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The Ribonucleoprotein HNRNPA2B1 Interacts With and Regulates Oncogenic KRAS in Pancreatic Ductal Adenocarcinoma Cells

Short title: HNRNPA2B1 modulates KRAS-dependent signaling in PDAC

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Abbreviations used in this paper: AKT, c-akt murine thymoma oncogene homolog 1; DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; GTP, guanosine triphosphate; HRAS, c-H-ras Harvey rat sarcoma oncogene; hnRNP A2/B1, heterogeneous nuclear ribonucleoprotein A2/B1; KRAS, c-Ki-ras2 Kirsten rat sarcoma oncogene homolog; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization- Time of Flight; MEK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; NRAS, c-N-ras, neuroblastoma rat sarcoma oncogene homolog; PDAC: pancreatic ductal adenocarcinoma; PDEδ, phosphodiesterase deltha; PI3K, phosphatidylinositide 3-kinase; PPλ, lambda protein phosphatase; RAF1, c-Raf rapidly accelerated fibrosarcoma oncogene; S6, 40S ribosomal protein S6; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean.

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ABSTRACT

Background & Aims: Development of pancreatic ductal adenocarcinoma involves activation of KRAS signaling, but little is known about the roles of proteins that regulate the activity of oncogenic KRAS. We investigated the activities of proteins that interact with KRAS in pancreatic ductal adenocarcinoma (PDAC) cells.

Methods: We used mass spectrometry to demonstrate that heterogeneous nuclear ribonucleoproteins (HNRNP) A2 and B1 (encoded by the gene *HNRNPA2B1*) interact with KRAS G12V. We used co-immunoprecipitation analyses to study interactions between HNRNPA2B1 and KRAS in KRAS-dependent and KRAS-independent PDAC cell lines. We knocked-down HNRNPA2B1 using small hairpin RNAs and measured viability, anchorage-independent proliferation, and growth of xenograft tumors in mice. We studied KRAS phosphorylation using the Phos-tag® system.

Results: We found that interactions between HRNPA2B1 and KRAS correlated with KRAS-dependency of some human PDAC cell lines. Knock down of HNRNPA2B1 significantly reduced viability, anchorage-independent proliferation, and formation of xenograft tumors by KRAS-dependent PDAC cells. HNRNPA2B1 knock down also increased apoptosis of KRAS-dependent PDAC cells, inactivated AKT signaling via mTOR, and reduced interaction between KRAS and PI3K. Interaction between HNRNPA2B1 and KRAS required KRAS phosphorylation at serine 181.

Conclusions: In KRAS-dependent PDAC cell lines, HNRNPA2B1 interacts with and regulates the activity of KRAS G12V and G12D. HNRNPA2B1 is required for KRAS activation of AKT–mTOR signaling, interaction with PI3K, and PDAC cell survival and tumor formation in mice. HNRNPA2B1 might be a target for treatment of pancreatic cancer.

KEYWORDS: signal transduction, carcinogenesis, oncogene, mouse model

INTRODUCTION

RAS proteins are well-known small GTPases involved in the regulation of key signal transduction pathways. Cycling from the inactive (GDP-bound) to the active (GTP-bound) state faithfully responds to extracellular signals due to its tight regulation by GTP-exchange factors (GEFs) and GTPase activating proteins (GAPs). Activating point mutations that render RAS proteins insensitive to the extracellular signals are crucial steps in the development of the vast majority of cancers.[1–3] Three different genes code for a total of four different Ras isoforms named HRAS, NRAS, KRAS4A and KRAS4B. KRAS4B (herein after referred to as KRAS) is the most frequently mutated oncogene in solid tumors and its oncogenic mutations occur mostly in pancreatic (95%), colon (40%) and adenocarcinomas of the lung (35%).[3–5] Mutational activation of KRAS in these tissues is sufficient to initiate neoplasia in mice.[6–8] The most prevalent oncogenic mutation in KRAS is at codon 12 (66% KRAS mutations)[4] and preserve the GTP-bound active state by inhibiting intrinsic GTPase activity or interfering with the action of GAPs. In the GTP-bound form, KRAS is able to interact with different effector proteins and consequently activates signal transduction pathways. Among those, the best characterized are the RAF1/MEK/ERK and the phosphatidylinositol-3-kinase (PI3K)/AKT.[9,10] Since oncogenic mutations of KRAS give rise to an always GTP-bound protein which constitutively binds to effectors to activate them, positive or negative physiological regulation of oncogenic KRAS was not initially expected. In recent years, emergence of new regulators either by direct interaction or by reversible post-translational modifications, proved to be crucial to fully display KRAS oncogenic phenotype. Interaction of KRAS with Galectin-3,[11] calmodulin,[12] and PDEδ[13,14] or with the initially-defined nuclear protein Nucleophosmin and Nucleolin,[15] proved to be necessary for correct activation of RAF/MEK/ERK signaling pathway.

As human cancers usually evolve through a multistage process in which tumors accumulate multiple oncogenic lesions, the presence of an oncogene could be often negligible for tumorigenesis, but, despite this complexity, tumor growth and survival can often be impaired by the inactivation of a single oncogene. This phenomenon, called ‘‘oncogene addiction,’’ provides a rationale to identify novel therapeutic molecular targets.[16,17] “KRAS addiction” has been deeply investigated in murine and human cancer models or in clinical studies.[18–25]

Pancreatic Ductal Adenocarcinoma (PDAC) is a nearly uniformly fatal disease despite intensive treatment, with less than 1-5% of patients surviving 5 years.[26,27] PDAC is one of the best-defined KRAS-driven human malignancies, with a wealth of molecular studies identifying mutant KRAS as the initiating event in the vast majority of cases.[28–32] Expression of KrasG12D or KrasG12V in the murine pancreas induces acinar cell dedifferentiation that progresses to metastatic PDAC,[29,30,33–35] and is associated with activation of the PI3K/PDK1/AKT signaling pathway.[36,37] The identification of KRAS-dependent (KRAS oncogenic “addicted”) versus KRAS-independent PDAC cell lines[18,38] allows the identification of KRAS-driven PDAC subtype-specific mechanisms of oncogenesis that can be exploited to therapeutic benefit.[13,39]

In a search for specific regulators of KRAS oncogenic activity in PDAC, we identified here HNRNPA2B1 as a novel oncogenic KRAS interactor, that was highly specific for previously validated KRAS dependent human PDAC cell lines.[18,38] By down-regulating HNRNPA2B1 in these cell lines, we found a KRAS-dependent specific requirement of HNRNPA2B1 for certain oncogenic features. Our data indicate that the interaction between HNRNPA2B1 and KRAS is important for PI3K/AKT activation in KRAS-dependent PDAC cells. Furthermore, this interaction is dependent on KRAS Ser181-phosphorylation status, suggesting that this post-translational modification could play a pivotal role in KRAS-dependent PDAC.

RESULTS

Identification of HNRNPA2B1 as a novel oncogenic KRAS interactor

In order to identify novel regulators of oncogenic KRAS, HeLa cells were transfected with a plasmid expressing the constitutively active HA-KRASG12V and immunoprecipitated with an anti-HA antibody using non-transfected cells as a control. A mild elution with hemagglutinin (HA)-peptide was performed to increase the specificity of the bound fraction .The bound fraction was resolved in a SDS-PAGE and stained with Flamingo®. The main bands reproducibly specific for HA-KRASG12V transfected cells were excised, digested with trypsin, and analyzed by MALDI-TOF. We identified 6 specific proteins (Fig S1). We focused on heterogeneous nuclear ribonucleoprotein A2/B1 (hereinafter referred to as HNRNPA2B1) (Mowse score= 3.89e+005; sequence coverage =35 %; peptides matched= 10/108) given the described involvement of its cytoplasmic accumulation in malignancies harboring oncogenic KRAS [40–43]

In order to confirm and further characterize the interaction, co-immunoprecipitation with the active form of the major RAS isoforms was studied. We observed a specific interaction with the K- and N-RAS isoforms, being excluded from the H-RAS (Fig 1A). To determine whether the activation status of KRAS was important for the interaction with HNRNPA2B1, we compared the co-immunoprecipitation either with the fully active mutant (V12) or the wild-type (WT) form of KRAS. We observed a specific interaction with the active oncogenic mutant (V12) (Fig 1B) that was further confirmed by the reverse co-immunoprecipitation (Fig 1C).

HNRNPA2B1 is a ribonucleoprotein that is involved in mRNAs processing that is mainly located in the cell nucleus.[44,45] Although a membrane-bound fraction has also been previously identified [44,46] the interaction with RAS proteins has not been previously described. Using cell fractionation and Western blotting, we found a substantial amount of HNRNPA2B1 protein in the membrane fraction (P100) (Fig. S2A). Furthermore, using immunofluorescence in fully intact cells, despite an extensive nuclear localization, HNRNPA2B1 was also found scattered throughout the cytoplasm and decorated plasma membrane (Fig S2B), thus co-localizing in the main KRAS compartment in normal conditions. Interestingly, when analyzing nuclear/cytoplasmic distribution of HNRNPA2B1 in tissue microarrays from PDAC patients, we found an exclusive nuclear staining in all premalignant stages analyzed, whereas a 28% of PDAC tumoral samples exhibited cytoplasmic staining (Table S1 and Fig S3).

To explore the role of HNRNPA2B1 in KRAS-driven oncogenesis, a subset of 6 Pancreatic Ductal Adenocarcinoma (PDAC) cell lines with oncogenic mutation of KRAS at codon 12 and previously characterized for bearing differential KRAS-dependency for survival (KRAS oncogenic “addiction”)[18,38] and revalidated here (Fig S4) was chosen to perform co-immunoprecipitation. We found that KRAS levels were elevated in the KRAS-dependent cell lines as previously described.[18,36] Intriguingly, we discovered that although total levels of HNRNPA2B1 were similar, the cytoplasmic pool was higher in KRAS-dependent cell lines. Interestingly, it was found that HNRNPA2B1 interacted with KRAS specifically in KRAS-dependent PDAC cell lines (Fig 2).

HNRNPA2B1 regulates cell proliferation in serum-limiting conditions and oncogenic capacity of KRAS-dependent PDAC

Next, we evaluated the effect of HNRNPA2B1 ablation in the 6 PDAC cell lines described above. We infected cells with previously validated HNRNPA2B1-specific shRNA (sh1 and sh2) or empty vector [MLP(-)] [47] and, after selection, knock-down was validated by western blot (Fig 4 and S5).

We found that cell proliferation was not affected in standard culture conditions (10%FCS) in any cell lines (data not shown). In order to recapitulate tumor growth conditions we evaluated cell

growth under serum-limiting conditions (0.1% FCS). After 48h of starvation, growth of KRAS-dependent cell lines was inhibited by approximately 50% (P<0.0001) after HNRNPA2B1 knock-down (both shRNA) compared to a control shRNA (MLP(-)). In contrast, HNRNPA2B1 depletion failed to produce any significant (P>0.05) change in cell growth under these conditions in KRAS-independent cell lines (Fig 3A). Furthermore, we found an induction of cleaved caspase-3 in KRAS-dependent cell lines when depleting HNRNPA2B1 in serum starving conditions (Fig 4).

Given off-target effects of shRNAs are known to commonly affect cell growth, we validated the specificity of the shRNAs used, by expressing exogenous HNRNPA2B1, using a cDNA which lacked the 3'UTR targeted by the shRNAs. Selected stable HPAF-II cells [expressing MLP(-), sh1 or sh2] were transfected with either empty GFP or GFP-HNRNPA2B1 and were starved for 48h with 0.1%FBS. Both cell growth (P<0.0001) and induction of caspase-3 were partially but significantly rescued, as GFP-HNRNPA2B1 transfected in both knock-down cells phenocopied HPAF-II MLP(-) cells transfected with GFP suggesting the shRNA effects are specific to HNRNPA2B1 depletion (Fig S5). Furthermore, since rescue was not complete, we further checked the specificity of the HNRNPA2B1 shRNA knock-down by transiently transfecting pooled siRNA directed against different sequences of HNRNPA2B1. siRNA against HNRNPA2B1 was transfected into two KRAS-dependent PDAC cell lines (MPanc-96 and HPAF-II) and into two KRAS-independent PDAC cell lines (SW-1990 and 8988T) and knock-down validated by western blot (Fig S6). Induction of caspase-3 only in KRAS-dependent cell lines after 48h of starvation with 0.1%FBS was again reproduced (Fig S6), conclusively confirming the specificity of the effects of HNRNPA2B1 knock-down.

Having confirmed the specificity of the HNRNPA2B1 shRNAs, we evaluated cell growth in anchorage-independent conditions at 10% FCS with and without HNRNPA2B1 depletion. Strikingly, KRAS-dependent PDAC cell lines exhibited a significant reduction (P<0.0001) in the number of colonies (>50% reduction) upon HNRNPA2B1-depletion, whereas changes were not significant (P>0.05) for KRAS-independent cell lines (Fig 3C).

Finally, to determine the importance of HNRNPA2B1 for PDAC tumor formation *in vivo*, nude mice were injected with two KRAS-dependent PDAC cell lines (MPanc-96 and HPAF-II) and two KRAS-independent PDAC cell lines (SW-1990 and 8988T) cells with and without knockdown of HNRNPA2B1. Again, when knocking down HNRNPA2B1, KRAS-dependent cell lines showed a significant reduction (P<0.001) in tumor growth while not significant changes were observed in the case of KRAS-independent cell lines. When tumors were allowed to develop for longer time differences in tumor size disappeared. Interestingly western blot analysis showed a re-expression of previously silenced HNRNPA2B1 protein in these tumors, suggesting that growth recovery was potentially due to a positive selection of HNRNPA2B1 expressing cells (data not shown),

Induction of apoptosis by HNRNPA2B1 downregulation in KRAS-dependent PDAC cell lines correlates with diminished activation of PI3K/AKT/mTOR signaling pathway

To explore the KRAS-driven signaling pathways that could be involved in the observed effects of HNRNPA2B1 depletion, we first checked the possible effect on the stability on KRAS itself. Importantly we found that HNRNPA2B1 downregulation had no significant effect on KRAS levels (Fig 4, Fig S8).

Given the relevance of growth factor limitation in the phenotype of HNRNPA2B1 knock-down, we wondered if the tyrosine kinase receptors RON and the Insulin receptor β, relevant in PDAC[48] and previously described to be altered by HNRNPA2B1 knock-down,[47] were affected. No significant changes in levels of both proteins were observed upon HNRNPA2B1 knock-down in any of the cell lines (Fig S7, Fig S8).

We also analyzed the effect of HNRNPA2B1 depletion on ERK activation and we found that its activation status was not significantly affected (phosphorylation of Thr202/Tyr204) in none of the PDAC cell lines analyzed (Fig 4, Fig S8).

Then, we evaluated the activation through the PI3K/AKT axis as it has been described as an important effector pathway of oncogenic KRAS together with MAPK axis in PDAC.[36,37,49–51] We found that at the serum-limiting conditions there was a significantly diminished activation of AKT in KRAS-dependent PDAC as assessed by Ser473 and Thr308- phosphorylation in the absence of HNRNPA2B1 (Fig 4, Fig S8). In contrast, and in agreement with the data above, HNRNPA2B1-depletion had no effect on AKT activation in KRAS-independent PDAC (Fig 4, Fig S8).

Since the activation of AKT was diminished upon HNRNPA2B1 depletion, we studied signaling downstream of AKT. Consistent with the data above, we found that the activation of both mTOR and its downstream effector S6 were dramatically diminished when HNRNPA2B1 was depleted in KRAS-dependent cell lines, while unaffected in KRAS-independent (Fig 4, Fig S8). Consistently, HNRNPA2B1 depletion by pooled siRNA reproduced the same signaling pattern (Fig S6).

Reduced interaction of KRAS with PI3K in hnRNA A2/B1-depleted KRAS dependent PDAC

Having shown that AKT activation by KRAS was dependent on HNRNPA2B1 in KRAS-dependent PDAC cells, we wondered whether HNRNPA2B1 mediated the physical interaction of the KRAS-PI3K complex. First, we demonstrated that the catalytic subunit of PI3K (p110α) interacted with KRAS in KRAS-dependent but not in the KRAS-independent cell lines (Fig S9). Interestingly, when performing KRAS immunoprecipitation in two KRAS-dependent cell lines, we found that PI3K (both p110α and p85 subunits) could not interact with KRAS when HNRNPA2B1 was depleted (Fig 5). Thus, HNRNPA2B1 is an essential cofactor of KRAS-mediated PI3K activation in KRAS-dependent PDAC.

HNRNPA2B1 interaction with KRAS depends on KRAS Ser181-phosphorylation status

To further understand the reason for the differential interaction of HNRNPA2B1 with KRAS in KRAS-dependent and independent PDAC tumors, we evaluated the possible influence of KRAS Ser181-phosphorylation, a previously described post-translational modification that affected KRAS-driven oncogenesis and signal transduction.[52–54] HeLa cells were transfected with the oncogenic KRAS phosphomutants for Ser181 phosphorylation ("phosphorylatable" wild-type (S), non-phosphorylatable (A) or phosphomimetic (D)) and cell lysates were immunoprecipitated with anti-HA antibody. We found that HNRNPA2B1 interacted with the "phosphorylatable" (S) and exhibited an enhanced interaction with the phosphomimetic (D), while no interaction was seen with the non-phosphorylatable (A) KRAS phosphomutant (Fig 6A). To evaluate the capacity of Ser-181 KRAS phosphorylation to recruit HNRNPA2B1, we studied the recruitment of HNRNPA2B1 to the cell membrane, the main biological compartment of KRAS. We confirmed that HNRNPA2B1 was efficiently recruited to the membrane, especially when overexpressing the phosphomimetic (D) KRAS, but no membrane localization was seen when overexpressing non-phosphorylatable (A) KRAS (Fig 6B).

Finally, we tested the prediction that HNRNPA2B1 –KRAS interaction was dependent on phospho-KRAS by evaluating the presence of phospho-KRAS in the subset of PDAC cells we used before. By a gel retardation Phos-tag™ approach specific for phosphorylated proteins, we found a band with lower mobility exclusively in KRAS-dependent cell lines (Fig.6C) which did not appear in a standard SDS-PAGE (Fig S10). The retarded band disappeared after dephosphorylation treatment with PPλ (Fig 6C). Thus, KRAS was phosphorylated in KRAS-

dependent but not in KRAS-independent PDAC cell lines in agreement with the requirement of this post-translational modification for the oncogenicaly relevant HNRNPA2B1 –KRAS interaction

DISCUSSION

There has been no major breakthrough in the treatment of PDAC in the past 30 years and no effective targeted therapies are currently available.[31] Nearly all PDAC harbor KRAS mutations and this is an initiating event, but efforts to develop effective KRAS inhibitors have generally failed. It is therefore essential to understand and define the importance of interactors/regulators of mutant KRAS to identify essential nodes of "nononcogene addiction"[36] defined as the dependence of mutated oncogenes on unmutated genes.[55] To assess the contribution of an interactor/regulator on oncogenic KRAS signaling, we compared here the growth and the tumor formation capacity of PDAC cells that are dependent on oncogenic KRAS for their survival to PDAC cell lines that are independent of oncogenic KRAS.[18,38] This model has been recently used to assess the effect of an inhibition of KRAS- PDEδ interaction.[13] Here we show that HNRNPA2B1 is a novel interactor/regulator of oncogenic KRAS that modulates the signaling and interaction with its main effector pathway PI3K/AKT/mTOR in KRAS-dependent PDAC.

HNRNPA2B1 is a member of the hnRNP family of RNA-binding proteins, which are expressed in most human tissues and play multiple functions such as mRNA processing or telomere biogenesis.[44,56] They undergo nucleocytoplasmic shuttling, which was originally proposed as a means of transporting mRNA out of the nucleus, but it has been proved to be also essential for the numerous cytoplasmic functions in which hnRNPs participate.[44,45] In fact, the interaction of some members of the hnRNPs family with plasma membrane proteins has been described. hnRNP M4 interacts with carcinoembryonic antigen, a cell membrane receptor that may contribute to metastasis in colorectal cancer.[44,57] hnRNP A1 can also interact with BCR/ABL, thus promoting leukemogenesis [58] and hnRNP K can bind Vav and c-Src in hematopoietic cells serving as a docking site to facilitate kinase crosstalk at the plasma membrane.[59,60] Moreover, it has been reported the interaction of the nucleocytoplasmic shuttling proteins Nucleophosmin and Nucleolin with KRAS to enhance nanocluster formation and signal gain in the MAPK pathway.[15] Thus, the functional interaction of the cytoplasm confined KRAS with HNRNPA2B1 could be understood in the context of a novel hnRNP cytoplasmic function.

Related to this point, in certain KRAS-driven human malignances, HNRNPA2B1 cytoplasmic accumulation has been linked to the dedifferentiation progress leading to a neoplasia, suggesting a role of HNRNPA2B1 as an oncogenic promoter when is in the cytoplasm. Its cytoplasmic localization is considered a bona fide biomarker of hepatocellular carcinoma[42,43] or lung cancer.[40,41] This could explain why HNRNPA2B1 is functionally relevant for the studied oncogenic features in PDAC cells with a higher cytoplasmic distribution of this protein (KRAS-dependent PDAC) rather than for the HNRNPA2B1-nuclear-confined KRAS-independent PDAC (Fig 2 and 3). Interestingly, we analyzed here for the first time the nuclear/cytoplasmic distribution of HNRNPA2B1 in tissue microarrays from PDAC patients. Samples from any premalignant stages were exclusively nuclear, while cytoplasmic staining was evident in PDAC tumoral samples (Fig S3). Therefore, the specificity of cytoplasmic staining for tumoral stage reinforces the idea of an oncogenic activity of HNRNPA2B1 when colocalizing with the cytoplasmicaly located KRAS.

Furthermore, although a role in proliferation of human cell lines was previously described,[61] and overexpression of HNRNPA2B1 has been found in PDAC cell lines[62] a direct role of HNRNPA2B1 in tumorogenesis was not proved until recently. Golan-Gerst and coworkers[47] showed, on the one hand, that NIH 3T3 overexpressing HNRNPA2B1 became transformed, formed colonies in soft agar and were tumorigenic in nude mice, and, on the other hand, that knock-down of HNRNPA2B1 in glioblastoma cells inhibited tumor formation. Moreover, it has

been recently shown that HNRNPA2B1 silencing enhanced chemotherapy sensitivity of several pancreatic cell lines to gemcitabine, 5-FU, and oxaliplatin.[63] Interestingly, a study by Biankin and coworkers[64] showed that all PDAC patients harboring copy-number variations in HNRNPA2B1 gene exhibited gain in HNRNPA2B1 copy number, thus suggesting a positive selection of HNRNPA2B1 amplification.

The effects of low serum (0.1% FCS) in cell proliferation observed in KRAS-dependent PDAC cell lines could be harmonized with previous observations. It has been described that HNRNPA2B1 knock-down in glioblastoma cell lines impaired growth in serum-limiting conditions.[47] Interestingly, similarly to the case of HNRNPA2B1-depleted KRAS-dependent cell lines described here (Fig 4, Fig 5), Samuels and coworkers[65] described, using colorectal cell lines with oncogenic KRAS mutations (HCT116 and DLD-1), that PI3K activity (AKT/mTOR signaling) and growth were compromised at 0.5% FCS. Interestingly, both were rescued upon addition of EGF or Insulin, suggesting that AKT/mTOR pathway activation could be compensated by tyrosine kinase receptor activity induced by growth factors. Importantly, here we found no upregulation of tyrosine kinase receptors RON or Insulin receptors when cells were deprived at 0.1% FCS for 48h (Fig 4, Fig S7, Fig S8) in HNRNPA2B1 depleted cells, suggesting that most AKT/mTOR activity could not be compensated and was still driven by oncogenic KRAS activation of PI3K, and thus, very sensitive to KRAS-PI3K disruption induced by HNRNPA2B1 depletion (Fig 5).

Our results demonstrate a selective requirement of KRAS Ser181-phosphorylation for the interaction with HNRNPA2B1. KRAS has, adjacent of the farnesylated C-terminal cysteine, a stretch of six contiguous lysines in a total of eight lysine residues- known as the polybasic domain - which promotes an electrostatic interaction with the negatively-charged phosphate groups of phospholipids.[66] Phosphorylation of KRAS at serine-181 by PKC within this domain has been described.[67] We previously reported a role of PKC-induced KRAS Ser181 phosphorylation for activation of the wild-type KRAS and regulation of oncogenic KRAS activity. In our model of NIH 3T3 transformed by oncogenic KRAS, the non-phosphorylatable KRAS significantly reduced its growth (P<0.0001) and failed to activate AKT at serum-limiting conditions but not at serum-saturating conditions,[52] suggesting that the role of KRAS phosphorylation was more prominent under serum starvation. Moreover we recently found that, when phosphorylation of KRAS was prevented, it did neither interact with PI3K (p110α) nor form clusters with PI3K.[53] Most importantly, we recently reported that inhibition of KRAS phosphorylation impaired tumor growth.[54] This is in agreement with the data presented here as we show that the cell lines with higher interaction of oncogenic KRAS with p110α (Fig S9) and with HNRNPA2B1(Fig 2) were the ones harboring phosphorylated KRAS (Fig 6) at serum-limiting conditions. Related to this point, in the current work we also proved that KRAS phosphorylation at Ser181 was sufficient to recruit more HNRNPA2B1 to the cytoplasmic cell membrane (Fig 6), thus suggesting that the higher cytoplasmic accumulation that exhibited KRAS-dependent PDAC was a consequence, instead of a cause, of its increased interaction with –Ser181 phosphorylated- KRAS (Fig 2).

Interestingly, this interaction occurs in the two most prevalent KRAS mutations of human cancer. In our model, HNRNPA2B1 is able to interact with both KRAS G12V-mutated (MPanc-96) and G12D-mutated (HPAF-II) KRAS-dependent cell lines, underlying the importance of this interaction for KRAS-driven malignancies.

Although further investigation about the requirement of KRAS phosphorylation in gastrointestinal malignancies needs to be performed, it is worth mentioning that the putative kinase PKCδ is elevated in PDAC[8,68] and several studies highlight PKC inhibition as an effective therapy in PDAC cell lines.[69,70]

Our data support a role for the new KRAS interactor HNRNPA2B1 as a regulator of KRAS-dependent tumorigenesis through the critical PDAC signaling pathway PI3K/AKT. Moreover, we demonstrate that this functionally relevant interaction depends on KRAS Ser181-phosphorylation status. Taken together, our results describe a PDAC subtype specific functional and regulatory process that can be exploited for novel targeted therapeutic interventions for this grave disease.

MATERIAL AND METHODS

Cells

HeLa cells were obtained from American Tissue and Cell Collection (ATCC) and routinely verified according to the specifications outlined in the ATCC Technical Bulletin. Pancreatic Ductal Adenocarcinoma cell lines (MPanc-96, HPAF-II, pa-tu-8902, SW-1990, 8988-T and PANC-1) were kindly provided by Prof. Dr. A. Kimmelman (Harvard Medical School, Boston, USA).

HeLa and the Pancreatic Ductal Adenocarcinoma cell lines (MPanc-96, HPAF-II, pa-tu-8902, SW-1990, 8988-T and PANC-1) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS, Biological Industries), penicillin, streptomycin and non-essential aminoacids.

Statistics

All analyses were performed with GraphPad Prism 5.0. Data represent mean ± SEM. Mann-Whitney test was used to analyze significance levels. Specific significance levels are found in figure legends. $P < 0.05$ was considered significant.

See also supplementary information

FIGURE LEGENDS

Fig 1. Identification of HNRNPA2B1 as a novel oncogenic KRAS interactor. **(A)** HeLa cells were transfected with HA-tagged RAS isoforms (either K-, H- or N-RAS) bearing the oncogenic (G12V) mutation. Cell lysates were immunoprecipitated with anti-HA antibody (IP: α- HA). A non-transfected lysate (NT) was used as a negative control. **(B)** To check the requirement of the KRAS activation status, HeLa cells were transfected with either wild type (WT) or oncogenic G12V (V12) HA-tagged KRAS and immunoprecipitated with anti-HA antibody. The input and the bound fractions were immunoblotted with the indicated antibodies. **(C)** The reverse coimmunoprecipitation was performed. HeLa cells were co-transfected with YFP-KRASG12V and either with (+) or without (-) HA-hnRNP A2 and cell lysates were immunoprecipitated with anti-HA antibody

Fig 2. **HNRNPA2B1 interaction with oncogenic KRAS correlates with KRAS dependency in 6 lines of human PDAC**. Cell lysates of 6 PDAC cell lines harboring oncogenic KRAS mutations in codon 12 were immunoprecipitated with anti-KRAS antibody (IP:KRAS). A previous cell fractionation step was performed to purify the cytoplasmic fraction, which was loaded into the IP (input). The distribution of HNRNPA2B1 in the whole cell lysate (WCL) is displayed to differentiate from the cytoplasmic fraction (Cyto) used as "input". The same amount of an unrelated antibody (IP:IgG) was used as a negative control.

Fig 3. HNRNPA2B1 is required for growth under serum starvation, soft-agar colony formation and tumour progression in KRAS dependent PDAC. (A) PDAC cell lines from figure 2 were infected with retrovirus encoding HNRNPA2B1-specific shRNA (sh1 and sh2) or empty vector [MLP(-)] and after selection knock-down was validated by western blot (Fig 4). Same amount of cells were seeded and were deprived for 48h with 0.1%FBS. Fold change relative to day 0 is displayed. **(B)**. Quantification of colony formation in soft agar of cells described in (A). **(C)** cells described in (A) were injected ($1.5x10^6$ cells/site) subcutaneously near both rear flanks of nude/nude mice, and tumor volume was measured biweekly. Error bars indicate SEM (n = 10) (***, $P<0.0001$; **, $P<0.001$; *, $P<0.01$; and, when not indicated differences were not-significant i.e. $P>0.05$; *P* for Mann-Whitney test; mean and SEM are represented).

Fig 4. Induction of apoptosis by HNRNPA2B1 downregulation in KRAS dependent PDAC cell lines correlates with diminished activation of AKT signaling. Cells described in Fig 3 were serum starved (0.1% FCS) for 48h and harvested. Cell lysates were incubated with the indicated antibodies

Fig 5. **KRAS interaction of with PI3K is dependent on HNRNPA2B1 in KRAS-dependent PDAC**. Cell lysates from the cells described in Fig3 were immunoprecipitated with anti-KRAS antibody (IP: KRAS). The same amount of an unrelated antibody was used as a negative control (IP:IgG)

Fig 6. Oncogenic KRAS – hnRNP A2 interaction is dependent on KRAS phosphorylation status. (A) HeLa cells were transfected with HA-KRASG12V phosphomutants for Ser181 phosphorylation: "phosphorylatable" wild-type (S), non-phosphorylatable (A) or phosphomimetic (D). Cell lysates were immunoprecipitated with anti-HA antibody. A non-transfected lysate (NT) was used as a negative control. **(B)** To verify the recruitment of HNRNPA2B1 to the membrane - main KRAS compartment - HeLa cells were transfected with the constructs described in (A) and a cell fractionation was performed in order to obtain cytoplasmic membrane fraction (P100). **(C)** Cellular extract from cells described in Fig2 were resolved in a Phos-Tag™-SDS-PAGE and immunoblot was performed using anti-KRAS antibody.

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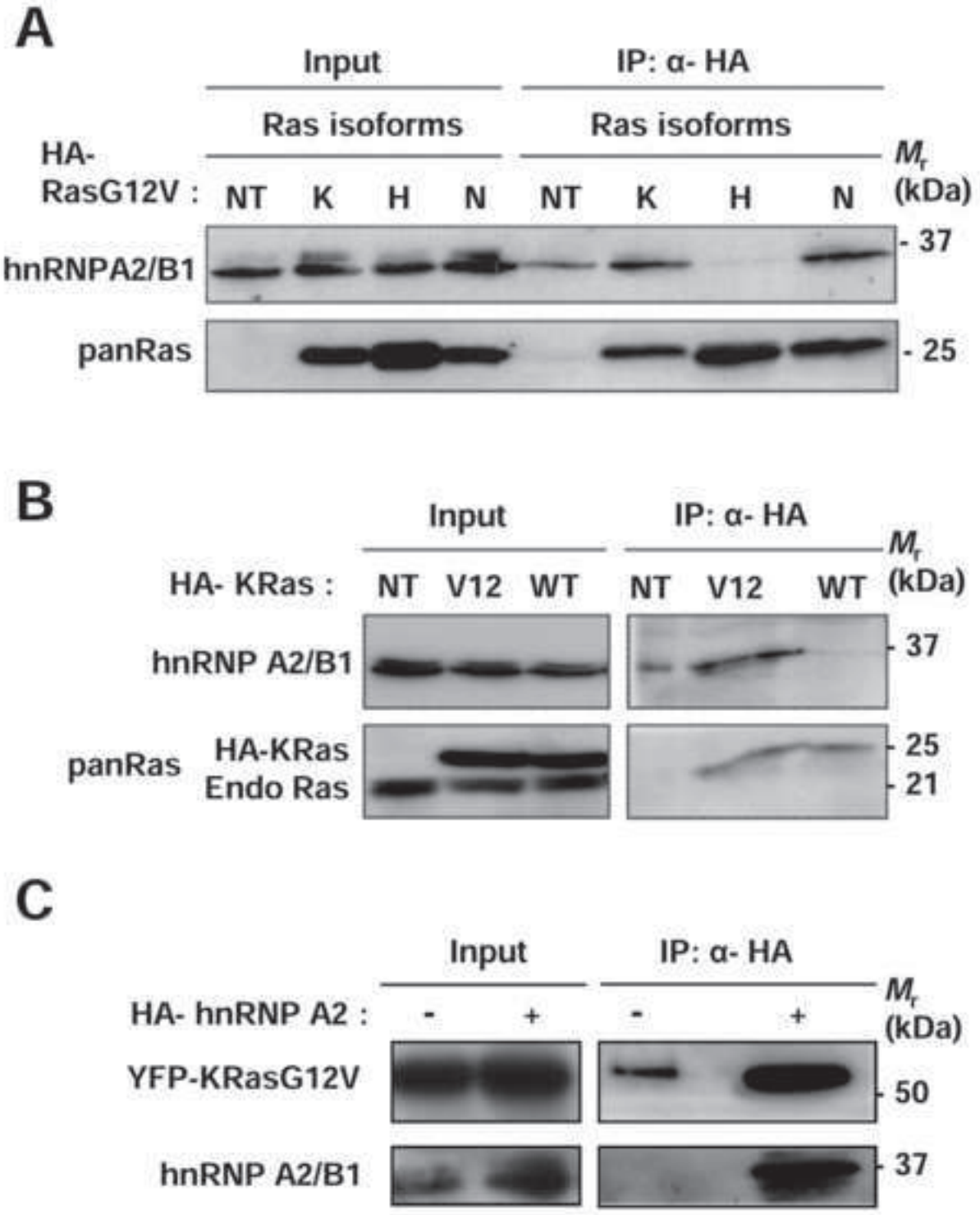

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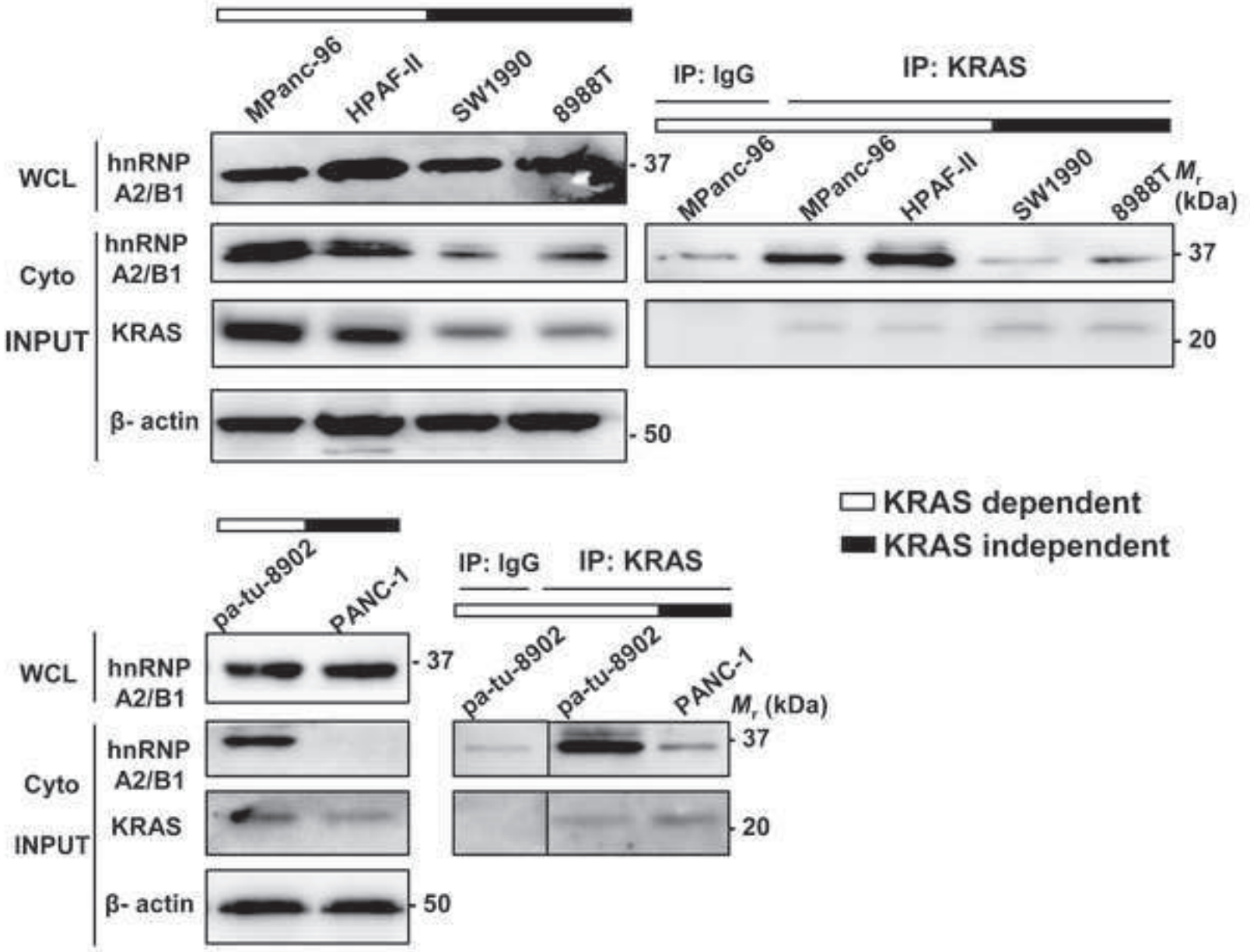

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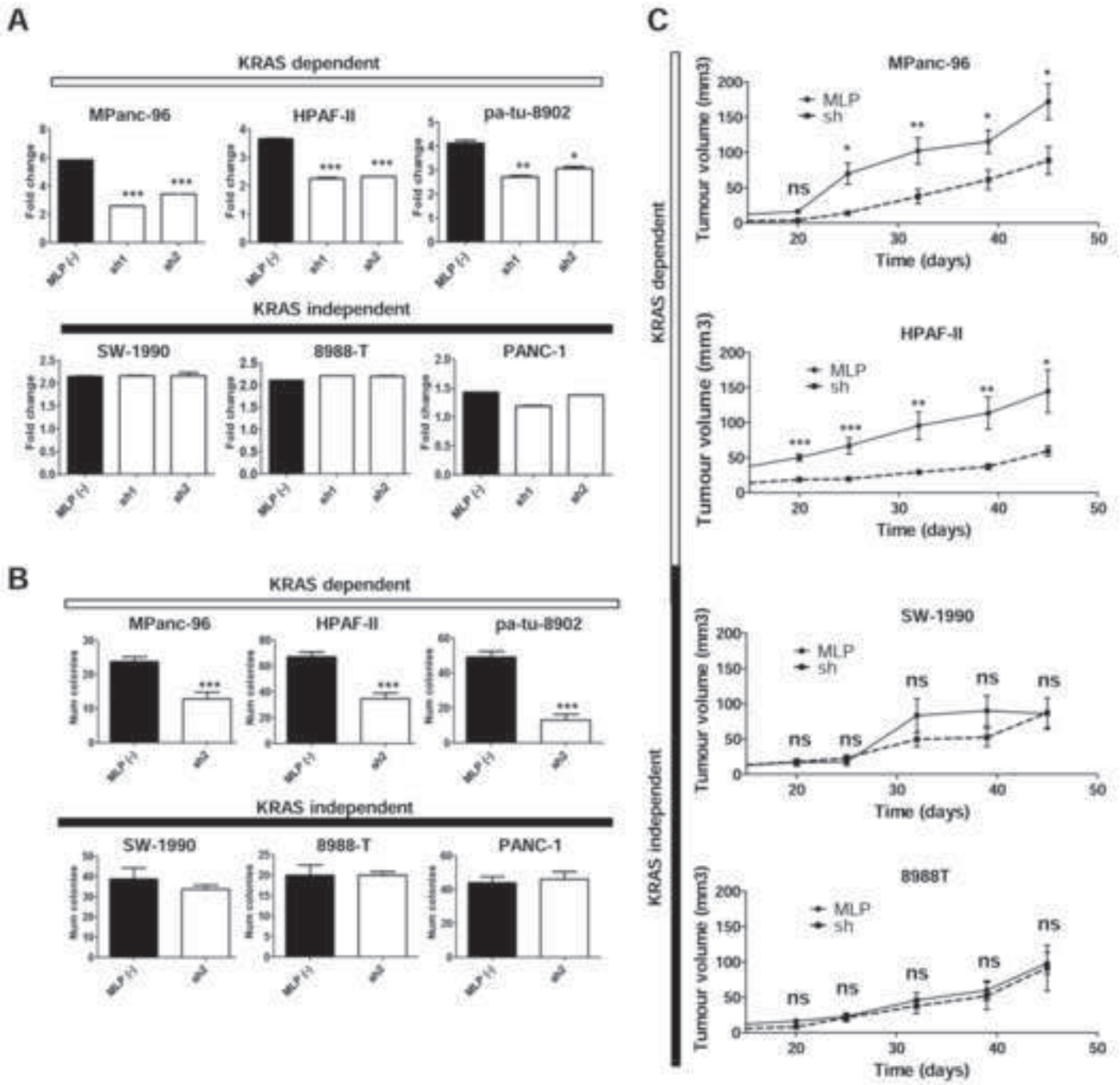

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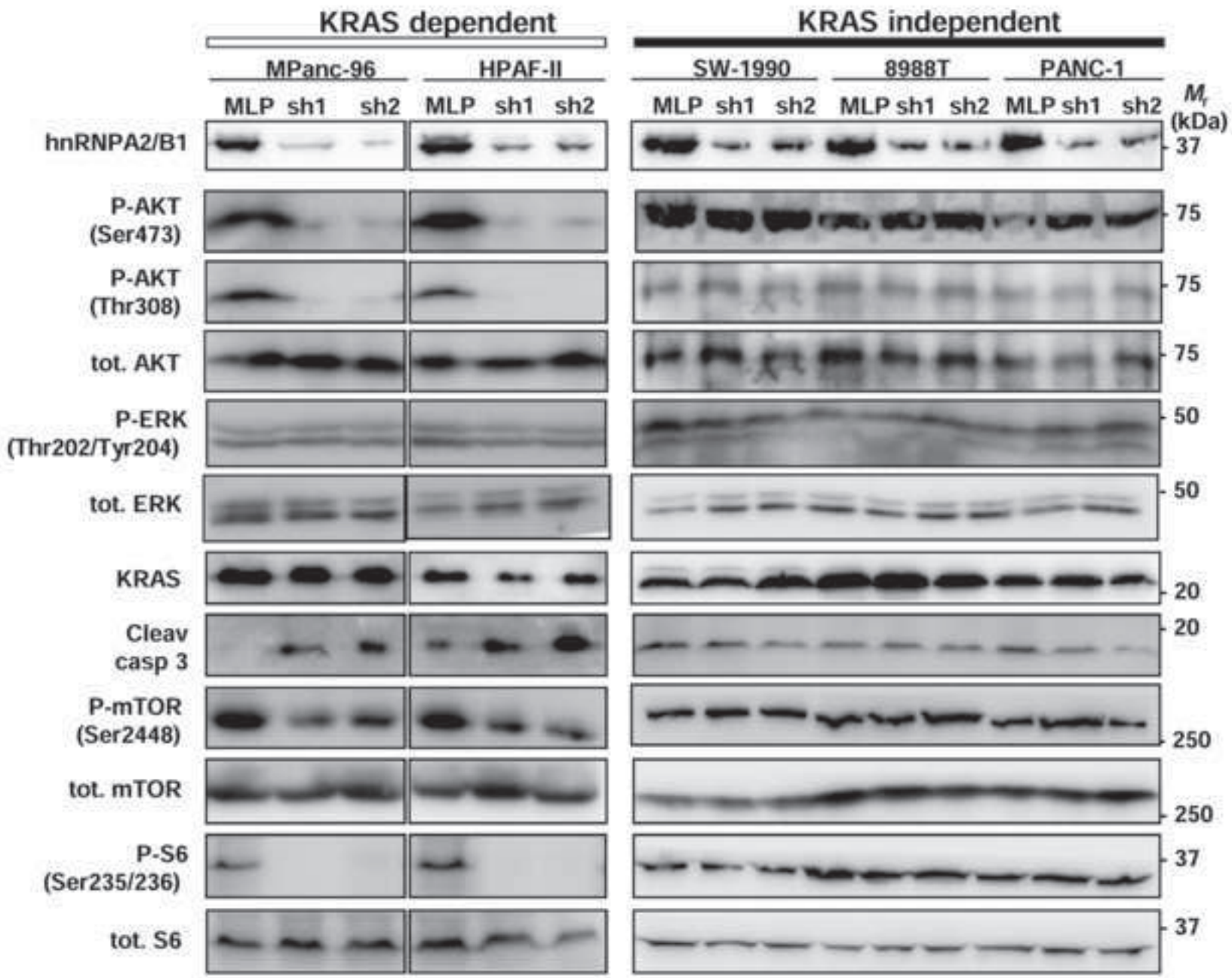

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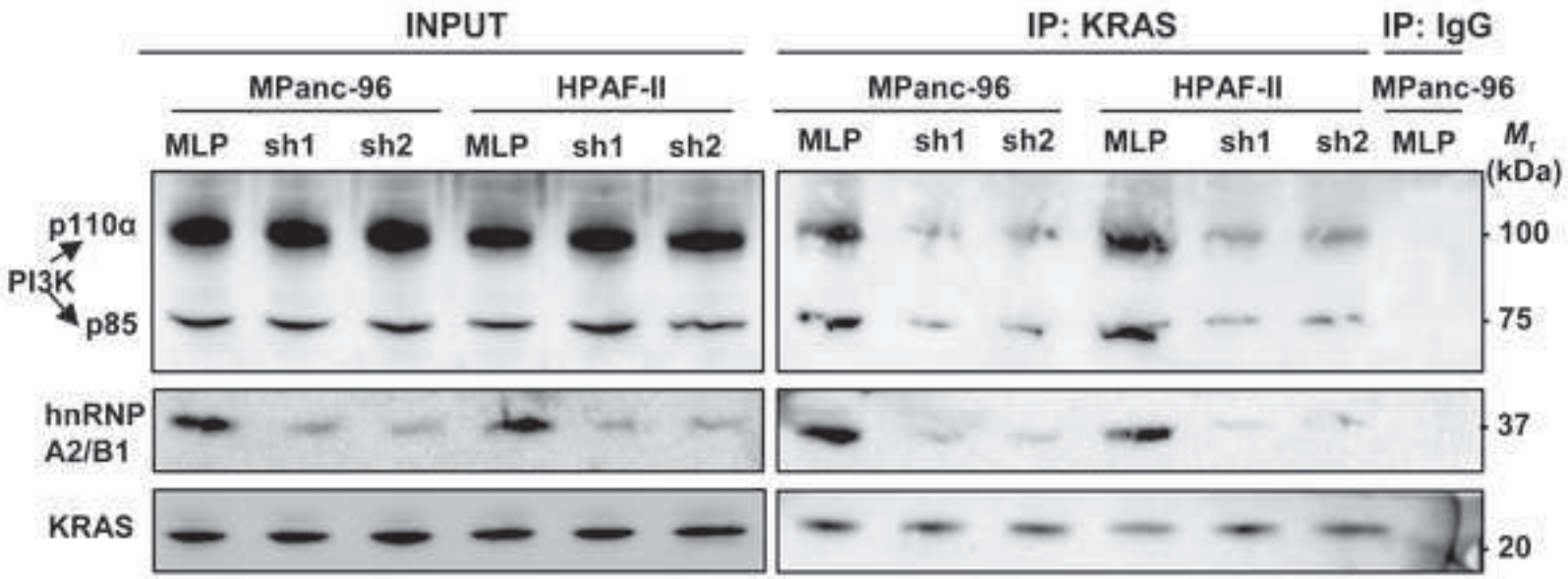

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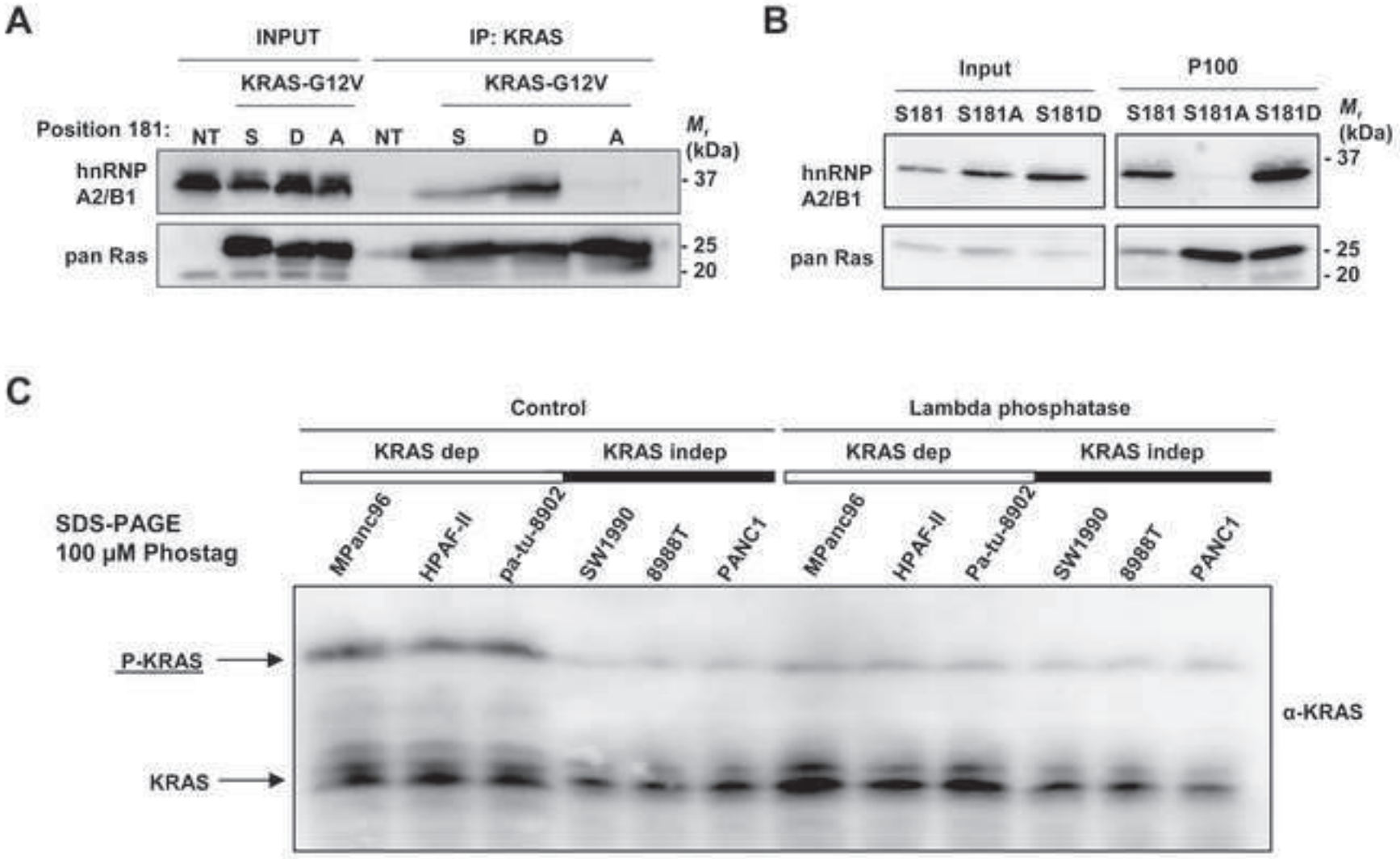

Supplementary methods and references
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SUPPLEMENTARY METHODS

Transfection

To generate stable transductant pools, Pancreatic Ductal Adenocarcinoma cell lines (MPanc-96, HPAF-II, pa-tu-8902, SW-1990, 8988-T and PANC-1) were infected with MLP-puro short hairpin RNA (shRNA) retroviral vectors targeting HNRNPA2B1 mRNA. Infected cells were selected with puromycin (2 µg/mL) for 96 hours as previously described[1] and used within low passages for experiments.

For RNAi experiments, each cell line was transfected with ON-TARGETplus SMARTpool siRNA against HNRNPA2B1 (#L-011690-01-0005, Dharmacon, Thermo Scientific, Rockford, IL USA)) or siRNA targeting KRAS[2] using Non-targeting siRNA pool as control (#D-001810-10-20, Dharmacon Thermo Scientific), Hi-PerFect (#301709, Qiagen) as transfection reagent according to manufacturer's recommendations.

For transient transfection, HeLa cells were transfected with Lipofectamine 2000® (Invitrogen) following the manufacturers protocol and harvested between 24-48 hours after transfection.

Cell viability assay

Pancreatic Ductal Adenocarcinoma cell lines (MPanc-96, HPAF-II, pa-tu-8902, SW-1990, 8988-T and PANC-1) were infected with the indicated retrovirus. After selection, 6000 cells per well were seeded in 96-well plates. After deprivation with 0.1% (v/v) FCS DMEM for 48h, they were washed with PBS and treated with Cell Titer Glo assay (#G7572, Promega, Madison, WI, USA) following the manufacturer's procedure.

Anchorage-independent growth

Colony formation in soft agar was assayed as previously described.[3] Briefly, cells were mixed with 0.4% top agar and layered over 0.6% bottom agar, in triplicate, in six-well plates (BD Biosciences, Franklin Lakes, NJ). The cells were allowed to grow for 2-3 weeks, at which time colonies larger than 200 µm were counted for each cell line with the use of the publicly available Image J software (version 1.41; http:// rsbweb.nih.gov/ij/index.html).

Tumorigenic assays in nude mice

$1{,}5x10^6$ Pancreatic Ductal Adenocarcinoma cell lines (MPanc-96, HPAF-II, SW-1990 and 8988-T) expressing either MLP empty (control) or MLP containing HNRNPA2B1 shRNAs (knock-down) were subcutaneously injected into each rear flank of nu/nu Swiss nude mice (foxn1 $^{-/-}$) by using a 26-gauge needle. Generated tumors were measured twice a week from day ~20 after injection (each group n=10).

Confocal microscopy

HeLa cells were transfected with YFP-KRASG12V. At 24h cells were fixed and incubated with anti-HNRNPA2B1 (F-16) (sc-10035, 1:20, Santa Cruz), washed and incubated with Alexa647-labeled anti-mouse secondary antibody. To determine YFP-KRASG12V and endogenous HNRNPA2B1 (Alexa647-labeled) colocalization, YFP and Alexa647-labeled images were acquired sequentially using 514 and 650 laser lines, emission detection ranges 525-573 nm and 580-700 nm respectively and the confocal pinhole set at 1 Airy units. Images were acquired at 400 Hz in a 1024 x 1024 pixels format, zoom at 4 and pixel size of 60 x 60 nm.

Cell fractionation and Immunoprecipitation

The cells were subjected to hypotonic lysis and the post-nuclear supernatant was isolated as previously described.[4] Briefly, 150-cm dish of cells was lysed in 400 µL Buffer A (10 mM Tris, pH 7.5, 25 mM NaF, 5 mM $MgCl_2$, 1 mM EGTA, 0.33 ng/mL aprotinin, 0.1 mM Na_3VO_4, 3.3 ng/mL leupeptin, 1 mM dithiothreitol) by 30 strokes in a 1mL syringe and 25G needle obtaining the whole-cell lysate (WCL). Post-nuclear supernatant (PNS) was obtained by centrifugation at 1000 g for 10 min at 4ºC. Where required for complete cell fractionation, cytosolic (S100) and membrane (P100) fractions were further fractionated from the post-nuclear supernatant by a 30 min, 100.000 g ultracentrifugation at 4°C.

To prepare the input for immunoprecipitation, the post-nuclear supernatants (PNS) were solubilized with 0.1% (v/v) Triton X-100 Buffer A for 1 h, and then non-soluble proteins were cleared by centrifugation at 100,000 g. For anti-KRAS immunoprecipitation, the appropriate antibody was cross-linked to protein M-270 Epoxy magnetic Dynabeads (#14302D, Invitrogen) and then incubated with the PNS balanced in 0.1% (v/v) Triton X-100 Buffer A for 90 min rotating at 4 °C. The beads were washed with the washing buffers and eluted with the elution buffer provided by the manufacturer For anti-HA immunoprecipitation, anti-HA was cross-linked to Dynabeads protein A (#10002D, Invitrogen) and then incubated with the PNS balanced in 0.1% (v/v) Triton X-100 Buffer A for 3h rotating at 4 °C. The beads were washed with 0.1% (v/v) Triton X-100 Buffer A and eluted with glycine 200 mM pH2.5. The eluates were neutralized to pH 7.5 and added sample buffer, resolved on SDS-PAGE gels, and transferred to polyvinylidene difluoride using semi-dry transfer. The membranes were probed with primary and secondary horseradish peroxidase antibodies. When required, band intensity was determined using the measurement tool of Multigauge 2.0 (FUJIFILM, Tokyo, Japan).

Proteomic identification of new oncogenic KRAS binding partners

The above anti-HA immunoprecipitation was performed. HeLa cells were transfected with HA-tagged KRAS bearing the oncogenic (G12V) mutation. Cell lysates were immunoprecipitated with anti-HA antibody in the presence of 5% (w/v) BSA to block non-specific binding. A mild elution with hemagglutinin (HA)-peptide was performed to increase the specificity of the bound fraction. Bound fractions of the HA-KRASG12V transfected cells and non-transfected (negative control) were resolved in 8-15 % acrylamide SDS-PAGE and stained with Flamingo® (#161-0492, BioRad, Hercules, CA, USA). Main specific bands were excised from the polyacrilamide gel and analyzed by MALDI-TOF Voyager DE PRO (Applied Biosystems) as previously described.[5] Proteins reproducibly identified with a Mowse score above e+005 were considered.

Antibodies

Primary antibodies used for immunoblotting were as follows: Anti-β-Actin (clone C4) (#691001; 1:1000; MP Biomedicals, Santa Ana, CA, USA); anti-HNRNPA2B1 (F-16) (sc-10035, 1:500, Santa Cruz); Anti-cleaved caspase-3 (Asp175) (#9661; 1:1000; Cell Signaling, Danvers, MA, USA); Anti-AKT (#9272; Cell Signaling); Anti-phospho-AKT (Ser473) (193H12) (#4058; 1:1000, Cell Signaling), Anti-phospho-AKT (Thr308) (#9275S; Cell Signaling), Anti- ERK 1/2 (#9102; 1:1000; Cell Signaling); Anti-phospho- ERK 1/2 (Thr202/Tyr204) (#9101; 1:1000; Cell Signaling) Anti-KRAS (clone Ab-1) mouse (#OP24, 1:400, Calbiochem); (Anti-Pan-Ras (clone Ab-3) mouse (#OP40; 1:400; Calbiochem); Anti-PI3K p110 alpha (C73F8) (#4249S; 1:1000, Cell Signaling); Anti-PI3K p85 (19H8) (#4257S; 1:1000, Cell Signaling), Anti-mTOR (#2972; Cell Signaling), Anti-phospho-mTOR (Ser2448) (#2971; Cell Signaling); Anti-pS6 (54D2) (#2317; Cell Signaling), Anti-phospho-S6 (Ser235/Ser236) (#2211S; Cell Signaling), Anti-RON (C81H9) (#2654; Cell Signaling), Anti-Insulin receptor β (4B8) Rabbit mAb (#3025; Cell Signaling).

For immuhistochemistry we used anti-HNRNPA2B1 antibody (Sigma, HPA001666, 1/100). For Immunoprecipitation we used Anti-HA (clone HA-7) (#A2095; Sigma, St. Louis, MO, USA); and Anti-KRAS (clone Ab-1) mouse (#OP24, 1:400, Calbiochem).

Immunohistochemistry on tissue microarrays

Immunohistochemical analyses were performed on tissue microarrays containing representative samples of normal tissue, pancreatic cancer and chronic pancreatitis. After deparafination and rehydration, endogenous peroxidase was blocked. Sections were incubated in boiling water for 10 min in citrate buffer pH 6. The slides were then incubated at 4 °C overnight with anti-hnRNP A2/b1antibody (Sigma, HPA001666, 1/100). After washing in PBS1X, the slides were incubated with a secondary antibody for 1 h at room temperature. Bound antibody was revealed with DAB and sections were counterstained with hematoxylin. The following variables were analyzed: intensity and proportion of cells displaying nuclear and cytoplasmic staining

Detection of phospho-KRAS

Phos-tag-PAGE. To detect phospho-KRAS from human Pancreatic Ductal Adenocarcinoma cell lines, cells from 100-cm dish were lysed in 0,4 mL of Lambda Phosphatase Lysis Buffer (50 mM Tris-HCl pH 8; 150 mM NaCl, 2 mM EDTA, 10% Glycerol, 1% Nonodet Nonidet P40, 5 mM DTT, 2mM $MnCl_2$) containing either protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, #87786, Thermo Scientific, Rockford, IL USA) alone or plus phosphatase inhibitors (0.2 mM Na_3VO_4, 5 mM NaF). Then, the sample homogenized with only protease inhibitors was treated with recombinant Protein Phosphatase Lambda (#539514-20KV; Calbiochem) for 30 minutes at 30ºC according to manufacturer instructions, and finally all tubes were balanced with phosphatase inhibitors in order to equalize both lysis buffers.

Protein content was assessed by Lowry method and tubes were balanced. 10 µg protein was loaded into a 12%-polyacrilamide SDS-PAGE gel supplemented with 100 µM Phos-tag and 100 µM $MnCl_2$. The gel was run over night at 5 mA/gel and soaked in a general transfer buffer containing 1 mM EDTA for 20 min followed by 10 minutes incubation with a transfer buffer without EDTA. Then, gels were transferred over night at 50 V into a PDVF membrane that was blocked and blotted with anti-KRAS (#OP24, Calbiochem).

References of Supplementary methods

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Table S1
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Table S1

	HNRNPA2B1 distribution in human biopsias			
	Normal	Pancreatitis	PanIN	Tumor
nucleus -	0	0	0	0
nucleus+ and cytoplasm-	19	7	10	13
nucleus+ and cytoplasm+	0	0	1	5
Total	19	7	11	18
% nucleus+ and cytoplasm+	0%	0%	9%	28%

Fig S1
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Protein	Mowse Score	Sequence coverage
hnRNP A2B1	3.89e+005	35%
Annexin A2	5.8e+005	33%
Clathrin heavy chain	4.85e+013	17%
Coiled-coil domain-containing protein 27	1.48e+004	19%
Liprin-beta-2	1.35e+004	13%
Nup88	1.22e+004	14%

Fig S2
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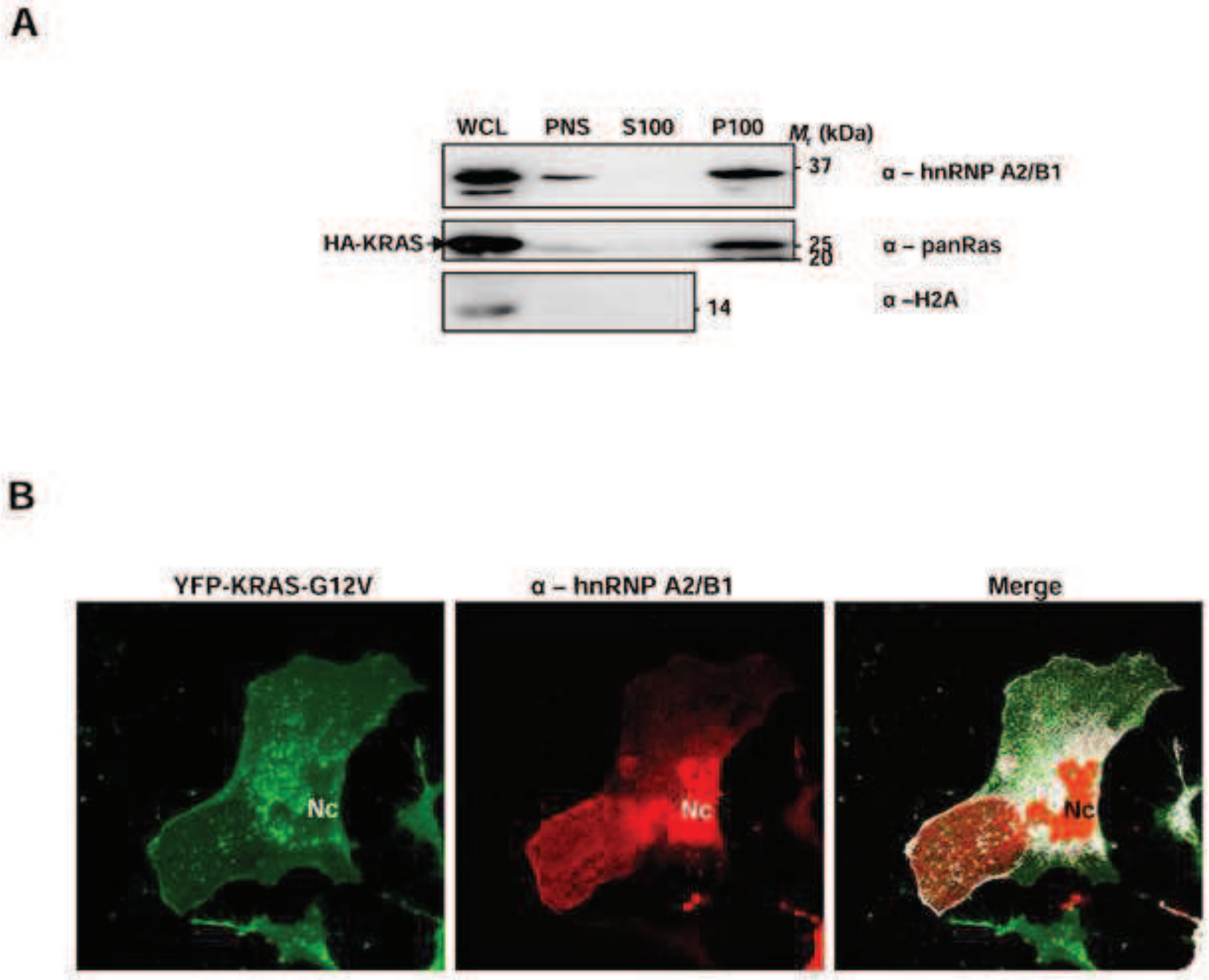

Fig S3
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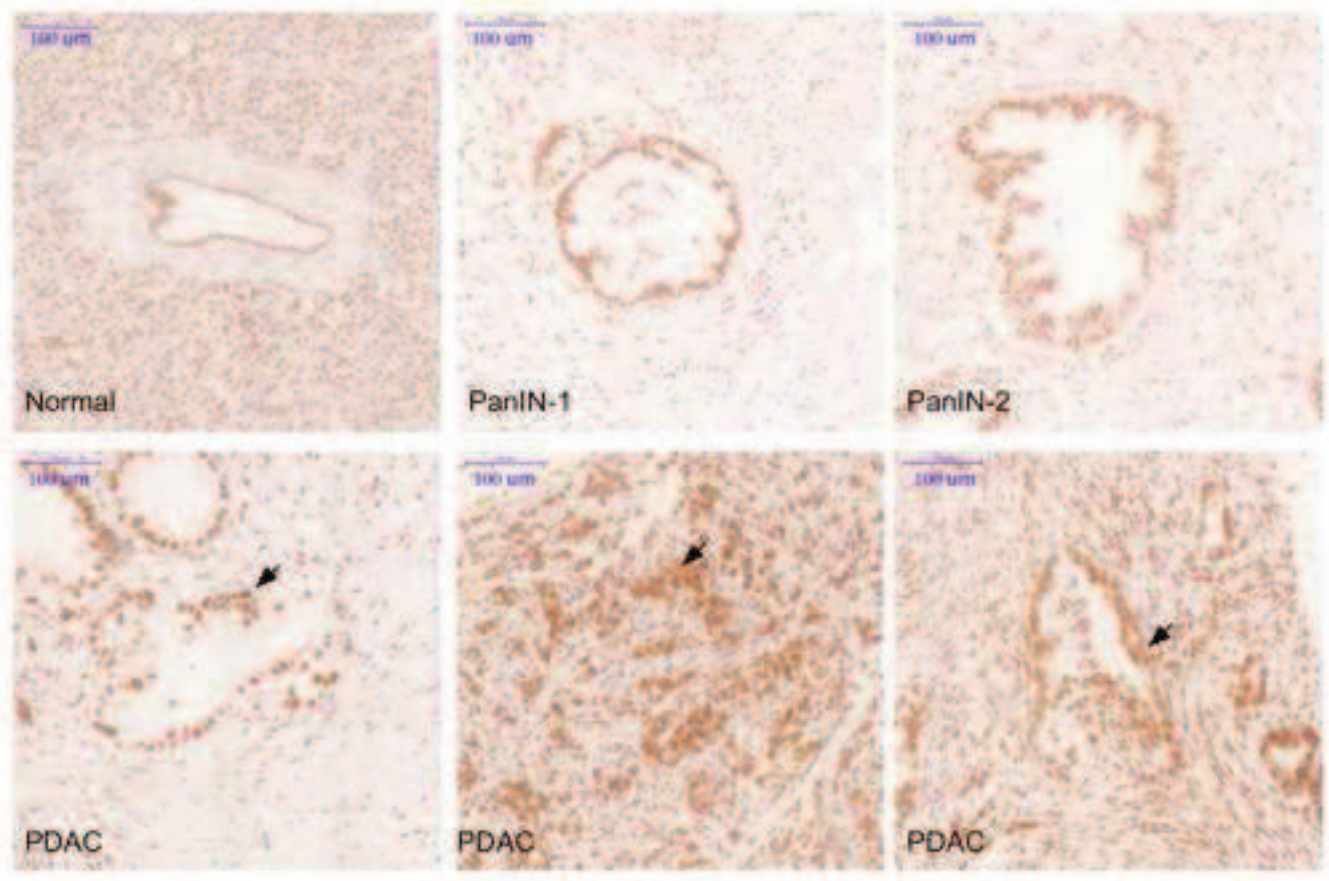

Fig S4

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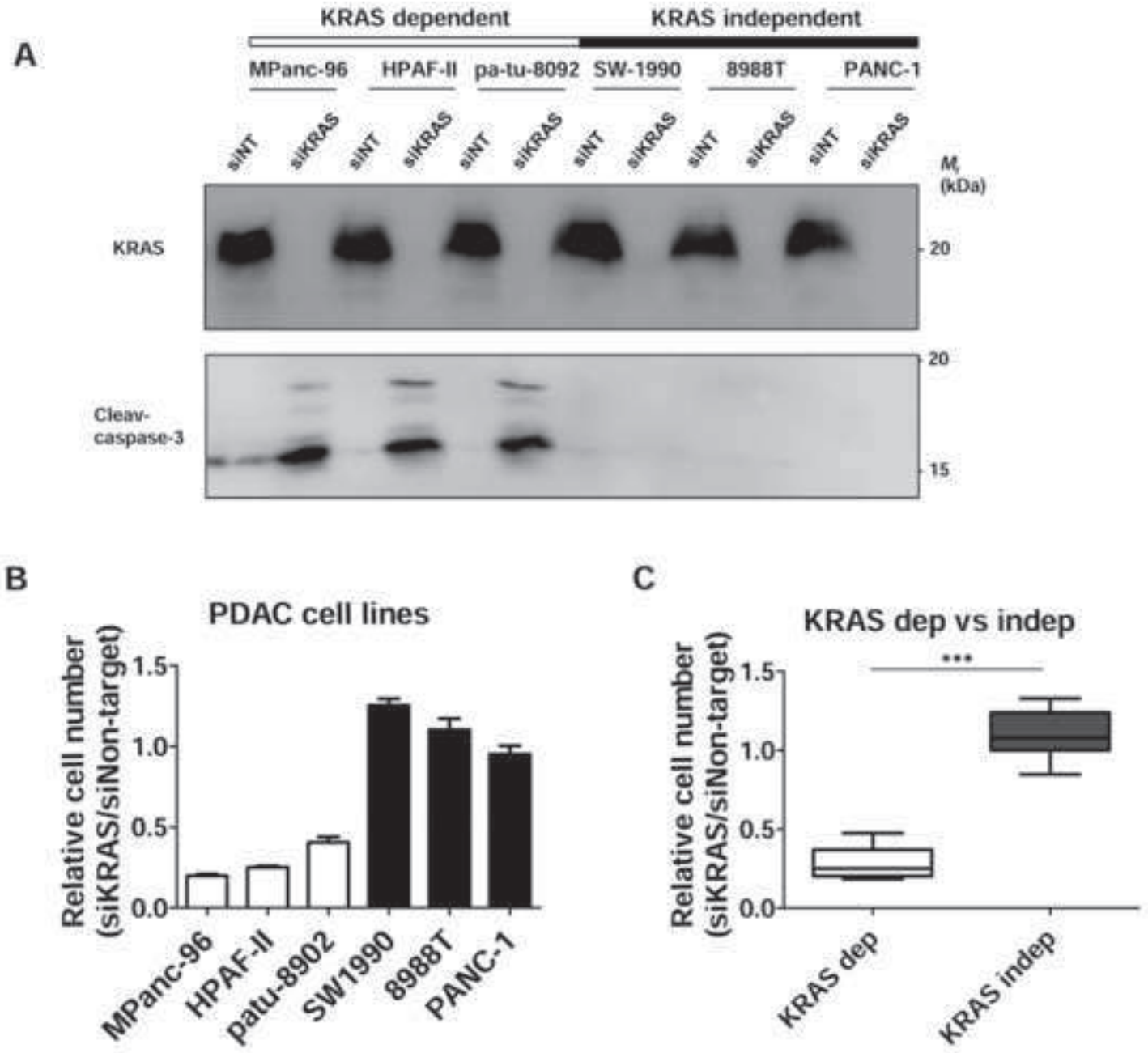

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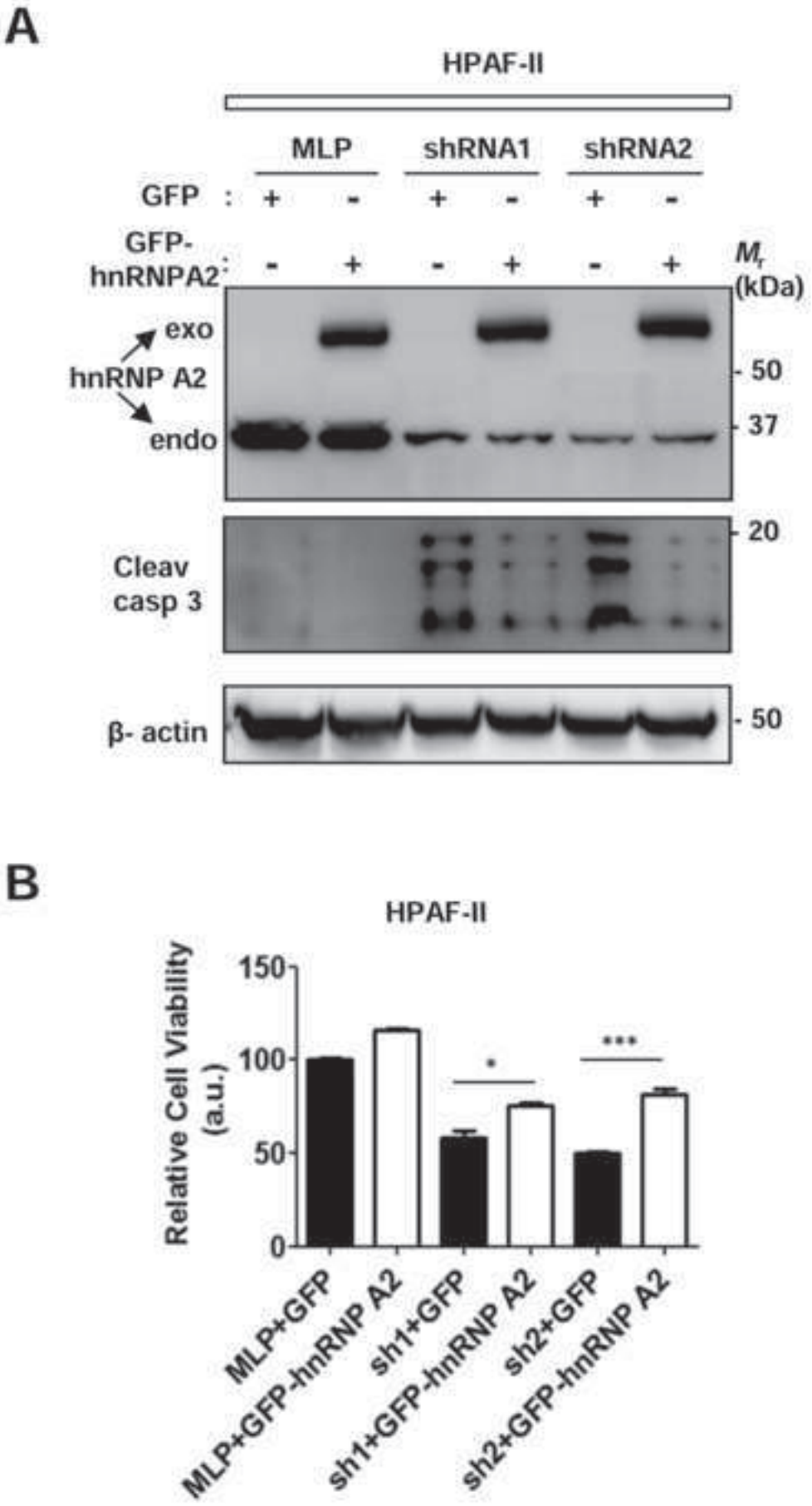

Fig S6
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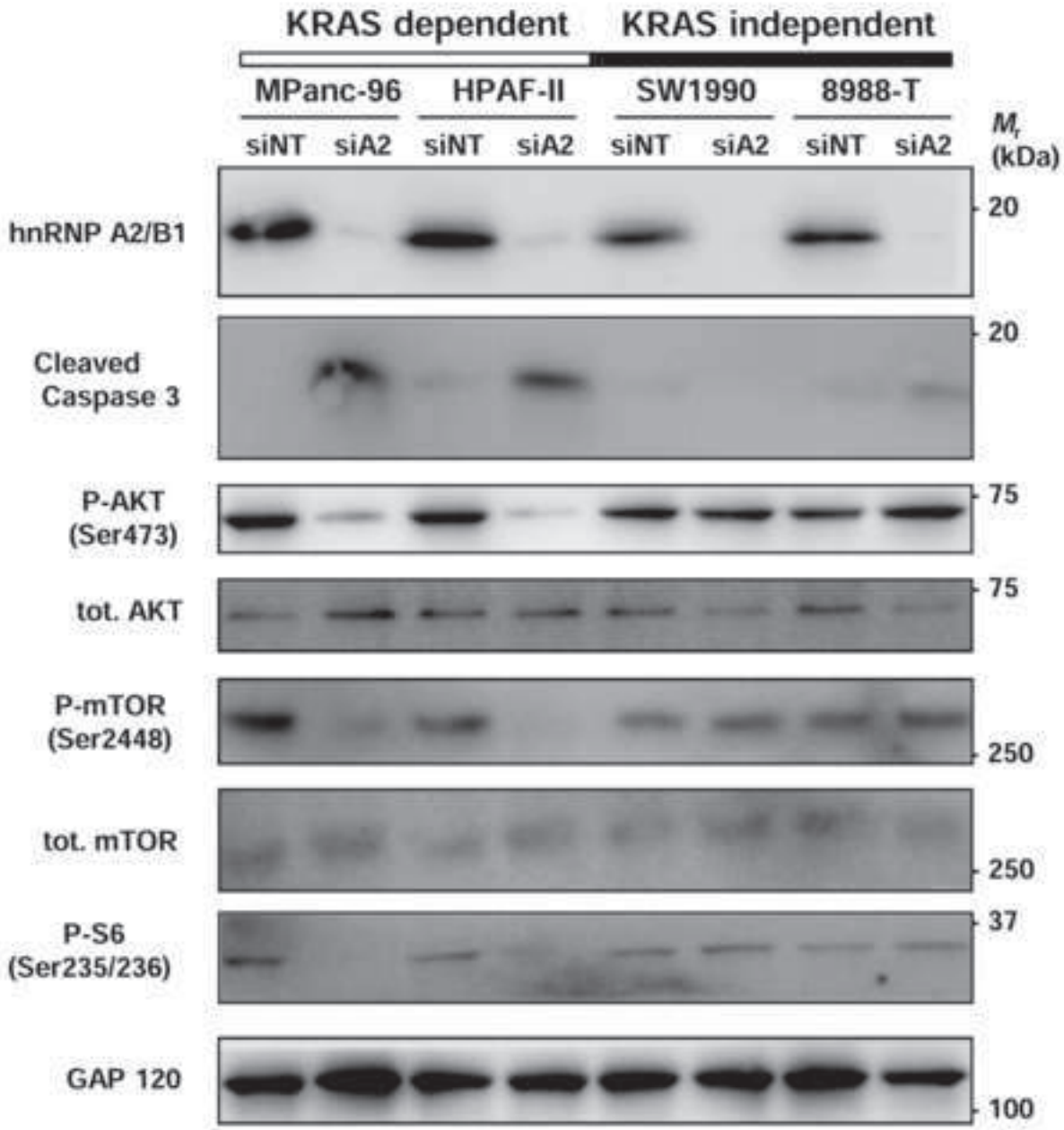

Fig S7

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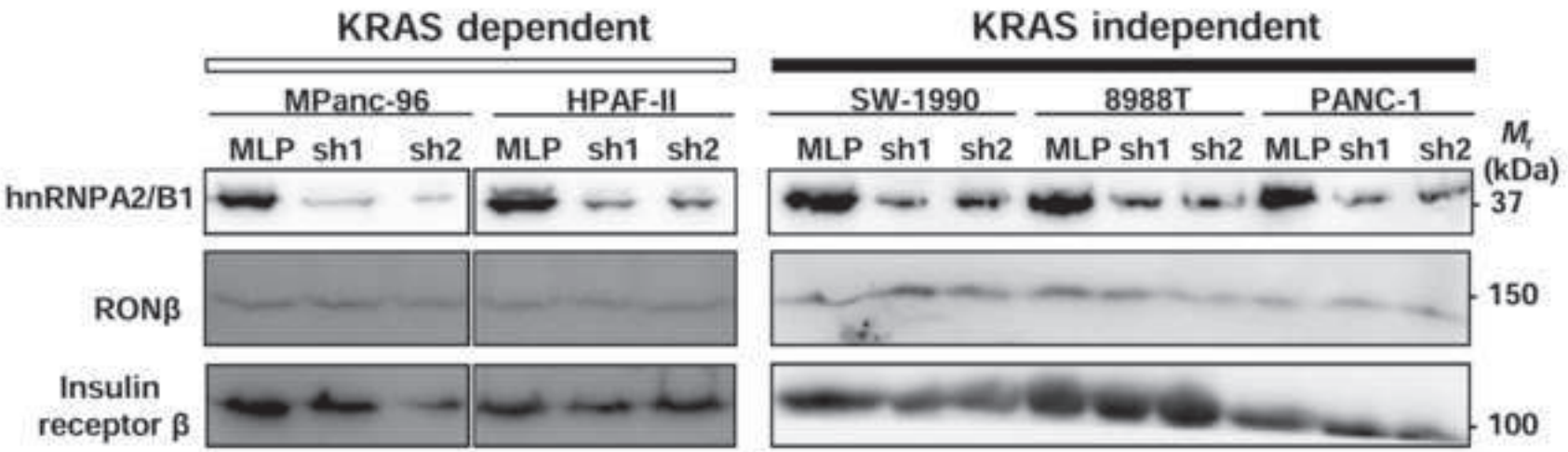

Fig S6A

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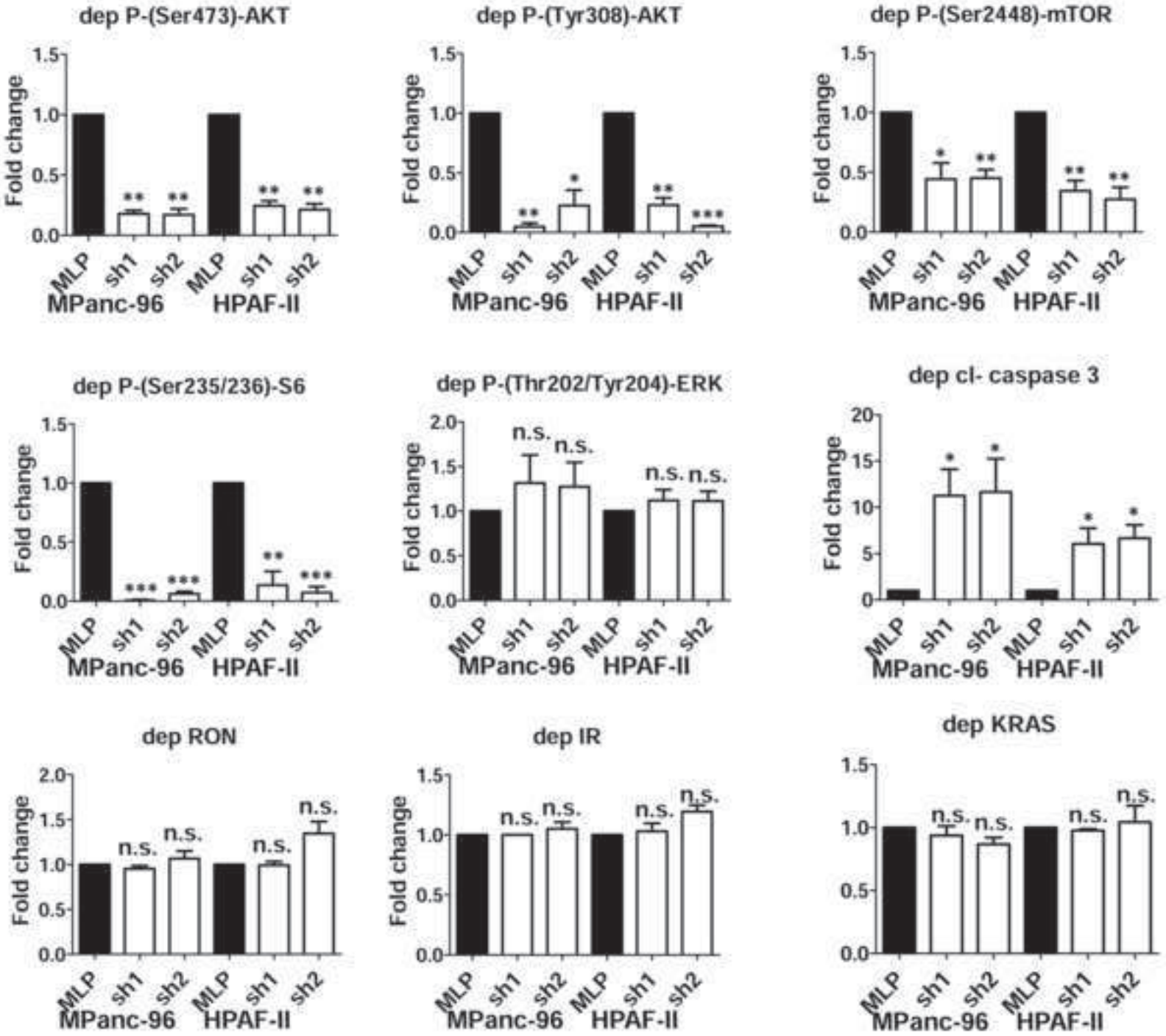

Fig S6B

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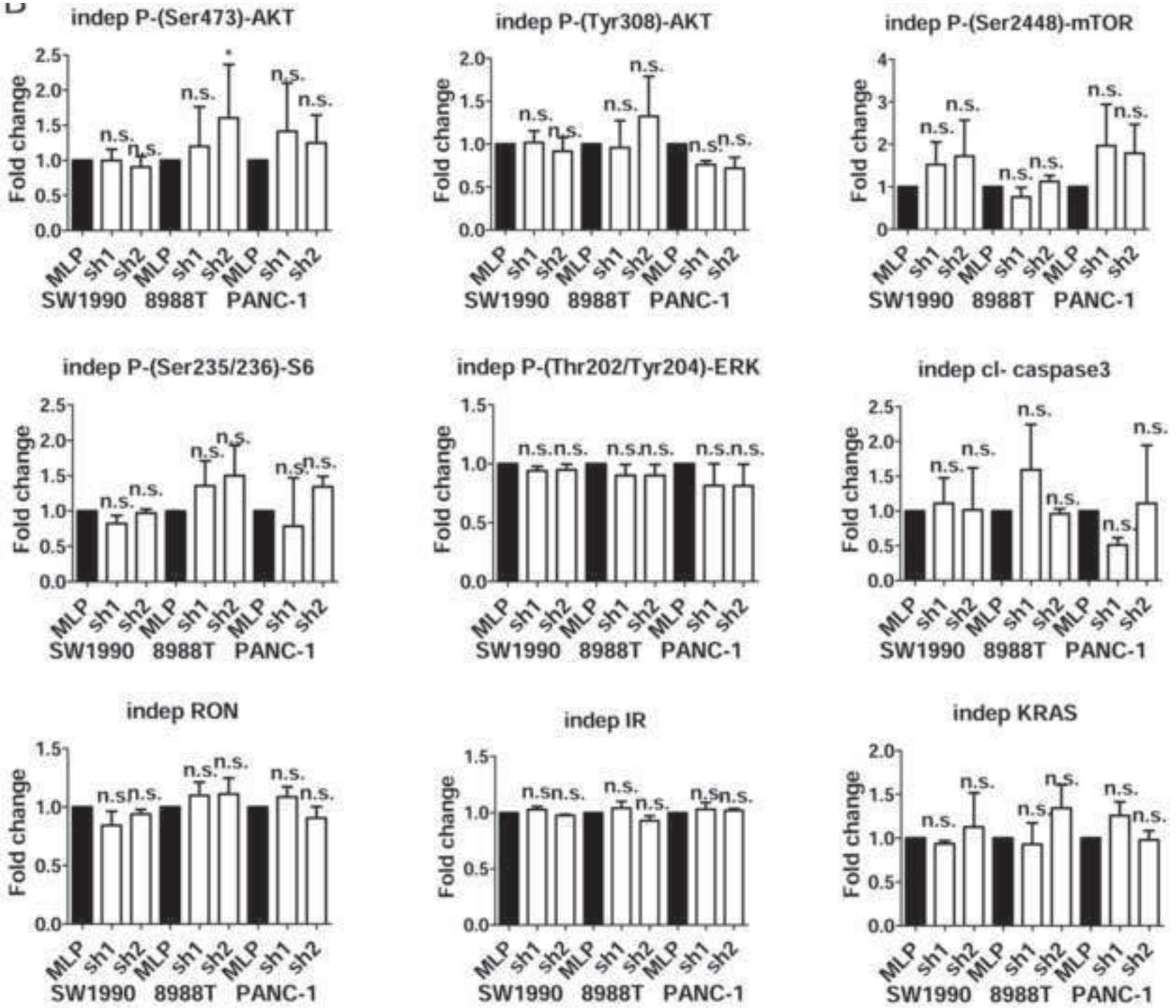

Fig S9

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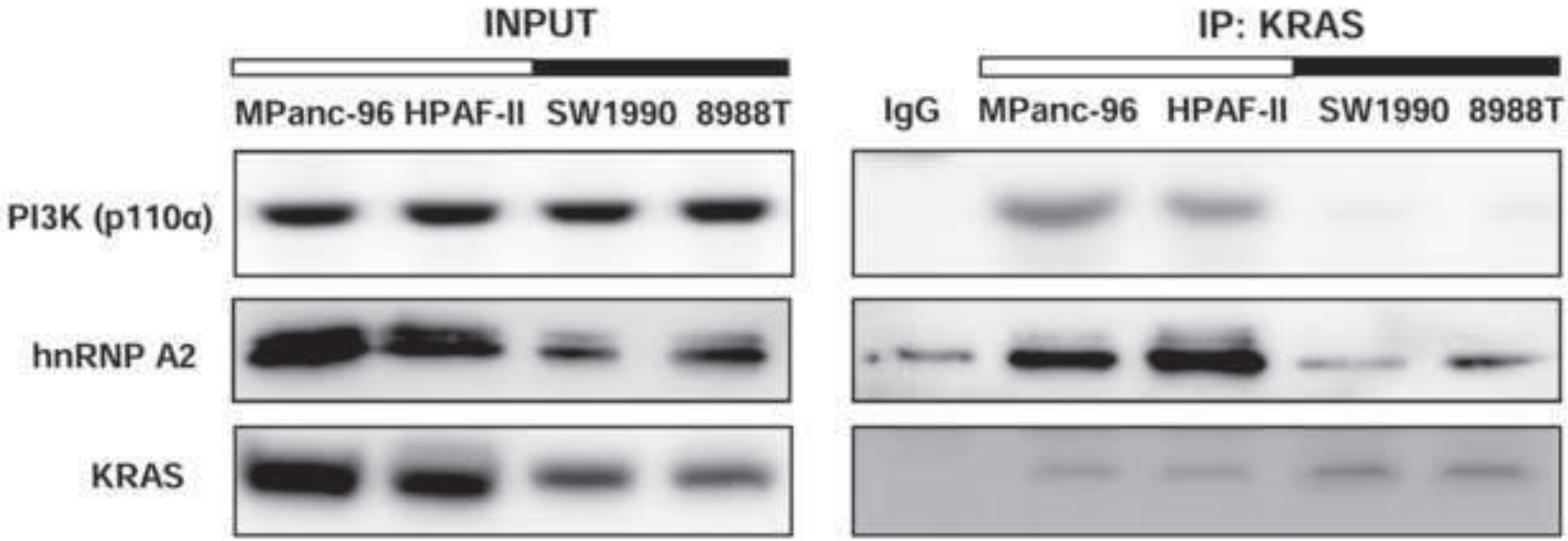

Fig S10

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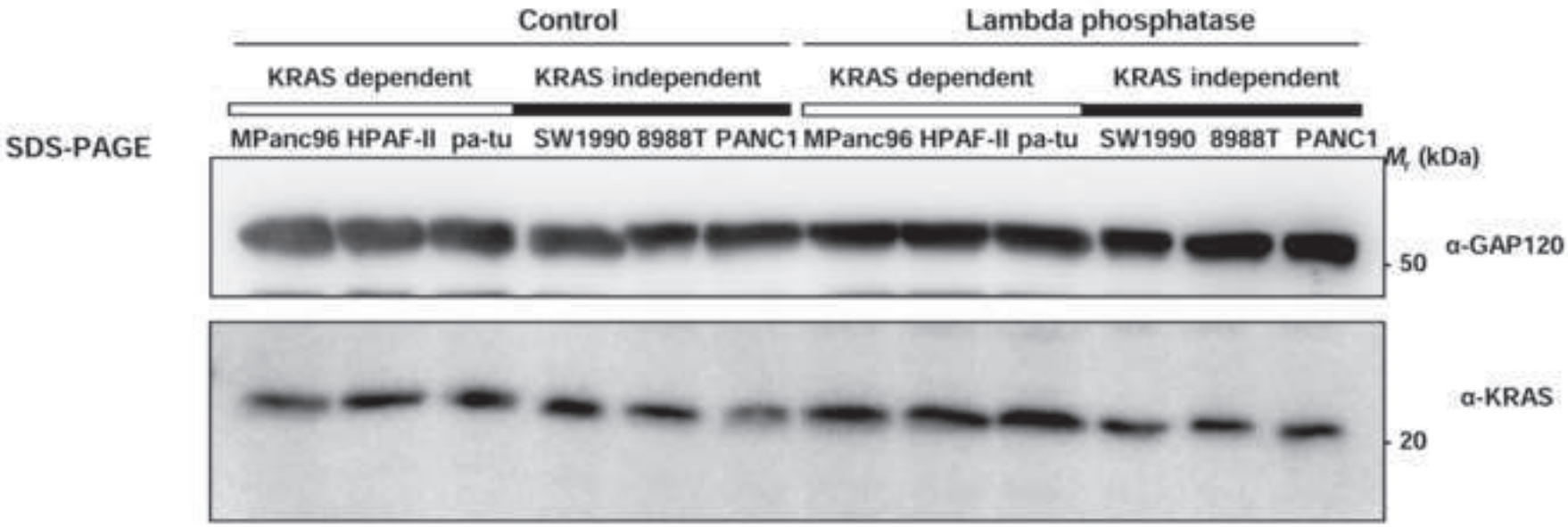

Supplementary figure legends (with changes marked)
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SUPPLEMENTARY FIGURE LEGENDS

Fig. S1. Identification of HNRNPA2B1 as a novel oncogenic KRAS interactor. HeLa cells were transfected with HA-tagged KRAS bearing the oncogenic (G12V) mutation. Cell lysates were immunoprecipitated with anti-HA antibody and non-transfected lysate was used as a negative control as indicated in supplementary methods. Bound fraction was resolved in a SDS-PAGE and stained with Flamingo® staining. Main specific bands were excised from the polyacrilamide gel and analyzed by MALDI-TOF Voyager DE PRO (Applied Biosystems) and by LCQ-DECA XP Plus ionic trap for peptide sequencing. Mowse score and sequence coverage for each identified proteins are shown in the table. Confirmation of proteins different than hnRNP A2/1 as specific KRAS binding proteins remains to be performed by other techniques.

Fig S2. **HNRNPA2B1 distribution within the cell compartments (A)** HeLa cells were transfected with HA-tagged KRASG12V and whole cell lysate (WCL) was further fractionated in order to obtain the post-nuclear supernatant (PNS), cytosolic (S100) or cytoplasmic membrane (P100) fractions. **(B)** HeLa cells transfected with YFP-KRASG12V were fixed and incubated with anti-HNRNPA2B1 antibody. Images were acquired by confocal microscopy. Nc: Nucleus

Fig S3. Nuclear/Cytoplasmatic expression of HNRNPA2B1 in different stages of human PDAC. Subcellular distribution of HNRNPA2B1 in human pancreatic tissue microarrays containing normal pancreas, pancreatitis, premalignant Pancreatic Intraepithelial Neoplasms (PanIN), and PDAC (Tumor), analyzed using immunohistochemistry as indicated in the methods section. Representative images obtained for normal pancreas, PanIN , and PDAC showing only nuclear (first PDAC sample image starting from the left) or both nuclear and cytoplasmic staining (second and third PDAC images). Arrows indicate examples of PDAC cells with only nuclear (lower left) or both nuclear and cytoplasmic (lower middle and right) HNRNPA2B1 expression.

Fig S4. Validation of KRAS-dependency in the PDAC cell lines used. (A) PDAC human cell lines (all harboring oncogenic KRAS mutations) were transfected either with non-targeting siRNA (siNT) or with siRNA targeting KRAS (siKRAS). Cells were harvested after 72h and KRAS and cleaved caspase 3 analyzed by western blot. **(B)** Relative proliferation is plotted and classified either into KRAS-dependent (white bars) or KRAS-independent (black bars) cell lines. **(C)** Box plot of relative proliferation of KRAS-dependent versus KRAS-independent PDAC human cell lines. (***, P<0.0001 in Mann-Whitney test; Error bars indicate SEM).

Fig S5. Validation of the specificity of HNRNPA2B1 knock-down by rescue of the endogenous HNRNPA2B1 ablation-induced cell death as assessed by caspase-3 cleavage. **(A)** Selected stable HPAF-II cells [MLP(-), sh1 or sh2] were transfected with either empty GFP or with GFP-HNRNPA2B1 and were deprived for 48h with 0.1%FBS. After that, cells were harvested and analyzed by western blot. β-actin was used as protein loading control **(B)** Quantification of cell viability of the rescue experiment described in (A).

Fig S6. Validation of the specificity of the effects of HNRNPA2B1 shRNA knock-down by pooled siRNA. Related to Fig 4. To further confirm the effects observed for the PDAC cell lines stably expressing shRNA against HNRNPA2B1, pooled siRNA targeting different sequences of HNRNPA2B1 was transfected into two KRAS-dependent PDAC cell lines (MPanc-96 and HPAF-II) and into two KRAS dependent PDAC cell lines (SW-1990 and 8988T). After 72h of expression, cells were harvested and analyzed by western blot.

Fig S7. Determination of the levels of the RONβ kinase and the Insulin receptor β . Related to Fig 4, levels of RONβ and the insulin receptor β were determined to establish prospective effects of HNRNPA2B1 knock down on these proteins.

Fig S8 Quantification of Western Blots of KRAS-dependent and KRAS-independent PDAC cell lines displayed in Fig 4 and Fig S7. Black bars represent MLP-empty vector expressing PDAC cancer cell lines, white bars represent levels of the determined protein in stable HNRNPA2B1 knock-down PDAC cell lines. Results are relative to the respective control cell line (MLP expressing cells; black). (***, $P<0.0001$; **, $P<0.001$; *, $P<0.01$; and, n.s., not-significant, i.e. $P>0.05$; P for Mann-Whitney test; Values are means ± SEM (n=3)). Dep: KRAS-dependent PDAC cell lines; Indep: KRAS-independent PDAC cell lines.

Fig S9. **Preferential interaction of PI3K(p110α) - KRAS in KRAS-dependent PDAC cell lines.** Related to Fig 5, the presence of PI3K (p110α) was checked in the immunoprecipitation displayed in Fig2 (upper panel).

Fig S10. Standard SDS-PAGE of samples loaded into the Phos-Tag™-SDS-PAGE. Related to Fig 6, samples loaded in the Phos-Tag™-SDS-PAGE were run into a standard SDS-PAGE as a control of the phosphorylation-dependent gel retardation.

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