Supporting Online Material for

PI(3,4,5)P₃ and PI(4,5)P₂ Lipids Target Proteins with Polybasic Clusters to the Plasma Membrane

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Materials and Methods

Cell culture and transfection. NIH3T3 and HeLa cells were purchased from ATCC. Cells were cultured in DMEM containing 10% FBS in 10% CO₂ and were split every three days by a 1/5 dilution at about 90% confluency. For confocal microscopy, 2.0 x 10⁴ cells were plated in each well of Lab-Tek 4-well chambered coverglasses (Nunc; #155383) coated with poly-D-lysine (0.1mg/ml) and cultured for 24 hrs prior to transfection. Cells were transfected with 0.25 μg/well of cDNA using Lipofectamine 2000 (Invitrogen).

Cloning of human small GTPases and generation of mutant constructs. A previously described library of human small GTPases (wildtype and constitutively active forms) (S1) was supplemented with twenty five additional human small GTPases using RT-PCR cloning with gene-specific primers containing attB sequences to create entry clones for the Gateway cloning system (Invitrogen). Wildtype and constitutively active forms were made for the 25 human small GTPases. Expression constructs were made by LR clonase (Invitrogen) to transfer the coding regions into CFP expression vectors. ARF subfamily members were N-terminally tagged and other subfamily members were C-terminally tagged with CFP. Tail mutants were designed using complementary oligonucleotides with restriction enzyme sites at both ends. p85 DN construct was kindly obtained from Dr. Daniel Storm (University of Washington). KRas tail was kindly provided by Dr. Marc Fivaz (Stanford).
**Microscopy.** All images were taken in live cells using Nipkow confocal and Zeiss epifluorescence microscopes (40x or 63x magnification). Images were analyzed using MetaMorph software (Molecular Devices). The detailed experimental protocol for the activation of the Inp54p PI(4,5)P$_2$ phosphatase is described in an accompanying paper (S2).

**In vitro lipid binding assay.** YFP-PLCδ-PH and YFP-conjugated to the respective polybasic peptides were transfected into 293F cells in 293 Freestyle media. As a negative control, YFP-HRas tail was expressed. 48hrs after transfection, the cells were collected and total proteins were extracted from the cells with a lysis buffer (50mM Tris, pH 8.0; 10mM EDTA, pH 8.0; 100mM NaCl; 0.5% Triton X-100; protease inhibitor cocktail). Each PIP Strip was preincubated for 1hr in 3% fatty-acid free BSA in TBS-T. Pre-incubated PIP strips were incubated with cell lysate in 3% BSA in TBS-T with gentle shaking overnight. After washing three times with 1% BSA in TBS-T, the strips were incubated with anti-GFP antibody (1:1000) in 1% BSA in TBS-T for an hour. After washing three times with 1% BSA in TBS-T, the strips were incubated with anti-rabbit IgG conjugated with alkaline phosphatase (Invitrogen; G-21079). The signals were detected with a NBT/BCIP detection kit (Biorad; 170-6432) after washing twice in 3% BSA in TBS-T, once in TBS-T, and once in 0.1M Tris, pH 9.5 for 5 min.
Supplementary Figure Legends

Fig. S1. Subcellular localization of 125 human CFP-conjugated CA small GTPases in NIH3T3 and HeLa cells. Fluorescent images were acquired using a Nipkow confocal microscope (40x or 63x objective) and processed using MetaMorph software (Molecular Devices). The localization of each small GTPase was visually compared in at least five independent images. One representative image was chosen from each cell line, NIH3T3 (left) and HeLa (right). Prenylation consensus sequences are indicated by a box, palmitoylation consensus sequences are underlined, basic amino acid residues are marked in red, hydrophobic amino acid residues are marked in blue, and myristoylation consensus sequences are marked in green. Scale bars, 10 μm.

Fig. S2. Differential localization of GDP and GTP bound ARF6. Arf6 was an outlier without a recognizable putative PM targeting domain. We tested whether PM targeting of Arf6 is regulated by a process that requires ARF6 GTP loading. CFP-tagged dominant negative ARF6 (T27N; GDP bound) was cotransfected in NIH3T3 cells with YFP-tagged constitutively active ARF6 (Q67L; GTP-bound) and the subcellular distribution of the two mutants was compared in the same cell using confocal imaging. Only the GTP bound Arf6 (Q67L) localized to the PM. The insets show a magnified part of the images and the arrow points to the PM. Scale bars, 10 μm.

Fig. S3. LY303511 (LY30), a kinase inactive analog of LY29, does not trigger the removal of Rin tail from the PM. (A) Structures of LY294002 (LY29) and LY303511 (LY30). LY30 contains a single atom substitution in the morpholine ring. LY30 has been reported to have similar chemical properties as LY29 but without a measurable inhibitory activity for PI3K (S3). (B) Effect of LY29 (50μM) and LY30 (50μM) on PDGF-induced
YFP-Akt-PH domain translocation. NIH3T3 cells were transfected with YFP-Akt-PH domains and cells were stimulated with 5nM PDGF for 3 min, followed by addition of LY29 or LY30. LY30 did not inhibit Akt-PH domain translocation or membrane ruffling induced by PDGF. (C) Effects of LY30, LY29, and iRap on Rin tail localization in the same cell. LY30 was first added to the cells, followed by LY29 and iRap. LY30 (50μM) did not trigger the removal of Rin tail from the PM, while LY29 (50μM) partially removed the Rin tail. Activation of CF-Inp by iRap removed most of the remaining Rin tail from the PM. Scale bars, 10 μm.

Fig. S4. Depletion of both PI(4,5)P₂ and PI(3,4,5)P₃ are required for the PM dissociation of MARCKS ED and Rin tail in HeLa cells. (A) Depletion of PI(4,5)P₂ by addition of iRap triggered complete dissociation of the PI(4,5)P₂ biosensor PLCδ-PH and only a small dissociation of MARCKS ED and Rin tail from the PM of HeLa cells. Joint depletion of PI(4,5)P₂ and PI(3,4,5)P₃ by addition of iRap and LY29 (iRap + LY29) triggered a near complete dissociation of MARCKS ED and Rin tail from the PM. (B) Quantitative analysis of the PM dissociation of PLCδ-PH domain, MARCKS ED, and Rin tail. PLCδ-PH domain, MARCKS ED, or Rin tail were cotransfected with CF-Inp and Lyn-FRB in HeLa cells. The dissociation index was analyzed using MetaMorph software as described in Fig. 2B. Scale bars, 10 μm.

Fig. S5. Kinetics of PM dissociation of different proteins with polybasic clusters. (A) Kinetics of the PM dissociation of Rit and Rin tails following the depletion of PI(4,5)P₂ and PI(3,4,5)P₃. (B) Kinetics of the PM dissociation of HRas tail (as a control), Arl7, Rab35, and DN RhoE (dominant negative version to minimize targeting by Rho-GTP binding proteins) following the depletion of PI(4,5)P₂ and PI(3,4,5)P₃.
**Fig. S6. Control experiments monitoring Rin tail dissociation for different protocols of PI(4,5)P$_2$ and PI(3,4,5) depletion.** (A) An alternative PI3K inhibitor, wortmannin (100nM), was added to the cells for 3 min, followed by the addition of iRap for 3 min and the change in localization of MARCKS ED and Rin tail were tracked over time. This protocol induced a similar PM dissociation as shown in Fig. 2 for the combination of iRap and LY29. (B) Image series showing that LY29 alone had only a small effect on Rin tail PM dissociation while the subsequent addition of iRap triggered a near complete PM dissociation of Rin tail. Scale bars, 10 μm.

**Fig. S7. Control experiments using expression of a peptide based inhibitor of PI3K, p85 DN.** (A) Control experiments showing that PDGF-induced translocation of Akt-PH domain, a biosensor for PI(3,4,5)P$_3$, was nearly completely suppressed in cells expressing the PI3K inhibitor construct, p85 DN. (B) Cells expressing p85 DN showed a near complete removal of Rin tail from the PM when PI(4,5)P$_2$ concentration was depleted using CF-Inp activation. Together with fig. S6, this further supports the model that PI3K inhibition has to be combined with PI(4,5)P$_2$ depletion for polybasic clusters to dissociate from the PM. Scale bars, 10 μm.

**Fig. S8. PI(3,4,5)P$_3$ synthesis at the PM enhances PM localization of Rin tail.** (A) Overexpression of a constitutively active PI3K (p110-CAAX) enhanced PI(3,4,5)P$_3$ synthesis at the PM and also enhanced the PM localization of Rin tail. CFP-Akt-PH domain and YFP-Rin tail were cotransfected in NIH3T3 cells together with p110-CAAX. In serum starved cells, p110-CAAX expression strongly increased Akt-PH domain localization at the PM. YFP-Rin tail also showed increased PM localization as evidenced by the recruitment of most nuclear Rin to the PM. There was also an
apparent co-localization of YFP-Rin with CFP-Akt-PH domain in lamellipodia. The presence of multiple lamellipodia correlated with p110-CAAX expression and Akt-PH localization to the PM. **(B)** Reversibility of the PI3K-dependent PM translocation of MARCKS ED and Rin tail. YFP-MARCKS ED or YFP-Rin tail was cotransfected with CFP-Akt-PH domain, together with p110-CAAX. To test for reversibility of PM targeting, cells were incubated with LY29 for 3min, followed by the removal of LY29 (in the presence of p110-CAAX). YFP-MARCKS ED and YFP-Rin tail partially dissociated from the PM by inhibition of p110-CAAX with LY29 (3min). After washout of LY29, PM targeting of YFP-MARCKS ED and YFP-Rin tail recovered within 1 min. The changes in the PM dissociation and PM binding of YFP-MARCKS ED or YFP-Rin tail coincided with the dissociation and PM binding of YFP-Akt-PH domains. Scale bars, 10 μm.

**Fig. S9. Effects of CF-Inp-activation and PDGF-stimulation on PI(3,4,5)P₃ production.** *(A)* Activation of CF-Inp lowered not only PI(4,5)P₂ but also mediated a partial reduction in PI(3,4,5)P₃ as was evident from a partial dissociation of YFP-Akt-PH. Pre-stimulation with PDGF was used for better visualization of the effect of CF-Inp activation on PI(3,4,5)P₃. *(B)* PDGF still increases PI(3,4,5)P₃ concentration (as evidenced by YFP-Akt-PH translocation) in cells in which CF-Inp has been activated before to lower PI(4,5)P₂ concentration. Together, these control experiments suggest that there is still sufficient PI(4,5)P₂ substrate in cells with activated CF-Inp for significant PI3K-mediated PI(3,4,5)P₃ production.

**Fig. S10. Polybasic clusters with only 4 positively charged amino acids still dissociate from the PM following PI(4,5)P₂ and PI(3,4,5)P₃ depletion.** *(A)* Rho E, which has only 4 positively charged amino acids in the 20 amino acid tail region, dissociated from the PM after depletion of PI(4,5)P₂ and PI(3,4,5)P₃. *(B)* Rho D, which
has also 4 positively charged amino acids, also dissociated from the PM after depletion of \( \text{PI}(4,5)\text{P}_2 \) and \( \text{PI}(3,4,5)\text{P}_3 \). Scale bars, 10 \( \mu \text{m} \).

**Fig. S11. Pharmacological inhibition of calmodulin and protein kinase C does not alter Rin-tail PM localization and dissociation. (A)** Rin-tail PM localization and dissociation by \( \text{PI}(4,5)\text{P}_2 \) and \( \text{PI}(3,4,5)\text{P}_3 \) is not affected by the calmodulin inhibitor W-7 (10\( \mu \text{M} \)). This supports the argument that increases in Ca\(^2+\) concentration and Ca\(^2+\)/CaM binding to the polybasic clusters are not responsible for the PM dissociation following \( \text{PI}(4,5)\text{P}_2 \) and \( \text{PI}(3,4,5)\text{P}_3 \) depletion. **(B)** Rin-tail PM localization and dissociation by \( \text{PI}(4,5)\text{P}_2 \) and \( \text{PI}(3,4,5)\text{P}_3 \) is not affected by the protein kinase C (PKC) inhibitor Ro-31-8220. This argues against the possibility that hydrolysis of \( \text{PI}(4,5)\text{P}_2 \) and inhibition of \( \text{PI}(3,4,5)\text{P}_3 \) may indirectly activate PKC and thereby phosphorylate polybasic clusters and cause their PM dissociation. A lack of such a role of protein phosphorylation is also supported by the finding that Rac1 (Fig. 4D) still dissociates from the PM even though it lacks a consensus phosphorylation site.

**Fig. S12. Polybasic peptides strongly bind phosphoinositides in vitro. (A)** Schematic view of the lipid positions of 15 different lipids on a phosphoinositide lipid strip (Echelon; P-6001). These PIP strips are 2 x 6 cm hydrophobic membranes that have been spotted with 15 different biologically active lipids. **(B)** Proteins with polybasic clusters bind phosphoinositides and show selectivity for \( \text{PI}(3,4,5)\text{P}_3 \) over \( \text{PI}(3,4,5)\text{P}_3 \). The images show binding of PLC\(\delta\)-PH domain and polybasic peptides to different phosphoinositides on the PIP Strip. YFP-PLC\(\delta\)-PH domain and YFP-conjugated polybasic peptides were expressed in 293F cells and total proteins were extracted. Total proteins were incubated with the PIP Strips and detected with anti-GFP antibody as described in Materials and Methods.
Fig. S13. Hydrophobic amino acids are necessary for the PM targeting of the small GTPases GEM and RAD. Identification of essential hydrophobic amino acids for PM targeting in the polybasic regions of the small GTPases GEM and RAD. (A) Two hydrophobic amino acid residues, Phe and Trp, were replaced in GEM with the less hydrophobic amino acid residue Ala. A marked loss in PM targeting was observed for this GEM mutant. (B) The hydrophobic amino acid residue Phe in the RAD tail was replaced with Ala which also led to a loss in PM targeting of this RAD mutant. Scale bars, 10 μm.
Supplementary References


Heo et al., Figure S1

Ras subfamily

NIH3T3

HeLa

NIH3T3

HeLa

HRAS (NP_005334 )

KLNPDESGPCMSPCKVLS

KRAS4A (NP_203524 )

KLSKEKTPGCVKIKCIIIM

RRAS (NP_006261)

PPSPPSAPRKKGGCCVLL

RRAS3 [MRAS] (NP_036382)

KTKWGRDRTGTHKLCVIL

NIH3T3

HeLa

NIH3T3

HeLa

NRAS (NP_002515 )

KLNSDDGTQGMGLPCVVM

KRAS4B (NP_004976 )

KMSKDGGGGGSKTKCVIM

RRAS2 (NP_036382)

PSPEPTREKDKGCCVIF

RIT [RIT1] (NP_008843)

KNSVWKRLKSPFRKKKDSVT

HeLa
NIH3T3  |  HeLa  |  NIH3T3  |  HeLa

**RIN [RIT2] (NP_002921)**
KDSLWKKLKGSLLKKREMT

**RALA (NP_005393)**
NGKKRKSLAKRIERCCIL

**RAP1A (NP_002875)**
INRKPVEKKFKKSCCLL

**RAP2A (NP_066361)**
MNYAAQPDKKDPCCASCNIQ

**RRP22 (NP_006468)**
PAHPALRLGALHPARCSIM

**RHEB (NP_005605)**
LEAEKMDGAASQGKSSCSSVM

**RALB (NP_002872)**
KNGKKSKNKSFKERCCLL

**RAP1B (NP_001010942)**
INRKPVPCKARKKSSCQIL

**RAP2B (NP_116235)**
MNYAAQSGDEGCCASCVIL

**NKIRAS1 (NP_065078)**
SKSSFPLGKRKNGSNSEN
**NIH3T3** | **HeLa** | **NIH3T3** | **HeLa**

**NKIRAS2 (NP_060065)**
QSKSAFLSLRKNKSGSLDG

**RERG (NP_116307)**
RSSTTHVRQAINKMLTKISS

**RRAGA (NP_006561)**
RKHFEKLERVDGPKHSLMR

**RRAGB (NP_006055)**
RKHFEKLERVDGPQCLLMR

**RRAGC (NP_071440)**
QTSASSLKALTHNGTPRNAI

**RRAGD (NP_067067)**
QNLRQKKRATPNTFRVLL

**GEM [GEM1] (NP_005252)**
RKARRFWGKIVAKNNKMAFKLKSKSCHDLSVL

**RAD [RRAD] (NP_004156)**
KKAKRFLGRIVARNSRKMMAFRAKSKSCHDLSVL

**REM (NP_054731)**
QRARRFLARLTARSAARRALKARSKSCHNLAVL

**RASL12 (NP_057647)**
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Rho subfamily

**NIH3T3**

- **RHOA (NP_001655)**
  - ATRAALQARRKKSGCLVL

- **RHOC (NP_786886)**
  - ATRAQLQRVRKKRGCPIL

- **RHO6 [RND1] (NP_055285)**
  - RSELISSTFKKEAKSCSIM

- **RHOH (NP_004301)**
  - NQARRRRRLFSINECKIF

**HeLa**

- **RHOB (NP_004031)**
  - AALQKRYGSNCINCKVL

- **RHO7 [RND2] (NP_005431)**
  - PDGNEGEIKDRAKSNLM

- **RHOE [RND3] (NP_005159)**
  - ELSAVATDLRDKAKSCTVM

- **RIF [RHOF] (NP_061907)**
  - ALSALKQRKKRRCLLLL
Rab subfamily

NIH3T3 | HeLa | NIH3T3 | HeLa
---|---|---|---
**RAB1A (NP_004152)**
EKSNVKIQSTPVKQSGGĆ

**RAB1B (NP_112243)**
ERPNLKIDSTPVKPGGGĆ

**RAB2 (NM_002865)**
ATNATHAGNQGGQAGGGĆ

**RAB3A (NP_002857)**
AKQGPLSDDQVPHQDCĆ

**RAB3B (NP_002858)**
SSKNTRLSDTPPLQQNĆ

**RAB3D (NP_004274)**
NGKPAGVDAPQPPSSĆ

**RAB4A (NP_004569)**
LRQLRSPRTQAPNAQECĆ

**RAB4B (NP_057238)**
LRQLRQPRAQAAPQPCĆ
NIH3T3 | HeLa | NIH3T3 | HeLa
--- | --- | --- | ---
RAB21 (NP_055814) | QIIDDEPQAQTSGGGC | RAB22A (NP_065724) | SGGKGFKLRRQFSEPKRSC
RAB22B [RAB31] (NP_006859) | GNNIGHTKEKPTMQASRRCC | RAB23 (NP_057361) | RPNKQRTKKRNPFSSCSIP
RAB24 (NP_570137) | DKGVDLGKKPNPYFYSCHH | RAB25 (P57735) | SAAQGEQPGEPKEACCTSL
RAB26 (NP_055168) | RFRLHDYVKREGRGASCRRP | RAB27A (NP_004571) | NGHASTQLSSEEKEGCSCC
RAB27B (NP_004154) | NGGNSGNLDEKPPEKCKIC | RAB28 (NP_004240) | YPEEEQHHTTSQSRICSVQ
ARF subfamily

NIH3T3    HeLa    NIH3T3    HeLa

ARF1 (NP_001649)  ARF3 (NP_001650)
MGNIFANLFK---SGDGLYEGLDWLSNLQKRNQK

ARF4 (NP_001651)  ARF5 (NP_001653)
MGLTISSLFS---TGTTGLYEGLDWLSNELSKR

ARF6 (NP_001654)  ARL1 (NP_001168)
MGKVLSSIFG---ATSGDVGLWLTWNYSLKS

ARL2 (NP_001658)  ARL3 (NP_004302)
MGLLTILKKKM---LLPGIDWLLDDISSRFTAD

MGFFISSIFK---KGTLDEAMEWLVRMKTSPQ
Other subfamily members

NIH3T3  HeLa  NIH3T3  HeLa

SAR1A (NP_064535)
SVLKRQGYGEGFWLSQYID

SAR1B (NP_057187)
SVLKRQGYGEGFWMAQYID

RAN (NP_006316)
YEHDLVQAQTALPDEDDDL
Heo et al., Figure S2
Heo et al., Figure S3

A

LY 294002

LY 303511

B

Akt-PH

Akt-PH

-LY29 -LY30

+LY29 +LY30

+PDGF

-C

Rin tail

Before LY30 LY29 Inp54

Bar
Heo et al., Figure S4

A

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B

PM dissociation index

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Heo et al., Figure S5

A

B

PM dissociation index

iRap+LY29

5 min

PM dissociation index

iRap + LY29

5 min

Rin tail
Rit tail

RhoE T37N
Rab35
Arf7
HRas tail
Heo et al., Figure S6

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B

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Heo et al., Figure S7
Heo et al., Figure S8

**A**

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**B**

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Heo et al., Figure S9

A

Before ↓ 3 min
CF-Inp
Akt-PH
PDGF (3min)  PDGF (6min)

B

Before ↓ PDGF 3min
CF-Inp
Akt-PH
iRap (3min) iRap (6min)
Heo et al., Figure S10

A
RhoE T37N
-1min 3min

iRap + LY29

B
RhoD T31N
-1min 3min

iRap + LY29
Heo et al., Figure S11

A

iRap + LY29

CF-Inp

-1min

3min

YFP-Rin tail

10μM W7 (Calmodulin inhibitor)

B

iRap + LY29

CF-Inp

-1min

3min

YFP-Rin tail

100nM Ro-31-8220 (PKC inhibitor)
Heo et al., Figure S12

A

<table>
<thead>
<tr>
<th></th>
<th>Lysophosphatidylcholine</th>
<th>Phosphatidylserine</th>
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<tbody>
<tr>
<td>Lysophosphatidic acid</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>PI(3)P</th>
<th>PI(4)P</th>
<th>PI(5)P</th>
<th>Phosphatidic acid</th>
<th>Phosphatidylserine</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingosine-1-phosphate</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>PI(3,4)P_2</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
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<tr>
<td>PI(3,5)P_2</td>
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<td>O</td>
<td>O</td>
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<td>O</td>
</tr>
<tr>
<td>PI(4,5)P_2</td>
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<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>PI(3,4,5)P_3</td>
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<td>O</td>
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</tr>
<tr>
<td>Phosphatidylcholine</td>
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<td>O</td>
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<td>O</td>
<td>O</td>
<td>O</td>
</tr>
</tbody>
</table>

B

PLC-δ-PH  HRAS tail  KRAS tail  MARCKS ED  Rin tail  Rit tail

Arrows:
- $\text{PI}(4,5)P_2$
- $\text{PI}(3,4,5)P_3$
Heo et al., Figure S13

A

GEM

GEM F269A W270A

B

RAD

RAD F281A

GEM: RKARRFWGKIVAKNNKNMAFKLKSCHDLSVL
RAD: KKAARKFGLGRIVARNSRKMFRASKSCHDLSVL
REM: QRARRFALARLTARSSRRLAKSRSNSNLAVL