Lab Meeting, May 5th, 2025:

Strept-tag stuff: Try lowering background binding of wild-type Qa and Qb

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

Try adding BSA

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

And also load the gels so the Unbound and Bound are same percentage, reducing confusion.

Floatations:

In experiments probing for Sec17 and Sec18 via the His tag, we continue to look for meaning.
One thing to clear up is the Sec17 oligomers.

Are they a real thing? I ran a colloidal Coomassie and western and probed for Sec17 vs His.
Conclusion, they are a real thing and kind of a pain in our neck. What are they doing there?

Try diff. buffers to reduce nonspecific binding of wt Qa, Qb to Mag strep beads

AO 25-17C
4-29-25

1,2,3	① DM150 + (20 mM Hepes, 150 mM NaCl, 1 mM EDTA, 0.1% DM
456	② DM300 " 300 mM NaCl " "
7,8,9	③ DM500 " 500 mM NaCl " "
10,11,12	④ DM150+BSA as ①, but add .2% BSA
13,14,15	⑤ RIPA (20 mM Hepes, 150 mM NaCl, .2% BSA, 1% Triton, 1% Nacholate, .1% SDS)
wt Qa step / Qb step	

Try with
- 2Q wt and
- 2Q (Qa-TWIN)
- 2Q (Qb-TWIN)

AO 25-17 new ward 5/8

Need ~~8~~ 9 mls of each buffer (6 washes of .5 ml each)

Not going to bother making the 2x versions. Just add 7.5 µl RPLs (in RB150+Mg) to 92.5 µl each buffer

Make 4 mls each buffer:

160	80 µl 1M Hepes 7.4 = 20 mM		
300	150 µl 4M NaCl = 150 mM	+ 3.642 mls water	7.284
600	300 µl 4M NaCl = 300 mM	+ 3.492 mls water	6.984
1000	500 µl 4M NaCl = 500 mM	+ 3.292 mls water	6.584
16	8 µl 500 mM EDTA = 1 mM		
80	40 µl 10% DM = 0.1%		= 4 mls each
160	80 µl 10% BSA = 0.2% (or water; for ① ② ③)		

ugh. needed 9. Made 8 more mls of each by doubling all the volumes I used to make 4 mls

Wash Magstrep beads (35 µl slurry = 1.75 µl beads) 3x w/ .5 ml buffer.
3 tubes for each of the 5 flavors of buffer.
Put 92.5 µl buffer in each tube w/ beads and add 7.5 µl of 2Q (wt) or 2Q (Qa-TWIN) or 2Q (Qb-TWIN).
Nutate 2 hrs (e.o.e.) at 4°C.
Remove + save 100 µl unbound (mix w/ 25 µl 5x SB + Bme, boil 5')
Wash beads 3x w/ appropriate buffer (.5 ml).
Elute in 125 µl 1x SB + Bme. Boil 95°C 2 min.

Now the unbound + bound are same volumes!

Run four, 15 lane gels 15 µl every lane

①

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	UN	B	UN	B	UN	B	UN	B	UN	B	UN	B		MW (dual color)	
	2Q wt ① u B		Qa step ② u B		2Q wt ④		Qa step ⑤		2Q wt ⑦		Qa step ⑧				
	DM150				DM300				DM500						

②

Well	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	UN	B	UN	B	UN	B	UN	B	UN	B	UN	B		MW MW (dual color)	
	wt ① u B		Qa step ② u B		wt ⑩		Qa step ⑪		wt ⑬		Qa step ⑭				
	DM150				DM150+BSA				RIPA						

③ as ① but alternate 2Q wt and 2Q Qb step
① u B ③ u B ④ ⑥ ⑦ ⑨

④ as ② " " " 2Q Qb step
① u B ③ u B ⑩ ⑫ ⑬ ⑮

} use all-blue MW markers

Complicated, but I did it.

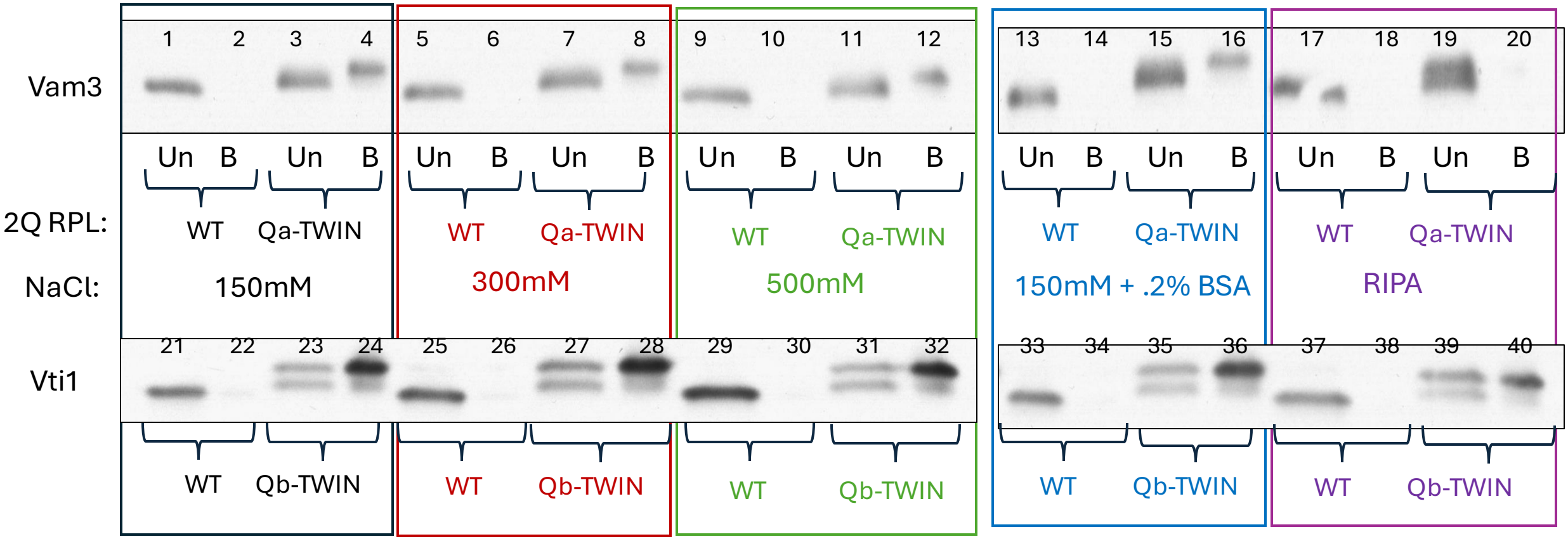
milk block
α Qa 8-26-24
α Qb 3-3-22) O/N 4°C

2° drabbit 1 hr

AO25-17C: Mag-strep pulldowns of 2Q wt vs 2Q with Qa or Qb-TWIN-strep tags. Different salt levels:

General Buffer: 20 mM HEPES-NaOH pH 7.4, varying salt, 1 mM EDTA, 0.1% DM

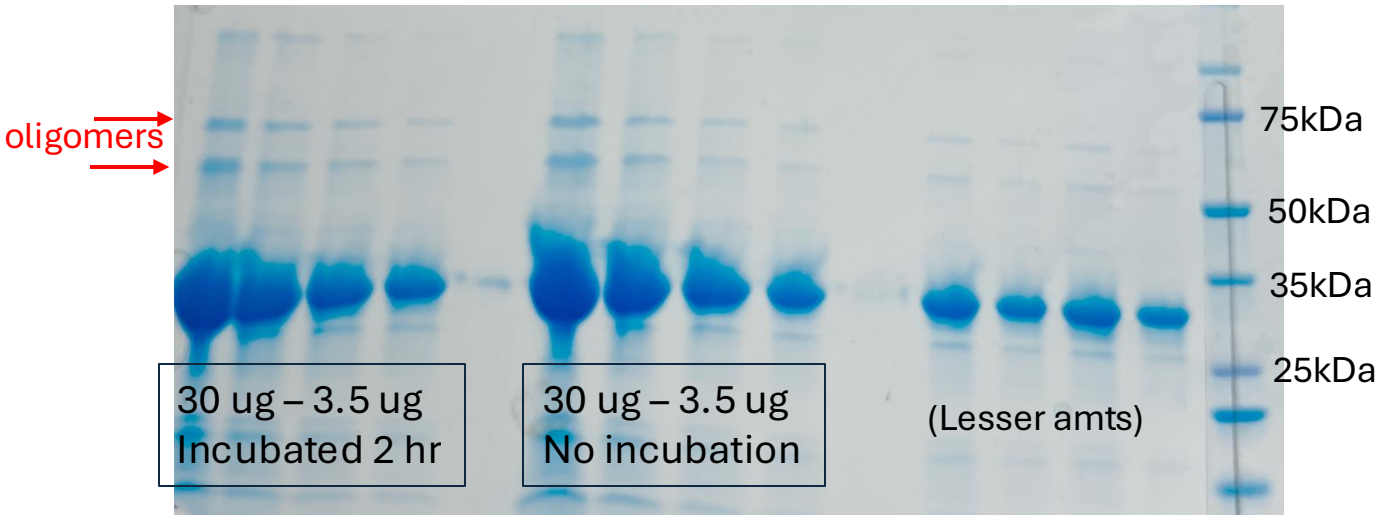
Unbound and Bound represent the same % of the prep so they are directly comparable.

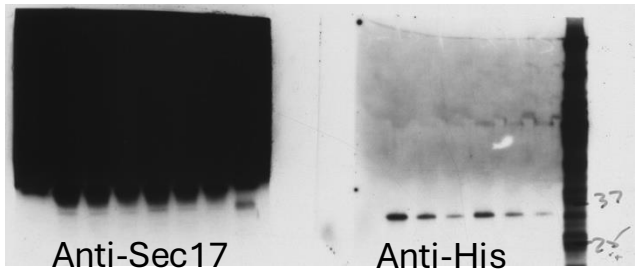


Bill points out that the 2 different MW bands with the TWIN-Strep proteins likely mean that proteases have cleaved off the tags! Let's figure out when that happened and prevent it!

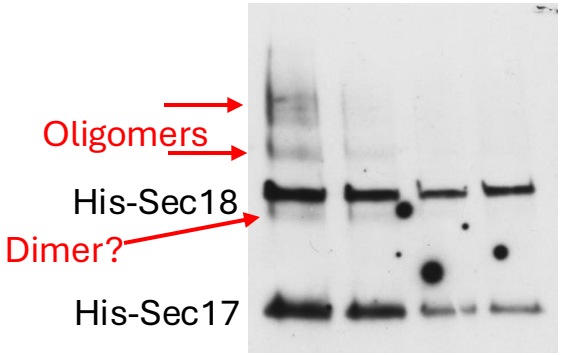
RIPA: 20mM HEPES 7.4, 150mM NaCl, 0.2% BSA, 1% Triton, 1%, NaCholate, 0.1% SDS

AO25-13F. Incubation did not yield more oligomers, but they are there!





The westerns were a bust.
Something in the buffer?
(400 mM Imidazole?)



AO25-13 F 4-29-25

Rerun colloidal coomassie vs westerns (αsec17 vs αHis) of His-Sec17 inc. 27°C for 2 hrs vs fresh from -80

His-Sec17 prep 539 box 354 @ 140 μM / 4.8 mg/ml.

Incubate 20 μl at 27°C for 2 hrs.
Remove a fresh aliquot from freezer.

Add 2x SB+Bme to each sample. Now 2.4 μg/μl. Boil 95°C 5'.
Load a 15 lane gradient gel w/
12 μl of each sample along w/ a 2-fold dilution series representing ~30 μg, 15 μg, 7.5 μg 3.25 μg.
The final dilution is ~3 μg/μl. (8.75 μM)
To that sample, do a 1:100 dilution in 1x SB+Bme.
Now .003 μg/μl. (87.5 nM)
Now do a 1:4 dilution series
.00075 μg/μl = .75 ng/ml (22 nM)

Run another gel with this diluted sample

Lane		
1	12 μl	27°C inc.
2	6 μl	
3	3 μl	
4	12 μl	fresh from -80
5	6 μl	
6	3 μl	
7	MW marker	
8	12 μl	27° inc.
9	6	
10	3	
11	12	fresh from -80
12	6	
13	3	
14	MW marker	
15		

αsec17 probe
αHis 7-24-24) O/N 4°C
αS17 3-19-25)
2° αrabbit for S17 (1:10,000)
αmouse for αHis (1:10,000)
1hr

αHis probe

Hmm... strange blots!
What's in the buffer?
20 mM Hepes KOH 7.0
400 mM Imidazole
10% glycerol

cut
αS17 / αHis

On the docket:

Vam3 pulldown: To complement Bill's phosphatase assay studies which show less ATPase activity with high Sec17:
Is 1uM Sec17, which is shown to inhibit fusion, causing less trans-complex to form?
Run a fusion assay and IP with no Sec17, 0.1uM Sec17, and 1 uM Sec17 +/- Sec18 ATPyS or ATP (9 tubes)
Actually, first, let's look through past experiments to see if I've done anything like this…

Floatations: We are putting a hold on floatation assays for the next paper until we know what we want to show.

Strept-tag stuff: Find out at what stage the Strept-tags are being cleaved from the protein….if it's already half-cleaved straight from the freezer after purification then we need to repurify with protease inhibitors!

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