

Lab Meeting April 28<sup>th</sup>, 2025

Last week:

Westerns of MagStrep pulldown experiment show that the TWIN-strep and the N-terminal Strep Qb seem to work a bit better (AO25-17B), but I think now the problem is background binding of Qa or Qb without the strept tags.

Floatation assays:

Repeat 2x the His-Sec17 and His-Sec18 co-floatation on PCPS liposomes +/- Qc AO25-13C,D,E).

Prepare prenyl Ypt7 liposomes with VML + Rh for floatations (AO25-15B)

Look for Sec18 floating on liposomes with Ypt7, Sec17, or SNAREs. (AO25-18)

Lets see if any of our strep-tagged  
RPLs are standouts at binding

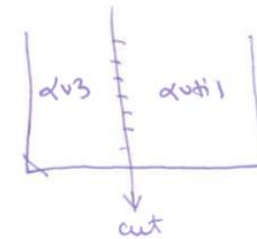
AD25-17B  
4-17-25

	1	2	3	4	5	MagStrep Beads	8	9
1								
2	Use Buffer DM = 20mM Hepes 7.4					<div>100 <math>\mu</math>l Reactions:</div> <div>2X Buffer DM : 50 <math>\mu</math>l water : 42.5 <math>\mu</math>l 2mM RPLs : 7.5 <math>\mu</math>l <hr/>100 <math>\mu</math>l</div>		
3	150mM NaCl							
4	1mM EDTA							
5	0.1% DDM							
6								
7								
8	Six flavors of RPLs							
9						<u>tulashree</u>		
10	(1) 2Q (wt)	AD24-34				blue		
11	(2) 2Q (Qa strep)	"				black		
12	(3) 2Q (Qa TWIN-strep)	AD25-17				orange		
13	(4) 2Q (Qb strep)	AD24-34				yellow		
14	(5) 2Q (Qb TWIN-strep)	AD25-17				purple		
15	(6) 2Q (strep Qb)	AD25-17				green dot		
16								
17								
18	Wash 20ul slurry of MagStrep beads (1ul settled beads)							
19	3x in .5ml Buffer DM							
20								
21	Add 2X Buffer, water, RPLs as indicated above.							
22	Nutate c.o.e. 2hrs @ 4°C							
23	Remove unbound (save 50ul) + boil w/ 2x SB+Bme 5'							
24	Wash beads 3x in .5ml Buffer DM.							
25	Elute in 50ul 1x SB+Bme. Boil 95°C <u>2min</u> .							
26	Store in -20.							
27	(Reboil — min before running gels:							
28								
29								

Protocol for binding strep-tagged RPLs to  
MagStrep beads:

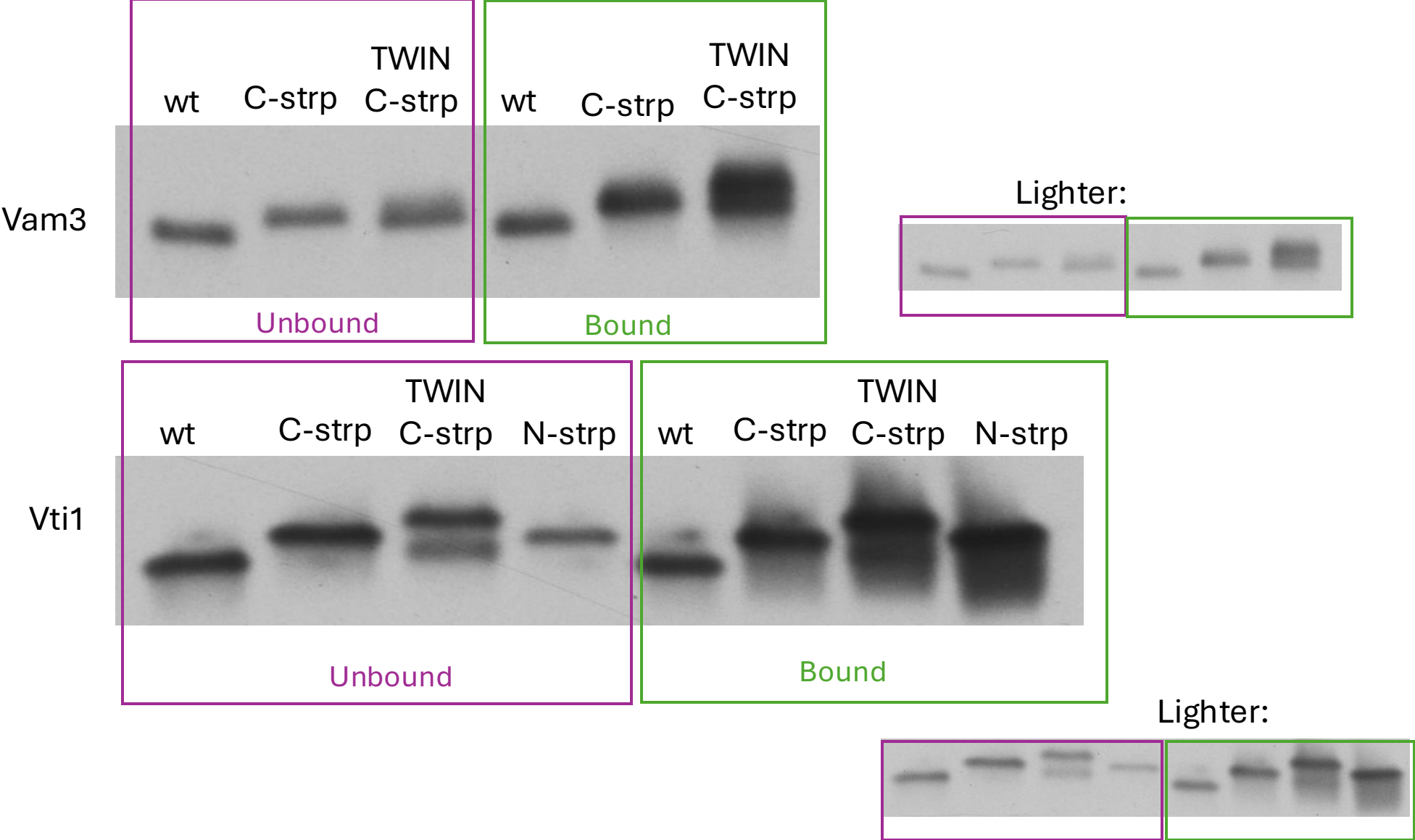
Nutate RPLs with 20ul bead slurry in DM Buffer  
(with 0.1% dodecyl-B-D-maltoside).  
Wash and elute in SB+BMe

Lane 1	Unbound (1)	15 $\mu$ l
2	(2)	
3	(3)	
4	Bound (1)	
5	(2)	
6	(3)	
7	MW marker	
8	Unbound (1)	
9	(4)	
10	(5)	
11	(6)	
12	Bound (1)	
13	(4)	
14	(5)	
15	(6)	



$\alpha v3$  8-26-24  
 $\alpha vti1$  3-3-22  
 20: 0/N 4°C  
 20: 2 rabbit 1hr RT

AO25-17B: At first look, it seems like the TWIN-tagged and the N-term tagged Qb are better, but the lighter burns make me wonder really how much better? There is a really high background of the untagged Qa and Qb binding.



## AO25-13C,D, and E: A 4-tube floatation assay

1. PCPS lipos+Sec18+Sec17 -----inc 10 min----- Add 4.8 ul Rb150 -----inc 60 min
2. PCPS lipos+Sec18+Sec17 -----inc 10 min-----Add 4.8 ul of Qc-----inc 60 min
  
- 3a. PCPS lipos+Sec18 -----inc 10 min-----Add 4.8 ul of **3b**-----inc 60 min
- 3b. Sec17 and Qc -----inc 10 min-----Add 4.8 ul to 3a
  
- 4a. PCPS lipos+Sec18 -----inc 10 min----- Add 3 ul of **4b** & 1.8 ul of **4c**-----inc 60 min
- 4b. Sec17 -----inc 10 min-----Add 3 ul to 4a
- 4c. Qc -----inc 10 min-----Add 1.8 ul to 4a

# AO25-13C, D, E

- 22.2  $\mu$ l 3  $\mu$ l 4.8  $\mu$ l = 30  $\mu$ l
1. PCPS + 1B + 17 — 10 min — + 4.8  $\mu$ l Pb
  2. PCPS + 1B + 17 — 10 min — + 4.8  $\mu$ l Qc Mix
  3. PCPS + 1B + Rb — 10 min — + 4.8  $\mu$ l Qc/17 Mix
  4. PCPS + 1B + Rb — 10 min — + 3  $\mu$ l sec17 and 1.8  $\mu$ l Qc

AO25-13 C 4-14-25  
D 4-22-25  
E 4-22-25

1. PCPS + 1B + 17 — 10 min — + 4.8  $\mu$ l Pb

2. PCPS + 1B + 17 — 10 min — + 4.8  $\mu$ l Qc Mix

3. PCPS + 1B + Rb — 10 min — + 4.8  $\mu$ l Qc/17 Mix

4. PCPS + 1B + Rb — 10 min — + 3  $\mu$ l sec17 and 1.8  $\mu$ l Qc

	hx	
Rb150	9.8	4.6x
10% BSA in Rb	0.6	45.08
30mM MgCl <sub>2</sub>	1.0	2.76
50mM ATP BS	0.6	4.6
20-16 RPS lipos naked	0.6	2.76
11.3 $\mu$ M S18	7.5	35.0
P. 469 box 293	2.7	12.42

Prepara 4.6 x Mix w/ Liposomes + Sec18 + put 22.2  $\mu$ l into tubes of PCR strip.

Prepara "Qc Mix" and Qc/17 Mix

Qc Mix	Qc/17 Mix
6 $\mu$ l Rb	6 $\mu$ l 10 $\mu$ M sec17
3.6 $\mu$ l 16.2 $\mu$ M Qc	3.6 $\mu$ l 16.2 $\mu$ M Qc

Add 3  $\mu$ l 10  $\mu$ M sec17 stock to tubes 1 + 2

Add 3  $\mu$ l Rb150 to tubes 3 + 4.

Inc. all tubes (including Mixes + stocks) 27°C 10'

To tube 1: add 4.8  $\mu$ l Rb150

tube 2: " " Qc Mix

tube 3: " " Qc/17 Mix

Tube 4: add 3  $\mu$ l sec17 stock and 1.8  $\mu$ l Qc stock

PREP 2

5  $\mu$ l S17 + 65  $\mu$ l Rb = 10  $\mu$ M sec17 stock

10  $\mu$ l Qc + 10  $\mu$ l Rb150 = 16.2  $\mu$ M Qc stock

27°C

- Incubate 30ul reaction at 30°C for 60 minutes.
  - Add 90ul 54% histodenz in iso-osmolar Rb150+Mg. Vortex gently.
  - Transfer 80ul to Beckman 7x20mm ultracent tubes (343775). Keep remaining starting samples on ice.
  - Overlay with 80ul 35% histodenz in iso-osmolar Rb150+Mg
  - Overlay with 80ul 30% histodenz in iso-osmolar Rb150+Mg
  - Overlay with 50ul Rb150+Mg
  - Spin in TLS-55 rotor, 55K rpm, 4°C, 30 min
  - Harvest 80ul at 0-30% interface.
  - Add 2ul 5% Thesit to each harvested sample and 1ul 5% Thesit to each starting sample
  - Bath sonicate 5min, nutate for 30min at RT (repeat if necessary).
  - Determine % lipid recovered by assaying fluorescence of 10ul of each starting sample vs each harvested sample. NBD (ex 460 em 538 co 515) or Rh (Ex560 Em580 CO 570)
  - Add 7.5ul 5x sample buffer + BME to the 30ul remaining starting samples
  - Add 17.5ul of 5x sample buffer + BME to the 70ul remaining harvested samples
  - Boil samples in sample buffer at 95°C for 5 min.
- Rb150+Mg: 20mM HEPES-NaOH pH 7.4, 150mM NaCl, 10% glycerol, 1mM MgCl<sub>2</sub>. Use 2% glycerol to make iso-osmolar Histodenz.



	start	harvested	% recovered	if 30ul is 100%	if 10 ul is 100%	if 20 ul is 100%
Rep D. 1	2288	1782	77.88	38.52	12.84	25.68
2	2455	1848	75.27	39.85	13.28	26.57
3	2387	1725	72.27	41.51	13.84	27.68
4	2460	1837	74.67	40.17	13.39	26.78
Rep E 1	2267	1745	76.97	38.97	12.99	25.98
2	2307	1754	76.03	39.46	13.15	26.31
3	2345	1838	78.38	38.28	12.76	25.52
4	2227	1758	78.94	38.00	12.67	25.34

His probe

QC probe

Sec17 probe

25-13 Diarr E

Run 2 gels like this.  
One for Rep D and one for Rep E

Gels 1 + 2  
Lane 1 start dil 1:10 30µl  
2 1:20  
3 1:40  
4 1:80  
5 1:160  
6 1:320  
7 sample (1)  
8 (2)  
9 (3)  
10 (4)  
as if 30µl = 100%.

(α His)

Diarr D  
Diarr E

Gel 3  
Lane 1 start dil 1:80 Rep D  
2 1:160  
3 1:320  
4 sample (1) Rep D  
5 (2)  
6 (3)  
7 (4)  
8 MW marker  
9 start dil 1:80 Rep E  
10 1:160  
11 1:320  
12 sample (1) Rep E  
13 (2)  
14 (3)  
15 (4)  
as if 10µl = 100%.

(α QC)

Diarr D  
Diarr E

Gel 4  
Lane 1 start dil 1:40 Rep D  
2 1:80  
3 1:160  
4 sample (1)  
5 (2)  
6 (3)  
7 (4)  
8 MW marker (blue)  
9 start dil 1:40 Rep E  
10 1:80  
11 1:160  
12 sample (1)  
13 (2)  
14 (3)  
15 (4)  
α Sec17

Gel 4

Lane 1 sample (1) Rep D  
2 (2)  
3 (3)  
4 (4)  
5 MW  
6 sample (1) Rep E  
7 (2)  
8 (3)  
9 (4)  
10 MW  
as if 20µl = 100%.

α Sec17

THG-His α : 7-24-24  
α S17 3-19-25  
α Sam 7 11-5-22

Diarr D  
Diarr E

0/N  
4°C

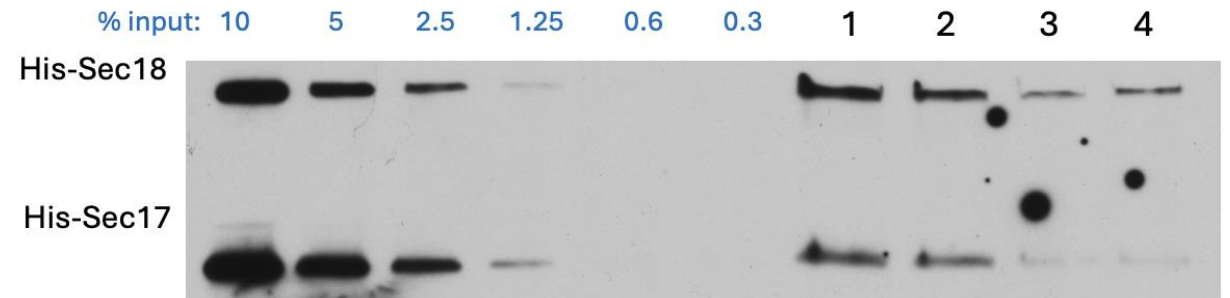
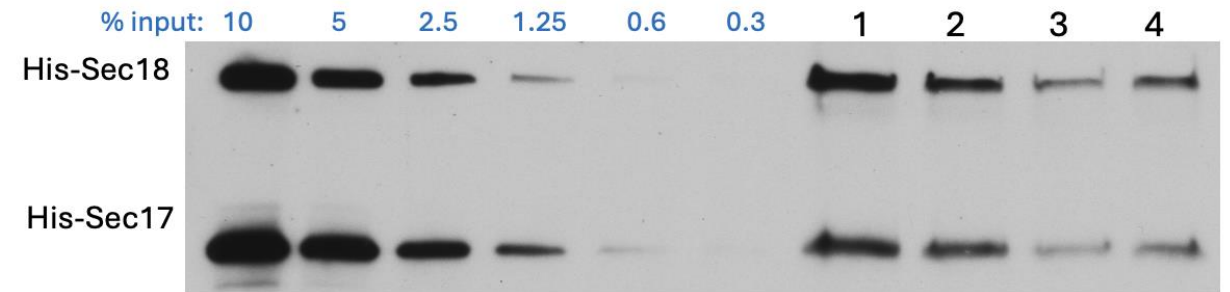
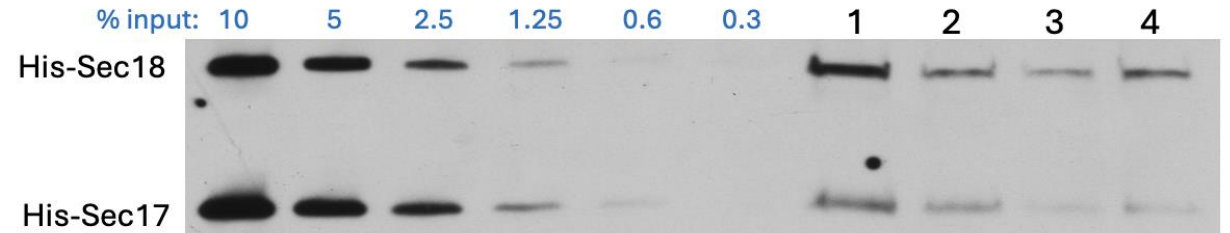
20  
α mouse!  
α rabbit  
α rabbit  
1 hr  
RT

AO25-13

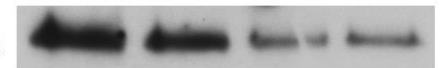
Reps C, D, and E

22.2  $\mu$ l 3  $\mu$ l 4.8  $\mu$ l = 30  $\mu$ l  
1. PCPS+18+17 — 10 min — + 4.8  $\mu$ l Pb  
2. PCPS+18+17 — 10 min — + 4.8  $\mu$ l Qc Mix  
3. PCPS+18+Pb — 10 min — + 4.8  $\mu$ l Qc/17 Mix  
4. PCPS+18+Pb — 10 min — + 3  $\mu$ l sec17 and 1.8  $\mu$ l Qc

1  $\mu$ M Sec18 (hex)  
= 160 nM Sec18  
1  $\mu$ M Sec17  
1  $\mu$ M Qc

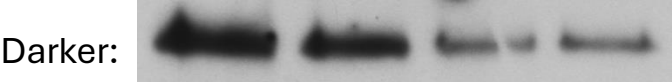
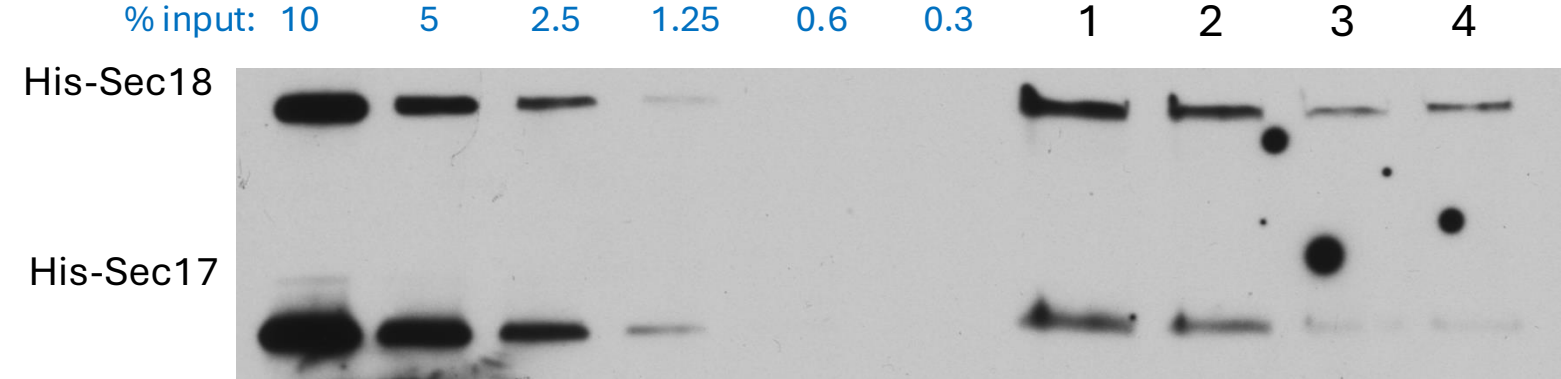
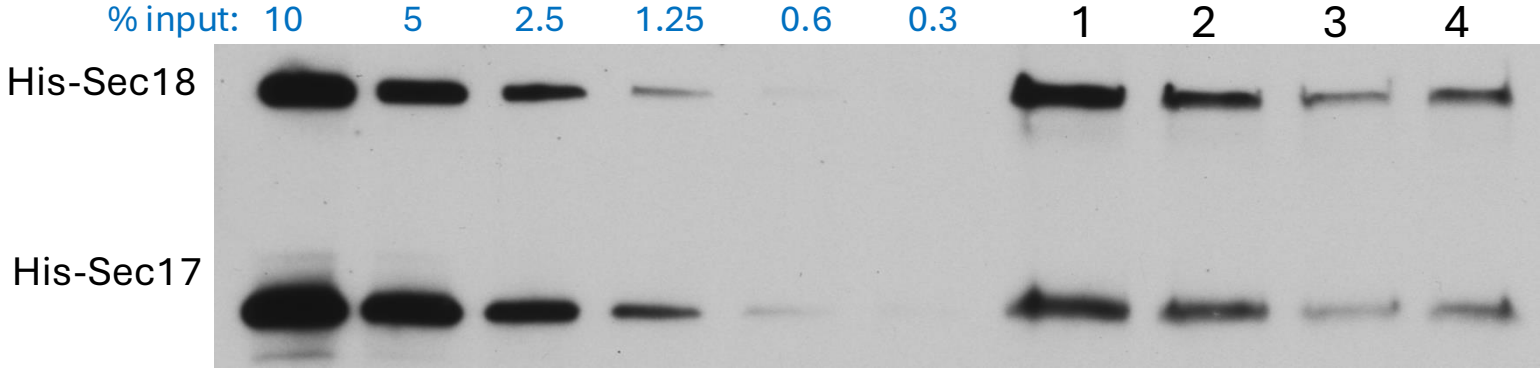
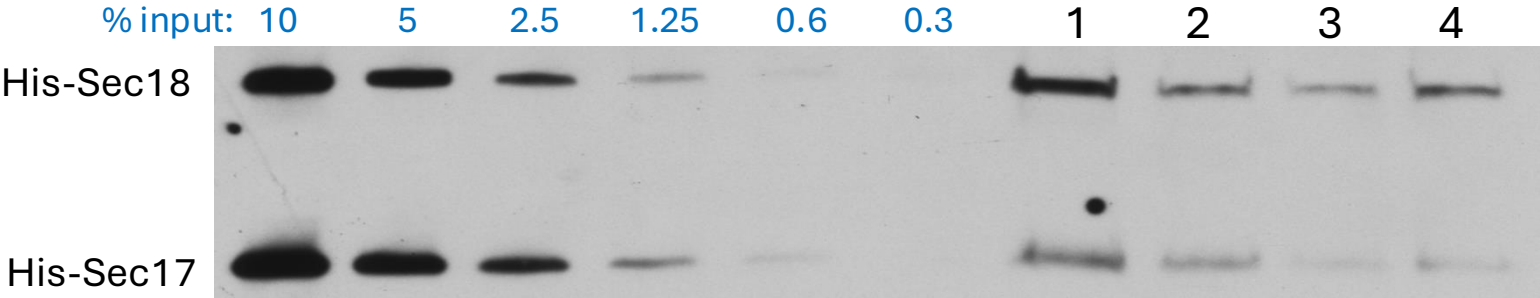


Darker:



AO25-13  
Reps C, D, and E

1uM Sec18 (hex)  
= 160 nM Sec18  
1 uM Sec17  
1 uM Qc



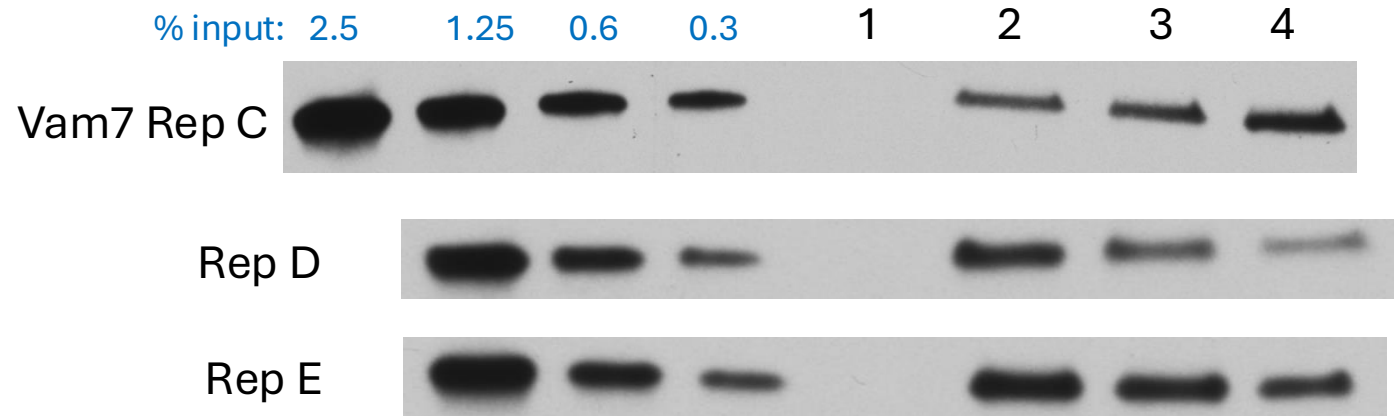


AO25-13

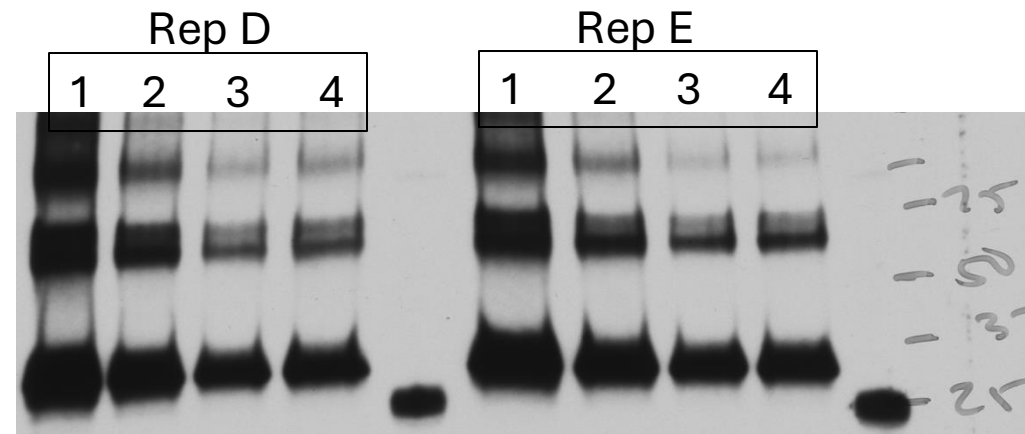
Reps C, D, and E

1  $\mu$ M Sec18 (hex)  
= 160 nM Sec18  
1  $\mu$ M Sec17  
1  $\mu$ M Qc

1: no Qc  
2: +Qc  
3: 17+Qc preinc together  
4: 17+Qc preinc separate



The Vam7 blots are less consistent. Is lane 4 more? Less? The same?



4-22am 4-23-25 25-15B

1	VML + Rh + prenyl Ypt7				
Protein	water		GST-Ypt7 (wt)	His-TEV	
Pro:Up Ratio			2	4	
SNARE/prep/box			DD040213/31	468/292	
conc (uM)			16.6	117	
volume	470.97		120.48	8.55	600.00
2	PC + Rh + prenyl Ypt7				
Protein	water		GST-Ypt7 (wt)	His-TEV	
Pro:Up Ratio			2	4	
SNARE/prep/box			DD040213/31	468/292	
conc (uM)			16.6	117	
volume	470.97		120.48	8.55	600.00

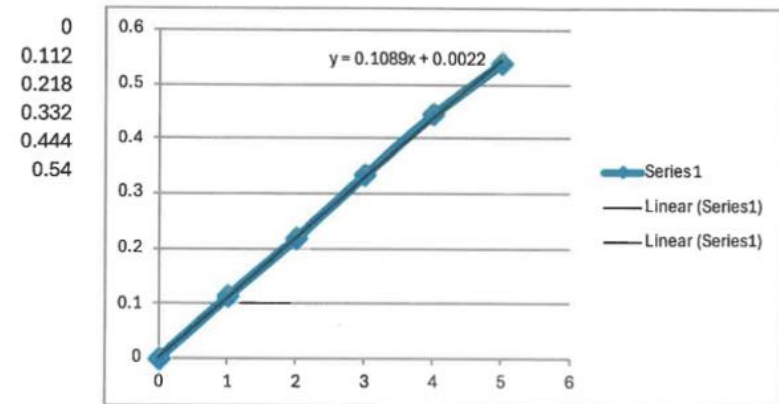
1:2K protein to lipid ratio in 1ml with 4mM lipid is 2 uM  
 1:4K protein to lipid ratio in 1ml with 4mM lipid is 1 uM  
 1:8K protein to lipid ratio in 1ml with 4mM lipid is 500 nM  
 1:16K protein to lipid ratio in 1ml with 4mM lipid is 250 nM  
 1:32K protein to lipid ratio in 1 ml with 4mM lipid is 125 nM

Lipid films:  
 PC+Rh (A023-10)  
 VML+Rh (A024-03)

0  
1  
2  
3  
4  
5

Details of VML+Rh liposomes  
 With prenylated Ypt7 for floatations.

25-15B



	lipids	SNARES all 1:2K	Ypt7	Ab 820	mM	vol (ul)	vol to = 2mM	Rb150+Mg vol to add
1	VML+Rh	none	Ypt7 prenyl 1:2k	0.443	4.04866603	585	1184.2	599.2
2	PC+Rh	none	Ypt7 prenyl 1:2k	0.456	4.16802961	585	1219.1	634.1

148 yellow tubes  
 152 blue tubes

25 uL or 33 uL  
 as written on  
 tube

Float sec18 on VML lipos w/ various proteins:

1. Naked : A025-03
2. Ypt7:GTP : A025-15B
3. Ppm1Ypt7:GDP : A025-15B
4. Sec17-tm A025-03
5. R A024-03
6. Qc-tm
7. 2Q
8. 4SN (Qc wt.)

Prep [Rb, EDTA ± GTP/GDP Mixes]  
Put 6.7 µl into 8 tubes  
Add 5 µl RPLS  
Inc. 10' 27°C  
Add MgCl<sub>2</sub> (2 µl)  
Add BSA/ATPES/sec18 Mix 6.3 µl.

1x  
Rb150 : 3.7  
20mM EDTA in Rb : 1.0  
± 1mM GTP/GDP b234 : 2.0/6.7  
2mM RPLS : 5.0  
30mM MgCl<sub>2</sub> 1/2 Rb : 2.0  
0.5% BSA in Rb : 1.6  
10mM ATPES bup 148 : 2.0  
7.4 µM sec18 : 2.7/6.3  
P 514 bup 329  
20.0

A025-18  
4-24-25

make 1:  
10x  
no GTP  
Rb: 57 µl  
GTA 10 µl

make 2:  
4x  
GDP or GTP  
Rb 14.8 µl  
GTA 4.0  
GXP 8.0

make 1:  
10x  
16 µl BSA  
20 µl ATPES  
27 µl sec18

25-18 4-24-25

				load
	start	harvested	% recovered	if 9ul is 100%
1	2917	2305	79.02	11.39
2	2862	2071	72.36	12.44
3	2946	2087	70.84	12.70
4	3087	1945	63.01	14.28
5	3048	2081	68.27	13.18
6	3283	2009	61.19	14.71
7	3066	2032	66.28	13.58
8	2962	1978	66.78	13.48

Prep 1:2 dilution series of start (8) in 1x SB+Bme

Spl = 45 µl, then 15+15

Load BioRad 4-15% gradient gels

Lane 1 start dil 1:10 = 9 µl = 10%  
Lane 2 1:20  
Lane 3 1:40  
Lane 4 1:80  
Lane 5 1:160  
Lane 6 1:320  
Lane 7 ①  
Lane 8 ②  
Lane 9 ③  
Lane 10 ④  
Lane 11 ⑤  
Lane 12 ⑥  
Lane 13 ⑦  
Lane 14 ⑧  
Lane 15 Hw marker

2 sec18 c/n 4°C

2-4-25

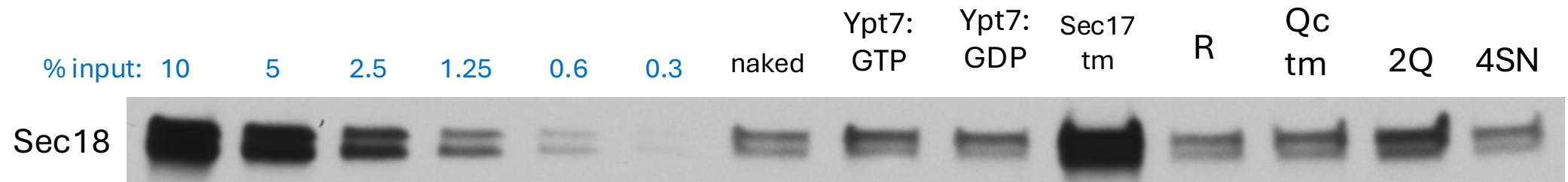
2° rabbit 1hr RT

Run gel(s) 200V for 35 minutes  
Transfer 250mAmps for 1hr 15 min.  
Milk block 30+ minutes  
Primary antibody:

- Incubate 20ul reaction at 30°C for 60 minutes.
- Add 60ul 54% histodenz in iso-osmolar Rb150+Mg. Vortex gently.
- Transfer 60ul to Beckman 7x20mm ultracent tubes (343775). Keep remaining starting samples on ice.
- Overlay with 90ul 35% histodenz in iso-osmolar Rb150+Mg
- Overlay with 90ul 30% histodenz in iso-osmolar Rb150+Mg
- Overlay with 50ul Rb150+Mg
- Spin in TLS-55 rotor, 55K rpm, 4°C, 30 min
- Harvest 60ul at 0-30% interface.
- Add 2ul 5% Thesit to each 60ul harvested sample and 0.67ul 5% Thesit to each 20ul remaining starting sample
- Bath sonicate 5 min (opt), then nutate for 30min at RT (repeat if necessary).
- Determine % lipid recovered by assaying fluorescence of 10 ul of each starting sample vs each harvested sample. NBD (ex 460 em 538 co 515) or Rh (Ex560 Em580 CO 570).
- Return those 10ul to the tubes they came from.
- Add 5ul 5x sample buffer +BMe to the 20ul remaining starting samples
- Add 15ul of 5x sample buffer +BMe to the 60ul remaining harvested samples
- Boil samples in sample buffer at 95°C for 5 min.

Rb150+Mg: 20mM HEPES-NaOH pH 7.4, 150mM NaCl, 10% glycerol, 1mM MgCl<sub>2</sub>.  
Use 2% glycerol to make iso-osmolar Histodenz.

AO25-18: Float Sec18 (Mg:ATPyS) on VML+Rh liposomes with various proteins.



With VML lipids, Sec17 is the only protein that causes Sec18 to float over background. I saw this also in AO25-04.

But I have seen Sec18 clearly binding to Ypt7 over background levels when lipids are PC only. See AO25-11B (next slide) .

AO25-11B: Does nucleotide state of Ypt7 affect Sec18's ability to bind?

Answer: doesn't look like it!

Float 1uM Sec18 on PC liposomes +/- Ypt7-tm exchanged with GDP, GTP, or GTPyS



(These were Ypt7-tm RPLs! NOT prenylated Ypt7)



On the docket:

Strept-tag stuff: How can I lower background binding of wild-type Qa and Qb?

Try higher NaCl... try 300mM and 500mM NaCl.

Try using RIPA Buffer just to see if it reduces the background, which would give us some hope.

Floatations: repeat Sec18 binding to VML? Try the same floatation assay with PC? Let's pause and think about this one.

: And what about the His-Sec17/Sec18 +/- Qc experiments? Let's regroup on what we want to show here.

I think it's that allowing Sec17 and Sec18 to incubate together without Qc means more 17:18 binding.

So then how about a time course of 17:18 together before adding Qc?

And what's up with Sec17 oligomers?? Are they a real thing? They don't show up with the anti-His blots, only the Sec17 probes. Let's run a colloidal Coomassie and a western and compare.

Repeat Michael's asymmetry experiments +/- Sec18 using my new R and 2Q (+/- prenyl Ypt7 GTP/GDP).

Try R + 3Q (+/- prenyl Ypt7 GDP/GTP) with HOPS vs GST-C1B as tether.

Try Hongki's fusion burst experiment again followed by IP looking for intermediate trans-complex without Qc