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Separable Roles for rent1/hUpf1 in Altered Splicing and Decay of Nonsense Transcripts

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The mechanism by which disruption of reading frame can influence pre–messenger RNA (pre-mRNA) processing is poorly understood. We assessed the role of factors essential for nonsense-mediated mRNA decay (NMD) in nonsense-mediated altered splicing (NAS) with the use of RNA interference (RNAi) in mammalian cells. Inhibition of rent1/hUpf1 expression abrogated both NMD and NAS of nonsense T cell receptor β transcripts. In contrast, inhibition of rent2/hUpf2 expression did not disrupt NAS despite achieving comparable stabilization of nonsense transcripts. We also demonstrate that NAS and NMD are genetically separable functions of rent1/hUpf1. Additionally, rent1/hUpf1 enters the nucleus where it may directly influence early events in mRNA biogenesis. This provides compelling evidence that NAS relies on a component of the nonsense surveillance machinery but is not an indirect consequence of NMD.

The most comprehensively studied consequence of a premature termination codon (PTC) is accelerated transcript degradation through NMD (*1*). Despite evidence that NMD requires translation, most nonsense transcripts are degraded in the nuclear fraction of mammalian cells (*2*). Additional evidence that disruption of reading frame can influence intranuclear RNA metabolism stems from the observed effects that PTCs can exert on splicing (*3–5*) and intranuclear trafficking of pre-mRNAs (*6*). In selected examples, altered splicing appears to be specifically dependent on disruption of reading frame rather than isolated inactivation of exonic splicing enhancers (ESEs) (*7–9*). The conclusion that nonsense codon recognition occurs in the nucleus is difficult to reconcile with existing tenets regarding the interpretation of reading frame. A prevailing model posits that recognition and degradation of nonsense transcripts by NMD indirectly influences the processing of pre-mRNAs derived from the same allele through an unknown mechanism (*9*, *10*).

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Substantial insight into the mechanism of mammalian NMD has come from studies of the trans-effectors that mediate the process including rent1/hUpf1, rent2/hUpf2, and hUpf3 (*3*). Assembly of these proteins, collectively referred to as the surveillance complex, on nonsense transcripts initiates NMD (*11*). Here, we sought to determine whether nonsense-mediated perturbations of pre-mRNA metabolism rely on the nonsense surveillance machinery. Additionally, we examined whether these effects are an indirect consequence of NMD or are the result of a distinct mechanism.

RNA interference (RNAi) using synthetic short-interfering RNA (siRNA) duplexes was used to inhibit expression of rent1/hUpf1 and rent2/hUpf2 in HeLa cells (*12*, *13*). Western blotting revealed siRNA sequence-specific and near complete (>10-fold) loss of expression of both targeted transcripts (Fig. 1). Transcripts derived from a previously described T cell receptor β (TCRβ) mini-gene (Fig. 2A) (*14*) were monitored under these conditions. In addition to being well-characterized substrates for the NMD pathway, TCRβ nonsense transcripts show translation-dependent alternative splicing that restores the open reading frame (*9*). In untreated cells or cells treated with siRNA directed against an irrelevant target, the PTC-containing transcript was reduced in abundance to less than 20% of wild-type levels, as assessed using a Northern blot probe specific for the VDJ exon (VDJ probe, Fig. 2, A and B). In contrast, RNAi directed against either rent1/hUpf1 or rent2/hUpf2 increased the level of the mutant transcript to greater than 50% of wild-type levels, extending existing evidence (*15–17*) that these factors are essential for NMD.

To assess the effect of diminished rent1/hUpf1 and rent2/hUpf2 expression on NAS, Northern blot analysis was performed with a probe (LV probe, Fig. 2A) that more efficiently detects the transcripts produced by alternative splicing (designated "TCR alt"). Northern blotting revealed that production of TCR alt was induced by the presence of a PTC, as described by Wang *et al.* (*18*) (Fig. 2C). Direct sequencing of reverse transcriptase–polymerase chain reaction (RT-PCR) products demonstrated that all TCR alt transcripts are generated by use of an alternative splice donor in the VDJ exon. In addition, approximately one-third of TCR alt transcripts use an alternative splice acceptor 22 nucleotides upstream of the bona fide splice acceptor for the VDJ exon (Fig. 2A). Both of these alternative transcripts, which cannot be

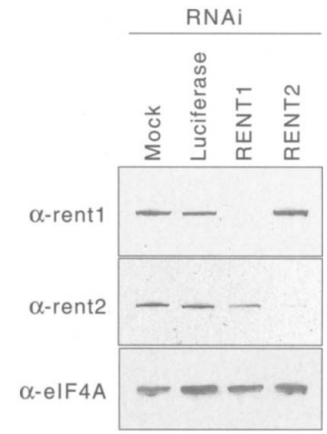


Fig. 1. Sequence-specific inhibition of gene expression with RNAi in mammalian cells. HeLa cells were mock transfected or transfected with siRNA duplexes directed against firefly luciferase (a negative control), rent1/hUpf1, or rent2/hUpf2. Seventy-two hours after transfection, cell lysates were analyzed by Western blotting with antisera specific for rent1/hUpf1 (*17*), rent2/hUpf2 (*11*), or eIF4A as a control for nonspecific effects of RNAi treatment.

individually resolved on Northern blots, extend the open reading frame into the final exon, precluding initiation of NMD.

In contrast to siRNA directed against an irrelevant target, treatment with siRNA directed against rent1/hUpf1 completely inhibited alternative splicing and stabilized the nonsense-containing TCRβ transcript (Fig. 2C). Inhibition of rent2/hUpf2 expression did not affect alternative splicing despite achieving comparable stabilization of nonsense-containing TCRβ mRNA. It is not possible to conclude, however, that NAS is rent2/hUpf2-independent because trace levels of residual protein may be sufficient to support the process. These results provide strong evidence that NAS is not a secondary consequence of NMD, as was previously proposed (*9*, *10*). Deficiency of the Upf proteins in *Saccharomyces cerevisiae* has been shown to increase translational readthrough of nonsense codons (nonsense suppression) (*18*, *19*). Nonsense suppression, assayed with a dual-luciferase reporter system, was not increased after RNAi-mediated inhibition of rent1/hUpf1 or rent2/hUpf2 expression in HeLa cells (fig. S1). This provides compelling evidence that abrogration of NAS is not a secondary consequence of increased nonsense codon readthrough. Taken together, these data suggest that rent1/hUpf1 has a primary role in regulating splice site selection for TCRβ nonsense transcripts.

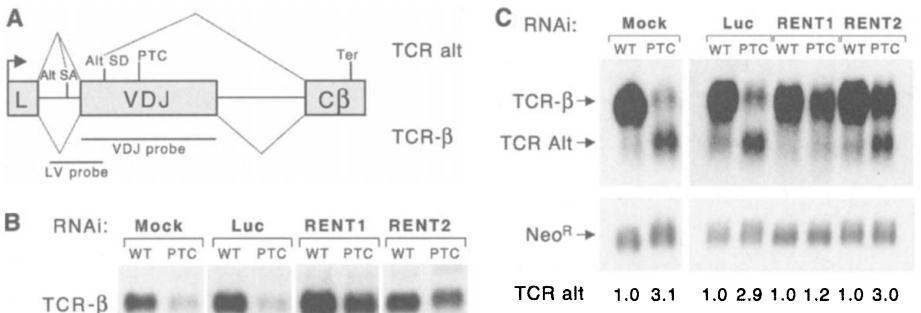


Fig. 2. Effect of diminished rent1/hUpf1 and rent2/hUpf2 expression on NMD and NAS. (**A**) Schematic representation of the TCRβ mini-gene used here. Alt SA and Alt SD indicate the position of the alternative splice acceptor and splice donor sites used in TCR alt transcripts, respectively. Dotted lines represent splicing of TCR alt transcripts (above) or TCRβ transcripts (below). (**B**) Northern blot analysis of polyadenylated [poly(A)] RNA hybridized with the VDJ probe. HeLa cells were treated with RNAi directed against firefly luciferase (Luc), rent1/hUpf1, or rent2/hUpf2 and were transfected with wild-type or PTC-containing TCRβ mini-gene constructs. The transcript encoding neomycin phosphotransferase II (Neo^R), also encoded by the TCRβ plasmid, served as a loading control. The level of the wild-type (WT) TCRβ transcript was normalized to 100 for each condition tested. For this and all subsequent Northern analyses, similar results were obtained for multiple independent experiments. Mean values are shown. (**C**) Northern blot analysis of poly(A) RNA hybridized with the LV probe. For each condition tested, the level of TCR alt produced from the WT transcript was normalized to 1.0.

In order to further investigate the requirement for rent1/hUpf1 in NAS, we developed a system that allowed the replacement of endogenous rent1/hUpf1 with wild-type or mutant recombinantly expressed protein. RNAi specifically silenced expression of both endogenous rent1/hUpf1 and a transiently expressed rent1–green fluorescent protein (GFP) fusion (Fig. 3A, lane 6). A two-nucleotide coding sequence substitution, which does not change the amino acid sequence of the protein, was introduced into the rent1-GFP expression construct within the site targeted by rent1/hUpf1-specific siRNA duplexes. This rent1-GFP variant (designated R1^R)

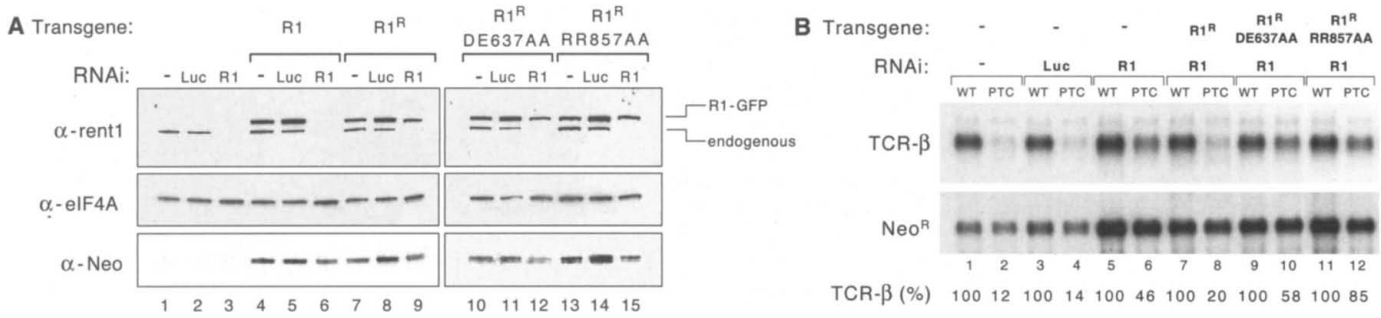
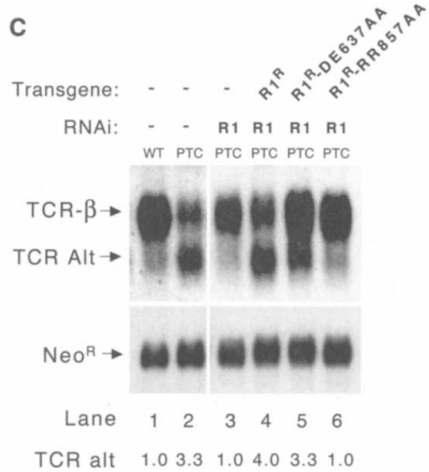


Fig. 3. Replacement of endogenous rent1/hUpf1 with recombinantly expressed isoforms through allele-specific RNAi. (**A**) HeLa cells were treated with siRNA directed against firefly luciferase (Luc) or rent1/hUpf1 (R1) and subsequently transfected with a rent1-GFP fusion construct (R1), a rent1-GFP fusion construct harboring a silent mutation in the siRNA targeting site (R1^R), or constructs encoding additional mutant forms of rent1-GFP (R1^R-DE637AA, R1^R-RR857AA). Cell lysates were analyzed by Western blotting with antisera specific for rent1/hUpf1. To control for equal loading, blots were probed for eIF4A. To control for transfection efficiency of rent1-GFP constructs, blots were probed with an antibody specific for Neo (Cortex Biochem, San Leandro, California) that is encoded on the GFP expression plasmid. (**B**) Northern blot analysis of poly(A) RNA derived from HeLa cells treated with the indicated RNAi and subsequently transfected with the wild-type or PTC-containing TCRβ mini-gene alone or in combination with the indicated rent1-GFP fusion construct. RNA was hybridized with the VDJ probe or with a probe that specifically detects the Neo^R transcript derived from the TCRβ mini-gene. The level of the WT TCRβ transcript was normalized to 100 for each condition tested. (**C**) Northern blot analysis of poly(A) RNA hybridized with the LV probe. Similar results were obtained in three independent experiments. Mean values are shown.



was completely resistant to RNAi treatment (Fig. 3A, lane 9). Northern blot analysis revealed that expression of $R1^R$ in cells pretreated with rent1/hUpf1 siRNA duplexes was sufficient to restore NMD and NAS (Fig. 3B, lanes 7 and 8; Fig. 3C, lane 4). These results provide definitive evidence that the effects of anti-rent1/hUpf1 siRNA duplexes on NMD and splicing are specifically attributable to diminished rent1/hUpf1 expression, as opposed to a non-sequence–specified effect.

We next examined the functional consequence of two dual amino acid substitutions in rent1/hUpf1 (introduced into the $R1^R$ background): $Asp^{637} \rightarrow Ala^{637}$, $Glu^{638} \rightarrow Ala^{638}$ (D637A,E638A or $R1^R$-DE637AA) (*20*) and $Arg^{857} \rightarrow Ala^{857}$, $Arg^{858} \rightarrow Ala^{858}$ (R857A,R858A or $R1^R$-RR857AA). These mutations were chosen because the analogous substitutions in *S. cerevisiae* Upf1p cause loss of NMD function (*21*). Expression of the DE637AA and RR857AA variants occurred at wild-type levels and was resistant to RNAi treatment (Fig. 3A, lanes 12 and 15). Consistent with observations in yeast, these mutant forms of rent1/hUpf1 failed to complement the defect in NMD induced by RNAi treatment directed against rent1/hUpf1 (Fig. 3B, lanes 9 to 12). $R1^R$-DE637AA restored alternative splicing despite failing to support NMD (Fig. 3C, lane 5). $R1^R$-RR857AA was not able to complement alternative splicing (Fig. 3C, lane 6). These data document that NMD and NAS are genetically separable functions of rent1/hUpf1 and provide compelling evidence that they represent distinct cellular processes.

It has been previously reported that rent1/hUpf1 is restricted to the cytoplasmic compartment of mammalian cells (*11*, *22*). Given our finding that this protein can influence pre-mRNA splicing, an intranuclear process, we sought to determine whether rent1/hUpf1 enters the nucleus. HeLa cells were treated with the specific inhibitor of CRM1-mediated nuclear export leptomycin B (LMB) (*23*), and endogenous rent1/hUpf1 was localized by immunofluorescence. After treatment with the drug, we observed dramatic nuclear accumulation of rent1/hUpf1, demonstrating that this protein shuttles between the nucleus and cytoplasm. rent1-GFP also accumulated in the nucleus after LMB treatment. As previously reported, a rent2-GFP fusion protein was not detectable in the nucleus after drug treatment (*24*). Although we cannot exclude the possibility that rent2/hUpf2 shuttles using a LMB-insensitive mechanism, the data are consistent with our finding that near-complete inhibition of rent2/hUpf2 expression does not abrogate NAS (Fig. 4, A and B).

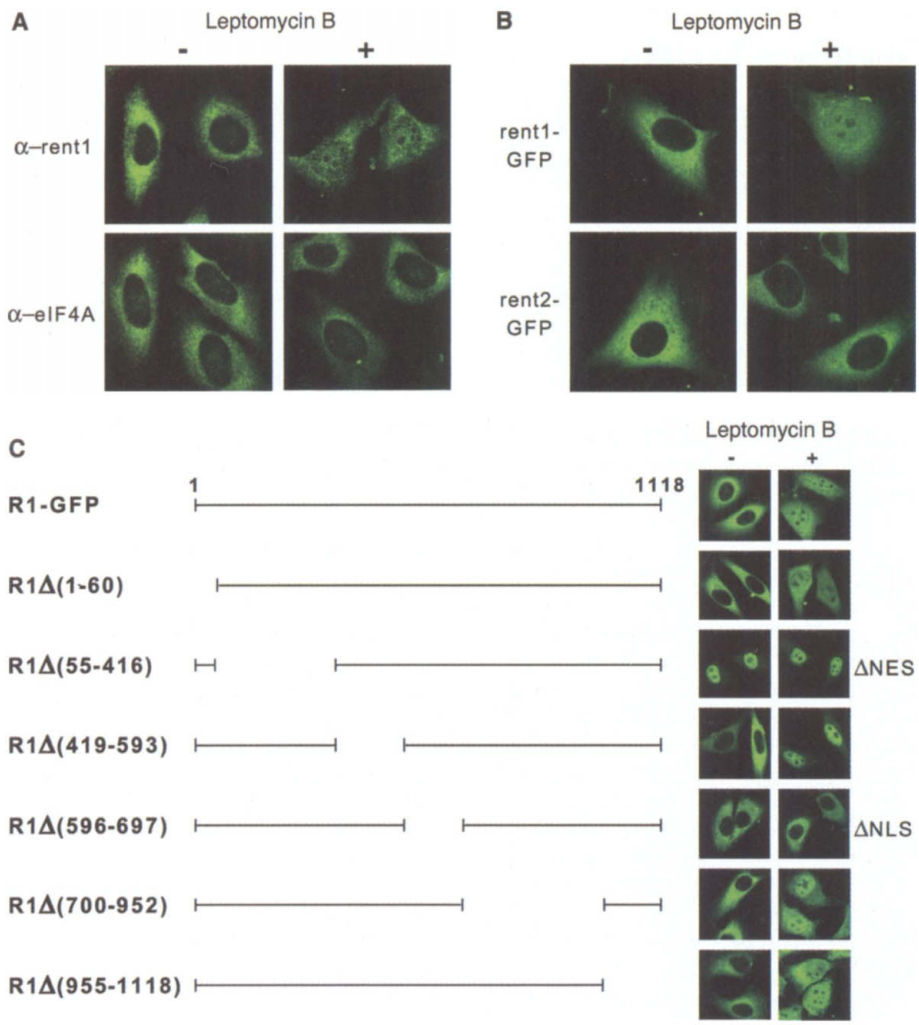


Fig. 4. rent1/hUpf1 shuttles between the nucleus and cytoplasm. (**A**) Subcellular localization of endogenous rent1/hUpf1 in untreated HeLa cells or in cells treated with leptomycin B. eIF4A served as a negative control. (**B**) Subcellular localization of transiently expressed rent1-GFP or rent2-GFP fusion proteins. (**C**) Subcellular localization of rent1-GFP fusion proteins containing contiguous deletions. The identity of rent1-GFP fusion constructs is indicated on the left with numbers referring to deleted residues. NES, nuclear export signal; NLS, nuclear localization signal.

In order to broadly define the domains required for nuclear import and export, the subcellular localization of rent1-GFP fusion proteins containing a series of contiguous deletions was determined in the presence or absence of LMB (Fig. 4C). rent1/hUpf1 lacking residues 55–416 [R1Δ(55-416)] showed near-complete nuclear localization in the absence of LMB, indicating that the nuclear export signal resides in this interval. This result also demonstrates that rent1/hUpf1 nuclear accumulation is not simply an indirect effect of LMB treatment. A deletion encompassing residues 596–697 [R1Δ(596-697)] abolished nuclear accumulation after LMB treatment, suggesting that the nuclear localization signal is contained in this region. Neither of these regions contains consensus nuclear import or export signals, suggesting an atypical trafficking mechanism.

It has been suggested that NAS is the result of direct disruption of cis-acting regulatory elements such as ESEs, which recruit serine-arginine (SR) rich proteins to transcripts (*10*). In order for this model to explain the generation of TCR alt, the disrupted ESE would have to regain the ability to bind trans-factors specifically in the absence of rent1/hUpf1, an unlikely scenario. It has also been proposed that NAS is a secondary consequence of NMD, perhaps resulting from consumption of a factor that regulates pre-mRNA metabolism (*9*) or through transcriptional upregulation of the transcript, allowing detectable accumulation of an alternatively spliced isoform (*10*). Evidence presented here suggests that NMD of TCRβ nonsense transcripts does not underlie production of TCR alt and documents that rent1/hUpf1 provides genetically separable functions essential for these distinct processes. Though these data are consistent with a model that invokes nuclear nonsense surveillance, many mechanistic details remain to be elucidated. For example, the distinguishing characteristics of exons that manifest NAS are unknown. In at least two examples of reading frame–depen-

dent NAS, there is evidence for an operative ESE in the susceptible exon (*25–27*). In view of our results, it is possible that rent1/hUpf1-mediated events selectively impair utilization of such vulnerable (ESE-dependent) exons.

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Fig. S1

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Modulation of Acetaminophen-Induced Hepatotoxicity by the Xenobiotic Receptor CAR

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We have identified the xenobiotic receptor CAR (constitutive androstane receptor) as a key regulator of acetaminophen metabolism and hepatotoxicity. Known CAR activators as well as high doses of acetaminophen induced expression of three acetaminophen-metabolizing enzymes in wild-type but not in CAR null mice, and the CAR null mice were resistant to acetaminophen toxicity. Inhibition of CAR activity by administration of the inverse agonist ligand androstanol 1 hour after acetaminophen treatment blocked hepatotoxicity in wild type but not in CAR null mice. These results suggest an innovative therapeutic approach for treating the adverse effects of acetaminophen and potentially other hepatotoxic agents.

Overdoses of acetaminophen (APAP; also known as 4′-hydroxyacetanilide, *N*-acetyl-*p*-aminophenol, and paracetamol) are the leading cause of hospital admission for acute liver failure in the United States (*1*). Ingestion of amounts of APAP only two to three times the maximum daily recommended dose can cause hepatotoxicity, and higher doses result in centrilobular necrosis that is potentially fatal (*2, 3*). The basis for this toxicity has been well studied. Particularly at high doses, cytochrome P-450 enzymes—especially CYP1A2, CYP2E1, and isoforms of CYP3A—convert APAP to a reactive quinone form, *N*-acetyl-*p*-benzoquinone imine (NAPQI) (*4–7*), that covalently binds to cellular macromolecules and also causes production of reactive oxygen species (*8, 9*). At subtoxic doses, NAPQI is inactivated by glutathione S-transferases (GSTs) via conjugation with reduced glutathione (GSH), but NAPQI accumulates when GSH levels are depleted. Among the numerous GST enzymes, the GSTPi isoforms are particularly effective at inactivating NAPQI (*10*). Their importance in APAP toxicity was confirmed by the unexpected demonstration that knockout mice lacking both GSTPi isoforms are relatively resistant to APAP hepatotoxicity because of a decreased rate of GSH depletion (*11*).

APAP toxicity is increased in both humans and rodents by pretreatment with various inducers of CYP gene expression, including ethanol, an inducer of CYP2E and CYP3A isoforms (*12, 13*), and phenobarbital (PB), a well-known inducer of CYP2B, CYP3A, and other isoforms (*14, 15*). Because the xenobiotic receptor CAR has recently been shown to mediate the effects of PB and other PB-like inducers (*16–19*), we examined the effect of PB and the potent CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on APAP toxicity in wild-type and CAR knockout mice. We treated both strains of mice with inducers or the vehicle control, followed by APAP (250 mg per kg of body weight) (*20*). Neither the inducers alone nor this dose of APAP induced hepatotoxicity, as indicated by either the serum levels of the liver enzyme alanine aminotransferase (ALT) or histologic examination, but animals treated with PB or TCPOBOP plus APAP showed elevated ALT levels and hepatic necrosis at 24 hours (Fig. 1, A and B). The CAR null mice showed no such hepatotoxicity.

Among genes associated with APAP toxicity, PB or TCPOBOP treatment modestly suppressed CYP2E1 mRNA levels but induced CYP1A2, CYP3A11, and GSTPi mRNAs in wild-type animals (Fig. 1C). We observed neither suppression of CYP2E1 nor induction of the other enzymes in CAR null mice. The strong induction of GSTPi expression by PB or TCPOBOP treatments in wild-type mice suggests that GSH depletion could contribute to the xenobiotic-induced toxicity, and wild-type mice pretreated with PB or TCPOBOP showed about a 50% decrease in hepatic GSH 2 hours after administration of APAP (Fig. 1D). To rule out the possibility that the CAR knockout animals are somehow resistant to NAPQI, we directly injected the metabolite into the livers of both wild-type and CAR null mice, and serum ALT levels increased in both by a factor of 5 to 10 (*21*). Hence, both increased NAPQI production and GSH depletion may contribute to xenobiotic-induced APAP toxicity.

To determine whether CAR null mice are

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