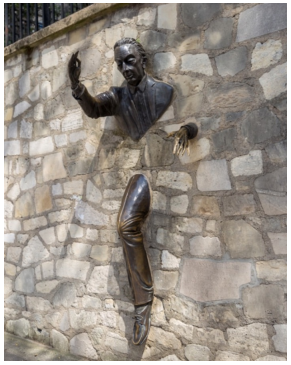
**aeis-2021 : LES SIGNATURES DES ÉTATS MÉSOSCOPIQUES DE LA MATIÈRE 28 & 29 Octobre 2021**

*28-29 oct. 2021 75005 Paris (France)*



Sculpture (1967, Jean Marais), représentant Marcel Aymé en « passe-murailles », installée en 1989 sur la place éponyme, quartier Montmartre, Paris

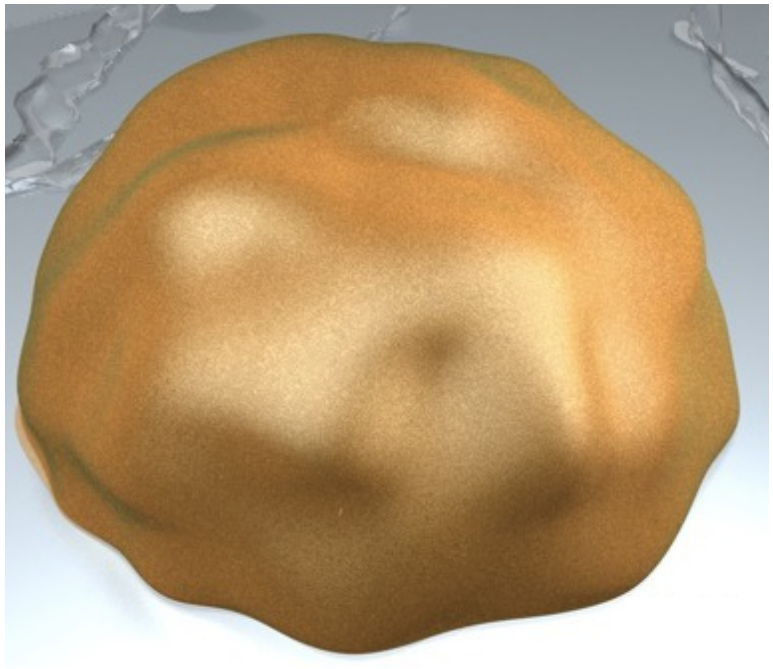
# Progrès récents dans le transport de molécules au travers des membranes cellulaires ou comment des molécules polaires de haut poids moléculaire peuvent traverser une barrière imperméable, sans systèmes de transport spécialisés

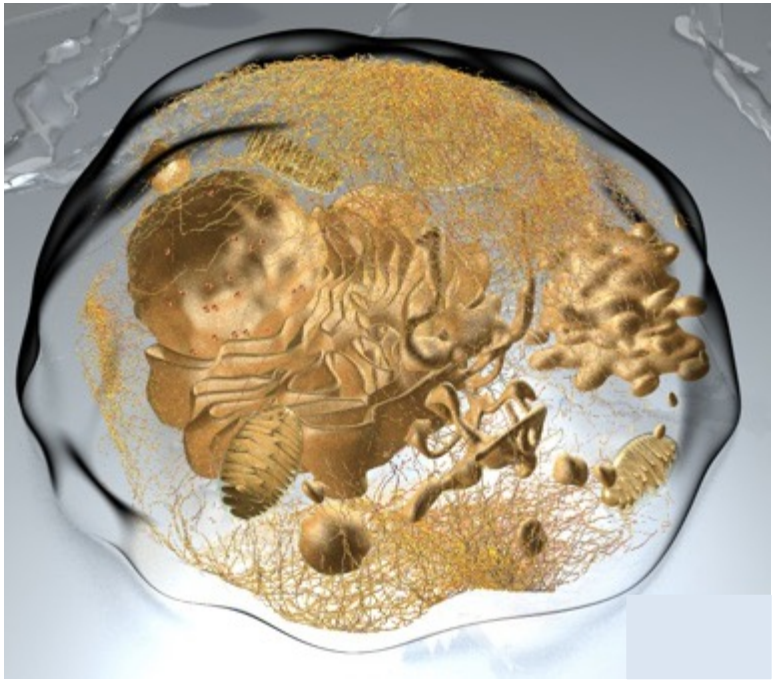
**Sandrine Sagan, Laboratoire des Biomolécules, Paris**

# At every level of organization, a living organism must maintain homeostasis.

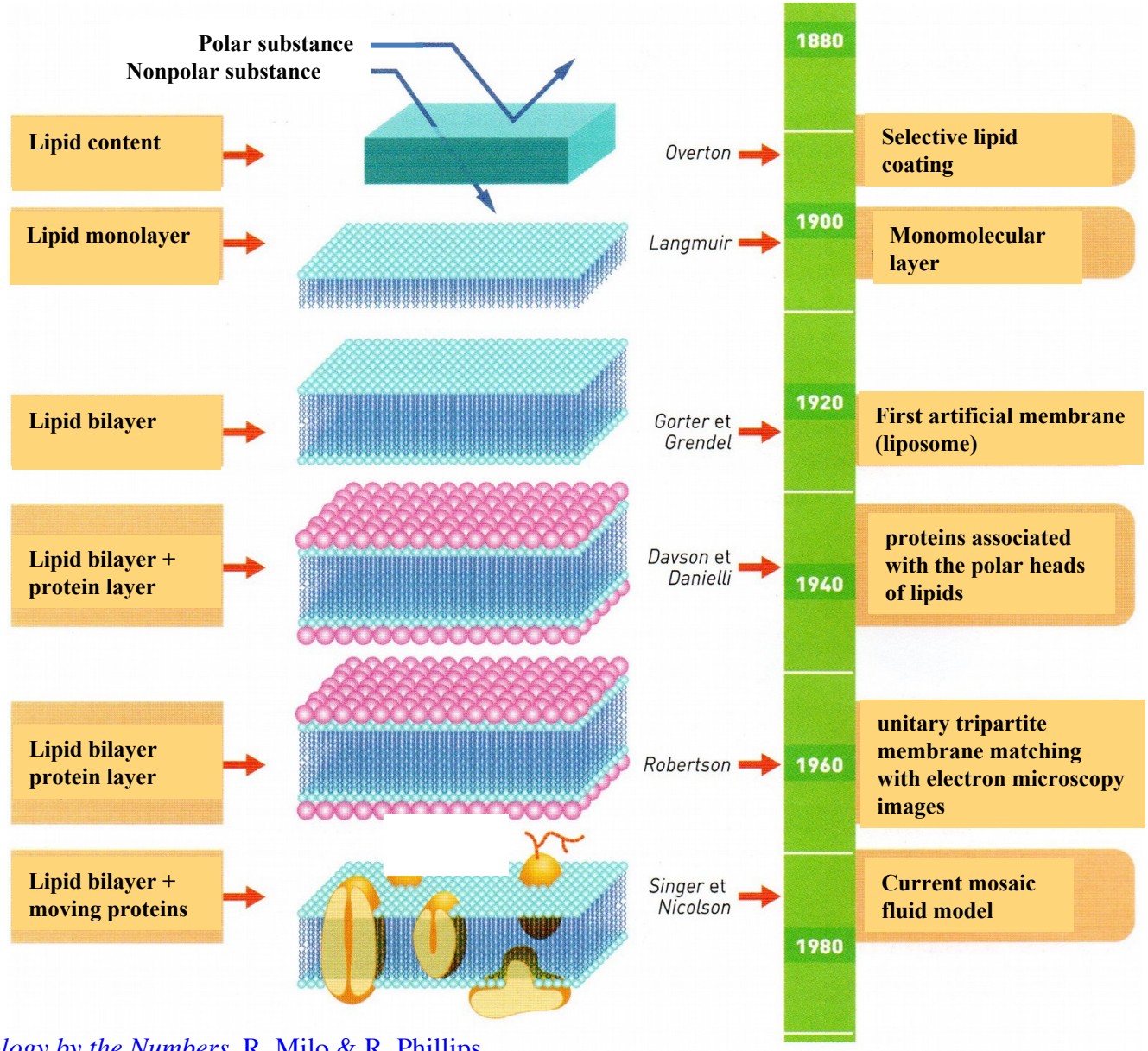
- Electrochemical potential
- Osmotic pressure
- pH
- Temperature
- Red-ox balance

**Cell membranes : barriers to uncontrolled diffusion**





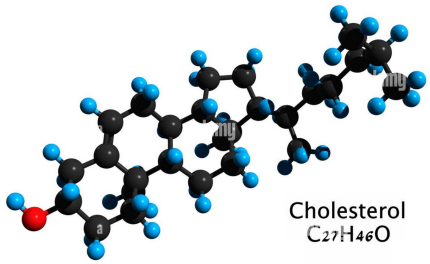
# Description of the eucaryotic cell membrane

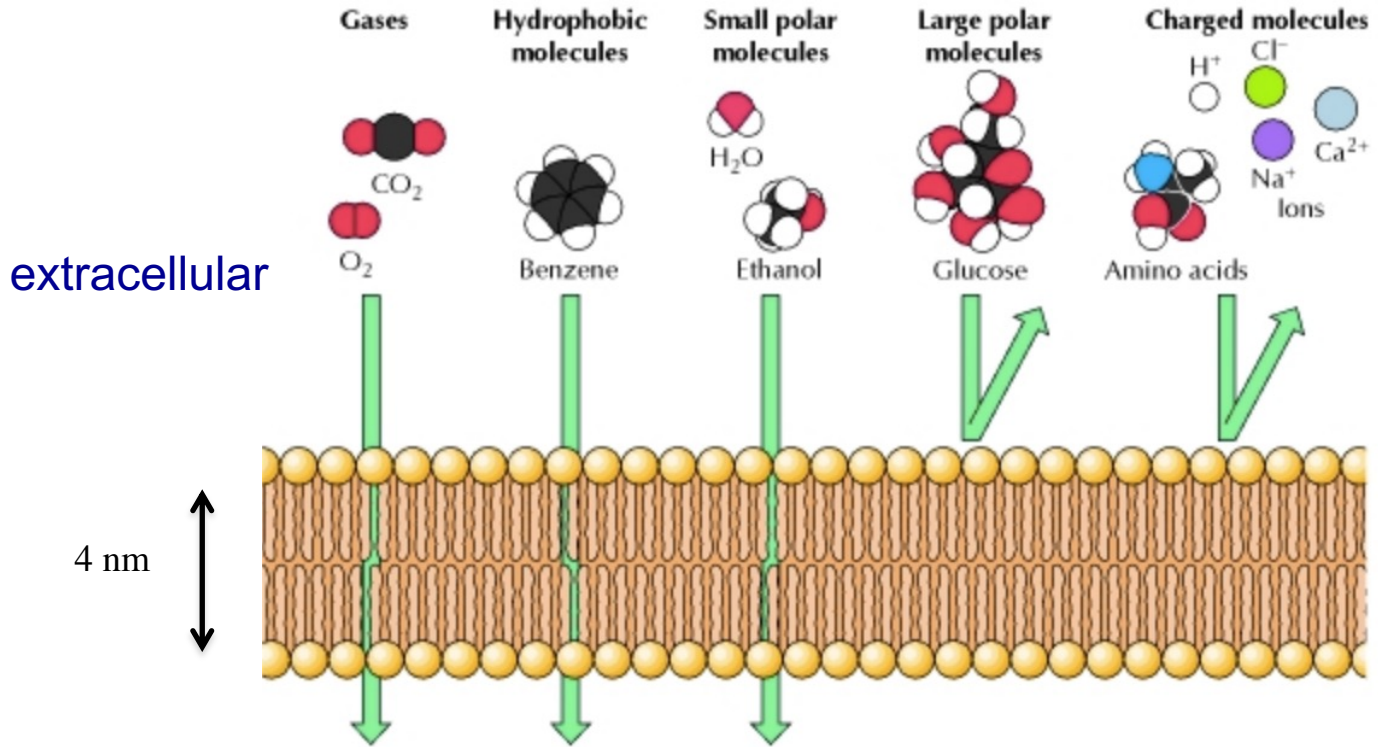



Adapted from *Cell Biology by the Numbers*, R. Milo & R. Phillips

# Lipid bilayer permeability

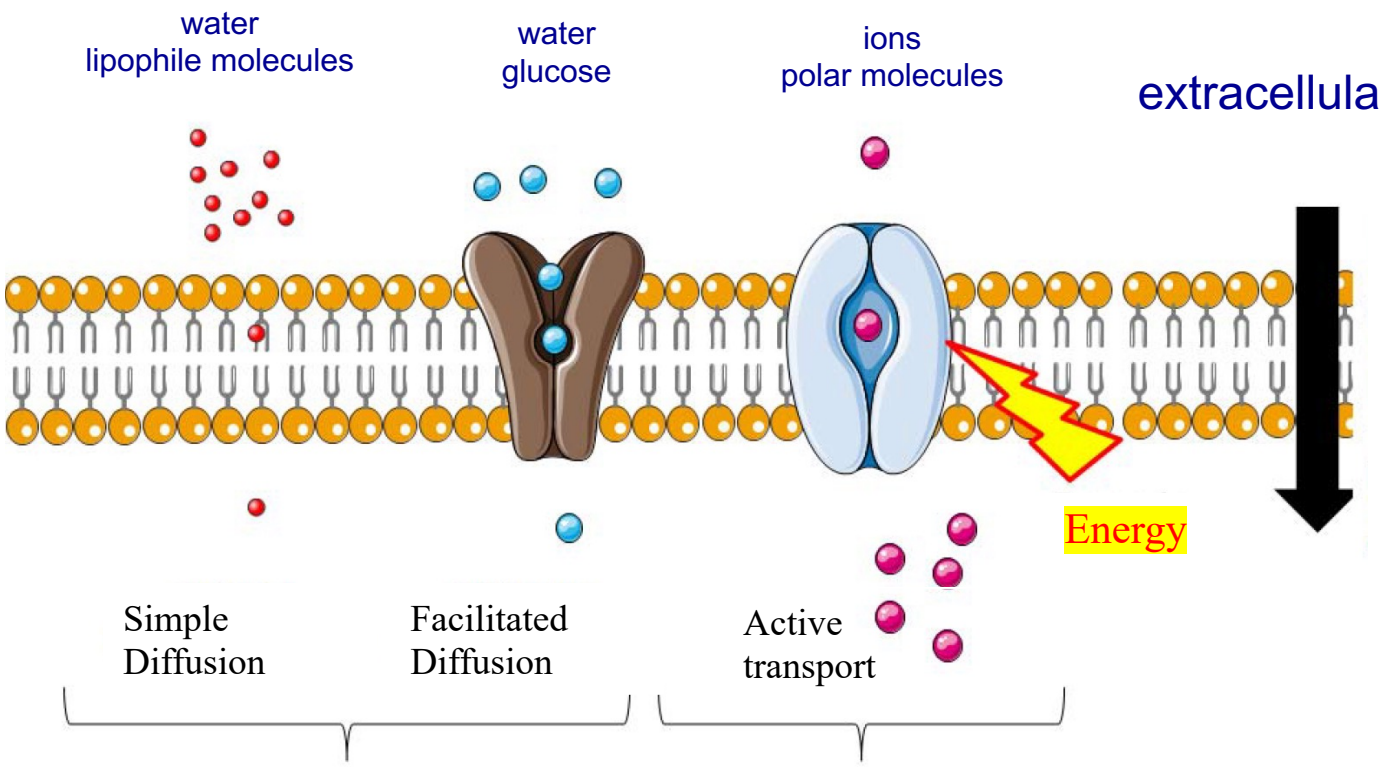
Passive diffusion



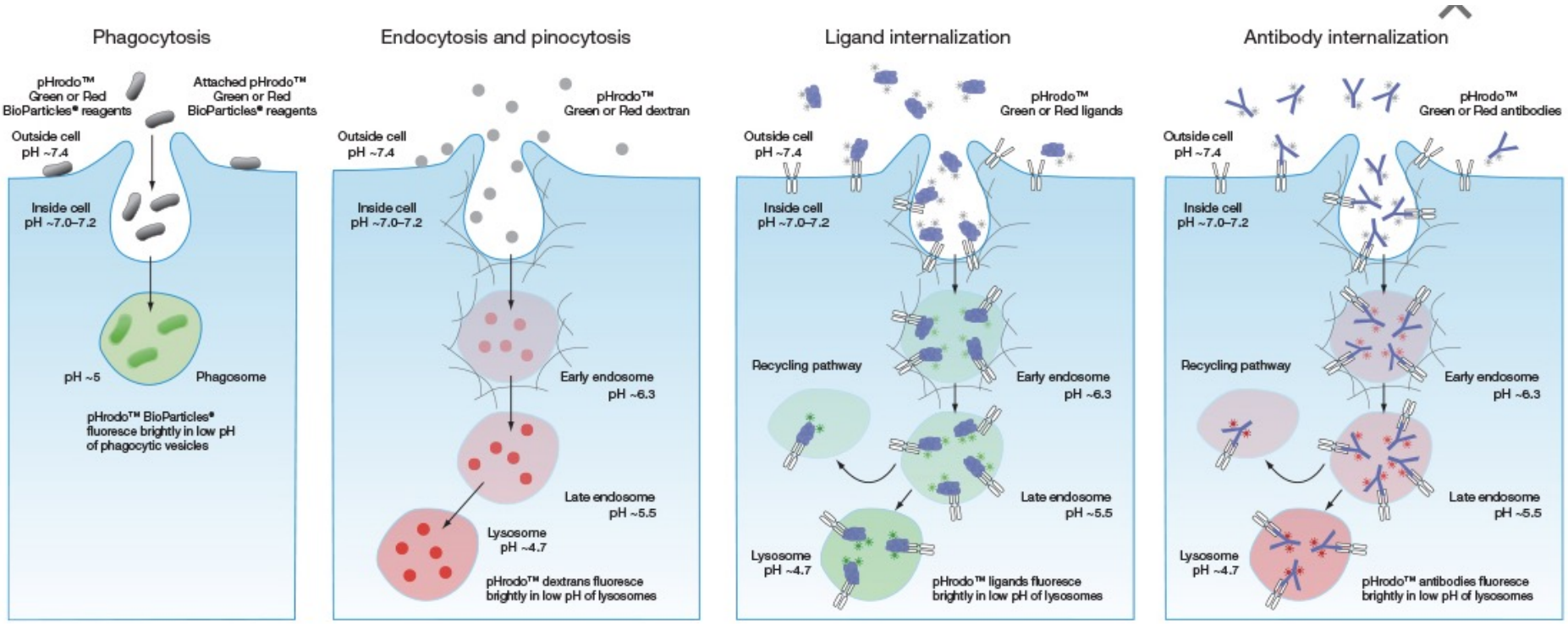



# Transport across cell membrane: how ?

Ions
Glucose
Amino acids
neurotransmitters



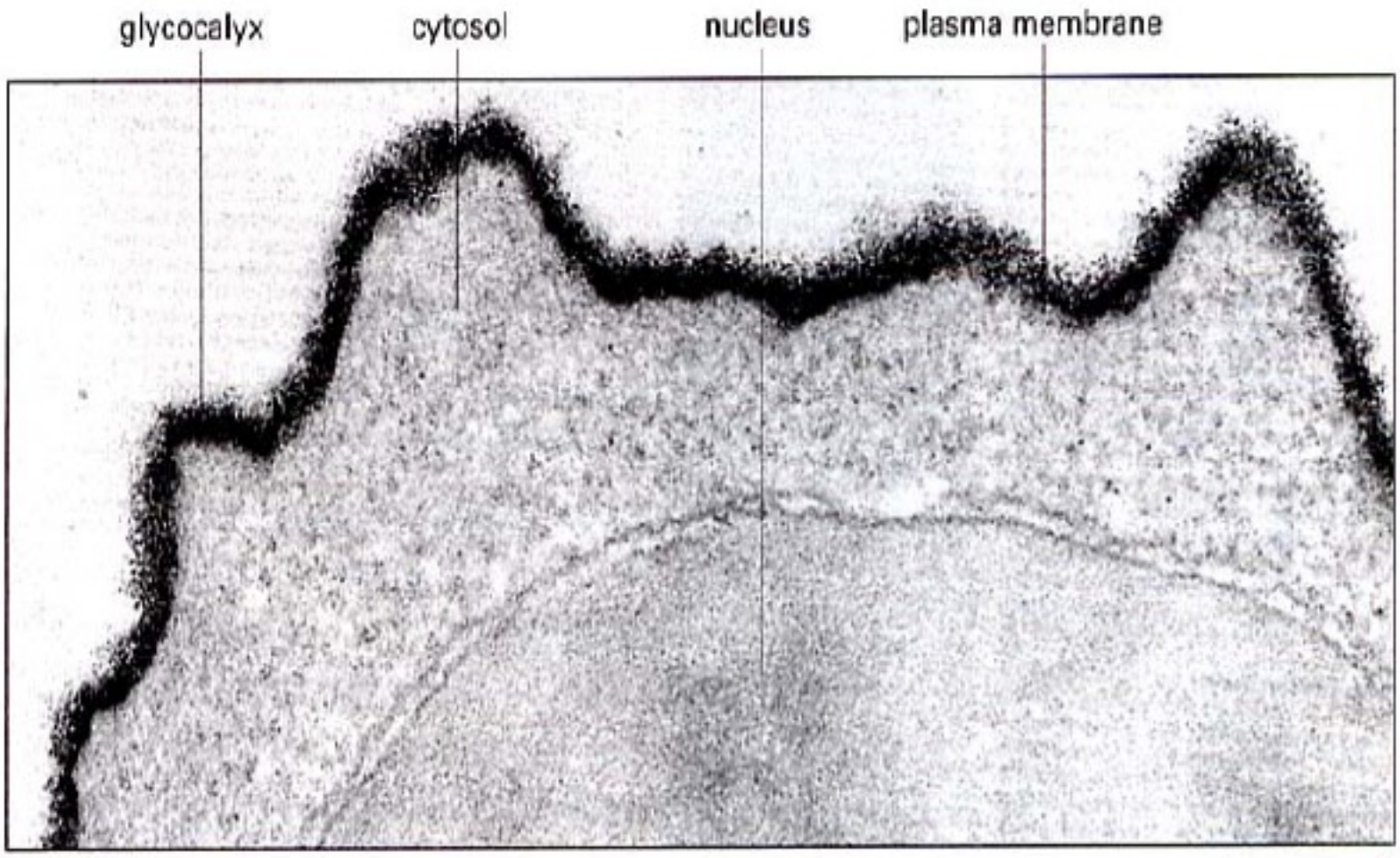

# Active transport pathways at the level of the lipid bilayer





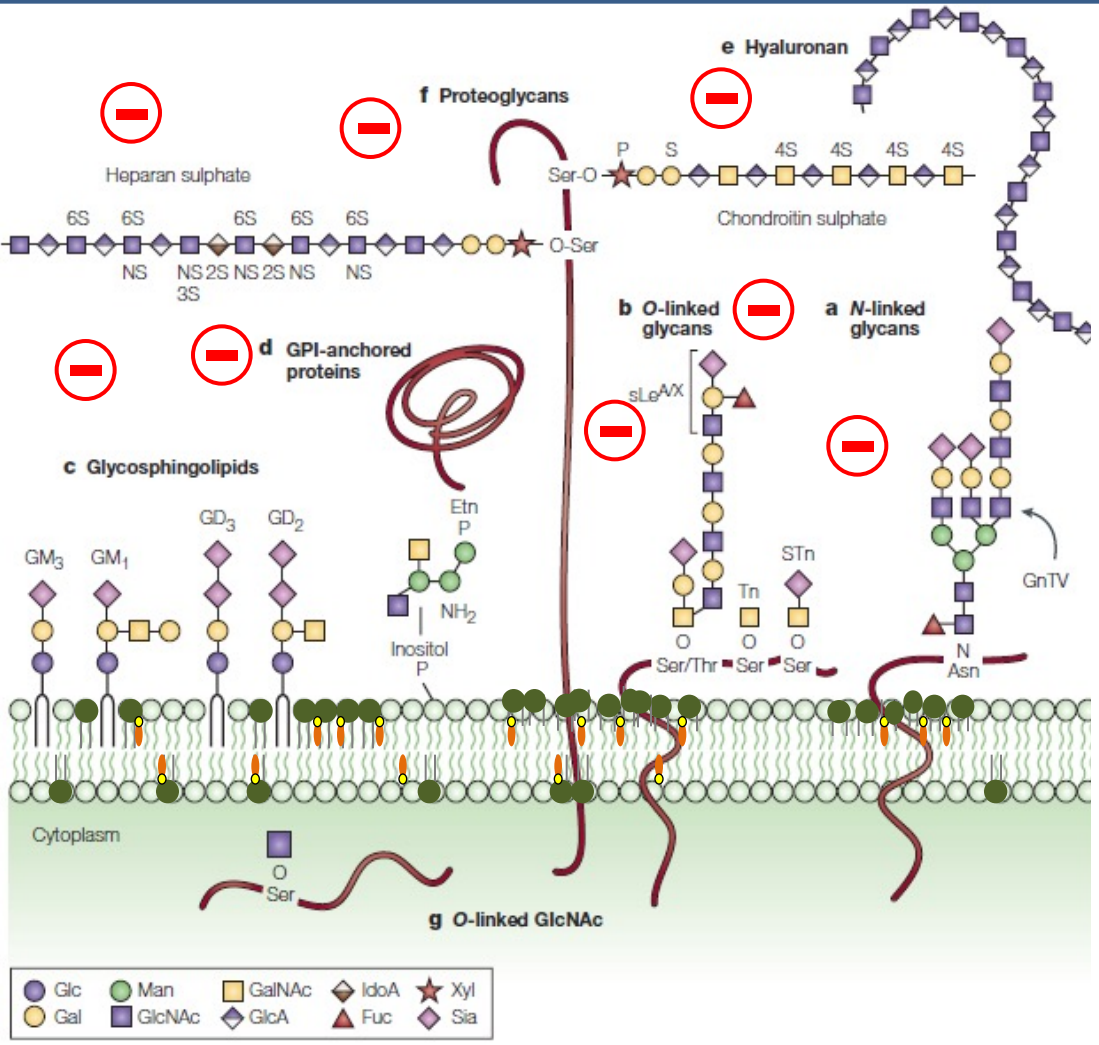
https://www.thermofisher.com/fr/fr/home/life-science/cell-analysis/cell-viability-and-regulation/endocytosis-exocytosis-and-phagocytosis.html

# Cell membrane : more than just a lipid bilayer and proteins




Molecular Biology of the Cell

# Cell membrane composition : lipids, proteins … and polysaccharides




**Glycocalyx**
- ✓Polysaccharides
- ✓Associated proteins

*NEGATIVELY charged*

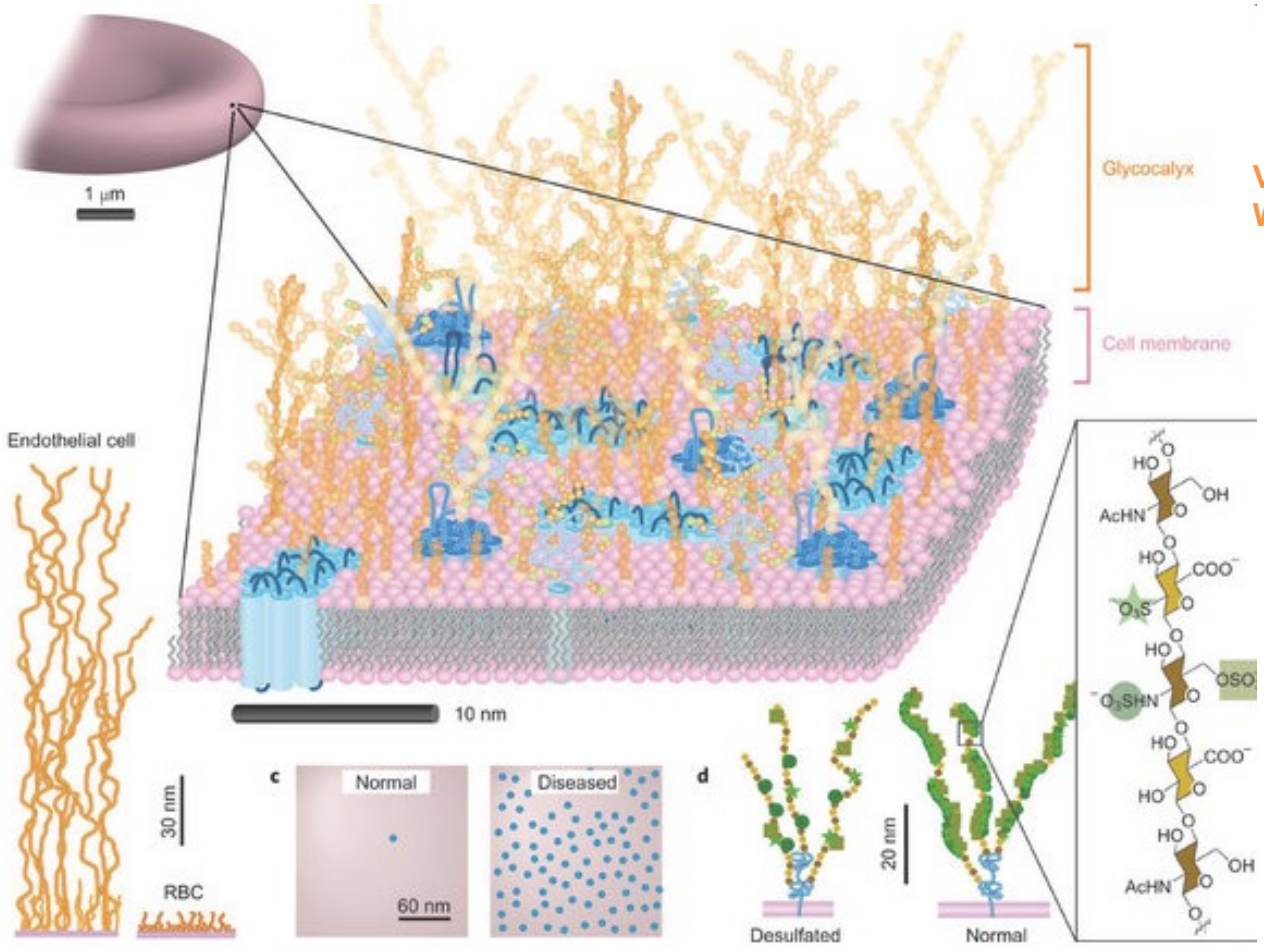
**Lipid Bilayer**
- ✓Heterogeneous (lateral/transverse asymmetry)
- ✓Dynamic

*HYDROPHOBIC boundary*

Fuster, Nat. Rev. Cancer (2005) 5:526

# Cell membrane : many exploited portals of entry

- Virus, bacteria
- Growth factors, homeoproteins, toxins
- Peptides (protein-transduction domains, cell-penetrating peptides …)




Viscous gel-like layer
Water and ions

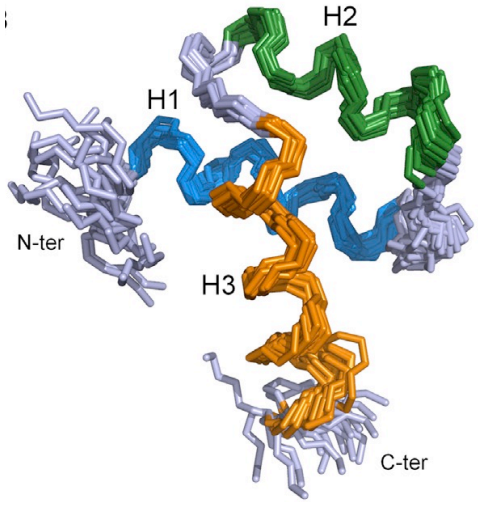


Mager, Nat Chem (2011) 3:582

# First evidence for cell transfer of an endogenous polypeptide

**60 amino acids basic polypeptide derived from *Antennapedia* homeoprotein (transcription factor)**

**KRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKENKTKGEPGSGGEGDEITPPNSPQ**



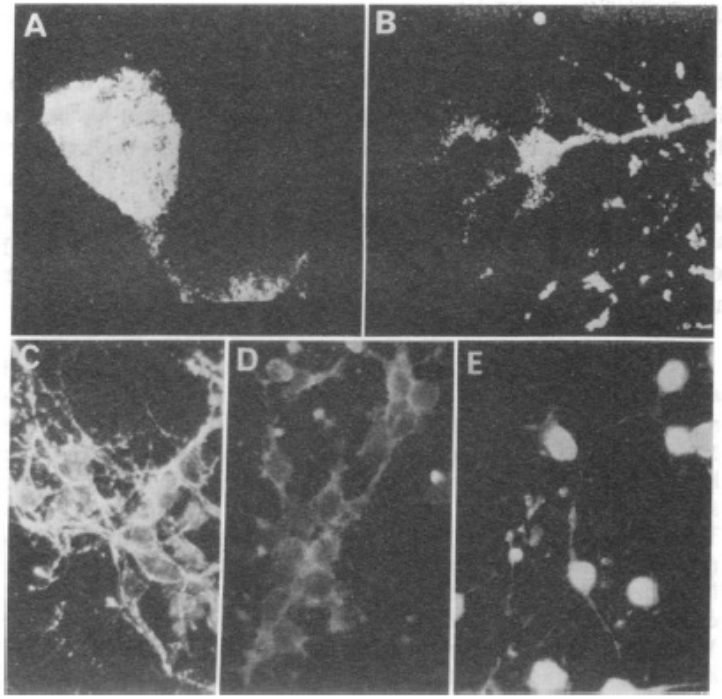




FIG. 5. Fluorescent pAntp penetrates live nerve cells. Confocal sections of living neurons at the soma (*A*) and the growth cone (*B*). Neural cell adhesion molecule immunostaining of live cells untreated (*C*) or treated with proteinase K (*D*). (×500.) (*E*) Intracellular and nuclear localization of pAntp in cells incubated for 1 hr with the fluorescent peptide, treated with proteinase K as in *D*, and fixed. (×260.)



Carlier (2013) Biophys J, 105:667

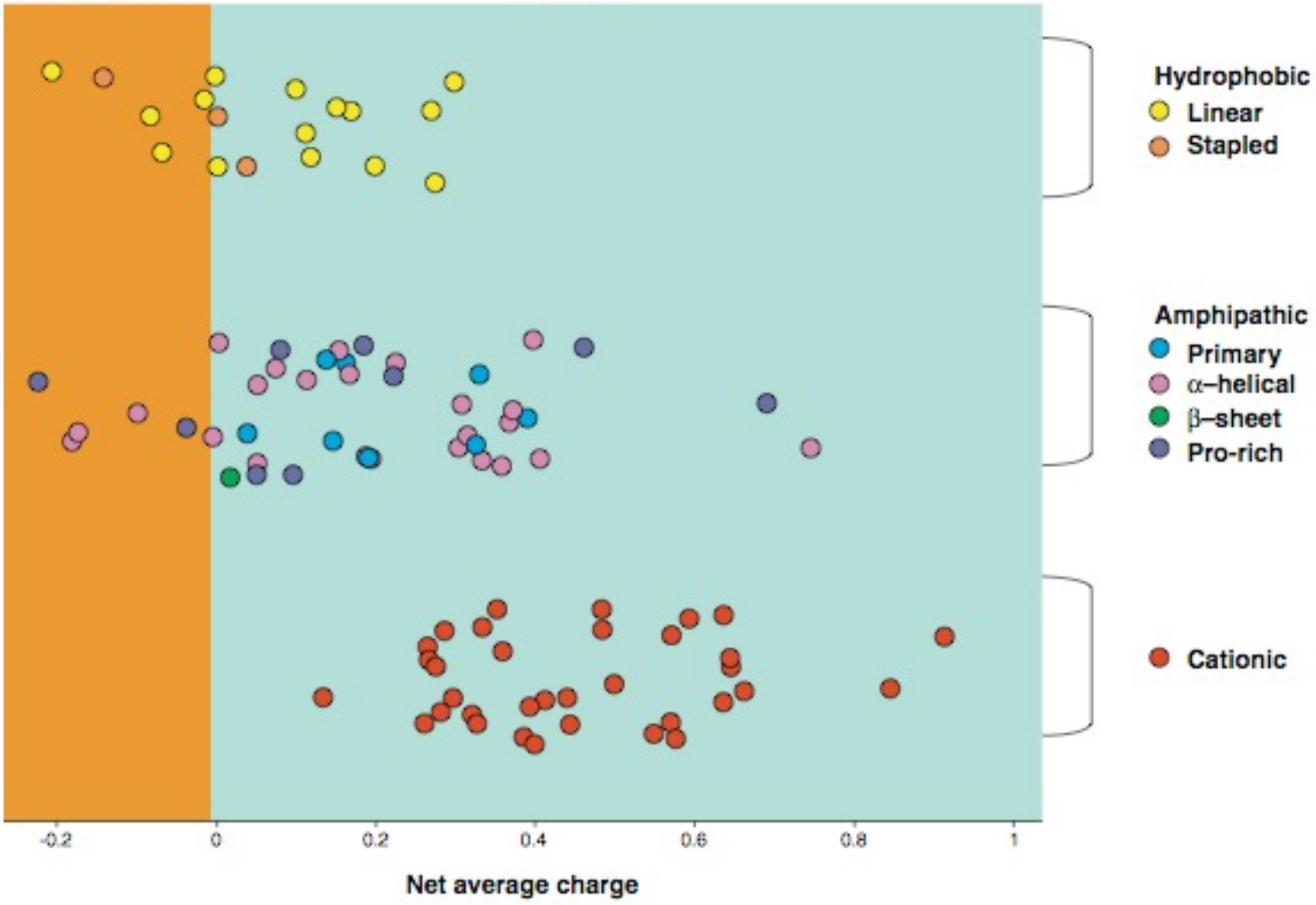
Joliot, Prochiantz, Proc Natl Acad Sci USA. (1991) 88:1864

# Cationic cell-penetrating peptides

| CPPs derived from heparan-, RNA- and DNA-binding proteins | | |
|---|---|---|
| *Cationic* | | Refs |
| **Heparan binding proteins** | | |
| RKKRRRESRKKRRRES | DPV3 | [77] |
| GRPRESGKKRKRKRLKP | DPV6 | [77] |
| GKRKKKGKLGKKRDP | DPV7 | [77] |
| GKRKKKGKLGKKRPRSR | DPV7b | [77] |
| RKKRRRESRRARRSPRHL | DPV3/10 | [77] |
| SRRARRSPRESGKKRKRKR | DPV10/6 | [77] |
| VKRGLKLRHVRPRVTRMDV | DPV1047 | [77] |
| SRRARRSPRHLGSG | DPV10 | [77] |
| LRRERQSRLRRERQSR | DPV15 | [77] |
| GAYDLRRRERQSRLRRRERQSR | DPV15b | [77] |
| **RNA binding proteins** | | |
| RKKRRQRRR | HIV-1 Tat | [34] |
| RRRRNRTRRNRRRVR | FHV coat | [35,97] |
| TRQARRNRRRRWRERQR | HIV-1 Rev | [35,97] |
| TRRQRTRRARRNR | HTLV-II Rex | [35,97] |
| KMTRAQRRAAARRNRWTAR | BMV Gag | [35,97] |
| NAKTRRHERRRKLAIER | P22 N | [35] |
| MDAQTRRRERRAEKQAQWKAAN | λN(1–22) | [35] |
| TAKTRYKARRAELIAERR | φ21N(12–29) | [35] |
| TRRNKRNRIQEQLNRK | Yeast PrP6 | [35] |
| **DNA binding proteins** | | |
| PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR | Protamine 1 | [98] |
| *Leucine zipper* | | |
| RIKAERKRMRNRIAASKSRKRKLERIAR | Human cJun | [35,97] |
| KRRIRRERNKMAAAKSRNRRRELTDT | Human cFos | [35,97] |
| *Transcription factors* | | |
| KRARNTEAARRSRARKLQRMKQ | Yeast GCN4 | [35] |
| *Homeoproteins* | | |
| RQIKIWFQNRRMKWKK | Penetratin | [75,99] |
| RVIRVWFQNKRCKDKK | Islet-1 | [100] |
| SKRTRQTYTRYQTLELEKEFHFNRYITRRRRIDI-ANALSLSERQIKIWFQNRRMKSKKDR | Fushi-tarazu | [101] |
| SQIKIWFQNKRAKIKK | Engrailed-2 | [99,101] |
| RQVTIWFQNRRVKEKK | HoxA-13 | [99] |
| KQINNWFINQRKRHWK | Knotted-1 | [99] |
| RHIKIWFQNRRMKWKK | PDX-1 | [102] |

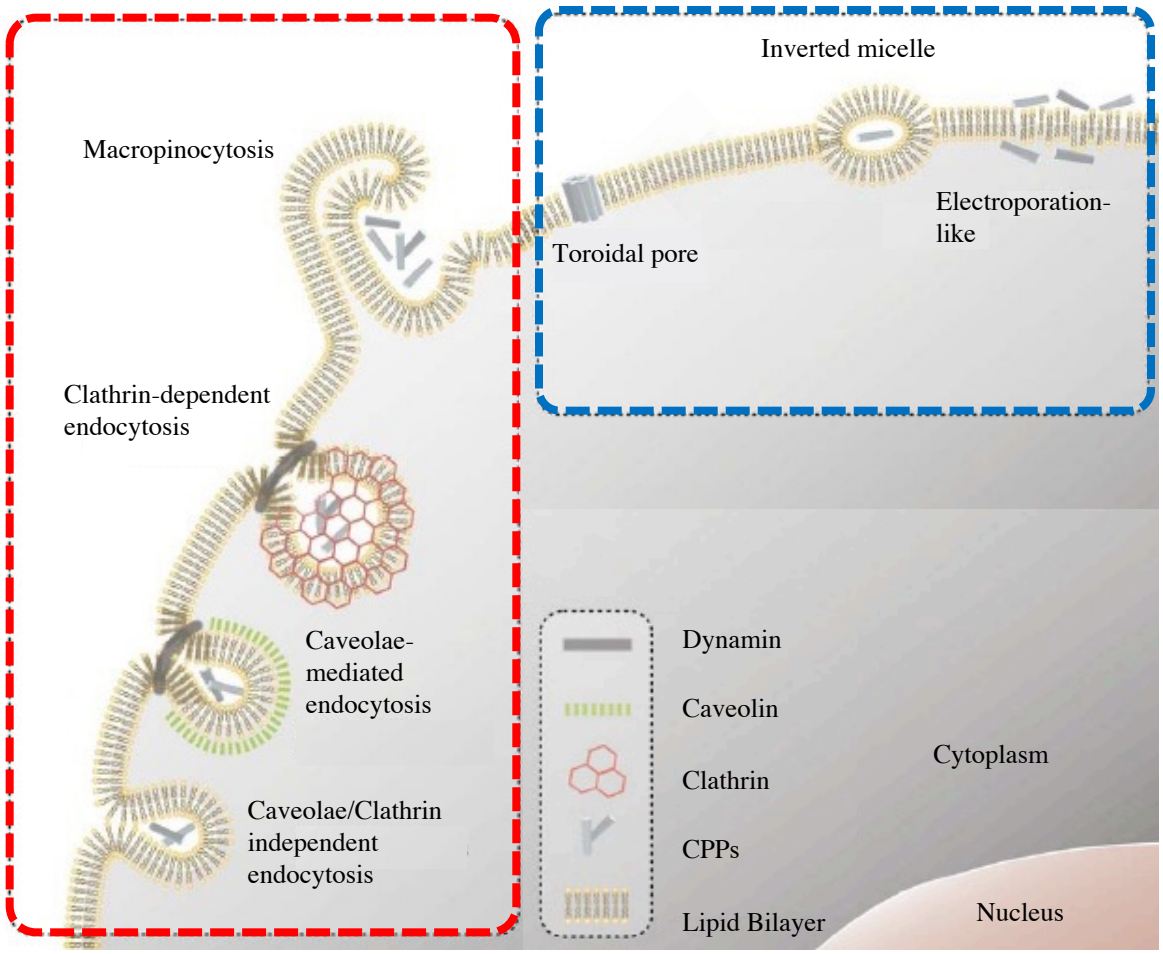
Milletti, Drug Discovery Today. (2012) 17:850

# CPPs : no particular molecular and structural features




Milletti, Drug Discov Today (2012), 17:850

# Internalization pathways for CPPs



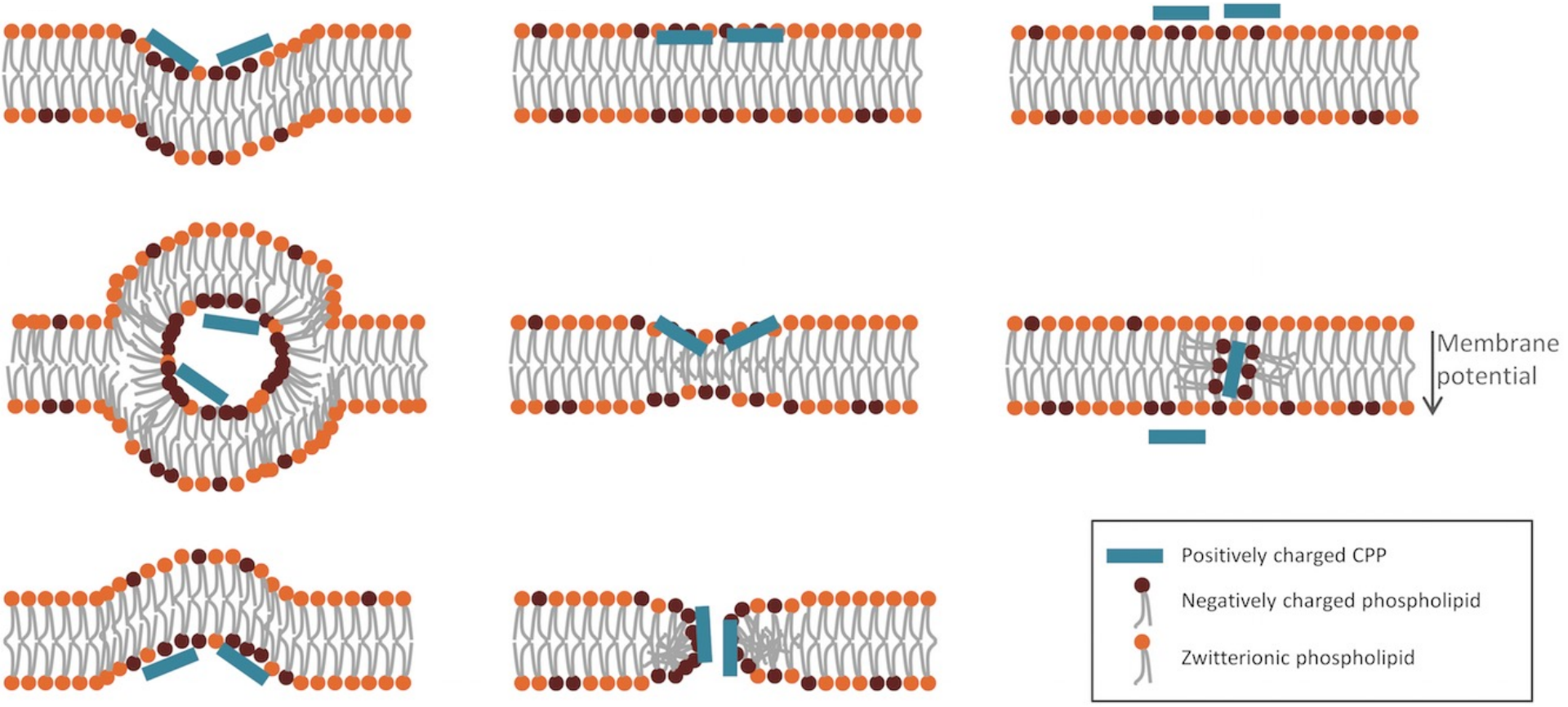

**Direct Translocation**
Less energy-dependent, still observed at 4 ° C (although decreased: altered membrane fluidity)

**Endocytosis**
energy dependent
blocked at 4 ° C

*Different mechanisms occur **simultaneously***

Adapted from Trabulo, Pharmaceuticals (2010) 3:961

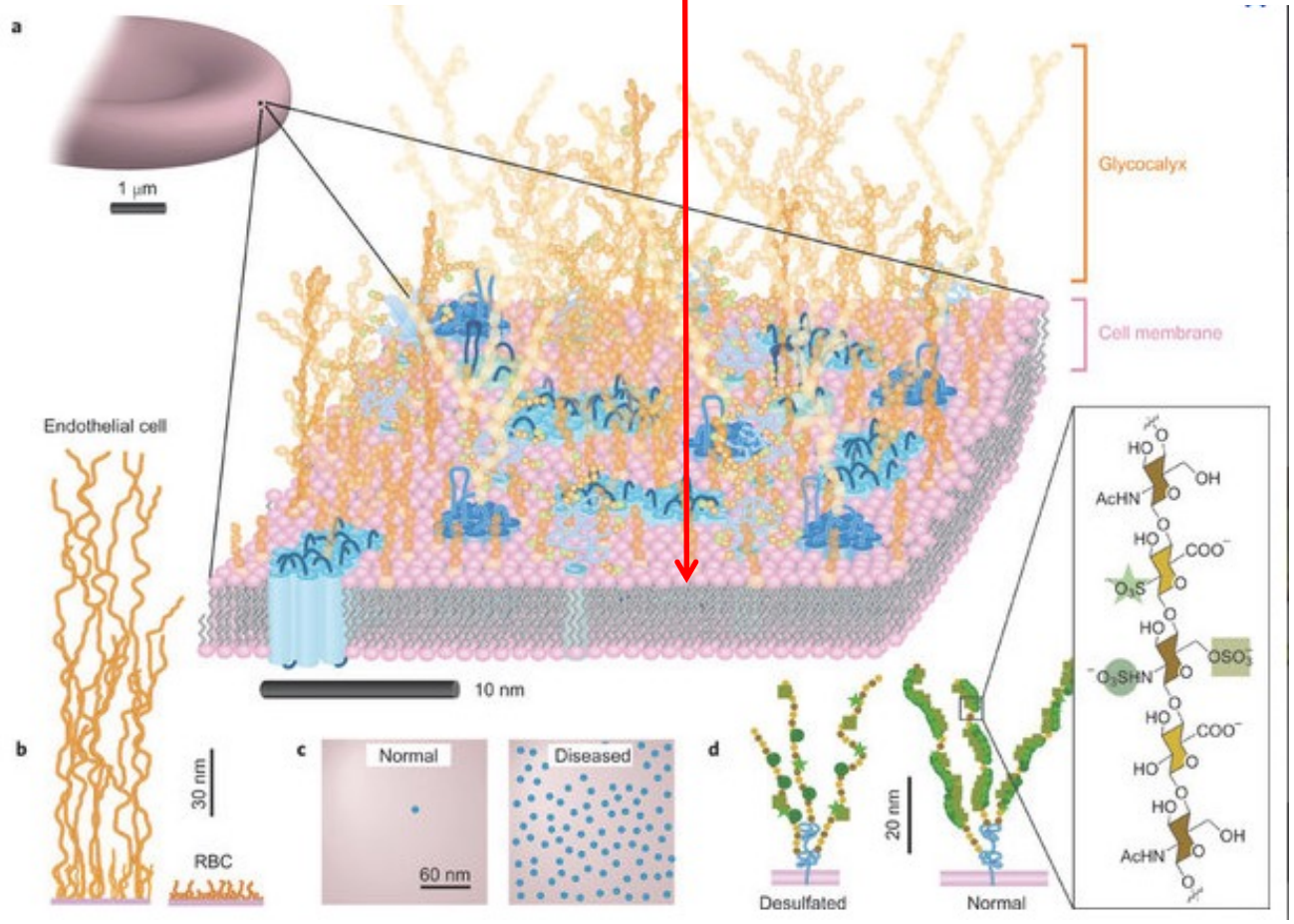
# Hypothetical current models for translocation




Bechara, FEBS (2013) 587: 1693

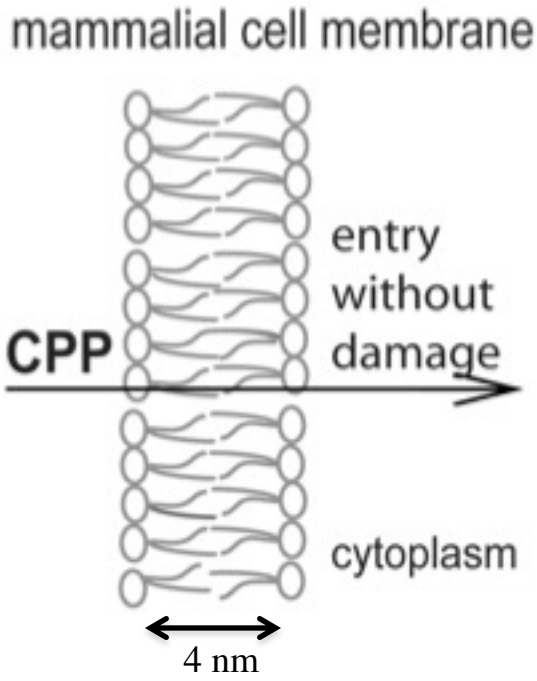
# Cell membrane : many exploited portals of entry

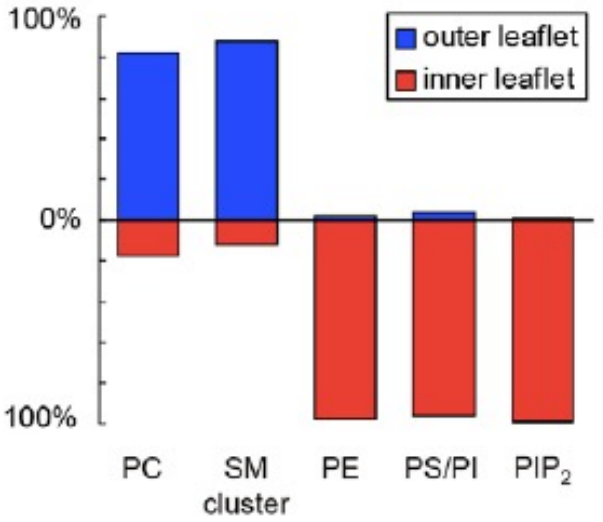
Focus on lipids




Mager, Nat Chem (2011) 3:582

# CPP passage does not damage cell plasma membrane




Net charge : 0

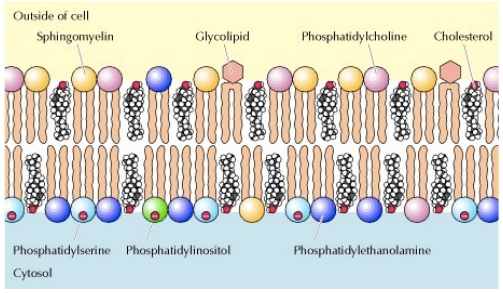
Net charge : negative

Absence of cell toxicity

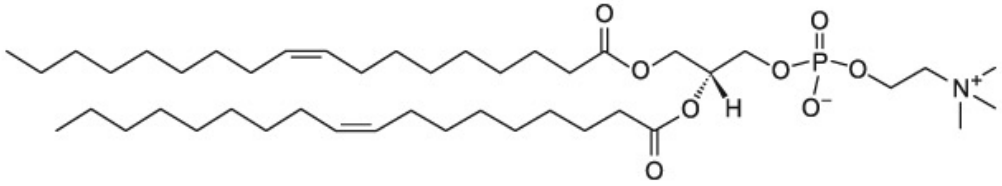
Nekhotiaeva, FASEB J (2004) 18: 394

Murate, J Cell Sci (2015) 128, 1627

# Membrane lipid partners for CPPs



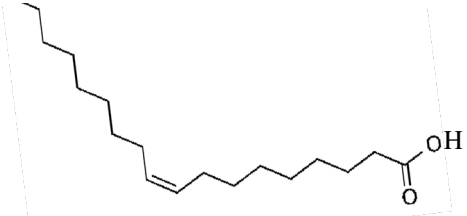

Free energy profiles as functions of the distance of the center of mass of the TAT peptide from the center of mass of the lipid bilayer (DOPC, oleic acid)



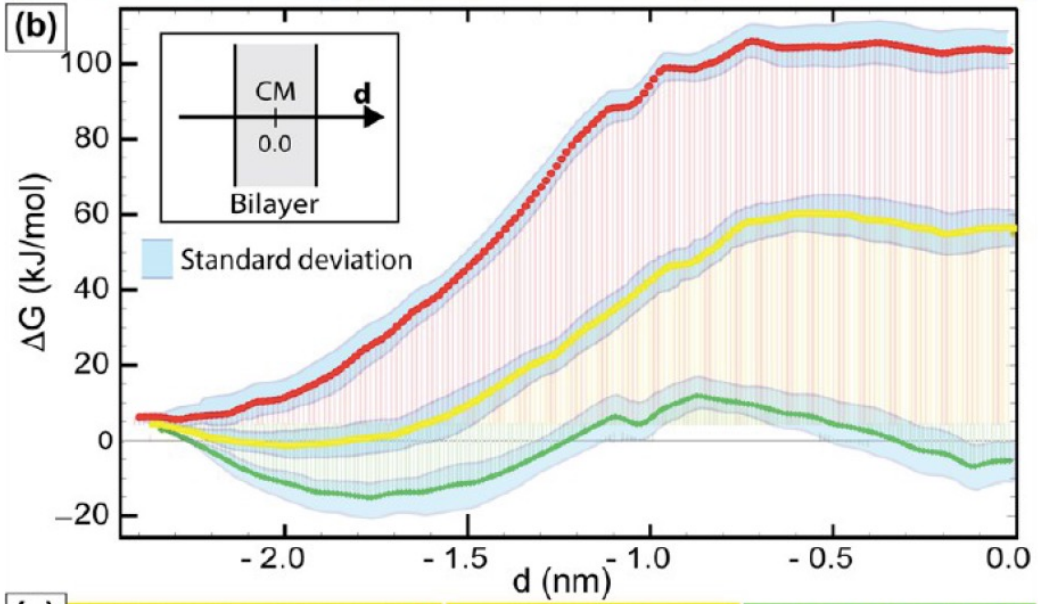
DOPC (18:1 Δ9-cis PC)

Molecular Simulation Dynamics

- 1 TAT (YGRKKRRQRRR)
- 8700 water molecules
- 68 DOPC
- 48 oleic acid



Oleic acid (C18:1)




Protonated COOH

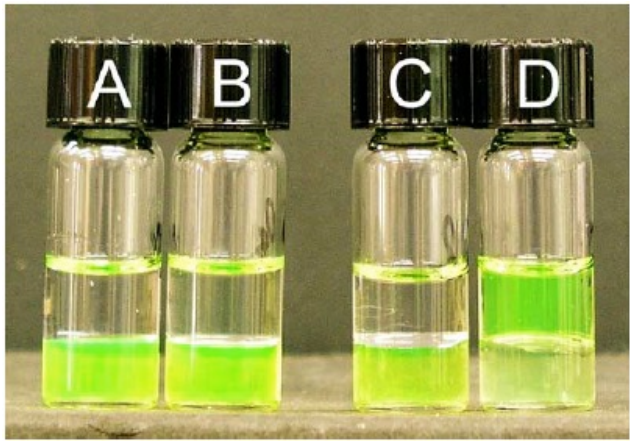
Half Protonated COOH / $COO^-$

Deprotonated $COO^-$

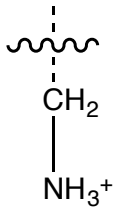
Herce, JACS (2014) 18: 394

# How does a cationic peptide penetrate a hydrophobic bilayer?

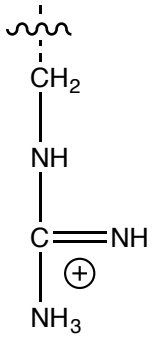
Octanol/water partition




A, C : CF-Lys8

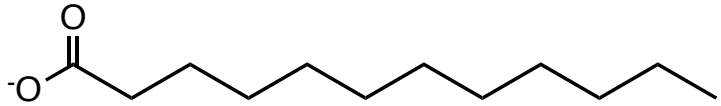



B, D : CF-Arg8

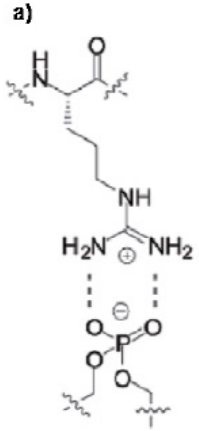


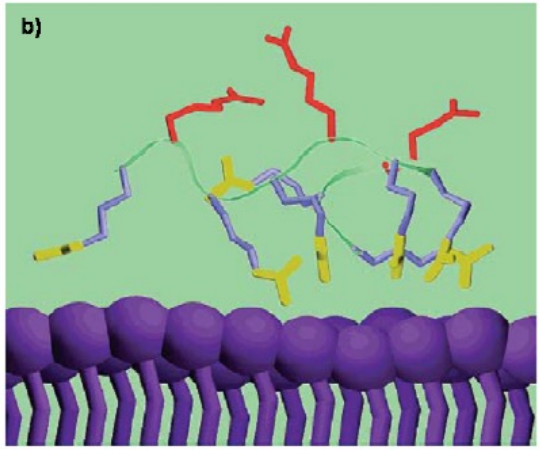

without

with sodium laurate (C12:0)




bidentate hydrogen bonds

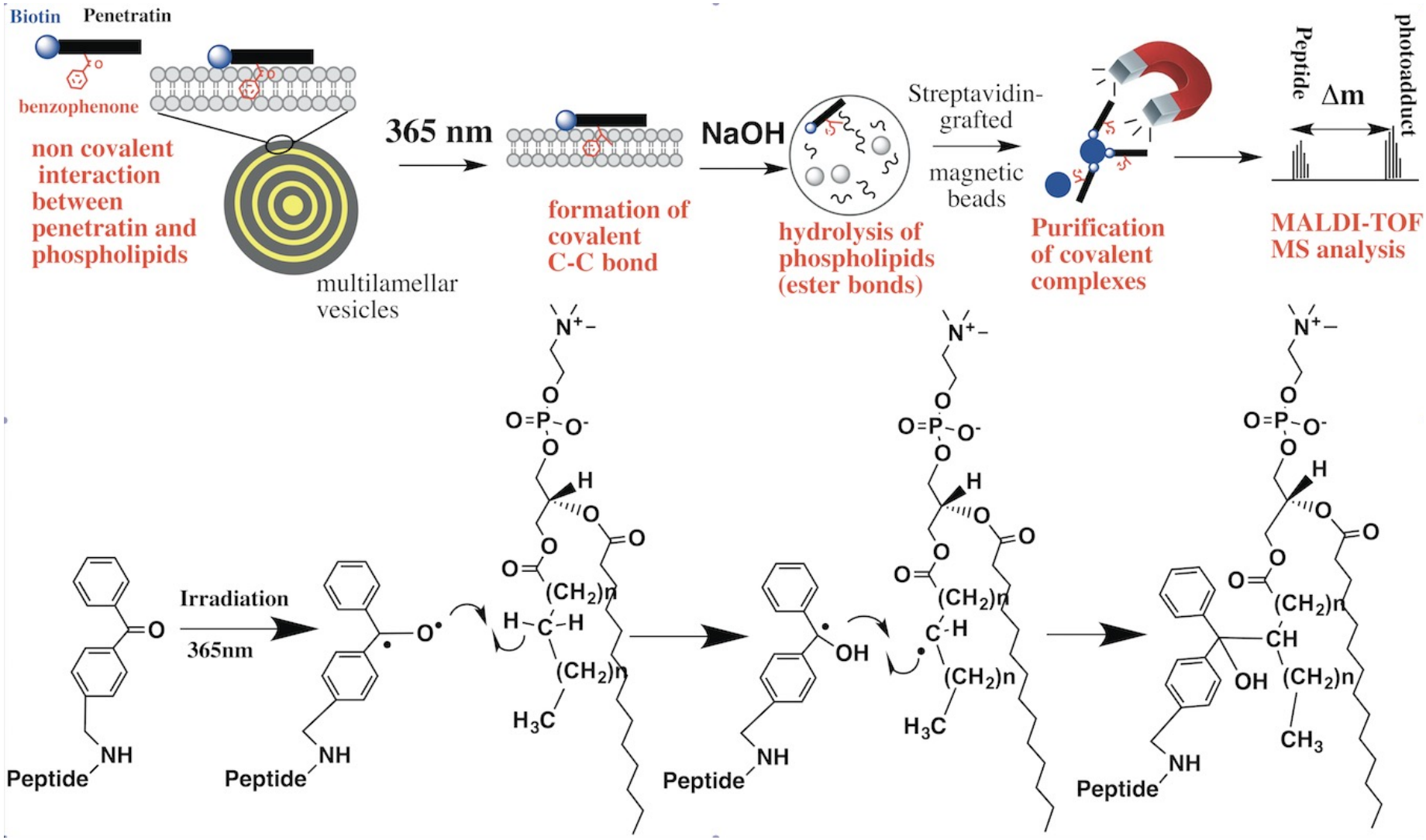



counter-ion phase transfer

Rothbard, Adv Drug Del Rev (2005) 57:495

# Lipid partners for translocation - Model membranes



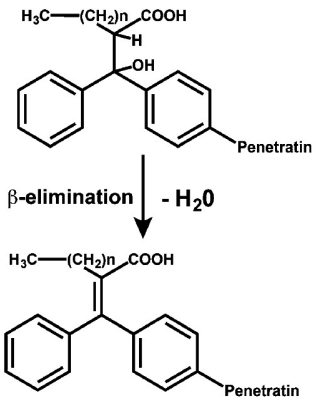

Jiao, Angew Chem (2017) 56:8226

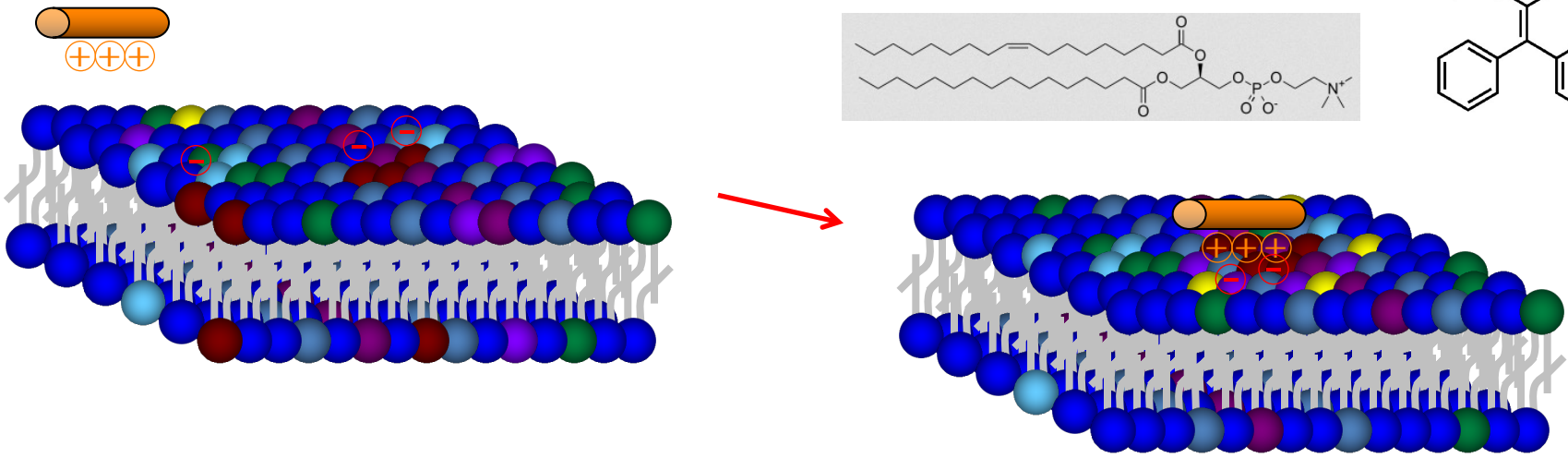
# Exploiting benzophenone photoreactivity to probe CPP insertion depth and surroundings

Penetratin has a preference for disordered phases :

- Negatively charged polar heads (PG > PC)
- Unsaturated fatty chains (C18:2 > C18:1 > C18)
- Short saturated fatty chains (C14 > C18)
- PC : photolabeling in α of carboxylate (water/lipid interface)

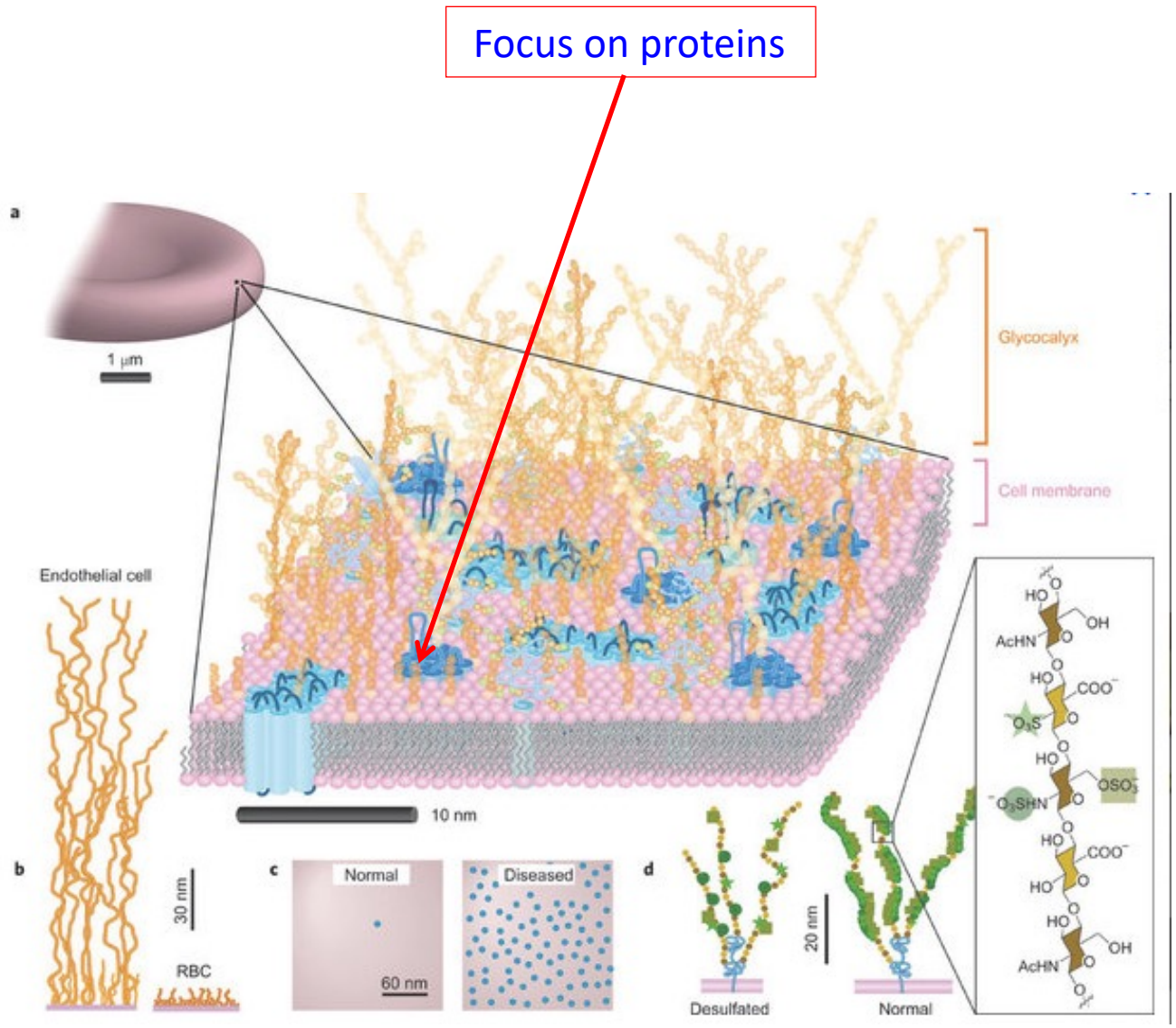
Pure PC vesicles



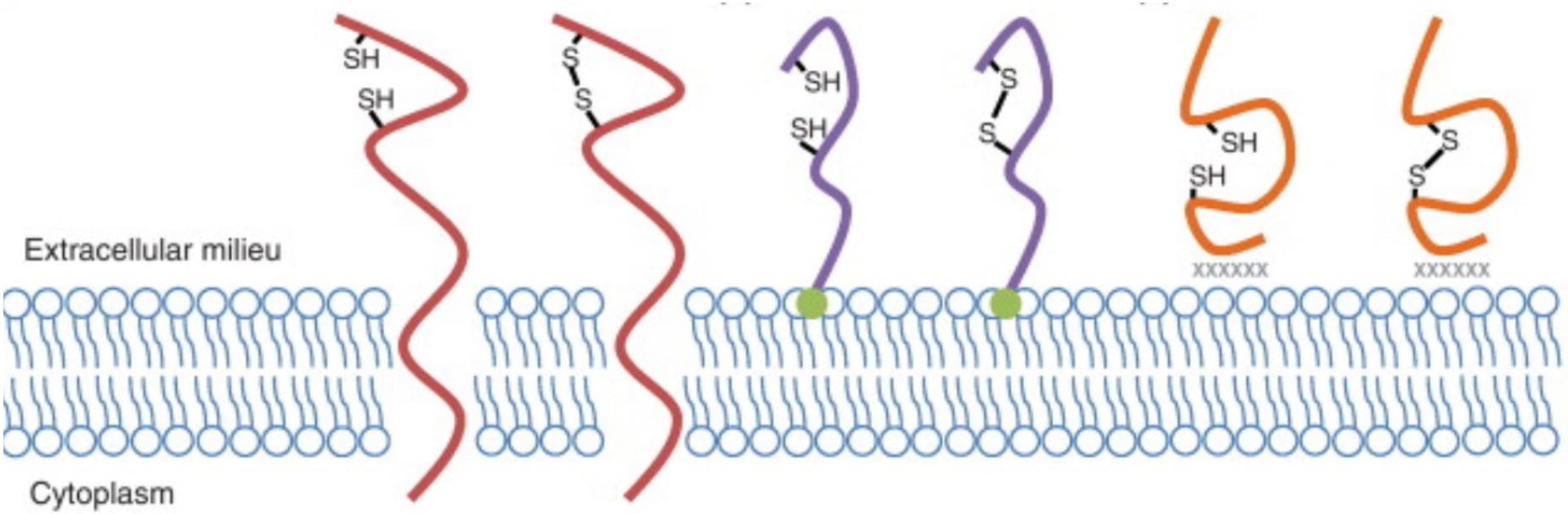
Jiao, Angew Chem (2017) 56:8226

# Cell membrane : many exploited portals of entry




Mager, Nat Chem (2011) 3:582

# Cell membrane : many exploited portals of entry




Membrane proteins expose thiol moieties to the extracellular milieu (exofacial thiols).

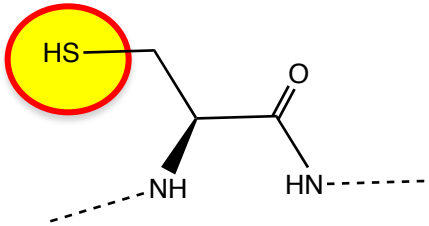
Thiols present in these proteins can be in reduced (-SH) or oxidized (S-S) form.



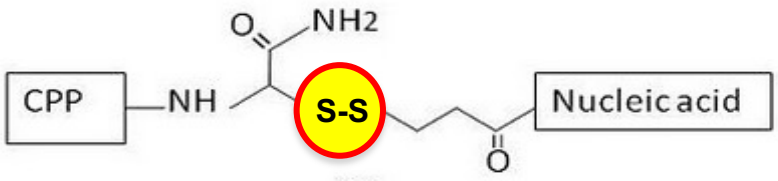
Torres, Trends Biotech (2012) 30:185

# Impact of cell surface thiols in CPP internalization

CRWRWKCCKK (CyLoP-1)



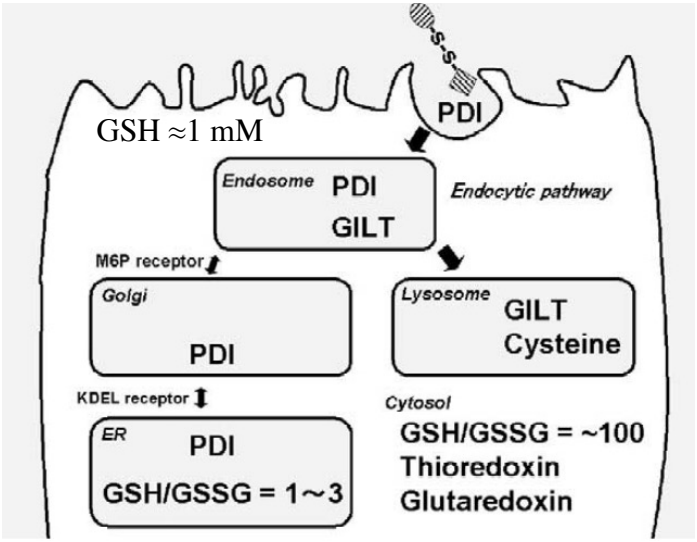

Cys-rich CPP (from crotamine toxin)




Disulfide conjugated CPP-cargo

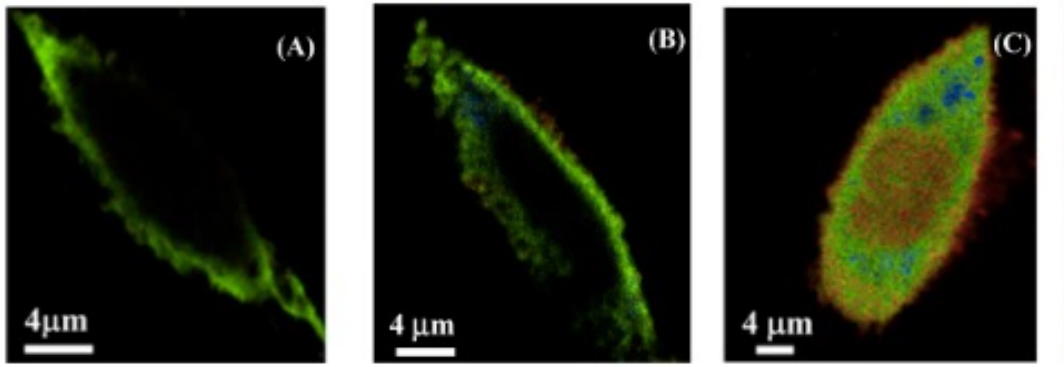
# Exofacial cell thiols

PDI : Protein Disulfure Isomerase
GILT : Gamma-interferon-inducible lysosomal thiol reductase

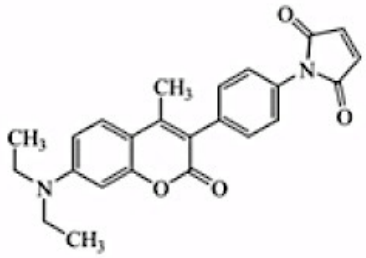



Saito, Adv Drug Del Rev (2003) 55:199

500 ps




Confocal image of CHO cells stained by (A) 10 nM CPM, (B) 200 nM CPM, (C) 800 nM CPM

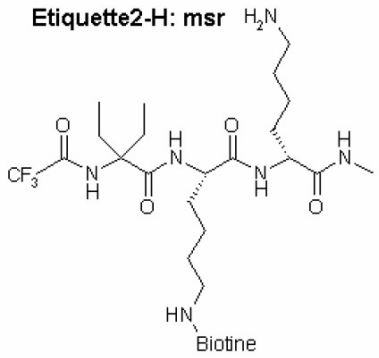


7-(diethylamino)-3-(4-maleimidophenyl)-4-methylcoumarin

Ghosh, J Phys Chem B (2014), 118:2949

# Impact of cysteinyl residues on CPP uptake efficiency

| Name | Sequence |
|---|---|
| **pAntp** | Biot-$G_4$RQIKIWFQNRRMKWKK-$NH_2$ |
| **Lin(pAntp)** | Biot-$G_4$CRQIKIWFQNRRMKWKKC-$NH_2$ |
| **Cyc(pAntp)** | Biot-$G_4$CRQIKIWFQNRRMKWKKC-$NH_2$ |
| **pAntp-2SAcm** | Biot-$G_4$C(Acm)RQIKIWFQNRRMKWKKC(Acm)-$NH_2$ |
| **$(R/W)_9$** | Biot-$G_4$RRWWRRWRR-$NH_2$ |
| **Lin$(R/W)_9$** | Biot-$G_4$CRRWWRRWRRC-$NH_2$ |
| **Cyc$(R/W)_9$** | Biot-$G_4$CRRWWRRWRRC-$NH_2$ |
| **$(R/W)_9$-2SAcm** | Biot-$G_4$C(Acm)RRWWRRWRRC(Acm)-$NH_2$ |
| **$^{msr}(R/W)_9$** | msr-CRRWWRRWRR-$NH_2$ |
| **PKCi-SAcm** | Biot-$G_4$C(Acm)RFARKGALRQKNV-$NH_2$ |
| **$^{msr}(R/W)_9$–PKCi** | msr-CRRWWRRWRR-$NH_2$<br>Biot-$G_4$CRFARKGALRQKNV-$NH_2$ |

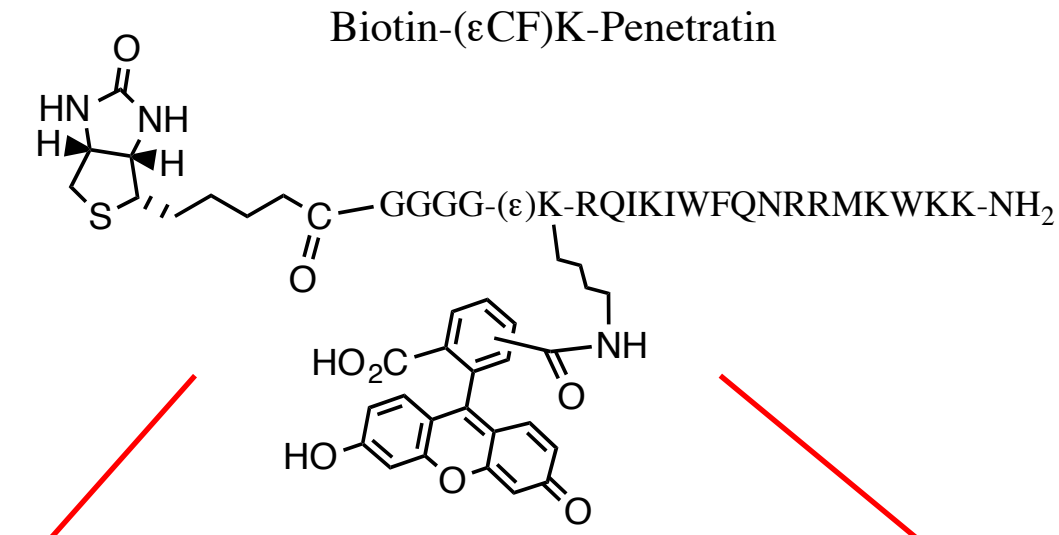



Study of the internalization efficacy of two CPP sequences without or with additional Cysteinyl residues in reduced or oxidized forms.



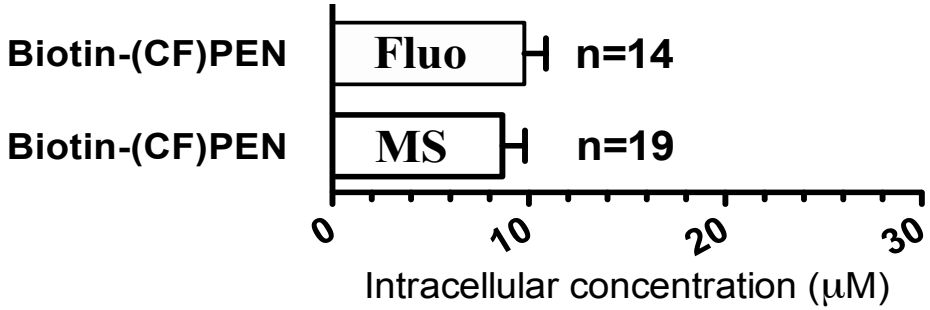
Aubry, FASEBJ (2009) 23: 2956-2967

# Methods to measure absolute intracellular peptide amounts




MALDI-TOF MS

Fluorescence spectroscopy

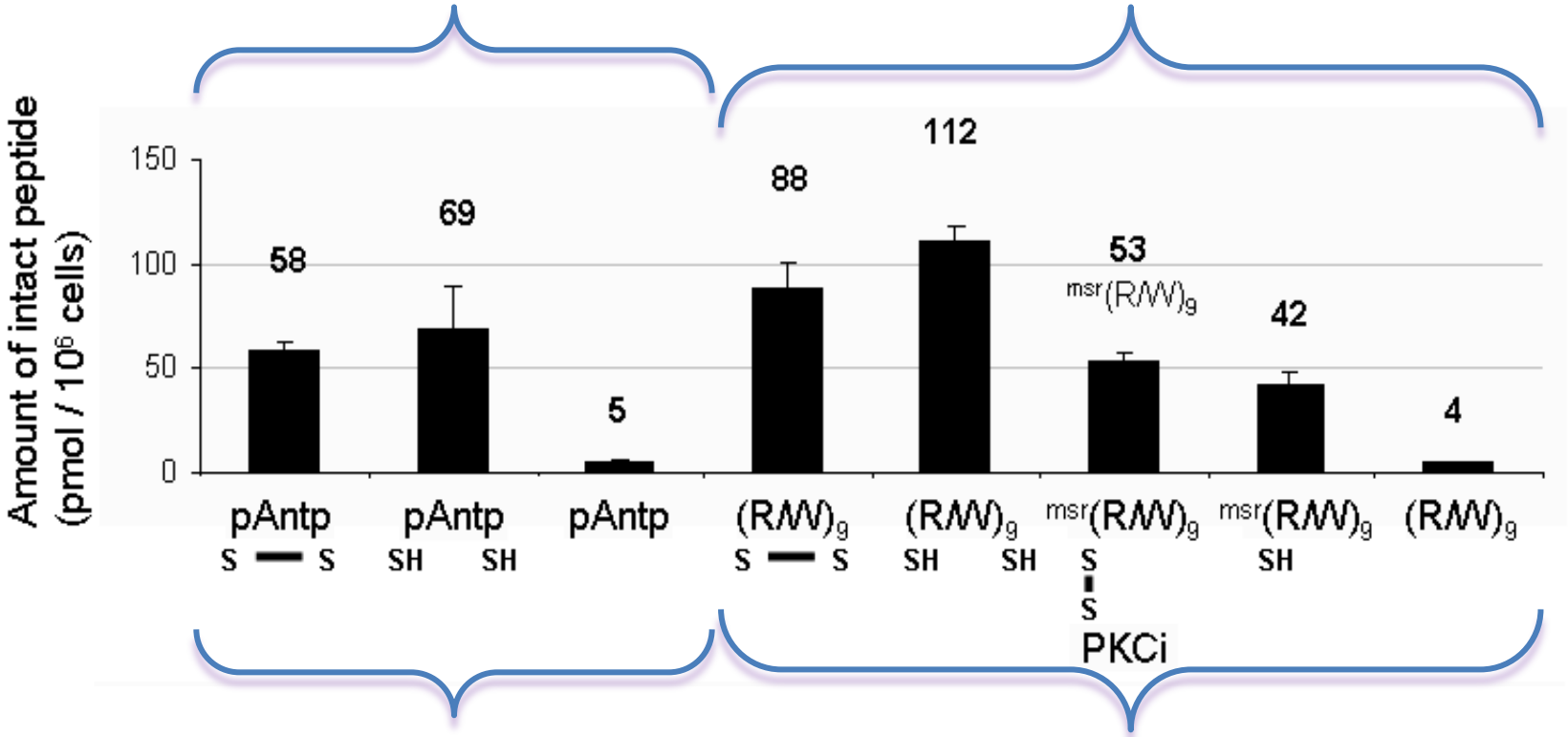





Burlina, Angew Chem. (2005) 44:4244; Nat Protoc. (2006) 1:200

Illien, Sci Rep. (2016) 6:36938

# Impact of cysteinyl residues on CPP uptake efficiency

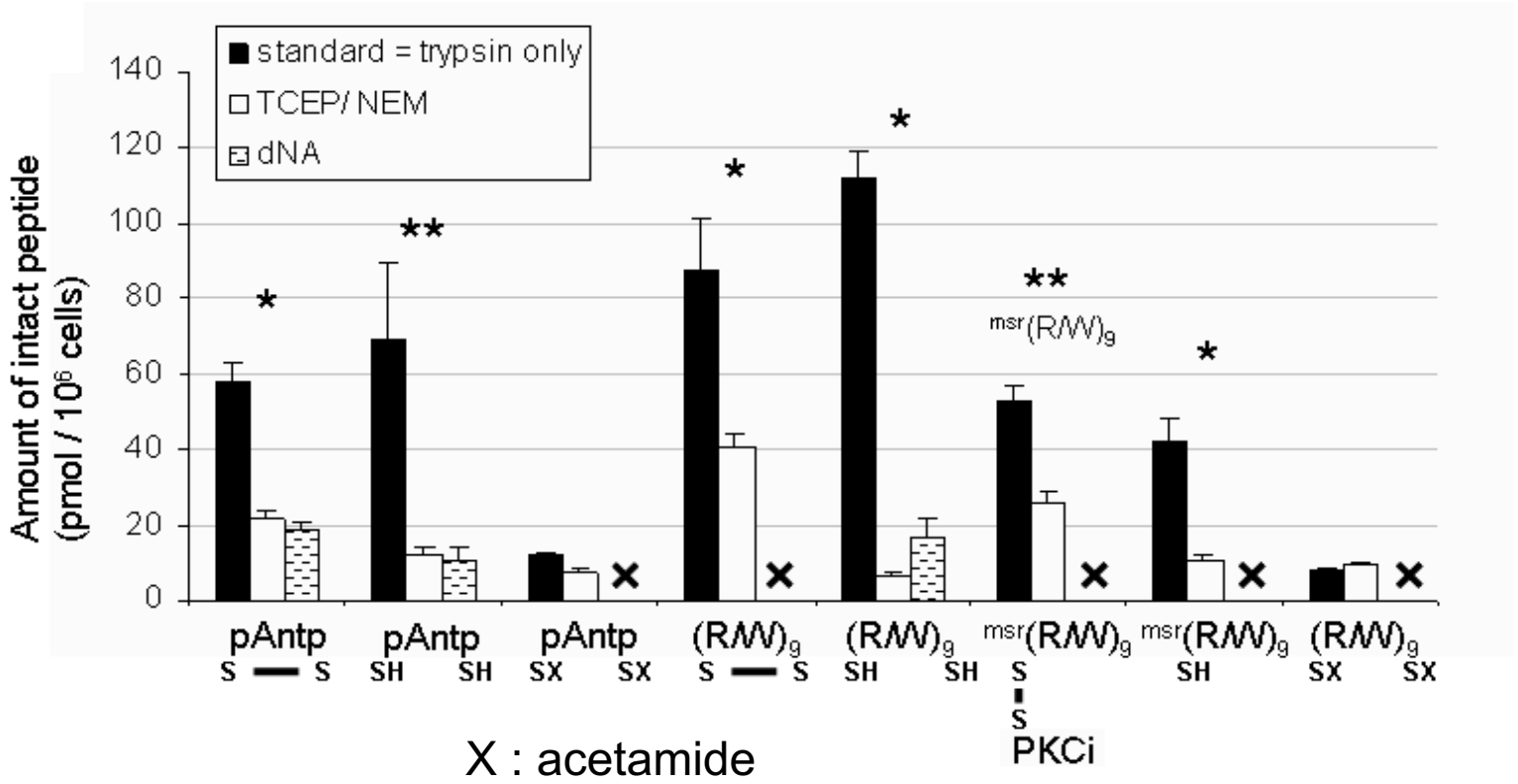



**Same CPP sequence with different internalization efficacy**

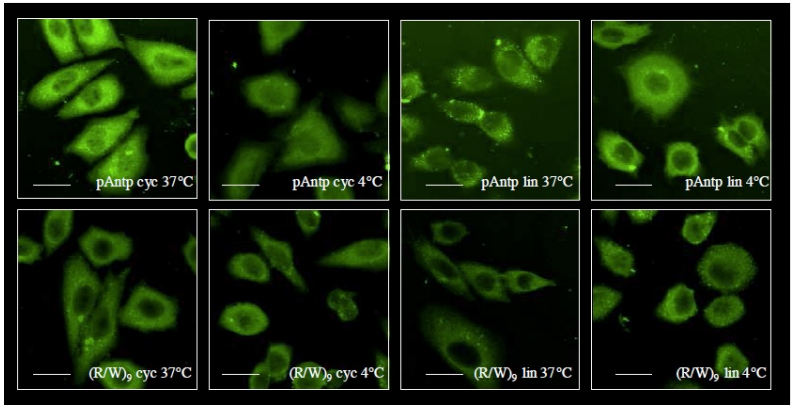


Aubry, FASEBJ (2009) 23: 2956-2967

# Impact of cysteinyl residues on CPP uptake efficiency

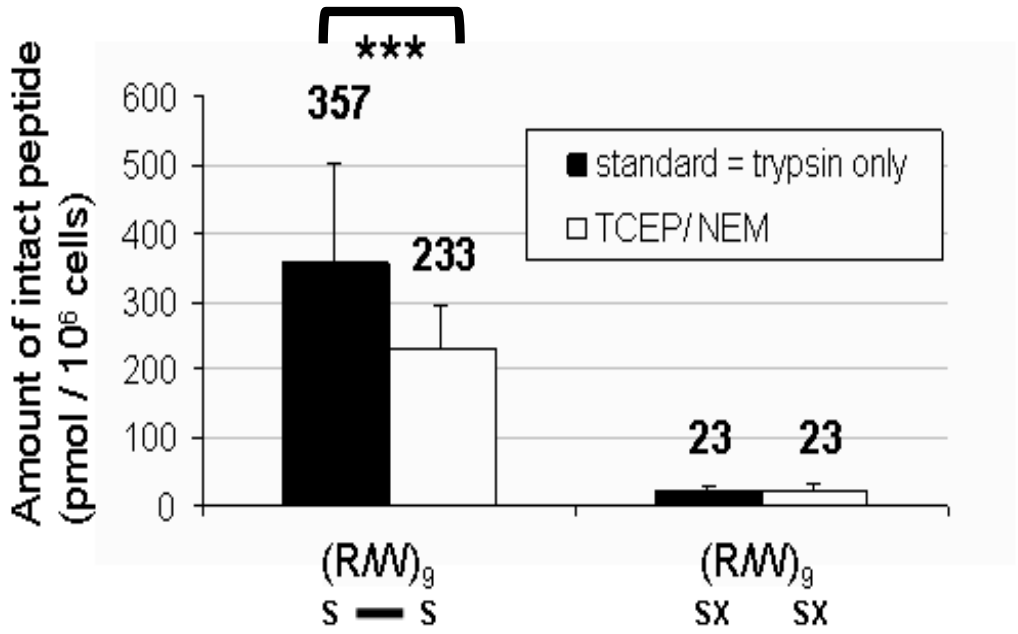



**Identical quantities are measured after chemical modification of membrane thiols**




Aubry, FASEBJ (2009) 23: 2956-2967

# Disulfide bridge enhances CPP uptake efficiency




X : acetamide



Aubry, FASEBJ (2009) 23: 2956-2967

# Exploiting cell surface thiols to enhance cellular uptake

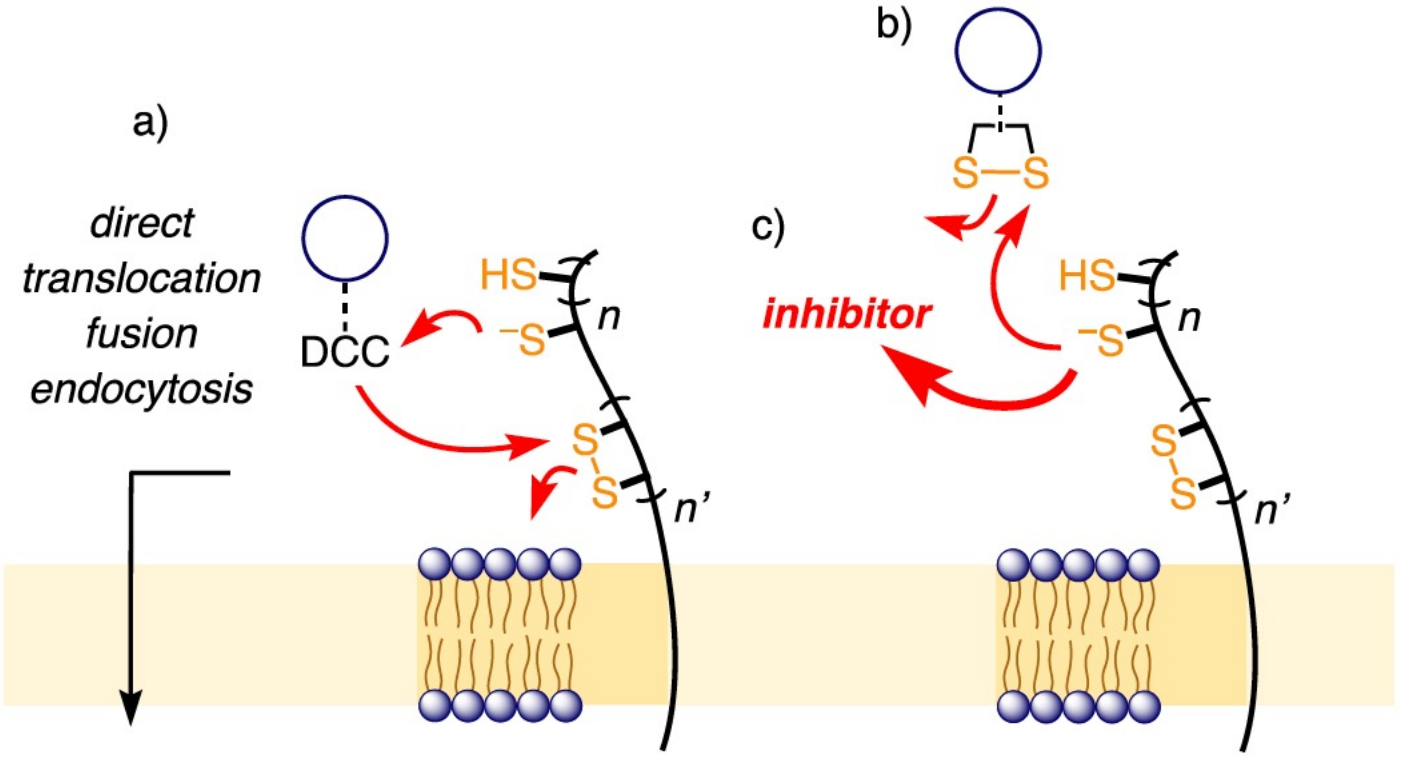
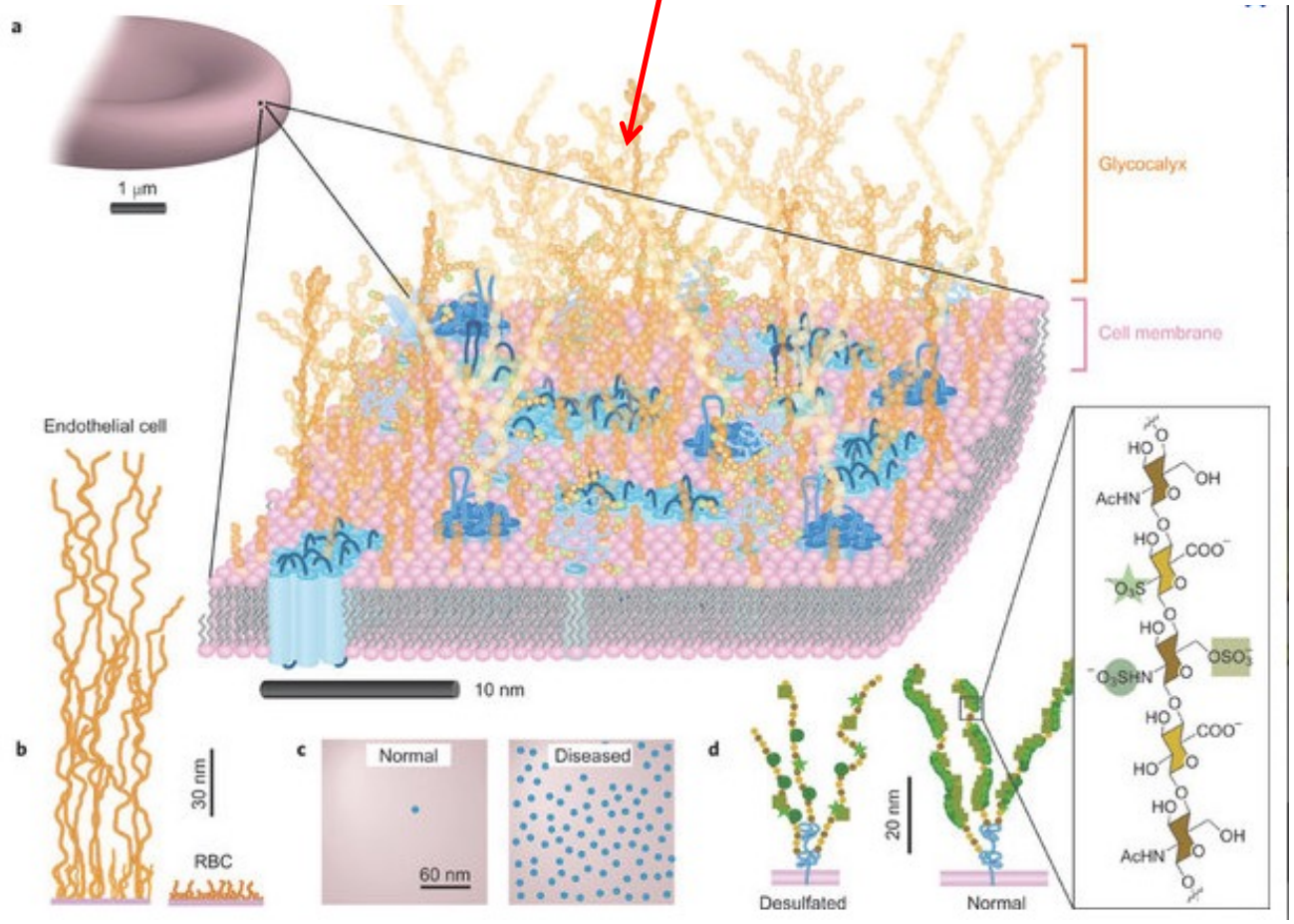



**Figure 1.** Thiol-mediated uptake (a) operates with the dynamic covalent chemistry (DCC) of chalcogenide exchange before or during cellular entry by direct translocation, endocytosis, or fusion, (b) usually involves thiol/disulfide exchange, and (c) can be inhibited with the same DCC.

Laurent, Matile, JACS Au 2021, 1, 710–728

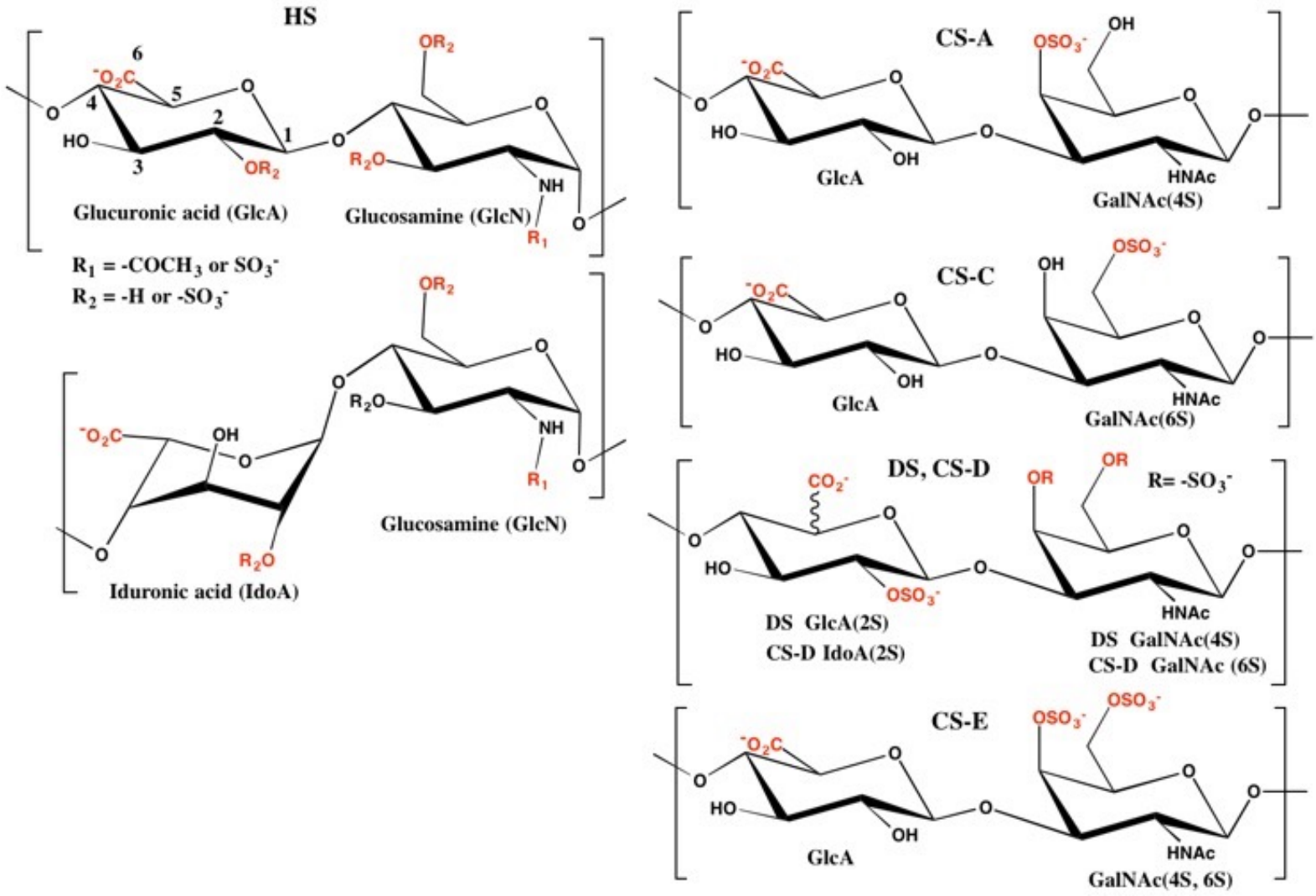
# Cell membrane : many exploited portals of entry

Focus on polysaccharides

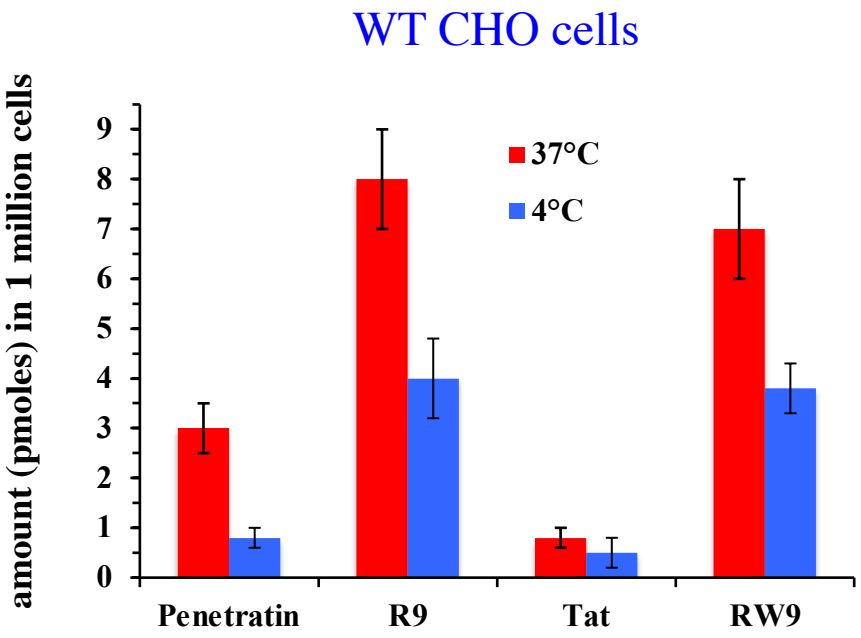



Mager, Nat Chem (2011) 3:582

# Role of negatively charged cell-surface polysaccharides in CPP uptake



# Internalization efficacy is sequence-dependent

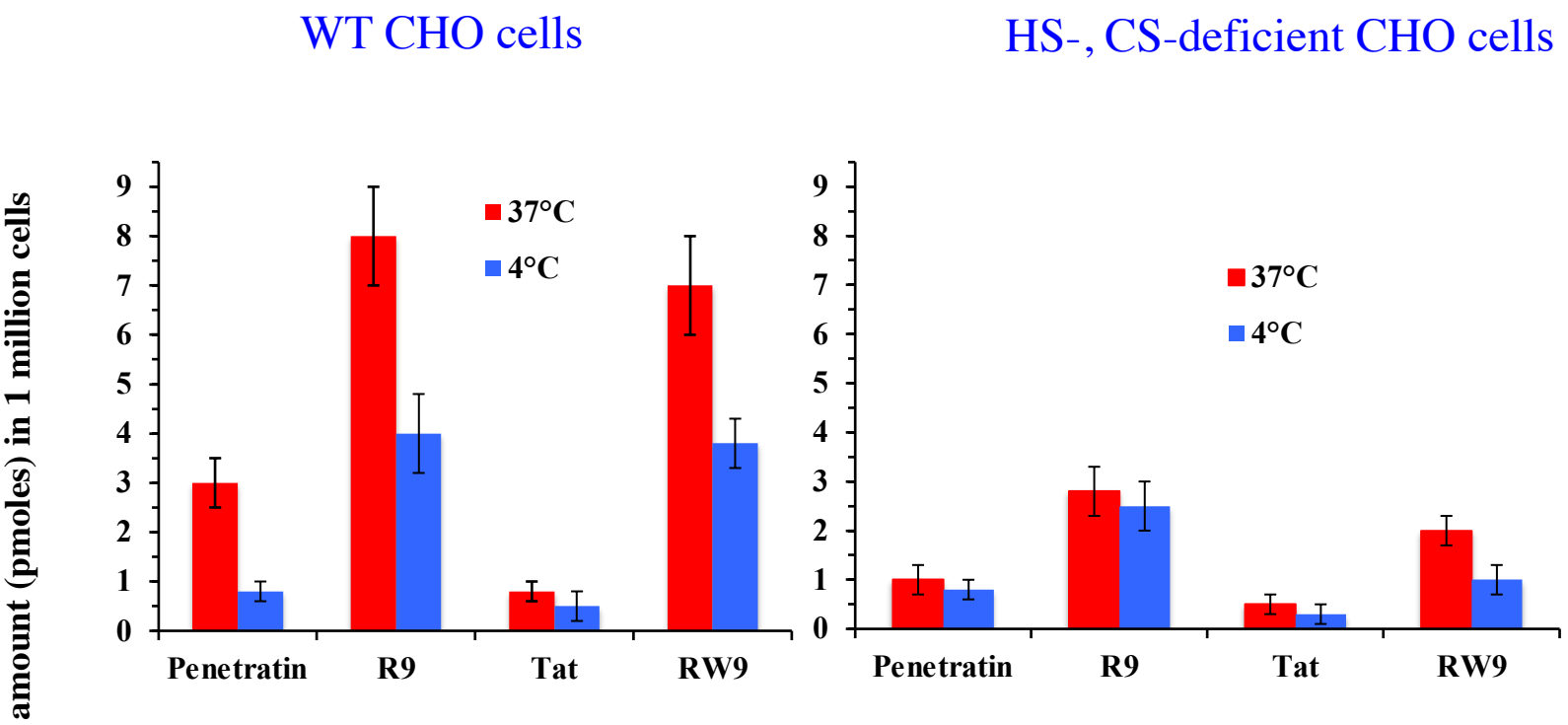



5 μM peptide concentration

| | | |
|---|---|---|
| Penetratin | RQIKIWFQNRRMKWKK | (+7) |
| R9 | RRRRRRRRR | (+9) |
| Tat | YGRKKRRQRRR | (+8) |
| RW9 | RRWWRRWRR | (+6) |

Jiao, J Biol Chem (2009) 284:33957

# Cell-surface HS and CS contribute differently to CPP internalization




5 μM peptide concentration

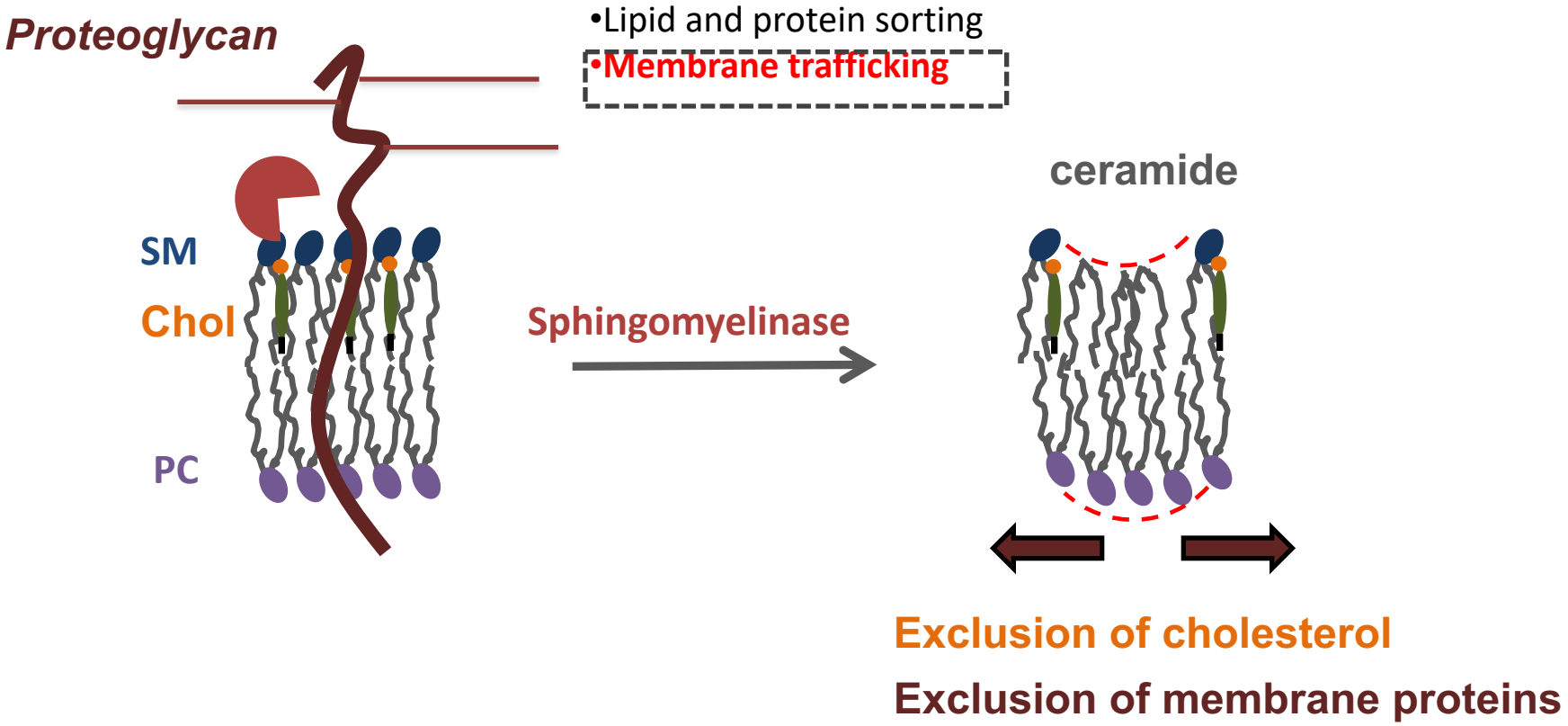
| | | |
|---|---|---|
| Penetratin | RQIKIWFQNRRMKWKK | (+7) |
| R9 | RRRRRRRRR | (+9) |
| Tat | YGRKKRRQRRR | (+8) |
| RW9 | RRWWRRWRR | (+6) |

Jiao, J Biol Chem (2009) 284:33957; Bechara, FASEB J (2013) 27:738

# GAGs and lateral heterogeneity of the lipid bilayer

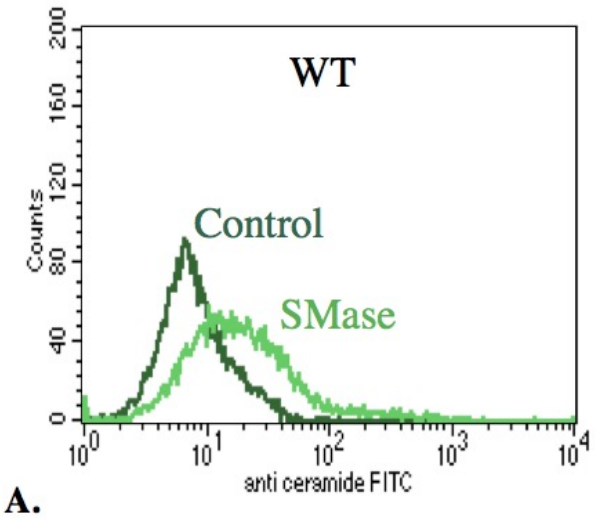
**Sphingomyelin** and **Cholesterol**-enriched domains

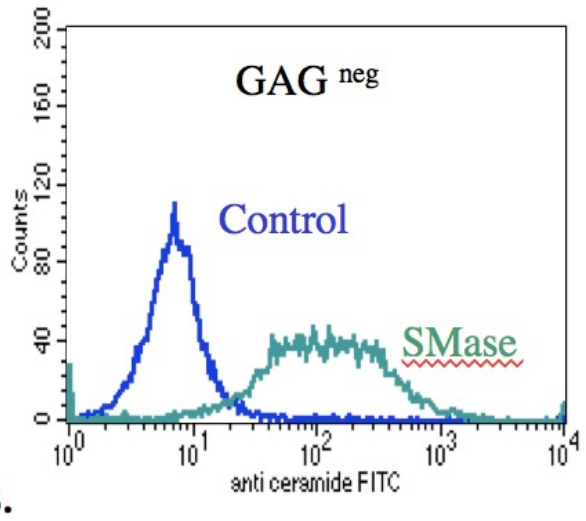
- Cellular signalling
- Lipid and protein sorting
- **Membrane trafficking**

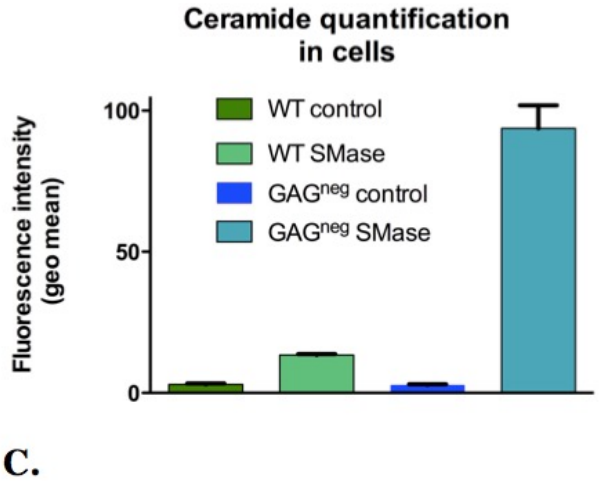



**Destabilization of the bilayer** (curvature, defects in lipid packing, …)

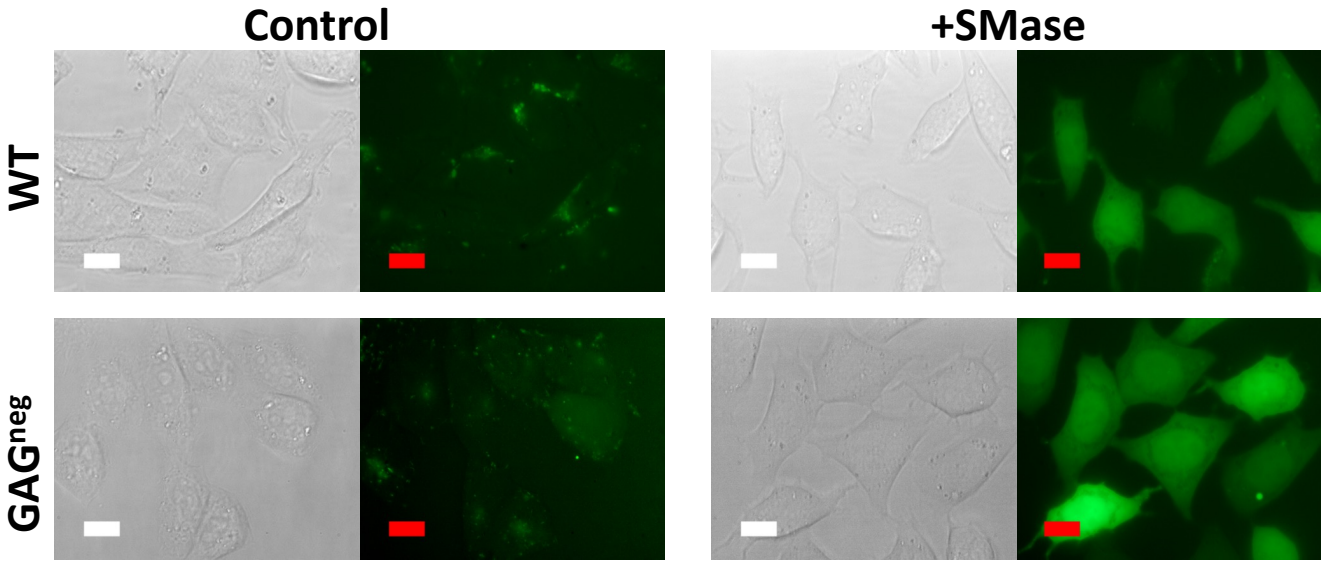
# Detection of sphingomyelin hydrolysis in the cell membrane




*4.4 KDa Dextran-FITC: fluid-phase endocytosis marker*



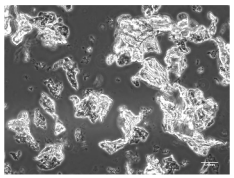


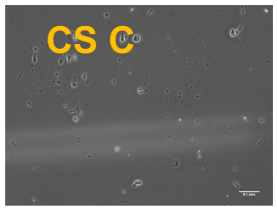

Bechara, Cell Mol Life Sci (2015) 72:809

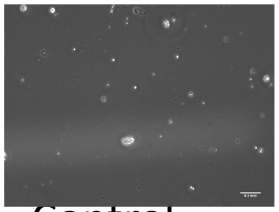
# Sphingomyelin hydrolysis: impact on CPP uptake

***Penetratin (2W; +7)***

*GAG-aggregates*





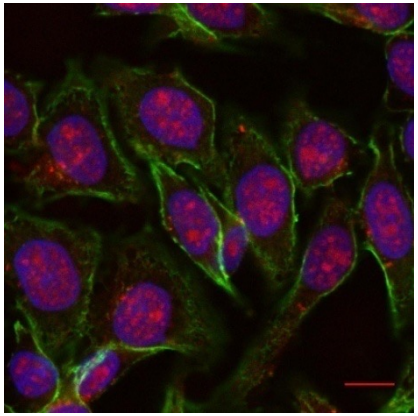



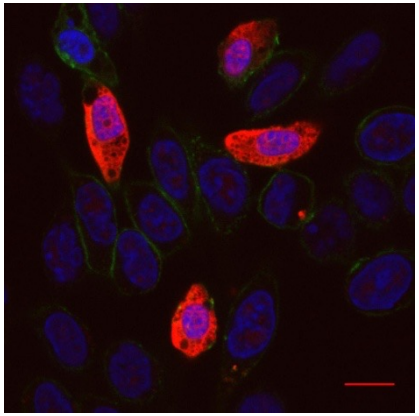
***TAT peptide (0W; +8)***

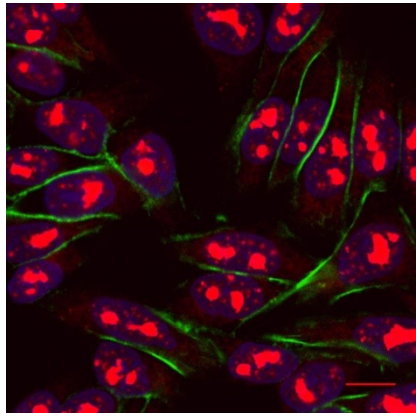
*No GAG-aggregates*

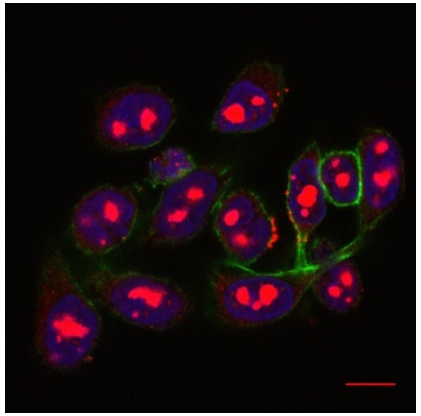
Control | +SMase | Control | +SMase

**WT**

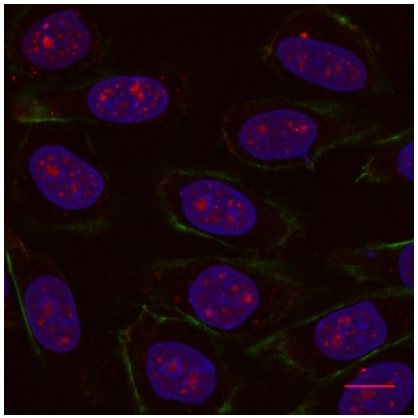


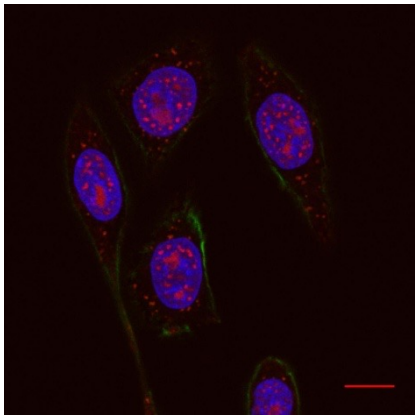


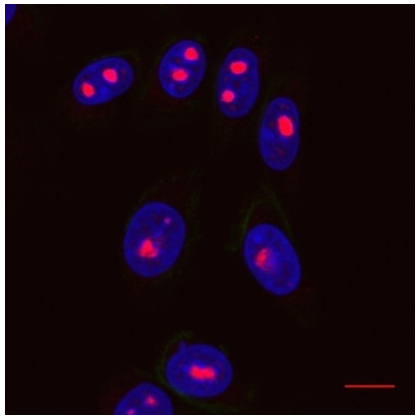


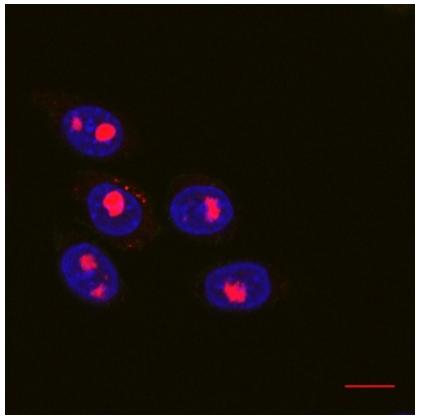


**GAG$^{neg}$**









*Red: CPP*; *Green: Actin filaments*; *Blue: Nucleus*

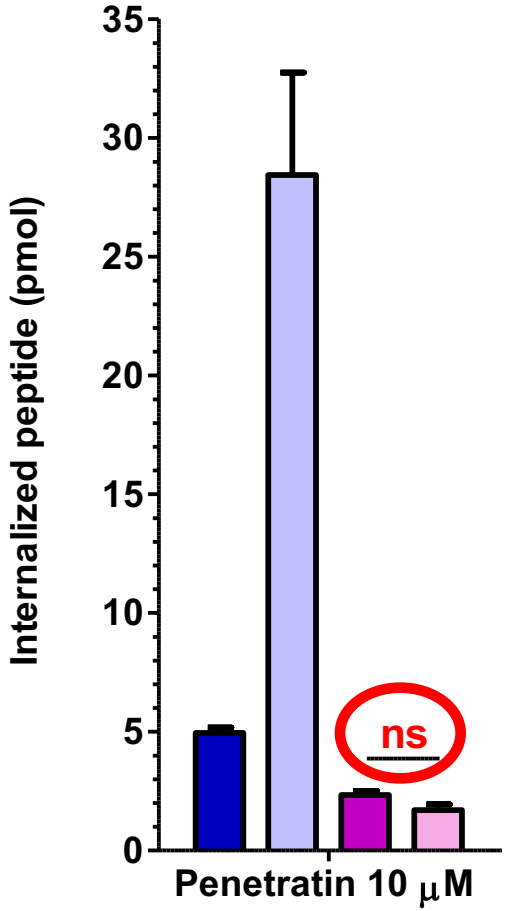
Scale bar: 10µm

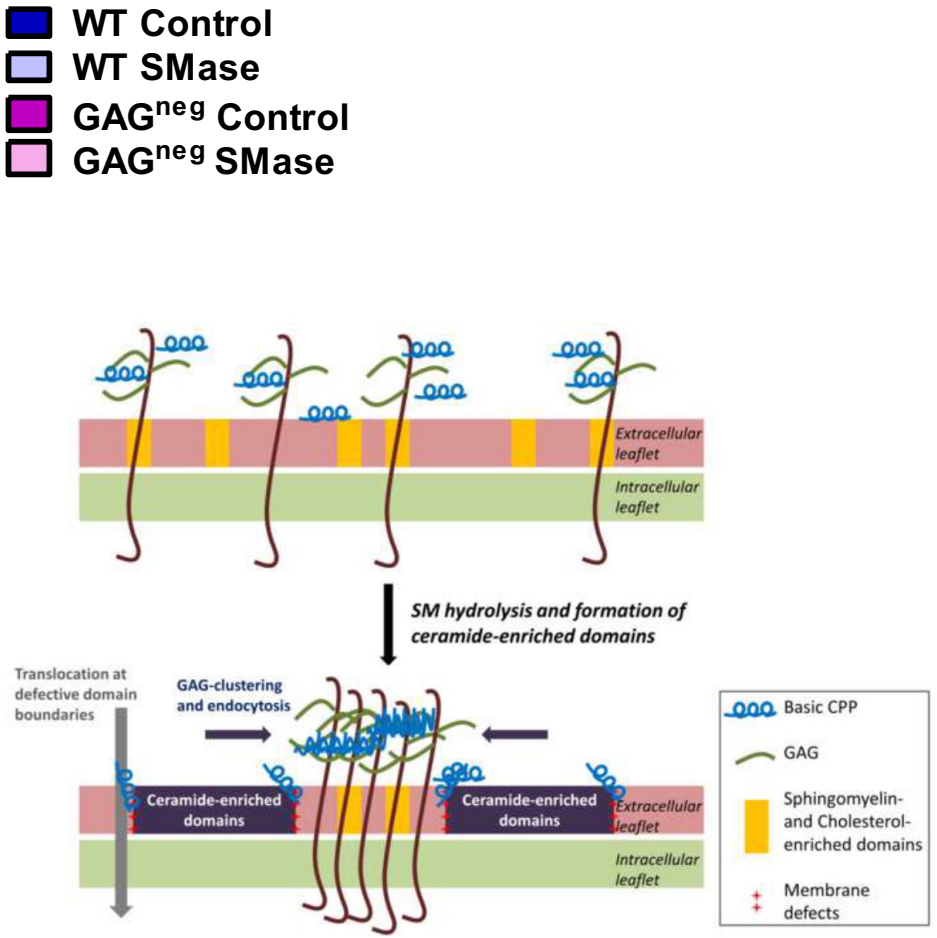


Bechara, Cell Mol Life Sci (2015) 72:809

# Sphingomyelin hydrolysis: quantification of CPP uptake

## SM hydrolysis increases uptake, mainly driven by GAGs

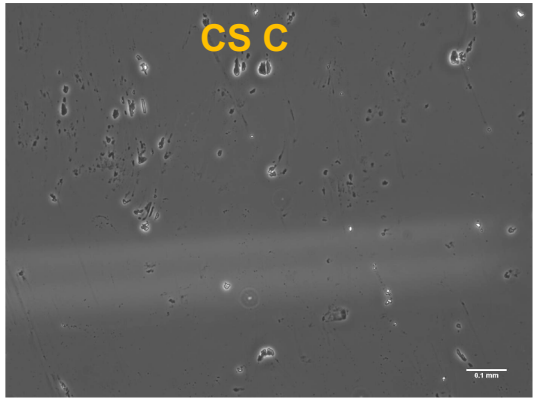


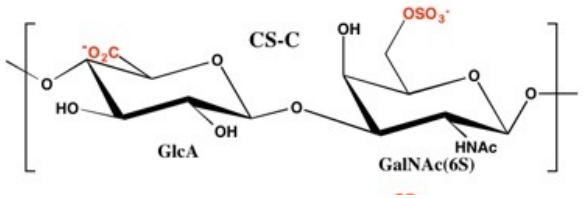




*n ≥ 6 independent*
*$10^6$ cells, 1 hour incubation, 37 ° C*

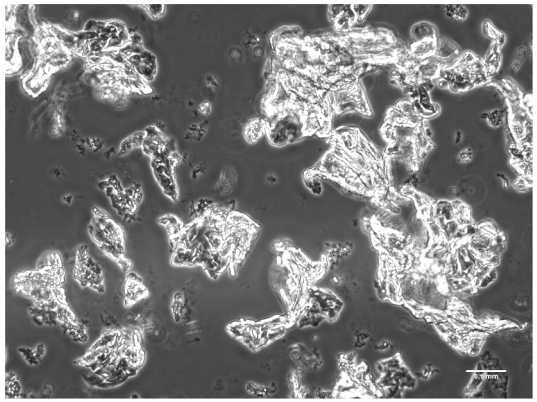
Bechara, Cell Mol Life Sci (2015) 72:809

# Role of Trp in peptide internalization



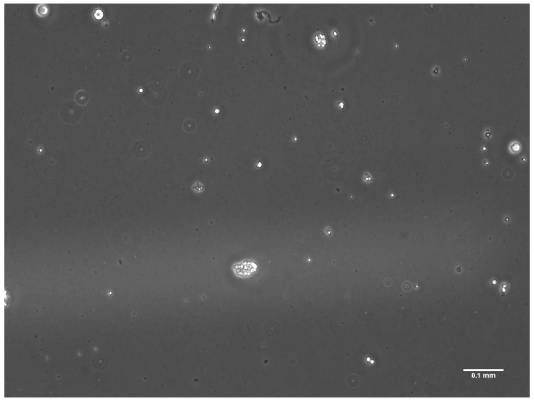




*GAG-aggregates*



***Penetratin (2W; +7)***

*No GAG-aggregates*



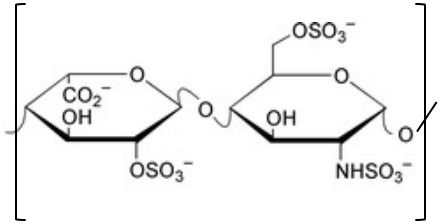
***TAT peptide (0W; +8)***

Scale bar: 10µm



Bechara, Cell Mol Life Sci (2015) 72:809

# In vitro binding to GAGs: Thermodynamics of Trp-rich peptides

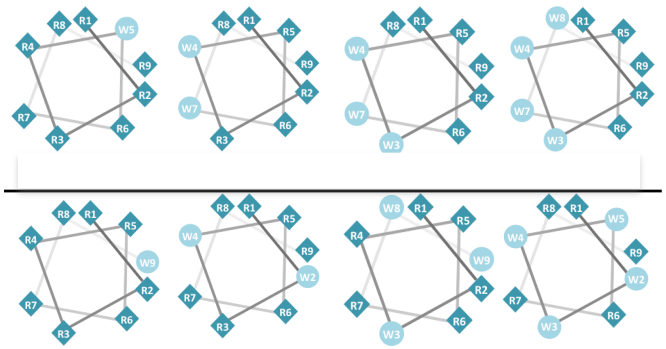



Main disaccharide motif of heparin

ITC: Injection of Heparin into peptide, 37 ° C
HI, average mass 12 kDa, 100 charges/chain

| Peptide | Net charge | N Trp | Hydrophobic moment μH (hydrophobicity) | $K_D$ (nM) | ΔH (kJ/mol) | n (peptide/HI chain) | Charge of the complex |
|---|---|---|---|---|---|---|---|
| R9 | +9 | 0 | 0.15 (-1.01) | 7 | -300 | 8 | -20 |
| aR8W | +8 | 1 | 0.22 (-0.65) | 30 | -342 | 10 | -20 |
| nR8W | +8 | 1 | 0.27 (-0.65) | 26 | -386 | 7 | -43 |
| aR7W2 | +7 | 2 | 0.73 (-0.29) | 18 | -405 | 11 | -23 |
| nR7W2 | +7 | 2 | 0.05 (-0.29) | 26 | -391 | 12 | -16 |
| aR6W3 | +6 | 3 | 0.96 (0.08) | 15 | -487 | 9 | -46 |
| nR6W3 | +6 | 3 | 0.05 (0.08) | 23 | -516 | 11 | -34 |
| aR5W4 | +5 | 4 | 0.98 (0.44) | 25 | -739 | 17 | -15 |
| nR5W4 | +5 | 4 | 0.08 (0.44) | 25 | -750 | 14 | -30 |

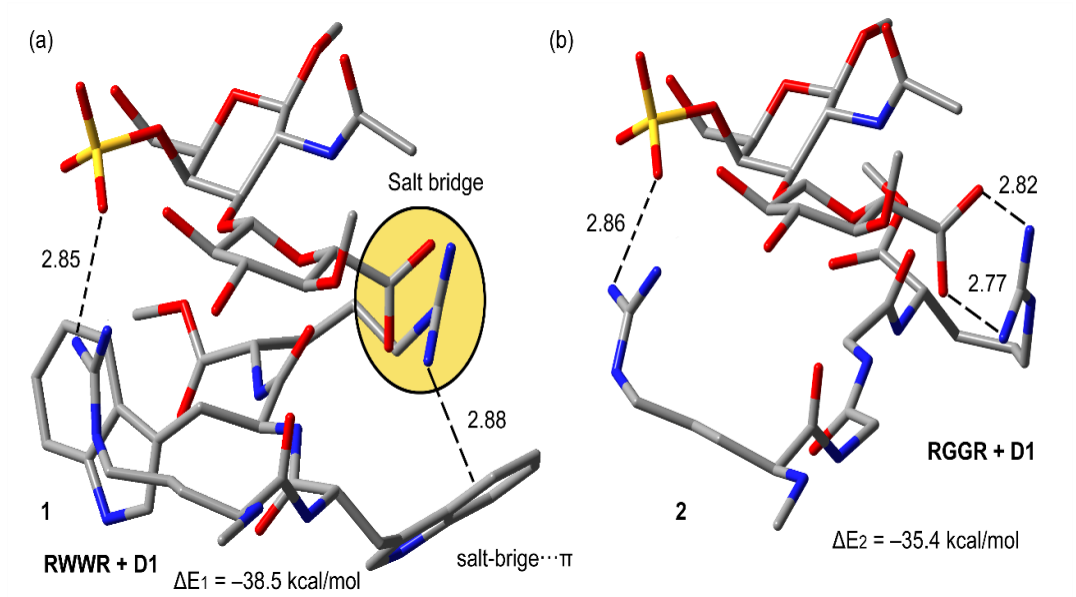
a: facial amphiphilicity

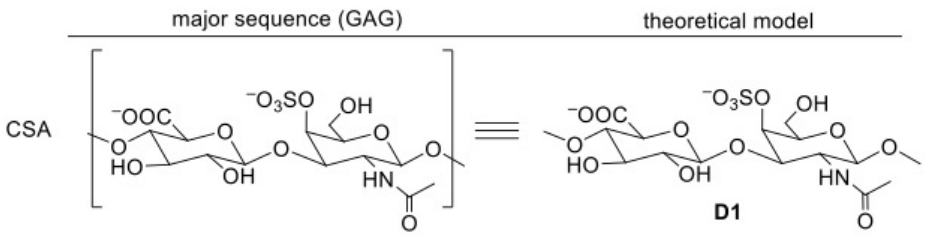


n: non facial amphiphilicity

Walrant, BBA-Biomemb (2020) 1862(2):183098

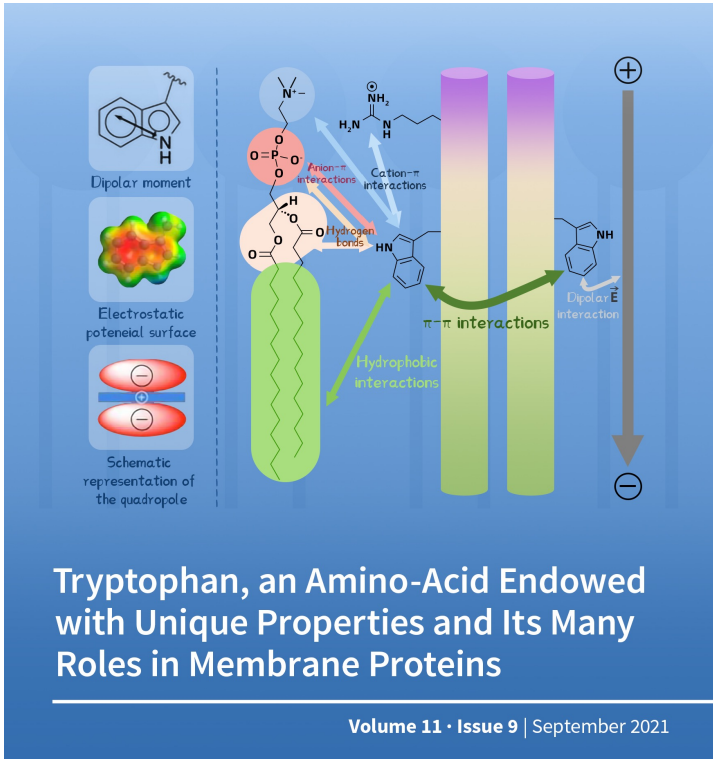
# Trp improves GAG-dependent entry via ionpair-π interactions




DFT calculations

Antonio Frontera

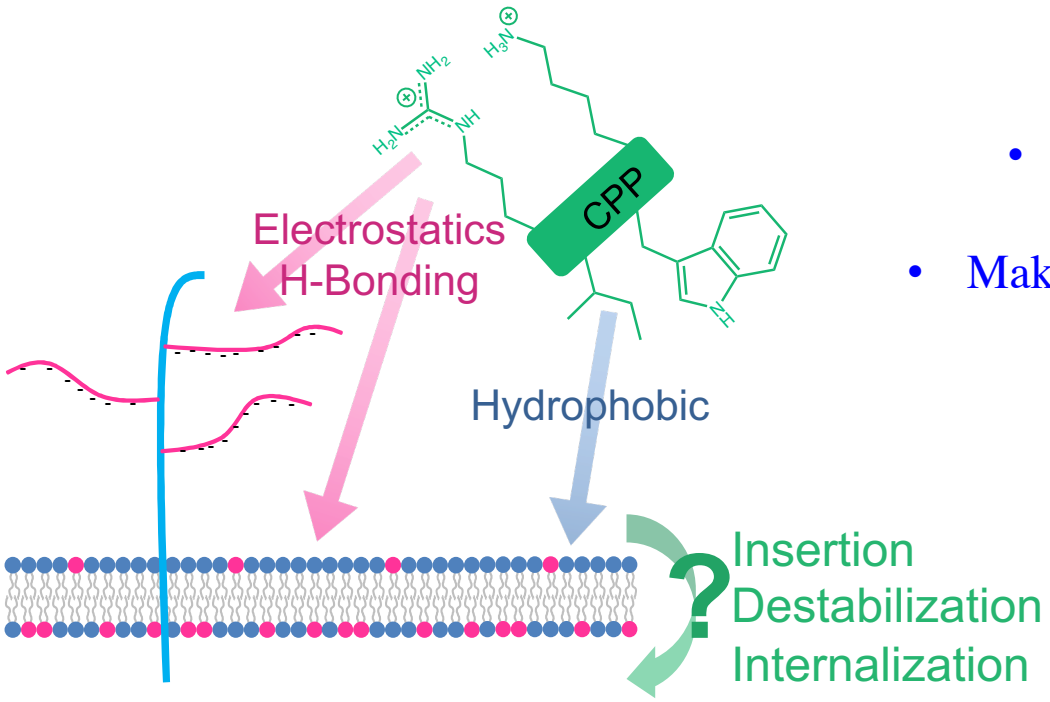





Walrant, BBA-Biomemb (2020) 1862(2):183098

Khemaissa, Crystals (2021) 11(9), 1032

# What we do really know is still little




Current and future research

- Role of GAGs in internalization
- Making targeting cell-penetrating peptides

Cardon et al., bioRXiv (July 2021) A cationic motif in Engrailed-2 homeoprotein controls its internalization via selective cell-surface glycosaminoglycans interactions

Walrant, Acc Chem Res (2017)

# Acknowledgments


- Sébastien Cardon (PhD)
- Bingwei He (PhD)
- Chen-Yu Jiao (PhD)
- Soline Aubry (PhD)
- Cherine Bechara (PhD)
- Sonia Khemaissa (PhD)
- Ludovic Carlier
- Astrid Walrant
- Olivier Lequin
- Fabienne Burlina
- Gérard Chassaing
- Gérard Bolbach
- Françoise Illien
- Delphine Ravault
- Emmanuelle Sachon
- Sophie Cribier
- Nicolas Rodriguez

Collaborators

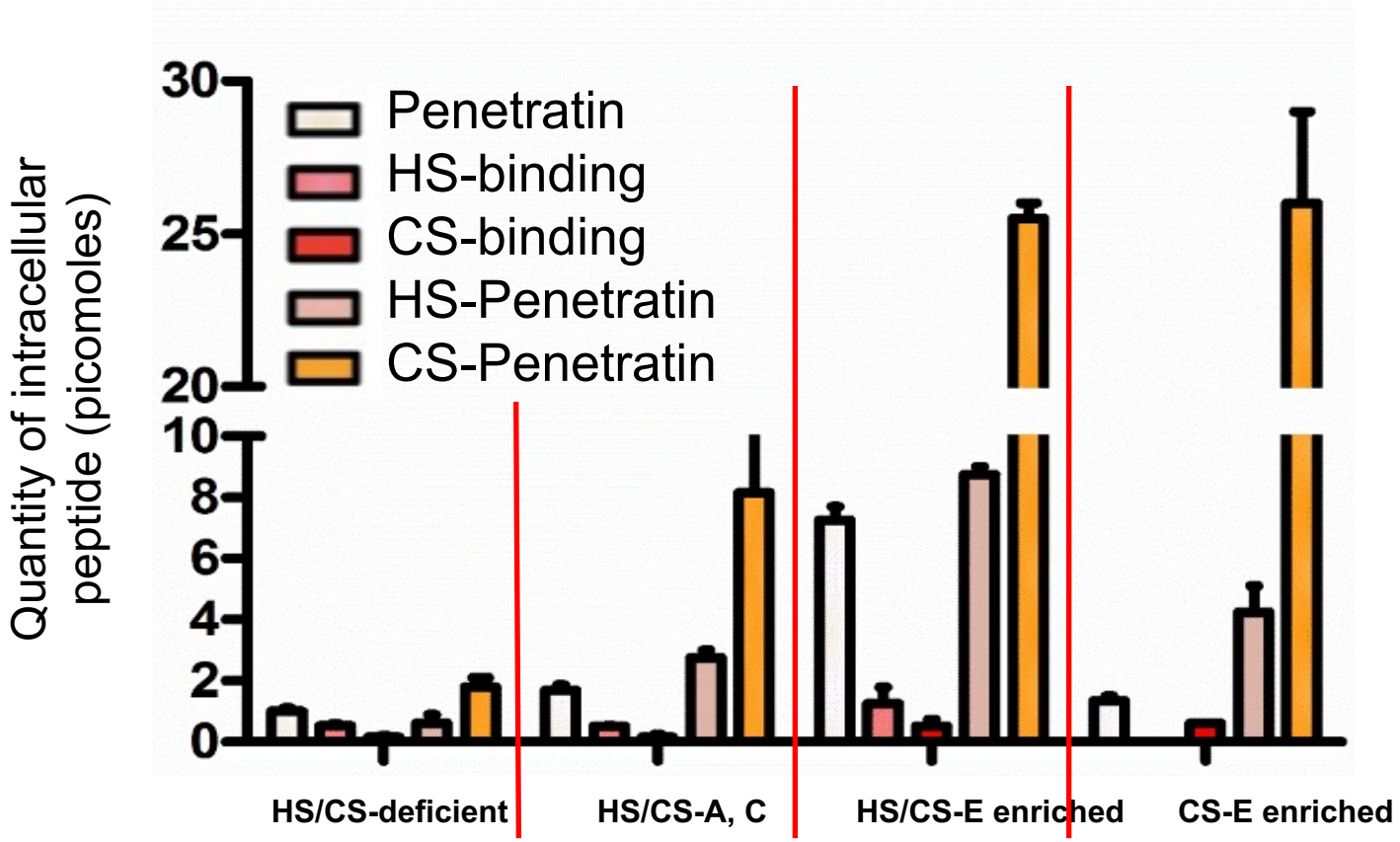
- Alain Joliot (Institut Curie - U932)
- Antonio Frontera (University of the Balearic Islands)
- Chrystel Lopin-Bon (ICOA, Orléans)

# Cell pH

| Cell structure | pH |
|---|---|
| cytosol | 7.3 |
| endoplasmic reticulum | 7.3 |
| Golgi apparatus | 6.5 |
| lysosome | 4.5 |
| mitochondria | 8.0 (matrix)<br>7.1 (IMS) |
| nucleus | 7.3 |
| peroxysome | 7.0 |
| secretory vesicle | 5.5 |

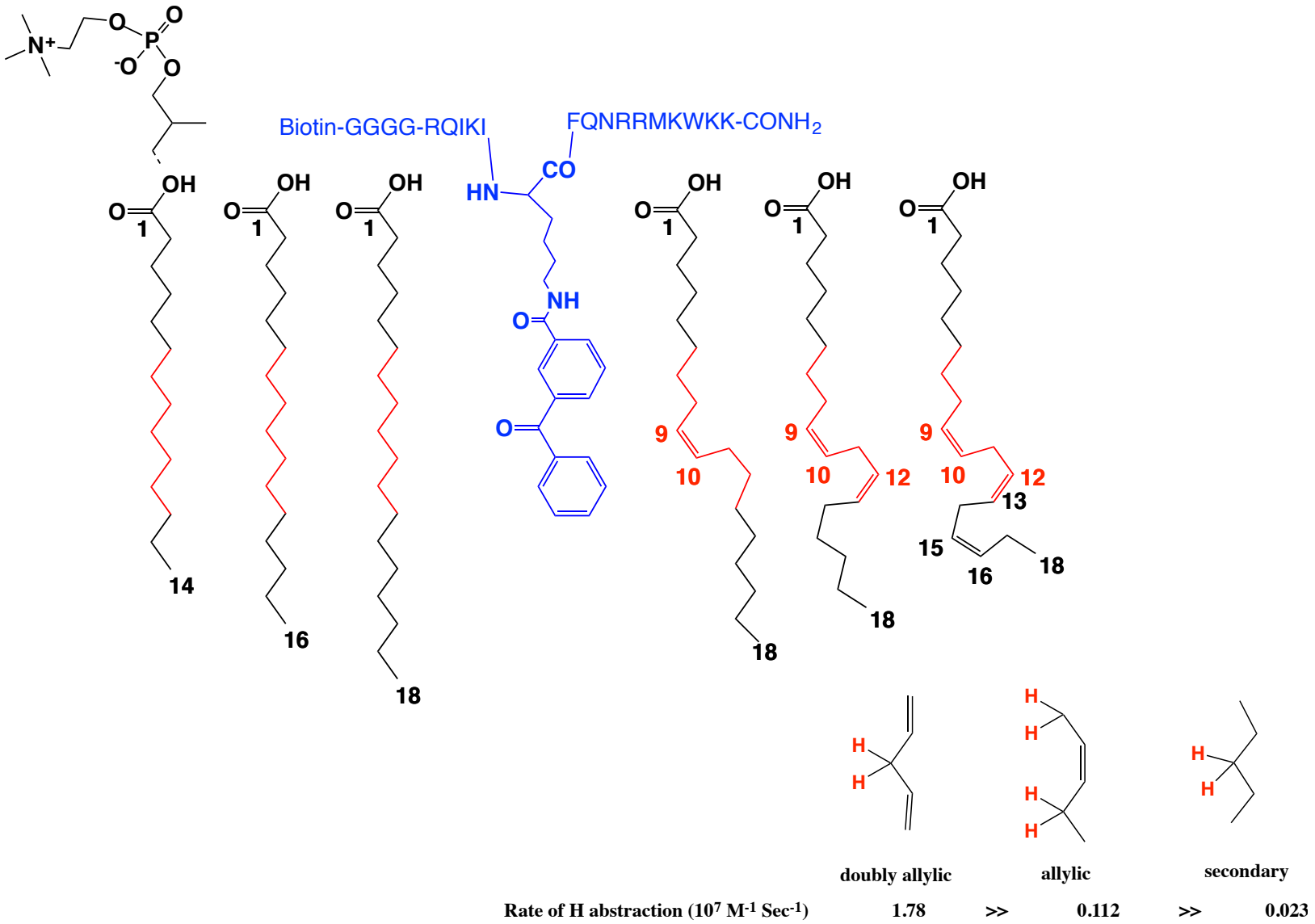
Cho, J Pharm Sci (2002) 91: 903

# Targeting cell-surface GAGs to improve cell-specific CPP internalization



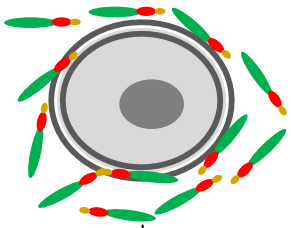

Bingwei He, unpublished

# Exploiting benzophenone photoreactivity to probe CPP insertion depth and lipid surroundings




Jiao, Angew Chem (2017) 56:8226

# Internalization efficacy of CPPs: a quantitative MS approach



$10^6$ cells

Washings

Peptide degradation

Cell Lysis
(+ [$^2$H]CPP in lysis buffer)

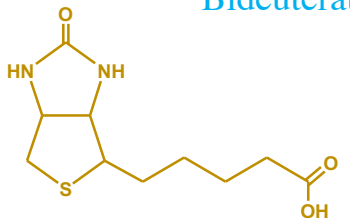
Purification
(Streptavidin-coated magnetic beads)

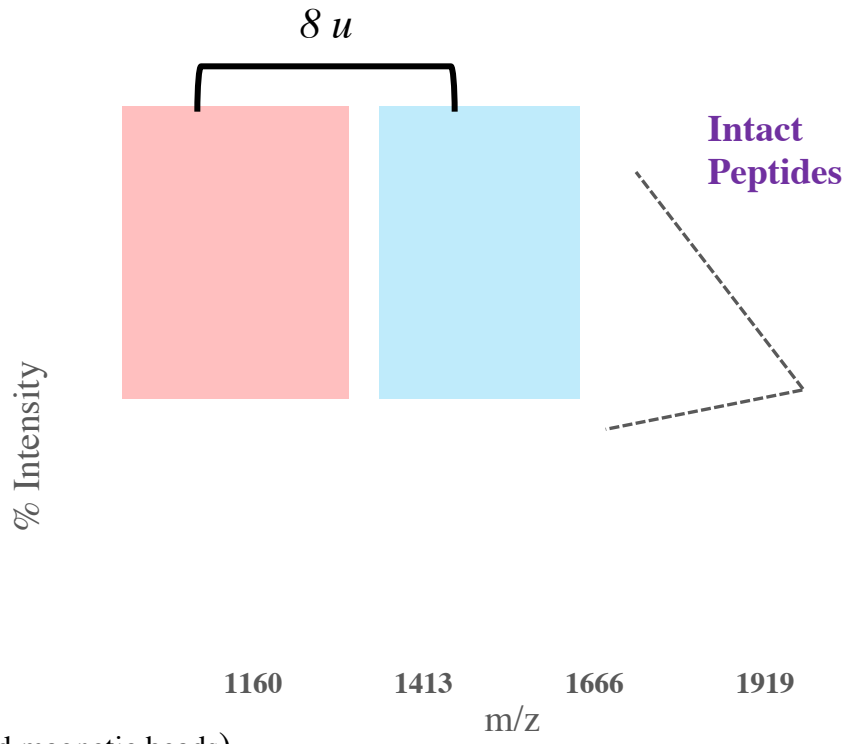
Biotin — GGGG — CPP

Non deuterated Glycine

Biotin — GGGG — CPP

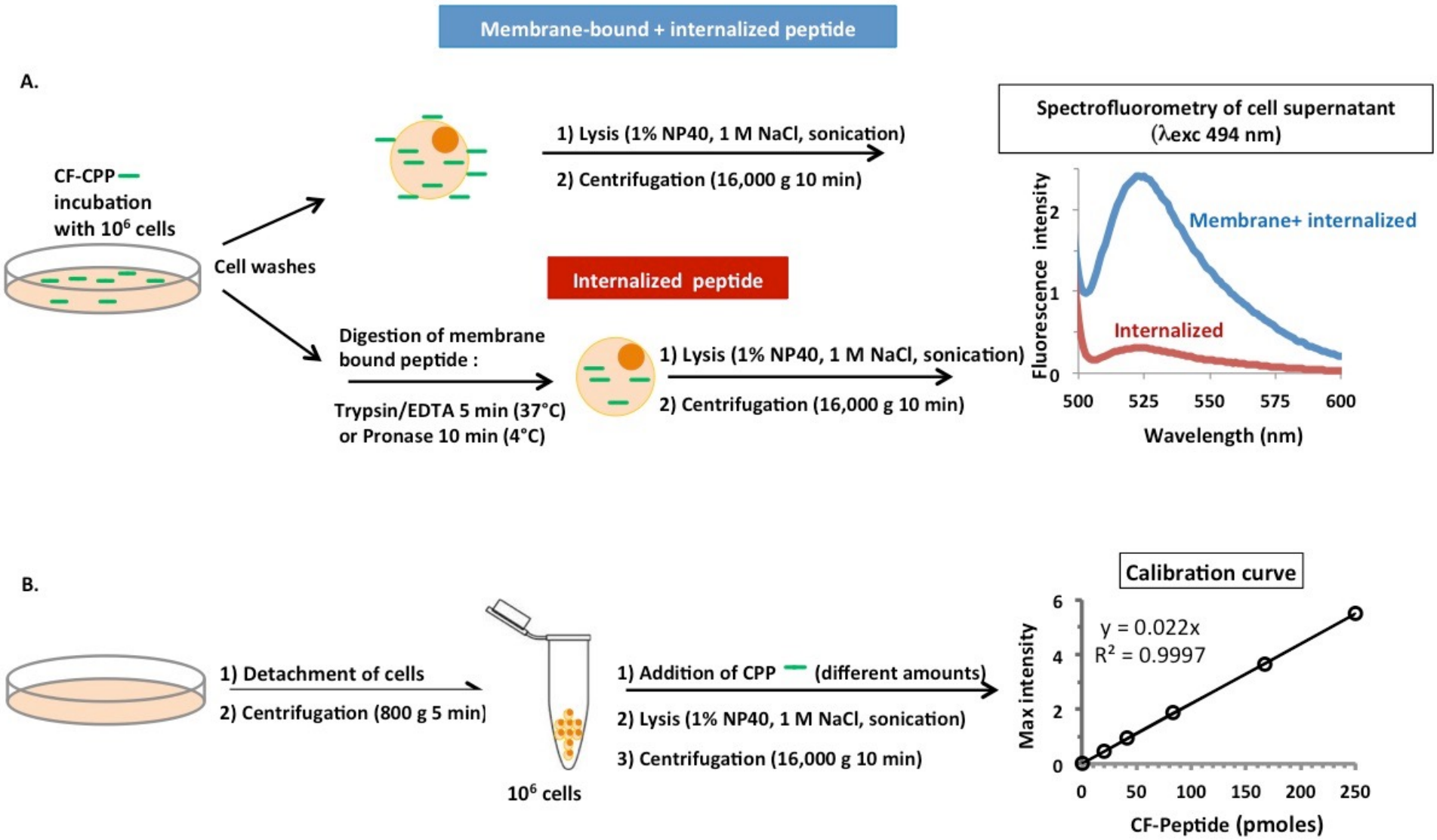
Bideuterated Glycine




Burlina, Angew Chem Int Ed Engl (2005) 44:4244; Nat Protoc. (2006) 1:200

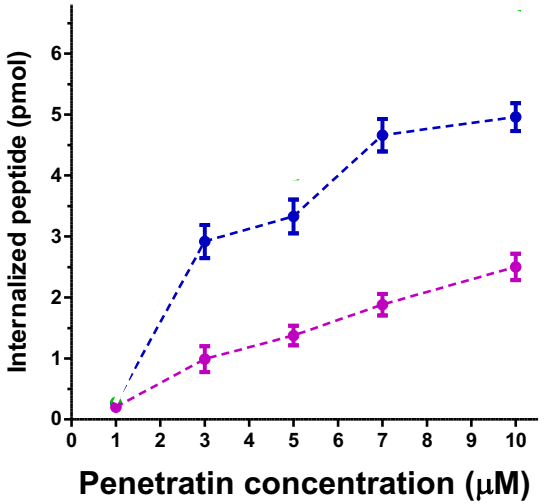
# Internalization efficacy of CPPs: a quantitative fluorescence approach




Illien, Sci Report (2016) 6:36938.

# Involvement of cell-surface GAGs in Penetratin internalization

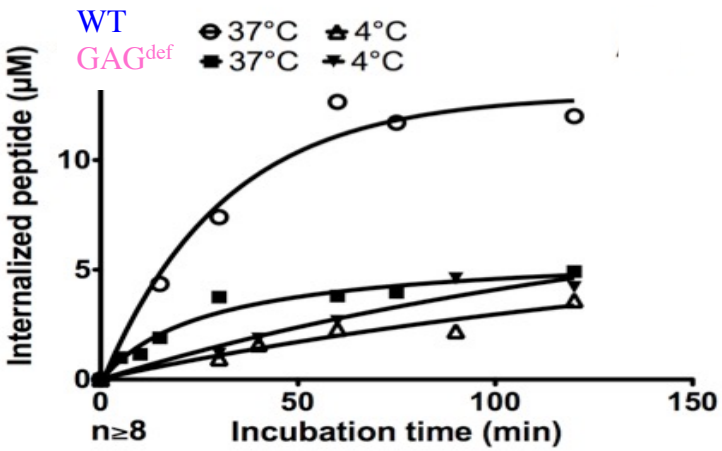
Penetratin: RQIKIWFQNRRMKWKK




WT

HS-, CS-deficient

37° C






Jiao, J Biol Chem (2009) 284:33957