The potential of a newly developed transgenic mouse liver platform in the investigation of hepatocellular carcinoma and perspectives on PARP inhibitor-based therapy

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List of abbreviations:

Afp	Alpha-fetoprotein
BRCA1/2	Breast Cancer gene 1/2
COSMIC	Catalogue of Somatic Mutations in Cancer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CDS	Coding sequence
DDX11	DEAD/H-Box Helicase 11
dsDNA	double-stranded DNA
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
FACS	Fluorescence-activated cell sorting
Fah	Fumarylacetoacetate hydrolase
FDA	Food and Drug Administration
GFP	Green fluorescent protein
Gpc3	Glypican-3
HADHA/B	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex alpha and beta subunits
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF	Hypoxia-inducible factor
HR	Homologous recombination
HU	Hydroxyurea
IHC	Immunohistochemistry
ITR	Inverted terminal repeat
KO	Knock out

NAD	Nicotinamide adenine dinucleotide
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining
NTBC	2-(2-Nitro-4-trifluoromethyl benzoyl)-1,3-cyclohexanedione
OIS	oncogene-induced senescence
PARP	Poly (ADP-ribose) polymerase
PCC	Propionyl-CoA carboxylase
RNAi	RNA-interference
Rpl27	Ribosomal protein 27
RT-qPCR	Quantitative reverse transcription PCR
SB	Sleeping Beauty transposon
shRNA	Short hairpin RNA
ssDNA	single-stranded DNA
TALEN	Transcription activator-like effector nuclease
TCGA	The Cancer Genome Atlas
TE	Transposable element
TME	tumor microenvironment
TTS	Transcriptional termination site
VEGF	vascular endothelial growth factor
WHO	World Health Organization
w/o	without
wt	wild-type

1. Introduction

1.1. Cancer as a multifactorial disease

Cancer is a multifactorial disease with high morbidity and mortality. According to the WHO's statistics, approximately 20 million new patients are diagnosed with cancer every year, and only half of them survive 5 years after the diagnosis (WHO, 2024). Cancer development is caused by the accumulation of genetic alterations. Usually, it can happen on an inherited mutational background combined with environmental factors, such as radiation, mutagenic chemical exposure, viral infection, or toxins (Y.-Y. Liu et al., 2025). Additionally, certain aspects related to lifestyle such as smoking, obesity, and long-term alcohol use can lead to chronic inflammation and provide a tumor-prone environment (Furman et al., 2019).

Cancer development is caused by genetic changes such as driver mutations, copy number variations, and epigenetic alterations (Sinkala, 2023). Driver mutations can be classified based on their role in cancer. They can occur in a tumor suppressor gene causing its loss of function phenotypes, for example, mutations make the p53 tumor suppressor inactive leading to the lack of its regulating role in cell cycle arrest and apoptosis initiating tumor formation (Rivlin et al., 2011). They can also be found in proto-oncogenes, causing their activation as oncogenes and resulting in gain of function phenotypes (E. Y. H. P. Lee & Muller, 2010). For instance, driver mutations of Ras proto-oncogenes prevent GTP hydrolysis and make them constitutively active leading to uncontrolled cell growth (Miller & Miller, 2012).

On average, 2 to 8 driver mutations are required for tumor initiation. The number of mutations can vary based on cancer type. For example, pediatric cancers originating from inherited genetic aberrations have the lowest prevalence of mutations. However, in cancer types caused by excessive mutagen exposure such as lung cancer or melanoma, hundreds of mutations can be detected (Vogelstein et al., 2013).

1.2. Hallmarks of Cancer

The paradigm of solid tumor development was described with the Armitage–Doll multi-stage model in 1954. According to the model, the subsequent accumulation of a certain number of distinct mutations is required for changing cell growth and proliferation and undergoing cell-to-malignant transformation(Armitage & Doll, 2004 - reprinted). In 2000, the multistep development of cancer was characterized by six particular features that are unlocked due to the accumulation of driver mutations what is called the Hallmarks of Cancer (Hanahan & Weinberg, 2000, Fig.1.).

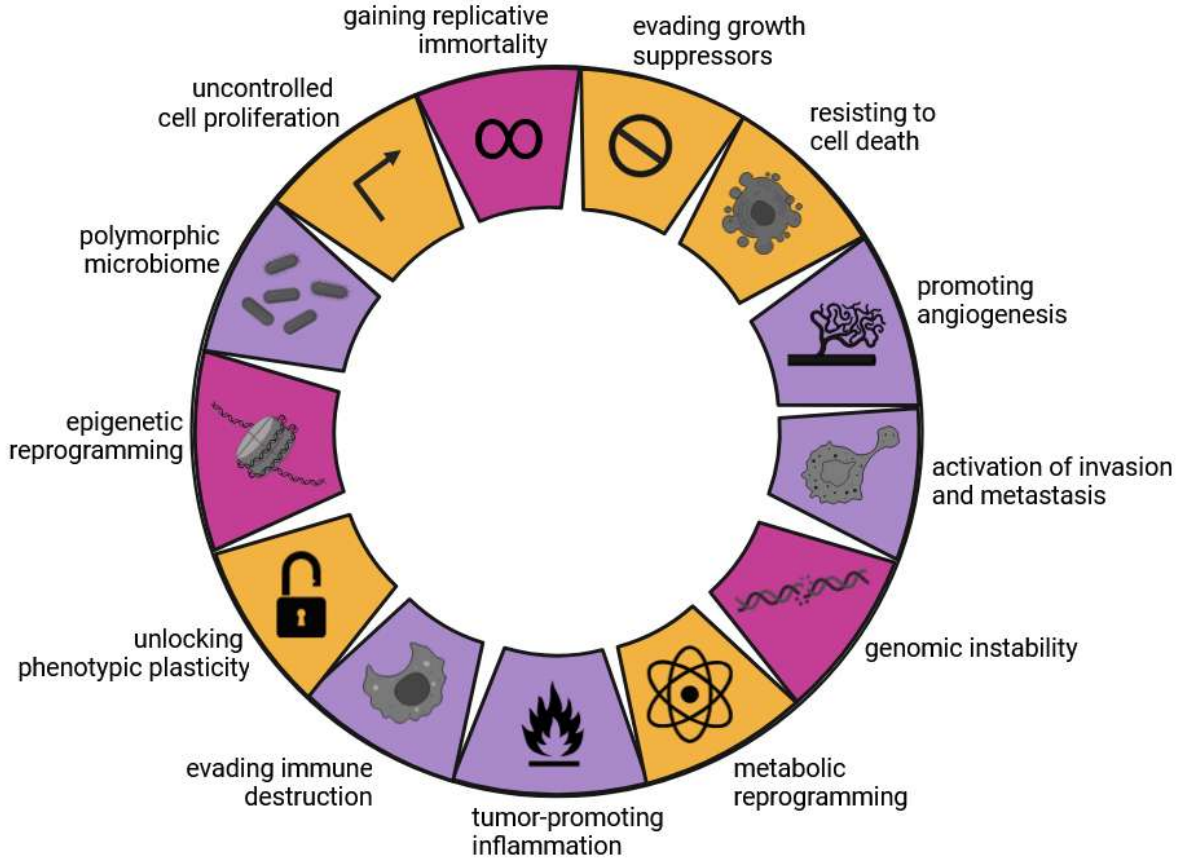


Fig.1. Hallmarks of Cancer – based on the publication Hallmarks of Cancer: New Dimensions (Hanahan, 2022)**.** Magenta background: hallmarks affect the level of chromosome, orange background: hallmarks on the level of cell homeostasis and physiology, purple background: hallmarks related to the interactions of tumor cells with surrounding tissue.

First, cells gain self-reliance in growth signals maintaining uncontrolled cell proliferation. However, telomeric DNA is shortened by rapid cell divisions and when it reaches a critically short length, it will be able to trigger senescence. Retrieving telomerase activity enables the replicative immortality of cancer cells by helping them to elongate their telomeric DNA. "Gatekeepers" are tumor suppressors that gather information about the extent of DNA damage, the level of nucleotide pool, nutrients, and growth-promoting factors and decide whether the cell can pass over cell cycle checkpoints. So evading growth suppressor gatekeepers is essential to gain the ability of perpetual progression of the cell cycle. By halting both regulators and effectors of the intrinsic apoptosis pathway, cancer cells become resistant to cell death. Reactivation of angiogenesis is a permanent characteristic of enlarging neoplastic masses to provide their nutrients and oxygen supply, remove metabolic wastes as well as spread pioneer tumor cells, and help them to metastasize. A group of advanced tumor cells can gain motility

to leave the surrounding stroma, enter the blood stream, and invade distant tissues all over the body. Tumor cells face challenges in adapting to the foreign tissue where tumor cells lack their supportive microenvironment. Thus, the formation of micrometastatic lesions is preceded by the establishment of pre-metastatic niches at distant sites of the body which can be colonized by the incoming tumor cells. Metastasis is finalized by the reconstitution of tumor microenvironment (TME) and secondary lesion formation (Hanahan & Weinberg, 2000, 2011, Fig.1.).

In 2011, the list of hallmarks was expanded with four additional traits. Genomic instability is the driving force of multistep tumor formation and is required for the collection of all the six traits enlisted above. Genomic instability is resulted by mutations in DNA damage checkpoint genes along with the ones coding DNA repair enzymes. Cancer cells usually reprogram their energy metabolism using so-called "aerobic-glycolysis". Under proper oxygen supply, normal cells utilize glucose for ATP production via oxidative phosphorylation which provides 18-fold more energy than glycolysis. However, since cancer cells rapidly grow and divide, they require a lot of pyruvate to synthesize biomolecules which will serve as building blocks of organelles. Additionally, due to the erratic regulation of angiogenesis, tumors bear with sprouting, twisted, and leaky blood vessels providing inefficient blood steam which leads to intense fluctuation between normoxia and hypoxia at the latter glycolysis is more favorable. Solid tumors are composed of a broad variety of cell types, such as cancer cells, cancer stem cells, immune cells, stromal cells, endothelial cells, and pericytes forming a complex tumor microenvironment. Cells of innate immunity always infiltrate into all types of cancer and they are recently considered to fuel a tumor-promoting inflammation. Paradoxically, while they fight against cancer and induce inflammation, they produce a sort of chemicals and signal molecules that facilitate proliferation, sustain angiogenesis, or further mutagenize tumor cells helping them to evolve faster. To evade immune destruction, cancer cells also can influence immune cells by inhibiting immune checkpoints or hiding from immune cells by decreasing antigen presentation (Hanahan & Weinberg, 2011).

By 2022, new hallmark dimensions emerged, spotlighting new aspects of tumorigenesis (Fig.1.). By unlocking phenotypic plasticity, cancer cells can avoid terminal differentiation and be capable of intense cell proliferation. Alternatively, through transdifferentiation, they can gain new traits that were originally unavailable considering the characteristics of the original lineage of differentiation. First among hallmarks, a non-mutational approach was enlisted where cancer cells can undergo epigenetic reprogramming by the tumor microenvironment. Not only healthy

human cells, but polymorphic human microbiome can also influence tumor initiation and progression versatilely. Tumor-promoting and cancer-protective species were reported which can produce mutagenic toxins or emit substances that impair the DNA repair machinery of human cells. Furthermore, tumor microbiome within immune and cancer cells can contribute to the malignant transformation, as well (Hanahan, 2022).

Although all type of solid tumors exhibits most of the hallmarks, both their order of appearance and their dominance can vary based on cancer stage, type, and microenvironmental influence (Compton, 2021; Hanahan & Weinberg, 2000). Moreover, a certain mutation can contribute to the development of multiple traits, respectively (Sinkala, 2023). The large number of driver gene combinations, the dominance of each trait depending on the stage of tumor progression as well as the huge variety of non-mutational factors highlight the importance of personalized cancer treatment.

1.3. Personalized cancer treatment

In traditional cancer treatment, chemotherapy is often combined with surgery and radiotherapy which is called multimodal treatment (Yarbro et al., 2004). Chemotherapeutic drugs are highly mutagenic cytotoxic agents that not only kill rapidly dividing cancer cells but also mutagenize the proliferating healthy ones leading to serious side effects and arising *de novo* tumor formation. Moreover, the efficiency of traditional chemotherapy highly depends on the underlying molecular causes of cancer, such as the unique combination of driver mutations(Tilsed et al., 2022). Therefore, there is an increasing need for the development of new targeted cancer therapies that can improve the effectiveness of traditional therapeutic approaches or even replace them.

Precision medicine in cancer treatment is based on the analysis of the unique genetic background of the patient and the genetic alterations in their tumor (Mateo et al., 2020). For decades, it was considered that only one drug targeting a certain pathway can be enough to selectively eliminate all the cancer cells. However, after a fast recovery with minimal side effects sharp relapse happened in many patients due to the development of drug resistant cancer cells (E. M. Kropp & Li, 2022; Mokhtari et al., 2017). The reason behind the observation was the redundancy of signaling pathways behind hallmarks and the heterogenic nature of cancer. Effective targeted therapy results in a strong selection pressure leading to an adaptive shift toward both hallmarks independent from the targeted pathway and parallel signaling pathways promoting the same hallmark. In this way, there always will be a few cells of the initial cancer

that can survive and cause the recurrence of therapy-resistant cancer (Lavi, 2015; Trusolino & Bertotti, 2012). This highlights the importance of following the principle of multimodal treatment during the application of targeted therapy, as well.

1.4. Challenges in cure of hepatocellular carcinoma

Liver cancer is the sixth most frequent type of cancer and the third most lethal one worldwide (Bray et al., 2024). Approximately 80% of primary liver cancer is classified as hepatocellular carcinoma (HCC) (Rumgay et al., 2022). There is a strong correlation between chronic hepatitis B or C (HBV or HCV) infection and subsequent HCC development making it the most common cause of HCC (El-Serag, 2012). HCC treatment lines up both traditional therapeutic approaches such as surgery followed by liver transplant or transarterial chemoembolization as well as different types of targeted cancer therapy, however, limited options are available for the cure of the advanced stage of HCC(Crissien & Frenette, 2014). Additionally, resistance against current therapy alongside poor survival within 5 years after the diagnosis raises HCC among the most pressing problems in healthcare and urges to find new therapeutic approaches that decrease relapse and increase the length of disease-free survival and patient's life quality (S. Liu et al., 2023).

1.5. Molecular basis of PARP inhibitor therapy

PARP inhibitors are emerging agents of targeted cancer therapy exploiting the genetic instability of cancer cells. PARP1 is a poly(ADP)-ribosyltransferase utilizing NAD+ to form poly(ADP)-ribose chains at the sites of DNA breaks recruiting factors involved in DNA repair (Ahel et al., 2009). In this way, PARP1 has an essential role in single-stranded DNA (ssDNA) break repair (Hanzlikova et al., 2016). However, when PARP1 is not available, ssDNA breaks accumulate and they are converted to double-stranded DNA (dsDNA) breaks which can be repaired via non-homologous end joining (NHEJ) or homologous recombination (HR). HR is the only error-free pathway which makes it essential in dsDNA break repair. When HR is compromised, the error-prone NHEJ can rescue the cells, however, it becomes insufficient alone to correct the high amount of dsDNA breaks when PARP1 activity is also missing. Neither HR deficiency nor impaired PARP1 activity is lethal alone, however, when both of them are compromised cell death is inevitable which is called synthetic lethality (Farmer et al., 2005). Interestingly, upon PARP1 inhibition, the increased PARP1 retention on the damaged DNA called PARP1-trapping is more likely to be considered the main reason of PARP inhibitor sensitivity rather than the inefficient recruitment of DNA repair factors (Murai et al., 2012).

PARP inhibitors have been used for almost a decade for the treatment of HR-deficient breast and ovarian cancer(Bondar & Karpichev, 2024). Despite their higher cancer cell specificity than classical chemotherapy, PARP inhibitors also have major side effects, such as nausea, headache, anemia, severe joint pain, and shortness of breath(Friedlander et al., 2023; LaFargue et al., 2019). Consequently, besides HR deficiency, there is a growing demand for the characterization of new synthetic lethal interactors to make the current therapy more potent and mitigate side effects by decreasing PARP inhibitor dosage. Additionally, there is increasing interest in the identification of alternative pathways where PARP1 is relevant to exploit the synergistic effect of PARP inhibition in more cancer types.

1.6. PARP inhibitors in hepatocellular carcinoma

Although PARP1 inhibitor-based synthetic lethality is usually reckoned in the treatment of breast and ovarian cancer, it could also be utilized for the cure of other cancer types, such as HCC. Although, there has been no example of applying PARP inhibitor-based synthetic lethality for the treatment of HCC in clinical practice yet, multiple promising *in vitro* and *in vivo* preclinical studies lean on the unique genetic alterations of HCC, especially the ones that cause impaired HR.

A good example of it is DDX11 DNA helicase which was shown to assist HR in HCC cells by recruiting RAD51 at the site of DNA damage. Q238H mutation of DDX11 affects the neighboring phosphorylation site which was shown to interfere with the biological function of DDX11 in facilitating the interaction between BRCA2 and RAD51 leading to compromised HR and making the tumor cells PARP1 inhibitor sensitive (Cao et al., 2024).

Not only certain genetic alterations of HCC but also non-mutational underlying conditions of the disease be exploited to induce synthetic lethality. Hepatitis B-associated HCC cells express HBx viral protein which promotes the degradation of Smc5/6 complex(Funato et al., 2022). Lack of Smc5/6 results in not just intense viral replication but also impaired HR since Smc5/6 is responsible for sister chromatid recombination(Potts et al., 2006).

Interestingly, PARP1 was shown to be upregulated in HCC which is in line with the data of TCGA where PARP1 amplification was detected in almost 8% of HCC samples compared to the adjacent liver tissue (Gerossier et al., 2021; Ossovskaya et al., 2010). Elevated PARP1 was shown to enhance hepatocyte proliferation in both normal liver as well as upon HCC development (Ju et al., 2019; Shiobara et al., 2001). Additionally, PARP1 facilitates many signaling pathways of HCC, such as ß-catenin, NF-kB, and HIF, promoting cancer cell

proliferation, tumor-prone chronic inflammation, angiogenesis, and adaptation to hypoxia, respectively (Paturel et al., 2022). Targeting multiple key pathways of HCC development at the same time hinder tumor cells to switch from one pathway to another and escape from toxicity of the treatment. In this way, PARP1 inhibitors combined with other targeted treatments can lead to synergistic cytotoxicity and reduce the risk of relapse (Zai et al., 2020).

In spite of the promising perspective of the application of PARP inhibitors in the cure of HCC, it is also important to highlight the possible downsides of these drugs. The clearance of PARP inhibitors happens via the liver. Compromised liver function leads to the accumulation of these drugs which causes liver failure. Consequently, only patients with normal liver function or with mild or moderate impairment are eligible for PARP inhibitor-based clinical trials (Rolfo et al., 2020). This excludes the vast majority of patients with advanced HCC who would need alternative combinational therapy the most. However, for patient who suffer from severe hepatic impairment, therapeutic drug monitoring alongside with the frequent investigation of signs of toxicity would be a good solution to choose the right PARPi dosage (Zhao et al., 2022).

In summary, the identification of new driver combinations of HCC alongside understanding their contribution to HCC development at the molecular level could be more helpful to extend the utility of PARP inhibitors and by exceeding preclinical models take a few steps toward the clinical applications of these drugs in the therapy of HCC.

1.7. POLE4 as a promising biomarker in cancer

Previously, we carried out a CRISPR-Cas9-based knockout screen by using Olaparib PARP inhibitor to identify new factors that can sensitize cells for PARP inhibitors when they are absent. In our screen, POLE4 was the third best candidate for being a synthetic lethal interactor of PARP1 (Juhász et al., 2020). Additionally, other screens also identified that the loss of POLE4 causes synthetic lethality in the presence of PARP inhibitors (Su et al., 2020). Thereafter, we successfully validated POLE4 as a new synthetic lethal interactor of PARP1 (Mamar, 2024), so our interest turned toward the understanding of the molecular mechanisms underlying their synthetic lethal interaction.

POLE4 is a histone-fold protein described as the part of PolE DNA polymerase complex (Li et al., 2000). PolE is a four-subunit enzyme complex composed of POLE catalytic subunit and POLE2,3 and 4 accessory subunits. PolE is responsible for leading strand synthesis during DNA replication (Cvetkovic et al., 2022). In yeast it was shown that the POLE4 homologue Dpb3 is necessary to maintain the stability of the PolE complex and increase its ability to bind template

DNA (Aksenova et al., 2010). Additionally, Dpb3 (orthologue of POLE4) and Dpb4 (orthologue of POLE3) were shown to play a crucial role in yeast chromatin inheritance which highlights the fact that the cellular role of POLE3/4 in mammalians is still poorly understood (He et al., 2017).

Among subunits of the PolE complex, the mutations of POLE were associated with a large variety of solid tumors such as endometrial cancer, colorectal cancer, lung adenocarcinoma, and melanoma (Li et al., 2019; Wang et al., 2019), while the role of genetic alterations of accessory subunits in cancer progression has been largely remained unknown. However, it was recently shown that all the PolE complex members are likely to be overexpressed in HCC which decreases both the disease-free and overall survival of cancer patients. Altogether these findings point out the biological importance of PolE complex in HCC and introduce all the subunits as new prognostic markers (Zhang et al., 2023).

1.8. The source of driver mutations

To identify genetic alterations that can drive tumorigenesis or can increase the selective toxicity of current treatments two main sources are available. New driver mutations can be identified by high-throughput sequencing performed on human tumor samples or from *in vitro* and *in vivo* screens. A large collection of genomic and transcriptomic data is available in cancer genome databases such as TCGA and COSMIC. The greatest challenge of the identification of cancer driver mutations is to distinguish them from the orders of magnitude more abundant passenger mutations accumulating via cancer progression but not giving selective growth advantage to the tumor. Consequently, driver mutations with lower frequency cannot be identified only with bioinformatical approaches based on mutational frequency analysis. Additionally, passenger mutations can also be useful in terms of the selection of the most appropriate cancer treatment as they can create the Achilles heel of tumors making them more vulnerable to therapy (Aparisi F et al., 2019). However, both the validation of driver genes facilitating tumorigenesis and the analysis of passenger mutations that can have an impact on therapeutic effectiveness require the appropriate choice of experimental systems.

1.9. Validation of driver mutations *in vitro*

For a long time, cancer cell lines have been used to investigate how driver genes are involved in cell cycle progression, proliferation, and survival. Since the widespread application of CRISPR/Cas9-based gene editing, driver mutations can be easily introduced to validate a myriad of potential cancer driver alterations which makes it possible to carry out a large number

of experiments in a short time. However, the use of cancer cell lines as well as non-tumorous immortalized cell lines also have disadvantages leading to caveats during the interpretation of experiments. During cell line establishment, cells undergo a set of stringent selection process, creating a strong bottleneck effect which makes most of the cell lines inconvenient for modeling the tumor heterogeneity (Gisselsson et al., 2019). Moreover, cell culture experiments are suitable only for showing the impact of a certain driver gene on fitness or cell division but not for deciding whether this gene is involved in tumor initiation. Additionally, adaptation of cells to the *in vitro* environment, and the subsequent culturing of commonly used laboratory cell lines for decades altogether result in the accumulation of background mutations that can influence the biological process of interest leading to artefacts or misinterpretation of the results (Hughes et al., 2007; M. Kim et al., 2017). Also studying certain hallmarks of cancer such as angiogenesis or metastasis as well as modeling interactions between cancer cells and tumor microenvironment requires a multicellular organism, pointing out the limitation of cell culture assays.

1.10. Validation of driver mutation in germline transgenic mice

Over decades, transgenic mice have been the powerful workhorses of cancer research. Originally, germline knock-in and knockout models were established to study gain of function and loss of function phenotypes in tumorigenesis (Hanahan et al., 2007). However, many cancer driver mutations are not compatible with embryogenesis leading to toxicity and making it impossible to establish the transgenic animal model of that certain genetic alteration (Suzuki et al., 1998; Yong et al., 2015). Additionally, germline transgenic mice cannot model the sporadic nature of cancer faithfully because they have already carried driver mutations in all the cells of their bodies (Kersten et al., 2017).

The era of conditional germline transgenic mice partially overcomes these problems, however, it arouses new challenges (Jonkers & Berns, 2002). Conditional germline transgenic mice are usually generated with the help of the Cre-loxP recombination system, but Dre-Rox or Flp-Frt systems also started to be favored (Friedel et al., 2011; Han et al., 2021; Zhu et al., 2023). To create conditional knock-in mouse models, a germline transgenic mouse is generated where a transcriptional termination site (TTS) flanking by loxP sites is inserted between the promoter and the oncogene carrying the mutation of interest. In the presence of recombination sites, transcription from the oncogene is blocked by TTS. Then these mice are crossed with a Cre recombinase-carrying strain wherein the expression of the enzyme is controlled by a tissue-specific or an inducible promoter. When Cre is expressed in a certain tissue, it mediates

recombination between loxP sites facilitating the expression of the oncogene. Conditional knockouts are designed based on a similar principle. In that case, loxP sites encompass certain exons of the tumor suppressor gene of interest. Upon Cre recombinase expression, these exons are recombined disrupting the gene(Lampreht Tratar et al., 2018).

The greatest advantage of this model is that it can faithfully mimic sporadic cancer development from a single cell surrounded by healthy tissue with normal cell proliferation (Kersten et al., 2017). Despite of all the benefits of conditional germline transgenesis, the major disadvantage of conditional mouse models is the long and time-consuming process of the generation of different Cre and loxP-carrying strains, as well as the final mouse model (Ruiter et al., 2018). Precise regulation of Cre expression is the key of successful conditional germline transgenesis, however, spontaneous ectopic expression of Cre is inevitable leading to the loss LoxP cassettes and mosaicism (Eckardt et al., 2004). Additionally, Cre recombinase can mediate unspecific recombination events at genomic sites similar to loxP generating germline recombination events and promoting toxicity(Kurachi et al., 2019; Schmidt et al., 2000).

These limitations can be ruled out by using somatically transgenic mouse models where the somatic cells of adult mice are genetically engineered.

1.11. Somatically transgenic mouse models

Over 90% of driver mutations occur solely in somatic cells which makes somatic transgenesis the most accurate way to mimic tumor initiation (Martincorena & Campbell, 2015). Upon somatic gene engineering, no germline cells are affected by the genetic modification which excludes toxicity issues interfering with embryogenesis. In comparison to the traditional germline transgenic mouse models, the establishment of somatically transgenic mouse models is a much faster and cost-effective way of transgenesis, resulting in the required model within one generation. However, somatic transgenesis has its own challenges which should have been considered to choose the most accurate method for our purposes. Key aspects of *in vivo* modification of somatic cells are the efficacious reach of the target organs alongside robust gene delivery. Additionally, the maintenance of long-lasting transgene expression is also the key to utilizing the model for deciphering the long-term parameters of tumorigenesis.

1.12. Molecular tools of somatic transgenesis

Driver genes carrying the mutation of our interest can be expressed transiently from plasmid DNA or in a stable manner by integrating them into the genome. Besides ectopic expression of mutated driver genes, the endogenous copy of the target gene can be directly edited by nucleases

such as Zn-finger or transcription activator-like effector nucleases (TALEN) as well as by the novel CRISPR/Cas9-based gene tailoring methods (Bedell et al., 2012; Chou et al., 2012; Xue et al., 2014). However, these approaches fail to modify multiple genes simultaneously, although tumor initiation usually requires the presence of 2-8 driver mutation (Vogelstein et al., 2013a).

Both viral vectors and DNA transposons are suitable to integrate multiple transcription cassettes into the genome of the same cell at the same time which ensures constitutive overexpression of oncogenes and procession of small non-coding RNAs that can silence tumor suppressors via RNA interference (RNAi)-based gene silencing (Ahronian & Lewis, 2014; Amy P Chiu et al., 2015).

To accomplish stable gene transfer, viral gene delivery systems such as retroviral and lentiviral vectors are considered to be the most prevailing methods due to their ability to integrate their cargo efficiently into the genome (Miller et al., 1995). Since they are able to maintain the genomic incorporation of transgenes, they can warrant long-lasting expression. Additionally, they can also be used to create a transgenic organ because they can be designed to recognize only the target cell type. In spite of these favorable features, the major drawbacks of viral vectors are the low cargo capacity (less than 8 kb) and their ability to trigger the host immune system with the presence of viral proteins which leads to unstable transgene expression.

Transposable elements (TE) are mobile genetic elements that can be classified based on the mechanism of transposition. They can be divided into class TE I called retrotransposons and TE II which are DNA transposons. DNA transposons are composed of transposase enzymes that mediate the transposition encompassed by terminal repeats. Retrotransposons drive transposition with a “copy and paste” mechanism via an RNA intermediate increasing transposon copy number by the end of the process while DNA transposons mediate transposition with a “cut and paste” mechanism where the excision of transposon is followed by its relocation(Wells & Feschotte, 2020). So only DNA transposons are suitable to utilize them as well-controllable tools of gene delivery by separating the transposase from the inverted terminal repeats surrounding the cargo DNA of interest (Munoz-Lopez & Garcia-Perez, 2010).

In the past decades, many inactive vertebrate DNA transposons such as Sleeping Beauty and Frog Prince were resurrected by reverse engineering and many insect transposons such as PiggyBac was utilized for gene delivery in mammalians(Ivics et al., 1997; Csaba Miskey et al., 2003; Ding et al., 2005). However, the use of the first active versions of these transposons was limited due to their very low transposition efficiency (Munoz-Lopez & Garcia-Perez, 2010).

The era of hyperactive transposases alongside optimized inverted terminal repeats raised transposition efficacy onto the level of viral gene delivery (Mátés et al., 2009; Yusa et al., 2011). Despite viral vectors, DNA transposons show a lot of advantages, such as higher cargo capacity, low immunogenicity, and stable transgene expression which makes them powerful tools for non-viral gene delivery(Nagy et al., 2015).

1.13. Exploiting tyrosinemia to create somatically transgenic liver

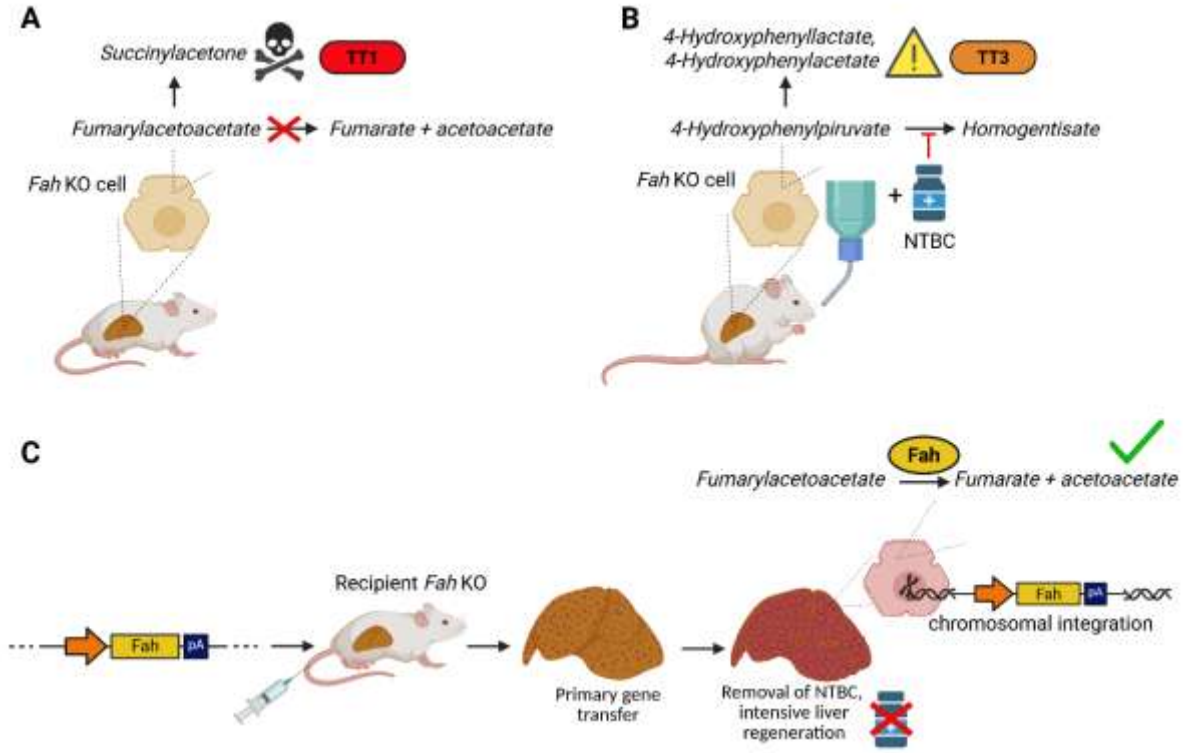


Fig.2. The Fah KO mouse model provides a basis to establish a somatically transgenic liver. A) The lack of Fah enzyme leads to the development of Tyrosinemia Type I (TT1). **B)** NTBC converts Tyrosinemia Type I to Tyrosinemia Type III (TT3). **C)** Somatic transgenesis on *Fah* KO background.

To create a somatically transgenic organ, it is important to maximize the number of modified cells as well as maintain long-term stable transgene expression. Although genomic integration partially helps to avoid transgene silencing, continuous loss of expression occurs because of heterochromatinization via CpG methylation in a long-term manner which highlights the importance of the strong selection pressure on transgene expression working against its silencing (Chevalier-Mariette et al., 2003).

The high regenerative potential of the liver eases the establishment of somatically transgenic liver models (Atta, 2010). To achieve this, metabolic diseases affecting liver function such as

tyrosinemia type I can provide a good basis. Tyrosinemia is a hereditary disease caused by the impairment of the tyrosine catabolic pathway. In tyrosinemia type I, the fumarylacetoacetate hydrolase coding *FAH* gene is mutated which leads to the accumulation of harmful metabolites such as the highly toxic succinyl acetone resulting in liver and kidney damage (Fig.2/A). Adverse effects can be avoided by the inhibition of 4-hydroxyphenylpyruvate dioxygenase with a drug nitisinone (NTBC) converting tyrosinemia type I to tyrosinemia type III(Grompe et al., 1995, Fig.2/B).

By introducing the *Fah* gene into the liver of *Fah* KO mice in a stable manner and withdrawing NTBC, the entire hepatocyte population can be replaced in 2-3 months (Lindstedt et al., 1992, Fig.2/C). If the *Fah* gene is connected to the oncogene of our interest or the RNAi element silencing the studied tumor suppressor, a high positive selection pressure can be reached which is enough to hinder transgene heterochromatinization and subsequent silencing.

1.14. Somatically transgenic mouse liver models of hepatocellular carcinoma

Over 15 years, there were examples of the establishment of HCC mouse liver models by utilizing hydrodynamic transfection of hepatocytes of *Fah*-deficient mice (Chung et al., 2016; Keng et al., 2011; Hu et al., 2016). Common features of these models are the utility of SB transposon system for transgene integration, application of RNA interference-based gene silencing with shRNA, and transgene expression was driven by strong synthetic promoters. However, all these systems show major shortcomings that obstruct their extensive use particularly to validate novel driver gene candidates having lower oncogenic potential.

In mouse liver, long-term high-level expression of shRNA was associated with toxicity and morbidity due to the oversaturation of endogenous small RNA pathways (Grimm et al., 2006). The utility of an artificial microRNA backbone can mitigate toxicity because it prevents oversaturation by elongating the maturing of small non-coding RNAs (McBride et al., 2008). Moreover, artificial microRNA processing can be guided by Pol-II which results in a safer alternative than Pol-III-based approaches (Xia et al., 2002).

Current HCC models use different transcriptional units indeed often different plasmids to introduce the oncogenes of interest and target the investigated tumor suppressors with small non-coding RNAs. This arrangement highly reduces the chance of co-expression of each transgene, however, ectopic expression of one driver gene is not enough to induce tumorigenesis (Vogelstein et al., 2013). Connected transcriptional units could ensure that all

the necessary changes of driver gene expression can happen at the same time in as many cells as it is possible to be targeted.

Upon establishment of a somatically transgenic organ, maximization of the number of cells in which chromosomal gene transfer happens is still a great challenge. Considering the development of HCC models, the validation of low-penetrance driver mutations which are the main priority of cancer research requires as many targeted hepatocytes as possible. By using hyperactive transposases and *Fah* gene replacement-induced liver regeneration in *Fah* knockout (KO) mice this limitation could be overcome.

2. Aims

In my doctoral thesis, I focused on two different aspects of cancer biology.

During ***Study 1#***, we aspired to make current PARP inhibitor therapies more potent. To achieve this, our aim was to understand the underlying molecular mechanism of synthetic lethal interaction between PARP1 and POLE4.

In ***Study 2#***, we wanted to broaden the repertoire of *in vivo* experimental systems suitable for the validation of new cancer driver genes. Therefore, we aimed to build up a transposon-based system that can be used to establish any tumor model in a single experimental animal. To connect the expression of the oncogene our interest with the artificial microRNAs silencing a certain tumor suppressor while avoiding toxic side effects, we wanted to utilize a strong endogenous bidirectional promoter.

First, we aimed to choose the most appropriate promoter which has well-balanced bidirectional activity *in vivo*. Thereafter, our aim was to utilize this bidirectional promoter to optimize RNA interference-based gene silencing in our mouse model. Lastly, we aimed to show how tumor formation can be demonstrated in our system creating $hRas^{G12V}$ overexpressing p53 silenced mice.

3. Materials and methods

3.1. Antibodies

	Host	Company	Catalog number	Dilution	Used in
anti-GFP	rabbit	Abcam	ab6556	1:4000	WB
anti-GAPDH	rabbit	ThermoFisher Scientific	MA5-15738-HRP	1:10 000	WB
anti-rabbit-HRP	goat	Sigma Aldrich	A0545	1:20 000	WB
anti-FAH	rabbit	ThermoFisher Scientific	PA5-42049	1:100	IHC
anti-glypican-3	rabbit	Abcam	ab186872	1:800	IHC
anti-Afp	rabbit	Abcam	ab46799	1:500	IHC
anti-rabbit-HRP	rabbit	DAKO	P0448	1:500	IHC
S9.6	mouse	Millipore	MABE1095	1:100	IF
anti-mouse IgG-Alexa Fluor 555	goat	Invitrogen	A21422	1:500	IF

Table 1. Antibodies are used in study 1# and study 2#

3.2. Cell culture

HeLa was cultured in DMEM (Biosera) supplemented with 10% FBS (Gibco) and 1% HyClone Penicillin-Streptomycin 100X solution and maintained at 37°C in a 5% CO_2 incubator. Our HeLa wild-type cell line underwent STR profiling (Eurofins Genomics) and the results were compared to the Cellosaurus cell line database which showed 100% identity with HeLa. *POLE4* KO cell lines were established by CRISPR/Cas9-based gene editing during this study.

3.3. Cell survival assay

$5x10^4$ POLE4 KO and wild-type cells were seeded into each well of 96-well plates. Thereafter cells were treated with HU (Sigma Aldrich) in a concentration range from 0mM to 8mM for 24h followed by washing and a one-week-long incubation in complete medium. After that, I removed the supernatant and incubated the cells in 25µg/ml resazurin dissolved in Leibowitz's L-15 (Gibco) for 30 min prior to the resorufin measurement. Resorufin fluorescence was detected by using the 530/590 filter set of Biotek Synergy H1 microplate reader.

3.4. Plasmid generation

We designed a T2 type transposon carrying the *HADHA/B* promoter, a multicloning site with NotI, BamHI, SpeI, KpnI, MluI, NheI, XhoI and PacI restriction sites at the HADHA side of the promoter, and the coding sequence mCherry linked to *Fah* coding sequence with the T2A peptide. mCherry coding sequence is discontinued by the first intron of the human eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) gene which provides intronic microRNA expression. Our newly designed transposon was synthesized by GeneScript and it was cloned into the pUC57 plasmid vector. cDNA transcribed from mouse total liver RNA was used to amplify *hRas* and G12V mutation was constructed by site-directed mutagenesis. Both GFP and *hRas* coding sequences were inserted into BamHI/PacI sites. Each microRNA was cloned with AgeI/XbaI or SacI/SalI restriction enzymes.

3.5. Animal maintenance and sample collection

Mice were housed and bred in the Central Animal House of the HUN-REN Biological Research Centre (Szeged, Hungary). Mouse studies were conducted according to the protocols of the Institutional Animal Care and Use Committee at the Biological Research Center (Szeged, Hungary) under the approval number of XVI. /801/2018. *Fah* KO mice were kept on 8mg/ml NTBC (Swedish Orphan Biovitrum) until the day of tail vein injection. Hydrodynamic tail vein injection of 6-8-week-old *Fah* KO mice were carried out by administering 50 µg of each SB transposon and 4 µg SB100X transpose coding plasmid in Ringer's solution (0.9% NaCl, 0.03% KCl, 0.016% $CaCl_2$). Mice were sacrificed at 5 weeks or 5 months after the administration of plasmid mixtures and livers were removed for stereomicroscopic imaging or sample collection. 50 mg liver tissue was homogenized in 2 ml TRI Reagent (MRC) for further RNA isolation or in 2 ml RIPA (radioimmunoprecipitation assay buffer) buffer for later immunoblotting. Whole livers were lysed in 150 ml Tris-HCl-based 0.2% SDS containing lysis buffer in the presence of 5mM EDTA for the subsequent DNA isolation and transposon copy number assessment. For later immunohistochemical staining livers were fixed in 4% paraformaldehyde overnight.

3.6. Nucleic acid isolation and quantitative PCR

RNA was isolated from liver tissue preserved in TRI (MRC) reagent according to the manufacturer's instructions. DNA content of RNA samples was removed using PerfeCTa DNase I (Quantabio) followed by cDNA synthesis with the help of RevertAid First Strand cDNA Synthesis Kit. DNA was precipitated with the classical phenol/chloroform extraction method. Triplicates of qPCR reactions were assembled by using PerfeCTa SYBR Green SuperMix (Quantabio). Both gene expression analysis and transposon copy number assessment were done by using according to the manufacturer's instructions.

		Gene expression analysis by **RT-qPCR**		Transposon copy number assessment by **qPCR**	
1X	Polymerase activation and initial denaturation	95°C	7 min	95°C	7 min
35X	Denaturation	95°C	20s	95°C	20s
	Annealing	60°C	20s	60°C	20s
	Extension	72 °C	20s	72 °C	20s
1X	Hold	4°C	∞	4°C	∞

Table 2. RT-qPCR and qPCR protocols of study 2#

3.7. Immunoblotting

Each homogenized liver tissue was sonicated for 3X10 seconds and was centrifugated with 13 000g for 15 min at 4°C. 80 µg protein of each supernatant was separated on 10% SDS-polyacrylamide gel and transferred onto 0.2 µm nitrocellulose membrane (Amersham). To verify the quality of blotting, membranes were stained with Ponceau, then they were blocked in 5% non-fat dry milk in Tris-buffered saline 0.1% Tween 20 (TBST) for 1h at room temperature (RT). This was followed by incubation with anti-GFP and anti-GAPDH for 2h at RT. HRP-conjugated anti-rabbit IgG incubation was performed for 1h at RT. All the antibodies were diluted in 5% BSA in TBST. Membranes were visualized with ECL Prime Western blotting Detection Reagent (Amersham) on ECL Hyperfilm (Amersham).

3.8. Immunohistochemistry

The formalin-fixed, paraffin-embedded median lobe of each transgenic liver was dissected into 5 µm slices and deparaffinized overnight at 56°C. Deparaffinization, rehydration, and epitope retrieval were accomplished in the PT Link machine (DAKO). Samples were incubated in anti-glypican-3 and anti-alpha 1 fetoprotein overnight or in anti-FAH for 120 minutes. Primary

antibody incubation was followed by 30 minutes-long HRP-conjugated anti-rabbit secondary antibody staining. Samples were visualized by EnVision FLEX DAB+ Chromogen System, and stained with hematoxylin-eosin for 5 minutes, followed by mounting. Slides were scanned with Panoramic Digital Slide Scanner and images were analyzed by using BIAS software.

3.9. Stereomicroscopy

After the removal of transgenic livers, two washing steps in 1X PBS were carried out to remove the remnant blood from the organs. Images of transgenic livers were taken with an Olympus SZX12 fluorescence stereozoom microscope fitted with a 100-W mercury lamp and filter sets for selective excitation and emission of GFP and mCherry.

3.10. Fluorescent Imaging

10^4 Hela wt/*POLE4* KO cells were plated onto 96-well imaging plates with fluorocarbon film bottom (MoBiTec). On the next day, Olaparib (10 μM) and hydroxyurea (2mM) treatment were carried out for 24h prior to the cells were washed with 1XPBS and fixed and permeabilized with ice-cold methanol at -20°C for 15 minutes. Then fixed cells were left untreated in 1XPBS or incubated with RNaseH (2.5 units per sample, New England Biolabs) for 2.5 hours at 37°C. Then cells were washed three times in 1XPBS and blocked with 3% BSA in 0.1% Triton-X100 for 45 min at room temperature. Incubation with S9.6 primary antibody was done overnight at 4°C followed by three times washing on the next day. Thereafter, incubation with an anti-mouse secondary antibody was performed for 1h at room temperature followed by 10-minute-long DAPI staining (1 μg/ml in PBS). Z-stacks of images were obtained on a VisiScope Spinning Disk confocal microscope with a 40X/0.6 objective lens. Imaging was carried out by using diode lasers of 405 and 561nm and an Andor Zyla 4.2 PLUS camera. After generating maximum intensity projection, Z-stacks of images were analyzed by using a custom CellProfiler pipeline.

3.11. Statistics

GraphPad Prism (version 7 and 8.4.3. for Windows, GraphPad Software) was used to perform statistical analysis and draw all the diagrams. All statistical tests were done on at least 3 biological replicates of each experiment. Figures were created by using BioRender.

4. Results

4.1. Results of Study 1#

4.1.1. PARP inhibition causes transcription-replication catastrophe due to R-loop accumulation upon the loss of POLE4

In our previous work, we showed the reduced replication fork speed upon the loss of POLE4 alongside the elevated ssDNA gaps after hydroxyurea (HU) treatment and the impairment of post-replicative repair (Mamar et al., 2024). To better explore the effect of HU-induced replicative stress, resazurin-based cell survival was performed on HeLa *POLE4* KO and wild-type cells, respectively (Fig.3/A). According to my data, the viability of *POLE4* KO cells was significantly lower compared to wild-type indicating the hypersensitivity of *POLE4* KO upon replication stress which is in line with our previous findings.

Hydroxyurea depletes the dNTP pool of cells by the inhibition of ribonucleotide reductase enzyme leading to replication fork stalling and accumulation of ssDNA gaps (Petermann et al., 2010). Transcribing RNA can hybridize with ssDNA gaps resulting in R-loop formation. Moreover, PARP1 was shown to be involved in the clearance of R-loops(Laspata et al., 2023). So, we hypothesized that the inhibition of PARP1 impairs the resolution of R-loops leading to genome instability and genotoxicity. To test our hypothesis, I performed DNA: RNA hybrid labeling with S9.6 antibody on both HeLa *POLE4* KO and wild-type cells and compared the effect of HU and PARP inhibitor treatments on the accumulation of R-loops (Fig.3/C). Both HU and PARPi treatments increased the mean nuclear intensity of S9.6 in the absence of POLE4 (Fig.3/B). To ensure that the increase of DNA: RNA hybrids in the nucleus were mostly caused by R-loop formation, I had RNaseH-treated control samples, where the mean nuclear intensity of S9.6 decreased suggesting the specificity of the S9.6 antibody for R-loops. My results indicate that the PARP inhibition caused impairment of R-loop clearance together with decreased replication fork speed leading to transcription-replication catastrophe which is the main reason for the high toxicity of PARPi treatment in *POLE4* KO cells.

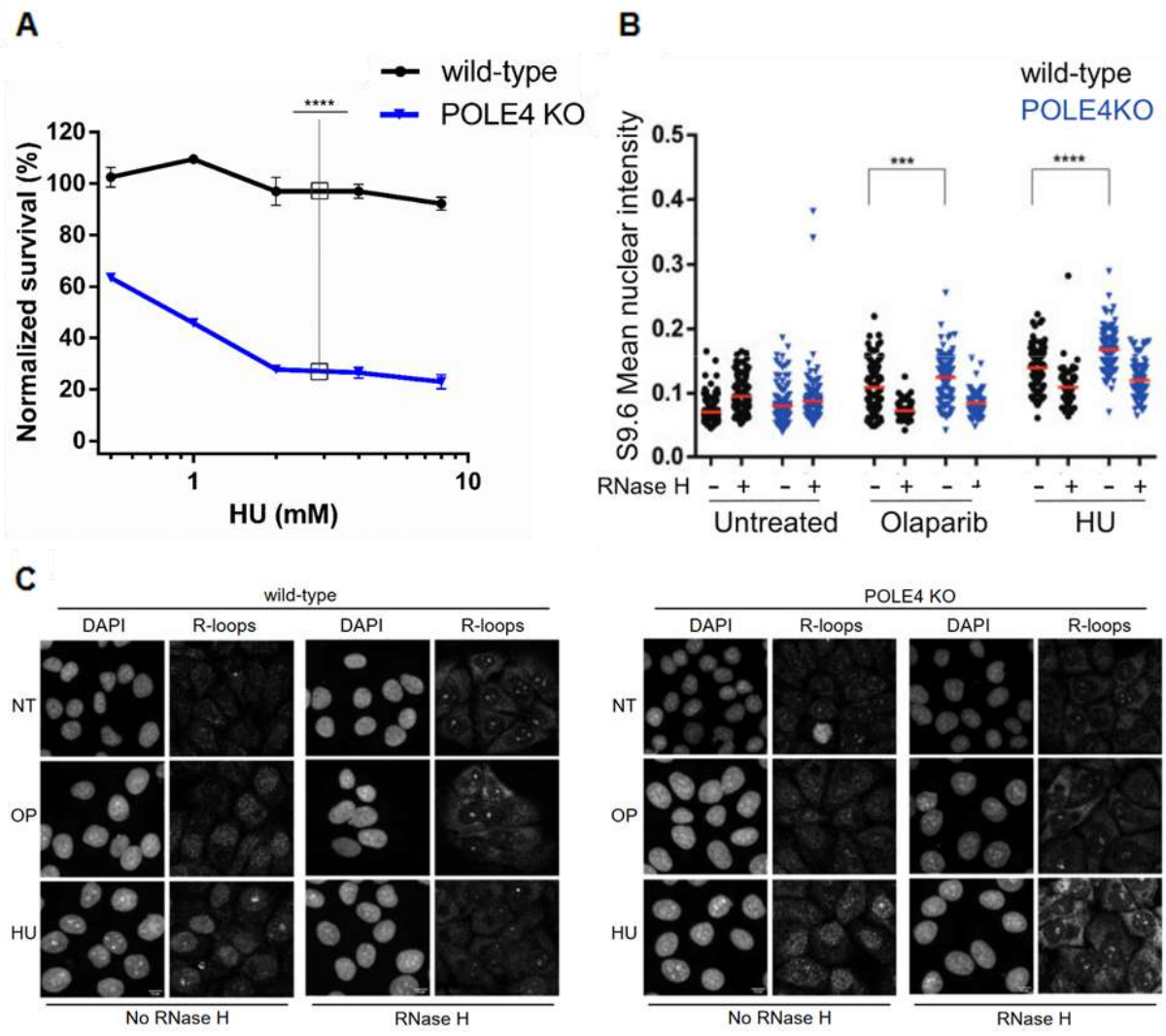


Fig.3. The loss of POLE4 leads to replication-transcription conflict upon HU or PARPi treatment. A) Resazurin-based cell survival assay to test cell viability upon HU treatment. Significance was calculated by two-way ANOVA, ****p<0.0001. **B)** Mean nuclear intensity of R-loop staining upon HU or PARPi treatment. Significance was calculated by one-way ANOVA, ***p<0.001, ****p<0.0001. **C)** Z-stacks of confocal microscopic images upon R-loop staining.

4.2. Results of Study 2#

4.2.1. Utilization of *HADHA/B* bidirectional promoter *in vivo*

To establish our *in vivo* experimental system, we had to select a well-balanced bidirectional promoter. To achieve this, we created a new Sleeping Beauty transposon vector with T2 type ITRs where *HADHA/B* promoter (which drives the expression of the human hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex alpha (HADHA) and beta (HADHB) subunits) maintains the expression of two transcriptional units in head-to-head orientation on opposite strands. In this arrangement, the A side of the promoter ensures the expression of mCherry-T2A-Fah transcript while the B side is followed by MCS to insert any oncogene of interest. In this way, Fah serves as a selectable marker gene providing a strong positive selection pressure while mCherry is a fluorescent marker enabling to monitor both Fah-based regeneration and potential tumor induction as well as possible metastasis of HCC by whole mouse imaging. Additionally, we applied the first intron of the *EEF1A1* gene to separate the mCherry CDS into two exons. This large intron is suitable for incorporating artificial pri-microRNA hairpins and allows their simultaneous processing with *HADHA* transcription. The connected A and B transcription units terminate with a bGH polyadenylation signal on both sides (Fig.4/A).

In our gene delivery platform, each transposon plasmid carrying the genetic elements of our interest can be introduced to *Fah* KO mice as follows. *Fah*-deficient mice are kept on the drug NTBC prior to the experiments to keep them alive. To deliver genetic material into their liver, each transposon is mixed with SB100x hyperactive transposase coding helper plasmid and it was diluted in Ringer's solution in a volume equivalent to 10% of mouse body weight. In this way, we can administer approximately as much Ringer's solution into the lateral tail vein of *Fah* KO mice within a few seconds which is equal to their blood volume. Consequently, due to the unique blood vessel arrangement and sinusoidal structure of the liver, mostly hepatocytes are able to uptake the delivered plasmids resulting in a highly efficient hydrodynamic transfection (Kamimura et al., 2015). After the injection, NTBC is withdrawn from mice which will result in the slow replacement of untransfected hepatocytes for the ones that got both the transposon and the transposase and have undergone chromosomal gene transfer. In this manner, within 2-3 months, liver regeneration is completed and all the hepatocytes will carry the genetic cargo of interest.

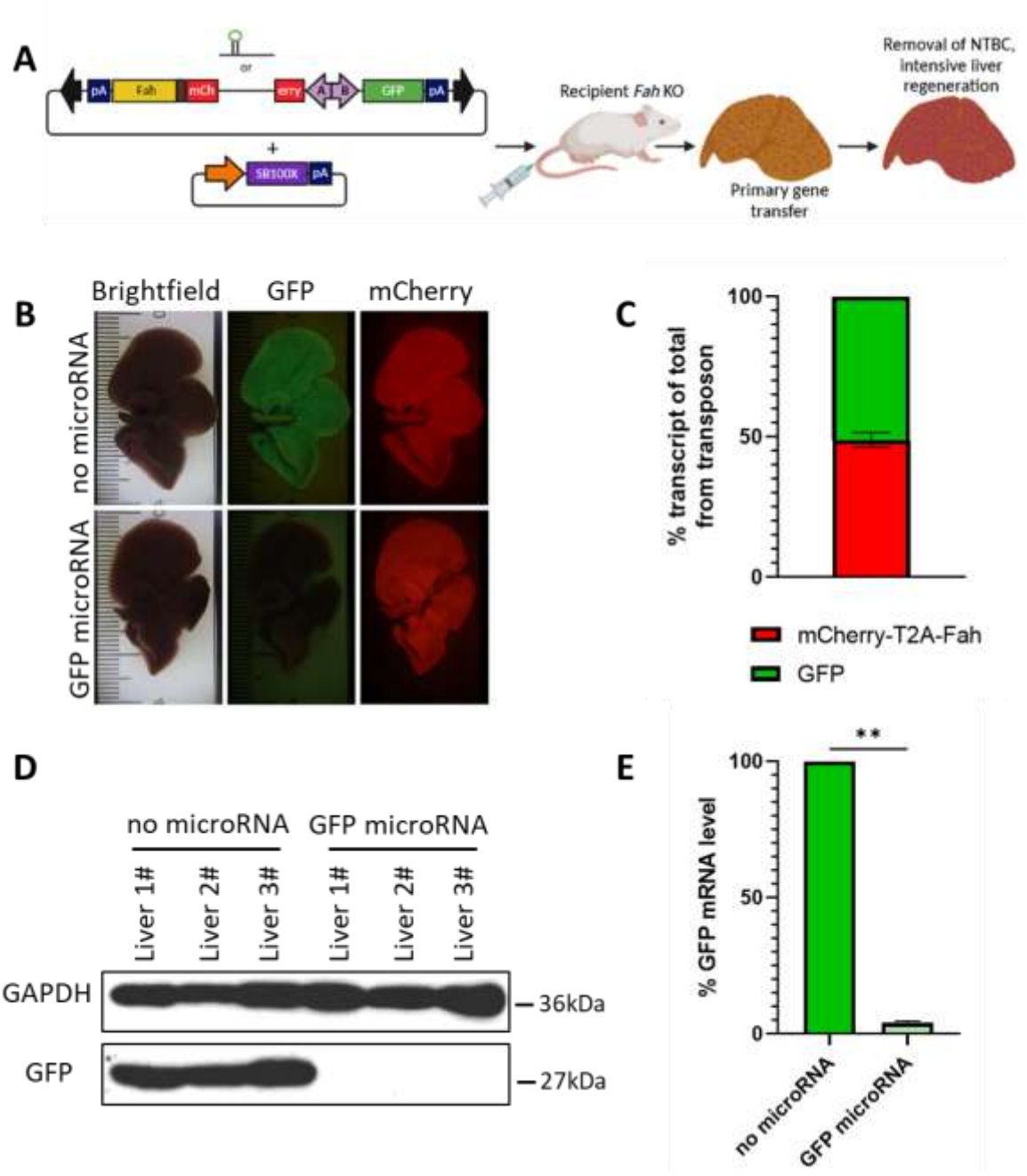


Fig.4. Optimization of somatic transgenesis in the mouse liver by creating transposon vectors carrying *HADHA/B* promoter. A) Schematic representation of *in vivo* workflow. **B)** Stereomicroscopic images of the transgenic livers. **C)** Percentages of mean relative mCherry-T2A-Fah and GFP transcript level (± SD) of total mRNA expression driven by *HADHA/B*. **D)** GFP and GAPDH protein levels of transgenic livers with and w/o GFP microRNA. **E)** Percent decrease in mean GFP mRNA level (± SD) upon using GFP microRNA. Significance was determined by Welch's t-test, **p<0.01.

The comparable expression level of the connected transcriptional units is essential to provide robust oncogene expression high enough to induce tumorigenesis and stable mCherry-T2A-Fah

expression high enough to rescue animals. To verify whether *HADHA/B* has a well-balanced activity *in vivo*, I cloned the GFP coding sequence after the B side of the promoter. Then this new transposon was introduced into *Fah* KO mice as described above (Fig.4/A). 5 months following the administration of plasmids, mice were sacrificed to investigate mCherry and GFP signals which were comparable based on the stereomicroscopic images (Fig.4/B). To get more precise information, I extracted RNA from liver tissue and determined the relative levels of both mCherry-T2A-Fah and GFP transcripts by RT-qPCR. Considering the total mRNA expression 100% from the two sides of the promoter, mCherry-T2A-Fah, and GFP mRNA percentages were given 48,9% and 51,1%, respectively (Fig.4/C). My RT-qPCR measurements clearly indicated that the *HADHA/B* promoter shows well-balanced activity *in vivo*.

To overcome toxicity caused by Pol-III-driven shRNA expression, we intended to investigate artificial pri-microRNA processing from *EEF1A1* intron in parallel with the maturation of mCherry-T2A-Fah mRNA. In this way, we anticipated that *HADHA/B* acts as a strong endogenous promoter and provides enough microRNAs to achieve robust gene silencing but not too many to oversaturate endogenous small RNA pathways. To test whether our hypothesis is true, I used my previous *HADHA/B* plasmid encoding GFP and incorporated an artificial microRNA element specific for the GFP transcript into the large intron of the mCherry-T2A-Fah. As the backbone of pri-microRNA, I utilized the human miR30a-based miR-E backbone optimized for the improvement of knockdown potency (Fellmann et al., 2013). The mixture of GFP microRNA coding plasmid and transposase coding helper plasmid was administered into the tail vein of *Fah*-deficient mice (Fig.4/A). 5 months after the injection stereomicroscopic images of the livers were taken which showed large decrease of GFP fluorescence (Fig.4/B). Thereafter, I determined both the relative GFP transcript level by RT-qPCR and GFP protein level by immunoblotting. Relative GFP mRNA levels of microRNA-carrying livers decreased by 96% compared to the ones without microRNA (Fig.4/E). The RT-qPCR data were also confirmed by the immunoblotting experiment (Fig.4/D).

In this experimental arrangement GFP expression was driven by the *HADHA/B* endogenous promoter. At the same time, artificial microRNA level against GFP was also maintained by *HADHA/B* promoter in association with transcription of mCherry-T2A Fah resulted in robust GFP knockdown *in vivo*. Consequently, these data indicate that our experimental arrangement is efficient enough to silence any tumor suppressor genes.

4.2.2. Knockdown of *p53* tumor suppressor in our somatically transgenic mouse model

In cancer research, the *p53* tumor suppressor is considered to be the main gatekeeper of tumorigenesis, whose absence contributes to the rise of all hallmarks of cancer (Levine, 1997). Although its mutations do not lead to HCC formation alone, however, in combination with other mutated driver genes *p53* facilitates the development of approximately 50% of liver cancer (Kunst et al., 2016).

So, we aimed to optimize the *in vivo* knockdown of the *p53* tumor suppressor to create a favorable environment for the investigation of other potential cancer driver alterations that in our system can be transcriptionally linked to this modification. To achieve silencing of the *p53* tumor suppressor, we designed microRNA guide sequences against *p53*, inserted them into the miR-E backbone, and incorporated either one or two different artificial *p53* pri-microRNA structures into the *EEF1A1* intron. In this way, we were able to compare the efficiency of one or two different microRNAs targeting the same gene of interest. Moreover, we could get a picture of whether toxicity would increase if we used multiple microRNA elements at the same time. To get answers, transposons in the presence of SB100X coding helper plasmid were injected into the tail vein of mice and 5 months later, animals were sacrificed to determine both the expression of mCherry-T2A-Fah and GFP transgenes and endogenous *p53* mRNA level (Fig.5/A).

We managed to significantly decrease *p53* expression with 47% upon using a single microRNA against *p53* and with 68% when we used two different *p53* pri-microRNA hairpins (Fig.5/B). The expression of transgenes from each side of the *HADHA/B* promoter showed a slight but not significant decrease by increasing the number of expressed microRNAs (Fig.5/C, D). This can be explained with a minor toxicity due to the overloaded small RNA pathways or it can be associated with the malfunction of cells upon decreased *p53* expression. Based on the transgene expression levels, we can anticipate that cells expressing transgenes at lower levels alongside less processed artificial microRNA gained a selection advantage during liver regeneration. This idea was also supported by the fact that the decreasing trend of the transposon copy number occurred in connection with the use of a higher number of pri-microRNA hairpins (Fig.5/E).

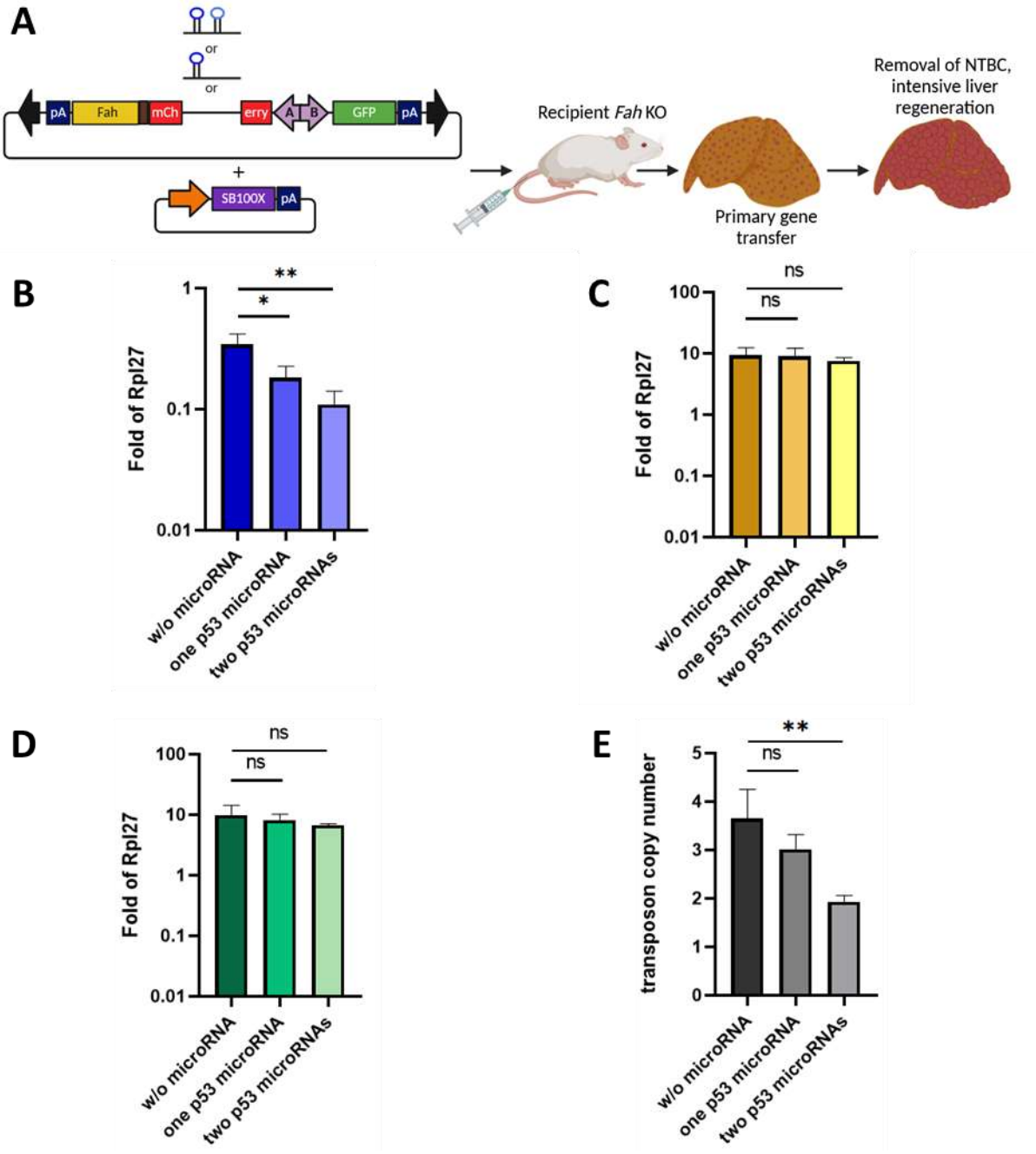


Fig.5. Silencing of p53 tumor suppressor in the mouse liver. A) Schematic representation of *in vivo* workflow. **B)** Mean of relative *p53* mRNA level (± SD). **C)** Mean of relative *Fah* mRNA level (± SD). **D)** Mean of relative GFP mRNA level (± SD). **E)** Mean transposon copy number integration (± SD). Significant change of each parameter was tested by one-way ANOVA, *p<0.05, **p<0.01, ns=not significant.

4.2.3. Creation of transgenic mouse livers overexpressing *hRas*^{G12V} oncogene

In HCC, it was recently reported that the Ras/Raf/MAPK signaling pathway is upregulated in 50% of HCC cases with the occurrence of *RAS* mutations in approximately 15% of HCC lesions

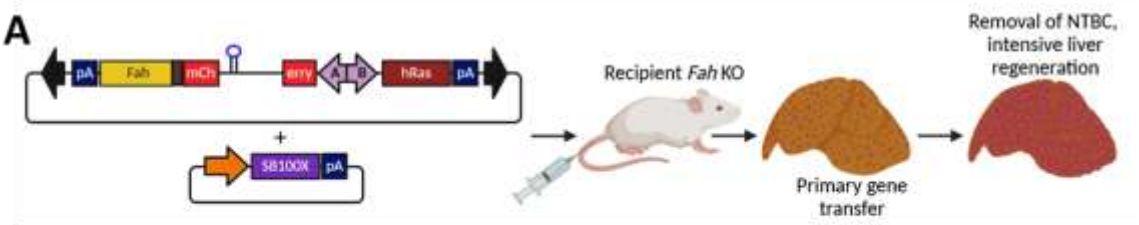
(Moon & Ro, 2021). This encouraged us to demonstrate the utility of our system for HCC establishment by creating transgenic livers overexpressing *hRas* harboring G12V mutation that results in the constitutively active state of the protein. Constitutively active *hRas* fuels cell cycle progression so strongly as to cause cellular senescence(Serrano et al., 1997). So, we invoked our well-designed artificial microRNA against *p53* to evade senescence and promote tumor cell growth by silencing *p53* and overcoming the G1/S cell cycle checkpoint.

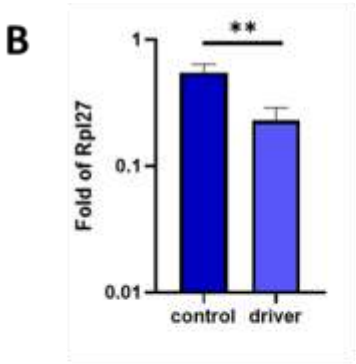
To achieve our goal, I created $hRas^{G12V}$ with site-directed mutagenesis and incorporated it into our newly designed transposon vectors already carrying pri-microRNA hairpin against *p53* in the large intron of mCherry-T2A-Fah. As a negative control, I always used transposon carrying neither *p53* microRNA nor $hRas^{G12V}$ but expressing GFP instead (Fig.6/A). 5 weeks after the injection of the transposon coding the mutant *hRas* CDS into the tail vein of *Fah* KO mice, animals had to be sacrificed because the thousands of uncontrollable multiplying hepatocytes proved to be incompatible with life. This meant that we also had to collect livers of control animals 5 weeks after transposon introduction.

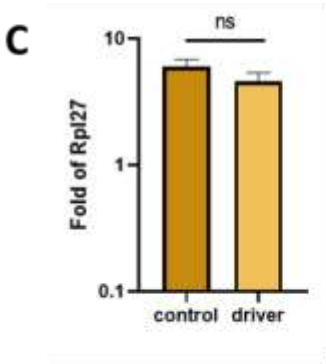
To ensure that our system worked well methodologically, I verified the gene expression modifications of our driver genes of interest by RT-qPCR. I was able to detect a 58% decrease in endogenous *p53* level in *p53* silenced $hRas^{G12V}$ overexpressing livers (Fig.6/B). Moreover, a slight but not significant decrease of Fah expression was detected in livers carrying the driver construct (Fig.6/C), while the $hRas^{G12V}$ transcript level provided by the B side of *HADHA/B* promoter was significantly reduced compared to the GFP level indicating the adverse effects of the upregulated Ras/Raf/MAPK pathway (Fig.6/D). The fact that we were able to express $hRas^{G12V}$ so efficiently is the result of that the strong positive selection on Fah expression was to some extent able to counterbalance the negative selection on expression of the $hRas^{G12V}$ which points out the fact that the two transcriptional units are compulsorily connected by *HADHA/B.*

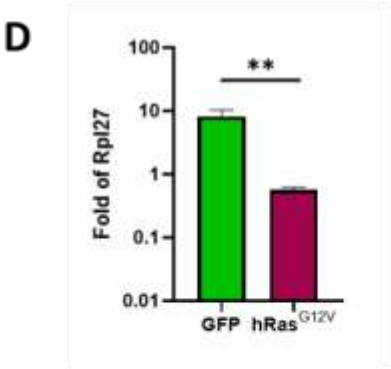
Although we had to collect livers only 5 weeks after transposons were administered, we attempted to demonstrate the onset of tumorigenesis. So first we determined the percentage of Fah-positive hepatocytes 5 weeks after treatment by a machine learning-based measurement to assess the relative proliferation rate in livers treated with the driver construct as compared to controls.

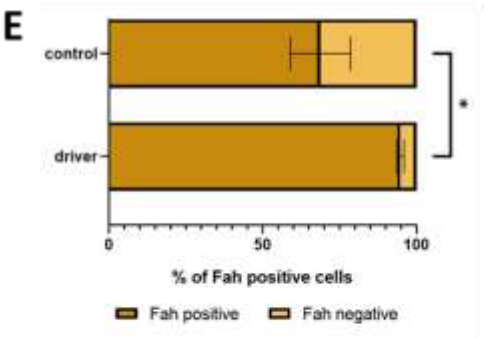
Based on the quantification of Fah immunostaining, the number of Fah-positive cells on a single field was significantly higher in *hRas*^{G12V} overexpressing animals than in the GFP overexpressing ones (Fig.6/E).











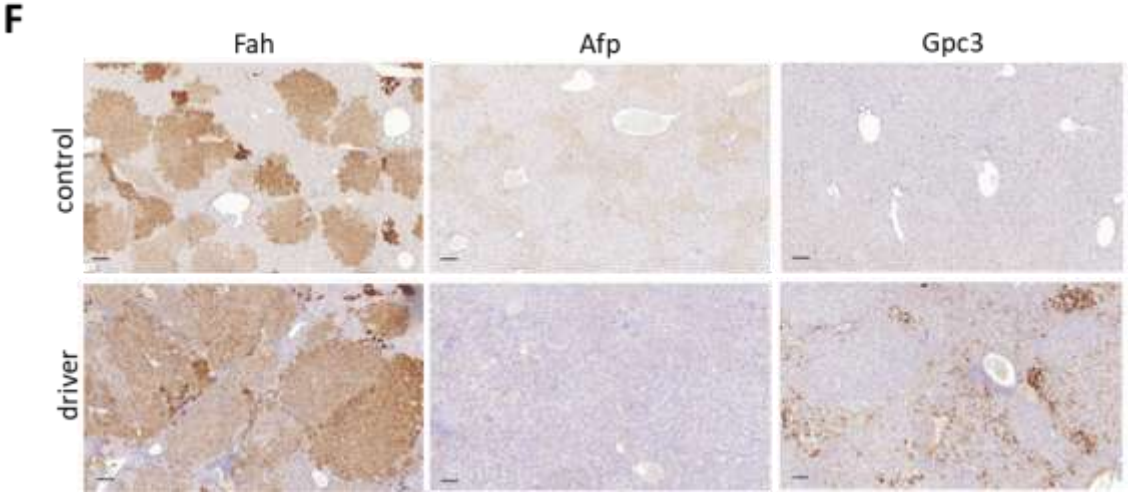


Fig.6. Overexpression of *hRas*^{G12V} oncogene in the mouse liver. **A)** Schematic representation of *in vivo* workflow. **B)** Mean of relative *p53* mRNA level (± SD). Significance was determined by Welch's t-test, **p<0.01. **C)** Mean of relative *Fah* mRNA level (± SD). Statistical significance was investigated by Welch's t-test, ns= not significant. **D)** Mean of relative GFP mRNA and *hRas*^{G12V} levels (± SD). Significance was determined by Welch's t-test, **p<0.01. **E)** Mean percentage of Fah positive hepatocytes (± SD) of control and driver transposon carrying livers 5 weeks after the injection. Welch's t-test was obtained to determine the statistical significance, *p<0.05. **F)** Fah, Afp and Gpc3 immunohistochemical staining of livers 5 weeks following the injection.

We were also looking for the shaping of the HCC-associated gene expression changes indicated by the elevated alpha-fetoprotein (Afp) level alongside increased glypican-3 (Gpc3) (Fig.6/F) (Guo et al., 2020; Lu et al., 2024). Repopulating hepatocytes did not show any Afp positivity in early HCC livers, however, we found *Fah* KO non-repopulated hepatocyte island positive for Afp in both early HCC and control livers. Afp is known to be not specific for the malignant transformation of hepatocytes but can be produced by hepatocytes upon certain noncancerous liver conditions such as tyrosinemia, too. Uponn nonpathogenic conditions, Gpc3 is crucial for fetal liver development(Guo et al., 2020). However, it is re-expressed early during the malignant transformation of the liver. Early HCC were proven to be Gpc3 positive in our experiments, while control livers showed no signs of Gpc3 re-expression. Altogether these findings are in line with our expectations because Gpc3 is a more specific marker of liver cancer elevated early in HCC while Afp detection has limitations in early-stage HCC (Tsuchiya, 2015).

4.2.4. Modelling long-term parameters of HCC development

To show liver tumor formation in an otherwise nontumorous cellular environment, we mixed driver construct harboring *hRas*^{G12V} and *p53* microRNA with a therapeutic construct promoting only liver regeneration but not tumor formation (Fig.7/A). 5 months following the injection, hypervascularized advanced liver tumors were formed (Fig.7/B). On hematoxylin-eosin staining of nodules (Fig.7/C), multiple histopathological features of HCC were detected such as lipid accumulation within the tumor cells (Currie et al., 2013). Additionally, abnormally segregating cells were also observed. Moreover, cancer cells had already penetrated nearby normal liver tissue indicating incipient metastasis. Immunohistochemical staining of liver tumors showed a high level of Gpc3 alongside intense expression of Afp in comparison with control repopulated livers indicating advanced HCC formation (Fig.7/D).

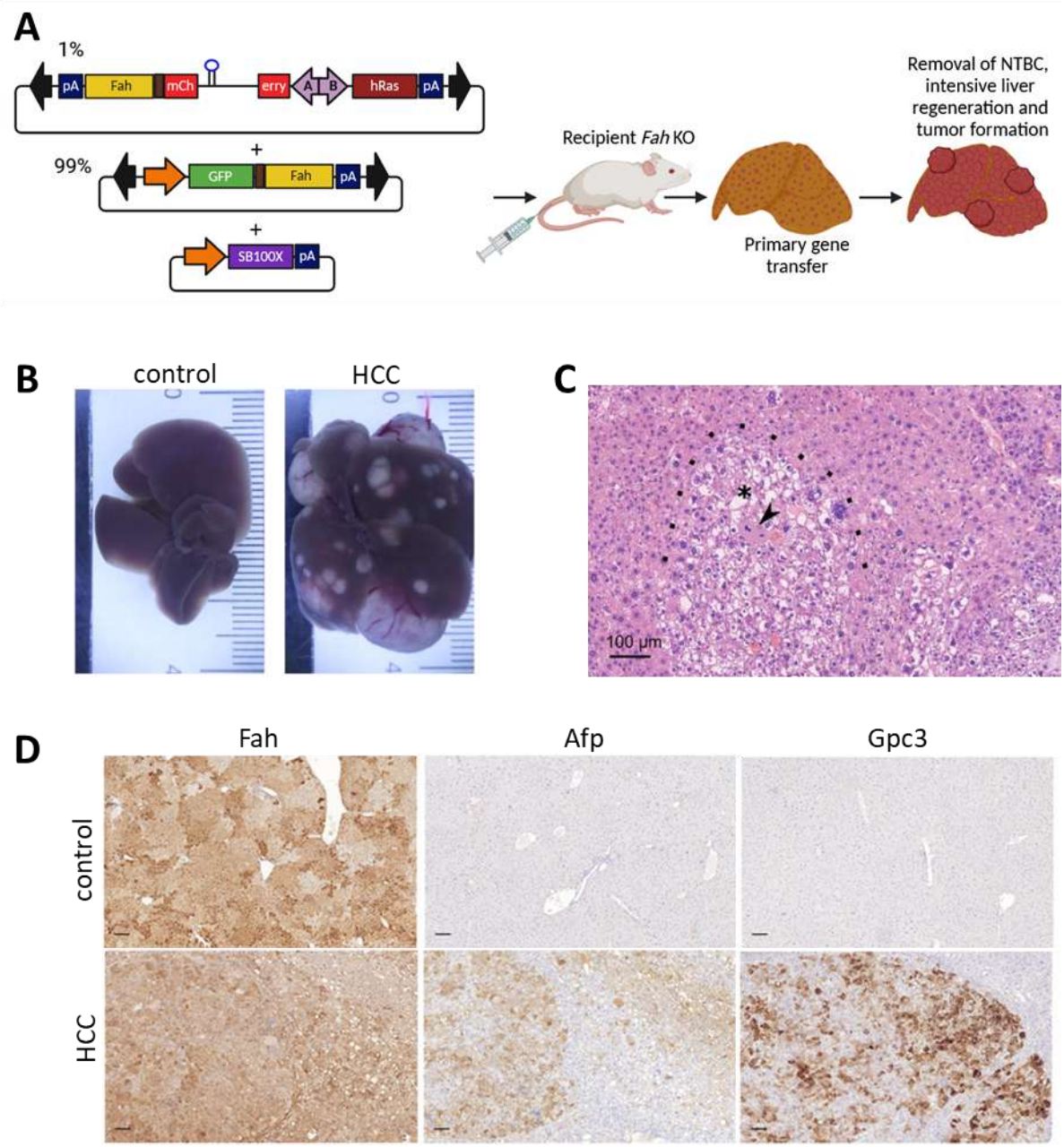


Fig.7. Development of advanced liver cancer. A) Schematic representation of *in vivo* workflow. **B)** Stereomicroscopic images of the transgenic livers. **C)** Hematoxylin-eosin staining of HCC. Asterix, lipid accumulation; arrowhead, abnormally segregating cells; dotted line, cancer cells penetrating into healthy liver tissue. **D)** Fah, Afp and Gpc3 immunohistochemical staining of livers 5 months following the injection.

5. Discussion

In study 2#, I successfully proved the well-balanced bidirectional activity of the *HADHA/B* promoter by utilizing this promoter to express mCherry-T2A-Fah and GFP mRNAs in a 1:1.05 ratio (Fig.4/C). Although viral and viral-based artificial promoters drive robust gene expression, the use of these promoters also has drawbacks such as toxicity caused by abnormally high expression level or the high likelihood of promoter methylation resulting in the sharp decrease of transgene expression or the shutting down of the transcription cassette (K. A. Kropp et al., 2014; Zhou et al., 2014). By using an endogenous promoter, potential toxicity can be avoided and we experienced stable transgene expression without any sign of mosaicism.

Such a versatile biotechnology tool can be valuable not just in cancer research but also in gene therapy where co-expression of therapeutic proteins is needed. To the present day, there were several attempts to fulfill this need by constructing many artificial bidirectional promoters. However, artificially designed and built promoters have high limitations. During the design of these elements, it is hard to estimate how close the opposite core promoters or core promoters and upstream enhancers should be placed (Amendola et al., 2005). Subsequently, due to their relatively large size, they do not leave enough room for coding sequences in lentiviral vectors where cargo size is critical (B. Liu et al., 2008). Additionally, artificial bidirectional promoters often carrying viral elements such as CMV enhancer leading to epigenetic silencing because they provide too robust gene expression and trigger the host's antiviral response (Golding & Mann, 2011; Harms & Splitter, 1995). All these drawbacks together point out the necessity of the compact and reliable endogenous bidirectional promoters. As *HADHA/B* is a liver-specific promoter, so it can be used for liver gene therapy approaches where the balanced co-epression of two protein coding gene is required. A good example for that is the gene therapy of propionic acidemia, a genetic metabolic disorder, when due the mutations of propionyl-CoA carboxylase (PCC), a mitochondrial enzyme composed of alpha (PCCA) and beta (PCCB) subunits, the conversion of propionyl-CoA is impaired leading to the accumulation of this metabolite alongside with life-threatening cardiac disease and stroke (Marchuk et al., 2023). A recent preclinical study showed that the PCC enzyme activity in mRNA-based gene therapeutic approach was the most efficient when PCCA:PCCB mRNA ratio was 1:1 which is highly supported by the data of the first clinical trial phase 1/2 (Jiang et al., 2020; Koeberl et al., 2024). In the therapy of propionic acidemia, our *HADHA/B* promoter could be game-changer due to its compact size, well-balanced nature and mitochondrial origin.

Herein to evade toxicity issues of U6-promoter-driven shRNA-based gene silencing which has been widely applied so far to downregulate gene expression *in vivo*, we successfully optimized artificial microRNA-based gene silencing avoiding major signs of liver toxicity. Upon optimization, it was crucial to achieve the right amount of microRNA bearing transcripts that upon maturing gets loaded into the RNA-induced silencing complex (RISC) for efficient gene silencing but is not causing unwanted side effects due to oversaturation of small RNA pathways. So, in our animal model, we used intronic microRNA expression wherein microRNA can be processed in parallel with transgene expression without hindering its translation.

HADHA and HADHB enzymes are responsible for catalyzing the last steps of beta-oxidation of long-chain fatty acids (Houten et al., 2016), which is an important metabolic pathway of hepatocytes, ensuring the the *HADHA/B* promoter serves as a strong endogenous promoter in hepatocytes. My RT-qPCR measurements showed that the *HADHA/B* promoter-driven expression of mCherry-T2A-Fah and GFP was approximately 9 times higher than the Rpl27 housekeeping gene (Fig.5/C and D). This expression level is high enough to ensure efficient knockdown *in vivo*, but not too high to burden small RNA pathways, as in the case of Pol-III-mediated expression driven by the U6 promoter.

Our experiments show an apparent alteration in the efficiency of microRNAs silencing GFP (96% knockdown) (Fig.4/E) versus *p53* (47% knockdown with a single microRNA) (Fig.5/B). The reason behind this difference can be explained by the cell population affected by the transgenesis. Our somatical transgenesis affects mostly hepatocytes which give on half of actual liver cells. Subsequently, GFP singal is produced by only transgenic hepatocytes, and it can be effectively silenced by GFP microRNA also processed by the same transgenic hepatocytes in parallel with GFP expression. In contrast, *p53* microRNA processed by only transgenic hepatocytes, however, all cell types in the liver expresses *p53* and I performed my RT-qPCR on total liver extracts. Thereafter, correction with the ratio of hepatocytes among all cell types found in the liver, rises the *p53* microRNA efficiency to the efficiency level of the GFP microRNA, proving the consistency of our system. Despite the efficient knockdown, we did not observe any signs of tumor formation in the transgenic livers which is in line with the published phenotype of *p53* KO mice. Heterozygous *p53* KO animals developed only a few lymphoid and testis tumors by 9 months of age, whereas approximately 75% of homozygous KO mice exhibited multiple tumor types already by 6 months. The most frequently observed tumors were malignant lymphomas, but no liver tumors were observed at all. (Donehower et al., 1992). This also implies that SB transposon-mediated random transgene integration has no

or very low oncogenic side effects, meaning that it is not sufficiently tumorigenic by itself to ruin our test system even upon silencing *p53* tumorsuppressor.

When a certain oncogene and the small non-coding RNA targeting the tumor suppressor of interest are expressed from different transcriptional units, in addition, from different plasmids, co-modification of all driver genes of interest cannot be warranted. In this way, the number of potential tumor initiation cores is much lower than the total number of targeted cells. Before the application of artificial microRNAs having Pol-II-mediated expression, Pol-III-mediated shRNA expression was used to silence tumor suppressors (Bric et al., 2009; Kleinhammer et al., 2011). However, Pol-II and Pol-III RNA polymerases act on different promoters (Barba-Aliaga et al., 2021), which was the major obstacle of the construction of connected transcriptional cassettes ensuring both the oncogene expression and small non-coding RNA production. In our transgenic animal model, the efficient intronic artificial microRNA-based gene silencing overcomes the aforementioned challenges. By utilizing the *HADHA/B* promoter to create bidirectional transcriptional units we managed to co-modify the expression of all driver genes of interest in all the targeted cells turning them into potential tumor initiation cores. Additionally, we applied the SB100x hyperactive transposase to maximize the number of targeted cells. Altogether, in our transgenic mouse liver model, cancer initiation can happen from orders of magnitude more cells than in any other model which was used before raising this system among the most accurate ones to validate the oncogenic potential of low-penetrance driver mutations.

Oncogene overexpression in parallel with tumor suppressor deactivation results in the transformation of hepatocytes to neoplastic cells if sufficient functional synergy exists between the given drivers. After neoplastic transformation starts, the immune system attempts to eliminate neoplastic cells. However, in the absence of NTBC, the survival of a certain number of Fah positive hepatocytes is crucial to regenerate the liver and secure the survival of the animals. Since Fah expression is connected to the expression of driver genes in all the cells, a significant portion of transformed hepatocytes should survive which will maintain neoplastic tissue formation and subsequent malignant progression.

We established a mutant *hRas* overexpressing *p53* silenced tumor model because both Ras oncogene activation and p53 deregulation contribute to the emergence of numerous hallmarks of cancer so this driver combination is suitable to show how each characteristic of HCC appears upon its development. The upregulation of the Ras/Raf/MAPK pathway is among the most common driving forces of the proliferation of HCC cells that we achieved by the overexpression

of mutant *hRas*^{G12V} (Moon & Ro, 2021). We successfully proved the emergence of the hallmark of uncontrolled cell proliferation, by detecting a significantly higher amount of Fah positive cells in the presence of the hRas^{G12V} mutant isoform (Fig.6/E). Intense cell proliferation acts as a genotoxic stress resulting in the activation of tumor suppressor pathways leading to a response called oncogene-induced senescence (OIS) (X. Liu et al., 2018). However, OIS can be overcome with the overexpression of a cooperating oncogene or the inactivation of a tumor suppressor (Hydbring et al., 2010; Serrano et al., 1997). We successfully silenced p53 which is the main coordinator of cell cycle arrest to evade OIS. Avoiding OIS provided the basis for functional synergy between the two applied drivers. Moreover, the downregulation of p53 made tumor cells resistant to cell death and led to advanced tumor formation by 5 months after the injection in mixed driver and therapeutic gene delivery experiments (Fig.7). This is in line with the fact that the lack of p53 also obstructs the activation of the extrinsic pathway of apoptosis and halts the intrinsic one, as well as the immune checkpoints altogether helping the tumor avoiding death (Cortez et al., 2016; Müller et al., 1998; Nakano & Vousden, 2001).

Both the constitutive activation of hRas and the loss of p53 function lead to the upregulation of VEGF and promote angiogenesis(Arbiser et al., 1997; Dameron et al., 1994),which was demonstrated by the appearance of sprouted fragile blood vessels on the tumors (Fig.7/B). Poor vascularization of the induced tumors caused hypoxia leading to high extent of lipid accumulation in our experiment which is in line with our current knowledge of main HCC features (Chen et al., 2019, Fig.7/C). Hematoxylin-eosin staining of the advanced tumors showed tumor cells with abnormal chromosome segregation which were the clear signs of the emergence of genomic instability hallmark (Fig.7/C).

Moreover, the staining showed cancer cells entering the healthy liver tissue which is the clear proof of the ability for metastasis (Fig.7/C). This is concordant with our current knowledge about *Ras* and *p53* driver genes because they can contribute to the epithelial-to-mesenchymal transition which makes tumor cells enable to migrate and invade and metastasize (Zhang et al., 2017).

Newly developed anti-cancer drugs usually undergo *in vitro* screens, before being tested on mouse and large animal models. However, classical 2D cell culture experiments fail to mimic the complexity of a human tumors and their interactions with TME, as well as probable side effects(van Rijt et al., 2023). Considering all the advantages enlisted above, our animal model would be suitable for testing the *in vivo* effectiveness of certain drug candidates on tumors with a different genetic background as well as in combination with other anti-cancer agents.

Historically, PARP inhibitors were associated with cancer therapy in connection with the synthetic lethal interaction of BRCA1/2 and PARP1 (J. Lee et al., 2014). In 2014, PARP inhibitor Olaparib was approved by the FDA and it can be used in the treatment of BRCA-mutant ovarian, breast, prostate, and pancreatic cancer. Additionally, three other FDA-approved PARPi, Rucaparib, Talazoparib, and Niraparib are also available as therapeutic options in the treatment of ovarian, breast, and prostate cancer. Notably, Niraparib is accepted to be used in any HR-deficient ovarian cancer regardless of its BRCA status (Rose et al., 2020).

It was recently described that more than 80% of HCC patients have HR-deficient tumors (H. Kim et al., 2022). Moreover, HBx viral protein causes impaired HR and is expressed in four HCC cases out of five because HBV infection is the most common underlying cause of HCC development (Rumgay et al., 2022). In this way, there is a growing demand to test the usefulness of synthetic lethality-based PARP1 therapy in the treatment of HCC carrying various driver combinations or carrying HBx viral protein (Fig.8/A). Besides the co-expression of an oncogene and silencing of a tumor suppressor, our experimental setup ensures the silencing of any gene causing defective HR. A good example can be the overexpression of *n-myc* and silencing of *Arid1a* (Fig.8/B), as both of them cause strong BRCAness phenotype but with a different underlying mechanism (Colicchia et al., 2017; Shen et al., 2015). The presence of mCherry makes it possible to follow both the appearance of tumors and their shrinking due to PARPi in a non-invasive way. Additionally, the overexpression of certain oncogenes and non-driver biomarkers also have a synergistic lethal effect in combination with PARP1 inhibitors (Carey et al., 2018; Zai et al., 2020). Their interaction with PARPi can be investigated in HCC generated by the silencing of two tumor suppressor genes. Alternatively, by redesigning our platform and replacing the mCherry fluorescent marker gene with the potential synergistic lethal interactor, its sensitizing effect for PARPi treatment also can be tested. HR-deficient PARP inhibitor therapy mostly affects mitotically active cells, because homologous recombination is the repair pathway of dividing cells, so it is mostly active in S/G2 phase when homologue template is available (Helleday, 2011; Mathiasen & Lisby, 2014). The limitation of this experimental arrangement is that all the cells carry the modification sensitizing them to PARPi therapy. However, by choosing the therapeutic window carefully after the liver regeneration when hepatocytes have already gone quiescent phase and not been dividing anymore, we could have the chance to specifically target only the rapidly proliferating cancer cells.

In study 1#, I took a significant step in understanding the molecular role of POLE4 in PARP inhibitor sensitivity. My data indicate that both PARP1 and POLE4 have a role in resolving R-loops, but on different pathways what underlies PARPi sensitivity when both of them are missing (Fig.3). This raises POLE4 among the promising targets of PARP inhibitor-based cancer therapy. Previously, POLE4 was described as a potential biomarker in HCC (Zhang et al., 2023). Similar to the exploitation of synthetic lethality in association with HR deficiency, our experimental system can be suitable for better exploring the role of POLE4 *in vivo* with particular interest in HCC development and its impact on PARPi therapeutic outcome in the future (Fig.8/C). By using many different driver combinations in parallel to induce HCC and test PARPi-based therapy of these tumors, we could have a powerful experimental system to investigate the underlying molecular mechanism of PARPi sensitivity/resistance in HCC on various genetic background (Fig.8/D, E).

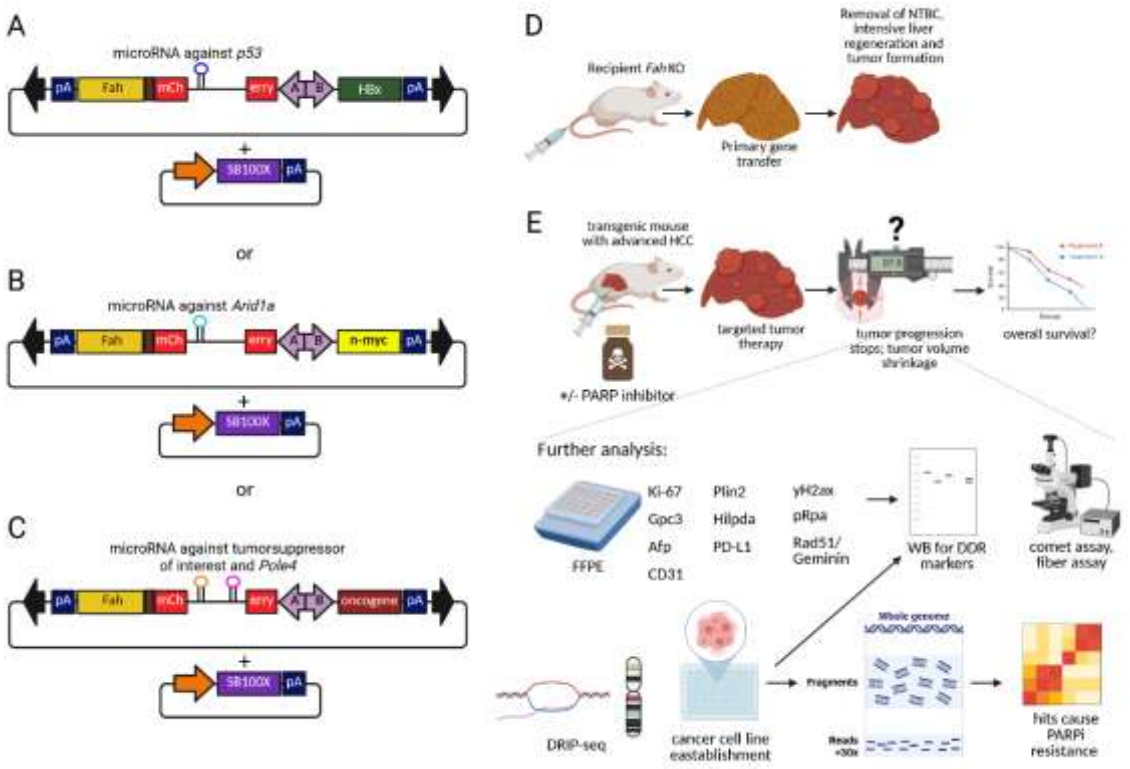


Fig.8. The potential of our transgenic mouse liver platform to study the underlying molecular mechanism of PARP inhibitor-based therapy in HCC treatment. A) Construction of a HBx viral protein overexpressing and *p53* silencing SB transposon. **B)** Construction of a *n-myc* oncogene overexpressing and *Arid1a* silencing transposon. **C)** Construction of a transposon plasmid which is overexpressing the oncogene of our interest and silencing both the tumorsuppressor of our interest and *Pole4* biomarker and potential sensitizer

for PARPi. **D)** Hydrodynamic injection of each driver contruct followed by NTBC withdrawel, intensive liver regeneration and mature tumor formation. **E)** PARPi treatment of mice with HCC and followed by monitoring of overall survival, tumor shrinkage, as well as, analysis of the underlying molecular mechanism of PARPi sensitivity/resistance in HCC.

6. Summary of new findings

- We described the role of R-loop formation in Olaparib sensitivity in the absence of POLE4.

- We successfully characterized the well-balanced bidirectional activity of *HADHA/B* promoter *in vivo.*

- We applied the HADHA/B promoter in a novel *in vivo* somatic transgenesis context.

- By using intronic artificial microRNA expression from *EEF1A1* intron during *in vivo* somatic transgenesis, we solved the widely reported toxicity issues of *in vivo* gene silencing and have turned it into a realistic technical approach in the mouse liver.

- We showed the utility of our transgenesis system in cancer research; providing an alternative high-throughput platform for validation of cancer driver mutations and for the fast and efficient establishment of multiple tumor models in parallel.

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