</sup> and that restoration of its expression resulted in inhibition of liver proliferation and reduction of the development of tumors after xenografting HCC cells into nude mice.<sup>33</sup> Exomic sequencing of hepatitis C virus (HCV)-associated HCCs has identified novel mutations in AT-Rich Interactive Domain 2 (ARID2) protein which has been further shown to be a liver tumor suppressor protein.<sup>34</sup> A family of Suppressors of Cytokine Signaling (SOCS), are inhibitors of cytokine signaling. It has been shown that the liver specific deletion of a member of this family, SOCS3, leads to the increased liver proliferation and formation of hepatocellular carcinoma.<sup>35</sup> Among more than 20 known tumor suppressor proteins of the liver, Rb, p53, HNF4 $\alpha$ , C/EBP $\alpha$  and p16, are investigated in great detail and have been shown to be most critical inhibitors of liver proliferation.

#### **TUMOR SUPPRESSOR PROTEIN P53**

P53 is a transcription factor which regulates expression of many genes by direct binding to their promoters.<sup>36</sup> Under conditions when liver is challenged by surgical resections or treatments with drugs, expression of p53 is elevated

leading to growth arrest, induction of apoptosis, or senescence.<sup>37,38</sup> It has been also shown that p53 regulates ploidy of hepatocytes. Using p53 KO mice, Barton's group has shown that ploidy levels increased during regeneration of both Wild-Type (WT) and p53(-/-) hepatocytes, but only WT hepatocytes were able to dynamically resolve ploidy levels and return to normal by the end of regeneration. Kurrina et al. identified multiple cell cycle and mitotic regulators (Foxm1, Aurka, Lats2, Plk2, and Plk4) as direct targets of p53 in the liver.<sup>37</sup> The expression and activity of p53 is significantly reduced in the majority of cancers including hepatocellular carcinoma.<sup>39,40</sup> In about 50% of patients with HCC, the reduction of p53 levels and activity is mediated by mutations within the coding region or within the p53 promoter.<sup>40</sup> However, a number of recent studies revealed that the elimination of p53 by ubiquitin proteasome system contributes to the loss of p53 tumor suppressor functions in cancers.<sup>41</sup> The main ligase that triggers p53 degradation is MDM2 which targets six key lysine amino acids on p53.<sup>42</sup> In addition to MDM2, there are other ligases that target p53 degradation such as CHIP (C-terminus of HSP70 interaction protein).<sup>41,43</sup> It is interesting that MDM2 is a transcriptional target for p53 which creates an auto regulation loop that works under conditions of DNA damage. The DNA damage stabilizes p53 protein, but it is degraded by MDM2-proteasome pathway by activation of its own inhibitor at the time when cells recover after stress and do not need p53 anymore.<sup>44-46</sup> The MDM2-dependent degradation of p53 involves other proteins which cooperate with MDM2<sup>47</sup> or control levels of MDM2. This review is focused on the one of these regulators, Gankyrin, which stabilizes MDM2 and facilitates degradation of p53 during development of liver cancer (see below).

#### **P16/RB/E2F PATHWAY IN LIVER PROLIFERATION AFTER PH AND IN LIVER CANCER**

Cell cycle progression in proliferating livers is stimulated by E2F transcription factors which activate several key S-phase specific genes.<sup>4</sup> The E2F family consists of eight members, five of which (E1F1-E2F5) interact with Rb, while E2F6-E2F8 do not and work as a repressor of E2F-dependent genes. It has been shown that E2F1 plays an overlapping role in HCC<sup>48</sup> and E2F2-E2F7 promote cancer.<sup>49</sup> E2F8 transcription factor is a unique member of the family which represses promoters without interactions with Rb. It has been shown that inactivation of both Rb and E2F8 works synergistically to trigger DNA replication.<sup>50</sup> In addition, E2F8 is essential for polyploidization in mammalian cells.<sup>51</sup> The detailed information for the role of E2F family in cancer has been described in a recent review.<sup>49</sup> Similar to other quiescent tissues, the activity of E2F transcription factors is inhibited in quiescent livers by retinoblastoma, (Rb) protein. Among several members of E2F family, E2F2 seems to be a most important regulator of liver proliferation and timely liver regeneration after PH.<sup>52</sup> It is important to emphasize that C/EBP $\alpha$  is one of the critical regulators of Rb-E2F complexes and that aged livers have a weak proliferation after PH due to C/EBP $\alpha$ -mediated enhancement of Rb-E2F repression function.<sup>53,54</sup> C/EBP $\alpha$  also regulates E2F complexes with another member

of Rb family, p107, which brings about growth arrest in hepatocytes.<sup>55</sup> Although C/EBP $\alpha$ -mediated regulation of Rb-E2F complexes is involved in the control of liver proliferation, the most significant pathway of regulation of Rb-E2F complexes is associated with cyclin dependent kinases cdk4 and cdk6. Upon stimulation of liver proliferation by surgical resections, cdk4/cdk6 kinases are activated by cyclin D1 and phosphorylate Rb leading to the dissociation of Rb-E2F complexes.<sup>56</sup> The activities of cdk4/6 are negatively regulated by a member of inhibitors of cdk (INK) proteins, p16. Despite numerous studies of p16 in the liver, very little is known about its role in liver proliferation after PH. Lee et al. showed that p16 undergoes methylation after PH which correlated with liver proliferation.<sup>57</sup> Another study of liver proliferation in aged mice revealed that p16 is elevated in livers of old mice and contributes to the weak proliferative response of livers to PH.<sup>58</sup> Studies of 130 old human patients who underwent hepatectomy showed that these patients had much higher levels of p16 and that these levels negatively correlated with liver regeneration.<sup>59</sup>

Examination of mutation/expression of p16 and Rb proteins in human liver cancer and in animal models of carcinogenesis strongly indicated that the loss of functions of these proteins is involved in development of severe liver cancer. It has been shown that p16 is inactivated at early stages of hepatocarcinogenesis.<sup>60</sup> It has been also shown that p16INK4a pathway is altered in rat liver tumors induced by NNK.<sup>61</sup> The inactivation of p16 and Rb in human HCC samples has been shown in many publications which are summarized in several reviews.<sup>62-64</sup> These reviews emphasized that p16, cyclin D1 and Rb pathways are commonly targeted in various cancers. To determine the role of the disruption of these three pathways in HCC, Azichi et al. have analyzed p16, pRB and cyclin D1 in 47 patients with HCCs. The authors have shown that inactivation of p16 was detected in 64% of HCCs; while Rb was inactivated in 28% of HCC samples. Importantly, several patients had inactivation both of these pathways.<sup>65</sup> In this study, over expression of cyclin D1 was detected in 11% of examined samples. These observations showed critical role of p16-Rb pathway in protection of liver from development of cancer. In agreement with these observations, Viatour et al. have deleted three members of Rb family (Rb, p107 and p130) and found that these triple knockout mice develop liver cancer with gene expression profile similar to that of human HCC.<sup>66</sup> Further studies from this group revealed that Hippo pathway is activated at later stages in these mice.<sup>67</sup>

#### **C/EBP $\alpha$ : A STRONG INHIBITOR OF LIVER PROLIFERATION AND A TUMOR SUPPRESSOR PROTEIN**

C/EBP $\alpha$  belongs to the C/EBP family of proteins,  $\beta$ ZIP proteins which contain basic region and leucine zipper region.<sup>4,68</sup> These proteins are transcription factors which dimerize with each other and control multiple functions in different tissues.

Numerous studies revealed that C/EBP $\alpha$  is a strong inhibitor of liver proliferation.<sup>69-74</sup> Despite the fact that C/EBP $\alpha$  is a transcription factor, its activities are regulated on the levels of protein-protein interactions and post-translational modifications. Growth inhibitory activity of C/EBP $\alpha$  is tightly regulated in the liver. One of the critical pathways that control the growth inhibitory activity of C/EBP $\alpha$  is phosphorylation at Ser193. It has been shown that ph-S193 isoform of C/EBP $\alpha$  is a strong growth inhibitory protein, while un-ph-193 isoform has reduced activity to inhibit liver proliferation.<sup>75-77</sup> Generation of C/EBP $\alpha$  knockin models with substitution of Ser193 to Ala (S193A) and to Asp (S193D) further confirmed the critical role of modifications of S193 in the biological functions of C/EBP $\alpha$ .<sup>13,14,15-18</sup> While liver proliferation after PH is almost completely inhibited in S193D mice, the S193A mice showed an early entry in cell cycle and lack of termination of proliferation after surgeries.<sup>13,15</sup> The tumor suppression activity of C/EBP $\alpha$  has been demonstrated in several animal models. Tan et al. have generated C/EBP $\alpha$  knockin mice in which C/EBP $\alpha$  is expressed from the alpha-fetoprotein promoter (which is active in HCC) and have shown that the elevated expression of C/EBP $\alpha$  inhibits liver carcinogenesis.<sup>74</sup> Examination of liver cancer in C/EBP $\alpha$  S193D mice under conditions of DEN-mediated carcinogenesis revealed that C/EBP $\alpha$  is a critical tumor suppressor protein because its degradation by Gankyrin causes early development of liver cancer.<sup>15</sup> A recent paper by Habib's group showed that activation of C/EBP $\alpha$  in cirrhotic livers with HCC inhibits liver cancer.<sup>78</sup> Regarding levels of C/EBP $\alpha$  in human cancer; C/EBP $\alpha$  was also examined in several reports of human HCC. Examination of levels of C/EBP $\alpha$  in liver tumor sections and non-tumor sections of the same patients has found a significant reduction of C/EBP $\alpha$  mRNA in tumor sections.<sup>79</sup> It has been also shown that the reduced expression of C/EBP $\alpha$  in hepatocellular carcinoma is associated with advanced tumor stage and with shortened patient survival.<sup>80</sup> In addition to transcriptional down-regulation of C/EBP $\alpha$  and degradation of the protein, liver cancer neutralizes the activity of C/EBP $\alpha$  by de-phosphorylation of C/EBP $\alpha$  at S193.<sup>75</sup> Taken together, these studies showed that C/EBP $\alpha$  is a tumor suppression protein and that elimination of growth inhibitory activity of C/EBP $\alpha$  is a critical step in development of liver cancer. C/EBP $\alpha$ -S193D mutant completely inhibits liver proliferation after PH<sup>15</sup> and given this strong growth inhibitory activity of S193D mutant in partial hepatectomy studies, one should assume that these mutant mice should be resistant to the development of liver cancer. However, further studies of DEN-mediated liver cancer in the S193D mice revealed that liver cancer developed a mechanism for complete elimination of C/EBP $\alpha$  by Gankyrin.

#### LIVER-SPECIFIC TUMOR SUPPRESSOR PROTEIN HNF4 $\alpha$

Hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), regulates several liver functions including proliferation and differentiation of hepatocytes. HNF4 $\alpha$  has been a subject of intensive investigations for almost 20 years. These studies demonstrated that HNF4 $\alpha$  is a master regulator of liver biology.<sup>81</sup> In addition to the

key role of HNF4 $\alpha$  in adult livers; HNF4 $\alpha$  is a critical regulator of pre-natal liver development. The studies by Duncan's group revealed that HNF4 $\alpha$  controls the development of a hepatic epithelium, liver morphogenesis and the sinusoidal organization of the liver during prenatal liver development.<sup>82,83</sup> The HNF4 $\alpha$  gene contains two promoters, P1 and P2, each produces 6 and 3 HNF4 $\alpha$  isoforms correspondingly by alternative splicing.<sup>81</sup> Although the functional relevance of these isoforms is unknown, examination of 450 human colon cancer specimens showed that P1-HNF4 $\alpha$  isoforms are lost or localized in the cytoplasm of 80% of examined samples.<sup>84</sup> This paper also showed that phosphorylation of HNF4 $\alpha$  by Src tyrosine kinase decreases stability of HNF4 $\alpha$  and that this mechanism is likely activated in patients with colon cancer.<sup>84</sup> These observations suggested that HNF4 $\alpha$  is involved in protection of cancer. In agreement with these results, the possible role of HNF4 $\alpha$  in development of human HCC has been demonstrated by examination of patients with HCC which showed that the expression of HNF4 $\alpha$  correlates with epithelial-mesenchymal transition which is involved in metastatic tumor formation.<sup>85</sup> A recent paper by Zhang et al. added additional evidence for the role of reduction of HNF4 $\alpha$  in development of HCC.<sup>86</sup> The role of HNF4 $\alpha$  in liver cancer was examined in WT mice and in several genetically modified animal models. The studies in mice have shown a critical role of HNF4 $\alpha$  in the liver functions of adult animals. These functions include regulation of expression of genes involved in lipid and bile acid synthesis, gluconeogenesis, blood coagulation, differentiation and proliferation. In this review, we focus on the discussion of HNF4 $\alpha$  functions in liver proliferation and cancer. Examination of liver biology in acute HNF4 $\alpha$  knockout mice demonstrated up-regulation of genes which are associated with liver proliferation and cell cycle control.<sup>87</sup> These studies identified several new direct targets of HNF4 $\alpha$  which include Bmp7 and Perp, a regulator of p53-dependent apoptosis. In agreement with these observations, it has been shown that the transient inhibition of HNF4 $\alpha$  initiates hepatocellular transformation through microRNA feedback loop circuit.<sup>88</sup> It is interesting that once this circuit is activated, it inhibits expression of HNF4 $\alpha$  leading to cancer. Tumor suppressor functions of HNF4 $\alpha$  have been demonstrated in rat and mouse livers. Ning et al. have found that HNF4 $\alpha$  levels are progressively decreased in the livers of DEN-induced rats and that forced expression of HNF4 $\alpha$  blocked development of HCC.<sup>89</sup> The mechanism of this inhibition of liver cancer involves the block of activation of  $\beta$ -catenin signaling. Consistent with this report, Apte's group has shown that hepatocyte-specific deletion of HNF4 $\alpha$  in adult mice causes increased hepatocyte proliferation and activation of cell cycle genes.<sup>90</sup> Examination of liver cancer in these hepatocyte-specific knockout mice after DEN injections showed that the deletion of HNF4 $\alpha$  significantly increases the number and size of hepatic tumors.<sup>90</sup> While in rat livers HNF4 $\alpha$  protected development of liver cancer through inhibition of  $\beta$ -catenin signalling,<sup>89</sup> it appears that in mouse livers HNF4 $\alpha$  represses tumor through inhibition of both  $\beta$ -catenin and c-myc expression.<sup>91,92</sup> In the liver, HNF4 $\alpha$  is under control of several pathways alterations of which might reduce levels of

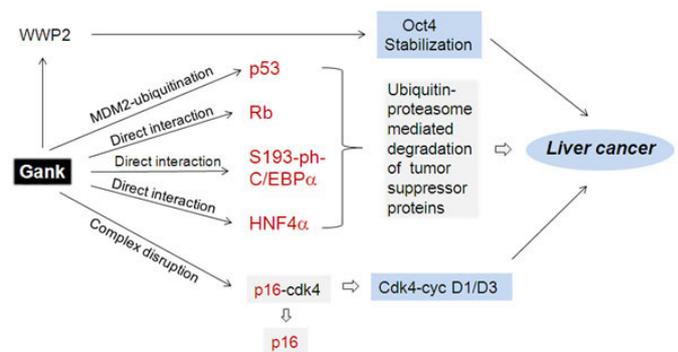
HNF4 $\alpha$  and cause liver cancer. One of these pathways is Hippo signaling. Using *in vivo* mouse liver development model, Alder et al. have recently shown that Hippo signaling affects hepatocyte differentiation through HNF4 $\alpha$ .<sup>93</sup> It has been also shown that mutations in isocitrate dehydrogenase 1 (IDH1) and IDH2 cause intrahepatic cholangiocarcinoma *via* complete silencing HNF4 $\alpha$  and subsequent impaired hepatocyte differentiation.<sup>94</sup>

### GANKYRIN: A POWERFUL ACTIVATOR OF LIVER CANCER

As we mentioned above, quiescent livers express more than 20 tumor suppressor genes. How does liver cancer eliminate activity of these TSGs? Examination of early events in the development of liver cancer in chemical models has identified elevation of Gankyrin.<sup>95,96</sup> Gankyrin (gann-ankyrin repeat protein; gann means cancer in Japanese; also known as p28, p28GANK, PSMD10, and Nas6p) is a non-ATPase subunit of the 26S proteasome and is an oncogene consisting of seven ankyrin repeats that is expressed in several cancer types, particularly HCC in which it was first discovered.<sup>95,97</sup> Recent studies have shown Gankyrin is up-regulated during initiation and progression of HCC and is correlated with capsular invasion, intrahepatic metastasis, and decreased apoptosis.<sup>95,98,99</sup> Furthermore, siRNA to Gankyrin has been shown to decrease tumor cell growth in nude mice and higher levels of Gankyrin expression have been correlated with poor prognosis in HCC.<sup>100,101</sup> It has been recently found that the histone deacetylase inhibitor panabinstat (LBH589) inhibits proliferation and metastasis of hepatocellular carcinoma through inhibition of Gankyrin.<sup>101</sup> Li et al. have recently identified microRNA-605 as a potent repressor of Gankyrin which also leads to inhibition of liver cancer.<sup>102</sup> Many studies have investigated the role of Gankyrin in HCC and several pathways have been elucidated. Jiang et al. have shown that Gankyrin is repressed by FXR in quiescent liver and FXR expression is decreased in HCC. This interaction depends on downstream targets of FXR: C/EBP $\beta$  and HDAC1, which form a complex to inhibit Gankyrin expression in quiescent tissue.<sup>103</sup> This paper also showed that FXR-mediated prevention of Gankyrin activation in DEN-mediated carcinogenesis inhibits liver cancer.<sup>103</sup> Taken together, these papers clearly demonstrated that the inhibition of Gankyrin leads to inhibition of liver cancer.

### MECHANISMS OF GANKYRIN-MEDIATED LIVER CANCER

Investigations of mechanisms by which Gankyrin causes development of HCC showed that Gankyrin has two main cancer-promoting activities. The first activity is associated with the neutralization of at least five tumor suppressor proteins and subsequent support of proteins that promote liver cancer. (Figure 1) summarizes signaling pathways which Gankyrin uses to diminish expression/activities of the tumor suppressor proteins and support high levels of cdk4 and Oct4 which promote liver cancer. It has been shown that Gankyrin binds to MDM2/HDM2 and enhances ubiquitination and degradation of p53.<sup>104</sup>

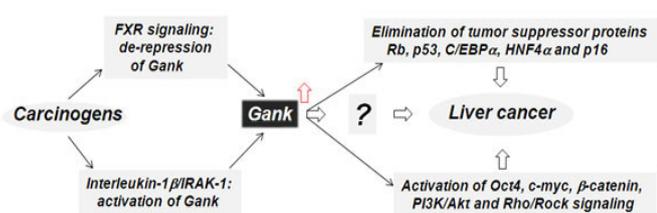


**Figure 1:** A summary of signaling pathways by which Gankyrin diminishes expression/activities of tumor suppressor proteins and by which it supports high levels/activities of cdk4 and Oct4 promoting liver cancer. Gankyrin directly interacts with C/EBP $\alpha$ , Rb and HNF4 $\alpha$  and triggers their degradation. Gankyrin causes degradation of p53 through stabilization of MDM2 ubiquitin ligase. Gankyrin-mediated neutralization of p16 is associated with the disruption of p16-cdk4 complexes and subsequent activation of cdk4 by cyclins D1 and D3. Gankyrin also stabilizes Oct4 by interaction with WWP2 which marks Oct4 degradation.

During the initial discovery of Gankyrin, it was discovered that it is capable of binding Rb through an LXCXE domain and that this leads to increased phosphorylation of Rb and its subsequent degradation.<sup>105</sup> This interaction is involved in conferring anchorage-independent growth in NIH 3T3 fibroblasts. In addition to the interaction with Rb, Gankyrin also binds to D-type kinase, cdk4, and replaces p16<sup>INK4a</sup> from cdk4 leading to the activation of cdk4.<sup>106</sup> The Gankyrin-mediated elimination of p53, Rb and p16 in liver cancer has been confirmed in many other reports.<sup>2,15,95,103</sup> Recent studies identified two additional targets of Gankyrin; tumor suppressor proteins C/EBP $\alpha$  and HNF4 $\alpha$ . As we noted above, C/EBP $\alpha$  is a strong tumor suppressor protein when it is phosphorylated at Ser193. Gankyrin specifically recognizes ph-Ser193 isoform of C/EBP $\alpha$  and S193D mutant and triggers their degradation through the ubiquitin proteasome system. During development of liver cancer in WT mice treated with DEN, C/EBP $\alpha$  is almost completely converted into ph-S193 isoform and becomes a target for Gankyrin.<sup>15</sup> In C/EBP $\alpha$ -S193D mice, Gankyrin eliminates the mutant C/EBP $\alpha$  much earlier leading to fast development of liver cancer.<sup>15,103</sup> Several recent publications from Dr. Wang's group identified HNF4 $\alpha$  as additional target of Gankyrin. Using established hepatoma cell lines, this group showed that down-regulation of Gankyrin promotes differentiation of hepatoma cells and that this differentiation is mediated by stabilization of HNF4 $\alpha$ . The inverse correlation of Gankyrin and HNF4 $\alpha$  was observed in DEN-mediated cancer and in human HCC.<sup>107</sup> In addition to degradation of HNF4 $\alpha$ , Gankyrin-dependent dedifferentiation of hepatocytes in tumor initiating cells includes stabilization of Oct4 through Gankyrin competitively binding to WWP2, the ubiquitin ligase that normally marks Oct4 for degradation.<sup>108</sup>

The second liver cancer promotion activity of Gankyrin is associated with activation of signaling pathways which initiate liver cancer. It has been shown that Gankyrin promotes liver tumor growth and metastases through activation of Il-6/STAT3

signaling.<sup>109</sup> Gankyrin also activates IL-8 during development of liver cancer.<sup>110</sup> Two key pathways of liver cancer,  $\beta$ -catenin and c-myc, are also activated by Gankyrin.<sup>111</sup> In addition, several reports showed that Gankyrin-mediated liver cancer includes activation of PI3K/Akt pathway and Rho/ROCK/PTEN signalling.<sup>112,113</sup> Interestingly, the activation of some of these pathways correlates with expression of stemness factors.<sup>114</sup> Although elevation of Gankyrin in HCC is well documented, very little is known about mechanisms by which liver cancer activates Gankyrin. Our work revealed that Gankyrin is expressed in normal livers at very low levels due to FXR-dependent silencing, but it is activated in liver cancer by the reduction of FXR signalling.<sup>103</sup> FXR supports high levels of chromatin-remodeling complexes C/EBP $\alpha$ -HDAC1 which bind and partially repress the Gankyrin promoter in quiescent liver. Upon treatments with DEN, FXR is reduced leading to de-repression of the promoter.<sup>103</sup> A recent paper suggested an additional mechanism of increase of Gankyrin which is associated with activation of interleukin-1 $\alpha$ /IRAK-1 inflammation signaling and subsequent activation of the Gankyrin promoter by NF-Y-p300 complexes.<sup>115</sup> (Figure 2) summarizes current knowledge about activation of Gankyrin in liver cancer and Gankyrin-dependent activities which contribute to development of liver cancer. The activation of Gankyrin in rodent models of carcinogenesis is mediated perhaps by two important events: de-repression of the Gankyrin promoter by reducing FXR signaling and subsequent activation by interleukin-1 $\alpha$ /IRAK-1 signaling. The elevation of Gankyrin causes elimination of 5 tumor suppressor proteins and activation of positive regulators of cancer such as  $\beta$ -catenin and c-myc. These global alterations contribute to the development of liver cancer.

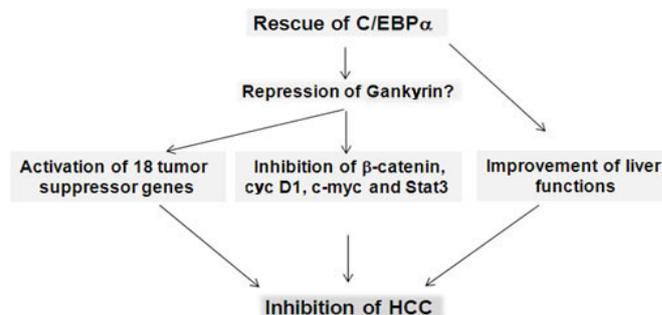


**Figure 2:** Activation of Gankyrin in liver cancer. Gankyrin is activated by carcinogens using two main pathways: 1) reduction of FXR signaling leading to a release of repression of the Gankyrin promoter; and 2) activation of Interleukin-1 $\alpha$ /IRAK-1 pathway and subsequent activation of the Gankyrin promoter by JNK and NF-Y/p300/CBP transcriptional complex. Once activated, Gankyrin displays two main cancer-promoting activities: 1) elimination of tumor suppressor proteins; and 2) activation of tumor-promoting Oct4, c-myc,  $\beta$ -catenin, PI3K-Akt and Rho/ROCK pathways.

### TREATMENTS AND PREVENTION OF LIVER CANCER BY INHIBITION OF GANKYRIN AND BY RESTORATION OF ACTIVITIES OF TSGs

Current studies of liver cancer using global profiling of gene expression, chromatin remodeling and proteomics revealed multiple alterations in the liver biology which are associated with each other. This situation suggests that it is unlikely to

generate a single-gene therapeutic approach to cure liver cancer. However, literature data also show that Gankyrin is one of the critical components of the development of liver cancer because it controls multiple pathways of liver cancer (Figures 1 and 2). This fact raises a unique possibility to correct/prevent liver cancer by targeting of Gankyrin or by activation of FXR/inhibition of interleukin-1 $\alpha$ /IRAK signaling. Among those possibilities, the promising approach might be the activation of FXR because it has been shown that long-lived little mice express high levels of FXR and do not develop liver cancer with age and after treatments with DEN.<sup>103</sup> It has been shown that high levels of FXR prevent activation of Gankyrin and rescue expression of tumor suppressor genes protecting from development of cancer.<sup>103</sup> Moreover, our unpublished results revealed that direct activation of FXR by specific ligand GW4064 rescues tumor suppressor proteins and prevents liver cancer (Lewis and Timchenko, unpublished results). Very promising observations have been recently found in the studies of liver cancer in rat models of cirrhosis and HCC by Habib's group. Using short activating RNA (saRNA) strategy, the authors activated C/EBP $\alpha$  in rats with severe cirrhosis and HCC and found significant inhibition of liver cancer and dramatic improvement of liver functions.<sup>78</sup> Examination of cancer pathways in hepatoma cell lines after activation of C/EBP $\alpha$  by saRNA revealed that correction of C/EBP $\alpha$  expression increased levels of 18 tumor suppressor gene including HNF4 $\alpha$ , p53, Rb, DLC1, ARID2 and SOCS3. saRNA-mediated activation of C/EBP $\alpha$  also down-regulated several canonical pathways of liver cancer such as HFG,  $\beta$ -catenin and c-myc signaling. Several critical drivers of liver proliferation were also down-regulated including cyclin D1 and Stat3.<sup>78</sup> Importantly, activation of C/EBP $\alpha$  by saRNA improved liver functions. (Figure 3) summarizes positive effects of activation of C/EBP $\alpha$  in livers with HCC on liver biology and functions.



**Figure 3:** Rescue of C/EBP $\alpha$  expression in HCC inhibits liver cancer. The diagram summarizes observations published in a recent paper<sup>78</sup> and suggests possible mechanisms of C/EBP $\alpha$ -mediated inhibition of liver cancer (see text).

These observations show that C/EBP $\alpha$  is a master regulator of many tumor suppressor genes, critical repressor of tumor promoting pathways, and a positive regulator of liver functions. These observations place C/EBP $\alpha$  in a unique position to be a therapeutic target for the treatments of patients with liver functions. How does the correction of one protein correct so many cancer associated dysfunctions in the liver?

Although this issue requires further examination of molecular pathways in livers after activation of C/EBP $\alpha$ , literature data and data in our lab suggest some of these pathways such as a possible feedback loop leading to down-regulation of Gankyrin. We have shown that the Gankyrin promoter contains two high affinity C/EBP sites.<sup>103</sup> Therefore, it is possible that activated C/EBP $\alpha$  represses the Gankyrin promoter in complexes with HDAC1 leading to the rescue of TGS and to repression of c-myc and  $\beta$ -catenin signaling (Figure 3). In agreement with this hypothesis, some of the up-regulated TSGs, c-myc and  $\beta$ -catenin are targets of Gankyrin see Figure 2. Regardless of the mechanisms, it is clear that C/EBP $\alpha$  is a key tumor suppressor protein in the liver.

## CONCLUSION

Development of liver cancer involves multiple alterations of liver biology on several levels of gene expression complicating development of therapeutic approaches to treat cancer. Although these multiple changes are not easy to correct, recent progress in investigations of tumor suppressor proteins and mechanisms of their elimination in cancer provides a possibility to develop approaches which might reduce liver cancer at advanced stages and improve liver functions. It is likely that tumor suppressor proteins communicate with each other through different signaling pathways and rescue/protection of one of them is sufficient for inhibition of liver cancer. In this regard, tumor suppressor protein C/EBP $\alpha$  is a promising candidate, correction of which inhibits liver cancer. We think that, similar to C/EBP $\alpha$ , correction of HNF4 $\alpha$  might also have beneficial effects on the liver since HNF4 $\alpha$  regulates liver differentiation and many liver functions. It is also interesting that activities of both these proteins are regulated by specific phosphorylation pathways which also might be considered as possible tools for correction of C/EBP $\alpha$  and HNF4 $\alpha$ . However, the most hopeful strategy seems to be activation of their promoters and prevention of their degradation by Gankyrin. Specifically, drug-mediated activation of FXR and subsequent block of Gankyrin elevation could be considered for inhibition of liver cancer in human patients. Some of the known drug-activators of FXR are already in trials for NAFLD and might be quickly incorporated in the trails for patients with HCC.

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