Inactivation of the Nuclear Orphan Receptor COUP-TFII by Small Chemicals

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Abstract

Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII/NR2F2) is an orphan member of the nuclear receptor family of transcription factors whose activities are modulated upon binding of small molecules into an hydrophobic ligand binding pocket (LBP). Although the LBP of COUP-TFII is filled with aromatic amino-acid side chains, alternative modes of ligand binding could potentially lead to regulation of the orphan receptor. Here, we screened a synthetic and natural compound library in a yeast one-hybrid assay and identified 4-methoxynaphthol as an inhibitor of COUP-TFII. This synthetic inhibitor was able to counteract processes either positively or negatively regulated by COUP-TFII in different mammalian cell systems. Hence, we demonstrate that the true orphan receptor COUP-TFII can be targeted by small chemicals which could be used to study the physiological functions of COUP-TFII or to counteract detrimental COUP-TFII activities in various pathological conditions.

Introduction

COUP-TFI (NR2F1) and COUP-TFII (NR2F2) belong to the nuclear receptor (NR) family which is made up of 48 members in human.¹ COUP-TFI/II regulate developmental processes and control important functions during adult life.²⁻⁷ COUP-TFs were first designated as « orphan » nuclear receptors in view of their homology with founding members of the family and their lack of known ligand. X-ray determination of the structure of the COUP-TFII ligand binding domain (LBD) revealed that it is probably a true orphan, due to an autorepressed conformation in which helix α 10/11 is bent into the putative hydrophobic ligand-binding pocket (PDB 3cjlw).⁸ True orphans are uncommon proteins in the family since among the 41 crystallized vertebrate receptors (see Suppl. Table I), only 7 of them (*i.e.* COUP-TFII, Nurr1, Nur77, PNR, TR4, REV-ERB α and REV-ERB β) show a ligand binding cavity which is fully occupied by bulky amino-acid side-chains.⁸⁻¹⁰ Surprisingly, despite the absence of a classical ligand binding cavity in the crystal structure of COUP-TFII, Kruse *et al.*⁸ showed that, under certain circumstances, COUP-TFII could be activated by retinoic acid and interpreted this observation as an indication that a ligand binding pocket might form in some cellular contexts, leading to an active conformation in which helix α 10/11 is straightened.⁸ NR ligand binding cavities have highly different volumes ranging from 100 Å³ (ERR1)¹¹ to 1600 Å³ (SF-1,¹² see Suppl. Table I). The hydrophobic

cavity found in the modeled putative active conformation of COUP-TFII LBD with a straight helix $\alpha 10/11$ was predicted to have a volume of 600-700 Å³ and could thus easily accommodate small ligands such as retinoic acid.⁸ Alternatively, activation of COUP-TFII by retinoic acid could result from binding to a cryptic site within the COUP-TFII LBD. Interestingly, Nurr1 can be activated by small chemicals that bind to hydrophobic grooves at the surface of the ligand-binding domain,^{13,14} suggesting that even true orphans can be drug targets. On the other hand, small LBP may accommodate large ligands, inducing conformational disturbances which promote alternative activation states.¹⁵ In opposition to the more classic transcriptional activation or repression model, conditioned by coactivator or corepressor recruitment, these alternative activation states may induce nuclear degradation and cytoplasmic or nuclear aggregation of the targeted NR, as demonstrated for the estrogen receptor alpha bound by antagonists.^{16,17} In the present report, we describe the discovery of small molecules regulating COUP-TFII and their use for the functional inspection of the COUP-TFII orphan receptor in adipocyte differentiation and liver gene expression.

Results and Discussion

Identification of a « surface » binding pocket within the hCOUP-TFII LBD.

Despite the absence of a buried ligand binding pocket, the published structure of hCOUP-TFII LBD (PDB 3cju) was interrogated for the presence of interaction surfaces by various on-line softwares. 3cju was first screened for putative ligand binding sites with Q-SiteFinder.¹⁸ Two sites, out of 10 predicted (see supplementary Fig. S1), displayed a volume of 271 Å³ (site 1) and a volume of 254 Å³ (site 2), potentially able to accommodate small molecules. This first analysis was followed by a search for protein pockets and cavities with Pocket-Finder, a software implementing LIGSITE.¹⁹ Pocket-Finder predicted that site 1 is associated with a cavity of 208 Å³, whereas no significant cavity was predicted at site 2 (Suppl. Fig. S1). Lastly, applying PPI-Pred, a software designed to predict protein-protein interaction surfaces,²⁰ predicted that site 2 is integrated into a large protein-protein interaction interface, whereas site 1 only partially overlaps with a protein-protein interaction domain (Suppl. Fig. S1). Hence, site 1 is likely to provide a docking site for a putative non-proteinaceous ligand. According to the above analysis, this molecule would dock into a cavity (hereafter called « surface pocket ») ranging from 208 to 271 Å³ and delineated by residues from helices $\alpha 3$, $\alpha 7$ and $\alpha 10/11$ (Fig. 1A, 1B and 1C). Residues lining the cavity are mostly hydrophobic, although a few polar aminoacids may

provide hydrogen bonds to putative ligands (C213, R385, H291). Nonetheless, it should be stressed that residues L269 to V285 between helices $\alpha 5$ and $\alpha 7$ are missing in the solved structure.⁸ Depending on their respective spatial positioning, these residues could eventually reduce the size of the detected cavity. However, superimposition of COUP-TFII, RXR α and HNF4 α LBD structures indicated that the missing residues in COUP-TFII LBD structure are not likely to fill the cavity since the corresponding space is mostly occupied by residues forming the start of $\alpha 3$ in RXR α and HNF4 α , residues which are positioned away from the cavity in COUP-TFII, a situation which occurs also in the structure of other autorepressed LBDs like those of PNR and TR4 (Suppl. Fig. S2).

We next mutated residues either found in the putative surface pocket or being buried in the filled classical pocket (Fig. 1D) and analyzed the residual activity of the mutants in a yeast one-hybrid system (Fig. 1E and 1F). In such a system, wild-type (wt) COUP-TFII was able to induce transcription of the reporter gene. The activity of mutants W249A, F253A and F295A, was severely compromised. Side chains of residues W249, F253 and F295 fill the classical pocket⁸ and hence are likely to be required for proper folding of the protein. Interestingly, mutants for residues found in the surface cavity (I212A, C213A, F288A and H291A) maintained high transcriptional activation, indicating that these three aminoacids do not play a major role in folding the LBD.

COUP-TFII is inactivated by naphthol derivatives.

The shape, size and nature of the predicted surface cavity in COUP-TFII LBD suggested that it could accommodate small natural or synthetic ligands composed of functionalized phenyl rings. We thus designed a focused library of 67 commercially available compounds, most of which met the above mentioned criteria (see Suppl. Table II). This library was screened in the yeast one-hybrid system measuring wtCOUP-TFII mediated transcriptional activity (Fig. 2A). No compound was able to increase transcription of the reporter gene. Conversely, 4-methoxynaphtalen-1-ol, 6-methoxynaphtalen-2-ol, 2-naphtol and, to a lesser 1,3-dihydroxynaphtalen (compounds 62, 64, 61 and 46 respectively Suppl. Table II) inhibited wtCOUP-TFII activity (Fig. 2A). Interestingly, these four compounds were structurally related to naphthol (Fig. 2B) and the most active molecule (4-methoxynaphthalene-1-ol, thereafter called 4-MNol) had an IC₅₀ of 100 μ M in the yeast one hybrid assay. Interestingly, the calculated volume of 4-MNol was about 180 $\text{\AA}^3$, thus below the estimated volume of the surface pocket of COUP-TFII (273 $\text{\AA}^3$). Binding of small compounds to nuclear receptor

surfaces has already been shown to occur in small hydrophobic grooves implicated in cofactor recruitment by the estrogen receptor α and Nurr1.^{13,14,21} However, the COUP-TFII surface cavity where 4-MNol docks is away from the known cofactor interaction site for NRs (opposite end of helix α 11) which is occupied by helix α 12 in COUP-TFII LBD structure.⁸ Hence, 4-MNol inhibition of COUP-TFII might not rely on a direct blockade of cofactor interaction, but could rather stabilize an autorepressed conformation. Docking simulations showed that 4-MNol could bind in the predicted pocket with the methyl group fitting deeply into the cavity and contacting F382, one of the residues involved in filling the classical ligand-binding pocket (Fig. 2C and 2D). Interestingly, the oxygen atom of the hydroxy group of 4-MNol could potentially form hydrogen bonds with C213 and R385 (Fig. 2D). Since the SH group of C213 is engaged in hydrogen bonding with F382 (oxygen atom of the peptide bond between F382 and F383 at the end of α 10/11) in the COUP-TFII LBD crystal,⁸ binding of 4-MNol to C213 is likely to destabilize this intramolecular bond, an event which could indirectly impact on the positioning of α 12. According to this hypothesis, 4-MNol was tested in yeast one-hybrid assay using COUP-TFII mutant C213A. Data showed a reduced sensitivity to the inhibitory action of the compound in the context of the mutated receptor (Fig. 2E), suggesting that 4-MNol can bind within the predicted cavity, an event which could lead to structural alterations reducing the transcriptional activity of COUP-TFII. To probe such structural modifications, *in vitro*-translated full-length wtCOUP-TFII was incubated with increasing amounts of chymotrypsin in a limited proteolysis assay. Short fragments resulting from the partial cleavage of the full-length protein were more amenable to degradation by chymotrypsin in the presence of 4-MNol (Fig. 2F). Since these fragments are likely to result from the cleavage of the ligand binding domain as described for nuclear receptors,²² these results indicate that 4-MNol might destabilize COUP-TFII LBD. Consequences of a direct interaction between 4-MNol and COUP-TFII were next investigated by gel shift experiments. Translation of the nuclear receptor in the presence of 4-MNol decreased wtCOUP-TFII binding to DNA while it did not affect the rate of *in vitro* synthesis (Fig. 3A and 3B). This was probably not due to a direct effect of 4MNol on the DNA binding domain of COUP-TFII since COUP-TFII transcriptional activity was inhibited in yeast one-hybrid assay in which COUP-TFII binds DNA through the LexA-DBD moiety. When 4-MNol was added after protein synthesis, during incubation with the DNA probe, the decrease in DNA binding was less pronounced (Fig. 3C). When dithiothreitol (DTT) was added in the gel-shift assay, DNA binding of COUP-TFII, *in vitro* translated in the presence of 4-MNol, was partially restored. These data, together with the

predicted interaction of 4-MNol with C213, indicate that the redox-state of COUP-TFII could be altered by 4-MNol. This observation point to a prominent role of sulfhydryl groups in the functionality of COUP-TFII. Hence, mutant C213A was tested in gel shift assay for its susceptibility to 4-MNol. In these assays, binding of C213A COUP-TFII to DNA was found to be less affected than binding of wtCOUP-TFII (Fig. 3D and Fig. S3). These data support the 3D docking model suggesting that 4-MNol anchoring to the receptor LBD is likely to rely, at least partly, on hydrogen bonding between C213 and the oxygen of the hydroxy group of 4-MNol. Nonetheless, it has been demonstrated that 4-MNol can undergo oxidation into Russig's blue.²³ Such a reaction would be slowed down by DTT *in vitro*, suggesting that active form of 4-MNol inhibiting COUP-TFII activity in our assays could actually be oxidized 4-MNol. We thus analyzed a putative interaction of Russig's blue with the surface pocket of COUP-TFII LBD through docking experiments. Data revealed that Russig's blue could fit in the pocket mainly through hydrophobic interaction and anchoring to C213 and R385 (Fig. S4A). Binding energies indicated that Russig's blue would have a higher affinity than 4-MNol for this site (Fig. S4B). Moreover, these docking experiments predicted that one phenyl ring of Russig's blue would protrude from COUP-TFII LBD hence possibly favoring aggregation and degradation. Collectively, these data favor a model in which 4-MNol induces a conformational switch of COUP-TFII LBD leading to aggregation and/or degradation of the protein.

4-MNol decreases COUP-TFII protein levels and activity in hepatoma cells.

COUP-TFII is highly expressed in liver and kidney where it has been proposed to regulate a number of metabolic pathways.²⁴ Co-transfection experiments have been used previously to investigate the regulatory functions of COUP-TFs (COUP-TFI and COUP-TFII) on putative target genes. It has been for instance established *in vitro* that COUP-TFs down-regulate genes such as *Apolipoprotein A1*, *AIV*, *B* and *CIII*,²⁵⁻²⁷ whereas COUP-TFII is positively involved in the HNF-4 α -dependent *Cyp7a1* gene activation.²⁸⁻³⁰ In this context, we analysed the expression of COUP-TFII target genes by RT-qPCR in human liver hepatoma cells (HepG2) treated with 4-MNol. The 4-MNol toxicity was found to be limited even at 100 μ M (Fig. 4A) and RT-qPCR data revealed that *Cyp7a1* mRNA levels gradually decreased with increasing 4-MNol concentrations whereas ApoA1 mRNA levels remained unchanged up to 50 μ M 4-MNol (Fig. 4B). In order to analyse whether the effects of 4-MNol on *Cyp7a1* gene expression were dependent on COUP-TFII, we next transfected HepG2 cells with siRNAs targeting COUP-TFII. In

these conditions, 4-MNol showed a reduced ability to inhibit *Cyp7a1* expression (Fig. 4C), indicating that its effects are at least partly conveyed through inhibition of the orphan receptor. COUP-TFII is known to co-activate HNF-4 α on the *Cyp7a1* gene²⁸ and its presence at the *Cyp7a1* promoter in liver cells requires HNF-4 α (Talianidis, unpublished data). To evaluate the impact of 4-MNol on molecular events occurring at the *Cyp7a1* promoter, we then run chromatin immunoprecipitation (ChIP) assays. The results demonstrated that COUP-TFII binding to the promoter of *Cyp7a1* was reduced by 4-MNol (Fig. 4D). Interestingly, the reduction in *Cyp7a1* promoter occupancy by COUP-TFII did not alter the engagement of HNF-4 α but was detrimental to the recruitment of activated RNA Pol II and the histone acetyl-transferase CBP (Fig. 4D). Accordingly, histone H3 acetylation at the *Cyp7a1* promoter was lower in 4-MNol-treated cells (Fig. 4D). In accordance with a lower engagement of COUP-TFII at the *Cyp7a1* promoter upon 4-MNol treatment, COUP-TFII protein levels were dramatically reduced by 4-MNol (Fig. 4E), despite a slightly increased COUP-TFII mRNA expression (Fig. 4C). In order to gain insights into the mechanisms leading to reduced COUP-TFII protein levels in HepG2 cells treated with 4-MNol, we first inhibited the proteasome by adding MG132 to HepG2 cells. Surprisingly, MG132 reduced COUP-TFII levels, and combining MG132 with 4-MNol led to further reduction in COUP-TFII (Fig. 4E). Hence, it is unlikely that 4-MNol induces degradation of COUP-TFII through the ubiquitin/proteasome pathway. Alternatively, aggregates of COUP-TFII might form upon 4-MNol binding and be degraded through autophagy. We then hypothesized that if such aggregates are induced by 4-MNol, they could be evidenced in the form of foci by immunofluorescence experiments. Results indicated that HepG2 cells treated with 4-MNol tend to have an increased number of fluorescent foci, specially in the cytosol (Fig. 4F), suggesting that indeed 4-MNol induces aggregation of COUP-TFII in HepG2 cells.

4-MNol enhances 3T3-L1 adipocyte differentiation.

Since 4-MNol reduced COUP-TFII activity in HepG2 cells, we hypothesized that 4-MNol could also affect adipocyte differentiation, a process inhibited by COUP-TFII, partly through binding to and repression of the *PPAR γ* gene.^{7,31,32} Hence, 3T3-L1 cells (a fibroblastic cell line able to differentiate into mature adipocytes) were treated with 4-MNol following the protocol displayed in Fig. 5A. Negative control was obtained from confluent cells cultured with regular medium without any stimuli (Fig. 5A, control -), whereas positive control corresponded to differentiation induced by dexamethasone and

insulin (Fig. 5A, control +). Adipocyte differentiation was then evaluated by Oil Red O (ORO) staining of triglycerides (TGs) and direct TG content determination 8 days after dexamethasone addition. As observed for HepG2 cells, the viability of 3T3-L1 cells was not affected by 4-MNol up to 100 μ M (Fig. 5B). As expected, dexamethasone alone induced partial adipocyte differentiation (Fig. 5C) and when 4-MNol and dexamethasone were combined, TG positive cells and TG content increased in a dose-dependent manner (Fig. 5D,E). These data indicate that 4-MNol enhanced dexamethasone-induced conversion of 3T3-L1 fibroblasts to adipocytes. To further analyze the effects of 4-MNol on *in vitro* adipocyte differentiation, mRNA expression levels of two main adipogenic markers (*PPAR γ* and *Adiponectin*) were determined by RT-qPCR. Cells were treated with increasing concentrations of 4-MNol and *PPAR γ* and *adiponectin* mRNA expression was monitored 2, 4, 6 and 8 days after induction of differentiation. In accordance with the observed increase in the percentage of adipocytes in 4-MNol treated cells, expression of both *PPAR γ* and *adiponectin* mRNAs was enhanced in a time and dose-dependent manner by 4-MNol (Fig. 5F). Taken together, these data demonstrate that 4-MNol enhanced adipocyte differentiation as well as mRNA expression of adipogenic markers such as *PPAR γ* and *adiponectin*. Despite 4-MNol reduced COUP-TFII protein levels in HepG2 cells, a similar effect of 4-MNol was not evidenced in 3T3-L1 (data not shown). This observation points to diverse molecular outputs of 4-MNol/COUP-TFII interaction in different cell types. Our EMSA analysis suggests that 4-MNol oxidation could generate more efficient inhibitors of COUP-TFII which could lead to protein aggregation in cells. Accordingly, 4-MNol could be oxidized in cells expressing cytochrome P450 enzymes like HepG2 cells.²³ Hence, several 4-MNol-derived molecular species might be at the origin of a seemingly differential behaviour of the COUP-TFII protein in different cell types.

Conclusion

We report here that activity of the orphan nuclear receptor COUP-TFII can be modulated by small chemicals belonging to the naphthol family. Challenging COUP-TFII-regulated processes with 4-MNol allowed us to conclude that this compound can efficiently target COUP-TFII not only in the yeast one-hybrid system but also in different mammalian cell types. Hence, 4-MNol can be used as a new tool to probe the role of COUP-TFII in various biological processes. Importantly, these data further reveal that COUP-TFII could be considered as a therapeutic target since the activity of this orphan nuclear receptor can be manipulated through the action of small chemicals.

Experimental procedures

Materials and cloning

All yeast media were from Sigma-Aldrich. The synthetic compound library was built from individual compounds from Sigma-Aldrich. For screening of compound activity we used the DupLex-A™ yeast two-hybrid system from OriGene Technologies, Inc. (USA). Full-length human HNF-4α1 and human COUP-TFII aa52-414 were fused in frame with the LexA DNA binding domain (DBD) in the one-hybrid vector pEG202 (OriGene Technologies, Inc.) as already described.³³ All COUP-TFII mutant plasmids were prepared with the QuickChange™ kit (Stratagene, USA) as described by the manufacturer with oligonucleotides from Proligo (France - see Suppl. information). All mutations were confirmed by sequencing.

One hybrid assays and library screening

Compounds from the targeted library were tested in a 96 well-plate format assay as described,³⁴ using the LexADBBD-COUP-TFII one-hybrid system in which the production of β-galactosidase in yeast cells reflects the transcriptional activity of the fusion protein. Cells were treated with compounds solubilized in DMSO (1% final concentration). Control experiments run with DMSO alone were used to correct for the solvent effect. After cell lysis, β-galactosidase activity was revealed with the fluorescent substrate 4-methylumbelliferyl β-D-galactopyranoside (4MUG, Sigma-Aldrich) at a final concentration of 40 μg/mL.

Electromobility shift assay

Electromobility shift assay were performed in 10 μl reaction consisting of 20 mM Hepes (pH 7.9), 350 mM KCl, 25 mM MgCl₂, 25% glycerol, 1 mM dithiothreitol, 100 μM ZnCl₂. In vitro translated COUP TFII was added to the binding buffer, followed by 2 μg poly (dI-dC) and 10000 cpm of ³²P-labeled double stranded DR1 probe (5'-TGCGAGGTCAAAGGTACCTG-3'). When required, 4-MNol was added at a final concentration of 100 μM. The mixture was incubated for 20 min on ice followed by 10 min at room temperature. Complexes were separated on 5 % nondenaturing polyacrylamide gel followed by autoradiography using PhosphorImager.

Western blotting

HepG2 cells were lysed by incubation on ice for 30 minutes in a buffer containing 50 mM Tris, pH 7.5, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 10% glycerol and protease inhibitor cocktail (Roche). The lysates were centrifuged at 12000 g for 15 minutes. The cleared supernatants were separated by 10% polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA-PBS and probed with antibodies against TFIIB (Santa Cruz, sc-225) and COUP-TFII. COUP-TFII antibodies were raised in rabbits using bacterially-expressed recombinant proteins as antigen. When required, cells were treated with 2 μ M MG132 for 18 hours.

In vitro translation of hCOUP-TFII and limited proteolysis assay

In vitro translation was performed in reticulocyte lysate using the TNT Promega kit (Promega, France) and [35 S]-methionine, in the presence of vehicle (DMSO) or 40 μ M of 4-MNol. Limited digestion of labeled receptor was carried out with chymotrypsin (SIGMA) at concentrations ranging from 1 to 10 μ g/mL. After 10 min incubation, digestion was stopped with the addition of Laemmli buffer. The samples were boiled 5 min and separated on a 10% SDS-PAGE. The radiolabeled products were visualized by autoradiography.

Chromatin immunoprecipitation

Chromatin immunoprecipitation with HepG2 cells was performed as described in Tatarakis *et al.* 2008,³⁵ using antibodies against acetylated H3 and H4 (Upstate/Millipore), RNA-PolIII (Abcam), HNF-4 α and COUP-TFII raised in the laboratory. The specificity of the antibodies was verified by IP-western blot assays using crosslinked cellular extracts. Quantitative PCR was performed using primers amplifying the human Cyp7A1 promoter: Sense: 5' GTATTGCAGGTCTCTGATTG 3'; Antisense: 5' CCAGAT CCATTAACCTTGAGC 3'.

Structure visualization, binding site prediction and docking analyses

COUP-TFII (3cjw), RXR α (1xdk) and HNF4 α (1m7w) LBD structures were visualized from pdb files with UCSF Chimera software (version 1.4.1). Q-SiteFinder, Pocket-Finder and PPI-pred on-line softwares (<http://www.bioinformatics.leeds.ac.uk>) were used with 3cjw pdb file to screen for putative

ligand binding sites, protein cavities and protein-protein interaction surfaces respectively.¹⁸⁻²⁰ Clean hCOUP-TFII (3cjw pdb) input file was generated by using the protein preparation wizard of Gold Suite V5.1 (Cambridge Crystallographic Data Centre, Cambridge). The bond orders were assigned and hydrogen atoms were added. The resulting receptor structure was saved to a mol2 file. The ligand input structures were generated and 3D optimized with MarvinSketch Academic Package (MarvinSketch 5.4.1.1, 2011, ChemAxon <http://www.chemaxon.com>). The ligand structures were saved as mol2 file. Docking was performed by using chemscore fitness function under “Standard default settings” of GOLD software. Early termination was allowed if 4 solutions were within 1.5 angstroms R.M.S.D.

3T3-L1 cell culture and adipocyte differentiation

3T3-L1 cells were maintained at subconfluence at 37°C and 5% CO₂ in proliferation medium (PM): Dulbecco's modified Eagle's medium (DMEM, Gibco) containing high glucose concentration (4.5 g/L), a mix of 100 U/mL penicilline and 100 µg/mL streptomycine (Gibco), supplemented with 10 % (v/v) calf serum (Cosmic Calf Serum, Hyclone). Before induction of adipocyte differentiation, cells were plated in 12-well plates (50.10³ cells/well) in PM. When cells reached confluence (day-2, D-2), PM was renewed and cells were maintained 2 days post-confluence until day 0 (D0). At D0, 3T3-L1 cells were induced to differentiate into adipocytes using differentiation medium (DM) using Cosmic Calf Serum into Foetal bovine Serum (Gibco) supplemented with 1µM dexamethasone (Dex). Two days later (day 2, D2), medium was replaced by DM with 1µg/mL bovine insulin (Ins). Two days later (day 4, D4), medium was replaced by DM and subsequently renewed every two days until adipocytes reached differentiation (day 8, D8).

Oil Red O staining and quantification of triglycerides

Eight days after differentiation induction (D8), Oil red O (ORO) staining and quantification was performed as previously described.³⁶ Additionally, intra-cellular triglycerides (TG) were also quantified as described hereafter. Cells were washed with cold PBS and harvested using 200 µL of lysis buffer (1X PBS with 1% SDS). 50 µL of lysate was used for protein quantification using the Bio-Rad DC Protein Assay according to the manufacturer's instructions. The remaining lysate was transferred into glass tubes on ice and diluted by adding 600 µL of ultra-pure water. Intracellular TG were extracted by

addition of 3mL of chloroform:methanol (2:1). After homogenization and centrifugation at 160 g for 5 minutes at room temperature, the upper phase and protein disk were discarded. TG were then precipitated by adding 1mL of chloroform and Triton-X100 (final concentration 1%) to the organic phase and samples were dried under nitrogen after homogenization. Pellets were resuspended into 80 μ L of ultra-pure water and TG were quantified using the TG PAP-1000 (BioMerieux) according to the manufacturer's instructions and CFAS (Roche Diagnostics) as standard. TG contents were normalized to total protein levels.

Gene expression analysis by RT-PCR and RT-qPCR

At indicated times, total RNAs were extracted using the Extract-all reagent (Eurobio, Courtabeuf, France) according to the manufacturer's protocol. 500 ng of total RNAs were reverse-transcribed using the High Capacity Reverse Transcription Kit (Applied Biosystem, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. A 1:20 dilution of cDNA was then amplified by real time qPCR cDNA either in a 7500 Fast real-time PCR system (Applied Biosystems) using TaqMan Gene Expression Assays as recommended by the manufacturer (3T3-L1 cells) or in a CFX96 real-time system (Biorad) using SYBR green (HepG2 cells). Relative gene expression was calculated by the $\Delta\Delta C_t$ method and final results were expressed as the fold difference relative to control conditions in gene expression normalized to 18S rRNA for 3T3-L1 and RSP28 in HepG2 cells. Primers were provided by Sigma (France) and are listed in Supporting information. Probes used with TaqMan Gene Expression Assays were provided by ThermoFisher Scientific and are listed in Supporting information. For siRNA experiments, HepG2 cells were transfected with scramble or COUP-TFII targeting pools of siRNAs (Trisilencer-27 siRNA duplexes, Origene) as recommended by the manufacturer. After 6 hours of transfection, cells were treated for 48 hours with DMSO or 50 μ M 4-MNol.

Immunofluorescence

HepG2 cells ($5 \cdot 10^5$ cells per well) were seeded on coverslips in 6-well plates 24 h before treatment. Cells were treated two times during 48 h with 50 μ M 4-MNol or 0.5 % DMSO as a negative control. Cells were then washed three times with PBS and fixed with 4% paraformaldehyde. After three washes in PBS, unspecific sites were saturated using blocking buffer (PBS with 0.1% triton, 5% foetal bovine serum 5 %). Then, cells were incubated with primary antibody (anti-COUP-TFII, 1/1000 in

blocking buffer) during 16 h at 4 °C. Cells were next incubated with secondary antibody (anti-rabbit antibody coupled to Alexafluor) during 2 h at room temperature. Following three washes in PBS, coverslips were mounted on glass slides with DAPI (Vectashield). Images were acquired on an Apotome microscope (Zeiss). For each condition, 14 microscopic fields with an average of 200 cells per field were analysed for the presence of 1 or several fluorescent foci in the nucleus or in the cytosol.

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Figure legends

Figure 1: Definition of a putative ligand binding site in COUP-TFII LBD and mutational analysis in yeast one-hybrid system. (A) Hydrophobicity surface view of COUP-TFII LBD (3cjl). Color code varies from blue for hydrophilic residues to red for hydrophobic residues. The position of a small cavity thereafter called "surface pocket" is indicated by an arrow. (B) Sequence of the LBD protein crystallized in 3cjl. Numbering on the left referred to the crystallized protein. Positions of the first and last residues with respect to the full-length COUP-TFII protein are indicated. Residues lining the predicted ligand binding site are highlighted in color (red for polar residues and green for non-polar residues). These residues are mainly included in helices $\alpha 3$, $\alpha 7$ and $\alpha 10/11$. (C) Close-up view of the surface pocket. (D) List of the mutated residues analyzed in yeast one-hybrid assay. Their inclusion in the filled classical ligand binding pocket or in the surface pocket is indicated. (E and F) One-hybrid assay of wt and mutated COUP-TFII. Bars indicate β -galactosidase activity relative to wt COUP-TFII. Results are shown as means $\pm$ SEM (n = 3).

Figure 2: Naphthol derivatives inhibit COUP-TFII transcriptional activity. (A) Dose-effect of naphthol derivatives in yeast one-hybrid assay. Beta-galactosidase activities are shown relative to untreated yeast cells as means $\pm$ SEM (n = 3). (B) Structures of two active compounds. (C) Docking of 4-MNol in the surface pocket. The pocket is shown as hydrophobicity surface (blue: hydrophilic, red: hydrophobic). 4-MNol atoms are colored as follow: carbon: white, hydrogen: white, oxygen: red, lone pairs of electrons: magenta. (D) Residues from the surface pocket predicted to contact 4-MNol. Pseudobonds were determined with Chimera and are shown as green lines. Hydrophobic contacts are

detected for I212, A287, I292, L381 and F382, whereas lone pairs of electrons could establish hydrogen bonds with C213 and R385. Color code for 4-MNol atoms is identical to (C) except for carbon atoms which are colored in blue. **(E)** C213A COUP-TFII mutant is less sensitive to 4-MNol inhibition. Dose-response curves of β -galactosidase activities obtained with wt or C213A COUP-TFII in yeast one-hybrid assays. Data are shown as in (A). **(F)** Limited proteolysis assay of wtCOUP-TFII, *in vitro* translated in the presence or not of 100 μ M 4-MNol and incubated with increasing concentrations of chymotrypsin (black triangles). The full-length protein is indicated by an arrow and a vertical bar indicates the degradation products resistant to protease.

Figure 3: Altered binding of COUP-TFII to DNA in the presence of 4-MNol. **(A)** 4-MNol does not inhibit *in vitro* translation of wtCOUP-TFII. Translation was run in the presence of 35 S-methionine in the presence or not of 100 μ M 4-MNol, and proteins were separated by SDS-PAGE before autoradiography. The positions of molecular mass markers are indicated on the left. **(B)** EMSA of wtCOUP-TFII *in vitro* translated in the presence of 100 μ M 4-MNol with a radio-labeled DR1 probe. Unprogrammed lysate (UPL) was used as a control. The free probe is indicated by a black arrowhead, a non-specific shifted band by a white arrowhead, and the COUP-TFII/DR1 probe complex by an asterisk. **(C and D)** EMSA of wt (C) and C213A (D) COUP-TFII binding to the DR1 probe in two different conditions: (1) COUP-TF was *in vitro* translated in the presence of 100 μ M 4-MNol or vehicle (ethanol) and (2) COUP-TFII, *in vitro* translated in the presence of ethanol, was incubated with increasing concentrations of 4-MNol before addition of the probe, whereas COUP-TFII translated in the presence of 4-MNol was incubated with increasing concentrations of DTT before addition of the probe. Black arrowheads indicate position of the free probe.

Figure 4: 4-MNol reduces expression of the COUP-TFII target gene *Cyp7a1*. **(A)** Toxicity assay of 4-MNol. HepG2 cells were treated for 48h with DMSO or increasing concentrations of 4-MNol. Cell viability was determined by trypan blue exclusion. **(B)** RT-qPCR analysis of ApoA1 and *Cyp7a1* mRNA levels. Data were expressed as $\Delta\Delta$ ct with RSP28 as a reference. Results are expressed as % of expression of the corresponding genes in cells treated with DMSO (mean $\pm$ SEM, n = 9). **(C)** RT-qPCR analysis of COUP-TFII *Cyp7a1* and ApoA1 mRNA levels in cells treated with DMSO (black bars) or 50 μ M 4-MNol (grey bars) for 48 hours after transfection of either scramble siRNA or siRNAs

targeting COUP-TFII (mean $\pm$ SEM, $n = 6$). Statistical significance was determined using unpaired t-test. **(D)** ChIP assay of the indicated proteins and histone marks at the Cyp7a1 promoter in HepG2 cells treated for 2 days with DMSO (black bars) or 25 μ M 4-MNol (grey bars). The scheme depicts the amplified region (primers figured as arrows) which includes the bile acid response element (BARE) known to bind HNF4 α and the TATA box. Statistical significance was determined using unpaired t-test (** $p < 0.01$, *** $p < 0.001$). **(E)** Western blot analysis of COUP-TFII and TFIIIB in HepG2 cells treated either with 4-MNol or the proteasome inhibitor MG132 or both. **(F)** Immunofluorescence analysis of COUP-TFII distribution in HepG2 cells treated or not with 50 μ M of 4-MNol. Cells either presented a homogeneous nuclear distribution of COUP-TFII or a punctuated distribution with the different phenotypes exemplified in the pictures: 1, a single foci per nucleus; 2, several foci per nucleus; 3, several foci in the cytosol. The occurrence of each phenotype was determined and data are presented as the ratio of the occurrence in 4-MNol- versus DMSO-treated cells (mean $\pm$ SEM, $n = 13$).

Figure 5: 4-MNol enhances conversion of 3T3-L1 fibroblasts to adipocytes *in vitro*. **(A)** Schematic representation of the protocol used to induce partial differentiation of 3T3-L1 cells in adipocytes. **(B)** Toxicity assay of 4-MNol. 3T3-L1 cells were treated with DMSO or increasing concentrations of 4-MNol as described in (A). Cell viability was determined by trypan blue exclusion. **(C)** ORO-staining of 3T3-L1 cells 8 days post-induction of differentiation [upper: macroscopic view; bottom: microscopic view (x20)]. **(D)** Quantification of ORO staining (mean $\pm$ SEM, $n = 3$). **(E)** Quantification of TG content of 3T3-L1 –derived adipocytes (mean $\pm$ SEM, $n = 3$). **(F)** RT-qPCR analysis of PPAR γ and Adiponectin mRNA levels. Results are displayed relatively to the mRNA expression levels of PPAR γ and adiponectin at each day post-induction of partial adipocyte differentiation without 4-MNol (set to 1). Histograms represent means $\pm$ SEM of a representative experiment performed in triplicates. Statistical significance was determined using unpaired t-test (* $p < 0.05$, ** $p < 0.01$).

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