



**EMORY**  
LIBRARIES &  
INFORMATION  
TECHNOLOGY

**OpenEmory**

## **Identification of the interactome of a palmitoylated membrane protein, phosphatidylinositol 4-Kinase type II Alpha**

[Avanti S Gokhale](#), *Emory University*

[Pearl V. Ryder](#), *Emory University*

[Stephanie Zlatic](#), *Emory University*

[Victor Faundez](#), *Emory University*

---

**Journal Title:** Methods in Molecular Biology

**Volume:** Volume 1376

**Publisher:** Humana Press | 2016-01-01, Pages 35-42

**Type of Work:** Article | Post-print: After Peer Review

**Publisher DOI:** 10.1007/978-1-4939-3170-5\_4

**Permanent URL:** <https://pid.emory.edu/ark:/25593/th0pm>

---

Final published version: [http://dx.doi.org/10.1007/978-1-4939-3170-5\\_4](http://dx.doi.org/10.1007/978-1-4939-3170-5_4)

### **Copyright information:**

© Springer Science+Business Media New York 2016.

*Accessed November 13, 2019 6:53 AM EST*



Published in final edited form as:

*Methods Mol Biol.* 2016 ; 1376: 35–42. doi:10.1007/978-1-4939-3170-5\_4.

## Identification of the interactome of a palmitoylated membrane protein, phosphatidylinositol 4-kinase type II alpha

Avanti Gokhale<sup>\*,a</sup>, Pearl V. Ryder<sup>\*,a</sup>, Stephanie A. Zlatic<sup>a</sup>, and Victor Faundez<sup>a,b,#</sup>

<sup>a</sup>Department of Cell Biology, Emory University, Atlanta, Georgia, 30322, USA

<sup>b</sup>Center for Social Translational Neuroscience, Emory University, Atlanta, Georgia, 30322, USA

### Abstract

Phosphatidylinositol 4-kinases (PI4K) are enzymes responsible for the production of phosphatidylinositol 4-phosphates, important intermediates in several cell signaling pathways. In mammalian cells PI4KII $\alpha$  is the most abundant membrane-associated kinase involved in a variety of essential cellular functions. However, the precise role(s) of PI4KII $\alpha$  in the cell is not deciphered. Here we present an experimental protocol that employs the use of a chemical crosslinker DSP combined with immunoprecipitation and immunoaffinity purification techniques that identifies novel PI4KII $\alpha$  interactors. PI4KII $\alpha$  predictably participates in transient, low-affinity interactions that are stabilized by the use of DSP. Using this optimized protocol we have successfully identified actin cytoskeleton regulators – the WASH complex and RhoGEF, as novel interactors of PI4KII $\alpha$ . While this chapter focuses on the PI4KII $\alpha$  interactome this protocol can and has been used to generate other membrane interactome networks.

### Keywords

Phosphatidylinositol; phosphatidylinositol kinase; phosphoinositide; phospholipid; endosome; interactome; mass spectrometry; crosslinking

## 1. Introduction

Phosphatidylinositol 4-kinases (PI4K) have fundamental roles in lipid signaling and membrane trafficking [1–4]. This kinase family is categorized into type II and III members, which are distinguishable by structure, function and subcellular localization [5,6]. The focus of this chapter is on the type II family of PI4K. Yeast, flies and worms express a single isoform of PI4KII, whereas mammals have two isoforms of type II PI4Ks (PI4KII $\alpha$ , PI4KII $\beta$ ) [2,4,7]. PI4KII $\alpha$  is the most abundant membrane-associated PI4K in mammalian cells and is largely found on the trans-Golgi network and endosomes [8–15]. Importantly, phosphatidylinositol 4 phosphate is present in Golgi and endosomes [16]. Biochemical and functional studies have demonstrated that PI4KII $\alpha$  and  $\beta$  have several diverse functions including, but not limited to, regulating endosomal sorting of specific cargo proteins and recruitment of adaptor proteins, signal transduction, and the regulation of synaptic vesicle

<sup>#</sup>Correspondence should be addressed to: Victor Faundez (vfaunde@emory.edu).

<sup>\*</sup>These authors contributed equally to this work.

biogenesis [11,12,7,15]. Mutations in PI4KII $\alpha$  are implicated in tumorigenesis, spastic paraplegia suggesting that PI4KII $\alpha$  is an important molecule in pathogenesis mechanisms and therefore an attractive clinical target [17,18,13].

To further dissect the precise role of PI4KII $\alpha$  in cellular pathways we sought to biochemically identify its molecular interactors [19]. One of the major challenges is that PI4KII $\alpha$  is associated to membranes and there are technical complications linked to isolating a membrane protein interactome. These include (1) a relatively low abundance of PI4KII $\alpha$  (2) the need for detergent solubilization which may only partially solubilize PI4KII $\alpha$  or its in vivo interactions and (3) predictably PI4KII $\alpha$  is involved in dynamic cellular signaling pathways and therefore it is likely that PI4KII $\alpha$  engages in low affinity interactions and this potentially excludes the use of stringent methods for the isolation of PI4KII $\alpha$  network proteomes [20].

Here we describe a method that employs the use of chemical crosslinkers to allow the selective stabilization of transient interactions, thus circumventing the limitations for biochemical isolation [21,20,22,19,23,24]. This protocol is applied to cells in culture and uses a homobifunctional membrane permeable crosslinker DSP (dithiobis-(succinimidyl propionate) with a 12 Å spacer arm [25]. DSP crosslinks amine-reactive ester groups to bind primary amines such as lysines or the amino acid terminus of proteins. Under denaturing conditions DSP is cleaved by reduction of a disulphide bond present in the molecule. Cross-linking followed by immunoprecipitation and/or immunoaffinity chromatography of proteins of interest (in this case PI4KII $\alpha$ ) with magnetic beads permits the isolation of protein complexes that otherwise would not be amenable to stringent purification techniques. This protocol is typically compatible with regular immunoblot techniques and it can be scaled up for protein identification by quantitative mass spectrometry.

This protocol has been successfully used to construct network proteomes using various cellular baits [21,20,22,19,23,24]. In particular, we have effectively isolated PI4KII $\alpha$  and its novel interactors by quantitative immunoaffinity purification from in vivo cross-linked cell lysates [19]. We found that PI4KII $\alpha$  interacted with proteins involved in actin cytoskeleton polymerization – primarily the WASH complex and RhoGEF1. These interactions were confirmed by alternate biochemical, genetic and imaging methods [19]. The interactions between PI4KII $\alpha$  and WASH are particularly interesting since both molecules have been independently implicated in onset of the neurodegenerative disorder in mice – spastic paraplegia [26,13]. This newly discovered molecular interaction between PI4KII $\alpha$  and the WASH subunits could begin to explain the cellular pathways that ultimately lead to the disease phenotype. The method presented here is applicable to any membrane protein anchored by palmitoyl moieties.

## 2. Materials

### 2.1. Crosslinking and cell lysis

1. Phosphate-buffered saline (Thermo Fisher Scientific) buffer with 0.1mM CaCl<sub>2</sub> and 1mM MgCl<sub>2</sub> (PBS/Ca/Mg). Store at 4°C.

2. 10X stock buffer for lysis and immuno-magnetic-precipitation (IP) buffers – (100mM HEPES, 1.5M NaCl, 10mM EGTA, 1mM MgCl<sub>2</sub>; pH 7.4 (10X Buffer A). The 10X buffer A solution is diluted to 1X as required.
3. Lysis Buffer - 1X Buffer A + 0.5% Triton X-100 (made from a 20% stock) + Complete Protease Inhibitor Cocktail (Roche) (50X solution - 1 tablet dissolved per ml of Milli-Q water stored at -20°C).
4. Stock Crosslinking solution – 100 mM solution of DSP (Thermo Fisher Scientific) (40mg of DSP dissolved in 1ml DMSO). Solution must be prepared fresh every time. Care must be applied to maintain DSP crystal stock free of moisture.
5. 50X Crosslinking quenching solution - 1M TRIS to pH 7.4. Store at room temperature.

## 2.2. Immunoprecipitation and elution (Immunoaffinity precipitation)

1. Immuno-Magnetic Precipitation Buffer (IP Buffer) 1X Buffer A + 0.1% Triton X-100. Store at 4°C.
2. Beads - Dynal magnetic beads (Invitrogen).
3. Antibodies and antigenic peptide: PI4KII $\alpha$  antibody used was raised against the sequence 51-PGHDRERQPLDRARGAAAQ-70. The antigenic peptide was diluted to a 20 mM stock in 0.5 M MOPS, pH 7.4, and stored at -80°C. Alternatively, we have triple FLAG-tagged proteins to purify their interactors [22].
4. Elution buffer: The PI4KII $\alpha$  peptide was diluted in lysis buffer to a final concentration of 200  $\mu$ M.

## 2.3. Analysis of results

Gel electrophoresis - Samples were resolved by SDS-PAGE electrophoresis, typically using a 4–20% gel, followed by either, MS/MS analysis, silver stain or immunoblotting using the PI4KII $\alpha$  antibody and antibodies against the identified interacting partners to detect individual proteins [22,19].

## 3. Methods [24]

### 3.1. Crosslinking and Cell Lysis

1. Cells were grown to ~75–90% confluency and placed directly on ice. The media was aspirated and cells washed twice in ice cold PBS/Ca/Mg buffer.
2. Cells were then incubated either in freshly made DSP crosslinker solution diluted to 1mM in the PBS/Ca/Mg buffer or a DMSO alone control for 2h in an ice bath. Gently swirl the solution and ensure all cell monolayer is covered with the DSP solution. Note that there is formation of DSP crystals on the solution yet this is not a concern (see notes below).

3. The crosslinking reaction is quenched by addition of a 25 mM Tris solution for 15 min on ice.
4. Cells were washed twice in ice-cold PBS/Ca/Mg and lysis buffer was added to the cells. Depending of the membrane protein the lysis buffer may need to be adjusted. Some membrane proteins may need extra salt, ionic detergent, or chaotropic agents. Additionally, sonication of samples may increase the yield of solubilized protein. Cell debris was scraped from the plates and put in Eppendorf tubes and placed on ice for 30minutes. Lysates were then spun at  $16,100 \times g$  for 15 min. The supernatant was recovered and diluted to 1 mg/ml for immunoprecipitation.

### 3.2. Preparation of beads for immunoprecipitation

1. 30ul of the Dynal beads were added to 500ul of the IP buffer in screw-top microcentrifuge tubes. To this slurry the PI4KII $\alpha$  antibody was added to coat the beads. Antibody-free and non-specific antibody tubes were prepared as negative controls. Additionally PI4KII $\alpha$  peptide competition controls were included where the PI4KII $\alpha$  antigenic peptide (final concentration 40uM) was included in the immunoprecipitation reaction.
2. The tubes were inserted in an end to end rotor for 2h at room temperature.
3. After 2h the beads were washed twice in IP buffer.

### 3.3. Immunoprecipitation and Immunoaffinity purification

1. 500ug of the lysate was added to each of the beads coated with antibodies as well as the control beads and the mixture was incubated at 4°C for 2h in end to end rotors.
2. After incubation the beads were washed 6 times for 5 minutes in the IP buffer. All the washes were done in ice cold conditions at 4°C.
3. Proteins were then eluted from the beads either by boiling in Laemmli sample buffer at 75°C for 5 min (immunoprecipitation). This will elute all IgG from beads and in addition proteins that may bind non-selectively to beads. Elution can be done by incubating with the antigenic peptide for 2 h on ice (immunoaffinity purification). In case of the peptide elution the antigenic PI4KII $\alpha$  peptide was diluted in lysis buffer to a final concentration of 200  $\mu$ M. This elution protocol completely eliminates bead IgG and non-selectively bound proteins.
4. Samples were then resolved by SDS-PAGE electrophoresis followed by MS/MS analysis, silver stains or immunoblotting protocols. We used commercially prepared gels and Laemmli buffer for mass spectrometry studies to minimize contaminants.

## Notes

1. The PBS/Ca/Mg buffer should be stored at 4°C. Calcium and magnesium ions in the PBS buffer are required to maintain adhesion of the cells to the tissue culture plate during the experiment.
2. For preparation of stock 20% Triton X-100 - 10g Triton X -100 is diluted in total volume of 50 ml Milli-Q Water. The stock solution is stored at 4°C. Do not use and store for more than a month.
3. The DSP crosslinking solution must be made fresh, right before adding it the cells. DSP is highly hydrophobic and is dissolved in DMSO before diluting with warm PBS/Ca/Mg buffer. Warming of the PBS/Ca/Mg buffer in a 37°C water bath prevents precipitation of the DSP crystals [24].
4. Since the cells need to maintain a temperature of 4°C, the DSP solution should be placed in an ice bath once the DSP is completely solubilized in the warm PBS/Ca/Mg buffer. In the event that DSP is not completely solubilized, significant amounts will precipitate when the solution cools. If this occurs, the solution should be reheated to 37°C for complete solubilization. In case DSP repeatedly falls out of solution, fresh DSP solution must be prepared to ensure effective crosslinking. Once DSP is applied to cells in the ice bath it is normal to see the development of a crystalline layer in the wells of DSP treated cells over time. The presence of this layer does not obstruct the cross-linking chemistry in cells [24].
5. 10µL of the DSP stock solution is added to every 1 mL of warm PBS/Ca/Mg buffer. The DSP stock solution must be added drop wise with repeated mixing until all the DSP has dissolved. A control solution of 10 µL DMSO added to every 1 mL of PBS/Ca/Mg buffer is also used.
6. Volumes required for each plate size.
  - a. 2ml per well of a 6 well plate
  - b. 10ml per plate of a 10 cm plate
  - c. 20ml per plate of a 15cm plate

Periodically check is the cells on the plate are fully submerged in the DSP crosslinking solution. If required tap the plate to make sure of even distribution of the crosslinking solution on the plate.

7. Dynal magnetic beads conjugated to sheep anti rabbit IgG were used to bind the PI4KII $\alpha$  antibody for isolation of PI4KII $\alpha$  associated proteins. The use of screwcap microcentrifuge tubes and end-to-end rotors is critical to the protocol.
8. This protocol has been applied to various cell types [21,22,19,23]. The number of plates required per experiment is subjective to the goal of the experiment and is dependent upon the yield of total protein the cell type provides. Each standard tube reaction required 500 ug of total protein. Samples from a single tube might

be sufficient for identification/confirmation of interactors by western blot analysis. However, for quantitative MS/MS analysis the number of experimental reactions should be increased at least 10 times for efficient identification [21,22,19,23]. In this latter case the samples are ultimately concentrated to workable volumes by TCA precipitation.

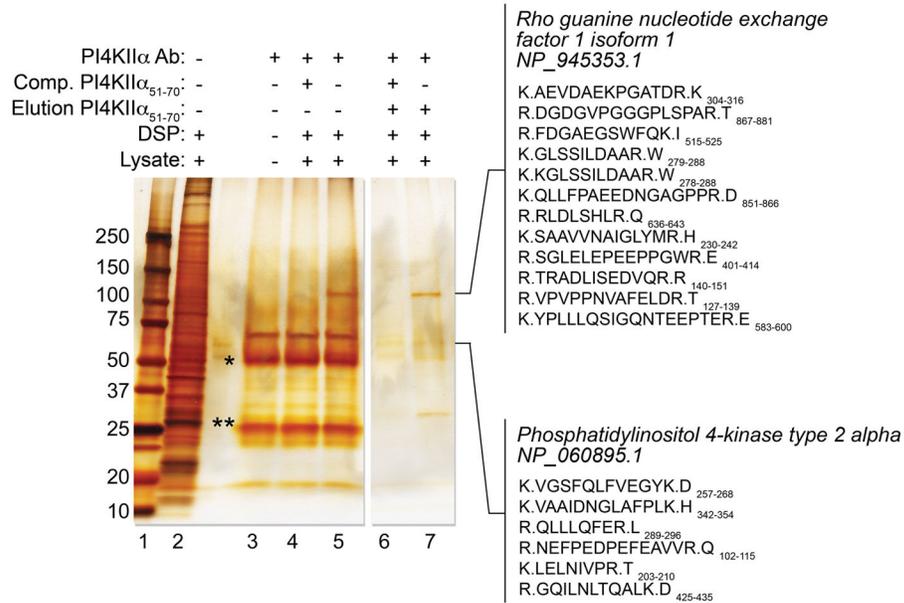
## Acknowledgments

This work was supported by grants from the National Institutes of Health (GM077569 and NS42599) and CHOA Children's Center for Neuroscience to V.F. P.V.R. was supported by National Research Service Award Fellowship F31NS0765.

## References

1. De Matteis MA, Di Campli A, Godi A. The role of the phosphoinositides at the Golgi complex. *Biochim Biophys Acta*. 2005; 1744(3):396–405. DOI: 10.1016/j.bbamcr.2005.04.013 [PubMed: 15979509]
2. Balla A, Balla T. Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol*. 2006; 16(7):351–361. [PubMed: 16793271]
3. Di Paolo G, De Camilli P. Phosphoinositides in cell regulation and membrane dynamics. *Nature*. 2006; 443(7112):651–657. [PubMed: 17035995]
4. Tan J, Brill JA. Cinderella story: PI4P goes from precursor to key signaling molecule. *Critical reviews in biochemistry and molecular biology*. 2014; 49(1):33–58. DOI: 10.3109/10409238.2013.853024 [PubMed: 24219382]
5. Balla T. Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiol Rev*. 2013; 93(3):1019–1137. DOI: 10.1152/physrev.00028.2012 [PubMed: 23899561]
6. Kim YJ, Hernandez ML, Balla T. Inositol lipid regulation of lipid transfer in specialized membrane domains. *Trends Cell Biol*. 2013; 23(6):270–278. DOI: 10.1016/j.tcb.2013.01.009 [PubMed: 23489878]
7. Wieffer M, Cibrian Uhalte E, Posor Y, Otten C, Branz K, Schutz I, Mossinger J, Schu P, Abdelilah-Seyfried S, Krauss M, Haucke V. PI4K2beta/AP-1-based TGN-endosomal sorting regulates Wnt signaling. *Curr Biol*. 2013; 23(21):2185–2190. DOI: 10.1016/j.cub.2013.09.017 [PubMed: 24206846]
8. Waugh MG, Minogue S, Chotai D, Berditchevski F, Hsuan JJ. Lipid and peptide control of phosphatidylinositol 4-kinase IIalpha activity on Golgi-endosomal Rafts. *J Biol Chem*. 2006; 281(7):3757–3763. DOI: 10.1074/jbc.M506527200 [PubMed: 16249177]
9. Balla A, Tuymetova G, Barshishat M, Geiszt M, Balla T. Characterization of type II phosphatidylinositol 4-kinase isoforms reveals association of the enzymes with endosomal vesicular compartments. *J Biol Chem*. 2002; 277(22):20041–20050. [PubMed: 11923287]
10. Barylko B, Gerber SH, Binns DD, Grichine N, Khvotchev M, Sudhof TC, Albanesi JP. A novel family of phosphatidylinositol 4-kinases conserved from yeast to humans. *J Biol Chem*. 2001; 276(11):7705–7708. [PubMed: 11244087]
11. Craigie B, Salazar G, Faundez V. Phosphatidylinositol-4-Kinase Type II Alpha Contains an AP-3 Sorting Motif and a Kinase Domain that are both Required for Endosome Traffic. *Mol Biol Cell*. 2008; 19:1415–1426. [PubMed: 18256276]
12. Wang YJ, Wang J, Sun HQ, Martinez M, Sun YX, Macia E, Kirchhausen T, Albanesi JP, Roth MG, Yin HL. Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell*. 2003; 114(3):299–310. [PubMed: 12914695]
13. Simons JP, Al-Shawi R, Minogue S, Waugh MG, Wiedemann C, Evangelou S, Loesch A, Sihra TS, King R, Warner TT, Hsuan JJ. Loss of phosphatidylinositol 4-kinase 2alpha activity causes late onset degeneration of spinal cord axons. *Proc Natl Acad Sci U S A*. 2009; 106(28):11535–11539. 0903011106 [pii]. DOI: 10.1073/pnas.0903011106 [PubMed: 19581584]

14. Minogue S, Anderson JS, Waugh MG, dos Santos M, Corless S, Cramer R, Hsuan JJ. Cloning of a human type II phosphatidylinositol 4-kinase reveals a novel lipid kinase family. *J Biol Chem.* 2001; 276(20):16635–16640. [PubMed: 11279162]
15. Minogue S, Waugh MG, De Matteis MA, Stephens DJ, Berditchevski F, Hsuan JJ. Phosphatidylinositol 4-kinase is required for endosomal trafficking and degradation of the EGF receptor. *J Cell Sci.* 2006; 119(Pt 3):571–581. [PubMed: 16443754]
16. Hammond GR, Machner MP, Balla T. A novel probe for phosphatidylinositol 4-phosphate reveals multiple pools beyond the Golgi. *J Cell Biol.* 2014; 205(1):113–126. DOI: 10.1083/jcb.201312072 [PubMed: 24711504]
17. Li J, Lu Y, Zhang J, Kang H, Qin Z, Chen C. PI4KIIalpha is a novel regulator of tumor growth by its action on angiogenesis and HIF-1alpha regulation. *Oncogene.* 2010; 29(17):2550–2559. DOI: 10.1038/onc.2010.14 [PubMed: 20154717]
18. Qin Y, Li L, Pan W, Wu D. Regulation of phosphatidylinositol kinases and metabolism by Wnt3a and Dvl. *J Biol Chem.* 2009; 284(34):22544–22548. DOI: 10.1074/jbc.M109.014399 [PubMed: 19561074]
19. Ryder PV, Vistein R, Gokhale A, Seaman MN, Puthenveedu M, Faundez V. The WASH Complex, an Endosomal Arp2/3 Activator, Interacts with the Hermansky-Pudlak Syndrome Complex BLOC-1 and its Cargo Phosphatidylinositol-4-kinase Type II Alpha. *Mol Biol Cell.* 2013; doi: 10.1091/mbc.E13-02-0088
20. Gokhale A, Perez-Cornejo P, Duran C, Hartzell HC, Faundez V. A comprehensive strategy to identify stoichiometric membrane protein interactomes. *Cell Logistics.* 2012; 2:1–8. doi:<http://dx.doi.org/10.4161/cl.22717>.
21. Gokhale A, Larimore J, Werner E, So L, Moreno-De-Luca A, Lese-Martin C, Lupashin VV, Smith Y, Faundez V. Quantitative proteomic and genetic analyses of the schizophrenia susceptibility factor dysbindin identify novel roles of the biogenesis of lysosome-related organelles complex 1. *J Neurosci.* 2012; 32(11):3697–3711. DOI: 10.1523/JNEUROSCI.5640-11.2012 [PubMed: 22423091]
22. Perez-Cornejo P, Gokhale A, Duran C, Cui Y, Xiao Q, Hartzell HC, Faundez V. Anoctamin 1 (Tmem16A) Ca<sup>2+</sup>-activated chloride channel stoichiometrically interacts with an ezrin-radixin-moesin network. *Proc Natl Acad Sci U S A.* 2012; 109(26):10376–10381. DOI: 10.1073/pnas.1200174109 [PubMed: 22685202]
23. Salazar G, Zlatic S, Craige B, Peden AA, Pohl J, Faundez V. Hermansky-Pudlak syndrome protein complexes associate with phosphatidylinositol 4-kinase type II alpha in neuronal and non-neuronal cells. *J Biol Chem.* 2009; 284(3):1790–1802. [PubMed: 19010779]
24. Zlatic SA, Ryder PV, Salazar G, Faundez V. Isolation of labile multi-protein complexes by in vivo controlled cellular cross-linking and immuno-magnetic affinity chromatography. *J Vis Exp.* 2010; (37):1855. [pii] 1810.3791/1855. 1855 [pii]. doi: 10.3791/1855 [PubMed: 20216526]
25. Lomant AJ, Fairbanks G. Chemical probes of extended biological structures: synthesis and properties of the cleavable protein cross-linking reagent [35S]dithiobis(succinimidyl propionate). *J Mol Biol.* 1976; 104(1):243–261. [PubMed: 957432]
26. Blackstone C, O’Kane CJ, Reid E. Hereditary spastic paraplegias: membrane traffic and the motor pathway. *Nat Rev Neurosci.* 2011; 12(1):31–42. nrn2946 [pii]. DOI: 10.1038/nrn2946 [PubMed: 21139634]



**Figure 1. Silver stain of Immunoprecipitation and elution (Immunoaffinity precipitation) using PI4KII $\alpha$  –specific reagents (PI4KII $\alpha$  antigenic peptide and antibody)**

Lane 1: Molecular weight standard (Biorad). Lane 2: Crosslinked soluble homogenate from SH-SY5Y (ATCC) neuroblastoma cells. Lane 3: Immunomagnetic beads incubated only with the PI4KII $\alpha$  antibody. This negative control predominantly depicts background IgG heavy (\*) and light (\*\*) bands eluted of the beads when heated with the Laemmli buffer. Lane 4–5: Immunoprecipitation with the PI4KII $\alpha$  antibody. Lane 4: Immunoprecipitation with the PI4KII $\alpha$  antibody in the presence of the excess PI4KII $\alpha$  peptide. This is an outcompetition control that represents a profile of non-specific peptides that may bind to the magnetic beads. Lane 5: Immunoprecipitation with the PI4KII $\alpha$  antibody. Note the presence of a prominent band at ~100kD in Lane 5 that is absent from the control lanes 3 and 4 depicting a putative PI4KII $\alpha$  specific interactor. Samples in lane 4 and 5 and prepared by elution with the Laemmli buffer. Lane 6–7: Immunoaffinity purification of PI4KII $\alpha$  interactors. Lane 6: Immunoprecipitation with PI4KII $\alpha$  with the PI4KII $\alpha$  antibody in the presence of antigenic peptide for outcompetition followed by elution with the excess PI4KII $\alpha$  peptide. Note the absence of bands in this control. Lane 7: Immunoprecipitation followed by elution with the PI4KII $\alpha$  peptide allowing for selective elution of putative PI4KII $\alpha$  interacting proteins with low background (compare lanes 6 and 7 with lanes 4 and 5). Note the absence of heavy (\*) and light (\*\*) IgG chains in lanes 6 and 7. MS/MS analysis of the sample in lane 7 identified highly enriched polypeptides that were absent in all the control samples. The two notable peptides shown here include (a) the PI4KII $\alpha$  peptide at ~55kDa as expected and (b) a completely novel PI4KII $\alpha$  interactor RhoGEF1 migrating at ~100kDa seen prominently in lanes 7 as well as lane 5.