Using split protein reassembly strategy to optically control PLD enzymatic activity

Yuanfa Yao[1, #], Xiayan Lou[1, #], Jianxu, Li[2, #], Min Zhu[3], Xiaohan Qian[4], Jingfang Liu[3], Luhao Zhang[3], Peng Zhang[2], Lian He[5, *], Hanbing Li[1, *] , Yingke Xu[3, 6 *]

1 Institute of Pharmacology, College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou, 310014, China

2 National Key Laboratory of Plant Molecular Genetics, Center for Excellence in Molecular Plant Sciences, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China

3 Department of Biomedical Engineering, Key Laboratory of Biomedical Engineering of Ministry of Education, State Key Laboratory of Extreme Photonics and Instrumentation, Zhejiang Provincial Key Laboratory of Cardio-Cerebral Vascular Detection Technology and Medicinal Effectiveness Appraisal, Zhejiang University, Hangzhou, 310027, China

4 State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Centre for Infectious Diseases, Collaborative Innovation Centre for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China.

5 Department of Pharmacology, School of Medicine, Southern University of Science and Technology, Shenzhen, 518055, China.

6 Department of Endocrinology, Children's Hospital of Zhejiang University School of Medicine, National Clinical Research Center for Children's Health, Hangzhou, Zhejiang 310051, China

# These authors contributed equally: Yuanfa Yao, Xiayan Lou, Jianxu Li;

*Correspondence should be addressed to **Prof. Yingke Xu**, **Drs. Hanbing Li**, and **Lian He**

Address for Prof. Yingke Xu (lead): Department of Biomedical Engineering, 38 Zheda Road, Yuquan Campus, Zhejiang University, Hangzhou, 310027, China

Email: yingkexu@zju.edu.cn ; Tel & Fax: +86-0571-87951091

Address for Dr. Hanbing Li: Institute of Pharmacology, College of Pharmaceutical Science, 18 Chaowang Road, Zhejiang University of Technology, Hangzhou, 310014, China

Email: hanbing.li@163.com; Tel & Fax: +86-13185064285

Address for Dr. Lian He: Department of Pharmacology, School of Medicine, 1088 Xueyuan Avenue, Southern University of Science and Technology, Shenzhen, 518055, China.

Email: hel3@sustech.edu.cn; Tel & Fax:+86-0755-880100

Abstract

Phospholipase D (PLD) and phosphatidic acid (PA) play a spatio-temporal role in regulating diverse cellular activities. Although current methodologies enable optical control of the subcellular localization of PLD and by which influence local PLD enzyme activity, the overexpression of PLD elevates the basal PLD enzyme activity and further leads to increased PA levels in cells. In this study, we employed a split protein reassembly strategy and optogenetic techniques to modify superPLD (a PLD_{PMF} variant with a high basal activity). We splited this variants into two HKD domains and fused these domains with optogenetic elements and by which we achieved light-mediated dimerization of the two HKD proteins and then restored the PLD enzymatic activity.

Keywords: Phospholipase D; Phosphatidic acid; Optogentics;

Phospholipase D (PLD) and phosphatidic acid (PA) have been shown to play crucial roles in diverse cellular physiological and pathological processes (Yao et al., 2020). However, the spatiotemporal regulatory mechanisms of both remain largely unknown in cells. To address this, there is a critical need for a highly efficient platform capable of activating PLD and detecting PA. The enzymatic activity of cellular PLD and the levels of intracellular PA are notably low under non-stimulated conditions. PLDs have been demonstrated to be activated by indirect agonists, including PMA, EGF, and co-expression with small G proteins ARF1/6, RHOA, ARL11/14 (Li et al., 2022). To directly increase the PLD activity in subcellular structure, Baskin et al. employed optogenetic techniques to manipulate the subcellular localization of bacterial PLD_{PMF} for PA production (Tei and Baskin, 2020). However, platforms for directly controlling PLD enzyme activity remains unsolved. Here, we propose a method for optogenetic control of PLD enzyme (Opto-PLDe) activity through split protein reassembly.

To achieve optogenetic control of PLD enzyme activity, we selected PLDα1, a PLD isoform with relatively high basal activity, and inserted the light-sensitive element LOV2 near the unique lid structure of the substrate pocket of PLDα1 (Li et al., 2020; Yao et al., 2021). We hypothesized that light-induced unwinding of LOV2 would alter the conformation of the lid, thereby regulating the activity of PLDα1. To our surprise, upon inserting LOV2 at residue Thr277 site (Opto-PLD (277)), blue light stimulation abolished the total PLD activity in Opto-PLD (277)-overexpressed stable cell lines, despite these cells displaying a modest increase in PLD activity in the dark. Given that the C2 domain is required for PLDα1 enzyme activity, we subsequently hypothesized that through splitting PLDα1 at the linker between the C2 domain and the catalytic domain (PLDCTD), we might be able to control the dimerization of C2 and PLDCTD domain based on optogenetic and consequently restoring the enzyme activity of the split PLDα1.We utilized the iLID system to fuse corresponding split PLDα1 fragments, specifically combining Tom20-C2-LOV2 (SsrA) and mCh-SspB-PLDCTD. The AlphaFold2 tool was employed to predict the assembled structure and assess the spatial accessibility of C2 and PLDCTD for reassembly. Upon co-expression of both constructs in HeLa cells, dynamic changes in the localization of PLDCTD to mitochondria were observed upon blue light stimulation. Despite the successful dimerization of the C2 and PLDCTD domains, enzymatic activity assays indicated no significant increase in total cellular PLD activity. This outcome suggests that the split PLDα1 activity was not restored, highlighting a functional limitation even with the established interaction between the C2 and PLDCTD domains. These findings underscore the intricacies of the regulatory mechanisms governing PLDα1 function. Furthermore, we noted the spontaneous dimerization of the two HKD domains of human PLD1 when we splited human PLD1 into two parts at Ser 584 residue. This finding suggests that human PLD1 may not be a suitable candidate for optogenetic modification.

Very recently, Baskin et al. undertook efforts to optimize PLDPMF activities through directed evolution, leveraging their ingenious phosphatidic acid (PA) detection technique (Tei et al., 2023). They successfully generated a range of PLD_{PMF} variants exhibiting significantly

enhanced enzyme activity. Notably, certain variants demonstrated a remarkable 100-fold increase in activity, referred to as superPLD. Hence, we selected superPLD as the target for our optogenetic modification. To identify potential splitting positions, we employed a flexible, long linker to connect two split subunits. We observed that superPLD with insertions of the linker at Pro 285 and Ala 290 residues maintained enzyme activity, resulting in the accumulation of PA probes (Opto-PASS, an optogenetic modification PA biosensor (Yao et al., 2023)) on mitochondria. Interestingly, In cells with overexpression of superPLD, we noted a significant modification in mitochondrial morphology, characterized by a punctate structure. This alteration persisted even in the absence of anchoring superPLD to mitochondria through a mitochondrial targeting sequence. In addition, some of these punctate mitochondria also co-localized with lysosomes. To exclude the possibility of mitochondrial autophagy in this subset, we employed the mitochondrial autophagy probe Mito-Rosella. We observed that the fluorescence signal of PHluorin on these punctate mitochondria persisted. Upon addition of the mitochondrial autophagy inducer CCCP, the fluorescence signal diminished. This suggests that the punctate mitochondria did not fuse with lysosomes, indicating a lack of mitophagy. The morphological changes, therefore, are likely attributed to elevated intracellular PA levels resulting from the overexpression of superPLD. This further underscores the importance of controlling the activity of this highly active PLD_{PMF} variant.

Subsequently, we splited superPLD at Pro 285 and Ala 290 residues and fused it with corresponding optogenetic tools, namely Tom20-HKD1(285)-LOV2(SsrA) and mCh-SspB-PLDCTD (referred to as Opto-PLDe(285)), and Tom20-HKD1(290)-LOV2 (SsrA) and mCh-SspB-PLDCTD (referred to as Opto-PLDe(290)). Following blue light stimulation, we observed an increase in PA levels on mitochondria using the Opto-PASS probe, which indicates dimerization of the two HKD domains of superPLD resulted in the restoration of enzyme activity. Additionally, we employed a high-resolution PA detection probe developed by our team (Yao et al., 2023). Upon exposure to blue light stimulation, this probe forms punctate structures and each cluster incorporated multiple copies of the PA-binding motif. This design enables the clear observation of subtle changes in PA levels and facilitates subsequent tracking. Using this enhanced probe, we observed that Opto-PLDe, upon

responding to blue light, displayed numerous points adhering to the surface of mitochondria with synchronized movement. This observation suggests the potential activation of Opto-PLDe, leading to the generation of PA on the mitochondria surface.

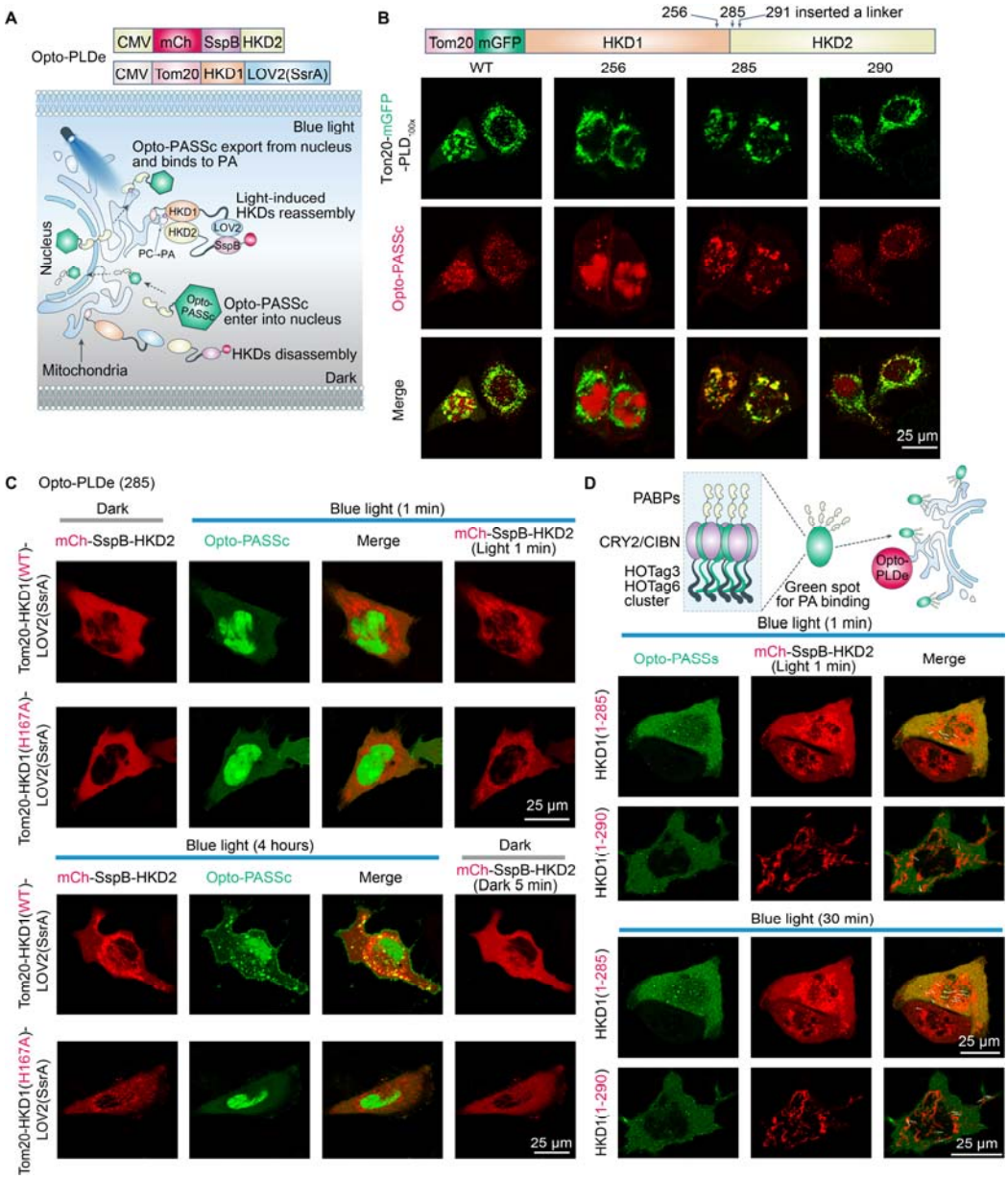
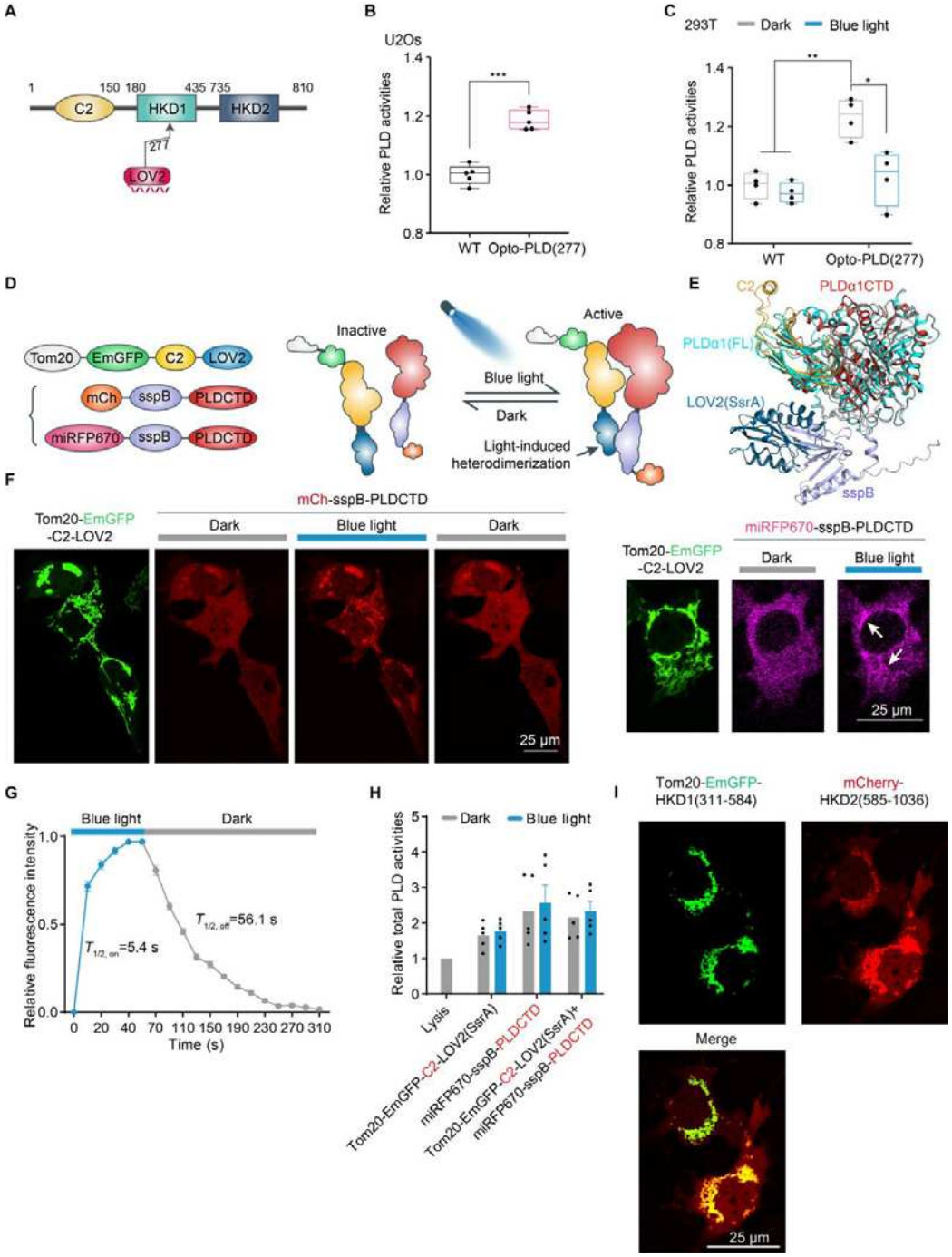


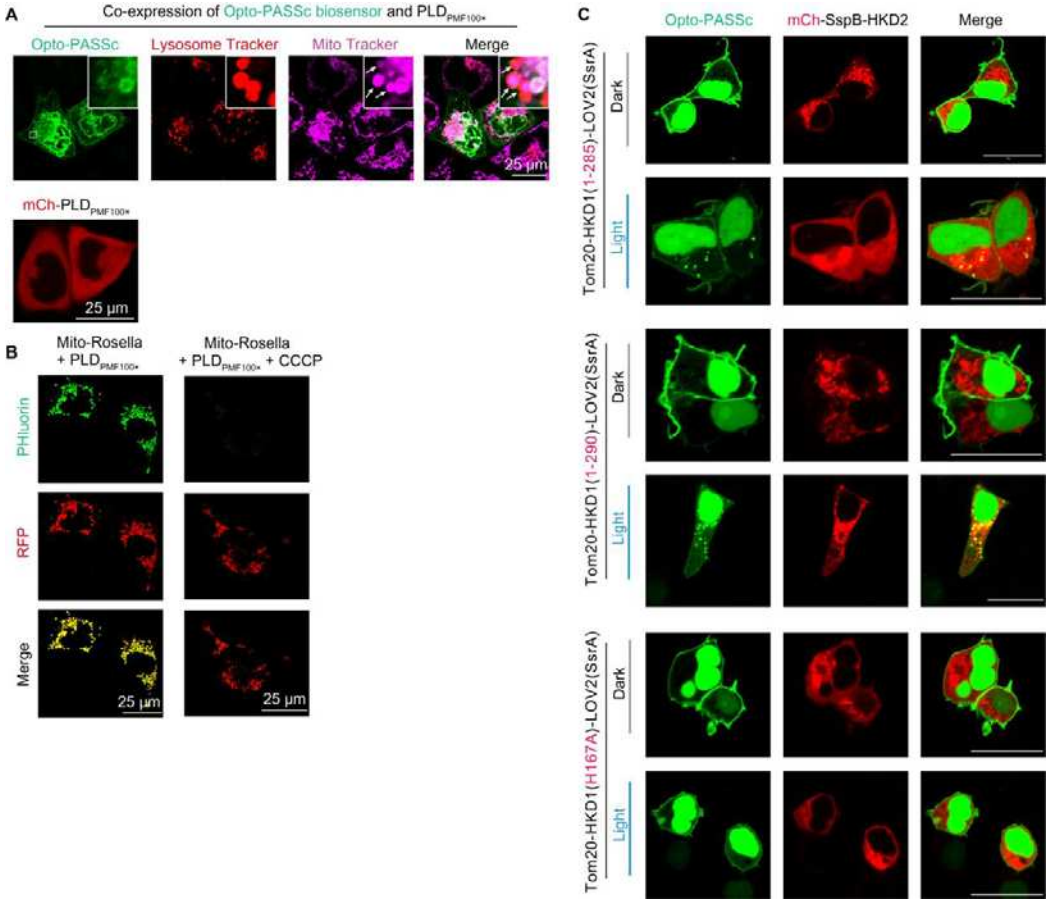
Figure1 Using the split protein reassembly strategy to modify PLD and control its activity via light. A, the conceptual diagram of optogenetic control of PLD enzyme activity. B, using PA probe Opto-PASSc to screen the split site of the superPLD by insertion of a linker. C, blue light stimulation induced the dimerization of split PLD on mitochondria and restored

its activity. D, a high resolution PA probe Opto-PASSs was used to monitor the Opto-PLDe activity.



Supplementary Figure 1 Optogenetic modification of Arabidopsis PLDα1. A-C, Insertion of LOV2 at Thr 277 residue caused an inhibitory effect of PLDα1 activity by blue light. D,

the optogenetic design of split PLDα1 and its fusion with iLID system. E, the predicted secondary structure of Opto-PLD by AlphaFold2. F-G, the light-induced dimerization of PLDCTD and C2 domain and quantitative analysis of its dynamic. H, biochemical analysis of Opto-PLD enzyme activity in response to light stimulation. I, spontaneous dimerization of split human PLD1.



Supplementary Figure 2 overexpression of superPLD altered the mitochondrial morphology. A, the mitochondria displayed a punctate structure in superPLD-overexpressed cells. B, using Mito-Rosella to assess the mitophagy of the punctate mitochodria. C, Opto-PASSc biosensor was used to assess Opto-PLDe activity under light illumination in 293T cells.

References

Li, F.L., Wu, Z., Gao, Y.Q., Bowling, F.Z., Franklin, J.M., Hu, C., Suhandynata, R.T., Frohman, M.A., Airola, M.V., Zhou, H., *et al.* (2022). Defining the proximal interaction networks of Arf GTPases reveals a mechanism for the regulation of PLD1 and PI4KB. The EMBO Journal *41*.

Li, J., Yu, F., Guo, H., Xiong, R., Zhang, W., He, F., Zhang, M., and Zhang, P. (2020). Crystal structure of plant PLDα1 reveals catalytic and regulatory mechanisms of eukaryotic phospholipase D. Cell Res *30*, 61-69.

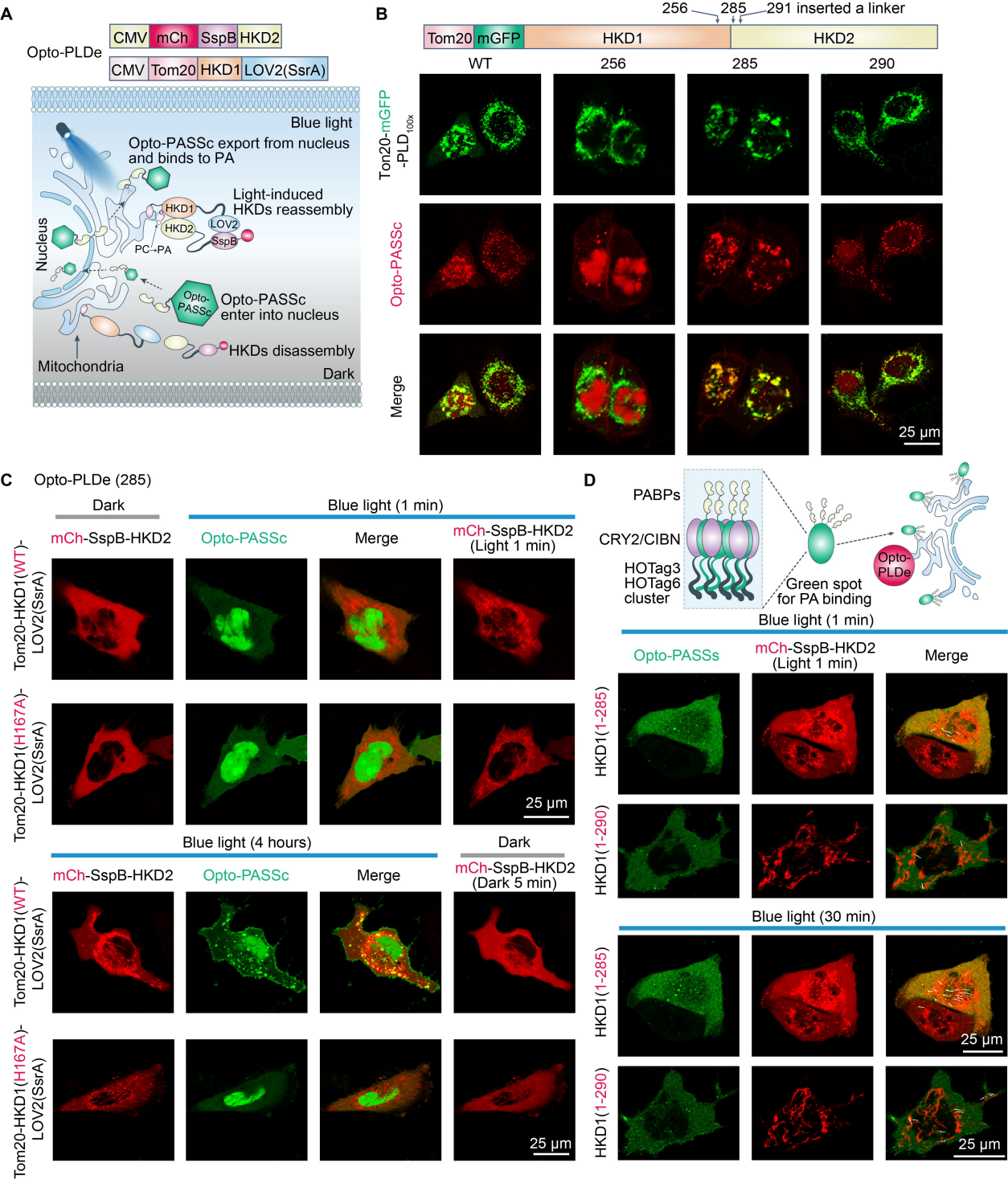
Tei, R., Bagde, S.R., Fromme, J.C., and Baskin, J.M. (2023). Activity-based directed evolution of a membrane editor in mammalian cells. Nature Chemistry *15*, 1030-1039.

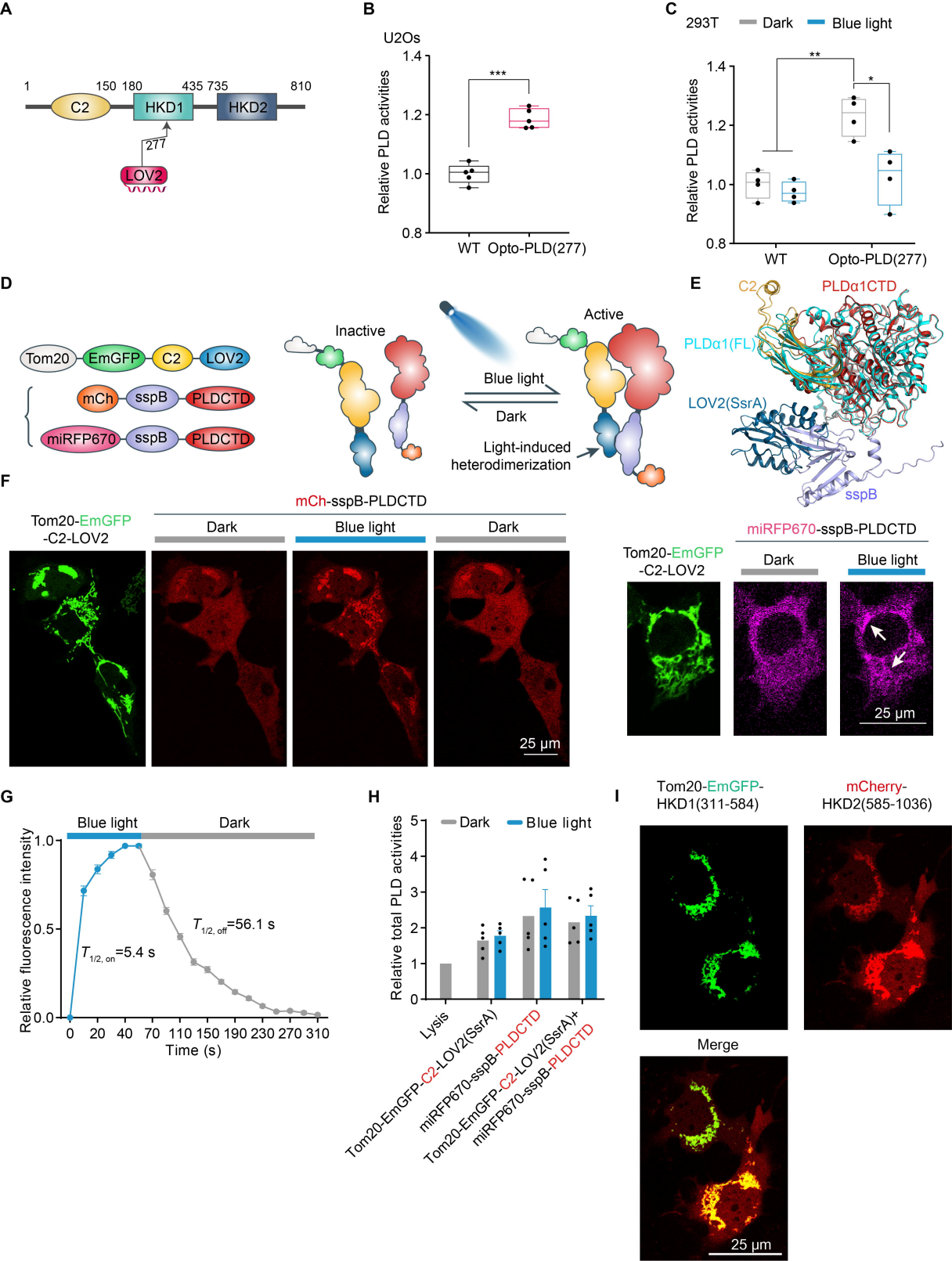
Tei, R., and Baskin, J.M. (2020). Spatiotemporal control of phosphatidic acid signaling with optogenetic, engineered phospholipase Ds. Journal of Cell Biology *219*.

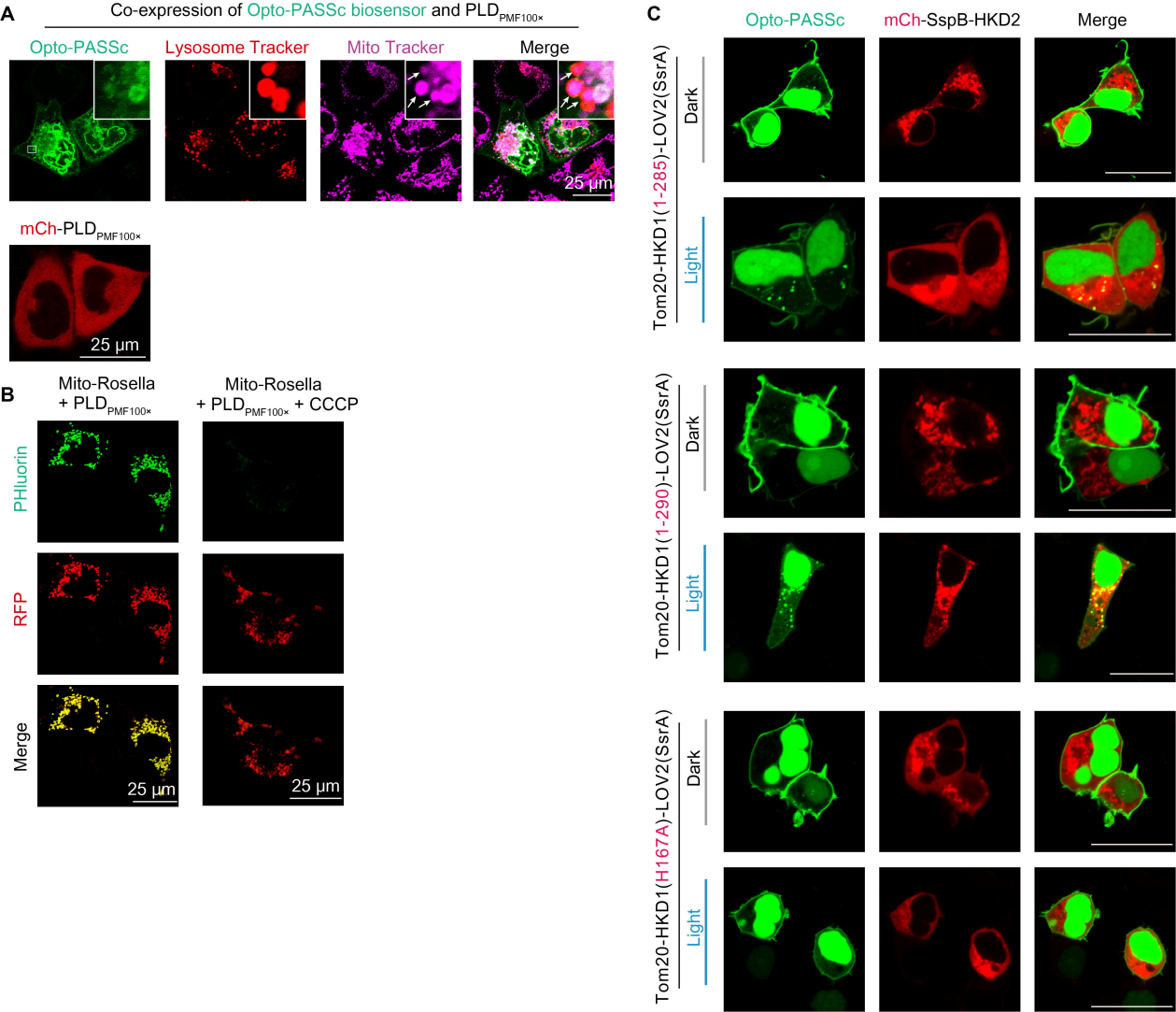
Yao, Y., Li, J., Lin, Y., Zhou, J., Zhang, P., and Xu, Y. (2021). Structural insights into phospholipase D function. Progress in Lipid Research *81*, 101070.

Yao, Y., Lou, X., Jin, L., Li, J., Liu, J., Chen, Y., Cheng, S., Zhao, T., Ke, S., Zhang, L., *et al.* (2023). Optogenetic strategies for optimizing the performance of biosensors of membrane phospholipids in live cells. bioRxiv, 2023.2008.2003.551799.

Yao, Y., Wang, X., Li, H., Fan, J., Qian, X., Li, H., and Xu, Y. (2020). Phospholipase D as a key modulator of cancer progression. Biological Reviews *95*, 911-935.


A
Opto-PLDe
CMV mCh SspB HKD2
CMV Tom20 HKD1 LOV2(SsrA)
Blue light
Opto-PASSc export from nucleus and binds to PA
Light-induced HKDs reassembly
HKD1
HKD2
LOV2
SspB
PC→PA
Nucleus
Opto-PASSc
Opto-PASSc enter into nucleus
HKDs disassembly
Mitochondria
Dark
B
256
285
291 inserted a linker
Tom20 mGFP HKD1 HKD2
WT
256
285
290
Ton20-mGFP -PLD100x
Opto-PASSc
Merge
25 μm
C
Opto-PLDe (285)
Dark
Blue light (1 min)
mCh-SspB-HKD2
Opto-PASSc
Merge
mCh-SspB-HKD2 (Light 1 min)
Tom20-HKD1(WT)-LOV2(SsrA)
Tom20-HKD1(H167A)-LOV2(SsrA)
25 μm
Blue light (4 hours)
Dark
mCh-SspB-HKD2 (Dark 5 min)
D
PABPs
CRY2/CIBN
HOTag3
HOTag6
cluster
Green spot for PA binding
Opto-PLDe
Blue light (1 min)
Opto-PASSs
mCh-SspB-HKD2 (Light 1 min)
Merge
HKD1(1-285)
HKD1(1-290)
Blue light (30 min)
25 μm


A
1
150 180
435 735
810
C2
HKD1
HKD2
277
LOV2
B
U2Os
Relative PLD activities
1.4
1.2
1.0
0.8
***
WT
Opto-PLD(277)
C
293T
Dark
Blue light
Relative PLD activities
**
*
WT
Opto-PLD(277)
D
Tom20
EmGFP
C2
LOV2
mCh
sspB
PLDCTD
miRFP670
Inactive
Active
Blue light
Dark
Light-induced heterodimerization
E
C2
PLDα1CTD
PLDα1(FL)
LOV2(SsrA)
sspB
F
Tom20-EmGFP -C2-LOV2
mCh-sspB-PLDCTD
Dark
Blue light
Dark
25 μm
miRFP670-sspB-PLDCTD
Tom20-EmGFP -C2-LOV2
Dark
Blue light
25 μm
G
Blue light
Dark
Relative fluorescence intensity
1.0
0.5
0.0
$T_{1/2, off}$=56.1 s
$T_{1/2, on}$=5.4 s
0 20 40 70 110 150 190 230 270 310
Time (s)
H
Dark
Blue light
Relative total PLD activities
5
4
3
2
1
0
Lysis
Tom20-EmGFP-C2-LOV2(SsrA)
miRFP670-sspB-PLDCTD
Tom20-EmGFP-C2-LOV2(SsrA)+ miRFP670-sspB-PLDCTD
I
Tom20-EmGFP-HKD1(311-584)
mCherry-HKD2(585-1036)
Merge
25 μm


A
Co-expression of Opto-PASSc biosensor and PLD$_{PMF100\times}$
Opto-PASSc
Lysosome Tracker
Mito Tracker
Merge
25 μm
mCh-PLD$_{PMF100\times}$
25 μm
B
Mito-Rosella + PLD$_{PMF100\times}$
Mito-Rosella + PLD$_{PMF100\times}$ + CCCP
PHluorin
RFP
Merge
25 μm
25 μm
C
Opto-PASSc
mCh-SspB-HKD2
Merge
Tom20-HKD1(1-285)-LOV2(SsrA)
Dark
Light
Tom20-HKD1(1-290)-LOV2(SsrA)
Dark
Light
Tom20-HKD1(H167A)-LOV2(SsrA)
Dark
Light