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Developing new tools for gene therapy of HCC

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and one of the most difficult to treat. HCCs, in fact, often develop on severe pre-existing chronic liver diseases, in particular fibrosis or cirrhosis, that impair organ function and make inappropriate any potentially curative approach.

Several studies suggested the high therapeutic potential of master regulators of hepatocyte differentiation belonging to the LETF family, mainly HNF4α, HNF1α and HNF6, whose loss represents a common feature of advanced-stage HCC. Moreover, preclinical data showed that the transduction of these proteins in vivo in mouse models, prevents tumor formation and induces regression of established tumors.

However these approaches, although promising, need to take in account the micro-environmental cues that can influence the effectiveness of therapies. Our recent data, in particular, indicated that the efficacy of HNF4α gene delivery can be limited by TGFβ, a cytokine known to induce tumor progression, angiogenesis and epithelial-to mesenchymal transition. These studies demonstrated that TGFβ impairs tumor suppressor activity of exogenous HNF4α through the inactivation of the kinase GSK-3β, which is needed for both HNF4α DNA binding and phosphorylation.

Taking into account all these observations, the aim of this work was to develop new molecular tools, insensitive to the presence of TGFβ in the tumor microenvironment, for the gene therapy of HCC, based on the restoration of HNF expression/activity.

On one hand, we attempted the characterization of the GSK-3β-mediated phosphorylation on HNF4α protein in order to develop HNF4α mutant proteins insensitive to TGFβ-induced inactivation. At the same time, we investigated the potential use of HNF1α and HNF6, analyzing their possible resistance to the TGFβ-induced impairment.

First, we demonstrated that HNF4α is a direct target of phosphorylation by GSK-3β. The residues involved in this phosphorylation were predicted by in silico studies and mutated to produce phosphomimetic mutants. After the

assessment of the in vivo functionality of mutant proteins we demonstrated that the HNF4α protein, mutated in three residues (Ser143, Thr422 and Ser426), was made resistant to the inactivation by both a chemical inhibitor of GSK-3β kinase and TGFβ, indicating the involvement of the identified residues i) in the GSK-3β -induced phosphorylation of HNF4α and ii) in the TGFβ- induced HNF4α functional inactivation. These results support the potential of our triple mutant as therapeutic tool for HCC treatment.

Next, we found that TGFβ was also able to override in vivo transcriptional activity of HNF1α and HNF6. However, no impairment of their DNA binding activity was observed, indicating that the mechanism involved in their functional inactivation is different from that observed for the HNF4α protein. In fact, we demonstrated that TGFβ induced a chromatin remodeling of HNF1α target gene promoters, indicative of a "closed" and inactive chromatin state. In particular, we observed the early loss of H3 acetylation, correlated with the displacement of CBP/p300 acetyl transferase from HNF1α binding sites. This result was confirmed by a reduced physical interaction of HNF1α protein with CBP/p300.
Collectively, data obtained in this work unveiled new mechanisms involved in the dominance of TGFβ over transcriptional activity of HNFs and identified potential therapeutic tools for the molecular therapy of HCC.

INTRODUCTION

1. HCC

Hepatocellular carcinoma (HCC) is one of the most common cancer worldwide; it is the main primary liver tumor and the third cause of cancer mortality [Parkin, 2001]. The epidemiologic features of HCC include marked variations among geographic regions, racial and ethnic groups, men and women [Yang and Roberts, 2010].
The most relevant aetiological factors leading to HCC include chronic hepatitis B and C viral infection, chronic alcohol consumption, aflatoxin-B1-contaminated food and all cirrhosis-inducing conditions, while non-alcoholic steato-hepatitis, diabetes and some metabolic disorders have been identified as minor factors (Fig 1) [Farazi and DePinho, 2006]. HCCs, in fact, are phenotypically and genetically heterogeneous tumors that frequently develop on a pathological background of pre-existing chronic liver diseases and cirrhosis (70%–90% of all detected HCC cases). Epidemiologic researches have shown that the majority of adult-onset HCC cases are sporadic and that many have at least one established non-genetic risk factor such as alcohol abuse or chronic HCV and HBV infection, even though most people with these known environmental risk factors never develop cirrhosis or HCC [El-Serag and Rudolph, 2007].

The therapeutic strategies for HCC treatment are limited and the survival of patients has not improved over the past three decades. Surgery, liver transplantation and percutaneous interventions are the approaches used for early stage HCC; however, most patients are diagnosed at advanced stage, when the high recurrence rate and the tendency to metastasize make these treatments ineffective. In the latter cases, the chemotherapy with the multi targeted kinase inhibitor *sorafenib*, since its approval in 2007, is the main treatment option [Spangenberg et al., 2009]. Based on the more recent

knowledge about the molecular alterations occurring in HCC, novel therapeutic strategies are being developed and proposed.

1.1. Molecular alterations

Despite the different aetiology and the high intratumor heterogeneity (as proliferative activity, histologic differentiation grade and cytological features) [Friemel et al., 2015], some common molecular mechanisms of hepatocarcinogenesis have been identified (Fig 1) [Farazi and DePinho, 2006].

An important pathway involved is the one of the tumor suppressor protein *p53*. p53 gene results mutated in 30-60% of HCCs [Hussain et al., 2007] but in other cases the tumors retain a wild-type p53, suggesting that the inactivation of this pathway is caused by other mechanisms or involves other molecules of its pathway [Nishida and Goel, 2011]. For example, the protein HBx, which is encoded by the HBV virus, can bind to p53, altering its nuclear localization and DNA binding ability [Wang et al., 1995] [Kim et al., 1991]. However, it has not been fully elucidated yet if p53 mutation is more important in cancer initiation, progression or both. In fact, HBV- and HCV-related HCCs display a higher frequency of p53 mutations in advanced stage samples (43%) than in regenerative nodules (7%) [Minouchi et al., 2002]; moreover, since some predisposing factors of HCC, such as alcohol abuse, imply oxidative stress and cycles of regeneration, p53 inactivation could promote HCC progression, enabling a high proliferative potential despite the DNA-damage signaling activation [Farazi and DePinho, 2006].

The Wnt pathway is also frequently altered in HCCs; its deregulation occurs early in hepatocarcinogenesis and is associated with an aggressive phenotype, since it is implicated in cell survival, proliferation, migration and invasion [Pez et al., 2013]. β-catenin is the main component of the Wnt signalling pathway: the binding of Wnt to its receptors inhibits the activity of Glicogen Synthase Kinase-3β (GSK-3β), responsible for β-catenin phosphorylation, the

recognition by the complex Axin/APC and the following degradation through the proteasome pathway. In the presence of Wnt, β-catenin is stabilized ant it can translocate into the nucleus where, in association with the TCF/LEF family of transcription factors, activates several genes related to cell proliferation and cancer (e.g. Myc, cyclin D1 and MMP7) [Rubinfeld et al., 1996]. 10-30% of HCCs have mutations in the CTNNB1 gene, encoding for β-catenin, which allow the accumulation of the protein in the nuclei; also the Axin1 gene presents mutations in 5-9% of human HCC while the major mechanism for the APC gene inactivation is its promoter hyper-methylation [Nishida et al., 2007]. In HCV-induced HCCs, β-catenin overexpression and mutations are more frequent than in the HBV-related ones, where HBx protein can stabilize the protein.

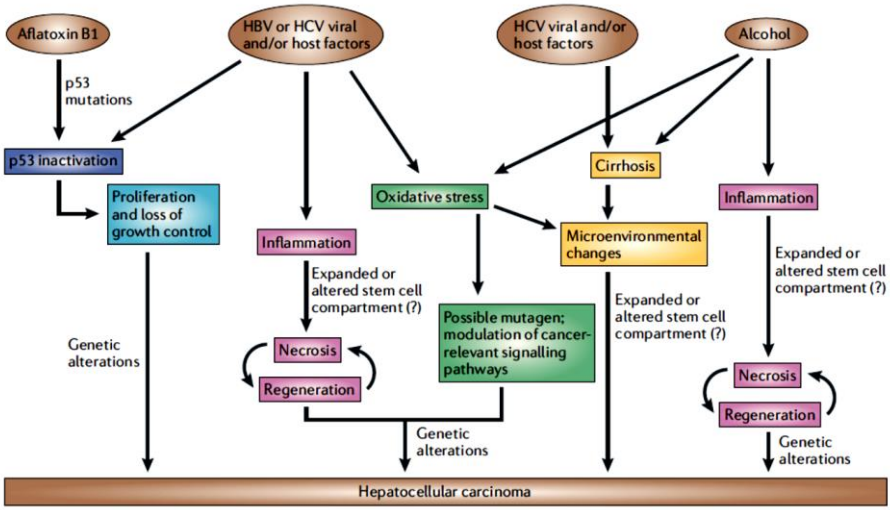

Figure 1. *Representation of the main aetiological factors and the common molecular mechanisms leading to hepatocarcinogenesis. Many HCCs develop on pre-existing chronic liver diseases, as hepatitis infection, fibrosis and cirrhosis. Despite the different origins, some common features, including inflammation, necrosis and regeneration or genetic alterations, have been identified* [From Farazi and DePinho, 2006].

The Hippo/YAP pathway, which is involved in the transduction of mechanical stimuli from extracellular matrix (ECM), presents dysregulations in HCCs, as well as in other tumor types, resulting in increased proliferation, survival and metastasis [Harvey et al., 2013]. In particular, YAP is overexpressed in some HCC samples and correlates with poor prognosis [Xu M. Z. et al., 2009].

A specific feature of HCC, is the reduced expression of the Liver Enriched Transcription Factors (LEFTs), a family of transcription factors, including five groups of proteins, whose expression controls liver differentiation during the embryogenesis and the maintenance of the differentiated state in adult hepatocytes. In HCCs samples a decrease or lack of expression of these factor was observed, which correlates with a reduced expression of specific liver genes, loss of the epithelial cell morphology, cell-cell and cell-extracellular matrix interactions, increased proliferation and invasivity and tendency to metastasize [Lazarevich et al., 2004].

Another characteristic of HCC is the genomic instability. In particular, at early stages of hepatocarcinogenesis, telomere shortening induces the chromosomal instability that leads to the accumulation of cancer promoting mutations; after that, the re-activation of telomerase provides cancer-cell viability [Plentz et al., 2004].
The high rate of genomic instability in HCCs leads to the amplification of oncogenes, as ERK5, and to loss of heterozygosity (LOH) in tumor suppressor genes as p53, BRCA2 (leading to a further increase in genomic instability) and Rb [Zen et al., 2009].

More recent data, have demonstrated also a significant role of epigenetic regulations in HCC. In particular, it has been shown how the DNA methylation signature is altered in many tumor samples, displaying a hypermethylated state of tumor suppressor gene promoters, which correlates to their inactivation, and hypomethylation of repetitive DNA regions, which increases chromosomal instability [Calvisi et al., 2007]. A specific analysis of

the methylation state of CpG island in several cancer related genes has allowed to develop different signatures of aberrant methylation in different subsets of genes that correlates with specific aetiologies and with different outcomes of the pathology [Hernandez-Vargas et al., 2010]. Consistently, higher expression levels of the DNA methyltransferases DNMT1, DNMT3A and DNMT3B has been described in HCC [Saito et al., 2014].
As regard the histone modifications, HCC samples present higher expression of HDAC and increased levels of H3K27me3, correlated with large tumor size, poor differentiation and worse outcome [Cai et al., 2011].

Also the expression profile of some miRNAs is altered, compared to that of normal liver tissues.
The importance of miRNAs alterations in HCC is revealed by a study that highlighted a subset of 20 miRNAs with important predictor functions on survival and metastasis formation [Budhu et al., 2008].
Among others, miR-122 is specifically expressed and highly abundant in the human liver and it is significantly downregulated in a subset of HCCs [Kutay et al., 2006]; miR-122 can modulate cyclin G1 expression so its downregulation results in an altered control of cell-cycle progression [Gramantieri et al., 2007]. Moreover, miR-122 plays a role also in the control of intrahepatic metastasis formation, suppressing angiogenesis through ADAM17 regulation [Tsai et al., 2009].
On the other hand, other miRs demonstrate a tumor promoting effect and are thereby upregulated in hepatocarcinoma: miR-221 and miR-222, for instance, promote proliferation targeting the cell cycle inhibitors p27 and p57 [Fornari et al., 2008]. The importance of miRNAs during tumor progression is also due to their role in the regulation of key modifying enzymes (e.g. miR29 family controls the expression of DNMT3A and DNMT3B) [Fabbri et al., 2007]. Also miR-34a is significantly downregulated in 76% of human HCC and, regulating c-Met, has a role in the control of migration and invasion [Li et al., 2009]. Several evidences demonstrate its tumor suppressor role in various cancer types and suggest its possible use as therapeutic target [Li et al., 2014]; nevertheless, recent findings suggest a controversial role of miR-34a, which

may exert even an oncogenic role, depending on the specific tumor genetic background [Gougelet et al., 2016].
MiR-200 family have also been found downregulated in HCCs and their levels inversely correlate with the expression of mesenchymal markers; moreover, mir-200 can act as a new diagnostic marker for HCC-related cirrhosis [Dhayat et al., 2014].

Furthermore, many lnc-RNAs show an altered expression in tumor samples and can act either as tumor suppressor or tumor promoting factors, mainly due to their ability to control the epigenetic status of the chromatin [Amicone et al., 2015]. For example, the lnc RNA Hotair is overexpressed in HCC tissues and can regulate gene expression acting as a scaffold, due to its ability to bind both to the methyltransferase EZH2 and the demethylase LSD1 [Tsai et al., 2010].
Finally, the progression of HCC towards more aggressive state often correlates with the process of epithelial-to-mesenchymal transition that will be further examined below [van Zijl et al., 2009].

1.2. Established therapies and target therapy of HCC

As discussed above, the current therapies adopted for HCC treatment are not sufficient to induce the regression of the tumor and to increase patient survival, so new target therapies and gene therapy are currently under investigations. The main difference from the traditional therapy strategies is that, while conventional therapies are aimed to basic cell mechanism (as DNA replication), target therapies are direct to tumor specific pathways found altered during carcinogenesis [Spangenberg et al., 2009].
The established therapies for HCC consist in surgery, percutaneous interventions, trans-arterial interventions and *Sorafenib* delivery. The latter is the only drug approved for HCC treatment, since other chemotherapeutic agents have proven ineffective in some clinical trials or are still involved in ongoing clinical trials [Spangenberg et al., 2009]. *Sorafenib* is a multitargeted tyrosine kinase inhibitor that blocks the Ras/Raf/MAPK pathway, decreasing

angiogenesis, cell proliferation, migration and resistance to apoptosis [Llovet and Bruix, 2008].

The complexity and heterogeneity of HCC and the presence of concurrent liver diseases in many cases have limited the number of clinical studies of target therapies. Those currently available target receptor tyrosine kinase and can be classified as monoclonal antibodies that block the receptor (as *Bevacizumab* and *Cetuximab*) or small inhibitor molecules that bind to the catalytic domain (as *Erlotinib*, *Gefitinib*) [Schiffer et al., 2005] [Thomas and Abbruzzese, 2005].
Overall, targeted therapies have been developed for the main signal transduction pathways implicated in the pathogenesis of HCC (i.e. Wnt/βcatenin, EGFR/RAS/MAPKK, c-MET, IGF signaling, Akt/mTOR, VEGF and PDGF signaling cascades) (Fig 2).

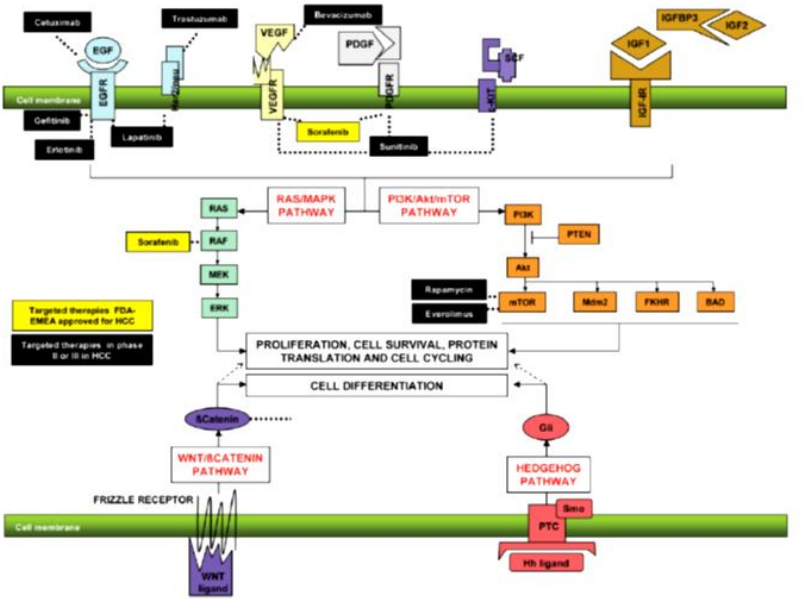

Figure 2. *Target therapy for HCC. Target therapies include monoclonal antibody and inhibitor molecules, designed against the main molecular pathway that result altered during HCC development* [from Llovet and Bruix, 2008].

Another therapeutic option for HCC treatment is the gene therapy, but it has not reached conspicuous achievement so far. In fact, the efficacy of this strategy depends on several factors that include: the appropriate choice of the therapeutic gene, of the most suitable and safe cell-entry strategy- viral or non-viral vectors- and of the delivery technique (systemic intravenous, intra-arterial, intra-tumoral, intra-portal, and intra-splenic injection, intra-biliary delivery) [Duan and Lam, 2013].
In particular, adenoviral gene therapy has been considered a promising treatment, because the delivery of vectors directly into cancer cells could reduce the potential side effects and could enhance the ability of host immune systems or increase the sensitivity of cancer cells to chemotherapeutic drugs. The adenovirus-mediated gene therapy was mainly aimed to restore cell death pathway, to improve immune response or to induce an anti-angiogenic effect [Lyra-Gonzalez et al., 2013].
Regarding the first strategy, a good tools is the delivery of the oncosuppressor p53, able to trigger apoptosis and to improve the response to chemotherapy [Tian et al., 2009].

Another advantageous possibility is to use differentiation-specific master genes that can induce a wide reprogramming of gene expression. In particular, Liver Enriched Transcription Factors (LETFs) seem to be the most suitable candidates in the context of the latter strategy, which will cause a lower toxicity than other drugs [reviewed in Marchetti et al., 2015]. A possible side effect could regard the induced differentiation of the liver stem cells, although their real involvement in the process of liver regeneration is still controversial.

1.3. Microenvironmental Cues

HCC development and progression is strongly influenced by micro-environmental cues, including soluble factors, matrix stiffness and interplay with stroma and cells of immune system.

Among the pre-existing pathologic background that foster HCC development and progression, cirrhosis mainly affects the microenvironment, as it is characterized by activation of stellate cells, resulting in increased production of extracellular matrix proteins, cytokines, growth factors and products of oxidative stress, thus altering hepatocytes proliferation and promoting tumor formation [Bataller and Brenner, 2005].

Recently, it has been demonstrated that the biophysical changes in extra-cellular matrix stiffness could influence tumor growth and progression; accordingly, fibro-cirrhotic livers are characterized by a significant increase of ECM stiffness [Mueller, 2010]. The molecular transducer of mechanical stimuli are the members of the Hippo/YAP signalling pathway that, as said before, results deregulated in hepatocarcinoma [Harvey et al., 2013] [Xu M. Z. et al., 2009].

A hallmark of HCC is the inflammatory microenvironment, which influences each step of HCC. Tumor-associated macrophages (TAMs) play a pivotal role between tumor cells and stromal cells: they can be recruited to tumor lesions and can secrete pro-inflammatory cytokines that amplify inflammation and accelerate angiogenesis, invasion and metastasis. Fundamental molecules involved in response to inflammation are the NF-kB, Il-6/STAT3 pathway but also several microRNAs [Jin et al., 2016].

One of the main component of liver tumor microenvironment involves cancer-associated fibroblasts (CAFs), as HCCs often occur in a fibrotic liver. CAFs promote tumor progression establishing a cross-talk with hepatocytes. They secrete chemokines that accelerate invasion and migration, inducing the Hedgehog pathway and enhancing TGFβ signaling [Liu et al., 2016] [Kubo et al., 2016].

Also exosomes play an important role in the regulation of tumor microenvironment since, carrying mRNAs, miRNAs and proteins, allow exchange of information among cells [Van der Pol et al., 2012]. Tumor cell-

derived exosomes can regulate epithelial-mesenchymal transition, angiogenesis, metastasis [Azmi et al., 2013]. Recently, it has been demonstrated that HCC-derived exosomes enhance *sorafenib* resistance, through the activation of the HGF/c-Met/Akt signaling pathways that result in the inhibition of *Sorafenib*-induced apoptosis [Qu et al., 2016].

2. Transforming growth factor β

The pleiotropic transforming growth factor beta (TGFβ) cytokine has emerged as a major micro-environmental factor playing a role in carcinoma progression.
TGFβ is a multifunctional cytokine that controls a plethora of cellular processes including proliferation, apoptosis, fibrosis, differentiation, specification of development fate, recognition, epithelial-mesenchymal transition and tumor progression [Shi and Massague, 2003]. The signal is conveyed differently in different cells depending on the state of responsiveness of the cell [Massague, 2000].

TGFβ belongs to a family of structurally related polypeptide growth factors, expressed in complex temporal tissue specific patterns. Comparing the sequences in the bioactive domains, TGFβ family is divided into subfamilies including BMP2 (bone morphogenetic protein), BMP5 and BMP3 subfamilies, GDF5 (growth and differentiation factor), activin and TGFβ subfamilies. The latter is composed in mammals by three different TGFβ (TGFβ1, TGFβ2 and TGFβ3) which are encoded by different genes and which all function through the same receptor signaling system [Massague, 1998].
The TGFβ protein is released as an inactive 'latent' complex, made of a TGFβ dimer in a non-covalent complex with two pro-segments, to which one of several 'latent TGFβ binding proteins' is often linked. Latent TGFβ is sequestered in the extracellular matrix, which thus acts as a reservoir from which TGFβ can readily be recruited without the need for new synthesis

[Miyazono et al., 1993]. So the activation of the latent complexes is a process finely regulated that depends on the activity of proteases, as plasmin [Lyons et al., 1990], metalloproteases 2 and 9 [Yu and Stamenkovic, 2000] or αvβ6 integrin that may induce a conformational change in TGFβ complexes [Munger et al., 1999].

2.1. Signaling

TGFβ, and related factors, signal through a family of transmembrane protein serine/threonine kinases, the TGFβ receptor family that, on the base of their structural and functional properties, is divided into two subfamilies: type I receptors (TβRI) and type II receptors [Massague 1998]. There is only one type II TGFβ receptor (TβRII) and three type I receptors [Derynck et al., 2001]. Type I and II receptors are both glycoproteins. Type I receptors differ for a highly conserved 30-amino acids region that, for the characteristic sequence, is called GS domain. Ligand induced phosphorylation in the GS sequence by the type II receptor is required for activation of signaling; GS is a key regulatory region that can control the catalytic activity and mutations in these residues allow a constitutive active signaling [Attisano et al., 1996].
The kinase domain of type I and II receptor is the canonical serine/threonine protein kinase domain; type I receptors phosphorylate their substrates on serine residues, whereas type II receptors phosphorylate themselves and type I receptors on serine and threonine residues [Mathews and Vale,1993]. The active form of TGFβ is a dimer, held together by hydrophobic interactions or by an inter-subunit disulfide bond [Sun and Davies,1995], but the signaling transduction needs the forming of hetero-tetrameric receptor complexes through a direct binding to TGFβIIR and a subsequent interaction with TGFβIR [Franzen et al., 1993].

After the binding of TGFβ to the receptor complex, the TβRII kinase phosphorylates TβRI in the ‘GS sequence’. This phosphorylation activates the

TβRI kinase that mediates TβRI autophosphorylation and phosphorylation of downstream target proteins [Derynck et al., 2001].
TGFβ transduces signaling through Smad and non-Smad signaling pathways, the first of which was the first to be described and characterized (Fig 3).

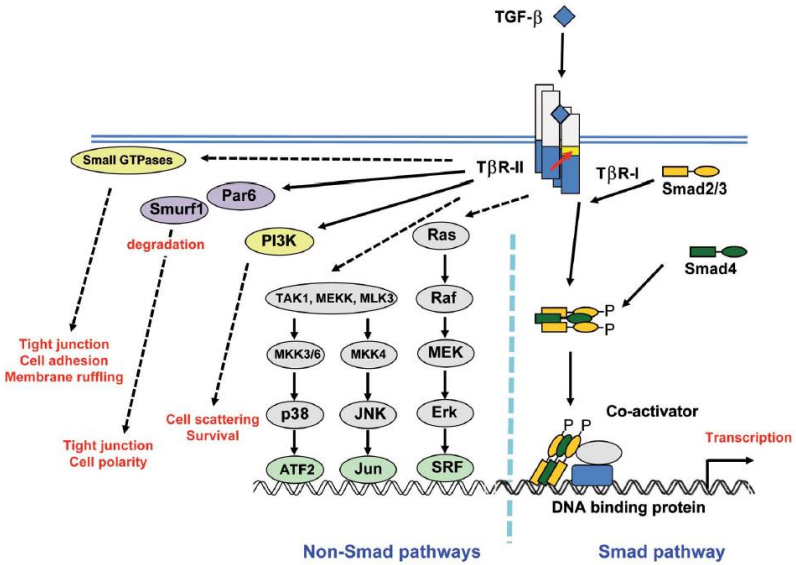

Figure 3. *TGFβ signaling pathway. TGFβ signals through a family of transmembrane serine/threonine kinase receptors divided in Type-I and Type-II receptors. The signal transduction pathway is classified as Smad or non-Smad pathway. The first one requires a phosphorylation cascade that leads to the formation of Smad-proteins complexes, which translocate into the nucleus and, interacting with other transcription factors, regulate gene expression. The non-Smad pathway involves different cellular signaling cascades, as Erk, PI3K and p38* [from Miyazono 2009].

In vertebrates, the receptors for TGFβ and Activin act through SMAD2 and SMAD3 which are referred to as receptor-phosphorylated SMADs (R-SMADs). Receptor-mediated phosphorylation at carboxy-terminal serine residues increases the affinity of R-SMADs for a particular member of the family, SMAD4. The SMAD4 protein is required for active transcriptional

complexes assembly. R-SMADs bind the transcriptional co-activators p300 and CBP [Massague 2000] and SMAD4 may allow this recruitment.
In the basal state, SMADs are retained in the cytoplasm, process that, in the case of SMAD2, is mediated by interactions with the SMAD anchor for receptor activation, SARA. The binding with SARA masks the nuclear import region of SMAD2; when phosphorylated, SMAD2 shows increased affinity for SMAD4 and decreases that for SARA, so its nuclear import region is unmasked and it can rapidly be accumulated into the nucleus [Xu et al., 2000].
The choice of target genes is circumscribed by the competence of each R-SMAD protein; both SMAD1 and SMAD2 (and the other members of either subgroup) are competent to access separate sets of target genes [Massague and Wotton, 2000].
Moreover, growing evidences indicate that activated SMADs achieve high affinity in their interactions with DNA by associating with partner DNA-binding cofactor - structurally diverse proteins that share the ability to simultaneously contact an R-SMAD and a specific DNA sequence. The fact that these proteins are functionally expressed in some cell types but not in others provides a basis for the cell-type specificity of TGFβ family gene responses [Massague 2000].
One mechanism for switching off the TGFβ signal involves SMAD ubiquitination in the nucleus, followed by proteasome-mediated degradation of the SMAD protein, while a separate ubiquitination mechanism controls the basal level of SMAD through the ubiquitin ligase SMURF1 [Lo and Massague, 1999].

In addition to the SMAD signaling pathways, TGFβ activates various types of non-SMAD signaling [Moustakas and Heldin, 2005].
Among them, it is reported that ERK, c-Jun N-terminal kinase (JNK), p38 MAP kinases, phosphatidylinsitol-3 kinase (PI-3K), and RhoA GTPase play important roles in TGFβ signaling [Yue and Mulder, 2000 A].
P38 and JNK are particularly important in driving the TGFβ-induced apoptosis; the type II receptor for TGFβ interacts with the proapoptotic adaptor protein Daxx, which leads to activation of JNK and induction of apoptosis in

epithelial cells [Perlman et al., 2001]. Moreover, JNK-mediated phosphorylation of SMAD3 enhances its activation and nuclear translocation [Engel et al., 1999].

Metastatic mammary cancer cells exploit autocrine produced TGFβ to induce their migratory capacities through the PI3K/Akt pathway [Dumont et al., 2003].

TGFβ signaling entails also the Rho GTPase, which is particularly important for cytoskeleton organization. In epithelial polarized cells, in fact, TGFβR is recruited to tight junctions through occludin and interacts also with the polarity protein Par6 which is phosphorylated by TGFβRII after its activation with consequent recruitment of the ubiquitin ligase Smurf1, leading to RhoA degradation and consequent local loss of tight junction and actin cytoskeleton disassembly [Ozdamar et al., 2005].

Anyway, the major non-SMAD signaling involves the MAPK pathway and was first described by Yue et al., who demonstrated the activation of Ras and ERK1/2 by TGFβ [Yue and Mulder, 2000 B].

Another member of the MAPK family, ERK5/MAPK7, was found activated by TGFβ through a Src dependent pathway, and involved in the inactivation of GSK-3β kinase [Marchetti et al., 2008].

GSK-3β is a serine/threonine kinase that was first identified for its ability to phosphorylate the enzyme glycogen synthase but, a part its role in metabolism regulation, it is involved also in cell cycle regulation and proliferation and is a key regulator of numerous signaling pathways, including Wnt (as described above), receptor tyrosine kinases and G protein-coupled receptors. GSK-3β presents the peculiarity to be usually constitutively active in cells and to be regulated through inhibition of its activity; moreover, GSK-3β often needs a priming phosphorylation of its substrates by another kinase, thus allowing additional regulatory mechanism [Doble and Woodgett, 2003]. The dysregulation of the signaling involving GSK-3β has been implicated in diabetes, Alzheimer's disease, bipolar disorder and cancer [Doble and Woodgett, 2003]. In hepatocytes, its inactivation by TGFβ was found responsible for the stabilization of the master gene of EMT program, Snail [Marchetti et al., 2008].

2.2. Functions

TGFβ regulates many important cellular processes both during embryogenesis and in adult tissues.

TGFβ controls the cell cycle progression in many cell types, determining growth arrest through the induction of CDK inhibitors (CKI) as p15INK4B or p21CIP1 [Reynisdottir et al., 1995]. The upregulation of these CKIs depends on the interaction of both SMAD signaling and the Ras/MAPK pathway [Hu 1999]. In particular, the transcription factor Sp1 is necessary for p21 induction, as it physically interacts with SMAD, binds the p21 promoter and recruits the co-activators CBP and p300 [Pardali et al., 2000]. However, *p21* is also induced by TGFβ through mechanisms that involve Ras, MEKK1 and ERK [Hu et al., 1999].
During differentiation of osteoblast from pluripotent progenitor cells, TGFβ and BMPs, through SMADs and p38 pathway, regulate expression of the osteoblastic differentiation protein Runx2 [Lee et al., 2002].

The role of TGFβ is particularly important on the immune system, where it suppresses growth and differentiation of B and T cells [Letterio and Roberts, 1998]. The same immune cells produce the cytokine that thus acts in an autocrine and paracrine manner. In the bone marrow and in the thyme, TGFβ regulates also the expression of cell adhesion and extracellular matrix proteins and it acts as a chemoattractant for monocyte/macrophages. Moreover, it inhibits immune cell activation, as confirmed by TGFβ1 knock-out mice models that exhibit an over production of auto-antibodies [Yaswen et al., 1996].

Recently, in our lab it has been demonstrated a novel role of TGFβ as a major inducer of hepatocyte binucleation both in adult hepatocytes and during embryonic development, working through Src/RhoA GTPase pathway, responsible for the cytokinesis failure [De Santis Puzzonia et al., 2016].

However, the best characterized function of TGFβ is the induction of epithelial-to mesenchymal transition.

2.3. Role of TGFβ in EMT

Epithelial-to-mesenchymal transition is a complex biological process during which epithelial cells undergo several molecular alterations that allow them to lose their polarity and cell-cell and cell-ECM interactions and to acquire a mesenchymal morphology, increased resistance to apoptosis, production of ECM, invasiveness and migratory capacities [Kalluri and Neilson, 2003].
So, the EMT process involves a complete reorganization of the cytoskeleton and the adhesion molecules, expression of specific transcription factors and microRNAs, synthesis of extra cellular matrix proteins (Fig 4) [Thiery and Sleeman, 2006] [Kalluri, Weinberg 2009].
An important feature of EMT is its complete reversibility through the opposite process named Mesenchymal-to-epithelial transition (MET) [Kalluri and Weinberg, 2009].

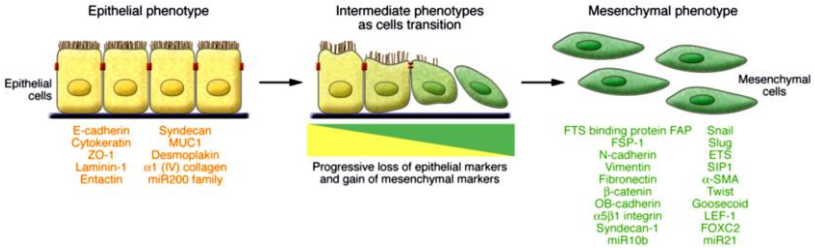

Figure 4. *Epithelial-to-mesenchymal transition. EMT is a complex biological process that involves a complete reorganization of the cytoskeleton, expression of specific transcription factors and microRNAs, synthesis of extracellular matrix proteins. These alterations induce a complete phenotypic change, so that epithelial cells acquire a mesenchymal phenotype* [from Kalluri and Weinberg, 2009].

EMT is implicated both in physiological and pathological processes: embryogenesis, fibrosis and tumor development.

These three types of EMT, even if involved in very different biological processes, share common genetic and molecular basis [Kalluri, 2009].
EMT was first described regarding the embryogenesis, when some epithelial cells undergo subsequently EMT and MET during organs development to move in the embryo and assume different specialized functions in different organ districts [Lee J. M. et al., 2006].
The EMT involved in the fibrosis process, the so called "type 2 EMT", entails wound healing and tissue regeneration processes and is often associated to a response to an injury that induce an inflammatory process.
Type 3 EMT, instead, regards cancer cells and enables epithelial cancer cells to acquire a malignant phenotype, with invasiveness properties, and a subsequent systemic spread. Once reached distant organs, cancer cells undergo the MET process, mainly due to the absence of the stimuli that have induced EMT in the primary tumor [Thiery, 2002].
The mechanisms involved are shared by three types of EMT.
One of the first steps involves the disruption of tight and adherens junctions with delocalization of ZO-1, Claudin and Occludin from tight junctions and E-cadherin and β-catenin from adherent junctions. Actin cytoskeleton organization changes from cortical localization to stress fibers and cells start to express mesenchymal markers as Vimentin, Fibronectin, α-SMA [Miyazono, 2009].
The main transcription factors that control the EMT are the zinc-finger factors Snail and Slug, the basic helix-loop-helix factor Twist and the two-handed zinc finger factors Zeb1 and Zeb2. One of the target of these factors is E-cadherin: Snail represses E-cadherin expression [Batlle et al., 2000] by directly binding to the E-box sequence on its promoter [Cano et al., 2000] and recruiting HDAC1 and HDAC2 [Peinado et al., 2004]; Zeb1 and Zeb2 form a repressor complex on the E-box region interacting with SMADs [Vandewalle et al., 2005].
Snail targets also tissue specific genes to induce the loss of epithelial polarity and dedifferentiation; in the liver, Snail directly represses the expression of HNF4α, the master gene of epithelial/hepatocyte differentiation [Cicchini et al., 2006].

Moreover, miR-200 family members regulate the process of EMT by targeting Zeb1 and Zeb2; their expression is down-regulated in cells undergoing EMT with concomitant acquisition of the EMT phenotype, while re-expression of miR-200 leads to the reversal of the process [Cano and Nieto, 2008].

TGFβ is a major inducer of EMT. The mechanism of its action is complex, and involves SMAD activation as well as Ras/PI-3K and RhoA signaling with distinct roles [Derynck et al., 2001].
The EMT transcription factors Snail, Zeb1 and Zeb2 are strongly upregulated by TGFβ both at transcriptional and at post-transcriptional level. In particular, Snail is transcriptionally upregulated by the activation of SMADs [Peinado et al., 2003] but also at post-translational level through SMAD-independent pathways: the inactivation by TGFβ of GSK-3β kinase, which phosphorylates Snail in two different consensus motifs, controlling its degradation and subcellular localization [Zhou et al., 2004], allows Snail protein stabilization [Marchetti et al., 2008]. As highlighted above, GSK-3β is also responsible for the phosphorylation and consequent proteasomal degradation of β-catenin: its inactivation, therefore, represents an important point of synergism between TGFβ and Wnt signaling pathways [Willert and Nusse, 1998]. Notably, the sequestration of β-catenin in the cytoplasm by E-cadherin at adhesion complexes is important for the preservation of epithelial features of cancer cells, and acquisition of the mesenchymal phenotype correlates with the movement of β-catenin to the nucleus, where it becomes part of TCF/LEF complexes [Gottardi et al., 2001].

2.4. Role in cancer

During cancer progression, TGFβ has a dual role depending on the stage and on the tumor type, acting either as a tumor suppressor or as a tumor promoting factor.

The tumor suppressor role is attested by the observation that in many tumor cells TGFβ is often downregulated or its availability at the cell surface is impaired (mechanisms used by cells to escape the growth inhibitory effects of TGFβ) [Kim et al., 2000]. As a matter of fact, the overexpression of TGFβR in tumor lacking the functional allele reduced tumor formation [Turco et al., 1999].

Although TGFβ can play a protective role at early tumor onset, on the other hand, at advanced stage, it promotes tumor progression [Cui et al., 1996]. Many tumor cells, in fact, show increased production of the cytokine and it is consistent with EMT induction, which allow the cells to acquire an invasive malignant phenotype. In fact, TGFβ can influence, in an autocrine manner, the differentiation of the tumor cells and, in a paracrine manner, the extracellular microenvironment [Derynck et al., 2011].
This is true also for hepatocellular carcinoma, where TGFβ provides a dual role, triggering cell cycle arrest and apoptosis in healthy liver and in the first phases of tumor development and, in contrast, inducing dedifferentiation and spreading of cancer cells at advanced tumor stage [van Zijl et al., 2009].

The growth inhibitory effects are mediated by c-myc, which is transcriptionally repressed by SMAD protein complexes, and by the cyclin-dependent kinase (CDK) inhibitors (CKI) p15 and p21 that, instead, are induced by SMAD, resulting in a G1 phase cell-cycle arrest [Reynisdottir et al., 1995].
These effects can be counteracted since many human tumors present c-myc gene amplification, resulting in its prolonged constitutive expression and in the c-myc mediated repression of p15 and p21 that thus overcome their SMAD-dependent induction. [Warner et al., 1999].
Various signals, including integrin, Notch, Wnt, TNF-α, and EGF, have been reported to cooperate or synergize with TGFβ signaling and stimulate tumor invasion and metastasis [Moustakas and Heldin, 2007]. In particular, synergism between TGFβ and Ras signaling has been extensively investigated. In mammary epithelial cells, hyper-activation of the Raf-MAP kinase pathway

synergizes with TGFβ signaling and, inducing EMT, accelerates the tumorigenesis and metastasis formation [Janda et al., 2002]. Also in hepatocytes, the expression of the oncogenic Ha-Ras induces a rapid conversion to a fibroblastoid phenotype in presence of TGFβ [Gotzmann et al., 2001]. Moreover, 70% of murine liver carcinomas express activated Ha-Ras, supporting the idea of its involvement in the induction of hepatocellular carcinoma [Saitoh et al., 1990].

TGFβ-induced EMT contributes also to liver fibrosis, causing the conversion of hepatocyte to fibroblast that contributes to progression of liver fibrosis [Zeisberg et al., 2007].

In HCCs, the expression of EMT master genes is associated with poor prognosis [Sugimachi et al., 2003] [Lee T.K. et al., 2006] and their expression in circulating tumor cells has been proposed as a prognostic marker [Li et al., 2013]. Moreover, HCC patients show high levels of TGFβ in the plasma [Shirai et al., 1994] and its signaling is constitutively activated [Lee et al., 2012].

3. Liver Enriched Transcription Factors (LETFs)

3.1. Liver development and hepatocyte differentiation

The liver is the largest organ of the body and it controls essential functions as detoxification, gluconeogenesis, glycogen synthesis, glucose, fat and cholesterol metabolism, production of plasma proteins, synthesis of bile acids. Hepatocytes, which constitute about 80% of adult parenchyma, form a typical polarized epithelial that account for liver functions, even if hepatocyte functions are not identical among all hepatocytes. The primary functional unit of the liver is the hepatic lobule; along the lobule perimeter there is the portal triad consisting of the portal vein and hepatic artery (where blood enters the liver) and the bile duct. From the triad, blood flows through liver sinusoids, which face the basolateral hepatocyte surface, toward the central vein; the

apical face of hepatocytes, instead, forms a bile canaliculus where bile is secreted and then drains toward bile ducts. Based on this anatomical organization, hepatocytes perform different roles depending on their physical location within the hepatic lobule. "Metabolic zonation" refers to the differential properties of periportal (adjacent to the portal triad) and pericentral (adjacent to the central vein) hepatocytes [Duncan and Dorrell, 2009].

Hepatocytes and bile duct cells originate from a common precursor, the hepatoblast, which derives from the definitive endoderm [Zhao and Duncan, 2005]. At E17 of mouse embryonic development, hepatocytes begin to establish the polarized epithelium that is an essential feature of the hepatic parenchyma and their role shift from a haematopoietic support one to become the primary cells controlling the levels of many metabolites and serum proteins in the bloodstream [Zaret, 2000].

The complex process of hepatogenesis requires a concerted functioning of regulatory mechanisms that respond to different signaling molecules, as FGF and BMP. The response of the endoderm to these inductive cues is to initiate a program of hepatic gene expression and some studies demonstrated that the homeobox transcription factor Hex is essential for expansion of the hepatoblast population [Zhao and Duncan, 2005].

Though hepatoblasts already express some genes specific of fully differentiated hepatocytes, such as serum albumin, in the fetal liver the hepatoblasts will give rise to definitive hepatocytes and bile duct cells (cholangiocytes). Regulator of liver lineages are Notch and TGFβ signaling. Downstream of signaling molecules that induce liver differentiation are the transcription factors that execute the liver program [Lemaigre and Zaret, 2004].

The analyses of regulatory regions of numerous hepatocyte-specific genes revealed that their expression is controlled by members of the Liver Enriched Transcription Factor (LEFT) family. The simultaneous binding of multiple, distinct LETFs to the gene regulatory region is required, providing synergistic transcriptional activation. Furthermore, LETFs show a cross regulation by each other.

On the basis of homology within DNA-binding domains, LETFs are grouped into five related families (Fig 5).

- The HNF3a, HNF3b and HNF3g proteins (renamed as Forkhead box a1 [**Foxa1**], Foxa2, and Foxa3 respectively) bind to DNA as a monomer using the winged helix DNA-binding domain, which also contains sequences essential for nuclear localization and transcriptional activation.
- The **HNF6** or Onecut1 (OC-1) contains a single cut domain and a homeodomain motif; it binds DNA through the cut domain that contains also sequences important for nuclear localization and transcriptional activation.
- The CCAAT/enhancer binding proteins (**C/EBP**) utilize an amino-terminal basic region leucine zipper (bZIP) bipartite DNA-binding domain consisting of a dimerization interface (composed of heptad-repeated leucine residues, termed the "leucine zipper") and a DNA binding interface (consisting of basic amino acids);
- The **HNF1α** uses a POU-homeodomain and a myosin-like dimerization domain located at the amino terminus of the protein to bind DNA as a dimer, stabilized through association with the dimerization cofactor DcoH. HNF1α can also form heterodimers with the isoform HNF1β.
- The orphan nuclear receptor **HNF4α** protein utilizes the zinc finger DNA-binding domain to recognize DNA while both the DNA- and ligand-binding domain are used to form homodimers or heterodimers with retinoic X receptor [Costa et al., 2003] [Cereghini, 1996].

These factors are expressed in liver at different stages of embryonic development showing distinct roles and operating during hepatocyte differentiation through a hierarchical and complex cross-regulatory network [Kyrmizi et al., 2006], which includes also autoregulation [Odom et al., 2006] (Fig 6).

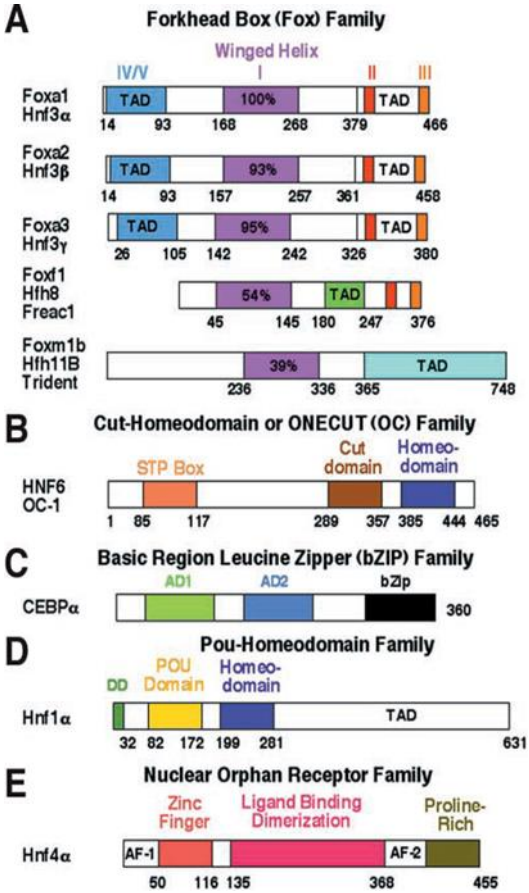

Figure 5. *Liver Enriched transcription Factors. LEFTs are grouped into five related families, structurally different. Forkhead box family (**A**) include winged helix proteins; Onecut-1 (**B**) proteins are characterized by a Cut-homeodomain; CEBPs (**C**) possess a basic Leucin Zipper domain; HNF1α (**D**) belongs to the Pou-Homeodomain family; Nuclear Orphan Receptor Family (**E**) presents a Zinc Finger domain* [from Costa et al., 2003].

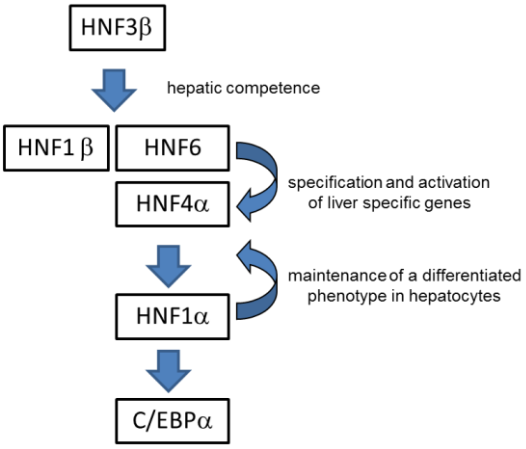

Figure 6. *Interactions among LEFTs. During hepatocyte differentiation and liver development, exist a hierarchical network among LEFTs, which includes also auto-regulation.*

Moreover, in the acquisition and maintenance of the hepatocyte's differentiated phenotype a dominant role is played by epigenetic events, involving interactions of LETFs with chromatin remodeling factors [Snykers et al., 2009].

3.2. Hepatocyte nuclear factor 4α

The hepatocyte nuclear factor 4α (HNF-4α) is a member of the orphan nuclear receptor superfamily and it is involved in the regulation of several metabolic pathways and developmental processes.

It can bind DNA exclusively as a homodimer and its DNA recognition site is a direct repeat element of the sequence CAAAGTCCA [Fang et al., 2012].

The Hnf4α gene can produce two series of transcript variants from alternative promoters, which are separated by 40kb on human chromosome 20, named proximal P1 and distal P2: the HNF4α1/6 and HNF4α7/9 transcripts,

respectively (Fig 7 A). In embryos, both HNF4α1 and HNF4α7 isoforms are mainly expressed, while in the adult liver α1 is the almost exclusively isoform detectable. Since the P2-driven isoforms are repressed by HNF4α1, increasing HNF4α1 expression levels throughout development cause a switch to exclusive P1 promoter activity in the adult liver [Briancon et al., 2004].
It has been notably demonstrated that the expression profile of P1 and P2 isoforms is modified in many cancers such as hepatocellular carcinoma where P1 isoforms expression is inhibited and P2 isoforms re-expressed [Tanaka et al., 2006].

3.2.1. Structure

The human and rat/mouse HNF4α1 proteins are highly conserved, with an overall similarity of 96%.
As others nuclear receptors, HNF4α exhibits a modular structure with six distinct regions (referred to as regions A–F), which correspond to functional domains (Fig 7 B). The N-terminal region A/B contains a ligand-independent activation domain AF-1; region C contains the DNA binding domain (DBD) composed of two zinc-finger modules and is responsible for specific binding to response elements. Regions D functions as a flexible connection. Region E is functionally composite, since it contains the LBD and the second activation domain AF-2. The dimerization domains of HNF4α map to both the DNA binding and the ligand binding domain [Jiang and Sladek, 1997]. HNF4α differs from other nuclear receptor for a proline-rich F region at C-terminal, which possesses regulatory functions: the two main isoforms, α1 and α2, differ for the presence or absence of a 10-amino acid segment in the middle of region F [Chartier et al., 1994].
While AF-1 consists of the extreme N-terminal 24 amino acids and functions as an autonomous acidic transactivator, AF-2 domain is very complex, spanning the 128–366 D/E region; its full transactivation potential is inhibited by sequences spanning region F [Hadzopoulou-Cladras et al., 1997]. In fact, F domain interferes with the interaction between AF-2 and coactivators as Src,

CBP and Grip1. The 10-aa insertion in HNF4α2 abrogates the interference by the F domain. One model to explain this inhibition states that the F domain of HNF4α1 inhibits transcription, contacting another portion of the protein, most likely the LBD, and this contact might cover an activation region such as AF-2 and thereby limit access to coactivators. In HNF4α2, the predicted structure of the region suggests that the 10-aa insert introduces a turn in the F domain, which might cause a partial displacement of the repressor region [Sladek et al., 1999].
HNF4α transcriptional activity can be also modulated by long-chain fatty acids that bind the LBD as acyl-CoA thioesters. This binding may shift the oligomeric-dimeric equilibrium of HNF4α or may modulate the affinity of HNF4α for its cognate promoter element, resulting in either activation or inhibition depending on the chain length and the degree of saturation of the fatty acyl-CoA ligands [Hertz et al., 1998].

3.2.2. Role in the hepatocyte differentiation

HNF4α is expressed in hepatocytes and in epithelial cells of the pancreas islets, intestine, stomach and kidney.
During mouse development, HNF4α is expressed very early in the primary endoderm prior to gastrulation and, consistently, HNF4α$^{-/-}$ embryos present severe defects that cause a failure to develop past 6.5 day [Duncan et al., 1994].

To examine the role of HNF4α in liver development past this stage, a Hnf4$^{loxP/loxP}$ Alfp.cre mice model was created; these embryos loose hepatocyte expression of cell adhesion/junction molecules as E-cadherin and ZO-1, the hepatic and the sinusoidal architecture is disrupted and hepatocytes fail to store glycogen with an associated decrease in glycogen synthase, Pepck (Pck1), and glucose-6-phosphatase (G6pc) expression [Parviz et al., 2003].
The crucial importance of HNF4α for development and proper function of liver was confirmed by a genome-wide promoter occupancy study that

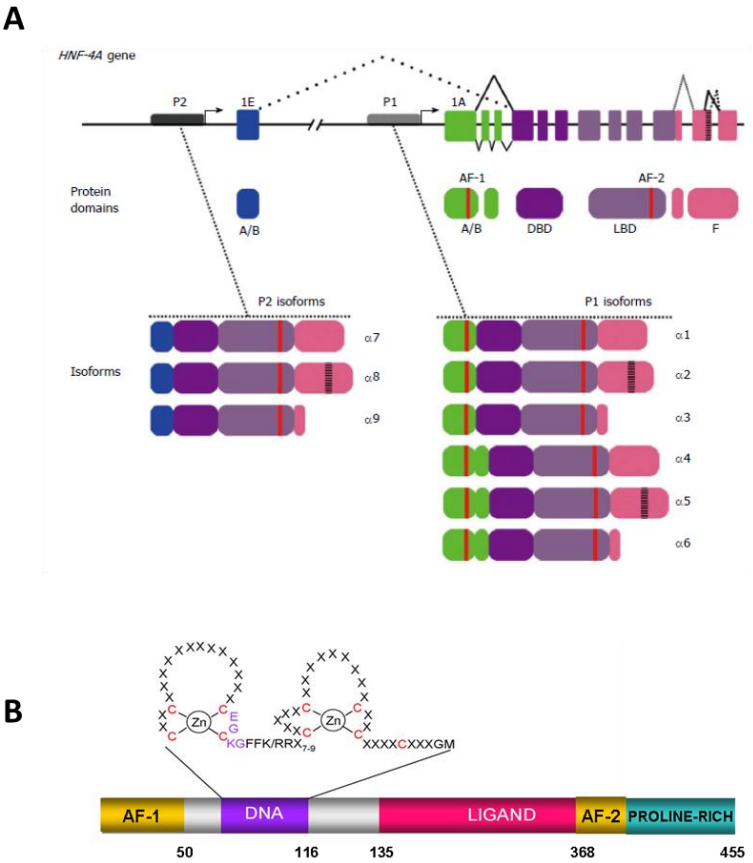

Figure 7. (**A**) *Structure of the human HNF4α gene. HNF4α transcripts can derive from the use of two alternative promoters, P1 and P2; each of them generates several splicing variants* [from Babeu 2014]. (**B**) *Protein structure. HNF4α belongs to the Orphan Nuclear Receptor Family, which is characterized by six different regions (A –F). Two zinc-fingers domains constitute the DNA binding domain that, together with the ligand binding domain, represent the dimerization domain* [from Costa et al., 2003].

demonstrated that half of the promoters of active genes are bound by HNF4α and most of the promoters bound by HNF1α or HNF6 are also occupied by HNF4α [Odom et al., 2004].

HNF4α is an epithelial morphogen because it induces the epithelial marker gene E-cadherin and establishes expression of the intermediate filament cytokeratin proteins [Spath and Weiss, 1998]; in this process, it can be considered a dominant regulator of the epithelial phenotype, as its ectopic expression in fibroblasts induces a mesenchymal-to-epithelial transition, with cells expressing HNF4α that acquire a epithelioid phenotype and express localized E-cadherin and ZO-1 [Parviz et al., 2003].

Furthermore, HNF4α is not only a positive regulator of epithelial genes but it acts also as a negative regulator of mesenchymal markers through the direct binding to the promoters of *snail*, *slug*, *hmga2*, *fibronectin*, *vimentin* and *desmin*. HNF4α can repress several of these mesenchymal genes recruiting the corepressor NcoR to its regulatory regions [Santangelo et al., 2011]. On the other hand, Snail is able to repress Hnf4α gene through a direct binding to its promoter, coherently with the observation that EMT in hepatocytes correlates with downregulation of LETFs and HNF4α in particular [Cicchini et al., 2006]. So in hepatocytes, the maintenance of the differentiated phenotype is regulated by a cross-regulatory circuit between Snail and HNF4α, being each factor able to repress the other thanks to the presence of repressor elements in their promoter [Cicchini et al., 2015].

Recently, the role of HNF4α in the control of the epigenetic state emerged both during EMT and for the regulation of the stem cell compartment. HNF4α, in fact, downregulates DNMT3A and B, which are important during TGFβ-induced EMT, through the direct transcriptional regulation of mir-29 [Cicchini, de Nonno et al., 2015]. Moreover, it contributes to the active repression of stem cells genes through the upregulation of miR34a and miR200 family members, which in turn target several stem cell genes [Garibaldi et al 2012].

3.2.3. Role in tumor suppression

During hepatocellular progression, LETFs – and in particular HNF4α – play an important regulatory role. In fact, from a comparison of expression in human HCC samples, resulted that most of the genes found downregulated in tumor cells are target of LETFs [Xu et al., 2001] and their expression correlates with the differentiation state of hepatocellular carcinoma [Hayashy et al., 1999]. During hepatocarcinogenesis, HNF4α expression decreases [Flodby et al., 1995] and it has been found downregulated in about 70% of HCCs not associated with viral infection. Interestingly, activation of HNF4α7 isoform, not characteristic of adult hepatocytes, was found in the 90% of cases and so this isoform is a marker of hepatocarcinogenesis. In late stage HCCs, the activation of HNF4α7 and the decrease of HNF4α1 expression correlated with unfavorable prognosis of the disease [Lazarevich et al., 2010].

The role of HNF4α as master gene in hepatocyte differentiation has been investigated in hepatoma cells, demonstrating that its expression is sufficient to direct differentiation of dedifferentiated rat hepatoma cells [Spath and Weiss, 1997], inducing the re-expression of E-cadherin and consequently allowing the formation of junctional complexes [Spath and Weiss 1998]. However, the consequences of forced expression depends on the properties of the recipient cells and whether they express molecules acting together with the overexpressed factors [Bailly et al., 1998].
Therefore, HNF4α can be considered a tumor suppressor gene controlling differentiation and proliferation [Hayashy et al., 1999]. Some studies elucidated its role not only in HCC onset but also in HCC progression, a process characterized by a decrease in differentiation, loss of tissue specific gene expression and epithelial morphology, increased proliferation and invasiveness. In fact, in an *in vivo* model of highly invasive fast growing dedifferentiated HCC, forced re-expression of HNF4α reversed the phenotype, inducing the reacquisition of an epithelial morphology and a liver specific gene transcription profile, reducing proliferation and tumor formation in mice models [Lazarevich et al., 2004]. Moreover, HNF4α directly controls the

expression of miRNAs with a tumor suppressor role in HCC, as miR122 [Li et al., 2001].
For these reasons, the gene delivery of HNF4α has been considered a good candidate tool for HCC treatment. Forced expression of HNF4α inversely correlates with EMT both in hepatocytes and hepatic stellate cells, resulting in suppression of the fibrosis correlated with HCC progression. Moreover, it further contributes to inhibit hepatocarcinogenesis suppressing cancer stem cells generation. A possible mechanism involved is the HNF4α-mediated suppression of β-catenin pathway, which is frequently aberrantly activated in HCC [Ning et al., 2010]. The proposed use of recombinant HNF4α adenovirus strategies as differentiation therapy demonstrated *in vivo* a protective role from liver metastatic tumor formation and it can regress established tumor growth [Yin et al., 2008].

However, since tumor onset and progression depends also on micro-environmental cues, these factors should be taken in account and in particular the role of TGFβ, which is important in HCC progression and is associated to a poor prognosis. At this regard, our recent data showed that in a TGFβ containing environment, the restoration of HNF4α function is not effective in suppressing the TGFβ -induced tumor promoting effects; this cytokine, indeed, overcomes both the anti-EMT and the tumor suppressor activity of HNF4α, thus indicating that the therapeutic use of HNF4α gene delivery can be limited *in vivo* by the presence of TGFβ in the tumor microenvironment [Cozzolino et al., 2013].
Another aspect that should be taken in account is the low expression in hepatoma of HNF4α coactivators, in particular PGC1α and SRC1, which can compromise the efficiency of its transcriptional activity [Martinez-Jimenez et al., 2006].

3.2.4. Co-activators and co-repressors

The transactivation potential of HNF4α depends also on the recruitment of coactivators and corepressors that modulate the local chromatin configuration through post-translational histone modification or participating in the assembly of the basal transcription machinery.

Among the coactivators, HNF4α directly interacts with the histone acetyl transferases (HATs) protein CBP, p300 and SRC1, resulting in its increased transcriptional activity. HNF4α1 interacts with the N-terminal region of CBP (amino acids 1–771) and the C-terminal region of CBP (amino acids 1812–2441) through the AF-1 and AF-2 regions [Dell 1999]. HNF4α7 shows similar properties, but only via the AF2 region, the AF1 being absent from this isoform [Torres-Padilla et al., 2002].
The coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is particularly important to modulate hepatic gluconeogenesis [Yoon et al., 2001] and HNF4α can activate properly glucose-6-phosphatase only in concert with PGC-1α [Rhee et al., 2003].

The F domain plays a key regulatory role and helps to discriminate between coactivators and corepressors. In fact, the corepressor SMRT directly interacts with HNF4α through a mechanism that involves the F domain, as demonstrated by the reduction of this interaction due to the removal of this domain. Moreover, SMRT competes for the interaction with the coactivators GRIP1 (glucocorticoid receptor interacting protein1), CBP and p300 [Ruse et al., 2002].
P53 may also act as a corepressor of HNF4α-mediated transactivation with a mechanism that involves the interaction with the ligand binding domain of HNF4α and the recruitment of histone deacetylase [Maeda et al., 2002].
Notably, the activities elicited by coregulators on HNF4α-dependent transcription are dependent on the target promoter [Torres-Padilla and Weiss, 2003].

3.2.5. Post-translational modifications

HNF4α protein is regulated through different post-translational modifications (PTMs), especially phosphorylation, acetylation and methylation that can influence the DNA binding ability, protein dimerization, transactivation and intracellular localization.
Proteomic analysis revealed the complexity of PTMs in the native HNF4α protein differentially identified in the various isoforms. Among the reported phosphorylations through a MS spectra analysis of the native HNF4α2 isoform the sites Ser133 and Ser134, Ser158, Ser427, and the double phosphorylation of Thr420 -Ser427 were identified [Daigo et al., 2011]. Regarding other HNF4α modifications, ubiquitination of Lys224 was also observed [Daigo et al., 2011]. Another mass spectrometry study identified totally 8 PTM sites, including ubiquitination and acetylation, which are the major and not transient PTMs [Yokoyama et al., 2011].

HNF4α is a phosphoprotein. The phosphorylation at tyrosine residues has a key role for DNA-binding activity and transactivation potential. Moreover, even if tyrosine phosphorylation do not affect the nuclear import, they strongly influence its subnuclear localization, probably altering the interaction with a nuclear matrix protein responsible for directing HNF4α to specific subnuclear sites [Ktistaky et al., 1995].

Nuclear import, instead, relies mainly on CBP acetylation, which acetylates HNF4α on lysine residues within the nuclear localization sequence and it enables the maintenance of the protein in the nucleus, inhibiting its active export to the cytoplasm. Acetylation increases also DNA binding activity and the affinity for CBP itself which, acting as a coactivator, acetylates histones, increasing HNF4α activating potential [Soutoglou et al., 2000 A].
Furthermore, the phosphorylation in several serine/threonine residues in HNF4α protein has been recently described both in the native protein and following different stimuli. Ser78, located in the DBD, is phosphorylated *in*

vitro by PKC and this phosphorylation is implicated in decreasing DNA binding, transactivation and protein stability [Sun et al., 2007].

Some PTMs can serve as specific regulation of determined pathway, as it is in the case of oxidative stress response. HNF4α, in fact, acts as an activator of redox-associated hepatocyte iNOS but its activity is associated with a unique serine/threonine kinase-mediated phosphorylation pattern. This means that a redox-sensitive kinase pathway targets HNF-4α to augment hepatocyte iNOS expression [Guo et al., 2003].

HNF4α is also a downstream target of AMPK, which directly phosphorylates the protein, reducing its ability to form homodimers and bind DNA and increasing its degradation rate *in vivo* [Hong et al., 2003], and of PKA which is involved in the transcriptional inhibition of liver genes by cAMP inducers, as PKA phosphorylation inhibits DNA binding activity [Viollet et al., 1997].

Moreover, HNF4α is methylated by PRMT1 on arginine R91, which is located within the DBD, enhancing the formation of homodimer and the affinity for its binding site. PRMT1 functions also as a coactivator: in a second step, it is recruited to the LBD of HNF4α and methylates histone H4 at arginine 3 at HNF4α binding sites within target promoters. This, together with recruitment of the histone acetyltransferase p300, leads to nucleosomal alterations and subsequent RNA polymerase II preinitiation complex formation [Barrero and Malik, 2006].

3.3. Hepatocyte nuclear factor 1α

Hepatocyte nuclear factor 1α is a fundamental protein for both hepatocyte differentiation and maintenance of hepatic functions, even if it is not required for specification of hepatic cell lineage. It binds and thus regulates almost 200 genes and controls many hepatic functions as carbohydrate synthesis, lipid

metabolism, detoxification and synthesis of serum proteins [Odom et al., 2004]. HNF1α is expressed, a part from hepatocytes, also in pancreas islet, intestine, stomach and kidney.

Hnf1α gene has been found mutated in patients affected by maturity onset diabetes of the young (MODY) type 3, a form of non-insulin dependent diabetes with autosomal dominant inheritance [Yamagata et al., 1996 B]. A similar pathology, MODY1, is instead related to HNF4α mutations [Yamagata et al., 1996 A].

3.3.1. Structure

HNF1α belongs to the POU-homodomain subfamily of LETFs being characterized by a homeobox-containing DNA binding domain that is well conserved throughout evolution and a POU domain that confers sequence specificity (Fig 8).

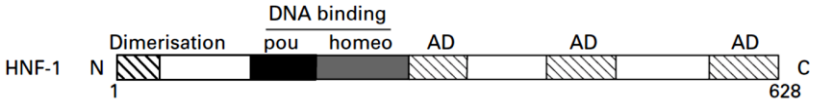

Figure 8. *Structure of the HNF1α protein. HNF1α is a POU-homeodomain protein. The DNA-binding domain contains the homeobox and the POU-domain, the dimerization domain is in the N-terminal region* [from Qin et al., 2009].

HNF1α differs from other homeodomain transcription factors for an extra 21-amino acid loop within the DNA-binding domain and dimerizes via the N-terminal dimerization domain [Baumhueter et al., 1990]. Because of similar dimerization domain in their N-terminal regions, HNF1α can dimerize with HNF1β isoforms. The dimerization domain can associate with DcoH, an 11-kDa protein that has been suggested to be involved in dimer stabilization [Johnen and Kaufman, 1997].

The C-terminal part of HNF1α contains three regions, ADI, ADII, and ADIII, which have been shown to be indispensable for transcription activation. Differences in the transactivation domains at C-terminal confer to HNF1α higher transactivation potential than HNF1β [Hayashi et al., 1999].

3.3.2. Role in the hepatocyte differentiation

In embryonic livers, HNF1α and HNF1β are expressed at comparable levels, while in the adult liver HNF1α expression further increases and HNF1β expression decreases [Kyrmizi et al., 2006]. During development, HNF1α expression follows that of HNF4α, leading to a reciprocal regulatory loop [Cereghini et al., 1992], even though the presence of one factor is not essential for the expression of the other, as hnf4α-deficient mice express hnf1α gene and *vice versa* [Duncan et al., 1997] [Pontoglio et al., 1996]. Nonetheless, in differentiated hepatocytes HNF4α is capable to activate HNF1α expression [Kuo et al., 1992] and HNF1αcan activate HNF4α [Zhong et al., 1994] as well as its expression can be self-sustained [Miura and Tanaka, 1993]. HNF1α cooperates also with other members of the LETF family, as with C/EBP in the induction of PEPCK [Yanuka-Kashles et al., 1994] or Albumin [Wu et al., 1994].

Hnf1α$^{-/-}$ mice died at the time of weaning due to a severe wasting syndrome with massive glucosuria, phosphaturia, and aminoaciduria from renal tubular dysfunction. In fact, even if HNF1α KO mice liver can develop normally and the overall liver phenotype is preserved, their distinctive trait is the complete loss of phenylalanine hydroxylase (PAH) expression, that causes a phenotype comparable to human phenylketonuria [Pontoglio et al., 1996]. Moreover, Hnf1α$^{-/-}$ mice are characterized by defective glycolytic signaling in pancreatic β-cells resulting in diminished insulin secretion [Pontoglio et al., 1998].

Several studies demonstrates HNF1α importance in cell reprogramming: functional induced- hepatocytes (iHeps) have been generated overexpressing

HNF1α in fibroblast, both in human [Du et al., 2014] [Simeonov and Uppal, 2014] and mice models, where transplanted iHeps have been able to repopulate liver and to rescue liver functions of recipient mice [Huang et al., 2011].

3.3.3. Role in tumor suppression

In HCC samples, HNF1α is expressed at lower levels in poorly differentiated tumors compared to the well differentiated ones, whereas HNF1β retains its expression also in dedifferentiated variants [Wang 1998] [Lazarevich et al., 2004]. Moreover HNF1α deficient mice display a tumor-like phenotype, with increased proliferation of hepatocytes and deficit in normal liver functions [Pontoglio et al., 1996]; mutation of HNF1α is also a critical event during the development of liver adenoma, where it has been found mutated in 84% of cases [Bluteau et al., 2002] [Bacq et al., 2003].

Starting from these evidences, some studies have investigated the tumor suppressing effect of forced re-expression of HNF1α in hepatomas. Exogenous HNF1α triggers the G2/M arrest in hepatoma cell lines, due to the accumulation of p21, which has been found up-regulated both at transcriptional and protein levels. Moreover, HNF1α induces differentiation (re-establishing the expression of liver specific genes and miRNAs as miR-192 and miR-194) and, more importantly, significantly inhibits xenograft growth *in vivo* [Zeng et al., 2011].

HNF1α tumor suppressor role is further validated by the observation that its inactivation leads to the activation of pathways involved in tumorigenesis and in particular the mTOR pathway [Pelletier et al., 2009]. Importantly, HNF1α inhibition is also related to EMT, with cells that loose cell-cell contacts, acquire migratory properties and express mesenchymal markers including EMT master genes [Pelletier et al., 2011]. This is consistent with the capacity of HNF1α to act as repressor of mesenchymal markers and to bind directly the promoter of *snail*, *slug*, *hmga2* and of mesenchymal genes as *vimentin* and *desmin* [Santangelo et al., 2011].

3.3.4. Co-activators and co-repressors

To accomplish its functions on specific targets, HNF1α often cooperates with co-activators or co-repressor.

Consistent with the important role of the CBP/p300 acetyltransferases in regulating transcription of hepatocyte-specific genes, HNF1α can directly bind these proteins. In particular, CBP and P/CAF interact with the N- and C-terminal domain of HNF1α, respectively, and operate a synergistic transactivation, since the interaction of CBP with HNF1α N-terminal domain greatly increases the affinity for P/CAF binding [Soutoglou et al., 2000 B]. Moreover, this interaction can somehow modulate also enzymatic activity of the coactivators, since two dominant negative mutants of HNF1α, found in Mody3 affected patients, have been found to possess a stronger interaction affinity but, in this case, CBP and P/CAF lack HAT activity [Soutoglou et al., 2001].
This interaction has been described on several genes, from those implicated in metabolism, as Glut2 [Ban et al., 2002] to plasma proteins, as Albumin [Dohda et al., 2004]. In the activation of Glut2, which is an important HNf1α target in pancreatic cells, where glucose metabolism is fundamental to induce insulin secretion, p300 may act as a transcriptional co-activator by bridging the activator to the basal transcriptional machinery and, with its HAT activity, modifying chromatin structure promoting a locally open and transcriptionally active configuration [Ban et al., 2002]. On the albumin promoter, both CBP and p300 interacts with HNF1α and form a preinitiation complex of Rna PolII [Dohda et al., 2004].
The ability to direct nucleosomal hyperacetylation to transcriptional target is fundamental for HNF1α. In fact, in a study conducted with hnf1$\alpha^{-/-}$ mice models, Parrizas et al. demonstrated that the organ specific induction of different targets does not rely only on promoter occupancy by HNF1α but is strongly dependent on nucleosomal acetylation. In the specific, HNF1α is necessary for the expression of *glut2* and *pklr* genes in pancreatic insulin-producing cells but not in liver even though HNF1α occupies these promoters

in both pancreatic islet and liver cells. However, it is indispensable for hyper-acetylation of histones in *glut2* and *pklr* promoter in pancreatic islets but not in liver cells. On the contrary, PAH is a specific HNF1α liver target that requires HNF1α for transcriptional activation and presents localized histone hyperacetylation only in liver tissue [Parrizas et al., 2001]. Moreover, in the liver of hnf1α deficient mice models the lack of pah expression correlates with a condensed chromatin state and with its promoter hypermethylation [Pontoglio et al., 1997].

HNF1α, through the recruitment of coactivators and corepressors, can influence also the histone methylation state. In hnf1α$^{-/-}$ cells, in fact, HNF1α depletion correlates with an increase of H3K27me3 (tri-methylated lysine 27 on histone H3), which is a marker of condensed and not active state of chromatin, and a concomitant decrease of active chromatin associate mark H3K4me2/3 (methylation of Lysine 4 on histone H3) [Luco et al., 2008].

HNF1α, through its homeodomain, interacts also with HMGB1, a non-histone architectural chromosomal protein that stabilizes nucleosomes and allows bending of DNA to facilitate gene transcription, interacting both with the basal transcription machinery and with individual transcription factors (such as p53 and NF-kB) [Yu et al., 2008].

Among corepressor, HDAC was found to interact with HNF1α through NcoR, reducing its transcriptional activity on its target gene promoters. Accordingly, inhibition of HDAC with tricostatin (TSA) inhibits the formation of the repressor complex -NcoR-HDAC with HNF1α and results in a significant increase of HNF1α-mediated transcription [Soutoglou et al., 2001].

Another corepressor of HNF1α is the Prospero-Related-Homeobox-Protein 1 (PROX1), which interacts through its N-terminal region with the DNA-binding domain of HNF1α. This interaction has been described to be relevant to the HNF1α-mediated repression of Hepatitis B virus genes and therefore for the inhibition of virus replication. The presence of Prox1, indeed, switches the

role of HNF1α from activator to inhibitor of target gene expression [Qin et al., 2009].

3.4. Hepatocyte nuclear factor 6

3.4.1. Structure

Hepatocyte nuclear factor 6 belongs to the One-Cut family and it contains two different DNA binding domain: a novel homeodomain and a domain homologous to the Drosophila cut domain, with whom it binds DNA as a monomer (Fig 9). It was first discovered as a regulator of the expression of the glucose metabolism enzyme 6-phosphofructo-2-kinase (PFK-2) [Lemaigre et al., 1996] and then it has been described as regulator of several cellular processes during development, differentiation, regeneration, metabolism, and inflammatory response.
During liver regeneration, HNF6 regulates S-phase progression in hepatocytes through the stimulation of TGFα, cyclinD1 and Cdk2 [Tan et al., 2006].

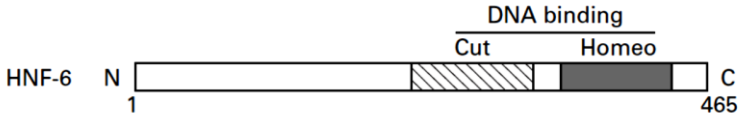

Figure 9. *Structure of the HNF6 protein. HNF6 contains a Cut and a Homeo-domain that constitute the DNA binding domain in the C-terminal region* [from Hayashy et al., 2009]

HNF6 interaction with other LETFs is important for its transactivating properties. In particular, HNF6 and FoxA2 interact synergistically to regulate hepatic specific genes expression. Therefore, HNF6 increases the expression of FoxA2 specific targets recruiting the acetyl transferases CBP or p300. Moreover, HNF6 forms a complex with C/EBPα to induce FoxA2 expression and their transcriptional synergy is abrogated by CBP inhibition, indicating its

requirement for the activation of FoxA2 [Yoshida et al., 2006]. On the contrary, FoxA2 exerts an inhibitor effect on HNF6 activation, as it impedes the binding of HNF6 to its recognized sites on the promoters [Rausa et al., 2003].

3.4.2. Role in development

HNF6 is expressed in tissues that originate from the endoderm cells. During development, HNF6 is expressed in hepatocytes and in the epithelial cells of the intrahepatic and extrahepatic bile ducts. *Hnf6*$^{-/-}$ mouse embryos fail to develop a gallbladder and exhibited severe abnormalities in both extrahepatic and intrahepatic bile ducts [Clotman et al., 2002]; moreover, they are diabetic with severe defects in pancreatic islets [Jacquemin et al., 2000].
In livers, HNF6 promoter is occupied by HNF4 at day 14 and later also by HNF1β and C/EBPα and by HNF6 itself postnatally [Kyrmizi et al., 2006].
During development, HNF6 binds to the promoter regions of FoxA2 and HNF4α, in particular on the HNF4α7 promoter [Odom et al., 2004] as well as in the liver specific genes transthyretin and alpha-fetoprotein.
The onset of HNF6 gene transcription is detected in the liver at embryonic day 9, then its expression disappears transiently from the liver between embryonic days 12.5 and 15, but it is present again in the liver after embryonic day 15. This pattern is paralleled by FoxA2. In addition, HNF6 and FoxA2 transcripts are expressed abundantly and co-localize in the exocrine acinar cells of the pancreas on day 18 of gestation and in the adult liver [Rausa et al., 1997].

A HNF6 important target for hepatocyte differentiation is miR-122. HNF6 induces miR-122 which, through a positive feedback-loop, positively regulates the expression of other LETFs, including HNF6 itself, through a direct or indirect mechanisms, allowing the progression of hepatocyte differentiation [Laudadio et al., 2012].

3.4.3. Role in tumor suppression

The role of HNF6 during tumor progression is controversial as it has been show that HNF6 regulates cell cycle, inducing S phase progression in hepatoma cells [Tan et al., 2006]. Moreover, the overexpression of functional HNF6 in hepatoma and colon cancer cell lines correlates with an inhibition of cell cycle progression in G2/M phase [Lehner et al., 2010].

HNF6 protein has been detected in liver colon cancer metastasis but, since healthy colon or primary colonic cancer do not express it, HNF6 induction is probably driven by the hepatic environment. Moreover, liver metastasis are characterized by a strong expression of the HNF6-direct target FoxA2 but not of other targets. These observations mean that the presence of HNF6 does not correlate with its transactivation activity, which could be instead ascribed at the presence of FoxA2 (whose interaction with HNF6 exerts an inhibitor effect on HNF6 activation) or at a lack of specific PTMs. In particular, the unacetylated form of HNF6 was found in metastasis where its DNA binding is abrogated [Lehner et al., 2007]. The acetylation on HNF6 is CBP-dependent and increases its stability and protein levels, without involving the proteasomal pathway inhibition. When CBP acetyl transferase activity is inhibited, the formation of the complex between HNF6 and FoxA2 is altered and their synergistic action abrogated [Rausa et al., 2004].

HNF6 expression is significantly reduced in human pancreatic tumors and its expression levels correlates with the progression of the disease [Pekala et al., 2014]; moreover, its tumor suppressor role has been observed in lung cancer cells. In this tumor model, in particular, its forced re-expression was found inhibit cell migration and reduce the formation of xenograft tumors *in vivo*. A possible explanation involves the activation of p53 oncosuppressor through a direct binding of HNF6 to its promoter. Interestingly, HNF6 expression also inversely correlates with EMT: it is downregulated by TGFβ and can induce E-cadherin expression [Yuan et al., 2013]. On the other side, HNF6 inhibits

TGFβ pathway during liver development, through the repression of TGFβRII [Plumb-Rudewiez et al., 2004].

4. LETFs in HCC gene therapy

The characteristics of LEFTs described above suggest their high potential as HCC therapeutic tool.
HNF4α in particular, being the master regulator of epithelial/hepatocyte differentiation and MET, but also HNF1α and HNF6, thanks to their reciprocal regulations, are able to suppress tumor onset and progression not only restoring the differentiation of tumor cells, but also inhibiting proliferation and negatively controlling EMT and stemness properties [reviewed in Marchetti et al., 2015 Disease] (Fig 10).

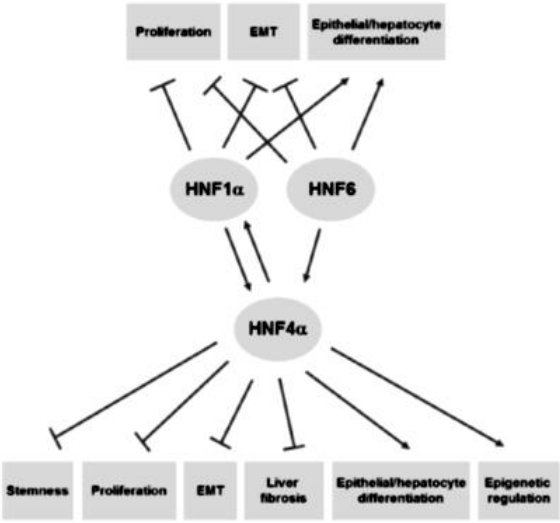

Figure 10. *Tumor suppressor properties of HNF4α, HNF1α, and HNF6 in HCCs. HNFs can regulate different cell functions associated with the HCC onset and progression, through the direct transcriptional activation/repression of target genes). The reciprocal regulation among HNFs is also shown* [from Marchetti et al., 2015].

MATERIALS AND METHODS

Cell cultures and treatments

Murine Hepatocyte cell lines used in this work are immortalised non-tumorigenic cell lines derived from livers at different stage of development (livers at 14,5 days post-coitum, MMH/E14 and WT/3A, or at 3 days post-birth, MMH/D3) [Amicone et al., 1997][Guantario et al., 2012) and largely characterized. Cells show an epithelial morphology, express LEFTs and hepatic markers and possess all the hepatic functions.
Cells were grown at 37°C, in a humidified atmosphere with 5% CO_2 on collagen I (*Collagen I, Rat Tail*; Gibco – Life Technologies) coated dishes in RPMI medium (Gibco – Life Technologies), supplemented with 10% FBS (Gibco – Life Technologies), 2 mM L-glutamine (EuroClone), 100 mg/ml penicillin, 100 mg/ml streptomycin (Gibco – Life Technologies), 50 ng/ml EGF (PeproTech), 30 ng/ml IGF II (PeproTech), 10 µg/ml insulin (Roche).
Where indicated, cells were treated with TGFβ1 (PeproTech) (5 ng/ml) or with *GSK3-inhibitor 6-bromoindirubin-30-oxime, BIO* (Calbiochem) (2.5 nmol/mL) for the indicated time.

Site-directed mutagenesis

Mutants HNF4α proteins were obtained with the *QuikChange II XL Site-Directed Mutagenesis Kit* (Agilent Technologies) on the pcDNA3 HNF4α vector.
The sequences of primers are specified in Table 1.
The mutant strands synthesis reaction was prepared adding 5 µl of 10X reaction buffer, 10 ng of dsDNA template, 125 ng of forward primer, 125 ng of reverse primer, 1 µl of dNTP mix, 3 µl of *QuikSolution*, ddH_2O to a final volume of 50 µl and 2.5 U of *PfuUltra HF DNA polymerase*.
The synthesis of mutant vectors was obtained running the mutant strands synthesis reaction with the following cycle parameters:

Cycles	Temperature	Time
1	95° C	1 minutes
18	95 ° C	50 seconds
	60 ° C	50 seconds
	68 ° C	7 minutes 20 seconds
1	68 ° C	7 minutes

Then, 10U of *DpnI restriction enzyme* were added in the amplification products for 1 hour at 37 °C, in order to digest non-mutated parental methylated and hemimethylated DNA.
2 µl of the reaction were used to transform 50 µl of *XL10-Gold Ultracompetent Cells* by heat-pulse; then 500 µl of NZY^+ broth were added, the transformation reaction was incubated at 37°C for 1 hour and 250 µl were spread on each of two ampicillin-agar plates overnight.
Single colonies were amplified and controlled by sequencing to verify the presence of the desired mutations.

Cell transfections

Transient overexpressing-cells were obtained by transfection with pcDNA3 HNF4α plasmids (coding for the wild-type or mutant proteins), pclBcx $HNF1\alpha^{Myc}$, pCMV $HNF6^{Flag}$ and the relative empty vectors.
Cells were transfected with *Lipofectamine 2000* (Invitrogen, Thermo Fisher) at 90% of confluence with 3.5 µg of vectors in 35mm plates in *Optimem medium* (Gibco) for 5 hours.
Cells were collected 48 hours after transfection.

Luciferase assay

For the luciferase assay, cells were transfected in 12 well dishes using *Lipofectamine 2000* (Invitrogen, Thermo Fisher) with 500 ng of the expression vector and 500 ng of the reporter construct containing ApoA1 promoter

sequence, or 250 ng of Snail reporter construct, fused to the firefly reporter gene. 100 ng of reporter construct containing Renilla gene coding sequence were co-transfected and used as an internal control for transfection efficiency. All transfections were performed in duplicate.
48 hours after transfection, cells were lysed with *Passive Lysis Buffer* (Promega) and underwent a freeze-thaw cycle to further lyse cells. Then, the lysate was centrifuged at 13000 rpm at 4° C for 5'; the supernatant was collected and 20 µl were used to analyse luciferase activity, according to the *Dual-Glo Luciferase Assay System* (Promega). 100 µl of *Dual-Glo Luciferase Assay Reagent* were added and the luciferase emission was measured with a luminometer for 10 seconds. Then, 100 µl of *Stop & Glo Reagent* were added, measured for further 10 second and used to normalise the luciferase emission.

RT-qPCR

Total RNA was extracted with *reliaPrep RNA Cell miniprep System* (Promega), according to manufacturer's protocol.
1 µg of RNA was reverse-transcribed using *iScript cDNA Synthesis Kit* (BioRad); the complete reaction mix was incubated at 25°C for 5', 42°C for 1 hour, 85°C for 5'.
RT-qPCR were performed using *GoTaq qPCR Master Mix* (Promega) and the reaction were carried out in *BioRad-iQ-iCycler* with 20 ng of cDNA used as template.
The cycling conditions were: 95°C for 3', followed by 40 cycles at 95°C for 10 seconds and 59°C for 30 seconds, then the temperature was raised from 65°C to 95°C with 0.5°C increase steps for 0.5 seconds. For Albumin a melting temperature of 57°C was used; for Transthyretin the cycling conditions adopted require 40 cycles at 95°C for 10 seconds, 57°C for 30 seconds and 80°C for further 30 seconds. The sequence of primer used are listed in Table 2.
The results were analysed with *CFX Manager software* (BioRad) and calculated with the ΔC(t) method.

SDS-PAGE and Western Blotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40) containing freshly added cocktail protease inhibitors (*cOmplete, EDTA-free Protease Inhibitor Cocktail*; SigmaAldrich) and phosphatase inhibitors (5 mM EGTA pH 8.0; 50 mM sodium fluoride; 5 mM sodium orthovanadate). Lysates were incubated on ice for 20' and then centrifuged at 13000 rpm for 30' at 4°C. Protein concentration was determined with *Protein Assay Dye Reagent* (BioRad), based on the Bradford assay.

Samples (20 µg of proteins) were prepared in Laemli Buffer (containing 2-β mercaptoethanol and SDS) and were loaded on 12% acrylamide gels.

Gels were electrophoresed at 100V in Running Buffer (25mM Tris, 190 mM glycine; 0.1% SDS) and then transferred to a nitrocellulose membrane (*Pure Nitrocellulose Membrane 0.45 µm;* Bio-Rad) at 100V for 1 hour and 30' in Transfer Buffer (50 mM Tris , 40 mM glycine; 0.1% SDS; 20% Methanol).

Blots were blocked in 5% non-fat milk prepared in TBS-Tween (10mM Tris-HCl pH 7.5; 150mM NaCl; 0.05% Tween 20) and incubated overnight with the primary antibody (α-Flag Mouse monoclonal, M2 Sigma, 1:2000; α-HNF1α Rabbit polyclonal, NBP1-33596, Novus, 1:2000; αSnail Mouse monoclonal, L70G2, Cell Signalling, 1:1000; α-CBP Rabbit polyclonal, 451, sc-1211X, Santa Cruz, 1.1000).

Then blots were incubated with HRP-conjugated species-specific secondary antibodies (*Goat Anti-Mouse IgG (H+L)-HRP Conjugate* or *Goat Anti-Rabbit IgG (H+L)-HRP Conjugate*, Bio-Rad) followed by enhanced chemiluminescence reaction (*WESTAR Nova 2.0*, Cyanagen) and the signal was revealed through autoradiography X-ray film.

EMSA assay

-Nuclear extracts

EMSA assays were carried out with the nuclear fraction of proteins.

To prepare nuclear extracts, cells were scraped in cold phosphate buffer saline (PBS) and pelleted at 1200 rpm for 5' at 4°C. The pellet was resuspended in Buffer A (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 10mM KCl, 0.1% NP40, 0.1mM EDTA) plus 0.5 mM DTT, 50mM sodium fluoride, 5mM sodium orthovanadate, 5mM EGTA and a cocktail of protease inhibitors (*cOmplete, EDTA-free Protease Inhibitor Cocktail*; SigmaAldrich) and incubated for 30' in rotation at 4°C. Then, the lysates were centrifuged at 13000 rpm for 10' at 4°C; the supernatant, containing the cytoplasmic protein fraction, was stored while the pellet, containing intact nuclei, was resusupended in buffer C (20 mM Hepes pH 7.9, 20% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% NP40), plus 0.5 mM DTT, 50mM sodium fluoride, 5mM sodium orthovanadate, 5mM EGTA and a cocktail of protease inhibitors. The nuclear lysis was conducted for 30' at 4°C in rotation and nuclear protein were extracted centrifuging the lysates at 13000 rpm for 10' at 4°C. Then protein concentrations were determined with the *Bio-Rad Protein Assay Dye Reagent.*

-Biotinylated oligo preparation

Oligo containing the binding site for the protein of interest were biotinylated with the *Biotin 3´ End DNA Labeling Kit* (Thermo Scientific). For each Forward and Reverse primer was prepared a mix containing TdT Reaction Buffer 1X, 0.5µM Biotin-11-UTP, 0.15 U/µL diluted TdT and 2.5 pmol of primer. The reaction was incubated for 30' at 37°C and then stopped with EDTA 10mM.

An equal volume of chloroform:isoamylic alcohol was added to each reaction to extract the biotynilated oligos.

Annealing was obtained incubating an equal volume of biotinylated forward and reverse primer for 5' at 90°C and then slowly cooled at room temperature.

At the same time, a 100-fold excess (250pmol) of unlabelled forward and reverse oligo were annealed.

The sequences of oligonucleotide used are listed in Table 4.

-EMSA assay

EMSA assay was performed with the *LightShift Chemiluminescent EMSA kit* (Thermo Scientific).

The binding reaction was prepared in a final volume of 20µL incubating 1X Binding Buffer, 2,5% Glycerol, 5mM $MgCl_2$, 50ng/µl PolydI-dC, 0,05% NP-40 and 10µg of nuclear extracts (except for the free probe sample) for 10' at 4ºC. Then 25fmol of the biotinylated probe were added and the reaction conducted for further 20' at RT. When specified, 5pmol of unlabeled annealed oligo or 5µg of antibody (α-Flag Mouse monoclonal, M2 Sigma; α-HNF1α Rabbit polyclonal, H-140 sc-10791X, Santa Cruz; α-HNF4α, Rabbit polyclonal, H-171 sc-8987X, Santa Cruz; α-Tubulin, Mouse monoclonal, TU-02 sc-8035, Santa Cruz) were added and incubated for 10' at 4ºC and further 10' at RT before adding the nuclear extracts.

The reaction was stopped with 5µl of 5X *Loading Buffer* and loaded on a 6% native polyacrilammide gel in 0.5X TBE (pre-electrophoresed for 60' at 100V), electrophoresed in 0.5 TBE for about 60', until the bromophenol blue dye, corresponding to the migration of the free-biotin probe, has migrated approximately to 3/4 down the length of the gel.

Then, binding reaction were transferred to a nylon membrane (*Biodyne B Nylon Membrane*, Thermo Scientific) at 100V for 30' in cooled 0.5X TBE.

Transferred DNA was cross-linked to the membrane at 120mJ/cm^2 for 1' with *UV stratalinker 1800* (Stratagene).

Biotin-labeled DNA was detected by chemiluminescence according to *Chemiluminescent Nucleic Acid Detection Module Kit* (Thermo scientific) and revealed through autography X-ray films.

ChIP

To crosslink protein complexes to DNA, fixation solution (11% formaldehyde, 50 mM Hepes pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA) was added directly in the cell culture medium in order to obtain a 1% final concentration of formaldehyde and incubated for 10' at 37ºC. The crosslinking reaction was stopped adding one tenth volume of 1.25 M glycine for 5' at 4ºC

and 5' with gentle shaking at RT. Then, cells were washed and scraped in cold Phosphate Buffer Saline (with cocktail protease inhibitor and 100 mM PMSF) and centrifuged at 1200 rpm for 5' at 4°C.

Pellet was resuspended and lysed in 10 volumes of L1 Buffer (50 mM Tris HCl pH 8.0, 2 mM EDTA pH 8.0, 0.1% NP-40, 10%, glycerol plus protease and phosphatase inhibitors) for 15' at 4°C in rotation. The lysates were homogenized by 15 dounce strokes and then centrifuged at 5000 rpm for 15' at 4°C, to separate the cytoplasmic from the nuclear fraction. The pellets, containing nuclei, was resuspended in L2 buffer (50 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 1% SDS plus protease and phosphatase inhibitors) and incubated for 20' at 4°C in rotation. The chromatin was sonicated on ice with 5 pulses for 10 seconds at 60% settings (VibraCell Sonicator) to obtain chromatin fragments of an average length of 200 to 500 base pairs. After that, chromatin was centrifuged at 10.000 for 10', supernatants were collected and chromatin concentration was determined.

For each sample, two 150 µg aliquots (one for each specific antibody and one for the specie-specific corresponding IgG) were diluted 1:10 in Dilution Buffer (20 mM Tris HCl ph 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% Triton X-100) plus protease inhibitor and precleared with 40 µl of *Protein A Sepharose* (Sigma Aldrich) (previously blocked with sonicated salmon sperm DNA (200 µg/ml) in 3% Bovine Serum Albumin) for 3h at 4°C in rotation.

Pre-cleared chromatin was centrifuged at 2000 rpm for 5' and the supernatant was incubated over night at 4°C in rotation with 5 µg of specific antibody (α-HNF6, Rabbit polyclonal, H-100 sc-13050, Santa Cruz; α-HNF1α Rabbit polyclonal, H-140 sc-10791X, Santa Cruz; α-CBP Rabbit polyclonal, 451, sc-1211X, Santa Cruz; α-acetyl H3, Rabbit polyclonal, 06-599, Millipore; α-H3K4me2, Rabbit polyclonal, ABE250, Millipore; α-H3K27me3, Rabbit polyclonal, 07499, Millipore), or *Normal Rabbit antiserum* (Millipore) as negative control, to proceed with immunoprecipitation.

Immunoprecipitated complexes were collected by incubation with 50 µl of *Protein A Sepharose* for 3 hours at 4°C in rotation. The samples were centrifuged at 3000 rpm for 3' at 4°C and, before washing, 300 µl of the supernatant of the IgG sample were collected and stored as Input sample.

Then the beads were washed in the following buffers with protease and phosphatase inhibitors:

- Low salt (20 mM Tris HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% TritonX-100)
- High salt Buffer (20 mM Tris HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100)
- LiCl wash Buffer (10 mM Tris HCl pH 8.0, 0.25M LiCl, 1% NP-40, 1% Na-deoxycholate, 1mM EDTA)
- TE wash Buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0)

After that, immune complexes were eluted twice from *Protein A Sepharose* with 150 µl of Elution buffer (1% SDS and 100mM $NaHCO_3$) for 15' with shaking at RT.

Then the samples were incubated with 10 µg of RNase for 10' at RT and after that, cross-linking was reversed incubating sample at 65ºC over night with gentle shaking.

In each sample was added 20 µl of 1M Tris-HCl pH 6.5 to neutralize NaHCO3 and then 12 µl of proteinaseK 20mg/ml was added and the reaction conducted for 2 hours at 56 ºC.

Finally, DNA was extracted with phenol-chloroform and chloroform and then precipitated in 1 volume of 100% isopropanol. Pellet was washed with cold 70% ethanol and resuspended in 50 µl of H_20 and chromatin concentration was determined. RT-qPCR was performed with 2 µl of 2 ng/µl diluted DNA. The utilized primers are listed in Table 4.

In vitro translation and kinase assay

The production of *in-vitro* translated (IVT) proteins was achieved with the *TNT Coupled Reticulocyte Lysate Systems Kit* (Promega). According to manifacturer's instruction, the following reaction was assembled:

- TNT Rabbit Reticulocyte Lysate 25µl
- TNT Reaction Buffer 2µl
- T7 TNT RNA Polymerase 1µl
- Amino Acid Mixture, Minus Leucine, 1ul

- Amino Acid Mixture, Minus Methionine, 1ul
- RNasin Ribonuclease Inhibitor (40u/µl) 1µl
- DNA Template(s) (0.5µg/µl) 2µl
- H_2O to a final volume of 50µl.

The reaction was incubated for 30' at 90°C

For kinase assay 5 µl of IVT protein were incubated with 100 ng of recombinant GSK3β (*GSK3β active*; SignalChem), 1µl ATP 10mM (Cell signalling) and 23µl of Kinase Buffer 1X (Cell Signalling) at 30°C for 30'.
When specified, 1µl of 10U/µL CIP (*Calf Intestinal Phosphatase*, New England BioLabs) were added for 1 hour at 37 °C

Co-immunoprecipitation

Cells were lysed with IP Lysis Buffer (150 mM NaCl; 50mM Tris-HCl pH7.5; 2mM EDTA; 1% Triton-X100; 10% glycerol) plus protease and phosphatase inhibitor. Lysates were incubated for 1 hour at 4°C in rotation and then centrifuged at 13000 rpm for 20' at 4°C.
1 mg of protein was precleared adding 40 µl of Protein A Sepharose (blocked in 3% Bovine serum Albumin) for 1 hour at 4°C in a total volume of 1 ml of IP Lysis Buffer. Then, beads were removed by centrifugation and the extracts were incubated with 5 µg of the primary antibody (α-HNF1α Rabbit polyclonal, H-140 sc-10791X, Santa Cruz) or Normal Rabbit antiserum (Millipore) at 4°C overnight. Immuno-complexes were collected adding 50 µl of Protein A Sepharose for 3 hours at 4°C; the beads were then washed trice with NetGel Buffer (150 mM NaCl; 50mM Tris-HCl pH7.5; 1mM EDTA; 0.1% NP-40; 0,25% gelatin) and finally immunoprecipitated proteins were separated from beads adding 50 µl of Laemli 2X. Samples were boiled at 95°C for 5', beads were eliminated by centrifugation and half of each sample was loaded on polyacrilammide gel and analysed by Western Blotting.

2-DE (two-dimensional gel electrophoresis)

2-DE was performed using IPGphor II (GE Healthcare). Proteins (90 µg) from IVT or nuclear extracts were precipitated with 100% acetone and then loaded on pH 3–10 IPG strips (IPGs) by in-gel rehydration for 9 h. Proteins were then electrofocused at 15,000 V/h at a maximum voltage of 5000 V. The second dimension separation was performed at a constant current of 50 mA for 2 h. Proteins from cell lysates were transferred to nitrocellulose membranes and blots were blocked in 5% non-fat dried milk in TBS-T buffer. Western blot was performed as described above with HNF4α (α-HNF4 α, Goat polyclonal, C-19: sc-6556, Santa Cruz) or HNF1α (α-HNF1α Rabbit polyclonal, NBP1-33596, Novus) antibodies.

AIM OF THE WORK

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the most frequent among the primary tumors of the liver. However, treatment options are limited and often ineffective since HCCs frequently develop on a pathological background of pre-existing chronic liver diseases, including liver fibrosis and cirrhosis, frequently associated to viral infections [Farazi and DePinho, 2006]. Moreover, most of the tumors are diagnosed at advanced stages, when the recurrence rate after therapy and the tendency to metastasize are high. Thus, new therapeutic strategies are needed to inhibit tumor progression and to improve the survival of patients. The targeted gene therapy, based on the restoration of tumor suppressor proteins lost during neoplastic transformation, seems to be the most appealing approach [Spangeberg et al., 2009].

Promising candidates for targeted gene therapy in HCC are represented by master transcriptional factors belonging to the family of Liver Enriched Transcription Factors (LETFs) [reviewed in Marchetti et al., 2015]. In particular, HNF4α is a master regulator of epithelial/hepatocyte differentiation during development and a key factor for the execution and the maintenance of the epithelial program in adult liver. Its reduction or lack of expression is associated with advanced stage HCCs [Lazarevich et al., 2010]. Preclinical data in mice suggests the use of HNF4α for the treatment of HCC. It has been shown, indeed, that the systemic administration or the intra-tumor injection of adenoviral HNF4α protected mice from liver metastatic tumor formation and displayed a significant regression of already established tumors [Yin et al., 2008]. Furthermore, data from our laboratory recently showed that HNF4α re-expression in HNF4α low-expressing hepatoma cell lines is able to trigger differentiation and to actively repress the epithelial-to-mesenchymal transition (EMT) program, a trans-differentiation process that results in the loss of epithelial polarity and in the acquisition of mesenchymal phenotype, motility and stemness properties. In particular, the HNF4α mediated tumor reversion towards a highly differentiated and less invasive phenotype appears mediated by its direct transcriptional repression of EMT master genes, such as Snail and

Slug [Santangelo et al, 2011]. Therefore, restoration of HNF4α functions in invasive HCCs represents a promising therapeutic strategy.

Tumor onset and progression, however, not only depend on the acquisition of genetic and/or epigenetic mutations by differentiated or stem/precursor cells but also on micro-environmental cues, including soluble factors, matrix stiffness and interplay with stroma and cells of immune system.
In particular, the pleiotropic transforming growth factor beta (TGFβ) has emerged as a major micro-environmental factor playing a role in carcinoma progression. Thus, regarding HCC, an unbalanced level of TGFβ in the tumor niche can drive transformed hepatocytes towards an EMT and, consequently, the acquisition of migration and invasive properties. Accordingly, in HCC patients was observed that TGFβ signaling activation contributes to tumor progression and it is associated to a poor prognosis [Lee et al., 2012].

Recent data from our laboratory showed that in a TGFβ containing environment, the restoration of HNF4α function by gene transfer in transformed hepatocytes is not effective in suppressing the malignant behavior. This cytokine, indeed, overrides both the anti-EMT and the tumor suppressor activity of the ectopically expressed HNF4α protein, thus indicating that the therapeutic use of HNF4α gene delivery can be limited *in vivo* by the presence of TGFβ in the tumor microenvironment. In particular, it has been shown that TGFβ impairs HNF4α DNA binding activity by displacing it from promoters of target genes and that HNF4α functional inactivation correlates with changes in the post-translational modification (PTM) profile, including the phosphorylation pattern mediated by the GSK-3β kinase that, in turn, is inactivated by TGFβ. The use of a constitutively active form of GSK-3β indeed, insensitive to the TGFβ-induced inactivation, causes a significant recovery of HNF4α functionality. On the contrary, the treatment of cells with a chemical inhibitor of GSK-3β induces HNF4α modifications compatible with dephosphorylation events and the loss of its capability to bind target gene promoters. This evidence suggested the involvement of GSK-3β

kinase in the maintenance of basal phosphorylations of HNF4α proteins that are needed for its transactivation activity [Cozzolino et al., 2013].

In the last few years other members of LETF family have been suggested as possible tumor suppressor in HCC and their potential as therapeutic tool highlighted.
In particular HNF1α, which has been found downregulated in hepatocarcinoma [Lazarevich et al., 2004], has been proposed as a valid therapeutic tool. It is able to induce liver differentiation and it can also act as an anti-EMT tool, since it can transcriptionally repress EMT master genes [Santangelo et al, 2011]. Noteworthy, its tumor suppressor role has been demonstrated also *in vivo*, where it inhibits cell proliferation and tumor growth [Zeng et al, 2011].
Another promising tool is HNF6, previously found downregulated during tumor progression [Lazarevich et al, 2004] [Pekala et al., 2014]. HNF6 is involved in hepatocyte differentiation during liver development and it presents tumor suppressor properties, since it can induce p53 expression, represses EMT, interferes with TGFβ pathway, and reduces the formation of xenografts lung tumor in mice models [Yuan et al., 2013].

Taking into account all these observations, the aim of this work was to develop new molecular tools, insensitive to the presence of TGFβ in the tumor microenvironment, for the gene therapy of HCC, based on the restoration of HNF expression/activity.
On one hand, we attempted the characterization of the GSK-3β-mediated phosphorylations on HNF4α protein in order to develop HNF4α mutant proteins insensitive to TGFβ-induced inactivation. At the same time, we investigated the potential use of HNF1α and HNF6, analyzing their possible resistance to the TGFβ-induced impairment.

RESULTS

Part I – Development of HNF4α proteins insensitive to TGFβ-induced inactivation

1. HNF4α is phosphorylated by GSK-3β *in vitro*

Previous data of our laboratory showed the involvement of the kinase GSK-3β in the functional regulation of HNF4α. Its pharmacological inhibition, indeed, elicits the loss of HNF4α DNA binding on target gene promoters and modifications of its PTM profile compatible with dephosphorylation events. Moreover, a physical interaction between the two proteins has been described [Cozzolino et al., 2013]. These data suggested that GSK-3β is somehow involved in the steady-state phosphorylations of HNF4α that, in turn, are necessary to bind DNA.

To assess whether GSK-3β is directly responsible of these HNF4α phosphorylations, we produced the *in vitro*-translated (IVT) HNF4α protein that was next subjected to a non-radioactive kinase assay in the presence of a recombinant GSK-3β protein. The product of the reaction was then analyzed by means of a two-dimensional gel electrophoresis (2-DE), where proteins are first separated according to their different isoelectric point (pI) and then according to their molecular weight (MW). This technique, therefore, allows the separation and the identification of distinct spots corresponding to proteins differing even for only one phosphate group (minimal change in MW, but significant change in pI). The detection of HNF4α protein was then carried by Western Blotting with HNF4α antibody.

From the comparison of the spots obtained from samples before and after kinase assay, resulted that the single spot corresponding to IVT HNF4α (with a MW of 52 kDa and a PI of 6.8) is subjected to a shift towards the acidic end of the pH gradient in the presence of GSK-3β and shows a pattern "spot trains" (several spots with similar MW but different pI) indicative of a number of phosphorylation events (Fig 11, upper and middle panels). To confirm that the observed spot profile was due to phosphorylations, the calf intestinal

phosphatase (CIP) was added in kinase reaction. In the presence of phosphatase treatment, the spot trains are no more detectable while the only spot present migrates at the same pI and MW of the non-phosphorylated protein (Fig 11, lower panel).
These results indicate that HNF4α is a direct target of GSK-3β and that its phosphorylation, differently from other GSK-3β substrates [Doble and Woodgett, 2003], does not require "priming" phosphorylation, at least *in vitro*.

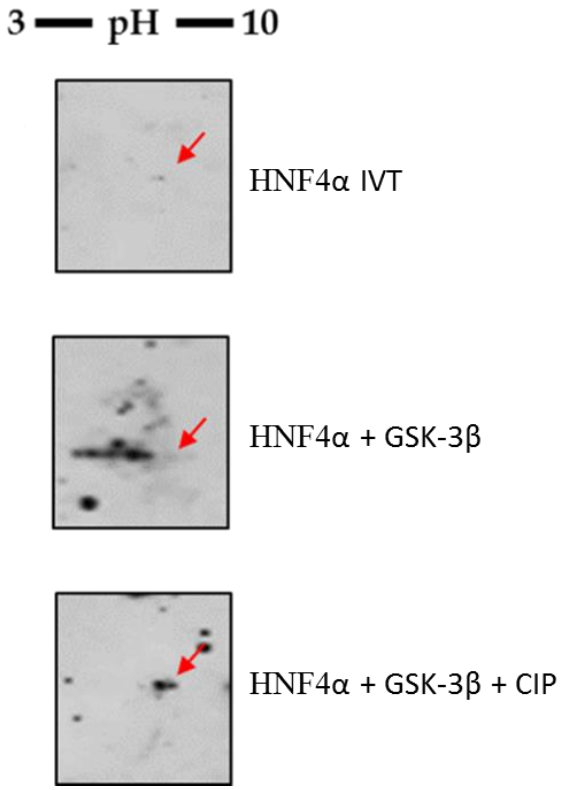

Figure 11. *Two-dimensional gel analysis of HNF4α. In vitro translated HNF4α protein was subjected to in vitro kinase assay with recombinant GSK-3β kinase in the presence of absence of alkaline phosphatase (CIP). Samples were separated on 2-DE gel followed by Western Blotting with a specific HNF4α antibody.*

2. Mapping of GSK-3β consensus motifs within HNF4α protein

Once established the direct phosphorylation *in vitro* of HNF4α by GSK-3β, we aimed to identify the aminoacidic residues involved. At this purpose, we performed a bioinformatic analysis of primary HNF4α protein sequence using two different software, GPS 3.0 and NetPhOS 3.1, both based on specific algorithms able to predict phosphorylatable residues and the putative kinases involved.

Being GSK-3β a serine/threonine kinase, we focused on these amino acids. The analysis allowed the identification of two residues, threonine 422 and serine 426 in HNF4α1 isoform (corresponding to Thr432 and Ser436 in HNF4α2 isoform), predicted as GSK-3β targets of phosphorylation with high score by both software being present within a canonical GSK-3β consensus site (S/T XXX/P S/T) (Fig 12 A and B; Fig 13; Fig 14A).
Tandem repeats of this consensus motif (SxxxSxxxSxxxSxxxS) (Fig 2C) were previously identified in the well-known GSK-3β substrates (i.e, β-catenin and Snail [Doble and Woodgett, 2003]), resulting in their processive phosphorylation [Xu C. et al., 2009]. In the primary HNF4α protein structure similar tandem repeats are also present (a.a.139-151) (Fig 12 A and B) and conserved between HNF4α isoforms from different species (human, rat, mouse and others). We focused on serine 143, recognized by GPS as possible GSK-3β target site and with high score of prediction by NetPhOS for unspecified kinases (Fig 13 and Fig 14B). Notably, Ser143 as well as Thr422 and Ser426, has been found among the steady-state phosphorylations in the native HNF4α protein in hepatocytes (as expected for putative phosphorylation by GSK-3β from our previous data) [Daigo et al., 2011] [Yokoyama et al., 2011] (Fig 15).

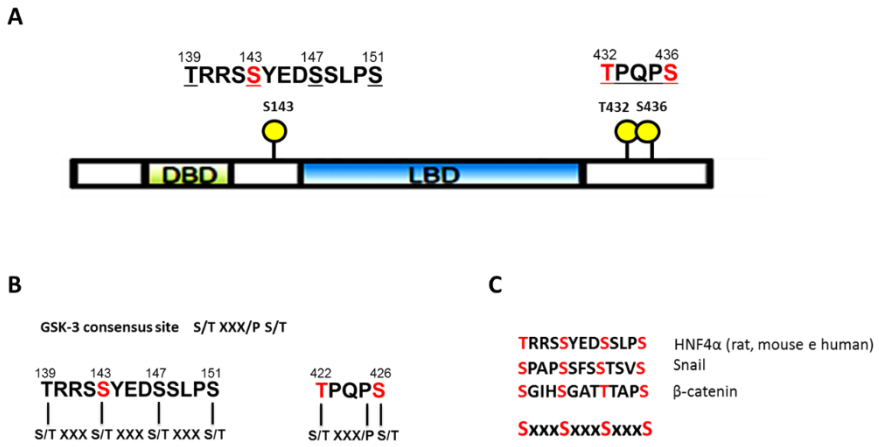

Figure 12. *(A) GSK-3 consensus sites in the native HNF4α1 protein. (B) The canonical GSK-3 consensus site in the C-terminal of HNF4α protein (right) and a stretch of hypothetical GSK-3 consensus sites at the N-terminal (left), conserved in other GSK-3 substrates (C) were shown.*

Position	Code	Kinase	Peptide	Score	Cutoff
139	T	CMGC/GSK	NERDRISTRRSSYED	2,189	1,884
143	S	CMGC/GSK	RISTRRSSYEDSSLP	2,074	1,884
422	T	CMGC/GSK	GQMSTPETPQPSPPS	9,891	1,884
426	S	CMGC/GSK	TPETPQPSPPSGSGS	2,2	1,884
422	T	CMGC/GSK/GSK-3B A	GQMSTPETPQPSPPS	10,529	4,488
426	S	CMGC/GSK/GSK-3B A	TPETPQPSPPSGSGS	9,294	4,488
422	T	CMGC/GSK/GSK-3B	GQMSTPETPQPSPPS	6,257	2,826
426	S	CMGC/GSK/GSK-3B	TPETPQPSPPSGSGS	5,762	2,826

Figure 13. *Putative GSK-3 phosphorylation sites in HNF4α primary sequence as predicted by the GPS 3.0 software with a medium threshold.*

A

Sequence	X	context	score	kinase	answer
# Sequence	422 T	STPETPQPS	0.757	unsp	YES
# Sequence	422 T	STPETPQPS	0.640	cdk5	YES
# Sequence	422 T	STPETPQPS	0.562	p38MAPK	YES
# Sequence	422 T	STPETPQPS	0.532	GSK-3β	YES
# Sequence	426 S	TPQPSPPGG	0.931	unsp	YES
# Sequence	426 S	TPQPSPPGG	0.607	cdk5	YES
# Sequence	426 S	TPQPSPPGG	0.530	cdc2	YES
# Sequence	426 S	TPQPSPPGG	0.520	GSK-3β	YES

B

Sequence	X	context	score	kinase	answer
# Sequence	138 S	RDRISTRRS	0.997	unsp	YES
# Sequence	139 T	DRISTRRSS	0.986	unsp	YES
# Sequence	142 S	STRRSSYED	0.998	unsp	YES
# Sequence	143 S	TRRSSYEDS	0.998	unsp	YES
# Sequence	147 S	SYEDSSLPS	0.520	cdc2	YES

Figure 14. *(A) Putative GSK-3 phosphorylation sites in HNF4α primary sequence as predicted by the NetPhOS 3.1 software with a medium score (>0.5). (B) Prediction of phosphorylation in the residues 139-151 of HNF4α. Of note, the residue S151 is not indicated since it presents low score (<0.5).*

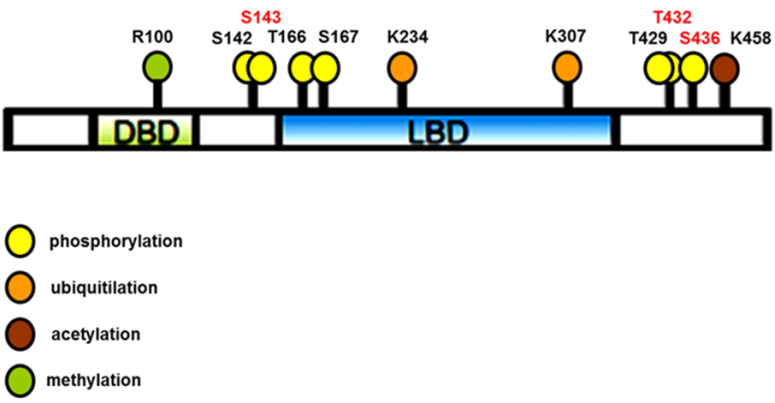

Figure 15 .*Steady-state PTMs of native HNF4α protein (α2 isoform) as described in literature and identified by proteomic studies. In red, the residues analyzed in this work.* [from Yokoyama et al., 2011]

3. Generation of phosphomimetic HNF4α mutants

To analyze the role of phosphorylation in the aminoacidic residues identified with the bioinformatics analysis, phosphomimetic mutants were created through site-directed mutagenesis.

First, we mutated Ser143, Thr422 and Ser426 into aspartic acid since this aminoacid, with the negative charge of its carboxylic group, mimics the negative charge of the phosphate group.
Once produced the single mutants in each of the putative sites (the mutants S143D, T422D and S426D), the double mutant in both T422 and S426 residues (S/T 2D) was generated. Finally, within the double-mutated HNF4α protein the mutation of serine 143 was added, obtaining the triple mutant (S/T

3D). All constructs were cloned in pcDNA3.1 vector, suitable for both *in vivo* expression and *in vitro* translation (Fig 16 A and B).

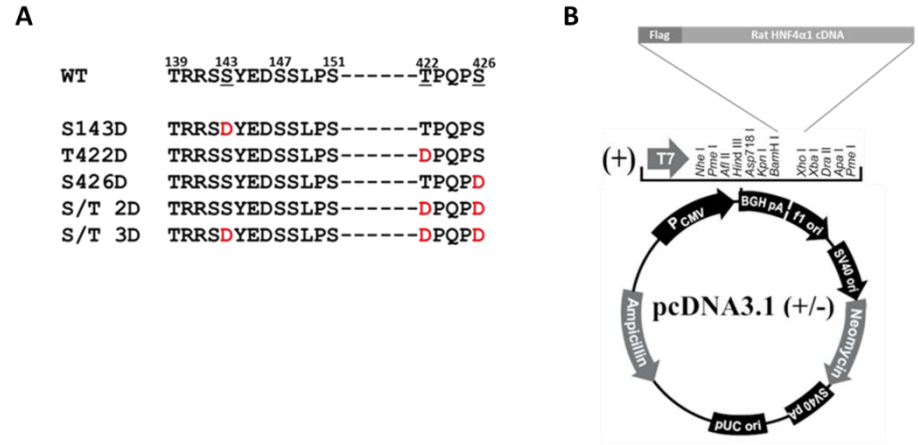

Figure 16. *Generation of phosphomimetic (Ser/Thr→Asp) HNF4α mutants in pcDNA3 expressing vector. (A) Residues of HNF4α protein, target of mutations. (B) pcDNA3.1 vector holding rat HNF4α gene under a strong promoter for in vivo gene expression (P_{CMV}) and T7 promoter for in vitro translation with T7 DNA Polimerase, utilized for site-directed mutagenesis.*

To verify that the introduced mutations did not alter the functionality of the mutant proteins, their transactivating properties were assessed in hepatocytes by means of a luciferase assay, with the luciferase gene under transcriptional control of ApoA1 and of Snail promoters (genes respectively induced and repressed by HNF4α).

Single, double and triple phosphomimetic mutants resulted all able to activate ApoA1 promoter and to repress Snail promoter without significant differences compared to the wild-type protein, demonstrating that the mutant protein's functionality is not altered in basal conditions (Fig 17 A and B).

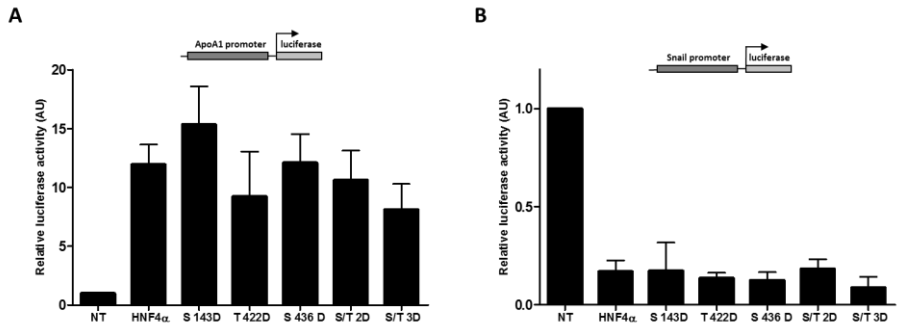

Figure 17. *Evaluation of transcriptional activity of HNF4α phosphomimetic mutants in hepatocytes. Murine hepatocytes were transiently transfected with empty vector or HNF4α wild-type and mutants expression vectors, ApoA1-luc (A) or Snail-luc promoter (B) and Renilla luciferase vector. Renilla luciferase activity was used to normalize transfections. Data were reported as mean ± SD of three independent experiments performed in duplicates. The differences in luciferase activity among HNF4α proteins are not statistically significant (p>0.05).*

4. GSK-3β phosphorylates HNF4α at Ser143, Thr422 and Ser426

The phosphorylation by GSK-3β at the identified putative sites was assessed analyzing the binding of the phosphomimetic mutants *in vitro*, with an EMSA assay, in presence of the chemical inhibitor of GSK-3β, BIO.

To this aim, nuclear extracts from untreated and BIO treated hepatocytes overexpressing wild-type or mutant HNF4α proteins were assessed by non-radioactive EMSA assay with a biotin-labeled oligo carrying the HNF4α binding sequence in the context of the ApoC3 promoter. The mobility shift of the complex protein-DNA was revealed by Western Blotting with streptavidin conjugated with the horseradish peroxidase and chemiluminescent reaction.
The inhibition of GSK-3β with BIO caused the impairment of DNA binding ability of Ser143D mutant (as the single mutations in Thr422 and Ser426D,

data not shown) and the double mutant, as well as the wild-type HNF4α, as shown in figure 18 A, B and C. Interestingly, the triple mutant maintained the DNA binding capacity in presence of BIO (Fig 18 D). This result suggests that all the three identified residues are target of GSK-3β phosphorylation and only when all of them have been made insensitive to GSK-3β inhibition, the protein rescues the binding to DNA.

5. HNF4α triple mutant maintains the ability to bind DNA in the presence of TGFβ

After proving that GSK-3β is responsible for HNF4α phosphorylation and having identified the residues involved, the resistance of phosphomimetic HNF4α mutants to TGFβ-induced inactivation was verified.
To this aim, we analyzed by EMSA assay the resistance of mutants to the impairment of DNA binding capacity in hepatocytes in the presence of TGFβ.
The HNF4α wild-type protein, as expected, showed an early (3 hours) inhibition of DNA binding ability by TGFβ (Fig 18A). The single mutation in the residue Ser143 (as well as the single mutations in Thr422 and Ser426D, data not shown) and the double mutations in Thr422/Ser426 were not sufficient to hamper the TGFβ-induced loss of DNA binding (Fig 18 B and C). The triple mutant, instead, was able to maintain its DNA binding ability in the presence of the cytokine (Fig 18 D). This result confirms the involvement of the three residues Ser143, Thr422 and Ser426 in TGFβ-induced HNF4α DNA binding inactivation.

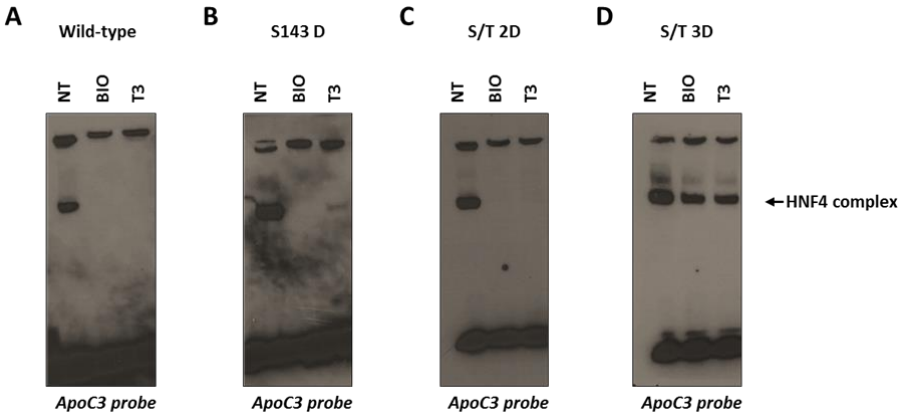

Figure 18. *Evaluation of HNF4α wild-type and mutant DNA binding activity after treatment with GSK-3 inhibitor BIO or TGFβ by EMSA. Nuclear extracts from untreated (NT), BIO-treated (for 5h) or TGFβ-treated (3h) hepatocytes transiently transfected with HNF4α wild-type and mutant were analyzed in EMSA for the binding to biotinylated probes containing the HNF4α consensus site within the ApoC3 promoter.*

Part II - Analysis of HNF1α and HNF6/OneCut1 transcriptional activity in the presence of TGFβ

In the second part of this study, in the attempt to identify new efficient tools for HCC molecular therapy, we investigated the potential tumor suppressing effect of other proteins belonging to LETF family, in the presence of TGFβ in tumor microenvironment. In particular, we focused on HNF1α and HNF6/One Cut1 since they are recently described as relevant tumor suppressor in HCC, downregulated during tumor onset and able to actively repress the EMT program in different tumors [Pelletier et al., 2011] [Santangelo et al., 2011] [Lazarevich et al., 2004] [Yuan et al., 2013] [Pekala et al., 2014]. Particularly, overexpression of HNF1α in murine model for fibrosis and

hepatocarcinogenesis highlighted the potential of this protein as therapeutic tool (Zeng et al., 2011].

6. HNF1α and HNF6 DNA binding activity is not affected by TGFβ both *in vitro* and *in vivo*

Since TGFβ was shown to inactivate HNF4α through the early impairment of its DNA binding ability, we first analyzed the *in vitro* DNA binding of HNF1α and HNF6 in the presence of TGFβ. Nuclear extracts from hepatocytes overexpressing HNF1α or HNF6 were assessed in EMSA on HNF6 consensus binding sites, embedded in HNF4α promoter and on HNF1α consensus binding sites, embedded in both HNF4α (Fig 19) and Snail promoters. As showed in figure 20 A and in figure 21 (A, C and D), the protein-DNA complexes observed for both proteins in untreated cells was maintained until 24h of TGFβ treatment, indicating that neither HNF1α nor HNF6 DNA binding ability is affected by TGFβ.
As control, in the same extracts, the binding of endogenous HNF4α on its consensus site within the ApoC3 promoter was analyzed, confirming the expected loss of HNF4α binding in presence of TGFβ (Fig 20 B and Fig 21 B).

Figure 19. *Schematic representation of HNF4α promoter. HNF1α and HNF6 binding sites on HNF4α promoter are represented by green and red boxes, respectively. Oligo used in EMSA assays are represented by black lane above the relative binding sites. Primers for HNF1α binding sites and HNF1α – HNF6 binding sites (within HNF4α promoter) used in ChIP experiments are indicated by blue arrows.*

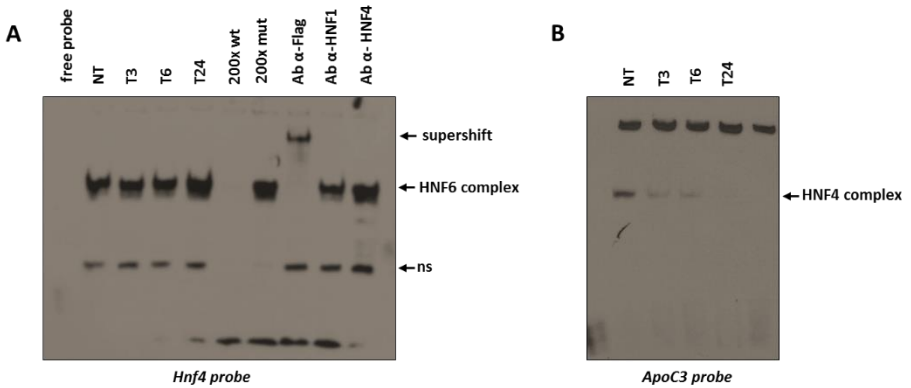

Figure 20. *Evaluation of HNF6 DNA binding activity after TGFβ treatment.(A) EMSA assays with biotinylated probes designed on the HNF6 consensus binding sites of murine HNF4α promoter. Nuclear extracts from untreated (NT) or TGFβ-treated (for 3h, 6h and 24h) HNF6 overexpressing hepatocytes were analyzed for the binding to HNF6 consensus. The specificity of binding was tested by means of wild-type (wt) and mutant (mut) competitor oligos, added in a 200-fold excess. The presence of the exogenous HNF6 protein in the complex was indicated by the band supershift obtained upon addition of anti-Flag antibody. The supershift is absent in the presence of HNF1α and HNF4α antibodies, indicating the absence of these protein in the complex. ns=non-specific band (B) In the same extracts, the HNF4α DNA binding activity to its consensus site within the ApoC3 promoter was analyzed.*

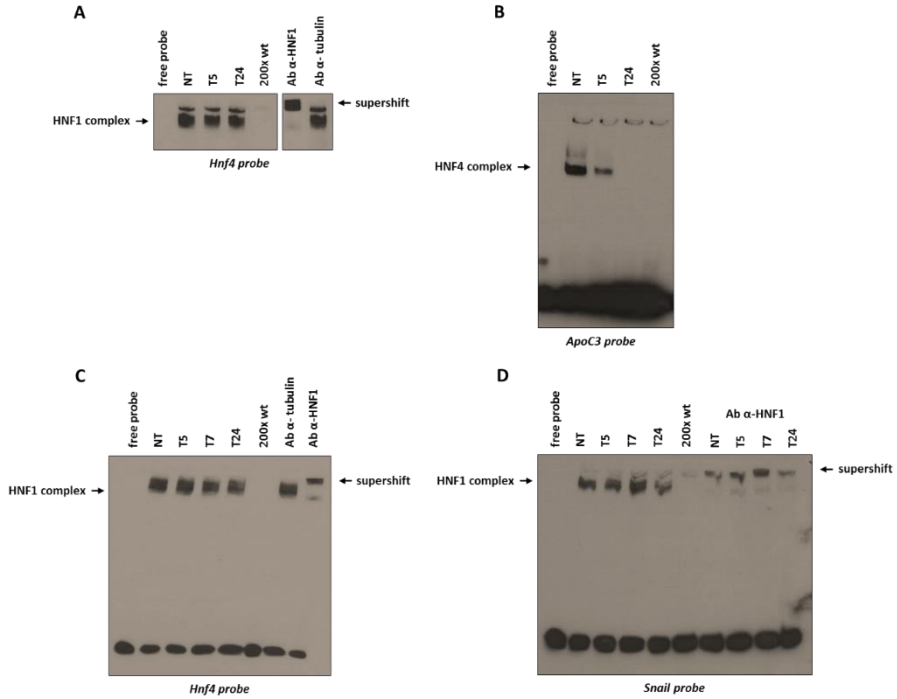

Figure 21. *Evaluation of HNF1α DNA binding activity after TGFβ treatment. (A) and (C) EMSA assays with biotinylated probes designed on the HNF1 consensus binding sites of murine HNF4α promoter and (D) Snail promoter. Nuclear extracts from untreated (NT) or TGFβ-treated (for the indicated time) HNF1α overexpressing hepatocytes were analyzed for the binding to HNF1 consensus. The specificity of binding was tested by means of wild-type competitor oligo, added in a 200-fold excess to the untreated extracts. The presence of the HNF1α protein in the complex was indicated by the band supershift obtained upon addition of anti-HNF1α antibody and the absence of the supershift with anti-tubulin antibody. (B) In the same extracts, the HNF4α DNA binding activity to its consensus site within the ApoC3 promoter was analyzed.*

Next, to analyze the binding of HNF1α and HNF6 *in vivo*, in a complex chromatin context, chromatin immunoprecipitation (ChIP) experiments have been performed.

HNF1α- or HNF6- chromatin complexes were immunoprecipitated from hepatocytes overexpressing HNF1α or HNF6, respectively, untreated and treated with TGFβ at different time points.

The HNF6 binding, analyzed by quantitative PCR with primers encompassing its binding site within *FoxA2, Transthyretin* and *Albumin* target gene promoters, remains unaltered after 3 and 6 hours of TGFβ treatment, (Fig 22 A, B and C) while a slight decrease was observed at 24 hours probably due to a lower efficiency of immunoprecipitation (Fig 22 D).

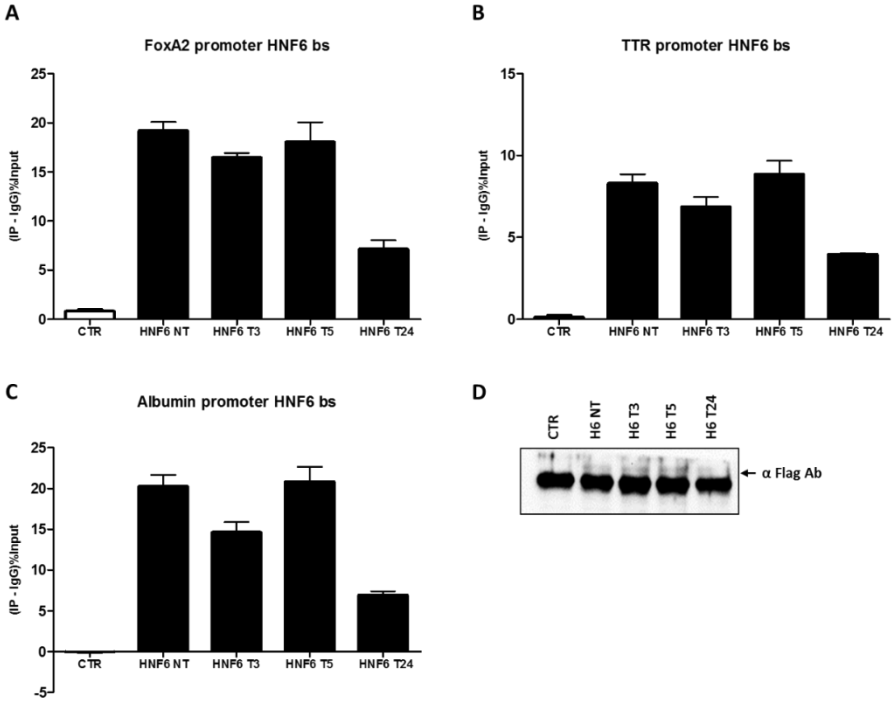

Figure 22. *<u>Analysis of in vivo HNF6 DNA binding activity by ChIP assay.</u> qPCR analysis of chromatin immunoprecipitated from parental (CTR) and HNF6 overexpressing hepatocytes with anti-HNF6 antibody. Specific HNF6 consensus regions in FoxA2, TTR and Albumin target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained in triplicate from one experiment are reported.*

Similarly, the binding of HNF1α was evaluated by qPCR, analyzing HNF1α binding sites within promoters of *Hnf4α* (Fig 23), *Pah*, *Ttr* (upregulated target genes) and of *Snail* (downregulated target gene). As showed in figure 23 the binding of HNF1α did not result impaired by TGFβ after 3 hours of treatment, accordingly to what previously observed in EMSA assays. In the same samples, as control, the endogenous HNF4α DNA binding was assessed resulting impaired by TGFβ, as expected (data not shown).

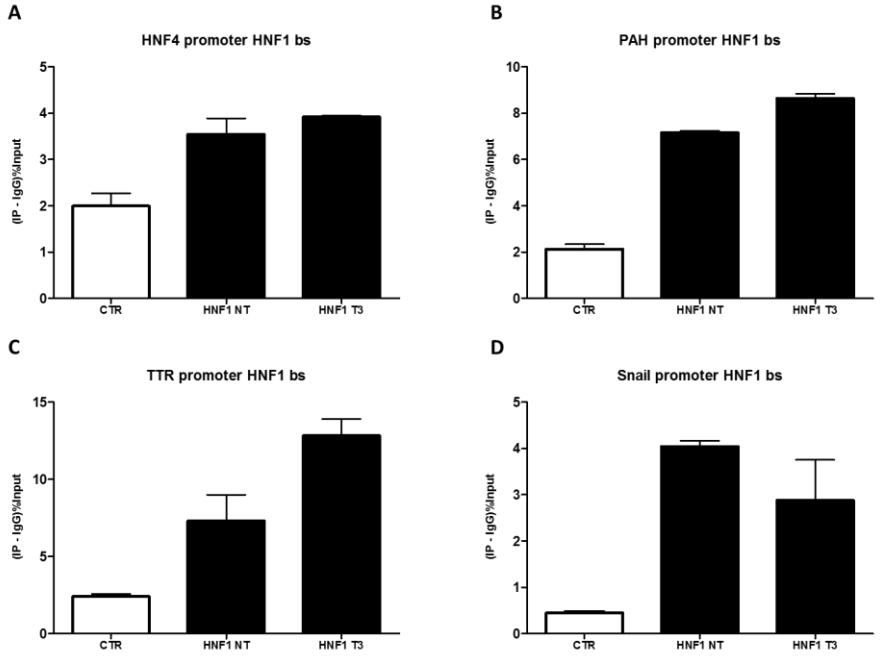

Figure 23. *<u>Analysis of in vivo HNF1α DNA binding activity by ChIP assay.</u> qPCR analysis of chromatin immunoprecipitated from parental (CTR) and HNF1α overexpressing hepatocytes with anti-HNF1α antibody. Specific HNF1α consensus regions in HNF4α (A), PAH (B), TTR (C) and Snail (D) target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained in triplicate from one of two independent experiments are reported.*

Since the endogenous HNF1α binding was also maintained after TGFβ treatment in EMSA assay (Fig 24), a second ChIP experiment was performed analyzing the binding of the endogenous protein at different time points. Notably, HNF1α maintains the binding also in presence of the GSK-3β inhibitor BIO in EMSA assay, indicating that the kinase is not involved in its DNA binding regulation. Data shown in figure 25 confirmed the maintenance of HNF1α binding to DNA in the presence of TGFβ.

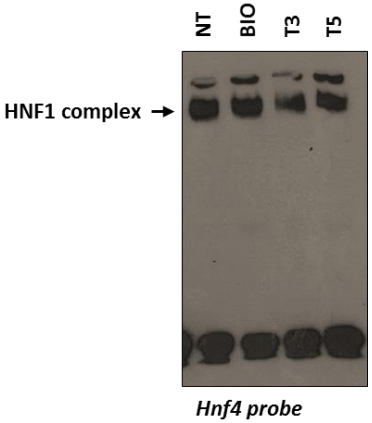

Figure 24. *Evaluation of endogenous HNF1α DNA binding activity after TGFβ treatment. Nuclear extracts from untreated (NT) or BIO (5h) TGFβ-treated (3h or 5h) hepatocytes were analyzed in EMSA for the binding to biotinylated probes containing the HNF1α consensus site within the HNF4α promoter.*

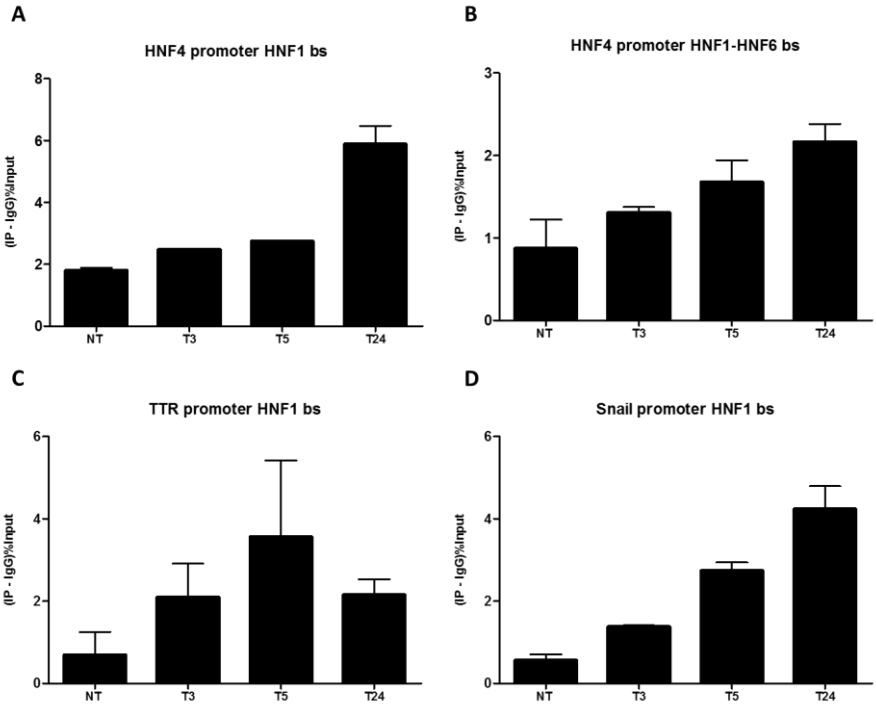

Figure 25. *Analysis of in vivo endogenous HNF1α DNA binding activity by ChIP assay.*
qPCR analysis of chromatin immunoprecipitated from hepatocytes with anti-HNF1α antibody. Specific HNF1α consensus regions in HNF4α (A and B), TTR (C) and Snail (D) target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained in triplicate from one experiment are reported.

7. TGFβ interferes *in vivo* with HNF1α and HNF6 transcriptional activity

The observation that TGFβ did not impair HNF6 and HNF1α DNA binding ability encouraged their possible use as tool for gene therapy of HCC. We therefore aimed to extend the analysis of their functionality, examining their transcriptional properties *in vivo*, in the presence of TGFβ.

To this aim, we first analyzed HNF6 target gene expression in hepatocytes overexpressing this protein and treated with the cytokine for 24 hours. RT-

qPCR analysis (showed in Figure 26) highlighted that the observed maintenance of HNF6 DNA binding did not correlate with a functional dominance *in vivo*. In fact, specific HNF6 target genes, as *Foxa2*, *Albumin*, *E-cadherin*, which were highly induced by HNF6 overexpression, were downregulated by TGFβ, indicating that TGFβ is dominant on HNF6 overexpression.

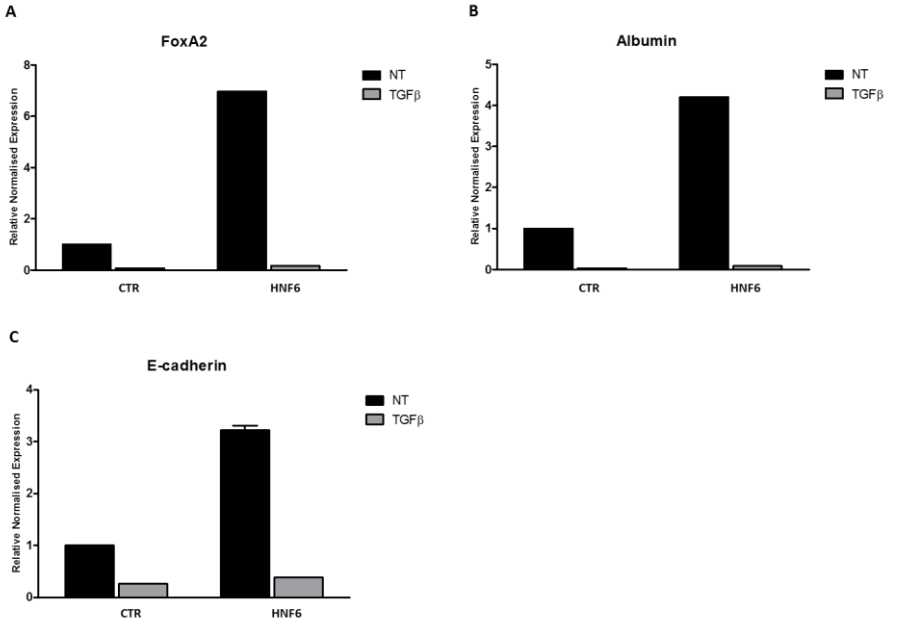

Figure 26. *<u>Gene expression analysis of HNF6 target genes in parental (CTR) and HNF6 overexpressing hepatocytes</u>**.** RT–qPCR analysis for the indicated genes after TGFβ treatment (24h). Data are expressed as relative gene expression in untreated (NT) and TGFβ-treated cells, normalized to the housekeeping gene, L32. The mean ± SD of qPCR data obtained in triplicate from one of two independent experiments are reported.*

The same analysis was carried out also in hepatocytes expressing ectopic HNF1α, treated or not with TGFβ for 24 hours. The observed induction of the epithelial/hepatocyte markers HNF4α, E-cadherin and Transthyretin in HNF1α overexpressing hepatocytes compared to parental hepatocytes was counteracted by the cytokine (Fig 27 A, B and C).

At the same time, HNF1α overexpression was no longer capable to repress Snail in presence of TGFβ, both at transcriptional and protein level (Fig 27 D and E).

Thus, while TGFβ did not impair DNA binding activity of exogenously expressed HNF1 and HNF6 proteins, it impaired their transcriptional activity on target genes.

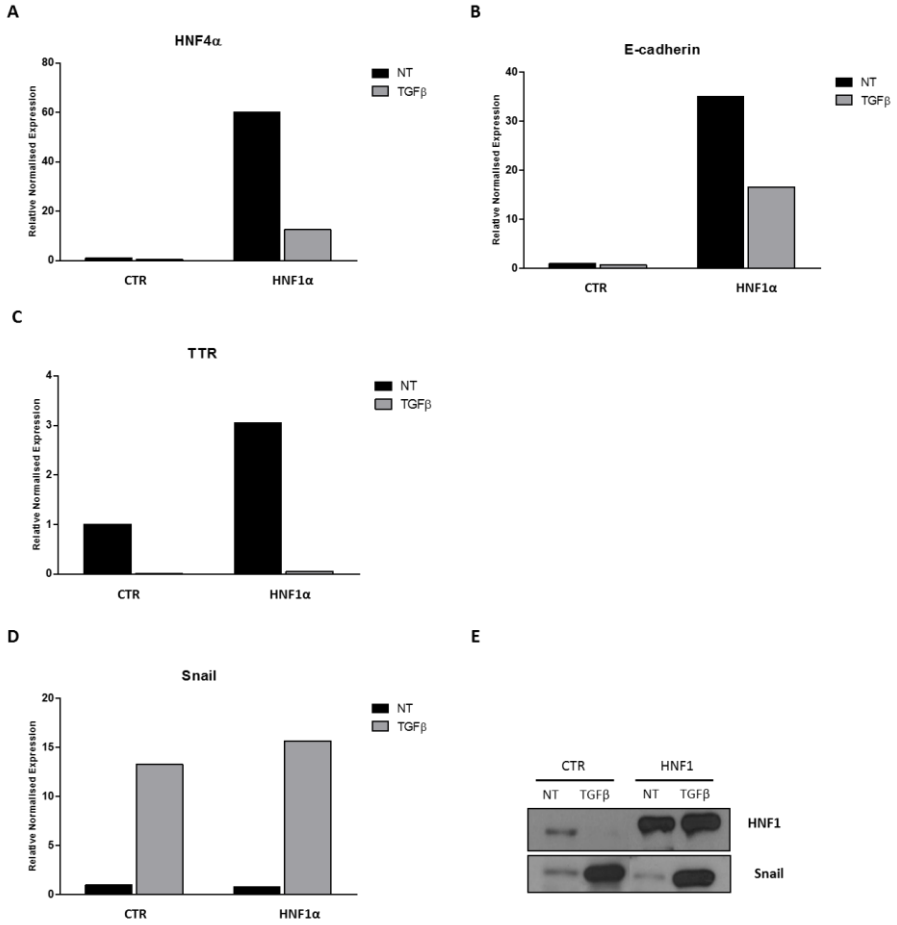

Figure 27. *Gene expression analysis of HNF1α target genes in parental (CTR) and HNF1α overexpressing hepatocytes. (A), (B) and (C) RT–qPCR analysis for the indicated genes after TGFβ treatment (24h). Data are expressed as relative gene expression in untreated (NT) and TGFβ-treated cells, normalized to the housekeeping gene, L32. The mean ± SD of qPCR data obtained in triplicate from one of two independent experiments are reported. (D) RT–qPCR analysis for Snail gene after TGFβ treatment as in (A). (E) Western Blot analysis of the indicated proteins in the same experiment.*

8. HNF1α overexpression is dominant on TGFβ in chromatin-free assay

Since both HNF1α and HNF6 proteins were found functionally inactivated by TGFβ, we decided to focalize our study on HNF1α, whose role in differentiation of adult hepatocytes and in tumor suppression is well-established and whose potential as therapeutic tool *in vivo* is more promising.

In the attempt to identify the mechanisms involved in HNF1α inactivation by TGFβ we analyzed its transcriptional activity in luciferase assays, in the presence or absence of the cytokine.
The analysis of HNF1α transactivation ability revealed that HNF1α is still able to repress the expression of luciferase reporter under the control of Snail promoter in the presence of TGFβ, resulting dominant on the cytokine (Fig 28).

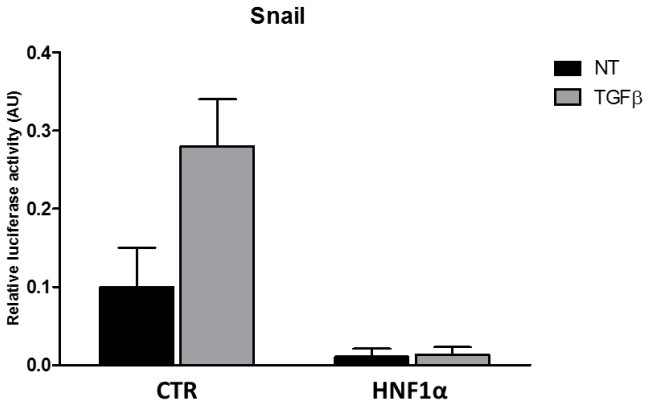

Figure 28. *Evaluation of transcriptional activity of HNF1α in hepatocytes upon TGFβ treatment. Murine hepatocytes were transiently transfected with empty vector (CTR) or HNF1α expression vectors, Snail-luc promoter and Renilla luciferase vector. Renilla luciferase activity was used to normalize transfections. Data were reported as mean ± SD of two independent experiments performed in duplicates.*

Since these data, showing the resistance of HNF1α to the TGFβ-induced inactivation, are obtained in a chromatin-free assay, they suggested that a chromatin remodeling induced by TGFβ could interfere with HNF1α activity *in vivo.*

9. A dynamic epigenetic remodeling of HNF1α target gene promoters was induced by TGFβ treatment

The differences observed in the reciprocal dominance among HNF1α and TGFβ between chromatin-free and *in vivo* analyses, in a native chromatin context, suggested the involvement of TGFβ-induced epigenetic regulations in the functional inactivation of HNF1α.
To deeper characterize the possible mechanisms responsible for the observed TGFβ dominance on HNF1α function *in vivo*, we first investigated the dynamics of chromatin remodeling at level of HNF1α binding sites.
In particular, we analyzed histone PTMs at early time points of TGFβ treatment that could interfere with the transcriptional regulation (activation or repression) by HNF1α of its target promoters despite the maintenance of the binding.

In order to verify the involvement of acetylation/deacetylation events in the TGFβ-induced HNF1α inactivation, we first analyzed by Chromatin immunoprecipitation the levels of an epigenetic mark, the acetyl-histone H3, indicative of an "open" and active chromatin state and thus correlated to active gene expression. ChIP assays for acetyl-histone H3 was performed and the HNF1α binding sites within HNF4α, TTR and Snail promoters were analyzed in control and HNF1α-overexpressing hepatocytes, in the presence or absence of TGFβ (3 hours of treatment). As shown in figure 29, the acetylation of histone H3 was found at higher levels in HNF1α overexpressing cells with respect to parental cells; interestingly, these levels are reduced in presence of TGFβ. These data suggest a role of HNF1α in driving histone acetyl transferases (HATs) on activated target genes and highlight an early

mechanism induced by TGFβ that could involve the displacement of histone acetyl transferases or the recruitment of histone de-acetylases (HDACs).

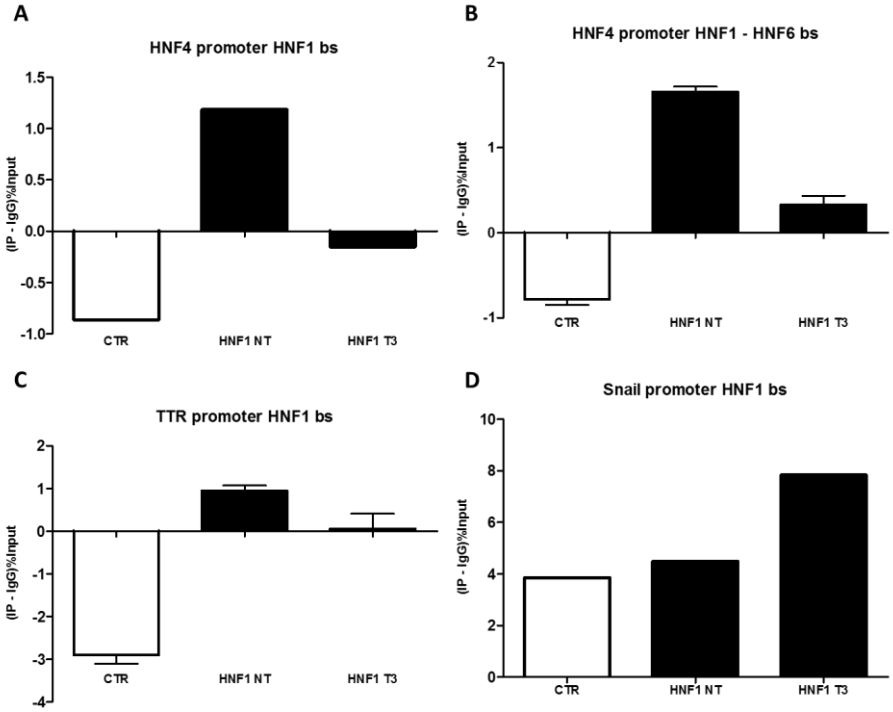

Figure 29. *<u>qPCR analysis of acetyl histone H3 by ChIP assay.</u> qPCR analysis of chromatin immunoprecipitated from parental (CTR) and HNF1α overexpressing hepatocytes with anti-acetyl H3 antibody. Specific HNF1α consensus regions in HNF4α, TTR, PAH and Snail target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained in triplicate from one experimetns are reported.*

Instead, the analysis of the histone acetylation levels on the HNF1α binding site on Snail promoter revealed no differences between the control and the

HNF1α overexpressing hepatocytes (Fig 29 D), suggesting that HNF1α did not alter the acetylation on this promoter in basal conditions. Besides, TGFβ is able to increase the acetylation in this site, according to the strong increase of Snail expression observed at that time, achievable also in the presence of HNF1α binding.

This analysis was extended to endogenous HNF1α binding in hepatocytes untreated and TGFβ-treated for 3 hours, confirming that the reduction of acetylation is already apparent at 3 hours of treatment in HNF1α binding sites within HNF4α and TTR promoters (Fig 30).

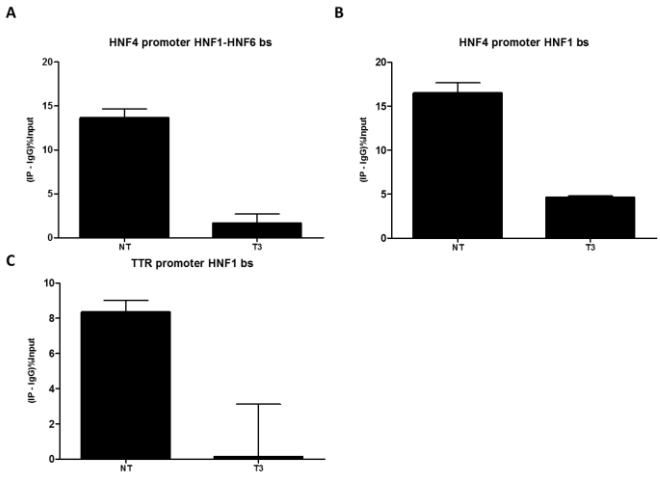

Figure 30. *<u>qPCR analysis of acetyl histone H3 by ChIP assay in untreated and TGFβ-treated hepatocytes</u>**.** Specific HNF1α consensus regions in HNF4α (A and B) and TTR (C) target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained from a single experiments are reported.*

Next, we analyzed on the same experiment the levels of di-methylated Lysine 4 on histone H3 (H3K4me2) that is another marker of open chromatin state. Chromatin immunoprecipitation of H3K4me2 revealed that TGFβ induces its

reduction on HNF4α and TTR promoter with a timing following the reduction of acetylation in the same sites (Fig 31). Instead, no difference was revealed by Chip experiment concerning the chromatin repressive mark tri-methylated Lysine 27 on histone H3 (H3K27me3), following TGFβ treatment (Fig 32).

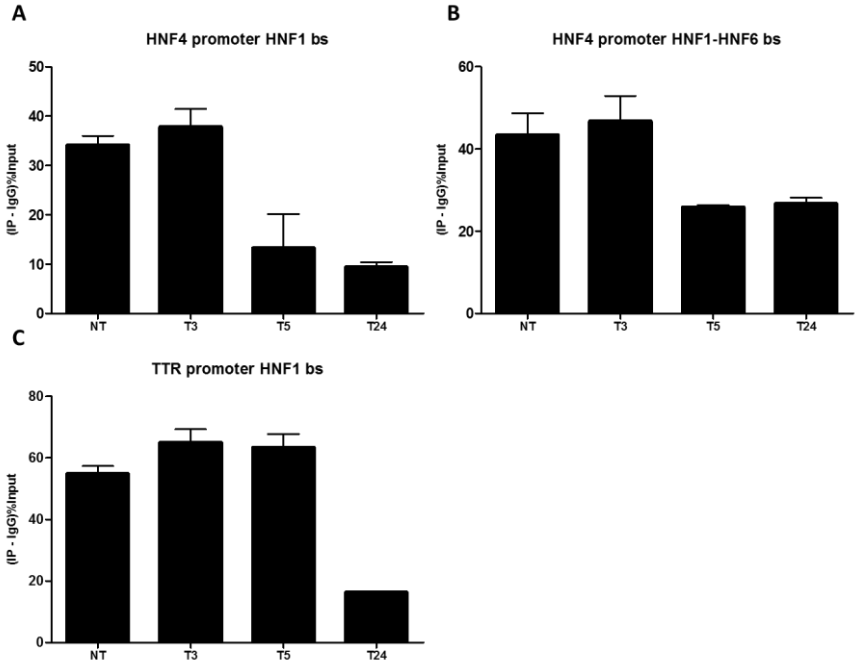

Figure 31. *qPCR analysis of di-methylated lysine 4 of histone H3 (H3K4me2) by ChIP assay in untreated and TGFβ-treated hepatocytes. Specific HNF1α consensus regions in HNF4α and TTR target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained from a single experiments are reported.*

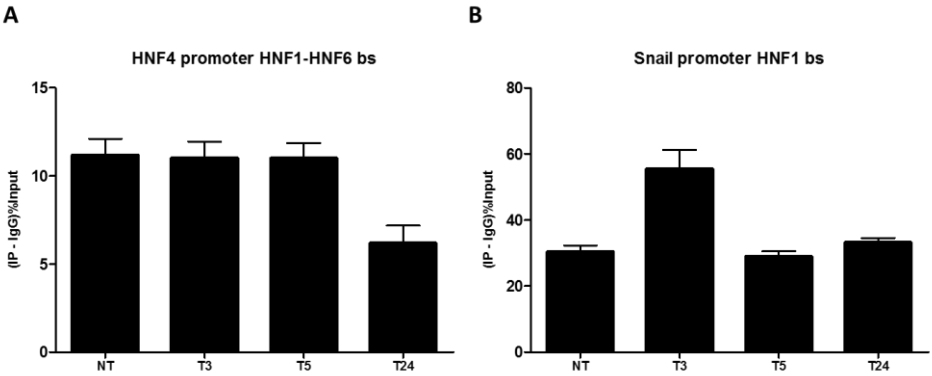

Figure 32. *qPCR analysis of tri-methylated lysine 27 of histone H3 (H3K27me3) by ChIP assay in untreated and TGFβ-treated hepatocytes. Specific HNF1α consensus regions in HNF4α and Snail target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained from a single experiments are reported.*

Altogether, these results indicate the TGFβ induced a dynamic epigenetic remodeling of HNF1α target gene promoters at level of its binding sites.

10. TGFβ-induced HNF1α transcriptional inactivation correlates with the early displacement of CBP/p300 acetyl transferase from its specific binding sites

Since among the earlier epigenetic modification induced by TGFβ we found there was the loss of histone acetylation, we investigated whether TGFβ could interfere with the recruitment of histone acetyl transferase on the HNF1α binding sites. It has been shown that HNF1α interacts with the histone acetyl transferases CBP/p300 [Ban 2002, Dohda 2004] in the activation of target genes. Thus, we analyzed by ChIP the CBP occupancy on HNF1α binding sites in the presence of TGFβ. Our results showed the presence of CBP/p300

in the untreated sample and the displacement of this protein starting at 3 hours of TGFβ treatment (Fig 33).

This result can account for the observed epigenetic modification and, ultimately, for the transcriptional inactivation of HNF1α target genes despite the maintenance of its DNA binding.

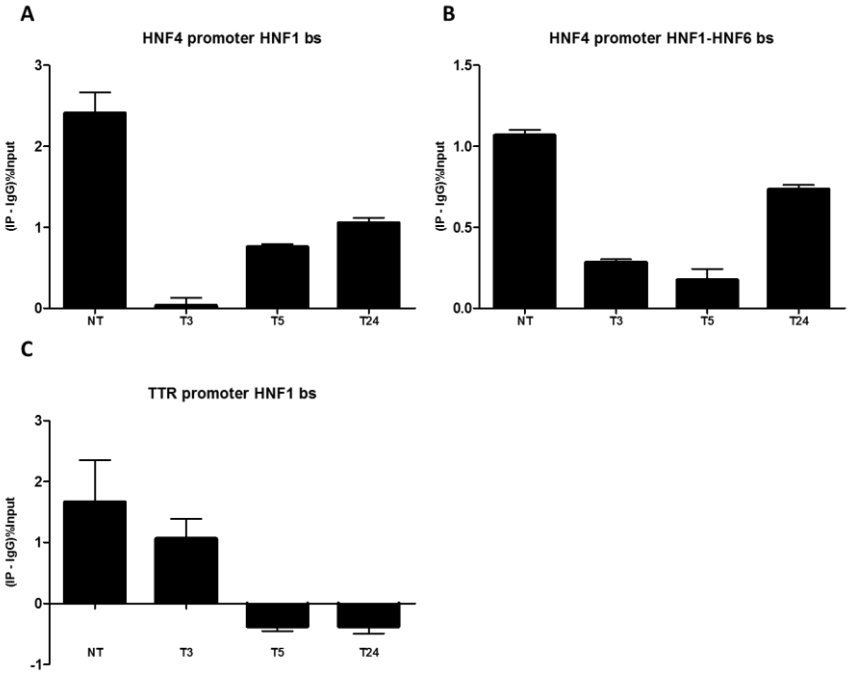

Figure 33. *Analysis of in vivo CBP/p300 DNA binding activity by ChIP assay. qPCR analysis of chromatin immunoprecipitated from hepatocytes with anti-CBP/p300 antibody. Specific HNF1α consensus regions in HNF4α and TTR target gene promoters were analyzed. Data are both normalized to total chromatin input and background (control immunoprecipitation with immunoglobulin G) and expressed as (Ip-IgG)%Input. Means ± SD of qPCR data obtained in one single experiment are reported.*

11. TGFβ reduces the interaction of HNF1α with CBP/p300 acetyl transferase

The observed displacement of CBP/p300 from HNF1α target gene promoters, observed in ChIP experiment, prompted us to investigate the protein-protein interaction between HNF1α and CBP/p300 in the presence of TGFβ. To explore this hypothesis, we expressed exogenous HNF1α in hepatocytes. The cells were lysed 48 hours after transfection and the cell lysates were immunoprecipitated with the rabbit monoclonal anti-HNF1α antibody. The immunoprecipitates were then analyzed by Western blotting with the rabbit anti-CBP/p300 antibody. As shown in figure 34, the anti-HNF1α antibody precipitates the acetyl transferase in untreated hepatocytes, confirming the *in vivo* interaction between the two proteins. However, starting from 3 hours of TGFβ treatment this interaction was reduced. This result suggests the loss of interaction with the CBP/p300 as the mechanism involved in HNF1α inactivation by TGFβ on positive target genes.

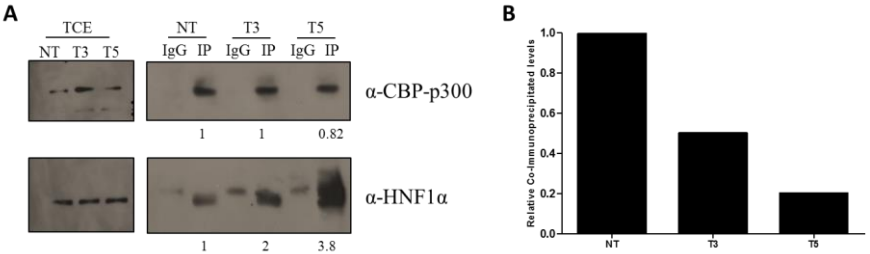

Figure 34. *<u>In vivo co-immunoprecipitation of HNF1α with CBP/p300 in hepatocytes.</u> (A) HNF1α^{Myc} were transiently transfected in hepatocytes untreated or treated with TGFβ (for 3h and 5h). Cells were lysed at 48h after transfection, immunoprecipitated with anti-HNF1α antibody (IP) and then analyzed for Western Blotting with anti-CBP/p300 antibody. The control immunoprecipitation was performed with normal rabbit antiserum(IgG). Densitometric analysis of gels was shown. (B) Levels of immunoprecipitated CBP/p300 protein normalized on immunoprecipitated HNF1α levels..*

To explore whether this reduced interaction was caused by PTMs in HNF1α protein that could influence its ability to bind to other proteins, we performed a two-dimensional gel electrophoresis with nuclear extracts from untreated and TGFβ-treated hepatocytes.

A preliminary experiment confirmed that TGFβ induces some PTMs on HNF1α protein, which may account for altered interactions with co-factors and, in particular, with CBP/p300. Further analysis will allow to identify these PTMs and define their role in the regulation of HNF1α transcriptional activity (Fig 35).

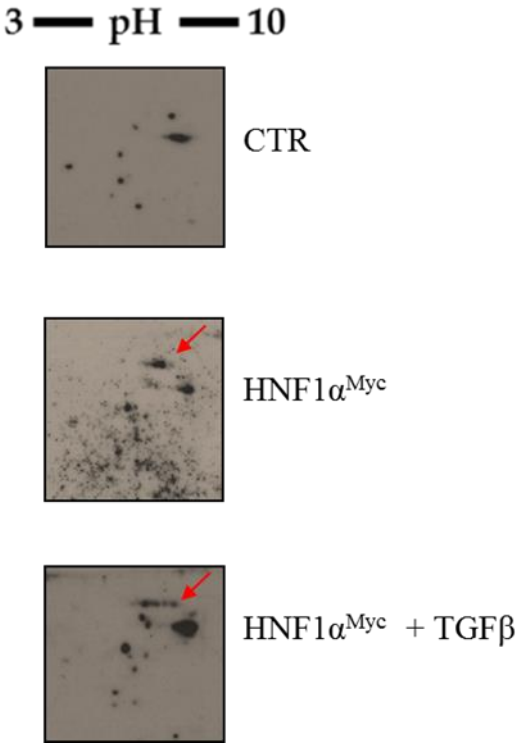

Figure 35. *Two-dimensional gel analysis of HNF1α. Nuclear extracts from control hepatocytes (A) and HNF1α^{Myc}-overexpressing hepatocytes untreated (B) or treated with TGFβ (3h). Samples were separated on 2-DE gel followed by Western Blotting with a specific α-Myc tag antibody.*

DISCUSSION

The treatment of hepatocellular carcinoma, one of the most common cancer worldwide, needs to be improved, since common therapeutic strategies are still often ineffective.

The most promising approach seems to be the targeted gene therapy based on the restoration of tumor suppressor proteins lost during tumor progression. In this context, the transcriptional factors belonging to the family of Liver Enriched Transcription Factors (LEFTs) are the most promising candidates [reviewed in Marchetti et al., 2015]. In particular HNF4α, the master regulator of epithelial/hepatocyte differentiation, is lost during neoplastic transformation and several studies indicated HNF4α re-expression as a novel therapeutic approach for HCC management and prevention. Its restoration in invasive HCC cell lines, indeed, can suppress tumor growth and progression; furthermore, the delivery of HNF4α *in vivo* prevents metastatic tumor formation and induces a significant regression of already established tumors [Ning et al., 2010]. More recently, the role as tumor suppressor of other members of LETF family, HNF1α and HNF6, has been highlighted. Their loss correlates with tumor progression and they are poorly expressed in dedifferentiated hepatomas [Lazarevich et al, 2004] or pancreatic tumors [Pekala et al., 2014]. Interestingly, HNF1α restoration in hepatoma cell lines induces cell cycle arrest and in xenograft mice models inhibits the tumor growth *in vivo* [Zeng et al., 2011]. HNF6 forced re-expression is associated with inhibition of cell cycle progression in hepatoma and colon cancer cell line [Lehner et al., 2010] and reduces migration and xenograft formation in lung cancer mice models [Yuan et al., 2013].

However, while promising, the *in vivo* experiments have limitations and the translational application is still remote. In particular, HCC model in mice, often rapidly induced by pharmacological liver damage, can hardly reproduce the tumor niche cues influencing tumor onset and progression, often established in HCC during long period of fibrosis and/or viral infection. In

human, it has been shown the presence of constitutive activation of TGFβ pathway, associated to high level of this cytokine in the blood of patients and to poor prognosis [Shirai et al., 1994] [Lee et al., 2012].
Recent data from our laboratory indicated that in similar context the effectiveness of a molecular therapy based on the restoration of HNFs function/expression, while encouraging, could be impaired. In particular, it has been shown that TGFβ overrides HNF4α tumor suppressing activity by impairing its DNA binding activity through the displacement from promoters of target genes and that the HNF4α functional inactivation correlates with its post-translational modifications (PTMs). The mechanism involved is based on the inactivation of the kinase GSK-3β by TGFβ through the Src/MEK5/ERK5 pathway [Marchetti et al., 2008], [Cozzolino et al., 2013].

The aim of this work was to develop new molecular tools, insensitive to the presence of TGFβ in the tumor microenvironment, for the molecular therapy of HCC.
On the one hand, we focused on the identification of the mechanisms involved in the TGFβ-induced inactivation through the analysis of the GSK-3β-mediated phosphorylations within HNF4α protein in order to develop HNF4α proteins insensitive to TGFβ. On the other hand, we aimed to investigate the effectiveness as tumor suppressor of other members of LETF family, specifically HNF1α and HNF6/OneCut1, in the presence of TGFβ.

Since our previous data demonstrated the requirement of GSK-3β activity for some of basal phosphorylations of HNF4α and for its DNA binding, we started to formally prove that GSK-3β can directly phosphorylate HNF4α.
We thus performed a kinase assay with the *in vitro* translated HNF4α protein and the recombinant GSK-3β kinase; the product of the reaction was analysed in a two-dimensional gel electrophoresis. This experiment evidenced the shift of the spot corresponding to HNF4α IVT toward the acidic pole of the gel, with a pattern "train of spots" compatible with phosphorylation events, as further demonstrated by the reversion of this pattern following treatment with a phosphatase.

This result demonstrated for the first time that GSK-3β is able to directly phosphorylate HNF4α, at least *in vitro*.
Since other targets of GSK-3β require a priming phosphorylation by specific kinases [Doble and Woodgett, 2003] we cannot exclude that *in vivo* a priming kinase could be needed to increase the efficiency of the reaction or the recognition between the kinase and its substrate. Further studies will be necessary to address this issue.

To identify the residues in HNF4α protein target of the GSK-3β-induced phosphorylation, we performed an *in silico* analysis of the primary sequence of the protein, utilizing two different bioinformatics software, NetPhOS 3.1 and GPS 3.0.
Two aminoacidic residues, threonine 422 and serine 426, present within the canonical GSK-3β consensus sequence, were recognized by both software as target of GSK-3β with a high score. In addition, we considered another site, Serine 143. It was predicted as GSK-3β target only by GPS and as a site with a high score of phosphorylation for unspecified kinase by NetPhOS but it is within a tandem repeat of the GSK-3β consensus sequence that is present also on other known target of GSK-3β and conserved among different species. Further, according to our working hypothesis about a role of GSK-3β - induced phosphorylation in the steady-state activity of HNF4α, all these sites have been found constitutively phosphorylated in the native protein [Daigo et al., 2011] [Yokoyama et al., 2011].

After identifying the putative residues involved in HNF4α phosphorylation by GSK-3β (based on the results of the *in silico* analysis), specific phosphomimetic HNF4α mutants have been created by site directed mutagenesis, substituting the serine or threonine with aspartic acid. In addition to single mutants, we created also the double mutant in both Thr422 and Ser426 residues (suggested as GSK-3β target by the bioinformatic analysis) and the triple mutant including substitution of Ser143.

A first characterization of these mutant proteins showed that the functionality of HNF4α protein (i.e. transactivation activity and DNA binding activity) had not been altered by the introduced mutations.
To confirm that the identified putative sites were target of GSK-3β phosphorylation, we analysed the binding of phosphomimetic mutants to DNA *in vitro*, by EMSA, in hepatocytes treated with the chemical inhibitor of GSK-3β, BIO.
We observed that, as well as the wild type HNF4α protein, the single and the double mutant loose the DNA binding ability in presence of BIO. In contrast, the binding of HNF4α-3D mutant is not altered, suggesting that all the three residues need to be phosphorylated by GSK-3β to maintain the binding to DNA.
This result confirms that the identified residues are target of GSK-3β phosphorylation, including the serine 143, not recognized by in silico analysis as GSK-3β target. To demonstrate that GSK-3β is responsible for their phosphorylation also *in vivo*, phospho-specific antibodies against the identified residues will be produced and tested in Western Blotting.

Next, we investigated the effects of TGFβ on the mutant proteins binding. The EMSA assay performed with extracts from cells treated with the cytokine and overexpressing the different mutants, indicated that only the triple mutant is able to maintain the binding to DNA. This result demonstrates the role of the three residues in the TGFβ-induced impairment of the HNF4α DNA binding ability.
Further analysis are required to deeper characterize the resistance of the triple mutant *in vivo*, to assess whether the constitutive phosphorylation of HNF4α by GSK-3β is sufficient to override TGFβ-induced inactivation and to maintain HNF4α tumor suppressive activity also in the presence of the cytokine.
In parallel, it will be interesting to characterize the non-phosphorylatable mutants in the three GSK-3β specific target residues (that have been already generated but not yet characterized), in order to study the role of these phosphorylations in the native HNF4α protein. Preliminary data indicated that,

at least in luciferase assays, the transactivation properties of these mutants are not altered. However, additional studies should be carried out to verify their functionality *in vivo*, in the presence of a complex chromatin context. Further studies about functional properties of non-phosphorylatable mutants will also be necessary to unveil the role of these phosphorylations in influencing HNF4α activity (e.g. interactions with cofactors, subcellular localization, stability or transcriptional activity in response to specific signals).

In the second part of the study, we focused on the characterization of the functionality of other proteins belonging to LETF family in presence of TGFβ. In particular, we analyzed HNF1α and HNF6 proteins, which are both found down-regulated during tumor progression, inversely correlated with EMT, and proposed as tumor suppressor of HCC [Lazarevich, 2004] [Santangelo, 2011], [Zeng 2011] [Yuan, 2013].

Since TGFβ counteracts HNF4α tumor suppressor activity impairing its DNA binding ability, we first wondered if a similar regulatory mechanism could exist also for HNF1α and HNF6. To this aim, we valued the *in vitro* DNA binding ability of these factors, through an EMSA assay. Notably, both HNF1α and HNF6 showed the maintenance of the binding to DNA in the presence of TGFβ. This result was also confirmed *in vivo*, by means of chromatin immunoprecipitation experiments. Both HNF1α and HNF6 binding was found unaltered by TGFβ treatment.

These interesting results encouraged further studies to verify the efficacy of a gene therapy strategy based on HNF1α or HNF6 restoration in hepatocarcinoma. To this aim we extended the analysis to their functionality *in vivo*, in the presence of the cytokine.
The analysis of gene expression, both in hepatocytes and hepatoma cell lines overexpressing HNF1α or HNF6 protein, showed that TGFβ overrides their transcriptional activity on target genes. However, HNF1α overexpression resulted dominant on TGFβ in a chromatin-free assay (luciferase assay), where

HNF1α was still able to repress the expression of its target Snail also in the presence of the cytokine.

The differences in the reciprocal dominance among HNF1α and TGFβ between a chromatin-free assay and a native chromatin context suggested the involvement of epigenetic regulations in the functional inactivation of HNF1α.
To deeper characterize the possible mechanisms responsible for the observed TGFβ dominance on HNF1α function *in vivo*, we first investigated the dynamics of chromatin remodeling at level of HNF1α binding sites.
In particular, we analyzed histone PTMs at early time points of TGFβ treatment that could interfere with the transcriptional regulation (activation or repression) by HNF1α of its target promoters despite the maintenance of the binding.
We first demonstrated the HNF1α-induced increase in histone 3 acetylation, indicative of an "open" and active state of chromatin, that was early lost after TGFβ treatment (3 hours) on activated promoters (HNF4α and TTR) and not observed in the repressed ones (Snail). The reduction of this epigenetic mark was correlated with the displacement of CBP/p300 histone acetyltransferase from HNF1α binding sites. Interestingly, another chromatin activation mark, the di-methylation of lysine 4 on histone H3 (H3K4me2), was found reduced in the same sites, following temporally the histone deacetylation. Instead, preliminary data showed that HNF1α transcriptional inactivation on positive targets was not associated to an increased chromatin repressive mark H3K27me3 (tri-methylated lysine 27 on histone H3).
Since a physical protein-protein interaction between HNF1α and CBP/p300 was previously shown, we have investigated, by co-immunoprecipitation experiments, the possibility that TGFβ could interfere with the formation of this complex. Our preliminary results suggest that the presence of TGFβ determines the reduction of HNF1α-p300/CBP interactions. Moreover, it is conceivable that the HNF1α PTMs, detected in a preliminary two-dimensional electrophoresis after TGFβ treatment, could be involved in this mechanism.

Furthermore, it will be interesting to analyze the involvement of lysine-specific histone demethylase 1A (LSD1) in the reduction of di-methylated lysine 4 on histone H3. H3K4me2, indeed, is the substrate of LSD1 protein, which is able to trigger a mono- or a di-demethylation, leading to a condensed chromatin state. While the link between LSD1 and HNF1α was not previously described, the hypothesis of a recruitment of LSD1 on HNF1α binding sites as a consequence of TGFβ is supported by the observation that the protein Prox1 was previously described as repressor of HNF4α transcriptional activity through the recruitment of LSD1 [Ouyang et al., 2013] and found to physically interact with HNF1α [Qin et al., 2009]. The involvement of LSD1 in HNF1α inactivation will be next analyzed.
A model of HNF1α inactivation mechanisms by TGFβ suggested by our data is depicted in figure 36.

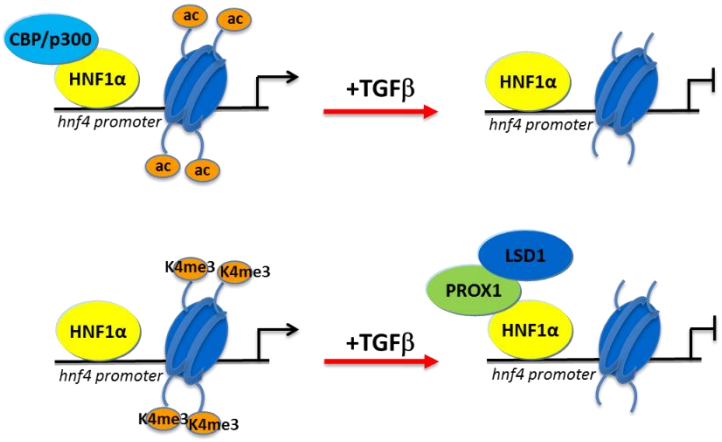

Figure 36. *Model of HNF1α inactivation by TGFβ.*

In conclusion, data described in this work identify the mechanisms involved in transcriptional inactivation of HNF proteins by TGFβ unveiling new regulatory levels and encouraging the use of our HNF4α triple mutant protein as genetic tools for the molecular therapy of HCC.

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APPENDIX

Mutant		Sequence
S143D	For	GCA CGC GGA GGT CAG ACT ACG AGG ACA GC
	Rev	GCT GTC CTC GTA GTC TGA CCT CCG CGT GC
T422D	For	GCC ACC CCT GAG GAT CCA CAG CCA TCA CC
	Rev	GGT GAT GGC TGT GGA TCC TCA GGG GTG GC
S426D	For	CCT GAG ACT CCA CAG CCA GAT CCA CCA AGT GGC TCT GG
	Rev	CCA GAG CCA CTT GGT GGA TCT GGC TGT GGA GTC TCA GG
S/T 2D	For	GCC ACC CCT GAG GAT CCA CAG CCA GAT CC
	Rev	GGA TCT GGC TGT GGA TCC TCA GGG GTG GC

Table 1: primers for site-directed mutagenesis

Primer		Sequence
mL32	For	AAG CGA AAC TGG CGG AAA C
	Rev	TAA CCG ATG TTG GGC ATC AG
mHNF4α	For	TCT TCT TTG ATC CAG ATG CC
	Rev	GGT CGT TGA TGT AAT CCT CC
mE-cadherin	For	CTA CTG TTT CTA CGG AGG AG
	Rev	CTC AAA TCA AAG TCC TGG TC
mFoxA2	For	TGA AGA TGG AAG GGC ACG AG
	Rev	CAC GGA AGA GTA GCC CTC GG
mTransthyretin	For	CCA TGA ATT CGC GGA TGT GG
	Rev	TCA ATT CTG GGG GTT GCT GA
mAlbumin	For	TTC CTG GGC ACG TTC TTG TA
	Rev	GCA GCA CTT TTC CAG AGT GG
mSnail	For	CCA CTG CAA CCG TGC TTT T
	Rev	CAC ATC CGA GTG GGT TTG G

Table 2: mouse primers for qRT-PCR

Primer		Sequence
ApoC3 HNF4α bs	For	CAGCAGGTGACCTTTGCCCAGCTCAC
	Rev	GTGAGCTGGGCAAAGGTCACCTGCTG
HNF4α HNF1α bs	For	CGGGGTGATTAACCATTAACTCCTACCCCT
	Rev	AGGGGTAGGAGTTAATGGTTAATCACCCCG
HNF4α HNF6 bs	For	TTGAGGATAGAAGTCAATGATCTGGGACG
	Rev	CGTCCCAGATCATTGACTTCTATCCTCAA
Snail HNF1αbs	For	AGGCAGAAGTTACTGATTCTTACCCCGGG
	Rev	CCCGGGGTAAGAATCAGTAACTTCTGCCT

Table 3: oligo for EMSA assay

Primer	Sequence	
FoxA2 prom HNF6 bs	For	CTC CTG AAG TCA TCC CAC AAG G
	Rev	GGT GCC CAA AGC ATT TCG TAA C
TTR prom HNF6 bs	For	TAA GGG AGA AGG CCG AGA AG
	Rev	GGA GGT GTC TTT GCT TAG CC
Albumin prom HNF6 bs	For	AAT CGT CTT TGA GGC ACC AG
	Rev	GCT CAA TCT TCC CAA ACA GG
HNF4 prom HNF1 bs	For	TAG CCA ACG CAC CTC GAC AG
	Rev	TCT CCT CCC AAG CCT CAG TT
HNF4 prom HNF1 – HNF6 bs	For	TCC GAA AGA CCC AAG TGT GG
	Rev	GCC AAT CAC GTC CCA GAT CA
Snail prom HNF1 bs	For	GGC AGA AGT TAC TGA TTC TTA CC
	Rev	GGT GTC TAT GAC TTC CTA GAG
TTR prom HNF1 bs	For	CTG GCT GTA TCT TCT CAT TGT TGC
	Rev	GGC TTT TAT ACC CCC TCC TTC C
PAH prom HNF1 bs	For	CAT TGC CAG GCC TGT CTG AGC
	Rev	GTT GCC CTG ACG TAG CAG TGG A

Table 4: mouse primers for ChIP experiments