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Article

S-Nitrosothiol Signaling Regulates Liver Development and Improves Outcome following Toxic Liver Injury

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SUMMARY

Toxic liver injury is a leading cause of liver failure and death because of the organ's inability to regenerate amidst massive cell death, and few therapeutic options exist. The mechanisms coordinating damage protection and repair are poorly understood. Here, we show that S-nitrosothiols regulate liver growth during development and after injury in vivo; in zebrafish, nitric-oxide (NO) enhanced liver formation independently of cGMP-mediated vasoactive effects. After acetaminophen (APAP) exposure, inhibition of the enzymatic regulator S-nitrosoglutathione reductase (GSNOR) minimized toxic liver damage, increased cell proliferation, and improved survival through sustained activation of the cytoprotective Nrf2 pathway. Preclinical studies of APAP injury in GSNOR-deficient mice confirmed conservation of hepatoprotective properties of S-nitrosothiol signaling across vertebrates; a GSNOR-specific inhibitor improved liver histology and acted with the approved therapy N-acetylcysteine to expand the therapeutic time window and improve outcome. These studies demonstrate that GSNOR inhibitors will be beneficial therapeutic candidates for treating liver injury.

INTRODUCTION

Acetaminophen (APAP) is the most widely used analgesic in the United States and the most common cause of acute liver failure (Bernal et al., 2010), caused by oxidative stress and necrosis (Jaeschke et al., 2012). The only available therapy is the antidote N-acetylcysteine (NAC). However, its use is limited to the first few hours after ingestion, and delayed treatment further increases mortality (Craig et al., 2012). Consequently, there is an unmet clinical need for therapeutic options for treating toxic liver injury. Compounds that exploit the liver's remarkable capacity to regenerate, rather than prevent toxic effects, may provide therapeutic opportunities for treating toxic liver injury.

Many of the signaling pathways involved in liver repair also play a critical role in the formation of liver progenitors and in liver growth during development (Goessling et al., 2008; Lade and Monga, 2011). The zebrafish has recently emerged as an attractive system to study liver development and regeneration, given that many of the molecular programs are highly conserved with mammals (Chu and Sadler, 2009). Further, we established a clinically relevant model of APAP-induced liver toxicity in zebrafish (North et al., 2010) and developed surgical resection models to study liver regeneration (Goessling et al., 2008; Sadler et al., 2007). In order to identify regulators of hepatic growth that could lead to therapeutic approaches for patients with liver failure, we conducted a chemical genetic screen for modifiers of liver development in vivo (Garnaas et al., 2012) using zebrafish and identified compounds targeting nitric oxide (NO) regulation.

NO is a well-characterized signaling molecule that plays a crucial role in regulating vasodilation and angiogenesis. NO can regulate signaling pathways by two distinct mechanisms. The best-known action of NO is to activate soluble guanylate cyclase, which causes cGMP accumulation and vasodilation (Murad, 2006). Many biological effects of NO, however,

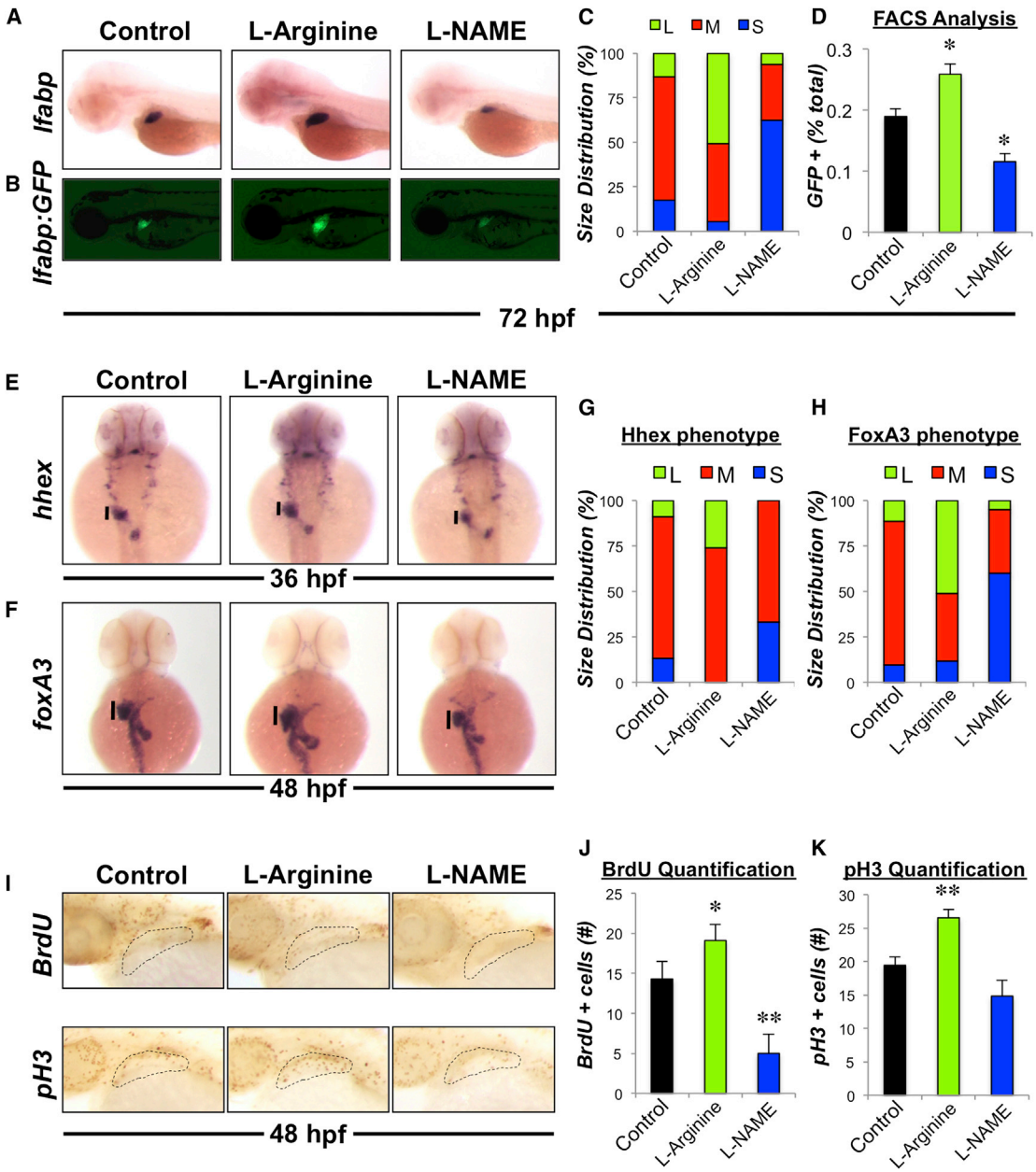

Figure 1. NO Signaling Regulates Liver Size during Development in Zebrafish

(A) Effect of chemical modulators of NO signaling on liver formation. Zebrafish were exposed to chemicals (10 μM) from 24 to 72 hpf and subjected to in situ hybridization for the hepatocyte gene *lfabp*. Representative photomicrographs were taken at 10× magnification.

(B) Effect of drug treatment on liver size in *Tg(lfabp:GFP)* embryos. Representative fluorescent photomicrographs were taken at 10× magnification.

(C) Phenotypic analysis of liver size as determined by *lfabp* in situ hybridization in treated embryos at 72 hpf (S, small; M, medium; L, large, n > 50 embryos per treatment).

(D) Effect of drug treatment on the percentage of hepatocytes specified during liver formation. Chemically treated *Tg(lfabp:GFP)* embryos were dissociated and the percentage of GFP positive hepatocytes was analyzed by fluorescence-activated cell sorting (FACS). n = 4; ANOVA, *p < 0.05 in comparison to control.

(E and F) Effect of drug treatment on hepatic progenitor cells. Zebrafish embryos were exposed to chemicals (10 μM) from 18 to 36 hpf or 24–48 hpf and subjected to in situ hybridization for the endodermal genes *hhex* and *foxA3*, respectively.

(G and H) Phenotypic analysis of hepatic bud size as determined by *hhex* and *foxA3 in situ* hybridization in treated embryos at 36 and 48 hpf, respectively (n > 25 embryos per treatment).

(I) Effect of drug treatment on hepatic progenitor cell proliferation as determined by BrdU incorporation or pH 3 staining, respectively.

(J and K) Quantification of hepatic progenitor cell proliferation.

Representative photomicrographs were taken and cell counts were performed. n = 10; ANOVA, *p = 0.042, **p < 0.001 in comparison to control. Results in (D), (J), and (K) show mean ± SEM.

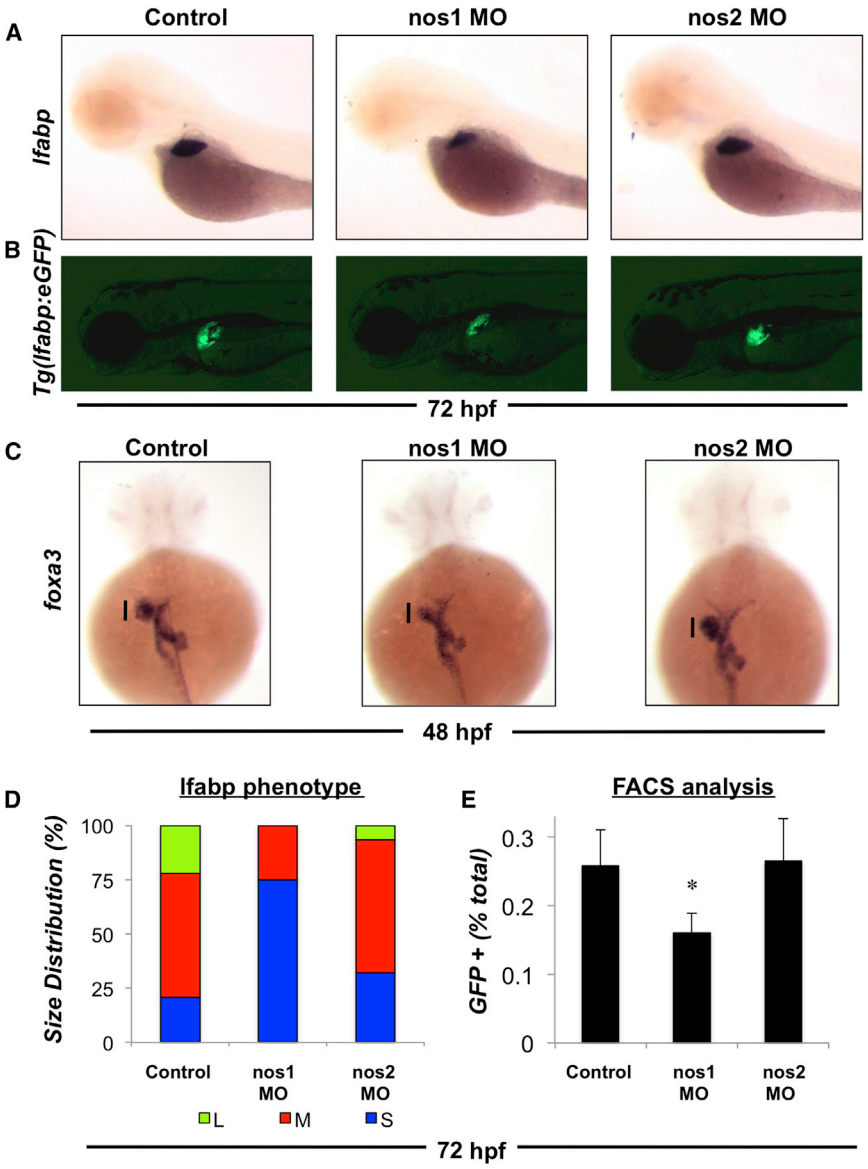

Figure 2. Constitutive NOS Activity Is Required for Optimal Liver Development

(A) Effect of morpholino-mediated knockdown of nos1 and nos2 gene expression on liver formation as assessed by in situ hybridization for *lfabp* at 72 hpf. Translation blocking ATG morpholinos against nos 1 and 2 were injected at a concentration of 50 μM. Representative photomicrographs were taken at 10× magnification.

(B) Effect of nos knockdown on liver size in *Tg(lfabp:GFP)* embryos. Representative fluorescent photomicrographs were taken at 10× magnification.

(C) Effect of nos knockdown on hepatic progenitor cells as assessed by in situ hybridization for *foxA3* at 48 hpf. Representative photomicrographs were taken at 10× magnification.

(D) Phenotype analysis of liver size (*lfabp*) in morphants at 72 hpf (n > 50 embryos per treatment).

(E) Effect of nos knockdown on the percentage of hepatocytes specified during liver formation. *Tg(lfabp:GFP)* morphants were dissociated, and the percentage of GFP positive hepatocytes was analyzed by FACS.

n = 8; ANOVA, *p = 0.033 in comparison to control. Results in (E) show mean ± SEM.

occur via a second mechanism involving direct binding of NO to cysteine residues of proteins to form S-nitrosothiols (S-nitrosylation), which can alter protein function and modulate signaling pathways (Lima et al., 2010). S-nitrosoglutathione (GSNO), the primary S-nitrosothiol, is catabolized in vivo by the enzyme GSNO reductase (GSNOR). Despite the wealth of knowledge regarding NO function in the vasculature, the involvement of NO regulation in liver development has not been examined.

The role of NO signaling in the context of liver injury has been controversial. Studies have shown that nitric oxide synthase 2 (*Nos2*, inducible isoform) is induced by APAP, and that nitrosative stress, as measured by nitrotyrosine, is increased in the centrilobular region of the liver (Hinson et al., 1998; Knight et al., 2001; Saito et al., 2010). Furthermore, mice deficient in either *Nos1* (neuronal isoform) or *Nos2* are protected from APAP, suggesting that the Nos isoforms may exacerbate liver injury (Agarwal et al., 2012; Michael et al., 2001). However, there is also evidence that NO signaling can be beneficial in the setting of ischemia-reperfusion injury (Cottart et al., 1999; Elrod et al., 2008) or during liver regeneration following partial hepatectomy (Kurokawa et al., 2012; Mei and Thevananther, 2011; Rai et al., 1998). Given these opposing results, a more detailed understanding of the functional role of NO signaling in liver injury, particularly during toxic insults, is needed.

In the present study, we discover that chemical modulators of NO signaling can regulate liver formation. Increased NO levels enhanced the proliferation of hepatic progenitor cells via a cGMP-independent mechanism involving S-nitrosylation. GSNOR inhibition, which enhances S-nitrosothiol signaling, activated the Nrf2 antioxidant response pathway, which enhanced survival in zebrafish exposed to APAP. In addition to its effects after toxic injury, GSNOR inhibition enhanced liver regrowth following partial hepatectomy. The effects of GSNOR were found to be evolutionarily conserved as GSNOR-deficient mice were similarly protected from APAP-induced liver injury. Finally, in translational experiments, we demonstrated the therapeutic potential of a chemical GSNOR inhibitor, N6547, which synergized with NAC to protect wild-type mice from APAP-induced liver injury. These findings demonstrate the great therapeutic promise of GSNOR inhibitors in protecting from liver injury and promoting organ repair.

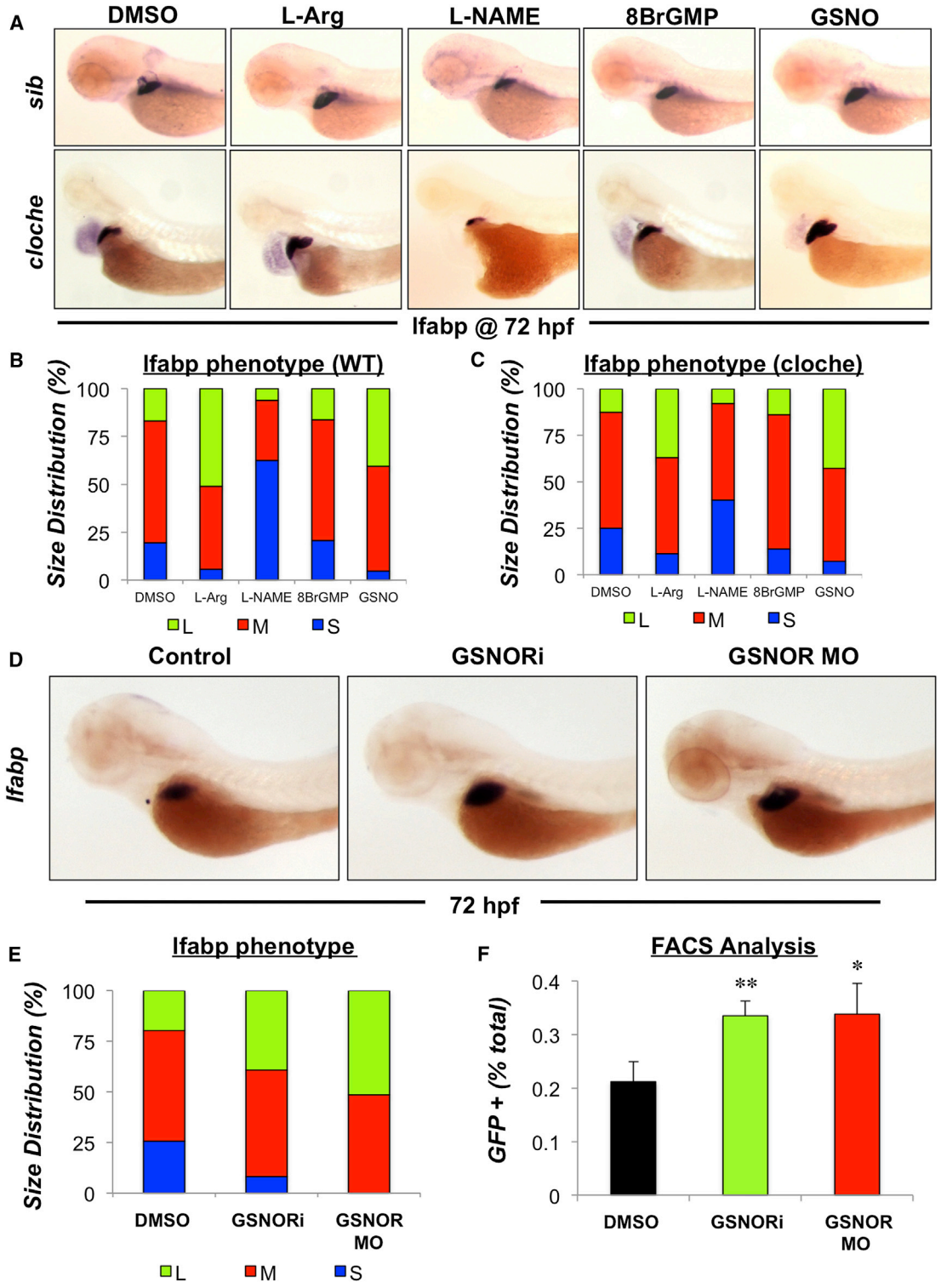

Figure 3. NO Signaling Regulates Liver Size in a cGMP-Independent Manner Involving Modulation of S-Nitrosothiol Homeostasis

(A) Effect of chemical modulators of NO signaling on liver formation in cloche mutants that lack a vasculature. Cloche mutants and siblings (sib) were exposed to chemicals (5 μM) from 24 to 72 hpf and subjected to in situ hybridization for the hepatocyte gene *lfabp*. Representative photomicrographs were taken at 10× magnification.

(B and C) Phenotype analysis of liver size in WT and cloche mutants exposed to the cGMP analog, 8BrGMP (10 μM), or the S-nitrosothiol GSNO (10 μM) from 24 to 72 hpf as determined by in situ hybridization for *lfabp* (n > 25 embryos per treatment).

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RESULTS

NO Signaling Regulates Liver Size during Development in Zebrafish

We previously performed a chemical genetic screen for modulators of liver formation in zebrafish embryos at 72 hr postfertilization (hpf) (Garnaas et al., 2012) and identified compounds that impact NO production and signaling. To confirm the screening results, we examined the effect of well-established modulators of NO signaling on liver formation: exposure to the NO precursor, L-arginine (L-Arg, 10 μM), or the NO donor diethylenetriamine-NONOate (Deta, 10 μM) from 24–72 hpf increased liver size, as determined by in situ hybridization for the hepatocyte-specific genes *liver fatty acid binding protein* (*lfabp*) and *transferrin* (Figures 1A, S1A, and S2B). Incubation with the NOS inhibitors, L-N^G-Nitroarginine methyl ester (L-NAME, 10 μM) or NG-amino-L-arginine (L-NMMA, 10 μM) from 24 to 72 hpf decreased liver size. The contrasting effects of L-Arg and L-NAME on liver formation were confirmed by in vivo fluorescent microscopy of *lfabp*:GFP transgenic zebrafish (Figure 1B). Of embryos exposed to L-Arg, 51% had a large liver, whereas L-NAME treatment diminished liver size in 62% of embryos examined (Figure 1C). Flow cytometric analysis of GFP^+ cells in *lfabp*:GFP embryos quantified the effects of L-Arg (37% increase) and L-NAME (39% decrease) on hepatocyte number (Figure 1D). Chemical modulation of NO signaling did not affect the formation of other endodermal organs as determined by in situ hybridization for *foxA3* (pan-endoderm), *trypsin* (exocrine pancreas), *insulin* (endocrine pancreas), and *ifabp* (gut). Similarly, heart formation (*cmlc2*), a known target tissue for NO modulation, was not affected by NO signaling (Figure S1C). Altogether, these studies demonstrate that NO signaling specifically regulates liver growth during development.

We further studied the temporal requirements for NO signaling during liver development. NO caused dramatic changes in liver formation after exposure from 24 to 48 hpf when liver progenitors are being formed (Figure S1D); however, no changes in liver size were observed after treatment from 48 to 72 hpf during hepatocyte differentiation (Figure S1E). The effect of NO on hepatic progenitors was examined with the early hepatic progenitor/endoderm markers *hhex*, *prox1*, and *foxA3*. L-Arg increased hepatic bud size (*hhex* and *prox1*) at 36 hpf in 26% (Figures 1E, 1G, S1F), and *foxA3* expression at 48 hpf in 51% of embryos (Figures 1F and 1H). In contrast, L-NAME substantially diminished hepatic bud size. Bromodeoxyuridine (BrdU) incorporation or staining for the mitotic marker pH3 indicated that L-Arg increased hepatic proliferation, whereas L-NAME inhibited cell-cycle progression (Figures 1I–1K). Alogether, these data demonstrate that NO enhances hepatic progenitor proliferation during development.

To genetically confirm the role of NO signaling in liver development, liver formation was assessed after morpholino mediated knockdown of *nos1* and *nos2*. Knockdown of *nos1* severely inhibited liver formation, whereas knockdown of *nos2* had no effect (Figures 2A, 2B, and S2A). Knockdown of *nos1*, but not *nos2*, also caused significant reduction in hepatic progenitors at 48 hpf (Figure 2C). These specific effects of *nos1* knockdown were quantified by phenotypic analysis of liver size (Figures 2D and S2B) and flow cytometric analysis of *lfabp*:GFP morphants (Figure 2E). Together, these results indicate that *nos1* is required for optimal liver formation.

S-Nitrosothiol Signaling Regulates Liver Formation Independently of cGMP

NO signaling can occur via two distinct mechanisms, namely, cGMP-dependent vasodilation and S-nitrosylation pathways. In order to clarify the importance of the well-known vascular effects of NO mediated by cGMP, we examined the impact of chemical modulators of NO signaling in *cloche* mutants that have no endothelial cells and therefore lack any vasculature. Exposure to L-Arg increased liver size in 37% of treated *cloche* embryos, whereas 40% of L-NAME treated *cloche* embryos had a smaller liver (Figures 3A–3C). Similarly, exposure of embryos to a cGMP analog (8BrGMP, 10 μM) had no effect on liver size, whereas the S-nitrosothiol S-nitrosoglutathione (GSNO, 5 μM) increased liver size in 41% of wild-type and 43% of *cloche* embryos (Figures 3A–3C). Together, these results demonstrate that NO signaling regulates liver size by S-nitrosothiol signaling independently of cGMP-mediated effects on the vasculature.

GSNOR is a highly conserved enzyme (Figure S3A) that metabolizes S-nitrosothiols and thereby negatively regulates S-nitrosothiol signaling. Embryos exposed to the specific GSNOR inhibitor N6547 (GSNORi, 1 μM, N30 Pharmaceuticals) from 24 to 72 hpf exhibited a dramatic increase in liver size in 40% of the embryos examined (Figures 3D, 3E, S3B, and S3C). Similarly, morpholino-mediated knockdown of GSNOR caused an enlarged liver in 51% of embryos (Figures 3D and 3E). GSNORi exposure from 24 to 48 hpf also increased hepatic bud size (*sid4*) (Figure S3D). Flow cytometry of GSNORi-treated or GSNOR-deficient *lfabp*:GFP embryos confirmed increased hepatocyte number (Figure 3F). Together, these findings demonstrate that enhanced S-nitrosothiol signaling due to GSNOR inhibition enhances growth in the developing liver.

GSNOR Inhibition Is Hepatoprotective and Enhances Survival in Embryos Exposed to APAP via Activation of the Nrf2 Pathway

Given the positive effects of S-nitrosothiol signaling on hepatic growth during development, we examined whether GSNORi

(D) Effect of chemical inhibition or morpholino-mediated knockdown of GSNOR on liver size as determined by in situ hybridization for the hepatocyte gene *lfabp* at 72 hpf. The translation blocking ATG morpholino against GSNOR was injected at a concentration of 50 μM. Representative photomicrographs were taken at 10× magnification.

(E) Phenotype analysis of liver size in GSNOR-deficient embryos at 72 hpf as determined by in situ hybridization for *lfabp* (n > 25 embryos per treatment).

(F) Loss of GSNOR function alters the percentage of hepatocytes specified during liver formation. *Tg(lfabp:GFP)* morphants were dissociated and the percentage of GFP positive hepatocytes was analyzed by FACS.

n = 4; ANOVA, *p = 0.041, **p = 0.002 in comparison to control. Results in (F) show mean ± SEM.

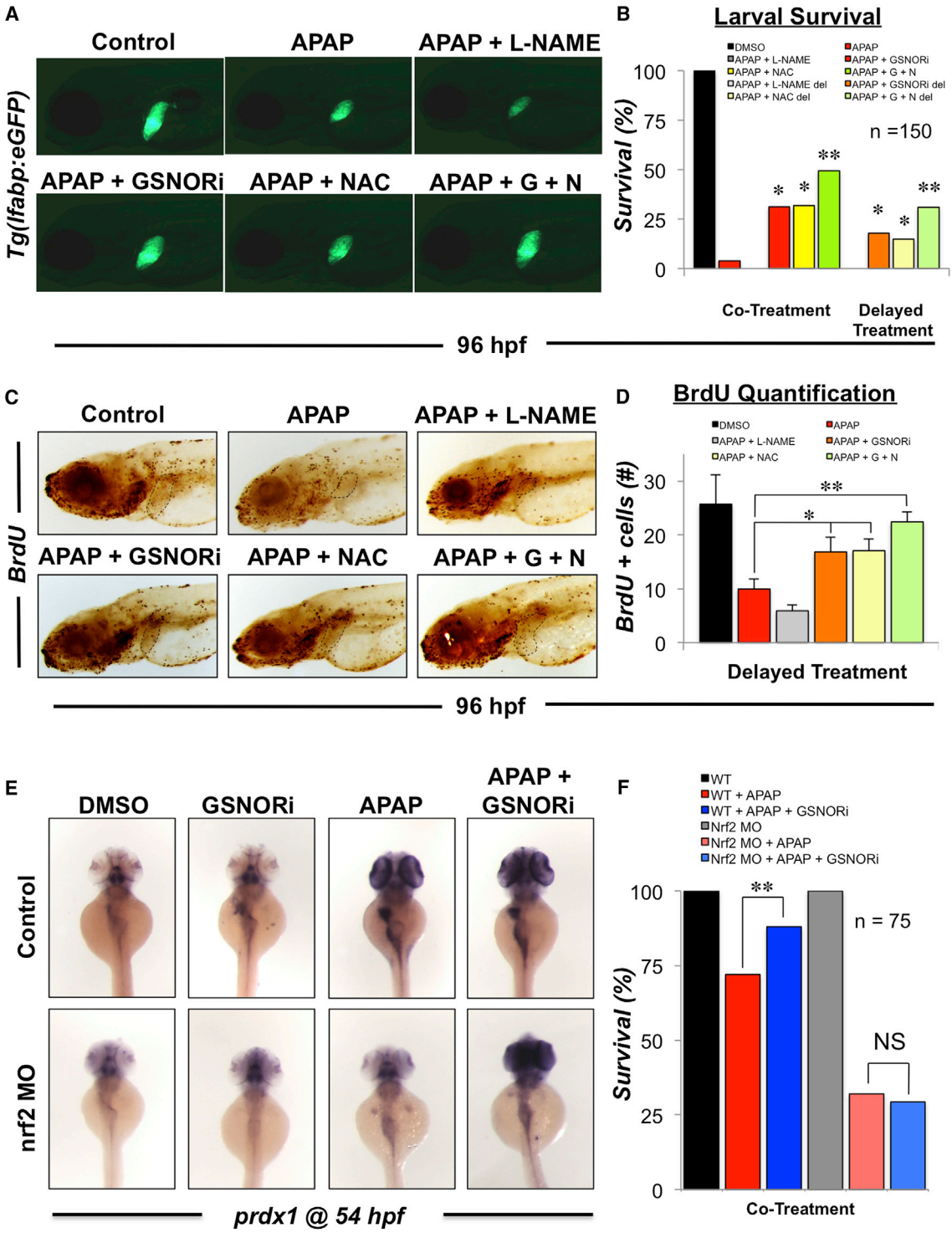

Figure 4. GSNOR Inhibition Is Hepatoprotective and Enhances Survival in Embryos Exposed to APAP via Activation of the Nrf2 Pathway

(A) Effect of GSNORi and NAC on recovery from APAP-induced liver injury in *Tg(lfabp:GFP)* embryos. *Tg(lfabp:GFP)* embryos were exposed to a sublethal dose of APAP (5 mM) from 48 to 60 hpf at which point embryos were washed and subsequently exposed to GSNORi (1 μM) and/or NAC (10 μM) from 60 to 96 hpf. Representative fluorescent photomicrographs were taken at 10× magnification.

(B) Survival in embryos exposed to a lethal dose of APAP (10 mM) from 48 to 96 hpf in the presence or absence of L-NAME (10 μM), GSNORi (1 μM), and NAC (10 μM). Under some conditions treatments were given after a 12 hr delay (del). n = 150; Fisher's exact test, *$p < 0.001$ compared to APAP alone, **$p < 0.001$ compared to APAP + NAC.

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improved outcome in a zebrafish model of acetaminophen (APAP)-induced liver injury (North et al., 2010). Transgenic *lfabp*:GFP embryos were exposed to a sublethal dose of APAP (5 mM) from 48 to 60 hpf, followed by treatment with L-NAME, GSNORi, and/or NAC during the recovery phase (60–96 hpf) (Figure 4A). APAP decreased liver size, and this reduction was exacerbated by L-NAME. In contrast, either NAC (10 μM) or GSNORi alone rescued liver size in APAP exposed embryos and acted synergistically to further stimulate hepatic repair. A lethal dose of APAP (10 mM) from 48 to 96 hpf caused a dramatic reduction in survival (Figure 4B, 4%). Treatment with GSNORi or NAC alone significantly increased survival to 32%, and combination therapy further improved survival to 49%. When GSNORi or NAC was given alone after a 12 hr delay to model a scenario often encountered in clinical practice, there was a modest increase in survival (18% and 15%, respectively); in contrast, combined treatment led to a doubling in survival rates (31%). In addition to GSNORi, cotreatment with the NO donors Deta and GSNO also protected from APAP-induced liver toxicity and improved survival (Figures S4A and S4B). In order to further investigate the proproliferative effect of treatments following APAP-induced liver injury, BrdU incorporation was examined during the recovery phase (Figure 4C). APAP exposure decreased the number of BrdU-positive cells, and this was further decreased by incubation with L-NAME (Figures 4C and 4D). GSNORi or NAC exposure increased BrdU incorporation, and the compounds together dramatically increased BrdU-positive cell number in the regenerating liver (Figures 4C and 4D). Together, these studies demonstrate that GSNORi may work in a synergistic or additive fashion with NAC to prevent APAP toxicity, stimulate organ repair, and enhance survival.

To identify the mechanism by which GSNORi enhances survival, we investigated whether S-nitrosothiol signaling activated the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which plays a pivotal role in the cell's adaptive response to stress (Taguchi et al., 2011). Exposure to GSNORi alone from 48 to 54 hpf induced hepatic expression of the nrf2 target *prdx1* (Figure 4E). Further, *nrf2* knockdown prevented this GSNORi-dependent induction of *prdx1*, indicating regulation of the Nrf2 pathway by S-nitrosothiol signaling. To demonstrate the response of Nrf2 signaling to toxic stress, zebrafish were exposed to APAP, which caused a dramatic increase in Nrf2-dependent expression of *prdx1* and hmox 1 both in the presence or absence of GSNORi at 54 and 72 hpf (Figures 4E, S4C, and S4D). Nrf2 signaling was essential for survival, because *nrf2* morphants were highly sensitive to APAP toxicity compared to control-injected siblings (Figure 4F). More importantly, the therapeutic effect of GSNORi on survival was abated in *nrf2* morphants. Together, these studies demonstrate that the hepatoprotective properties of GSNORi in APAP-induced liver injury are mediated by the Nrf2 pathway.

GSNOR Inhibition Protects Adult Zebrafish from APAP-Induced Toxicity and Enhances Regeneration following Liver Resection

Having shown that S-nitrosothiol signaling was hepatoprotective and proregenerative in APAP-exposed larvae, we examined whether GSNORi remained effective in adult zebrafish. Adult transparent (*casper*) adult *lfabp*:GFP transgenic fish were exposed to APAP in the presence or absence of GSNORi for 24 hr and examined at 72 hr postexposure (hpe) by in vivo fluorescence microscopy. APAP exposure reduced GFP fluorescence, suggestive of hepatocyte necrosis, whereas coexposure with GSNORi prevented loss in fluorescence (Figure 5A). Histologically, GSNORi ameliorated hepatocellular necrosis (TUNEL stain), sinusoidal widening, and hemorrhage (Figures 5B and S5A). Circulating liver aminotransferase (ALT) levels in APAP-exposed fish peaked at 24 hpe with 196 ± 35 U/l from a baseline level of 47 ± 5 U/l, whereas GSNORi suppressed ALT levels to 55 ± 3 U/l (Figure 5C). Survival studies revealed that APAP exposure caused a time-dependent loss in survival reaching 39% by 72 hpe; conversely, fish treated with GSNORi exhibited a markedly enhanced survival rate of 65% by 72 hpe (Figure 5D). Similarly, the NO donors Deta and GSNO increased overall survival compared to APAP alone, whereas treatment with L-NAME exacerbated death (Figure S5B). These results demonstrate that GSNORi improves liver enzymes, histology, and survival after APAP exposure.

Treatment for APAP ingestion generally occurs after considerable delay in the clinical setting. Here, we tested whether GSNORi remain beneficial when treatment was delayed. Treatment with GSNORi or NAC alone after delay increased survival at 72 hpe from 57% to ~80% (Figure 5E). Combined treatment with GSNORi and NAC, however, greatly enhanced survival further to 95%. Immunohistochemical staining for PCNA after delayed GSNORi treatment revealed that GSNORi stimulated cell proliferation in the APAP-injured liver by 2-fold (Figures 5G and S5A), demonstrating that it is not only hepatoprotective, but also proproliferative as observed during development. To test the requirement of the Nrf2 pathway, we exposed adult *nrf2* mutant zebrafish to APAP in the presence or absence of GSNORi, which demonstrated that the *nrf2* mutants were highly susceptible to APAP toxicity and they did not respond to GSNORi treatment (Figures 5F and S5C). To directly examine the proproliferative properties of GSNORi, we employed a surgical resection assay in adult zebrafish, removing the inferior lobe of the liver and treating fish during the regenerative phase (Goessling et al., 2008). GSNORi exposure significantly increased the

(C) Effect of GSNORi and NAC on proliferation following APAP-induced liver injury. Embryos were exposed to a sublethal dose of APAP (5 mM) from 48 to 60 hpf at which point embryos were washed and subsequently exposed to GSNORi (1 μM) and/or NAC (10 μM) from 60 to 96 hpf. Embryos were pulsed for 10 min in 10 mM BrdU just prior to fixation. Representative photomicrographs were taken at 10× magnification.

(D) Quantification of BrdU positive cells in the regenerating liver of APAP exposed embryos exposed to GSNORi and/or NAC. n = 6; ANOVA, *$p < 0.05$, **$p < 0.001$ in comparison to APAP alone.

(E) Effect of GSNORi (1 μM) and/or APAP (5 mM) exposure from 48 to 54 hpf on induction of the Nrf2 target gene, *prdx1*, as determined by in situ hybridization. Translation blocking ATG morpholino against nrf2 was injected at a concentration of 50 μM.

(F) Survival in WT embryos and nrf2 morphants exposed to a lethal dose of APAP (10 mM) from 48 to 80 hpf in the presence or absence of GSNORi (1 μM). n = 75; Fisher's exact test, **$p = 0.012$. Results in (D) show mean ± SEM.

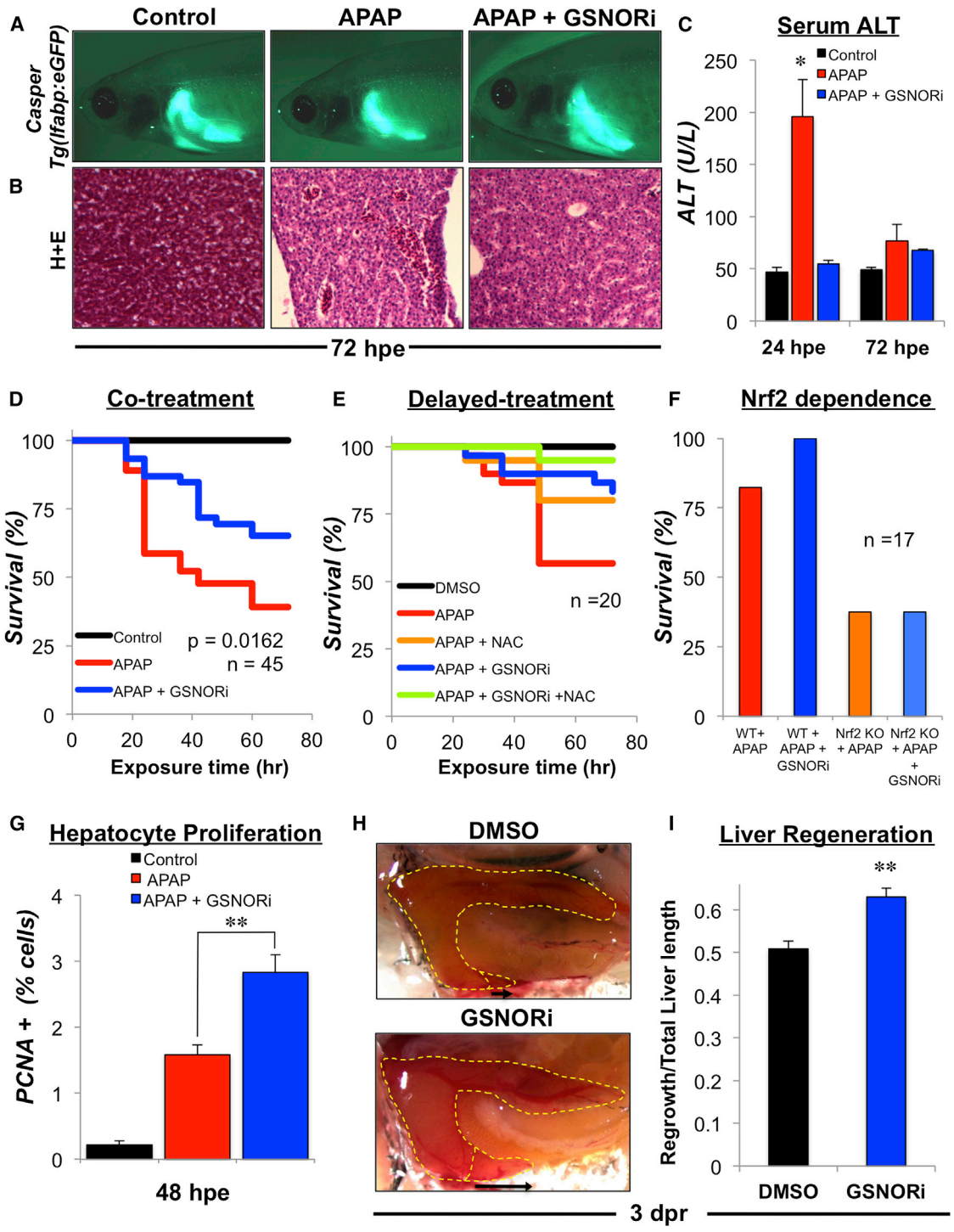

Figure 5. GSNOR Inhibition Protects Adult Zebrafish from APAP-Induced Toxicity and Enhances Regeneration following Liver Injury

(A) In vivo fluorescent imaging of the liver in adult casper *Tg(lfabp:GFP)* fish exposed to 10 mM APAP for 18 hr before being exposed to GSNORi (1 μM). Adult fish were anaesthetized at 72 hpe and immediately imaged by fluorescent microscopy. Representative fluorescent photographs were taken at 10× magnification.

(B) Hepatic histology (H&E) in fish exposed to APAP (10 mM) in the presence or absence of GSNORi (1 μM) at 48 hpe.

(C) Serum alanine aminotransferase (ALT) activity in fish exposed to APAP (10 mM) in the presence or absence of GSNORi (1 μM) at 24 or 72 hpe. n = 6; ANOVA, *p < 0.05 in comparison to control.

(D) Kaplan-Meier plot of survival in adult fish exposed to APAP (10 mM) for 24 hr in the presence or absence of GSNORi (1 μM). Chemicals were removed 24 hpe, fish were reintroduced into fresh water and survival was monitored over 72 hr. n = 45; Kaplan-Meier estimate, p = 0.0162, Cox regression hazard ratio (HR) = 1.95.

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length of regrowth in the inferior lobe at 3 dpr by 25% (Figures 5H, 5I, and S5E). Similarly, L-Arg increased the rate of liver regeneration following partial hepatectomy, whereas L-NAME diminished the regenerative response (Figures S5D–S5F). Collectively, these data reveal that GSNORi is both hepatoprotective and proproliferative in the context of liver damage, reducing toxicity, and promoting liver regeneration.

GSNOR-Deficient Mice Are Protected from APAP Injury

We next tested whether GSNOR-dependent hepatoprotection is conserved in mice. WT and *GSNOR* KO C57Bl/6 mice were injected with a sublethal dose of APAP (300 mg/kg) and examined at 6 and 24 hr. WT mice displayed significant centrilobular hepatocyte necrosis coincident with increased TUNEL-positive cells, whereas *GSNOR* KO mice showed a significant decrease in hepatocellular necrosis at 6 and 24 hr (Figure 6A). The circulating ALT values in APAP-injected WT mice increased to 1,431 ± 332 U/l at 6 hr and rose to 2,584 ± 299 U/l at 24 hr, whereas *GSNOR* KO mice had markedly lower ALT values averaging 543 ± 176 U/l at 6 hr and 1,969 ± 227 U/l at 24 hr (Figure 6B). Quantification of liver necrosis from histological sections confirmed that *GSNOR*-deficient mice were protected from APAP (Figure 6C). Expression analysis of the APAP-metabolizing enzyme *Cyp2e1* by RT-PCR confirmed no differences between WT and GSNOR KO mice, suggesting that the metabolic activation of APAP was not altered in the *GSNOR*-deficient mice (Figure S6). To see whether GSNOR-dependent regulation of the Nrf2 pathway was conserved in mice, we monitored the nuclear translocation of Nrf2, as an indication of Nrf2 activation, and found that *GSNOR* KO mice exhibited a more sustained accumulation of nuclear Nrf2 at 24 hr (Figures 6D and 6E). We examined expression of Nrf2 target genes by quantitative PCR (qPCR), and this revealed sustained *Hmox1* and *Prdx1* induction in *GSNOR* KO mice (Figures 6F and 6G). Together, these studies demonstrate that the GSNOR regulation of the hepatoprotective Nrf2 pathway is conserved across species.

Therapeutic Administration of GSNORi and NAC after APAP Injury in Mice Indicates Significant Additive Hepatoprotective Effects In Vivo

To directly evaluate the therapeutic properties of GSNORi in translational studies in mammalian models, WT mice were injected with a sublethal dose of APAP followed 2 hr later by GSNORi (5 mg/kg) and/or NAC (300 mg/kg). Histological evaluation showed that GSNORi provided a small yet significant level of protection from APAP-induced centrilobular necrosis at both 6 and 24 hr (Figure 7A). As anticipated from clinical observations, NAC prevented liver necrosis; however, the combination therapy of GSNORi and NAC was significantly more effective (Figure 7A). GSNORi or NAC alone reduced circulating ALT to 72% and 43% of the level of APAP alone, respectively, whereas GSNORi and NAC combined reduced ALT levels to 25% of the level of APAP alone (Figure 7B). The extent of liver necrosis reflected the differences seen in serum ALT values, with GSNORi reducing liver necrosis, particularly when combined with NAC (Figure 7C). Collectively, these experiments reveal that GSNORi acts in combination with NAC to protect mice after APAP exposure, highlighting the therapeutic potential of GSNORi in toxic liver injury.

DISCUSSION

In this study, we identified NO and S-nitrosothiol signaling as critical regulators of hepatic growth during development and following toxic liver injury. Originating from its discovery as a modulator of organ development in a chemical genetic screen, we demonstrate the conserved action of S-nitrosothiol signaling as a proproliferative and hepatoprotective pathway. Importantly, we reveal the therapeutic potential of GSNOR inhibitors in preclinical zebrafish and murine models of toxic and surgical liver injury.

S-Nitrosothiol Signaling Is a Universal Injury Response Pathway

The role of S-nitrosothiols may extend beyond the liver as a universal response to injury. Most forms of liver injury, including partial hepatectomy (Ronco et al., 2004), APAP (Hinson et al., 1998; Knight et al., 2001; Saito et al., 2010), thioacetamide (Díez-Fernández et al., 1997), carbon tetrachloride (Mizumoto et al., 1997), or ethanol exposure (Venkatraman et al., 2004), lead to iNOS induction, suggesting that NO and S-nitrosothiol signaling may be universal components of the stress response in the liver. From a teleological perspective, it would be logical for an organism to respond to injury by inducing expression of enzymes whose products play an integral role in the regenerative response. The cytoprotective properties of S-nitrosothiol signaling are not limited to the liver, as previous work has shown that *GSNOR*-deficient mice are protected from myocardial injury (Lima et al., 2009), indicating that S-nitrosothiol signaling may play an important role in the injury response. Comparative studies in sunflower seedlings and *Arabidopsis thaliana* demonstrate that mechanical wounding downregulates *GSNOR* and provokes an increase in S-nitrosothiols, which are necessary for an appropriate response to injury (Chaki et al., 2011; Espunya et al., 2012). Together, these studies suggest that GSNOR-dependent S-nitrosothiol signaling may be a phylogenetically

(E) Kaplan-Meier plot of survival in adult fish exposed to APAP (10 mM) for 18 hr followed by a delayed treatment with GSNORi (1 mM) and/or NAC (10 mM). n > 20 fish per treatment.
(F) Survival in adult WT fish and Nrf2 mutant fish exposed to APAP (10 mM) in the presence or absence of GSNORi (1 μM) for 18 hr. n > 17 fish per treatment.
(G) Hepatocyte proliferation was determined by immunohistochemical staining of PCNA positive hepatocytes in liver sections from WT fish exposed to APAP (10 mM) followed by delayed treatment with GSNORi (1 μM) at 48 hpe. n = 10; ANOVA, **p < 0.001.
(H) Liver regeneration following partial hepatectomy in adult zebrafish at 3 days post resection (dpr). Adult fish were exposed to DMSO or GSNORi (1 μM) 6 hr after surgery for 24 hr. Representative en bloc dissection photographs were taken at 2.5× magnification.
(I) Quantification of liver regeneration as determined by the length of regrowth from the resection margin divided by the total length of the inferior lobe. n = 20; ANOVA, **p < 0.001 in comparison to control. Results in (C), (G), and (I) show mean ± SEM.

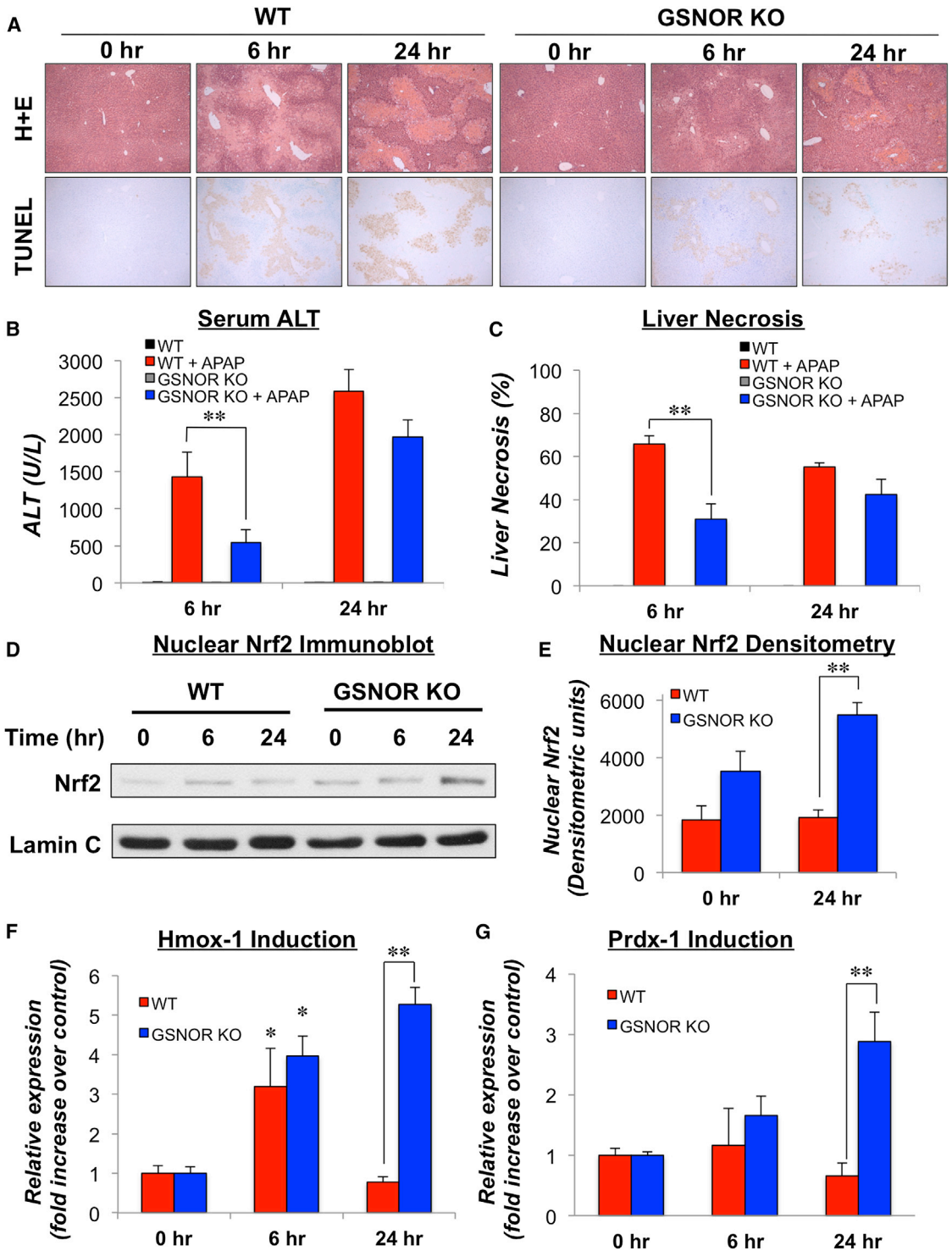

Figure 6. GSNOR Deficiency Protects Mice from APAP Injury

(A) Hepatic histology in WT and GSNOR KO mice injected with a sub-lethal dose of APAP (300 mg/kg) at 6 or 24 hpe. Mice were sacrificed at 6 or 24 hpe. Livers were sectioned and stained with H&E in conjunction with TUNEL stain in order to highlight centrilobular necrosis. Representative sections were taken at 10× magnification. n = 4–5 mice per group.

(B) Serum alanine aminotransferase (ALT) activity in WT and GSNOR KO mice injected with APAP (300 mg/kg) at 6 or 24 hpe. n = 4–5; ANOVA, $^{**}p < 0.05$.

(C) Quantification of liver necrosis in histological sections of WT or GSNOR KO mice injected with APAP (300 mg/kg) at 6 or 24 hpe. Areas of necrosis were highlighted and quantified using ImageJ software. n = 4–5; ANOVA, $^{**}p < 0.01$.

(legend continued on next page)

conserved and universal mechanism across kingdoms by which multicellular organisms respond to and recover from injury.

S-Nitrosothiol Signaling Orchestrates Protective and Proliferative Pathways after Injury

Our data revealed that the hepatoprotective properties of GSNOR inhibition were due at least in part to activation of the Nrf2 pathway. Nrf2 reacts to xenobiotic stress by inducing production of proteins that promote detoxification and cytoprotection (Taguchi et al., 2011); this mechanism is highly conserved in zebrafish (Kobayashi et al., 2009; Mukaigasa et al., 2012). Previous studies in mice have shown that Nrf2 activation protects from APAP injury (Okawa et al., 2006), whereas Nrf2 deficiency sensitizes mice to APAP toxicity (Chan et al., 2001; Enomoto et al., 2001) and impairs liver regeneration following partial hepatectomy (Beyer et al., 2008; Wakabayashi et al., 2010). Our data demonstrate that, in contrast to exogenous activators of the Nrf2 pathway, GSNOR inhibition may lead to transient nitrosylation of the Nrf2 binding partner Keap1, thereby amplifying the availability of Nrf2. GSNOR inhibitors therefore may play an important role in regulating the Nrf2 activation threshold and extending the duration of the injury response.

Although our studies focused on the GSNOR-mediated regulation of the Nrf2 pathway, GSNOR likely regulates the activity of additional regenerative pathways through nitrosylation of other protein targets. For example, Snyder and colleagues have shown that S-nitrosylation of Cox-2 enhances PGE2 production (Kim et al., 2005), which is intriguing, because we have previously shown that PGE2 is hepatoprotective in APAP-induced liver injury (North et al., 2010). There is also evidence that other transcription factors, including Hif1α (Lima et al., 2009), β-catenin (Thibeault et al., 2010), and NF-κB (Kelleher et al., 2007; Sen et al., 2012), are subject to S-nitrosylation under certain conditions. The transient nature of these nitrosylation processes has posed a challenge for their detection, but may represent a fast and highly flexible way to rapidly initiate and terminate the desired regenerative effects. Consequently, one could envisage a model in which iNOS induction during liver injury facilitates the S-nitrosylation of numerous targets to coordinate the regenerative response. Thus, GSNOR inhibition may stimulate organ repair by orchestrating the activity of several protective and proliferative pathways in unison (Figure S7).

GSNOR Inhibitors as Hepatoprotective Agents

We have demonstrated that a GSNOR inhibitor, N6547, can protect from APAP-induced liver toxicity in vivo. Further, the conserved effects in zebrafish and mice, the synergistic action with the clinically used antidote NAC, and the widening of the therapeutic time window, indicate the imminent translation potential toward a clinical trial. The discovery that this NO-induced process is distinct from the classic cGMP-dependent pathway has important clinical relevance: loss of GSNOR function does not affect baseline blood pressure (Beigi et al., 2012; Liu et al., 2004), which is often critically low in patients with liver failure. Therefore, GSNOR inhibitors emerge as highly specific drug candidates to provide enhanced hepatoprotection without systemic side effects. To date, chemical screens in zebrafish have led to clinical trials in blood stem cell therapy (Goessling et al., 2011; North et al., 2007) and melanoma (White et al., 2011). Here, we reveal a possible clinical application of GSNOR inhibitors in regenerative medicine with the potential to extend to other causes of liver failure and injury of other organs.

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Zebrafish were maintained according to institutional animal care and use committee (IACUC-BIDMC) protocols. Lines used in this study include wild-type (AB), *Tg(−2.8fabp10:GFP)*^{as3}, *mitfa*$^{w2/w2}$;*roy*$^{a9/a9}$;*Tg(−2.8fabp10:GFP)*^{as3}, and *nfe2l2a*^{m318} as previously described (Her et al., 2003; Mukaigasa et al., 2012; White et al., 2008).

Mice

C57BL/6 and *GSNOR*$^{-/-}$ mice (Liu et al., 2004) were maintained in barrier animal facilities. After overnight fasting, male mice were injected intraperitoneally with 300 mg/kg APAP dissolved in warm saline or saline alone. Mice were injected intraperitoneally with chemical treatments (5 mg/kg GSNORi and/or 300 mg/kg NAC) dissolved in warm saline 1.5 hr after APAP injection. Mice were sacrificed and blood and livers were harvested. Animal care and procedures were reviewed and approved by the Harvard Medical School Standing Committee on Animals.

Chemical Exposure

Embryos and adult zebrafish were exposed to L-arginine (L-Arg), D-arginine (D-Arg), Deta-NONOate (Deta), L-NG-nitroarginine methyl ester (L-NAME), L-N^{G}-monomethyl Arginine citrate (L-NMMA), 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8BrGMP), S-nitrosoglutathione (GSNO), GSNORi (N6547, N30 Pharmaceuticals), and acetaminophen (APAP) as described. Unless otherwise indicated, all chemicals were obtained from Cayman Chemicals or Sigma-Aldrich.

Morpholino Injection

Validated morpholinos (MOs) (GeneTools) designed against the ATG and exon 1 splice site of *nos1* (5′-ACGCTGGGCTCTGATTCCTGCATTG-3′; 5′-TTAATG ACATCCCTCACCTCTCCAC-3′) (North et al., 2009), the ATG site of *nos2* (5′-AGTGGTTTGTGCTTGTCTTCCCATC-3′) (North et al., 2009), the ATG site of *nrf2* (5′-CATTTCAATCTCCATCATGTCTCAG-3′) (Kobayashi et al., 2002), the ATG site of *gsnor* (5′-TGATCACTTTCCCAGTTGTGTCCAT-3′) (this study) or mismatched controls were injected into AB or Tg(−2.8fabp10:GFP)^{as3} embryos at the one-cell stage.

In Situ Hybridization

Paraformaldehyde (PFA)-fixed embryos were processed for in situ hybridization according to standard zebrafish protocols (http://zfin.org/). The following RNA probes were used: *lfabp*, *transferrin*, *foxa3*, *hhex*, *sid4*, *trypsin*, *insulin*, *cmlc2*, *ifabp*, *prdx1*, and *hmox1*. Changes in expression pattern (i.e., lfabp – liver size) were typically scored as small, medium, or large. Expression patterns represent the average of >50 embryos per treatment.

(D) Nuclear translocation of Nrf2 in the liver of WT and GSNOR KO mice as determined by immunoblot analysis.
(E) Quantification of nuclear Nrf2 in liver as determined by densitometry. n = 4; ANOVA, **p < 0.001.
(F) Expression of the Nrf2 target gene Hmox-1 in liver as determined by qPCR in WT and GSNOR KO mice injected with APAP (300 mg/kg) at 6 or 24 hpe. n = 4–5; ANOVA, *p < 0.05 in comparison to control, **p < 0.001.
(G) Expression of the Nrf2 target gene Prdx-1 in liver as determined by qPCR in WT and GSNOR KO mice injected with APAP (300 mg/kg) at 6 or 24 hpe. n = 4–5; ANOVA, **p < 0.01. Results in (B), (C), and (E)–(G) show mean ± SEM.

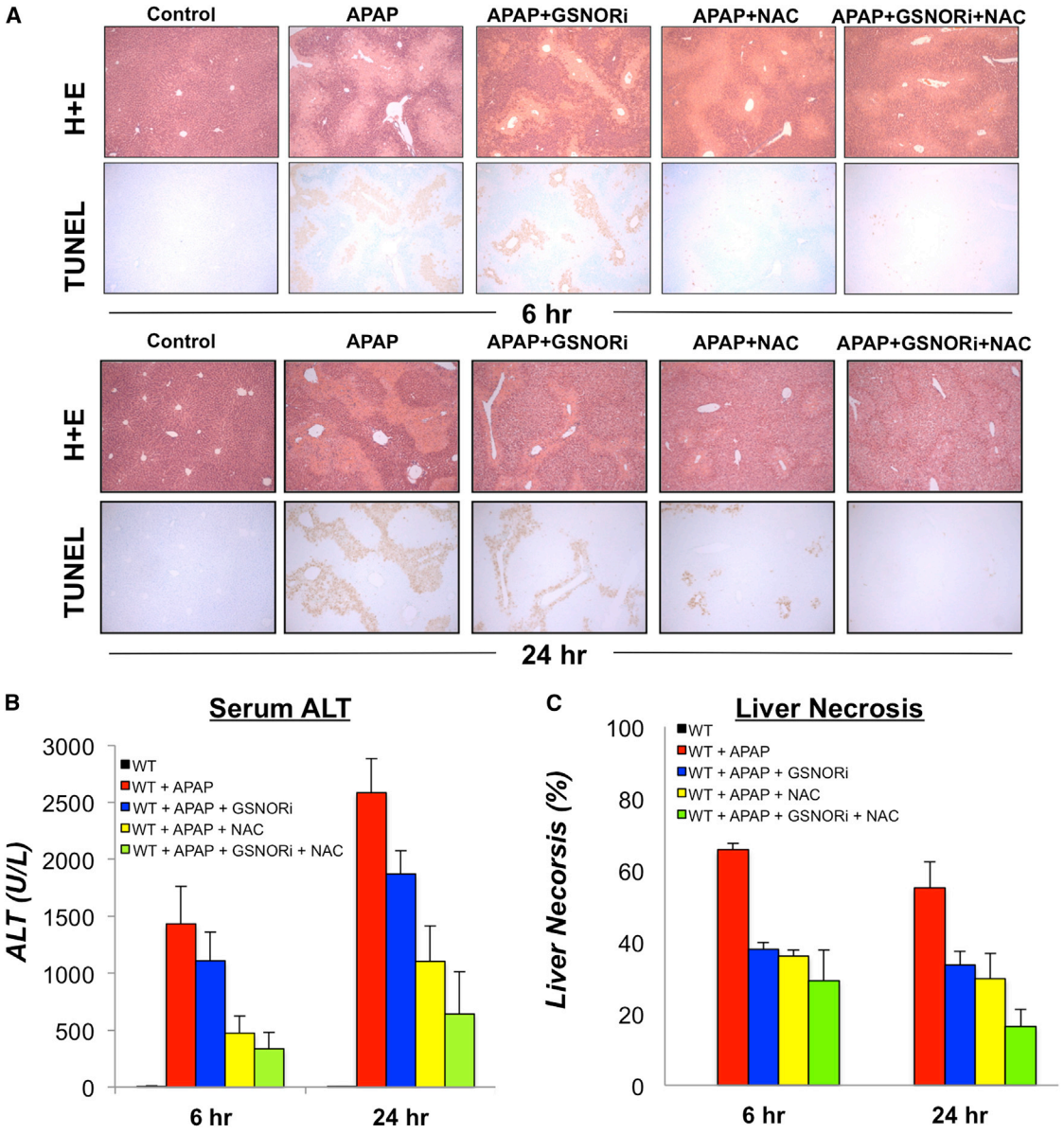

Figure 7. GSNORi and NAC Are Synergistically Hepatoprotective in APAP-Induced Liver Injury in Mice

(A) Hepatic histology in mice injected with a sublethal dose of APAP (300 mg/kg) followed by delayed (2 hpe) treatment with GSNORi (5 mg/kg) and/or NAC (300 mg/kg). Mice were sacrificed at 6 or 24 hpe. Livers were sectioned and stained with H&E in conjunction with TUNEL stain in order to highlight centrilobular necrosis. Representative sections were taken at 10× magnification. n = 4–5 mice per group.

(B) Serum alanine aminotransferase (ALT) activity in WT mice exposed to APAP (300 mg/kg) in the presence or absence of GSNORi (5 mg/kg) and/or NAC (300 mg/kg) at 6 or 24 hpe. n = 4–5 mice per group.

(C) Quantification of liver necrosis in histological sections of WT mice injected with APAP (300 mg/kg) in the presence or absence of GSNORi (5 mg/kg) and/or NAC (300 mg/kg) at 6 or 24 hpe. Areas of necrosis were highlighted and quantified using ImageJ software.

n = 4–5 mice per group. Results in (B) and (C) show mean ± SEM.

Fluorescence Microscopy

Fluorescent liver reporter [*Tg(−2.8fabp10:GFP)*^{as3}] embryos or adults were exposed to drugs as indicated, and microscopy was performed following anesthesia with 0.04 mg/ml Tricaine-S.

Proliferation Assays

Proliferation was assessed in zebrafish embryos by whole-mount immunohistochemistry against either pH 3 or incorporated BrdU. In adult fish, cell proliferation was assessed in paraffin sections by performing immunohistochemistry against PCNA.

Fluorescence-Activated Cell Sorting Analysis

Fluorescent liver reporter (*Tg(−2.8fabp10:GFP)*^{as3}) embryos were dissociated by enzymatic disaggregation in TrypLE Express (Invitrogen) combined with manual disruption. Upon addition of Dulbecco's modified Eagle's medium with 10% FCS, the cells were strained through a 35 μM nylon

mesh and then analyzed using a BD FACSAria II flow cytometer (BD Biosciences).

Histology

PFA-fixed fish or mice livers were paraffin embedded, cut into serial sections, and stained with Hematoxylin and eosin (H&E) with standard techniques.

Partial Hepatectomy

Liver resections and regeneration analysis were conducted as described previously (Goessling et al., 2008, 2009). Drug exposure occurred from 6 to 30 hr postresection (hpr), and liver regrowth was quantified in en bloc dissected specimens at 3 dpr.

Serum ALT

Blood was collected from mice or zebrafish with a heparinized microcapillary tube (Sarstedt) with standard methods (Retro-orbital or cardiac puncture). Plasma ALT was measured using a microplate-based ALT activity assay kit (Pointe Scientific).

Immunoblot analysis

Nuclear extracts were prepared from mice liver tissue with the NE-PER nuclear extraction kit according to the manufacturer's directions (Pierce Biotechnology). Nuclear lysates were resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose. Membranes were probed with anti-Nrf2 (Abcam) or antilamin A/C (Cell Signaling Technology) overnight and detected with secondary antibody conjugated with HRP. Antibody complexes were visualized by enhanced chemiluminescence using X-ray film. Nrf2 band intensity was quantified using ImageJ.

qPCR Analysis

qPCR was performed using primer sets for murine hmox1, prdx1, gapdh, b-actin, and cyp2e1 (Table S1). RNA was extracted from dissected liver with Trizol (Invitrogen). qPCR was performed using SYBR Green Supermix (QIAGEN) on the iQ5 Multicolor RTPCR Detection System (Bio-Rad).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.12.007.

AUTHOR CONTRIBUTIONS

A.G.C., T.E.N., and W.G. conceived of experiments. A.G.C. performed the experiments aided by experimental input from D.C.S., P.K., A.A.C., Y.T., J.F.M., and K.K.B., and intellectual input from J.S.S, D.B.C., G.J.R., and K.J.C. A.G.C., T.E.N., and W.G. wrote the manuscript. All authors reviewed and edited the manuscript.

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