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ORIGINAL ARTICLE

Wild type Kirsten rat sarcoma is a novel microRNA-622-regulated therapeutic target for hepatocellular carcinoma and contributes to sorafenib resistance

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ABSTRACT

Objective Sorafenib is the only effective therapy for advanced hepatocellular carcinoma (HCC). Combinatory approaches targeting mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)- and phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K)/protein-kinase B(AKT) signalling yield major therapeutic improvements. RAS proteins regulate both RAF/MAPK and PI3K/AKT signalling. However, the most important RAS isoform in carcinogenesis, Kirsten rat sarcoma (KRAS), remains unexplored in HCC.

Design Human HCC tissues and cell lines were used for expression and functional analysis. Sorafenib-resistant HCC cells were newly generated. RNA interference and the novel small molecule deltarasin were used for KRAS inhibition both in vitro and in a murine syngeneic orthotopic HCC model.

Results Expression of wild type KRAS messenger RNA and protein was increased in HCC and correlated with extracellular-signal regulated kinase (ERK) activation, proliferation rate, advanced tumour size and poor patient survival. Bioinformatic analysis and reporter assays revealed that KRAS is a direct target of microRNA-622. This microRNA was downregulated in HCC, and functional analysis demonstrated that KRAS-suppression is the major mediator of its inhibitory effect on HCC proliferation. KRAS inhibition markedly suppressed RAF/ERK and PI3K/AKT signalling and proliferation and enhanced apoptosis of HCC cells in vitro and in vivo. Combinatory KRAS inhibition and sorafenib treatment revealed synergistic antitumorigenic effects in HCC. Sorafenib-resistant HCC cells showed elevated KRAS expression, and KRAS inhibition resensitised sorafenib-resistant cells to suppression of proliferation and induction of apoptosis.

Conclusions KRAS is dysregulated in HCC by loss of tumour-suppressive microRNA-622, contributing to tumour progression, sorafenib sensitivity and resistance. KRAS inhibition alone or in combination with sorafenib appears as novel promising therapeutic strategy for HCC.

Significance of this study

What is already known on this subject?

- RAS proteins regulate both RAF/MAPK and PI3K/AKT signalling. The RAS isoform Kirsten rat sarcoma (KRAS) is rarely mutated and therefore widely unexplored as an oncogenic target in hepatocellular carcinoma (HCC).
- The RAF/MAPK pathway is a major target of sorafenib, the only approved therapy for advanced HCC. However, even with positive initial response, most patients develop disease progression, and there is no effective second-line therapy approved for these patients.
- Sorafenib resistance is the main reason for therapeutic failure. The underlying mechanisms for (acquired) resistance to sorafenib are not yet elucidated.

What are the new findings?

- Wild type KRAS expression is enhanced in HCC and correlates with MAPK pathway activation and poor patient survival.
- KRAS expression in HCC is caused by downregulation of microRNA-622, which exhibits its suppressive function on HCC growth mainly by targeting KRAS.
- Acquired resistance to sorafenib leads to enhanced KRAS expression, and KRAS inhibition can overcome resistance to sorafenib.

How might it impact on clinical practice in the foreseeable future?

- This study provides experimental evidence that enhanced (wild type) KRAS promotes HCC progression and outlines a potential mechanism underlying resistance to sorafenib.
- Eventually, these findings can contribute to the design of clinical trials in patients with HCC to evaluate KRAS-inhibiting drugs alone or in combination with sorafenib in second-line treatment, a currently unmet medical need.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is commonly diagnosed at advanced stages, and in these cases, the multitarget tyrosine kinase inhibitor sorafenib is the only approved systemic therapy.[1 2] The time to radiological progression is merely ~3 months longer for patients treated with sorafenib than for those given placebo.[3–5] Moreover, almost every patient shows disease progression after initial drug response.[6–8]

The RAF/MAPK pathway is a major target of sorafenib.[5 9] Next to RAF/MAPK, the PI3K/AKT/

mTOR signalling axis is an emerging target in HCC contributing to disease progression and development of sorafenib resistance.[10] Therefore, combinatory approaches targeting both RAF/MAPK and PI3K/AKT signalling may yield major improvements in the management of HCC.[10]

Common upstream mediators of both RAF/MAPK and PI3K/AKT pathways are RAS proteins. RAS signalling in HCC is correlated with poor prognosis; however, mechanisms of RAS pathway activation in HCC and the potential therapeutic implications are just beginning to be elucidated.[1 11–13] Notably, the expression and the exact functions of the most important RAS isoform in carcinogenesis, Kirsten rat sarcoma (KRAS), are unknown in HCC progression. This may be explained by the fact that—compared with other types of cancer—KRAS is rarely mutated and therefore widely unexplored as an oncogenic target in HCC.[14]

In the present study, the expression, regulation and function of wild type KRAS in HCC was analysed. We found that KRAS is dysregulated in HCC by loss of the tumour-suppressive microRNA-622, which contributes to tumour progression. KRAS inhibition reduced HCC growth in vitro and in an experimental mouse model of HCC. Moreover, we found that KRAS modulates sorafenib sensitivity and resistance, indicating KRAS inhibition as a novel therapeutic strategy for HCC.

MATERIALS AND METHODS

Cells and cell culture

Primary human hepatocytes (PHH) were isolated and cultured as described.[15] The human HCC cell lines PLC (ATCC CRL-8024), Hep3B (ATCC HB-8064), HepG2 (ATCC HB-8065) and Huh-7 (ATCC PTA-4583) and the murine HCC cell line Hepa129 (National Cancer Institute at Frederick Cancer Research and Development Centre) were used as described before.[16] We further established sorafenib resistant (SR) Hep3B (Hep3B-SR) and HepG2 (HepG2-SR) cells by incubation with increasing doses of sorafenib (up to 10 µM) for approximately 4 months.

KRAS inhibition

Two different approaches of RNA interference—a single small interfering RNA (si-RNA) (Hs_KRAS2_8 FlexiTube siRNA: functionally verified si-RNA directed against human KRAS, Qiagen, Hilden, Germany) and a si-RNA-Pool (si-POOL-KRAS: functionally verified pool of 30 si-RNAs against KRAS, siTOOLs Biotech GmbH, Planegg, Germany)—were used to avoid any bias or off-target effects. For reasons of clarity, images in the main manuscript are labelled with 'si-KRAS' independent of which approach was used. Both approaches confirmed similar results. Detailed information for which approach was used in each experiment is depicted in the figures in the online supplementary file 1.

For pharmacological inhibition of KRAS signalling, we used deltarasin (DR) (Cayman Chemicals, Ann Arbour, Michigan, USA), a recently developed small molecule inhibitor of KRAS processing, which inhibits cyclic guanosine monophosphate phosphodiesterase delta subunit (PDE-delta, PDE6D) mediated KRAS trafficking to the cell membrane.[17]

Murine orthotopic HCC allograft and DR application

A cell suspension of 5 µL containing 2.5×10^5 Hepa129 cells was injected into the right median lobe of the liver of male syngeneic C3H/HeN mice. Starting on day 1 after tumour cell injection, mice of the DR group received a single dose of DR (20 mg/kg bodyweight) every 24 hours by intraperitoneal injection. Mice of the control group received the vehicle (dimethyl sulfoxide (DMSO)) only. Experimental protocols were approved by the Committee on Animal Health and Care of the local government, and conformed to international guidelines on the ethical use of animals.

Human liver tissues

Paired human HCC tissues and corresponding non-tumorous liver tissues were obtained from patients after partial hepatectomy. Furthermore, a tissue microarray of paraffin-embedded human HCC tissue samples[16 18] was analysed. Human liver tissues were obtained and experimental procedures were performed according to the guidelines of the non-profit state-controlled HTCR (Human Tissue and Cell Research) foundation[18] with the informed patients' consent.

Statistical analysis

Results are expressed as mean±SEM. The Student's t-test or one-way analysis of variance, if appropriate, were used for comparisons between groups. For analysis of tissue microarrays, the Fisher's exact test was used. The level of significance was $P<0.05$ (using the abbreviations 'ns', not significant and '*', $P<0.05$). The number of independent experiments was $n\geq3$ (if not depicted otherwise). Calculations were performed using the GraphPad Prism Software (GraphPad Software, San Diego, California, USA) and SPSS V.23.

Additional methods

Detailed methodology is described in the 'Methods' section of the online supplementary file 2.

RESULTS

Increased KRAS expression correlates with HCC progression

First, we examined the expression of (wild type) KRAS in four different human HCC cell lines (Hep3B, PLC, HepG2 and Huh7) and observed enhanced messenger RNA (mRNA) expression as compared with PHH (figure 1A). Western blot and immunofluorescence analysis confirmed elevated KRAS protein in HCC cells compared with hepatocytes (figure 1B, C; figure S1A in the online supplementary file 1). In silico analysis of patient datasets (Roessler liver[19] (n=445); Wurmbach liver[20] (n=75)) using the Oncomine human cancer microarray database[21] revealed significant upregulation of KRAS in human HCC tissues as compared with non-tumorous livers (figure 1D). Analysis of Gene Expression Omnibus (GEO) datasets and further evaluation of Oncomine-derived data[20 22] revealed that KRAS expression is already enhanced in preneoplastic lesions compared with non-neoplastic livers and further increases during HCC progression (see figure S1B, C in the online supplementary file 1). Next, we performed immunohistological analysis of KRAS protein expression using a tissue microarray comprising human HCC tissues and corresponding non-tumorous liver samples (n=88).[16 23] KRAS expression was strongly elevated in HCC as compared with non-tumorous liver (figure 1E; figure S2A, B in the online supplementary file 1). KRAS expression positively correlated with tumour size and tumour stage (figure 1F, G; figure S2C, D and table S1 in the online supplementary file 1). No correlation was found with age, gender, aetiology of underlying liver disease, histological grading or the presence of a pre-existing cirrhosis (see table S1 in the online supplementary file 1). Next, The Cancer Genome Atlas (TCGA) data analysis using the 'SurvExpress-Biomarker validation for cancer gene expression' database was

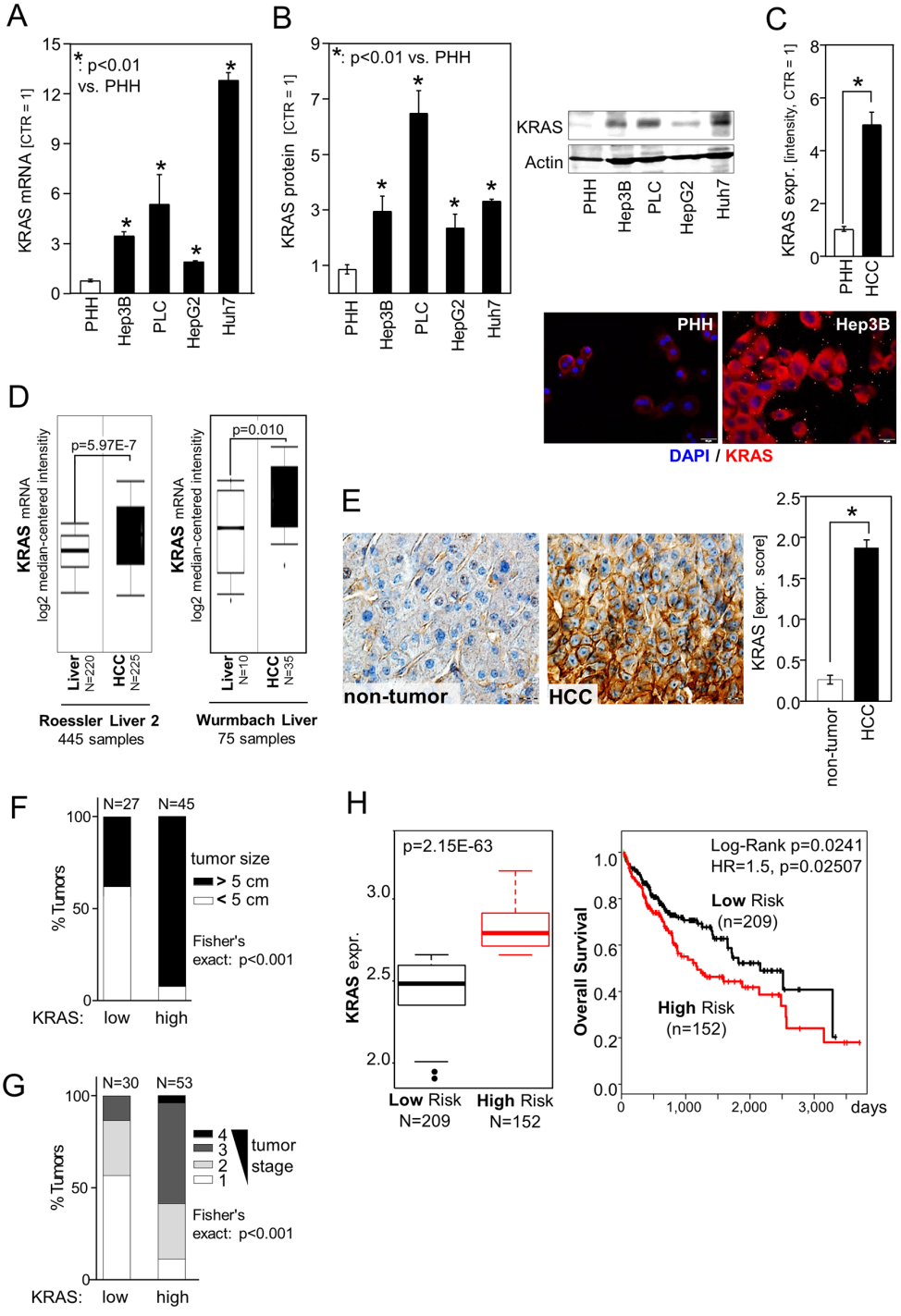

Figure 1 KRAS expression in HCC. (A) KRAS mRNA levels in human HCC cell lines (Hep3B, PLC, HepG2, Huh7) compared with primary human hepatocytes (PHH). (B) Densitometric quantification and exemplary image of western blot analysis of KRAS protein levels in HCC cell lines compared with PHH. (C) Immunofluorescence analysis of KRAS expression in PHH and HCC cells (exemplary image for Hep3B). (D) Oncomine human cancer microarray database analysis of two patient datasets depicting KRAS mRNA expression levels in HCC compared with non-tumorous livers. (E) Immunohistological analysis of KRAS protein expression of human HCC samples and corresponding non-tumorous liver tissues applying a TMA. The immunohistochemical score is described in detail in the Methods section in the online supplementary file 2. (F, G) TMA analysis of tumour sizes (F) and tumour stages (G) according to low (score 0–1) and high (score 2–3) KRAS expression. (H) SurvExpress-Biomarker validation for cancer gene expression database quantification of KRAS expression (left panel) and according to Kaplan-Meier curve of overall survival (right panel). Stratification into 'low-risk' and 'high-risk' patient groups was based on prognostic index (*P<0.05 compared with PHH). CTR, control; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; mRNA, messenger RNA; PHH, primary human hepatocyte; TMA, tissue microarray.

performed.[24] Stratification into low-risk (n=209) and high-risk (n=152) patient groups (based on prognostic index) revealed markedly enhanced KRAS expression and reduced overall survival in the high-risk group (figure 1H; figure S3 in the online supplementary file 1). In summary, expression of wild type KRAS is increased in HCC and KRAS expression

correlates with advanced tumour size, tumour stages and poor patient survival.

KRAS is a target of downregulated microRNA-622 in HCC

Changes in the expression of microRNAs (miRs) are increasingly recognised as functional regulators of HCC development and progression.[25–27] We hypothesised that also overexpression of KRAS in HCC is caused by loss of suppression by microRNAs. A multistep approach using an experimentally validated miRNA-target interactions database (miRTarBase)[28] revealed a subset of five microRNAs targeting KRAS that are downregulated in HCC (miR-216b-5p, miR-126–3p, miR-217, miR-622 and miR-181a-5p) (see figure S4A in the online supplementary file 1). Further selection for potential tumour-suppressive microRNAs was performed by analysis of TCGA datasets (using the MIRUMIR/GEO2R database[29 30]). Elevated expression of four of the five miRs (miR-216b-5p, miR-126–3 p, miR-217 and miR-181a-5p) revealed only inconsistent or even negative effects on patient survival in different studies and types of cancer (see figure S4B in the online supplementary file 1). Herewith, potential regulation of KRAS expression by these four microRNAs was not consistent with our hypothesis. In contrast, high miR-622 was associated with longer patient survival in several cancer types (see figure S4B, C in the online supplementary file 1) and recently, this microRNA has also been described to act as a tumour suppressor in HCC.[31 32] However, the potential relevance of a functional miR-622-KRAS interaction in HCC was unknown.

Therefore, we first specified the strong downregulation of miR-622 in human HCC cell lines and tissues compared with PHH and corresponding non-tumorous liver tissues, respectively (figure 2A, B; figure S5A in the online supplementary file 1). In silico analysis predicted canonical microRNA response elements (MREs) for miR-622 in the 3′UTR of both KRAS isoforms (figure 2C). Transfection with a miR-622-mimic reduced KRAS mRNA levels, while anti-miR-mediated miR-622 suppression (further) enhanced KRAS levels in HCC cells (figure 2D; figure S5B, C in the online supplementary file 1). Significant miR-622-induced KRAS suppression was also confirmed on the protein level (figure 2E; figure S5D in the online supplementary file 1). In line with this, luciferase reporter assays using a vector encoding the KRAS 3′UTR containing the miR-622 MREs showed that overexpression of miR-622 led to decreased luciferase activity in HCC cells (figure 2F; figure S5E in the online supplementary file 1). Moreover, KRAS and miR-622 expression levels were inversely correlated in vivo in HCC tissues (figure 2G) and in cell lines (see figure S5F in the online supplementary file 1). Although conventional RAS isoforms (ie, NRAS, HRAS, KRAS) have high sequence homology,[33] HRAS and NRAS revealed no conserved miR-622 MREs and miR-622 transfection did not alter HRAS and NRAS expression in HCC cell lines (see figure S5G in the online supplementary file 1). Together, these data reveal that miR-622 is strongly downregulated in HCC and specifically targets the KRAS isoform.

KRAS rescues tumour-suppressive miR-622 effects in HCC

In line with previous studies,[31 32] we found that re-expression of miR-622 strongly reduced proliferation, while inhibition of endogenous miR-622 even further enhanced proliferation of HCC cells (figure 3A, B; figure S6A in the online supplementary file 1). Additionally, fluorescence activated cell sorting (FACS) analysis revealed that enforced miR-622 expression enhanced G1/G0 and reduced G2 cell cycle fractions (see figure S6B, C in the online supplementary file 1). Furthermore, we found that miR-622 exerts a strong inhibitory effect on the migratory activity of HCC cells in time lapse Scratch and Boyden chamber assays (see figure S7A, B in the online supplementary file 1). Moreover, anchorage-dependent and anchorage-independent clonogenicity assays revealed strong tumour-suppressive functions of miR-622 in HCC (figure 3C, D; figures S8 and S9 in the online supplementary file 1).

To analyse if miR-622 mediates its tumour-suppressive functions via targeting KRAS, we used a pCMV6-Entry cDNA vector system containing the open reading frame (ORF) of the KRAS gene (ORF-KR) for KRAS overexpression in HCC cell lines. This ORF-KR vector did not contain the KRAS 3′UTR (harbouring the miR-622 MREs). Transfection of ORF-KR caused a significant increase in KRAS mRNA levels in control cells as well as in HCC cells co-transfected with miR-622 (figure S10A in the online supplementary file 1). Functionally, ORF-KR transfection significantly attenuated miR-622 induced inhibition of proliferation (figure 3E; figure S10B in the online supplementary file 1). Moreover, ORF-KR completely ‘rescued’ the strong inhibitory effects of miR-622 on clonogenicity (figure 3F, G; figures S10C–S12 in the online supplementary file 1). In contrast, ORF-KR transfection did not significantly affect the inhibitory miR-622 effects on HCC cell migration (see figure S13 in the online supplementary file 1). These experiments indicate that KRAS suppression is the major mediator of the inhibitory effect of miR-622 on HCC proliferation and clonogenicity.

KRAS knockdown inhibits clonogenicity, proliferation and oncogenic signalling in HCC

To further unravel the role of increased KRAS expression in HCC cells, we next analysed the functional impact of KRAS suppression in HCC cells using specific RNA interference (see figure S14 in the online supplementary file 1). Knockdown of KRAS expression strongly suppressed clonogenicity (figure 4A; figures S15 and S16 in the online supplementary file 1) and proliferation (figure 4B; figure S17 in the online supplementary file 1) of HCC cells. Furthermore, KRAS expression significantly correlated with the number of Ki-67-positive cells in patient-derived HCC tissue samples (figure 4C; table S1 in the online supplementary file 1).

In some tumour entities such as in colon cancer,[34] mutant KRAS signalling has been described to transcriptionally regulate epithelial–mesenchymal transition (EMT). Regarding wild type KRAS in HCC, we found that EMT markers were only partly regulated by KRAS (see figure S18A in the online supplementary file 1). Also, the migratory potential of HCC cells was not affected by KRAS suppression (see figure S18B in the online supplementary file 1).

RAS proteins are acting upstream of the RAF/MAPK and PI3K/AKT pathways, both known to be critical effectors of tumorigenicity in HCC.[5 9 10] Western blot analysis showed that KRAS knockdown markedly attenuated activation of extracellular-signal regulated kinase (ERK) and protein kinase B (AKT) (figure 4D; figure S19A in the online supplementary file 1). Similar effects on pERK-and pAKT-signalling in HCC cells were observed after miR-622-mediated KRAS suppression (figure 4E; figure S19B in the online supplementary file 1). Membranous KRAS localisation is required for active KRAS signalling. Accordingly, immunohistochemical analysis of human HCC tissues applying tissue micro array technology[16 23] revealed a significant correlation between ERK activation and KRAS membrane localization (figure 4F; figure S20 in the

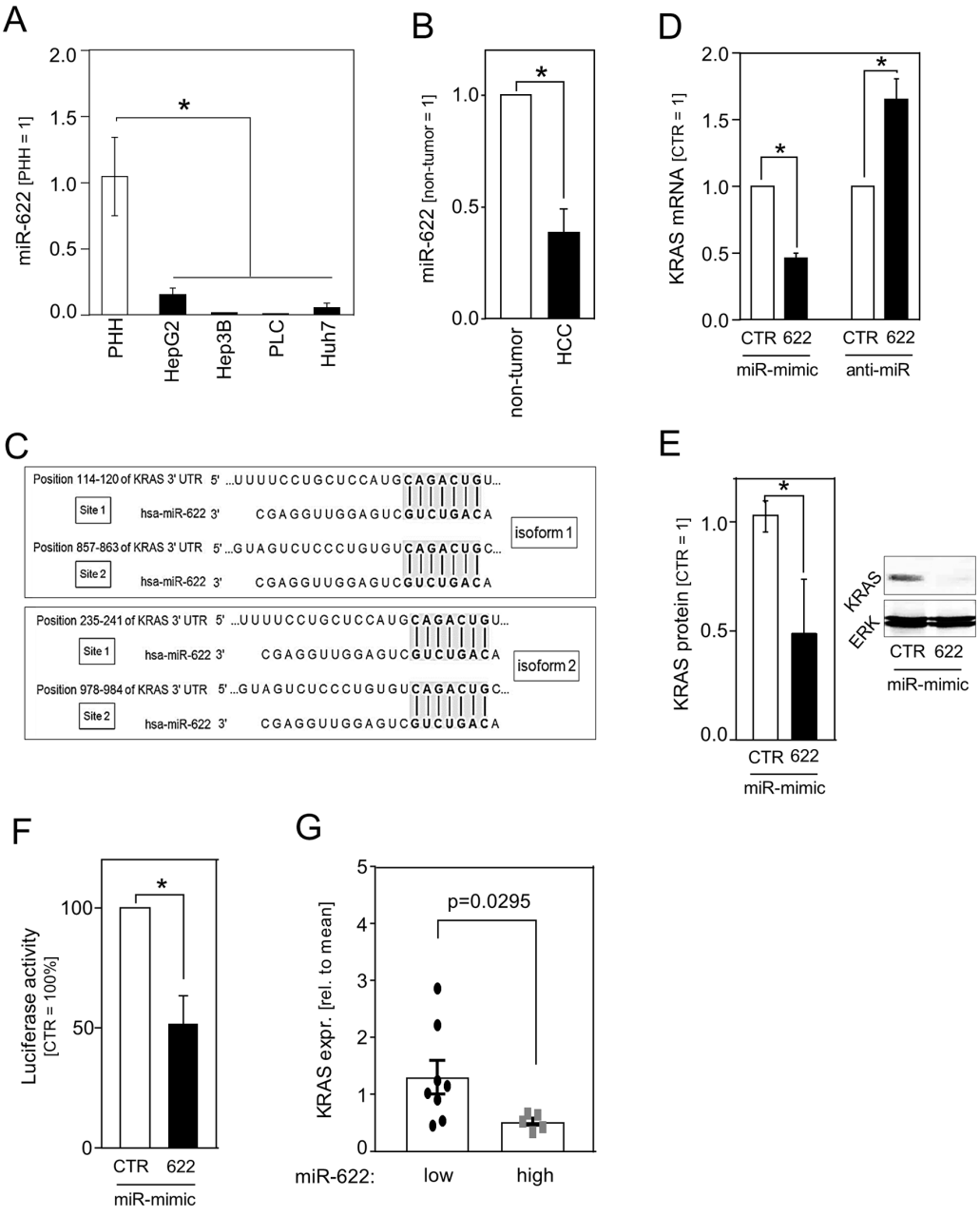

Figure 2 MiR-622-mediated regulation of KRAS expression in HCC. (A) MiR-622 expression levels in HCC cell lines (HepG2, Hep3B, PLC, Huh7) as compared with PHHs. (B) MiR-622 expression levels in pairs (n=14) of patient-derived HCC samples and corresponding non-tumorous liver tissues. (C) Predicted MREs for miR-622 in the 3'UTR of both KRAS isoforms (TargetScan V.7.1). (D and E) HCC cell lines were transfected with control-miR-mimics and anti-miRs (CTR) or miR-622-mimics and anti-miRs (622); (D) depicts KRAS mRNA levels (eg, PLC) and (E) depicts western blot analysis (quantification and exemplary western blot image) of protein expression levels (eg, Hep3B). (F) Luciferase KRAS 3'UTR-reporter activity in control-miR (CTR) and miR-622-mimic (622)-transfected cells (PLC). (G) KRAS mRNA expression in HCC patient samples with high and low miR-622 expression (as compared with mean) (*P<0.05). ERK, extracellular-signal regulated kinase; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; miR, microRNA; MRE, microRNA response elements; PHH, primary human hepatocyte.

online supplementary file 1). Together these data indicated (wild type) KRAS as a potent therapeutic target in HCC.

Small molecule KRAS inhibition strongly reduces tumorigenicity in HCC in vitro

By now, all attempts to target KRAS failed in clinical studies.[35] However, recently, the novel small-molecule KRAS inhibitor deltarasin (DR) has been identified (figure 5A).[17] DR binds to the delta subunit of rod-specific photoreceptor phosphodiesterase (PDE6D), a protein that regulates the antegrade trafficking of KRAS to the plasma membrane.[17] Quantitative real time-PCR analysis revealed that this KRAS-chaperon is strongly expressed in HCC cells compared with PHHs (see figure S21A in the online supplementary file 1). In vitro, DR exhibited acute cytotoxic effects (within 24 hours) in HCC cells at doses of 16 µM and higher (figure 5B, D; figure S21B, C in the online supplementary file 1). In contrast, doses up to 32 µM DR did not induce any signs of toxicity in PHHs (figure 5C, D).

FACS analysis (annexin/propidium iodide) revealed that DR induced significant apoptosis in HCC cells (figure 5E; figure

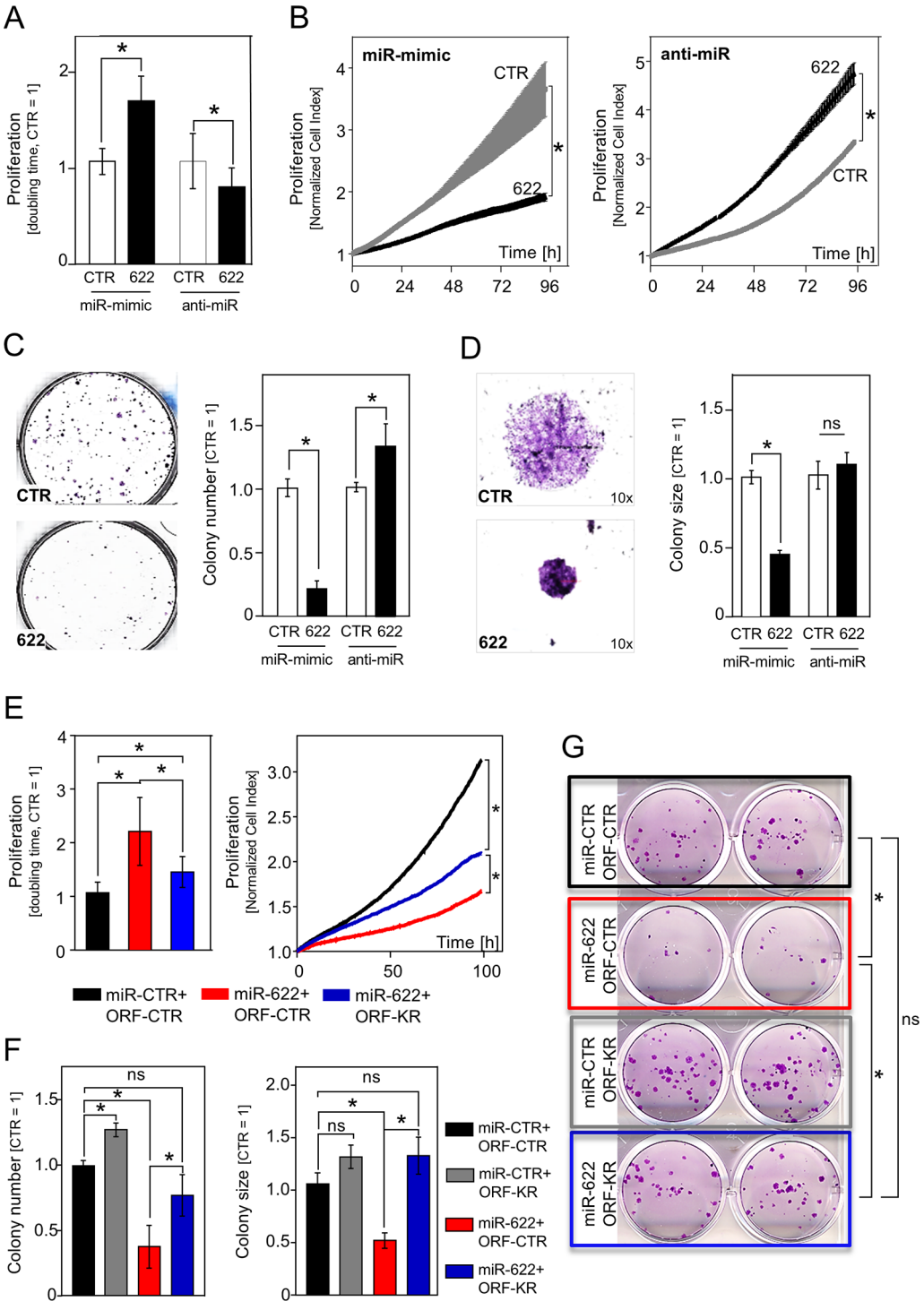

Figure 3 Analysis of miR-622 function in HCC and KRAS 'rescue' assays. Functional analysis of miR-622 effects on HCC cell lines was performed after re-expression of miR-622-mimics or inhibition of endogenous miR-622 activity by using transfection of an anti-miR-622 (622) and according to controls (CTR), respectively. (A, B) Real-time cell proliferation. 'Doubling time' (A) summarises the proliferative ability of tumour cells. (B) Depicts exemplary proliferation curves. Data are shown for PLC cells. (C, D) Anchorage-dependent clonogenic assay with exemplary images and quantified colony numbers (A) (eg, PLC) and colony sizes (B) (eg, Hep3B). (E–G) HCC cell lines were transfected in four different groups: (1) control miR+control vector (miR-CTR+ORF CTR), (2) control miR+KRAS Open Reading Frame (miR-CTR+ORF KR) containing vector for KRAS overexpression, (3) miR-622+control vector and (4) miR-622+KRAS ORF vector. Quantification of real-time cell proliferation (left panel) and exemplary proliferation curves (right panel) are shown in (E) (eg, PLC). Quantified colony number and colony size (F) and exemplary images (G) are shown for anchorage-dependent clonogenic assays (eg, Hep3B) (*P<0.05). HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; miR, microRNA; ns, non-significant.

S21D in the online supplementary file 1). Moreover, subtoxic doses of DR induced a G2/M cell cycle arrest in HCC cells, eventually leading to enhanced SubG1 fractions (figure 5F). In line with these findings, DR strongly inhibited HCC cell proliferation with half maximal inhibitory concentration (IC_{50}) values in subtoxic dose ranges (figure 5G; figure S22A in the online

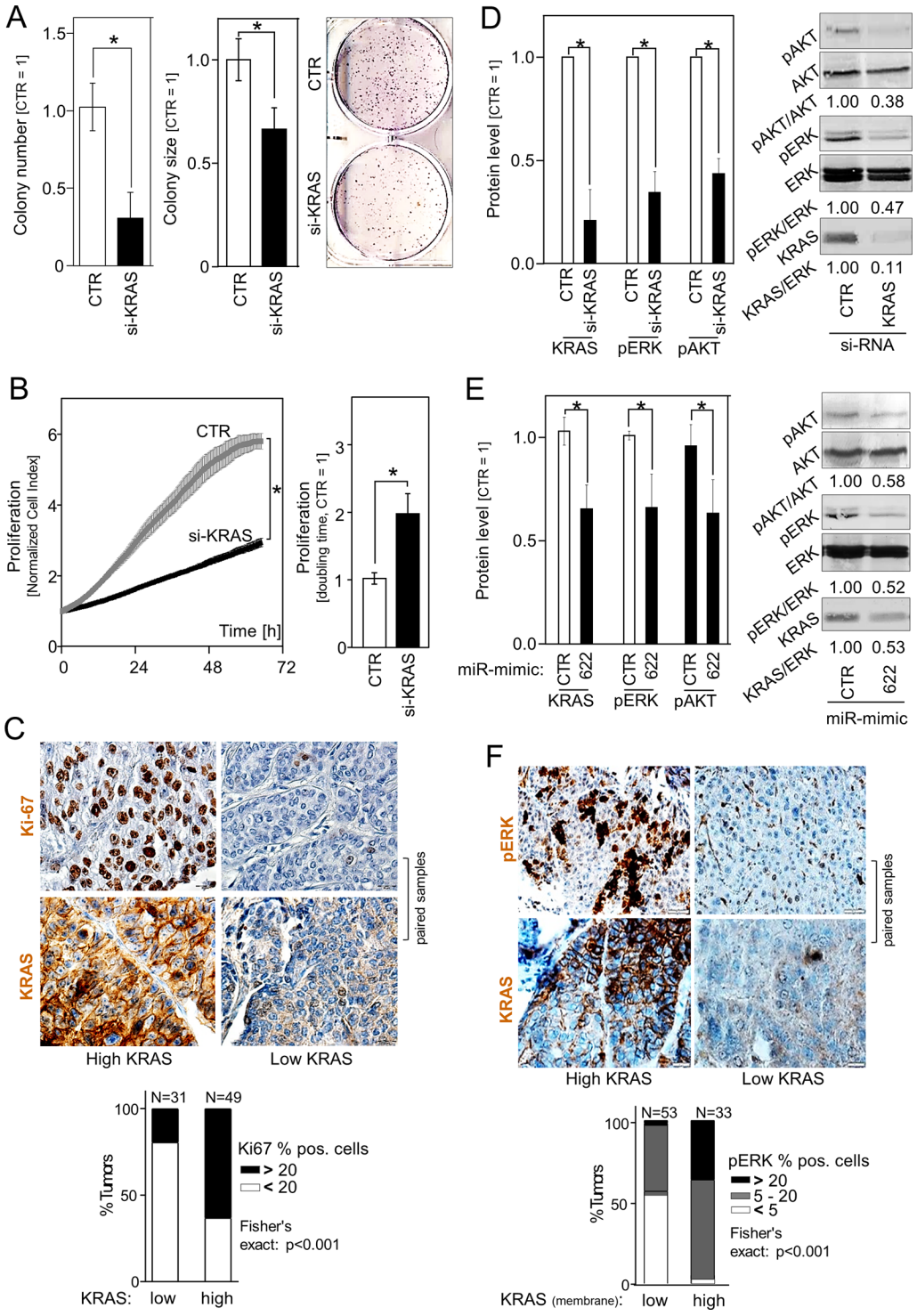

Figure 4 Effects of KRAS knockdown on clonogenicity, proliferation and oncogenic signalling in HCC. Prior to functional experiments, HCC cell lines were transfected with si-RNAs against KRAS (si-KRAS), microRNA-622-mimics (622) or the according control-si-RNAs or control-miR-mimics (CTR). (A) Quantification of colony number and size as well as exemplary images in anchorage-dependent clonogenic assays (eg, Hep3B). (B) Real-time cell proliferation. Exemplary proliferation curves (left panel) and quantified 'doubling times' (summarising the proliferative ability) (right panel) are depicted for Hep3B. (C) Exemplary images of paired KRAS and Ki-67 immunohistochemistry staining of HCC tissues with strong (left side) and weak (right side) KRAS expression and according statistical analysis (below). The immunohistochemical scores are described in detail in the Methods section in the online supplementary file 2. (D, E) Densitometric analysis of KRAS, pERK/ERK and pAKT/AKT protein expression and exemplary western blot images after si-RNA-mediated KRAS repression (D) or miR-622-mimic (622)-transfection (E) (eg, Hep3B). (F) Exemplary images of paired KRAS and pERK immunohistochemistry staining of HCC tissues with strong (left side) and weak (right side) KRAS membrane localisation and according to statistical analysis. The immunohistochemical scores are described in detail in the Methods section in the online supplementary file 2 (*P<0.05). AKT, protein kinase B; ERK, extracellular-signal regulated kinase; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; miR, microRNA; si-RNA, small interfering RNA.

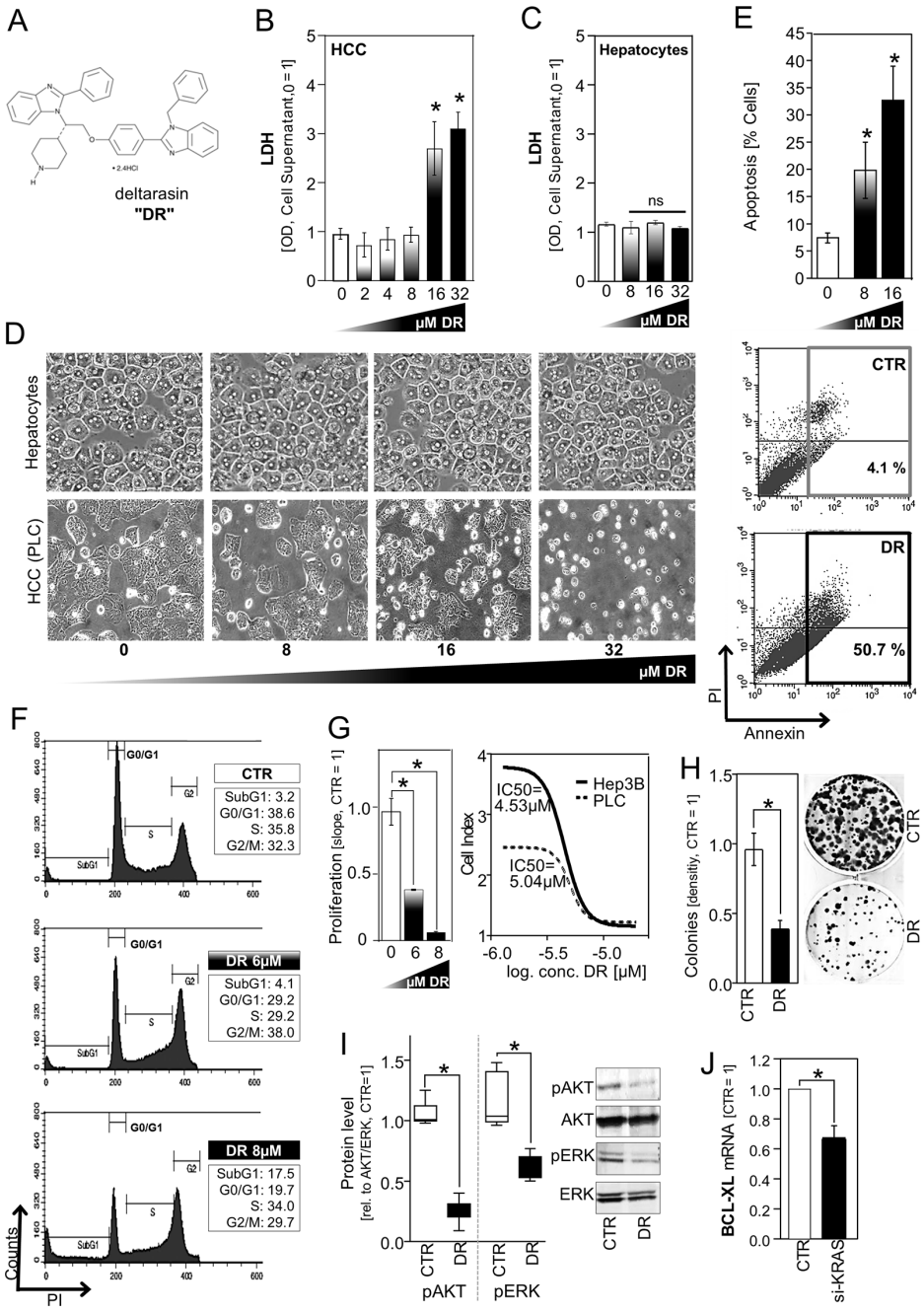

Figure 5 Function of small molecule-induced KRAS inhibition in HCC. HCC cells were treated with different doses of the small molecule KRAS inhibitor DR. (A) Chemical formula of DR. (B–D) LDH release into cell supernatants of DR-treated (48 hours) HCC (eg, Hep3B) cells (B) and primary human hepatocytes (C). Exemplary images are depicted (D) for primary human hepatocytes and HCC (eg, PLC) cells. (E) FACS analysis after 24 hours of DR treatment. Quantification of annexin-positive apoptotic cells (upper panel) and exemplary images (lower panel) are depicted (eg, Hepa129 cells). (F) FACS-based cell cycle analysis of HCC cells (eg, Hepa129 cells). Depicted are mean percentage numbers of cell cycle fractions according to different DR doses (0 µM (CTR), 6 µM, 8 µM) (24-hour treatment). (G) Real-time cell proliferation, depicted by quantification of the slopes of the cell index (left panel) (eg, PLC) and according to IC_{50} values (right panel). (H) Quantification of the number of colonies and representative image for an anchorage-dependent clonogenic assay (10 days, 5 µM DR) (eg, Hep3B). (I) Densitometric western blot analysis of pAKT/AKT and pERK/ERK levels and representative western blot images (eg, PLC). After starvation for 24 hours, cells were preincubated with 5 µM DR for 5 min, with subsequent stimulation (10 min) using fetal calf serum and basic Fibroblast Growth Factor (5.0 ng/mL). (J) BCL-XL mRNA expression (eg, Hep3B cells) 24 hours after transfection with si-KRAS or control si-RNA (CTR) (*P<0.05 compared with control). AKT, protein kinase B; BCL-XL, B-cell lymphoma-extra large; CTR, control; DR, deltarasin; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; IC_{50}, half maximal inhibitory concentration; KRAS, Kirsten rat sarcoma; LDH, lactate dehydrogenase; mRNA, messenger RNA; ns, non-significant.

supplementary file 1). KRAS inhibition by DR also strongly reduced clonogenicity and RAF/MAPK and PI3K/AKT signalling in HCC cells (figure 5H, I; figure S22B, C in the online supplementary file 1).

DR-mediated induction of a G2 arrest, activation of apoptosis and ERK and AKT signalling inhibition are in line with the fact that both ERK and AKT signalling were shown to promote transcription of apoptosis-inhibiting genes of the B-cell lymphoma 2

(BCL-2) family such as BCL-XL and BCL-2.[36–39] Accordingly, we found that specific KRAS knockdown significantly reduced the expression of antiapoptotic BCL-XL and BCL-2 and enhanced the expression of proapoptotic BCL-2 associated X protein (BAX) and BCL-2 binding component 3 (PUMA) in HCC cells (figure 5J; figure S22D in the online supplementary file 1). BCL-XL, BCL-2, BAX and PUMA were shown to have crucial roles in HCC progression.[40 41]

Together, these results indicated that KRAS inhibition using the small compound DR exhibits strong inhibitory effects on the tumorigenicity of HCC cells.

Pharmacological KRAS inhibition is effective in an experimental HCC model in vivo

The strong antitumour effects of DR on human HCC cells prompted us to further analyse the therapeutic effects of this pharmacological KRAS inhibitor using an experimental HCC model. We used an orthotopic HCC model, in which murine Hepa129 HCC cells were implanted into the liver of syngeneic C3H/HeN mice. Before, we had validated that murine HCC cells including Hepa129 have enhanced KRAS expression levels as compared with primary murine hepatocytes (see figure S23A in the online supplementary file 1). Moreover, we had confirmed that DR is effective in murine HCC cells in vitro with similar effects and in the same dose ranges as seen in human HCC (see figure S23B, C in the online supplementary file 1). Twenty-four hours after intrahepatic tumour cell injection, mice were randomised into a DR group (receiving 20 mg/kg body weight DR every 24 hours intraperitoneally, for 7 days) and a control group (CTR, receiving solvent (DMSO) only) (figure 6A). Tumour volumes/sizes were significantly reduced in the DR group as compared with the control group (figure 6B). Immunohistological analysis revealed reduced AKT and ERK activation in tumours formed in DR-treated mice (figure 6C, E; figures S24 and S25A in the online supplementary file 1). The apoptosis marker cleaved-caspase 3 was elevated in tumours of the DR group (figure 6C, F; figures S24 and S25A in the online supplementary file 1). In contrast, CyclinD1 staining was reduced in tumours of the DR group (figure 6C, G; figures S24 and S25A in the online supplementary file 1) and these tumours also showed a reduced number of mitotic cells (see figure S25B in the online supplementary file 1). In summary, these data indicate small molecule inhibition of KRAS as a promising novel therapeutic strategy for HCC.

KRAS inhibition enhances antitumorigenic efficacy of sorafenib in HCC cells

Currently, sorafenib is the only clinically established pharmacological therapy for HCC. Sorafenib inhibits intracellular tyrosine and serine/threonine protein kinases (vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), Raf-1 proto-oncogene (CRAF) and B-Raf proto-oncogen (BRAF)) that are mainly involved in RAF/MAPK and (via RAS) also PI3K/AKT signalling.[7] The strong inhibitory effect of KRAS inhibition on these two signalling pathways prompted us to study whether the combination of KRAS inhibition with sorafenib could be an effective approach. To address this question, we used DR in a low, subtoxic concentration (4 µM) in combination with sorafenib treatment. Even in this low concentration, DR significantly enhanced the dose-dependent toxicity of sorafenib in HCC cells (figure 7A, B). Moreover, combined application of sorafenib and DR in non-apoptosis-inducing doses for each single compound markedly enhanced tumour cell apoptosis (figure 7C; figure S26A in the online supplementary file 1). Furthermore, DR enhanced sorafenib-induced inhibition of HCC cell proliferation (figure 7D). These inhibitory effects were confirmed by both si-RNA-mediated KRAS inhibition and miR-622-mediated KRAS inhibition (figure 7E, F; figure S26B, D in the online supplementary file 1). Interestingly, sorafenib treatment caused a dose-dependent upregulation of KRAS expression in HCC cells (figure 7G; figure S26E in the online supplementary file 1). Together, these data suggested that in the presence of sorafenib, HCC cells depend on KRAS signalling, and thus indicate KRAS inhibition as a potential therapeutic strategy to enhance the antitumorigenic efficacy of sorafenib.

KRAS expression and effects of KRAS inhibition in acquired sorafenib resistance in HCC

To analyse whether KRAS also plays a role in acquired drug resistance to sorafenib, we established sorafenib resistant (SR) Hep3B (Hep3B-SR) and HepG2 (HepG2-SR) HCC cells. Acquired drug resistance was established by applying increasing doses of sorafenib over 3–4 months. The resistant cell lines (results are shown for Hep3B-SR) proliferated properly in the presence of up to 10 µM sorafenib (figure 8A; figure S27A, B in the online supplementary file 1). Sorafenib induced a dose-dependent dynamic upregulation of KRAS expression also in resistant cells as described above for non-resistant cells (figure 8B). Importantly, both sorafenib-induced (figure 8B) and basal KRAS expression (figure 8C, D) was even enhanced in resistant cells as compared with non-resistant cells. Accordingly, miR-622 levels (which are already strongly suppressed in HCC cells) tended to be even further downregulated in sorafenib-resistant compared with non-resistant HCC cells (figure 8E). Similarly as observed in non-resistant HCC cells, DR was sufficient to strongly inhibit clonogenicity and proliferation in sorafenib-resistant cells (figure 8F, G; figure S27C in the online supplementary file 1). Moreover, in sorafenib-resistant HCC cells, application of DR in subtoxic doses (≤4 µM) restored sorafenib-induced toxicity (figure 8H, I) and enhanced sorafenib-induced inhibition of proliferation (figure 8J). Particularly, in the light of potential long-term (combinatory) treatment options, we evaluated whether HCC cells can be easily reprogrammed to become resistant to DR by stepwise and slow dose escalation (similar to acquisition of a sorafenib resistance). However, even moderate doses (1× IC_{50}) of DR were sufficient to avoid the emergence of surviving tumour cells (see figure S28 in the online supplementary file 1). Together, these data indicate that the miR-622-KRAS axis contributes to chemosensitivity and resistance in HCC.

DISCUSSION

KRAS is one of the most frequently mutated oncogenes in cancer[42] but is only rarely mutated in HCC.[14] Therefore, the expression and function of KRAS in HCC were widely unexplored. In this study, we directly and functionally uncover the importance of wild type KRAS in HCC and provide evidence that KRAS is a novel therapeutic target in this aggressive form of liver cancer.

We found that KRAS mRNA and protein expression are strongly upregulated in HCC and that increased KRAS expression correlates with tumour progression, proliferation rate and a poor survival of patients. Exploring KRAS regulation in HCC, we unravelled that microRNA-622 (miR-622) directly affects KRAS expression in HCC. We observed that miR-622 exhibits a strong antitumorigenic effect on HCC cells and revealed that the inhibitory effect of miR-622 on proliferation as well as anchorage-dependent and anchorage-independent growth of HCC cells was dependent on

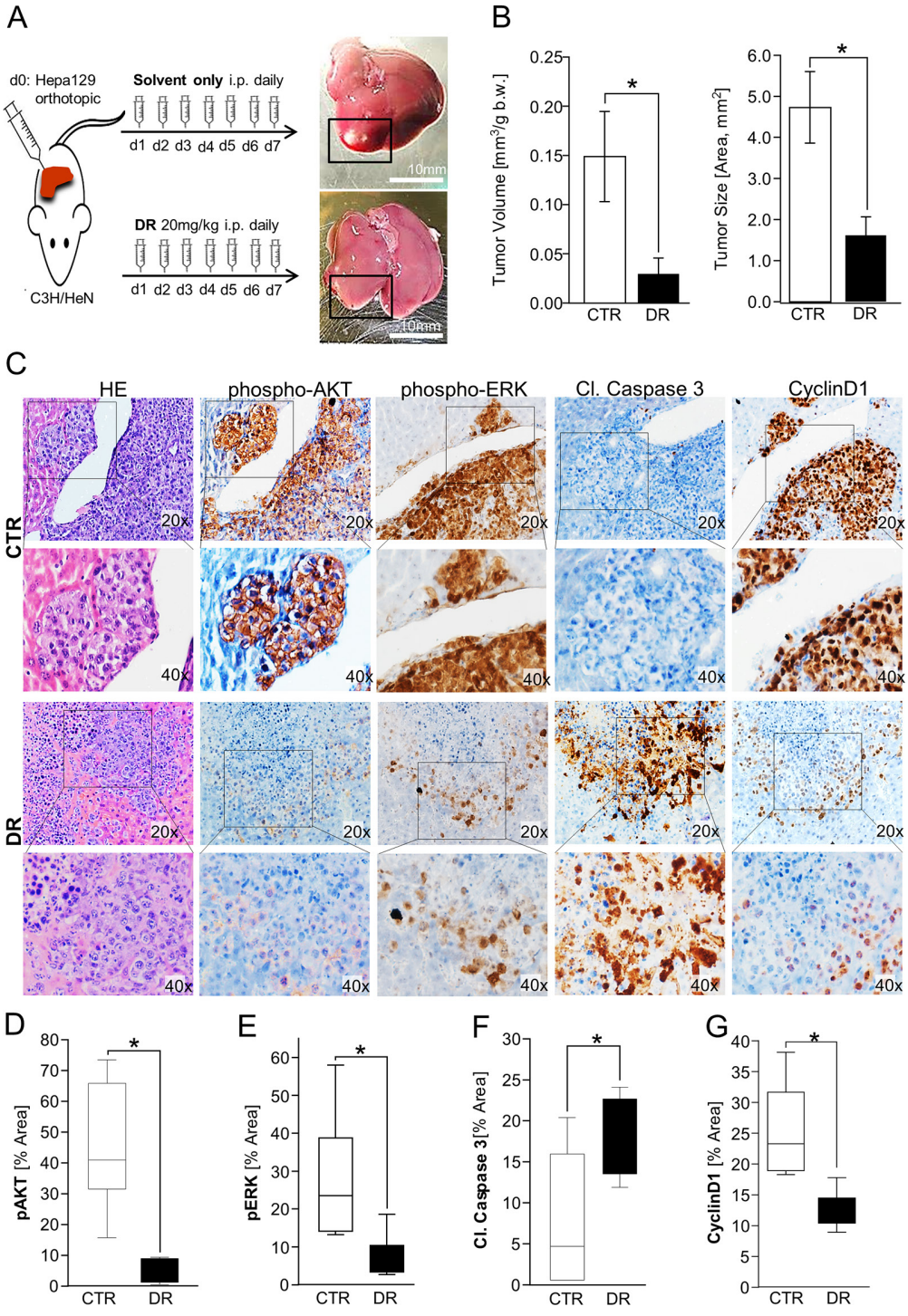

Figure 6 Effects of small molecule KRAS inhibition in HCC in vivo. (A) Murine Hepa129 HCC cells were implanted into the liver of syngeneic C3H/HeN mice. Twenty-four hours after intrahepatic tumour cell injection, mice were randomised into a DR group (receiving 20 mg/kg b.w. DR every 24 hours intraperitoneally, for 7 days, n=7), and a control group (CTR, receiving solvent (DMSO) only, n=9). (B) Tumour volumes per b.w. (left panel). One minimal tumour had not been observed macroscopically in the DR group but was detected histologically (CTR: 100%, DR: 85.7% tumour formation). Therefore, tumour sizes (mean area) were also measured microscopically in histological sections for confirmation (right panel). (C) Exemplary images showing H&E, phospho-AKT (pAKT), phospho-ERK (pERK), Cleaved (Cl.) Caspase 3 and CyclinD1 staining in histological tumour sections of a CTR and a DR-treated mice. (D–G) Analysis of phospho-AKT (pAKT), phospho-ERK (pERK), Cleaved (Cl.)-Caspase 3 and CyclinD1 staining in histological tumour sections of CTR and DR-treated mice. Depicted is the percentage of stained area as analysed using Cells Dimensions software (described in detail in the Methods section in the online supplementary file 2) (*P<0.05). AKT, protein kinase B; b.w., body weight; CTR, control; DMSO, dimethyl sulfoxide; DR, deltarasin; ERK, extracellular-signal regulated kinase; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma.

KRAS suppression. In contrast, miR-622's inhibitory effect on the migratory activity of HCC cells was independent of KRAS suppression. These data are in line with two recent reports that also described a tumour-suppressive function of miR-622 in HCC. Liu *et al* identified miR-622 as negative regulator of CXC chemokine receptor 4 (CXCR4) in HCC and showed that the inhibitory

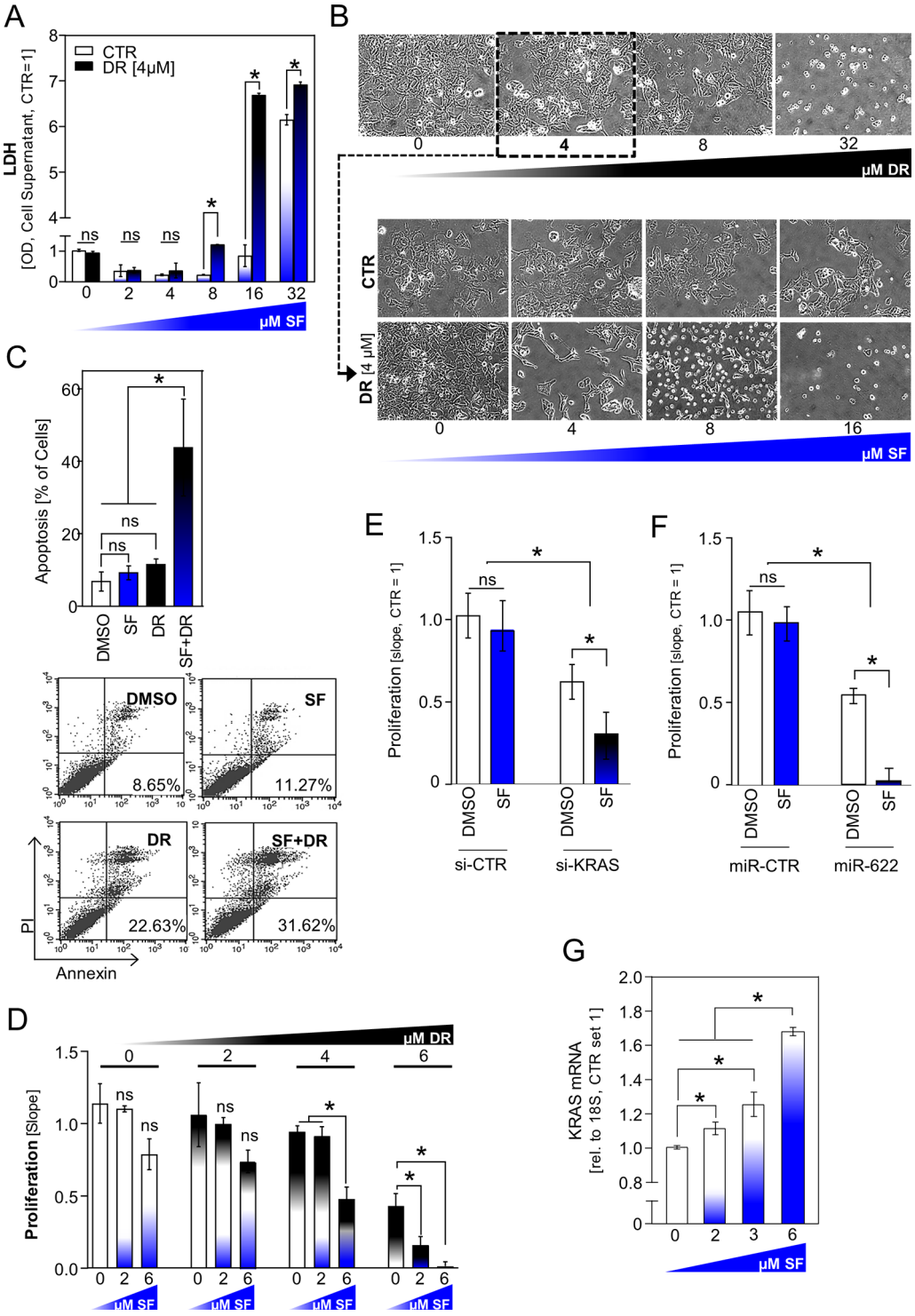

Figure 7 Effects of combined sorafenib treatment and KRAS inhibition in HCC. (A) LDH quantification in supernatants and (B) microscopic images of HCC cells (eg, PLC) treated with different doses of SF only (CTR) or in combination with subtoxic DR doses (4 µM) for 24 hours. (C) FACS analysis of apoptotic cells after treatment with DMSO, SF (10 µM), DR (4 µM) or a combination of SF+DR. Treatment was performed for 16 hours on confluent cells (eg, Hep3B). (D) Real-time cell proliferation (slope) of HCC cells (eg, PLC) treated with the depicted doses of DR and SF. (E) Real-time cell proliferation (slope) of SF (4 µM)-treated HCC cells (eg, Hep3B), with (si-KRAS) or without (si-CTR) si-RNA-mediated KRAS repression. (F) Real-time cell proliferation (slope) of SF (4 µM)-treated HCC cells (eg, PLC), with combined miR-control (miR-CTR) or miR-622 transfection. (G) KRAS mRNA expression in SF-treated (24 hours) HCC cells (eg, Hep3B) (*P<0.05). CTR, control; DMSO, dimethyl sulfoxide; DR, deltarasin; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; LDH, lactate dehydrogenase; mRNA, messenger RNA; miR, microRNA; ns, non-significant; SF, sorafenib; si-RNA, small interfering RNA.

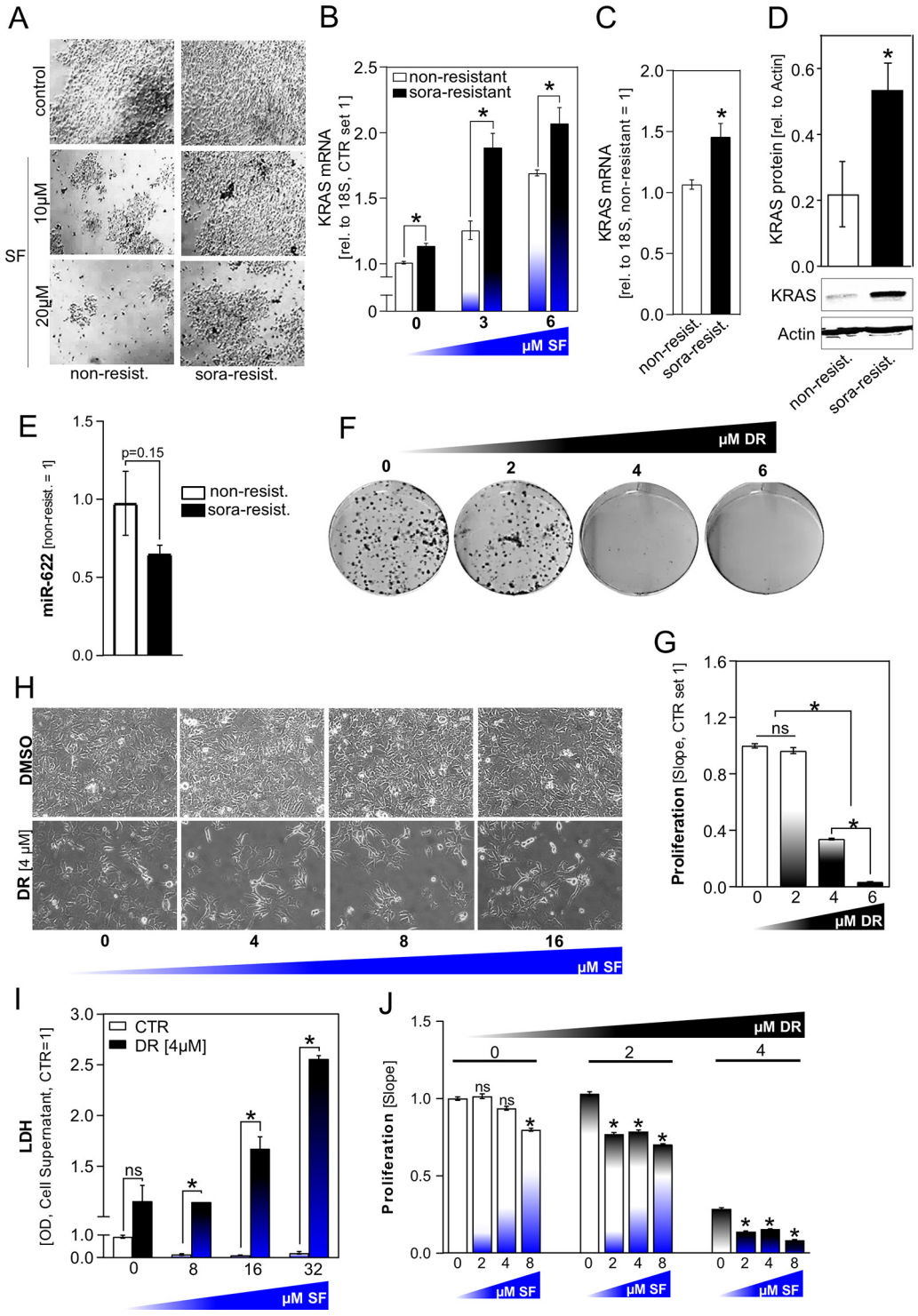

Figure 8 KRAS expression and function in SF-resistant HCC. (A) Microscopic images of SF-treated non-resistant and SF (sora)-resistant Hep3B cells. (B) KRAS mRNA expression in SF-treated (24 hours) non-resistant and sora-resistant Hep3B cells. (C, D) (Basal) expression of KRAS mRNA (C) and protein (D) in untreated non-resistant and sora-resistant Hep3B cells. (E) MiR-622 expression (quantitative reverse transcription-PCR analysis) in non-resistant HCC cells as compared with sora-resistant Hep3B cells. (F) Exemplary clonogenic assay images of DR-treated sora-resistant Hep3B cells. (G) Real-time cell proliferation of DR-treated sora-resistant Hep3B cells. (H) Microscopic images and (I) LDH quantification in supernatants of sora-resistant Hep3B cells treated with SF alone or in combination with low dose (4 µM) DR. (J) Real-time cell proliferation of sora-resistant Hep3B cells treated with combined SF and DR (*P<0.05 compared with control). CTR, control; DR, deltarasin; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; LDH, lactate dehydrogenase; mRNA, messenger RNA; miR, microRNA; ns, non-significant; SF, sorafenib.

effect of miR-622 on migration of HCC cells strongly depends on CXCR4 suppression.[32] In contrast, growth-suppressive effects of miR-622 on HCC cells were only slightly affected by its effect on CXCR4 expression.[32] Song *et al* found that miR-622 negatively regulates mitogen-activated protein 4 kinase 4 (MAP4K4) in HCC, but overexpression of MAP4K4 only partially reversed

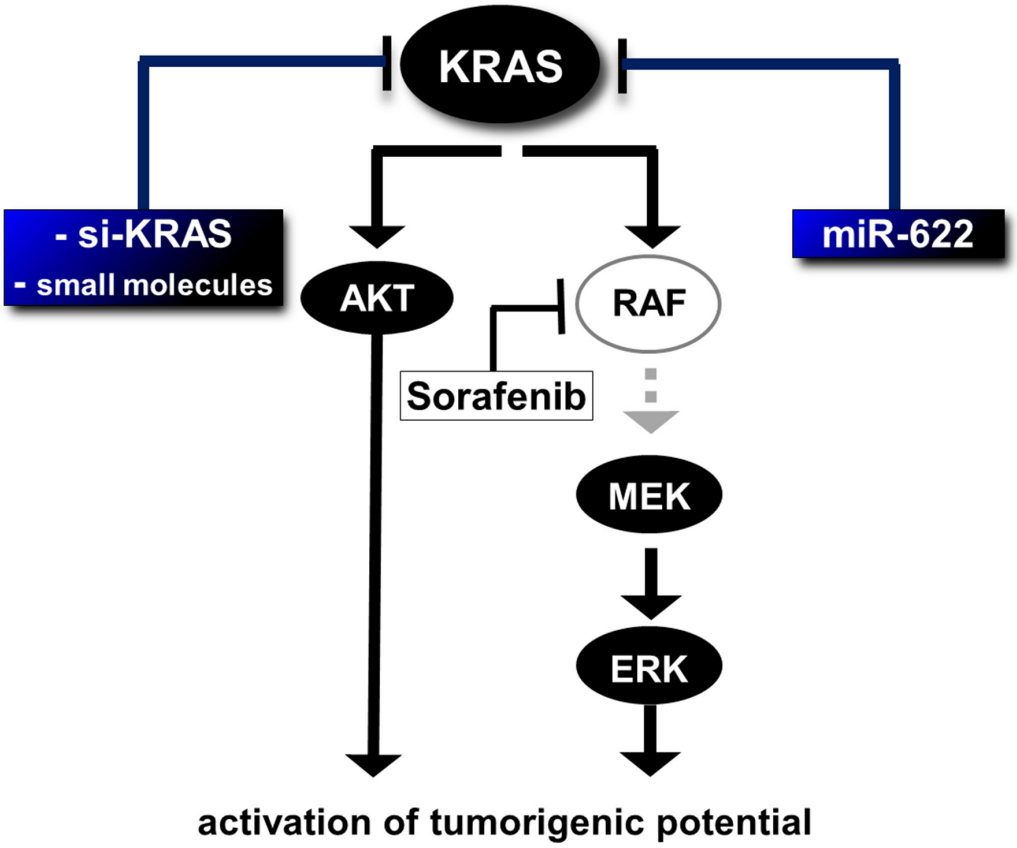

Figure 9 Summarised hypothesis on the microRNA-622-KRAS axis in HCC. Wild type KRAS mediates activation of tumorigenic potential (ie, proliferation, clonogenicity, resistance to apoptosis) and therapy resistance to sorafenib by activation of two of its major downstream signalling pathways: RAF/MEK/ERK and PI3K/AKT signalling. In case of sorafenib-mediated RAF inhibition, KRAS upregulation and KRAS induced AKT activation ensure survival and emergence of therapy resistance. Upregulation of KRAS is mediated by downregulated tumour-suppressive miR-622. Increasing miR-622 or (direct) targeting of KRAS (by RNA interference or small molecule approaches (eg, deltarasin)) are potential therapeutic strategies to inhibit HCC progression and to break sorafenib resistance.AKT, protein kinase B; ERK, extracellular-signal regulated kinase; HCC, hepatocellular carcinoma; KRAS, Kirsten rat sarcoma; miR, microRNA.

the growth-suppressive effects of miR-622 on HCC cells.[31] In a recent study, the same group demonstrated that MAP4K4 promotes the epithelial–mesenchymal transition and invasiveness of HCC cells largely via activation of c-Jun N-terminal kinases and nuclear factor kappa-light-chain-enhancer of activated B cells signalling.[43] Together, these data indicate that miR-622 exhibits its tumour-suppressive function in HCC via several relevant target genes and mechanisms, respectively, with KRAS being the major target responsible for miR-622's inhibitory effect on HCC proliferation and clonogenicity. Potentially, miR-622 serum levels might be used as a predictive marker for HCC (progression); however, detection of a strongly downregulated microRNA would be technically demanding, while quantification of increased microRNAs has potential as serum marker in HCC.[44]

Pharmacologically, KRAS has been suggested to be 'undruggable' for many years. The main reason for this is that RAS proteins do not present suitable pockets for drug binding, except for the guanosine diphosphate (GDP)/guanosine triphosphate (GTP) binding site, which adheres GDP/GTP very tightly in picomolar affinities.[17 35 45–48] Recently, a number of new approaches to address RAS activity have led to the revival of KRAS as a molecular target.[49 50] Here, we investigated the effect of the recently developed inhibitor of KRAS trafficking DR.[17] We demonstrate strong antitumour effects of DR in HCC cells and in a murine orthotopic HCC model, and even after long-term incubation, we did not detect resistant cells. Moreover, we show for the first time that combinatory approaches of KRAS inhibition and sorafenib reveal synergistic antitumorigenic effects in HCC. Future studies need to assess whether analysis of KRAS or miR-622 expression could serve as a companion diagnostic tool to predict response to sorafenib therapy in patients with HCC.

Until today, sorafenib is the only effective therapeutic option for patients with HCC, and significant improvement of sorafenib efficacy is a major goal in HCC management.[3 5] Activation of escape pathways from RAS/RAF/ERK is considered to be a crucial mediator of chemoresistance, underlining the importance of RAS/RAF/ERK signalling in HCC.[6 51] In our study, we discovered that endogenous KRAS is a therapeutic target contributing to sorafenib resistance in HCC. KRAS inhibition resensitised sorafenib-resistant cells to inhibition of proliferation and induction of apoptosis. The proposed mechanisms are outlined in figure 9. Together, our findings further foster the concept that targeting KRAS or combined KRAS inhibition and sorafenib could be rational approaches for HCC treatment. Still, further preclinical studies are required to determine optimal therapeutic regimens .

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Contributors PD, AKB, and CH conceived the project, analyzed the data, and wrote the manuscript. PD designed and performed most of the experiments. AK helped panning, performing, and analyzing mouse experiments. VF was involved in functional cell culture experiments. AH provided material and contributed to data anlysis. All authors approved the final version of the manuscript.

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Disclaimer None of the pictures/images has been published before. All data/pictures shown have been newly generated by the authors.

Competing interests None declared.

Patient consent Obtained.

Ethics approval HTCR (Human Tissue and Cell Research) foundation and the local ethics commitee of the University of Regensburg.

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Data sharing statement There are no additional data.

REFERENCES

1 Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. *Hepatology* 2008;48:2047–63.
2 Mirzaei HR, Sahebkar A, Mohammadi M, *et al*. Circulating microRNAs in hepatocellular carcinoma: potential diagnostic and prognostic biomarkers. *Curr Pharm Des* 2016;22:5257–69.
3 Llovet JM, Ricci S, Mazzaferro V, *et al*. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.
4 Cheng AL, Kang YK, Chen Z, *et al*. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;10:25–34.
5 Pascual S, Herrera I, Irurzun J. New advances in hepatocellular carcinoma. *World J Hepatol* 2016;8:421–38.
6 Nishida N, Kitano M, Sakurai T, *et al*. Molecular mechanism and prediction of sorafenib chemoresistance in human hepatocellular carcinoma. *Dig Dis* 2015;33:771–9.
7 Mazzoccoli G, Miele L, Oben J, *et al*. Biology, epidemiology, clinical aspects of hepatocellular carcinoma and the role of sorafenib. *Curr Drug Targets* 2016;17:783–99.
8 Makarova AS, Lazarevich NL. Deregulation of signaling pathways involved in sorafenib resistance of hepatocellular carcinoma. *Klin Lab Diagn* 2013;8:34–7.
9 Wilhelm SM, Carter C, Tang L, *et al*. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. *Cancer Res* 2004;64:7099–109.
10 Gao JJ, Shi ZY, Xia JF, *et al*. Sorafenib-based combined molecule targeting in treatment of hepatocellular carcinoma. *World J Gastroenterol* 2015;21:12059–70.
11 Calvisi DF, Ladu S, Gorden A, *et al*. Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. *Gastroenterology* 2006;130:1117–28.
12 Yoshida T, Hisamoto T, Akiba J, *et al*. Spreds, inhibitors of the Ras/ERK signal transduction, are dysregulated in human hepatocellular carcinoma and linked to the malignant phenotype of tumors. *Oncogene* 2006;25:6056–66.
13 Delire B, Stärkel P. The Ras/MAPK pathway and hepatocarcinoma: pathogenesis and therapeutic implications. *Eur J Clin Invest* 2015;45:609–23.
14 Turhal NS, Savaş B, Çoşkun Ö, *et al*. Prevalence of K-Ras mutations in hepatocellular carcinoma: a Turkish oncology group pilot study. *Mol Clin Oncol* 2015;3:1275–9.
15 Lee SM, Schelcher C, Demmel M, *et al*. Isolation of human hepatocytes by a two-step collagenase perfusion procedure. *J Vis Exp* 2013.doi: 10.3791/50615. [Epub ahead of print 3 Sep 2017].
16 Bauer R, Valletta D, Bauer K, *et al*. Downregulation of P-cadherin expression in hepatocellular carcinoma induces tumorigenicity. *Int J Clin Exp Pathol* 2014;7:6125–32.
17 Zimmermann G, Papke B, Ismail S, *et al*. Small molecule inhibition of the KRAS-PDEδ interaction impairs oncogenic KRAS signalling. *Nature* 2013;497:638–42.
18 Thasler WE, Weiss TS, Schillhorn K, *et al*. Charitable state-controlled foundation human tissue and cell research: ethic and legal aspects in the supply of surgically removed human tissue for research in the academic and commercial sector in Germany. *Cell Tissue Bank* 2003;4:49–56.
19 Roessler S, Jia HL, Budhu A, *et al*. A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients. *Cancer Res* 2010;70:10202–12.
20 Wurmbach E, Chen YB, Khitrov G, *et al*. Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma. *Hepatology* 2007;45:938–47.
21 Rhodes DR, Yu J, Shanker K, *et al*. ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* 2004;6:1–6.
22 Archer KJ, Mas VR, David K, *et al*. Identifying genes for establishing a multigenic test for hepatocellular carcinoma surveillance in hepatitis C virus-positive cirrhotic patients. *Cancer Epidemiol Biomarkers Prev* 2009;18:2929–32.
23 Hellerbrand C, Amann T, Schlegel J, *et al*. The novel gene MIA2 acts as a tumour suppressor in hepatocellular carcinoma. *Gut* 2008;57:243–51.
24 Aguirre-Gamboa R, Gomez-Rueda H, Martínez-Ledesma E, *et al*. SurvExpress: an online biomarker validation tool and database for cancer gene expression data using survival analysis. *PLoS One* 2013;8:e74250.
25 Wang L, Yue Y, Wang X, *et al*. Function and clinical potential of microRNAs in hepatocellular carcinoma. *Oncol Lett* 2015;10:3345–53.
26 Afonso MB, Rodrigues PM, Simão AL, *et al*. Circulating microRNAs as potential biomarkers in non-alcoholic fatty liver disease and hepatocellular carcinoma. *J Clin Med* 2016;5:30.
27 Sugimachi K, Matsumura T, Hirata H, *et al*. Identification of a bona fide microRNA biomarker in serum exosomes that predicts hepatocellular carcinoma recurrence after liver transplantation. *Br J Cancer* 2015;112:532–8.
28 Chou CH, Chang NW, Shrestha S, *et al*. miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. *Nucleic Acids Res* 2016;44:D239–D247.
29 Antonov AV, Knight RA, Melino G, *et al*. MIRUMIR: an online tool to test microRNAs as biomarkers to predict survival in cancer using multiple clinical data sets. *Cell Death Differ* 2013;20:367.
30 Antonov AV. BioProfiling.de: analytical web portal for high-throughput cell biology. *Nucleic Acids Res* 2011;39:W323–W327.
31 Song WH, Feng XJ, Gong SJ, *et al*. microRNA-622 acts as a tumor suppressor in hepatocellular carcinoma. *Cancer Biol Ther* 2015;16:1754–63.
32 Liu H, Liu Y, Liu W, *et al*. EZH2-mediated loss of miR-622 determines CXCR4 activation in hepatocellular carcinoma. *Nat Commun* 2015;6:8494.
33 Hobbs GA, Der CJ, Rossman KL. RAS isoforms and mutations in cancer at a glance. *J Cell Sci* 2016;129:1287–92.
34 Shao DD, Xue W, Krall EB, *et al*. KRAS and YAP1 converge to regulate EMT and tumor survival. *Cell* 2014;158:171–84.
35 McCormick F. KRAS as a therapeutic target. *Clin Cancer Res* 2015;21:1797–801.
36 Sale MJ, Cook SJ. That which does not kill me makes me stronger; combining ERK1/2 pathway inhibitors and BH3 mimetics to kill tumour cells and prevent acquired resistance. *Br J Pharmacol* 2013;169:1708–22.
37 Wilson BE, Mochon E, Boxer LM. Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis. *Mol Cell Biol* 1996;16:5546–56.
38 Boucher MJ, Morisset J, Vachon PH, *et al*. MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1 and promotes survival of human pancreatic cancer cells. *J Cell Biochem* 2000;79:355–69.
39 Yang J, Song Q, Cai Y, *et al*. RLIP76-dependent suppression of PI3K/AKT/Bcl-2 pathway by miR-101 induces apoptosis in prostate cancer. *Biochem Biophys Res Commun* 2015;463:900–6.
40 Yang J, Yao S. JNK-Bcl-2/Bcl-xL-Bax/Bak pathway mediates the crosstalk between matrine-induced autophagy and apoptosis via interplay with beclin 1. *Int J Mol Sci* 2015;16:25744–58.
41 Azumi J, Tsubota T, Sakabe T, *et al*. miR-181a induces sorafenib resistance of hepatocellular carcinoma cells through downregulation of RASSF1 expression. *Cancer Sci* 2016;107:1256–62.
42 Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. *Cell* 2017;170:17–33.
43 Feng XJ, Pan Q, Wang SM, *et al*. MAP4K4 promotes epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma. *Tumour Biol* 2016;37:11457–67.
44 Nishida N, Arizumi T, Hagiwara S, *et al*. MicroRNAs for the prediction of early response to sorafenib treatment in human hepatocellular carcinoma. *Liver Cancer* 2017;6:113–25.
45 The KRAS-PDEδ interaction is a therapeutic target. *Cancer Discov* 2013;3:OF20.
46 Schmick M, Vartak N, Papke B, *et al*. KRas localizes to the plasma membrane by spatial cycles of solubilization, trapping and vesicular transport. *Cell* 2014;157:459–71.
47 Cox AD, Der CJ, Philips MR. Targeting RAS membrane association: back to the future for anti-RAS drug discovery? *Clin Cancer Res* 2015;21:1819–27.
48 Stephen AG, Esposito D, Bagni RK, *et al*. Dragging ras back in the ring. *Cancer Cell* 2014;25:272–81.
49 Milroy LG, Ottmann C. The renaissance of Ras. *ACS Chem Biol* 2014;9:2447–58.
50 Ledford H. Cancer: the Ras renaissance. *Nature* 2015;520:278–80.
51 D'Alessandro R, Messa C, Refolo MG, *et al*. Modulation of sensitivity and resistance to multikinase inhibitors by microenvironmental platelet factors in HCC. *Expert Opin Pharmacother* 2015;16:2773–80.

Wild type Kirsten rat sarcoma is a novel microRNA-622-regulated therapeutic target for hepatocellular carcinoma and contributes to sorafenib resistance

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