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Rapid Disruption of Genes Specifically in Livers of Mice Using Multiplex CRISPR/Cas9 Editing

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Abstract

Despite advances in gene editing technologies, generation of tissue-specific knockout mice is time consuming. We used CRISPR/Cas9-mediated genome editing to disrupt genes in livers of adult mice in just a few months, which we refer to as somatic liver knockouts. In this system, *Fah*−/− mice are given hydrodynamic tail vein injections of plasmids carrying CRISPR/Cas9 designed to excise exons in *Hpd*; the *Hpd*-edited hepatocytes have a survival advantage in these mice. Plasmids that target *Hpd* and a separate gene of interest can therefore be used to rapidly generate mice with liver-specific deletion of nearly any gene product. We used this system to create mice with liver-specific knockout of argininosuccinate lyase, which develop hyperammonemia—observed in humans with mutations in this gene. We also created mice with liver-specific knockout of ATP binding cassette subfamily B member 11, which encodes the bile salt export pump. We found that these mice have a biochemical phenotype similar to that of *Abcb11*−/− mice. We then used this system to knock out expression of 5 different enzymes involved in drug metabolism

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AUTHOR CONTRIBUTIONS

K.D.B., F.P.P. and D.D.M. designed the experiments. F.P.P. and B.B.-C. performed *in vivo* experiments. M.B. and C.S.M. did immunostaining. X.L. did Western blotting. F.F.P., M.B. W.R.L. and K.D.B. designed and prepared CRISPR/Cas9 knock-out approach. K.K and C.R.W-K. performed bile acid analysis and S.H.E. and J.C. M. analysis of other metabolites. All authors read and approved the final manuscript.

COFLICT OF INTERESTS

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within the same mouse. This approach might be used to develop new models of liver diseases and study liver functions of genes that are required during development.

While advances in gene editing technologies, particularly CRISPR/Cas9, allow for the generation of knockout mice faster than ever, this process still involves months of crossings and genotyping with no guarantee that the resulting mouse will be viable. Conditional knockouts using the Cre/*lox*P system can alleviate certain pitfalls, but this process is even more time-consuming, necessitating a more extensive breeding strategy and crossing of multiple mouse lines[1, 2]. To circumvent these issues, we have developed a method to rapidly knockout any gene from the livers of adult mice, utilizing selection pressure in the murine liver.

Hepatocytes in mice lacking the fumarylacetoacetate hydrolase (*Fah)* gene undergo apoptosis due to the accumulation of toxic tyrosine catabolites[3–5]. *Fah*$^{-/-}$ mice die as neonates due to hepatotoxicity, but can be rescued using the small molecule drug nitisinone, which inhibits the protein hydroxyphenylpyruvate dioxygenase (HPD) in order to prevent toxicity [6, 7]. We previously demonstrated that using CRISPR-Cas9 to excise critical exons of the *Hpd* gene confers resistance to hepatotoxicity in *Fah*$^{-/-}$ mice, resulting in a selection advantage for *Hpd*-edited cells[8] (Fig. 1a). We found the edited hepatocytes almost completely replaced the murine liver in as little as two months, and were maintained twelve months after genome editing with no obvious signs of liver injury (Supplementary Fig. 1-3). We reasoned that by simultaneously targeting *Hpd* and a separate gene of interest (*GOI*) in *Fah*$^{-/-}$ mice, we could use the selection advantage conferred by *Hpd* editing to rapidly generate a liver-specific knockout for nearly any desired *GOI*, a technique we refer to as Somatic Liver Knockout (SLiK) (Fig. 1b).

To introduce CRISPR-Cas9 plasmids into the liver, SLiK uses hydrodynamic tail vein injection, a technique capable of transfecting up to 30% of hepatocytes without the need for viral or other complex delivery mechanisms[9, 10]. A growth advantage is conferred to hepatocytes in *Fah*$^{-/-}$ mice using two gRNAs targeted to introns flanking critical exons in the *Hpd* gene (Supplementary Fig. 4). Simultaneously, two gRNAs targeting early exons of the desired *GOI* are used to introduce exonic mutations. It is likely that every cell that gains a growth advantage from *Hpd* excision is also deficient in the *GOI*, since each *GOI* gRNA can cause a frameshift mutation, whereas both *Hpd* gRNAs must cut simultaneously and excise the intervening exons in order to gain the selective advantage of *Hpd* deletion[8, 11]. We performed proof-of-concept studies for SLiK using two different genes.

Argininosuccinate Lyase (ASL) is a critical protein in the urea cycle, and is produced mainly in the liver, and to a lesser amount in the kidneys. ASL deficiency causes increased ammonia concentration in the blood, leading to lethargy, seizures, and eventually death[12]. We generated *Asl*^{SLiK} mice by hydrodynamic injection of the CRISPR-Cas9 plasmids targeting both *Hpd* and *Asl*. In accord with the severity of the human disease, *Asl*^{SLiK} mice survived ~10 weeks after application of selection pressure, at which point they exhibited hyperammonemia and had to be euthanized with clinical symptoms (somnolence and abnormal behavior). This phenotype parallels that of the *Asl* knockout mouse (*Asl*$^{-/-}$), which is neonatally lethal[13]. Western blot analysis confirmed liver protein depletion for both

HPD and ASL in these mice (Fig. 1c), and biochemical analysis of plasma showed higher levels of ammonia and argininosuccinate, the substrate of ASL, in Asl^{SLiK} mice compared to Hpd^{SLiK} control mice (Fig. 1d). Argininosuccinate was also increased in the urine of Asl^{SLiK} mice (Supplementary Fig. 5). In contrast to the $Asl^{-/-}$ mouse, SLiK relies on the clonal expansion of edited cells, which have a growth advantage and replace non-edited apoptotic cells. We therefore provided residual ASL function by rescuing non-edited hepatocytes with nitisinone, thus generating mice with a nearly complete knockout for *Asl* while mitigating lethality (Fig. 1e,f). However, all HPD-negative cells were also negative for ASL, which suggests that, given sufficient time and a non-lethal phenotype, a complete liver knockout might be achievable.

Progressive familial intrahepatic cholestasis-2 (PFIC2) is caused by deficiency of the Bile Salt Export Pump (BSEP), encoded by the *Abcb11* gene [14]. $Abcb11^{-/-}$ mice have been generated by traditional means, however, offspring of these mice often die due to maternal cholestatis[15]. As such, generating sufficient numbers of mice to study BSEP knockout is difficult. To bypass this hurdle, we used SLiK to eliminate BSEP from the livers of adult mice. $Abcb11^{SLiK}$ mice appear healthy, and both western blot and immunohistochemistry confirmed depletion of BSEP from the liver 8 weeks after injection with CRISPR-Cas9 constructs (Fig. 2a-b). Biochemically, serum bile acid concentrations in $Abcb11^{SLiK}$ mice achieved levels similar to those in conventionally generated $Abcb11^{-/-}$ mice (Fig. 2c). From these data, we can conclude that a full liver knockout is possible using SLiK, and the biochemical phenotype is comparable to the traditional knockout mouse model.

Traditional knockout models are impractical for multiple genes, however many hepatocellular functions depend on protein networks and redundant pathways. To demonstrate how SLiK can also be used to study such complex systems, we were able to simultaneously target five different enzymes implicated in drug metabolism within the same mouse (Fig. 2d). Immunohistochemistry confirmed that areas of expanding $Hpd^{-/-}$ hepatocytes were also negative for all five targeted proteins, and 6 month analysis showed not only extensive deletion of all genes, but also replicated the heterogeneous phenotype previously observed with hepatic *Por* deletion[16].

In summary, SLiK is a novel technique to rapidly generate liver-specific knockout models without the need for embryonic gene targeting or prolonged breeding schemes. Furthermore, this technique does not require the expertise and laborious production of viral vectors. The degree of liver knockout can be controlled with a small molecule drug, and the method is amenable to multiplexing knockout of several genes simultaneously. It is important to note, however, that poor gRNA binding can influence *GOI* knockout efficiency, and that knockouts of genes required for cell survival or proliferation cannot be generated using SLiK. Despite these caveats, SLiK is a highly versatile method and should be valuable to biomedical research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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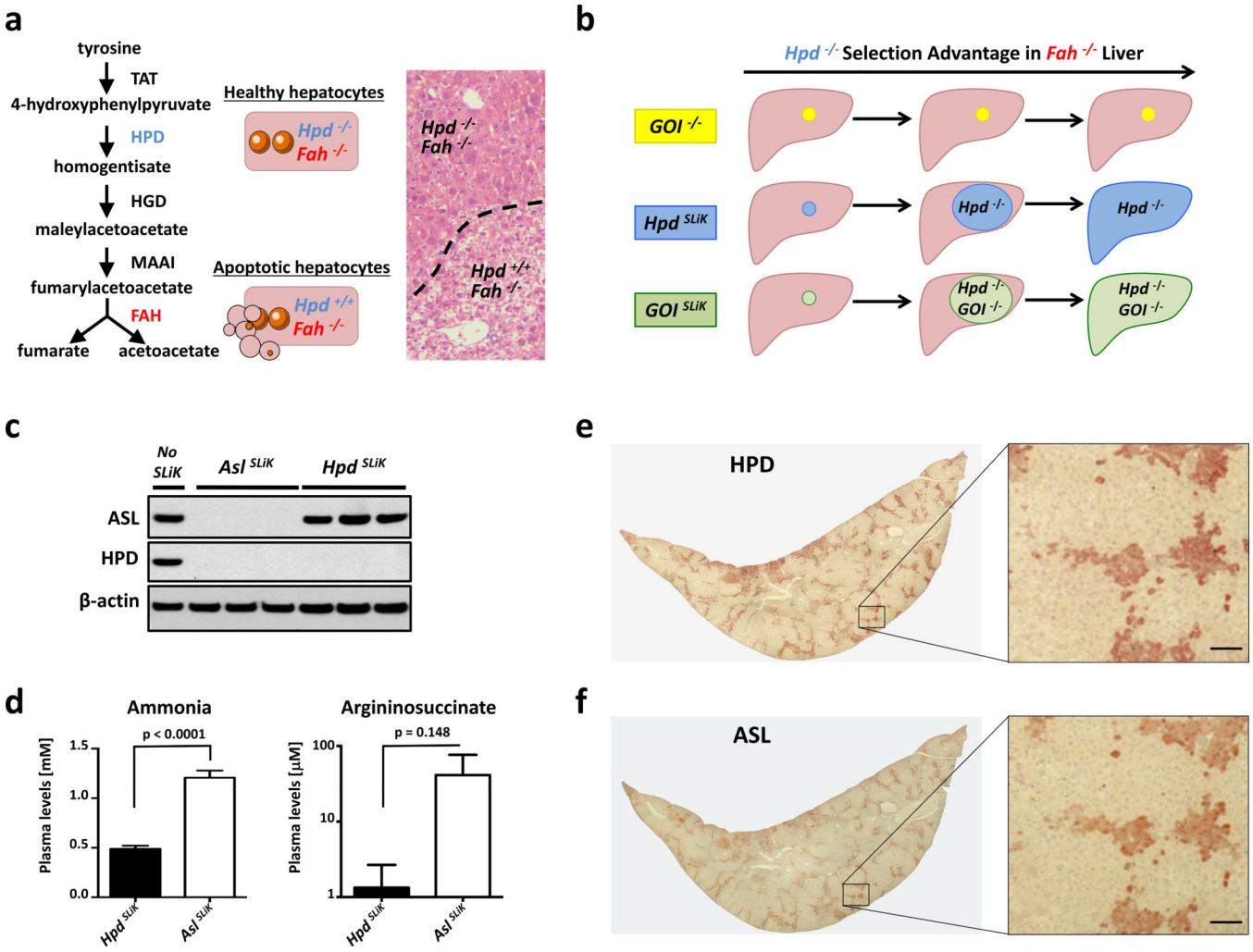

Figure 1.

Somatic Liver Knockout (SLiK) by multiplex CRISPR-Cas9 editing. (**a**) The toxic effect of *Fah* deficiency can be counteracted by deleting *Hpd*. (**b**) Schematic for SLiK showing the expansion of GOI- edited cells using the growth advantage of $Hpd^{-/-}$ hepatocytes in $Fah^{-/-}$ mice. (**c**) Western blot for HPD and ASL ten weeks after targeted editing in Hpd^{SLiK} (*Hpd*-deleted) or Asl^{SLiK} (*Hpd*- and *Asl*-deleted) mice. (**d**) Serum analysis of ammonia and argininosuccinate for Hpd^{SLiK} and Asl^{SLiK} mice (n=5). (**e,f**) Serial sections of Asl^{SLiK} liver lobes stained for either HPD (**e**) or ASL (**f**) in brown. Inset boxes are shown in higher magnification. Error bars indicate SEM. Scale bars are 50 μm.

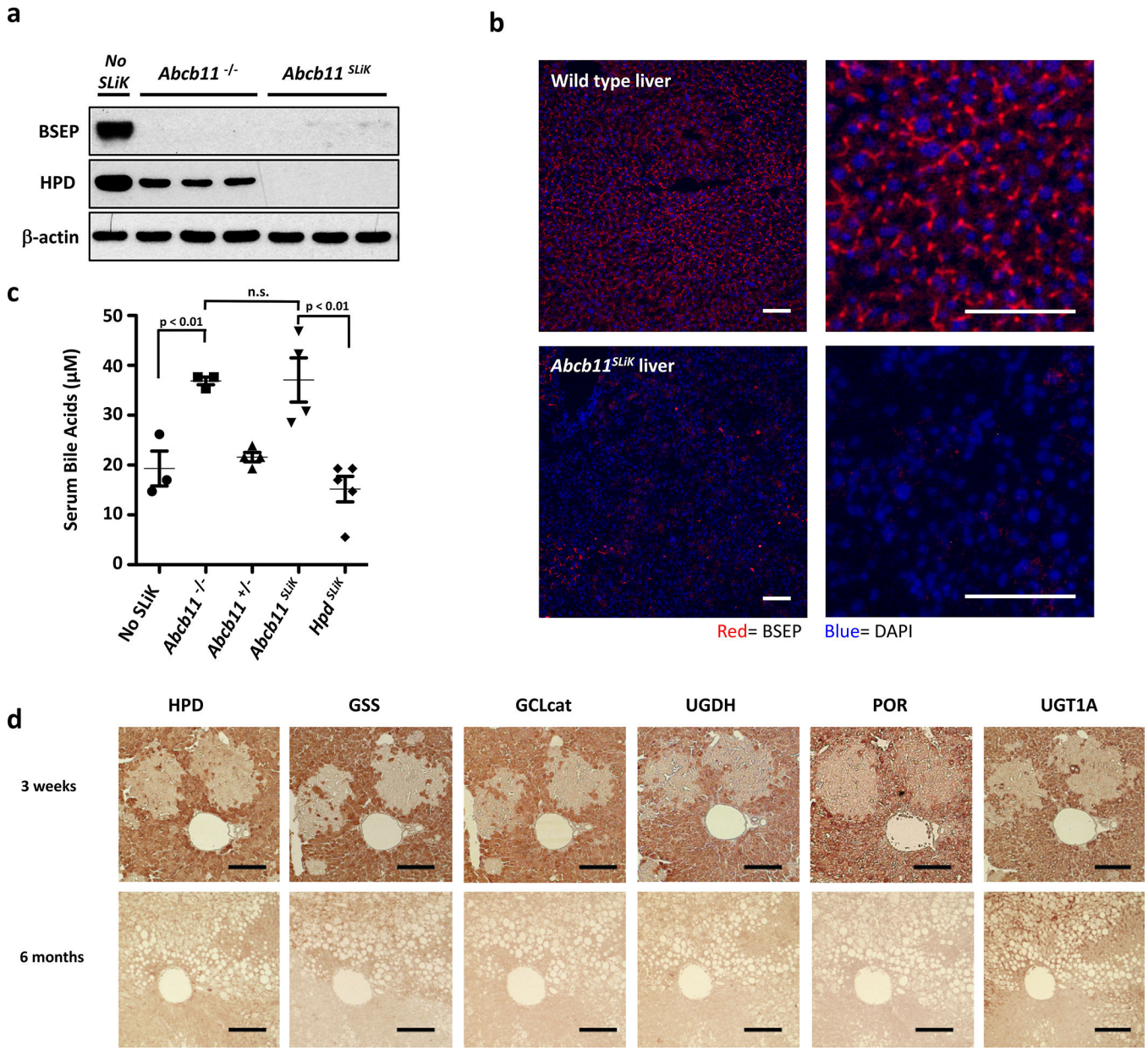

Figure 2.

SLiK of *Abcb11* and multiplexed SLiK (**a**) Western blot of BSEP in *Abcb11*$^{-/-}$ knockout mice and *Abcb11*^{SLiK} mice. (**b**) Immunofluorescent staining for BSEP (red) in wild-type (upper panels) and *Abcb11*^{SLiK} (lower panels), counterstaining DAPI (blue). (**c**) Total serum bile acids in *Abcb11*$^{-/-}$ and *Abcb11*^{SLiK} mice. **(d)** Multiplex SLiK of glutathione synthetase (GSS), glutamate cysteine ligase catalytic subunit (GCLcat), UDP-glucose-6'-dehydrogenase (UGDH), cytochrome P450 oxidoreductase (POR), and UDP glucuronosyltransferase family 1 member A (UGT1A), shown by immunostaining serial liver sections (brown) at 3 weeks and 6 months after nitisinone withdrawal (n=6 for each time point). Error bars represent SEM. Scale bars are 50 μm.