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# MEK-ERK is an endogenous signal retaining the nuclear receptor CAR in the cytoplasm of mouse primary hepatocytes

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Running title; ERK regulation of CAR nuclear translocation

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Abbreviations used:

CAR, constitutive active/adrostane receptor; TCPOBOP, 1, 4-Bis[2-(3,5-dichloropyridyloxy)]benzene; EGF, epidermal growth factor; HGF, hepatocytes growth factor; MEK, mitogen-activated protein/exteracellular signal-regulated kinase kinase; ERK, exteracellular signal-regulated kinase; CCRP, cytoplasmic CAR retention protein.

**Abstract**

The nuclear receptor CAR is sequestered in the cytoplasm of liver cells before its activation by therapeutic drugs and xenobiotics such as phenobabrital (PB) and 1, 4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in mouse liver, the regulatory mechanism of which remains poorly understood. Given the finding that epidermal growth factor repressed PB activation of CAR-mediated transcription (Bauer et. al., *Mol. Pharmacol*., *65,* 172-180, 2004), here we investigated the regulatory role of hepatocytes growth factor (HGF)-mediated signal in sequestering CAR in the cytoplasm of mouse primary hepatocytes. HGP treatment effectively repressed the induction of endogenous *CYP2b10* gene by PB and TCPOBOP in mouse primary hepatocytes. On the other hand, inhibition by U0126 of a HGF-down stream kinase MEK induced the *Cyp2b10* gene and up-regulated the CAR-regulated promoter activity in the absence of TCPOBOP. HGF treatment increased phosphorylation of ERK1/2 in the cytosol, thus decreasing the TCPOBOP-induced nuclear accumulation of CAR. In contrast, U0126 dephosphorylated ERK1/2 and increased nuclear CAR accumulation in the absence of TCPOBOP. These results are consistent with the conclusion that the HGF-dependent phosphorylation of ERK1/2 is the endogenous signal sequestering CAR in the cytoplasm of mouse primary hepatocytes.

**Introduction**

The nuclear constitutive/androstane receptor CAR is a xeno-sensing transcription factor that regulates numerous hepatic genes in response to a large group of chemicals and therapeutic drugs. Phenobarbital (PB) represents this group of xenobiotics and not only induces drug metabolism and secretion but also elicits pleiotropic effects on liver functions. These effects include metabolism and secretion of endobiotics such as bilirubin, charges in energy metabolism, cell growth, cell-cell communication, and also includes tumor promotion (Honkakoski and Negishi, 1998a). Activation of CAR by drugs like PB is paramount to elicit these effects by up- or down-regulating the genes that encode the key proteins and enzymes for these liver functions (Kodama and Negishi, 2006). As the function of CAR has expanded, deciphering the molecular mechanism of its activation by drugs is an urgent subject of current investigations.

Mouse hepatocytes retain CAR in the cytoplasm, thus making the nuclear translocation an initial step of its activation by drugs. CAR forms a complex with Hsp90 and co-chaperone CCRP (cytoplasmic CAR retention protein) in the cytoplasm. In response to PB, the complex recruits protein phosphatase 2A (PP2A) prior translocation of CAR into the nucleus. The protein phosphatse inhibitor okadaic acid (OA) represses PB-induced nuclear translocation of CAR in mouse primary hepatocytes (Kawamoto et. al., 1999). PB triggers the nuclear translocation without directly binding to the receptor (Swales and Negishi, 2004). These facts are indicative of that cellular signals are involved in regulating the cytoplasmic retention and nuclear translocation of CAR. Recently, two

observations providing insight into such a signal have been reported: the attenuation by epidermal growth factor (EGF) CAR-mediated trans-activation of PBREM by PB and the augmentation by the MEK inhibitor U0126 of PB induction of the *CYP2B* gene in rat primary hepatocytes (Bauer et. al., 2004; Joannard et. al., 2006). MEK is a downstream protein kinase of EGF signaling and activates ERK, Exteracellular Signal-Regulated Kinase. Here we have investigated MEK-ERK signal for its ability to regulate the intracellular localization of CAR in mouse primary hepatocytes. The experimental results presented here suggest that the activation of ERK is the signal retaining CAR in the cytoplasm.

## Materials and Methods

**Reagents and plasmids:** PB, TCPOBOP and ITS liquid media supplement were obtained from Sigma (St. Louis, MO); HGF form Chemicon (Temecula, CA); U0126 from Promega (Madison, WI); EGF and U0124 from Calbiochem (San Diego, CA); anti-rabbit IgG from Santa Cruz biotechnology (Santa Cruz, CA); anti-ERK1/2 and anti-phospho-ERK1/2 from Cell Signaling (Danvers, MA); androstenol from Steraloids (Newport, RI). All plasmids used were previously constructed; pcDNA3.1/V5-mCAR, $(NR1)_5$-tk-luciferase reporter, and -1.8 kb *CYP2B6* promoter in pGL3, phRL-tk (Sueyoshi et al., 1999; Zelko et al., 2001; Kobayashi et al., 2003; Swales et al., 2005).

**Primary hepatocytes:** Mouse primary hepatocytes were isolated from 6-8 weeks- old $Car^{-/-}$ and $Car^{+/+}$ males using a two step collagenese perfusion as previously described (Honkakoski et. al., 1996; 1998b; Yamamoto et al., 2004). Hepatocytes were suspended in Hanks' balanced salt solution containing collagenese (0.4-0.5 mg/ml), 10 mM Hepes, pH 7.4 and porcine insulin (1 μM), collected by low speed centrifugation, resuspended in pre-warmed Williums' E media supplemented with 7% Fetal bovine serum, porcine insulin (0.1 mg/ml), transferrin (55 μg/ml), sodium selenite (50 ng/ml), streptomycin (0.1 mg/ml) and penicillin G (100 unit/ml) and seeded on 6 well plates or 10cm dishes. One hour after seeding, the medium was changed to prewarmed Williums' E media supplemented with dexamethasone (5 nM), sodium selenite (50 ng/ml), streptomycin (0.1mg/ml) and penicillin G (100 unit/ml) with or without a given chemical.

**RNA preparation and RT-PCR:** Total RNAs were extracted from the hepatocytes treated PB (1 mM), TCPOBOP (250 nM), EGF (10 μg/ml), HGF (10 μg/ml), U0126 (2.5 μM, or 25 μM), or U0124 (25 μM) for 8 h, using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) and real time PCR was performed using ABI Prism 7700 (Applied Biosystems, Foster City, CA). The CYP2B10 cDNA was amplified using 5'-AAAGTCCCGTGGCAACTTCC-3' and 5'-TCCCAGGTGCACTGTGAACA-3' for 5'- and 3'-primers, respectively. Amplified cDNA was measured using 6FAM-ACCCCGTCCCCTGCCCCTCTT-TAMRA as a CYP2B10 probe. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified as an internal control using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The levels of a given mRNA were normalized to the GAPDH mRNA level.

**Luciferease reporter assays:** HepG2 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum. Mouse CAR (mCAR) expression plasmids (0.1 μg) were cotransfected with –1.8k-pGL3 (0.1 μg) and phRL-tk (0.1 μg) into HepG2 cells using FuGENE6 transfection reagent (Roche, Indianapolis, IN). Mouse primary hepatocytes were co-transfected with -1.8 kb-CYP2B6 promoter-pGL3 (10 μg) and phRL-tk (5 μg) using electroporation. After being treated with DMSO or a given cheimcal at the concrntrations indicated, the cells were lysed and subjected to luciferase assays using the dual-luciferase reporter assay system (Promega, Madison, WI).

**Western blot:** The nuclear extracts and cytosolic fractions were prepared from these hepatocytes using the methods previously established (Dignam et. al., 1983). Nuclear and cytosolic proteins were resolved on a SDS-10% polyacrylamide gel and transferred to PVDF membrane using Hoefer SemiPhior (Amersham Pharmacia Biotech., Piscataway, NJ) with NuPAGE transfer buffer (Invitrogen). See Blue Plus2 Pre- Stained Standard (Invitrogen) was used as molecular weight markers. Western blot analysis was performed by using anti-rabbit IgG, anti-ERK1/2, anti-phospho-ERK1/2 or anti-CAR polyclonal antibody (Kobayashi, et al., 2003). Protein bands were visualized using ECL Plus Western blotting detection reagent (Amersham Bioscience, Little Chalfont, Buckinghamshire, UK).

**Results and Discussion**

**ERK-mediated regulation of *Cyp2b10* gene:** First, we treated mouse primary hepatocytes with EGF or HGF (hepatocyte growth factor) and confirmed the fact that growth factors repression of the *Cyp2b10* gene. Both EGF and HGF repressed the induction of *Cyp2b10* gene by PB and TCPOBOP in the experimental conditions of our mouse primary hepatocytes (Fig. 1A). We then investigated whether or not the downstream kinase ERK1/2 played the role in this repression. Both HGF and EGF transduce their signal by activating MAP kinase kinase (MEK1/2) that phosphorylates ERK1/2 to further transfer the signal (Cobb et. al., 1991; Seger and Krebs, 1995). U0126 is the specific MEK1/2 inhibitor commonly used to inactivate ERK1/2, thus repressing growth factor signaling (Favata et. al., 1998; DeSilva et. al., 1998). Mouse primary hepatocytes were treated with 2.5 μM or 25 μM of U0126, from which cytosols were prepared for Western blot to measure the degree of the MEK inhibition as dephosphorylation of ERK1/2 using anti-phosphorylated ERK antibody. To significantly decrease the phosphorylation of ERK1/2 25 μM was the concentration of U0126 required (Fig. 1B, inset). From these hepatocytes, RNAs were isolated and subjected to real time PCR to measure CYP2B10 mRNA. In the absence of TCPOBOP, U0126 increased the mRNA 4-fold in the hepatocytes prepared from $Car^{+/+}$ but not from $Car^{-/-}$ mice (Fig. 1B). This mRNA increase occurred only at 25 μM of U0126, correlating with the dephophorylation of ERK1/2. Taken together, growth factors appeared to repress the CAR-mediated activation of *Cyp2b10* gene through the MEK-ERK pathway. As expected, TCPOBOP induced the CYP2B10 mRNA 8-fold in $Car^{+/+}$ primary hepatocytes

only; U0126 did not further increase this induction, suggesting that ERK regulated a step of the TCPOBOP-activation of CAR (Fig. 1B).

**ERK regulation of CAR-mediated transcription:** The -1.8 kb *CYP2B6* promoter was transfected into mouse primary hepatocytes to investigate the effect of U0126 on the CAR-mediated transcription activity. In the $Car^{+/+}$ primary hepatocytes, U0126, but not U0124 activated the promoter approximately 7-fold, whereas this activation was statistically insignificant in the $Car^{-/-}$ primary hepatocytes (Fig. 2A). These activation patterns of CAR-mediated transcription by U0126 were reminiscent of the corresponding induction of the *Cyp2b10* gene in the mouse primary hepatocytes (Fig. 1B). Since primary hepatocytes are not a suitable system to examine whether U0126 directly activates CAR, we employed transient transfection assays using the CAR-responsive enhancer module (PBREM)-Luc reporter in HepG2 cells (Fig. 2B). U0126 did not further activate the PBREM in the presence or absence of TCPOBOP. We also employed androstenol, the repressor of the constitutive activity of CAR that is routinely used to demonstrate whether a given chemical is a direct CAR activator: such as that TCPOBOP directly bind to CAR and re-activates the androstenol-repressed CAR (Sueyoshi et. al., 1999; Tzameli et al., 2000). No re-activation of the androstenol-repressed PBREM activity was observed in the HepG2 cells (Fig. 2B). Thus, these results suggested that U0126 indirectly activate the CAR-mediated transcription.

**ERK-mediated repression of CAR translocation:** CAR is retained in the cytoplasm of unexposed mouse primary hepatocytes and translocates into the nucleus in response to

CAR activators such as PB and TCPOBOP, thus making the nuclear translocation the initial step of CAR activation (Kawamoto et al., 1999). Since CAR is spontaneously accumulated in the nucleus of HepG2 cells (Kawamoto et al., 1999) in which U0126 did not alter the CAR-mediated transcription, we examined the possibility that U0126 might inhibit the nuclear translocation of CAR in the mouse primary hepatocytes. To this end, mouse primary hepatocytes were treated with U0126 and/or TCPOBOP, from which the cytosols and nuclear extracts were prepared. Western blot analyses of the nuclear extracts showed the accumulation of CAR after the treatment with U0126 in the manner associating with the dephopshorylation of ERK1/2, while the nuclear CAR accumulation by TCPOBOP was independent of the function of ERK1/2 (Fig. 3). Treatment with the inactive inhibitor U0124 neither caused CAR to accumulate in the nucleus nor caused dephosphorylation of the ERK1/2. Thus, the ERK dephopshorylation by U0126 was sufficient to translocate CAR into the nucleus, activating the CAR-mediated transcription and inducing the *Cyp2b10* gene in the absence of CAR activators. Consistent with the repressive role of ERK, HGF increased phosphorylation of ERK, thereby repressing CAR nuclear accumulation of in the mouse primary hepatocytes (Fig. 3B).

We have now shown that ERK is the signal molecule that represses the nuclear translocation of CAR in mouse primary hepatocytes. This finding can explain why EGF repressed the CAR-mediated activation of *CYP2B* genes in rat primary hepatocytes (Bauer et al., 2004). It also agrees with the observation that U0126 augmented the induction of the *CYP2B* gene by PB in rat primary hepatocytes (Joannard et al., 2006). Taken in sum, these evidences are consistent with the conclusion that ERK is a

repressive signal for CAR activation. The fact intriguing us is that once ERK1/2 was de-phosphorylated, CAR moved into the nucleus and activated the transcription of the *Cyp2b10* gene in the absence of TCPOBOP. If CAR retains a high constitutive activity in the primary hepatocytes, as it does in the cell-based transfection assays, then CAR should be able to activate transcription once it is in the nucleus. However, our previous works showed that the $Ca^{+}$/calmodulin kinase inhibitor KN-62 did not prevent the PB-induced nuclear accumulation of CAR but could repress the induction of the *Cyp2b10* gene in mouse primary hepatocytes (Yamamoto et al., 2003). Taking the opposite effects of U0126 and KN-62 into consideration, CAR requires a distinct activation signal in the nucleus and ERK only regulates the nuclear translocation of CAR. The bulk of ERK1/2 was phosphorylated under the conditions used for culturing mouse primary hepatocytes, which might be the reason for why the de-phosphorylation of ERK by PB was not detected (unpublished data). Thus, this kinase provides us with an excellent tool to further investigate the ligand-independent, signal-regulated mechanism of CAR activation by drugs including PB.

In addition to MEK-ERK, AMP-activated protein kinase (AMPK) has recently been suggested to activate CAR to induce the *CYP2B* genes by PB in the human and mouse primary hepatocytes (Rencurel et al., 2005; Rencurel et al., 2006; Shindo et al., 2006). However, the process by which AMPK activates CAR is not clear at the present time. One study, using AMPK KO ($\alpha1/\ \alpha2^{\ LS-/-}$) mice, demonstrated that while AMPK does not regulate the PB-induced nuclear translocation of CAR, it may be involved in the activation of CAR in the nucleus (Rencurel et al., 2006). Another study demonstrated that

the activation of AMPK resulted in the nuclear accumulation of CAR but was not sufficient to activate CAR-mediated transcription (Shindo et al., 2006). PB did not induce the *Cyp2b10* gene in the AMPK KO mice, providing the basis of support for the notion that AMPK mediates PB induction. However, the apparent reason that PB could not induce the *Cyp2b10* gene was that the expression levels of the *Cyp2b10* gene in the livers of non-treated AMPK KO mice was already elevated to the levels that are close to those observed in the PB-treated wild type mice (Rencurel et al., 2006). While AMPK is the activating signal for PB induction, MEK-ERK appears to be the repressive signal in our study. Nevertheless, it is an intriguing question as to whether AMPK and ERK cross talk to generate a cellular signal for CAR activation. We used the inhibitors LY294002 and found that PI3K does not regulate the nuclear translocation of CAR in the mouse primary hepatocytes (data not shown). PKA has also been proposed as a possible signal molecule repressing PB induction in rat primary hepatocytes (Sidhu et al., 1995), but its molecular mechanism is poorly understood. Recent reports have suggested that the activation of PKA and PKC modulated the PXR-mediated induction of *Cyp3a* gene in mouse primary hepatocytes by altering the receptor interactions with co-regulators (Ding and Staudinger, 2004a and 2004b). For all, exciting research remains for the future in the signal-regulated mechanism of activation of the xenobiotic receptors CAR and PXR.

CAR forms a complex with Hsp90 and co-chaperone CCRP in the cytoplasm (Kobayashi et. al., 2003). In response to PB, the complex recruits protein phosphatase 2A (PP2A) prior to translocate CAR into the nucleus, making PB-induced CAR nuclear translocation sensitive to okadaic acid, a protein phosphatase inhibitor in mouse primary hepatocytes

(Yoshinari et. al., 2003; Kawamoto et. al., 1999). Recently, de-phosphorylation of serine-202 of mouse CAR was found to be prerequisite for the nuclear translocation occurred (Hosseinpour et. al., 2005). If ERK directly targets CAR serine-202 can be a possible candidate of ERK phosphorylation, although the target of ERK can be the other components of the cytoplasmic CAR complex such as CCRP and Hsp90. Identifying the phorphorylation site (s) will help us deciphering the molecular basis for the signal-regulated mechanism of CAR activation, and is now the major objective of our research.

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Footnotes

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**Figure Legends**

**Figure 1.** Growth factors regulation of *Cyp2b* gene in mouse primary hepatocytes. (A) Primary hepatocytes were prepared from $Car^{+/+}$ mice and were treated with DMSO (open bar), EGF (10 μg/ml; stippled bar) or HGF (10 μg/ml; closed bar) in the presence or absence of PB (1 mM) and TCPOBOP (250 nM) for 8 hours. From these hepatocytes, total RNAs were isolated for RT-PCR for quantitative measurement of CYP2B10 mRNA. Fold induction was expressed by taking that of the DMSO-treated control hepatocytes as one. Bars mean ± S.D. (B) Primary hepatocytes were prepared from $Car^{-/-}$ and $Car^{+/+}$ mice and were treated with DMSO (open bar), U0126 (2.5 μM; stippled bar and 25 μM; solid bar) or U0124 (25 μM; hatched bar) in the presence and absence of TCPOBOP for 8 hours. Total RNAs were prepared and subjected to RT-PCR for quantitative measurement of CYP2B10 mRNA. Fold induction was expressed by taking that of the DMSO-treated control hepatocytes as one. Bars mean ± S.D. The inset is Western blot showing the de-phophorylation of ERK1/2 by the MEK inhibitor U0126 and its inactive derivative U0124. The cytosols were prepared from the mouse primary hepatocytes and subjected to Western blot analyses using anti-ERK and anti-P-ERK antibodies. Numbers above bands are the concentrations of inhibitors at μM.

**Figure 2.** Regulation of CYP2B promoter activity by MEK-ERK in mouse primary hepatocytes. (A) The -1.8 kb *CYP2B6* promoter-pGL3 (10 μg) were co-transfected with phRL-tk (5 μg) into the mouse primary hepatocytes prepared from $Car^{-/-}$ or $Car^{+/+}$ mice using electroporation, and were treated with DMSO (open bar), U0126 (2.5 μM; stippled

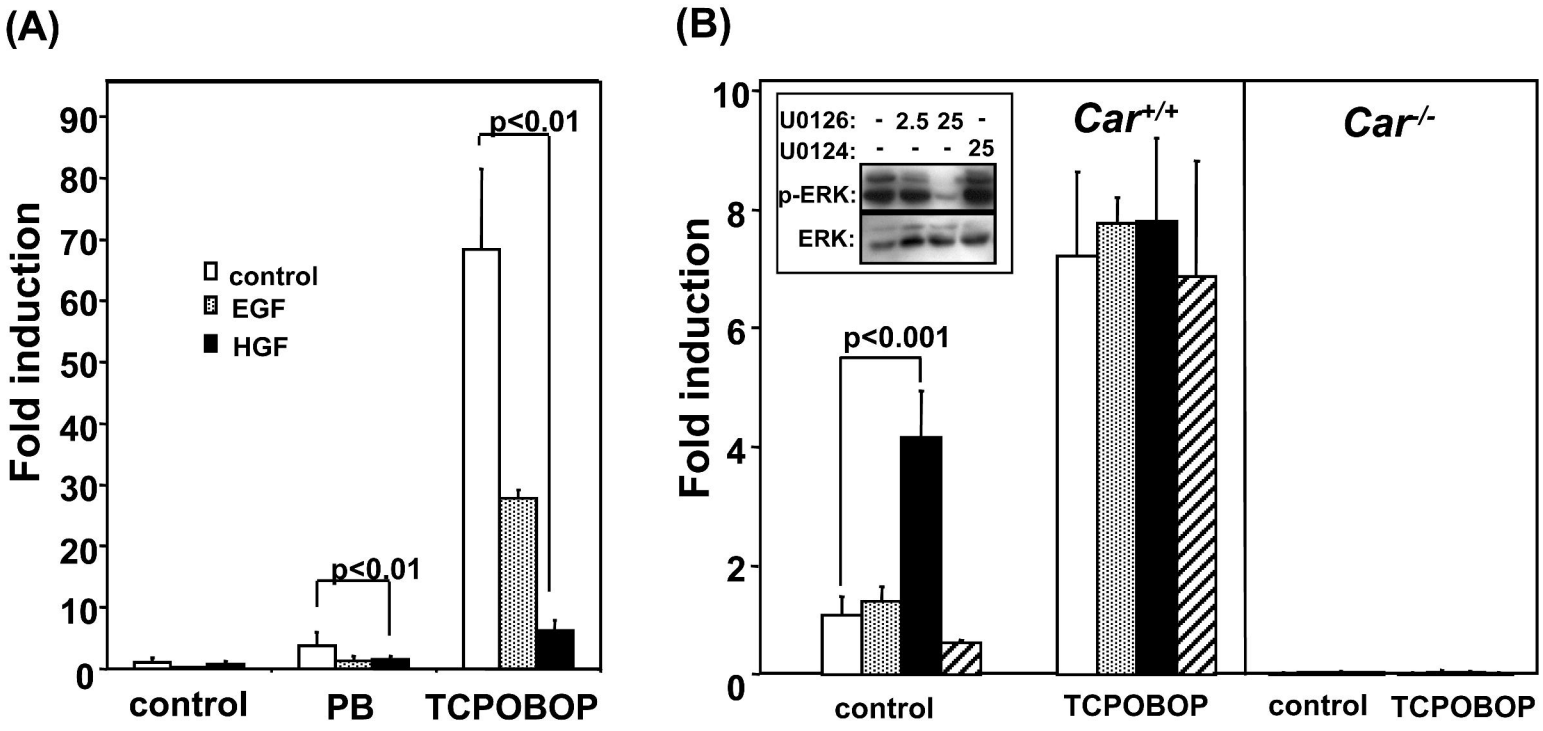
bar and 25 μM; closed bar) or U0124 (25 μM; hatched bar) in the presence or absence of TCPOBOP (250 nM) for 24 hours. These hepatocytes were lysed and subjected to luciferase assays. Fold activation was expressed by taking that of the DMSO-treated control hepatocytes as one. Bars mean ± S.D. (B) mCAR-pcDNA3 (0.1 μg) were co-transfected with PBREM-tk-Luc (0.1 μg) and phRL-tk (0.1 μg) into HepG2 cells for 24 hours. Subsequently, the cells were treated with DMSO (open bar), U0126 (2.5 μM; stippled bar and 25 μM; closed bar) and/or U0124 (25 μM; hatched bar) in the presence of absence of TCPOBOP (250 nM) or androstenol (5 μM) for additional 24 hour and were lysed for luciferase assays. Fold activation was expressed by taking that of the DMSO-treated control hepatocytes as one. Bars mean ± S.D.

**Figure 3.** ERK-mediated regulation of CAR nuclear translocation. (A) Mouse primary hepatocytes were prepared from the *Car*$^{+/+}$ mice and were treated with DMSO, TCPOBOP (250 nM), U0126 (25 μM), U0124 (25 μM) or TCPOBOP plus U0126 for 30 min. For (B), mouse primary hepatocytes were treated with DMSO, TCPOBOP (250 nM), HGF (10 μg/ml) or TCPOBOP plus HGF for 30 min. Both cytosols and nuclear extracts were prepared from these hepatocytes and subjected to Western blot analysis using anti-mCAR, anti-ERK1/2 and anti-phospho-ERK1/2 antibodies.

**Figure 4**. Model of MEK-ERK regulation of CAR translocation. Growth factors, such as HGF, stimulate MEK1/2 phosphorylating ERK1/2 to activate it. Activated ERK1/2 represses the translocation of CAR into the nucleus. U0126 inhibits the activation of ERK1/2 by MEK1/2, making CAR translocating into the nucleus. In the nucleus, CAR

forms a complex with RXR and activates the transcription of *Cyp2b10* gene in mouse primary hepatocytes.

# Figure 1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(B)
Fold induction
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Car+/+
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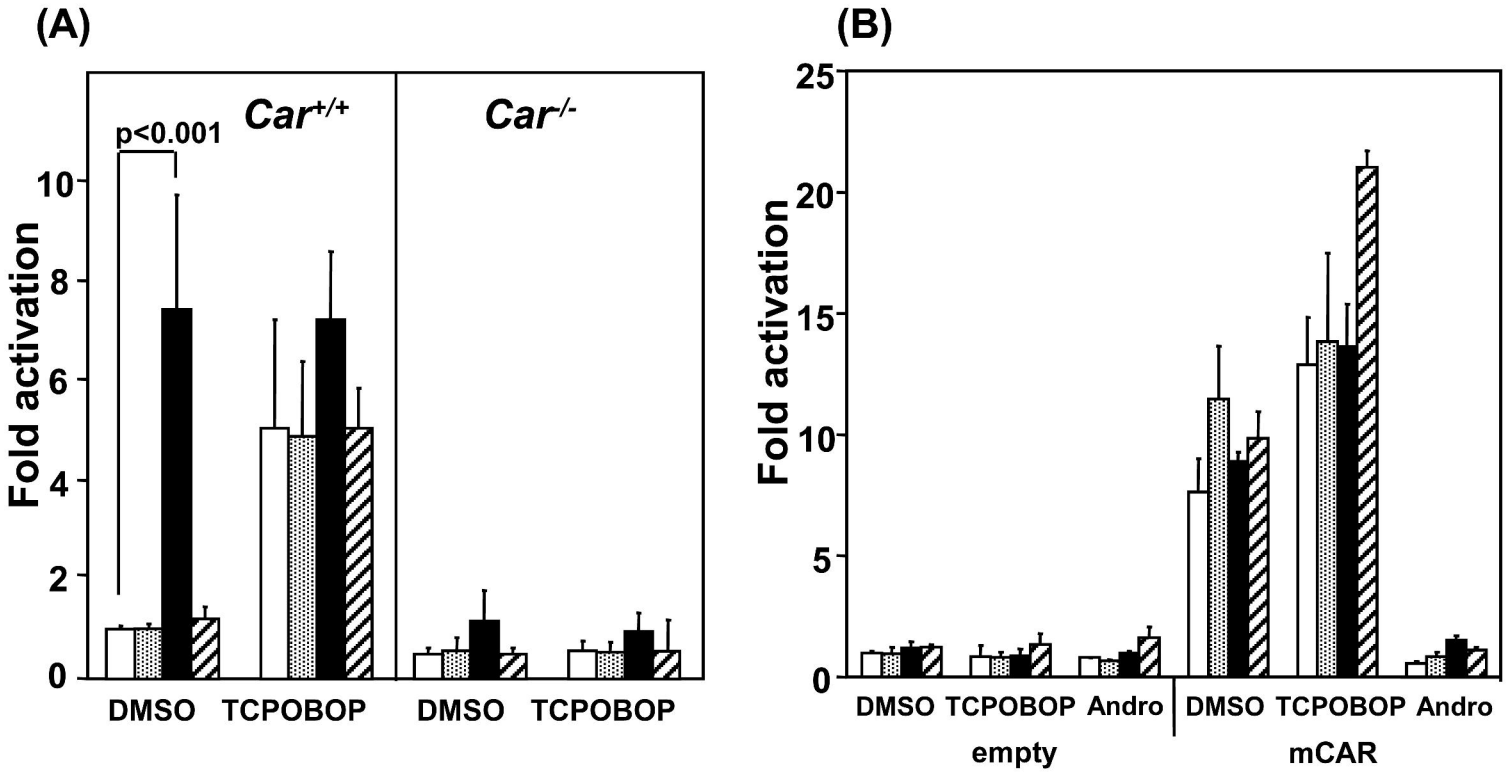
control
TCPOBOP
control
TCPOBOP

# Figure 2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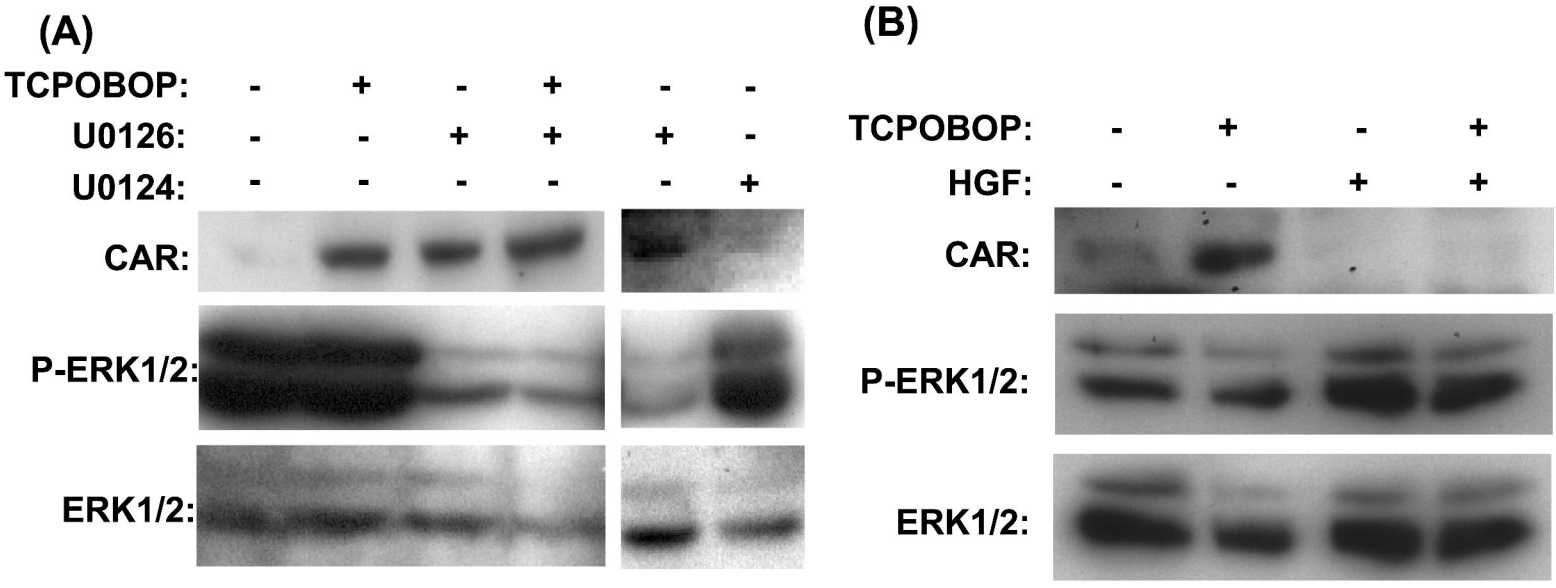
# Figure 3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ERK1/2:

Figure 4