



# Coupled Diagnostic-Therapeutic Regimen for Neuroendocrine Cancer

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## Introduction

Neuroendocrine (NE) cancers originate from select secretory cells of the body's various endocrine structures. Some patients develop two or more NE cancers, a syndrome termed multiple endocrine neoplasia (MEN). DNA sequencing has led to breakthroughs in identification of the genes mutated in four types of familial MEN syndromes. Mutations in the protein menin are associated with MEN1<sup>1</sup>. Mutations in the receptor tyrosine kinase RET are linked to MEN2 and MEN3<sup>2,3</sup>. The cyclin-dependent kinase inhibitor, CDNK1B, is mutated in MEN4<sup>4</sup>. However, a large percentage of NE cancers are sporadic and a high percentage of these do not harbor menin, RET, or CDNK1B mutations. Cyclin-dependent kinase 5 (Cdk5) is the regulator of an important tumorigenic signaling network in medullary thyroid carcinoma<sup>5</sup>, one type of NE cancer. As NE cancers share many common features, we asked 1) if Cdk5 plays a role in other types of NE cancers, 2) if biomarkers of Cdk5 driven tumors can be identified, and 3) if inhibitors targeting Cdk5 are effective as therapeutics *in vivo*.

## Material & Methods

FFPE human NETs were analyzed by H&E and immunohistochemistry. Cell lines treated with inhibitors and SIPs were monitored for effects on growth. Phosphoproteomic analysis was conducted on growing and arrested mouse tumors. Phosphorylation state-specific antibodies were generated to a set of candidate biomarker phosphoproteins. Cell lines, mouse tumors, and human tumors were evaluated for these phosphoproteins by western blot. Transgenic and human xenograft mouse models of NE cancers were treated with Cdk5 inhibitors and monitored for effects on tumor growth.

## Results

Cdk5 pathway components are present in multiple types of human NETs and growth of human NE cell lines is dependent on Cdk5 activity. Using phosphoproteomic analysis and cell-based growth screens a set of potential downstream targets of Cdk5 was identified. Phosphorylation state-specific antibodies were generated to interrogate these targets *in vivo*. Phosphorylation of these proteins is dependent on Cdk5 activity in cell lines and elevated in mouse tumors generated by transgenic expression of Cdk5 activators, validating the phosphosites as biomarkers of Cdk5 activity. Additionally, these biomarkers are present in a subset of tumors derived from human patients. Preclinical studies in three NE mouse models that contain biomarkers of aberrant Cdk5 activity demonstrate that treatment with Cdk5 inhibitors blocks tumor growth.

## Conclusion

Aberrant activation of Cdk5 drives tumor growth in a subset of human NE patients. Biomarkers of Cdk5 activity, identified here, could potentially be utilized clinically to distinguish this group of patients as expected responders to Cdk5-targeted therapies.

## References

1. Lemos, M.C. & Thakker, R.V. Multiple endocrine neoplasia type 1 (MEN1): analysis of 1336 mutations reported in the first decade following identification of the gene. *Hum Mutat* 29, 22-32 (2008).
2. Mulligan, L.M., et al. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 363, 458-460 (1993).
3. Donis-Keller, H., et al. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum Mol Genet* 2, 851-856 (1993).
4. Pellegata, N.S., et al. Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *Proc Natl Acad Sci U S A* 103, 15558-15563 (2006).
8. Pozo, K., et al. The role of Cdk5 in neuroendocrine thyroid cancer. *Cancer cell* 24, 499-511 (2013).

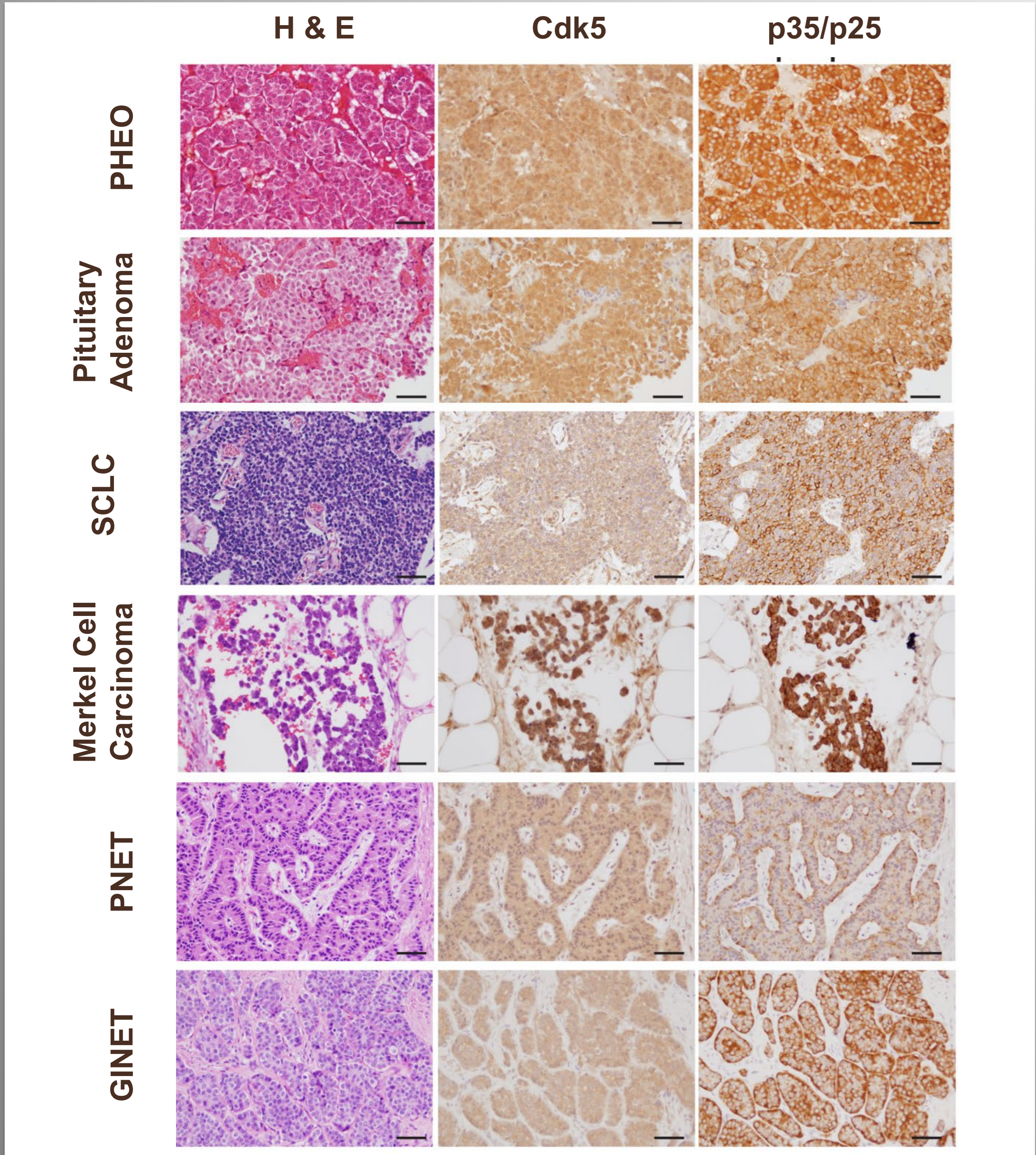
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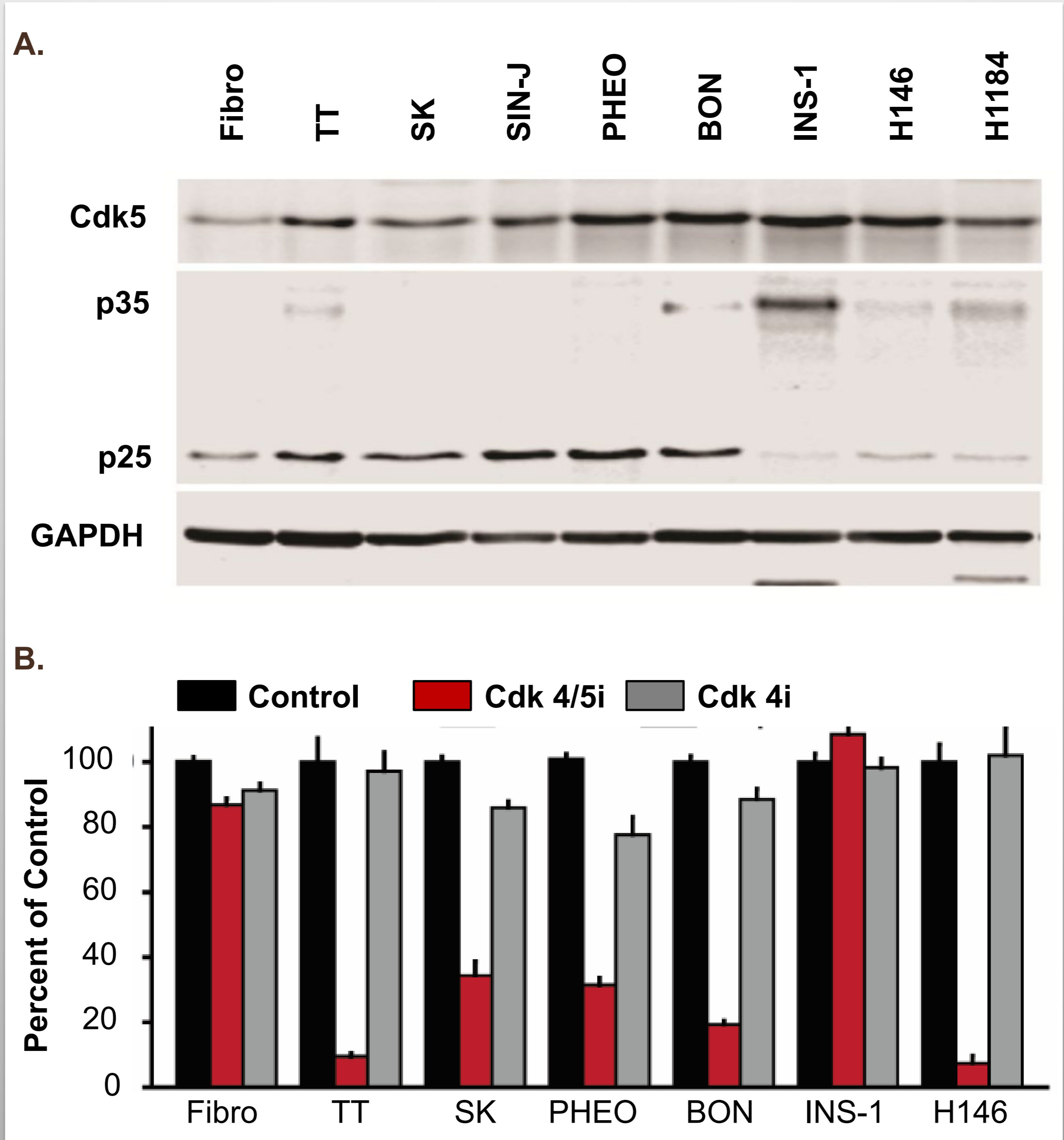
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**Figure 1. Cdk5 pathway components are present in human neuroendocrine tumors.**

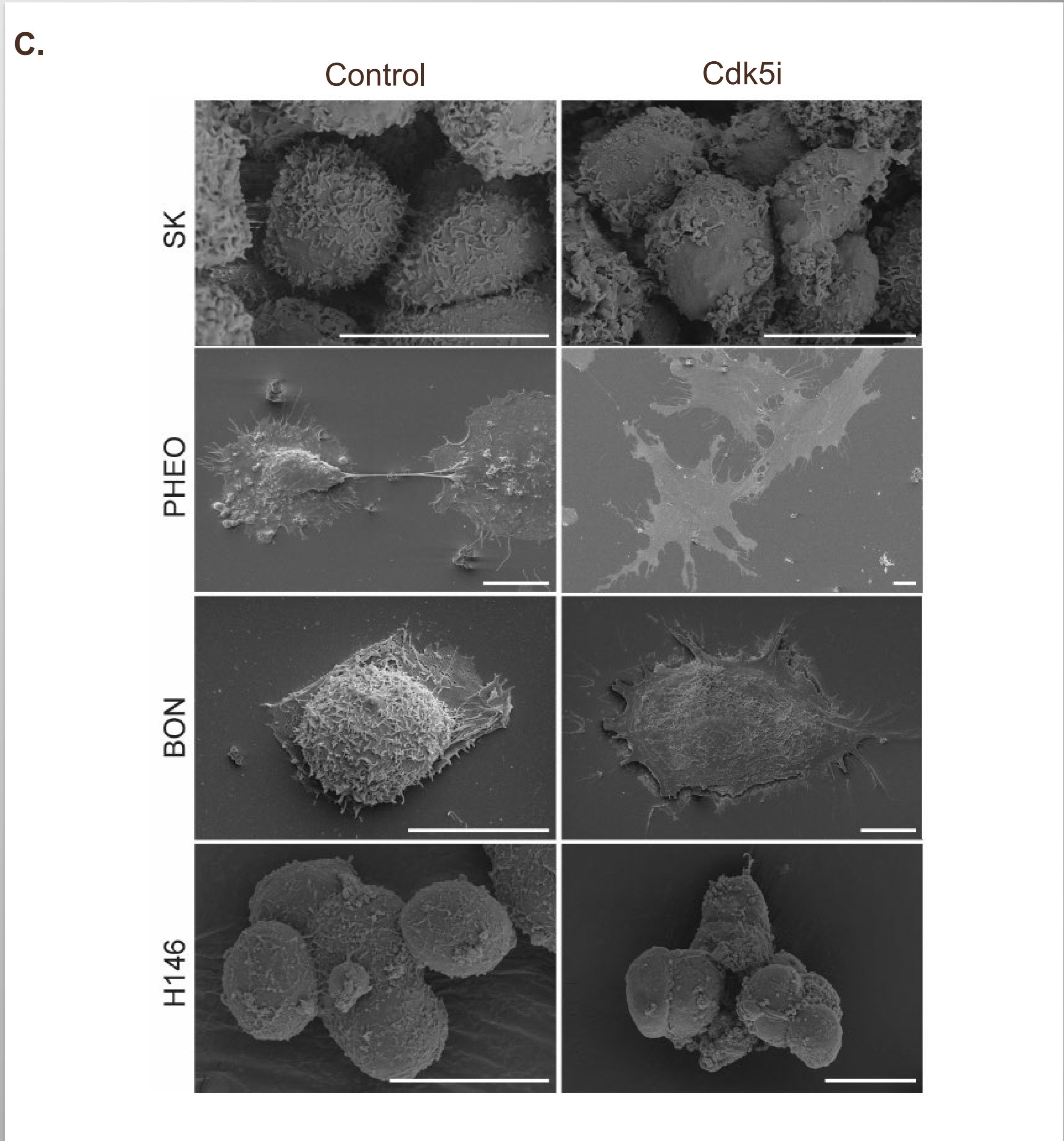


**Figure 1: Cdk5 pathway components are present in human neuroendocrine tumors.** Human neuroendocrine tumors were fixed in 10% formalin, embedded with paraffin, and analyzed by IHC; scales bars = 50  $\mu$ m.

**Figure 2. Cdk5 promotes tumor cell growth.**



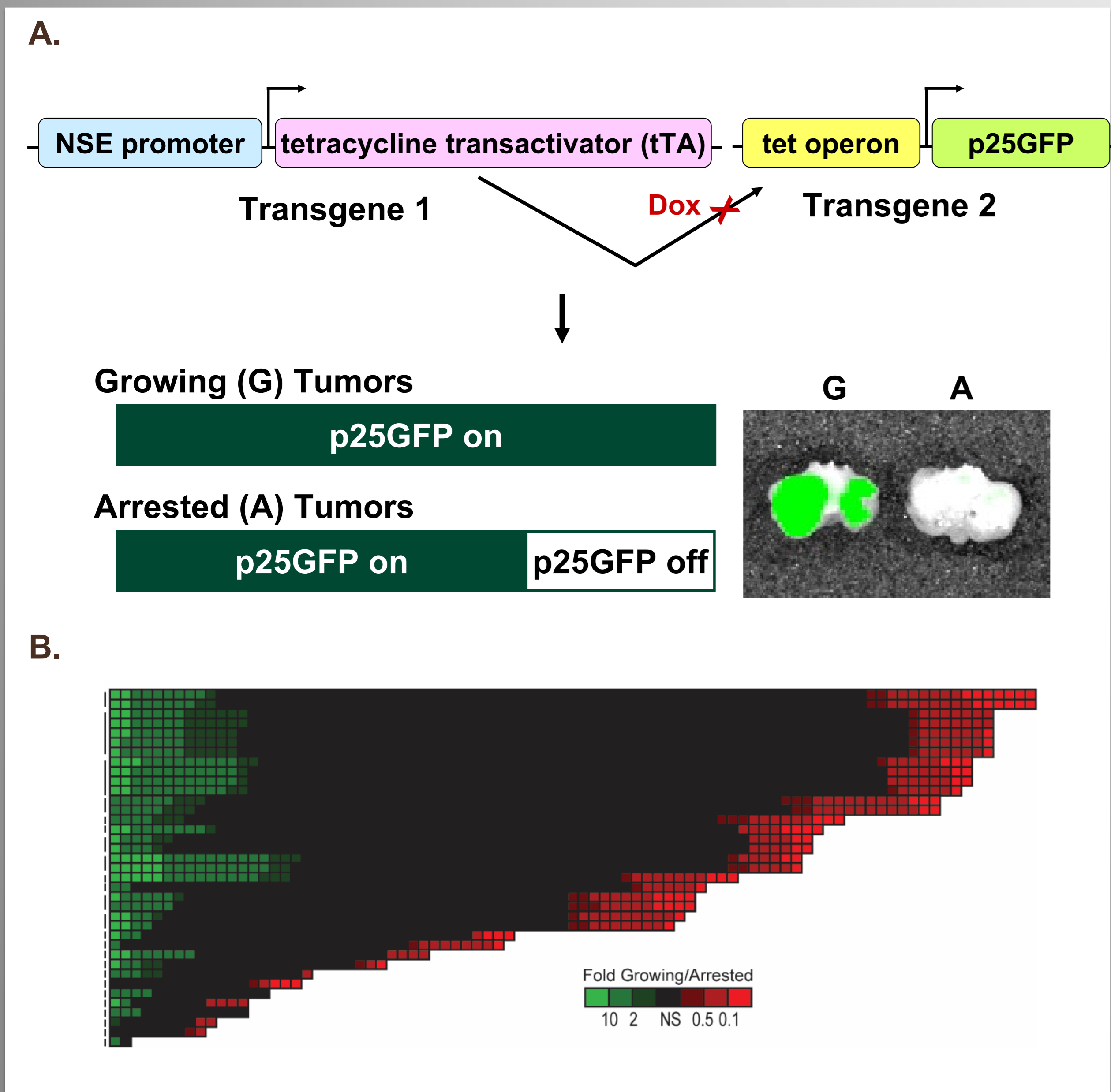
**Figure 2: Cdk5 promotes tumor cell growth.** A. Immunoblot of various NE cell lines. B. Cells were treated with 0.3  $\mu$ M Indo A (4/5i) or 0.3  $\mu$ M Indo B (4i) for 2-6 days. Cell number was assessed using CyQuant Direct Proliferation Assay.



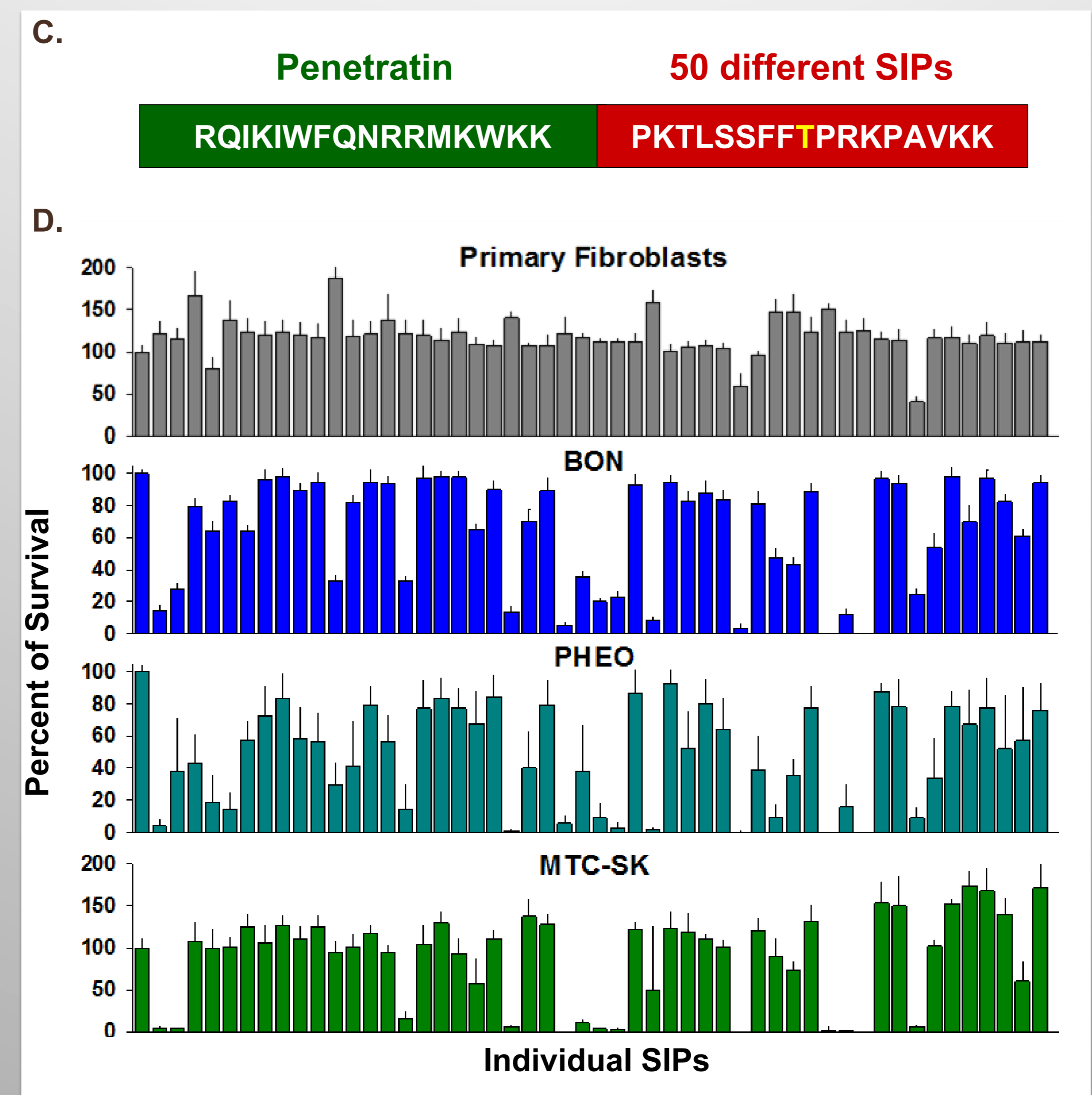
**Figure 2: Cdk5 promotes tumor cell growth.** C. SEM of NE cells treated with DMSO (Control) or Cdk 5 inhibitor (Cdk5i).



Figure 3. Identification of potential tumorigenic phosphoprotein signaling pathways.



**Figure 3: Identification of potential tumorigenic phosphoprotein signaling pathways.** A. Schematic of bi-transgenic mouse model of MTC and protocol for generation of growing and arrested tumors. B. Heat map comparing phosphopeptides detected by LC-MS/MS in growing vs. arrested mMTC tumors.

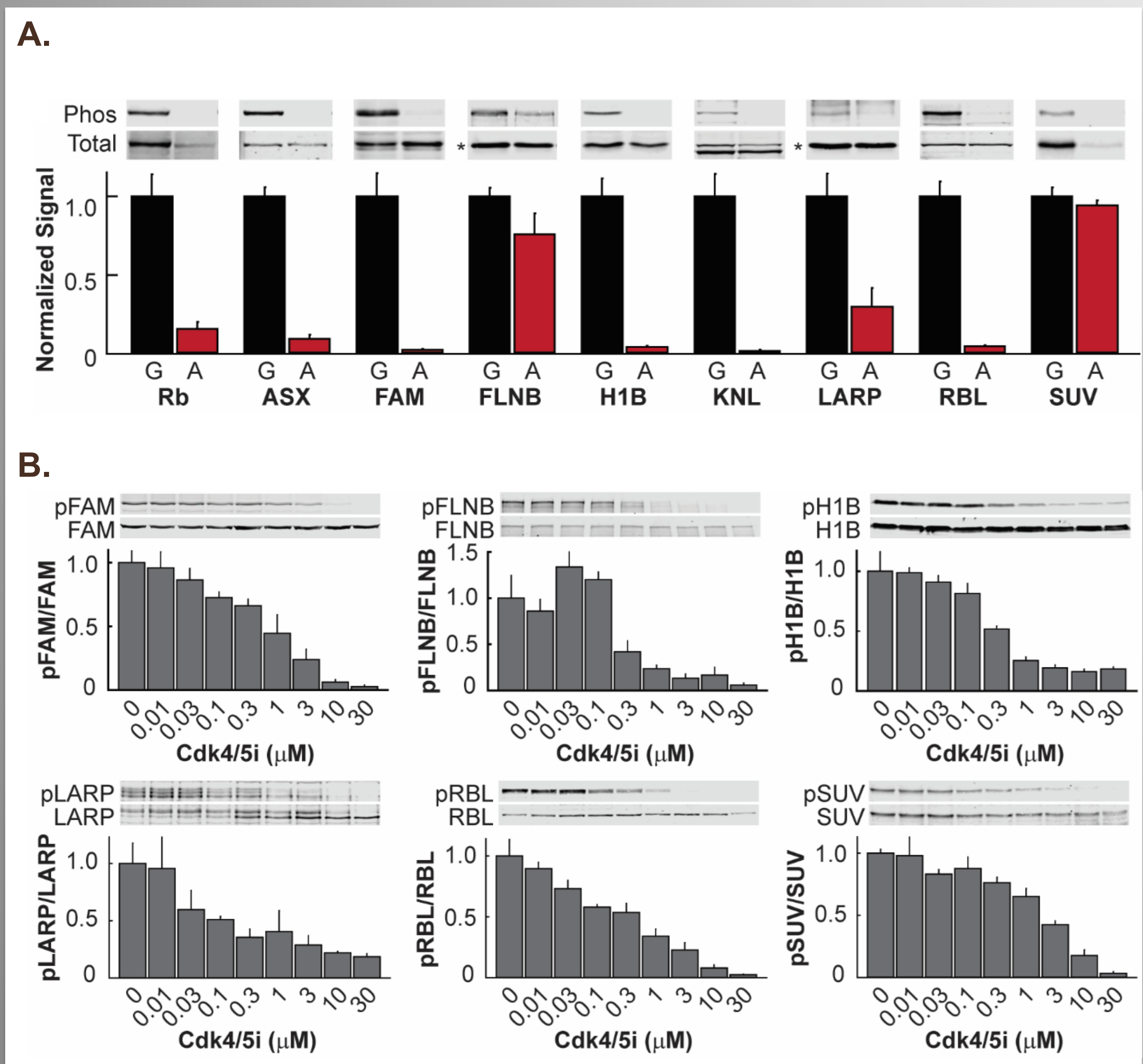


**Figure 3: Identification of potential tumorigenic phosphoprotein signaling pathways.** C. Diagram of short interfering peptides (SIPs) designed to inhibit phosphorylation of candidate proteins. D. High throughput screen using SIPs to identify phosphoproteins required for NE cell survival. Cells were treated with 30  $\mu$ M SIPs for 2-6 days. Cell number was assessed using CyQuant Direct Proliferation Assay.

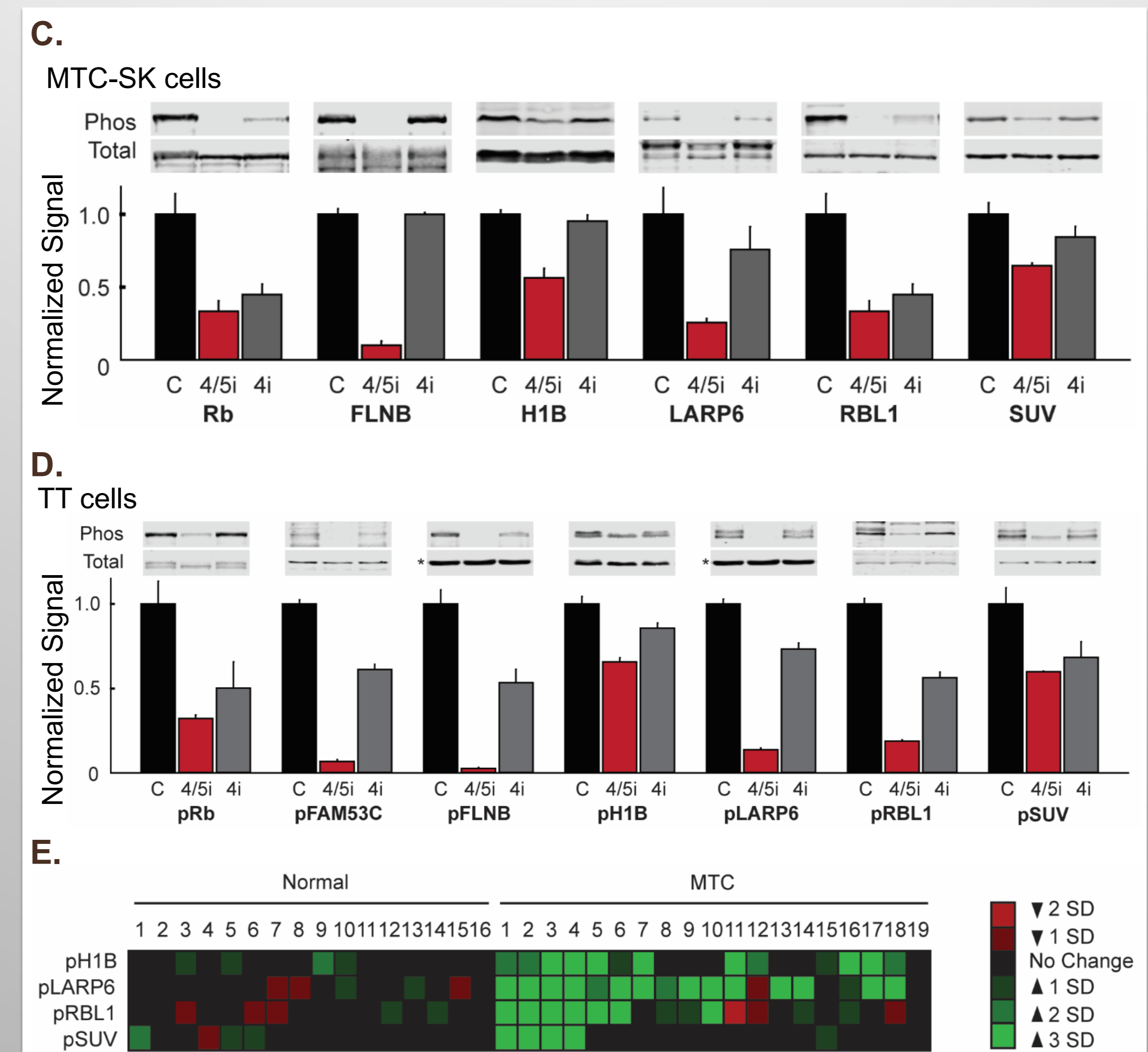
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Protein	Inhibition	Type	Function
RIZ	100	Methyltransferase	Tumor Suppressor, Neuronal Diff.
FBXL17	98-100	F-box	Ubiquitin ligation
LIG1	85-99	DNA Ligase	DNA Rep., Base Excision Repair
MTA2	86-99	Scaffold?	Member of HDAC complex, Homolog of MTA1
FLNB	78-99	Structural	Repair of Vascular Injuries
SLBP	84-98	RNA Binding	Histone mRNA Processing
SUV39H 1	77-98	Methyltransferase	Est. of Heterochromatin, Interacts w/ Rb1
ASXL2	80-97	Methyltransferase	Maintenance of Transcriptional Repression
KNL2	50-98	?	Chromosomal Segregation
LARP6	67-95	Ribonucleoprotein	Translation of collagen mRNA
POLE	49-99	DNA polymerase	DNA replication and repair, Colorectal Cancer
MTA1	10-90	Scaffold?	Member of HDAC complex, Associated with Metastasis
FAM53C	33-80	?	Neural Development
H1B	0-71	Histone	Chromatin Structure
RBL1	0-86	Scaffold?	Transcriptional Regulation, Homolog of Rb

**Figure 4. Phosphoproteins are biomarkers of Cdk5 dependent NE cancers.**

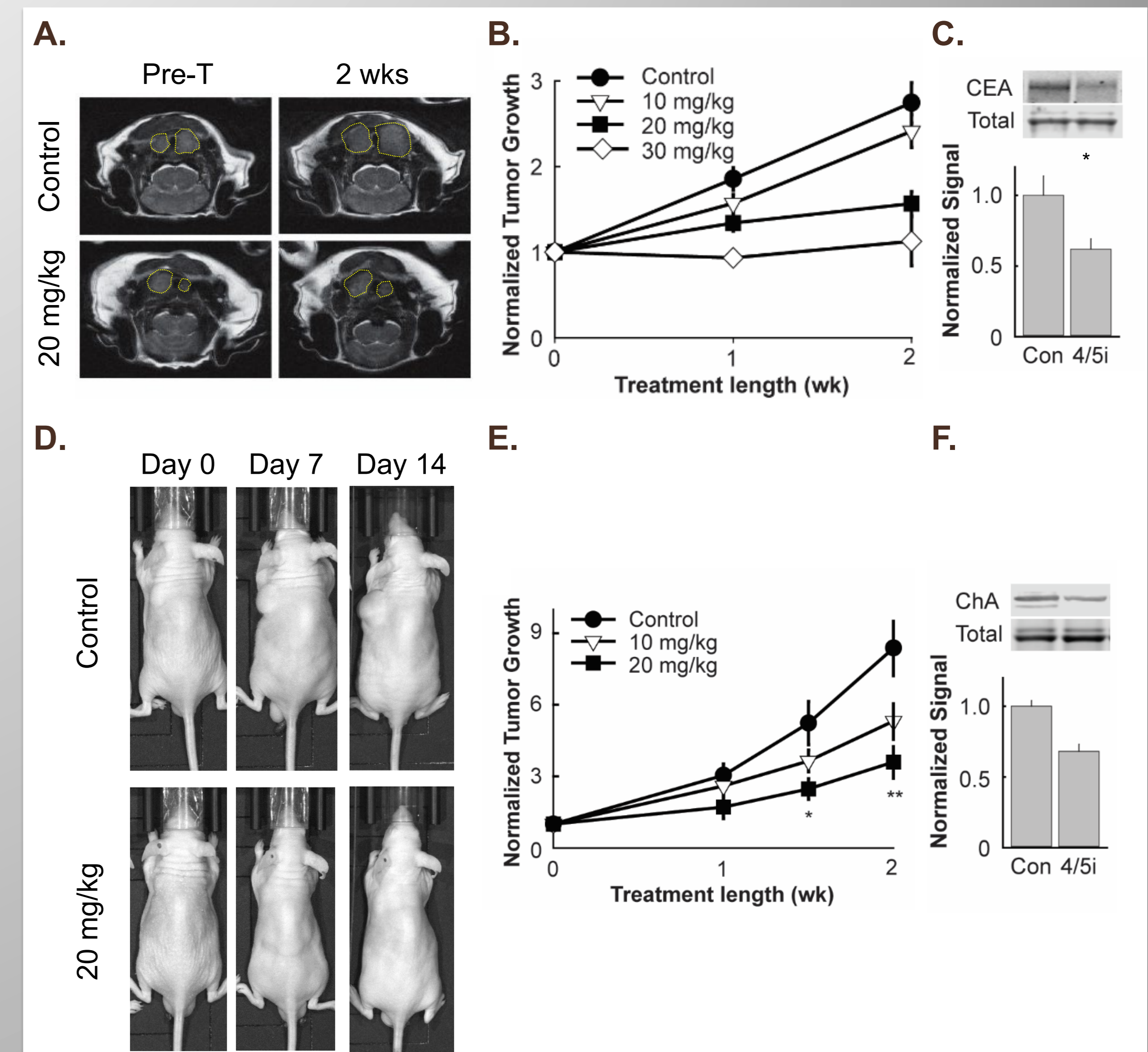


**Figure 4: Phosphoproteins are biomarkers of Cdk5 dependent NE cancers.** A. Phosphoproteins in growing (G) and arrested (A) mouse MTC tumors. B. Phosphoproteins in PHEO cells treated with increasing concentrations of Indo A (Cdk4/5i).



**Figure 4: Phosphoproteins are biomarkers of Cdk5 dependent NE cancers.** C. Phosphoproteins in SK cells treated with 0.3% DMSO (C), 0.3 μM Indo A (4/5i), or 0.3 μM Indo B (4i) for 4 hours. D. Phosphoproteins in TT cells treated with 0.3% DMSO (C), 0.3 μM Indo A (4/5i), or 0.3 μM Indo B (4i) for 4 hours. E. Heat map of phosphoproteins in normal human thyroid tissue and MTC tumors.

**Figure 5. Targeted Therapy.**



**Figure 5: Targeted therapy.** A. MRI of NSE-p25 animals treated with vehicle (Control) or 20 mg/kg BW Indo A (CDK4/5i). B. Quantitation of tumor growth from MRI of animals treated with vehicle (Control), 10 mg/kg BW Indo A, 20 mg/kg BW Indo A, or 30 mg/kg BW Indo A. C. Western of plasma from animals in A and B. D. Photos of human TT xenograft animals treated with vehicle (Control) or 20 mg/kg BW Indo A (Cdk4/5i). E. Quantitation of tumor growth from caliper measurement of animals treated with vehicle (Control), 10 mg/kg BW Indo A or, 20 mg/kg BW Indo A. F. Western of plasma from animals in D and E.