Alternative splicing rewires Hippo signaling pathway in hepatocytes to promote liver regeneration

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During liver regeneration, most new hepatocytes arise via self-duplication; yet, the underlying mechanisms that drive hepatocyte proliferation following injury remain poorly defined. By combining high-resolution transcriptome and polysome profiling of hepatocytes purified from naive and toxin-injured mouse livers, we uncover pervasive alterations in messenger RNA translation of metabolic and RNA-processing factors, which modulate the protein levels of a set of splicing regulators. Specifically, downregulation of the splicing regulator ESRP2 activates a neonatal alternative splicing program that rewires the Hippo signaling pathway in regenerating hepatocytes. We show that production of neonatal splice isoforms attenuates Hippo signaling, enables greater transcriptional activation of downstream target genes, and facilitates liver regeneration. We further demonstrate that ESRP2 deletion in mice causes excessive hepatocyte proliferation upon injury, whereas forced expression of ESRP2 inhibits proliferation by suppressing the expression of neonatal Hippo pathway isoforms. Thus, our findings reveal an alternative splicing axis that supports regeneration following chronic liver injury.

The mammalian liver possesses remarkable ability to regenerate after injury with the potential to restore up to 70% of its lost mass and function in just a few weeks1–2. Under normal circumstances, the liver sustains the turnover of its fully differentiated parenchymal cells—hepatocytes and cholangiocytes—through self-duplication3–4. Furthermore, upon acute injury such as partial hepatectomy, residual mature hepatocytes undergo hypertrophy or re-enter the cell cycle to proliferate and restore liver functionality. Yet, if the liver is severely damaged and hepatocyte proliferation is compromised by certain drugs or toxins, other cells, such as the bipotential hepatobiliary progenitors, may contribute towards regeneration by giving rise to new hepatocytes5–9. These progenitor cells are thought to serve as facultative stem cells that can differentiate into hepatocytes or cholangiocytes during chronic injury conditions5–9. Recent cell-fate and lineage-tracing studies, however, found no evidence for existence of such facultative stem cells in mouse livers, and demonstrated that, under both acute and chronic injury conditions, virtually all new hepatocytes are derived from pre-existing hepatocytes10–16. These findings indicate that adult hepatocytes can proliferate even after severe liver damage, but the core mechanisms that trigger a quiescent hepatocyte to re-enter the cell cycle remain elusive.

Here, we sought to determine how hepatic gene expression programs are reprogrammed following injury to support proliferation while maintaining essential liver functions, and which factor(s) regulate these remodeling events. We developed a cell type-specific polyribosome-profiling method and combined it with deep transcriptome analyses to probe messenger RNA translation at a genome-wide scale. Comparing ribosome occupancies of hepatocyte mRNAs from naive and toxin-injured adult mouse livers revealed global translation reprogramming in regenerating hepatocytes, which mimicked a neonatal-like gene expression program. We found that downregulation of Epithelial Splicing Regulatory Protein 2 (ESRP2) is essential for reactivating the neonatal splicing program for a large set of mRNAs associated with cell proliferation, including the Hippo signaling pathway. We also demonstrated that deletion of ESRP2 in mouse livers results in excessive hepatocyte proliferation upon injury, whereas maintaining high ESRP2 expression during toxin exposure blocks proliferation by inhibiting the adult-to-neonatal switch in splicing of core Hippo pathway genes. Thus, our results identify remodeling of mRNA splicing and translation as part of the molecular network that controls hepatocellular plasticity during regeneration and provide direct evidence that alternative splicing modulates Hippo signaling to tune hepatocyte proliferation in response to chronic liver injury.

Results
Regenerating hepatocytes reactivate neonatal gene expression patterns. To study transcriptome changes in regenerating hepatocytes, we used a toxin-induced liver injury model17. Adult mice were fed a 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-supplemented diet for 4 weeks followed by whole liver tissue collection for histological analysis and hepatocyte isolation for RNA sequencing (RNA-seq) (Fig. 1a). Gross morphology of injured livers exhibited the expected pathology18, including enlarged liver size, change in color from pale to dark red, and elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Fig. 1a and Supplementary Fig. 1a,b). Additional characteristic features of DDC injury such as the presence of porphyrin plugs, hepatic necrosis, cholestasis, inflammation, and ductal hyperplasia...
were also observed (Supplementary Fig. 1c). Furthermore, we detected a substantial increase in hepatocyte proliferation indicated by both 5-ethyl-2′-deoxyuridine (EdU) labeling of new DNA synthesis and immunostaining using hepatocyte nuclear factor 4-alpha (Hnf4α) and phospho-histone-3 (PH3) (Fig. 1b,c and Supplementary Fig. 1e) and performed RNA-seq on poly(A)-selected RNA from hepatocytes during development (E18-Adult CHOW) and regeneration (Adult CHOW-DDC). Statistically significant genes (>0.05) were obtained (<927)

Hepatocytes were isolated from CHOW-fed or DDC-injured adult livers and their relative purity determined by robust de-enrichment of non-parenchymal cell markers and a reciprocal enrichment of adult hepatocyte markers (Fig. 1d and Supplementary Fig. 1d).

Because the hepatic transcriptome is extensively remodeled during postnatal liver maturation, we hypothesized that in order to proliferate a mature hepatocyte might utilize similar gene expression programs that are active in a neonatal hepatocyte. So, we isolated hepatocytes from embryonic day 18 (E18) livers in parallel (Supplementary Fig. 1e) and performed RNA-seq on poly(A)-selected RNA of adult hepatocyte markers (Fig. 1d and Supplementary Fig. 1d).

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RNA from all three sample groups (E18, adult CHOW-fed, adult DDC-fed). Excellent mapping rates and reproducibility between biological replicates were achieved (Supplementary Table 1 and Supplementary Fig. 1f–h).

Injury-induced liver regeneration affected the mRNA abundance of approximately 21% of genes expressed in hepatocytes (>1 transcript per million (TPM)), as we identified 1,214 upregulated and 927 downregulated mRNAs (fold change >2.0, false discovery rate (FDR) <0.05) between quiescent and toxin-injured hepatocytes (Fig. 1e). Comparative analysis of the developmental [E18 (CHOW)-Adult (CHOW)] and regenerative [Adult (CHOW)-Adult (DDC)] transcriptomes revealed that nearly 76% of differentially expressed mRNAs in regeneration (1,631 of 2,141) revert their expression pattern towards the neonatal stage (Fig. 1f). The other 24% of mRNAs (510 of 2,141) changed exclusively in regenerating hepatocytes, as their abundance was unaltered through liver development (Fig. 1g).

A larger but separate cohort of developmentally regulated mRNAs, however, showed only a modest adult-to-neonatal shift in expression upon injury (Fig. 1h). Thus, our global analysis revealed portions of the hepatocyte transcriptome that undergo dampening of the adult, or reactivation of the neonatal, gene expression program in response to injury (Supplementary Fig. 2a). Further, we noted that the transcripts exhibiting decreased expression upon injury form highly connected gene networks of biological functions associated with mature hepatocytes, such as metabolic and biosynthetic processes (Supplementary Fig. 2b,c). In contrast, the transcripts exhibiting increased mRNA expression clustered into the cell cycle and growth factor signaling categories. Interestingly, while the development-specific group was similarly enriched for metabolic and cell cycle-related genes (Supplementary Fig. 2b,e), the regeneration-specific group formed uniquely enriched clusters of mRNAs involved in immune or endoplasmic reticulum stress response, cell proliferation, and regulation of mRNA translation (Supplementary Fig. 2d). Overall, these results illustrate that, in response to injury, mature hepatocytes selectively reprogram a portion of their transcriptome to a less differentiated, ‘neonatal-like’ state.

Reprogramming of mRNA translation in regenerating hepatocytes. Because the mRNA translation process was enriched within the ‘regeneration-specific’ gene cluster (Supplementary Fig. 2d), we hypothesized that, in addition to the transcriptome, hepatocytes may also reprogram their translatome following injury. We systematically probed the translation output of naive and regenerating hepatocytes by combining cell type-specific in vivo polyribosome profiling with deep transcriptome analyses. To ensure proper isolation of intact mRNA and ribosomal complexes from hepatocytes, we first perfused the liver with cycloheximide to stall the translating ribosomes (Supplementary Fig. 3a). Addition of cycloheximide at the start of the isolation procedure circumvents any shifts in ribosomal occupancies due to the stress of the procedure itself, as stress responses can influence gene-specific translation within minutes. RNAs cofractionating with monosomes, light polysomes (2–4 ribosomes), and heavy polysomes (>4 ribosomes) were purified, poly(A) selected, and sequenced to obtain transcript-level abundances. For each fraction, high reproducibility between biological replicates was obtained (Supplementary Fig. 3b–i).

To determine the changes in ribosomal occupancy of each transcript, we calculated an index we refer to as the fractional shift ratio (FSR), which involved determining log, ratios of the fractional abundance of transcripts present between CHOW- and DDC-fed mice. This calculation was performed on all expressed transcripts from each fraction, resulting in a distribution of transcript shift ratios (Fig. 2a–c). A null set was generated using the control CHOW samples to allow for the determination of significant shifts in ribosome occupancy (P value <0.01, FDR <0.1). We identified 1,486 transcripts within 1,326 (~9%) protein-coding genes that undergo robust ribosomal occupancy shifts in regenerating hepatocytes. Specifically, 796 transcripts showed increased ribosomal occupancy (translational activation), whereas 530 transcripts showed decreased ribosomal occupancy (translational repression). Among the groups of mRNAs with increased and decreased translation, we found further subclusters. For instance, while most mRNAs increasing in ribosomal occupancy shifted from monosomes directly to heavy polysomes, there were also groups of mRNAs that shifted from monosomes to light polysomes or light to heavy polysomes and vice versa (Fig. 2d). Hence, we conclude that the hepatocyte translatome is extensively reprogrammed after injury and, for many mRNA transcripts, this reprogramming varies greatly in terms of magnitude and regulatory dynamics.

We next asked whether there was any overlap between gene sets shifting in ribosomal occupancy and/or mRNA abundance after injury. Only a small number of translationally regulated mRNAs showed a concurrent change in abundance, implying that these two modes of regulation occur independently in regenerating hepatocytes (Fig. 2e). The most significant network of translationally upregulated mRNAs belonged to ‘RNA processing and translation’ categories, whereas the translationally repressed genes clustered into the ‘glucose and lipid metabolism’-related pathways (Fig. 2d). Intriguingly, these pathways are similarly enriched within gene sets that differ in mRNA abundance (Supplementary Fig. 2b); yet, the actual genes regulated via changes in either abundance or ribosome occupancies are mutually exclusive (Fig. 2e). These data, therefore, demonstrate that although the mRNA expression and translation regulatory networks appear to work independently at the individual gene level, their cumulative effects at the pathway level are synergistic during liver regeneration.

Regenerating hepatocytes alter translation of splicing factors and activate a neonatal splicing program. Transcripts coding for mRNA translation and splicing factors were enriched for ribosome occupancy shifts following injury (Fig. 2d and Supplementary Fig. 3i). We quantified individual transcript levels of eight developmentally regulated splicing factors in the liver by quantitative (qPCR) and observed significant shifts in ribosome occupancies for seven of these eight factors (Fig. 3a). We also examined the protein abundance of these factors by western blots in hepatocytes and detected substantial changes in their steady-state levels (Fig. 3b). Thus, the mRNAs encoding splicing factors, which differed significantly in their ribosome occupancies upon toxin injury, exhibited a corresponding change at the protein level, verifying that our polysome profiling method can positively predict changes in protein abundance through translational regulation.

Alternative splicing decisions are influenced by splice site strength, cis-acting regulatory elements within pre-mRNAs that promote or inhibit exon recognition, and activity of trans-acting factors that bind to these cis elements and regulate the accessibility of the spliceosome to splice sites. Given the notable changes in protein abundance for a number of splicing factors in regenerating hepatocytes, we further analyzed our RNA-seq data for changes in alternative splicing. We identified 420 significantly changing splicing events (difference in percent spliced index (PSI) >15%, FDR < 0.10) within 349 genes, with an expected distribution of splicing event type (Fig. 3c) and relatively little overlap with genes regulated at the mRNA abundance levels (Fig. 3d). Of the regeneration-regulated events, >80% were also regulated during development, with high similarity between the splicing patterns of neonatal and regenerating hepatocytes (Fig. 3e,f). Moreover, these alternatively spliced transcripts were enriched in functions such as chromatin modification and regulation of transcription and splicing (Fig. 3g). Collectively, our data suggest that, following injury, hepatocytes redeploy a neonatal splicing program by modulating the protein abundance of key splicing factors through translational reprogramming.
mRNA and protein features of regeneration-regulated exons. Global interactome studies have shown that alternatively spliced isoforms tend to generate distinct proteins rather than minor variants of each other, and these isoforms can be further post-translationally modified to produce even more diverse proteomes. To explore the functional impact of programmed changes in splicing, we first investigated the spatial distribution of regeneration-regulated cassette exons along with their associated transcripts. Of the alternatively spliced events detected from our analysis, 95% (399 of 420) occurred in protein-coding genes. Furthermore, 70% of differentially spliced exons were located within coding sequences (CDSs) and a significant number of those (24%) encoded an alternate START or STOP codon. The CDS-mapped exons were further separated based on whether they were open reading frame (ORF) preserving (exon length is a multiple of three) or non-preserving. We found that the majority (~64%) of CDS events preserve the ORF, thus adding or removing a peptide segment in the protein. Intriguingly, only 11 of the 66 (~17%) ORF non-preserving regions within the CDS were predicted to be nonsense mediated decay targets. These results suggest that a number of regeneration-regulated exons may be altering the intrinsic features of respective proteins. Next, we selected regeneration-regulated cassette exons from a database with at least 80% sequence conservation between mice and humans (n = 220) and probed their functional properties. Our analysis revealed many distinct protein features encoded by these exons, including structural and binding elements, cellular localization, and post-translational modifications. Within the localization category, a significant number of exons (n = 52) contain nuclear export and membrane localization signals, suggesting that programmed changes in splicing after injury may be a common mechanism to regulate subcellular targeting of proteins. Amid different domains, ‘protein-binding’ segments predominated...
Fig. 3 | Altered translation of alternative splicing factors and activation of a neonatal splicing program in regenerating hepatocytes. a, qPCR-based expression of select alternative splicing factors in different polysomal fractions of hepatocytes isolated from CHOW- and DDC-fed mouse livers. RNP, ribonucleoparticles. Data are mean ± s.d. (n = 3 animals per condition). b, Western blots and their respective quantification showing changes in protein abundance of select alternative splicing factors. Representative examples (n = 3 animals per condition) are shown in the blots; quantification was derived from 3 experiments on n = 5 animals per condition. Uncropped gel images are provided in Supplementary Data Set 1. c, Violin distribution plots for significantly changing alternative splicing events after DDC injury according to the event types (P < 0.05, FDR < 0.10 from rMATS; adjusted for multiple testing, ΔPSI ≥ 15%, and junction counts ≥ 10) obtained from RNA-seq (n = 2 animals per condition). d, Overlap of mRNA abundance and alternative splicing changes following DDC injury. e, Heatmap of PSI values showing splicing transitions (row normalized, PSI) overlapping between development (E18 CHOW-Adult CHOW) and regeneration (Adult CHOW-Adult DDC). Orange color indicates reduced exon inclusion and purple color indicates higher exon inclusion following DDC injury. Each paired column is an independent biological replicate. f, Venn diagram showing number of alternative splicing events in regenerating hepatocytes that are reciprocally regulated during normal development. g, Gene ontology analysis for significantly enriched pathways and molecular functions for genes undergoing alternative splicing following DDC injury (P < 0.05, hypergeometric test with P value adjusted using Benjamini method for multiple testing). Source data for panel a are available in Supplementary Data Set 3. Data underlying the analyses in panels c and e are available in Supplementary Data Set 2.
within the regeneration-regulated exons (Fig. 4d) and, when compared with randomized sets of constitutive or non-regulated alternative exons, the regulated exons were significantly enriched for intrinsically unstructured regions (Fig. 4e). We further noted that these unstructured regions were enriched for phosphorylation compared to other post-translational modification sites, and they frequently encoded conserved serine/threonine but not tyrosine residues (Fig. 4e). Altogether, these results suggest that through alternative inclusion of peptide segments—which contain protein-binding domains or disordered binding motifs with phosphosite—the regenerating hepatocytes may effectively rewire their protein–protein interactions.

**ESRP2 downregulation following injury supports hepatocyte proliferation.** Our next goal was to identify the regulatory factor(s) responsible for reactivating the neonatal splicing program and test whether they serve any direct roles in liver regeneration. Amongst the various splicing factors that differed in expression after DDC injury (Fig. 3a, b), we elected to focus on ESRP2 because: (1) ESRP2-binding motifs were highly enriched around regeneration-regulated exons, and (2) ESRP2 is a key hepatocyte factor that controls ~20% of splice isoform transitions occurring naturally during postnatal liver development. To determine whether injury-induced ESRP2 downregulation is reversible, we administered DDC diet to wild-type (WT) mice for four weeks (injury phase) and then switched them to regular CHOW diet for the next four weeks (recovery phase) (Fig. 5a). As expected, the characteristic hepatocellular damage and regeneration response abated considerably when the animals were recovered (Supplementary Fig. 4a–d). Notably, ESRP2 displayed a dynamic expression pattern during the injury and recovery phases of liver regeneration. ESRP2 protein levels decreased considerably when mice were fed DDC diet but were fully restored upon toxin removal (Fig. 5b). Also, the downregulation occurred relatively early as ESRP2 protein levels started to decline within 5 days of DDC administration (Supplementary Fig. 4e).

To evaluate the physiological relevance of ESRP2 downregulation in liver regeneration, we used Esrp2 knockout mice. The mice lacking ESRP2 have relatively normal growth and are fertile, but their hepatocytes are immature, smaller in size, and a significant number fail to fully exit the cell cycle. Adult littersmates of WT and Esrp2 knockout mice were subjected to the same DDC injury–recovery protocol (Fig. 5a). While the liver damage (necrosis, inflammation, and presence of porphyrin plugs) and systemic symptoms (weight loss and jaundice) after DDC treatment were comparable between WT and Esrp2 knockout (Fig. 5c, top row), the hepatosomatic index (liver/body weight ratio) of the knockouts was substantially higher under both injury and recovery conditions (Fig. 5d). We reasoned that higher hepatosomatic index in the absence of ESRP2 might be due to excessive hepatocyte proliferation during the injury-recovery circumstances. We quantified the number of proliferating hepatocytes by co-immunostaining the DDC-injured and recovered liver sections from WT and Esrp2 knockout mice with the mitotic marker PH3 and the hepatocyte marker Hnf4α. We detected significantly more PH3+ proliferating cells among Hnf4α+ cells in Esrp2 knockouts compared to WT mice under both injury and recovery conditions (Fig. 5c, middle row, and 5e). Quantification of the ductular reaction (KRT19+ cholangiocytes) throughout the injury-recovery circumstances, however, showed no difference between WT and Esrp2 knockout livers (Fig. 5c, bottom row, and 5f).

To further investigate whether ESRP2 downregulation is required for hepatocyte proliferation, we generated tetracycline-inducible, hepatocyte-specific FLAG-tagged ESRP2 transgenic mice (Supplementary Fig. 5a). As anticipated, the TRE-ESRP2 (TRE, tetracycline response element; ApoE-rtTA bitransgenics, hereafter referred to as ESRP2 overexpression (ESRP2 OE)) mice, allowed conditional, doxycycline (Dox)-dependent expression of ESRP2 (Supplementary Fig. 5b). We titrated the dose of Dox needed in the diet to restore ESRP2 protein expression in DDC-injured livers (Supplementary Fig. 5c,d). Remarkably, sustaining higher ESRP2 protein levels inhibited the increase in liver mass typically observed in response to DDC injury (Fig. 5d). Consistent with this observation, we witnessed a significant block in hepatocyte proliferation as revealed by the reduced number of PH3+ and Hnf4α+ cells in ESRP2 OE mouse livers following injury when compared to uninjured littersmate controls (Fig. 5c, middle row, and 5e). Maintaining high ESRP2 protein levels, however, had little effect on overall ductal hyperplasia (Fig. 5c, bottom row, and 5f). Next, we performed serum biochemical tests to measure the extent of liver function and hepatocellular damage in DDC-fed control and ESRP2 OE mice. Both total and direct serum bilirubin and ALT levels were significantly elevated in ESRP2 OE mice when compared with littermate controls (Fig. 5g,h), indicating that impaired hepatocyte proliferation following injury compromises their liver function. Collectively, these data provide compelling evidence that downregulation of ESRP2 supports the proliferative capacity of hepatocytes in response to chronic liver injury.

**ESRP2 downregulation generates neonatal isoforms of Hippo pathway proteins.** We next examined whether ESRP-binding motifs are overrepresented near alternative exons that undergo a robust change in splicing upon liver injury. We found that UGG-rich ESRP2 motifs, UGGUG and UUGGG, are significantly enriched (blue line) in the upstream introns of exons that exhibit enhanced inclusion in response to DDC injury (Fig. 6a). The ESRP motif UUGGG was also enriched in the downstream introns of exons (red line) that are repressed upon injury (Fig. 6a). These observations corroborate with ESRP2 downregulation in regenerating hepatocytes and the typical ESRP RNA map where the upstream binding represses and the downstream binding promotes alternative exon inclusion.

To assess the functional impact of ESRP2 downregulation in reprogramming alternative splicing, we used PCR with reverse transcription (RT-PCR) to screen the top 25 ESRP2-sensitive exons—obtained from ref. 19—in the livers of Esrp2 knockout, ESRP2 OE, and littermate control mice fed either CHOW or DDC diet. These exons showed a reciprocal switch in splicing in Esrp2 knockout and ESRP2 OE when compared with WT mice (Fig. 6b). The gel images of RT-PCR products from two representative examples—Arhgef10l 15-nucleotide (nt) exon and Lsm14b 78-nt exon—are shown in Supplementary Fig. 6a. Consistent with reduced ESRP2 protein expression following injury, all splicing events in the DDC-fed WT and CHOW-fed Esrp2 knockout mice were highly correlated in both pattern and directionality. Strikingly, sustaining ESRP2 protein levels blocked the injury-induced shift in splicing, indicating that ESRP2 downregulation is key for stimulating these splicing transitions (Fig. 6b and Supplementary Fig. 6a).

Among ESRP2 splicing targets, we found several genes involved in cell proliferation, including core components of the Hippo signaling pathway such as Neurofibromatosis 2 (NF2), Casein kinase 1 delta (Cskcd1), Yes-associated protein 1 (Yap1), and Tea domain transcription factor 1 (Tead1) (Fig. 6b). Hippo signaling is a conserved pathway that governs organ size, tissue homeostasis, and regeneration in metazoans, and its deregulation results in uncontrolled cell proliferation and malignant transformation. Furthermore, Hippo signaling is required for maintaining the differentiated state of hepatocytes and serves essential roles for cell fate determination in normal and regenerating livers.

When Hippo signaling is on, NF2 acts as an upstream activator of the MST1/2–WW45 complex, which phosphorylates and activates LATS1/2 kinases (Supplementary Fig. 6b). Activated LATS1/2 phosphorylates YAP1 to sequester it in the cytoplasm by 14-3-3 proteins, or prime it for additional phosphorylation by CSNK1D, which
promotes β-TrCP-mediated degradation of YAP1. Upon inactivation of Hippo signaling, YAP1 accumulates in the nucleus and partners with the TEAD family of transcriptional factors, stimulating expression of pro-proliferative genes. Interestingly, NF2, CSNK1D, Yap1, and Tead1 each encode a conserved developmentally regulated exon that is predominantly skipped in neonatal but included in the adult hepatocytes (Fig. 6c). We found that ESRP2 coordinates the postnatal inclusion of these in-frame exons to generate adult protein isoforms. ESRP2 deletion promoted their skipping and, therefore, production of neonatal isoforms in the adult liver, whereas ESRP2 OE enhanced the production of adult isoforms (Fig. 6d and Supplementary Fig. 6c). The presence of nearby ESRP2 binding sites along with skipping of all four exons following transient depletion of ESRP2 in cultured hepatocytes further confirmed ESRP2 as their primary regulator (Supplementary Fig. 6d–f). Importantly, similar to ESRP2 knockouts, DDC-injury in WT mice evoked an adult-to-neonatal shift in splicing, whereas sustaining ESRP2 expression during injury preserved the adult splicing pattern for all four genes (Fig. 6d and Supplementary Fig. 6c). As was previously reported, liver injury inhibited the Hippo pathway and upregulated the expression of its gene targets (Fig. 6c). Notably, the injury-induced upregulation of target genes was considerably higher in ESRP2 knockouts, but was largely blunted in ESRP2 OE (Fig. 6c). Together, these data reveal that ESRP2 downregulation following injury reprograms the splicing of core Hippo pathway components, probably modulating their signaling and downstream gene expression activity.

**Neonatal isoforms attenuate Hippo signaling and promote hepatocyte proliferation.** We next explored the functional differences between neonatal and adult protein isoforms of NF2, CSNK1D, Yap1, and TEAD1. The neonatal NF2 protein isoform exists in two functionally distinct conformations. In the open and active form, the LATS1/2 binding site is freely accessible, and thus Hippo signaling is enabled (Fig. 7a, panel 1). In the closed and inactive form, the C-terminal domain (CTD) binds to the N-terminal FERM domain and blocks access to the LATS1/2 binding site, thereby inhibiting Hippo signaling. The adult NF2 protein isoform, however, includes a 43-nt exon at the end of the CTD, which encodes an early STOP codon resulting in truncation of 5 amino acid residues, 4 of which are necessary for FERM binding (Fig. 7a, panel 1). The alternative exon also alters the composition of 11 other amino acids from primarily hydrophobic to charged residues, which are expected to create a repulsive force between the CTD and the hydrophobic binding pockets in the FERM domain. Thus, the adult NF2 isoform probably adopts a constitutively open state providing uninterrupted access to LATS1/2, thereby promoting YAP phosphorylation and degradation.

The neonatal CSNK1D protein isoform encodes a C-terminal tail containing a stretch of serine residues (Fig. 7a, panel 2), which when phosphorylated lower its enzymatic activity. Elimination of these auto-inhibitory phosphorylation sites through truncation or site-directed mutagenesis enhances the CSNK1D kinase activity towards its substrates. The adult CSNK1D protein isoform includes a 63-nt exon in the C-terminal tail, which, like NF2, encodes an early STOP codon truncating six amino acid residues (Fig. 7a, panel 2). Notably, this eliminates three serine residues in the adult CSNK1D isoform, which should relieve the auto-inhibition and thus generate a more active kinase for YAP phosphorylation and degradation.

YAP1 encodes a leucine zipper within its transcription activation domain that is important for protein–protein interactions. The neonatal YAP1 isoform has an intact leucine zipper with 4 heptads; however, the inclusion of a 48-nt exon in the adult YAP1 isoform inserts a 16-amino-acid-long intrinsically unstructured segment between heptad H3 and H4, leading to its thermodynamic destabilization (Fig. 7a, panel 3). Consequently, the inability of the...
adult YAP1 protein isoform to form a functional leucine zipper is expected to hinder the protein–protein interactions needed for its hetero-association with other cellular partners50–52.

TEAD proteins share a highly conserved N-terminal TEA domain that binds to DNA and a C-terminal transactivation domain, which binds the cofactor YAP1 to induce target gene expression53.
The TEA domain is composed of a three-helix bundle, where the H3 helix provides the interface for DNA binding. The 12-nt microexon in the adult TEAD1 protein isoform adds 4 amino acids just downstream of the DNA binding helix H3 (Fig. 7a, panel 4). Amongst these four amino acids is a conserved serine residue, which when phosphorylated by protein kinase C strongly inhibits TEAD1's DNA-binding ability. Thus, inclusion of this microexon should serve as an alternate mechanism for reducing proliferative capacity of cultured hepatocytes. Compared to control antisense morpholino oligonucleotides (ASOs), AML12 cells treated with a mixture of NF2-, Csnk1d-, Yap1-, and Tead1-targeted ASOs elicited a near-complete switch to the exon-skipped neonatal isoforms (Fig. 7b). Three non-targeted alternative exons were tested as control and all were unaffected, demonstrating absence of non-specific effects (Supplementary Fig. 7b). Also, the steady-state mRNA levels of NF2, Csnk1d, Yap1, and Tead1 were unaltered after ASO treatment, confirming that the switch in splicing occurred without a change in total mRNA levels (Supplementary Fig. 7c).

To determine whether redirection of splicing affected the output of the Hippo signaling pathway, we first evaluated the phosphorylation status of key serine/threonine residues within mouse MST1/2 (T183/T180), LATS1 (S958), and YAP1 (S112) that are known to restrict YAP/TEAD-mediated gene transcription. Consistent with our hypothesis, selective expression of neonatal splice isoforms led to a significant decrease in the ratio of pLATS1/LATS1 and pYAP1/YAP1 but not pMST/MST proteins (Fig. 7c). Next, we transfected the control- and ASO mix-treated AML12 cells with a

Fig. 6 | ESRP2 downregulation reprograms alternative splicing to generate neonatal isoforms of Hippo pathway proteins. a, Position and relative enrichment of ESRP2-binding motifs within exonic and 250-nt intronic sequences surrounding the regeneration-regulated exons. b, Heatmap of differences in RT-PCR-derived exon inclusion (row-normalized, ΔPSI) values in WT, Esrp2 KO, and ESRP2 OE livers under CHOW and DDC conditions (n = 3 animals per group). Number after gene name signifies the size of alternative exon in base pairs. 

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Taken together, the developmentally regulated exons introduce functionally relevant protein segments in NF2, Csnk1d, Yap1, and TEAD1 that appear to enhance Hippo signaling and thereby allow adult hepatocytes to enter quiescence. Given that ESRP2 promotes the inclusion of these alternative exons, we reasoned that ESRP2's influence on hepatocyte proliferation upon injury might be driven in part by the production of neonatal isoforms of the Hippo signaling pathway. We therefore decided to simultaneously redirect splicing of all four exons in AML12 cells (Supplementary Fig. 7a), a non-transformed mouse cell line that exhibits differentiated hepatocyte features and expresses high levels of hepatic mRNAs. Serum starvation-induced quiescence in AML12 cells led to high inclusion of NF2 and Tead1 alternative exons and moderate inclusion of Csnk1d and Yap1 alternative exons. This observation allowed us to gauge the combined effect of these exons on Hippo signaling and proliferative capacity of cultured hepatocytes. Compared to control antisense morpholino oligonucleotides (ASOs), AML12 cells treated with a mixture of NF2-, Csnk1d-, Yap1-, and Tead1-targeted ASOs elicited a near-complete switch to the exon-skipped neonatal isoforms (Fig. 7b). Three non-targeted alternative exons were tested as control and all were unaffected, demonstrating absence of non-specific effects (Supplementary Fig. 7b). Also, the steady-state mRNA levels of NF2, Csnk1d, Yap1, and Tead1 were unaltered after ASO treatment, confirming that the switch in splicing occurred without a change in total mRNA levels (Supplementary Fig. 7c).

To determine whether redirection of splicing affected the output of the Hippo signaling pathway, we first evaluated the phosphorylation status of key serine/threonine residues within mouse MST1/2 (T183/T180), LATS1 (S958), and YAP1 (S112) that are known to restrict YAP/TEAD-mediated gene transcription. Consistent with our hypothesis, selective expression of neonatal splice isoforms led to a significant decrease in the ratio of pLATS1/LATS1 and pYAP1/YAP1 but not pMST/MST proteins (Fig. 7c). Next, we transfected the control- and ASO mix-treated AML12 cells with a

![Fig. 6](image-url)
Fig. 7 | Exclusive production of neonatal protein isoforms attenuates Hippo signaling, activates gene expression, and promotes hepatocyte proliferation. a, Cartoons representing the impact of alternative exons on structure, activity, and function of core Hippo pathway proteins: NF2, CSNK1D, YAP1, and TEAD1. Red bar shows the relative location of the alternative exon within individual transcripts. (−) and (+) signs show exclusion (neonatal) and inclusion (adult) isoform amino acid sequences. The amino acid sequences encoded by alternative exons are indicated in red. Residues highlighted with a purple asterisk can be phosphorylated under specific conditions. Red regions within adult protein cartoons signify the location of resistive segments encoded by respective alternative exons. b, Simultaneous skipping of NF2, CSNK1D, YAP1, and TEAD1 alternative exons in AML12 cells after treatment with a mixture of ASOs. The band corresponding to (+) indicates exon inclusion and (−) indicates exon skipping. c, Western blots of core Hippo pathway proteins and their phosphorylated forms, and quantification of phosphorylated/total protein ratios in AML12 cells following treatment with control or a mixture of Hippo targeting ASOs. All values (mean ± s.d.) were normalized to β-tubulin (TBP); n = 3 biologically independent cell culture replicates. Uncropped gel images are provided in Supplementary Data Set 1. d, Schematic (top) and relative luciferase activity (bottom) derived from the TEAD reporter construct transfected in AML12 cells following treatment with control or a mixture of Hippo targeting ASOs. All values (mean ± s.d.) were normalized to β-galactosidase activity produced from a cotransfected control plasmid (n = 6 independent transfections). e, Relative mRNA expression of indicated Hippo target genes normalized to Tbp (qPCR); n = 3 biologically independent cell culture experiments. f–g, Representative images (f) and quantification (g) of EdU labeling. h, MTT assay-based quantification of AML12 cell proliferation following treatment with control or a mixture of Hippo-targeted ASOs from three biologically independent cell culture experiments. For g, n = 7 images fields were quantified per experiment; for h, n = 4 technical replicates were used per condition. Data are mean ± s.d.; *P < 0.05, **P < 0.01, and ****P < 0.0001 from unpaired two-tailed t-test. Source data for panels d and e are available in Supplementary Data Set 3.
Articles

demonstrated that neonatal splicing program to rewire the Hippo signaling pathway showed that, following injury, ESRP2 downregulation reactivates a Birc5 target that is repressed in the fetal and early postnatal periods of development. After birth, ESRP2 controls the splicing of developmentally regulated exons in NF2, CSNK1D, YAP1, and TEAD1 to introduce functionally relevant protein segments that enhance Hippo signaling. We found that, in comparison to the adult, the neonatal Hippo splice isoforms abate Hippo signaling, which enables greater transcriptional activation of YAP1/TEAD1 target genes promoting hepatocyte proliferation.

Discussion

How changes in the transcriptome and/or translome influence the proliferative capacity of hepatocytes following injury and how post-transcriptional gene regulatory mechanisms integrate into these paradigms are still unresolved questions. In this study, we uncovered that dynamic remodeling of mRNA splicing and translation programs is critical for hepatocyte proliferation in response to chronic liver injury. Our work further revealed that dynamic regulation of ESRP2 coordinates the adult-to-neonatal splicing switch of many proliferation-related genes, including core components of the Hippo signaling pathway, which may constitute a fundamental mechanism used by hepatocytes to adapt to injury. Importantly, we identified that ESRP2 controls the splicing of developmentally regulated exons in NF2, CSNK1D, YAP1, and TEAD1 to introduce functionally relevant protein segments that enhance Hippo signaling. We found that, in comparison to the adult, the neonatal Hippo splice isoforms have innate lower signaling capacity, which allows higher transcriptional activity of the downstream YAP1 and TEAD1 effectors, thereby sustaining hepatocyte proliferation during the fetal and early postnatal periods of development. After birth, ESRP2 protein levels rise in hepatocytes to promote the generation of adult isoforms of Hippo pathway that favor quiescence. We also showed that, following injury, ESRP2 downregulation reactivates a neonatal splicing program to rewire the Hippo signaling pathway and enable quiescent hepatocytes to proliferate. Accordingly, we demonstrated that ESRP2 knockout mice manifest excessive hepatocyte proliferation after injury, whereas sustaining high ESRP2 expression during toxin exposure inhibits hepatocyte proliferation by impeding the formation of neonatal splice isoforms of the Hippo signaling pathway. In the future, it will be important to develop isoform-specific antibodies and/or mass spectrometry-based methods to generate reliable, quantitative measurements of alternatively spliced Hippo pathway proteoforms and further interrogate their biological significance.

In conclusion, we have identified a novel ESRP2-Hippo pathway alternative splicing axis that tunes hepatocyte proliferation and facilitates regeneration in response to chronic liver injury. It remains to be seen whether this axis is also utilized to regenerate after acute liver injuries such as partial hepatectomy, and whether this axis can be therapeutically harnessed to stimulate regeneration during acute liver failure or end-stage liver disease.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability, and associated accession codes are available at https://doi.org/10.1038/s41594-018-0129-2.

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**Author contributions**

S.B., W.A., J.S., and A.K. conceived the project and designed the experiments. S.B., W.A., J.S., and A.K. interpreted results and wrote the manuscript. All authors performed experiments. R.P.C. and S.A. provided reagents. S.B., W.A., J.S., and A.K. interpreted results and wrote the manuscript. All authors discussed the results and edited the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Methods

Animal models. National Institutes of Health (NIH) and institutional guidelines were followed in the use and care of laboratory animals and experimental protocols were performed as approved by the Institutional Animal Care and Use Committee at University of Illinois at Urbana-Champaign (UIUC). Animals used in the study were identified using ear tags after being genotyped at weaning age. The following study is not gender-specific and specimens include both male and female animals. Whole liver tissues and hepatocytes were isolated from mice following guidelines for euthanization and/or anesthesia.

FVB/NJ mouse strain was used primarily for all experiments including RNA-seq. RNA isolation, isolation, western blotting, and polyosome profiling. For ESR2P loss-of-function studies, ESR2P (−/−) and ESR2P (+/+). For gain-of-function studies, mouse ESR2P complementary DNA containing an N-terminal 1× FLAG-tag was expressed from a transgene with a TRE/methylcysteovirus (CMV) promoter, ESR2P (K25E); and Bovine growth hormone polyadenylation site, and 3′ flanking genomic segment for mRNA 3′ end formation. The linearized transgene construct was subjected to pronuclear injection using standard methods to generate TRE-ESR2P transgenic mice that were maintained on a C57BL/6J background. ApoE-rTAT transgenic mice expressing an rTAT variant specifically in hepatocytes were obtained courtesy of Dr. D. Pan (University of Texas Southwestern Medical Center)69. All mice reported in this study were the F1 progeny of TRE-ESR2P and ApoE-rTAT transgenic mice mating, and are hemizygous for both transgenes.

Murine diet scheme. For toxin-mediated liver injury, the FVB/NJ strain of mice at 8–10 weeks of age was used. For all experiments the mice were fed a diet containing 0.1% DDC (Teklad, Environ) for 4 weeks, unless mentioned otherwise. Additionally, for the purposes of ESR2P gain-of-function studies, mice were fed a diet containing 0.5–2.0 kg/g−1 d−1 dexamethasone in addition to 0.1% DDC for 4 weeks (Teklad, Environ).

Isolation of neonatal and adult hepatocytes. Hepatocytes from neonatal and DDC-fed mouse livers were isolated using adapted protocols from previously described methods70,71. Briefly, adult FVB mice were anesthetized in a chamber supplied with isoflurane and oxygen (2.5% isoflurane in oxygen, 1 l min−1) and maintained on the anesthetic during the procedure. The liver was perfused via cannulation of the portal vein with 50 ml Solution A (1× HBSS, without Ca2+ and Mg2+), 0.5 mM EDTA) followed by 50 ml of Solution B (1× HBSS (Hank’s balanced salt solution) (with Ca+), 5.4 mM CaCl2, 0.04 mg/ml 0.04 mg/ml soybean trypsin inhibitor, and 4,500 units of collagenase type I (Worthington Chemicals)). Subsequently, the liver was massaged in a petridish containing 1× HBSS to release cells from the liver capsule, and then the cell suspension was passed successively through and 70- and 40-μm filters to obtain a single-cell suspension. The cells were then centrifuged at 50×g for 5 min (4°C) to separate live hepatocytes from non-parenchymal cells and dead cells. The cells were further washed 3 times in 1X HBSS as above, and then flash frozen in liquid N2 and stored at −80°C till further use.

RNA-seq analysis. RNA was isolated from experiment specific hepatocytes using RNeasy tissue mini-kit (Qiagen). Downstream RNA quality was assessed using an Agilent Bioanalyzer, and quantified using a Qubit Fluorometer by the Unit, UIUC. RNA-seq reads were processed for quality and read length filters using RNeasy tissue mini-kit (Qiagen). Downstream RNA quality was assessed using an Agilent Bioanalyzer, and quantified using a Qubit Fluorometer by the Unit, UIUC. RNA-seq reads were further aligned to the mouse ESRP2 complementary DNA containing an N-terminal 1× FLAG-tag was expressed from a transgene with a TRE/methylcysteovirus (CMV) promoter, ESR2P (K25E); and Bovine growth hormone polyadenylation site, and 3′ flanking genomic segment for mRNA 3′ end formation. The linearized transgene construct was subjected to pronuclear injection using standard methods to generate TRE-ESR2P transgenic mice that were maintained on a C57BL/6J background. ApoE-rTAT transgenic mice expressing an rTAT variant specifically in hepatocytes were obtained courtesy of Dr. D. Pan (University of Texas Southwestern Medical Center)69. All mice reported in this study were the F1 progeny of TRE-ESR2P and ApoE-rTAT transgenic mice mating, and are hemizygous for both transgenes.

RNA was isolated from experiment specific hepatocytes using RNeasy tissue mini-kit (Qiagen). Downstream RNA quality was assessed using an Agilent Bioanalyzer, and quantified using a Qubit Fluorometer by the Unit, UIUC. RNA-seq reads were further aligned to the mm10 annotated mouse exons were converted to corresponding mRNA regions.

Additionally, to perform the exon ontology analysis, the spliced exons regulated in regeneration obtained from the above analysis were used. The cassettes/spliced exon class of these mm10 annotated mouse exons were corresponded to corresponding human exons in the hg19 annotation using UCSC cliufover with minimum ratio of bases matching as 0.8. Additionally, the exons in hg19 reported by UCSC cliufover were checked for gene identity match to the mouse exons parent gene. These exons were then analyzed for ontology using the exon ontology and FasterDB packages69.

Polysome gradient fractionation. Mice on normal Chow and 0.1% DDC diet as described before were used for polysome fractionation from hepatocytes. A modified perfusion protocol was developed wherein the animals were firstly perfused via portal vein cannulation with 75 ml Solution I (300 μg/ml cycloheximide in 1X HBSS (with phenol red) at a rate of 3.5 ml min−1). EDTA was excluded from Solution I due to its dissociative effects on ribosomes and associated complexes. Instead, liver perfusion and hepatocyte wash buffers were supplemented with cycloheximide (300 μg/ml) to arrest translating ribosomes69.

Purified hepatocytes were thawed in polysome lyisis buffer (10 mM Tris-HCl at pH 7.6, 150 mM NaCl, 5 mM MgCl2, 1 mg/ml heparin, 1% Nonidet P-40, 0.5% deoxycholate, 40 mM dithiothreitol, 1 μl/ml SUPERasein RNAse inhibitor (Thermo Fisher), and 150 μg/ml cycloheximide) and lysed by pipetting gently 10 times. The cell nuclei and cell debris were removed by centrifugation (12,000×g, 1 min, at 4°C). The supernatant was transferred to a fresh tube and then centrifuged again to remove remaining cell debris (10,000×g, 7.5 min, at 4°C). The resulting supernatant was transferred to a fresh tube and 400 μl supernatant was layered onto a 10-ml linear sucrose gradient (15–45% sucrose (v/v) made using a Biocomp Gradient Master) and centrifuged in a SW41 Ti rotor (Beckman) for 125 min at 38,000 r.p.m. and 4°C, as previously described70. Polysome profiles were recorded using a UA-6 absorbance (ISCO) detector at 254 nm and fractions were collected along the gradient corresponding to the ribonucleoparticles, monosomes, light polysomes (2–4 ribosomes), and heavy polysomes (5+ ribosomes).

RNA for downstream sequencing and validations was purified using an adapted protocol70. The sucrose gradient fractions were mixed with 0.05 volumes of 3 M sodium acetate and 2 μl glycogen followed by addition of 2 volumes of 100% EtOH, and stored at −80°C overnight to precipitate RNA/protein. The pellet was suspended in molecular grade water and, post DNase digestion, the sample was phase separated using 3 volumes of acid phenol/chloroform (5:1). The aqueous phase was again phase separated using chloroform/isooamyl alcohol (24:1). Following this, the aqueous phase was mixed with 0.1 volumes 3 M sodium acetate and 2 μl glycogen, and precipitated with 2.5 volumes EtOH overnight at −80°C. The pelleted RNA was then used for downstream poly(A)+-selected deep sequencing or qPCR as previously described70.

Analysis of polyribosome sequencing. Reads were checked for quality using FastQC software, and biological replicates were checked for reproducibility using spearman correlation coefficients. Reads were mapped to the mm10 genome using STAR72. Transcript count abundances were estimated and normalized across conditions using Cuffnorm73, and the Cuffnorm-calculated fragments per kilobase per million (FPKM) values for individual transcripts were converted to TPM using the following74: 

\[
TPM_{transcript} = \frac{FKPM_{transcript} \times 10^6}{ \sum FPKM} 
\]

TPM values were averaged across biological duplicates for all three pools of monosome (M), light (LP), and heavy (HP) polyribosomal fractions. Transcript shift across polysomal fractions was performed based on previous described methodology75. Using the TPM values, an index was calculated called the polysome fraction index (PFI) as follows:

\[
PFI_i = \frac{TPM_A}{TPM_M + TPM_L + TPM_H}
\]

Further, to calculate the shift in transcript distribution in a fraction between two conditions, a polysome shift ratio was calculated as follows for all fractions separately:
To test whether a calculated polysome shift ratio (PSR) value for a given transcript is significantly changing between conditions, a null set was generated using the sequencing data from the CHOW samples. CHOW-specific fastq sequence files were randomly partitioned into two separate and non-overlapping subsets with a 2:1 ratio. Transcript PSR values calculated from these partitions resulted in a normal distribution ($\mu = 0$, and $\sigma = 0$). With the empirical null distribution, $P$ values were calculated for all fraction-specific transcript PSRs using the Student’s $t$-test ($cutoff P < 0.01$). Additionally, FDR values were calculated using the Benjamini–Hochberg method, and significance was deemed as FDR < 0.05. Finally, transcripts with significant PSR values based on above conventions, cutoffs, in at least two fractions were considered to be significantly regulated. Clustering of transcripts was performed using heatmap 2 package in R, with the ward clustering method.

**Histology and immunofluorescence staining.** Post harvesting, tissues were fixed in 10% buffered formalin for 24 h, and then processed to be embedded in paraffin. Tissue sections (5 μm) were cut and deparaffinized in xylene, then moved through serial alcohol (100%, 95%, 80%, 50%) washes to rehydrate. For hematoxylin and eosin staining, sections were washed in hematoxylin (2 min) and eosin (1 min) successively, coverslipped with permount medium, and imaged on a Hamamatsu Nanozoomer Z2 (Agilent Technologies, Inc.) with the plug-in for genomic biology (IGB) core facility, UICUC. For immunofluorescent staining, deparaffinized and rehydrated sections were antigen retrieved in a slow cooker at 120 °C for 10 min. The retrieval was performed in either Tris-EDTA (pH 8.0) or citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). The sections were then washed in buffer A (Tris-buffered saline (TBS) +0.05% Triton X-100) and blocked (2 h) in 10% normal goat serum and 1% BSA at room temperature. The primary antibodies were applied to the sections at standardized concentrations and incubated overnight at 4 °C. Following this, the sections were washed in buffer A, and secondary fluorescent antibodies were applied for 1 h at room temperature. Lastly, ToPro 3 nuclear stain was applied for 15 min at room temperature. The sections were then coveredslipped with CC/Mount aqueous mounting media (Sigma). All sections were imaged on a Zeiss LSM 710 microscope at Institute for Genomic Biology (IGB) core facility, UICUC. All antibodies used and respective dilutions are listed in Supplementary Table 2.

To stain for nascent DNA synthesis in regenerating livers, normal CHOW- and 0.1% DD-pulzed mice (4-week treatment) were treated with a 4-h pulse of EdU administered intraperitoneally (100 μg per g body weight). Mice were then killed, and livers were harvested and paraffin embedded. Sections (5 μm) were deparaffinized and rehydrated, and then antigen retrieved in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). The sections were then washed in buffer A (Tris-buffered saline (TBS) +0.05% Triton X-100), and blocked (2 h) in 10% normal goat serum and 1% BSA at room temperature. Edu- labelled DNA was stained with Alexa Fluor 488 using Click-iT Edu Alexa Fluor Kit (Thermo Fisher) as per the manufacturer’s protocol. Following this, the abovementioned protocol for immunofluorescence was followed to stain for β-Gal for nuclear receptor and ToPro nuclear stain.

**Protein isolation and western blot analysis.** Protein isolation was performed on frozen liver tissue or isolated hepatocyte fractions using homogenization in bullet blender followed by sonication. Homogenization buffer (10 mM HEPES-KOH, pH 7.5, 0.32 M sucrose, 5 μM MG132, 5 mM EDTA, and Pierce proteinase inhibitor tablet (1 tablet per 20 ml buffer volume)) was used to perform the abovementioned isolation. Before sonication, 20% SDS to a final concentration of 1% (v/v) was added. Protein concentration was measured using the Thermo Scientific BCA Assay Kit. Approximately 50 μg of total protein sample was loaded onto a 10% SDS-PAGE gel, and then transferred onto a polyvinylidene difluoride membrane. Membranes were visualized for equal loading using Ponceau (Pons) staining solution (0.5% w/v Pons, 1% acetic acid). Membranes were blocked using 5% milk powder (w/v) in TBST (Tris-buffered saline, 0.1% Tween 20) for 2 h at room temperature. Blots were incubated in primary antibodies at predetermined concentrations overnight at 4 °C. Blots were washed in TBST, and then incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were developed using Clarity Western ECL Kit (BioRad). All antibodies used and respective dilutions are listed in Supplementary Table 2.

**Gene expression and splice isoform analysis.** Total RNA from mouse livers and hepatocytes was isolated using either the RNasy Kit (Qiagen) or TRIzol reagent (Life Tech). Following 1 treatment, tissue data were reverse transcribed to cDNA using random hexamers and Maxima Reverse Transcriptase Kit (Thermo Scientific). The cDNA was diluted to a final concentration of 25 ng/μl to be used downstream for qPCR- or RT-PCR-based alternative splicing assays as described previously. Downstream analyses for PSI and log2(fold change) calculations were performed as described previously. All primers used for alternative splicing and qPCR are listed in Supplementary Table 3.
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# Experimental design

## 1. Sample size

Describe how sample size was determined.

No sample sizes calculations were performed. For the mouse studies, 4-6 animals/genotype were used based on previous experience to obtain statistical significance. For deep sequencing experiments, two biological replicates were used for each conditions.

## 2. Data exclusions

Describe any data exclusions.

None of the data points were excluded from any of the analysis.

## 3. Replication

Describe the measures taken to verify the reproducibility of the experimental findings.

Cell culture experiments holding consistent results after three repetitions were considered significant. All western blots, and qPCR experiments were repeated at least three times.

## 4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Randomization was done manually. FVB/NJ and C57BL/6J strains of mice were obtained directly from the Jackson laboratories.

## 5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Transgenic and wildtype mice were ear tagged with serial numbers, and were used to mark serum and tissue samples. Samples were only identified once the final data were acquired.

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### 6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
| ☐   | ☑         |

- **YES** The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided
  
  *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- Test values indicating whether an effect is present
  
  *Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.*
- A clear description of statistics including **central tendency** (e.g. median, mean) and **variation** (e.g. standard deviation, interquartile range)
- Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

*See the web collection on [statistics for biologists](#) for further resources and guidance.*
7. Software

Describe the software used to analyze the data in this study.

Statistical analysis was done using Graphpad prism. All bioinformatics analyses performed are discussed in detail in the Methods section and the following softwares/scripts were used: Trimmomatic, STAR, Cufflinks, rMATS, Exon Ontology, UCSC Liftover, Enrichment Maps (Cytoscape), DAVID Gene Ontology, Image Lab 5.2.1, DESeq2 and ggplot2.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

All materials used in the study are freely available upon request.

The antibody information is provided in supplementary Table 2. The Table lists the supplier and catalogue number, as well as dilutions used in all experiments. Detailed protocols are provided in the Methods section.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

AML12 mouse hepatocyte cell line (ATCC CRL-2254)

AML12 cells were obtained directly from ATCC. FACs analysis was performed on propidium iodide stained AML12 cells, and it was determined that there was no contamination from a secondary cell type.

b. Describe the method of cell line authentication used.

Cells were tested using staining of fixed cells with Hoechst/DAPI, and tested for presence of small mycoplasma spots around the nuclei.

c. Report whether the cell lines were tested for mycoplasma contamination.

No commonly misidentified cell lines were used in this study.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used in this study.

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

Species: Mus musculus, Age: 8-10 Weeks, Sex: Male/Female

1. FVB/NJ (Jackson Laboratories, 001800)
2. C57BL/6j (Jackson Laboratories, 000664)
3. ESRP2 KO (Bhate et al., 2015, Nat. Comm. 6, 8768)
4. TRE-ESRP2+/- ; ApoE-rtTA+/- these mouse lines were newly generated for this study
5. ApoE-rtTA+/- (Dong et al., Cell 130, 1120-1133(2007))
6. Embryonic Day 18 old pups were derived from mating FVB/NJ mice.

This study did not involve any human research participants.