DMD Fast Forward. Published on October 7, 2009 as doi:10.1124/dmd.109.029025
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TITLE: Gene Regulation of *CYP4F11* in Human Keratinocyte HaCaT Cells

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RUNNING TITLE: Mechanisms of *CYP4F11* regulation by retinoids and cytokines

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NUMBER OF TEXT PAGES: 28

NUMBER OF TABLES: 1

NUMBER OF FIGURES: 7

NUMBER OF REFERENCES: 39

NUMBER OF WORDS IN ABSTRACT: 187

NUMBER OF WORDS IN INTRODUCTION: 489

NUMBER OF WORDS IN DISCUSSION: 1201

ABBREVIATIONS: CYP, cytochrome P450; CYP4F, cytochrome P450 4F; AP-1, the activator protein 1; AP-1 RE, AP-1 responsive element; HRE, hormone responsive element; RXRs, retinoid X receptors; RARs, retinoic acid receptors; NR, nuclear receptors; PPARs, peroxisome proliferator-activated receptors; FXR, farnesoid X receptor; LXR, liver X receptor; TR, thyroid receptor; Nurr1, nuclear receptor related 1; PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; QRT-PCR, quantitative real time polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; RA, retinoic acids; TNFα, tumor necrosis factor-alpha; IL-1β, interleukin-1-beta; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; TTNPB, 4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid; LG268, 6-(1(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl) nicotinic acid; SP600125, 1,9-pyrazoloanthrone; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; ANOVA, analysis of variance.

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ABSTRACT

Mechanisms regulating *CYP4F* genes remain under investigation, although characterization of *CYP4F* regulatory modalities would facilitate the discovery of new drug targets. This present study shows that all-*trans* and 9-*cis* retinoic acids can inhibit *CYP4F11* expression in human keratinocyte-derived HaCaT cells. Transrepression of many genes by retinoic acids is mediated by interactions between retinoid receptors and the AP-1 complex. Pro-inflammatory cytokines TNFα and IL-1β, which can activate the AP-1 complex, induce *CYP4F11* transcription in HaCaT cells. The JNK-specific inhibitor SP600125 blocked the induction of *CYP4F11* by both cytokines, indicating involvement of the JNK pathway. Furthermore, TNFα failed to induce *CYP4F11* transcription when HaCaT cells were pre-incubated with retinoic acids. Retinoic acids are ligands for nuclear receptors RARs (the retinoic acid receptors) and RXRs (the retinoid X receptors). The RXR agonist LG268 greatly induced *CYP4F11* transcription, whereas the RAR agonist TTNPB markedly inhibited *CYP4F11* transcription, indicating that down-regulation of *CYP4F11* transcription by retinoic acid is mediated by RARs and may also be related to ligand competition for RXR receptors. Thus, the *CYP4F11* gene is positively regulated by multiple signaling pathways in HaCaT keratinocytes including RXR and JNK signaling pathways.

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INTRODUCTION

Cytochrome P450 (CYP) 4F enzymes are involved in cellular protection and metabolism of numerous small molecules including drugs, toxins and eicosanoids. To date, studies have been focused on CYP4F functions rather than transcriptional regulatory mechanisms. This is partially due to the apparent complexity of *CYP4F* gene regulation and differences observed among the various model systems studied.

In spite of the seeming complexity of *CYP4F* regulation, some research groups have made substantive contributions to this puzzle. Zhang and colleagues showed that retinoic acids and peroxisome proliferators can regulate *CYP4F2* gene activities in HepG2 cells, and that RXRα heterodimers stimulated and RARα repressed *CYP4F2* expression (Zhang *et al.*, 2000; Zhang and Hardwick, 2000). Hsu and colleagues showed that statins induced *CYP4F2* in primary human hepatocytes and HepG2 cells (Hsu *et al.*, 2007). Recently we showed that retinoic acids induced the expression of *CYP4F2*, *CYP4F3A*, *CYP4F3B* and *CYP4F11* in primary human epidermal keratinocytes, and that RXR (but not RAR) nuclear receptors mediate retinoic acid-induced up-regulation of *CYP4F2* and *CYP4F3A* (Kalsotra *et al.*, 2008). In a preliminary study (data not shown) using a transformed immortal human keratinocyte cell line (HaCaT), a different story unfolded. Lovastatin did not affect HaCaT cell *CYP4F2* expression, which is in contrast to its up-regulatory effects on *CYP4F2* in human hepatic cells (Hsu *et al.*, 2007). In addition, 9-*cis* retinoic acid failed to induce *CYP4F11* in HaCaT cells; rather, it significantly decreased CYP4F11 transcript levels.

Retinoic acids are derived from retinol, the animal form of vitamin A. Retinoic acids play a role in gene regulation via two groups of nuclear receptors, RARs and RXRs

(Mangelsdorf *et al*., 1995), each of which consists of three isotypes, α, β and γ (Mangelsdorf *et al*., 1990; Leid *et al*., 1993). Retinoic acids can up-regulate or down-regulate certain genes by three mechanisms: transactivation, transrepression, and competition with other nuclear receptors for heterodimerization with RXR. Transrepression by retinoic acids is the cross-talk between retinoid receptors and the AP-1 complex (Saatcioglu *et al*., 1994; Chen *et al*., 1995). Cytokines, oncogenes and growth factors that activate protein kinase C modulate the activities of the AP-1 complex composed of c-Jun and c-Fos proteins (Zhang and Pfahl, 1993). Active RAR-RXR heterodimers interact with AP-1 complexes thereby inhibiting the transcription of genes regulated by AP-1 (Fisher *et al*., 1998). Transrepression of AP-1 is a major mechanism of the pharmacological anti-inflammatory and anti-proliferative effects of retinoids (Fisher and Voorhees, 1996).

RXR can form heterodimers with other nuclear receptors including PPARs (peroxisome proliferator-activated receptors) (Green, 1995; Qi *et al*., 2000; Lazar, 2005), FXR (farnesoid X receptor) (Laffitte *et al*., 2000; Desvergne, 2007), LXR (liver X receptors) (Willy *et al*., 1995; Willy and Mangelsdorf, 1997; Desvergne, 2007), TR (thyroid receptor) (Apriletti *et al*., 1998; Barra *et al*., 2004) and Nurr1 (nuclear receptor related 1) (Perlmann and Jansson, 1995; Perlmann and Wallen-Mackenzie, 2004). RXRs can also form homodimers under certain conditions (Zhang and Pfahl, 1993). Thus, when an RAR-specific agonist stimulates the interaction of RXR with RARs, it can act to negatively regulate the activity of other nuclear receptors that partner with RXR in favor of RAR-mediated responses (Heinz Nau, 2007). The relative abundance of classical retinoid receptors and other RXR-interacting nuclear receptors, which have varying

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affinities for RXR, influences RXR dimerization and thereby gene regulation mediated by these nuclear receptors (Fisher and Voorhees, 1996). The aim of the current study is to clarify the mechanism and factors responsible for the down-regulation of HaCaT *CYP4F11* expression by retinoic acids.

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METHODS

Chemicals

9-*cis* and *all-trans* retinoic acids, TTNPB [4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl)benzoic acid] and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were from Sigma-Aldrich Corp. (St. Louis, MO). LG268 [6-(1(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydronaphthalen-2-yl)cyclopropyl) nicotinic acid] was a gift from Dr. Peter J.A. Davies (University of Texas-Houston). Human TNFα and IL-1β were from Invitrogen Inc. (Carlsbad, CA). SP600125, SB203580 were produced by Calbiochem Inc. (San Diego, CA). Optimal concentrations of drugs were chosen according to literature citations. Polyclonal anti-CYP4F11 antibody was from Proteintech Group, Inc. (Chicago, IL). Polyclonal antibodies against human RARs and RXRs were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal c-Jun and phosphor-c-Jun antibodies were from Cell Signaling Technology Inc. (Danvers, MA). All other chemicals utilized, were of reagent grade quality or higher.

Cell culture

The HaCaT cell line was from CLS- Cell Line Service (Germany) (Boukamp *et al.*, 1988). Cells were grown at 37°C in a humidified incubator with 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (Gibco, CA) supplemented with 10% fetal bovine serum (Atlas Biologicals, CO), 1% L-glutamine (Gibco), 1% sodium pyruvate (Gibco) and 1% Antibiotic-Antimycotic (Gibco). Cells were passaged at 1:10 ratio after they had been 100% confluent for 6-7 days and used for experiments at 70-80% confluency.

Protein isolation and Western blot analyses

Cells were washed once with cold PBS and then quickly detached by gently scraping. Cell pellets were collected by centrifugation for 2 minutes at 4500 rpm and stored at -80°C. Whole protein lysate buffer was prepared using 50mM Tris-HCl buffer (pH 6.8) with 2% sodium dodecyl sulfate (SDS). Cell pellets were dissolved in the lysate buffer by pipetting the suspension up and down and then boiled at 95°C for 5 minutes. The cell lysate was cleared by centrifugation at 13,000 rpm for 2 minutes. Protein concentrations of the clear supernatant fractions were measured using the BCA assay kit (Pierce, Rockford, IL) and subjected to Western blot assays.

Protein samples were boiled in Laemmli buffer and resolved electrophoretically on 4-15% gradient Tris-glycine-SDS-polyacrylamide gels (Bio-Rad laboratories, CA). Proteins were transferred to PVDF membranes (Bio-Rad wet transfer apparatus). Membranes were blocked for 1 hour at room temperature with 5% dried non-fat milk followed by incubation for 3 hours at room temperature with antibodies raised against CYP4F11 (1:1000 dilution) or c-Jun (1:500 dilution). Membranes were then washed and incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 dilution) for 1 hour. Immunoreactivity was visualized using a HRP chemiluminescence system (Pierce, Rockford, IL).

Quantitative real time polymerase chain reaction (qRT-PCR)

Cells were washed with PBS, and total RNA was isolated using Trizol reagent (Invitrogen, CA). All samples were treated with DNase I (Invitrogen, CA). Aliquots (200ng) of total RNA were reverse-transcribed by SuperScript™ II Reverse Transcriptase (Invitrogen, CA) in triplicate including an RT blank to evaluate presence of contaminating genomic DNA. Amplification was performed using *Taq* DNA Polymerase

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(Invitrogen, CA) with an ABI Prism 7700 (Applied Biosystems, Norwalk, CT) at 95°C for 1 min, followed by 40 cycles at 95°C for 12s and 60°C for 30s. CYP4F11 mRNA levels were measured using standard curves generated by plotting Ct versus the log of the amount of purified amplicon for *CYP4F11* (custom synthesis by IDT, Coralville, IA) (200 ag to 2 pg). Abundance of human 18S RNA was used as the internal control. Primers and fluorescent probe sequences for *CYP4F11* and 18S RNA were reported (Kalsotra *et al*., 2008).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Aliquots (200 ng) of total RNA were reverse transcribed and amplified using Superscript One-step RT-PCR kit (Invitrogen, CA) and transcript-specific oligonucleotides designed using Primer3 (version 0.4.0) (Supplemental Table 1). For RAR and RXR, the reverse transcription reaction (50°C, 30 min) was followed by 40 cycles of amplification (94°C, 15 sec; 60°C, 30 sec and 72°C, 1 min). For CYP4F11, the reverse transcription reaction (60°C, 30 min) was followed by 40 cycles of amplification (94°C, 1 min and 65°C, 1.5 min). Products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

Plasmids

To construct pGL3-CYP4F11 plasmid, *CYP4F11* promoter was cloned by PCR, using human genomic DNA as a template with the forward primer 5'TCT TAC GCG TGC TAG CTA CCC AGC ACC CAG AGT AGG3' and the reverse primer 5'CCG GAA TGC CAA GCT TGC TCC AAG GAC AGT GGA AAG3'. The resulting 1729bp product was the promoter region of CYP4F11 (NG_0008335) including exon1 due to the fact that the coding start site is in exon2. The PCR product was then cloned into HindIII and NheI

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digested linear pGL3 Basic luciferase vector (Promega, WI) using the infusion Advantage PCR Cloning Kit (Clontech, CA).

Transfection and luciferase assays

Cells were transfected by Attractene Transfection Reagent (Qiagen, CA) according to the manufacturer's protocol. Cells were seeded onto six-well plates (3×10^5 cells per well). Immediately following the seeding, cells were transfected with 4.5 μl/well of Attractene and 1.2 μg of DNA per well. Transfection efficiency was monitored by simultaneous co-transfection with a β-galactosidase reporter construct at the concentration of 20 ng/well. The culture medium was changed at 6 hrs post transfection. Twenty-four hours following transfection, cells were treated with agonists for RARs or RXRs with or without TNFα. After the treatment, cells were lysed in passive lysis buffer (Promega, WI). Luciferase activity was measured for 10 seconds in a luminometer. β-galactosidase activity was measured to normalize for variations in transfection efficiency. Promoter activity of each construct was expressed as firefly luciferase/β-gal activity. Each experiment was independently performed on three separate occasions with at least triplicate wells in each experiment.

Statistical analysis

Data are presented as Mean ± S.E. One way ANOVA followed by Tukey's multiple comparison test was used for the statistical analysis. Statistical differences were considered significant if $P < 0.05$.

Database Sequence Analysis

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The Transfac database was searched using AliBaba 2.1 for putative AP-1 binding sites and hormone responsive elements on the 5' flanking sequence (2000 bps from the ATG start codon) of CYP4F11 gene.

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RESULTS

Expression of retinoid receptors in HaCaT cells and inhibitory effects of retinoic acids on *CYP4F11* expression

We first determined which RXR and RAR isoforms are expressed in HaCaT cells. Results by RT-PCR show that HaCaT cells express RARα, RARγ, RXRα and RXRβ, whereas neither RARβ nor RXRγ was detectable (Figure 1a). To elucidate mechanisms by which retinoic acids regulate *CYP4F11* expression, we treated HaCaT cells with 9-*cis* and *all-trans* retinoic acids for 3 days at 0.1 μM, 1 μM and 10 μM concentrations or vehicle (DMSO) alone. Both retinoic acids resulted in dose-dependent suppression of CYP4F11 transcript levels in HaCaT cells, measured by qRT-PCR (Fig.1b). The reductions in CYP4F11 mRNA at the 0.1 μM, 1 μM and 10 μM dose levels were: 89.1%, 93.9%, and 98.3% for 9-*cis* retinoic acid and 21.1%, 86.5%, and 94.8% for *all-trans* retinoic acid, respectively. Data from luciferase assay indicated that at the concentration of 1 μM, 9-*cis* and all-*trans* retinoic acids were able to decrease CYP4F11 promoter activity by 83.0% and 70.9% respectively (Supplemental Fig. 1). Further, Western blot analyses demonstrated the down-regulation of CYP4F11 protein by both retinoic acids at 10 μM (Fig. 1c). Western blots against RARα, RARγ, RXRα and RXRβ were performed to study the potential effects of retinoic acids on the expression levels of retinoid receptors. As shown in Fig. 1d, cells treated with high concentrations (1 μM and 10 μM) of 9-*cis* retinoic acid had reduced levels of RARγ and RXRα. Cells treated with 10 μM all-*trans* retinoic acid demonstrated a lower level of RARγ. In contrast, three-day treatment of 0.1 μM 9-*cis* retinoic acid, 0.1 μM all-*trans* retinoic acid or 1 μM all-*trans* retinoic acid did not affect the protein level of RARγ and RXRα. The data also

demonstrated that retinoic acid treatments could not decrease the protein levels of RARα and RXRβ.

TNFα and IL-1β, but not TPA, can induce CYP4F11 mRNA levels in HaCaT cells through the JNK pathway

We investigated the ability of TNFα and IL-1β or TPA to induce *CYP4F* transcription in HaCaT cells through mechanisms involving AP-1, as reported for other cell systems (Chedid *et al.*, 1991; Kyriakis, 1999; Yu *et al.*, 2008; Lamph *et al.*, 1988; Sassone-Corsi *et al*., 1990). We treated HaCaT cells with TNFα (10 ng/ml) and IL-1β (10 ng/ml) or TPA (100 ng/ml) for 24 hours. Both cytokines induced *CYP4F11* mRNA levels significantly, whereas TPA had no effect compared with the vehicle control (Fig. 2a). Luciferase assay also indicated the induction of *CYP4F11* promoter activity by the treatment of 10 ng/ml TNFα for 24 hours (Supplemental Fig. 1). Cytokines and many other factors activate the AP-1 complex through multiple signaling pathways such as the JNK, P38, and ERK pathways (Whitmarsh and Davis, 1996). Hence, we pre-incubated HaCaT cells with the JNK inhibitor SP600125 or the p38 inhibitor SB203580 for 4 hours before administering TNFα or IL-1β. Inhibiting JNK completely blocked the cytokine induced *CYP4F11* transcription (Fig. 2b-c). Inhibiting p38 had no effect on CYP4F11 expression. After administering TNFα, phosphorylated c-Jun was observed within 15 minutes; it was and remained elevated for at least 3 hours (Fig. 2e and 2f). Inhibiting JNK by SP600125 pretreatment markedly blocked the phosphorylation of c-Jun protein in HaCaT cells (Fig. 2e and 2f). In contrast, TPA (100ng/ml) had no effect on c-Jun phosphorylation (Fig. 2d). Cells were also pre-incubated with 1 μM 9-*cis* or all-*trans* retinoic acid for 24 hours and then treated with TNFα. As shown in Fig. 2f, both retinoic

acids completely blocked the phosphorylation of c-Jun at 15 min and 30 min post TNFα administration.

JNK pathway is not the sole mechanism mediating *CYP4F11* regulation

To determine whether the JNK pathway is the primary mechanism regulating *CYP4F11* expression, we treated HaCaT cells with cytokine TNFα (10 ng/ml) for 24 hours after a 3-day pretreatment with 9-*cis* or *all-trans* retinoic acid at 10 μM concentration. TNFα alone increased *CYP4F11* mRNA levels, and pretreatment with either retinoic acid blocked this induction (Fig. 3a). Hence, TNFα could not reverse the inhibitory effects of retinoic acids on *CYP4F11* transcription. When we treated HaCaT cells with the JNK inhibitor SP600125 alone (20 μM for 24 hours), *CYP4F11* mRNA levels decreased by only 50% (Fig. 3b). At this concentration, SP600125 completely blocked the TNFα-induced *CYP4F11* transcription (Fig. 2b). Inhibiting the JNK pathway did not totally suppress the transcription of *CYP4F11*, indicating other signaling pathways positively regulate *CYP4F11* expression in HaCaT cells.

Opposing effects of RAR and RXR agonists on *CYP4F11* expression in HaCaT cells

Since retinoic acids activate both RARs and RXRs, we investigated which nuclear receptor(s) mediate the inhibitory effects of retinoic acids on *CYP4F11* expression. We treated HaCaT cells for 2 or 3 days with the pan-agonists for RXR (1 μM LG268, also known as LG100268) and RAR (10 μM TTNPB). The RXR pan agonist LG268 increased *CYP4F11* mRNA levels 12-fold versus vehicle (Fig. 4a). We confirmed this induction by visualizing RT-PCR products that were amplified using different primer sequences (Fig. 4b) and by Western blot analyses (Fig. 4c). CYP4F11 protein levels were greater in cells treated with LG268 for 2 days and 3 days compared with vehicle control.

In HaCaT cells treated with the RAR pan agonist TTNPB, *CYP4F11* mRNA levels were greatly decreased versus vehicle, and combined treatment with both pan agonists for 3 days decreased *CYP4F11* mRNA levels even further (Fig. 5a). Western blot analyses confirmed the markedly decreased CYP4F11 protein levels elicited by TTNPB treatment with or without LG268 (Fig. 5b).

DISCUSSION

CYP4Fs catalyze the metabolism of both endogenous and exogenous molecules (Kalsotra and Strobel, 2006). For instance, they inactivate the leukotriene and prostaglandin prompts for the inflammation cascade playing an anti-inflammatory role (Kikuta *et al.*, 2002) and they also catalyze the metabolism of many drugs (Hashizume *et al.*, 2002; Kalsotra *et al.*, 2004). Among the human CYP4F enzymes, CYP4F11 is most active in metabolizing therapeutic drugs. In order to elucidate the role of CYP4F11, it is necessary to decipher the mechanism of *CYP4F11* gene regulation under various physiological and pathological conditions. The results of our present study have shown that several distinct but interacting pathways of gene regulation impinge upon the regulatory region of the *CYP4F11* gene in HaCaT cells. We report for the first time that two possible pathways, the JNK/AP-1 pathway and the RXR-mediated pathway, are involved in the regulation of *CYP4F11* in HaCaT cells. These pathways and their interactions are depicted in the scheme as shown in Fig. 6b.

Our present study shows that retinoids down-regulate *CYP4F11* expression in HaCaT cells. The anti-AP-1 activity of retinoids supports the positive regulation of *CYP4F11* through the AP-1 complex by inflammatory cytokines TNFα and IL-1β. Moreover, this induction of *CYP4F11* was shown to be mediated by the JNK pathway as indicated by the loss of cytokine induction of *CYP4F11* as well as c-Jun phosphorylation in the presence of JNK inhibitor SP600125. TPA had no effect on *CYP4F11* mRNA levels in HaCaT, which is consistent with previous findings that TPA did not induce JNK activity in HaCaT cells (Zhou *et al.*, 2000). CYP4F11 expression was strongly induced by addition of the cytokines TNFα and IL-1β but not TPA indicating the efficacy of AP-1

sites in regulation of *CYP4F11* mRNA expression. The blockage of TNFα-induced *CYP4F11* expression and c-Jun phosphorylation by 9-*cis* or *all-trans* retinoic acid confirms the role of AP-1 transrepression in down-regulating *CYP4F11*. Furthermore, a total of five AP-1 sites were found using AliBaba2.1 Program within the first 2000 bases upstream of the start site for *CYP4F11* (Fig. 6a). These results support transrepression of AP-1 complexes as a mechanism whereby retinoic acids suppress *CYP4F11* transcription, as shown for other gene systems (Saatcioglu *et al*., 1994; Chen *et al*., 1995).

The transrepression mechanism proposed here is not adequate to explain the findings that 9-*cis* or *all-trans* retinoic acid almost completely eliminated *CYP4F11* expression whereas SP600125 alone only demonstrated moderate inhibition,although SP600125 pre-incubation could completely block the induction of *CYP4F11* by TNFα. It appears that the inducible regulation is different from the basal regulation through JNK activity. As also proposed in the scheme of Fig. 6b, retinoic acids bind to retinoid receptors and activate the formation of RAR/RXR heterodimers. RXRs, in conjunction with one or more other nuclear receptors (NR), are proposed to stimulate *CYP4F11* transcription by acting through the hormone responsive element (HRE). The formation of RAR/RXR heterodimers on the other hand reduces the number of RXRs available for interaction with NRs, thereby suppressing the transcription of *CYP4F11* through competition for RXRs. Thus retinoid treatment of proliferating HaCaT cells leads to reduced expression of *CYP4F11* through transrepression of the AP-1 pathway and/ or by competition for RXR receptors. RAR/RXR heterodimers lead to down-regulation while NR/RXR heterodimers would lead to up-regulation of *CYP4F11* expression.

Our results indicate that simultaneous activation of RARs and RXRs intensifies the inhibition of *CYP4F11* in HaCaT, which is consistent with data in Fig. 1b given the fact that 9-*cis* retinoic acid is able to activate both RARs and RXRs while *all-trans* retinoic acid only activates RARs (Leid *et al*., 1993; Fisher and Voorhees, 1996). It is worth noting that retinoids are able to activate retinoic acid 4-hydroxylase within hours (Marikar *et al.,* 1998). Retinoic acid 4-hydroxylase hydroxylates all-*trans* but not 9-*cis* retinoic acid. Therefore, the activation of retinoic acid 4-hydroxylase may result in the reduced level of all-*trans* retinoic acid, which may make all-*trans* retinoic acid less effective than 9-*cis* retinoic acid in the inhibition of *CYP4F11*. However, the distinct effects of TTNPB and LG268 on *CYP4F11* expression do increase the possibility of a competition mechanism and allow for the potential existence of a nuclear receptor that can directly activate *CYP4F11* transcription. It seems unlikely that RXR homodimers are involved in the induction of *CYP4F11* because 9-*cis* retinoic acid, which is able to promote the formation of RXR homodimers (Zhang and Pfahl, 1993), inhibited *CYP4F11* to a greater extent than the specific RARs ligand *all-trans* retinoic acid did. In addition, it has been reported that endogenous RAR/RXR heterodimers are the major functional forms regulating retinoid-responsive elements in human keratinocytes (Xiao *et al*., 1995), which further seems to exclude RXR homodimers as primary regulator for *CYP4F11* expression in HaCaT cells. Therefore, the partner of RXR in the up-regulation of *CYP4F11* could be other nuclear receptors which were not tested in this study or some unknown orphan nuclear receptors whose interaction with RXRs has not yet been clarified. It is worth noting that data from the luciferase assay (Supplemental Fig. 1) demonstrated the down-regulation of CYP4F11 promoter activity by retinoic acids and

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RAR agonist TTNPB whereas RXR agonist LG268 was not able to induce the promoter activity. This may be ascribed to the limited length of the promoter sequence we used in this assay. Two putative RXRβ binding sites were found in this promoter region (Fig. 6a). However, it is possible that other untested or unknown RXR binding sites beyond the current sequence are essential for the regulation of CYP4F11 by RXR agonists.

It is also worth noting that differences in cell type and cell status may greatly affect the regulation pattern of *CYP4F11*. We have previously reported that retinoid treatment of differentiated human primary keratinocytes induced the expression of CYP4F11 as well as other CYP4F subfamily members and this induction response seems to be mediated by RAR/RXR heterodimers (Kalsotra *et al*., 2008). These differences in response to retinoid treatments are likely attributable to the fact that HaCaT cells are a spontaneously arising cell line derived from keratinocytes. Culture state whether proliferating or differentiated plays a significant role in the type of response to retinoid treatment, although the expression pattern of retinoid receptors in HaCaT cell (Fig. 1a) appears to be the same as primary human keratinocytes (Elder *et al*., 1992). Such differences are also seen in the comparison of expression levels of CYP4Fs among various tissues. For instance, in rat lung CYP4F1 is absent and CYP4F6 comprises 95% of CYP4F expression while in kidney CYP4F1 expression comprises 90% of CYP4F expression while CYP4F6 expression accounts for only 8% (Kalsotra and Strobel, 2006).

Retinoic acids are important for skin development and they are also therapeutic agents against many skin diseases such as inflammation and cancer. Therefore, the regulation of *CYP4F11* by retinoic acids may result in the drug-drug interaction between retinoic acids and CYP4F11-targeted drugs. In addition, inflammation and cancer which stimulate

JNK/AP-1 pathway may also affect CYP4F11 drug metabolizing activity. Although CYP4F11 is the most active drug metabolizer in the CYP4F subfamily, no primary endogenous substrate for CYP4F11 has been uncovered. As the functions of CYP4F11 in normal physiological conditions and under disease conditions are clarified, *CYP4F11* regulatory mechanisms will be known more completely and their significance will become clearer.

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ACKNOWLEDGEMENTS

We acknowledge provision of chemicals by Dr. Peter J.A. Davies.

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FOOTNOTES

This work was supported by the National Institutes of Health [Grants NS44174, AR45603].

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DMD Fast Forward. Published on October 7, 2009 as DOI: 10.1124/dmd.109.029025
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LEGENDS FOR FIGURES

Figure 1. Expression of nuclear receptors RARs and RXRs in HaCaT cells and inhibition of *CYP4F11* expression by retinoic acids. (a). Expression of RARs and RXRs in HaCaT cells. Total RNA was isolated from HaCaT cells and an aliquot of 200ng of the total RNA was used to run RT-PCR assay with primers specific for each RAR and RXR isoform. The size of each PCR product is indicated in the Supplemental Table 1. **(b).** Inhibition of *CYP4F11* transcripts by retinoic acids. Cells were treated with 0.1μM, 1μM or 10μM 9-*cis* or all-*trans* retinoic acid for 3 days. Cells treated with 0.1% DMSO were used as the vehicle control. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents n=5. ** $P< 0.01$ compared with the relative control group. **(c).** Inhibition of CYP4F11 protein expression by retinoic acids. Cells were treated with 10μM 9-*cis* or all-*trans* retinoic acid for 3 days. Whole cell lysates were isolated and totally 30μg protein from each sample was subjected to western blot assay using CYP4F11 antibody. The amount of β-actin expression from each sample was used as the loading control. **(d).** Time-course and dose effects of retinoic acids on the protein expression of retinoid receptors. Cells were treated with 0.1μM, 1μM or 10μM 9-*cis* or all-*trans* retinoic acid and collected at different time points as indicated in the figure. Cells treated with 0.1% DMSO were used as the vehicle control. Whole cell lysates were isolated and totally 20μg protein from each sample was subjected to western blot assay using antibodies specific for RARα, RARγ, RXRα, RXRβ, respectively. The amount of β-actin expression from each sample was used as the loading control.

Figure 2. Induction of *CYP4F11* by cytokines through the JNK pathway. (a). Effects of cytokines and TPA on *CYP4F11* transcripts in HaCaT cells. Cells were treated with

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10ng/ml TNFα, 10ng/ml IL-1β or 100ng/ml TPA for 24 hours. Cells treated with 0.1% DMSO or 0.1% BSA were used as the vehicle control. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents n=5. **P*< 0.05, ** *P*< 0.01 compared with the control group. **(b, c).** Effects of pathway inhibitors on the induction of *CYP4F11* by cytokines. HaCaT cells pre-incubated with or without SP600125 (JNK inhibitor) or SB203580 (p38 inhibitor) were treated with 10ng/ml TNFα (b) or 10ng/ml IL-1β (c). At 24h post treatment, total RNA was isolated and the expression of *CYP4F11* was quantitated by qRT-PCR. Cells pre-incubated with 0.1% DMSO and then treated with 0.1% BSA were used as the vehicle control. Each data point represents n=5. ***P*< 0.01 compared with the relative control group. **(d).** TPA had no effect on c-Jun phosphorylation in HaCaT cells. Cells were treated with 100ng/ml TPA and collected at different time points as indicated in the figure. Whole cell lysate was isolated from each sample and 20μg proteins of each sample were loaded for western blot assay. β-actin was used as the loading control. **(e).** Effects of TNFα on c-Jun phosphorylation in HaCaT cells. Cells pre-incubated with or without 20μM SP600125 were treated with 10ng/ml TNFα and collected at different time points as indicated in the figure. Whole cell lysate was isolated from each sample and 15μg proteins of each sample were loaded for western blot assay. β-actin was used as the loading control. **(f).** Inhibition effects of SP600125, 9-*cis* retinoic acid and all-*trans* retinoic acid on TNFα-induced c-Jun phosphorylation in HaCaT cells. Cells pre-incubated with or without 20μM SP600125, 1μM 9-*cis* retinoic acid or 1μM all-*trans* retinoic acid were treated with 10ng/ml TNFα and collected at different time points as indicated in the figure. Whole cell lysate was isolated from each

sample and 15μg proteins of each sample were loaded for western blot assay. β-actin was used as the loading control.

Figure 3. JNK pathway is not the sole mechanism mediating *CYP4F11* regulation. (a). Effects of TNFα on cells treated with retinoic acids. Cells pre-incubated with or without 10μM 9-*cis* or all-*trans* retinoic acid were treated with 10ng/ml TNFα for 24 hours. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents n= 5. **P*<0.05 compared with the negative control. **(b).** Effects of JNK inhibitor SP600125 on CYP4F11 transcription in HaCaT cells. Cells were treated with 0.1% DMSO (vehicle control) or 20μM SP600125 for 24 hours. The expression of *CYP4F11* was quantitated by qRT-PCR. Each data point represents n=6. **P*< 0.05 compared with the control group.

Figure 4. Effects of LG268 on *CYP4F11* expression in HaCaT cells. (a, b). Effects of LG268 on the transcription of *CYP4F11*. HaCaT cells were treated with DMSO (vehicle control, n=5) or 1μM LG268 (n=6) for three days. The *CYP4F11* mRNA level was quantitated by qRT-PCR (a). ***P*< 0.01 compared with the control group. Aliquots of 200ng of total RNA from each sample were used to run RT-PCR (b) with primers specific for *CYP4F11*. **(c).** Effects of LG268 on expression of CYP4F11 protein. HaCaT cells were treated with or without 1μM LG268 for two or three days. Cells treated with 0.1% DMSO were used as the negative control. Whole cell lysate was isolated and 20μg of total protein from each sample was loaded to run western blot assay. β-actin was used as the loading control.

Figure 5. Effects of TTNPB on *CYP4F11* expression in HaCaT cells. HaCaT cells were treated with 0.1% DMSO, 1μM TTNPB or 1μM TTNPB plus 1μM LG268 for three

days. **(a).** The *CYP4F11* mRNA level was quantitated by qRT-PCR. Each data point represents n= 6. ***P*< 0.01 compared with the control group. **(b).** Whole cell lysate was isolated and 30μg of total protein from each sample was loaded to run western blot assay. β-actin was used as the loading control.

Figure 6. Scheme for CYP4F11 regulation. (a). Putative HRE and AP-1 binding sites in the promoter region of CYP4F11 gene. Sites shown were predicted using AliBaba2.1. Figure was generated using Biomatters Geneious Pro 4.5.3 software. **(b).** CYP4F11 regulation model. The JNK/ AP-1 pathway and the RAR/RXR pathway modulate the transcription of *CYP4F11* in HaCaT cells. Adobe Illustrator CS4 was used to draw this model.

Supplemental Figure 1. Effects of TNFα and retinoid receptor agonists on the CYP4F11 promoter activity. The effect of TNFα and retinoid receptor agonists were tested in HaCaT cells using a luciferase assay. Promoter fragments were generated by PCR as described under materials and methods, ligated and cloned upstream of the promoterless luciferase reporter gene ** vector as indicated. Twenty-four hours after transfection, cells were treated with or without retinoid agonists (all at the concentration of 1μM) for 2 days and then treated with 10 ng/ml TNFα for 24 hours. Data are presented as the mean luciferase activity adjusted for ß-gal activity (±SEM) and compared with control wells of three independent transfections. Each data point represents n=3. **P*< 0.05, **P< 0.01 compared with the control group.

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Table 1. Sequences of primers for RARs, RXRs and CYP4F11 for RT-PCR.

Transcript	Oligonucleotide sequences (5'-3')		Product size / Annealing temperature
RAR-α	Forward	GGCATGTCCAAGGAGTCTGT	420 bp / 60ºC
	Reverse	GTCCGAGAAGGTCATGGTGT	
RAR-β	Forward	GAGAGGTGGCATTGATCCAGG	435 bp / 62ºC
	Reverse	GGCCTGGGCCAGCCTGACCTC	
RAR-γ	Forward	TCGAGATGCTGAGCCCTAGCTTCC	351 bp / 58ºC
	Reverse	CATGCCCACTTCGAAGCACTTCTGT	
RXR-α	Forward	AATGAGGTGGAGTCGACCAG	400 bp / 60ºC
	Reverse	TCAGCACCCTGTCAAAGATG	
RXR-β	Forward	GGACAGAAGCTCAGGCAAAC	540 bp / 60ºC
	Reverse	AATGGATCGGTGTGAGAAGG	
RXR-γ	Forward	TGTGGTCAACAGTGTCAGCA	392 bp / 60ºC
	Reverse	TCTTGCACAGCTTCCCTCTT	
CYP4F11	Forward	GCCTCAGGATCCCACCCTCCAT	290 bp / 65ºC
	Reverse	ATGTGGTCACCAGCTGGGTCAATGT	

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Figure 1

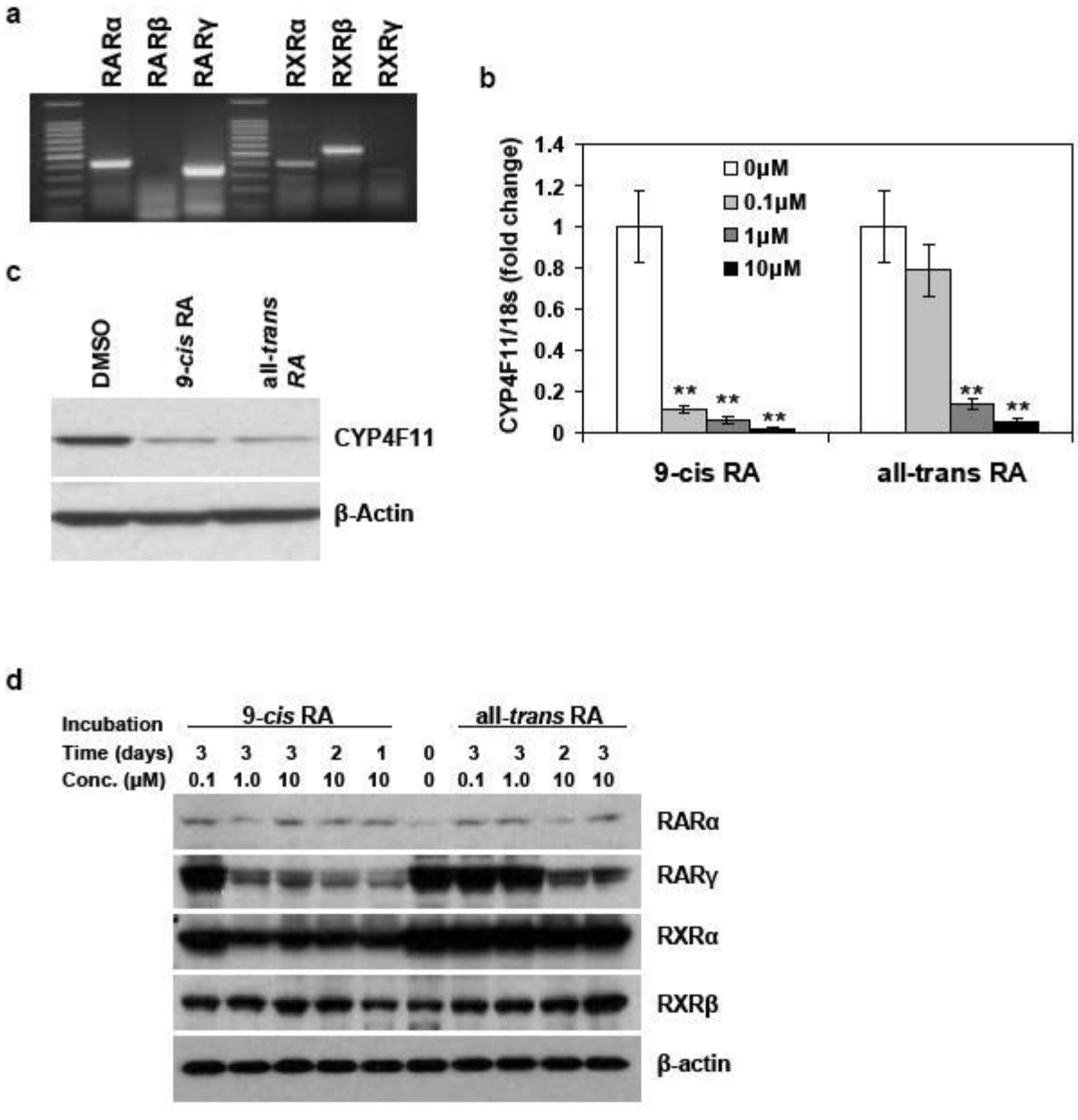

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Figure 2

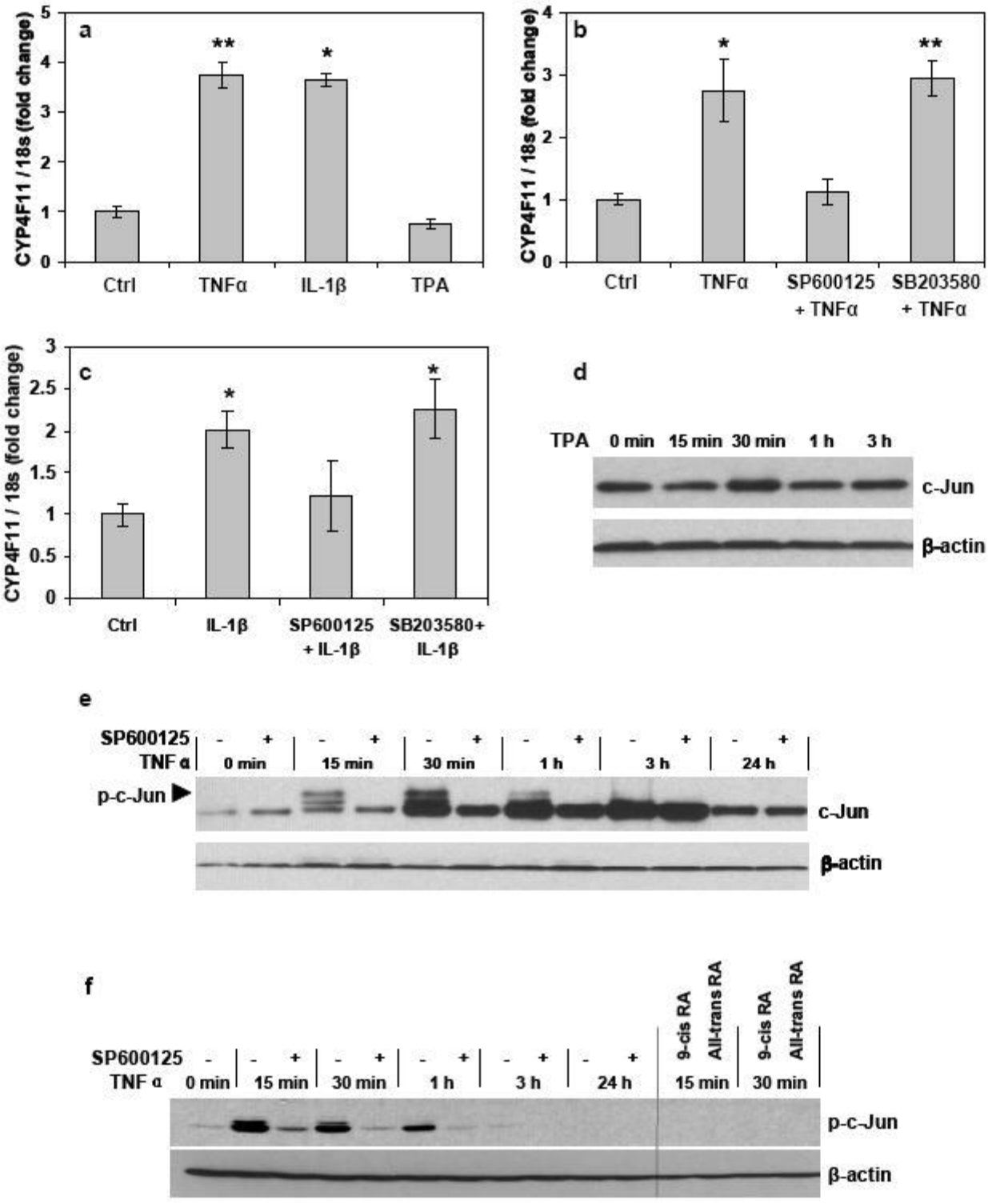

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Figure 3

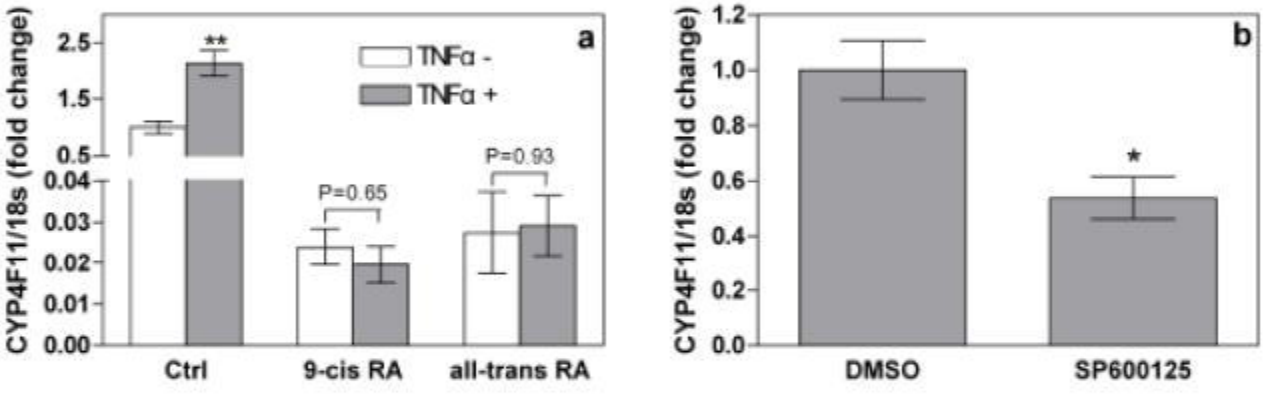

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Figure 4

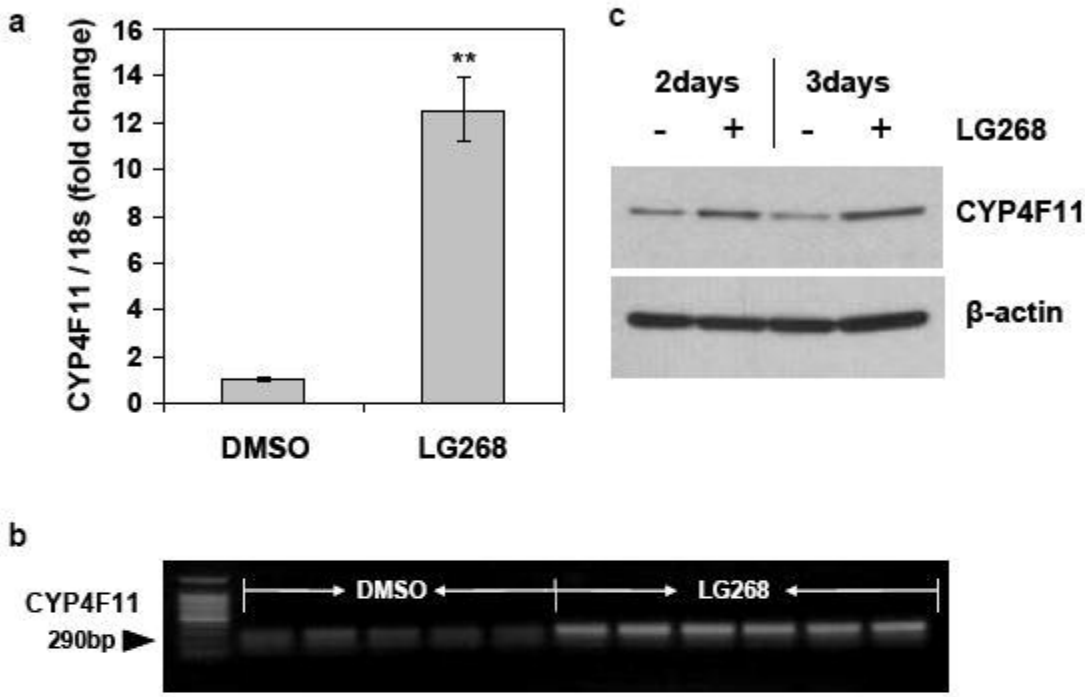

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Figure 5

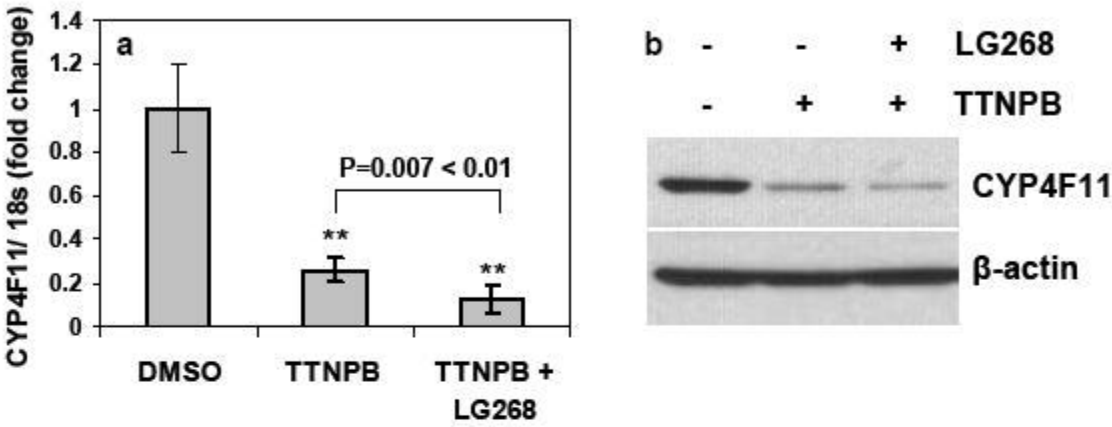

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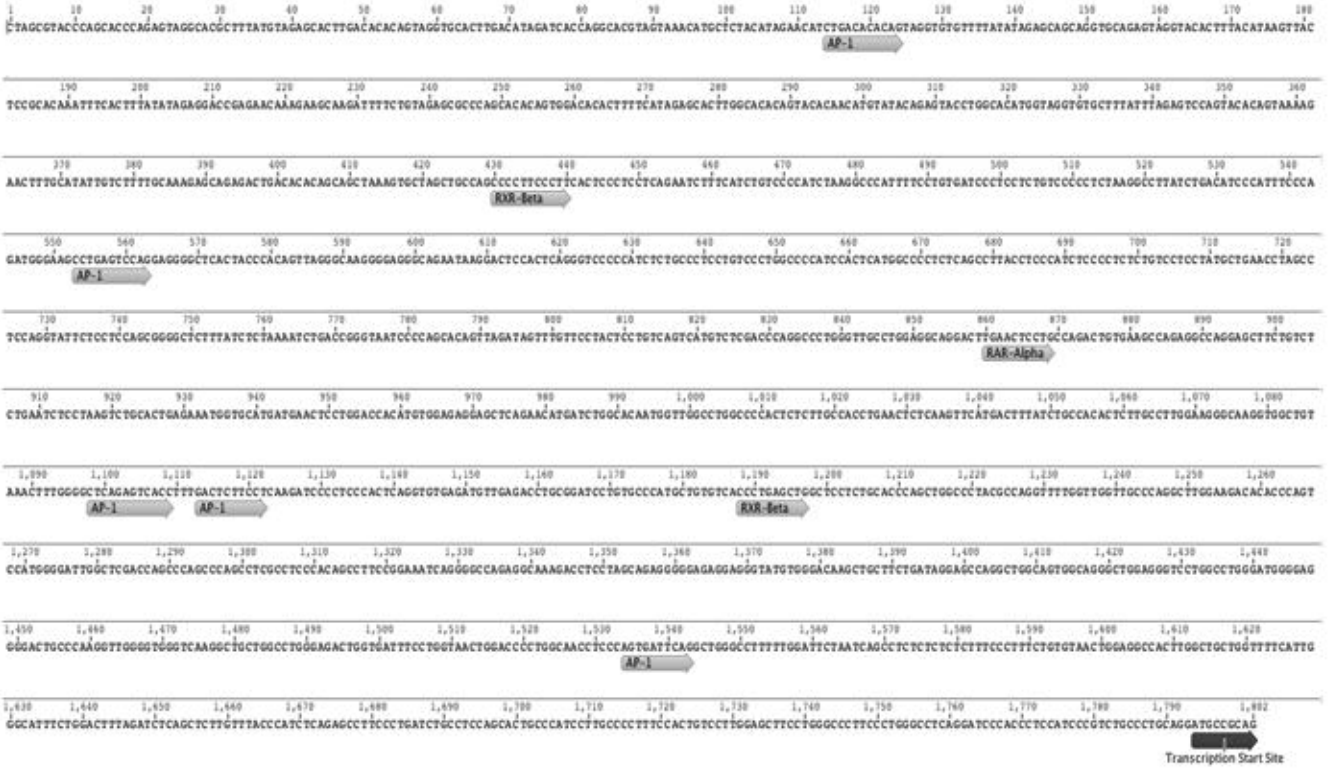
Figure 6

a



b