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NOVEL GENETIC ACTIVATION SCREENING IN LIVER REPOPULATION AND CANCER; NOW CRISPR THAN EVER!

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Liver cancer, in particular hepatocellular carcinoma (HCC), is unique in its association with chronic hepatic injury, as 80% of cases arise in cirrhotic livers resulting from chronic liver diseases. In the United States, high prevalence of hepatitis C and increasing rates of nonalcoholic fatty liver disease are contributing factors to increasing incidence rates of HCC (1, 2). Chronic liver injury leading to cell death, impaired hepatocyte proliferation, inflammation and stellate cell activation, results in a microenvironment conducive for genomic alterations in hepatocytes. While extensive work has been done to elucidate common oncogenic drivers in HCC, including mutated β -catenin, P53, and Myc amplification, additional molecular characterization of HCC is essential due to heterogeneity in signaling. New mutations are being identified via next generation sequencing, however their cooperative roles and identities as driver mutations versus passenger mutations remain unclear (2). In order for development of improved and effective therapies, more precise, comprehensive and functional alterations in the genetic landscape of HCC need to be identified and characterized.

CRISPR-Cas9 remains a cutting-edge genome editing tool. The Cas9 protein was initially discovered in bacteria as an immunoprotective mechanism by targeting virus phage and plasmid DNA, and thus interfering with viral replication. Cas9 was later identified to have nuclease domains capable of cleaving DNA, regulated by an RNA duplex (CRISPR RNA and Transactivating CRISPR RNA) which serves as a guide to direct Cas9 to specific DNA sites (3). Cas9 then causes double stranded breaks that lead to genetic loss of function once repaired (4). These CRISPR RNAs have since been modified into single guide RNAs (gRNA), of approximately 20 nucleotides enabling direct targeting of Cas9 to a gene of interest. Cas9 is an attractive option for excising genes, as it is easily modifiable unlike

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TALEN and Zinc Finger Nucleases (3). Further, CRISPR-Cas9 provides opportunities for high-content genome editing, as gRNA libraries can be generated to target over 20,000 genes simultaneously (4). A caveat of CRISPR-Cas9 is therefore the permanent disruption of the genome and limitation to loss-of-function studies. To circumvent this, a nuclease-deficient Cas9 (dCas9) has been generated and fused with a VP64 transactivation domain. The addition of a gRNA is able to guide dCas9 to the complementary DNA, and the transactivation domain is able to recruit transcriptional machinery to the site of interest (5), thus establishing an activating CRISPR system (CRISPRa). To date, CRISPRa-dCas9 has been used to induce gene expression *in vitro*, or to manipulate lymphomas *ex vivo* prior to injection into mice (5, 6). However, CRISPRa-dCas9 has not been utilized to study tumor initiation *in vivo* or to study the genetic landscape of liver repopulation leading to HCC.

In the current study in Hepatology, Wangenstein *et al* developed an *in vivo* CRISPRa-dCas9 murine model to perform a genetic screen in the context of hepatic repopulation following liver injury (7). For successful CRISPRa *in vivo*, the system needed dCas9 fused to a transactivation domain, appropriate gRNAs to target genes of interest, and a transcriptional activator. To satisfy the first criteria, a plasmid containing dCas9 fused to the 'SunTag' transactivation domain was developed and used to generate a Rosa26 knock-in mouse, enabling dCas9 expression in any tissue targetable by Cre recombinase. Accompanying plasmids containing gRNAs, described in more detail below, contained the transcriptional activator VP64, which binds SunTag and facilitates recruitment of RNA Polymerase II to the intended site (8) (Figure 1A). The homozygous dCas9^{+/+} mice should be available shortly through the Mutant Mouse Regional Resource. They utilized heterozygous dCas9⁺ mice to confirm dCas9 activation in hepatocytes after injection of adeno-associated virus expressing Cre under the liver-specific thyroxine-binding globulin promoter (AAV-TBG-Cre).

To generate a model of controlled hepatocyte repopulation, dCas9⁺ mice were bred with FAH^{-/-} mice. FAH^{-/-} mice display a lethal hereditary tyrosinemia phenotype, resulting from toxic metabolite accumulation and hepatocyte injury. Toxicity and subsequent death can be prevented by continuous administration of 2-(2-nitro-4-trifluoromethylbenzyl)-1,3-cyclohexanedione (NTBC). In order to induce hepatocyte repopulation in their FAH^{-/-};dCas9⁺ mice, the authors designed an elegant system by promoting clonal expansion of hepatocytes expressing their genes of interest to repopulate the injured liver. Using hydrodynamic tail vein injection (HTVI) and sleeping beauty (SB) plasmids containing a transposon, transposase, gRNA of interest, the VP64 transcriptional activator, and FAH cDNA were injected three days after administration of AAV-Cre to activate dCas9 in FAH^{-/-};dCas9⁺ mice. SB allows plasmid integration into the genome, resulting in stable gRNA expression and FAH in hepatocytes that receive the plasmid due to HTVI (9). NTBC is ceased at time of injection, and hepatocytes expressing FAH (and the subsequent gRNA and transcriptional activator) repopulate the liver and replace dying FAH^{-/-} hepatocytes (Figure 1B).

Two gRNA libraries were generated (termed Lib1 and Lib2) based off a published computational analysis involving the development of a genome-scale gRNA library (10). Lib1 contained 10 gRNAs specific for cMyc, 10 gRNAs for Tnfrsf1a, and 20 nonspecific control gRNAs. The rationale was a previous study Wangenstein *et al* demonstrating cMyc

upregulation and *Tnfrsf1a* suppression after hepatocyte repopulation in the *FAH*^{-/-} mice (11). Further, multiple gRNAs per gene were used as it created a synergistic effect (5). In Lib2, the authors maintain gRNAs targeted towards cMyc, *Tnfrsf1a*, and control, and introduced gRNAs specific for *Slc7a11*, another gene upregulated during repopulation in *FAH*^{-/-} mice, and *Tp53*, a tumor suppressor. *FAH*-positive hepatocytes repopulated *FAH*^{-/-};dCas9⁺ livers after addition of Lib1 or Lib2 and removal of NTBC. Both Myc-positive and Myc-negative repopulating nodules were evident. DNA sequencing identified cMyc enrichment after repopulation compared to gRNAs for controls or other genes. Moreover, mice injected with Lib1 or Lib2 developed tumors that positively selected for cMyc expression (300-fold increase), and to a lesser extent *Slc7a11*. Tumors maintained 2–7 unique gRNAs for cMyc, and gRNAs for *Tp53* and *Tnfrsf1a* were depleted (Figure 1C).

While this particular result is not surprising, as repopulating cells with cMyc and *Slc7a11* will have advantage over *Tp53* and *Tnfrsf1a*, it underscores the relevance of the *in vivo* CRISPRa system. Relationships between any genes of interest can be contextually assessed after hepatic injury and repopulation, as for example the authors note Myc-positive *Tnfrsf1a*-positive foci do not become oncogenic, supporting that *Tnfrsf1a* blocks Myc-driven tumorigenesis. Further, the authors estimate up to 10,000 gRNAs targeting 2,000 genes (with 5 gRNAs per gene) can be used to conduct large scale parallel genetic screens and pathway analyses, thus surpassing the capabilities of overexpression models including hydrodynamic tail vein injections of Sleeping Beauty transposons and oncogenes. Specific roles of genes can be readily assessed via CRISPRa, and one can determine whether known genetic alterations can initiate HCC, or are secondary oncogenic events that likely arise after established tumors. One caveat is that transcriptional activation complexes such as synergistic activation mediator (SAM) may have greater efficiency than Suntag *in vivo* and require fewer gRNAs to achieve targeted effects (12, 13). Further, while the dCas9⁺ mouse can be useful for any cell or tissue type with a specific Cre, methods of gRNA delivery to the required locations may be lacking, limiting the efficacy of this system across organs. However, this innovative model is the first to allow parallel activation of potentially thousands of genes to assess their additive roles in HCC initiation and will enable massive genetic screens to elucidate the genetic landscape of liver repopulation and cancer. This model is likely to lead to the discovery of novel signaling pathway aberrations and hence new druggable targets.

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Abbreviations

HCC	hepatocellular cancer
dCas9	nuclease-deficient Cas9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRa	CRISPR-activation

gRNA	guide RNA
FAH	fumarylacetoacetate hydrolase
NTBC	2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexanedione

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Figure 1.

Figure 1A: Schematic comparison of Cas9 and dCas9-mediated genome editing. Cas9 is guided to an area of DNA based on the complementary guide RNA (gRNA), where its nucleolytic activity induces double-stranded breaks (top panel). To utilize CRISPRa, a nuclease-deficient Cas9 (dCas9) protein is fused to a SunTag transactivation domain. A gRNA and a transactivator, VP64, are incorporated to allow gRNA-mediated translocation to the DNA of interest. VP64 binds SunTag and recruits RNA polymerase II (RNA Pol) to induce transcription at the targeted site (bottom panel).

Figure 1B: Schematic representation of gRNA-regulated liver repopulation and tumorigenesis in FAH^{-/-};dCas9⁺ mice. Liver injury is prevented in FAH^{-/-};dCas9⁺ mice with 2-(2-nitro-4-trifluoro-methylbenzyl)-1,3-cyclohexanedione (NTBC) administration. First, AAV-Cre is injected intravenously to activate dCas9 expression in hepatocytes. The nuclease-deficient dCas9 protein is fused to a SunTag transactivation domain, required for complete CRISPRa. Three days following AAV-Cre, NTBC is removed and plasmids are injected via hydrodynamic tail vein injections. A single plasmid contains cDNA for FAH to promote FAH⁺ hepatocyte repopulation in FAH-negative livers, a transcriptional activator (TA) that binds the SunTag domain of dCas9, gRNA to target dCas9 near the transcriptional start site of the gene of interest, and the sleeping beauty transposase (SB), which allows for

transposon-mediated plasmid entry into the genome in affected hepatocytes. Mice are sacrificed 1–2 months later, and hepatic repopulation and presence of hepatocellular carcinoma are assessed.

Figure 1C: Positive selection of tumors in the model. After addition of gRNAs for cMyc, Tnfrsf1a, Tp53, and Slc7a11, tumors positively select for cMyc and Slc7a11 and negatively select for Tp53 and Tnfrsf1a.

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