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# Signalling cross-talk between hepatocyte nuclear factor 4α and growth-hormone-activated STAT5b

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In the present study, we have characterized signalling cross-talk between STAT5b (signal transducer and activator of transcription 5b) and HNF4α (hepatocyte nuclear factor 4α), two major regulators of sex-dependent gene expression in the liver. In a HepG2 liver cell model, HNF4α strongly inhibited β-casein and *ntcp* ($Na^+$/taurocholate cotransporting polypeptide) promoter activity stimulated by GH (growth hormone)-activated STAT5b, but had no effect on interferon-γ-stimulated STAT1 transcriptional activity. By contrast, STAT5b synergistically enhanced the transcriptional activity of HNF4α towards the *ApoCIII* (apolipoprotein CIII) promoter. The inhibitory effect of HNF4α on STAT5b transcription was associated with the inhibition of GH-stimulated STAT5b tyrosine phosphorylation and nuclear translocation. The short-chain fatty acid, butyrate, reversed STAT5b transcriptional inhibition by HNF4α, but did not reverse the inhibition of STAT5b tyrosine phosphorylation. HNF4α inhibition of STAT5b tyrosine phosphorylation was not reversed by pervanadate or by dominant-negative phosphotyrosine phosphatase 1B, suggesting that it does not result from an increase in STAT5b dephosphorylation. Rather, HNF4α blocked GH-stimulated tyrosine phosphorylation of JAK2 (Janus kinase 2), a STAT5b tyrosine kinase. Thus STAT5b and HNF4α exhibit bi-directional cross-talk that may augment HNF4α-dependent gene transcription while inhibiting STAT5b transcriptional activity via the inhibitory effects of HNF4α on JAK2 phosphorylation, which leads to inhibition of STAT5b signalling initiated by the GH receptor at the cell surface.

Key words: growth hormone (GH), hepatocyte nuclear factor 4α (HNF4α), Janus kinase 2 (JAK2), liver sexual dimorphism, signal transducer and activator of transcription 5b (STAT5b).

## INTRODUCTION

HNF4α (hepatocyte nuclear factor 4α) is a liver-enriched member of the nuclear receptor superfamily that regulates the expression of genes involved in fatty acid, cholesterol and glucose metabolism, apolipoprotein synthesis and liver development [1–3]. HNF4α regulates the expression of genes in liver, both directly and indirectly, through interaction with other HNFs, including the variant homeodomain protein HNF1α, C/EBPs (CCAAT/enhancer-binding proteins), HNF3 winged helix factors and the one-cut homeoprotein, HNF6 [4]. These HNFs are, in turn, regulated through a complex transcriptional-control hierarchy that determines the relative expression and activity of other HNF family members. Among the HNFs, HNF4α is proposed to play a central role in the regulation of liver-enriched transcription factors and their liver-specific targets, including liver *CYP* (cytochrome P450) genes [5]. The key regulatory role of HNF4α was demonstrated in a liver-HNF4α-deficient mouse model [2], in which HNF4α was shown to control the expression of HNFs *in vivo* in both a positive manner (HNF1α, C/EBPα and C/EBPβ) and a negative manner {HNF3α, HNF3β, HNF6 and the HNF4α co-activator, PGC-1α [PPARγ (peroxisome-proliferator-activated receptor γ) co-activator-1 α]} [6].

HNF4α is also a key regulator of many hepatic *CYP* genes, as demonstrated by *in vitro* promoter analyses [7–10], by expression of HNF4α antisense transcripts [11] and by characterization of mice with a liver-specific deficiency of HNF4α [2,6]. In particular, HNF4α was shown to determine the expression of a unique subset of mouse liver genes which differs markedly between the sexes. Notably, HNF4α was shown to positively regulate several male-specific genes whereas it negatively regulates certain female-predominant genes through a mechanism that is operative in male, but not female, mouse liver [6,12].

The transcription of sex-dependent liver *CYP* genes is regulated by GH (growth hormone), which is secreted into the bloodstream in a sex-dependent manner. The resultant plasma GH profiles, pulsatile in males and more continuous in females, regulate the sexually dimorphic expression of liver *CYP*s through a mechanism that is proposed to involve the GH-activated transcription factor, STAT5b (signal transducer and activator of transcription 5b) [13]. GH signalling is initiated by the binding of GH to its plasma membrane receptor, which induces activation/tyrosine phosphorylation of the GH-receptor-associated tyrosine kinase, JAK2 (Janus kinase 2). JAK2, in turn, phosphorylates a tyrosine residue on the GH receptor, followed by tyrosine phosphorylation and nuclear translocation of STAT5b [14]. This pathway for STAT5b activation is uniquely responsive to the male (pulsatile) plasma GH pattern; it results in a high level of active STAT5b in adult male, but not female, rat liver, coincident with each plasma GH pulse [15–17]. GH induces a similar sex-dependent activation of STAT5b in mouse liver [18]. The importance of this pathway is evident from the characterization of STAT5b-deficient male mice [19,20], which exhibit impaired body growth from approx. 4 weeks of age and a global loss of sex-dependent liver *CYP* expression [21].

*In vivo* studies, as well as *in vitro* promoter analyses of liver *CYP* genes, indicate that STAT5b may require collaborative interaction with other factors, in particular HNFs, to achieve the strong male *CYP* transcriptional responses observed *in vivo* [5]. Signalling cross-talk between STAT5b and HNF3β has been reported,

Abbreviations used: ApoCIII, apolipoprotein CIII; C/EBP, CCAAT/enhancer-binding protein; CYP, cytochrome P450; GH, growth hormone; HNF, hepatocyte nuclear factor; JAK, Janus kinase; PPAR, peroxisome proliferator-activated receptor; PTP, phosphotyrosine phosphatase; SOCS, suppressor of cytokine signalling; STAT5b, signal transducer and activator of transcription 5b; TC-PTP, T cell-PTP.

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whereby STAT5b inhibits HNF3$\beta$-dependent *trans*-activation of a male-specific *CYP* promoter, while HNF3$\beta$ blocks STAT5b *trans*-activation by inhibiting GH-stimulated STAT5b tyrosine phosphorylation [22]. Co-operative interaction between STAT5b and HNF4$\alpha$ leading to regulation of a female-specific *CYP* promoter has also been described [23]. Finally, both stimulatory and inhibitory cross-talk may occur between STAT5b and certain nuclear receptors [24–26].

In the present study, we have used a liver cell model to investigate signalling cross-talk between STAT5b and HNF4$\alpha$. We demonstrate that STAT5b enhances HNF4$\alpha$-dependent *trans*-activation of the *ApoCIII* (apolipoprotein CIII) promoter. By contrast, HNF4$\alpha$ is shown to inhibit GH- and STAT5b-stimulated $\beta$-casein and *ntcp* ($Na^+$/taurocholate cotransporting polypeptide) promoter activity by blocking JAK2 tyrosine phosphorylation, which leads to inhibition of STAT5b tyrosine phosphorylation and nuclear translocation. Together, these findings suggest that metabolic and other factors associated with elevated HNF4$\alpha$ activity in liver may suppress STAT5b activation and transcriptional activity, and that hormonal factors associated with a high level of STAT5b activity may lead to an increase in HNF4$\alpha$-dependent gene transcription.

## MATERIALS AND METHODS

### Antibodies

A rabbit polyclonal anti-STAT5b antibody (sc-835), raised against STAT5b residues 776–786, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). A rabbit polyclonal anti-pTyr$^{699}$-STAT5b (where pTyr is phosphorylated tyrosine) antibody, raised against a synthetic phosphotyrosine peptide (keyhole-limpet-haemocyanin-coupled) surrounding Tyr$^{699}$ of mouse STAT5b, was purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Recombinant proteins containing a V5 epitope were detected using a mouse monoclonal anti-V5 antibody (Invitrogen, Carlsbad, CA, U.S.A.). A rabbit anti-HNF4$\alpha$ antibody raised against a synthetic peptide corresponding to amino acids 445–455 of rat HNF4$\alpha$1 was obtained from Dr F. Sladek (Department of Pharmacology, University of California, Riverside, CA, U.S.A.). Rabbit polyclonal antibodies against JAK2 and pTyr$^{1007/1008}$-JAK2 were purchased from Upstate Biotech (Lake Placid, NY, U.S.A.).

### Expression and reporter plasmids

An expression plasmid encoding rat HNF4$\alpha$, corresponding to the $\alpha$1 splice variant, was obtained from Dr F. Sladek and was used in all experiments, except where human HNF4$\alpha$ was used, as has been noted. Human HNF4$\alpha$ cloned into pCDNA3 was obtained from Dr T. Leff (Department of Pathology, Wayne State University, Detroit, MI, U.S.A.). Expression plasmids for mouse STAT5b (Dr A. Mui, DNAX Corporation, Palo Alto, CA, U.S.A.), mouse STAT5b-Y699F (Dr Hallegeir Rui, Lombardi Cancer Center, Georgetown University, Washington, U.S.A.), rat GH receptor (Dr N. Billestrup, Signal Transduction, Hagedon Research Institute, Gentofe, Denmark), HNF1$\alpha$ (Dr F. Gonzalez, National Cancer Institute, Bethesda, MD, U.S.A.), PTP (phosphotyrosine phosphatase)-1B and the dominant-negative mutant PTP-1B-D181A (Dr N. Aoki, Department of Applied Biological Sciences, Nagoya University, Nagoya, Japan), and TC (T cell)-PTP and the dominant negative mutant TC-PTP-C216S (Dr T. Mustelin, Signal Transduction Laboratory, Burnham Institute, La Jolla, CA, U.S.A.) were obtained from the indicated individuals. The STAT5 reporter plasmids 4x-ntcp-Luc (Dr M. Vore, Department of Toxicology, University of Kentucky, Lexington, KY, U.S.A.) and pZZ1-Luc ($\beta$-casein promoter-Luc reporter; Dr B. Groner, Chemotherapeutisches Forschungsinstitut George-Speyer-Haus, Institute for Biomedical Research, Frankfurt, Germany), the STAT1 reporter 8x-GAS-Luc (Dr C. K. Glass, Department of Medicine, University of California, San Diego, CA, U.S.A.) and the HNF4$\alpha$ reporter ApoCIII ($-854/+22$)-Luc (Dr T. Leff) were obtained from the indicated sources. C-terminal derivatives of HNF4$\alpha$ and HNF1$\alpha$ tagged with a V5-epitope were subcloned into pcDNA3.1D/V5-His-TOPO© using the pcDNA3.1© Directional TOPO [27].

### Cell culture and transfections

Human hepatoma HepG2 cells, and African green monkey kidney COS-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% BSA, 50 units/ml penicillin and 50 $\mu$g/ml streptomycin. For transient transfection studies, HepG2 cells were seeded at $7 \times 10^4$ cells/well and COS-1 cells were seeded at $3 \times 10^4$ cells/well in a 48-well plate. FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) was used as described in the manufacturer's protocol at a FuGENE™ 6/DNA ratio of 1.3:1 (v/w). Typically, each well received a total of 300 ng of DNA, including 100 ng of luciferase reporter plasmid, 10 ng of GH receptor expression plasmid, 10 ng of STAT5b expression plasmid and various amounts of HNF4$\alpha$ expression plasmid, as indicated. The above amounts were scaled up approx. 12-fold for transfection experiments for Western blotting, which were carried out in 6-well plates. All experiments used V5-tagged rat HNF4$\alpha$ except where human HNF4$\alpha$ is specified. In some experiments, a JAK2 expression plasmid was included to increase the sensitivity for Western blot detection of pTyr$^{1007/1008}$-JAK2. For transfections involving the ApoCIII-Luc reporter, each well of a 48-well plate received a total of 350 ng of DNA, including 50 ng of ApoCIII-Luc reporter plasmid, 100 ng of the HNF4$\alpha$ expression plasmid and 25 ng of GH receptor, 100–150 ng of STAT5b and/or 100 ng of STAT5b-Y699F expression plasmid, as indicated. A *Renilla* luciferase expression plasmid pRL-tk-Luc (25 ng) was included in each sample as an internal control for transfection efficiency. After transfection for 24 h, cells were treated with rat GH (200 ng/ml; National Hormone and Peptide Program, UCLA, Torrance, CA, U.S.A.) for either 30 min (Western blotting analysis of STAT5b tyrosine phosphorylation) or for 18–24 h (luciferase reporter assays).

Promega lysis buffer (1×) (Promega, Madison, WI, U.S.A.) was used to prepare crude cell lysates for luciferase activity assays. Firefly and *Renilla* luciferase activity was measured using a Dual-Reporter Assay System (Promega) and a Monolight 2010 luminometer (Analytical Luminesence Laboratory, San Diego, CA, U.S.A.). Data shown in the individual Figures are based on normalized luciferase activity values (i.e. firefly/*Renilla* luciferase activity). Values are the means $\pm$ S.D. for three replicates from a single experiment, and are representative of at least two to three independent experiments.

### Western blotting

Cell lysates used for Western blotting were centrifuged for 30 min at 15000 ***g***, and samples (20 $\mu$g/well) were electrophoresed on 7.5% Laemmli SDS gels, electrotransferred on to a nitrocellulose membrane and then probed with an antibody against STAT5b or pTyr$^{699}$-STAT5b, as described in the manufacturer's protocol. Antibody binding was visualized on X-ray film by enhanced chemiluminescence using the ECL® kit from Amersham Biosciences. Nitrocellulose membranes were heated in stripping buffer [62.5 mM Tris/HCl (pH 7.6), 2% SDS and 50 mM 2-mercaptoethanol] for 20 min at 50 °C and then probed with a rabbit

polyclonal anti-HNF4α or mouse monoclonal anti-V5 antibody. Membranes were blocked in Solution I (0.3 % Tween-20 in 1 × PBS) containing 1 % BSA and 1 % non-fat dried milk for 1 h at 37 °C and then incubated overnight with an anti-HNF4α antibody (1:2000 dilution in blocking solution) at 4 °C. Membranes probed with other antibodies were blocked for 1 h at 37 °C in blocking solution containing 5 % non-fat dried milk, then incubated overnight at 4 °C with anti-V5 (1:5000), anti-JAK2 (1:1000) or anti-pTyr$^{1007/1008}$-JAK2 (1:1000) antibodies diluted in blocking solution.

### Immunofluorescence studies

Growth and passage of CWSV-1 cells was carried out as described [28]. Confocal immunofluorescence analysis of STAT5b and pTyr$^{699}$-STAT5b, and the visualization of GH-stimulated STAT5b nuclear translocation were carried out as described [29], with the following modifications. Methanol-fixed cells were blocked with 3 % charcoal-stripped BSA in PBS for 1 h at room temperature then incubated overnight at 4 °C in blocking solution containing an anti-STAT5b antibody (1:500 dilution). For anti-pTyr$^{699}$-STAT5b immunostaining, methanol-fixed cells were blocked with 5.5 % charcoal-stripped BSA in TBST [50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.1 % Triton X-100] for 1 h at room temperature and then incubated for 24 h in a humidified environment at 4 °C with the anti-pTyr$^{699}$-STAT5b antibody (1:500 dilution) in TBS [50 mM Tris/HCl (pH 7.4) and 150 mM NaCl] containing 3 % BSA. The samples were then washed three times with PBS containing 3 % BSA (for anti-STAT5b antibody) or with TBST (for anti-pTyr$^{699}$-STAT5b antibody) (5 min/wash). For V5 immunostaining, fixed cells were blocked for 30 min at room temperature with 1 % BSA in PBS (blocking buffer) and incubated with an anti-V5 antibody (diluted in 1:200 in blocking buffer) either for 1 h at room temperature or overnight at 4 °C. Cells were then incubated for 1 h at 37 °C with an FITC-conjugated goat anti-(rabbit IgG) antibody(1 μg/ml; Molecular Probes, Eugene, OR, U.S.A.). Cells were counterstained with 10 ng/ml propidium iodide (Sigma) to localize nuclei.

## RESULTS

### HNF4α inhibition of STAT5b tyrosine phosphorylation

HNF4α is required for the expression of certain sex-specific GH-regulated liver *CYP* genes, several of which also require STAT5b [6,12]. We therefore investigated the possibility of signalling cross-talk between these two liver-expressed transcription factors. GH-stimulated STAT5b signalling was reconstituted in HepG2 cells by transfection with the GH receptor and STAT5b, together with the STAT5 reporter plasmid 4x-ntcp-Luc, which contains four copies of a STAT5-response element derived from the GH-responsive rat *ntcp* gene. Co-transfection of HNF4α resulted in a dose-dependent inhibition of GH-stimulated reporter activity (Figure 1A, left). The inhibitory effect of HNF4α was verified using a second STAT5 reporter, pZZ1-Luc, which contains a 345 nt fragment of the prolactin-responsive β-casein gene and includes a pair of STAT5 sites in their natural promoter context (Figure 1B). The specificity of the HNF4α inhibitory response was apparent from the absence of STAT5b inhibition in cells transfected with HNF1α in place of HNF4α (Figure 1A, right) and from the fact that HNF4α did not inhibit STAT1 transcriptional activity in cells stimulated with interferon-γ (Figure 1C). More potent inhibition of STAT5b transcriptional activity was observed using a different expression plasmid, which codes for human HNF4α (Figure 1D) and gave a several fold higher level of HNF4α protein expression in the transfected cell lysates compared with the rat HNF4α expression plasmid used in Figure 1(A) (results not shown).

Western blot analysis was carried out to ascertain whether HNF4α affects STAT5b tyrosine phosphorylation, which is stimulated by GH treatment and is required for STAT5b transcriptional activity. Figure 1(E) demonstrates that HNF4α inhibited the GH-stimulated phosphorylation of STAT5b on Tyr$^{699}$, as indicated by the substantial decrease in pTyr$^{699}$-STAT5b immunoreactivity (lane 6 versus lane 4) and by the decrease in the ratio of tyrosine phosphorylated STAT5b to unphosphorylated STAT5b, detected with an anti-STAT5b antibody. This inhibitory effect on STAT5b tyrosine phosphorylation was dose-dependent, as shown using human HNF4α (Figure 1E, lanes 10 and 12 versus lane 8).

### Stimulatory effect of STAT5b on the HNF4α-dependent *ApoCIII* promoter

We next investigated whether the inhibitory effect of HNF4α on STAT5b transcription is mutual, as reported for STAT5b and another nuclear receptor, PPAR [30]. To examine the effect of STAT5b on HNF4α-dependent transcription, we used a reporter gene based on *ApoCIII*, a well-established HNF4α target gene. ApoCIII-Luc reporter activity was stimulated by HNF4α, both in HepG2 cells (an approx. 3–4-fold increase) and in COS-1 cells (an approx. 14–16-fold increase) (Figure 2A). Although STAT5b alone had little or no effect on *ApoCIII* promoter activity, HNF4α-stimulated *ApoCIII* activity was increased approx. 4-fold further upon co-transfection with STAT5b in HepG2 cells and in COS-1 cells. STAT5b had no effect on the expression of HNF4α protein under the conditions of these experiments (results not shown). Moreover, in the case of HepG2 cells, *ApoCIII* promoter activity was further enhanced by GH treatment. The enhanced activity of HNF4α by STAT5b was markedly decreased when STAT5b was replaced with STAT5b-Y699F (Figure 2B), which is defective in DNA binding and transcriptional activity owing to mutation of the tyrosine phosphorylation site to phenylalanine.

### Effect of a short-chain fatty acid on HNF4α inhibitory effects

Structural studies have revealed that fatty acids serve as endogenous ligands of HNF4α [31,32]. Moreover, short-chain fatty acids, such as butyrate, can enhance GH-induced STAT5b activity [33]. We therefore investigated whether the inhibitory effect of HNF4α on STAT5b activation (Figure 1) is influenced by butyrate. Figure 3(A) shows that butyrate treatment of HepG2 cells induced a modest dose-dependent increase in STAT5b transcriptional activity. In cells expressing HNF4α, the magnitude of the stimulatory effect from butyrate was increased more substantially, such that the inhibitory effect of HNF4α on STAT5b-dependent transcription was abolished at 1000 μM butyrate. Western blot analysis showed, however, that butyrate did not reverse the inhibition of STAT5b tyrosine phosphorylation by HNF4α (Figure 3B, lane 8 versus lane 6). Thus butyrate renders STAT5b hyperactive, by a mechanism that does not alter STAT5b tyrosine phosphorylation or reverse the inhibitory effect of HNF4α.

### Impact of HNF4α on STAT5b nuclear translocation

GH-activated STAT5b translocates from the cytosol to the nucleus, where it binds to STAT5 response elements and *trans*-activates GH-responsive target genes. We therefore investigated whether inhibition of STAT5b transcriptional activity by HNF4α is associated with the inhibition of STAT5b nuclear translocation. GH-stimulated STAT5b nuclear translocation was visualized in HepG2 cells by confocal immunofluorescence microscopy

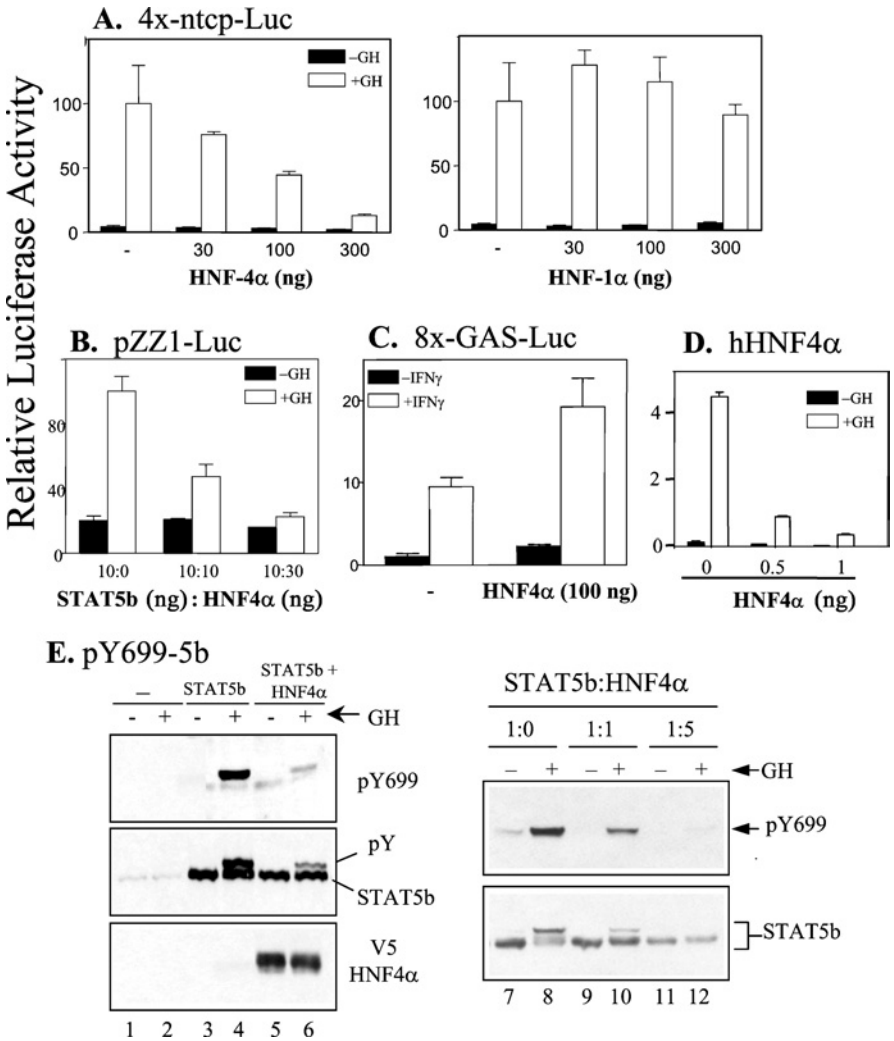


**Figure 1 HNF4α inhibits GH-stimulated STAT5b reporter activity and STAT5b tyrosine phosphorylation**

HepG2 cells were transfected with STAT5 reporter plasmid 4x-ntcp-Luc (**A**) or pZZ1-Luc (**B**) together with plasmids encoding the GH receptor (10 ng) and STAT5b (10 ng) and the indicated amount of each V5-tagged HNF expression plasmid. Cells were treated with GH, and luciferase reporter activity was assayed as described in the Materials and methods section. (**C**) HepG2 cells were transfected with the STAT1 reporter plasmid, 8x-GAS-Luc, in the presence or absence of HNF4α, as indicated, and then treated overnight with interferon (IFN)-γ. Reporter activity reflects interferon-γ activation of endogenous STAT1, via the endogenous interferon-γ receptor. (**D**) Effect of human HNF4α on GH-stimulated 4x-ntcp reporter activity, assayed as in (**A**). The HNF4α to STAT5b expression plasmid ratio was 0, 0.5:1 or 1:1 (w/w), as indicated. Data shown are firefly luciferase activities normalized to *Renilla* luciferase activity (internal standard), means ± S.D. values for three replicates, with the STAT5b reporter activity in the absence of HNF set to 100 in (**A**) and (**B**). (**E**) HNF4α inhibition of STAT5b tyrosine phosphorylation detected on a Western blot of extracts prepared from HepG2 cells co-transfected with 10 ng of the GH receptor, 20 ng of STAT5b and 60 ng of V5-tagged HNF4α expression plasmid after a 30 min stimulation with GH (lanes 1–6). Lanes 7–12 show the effect of human HNF4α on GH-stimulated STAT5b tyrosine phosphorylation, at the indicated STAT5b/HNF4α expression plasmid ratio (w/w). Blots were sequentially probed with each of the indicated antibodies; $pTyr^{699}$ is an anti-$pTyr^{699}$-STAT5b antibody. The anti-STAT5b antibody detects an upper tyrosine-phosphorylated STAT5b band (marked pY, lane 6) and a lower non-pTyr-STAT5b band, as characterized previously [28,29].

(Figure 4A, lane 2 versus lane 1). HNF4α was constitutively expressed in the nucleus, independent of GH treatment (lane 4 versus lane 3). Moreover, HNF4α blocked STAT5b nuclear translocation in GH-treated cells (lane 4 versus lane 2). These findings were confirmed using the GH-responsive liver cell line, CWSV-1, in which the inhibitory effect of HNF4α could be evaluated under conditions where GH receptor and STAT5b are produced endogenously and are not overexpressed. GH induced tyrosine phosphorylation and nuclear translocation of STAT5b, as revealed by the appearance of immunoreactive $pTyr^{699}$-STAT5b in the nucleus (Figure 4B, top, lane 6 versus lane 5). Transfection of HNF4α resulted in the expression of HNF4α protein in a subset of the cells (Figure 4B; lane 6, bottom), all of which were devoid of nuclear $pTyr^{699}$-STAT5b, as revealed by an overlay of the HNF4α immunofluorescence signal with that of $pTyr^{699}$-STAT5b (lane 6, top). Thus HNF4α inhibits the endogenous CWSV-1 cell pathway of STAT5b tyrosine phosphorylation and nuclear translocation.

### Effect of HNF4α on the time course of STAT5b phosphorylation and dephosphorylation

The inhibitory effect of HNF4α on STAT5b tyrosine phosphorylation could result from a decrease in the rate, and/or the maximal extent of STAT5b tyrosine phosphorylation. Alternatively, HNF4α could activate (or induce) tyrosine phosphatases that down-regulate GH signalling to STAT5b by catalysing dephosphorylation of the GH receptor, JAK2 or STAT5b itself. To address these questions, we first investigated the impact of HNF4α on the kinetics of STAT5b tyrosine phosphorylation in GH-stimulated HepG2 cells. STAT5b tyrosine phosphoryation was

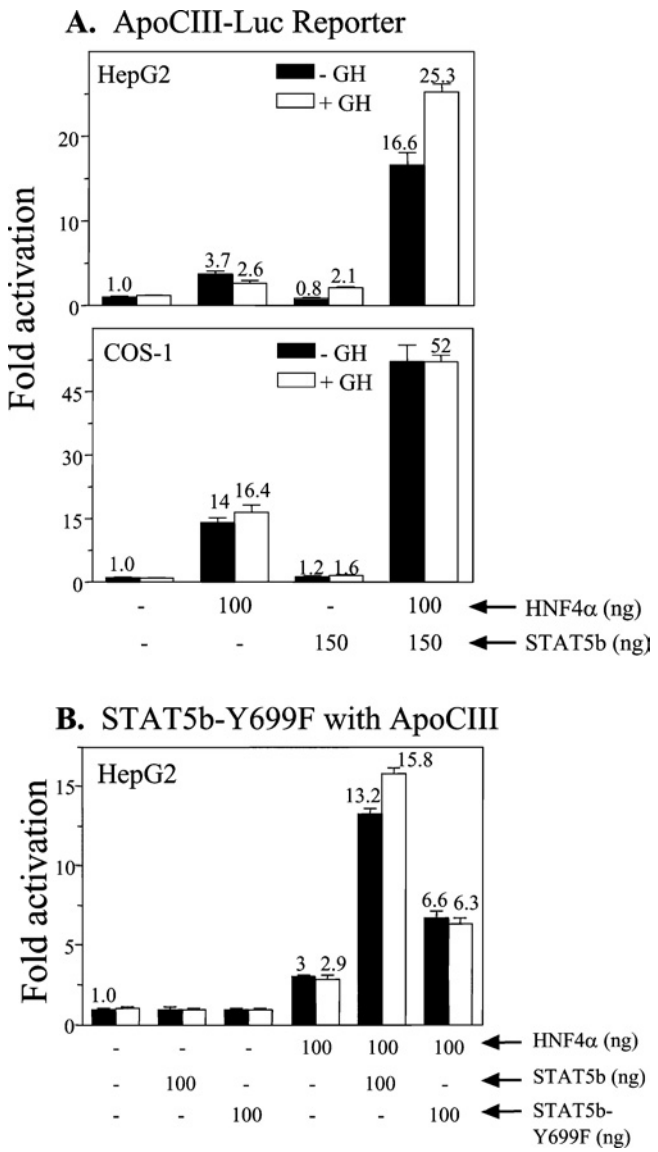


**Figure 2 STAT5b stimulation of the HNF4α-responsive *ApoCIII* promoter**

HepG2 cells (**A**, top panel) and (**B**) and COS-1 cells (**A**, bottom panel) were transfected with the *ApoCIII*-Luc reporter plasmid in the presence of HNF4α and/or STAT5b expression plasmids, individually or in combination as indicated at the bottom of each panel, together with the GH receptor expression plasmid. After transfection (24 h), the cells were treated with 200 ng/ml GH for 16 h or were left untreated. Data are the normalized luciferase reporter activities relative to reporter activity measured in the absence of transfected STAT5b or HNF4α ('fold-activation' values, means ± S.D., $n = 3$), as shown above each bar.

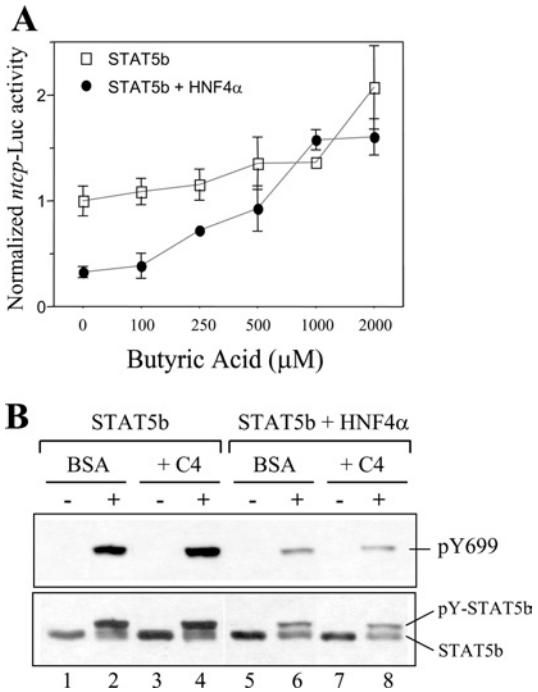


**Figure 3 Impact of HNF4α activator butyrate on STAT5b inhibition**

HepG2 cells seeded in 48-well plates (**A**) or in 6-well plates (**B**) were transfected with STAT5b and GH receptor expression plasmids and a 4x-ntcp-luciferase reporter plasmid, alone or in combination with the HNF4α expression plasmid. After 24 h the cells were treated for a further 16 h with BSA (vehicle control for fatty acid, 1% final concentration) or with butyrate (C4) dissolved in BSA to give the final concentration indicated. The STAT5b/HNF4α plasmid ratio was 1:3 (10 ng of STAT5b, 30 ng of HNF4α; **A**) or 1:5 (160 ng of STAT5b, 800 ng of HNF4α; **B**). Cells were treated with GH (200 ng/ml) for 16 h for luciferase assays (**A**) or for 30 min before preparation of cell extracts for Western blot analysis (**B**). Data shown in (**A**) are normalized ntcp-luciferase activities, means ± S.D. values for three replicates, with the activity in the absence of HNF4α and the absence of butyrate set at 1. (**B**) Western blots probed sequentially with anti-pTyr$^{699}$-STAT5b (top) and anti-STAT5b antibodies (bottom) as in Figure 1(E).

first detected after 10 min, became near-maximal after 20–45 min, and then began a slow decline that extended from approx. 2 h to 8–16 h (Figure 5A, lanes 1–5; Figure 5B, lanes 1–4, and results not shown). The time course for STAT5b phosphorylation was very similar in cells expressing HNF4α (Figure 5A, lanes 6–8 versus lanes 1–3). However, the maximal extent of phosphorylation was substantially lower, as indicated by the lesser intensity of the pTyr-STAT5b band (upper panel) and by the higher ratio of unphosphorylated to tyrosine phosphorylated STAT5b protein bands (middle panel), which is in agreement with Figure 1(E). Examination of the time course of STAT5b dephosphorylation suggested that HNF4α expression might accelerate STAT5b dephosphorylation. This is indicated by the decrease in pTyr$^{699}$-STAT5b intensity after 45 min and 2 h compared with the corresponding 20 min control (Figure 5A, top panel; lanes 9 and 10 versus lane 8, compared with lanes 4 and 5 versus lane 3) and was verified by a decrease in the ratio of phosphotyrosine-STAT5b to STAT5b protein in the same protein samples (Figure 5A, middle panel). This finding was confirmed in a second study in which the decline in STAT5b tyrosine phosphorylation was examined after 45 min to 16 h, as revealed by the effect of HNF4α on the change in the STAT5b band ratio after 45 min (Figure 5B, lane 6 versus lane 2) to 8 h (lane 7 versus lane 3).

To further investigate whether an increase in STAT5b tyrosine phosphatase activity may contribute to the HNF4α-dependent decrease in maximal pTyr-STAT5b signal, HepG2 cells were treated with GH in the presence of pervanadate, a general PTP inhibitor, in an effort to dampen down the inhibitory effect of HNF4α. Western blot analysis revealed, however, that STAT5b tyrosine phosphorylation was substantially inhibited by HNF4α, independently of the presence of pervanadate (Figure 6A, lane 8 versus lane 4). We therefore investigated the effect of PTP-1B-D181A, a dominant-negative mutant of the STAT5b tyrosine phosphatase PTP-1B [34]. pTyr$^{699}$-STAT5b is a good substrate for PTP-1B, which may contribute to the dephosphorylation and deactivation of STAT5b that occurs in hormone-stimulated cells. PTP-1B may also contribute to the down-regulation of GH receptor signalling by dephosphorylation of the GH receptor

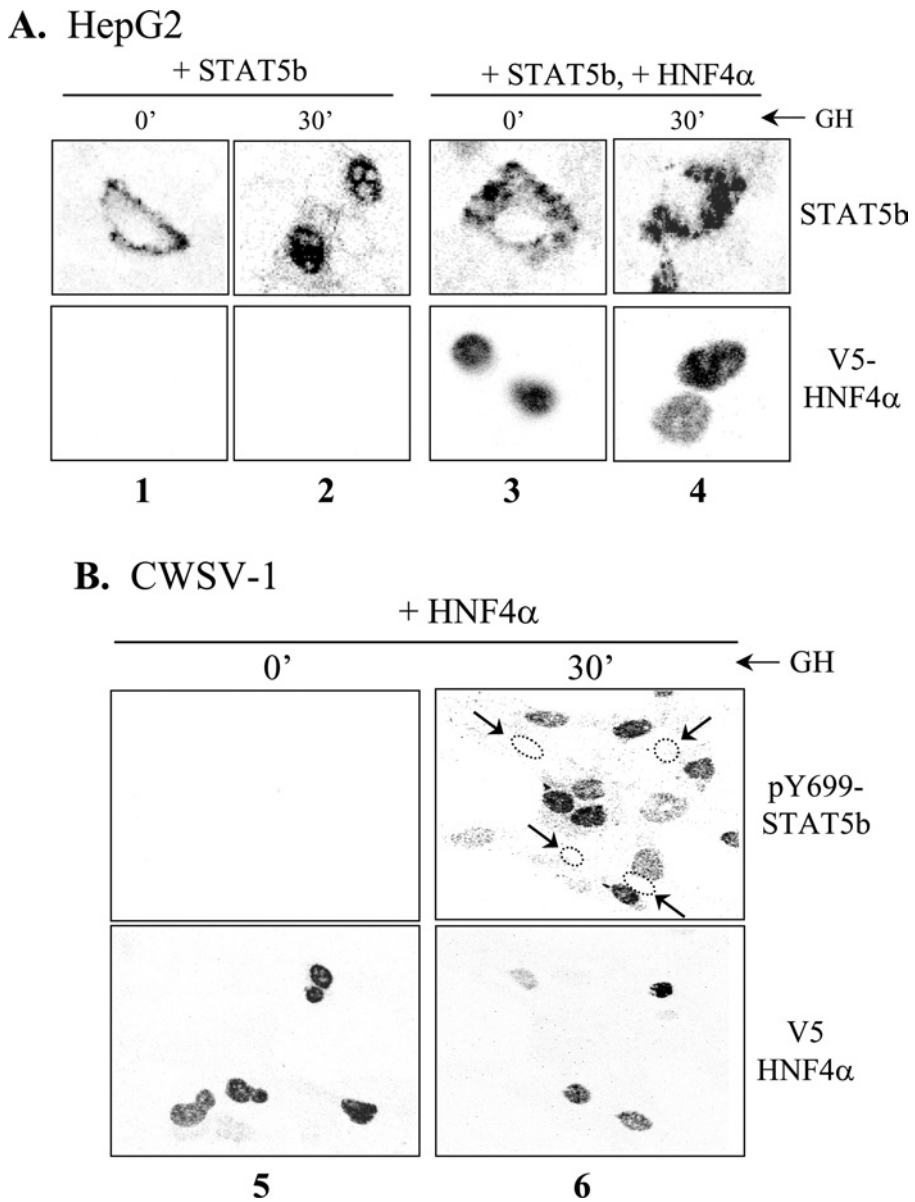


**Figure 4 HNF4α inhibits GH-induced STAT5b nuclear translocation**

Confocal immunofluorescence images of cells stained with antibodies against STAT5b, HNF4α and pTyr$^{699}$-STAT5b. (**A**) HepG2 cells transfected with the GH receptor and STAT5b, alone or in combination with V5-tagged HNF4α, were either untreated or were stimulated with GH for 30 min. Cells were fixed and parallel samples were analysed by confocal immunofluorescence microscopy using anti-STAT5b (top) or anti-V5 antibodies (bottom). Nuclei were visualized by propidium iodide staining (results not shown). GH-stimulated STAT5b nuclear translocation (lane 2 versus lane 1) was blocked in samples that were transfected with HNF4α (lane 4). GH had no effect on the nuclear localization of HNF4α (lanes 3 and 4, bottom). (**B**) Double immunofluorescence images of CWSV-1 liver cells demonstrating the inhibitory effect of HNF4α on GH-stimulated tyrosine phosphorylation of endogenous STAT5b. STAT5b tyrosine phosphorylation, visualized with an antibody against pTyr$^{699}$-STAT5b, was blocked in the four individual HNF4α-transfected cells in the field of view shown but not in untransfected cells present in the same field of view. HNF4α-transfected cells were identified using an anti-V5 antibody (lane 6, bottom) and are marked by dotted circles and arrows (lane 6, top), as determined from an image of the green-fluorescence-labelled STAT5b signal overlaid with the red-fluorescence-labelled HNF4α signal (results not shown). No STAT5b tyrosine phosphorylation was seen in the absence of GH treatment (lane 5, top).

[35] and/or JAK2 [36]. We first confirmed that PTP-1B blocks GH-stimulated STAT5b transcriptional activity (Figure 6B, bar 2 versus bar 1). Next, we demonstrated that PTP-1B-D181A reverses the inhibitory effect of PTP-1B in a dose-dependent manner (bars 3 and 4 versus bar 2). Finally, we examined the effect of PTP-1B-D181A on the HNF4α-dependent inhibition of STAT5b activity. No reversal of HNF4α inhibition was observed (Figure 6B, bar 6 versus bar 5). A similar result was obtained using TC-PTP-C216S, a dominant-negative inhibitor of TC-PTP [37], which also catalyses pTyr-STAT5b dephosphorylation (results not shown).

### HNF4α inhibits JAK2 tyrosine phosphorylation

Given the role of tyrosine-phosphorylated JAK2 in catalysing GH-induced STAT5b tyrosine phosphorylation [14], we investigated whether JAK2 tyrosine phosphorylation is altered by HNF4α. These experiments were carried out in HepG2 cells transfected with JAK2 in order to increase the sensitivity for detection of pTyr-JAK2. HNF4α effected a dose-dependent inhibition of JAK2 tyrosine phosphorylation, as revealed by Western blotting using an antibody against pTyr$^{1007/1008}$-JAK2 (Figure 7A). This inhibition paralleled that of STAT5b tyrosine phosphorylation and was more

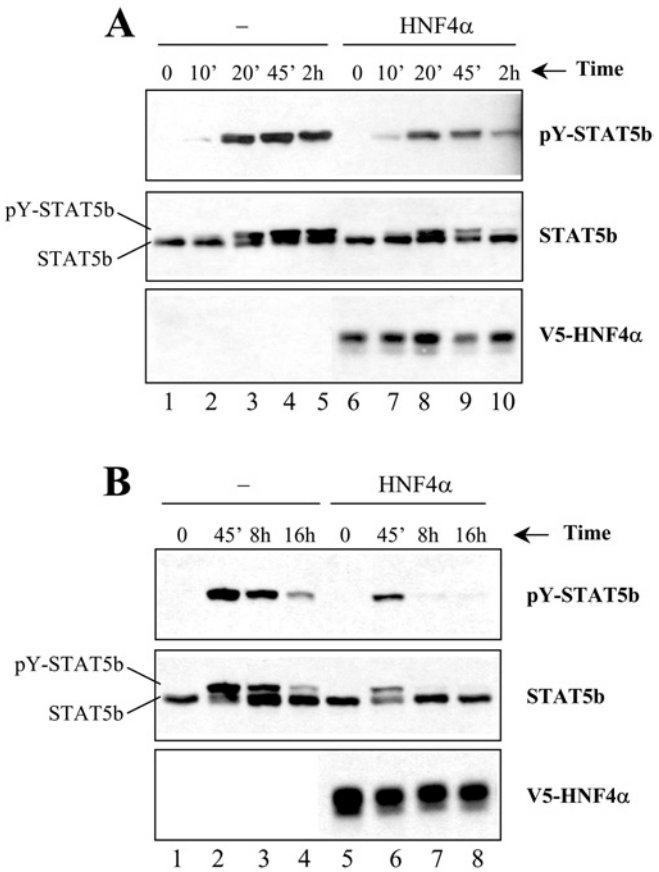


**Figure 5 Effect of HNF4α on the time course for activation (A) and deactivation (B) of STAT5b tyrosine phosphorylation**

HepG2 cells were transfected with the GH receptor and STAT5b, alone or in combination with a V5-HNF4α expression plasmid [STAT5b/HNF4α, 1:5 (w/w)]. Cells were stimulated with 200 ng/ml GH for the time period indicated. Cell extracts were then prepared and analysed on a Western blot probed sequentially for pTyr$^{699}$-STAT5b, total STAT5b protein and V5-HNF4α. HNF4α inhibited the maximal level, but had no effect on the rate, of STAT5b tyrosine phosphorylation (**A**, lanes 6–10 versus lanes 1–5). The rate of STAT5b dephosphorylation was apparently increased somewhat in the presence of HNF4α (**B**, lanes 6–8 versus lanes 2–4). The time course for GH-induced STAT5b phosphorylation and dephosphorylation in HepG2 cells is slower than in CWSV-1 cells [46].

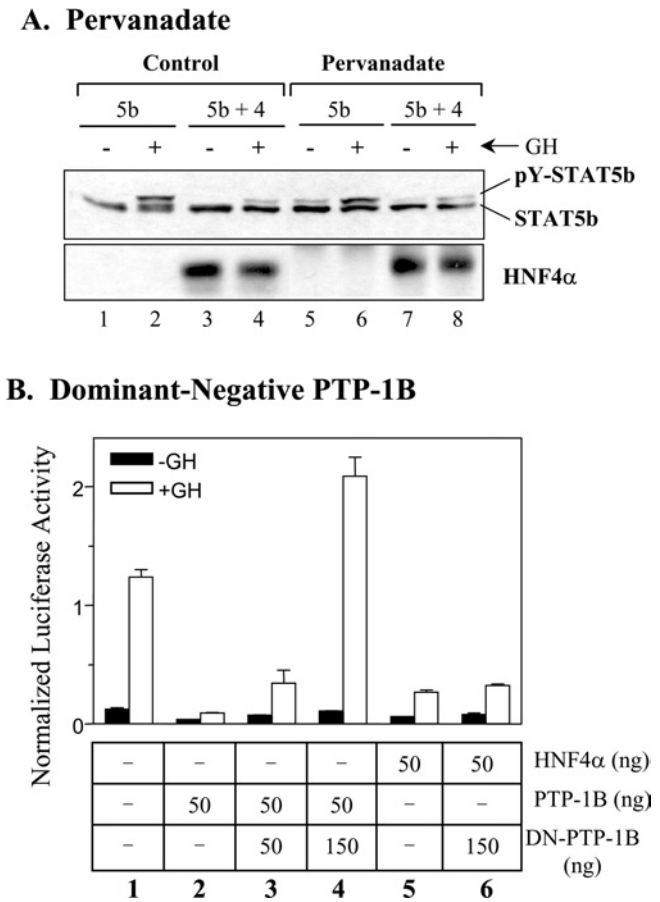


**Figure 6 Tyrosine phosphatase inhibitors do not block HNF4α inhibition of STAT5b activation**

(**A**) HepG2 cells transfected with expression plasmids for STAT5b (5b) alone, or STAT5b + HNF4α (5b + 4) (1:5, w/w), were untreated or were pre-treated with 60 μM pervanadate for 30 min. Cells were then stimulated with GH for a further 30 min, as indicated. Cell extracts were assayed by Western blotting using antibodies against STAT5b (upper panel) or HNF4α (lower panel). The effectiveness of pervanadate was verified by the increase in basal pTyr-STAT5b (note upper pTyr-STAT5b band in lane 5 versus lane 1) and by the general increase in tyrosine-phosphorylated cellular proteins detected with an anti-phosphotyrosine antibody 4G10 (results not shown). (**B**) Expression plasmids encoding HNF4α, the tyrosine phosphatase PTP-1B, or the dominant-negative (DN) mutant PTP-1B-D181A were transfected into HepG2 cells using the indicated amount of plasmid DNA, together with the STAT5 reporter plasmid 4x-ntcp-Luc. Cells were stimulated with GH for 16 h and reporter activity was assayed. Data shown are the normalized luciferase activities, means ± S.D., $n = 3$. PTP-1B-D181A reversed the inhibitory effects of PTP-1B (bars 3 and 4 versus bar 2) but not the inhibition by HNF4α (bar 6 versus bar 5).

extensive than the non-specific decrease in the level of JAK2 protein seen at higher levels of HNF4α plasmid. The latter non-specific decrease did not require the GH receptor (Figure 7A, lanes 3 and 4 versus lanes 1 and 2) and was independent of GH treatment (Figure 7A lanes 7, 9, 11 and 13 versus lanes 8, 10, 12 and 14). The inhibition of JAK2 tyrosine phosphorylation was also observed using human HNF4α (Figure 7B, lane 2 and lane 4 versus lane 6). We conclude that HNF4α inhibits JAK2 signalling to STAT5b in a manner that decreases STAT5b tyrosine phosphorylation, nuclear translocation and transcriptional activity.

## DISCUSSION

In the present study we have characterized bi-directional cross-talk between STAT5b and HNF4α, which both play important roles in liver gene expression, in particular sex-dependent, GH-regulated gene expression, as revealed by the analysis of knockout mouse models [6,12,21]. We also demonstrate that STAT5b enhances HNF4α-dependent gene transcription, while HNF4α is shown to inhibit GH-stimulated STAT5b tyrosine phosphorylation, nuclear translocation and transcriptional activity. Consequently, the relative functional expression levels of HNF4α and STAT5b may be an important determinant of the activity of both transcription factors, with conditions that are associated with a high level of HNF4α activity inhibiting STAT5b activation and STAT5b transcriptional activity, and conditions associated with high STAT5b activity augmenting HNF4α, as well as STAT5b transcriptional activity. STAT5b is repeatedly activated, and then deactivated, approx. every 3.5–4 h in direct response to each plasma GH pulse in the adult male rat [16,17], raising the possibility that STAT5b- and HNF4α-stimulated transcriptional events may both be influenced by the pulsatile, male plasma-GH rhythm. This latter possibility is consistent with the sex-dependent effects that HNF4α has on certain GH-dependent hepatic genes [6,12].

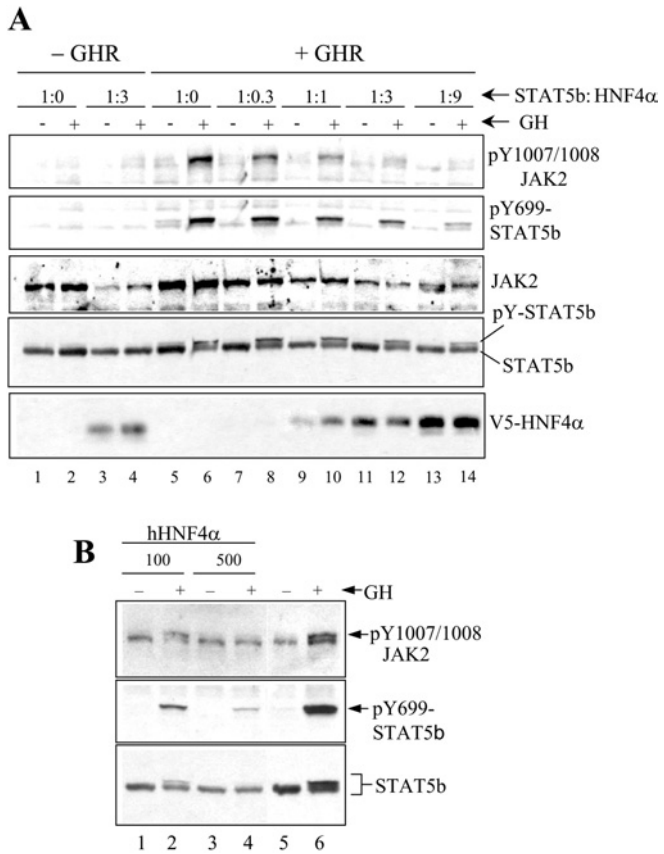


**Figure 7 HNF4α inhibits JAK2 tyrosine phosphorylation**

(**A**) HepG2 cells were transfected with the GH receptor (GHR), JAK2, STAT5b and HNF4α expression plasmids at STAT5b/HNF4α plasmid ratios from 1:0 to 1:9, as indicated. Cells were stimulated with GH for 30 min. Extracts were then prepared and analysed on a Western blot probed sequentially with each of the indicated antibodies. HNF4α inhibited the GH- and GH-receptor-dependent tyrosine phosphorylation of JAK2 and STAT5b in parallel and in a dose-dependent manner. Transfection of of HepG2 cells with HNF4α also caused a partial decrease in the expression of JAK2, which was non-specific and independent of the GH receptor (lanes 3 and 4 versus lanes 1 and 2) and independent of GH stimulation (lane 3 versus lane 4). (**B**) An experiment similar to (**A**) except that human HNF4α was used at either 100 or 500 ng, together with 100 ng of STAT5b. Upper band shown in lanes 2 and 6 of the top panel corresponds to tyrosine-phosphorylated JAK2.

In the present study, HNF4α has been shown to inhibit STAT5b transcriptional activity by blocking STAT5b tyrosine phosphorylation that is catalysed by the GH-receptor-associated tyrosine kinase JAK2. This inhibition was observed when using both rat and human HNF4α. The inhibitory effect of HNF4α was manifested at the level of GH-stimulated JAK2 phosphorylation of $Tyr^{1007/1008}$, which is causally linked to the activation of JAK2 catalytic activity [38]. By contrast, HNF4α did not inhibit interferon-γ-stimulated STAT1 transcriptional activity, which is mediated by the interferon-γ receptor and requires both JAK1 and JAK2 [39,40]. Further evidence for the specificity of the HNF4α inhibitory response includes our finding that STAT5b activation was not inhibited by HNF1α. In previous studies, we found that HNF3β, a forkhead transcription factor unrelated to HNF4α, can also inhibit STAT5b tyrosine phosphorylation [22], however, the mechanism for that inhibition has not been determined.

The mechanism by which HNF4α inhibits JAK2 phosphorylation is unknown. Given the major role of HNF4α in regulating liver gene expression [2,6,41], HNF4α may act by inducing the expression of one or more factors that block JAK2 activation or stimulate JAK2 deactivation (dephosphorylation) in GH-stimulated cells. For example, HNF4α may induce the expression of one or more JAK2-inhibitory cytokine receptor signalling inhibitors, such as SOCS (suppressor of cytokine signalling) 1 and SOCS3 [42]. Although SOCS induction is classically stimulated in liver cells by GH and other cytokine receptor ligands, another nuclear receptor family member, oestrogen receptor-α, can mediate oestradiol-stimulated SOCS induction [43]. Alternatively, HNF4α may induce, or activate, tyrosine phosphatases, such as PTP-1B [36] or SHP-2 (Src homology 2 domain-containing PTP) [44]. The latter possibility is suggested by the increase in the apparent rate of STAT5b tyrosine dephosphorylation observed in the presence of HNF4α. However, HNF4α inhibition was not reversed by pervanadate, a general tyrosine phosphatase inhibitor, or by the expression of dominant-negative inhibitors of PTP-1B and TC-PTP, which catalyse dephosphorylation of activated GH receptor [35], JAK2 [36] and STAT5b [34,45]. It is probable that the apparent increase in the rate of STAT5b dephosphorylation reflects the upstream inhibition of JAK2-catalysed STAT5b tyrosine phosphorylation, which has the effect of rapidly decreasing the steady-state pool of pTyr-STAT5b [46]. Finally, HNF4α inhibition of JAK2 phosphorylation could also involve 'non-genomic' (i.e. non-transcriptional), membrane-associated signalling events analogous to those described for other members of the nuclear receptor superfamily [47].

Cross-talk between STAT5b and several other nuclear receptors has been reported. Bi-directional inhibitory cross-talk between STAT5 and oestrogen receptor-α [48,49], thyroid hormone receptor [24,50] and PPARα and PPARγ have been described [25,30,51]. In contrast with the present findings using HNF4α, however, the inhibition of STAT5 transcriptional activity by PPARs occurs at a step downstream from the JAK2-catalysed tyrosine phosphorylation step [25]. In the case of oestrogen receptor-α, direct interaction with STAT5, requiring the oestrogen receptor-α DNA-binding domain, has been reported [49]. Finally, in direct contrast with our findings using HNF4α, cross-talk between STAT5 and the glucocorticoid receptor that is synergistic towards STAT5 transcription but is inhibitory towards glucocorticoid receptor-dependent transcription has been described [26].

HNF4α is widely regarded as a constitutively active (i.e. ligand-independent) nuclear receptor. Structural studies have revealed the presence of a previously unrecognized fatty acid docked in the ligand-binding domain of HNF4α [31,32], consistent with the finding that fatty acids regulate HNF4α-dependent gene expression [52,53]. Fatty acids may also modulate STAT5 activity [54,55]. We therefore investigated whether fatty acids could modulate the inhibitory cross-talk between HNF4α and STAT5b. Indeed, we found that the short-chain fatty acid, butyrate, stimulated STAT5b transcriptional activity to a greater extent in cells expressing HNF4α than in its absence, effectively reversing the inhibition of STAT5b transcriptional activity. However, butyrate did not reverse HNF4α inhibition of STAT5b tyrosine phosphorylation, indicating that it acts by a distinct mechanism, and suggesting that STAT5b transcriptional activity is hyperactive in its presence.

*ApoCIII* is an HNF4α target gene, as demonstrated by *in vitro* promoter analyses [56,57] and verified by the decreased expression of *ApoCIII* in a liver HNF4α-deficient mouse model [2,6] and in human patients with mutations in *HNF4α* [58]. In the present study, HNF4α activated the human *ApoCIII* promoter in HepG2 hepatoma cells, and to an even greater extent in COS-1 cells, which are devoid of the low level of endogenous HNF4α found in HepG2 cells. STAT5b enhanced the *trans*-activation of *ApoCIII* by HNF4α in a manner that was apparently synergistic and analogous to that observed previously using two male-specific *CYP* genes [27]. The *ApoCIII* promoter fragment used in these studies (nt −852 to +22) contains a consensus HNF4α-binding

site, nt $-465$ to $-457$, but does not include a binding site matching the classic STAT5 consensus sequence, TTC-NNN-GAA. Although STAT5b binding to non-consensus sites may occur [59], it is possible that the stimulatory effects of STAT5b on HNF4$\alpha$-activated *ApoCIII* described here occur in the absence of direct STAT5b–DNA binding. This possibility is supported by the partial stimulation of *ApoCIII* promoter activity by the DNA-binding-deficient STAT5b-Y699F (Figure 2B). Further studies are required to establish the mechanism for this stimulatory effect of STAT5b and its overall importance in the regulation of HNF4$\alpha$-dependent gene expression in liver. The possibility that STAT5b may play an important role in HNF4$\alpha$-directed gene expression is supported by the co-dependence of several *CYP*s and other GH-regulated, sex-specific genes on HNF4$\alpha$ and STAT5b that have been recently described in mouse liver [12].

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