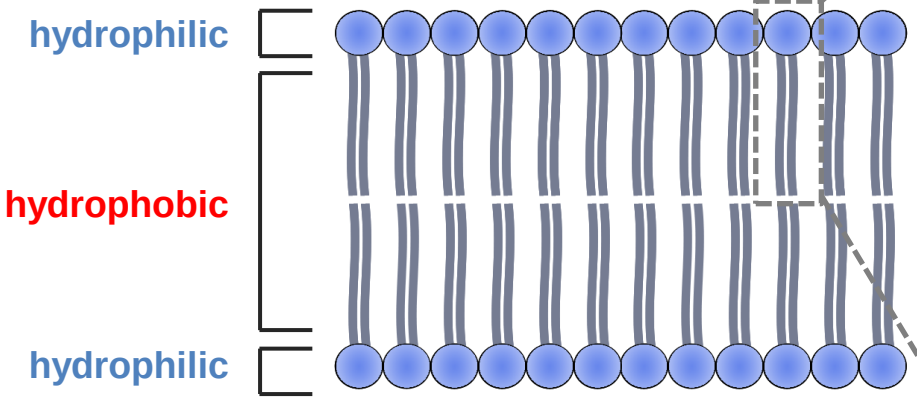
Crossing the Biological Membrane

Hiroaki Itoh

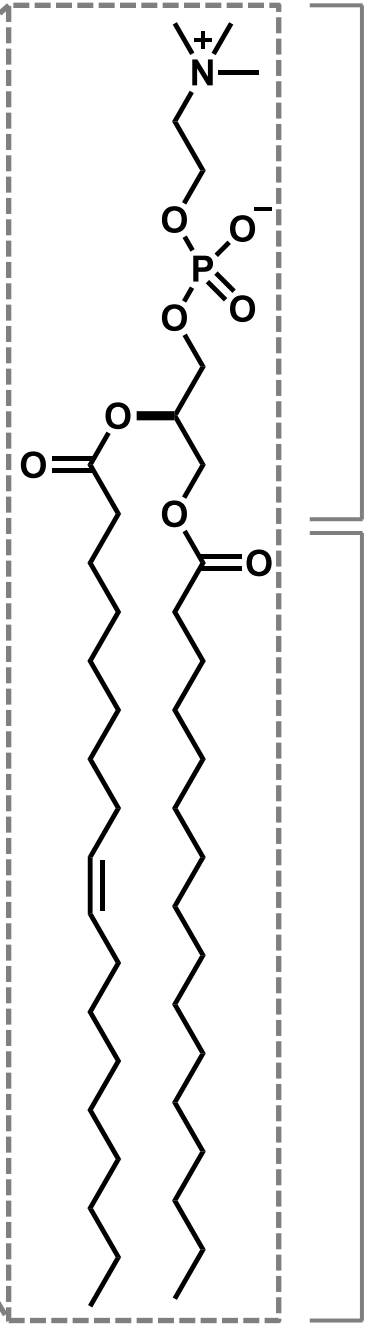
Mar. 8, 2025 | Literature Seminar

Biological Membrane

- Cells are surrounded by a lipid bilayer separating the insides of the cell from its outside



lipid bilayer



lipid polar head

hydrophobic acyl chain

(e.g. POPC)

To approach intracellular molecules, it is necessary to overcome this barrier function

Cell-Penetrating Peptides (CPPs)

CPP name	Sequence	Origin
HIV-1 TAT protein, TAT_{48-60}	GRKKRRQRRRPPQ	HIV-1 TAT protein
HIV-1 TAT protein, TAT_{49-57}	RKKRRQRRR	HIV-1 TAT protein
Penetratin, pAntp(43–58)	RQIKIWFQNRRMKWKK	Antennapedia *Drosophila melanogaster*
Polyarginines	Rn	Chemically synthesized
DPV1047	VKRGLKLRHVRPRVTRMDV	Chemically synthesized
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	HIV glycoprotein 41/ SV40 T antigen NLS
Pep-1	KETWWETWWTEWSQPKKKRKV	Tryptophan-rich cluster/SV40 T antigen NLS
pVEC	LLIILRRRIRKQAHAHSK	Vascular endothelial cadherin
ARF(1–22)	MVRRFLVTLRIRRACGPPRVRV	p14ARF protein
BPrPr(1–28)	MVKSKIGSWILVLFVAMWSDVGLCKKRP	N terminus of unprocessed bovine prion protein
MAP	KLALKLALKALKAALKLA	Chemically synthesized
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Chimeric galanin–mastoparan
p28	LSTAADMQGVVTDGMASGLDKDYLKPDD	Azurin
VT5	DPKGDPKGVTVTVTVTVTGKGDPKPD	Chemically synthesized
Bac 7 (Bac $_{1-24}$)	RRIRPRPPRLPRPRPRPLPFPRPG	Bactenecin family of antimicrobial peptides
C105Y	CSIPPEVKFNKPFVYLI	α1-Antitrypsin
PFVYLI	PFVYLI	Derived from synthetic C105Y
Pep-7	SDLWEMMMVSLACQY	CHL8 peptide phage clone

From the discovery of TAT and penetratin, a number of cell-penetrating peptides (CPPs) were identified (This table was taken from ref. 1)

cell-penetrating peptides (CPPs)[1,2)]

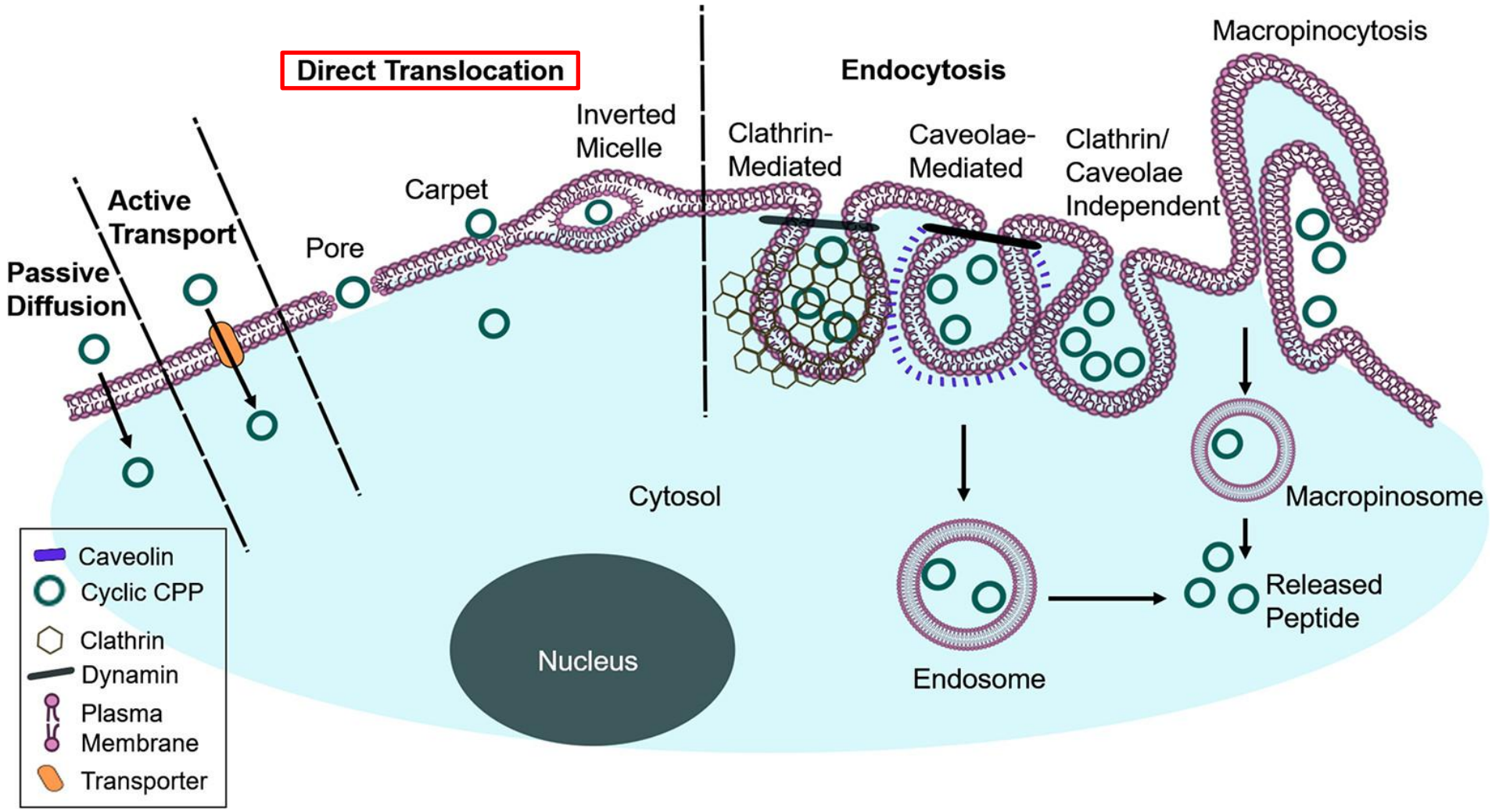
- **peptides typically comprising 5–30 amino acids**
- **CPPs pass through tissue and cell membrane with no interactions with specific receptors**

- **Several preclinical and clinical trials studies have been performed[1)]**

For a reviews, see: 1) Guidotti, G.; Brambilla, L.; Rossi, D. *Trends Pharmacol. Sci.* **2017**, *38*, 406.
2) Bechara, C.; Sagan, S. *FEBS Lett.* **2013**, *587*, 1693.

Four Possible Mechanisms of Cell Entry of Peptides

1)

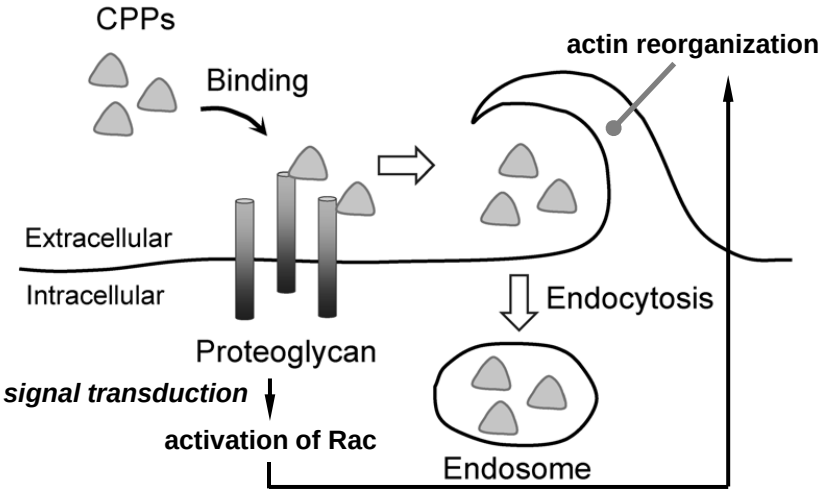


1) Dougherty, P. G.; Sahni, A.; Pei, D. *Chem. Rev.* **2019**, *119*, 10241.

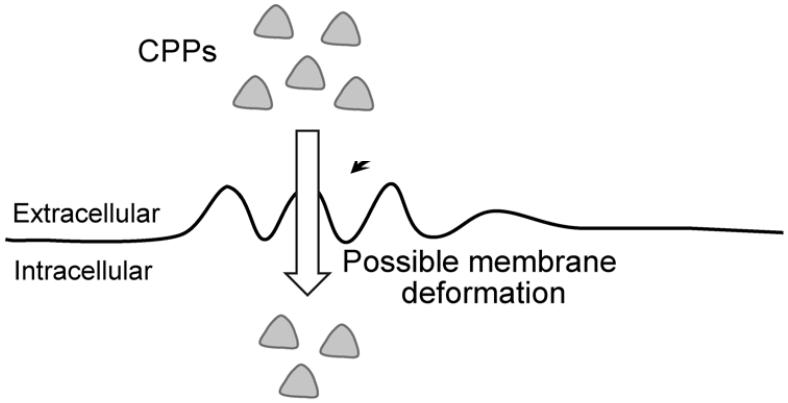
Internalization Modes of Arg-Rich CPPs

- The CPPs are usually polycationic and highly hydrophilic (e.g. TAT and polyR) and are not expected to passively diffuse across the lipid bilayer
- Arg-rich CPPs are internalized into the cells through two different modes[1-3)]

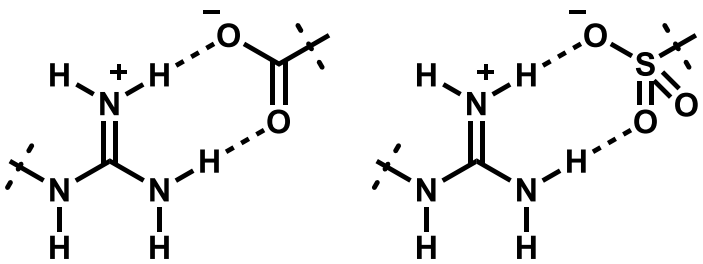
endocytosis-dependent internalization



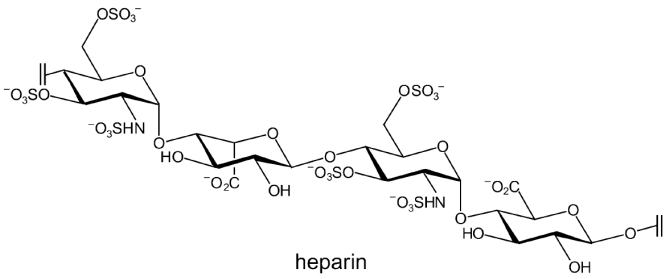
direct translocation (typically >10 μM)

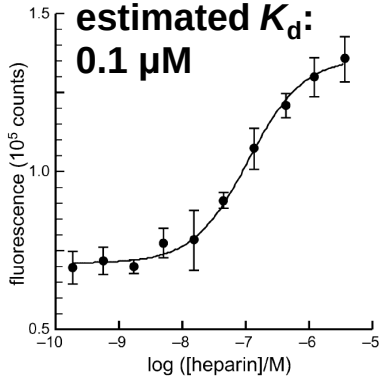


- interaction between anionic proteoglycan and cationic guanidium



- affinity of R9 to heparin[4)]





1) Takeuchi, T.; Futaki, S. *Chem. Pharm. Bull.* **2016**, *64*, 1431. 2) Perret, F.; Nishihara, M.; Takeuchi, T.; Futaki, S.; Lazar, A. N.; Coleman, A. W.; Sakai, N.; Matile, S. *J. Am. Chem. Soc.* **2005**, *127*, 1114. 3) Futaki, S.; Nakase, I. *Acc. Chem. Res.* **2017**, *50*, 2449. 4) Fuchs, S. M.; Raines, R. T. *Biochemistry* **2004**, *43*, 2438.

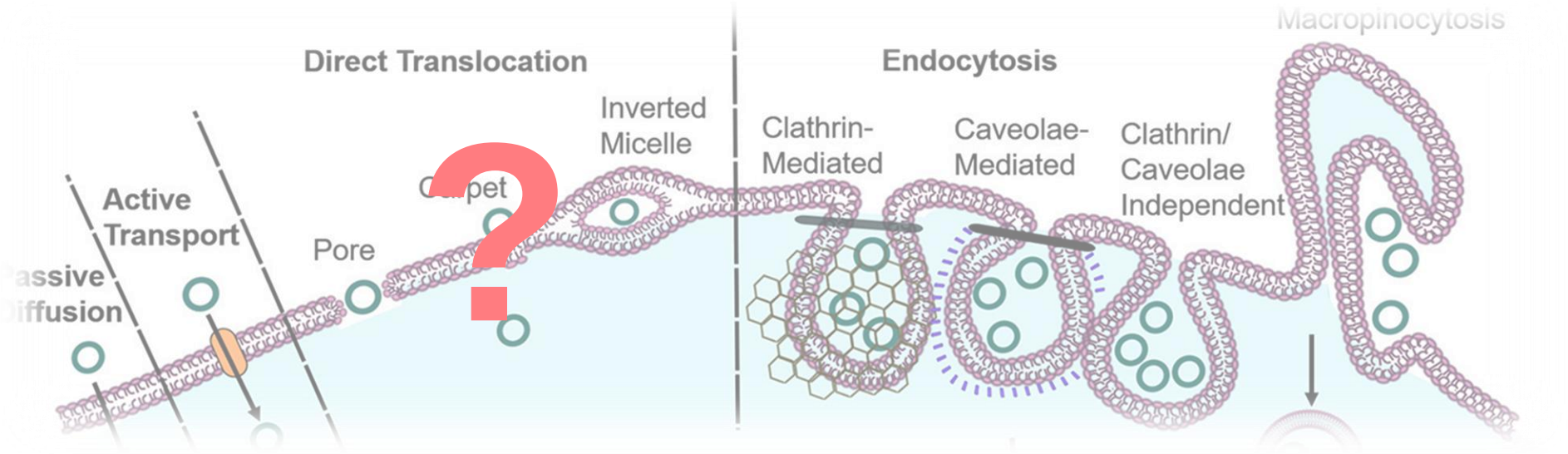
What is the Direct Translocation?

- ***key features of direct translocation*:**

(1) energy-independent and can occur at 4 ^{o}C (c.f. endocytosis)

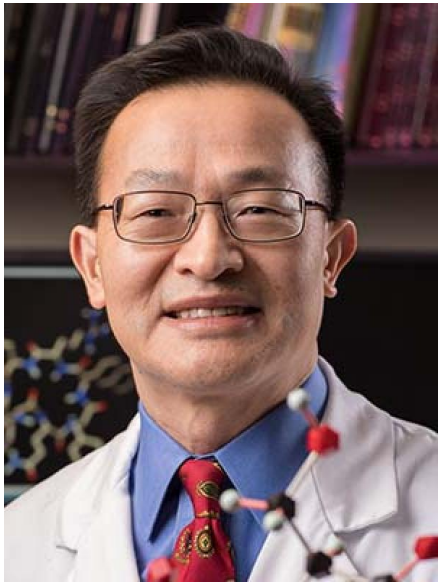
(2) faster than endocytosis (within a few minutes)

The mechanism of
the direct translocation is controversial



Prof. Dehua Pei

2004-present	**Professor, Ohio State University**
2001-2004	**Associate Professor, Ohio State University**
1995-2004	**Assistant Professor, Ohio State University**
1991-1995	**Postdoctoral Fellow, Harvard Medical School (advisor: Prof. Christopher T. Walsh)**
1991	**Ph.D. in organic chemistry from University of California, Berkeley (advisor: Prof. Peter G. Schultz)**
1986	**B.S. in Chemistry from Wuhan University, China**

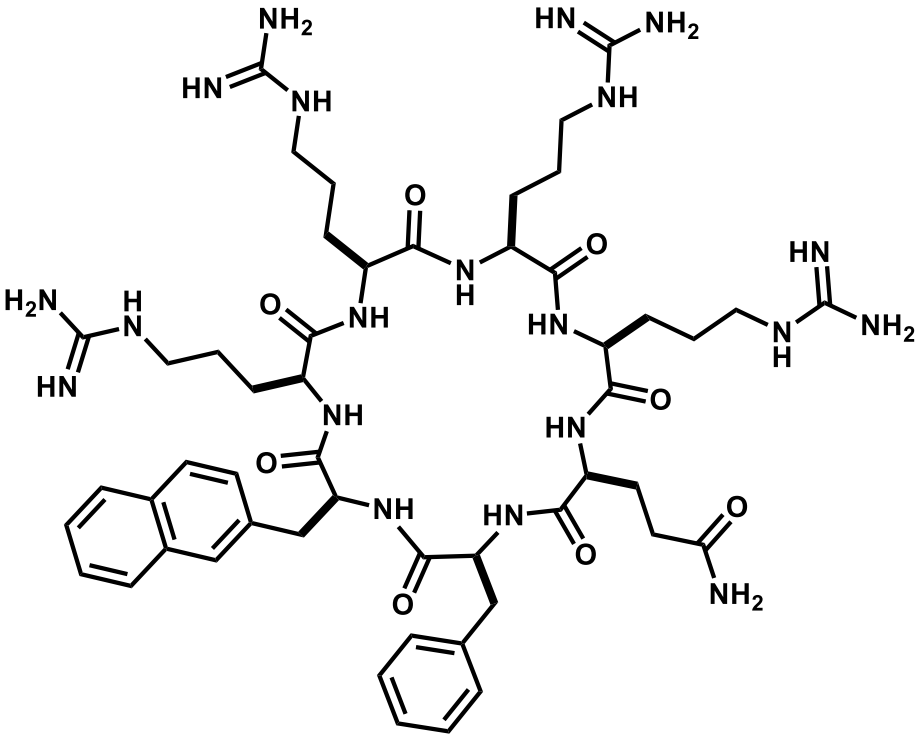


Research field: biochemistry/chemical biology/drug discovery/organic chemistry

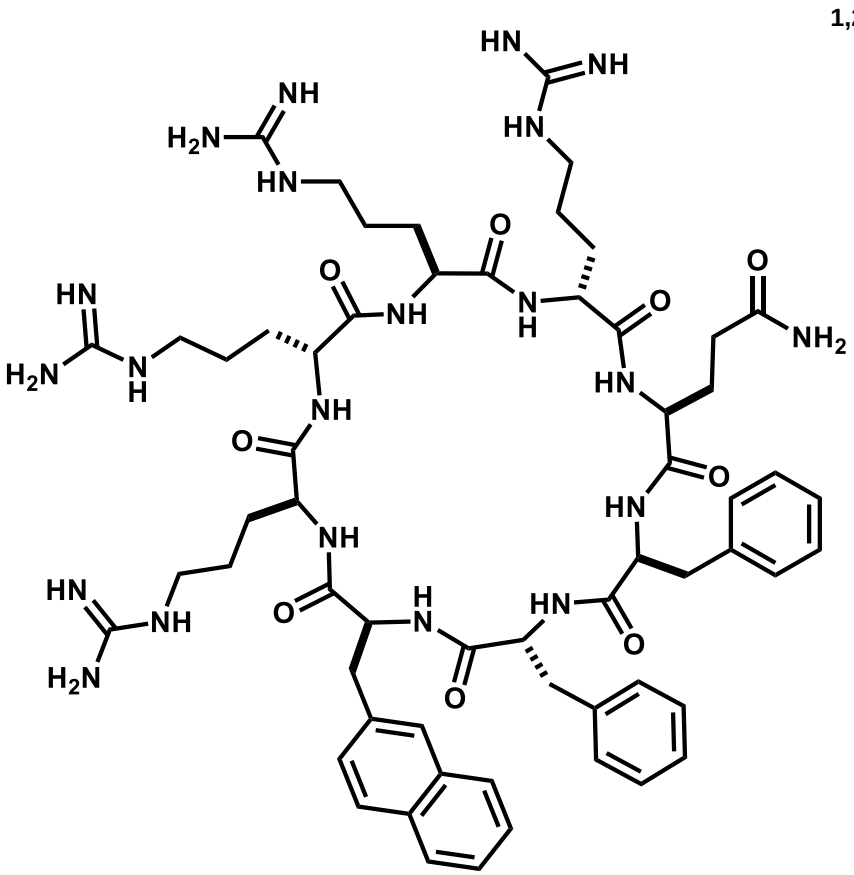
focuses

- ***How Do Biomolecules Cross the Cell Membrane?***
- ***Macrocyclic Peptides as Protein-Protein Interaction Inhibitors***
- ***Development of Intracellular Biologics and Chemical Probes***

Cyclic CPPs Developed by Pei Group



CPP1



CPP12

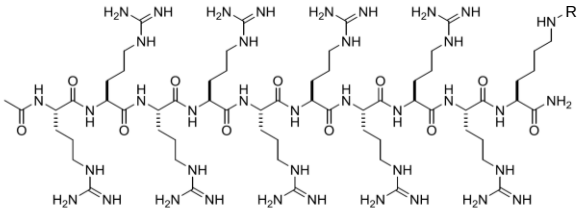
1,2)

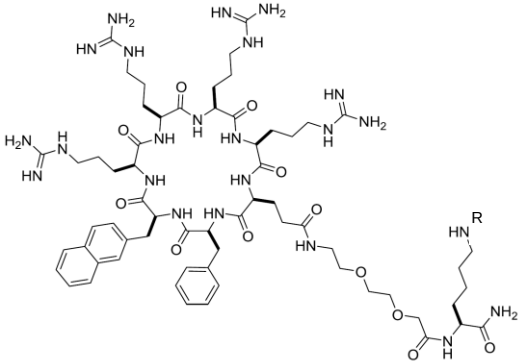
1) Qian, Z.; Martyna, A.; Hard, R. L.; Wang, J.; Appiah-Kubi, G.; Coss, C.; Phelps, M. A.; Rossman, J. S.; Pei, D. *Biochemistry* **2016**, *55*, 2601. 2) Qian, Z.; LaRochelle, J. R.; Jiang, B.; Lian, W.; Hard, R. L.; Selner, N. G.; Luechapanichkul, R.; Barrios, A. M.; Pei, D. *Biochemistry* **2014**, *53*, 4034.

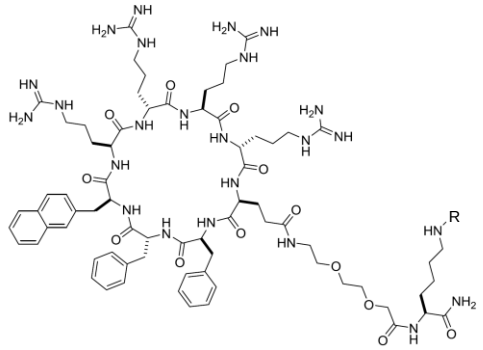
Cyclic CPPs Developed by Pei Group

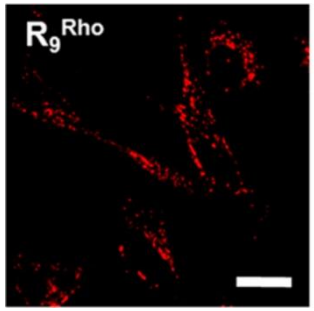
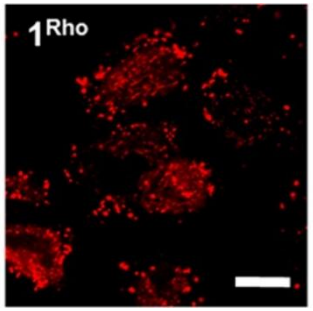
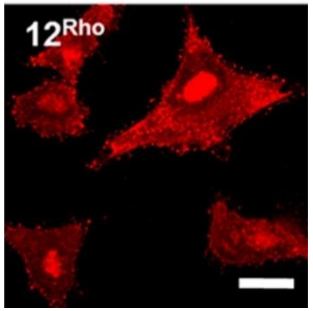
[1,2)]

fluorophore (R)-conjugated CPPs







R_9	CPP1	CPP12
Lissamine rhodamine B-labeled CPPs		
		
relative cellular uptake of FITC-labeled CPPs (standardized against CPP1 by using flow cytometry)		
35%	**100%**	**681%**

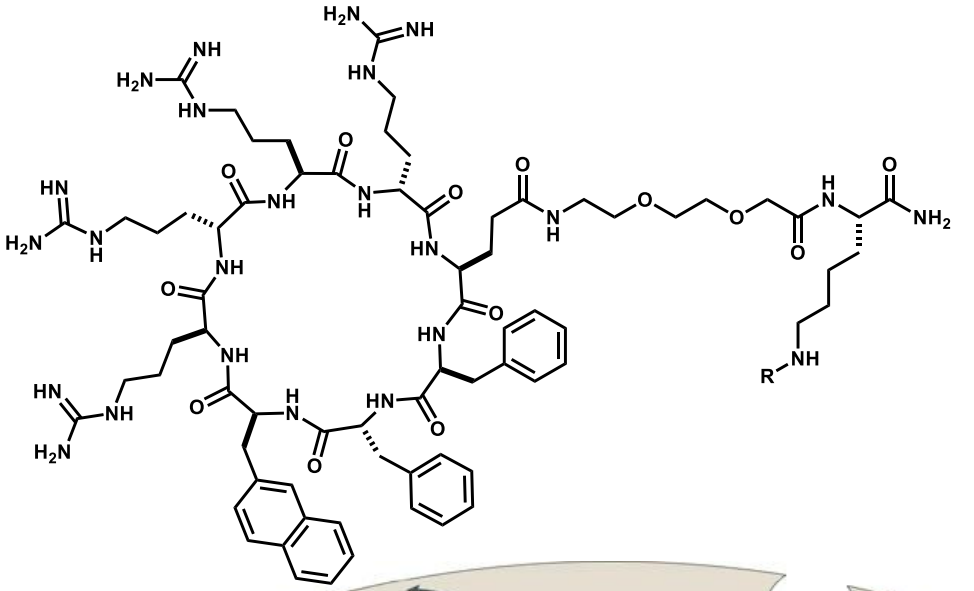
- **Pei group reported multiple cyclic CPPs[1,2)]**
- **CPP1 is demonstrated to be orally bioavailable (4% oral bioavailability)**

c.f. most orally administered peptidic drugs: <1−2%

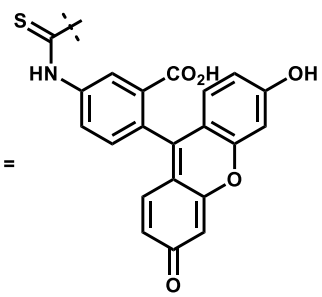
1) Qian, Z.; Martyna, A.; Hard, R. L.; Wang, J.; Appiah-Kubi, G.; Coss, C.; Phelps, M. A.; Rossman, J. S.; Pei, D. *Biochemistry* **2016**, *55*, 2601. 2) Qian, Z.; LaRochelle, J. R.; Jiang, B.; Lian, W.; Hard, R. L.; Selner, N. G.; Luechapanichkul, R.; Barrios, A. M.; Pei, D. *Biochemistry* **2014**, *53*, 4034.

Investigating the Mechanism of Endosomal Escape

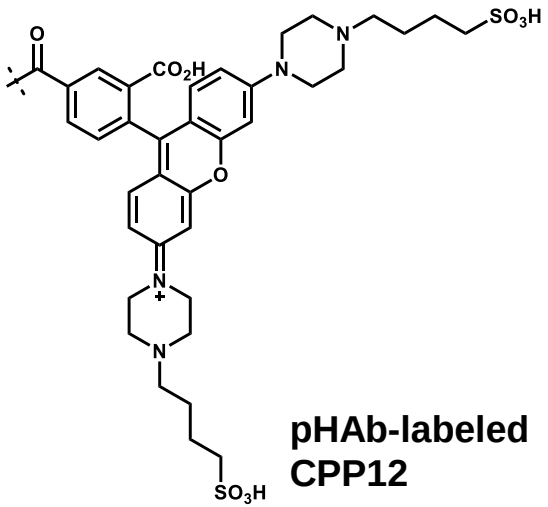
- The previous Pei's study focused on the mechanism of endosomal escape of the CPPs



R =

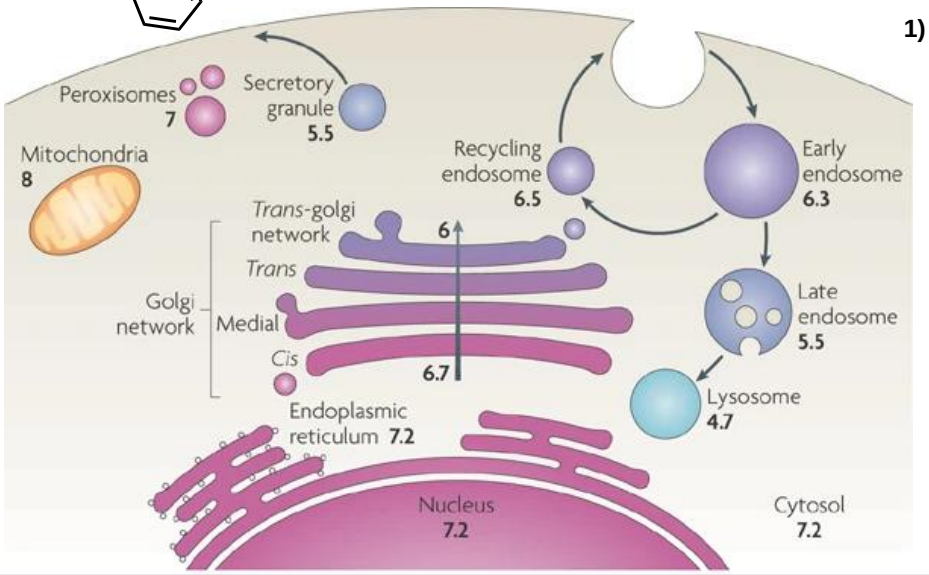


FITC-labeled CPP12



pHAb-labeled CPP12

1)



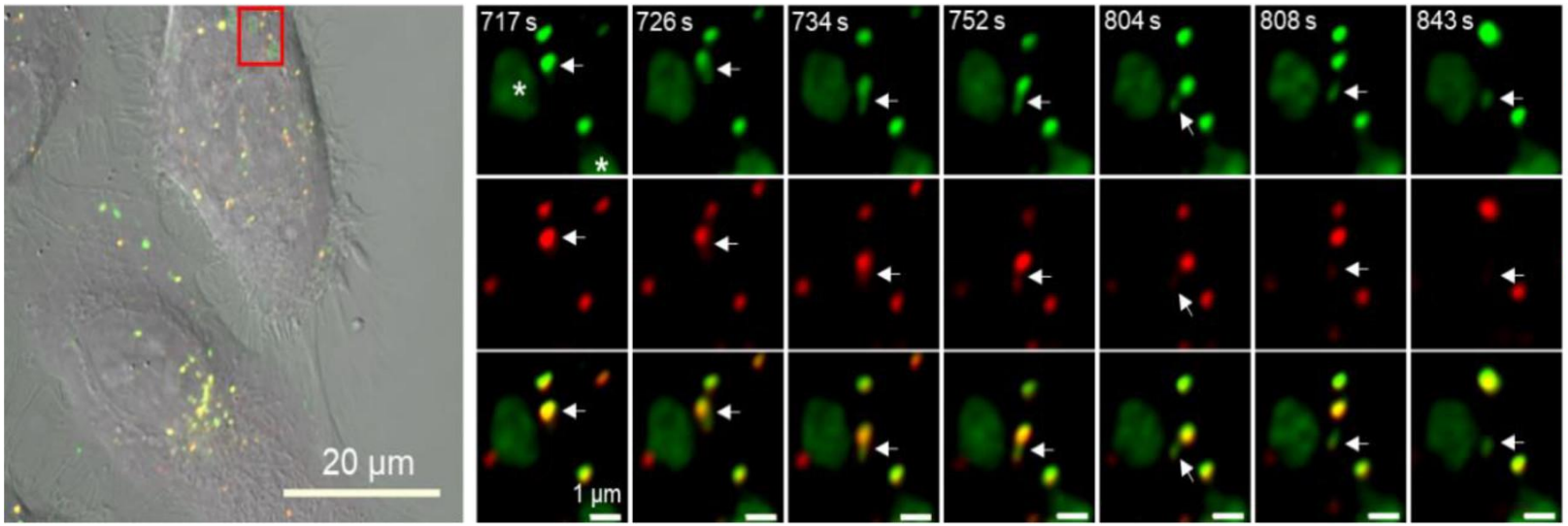
pHAb[2]:
p*Ka* 6.2
strong fluorescence in endosome (pH 5.5−6.5) /lysosome (pH 4.5−5.5)

weak fluorescence in cytosolic/nuclear or extracellular space (pH 7)

1) Casey, J. R.; Grinstein, S.; Orlowski, J. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 50. 2) Robers, M. B.; Binkowski, B. F.; Cong, M.; Zimprich, C.; Corona, C.; McDougall, M.; Otto, G.; Eggers, C. T.; Hartnett, J.; Machleidt, T.; Fan, F.; Wood, K. V. *Anal. Biochem.* **2015**, *489*, 1.

Endosomal Escape: Vesicle Budding in Endosomes

1)

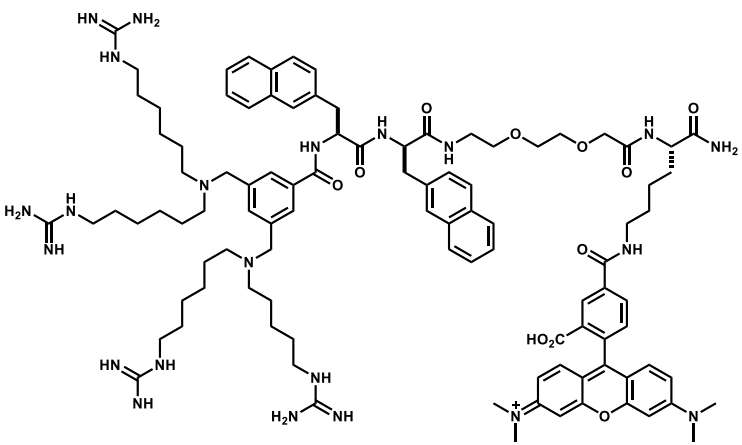


cell line: HeLa
compounds: FITC-labeled CPP12 (2 μM, green)
pHAb-labeled CPP12 (2 μM, red, pH dependent)
white arrow: the endosome undergoing vesicle budding and collapse (VBC)
***: lipid/peptide aggregates derived collapsed vesicles or intracellular organelles bound with cytosolic FITC-labeled CPP**
scale bar: 20 (left) or 1 (right) μm

- **Doubly labeled endosomes visualized vesicle budding and collapse (VBC)**

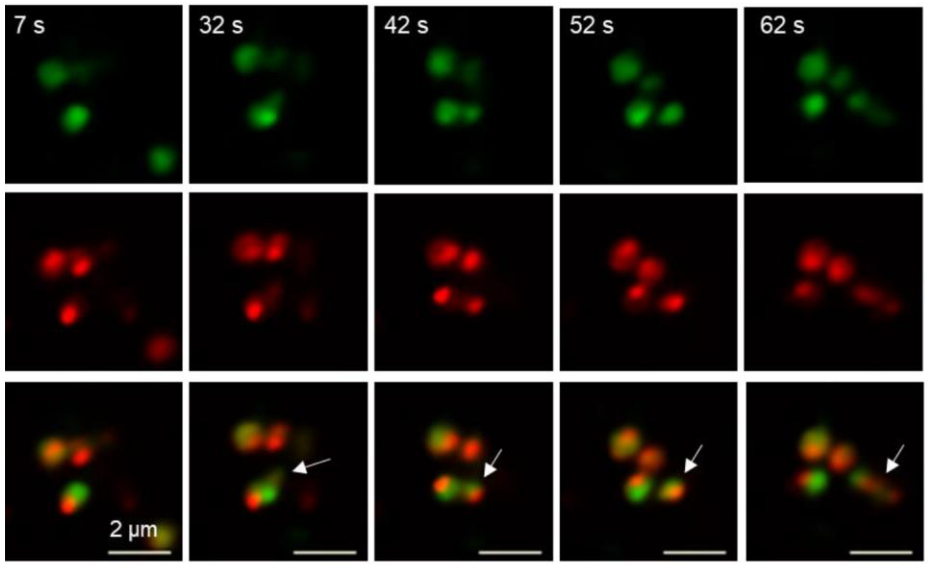
1) Sahni, A.; Qian, Z.; Pei, D. *ACS Chem. Biol.* **2020**, *15*, 2485.

Vesicle Budding in Endosomes Induced by Other Cell-Penetrating Molecules



TAMRA-labeled CPM3[1)]

2)



Alexa Fluor 488-labeled dextran

TAMRA-labeled CPM3

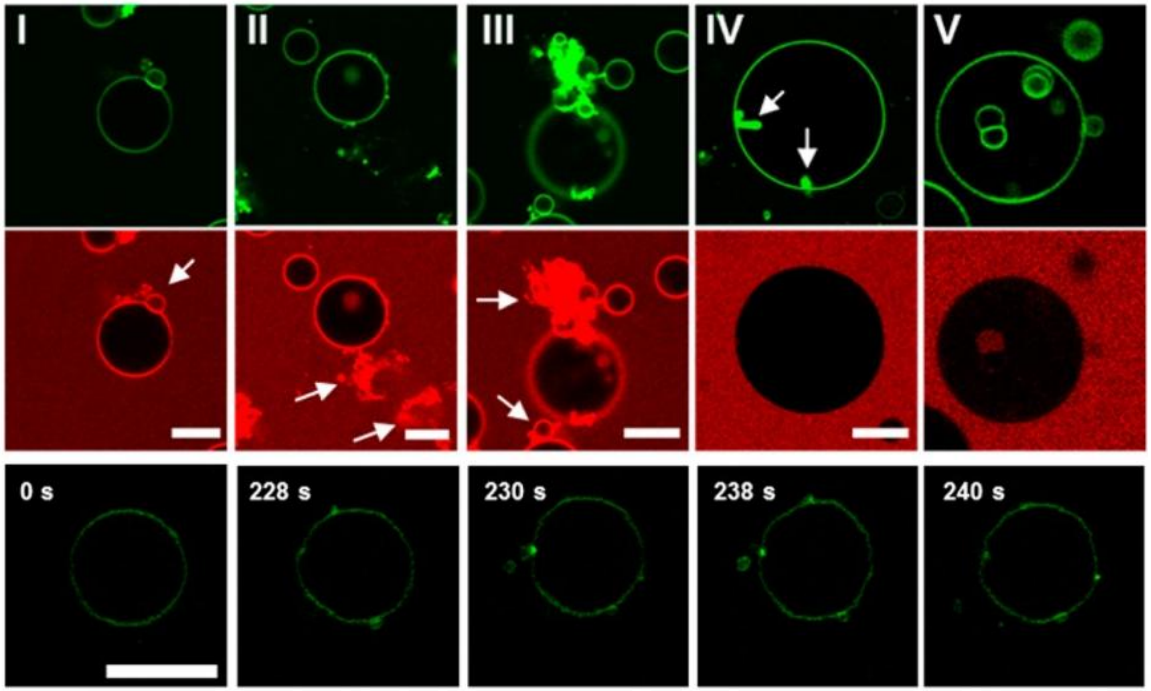
merged

cell line: HeLa treated with YM201636 for 2 h (800 nM, for endosomal enlargement)
compounds: Alexa Fluor 488-labeled dextran (50 μg/mL, green)
TAMRA-labeled CPM3 (2 μM, red)
scale bar: 2 μm

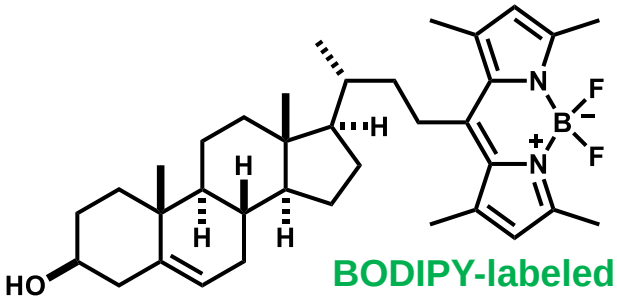
- **CPM3 caused the similar vesicle budding events in endosomes**
- **CPM3 and other Arg-rich CPPs likely share the same mechanism in the endosomal escape**

1) Kubi, G. A.; Qian, Z.; Amiar, S.; Sahni, A.; Stahelin, R. V.; Pei, D. *Angew. Chem. Int. Ed.* **2018**, *57*, 17183.
2) Sahni, A.; Qian, Z.; Pei, D. *ACS Chem. Biol.* **2020**, *15*, 2485.

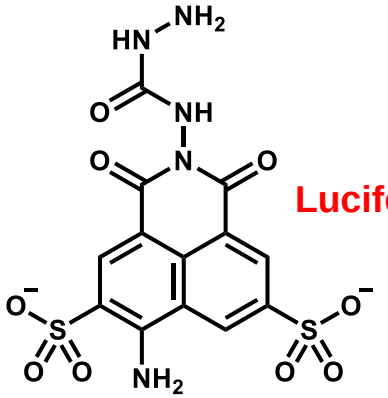
VBC Observed in Giant Unilamellar Vesicles (GUVs)



1)



BODIPY-labeled cholesterol



Lucifer Yellow

GUV composition (mimicking late endosomes):
50% phosphatidyl choline (PC), 20% phosphatidyl ethanolamine (PE),
10% phosphatidylinositol (PI), 20% bis(monooleoylglycero)phosphate (BMP)

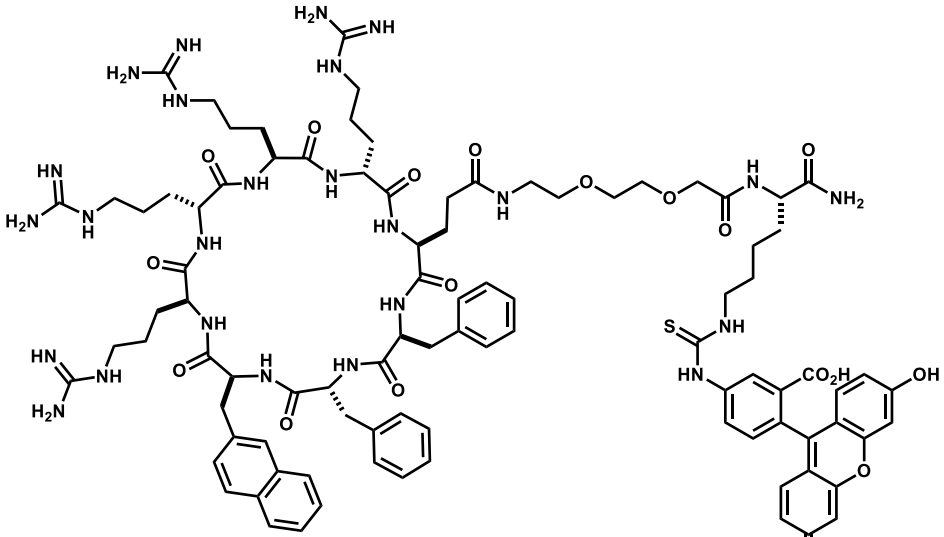
BODIPY-labeled cholesterol (green) as a membrane marker
Lucifer Yellow (red) for visualizing the solution outside of the GUVs
scale bar: 10 µm

- **This GUV-based experiment indicated that the VBC is an energy-independent process**

➡ **It was assumed that VBC also occurs on the plasma membrane**

1) Qian, Z.; Martyna, A.; Hard, R. L.; Wang, J.; Appiah-Kubi, G.; Coss, C.; Phelps, M. A.; Rossman, J. S.; Pei, D. *Biochemistry* **2016**, *55*, 2601.

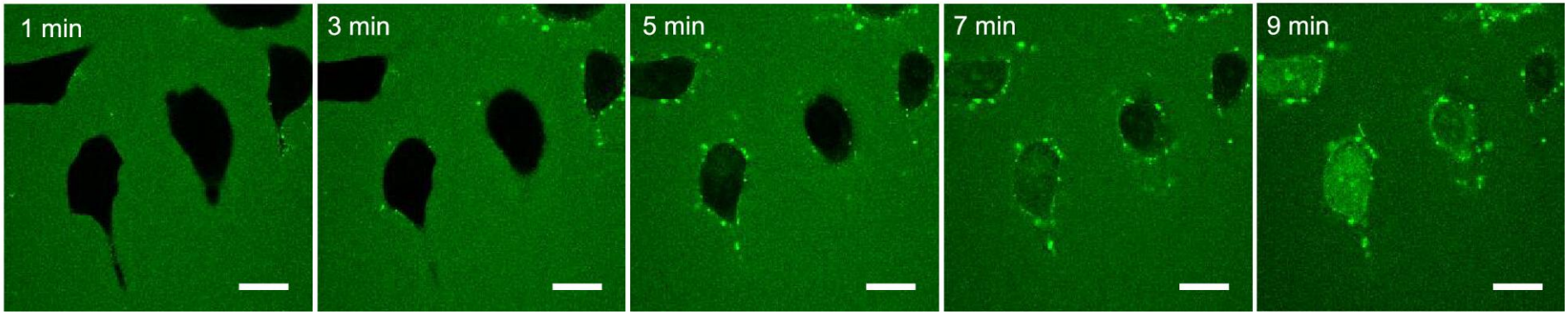
Cell Entry of CPP12 via Direct Translocation



FITC-labeled CPP12

cell line: HeLa
compounds: FITC-labeled CPP12 (5 μM, green)
CPP12 (20 μM)
scale bar: 20 μm

1)

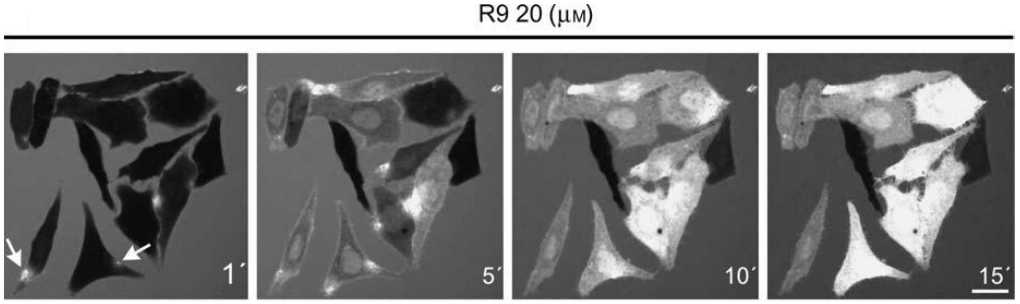


- **Based on the fast cell entry, CPP12 (total 25 μM) entered the cells through the direct translocation**
- **Nucleation zones (NZs) were observed before diffusion of FITC-labeled CPP12 into cytosol**

1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

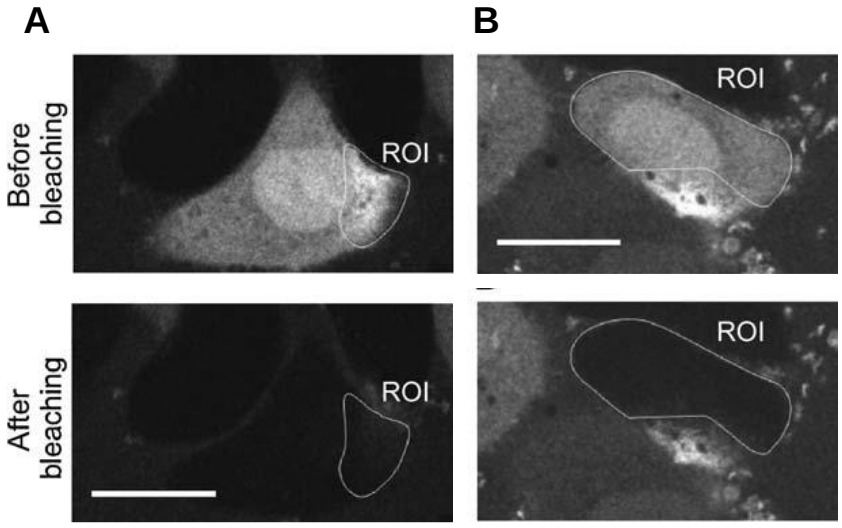
“Nucleation Zone”

- **Brock group reported that the rapid uptake of R9 were associated with the formation of “nucleation zone (NZ)” in plasma membrane[1)]**



cell line: HeLa cells
compounds: fluorescein-labeled R9 (20 μM)
time-lapse imaging: four images at 1, 5, 10 and 15 min are shown
white arrow: nucleation zone

- **fluorescence loss in photobleaching (FLIP) experiment**



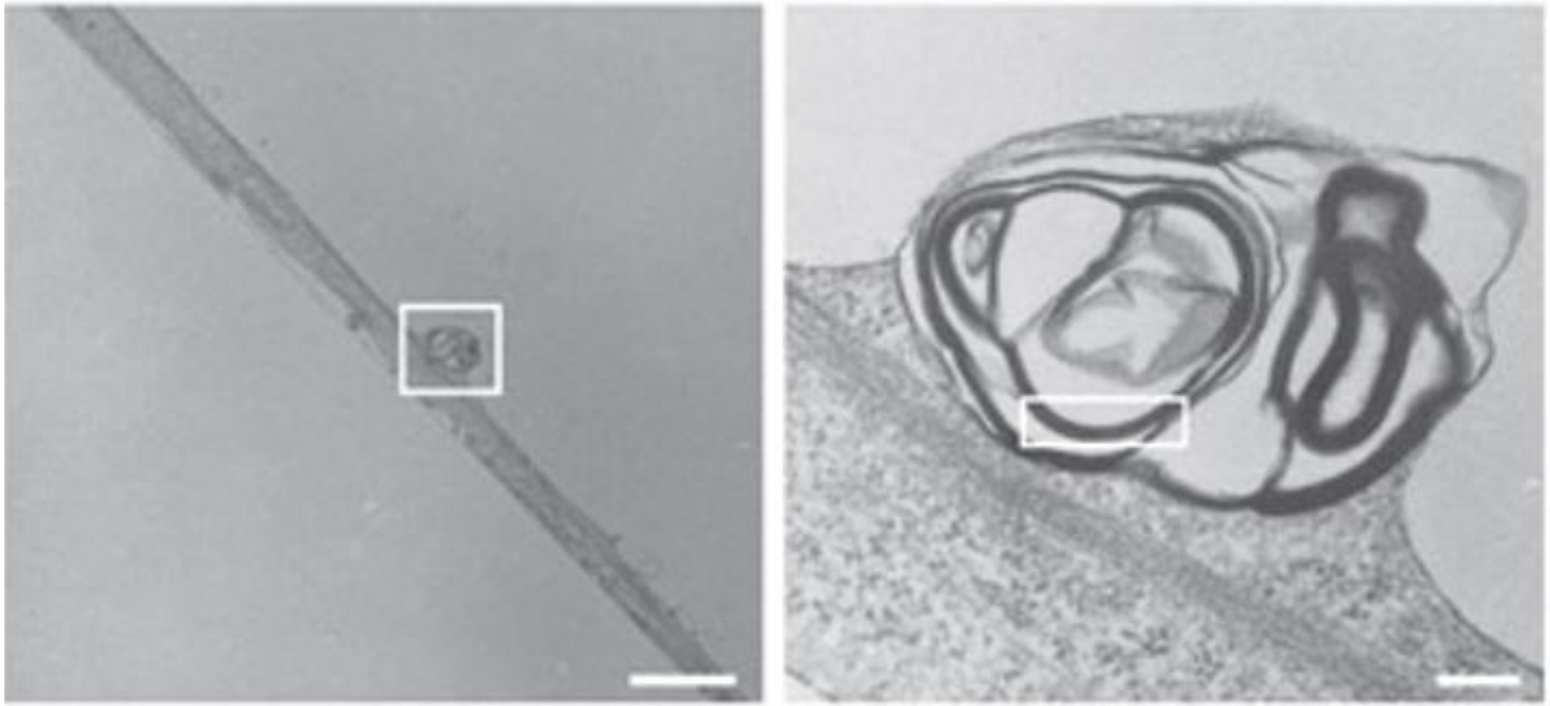
fluorescein-labeled R9 was bleached within the regions of interest (ROIs) for 60 s (488 nm)

- **The result suggested that R9 are transiently confined in the NZs**

1) Duchardt, F.; Fotin-Mleczek, M.; Schwarz, H.; Fischer, R.; Brock, R. *Traffic* **2007**, *8*, 848.

TEM Analysis of NZ

1)



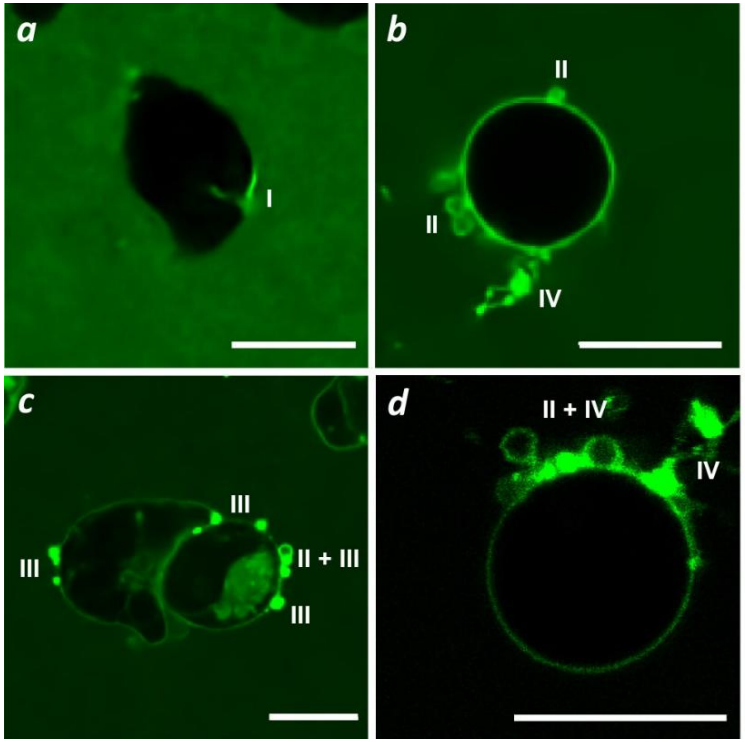
HeLa cells treated with Alexa Fluor 488-labeled R12 (6 μM) for 5 min, then fixed by 2% glutaraldehyde in 30 mM HEPES
scale bars: left: 2 μm, right: 200 nm

- **The "particle-like structure" (1–3 μm in diameter) forms even in low temperature (4 °C)**
  ➡ **energy independent and do not require active transport machineries, including macropinocytosis**

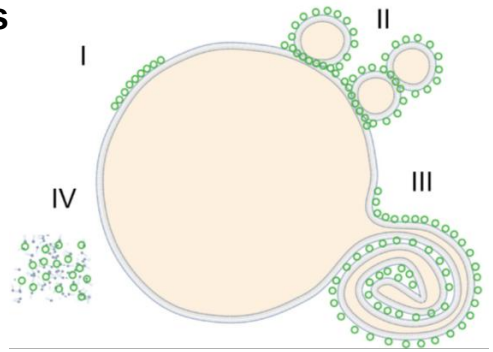
1) Hirose, H.; Takeuchi, T.; Osakada, H.; Pujals, S.; Katayama, S.; Nakase, I.; Kobayashi, S.; Haraguchi, T.; Futaki, S. *Mol. Therapy* **2012**, *20*, 984.

Classification of the NZs

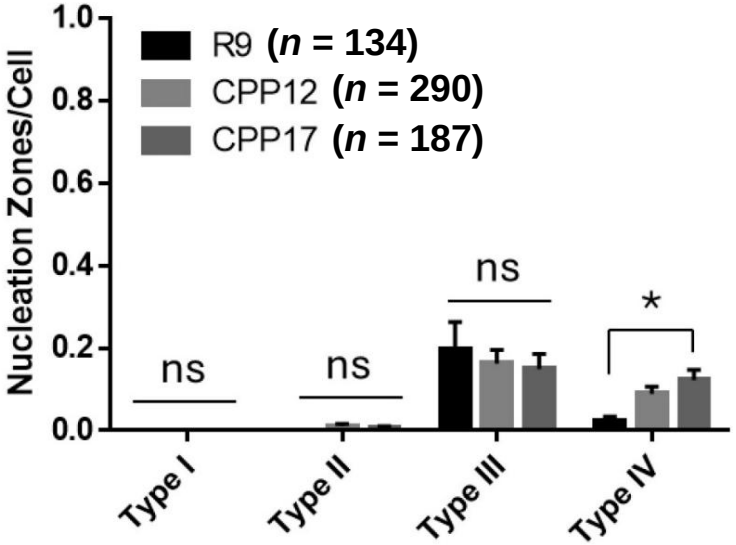
- representative images of Jurkat cells



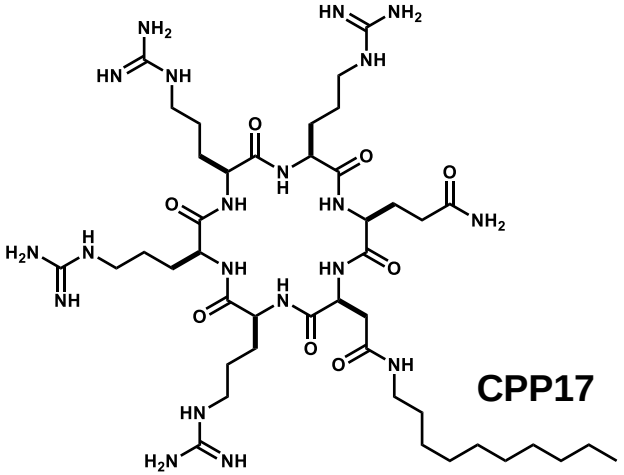
cell line: Jurkat cells
compounds:
FITC-labeled CPP12
(5 μM, green)
CPP12 (20 μM)
scale bar: 5 μm



- abundance of types I-IV NZs [1)]



cell line: Jurkat cells
compounds: 2.5 μM FITC-labeled CPP/10 μM CPP
incubation time: 2 min

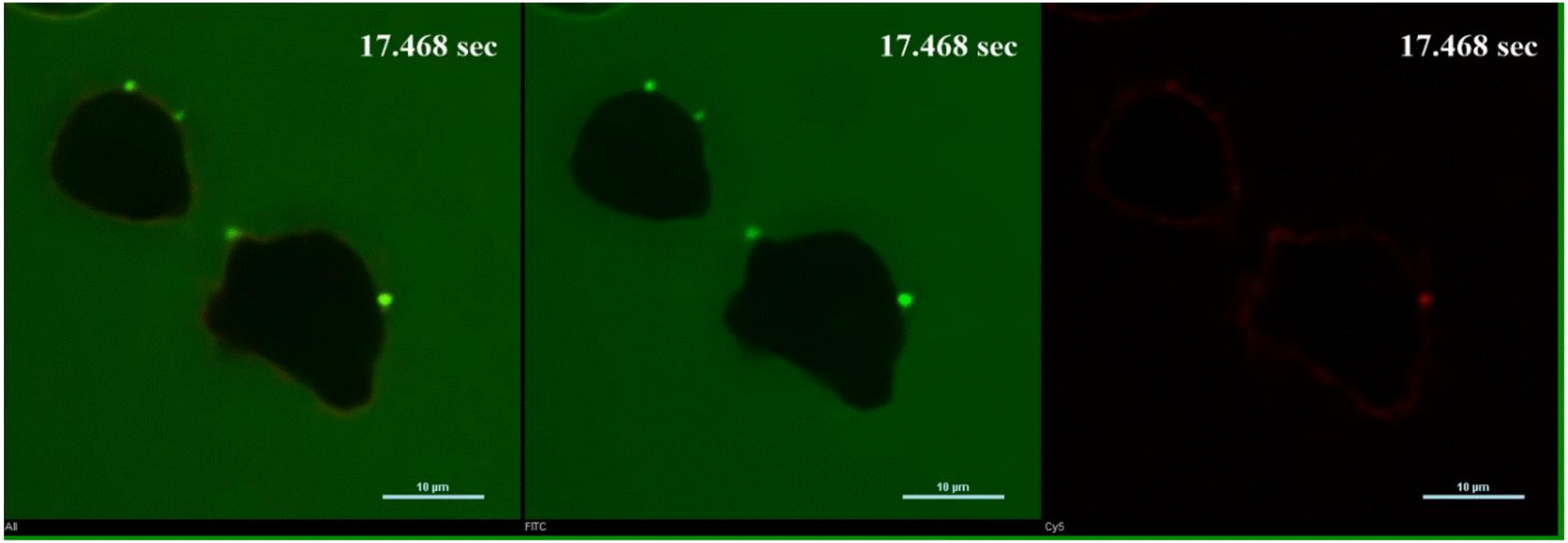


- NZ type III are the most frequently observed

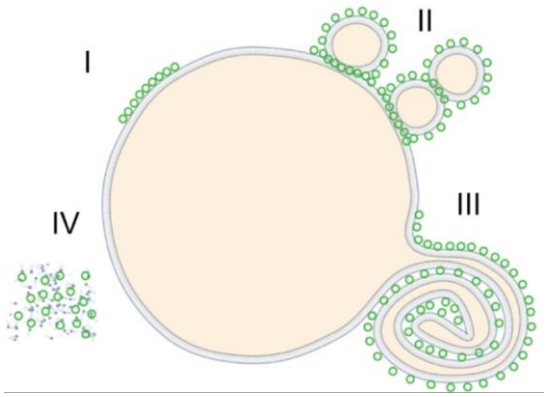
1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

Interconversion of NZs

1)



cell line: Jurkat cells
compounds:
FITC-labeled CPP12 (5 µM, green)
CPP12 (20 µM)
scale bar: 10 µm

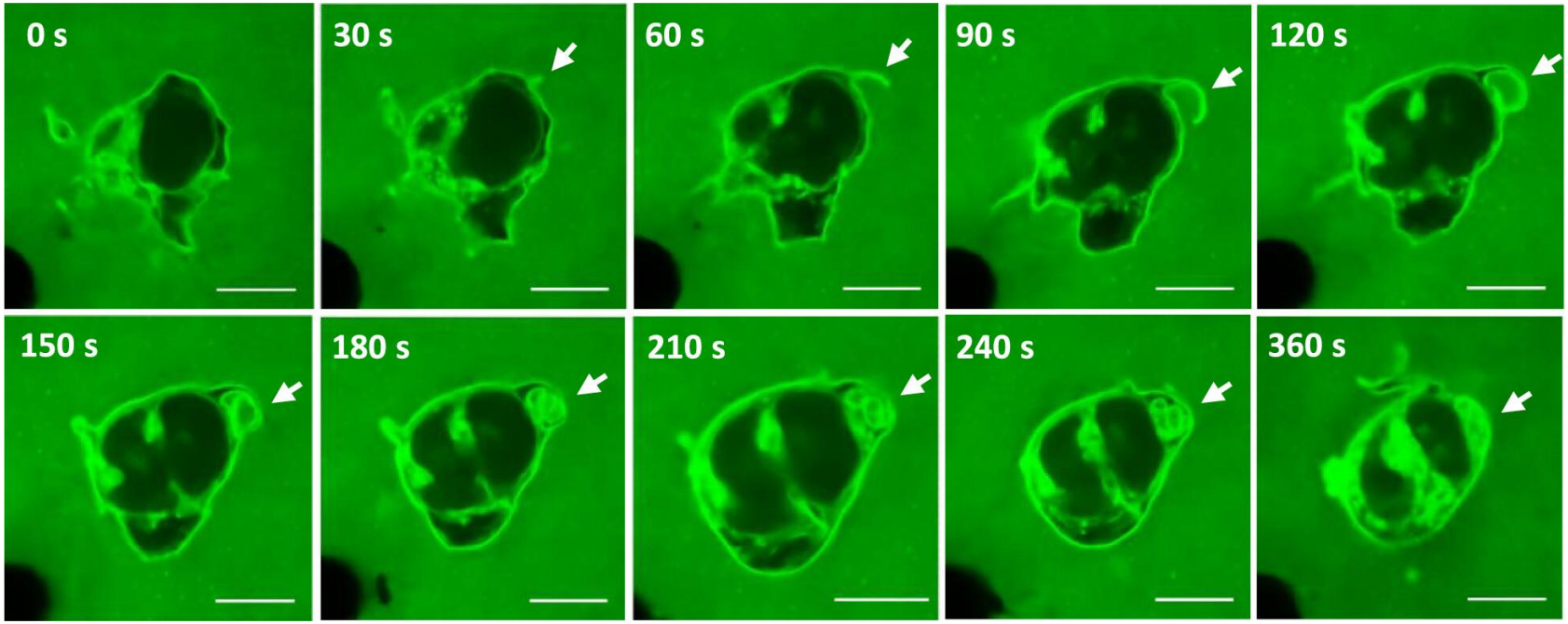


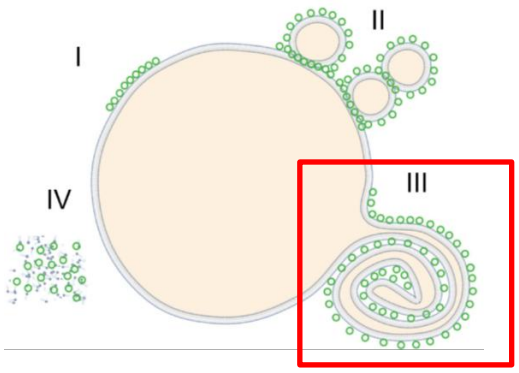
■ **NZ types I, II, and III are interconvertible**

1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

Time-Lapse Imaging of Type III NZ Formation

1)





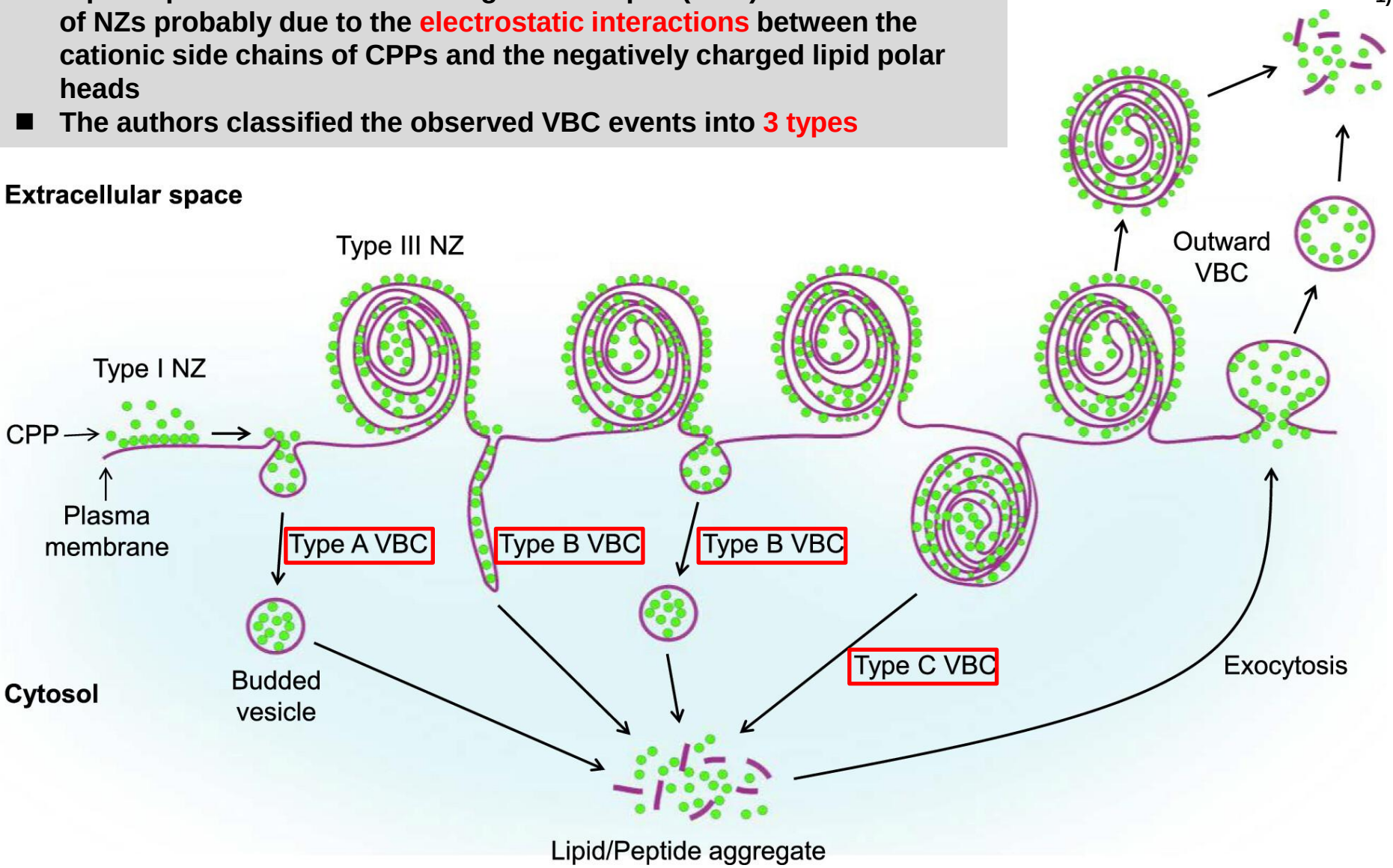
cell line: Jurkat cells
compounds:
FITC-labeled CPP12 (5 μM, green)
CPP12 (20 μM)
scale bar: 5 μm
white arrow: type III NZ formation

- **The most frequently observed type III NZs were formed within 6 min and were structurally similar to the "particles" reported previously (see also page 16)**

1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

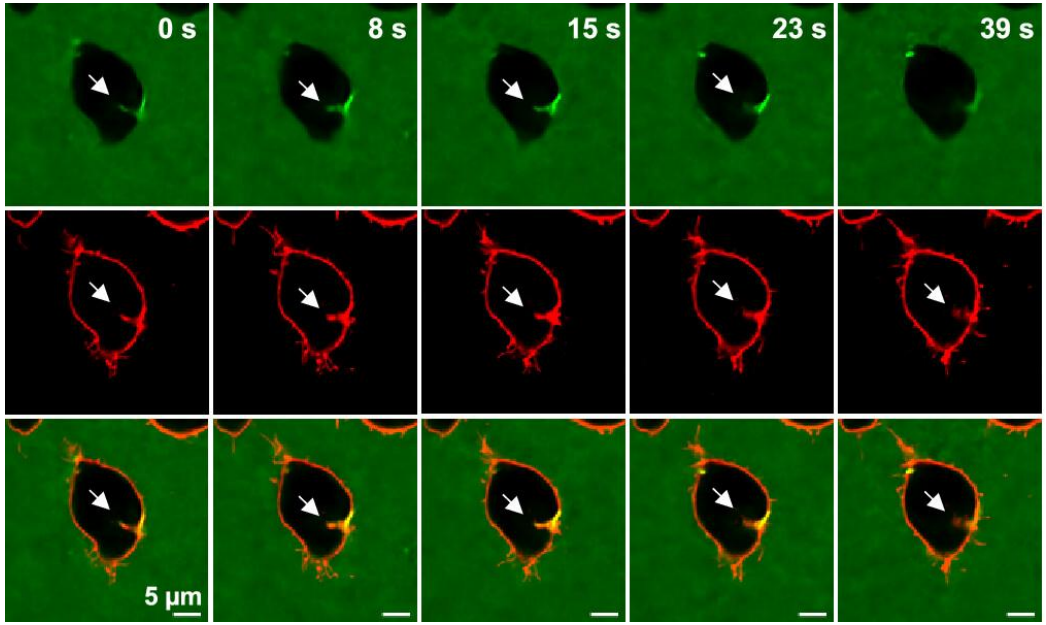
Types A-C VBC Events

- A prerequisite of vesicle budding and collapse (VBC) is the formation of NZs probably due to the electrostatic interactions between the cationic side chains of CPPs and the negatively charged lipid polar heads
- The authors classified the observed VBC events into 3 types



1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

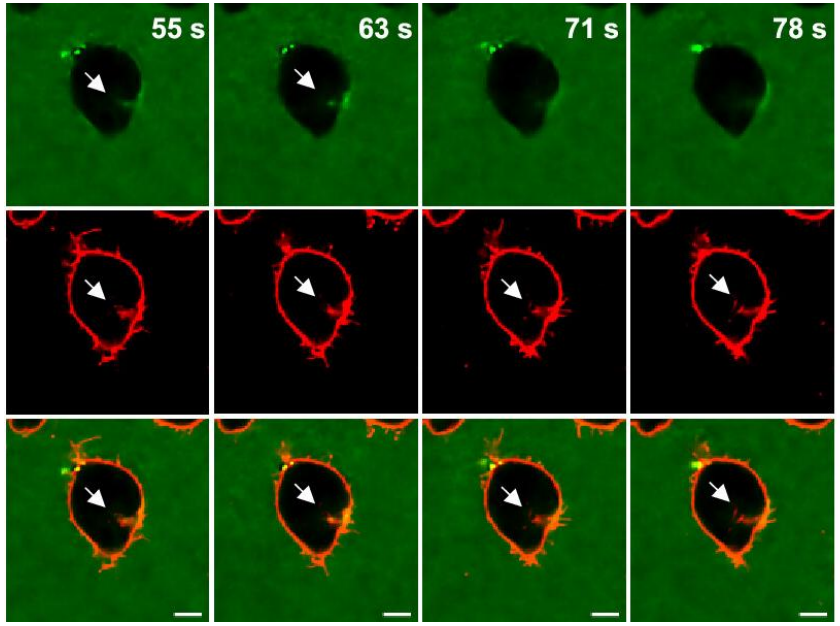
Type A VBC



FITC-labeled CPP12

plasma membrane

merged



FITC-labeled CPP12

plasma membrane

merged

cell line: Jurkat cells
compounds:
FITC-labeled CPP12 (5 μM, green)
CPP12 (20 μM)
CellMask Deep Red for staining plasma membrane
scale bar: 5 μm
white arrow: Type A VBC event

- By using CPP12, type A VBC was observed in the plasma membrane of Jurkat cells

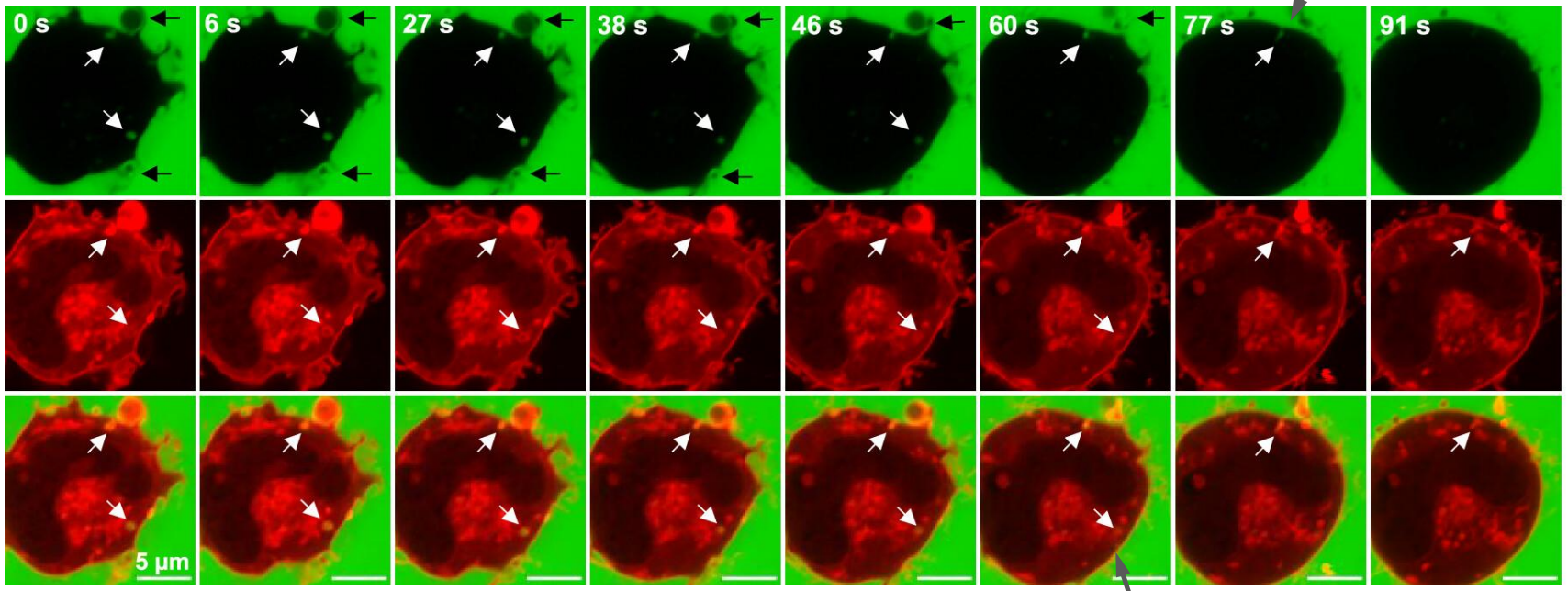
1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

Type B VBC

- The extracellular region was labeled by Alexa Fluor 488

detachment of the vesicle from membrane

1)



loss of green fluorescence: collapse of the vesicle

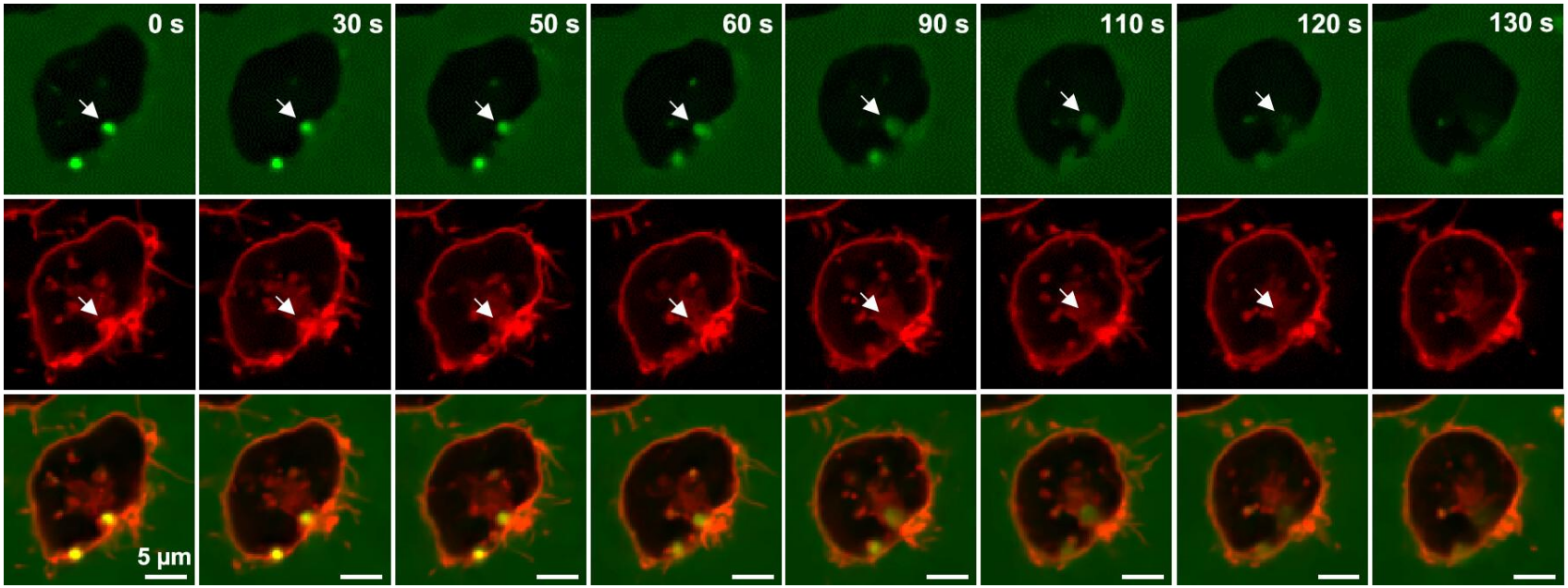
cell line: Jurkat cells
compounds: TAMRA-labeled CPP12 (3 μM, red)
CPP12 (20 μM)
Alexa Fluor 488 (20 μM, green)
The cells were incubated for 3 min
and time-lapse imaging was conducted
scale bar: 5 μm
white arrow: Type B VBC events

- The authors reported that vesicle budding events were frequently observed around type III NZs
- The formation of a type III NZ likely induces negative membrane curvature in its surrounding area and facilitates type B VBC events

1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

Type C VBC

1)



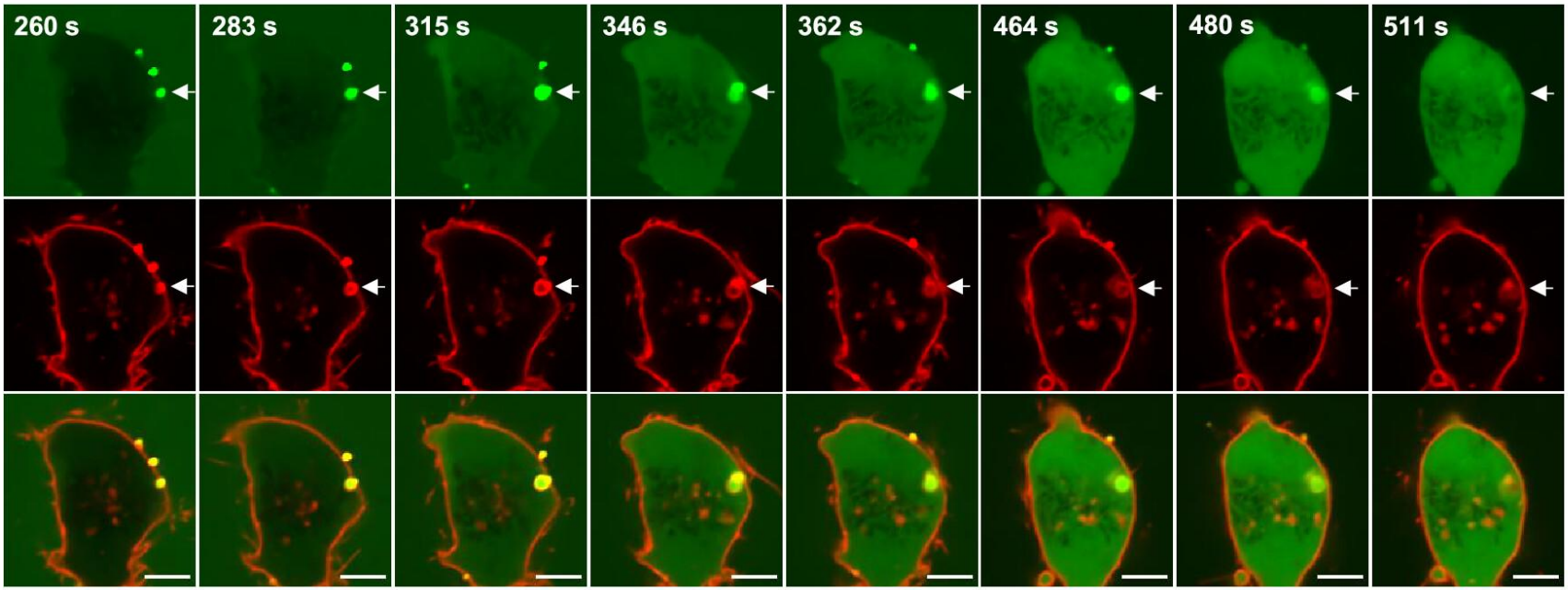
cell line: Jurkat cells
compounds: Alexa Fluor 488-labeled CPP17 (1 μM, green)
CPP17 (4 μM)
CellMask Deep Red for staining plasma membrane
The cells were incubated for 3 min and time-lapse imaging was conducted
scale bar: 5 μm
white arrow: Type C VBC events

- **CPP17[2] was also confirmed to induce VBC at low concentration**
- **Type C VBC was observed**

1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.
2) Song, J.; Qian, Z.; Sahni, A.; Chen, K.; Pei, D. *ChemBioChem* **2019**, *20*, 2085.

Direct Translocation of R9

1)



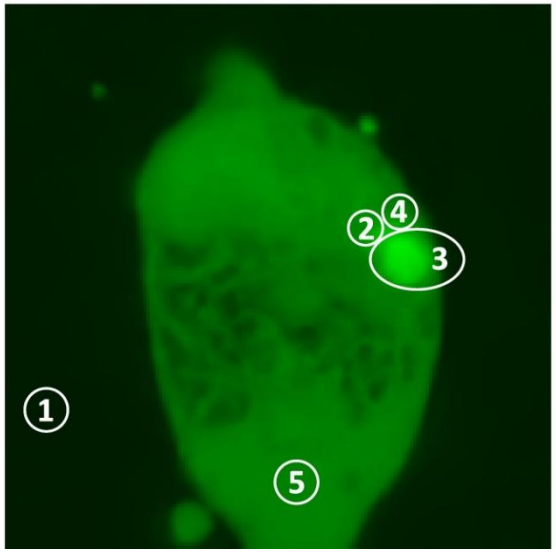
cell line: Jurkat cells
compounds: FITC-labeled R9 (5 μM, green)/R9 (20 μM)
CellMask Deep Red for staining plasma membrane
The cells were incubated for 3 min and time-lapse imaging was conducted
scale bar: 5 μm
white arrow: Type C VBC events

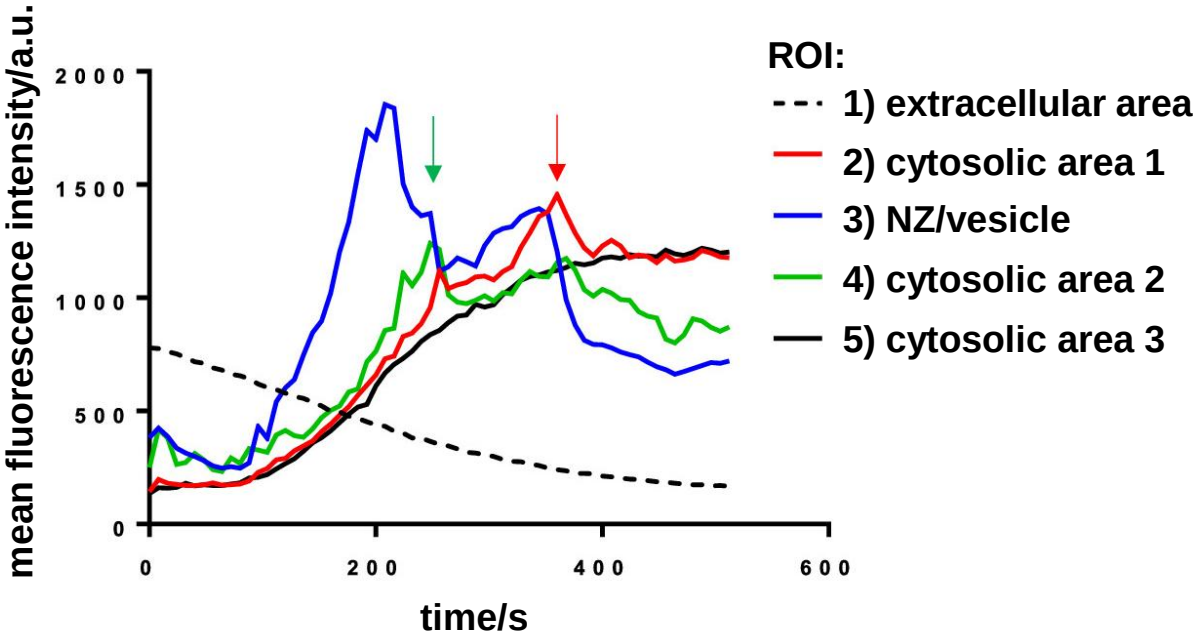
- **VBC is likely general mechanism of direct translocation of Arg-rich CPPs**

1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, *146*, 25371.

NZ Fluorescence Change is Relevant with the Fluorescence of Surrounding Region

ROI of Jurkat cell 1-5:





[1)]

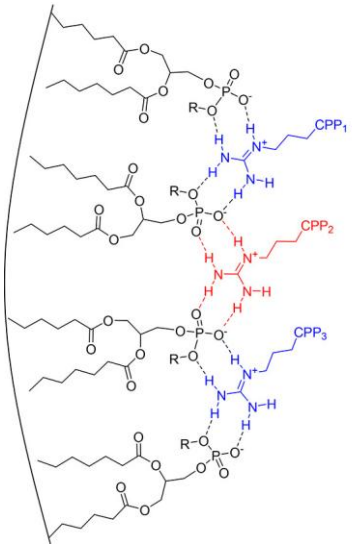
cell line: Jurkat cells
compounds: FITC-labeled R9 (5 μM, green)/R9 (20 μM)
The cells were incubated for 2 min and time-lapse imaging was conducted

- **Time-course of fluorescence intensity of ROI indicated that the collapse of NZ releases R9 into its surrounding region, followed by diffusion into the cytosol**

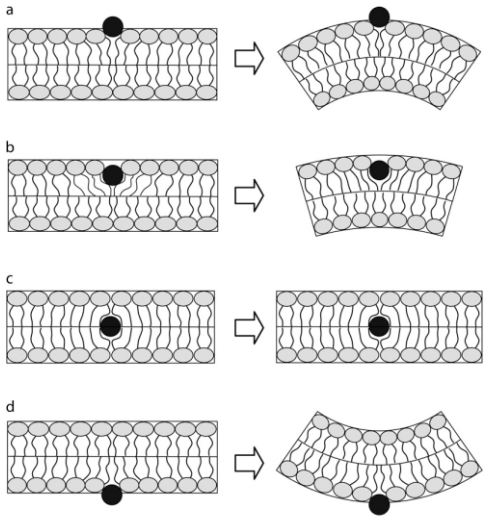
1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, 146, 25371.

VBC and Membrane Curvature

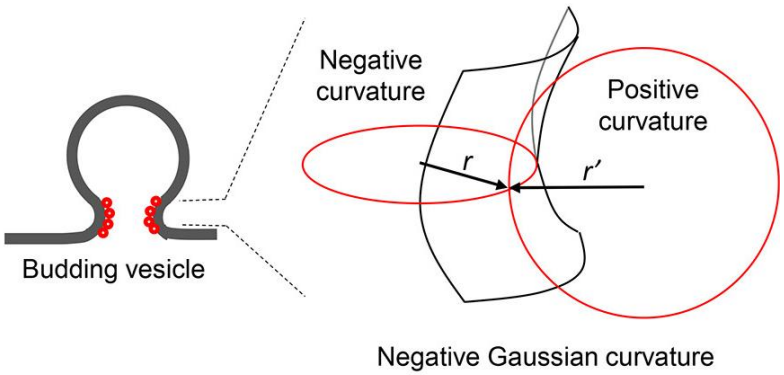
- **Arg residues induce curvature by hydrogen bonding to the lipid polar heads[1] (the diagram corresponds to negative curvature)**



- **The (shallow) insertion of hydrophobic groups in membrane generates positive curvature[2]**



- **Hydrogen-bonding and hydrophobic interaction between CPPs and phospholipids could induce negative Gaussian curvature at the budding neck[3]**

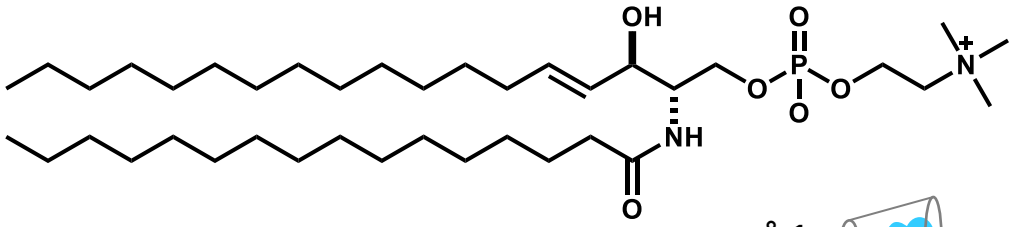


1) Mishra, A.; Lai, G. H.; Schmidt, N. W.; Sun, V. Z.; Rodriguez, A. R.; Tong, R.; Tang, L.; Cheng, J.; Deming, T. J.; Kamei, D. T.; Wong, G. C. L. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 16883. 2) Campelo, F.; McMahon, H. T.; Kozlov, M. M. *Biophys. J.* **2008**, *95*, 2325. 3) Pei, D. *Acc. Chem. Res.* **2022**, *55*, 309.

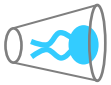
Effect of Sphingomyelinase

- Cytosolic entry of R9 into HeLa cells via the direct translocation is known to be facilitated by exogenous sphingomyelinase
- Conversion of sphingomyelin into ceramide (C_0 < 0) could facilitate the formation of the budding neck

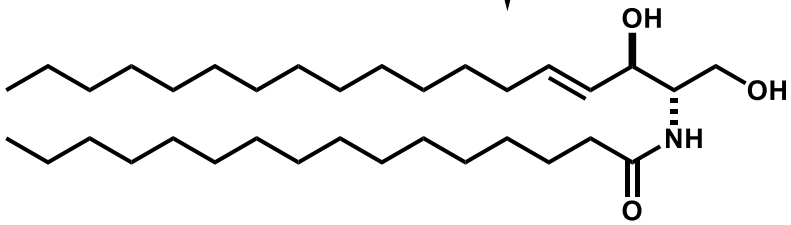
intrinsic curvatures of lipids at 35 °C:[1,2)]



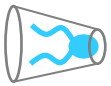
palmitoyl sphingomyelin (C_0 = 0.01 Å^{-1})



cone-shaped



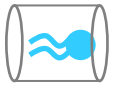
ceramide (C_0 = -0.11 Å^{-1})



inverted cone-shaped

intrinsic curvature $C_0 = 1/R_0$
R_0 can be experimentally measured as the radius of the inverted micellar structure (hexagonal phase) in water

c.f. POPC (C_0 = 0.001 Å^{-1})

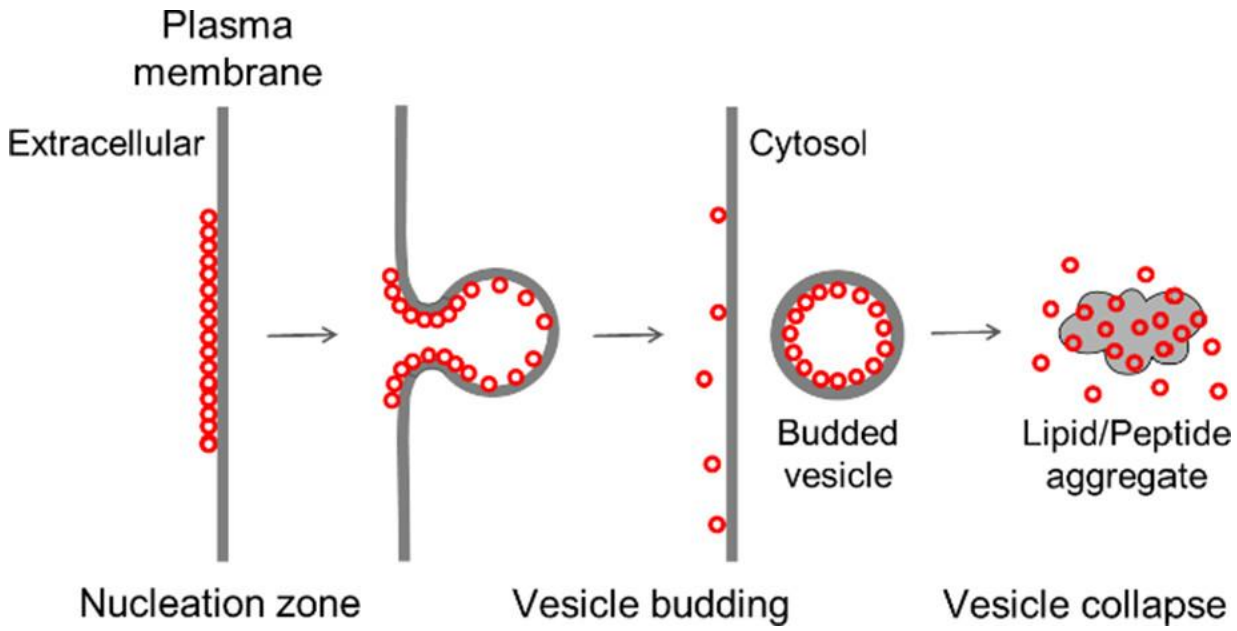


cylindrical

1) Hamai, C.; Yang, T.; Kataoka, S.; Cremer, P. S.; Musser, S. M. *Biophys. J.* **2006**, *90*, 1241. 2) Kaltenegger, M.; Kremser, J.; Frewein, M. P. K.; Ziherl, P.; Bonthuis, D. J.; Pabst, G. *Biochim. Biophys. Acta Biomembr.* **2021**, *1863*, 183709.

Conclusion

- **The extensive imaging studies by Pei group suggested that the mechanism of direct translocation of CPPs is vesicle budding and collapse (VBC) at plasma membrane[1)]**



1) Sahni, A.; Ritchey, J. L.; Qian, Z.; Pei, D. *J. Am. Chem. Soc.* **2024**, 146, 25371.