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Chronic liver injury alters driver mutation profiles in hepatocellular carcinoma

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Abstract

Most hepatocellular carcinomas (HCCs) develop in a chronically injured liver, yet the extent to which this microenvironment promotes neoplastic transformation or influences selective pressures for genetic drivers of HCC remains unclear. We sought to determine the impact of hepatic injury in an established mouse model of HCC induced by *Sleeping Beauty* transposon mutagenesis. Chemically-induced chronic liver injury dramatically increased tumor penetrance and significantly altered driver mutation profiles, likely reflecting distinct selective pressures. In addition to established human HCC genes and pathways, we identified several novel injury-associated candidates that represent promising loci for further study. Among them, we found that *FIGN* is overexpressed in human HCC and promotes hepatocyte invasion. We also validated *Gli2*'s oncogenic potential *in vivo*, providing direct evidence that Hedgehog signaling can drive liver tumorigenesis in the context of chronic injury. Finally, we show that a subset of injury-associated candidate genes identifies two distinct classes of human HCCs. Further analysis of these two subclasses revealed significant trends among common molecular classification schemes of HCC. The genes and mechanisms identified here provide functional insights into the origin of HCC in a chronic liver damage environment.

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Conclusion—A chronically damaged liver microenvironment influences the genetic mechanisms that drive hepatocarcinogenesis.

Keywords

Gli2; Fign; mutagenesis; cancer genetics; mouse model

Each year ~745,000 people worldwide die from liver cancer, making it the second leading cause of cancer-related death (1). Hepatocellular carcinoma (HCC) accounts for >80% of cases, generally occurring in patients with chronic liver injury (2). This condition typically includes sustained inflammation, compensatory hepatocyte proliferation, and fibrosis – an accumulation of extracellular matrix (ECM) that displaces parenchymal cells and increases liver stiffness. Induction of fibrosis, which is essentially scarring of the liver, represents a wound-healing response. While acute injury can activate inflammation and mechanisms of fibrogenesis, a sustained stimulus is required for significant ECM accumulation and long-term damage to occur. All of the major risk factors for HCC, which include HBV or HCV infection, alcohol-induced liver damage, and non-alcoholic fatty liver disease (NAFLD), provide a chronic injury stimulus that leads to significant hepatic inflammation and fibrosis. Without resolution of the underlying injury stimulus, liver fibrosis progressively increases in severity and can eventually lead to cirrhosis. One third of cirrhotic patients will develop HCC in their lifetime, with an annual risk of ~4% (3).

A strong association between liver fibrosis and HCC is clear, as ~90% of cases develop in this context (4). While this association has been recognized for decades, the extent to which fibrosis directly contributes to HCC pathogenesis remains unclear. Whether the two conditions co-occur due to shared etiologies or a direct role for fibrosis in promoting HCC is a question without a conclusive answer. Several potential fibrosis-dependent mechanisms of hepatocarcinogenesis have been proposed (including enhanced mitogenic and anti-apoptotic signaling through integrins, growth factor secretion by activated hepatic stellate cells, increased stromal stiffness leading to proliferation, and growth factor sequestration by the ECM resulting in autocrine and paracrine signaling (5)), but they require definitive experimental validation. It is also unclear how nascent HCC responds to the fibrotic microenvironment once initiated. Assessing the relative importance of fibrosis and other chronic injury-associated conditions during HCC development and progression has great potential to identify molecular pathways that may be targeted prophylactically in patients to prevent or delay HCC.

Modeling cancer in mice is an effective way to improve understanding of the molecular drivers of human disease. When engineering such models, it is essential to recapitulate the pathogenesis of human disease as closely as possible to maximize the relevance and utility of experimental findings (6). Insertional mutagenesis screens using the *Sleeping Beauty* (SB) transposon system mimic the random, stepwise accumulation of somatic mutations that occurs in human cancer and are thus useful for cancer gene discovery (7). We present here the results of a liver-specific SB mutagenesis screen conducted in the presence or absence of chronic hepatic injury induced by carbon tetrachloride (CCl₄). Unlike most mouse models of HCC, our SB-induced model takes into account the damaged microenvironmental context in

which the majority of human HCC cases develop. By comparing transposon insertion profiles between tumors developed in normal or damaged livers, we have identified several mutation events that are subject to differential selective pressure depending on the microenvironmental context. Encouragingly, genes known to be involved in human HCC (Hippo, HGF, WNT pathway members) were commonly mutated in injury-associated tumors. This result highlights the relevance of our model to human cancer and supports the hypothesis that other recurrently mutated genes, including *Gli2* and *Fign*, may be similarly relevant cancer genes.

Materials and methods

Mice

Alb-Cre^{Tg/Tg} mice (8) (Jackson Laboratory) were crossed to *RosaSBase^{LsL/LsL};T2/Onc3^{Tg/Tg}* mice (TG12740 or TG12775 strain, which contain ~11 or 28 transposon copies on Chr9 or Chr12, respectively) (9) to induce HCC. Control cohort tumors from previous SB screens (9, 10) were generated by crossing T2/*Onc3^{Tg/Tg}* strains to mice ubiquitously expressing SBase from the *ROSA26* locus (11). Male offspring were aged up to 65 weeks and monitored for liver tumor development. Starting at eight weeks of age, mice received intraperitoneal injections of 10% CCl₄ in mineral oil (2.5μl/g body weight twice a week for twelve weeks). Hydrodynamic tail vein injection and liver repopulation in *Fah*-deficient mice was performed as described (12). All animal studies were conducted using procedures approved and monitored by the Institutional Animal Care and Use Committees at the University of Iowa and University of Minnesota.

Candidate gene comparison across SB-induced HCC models

Candidates from five previously reported SB-induced HCC models (12-16) were compared. To normalize candidate-calling stringency across screens, gene lists from two studies (15, 16) were filtered based on criteria used in one of the studies (15) to identify “trunk” drivers (requiring at least four sequencing reads to include an insertion site in the CIS analysis). The R package UpSet (17) was used to generate a figure depicting overlaps.

Immunohistochemistry

Antigen unmasking was performed with citrate buffer (pH 6), endogenous peroxidase was quenched with 3% H₂O₂, and 2.5% horse serum was used to block nonspecific staining. Sections were incubated with 1:100 dilutions of goat anti-GLI2 (AF3635-SP, Novus) or rabbit anti-FIGN (SAB1304554, Sigma Aldrich) overnight at 4°C. Secondary antibodies: Vector Laboratories ImPRESS anti-goat (GLI2) or anti-rabbit (FIGN). Slides were developed with the Vector Laboratories NovaRED substrate reagent mix for seven minutes and counterstained with hematoxylin. For the liver tissue microarray, each sample was assigned an intensity value for hepatocyte FIGN staining in a blinded manner: 4 = strong cytoplasmic staining; 3 = reduced cytoplasmic staining; 2 = faint, discontinuous staining; and 1 = essentially absent staining.

Comparison to human HCC

Human HCC expression data for all orthologues of injury-associated driver genes were obtained through the LIHC project within The Cancer Genome Atlas (n=371). RSEM values were log₂-transformed, a 1.5 standard deviation cutoff was applied, and mean centering of both genes and tumors was performed. Next, k-means clustering (k = 2) was used to identify two subtypes of human HCC based on expression of injury-associated candidates. Correlations were identified between these subtypes and selected clinical and molecular categories recently described (18). A chi-squared test was performed to compare the category distribution within each injury-associated subtype to the entire HCC population.

Results

Chronic liver injury increases severity of SB-induced HCC

A model of spontaneous HCC was produced using mice with liver-specific SB mutagenesis (RosaSBBase^{LsL/+}, T2/Onc3^{Tg/+}, Alb-Cre^{Tg/+}) (8, 9). Only males were aged to assess tumor formation, as a similar SB HCC model reported tumor resistance in females (14). Starting at eight weeks of age, chronic liver injury was induced in half (n=77) of the mutagenized cohort by intraperitoneal injection of carbon tetrachloride (CCl₄) twice weekly for twelve weeks. Injected and control untreated mice (n=77) were aged to 65 weeks or until moribund. Histological analysis by Masson's trichrome staining revealed persistent liver fibrosis at the time of euthanasia for all analyzed CCl₄-treated mice (Supporting Table S1, Supporting Fig. S1). Over 95% of tumors from CCl₄-treated animals evaluated via histopathology were clearly hepatocellular carcinoma (23/24), as expected.

Induction of chronic liver injury with CCl₄ increased tumor penetrance and multiplicity, while decreasing latency (Fig. 1). All CCl₄-treated mice developed liver tumors, with an average latency of 48.5 weeks. Only 44% of control mice developed tumors within the 65-week aging period, with an average latency of 64.5 weeks. Of the mice that developed HCC, average multiplicities of 5.0 and 1.7 tumors per mouse were observed for the CCl₄-treated and control cohorts, respectively, with slight differences between the two SB transposon strains used. A control cohort (n=17) lacking SB-induced mutagenesis (Cre-negative) was subjected to CCl₄-induced liver damage to estimate the frequency of spontaneous tumors in this context. When euthanized at 65 weeks of age, only ten liver tumors were collected from three of the mice (~18%).

Damaged liver microenvironment influences mutation profile in SB-induced HCCs

To identify recurrent mutation events implicated as drivers of hepatocarcinogenesis, transposon integration sites were profiled in 343 and 55 liver tumors induced in the presence or absence of CCl₄-induced hepatic injury, respectively. To increase the statistical power in detecting differences in driver mutation frequency between the two cohorts, the control cohort was expanded using 42 liver tumors (total n = 97) generated by two previous SB screens (9, 10). Absence of hepatic fibrosis in each group of untreated SB-mutagenized liver tumors was verified prior to downstream analysis. DNA fragments containing transposon/genome junctions were amplified by ligation-mediated (LM)-PCR and sequenced using the Illumina HiSeq platform. A list of clonal integration sites was generated for each sample

based on criteria including relative abundance within the sample (19). Only these clonally expanded sites were included in subsequent analyses, as they most confidently represent mutation events that have been subject to positive selection during tumor development and progression. A gene-centric common insertion site (gCIS) analysis (19) was performed for each cohort independently to identify genes with higher frequencies of transposon-induced mutation than predicted based on a random integration pattern.

Table 1 lists the candidate driver genes mutated in ≥2% of tumors in either cohort that were preferentially mutated in one cohort and all driver genes common to both cohorts. Overall, we identified 419 and 49 candidate genes in injury-associated and control tumors, respectively (Supporting Tables S2-S3), with 21 genes implicated in both cohorts. Insertion within the *Rtl1* locus was the most commonly observed event in both cohorts, consistent with its validated role as an oncogene (20) and with what we and others have previously observed (9, 15). Additional genes mutated in >5% of tumors include *Met*, *Figl*, *Gli2*, and *Dpyd* in the CCl₄ cohort and *Hras*, *Dpyd*, *Kras*, *Stag2*, *Snd1*, *Dennd5b*, and *Fndc3b* in the untreated cohort.

Fisher's exact test was used to identify candidate driver genes significantly enriched for recurrent mutation in one cohort or the other (Table 1). *Met*, *Figl*, and *Gli2* mutations were significantly enriched in the CCl₄ cohort, while *Hras* and *Dennd5* mutations were significantly more common in control tumors. Notably, *Figl* mutations were detected in 8% (27/343) of injury-associated tumors but were never detected in control tumors. We identified three classes of driver genes based on relative abundance in tumors from each cohort: higher in the CCl₄ cohort, higher in the control cohort, and common to both cohorts. Figure 2 shows the distribution of driver mutations across these three classes for both tumor cohorts. Interestingly, tumors in the CCl₄ cohort with mutations in *Figl* or *Gli2* had fewer driver gene mutations than other tumors ($p = 0.0022$) (Supporting Fig. S2), suggesting that *Figl* and *Gli2* mutations may be particularly strong drivers of hepatic transformation in a damaged microenvironment.

Comparison to other SB-induced HCC models

To increase functional understanding of the genetic mechanisms driving injury-associated tumors, we compared candidate genes from the CCl₄ cohort to those identified by other SB-induced HCC models conducted in a variety of genetic contexts (see *Materials and methods* for details). Though limited by differences in sample numbers, sequencing methodologies, and bioinformatics strategies across studies, this analysis revealed some overlap with each of the previously reported HCC models (Fig. 3). A major determinant of genetic similarity was the version of SB transposon utilized (T2/Onc3 versus T2/Onc or T2/Onc2), indicating that the internal promoter (CAG or MSCV, respectively) strongly influences the transposon's mutagenic activity, consistent with previous results (9). Additionally, we found substantial overlap with models of hepatitis B virus (HBV)-associated HCC. Overall, 14% (57/419) of injury-associated candidates were also reported by two recent SB screens in which HBV surface antigen (HBsAg) was expressed hepatically (15, 16). An additional 35% (148/419) were identified in one of the HBV screens, bringing the total overlap with our chronic injury model to 49% of candidate genes. Of the genes mutated in ≥2% of CCl₄-associated tumors,

88% (50/57) were also identified as candidates in HBV-associated tumors. Eight of these top candidates (*Dpyd*, *Snd1*, *Adk*, *Zbtb20*, *Gsk3b*, *Psd3*, *Trip12*, and *Stard13*) were drivers in more than half of the SB-induced HCC models, providing strong support for potentially broad roles in hepatocarcinogenesis.

Gli2 is a strong driver of hepatocarcinogenesis in vivo

Hedgehog (Hh) signaling pathway activation has been proposed to contribute to human HCC (21, 22). Although direct evidence for a functional role in hepatic transformation is scarce, several studies have detected strong, tumor-specific GLI2 expression in ~60-80% of HCC samples by immunohistochemistry (23-25). Others have suggested a role of the Hh pathway and Gli2 in HCC (24, 26), but there is no evidence that Gli2 overexpression itself is capable of initiating hepatocarcinogenesis. Given its high mutation rate in injury-associated SB-induced liver tumors, we hypothesized that Gli2 overexpression cooperates with CCl₄-induced liver damage to initiate HCC. To test this hypothesis, mice with stable hepatic expression of *Gli2* were generated via hydrodynamic tail vein injection and liver repopulation. A subset of these mice was subsequently treated with CCl₄ to induce chronic liver injury. At 150 days post-injection, all surviving untreated *Gli2*-expressing mice developed tumors, with an average of 39.5 per mouse (Gli2 vs. mCherry, ANOVA $p < 0.0001$, Fig. 4A). As expected, CCl₄ treatment increased tumor multiplicity to 57.0 tumors/mouse (Gli2 CCl₄ vs. Gli2 untreated, Tukey post-hoc $p = 0.006$).

Unexpectedly, 89% of mice (59/66) receiving the *Gli2* transgene died within 75 days of hydrodynamic injection without tumor formation. Morbidity in this cohort did not likely result from failure to rescue the *Fah* deficiency since *Fah* transgene delivery was verified in a subset of animals via a luciferase reporter included in the transgene cassette. Lethality may instead result from interference of Gli2 overexpression with liver repopulation and/or function. We hypothesized that reducing the strong overexpression observed in livers of injected mice (Fig. 4D,E) by decreasing the amount of injected Gli2 transgene would eliminate lethality by producing a mosaic liver in which a smaller percentage of hepatocytes overexpress Gli2. A small cohort of mice was generated in which the Gli2 transgene was diluted 1:10 relative to the *Fah* transgene. As expected, all mice survived and developed spontaneous tumors, albeit with decreased multiplicity (Fig. 4F).

While CCl₄ treatment significantly increased Gli2-mediated hepatocarcinogenesis *in vivo*, Gli2 overexpression alone produced an unexpectedly high tumor burden, perhaps in conflict with the results of our genetic screen (Fig. 2). However, the hydrodynamic delivery model utilized for this experiment involves substantial hepatic injury, as the livers of *Fah*-null mice must regenerate from a small population of *Fah*⁺ hepatocytes (27). We also speculated that this process might impact endogenous Gli2 expression even in the absence of transgene expression. We performed Gli2 staining on liver sections from mice undergoing liver regeneration following hydrodynamic injection with a control plasmid expressing mCherry. Consistent with previous reports (22), age-matched control mice did not detectably express Gli2 protein (Fig. 4B). However, variable levels of Gli2 expression were apparent in hepatocytes of regenerating livers, indicating that endogenous Gli2 expression is activated during liver repopulation following damage (Fig. 4C).

Fign is overexpressed in regenerating liver and human liver tumors

Fign was among the most common driver mutations in the CCl₄-treated tumor cohort (Fig. 2). Interestingly, *Fignl2* was identified as a driver in the same tumor cohort, although its mutation frequency was significantly lower (Supporting Table S2). The pattern of transposon insertions within these loci suggests that overexpression of *Fign* or *Fignl2* can promote hepatocarcinogenesis in the context of liver injury. As with *Gli2*, *Fign* staining on sections from regenerating *Fah*-null mouse livers revealed moderate to strong expression in sporadic hepatocytes (Supporting Fig. S3), suggesting a potential role in this process.

RNA-Seq data from 371 human HCCs in The Cancer Genome Atlas indicates that 15% of human HCCs overexpress ($z \geq 2.0$) *FIGN* and/or *FIGNL2* (Supporting Fig. S4A). We also performed *FIGN* staining on a panel of 100 human HCC samples, evaluating four independent core biopsies from both tumor and normal adjacent liver for each patient (Supporting Fig. S4B-E). Consistent with RNA-Seq results, 15% of human HCCs were found to have strong expression of *FIGN* protein at levels significantly higher than the normal matched tissue ($\chi^2 = 35.647$, $df = 3$, $p < 0.0001$). No significant association was detected between *FIGN* expression and survival (data not shown).

Our results suggest that *Fign* acts as an oncogene to drive hepatocarcinogenesis in the context of chronic liver injury. Little is known about *Fign* and any potential HCC-promoting mechanisms, although a microtubule-severing enzymatic activity has been described (28). Thus we hypothesized that overexpression may promote increased proliferation via disruption of normal anaphase, resulting in copy number alterations following mitosis. However, overexpression of *Fign* in an immortalized mouse hepatocyte cell line (TIB-73) had no effect on ploidy (Supporting Fig. S4F) or proliferation rate in cells overexpressing *Fign* under either normal or low serum conditions (data not shown).

A recent publication showed that *Fign* contributes to astrocyte migration by severing tyrosinated microtubules at the leading edge of the cell (29), leading us to hypothesize that *Fign* overexpression increases hepatocyte invasion through extracellular matrix. We have previously shown that TIB-73 cells are capable of generating spheroids in Matrigel suspension cultures (20). As expected, cells transfected with empty vector produced non-invasive spheroids in Matrigel after one week in culture (Fig. 5A,B). In contrast, TIB-73 cells overexpressing *Fign* displayed a more invasive phenotype, exhibiting altered colony morphology with collective invasion into the Matrigel (Fig. 5C,D). *Fign* overexpression also promoted invasion through Matrigel in response to an HGF gradient (Fig. 5E).

Comparison to human HCC

To assess the broader relevance of our model to human HCC, we analyzed expression of genes orthologous to injury-associated candidates from our screen in 371 human HCCs in The Cancer Genome Atlas LIHC project. Unsupervised k-means clustering identified a set of 24 genes that reliably segregated human HCCs into two groups that we designated injury-associated subtypes 1 and 2 (Fig. 6A). Interestingly, injury-associated subtype 1 is defined by elevated expression of a gene set that includes *FIGN*, *FIGNL2*, and *GLI2* – drivers specific to HCCs that emerged from our CCl₄-treated cohort (Fig. 3).

We next performed correlation analyses to identify clinical characteristics shared by tumors in the injury-associated subtypes we identified. For these comparisons, we relied on a recent publication that described a comprehensive clinical and molecular analysis of 191 human HCCs (18). We assessed correlation with twelve distinct parameters and discovered that three different gene expression classification schemes showed significant correlations among the injury-associated subtypes we identified (Fig. 6B, Supporting Table S4). Most compelling was the association between injury-associated subtype 1 and a cholangiocarcinoma-like gene expression signature first identified by Woo *et al.* (30). Figure 6C shows the strong associations between the injury-associated subtypes we identified and different gene expression subtypes previously described.

Finally, we examined somatic mutation data from tumors within each injury-associated subtype for associations with recently reported HCC drivers (18). Significant associations were observed between injury-associated subtype 2 and mutations in *TERT* promoter ($q=0.0003$), *CTNNB1* ($q=0.003$), and *ALB* ($q=0.023$), while *BAP1* mutations were associated with injury-associated subtype 1 ($q=0.006$) (Supporting Table S4). These observations suggest our genetic screen identified genetic trends relevant for human HCC.

Discussion

We describe here a forward genetic mutagenesis screen conducted in HCCs developing in the presence or absence of chronic liver injury. This approach identified several candidate genes predicted to be involved in HCC development, many of which were differentially detected in tumors depending on the microenvironmental context. The list of putative liver cancer genes includes known effectors of human HCC, as well as novel drivers that represent good candidates for further study.

Induction of chronic liver injury via CCl_4 administration greatly increased the severity of HCC resulting from SB insertional mutagenesis. We observed 100% tumor penetrance in the CCl_4 cohort, compared to 44% in the untreated cohort, and multiplicity was 3-fold higher with injury. Our power to identify even relatively rare injury-associated driver mutations was enhanced by the large number of tumors developed in this cohort (the largest SB screen conducted to date). While 18% of CCl_4 -treated mice developed tumors in the absence of transposon mobilization, it is clear that SB mutagenesis is the primary driver of tumorigenesis in the model. Additionally, it should be noted that CCl_4 is not directly mutagenic when used in mouse studies (although indirect DNA damage from oxidative stress can occur)(31), so although background tumorigenesis is slightly increased by CCl_4 alone, our ability to identify driver mutations is unlikely to be significantly affected by the presence of chemically-induced mutations. Based on the injury stimulus and induction of hepatocyte turnover provided by CCl_4 treatment, it may be expected that fewer mutations would be required to transform hepatocytes as compared to undamaged liver. This does not seem to be the case in our model, as the average number of clonally expanded mutations per tumor did not significantly differ between CCl_4 -treated (23.8 clonal mutations) and untreated (19.5 clonal mutations) mice ($p=0.34$). This result is consistent with our assumption that CCl_4 provides a minimal mutagenic contribution to tumor development in the model.

We observed a striking degree of overlap between injury-associated driver mutations and drivers identified by HBV-associated, SB-induced HCC screens (15, 16)(Fig.3). The mouse model utilized in these screens expresses HBV surface antigen in the liver, leading to chronic inflammation and hepatocyte damage. Importantly, mice do not develop fibrosis (15, 32). In contrast, CCl₄ treatment induces inflammation, cellular damage, steatosis, and fibrosis in mice (33). We speculate that candidate genes shared by CCl₄- and HBV-associated models of SB-induced HCC may reflect selective pressures for neoplastic transformation imparted by chronic hepatocyte damage and liver inflammation. In contrast, the top candidates identified in CCl₄-, but not HBV-associated tumors (*Fign*, *Gli2*, *Nfe2*, *Med13b*) may represent strong drivers of tumorigenesis specifically in the context of liver fibrosis.

Our detection of recurrent *Met* mutations preferentially in injury-associated HCCs makes intuitive sense. Inserted SB transposons clustered upstream of and in the same transcriptional orientation as the ORF, a pattern indicative of overexpression (Supporting Fig. S5)(20, 34). Importantly, the overexpressed Met protein is predicted to be full-length (Supporting Fig. S6) and thus still dependent on HGF ligand binding for activation of its tyrosine kinase activity and downstream proliferative signaling pathways. In a basal state, there is little hepatic HGF/Met signaling, demonstrated by the fact that liver-specific knockout of *Met* produces no overt phenotype (35). Under these conditions, a mutation resulting in overexpression of ligand-dependent Met would not be subject to strong positive selection. Upon induction of liver injury and fibrosis, hepatic stellate cells are dramatically expanded and activated to secrete cytokines, including HGF (36). In this environment, overexpressed Met would be functionally active, allowing it to promote tumorigenesis. CCl₄-treatment may thus provide a strong positive selective pressure for mutations causing Met overexpression.

Mutations predicted to drive *Gli2* overexpression were also detected preferentially in injury-associated tumors. *Gli2* is a transcription factor activated downstream of Hedgehog (Hh) signaling. The Hh pathway is activated in response to many types of liver injury, where it plays a role in regeneration (37). Mutations causing *Gli2* overexpression could uncouple activation of Hh pathway transcriptional targets from upstream receptor signaling. Such mutations may confer a particularly strong selective advantage in a liver environment with an initial injury stimulus to activate Hh signaling, preventing subsequent cessation of signaling and promoting increased fibrosis and eventual tumor development. Another possibility is that *Gli2* activation may be strongly oncogenic in cell populations expanded during the liver's response to damage but not in quiescent hepatocytes. These expanded populations include hepatic stellate cells, myofibroblasts, and liver progenitors, cell types known to be highly responsive to Hh signaling and to contribute to both fibrotic and parenchymal mass in regenerated livers (38, 39). Increased GLI2 protein expression and nuclear localization have been reported in human HCCs, particularly those with poor prognosis (23, 25). Interestingly, a recent comprehensive molecular analysis of human HCCs identified an HBV insertion in the *GLI2* promoter region associated with overexpression (18). There is also evidence that other upstream signaling pathways, including EGF and HGF, can increase GLI2-mediated transcription (40), suggesting that GLI2 may represent a convergence point that integrates multiple upstream signaling pathways to drive proliferation

during liver regeneration and transformation. Importantly, driver mutations in other Hh pathway genes (*e.g. Smo, Ptch*) were not identified in our screen, unlike other key pathways (*e.g. Wnt, Hippo, Met*) from which multiple members were identified as drivers. This result suggests GLI2 may function independently from canonical Hh signaling in HCC.

Another novel finding is our identification of *Fign* as a candidate HCC oncogene. In addition to being a common driver in SB-induced tumors, we identified a substantial subset of human HCCs overexpressing FIGN at both the mRNA and protein levels. An independent transposon mutagenesis screen also identified *Fign* as a candidate oncogene in a mouse model of pancreatic cancer (41). Interestingly, pancreatic tumors harboring *Fign* mutations exhibited hepatoid histology, suggesting potential shared etiological characteristics beyond simple histology between HCC and hepatoid adenocarcinomas. The mechanism of *Fign*-induced transformation may involve microtubule regulation. While *Fign* overexpression did not affect ploidy in immortalized mouse hepatocytes (Supporting Fig. S4F), it did induce an invasive phenotype (Fig. 5), consistent with *Fign*'s reported role as a regulator of microtubule dynamics at the leading edge of the cell during astrocyte migration (29). Additional experimentation is needed to determine if *Fign* overexpression can drive transformation in chronically injured liver, perhaps through increased invasiveness that facilitates proliferation in a fibrotic microenvironment.

Our results demonstrate that chronic liver injury significantly influences the driver mutation profile in a mouse model of HCC. Similar influence likely exists in human HCC given the significant overlap between our injury-associated model and human tumors. We identified several candidate HCC driver mutations, with many subject to differential selection in tumors depending on microenvironmental context. Recurrently mutated genes include known effectors of human HCC and novel putative drivers that represent good candidates for further study. Our results provide some insight into the mechanistic role of injury and fibrogenesis in HCC, suggesting that oxidative stress and growth factor signaling from hepatic stellate cells may be important mediators of fibrosis-associated hepatocarcinogenesis. Future studies could individually dissect contributions of fibrosis and other CCl₄-induced liver injury phenotypes, including inflammation and hepatocyte proliferation, to HCC development.

To our knowledge, this study presents the first direct evidence that *Gli2* overexpression drives liver tumorigenesis *in vivo*. This result combined with prior observations of aberrant GLI2 staining in ~80% of human tumors (24, 25) suggests that GLI2 may be a common HCC driver. It is important to note that data from The Cancer Genome Atlas indicates that GLI2 expression in human HCC is not generally driven by direct mutation or copy number gain. Furthermore, *GLI2* transcript is not overexpressed in tumors relative to normal adjacent liver samples (Supporting Fig. S7). We also did not detect increased *Gli2* transcript during liver regeneration in the mouse *Fah* model (data not shown), despite observing increased protein expression (Fig. 4C,D). These observations suggest that altered GLI2 protein stability may underlie elevated protein levels observed in human HCC.

Finally, we discovered that human HCCs can be categorized into two distinct subtypes based on expression of a subset of our injury-associated driver genes (Fig. 6A). These subtypes

have distinct molecular features, including mutation and expression profiles (Fig. 6B). Interestingly, the strongest association we observed was between the CC-like class of HCC (30) and our injury-associated subtype 1 ($p=1.83E-24$), leading us to speculate that the injury-associated subtypes may have distinct cells of origin that are transformed via different mechanisms (Fig. 7).

The injury-associated subtype 2 is enriched for many features commonly observed in HCC. It shows a typical gender bias of ~3:1 male-to-female ratio and frequent gain-of-function mutations in *CTNNB1*. Mutations in *ALB* and *TERT*, recently described in HCC (18), are also enriched in injury-associated subtype 2. These observations fit a model in which tumors originate from hepatocytes. Restored *TERT* expression would be necessary to maintain proliferation of a differentiated hepatocyte. Inactivation of *ALB* expression, which accounts for ~20% of cellular RNA in normal hepatocytes (42), would allow HCC to direct energy elsewhere and increase proliferation (18).

By contrast, HCCs in the injury-associated subtype 1 class have a cholangiocarcinoma-like gene expression pattern (30). Tumors are not enriched for *TERT* or *ALB* mutations, suggesting that selective pressures differ from typical HCCs. Instead, tumors of this subtype overexpress *FIGN*, *FIGNL2*, and *GLI2* – genes that are unique drivers of our CCl₄-treated mouse model of HCC (Fig. 2, 3). One possible explanation for these associations is that tumors emerge from progenitor cells expanded during chronic liver injury, such as those described by Tarlow and colleagues (43). Transformation of progenitors could involve mechanisms distinct from those necessary to transform differentiated hepatocytes. These differences in cell of origin could contribute to some of the variable associations and gene expression signatures described in human HCC.

Further study of the candidate driver genes and mechanisms we identified may provide diagnostic and therapeutic benefits for patients with HCC. In addition, experiments examining potential differences in cell of origin for injury-associated HCC could provide insights into more effective therapies that disrupt key mechanisms driving tumor progression. More work is needed to understand the biology underlying the injury-associated driver mutations we report here. Nevertheless, we clearly show that selection of drivers during HCC initiation and/or progression is a complex process that is impacted by the liver microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015; 136:E359–386. [PubMed: 25220842]
2. Schutte K, Bornschein J, Malfertheiner P. Hepatocellular carcinoma--epidemiological trends and risk factors. *Dig Dis*. 2009; 27:80–92.
3. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet*. 2003; 362:1907–1917. [PubMed: 14667750]
4. Seitz HK, Stickel F. Risk factors and mechanisms of hepatocarcinogenesis with special emphasis on alcohol and oxidative stress. *Biol Chem*. 2006; 387:349–360. [PubMed: 16606331]
5. Zhang DY, Friedman SL. Fibrosis-dependent mechanisms of hepatocarcinogenesis. *Hepatology*. 2012; 56:769–775. [PubMed: 22378017]
6. Frese KK, Tuveson DA. Maximizing mouse cancer models. *Nat Rev Cancer*. 2007; 7:645–658. [PubMed: 17687385]
7. Moriarity BS, Largaespada DA. Sleeping Beauty transposon insertional mutagenesis based mouse models for cancer gene discovery. *Curr Opin Genet Dev*. 2015; 30:66–72. [PubMed: 26051241]
8. Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem*. 1999; 274:305–315. [PubMed: 9867845]
9. Dupuy AJ, Rogers LM, Kim J, Nannapaneni K, Starr TK, Liu P, et al. A modified sleeping beauty transposon system that can be used to model a wide variety of human cancers in mice. *Cancer Res*. 2009; 69:8150–8156. [PubMed: 19808965]
10. Rogers LM, Olivier AK, Meyerholz DK, Dupuy AJ. Adaptive immunity does not strongly suppress spontaneous tumors in a Sleeping Beauty model of cancer. *J Immunol*. 2013; 190:4393–4399. [PubMed: 23475219]
11. Dupuy AJ, Akagi K, Largaespada DA, Copeland NG, Jenkins NA. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. *Nature*. 2005; 436:221–226. [PubMed: 16015321]
12. Keng VW, Villanueva A, Chiang DY, Dupuy AJ, Ryan BJ, Matise I, et al. A conditional transposon-based insertional mutagenesis screen for genes associated with mouse hepatocellular carcinoma. *Nat Biotechnol*. 2009; 27:264–274. [PubMed: 19234449]
13. O'Donnell KA, Keng VW, York B, Reineke EL, Seo D, Fan D, et al. A Sleeping Beauty mutagenesis screen reveals a tumor suppressor role for Ncoa2/Src-2 in liver cancer. *Proc Natl Acad Sci U S A*. 2012; 109:E1377–1386. [PubMed: 22556267]
14. Keng VW, Sia D, Sarver AL, Tschida BR, Fan D, Alsinet C, et al. Sex bias occurrence of hepatocellular carcinoma in Poly7 molecular subclass is associated with EGFR. *Hepatology*. 2013; 57:120–130. [PubMed: 22899566]
15. Bard-Chapeau EA, Nguyen AT, Rust AG, Sayadi A, Lee P, Chua BQ, et al. Transposon mutagenesis identifies genes driving hepatocellular carcinoma in a chronic hepatitis B mouse model. *Nat Genet*. 2014; 46:24–32. [PubMed: 24316982]
16. Kodama T, Bard-Chapeau EA, Newberg JY, Kodama M, Rangel R, Yoshihara K, et al. Two-Step Forward Genetic Screen in Mice Identifies Ral GTPase-Activating Proteins as Suppressors of Hepatocellular Carcinoma. *Gastroenterology*. 2016
17. Lex A, Gehlenborg N, Strobel H, Vuilleumot R, Pfister H. UpSet: Visualization of Intersecting Sets. *IEEE Trans Vis Comput Graph*. 2014; 20:1983–1992. [PubMed: 26356912]
18. Cancer Genome Atlas Research Network. Electronic address wbe, Cancer Genome Atlas Research N. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell*. 2017; 169:1327–1341 e1323. [PubMed: 28622513]
19. Brett BT, Berquam-Vrieze KE, Nannapaneni K, Huang J, Scheetz TE, Dupuy AJ. Novel molecular and computational methods improve the accuracy of insertion site analysis in Sleeping Beauty-induced tumors. *PLoS One*. 2011; 6:e24668. [PubMed: 21931803]

20. Riordan JD, Keng VW, Tschida BR, Scheetz TE, Bell JB, Podetz-Pedersen KM, et al. Identification of rtl1, a retrotransposon-derived imprinted gene, as a novel driver of hepatocarcinogenesis. *PLoS Genet.* 2013; 9:e1003441. [PubMed: 23593033]
21. Huang S, He J, Zhang X, Bian Y, Yang L, Xie G, et al. Activation of the hedgehog pathway in human hepatocellular carcinomas. *Carcinogenesis.* 2006; 27:1334–1340. [PubMed: 16501253]
22. Sicklick JK, Li YX, Jayaraman A, Kannangai R, Qi Y, Vivekanandan P, et al. Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis. *Carcinogenesis.* 2006; 27:748–757. [PubMed: 16339184]
23. Lin M, Guo LM, Liu H, Du J, Yang J, Zhang LJ, Zhang B. Nuclear accumulation of glioma-associated oncogene 2 protein and enhanced expression of forkhead-box transcription factor M1 protein in human hepatocellular carcinoma. *Histol Histopathol.* 2010; 25:1269–1275. [PubMed: 20712011]
24. Shi C, Huang D, Lu N, Chen D, Zhang M, Yan Y, et al. Aberrantly activated Gli2-KIF20A axis is crucial for growth of hepatocellular carcinoma and predicts poor prognosis. *Oncotarget.* 2016; 7:26206–26219. [PubMed: 27036048]
25. Zhang D, Cao L, Li Y, Lu H, Yang X, Xue P. Expression of glioma-associated oncogene 2 (Gli 2) is correlated with poor prognosis in patients with hepatocellular carcinoma undergoing hepatectomy. *World J Surg Oncol.* 2013; 11:25. [PubMed: 23356443]
26. Kim Y, Yoon JW, Xiao X, Dean NM, Monia BP, Marcusson EG. Selective down-regulation of glioma-associated oncogene 2 inhibits the proliferation of hepatocellular carcinoma cells. *Cancer Res.* 2007; 67:3583–3593. [PubMed: 17440069]
27. Wangenstein KJ, Wilber A, Keng VW, He Z, Matise I, Wangenstein L, et al. A facile method for somatic, lifelong manipulation of multiple genes in the mouse liver. *Hepatology.* 2008; 47:1714–1724. [PubMed: 18435462]
28. Mukherjee S, Diaz Valencia JD, Stewman S, Metz J, Monnier S, Rath U, et al. Human Fidgetin is a microtubule severing the enzyme and minus-end depolymerase that regulates mitosis. *Cell Cycle.* 2012; 11:2359–2366. [PubMed: 22672901]
29. Hu Z, Feng J, Bo W, Wu R, Dong Z, Liu Y, et al. Fidgetin regulates cultured astrocyte migration by severing tyrosinated microtubules at the leading edge. *Mol Biol Cell.* 2016
30. Woo HG, Lee JH, Yoon JH, Kim CY, Lee HS, Jang JJ, et al. Identification of a cholangiocarcinoma-like gene expression trait in hepatocellular carcinoma. *Cancer Res.* 2010; 70:3034–3041. [PubMed: 20395200]
31. Eastmond DA. Evaluating genotoxicity data to identify a mode of action and its application in estimating cancer risk at low doses: A case study involving carbon tetrachloride. *Environ Mol Mutagen.* 2008; 49:132–141. [PubMed: 18213651]
32. Chisari FV, Klopchin K, Moriyama T, Pasquinelli C, Dunsford HA, Sell S, et al. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. *Cell.* 1989; 59:1145–1156. [PubMed: 2598264]
33. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol.* 2003; 33:105–136. [PubMed: 12708612]
34. Rogers LM, Riordan JD, Swick BL, Meyerholz DK, Dupuy AJ. Ectopic expression of Zmiz1 induces cutaneous squamous cell malignancies in a mouse model of cancer. *J Invest Dermatol.* 2013; 133:1863–1869. [PubMed: 23426136]
35. Huh CG, Factor VM, Sanchez A, Uchida K, Conner EA, Thorgeirsson SS. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc Natl Acad Sci U S A.* 2004; 101:4477–4482. [PubMed: 15070743]
36. Friedman SL. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev.* 2008; 88:125–172. [PubMed: 18195085]
37. Omenetti A, Choi S, Michelotti G, Diehl AM. Hedgehog signaling in the liver. *J Hepatol.* 2011; 54:366–373. [PubMed: 21093090]
38. Michelotti GA, Xie G, Swiderska M, Choi SS, Karaca G, Kruger L, et al. Smoothed is a master regulator of adult liver repair. *J Clin Invest.* 2013; 123:2380–2394. [PubMed: 23563311]

39. Swiderska-Syn M, Syn WK, Xie G, Kruger L, Machado MV, Karaca G, et al. Myofibroblastic cells function as progenitors to regenerate murine livers after partial hepatectomy. *Gut*. 2014; 63:1333–1344. [PubMed: 24173292]
40. Grzelak CA, Martelotto LG, Sigglekow ND, Patkunanathan B, Ajami K, Calabro SR, et al. The intrahepatic signalling niche of hedgehog is defined by primary cilia positive cells during chronic liver injury. *J Hepatol*. 2014; 60:143–151. [PubMed: 23978713]
41. Rad R, Rad L, Wang W, Strong A, Ponstingl H, Bronner IF, et al. A conditional piggyBac transposition system for genetic screening in mice identifies oncogenic networks in pancreatic cancer. *Nat Genet*. 2015; 47:47–56. [PubMed: 25485836]
42. Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome *Science*. 2015; 347:1260419.
43. Tarlow BD, Pelz C, Naugler WE, Wakefield L, Wilson EM, Finegold MJ, Grompe M. Bipotential adult liver progenitors are derived from chronically injured mature hepatocytes. *Cell Stem Cell*. 2014; 15:605–618. [PubMed: 25312494]

Abbreviations

SB	Sleeping Beauty
HCC	hepatocellular carcinoma
CCl4	carbon tetrachloride
ECM	extracellular matrix
NAFLD	non-alcoholic fatty liver disease
HBV	hepatitis B virus
HCV	hepatitis C virus
LM-PCR	ligation-mediated PCR
gCIS	gene-centric common insertion site
Hh	hedgehog
HGF	hepatocyte growth factor

Figure 1. Comparison of tumor phenotype in CCl₄-treated and control cohorts

Slight differences in tumor multiplicity were seen in the two Sleeping Beauty transposon strains used, TG12740 (**A**) and TG12775 (**B**). In both cases, CCl₄-treatment significantly increased tumor multiplicity ($p < 0.0001$).

Figure 2. Distribution of transposon-induced driver mutations in liver tumor cohorts

Three general classes of driver genes were identified: those mutated more often in the CCl₄-treated cohort (top), those mutated more often in the control cohort (middle), and those common to both cohorts (bottom). Rows indicate the mutation status of individual genes and columns represent individual tumors. A shaded box indicates the presence of a transposon-induced mutation in the corresponding gene and tumor.

Figure 3. Genetic comparison to other SB-induced HCC models

Vertical bars show the number of CCl₄-associated candidate genes overlapping with other studies. Connected black dots below indicate which studies share the candidates. Horizontal bars show the total number of candidates from each screen utilized for analysis. The inset heat map displays the relative frequency for each CIS identified in 2% of CCl₄-associated tumors across studies and indicates which SB transposon was utilized for each screen.

Figure 4. Validation of *Gli2* as an oncogene in liver cancer

Hydrodynamic gene delivery was used to stably express *Gli2* or mCherry in hepatocytes of *Fah*-null mice along with an *Fah* rescue transgene. Following selective liver regeneration by *Fah*⁺ hepatocytes, mice developed spontaneous liver tumors in a *Gli2*-dependent manner (A). CCl₄ treatment increased tumor multiplicity, indicating cooperation between *Gli2* expression and chronic liver damage. While not detected in normal liver sections (B), the liver regeneration method led to sporadic *Gli2* protein expression even in the absence of the *Gli2* transgene (C). Transgene delivery induced strong *Gli2* protein expression (D,E) associated with frequent lethality during liver regeneration. Mice with mosaic liver *Gli2* expression survived normally and developed spontaneous tumors (F).

Figure 5. Fign overexpression causes hepatocyte invasion in three-dimensional culture
TIB-73 hepatocytes embedded in Matrigel culture produce spheroids after one week (A-B). Fign overexpression alters cell morphology (C-D) and increases matrix invasion in response to HGF (E).

(A) Unsupervised clustering using expression values for the human orthologues of 24 injury-associated candidate genes defined two subtypes of human HCC (injury-associated subtypes 1 and 2). (B) Injury-associated subtypes show significant associations with molecular classes defined by a recent comprehensive analysis of human HCC (Supporting Table S4)(18). (C) A dot plot shows individual TCGA LIHC samples in each injury-associated subtype.

injury-associated subtype 1

injury-associated subtype 2

Figure 7. Proposed origin and enriched features for injury-associated HCC subtypes 1 and 2

Table

Gene	Driver status		mutation frequency(%)		FET p-value
	CCl ₄	untreated	CCl ₄	untreated	
drivers more abundant in CCl ₄ -treated or untreated cohort					
<i>Fign</i>	x		7.9	0.0	0.0158
<i>Gli2</i>	x		6.4	1.0	0.0370
<i>Met</i>	x		9.3	2.1	0.0165
<i>Hras</i>	x	x	1.2	9.3	0.0003
<i>Dend5b</i>		x	0.6	5.2	0.0068
<i>Junj1c</i>		x	0.9	4.1	0.0451
<i>Gbp1</i>		x	0.6	4.1	0.0232
<i>Insr</i>		x	0.6	4.1	0.0232
<i>Smg7</i>		x	0.3	4.1	0.0093
<i>Uqcc1</i>		x	0.3	3.1	0.0351
<i>Cacull</i>		x	0.3	3.1	0.0351
<i>Kif1b</i>		x	0.3	3.1	0.0351
<i>Memo1</i>		x	0.3	3.1	0.0351
<i>Nr5a2</i>		x	0.0	3.1	0.0105
<i>Lrppc</i>		x	0.0	3.1	0.0105
<i>Atp6v1h</i>		x	0.3	3.1	0.0351
drivers exclusive to CCl ₄ -treated cohort					
<i>Magi1</i>	x		4.4	1.0	---
<i>Piprk</i>	x		4.4	2.1	---
<i>Pard3</i>	x		4.1	3.1	---
<i>Nfia</i>	x		3.8	3.1	---
<i>Zbtb20</i>	x		3.5	3.1	---
<i>Thrb</i>	x		3.2	0.0	---
<i>Nfe2</i>	x		2.9	0.0	---
<i>Ghr</i>	x		2.9	1.0	---
<i>Rabgap1l</i>	x		2.9	1.0	---
<i>Vps13b</i>	x		2.9	2.1	---

Gene	Driver status		mutation frequency(%)		FET p-value
	CCl ₄	untreated	CCl ₄	untreated	
<i>Cul3</i>	x		2.9	1.0	---
<i>Fto</i>	x		2.6	1.0	---
<i>Vwa8</i>	x		2.6	1.0	---
<i>Gsk3b</i>	x		2.6	1.0	---
<i>Rfwd2</i>	x		2.6	1.0	---
<i>Usp34</i>	x		2.3	0.0	---
<i>Magi3</i>	x		2.3	0.0	---
<i>Pid1</i>	x		2.3	1.0	---
<i>Klf12</i>	x		2.3	0.0	---
<i>Psd3</i>	x		2.3	1.0	---
<i>Nbeal1</i>	x		2.3	2.1	---
<i>Myo1b</i>	x		2.3	2.1	---
<i>Fam35a</i>	x		2.0	0.0	---
<i>Kans11</i>	x		2.0	0.0	---
<i>Zfp148</i>	x		2.0	0.0	---
<i>Stard13</i>	x		2.0	0.0	---
<i>Gtgyf2</i>	x		2.0	0.0	---
<i>Med13l</i>	x		2.0	0.0	---
<i>Milt10</i>	x		2.0	0.0	---
<i>Suc1g2</i>	x		2.0	0.0	---
<i>Ext1</i>	x		2.0	0.0	---
<i>Snb1</i>	x		2.0	1.0	---
<i>Macf1</i>	x		2.0	0.0	---
<i>Atxn1</i>	x		2.0	1.0	---
<i>Eml4</i>	x		2.0	1.0	---
<i>Usp24</i>	x		2.0	1.0	---
<i>Rictor</i>	x		2.0	1.0	---
<i>Chchd3</i>	x		2.0	2.1	---

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Gene	Driver status		mutation frequency(%)		FET p-value
	CCl ₄	untreated	CCl ₄	untreated	
<i>Cdh2</i>	x		2.0	2.1	---
<i>Iqgap2</i>	x		2.0	2.1	---
<i>Tmem135</i>	x		2.0	2.1	---
<i>Tnrc6b</i>	x		2.0	2.1	---
<i>Mus1</i>	x		2.0	2.1	---
common drivers					
<i>Rtl1</i>	x	x	10.8	16.5	---
<i>Dpyd</i>	x	x	6.1	9.3	---
<i>Kras</i>	x	x	2.6	7.2	---
<i>Snd1</i>	x	x	3.8	6.2	---
<i>Stag2</i>	x	x	2.6	6.2	---
<i>Adk</i>	x	x	3.5	4.1	---
<i>Arid1b</i>	x	x	2.3	3.1	---
<i>Rbfox2</i>	x	x	2.3	3.1	---
<i>Trip12</i>	x	x	2.3	3.1	---
<i>Itch</i>	x	x	2.3	3.1	---
<i>Ap3b1</i>	x	x	2.0	3.1	---