

RABL6A Inhibits Tumor-Suppressive PP2A/AKT Signaling to Drive Pancreatic Neuroendocrine Tumor Growth

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Background

Introduction

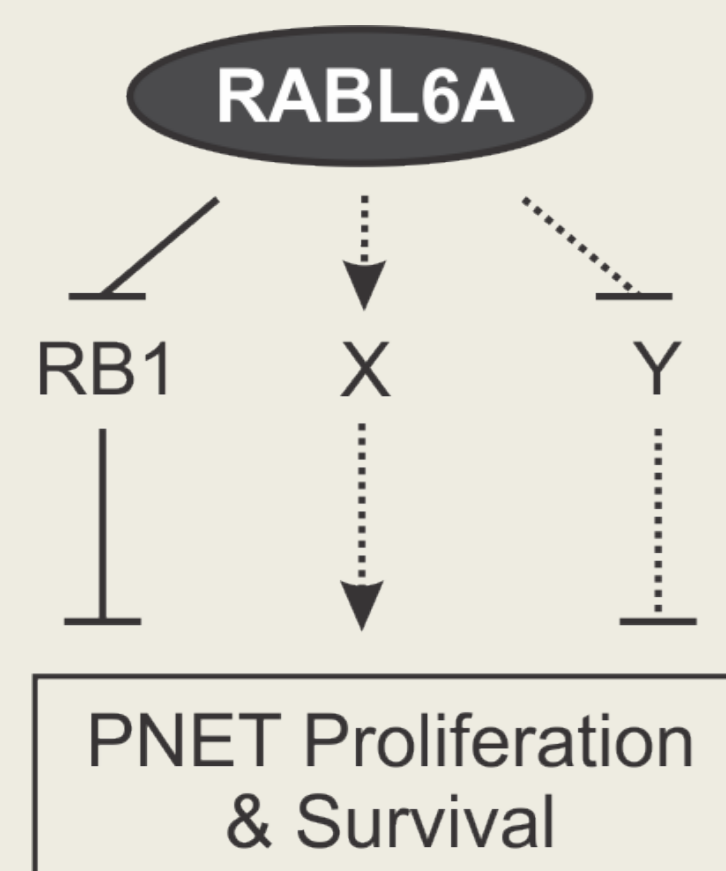
Our goal is to better understand key pathways underlying pancreatic neuroendocrine tumors (PNET) pathogenesis and thereby identify novel PNET biomarkers and drug targets to improve patient diagnosis and treatment.

PI3K/AKT/mTOR pathway is aberrantly activated in a high percentage of PNETs due to gene mutation and altered expression / activity of factors in the pathway.

We discovered a new oncogenic GTPase, RABL6A (RAB like protein 6A), which is amplified in PNETs. RABL6A is required for PNET cell proliferation and survival through regulation of retinoblastoma (RB1) activity and other undefined cancer pathways.

Here, we show that RABL6A inhibits PP2A, which then activates AKT-mTOR signaling to drive PNET tumorigenesis.

RABL6A expression in tumor cells dictates the cellular responses to clinically relevant drugs that inhibit AKT and activate PP2A.



- RABL6A promotes PNET cell proliferation and survival by negatively regulating RB1 signaling, but other undefined factors are also required for its oncogenic function in PNETs.
- Microarray analyses of RABL6A knockdown cells suggest it may be a master regulator of several clinically important PNET pathways, including AKT-mTOR signaling.

Hypothesis

RABL6A promotes PNET cell proliferation and survival through activation of AKT and downstream mTOR signaling

RABL6A

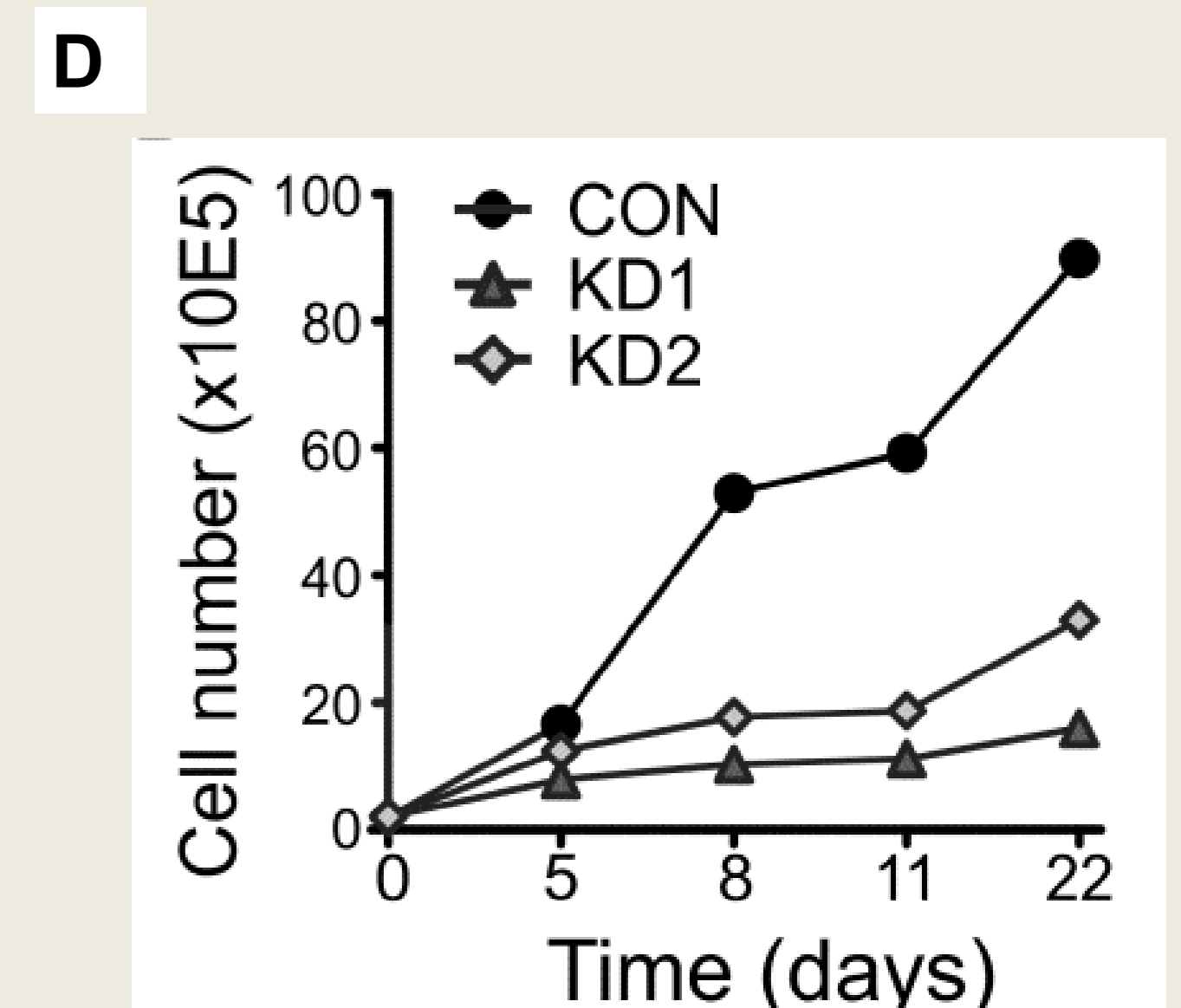
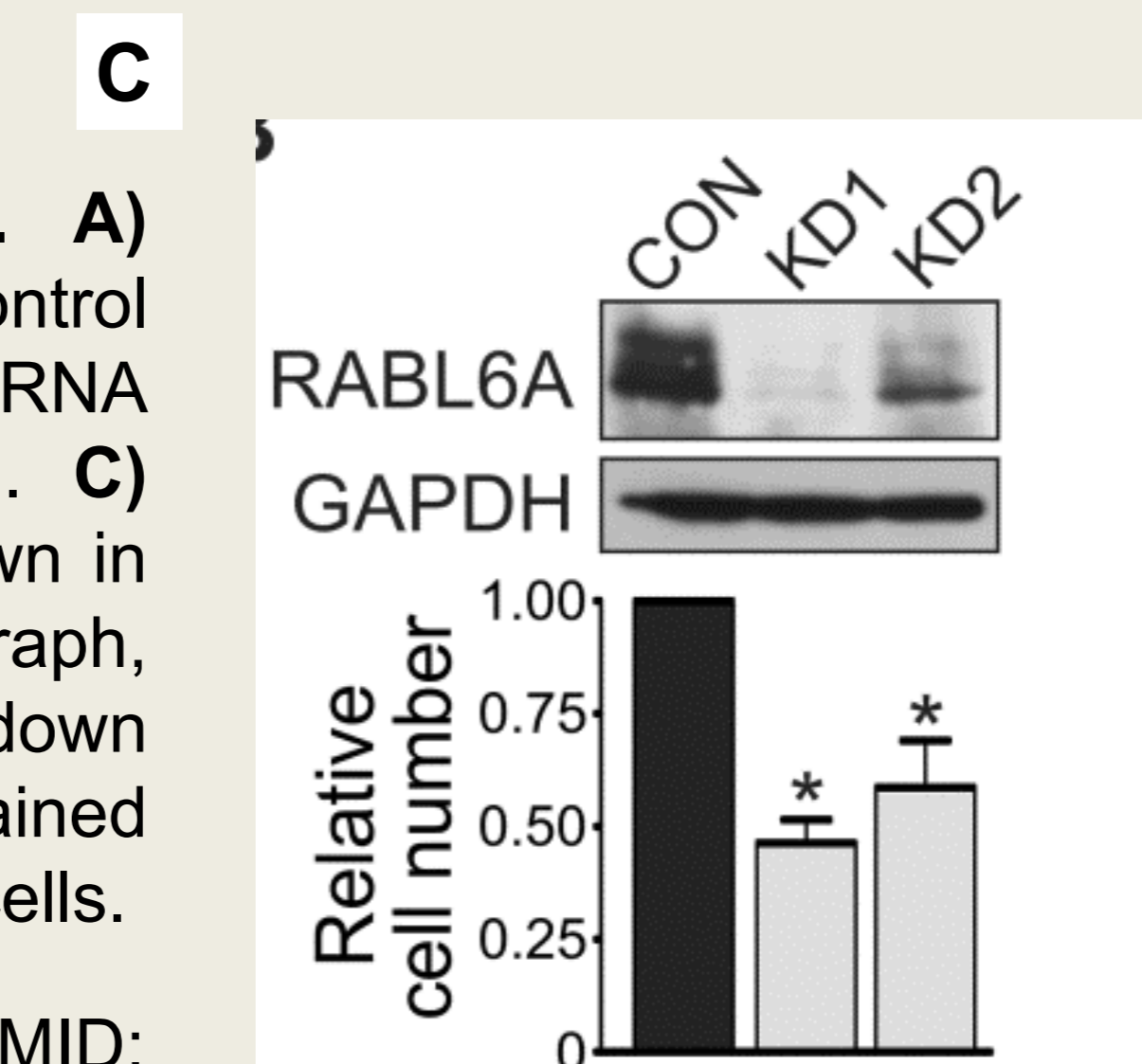
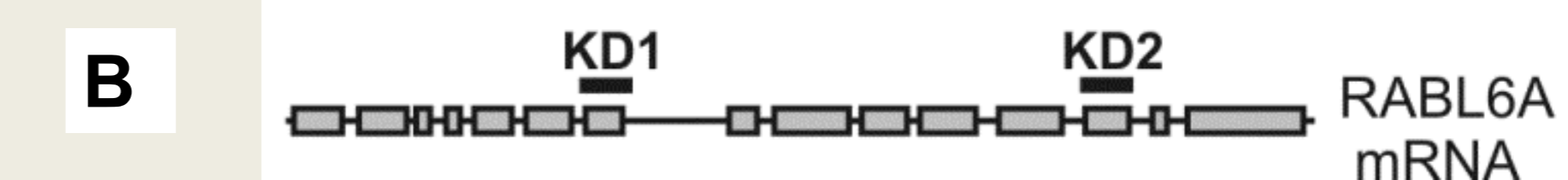
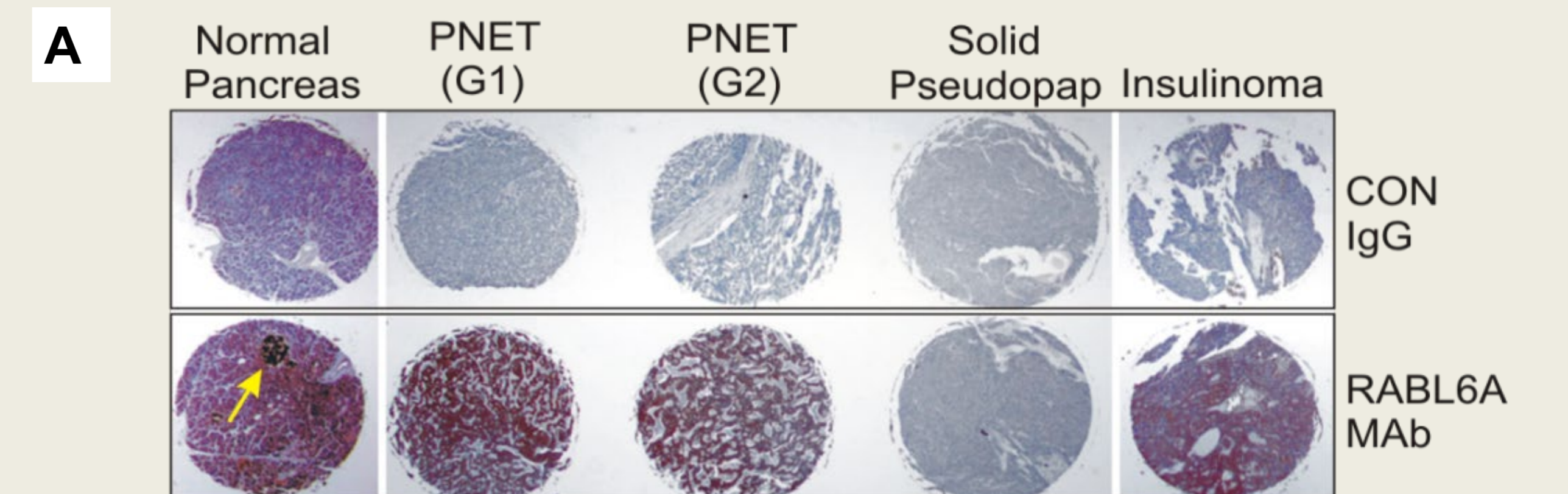
- Oncogenic GTPase
- Marker of poor survival in PDAC (Muniz, 2013) and breast cancer (Li, 2013)
- Unknown function

PNETs

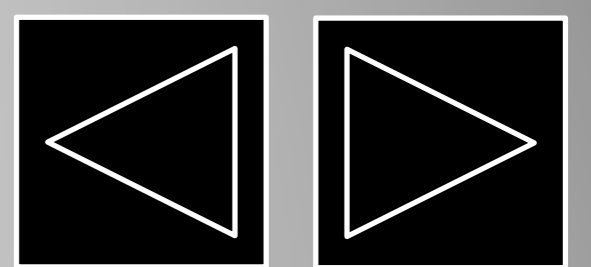
- Growing clinical challenge
- Mechanisms underlying PNET development are poorly understood, biomarkers needed
- AKT amplified and mTOR signaling activated in PNETs, targeted clinically

RABL6A is essential for PNET cell proliferation. **A)** Pancreatic tissue microarray after staining with control IgG and RABL6A. **B)** Schematic of RABL6A mRNA regions targeted by the shRNAs KD1 and KD2. **C)** Western blotting shows effective RABL6A knockdown in BON PNET cells compared with control (CON). Graph, cell numbers are reduced after RABL6A knockdown relative to CON cells. (*, $p < 0.05$). **D)** Sustained suppression of proliferation in RABL6A knockdown cells.

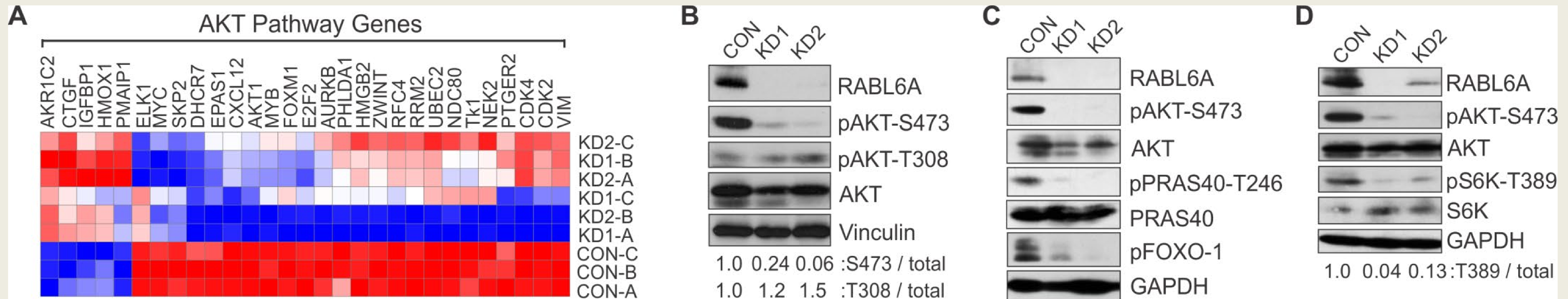
Hagen, J.; et al., Cancer Res. (2014), 74(22), PMID: 25273089



Results

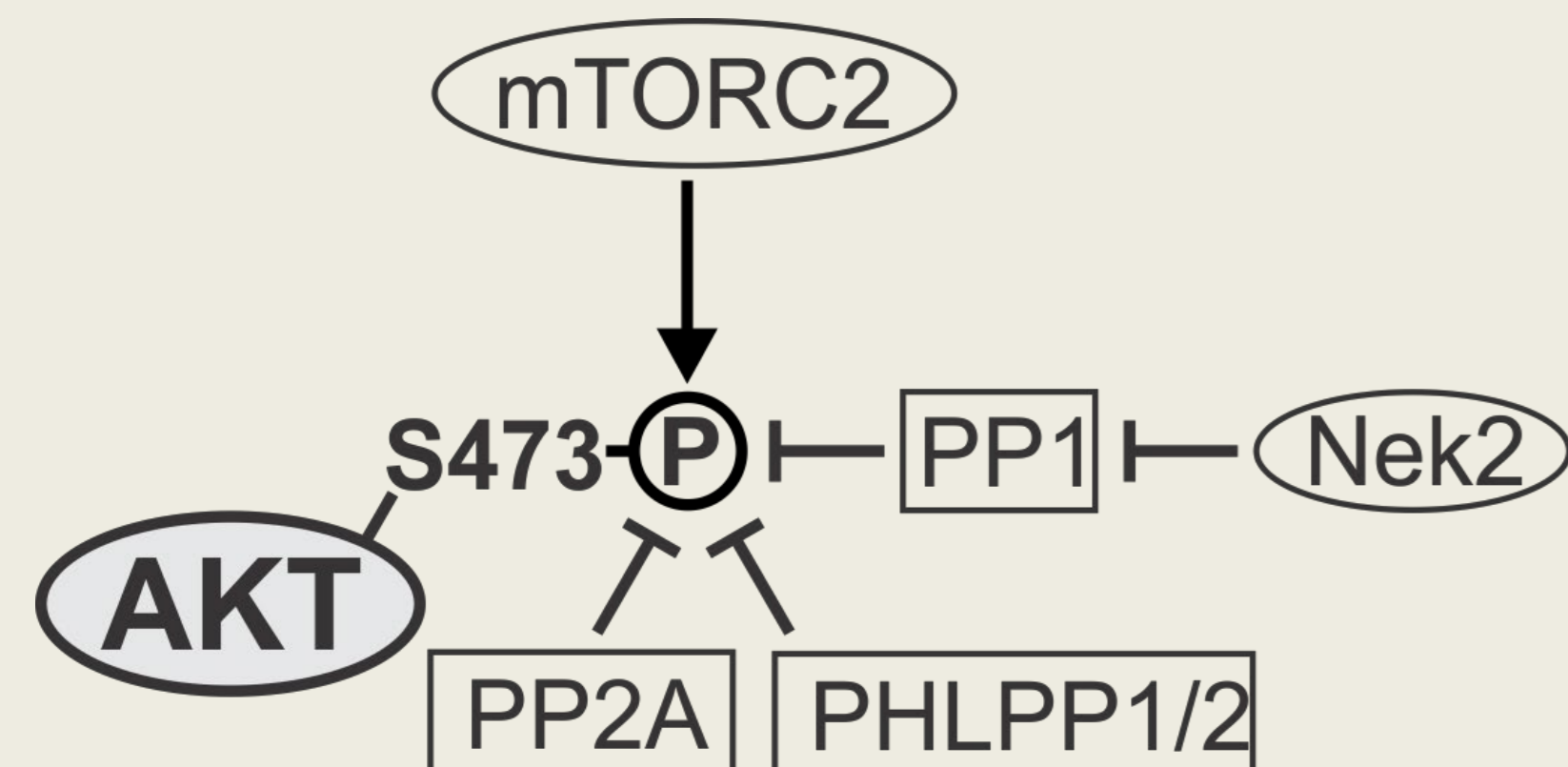


RABL6A depletion significantly impairs AKT-S473 phosphorylation and AKT-mTOR signaling in PNETs



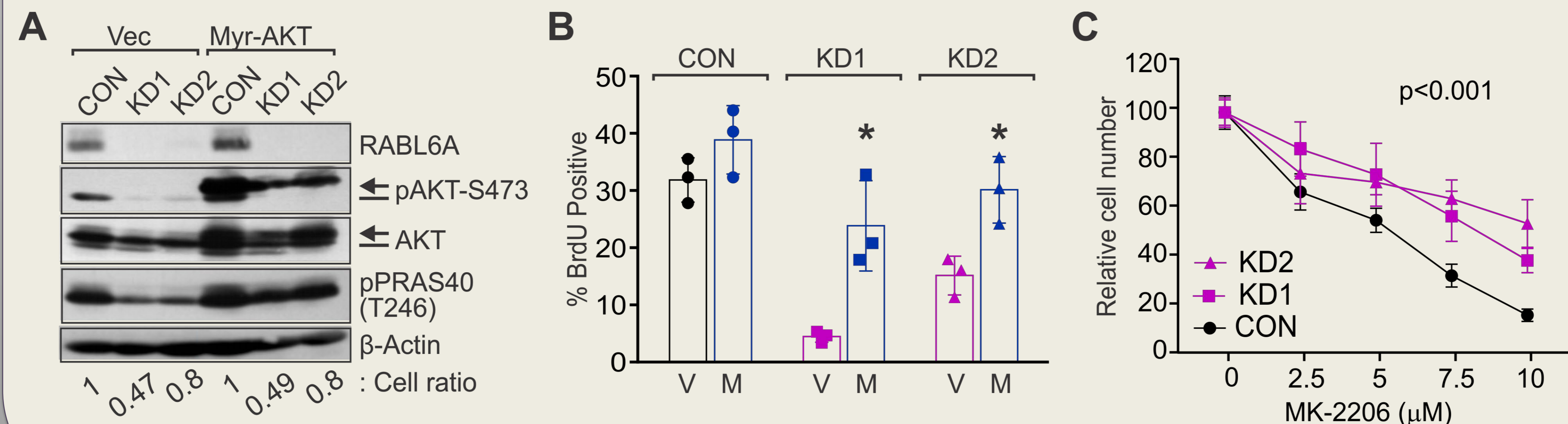
BON-1 cells expressing vector control (CON) or shRNAs targeting RABL6A (KD1 and KD2) were examined by microarray and western analyses. **A**) Heat map shows RABL6A depletion significantly alters the expression of genes involved in Akt signaling; data from 3 experiments, designated A-C. Genes were categorized by IPA software and displayed 2-fold or greater changes in expression ($p < 0.05$). **Red**, relatively increased expression; **blue**, relatively decreased expression. **B-D**) Western blots show that loss of RABL6A reduces the activating phosphorylation of Akt at S473. Effects on Akt-S473 are specific since T308 phosphorylation is unaffected (**B**). Inactivation of Akt coincides with loss of phosphorylation of Akt substrates, PRAS40 and FOXO-1 (**C**) and downstream mTORC1 inactivation, as measured by decreased S6K phosphorylation at T389 (**D**).

Factors regulating AKT phosphorylation



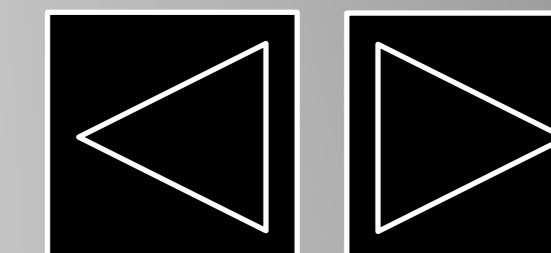
Kinases and phosphatases regulating AKT activation. P, phosphorylation; arrows, activating events; perpendicular bars, inhibiting events. RABL6A loss reduces AKT phosphorylation at S473.

RABL6A-AKT signaling is required for PNET cell cycle progression and response to AKT inhibitors

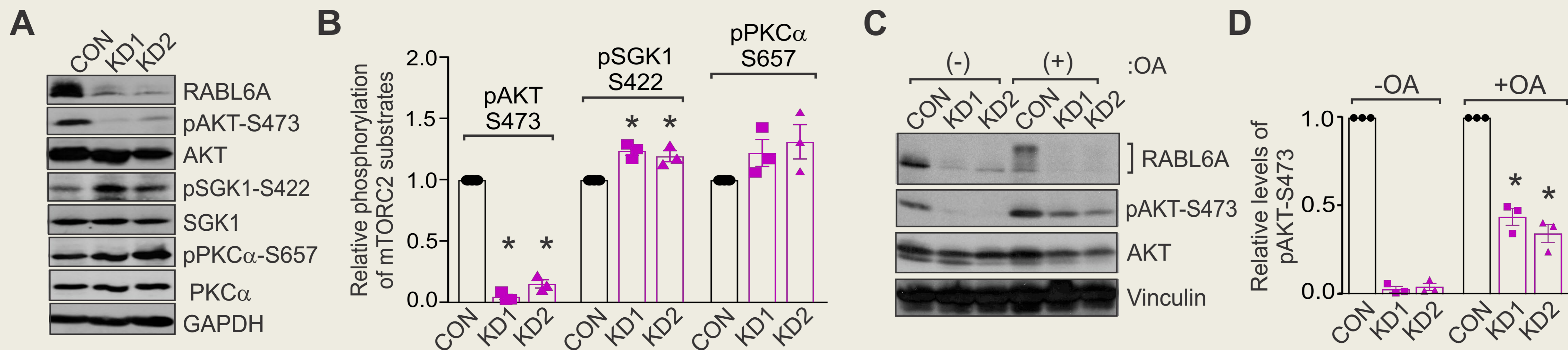


BON-1 cells expressing vector (Vec) or activated AKT (Myr-AKT) were infected with control (CON) or RABL6A shRNAs (KD1, KD2). **A**) Western blots show Myr-AKT expression and activation (lanes 4-6). β -actin is loading control. **B**) BrdU incorporation assay shows Myr-AKT (M) promotes DNA synthesis in RABL6A knockdown cells relative to vector (V) control cells. *, $p < 0.05$, Student's t-test. **C**) BON-1 cells expressing CON, KD1 or KD2 shRNAs were exposed for five days to increasing concentrations of the AKT inhibitor, MK-2206, and relative cell proliferation assayed using Cell-Quant™. *, $p < 0.001$ for KD1/2 versus CON, Student's t test.

Results

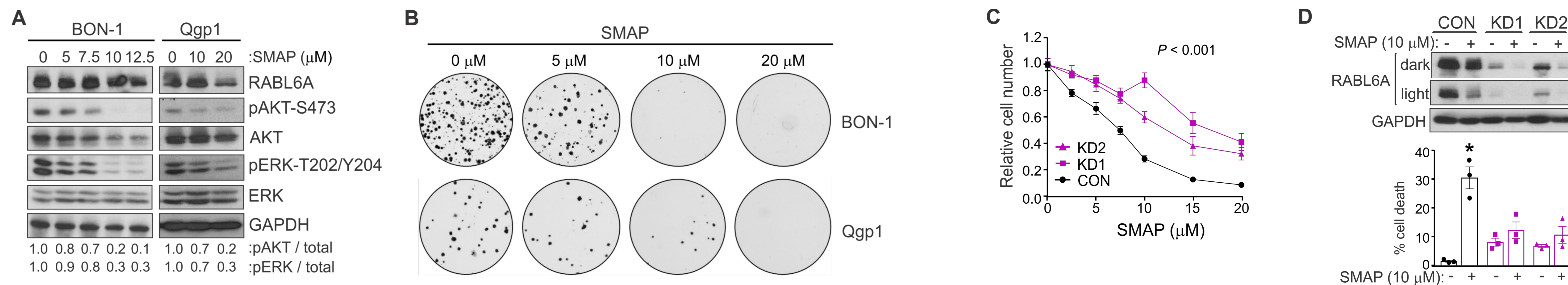


RABL6A regulates AKT-S473 phosphorylation through protein phosphatase, PP2A, not through regulation of mTORC2



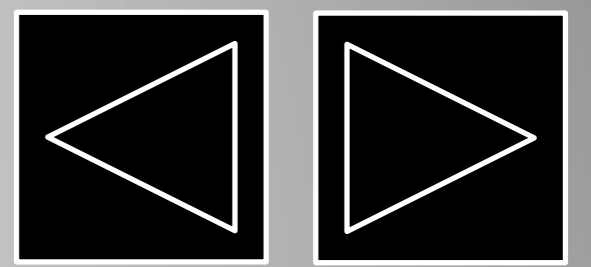
A) Western analyses showed selective loss of AKT-S473 phosphorylation in RABL6A depleted cells while phosphorylation of other mTORC2 substrates, SGK1 and PKC α , was moderately increased. **B)** Relative phosphorylation of mTORC2 substrates was quantified by ImageJ; error bars represent the deviation from the mean for data from three independent experiments (*, $p < 0.05$ compared to CON cells, Student's t test). **C)** BON-1 cells expressing CON, KD1 or KD2 shRNAs were treated with 100 nM okadaic acid (OA) for 20 hrs. Westerns show that OA inhibition of PP2A significantly reverses the effects of RABL6A loss on pAKT-S473. **D)** Relative phosphorylation of AKT-S473 was quantified from 3 experiments (as in C). *, $p < 0.05$ compared to untreated counterparts.

PP2A re-activation with a new drug, SMAP, reduces AKT-S473 phosphorylation, downregulates RABL6A levels, and induces PNET cell death in a RABL6A dependent manner

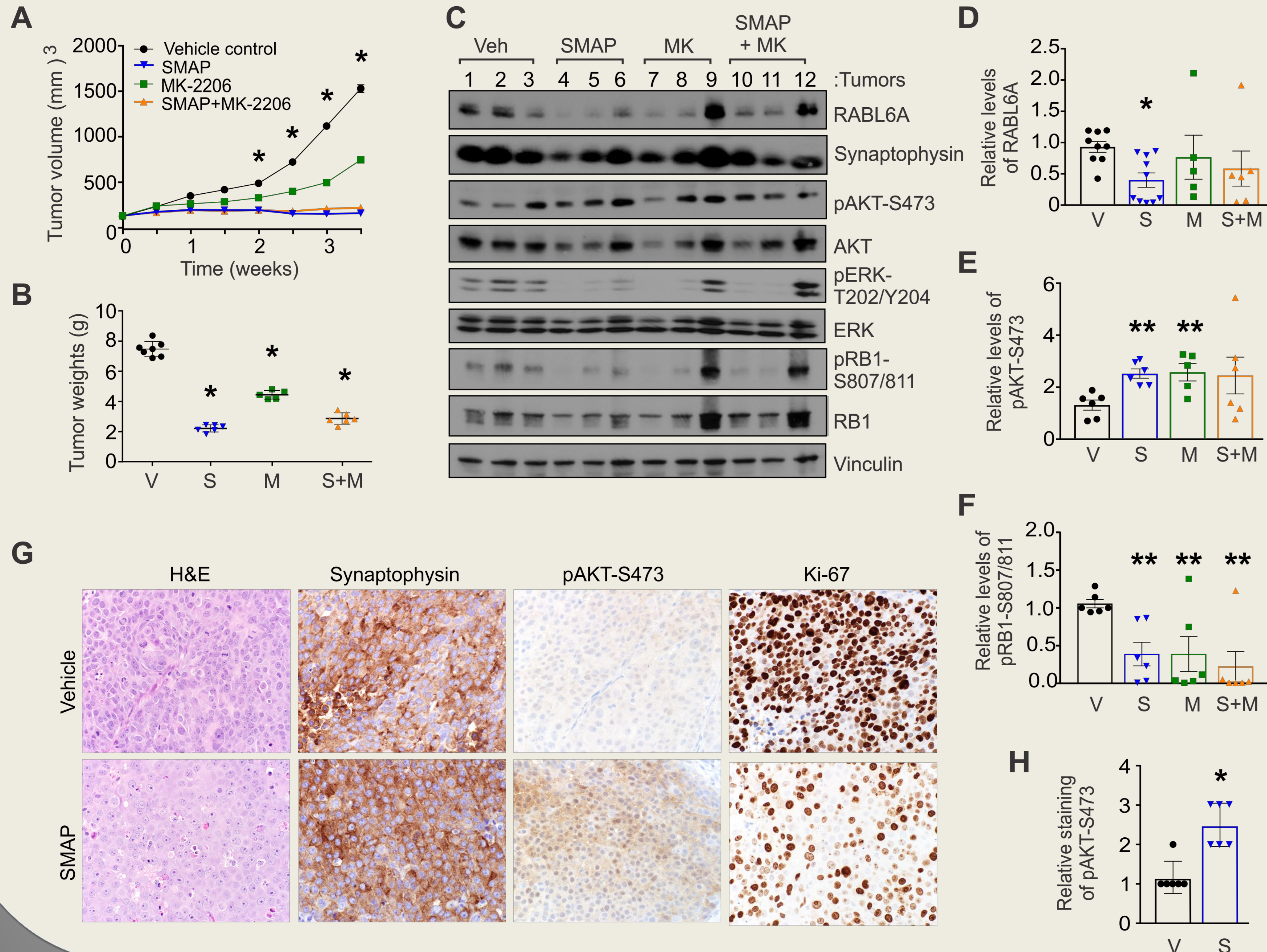


A) Westerns of BON-1 and Qgp1 parental cells treated for 20 hrs with SMAP (PP2A activator) showed SMAP effectively reduced AKT-S473 phosphorylation and ERK1/2 phosphorylation. **B)** Clonogenic assay of BON-1 and Qgp1 treated with the indicated concentrations of SMAP for 3 weeks. **C)** BON-1 cells expressing CON, KD1 or KD2 shRNAs were exposed for 3 days to SMAP, and relative cell proliferation assayed using Cell-QuantTM. $P < 0.001$ for KD1/2 versus CON, two-way ANOVA and Bonferroni correction. **D)** BON-1 cells expressing CON, KD1 or KD2 shRNAs were treated with 10 μ M SMAP for 20 hrs. Westerns show that PP2A activation decreased RABL6A levels and altered its migration on gels in both control and RABL6A knockdown cells. *, $p < 0.05$ for + versus - SMAP. For C and D, error bars represent SEM for data from three independent experiments.

Results



Therapeutic reactivation of PP2A with SMAP effectively suppresses PNET growth *in vivo*



For these *in vivo* studies, BON-1 cells (5×10^6) were injected s.c. into NOD.SCID mice to generate PNET xenograft tumors.

A) Tumors were allowed to grow to an average of 200mm³ before beginning drug treatments. Tumor volumes were measured over a 4 week period in which mice were treated by oral gavage with vehicle control, SMAP 5mg/kg (twice a day), MK2206 30mg/kg (three times a week) and a combination of SMAP plus MK2206. SEM for at least n=5 mice; *, p<0.001, two-way ANOVA with Bonferroni correction.

B) Comparison of tumor weights for each group after the final treatment. Error bars, SEM: *, p<0.001, two-way ANOVA with Bonferroni correction.

C) Western analyses of tumor lysates showing that SMAP treatment effectively reduces RABL6A and pERK levels in most tumors. Unexpectedly, AKT-S473 phosphorylation was increased by SMAP and MK2206 treatments, possibly reflecting loss of negative feedback regulation by sustained exposure to each drug.

D-F) Quantification of relative levels of RABL6A, pAKT-S473, and pRB1-S807/811 in xenograft tumors respectively from ImageJ analysis of western blots (as shown in C). Mean +/- SEM; *, p<0.05 ** p<0.01 Student's t test.

G) Representative H&E and IHC staining for the indicated proteins in BON-1 xenograft tumors from vehicle control and SMAP treated mice. Images were taken at 400X.

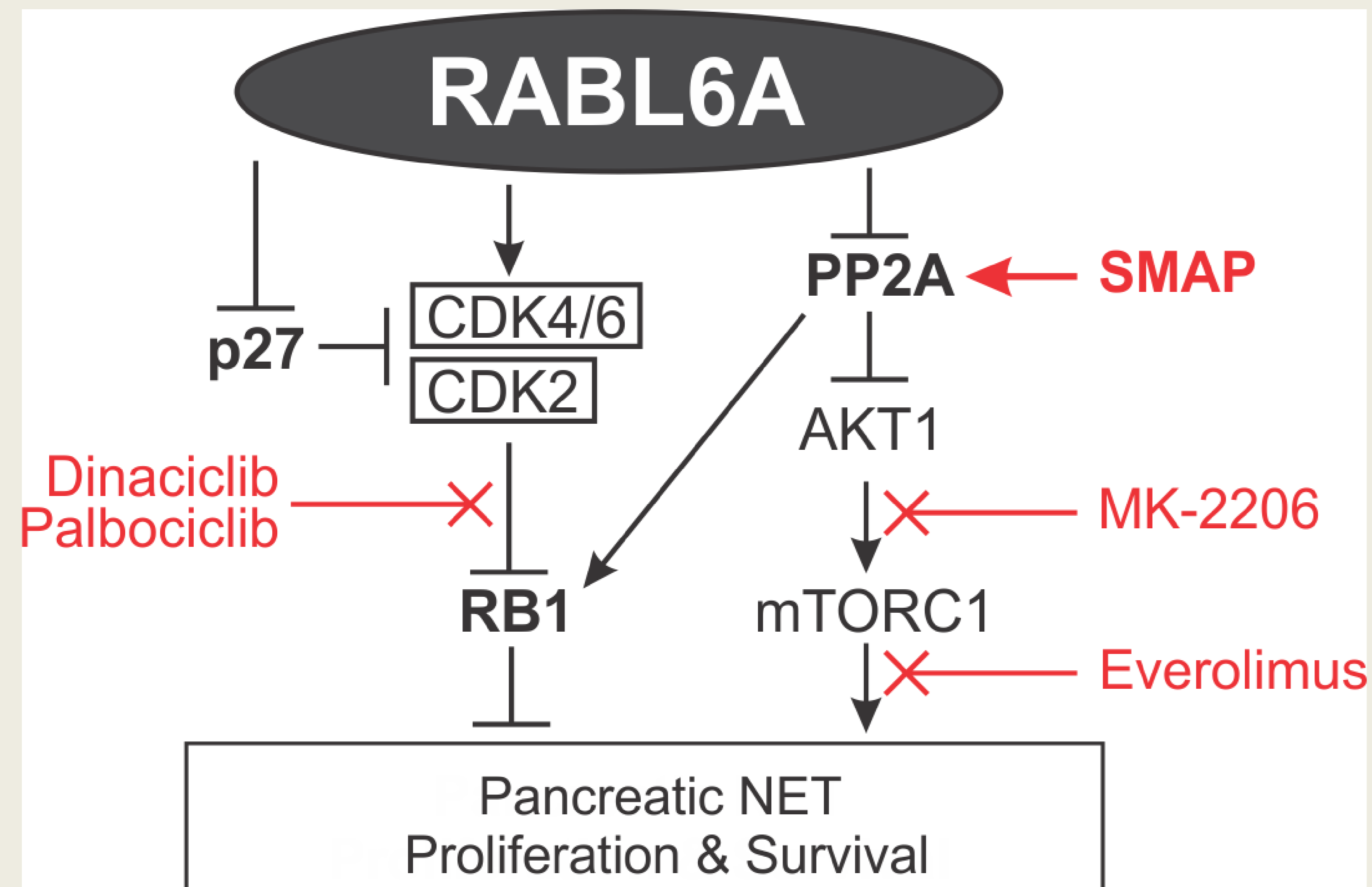
H) Quantification of pAKT-S473 staining (assessed as weak=1, moderate+2, strong+3) in tumors from vehicle (V) control and SMAP (S) treated mice. Mean +/- SEM; *, p<0.05, Student's t test.

Conclusions

RABL6A is a new regulator of PP2A, AKT and mTOR

RABL6A controls multiple, clinically relevant cancer pathways essential for PNET cell proliferation and survival.

PP2A reactivating drugs may represent a new therapeutic strategy for treating PNET patients.



Future Directions

- Determine the mechanisms by which RABL6A controls PP2A
 - Direct association and interference of PP2A complex formation
 - Upregulation of PP2A inhibitory proteins (CIP2A)
- Determine how PP2A regulates RABL6A protein expression and stability
- Determine if RABL6A levels in patient NETs correlate with PP2A and AKT-mTOR status in tissue microarrays (TMAs)
- Testing novel drug combination therapies for treating advanced PNETs.

Acknowledgements

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