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List of Abbreviations: AAV-Cre (serotype 8 adeno-associated virus with thyroxin-binding globulin promoter driving Cre recombinase enzyme expression), AFP (alpha-fetoprotein), AKT (thymoma viral proto-oncogene 1), ARF (alternative reading frame protein; human: p14ARF, mouse: p19ARF), Apc (adenomatous polyposis coli), B2m (beta-2 microglobulin), Cas9 (CRISPR associated protein 9), CDK7 (cyclin-dependent kinase 7), Cdkn2a (cyclin dependent kinase inhibitor 2A), cDNA (complementary DNA), CIS (common insertion site), CRISPR (clustered regularly interspaced short palindromic repeats), CRISPRa (CRISPR activation), CRISPRi (CRISPR inhibition), dCas9 (catalytically dead Cas9), Dpyd (dihydropyrimidine dehydrogenase), EGFR (epidermal growth factor receptor), FAH (fumarylacetoacetate hydrolase), GeCKO (genome-scale CRISPR knockout), GEMM (genetically engineered mouse model), Gsk3b (glycogen synthase kinase 3 beta), gRNA (guide RNA), HBsAg (HBV surface antigen), HBV (hepatitis B virus), HCC (hepatocellular carcinoma), HCV (hepatitis C virus), HTVI (hydrodynamic tail vein injection), HSF1 (heat shock factor 1), HTS (high-throughput sequencing), Kansl1 (KAT8 regulatory NSL complex subunit 1), KRAB (Kruppel-associated box), Kras (Kirsten rat sarcoma viral oncogene homolog), Mapk14 (mitogen-activated protein kinase 14), Mkk4 (mitogen-activated protein kinase kinase 4; also called Map2k4), mRNA (messenger RNA), Nf1 (neurofibromin 1), Nras (neuroblastoma ras oncogene), PB (piggyBac), PH (partial hepatectomy), Pten (phosphate and tensin homolog), RISC (RNA-induced silencing complex), RNAi (RNA interference), SAM (synergistic activation mediator), SB (Sleeping Beauty), scFv (single-chain variable fragment), shRNA (short hairpin RNA), Slc7a11 (solute carrier family 7 member 11), Snd1 (staphylococcal nuclease and tudor domain containing 1), Stard13 (StAR-related lipid

transfer (START) domain containing 13; also known as deleted in liver cancer 2), TA (transcriptional activator), TBG (thyroxin-binding globulin), Tet2 (tet methylcytosine dioxygenase 2), TGFα (transforming growth factor α), Tnfrsf1a (tumor necrosis factor superfamily member 1a), TP53 (tumor protein 53; ortholog of Trp53), Trp53 (transformation related protein 53), Trip12 (thyroid hormone receptor interactor 12), tTA/TRE (tetracycline transactivator/tetracycline response element), VP16 (Herpes Simplex Viral Protein 16), Xpo4 (exportin 4)

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ABSTRACT

Hepatocellular carcinoma (HCC) is a devastating and prevalent cancer with limited treatment options. Technological advances have enabled genetic screens to be employed in HCC model systems to characterize genes regulating tumor initiation and growth. Relative to traditional methods for studying cancer biology, such as candidate gene approaches or expression analysis, genetic screens have several advantages: they are unbiased, with no *a priori* selection, can directly annotate gene function, and can uncover gene-gene interactions. In HCC, three main types of screens have been conducted and are reviewed here: (1) Transposon-based mutagenesis screens, (2) knock-down screens using RNA interference (RNAi) or the CRISPR/Cas9 system, and (3) overexpression screens using CRISPR activation (CRISPRa) or cDNAs. These methods will be valuable in future genetic screens to delineate the mechanisms underlying drug resistance and to identify new treatments for HCC.

Main Concepts and Learning Points

- Genetic screens in animal models shed light on functional alterations in HCC
- Advances in sequencing technology allow for increasingly complex screens
- Liver genetic screens are revealing the heterogeneity of genetic alterations in HCC patients
- Future work may apply genetic screening to discover new treatments for HCC

HCC is a unique cancer that needs unique approaches to treatment

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related mortality worldwide and is growing in incidence[1,2]. HCC is unique among cancers: a recent analysis of 17 human cancer types revealed substantial overlap in the gene expression patterns of all the cancers with the single exception of HCC[3]. This is consistent with the observation that several treatments, though effective against other cancers, have largely failed to improve outcomes for patients with HCC[4]. Therefore, the discovery of new treatments will depend on the development of models that accurately recapitulate HCC.

Most cases of HCC arise in cirrhotic livers; hence it is not surprising that several major underlying risk factors are characterized by chronic liver injury. Worldwide, the most common etiologies are chronic hepatitis B or C infections. Though the prevalence of these infections is decreasing in many countries due to the availability of an effective vaccine and treatment, the incidence of Hepatitis C-associated HCC in the United States is still not falling due to a large number of 'baby boomers' who have been chronically infected for decades. Non-alcoholic fatty liver disease is also rising in incidence in Western countries and is likely to soon overtake Hepatitis C as the major cause of HCC in the United States[5–7]. Additional etiologies of HCC include biliary tract diseases and exposure to toxins such as alcohol.

The advent of high throughput sequencing (HTS) technology has accelerated the identification of mutations and gene expression changes in cancers. The genomes of thousands of patients' cancers have been sequenced to date, including hundreds of HCCs (a large collection of data is available publicly from the Cancer Genome Atlas at

http://cancergenome.nih.gov/). These data have helped define the diverse genetic landscape of mutations and expression changes that occur in HCCs[8–11]. Nevertheless, additional complementary data are necessary to provide evidence of causation, i.e. that genetic changes induce tumor formation rather than occurring secondarily during the process of cancer development[12].

HCC is challenging to treat due to several factors including its genetic diversity, the underlying liver dysfunction in most patients, the difficulty in detecting early stage disease, and the lack of effective treatments. Only 30% of patients with HCC are eligible for potentially curative interventions like liver transplantation at the time of diagnosis[13]. In general, classical chemo- and radiotherapies have either low efficacy or undesirable toxicity[4,13]. Only a single drug is approved in the United States to treat HCC as a first-line therapy: sorafenib, a multi-specific kinase inhibitor[14,15]. Sorafenib treatment has a marginal median survival benefit of just 2-3 months, and there are no good predictors of response[16]. In patients whose disease progresses during sorafenib treatment, second-line options were recently approved. One is another multikinase inhibitor, regorafenib, which improves survival over placebo by just under three months[17]. The other second-line option is Nivolumab, an immune-therapeutic with remarkable efficacy in a fraction of patients[18–20]. However, the search must continue for more broadly-effective therapies.

The goal of this review is to provide an overview of how innovations in gene delivery and in genetic screening technology in mice can significantly expand our knowledge of the genes driving or modulating the development of HCC, which can be applied to discover new therapeutic targets. The main methods and most notable studies are summarized in **Table 1**.

Genetic screens are a powerful method for annotating gene function

A genetic screen is an assay in which the genetic codes are mutated or gene expression is systematically perturbed in order to identify which changes result in a phenotype of interest[21]. Whole organism genetic screening has been employed frequently in the nematode *Caenorhabditis elegans*, in the fruit fly *Drosophila melanogaster*, in the zebrafish *Danio rerio*, and in mice (reviewed in 22). Internet resources for many model organisms, including wormbase.org, flybase.org, and ZFIN.org, catalog detailed information linking specific genetic changes to precise phenotypic manifestations. Screens have used a variety of methods to manipulate gene expression, including chemical mutagenesis, transposon insertional mutagenesis, RNA interference (RNAi), complementary DNA (cDNA) expression, and the prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system. More recent efforts have combined genetic screens with chemical library screens to explore therapeutic potential or identify mechanisms responsible for drug efficacy[22]. While useful in identifying phenotypes in a whole organism, these types of studies do not easily translate to complex, tissue-specific human diseases such as HCC.

Genetic screens in cancer cells can provide mechanistic evidence linking genetic changes to the important phenotypes of tumor initiation, morphology, growth, or drug sensitivity or resistance. Specific functional insights, as compared to correlative genetic associations, better facilitate therapeutic development and selection.

To efficiently identify cancer-driving genes, transposon mutagenesis, cDNA, RNAi, and CRISPR screens all heavily rely on high-throughput sequencing (HTS). For

transposon mutagenesis, the molecular readout is the flanking genomic sequence, which precisely identifies the insertion site of the transposon. cDNA screens may use barcoding of plasmids to enable precise quantification of the proportion of plasmids before and after the screen[23]. RNAi and CRISPR screens depend on identifying and quantifying the RNA coding sequences.

Data generated by genetic screens in mice can be integrated with the large collection of available human cancer databases to provide insights into the multiple alterations found in HCC. Such studies can significantly contribute to the identification of potential new drug targets and to the ability to predict which signals should be targeted in patients.

Mouse models of HCC

While most strains of mice do not spontaneously develop HCC, liver cancer in mice can be induced through chemical stimulus, transplantation of tumor cells, or genetic alteration. Administration of the chemical diethylnitrosamine (DEN) causes DNA damage, which leads to the formation of tumors by proliferating hepatocytes harboring mutations. DEN does not induce tumorigenesis in adult mice unless it is combined with an additional toxin, such as carbon tetrachloride (CCl_4), a hepatotoxin that triggers inflammation and fibrosis. Chemical stimulus is also frequently used in combination with genetically engineered mouse models (GEMMs) of HCC.

A number of GEMMs have been generated to study tumorigenesis in the liver. Due to the numerous etiologies contributing to hepatocellular carcinogenesis, no single GEMM is capable of completely recapitulating the disease. However, by reproducing genetic

alterations that are common in HCC (**Figure 1A)**, such as by inactivating tumor suppressors, including transformation related protein 53 (*Trp53*) and adenomatous polyposis coli (*Apc*), or ectopically expressing oncogenes, including *Myc* and catenin beta 1 (*Ctnnb1*), in hepatocytes, transgenic mice develop tumors that mimic features of the disease (reviewed in 24–27).

The use of GEMMs for combinatorial studies has led to the discovery of important genetic synergisms, such as concomitant MYC and transforming growth factor α (TGFα) overexpression, which accelerates liver carcinogenesis[28]. A GEMM with temporally controlled oncogene expression was used to demonstrate that activated MYC drives the development of HCC and that MYC-driven tumors involute when MYC expression is removed[29]. Several mouse models carrying alterations of receptor tyrosine kinase (RTK) signaling components recapitulate alterations frequently observed in HCC patient subgroups, including transcriptional, epigenetic, and signaling modifications. Recently, a unique genetic system found that mice with slightly enhanced wild-type MET expression (*Alb-R26^{Met}* mice) spontaneously develop HCC, illustrating a striking vulnerability of the liver to subtly increased RTK levels[30].

The downside of the GEMM approach is that models can take months to develop and often require complicated breeding schemes in order to examine gene-gene interactions in cancer development. Furthermore, the expression of oncogenes may not be restricted to the adult stage of development, as the promoters – most commonly alpha-fetoprotein (AFP) or albumin (Alb) – may be active during early liver development, unless expression is controlled with inducible systems such as tTA/TRE or Cre-LoxP **(Figure**

1A). Thus, while transgenic mouse models are useful in studying liver cancer, several technical challenges must be taken into consideration prior to implementation.

A powerful method for transgene delivery to the mouse liver is the hydrodynamic tail vein injection (HTVI) technique **(Figure 1B)**[31–34]. HTVI introduces DNA sequences into the nuclei of up to 40% of hepatocytes upon the injection of a large volume of the DNA in saline or lactated ringer's solution (a 1:10 ratio of volume to mouse mass) into the tail vein of mice. The solution is delivered rapidly, in fewer than 10 seconds, which is believed to cause a transient right-sided venous congestion that engorges and floods the liver with fluid and mechanically disrupts cell membranes[35–39]. The technique is highly specific to hepatocytes, with little expression in other cell types of the liver or in other organs. When the HTVI technique is used to inject plasmids encoding transposon systems such as *Sleeping Beauty* (*SB*)[37,40,41], *piggyBac* (PB)[42], or *Tol2*[43], the transposon sequences and the "cargo" – i.e. the genes of interest – are integrated into the hepatocyte genome, which enables stable expression for the life of the cell. This achieves stable integration in 0.1% to 2% of all hepatocytes with *SB*[37,44]. In an adult mouse there are roughly 10^8 hepatocytes; therefore, HTVI of transposons could in theory examine more than 10^6 independent clonal events in a single mouse[45].

In previous studies of liver cancer, HTVI was used to transfect hepatocytes *in vivo* in order to assess the effects of specific changes in gene expression on tumorigenesis. For example, in one study, wild type mice underwent HTVI with an *SB*-overexpression plasmid encoding activated thymoma viral proto-oncogene 1 (*Akt*), which was stably integrated into the hepatocyte genomic DNA[46]. The upregulation of AKT induced increased lipogenesis and hepatocarcinogenesis. A subsequent study in which an *SB*-

overexpression plasmid encoding the intracellular domain of the NOTCH1 receptor was coinjected with the AKT overexpression plasmid showed that intrahepatic cholangiocarcinomas arise from mature hepatocytes that have undergone malignant transformation and not from biliary epithelial or liver progenitor cells[47]. These studies discovered mechanisms of HCC tumorigenesis and pointed toward new target genes for the development of new therapies. Thus, HTVI is a powerful alternative to generating genetically modified animals in order to study the role of hepatocytes in liver cancer.

In combination with HTVI, a useful tool for performing high throughput genetic screens in the mouse liver is the murine genetic model of hereditary tyrosinemia type 1 (HT-1)[48]. Humans with HT-1 have mutations in the gene encoding fumarylacetoacetate hydrolase (*FAH*), an enzyme required for proper tyrosine metabolism. Lack of FAH activity results in severe liver injury from the accumulation of the toxic metabolite fumarylacetoacetate (FAA) in hepatocytes. The drug nitisinone (chemical name 2-(2-nitro-4-(trifluoromethyl)benzoyl)cyclohexane-1,3-dione, or NTBC) restores healthy liver function in HT-1 patients by inhibiting an upstream enzyme in tyrosine catabolism and preventing the formation of FAA[49].

In the *Fah*$^{-/-}$ mouse, liver toxicity can be alleviated by the stable genomic integration of a transgene encoding the functional FAH enzyme[32,44]. Transposons expressing *Fah* are delivered by HTVI to *Fah*$^{-/-}$ mice that have been continuously administered nitisinone, which is then withdrawn to induce liver injury and create a selective environment for any *Fah*-corrected hepatocytes to repopulate the liver. Although the initial integration of the *Fah* transgene by *SB* transposase is estimated to occur in approximately 0.1-2% of hepatocytes, these FAH-positive cells are able to expand and restore liver function

without tumor formation[44]. The rescue of *Fah*-null hepatocytes by functional FAH expression has been leveraged to perform genetic screens by linking a functional copy of *Fah* to libraries of cDNAs, short hairpin RNAs (shRNAs), or guide RNAs (gRNAs) in order to study their effects on liver repopulation and cancer[41,50,51].

Transposon-based mutagenesis

Several forward genetic screens aimed at identifying HCC drivers have been performed via the mobilization of mutagenic transposons by the *SB* transposase **(Figure 2)**. *SB* excises transposon DNA that is flanked by inverted repeat/direct repeat sequences and reintegrates the transposon at random TA target dinucleotides[52]. The system is well-suited for insertional mutagenesis screens in mice using engineered transposon DNA cassettes, as the transposase is highly active in mammalian cells.

Three different iterations of the *SB* mutagenic transposon have been engineered, T2/Onc, T2/Onc2, and T2/Onc3, all of which are capable of inducing both gain- and loss-of-function mutations depending on the orientation of their insertion relative to coding sequence. These transposons all comprise the following elements: (a) a strong promoter and enhancer followed by a splice donor site, (b) a splice acceptor site joined to a polyadenylation signal positioned upstream of the promoter/enhancer in the sense orientation, and (c) a second splice acceptor site joined to a polyadenylation signal, which is positioned downstream of the promoter/enhancer in the antisense orientation **(Figure 2)**. Integration of a transposon cassette in the sense orientation can drive overexpression of downstream coding sequence, while its insertion in either orientation downstream of coding sequence truncates the transcript. By altering the gene sequence, the transposon

mutagenesis system can uncover genomic mutations that lead to oncogenic transformation, a unique advantage over RNAi or cDNA screens, which instead aim to modulate expression levels of endogenous genes.

Thousands of disruptive transposition events can occur in a tissue expressing the *SB* transposase as a consequence of multiple transposon mobilizations from the parent transposon concatemer[53–55]. Sites where transposons integrate more often than would be expected by chance in independent tumors are termed common insertion sites (CISs). Potential drivers of tumorigenesis are identified based on increased frequency of CISs in tumors, which is indicative of selection for a tumorigenic event.

The first version of the *SB* mutagenesis screen used the SB/T2Onc transposon in a p19Arf-deficient (*Arf*$^{-/-}$) mouse strain that ubiquitously expresses the *SB* transposase (*SB10*). The majority of mice formed tumors, mostly sarcomas, by 6 months of age. The most common CIS identified was in the ninth intron of *Braf*, an alteration predicted to produce a constitutively activated form of this known oncogene. This result demonstrated that *SB* insertional mutagenesis can identify disease-causing mutations that synergize with common mutations in cancer models[53].

Subsequent refinements made to the *SB*-T2/Onc system have improved its ability to promote tumorigenesis. The second-generation mutagenic transposon (T2/Onc2) included a larger splice acceptor and optimized *SB* transposase binding sites, along with an improved SB allele (*SB11*). *T2/Onc2*;*SB11* mice showed insertional mutations at the embryonic stage, exhibited high prenatal lethality, and spontaneously developed up to three separate, metachronous tumors by 17 weeks of age, the most common of which were T-cell lymphomas[54]. However, when investigating epithelial-derived tumors, this

high frequency of lymphomas is undesirable. To increase the frequency of carcinomas, the chicken β-actin (CAG) promoter, which is highly active in epithelial cells, was introduced in the T2/Onc3 system[55]. This change increased the frequency of tissue-specific carcinomas, including HCC. More recently, the Rosa26-lsl-SB11 mouse was developed to enable spatial and temporal activation of *SB11 allele* using Cre recombinase enzyme[56].

To identify mutations that synergize with loss of function of tumor protein 53 (*TP53;* ortholog of mouse *Trp53*), which is often mutated in human HCCs, insertions of *T2/Onc* were screened in mice expressing a dominant negative *Trp53* specifically in the liver[56]. Similar to what was seen in the $Arf^{-/-}$ genetic background, mice expressing mutant *Trp53* together with the transposon/transposase displayed a significantly increased tumor burden compared to mice expressing mutant *Trp53* alone. Importantly, as is observed in human HCC, there was a strong sex bias toward male mice, which showed greater tumor incidence and decreased latency. Epidermal growth factor receptor (*Egfr*) had the highest number of insertions, most of which truncated the transcript and resulted in increased kinase activity of the translated protein[56]. HCC tumors in males displayed higher rates of chromosome 7 polysomy (the locus encoding EGFR) and higher EGFR mRNA levels compared to females, suggesting elevated susceptibility to EGFR-driven HCC in male patients[57]. As both p53 and EGFR are linked to human HCC, these results are noteworthy and suggest that simultaneous dysregulation of these genes can promote HCC in a sex-specific manner.

Transposon insertional mutagenesis has also been used to examine epithelial-mesenchymal transition (EMT), which is thought to play a prominent role in HCC

metastasis and resistance to cancer drugs like sorafenib[58]. Kodama et al. generated immortalized hepatoblast cell lines from fetal liver cells harvested from mice with liver-specific transposase activation. When injected into the flanks of immunodeficient mice, these cells produced tumors that coexpressed epithelial and mesenchymal markers, including Epcam and vimentin, respectively, despite their epithelial origin. The authors examined CIS-associated genes from the tumors using KEGG pathway analysis and identified the involvement of pathways already known to be involved in EMT, supporting the accuracy of their model. Unexpectedly, however, the most highly enriched pathway was ubiquitin-mediated proteolysis, which was not previously associated with EMT. The authors went on to confirm that this pathway is involved in EMT using *in vitro* assays, and showed that inactivation of the HUWE1, KDM6A, and PTPN12 tumor suppressors led to the EMT phenotype of sorafenib resistance. Thus, the use of an insertional mutagenesis screen led to the identification of candidate genes driving EMT in human HCC[58].

Although clinically relevant discoveries have been made through SB transposon insertional mutagenesis, the system has several drawbacks. One disadvantage is the long latency to tumor development, which can take well over 100 days[53,57,59]. Additionally, the system requires that engineered mice express the mutagenic transposon, the transposase, and a liver-specific Cre recombinase for the system to be functional. Performing these screens on a cancer-predisposed genetic background adds another layer of complexity. Furthermore, in order to obtain sufficient statistical power, large cohorts of mice must be used. For example, a screen by Collier et al. utilized 64 *T2/Onc*;*SB10*;*Arf*$^{-/-}$ animals[53]. Given the complicated mating schemes required for such

experiments, obtaining sufficient animals of the correct genotype requires significant effort and time.

Another issue to consider while implementing insertional mutagenesis screens is the “local hopping” phenomenon in which transposition is more likely to occur near the locus of the parent concatemer (the series of multiple plasmids inserted into a genomic locus), than would be expected by chance. Insertions that occur on the same chromosome as the parent transposon concatemer must be excluded in downstream analysis of CIS loci to reduce false-positive CISs, except when supported by another transposon line in which the concatemer is located on a different chromosome. The exclusion of a chromosome from analysis imposes limits on the ability to fully exploit the findings of a screen. For example, in a study using *SB* and T2/Onc2 to identify drivers of liver tumorigenesis in the context of HBV surface antigen (HBsAg) expression, CISs identified in chromosome 1 were filtered from the results to account for local hopping[60]. Thus, a large number of genes on chromosome 1, a gene-rich chromosome harboring the orthologs of numerous known human cancer genes, could not be assessed. Therefore, a follow-up screen was performed in HBsAg mice in which the transposons were mobilized from a transposon concatemer located on chromosome 9[61]. As an alternative to *SB*, the *PB* transposon system has less local hopping and may be an improved insertional mutagenesis transposon system, but it has not yet been characterized in HCC models[62].

Collectively, these studies using *SB* to study HCC have shown that the identification of candidate cancer genes is dependent on the components and genetic background used in the screen. A comprehensive comparison of the genes identified by

several *SB* HCC screens found dissimilarities in sample number, genetic background, *SB* transposon version, sequencing, and bioinformatics methodologies[63]. Despite these limitations, eight candidate genes were identified as significantly enriched for transposon insertions in at least five of the eight *SB*-induced HCC models examined[63]. Human homologs of three of the genes, staphylococcal nuclease and tudor domain containing 1 (*Snd1*), StAR-related lipid transfer (START) domain containing 13 (*Stard13*; also known as *deleted in liver cancer 2*), and thyroid hormone receptor interactor 12 (*Trip12*), have already been implicated in HCC[64–66]. Two other candidate genes, dihydropyrimidine dehydrogenase (*Dpyd*) and glycogen synthase kinase 3 beta (*Gsk3b*) may mediate tumor chemoresistance[67,68]. While the relevance of these eight genes to HCC is not yet entirely clear, the regularity with which they are associated with CISs, as well as previous studies linking many to cancer, strongly supports their involvement in the disease. Future studies will be needed to identify cooperative interactions, genetic context dependence, and strengths of these candidate genes as drivers of HCC. Mapping of cooperative interactions has been described in mouse models of lung adenocarcinoma[69], in which a variety of tumor-predisposing GEMMs were used for CRISPR-based tumor suppressor inactivation screens in the lung to interrogate gene-gene interactions.

RNAi screening

Rather than disrupting gene expression at the DNA level, RNA interference (RNAi) knocks down expression of specific genes by targeting degradation of their transcripts (**Figures 1B and 3A**). In an RNAi screen, mRNAs are targeted for degradation by RISC by base pairing with the processed products of shRNAs expressed from a vector library

or small interfering RNAs (siRNAs) transfected as a pool into cells[70]. This method has been used in numerous large genetic screens in cancer cell lines to identify tumor suppressor genes and genes that confer resistance to drug treatment (reviewed in 71).

RNAi screens have been performed *in vivo* to study liver cancer as well. Zender *et al.* tested the hypothesis that tumor suppressor genes are likely to reside in chromosomal regions that are deleted in HCC[72]. They first analyzed DNA copy number variations in 100 human HCCs that resulted from different etiologies, then identified the mouse orthologs of the genes of interest in deleted regions. Candidate genes were targeted using mir30-based shRNA sequences. Pools of Retroviruses expressing shRNAs were used to infect *Trp53*$^{-/-}$ embryonic hepatocytes overexpressing MYC, which were then injected intrasplenically into mice to generate a "mosaic" liver. The tumor lesions resulting in these animals were collected in order to identify the shRNAs through sequencing. The most highly enriched shRNA targeted was exportin 4 (*Xpo4*), which follow-up studies in human HCC cell lines and tissues have confirmed functions as a tumor suppressor[73,74]. *Xpo4* encodes exportin 4, which is required for the nuclear export of the signal transduction molecule SMAD3, a mediator of TGF-β signaling[75]. Thus, this report demonstrated the utility of RNAi screens in identifying bona fide tumor suppressor genes.

A separate investigation used the same shRNA library to identify gene targets that could increase the regenerative capacity of hepatocytes in the *Fah*$^{-/-}$ mouse model[50]. The shRNAs were subcloned into *Fah-SB* transposon plasmids. To further model chronic liver damage beyond the induction of tyrosinemia, following repopulation, the *Fah*$^{-/-}$ mice were treated for six weeks with CCl_4. Hairpins targeting mitogen-activated protein kinase kinase 4 (*Mkk4*; also called *Map2k4*) became enriched several thousand-fold during both

repopulation and CCl_4 treatment. Furthermore, the hepatocytes of mice that were injected with an *Mkk4*-shRNA transposon and then underwent partial hepatectomy (PH) re-entered the cell cycle more quickly than those that were injected with a noncoding shRNA transposon. Hepatocytes that expressed *Mkk4*-shRNA were also more resistant to induced apoptosis, had an increased proliferation rate, and an increased survival rate *in vitro*, after which they could repopulate the mouse liver. Notably, *Mkk4*-shRNA-expressing mice did not develop *Mkk4*-shRNA-expressing tumors, even after a year[50], illustrating how, in some genetic contexts, knockdown of a tumor suppressor can improve liver regeneration without causing cancer.

In contrast to these studies examining genes that are deleted in HCC, Rudalska et al. targeted genes that undergo amplifications[76]. In an *in vivo* RNAi screen, HCC-susceptible $Arf^{-/-}$ mice underwent HTVI with a plasmid expressing *SB* transposase and a transposon vector expressing oncogenic neuroblastoma ras oncogene ($Nras^{G12V}$) plus a library of shRNAs against the genes of interest in order to uncover a possible mechanism leading to sorafenib resistance. Half of the mice receiving shRNA libraries were treated with sorafenib, while the other half were treated with vehicle. Notably, two mitogen-activated protein kinase 14 (*Mapk14*) shRNAs were depleted over 100-fold in sorafenib-treated livers, suggesting that *Mapk14* expression promotes tumor growth and sorafenib resistance. Encouragingly, $Arf^{-/-}$ mice with tumor growth triggered by $Nras^{G12V}$ that were treated with a combination of sorafenib and the MAPK14 inhibitor BIRB796 had a lower tumor burden and longer survival than mice treated by sorafenib alone. Together, this suggests that sorafenib does not completely inhibit kinase signaling in rodent models and that the addition of a second kinase inhibitor is a potential strategy to treat HCC[76].

RNAi screening has led to a better understanding of HCC mechanisms and possible treatments, but like insertional mutagenesis, it has limitations that must be considered. In particular, RNAi activation *in vivo* has the potential to overwhelm the endogenous RNAi machinery, leading to interference with normal microRNA processing that may be critical for hepatocyte function, causing severe hepatotoxicity[77–79]. Furthermore, there may be off-target effects from binding of shRNAs to similar target sequences. Therefore, all of the most promising hits from these screens must undergo extensive validation, such as with additional specific shRNAs[80]. A potential advantage of RNAi is that it could potentially translate to a direct therapeutic in the form of siRNAs that are targeted to genes in the liver (reviewed in 81).

CRISPR-based screening

Due to its ease of use, specificity, and scalability, the CRISPR/Cas9 system is now widely used for gene editing and to modulate endogenous gene expression in cancer studies, including studies on tumor initiation and progression in mice **(Figure 3B)**.

The CRISPR/Cas9 system was adapted from bacteria for genome and epigenome editing in eukaryotes (reviewed in depth in 82 and 83) and in genome-wide screens, particularly in cancer (reviewed in 84 and 85). Briefly, wild type Cas9 is an RNA-guided DNA endonuclease that forms a complex with a gRNA molecule, which then targets the complex to a complementary DNA sequence where it cleaves both strands of DNA. This system been adapted to target the induction of double strand breaks (DSBs) in order to precisely edit the genome by homology-directed repair, or to mutate the genome by insertion or deletions of local nucleotides (indels) introduced by non-homologous end

joining[82]. A catalytically dead Cas9 (dCas9), which cannot cleave DNA, can be used to target genomic loci for activation (known as CRISPR activation, or CRISPRa) or for inhibition (known as CRISPR inhibition or CRISPRi).

CRISPR screens performed *in vitro* are capable of identifying mediators of cell viability and potential drug targets in HCC. For example, a recent screen used the human genome-scale CRISPR knockout library version 2 (GeCKO v2), which comprises gRNAs targeting 19,050 genes, to perform a loss-of-function screen in human HCC-derived Huh7 cells being treated with sorafenib[86]. Among cells that were resistant to sorafenib treatment, gRNAs targeting shugoshin 1 (*SGOL1*) were the most enriched, suggesting that expression of this gene is necessary for sorafenib to induce cell death. Intriguingly, the authors found that high SGOL1 expression in HCCs predicts worse survival. Future studies will need to examine whether patients with high SGOL1 are more likely to respond to sorafenib. Another recent screen used the HCC cell lines Hep3B and Huh7 and transduced them with a lentiviral gRNA library targeting the full complement of human kinases[87]. gRNAs targeting cyclin-dependent kinase 7 (*CDK7*) were significantly depleted in both cell lines, suggesting that this gene is essential for HCC cell survival. Consistent with these results, CDK7 is upregulated in human HCC, and the expression correlates with worse survival. Importantly, HCC cell lines and mouse HCC xenografts were sensitive to a CDK7 inhibitor as long as MYC was upregulated in these cells, which is consistent with previous studies of CDK7 inhibitors and MYC-driven cancer[88–90]. Thus, these studies highlight the power of *in vitro* screens to identify promising therapeutic targets, but the results must be validated by comparison to human data, such as datasets from TCGA, in order to determine their clinical relevance.

A number of very large genetic screens using CRISPR have been performed in *ex vivo* transduced cancer cell lines, derived from leukemia, melanoma, intestine and lung, which could be xenografted into recipient mice to examine genes associated with phenotypes such as metastasis[91–94]. In these cases, the cell lines undergo a drug selection step to ensure that the gRNAs are linked to the resultant tumors, which can be easily sequenced and used as a surrogate for the genetic changes that become enriched in resultant tumors. The disadvantage of this approach is that these screens cannot examine tumor initiation, as the cells have already been transformed.

An *ex vivo* knockout screen was performed by Song *et al.* using cancer predisposed *Trp53*$^{-/-}$ mouse embryonic liver progenitor cells expressing oncogenic MYC and Cas9[95]. The cells were stably transduced with the mGeCKOa lentiviral library expressing gRNAs targeting 20,611 mouse genes and transplanted subcutaneously into nude mice. The relative enrichment of gRNAs in the resultant tumors was measured by high throughput sequencing of pre- and post-implantation cells, revealing significant enrichment of all three gRNAs for only one target, neurofibromin 1 (*Nf1*). Mutations in *NF1* cause neurofibromatosis and have been reported in cholangiocarcinoma. To validate the function of *Nf1* as a tumor suppressor in hepatocytes, the authors examined human HCC data and identified point mutations in *NF1*. Accelerated tumorigenesis in mice injected with plasmids encoding *Myc*, *Cas9*, and *Nf1*-gRNA in multiple tumor-predisposed genetic backgrounds further validated the tumor suppressor function of *Nf1*. Notably, HCC patients with low NF1 mRNA levels also had shorter survival times than those with high NF1 mRNA levels. Despite the clinical relevance of this discovery, tumor initiation

may be influenced by the liver microenvironment. Thus, *in vivo* screens with the same lentiviral library may result in enrichment of gRNAs against a different set of targets[95].

In vivo modeling of oncogenesis using CRISPR systems has developed rapidly over the past few years. In a few of the first studies, mice were shown to develop lung cancer after intra-tracheal injection of viruses delivering CRISPR/Cas9 components that targeted a variety of tumor suppressor genes, caused chromosomal translocation, or activated Kirsten rat sarcoma viral oncogene homolog (*Kras*) via homology directed repair to a mutant form[96–98].

In the liver, HTVI of CRISPR plasmids targeting mutations in *Trp53* and phosphate and tensin homolog (*Pten*) led to the development of liver tumors by 3 months[99]. The liver tumors had bile duct features that were similar to tumors that develop in mouse models of ablation of these two genes. CRISPR/Cas9 was also used to generate activated β-catenin, one of the most common features of HCC, by HTVI of plasmids providing Cas9, a gRNA targeting the β-catenin gene, *Ctnnb1*, and a repair cassette to insert four activating point mutations into the gene via homology-directed repair. After injection, nuclear β-catenin, a marker of activation, was detected in a number of hepatocytes, demonstrating the ability of CRISPR systems to model HCC[99].

The first true *in vivo* multiplexed CRISPR/Cas9 screen was performed by HTVI of plasmids into cancer predisposed *Kras*^{G12D} mice[100]. The plasmids consisted of *SB* transposase and transposons containing a Cas9 expression cassette plus gRNAs targeting up to 18 different putative liver cancer genes. The mice developed multiple tumors by 20-30 weeks of age. Similarly, wild type mice injected with the Cas9-gRNA library and treated with CCl_4 developed multiple tumors. Interestingly, the transposon

sequences were found to be integrated in the genomic DNA of only 5% of tumors, indicating that transient gRNA expression could lead to tumor formation. Therefore, rather than determining the enrichment of the gRNA sequences in each tumor, the target sites were instead examined for indels. Numerous indels were found in each tumor, with significant enrichment of mutations at *Pten* and tet methylcytosine dioxygenase 2 (*Tet2*) loci, which was correlated with a cholangiocarcinoma-type tumor phenotype. Sequencing also showed that Cas9-induced large intrachromosomal deletions between gRNA target sites. Thus, this was the first demonstration that CRISPR/Cas9 could be used for a multiplexed screen *in vivo* to induce specific genetic mutations, and to correlate the genetic changes with the tumor phenotype. Importantly, however, the study highlighted the challenges to scaling up, as even transient gRNA expression could lead to dramatic changes to the genome. Use of larger gRNA libraries with wild type Cas9 would potentially require whole exome sequencing of tumors to characterize all of the target site changes, which is costly and labor-intensive.

A large, fully *in vivo* loss-of-function CRISPR screen in HCC was performed by Xu et al. more recently[101]. They built a *PB* transposon library using the GeCKOv2 genome-scale mouse CRISPR/Cas9 knockout library containing over 130,000 gRNAs targeting all mouse protein coding genes and miRNAs, which was delivered by HTVI to recipient mice together with vectors expressing cyclin dependent kinase inhibitor 2A (*Cdkn2a*)-gRNA and $Nras^{G12V}$ to accelerate tumorigenesis. This resulted in the development of tumors in 9 out of 27 mice at 45 weeks. The gRNAs linked to tumors were identified by sequencing, revealing that 271 gRNAs were present in tumor genomic DNA, including 26 gRNAs targeting 21 known tumor suppressor genes. In support of the efficacy of this technique,

two gRNAs targeted the well characterized tumor suppressor *Trp53*. Unexpectedly, *Cdkn2b*, which was not previously recognized as having a role in liver cancer, was targeted by three gRNAs. Mutations in the target region of *Cdkn2b*, as well as the development of tumors in mice injected with *Cdkn2b*-gRNA in the absence of a sensitizing background, confirmed that *Cdkn2b* functions as a tumor suppressor gene in the mouse liver. The identification of a new tumor suppressor gene supports the potential for CRISPR/Cas9 to be used for large-scale screens *in vivo* to identify drivers of liver cancer[102]. What was not clear in this manuscript was whether, similar to what was seen in the first *in vivo* multiplexed CRISPR/Cas9 screen[103], substantial genetic changes from gRNA-mediated chromosomal cleavages were missed because of transient gRNA expression[102].

With a similar aim of targeting multiple tumor suppressors throughout the liver to study HCC, Wang et al. developed a CRISPR system that used pools of adeno-associated viruses (AAVs)[104]. AAV virus is highly effective at infecting hepatocytes, approaching expression in 100% hepatocytes for certain serotypes[105,106]. As it only rarely integrates sequences into the genome, expression with AAV is transient. In the Wang study, each AAV was designed to express Cre, a gRNA targeting *Trp53*, and a gRNA targeting one of 49 putative tumor suppressor genes or 7 housekeeping control genes, with 4-5 gRNAs for each target[104]. The authors injected the AAV library into *lox-stop-lox-Cas9* mice, which developed multiple large liver tumor nodules within four months. Because gRNA sequences likely had dissipated, the target genomic sites were examined to discover which mutations were linked to the tumors. The authors used an approach called molecular inversion probe (MIP) capture sequencing[107] to assess for mutations in

the ±70–base pair regions surrounding the predicted cut site for each of the gRNAs in their library. Analysis of co-occurring mutations revealed that beta-2 microglobulin (*B2m*) and KAT8 regulatory NSL complex subunit 1 (*Kansl1*) mutations were often found together. Notably, individually targeting *B2m* or *Kansl1* using AAV-CRISPR did not induce tumorigenesis, while targeting both together accelerated tumorigenesis, validating their approach for the identification of gene-gene interactions that drive HCC. The drawbacks of this approach are that many mutations – such as those secondary to *Trp53* knockout or due to large chromosomal deletions or off target effects – may be missed by this technique. Indeed, the causative mutations were undetectable by MIP capture sequencing in a number of the tumors analyzed in this study. Furthermore, scale up to include hundreds or thousands of genes may not be feasible with this methodology.

Instead of inducing permanent alterations in the genome with CRISPR/Cas9 to induce loss of function, or overexpressing cDNAs to assess gain of function, expression of specific genes can either be repressed (CRISPRi) or activated (CRISPRa) through systems employing dCas9[82,84] **(Figure 3B)**. CRISPRi, which comprises dCas9 and gRNAs complementary to the target gene promoters, represses transcription either by steric inhibition of the transcriptional machinery by dCas9 alone or through fusion of dCas9 to the Kruppel-associated box (KRAB) transcriptional repressor[82]. Conversely, in the CRISPRa system, a transcriptional activator (TA) is either tethered to the dCas9 or recruited to minimal hairpin aptamers appended to the to the stem loop regions of the gRNA. CRISPRa induces the expression of the endogenous gene, which circumvents limitations of cDNA overexpression screens such as difficulty in building comprehensive libraries due to the variability in length of the cDNA sequences, and in capturing all of the

gene isoforms[82,108–110].

The “first-generation” CRISPRa system consists of the TA VP64, derived from four tandem copies of the Herpes Simplex Viral Protein 16 (VP16), fused directly to the C terminus of dCas9. Subsequent refined versions are referred to as “second-generation” activators[111]. Two significant “second-generation” activators are the SunTag system and synergistic activation mediator (SAM). The SunTag version is composed of dCas9 fused to a multimer of short peptide epitopes that binds multiple copies of its cognate single-chain variable fragment (scFv) domain, which is fused to VP64[112]. SAM harnesses the synergistic effects of multiple activation domains: VP64 is tethered to dCas9, while NF-kB trans-activating subunit p65 and human heat shock factor 1 (HSF1) are bound to MS2, a bacteriophage coat protein that selectively binds the hairpin aptamers that are appended to the gRNA scaffold[109]. Both SAM and SunTag produce robust transcriptional activation from a single gRNA, making them practical for multiplexed, genome-wide gain-of-function screens[111].

In order to streamline the CRISPRa system for use in *in vivo* screens targeted to specific tissues, including hepatocytes, we derived mice encoding a nuclease-deficient *dCas9* allele fused to the ‘SunTag’ domain at the Rosa26 locus, termed *dCas9*$^{+}$, with an upstream floxed stop cassette[45]. We then crossed them to *Fah*$^{-/-}$ mice and activated dCas9 expression specifically in hepatocytes by injecting AAV-Cre. Using *SB* transposon vectors, we generated a library of plasmids containing the TA scFv-VP64, an *Fah* expression cassette, and gRNAs targeting the promoters of *Myc*, tumor necrosis factor superfamily member 1a (*Tnfrsf1a*), solute carrier family 7 member 11 (*Slc7a11*), and *Trp53*, which we intravenously injected into the *Fah*$^{-/-}$;*dCas9*$^{+}$ mice. Nodules of MYC-

expressing, FAH-positive hepatocytes were found in *Fah*$^{-/-}$;*dCas9*$^{+}$ livers, indicating a robust and specific activation of expression of the endogenous gene. *Myc* gRNA sequences were highly significantly enriched in tumors, while *Trp53* and *Tnfrsf1a* gRNAs were significantly depleted. Thus, the *in vivo* dCas9 system activated the *Myc* locus in hepatocytes and accelerated repopulation by these cells. The potent activation of MYC expression by CRISPRa strongly supports the potential for this system to be expanded to perform large scale screens of sets of genes belonging to a specific pathway simultaneously *in vivo*[45]. Furthermore, the *dCas9*$^{+}$ mouse can be crossed to GEMMs of HCC, such as the HBsAg transgenic mouse[113], to identify context-specific drivers of tumorigenesis associated with the multitude of HCC risk factors, as well as potential drug targets and mediators of resistance to chemotherapeutics.

General considerations with genetic screens

The following are important considerations for genetic screening in general:

1. Genetic screens must take into account statistical power and the rate of false discovery. In the case of transposon insertional mutagenesis, mutation events in a particular gene must be observed in independently a number of times[114]. For cDNA, CRISPR, and RNAi experiments, the results also need to be observed independently in multiple replicates. There is the additional consideration of the "coverage" of the sequencing. In producing a screening library, there is a natural distribution of the components of the screen, and the lowly represented components may be lost by chance[115]. The standard for CRISPR screening on a genome scale, which often includes 100,000 plasmids or more[84,116], is to screen

and sequence to a depth of greater than 100-fold of the median amount of the plasmid[117,118].

2. Off-target or non-specific effects also need to be considered. In the case of transposons, “local hopping” is overcome by excluding genes located on the same chromosome as the donor transposon from the analyses, or by using independent transgenic mice with transposon donors on different chromosomes. In the case of RNAi and CRISPR screening, multiple shRNA or gRNA sequences are typically designed to target the same gene, which helps ensure that the observed effect is specific[80,118].
3. Finally, positive hits from a screen should be further validated in isolation, ideally with multiple modalities such as gene deletion, temporal or conditional control, or drug targeting[117].

Future directions

Techniques for performing high-throughput genetic screens of cancer candidate genes have evolved dramatically over the past decade, with new technology enabling an exponential growth in the number and types of genes that can be screened. Liver cancer screens will continue to evolve as we develop new strategies to more accurately model HCC, classify its subtypes, and determine the responses to specific drug treatments. Transposon mutagenesis studies on different cancer-predisposed backgrounds have demonstrated the power of a predisposing mutation to influence which specific mutations become enriched in tumors. *SB* transposon and RNAi screens have provided clues to the mechanisms of sorafenib resistance, and CRISPR screens will likely be powerful tools in

future studies to examine drivers and tumor suppressors in the setting of different types of liver injury, as well as drug resistance and sensitivity patterns. Re-framing the concept of HCC as a group of diseases, based on discoveries from these previous studies, will increase the power of screens to identify the clusters of genes cooperatively driving HCC in subsets of patients, and the alterations in genes leading to sensitivity or resistance to specific treatments, enabling a personalized approach to HCC treatment with pharmaceuticals.

Finally, the technologies that have been applied so far – HTVI of plasmids, transposable elements, RNAi, and various types of CRISPR – are likely to evolve and improve in the decades to come. For example, future screens may examine cooperation between technologies to discover how combinations of gene activators and gene inhibitors interact. They may also include screens of libraries of expressed peptides or RNA molecules, which could accelerate the development of drugs that significantly improve the survival of patients with HCC.

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Figure 1

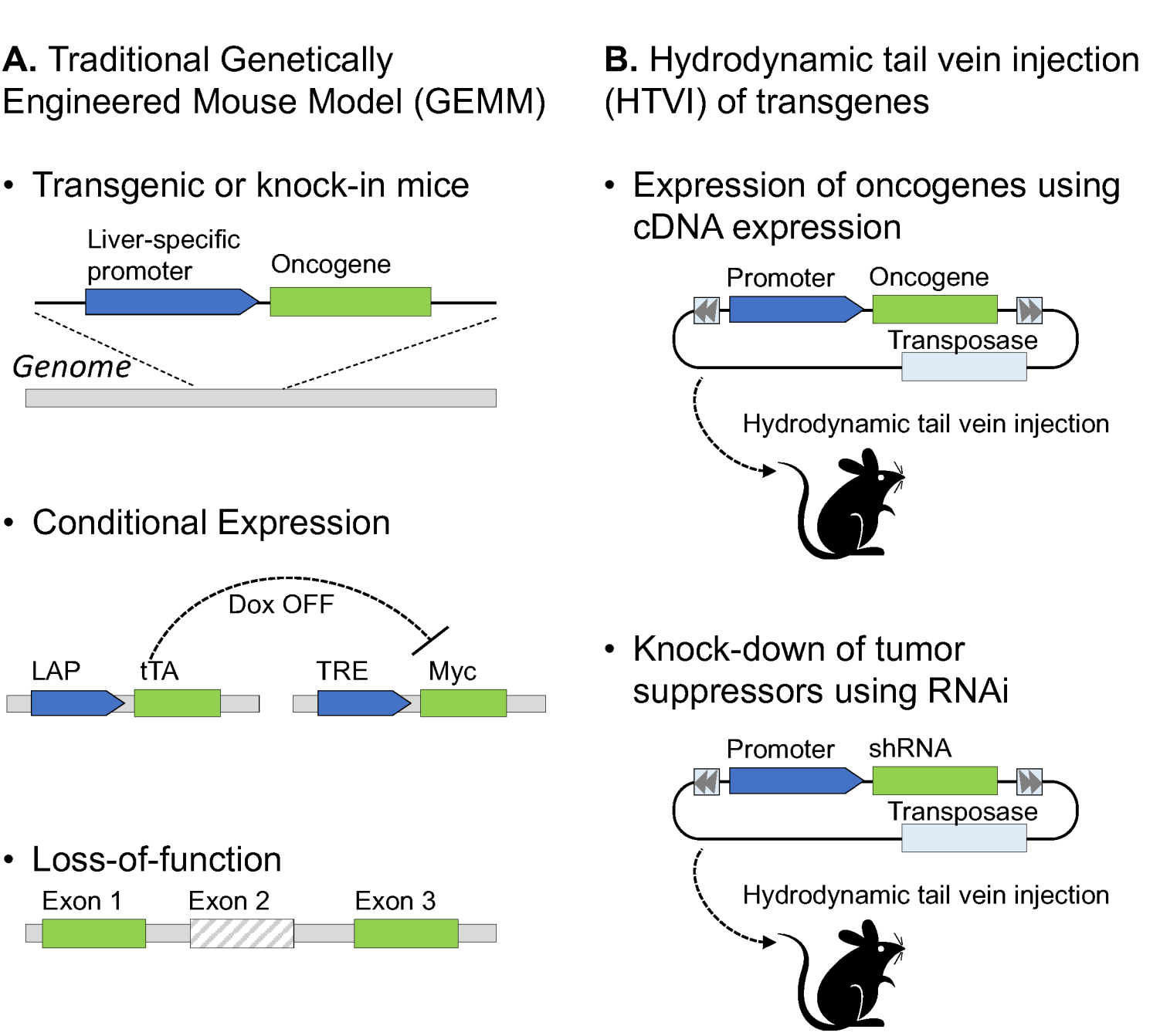

Figure 1: Mouse models to examine specific genes involved in the initiation and progression of hepatocellular carcinoma. (A) Examples of traditional genetically engineered mouse models (GEMMs). Transgenic and knock-in mice express oncogenes in the liver, often using liver-specific promoters (Top). Conditional expression systems such as the tTA/TRE system, in which administration of doxycycline turns off expression (middle). Loss-of-function models target tumor suppressor genes for mutations such as deletion of an exon (bottom). (B) The hydrodynamic tail vein injection (HTVI) technique leads to expression of oncogene cDNA (top) or knock-down of tumor suppressors using shRNA (bottom) in hepatocytes. The transposon systems enable integration and stable expression of the oncogenic sequences in a subset of the hepatocytes.

Figure 2

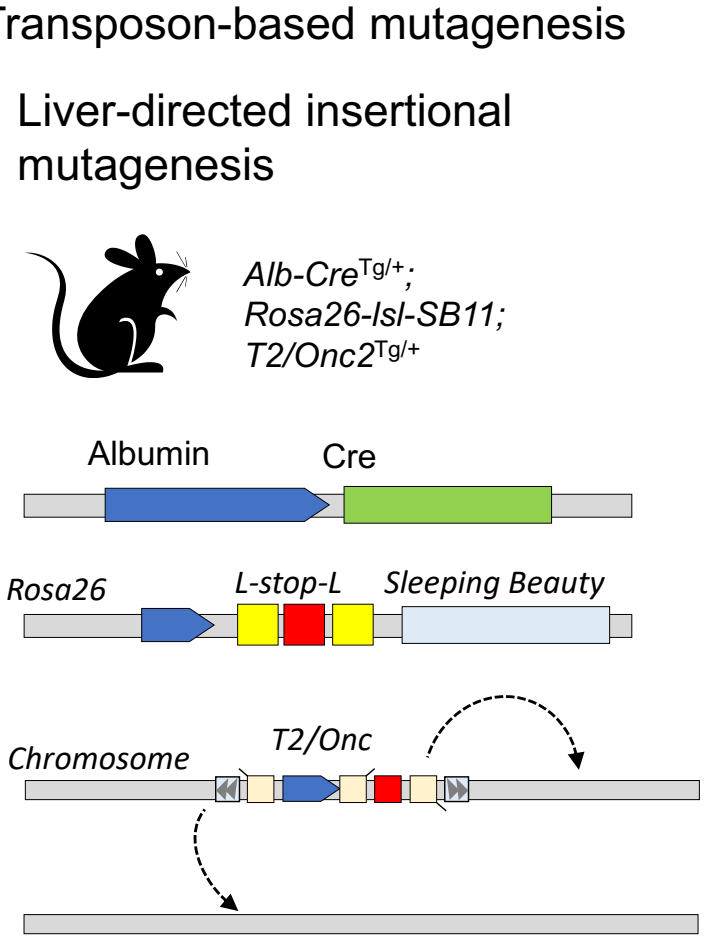

Figure 2: Transposon-based mutagenesis systems. The transposase enzyme mobilizes an engineered mutagenic transposon (e.g. T2/onc), which can disrupt tumor suppressor gene expression or activate oncogene expression, according to the position and the orientation of its insertion. Liver-specific expression of the transposase is regulated by albumin promoter-driven Cre recombinase.

Figure 3

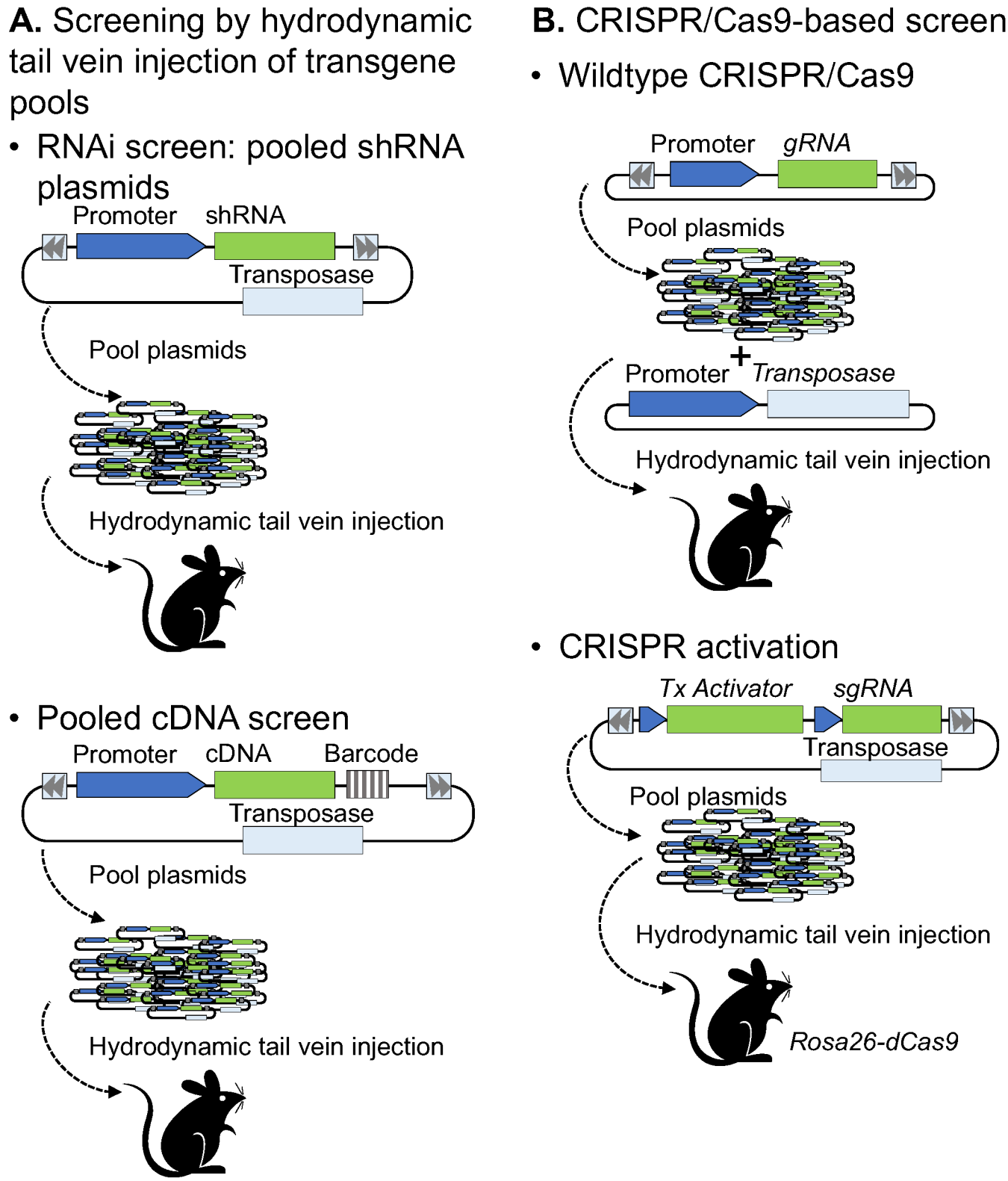

Figure 3: Genetic screens performed by hydrodynamic tail vein injection (HTVI) of plasmid pools. (A) RNAi screens by HDI of a pool of plasmids, each carrying a unique shRNA sequence (top) or a cDNA expression vector (bottom). The shRNA or cDNA linked to the resultant tumors is determined by sequencing the shRNA coding sequence or the unique barcode linked to each cDNA. (B) CRISPR/Cas9 screening systems use either a wild type Cas9 enzyme to cleave target gene DNA (for loss-of-function studies, top), or a dead Cas9 (dCas9) that binds to transcriptional activators (to enhance target gene expression, bottom).