A Phosphokinome-Based Screen Uncovers New Drug Synergies for Cancer Driven by Liver-Specific Gain of Nononcogenic Receptor Tyrosine Kinases

Yannan Fan,1* Maria Arechederra,1* Sylvie Richelme,1 Fabrice Daian,1 Chiara Novello,2 Julien Calderaro,3,4 Luca Di Tommaso,2 Guillaume Morcrette,5 Sandra Rebouissou,5 Matteo Donadon,6 Emanuela Morenghi,7 Jessica Zucman-Rossi,5 Massimo Roncalli,2 Rosanna Dono,1 and Flavio Maina1

Genetic mutations leading to oncogenic variants of receptor tyrosine kinases (RTKs) are frequent events during tumorigenesis; however, the cellular vulnerability to nononcogenic RTK fluctuations has not been characterized. Here, we demonstrated genetically that in the liver subtle increases in wild-type Met RTK levels are sufficient for spontaneous tumors in mice (Alb-R26Met), conceptually illustrating how the shift from physiological to pathological conditions results from slight perturbations in signaling dosage. By analyzing 96 different genes in a panel of tumor samples, we demonstrated that liver tumorigenesis modeled by Alb-R26Met mice corresponds to a subset of hepatocellular carcinoma (HCC) patients, thus establishing the clinical relevance of this HCC mouse model. We elucidated the regulatory networks underlying tumorigenesis by combining a phosphokinome screen with bioinformatics analysis. We then used the signaling diversity results obtained from Alb-R26Met HCC versus control livers to design an “educated guess” drug screen, which led to the identification of new, deleterious synthetic lethal interactions. In particular, we report synergistic effects of mitogen-activated protein kinase kinase, ribosomal S6 kinase, and cyclin-dependent kinase 1/2 in combination with Bcl-XL inhibition on a panel of liver cancer cells. Focusing on mitogen-activated protein kinase kinase and Bcl-XL targeting, we mechanistically demonstrated concomitant down-regulation of phosphorylated extracellular signal–regulated kinase and myeloid cell leukemia 1 levels. Of note, a phosphorylated extracellular signal–regulated kinase+/BCL-XL+/myeloid cell leukemia 1+ signature, deregulated in Alb-R26Met tumors, characterizes a subgroup of HCC patients with poor prognosis.

Conclusion: Our genetic studies highlight the heightened vulnerability of liver cells to subtle changes in nononcogenic RTK levels, allowing them to acquire a molecular profile that facilitates the full tumorigenic program; furthermore, our outcomes uncover new synthetic lethal interactions as potential therapies for a cluster of HCC patients. (HEPATOLOGY 2017;66:1644–1661).

Receptor tyrosine kinases (RTKs) are frequently mutated in different types of cancer.1 Through their aberrant activation, RTKs confer upon cancer cells a range of biological advantages and, in some cases, addiction. This is illustrated by the deleterious effects of agents targeting oncogenic RTKs on cancer cells.1,2 The relevance of RTKs in cancer also derives from bioinformatics methods applied in

Abbreviations: Cdk, cyclin-dependent kinase; ERK, extracellular signal–regulated kinase; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HLF, human lung fibroblast; Mcl1, myeloid cell leukemia 1; MEK, mitogen-activated protein kinase kinase; p-, phosphorylated; RSK, ribosomal S6 kinase; RTK, receptor tyrosine kinase.

Received November 30, 2016; accepted June 2, 2017.

Additional Supporting Information may be found at online library.wiley.com/doi/10.1002/hep.29304/suppinfo.

*These authors contributed equally to this work.

Supported by Institut National du Cancer (PL06_078 and PLBIO12-057 to EM.), Fondation de France (2003_012843, 2014_00051580, and 2016_00067080 to EM. and MA.); Association pour la Recherche contre le Cancer (SFI2011_1203807 and 2006_3146 to EM.); Fondation pour la Recherche Médicale (DLC2006_0206414 to EM.); and Fondation Bettencourt-Schueller (0504222 to EM.); the China Scholarship Council (2012065350070 to Y.F.); and the Associazione Italiana Ricerca Cancro (15437, to M.R.). The contributions of the Region Provence Alpes Côte d’Azur and of Aix-Marseille Université to the IBDM animal facility and of the France-BioImaging/PLC infrastructure (ANR-10-INBS-04-01) to the imaging facility are also acknowledged. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Combination with genome-wide profiling and protein network-based studies, which have highlighted RTK signaling as one of the few core pathways that impacts tumor evolution. In this context, we previously showed how alterations in RTK signaling influence other core pathways through interconnecting signaling nodes, which are vulnerable targets in cancer cells. Although genetic strategies have demonstrated how oncogenic RTK signaling mutations found in human patients are capable of triggering cancer, they largely recapitulate situations in which cancer cells are addicted to these drastic genetic oncogenic forms, which, in most cases, act as dominant signaling alterations. Nevertheless, enhanced RTK activation levels are often observed in tumors despite the absence of genetic mutations. Their significance in cancer biology and as a signature remains unclear.

The multitude of signaling alterations orchestrated by deregulated RTKs have been largely explored by focusing on putative candidate signaling circuits. Recent large-scale screens have been carried out in an attempt to gain broader knowledge regarding altered signal transduction systems operating in cancer cells and identify vulnerable nodes to target. For example, as determined using cancer cell lines characterized by oncogenic Met addiction, the Ras and phosphoinositide 3-kinase pathways are required to sustain addiction. Another elegant example of synergistic drug discovery in RTK-driven tumorigenesis involved a drug screen in combination with network modeling, leading to the identification of synergistic responses targeting cyclin-dependent kinase 4 (Cdk4) with insulin-like growth factor 1 receptor, epidermal growth factor receptor, or Akt in dedifferentiated liposarcoma. Alternatively, pooled short hairpin RNA screening strategies permitted vulnerable points in drug resistance to be uncovered, exemplified by identification of the ability of p38x mitogen-activated protein kinase to elevate mitogen-activated protein kinase (Mek)–extracellular signal–regulated kinase (Erk) signaling in sorafenib-resistant cells. Although all of these studies highlight examples of critical RTK pathways that participate in mediating the oncogenic properties of cells, major limitations are associated with the biological systems that are often used: the addiction of cancer cells to oncogenic RTK forms and/or downstream signaling component(s). Little is known about the signaling platforms originated by and operating in cancer cells with subtle, yet still functionally relevant, increases in RTK inputs as well as whether these platforms possess vulnerabilities. Such knowledge deficiencies are also attributable to a lack of proper genetic systems that model the biological context of nonchronic RTK activation.

We recently explored the ability of cells to handle subtle changes in RTK levels in vivo using conditional transgenic mice in which the expression of wild-type Met is slightly enhanced above endogenous levels (R26stopMet mice) and demonstrated the vulnerability of restricted cell types during embryogenesis. In the present study, we used the R26stopMet system to genetically explore cell vulnerability versus resilience to enhanced RTK levels in adulthood. Although most...
cells are resistant, liver cells (Alb-R26<sup>Met</sup>) are sensitive to enhanced wild-type Met levels and develop spontaneous hepatocellular carcinoma (HCC). We discovered new, deleterious synthetic lethal interactions for a panel of HCC cells. We also established the clinical relevance of our findings by identifying a signaling signature characterizing a subgroup of HCC patients with poor prognosis.

Materials and Methods

More detailed procedures can be found in the Supporting Information.

TRANSGENIC LINES AND GENOTYPE ANALYSIS

The generation of R<sup>26</sup>stopMet mice (international nomenclature Gt(ROSA)26Sortm1(Actb-Met)Fmai) carrying a conditional mouse–human chimeric Met transgene in the Rosa26 locus has been reported. (12-14) R<sup>26</sup>stopMet-Luc mice (international nomenclature Gt(ROSA)26Sortm1(Actb-Met-IRES-Luc)Fmai) were generated by following the same strategy used for R<sup>26</sup>stopMet mice.

CELL CULTURES

Culture conditions for primary embryonic hepatocytes have been described (6,15-17); the protocol was adapted to establish Alb-R26<sup>Met</sup> HCC cell lines.

WESTERN BLOTTING

Protein extracts were prepared, and western blot analysis was performed as described (7,13,18). The antibodies used are reported in Supporting Table S1.

STATISTICAL ANALYSIS

Results are expressed as the median or as the mean ± standard error of the mean, according to sample distributions. Statistically significant differences were estimated by applying unpaired Student t tests to data showing normal distributions and Mann-Whitney tests in all other situations (e.g., for xenografts studies). P values are indicated in figures. The cumulative overall survival and disease-free survival rates were calculated using the Kaplan-Meier method. P < 0.05 was considered significant. All tests were two-sided. All analyses were performed with Stata 13.

Results

ENHANCED NONONCOGENIC RTK Met LEVELS IN THE LIVER CAUSE HCC PATHOGENESIS

To enhance Met–RTK levels in all tissues, we crossed R<sup>26</sup>stopMet with Deleter-Cre transgenics to obtain Deleter-Cre;R26<sup>Met</sup> mice (referred to as Del-R26<sup>Met</sup>), in which the Met transgene (Met<sup>tg</sup>) is expressed in all tissues following LacZ-stop cassette excision (Supporting Fig. S1A). Whereas fully recombined Del-R26<sup>Met</sup> mice at birth exhibit a hyperflexed forelimb and weak hind limb phenotype, (13) pups with a mosaic deletion of the LacZ-stop cassette appeared normal. However, some died beginning at 25 weeks of age (Supporting Fig. S1B). A proportion of Del-R26<sup>Met</sup> mice spontaneously developed liver-specific tumors (Supporting Fig. S1C), which were identified as HCC through histopathological analysis (Supporting Fig. S1D). Based on these findings, whereas most tissues are competent to buffer enhanced RTK Met levels, liver cells are sensitive. Moreover, the wild-type form of Met, not just its oncogenic forms, is sufficient to induce spontaneous tumorigenesis when its expression is enhanced above endogenous levels.

To generate hepatocyte-specific conditional R26<sup>Met</sup> mice, we crossed Alb-Cre transgenics with R26<sup>stopMet</sup> mice (referred to as Alb-R26<sup>Met</sup>). The specificity of LacZ-stop cassette deletion in the liver was supported by the use of mice carrying a modified version of the R26<sup>stopMet</sup> construct, in which Met<sup>tg</sup> was followed by an internal ribosome entry site–luciferase reporter (R26<sup>stopMet-IRES-Luciferase</sup>, referred to as R26<sup>stopMet-Luc</sup>; Supporting Fig. S2A). In vivo imaging of Alb-R26<sup>Met-Luc</sup> mice confirmed liver-specific luciferase expression (Supporting Fig. S2B). Western blot analysis revealed increased Met<sup>tg</sup> expression in Alb-R26<sup>Met</sup> livers over time after birth (Supporting Fig. S2C), consistent with the reported Alb-Cre expression. Macroscopic tumors were observed in 42%, 66%, and 79% of Alb-R26<sup>Met</sup> mice at the ages of 40-48, 49-67, and >67 weeks, respectively (Fig. 1A,B). According to anatomo-pathological analysis, the majority of tumors range from poorly differentiated to well-differentiated HCC (Supporting Fig. S2D,E and Table S2). Histological analyses showed that Alb-R26<sup>Met</sup> tumors express Met<sup>tg</sup>.
and contain a proportion of cells with active Met (Supporting Fig. S3A). We compared Met expression levels in Alb–R26\textsuperscript{Met} (n = 32) and human (n = 249; from Tao et al\textsuperscript{(19)} liver tumors as well as in Alb–R26\textsuperscript{Met} (n = 6; see below) and human (n = 8) HCC cell lines. Met levels in the Alb–R26\textsuperscript{Met} genetic setting (3.16 ±
0.06 versus control livers) correspond to those found in 20% of HCC patients (48/249; Fig. 1C; Supporting Table S3). Quantitative RT-PCR analyses of 32 Alb-R26Met HCC compared to 5 control livers facilitated accurate marker characterization, revealing consistent up-regulation of Mki67 (proliferative marker), alphafetoprotein, and glypican3 (HCC markers; the former when expressed in adult livers), as well as Igf2bp3, Nream, Cd20, Cd24 (HCC prognosis markers), Prom1, Cadb1, Notch3, Notch4, Vim, Jag1, and Sox9 (progenitor markers); however, Glut, Oat, Lgr5, Tbx3, Pek1, and Reg3a (Wnt/β-catenin pathway) were down-regulated (Fig. 1D–F; Supporting Table S4). Based on these findings, Alb-R26Met tumors belong to the so-called proliferative HCC group; and within this group, they correspond to the progenitor subclass rather than to the Wnt subgroup. (20)

Next, we performed a series of biochemical studies to characterize Met-signaling levels. In Alb-R26Met tumors, Met is expressed, and its expression correlates with a concomitant increase in Met phosphorylation levels on Tyr1234-1235, Tyr1003, and Tyr1349 (Fig. 1G). (21,22) This is accompanied by increased phosphorylation of Erk, but not Akt, compared to control livers (Fig. 1G). We then explored quantitative and qualitative Met levels in cells prior to tumorigenesis using primary hepatocytes from E15.5 wild-type and Del-R26Met livers. Quantification analysis indicated an approximately 3-fold increase in Met expression levels in Del-R26Met hepatocytes compared to controls (Fig. 1H), consistent with quantitative RT-PCR data shown in Fig. 1F and with Fan et al. (13) Interestingly, qualitative analysis of the phosphorylation status of Met revealed (1) high phosphorylation levels of Tyr1234-1235, which are not further enhanced upon hepatocyte growth factor (HGF) stimulation, and (2) that phosphorylation of Tyr1349 and Tyr1356 is dependent on HGF stimulation (Fig. 1I,J). Thus, subtle increases in Met expression in hepatocytes (3-fold) confer a poised state upon the receptor: Met is active (as shown by high phosphorylated Tyr1234-1235 [pTyr1234-1235] levels) but not fully competent for signaling, which is conditioned by HGF stimulation. Based on these data, Alb-R26Met mice represent a genetic model of tumorigenesis in which vulnerable cells are challenged by subtle increases in RTK levels rather than the overexpression of an oncogenic form.

TUMORIGENESIS IN Alb-R26Met IS NOT Met-ADDICTED

To assess whether Alb-R26Met HCC correspond to Met-addicted tumorigenesis, we generated several HCC cell lines from individual Alb-R26Met tumors (referred to as Alb-R26Met HCC cells; Fig. 2A). Alb-R26Met HCC cells are of liver origin and express alpha-fetoprotein in contrast to mouse embryonic fibroblasts, which were used as a negative control (Fig. 2B; Supporting Table S5). A consistent 3-fold increase in Met expression; the up-regulation of Mki67, Prom1, Cadb1, Igf2bp3, Cd20, Nream, and Cd24; and the down-regulation of Lgs, Tbx3, Glut,
FIG. 2. Tumorigenesis modeled by Alb-R26<sup>Met</sup> mice is not Met-addicted. (A) Alb-R26<sup>Met</sup> liver tumors (arrowheads) were used either for the phosphokineome screen or to establish Alb-R26<sup>Met</sup> HCC cell lines. After characterization, Alb-R26<sup>Met</sup> HCC cells were used for drug screening, biochemistry, biological assays, and xenografts. (B) Quantitative RT-PCR analysis showing that Alb-R26<sup>Met</sup> HCC cells are of liver origin because they express Albumin, and Alpha-fetoprotein in contrast to mouse embryonic fibroblasts. (C) Expression levels of the indicated markers in six different Alb-R26<sup>Met</sup> HCC cell lines compared to control livers (the same controls used for analysis reported in Fig. 1F; green, down-regulated genes; violet, up-regulated genes). (D) Western blot analysis of total protein extracts from six different Alb-R26<sup>Met</sup> HCC cells. Note consistent phosphorylation levels of pSer217-221Mek, pThr202Tyr204Erks, pSer473Akt, and pThr421-424p70S6K in different Alb-R26<sup>Met</sup> HCC cell lines. (E) In vitro and in vivo characterization of Alb-R26<sup>Met</sup> HCC cell tumorigenic properties using anchorage-independent growth assays (soft agar) and xenografts, respectively. (F) Dose–response effects of Alb-R26<sup>Met</sup> HCC cells to the Met inhibitor PHA-665752 or crizotinib. In all figures, values indicate the percentage of cell viability in the presence of drugs compared to controls (untreated cells); values are expressed as the mean ± standard error of the mean; cell number is also reported in different colors corresponding to drug effects (scale is shown on the right and is used as reference in all studies); gray squares correspond to untested drug effects. Abbreviation: EMFI, mouse embryonic fibroblast.
and Oat characterized \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cells compared to control livers (Fig. 2C; Supporting Table S6), similar to \(\text{Alb}–\text{R}26^{\text{Met}}\) tumors (Fig. 1F). Western blot analyses confirmed Met expression and phosphorylation of Met as well as downstream signals in \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cells (Fig. 2D). According to anchorage-independent growth assays and xenografts in nude mice, \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cells retain tumorigenic properties (Fig. 2E). We then explored whether \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cells are addicted to Met and found that none of them are fully sensitive to Met inhibitors (Fig. 2F; Supporting Fig. S3B). This is consistent with our genetic strategy, which was based on use of the wild-type, rather than the oncogenic, form of Met as well as a subtle increase in Met expression levels. Given these results, the tumorigenesis modeled by \(\text{Alb}–\text{R}26^{\text{Met}}\) mice is characterized not by Met addiction but rather by a signaling context originating from, and associated with, subtle increase in RTK levels.

**SIGNALLING PERTURBATIONS IN RTK-DRIVEN TUMORIGENESIS IDENTIFIED IN A PHOSPHOKINOME SCREEN, LEADING TO THE DISCOVERY OF NEW SYNTHETIC LETHAL INTERACTIONS**

To undertake an unbiased analysis of hundreds of signaling proteins in tumors linked to subtle enhanced RTK levels, we employed a phosphokinome screen based on protein antibody microarrays (Kinex Antibody Microarray). We performed these experiments using liver tumors dissected from \(\text{Alb}–\text{R}26^{\text{Met}}\) mice rather than \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cells to identify signaling changes occurring \textit{in vivo} and to take into account the context of intrinsic tumor heterogeneity (Fig. 3A). The signaling profiles of \(\text{Alb}–\text{R}26^{\text{Met}}\) tumor (n = 19) versus control (n = 7) pools were analyzed by comparing the levels of phosphorylated signals (~300 epitopes) and kinase expression levels (~500). Among 118 identified changes, 43 were top-ranked for further validation based on their fold change, low error rate, and high antibody quality (Supporting Table S7). By performing a Kinetworks custom multiantibody screen, we confirmed changes in 26 signals between control and tumor pools by western blotting (Supporting Table S7). Analyses of expression and/or phosphorylation levels of 23 signals in individual tumor and control samples revealed consistent trends in the changes for distinct signals (using Kinetworks custom multisample comparison; Supporting Fig. S4A and Table S8). When we bioinformatically analyzed interactions between the 43 top-ranked signaling components, the majority formed an interactome characterized by multiple links between signals, a “signaling node” (p53), and several components of the Ras pathway (Fig. 3B). We next asked whether the \(\text{Alb}–\text{R}26^{\text{Met}}\) interactome could be used to predict vulnerable signaling points for HCC cells, taking into account three hypotheses. (1) The intense network of interactions between components may reflect the redundancy of certain signals during inhibition, and it may therefore be necessary to target several points to destabilize the interactome. (2) The interactome may be composed of a very restricted set of sensitive points required for the tumorigenic properties of cells among the most abundant changes that may be either irrelevant or linked to specific biological properties. (3) As tumor cells demonstrate a greater dependence on stress support pathways than normal cells, it may be necessary to simultaneously destabilize the interactome while targeting stress support pathways. Taking into account these three hypotheses, we designed an “educated guess” drug screen and assessed the effects of 42 treatments (as single drugs or two-drug combinations; Supporting Tables S9 and S10). \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC1 cells (not responding to Met inhibitors) were used for an unbiased search of drugs targeting HCC modeled by the \(\text{Alb}–\text{R}26^{\text{Met}}\) genetic setting. A dose–response screen was performed by administering drugs under optimal culture conditions (10% serum) for 48 hours; cell numbers were measured in a metabolic activity–based cell viability assay. Two out of the 42 treatments were deleterious for cells: the combined inhibition of Mek (by PD184161) or Cdk1/2 (by JNJ-7706621) together with the mitochondrial stress support pathway (by ABT-737) was not permissive for \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cell viability, whereas single treatments did not have significant effects (the Mek inhibitor elicited only a partial response at higher doses; Fig. 3C,D; Supporting Fig. S4B,C). Importantly, combined treatments of ABT-737 and Fas ligand or tumor necrosis factor-α did not induce \(\text{Alb}–\text{R}26^{\text{Met}}\) HCC cell death, excluding the possibility that ABT-737 sensitizes cells to any type of death-inducing signal (Supporting Fig. S5A). Overall, our phosphokinome screen combined with functional analyses identified Mek or Cdk1/2 together with the stress support pathway as a druggable synthetic lethal interaction.
FIG. 3. Phosphokinome screening identifies changes in interconnected signals in Alb-R26<sup>Met</sup> tumors, leading to the discovery of new synthetic lethal interactions. (A) Schematic diagram summarizing the strategy employed to identify signaling changes between Alb-R26<sup>Met</sup> tumor and control liver samples and the obtained results. (B) The interactome of top-ranked signals altered in Alb-R26<sup>Met</sup> tumors. Altered signals that are not part of this interactome are also reported (bottom right). Signals for which inhibitory effects were tested in Alb-R26<sup>Met</sup> HCC cells are highlighted in blue. (Top right) Additional signals not part of the interactome that were also targeted. (C) Schematic representation of the drug screen outcomes, highlighting single and combined treatments tested. (D) Percentage of cell viability following the drug screen (3 μM) performed in Alb-R26<sup>Met</sup> HCC cells. In (C) and (D), note that two drug combinations (in orange) are deleterious for Alb-R26<sup>Met</sup> HCC cells among others exhibiting no or minimal effects (green).
Mek, RIBOSOMAL S6 KINASE, OR Cdk1/2, TOGETHER WITH Bcl-XL, ARE SYNERGISTIC DRUG TARGETS IN HCC CELLS MODELED BY THE \textit{Alb-R26}^{\text{Met}} GENETIC SYSTEM

Next, we explored the net contribution of distinct components of the Ras and stress support pathways in a panel of \textit{Alb-R26}^{\text{Met}} HCC cell lines. In terms of the Ras pathway, the inhibition of Mek and ribosomal S6 kinase (Rsk), but not Raf and Erk2, together with the stress support pathway is deleterious for all \textit{Alb-R26}^{\text{Met}} HCC cell lines tested (Fig. 4A; Supporting Fig. S5B). Notably, the \textit{Alb-R26}^{\text{Met}} HCC cell lines are resistant to sorafenib, a standard care for HCC (Fig. 4A). Regarding the mitochondrial stress support pathway, we compared the effectiveness of ABT-737 (targeting Bcl-2, Bcl-XL, Bcl-w) with that of ABT-199 (targeting Bcl-2) and WEHI-539 (targeting Bcl-XL). The inhibition of Bcl-XL, but not Bcl-2, together with Mek or Cdk1/2 was detrimental for \textit{Alb-R26}^{\text{Met}} HCC cells (Fig. 4B). Thus, Bcl-XL inhibition confers \textit{Alb-R26}^{\text{Met}} HCC cell sensitivity to Mek or Cdk1/2 targeting. Based on the Chou-Talalay additivity–based combination index score, combinations of Mek, Rsk, or Cdk1/2 together with Bcl-XL inhibition to counteract HCC cell tumorigenesis and raise the possibility of applying these new synthetic lethal interactions to human HCC subgroups.

COMBINED Mek AND Bcl-XL INHIBITION TRIGGERS THE DOWN-REGULATION OF PHOSPHO-Erk AND MYELOID CELL LEUKEMIA 1 AND CAUSES \textit{Alb-R26}^{\text{Met}} HCC CELL DEATH

We next focused on the lethal synergistic effects of Mek and Bcl-XL inhibition on \textit{Alb-R26}^{\text{Met}} HCC cells and investigated the underlying mechanism of action. Treatment of cells with ABT-737 led to a strong increase in Erk phosphorylation levels, which was abrogated in the presence of selumetinib (Fig. 6A; Supporting Fig. S7C). Concomitantly, ABT-737 predisposed cells to apoptosis as revealed by cleaved Caspase3, levels of which were significantly enhanced by selumetinib cotreatment (Fig. 6A; Supporting Fig. S7C). Analysis of several proapoptotic and antiapoptotic regulators revealed drastic down-regulation of the antiapoptotic myeloid cell leukemia 1 (Mcl1) protein following combined ABT-737 and selumetinib treatment, whereas other Bcl-2 family members were not affected (Fig. 6A; Supporting Fig. S7C). Analysis of several proapoptotic and antiapoptotic regulators revealed drastic down-regulation of the antiapoptotic myeloid cell leukemia 1 (Mcl1) protein following combined ABT-737 and selumetinib treatment, whereas other Bcl-2 family members were not affected (Fig. 6A; Supporting Fig. S7C). Next, we analyzed the impact of drug treatments on \textit{Alb-R26}^{\text{Met}} HCC cell cycle progression. The percentage of cells in \text{G}_{0}/\text{G}_{1} phase was increased at the expense of those in \text{S} phase in the presence of selumetinib or ABT-737 plus selumetinib but not of ABT-737 alone (Fig. 6B). Thus, this new synthetic lethal interaction is likely to
FIG. 4. Mek, Rsk, or Cdk1/2 together with Bcl-XL are synergistic drug targets that are lethal for Alb-R26Met HCC cells. (A) Dose-response effects of ABT-737 alone or in combination with drugs targeting distinct Ras effectors on Alb-R26Met HCC3 and HCC13 cells after 48 hours of treatment. In (A) and (B), single drug effects are reported on the right. Note that the effects of Mek inhibition were tested using PD184161 and selumetinib compared to Raf inhibition, which was tested using GW5047 and sorafenib (which also targets vascular endothelial growth factor receptor, Kit, Flt3, and platelet-derived growth factor receptor). (B) Dose–response effects of WEHI-539 (targeting Bcl-XL) and ABT-199 (targeting Bcl-2) alone or in combination with selumetinib, BI-D1870, and JNJ-7706621 on Alb-R26Met HCC3 and HCC13 cells. (C,D) Drug effects were measured by calculating the Chou-Talalay additivity–based combination index score to categorize effects as antagonistic (>1), additive (1), or synergistic (<1). Using data obtained from cell viability measurements of single and combined drug effects, we used Compusyn software to calculate dose–response curves for individual and combined drugs (C) and for the computed simulation of different combination index values for each affected fraction (D). In (C), dose–response curves show the effects of single and combined drug doses (x axis on a logarithmic scale; in micrometers) according to different drug doses (x axis on a logarithmic scale; in micrometers). In (D), plots show the simulated calculation of the combination index (y axis) for each of the affected fractions (x axis, from 0 to 1) treated with drug combinations. Each black dot corresponds to a tested dose. Based on the combination index scores, combinations of Mek, Rsk, and Cdk1/2 with Bcl-XL inhibition resulted in strong synergistic interactions. (E,F) Combined ABT-737 and selumetinib treatment significantly reduces tumor volume (E) in nude mice injected subcutaneously with Alb-R26Met HCC13 cells compared to mice that were either untreated or treated with individual drugs. Tumor volume changes are shown in (F). Dots correspond to tumors from individual mice (vehicle, 15 mice; ABT-737, 16 mice; selumetinib, 11 mice; ABT-737 + selumetinib, 15 mice). Significant differences between groups are indicated at the top. Abbreviations: A, ABT-737; BI, BI-D1870; Cay, Cay10561; GW, GW5047; JNJ, JNJ-7706621; PD, PD184161; S, selumetinib; Selu, selumetinib; Sora, sorafenib.
ensure concomitant impairment of Erk signaling and cell cycle progression while potentiating apoptosis through Mcl1 down-regulation. The functional relevance of Mcl1 down-regulation for the synthetic lethal interaction between Mek and Bcl-XL inhibition was investigated through rescue experiments. Mcl1...
FIG. 6. Combined Mek and Bcl-XL inhibition leads to the concomitant down-regulation of phospho-Erk and the antiapoptotic Mcl1 protein in Alb-R26<sup>Met</sup>HCC cells. (A) Western blots showing the effects of ABT-737, selumetinib, and combined treatments at 3 μM on different signals in Alb-R26<sup>Met</sup>HCC1 (left) and HCC3 (right) cells after 3 and 6 hours of treatment. (Top) pErk, Erk, and cleaved Caspase3 levels (pErk panels correspond to short exposure times during which basal levels, documented in Figs. 1G and 2D, are barely visible). (Bottom) Analysis of different proapoptotic and antiapoptotic regulators. (B) Representative graphs for cell cycle analysis of Alb-R26<sup>Met</sup>HCC1 and HCC3 cells untreated or treated with the indicated drugs. (C) Mcl1 protein levels in Alb-R26<sup>Met</sup>HCC cells transfected with Mcl1 expression plasmid compared to controls (Alb-R26<sup>Met</sup>HCC<sub>overMcl1</sub>). Stable clones were used to assess molecular and functional properties of Alb-R26<sup>Met</sup>HCC<sub>overMcl1</sub> compared to control cells. (D) Cleaved Caspase3 and Mcl1 protein levels in Alb-R26<sup>Met</sup>HCC13 and Alb-R26<sup>Met</sup>HCC13<sub>overMcl1</sub> cells. (E) Combined Mek and Bcl-XL inhibition interferes with the viability of Alb-R26<sup>Met</sup>HCC13 cells but not Alb-R26<sup>Met</sup>HCC13<sub>overMcl1</sub> cells. Abbreviations: A, ABT-737; ABT, ABT-737; cDNA, complementary DNA; S, selumetinib; Sel/Selu, selumetinib.
overexpression interfered with Caspase3 activation and cell death triggered by ABT-737 plus selumetinib (Fig. 6C-E; Supporting Fig. S7D).

**HIGH LEVELS OF pERK, BCL-XL, AND MCL1 CHARACTERIZE Alb-R26<sup>Met</sup> TUMORS AND A SUBGROUP OF HCC PATIENTS**

High levels of pMEK1 have been detected in 49% of HCC samples. High MCL1 levels have been reported in approximately 50% of HCC patients, with an intriguing significant correlation with BCL-XL expression. However, a putative correlation between high levels of MCL1/BCL-XL and MEK/ERK deregulation has never been explored. Remarkably, Alb-R26<sup>Met</sup> tumors were characterized by increased levels of pMek, pErk, Bcl-XL, and Mcl1 (Fig. 7A). Comparable levels of two other antiapoptotic members of the Bcl-2 family, Bcl-2 and Bcl-w, in control livers and tumors corroborate the impact of targeting Bcl-XL (Fig. 7A). Next, we asked whether there is a clinical correlation between high levels of pMek/pErk, Bcl-XL, and Mcl1 in human HCC by analyzing a total of 116 patients. pERK, rather than pMEK, levels were analyzed as antibodies were of superior quality and reliability for sample evaluation by immunostaining (both pMek and pErk are up-regulated in Alb-R26<sup>Met</sup> tumors). Eighty-one HCC samples were positive for pERK (70%), 86 for BCL-XL (~74%), and 62 for pERK (~53%; Fig. 7B,C). When considering triple-positives for pERK/<sup>+</sup>/BCL-XL/<sup>+</sup>/MCL1<sup>+</sup>, we identified 38 HCC patients (~33%; Fig. 7B,C). Next, we looked at the association of triple-positives with Ki67 index and the main recognized molecular categories of HCC (p53<sup>+</sup>, glutamine synthetase (GS)/β-catenin<sup>+</sup>, double-positive p53 and GS/β-catenin, and null; Supporting Fig. S8). For triple-positive cases, 42% are also positive for Ki67, although this correlation is not statistically significant. In addition, triple-positive cases had an association with poor HCC differentiation (grade 3-4 according to Edmondson; P = 0.008). Moreover, there is a statistically significant positive correlation with p53<sup>+</sup> HCC and a negative correlation with null phenotype (Supporting Table S11). Remarkably, there is a striking correlation between pERK<sup>+</sup>/BCL-XL<sup>+</sup>/MCL1<sup>+</sup> triple cases with poor overall survival (hazard ratio, 2.12; 95% confidence interval, 1.10-4.07; P = 0.023) and with disease-free survival (hazard ratio, 2.08; 95% confidence interval, 1.23-3.49; P = 0.006; Fig. 7D,E). These results are supported by bioinformatics integrative analyses using outcomes from microarray studies revealing that ~27% and ~22% of patients (in GSE14323 and GSE14520, respectively) are MCL1<sup>+</sup>/BCL-XL<sup>+</sup>, and all of them (except one patient in GSE14323) are positive also for ERK1/ERK2 (Supporting Table S12). Thus, the pERK<sup>+</sup>/BCL-XL<sup>+</sup>/MCL1<sup>+</sup> signature characterizes a subgroup of HCC patients with poor prognosis.

**Discussion**

Modeling human tumors in mice in combination with genome-wide screening and bioinformatics enables the highly precise tracking of molecular mechanisms underlying tumorigenesis. Genetically modified mice carrying loss-of-function mutations in key tumor suppressors and/or overactivated forms of oncogenes mimic certain types of human tumors. However, such acute manipulations generally do not recapitulate the physiopathology observed in patients, in whom progressive, subtle alterations impact tumor initiation, maintenance, and evolution. Here, we show that nononcogenic RTKs are competent to trigger tumorigenesis when their expression is moderately enhanced above endogenous levels. However, vulnerability to enhanced RTK inputs is restrained to very specific cell types, as observed in Del-R26<sup>Met</sup> mice. These results demonstrate the unique sensitivity of each tissue to subtly increased RTK levels, which discriminates resilience versus vulnerability during tissue homeostasis perturbation and cell transformation. Also, they show how slight changes in nononcogenic signaling inputs may have profound consequences in cells, which become pathological by triggering the full tumorigenic program in the liver. Based on our biochemical studies in hepatocytes, the Met receptor exists in a poised state: the strong phosphorylation status of the two tyrosine residues in the kinase domain indicates that Met is active, although not fully signaling-competent, as phosphorylation of the multifunctional docking site <sup>21,22</sup> is conditioned by HGF stimulation. This poised state of Met likely challenges basal signaling mechanisms in hepatocytes, generating instability over time as revealed by large cell dysplasia foci at early stages. The tumorigenic program in Alb-R26<sup>Met</sup> mice likely results from the reprogramming of molecular settings triggered by enhanced RTK levels rather than from the acquisition of Met addiction. The competence of Met to reprogram signaling and biological events is consistent with its pleiotropic functions in...
developmental programs as well as in tumor evolution/resistance to therapies. The implications for oncogenic Met in liver cancer are supported by reports showing: (1) Met activation in approximately 50% of HCC patients, correlating with poor prognosis, although Met genetic mutations are rare; (2) a cell–cell crosstalk in which deregulated vascular endothelial growth factor A in HCC cells signals to HGF-producing macrophages to trigger tumor cell proliferation; (3) tumorigenesis in mice carrying either oncogenic Met forms or concomitant alterations of HGF/Met with other genes; (4) accelerated
in 20% of HCC patients. Therefore, the Alb–R26Met genetic setting demonstrates the dramatic consequence of moderately increased levels of wild-type Met for triggering the tumorigenic program. Moreover, in the context of high HCC heterogeneity, Alb–R26Met mice exemplify HCC patients corresponding to the progenitor cell group of the proliferative subset, which is associated with poor outcomes.

The challenge to identify cancer cell vulnerabilities is evidenced by their resilience to the blockade of several operating signaling circuits and by their capacity to acquire drug resistance over time. These features are critical limits to the broadening of anticancer-targeted treatments. In most cases, the effectiveness of molecular therapies is conditioned by the identification of oncogenic alterations that contribute to the addiction of cancer cells. Exceptional cases of effectiveness are represented by the inhibition of BCR-ABL for the treatment of chronic myeloid leukemia, ERBB2 for breast cancer, ERBB1 for non-small-cell lung cancer, and B-RAF for metastatic melanoma. However, in most other types of cancer, the long-term efficiency of targeted treatment remains unsatisfactory for several reasons, including the underestimated relevance of certain protein functions compared to others, the existence of redundancy and crosstalk between pathways, and the cytostatic rather than cytotoxic effects elicited by drugs. Several treatments involving drug combinations lead effective responses in cancer cells. A promising strategy to broaden the use of molecularly targeted therapies for cancer treatment is inspired by the concept of synthetic lethality, which reflects the deleterious effects of simultaneously targeting separate signals that are individually nonlethal on cells. The synthetic lethality approach may be particularly promising for the treatment of HCC, one of the most heterogeneous cancers characterized by poor clinical outcomes and a lack of effective therapies. In contrast to other carcinomas, in which tumor initiation and progression are triggered by mutations in a subset of oncogenes and/or tumor suppressors, a wide range of (epi)genetic mutations have been identified in HCC. How to translate this knowledge into therapeutic interventions remains unclear. The identification of functionally relevant targets in HCC is complicated because signals such as RTK pathways are rarely genetically mutated, although their activation is observed in a high proportion of HCC. Mice carrying genetic alterations found in human HCC have been instrumental for validating the roles of certain signaling mechanisms in this disease. However, agents targeting these pathways have been unsatisfactory in clinical trials due to their predominantly cytostatic rather than cytotoxic effects. The unbiased phosphokinome screen we performed in Alb–R26Met mice highlighted vulnerable nodes and identified synthetic lethal interactions between Mek, Rsk, or Cdk1/2 and Bcl-XL inhibition in HCC. These combined treatments elicit strong synergistic effects, whereas single blockage induces minimal effects. Based on our mechanistic studies, combined Mek and Bcl-XL inhibition suppresses Erk and Mcl1 signaling, whereas individual targeting exerts different effects (Fig. 8). Bcl-XL inhibition alone primes cells toward cell death, as shown by slight Mcl1 down-regulation and moderate Caspase3 activation, although proliferating cells have an enhanced pErk. Instead, Mek inhibition alone impairs Erk signaling and affects cell cycle progression, without influencing cell death. Thus, the combined effects of Mek and Bcl-XL inhibition are effective in HCC cells through the triggering of cytotoxic effects. Our results are consistent with studies showing that antibodies targeting the calcium channel zδ1 subunit interfere with tumor-initiating properties through pErk and Bcl-2 down-regulation. The functional relevance of the deregulated Ras pathway and Bcl-XL inputs in the context of Mcl1 overexpression in HCC biology is consistent with their concomitant alteration in a subgroup of HCC patients with poor prognosis. Thus, the synergistic effects of Mek and Bcl-XL inhibition previously observed in K-Ras mutant cancer cell lines may represent a therapeutic strategy that is also effective for the treatment of HCC subgroups.

According to our studies, inhibition of the Ras pathway at distinct vertical levels does not exert equivalent effects, at least in terms of HCC tumorigenesis modeled by the Alb–R26Met system. This is consistent with knowledge that the Ras pathway is not a simple linear cascade and that each component receives distinct regulatory inputs and is exposed to compensatory circuits. Sorafenib, which targets Raf-1 and B-Raf as well as several RTKs such as vascular endothelial growth factor receptor, Kit, Flt3, and platelet-derived growth factor receptor, is a standard treatment for HCC. In light of our findings, it may be relevant to assess the effectiveness of selumetinib or sorafenib treatment in combination with Bcl-XL inhibition in enriched trials.
The synthetic lethal interaction we identified reveals how the inhibition of multiple pathways with combined drug cocktails may be a successful strategy for HCC treatment and deserves further studies to optimize doses and to establish the potential (and limits) for the treatment of HCC subgroups. Drugs targeting vulnerable points emerging from the Alb-R26Met cancer model are very promising anticancer agents for molecularly targeted therapies and should be actively explored in clinical trials. Therefore, our discoveries may widen their use as synergistic treatments for HCC patient subgroups. It will be essential to stratify HCC patients who may benefit from such synergistic treatments, although patient stratification is not a simple issue. Sensitivity versus resistance to this treatment is likely linked not only to the presence of the pErk⁺/Bcl-XL⁺/Mcl1⁺ signature, found in Alb-R26Met mice and in a proportion of HCC patients, but also to other signaling regulators of HCC biology. In conclusion, our studies illustrate how the use of cancer mouse models such as Alb-R26Met mice, in which cell homeostasis is challenged by signaling fluctuations over time, uncovers “model-guided signatures” for patient stratification as a complementary strategy to broad sequencing screens in human cancer. In addition to recapitulating the molecular mechanisms underlying tumorigenesis and highlighting vulnerable signals, defined genetic settings may be valuable systems to explore signaling cooperation during the transition from healthy cells to transformation.

Acknowledgment: We are particularly grateful to F. Helmbacher for extremely valuable feedback on the study. We thank all members of our labs for helpful discussions and comments; V. Girod-David and L. Jullien for excellent help with mouse husbandry; M. Buferne for in vivo bioluminescence imaging; P. Grenot (CIPHE US12) for fluorescence-activated cell sorting analysis; C. Brun for advice and discussions about interactome analyses; E. Charabaszcz for contributions to the molecular characterization of Alb-R26Met HCC cell lines and to anchorage-independent growth assays with HLF cells; G. Couchy for marker characterization of Alb-R26Met tumors and HCC cells; H. Hassani for contributing to mouse model generation; N. Gadot and J.Y. Scoazec at ANIPATH, Université Lyon 1 (Lyon, France), for their initial help regarding the histopathological analysis of Alb-R26Met tumors; I. Fabregat for providing HLF cells; and A. Fatmi for advise on quantitative PCR analysis.
REFERENCES

1) Sun C, Bernards R. Feedback and redundancy in receptor tyrosine kinase signaling: relevance to cancer therapies. Trends Biochem Sci 2014;39:465-474.

2) Maina F. Strategies to overcome drug resistance of receptor tyrosine kinase addicted cancer cells. Curr Med Chem 2014;21:1607-1617.

3) Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008;455:1061-1068.

4) Bild AH, Yao G, Chang JT, Wang Q, Potti A, Chasse D, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature 2006;439:353-357.

5) Logue JS, Morrison DK. Complexity in the signaling network: insights from the use of targeted inhibitors in cancer therapy. Genes Dev 2012;26:641-650.

6) Furlan A, Lamballe F, Stagni V, Hussain A, Richelme S, Prodosno A, et al. Met acts through Abl to regulate p53 transcriptional outcomes and cell survival in the developing liver. J Hepatol 2012;57:1292-1298.

7) Furlan A, Stagni V, Hussain A, Richelme S, Conti F, Prodosno A, et al. Abl interconnects oncogenic Met and p53 core pathways in cancer cells. Cell Death Diff 2011;18:1608-1616.

8) Lamballe F, Toscano S, Conti F, Areschiderra M, Baesa N, Figarella-Branger D, et al. Coordination of signalling networks and tumorigenic properties by ABL in glioblastoma cells. Oncotarget 2016;7:17477-17476.

9) Bertotti A, Burbridge MF, Gastaldi S, Galimi F, Torti D, Medico E, et al. Only a subset of Met-activated pathways are required to sustain oncogene addiction. Sci Signal 2009;2:e211.

10) Miller ML, Molinelli EJ, Nair JS, Sheikh T, Samy R, Jing X, Hussain A, et al. Combined drug action of 2-phenylimidazo[2,1-b]benzothiazole derivatives on cancer cells according to their oncogenic molecular signatures. PLoS One 2012;7:e46738.

11) Tao J, Xu E, Zhao Y, Singh S, Li X, Coughy G, et al. Modeling a human hepatocellular carcinoma subset in mice through co-expression of Met and point-mutant beta-catenin. Hepatology 2016;64:1587-1605.

12) Llovet JM, Villamena A, Lachenmayer A, Finn RS. Advances in targeted therapies for hepatocellular carcinoma in the genomic era. Nat Rev Clin Oncol 2015;12:408-424.

13) Ponzetto C, Bardelli A, Zhen Z, Maina F, dalla Zonca P, Giordano S, et al. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell 1994;77:261-271.

14) Maina F, Casagranda F, Audero E, Simeone A, Comoglio P, Klein R, et al. Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. Cell 1996;87:531-542.

15) Chen L, Shi Y, Jiang CY, Wei LX, Wang YL, Dai GH. Expression and prognostic role of pan-Ras, Raf-1, pMEK1 and pERK1/2 in patients with hepatocellular carcinoma. Eur J Surg Oncol 2011;37:513-520.

16) Sieghart W, Losert D, Strommer S, Cejka D, Schmid K, Rassoul-Rockenschaub S, et al. Mc1r overexpression in hepatocellular carcinoma: a potential target for antisense therapy. J Hepatol 2006;44:151-157.

17) Maina F, Hilton MC, Andres R, Wyatt S, Klein R, Davies AM. Multiple roles for hepatocyte growth factor in sympathetic neuron development. Neuron 1998;20:835-846.

18) Maina F, Hilton MC, Ponzetto C, Davies AM, Klein R. Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. Genes Dev 1997;11:3341-3350.

19) Maina F, Klein R. Hepatocyte growth factor—a versatile signal for developing neurons. Nat Neurosci 1999;2:213-217.

20) Lamballe F, Genestine M, Caruso N, Arce V, Klein R, Helmbacher F, et al. Pool-specific regulation of motor neuron survival by neurotrophic support. J Neurosci 2011;31:11144-11158.

21) Caruso N, Herberth B, Lamballe F, Arce-Gorvel V, Maina F, Helmbacher F. Plasticity versus specificity in RTK signalling modalities for distinct biological outcomes in motor neurons. BMC Biol 2014;12:56.

22) Knudsen BS, Vande Woude G. Showering c-MET-dependent cancers with drugs. Curr Opin Genet Dev 2008;18:87-96.

23) Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. Nat Rev Mol Cell Biol 2010;11:834-848.

24) Gherardi E, Birchmeier W, Birchmeier C, Vande Woude G. Targeting MET in cancer: rationale and progress. Nat Rev Cancer 2012;12:89-103.

25) Kaposi-Novak P, Lee JS, Gomez-Quiroz L, Coulouarn C, Factor VM, Thorgeirsson SS. Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype. J Clin Invest 2006;116:1582-1595.

26) Goyal L, Muzzumdar MD, Zhu AX. Targeting the HGF/c-MET pathway in hepatocellular carcinoma. Clin Cancer Res 2013;19:2310-2318.

27) Park WS, Dong SM, Kim SY, Na EY, Shin MS, Pi JH, et al. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinoma. Cancer Res 1999;59:307-310.

28) Horwitz E, Stein I, Andreozzi M, Nemeth J, Shoham A, Pappo O, et al. Human and mouse VEGFα-amplified hepatocellular carcinomas are highly sensitive to sorafenib treatment. Cancer Discov 2014;4:730-743.
Hu J, Che L, Li L, Pilo MG, Cigliano A, Ribback S, et al. Co-activation of AKT and c-Met triggers rapid hepatocellular carcinoma development via the mTORC1/FASN pathway in mice. Sci Rep 2016;6:20484.

Ivanovska I, Zhang C, Liu AM, Wong KF, Lee NP, Lewis P, et al. Gene signatures derived from a c-MET-driven liver cancer mouse model predict survival of patients with hepatocellular carcinoma. PLoS One 2011;6:e24582.

Sakata H, Takayama H, Sharp R, Rubin JS, Merlino G, LaRochelle WJ. Hepatocyte growth factor/scatter factor overexpression induces growth, abnormal development, and tumor formation in transgenic mouse livers. Cell Growth Differ 1996;7:1513-1523.

Tward AD, Jones KD, Yant S, Cheung ST, Fan ST, Chen X, et al. Distinct pathways of genomic progression to benign and malignant tumors of the liver. Proc Natl Acad Sci USA 2007;104:14771-14776.

Takami T, Kaposi-Novak P, Uchida K, Gomez-Quiroz LE, Conner EA, Factor VM, et al. Loss of hepatocyte growth factor/c-Met signaling pathway accelerates early stages of N-nitrosodiethylamine induced hepatocarcinogenesis. Cancer Res 2007;67:9844-9851.

Schulze K, Imbeaud S, Letouze E, Alexandrov LB, Calderaro J, Rebouissou S, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nat Genet 2015;47:505-511.

Janne PA, Gray N, Settleman J. Factors underlying sensitivity of cancers to small-molecule kinase inhibitors. Nat Rev Drug Discov 2009;8:709-723.

Prahallad A, Bernards R. Opportunities and challenges provided by crosstalk between signalling pathways in cancer. Oncogene 2016;35:1073-1079.

Li Y, Tang ZY, Hou JX. Hepatocellular carcinoma: insight from animal models. Nat Rev Gastroenterol Hepatol 2012;9:32-43.

Zhao W, Wang L, Han H, Jin K, Lin N, Guo T, et al. B50-1, a mAb raised against recurrent tumor cells, targets liver tumor-initiating cells by binding to the calcium channel alpha2-delta1 subunit. Cancer Cell 2013;23:541-556.

Corcoran RB, Cheng KA, Hata AN, Faber AC, Ebi H, Coffee EM, et al. Synthetic lethal interaction of combined BCL-XL and MEK inhibition promotes tumor regressions in KRAS mutant cancer models. Cancer Cell 2013;23:121-128.

Samatar AA, Poulikakos Pl. Targeting RAS-ERK signalling in cancer: promises and challenges. Nat Rev Drug Discov 2014;13:928-942.

Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008;359:378-390.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29304/suppinfo.