

PROTEOMIKA

2023

- Proteomika, Metody práce s bílkovinami (Petrák 16/10)
- Separační metody, digesce a principy ID bílkovin pomocí MS (Petrák 23/10)
- Principy hmotnostní spektrometrie, instrumentace (Man 30/10)
- Hmotnostní spektrometrie v proteomice, analýza PTM (Man 6/11)
- ID proteinů, DDA, DIA, databáze, FDR (Talacko 13/11)
- Kvantifikace, isotopy, LFQ, cílená proteomika (Harant 20/11)
- Design experimentu, zpracování dat, statistika, bioinformatika (Harant 27/11)
- **Proteomika membránových proteinů, proteinové komplexy (Petrák 4/12)**
- **Klinická proteomika, speciální metody (Petrák 11/12)**

Příprava vzorků pro proteomické experimenty

Proteomika membránových proteinů

Analýza proteinových komplexů

shot-gun metody

(pros and cons)

- **až 10 000 proteinů v jednom experimentu**
- izotopická nebo label-free kvantifikace
- náročnost na instrumentaci a (bio)informatiku
- problém s inferencí proteinu (stejně peptidy v různých proteinech)
- analýza PTM je možná
- **ztráta většiny informace o proteoformách**



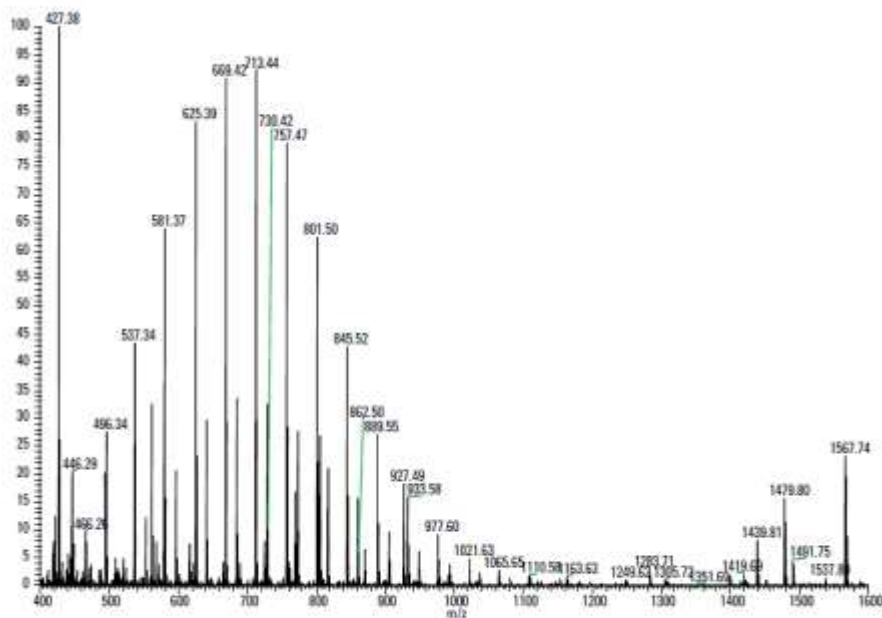
Filozofie přípravy vzorků v roztoku:

Dosáhnout rozbití buněk a maximální rozpustnosti všech bílovin/peptidů při zachování kompatibility se separační metodou a MS analýzou.

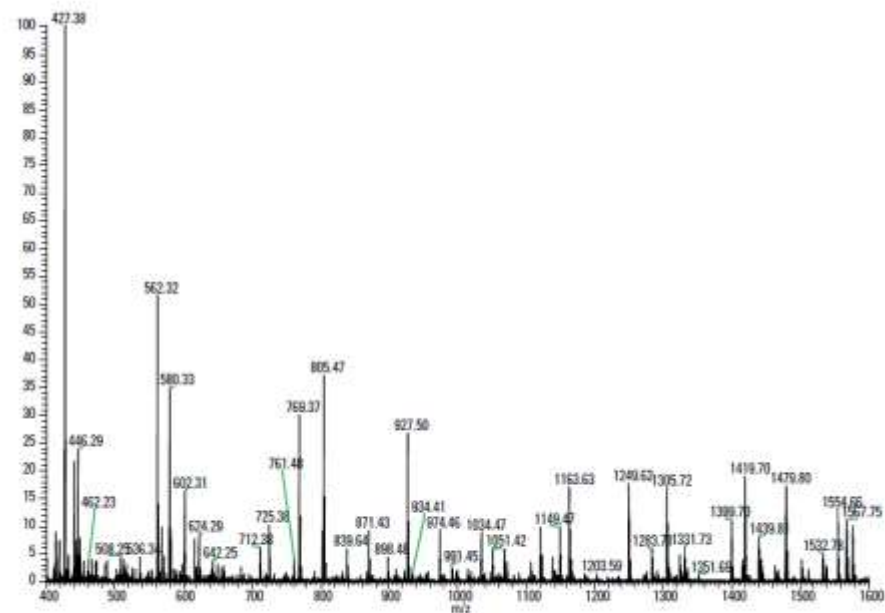
Detrgenty a močovina umožňují solubilizovat a denaturovat vzorek ale jsou nekompatibilní s digescí a/nebo LC-MS analýzou

Jak je využít a jak se jich zase rychle zbavit?

Odstranění detergentů



Triton X-100, Unprocessed



Triton X-100, Processed

Piercenet.com

Odstranění detergentů

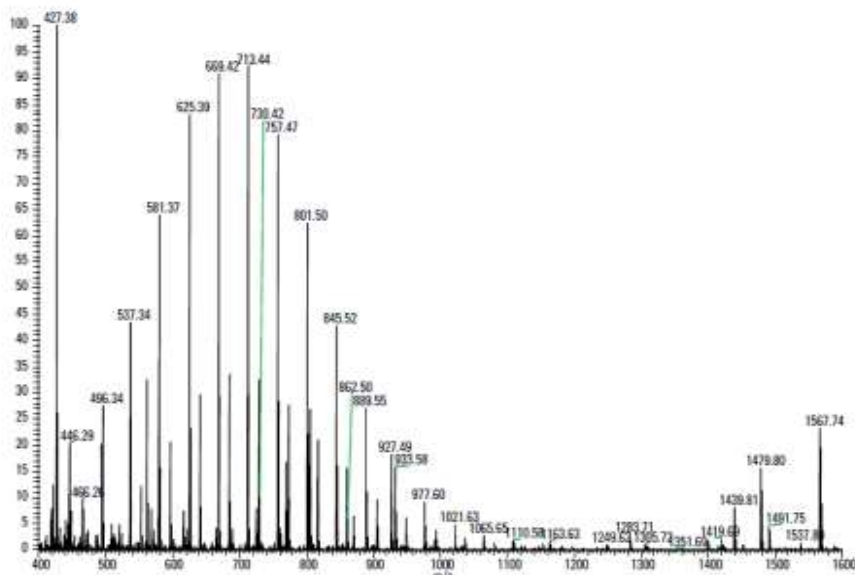
Deoxycholate (SDC)

SDS

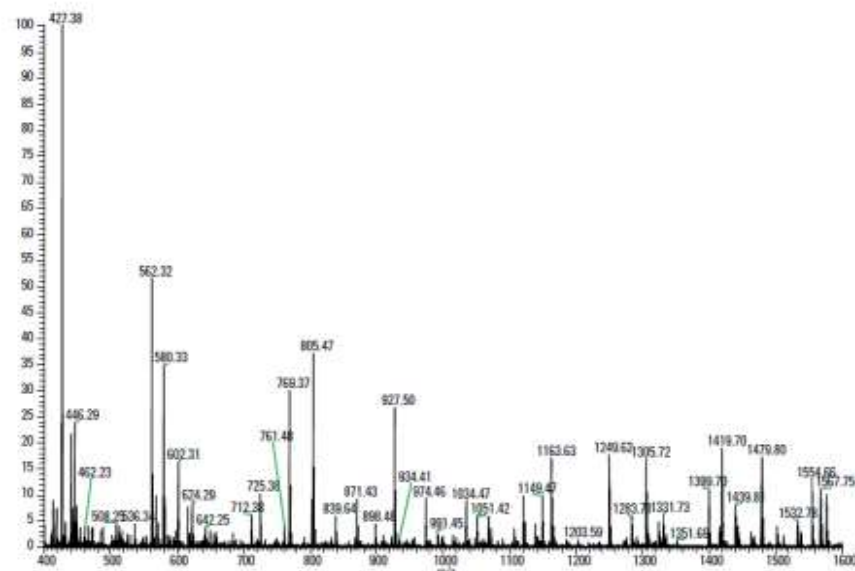
Triton X100

Rapigest

Kyselinou štěpitelné detergenty * Fázová separace * výměna pufru * SP3



Triton X-100, Unprocessed



Triton X-100, Processed

Odstranění detergentů

Deoxycholate (SDC)

Fázová separace, výměna pufru (FASP), SP3

SDS

výměna pufru (FASP)

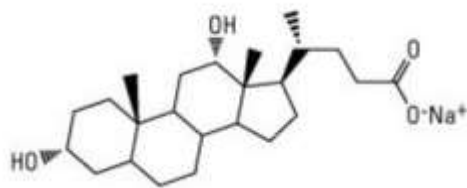
Triton X100

SP3

Odstranění detergentů

Deoxycholát sodný (SDC)

Fázová separace * výměna pufru * SP3



Deoxycholát sodný (SDC)

Silný anionický detergent,
tolerovaný trypsinem až do 1%

Lze snadno odstranit po štěpení:

- 1) Okyselení (pH ~ 2)
- 2) Přídavek octanu etylnatého 1:1
- 3) Fázový transfer (vytřepání) do octanu etylnatého
- 4) Odstranění horní fáze (octanu) a následné odsolení peptidů

Filter Assisted Sample Preparation - FASP

Odstranění SDS, SDC, ale nefunguje pro Triton

Výměna pufru, koncentrace vzorku, zbavení se detergentu, digesce

TÉMĚŘ UNIVERZÁLNÍ ŘEŠENÍ - FASP



Vhodné filtry s cut off 10-30 kDa

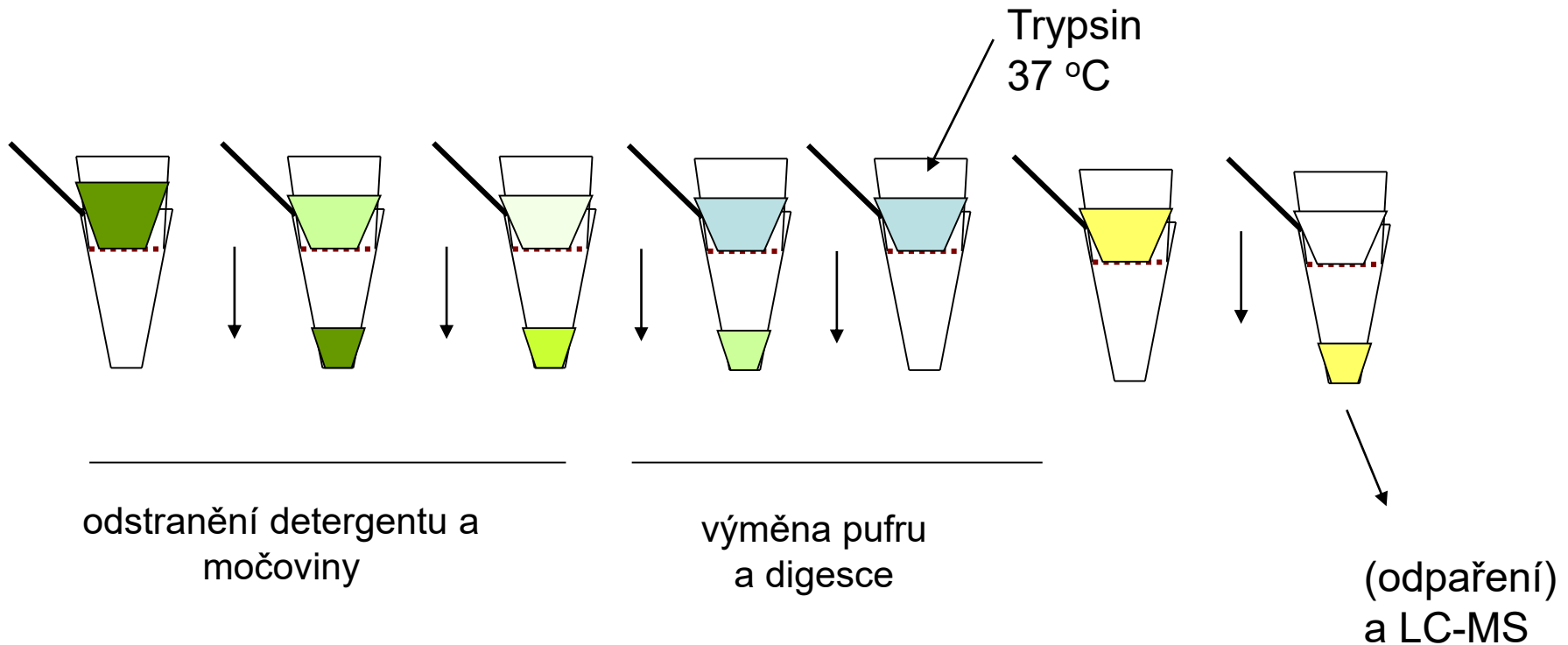
Manza LL, et al. Proteomics. 2005 May;5(7):1742-5.

Wiśniewski JR, et al. Nat Methods. 2009 May;6(5):359-62.

Filter Assisted Sample Preparation - FASP

Vzorek proteinů s vysokou koncentrací močoviny a/nebo detergentu
(nelze štěpit trypsinem)

Vhodné filtry s cut off 10-30 kDa

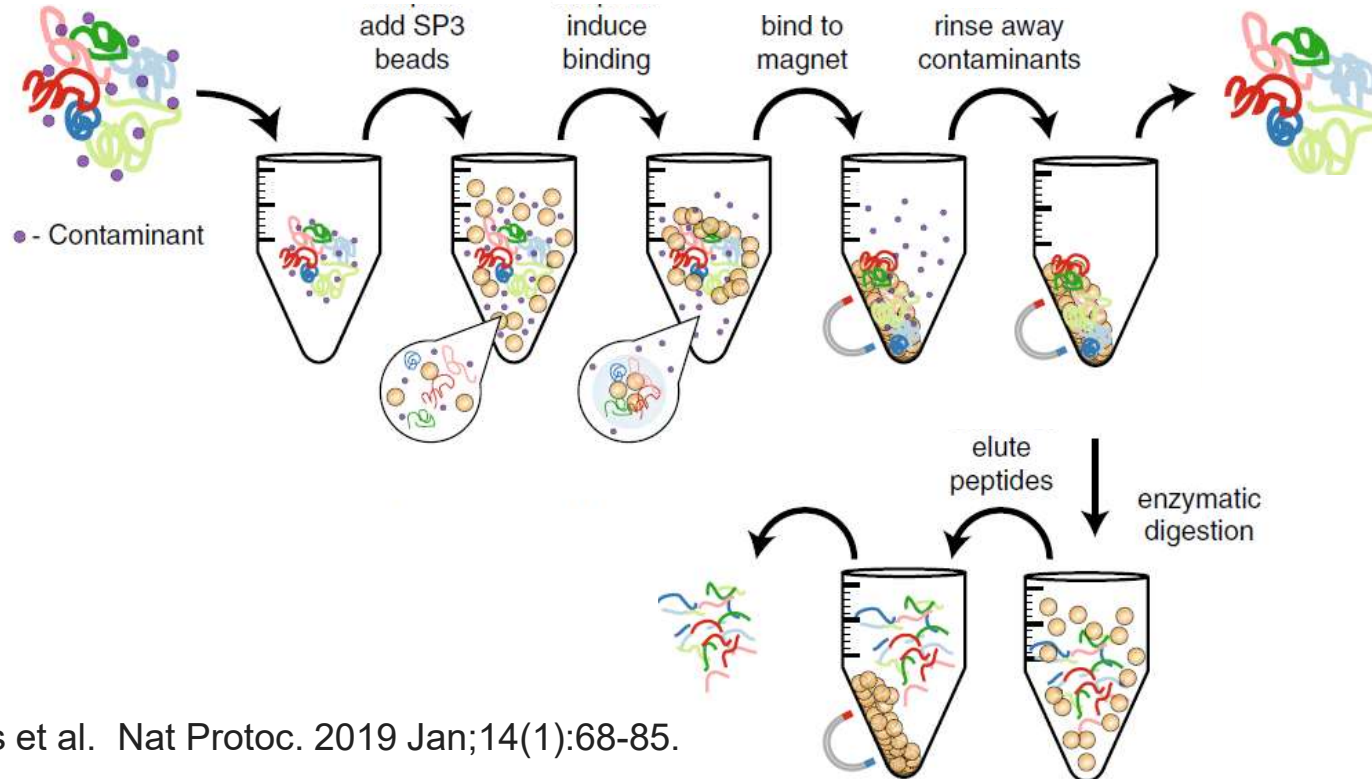


Single-pot, solid-phase-enhanced sample preparation for proteomics experiments

SP3

Odstranění SDS, SDC, Tritonu

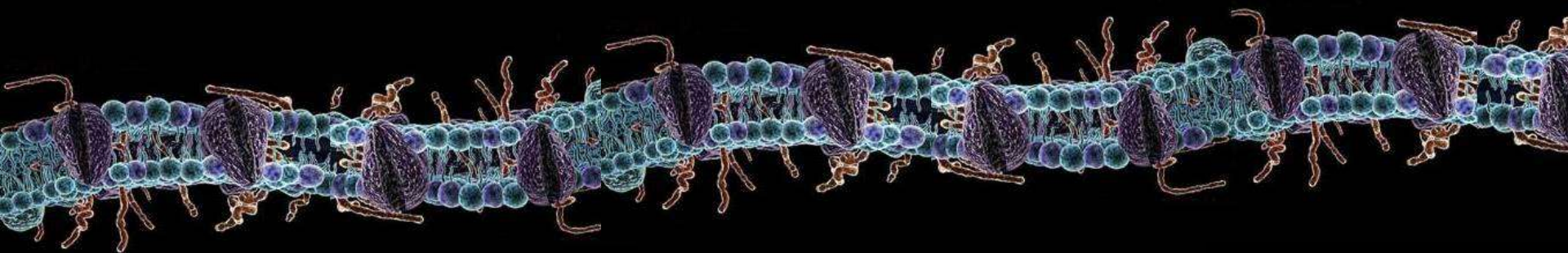
- Zachycení proteinů pomocí HILIC chromatografie na magnetických kuličkách
- odmytí detergentu
- štěpení na kuličkách
- eluce peptidů



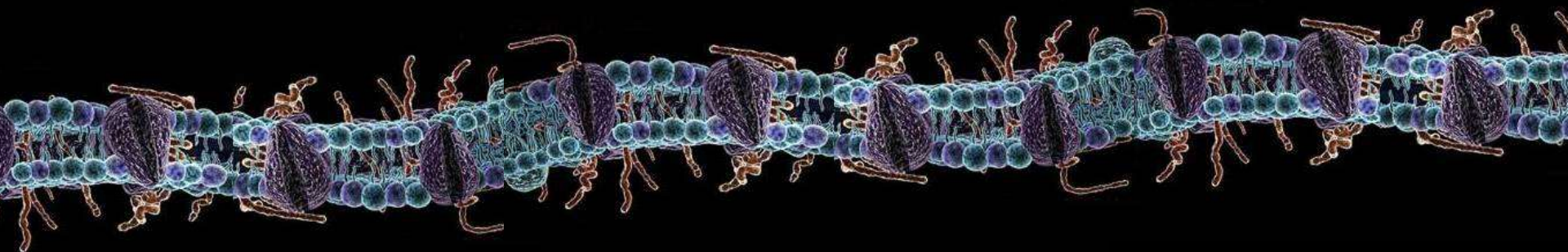
shot-gun metody

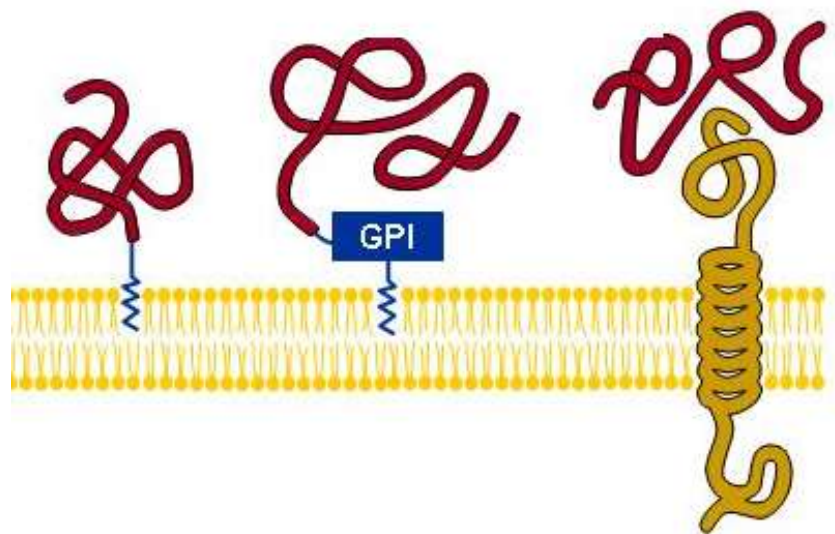
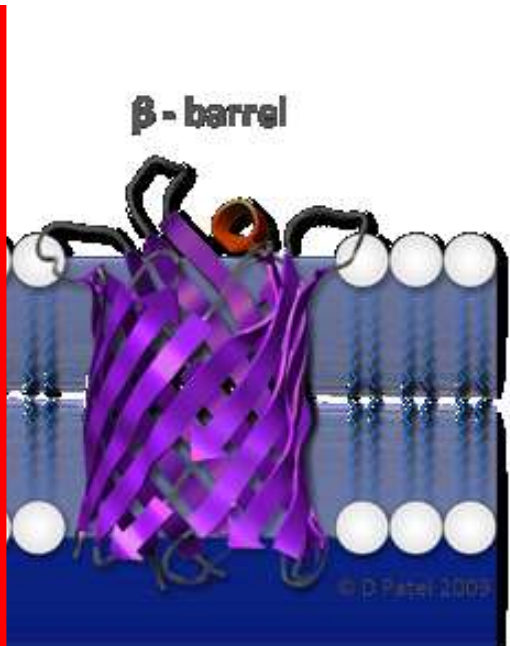
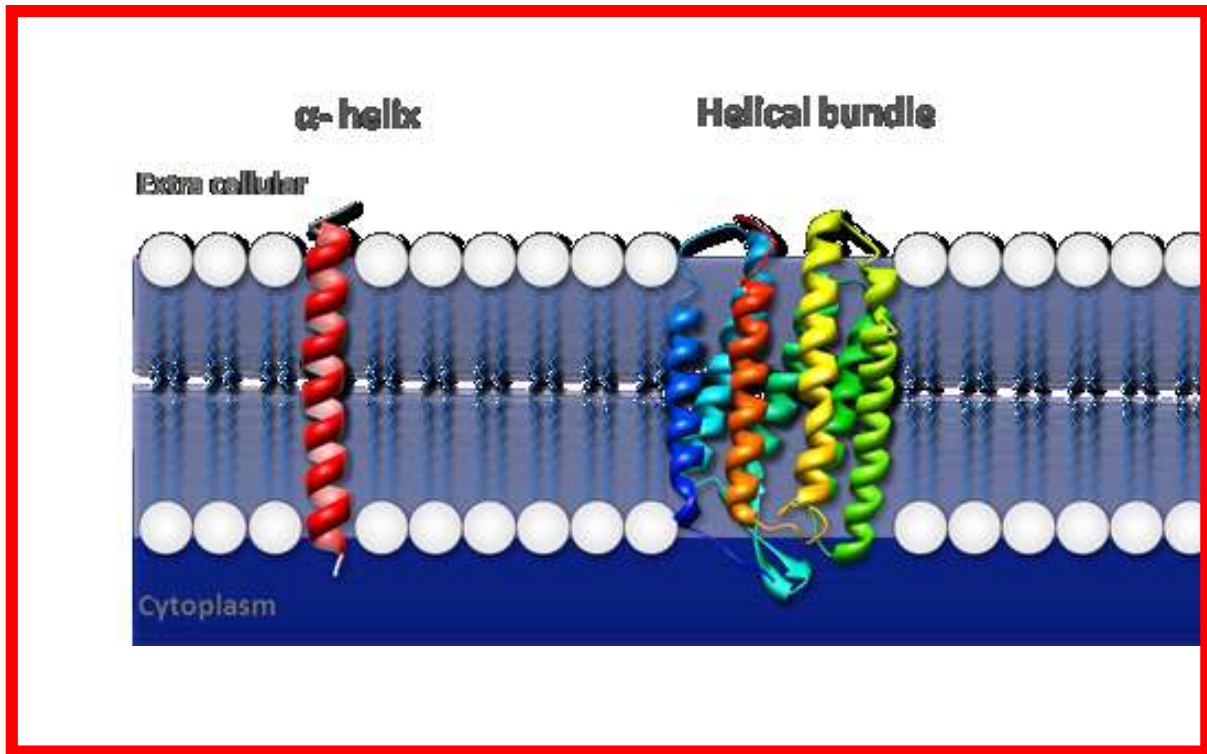
(pros and cons)

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- izotopická nebo label-free kvantifikace
- náročnost na instrumentaci a (bio)informatiku
- problém s inferencí proteinu (stejně peptidy v různých proteinech)
- analýza PTM je možná
- **ztráta většiny informace o proteoformách**
- **některé typy bílkovin jsou nedostatečně zastoupeny**

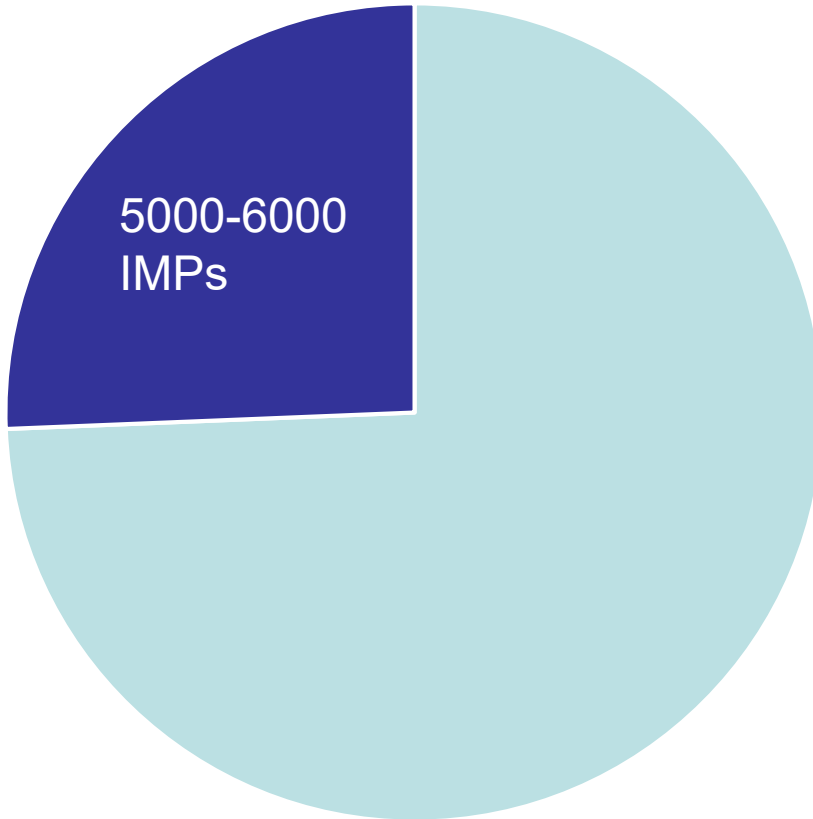


MEMBRÁNOVÉ PROTEINY

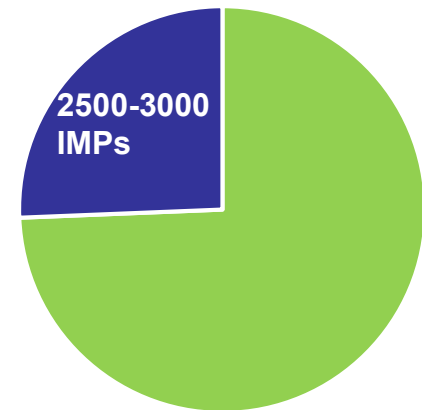


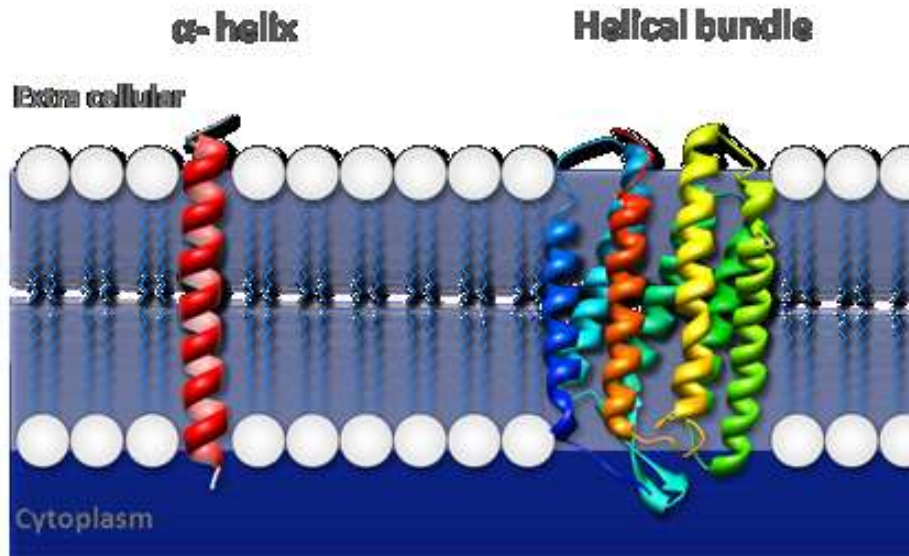


~ 20 000 human protein coding genes



10 000 genes actively expressed by an average cell type

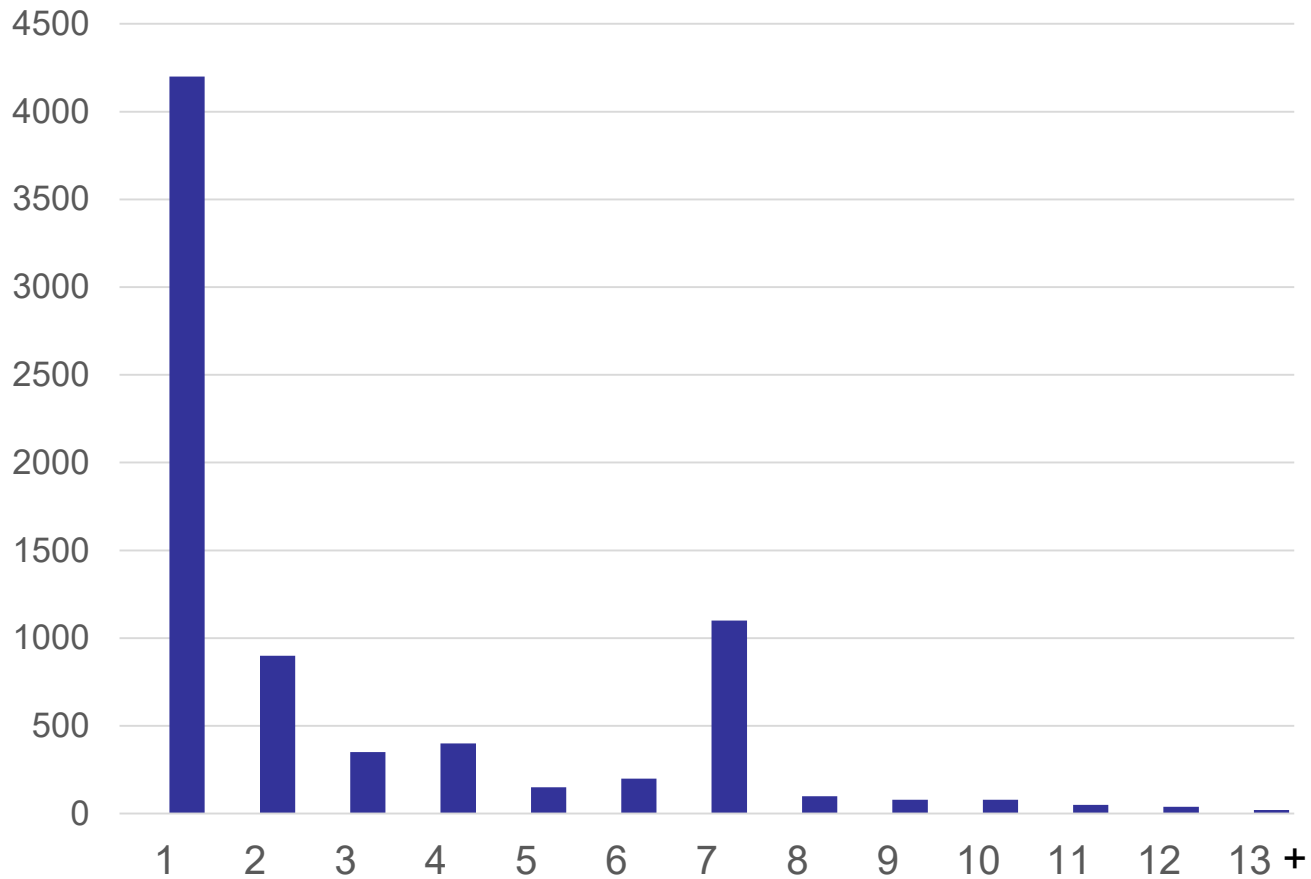


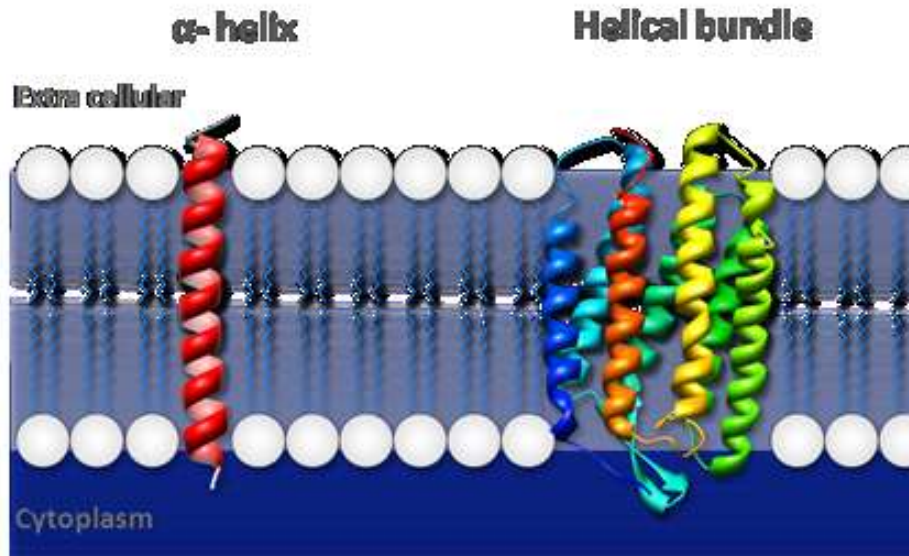


INTEGRAL MEMBRANE PROTEINS

- alpha helix TM domain(s) (20-25 AA) + soluble domains

Number of predicted TM segments in human integral membrane proteins

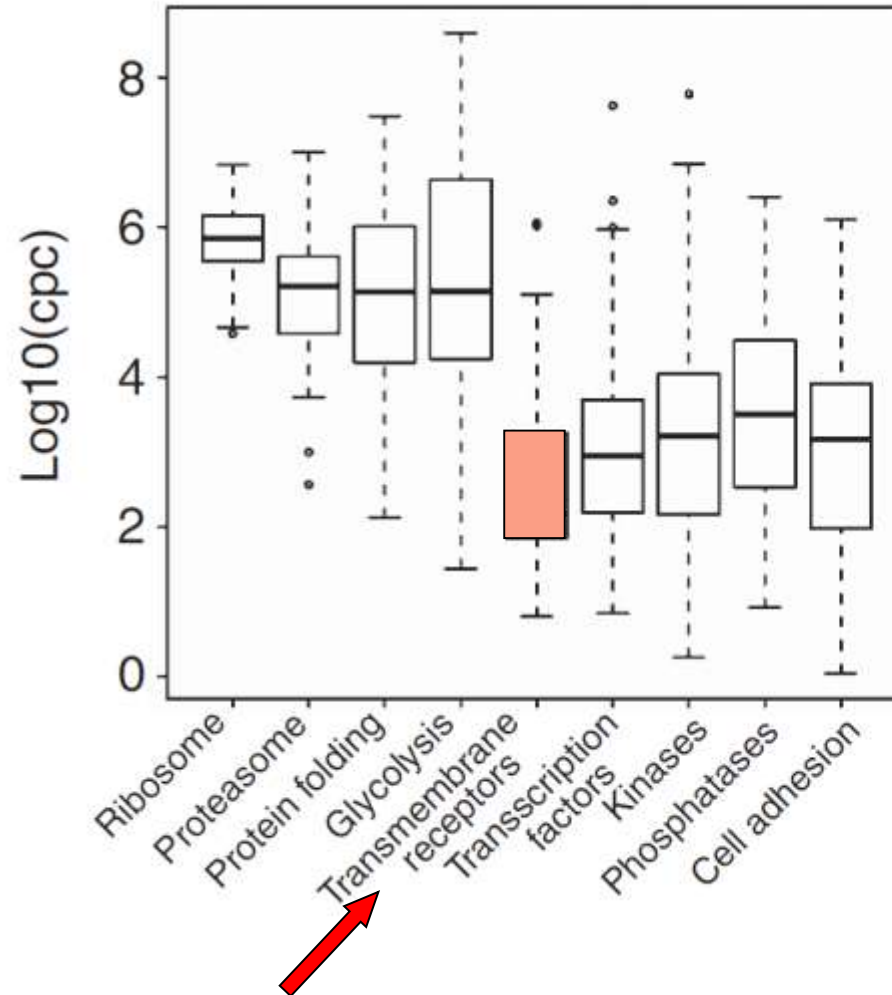


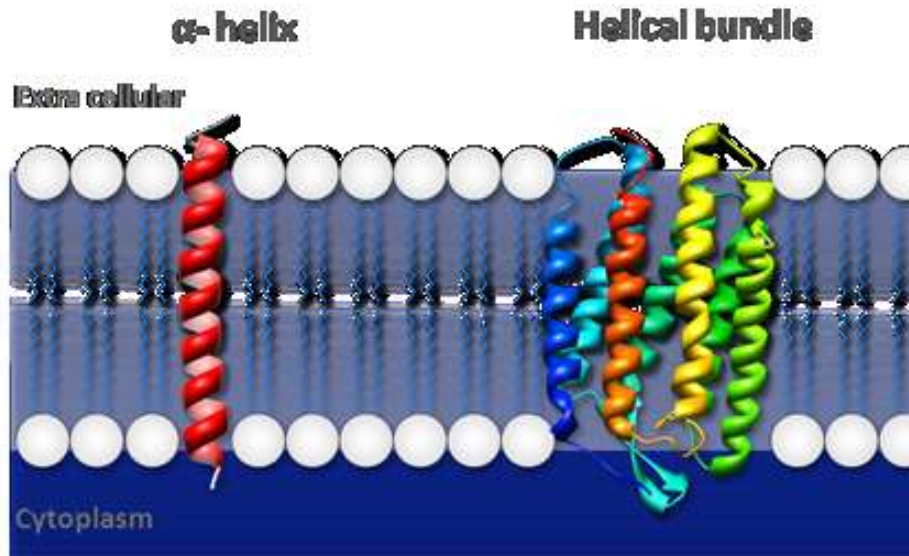


INTEGRAL MEMBRANE PROTEINS

- alpha helix TM domain(s) (20-25 AA) + soluble domains
- low expression

Cellular abundance of transmembrane proteins is LOW (100-1000 copies/cell)





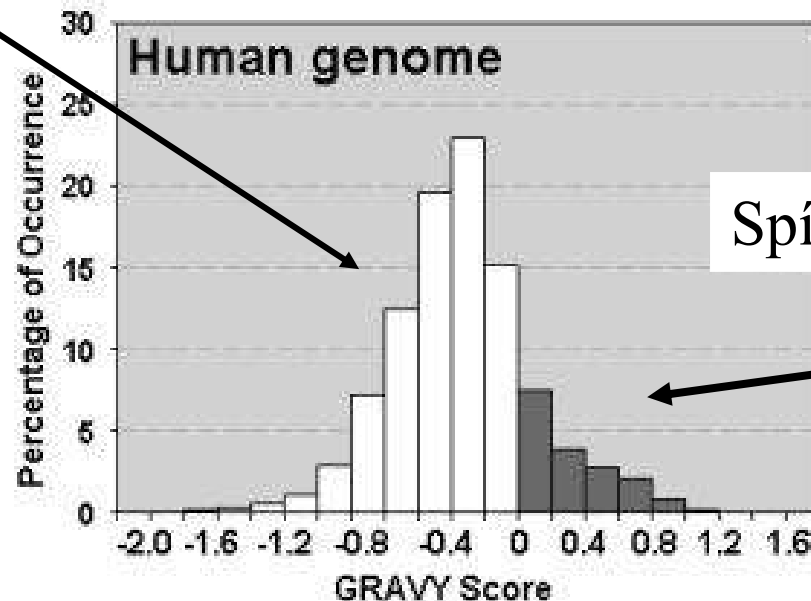
INTEGRAL MEMBRANE PROTEINS

- alpha helix TM domain(s) (20-25 AA) + soluble domains
- low expression
- hydrophobic/amphipathic nature

Hydrofobicita proteinu

GRAVY SCORE – Grand average hydropathy
(součet „hydrofobicity“ (-4.6 až 4.6) jednotlivých aminokyselin
dělený počtem aminokyselin)

Spíš rozpustné



Spíš transmembránové

Kyte, J., and Doolittle, R.F. (1982) J.Mol.Biol. 157, 105-132

Hydrofobicita proteinu

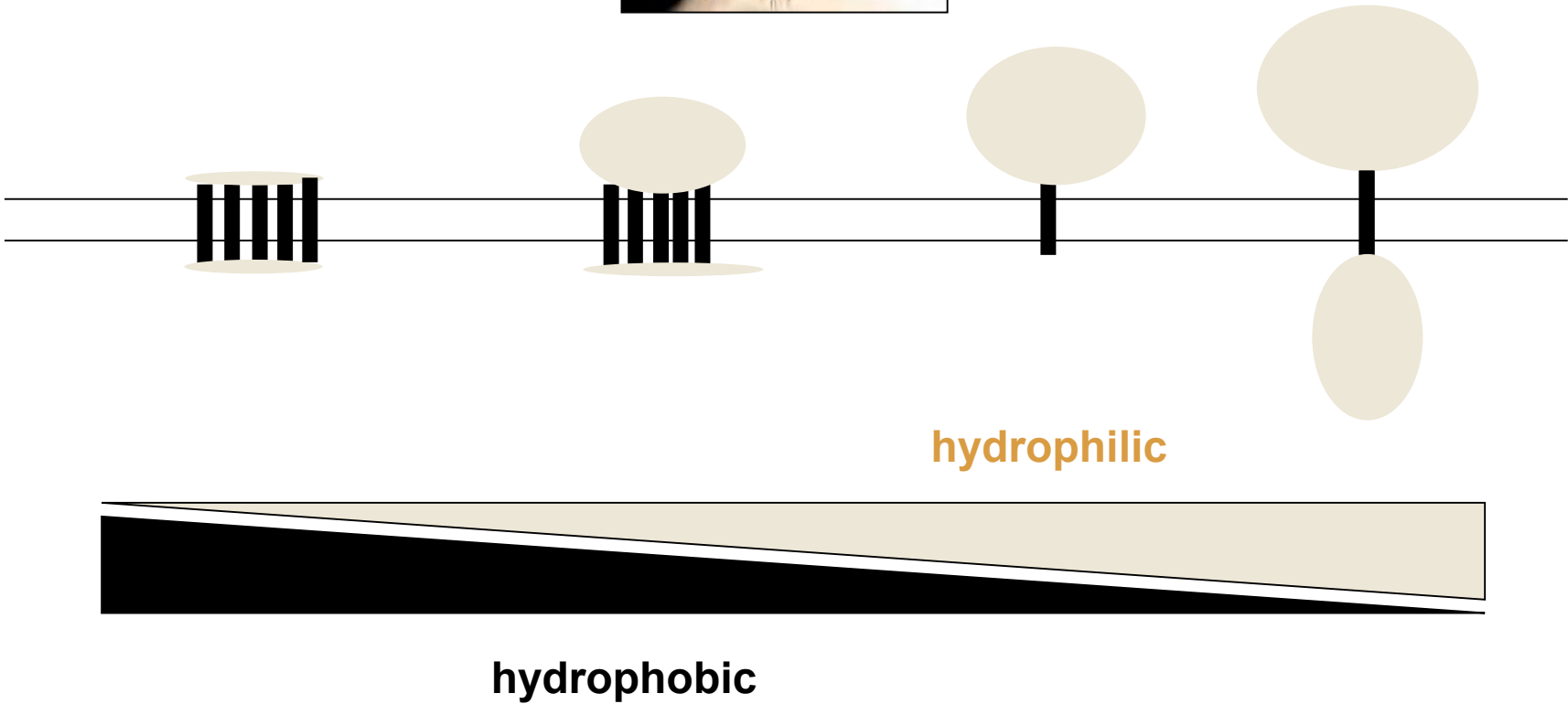
Amino Acid Name	One Letter Code	Hydropathy Score
Isoleucine	I	4.5
Valine	V	4.2
Leucine	L	3.8
Phenylalanine	F	2.8
Cysteine	C	2.5
Methionine	M	1.9
Alanine	A	1.8
Glycine	G	-0.4
Threonine	T	-0.7
Tryptophan	W	-0.9
Serine	S	-0.8
Tyrosine	Y	-1.3
Proline	P	-1.6
Histidine	H	-3.2
Glutamic acid	E	-3.5
Glutamine	Q	-3.5
Aspartic acid	D	-3.5
Asparagine	N	-3.5
Lysine	K	-3.9
Arginine	R	-4.5

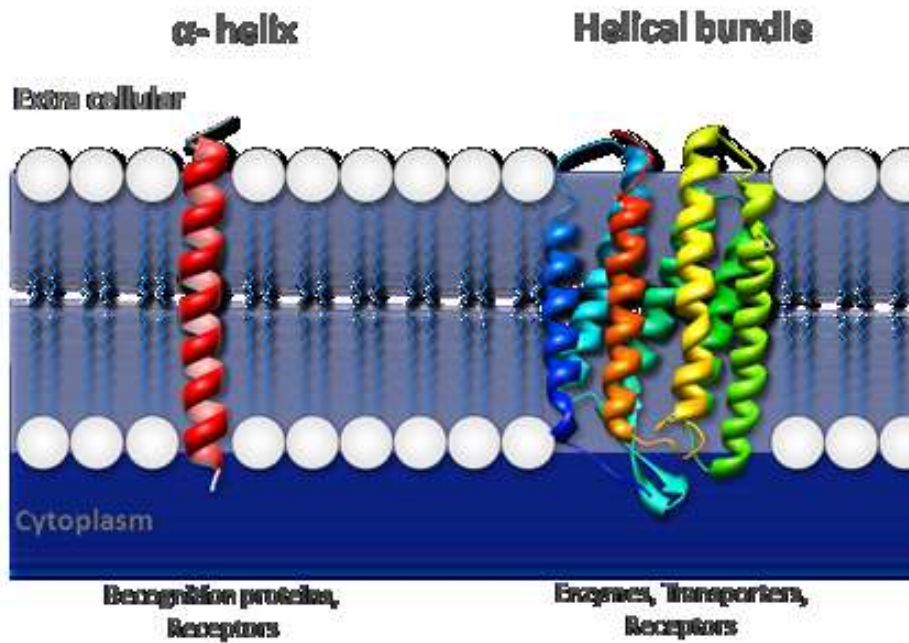
GRAVY SCORE – Grand average
hydropathy
(součet „hydrofobicity“ (-4.5 až 4.5)
jednotlivých aminokyselin dělený
počtem aminokyselin)

Aminokyseliny typické pro α -helixy:

„**FAMILY VW**“ (+S)

IMPs - molecules with split personalities

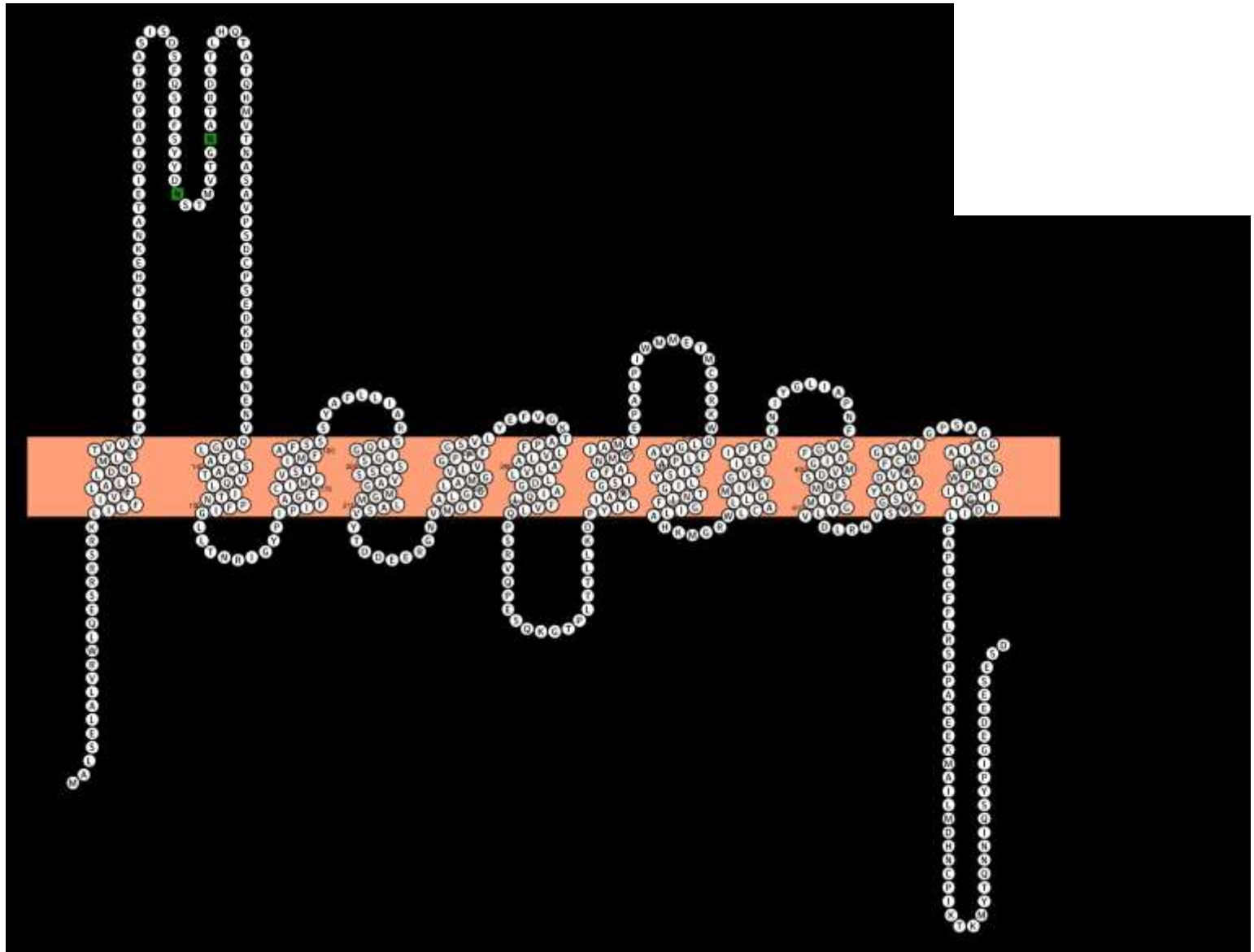




INTEGRAL MEMBRANE PROTEINS

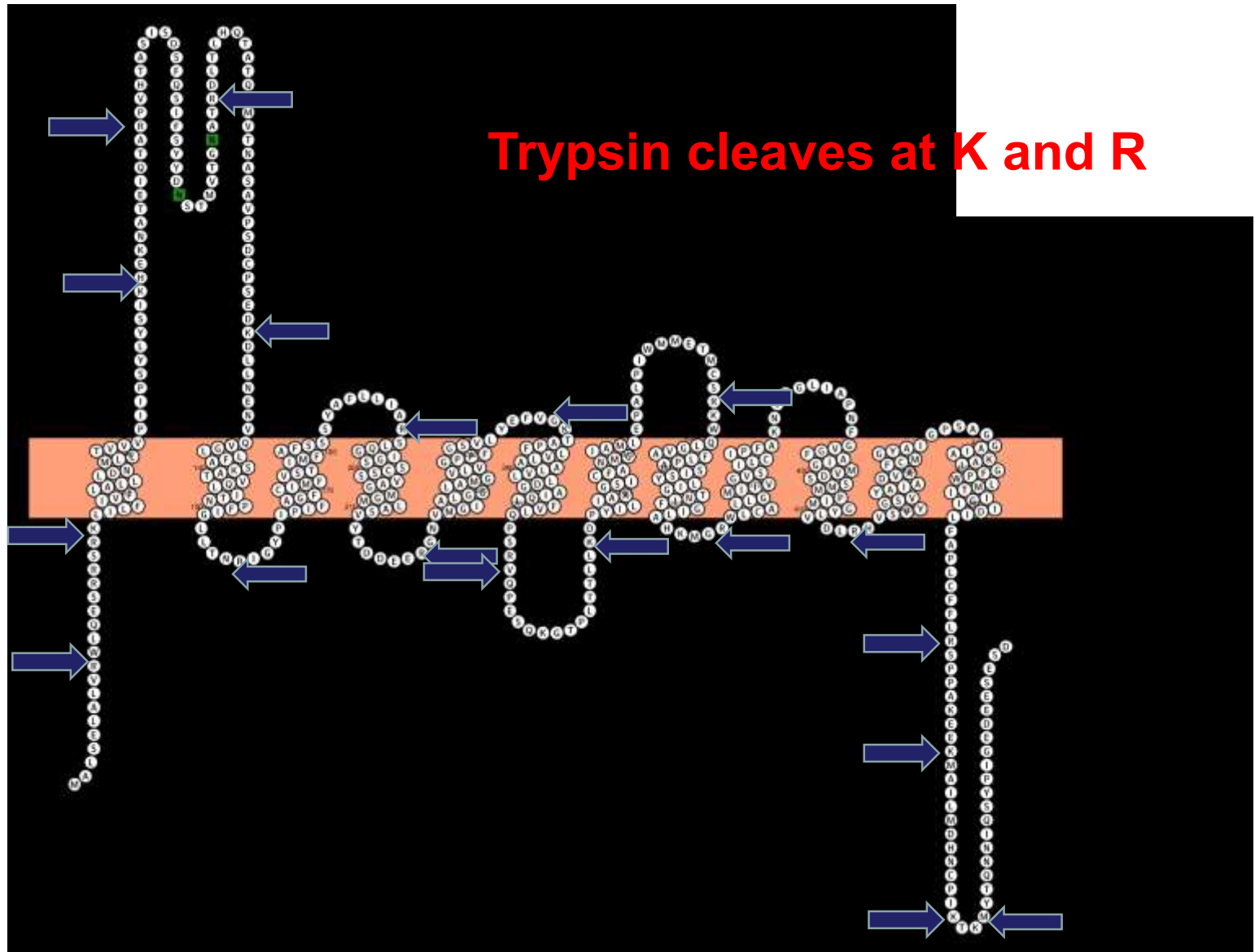
- alpha helix TM domain(s) (20-25 AA) + soluble domains
- low expression
- hydrophobic/amphipathic nature
- no Arg, Lys in TM domains

Synaptic vesicular amine transporter (Slc18a2)

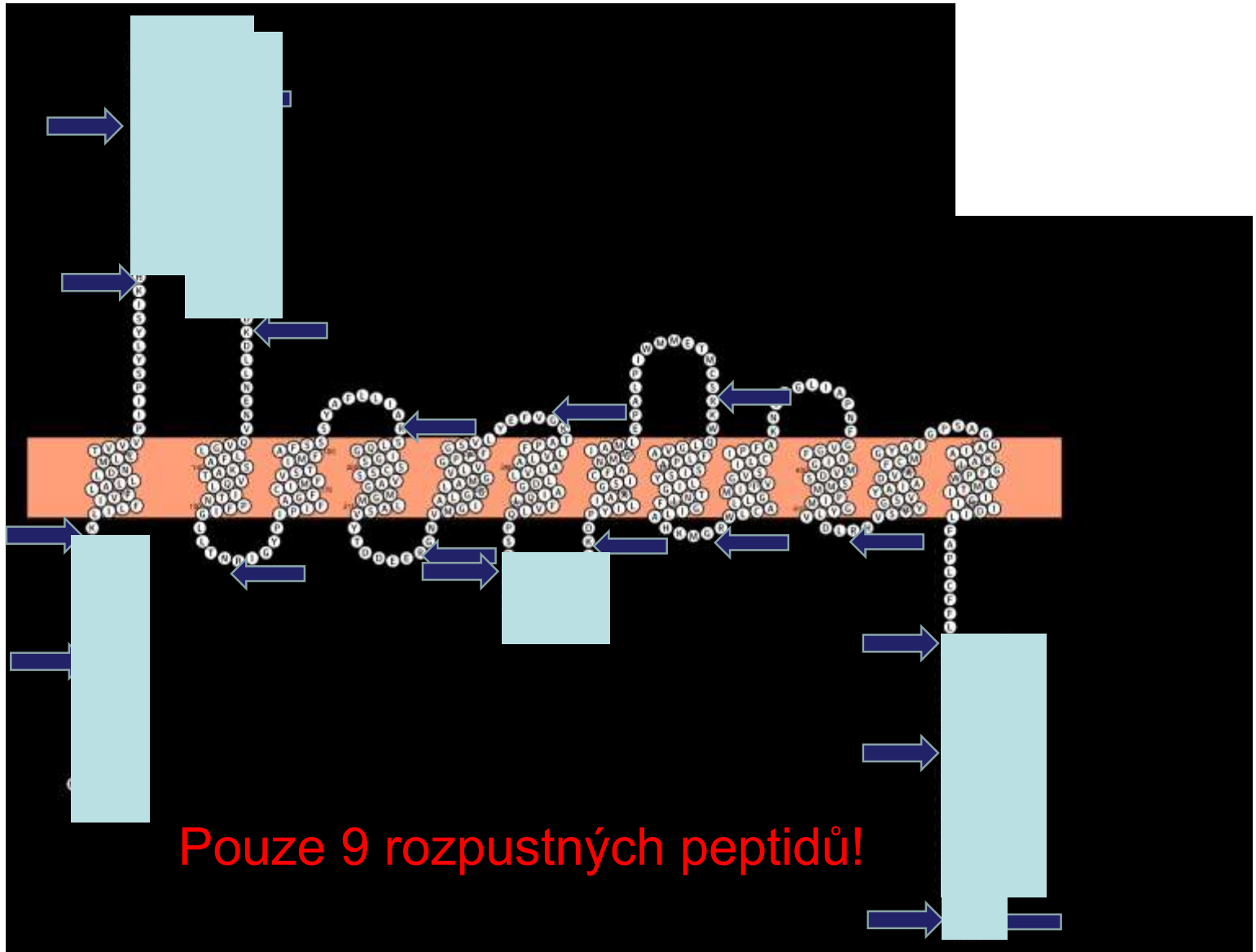


Visualization by Protter (Omasits et al., *Bioinformatics*. 2013)

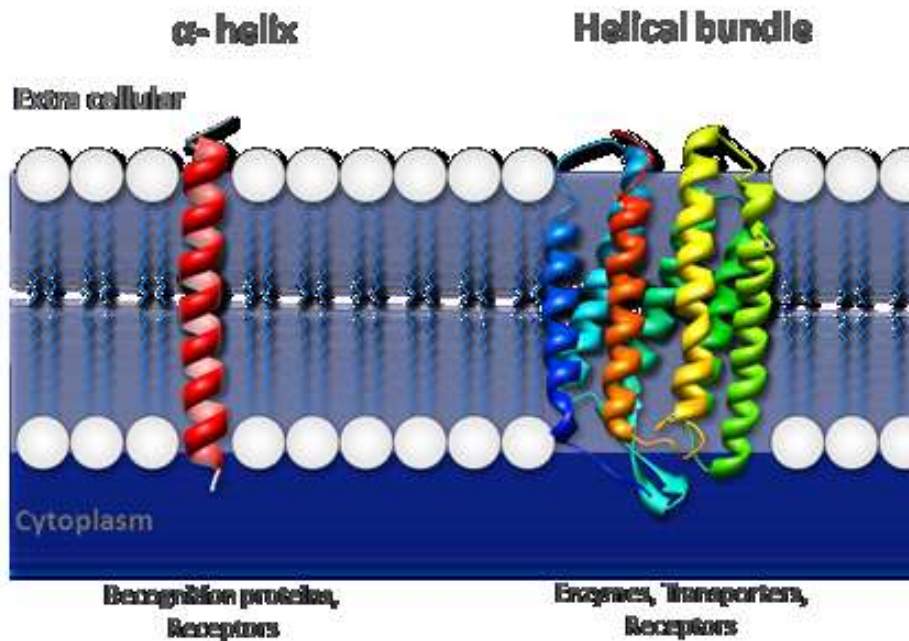
Synaptic vesicular amine transporter (Slc18a2)



Synaptic vesicular amine transporter (Slc18a2)



Pouze 9 rozpustných peptidů!



INTEGRAL MEMBRANE PROTEINS

- alpha helix TM domain(s) (20-25 AA) + soluble domains
- low expression
- hydrophobic/amphipathic nature
- no Arg, Lys in TM domains
- detergents interfere with digestion and/or LC-MS
- **UNDER-REPRESENTED IN PROTEOMIC ANALYSES**

Překonávání nízké abundance a amfipatie TM proteinů

Izolace (obohacení) membrán

- centrifugace

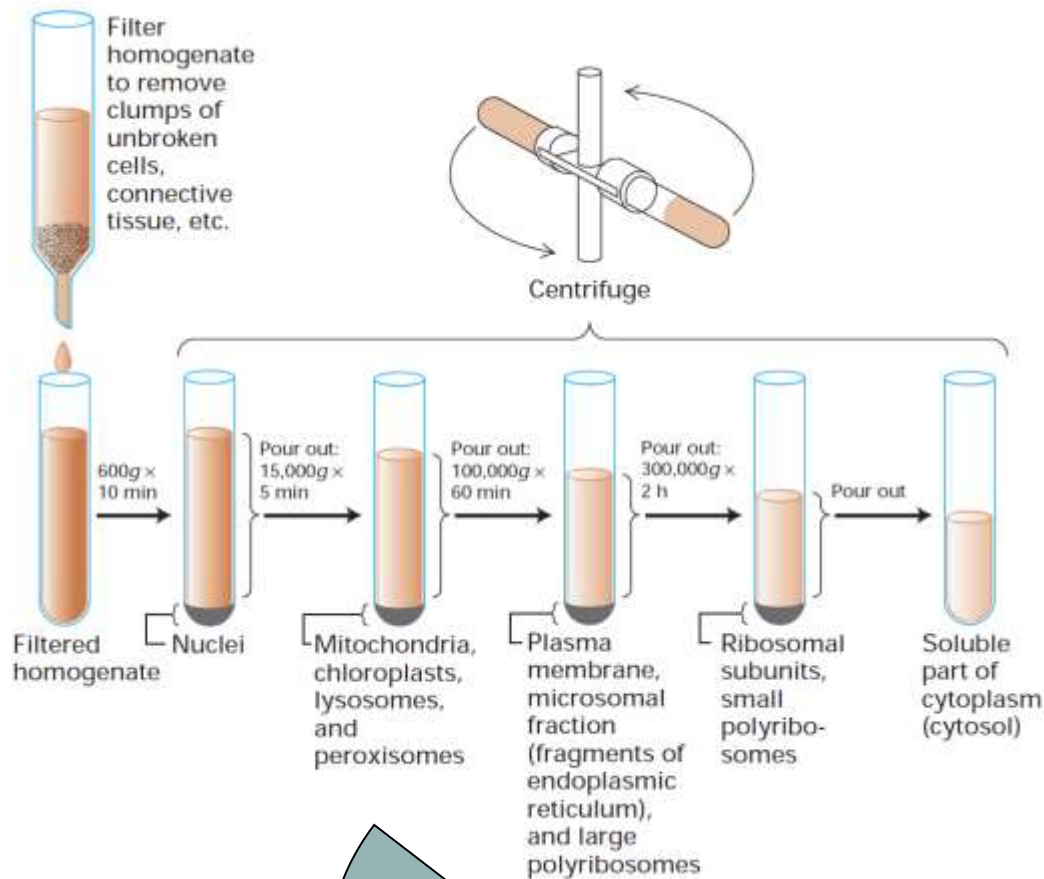
Obohacení/izolace TM proteinů

- „carbonate stripping“ - uhličitan sodný, vysoké pH
- delipidace (MetOH/chloroform)
- izolace povrchově značených (Ab, biotin, hydrazid, lektiny)

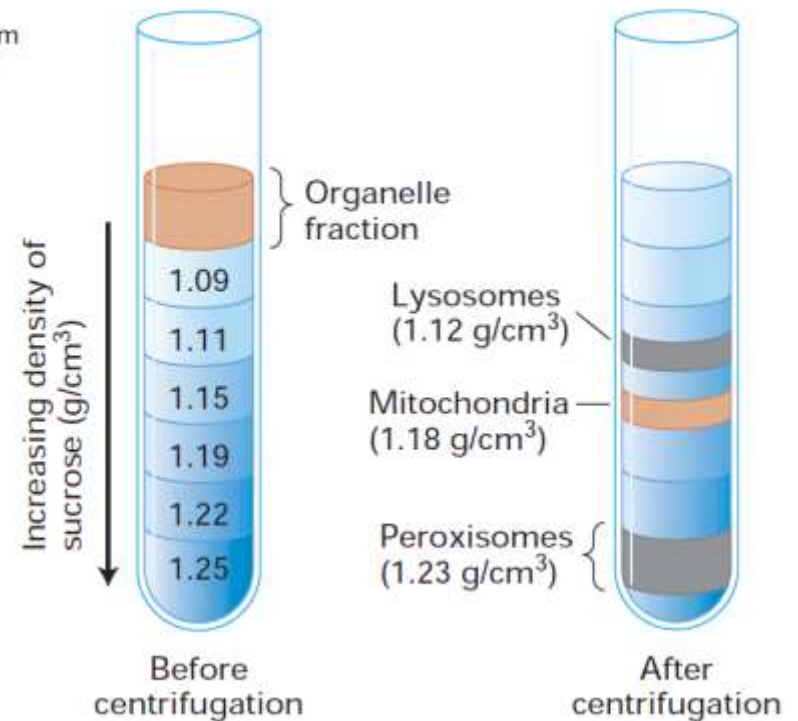
Solubilizace TM

- chaotropy
- detergenty (častá nekompatibilita s digescí, LC a MS!)
- organická rozpouštědla

Obohacení „membránových“ frakcí



- + majoritní cytosolické proteiny
- + cytoskelet
- + proteiny asociované s membránou





**Standard strategy
(targets intact proteins)**

Membrane fraction



Solubilization



Tryptic digestion



LC-MS/MS

**LOW ENRICHMENT
(10-15%)**

„Divide and conquer“
metody



**Standard strategy
(targets intact proteins)**



**Only the hydrophilic
segments**

Membrane fraction



Solubilization

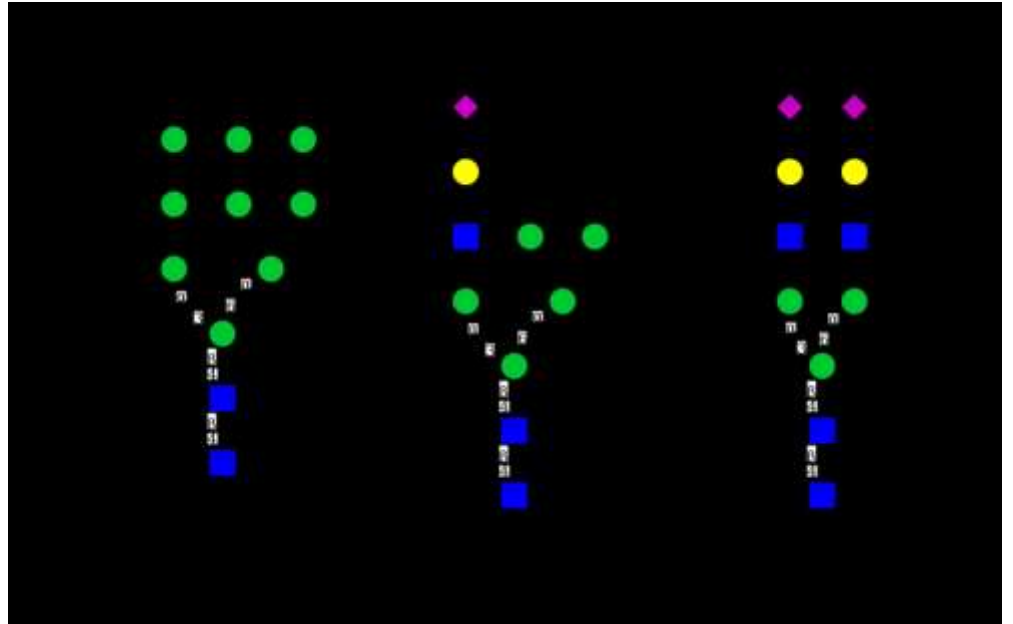
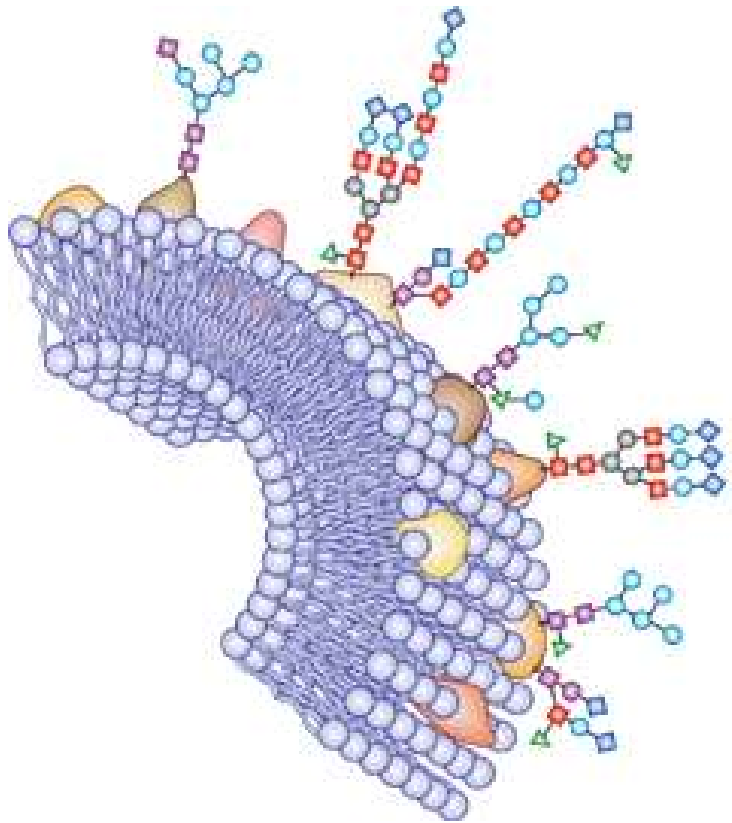


Tryptic digestion



LC-MS/MS

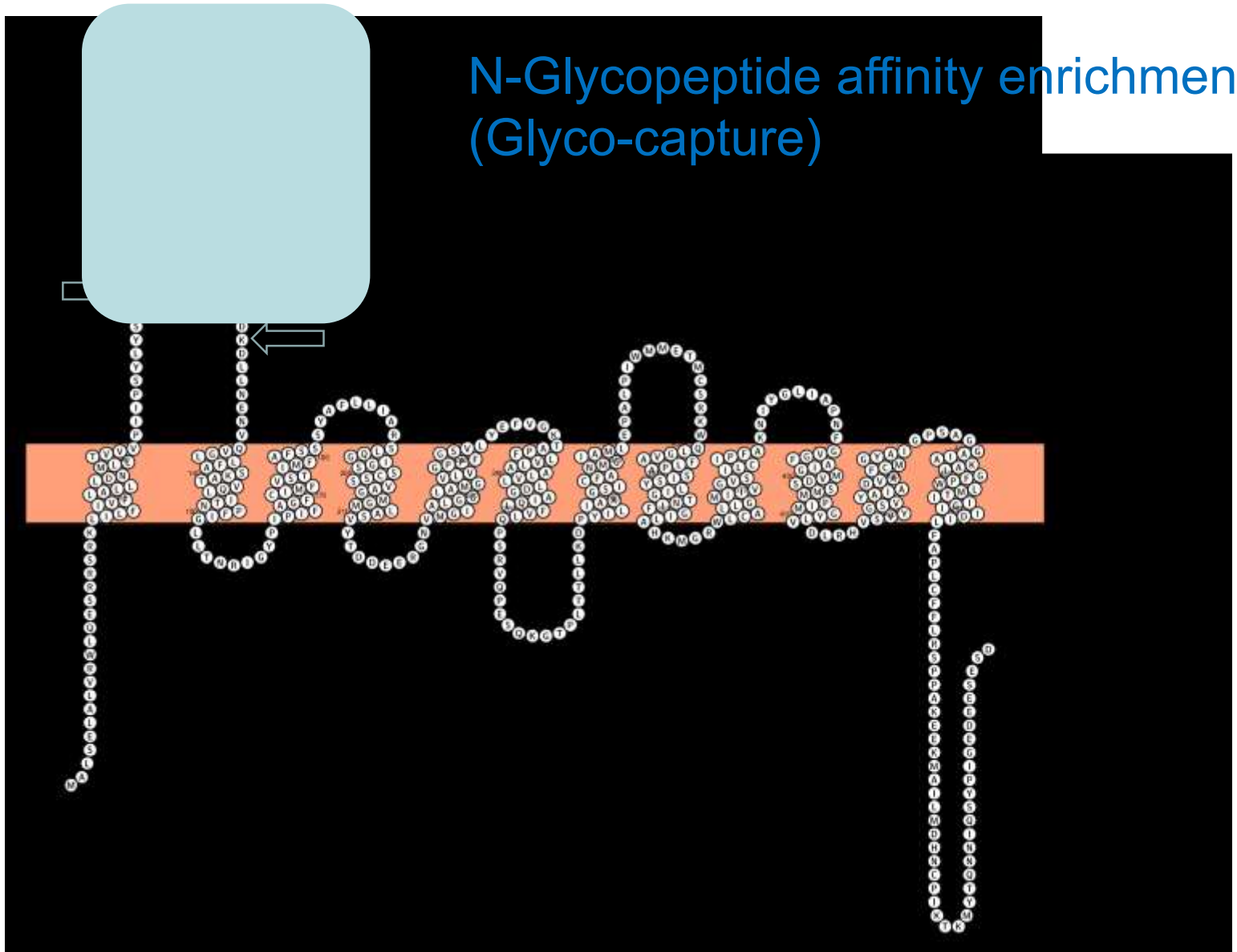
**LOW ENRICHMENT
(10-15%)**



Asn-Xaa-Ser/Thr

“DIVIDE AND CONQUER“ METHODS

N-Glycopeptide affinity enrichment
(Glyco-capture)



N-GLYCOCAPTURE

Capture of N-glycopeptides
by immobilized **LECTINS**



Peptides released
by PNGase F
N-Glyco-FASP

Zielinska et al. Cell, 2010

Capture of N-glycopeptides
using **hydrazide chemistry**



Peptides released
by PNGase F
SPEG

Zhang et al.
Nature Biotechnology, 2003

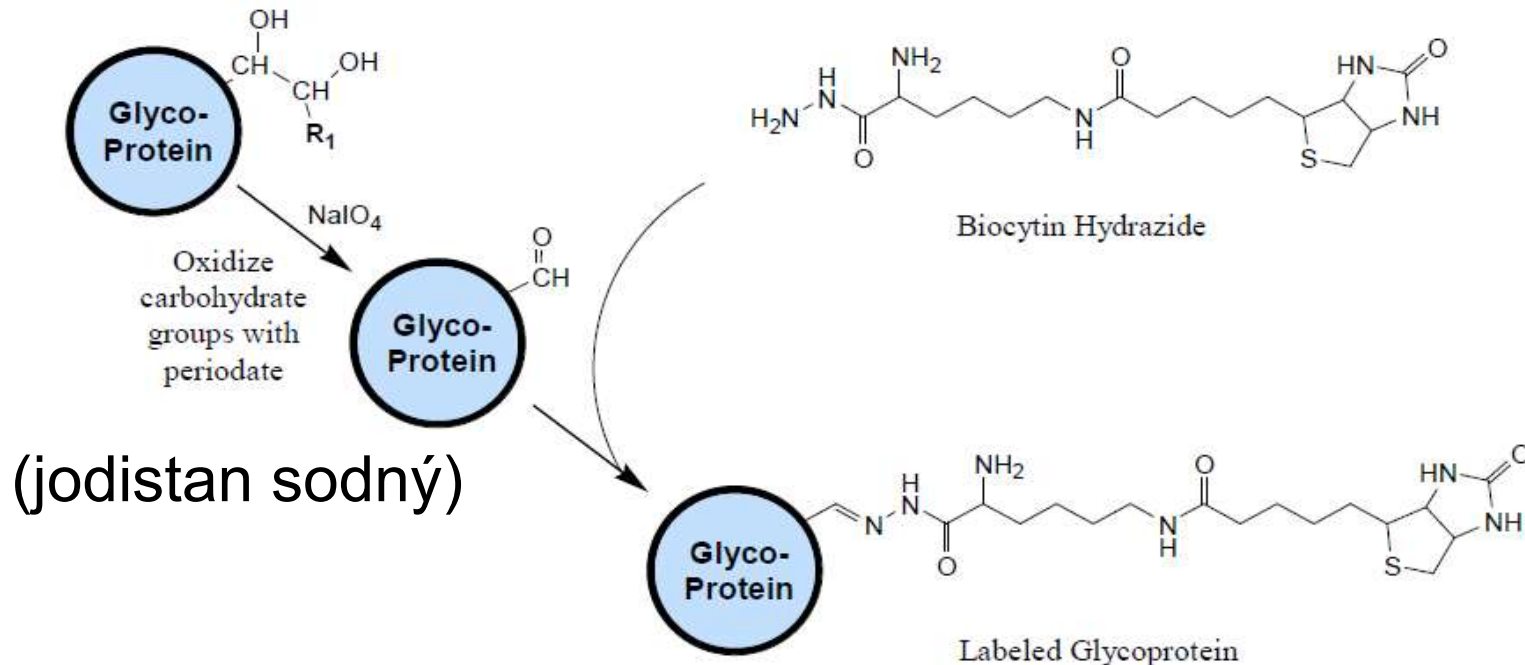
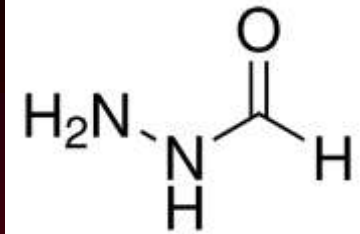
Glyco-capture

záchyt glykopeptidů z lyzátu

GLYCO-FASP — po digesci vzorku pomocí FASP jsou peptidy smíchány na filtru s **lektiny**. Neglykosylované peptidy jsou odmyty a glykopeptidy následně uvolněny PNGázou (**concanavalin A, WGA (wheat germ agglutinin), RCA (Ricinus communis agglutinin)**)

Cukry

hydrazid



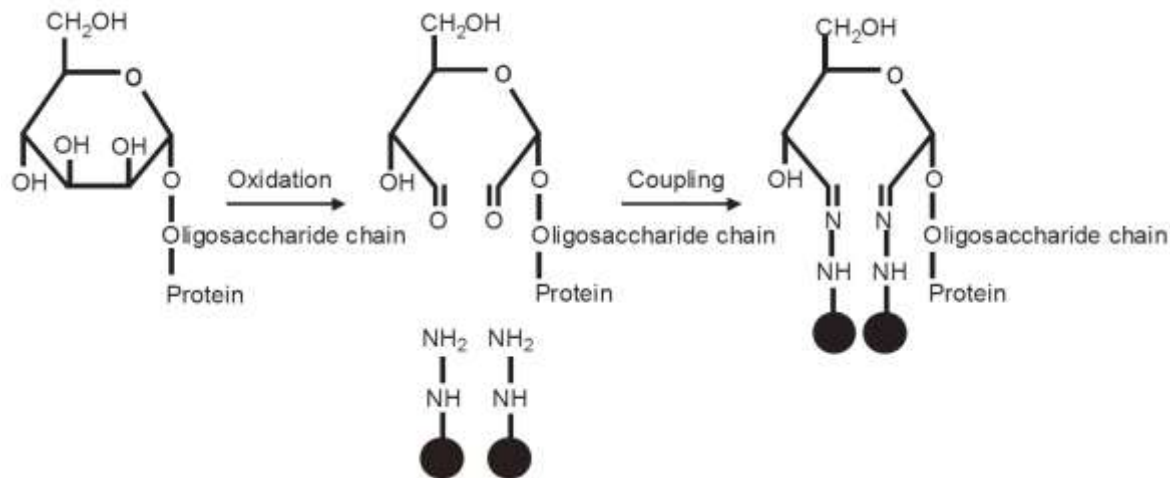
Biotin Hydrazide **bind to oxidized carbohydrates through the hydrazide group ($-\text{NH}-\text{NH}_2$), forming a hydrazone linkage.** Oxidation of glycoproteins generates reactive aldehydes that react specifically with hydrazide groups.

Glyco-capture

záchyt glykopeptidů z lyzátu

GLYCO-FASP — po digesci vzorku pomocí FASP jsou peptidy smíchány na filtru s **lektiny**. Neglykosylované peptidy jsou odmyty a glykopeptidy následně uvolněny PNGázou.
(**concanavalin A**, **WGA (wheat germ agglutinin)**, **RCA (Ricinus communis agglutinin)**)

SPEG — vychytání **oxidovaných** glykopeptidů na **kuličky s hydrazidem**, odmytí neglykosylovaných peptidů a následná eluce PNGázou





Classic strategy

Membrane fraction



Solubilization



Tryptic digestion



LC-MS/MS



Only the hydrophilic segments (GLYCOCAPTURE)

Solubilization



Digestion



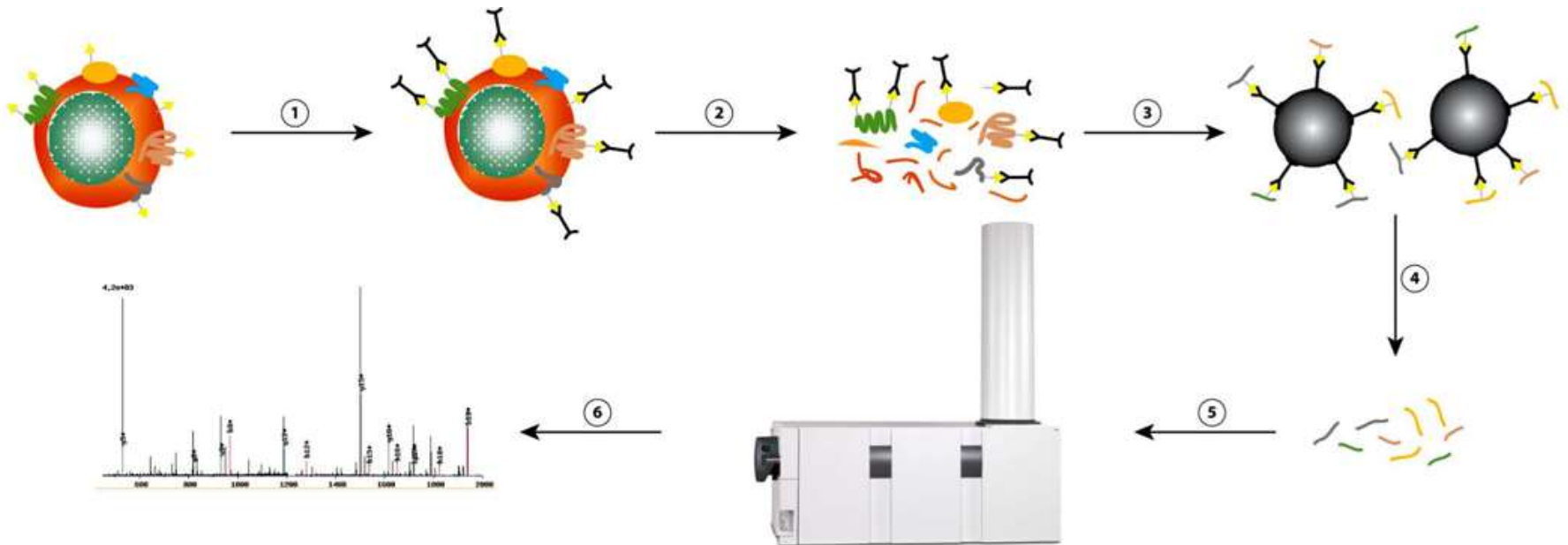
Isolation of N-glycopeptides



LC-MS/MS

Cell Surface Capturing

biotin hydrazide and avidin beads



200-800 identified cell surface membrane proteins



Only the hydrophobic segments

?



Classic strategy

Membrane fraction



Solubilization



Tryptic digestion



LC-MS/MS



**Only the hydrophilic segments
(GLYCOCAPTURE)**

Solubilization



Digestion



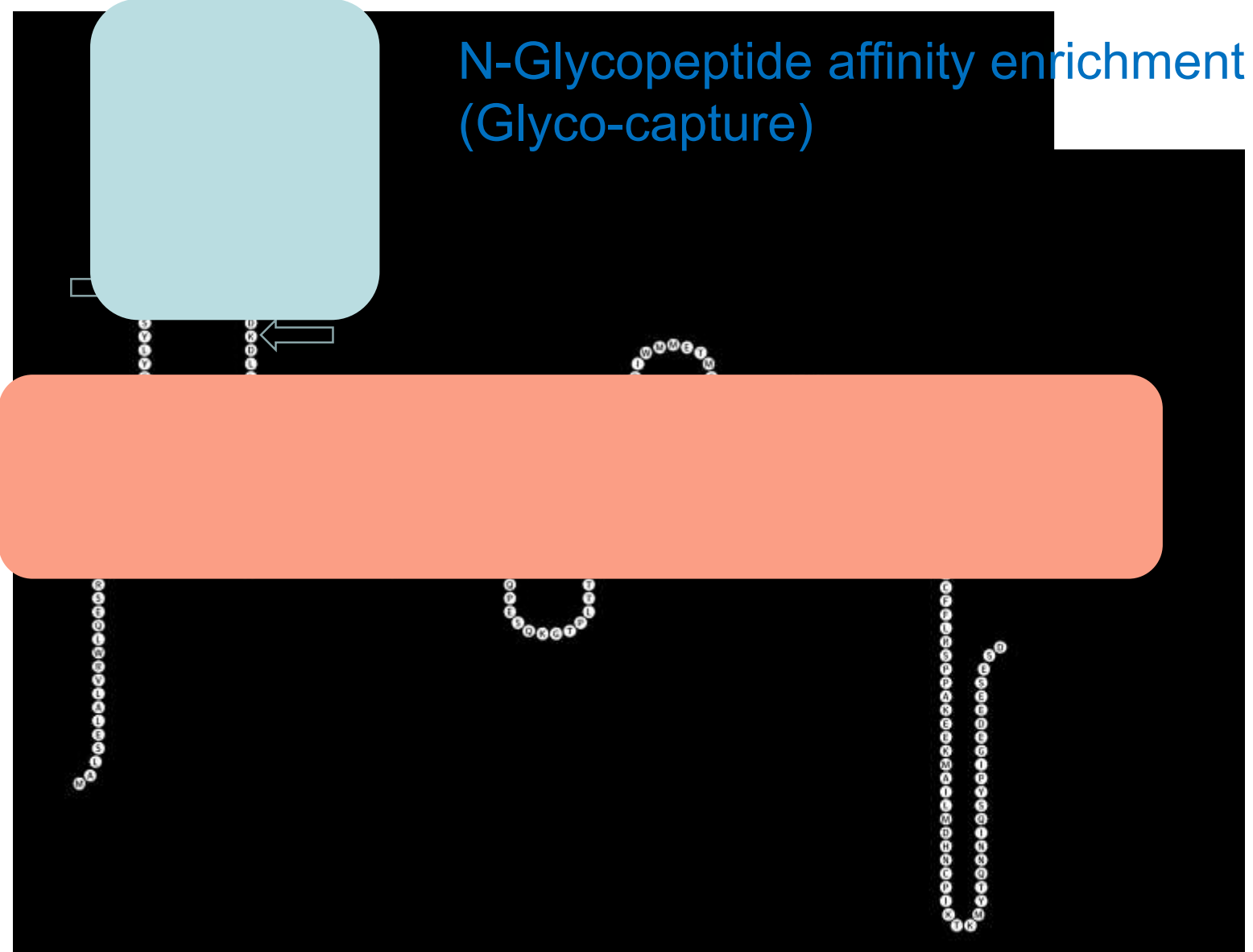
Isolation of N-glycopeptides



LC-MS/MS

“DIVIDE AND CONQUER“ METHODS

N-Glycopeptide affinity enrichment
(Glyco-capture)



Identification of IMPs via
enrichment of membrane-embedded segments
hpTC method
(High pH-Trypsin-CNBr)

Vit O, et al. J Proteomics. 2016, 21;149:15-22.

Blackler AR, et al. J Proteome Res. 2008, 7(7):3028-34.

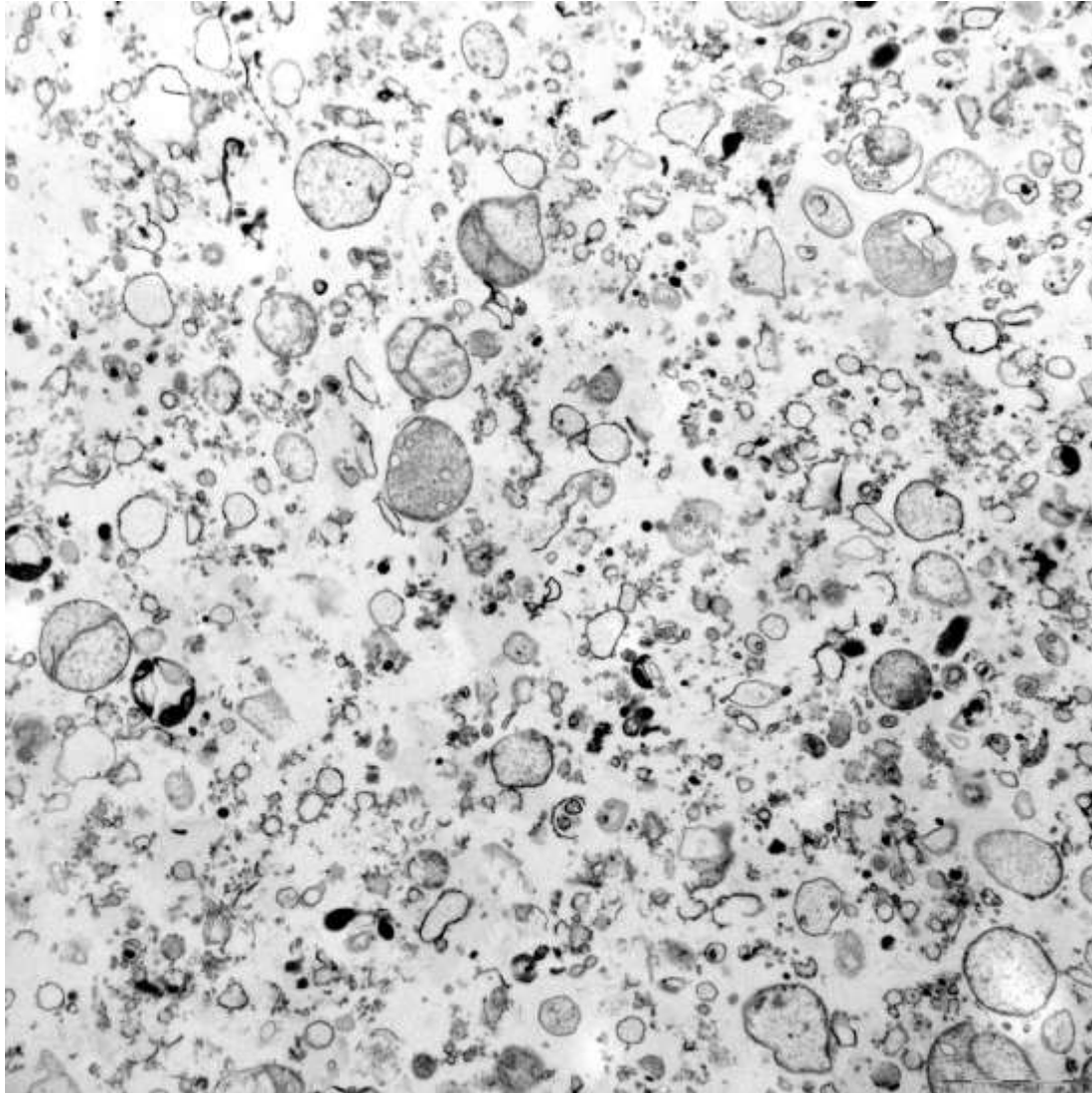
hpTC method (high pH-Trypsin-CNBr)



Membrane-enriched fraction

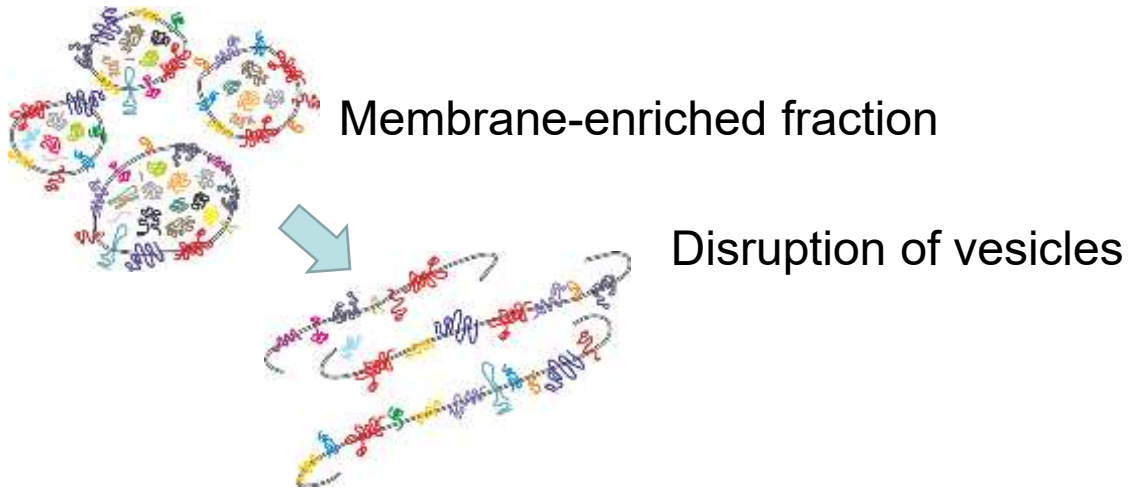


Vit O. et al., *Journal of Proteomics* 2016
Blackler A et al., *J Proteome Res.* 2008

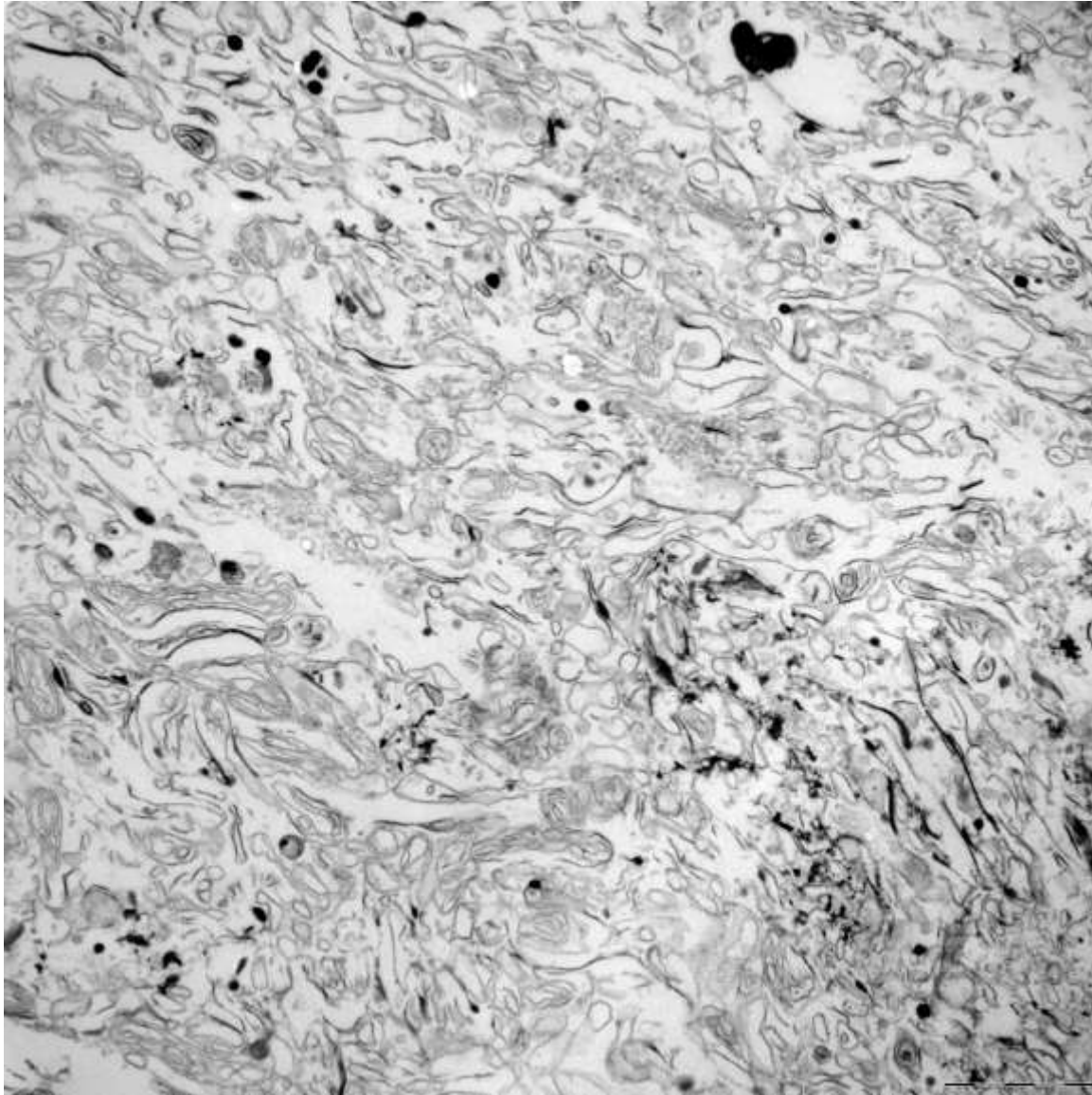


pH 7.4

hpTC method (high pH-Trypsin-CNBr)

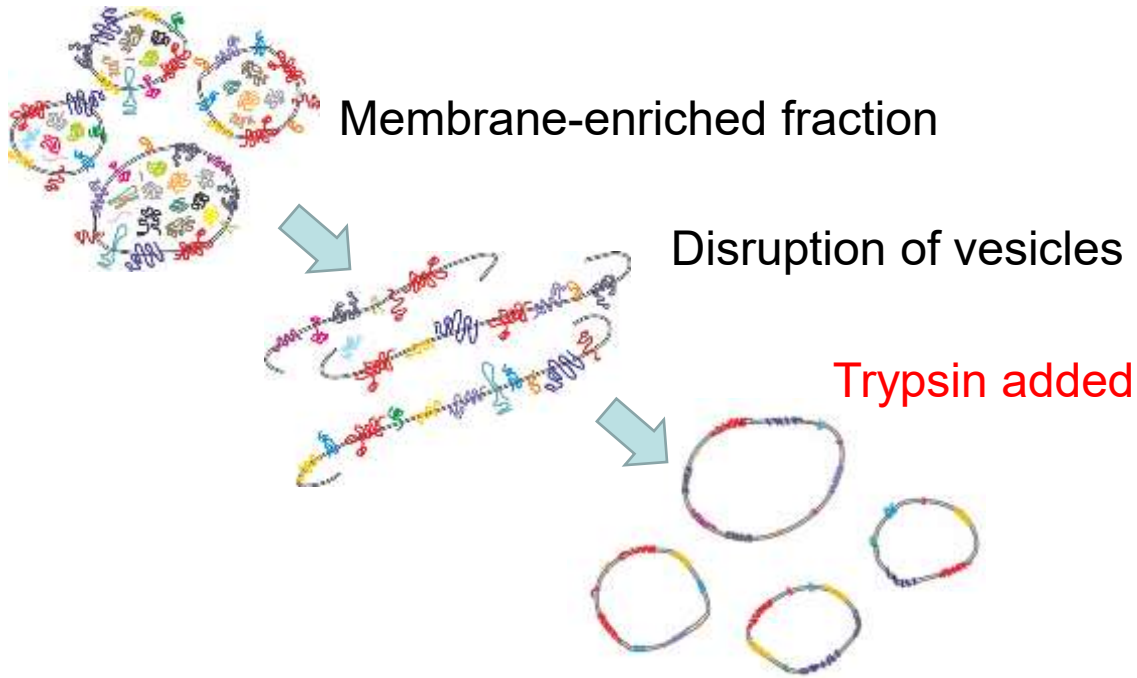


Vit O. et al., *Journal of Proteomics* 2016
Blackler A et al., *J Proteome Res.* 2008



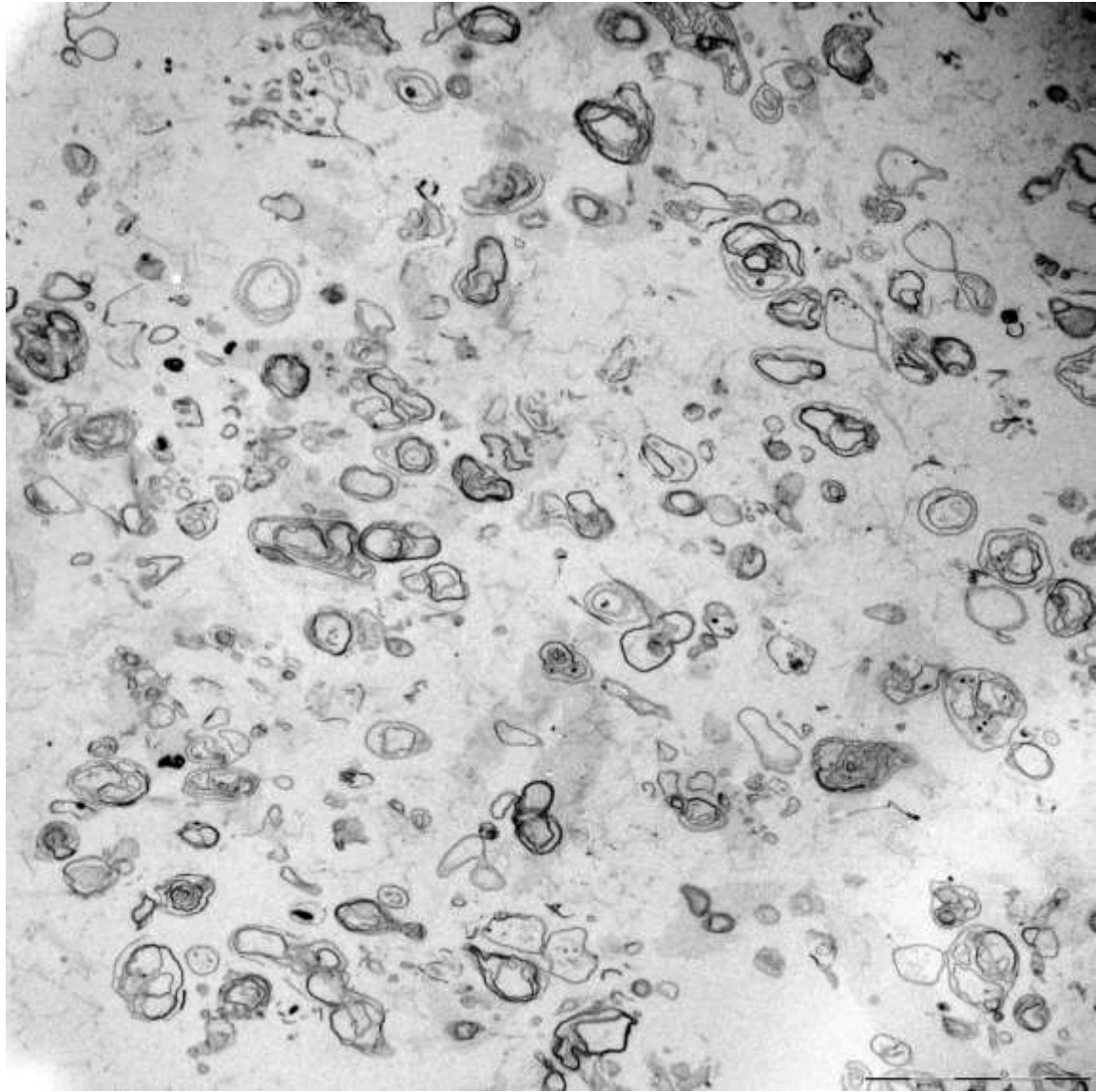
Na ledu
 Na_2CO_3
pH 11

hpTC method (high pH-Trypsin-CNBr)



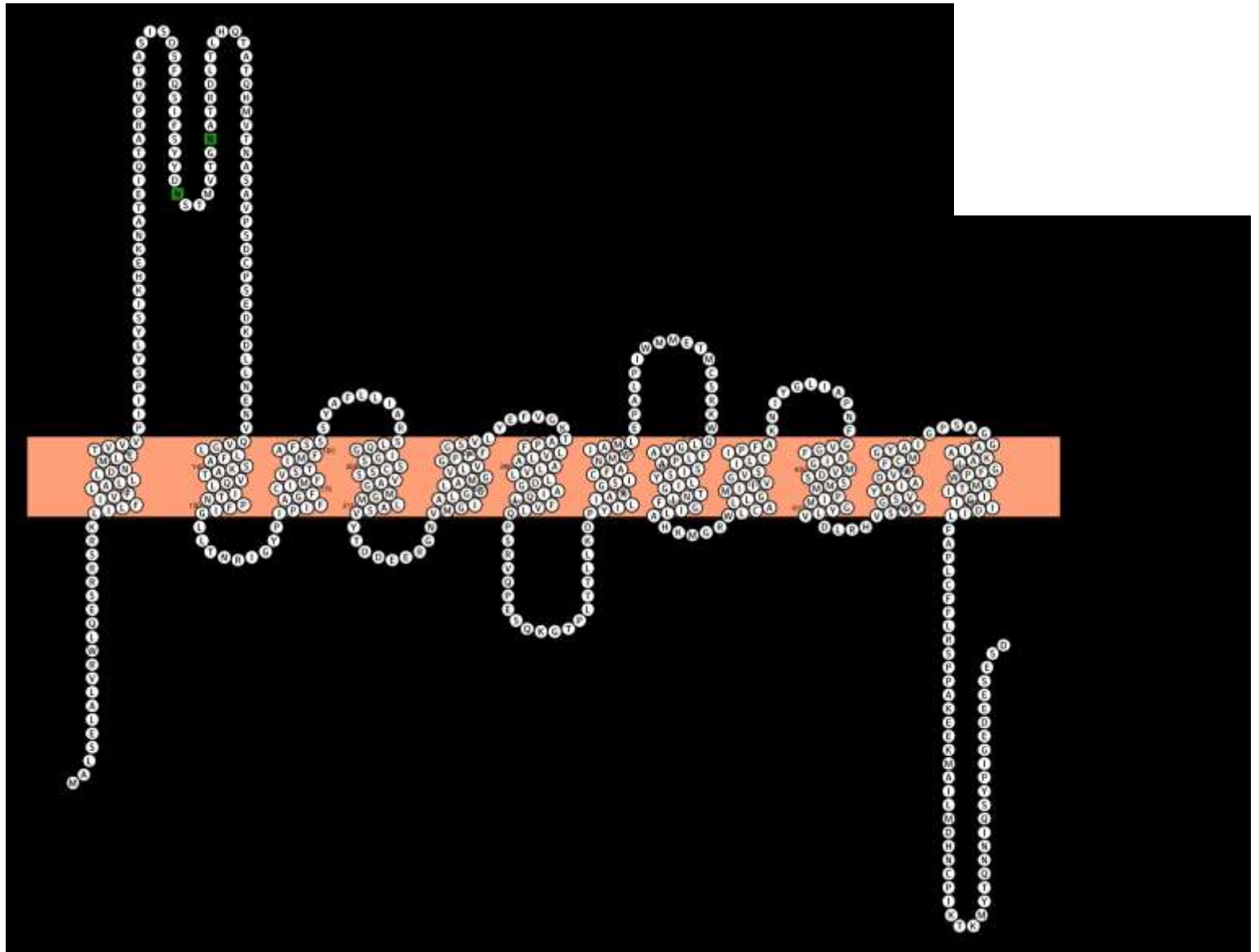
Vit O. et al., *Journal of Proteomics* 2016
Blackler A et al., *J Proteome Res.* 2008





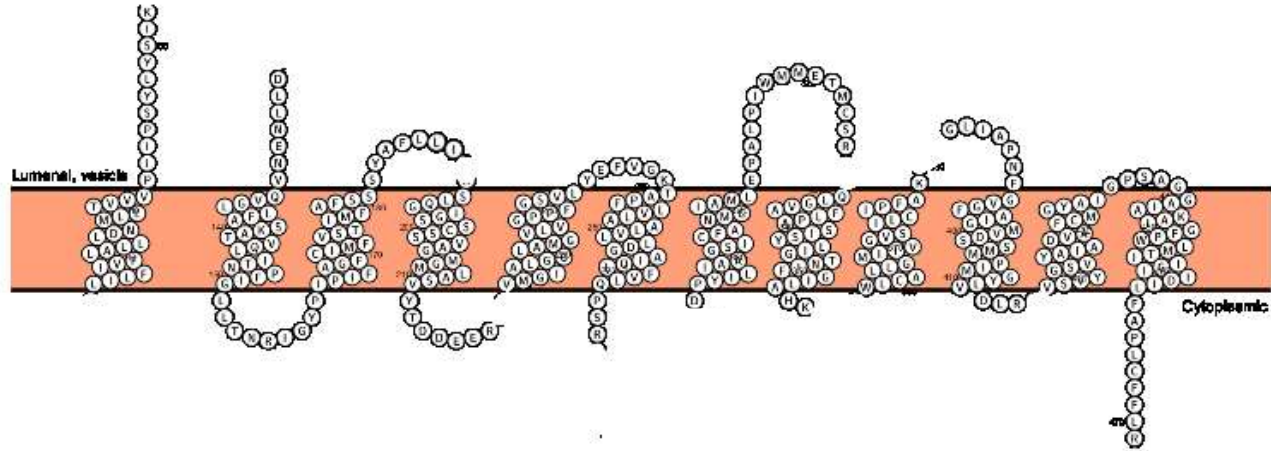
37 °C
trypsin

Synaptic vesicular amine transporter (Slc18a2)

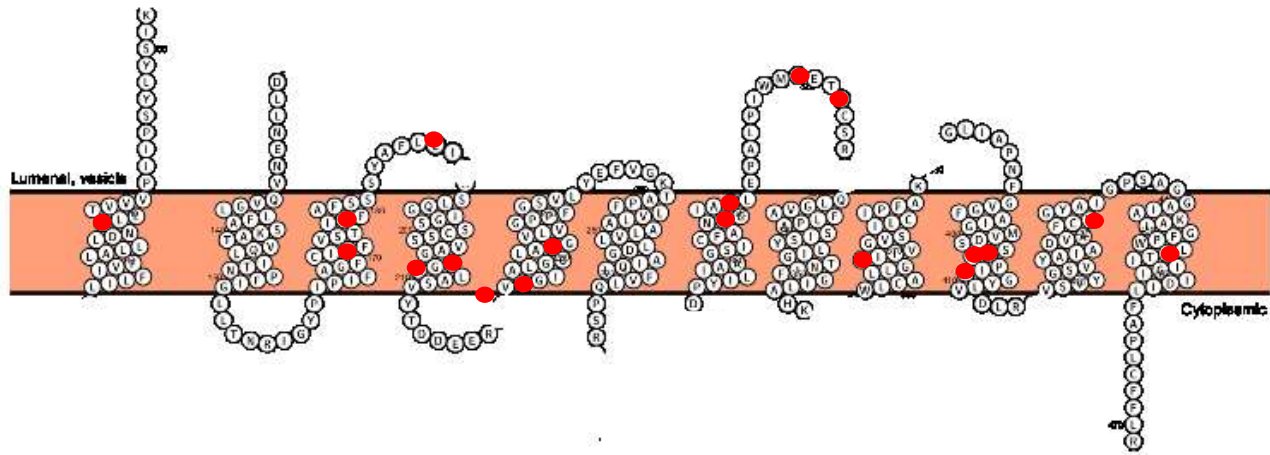


Visualization by Protter (Omasits et al., *Bioinformatics*. 2013)

Synaptic vesicular amine transporter (Slc18a2)



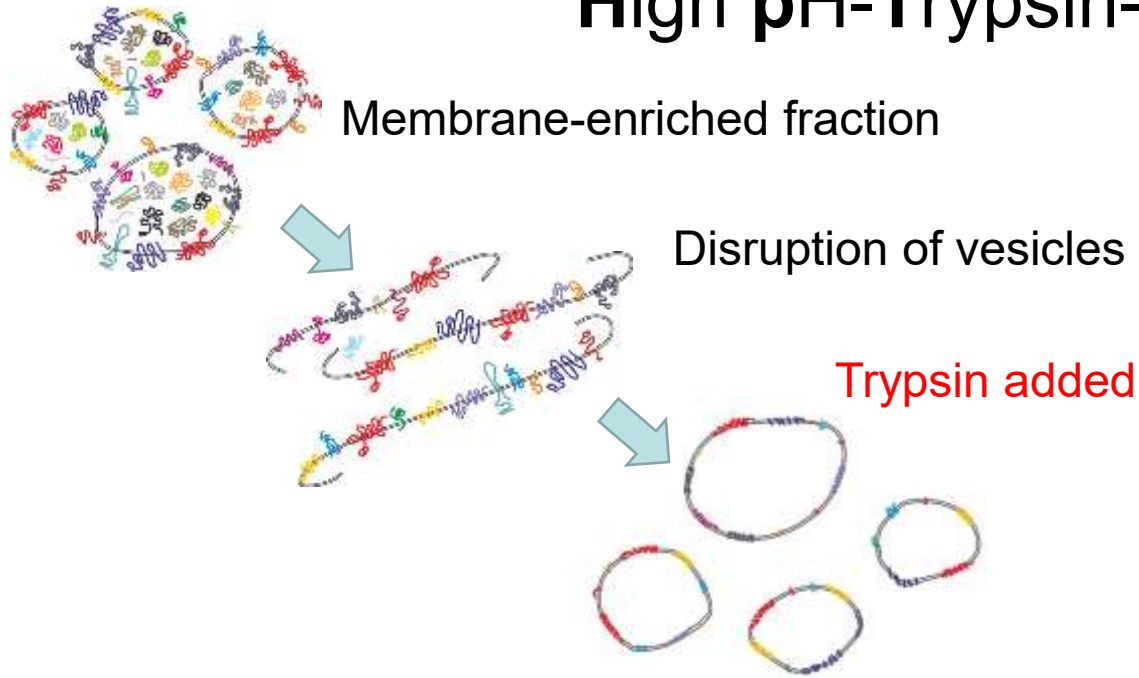
Synaptic vesicular amine transporter (Slc18a2)



Chemical cleavage of peptides by **CNBr** at **Met**

HpTC method

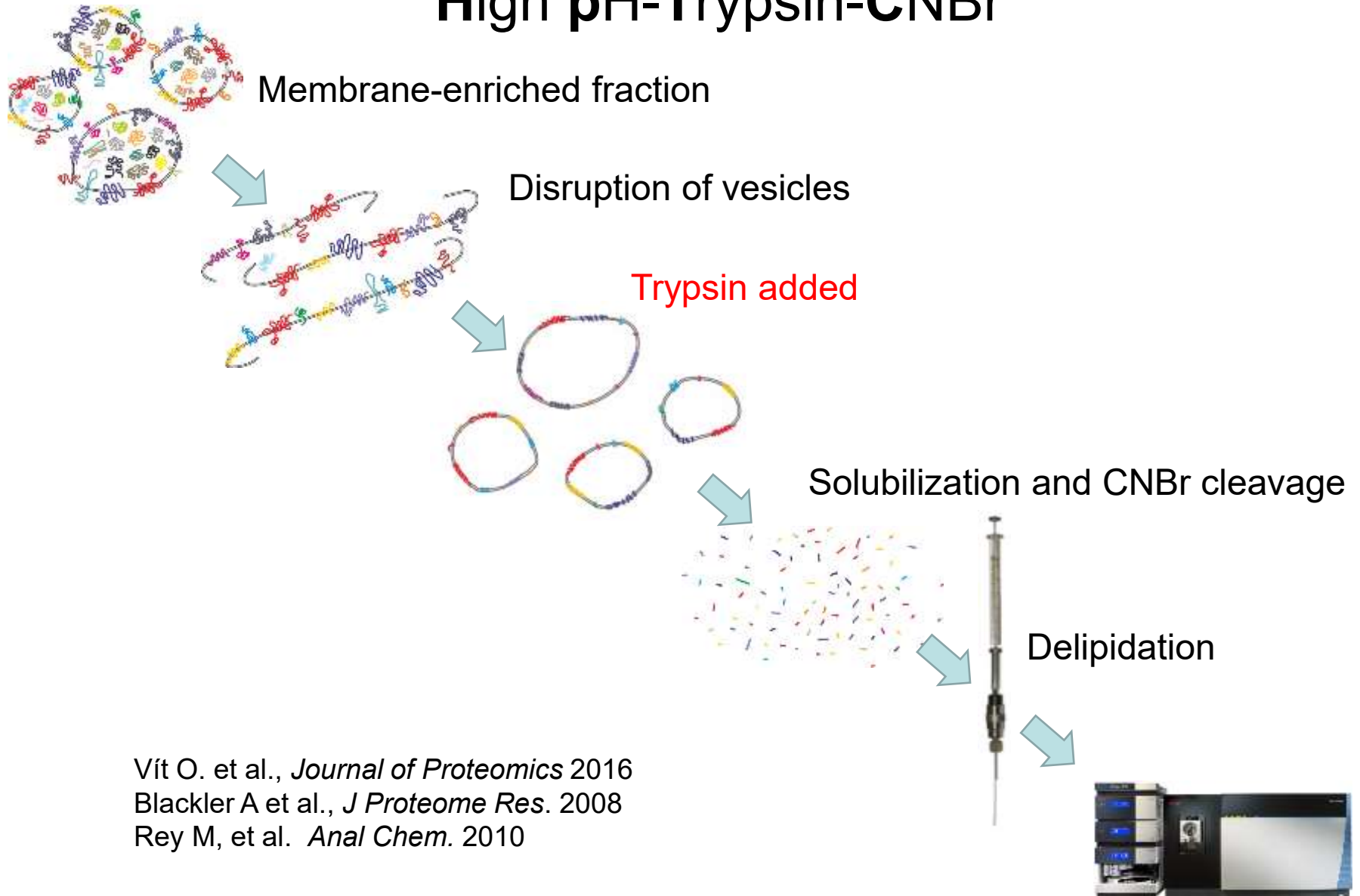
High pH-Trypsin-CNBr



Vit O. et al., *Journal of Proteomics* 2016
Blackler A et al., *J Proteome Res.* 2008

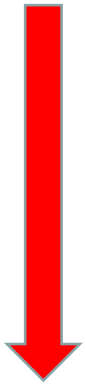
HpTC method

High pH-Trypsin-CNBr



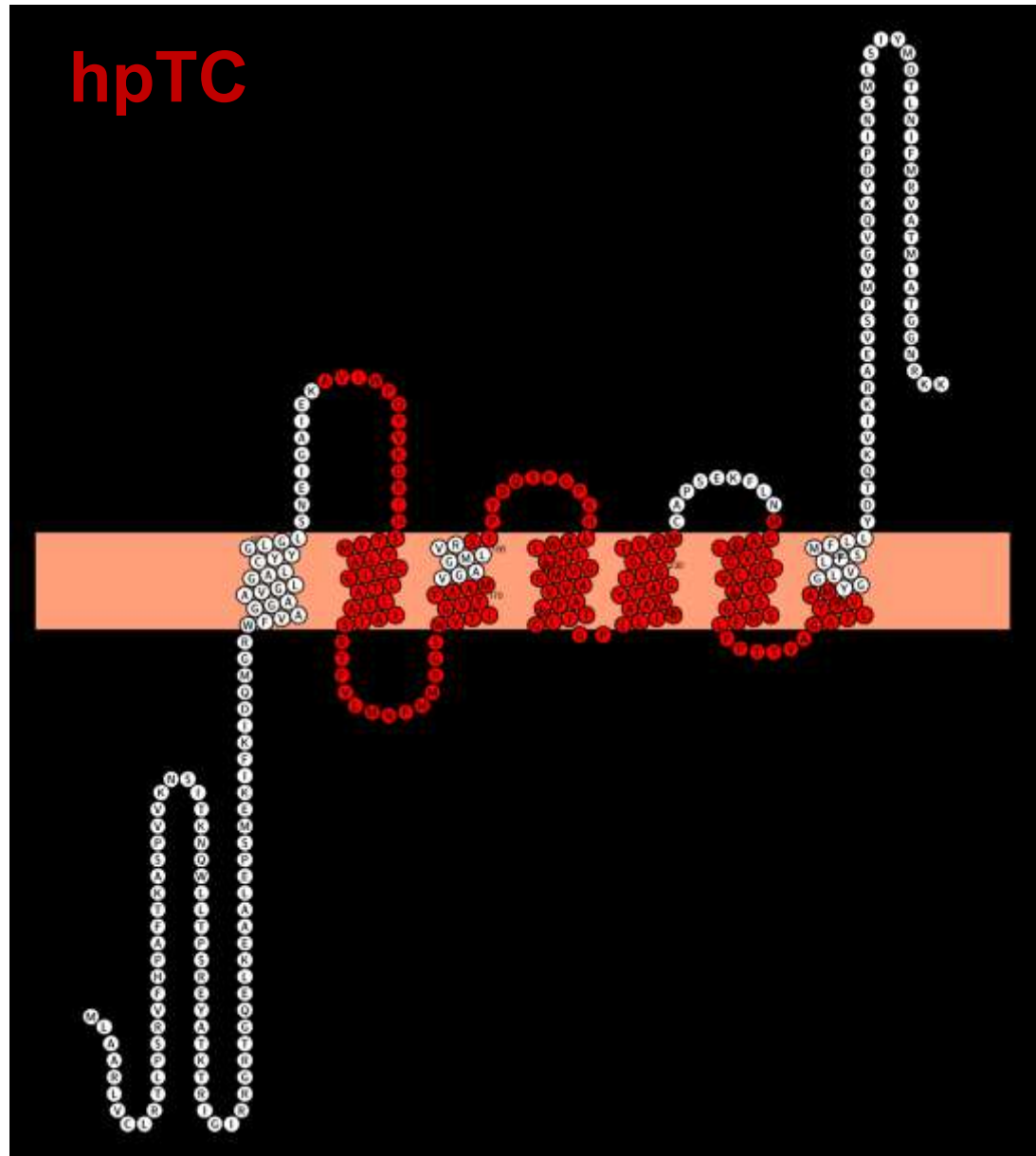
Vit O. et al., *Journal of Proteomics* 2016
Blackler A et al., *J Proteome Res.* 2008
Rey M, et al. *Anal Chem.* 2010

Only the hydrophobic
segments
(hpTC)



600-1000 IMPs
in various tissues

IMP Enrichment
40-60%

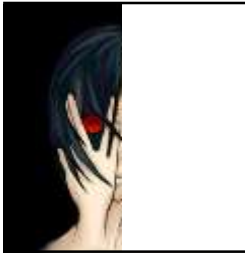


“DIVIDE AND CONQUER“ METHODS

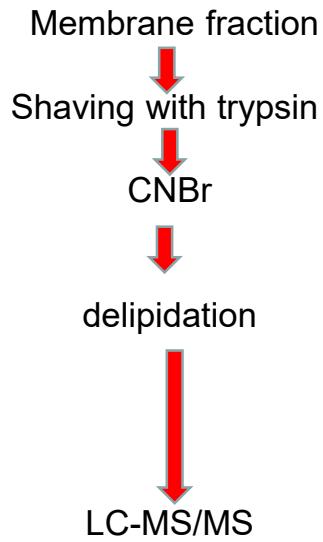
N-Glycopeptide affinity enrichment
(Glyco-capture)

hpTC method
targets the hydrophobic alpha helices

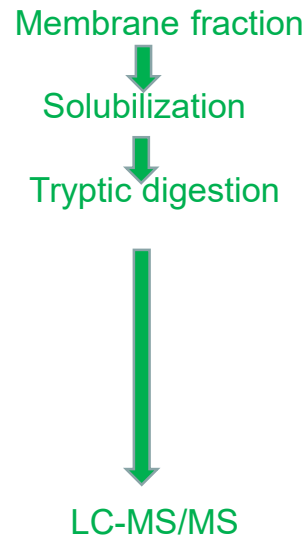
A standard trypsin-based method



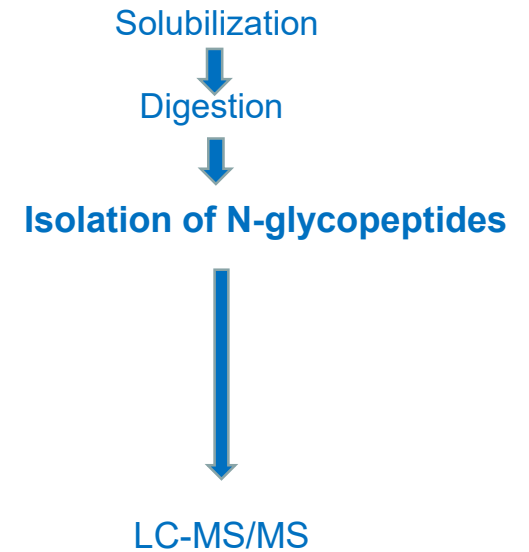
Only the hydrophobic segments



Classic strategy



**Only the hydrophilic segments
(GLYCOCAPTURE)**



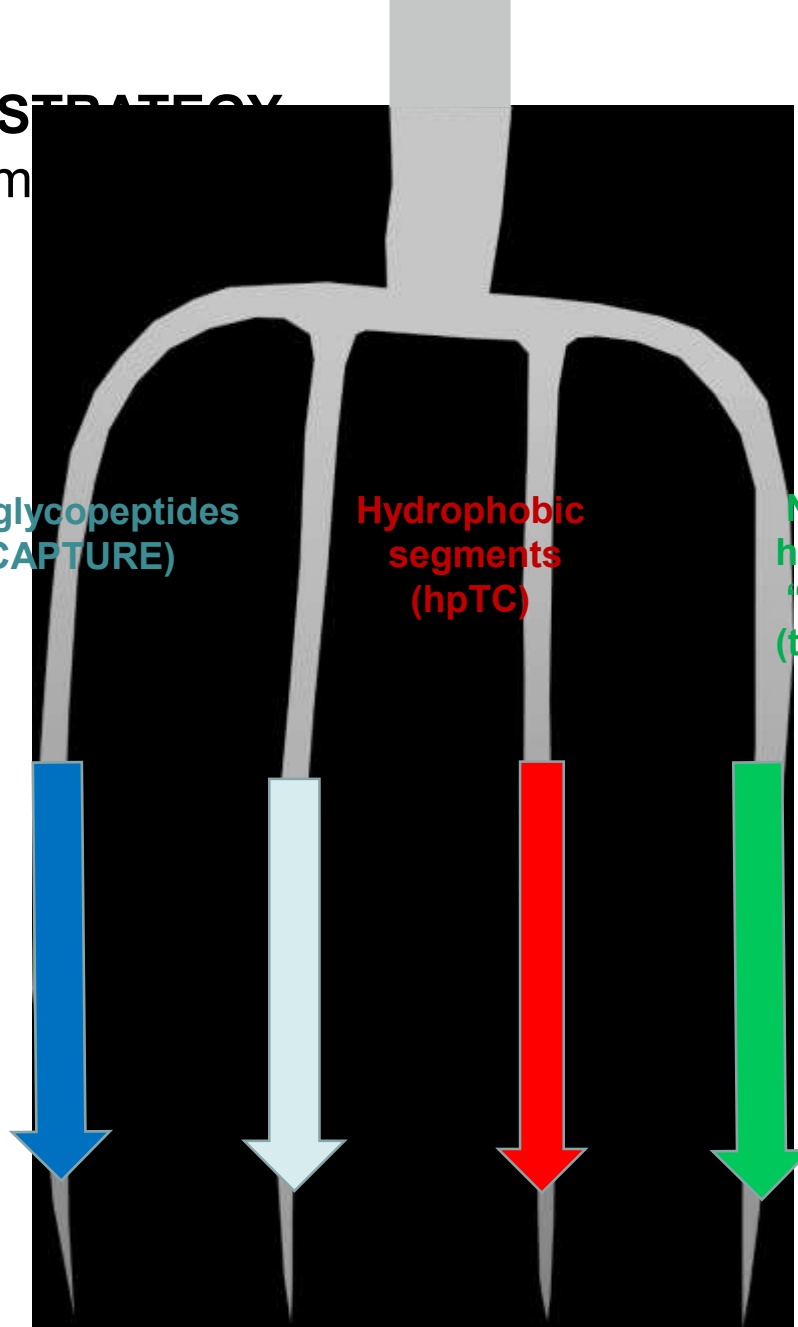
THE PITCHFORK STRATEGY

(Vit et al, J. Proteom

Hydrophilic/glycopeptides
(GLYCO-CAPTURE)

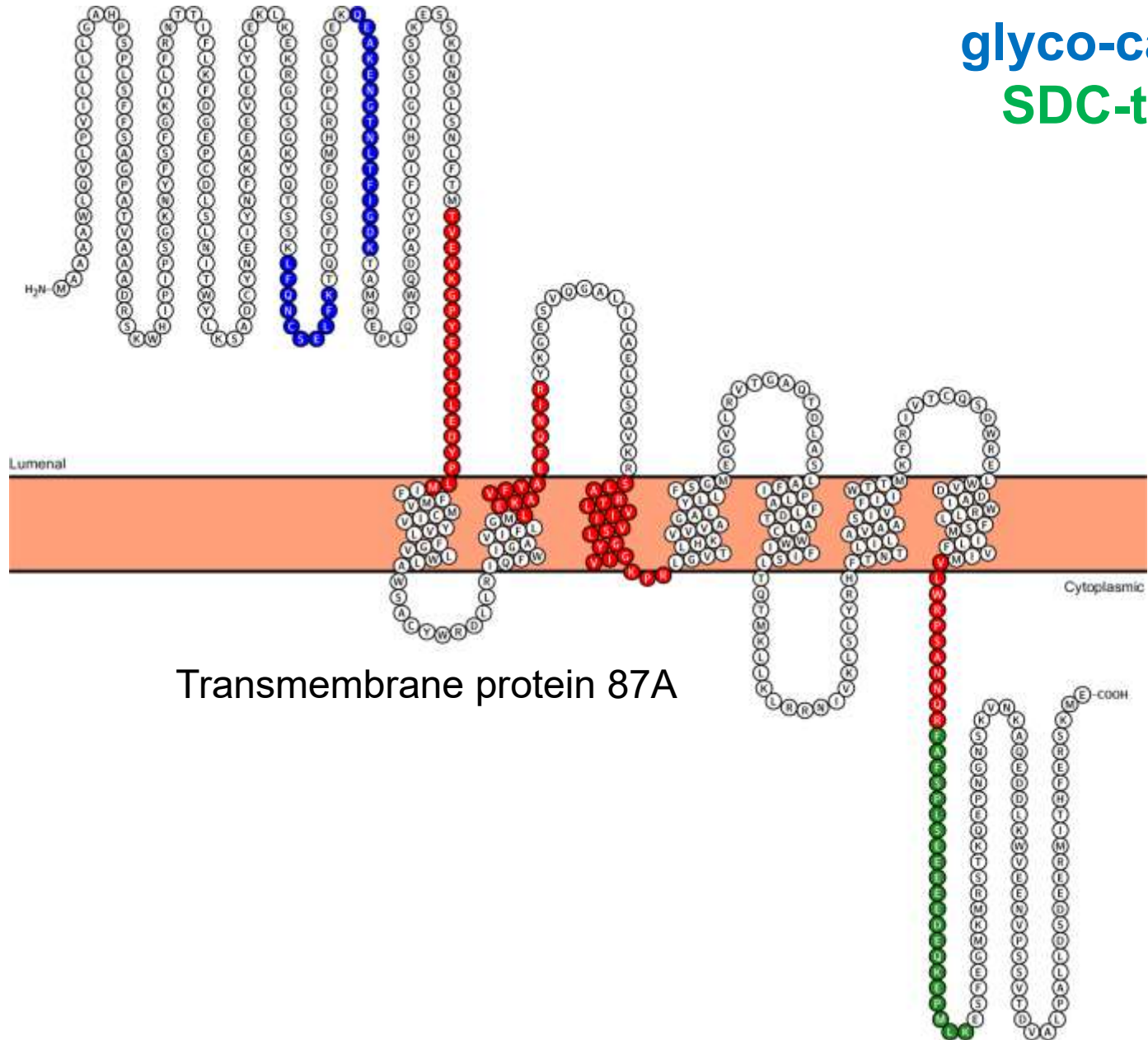
Hydrophobic
segments
(hpTC)

Non-glycosylated
hydrophilic peptides
"Classic strategy"
(trypsin and detergent)



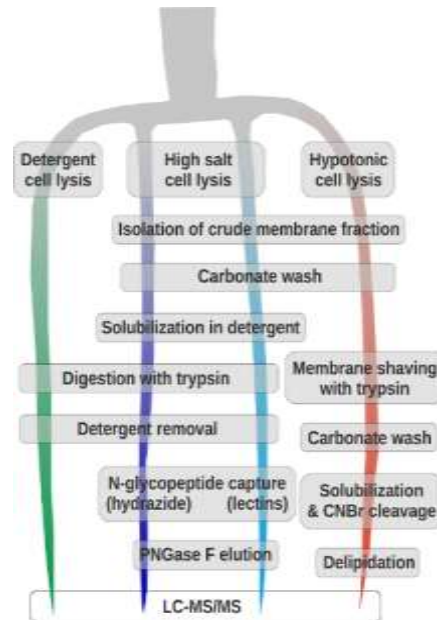
LC-MS/MS

hpTC
glyco-capture
SDC-trypsin



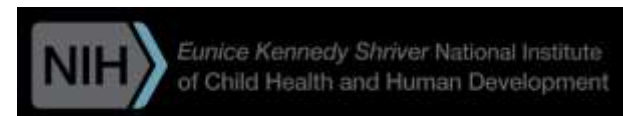
Transmembrane protein 87A

The Pitchfork strategy



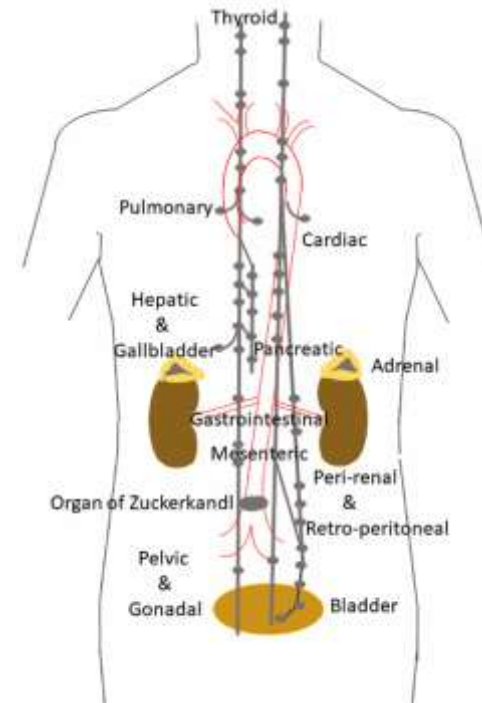
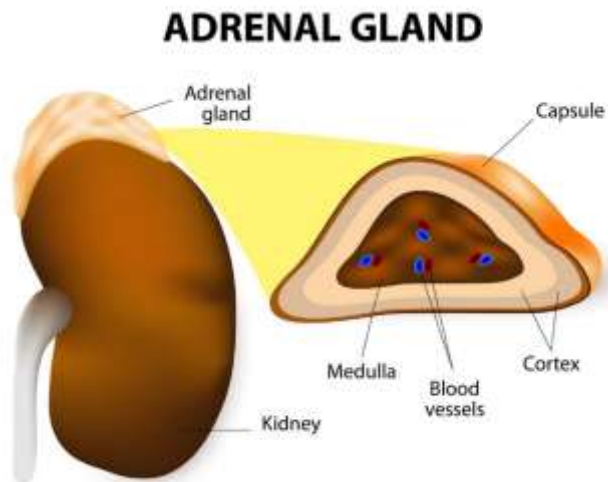
- 800-1300 IMPs identified in various human tissue samples
- IMPs from all compartments
- Applicable to any cellular material, fresh or frozen
- No bias toward number of TM domains

Looking for new theranostic targets in human Pheochromocytoma and Paraganglioma



PHEOCHROMOCYTOMA and PARAGANGLIOMA

- Rare neuro-endocrine tumors (0.8/100,000)
- From **chromaffin tissue of adrenal medulla (PHEO)** or **sympathetic ganglia (PGL)**
- From parasympathetic ganglia (PGL)



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- Catecholamine producing tumors (dopamine, noradrenaline, adrenaline)
- Up to 25 % are malignant, even benign disease has high mortality
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NEW DRUG TARGETS ARE NEEDED

INTEGRAL MEMBRANE PROTEINS ARE EXCELLENT DRUG TARGETS

PHEOCHROMOCYTOMA and PARAGANGLIOMA

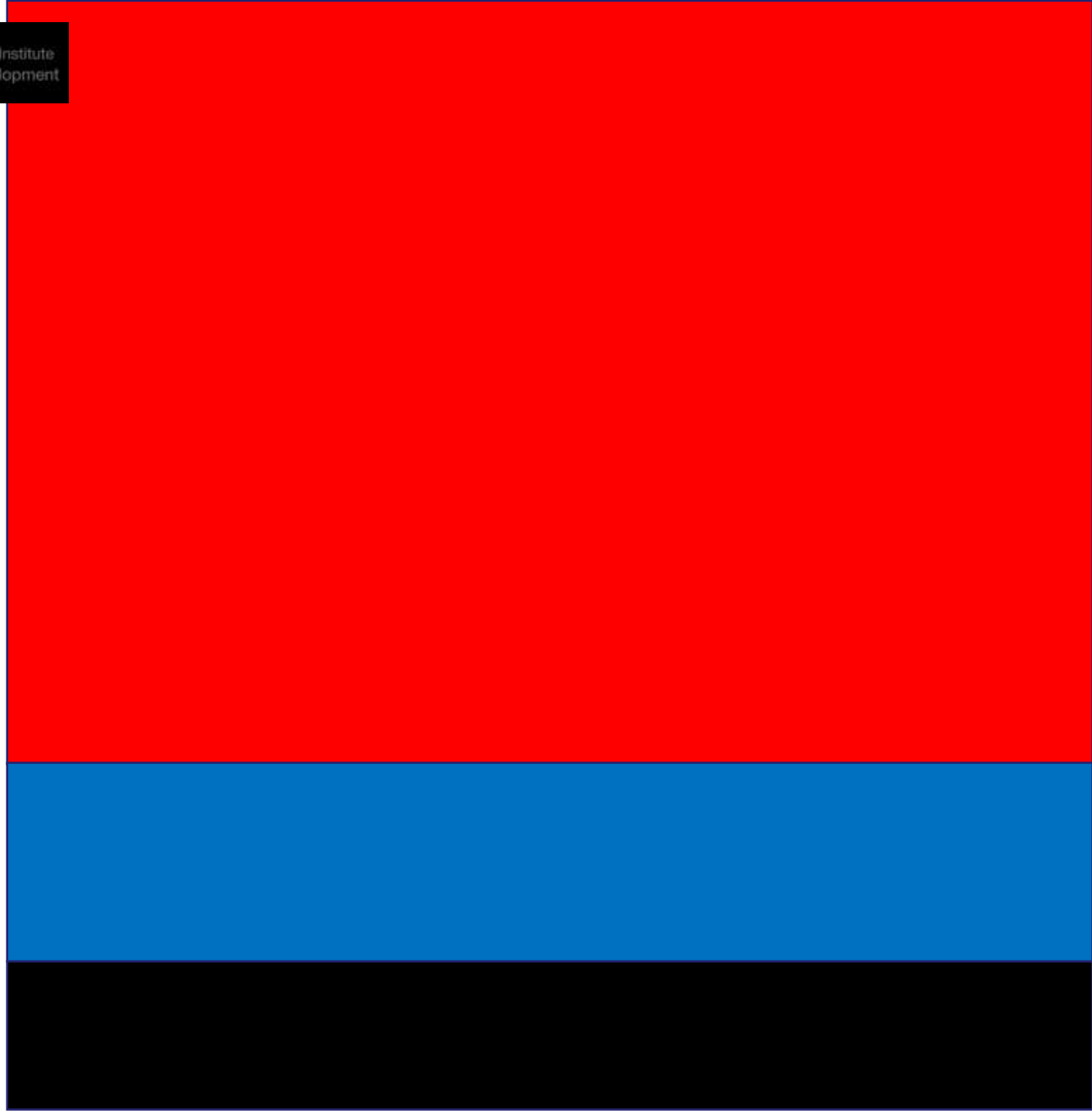
Distinct molecular subtypes of based on mutations, mRNA expression...

Cluster 1.	Pseudohypoxia (<i>SDHx, VHL, FH, HIF2A, EGLN1...</i>)
Cluster 2.	Kinase signaling (<i>RET, MAX, NF1, HRAS, TMEM127</i>)
Cluster 3.	Wnt altered (<i>UBTF-MAML3, CSDE1</i>)
Unassigned	Patients with no mutation in the PPGL susceptibility genes

No
mutation

Cluster 2

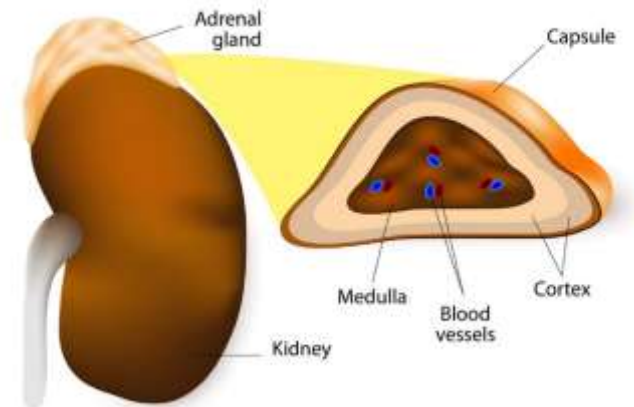
Cluster 1



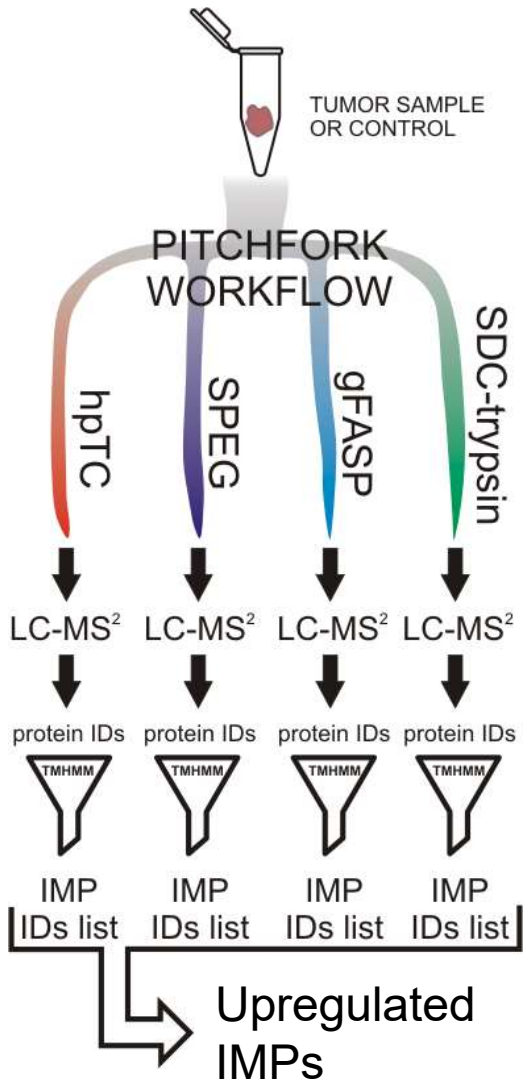
ADRENAL MEDULLA – THE CONTROL CHROMAFFIN TISSUE



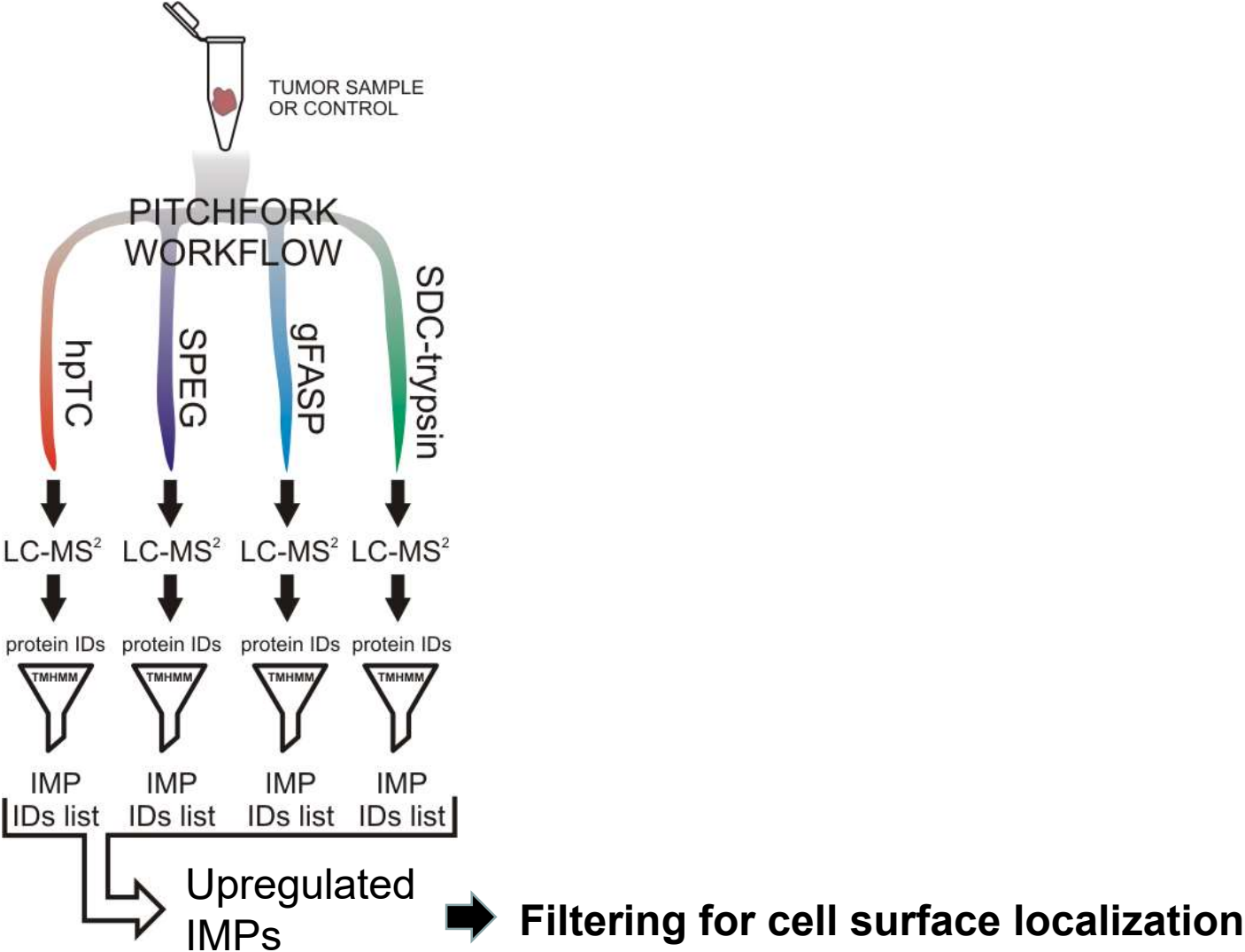
ADRENAL GLAND



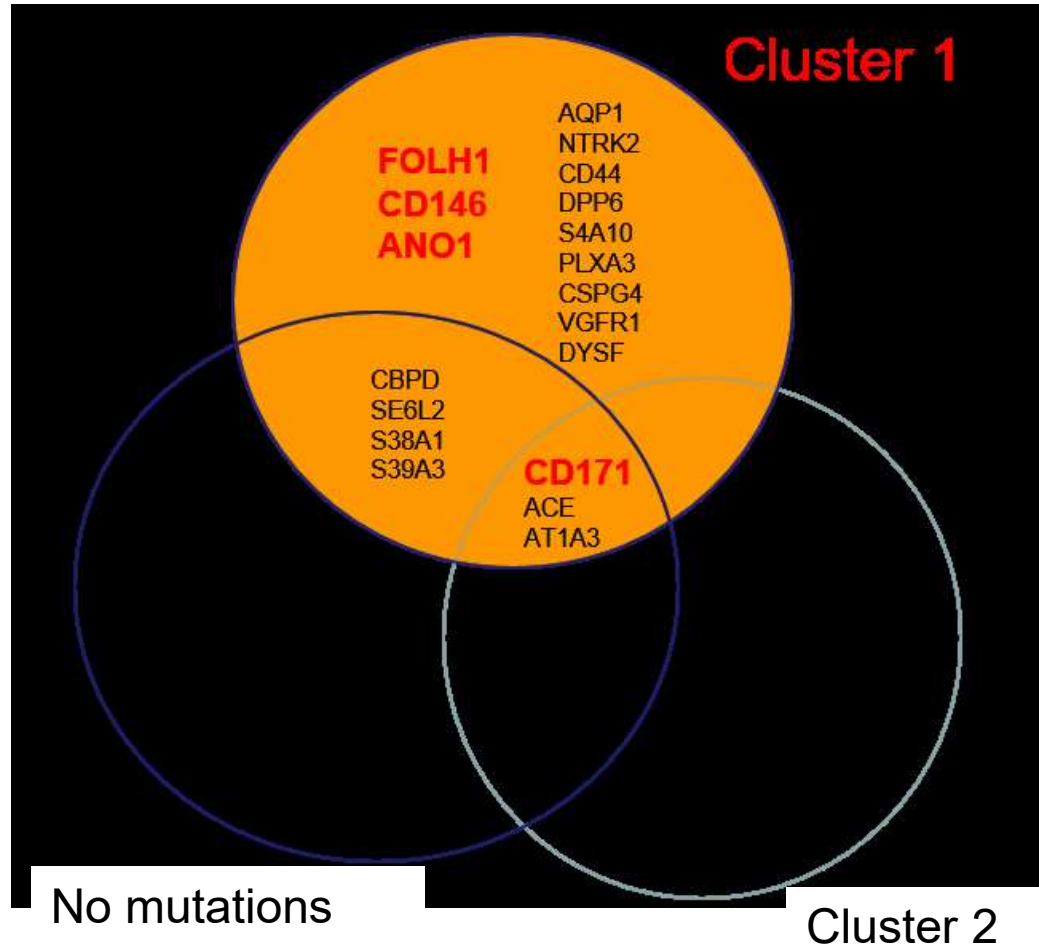
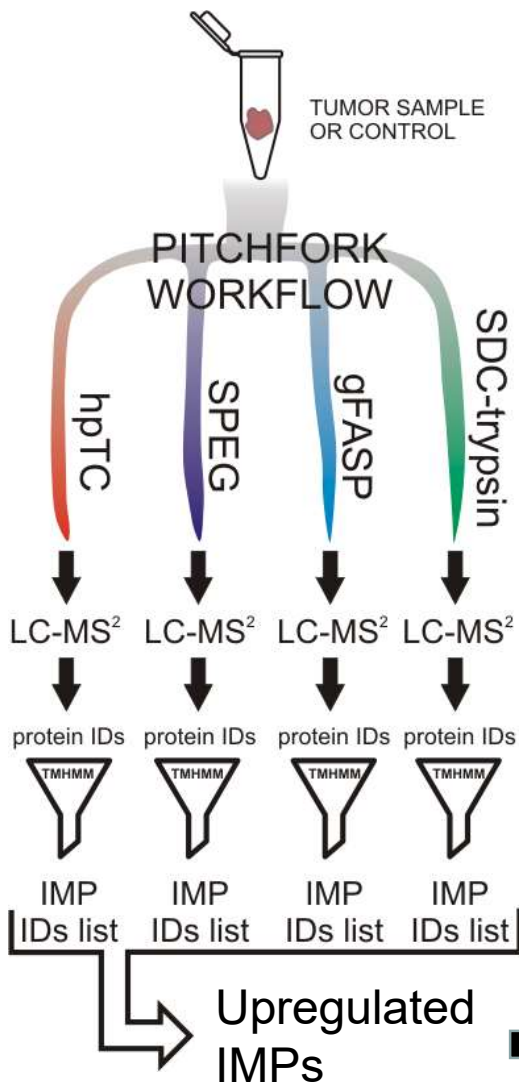
PROTEOMIC ANALYSIS OF PPGL MEMBRANE PROTEOME



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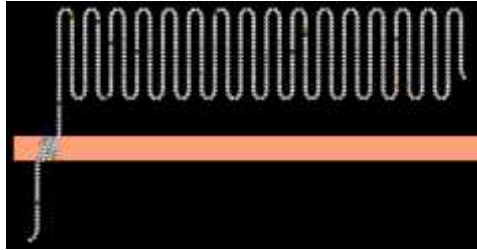


PROTEOMIC ANALYSIS OF PPGL MEMBRANE PROTEOME

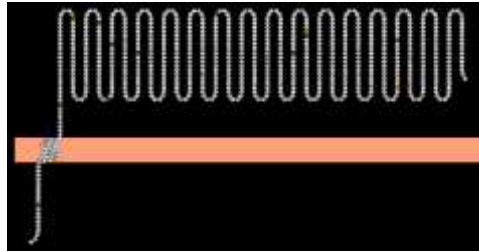


Filtering for cell surface localization

GLUTAMATE CARBOXYPEPTIDASE 2 (FOLH1)

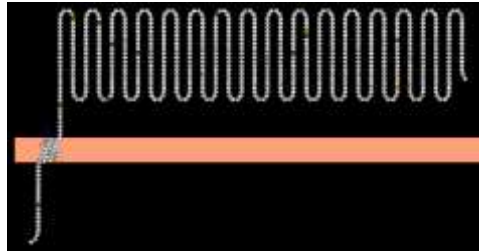


GLUTAMATE CARBOXYPEPTIDASE 2 (FOLH1)
PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA)



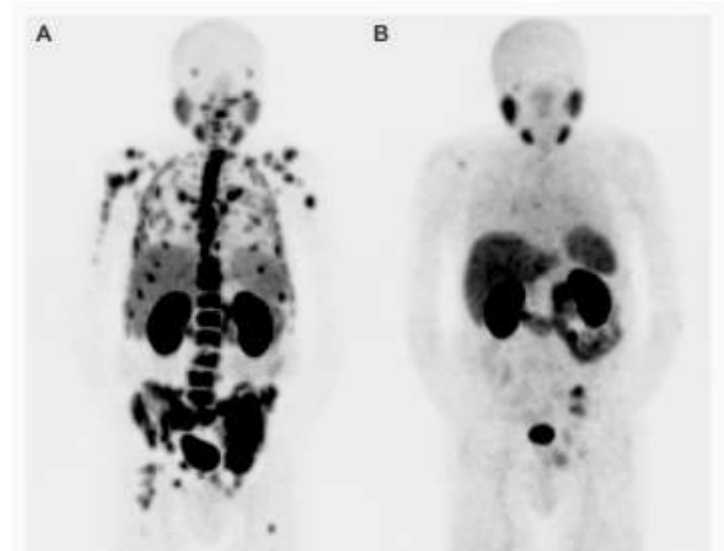
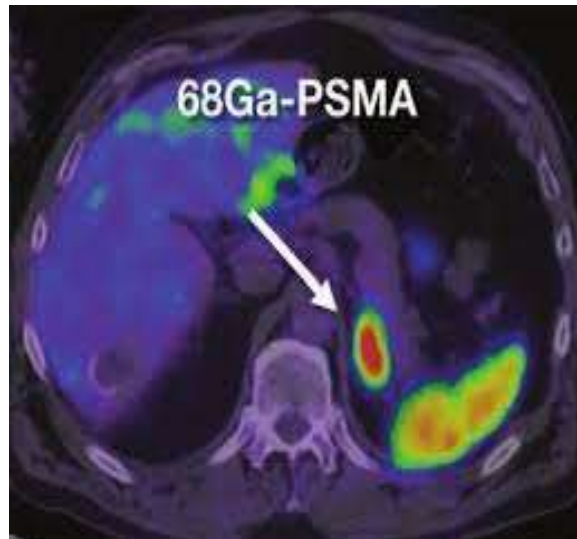
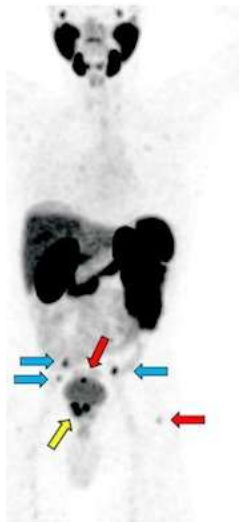
OVEREXPRESSED IN PROSTATE CANCER CELLS

GLUTAMATE CARBOXYPEPTIDASE 2 (FOLH1) PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA)

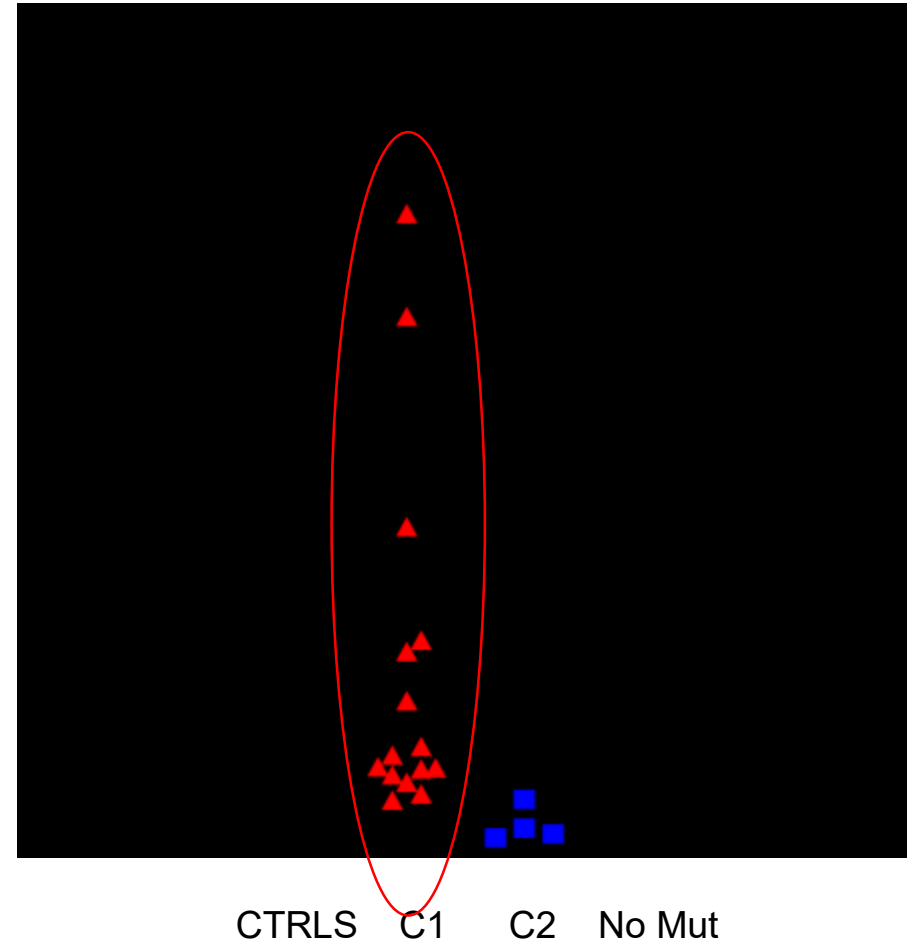
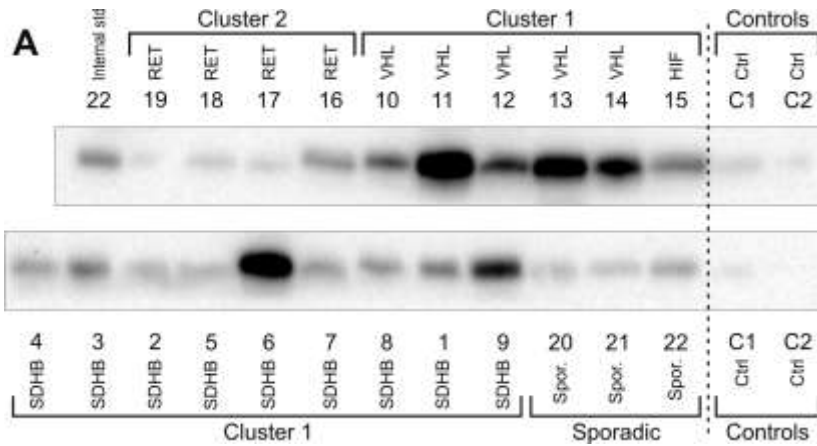


OVEREXPRESSED ON PROSTATE CANCER CELLS

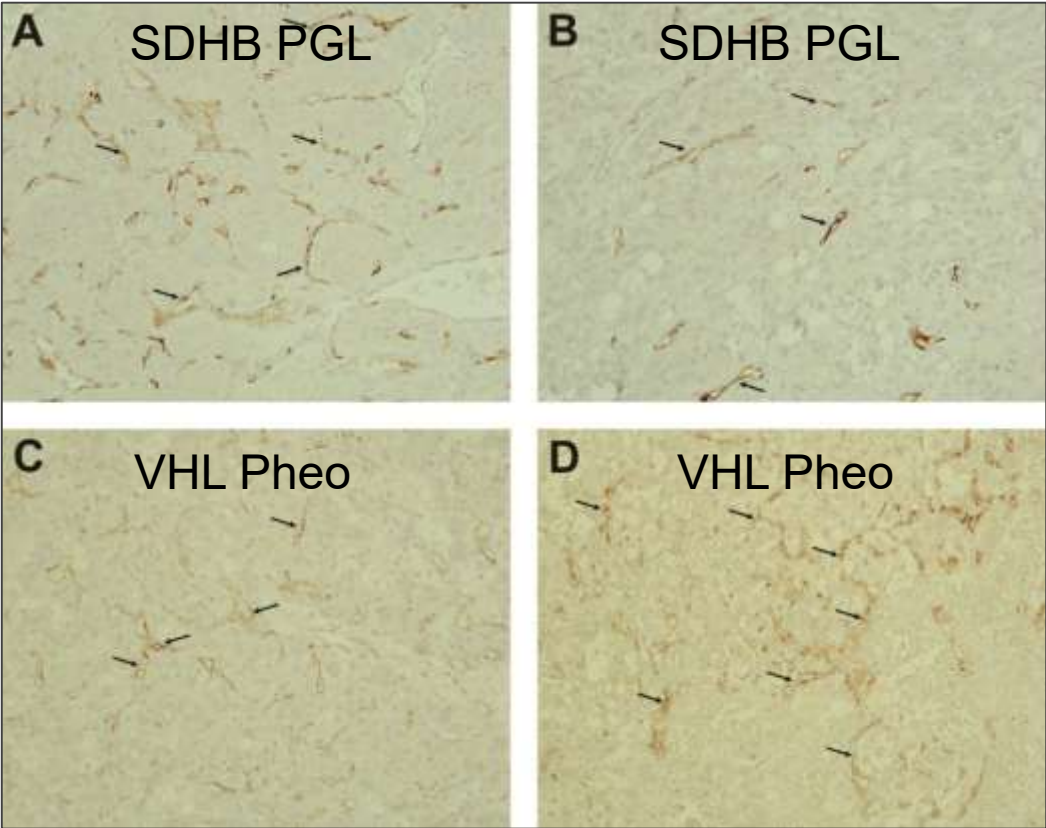
Anti-PSMA radio-conjugates approved for PC tumor imaging
and therapy of advanced PC



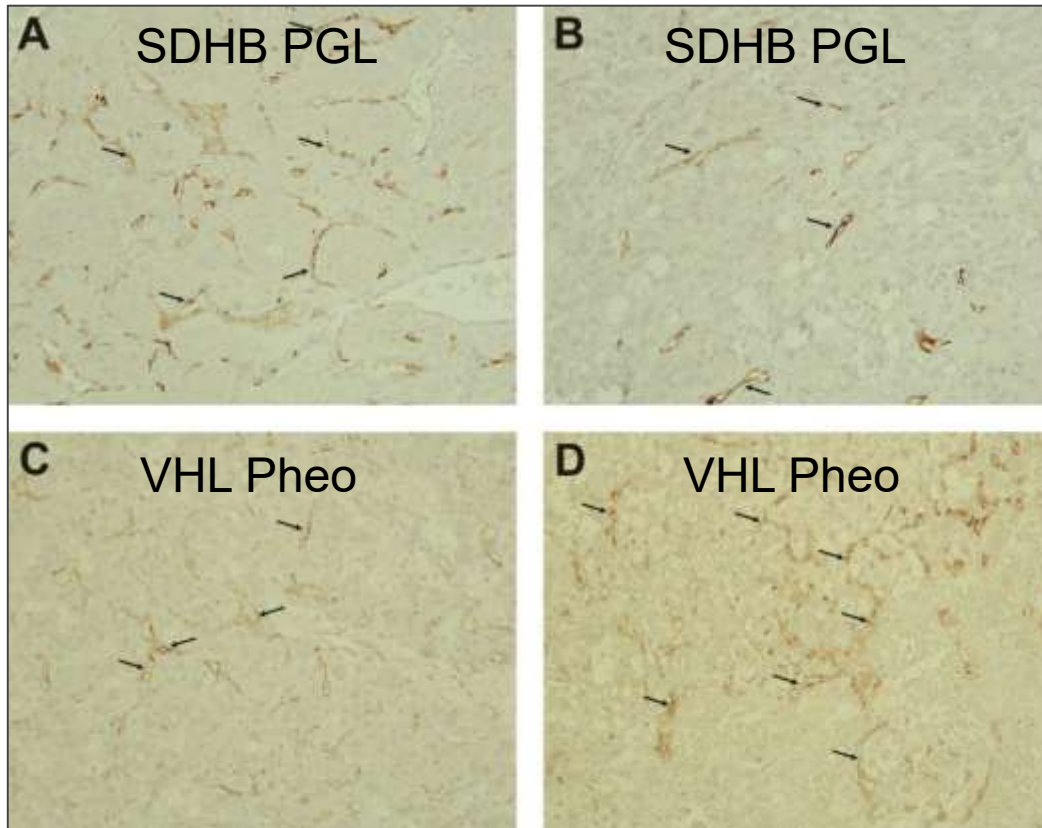
PSMA EXPRESSION IN HUMAN PPGL



PSMA EXPRESSION IN TUMOR VASCULATURE IN CLUSTER 1 PPGL

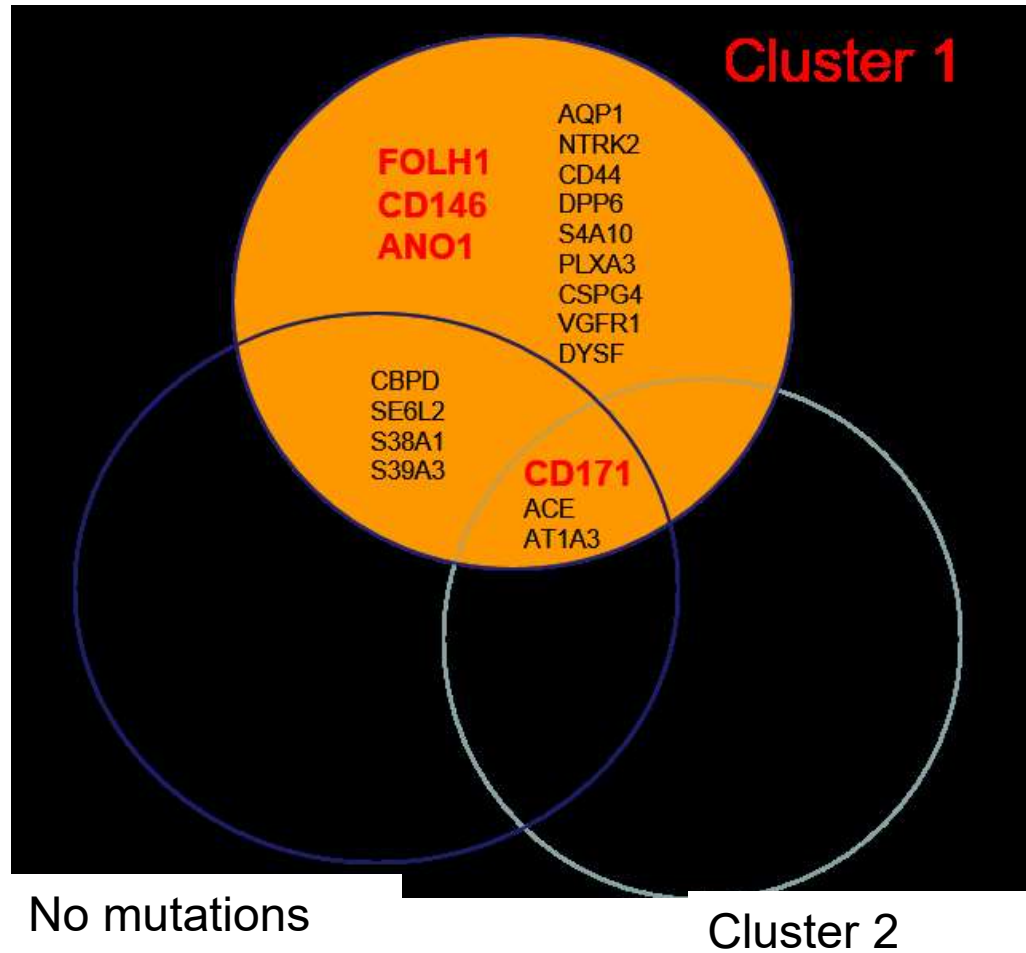


PSMA EXPRESSION IN TUMOR VASCULATURE IN CLUSTER 1 PPGL

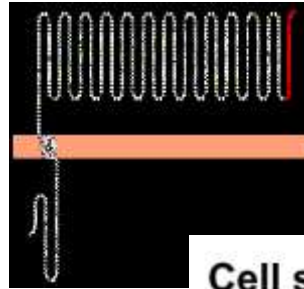


- PPGL imaging using ^{68}Ga -PSMA?
- PPGL therapy with ^{177}Lu -PSMA?

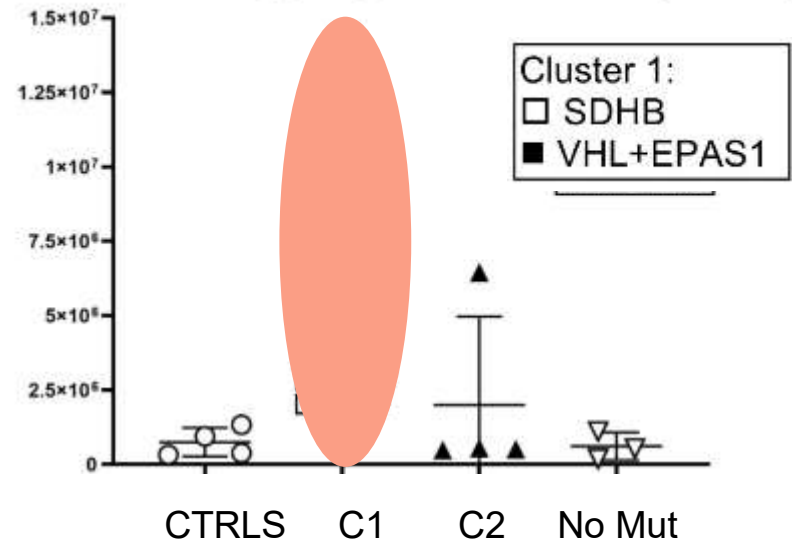
PROTEOMIC ANALYSIS OF PPGL MEMBRANE PROTEOME



CD146 (Melanoma cell adhesion molecule, MCAM, MUC18)

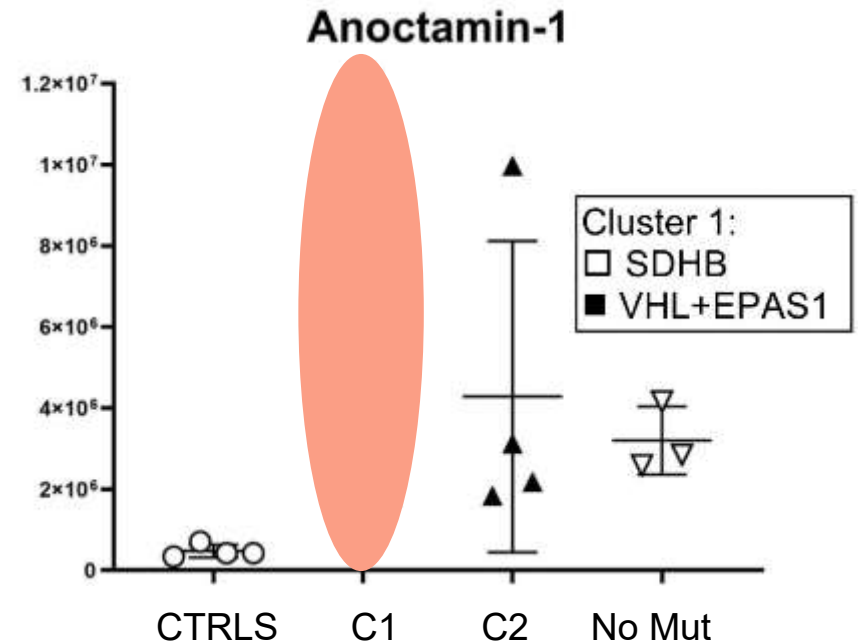
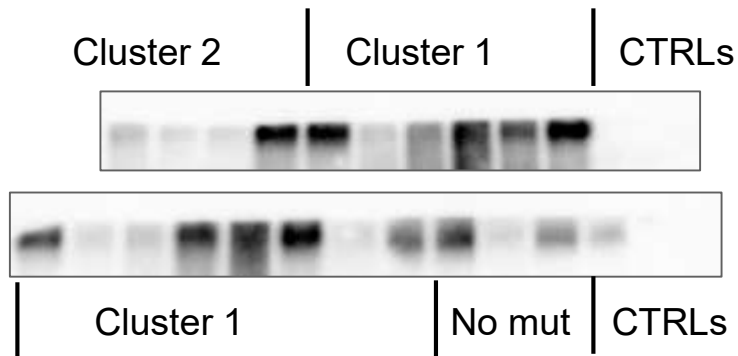
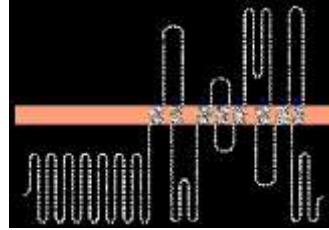


Cell surface glycoprotein MUC18 (CD146)



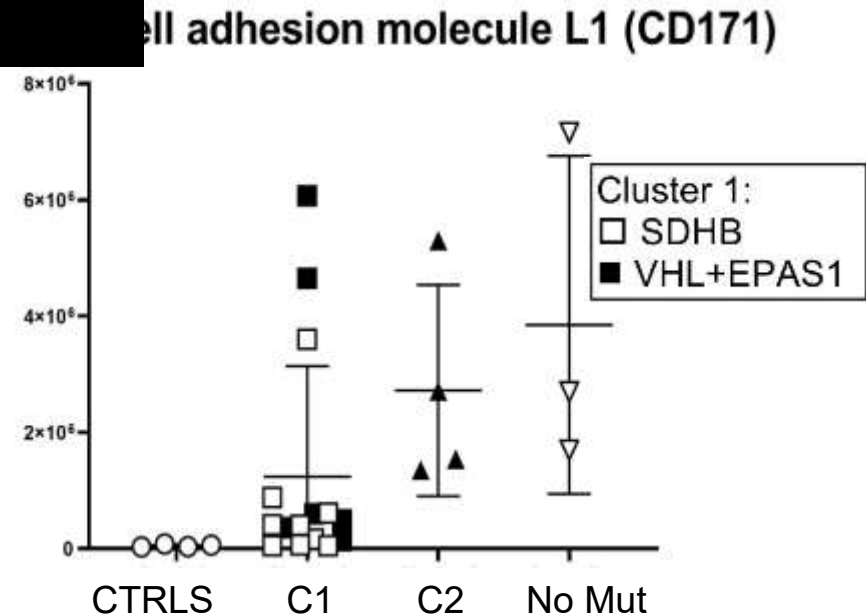
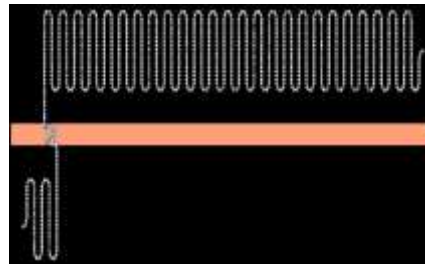
- A cell adhesion molecule, role in endothelial permeability
- Overexpressed in several cancers, expression correlates with progression
- Anti-CD146 antibody inhibited tumor growth in mouse xenograft models
- Tested as a drug target and imaging target for several tumors in preclinical studies

Anoctamin-1 (DOG1, TMEM16A)



- A Ca^{2+} -activated Cl^- channel
- Overexpressed in several cancers, correlates with poor prognosis
- Function in cancer unknown
- Inhibitor reduced growth of cancer cells
- Inhibitors in preclinical studies
- NIH-approved anti-asthma drug zafirlucast is ANO-1 inhibitor

CD171 (Neural cell adhesion molecule L1, NCAM-L1)



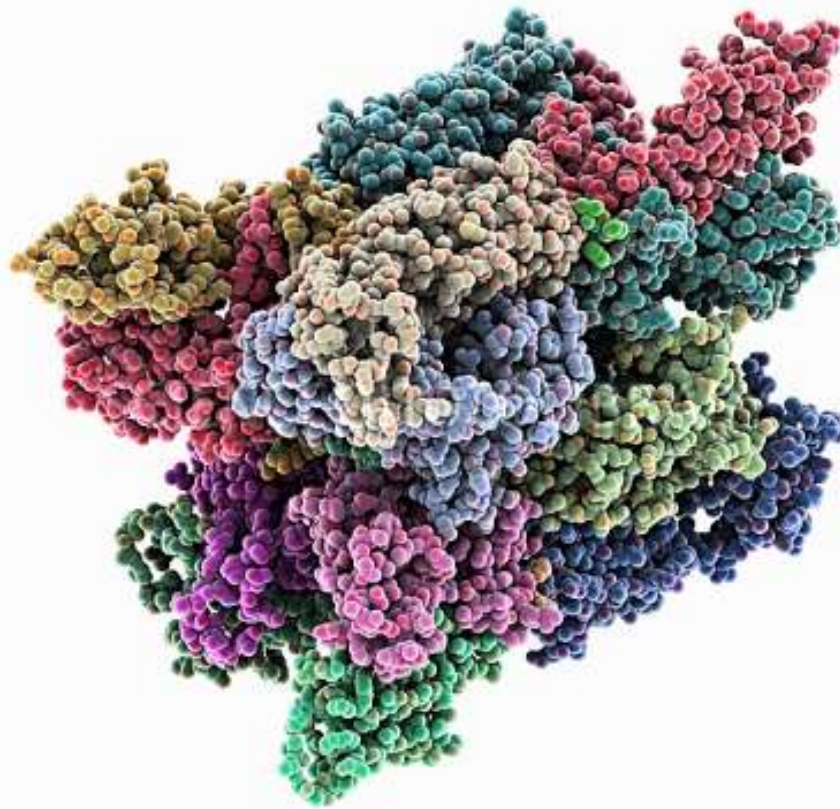
- Cell adhesion molecule, essential for neural development and regeneration
- Overexpressed in numerous cancers, expression correlates with disease progression
- Pro-angiogenic roles in the endothelial cells of tumor-associated vessels
- Anti-CD171 antibody decreased tumor vascularization and progression
- CAR-T cells recognizing CD171 in clinical trials for neuroblastoma

Příprava vzorků pro proteomické experimenty

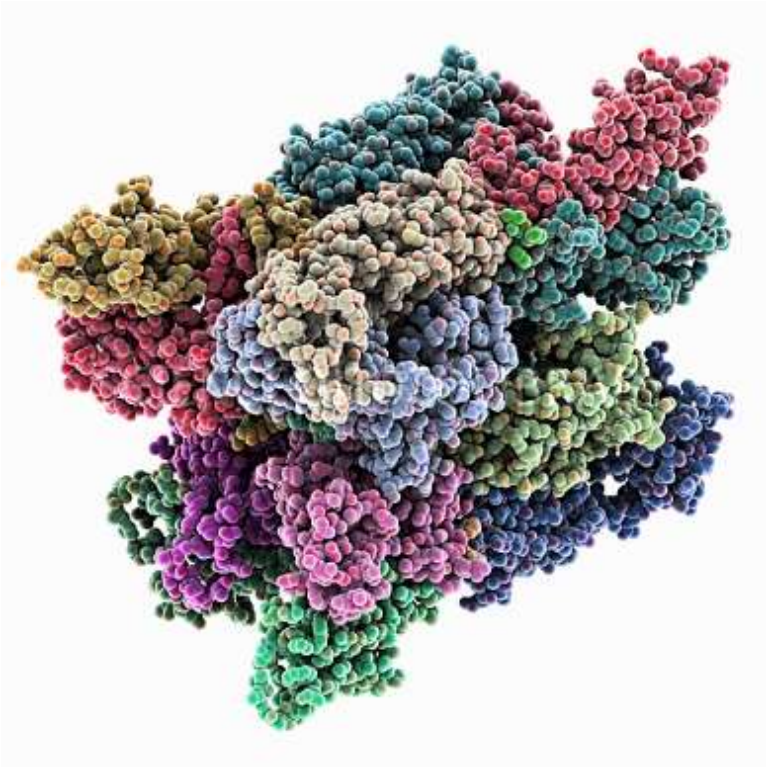
Proteomika membránových proteinů

Analýza proteinových komplexů

ANALÝZA PROTEINOVÝCH KOMPLEXŮ



ANALÝZA PROTEINOVÝCH KOMPLEXŮ



Afinitní purifikace komplexů

- s pomocí protilátky
- přes „tagované“ proteiny
- identifikace pomocí LC-MS/MS

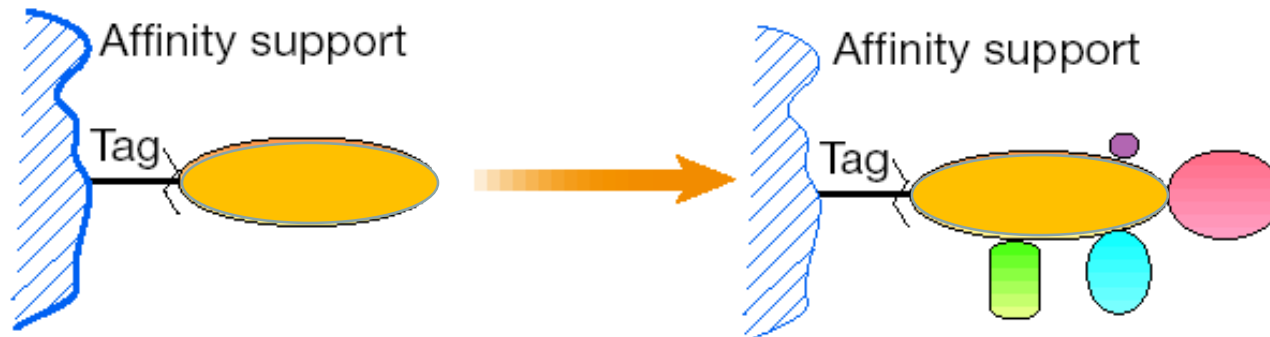
Proximity labeling

Nativní (vícerozměrné) separace

- Blue native/2D elektroforéza
- Clear native/2D elektroforéza

IMUNOAFINITNÍ IZOLACE PROTEINOVÝCH KOMPLEXŮ

- 1) matrix s **protilátkou** proti jedné složce komplexu
- 2) matrix s **rekombinantním proteinem** (složkou komplexu) nebo jinou „návnadou“



AFINITNÍ MATRIX

Aktivované matrice:

NHS Sepharose.....lze vázat za aminoskupinu (succinimid)

CNBr Sepharose.....lze vázat za aminoskupinu

EAH Sepharoselze vázat protein za karboxyl (karbodiimid)

Thiol sepharose.....lze vázat za SH cysteinu

Matrice s afinitou pro IgG (Fc fragment)

Protein G Sepharose

Protein A Sepharose

Protein A, G magnetic beads

Matrice s afinitou pro glykoproteiny

ConA Sepharose

Velké ligandy (DNA, protein) lze vázat přímo na matrix.

Malé ligandy (nukleotid, NADP, hormon...) se váží přes inertní „spacer arm“.

Typy možných interakcí při imunoprecipitaci

