RABL6A, a Novel Critical Regulator of Akt-mTOR Signaling in Pancreatic Neuroendocrine Tumor Cells

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SUPPORTED BY THE IOWA NEUROENDOCRINE TUMOR SPORE

Background

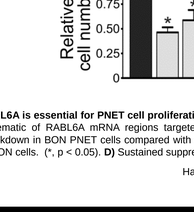
Introduction

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 and activity of factors in the pathway.

We discovered a new oncogenic GTPase, RABL6A (RAB like protein 6 A), and found it 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 is a new essential activator of AKT-mTOR signaling in PNET cells whose expression dictates response to clinically relevant AKT inhibitors.



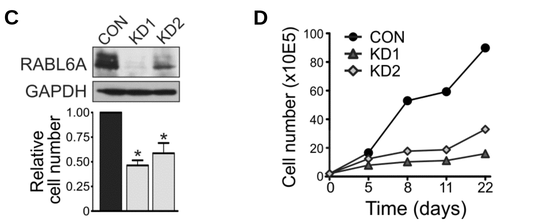
- RABL6A promotes PNET cell proliferation and survival by negatively regulating Rb1 signaling, but other undefined factors are also required for its oncogenic function in PNETs.
- Microarray analyses of RABL6A knockdown cells suggest it may be a master regulator of several clinically important PNET pathways, 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.

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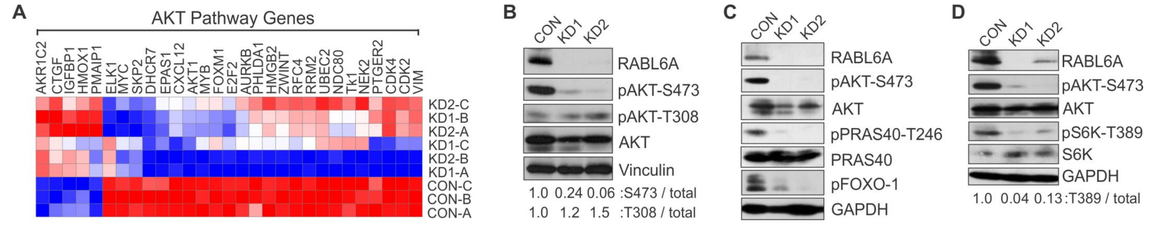
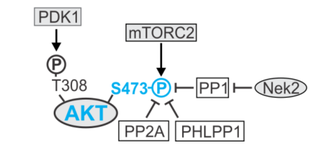
Results

RABL6A depletion significantly impairs AKT activation (selectively thru reduced S473 phosphorylation) and signaling



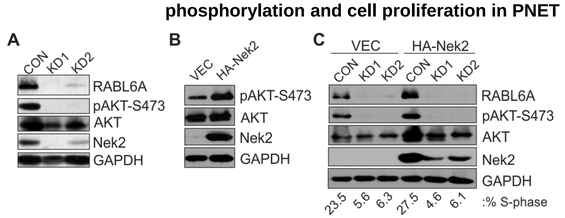
BON-1 PNET cells expressing vector control (CON) or shRNAs targeting RABL6A (KD1 and KD2) were examined by microarray and western analyses. **A)** Heat map shows RABL6A depletion significantly alters the expression of genes involved in Akt signaling; data from 3 experiments, designated A-C. Genes were 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



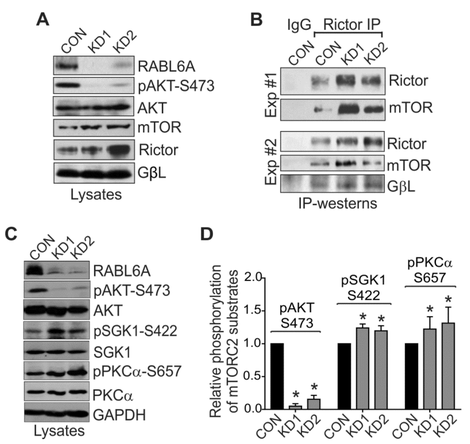
Kinases (gray) and phosphatases (white boxes) regulating AKT activation. P, phosphorylation; arrows, activating events; perpendicular bars, inhibiting events. RABL6A knockdown reduces AKT phosphorylation at S473 (**blue highlight**).

Nek2 kinase overexpression fails to rescue the effects of RABL6A loss on AKT phosphorylation and cell proliferation in PNET cells



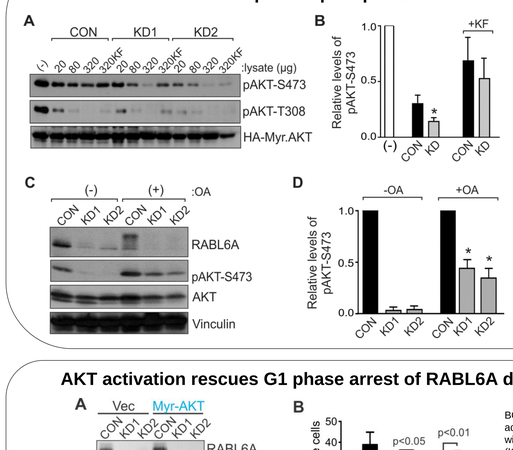
A) Loss of RABL6A in BON-1 KD1 and KD2 cells reduces levels of endogenous Nek2. **B)** BON-1 cells overexpressing HA-tagged Nek2 (HA-Nek2) display increased Akt-S473 phosphorylation, as expected. **C)** BON-VEC control and BON-HA-Nek2 cells were infected with CON, KD1 or KD2 viruses. Westerns show that Akt phosphorylation at S473 was not restored by HA-Nek2 overexpression in RABL6A deficient cells. Likewise, HA-Nek2 failed to override the cell cycle arrest induced by RABL6A loss (indicated by % S-phase).

mTORC2 complexes remain intact and active in RABL6A-depleted PNET cells



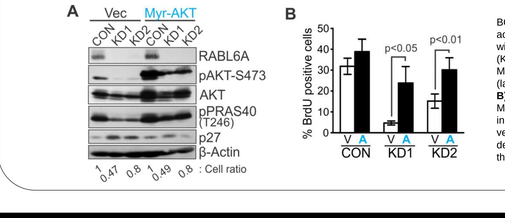
A) Western blots show expression of endogenous mTORC2 components (mTOR, Rictor and GβL) is largely unchanged by RABL6A depletion in BON-1 cells. **B)** IP-western analyses of endogenous mTORC2 complexes. Rictor antibodies (versus IgG control) detected intact mTORC2 complexes in RABL6A-depleted cells. Results from two experiments are shown, with lysates from (A) used in the Exp #1. **C)** Western analyses showed selective loss of AKT-S473 phosphorylation in RABL6A depleted cells while phosphorylation of other mTORC2 substrates, SGK1 and PKCα, was moderately increased. **D)** 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).

RABL6A regulates AKT-S473 phosphorylation via protein phosphatase PP2A



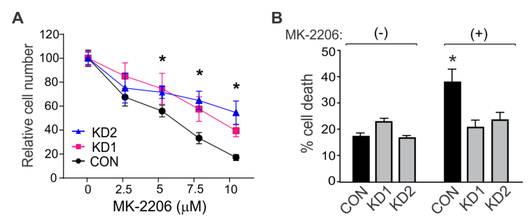
A) In vitro phosphatase assays. Phospho-AKT substrate (HA-tagged myristoylated AKT, HA-Myr.Akt) was mixed with increasing amounts of BON-1 CON, KD1 or KD2 lysates. As controls, no lysate (-) or KF was added to substrate prior to the phosphatase reaction. Westerns showed heightened phosphatase activity against AKT at Ser473, but not T308, in RABL6A-depleted cells using 80 and 320 ug of lysates. **B)** Relative levels of pAkt-Ser473, normalized to total HA-Myr.Akt, from 3 or more experiments using 320 ug of lysate. *, p<0.05 versus CON. **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 / PP1 phosphatases 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 cells.

AKT activation rescues G1 phase arrest of RABL6A depleted PNET cells



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 (A) promotes DNA synthesis in RABL6A knockdown cells relative to vector (V) control cells. Error bars, deviation from the mean for data from three separate experiments.

RABL6A sensitizes PNET cells to death induced by the AKT inhibitor, MK-2206

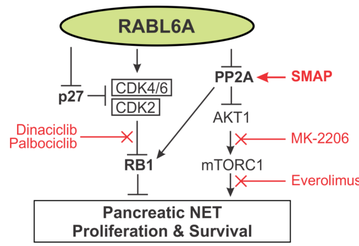


A) BON-1 cells expressing CON, KD1 or KD2 shRNAs were exposed for five days to increasing concentrations of the Akt inhibitor, MK-2206, and relative cell proliferation assayed using alamarBlue^R (Thermo Fisher Scientific) or Cell-Quant™ (Genecopoeia). **B)** Cells were treated for three days with MK2206 (10 μM) and cell death was quantified by trypan blue staining. For A and B, data were obtained from three independent experiments and error bars represent the deviation from the mean (*, p<0.05 compared to untreated cells in each group).

Conclusions

RABL6A is a novel regulator of AKT phosphorylation and is required for its activation.

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



Future Directions

Test novel combination PNET therapies targeting AKT, mTOR and/or CDKs, and determine how RABL6A status affects therapy responses

Determine if RABL6A levels in patient NETs correlates with AKT-mTOR status in tissue microarrays (TMAs)

Test how RABL6A influences the response of PNETs to SMAP (PP2A activator) compound

Test if SV40 small t antigen (a specific PP2A inhibitor) rescues the RABL6A knockdown phenotype

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