DEVELOPMENT OF PRE-CLINICAL MURINE MODELS FOR FIBROLAMELLAR HEPATOCELLULAR CARCINOMA

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Keywords: fibrolamellar carcinoma, liver, cancer, PKA, organoids, mouse models

ABSTRACT

Fibrolamellar hepatocellular carcinoma (FLC) is a rare form of cancer that affects primarily adolescents and young adults. FLC tumors are typically associated with an intrachromosomal deletion resulting in expression of a fusion protein between the chaperone DNAJ1B and the protein kinase PKA. FLC is challenging to study because of its rarity and limited pre-clinical models. Here we developed a novel transgenic mouse model of FLC. In this model, DNAJ1B-PKA expression in the liver of mouse embryos results in perinatal lethality while DNAJ1B-PKA expression in the liver of adult mice initiates tumors resembling FLC at low penetrance. Some of these tumors can be serially propagated in 3D cultures and in allografts, including in syngeneic hosts. One such model shows growth inhibition upon treatment with the CDK4/6 inhibitor palbociclib. New pre-clinical models of FLC will provide novel insights into the biology of this rare cancer and may help identify novel therapeutic strategies.

INTRODUCTION

While hepatocellular carcinoma (HCC) in adults is a leading cause of mortality worldwide [1], liver cancer is much rarer in children and young adults. A majority of pediatric liver cancer cases are hepatoblastomas but primary HCCs are sometimes diagnosed [2]. In particular, fibrolamellar hepatocellular carcinoma (FL-HCC, or FLC) is a very rare and distinct subtype of HCC that affects adolescents and young adults. FLC develops in the setting of normal healthy livers, with no known genetic predisposition factor [3-5]. FLC is associated with better survival than HCC in adults, presumably due to the young age of the patients and the lack of cirrhosis, which makes more aggressive surgery possible [6]. However, few therapeutic options have been successfully explored beyond surgery. The 5-year survival rate ranges from 35-75% in patients treated with liver transplantation or resection. In cases where surgery is not possible, the survival drops to 12-14 months [7-10].

Morphologically, FLC tumors are characterized by pleomorphic malignant hepatocytes with large central nucleoli and abundant eosinophilic cytoplasm. The cancer cells are arranged in variably sized nests or cords set within a meshwork of lamellated collagen fibers. In the majority of cases, FLC cells contain hyaline droplets and some isolated cells have round cytoplasmic inclusions known as “pale bodies” [11, 12]. FLC express markers associated with biliary (e.g., CK7 and MUC1) and hepatocytic (e.g., Heppar-1, Albumin, α-1-antitrypsin, and Glypican-3) differentiation, as well as hepatic progenitor/stem cells (e.g., CK19, EPCAM) [3, 13-19].

At the genetic level, a breakthrough in the field was the discovery of a fusion event resulting in high levels of the catalytic subunit of PKA (protein kinase A) in FLC tumors [20, 21]. This finding was confirmed in other studies [22-24]. Rare cases of FLC have been associated with genetic inactivation of *PRKAR1A*, which encodes a regulatory subunit of PKA [25]. Rare cases of *BAP1* mutant HCC also have features of FLC and show amplification of the *PRKACA* gene coding for PKA with no fusion event [26]. Thus, high PKA activity may be sufficient to drive FLC development. Indeed, expression of PKA in the liver of young adult mice is sufficient to initiate tumors resembling FLC [27, 28]. In addition, inhibition of PKA kinase activity has anti-tumor activity against pre-clinical

models of FLC [29] and knock-down of the fusion protein inhibits the growth of patient-derived xenografts [30]. However, the small part of DNAJ1B fused to PKA in FLC tumors also contributes to tumor development in mice [27], an observation supported by biochemical and structural studies [31-36]. Few additional alterations have been found in the genome of FLC tumors, but accumulating evidence suggests a role for telomerase activity, MAPK signaling, and WNT signaling [26, 27, 35, 37-41].

The very low incidence rate of 0.02 per 100,000 has kept FLC an understudied cancer [42]. Very few cell lines and patient-derived xenograft models remain available, and these models often do not propagate easily, even though they can provide important model systems [16, 43, 44]. Similarly, mouse models have also been developed using CRISPR/Cas9 approaches; in these models, tumors recapitulate some key features of human FLC, but they grow slowly, limiting their use [27, 28]. Organoids derived from tumors have only been recently described [45, 46]. Here we engineered a new mouse allele in which the DNAJ1B-PKA fusion can be induced following Cre-mediated recombination in transgenic mice. We used this new model to investigate the consequences of DNAJ1B-PKA expression in the adult liver. We were able to develop allografts and 3D culture systems that are amenable to investigations exploring further the biology of FLC and identifying new therapeutic approaches.

RESULTS

Transgenic mice expressing DNAJ1B-PKA

We generated transgenic mice by inserting a *DNAJB1-PRKACA-GFP* cDNA into the *Rosa26* locus. Upon Cre-mediated recombination and self-cleaving of the fusion protein, this construct allows expression of the DNAJB1-PKA fusion found in human FLC and the GFP reporter (**Fig. 1A**). *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA}$ transgenic mice were healthy and fertile, as would be expected for a conditional allele.

The cell of origin of FLC cannot be determined conclusively from patients. While mouse models have shown that adult hepatocytes can initiate FLC [27, 28], the development of FLC in young patients and the expression of stem cell markers in these tumors [3, 13-17, 44] are also suggestive of an initiating event early in liver development. We wondered if expression of the DNAJB1-PKA fusion in the developing liver may initiate FLC more frequently and more rapidly than in the adult setting. To test this idea, we crossed *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ mice to *Alb-Cre* mice where the Cre recombinase is expressed under the control of the *Albumin* promoter and is turned on at mid-gestation in the liver. Analysis of GFP expression showed positive signal in the liver of mice also expressing Cre at day E18.5 (**Fig. 1B**). Accordingly, we detected increased expression of *PRKACA* mRNA molecules in transgenic mice expressing Cre at the same time point (**Fig. 1C**). These observations indicated proper expression of the transgene and led us to investigate the consequences of DNAJB1-PKA expression in the embryonic liver for possible tumor development later in adult mice. However, no *Alb-Cre*;*Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ pups from this cross survived past weaning (**Fig. 1D**). While the cause underlying this perinatal death is not known, these observations provided an initial validation of this new transgenic model to express the DNAJB1-PKA fusion in the liver of mice.

DNAJ1B-PKA expression in the adult liver of mice results in the development of slow-growing tumors with low penetrance

Fluorescence in situ hybridization (FISH) shows that the gene fusion is only present in FLC cells and not in surrounding liver cells [47]. To introduce the oncogenic fusion in a subset of adult liver cells and allow for clonal growth, we performed hydrodynamic tail

110 vein tail injections of *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ mice with a plasmid expressing the Cre
111 recombinase (**Fig. 2A**), generating a cohort of 38 animals (28 males and 10 females). We
112 aged these mice and waited for signs of disease before analysis. We found that 10 mice
113 developed tumors (3 spleen tumors, which were not further studied, and 7 liver tumors –
114 **Table S1** and **Fig. 2B**). The low penetrance of FLC development and the slow growth of
115 these tumors in this transgenic model is consistent with previously described mouse
116 models [27, 28], and makes it difficult to use these mice as pre-clinical models of FLC
117 development and response to candidate therapies.

118 We thus wondered if some of the most aggressive tumors in transgenic mice may be
119 able to be transplanted into recipient mice in allograft models. We were able to generate
120 single-cell suspensions from tumors in 5 out 7 of the mice with liver tumors, 2 of which
121 grew in NSG immunodeficient mice upon subcutaneous injection. One allograft model
122 had a fibrosarcoma histology and was not pursued further (**Fig. S1A,B**). The other tumor,
123 FL1 (shown in **Fig. 2B**), had histopathological features of FLC and some GFP expression
124 by immunostaining (**Fig. S1C,D**); the first allograft generated from this primary tumor
125 stopped growing when its size was still small and did not further expand in subsequent
126 passages, and it was not further studied.

127 Based on this low efficiency of tumor take from single cells, we also directly implanted
128 small pieces from 2/7 tumors subcutaneously into NSG mice. These two models, FL2 and
129 FL8, grew and could be propagated into new NSG hosts. Cells in these tumors were
130 positive for the liver marker HNF4α (Hepatocyte nuclear factor 4α) [48] and GFP (**Fig. 2C**
131 and **Fig. S2A**). Similar to the two other mouse models previously described [27, 28], FL2
132 and FL8 tumors were largely negative for the expression of cytokeratins 17 and 19
133 (CK17/19) (**Fig. S2B**). These same two mice had lung metastases (**Fig. S2C**), which we
134 also microdissected to generate two additional models, FL2M and FL8M, which were
135 expanded in NSG mice. For FL2 and FL8, tumor growth was slow and variable, from 3-6
136 months, but these tumors retained their initial histology (**Fig. 2D-H**).

137 We attempted to grow FL2 and FL8 cells into immunocompetent mice (n=3 tumor
138 pieces for FL2 and FL8, n=5 each for FL2 and FL8 for cell suspensions). To increase the
139 probability that some of the cancer cells may grow, we performed orthotopic

transplantations: for both FL2 and FL8, we sutured tumor fragments into the liver of 3 mice per line, and injected cell suspensions into the liver of 5 mice per line. We chose F1 mice between pure C57BL/6 and 129Sv/J to recapitulate the MHC alleles in the *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ mice (which are in a mixed background between these two mouse strains). Both the FL2 and the FL8 models grew in the liver of F1 hosts upon injection of sutured tumor fragments (**Fig. 2I,J** and **Fig. S2D,E**).

Overall, the development of liver tumors in this mouse model is slow and has low penetrance but FLC allografts derived from our transgenic mice can be serially passaged.

3D culture models for mouse FLC tumors

In the past few years, the development of 3D culture systems has enabled the analysis of normal tissues and tumors *ex vivo* under conditions more similar to *in vivo* conditions than 2D cultures. Human liver organoids have recently been described from human FLC tumors [45, 46]. We found that mouse FLC cells could be isolated from allografts and grown in 3D organoids in an air-liquid interface (ALI) system (**Fig. S3A**). However, these cells did not expand beyond 3 passages in this context (~1 passage per month), suggesting that these culture conditions are not optimal. Still, we were able to re-inject FL2 and FL8 cells from passages 2 and 1, respectively, in ALI organoids using orthotopic injections (cell suspensions) into the liver of NSG mice (**Fig. S3B**) (we chose orthotopic injections because of failure to grow from single cell suspensions in subcutaneous injections). The resulting allografts had retained FLC histology (**Fig. S3C**), indicating that *ex vivo* culture followed by re-implantation is possible for these models.

The growth of FLC allografts can be inhibited by palbociclib treatment

While FLC cells could grow orthotopically, tumor development was variable and unreliable in this assay. We sought to develop a subcutaneous model in which tumor initiation and tumor growth may be more consistent than single cell injections (rare tumor growth) and injections of tumor fragments (reliable tumor growth but variable growth rate). We wondered if a matrix that could recapitulate the stiffness of the liver microenvironment may provide a better environment for FLC cells to grow, as observed in other experiments to grow liver cells in 3D models [49-52]. We embedded mouse FLC cells in methacrylated gelatin (GelMA). GelMA is a biocompatible matrix with modular mechanical properties

170 [53] and it is compatible with the growth of liver epithelial cells [54]. FLC cells grew in
171 GelMA (**Fig. 3A**) and FL8 cells embedded in GelMA were implanted subcutaneously in
172 recipient mice (**Fig. 3A**) We found that tumor development was not visibly different
173 between this method and the implantation of tumor fragments (compare controls in **Fig.**
174 **S3D** to tumor growth **Fig. 2E,G**), but reproducible enough to perform a treatment
175 experiment. We observed a mild tumor inhibitory effect of the CDK4/6 inhibitor palbociclib
176 in this experiment (**Fig. 3C** and **Fig. S3D**).

177 We performed bulk RNA sequencing (RNA-seq) of tumors at the end of the
178 experiment. When we analyzed the expression of genes that have been used as markers
179 of FLC and other liver tumors (**Fig. 3D**), we found that the FL8 model in this context
180 expressed genes such as *Alb* (coding for albumin), *Arg1* (coding for Arginase-1), *Krt8/18*
181 (coding for cytokeratins 8 and 18), or *Serpin1a/b* (coding for Alpha-1-Antitrypsin) as
182 expected for a liver cancer originating from hepatocytes [18, 55, 56]. Detection of *Krt5*
183 (coding for cytokeratin 5) and *Muc1* (coding for Mucin1) in this analysis was more
184 indicative of bile duct differentiation and could be a sign of dedifferentiation [14, 57].
185 Accordingly, expression of *Afp* (coding for Alpha-fetoprotein) was high in the FL8 model,
186 and is a feature of FLC tumors with a worse prognosis [58, 59]. Expression of *Fgb* is a
187 marker of FLC, with the encoded protein Fibrinogen B being present in pale bodies
188 characteristic of this cancer type [55]. Palbociblib has recently be shown to promote
189 differentiation in neuroblastoma [60], but we did not observe any significant changes in
190 the expression of these "liver" genes in response to palbociclib (**Fig. 3D**). When we
191 analyzed up- and down-regulated genes in tumors treated with palbociclib compared to
192 controls, we found very few genes (n=8) with a significantly different expression (p-
193 adj<0.05) between controls and palbociclib-treated tumors (**Table S2**). When we
194 performed a gene ontology (GO) enrichment analysis of the 952 genes differentially
195 expressed between the two conditions (p-value ≤0.05), we found an enrichment for cell
196 cycle terms, as would be expected for treatment with a cell cycle inhibitor (**Fig. 3E** and
197 **Table S3**) Overall, this analysis further supports that FL8 cells may represent an FLC-like
198 model and suggests that FLC tumors may respond to CDK4/6 inhibitors such as
199 palbociclib.

DISCUSSION

Here we present a new transgenic mouse model for human FLC based on expression of the DNAJ1B-PKA fusion protein. While a limitation of this mouse model is the low frequency and the slow development of tumors, these limitations can be in part circumvented by the expansion *ex vivo* of cell models derived from these tumors.

The *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA}$ transgenic model is versatile as Cre can be delivered at different times and in different cell types using other mouse alleles or viral vectors. When we activated DNAJ1B-PKA during embryonic liver development, mice did not survive to adulthood, suggesting that expression of the fusion protein at this stage is detrimental to some aspect of liver function. This issue could be possibly alleviated by decreasing the number of liver cells expressing Cre and may help initiate tumors more efficiently than Cre delivery in the adult liver, for example using an *Alb-CreER* allele with tamoxifen-inducible Cre expression [61], using low doses of tamoxifen during embryogenesis. The *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA}$ transgenic model could also be used to model other cancer types in which DNAJ1B-PKA is thought to be oncogenic, including rare cases of pancreatic cancer [62], which may be modeled using the appropriate Cre driver.

A growing number of organoid and patient-derived xenograft models are being developed from patients with FLC to investigate key features of this disease (see [29, 30, 45, 46, 63-66] for recent examples). We describe the growth of mouse FLC-like cell models in culture and as allografts. These murine models, which grow in immunocompetent hosts as primary tumors or allografts, may prove useful in the future to investigate specific aspects of FLC biology, including for example how these tumors interact with immune cells. We note that one possible limitation of our transgenic model is the expressing of GFP together with the fusion protein, as GFP is immunogenic [67]; this may confound some analyses focusing on the response of immune cells to cancer cells expressing DNAJ1B-PKA in this context. Mouse models also provide a simpler genetic setting to explore mechanisms of tumorigenesis, and it would be interesting in the future to determine how expression of the fusion protein in mouse hepatocytes or in cell models such as FL2 and FL8 or their metastatic derivatives FL2M and FL8M affects the biology of these cells compared to recent observations made in human models [34, 68].

230 We chose palbociclib as a proof-of-principle compound because genomic analyses
231 of FLC tumors indicate that these tumors are wild-type for the *RB1* gene (coding for the
232 RB tumor suppressor) [20-24] and our transgenic model was also engineered to be wild-
233 type for *Rb1*. CDK4/6 inhibitors are approved for the treatment of breast cancer and
234 presence of RB is required for tumor inhibition [69, 70]. The anti-tumor effects observed
235 with the FL8 model were not very strong, which is possibly due to the fact that FL8 tumors
236 do not expand very fast in the context studied. Future experiments may combine CDK4/6
237 inhibitors with other strategies such as chemotherapy or radiotherapy to induce more
238 potent tumor inhibition.

239 It has often been difficult for rare diseases such as FLC to benefit from new therapies
240 because pre-clinical models often lag compared to more frequent tumor types. The
241 development of new mouse models, but also fish models [71], may help provide pre-
242 clinical data that lead to the design of new clinical trials in patients with FLC.

243 **Methods**

244 **Mice and generation of tumors**

245 Mice were maintained according to practices prescribed by the NIH at Stanford's
246 Research Animal Facility (approved protocol #32398). Additional accreditation of
247 Stanford animal research facilities was provided by the Association for Assessment and
248 Accreditation of Laboratory Animal Care (AAALAC).

249 A *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ C56BL/6 founder mouse was generated and validated by
250 Applied StemCell using a site-specific integrase via pronuclear injection [72]. *Alb-Cre*
251 mice were described before [73] and obtained from the Jackson Laboratory (Stock No:
252 035593). Primers for genotyping are available upon request.

253 To generate tumors in the liver of mice, 10-16-week-old mice were anesthetized using
254 isoflurane, the tail vein was dilated with a heat pad, and 10 µg of Turbo-Cre plasmid was
255 delivered in a solution of sterile PBS using a hydrodynamic tail-vein injection protocol.
256 The Turbo-Cre plasmid was a gift from the laboratory of Dr. Steven Artandi at Stanford
257 University. Mice were housed for 1-2 years before subsequent analysis.

258 **RT-qPCR**

259 Liver tissue was isolated by microdissection, minced with a razor blade, and total RNA
260 was collected using RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA synthesis was
261 performed using the iScript cDNA synthesis kit (Bio-Rad) and RT-qPCR was performed
262 using iQ SYBRGreen supermix (Bio-Rad). The following RT-qPCR primers were used
263 DNAJPRKACA F: 5'-TTACTACCAGACGTTGGGCCT-3', DNAJPRKACA R: 5'-
264 ATAGTGGTTCCCGGTCTCCT-3', mPRKACA-aF: 5'-AGATCGTCCTGACCTTTGAGT-
265 3', mPRKACA-aR: 5'-GGCAAAACCGAAGTCTGTCAC-3', mPRKACA-bF: 5'-
266 GGTGACAGACTTCGGTTTTGC-3', mPRKACA-bR: 5'-
267 CACAGCCTTGTTGTAGCCTTT-3'.

268 **Immunostaining**

269 Tissues were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS).
270 Liver tissues (normal or tumor) were initially perfused with 10 mL PFA through the inferior

271 vena cava. All tissues were submerged in 4% PFA for 48 hr before being switched to 70%
272 ethanol and finally processed and embedded. Slides were deparaffinized in 2x 5 min
273 changes of Histo-Clear (National Diagnostics HS-202), and rehydrated in 2x 1 min
274 changes of 100% ethanol, 2x 1 min changes of 70% ethanol, and finally 2x 5 min changes
275 of distilled water. Antigen retrieval was performed in a microwave for 10 min with citrate-
276 based buffer (Vector Labs H-3300). Slides were cooled at room temperature, quenched
277 for endogenous enzyme activity (SP-6000), and washed in 3x 3 min changes of PBS with
278 0.1% Tween-20 (PBST) (Sigma P1379). Slides were blocked with secondary antibody
279 serum for 1 hr at room temperature (RT). GFP antibody (Cell Signaling Technology
280 #2956) was diluted to 1:200 in PBST and applied to slides for 1 hr at 37 ºC. Slides were
281 washed in 3x 3 min changes of PBST. Horse anti-rabbit secondary (Vector Labs MP-
282 7401) was applied to slides for 1 hr at 37 ºC. Slides were washed in 3x 3 min changes of
283 PBST. The chromogen was developed using a DAB substrate kit (Vector SK-4100) for 5
284 min before quenching in distilled water for 5 min. Slides were counterstained with
285 hematoxylin (Newcomer Supply 1201B, dehydrated in 2x 1 min changes of 70% ethanol,
286 2x 1 min changes of 100% ethanol, and finally cleared in 2x 5 min changes of xylene
287 before mounting.

288 Embryos were dissected from timed pregnant females, fixed in 4% PFA overnight and
289 sections were generated as described above. Sections were depaffarinized, rehydrated,
290 and tissue was blocked in 5% goat serum for 1 hr. Anti-GFP was added overnight. The
291 following day, samples were washed before incubation with Goat anti-Rabbit - Alexa
292 Fluor™ 488 (Thermo Fisher) and DAPI (Sigma). Samples were washed and mounted in
293 ProLong Gold mounting media (Thermo Fisher) before confocal imaging.

294 **Tumor transplantation studies**

295 Nod.Cg-Prkdc^{scid}IL2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratories, Stock No: 005557)
296 were used for all experiments in immunodeficient recipients. For experiments in
297 immunocompetent hosts, F1 mice between pure C57BL/6 and 129Sv/J were used
298 (Jackson Laboratories, B6129SF1/J Stock No: 101043).

299 Tumors from *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ mice were microdissected from livers or lungs.
300 Tumors were bisected once to increase surface area exposed to transplant media

301 (Kubota's medium) and kept on ice until ready for transplant. Recipient mice were
302 anesthetized and shaved in the flank. The shaved flank was sterilized with one swab of
303 betadine, one swab of 70% ethanol repeated 3 times. After confirming adequate depth of
304 anesthesia by toe-pinch, a 5 mm incision was made in the shaved flank. Blunt dissection
305 under the skin was used to separate the skin from the muscle, creating a pocket between
306 the 2 layers. Tumor pieces were implanted (bisected side facing the muscular wall, ~5
307 mm piece) near macroscopically visible capillaries on muscle wall. The incision was
308 closed using 9 mm wound clips. For subcutaneous tumors, tumor volume was calculated
309 following this formula:

310 $$Tumor\ Volume = \frac{4}{3}\pi\left(\frac{L+W}{4}\right)^3$$

311 **Cell Culture**

312 Allografts were minced into 1-2 mm^3 fragments and incubated for 10 min at 37 ºC in an
313 enzymatic cocktail of Collagenase 1 (0.85 mg/mL, Sigma C0130) Collagenase 2 (0.28
314 mg/mL, Sigma C6885), Collagenase IV (0.85 mg/mL, Sigma 5138), Elastase (0.125
315 mg/mL Worthington LSL002292), DNAse 1 (0.125 mg/mL Roche 1014159001) in L-15
316 Medium (Sigma L1518).

317 To generate organoids, cells were pelleted and resuspended in Matrigel (Fisher 356213)
318 at 10^4-10^5 cells/mL. One volume of Matrigel with no cells was pipetted into a transwell
319 insert to gel at 37 ºC for 10 min. One volume of Matrigel with cell suspension was pipetted
320 onto the top of the acellular Matrigel layer and incubated for an additional 10 min at 37
321 ºC. Kubota's Medium (Phoenix Songs Biologicals 35101) was added outside the transwell
322 to a volume that submerged the acellular Matrigel layer and but left the cellular layer
323 exposed to air. Cells were fed 3 times a week for 1-2 months.

324 To passage cells, wells were washed 3x with PBS. Matrigel layers were collected into a
325 50 mL conical and incubated with an equivalent volume of Cell Recovery Solution
326 (Corning 354253). Layers and Recovery solution were incubated at 4 ºC for 30 min with
327 vigorous pipetting every 5 min. Cells were pelleted and resuspended in Matrigel for
328 following passages.

329 For GelMA experiments, 20% w/v Gelatin (Sigma G1890) in 0.25 M carbonate-
330 bicarbonate (CB) buffer was mixed with methacrylic anhydride (MA) (Thermo Fisher 760-
331 93-0) at 0.1mL/1g MA to gelatin for 1 hr at 37 ºC. This solution was henceforth referred
332 to as GelMA. Unreacted MA was removed by spinning solution at 3500 g for 5 min at RT.
333 Supernatant was collected with unreacted MA pellet discarded. GelMA was diluted three-
334 fold and dialyzed in 10 K MWCO tubing (Thermo Fisher 88245) for 7 days at 37 ºC against
335 Milli-Q water. Water was changed twice a day. Dialyzed GelMA was frozen at -80 ºC
336 overnight before lyophilization. Lyophilized GelMA was reconstituted at 20% w/v in RPMI,
337 0.005 N NaOH, 20 mM HEPES, 0.22 g NaHCO3, and sterile filtered with 0.45 µm filters.
338 To embed cells in GelMA, 20% w/v aliquots of GelMA were diluted 1:2 with RPMI to form
339 10% w/v solution. Cells were resuspended at appropriate concentrations. Lithium phenyl-
340 2,4,6-trimethylbenzoylphosphinate (LAP) (Sigma 900889) was mixed into solution to final
341 concentration of 0.67% w/v LAP. Gel was crosslinked with 405 nm light for 3 min.

342 **Orthotopic Injection and Implantation**

343 To generate tumors, cells were disaggregated from Matrigel using the protocol described
344 above**.** Cell pellets were resuspended at a concentration of 10^4-10^5 cells in 25 µL. These
345 cell suspensions were mixed with an equivalent volume of Matrigel to a total volume of
346 50 µL. Mice were anesthetized and shaved in the abdomen. Lateral incision was made in
347 the skin directly inferior to xyphoid process. Blunt dissection was used to separate
348 surrounding skin from parenchyma and musculature. Incision was made in caudal
349 direction originating from xyphoid process. The largest liver lobe available was exposed
350 and stabilized. For cell injection, 20 µL of cells mixed 1:1 with Matrigel were injected. For
351 tissue implantation, tissue 3-8 mm-wide at longest axis was sutured to the liver lobe using
352 5-0 vicryl suture. Liver lobe was returned to original site. Mouse musculature was closed
353 with 5-0 vicryl running suture, skin was closed with 9mm wound clips.

354 **Drug Study**

355 FL8 cells were suspended at concentration of 10^6 cells/100 µL in 10% w/v methacrylated
356 gelatin (Sigma 276685) and 0.067% w/v LAP (Sigma 900889) suspended in RPMI (Sigma
357 R0883). 200 µL aliquots in 96-well plates were photo-crosslinked into solid capsules at

405 nm light (Amazon 0191673174324) for 3 min at room temperature. Capsules were implanted in the subcutaneous flanks of NSG mice. When average tumor size reached 75 mm^3 mice were randomized into treatment groups. Mice either received vehicle (0.5% methocel Sigma 94378) or palbociclib (LC Laboratories P-7788) at a dose of 75 mg/kg via oral gavage for 3 consecutive days and given 4 days with no treatment.

RNA sequencing analysis

Frozen tumor samples were sent to Novogene for RNA extraction and library preparation. Raw sequencing reads were demultiplexed by the vendor to generate approximately 20 million paired-end reads per sample. Fastq files were trimmed using CutAdapt (v2.10) [74] using TruSeq sequencing adapter (5'-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'), and a minimum read length of at least 25. Reads were then aligned to the ENSEMBL mm10 genome using HiSat2 [75] using reverse strandedness and discarding unaligned reads. Counts were assigned to genes using featureCounts [76] at ENSEMBL gene annotation (v93). Differential expression analysis was conducted using DESeq2 [77] on R v4.1.2 (https://www.R-project.org/).

Statistics and reproducibility

Statistical significance was assessed using the Prism GraphPad software. The specific tests used are indicated in the figure legends. Mice for *in vivo* studies were randomized where applicable and sample sizes determined by pilot experiments or previous experiments using similar models. Investigators were not blinded to allocation during experiments and outcome assessment.

Data availability

All RNA sequencing datasets generated in this study are available at Gene Expression Omnibus (GEO) under Super Series GSE249325 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE249325). All other data are available in the article and supplementary materials, or from the corresponding author upon reasonable request.

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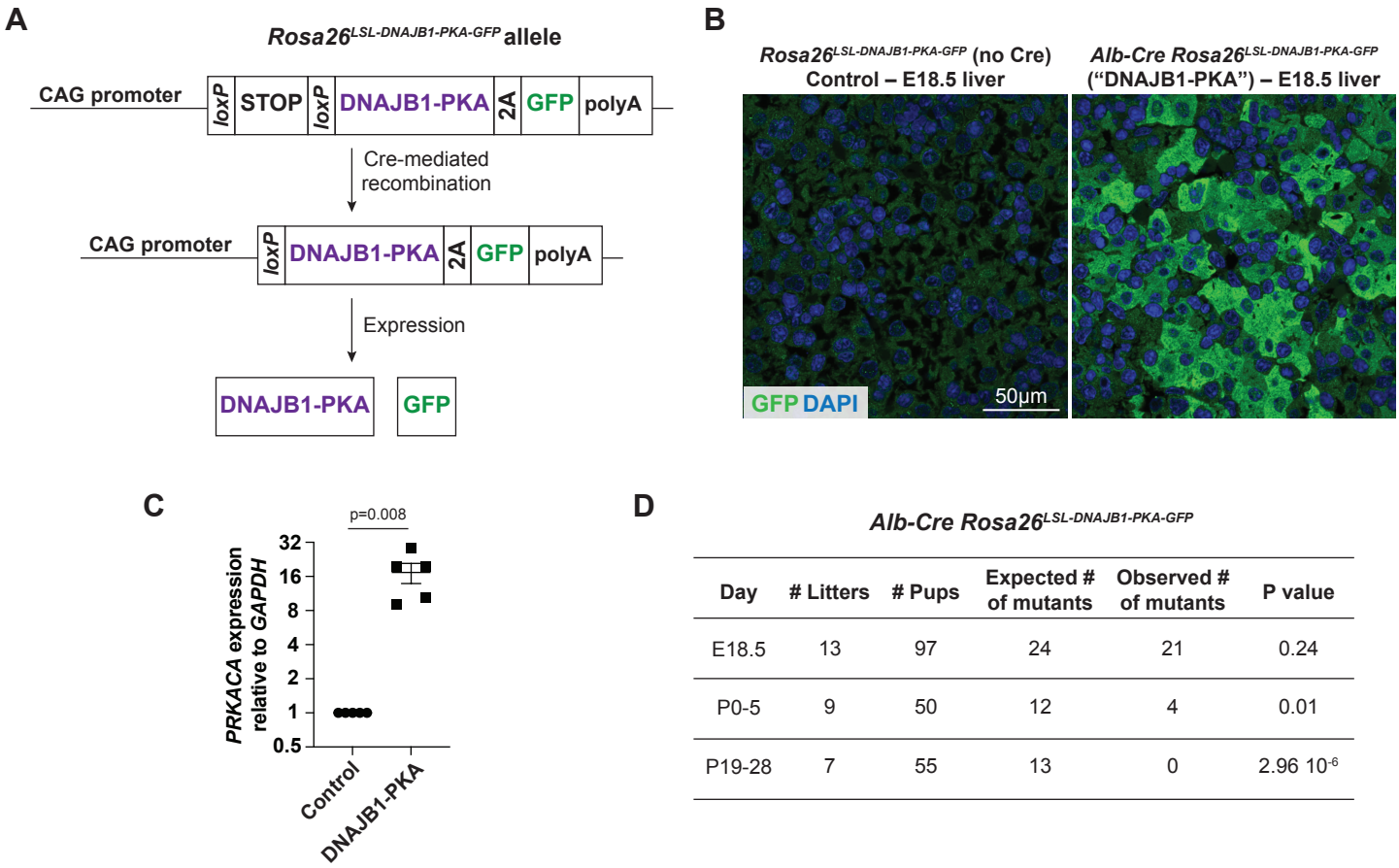
FIGURE 1



Alb-Cre Rosa26$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$

Day	# Litters	# Pups	Expected # of mutants	Observed # of mutants	P value
E18.5	13	97	24	21	0.24
P0-5	9	50	12	4	0.01
P19-28	7	55	13	0	$2.96\ 10^{-6}$

Figure 1. Generation of a transgenic mouse model to express the DNAJ1B-PRAKA fusion

A. Schematic representation of the transgene inserted into the *Rosa26* locus. The fusion cDNA is under the control of the CMV early enhancer/chicken β actin (CAG) promoter but is only expressed after Cre-mediated recombination of a transcriptional STOP cassette flanked by *loxP* sites. Upon translation, a self-cleaving 2A peptide separates the DNAJB1-PKA fusion protein from the GFP reporter. **B.** Representative confocal immunofluorescence images for GFP expression (green) from sections of control mice (transgenic mice with no Cre expression) or mice expression the fusion protein and GFP at E18.5. DAPI shows DNA staining (blue). Scale bar: 50µm. **C.** Expression levels of *PRKCA* transcripts analyzed by RT-qPCR analysis relative to *GAPDH* and to controls (n=5 E18.5 embryonic liver samples per genotype). Data shown as mean and S.E.M.; P value calculated by Mann-Whitney t-test. **D.** Analysis of expected and observed numbers of mutant mice before and after birth. P value calculated by a X^2 test.

FIGURE 2

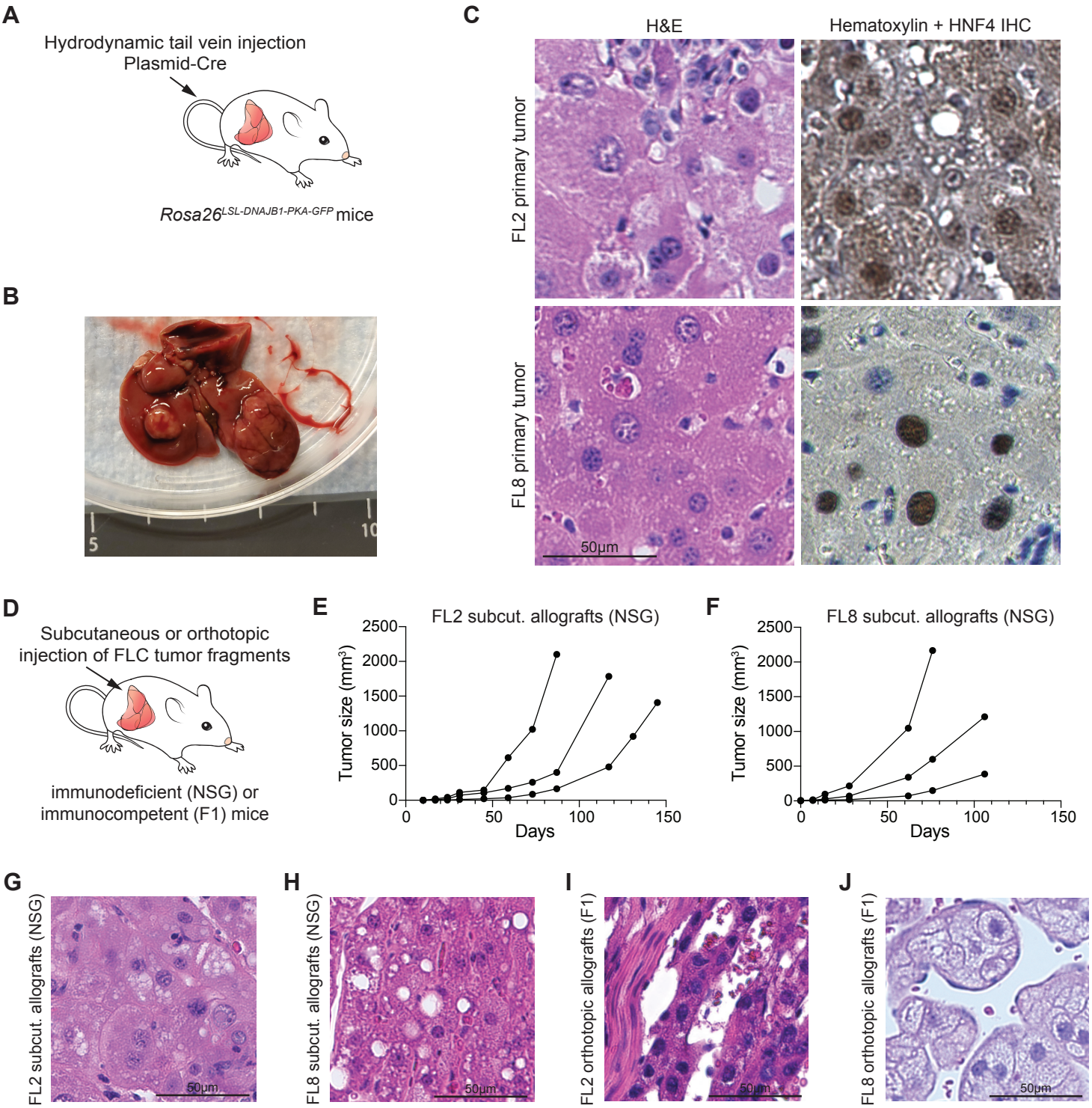


Figure 2. Propagation of tumors from transgenic mice expressing the DNAJ1B-PRAKA fusion

A. Schematic representation of the protocol to induce liver tumors in adult *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ mice upon delivery of a plasmid expressing the Cre recombinase to the liver. **B.** Representative photograph of liver tumors in the dissected liver of a *Rosa26*$^{LSL\text{-}DNAJB1\text{-}PKA\text{-}GFP}$ mouse 24.5 months after delivery of the Cre recombinase (FL1 tumor shown). **C.** Hematoxylin and eosin and hematoxylin (H&E) staining (left) and anti-HNF4α immunohistochemistry (right) on sections from the FL2 and FL8 primary tumors. Scale bar: 50µm. **D.** Schematic representation of the protocol to transplant tumor fragments from FLC models into immunodeficient or immunocompetent hosts. **E, F.** Growth of FL2 (E) and FL8 (F) subcutaneous allografts in NSG mice. **G,H**. Representative images of FL2 (G) and FL8 (H) subcutaneous allograft sections (H&E) from (E) and (F), respectively. Scale bar: 50µm. **I,J.** Representative images of FL2 (I) and FL8 (J) allograft sections (H&E) growing in the liver of C57BL/6x129Sv/J F1 immunocompetent hosts (F1). Scale bar: 50µm.

FIGURE 3

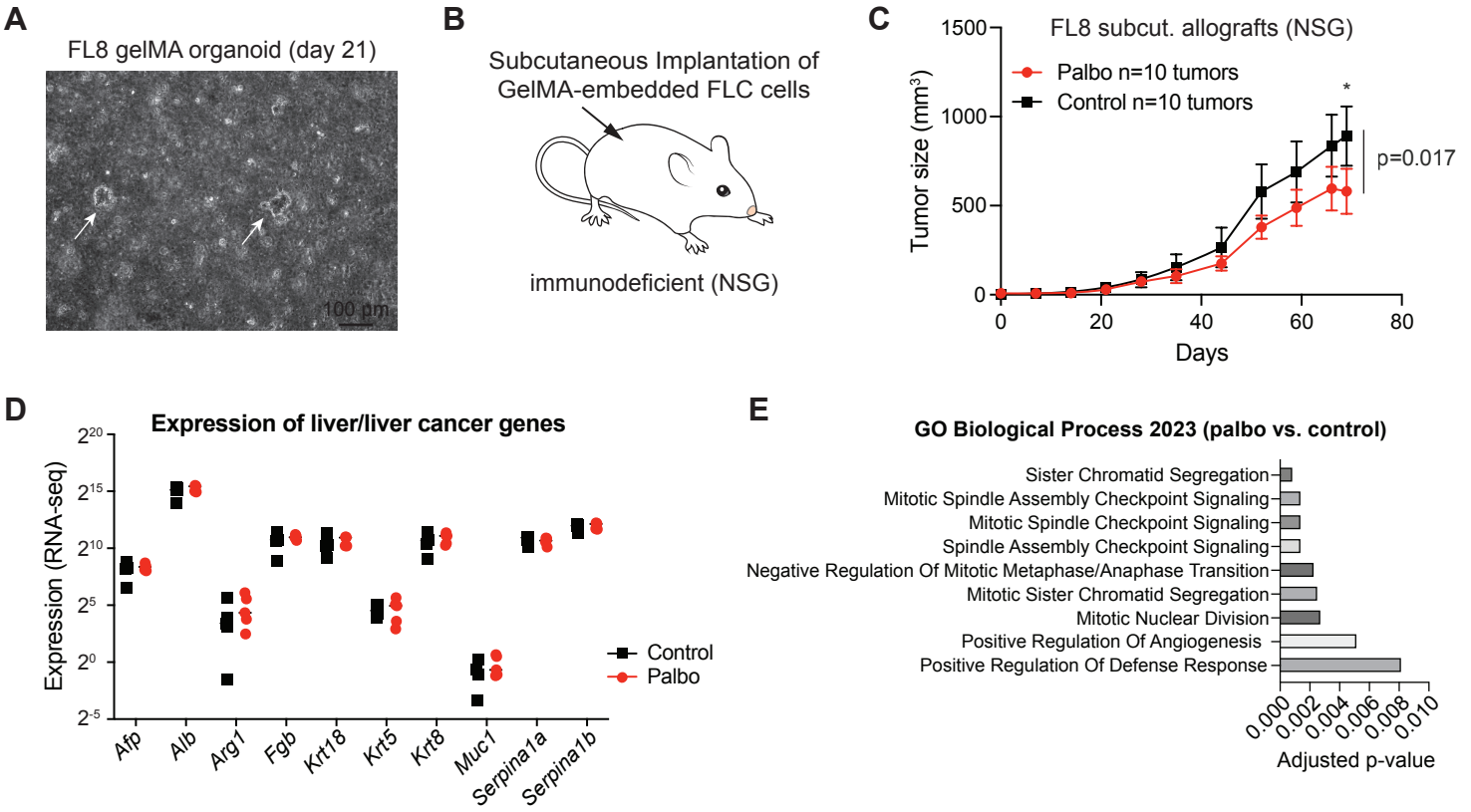


Figure 3. Inhibition of tumor growth by palbociclib

A. Representative photograph of FL8 cells growing in a GelMA matrix. Scale bar: 100µm. **B.** Schematic representation of the protocol to transplant FLC cells embedded into GelMA into immunodeficient or hosts. **C.** Growth of FL8 subcutaneous allografts treated with palbociclib (palbo) or control carrier. Data shown as mean and S.E.M.; P-value at the last time point calculated by t-test. **D.** Analysis of genes related to liver and liver cancer in control and palbociclib-treated tumors at endpoint. Data shown as mean and range. **E.** Gene Ontology (GO) analysis for biological process comparing control and palbociclib-treated tumors at endpoint.