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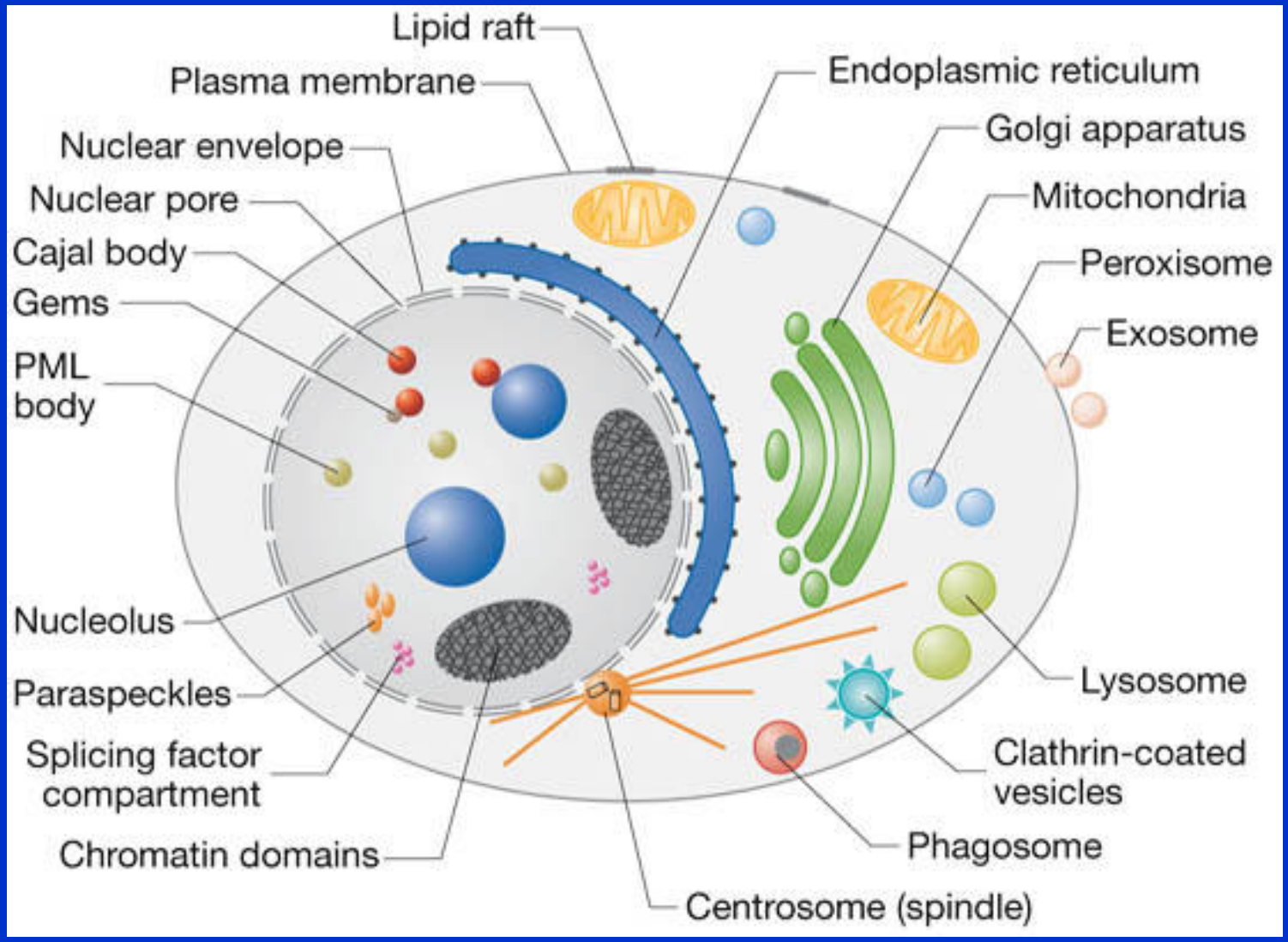
Molecular Architecture And Dynamics Of The Mammalian Secretory Pathway

- insights from the functional organeller proteomics studies

Xuequn Chen, Ph.D.

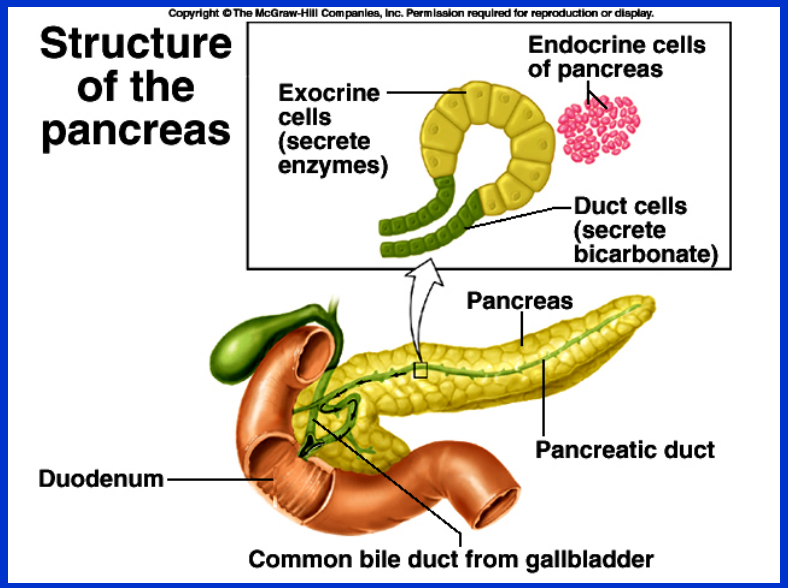
Department of Physiology
School of Medicine
Wayne State University

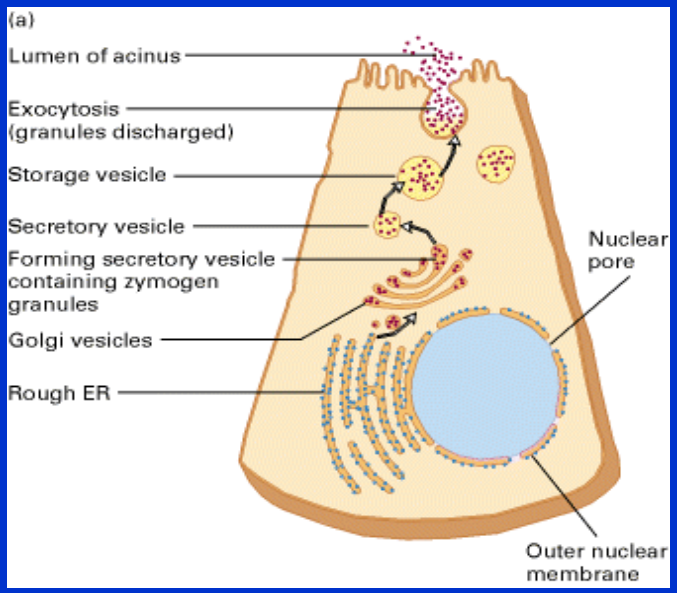
Overview of organellar proteomics



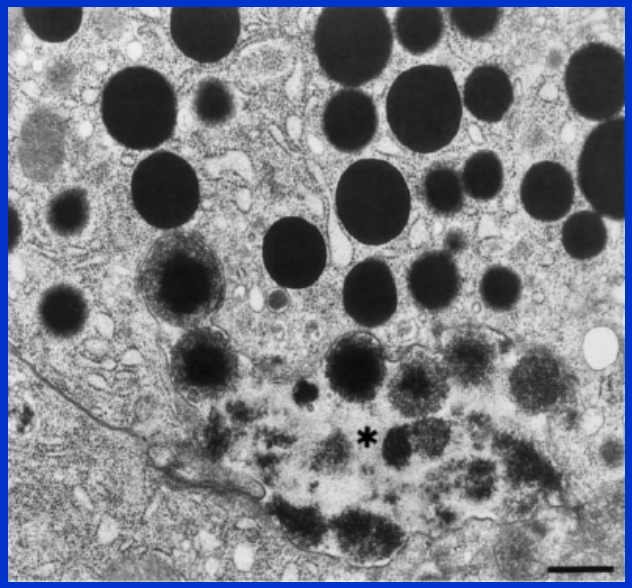
EMBO Rep. 2006: 874-9

Exocrine pancreas, acinar cells and zymogen granules



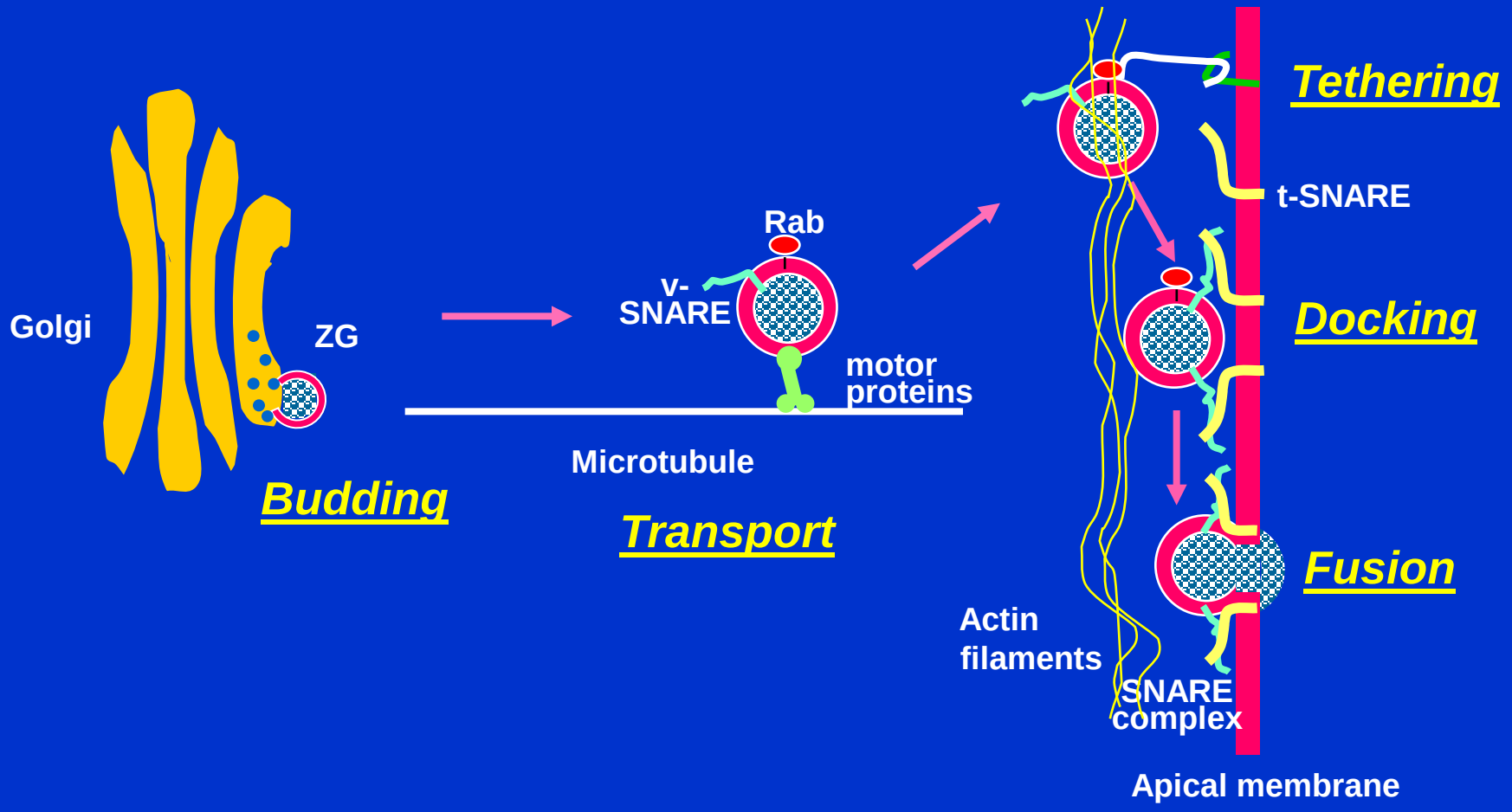


Modified from Lodish et. al. *Molecular Cell Biology*



Jamieson, JD *BBRC* 1999

A working model for ZG biogenesis, trafficking and exocytosis



AIM - ZG proteomics study

Develop a quantitative model of ZGM molecular architecture and dynamics

- Individual components
- The topology, copy numbers of ZGM proteins
- Protein complexes on ZGM
- Dynamic chagnes upon stimulation and in diseases

Purification of ZG membrane

Rat pancreatic homogenates
(~ 1 g)

S2 P2 (~100 mg)

Percoll gradient

highly purified ZGs
(~20 mg)

nigericin

Lysis of ZGs

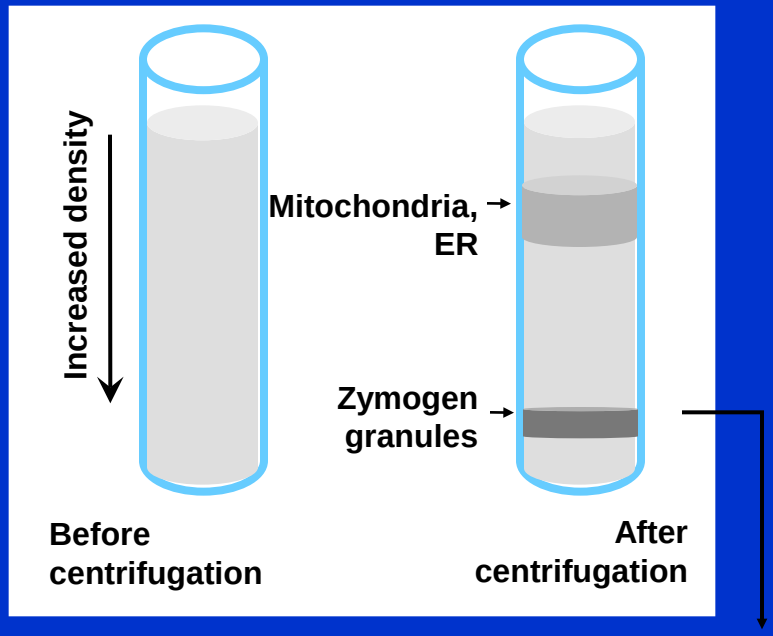
contents membrane
(~1.2 mg)

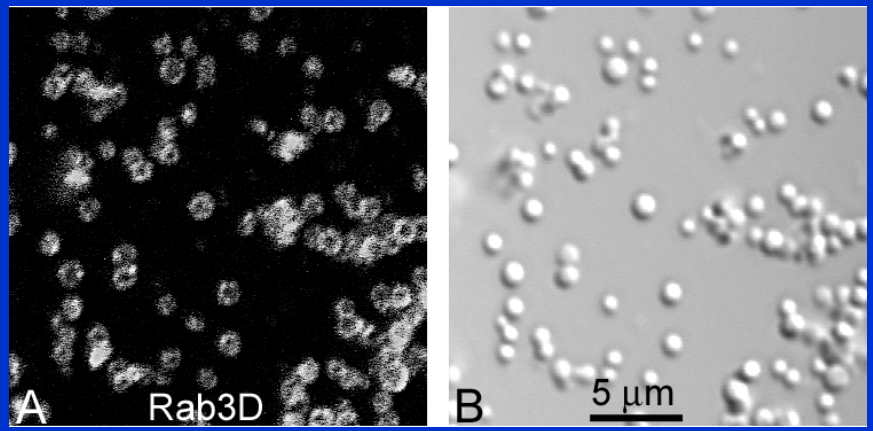
KBr wash

purified membrane

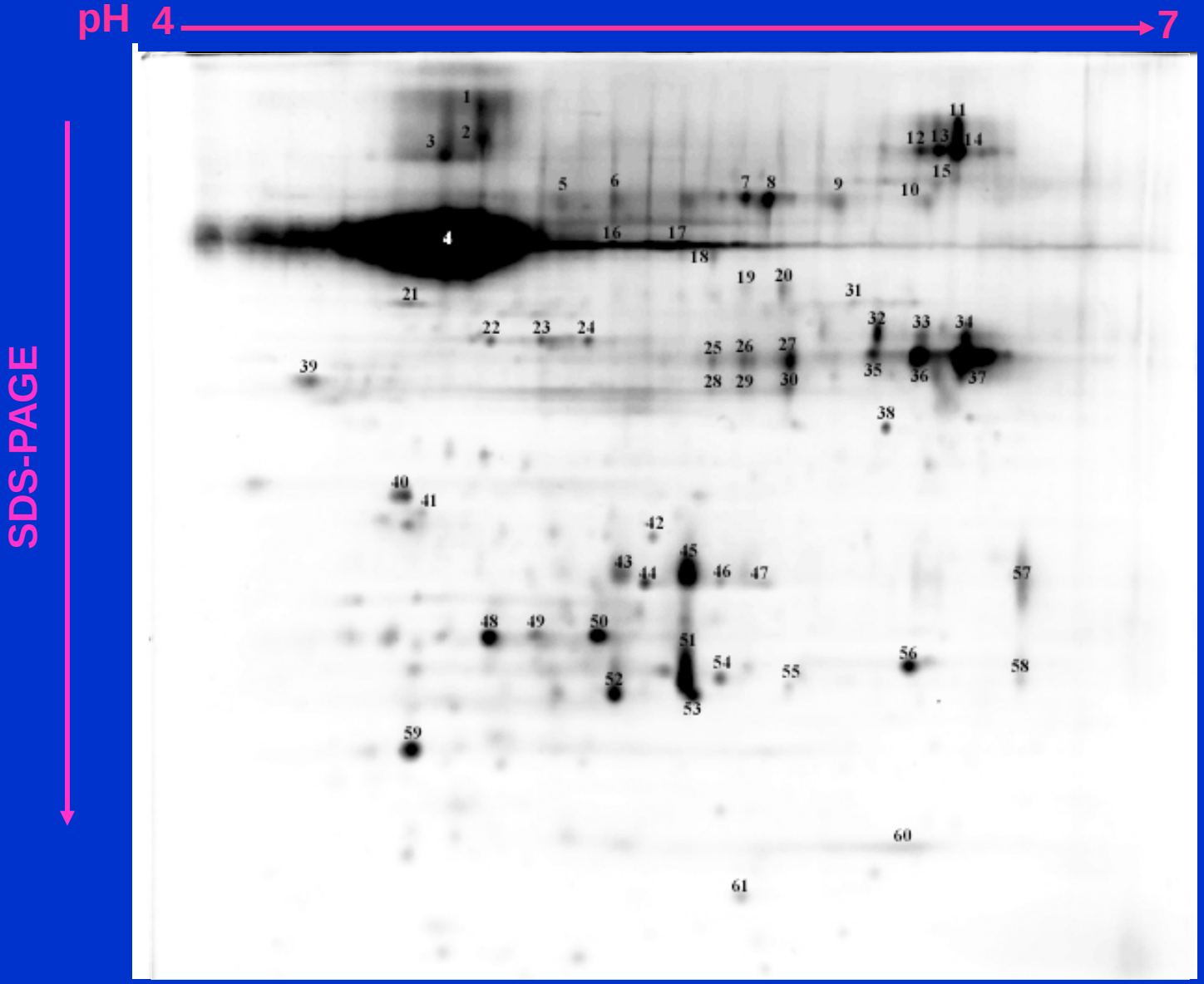
Na_2CO_3 wash

Highly purified membrane
(~0.3 mg)

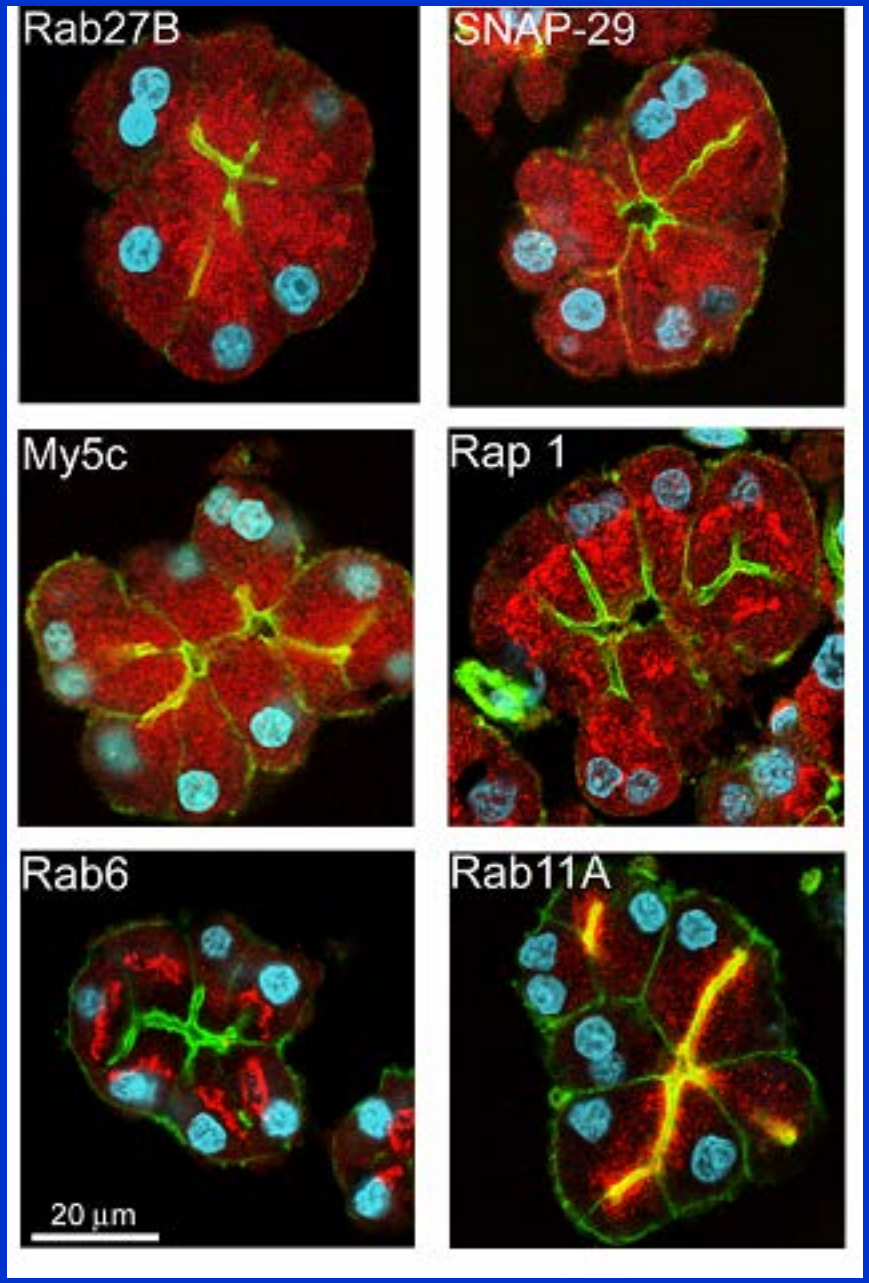




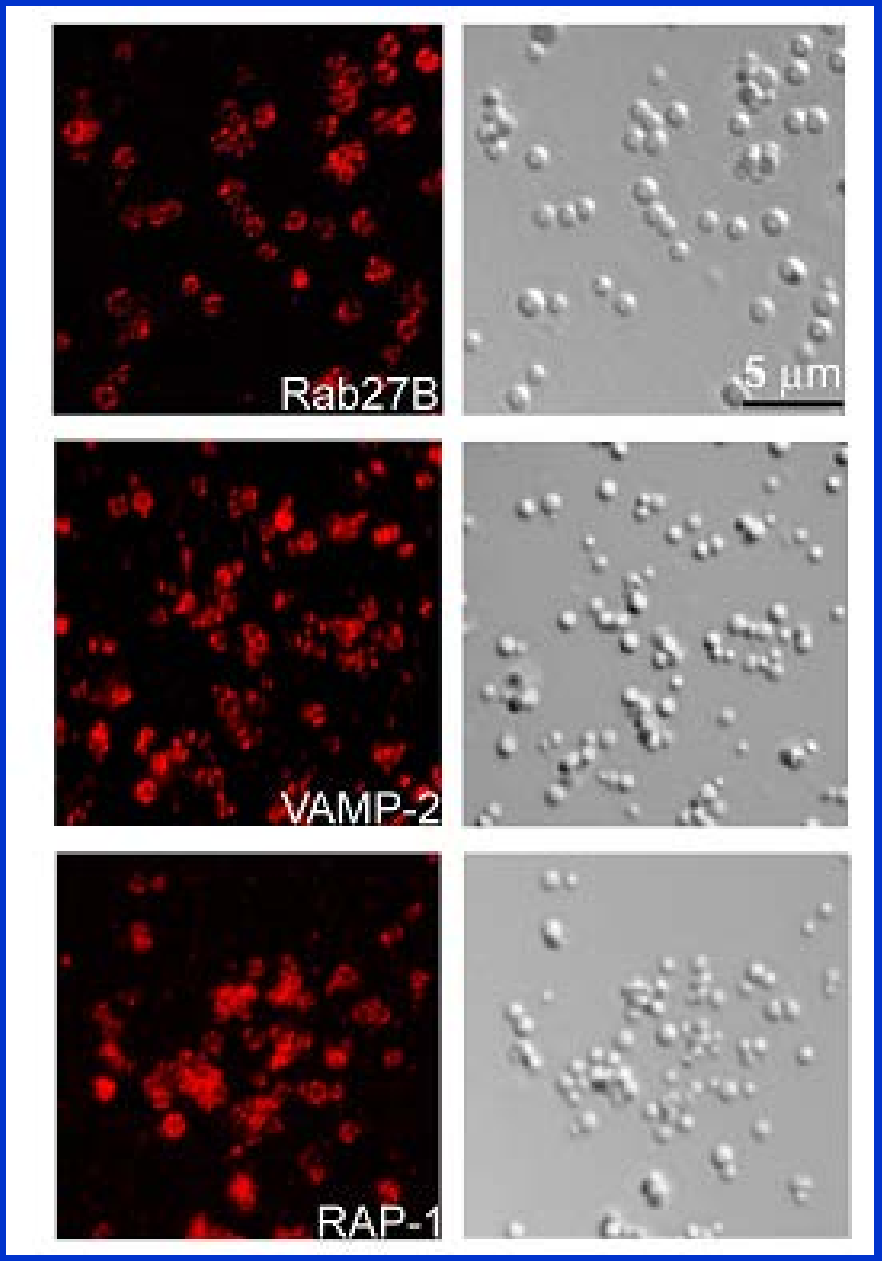
2D gel map of Na_2CO_3-washed ZGM proteins

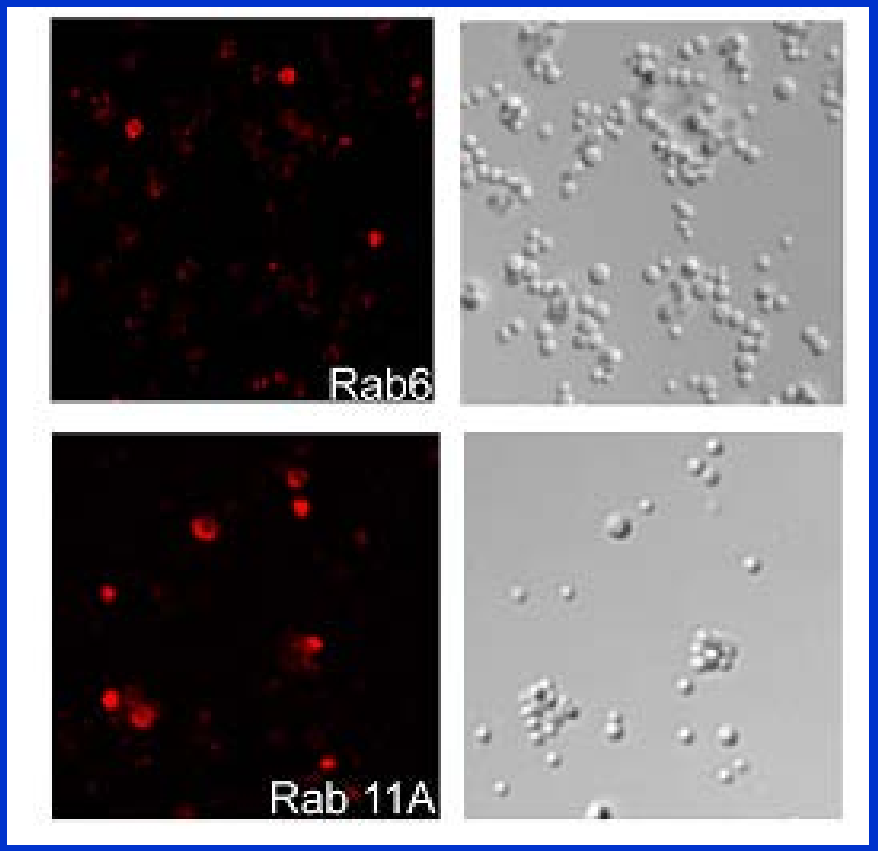


Localization of representative proteins in isolated acini

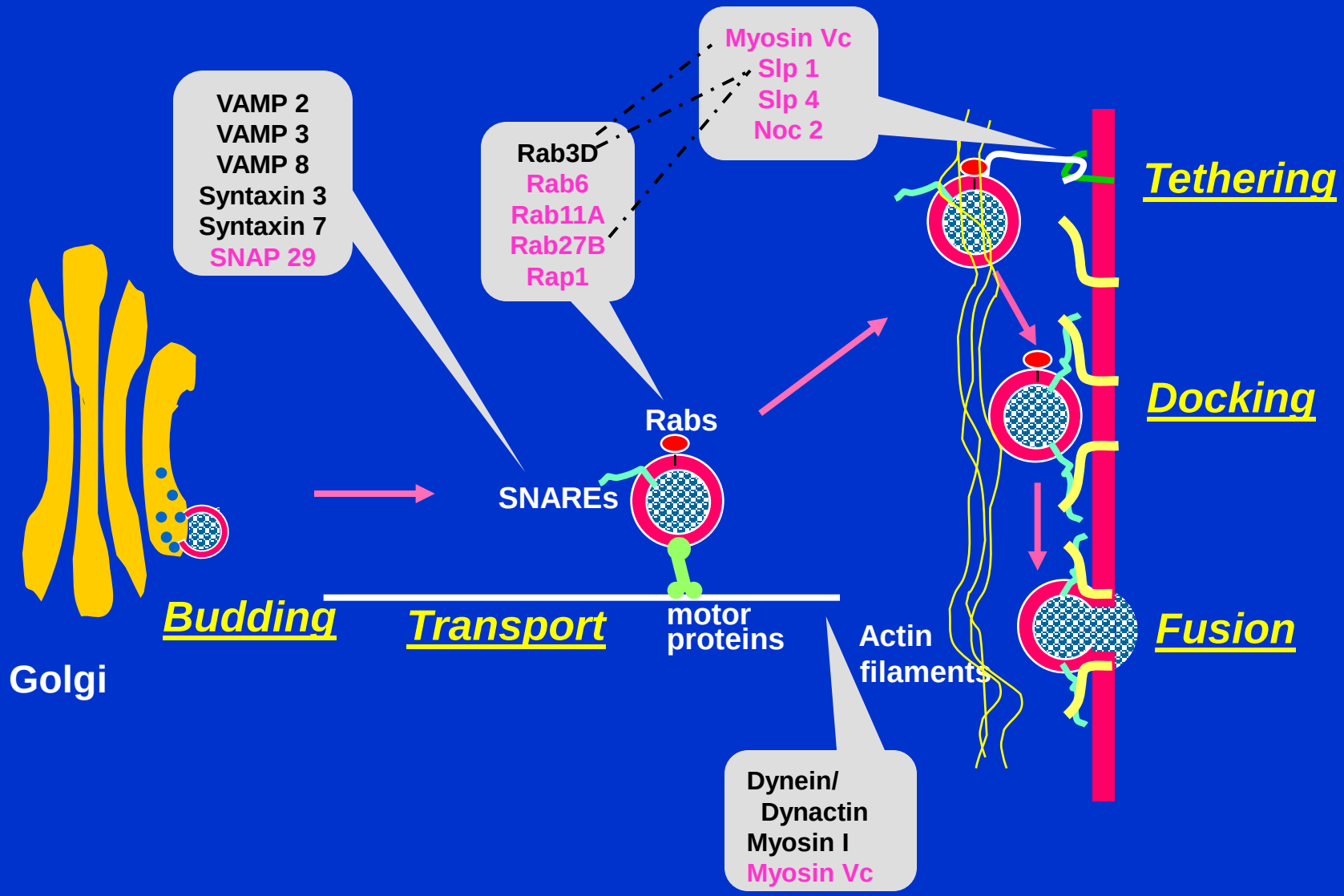


Localization of representative proteins on isolated ZGs

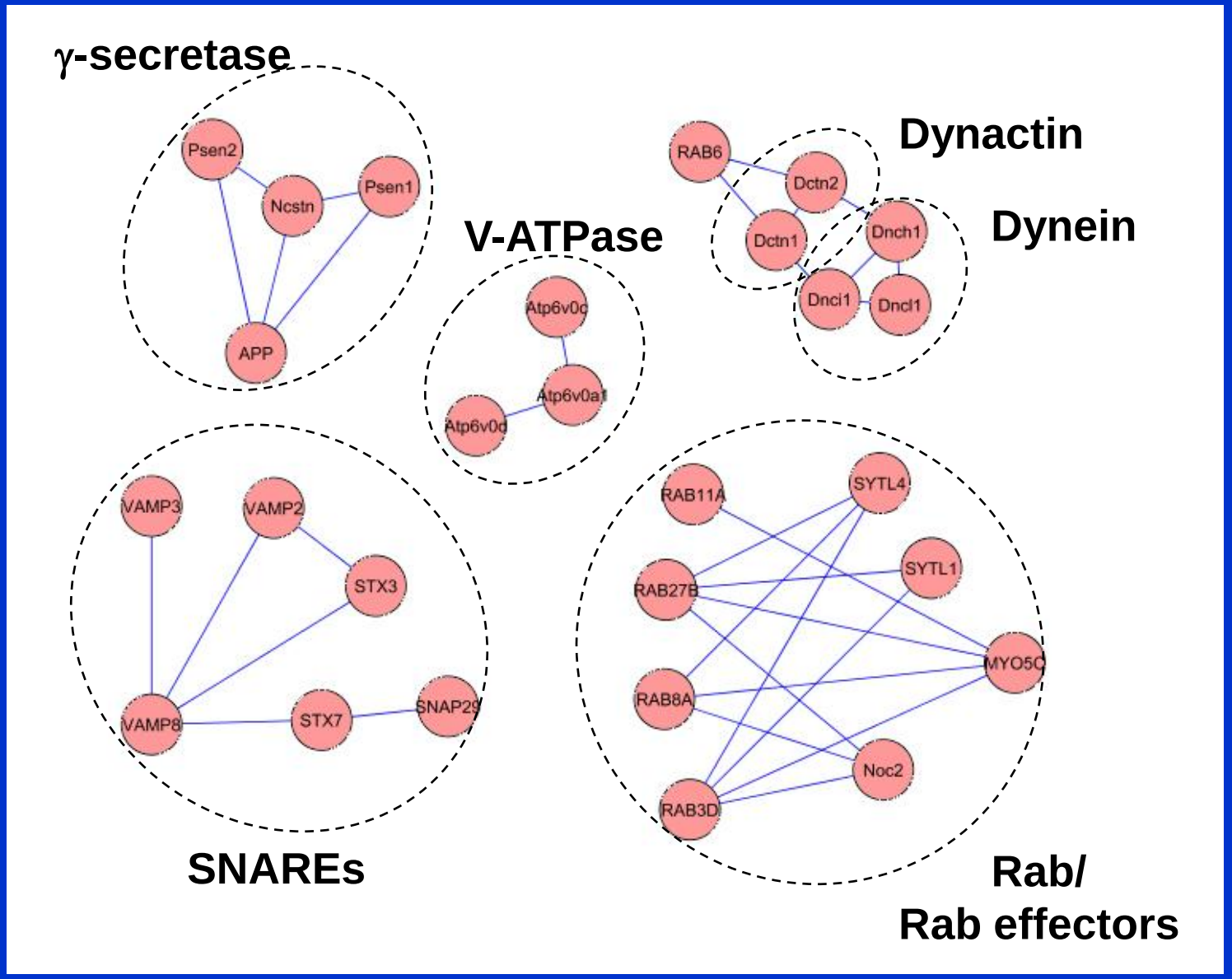




Summary: new insights in ZG proteins and their functional organizations

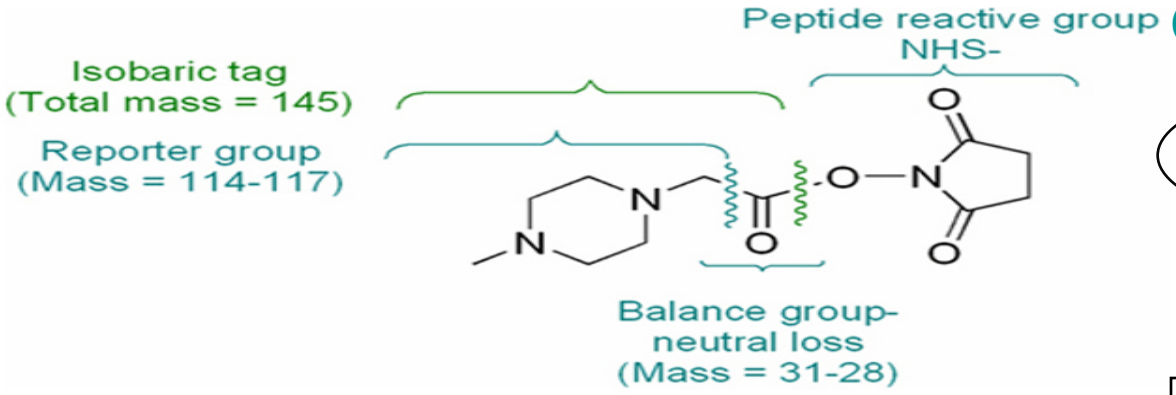


Protein-protein interaction network analysis of identified ZGM proteins

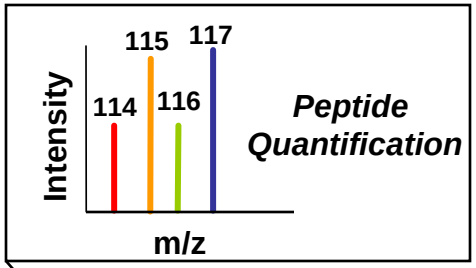


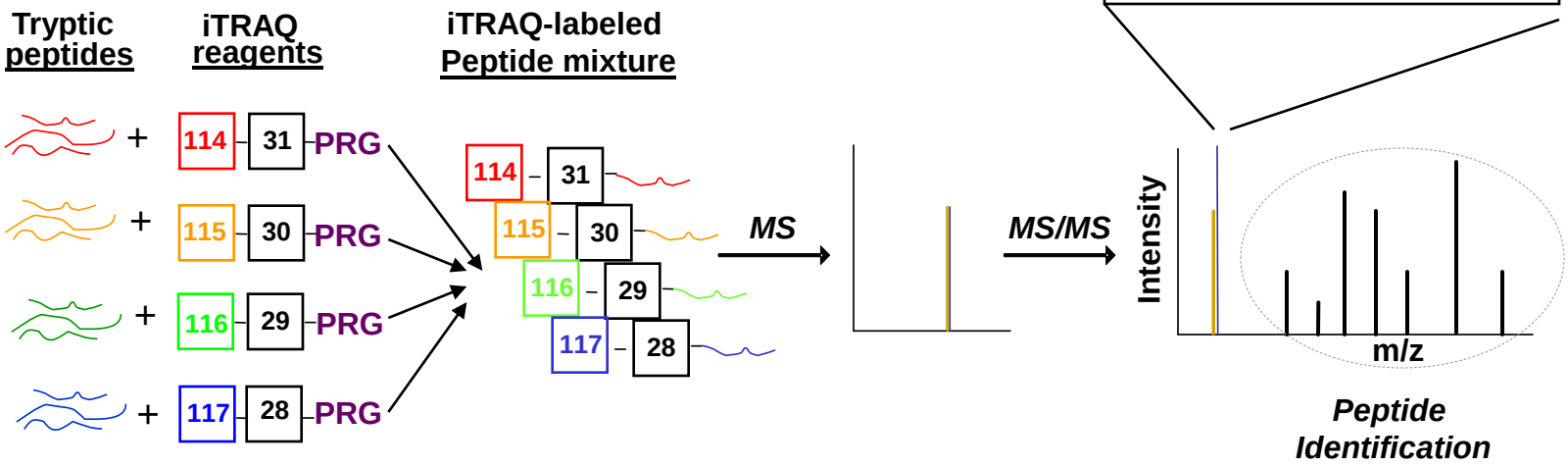
Global topology analysis of ZG membrane

iTRAQ: reagent design and workflow

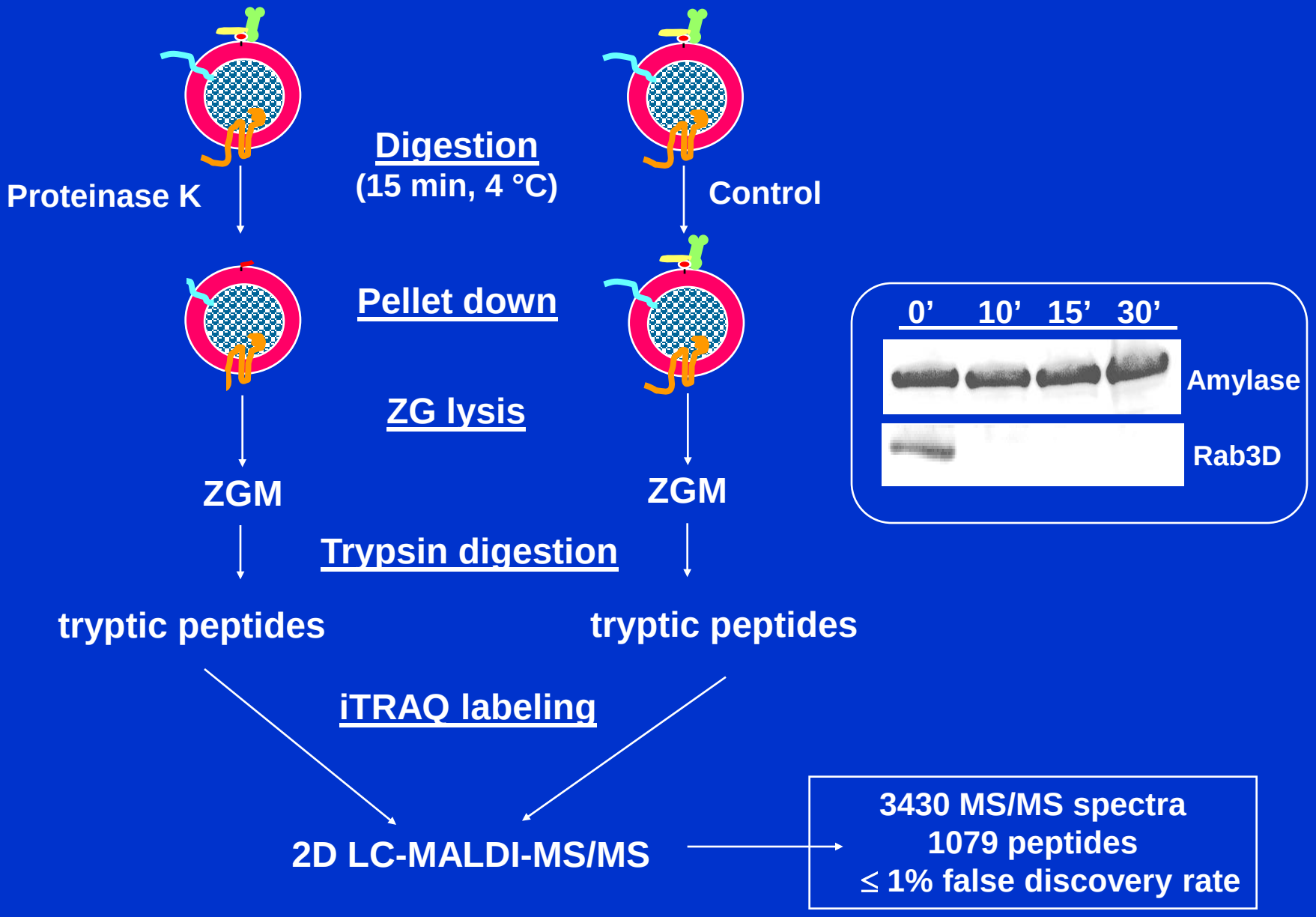


Isobaric tags for relative and absolute quantification

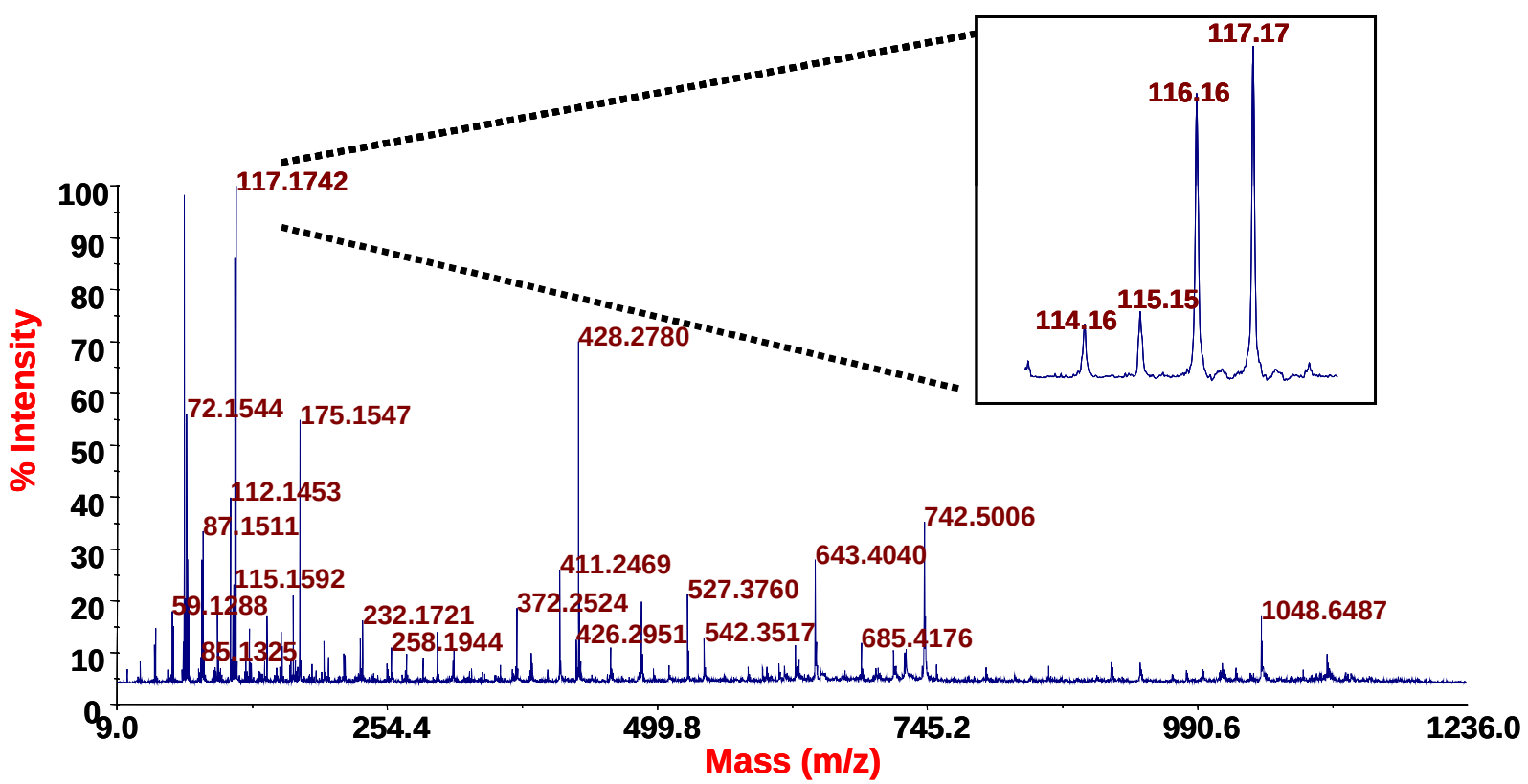




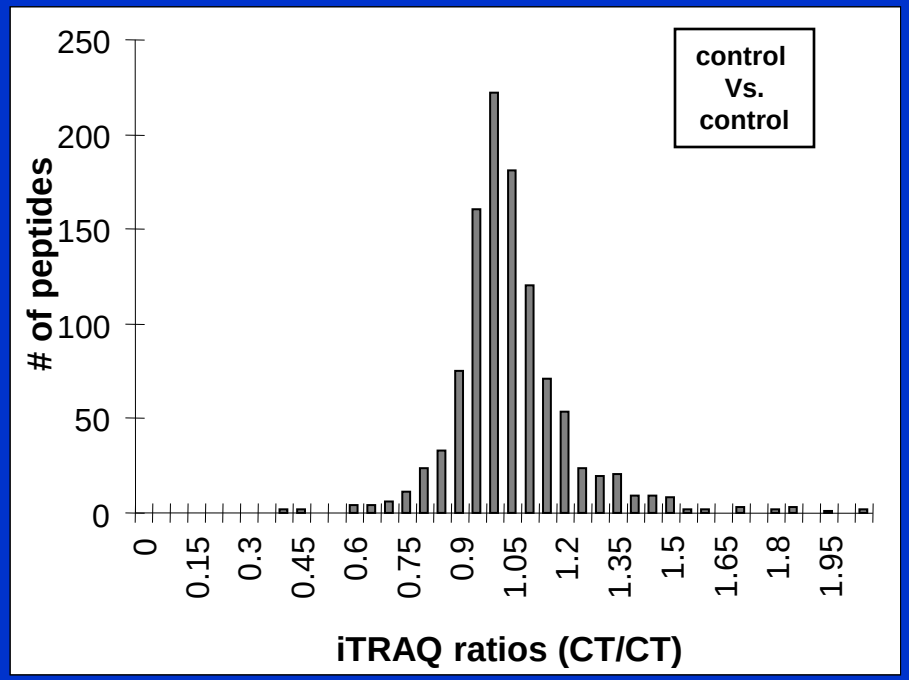
iTRAQ-based ZGM topology analysis

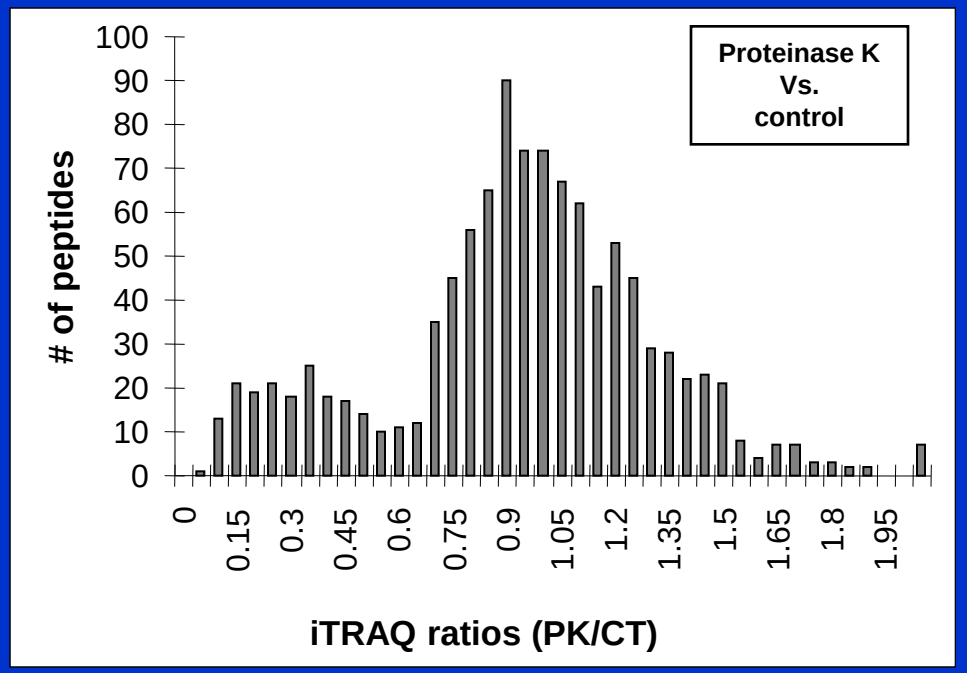


An example MSMS spectrum of iTRAQ-labeled peptide

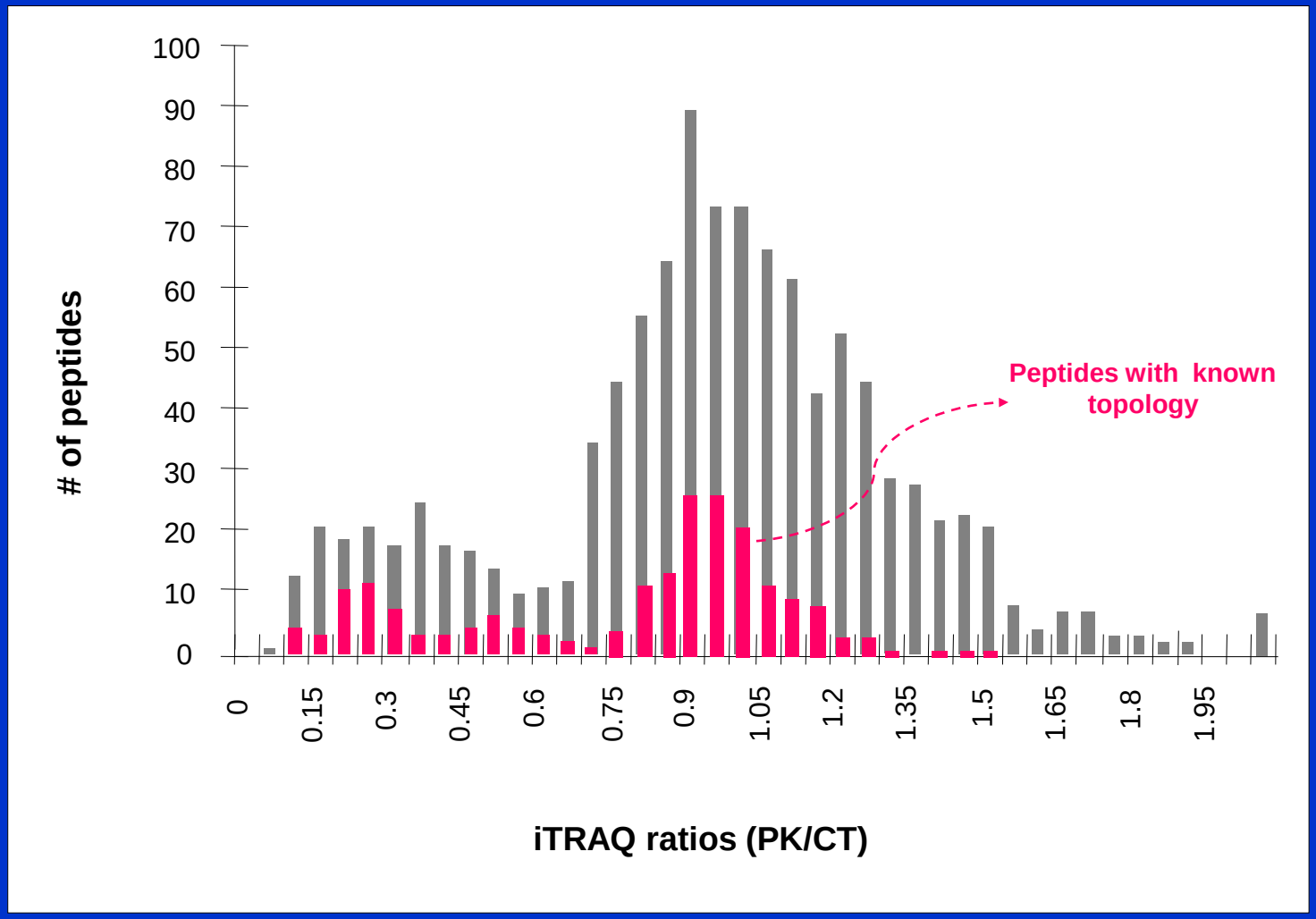


Peptide distributions on different iTRAQ ratios





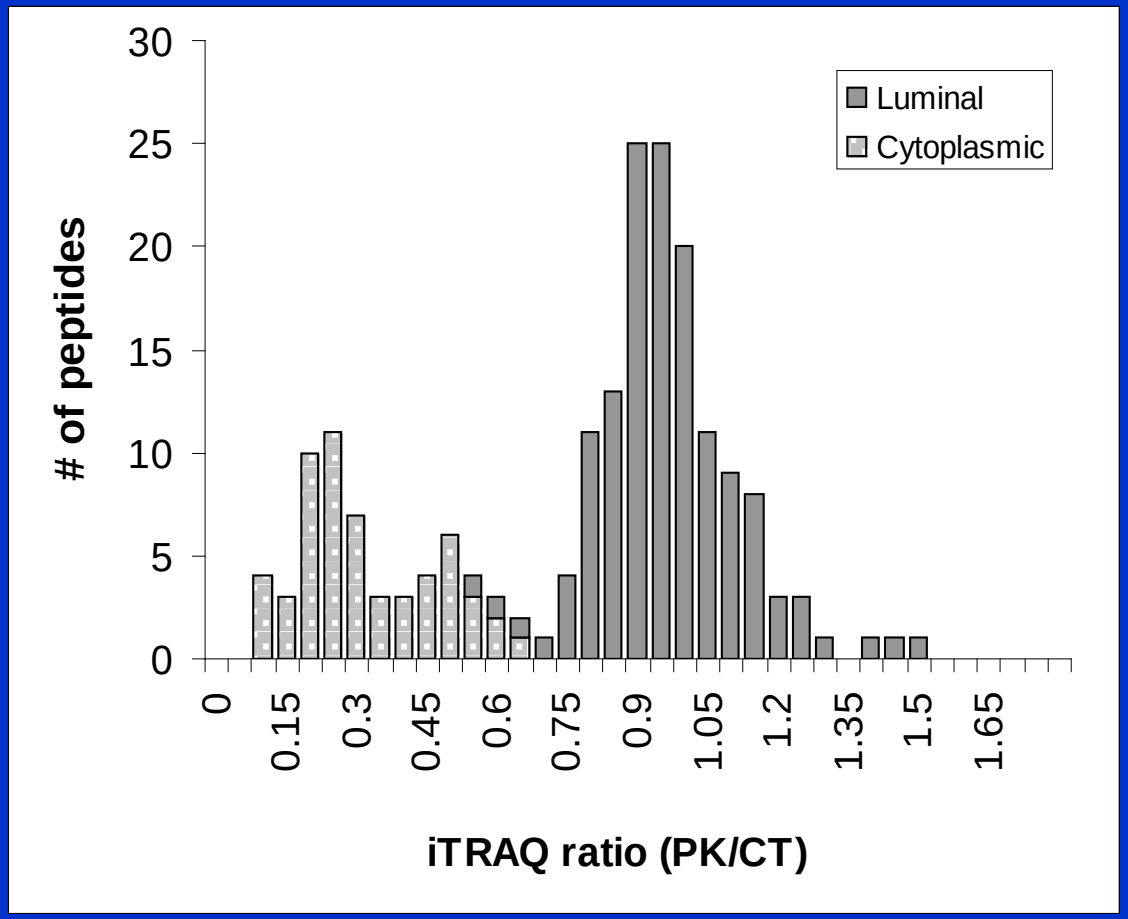
Peptide distributions on iTRAQ ratios (PK/CT)



Cytoplasmic and luminal peptides had distinct iTRAQ ratios

Cytoplasmic

- Dynactin 1
- Dynein
- Myosin I
- Rab 1A
- Rab 2A
- Rab 3D
- Rab 5B
- Rab 11A
- Rap 1
- VAMP 2
- VAMP 8



Luminal

- Amylase
- Anionic trypsin 1
- Carboxypeptidase A1
- Carboxypeptidase A2
- Chymotrypsinogen B
- Colipase
- Elastase 1
- GP 2
- GP 3
- Itmap 1
- Sterol esterase
- Syncollin
- ZG 16

The topology model of SCAMP1

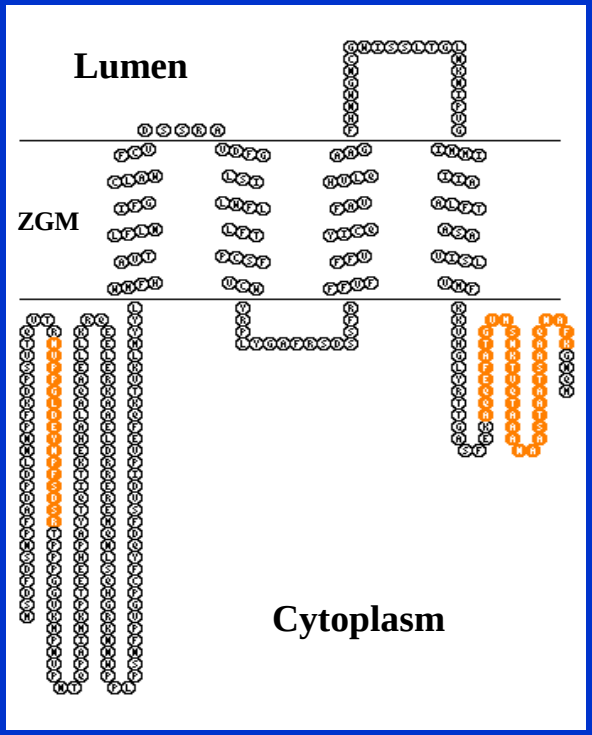
A

MSDFDSNPFADPDLNNPFKDPSVTQVTRNVPPGLDE
YNPFSDSRTPPPGGVKMPNVPNTQPAIMKPTEEHPA
YTQITKEHALAQAELLKRQEELERKAAELDRRERE
MQNLSQHGRKNNWPPLPSNFPVGPCFYQDFSVDIP
VEFQKTVKLMYYLWMFHAVTLFLNIFGCLAWFCV
DSSRAVDFGLSILWFLLFTPCSFVCWYRPLYGAFRS
DSSFRFFVFFFVYICQFAVHVLQAAGFHNWGNCGW
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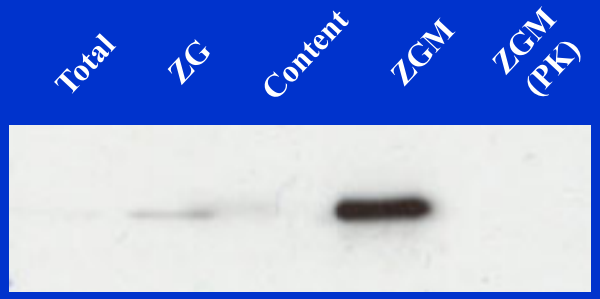
B

Peptide position	Ion Score C.I. %	Exp. 1 PK/CT	Exp. 2 PK/CT
29-44	100	0.25 ± 0.07	0.12 ± 0.03
299-311	100	0.12	0.16 ± 0.07
312-334	93.1	0.23	NA

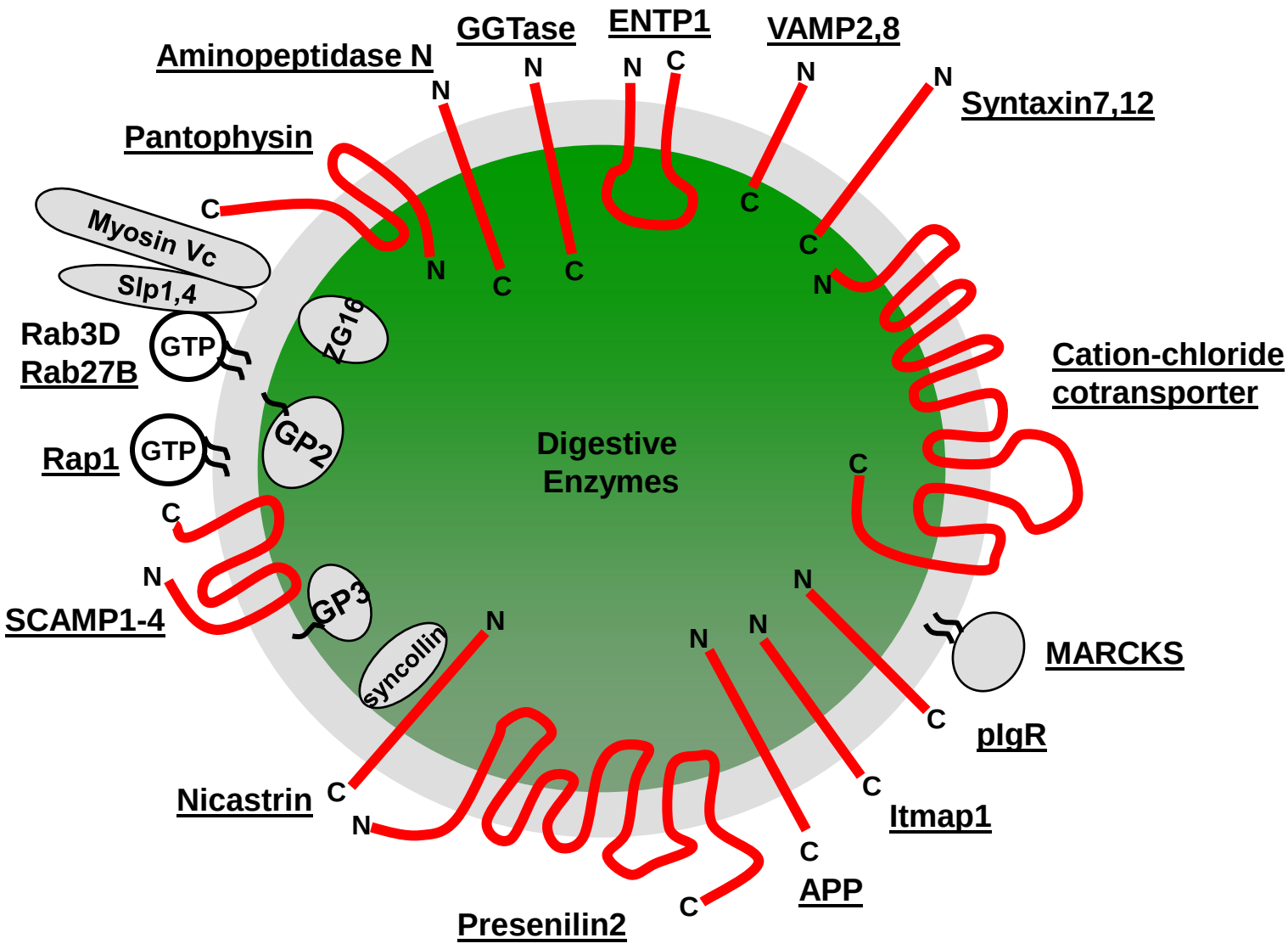
C



D

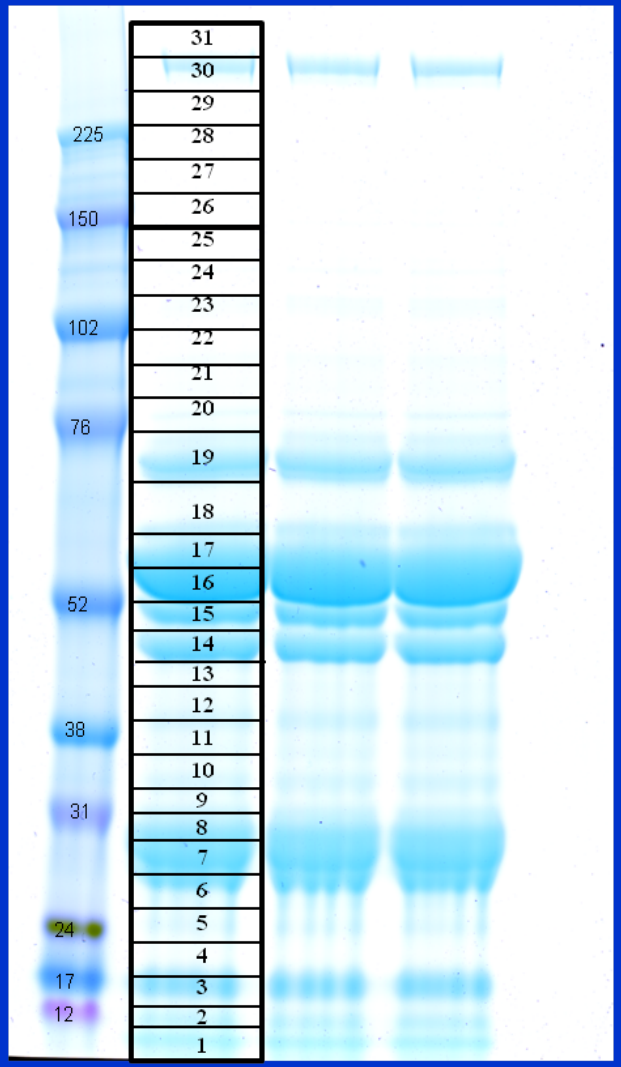


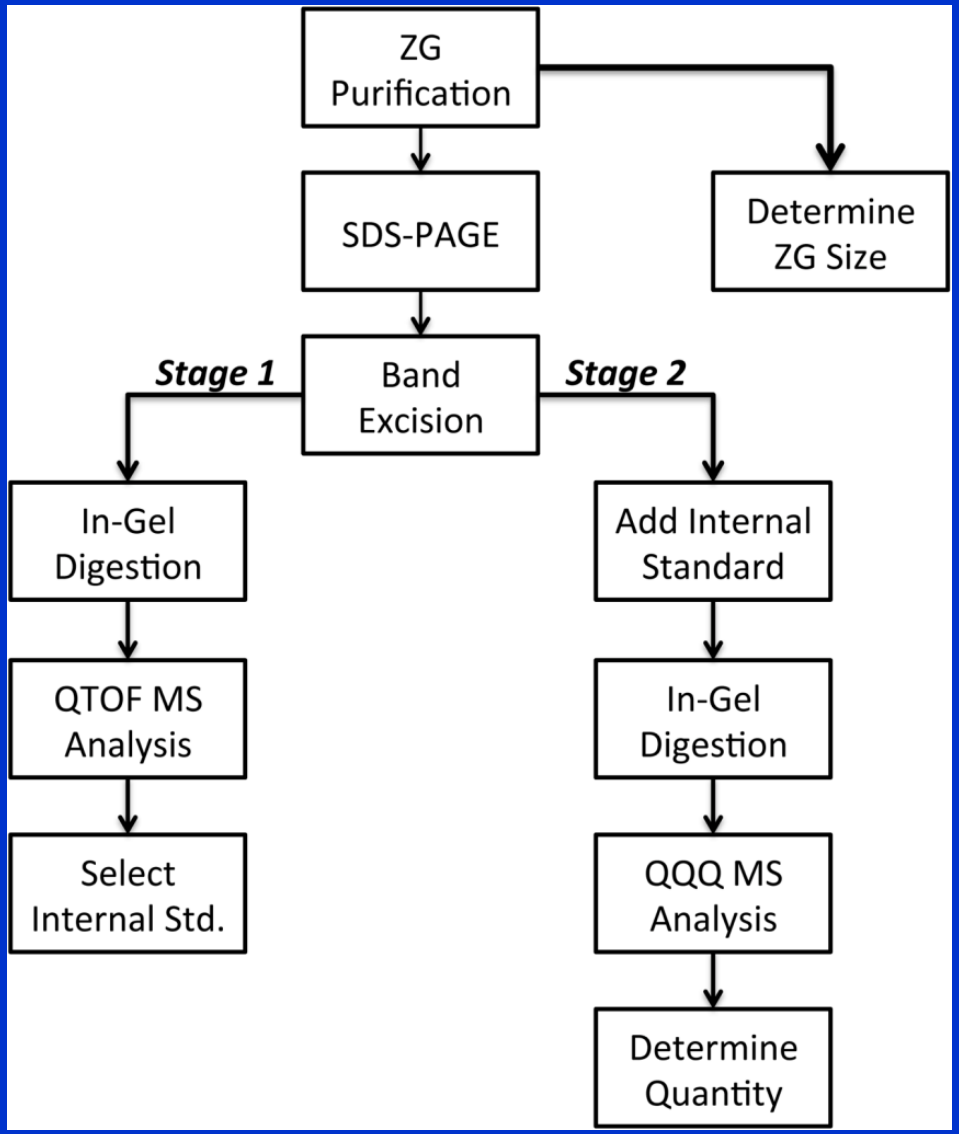
Molecular architecture of pancreatic zymogen granule



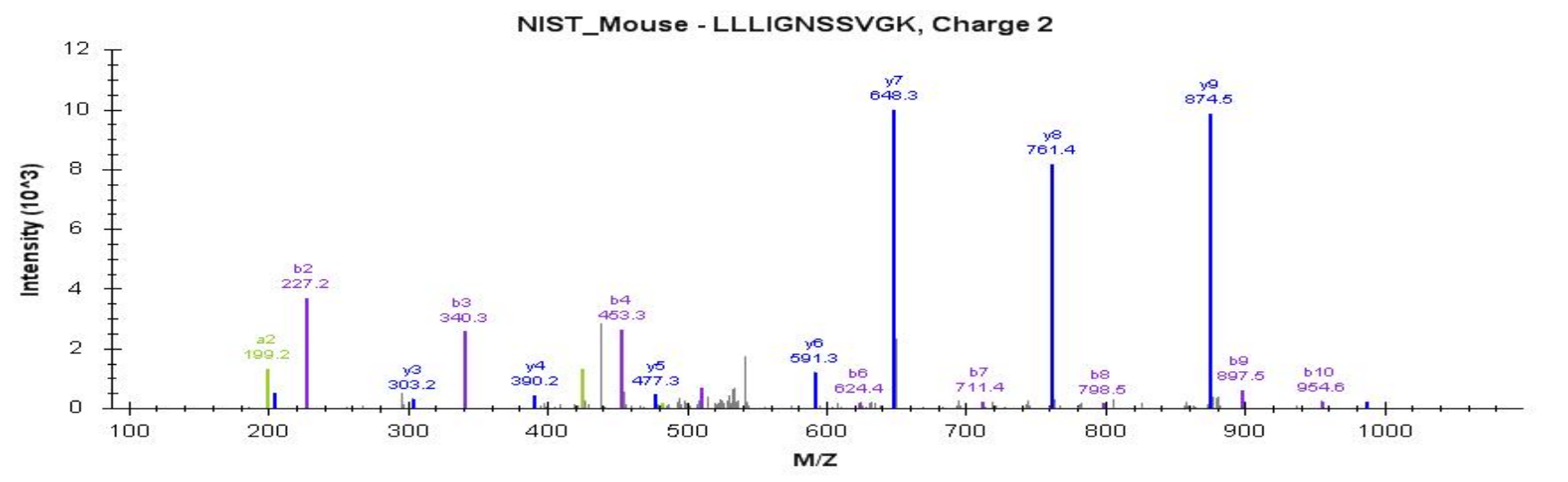
ZG proteins and their copy numbers

GeLC-MRM strategy for ZG copy number determination

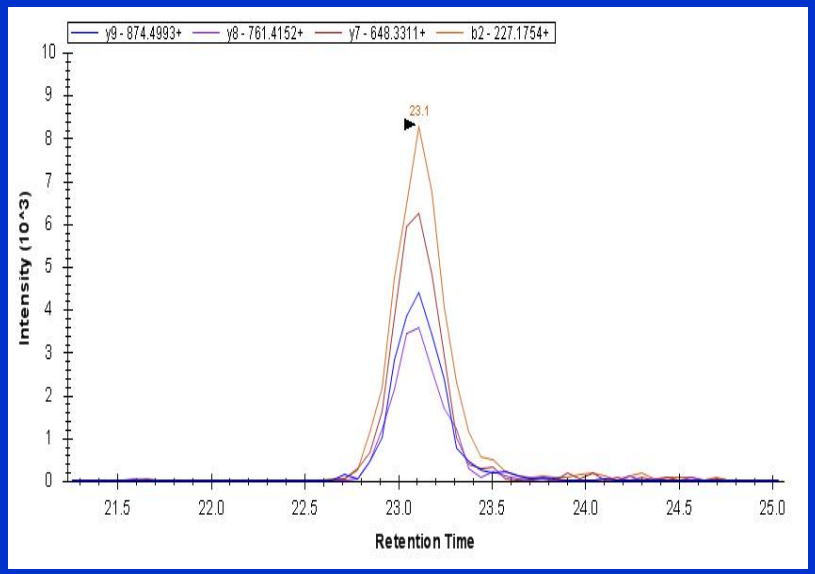




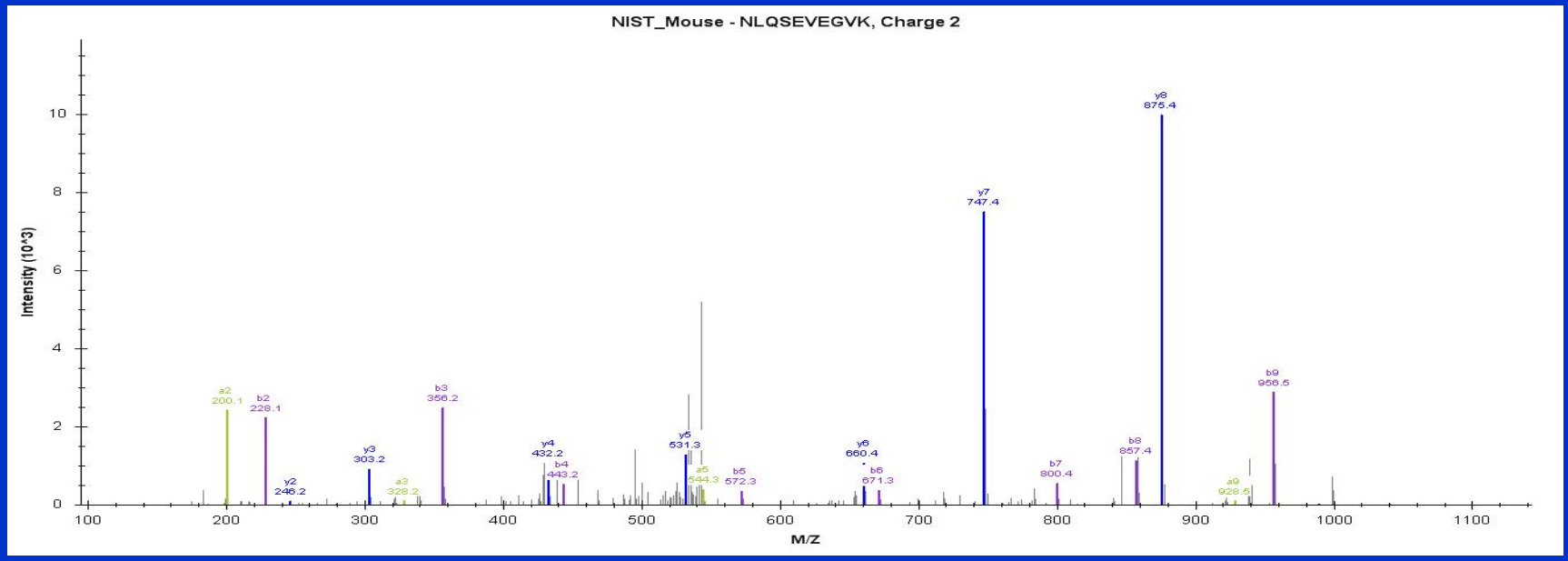
Development of MRM transitions for a unique Rab3D peptide



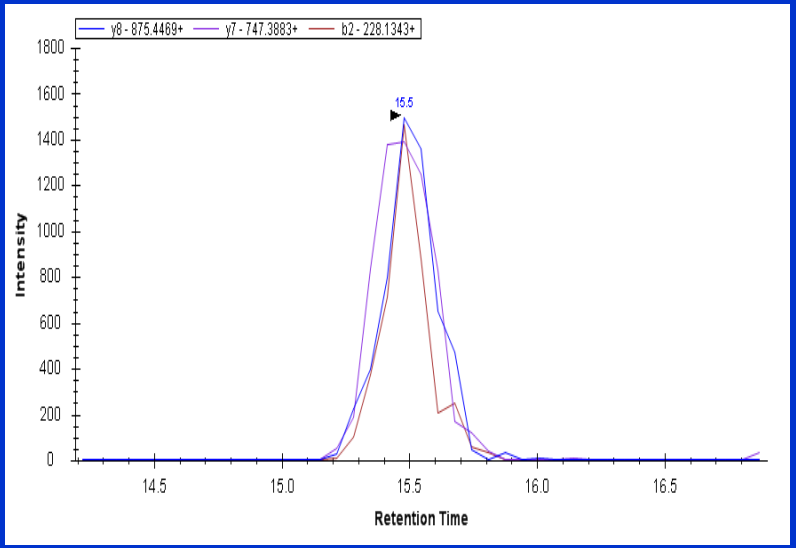
Protein of Interest	Amino Acid Sequence	Form	Precursor Ion (monoisotopic)	SRM Ion
RAB 3D [24,34]	LLLIGNSS VGK	Native-Light	550.8373	648.3311 (y7)
RAB 3D [24,34]	LLLIGNSS VGK	Native-Light	550.8373	874.4993 (y9)
RAB 3D [24,34]	LLLIGNSS VGK	Native-Light	550.8373	761.4152 (y8)
RAB 3D [24,34]	LLLIGNSS VGK	Native-Light	550.8373	227.1754 (b2)



Development of MRM transitions for a unique VAMP8 peptide

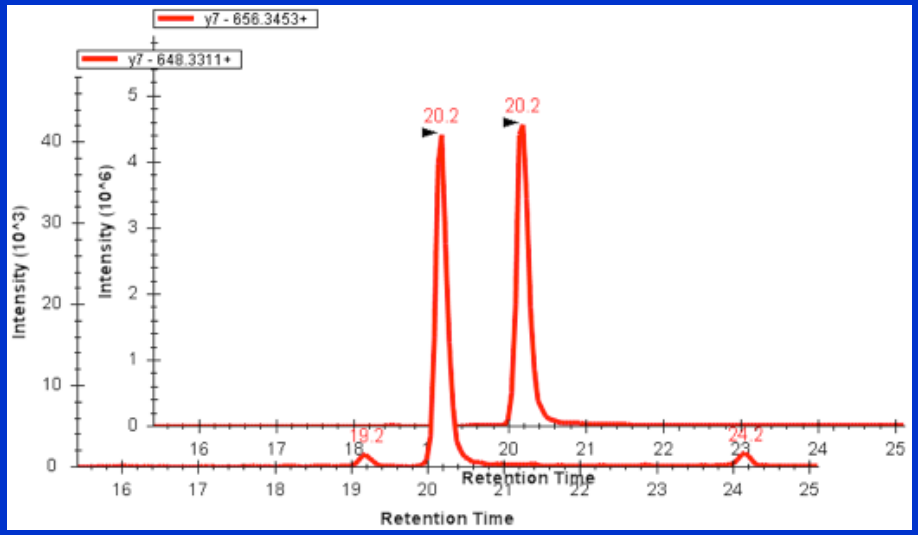


Protein of Interest	Amino Acid Sequence	Form	Precursor Ion (monoisotopic)	SRM Ion
VAMP8 [14,23]	NLQSEVEGVK	Native-Light	551.7906	747.3883 (y7)
VAMP8 [14,23]	NLQSEVEGVK	Native-Light	551.7906	875.4469 (y8)
VAMP8 [14,23]	NLQSEVEGVK	Native-Light	551.7906	228.1343 (b2)

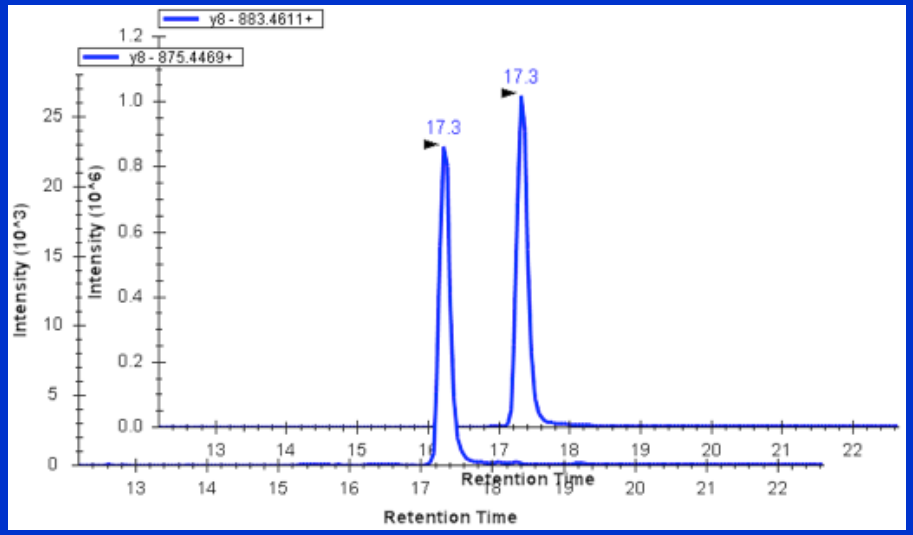


Absolute quantification of Rab3D and VAMP8 using AQUA peptides

Protein of Interest	Amino Acid Sequence	Form	Precursor Ion (monoisotopic)	SRM Ion
VAMP8 [14,23]	NLQSEVEGVK	Native- Light	551.7906	875.4469 (y8)
VAMP8 [14,23]	NLQSEVEGV**K**	AQUA- Heavy	555.7977	883.4469 (y8)
RAB 3D [24,34]	LLLIGNSSVGK	Native- Light	550.8373	648.3311 (y7)
RAB 3D [24,34]	LLLIGNSSVG**K**	AQUA- Heavy	554.8444	656.3453 (y7)

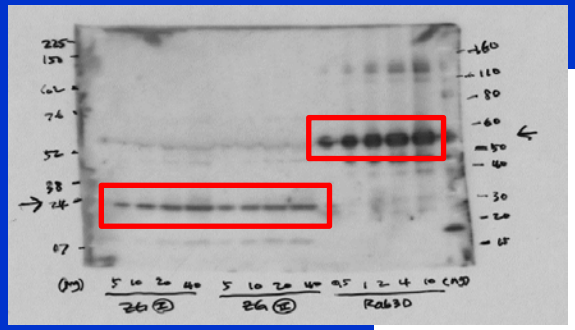


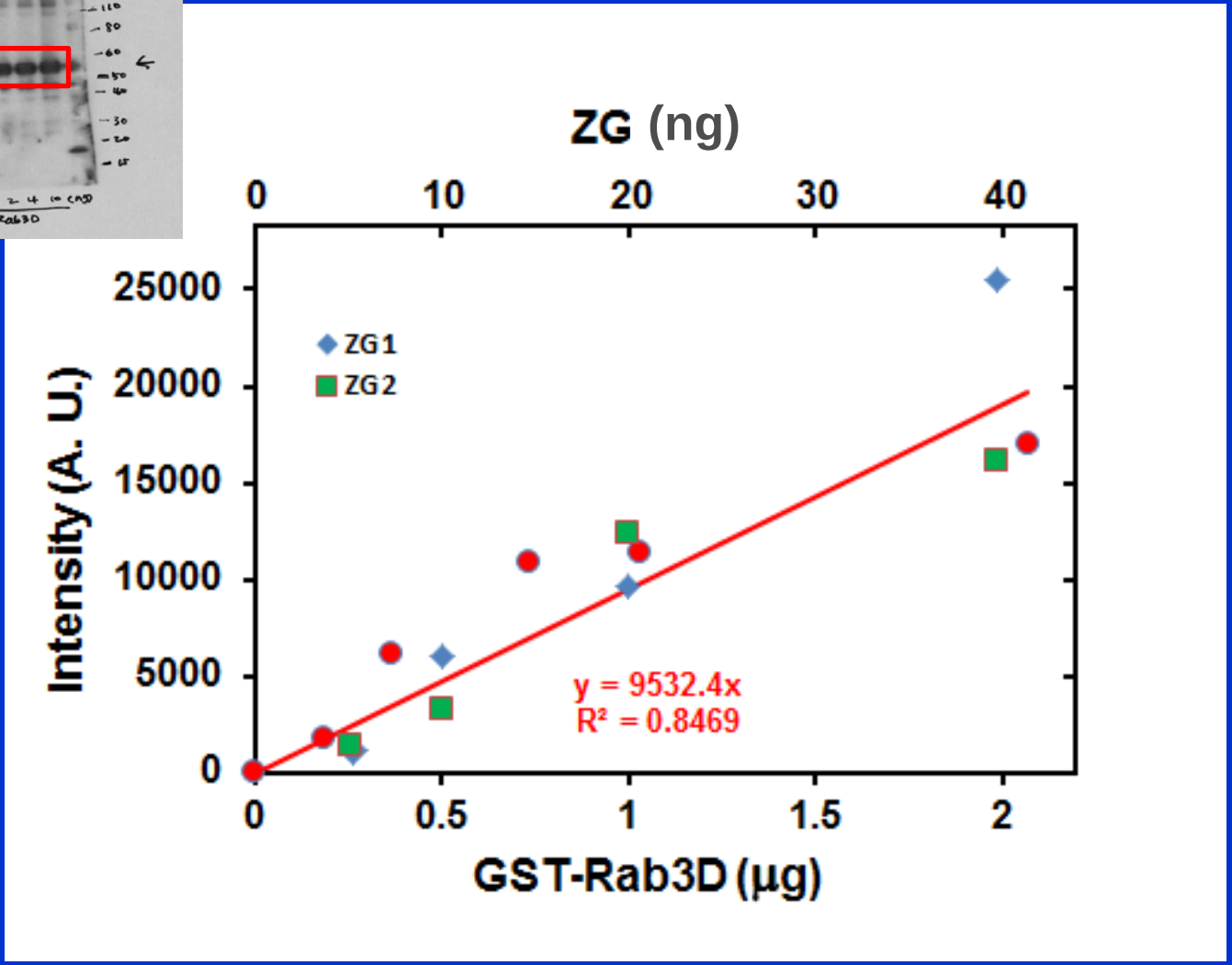
RAB3D_[24,34] (554.8444++)/(550.8373++)
Combined Chromatogram.



VAMP8_[14,23] (555.7977++)/(551.7906++)
Combined Chromatogram

Quantification of Rab3D by WB using GST-Rab3D as internal standard

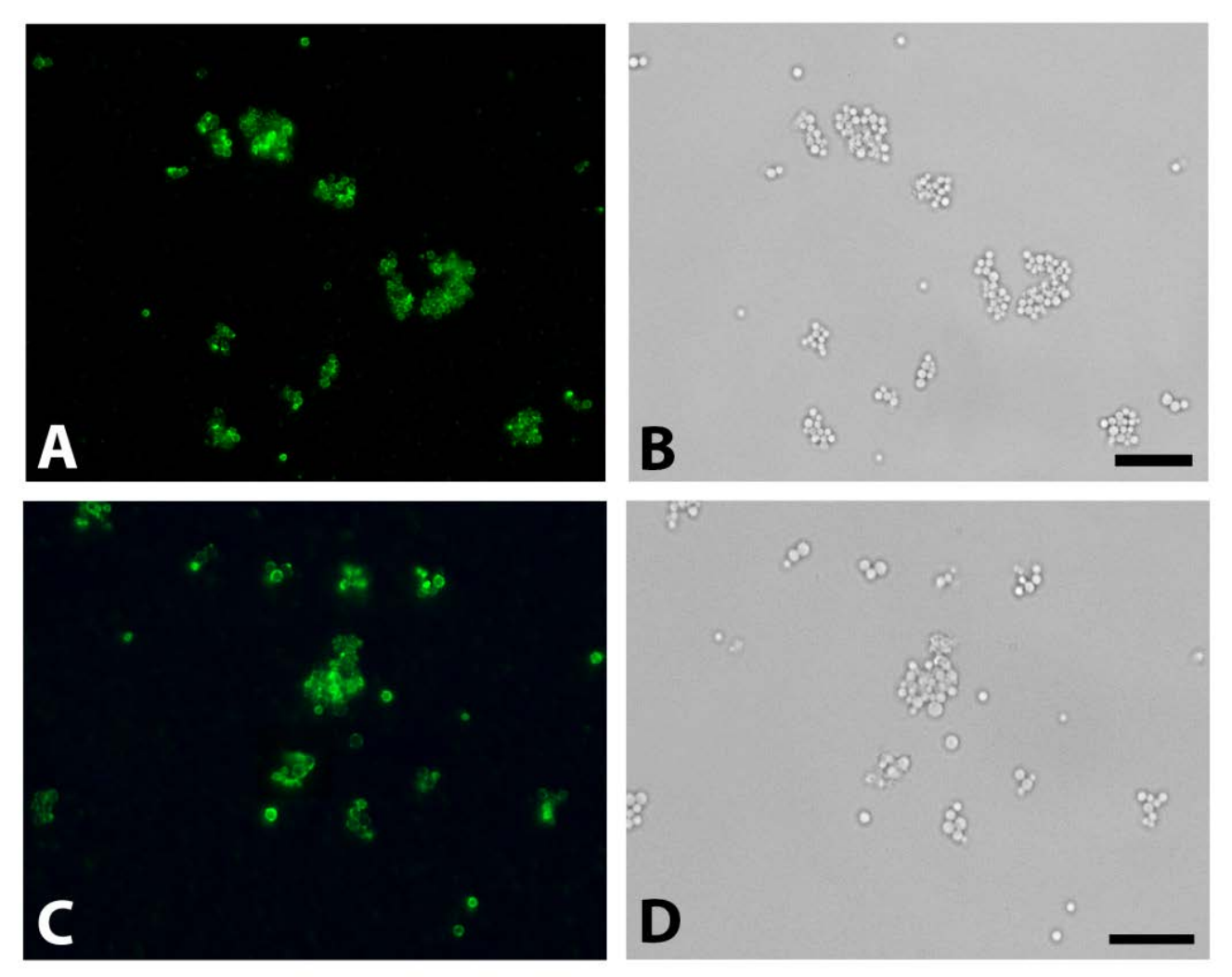




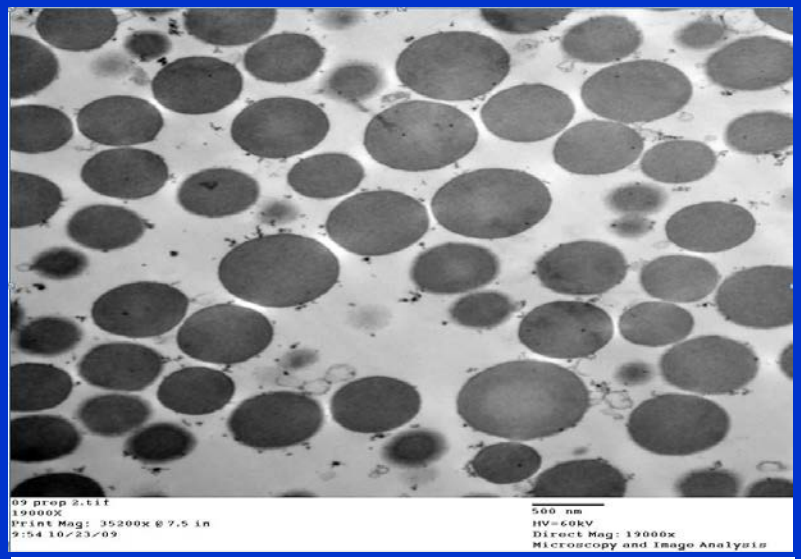
Immunofluorescence and bright field imaging of isolated ZGs

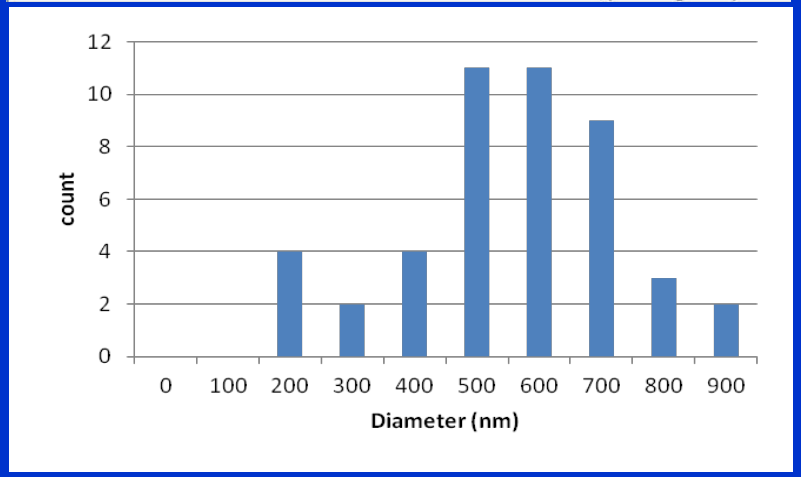
Rab3D

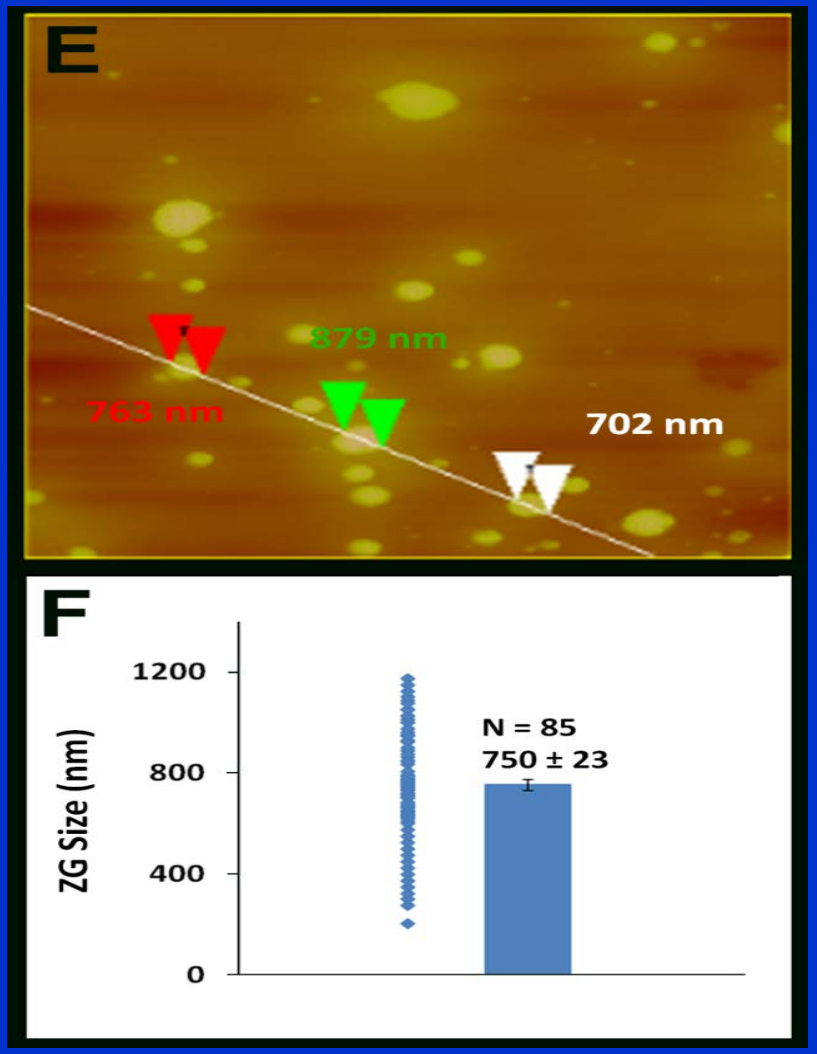
VAMP8



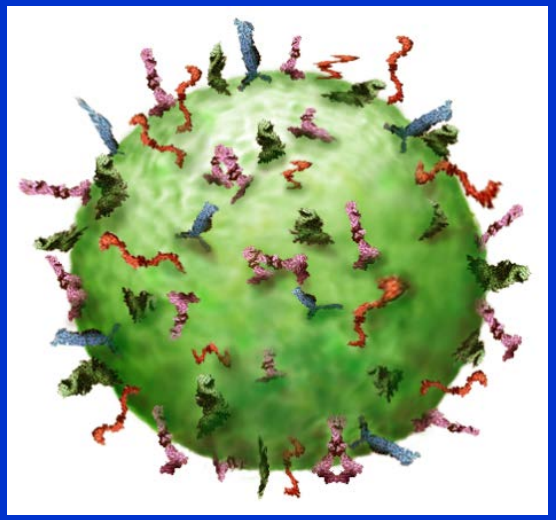
Determination of the size distribution of ZGs by EM and AFM







Comparison between pancreatic ZGs and neuronal SVs



ZG



SV

Secretory vesicles	Average size (nm)	Copy numbers per vesicle	Density (molecules / μm^2)
ZG	750	VAMP8: 6500	3678
		Rab3D: 7400	4187
SV	45	VAMP2: 70	11003
		Rab3A: 10	1572

Takamori et al. *Cell* 2006

Summary

- A total of 231 proteins identified using multiple separation techniques
- ZGM localization confirmed for some novel observations including Rab11A, Rab27B, Rap1, SNAP 29, Myosin Vc and Slp 1
- iTRAQ-based quantitative proteomics approach used to systematically ananlyze the enrichment and topology of ZG membrane proteins
- Copy numbers of selected ZG proteins determined by LC-MRM and AQUA peptides

Future directions

- **ZG protein complexes by BN-PAGE, co-IP and xlinking**
- **Dynamic changes of ZG composition upon stimulation and in diseases (e.g. pancreatitis)**

Acknowledgements

Dr. Chen's Lab
- Dr. Jin-sook Lee
- Patricia Skallos
- Garrett Hubbs

Dr. Stemmer's Lab
- Dr. Paul Stemmer
- Dr. Joseph Caruso

Dr. Jena's Lab
- Dr. Bhanu Jena

University of Michigan
Dr. Philip Andrews' lab
Dr. John Williams' lab

Fundings

WAYNE STATE

Michigan Diabetes Research and Training Center

Selection of Rab3D and VAMP8 for Absolute Quantification

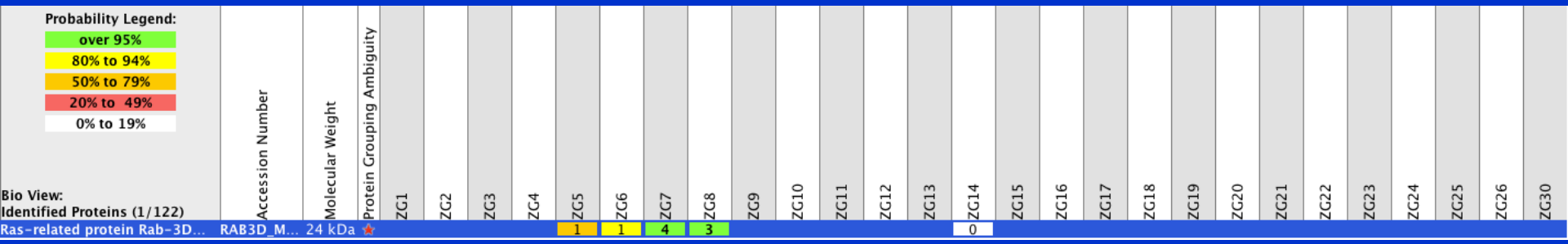


Figure 5: RAB3D Scaffold View.

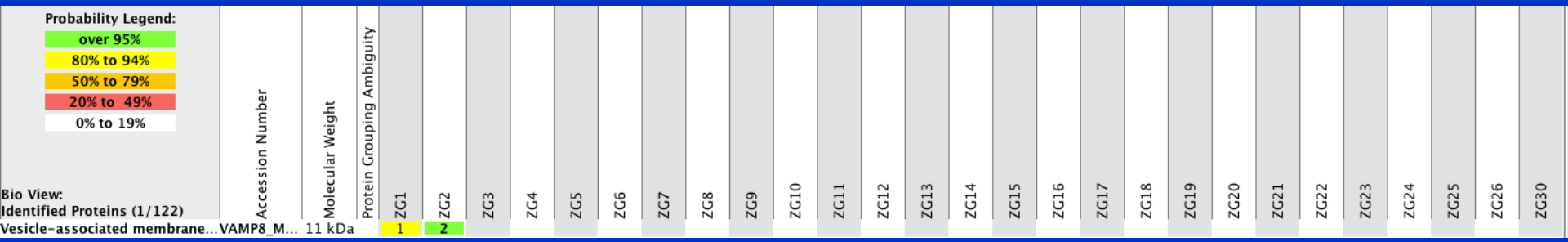


Figure 6: VAMP8 Scaffold View

The topology model of Tm63A

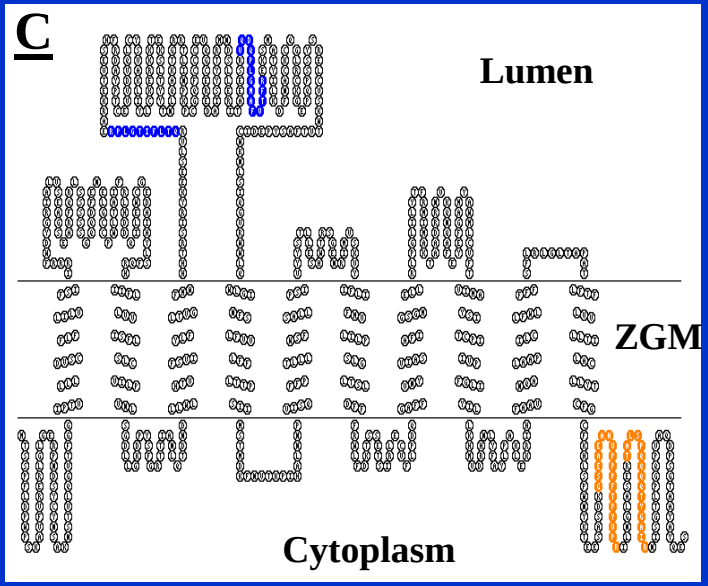
A

MTSSPFLDPW PSKAVSIRER LGLGDRPNDS YCYNSAKNST VLQGVTFGGI PTVLLLDVGC
FLVLILVFSI IRRKFWDYGR IALVSEAGSE ARFRRLSSSS SGQQDFESEL GCCSWLSAIF
RLHDDQILEW CGEDAIHYLS FQRHIIFLLV VVSFLSLCVI LPVNLSGDLL DKDPYSFGRT
TIANLQTDND LLWLHTVFSV IYLFLTVGFM WHHTRSIRYK EESLVRQTLF ITGLPREARK
ETVESHFRDA YPTCEVVDVQ LCYSVAKLIN LCKERKKTEK SLTYYTNLQV KTGRRTLINP
KPCGQFCCCE VQGCEREDAI SYYTRMNDSL TERITAEECR VQDQPLGMAF VTFREKSMAT
FILKDFNACK CQGLRCKGEP QPSSYSRELC VSKWSVTFAS YPEDICWKNL SIQGVRWWLQ
CLGINFSLFV VLFFLTTPSI IMSTMDKFNV TKPIHALNNP VISQFFPTLL LWSFSALLPT
IVYYSTLLES HWTRSGENRI MVSKVYIFLI FMVLILPSLG LTSLDFFFRW LFDKTSSDTS
IRLECVFLPD QGAFFVNYVI ASAFIGSGME LLRLPGLILY TFRMIMAKTA ADRRNVKQNQ
AFEYEFGAMY AWMLCVFTVI MAYSITCPII VPFGLIYILL KHMVDRHNLY FAYLPAKLEK
RIHFAAVNQA LAAPILCLFW LFFFSFLRLG LTAPATLFTF LVALLAILAC LLYTCFGCFK
HLSPWNYKTE EPVGDKRNEA EAHAPPPFTP YVPRILNGLT SERTALSPQQ QQTYGAIRNI
SGTLPGQLVA QDPSDTVAGV YQES

B

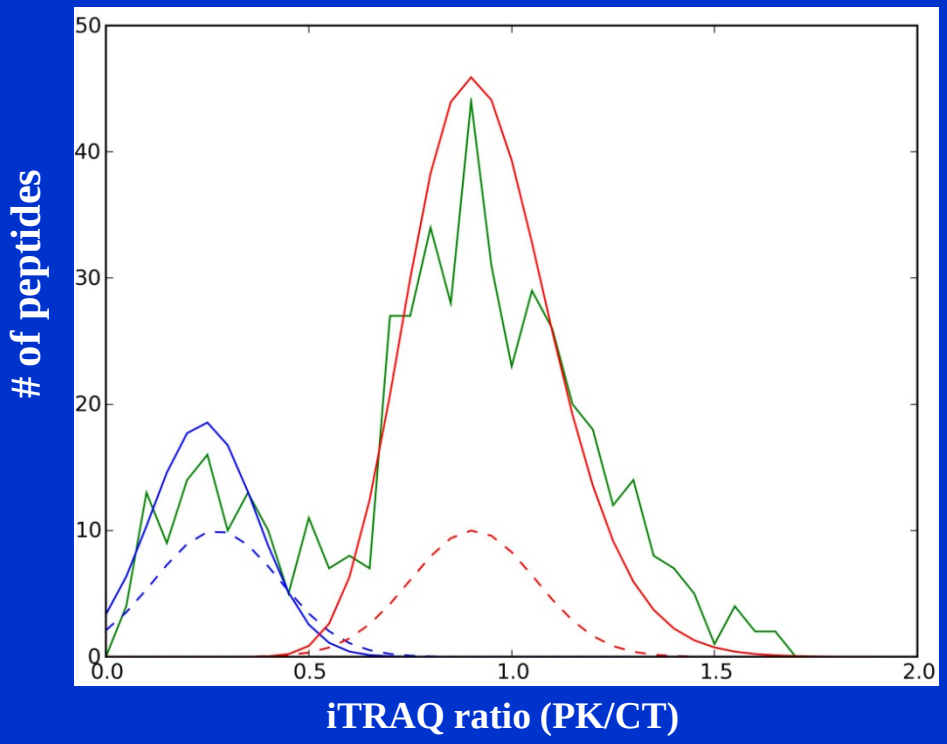
Peptide position	Ion Score C.I. %	Exp. 1 PK/CT	Exp. 2 PK/CT
227-236	100	0.84 ± 0.10	0.93
341-354	100	0.63 ± 0.19	0.68 ± 0.20
738-754	100	0.32 ± 0.12	0.29 ± 0.10
764-778	100	0.12 ± 0.04	0.18 ± 0.01

C

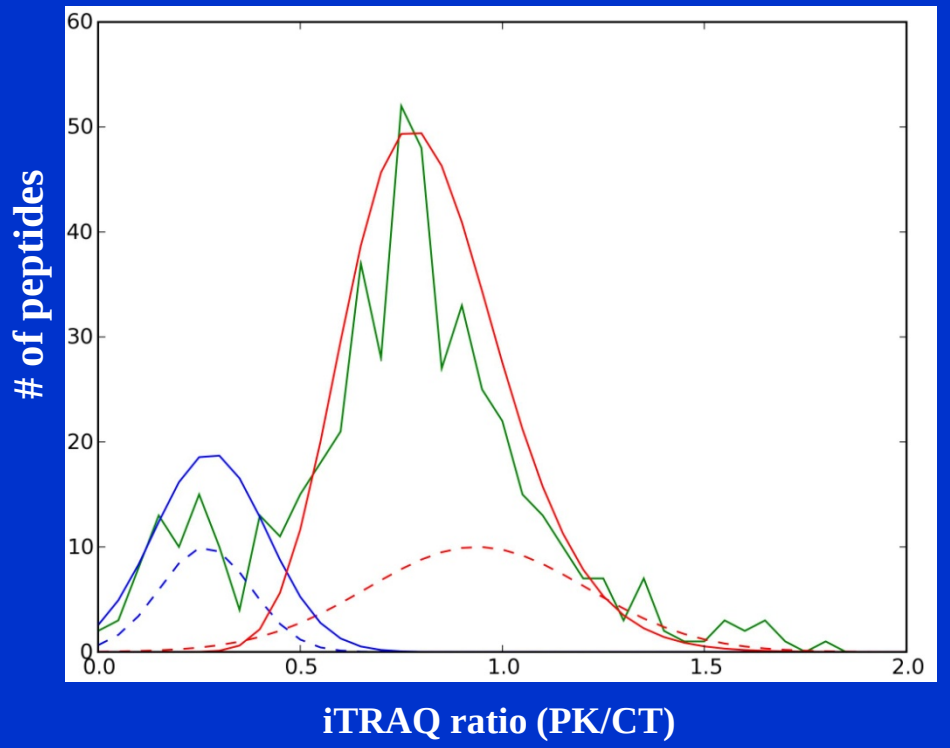


A mixture model for estimating peptide topology

Experiment #1



Experiment #2



Green: experimental distribution
Red: model distribution – luminal

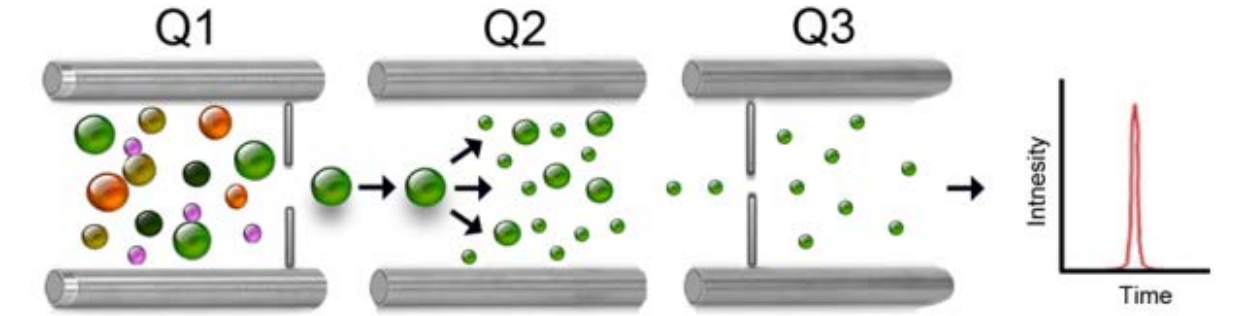
Blue: model distribution – cytoplasmic
Dashed: training set distribution

Probability Legend:

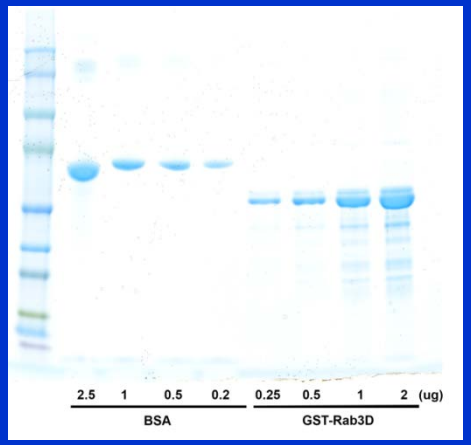
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

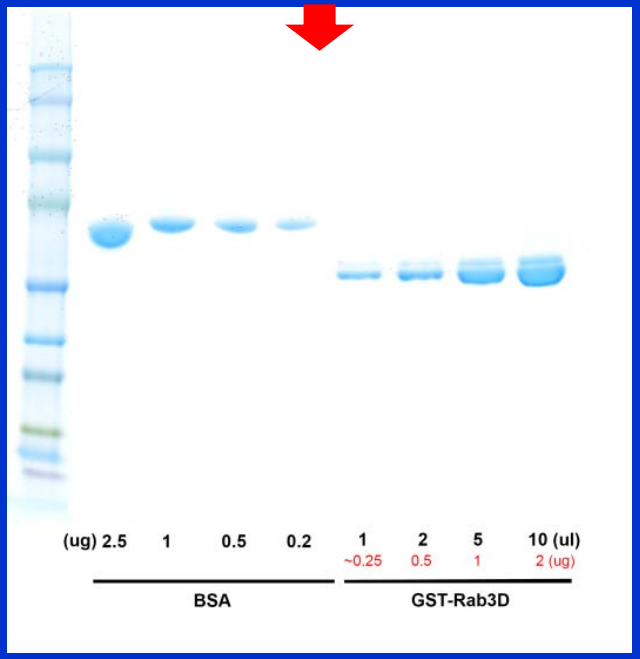
Bio View:
Identified Proteins (4)

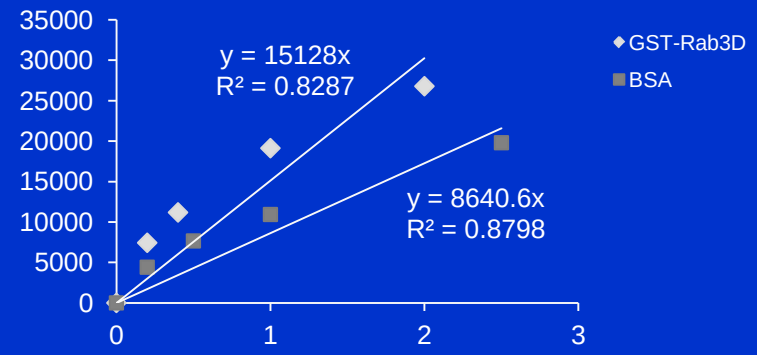
#	Visible?	Starred?		Accession Number	Molecular Weight	Protein Grouping Ambiguity	ZG1	ZG2	ZG3	ZG4	ZG5	ZG6	ZG7	ZG8	ZG9	ZG10	ZG11	ZG12	ZG13	ZG14	ZG15	ZG16	ZG17	ZG18	ZG19	ZG20	ZG21	ZG22	ZG23	ZG24	ZG25	ZG26	ZG31	ZG all bands
1	☑	☆	Ras-related protein Rab-3D OS=Mus musculus GN=R...	RAB3D...	24 kDa	★					1	1	2	5	2																		6
2	☑	☆	V-type proton ATPase 116 kDa subunit a isoform 1 O...	VPP1_...	96 kDa																						1	4	1					4
3	☑	☆	Vesicle-associated membrane protein 8 OS=Mus mus...	VAMP8...	11 kDa		1	3																										3
4	☑	☆	Secretory carrier-associated membrane protein 1 OS...	SCAM1...	38 kDa												2																	2

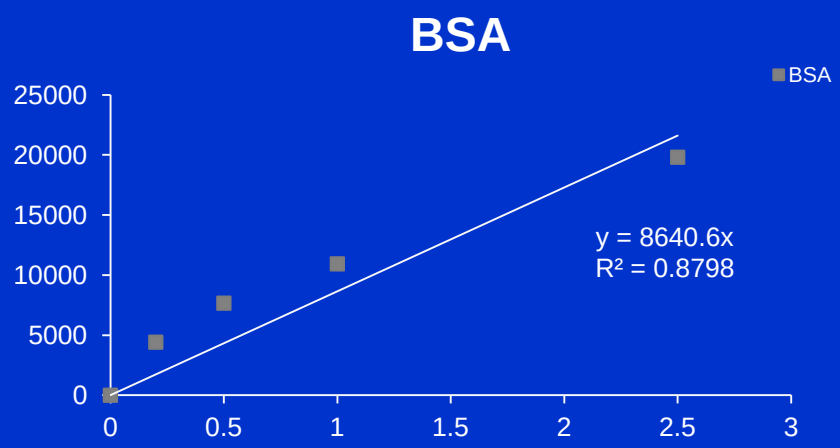


Quantification of BSA std & GST-Rab3D using Image J



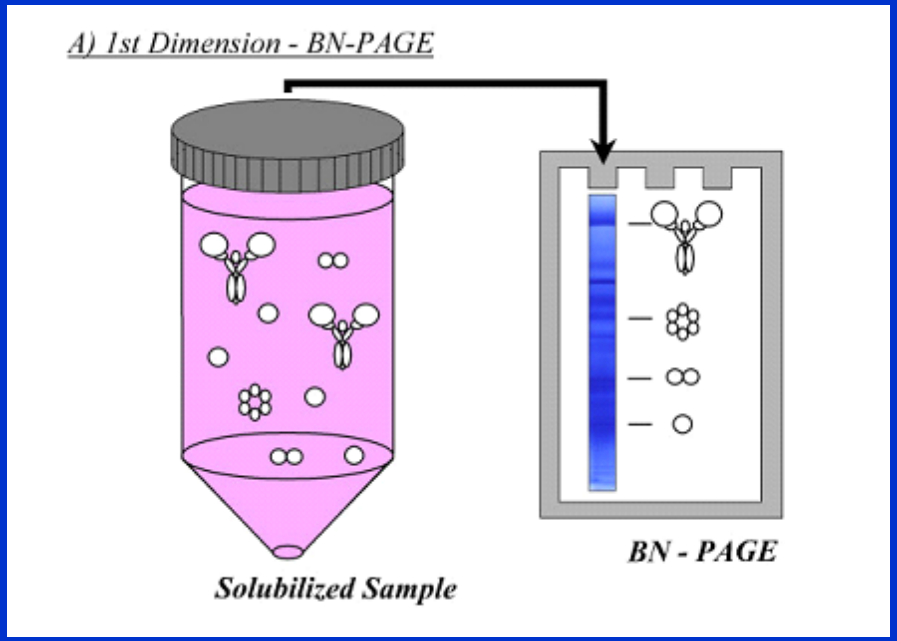


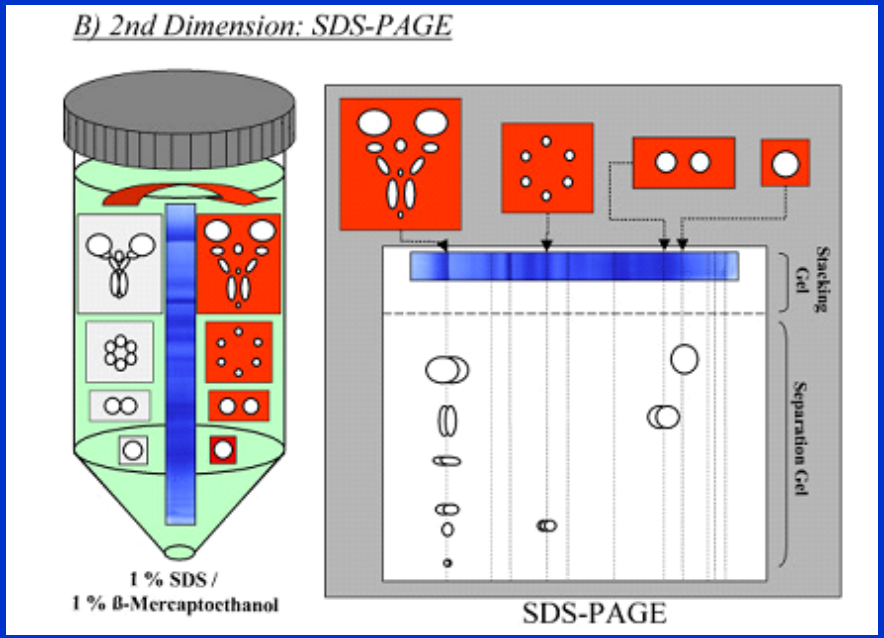





Resuspend mouse ZGs in 160 ul H buffer
Based on "Counting 1"
80 ul for characterizing ZGs with different imaging approaches
80 ul for SDS-PAGE
0.977 x 10⁶ ZGs/ ul
Fixed
Dilution factor
2.625
ZG size distribution:
D = 750 ± 120 nm
ZG density by hemocytometer:
0.977 x 10⁶ / ul
Add 80 ul lysis buffer, 4xSDS buffer etc to 210 ul (~ 2ug/ul protein)
0.372x 10⁶ ZGs/ ul
Run 50 ul ZG lysate on SDS-PAGE
0.372x 10⁵ ZGs/ ul
Cut gel slices and in-gel digestion
?% recovery is unknown
For Rab3D in band 9, resuspend tryptic peptide in 108ul solvent A and injected 5 ul for LC-MS/MS
dilution factor 21.6
For VAMP8 in band 2 & 3, resuspend tryptic peptide in 32ul solvent A and injected 5 ul for LC-MS/MS
dilution factor 6.4
AQUA:
5 fmole
AQUA:
50 fmole
AQUA:
5 fmole
AQUA:
50 fmole
Rab3D: 10 fmole for
0.87x 10⁶ ZGs
VAMP8: 11.5 fmole for
0.87 x 10⁶ ZGs
VAMP8: 27.4 fmole for
2.91x 10⁶ ZGs
VAMP8: 36 fmole for
2.91 x 10⁶ ZGs
VAMP8: 7439 copies per ZG
VAMP8: 6564 copies per ZG

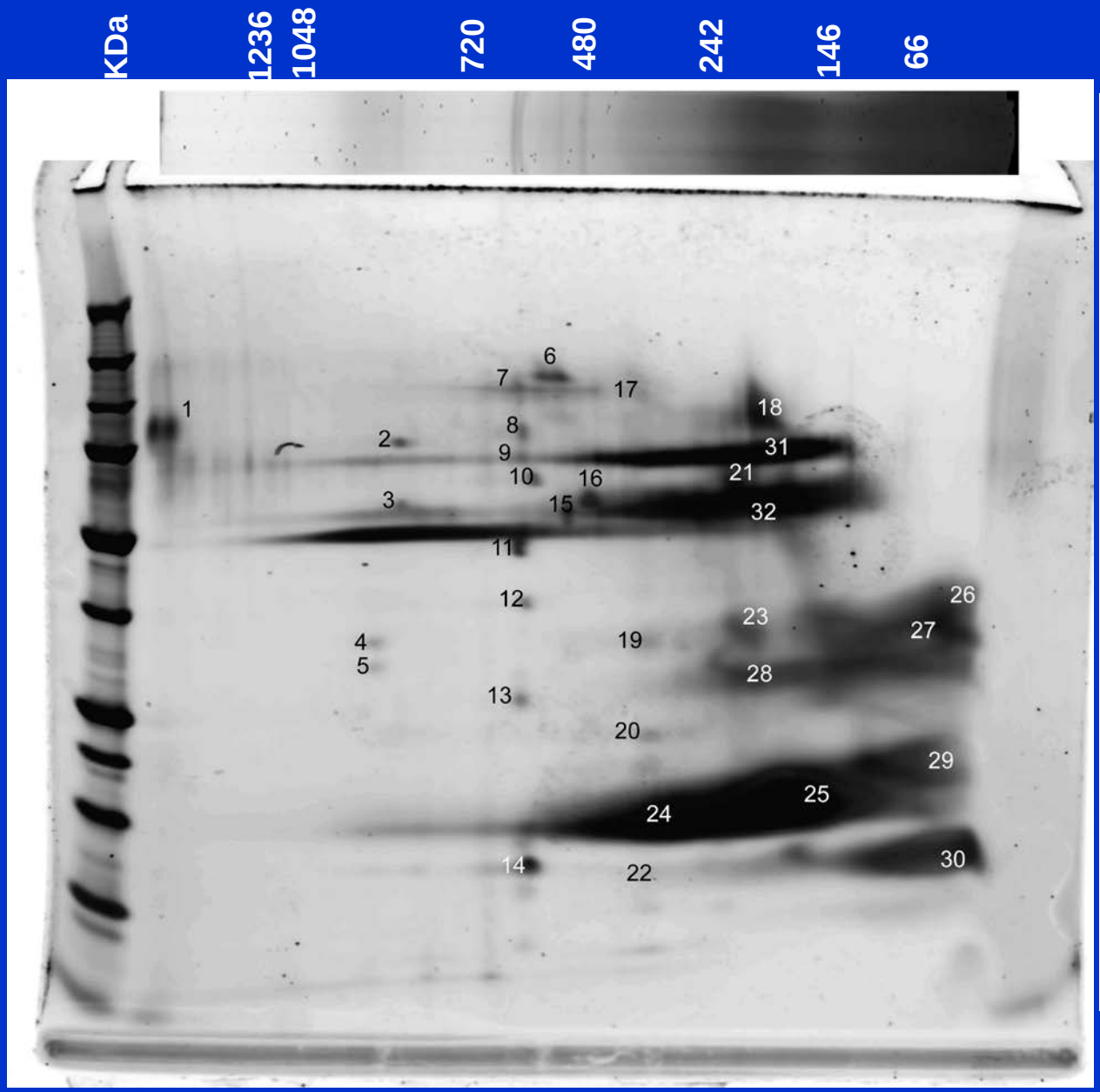
The working principles of Blue native/SDS-PAGE





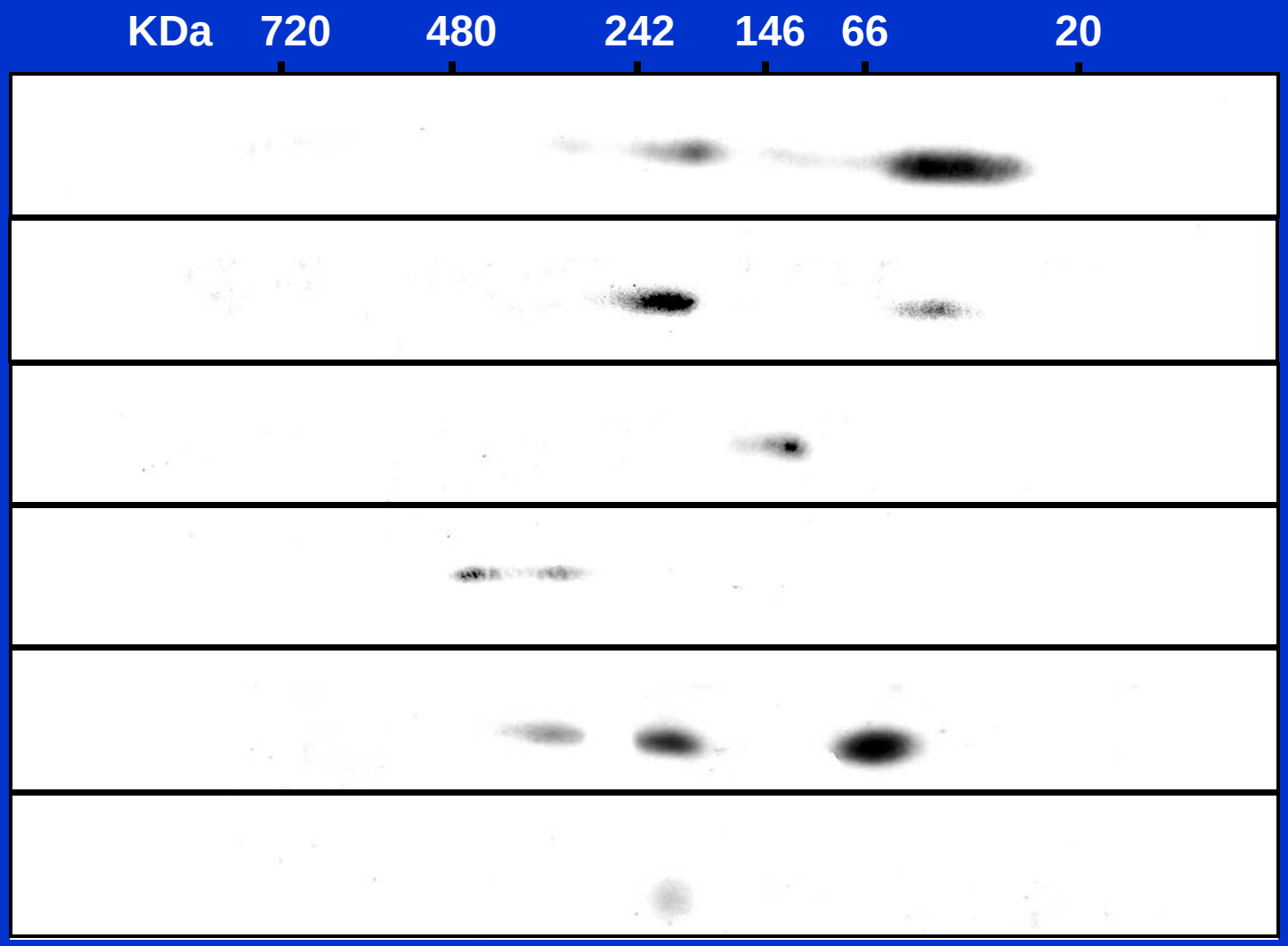
Plant Methods. 2005 Nov 16;1(1):11.

Two dimensional separation of ZGM by BN/SDS-PAGE

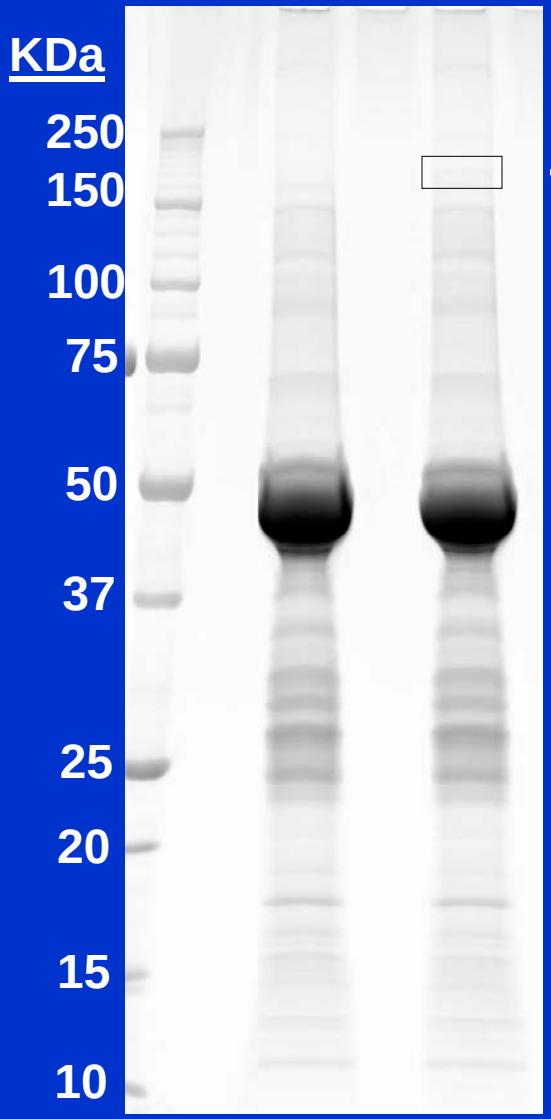


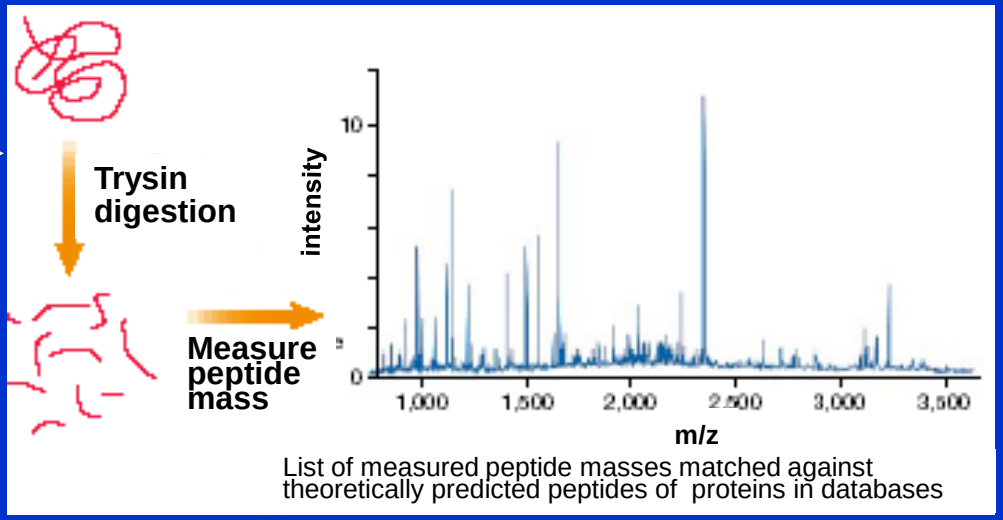
1.Itmap 1
2. Propionyl-CoA carboxylase α chain
3. Propionyl-CoA carboxylase β chain
4. Prohibitin 2
5. Prohibitin
6. Phosphodiesterase 3
7. NADP transhydrogenase
11. Ubiquitol cytochrome c-reductase
13. x
8. VATPase a1 subunit
12. VATPase V0 subunit d
14. VATPase V0 subunit c
10. Heat shock protein 65
15. ATPase β subunit
16. Glutamate dehydrogenase
18. GP2
17. x
19. X
20. X
22. X
21.Gamma glutamyl transpeptidase
23.Gamma glutamyl transpeptidase
24, 25. Syncollin
28. Elastase 2
31. Sterol esterase
32. GP3

WB results from 2nd dimension BN/SDS-PAGE



GST-Rab3D affinity purification to identify interacting partners





Myosin Vc (204 KDa)

Observed	Delta	Score	Peptide
1286.60	0.00	26	K.LGSAEEFNYTR.M
1468.71	-0.00	31	R.LIQDSNQYQFGR.T

Proteomics analysis leads to new hypotheses about ZG functions

- **Rab27B localized to ZG membrane and plays a positive role in regulated exocytosis**
- **Two potential Rab interacting proteins identified and their interactions with Rab3D and Rab27B confirmed**

Identification and confirmation of Rab27B on ZGM

A.

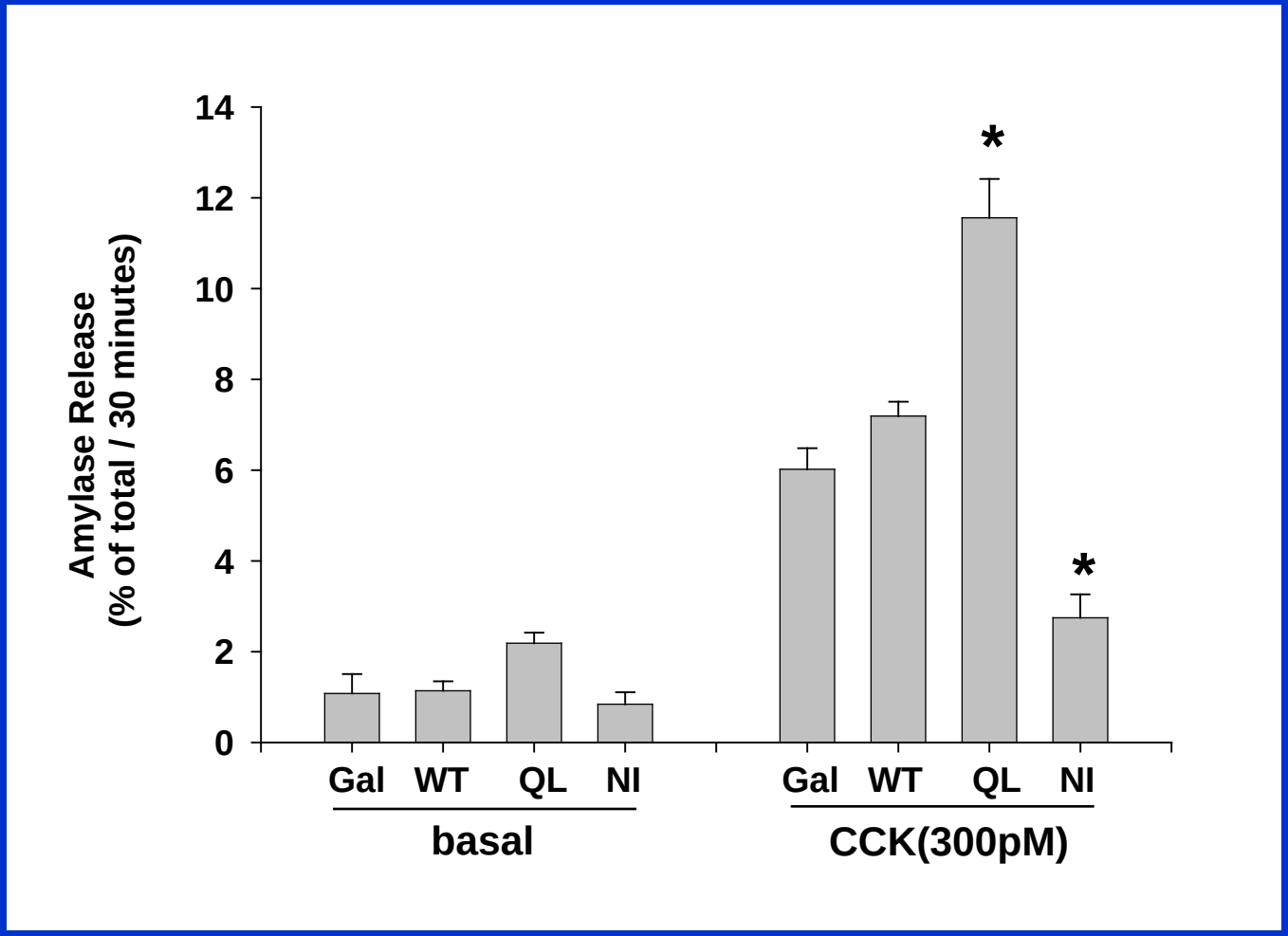
Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
12 - 22	1029.58	1028.57	1028.59	-0.01	0	LLALGDSGVGK
38 - 47	1168.64	1167.63	1167.63	0.00	0	FITTVGIDFR*
51 - 64	1383.66	1382.65	1382.63	0.02	0	VVYDTQGADGSSGK*
83 - 90	942.50	941.49	941.50	-0.00	0	SLTTAFFR
112 - 134	2678.21	2677.20	2677.26	-0.06	0	NWMSQLQANAYCENPDIVLIGNK
155 - 172	1974.93	1973.93	1973.94	-0.01	0	YGIPYFETSAATGQNVEK*
173 - 183	1277.70	1276.69	1276.69	-0.01	0	SVETLLDLIMK Oxidation(M)

B.

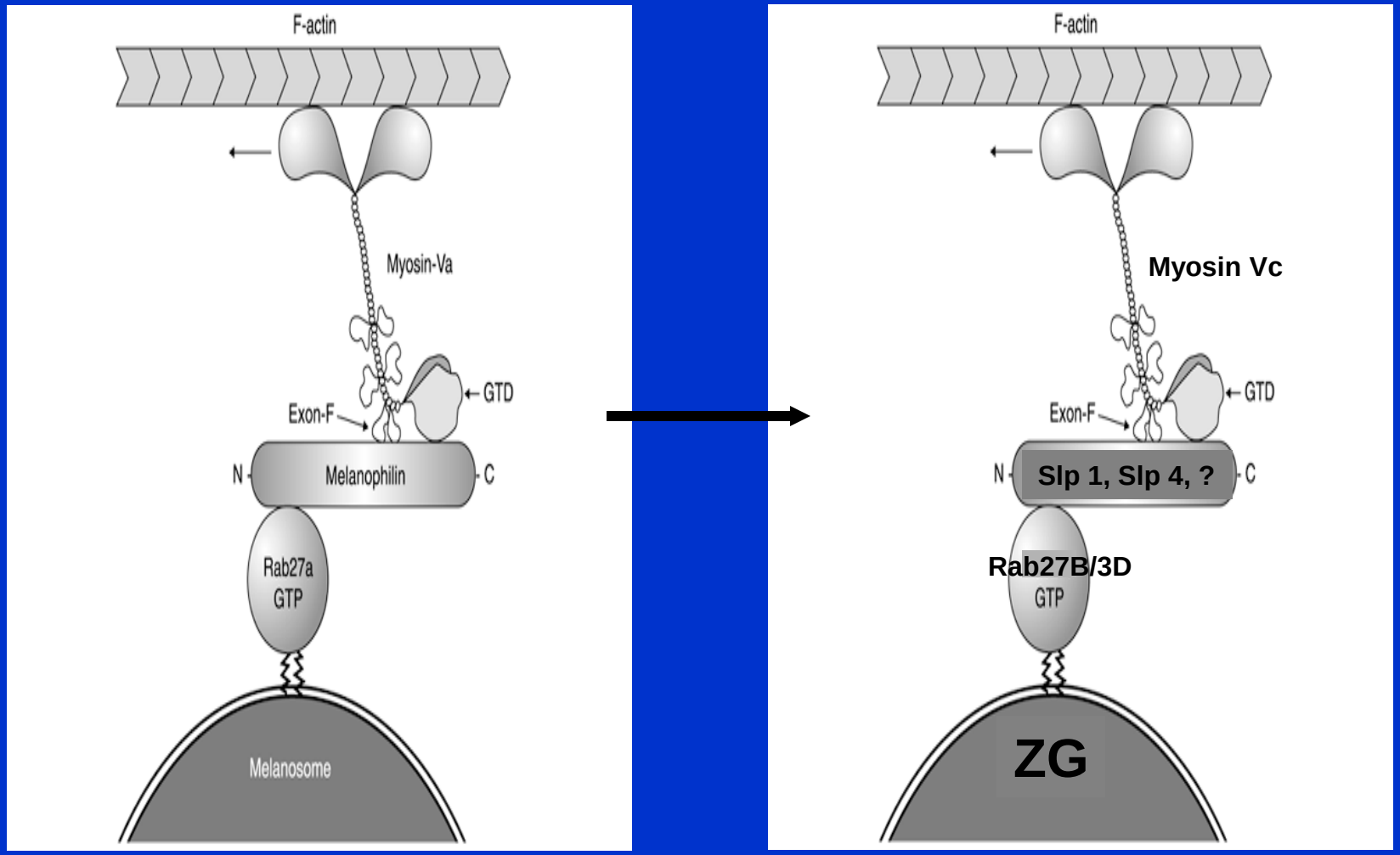
1 MTDGDYDYLI K**LLALGDSGV GK**TTFLYRYT DNKFNPK**FIT TVGIDFR**EKR
51 **VVYDTQGADG SSGK**AFKVHL QLWDTAGQER FR**SLTTAFFR** DAMGFLLMFD
101 LTSQQSFLNV R**NWMSQLQAN AYCENPDIVL IGNK**ADLLDQ REVNERQARE
151 LAEK**YGIPYF ETSAATGQNV EKSVETLLDL IMK**RMEKCVE KTQVPDTVNG
201 VNSGKVDGEK PAEKKCAC



Rab27B plays an important role in acinar secretion



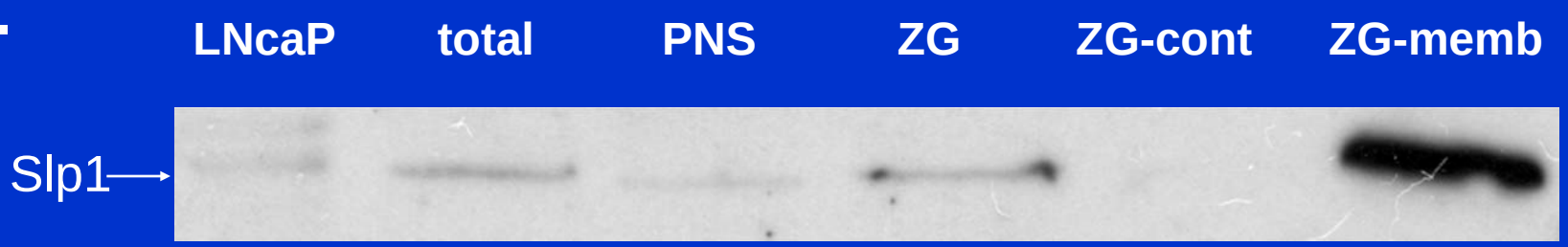
Proteomic analysis of ZG membrane helps to develop hypothesis on functional protein complex



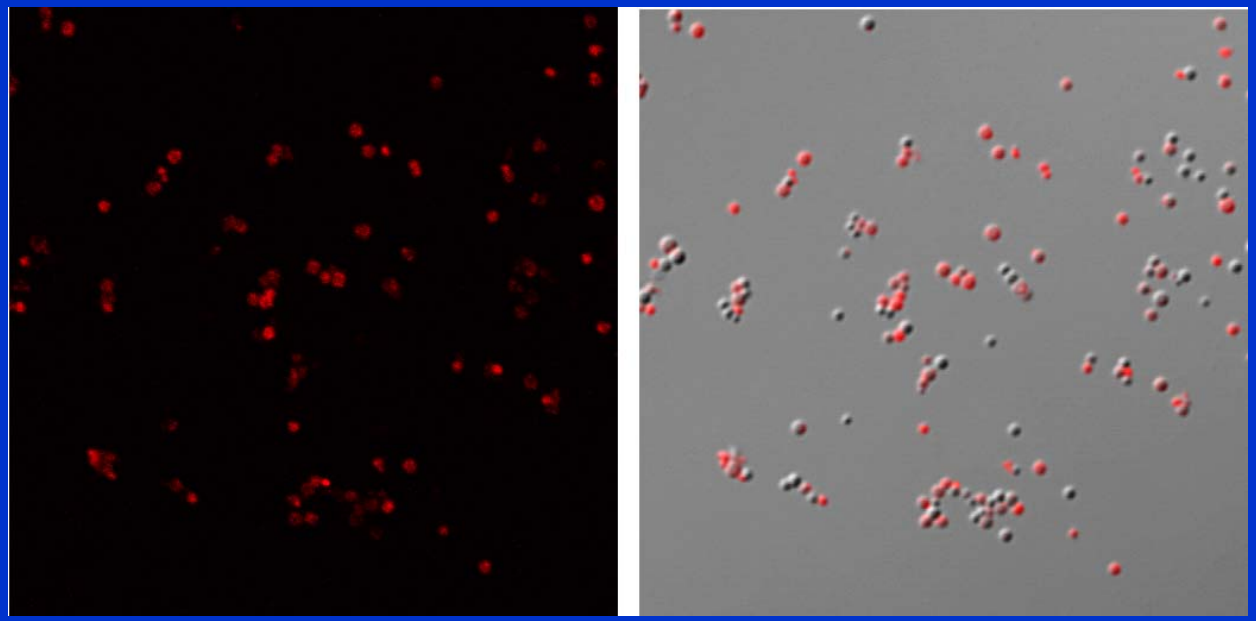
Modified from Nat Cell Biol. 2002 Apr;4(4):271-8

Slp1 is localized on ZG membrane

A.



B.



Anti-Slp1 (red)

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