# Tool on Protein Translocation

2023/10/12

M2 Hiroki Umeda

# Outline



1. Introduction
2. Latest Finding
    1. Tools to analyze protein translocation
    2. Tools to control protein translocation

# Introduction: Protein localization



## Localization:

- Many proteins are transported to specific organelles where they work.
- This localization is achieved mainly by 2 trafficking systems.

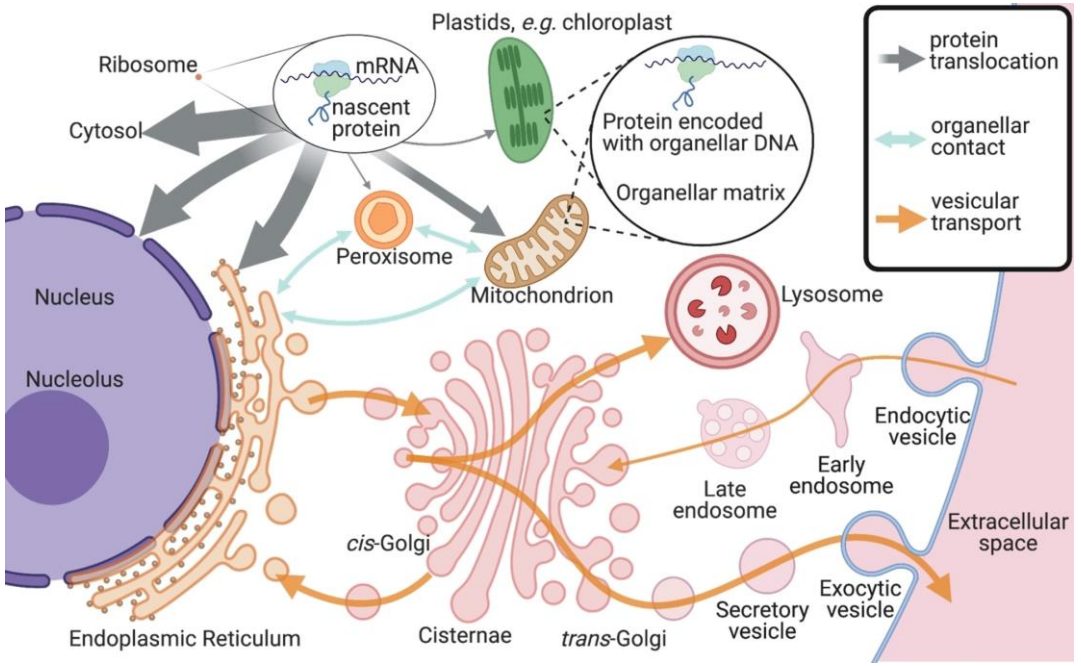
① **Signal Sequence** (gray arrow)

- nuclear localization signal (**NLS**)
- mitochondrial matrix targeting signal (**MTS**)
- ER signal sequences

etc.

② **Vesicular Transport** (orange arrow)

- Rab / tethering protein
  + v-SNARE / t-SNARE interaction




Genereux, J. C. *et al. ChemPlusChem* **2021**, *86*, 1397.

These trafficking systems sequester proteins into compartments, thereby allowing parallel processing of various reactions.

# Introduction: Protein Translocation

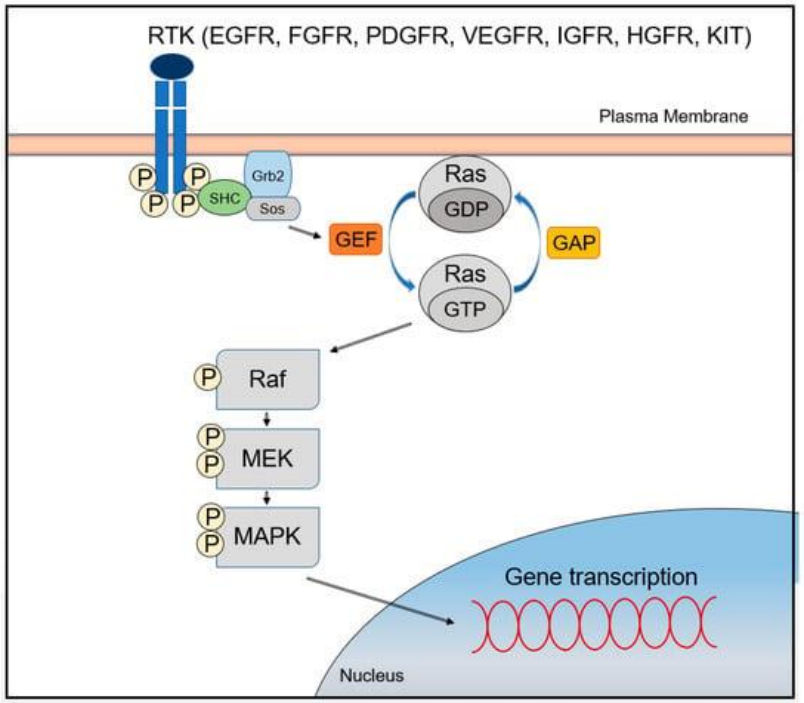


Protein localization is *NOT static event*

→ **Protein Translocation :**

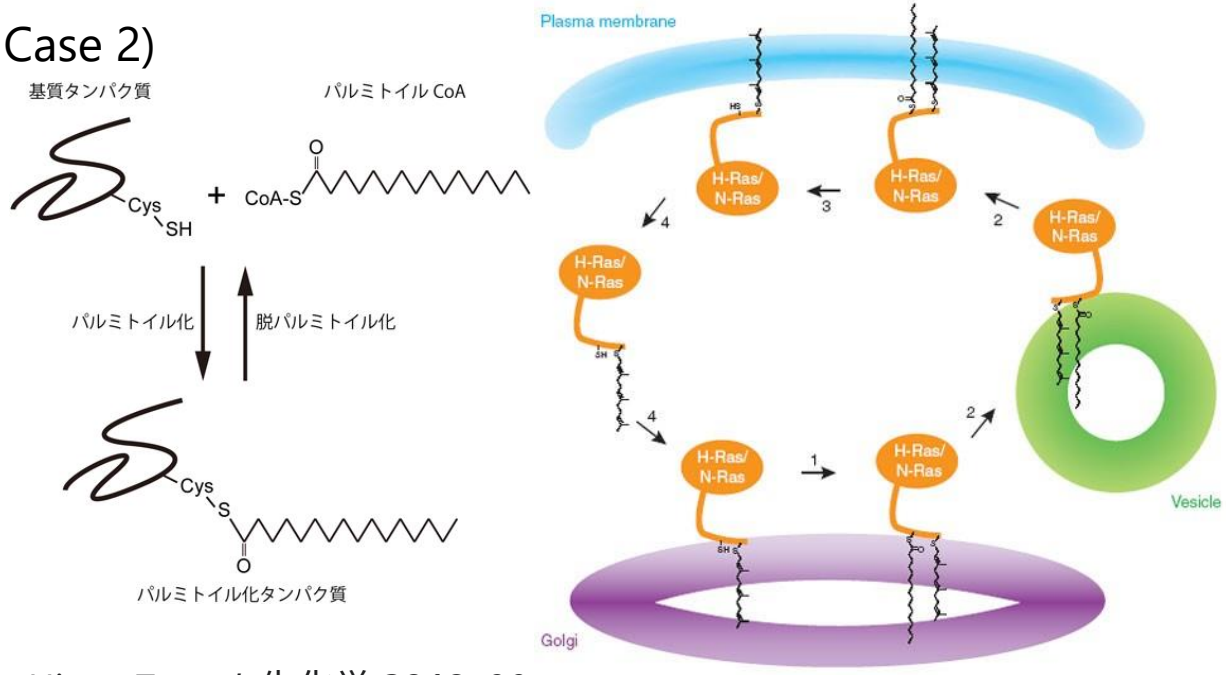
proteins localization is *dynamically regulated* in response to external stimuli or cellular state

Case 1)




Ro, S. W. *et al. Cancers* **2021**,*13*, 3026.

Case 2)




Hirata,T. *et al.* 生化学 **2018**, *90*, 125.
Waldmann, H. *et al. Nat. Chem. Biol.* **2006**, *2*, 518.

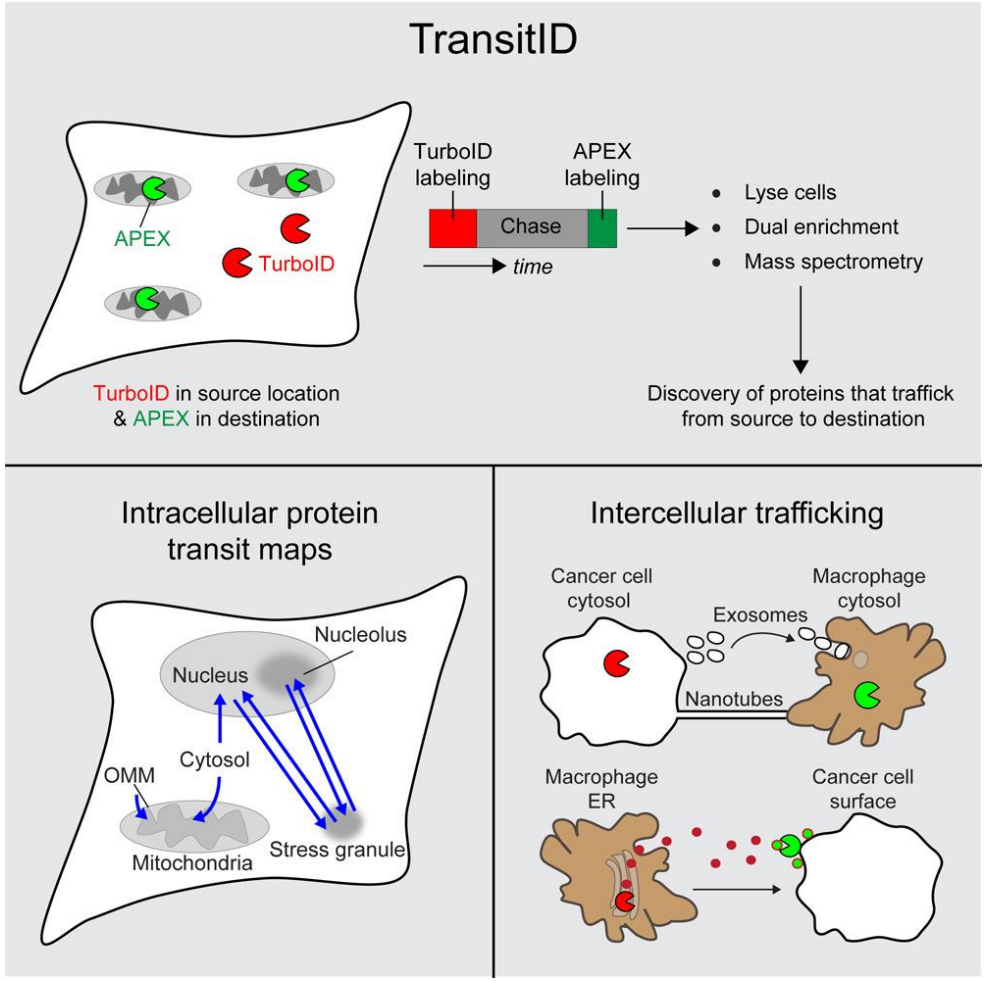
Protein translocation are closely related to cellular function
→ Chemical tools on analyze or control protein translocation is important.

# Today's Contents



## ① TransitID

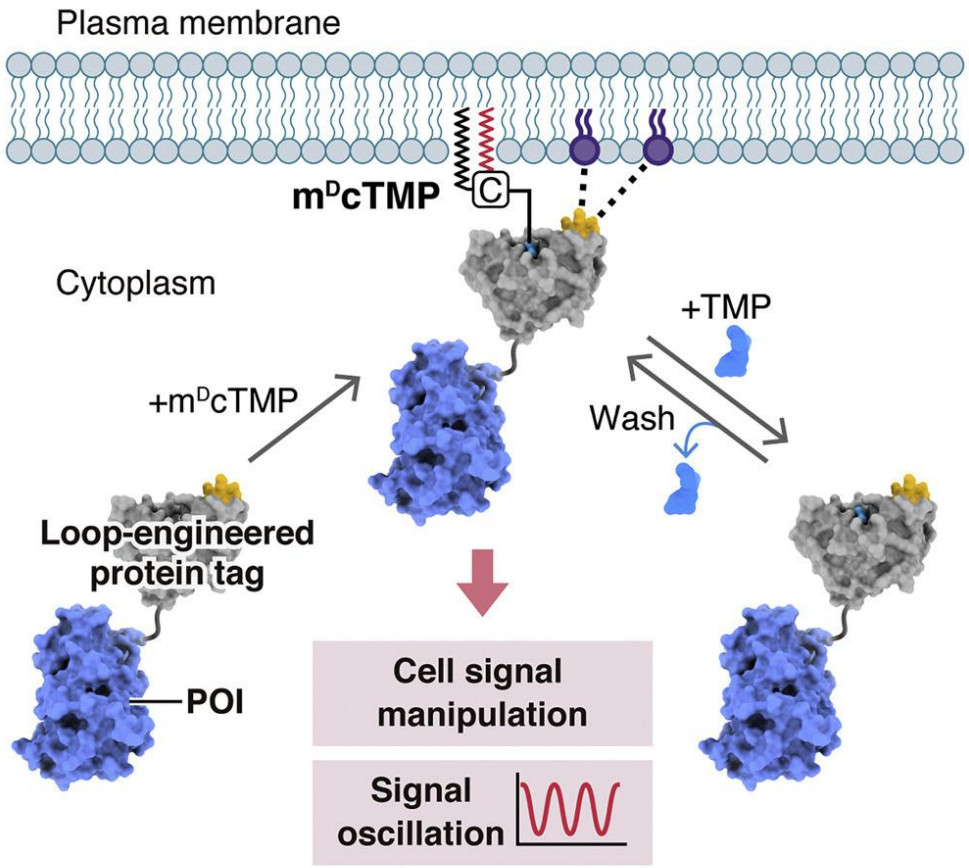
As Tools to analyze protein translocation




Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

## ② SLIPT

As Tools to control protein translocation




Tsukiji, S. *et al. Cell Chem. Biol.* **2022**, *29*, 1446.

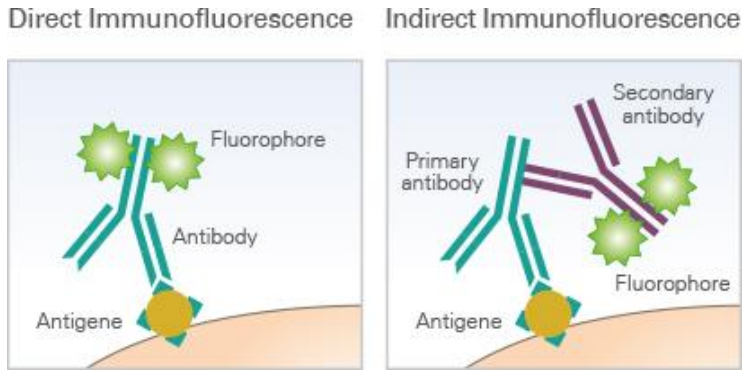
# Outline

# Conventional Method



## ① Immunofluorescence (IF)

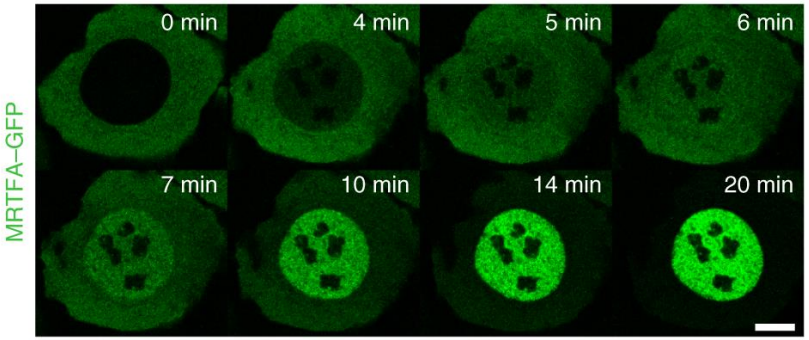
- ✓ No genetic manipulation
- ✗ Fixation of tissue
- ✗ Non-specificity and availability of some antibodies
- ✗ **Small number of proteins observed at once**




Taken from "ibidi" website
(https://ibidi.com/content/364-the-principle-of-immunofluorescence-assays)

## ② Genetically engineered fusions with fluorescent proteins

- ✓ Direct observation over time
- ✗ Genetic manipulation
- ✗ Possible effects of fused protein on target protein
- ✗ **Small number of proteins observed at once**




Garcia-Manyes, S. *et al. Nat. Phys.* **2019**, *15*, 973

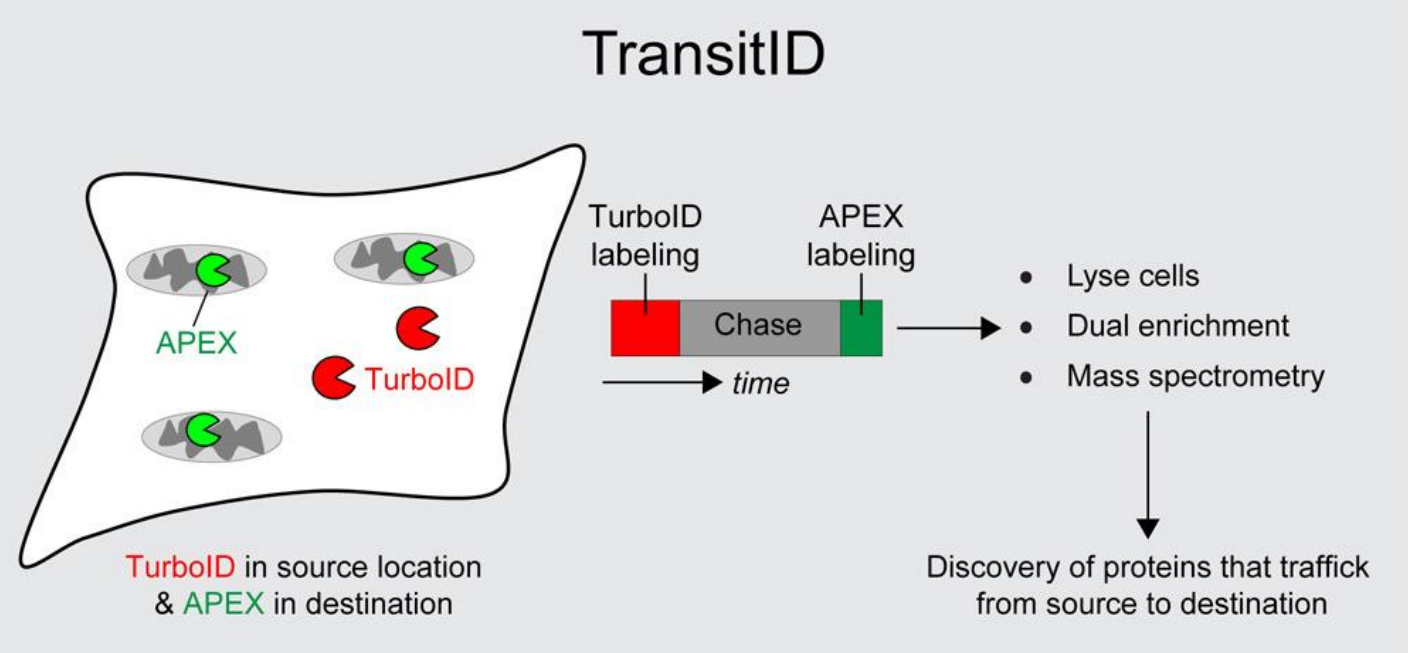
**Challenge: No method for analyzing protein tranlsocation without bias.**

# This Time's Method: "TransitID"



**TransitID (Trafficking Analysis by Sequential Incorporation of Tags for Identification)**

- Tool for unbiased analysis of protein translocation.
- Proximity Labeling (PL) with different methods (TurboID, APEX) at each of two points where translocation is to be observed.

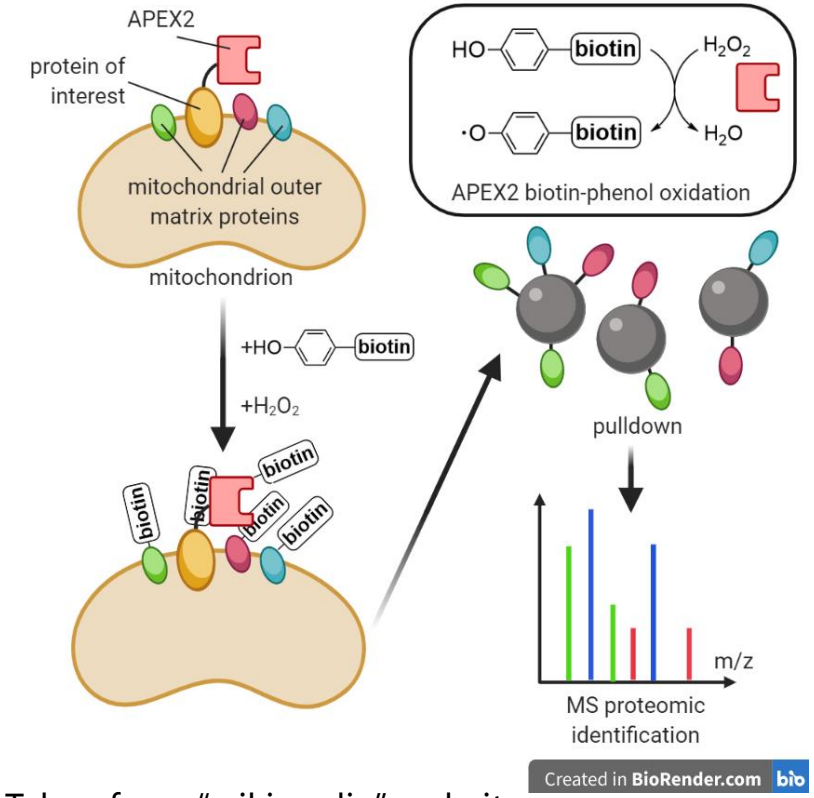



Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

## Proximity Labeling (PL)

Random labeling of proteins in proximity to the protein of interest (POI) for the purpose of protein-protein interaction analysis, etc.

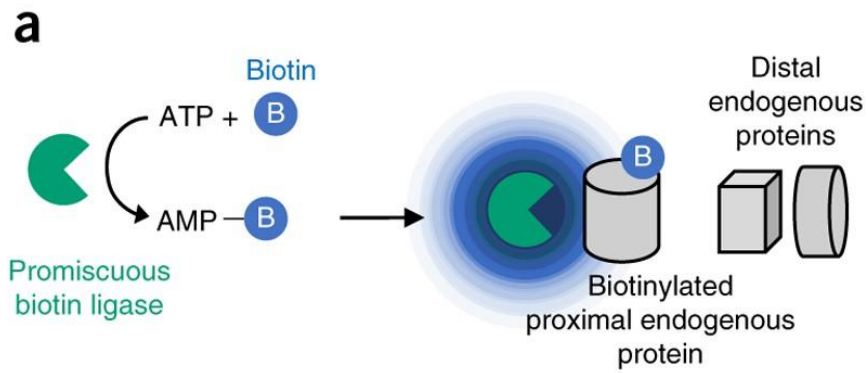
### APEX2



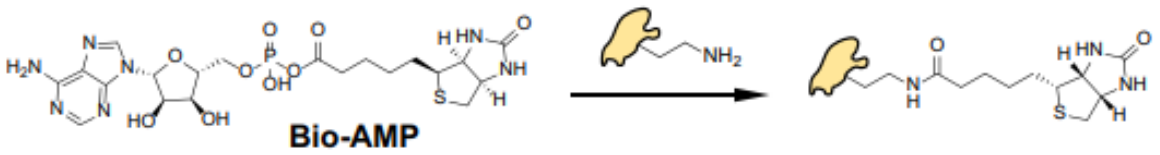

Taken from "wikipwdia" website

Kehlenbach, R. H. *et al. J. Biol. Chem.* **2019**, *294*, 16241

### TurboID




Ting, A. Y. *et al. Nat. Biotechnol.* **2018**, *36*, 880.




Zou, P. *et al. Curr. Opin. Chem. Biol.* **2021**, *60*, 30.

# Procedure of TransitID



## Basic Procedure

① Designate two points to be analyzed for translocation

② Express **TurboID** at the **start point** and **APEX2** at the **end point**

③ Add biotin before translocation for **biotin** labeling at the **start point**

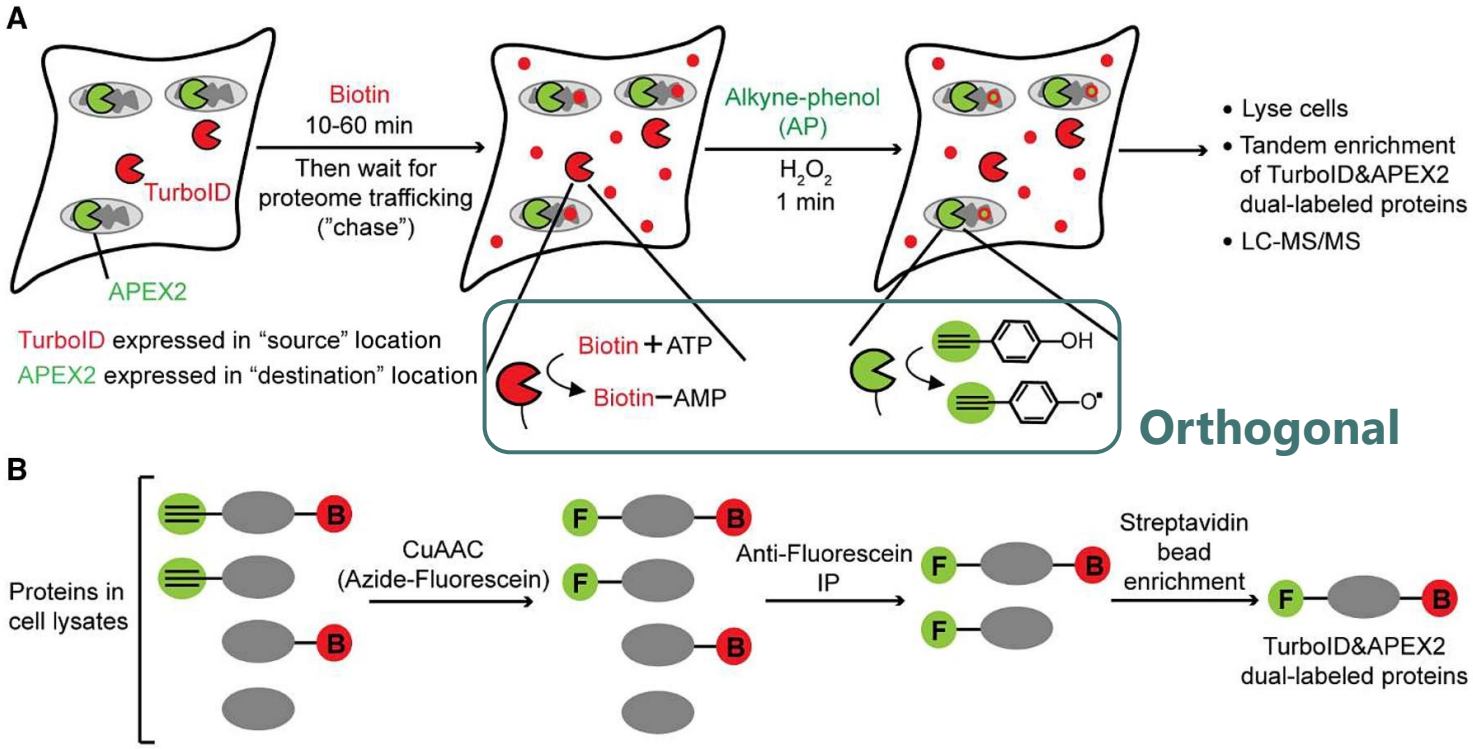
④ Wait for translocation

⑤ Add $H_2O_2$ and alkyne-conjugated phenol for **alkyne** labeling at **end point**

⑥ Perform click reaction to convert **alkyne** to **fluorescein** in the lysate

⑦ Proteins dual-labeled with **biotin** and **fluorescein** are enriched by sequential purification

⑧ Analyze with mass spectrometry




**Dual Labelled Proteins**

**||**

**Translocated Protein !**

Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

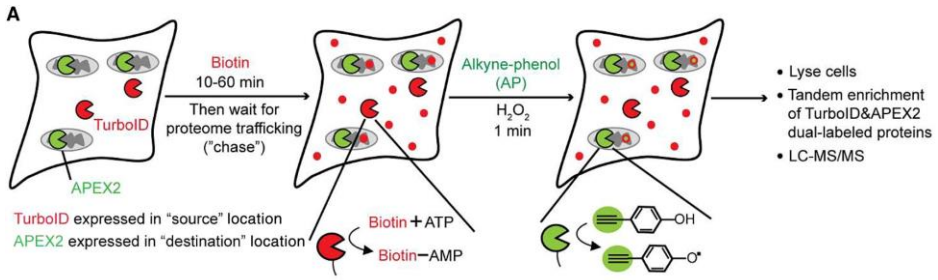
# Validation of TransitID

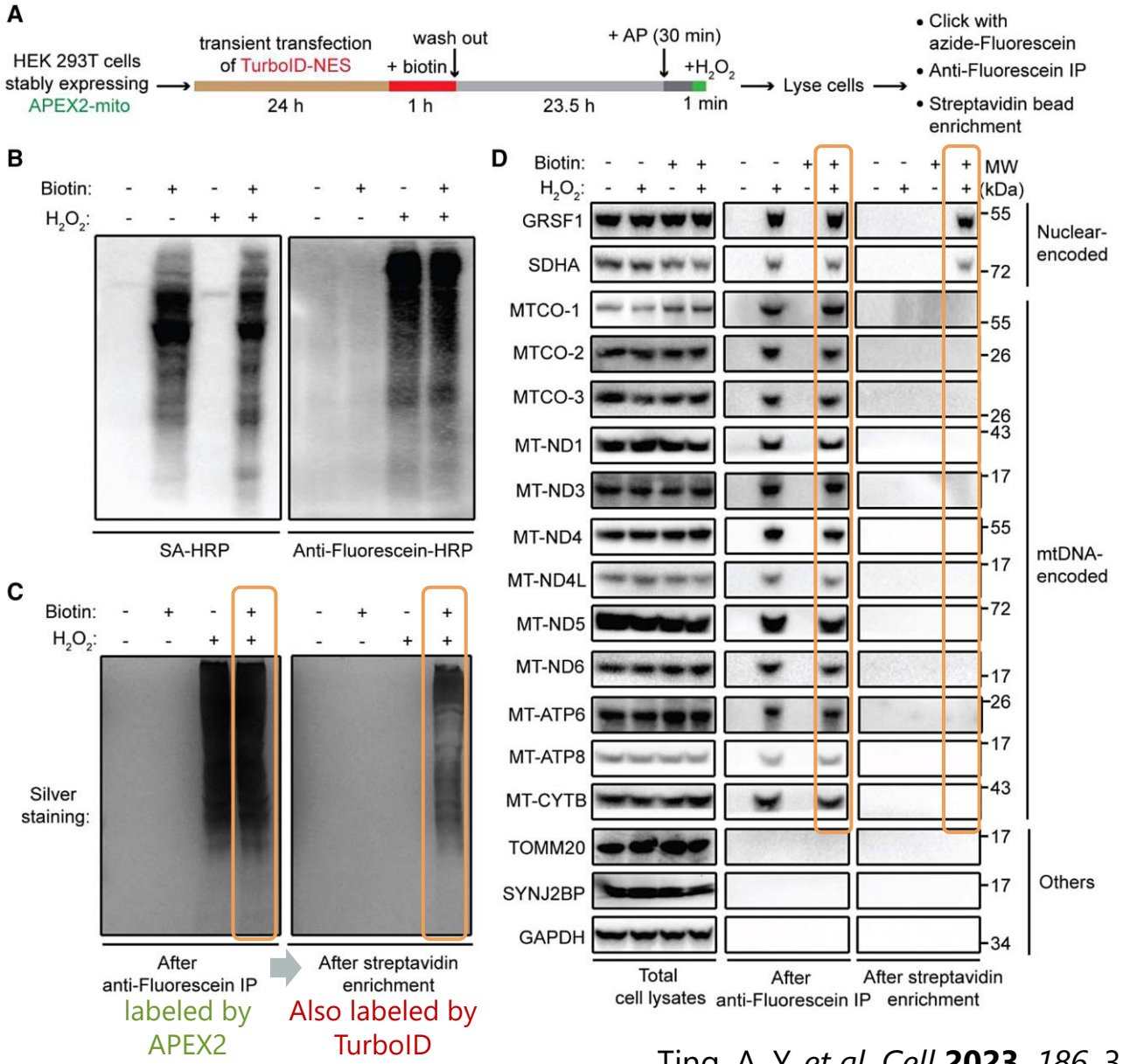


## Detection of protein translocation from cytoplasm to mitochondria

Start point: Turbo-ID NES @ cytoplasm
End point: APEX2-mito @ mitochondrial matrix

- Compared to proteins labeled by APEX2, proteins **dual labeled** by APEX2 and turboID were **less abundant** in SDS-PAGE (fig. C).
- Proteins that were **NOT visible** after the second purification were proteins derived from **mitochondrial DNA** (fig. D)
- Proteins that were **visible** after the second purification were proteins derived from **nuclear DNA** (fig. D)



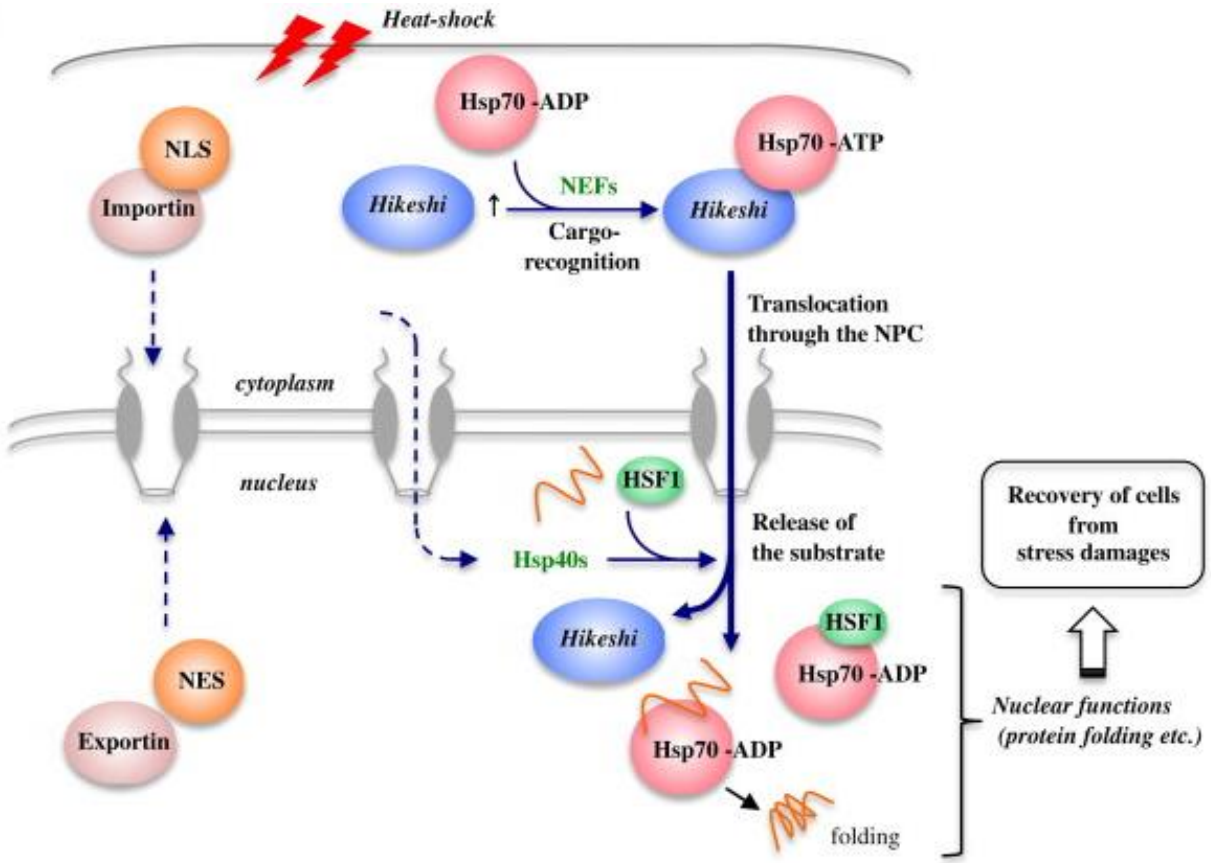




Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

# Application: Translocation under Stress

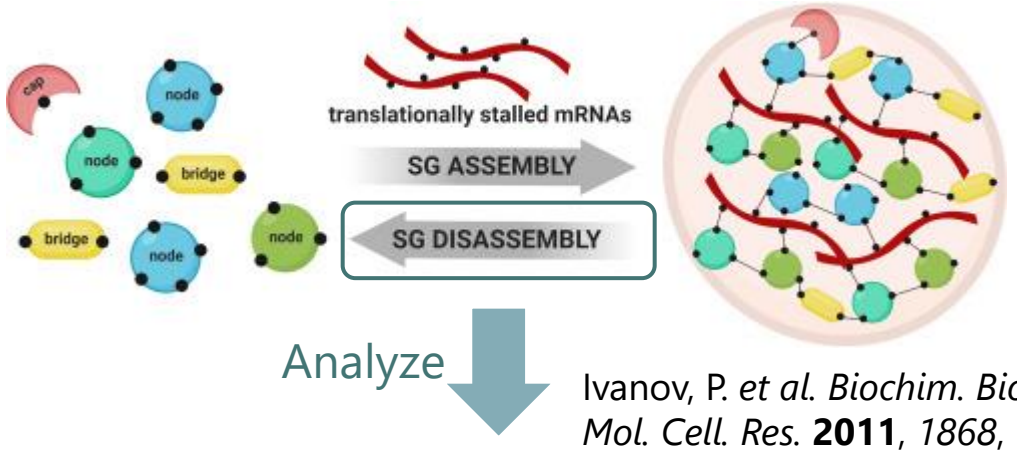
## Translocation under Stress

Case1: Hikeshi-Hsp70 translocation



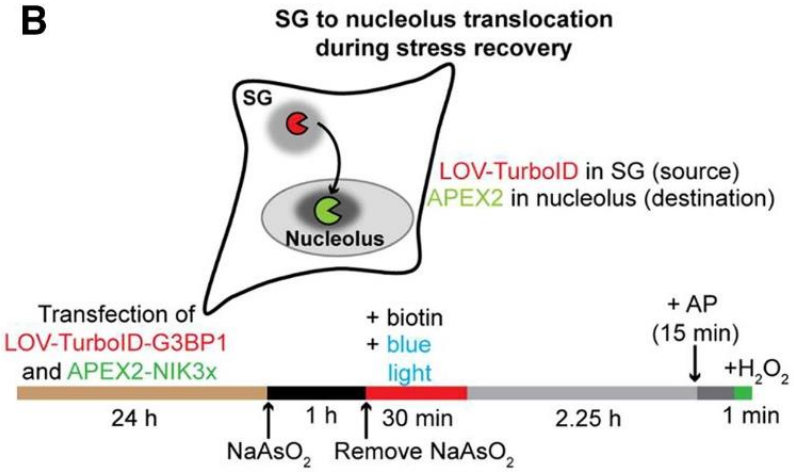

Imamoto, N. *et al. Cell* **2012**, *149*, 578.

Case2: **Stress Granule (SG)** formation




Analyze

Ivanov, P. *et al. Biochim. Biophys. Acta Mol. Cell. Res.* **2011**, *1868*, 118876.




Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

# Application: Translocation under Stress



## Detection of protein translocation from Nucleolus/Nucleus to SG

Start point: LOV-Turbo-G3BP1 @SG
End point: APEX2-NIK3x @Nucleolus
or APEX2-NLS @Nucleus

(Comparison of **blue** and **red** samples)

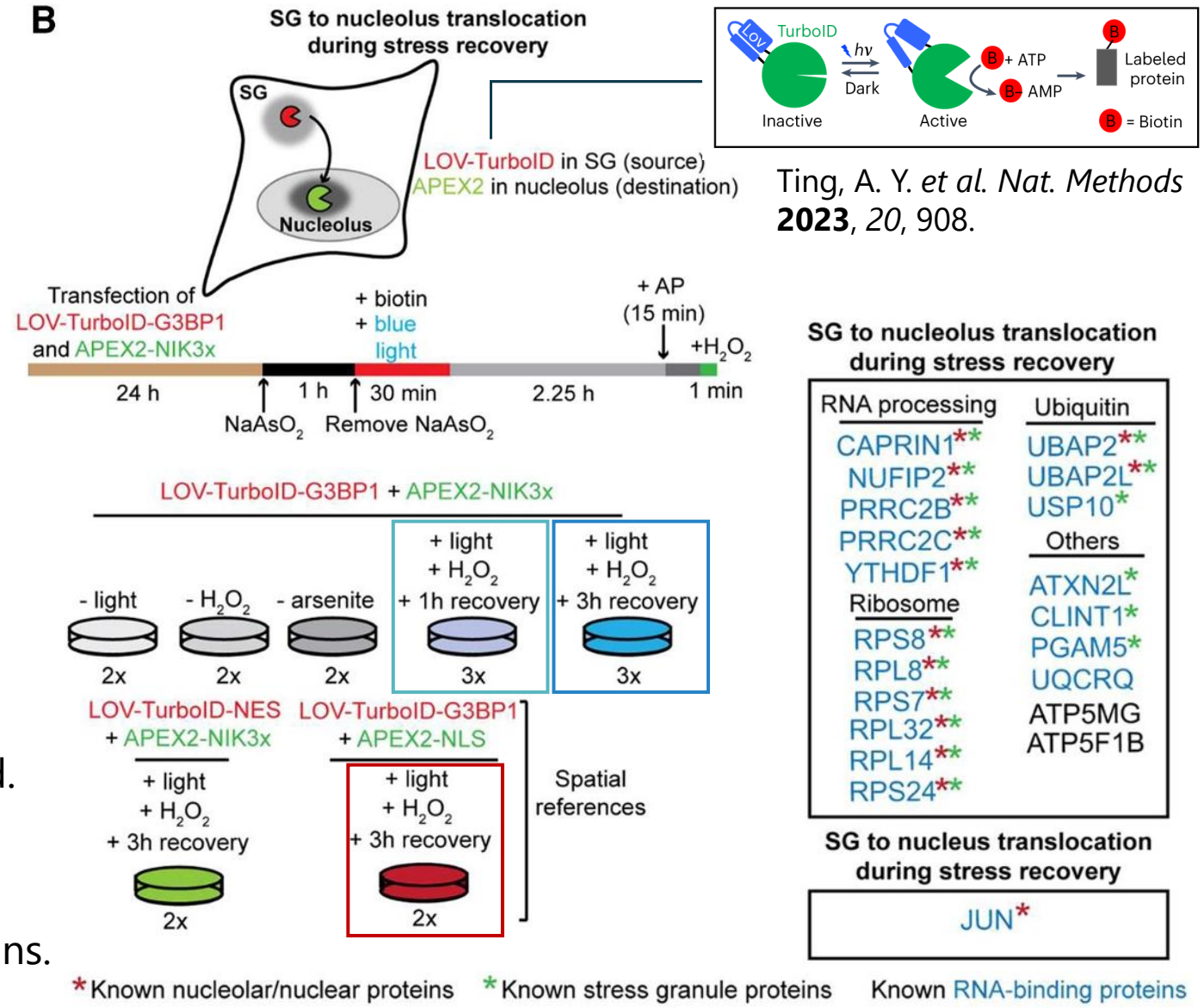
- **20 proteins** were identified that translocate from SG to **nucleolus**
- **1 protein (JUN)** was identified that translocate from SG to **nuclear** (other than the nucleolus).

  → Many of known SG proteins were identified, supporting **the reliability of the results**.

  → **Unreported SG proteins** were also identified.

(Comparison of **blue** and **light-blue** samples)

- Time scale of translocation differs among proteins.



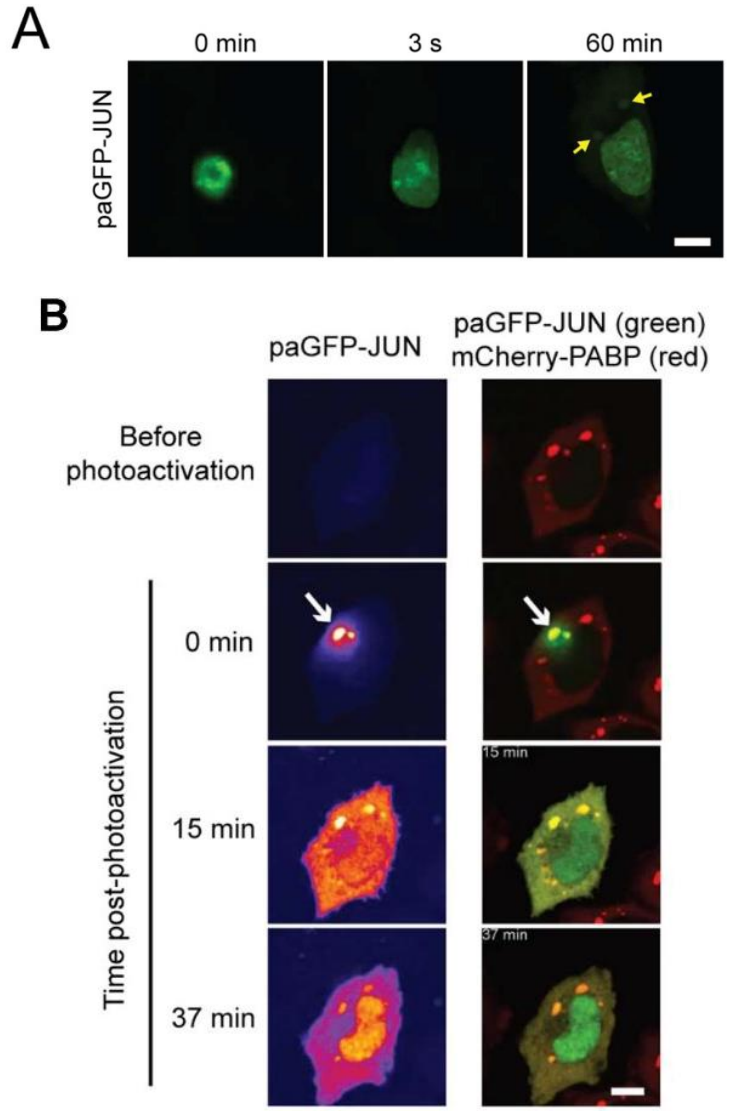

Ting, A. Y. *et al. Nat. Methods* **2023**, *20*, 908.

Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

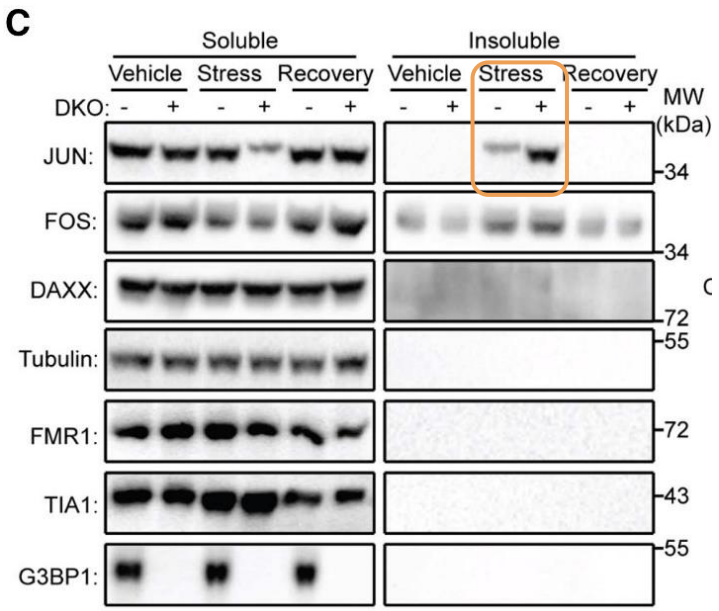
# Validation: Is JUN truly unreported SG proteins ?

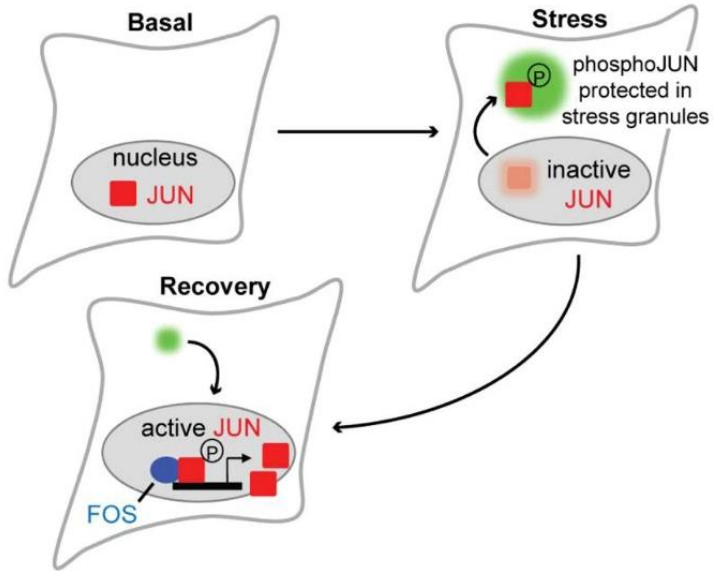


## Validation of whether JUN is SP protein




## The role of JUN under stress



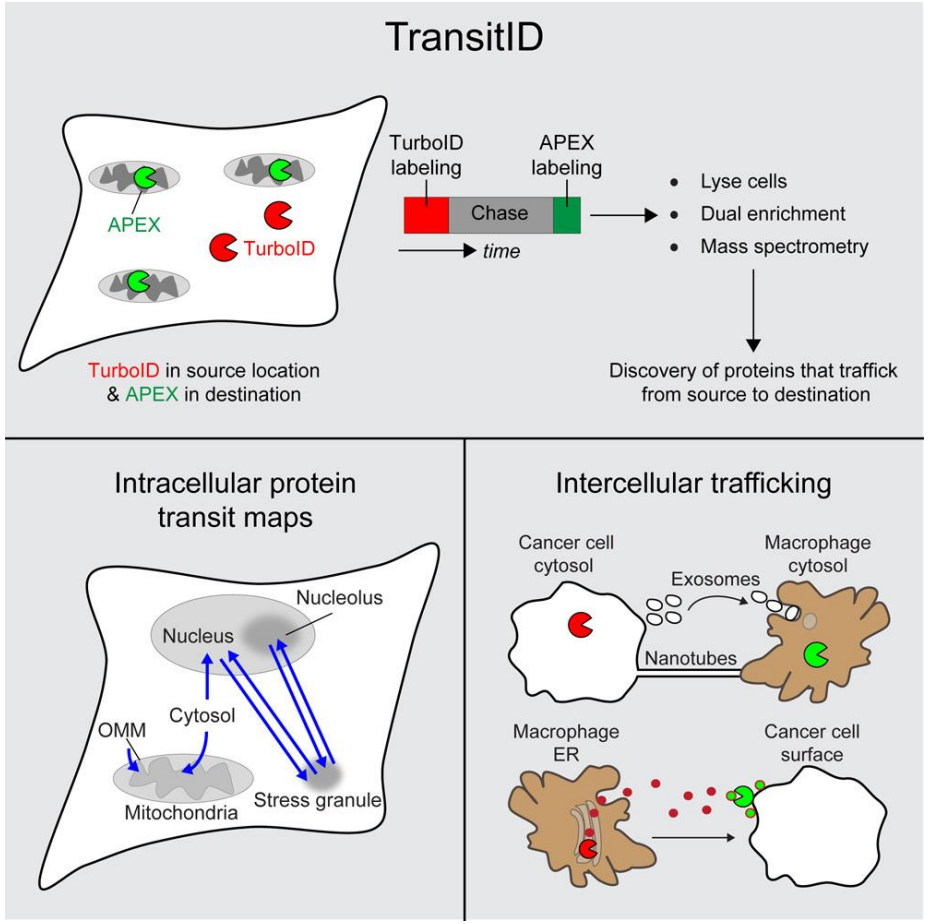

- The observation of fusions with photoactivatable GFP (paGFP) indicates that **JUN is an SG protein**
- JUN translocation to SG during stress **suppress its aggregation**.
- It enables JUN to **quickly re-localizes** to the nucleus and **get active** after stress.

Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

# Summary and Outlook



- Ting *et al.* have developed a general method for **unbiased analysis of proteins translocation** in an arbitrary spatio-temporal setting: "**TransitID**."
- This was accomplished using an **orthogonal proximity labeling** with modified APEX2 and TurboID.
- This method was successful in identifying a variety of proteins, including JUN, whose localization has not been known before.
- This method can also be used to analyze protein translocation between **cells** (omitted this time).
- This method inevitably inherits the issues of parental methods.
  Turbo ID : **low resolution** due to long labeling time (10-60 min.)
  APEX2 : **toxicity of $H_2O_2$**
- Development of improved proximity-labeling enzymes also improve this method.




Ting, A. Y. *et al. Cell* **2023**, *186*, 3307.

# Outline



1. Introduction
2. **Latest Finding**
    1. Tools to analyze protein translocation
    2. **Tools to control protein translocation**

# Conventional Method: CID

## Artificial control of the protein translocation

Useful as a means of elucidating the function of the protein

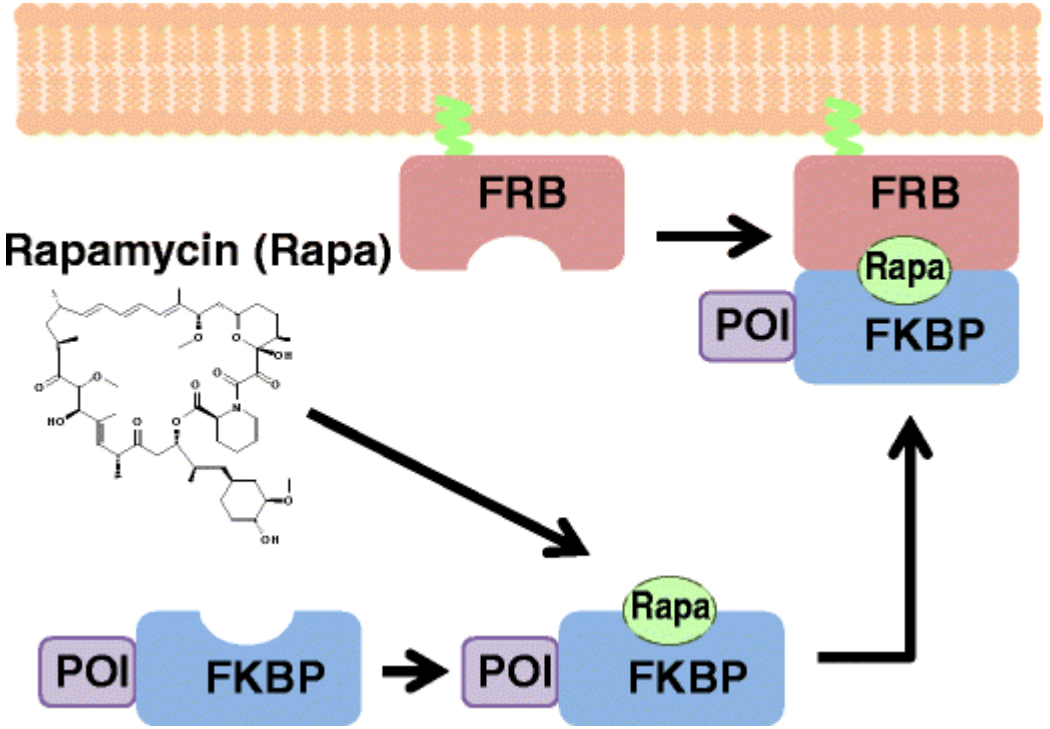
## Chemically-Inducible Dimerization (CID)

A method of dimerizing two proteins,
with an organic molecule (**dimerizer**)

*1st generation;*

**FKBP12/FRB dimerization with Rapamycin**

- ✓ Versatility
- ✗ Binding to endogenous FKBP12 and mTOR
- ✗ Almost irreversible binding ($K_d$=200 pM)

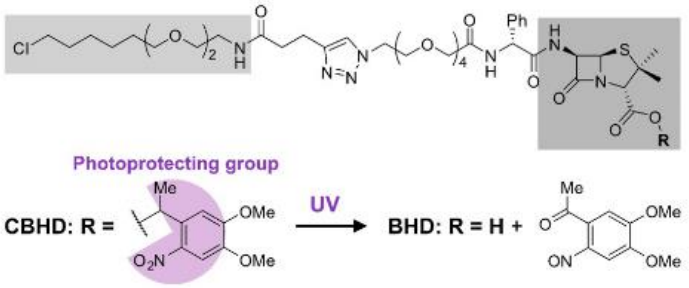


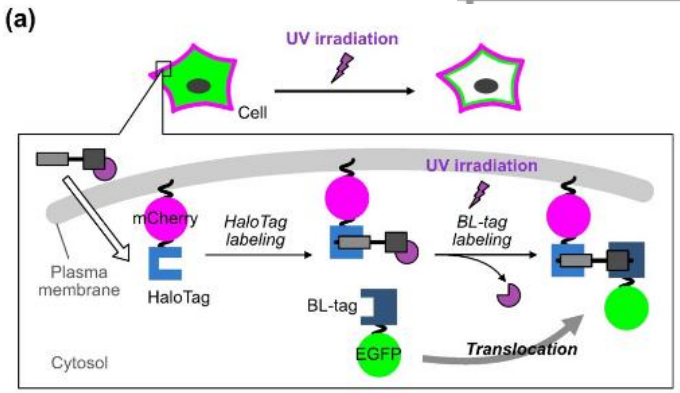

Inoue, T. *et al. Pflugers. Arch.* **2013**, *465*, 409.

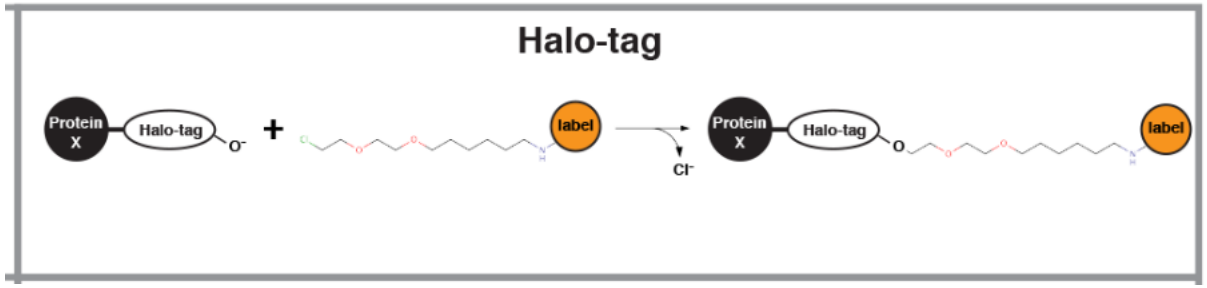
# Conventional Method: Improved CID

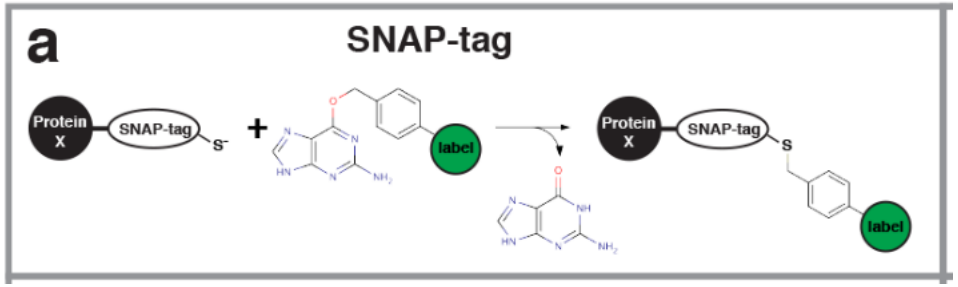
## Challenge 1: Bio-orthogonality

- **F36V mutant of FKBP12** and **SLF'** ligands for it
- Use of different types of tags such as **Halo, SNAP, BL tags**, etc.



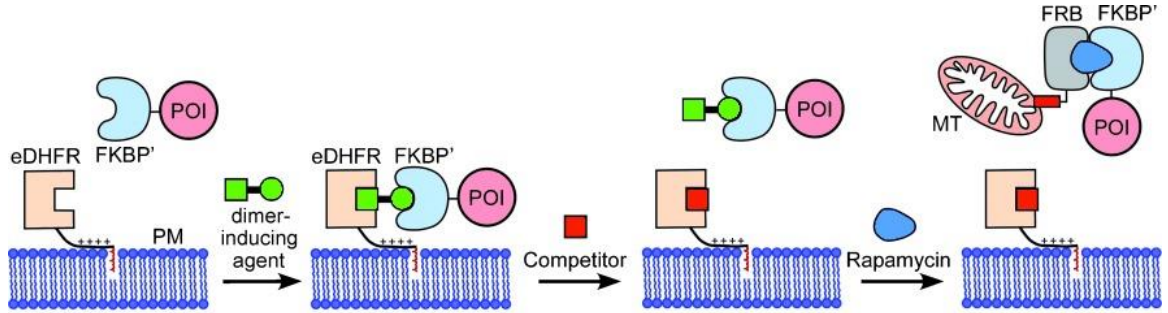



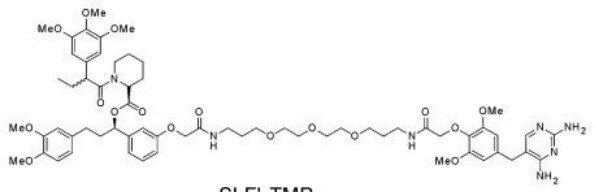

taken form "Bloomington Drosophila Stock Center" website
Mizukami, S. *et al. Angew. Chem. Int. Ed.* **2021**, *60*, 11378.

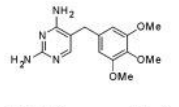
## Challenge 2: Reversibility

Combination of **TMP** and **eDHFR**

- Competitively dissociates dimerizers by adding an excess of free TMP
- Small concern for bio-orthogonality
  (for eDHFR; $K_I$=1nM vs. for mDHFR; $K_I$=4~8μM)




Wu, Y. *et al. Angew. Chem. Int. Ed.* **2014**, *53*, 10049.

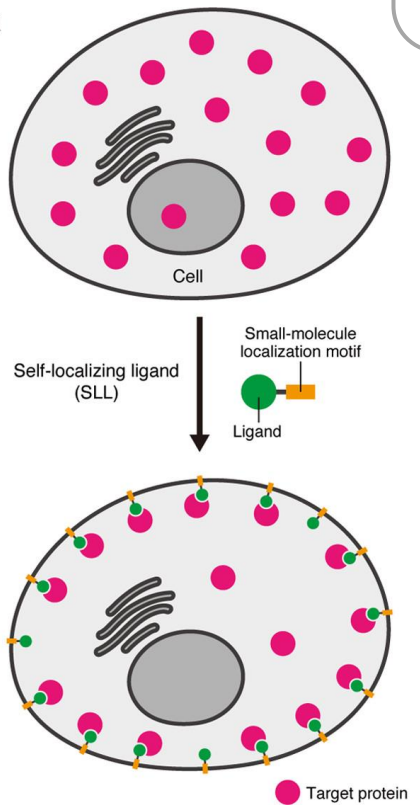
# This Time's Method: "SLIPT"

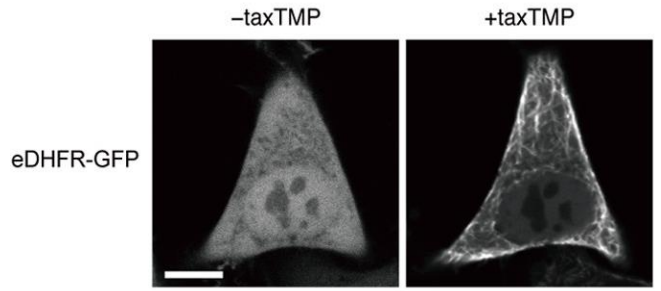
## Intrinsic problems with CID

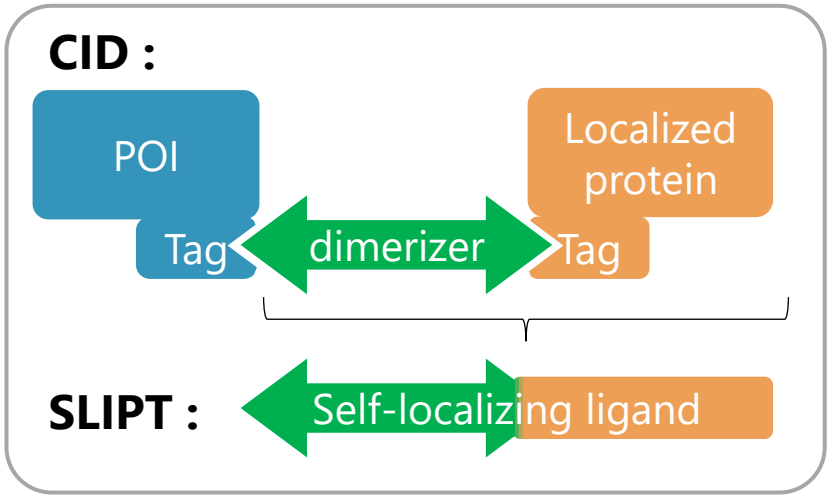
✗ Strict concentration control of dimerizers is required.
✗ Genetic manipulation is performed on two or more proteins.

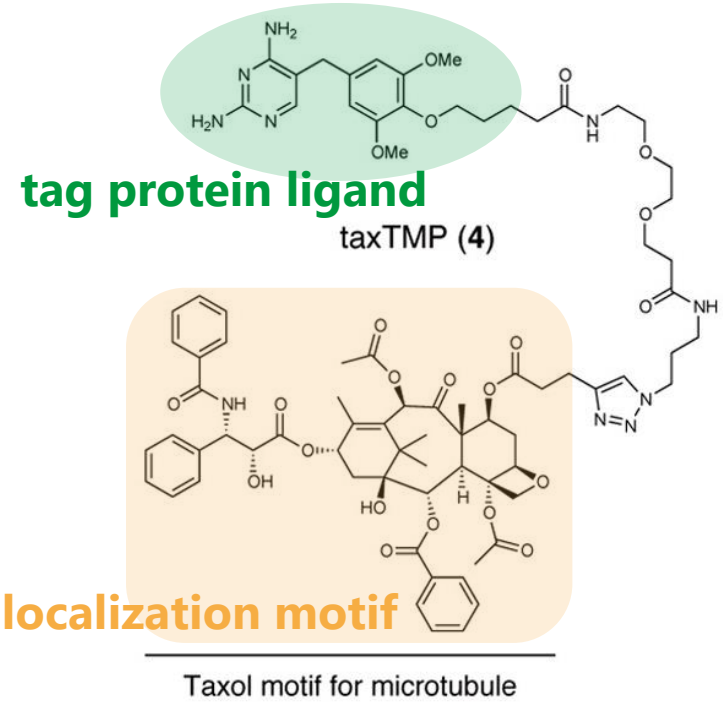
## SLIPT (Self-localizing Ligand-Induced Protein Translocation)

- Localization is controlled by 1-to-1 binding of the **protein of interest (POI)** to the **self-localizing ligand**
- **Self-localizing ligands (SL)**
  = **tag protein ligand** + **localization motif**
  e.g.) TMP for eDHFR  e.g.) Hoechst for nucleus, Taxol for microtubule



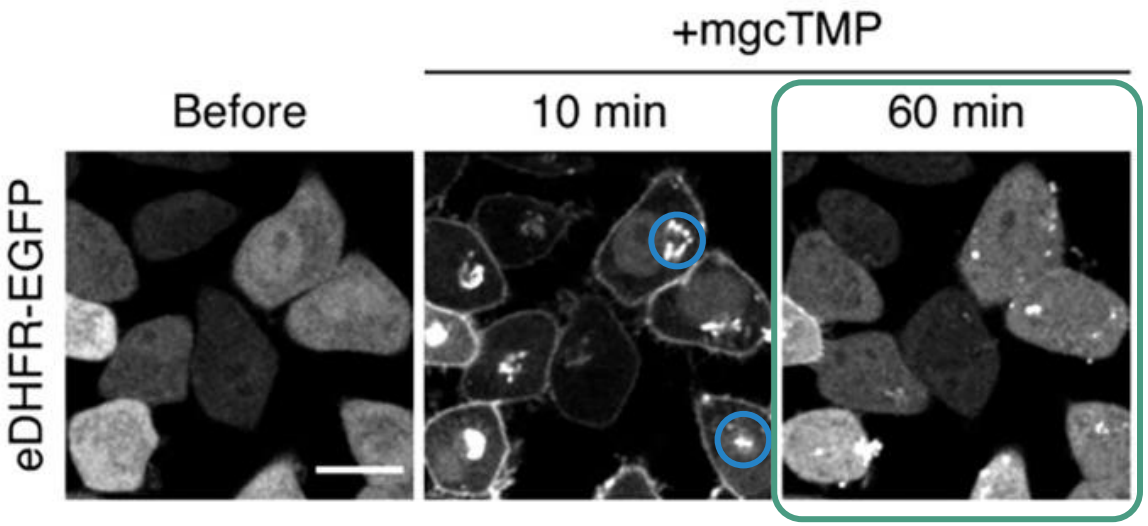



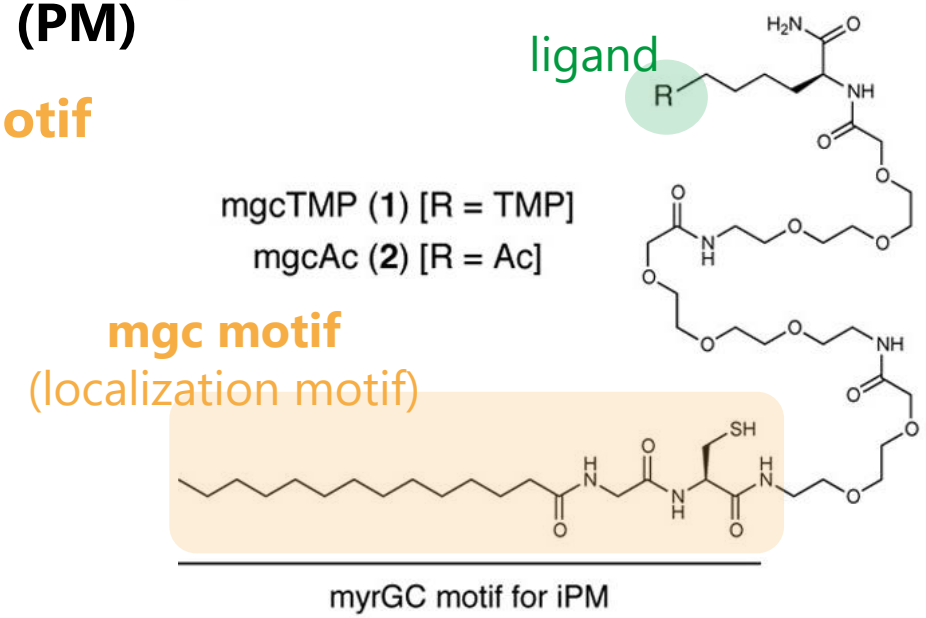




Tsukiji, S. *et al. J. Am. Chem. Soc.* **2013**, *135*, 12684.

## SLIPT-PM

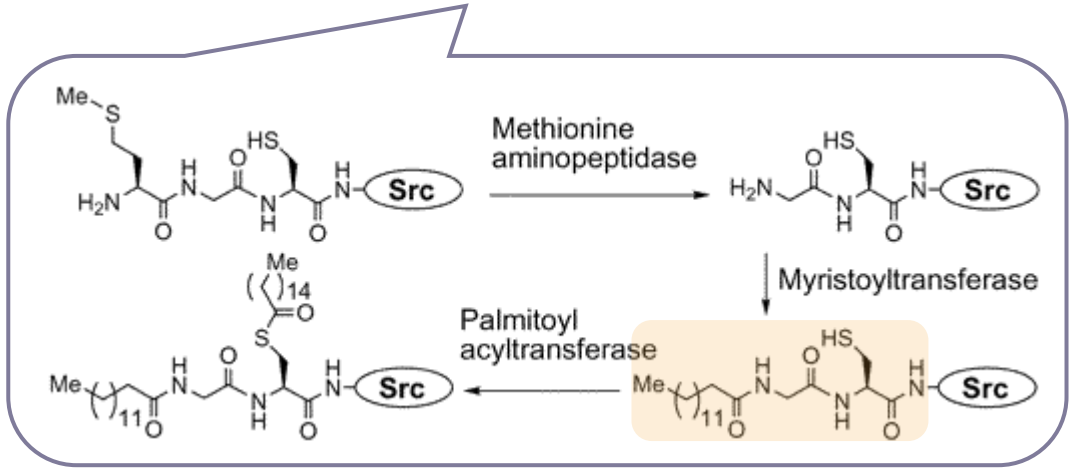
- SLIPT system to translocate POI to the **plasma membrane (PM)**
- translocation to the PM is achieved **palmitoylated mgc motif**




**Problem 1) Transient localization**

**Problem 2) Low selectivity of localization**




Tsukiji, S. *et al. J. Am. Chem. Soc.* **2013**, *135*, 12684.
Tsukiji, S. *et al. ACS Chem. Biol.* **2020**, *15*, 837.

Peterson, B. R. *et al. J. Am. Chem. Soc.* **2002**, *124*, 2444.

# Improved Selectivity in SLIPT-PM (Solution to Problem 1)



**Problem:**

**Transient** localization to the PM (plasma membrane)

**Cause:**

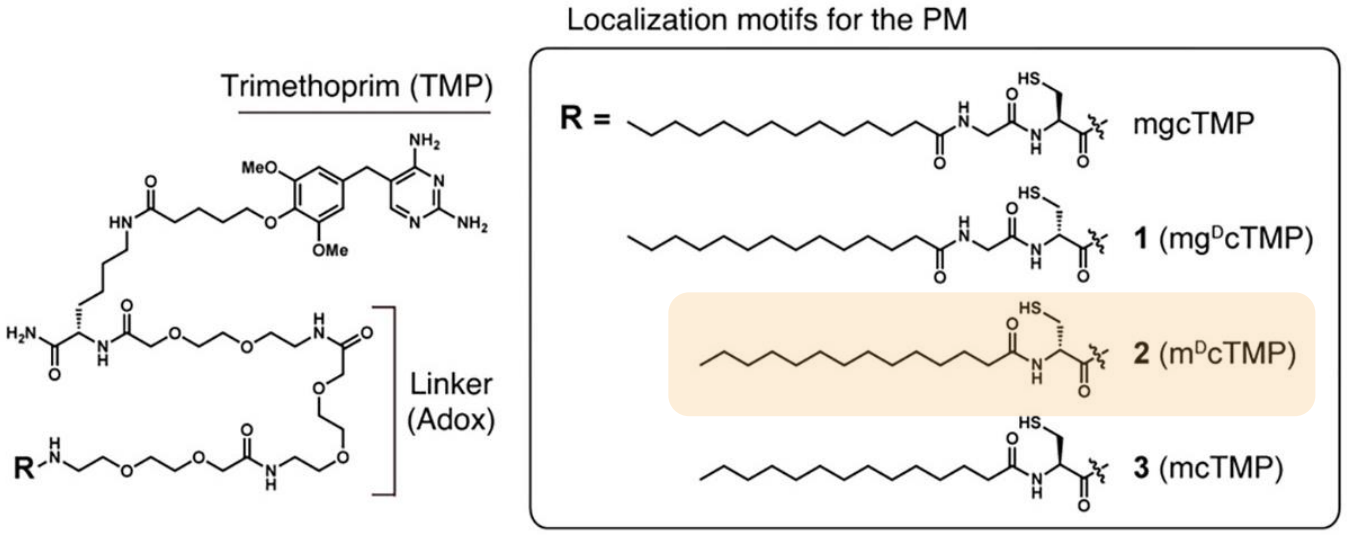
**Cleavage of amide bond** between Cys in mgc motif and linker in cell

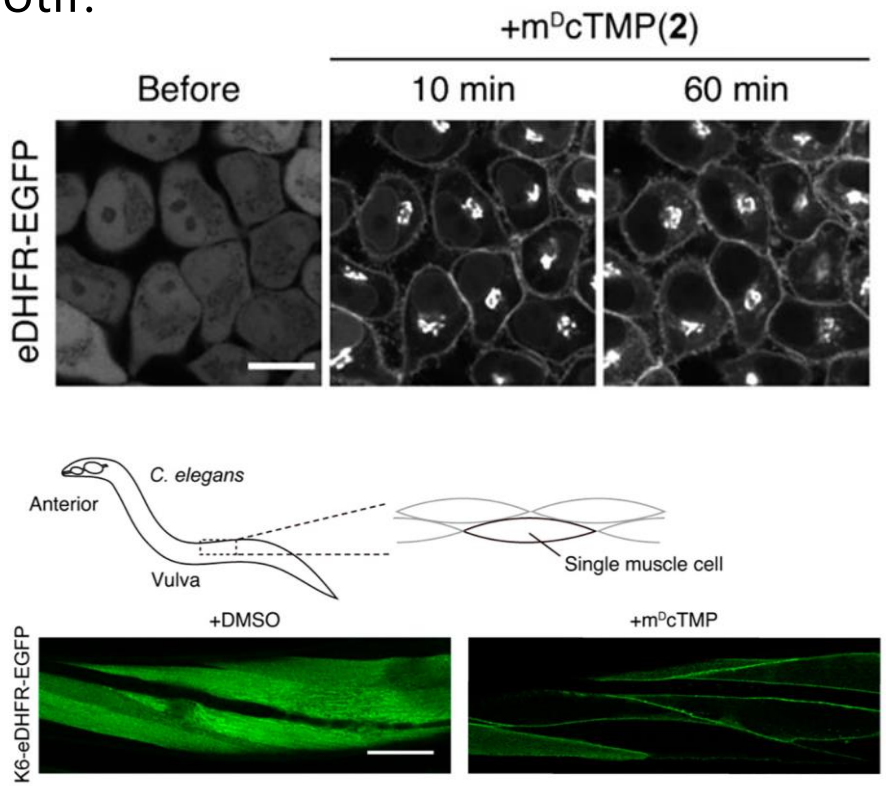
**Solution:**

Modification of the motif to a more degradation-resistant motif:

- Conversion of L-Cys to **D-Cys**
- Removal of **Gly**

} **m$^{D}$c motif** is the best




Tsukiji, S. *et al. ACS Chem. Biol.* **2020**, *15*, 837.

# Improved Persistence in SLIPT-PM (Solution to Problem 2)



**Problem:**

Localized to the **Golgi surface** as well as the PM (plasma membrane)

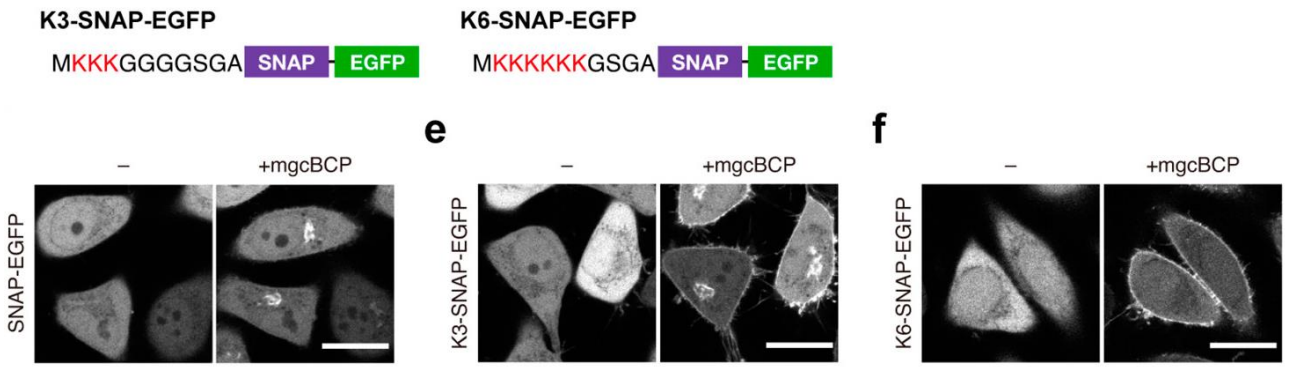
**Cause:**

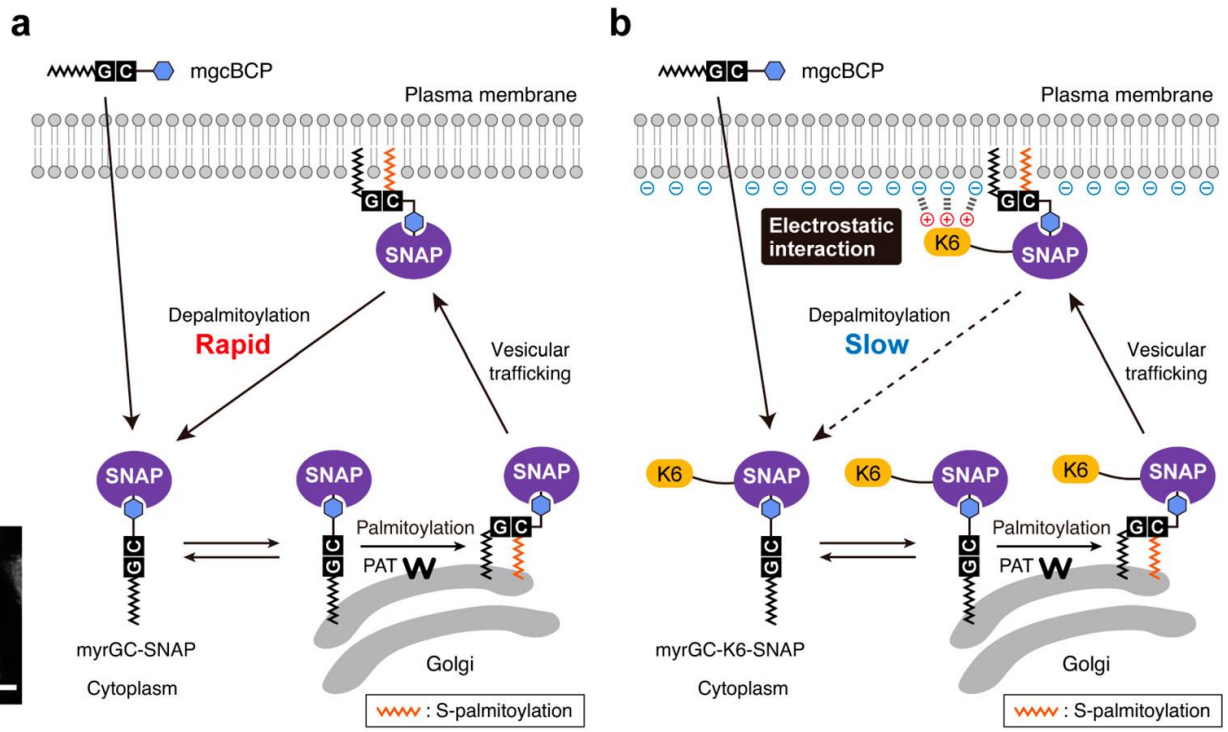
**Rapid depalmitoylation reaction**

**Solution:**

**Reduces the rate of dissociation** from the PM

→ Add a domain to the tag protein
that enhances its interaction with the PM
= Cationic **Lys** residues; **K6** is the best




Tsukiji, S. *et al. ACS Chem. Biol.* **2020**, *15*, 1004.

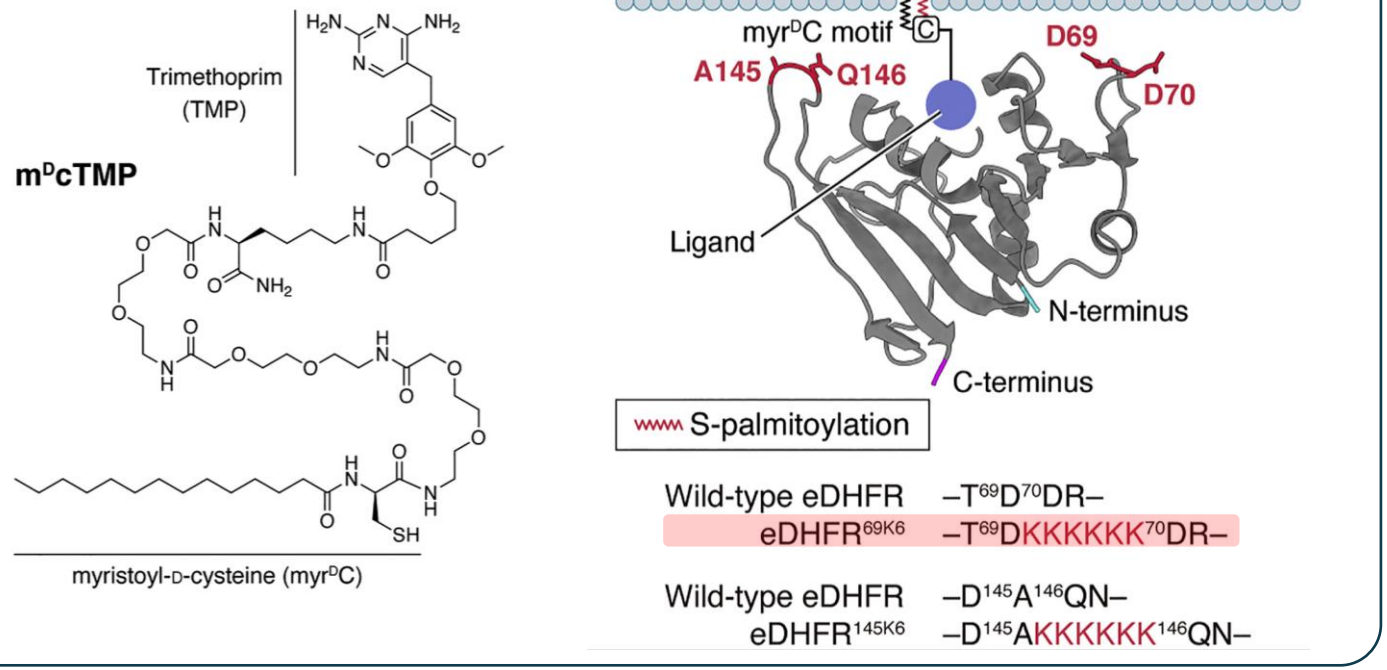
# Latest Version of SLIPT-PM

**Self-localizing ligands:** TMP with **m$^{D}$c motif**

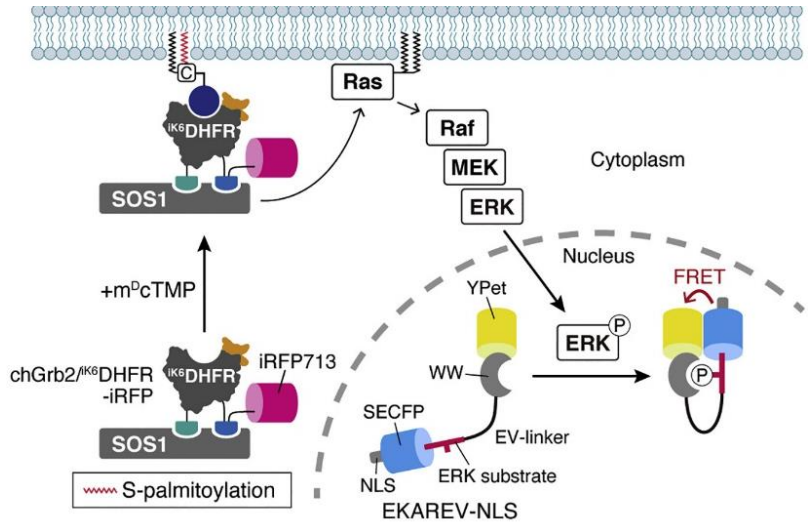
**Tag fused to POI:** eDHFR with K6 *grafted* between D69 and D70; **$^{iK6}$DHFR**

**more versatile tag** that can fuse proteins to the **N- as well as the C-terminus** of eDHFR
(Previous version cannot fuse protein at the C-terminus.)

## Optimized SLIPT-PM




*Application*




Tsukiji, S. *et al. Cell Chem. Biol.* **2022**, *29*, 1446.

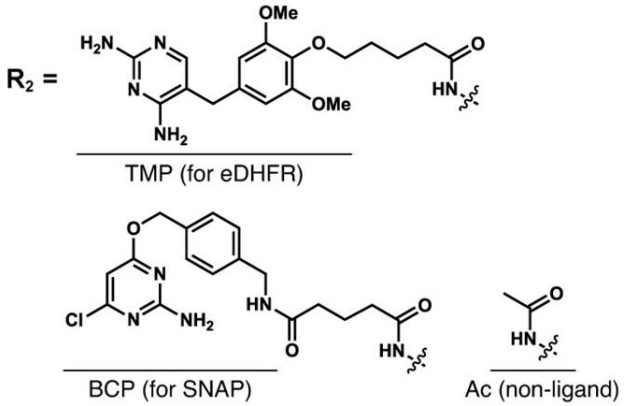
# Application: Multiplex Control of Signal Transduction

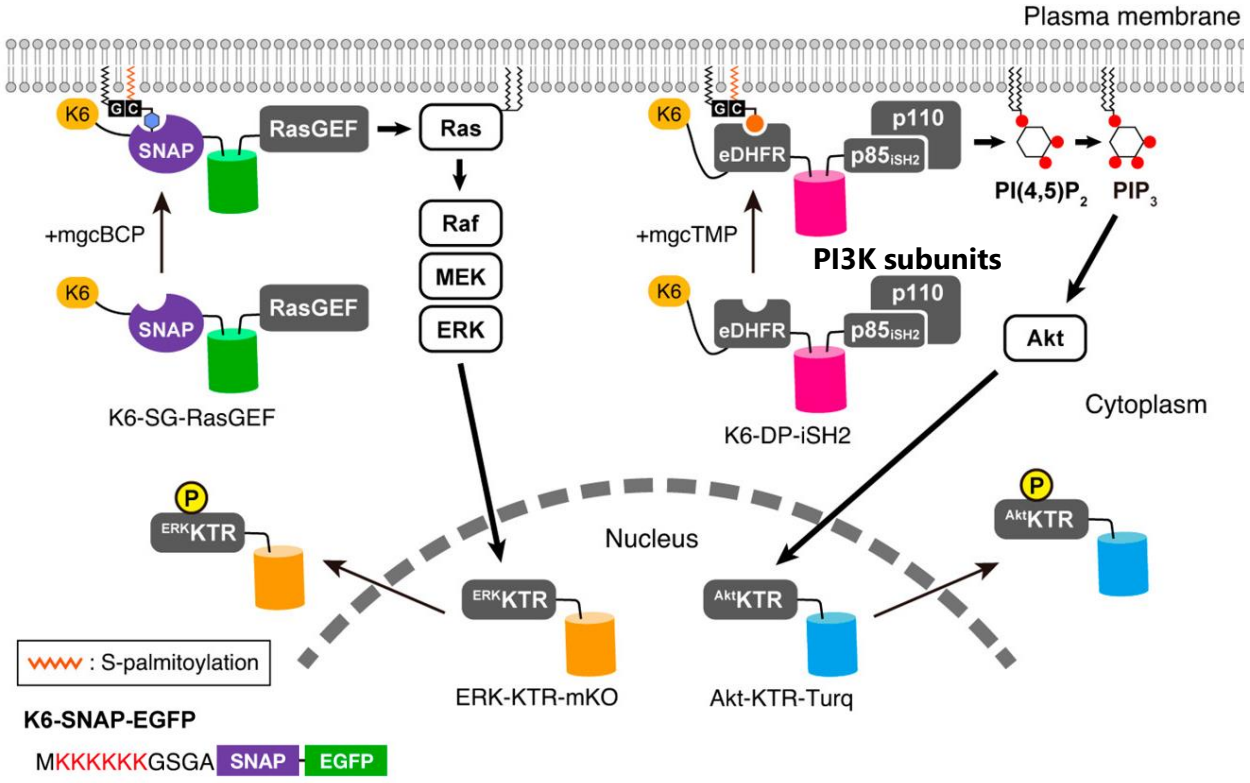


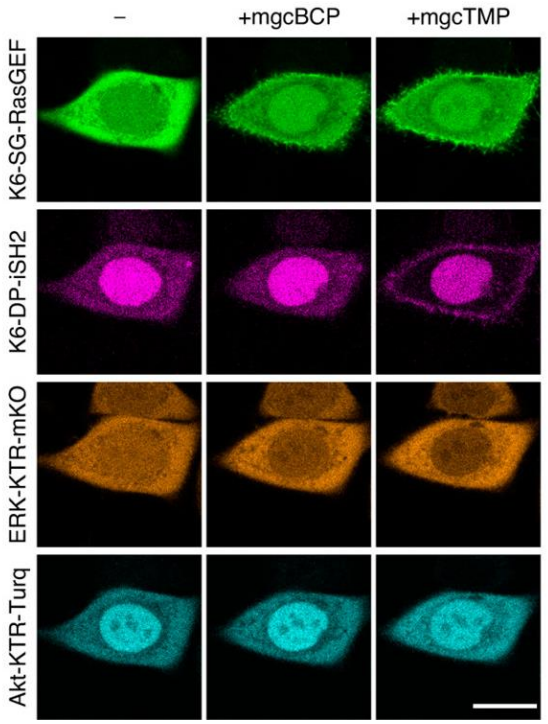
## Observation of the interaction of two signaling pathways

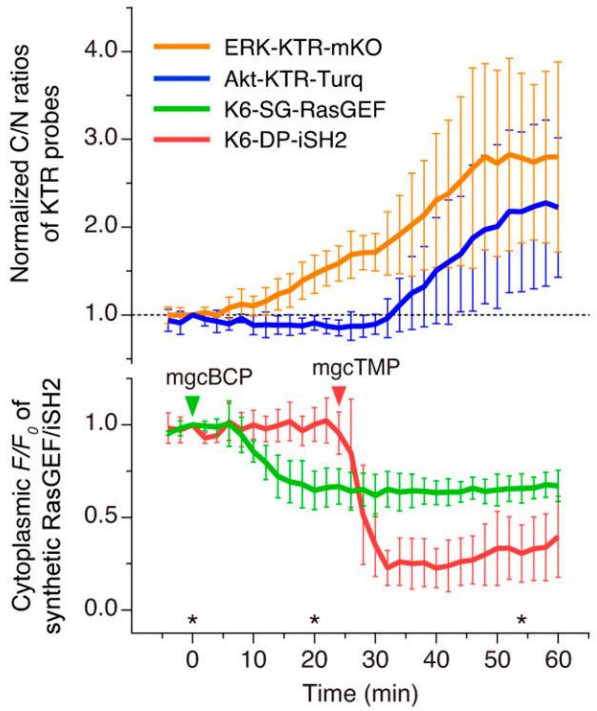
① **Add mgcBCP** → Activation of Ras/Erk pathway
(*No* activation of PI3K/Akt pathway)

② **Add mgcTMP** → Activation of PI3K/Akt pathway
→ **Activation of Ras/Erk pathway !** (positive feedback)




Tsukiji, S. *et al. ACS Chem. Biol.* **2020**, *15*, 1004.

# Application: Induction of Signal Oscillation



## Procedure for Signal Oscillation

- *Reversible* binding of TMP-eDHFR
- *Strong* binding of mgc motif - PM

① **Add m$^{D}$cTMP**

→ Raf translocation to PM leading to ERK activation (Nucleus translocation)

② **Add free TMP**

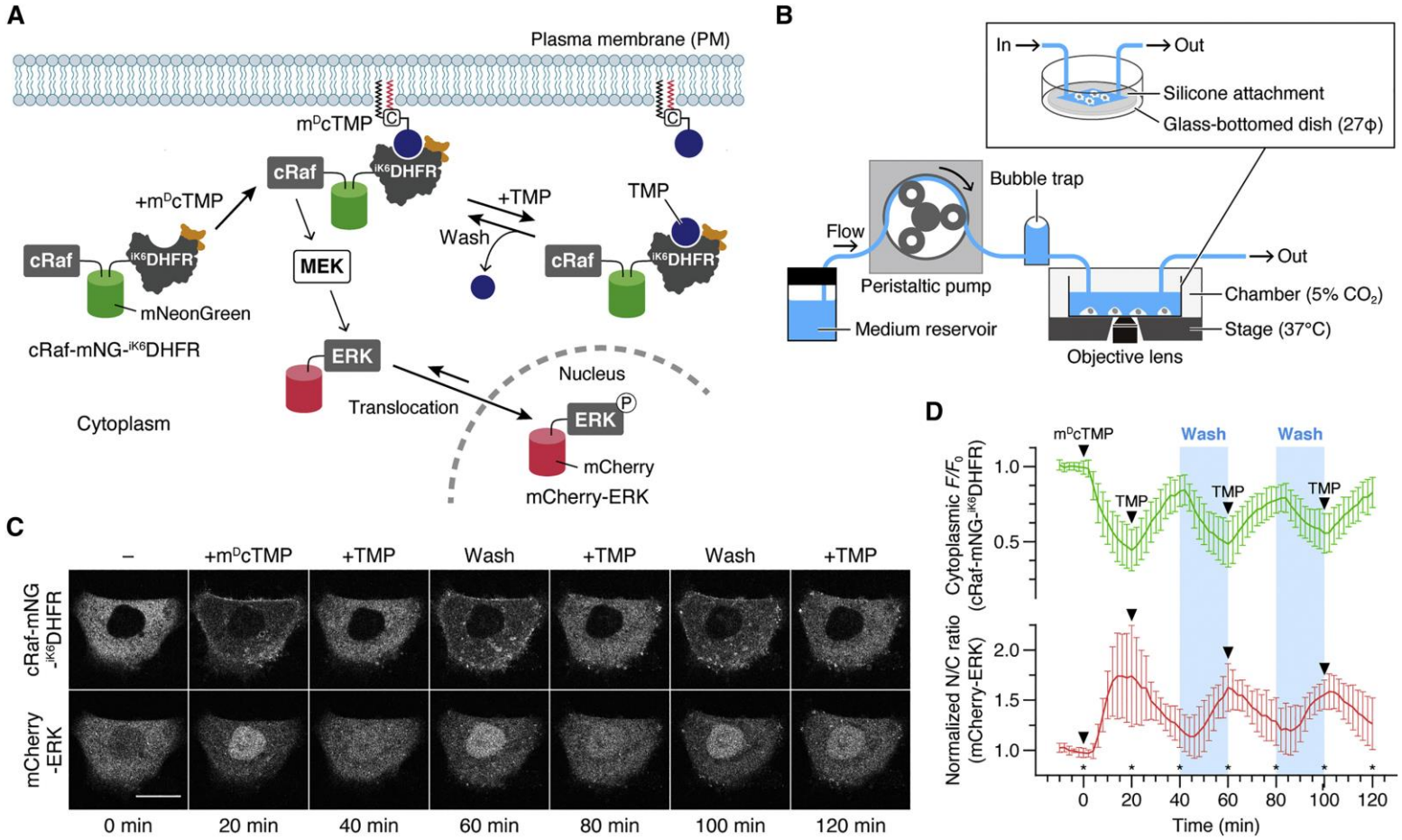
→ Raf dissociation from PM leading to ERK deactivation (Cytoplasm translocation)

③ **Wash (Remove free TMP)**

→ Raf re-translocation to PM leading to ERK activation (Nucleus translocation)

**Repetition of ② and ③ induce signal oscillation**

(≈ repetitive activation)

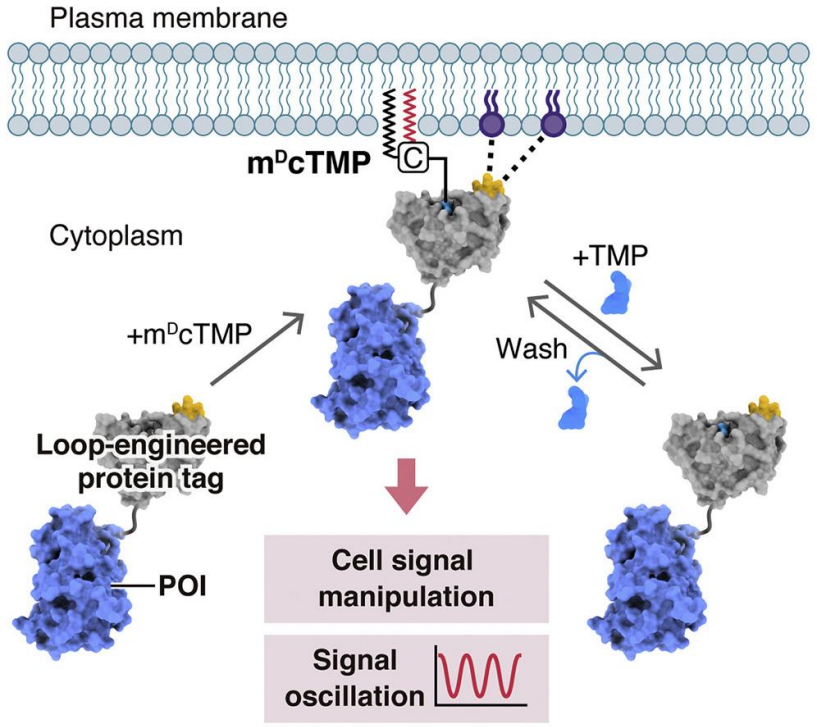



Tsukiji, S. *et al. Cell Chem. Biol.* **2022**, *29*, 1446.

# Summary and Outlook

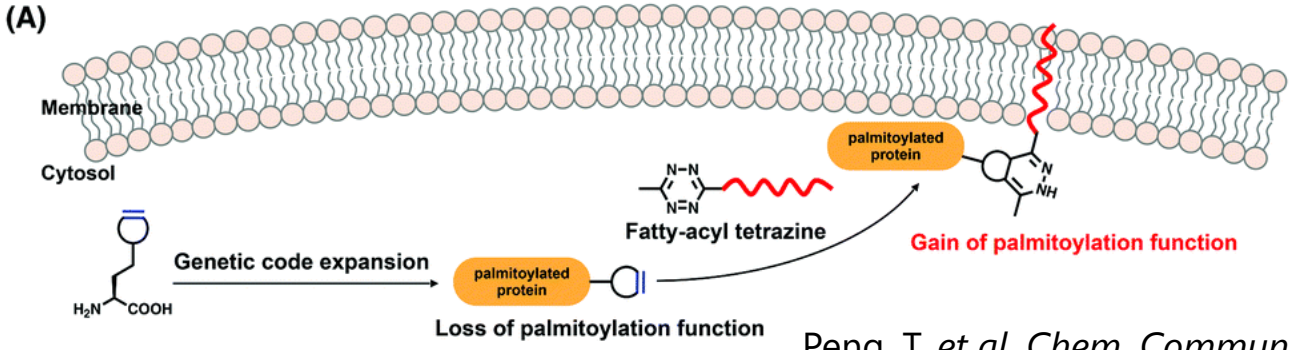


- Tsukiji *et al.* have developed a useful tool to **control proteins translocation** (especially to PM)**:** "**SLIPT**(-PM)."
- Latest tags; **$^{iK6}$DHFR are universal** and can be inserted in a variety of positions, including inside proteins
- SLIPT was successful in **multiplex** or **repetitive** activation of signaling pathways.
- SLIPT systems are **slower** than CID systems ($t_{1/2}$ in SLIPT; 2.6~6.1min. vs. $t_{1/2}$ in CID; 20~50 sec.)
- **localization motif is limited** to a few types.
  → Vigorous search for localization motifs will expand the versatility.

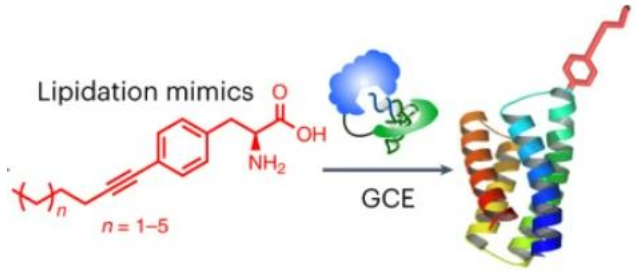



Tsukiji, S. *et al. Cell Chem. Biol.* **2022**, *29*, 1446.

cf.) Other methods of protein localization to the PM; **GCE**




Peng, T. *et al. Chem. Commun.* **2020**, *56*, 13880.

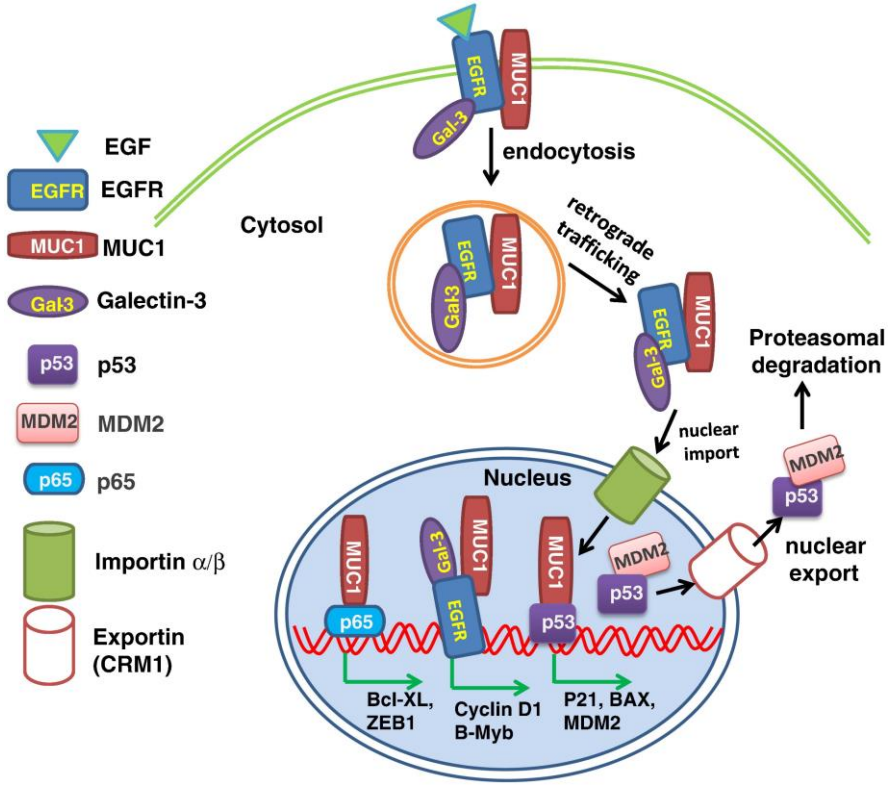



Lin, S. *et al. Nat. Chem. Biol.* **2023**, https://doi.org/10.1038/s41589-023-01400-8.

# Translocation in Disease

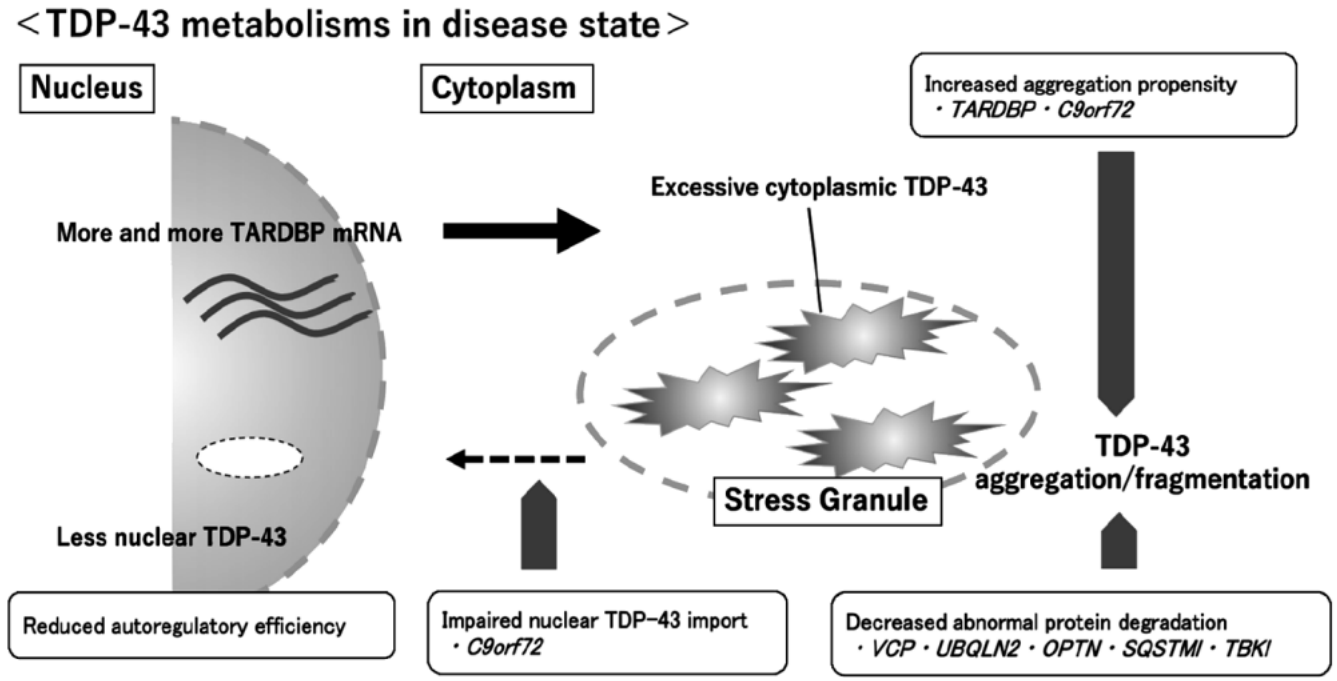
## Disruption of protein translocation leads to several disease.

### Case 1 ) Cancer




Li, S. *et al. Biochim. Biophys. Acta Rev. Cancer* **2014**, *1846*, 13.

### Case 2 ) ALS




Onodera, O. *et al.* 臨床神経, **2020**, *60*, 109.

**Tools to normalize protein mislocalization can be therapeutic.**

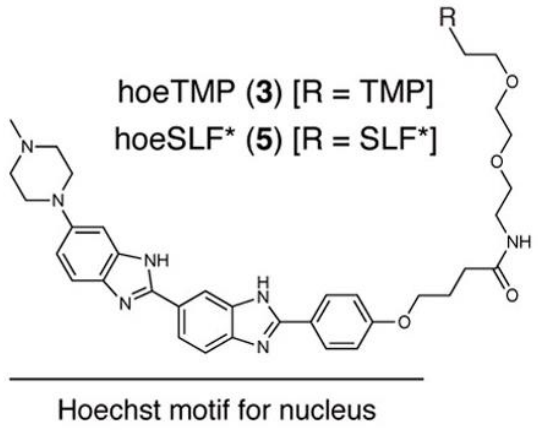
# Application to Therapy

## Related Works

Translocation of *endogenous* proteins

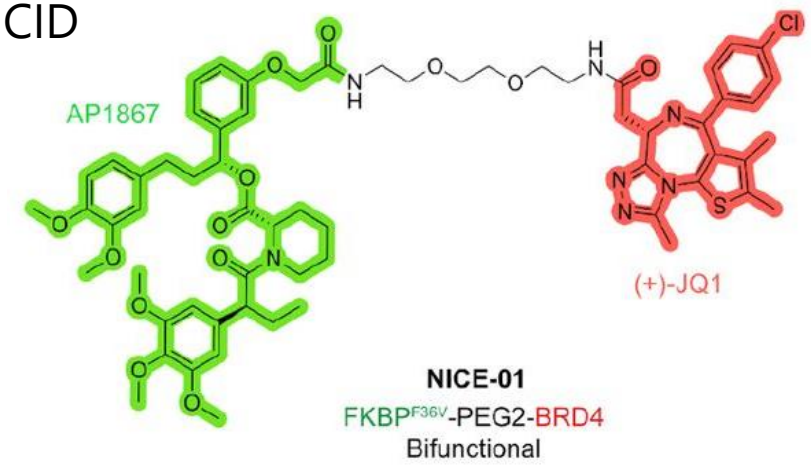
→ However, regulated protein is specific ones used as tags.

Case 1) SLIPT




Tsukiji, S. *et al. J. Am. Chem. Soc.* **2013**, *135*, 12684.

Case 2) CID




Schreiber, S. L. *et al. bioRxiv*, **2023**.
https://doi.org/10.1101/2023.07.07.548101.

## Challenge

Need small $K_d$ ↓ ↓ inhibitor cannot be used

Difficulty in developing ligands that can **translocate POI without impairing its function**

→ Covalently introduce localization motifs at sites with minimal effect on POI (with LDC) ?

*Thank you for your kind attention.*